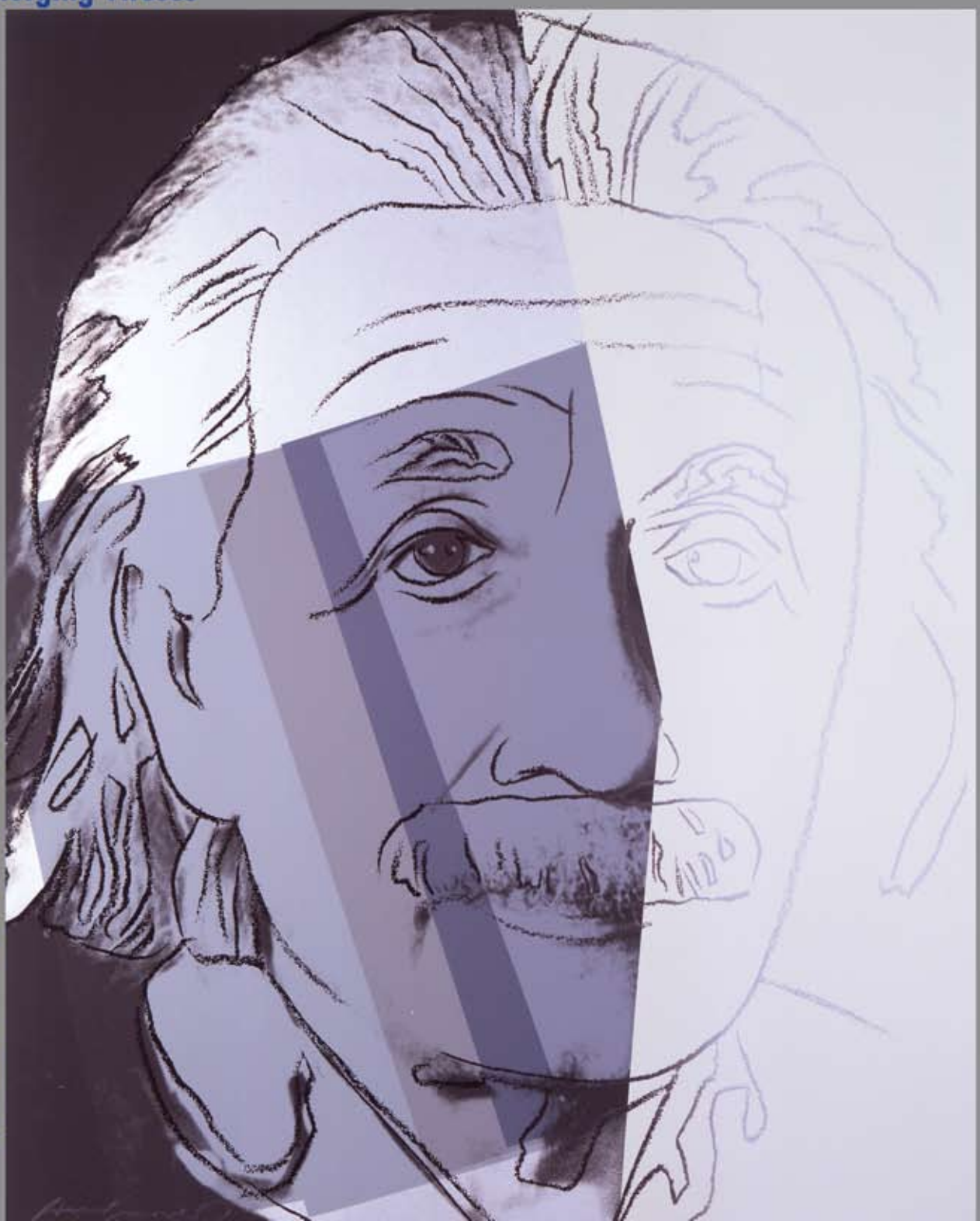
EMERGING INFECTIOUS DISEASES®

June 2010

Emerging Viruses



Andy Warhol Foundation for the Visual Arts/ARS, New York. Ronald Feldman Fine Arts, New York

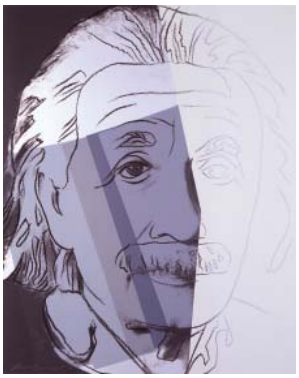
EMERGING
INFECTIOUS DISEASES®

www.cdc.gov/eid

Emerging Infectious Diseases
Emerging Infectious Diseases is published monthly by the Centers for Disease Control and Prevention, 1600 Clifton Road, Mailstop D61, Atlanta, GA 30333, USA. Telephone 404-639-1960, fax 404-639-1954, email eideditor@cdc.gov.

EMERGING INFECTIOUS DISEASES

June 2010



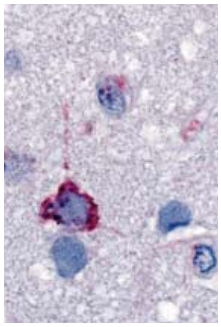
On the Cover

Andy Warhol (1928–1987)
Albert Einstein (1980)
Screen Print on Lenox Museum Board
(101.6 cm × 81.3 cm)

Andy Warhol Foundation for the Visual Arts/ ARS, New York. Ronald Feldman Fine Arts, New York, NY, USA

About the Cover p. 1052

Research



p. 924

p. 990

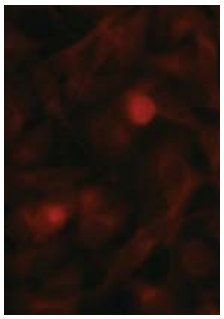
Dispatches

EMERGING INFECTIOUS DISEASES
June 2010

Commentaries

Photo Quiz

Letters



p. 993

p. 1025

Book Reviews

About the Cover

Evolution of Northeastern and Midwestern *Borrelia burgdorferi*, United States

Dustin Brisson, Mary F. Vandermause, Jennifer K. Meece, Kurt D. Reed, and Daniel E. Dykhuizen

The per capita incidence of human Lyme disease in the northeastern United States is more than twice that in the Midwest. However, the prevalence of *Borrelia burgdorferi*, the bacterium that causes Lyme disease, in the tick vector is nearly identical in the 2 regions. The disparity in human Lyme disease incidence may result from a disparity in the human invasiveness of the bacteria in the Northeast and Midwest caused by fundamentally different evolutionary histories. *B. burgdorferi* populations in the Northeast and Midwest are geographically isolated, enabling evolutionary divergence in human invasiveness. However, we found that *B. burgdorferi* populations in the Northeast and Midwest shared a recent common ancestor, which suggests that substantial evolutionary divergence in human invasiveness has not occurred. We propose that differences in either animal ecology or human behavior are the root cause of the differences in human incidence between the 2 regions.

Lyme disease, caused by the bacterium *Borrelia burgdorferi*, is the most common vector-borne disease in the United States (*1*). *B. burgdorferi* is transmitted to humans during the blood meal of an infected *Ixodes* tick, predominantly *Ixodes scapularis* in North America (*2*). The prevalence and density of *B. burgdorferi*–infected *I. scapularis* ticks are nearly identical in the northeastern and midwestern United States, the regions with the highest incidence of Lyme disease in humans (*3*–*6*); however, the number of human Lyme disease cases reported in the Northeast and Midwest is not (*1*). The overwhelming majority of Lyme disease cases in the United States are reported from the Northeast (82%), followed distantly by the Midwest (9%) (*1*). Similarly, per capita Lyme disease incidence is >2× greater in the Northeast than the Midwest. We address the hypothesis that *B. burgdorferi* populations in the Northeast and Midwest have fundamentally different evolutionary histories, which may result in differing degrees of human invasiveness.

The evolutionary and demographic histories of *B. burgdorferi* in the Northeast have been intensively studied. The effective population size of northeastern *B. burgdorferi* is small because of its recent colonization of the northern United States and its life-history strategy (*7*,*8*). The strikingly impoverished neutral genetic diversity and high linkage disequilibrium within *B. burgdorferi* populations likely result from small effective population sizes (*9*–*11*). Genetic loci are found in perfect or near-perfect association in *B. burgdorferi* in the Northeast (*9*–*12*). Strong linkage disequilibrium among genetic loci can result from several evolutionary and ecologic forces in addition to small population size (drift), such as lack of recombination machinery or limited opportunity for gene exchange (*13*). Genetically diverse strains of *B. burgdorferi* often are found within the same tick or same vertebrate host, suggesting ample opportunity for genetic exchange (*4*,*14*). Evidence is strong that recombination occurs within a genomic lineage of *B. burgdorferi* (*15*–*17*). Thus, *B. burgdorferi* has the opportunity and the recombination system needed for genetic exchange. A historically small effective population size is a parsimonious explanation for the low neutral genetic diversity and strong linkage disequilibrium.

The evolutionary and demographic histories of *B. burgdorferi* in the Midwest are comparatively understudied. On the coarse scale, the evolutionary history and ecol-

Author affiliations: University of Pennsylvania, Philadelphia, Pennsylvania, USA (D. Brisson); Marshfield Clinic Research Foundation, Marshfield, Wisconsin, USA (M.F. Vandermause, J.K. Meece); Northwestern University/Feinberg School of Medicine, Chicago, Illinois, USA (K.D. Reed); and Stony Brook University, Stony Brook, New York, USA (D.E. Dykhuizen)

DOI: 10.3201/eid1606.090329

ogy of *B. burgdorferi* in the Northeast and Midwest appear similar. Both regions have oak-maple–dominated forests ideal for deer and the small mammals that maintain the *I. scapularis* and *B. burgdorferi* populations, were under the Pleistocene ice sheet, and were recently colonized by *B. burgdorferi*. The differences accounting for a lower Lyme disease incidence in the Midwest than the Northeast are not clear but most likely can be found on a finer scale in the evolutionary history of *B. burgdorferi* (this study), the tick vector (*8*), or human exposure.

The differences accounting for a lower Lyme disease incidence in the Midwest than the Northeast are not clear but are likely to be found on a finer evolutionary or ecologic scale. A recent report suggested that a lower proportion of the 16S–23S rRNA intergenic spacer (IGS) type 1 (RST-1) allele in midwestern *B. burgdorferi* populations, an allele associated with human invasiveness in the northeastern United States, may account for the differences in human Lyme disease incidence (*8*). However, the 16S–23S rRNA IGS does not directly influence *B. burgdorferi* invasiveness; it is in linkage disequilibrium with a gene of major effect in the Northeast. The linkage patterns in midwestern *B. burgdorferi* are as yet unstudied. A fundamentally different evolutionary history would result in divergent linkage disequilibrium patterns between the Northeast and the Midwest and potentially result in differing degrees of human invasiveness associated with alleles at the 16S–23S rRNA spacer.

For this study, we used a phylogenetic framework to analyze the evolutionary and demographic histories of *B. burgdorferi* in the midwestern and northeastern United States (*18*). A previous multilocus sequencing typing study of 4 *B. burgdorferi* loci (outer surface protein C [*ospC*], 16S–23S rRNA (*rrs-rrl-A*), *ospA*, and outer membrane protein [*p66*]) from strains isolated in the Northeast identified 9 distinct lineages with complete linkage among alleles at the 4 loci (*10*). That study analyzed statistical associations of haplotypes at each locus without regard to the underlying evolutionary relationships of sequences. In this study, we analyzed *ospC*, *rrs-rrlA*, *ospB*, and *ospA* (p66 contains little evolutionary information [*10*]) from midwestern and northeastern populations in a phylogenetic framework to investigate the shared and vicariant evolutionary and demographic histories of *B. burgdorferi* from geographically isolated regions.

Methods

B. burgdorferi Isolates

All isolates were derived from skin biopsy specimens of 47 adult patients who had erythema migrans, at the Marshfield Clinic in central Wisconsin during 1995–2001. Specimens were collected and cultured as described elsewhere (*19*).

DNA Extraction and Amplification

The DNA sequences of *ospA*, *ospB*, *rrs-rrlA*, and *ospC* were determined for use in our phylogenetic analysis. Cultivated isolates were harvested by centrifugation at 5,000 × *g* for 15 min, resuspended in sterile water, and lysed by boiling for 5 min. PCR conditions are described by Bunikis et al. (*10*) for *ospA* and *rrs-rrlA*, by Caporale and Kocher (*20*) for *ospB*, and by Brisson and Dykhuizen (*4*) for *ospC*. Negative controls were included for DNA extraction and PCR procedures to monitor for contamination. Amplified PCR products were sequenced in both directions. *ospC* products were subject to the reverse-line blot procedure as described previously (*4,7*). *ospC* amplicons that could not be classified to a major allelic group were sequenced.

Analyses

All analyses included sequence data collected in this study and reported in Bunikis et al. (*10*). DNA sequences were aligned using the Clustal X algorithm (*21*) with default settings, and alignments were refined manually where necessary. Descriptive statistics (π, synonymous and nonsynonymous polymorphisms) were determined using DNAsp (ver. 4.50.3) (*22*). Two tests for recombination within genes (Sawyer test and maximum χ^2 test) and a test for allelic association (index of association [I_A]) were performed in START2 (*23*). Sawyer runs test compares pairs of alleles to determine whether regions of the sequence space have more consecutive identical polymorphisms (runs) than expected by chance (*24*). The maximum χ^2 test uses the distribution of polymorphic sites to identify potential recombination events between pairs of alleles (*25*). The I_A assesses the extent of association between loci using allele frequencies without regard to the underlying sequences (*26*).

Phylogenetic reconstruction was performed using Bayesian (MrBayes; http://mrbayes.csit.fsu.edu) and maximum likelihood (PAUP*, version 4.0; http://paup.csit.fsu.edu) approaches. Trees were constructed from the sequence data from each gene individually and from combinations of genes. IGS, *ospA*, and *ospB* provide reliable data for phylogenetic inference and can be easily compared with previous analyses (*10*). *ospC* DNA sequences provide information about disease invasiveness (*27*,*28*) but were not used in phylogenetic reconstruction because the gene tree topology is star-shaped with short and unsupported internal branches, long terminal branches, and many polytomies. This topology provides no information about the evolutionary history of this gene or the relationships among alleles. When information about *ospC* was included in the data set, it was included as a heavily weighted morphologic character in MrBayes, not as DNA sequence data. The morphologic data constrain the isolates with the same *ospC* allele cluster but do not affect the topology of the internal branches of the tree, which depend only on the DNA sequences of

the other genes in the data set. For each data set, we used Modeltest 3.4 (*29*) to select the appropriate model of molecular evolution. We used this model to find the Bayesian tree and the maximum likelihood tree.

We used the Shimodaira-Hasegawa (SH) test as implemented in PAUP to test for differences in evolutionary histories among genes using the resampling of estimated log-likelihoods approximation with 1,000 bootstrap replicates (*18*). This method tests for significant differences in the likelihood of several given tree topologies given a data set. The likely tree topologies produced by each locus analyzed in this study were used as the set of topologies. If no true difference exists, each data set is equally likely to produce any of the given tree topologies, and all genes in this study share similar evolutionary histories. Alternatively, a significantly lower likelihood suggests that recombination has occurred between the genes in the data set and the gene used to create the lower-likelihood tree topology.

Results

Sequence Diversity

B. burgdorferi isolates were cultured from primary or secondary skin legions of 47 adults visiting the Marshfield Clinic during 1995–2001. The sequences at the IGS, *ospA*, *ospB*, and *ospC* loci were determined from 39, 31, 44, and 44 strains of these *B. burgdorferi* isolates, respectively. Erythema migrans represented the bulk of the diversity found in tick samples (*27*) and provided a representative sample of *B. burgdorferi* bacteria in the Midwest. Furthermore, we found all of the IGS types found by Bunikis et al. (*10*), suggesting the sample from the Midwest was not biased in terms of the types found. *ospA* showed little evolutionarily information and was not determined from 14 of the isolates. One sequence was found at each locus in each culture.

Nucleotide diversity varied considerably among loci (Table 1). We found 47 polymorphic sites in the IGS sequence sample, resulting in 16 unique haplotypes (online Technical Appendix, www.cdc.gov/EID/content/16/6/911-Techapp.pdf). Six haplotypes were identical to haplotypes previously found in the Northeast, and 38 of the 47 polymorphic sites found in the Northeast were present in this sample from the Midwest, supporting a recent shared ancestry of *B. burgdorferi* from the 2 populations (online Technical Appendix). The 10 unique haplotypes in the Midwest and 9 unique polymorphic sites suggest some isolation by distance and an emerging evolutionary divergence.

ospA and *ospB* are considerably less diverse than IGS (Table 1). Nevertheless, there are 14 unique *ospA* haplotypes and 14 unique *ospB* haplotypes in this sample (online Technical Appendix). Nine of the *ospA* haplotypes in the samples from the Midwest also are found in *B. burgdorferi* in the Northeast. The diversity of *ospB* has not been examined in the Northeast. Amino acid sequence evolution in *ospA* and *ospB* appears to be constrained because synonymous changes greatly exceed nonsynonymous changes ($dN_{ospA}/dS_{ospA} = 0.334$; $dN_{ospB}/dS_{ospB} = 0.303$).

The mean pairwise diversity at *ospC* was much greater than at the other loci analyzed ($\pi = 0.207$). Sixty-three changes resulted in amino acid substitutions. However, we found no evidence of directional selection (dN/dS = 0.591). Eighteen major groups of alleles, which differ by >8% in sequence, are represented in the isolates from the Midwest (online Appendix Table, www.cdc.gov/EID/content/16/6/911-appT.htm). Fourteen of the 18 major group alleles are identical to those found in the Northeast, although types L and O are rare in the Northeast. We found 4 novel *ospC* major group alleles (W, V, Y, Z) in these samples (online Appendix Table). *ospC* major groups W, V, and X also were detected in tick populations in the Midwest (D. Brisson, unpub. data), although we did not see type X in this set of human isolates. Strains with *ospC* major groups I, J, and T, which are present in tick and human samples in the Northeast, were absent in this sample from the Midwest.

Phylogenies

We used Bayesian and likelihood algorithms to reconstruct phylogenies for each gene. The 2 approaches resulted in identical topologies for each gene except *ospC*. The IGS gene tree forms a strongly supported phylogeny with most nodes bifurcating (Figure). Polychotomies occur only at sequences that are identical or differed by 1 nucleotide. The phylogeny generally supports the previously described divisions of IGS sequences into the 3 restriction fragment length subgroups, arbitrarily called RST 1, RST2, and RST3 (*31*). However, the diverse RST3 group is not monophyletic. The IGS sequences from strains in the Northeast and Midwest are interdigitated, suggesting a recent shared history.

The gene trees for *ospA* and *ospB* individually have many weakly supported nodes and polychotomies because

Table 1. Genetic markers and population diversity of *Borrelia burgdorferi*, midwestern and northeastern United States, 1999–2001*

Marker	No. samples	π	dN/dS	Maximum χ^2, p value	Sawyer test, significant fragments
IGS	39	0.014	NA	107, p<0.01	0
ospA	31	0.0033	0.334	0	0
ospB	44	0.0042	0.303	739, p<0.05	0
ospC	44	0.207	0.591	74, 83, 90, 137, 338; p<0.001	4

*dN/dS, directional selection; IGS, intergenic spacer; NA, not applicable; osp, outer surface protein.

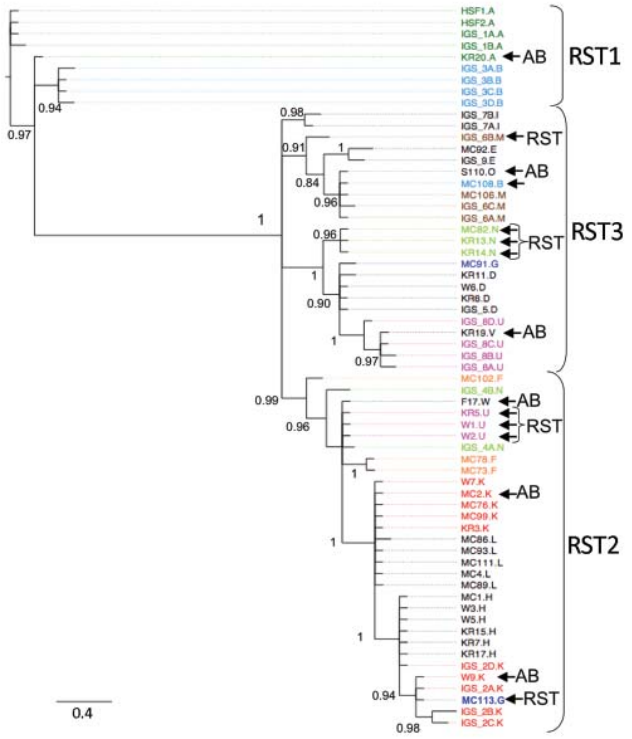


Figure. Phylogeny of *Borrelia burgdorferi* isolates in the northeastern and midwestern United States based on intergenic spacer (IGS) sequence. operational taxanomic unit names beginning with IGS were isolated in the northeastern United States (*10*); all other isolates are from patients in the Midwest. The letter after period designates the outer surface protein C (*ospC*) major allele of the isolate. Colored isolate names highlight isolates with the same *ospC* major group that cluster in different clades, which suggests horizontal gene transfer. The *ospC* of several strains is not linked to the IGS ribosomal spacer type (RST) to which it is commonly linked in the Northeast (*10,34*). AB indicates differences between the *ospAB* tree and the IGS tree. This tree is midpoint rooted. Scale bar indicates number of substitutions per site.

of the limited number of phylogenetically informative sites. The phylogeny reconstructed from the *ospA/ospB* operon (concatenated data set) result in much stronger support for internal nodes. Nevertheless, the *ospAB* phylogeny contains 1 polychotomy. Concatenating the data sets for *ospA* and *ospB* sequences is appropriate because these loci lie contiguously in a single operon, and little evidence exists for recombination either within or between these genes (see below). The northeastern and midwestern strains were interdigitated on the *ospA*, *ospB*, and *ospAB* trees, which suggests a recent, shared population history.

Analysis of the *ospC* data set yielded a phylogeny with short internal branches, long terminal branches, and no supported internal nodes, commonly referred to as a star phylogeny (*10*,*13*). *ospC* thus appeared to have evolved by recombination (*9*,*32*). Phylogenies of highly recombining loci are poorly supported because the evolutionary history differed in different segments of the allele (*33*).

Intragenic Recombination

We found little evidence of recombination within IGS, *ospA*, or *ospB* in the Midwest data set or the combined northeastern/midwestern data set (Table 1). Sawyer runs test found no runs of polymorphisms that are significantly incongruous within the sequence. Maynard Smith's maximum χ^2 test found 1 recombination event within the IGS sequence at nt 107 (position compared with B31 type strain) in both data sets. We found no evidence for recombination within *ospA* and only 1 potential site of recombination at the extreme 3′ end of *ospB*. Neither maximum χ^2 nor Sawyer test found evidence for recombination between *ospA* and *ospB* in the *ospAB* operonic data set. However, *ospA* haplotypes are often associated with multiple *ospB* haplotypes suggesting recombination or independent evolution (online Technical Appendix). Intragenic recombination was evident within *ospC* sequences. Sawyer test identified 4 runs of polymorphisms that appear to be transferred from another *ospC* major allelic group. Maynard Smith maximum χ^2 test found evidence of recombination at 5 locations along the sequence (Table 1).

Intergenic Recombination

We assessed intergenic recombination using the I_A and the SH test. The index of association detects nonrandom associations of haplotypes among genes but does not account for sequence variation or relatedness. The I_A indicated that all loci examined in this study were significantly nonrandomly associated, i.e., in linkage disequilibrium ($p<0.0001$) (Table 1).

The SH test uses phylogenetic information to identify horizontal transfer events by comparing the evolutionary histories of genes as represented by the gene-tree topologies. The likelihood of the IGS sequence data resulting in the *ospAB* tree topology is significantly less likely than these data resulting in the IGS topology (Table 2). That is, the evolutionary history of the IGS locus is not congruent with the *ospAB* evolutionary history, providing evidence of lateral gene transfers. Strains found in different strongly supported clades on the *ospAB* and the IGS trees are identified in the Figure ($p<0.0001$).

The phylogenies reconstructed from midwestern *ospAB* sequences were compared with *ospAB* phylogenies that constrain strains with the same *ospC* allele to cluster (*ospABC* tree). We found no statistical support for recombination between *ospC* and *ospAB* ($p = 0.313$). However, 4 *ospC* type K strains (W9, W7, MC2, KR3) are separated from the other K strains by supported nodes in the *ospAB* tree, and 1 *ospC* type F strain (MC102) is separated from the other *ospC* type F strains. We also found no evidence of

Table 2. Summary of Shimodaira-Hasegawa test results of potential horizontal gene transfer events in *Borrelia burgdorferi*, midwestern and northeastern United States*

Test comparison	Data set	Δ lnL	p value
ospAB vs.			
IGS	*ospAB*	8.50709	0.390
ospAB/ospC	*ospAB*	21.26004	0.313
IGS vs.			
ospAB	IGS	642.826545	<0.0001
IGS/*ospC*	IGS	67.53033	0.006
IGS/*ospC*†	IGS	388.21228	<0.0001
ospB vs. *ospB/ospC*	*ospB*	18.91326	0.338
ospA vs. *ospA/ospC*	*ospA*	45.30135	0.146
ospA vs. *ospA/ospC*†	*ospA*	79.62589	0.080

*Δ lnL, difference in log-likelihood; *osp*, outer surface protein; IGS, intergenic spacer.
†Included sequence data from Bunikis et al. (*10*).

recombination between *ospA* and *ospC* or *ospB* and *ospC* (p>0.1). However, the *ospA* tree is nearly significantly more likely than the *ospAC* tree when strains from the Northeast are included in the analysis (p = 0.08).

Several instances of recombination are evident between IGS and *ospC*. The differences in evolutionary history were observable by using the midwestern IGS data set alone (p = 0.006) and the combined midwestern/northeastern IGS data set (p<0.001). These analyses provide evidence that linkage patterns in *B. burgdorferi* from the Northeast differ from that of the linkage patterns in *B. burgdorferi* from the Midwest.

Discussion

The divergence in human Lyme disease incidence between the Northeast and Midwest does not result from independent evolution of human invasiveness because of geographic isolation. Although pathogen populations in the Midwest appear geographically isolated from those in the Northeast (*6*), evolutionary and demographic analyses indicate that they share a recent common ancestor. Both populations have little standing genetic variation, as indicated by the limited number of polymorphic sites, suggesting small effective population sizes and similar life-history strategies. The combination of linked alleles also is similar in both regions, supporting the recent shared ancestor hypothesis. *B. burgdorferi* strains isolated in the Midwest are interleaved with northeastern strains on phylogenetic trees evincing their close evolutionary relationship. However, there is some genetic divergence and differing linkage groups between the regions, intimating that gene flow is limited between these populations, allowing them to differentiate. The recent common ancestor in the northeastern and midwestern *B. burgdorferi* and limited genetic divergence suggests that human Lyme disease incidence cannot be explained by fundamentally different evolutionary histories resulting in differing degrees of human infectiousness.

None of the loci investigated show substantial genetic divergence between regions, suggesting a recent common ancestor and similar phenotypes. Northeastern haplotypes are interleaved with midwestern haplotypes such that the time to coalescence of alleles within a region is equivalent to the time to coalescence for alleles from both regions (Figure). These data suggest that northeastern and midwestern strains have a recent common ancestor. The limited genetic diversity in *B. burgdorferi* in the Midwest and Northeast (online Technical Appendix) suggests that the populations have retained the life-history strategy of their common ancestor (*10*,*12*). However, isolation by distance and subsequent divergence resulted in unique alleles in each region.

The IGS gene tree reconstructed from midwestern and northeastern data broadly supports the RST system described using northeastern populations (*31*). RST types 1 and 2 form strongly supported monophyletic groups. RST3 is polyphyletic and should be split into 3 groups as defined by the strongly supported clades (Figure). Supporting this suggestion, RST3 is diverse genetically and phenotypically (*34*). Interestingly, this division would separate *ospC* major group I bearing strains, a particularly invasive group in humans, from the other RST3 strains that rarely cause disseminated infections in humans (*27*,*28*,*30*).

The *ospC* data support the hypothesis that the strains from the Northeast and Midwest have a common ancestor but are currently isolated and have begun to diverge. Most *ospC* major groups are found in both regions. Given the genetic distance between major group alleles, the exact set of alleles is unlikely to have occurred twice independently. Additionally, the linkage relationships between *ospC* alleles and IGS alleles are similar in both regions. Both lines of evidence suggest that most of the diversity at *ospC* originated before the northeastern and midwestern populations diverged. Differences in invasiveness between *B. burgdorferi* in the Northeast and Midwest do not result from fundamentally different evolutionary histories.

Four novel *ospC* major group alleles appear to be unique to the Midwest (Table 2). In addition to these novel *ospC* major groups, a type C–like allele appears to have been generated independently in the Midwest and the Northeast. The group C allele in the Midwest shares 96.8% similarity with the group C allele in the Northeast. Whether the unique *ospC* major group alleles were generated recently in only 1 region or whether they were shared in the ancestral population and subsequently lost in only 1 region is not clear.

B. burgdorferi lineages exchange DNA, contrary to previous reports (*10*,*11*,*13*). We found at least some evidence of recombination between all genetic loci examined; even the *ospAB* operon has several homoplasious mutations, suggesting potential recombination (online Technical Appendix). However, recombination between *ospA*

and *ospB* is not statistically supported and may have arisen from recurrent mutation (online Appendix Table). Despite evidence for recombination, the linkage relationships are similar in the Northeast and the Midwest, supporting the recent common ancestry of these populations.

Recombination is more apparent in Lyme disease foci in the Midwest than in the Northeast (Figure; online Appendix Table). This is likely to be caused by neutral divergence of linkage patterns resulting from small effective population sizes in both regions coupled with gene flow from the Northeast to the Midwest but not in the other direction. Small effective population sizes eliminate most of the linkage combinations in each region such that they are in perfect linkage disequilibrium. Gene flow from the northeastern population then introduces linkage pattern variation into the midwestern population. Linkage patterns unique to the Midwest are absent from the Northeast, suggesting that gene flow from the Midwest to the Northeast is rare.

B. burgdorferi in the Northeast and Midwest share a remarkably similar evolutionary history. Independent evolution of human invasiveness in the 2 regions does not explain the lower human Lyme disease incidence in the Midwest. Other potential causes for the differences in human Lyme disease incidence include differences in human exposure to *B. burgdorferi*–infected ticks and ecologic differences in the reservoir host community. Lyme disease typically is contracted peridomestically in the Northeast (*35*), but similar studies reporting the peridomestic acquisition of *B. burgdorferi* have not been reported from the Midwest. Human risk for exposure to Lyme disease also may be exaggerated in the Northeast because of the immense suburban populations around the major metropolitan areas. Current ecologic conditions yielding differences in the composition of reservoir host species could alter the prevalence of *B. burgdorferi* lineages that are particularly invasive in humans (*27*,*28*,*30*,*36*,*37*). For example, midwestern ticks are rarely infected with *ospC* genotypes A or B (RST I) (*3*), 2 of the 4 genotypes that are common in the Northeast and regularly cause human Lyme disease (*27*,*28*,*30*).

This work was supported by National Institute of Health grants AI076342 (D.B.) and GM31912 (D.E.D) and Centers for Diseases Control and Prevention grant U01CK000170 (D.B.).

Dr Brisson is an assistant professor in the biology department at the University of Pennsylvania. His primary research interests focus on the ecology and evolution of infectious diseases.

References

1. Bacon RM, Kugeler KJ, Mead PS. Surveillance for Lyme disease—United States, 1992–2006. MMWR Surveill Summ. 2008;57:1–9.
2. Burgdorfer W, Barbour AG, Hayes SF, Benach JL, Grunwaldt E, Davis JP. Lyme disease—a tick-borne spirochetosis. Science. 1982;216:1317–9. DOI: 10.1126/science.7043737
3. Gatewood AG, Liebman KA, Vourc'h G, Bunikis J, Hamer SA, Cortinas R, et al. Climate and tick seasonality are predictors of *Borrelia burgdorferi* genotype distribution. Appl Environ Microbiol. 2009;75:2476–83. DOI: 10.1128/AEM.02633-08
4. Brisson D, Dykhuizen DE. ospC diversity in *Borrelia burgdorferi*: different hosts are different niches. Genetics. 2004;168:713–22. DOI: 10.1534/genetics.104.028738
5. Caporale DA, Johnson CM, Millard BJ. Presence of *Borrelia burgdorferi* (Spirochaetales: Spirochaetaceae) in southern Kettle Moraine State Forest, Wisconsin, and characterization of strain W97F51. J Med Entomol. 2005;42:457–72. DOI: 10.1603/0022-2585(2005)042[0457:POBBSS]2.0.CO;2
6. Diuk-Wasser MA, Gatewood AG, Cortinas MR, Yaremych-Hamer S, Tsao J, Kitron U, et al. Spatiotemporal patterns of host-seeking *Ixodes scapularis* nymphs (Acari: Ixodidae) in the United States. J Med Entomol. 2006;43:166–76. DOI: 10.1603/0022-2585(2006)043[0166:SPOHIS]2.0.CO;2
7. Qiu WG, Dykhuizen DE, Acosta MS, Luft BJ. Geographic uniformity of the Lyme disease spirochete (*Borrelia burgdorferi*) and its shared history with tick vector (*Ixodes scapularis*) in the northeastern United States. Genetics. 2002;160:833–49.
8. Humphrey PT, Caporale DA, Brisson D. Uncoordinated biogeography of the Lyme disease pathogen, *Borrelia burgdorferi*, and its tick vector, *Ixodes scapularis.* Evolution. 2010. In press.
9. Qiu WG, Schutzer SE, Bruno JF, Attie O, Xu Y, Dunn JJ, et al. Genetic exchange and plasmid transfers in *Borrelia burgdorferi* sensu stricto revealed by three-way genome comparisons and multilocus sequence typing. Proc Natl Acad Sci U S A. 2004;101:14150–5. DOI: 10.1073/pnas.0402745101
10. Bunikis J, Tsao J, Berglund J, Fish D, Barbour AG. Sequence typing reveals extensive strain diversity of the Lyme borreliosis agents *Borrelia burgdorferi* in North America and *Borrelia afzelii* in Europe. Microbiology. 2004;150:1741–55. DOI: 10.1099/mic.0.26944-0
11. Dykhuizen DE, Polin DS, Dunn JJ, Wilske B, Preac-Mursic V, Dattwyler RJ, et al. *Borrelia burgdorferi* is clonal: implications for taxonomy and vaccine development. Proc Natl Acad Sci U S A. 1993;90:10163–7. DOI: 10.1073/pnas.90.21.10163
12. Attie O, Bruno JF, Xu Y, Qiu D, Luft BJ, Qiu WG. Co-evolution of the outer surface protein C gene (ospC) and intraspecific lineages of *Borrelia burgdorferi* sensu stricto in the northeastern United States. Infect Genet Evol. 2007;7:1–12. DOI: 10.1016/j.meegid.2006.02.008
13. Dykhuizen DE, Baranton G. The implications of a low rate of horizontal transfer in *Borrelia.* Trends Microbiol. 2001;9:344–50. DOI: 10.1016/S0966-842X(01)02066-2
14. Guttman DS, Wang PW, Wang IN, Bosler EM, Luft BJ, Dykhuizen DE. Multiple infections of *Ixodes scapularis* ticks by *Borrelia burgdorferi* as revealed by single-strand conformation polymorphism analysis. J Clin Microbiol. 1996;34:652–6.
15. Casjens S, Palmer N, van Vugt R, Huang WM, Stevenson B, Rosa P, et al. A bacterial genome in flux: the twelve linear and nine circular extrachromosomal DNAs in an infectious isolate of the Lyme disease spirochete *Borrelia burgdorferi.* Mol Microbiol. 2000;35:490–516. DOI: 10.1046/j.1365-2958.2000.01698.x
16. Stevenson B, Miller JC. Intra- and interbacterial genetic exchange of Lyme disease spirochete erp genes generates sequence identity amidst diversity. J Mol Evol. 2003;57:309–24. DOI: 10.1007/s00239-003-2482-x
17. Zhang J-R, Norris SJ. Genetic variation of the *Borrelia burgdorferi* gene *vlsE* involves cassette-specific, segmental gene conversion. Infect Immun. 1998;66:3698–704.

18. Shimodaira H, Hasegawa M. Multiple comparisons of log-likelihoods with applications to phylogenetic inference. Mol Biol Evol. 1999;16:1114–6.
19. Wormser GP, Liveris D, Nowakowski J, Nadelman RB, Cavaliere LF, McKenna D, et al. Association of specific subtypes of *Borrelia burgdorferi* with hematogenous dissemination in early Lyme disease. J Infect Dis. 1999;180:720–5. DOI: 10.1086/314922
20. Caporale DA, Kocher TD. Sequence variation in the outer-surface-protein genes of *Borrelia burgdorferi.* Mol Biol Evol. 1994;11:51–64.
21. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 1994;22:4673–80. DOI: 10.1093/nar/22.22.4673
22. Rozas J, Rozas R. DnaSP version 3: an integrated program for molecular population genetics and molecular evolution analysis. Bioinformatics. 1999;15:174–5. DOI: 10.1093/bioinformatics/15.2.174
23. Jolley KA, Feil EJ, Chan MS, Maiden MC. Sequence type analysis and recombinational tests (START). Bioinformatics. 2001;17:1230–1. DOI: 10.1093/bioinformatics/17.12.1230
24. Sawyer S. Statistical tests for detecting gene conversion. Mol Biol Evol. 1989;6:526–38.
25. Smith JM. Analyzing the mosaic structure of genes. J Mol Evol. 1992;34:126–9.
26. Smith JM, Smith NH, O'Rourke M, Spratt BG. How clonal are bacteria? Proc Natl Acad Sci U S A. 1993;90:4384–8. DOI: 10.1073/pnas.90.10.4384
27. Seinost G, Dykhuizen DE, Dattwyler RJ, Golde WT, Dunn JJ, Wang IN, et al. Four clones of *Borrelia burgdorferi* sensu stricto cause invasive infection in humans. Infect Immun. 1999;67:3518–24.
28. Dykhuizen DE, Brisson D, Sandigursky S, Wormser GP, Nowakowski J, Nadelman RB, et al. The propensity of different *Borrelia burgdorferi* sensu stricto genotypes to cause disseminated infections in humans. Am J Trop Med Hyg. 2008;78:806–10.
29. Posada D, Crandall KA. MODELTEST: testing the model of DNA substitution. Bioinformatics. 1998;14:817–8. DOI: 10.1093/bioinformatics/14.9.817
30. Wormser GP, Brisson D, Liveris D, Hanincová K, Sandigursky S, Nowakowski J, et al. *Borrelia burgdorferi* genotype predicts the capacity for hematogenous dissemination during early Lyme disease. J Infect Dis. 2008;198:1358–64. DOI: 10.1086/592279
31. Liveris D, Wormser GP, Nowakowski J, Nadelman R, Bittker S, Cooper D, et al. Molecular typing of *Borrelia burgdorferi* from Lyme disease patients by PCR-restriction fragment length polymorphism analysis. J Clin Microbiol. 1996;34:1306–9.
32. Livey I, Gibbs CP, Schuster R, Dorner F. Evidence for lateral transfer and recombination in OspC variation in Lyme disease *Borrelia.* Mol Microbiol. 1995;18:257–69. DOI: 10.1111/j.1365-2958.1995.mmi_18020257.x
33. Ragan MA. Detection of lateral gene transfer among microbial genomes. Curr Opin Genet Dev. 2001;11:620–6. DOI: 10.1016/S0959-437X(00)00244-6
34. Wang G, Ojaimi C, Wu H, Saksenberg V, Iyer R, Liveris D, et al. Disease severity in a murine model of Lyme borreliosis is associated with the genotype of the infecting *Borrelia burgdorferi* sensu stricto strain. J Infect Dis. 2002;186:782–91. DOI: 10.1086/343043
35. Cromley EK, Cartter ML, Mrozinski RD, Ertel SH. Residential setting as a risk factor for Lyme disease in a hyperendemic region. Am J Epidemiol. 1998;147:472–7.
36. Brisson D, Dykhuizen DE. A modest model explains the distribution and abundance of *Borrelia burgdorferi* strains. Am J Trop Med Hyg. 2006;74:615–22.
37. Brisson D, Dykhuizen DE, Ostfeld RS. Conspicuous impacts of inconspicuous hosts on the Lyme disease epidemic. Proc Biol Sci. 2008;275:227–35. DOI: 10.1098/rspb.2007.1208

Address for correspondence: Dustin Brisson, Department of Biology, University of Pennsylvania, Leidy Laboratories 209, 433 S University Ave, Philadelphia, PA 19104-6018, USA; email: dbrisson@sas.upenn.edu

Astrovirus Encephalitis in Boy with X-linked Agammaglobulinemia

Phenix-Lan Quan, Thor A. Wagner, Thomas Briese, Troy R. Torgerson, Mady Hornig, Alla Tashmukhamedova, Cadhla Firth, Gustavo Palacios, Ada Baisre-De-Leon, Christopher D. Paddock, Stephen K. Hutchison, Michael Egholm, Sherif R. Zaki, James E. Goldman, Hans D. Ochs, and W. Ian Lipkin

Encephalitis is a major cause of death worldwide. Although >100 pathogens have been identified as causative agents, the pathogen is not determined for up to 75% of cases. This diagnostic failure impedes effective treatment and underscores the need for better tools and new approaches for detecting novel pathogens or determining new manifestations of known pathogens. Although astroviruses are commonly associated with gastroenteritis, they have not been associated with central nervous system disease. Using unbiased pyrosequencing, we detected an astrovirus as the causative agent for encephalitis in a 15-year-old boy with agammaglobulinemia; several laboratories had failed to identify the agent. Our findings expand the spectrum of causative agents associated with encephalitis and highlight unbiased molecular technology as a valuable tool for differential diagnosis of unexplained disease.

The economic cost of encephalitis is profound. Among the general population of western industrialized countries, the annual incidence of acute encephalitis is 7.3 cases per 100,000 persons (*1*). Although some persons recover from encephalitis without apparent sequelae, up to 71.0% experience lasting sequelae and up to 7.4% die (*1*,*2*). Khetsuriani et al. reported that each year in the United States alone, encephalitis is associated with ≈19,000 hospitalizations (average hospital stay 12 days), 1,400 deaths, and a cost of ≈$650 million for encephalitis-associated hospitalization (*1*).

Encephalitis is associated with a wide spectrum of infectious agents, including viruses, bacteria, fungi, and parasites (*3*). The most commonly implicated viruses are herpes simplex, varicella-zoster, Epstein-Barr, mumps, measles, and enteroviruses (*4*). Despite the use of various diagnostic methods (culture, molecular, immunohistochemical, or serologic), a causative agent is not identified for a high proportion of encephalitis cases (up to 75%) (*5*). This diagnostic failure may reflect the absence of a known agent or its molecular footprint at time of sampling, suboptimal specimen handling, lack of assay sensitivity, or presence of an unexpected or novel agent not considered in conventional assays. A better understanding of emerging and reemerging pathogens implicated in outbreaks of encephalitis (e.g., West Nile virus, Hendra virus, Nipah virus, Australian bat lyssavirus, and enterovirus 71) indicates an urgent need for novel tools for rapid differential diagnostic testing and surveillance (*6*,*7*).

The advent of unbiased molecular discovery technologies offers new opportunities to identify novel pathogens without the constraints imposed by assays selective for known or expected agents. We used unbiased high-throughput pyrosequencing to detect an astrovirus in a patient who died with unexplained encephalitis.

Materials and Methods

The Patient

In 2007, a 15-year-old boy with X-linked agammaglobulinemia (XLA) caused by a missense mutation (Thr35Pro) in the Bruton tyrosine kinase (*Btk*) gene was admitted to a

Author affiliations: Columbia University, New York, New York, USA (P.-L. Quan, T. Briese, M. Hornig, A. Tashmukhamedova, G. Palacios, A. Baisre-De-Leon, J.E. Goldman, W.I. Lipkin); University of Washington, Seattle, Washington, USA (T.A. Wagner, T.R. Torgerson, H.D. Ochs); Seattle Children's Hospital, Seattle (T.A. Wagner, T.R. Torgerson, H.D. Ochs); Pennsylvania State University, Pittsburgh, Pennsylvania, USA (C. Firth); Centers for Disease Control and Prevention, Atlanta, Georgia, USA (C.D. Paddock, S.R. Zaki); and 454 Life Sciences, Branford, Connecticut, USA (S.K. Hutchison, M. Egholm)

DOI: 10.3201/eid1606.091536

psychiatric facility in Seattle, WA, USA, because of suicidal and homicidal ideation, headache, memory loss, and ataxia. He had progressive cognitive decline, was unable to walk or communicate within 4 weeks of admission, became comatose, and died 71 days after admission.

Samples

Patient samples available for examination were fresh-frozen biopsy specimens of frontal cortex and postmortem tissues (brain stem, frontal lobe, kidney, liver, and spleen). Also available for immunohistochemical and neuropathologic examination were formalin-fixed postmortem samples of cerebral cortex, basal ganglia, and cerebellum from the patient and from other persons without encephalitis or brain inflammation.

Unbiased High-Throughput Sequencing

RNA (0.5 μg) from the frontal cortex biopsy specimen was treated with DNase I (Ambion DNA-*free*; Austin, TX, USA) and reverse transcribed by using Superscript II (Invitrogen, Carlsbad, CA, USA) with random octamer primers linked to an arbitrary defined 17-mer primer sequence (Eurofins MWG Operon, Huntsville, AL, USA). cDNA was treated with RNase H before random PCR amplification with a 9:1 mixture of a 17-mer random sequence primer and an octamer-linked 17-mer random sequence primer (*8*). Products with >70 bp were purified by using MinElute (QIAGEN, Valencia, CA, USA) and ligated to linkers for sequencing on a GSL FLX Sequencer (454 Life Sciences, Branford, CT, USA). After primers were trimmed and highly repetitive sequences eliminated, reads were clustered and assembled into contiguous fragments for comparison by BLAST (*9*) with the GenBank database at the nucleotide and translated amino acid levels. We used custom software applications written in Perl (BioPerl 5.8.5) and programs available through the GreenePortal website (http://tako.cpmc.columbia.edu/Tools/).

Rapid Amplification of cDNA Ends

Virus-specific primers for 5′ rapid amplification of cDNA ends were 5′-ACGCTCAAGCTCATGTCT GA-3′ for reverse transcription, 5′-GATGAGCGCTCTG TTTTCAA-3′ for the first PCR with UAP (Invitrogen), and 5′-TCAACCTCAACCCAATCGTT-3′ for the second PCR with AUAP (Invitrogen). Primers for 3′ rapid amplification of cDNA ends were 5′-CTCGCAAGGCATATGAGTGA-3′ and UAP (Invitrogen) for the first PCR and 5′-CTGGCTTGGTTGCAAAAGTT-3′ and AUAP (Invitrogen) for the second PCR. Final concentration of all primers was 0.2 mmol/L. PCR products were purified with QIAquick PCR Purification kits (QIAGEN) and directly dideoxy sequenced in both directions.

Quantitative Real-Time PCR

Reactions were performed in an ABI 7300 cycler by using SYBR-Green Master Mix (Applied Biosystems, Foster City, CA, USA) with primers 5′-CCATGTGTCTGATGGTGCTG-3′ and 5′-TTGATC ATATCAATCACCAAATCA-3′ in a volume of 25 μL. Cycling conditions were 50°C for 2 min and 95°C for 10 min, followed by 45 cycles at 95°C for 15 s and 60°C for 1 min.

Cloning, Expression, and Purification of VP29

The VP29 variable region of the capsid gene (nt 5231–6562) was amplified by PCR with forward VP29-*Kpn*I primer 5′-CGGGGTACCTGCTAGGTAAATCAGCAA ATACT-3′ and reverse VP29-XhoI primer 5′-CCGCTCG AGTTGATCATATCAATCACCAAATCA-3′. Histidine 6–tagged VP29 was expressed in *Escherichia coli* (Gateway vector pDEST 17; Invitrogen) and purified by using 1 mL Histrap column (GE Healthcare Life Sciences, Piscataway, NJ, USA) in denaturing binding buffer (20 mmol/L Tris-HCl [pH 8], 6 M guanidine, 0.5 M NaCl, 20 mmol/L imidazole), washed with 5 column volumes of denaturing binding buffer followed by 5 column volumes of denaturing wash buffer (20 mmol/L Tris-HCl [pH 8], 6 M urea, 0.5 M NaCl, 20 mmol/L imidazole), and eluted in denaturing elution buffer (20 mmol/L Tris-HCl [pH 8], 6 M urea, 0.5 M NaCl, 0.5 M imidazole). Peak fractions were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, pooled, and dialyzed against 20 mmol/L Tris-HCl (pH 8) and 3 M urea. Purification of VP29 was confirmed by Western blot with anti-histidine antibody (Genscript, Piscataway, NJ, USA) and mass spectroscopy of a trypsin-digested purified sample.

Rabbit antiserum against VP29 was generated by injecting rabbits with recombinant VP29 (3 injections of 0.5 mg each in Freund complete/incomplete adjuvant). Immunoglobulin (Ig) G was purified by using protein A-Sepharose (Lampire Biologic Laboratories, Pipersville, PA, USA).

Immunohistochemistry and Immunofluorescence

Formalin-fixed, paraffin-embedded brain sections were heated at 56°C for 10 min, deparaffinized in a citrus clearing agent, and rehydrated through decreasing concentrations of ethanol. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 15 min. Heat-induced antigen retrieval was performed in Trilogy antigen retrieval solution (Cell Marque, Rocklin, CA, USA) for 20 min at 95°C, after which the solution was cooled for 30 min. After blocking with Background Sniper solution (BS966H; Biocare Medical, Concord, CA, USA) for 10 min at room temperature, sections were incubated with primary antibodies overnight at 4°C. Slides were washed with

wash buffer (Dako, Carpinteria, CA, USA) and incubated with appropriate secondary antibody for either immunohistochemical or immunofluorescence examination.

For immunohistochemical examination, Vectastain Elite ABC kits (PK-6101, AK5002; Vector Laboratories, Burlingame, CA, USA) were used to develop diaminobenzidine tetrahydrochloride chromogen. Tissue sections were incubated with either biotinylated goat antirabbit or biotinylated horse antimouse IgG (1:200, Vector Laboratories) for 1 h at 37°C, after which ABC reagents were added. Sections were counterstained with hematoxylin and dehydrated with 100% ethanol. Sections were affixed to slides by using Permount histologic mounting medium (Fisher, Fair Lawn, NJ, USA), and coverslips were placed.

For immunofluorescence assays, sections were incubated with secondary Cy3-conjugated goat antirabbit antibody or Cy2 goat antimouse antibody (1:200, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 h at room temperature. Sections were mounted on slides by using ProLong Gold antifade reagent with DAPI (Invitrogen). Images were viewed on a Zeiss LSM 510 multiphoton confocal microscope and analyzed by using AIM Software (Carl Zeiss GmbH, Thornwood, NY, USA). Primary antibodies used were mouse antiglial fibrillary acidic protein cocktail (1:100, BD Bioscience Pharmagen, San Jose, CA, USA), mouse anti-CD3 (1:350, Dako), and mouse anti-CD68 (1:50, Dako).

Phylogenetic Analysis

Representative capsid gene (open reading frame 2) sequences were downloaded from GenBank, and aligned with the capsid gene sequence of the novel astrovirus by using Se-Al version 2.0a11 (http://tree.bio.ed.ac.uk/software/seal/). A Bayesian phylogenetic tree based on the full-length amino acid alignment of the capsid protein was generated by using MrBayes version 3 (*10*) and the WAG amino acid transition model. Two independent runs were allowed to converge over 10 million generations, burn-in setting was 10%, and every 1,000 generations were sampled. The robustness of the resultant phylogenetic analysis was assessed by using Bayesian posterior probability values.

Results

During the patient's hospitalization, Gram stain, bacterial cultures, PCR, and cryptococcal antigen tests of 2 cerebrospinal fluid samples performed on days 6 and 12 after hospitalization failed to detect any agents that might be implicated in disease (Tables 1–4). Examination of a frontal cortex biopsy found diffuse astrogliosis and microgliosis of gray and white matter, perivascular and parenchymal CD3+ T-cell infiltrates, neuronal loss, and axonal swelling. No Negri bodies, Cowdry inclusions, or evidence of prion disease were found. No infectious agents were detected by electron microscopy or immunohistochemistry when a panel of antiserum for detection of herpes simplex virus, polyomavirus, and adenovirus was used. Bacterial and fungal cultures, broad-based 16S rRNA PCR, viral culture, and PCR for multiple viruses were negative (Tables 2–4).

Histologic examination of postmortem brain specimens showed diffuse neuronal loss, cortical thinning, and vacuolation of the gray matter of the cortices and deep nuclei (Figure 1, panels A, B) accompanied by astrogliosis in cortex and subcortical white matter (Figure 1, panels C, D). $CD3^+$ T-lymphocytes were identified in the perivascular spaces, infiltrating the parenchyma (Figure 1, panel E), and in microglial nodules (Figure 1, panel F). No $CD20^+$ B cells were noted. The white matter showed vacuolation and myelin clumps consistent with myelin degeneration (Figure 1, panel G), axonal loss (Figure 1, panel H), and microgliosis (Figure 1, panel I). Microcalcifications were seen in the globus pallidus (Figure 1, panel J).

Because the histologic findings were consistent with virus infection and because molecular, serologic, and morphologic methods failed to identify an infectious agent, we pursued unbiased pyrosequencing of RNA from the frontal cortex biopsy specimen (*11*,*12*). From 2 cDNA libraries that mapped either to human (host) sequences or were uninformative in GenBank searches at the nucleotide

Table 1. Results of testing of cerebrospinal fluid from 15-year-old boy with X-linked agammaglobulinemia and astrovirus encephalitis*

Hospitalization day	Nucleated cells, cells/mm^3 (ref <5)	Erythrocytes, cells/mm^3	Neutrophils, %	Protein, mg/dL (ref <40)	Glucose, mg/dL (ref 45%–60% of glucose in serum)	Immunoglobulin G, mg/dL (ref 0.8–7.7)
6	4	2	2	76	50	ND
12	6	107	1	48	57	3.7

*Ref, reference range; ND, not done.

Table 2. Staining and culture results for 15-year-old boy with X-linked agammaglobulinemia and astrovirus encephalitis*

Sample	AFB stain	Gram stain	Cryptococcal antigen	Bacterial culture†	Fungal culture	Viral culture
Cerebrospinal fluid	ND	Neg	Neg	Neg	ND	ND
Brain biopsy	Neg	ND	ND	Neg	Neg	Neg

*AFB, acid-fast bacteria; ND, not done; neg, negative.
†Includes *Mycobacteria* spp.

Table 3. PCR results for bacteria, fungi, parasites, and DNA viruses in 15-year-old boy with X-linked agammaglobulinemia and astrovirus encephalitis*

Sample	Bacteria (16S)	Fungi	*Toxoplasma gondii*	Adenovirus	BK virus	CMV	Epstein-Barr virus	JC virus	Parvovirus B19
Cerebrospinal fluid	ND	ND	ND	ND	Neg	Neg	Neg	Neg	ND
Brain	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg

*CMV, cytomegalovirus; ND, not done; neg, negative.

Table 4. PCR results for RNA viruses in 15-year-old boy with X-linked agammaglobulinemia and astrovirus encephalitis*

Sample	Coronavirus	Enterovirus	HMPV	HPIV 1 to 4	HSV 1 or 2	Influenza virus†	Rhinovirus	RSV	VZV	WNV
Cerebrospinal fluid	ND	Neg	Neg	ND	Neg	ND	ND	ND	Neg	ND
Brain	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg

*HMPV, human metapneumovirus; HPIV, human parainfluenza virus; HSV, herpes simplex virus; RSV, respiratory syncytial virus; VZV, varicella zoster virus; WNV, West Nile virus; ND, not done; neg, negative.
†Type A or B.

level (BLASTn), we obtained an average of 102,000 sequence fragments with a mean length of 180 nt. However, analysis at the deduced amino acid level (BLASTx) identified 12 sequences homologous to astroviruses (Figure 2; Table 5). The complete 6,584-nt genomic sequence of the virus (GenBank accession no. GQ89199) was determined in a 2-step procedure in which gaps between sequence fragments identified through pyrosequencing were filled by PCR amplification and sequencing, and the genomic termini were cloned and sequenced by rapid amplification of cDNA ends (Figure 2; Table 6). Phylogenetic analyses placed the virus, tentatively named human astrovirus Puget Sound (HAstV-PS), apart from known human astroviruses. Analysis of the full-length capsid protein sequence showed HAstV-PS in a clade together with ovine, mink, and bat astroviruses (Figure 3); analyses of polymerase or protease protein sequences gave comparable results (data not shown).

Real-time PCR indicated that viral load was higher in the biopsy specimen (1.53×10^7 RNA molecules per reaction) than in postmortem specimens (cerebellum [5.39×10^2], frontal lobe [1.14×10^2], and brain stem [1.92×10^4]).

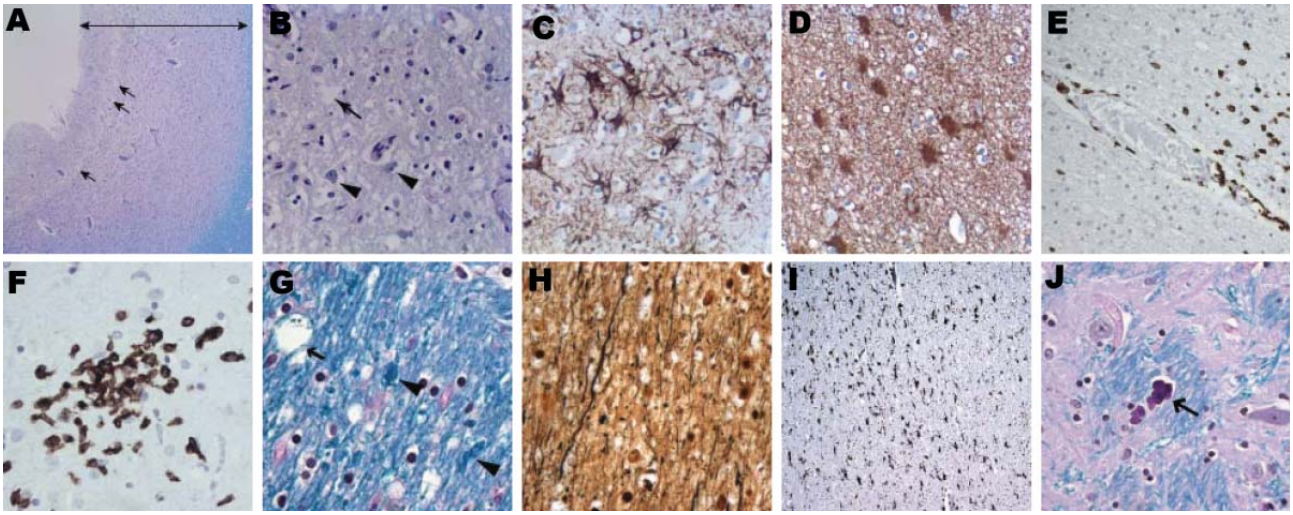


Figure 1. Histologic findings from brain of 15-year-old boy with X-linked agammaglobulinemia and astrovirus encephalitis. A) Frontal cortex with cortical thinning (double-headed arrow) and vacuolation (arrows) (Luxol fast blue stain with periodic acid–Schiff method [LFB/PAS], original magnification ×10). B) Frontal cortex with vacuolation (arrow) and rare residual neurons (arrowheads) (LFB/PAS, original magnification ×50). C) Marked astrogliosis in the frontal cortex (glial fibrillary acidic protein [GFAP] immunostain, original magnification ×100). D) White matter with marked astrogliosis (GFAP immunostain, original magnification ×100). E) Penetrating artery with abundant CD3+ T-cells in the perivascular space and adjacent brain parenchyma ($CD3^+$ immunostain, original magnification ×25). F) $CD3^+$ T-cells as part of microglial nodules ($CD3^+$ immunostain, original magnification ×100). G) White matter in the internal capsule showing myelin clumps (arrowheads) and vacuolation (arrow) (LFB/PAS, original magnification ×100). H) Loss of axons in the internal capsule (Bielschowsky nerve fiber silver stain, original magnification ×100). I) Internal capsule with marked microgliosis ($CD68^+$ immunostain, original magnification ×40). J) Microcalcifications (arrow) in the globus pallidus (LFB/PAS, original magnification ×100). All paraffin sections were counterstained with hematoxylin.

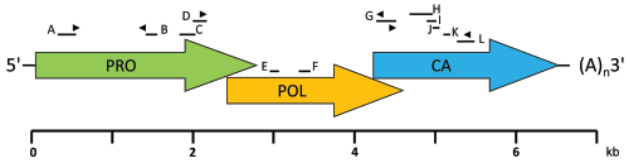


Figure 2. Schematic genome organization of human astrovirus Puget Sound (HAstV-PS). Arrows represent the 3 open reading frames of the 6,584-nt single-strand, positive-sense genome. Bars above the schematic indicate the 12 contiguous fragments (contigs A–L) generated through unbiased high-throughput sequencing. PCR primers for amplification across sequence gaps were designed based on the unbiased high-throughput sequencing data, and the draft genome was resequenced by overlapping PCR products that covered the entire genome except for terminal sequences. Genomic termini were characterized with 5′ and 3′ rapid amplification of cDNA ends kits (Invitrogen, Carlsbad, CA, USA). Arrowheads indicate primer locations. PRO, protease; POL, polymerase; CA, capsid; $(A)_n$, poly-A tail.

No viral RNA was found in postmortem kidney, liver, or spleen specimens.

Commercial antibodies to human astroviruses did not stain infected brain (data not shown). Therefore, polyclonal antibodies were generated by injecting rabbits with recombinant capsid protein of HAstV-PS. Indirect double-immunofluorescence staining of postmortem tissue sections demonstrated capsid protein in hypertropic astrocytes throughout the subcortical white matter and cortex (Figure 4, panel A). Astrocytes had swollen cell bodies and showed intense immunostaining of the glial fibrillary acidic protein. Indirect immunohistochemical staining of a frontal cortex brain biopsy specimen demonstrated intracytoplasmic capsid protein in only 1 cell; morphologic appearance was consistent with that of a neuron (Figure 4, panel B). No evidence of infection was found in oligodendrocytes or macrophages.

Discussion

The astrovirus identified in the central nervous system (CNS) of an immunocompromised patient with XLA and encephalitis was discovered through unbiased high-throughput pyrosequencing after conventional methods failed to identify an infectious agent. The astrovirus infection was confirmed by specific PCR and antigen detection. To prove causation according to the Koch postulates, the infectious agent must be propagated and must reproduce disease in a previously unexposed host (*13*); to prove causation according to Rivers, a specific humoral immune response to infection must be found (*14*). Because we have not been able to grow the astrovirus in culture and because persons with XLA do not generate specific antibodies, these criteria have not been met. Nonetheless, the neuropathologic findings consistent with viral encephalitis in conjunction with the high viral load found in the CNS suggest a causative association between HAstV-PS and disease. The most prominent histologic lesions in the brain consisted of severe neuronal degeneration, hypertrophic astrocytes, and infiltration by T-lymphocytes and macrophages. Although we cannot exclude prominent neuronal infection earlier in the course of infection, immunohistochemical and immunofluorescence studies localized astroviral protein mainly in astrocytes.

Astrocytes are essential to neuronal function and viability (*15*); they are critical for maintenance of the blood–brain barrier; they provide axon guidance during development and structural support to neural elements; and they are involved in CNS homeostasis as regulators of extracellular glutamate, ionic environment, and pH (*16*). Astrocyte dysfunction is implicated in the pathogenesis of acute and chronic CNS disorders (*16*,*17*). Recent data indicate that infected astrocytes play a role in the pathogenesis of HIV-associated dementia, a neurodegenerative disorder (*18*). The essential features of this disorder are cognitive and motor impairment, speech problems, and behavioral changes.

Table 5. Genomic location of HAstV-PS contigs identified by 454 pyrosequencing and their relationship to known astroviruses*

Contiguous fragment	Genome position in HAstV-PS	Highest amino acid identity, %	GenBank accession no.†	Astrovirus species
A	238–504	52	NP_795334	Mink
B	1765–1839	64	NP_795334	Mink
C	2012–2184	67	NP_795334	Mink
D	2308–2466	42	NP_059945	Ovine
E	2763–2945	48	AAO32082	Mink
F	3360–3728	65	NP_059945	Ovine
G	4558–4679	65	NP_795336	Mink
H	4558–5140	70	NP_059946	Ovine
I	4708–4944	72	NP_795336	Mink
J	4801–5110	72	NP_059946	Ovine
K	4819–4929	76	NP_059946	Ovine
L	5104–5331	70	NP_059946	Ovine

* HAstV-PS, human astrovirus Puget Sound.
†Accession number for BLASTX (www.ncbi.nlm.nih.gov/blast/Blast.cgi) match with highest amino acid identity (GenBank database December 2007).

Table 6. Primers used in cloning and sequencing the human astrovirus Puget Sound genome

Sequence (5′ → 3′)	Position
TCATGGAGCGCTCATACAAG	38–57
GTGTAAGCGAAGCCAAAAGC	815–796
GATTGGGTTGAGGTTGATGC*	340–359
TCCAGTGGTGGCTTGATGTA	1797–1778
GAAGTGGGATGGTGGAGTTG	666–685
TCCAGAAATCCGATTCAACC	1314–1295
TGGAGCAGTTGTTGGTGAAA	1215–1234
TTCTCAAGGTCTATCTCCCTTTGT	1877–1854
TACATCAAGCCACCACTGGA*	1778–1797
TCCGCCTCTCTAAGCACTTC	2159–2140
TACATCAAGCCACCACTGGA	1778–1797
TTGGATTGACTCCCTCAAGC	2533–2514
ATCACCACAAGAGGCGTAGG*	2039–2058
CCAGTAACTGCTGATGGACCAACAA	4649–4625
CGTGATTTGCAGGAATACCA	2446–2465
CCCATACGTGTCAGGGTTCT	3343–3324
TCTGGAGAGAGGCCTGATGT	3159–3178
TCTCTCTTGACCCACATCCC	3865–3846
CCTCTGGGCAAATATCCACA	3625–3644
TAGGGATATGCGGAAAACAGA	4671–4651
GTTTGTGGCGCTTGAAAAGT	4583–4602
TTGCGTGGAAGAAAGTGTTG	5365–5345
TTGTTGGTCCATCAGCAGTTACTGG*	4 625–4649
AACTTTTGCAACCAAGCCAG	5175–5156
CTGGCTTGGTTGCAAAAGTT	5156–5175
GAGTATTGTGCCCGCAAAGT	6041–6022
CGGCTACACCAGCCTACATT	5833–5852
CAGCACCATCAGACACATGG	6442–6423

*Primer pairs designed for amplification between ultra–high-throughput sequencing contiguous fragments (Figure 2); primer pairs without asterisk were applied for resequencing of the draft genome.

Similar to findings for the patient described here, HIV-associated dementia is characterized by infiltration of macrophages into the CNS, gliosis, pallor of myelin sheaths, and loss of neurons (*19*).

Astroviruses have not previously been associated with CNS disease. They are nonenveloped, single-stranded RNA viruses, are typically transmitted by the fecal–oral route, and cause mild gastrointestinal disease. Antibodies to human astrovirus-1 have been found in >90% of the human population (*20*). Immunocompromised persons are prone to astrovirus infections of the gastrointestinal tract (*21*). Postmortem analysis of the gastrointestinal tract of the patient described in this article found no evidence of astrovirus infection (data not shown); however, we cannot exclude the possibility of a gastrointestinal infection that cleared before the patient died.

The fact that the patient had XLA may explain dissemination of the astrovirus to the CNS. XLA is a primary immunodeficiency disorder caused by mutations in the *Btk* gene, which results in absence of B lymphocytes and serum immunoglobulins (*22*). Several recent reports demonstrate that Btk is required for Toll-like receptor 8–mediated production of interleukin-6 and production of tumor necrosis factor-α by peripheral blood mononuclear cell–derived dendritic cells (*23*,*24*). Hence, Btk deficiency may impair innate immune responses after a person is infected with single-stranded RNA viruses known to cause fatal CNS infection in those with XLA (*25*,*26*), such as enteroviruses, and now, potentially, astroviruses.

The source of infection for the patient described here remains unknown. Exposure to mink was a potential source,

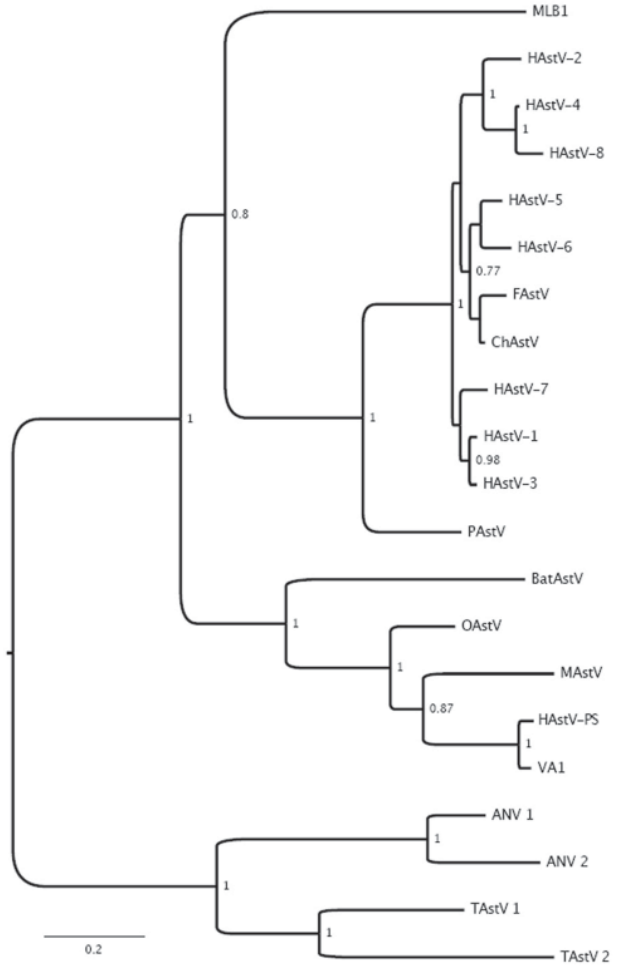


Figure 3. Phylogenetic analysis of full-length capsid protein sequences showing the relationship between human astrovirus Puget Sound (HAstV-PS) identified in brain of 15-year-old boy with X-linked agammaglobulinemia and encephalitis and other astroviruses. GenBank accession numbers in parentheses: MLB1 (FJ22245), VA1 (FJ973620), HAstV-1 (AB000295), HAstV-2 (L06802), HAstV-3 (DQ630763), HAstV-4 (AB025803), HAstV-5 (U15136), HAstV-6 (Z46658), HAstV-7 (Y08632), HAstV-8 (Z66541), MAstV (AY179509), OAstV (NC_002469), BatAstV (FJ571074), FAstV (AF056197), ChAstV (EU650331), PAstV (Y15938), ANV 1 (AB033998), ANV 2 (AB046864), TAstV 1 (Y15936), and TAstV 2 (AY769615). Bayesian posterior probability values >75% are shown at respective nodes. FAstV, feline astrovirus; ChAstV, cheetah astrovirus; PAstV, porcine astrovirus; OAstV, ovine astrovirus; MAstV, mink astrovirus; ANV, avian nephritis virus; TAstV, turkey astrovirus. Scale bar indicates number of amino acid substitutions per site.

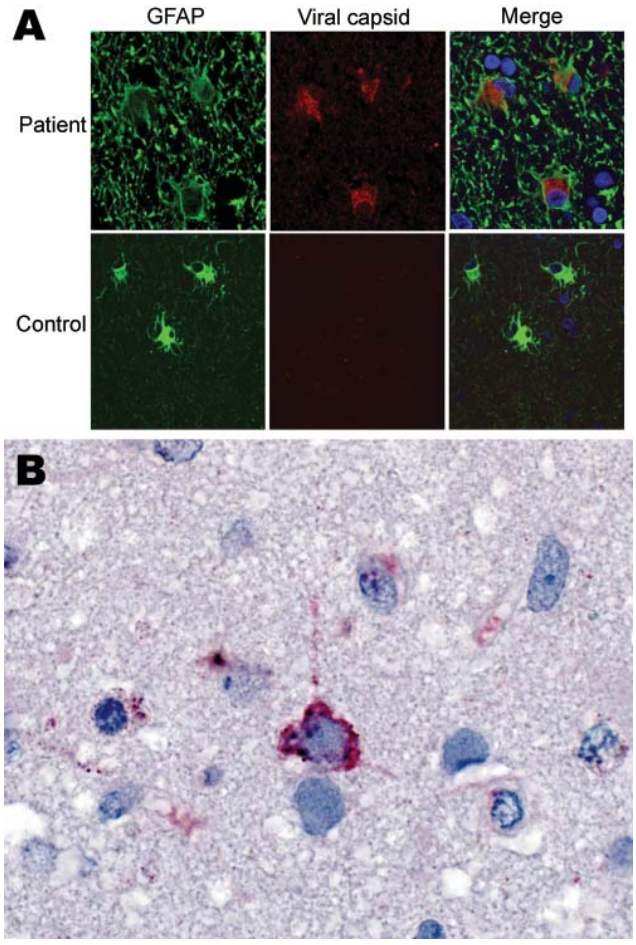


Figure 4. Immunofluorescence and immunohistochemical analyses with human astrovirus Puget Sound capsid antibodies. A) Indirect double immunofluorescence–stained, formalin-fixed, paraffin-embedded tissue sections from 15-year-old boy with X-linked agammaglobulinemia and astrovirus encephalitis and a control with astrogliosis not caused by astrovirus infection. The sections were stained for the astrocyte marker glial fibrillary acidic protein (GFAP, green) and for viral capsid protein (rabbit serum 1:1,000, red). Viral capsid protein is present in hypertropic astrocytes throughout the subcortical white matter and cortex; astrocytes have swollen cell bodies with intense GFAP immunostaining. Blue signal (DAPI) indicates nuclear counterstaining. Original magnification ×100. B) Immunohistochemical localization of viral antigen in a frontal cortex biopsy specimen. Immunoalkaline phosphatase stain with viral capsid antibodies (rabbit serum 1:1,000) and naphthol-fast red with hematoxylin counterstain. Original magnification ×158.

suggested by the phylogenetic relationship of HAstV-PS to mink astroviruses and the proximity of the patient's residence to a mink farm. Another possible source was the patient's monthly treatment with intravenous immunoglobulin. Several reports describe progressive neurodegeneration of unknown cause in immunosuppressed patients who received long-term intravenous immunoglobulin therapy (*26*,*27*). Some of these patients had neuropathologic findings similar to those reported here (*26*,*27*). Intravenous immunoglobulin preparations from 5 companies (Vivaglobin [CSL Behring GmbH, Marburg, Germany], Carimune [CSL Behring GmbH], Gammagard [Baxter, Westlake Village, CA, USA], Gamimune N [Bayer Healthcare Pharmaceuticals, West Haven, CT, USA], Flebogamma [Instituto Grifols, S.A. Barcelona, Spain]) were negative for the newly identified astrovirus, according to ELISA and PCR (data not shown).

The recent finding of an astrovirus closely related to HAstV-PS in fecal samples from children from Virginia with acute gastroenteritis (*28*) suggests that these novel viruses are circulating widely in humans across the United States. They should be considered in the differential diagnosis of encephalitis, particularly for immunosuppressed patients.

Despite extensive microbiologic investigation, the causes of up to 75% of encephalitis cases remain elusive. These undiagnosed cases pose a challenge for clinical medicine and public health and underscore the need to invest in developing new methods for investigating these debilitating, frequently fatal, disorders. To address the challenge of unexplained encephalitis, public health practitioners and diagnosticians need a more comprehensive armamentarium and methods that would enable them to discover new and unexpected pathogens associated with encephalitis. Our findings emphasize the value of unbiased pyrosequencing as a powerful tool for diagnosing the cause of encephalitis and the need to consider astroviruses as CNS pathogens, particularly in immunosuppressed persons. Early recognition of the causative agent of unexplained encephalitis cases will enable specific interventions that reduce illness and death and facilitate the recognition of outbreaks that threaten public health.

Acknowledgments

We are grateful to Eric Brouzes and Katrina Ciraldo for providing critical comments and to Raj Kapur for providing clinical information and brain tissues. We thank Alexandra Petrosov for excellent experimental assistance; Sean Conlan, Omar Jabado, and Craig Street for help with bioinformatics analyses; and Karen Chave and Marcella Powell for expression of VP29 recombinant protein.

This study was supported by National Institutes of Health grants AI57158 (Northeast Biodefense Center to W.I.L.), AI070411, EY017404-02; Google.org; and the Department of Defense.

Dr Quan is an associate research scientist at the Center for Infection and Immunity in the Mailman School of Public Health of Columbia University. Her research focuses on emerging zoonoses, pathogen surveillance, and discovery of emerging pathogens.

References

1. Khetsuriani N, Holman RC, Anderson LJ. Burden of encephalitis-associated hospitalizations in the United States, 1988–1997. Clin Infect Dis. 2002;35:175–82. DOI: 10.1086/341301
2. Jmor F, Emsley HC, Fischer M, Solomon T, Lewthwaite P. The incidence of acute encephalitis syndrome in western industrialised and tropical countries. Virol J. 2008;5:134. DOI: 10.1186/1743-422X-5-134
3. Glaser CA, Honarmand S, Anderson LJ, Schnurr DP, Forghani B, Cossen CK, et al. Beyond viruses: clinical profiles and etiologies associated with encephalitis. Clin Infect Dis. 2006;43:1565–77. DOI: 10.1086/509330
4. Khetsuriani N, Anderson LJ. Epidemiology of viral encephalitis. In: Reiss CS, Editor. Neurotropic viral infections. Cambridge (UK): Cambridge University Press; 2008. p. 315–33.
5. Glaser CA, Gilliam S, Schnurr D, Forghani B, Honarmand S, Khetsuriani N, et al. In search of encephalitis etiologies: diagnostic challenges in the California Encephalitis Project, 1998–2000. Clin Infect Dis. 2003;36:731–42. DOI: 10.1086/367841
6. Tyler KL. Emerging viral infections of the central nervous system: part 1. Arch Neurol. 2009;66:939–48. DOI: 10.1001/archneurol.2009.153
7. Tyler KL. Emerging viral infections of the central nervous system: part 2. Arch Neurol. 2009;66:1065–74. DOI: 10.1001/archneurol.2009.189
8. Palacios G, Quan PL, Jabado OJ, Conlan S, Hirschberg DL, Liu Y, et al. Panmicrobial oligonucleotide array for diagnosis of infectious diseases. Emerg Infect Dis. 2007;13:73–81. DOI: 10.3201/eid1301.060837
9. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990;215:403–10.
10. Ronquist F, Huelsenbeck JP. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics. 2003;19:1572–4. DOI: 10.1093/bioinformatics/btg180
11. Cox-Foster DL, Conlan S, Holmes EC, Palacios G, Evans JD, Moran NA, et al. A metagenomic survey of microbes in honey bee colony collapse disorder. Science. 2007;318:283–7. DOI: 10.1126/science.1146498
12. Palacios G, Druce J, Du L, Tran T, Birch C, Briese T, et al. A new arenavirus in a cluster of fatal transplant-associated diseases. N Engl J Med. 2008;358:991–8. DOI: 10.1056/NEJMoa073785
13. Koch R. Über bakteriologische Forschung Verhandlung des X Internationalen Medichinischen Congresses, Berlin, 1890,1,35. August Hirschwald, Berlin [in German]. Xth International Congress of Medicine, Berlin, Germany; 1891.
14. Rivers TM. Viruses and Koch's postulates. J Bacteriol. 1937;33:1–12.
15. Rossi D, Volterra A. Astrocytic dysfunction: insights on the role in neurodegeneration. Brain Res Bull. 2009 Jul 22.
16. Volterra A, Meldolesi J. Astrocytes, from brain glue to communication elements: the revolution continues. Nat Rev Neurosci. 2005;6:626–40. DOI: 10.1038/nrn1722
17. De Keyser J, Mostert JP, Koch MW. Dysfunctional astrocytes as key players in the pathogenesis of central nervous system disorders. J Neurol Sci. 2008;267:3–16. DOI: 10.1016/j.jns.2007.08.044
18. Churchill MJ, Wesselingh SL, Cowley D, Pardo CA, McArthur JC, Brew BJ, et al. Extensive astrocyte infection is prominent in human immunodeficiency virus–associated dementia. Ann Neurol. 2009;66:253–8. DOI: 10.1002/ana.21697
19. Gray F, Adle-Biassette H, Chretien F, Lorin de la Grandmaison G, Force G, Keohane C. Neuropathology and neurodegeneration in human immunodeficiency virus infection. Pathogenesis of HIV-induced lesions of the brain, correlations with HIV-associated disorders and modifications according to treatments. Clin Neuropathol. 2001;20:146–55.
20. Koopmans MP, Bijen MH, Monroe SS, Vinje J. Age-stratified seroprevalence of neutralizing antibodies to astrovirus types 1 to 7 in humans in the Netherlands. Clin Diagn Lab Immunol. 1998;5:33–7.
21. Cubitt WD, Mitchell DK, Carter MJ, Willcocks MM, Holzel H. Application of electron microscopy, enzyme immunoassay, and RT-PCR to monitor an outbreak of astrovirus type 1 in a paediatric bone marrow transplant unit. J Med Virol. 1999;57:313–21. DOI: 10.1002/(SICI)1096-9071(199903)57:3<313::AID-JMV16>3.0.CO;2-A
22. Smith CIE, Satterthwaite AB, Witte ON. X-linked agammaglobulinemia: a disease of Btk tyrosine kinase. In: Ochs HD, Smith CIE, Puck JM, editors. Primary immunodeficiency diseases: a molecular and genetic approach. 2nd ed. New York: Oxford University Press; 2007. p. 279–303.
23. Doyle SL, Jefferies CA, Feighery C, O'Neill LA. Signaling by Toll-like receptors 8 and 9 requires Bruton's tyrosine kinase. J Biol Chem. 2007;282:36953–60. DOI: 10.1074/jbc.M707682200
24. Sochorova K, Horvath R, Rozkova D, Litzman J, Bartunkova J, Sediva A, et al. Impaired Toll-like receptor 8–mediated IL-6 and TNF-alpha production in antigen-presenting cells from patients with X-linked agammaglobulinemia. Blood. 2007;109:2553–6. DOI: 10.1182/blood-2006-07-037960
25. Winkelstein JA, Marino MC, Lederman HM, Jones SM, Sullivan K, Burks AW, et al. X-linked agammaglobulinemia: report on a United States registry of 201 patients. Medicine (Baltimore). 2006;85:193–202. DOI: 10.1097/01.md.0000229482.27398.ad
26. Ziegner UH, Kobayashi RH, Cunningham-Rundles C, Espanol T, Fasth A, Huttenlocher A, et al. Progressive neurodegeneration in patients with primary immunodeficiency disease on IVIG treatment. Clin Immunol. 2002;102:19–24. DOI: 10.1006/clim.2001.5140
27. Rudge P, Webster AD, Revesz T, Warner T, Espanol T, Cunningham-Rundles C, et al. Encephalomyelitis in primary hypogammaglobulinaemia. Brain. 1996;119:1–15. DOI: 10.1093/brain/119.1.1
28. Finkbeiner SR, Li Y, Ruone S, Conrardy C, Gregoricus N, Toney D, et al. Identification of a novel astrovirus (astrovirus VA1) associated with an outbreak of acute gastroenteritis. J Virol. 2009;83:10836–9.

Address for correspondence: W. Ian Lipkin, Center for Infection and Immunity, Mailman School of Public Health, Columbia University, 722 W 168th St, New York, NY 10032, USA; email: wil2001@columbia.edu

Oseltamivir-Resistant Influenza Viruses A (H1N1) during 2007–2009 Influenza Seasons, Japan

Makoto Ujike, Kozue Shimabukuro, Kiku Mochizuki, Masatsugu Obuchi, Tsutomu Kageyama, Masayuki Shirakura, Noriko Kishida, Kazuyo Yamashita, Hiroshi Horikawa, Yumiko Kato, Nobuyuki Fujita, Masato Tashiro, Takato Odagiri, and the Working Group for Influenza Virus Surveillance in Japan[1]

To monitor oseltamivir-resistant influenza viruses A (H1N1) (ORVs) with H275Y in neuraminidase (NA) in Japan during 2 influenza seasons, we analyzed 3,216 clinical samples by NA sequencing and/or NA inhibition assay. The total frequency of ORVs was 2.6% (45/1,734) during the 2007–08 season and 99.7% (1,477/1,482) during the 2008–09 season, indicating a marked increase in ORVs in Japan during 1 influenza season. The NA gene of ORVs in the 2007–08 season fell into 2 distinct lineages by D354G substitution, whereas that of ORVs in the 2008–09 season fell into 1 lineage. NA inhibition assay and M2 sequencing showed that almost all the ORVs were sensitive to zanamivir and amantadine. The hemagglutination inhibition test showed that ORVs were antigenetically similar to the 2008–09 vaccine strain A/Brisbane/59/2007. Our data indicate that the current vaccine or zanamivir and amantadine are effective against recent ORVs, but continuous surveillance remains necessary.

Influenza A and B viruses are major pathogens that represent a threat to public health with subsequent economic losses worldwide (*1*). Vaccination is the primary method for prevention; antiviral drugs are used mainly for prophylaxis and therapy. Currently, 2 classes of drugs, matrix 2 (M2) blockers and neuraminidase inhibitors (NAIs) are available, but M2 blockers such as amantadine and rimantadine are not commonly used because of the rapid generation of resistance and lack of efficacy against influenza B virus (*2–4*). The NAIs zanamivir and oseltamivir are widely used because of effects against influenza A and B viruses and a low frequency of resistance. NAI virus surveillance studies by several groups have demonstrated that <1% of viruses tested show naturally occurring resistance to oseltamivir as of 2007 (*5–10*), indicating limited human-to-human transmission of these viruses.

At the beginning of the 2007–08 influenza season, however, detection of a substantially increased number of oseltamivir-resistant influenza viruses A (H1N1) (ORVs) was reported, mainly in countries in Europe where the prevalence varies, with the highest levels in Norway (67%) and France (47%) (*11–14*). These viruses showed a specific NA mutation with a histidine-to-tyrosine substitution at the aa 275 position (N1 numbering, H275Y), conferring high-level resistance to oseltamivir. Most of these ORVs were isolated from NAI-untreated patients and retained similar ability of human-to-human transmission to oseltamivir-sensitive influenza viruses A (H1N1) (OSVs) (*10*,*15*). In response to public health concerns about ORVs, the World Health Organization (WHO) directed Global Influenza Surveillance Network laboratories to intensify NAI surveillance and announced regularly updated summaries of ORV data collected from each laboratory on its website (*16*). This site reported that the global frequency increased from 16% (October 2007–March 2008) to 44% (April 2008–September 2008) to 95% (October 2008–January 2009), indicating that ORVs have spread rapidly around the world.

Author affiliations: National Institute of Infectious Diseases, Tokyo, Japan (M. Ujike, K. Shimabukuro, K. Mochizuki, M. Obuchi, T. Kageyama, M. Shirakura, N. Kishida, K. Yamashita, M. Tashiro, T. Odagiri); and National Institute of Technology and Evaluation, Tokyo (H. Horikawa, Y. Kato, N. Fujita)

DOI: 10.3201/eid1606.091623

[1]Members of the Working Group for Influenza Virus Surveillance in Japan are listed at the end of this article.

Japan has the highest annual level of oseltamivir usage per capita in the world, comprising >70% of world consumption (*10*). Such high use of oseltamivir has raised concerns about emergence of OSVs with increased resistance to this drug. Moreover, in Japan, 2 recent influenza seasons were dominated by influenza viruses A (H1N1) (Figure 1). If a high prevalence of ORVs is observed, primary selection of oseltamivir treatment for influenza patients should be reconsidered. Thus, monitoring ORVs is a serious public health issue.

To estimate the frequency of ORVs and characterize these viruses, we analyzed 1,734 clinical samples isolated from the 2007–08 season and 1,482 isolates from the 2008–09 season by NA sequencing and/or NAI inhibition assay. The total frequencies were 2.6% in the 2007–08 season and 99.7% in the 2008–09 season, indicating that ORVs increased dramatically in Japan.

Materials and Methods

Virus Testing

Influenza sentinel clinics send clinical specimens to local public health laboratories for virus isolation. Several culture tissues, including MDCK, Caco-2, and LLC-MK2, are used for virus isolation. Without successful viral isolation, clinical specimens are analyzed directly. Influenza viruses were collected from all 47 prefectures in Japan for this study; 1,734 samples of influenza A (H1N1) were isolated during the 2007–08 season (September 2007–August 2008**)** and 1,482 samples of influenza A (H1N1) were isolated in the 2008–09 season (September 2008–April 2009). During the 2007–08 season, viruses were isolated primarily after December 2007. All influenza viruses A (H1N1) were subjected to full or partial (nt 615–1076) NA sequencing to detect H275Y substitution on the N1 NA protein. Representative influenza viruses A (H1N1), including ORVs and OSVs, were subjected to NA inhibition assay (number of tested viruses isolated during the 2007–08 and 2008–09 seasons was 306 and 58, respectively), full NA sequencing (891 and 83), hemagglutinin (HA) 1 sequencing (299 and 83), M2 sequencing (288 and 79), and hemagglutinin inhibition (HI) test (187 and 59).

Sequence Analysis

The phylogenetic tree of NA and HA1 genes was constructed by neighbor-joining methods. The phylogenetic tree was described by representative ORVs and OSVs isolated from several prefectures in Japan. Sequence information for isolates from other countries was obtained from the Global Initiative on Sharing Avian Influenza Data and the Los Alamos National Laboratory database. All amino acid positions in the phylogenetic tree were described by N1 numbering.

NA Inhibition Assay

The chemiluminescent NA inhibition assay was per-

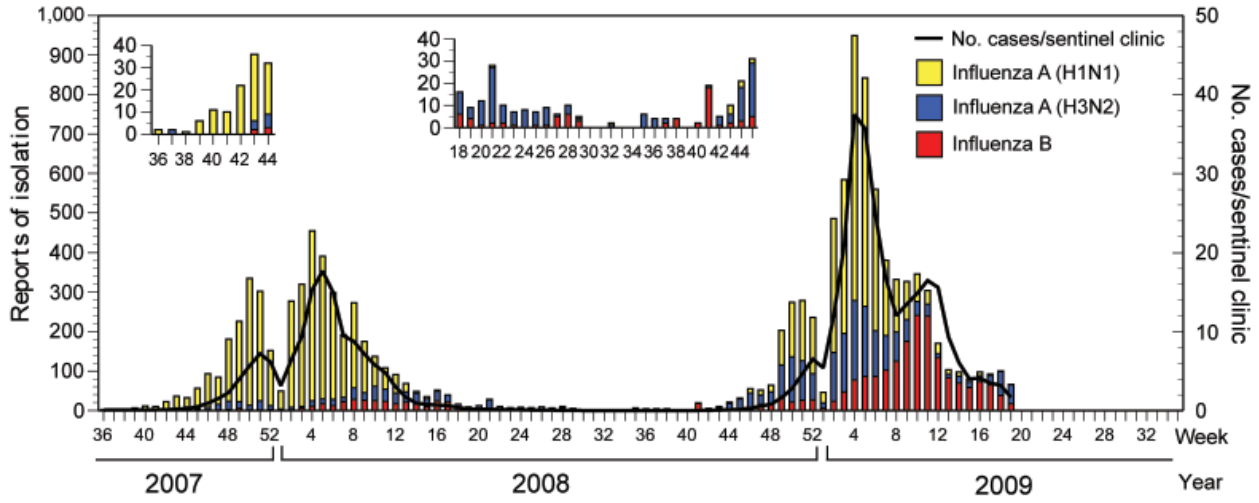


Figure 1. Weekly cases of influenza and isolation of influenza viruses in the 2007–08 and 2008–09 seasons in Japan (as of July 2, 2009). The National Epidemiologic Surveillance of Infectious Diseases (NESID) Network comprises the Ministry of Health, Labor and Welfare; the National Institute of Infectious Diseases; 76 local public health laboratories; ≈3,000 pediatric clinics; and 2,000 internal medical clinics. The NESID Network monitored influenza activity during the 2007–08 season (week 36, September 2007–week 35, August 2008) and 2008–09 season (week 36, September 2008–week 22, May 2009). Clinically diagnosed influenza-like cases were reported weekly by influenza sentinel clinics. **Boldface** line indicates weekly cases of influenza-like illness per influenza sentinel clinic (values shown in right bar). Bars indicate numbers of influenza A (H1N1) (yellow), A (H3N2) (blue), and B (red) isolates (values shown in left bar). Influenza activity started week 47 of 2007 and finished in week 14 of 2008 in the 2007–08 season and started week 49 of 2008 and finished in week 22 of 2009 in the 2008–09 season. Among all influenza isolates, influenza A (H1N1) consisted of 81% during 2007–08 and 49% during 2008–09. Seasonal influenza surveillance showed that influenza viruses A (H1N1) dominated the 2 recent influenza seasons in Japan.

formed by using the NA Star Kit (Applied Biosystems, Tokyo, Japan) with slight modifications of the instructions provided by the manufacturer. The final drug concentration ranged from 0.03 nmol/L to 6,500 nmol/L for oseltamivir and from 0.03 nmol/L to 12,500 nmol/L for zanamivir. Chemiluminescent light emission was measured by using an LB940 plate reader (Berthhold Technologies, Bad Wildbad, Germany). Drug concentrations required to inhibit NA activity by 50% (IC_{50}) were calculated by a 4-parameter method using MikroWin 2000 version 4 software (Mikrotek Laborsysteme GmbH, Overath, Germany).

Hemagglutination Inhibition Test

The HI test was performed to evaluate the reactivity of ferret antiserum against 2008–09 vaccine strain A/Brisbane/59/2009, as described by the WHO manual (*17*). Antiserum was treated by receptor-destroying enzyme II (Denka Seiken, Tokyo, Japan) and adsorbed with packed turkey erythrocytes before testing to prevent nonspecific reaction. A 0.5% suspension of turkey erythrocytes was used for the HI test. Viruses with >8-fold reduced HI titer to the homologous titer of A/Brisbane/59/2009 antiserum were regarded as antigenic variants.

Statistical Analysis

To determine the cutoff value between NAI-resistant (outlier) and -sensitive viruses, box-and-whisker plots were used. The cutoff value was defined as upper quartile + 5.0×interquartile range from the 25th to 75th percentile. In this study, ORVs with H275Y were excluded from the overall population for statistical analysis. Outliers were excluded from the calculation of mean values and standard deviations for IC_{50}.

Results

Geographic Distribution of ORVs during the 2007–08 and 2008–09 Influenza Seasons

To estimate the frequency of influenza A (H1N1) ORVs in each prefecture of Japan, 1,734 isolates during the 2007–08 season and 1,482 isolates during the 2008–09 season were collected from all prefectures and examined by NA sequencing to detect the H275Y mutation in NA protein. In the 2007–08 season, 45 viruses possessing H275Y mutation (total frequency of ORVs 2.6%; Figure 2, panel A) were observed in 10 prefectures, indicating that the frequency of ORVs was significantly lower than that in countries in Europe and the United States (*8*,*11*–*14*). In Tottori prefecture, however, 22 of 68 influenza viruses A (H1N1) tested possessed H275Y, showing a markedly higher frequency (32.4%) than that in other prefectures. In the 2008–09 season, however, ORVs were observed nationwide. Of 1,482 influenza viruses A (H1N1), 1,477 viruses possessed a H275Y mutation, for a total frequency of 99.7% (Figure 2, panel B). These data show that ORVs increased dramatically in Japan from the 2007–08 season to the 2008–09 season.

Genetic Analysis

Influenza viruses A (H1N1) during 2007–08 fell into either clade 2B, including the current vaccine strain A/

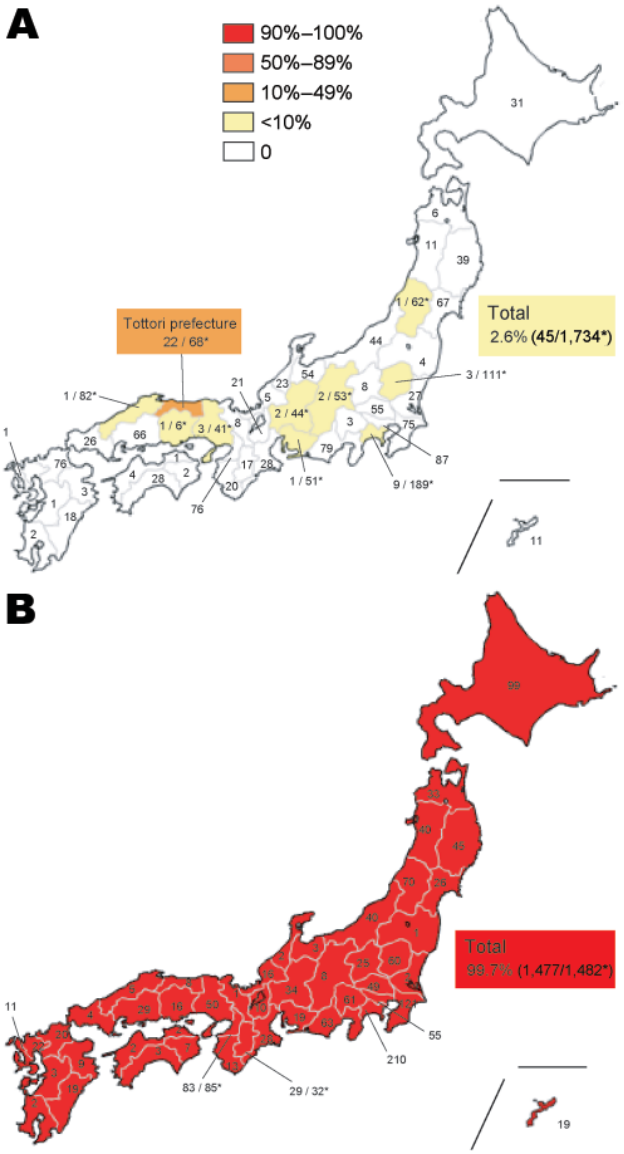


Figure 2. Geographic distribution of oseltamivir-resistant influenza viruses A (H1N1) (ORVs) with H275Y in Japan during the 2007–08 and 2008–09 seasons. The total number of influenza A (H1N1) isolates tested is described inside each prefecture. Total frequency in Japan was 2.6% (45/1,734) during the 2007–08 season, although a high frequency (32.4%) of ORVs was observed in Tottori prefecture (A). On the other hand, total frequency was 99.7% (1,477/1,482) during the 2008–09 season (B), indicating a drastic increase in ORVs in Japan from the 2007–08 season to the 2008–09 season. *Number of ORVs/number of subtype H1N1 isolates tested.

Brisbane/59/2007, or clade 2C, and almost all influenza viruses A (H1N1) during 2008–09 fell into clade 2B. Most ORVs with H275Y belong to clade 2B, which can be further divided into 2 distinct lineages by an aspartic acid to glycine substitution at aa 354: a Northern-Eu lineage sharing 354G, which was first isolated from countries in northern Europe and now represents most ORVs worldwide; and a Hawaii lineage sharing 354D, which was first detected in Hawaii and was rarely isolated in a few countries during the 2007–08 season (online Appendix Figure, www.cdc.gov/EID/content/16/6/926-appF.htm). In the 2007–08 season, of 45 ORVs, 1 virus (A/Yokohama/91/2007) isolated in November 2007 belonged to clade 2C, and 44 viruses fell into either the Hawaii lineage or Northern-Eu lineage. Conversely, in the 2008–09 season, all ORVs belonged to the Northern-Eu lineage, indicating that ORVs of the Northern-Eu lineage dominated in the 2008–09 season in Japan.

In the Hawaii lineage, OSVs genetically close to ORVs were observed. The NA gene of some ORVs had only 1 nucleotide difference from that of OSV counterparts (i.e., A/Tochigi/8/2008 and A/Tochigi/9/2008, A/Nagano/1100/2008 and A/Nagano/1071/2008, A/Yamagata/68/2008 and A/Yamagata/41/2008, respectively), and the other ORVs are also genetically close to OSVs from Japan (online Appendix Figure). These HA genes were also genetically identical or close together (online Appendix Figure), suggesting that almost all ORVs from Japan with the Hawaii lineage are derived from OSVs from Japan. On the other hand, in the Northern-Eu lineage, OSV counterparts were not observed, but foreign ORVs genetically close to ORVs from Japan were observed. During the 2007–08 season, the NA gene of ORVs from Japan was close to that of ORVs isolated from countries in Europe (i.e., A/Paris/0341/2007 and A/England/26/2008). During the 2008–09 season, the ORVs from Japan, which shared A189T on HA protein, were further divided into 4 subclades (C-1 to C-4) by common amino acid changes on HA and/or NA (online Appendix Figure). ORVs from Japan in C-2 and C-3 were genetically close to the ORVs isolated from North America or Hawaii (e.g., A/Memphis/03/2008 and A/Hawaii/19/2008), whereas ORVs in C-1, representing most influenza A (H1N1) viruses from the 2008–09 season in Japan, and ORVs in C-4 were close to ORVs isolated from South Africa and Australia in the Southern Hemisphere (e.g., A/Kenya/1432/2008 and A/Victoria/501/2008). All ORVs except C-3 were isolated before the emergence of ORVs from Japan in each subclade. These findings suggest that ORVs from Japan within a Northern-Eu lineage would not have emerged domestically but instead may have been introduced from various countries.

Antiviral Drug Susceptibility

Of the 364 viruses (306 isolates in the 2007–08 season and 58 isolates in the 2008–09 season) tested by NA inhibition assay, 101 possessed a H275Y substitution. With the NA inhibition assay, although precise IC_{50} values were calculated from a normal sigmoid curve (Figure 3, panels A and B), some viruses generated 2 types of unusual sigmoid curves (Figure 3, panels C and D) resulting from the mixed population of NAI-resistant and -sensitive viruses, as previously reported (*18*). Tentative IC_{50} values were calculated from type A curves (Figure 3, panel C) and included in overall statistical analysis, but values could not be calculated from type B curves (Figure 3, panel D). Later viruses were regarded as resistant candidates.

In the NA inhibition assay for oseltamivir, OSVs showed a mean IC_{50} ± SD of 0.10 ± 0.05 nmol/L (range 0.01–0.35 nmol/L), and ORVs had a mean ± SD IC_{50} of 67.7 ± 44.1 nmol/L (range 26.1–239.2 nmol/L), showing a reduction of >260-fold in susceptibility to oseltamivir. One OSV identified as a statistical outlier (cutoff IC_{50}, >0.40 nmol/L; upper quartile + 5.0× interquartile range) showed a D151E substitution on the NA protein (Table 1).

In the NA inhibition assay for zanamivir, statistical analysis showed that 341 viruses were regarded as the zanamivir-sensitive viruses, with a mean ± SD IC_{50} of 0.40 ± 0.26 nmol/L (range 0.01–1.92 nmol/L), and 16 viruses (10 ORVs and 6 OSVs) were identified as outliers (cutoff IC_{50}, >1.99 nmol/L) (Table 1). Seven viruses (2 ORVs and 5 OSVs) were regarded as resistant candidates from curve fit patterns. NA-sequencing for these 23 viruses (12 ORVs and 11 OSVs) showed 2 types of amino acid changes on the NA protein. One virus, A/Tottori/16/2008 (OSV), possessed a Q136K substitution, showing a high IC_{50} (41.89 nmol/L), and 19 of the other 22 viruses displayed an amino acid change G, N, or V at the D151 position (Table 1). These data suggest that D151 changes have a substantial effect on sensitivity to zanamivir (and oseltamivir). Moreover, A/Tottori/44/2008 with H275Y and D151D/G substitutions conferred high-level resistance to both NAIs (Figure 3, panels A and B). However, a recent study reported that a D151E change was detected only after virus propagation in cell culture, but not in the original clinical specimen (*19*), suggesting a possible role of cell culture in selecting these D151 variant viruses. To further investigate D151 variants, available original clinical specimens of viruses with D151 variation were subjected to NA sequencing, so that all D151 variations (D151G/E/N) were not detected in the original clinical specimens (Table 1). We thus concluded that D151 variants might not have emerged as a natural occurrence and all recent ORVs would retain sensitivity to zanamivir.

Susceptibility to M2 inhibitors was determined to find established-resistant markers by M2-sequencing. The 367

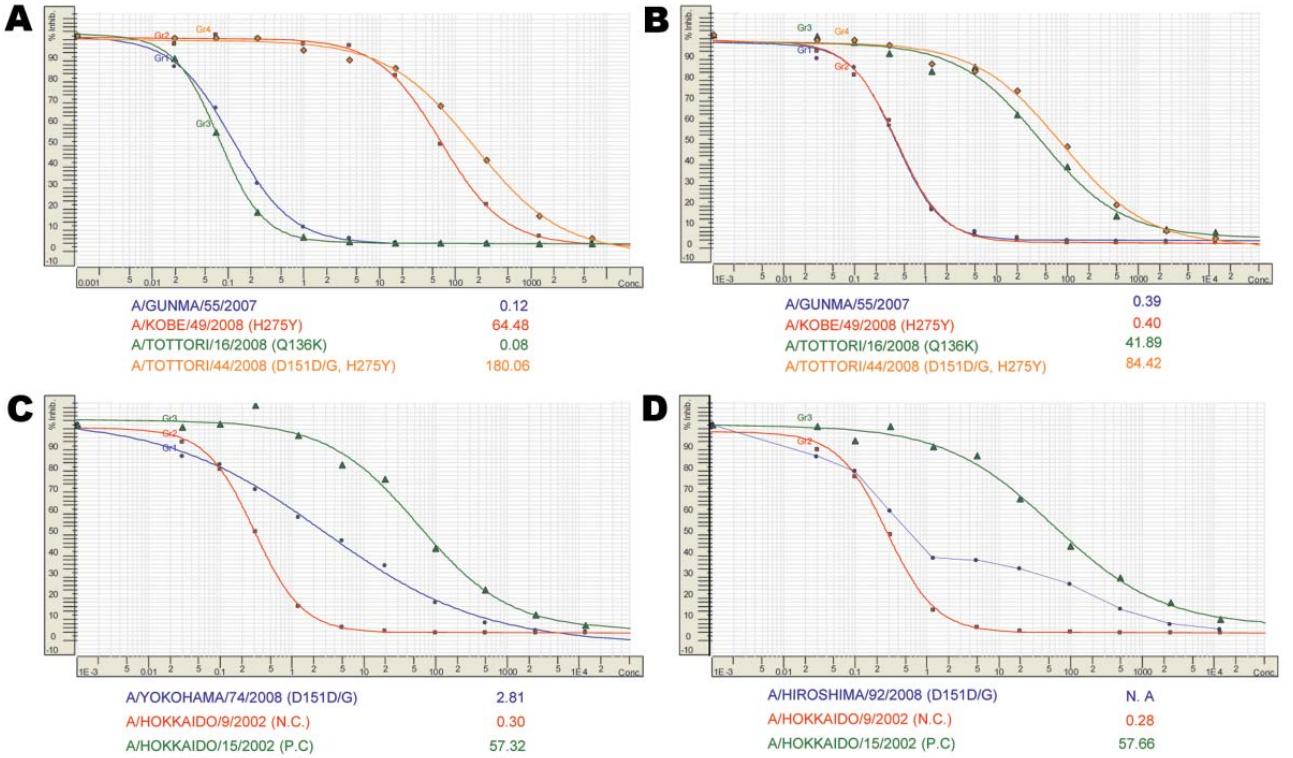


Figure 3. Assessment of drug concentrations required to inhibit neuraminidase activity by 50% (IC_{50}) for neuramindase inhibitors (NAIs). Normal sigmoid curves were generated for most tested viruses by a neuraminidase inhibition assay for oseltamivir (A) and zanamivir (B). Sensitive A/Gunma/55/2007 (blue), oseltamivir-resistant A/Kobe/49/2008 (red) with H275Y, zanamivir-resistant A/Tottori/16/2008 (green) with Q136K, and oseltamivir/zanamivir-resistant A/Tottori/44/2008 (orange) with H275Y and D151D/G are shown. Unusual sigmoid curves were sometimes generated by the mixed population of NAI-resistant and -sensitive viruses for zanamivir: A/Yokohama/74/2008 with D151D/G (C, type A curve); and A/Hiroshima/92/2008 with D151D/G (D, type B curve). Tentative IC_{50} values (nM), shown below each panel, were obtained from type A curves but not from type B curves. NA, not available; NC, negative control; PC, positive control.

viruses (288 isolates in the 2007–08 season; 79 isolates in the 2008–09 season) including 123 ORVs (45 and 78, respectively) and 244 OSVs (243 and 1, respectively) were tested. Viruses belonging to clade 2B were sensitive to M2 inhibitors, and viruses belonging to clade 2C were resistant to M2 inhibitors, so all ORVs except A/Yokohama/91/2007 were sensitive to M2 inhibitors. A/Yokohama/91/2007 belonged to clade 2C and was the only virus resistant to both oseltamivir and M2 inhibitors.

Antigenic Characteristics

To estimate the reactivity of ORVs and OSVs to ferret antiserum against 2008–09 vaccine strain A/Brisbane/59/2009, the HI test was performed. Good inhibition was achieved in 76% of OSVs and 69% of ORVs by A/Brisbane/59/2009 ferret antiserum, and 22% of OSVs and 28% of ORVs showed a 4-fold reduction in HI titer to the homologous titer, respectively (Table 2). Only 2% and 3% of OSVs and ORVs showed a >8-fold reduction in HI titer to A/Brisbane/59/2009 ferret antiserum. These data demonstrated that OSVs and ORVs were anitigenitically indistinguishable from each other and were similar to the 2008–09 vaccine strain A/Brisbane/59/2009.

High Frequency of ORVs in Tottori Prefecture during the 2007–08 Season

Tottori Prefecture is located in the western part of the main island of Japan. Comprising 19 cities and geographically divided into 3 areas, this prefecture has the lowest population in Japan (Figure 4, panel B). Despite a low frequency of only 2.6% in Japan during 2007–08 season, an unexpectedly high frequency (32.4%) of ORVs was observed in Tottori prefecture (Figure 2, panel A). ORVs from Tottori were collected from 4 cities in 2 areas with no systematic bias apparent in the sampling process (Figure 4, panel B).

Phylogenetic analysis of NA genes showed that these ORVs formed 3 subclades (Figure 4, panel A): the first with a Northern-Eu lineage sharing V75A and D354G (T-1); the second with a Hawaii lineage without common changes (T-2); and the third with a Hawaii lineage and sharing M188L (T-3). Among these, only OSVs genetically close

to ORVs were observed in T-2, suggesting that ORVs in T-2 would be derived from OSVs in Tottori prefecture.

A mapping study for ORVs showed that all ORVs in the Hawaii lineage were collected from Tottori city only, primarily at the end of January, whereas ORVs with the Northern-Eu lineage were collected from 4 cities, including Tottori city, during February and March. Genetically diverse ORVs belonging to T1-T3 were cocirculating only in Tottori city in the eastern area (Figure 4, panel B). The Tottori case raised concern about the possibility that these Tottori ORVs could survive to become an origin ORV for the 2008–09 season in Japan. However, phylogenetic analysis showed that all ORVs isolated during the 2008–09 season were not genetically close to ORVs from Tottori (online Appendix Figure). As a result, all ORVs from Tottori seemed to have been eliminated in the 2007–08 season, and ORVs that may have been introduced from other counties were circulating during 2008–09 in Japan.

Discussion

Our study demonstrated that ORVs dramatically increased in Japan from the 2007–08 season (2.6%) to the 2008–09 season (99.7%). All tested ORVs showed a reduction of >260-fold in susceptibility to oseltamivir by NA inhibition assay. On the other hand, almost all ORVs remained sensitive to the other antiviral-drugs, e.g., zanamivir, and M2 inhibitors. HI testing suggested that the current vaccine, A/Brisbane/59/2008, would be effective against recent ORVs. In addition, recent studies have reported that symptoms and hospitalization rates of patients infected with ORVs are no different from those seen with OSVs (*14*,*20*).

Table 1. Influenza virus A (H1N1) outliers to oseltamivir and/or zanamivir, Japan*

Strain	Sequence change(s)			Clinical specimen	Curve fit‡	IC_{50}, nmol/L	
	D151	H275	Q136	D151		Oseltamivir	Zanamivir
Oseltamivir outlier							
A/YAMAGATA/28/2008	D/E†	H	Q	NA	Normal	0.55	0.60
Zanamivir outlier candidates							
A/TOTTORI/16/2008	D	H	K	NA	Normal	0.08	41.89
A/TOTTORI/60/2008	D	Y	Q	NA	Normal	113.86	3.64
A/KOBE/31/2008	D	Y	Q	NA	Type A	26.05	2.75
A/KOBE/32/2008	D	Y	Q	NA	Type A	135.85	3.56
A/MIE/13/2008	D/G	H	Q	D	Type A	0.18	14.80
A/YOKOHAMA/75/2007	D/G	H	Q	NA	Type A	0.13	6.53
A/HAMAMATSU/33/2008	D/G	H	Q	NA	Type A	0.13	6.15
A/TOCHIGI/30/2008	D/G	H	Q	NA	Type A	0.13	4.32
A/YOKOHAMA/74/2007	D/G	H	Q	NA	Type A	0.12	2.81
A/HIROSHIMA/92/2007	D/G	H	Q	NA	Type B	0.07	NA
A/MIE/9/2008	D/G	H	Q	D	Type B	0.08	NA
A/MIE/1/2008	D/G	H	Q	D	Type B	0.16	NA
A/MIE/14/2008	D/G	H	Q	D	Type B	0.08	NA
A/YAMAGATA/60/2008	D/G	H	Q	NA	Type B	0.19	NA
A/SAPPORO/64/2008	D/G	Y	Q	NA	Type B	147.90	NA
A/TOTTORI/44/2008	D/G	Y	Q	NA	Normal§	180.06	84.42
A/HIROSHIMA/44/2008	D/N	Y	Q	NA	Type A	239.23	2.26
A/YOKOHAMA/79/2008	D/N	Y	Q	NA	Type A	167.66	2.28
A/HIROSHIMA/46/2008	D/N	Y	Q	NA	Type A	190.35	2.40
A/HIROSHIMA/45/2008	D/N	Y	Q	NA	Type A	169.92	3.34
A/MIE/1/2009	D/N	Y	Q	NA	Type A	231.78	3.55
A/HIROSHIMA/47/2008	D/N	Y	Q	NA	Type A	106.19	4.24
A/YOKOHAMA/96/2008	D/V	Y	Q	NA	Type B	126.50	NA
Zanamivir sensitive							
A/MIE/18/2008	D/E¶	H	Q	D	Normal	0.35	1.06
A/MIE/21/2008	D/N	H	Q	D	Normal	0.22	1.18
IC_{50} mean of sensitive viruses						0.10 ± 0.05	0.40 ± 0.26
Cutoff IC_{50} values (UQ +5.0 IQR)						0.40	1.99

*IC_{50}, drug concentrations required to inhibit neuraminidase activity by 50%; UQ, upper quartile; IQR, interquartile range; NA, not available. Oseltamivir-resistant viruses with H275Y were excluded from overall population in statistical analysis of oseltamivir.
†Mixture of D151 and D151 variants.
‡Curve fit patterns were evaluated based on Figure 3, panels C (Type A) and D (Type B). Although the viruses with D151D/G tend to generate both patterns from repeat testing for the same samples, type B was selected in this case.
§Although A/TOTTORI/44/2008 showed mixed population of D151D/G, it tended to show a normal curve fit (Figure 3, panel B).
¶The IC_{50} values of most viruses with D151D/E tend to be higher than mean IC_{50} values but do not exceed the cutoff value.

Table 2. Antigenic characterization of oseltamivir-resistant and oseltamivir-sensitive influenza virus A (H1N1), Japan, 2007–2009

Antiserum	Low to homologous titer, -fold*	No. (%) sensitive, n = 169	No. (%) resistant, n = 77
A/Brisbane/59/2007	<2	128 (76)	53 (69)
	4	36 (22)	22 (28)
	>8	3 (2)	2 (3)

*Viruses with >8-fold reduced hemagglutinin inhibition titer to homologous titer were regarded as an antigenic variant.

Japan has the largest per capita use of oseltamivir (>70%) in the world (*10*). Because this use could cause efficient selection of ORVs in individual patients, Japan might be the initial site of worldwide spread of ORVs. However, long-term NAI surveillance in Japan during 1996–2007 and recent surveillance showed a low frequency of NAI-resistant viruses for any strains and subtypes (*10*,*21*,*22*), suggesting that transmissibility of ORVs selected by drug pressure was remarkably decreased. In addition, previous NAI surveillance (*5*–*10*) and several animal studies (*23*–*26*) also suggested that NAI-resistant viruses would become defective viruses with attenuated infectivity and transmissibility to human. In contrast, despite little NAI use, a high emergence of ORVs has been detected in several countries in Europe since November 2007. These ORVs had as efficient transmissibility as OSVs in human-to-human transmission, resulting in worldwide spread in a short period of time. Although whether the initial ORV detected in Norway in the 2007–08 season appeared because of NAI drug pressure is unknown, those ORVs may have obtained amino acid changes on NA and/or other proteins to compensate for the defect, in addition to the H275Y substitution on the NA protein. Most ORVs belong genetically to the Northern-Eu lineage in clade 2B, suggesting that the gene constellation may contain a big advantage to retain infectivity and transmissibility.

An interesting question arose as to where the ORVs in Japan originated. In the Hawaii lineage, almost all ORVs in Japan would be derived from OSVs in Japan because the NA gene of ORVs was similar to OSV counterparts isolated at similar times or from similar regions (online Appendix Figure). On the other hand, in the Northern-Eu lineage, ORVs in Japan would have been introduced from other countries. In 2007–08, almost all ORVs would be imported from countries in Europe. In 2008–09, the ORVs in C-1, which comprised most isolates in 2008–09, and ORVs in C-4 were genetically similar to ORVs isolated from the Southern Hemisphere. Because influenza activity in the Southern Hemisphere occurs half a year earlier than that in the Northern Hemisphere, most ORVs in Japan conceivably could have been imported from the Southern Hemisphere. ORVs in C-2 and C-3 were genetically similar to ORVs isolated in North America and Hawaii, but the collection month of ORVs in C-3 were similar to each other, suggesting that ORVs in C-3 might be derived from an unknown common origin ORV. The ORVs obtained during 2008–09 were not genetically similar to any ORVs isolated in Tottori during 2007–08, indicating that ORVs from Tottori had been eliminated and had not formed the origin ORVs for the 2008–09 season in Japan. As for A/Yokohama/91/2007 belonging to clade 2C, the patient from which this virus was isolated was known to have taken oseltamivir before sampling (*22*), indicating that selective drug pressure in this person might have selected for this ORV.

In the NA inhibition assay for zanamivir, some viruses, including ORVs and OSVs, showed reduced sensitivity to zanamivir. NA sequencing of these viruses showed 2 types of amino acid changes. One virus, A/Tottori/16/2008 (OSV), possessed a Q136K substitution, which reportedly confers resistance to zanamivir (*27*,*28*). Conversely, most of the other viruses possessed D151 G/V/N. The amino acid changes D151 to N or E among subtype H1N1 viruses and to A, G, E, N, or V among H3N2 have been reported (*7*,*8*,*19*), and viruses with D151 substitutions often exhibit reduced sensitivity to NAIs (*8*,*19*,*29*). However, a recent study reported a possible role for cell culture in selecting these D151 variant viruses (*19*). In the present study, D151 variations (D151G/E/N) also were not detected from available original clinical specimens (Table 1), supporting the previous finding. We thus concluded that viruses with D151 variations would not have emerged naturally, and all ORVs would remain sensitive to zanamivir.

By sequencing of M2 gene, we confirmed that almost all Japanese ORVs belonging to clade 2B retained sensitive genotype to M2 inhibitors, consistent with previously reports that recent clade 2B viruses are sensitive to M2 inhibitors, but clade 2C viruses are resistant (*27*).

During the 2007–09 seasons, we also addressed NAI surveillance for A/H3N2 and type B circulating in Japan and identified no viruses resistant to both NAIs. Conversely, in March and early April 2009, a new swine-origin influenza virus A (H1N1) (now known as pandemic [H1N1] 2009 virus) emerged in Mexico and the United States and spread rapidly to many countries, including Japan (*30*–*33*). In June 2009, detection of pandemic (H1N1) 2009 virus with H275Y on the NA protein was reported from Denmark, Hong Kong Special Administrative Region, People's Republic of China, and Japan, but all ORVs of pandemic (H1N1) 2009 virus emerged as sporadic cases with no evidence of efficient human-to-human transmission (*34*). Although oseltamivir remains a valuable drug for treatment of pandemic (H1N1) 2009, many ORVs were isolated after prophylaxis with a half dose of the drug. Therefore, prophylaxis with oseltamivir may not be recommended as

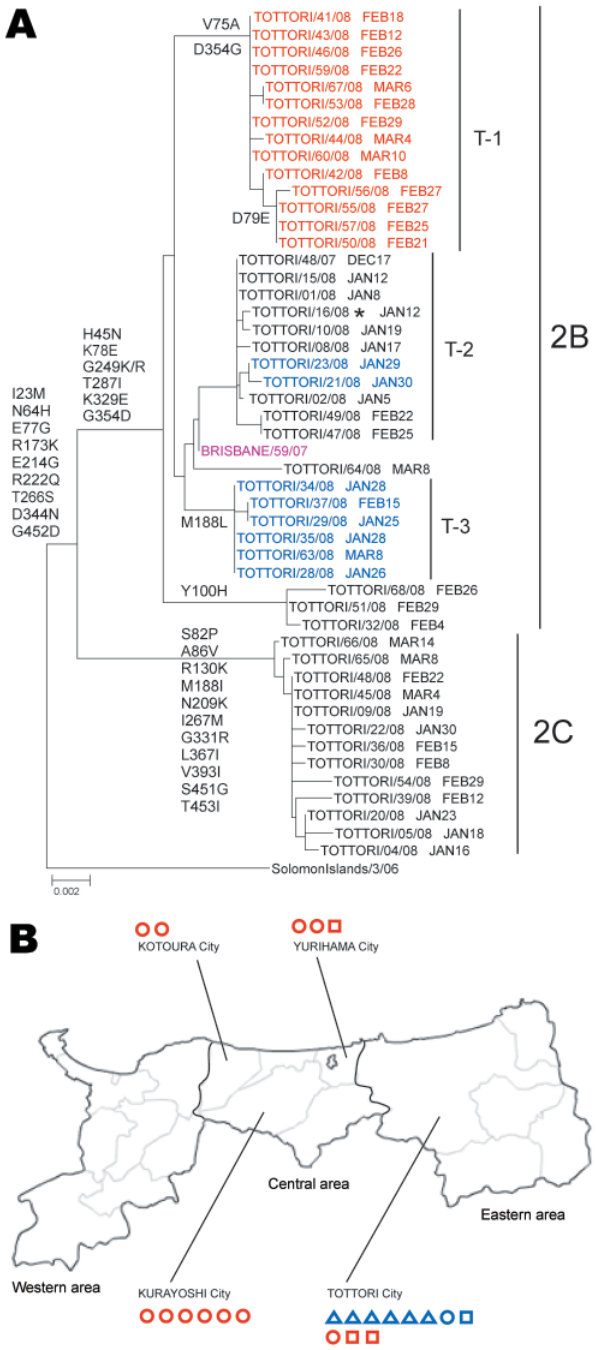


Figure 4. Phylogenetic analysis of influenza A (H1N1) neuraminidase genes (A) and geographic distribution of oseltamivir-resistant viruses (ORVs) (B) isolated from Tottori Prefecture, Japan, 2007–08. ORVs fell into either Northern-Eu lineage (red) or Hawaii lineage (blue); Tottori ORVs and current vaccine strains are indicated by black and purple, respectively. A) ORVs formed 3 subclades: T-1, sharing V75A and D354G; T-2, without common changes; and T-3, sharing M188L. Sampling dates are given after each strain name. Scale bars indicates nucleotide substitutions per site. B) Tottori Prefecture is geographically divided into 3 areas, comprising 19 cities. ORVs from Tottori were collected from 4 cities over 2 areas. The sampling month for each ORV is indicated by a triangle (January), circle (February), or square (March). *Zanamivir resistant.

stated by WHO (*35*). Rapid and continuous monitoring of NAI-resistant viruses, including pandemic (H1N1) 2009 virus, and dissemination of the findings in timely manner remains essential.

Members of the Working Group for Influenza Virus Surveillance in Japan: Hideki Nagano (Hokkaido Institute of Public Health), Masayuki Kikuchi (Sapporo City Institute of Public Health), Masaki Takahashi (Research Institute for Environmental Sciences and Public Health of Iwate Prefecture), Yuki Sato (Miyagi Prefectural Institute of Public Health and Environment), Masanori Katsumi (Sendai City Institute of Public Health), Hiroyuki Saito (Akita Research Center for Public Health and Environment), Katsumi Mizuta (Yamagata Prefectural Institute of Public Health), Syoko Hirose (Fukushima Prefectural Institute of Public Health), Setsuko Fukaya (Ibaraki Prefectural Institute of Public Health), Asumi Hirata(Tochigi Prefectural Institute of Public Health and Environmental Science), Hiroyuki Tsukagoshi (Gunma Prefectural Institute of Public Health and Environmental Sciences), Yuka Uno (Saitama City Institute of Health Science and Research), Hiromi Maru (Chiba Prefectural Institute of Public Health), Hajime Yokoi (Chiba City Institute of Health and Environment), Mami Nagashima (Tokyo Metropolitan Institute of Public Health), Sumi Watanabe (Kanagawa Prefectural Institute of Public Health), Hideaki Shimizu (Kawasaki City Institute of Public Health), Sumiko Ueda (Sagamihara City Laboratory of Public Health), Chika Hirokawa (Niigata Prefectural Institute of Public Health and Environmental Sciences), Gen Kobayashi and Yoko Miyajima (Niigata City Institute of Public Health and Environment), Sanae Kuramoto and Hiroe Kodama (Ishikawa Prefectural Institute of Public Health and Environmental Science), Masako Nakamura (Fukui Prefectural Institute of Public Health), Hiroyoshi Asakawa (Yamanashi Institute for Public Health), Seiko Sawatari (Gifu Prefectural Institute of Health and Enviromental Sciences), Yasunori Tanaka (Gifu Municipal Institute of Public Health), Yoshinobu Miwa (Shizuoka Institute of Environment and Hygiene), Shinobu Ide (Shizuoka City Institute of Environmental Sciences and Public Health), Yoshinori Kohno and Ryu Hibino (Hamamatsu City Health Environment Research Center), Mami Hata (Aichi Prefectural Institute of Public Health), Noriko Goto (Nagoya City Public Health Research Institute), Takuya Yano (Mie Prefecture Health and Environment Research Institute), Fumie Matsumoto (Shiga Prefectural Institute of Public Health), Tohru Ishizaki (Kyoto Prefectural Institute of Public Health and Environment), Satoshi Hiroi (Osaka Prefectural Institute of Public Health), Hideyuki Kubo (Osaka City Institute of Public Health and Environmental Sciences), Kiyoko Uchino (Sakai City Institute of Public Health), Tomohiro Oshibe (Hyogo Prefectural Institute of Public Health and Consumer Sciences), Ai Mori and Tomoko Suga (Kobe Institute of Health), Yoshiteru Kitahori (Nara Prefectural Institute for Hygiene and Environment), Fumio Terasoma (Wakayama Prefectural Research Center

of Environment and Public Health), Yoshiaki Kimura and Naomi Matsumoto (Tottori Prefectural Institute of Public Health and Environmental Science), Tamaki Omura (Shimane Prefectural Institute of Public Health and Environmental Science), Shinichi Takao (Center for Public Health and Environment, Hiroshima Prefectural Technology Research Institute), Katsuhiko Abe (Hiroshima City Institute of Public Health), Yumiko Kawakami (Tokushima Prefectural Centre for Public Health and Environmental Sciences), Ootsuka Yuka (Ehime Prefecture Institute of Public Health and Environmental Science), Daisuke Kawamoto (Fukuoka City Institute for Hygiene and the Environment), Takayuki Hirano and Hisato Masumoto (Saga Prefectural Institute of Public Health and Pharmaceutical Research), Manabu Hirano and Akinori Yamaguchi (Nagasaki Prefectural Institute for Environment Research and Public Health), Seiya Harada and Koichi Nishimura (Kumamoto Prefectural Institute of Public Health and Environmental Science), Miki Kato (Oita Prefectural Institute of Health and Environment), Miho Miura (Miyazaki Prefectural Institute for Public Health and Environment), Kanji Ishitani and Akihide Kamimura (Kagoshima Prefectural Institute for Environmental Research and Pubulic Health), Katsuya Taira (Okinawa Prefectural Institute of Health and Environment), Mitsutaka Kuzuya (Okayama Prefectural Institute for Environmental Science and Public Health), Shoichi Toda (Yamaguchi Prefectural Institute of Public Health and Environment), Chiharu Kawakami (Yokohama City Institute of Health), Mayumi Konno (Kyoto City Institute of Health and Environmental Sciences), Hiroya Komoda (Kagawa Prefectural Research Institute for Environmental Sciences and Public Health), Tae Taniwaki (Kochi Public Health and Sanitation Institute), Toshitaka Minegishi (Saitama Institute of Public Health), Rika Tsutsui (Aomori Prefectural Institute of Public Health and Environment), Shizuko Kasuo (Nagano Environmental Conservation Research Institute), Yuichiro Okamura (Nagano City Health Center), Eiji Horimoto (Toyama Institute of Health), Nobuyuki Sera (Fukuoka Institute of Health and Environmental Sciences), and Hiromi Yoshikawa (Kitakyushu City Institute of Environmental Sciences)

Acknowledgments

We thank Miho Ejima and Mariko Tokunaga for the production of several figures.

This study was supported by Grants-in-Aid for Seisaku-Soyaku-Sougo (H18-Iyaku- Ippan 033), and Emerging and Re-emerging Infectious Diseases (H20-Shinko-Ippan 005) from the Ministry of Health, Labour and Welfare of Japan.

Dr Ujike is a virologist and the senior researcher of Influenza Virus Research Center of the National Institute of Infectious Diseases, Tokyo, Japan. His research interests are influenza virus and severe acute respiratory syndrome–coronavirus.

References

1. Cauchemez S, Valleron AJ, Boëlle PY, Flahault A, Ferguson NM. Estimating the impact of school closure on influenza transmission from sentinel data. Nature. 2008;452:750–4. DOI: 10.1038/nature06732
2. Fleming DM. Managing influenza: amantadine, rimantadine and beyond. Int J Clin Pract. 2001;55:189–95.
3. Saito R, Li D, Suzuki H. Amantadine-resistant influenza A (H3N2) virus in Japan, 2005–2006. N Engl J Med. 2007;356:312–3. DOI: 10.1056/NEJMc062989
4. Saito R, Suzuki Y, Li D, Zaraket H, Sato I, Masaki H, et al. Increased incidence of adamantane-resistant influenza A(H1N1) and A(H3N2) viruses during the 2006–2007 influenza season in Japan. J Infect Dis. 2008;197:630–2. DOI: 10.1086/525055
5. World Health Organization. Monitoring of neuraminidase inhibitor resistance among clinical influenza virus isolates in Japan during 2003–2006 influenza seasons. Wkly Epidemiol Rec. 2007;82:149–50.
6. Monto AS, McKimm-Breschkin JL, Macken C, Hampson AW, Hay A, Klimov A, et al. Detection of influenza viruses resistant to neuraminidase inhibitors in global surveillance during the first 3 years of their use. Antimicrob Agents Chemother. 2006;50:2395–402. DOI: 10.1128/AAC.01339-05
7. McKimm-Breschkin J, Trivedi T, Hampson A, Hay A, Klimov A, Tashiro M, et al. Neuraminidase sequence analysis and susceptibilities of influenza virus clinical isolates to zanamivir and oseltamivir. Antimicrob Agents Chemother. 2003;47:2264–72. DOI: 10.1128/AAC.47.7.2264-2272.2003
8. Sheu TG, Deyde VM, Okomo-Adhiambo M, Garten RJ, Xu X, Bright RA, et al. Surveillance for neuraminidase inhibitor resistance among human influenza A and B viruses circulating worldwide from 2004 to 2008. Antimicrob Agents Chemother. 2008;52:3284–92. DOI: 10.1128/AAC.00555-08
9. Kramarz P, Monnet D, Nicoll A, Yilmaz C, Ciancio B. Use of oseltamivir in 12 European countries between 2002 and 2007–lack of association with the appearance of oseltamivir-resistant influenza A(H1N1) viruses. Euro Surveill. 2009;14. pii: 19112.
10. Tashiro M, McKimm-Breschkin JL, Saito T, Klimov A, Macken C, Zambon M, et al. Surveillance for neuraminidase-inhibitor-resistant influenza viruses in Japan, 1996–2007. Antivir Ther. 2009;14:751–61. DOI: 10.3851/IMP1194
11. Rameix-Welti MA, Enouf V, Cuvelier F, Jeannin P, van der Werf S. Enzymatic properties of the neuraminidase of seasonal H1N1 influenza viruses provide insights for the emergence of natural resistance to oseltamivir. PLoS Pathog. 2008;4:e1000103. DOI: 10.1371/journal.ppat.1000103
12. Meijer A, Lackenby A, Hungnes O, Lina B, van-der-Werf S, Schweiger B, et al. European Influenza Surveillance Scheme. Oseltamivir-resistant influenza virus A (H1N1), Europe, 2007/08 season. Emerg Infect Dis. 2009;15:552–60. DOI: 10.3201/eid1504.081280
13. Lackenby A, Hungnes O, Dudman SG, Meijer A, Paget WJ, Hay AJ, et al. Emergence of resistance to oseltamivir among influenza A(H1N1) viruses in Europe. Euro Surveill. 2008;13. pii: 8026.
14. Hauge SH, Dudman S, Borgen K, Lackenby A, Hungnes O. Oseltamivir-resistant influenza viruses A (H1N1), Norway, 2007/08. Emerg Infect Dis. 2009;15:155–62. DOI: 10.3201/eid1502.081031
15. World Health Organization. WHO/ECDC frequently asked questions for oseltamivir resistance. 2008 Feb 15 [cited 2009 Oct 20]. http://www.who.int/csr/disease/influenza/oseltamivir_faqs/en/index.html
16. World Health Organization. Influenza A(H1N1) virus resistance to oseltamivir. 2009 Mar 21 [cited 2009 Oct 20]. http://www.who.int/csr/disease/influenza/h1n1_table/en/index.html

17. World Health Organization. 2002. Manual on animal influenza diagnosis and surveillance, 2nd ed. [cited 2009 Oct 20]. http://www.wpro.who.int/NR/rdonlyres/EFD2B9A7-2265-4AD0-BC98-97937-B4FA83C/0/manualonanimalaidiagnosisandsurveillance.pdf
18. Wetherall NT, Trivedi T, Zeller J, Hodges-Savola C, McKimm-Breschkin JL, Zambon M, et al. Evaluation of neuraminidase enzyme assays using different substrates to measure susceptibility of influenza virus clinical isolates to neuraminidase inhibitors: report of the neuraminidase inhibitor susceptibility network. J Clin Microbiol. 2003;41:742–50. DOI: 10.1128/JCM.41.2.742-750.2003
19. Deyde VM, Okomo-Adhiambo M, Sheu TG, Wallis TR, Fry A, Dharan N, et al. Pyrosequencing as a tool to detect molecular markers of resistance to neuraminidase inhibitors in seasonal influenza A viruses. Antiviral Res. 2009;81:16–24. DOI: 10.1016/j.antiviral.2008.08.008
20. Dharan NJ, Gubareva LV, Meyer JJ, Okomo-Adhiambo M, McClinton RC, Marshall SA, et al. Infections with oseltamivir-resistant influenza A(H1N1) virus in the United States. JAMA. 2009;301:1034–41. DOI: 10.1001/jama.2009.294
21. Tamura D, Mitamura K, Yamazaki M, Fujino M, Nirasawa M, Kimura K, et al. Oseltamivir-resistant influenza a viruses circulating in Japan. J Clin Microbiol. 2009;47:1424–7. DOI: 10.1128/JCM.02396-08
22. Kawakami C, Obuchi M, Saikusa M, Noguchi Y, Ujike M, Odagiri T, et al. Isolation of oseltamivir-resistant influenza A/H1N1 virus of different origins in Yokohama City, Japan, during the 2007–2008 influenza season. Jpn J Infect Dis. 2009;62:83–6.
23. Ives JA, Carr JA, Mendel DB, Tai CY, Lambkin R, Kelly L, et al. The H274Y mutation in the influenza A/H1N1 neuraminidase active site following oseltamivir phosphate treatment leave virus severely compromised both in vitro and in vivo. Antiviral Res. 2002;55:307–17. DOI: 10.1016/S0166-3542(02)00053-0
24. Carr J, Ives J, Kelly L, Lambkin R, Oxford J, Mendel D, et al. Influenza virus carrying neuraminidase with reduced sensitivity to oseltamivir carboxylate has altered properties in vitro and is compromised for infectivity and replicative ability in vivo. Antiviral Res. 2002;54:79–88. DOI: 10.1016/S0166-3542(01)00215-7
25. Herlocher ML, Truscon R, Elias S, Yen HL, Roberts NA, Ohmit SE, et al. Influenza viruses resistant to the antiviral drug oseltamivir: transmission studies in ferrets. J Infect Dis. 2004;190:1627–30. DOI: 10.1086/424572
26. Herlocher ML, Carr J, Ives J, Elias S, Truscon R, Roberts N, et al. Influenza virus carrying an R292K mutation in the neuraminidase gene is not transmitted in ferrets. Antiviral Res. 2002;54:99–111. DOI: 10.1016/S0166-3542(01)00214-5
27. Barr IG, Deng YM, Iannello P, Hurt AC, Komadina N. Adamantane resistance in influenza A(H1) viruses increased in 2007 in South East Asia but decreased in Australia and some other countries. Antiviral Res. 2008;80:200–5. DOI: 10.1016/j.antiviral.2008.06.008
28. Hurt AC, Holien JK, Parker M, Kelso A, Barr IG. Zanamivir-resistant influenza viruses with a novel neuraminidase mutation. J Virol. 2009;83:10366–73. DOI: 10.1128/JVI.01200-09
29. Yen HL, Hoffmann E, Taylor G, Scholtissek C, Monto AS, Webster RG, et al. Importance of neuraminidase active-site residues to the neuraminidase inhibitor resistance of influenza viruses. J Virol. 2006;80:8787–95. DOI: 10.1128/JVI.00477-06
30. Garten RJ, Davis CT, Russell CA, Shu B, Lindstrom S, Balish A, et al. Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. Science. 2009;325:197–201. DOI: 10.1126/science.1176225
31. Novel Swine-Origin Influenza A (H1N1) Virus Investigation Team, Dawood FS, Jain S, Finelli L, Shaw MW, Lindstrom S, et al. Emergence of a novel swine-origin influenza A (H1N1) virus in humans. N Engl J Med. 2009;360:2605–15. DOI: 10.1056/NEJMoa0903810
32. Shinde V, Bridges CB, Uyeki TM, Shu B, Balish A, Xu X, et al. Triple-reassortant swine influenza A (H1) in humans in the United States, 2005–2009. N Engl J Med. 2009;360:2616–25. DOI: 10.1056/NEJMoa0903812
33. Smith GJ, Vijaykrishna D, Bahl J, Lycett SJ, Worobey M, Pybus OG, et al. Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. Nature. 2009;459:1122–5. DOI: 10.1038/nature08182
34. World Health Organization. Viruses resistant to oseltamivir (Tamiflu) identified. 2009 Jul 8 [cited 2009 Oct 20]. http://www.who.int/csr/disease/swineflu/notes/h1n1_antiviral_resistance_20090708/en/index.html
35. World Health Organization. Antiviral use and the risk of drug resistance. Pandemic (H1N1) 2009 briefing note 12 [cited 2009 Oct 20]. http://www.who.int/csr/disease/swineflu/notes/h1n1_antiviral_use_20090925/en/index.html

Address for correspondence: Takato Odagiri, Influenza Virus Research Center, National Institute of Infectious Diseases, 4-7-1,Gakuen, Musashi-Murayama, Tokyo, 208-0011, Japan; email: todagiri@nih.go.jp

Increased Prevalence of *Trichinella* spp., Northeastern Germany, 2008

Gunter Pannwitz,[1] Anne Mayer-Scholl,[1] Aleksandra Balicka-Ramisz, and Karsten Nöckler

In 2008, a *Trichinella* spp. outbreak occurred on a small family-owned pig farm in Mecklenburg–Western Pomerania in northeastern Germany. To obtain epidemiologic information on this outbreak, we determined that after 2005 the prevalence of *Trichinella* spp. in wild boars has increased in this region of Germany. We discuss the potential role of the raccoon dog in the increase in *Trichinella* spp. prevalence in the sylvatic cycle in this region. We believe that this increase could pose a threat to pigs kept in back yard conditions, and we provide recommendations to ensure public health safety.

Nematodes of the genus *Trichinella* infect a broad variety of mammals, birds, and reptiles and are distributed worldwide (*1*). Trichinellosis is a foodborne zoonotic disease caused by a parasite. Human infections occur after ingestion of raw or inadequately cooked meat containing parasite larvae (*2*). Pigs represent a major source of human infection, but meat from horses, wild boars, bears, and badgers have also played a major role during outbreaks (*3*,*4*).

Trichinella spp. can be transmitted by domestic and sylvatic cycles. The domestic cycle is maintained by feeding of swill to pigs and pigs feeding on animal carcasses or on synanthropic animals (e.g., rats, mice). In Germany, the domestic cycle disappeared >30 years ago (*5*). During 1998–2007, ≈436 million pigs were slaughtered and tested for *Trichinella* spp. by using artificial digestion according to regulation (European Commission [EC]) no. 2075/2005 (*6*). However, in 2003, 1 positive case was reported; it was in a pig kept in a back yard (*7*).

Author affiliations: Veterinär-und Lebensmittelüberwachungsamt Ostvorpommern, Anklam, Germany (G. Pannwitz); Federal Institute for Risk Assessment, Berlin, Germany (A. Mayer-Scholl, K. Nöckler); and West Pomeranian University, Szczecin, Poland (A. Balicka-Ramisz)

DOI: 10.3201/eid1606.091629

Currently, the major *Trichinella* spp. reservoir and source of infection for domestic pigs in Germany is wild boars (*Sus scrofa*), raccoon dogs (*Nyctereutes procyonoides*), and foxes (*Vulpes vulpes*). The most prevalent *Trichinella* spp. is *T. spiralis*, followed by *T. britovi* and *T. pseudospiralis* (*8*).

In the past 30 years, sporadic human infections with *Trichinella* spp. (0–50 reported cases per year) have occurred in Germany (*7*,*9*). These infections are usually linked to consumption of contaminated meat during holiday visits to high-risk countries (*10*). Autochthonous outbreaks occur infrequently, such as during 2005–2006, when 17 members of a large family in Mecklenburg–Western Pomerania were infected with *Trichinella* spp. after consumption of meat products from a pig reared and slaughtered at home (*11*).

We report a *Trichinella* spp. outbreak on a small family-owned pig farm in Mecklenburg–Western Pomerania in northeastern Germany during December 2008. We show that after 2005, the prevalence of *Trichinella* spp. in wild boars has increased in this region of Germany. Furthermore, we discuss the possibility that increased *Trichinella* spp. prevalence in wild boars is the result of high prevalence of the disease in neighboring Poland. The potential role of migration of raccoon dogs from Poland into Germany is also considered as a factor of increased prevalence.

Materials and Methods

Outbreak Investigations

Veterinary inspections of the outbreak farm were performed with emphasis on production type, location, potential contacts with wild animals (including rodents), feeding habits, *Trichinella* spp. status of animals on origin farms, and human factors. Meat and blood samples of 5 slaughtered pigs from the outbreak farm were sent to the National

[1]These authors contributed equally to this article.

Reference Laboratory for Trichinellosis at the Federal Institute for Risk Assessment in Berlin, Germany.

Laboratory Examinations

Muscle Samples

All muscle samples from Germany and Poland were examined by artificial digestion (magnetic stirrer method) according to regulation (EC) no. 2075/2005 (*6*). A mouse caught in a trap on the outbreak farm was examined by using the same method.

PCR

Larvae were isolated from muscle tissues and washed 4× with distilled water on ice and stored in 5 μL of distilled water at –20°C. DNA extraction and PCR were performed as described by Pozio and La Rosa (*12*).

Serologic Testing of Blood and Meat Juice

Venous blood was collected from live pigs. Meat was obtained from the diaphragm pillar of slaughtered pigs, cut in small pieces, put in plastic bags, and frozen at –20°C for 3 days. After thawing, the meat was squeezed and meat juice was collected and stored at –20°C. Serum samples were examined by using an in-house ELISA as described by Nöckler et al. (*13*). This assay is based on the excretory–secretory antigen (*14*).

Sampling, Data Management, and Statistical Analysis

There are currently no reliable estimates of the number of wild boars in Germany. However, the number of wild boars hunted during a hunting year in a specific region is considered proportional to the size of the wild boar population (*15*). To optimally control the wild boar population and prevent damage to land or crops and the spread of disease, wild boars in Germany are hunted annually (*16*). Depending on climate, reproduction rate, and hunting success, ≈50%–100% of the animals born in a given year are hunted (killed) in Germany each year (*17*). According to specifications of German hunting associations, the number of wild boars hunted should consist of 80% piglets (maximum age 1 year), 10% juveniles (1–2 years of age), and 10% older sows. However, because hunters generally focus on older piglets and juveniles, the suggested number of wild boars hunted is not representative of the population and is biased toward younger animals. This factor could lead to an underestimation of the *Trichinella* spp. prevalence in wild boars because young animals only have a short time span during which they can become infected.

The number of foxes and raccoon dogs hunted is also thought to reflect changes in the sizes of the populations of these species. Raccoon dogs and foxes are not examined for *Trichinella* spp. according to regulation (EC) no. 2075/2005, and currently, Germany has no countrywide monitoring program for *Trichinella* spp. in these animals. Animals examined were part of research initiatives. Therefore, sampling of raccoon dogs and foxes is less representative than that of wild boars. Information on wild boars examined each year for *Trichinella* spp. in the German Federal States during 2002–2008 was supplied by the German Federal Statistics Office (*18*).

During 2002–2008, prevalences of *Trichinella* spp.–positive wild boars in Ostvorpommern, Germany, were compared with those in Mecklenburg–Western Pomerania and in the remaining German Federal States. Data for *Trichinella* spp. prevalences in foxes and raccoon dogs hunted in Mecklenburg–Western Pomerania during 1993–2008 and in wild boars and foxes hunted during 2004–2008 on Island Wolin, Poland, which borders Ostvorpommern, were also analyzed. We determined 95% confidence intervals from the binomial distribution as described by Clopper and Pearson (*19*). Descriptive statistics were calculated by using available online resources (www.statpages.org).

Results

Outbreak Investigations

In November 2008, an 11-month-old pig (German Landrace × Large White) was declared positive for *Trichinella* spp. at an abattoir in Ostvorpommern, a district in Mecklenburg–Western Pomerania. The day after detection of *Trichinella* larvae, veterinarians of the Veterinary and Food Safety Authority visited the outbreak farm and conducted epidemiologic investigations. A blood sample obtained for swine fever monitoring shortly before slaughtering was sent to the National Reference Laboratory for Trichinellosis. The remaining 4 pigs at this site were slaughtered ≈2 weeks after the first case of *Trichinella* infection was identified.

Description of Farm

The farm was a small holding of ≈0.5 acres at the edge of a village bordering a large field. In addition to the 5 pigs, sheep, poultry, cats, and a dog also lived on the farm. The pigs were penned in an old, closed building and allowed to enter a small outdoor yard once a week. The farm was run in accordance with the German Pig-Keeping Hygiene Ordinance.

Rodent Manifestations

During the summer of 2008, the farmer observed some rats on the premises. A professional pest control company was contacted after the slaughtering of all pigs, but no rodent infestation was detected.

Feeding

The pigs were routinely fed steamed potatoes, potato ensilage, fodder beets, feed grain, sugar beet pellets, and water. According to the farmer, the pigs were not fed swill.

Trichinella spp. Status of Origin Farm

At the beginning of 2008, a total of 3 *Trichinella* spp.–positive animals had been bought as weaned pigs from a small breeding farm, which had 8 breeding sows, 2 boars, and ≈60 piglets ≈3 km from the outbreak farm. Blood samples were taken from 16 animals (including 8 breeding sows) for testing by ELISA. All pigs sold from and slaughtered at this breeding farm in 2008 were traced and confirmed to have been negative for *Trichinella* spp.

The 2 *Trichinella* spp.–negative pigs from the outbreak farm were obtained as finishers from a large commercial fattening unit that delivered hundreds of finished pigs per week to an EU-licensed abattoir. To date, all pigs from the commercial piggery have been negative for *Trichinella* spp.

Human Factors

Until the fall of 2008, a family member living on the outbreak farm was employed as a cook in a restaurant near the border with Poland. The restaurant is well known for game dishes and has its own meat-cutting room and area for selling meat. During an inspection of the restaurant, veterinarians found evidence that meat offal was being supplied to unknown owners of animals. However, additional information (number or identity of owners of animals) could not be obtained.

Laboratory Examinations

Muscle Examination

One *Trichinella* spp.–positive pig slaughtered had 299 larvae/g of tissue, and the 2 other pigs kept in the area had 1.2 and 1.3 larvae/g of tissue, respectively (Table 1). The other 2 pigs kept in a separate area on the farm were negative for *Trichinella* spp. Larvae from all 3 *Trichinella* spp.–positive pigs were examined by PCR and identified as *T. spiralis*. All 3 *Trichinella* spp.–positive carcasses were classified as unfit for human consumption.

Serologic Examination

ELISAs were conducted for blood and meat juice samples from the 5 pigs on the outbreak farm. Results were consistent with those of the magnetic stirrer method (Table 1). All 16 blood samples from the 3 *Trichinella* spp.–positive pigs from the origin farm were negative for *Trichinella* spp. by ELISA. Antibodies against *Trichinella* spp. were not detected in the serum of the farmer (immunoglobulin G titer <10, immunoglobulin M titer <40).

Prevalence of *Trichinella* spp. in Wild Boars in Germany, 2002–2008

In Germany, examination of wild boar carcasses intended for human consumption is compulsory (*6*). Because reporting to the German Federal Statistical Office is obligatory, the number of wild boars examined per year was considered a representative sampling unit.

The yearly prevalence of *Trichinella* spp. in wild boars in Germany during 2002–2008 ranged from 0.0027% to 0.0032% (Table 2). In 2005, there was a sudden increase in *Trichinella* spp. prevalence in wild boars in Mecklenburg–Western Pomerania compared with the rest of Germany. During 2005 and 2008 (Figure 1, panel A) and during 2005–2008, more *Trichinella* spp.–positive wild boars were detected in Mecklenburg–Western Pomerania than in the rest of Germany.

Within Mecklenburg–Western Pomerania, 21 (87.5%) of 24 *Trichinella* spp.–positive results were in wild boars in Ostvorpommern, the district where the outbreak farm was located. *Trichinella* spp. prevalence in Oatvorpommern was higher in 2005, 2007, and 2008 than in the rest of Mecklenburg–Western Pomerania (Figure 1, panel B).

For verification and species identification, 20 *Trichinella* spp.–positive wild boar samples obtained during 2005–2008 were sent to the National Reference Center for Trichinellosis. A total of 80% (14) were identified as *T. spiralis*, 15% (3) as *T. pseudospiralis*, and 1 as a mixed infection (*T. spiralis* and *T. pseudospiralis*) (*20*). Larval load ranged from 2 to 922 larvae/g of tissue.

Trichinella spp.–Positive Raccoon Dogs and Foxes, Mecklenburg–Western Pomerania

The number of raccoon dogs and red foxes hunted in Mecklenburg–Western Pomerania is shown in Table 3.

Table 1. Results of laboratory examinations of 5 pigs for *Trichinella* spp. on outbreak farm, Germany, 2008*

	Diaphragm pillar		IgG ELISA (meat juice)		IgG ELISA (serum)	
Animal no.	Artificial digestion	Larvae/g	Titer	Result	Titer	Result
1	+	299	≥128	+	>1,280	+
2	+	1,2	32	+	320	+
3	+	1,3	64	+	320	+
4	–	0	<10	–	10	–
5	–	0	<10	–	<10	–

*Ig, immunoglobulin.

Table 2. Western blot results for prevalence of *Trichinella* spp. in wild boars, Mecklenburg– Western Pomerania and Ostvorpommern, Germany

Year	Germany, no. positive/no. tested (%)	Mecklenburg–Western Pomerania, no. positive/no. tested (%)	Ostvorpommern District, no. positive/ no. tested (%)
2002	12/397,425 (0.0032)	0/31,667 (0)	0/3,616 (0)
2003	10/370,187 (0.0027)	0/30,632 (0)	0/3,201 (0)
2004	11/ 390570 (0.0028)	0/29,592 (0)	0/2,992 (0)
2005	11/402,996 (0.0027)	6/32,227 (0.0186)	4/2,760 (0.1449)
2006	8/272,258 (0.0029)	2/28,764 (0.0070)	2/2,577 (0.0776)
2007	9/282,442 (0.0032)	4/27094 (0.0148)	4/2,983 (0.1341)
2008	16/354,118 (0.0045)	12/37,880 (0.0317)	11/3,921 (0.2805)

During 1993–2008, a sharp increase in raccoon dogs hunted was observed; the number of foxes hunted remained constant over this period. No difference was found between the number of foxes and raccoon dogs hunted in Ostvorpommern and those hunted in more westward districts of similar sizes in Mecklenburg–Western Pomerania.

In Germany, raccoon dogs and foxes are not routinely examined for *Trichinella* spp. In a monitoring program conducted in Mecklenburg–Western Pomerania during February 2006–January 2007, a total of 100 raccoon dogs and foxes were examined by using the magnetic stirrer method. *Trichinella* spp. prevalence was 4.0% in raccoon dogs and 1.0% in foxes. In a smaller district-level monitoring study during February–August 2006, a total of 3 of 46 raccoon dogs from Ostvorpommern and a neighboring district were positive for *Trichinella* spp. (prevalence 6.5%). Larval load ranged from 0.06 to 65 larvae/g of tissue. Four of 7 raccoon dogs were infected with *T. spiralis* and 2 with *T. pseudospiralis*. One raccoon dog from Ostvorpommern had a mixed infection (*T. spiralis* and *T. pseudospiralis*).

Trichinella spp. Prevalence in Wild Boars and Raccoon Dogs, Ostvorpommern

Ostvorpommern is located in the eastern part of Mecklenburg–Western Pomerania, Germany (Figure 2). Part of this district is on Usedom Island, an island in the Baltic Sea. The western part of Usedom Island is in Germany and the eastern part is in Poland. Of the 26 reported *Trichinella* spp. infections in wild animals (21 in wild boars and 5 in raccoon dogs) in Ostvorpommern during 2005–2008, a total of 80.7% were found on Usedom Island (Figure 2).

Trichinella spp. Prevalence in Wild Boars and Foxes, Wolin Island, Poland, 2004–2008

On Wolin Island, which borders Usedom Island, 22 (3.27%) of 672 wild boars examined during 2004–2008 were positive for *Trichinella* spp. During the same period, 6 (4.22%) of 142 foxes were positive for *Trichinella* spp. (Table 4).

Discussion

In December 2008 a *Trichinella* spp. outbreak occurred on a pig farm in Mecklenburg–Western Pomerania, Germany. Although the affected animals originated from a farm with similar biosecurity levels as the outbreak farm, negative serologic results for pigs at the origin farm and the longer period spent on the outbreak farm make infection on the outbreak farm more likely.

Potential infection sources were manifold. Although trichinellosis is not a contagious disease, all pigs kept in 1 area were positive for *Trichinella* spp. This finding suggests a common source of infection. At the time of outbreak investigations in December 2008, no evidence of rodents was found on the farm. However, the role of rats as a *Trichinella* spp. maintenance host is unclear, and infection in rats is considered a marker for infection in pigs (*21*).

Feeding of infectious meat (e.g., wild boar) can also lead to *Trichinella* spp. infection in pigs. The farmer on the

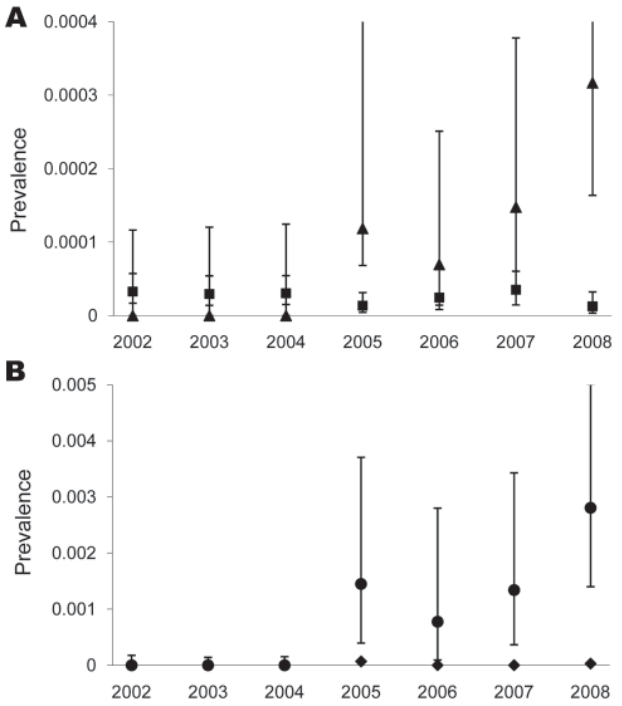


Figure 1. Yearly prevalence of *Trichinella* spp. in wild boars in A) Mecklenburg–Western Pomerania and B) Ostvorpommern, Germany. Nonoverlapping error bars indicate significance at $p \leq 0.05$. Squares, Germany other than Mecklenburg–Western Pomerania; triangles, Mecklenburg–Western Pomerania; circles, Ostvorpommern; diamonds, Mecklenburg–Western Pomerania.

Table 3. Number of raccoon dogs and red foxes hunted, Mecklenburg–Western Pomerania, Germany

Years	Raccoon dogs	Red foxes
1993–1996	541	123,316
1997–2000	9,324	144,260
2001–2004	43,259	122,820
2005–2008	78,311	116,322

outbreak farm had access to game meat because a family member worked at a restaurant in which game meat offal was given to owners of animals. Although the direct circumstances could not be elucidated, game meat offal seems to be the most likely source of infection.

The yearly prevalence of *Trichinella* spp. in wild boars in Germany ranged from 0.0027% in 2002 to 0.0045% in 2008. In contrast, during 1975–2005, *Trichinella* spp. were not detected in wild boars in Mecklenburg–Western Pomerania. Since 2005, *Trichinella* spp. prevalence has increased in Mecklenburg–Western Pomerania in wild boars in comparison with the rest of Germany. Mecklenburg–Western Pomerania, the district in which Ostvorpommern is located, has been predominantly affected. A total of 80.7% of all wild boars and raccoon dogs positive for *Trichinella* spp. were found on Usedom Island.

Since 2005, the trichinoscopic method for larvae detection has been replaced by more sensitive methods of artificial digestion (*6*). Because new legislation regarding trichinellosis was implemented at the same time in Germany, it is unlikely that higher *Trichinella* spp. prevalence in wild boars in Mecklenburg–Western Pomerania can be attributed to the use of this improved diagnostic technique.

Human trichinellosis is regularly reported in Poland. Thirty-five human outbreaks and 702 cases were reported in Poland during 2002–2007 (*22*). Because of stringent control measures in recent years, the primary source of human infection has changed from pork to wild boar meat (*23*). During 1999–2004, Balicka-Ramisz et al. (*24*) examined >56,000 wild boars in West Pomerania, a province in northwestern Poland bordering Mecklenburg–Western Pomerania, Germany. *Trichinella* spp. prevalence in wild boars in this region increased 8-fold from 0.12% to 1.48% during this period. In the same study, >500 foxes from West Pomerania were tested; *Trichinella* spp. prevalence was 4.4%. *Trichinella* spp. prevalence in wild boars on Wolin Island Poland was even higher (6.12% in 2008).

In Germany, the *Trichinella* spp. prevalence in foxes is <1% (*25*–*27*). However, the raccoon dog can also play a major role in maintenance of the *Trichinella* spp. sylvatic cycle (*28*). Raccoon dogs were first reported in Poland in 1955 (*29*); this species has become established in eastern Europe and has extended its settlement area to the west and south. The raccoon dog population in Germany has increased dramatically in recent years. In the former German Democratic Republic, only 58 raccoon dogs were hunted before 1987 (*30*). In 2001–2002, a total of ≈12,000 raccoon dogs were hunted in Germany; >96.0% were hunted in Mecklenburg–Western Pomerania and Brandenburg, the most eastern German Federal States (*31*). Studies in eastern Germany showed that 25.9%–35.1% of the raccoon dog diet is carrion (*32*). When intensity of infection in these animals was compared with that of other carnivores, the raccoon dog had higher larvae loads (*33*,*34*), which indicates the role of this species as a *Trichinella* spp. reservoir. In addition, *T. spiralis* can survive in rat carcasses during the summer in northern Europe for 2–4 weeks (*33*). Thiess et al. (*28*) examined 120 raccoon dogs from the northern part of Brandenburg; *T. spiralis* was identified in 5.0%. Results of that study support our findings in Mecklenburg–Western Pomerania.

Our data are insufficient to fully ascertain risk factors specific for the increase in *Trichinella* spp. prevalence in this region. Although for some years the raccoon dog has become widespread in Mecklenburg–Western Pomerania and Brandenburg in recent years, the *Trichinella* spp. prevalence in wild boars has increased mainly on Usedom Island. As for wild boars, the number of raccoon dogs hunted is considered to be proportional to the size of the total population. During 2005–2008, ≈34,000 raccoon dogs (1.15 raccoon dogs/km^2) were hunted in Brandenburg. During the same period, 3.38 raccoon dogs/km^2 were hunted in Mecklenburg–Western Pomerania, which indicated that the raccoon dog population in this region is larger than that in Brandenburg (*35*–*38*). During this time, numbers of wild boars and foxes did not differ between these 2 regions of Germany, and the hunting activity was expected to be similar in both regions.

The optimal habitat for raccoon dogs is wet areas and fields, small forests, and large ditches bordered by thick

Figure 2. Region in Germany and Poland investigated for *Trichinella* spp. 1, Ostvorpommern, Germany; 2, Usedom Island, Germany; 3, Wolin Island, Poland. Triangles, *Trichinella* spp.–positive cases in raccoon dogs; circles, cases in wild boars; square, location of outbreak farm; white circles and triangles, cases in 2005; light gray circles, cases in 2006; dark gray circles, cases in 2007; black circles, cases in 2008.

Table 4. Western blot results for *Trichinella* spp. in wild boars and red foxes, Wolin Island, Poland

Year	Wild boars, no. positive/ no. tested (%)	Red foxes, no. positive/ no. tested (%)
2004	1/107 (0.93)	0/21 (0)
2005	1/150 (0.67)	2/32 (6.25)
2006	2/84 (2.38)	1/26 (3.85)
2007	3/86 (3.490	1/25 (4.00)
2008	15/245 (6.12)	2/38 (5.26)

vegetation (*39*); this habitat corresponds to the landscape in northeastern Germany and northwestern Poland. Also, since 1960, large areas of the Wolin Island have been designated as a national park, which may have led to an increase in the raccoon dog population. Because the body of water separating Wolin Island from Usedom Island is narrower than the Stettiner Haff Lagoon and the Untere Oder (part of the Oder River) to the south, raccoon dog migration from east to west might have occurred through this route. Whether future increased spread of raccoon dogs in Brandenburg will result in increased *Trichinella* spp. prevalence in wild boars is unclear.

In summary, since 2005, an increase in *Trichinella* prevalence spp. in wild boars has occurred in Mecklenburg–Western Pomerania, Germany. This increase in *Trichinella* spp. prevalence in the sylvatic cycle may be associated with spread of raccoon dogs in this region. This increase poses a threat to public health because of pigs kept in backyards. Backyard farming is fairly common in the region and because of economic reasons has been traditionally associated with feeding of kitchen scraps to pigs.

For intensive pig production units that practice high levels of biosecurity, risk for *Trichinella* spp. infection is low. Although feeding with swill is illegal (*40*), feeding of kitchen scraps including wild boar offal still occurs, which increases risk for human outbreaks. Although scavenging of wild boar, fox, or raccoon dog carcasses by pigs is rare, exposure of domestic pigs kept outdoors to *Trichinella* spp. cannot be excluded. Therefore, farmers should be made aware of increased potential risk factors, especially feeding with swill. Also, hunters should be encouraged to remove carcasses from the forest or bury them at an appropriate depth. It would also be advisable to monitor whether the western and southern migration of raccoon dogs is indicative of increased *Trichinella* spp. prevalence in the sylvatic cycle in newly settled areas. Furthermore, programs are needed that emphasize the necessity of ensuring testing for *Trichinella* spp. infection in all wild boars intended for human consumption and promoting education of humans regarding thorough cooking of meat to guarantee food safety.

Acknowledgments

We thank Sascha Gerst for providing data on foxes and raccoon dogs; Nicole Awe, Holger Vogel, Heike Kerlikowsky, and Gaby Splittgerber for providing hunting statistics and data on wild boars; and Sabine Reckinger for performing serologic, parasitologic, and molecular examinations.

Dr Pannwitz is district veterinary officer in Ostvorpommern, Mecklenburg–Western Pomerania. His main interests include zoonoses, veterinary epidemiology, and veterinary public health.

References

1. Gottstein B, Pozio E, Nockler K. Epidemiology, diagnosis, treatment, and control of trichinellosis. Clin Microbiol Rev. 2009;22:127–42. DOI: 10.1128/CMR.00026-08
2. Dorny P, Praet N, Deckers N, Gabriel S. Emerging food-borne parasites. Vet Parasitol. 2009;163:196–206. DOI: 10.1016/j.vetpar.2009.05.026
3. Ozeretskovskaya NN, Mikhailova LG, Sabgaida TP, Dovgalev AS. New trends and clinical patterns of human trichinellosis in Russia at the beginning of the XXI century. Vet Parasitol. 2005;132:167–71. DOI: 10.1016/j.vetpar.2005.05.056
4. Boireau P, Vallee I, Roman T, Perret C, Mingyuan L, Gamble HR, et al. *Trichinella* in horses: a low frequency infection with high human risk. Vet Parasitol. 2000;93:309–20. DOI: 10.1016/S0304-4017(00)00348-4
5. Pozio E, Hoberg E, La Rosa G, Zarlenga DS. Molecular taxonomy, phylogeny and biogeography of nematodes belonging to the *Trichinella* genus. Infect Genet Evol. 2009;9:606–16. DOI: 10.1016/j.meegid.2009.03.003
6. Commission Regulation (EC) no. 2075/2005 of 5 December 2005. Laying down specific rules on official controls for *Trichinella* in meat [cited 2010 Mar 21]. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2005:338:0060:0082:EN:PDF
7. Nöckler K. Vorkommen und bedeutung von *Trichinella* spp. in Deutschland. Veterinary Medicine Austria. 2005;92:301–7.
8. International Trichinella Reference Center [cited 2010 Feb 10]. http://www.iss.it/site/Trichinella/index.asp
9. Robert Koch Institute. SurvStat@RKI [cited 2009 Jan 1]. http://www3.rki.de/SurvStat/
10. Jansen A, Schöneberg I, Stark K, Nöckler K. Epidemiology of trichinellosis in Germany, 1996–2006. Vector Borne Zoonotic Dis. 2008;8:189–96. DOI: 10.1089/vbz.2007.0183
11. Littmann M, Nöckler K. Trichinellose: Zu einer Häufung in Mecklenburg-Vorpommern. [RKI]. Epidemiologisches Bulletin. 2006;18:139–40.
12. Pozio E, La Rosa G. PCR-derived methods for the identification of *Trichinella* parasites from animal and human samples. Methods Mol Biol. 2003;216:299–309.
13. Nöckler K, Voigt WP, Protz D, Miko A, Ziedler K. Diagnosis of trichinellosis in living pigs using indirect ELISA [in German]. Berl Munch Tierarztl Wochenschr. 1995;108:167–74.
14. Gamble HR, Anderson WR, Graham CE, Murrell KD. Diagnosis of swine trichinosis by enzyme-linked immunosorbent assay (ELISA) using an excretory–secretory antigen. Vet Parasitol. 1983;13:349–61. DOI: 10.1016/0304-4017(83)90051-1
15. Leuenberger R, Boujon P, Thür B, Miserez R, Garin-Bastuji B, Rüfenacht J, et al. Surveillance of wild boar in Switzerland: prevalence of classical swine fever, Aujeszky's disease and brucellosis. Vet Rec. 2007;160:362–8.
16. Praxishilfe Wildschweinmanagement. Schweizerische eidgenossenschaft, bundesamt für umwelt BAFU. Teil A [cited 2010 Feb 10]. http://www.wildschwein-sanglier.ch/ws_start.php?la=d&th=prax&st=1
17. Ökologischer Jagdverband Sachsen [cited 2010 Feb 10]. http://www.sachsen-oejv.de/index.html

18. German Federal Statistical Office. Fachserie 3 Reihe 4.3 (2001–2007) [cited 2010 Mar 21]. http://www.destatis.de/jetspeed/portal/cms/Sites/destatis/Internet/EN/press/pr/2008/01/PE08__015__811,templateId=renderPrint.psml
19. Clopper CJ, Pearson ES. The use of confidence or fiducial limits illustrated in the case of the binomial. Biometrika. 1934;26:404–13. DOI: 10.1093/biomet/26.4.404
20. Nöckler K, Reckinger S, Pozio E. *Trichinella spiralis* and *Trichinella pseudospiralis* mixed infection in a wild boar (*Sus scrofa*) of Germany. Vet Parasitol. 2006;137:364–8. DOI: 10.1016/j.vetpar.2006.01.031
21. Pozio E, Zarlenga DS. Recent advances on the taxonomy, systematics and epidemiology of *Trichinella*. Int J Parasitol. 2005;35:1191–204. DOI: 10.1016/j.ijpara.2005.07.012
22. Sadkowska-Todys M, Golab E, Rosinska M. Geographical distribution of human and animal trichinellosis in Poland: is there a connection? 5th Annual Scientific Meeting of MedVetNet, 2009 May 5; Madrid, Spain. Maisons-Alfort (France): Agence Française de Sécurité Sanitaire des Aliments. Abstract book; 2009. p. 32.
23. Golab E, Sadkowska-Todys M. Epidemiology of human trichinellosis in Poland—currently and in the past [in Polish]. Wiad Parazytol. 2006;52:181–7.
24. Balicka-Ramisz A, Grupinski T, Ramisz A, Pilarczyk B, Laurans L. Prevalence of *Trichinella* spp. in red foxes and wild boars in the northwestern part of Poland [in German]. Dtsch Tierarztl Wochenschr. 2007;114:354–7.
25. Wacker K, Rodriguez E, Garate T, Geue L, Tackmann K, Selhorst T, et al. Epidemiological analysis of *Trichinella spiralis* infections of foxes in Brandenburg, Germany. Epidemiol Infect. 1999;123:139–47. DOI: 10.1017/S0950268899002617
26. Wagner JA, Frank W. Recent research on the prevalence of *Trichinella* in wild mammals in the Federal Republic of Germany. In: Tanner CE, Martinez-Fernandez AR, Bolas-Fernandez, F, editors. Trichinellosis. Proceedings of the Seventh International Conference on Trichinellosis, 1988 May 2–6; Alicante, Spain. Madrid: CSIC Press; 1989. p. 416–22.
27. Hoffmann L, Klengel K, Worbes H, Orthey G. Zur Prävalenz von *Echinococcus multilocularis* und *Trichinella* spp. beim Rotfuchs in Thüringen. Neue Methoden und Ergebnisse zur Epidemiologie von Parasitosen. Hannover (Germany): DVG Tagung, 10–12.03; 1999.
28. Thiess A, Schuster R, Nöckler K, Mix H. Helminth findings in indigenous raccoon dogs *Nyctereutes procyonoides* (Gray, 1843) [in German]. Berl Munch Tierarztl Wochenschr. 2001;114:273–6.
29. Dudzinski W, Haber A, Matuszewski G. Die Verbreitung des Marderhundes (*Nyctereutes procyonoides* Gray) in Polen. Zeitschrift fur Jagdwissenschaft. 1963;9:98. DOI: 10.1007/BF01956831
30. Stubbe M. Neue Erkenntnisse zur Ökologie und Verbreitung der Marderhundes Nyctereutes procyonoides (Gray, 1834) in der DDR. Beiträage zur Jagd- und Wildforschung. 1989;16:261–7.
31. Goretzki J. Invasoren auf vier pfoten. Forschungsreport. 2003;2:24–7.
32. Thiess A. Untersuchungen zur Helminthenfauna und zum Vorkommen von *Trichinella* sp. beim Marderhund (*Nyctereutes procyonoides*) in Brandenburg [dissertation]. Berlin: Federal University; 2004.
33. Oivanen L, Kapel CMO, Pozio E, La Rosa G, Mikkonen T, Sukura A. Associations between *Trichinella* species and host species in Finland. J Parasitol. 2002;88:84–8.
34. Malakauskas A, Paulauskas V, Jarvis T, Keidans P, Eddi C, Kapel CM. Molecular epidemiology of *Trichinella* spp. in three Baltic countries: Lithuania, Latvia and Estonia. Parasitol Res. 2007;100:687–93. DOI: 10.1007/s00436-006-0320-y
35. Jagdbericht Mecklenburg-Vorpommern 2006/07 [cited 2009 Apr 17]. http://www.ljv-mecklenburg-vorpommern.de/Jagdbericht%202006-2007.pdf
36. Jagdbericht Mecklenburg-Vorpommern 2005/06 [cited 2009 Apr 17]. http://www.ljv-mecklenburg-vorpommern.de/Jagdbericht_2005_2006.pdf
37. Jagdbericht Mecklenburg-Vorpommern 2004/2005 [cited 2009 Apr 17]. http://www.kora.ch/malme/05_library/5_1_publications/R/Rackwitz_et_al_2006_13.Jagdbericht_Mecklenburg_Vorpommern.pdf
38. Jagdberichte des Landes Brandenburg [cited 2009 Apr 17]. http://www.mluv.brandenburg.de/cms/detail.php/124304
39. Holmala K, Kauhala K. Ecology of wildlife rabies in Europe. Mammal Review. 2006;36:17–36. DOI: 10.1111/j.1365-2907.2006.00078.x
40. Regulation (EC) no. 1774/2002 of the European Parliament and of the Council of 3 October 2002. Laying down health rules concerning animal products not intended for human consumption [cited 2010 Mar 21]. http://europa.eu/legislation_summaries/food_safety/specific_themes/f81001_en.htm

Address for correspondence: Anne Mayer-Scholl, Federal Institute for Risk Assessment, Diedersdorfer Weg 1, Berlin 12277, Germany; email: anne.mayer-scholl@bfr.bund.de

New Measles Virus Genotype Associated with Outbreak, China

Yan Zhang,[1] Zhengrong Ding,[1] Huiling Wang, Liqun Li, Yankun Pang, Kevin E. Brown, Songtao Xu, Zhen Zhu, Paul A. Rota, David Featherstone, and Wenbo Xu

To determine the origin of the virus associated with a measles outbreak in Menglian County, Yunnan Province, People's Republic of China, in 2009, we conducted genetic analyses. Phylogenetic analyses based on nucleoprotein (N) and hemagglutinin (H) gene sequences showed that these Menglian viruses were not closely related to sequences of any World Health Organization (WHO) reference strains representing the 23 currently recognized genotypes. The minimum nucleotide divergence between the Menglian viruses and the most closely related reference strain, genotype D7, was 3.3% for the N gene and 3.0% for the H gene. A search of the databases of GenBank, WHO, and the Health Protection Agency Measles Nucleotide Surveillance showed that the Menglian viruses, together with the 2 older non-Menglian viruses, could be members of a new proposed measles genotype, d11. The new genotype designation will allow for better description of measles transmission patterns, especially in the Southeast Asian and Western Pacific regions.

Measles virus is a negative-sense, single-stranded RNA virus in the family *Paramyxoviridae,* genus *Morbillivirus*. Infection with this virus typically causes high fever, maculopapular rash, conjunctivitis, cough, and coryza (*1*). Measles virus is monotypic, but genetic variation in the hemagglutinin (H) and nucleoprotein (N) genes can be analyzed by molecular epidemiologic techniques and used to study virus transmission patterns (*2*–*4*). The World Health Organization (WHO) currently recognizes 23 genotypes of measles virus and has established guidelines for the designation of new genotypes (*3*–*7*).

Although deaths attributed to measles have declined by 78% worldwide, from ≈733,000 deaths in 2000 to ≈164,000 in 2008, risk for illness and death from measles still exists in countries with poor routine vaccination coverage, and outbreaks are a threat in most of these countries (*6*,*8*). When the Universal Childhood Immunization goals were attained in the 1990s, illness and death from measles in the People's Republic of China decreased dramatically. During 1995–2009, measles incidence was 5–10/100,000 population, and <250 measles-associated deaths were reported each year (*9*–*11*; National Notifiable Disease Reporting System of China [http://202.106.123.35/]). Outbreaks of measles continued to occur because of increased numbers of susceptible children, especially in areas with low routine vaccination coverage. At the end of 2009, a measles outbreak in Menglian County was reported to the National Measles Laboratory of the Chinese Center for Disease Control and Prevention. Menglian County is situated on the western side of Yunnan Province and shares a border with Myanmar. As part of routine surveillance activities, the measles outbreak was investigated. Cases were confirmed through detection of immunoglobulin (Ig) M, and virus isolates were obtained for genetic analysis.

Materials and Methods

Specimen Collection

Staff members from the Yunnan Center for Disease Control and Prevention collected serum and throat swab or

Author affiliations: National Institute for Viral Disease Control and Prevention, Beijing, People's Republic of China (Y. Zhang, H. Wang, S. Xu, Z. Zhu, W. Xu); Yunnan Center for Disease Control and Prevention, Kunming, People's Republic of China (Z. Ding, L. Li, Y. Pang); Capital Medical University, Beijing (H. Wang); Beijing Children's Hospital, Beijing (H. Wang); Health Protection Agency, London, UK (K.E. Brown); Centers for Disease Control and Prevention, Atlanta, Georgia, USA (P.A. Rota); and World Health Organization, Geneva, Switzerland (D. Featherstone)

DOI: 10.3201/eid1606.100089

[1]These authors contributed equally to this study.

urine specimens from patients during the outbreak (14 from Myanmar and 16 from Menglian). Serum samples were collected from 9 Myanmar and 7 Menglian case-patients. Throat swabs or urine specimens, from the first 6 Menglian patients, were obtained according to the WHO procedures for laboratory diagnoses of measles and rubella virus infections (*12*) and transported to the National Measles Laboratory for processing by standard procedures. To confirm all suspected cases, we used ELISA kits (Virion/Serion GmbH, Würzburg, Germany) to detect measles and rubella virus IgM.

Virus Isolation, PCR, and Sequencing

Measles virus was isolated by using the Vero/hSLAM cell line (*13*), and infected cells were harvested when >75% of the culture showed cytopathic effect (*12*). Meanwhile, RNA was extracted from all clinical specimens by using the QIAamp Viral RNA Mini Kit (QIAGEN, Beijing, China) according to manufacturer's instructions. Reverse transcription–PCR (RT-PCR) was used to amplify either the 550 nt coding for the COOH terminus of the N gene or the full-length open reading frame for the H gene (*14*). PCR products were purified by using a QIAquick Gel Extraction kit (QIAGEN). Sequences of the amplicons were obtained by using BigDye terminator version 2.0 chemistry according to the manufacturer's protocol for both sense and antisense strands on an automated ABI PRISMTM 3100 DNA Sequencer (PerkinElmer, Beijing, China). Sequences were analyzed by using SequencerTM (Gene Codes Corporation, Ann Arbor, MI, USA) and version 7.0 of BioEdit (www.mbio.ncsu.edu/BioEdit/BioEdit.html). Phylogenetic analyses were performed and trees were generated by using MEGA4 (www.megasoftware.net). The robustness of the groupings was assessed by using bootstrap resampling of 1,000 replicates.

Results

During 2008–2009, routine measles vaccine coverage in Menglian County was as high as 95%; the last local measles case before this outbreak had been reported in June 2008. The outbreak began when 14 persons from Myanmar became ill with fever and rash and sought healthcare in Menglian during October 10–November 28, 2009. The first measles case in a person from Menglian occurred on November 5, 2009; the last occurred on December 20. On December 28, the number of measles cases from the outbreak totaled 14 persons from Myanmar and 16 from Menglian. Measles was confirmed by laboratory detection of IgM in all 16 patients from whom serum samples were available (9 from Myanmar and 7 from Menglian). No IgM against rubella virus was detected (Table 1).

Two measles virus isolates, MVi/Menglian.Yunnan.CHN/47.09 and MVi/Menglian.Yunnan.CHN/51.09, were obtained from urine and throat swab specimens of 2 persons from Menglian. Positive RT-PCR products of the partial N gene were obtained from clinical specimens from 5 of 6 case-patients, and the entire H gene was amplified from the 2 isolates (Table 2). The sequence of the 450 nucleotides coding for the 150 amino acids at the COOH terminus of the N gene was obtained for all 5 case-patients, and the entire coding region of the H gene was sequenced for 1 representative isolate. Nucleotide sequence data for the strains from the Menglian case-patients were deposited in GenBank under accession nos. GU440571–GU440576. Sequence analysis of the C-terminal 450 nucleotides of the N gene of 2 isolates and 5 clinical specimens revealed identical sequences, suggesting a single chain of transmission. Phylogenetic and genetic distance analyses based on both N and H gene sequences showed that these viruses from Menglian case-patients were members of clade D (Figures 1, 2; Table 3).

Table 1. Characteristics of patients with laboratory-confirmed measles, 2009*

Patient no.	Patient age	Residence†	Date of most recent vaccination	Date of disease onset
1	9 y	Myanmar	Unknown	Nov 4
2	9 y	Myanmar	Unknown	Nov 4
3	8 y	Myanmar	Unknown	Nov 4
4	7 y	Myanmar	Unknown	Nov 6
5	2 y	Myanmar	Unknown	Nov 3
6	2 y	Menglian	Unknown	Nov 9
7	39 y	Menglian	Unknown	Nov 9
8	21 y	Menglian	Unknown	Nov 5
9	18 mo	Myanmar	Unknown	Nov 13
10	6 mo	Menglian	No vaccination	Nov 14
11	13 y	Myanmar	Unknown	Nov 6
12	8 y	Myanmar	No vaccination	Nov 22
13	20 y	Myanmar	No vaccination	Nov 20
14	2 y	Menglian	2008 Jan15	Nov 19
15	5 y	Menglian	2006 Jun 7	Dec 9
16	5 y	Menglian	2008 Oct 27	Nov 5

*For all patients, immunoglobulin M against measles virus was positive and against rubella virus was negative.
†Menglian, Menglian County, Yunnan Province, People's Republic of China.

Table 2. Description of measles viruses, proposed genotype d11, detected in Menglian County, Yunnan Province, People's Republic of China, 2009

Patient no.	Patient age	Result, by test		Strain name	GenBank accession no.
		RT-PCR	Virus isolation		
YN09–1	6 mo	+	+	MVi/Menglian.Yunnan.CHN/47.09	GU440571,† GU440576‡
YN09–2	45 y	+	–	MVs/Menglian.Yunnan.CHN/47.09/1	GU440572†
YN09–3	21 y	+	–	MVs/Menglian.Yunnan.CHN/47.09/2	GU440573†
YN09–4	6 mo	+	+	MVi/Menglian.Yunnan.CHN/51.09	GU440574†
YN09–5	5 y	+	–	MVs/Menglian.Yunnan.CHN/51.09	GU440575†

*RT-PCR, reverse transcription–PCR; MVi, measles virus sequence from isolates; MVs, measles virus sequence from clinical specimens.
†Sequence of N partial gene.
‡Sequence of H gene.

The sequences of these viruses were not closely related to the sequences of any of the WHO reference strains that represented the 23 currently recognized genotypes. These sequences were closest to the sequence of the genotype D7 reference strain and the contemporary genotype D7 strains available from GenBank. The minimum nucleotide divergence between the Menglian viruses and the D7 WHO reference strain (Victoria.AUS/16.85) was 3.3% for the N gene and 3.0% for the H gene (Table 3). Bootstrap analysis of Menglian H-gene sequences and the WHO reference sequences showed 100% confidence in the group containing the Menglian viruses. When the N-gene sequences were compared with WHO reference sequences and contemporary genotype D7 sequences, bootstrap support for the Menglian branch was 100% (Figure 1).

A search of the databases of GenBank, WHO, and the Health Protection Agency Measles Nucleotide Surveillance (www.who-measles.org) identified only 2 closely related sequences. The most closely related sequence was from a measles virus isolate, MVi/WA.AUS/12.01 (GenBank accession no. AF481492), previously designated as genotype D7, which had been imported from Myanmar into Australia in 2001 (*15*). The second, from a clinical specimen, MVs/Uvbridge.GBR/19.04 (GenBank accession no. GU937234), was detected in an oral fluid sample collected in 2004 from a person who had recently returned to the United Kingdom from Bangladesh. Both of these related sequences shared 98.4% nucleotide homology with the Menglian virus over the

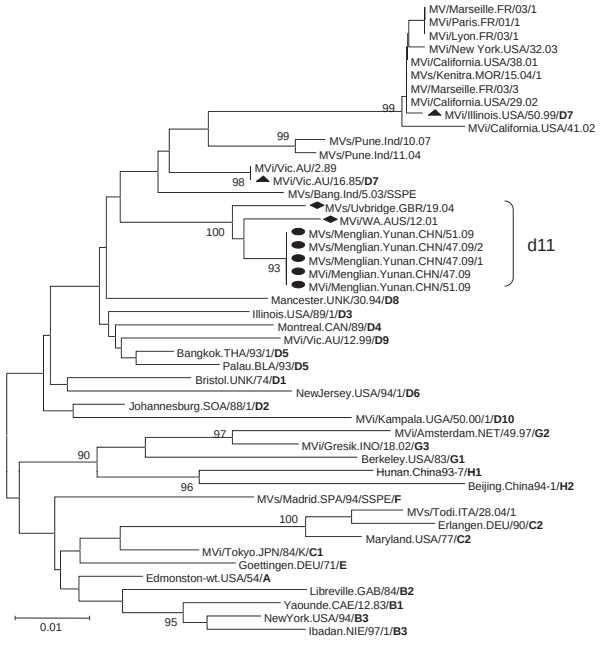


Figure 1. Phylogenetic analysis of the sequences of the nucleoprotein genes (450 nt) of the strains of measles virus from Menglian County, Yunnan Province, People's Republic of China. The unrooted tree shows sequences from Menglian viruses (circles) compared with World Health Organization (WHO) reference strains for each genotype. Triangles indicate D7 WHO reference strains; diamonds, the 2 older non-Menglian strains. Genotype designation is in **boldface.** MV, measles virus; MVi, measles virus sequence from isolates; MVs, measles virus sequence from clinical specimens. Scale bar indicates base substitutions per site.

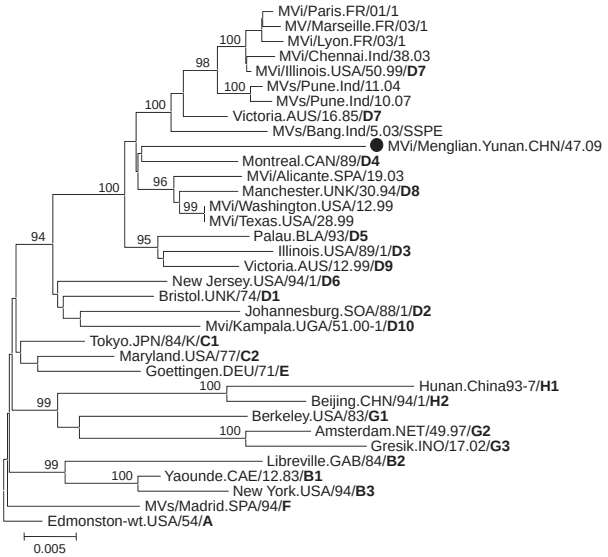


Figure 2. Phylogenetic analysis of the sequences of the hemagglutinin genes of the strains of measles virus from Menglian County, Yunnan Province, People's Republic of China. The unrooted tree shows sequences from the Menglian viruses (circles) compared with World Health Organization reference strains for each genotype. Genotype designation is in **boldface.** MVi, measles virus sequence from isolates; MV, measles virus; MVs, measles virus sequence from clinical specimens; wt, wild type. Scale bar indicates base substitutions per site.

Table 3. Genetic distances between proposed reference strain for genotype d11 (MVi/Menglian.Yunnan.CHN/47.09) and reference strains*

WHO reference strain, genotype: virus name	Genotype d11, % nucleotide divergence	
	Nucleoprotein gene	Hemagglutinin gene
A: Edmonston-wt.USA/54	5.8	3.7
B1:Yaounde.CAE/12.83	7.3	4.7
B2: Libreville.GAB/84	7.8	5.6
B3: New York.USA/94	7.1	5.2
C1: Tokyo.JPN/84/K	6.0	3.9
C2: Maryland.USA/77	7.8	4.4
D1: Bristol.UNK/74	4.4	4.0
D2: Johannesburg.SOA/88/1	4.4	4.6
D3: Illinois.USA/89/1	4.2	3.7
D4: Montreal.CAN/89	4.7	3.0
D5: Palau.BLA/93	3.8	3.5
D6: New Jersey.USA/94/1	5.8	4.2
D7: Victoria.AUS/16.85	3.3	3.0
D8: Manchester.UNK/30.94	4.4	3.0
D9: Victoria.AUS/12.99	4.9	3.5
D10: Kampala.UGA/51.00/1	7.3	4.3
E: Goettingen.DEU/71	6.4	4.5
F: MVs/Madrid.SPA/94 SSPE	6.4	4.4
G1: Berkeley.USA/83	8.0	5.2
G2: Amsterdam.NET/49.97	8.7	5.8
G3: Gresik.INO/17.02	7.6	6.3
H1: Hunan.CHN/93/7	7.6	6.6
H2: Beijing.CHN/94/1	9.1	5.7

*WHO, World Health Organization; CAE, Cameroon; GAB, Gabon; JPN, Japan; UNK, United Kingdom; SOA, South Africa; CAN, Canada; AUS, Australia; UGA, Uganda; DEU, Germany; SPA, Spain; NET, the Netherlands; INO, Indonesia; CHN, China.

450 nucleotides coding for the 150 amino acids at the COOH terminus of the N gene. Phylogenetic analysis showed that the 2 older non-Menglian strains (MVi/WA.AUS/12.01 and MVs/Uvbridge.GBR/19.04) formed a unique cluster combined with 5 Menglian strains (Figure 1).

Discussion

The Menglian measles viruses, together with the 2 closely related viruses, MVi/WA.AUS/12.01 and MVs/Uvbridge.GBR/19.04, should be considered as members of a newly proposed measles genotype, d11. MVi/Menglian.Yunnan.CHN/47.09 (GenBank accession nos.: N, GU440571; H, GU440576) was chosen as the reference strain because an isolate is available, it grows to high titer in cell culture, and the sequence is representative of the Menglian strains. Although designation as a genotype is not permanent until after the new genotype has been acknowledged and formally designated by WHO, the percentage sequence divergence between the N and H gene sequences of the Menglian isolates and the sequences of the reference strains exceed the recommended threshold for designation of a new measles genotype, 2.5% and 2.0%, respectively (*3*–*5*). Phylogenetic analyses indicated that these viruses form a unique cluster compared with all previously characterized wild-type measles viruses. Because the Australia virus was imported from Myanmar and the sequence from the United Kingdom originated in a country adjacent to Myanmar, these sequences should be included as members of the proposed d11 genotype.

The most useful criterion for a new genotype is that it must have some epidemiologic utility for describing the transmission pathways of measles virus. The Menglian viruses responsible for the measles outbreak in China represent strains that are probably associated with endemic transmission of virus in Myanmar. When we consider the sequence of the Australia virus, we believe that the genotype d11 measles virus might have been circulating in Myanmar since 2001. The d11 sequences are easily distinguished from the sequences of viruses in other genotypes (e.g., D8, D4, H1, D5, D9, G3) that are circulating in neighboring countries. Therefore, the Menglian viruses are providing baseline genetic information about viruses endemic to Myanmar and possibly neighboring countries. The new genotype designation will enable better description of measles transmission patterns, especially in the Southeast Asian and Western Pacific regions of WHO.

Molecular epidemiologic data, when analyzed in conjunction with standard epidemiologic data, can help document viral transmission pathways, identify whether a virus is endemic or imported, and aid in case classification, thus enhancing control and elimination programs (*16*–*20*). Genetic characterization of measles viruses circulating in China from 1993 through 2009 demonstrated that genotype H1 was widely distributed throughout the country and

that China has a single, endemic genotype (*9*–*11*; National Measles Laboratory database, unpub. data) However, data on the circulating genotypes in some other countries in the Southeast Asian and Western Pacific regions of WHO are limited. Further sequence analysis of measles virus strains circulating in Myanmar and neighboring countries should clarify the distribution pattern of this newly recognized genotype and may enable recognition of other new genotypes. Countries in the Western Pacific Region, including China, have committed to 2012 as the target year for measles elimination (*21*). However, rapidly identifying imported measles cases and controlling spread are major challenges for achieving this goal. Enhancing measles virus surveillance to quickly identify imported cases of measles will become more critical as measles elimination goals are achieved throughout the world.

Acknowledgments

We thank the Yunnan provincial, prefectural, and county staff members who collected the clinical specimens for this study.

This study was supported by the Key Technologies Research and Development Program of the National Ministry of Science (grant nos. 2009ZX10004-201, 2009ZX10004-202, and 2008ZX10004-014-5) from China and the grants from the WHO fund for the Western Pacific Regional Office of the Measles Regional Reference Laboratory.

Dr Zhang is an associate researcher at the National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention. Her major research interests are the epidemiology and molecular biology of respiratory viruses.

References

1. Griffin DE. Measles virus. 4th ed. Philadelphia: Lippincott Williams and Wilkins; 2001.
2. Rota PA, Rota JS, Bellini WJ. Molecular epidemiology of measles virus. Seminars in Virology. 1995;6:379–86. DOI: 10.1016/S1044-5773(05)80015-0
3. World Health Organization. Nomenclature for describing the genetic characteristics of wild-type measles viruses (update). Part 2. Wkly Epidemiol Rec. 2001;76:249–51.
4. World Health Organization. Nomenclature for describing the genetic characteristics of wild-type measles viruses (update). Part 1. Wkly Epidemiol Rec. 2001;76:241–7.
5. World Health Organization. Update of the nomenclature for describing the genetic characteristics of wild-type measles viruses: new genotypes and reference strains. Wkly Epidemiol Rec. 2003;78:229–32.
6. World Health Organization. Global measles and rubella laboratory network–update. Wkly Epidemiol Rec. 2005;80:384–8.
7. World Health Organization. New genotype of measles virus and update on global distribution of measles genotypes. Wkly Epidemiol Rec. 2005;80:347–51.
8. World Health Organization. Global reductions in measles mortality 2000–2008 and the risk of measles resurgence. Wkly Epidemiol Rec. 2009;84:505–16.
9. Zhang Y, Zhu Z, Rota PA, Jiang X, Hu J, Wang J, et al. Molecular epidemiology of measles viruses in China, 1995–2003. Virol J. 2007;4:14. DOI: 10.1186/1743-422X-4-14
10. Zhang Y, Ji Y, Jiang X, Xu S, Zhu Z, Zheng L, et al. Genetic characterization of measles viruses in China, 2004. Virol J. 2008;5:120. DOI: 10.1186/1743-422X-5-120
11. Ji Y, Zhang Y, Xu S, Zhu Z, Zuo S, Jiang X, et al. Measles resurgence associated with continued circulation of genotype H1 viruses in China, 2005. Virol J. 2009;6:135. DOI: 10.1186/1743-422X-6-135
12. World Health Organization. Manual for the laboratory diagnosis of measles and rubella viral infection. 2nd ed. 2007 [cited 2010 Apr 8]. http://www.who.int/vaccines-documents
13. Ono N, Tatsuo H, Hidaka Y, Aoki T, Minagawa H, Yanagi Y. Measles viruses on throat swabs from measles patients use signaling lymphocytic activation molecule (CDw150) but not CD46 as a cellular receptor. J Virol. 2001;75:4399–401. DOI: 10.1128/JVI.75.9.4399-4401.2001
14. Liffick SL, Thi Thoung N, Xu W, Li Y, Phoung Lien H, Bellini WJ, et al. Genetic characterization of contemporary wild-type measles viruses from Vietnam and the People's Republic of China: identification of two genotypes within clade H. Virus Res. 2001;77:81–7. DOI: 10.1016/S0168-1702(01)00268-4
15. Chibo D, Riddell M, Catton M, Lyon M, Lum G, Birch C. Studies of measles viruses circulating in Australia between 1999 and 2001 reveals a new genotype. Virus Res. 2003;91:213–21. DOI: 10.1016/S0168-1702(02)00273-3
16. Bellini WJ, Rota PA. Genetic diversity of wild-type measles viruses: implications for global measles elimination programs. Emerg Infect Dis. 1998;4:29–35. DOI: 10.3201/eid0401.980105
17. Rota JS, Rota PA, Redd SB, Redd SC, Pattamadilok S, Bellini WJ. Genetic analysis of measles viruses isolated in the United States, 1995–1996. J Infect Dis. 1998;177:204–8. DOI: 10.1086/513825
18. World Health Organization. Expanded Programme on Immunization (EPI). Standardization of the nomenclature for describing the genetic characteristics of wild-type measles viruses. Wkly Epidemiol Rec. 1998;73:265–72.
19. Rota PA, Liffick SL, Rota JS, Katz RS, Redd S, Papania M, et al. Molecular epidemiology of measles viruses in the United States, 1997–2001. Emerg Infect Dis. 2002;8:902–8.
20. Rota PA, Bellini WJ. Update on the global distribution of genotypes of wild-type measles viruses. J Infect Dis. 2003;187(Suppl 1):S270–6. DOI: 10.1086/368042
21. World Health Organization. WHO regional EPI targets: eliminate measles and control hepatitis B by 2012. Fifty-sixth session of the WHO Regional Committee for the Western Pacific; 2005 Sep 19–23; Noumea, New Caledonia. Press release [cited 2010 Apr 8]. http://www.wpro.who.int/rcm/en/archives/rc56/press_releases/pr_20050922+RCM.htm

Address for correspondence: Wenbo Xu, National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Rm 509, 27 Nan Wei Rd, Xuan Wu District, Beijing, China 100050; email: wenbo_xu1@yahoo.com.cn

Clonal Expansion of Multidrug-Resistant and Extensively Drug-Resistant Tuberculosis, Japan

Yoshiro Murase, Shinji Maeda, Hiroyuki Yamada, Akihiro Ohkado, Kinuyo Chikamatsu, Kazue Mizuno, Seiya Kato, and Satoshi Mitarai

The emergence and spread of multidrug-resistant (MDR) and extensively drug-resistant (XDR) tuberculosis (TB) has raised public health concern about global control of TB. To estimate the transmission dynamics of MDR and XDR TB, we conducted a DNA fingerprinting analysis of 55 MDR/XDR *Mycobacterium tuberculosis* strains isolated from TB patients throughout Japan in 2002. Twenty-one (38%) of the strains were classified into 9 clusters with geographic links, which suggests that community transmission of MDR/XDR TB is ongoing. Furthermore, the XDR *M. tuberculosis* strains were more likely than the non–XDR MDR strains to be clustered (71% vs. 24%; p = 0.003), suggesting that transmission plays a critical role in the new incidence of XDR TB. These findings highlight the difficulty of preventing community transmission of XDR TB by conventional TB control programs and indicate an urgent need for a more appropriate strategy to contain highly developed drug-resistant TB.

The epidemic of drug-resistant tuberculosis (TB) has raised public health concern about the global control of TB. The World Health Organization estimated that 0.5 million cases of multidrug-resistant TB (MDR TB) (i.e., *Mycobacterium tuberculosis* resistant to ≥2 of the most potent TB drugs, rifampin and isoniazid) occurred in 2007 (*1*). Some countries have extraordinarily high rates of this disease, but the problem is universal, and the extent varies from 1 country to another.

Another recent alarming issue is the emergence of extensively drug-resistant TB (XDR TB) (i.e., *M. tuberculosis* with MDR plus resistance to any fluoroquinolone and >1 injectable drug, thus posing even greater management challenges than MDR TB alone). The treatment outcome of XDR TB is worse than that of simple MDR TB, and the death rate is particularly high among HIV-infected patients (*2*). Also, because XDR TB is much more expensive to manage in terms of prolonged medication and prolonged period of infectivity to other persons (*3*), it has the potential to exhaust human and financial resources of the public health system for TB control. Although this new life-threatening disease had been reported from 49 countries as of June 2008 (*4*), its transmissibility among immunocompetent persons is not well known (*5*).

In Japan, TB remains a major infectious disease; in 2008, a total of 19.4 cases/100,000 population were reported (*6*), and Japan is generally classified as a country with intermediate TB incidence. According to the most recent nationwide drug-resistance survey, the prevalence of MDR TB and XDR TB were 1.9% and 0.5%, respectively (*7*). Approximately one third of MDR and XDR (MDR/XDR) *M. tuberculosis* strains were isolated from new TB patients, implying ongoing transmission of MDR/XDR TB in Japan.

Our purpose was to evaluate the transmission dynamics of MDR/XDR TB by using strains from the most recent (2002) nationwide drug-resistance survey in Japan, an industrialized country with low HIV incidence and intermediate TB incidence. We did so by analyzing the MDR/XDR strains by molecular genotyping methods, i.e., insertion sequence *6110* restriction fragment length polymorphism (IS*6110*-RFLP), spacer-oligonucleotide genotyping (spoligotyping), and variable number tandem repeats (VNTR).

Materials and Methods

We used data and culture isolates obtained in the 2002 nationwide drug-resistance survey, as previously reported (*7*). Briefly, during June–November 2002, a total of 3,122

Author affiliation: The Research Institute of Tuberculosis/Japan Anti-Tuberculosis Association, Tokyo, Japan

DOI: 10.3201/eid1606.091844

clinical strains were collected from different patients who had started treatment in 99 hospitals throughout Japan. The number of patients enrolled represented 36.0% of all new reported TB cases during the study period. The sampling of the hospital was not randomized but was based on voluntary participation. The survey identified 60 MDR/XDR *M. tuberculosis* strains, 55 of which were analyzed in this study; the other 5 strains were unavailable for use in this study.

Patient Information

We used patient information collected from a standard data collection form in the drug-resistance survey in 2002 (*7*). The information included demographic data (age, sex, nationality, hospital, geographic area of the hospital), clinical data (history of TB treatment, site of TB disease, chest radiograph findings, underlying disease), and bacteriologic data (results of sputum smear test for acid-fast bacilli). Patients were classified as new if they had never been treated for TB for >4 weeks and as previously treated if they had ever been treated for TB for ≥4 weeks. The survey protocol conformed to the national guidelines for epidemiologic research (*8*).

Drug Susceptibility Testing

Drug susceptibility testing was performed at the Research Institute of Tuberculosis, Tokyo, by using the proportion method on standard 1% Ogawa egg-based slants (*7*) and the following drug concentrations: isoniazid 0.2 μg/mL, rifampin 40 μg/mL, streptomycin 10 μg/mL, ethambutol 2.5 μg/mL, ethionamide 20 μg/mL, kanamycin 20 μg/mL, cycloserine 30 μg/mL, *p*-aminosalicylic acid 0.5 μg/mL, and levofloxacin 1 μg/mL. Pyrazinamide susceptibility was tested by using MGIT AST (Becton Dickinson, Sparks, MD, USA) at a concentration of 100 μg/mL. All compounds were obtained from Sigma (St. Louis, MO, USA).

Definition of XDR TB

We defined XDR strains according to the World Health Organization definition of XDR (*1*) and drug availability for TB treatment in Japan. XDR strains were defined as *M. tuberculosis* strains with resistance to at least isoniazid, rifampin, levofloxacin, and kanamycin.

Molecular Genotyping

Three molecular genotyping methods based on IS*6110*-RFLP, spoligotyping, and VNTR were performed on the 55 MDR/XDR strains. IS*6110*-RFLP typing was performed according to the standardized protocol (*9*). The RFLP band patterns were compared by using the BioNumerics software package version 5.1 (Applied Maths, Kortrijk, Belgium). Strains with an identical band pattern were classified as an RFLP cluster. Spoligotyping was also performed according to the standard protocol (*10*). Classification of the spoligotype family was performed according to the international database, SpolDB4 (*11*). The VNTR analysis was conducted as described elsewhere (*12*) by using the standard 15 mycobacterial interspersed repetitive unit–VNTR proposed by Supply et al. (*13*), i.e., VNTRs 0424, 0577, 0580, 0802, 0960, 1644, 1955, 2163b, 2165, 2401, 2996, 3192, 3690, 4052, and 4156 plus 4 other loci, VNTRs 2074, 2372, 3155, and 3336. The latter 4 loci were added to increase discrimination for the Beijing family strains because of their high prevalence in Japan (*12*).

Statistical Analysis

Statistical analysis was performed with Epi Info software 3.51 (Centers for Disease Control and Prevention, Atlanta, GA, USA), by using χ^2 test or Fisher exact test for the comparison of proportions. A p value <0.05 was considered significant.

Results

Epidemiologic Characteristics of Patients

A total of 55 MDR/XDR cases were analyzed. The characteristics of patients with MDR/XDR TB are summarized and compared with those of patients with pansusceptible strains (n = 2,782) in Table 1. Patients with MDR/XDR TB were significantly more likely to be younger (odds ratio [OR] 5.69 for age 21–40 years; 4.11 for age 41–60 years) and foreigners (OR 6.41) and to have been previously treated (OR 10.55). All MDR/XDR TB patients had pulmonary disease, and these patients were significantly more likely to have cavitary lesions (OR 3.24) and to have positive sputum smear test results (OR 2.20).

Of the 55 MDR/XDR TB cases, 17 (31%) were XDR TB. We found no significant differences between patients with XDR TB and patients with MDR but not XDR (non–XDR MDR) TB. XDR TB patients tended to be female, although the difference was not statistically significant (p = 0.06, Fisher exact test).

Analysis by Spoligotyping

We performed spoligotyping to determine the population structure of the 55 MDR/XDR strains (Table 2). The most dominant spoligotype family in the MDR/XDR cases was the Beijing family genotype (62%, n = 34), followed by the T (13%, n = 7), Latino-American and Mediterranean (5%, n = 3), U (2%, n = 1), East-African Indian (2%, n = 1), and X (2%, n = 1) family genotypes. Eight (15%) strains were unclassified according to the database.

The proportion of the Beijing family, which is frequently reported to be associated with drug resistance (*14*), did not significantly differ between the non–XDR MDR *M.*

Table 1. Comparison of characteristics between TB patients with XDR TB and non-XDR MDR TB from the most recent (2002) nationwide drug susceptibility survey, Japan*

Characteristic	No. (%) cases				Odds ratio (95% confidence interval)	
	XDR, n = 17	Non–XDR MDR, n = 38	MDR/XDR, n = 55	Drug susceptible, n = 2,782	MDR/XDR vs. drug susceptible	XDR vs. non–XDR MDR
Age, y						
0–20	0	1 (3)	1 (2)	51 (2)	2.57 (–)	–
21–40	6 (35)	14 (37)	20 (36)	460 (17)	**5.69 (2.63–12.45)**	0.51 (0.12–2.18)
41–60	10 (59)	12 (32)	22 (40)	701 (25)	**4.11 (1.93–8.85)**	1
≥61	1 (6)	11 (29)	12 (22)	1,570 (56)	1	0.11 (0.00–1.11)
Sex						
M	9 (53)	30 (79)	39 (71)	1,973 (71)	1	1
F	8 (47)	8 (21)	16 (29)	809 (29)	1.00 (0.53–1.86)	3.33 (0.83–13.77)
Nationality						
Japanese	16 (94)	31 (82)	47 (85)	2,710 (97)	1	1
Foreigner	1 (6)	7 (18)	8 (15)	72 (3)	**6.41 (2.69–14.72)**	0.28 (0.01–2.64)
Treatment history						
New	8 (47)	17 (45)	25 (45)	2,498 (90)	1	1
Previous	9 (53)	21 (55)	30 (55)	284 (10)	**10.55 (5.93–18.83)**	0.91 (0.25–3.33)
Site of TB						
Pulmonary	17 (100)	38 (100)	55 (100)	2,687 (97)	–	–
Extrapulmonary	0	0	0	95 (3)	–	–
Chest radiograph finding						
Noncavitary	5 (29)	8 (21)	13 (24)	1,394 (50)	1	1
Cavitary	12 (71)	30 (79)	42 (76)	1,388 (50)	**3.24 (1.68–6.38)**	0.64 (0.15–2.84)
Sputum smear test result						
Negative	3 (18)	6 (16)	9 (16)	838 (30)	1	1
Positive	14 (82)	32 (84)	46 (84)	1,944 (70)	**2.20 (1.03–4.85)**	0.88 (0.16–5.23)
Complication						
None	6 (35)	21 (55)	27 (49)	1,344 (48)	1	1
Diabetes mellitus	4 (24)	7 (18)	11 (20)	438 (16)	1.25 (0.58–2.65)	2.00 (0.34–11.88)
Malignancy	3 (18)	2 (5)	5 (9)	180 (6)	1.38 (0.46–3.83)	5.25 (0.52–61.86)

*TB, tuberculosis; XDR, extensively drug-resistant; MDR, multidrug-resistant; MDR/XDR, MDR TB and XDR TB; –, not available. **Boldface** indicates significance.

tuberculosis strains and XDR *M. tuberculosis* strains (68% vs. 47%; p = 0.14), and distribution of the other spoligotype families did not differ significantly (data not shown).

Cluster Analysis by IS*6110*-RFLP

All 55 MDR/XDR strains were genotyped by IS*6110*-RFLP, and 21 (38%) were classified into 9 clusters, each with identical RFLP patterns (clusters 1–9) (online Appendix Figure, www.cdc.gov/EID/content/16/6/948-appF.htm). All members of each cluster belonged to the same spoligotype family. The remaining 34 strains exhibited unique RFLP patterns. Cluster size ranged from 2 to 4, and 7 clusters had 2 members, 1 with 3 members and 1 with 4 members. IS*6110* copy numbers ranged from 1 to 18. Four (7%) strains had <5 copies of IS*6110*, and these strains were discriminated as unique strains by the subsequent VNTR analysis. Of the 21 RFLP-clustered strains, 13 (62%) were classified as XDR *M. tuberculosis*, and 8 (38%) were isolated from new TB patients. Of the 17 XDR *M. tuberculosis* strains, 4 were resistant to all drugs tested, and 2 belonged to cluster 8. Among the 9 RFLP-clusters, 7 were from Japanese patients exclusively; the other 2 clusters were from both Japanese and foreign-born patients.

Cluster Analysis by VNTR

The results of the 19-locus VNTR (15 mycobacterial interspersed repetitive unit–VNTR + additional 4 loci) analysis showed that 7 of the 9 RFLP-clusters were identical according to the 19-locus VNTR (online Appendix Figure). A difference in only 1 locus was observed in the remaining 2 RFLP-clusters: 1 at VNTR1955 in cluster 6, and 1 at VNTR2163b in cluster 9 (online Appendix Figure).

Geographic Distribution of Hospitals with MDR/XDR TB Patients among Each Cluster

To estimate the possible clonal expansion of MDR/XDR TB in communities, we compared the geographic areas of patients' hospitals among each cluster (Table 3). The 55 MDR/XDR TB patients were treated by 23 hospitals, which were located in 16 of the 47 prefectures in Japan. Of the 9 clusters, 7 consisted of patients whose hospitals were located in the same or neighboring prefectures, and the remaining 2 clusters consisted of patients whose hos-

Table 2. Distribution of *Mycobacterium tuberculosis* spoligotype families among 55 persons with MDR/XDR TB, Japan*

	No. (%) cases		
Spoligotype family	XDR TB, n = 17	Non-XDR MDR TB, n = 38	MDR and XDR TB, n = 55
Beijing†	8 (47)	26 (68)	34 (62)
T	2 (12)	5 (13)	7 (13)
LAM	3 (18)	0	3 (5)
U	0	1 (3)	1 (2)
EAI	0	1 (3)	1 (2)
X	0	1 (3)	1 (2)
Unclassified‡	4 (24)	4 (11)	8 (15)

*MDR, multidrug-resistant; XDR, extensively drug-resistant; TB, tuberculosis; LAM, Latino-American and Mediterranean; EAI, East-African Indian.
†Includes Beijing-like strains.
‡Unclassified according to the SpolDB4.

pitals were located in the same region (i.e., a geographic and administrative area with several prefectures). Clusters with geographic links suggest the clonal expansion of MDR/XDR TB occurred in local areas. Although patients in clusters 4 and 7 were from the same hospital, further information about their contact was unavailable because an epidemiologic survey was not performed.

Characteristics of Clustered Cases

We analyzed the associations of genetic clustering by IS*6110*-RFLP with patients' characteristics and bacteriologic factors (Table 4). XDR TB occurred more frequently among the clustered cases than among the unique cases (OR 7.73), but differences between the 2 groups were not significant for any of the remaining factors i.e., age, sex, nationality, treatment history, site of TB disease, chest radiographic findings, sputum-smear test results, underlying disease, or member of the Beijing family genotype.

Discussion

Our study described the transmission dynamics of MDR/XDR TB in Japan on a national scale. Analysis of the 55 MDR/XDR TB cases showed that MDR/XDR TB was more frequent among younger patients, previously treated patients, and foreign-born patients than among patients with drug-susceptible TB (Table 1). In addition, the XDR TB cases, which represented 31% of the total MDR TB cases, were more likely to be associated with clustering than were the non–XDR MDR TB cases (Table 4), suggesting that ongoing transmission plays a critical role in new cases of XDR TB. We also found that the Beijing family genotype predominated in MDR/XDR *M. tuberculosis* (Table 2) and in drug-susceptible *M. tuberculosis* in this setting (*12*).

Although IS*6110*-RFLP analysis has been the standard for strain typing in studies of *M. tuberculosis* transmission, false clusters comprising strains without any epidemiologic link, and thus not reflecting clonal transmission, have been reported (*15*–*17*). This use of IS*6110*-RFLP analysis is especially problematic in area in which strains with stable RFLP patterns are endemic (*18*,*19*). In this context, because of its post hoc nature, a limitation of our study is its lack of information about epidemiologic links among clustered patients.

We therefore evaluated the genetic clonality of RFLP-clustered strains more rigorously by using the 19-locus VNTR. Our previous study demonstrated that most of the RFLP-clustered strains without epidemiologic links were discriminated by the 19-locus VNTR (*12*). In this study, the results correlated strongly between RFLP and VNTR in terms of cluster formation; 7 of the 9 RFLP-clusters had completely identical 19-locus VNTR profiles (online Appendix Figure). Each of the remaining 2 clusters contained a single-locus variant, i.e., the difference was at only 1 locus of 1 strain in each cluster. This minor difference in VNTR profile is highly likely to have occurred as a random variation in strains from the same source, as argued by several investigators (*13*,*20*). In addition to analyzing by RFLP and VNTR, we confirmed that all of the 9 clusters shared identical mutations on drug resistance genes for rifampin and isoniazid (i.e., *rpoB* and *katG*, respectively, data not shown).

Geographic distribution of the hospitals also supports the clonal expansion of MDR/XDR TB (Table 3). Isolation of most of the clustered strains from hospitals in the same or neighboring prefectures may indicate that transmission is occurring in the communities. Furthermore, we assume that some of the clustering in our study did not result from direct transmission among the members but rather resulted from exposure of the members to a common infection source or infection with different sources sharing a near ancestor. The discordance of drug resistance other than isoniazid and rifampin resistance among clusters implies that the stepwise acquisition of drug resistance had occurred chronologically during successive transmissions (online Appendix Figure). Thus, all these findings support the assumption of ongoing community transmissions of MDR/XDR TB.

A high proportion (71%, 12/17) of the XDR strains were involved in clusters, a finding consistent with the results of a hospital-based study in Osaka, Japan (*21*). Of the 12 clustered cases, 4 were new cases and 8 were among previously treated patients. The clustered XDR TB cases among the new cases strongly indicated that these persons had been primarily infected with XDR *M. tuberculosis*

Table 3. Geographic distribution of hospitals among each cluster of MDR and XDR TB, Japan*

Cluster no.	Patient ID	Type of TB	Hospital	Geographic link of the hospitals in clusters
1	DR43	MDR	A	
	DR42	XDR	B	A and B located in same prefecture
2	DR14	MDR	C	
	DR53	MDR	D	C located 103 km from D
3	DR54	XDR	D	
	DR58	XDR	D	
	DR18	XDR	E	D and E located in same prefecture
4	DR03	MDR	F	
	DR04	MDR	F	Same hospital
5	DR11	MDR	G	
	DR10	MDR	H	G located 221 km from H
6	DR51	XDR	D	
	DR50	XDR	D	
	DR55	MDR	D	
	DR16	XDR	E	D and E located in same prefecture
7	DR38	XDR	D	
	DR39	MDR	D	Same hospital
8	DR13	XDR	I	
	DR56	XDR	D	I and D located in neighboring prefectures
9	DR12	XDR	I	
	DR49	XDR	D	I and D located in neighboring prefectures

*MDR, multidrug-resistant; TB, tuberculosis; XDR, extensively drug-resistant.

strains. Also, TB may have developed in the clustered XDR TB patients with previous TB treatment as a result of exogenous reinfection with XDR TB, as described in a report of fatal TB in a patient infected with an MDR strain when his disease had been almost cured (*22*).

One possible explanation for the high clustering rate of XDR TB is that new cases of XDR TB are more likely to be caused by transmission than by acquisition of multiple drug resistance as a result of treatment failure. Shah et al. reported that patients with XDR TB were more likely than those without XDR TB to be infectious in terms of duration and proportion of sputum smear positivity (*3*). Furthermore, at least 142 non–XDR MDR TB and 180 XDR TB patients were reported to be in Japan as of 2000 (*23*). All were culture positive, and a considerable number were treated as outpatients despite their infectiousness and drug resistance. Thus, we assume that some of those patients with chronic MDR/XDR TB may have played a role as a source of transmission, as described in this study.

Because these findings and our cluster analysis suggest that the current TB control strategy is insufficient to prevent community transmission of MDR/XDR TB, more detailed investigations of MDR/XDR TB transmission based on contact tracing are urgently needed to improve the infection control strategy, including an isolation policy for highly infectious patients with refractory drug-resistant strains. At the same time, ethical issues, such as the human rights of these patients, should be carefully considered.

Only a few cases of XDR TB transmission have ever been reported. A large group of XDR TB cases, mainly among HIV-infected patients, was reported in South Africa (*2*). Another study in South Africa reported 12 cases of exogenous reinfection with XDR TB (*24*). In Iran, DNA fingerprinting analysis suggested 2 outbreaks of XDR TB involving 12 patients in 1 family and their close contacts (*25*). In Norway, a patient who was lost to follow-up has been transmitting an XDR *M. tuberculosis* strain for 12 years, and that transmission has ultimately resulted in 15 XDR TB cases (*26*). Consistent with these previous reports, our study has added novel evidence for clonal expansion of XDR *M. tuberculosis* strains even in an industrialized country with intermediate TB incidence and low incidence of HIV.

Another limitation of this study is a possible sampling bias, which could be caused by the following factors. First, the sampling of the survey participants was not randomized but was based on voluntary participation of the hospitals, which may be likely to include more serious TB cases (*7*). Second, the sampling fraction is low (38%) and the study period is short (6 months), either of which may produce a biased subset of the total cases. In addition, the low sampling fraction and the short study period may lead to reduced clustering of cases (*27*,*28*). A more complete understanding of transmission dynamics of MDR/XDR TB requires a real-time DNA fingerprinting method such as VNTR on a nationwide scale.

Four strains from the 55 MDR/XDR TB cases were identified as totally drug-resistant strains (*29*), indicating that they were resistant to all 10 drugs tested (online Appendix Figure). Of these strains, 2 in 1 cluster (cluster 8) were from new cases, and both patients were women in their 20s who were full-time workers and had no underlying disease.

Table 4. Comparison of MDR/XDR TB patients and bacteriologic characteristics between clustered and nonclustered cases by IS*6110*-RFLP, Japan*

Characteristic	No. (%) cases		Odds ratio (95% confidence interval)
	Clustered, n = 21	Unique, n = 34	Clustered vs. unique
Age, y			
0–20	1 (5)	0	–
21–40	8 (38)	12 (35)	0.96 (0.23–3.95)
41–60	9 (43)	13 (38)	1
≥61	3 (14)	9 (26)	0.48 (0.08–2.83)
Sex			
M	12 (57)	27 (79)	1
F	9 (43)	7 (21)	2.89 (0.75–11.45)
Nationality			
Japanese	19 (90)	28 (82)	1
Foreigner	2 (10)	6 (18)	0.49 (0.06–3.18)
Treatment history			
New	8 (38)	8 (24)	1
Previous	13 (62)	26 (76)	0.50 (0.13–1.90)
Site of TB			
Pulmonary	21 (100)	34 (100)	–
Extrapulmonary	0	0	–
Chest radiograph finding			
Noncavitary	6 (29)	7 (21)	1
Cavitary	15 (71)	27 (79)	0.65 (0.15–2.71)
Sputum smear test result			
Negative	4 (19)	5 (15)	1
Positive	17 (81)	29 (85)	0.73 (0.14–3.86)
Complication			
None	9 (43)	18 (53)	1
Diabetes mellitus	5 (24)	6 (18)	1.67 (0.32–8.78)
Malignancy	2 (10)	3 (9)	1.33 (0.13–12.94)
XDR TB			
No	9 (43)	29 (85)	1
Yes	12 (57)	5 (15)	**7.73 (1.84–34.83)**
Beijing family genotype			
No	6 (29)	15 (44)	1
Yes	15 (71)	19 (56)	1.97 (0.57–7.46)

*MDR, multidrug-resistant; XDR, extensively drug-resistant; TB, tuberculosis; IS*6110*-RFLP, insertion sequence *6110* restriction fragment length polymorphism –, not available. **Boldface** indicates significance.

Both patients were registered in the same area (no further data available); TB as formidable as totally drug-resistant TB can affect otherwise healthy young adults even on a mass basis.

The results of this study showed that transmission of MDR TB, especially XDR TB, is currently occurring in communities of Japan. Further studies to prospectively identify the transmission route through contact tracing and real-time DNA fingerprinting methods on a population basis are required. Although the MDR/XDR TB problem is not great in Japan, our findings highlight the relevance of proper infection control, as well as effective treatment, to further contain highly developed drug-resistant TB.

Acknowledgments

We are sincerely grateful to the members of the Tuberculosis Research Committee (RYOKEN) for the collection of TB strains. We thank Toru Mori for his valuable review of the manuscript.

This study was partly funded by the research project on *M. tuberculosis* in the "Emerging and Reemerging Infectious Diseases Research Fund," Ministry of Health, Labour and Welfare, Japan (principal investigator, Seiya Kato).

Dr Murase is a microbiologist at the Molecular Epidemiology Division of the Research Institute of Tuberculosis, Tokyo, Japan. His primary research interests include molecular epidemiology, drug resistance, and international control of tuberculosis.

References

1. World Health Organization. Anti-tuberculosis drug resistance in the world: fourth global report (WHO/HTM/TB/2008.394); 2008 [cited 2010 Feb 10]. http://whqlibdoc.who.int/hq/2008/WHO_HTM_TB_2008.394_eng.pdf
2. Gandhi NR, Moll A, Sturm AW, Pawinski R, Govender T, Lalloo U, et al. Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. Lancet. 2006;368:1575–80. DOI: 10.1016/S0140-6736(06)69573-1

3. Shah NS, Pratt R, Armstrong L, Robison V, Castro KG, Cegielski JP. Extensively drug-resistant tuberculosis in the United States, 1993–2007. JAMA. 2008;300:2153–60. DOI: 10.1001/jama.300.18.2153
4. Raviglione MC. Facing extensively drug-resistant tuberculosis—a hope and a challenge. N Engl J Med. 2008;359:636–8. DOI: 10.1056/NEJMe0804906
5. Borrell S, Gagneux S. Infectiousness, reproductive fitness and evolution of drug-resistant *Mycobacterium tuberculosis*. Int J Tuberc Lung Dis. 2009;13:1456–66.
6. Ministry of Health, Labour and Welfare. Statistics of tuberculosis 2008 [in Japanese]. Tokyo: Japan Anti-Tuberculosis Association; 2009.
7. Tuberculosis Research Committee (Ryoken). Drug-resistant *Mycobacterium tuberculosis* in Japan: a nationwide survey, 2002. Int J Tuberc Lung Dis. 2007;11:1129–35.
8. Ministry of Health, Labour and Welfare. National guidelines for the epidemiological research [in Japanese]. 2002 [cited 2009 Nov 20]. http://www.mhlw.go.jp/shingi/2002/12/s1211-9e.html
9. van Embden JD, Cave MD, Crawford JT, Dale JW, Eisenach KD, Gicquel B, et al. Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: recommendations for a standardized methodology. J Clin Microbiol. 1993;31:406–9.
10. Kamerbeek J, Schouls L, Kolk A, van Agterveld M, van Soolingen D, Kuijper S, et al. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. J Clin Microbiol. 1997;35:907–14.
11. Brudey K, Driscoll JR, Rigouts L, Prodinger WM, Gori A, Al-Hajoj SA, et al. *Mycobacterium tuberculosis* complex genetic diversity: mining the fourth international spoligotyping database (SpolDB4) for classification, population genetics and epidemiology. BMC Microbiol. 2006;6:23. DOI: 10.1186/1471-2180-6-23
12. Murase Y, Mitarai S, Sugawara I, Kato S, Maeda S. Promising loci of variable numbers of tandem repeats for typing Beijing family *Mycobacterium tuberculosis*. J Med Microbiol. 2008;57:873–80. DOI: 10.1099/jmm.0.47564-0
13. Supply P, Allix C, Lesjean S, Cardoso-Oelemann M, Rusch-Gerdes S, Willery E, et al. Proposal for standardization of optimized mycobacterial interspersed repetitive unit–variable-number tandem repeat typing of *Mycobacterium tuberculosis*. J Clin Microbiol. 2006;44:4498–510. DOI: 10.1128/JCM.01392-06
14. European Concerted Action on New Generation Genetic Markers and Techniques for the Epidemiology and Control of Tuberculosis. Beijing/W genotype *Mycobacterium tuberculosis* and drug resistance. Emerg Infect Dis. 2006;12:736–43.
15. Alonso-Rodriguez N, Martinez-Lirola M, Sanchez ML, Herranz M, Penafiel T, Bonillo Mdel C, et al. Prospective universal application of mycobacterial interspersed repetitive-unit–variable-number tandem-repeat genotyping to characterize *Mycobacterium tuberculosis* isolates for fast identification of clustered and orphan cases. J Clin Microbiol. 2009;47:2026–32. DOI: 10.1128/JCM.02308-08
16. Oelemann MC, Diel R, Vatin V, Haas W, Rusch-Gerdes S, Locht C, et al. Assessment of an optimized mycobacterial interspersed repetitive-unit–variable-number tandem-repeat typing system combined with spoligotyping for population-based molecular epidemiology studies of tuberculosis. J Clin Microbiol. 2007;45:691–7. DOI: 10.1128/JCM.01393-06
17. van Deutekom H, Supply P, de Haas PE, Willery E, Hoijng SP, Locht C, et al. Molecular typing of *Mycobacterium tuberculosis* by mycobacterial interspersed repetitive unit–variable-number tandem repeat analysis, a more accurate method for identifying epidemiological links between patients with tuberculosis. J Clin Microbiol. 2005;43:4473–9. DOI: 10.1128/JCM.43.9.4473-4479.2005
18. Godfrey-Faussett P, Stoker NG. Aspects of tuberculosis in Africa. 3. Genetic 'fingerprinting' for clues to the pathogenesis of tuberculosis. Trans R Soc Trop Med Hyg. 1992;86:472–5. DOI: 10.1016/0035-9203(92)90072-K
19. Braden CR, Templeton GL, Cave MD, Valway S, Onorato IM, Castro KG, et al. Interpretation of restriction fragment length polymorphism analysis of *Mycobacterium tuberculosis* isolates from a state with a large rural population. J Infect Dis. 1997;175:1446–52. DOI: 10.1086/516478
20. Savine E, Warren RM, van der Spuy GD, Beyers N, van Helden PD, Locht C, et al. Stability of variable-number tandem repeats of mycobacterial interspersed repetitive units from 12 loci in serial isolates of *Mycobacterium tuberculosis*. J Clin Microbiol. 2002;40:4561–6. DOI: 10.1128/JCM.40.12.4561-4566.2002
21. Ano H, Matsumoto T, Suetake T, Nagai T, Tamura Y, Takamatsu I, et al. Relationship between the isoniazid-resistant mutation katGS315T and the prevalence of MDR-/XDR-TB in Osaka, Japan. Int J Tuberc Lung Dis. 2008;12:1300–5.
22. Tsuyuguchi K. Exogenous re-infection in tuberculosis [in Japanese]. Kekkaku. 2006;81:80–1.
23. Kazumi Y, Itagaki N, Ohmori M, Wada M, Hoshino H, Mitarai S, et al. Frequency of MDR-TB/XDR-TB strains isolated from chronic pulmonary tuberculosis patients in Japan [in Japanese]. Kekkaku. 2007;82:891–6.
24. Andrews JR, Gandhi NR, Moodley P, Shah NS, Bohlken L, Moll AP, et al. Exogenous reinfection as a cause of multidrug-resistant and extensively drug-resistant tuberculosis in rural South Africa. J Infect Dis. 2008;198:1582–9. DOI: 10.1086/592991
25. Masjedi MR, Farnia P, Sorooch S, Pooramiri MV, Mansoori SD, Zarifi AZ, et al. Extensively drug-resistant tuberculosis: 2 years of surveillance in Iran. Clin Infect Dis. 2006;43:841–7. DOI: 10.1086/507542
26. Dahle UR. Extensively drug resistant tuberculosis: beware patients lost to follow-up. BMJ. 2006;333:705. DOI: 10.1136/bmj.333.7570.705
27. Glynn JR, Bauer J, de Boer AS, Borgdorff MW, Fine PE, Godfrey-Faussett P, et al. Interpreting DNA fingerprint clusters of *Mycobacterium tuberculosis*. European Concerted Action on Molecular Epidemiology and Control of Tuberculosis. Int J Tuberc Lung Dis. 1999;3:1055–60.
28. Houben RM, Glynn JR. A systematic review and meta-analysis of molecular epidemiological studies of tuberculosis: development of a new tool to aid interpretation. Trop Med Int Health. 2009;14:892–909. DOI: 10.1111/j.1365-3156.2009.02316.x
29. Velayati AA, Masjedi MR, Farnia P, Tabarsi P, Ghanavi J, Ziazarifi AH, et al. Emergence of new forms of totally drug-resistant tuberculosis bacilli: super extensively drug-resistant tuberculosis or totally drug-resistant strains in iran. Chest. 2009;136:420–5. DOI: 10.1378/chest.08-2427

Address for correspondence: Yoshiro Murase, Molecular Epidemiology Division, The Research Institute of Tuberculosis, Japan Anti-Tuberculosis Association, Kiyose, Matsuyama 3-1-24, Tokyo, Japan, email: ymurase@jata.or.jp

Increase in Pilus Islet 2–encoded Pili among *Streptococcus pneumoniae* Isolates, Atlanta, Georgia, USA

Dorothea Zähner, Aditya Gudlavalleti, and David S. Stephens

To define the prevalence of pilus islet 2 (PI-2)–encoded pili in *Streptococcus pneumoniae* in a geographically defined area, we examined 590 *S. pneumoniae* isolates from population-based surveillance of invasive pneumococcal disease in Atlanta, Georgia, USA, 1994–2006. In 2006, PI-2 was present in 21% of all invasive isolates, including serotypes 1 (100%), 7F (89%), 11A (21%), 19A (40%), and 19F (75%). Only serotype 19F is included in the 7-valent pneumococcal conjugate vaccine that is in use worldwide. In 1999, PI-2-containing isolates were of the same serotypes but accounted for only 3.6% of all invasive isolates. The increase of PI-2 in 2006 resulted predominantly from the emergence of serotype 19A isolates of sequence type 320 and the expansion of serotype 7F isolates. The increase in PI-2-containing isolates and the finding that isolates of all identified serotypes expressed highly conserved PI-2 pili supports their potential as a vaccine candidate.

Streptococcus pneumoniae is a major human pathogen, which causes pneumonia, sinusitis, otitis, bacterial meningitis, and septicemia. Introduction of the 7-valent pneumococcal conjugate vaccine (PCV7, Prevnar; Wyeth, Madison, NJ, USA) in 2000 has dramatically decreased invasive pneumococcal disease in the United States in children and in adults (*1*) and is now in use worldwide. PCV7 includes capsule polysaccharide serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F, serotypes that were responsible for >80% of cases of invasive pneumococcal disease in the United States before the introduction of the conjugate vaccine (*1*). In recent years other non-PCV7 serotypes, including 7F and 19A, have been emerging in the United States, (*2–4*).

Pili have recently been identified on several gram-positive bacteria (*5–7*). To date, 2 different pilus islets (PIs) have been described in *S. pneumoniae* that encode the structural and biosynthetic genes for 2 antigenically different types of pili, PI-1 (*8*) and recently described PI-2 (*9*). The so-called *rlrA* pathogenicity islet (*10*) or PI-1 is a 14-kb genetic region present in 21%–27% of clinical isolates, depending on the geographic region analyzed. It is most prevalent in, but not restricted to, the PCV7-vaccine serotypes 4, 6B, 9V, 14, and 19F (*11–13*).

PI-2 is a 7-kb region located between the genes that encode peptidase T (PepT) and ferrochelatase (HemH) (*9*). The region is composed of 5 genes, which encode 2 surface proteins, PitA and PitB, with *pitA* a pseudogene due to a stop-codon in the N terminus, a signal peptidase-like protein (SipA), and 2 sortases (SrtG1 and SrtG2); the latter is nonfunctional in most of the strains (9). This PI has been reported to be present in ≈16% of the analyzed isolates belonging to serotypes 1, 2, 7F, 19A, and 19F (*9*). The presence of PI-2 pili appears to be a clonal property, rather than serotype associated, an observation that has been described for PI-1 as well (*11–13*). For isolates of serotype 19A, PI-2–containing isolates have been shown to be associated with sequence type (ST) ST320, a clone related to the worldwide distributed multidrug-resistant serotype 19F clone Taiwan^{19F}-14 (*4*,*14*). ST320 belongs to clonal complex (CC) CC271 (with ST271 as the predicted founder of this CC); isolates of this CC have been shown to encode both PIs and express both pili concomitantly on their surface (*9*).

The PI-2 pilus is composed of polymers of the major pilus protein PitB (*9*). In contrast to other pili in gram-

Author affiliations: Emory University School of Medicine, Atlanta, Georgia, USA (D. Zähner, A. Gudlavalleti, D.S. Stephens); and Department of Veterans Affairs Medical Center, Atlanta (D. Zähner, D.S. Stephens)

DOI: 10.3201/eid1606.091820

positive bacteria that contain accessory pilus proteins, that serve as adhesins (*15*–*17*) or adaptors for pilus attachment to the cell wall (*18*,*19*), PI-2 pili appear to consist solely of PitB polymers, and these polymers themselves have been shown to mediate adhesion of *S. pneumoniae* to eukaryotic cells (*9*).

Bagnoli et al. identified PI-2–containing isolates in a random, worldwide collection of pneumococcal isolates (*9*). We describe the distribution of PI-2 pili in a well-documented, comprehensive collection from a defined geographic region, i.e., in invasive pneumococcal isolates collected as part of a population-based surveillance program conducted in the metropolitan area of Atlanta, Georgia, USA.

Materials and Methods

Strain Collection

A set of 590 strains of *S. pneumoniae* from population-based surveillance of invasive pneumococcal disease isolated between 1994 and 2006 in the 8-county metropolitan Atlanta area, Georgia Health District 3 formed the basis for this study. The isolates were obtained as a part of the Centers for Disease Control and Prevention–sponsored Active Bacterial Core surveillance of the Georgia Emerging Infections Program (*1*,*20*). Information about serotype and antimicrobial drug susceptibility was determined for all isolates as described (*21*). Only viable strains with documented serotype and available antibiogram were included in the study. Laboratory strain *S. pneumoniae* R6 is a nonencapsulated derivative of the serotype 2 strain D39 (*22*).

PCR-based Screening for PI-2

To ascertain whether PI-2 was absent from the described insertion site (*9*), we designed primers pepT_F and hemH_R (Table 1) against the flanking genes *pepT* and *hemH*. To determine whether PI-2 was present, we used primers sipA_up and sipA_dn (Table 1) designed against the PI-2–specific gene *sipA*. We tested for PI-1 using primers Rlr_up_F and Rlr_do_R for its absence and Rlr_SrtC_F and Rlr_srtD_R for its presence, respectively (Table 1).

Multilocus Sequence Typing and Clonal Complexes

Multilocus sequence typing (MLST) was performed as described (*23*). CCs were assigned by using the eBurst program (*24*) to partition the MLST database (http://spneumoniae.mlst.net). The predicted founder of a CC was assigned by eBurst to the ST with the highest number of single-locus variants.

Construction of a *pitB*-Deletion Mutant

The 3′- and 5′-flanking regions of *pitB* were amplified by using primer pairs sipA_up_F/PilA_del_3 and PilA_del_2/SrtC_do_R (Table 1). Both PCR products were used in a PCR-based overlap-extension reaction, which introduced an *Mfe*I restriction site between the 2 fused fragments. The PCR product was cloned into pCR-Blunt II (Invitrogen, Carlsbad, CA, USA), resulting in pCR-*pitB*. The erm-cassette of pSK-erm (*25*) was amplified by using primers erm_F and erm_R (Table 1) and ligated into *Mfe*I-digested pCR-*pitB*. The resulting plasmid, pCR-*pitB*:erm, was used to transform GA41070 according to established protocols (*26*), which resulted in mutant GA41070Δ*pitB*.

Production of Recombinant PitB and Antiserum

The region of *pitB* that encoded the mature protein (bp 126–bp 1128) was amplified from chromosomal DNA of serotype 1 strain GA19686 and cloned into the *Escherichia coli* expression vector pET-32a+ (Stratagene, La Jolla, CA, USA). The thioredoxin/6xHis-PitB fusion protein was purified by using the B-PER 6xHis Fusion Purification Kit (Pierce, Rockford, IL, USA). The N-terminal thioredoxin/6xhis-tag was removed by cleavage with recombinant enterokinase (Novagen, Madison, WI, USA)

Table 1. Primers used in this study of invasive *Streptococcus pneumoniae* isolates, Atlanta, Georgia, USA, 1994–2006*†

Region	Primer	Sequence (5′ → 3′)‡
Erm cassette	erm_F	gctctagaCGTTAGATTAATTCCTACCAGTGAC
	erm_R	gctctagaCTCCATTCCCTTTAGTAACGTGTAAC
PI-2	pepT_F	TAAGAAGCGGTCCAAGAGATTTGG
	hemH_R	AATAATGGGGCTCCAAAATCAAGC
	sipA_up_F	CTCTAGGAGGGATCTTCTTTATCATC
	sipA_do_R	CTACAGCCGTTGTTCGATTGTCC
	PilA_del_3	gcaattgcccgggcctagCCTGTATAGGGATGGTTCCAAAAG
	PilA_del_2	ctaggcccgggcaattgcGCTGGGGGCAGATGATG
	Rlr_up_F	CTTCCACGAAGTTCTTTCAATGG
PI-1	Rlr_do_R	GTCTTAGAATATCATGGTTTACGTGC
	Rlr_srtC_F	GGGGAAGATTATGCGACCTT
	Rlr_srtD_R	GCTTGGCTCTGCACGGTGCC

*PI, pilus islet.
†Not included are primers used for sequencing and multilocus sequence typing.
‡Lowercase letters represent bases added for mutagenesis; uppercase letters represent bases complementary to *S. pneumoniae*.

according to the manufacturer's recommendations. Purified PitB was used to produce a rabbit polyclonal antiserum (Covance Research Products Inc., Princeton, NJ, USA).

Preparation of Cell Wall Extracts from *S. pneumoniae*

Cultures were grown to mid-exponential phase (optical density$_{600}$ 0.5–0.6) in Todd-Hewitt medium containing 0.5% yeast extract. After centrifugation, the cell pellets were resuspended in 1/20 of the culture volume in extraction buffer (50 mmol/LTris-HCl; pH 8.0) containing 200 U/mL mutanolysin (Sigma-Aldrich, St. Louis, MO, USA) and 30% raffinose. After 2 h of incubation at 30°C, cell wall extracts were collected by centrifugation at 12,000 × *g* for 15 min; 20 μL cell wall extract were mixed with sodium dodecyl sulfate–polyacryamide gel electrophoresis sample buffer and boiled for 10 min immediately before separation on a 4%–12% Tris-HCl polyacrylamide gel (NuPAGE; Invitrogen). For immunostaining, proteins were transferred to a nitrocellulose membrane and detected with polyclonal anti-PitB antiserum at a dilution of 1:20,000.

Statistical Analysis

Proportions were compared by using the Fisher exact test. p values <0.005 were considered significant.

Results

PI-2 in Strains of Serotypes 1, 7F, 11A, 19A, and 19F

To determine the post PCV-7 prevalence of PI-2 in *S. pneumoniae*, we analyzed all 381 viable and documented invasive isolates collected in 2006 in metropolitan Atlanta (381/409) in a PCR-based screen. Two PCRs were performed with each strain; 1 resulted in a PCR product in strains that lacked PI-2, and a second resulted in a product only in strains that contained PI-2. Strains that produced a PCR product for PI-2 were further characterized by amplification of *pitB*, which encoded the pilus backbone protein. For all strains, 1 of the 2 PCRs resulted in a PCR product. No strain gave a PCR product for both PCRs, which could have indicated the presence of the PI in an alternative integration site. These results strongly suggest that the *pepT-hemH* intergenic region is the only integration site of PI-2 in *S. pneumoniae*. Overall, 21% of all viable isolates collected in 2006 contained PI-2. The PI-2 of *S. pneumoniae* strain GA47784 was assigned the GenBank accession no. GU256423.

PI-2 was identified only in isolates of serotypes 1, 7F, 11A, 19A, and 19F (Figure 1). Serotypes 19A and 7F were the most frequent PI-2–containing serotypes isolated in 2006, with a PI-2 prevalence of 40% and 89%, respectively. In serotype 11A, PI-2 was identified sporadically, whereas for serotype 19F, 75% of the isolates contained PI-2. Serotype 1 was represented by a single isolate in 2006. To determine the prevalence of PI-2 in serotype 1 isolates, we analyzed an additional 20 invasive isolates, collected in Atlanta from 1994 through 2005; all contained PI-2. Serotype 1 had the highest presence of PI-2 of all analyzed serotypes. Except for serotype 19F, the PI-2–containing serotypes are not included in PCV7.

To further characterize PI-2 of a serotype 11A strain, we sequenced the 7-kb region between the flanking genes *pepT* and *hemH* of strain GA47784. The sequence was 99.9% identical to the published PI-2 sequences, with the highest sequence identity to serotype 1 strain PN110, with only 1-bp difference over the entire PI-2 region. The mutation was located downstream of the stop-codon in *pitA*, which results in premature termination of PitA, as has been reported (*9*).

Effects of PCV7 Introduction on PI-2 Distribution

To assess whether PI-2 was associated with the emergence of serotypes in the aftermath of PCV7 introduction (2000), the PI-2 distribution was determined in invasive isolates from 1999 that belonged to the PI-2–containing serotypes (Figure 2). The numbers of isolates for the different serotypes in the 2 periods reflected the expansion of isolates of the nonvaccine serotypes 7F and 19A and the decline in 19F isolates (Figure 2) that has been described in

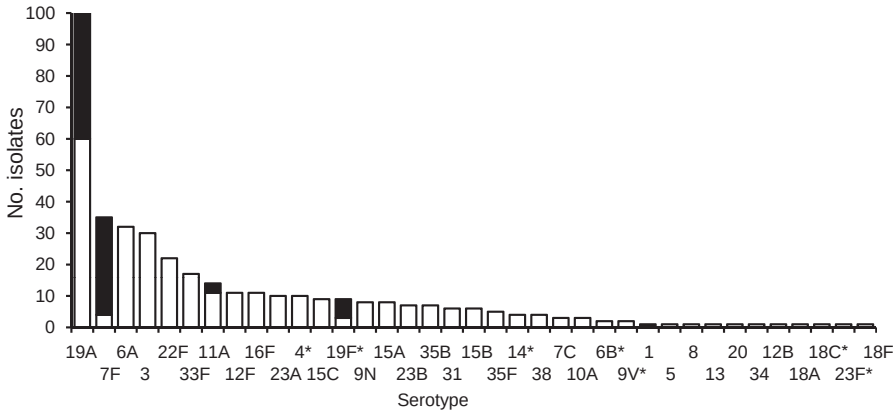


Figure 1. Frequency of pilus islet 2 (PI-2)–containing *Streptococcus pneumoniae* invasive isolates in metropolitan Atlanta, Georgia, USA, 2006. Black, PI-2–containing isolates; white, PI-2–lacking isolates. *Serotypes included in the 7-valent pneumococcal conjugate vaccine.

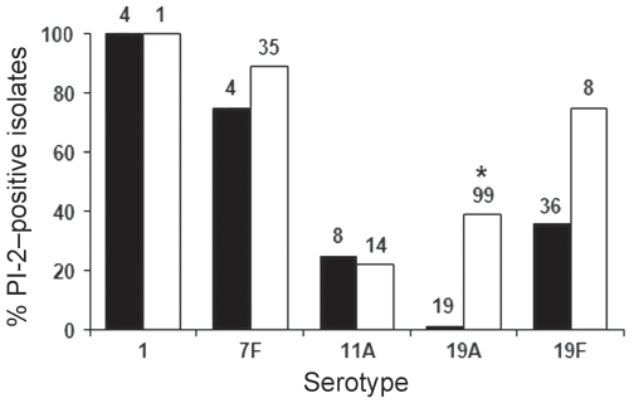


Figure 2. Percentage of pilus islet 2 (PI-2)–containing *Streptococcus pneumoniae* invasive isolates among serotypes associated with PI-2 in metropolitan Atlanta, Georgia, USA, 1999 and 2006. The total number of isolates for each serotype is shown at the top of the column. *Significant difference between 1999 and 2006 19A isolates ($p \leq 0.005$).

the United States since the introduction of PCV7 (*4*). PI-2 was present in isolates of all 5 serotypes before the introduction of PCV7; hence, PI-2 was not recently acquired. For serotype 7F, the ratio between PI-2–containing and PI-2–lacking isolates in 2006 compared to the ratio in 1999 remained essentially the same (Figure 2), which suggests that PI-2 pili may not provide a major selective advantage to invasive 7F isolates. Serotype 19A showed a significant increase in PI-2–containing isolates. To assess the presence of PI-2 in other serotypes in 1999, we analyzed isolates of all represented serotypes of PI-2: all isolates of infrequent serotypes (1, 5, 7C, 7F, 8, 9A, 9N, 10A, 13, 15B, 15C, 16, 16F, 18F, 20, 23A, 24B, 31, 33F, 34, 35B, 35F, 37) and at least 10% of the isolates of serotypes with >10 isolates (3, 4, 6A, 6B, 9V, 11A, 12F, 14, 18C, 19A, 19F, 22F, 23F). No additional PI-2–containing isolates were detected, which suggests that serotypes 1, 7F, 11A, 19A, and 19F were the only serotypes that contained substantial numbers of PI-2–containing isolates. These PI-2–containing isolates accounted for ≈3.6% of all viable isolates collected in 1999. The estimation of total viable isolates in 1999 was based on the known proportion of viable isolates to total collected isolates of the PI-2–containing serotypes of 1999 (93%).

Genetic Relationships Between PI-2–containing Strains

To analyze the clonality of the PI-2–containing isolates, we performed MLST for all PI-2–containing isolates from 1999 and 2006 (Table 2). For serotype 1, the isolates from 1999 and 2006 belonged to ST304 and ST227, respectively (Table 2). Both STs have been described as the most prevalent serotype 1 STs in the United States (*27*,*28*). PI-2–containing serotype 7F isolates of 2006 belonged to either ST191 or ST1176, and ST191 isolates were responsible for the strong expansion of this serotype in 2006. 7F isolates lacking PI-2 were tested and found to be of ST191 (data not shown), which indicated that PI-2 is present in only a subpopulation of this ST. ST1176 is unrelated to ST191, and 7F isolates of this ST were not present in 1999. All PI-2–containing isolates of serotype 11A belonged to ST62. Analysis of additional serotype 11A isolates that lacked PI-2 showed that they all belonged to ST62 (data not shown). This finding suggests that the structure of this serotype is clonally homogeneous and that PI-2 is present in a subset of this ST.

For serotype 19A, 27 of the 39 PI-2–containing 19A isolates in 2006 were ST320 (Table 2). This ST was absent in 1999 (*29*); thus these isolates were the major contributor to the increase of PI-2–containing isolates of serotype 19A in 2006. Additionally, PI-2 was identified in 19A isolates of ST1339 and ST2268, and both sequence types belonged to CC2090. One ST1339 isolate was present in 1999, which indicated that PI-2 was present in this ST before PCV7 was introduced. 19A isolates of 2006 that lacked PI-2 were tested and found to belong to other STs, e.g., ST199 and ST695, STs consistent with the described population structure of 19A isolates in the United States (*4*).

ST analysis of the remaining isolates of 19F isolates in 2006 showed that PI-2 was present in isolates that belonged to CC271 (ST236, ST651, ST3039) and also in the unrelated CC251 (ST251, ST654, ST1258) (Table 2). However, analysis of several 19F isolates that lacked PI-2 revealed isolates of ST3039 (data not shown); this finding indicated either a loss of PI-2 or a recent acquisition of the islet in a subset of isolates of this ST. Isolates of CC271 have been described to encode and express PI-1 and PI-2 concomitantly (*9*). Analysis for the presence of PI-1 in our

Table 2. Clonal distribution of PI-2-containing *Streptococcus pneumoniae* isolates, Atlanta, Georgia, USA, 1994–2006*

Serotype	CC	ST	No. PI-2-containing isolates 1999	2006
1	304	304	1	1
	306	227 (DLV)	3	–
7F	191	191	3	28
	218	1176 (DLV)	–	3
11A	53	62	2	3
19A	2090	1339 (SLV)	1	9
		2268 (DLV)	–	3
	271	320 (SLV)	–	27
19F	271	271	1	–
		236 (SLV)	3	1
		3039 (SLV)	–	1
		651 (DLV)	3	2
	251	251	1	1
		654 (SLV)	4	1
		1258 (DLV)	2	–

*CC, clonal complex; ST, sequence type; PI, pilus islet; DLV, double-locus variant; SLV, single-locus variant.

population confirmed PI-1 in isolates of CC271 (ST320, ST271, ST3039, ST236, ST651). Additionally, PCR results showed that PI-1 was in ST1339 isolates but not in isolates of the single locus variant ST2268. Isolates of all other PI-2–containing serotypes or STs did not contain PI-1.

Antimicrobial Drug Resistance and PI-2–containing Isolates

19F and 19A isolates of CC271 are closely related to the globally distributed multidrug-resistant clone Taiwan^{19F}-14, and all were resistant to penicillin, erythromycin, cotrimoxazole, tetracycline, and chloramphenicol. In addition, serotype 19A isolates of CC2090 (ST1339, ST2268) were resistant to penicillin, erythromycin, and cotrimoxazole. All PI-2–containing 19A isolates in 2006 were multidrug resistant. In contrast, PI-2–containing isolates of serotypes 1, 7F, 11A, and 19F (ST654, ST251) were susceptible to all tested antimicrobial drugs. Hence, antimicrobial drug resistance was not a consistent feature of PI-2–containing isolates.

Expression of High Molecular Weight PitB on the Surface of PI-2–containing Isolates

The first report of PI-2 pili described mutations in several genes encoded by PI-2 (namely, *pitA* and *srtG2*), which resulted in their inactivation (*9*). To confirm that PI-2–containing isolates indeed express PitB polymers on their surface, we produced an anti-PitB antiserum to detect PitB polymers. The specificity of the PitB antiserum was tested against cell wall extracts of strain GA41070 and its isogenic *pitB* deletion mutant, GA41070Δ*pitB* (Figure 3). In the wild type, the antibody reacted with several bands that produced the high molecular weight (HMW) banding pattern characteristic of gram-positive pili (*30*,*31*) (Figure 3). As expected, the PitB monomer and the HMW banding pattern were absent in the *pitB*-deletion mutant (Figure 3).

To analyze whether the PI-2–containing isolates of the different STs produced PitB polymers, we used Western blots to examine their cell wall extracts (Figure 4). All PI-2–containing strains showed the same HMW banding pattern (Figure 4, lanes 2–15). In addition, all 81 PI-2–containing isolates from 2006 were analyzed by whole cell dot blots that confirmed that all isolates expressed PitB on their surface (data not shown).

High Conservation of PitB among Different STs

To determine the degree of variation in PitB, we sequenced *pitB* in isolates representing the different STs (Table 2). The *pitB* sequences were 100% identical for all strains, except the 7F isolates GA47077 (ST191) and GA47340 (ST1176), which have a single point mutation that has been observed in other serotype 7F strains (*9*). The high conservation of PitB was consistent with the reported PitB sequences by Bagnoli et al. (*9*) and is an essential feature of PI-2 as a potential vaccine candidate. Additionally, we analyzed the gene for the second surface protein encoded in PI-2, *pitA,* and confirmed the presence of the described stop-codon in all strains representing the different STs (Table 2).

Discussion

PI-1 pili on *S. pneumoniae* have been reported as promising vaccine candidates for this important human pathogen (*13*,*32*). One limitation to this approach is that PI-1 has been found in only ≈25% of strains; it is most frequently found in pneumococcal isolates of serotypes covered by PCV7, which are decreasing in countries that have introduced the vaccine. The recent identification of PI-2 in serotypes not covered by PCV7 has raised the possibility that broad coverage of strains can be achieved by a bivalent pilus-based vaccine.

The first report on PI-2 pili described the serotypes and sequence types associated with PI-2 (*9*). In the current study, we defined the prevalence of PI-2–expressing isolates in a defined geographic region and population and assessed whether the introduction of PCV7 had an effect on the distribution of PI-2–containing isolates. Twenty-one

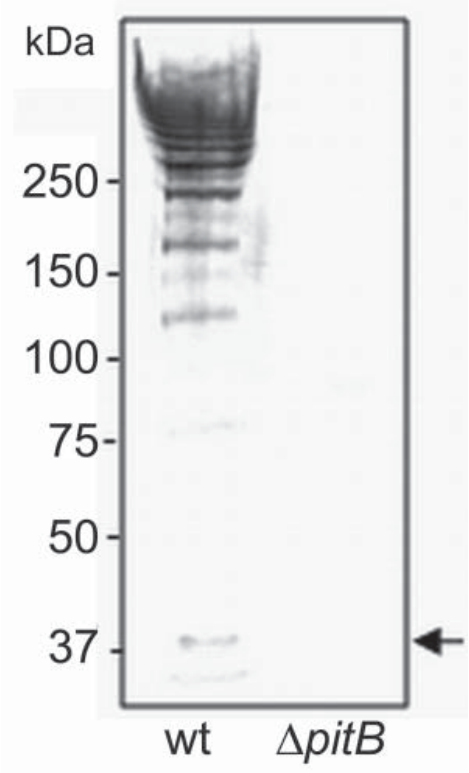


Figure 3. Detection of high molecular weight PitB polymers in invasive isolates of *Streptococcus pneumoniae*. Western blot of cell wall extracts from strains GA41070 (lane 1) and GA41070Δ*pitB* (lane 2) detected with anti-PitB antiserum. Monomeric PitB (arrow) and the marker sizes are indicated.

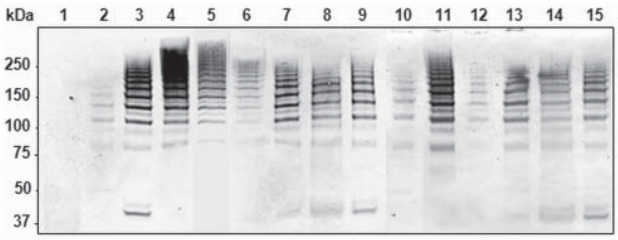


Figure 4. Detection of high molecular weight PitB polymers in invasive isolates of *Streptococcus pneumoniae*. Western blot of cell wall extract from strains R6 (lane 1), GA47901 (lane 2), GA13444 (lane 3), GA47077 (lane 4), GA47340 (lane 5), GA47784 (lane 6), GA47368 (lane 7). GA47751 (lane 8), GA47187 (lane 9), GA11293 (lane 10), GA47628 (lane 11), GA49138 (lane 12), GA47434 (lane 13), GA47373 (lane 14), and GA47105 (lane 15) detected with anti-PitB antiserum. Marker sizes are indicated.

percent of the 381 viable invasive pneumococcal isolates collected in 2006 in metropolitan Atlanta expressed PI-2 pili. This percentage is higher than the PI-2 incidence reported from a global strain collection (*9*) and is similar to the reported percentage for PI-1 (*11–13*). The higher incidence rate of PI-2 in our study refers to a post-PCV7-era population, whereas the earlier report was based on a random strain collection, including pre- and post-PCV7 isolates. Therefore, our higher results are consistent with the assessment of Bagnoli et al. (*9*) that PI-2 is most prevalent in emerging, non-PCV7 serotypes.

Notably, all serotype 1 strains had PI-2. This serotype does not play a large role in pneumococcal invasive disease in the United States (*27*), a conclusion reflected by the low number of serotype 1 isolates in our dataset. However, serotype 1 isolates have been identified in several studies as particularly invasive, in contrast to other serotypes that are more frequently associated with carriage (*33–35*) and associated with epidemic outbreaks (*27*,*36*,*37*). Further study of PI-2 prevalence in serotype 1 requires a geographically different dataset with a higher serotype 1 prevalence.

In contrast to serotype 1, serotype 7F, together with 19A, has been a major emerging serotype in the post-PCV7 era (*3*,*4*), a fact reflected in our dataset, with a 5-fold increase between 1999 and 2006. Serotype 7F causes primary invasive disease in otherwise healthy patients and is found less frequently in carriers (*33*,*35*,*38*). As most of the serotype 7F isolates belong to ST191, the genetic structure of this serotype is very homogeneous (*35*). However, 3 isolates of ST1176 were found in 2006. ST1176 belongs to CC218 and is unrelated to ST191. A 7F isolate of CC218 had been observed in 1999 (*29*), a CC previously associated with isolates of serotype 12F. The ratio between PI-2–containing and –lacking isolates of serotype 7F was essentially unchanged from 1999 to 2006; only the overall number of cases increased. Thus, PI-2 does not appear to provide a selective advantage to invasive serotype 7F isolates, i.e., contribute to the emergence of this serotype.

Serotype 11A has been described as an opportunistic serotype often found in asymptomatic carriers and a cause of disease in patients with underlying disease (*33*,*35*). However, when serotype 11A does cause invasive disease, it has been associated with a high number of deaths (*35*). Isolates that cause invasive disease have been identified as belonging to ST62, the ST most common among serotype 11A (*35*). The low prevalence of PI-2 in serotype 11A suggests that PI-2 pili are not essential for isolates of this serotype to cause invasive disease.

The genetic profile of the serotype 19A isolates in Atlanta is consistent with the described clonal distribution of this serotype in the United States in the post-PCV7 period (*4*). Several capsular switching events have contributed to the emergence of serotype 19A, including (but not limited to) ST320, originating from a multidrug-resistant derivative of clone Taiwan^{19F}-14(ST236), and CC2090 (ST1339, ST2289) from the highly related clone North Carolina^{6A}-23 (*39*). Unlike serotype 7F, 19A is characterized by a heterogeneous clonal structure (*4*), and the presence of PI-2 is a feature of some of the clones.

Most of the PI-2–containing isolates that we found do belong to the serotypes reported for PI-2 by Bagnoli et al. (*9*). Serotype 2 was absent from our population during the surveillance period of 1994 to 2006, and in addition we identified PI-2 in serotype 11A. Overall, we confirmed the presence of PI-2 in emerging non-PCV7 serotypes. However, other non-PCV7 serotypes that do not have PI-2 are emerging as well, e.g., 15BCF, 22F, 33F, and 38 (*2*,*40*), which indicates that other mechanisms may influence the emergence of new serotypes.

Of note, 3 of the 4 non-PCV7 serotypes with PI-2–containing isolates (serotypes 1, 7F, 19A) are included in the 13-valent pneumococcal conjugate vaccine now being introduced in Europe, Canada, and the United States. Use of this vaccine is expected to greatly reduce the frequency of all included serotypes and may thus reduce the proportion of *S. pneumoniae* isolates that contain PI-2. In summary, the prevalence of PI-2 increased from ≈3.6% of the invasive pneumococcal isolates in 1999 to 21% in 2006 in metropolitan Atlanta, Georgia, especially in the emerging serotypes 7F and 19A. PI-2-containing isolates of all identified sequence types expressed polymers of the highly conserved pilus protein PitB on their surface. These findings support the potential of PI-2 pili as a vaccine candidate.

Acknowledgments

We are grateful to Monica M. Farley, Wendy Baughman, Sarah W. Satola, and the Georgia Emerging Infections Program

for providing the clinical isolates and the corresponding documentation. We thank Leslie McGee and Scott T. Chancey for sequence type data of serotype 19A isolates.

This work was supported by funds from National Institutes of Health grant #R01AI 070829 (D.S.S.) and a Veterans Administration Merit Award from the Department of Veterans Affairs (D.S.S.).

Dr Zähner is a senior research associate at Emory University School of Medicine, Atlanta. Her main research interests are surface structures and antimicrobial resistance in *S. pneumoniae*.

References

1. Whitney CG, Farley MM, Hadler J, Harrison LH, Bennett NM, Lynfield R, et al. Decline in invasive pneumococcal disease after the introduction of protein-polysaccharide conjugate vaccine. N Engl J Med. 2003;348:1737–46. DOI: 10.1056/NEJMoa022823
2. Hicks LA, Harrison LH, Flannery B, Hadler JL, Schaffner W, Craig AS, et al. Incidence of pneumococcal disease due to non-pneumococcal conjugate vaccine (PCV7) serotypes in the United States during the era of widespread PCV7 vaccination, 1998–2004. J Infect Dis. 2007;196:1346–54. DOI: 10.1086/521626
3. Singleton RJ, Hennessy TW, Bulkow LR, Hammitt LL, Zulz T, Hurlburt DA, et al. Invasive pneumococcal disease caused by nonvaccine serotypes among Alaska Native children with high levels of 7-valent pneumococcal conjugate vaccine coverage. JAMA. 2007;297:1784–92. DOI: 10.1001/jama.297.16.1784
4. Moore MR, Gertz RE Jr, Woodbury RL, Barkocy-Gallagher GA, Schaffner W, Lexau C, et al. Population snapshot of emergent *Streptococcus pneumoniae* serotype 19A in the United States, 2005. J Infect Dis. 2008;197:1016–27. DOI: 10.1086/528996
5. Lauer P, Rinaudo CD, Soriani M, Margarit I, Maione D, Rosini R, et al. Genome analysis reveals pili in group B *Streptococcus*. Science. 2005;309:105. DOI: 10.1126/science.1111563
6. Mora M, Bensi G, Capo S, Falugi F, Zingaretti C, Manetti AG, et al. Group A streptococcus produce pilus-like structures containing protective antigens and Lancefield T antigens. Proc Natl Acad Sci U S A. 2005;102:15641–6. DOI: 10.1073/pnas.0507808102
7. Ton-That H, Schneewind O. Assembly of pili on the surface of *Corynebacterium diphtheriae.* Mol Microbiol. 2003;50:1429–38. DOI: 10.1046/j.1365-2958.2003.03782.x
8. Barocchi MA, Ries J, Zogaj X, Hemsley C, Albiger B, Kanth A, et al. A pneumococcal pilus influences virulence and host inflammatory responses. Proc Natl Acad Sci U S A. 2006;103:2857–62. DOI: 10.1073/pnas.0511017103
9. Bagnoli F, Moschioni M, Donati C, Dimitrovska V, Ferlenghi I, Facciotti C, et al. A second pilus type in *Streptococcus pneumoniae* is prevalent in emerging serotypes and mediates adhesion to host cells. J Bacteriol. 2008;190:5480–92. Epub 2008 May 30.
10. Hava DL, Camilli A. Large-scale identification of serotype 4 *Streptococcus pneumoniae* virulence factors. Mol Microbiol. 2002;45:1389–406.
11. Basset A, Trzcinski K, Hermos C, O'Brien KL, Reid R, Santosham M, et al. Association of the pneumococcal pilus with certain capsular serotypes but not with increased virulence. J Clin Microbiol. 2007;45:1684–9. DOI: 10.1128/JCM.00265-07
12. Aguiar SI, Serrano I, Pinto FR, Melo-Cristino J, Ramirez M. The presence of the pilus locus is a clonal property among pneumococcal invasive isolates. BMC Microbiol. 2008;8:41. DOI: 10.1186/1471-2180-8-41
13. Moschioni M, Donati C, Muzzi A, Masignani V, Censini S, Hanage WP, et al. *Streptococcus pneumoniae* contains 3 rlrA pilus variants that are clonally related. J Infect Dis. 2008;197:888–96. DOI: 10.1086/528375
14. McGee L, McDougal L, Zhou J, Spratt BG, Tenover FC, George R, et al. Nomenclature of major antimicrobial-resistant clones of *Streptococcus pneumoniae* defined by the pneumococcal molecular epidemiology network. J Clin Microbiol. 2001;39:2565–71. DOI: 10.1128/JCM.39.7.2565-2571.2001
15. Abbot EL, Smith WD, Siou GP, Chiriboga C, Smith RJ, Wilson JA, et al. Pili mediate specific adhesion of *Streptococcus pyogenes* to human tonsil and skin. Cell Microbiol. 2007;9:1822–33. DOI: 10.1111/j.1462-5822.2007.00918.x
16. Mandlik A, Swierczynski A, Das A, Ton-That H. *Corynebacterium diphtheriae* employs specific minor pilins to target human pharyngeal epithelial cells. Mol Microbiol. 2007;64:111–24. DOI: 10.1111/j.1365-2958.2007.05630.x
17. Konto-Ghiorghi Y, Mairey E, Mallet A, Dumenil G, Caliot E, Trieu-Cuot P, et al. Dual role for pilus in adherence to epithelial cells and biofilm formation in *Streptococcus agalactiae.* PLoS Pathog. 2009;5:e1000422. DOI: 10.1371/journal.ppat.1000422
18. Nobbs AH, Rosini R, Rinaudo CD, Maione D, Grandi G, Telford JL. Sortase A utilizes an ancillary protein anchor for efficient cell wall anchoring of pili in *Streptococcus agalactiae.* Infect Immun. 2008;76:3550–60. DOI: 10.1128/IAI.01613-07
19. Mandlik A, Das A, Ton-That H. The molecular switch that activates the cell wall anchoring step of pilus assembly in gram-positive bacteria. Proc Natl Acad Sci U S A. 2008;105:14147–52. DOI: 10.1073/pnas.0806350105
20. Whitney CG, Farley MM, Hadler J, Harrison LH, Lexau C, Reingold A, et al. Increasing prevalence of multidrug-resistant *Streptococcus pneumoniae* in the United States. N Engl J Med. 2000;343:1917–24. DOI: 10.1056/NEJM200012283432603
21. Gay K, Baughman W, Miller Y, Jackson D, Whitney CG, Schuchat A, et al. The emergence of *Streptococcus pneumoniae* resistant to macrolide antimicrobial agents: a 6-year population-based assessment. J Infect Dis. 2000;182:1417–24. DOI: 10.1086/315853
22. Ottolenghi E, Hotchkiss RD. Release of genetic transforming agent from pneumococcal cultures during growth and disintegration. J Exp Med. 1962;116:491–519. DOI: 10.1084/jem.116.4.491
23. Enright MC, Spratt BG. A multilocus sequence typing scheme for *Streptococcus pneumoniae*: identification of clones associated with serious invasive disease. Microbiology. 1998;144:3049–60. DOI: 10.1099/00221287-144-11-3049
24. Feil EJ, Li BC, Aanensen DM, Hanage WP, Spratt BG. eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. J Bacteriol. 2004;186:1518–30. DOI: 10.1128/JB.186.5.1518-1530.2004
25. Biswas I, Scott JR. Identification of *rocA*, a positive regulator of *covR* expression in the group A streptococcus. J Bacteriol. 2003;185:3081–90. DOI: 10.1128/JB.185.10.3081-3090.2003
26. Alloing G, Martin B, Granadel C, Claverys JP. Development of competence in *Streptococcus pneumonaie*: pheromone autoinduction and control of quorum sensing by the oligopeptide permease. Mol Microbiol. 1998;29:75–83. DOI: 10.1046/j.1365-2958.1998.00904.x
27. Brueggemann AB, Spratt BG. Geographic distribution and clonal diversity of *Streptococcus pneumoniae* serotype 1 isolates. J Clin Microbiol. 2003;41:4966–70. DOI: 10.1128/JCM.41.11.4966-4970.2003
28. Gonzalez BE, Hulten KG, Kaplan SL, Mason EO Jr. Clonality of *Streptococcus pneumoniae* serotype 1 isolates from pediatric patients in the United States. J Clin Microbiol. 2004;42:2810–2. DOI: 10.1128/JCM.42.6.2810-2812.2004

29. Beall B, McEllistrem MC, Gertz RE Jr, Wedel S, Boxrud DJ, Gonzalez AL, et al. Pre- and postvaccination clonal compositions of invasive pneumococcal serotypes for isolates collected in the United States in 1999, 2001, and 2002. J Clin Microbiol. 2006;44:999–1017. DOI: 10.1128/JCM.44.3.999-1017.2006
30. Telford JL, Barocchi MA, Margarit I, Rappuoli R, Grandi G. Pili in gram-positive pathogens. Nat Rev Microbiol. 2006;4:509–19. DOI: 10.1038/nrmicro1443
31. Scott JR, Zähner D. Pili with strong attachments: Gram-positive bacteria do it differently. Mol Microbiol. 2006;62:320–30. DOI: 10.1111/j.1365-2958.2006.05279.x
32. Gianfaldoni C, Censini S, Hilleringmann M, Moschioni M, Facciotti C, Pansegrau W, et al. *Streptococcus pneumoniae* pilus subunits protect mice against lethal challenge. Infect Immun. 2007;75:1059–62. DOI: 10.1128/IAI.01400-06
33. Brueggemann AB, Griffiths DT, Meats E, Peto T, Crook DW, Spratt BG. Clonal relationships between invasive and carriage *Streptococcus pneumoniae* and serotype- and clone-specific differences in invasive disease potential. J Infect Dis. 2003;187:1424–32. DOI: 10.1086/374624
34. Sandgren A, Sjöström K, Olsson-Liljequist B, Christensson B, Samuelsson A, Kronvall G, et al. Effect of clonal and serotype-specific properties on the invasive capacity of *Streptococcus pneumoniae*. J Infect Dis. 2004;189:785–96. DOI: 10.1086/381686
35. Sjöström K, Spindler C, Ortqvist A, Kalin M, Sandgren A, Kuhlmann-Berenzon S, et al. Clonal and capsular types decide whether pneumococci will act as a primary or opportunistic pathogen. Clin Infect Dis. 2006;42:451–9. DOI: 10.1086/499242
36. Dagan R, Gradstein S, Belmaker I, Porat N, Siton Y, Weber G, et al. An outbreak of *Streptococcus pneumoniae* serotype 1 in a closed community in southern Israel. Clin Infect Dis. 2000;30:319–21. DOI: 10.1086/313645
37. Antonio M, Hakeem I, Awine T, Secka O, Sankareh K, Nsekpong D, et al. Seasonality and outbreak of a predominant *Streptococcus pneumoniae* serotype 1 clone from The Gambia: expansion of ST217 hypervirulent clonal complex in West Africa. BMC Microbiol. 2008;8:198. DOI: 10.1186/1471-2180-8-198
38. Rückinger S, von Kries R, Siedler A, van der Linden M. Association of serotype of *Streptococcus pneumoniae* with risk of severe and fatal outcome. Pediatr Infect Dis J. 2009;28:118–22. DOI: 10.1097/INF.0b013e318187e215
39. Pai R, Moore MR, Pilishvili T, Gertz RE, Whitney CG, Beall B. Postvaccine genetic structure of *Streptococcus pneumoniae* serotype 19A from children in the United States. J Infect Dis. 2005;192:1988–95. DOI: 10.1086/498043
40. Gertz RE Jr, McEllistrem MC, Boxrud DJ, Li Z, Sakota V, Thompson TA, et al. Clonal distribution of invasive pneumococcal isolates from children and selected adults in the United States prior to 7-valent conjugate vaccine introduction. J Clin Microbiol. 2003;41:4194–216. DOI: 10.1128/JCM.41.9.4194-4216.2003

Address for correspondence: Dorothea Zähner, Department of Medicine, Division of Infectious Diseases, Emory University School of Medicine, Atlanta, GA 30322, USA; email: dzahner@emory.edu

Rift Valley Fever during Rainy Seasons, Madagascar, 2008 and 2009

Soa Fy Andriamandimby, Armand Eugène Randrianarivo-Solofoniaina, Elisabeth M. Jeanmaire, Lisette Ravololomanana, Lanto Tiana Razafimanantsoa, Tsanta Rakotojoelinandrasana, Josette Razainirina, Jonathan Hoffmann, Jean-Pierre Ravalohery, Jean-Théophile Rafisandratantsoa, Pierre E. Rollin, and Jean-Marc Reynes[1]

During 2 successive rainy seasons, January 2008 through May 2008 and November 2008 through March 2009, Rift Valley fever virus (RVFV) caused outbreaks in Madagascar. Human and animal infections were confirmed on the northern and southern coasts and in the central highlands. Analysis of partial sequences from RVFV strains showed that all were similar to the strains circulating in Kenya during 2006–2007. A national cross-sectional serologic survey among slaughterhouse workers at high risk showed that RVFV circulation during the 2008 outbreaks included all of the Malagasy regions and that the virus has circulated in at least 92 of Madagascar's 111 districts. To better predict and respond to RVF outbreaks in Madagascar, further epidemiologic studies are needed, such as RVFV complete genome analysis, ruminant movement mapping, and surveillance implementation.

Rift Valley fever virus (RVFV) belongs to the family *Bunyaviridae*, genus *Phlebovirus*, and was first isolated in 1930 during an investigation of a large epizootic in Kenya. Virions are enveloped and contain 3 single-stranded RNA genome segments designated large (L), medium (M), and small (S) coding for the viral proteins.

Rift Valley fever (RVF) is an arthropod-borne zoonosis; it affects ruminants and is characterized by high rates of abortion and death in young and adult animals. Economic consequences of this disease can be devastating. In humans, symptoms are usually mild, but in severe cases hemorrhage, meningoencephalitis, retinopathy, and death can occur. RVFV has been detected across Africa, from Senegal to Madagascar and from Egypt to South Africa. In 2000, RVFV reached the Arabian Peninsula (*1*).

Animals are typically infected before humans. RVFV is transmitted between ruminants primarily by bites of mosquitoes of numerous genera and species. Humans can also be infected by these vectors as well as by contact or inhalation of aerosols generated when handling sick or dead infected animals or their fresh tissues. Treatment of human patients is based on signs and symptoms; a commercial vaccine is available for animals only. RVFV outbreaks are periodic and occur every 10–15 years. Between epidemics, the virus is believed to be maintained through vertical transmission by mosquitoes of the genus *Aedes*. Outbreaks are closely linked to climate variations, especially widespread increased rainfall, that favor the hatching of mosquito eggs and the subsequent emergence of a large number of adult mosquitoes (*2*). Moderate or large outbreaks that have been documented in the Horn of Africa (1989, 1997–1998, 2006–2007) were associated with widespread rainfall. For the purpose of predicting RVF outbreaks in this area, a model based on several satellite-derived observations has been proposed (*3*).

RVFV has also been detected in Madagascar. The first isolate was obtained from mosquitoes caught during the March 1979 rainy season in a forest area in the Moramanga district (no. 514; online Appendix Figure, www.cdc.gov/EID/content/16/6/963-appF.htm), 120 km east of Antananarivo (*4*). Then in March 1990, an RVF epizootic

Author affiliations: Institut Pasteur, Antananarivo, Madagascar (S.F. Andriamandimby, T. Rakotojoelinandrasana, J. Razainirina, J. Hoffmann, J.-P. Ravalohery, J.-T. Rafisandratantsoa, J.-M. Reynes); Ministère de la Santé et du Planning Familial, Antananarivo (A.E. Randrianarivo-Solofoniaina, L. Ravololomanana); Food and Agriculture Organization, Antananarivo (E.M. Jeanmaire); Ministère de l'Agriculture, d'Elevage et de la Pêche, Antananarivo, (L.T. Razafimanantsoa); and Centers for Disease Control and Prevention, Atlanta, Georgia, USA (P.E. Rollin)

DOI: 10.3201/eid1606.091266

[1]Current affiliation: Centre Pasteur du Cameroon, Yaounde, Cameroon.

occurred in Fenoarivo Atsinana (district 509) on the east coast, where an abnormally high incidence of abortions and disease in humans was reported (*5*,*6*). A year later, from February through April 1991, RVFV was responsible for abortions and deaths of cattle in the central highlands. Human cases were also confirmed (*7*,*8*). After the outbreaks of RVF in 2006 and 2007 in the Horn of Africa (*9*), 17 years later, the virus was again detected in Madagascar during a major outbreak. We report some features of this outbreak and the results of preliminary molecular characterization of the circulating virus. We also performed a nationwide serosurvey to determine the range of past and recent RVFV circulation.

Materials and Methods

Human Surveillance Systems

In 1996, in accordance with World Health Organization resolution AFR/RC43/R7, the Integrated Diseases Surveillance and Response system was implemented by the Direction des Urgences et de la Lutte contre les Maladies (DULM) from the Malagasy Ministry of Health. Hemorrhagic fevers are among the reportable diseases. Each week, basic health centers and hospitals (district and regional) must notify DULM about cases or absence of cases.

In addition, in 2007 the DULM set up, in collaboration with the Institut Pasteur de Madagascar (IPM), a sentinel surveillance system including 19 clinical sites (basic health centers). Each site reports daily to the central level (DULM and IPM) the number of patients, persons with fever, confirmed malaria cases, suspected arboviral disease cases, and suspected influenza cases. Suspected arboviral disease cases were defined as cases in patients with axillary temperature >37.5°C and ≥2 of the following signs: headache, myalgia, arthralgia, retro-orbital pain, and cutaneous rash or hemorrhagic signs. Of the 19 centers, 4 are also biological surveillance sites. Serum samples from patients with suspected arboviral disease are sent weekly in liquid nitrogen to the IPM.

During the outbreak, a suspected case of RVF was defined as illness in a person with a hemorrhagic syndrome and history of fever or as encephalitis and a dengue-like syndrome after exposure to sick or dead ruminants or exposure to ruminants in a village where sick or dead animals had been reported since detection of the first cases.

Laboratory Diagnosis

Serologic Assays

RVFV immunoglobulin (Ig) M and IgG ELISAs were performed as described previously (*10*). After heat and detergent inactivation, serum samples were tested by anti-RVFV–specific IgM and IgG ELISAs. The assays were completed by using inactivated RVFV-infected Vero E6 cell antigens and uninfected Vero E6 cell antigens; 4 dilutions of each serum (1:100, 1:400, 1:1,600, 1:6,400) were used. Titers and the cumulative sum of optical densities of each dilution (SUM_{OD}) minus the background absorbance of uninfected control Vero E6 cells (adjusted SUM_{OD}) were recorded. Results of the assays for serum samples were considered positive only if the adjusted SUM_{OD} and titer were above preestablished conservative cutoff values, which were set for IgM ELISA (≥0.75 and ≥400, respectively) and IgG ELISA (≥0.95 and ≥400, respectively). A probable RVFV infection was one in which no RVFV was detected but antibodies were detected. Infection was considered recent when IgM against RVFV was detected and past when only IgG against RVFV (no IgM against RVFV) was detected.

Virologic Assays

Virus isolation was performed on mosquito cell lines (AP61 and Vero E6) by using acute-phase serum samples (diluted 1:10), and virus identification was performed by an indirect immunofluorescence assay that used pools of mouse immune ascetic fluids (*11*). These fluids reacted against several arboviruses previously isolated from Madagascar, including RVFV.

Molecular Assays

Liver or spleen specimens (50–100 mg) from dead animals with suspected RVF were homogenized at a dilution of 1:10 in culture medium containing 30% fetal bovine serum. The supernatant was collected after centrifugation. Viral RNA was extracted from the serum samples of patients and animals suspected of having infection by using the QIAamp Viral RNA Mini Kit (QIAGEN GmbH, Hilden, Germany) and from organ supernatants by using TRIzol LS reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturers' instructions.

The molecular detection of the virus was performed by using a nested reverse transcription–PCR (RT-PCR) described by Sall et al. (*12*) or a real-time RT-PCR described by Weidmann et al. (*13*). The minimum level of detection was 25 transcript RNA copies per assay. A confirmed RVFV infection was an infection in which RVFV was isolated or RVFV RNA was detected.

Molecular Characterization

Parts of the S, M, and L segments of RVFV were amplified and sequenced. S-amplified product (portion of nonstructural protein) was obtained by using the nested RT-PCR technique published by Sall et al. *(12)*. M-amplified product (portion of *G2*) was obtained by using RT-PCR primers M-F675 (5′-ACCATCATTGCAAAGGCTGA-3′) and M-R1645 (5′-GCCATGTGAACCCCTATGTC-3′)

and nested PCR primers MRV1a and MRV2g used by Sall et al. (*14*). L-amplified product (portion of L) was obtained by using RT-PCR primers L-F4209 (5′-GCGCATTGCAGAGAAAGTC) and L-R5113 (5′-CAA CGTGATCACCATCTAGAAA-3′) and nested PCR primers L-F4273 (5′-TGTAAAGTCATGGCCTCAGC-3′) and L-R4878 (5′-CATCCGGGAGAAATTGTCA-3′). M-F675, M-R1645, L-F4209, L-R5113, L-F4273, and L-R4878 were designed to obtain M- and L-nested PCR products >600 bp; Primer3Plus software (*15*) was used according to 33 published complete RVFV M or L sequences (*16*).

Amplification products were sequenced on both strands by Cogenics (Meylan, France). Unverified sequences and chromatograms were compared and corrected when needed. Sequences from the same segment were compared when aligned, and a phylogenetic analysis was conducted by using MEGA version 4 software (*17*). The partial S, M, and L sequences obtained in this study are available from GenBank under accession nos. GQ443126–GQ443256.

Nationwide Serologic Survey

A nationwide cross-sectional serologic survey was conducted among persons at risk for RVF. In all 111 districts of Madagascar (online Appendix Figure), persons were invited to participate in the survey if they had worked in slaughterhouses within the administrative center of the district since 2007, had been exposed to fresh meat or blood of ruminants, and had been residents of the district when they started this work. The study was approved by the Malagasy National Ethical Committee. From those who gave written informed consent, 5 mL of blood was collected into red-top tubes. Samples; informed consent forms; and data sheets recording age, sex, location, date of sampling, and criteria of sampling were sent to the IPM, where RVFV IgG and IgM ELISAs were performed.

Results

Outbreaks

The first RVF case of 2008 was detected during routine activity of the biological surveillance sentinel center in Tolagnaro city (district no. 614) in southern Madagascar (Figure 1; online Appendix Figure). The virus was isolated on AP61 and Vero E6 cells from an acute-phase serum sample collected on January 30, 2008, from a pregnant woman who had had a dengue-like syndrome for 2 days. Retrospective investigation showed that each day she had collected fresh meat from a local slaughterhouse to make and sell meat kebabs. On February 5, 2008, the DULM received an alert through the Integrated Diseases Surveillance and Response system. Cases of hemorrhagic fever and dengue-like fever, associated with deaths of farmers and with abnormal mortality rates for zebus, were reported from the Anjozorobe district (no. 107), 80 km north of Antananarivo. IgM against RVFV was detected in 16 of 23 persons sampled on February 9, 2008. These patients had been ill 1–3 weeks earlier. Retrospective investigation suggested that the virus had been circulating among livestock since December 2007.

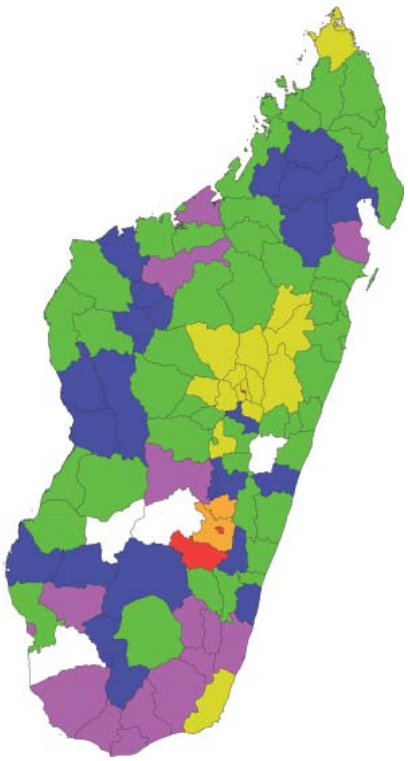


Figure 1. Distribution of Rift Valley fever in the 111 administrative districts in Madagascar, 2008 and 2009. Districts with laboratory-diagnosed confirmed or probable cases in humans and/or animals are indicated by yellow (2008), orange (2009), or red (both years). In districts without confirmed or probable cases, antibody data for Rift Valley fever virus immunoglobulin (Ig) levels in serum samples from at-risk professionals are indicated by green (IgM positive only), blue (IgG positive, IgM negative), or violet (IgG and IgM negative). No samples were received from districts shown in white.

Consequently, the DULM encouraged notification and sampling of RVF suspected cases by the basic health centers and district and regional hospitals. Overall, from January 28 through June 15, 2008, when the active surveillance was stopped, 476 suspected cases (19 of which were fatal) from 15 districts, mostly from the central highlands, were reported to the DULM.

Serum samples were received at IPM from 134 persons with suspected cases who lived in 16 districts: 36 cases were confirmed, and 31 were considered probable. Laboratory analysis results were negative for the other 67. Most of the persons with probable and confirmed cases had occupational risk; 54 were farmers and 5 were butchers.

Additionally, serum, organs, or fetuses were received from 119 animals sampled by the Direction des Services Vétérinaires in 19 districts. Of the 119, cases were confirmed for 15, considered probable for 7, and were negative for the other 97. Overall, RVFV confirmed and probable cases were identified in 19 districts (Figure 1). The last laboratory-confirmed case consisted of a fatal hemorrhagic disease that began on May 23, 2008, in a person from the Amparafaravola district (no. 504), near Lake Aloatra, 180 km north of Antananarivo.

Six months later, during the rainy season (October 2008–May 2009), an alert was launched by the Direction des Services Vétérinaires when abnormally high death rates among cattle were reported from Fianarantosoa-I (no. 301) and Fianarantsoa-II (no. 302) districts (located in the central highlands). Livers from 3 cows sampled on November 28, 2008, and sent to IPM were infected with RVFV. Suspected human RVF cases from this area were confirmed. Overall, from December 1, 2008, through May 15, 2009, 236 suspected cases (including 7 deaths) in humans, were reported to the DULM: 1 from Antananarivo (no. 101) and the rest from 4 neighboring districts: 74 cases from Fianarantsoa-I (no. 301), 152 from Fianarantsoa-II (no. 302), 4 from Ambohimahasoa (no. 305), and 5 from Ambalavao (no. 303). Of the 47 suspected cases sampled, 10 were confirmed and 9 were probable. Laboratory analysis results were negative for the other 28 cases. More than half of the 19 persons with confirmed and probable cases were at high risk: 8 were farmers, 1 a butcher, 1 a veterinarian, and 1 (from Antananarivo) a cattle trader who transported animals from the south through the infected area to Antananarivo. Additionally, confirmed or probable infection was detected in serum samples or livers from 24 ruminants among 88 sampled in Fianarantsoa-I and Fianarantsoa-II districts during the period. The last laboratory-confirmed RVFV case was in a person who had hemorrhagic manifestations and lived in the Ambalavao district (no. 303); disease onset was March 13, 2009.

Genetic Analysis

Verified partial S sequences encompassing 627 nt (positions 49–675 of the coding domain) were obtained from 46 of the 51 RVFV-positive samples collected during the January–May 2008 outbreak. These 46 samples originated from 14 of the 17 districts where the virus was detected, including the 3 main areas concerned (northern coast, southern coast, and central highlands) (Figure 1; Table). Of these 46 samples, 34 were collected from humans, 10 from cattle, 1 from a sheep, and 1 from a goat (Table). The maximum nucleotide percent identity difference between the 46 sequences was low (0.96%; 6/627). Of 11 haplotypes of sequences detected, 1 included 35 identical sequences from strains originating from 5 districts (central highlands). At the protein level, the maximum percent identity difference was 1.4% (3/209): 7 haplotypes of sequences were detected (data not shown). The virus sequences of cattle strains were identical to those of human strains in the 2 districts where RVFV strains had been obtained from cattle and humans. Phylogenetic analysis, using the neighbor-joining method with the Kimura 2-parameter model, was undertaken for the 11 Madagascar sequences representative of all the diversity, the corresponding part of the 33 complete S sequences described by Bird et al. (*16*), and the 6 sequences representative of the lineages Kenya-1, -1a and -2 recently described during the 2006–2007 Kenya outbreak (*18*). Although the bootstrap values did not support an unambiguous phylogenetic classification, the analysis showed that the Madagascar sequences were close to the Kenya sequences obtained from strains circulating in 2006–2007, especially to Kenya-1 and -1a lineages.

Partial M sequences (nt positions 781–1536 of the coding domain) and partial L sequences (nt positions 1276–1839 of the coding domain) were available, respectively, from 39 and 42 of the 46 strains from which we obtained the S sequence. Phylogenetic analysis performed as described above, and including the M or L sequences of the strains used for the S analysis, confirmed that these sequences were closer to sequences obtained from Kenya strains circulating in 2006–2007 (data not shown).

Analysis of sequences from strains detected in during the second outbreak November and December 2008 was limited to 4 strains (6510–08, 6546–08, 6547–08, 6660–08) and to the S segment (627 nt). All were close or identical to the Madagascar sequences detected during the first outbreak and clustered within the Kenya-1 and -1a lineages (Figure 2).

Cross-Sectional Serologic Survey

Confirmed and probable cases were detected in the central highlands and in 2 nonadjoining and well-separated areas, the southern and northern coasts of the island (Figure 1). Geographic distribution of RVFV recent circulation was hypothesized to be larger. To confirm this hypothesis, a nationwide serologic survey was organized. Serum was collected from September 27, 2008, through May 27, 2009, from 1,995 volunteers, who were at high risk for RVFV infection, from 106 of the 111 Malagasy districts. Probable recent RVFV infection was detected for 214 participants, probable past RVFV infection was detected for 219 participants, and recent or past RVFV infection was ruled out for the rest (online Appendix Table, www.cdc.gov/EID/content/16/6/963-appT.htm). Overall (considering the location of the persons with confirmed and probable cases), recent circulation of RVFV was detected in 70 districts and recent or past infection in 92 districts, which indicated wide circulation of RVFV in Madagascar (Figure 1).

Discussion

Since the first field and laboratory investigations conducted by IPM in the 1970s, 15 arboviruses have been isolated in Madagascar, 10 of which are known to be pathogenic for humans and 3 of which (dengue virus type 1, chikungunya virus, and RVFV) have been responsible for large outbreaks (*4–8;19–22*). The RVF outbreak in 2008–2009 is the largest detected in the country, although during this outbreak the reporting and sampling for suspected cases in humans and animals were not optimal. RVF is a rural

Table. Features of 46 sequenced Rift Valley fever viruses, Madagascar, 2008–2009

					Coding sequences		
Code	Species	Sample type	Sampling date	District of sampling	Small	Medium	Large
212-08	Human	Serum	2008 Jan 30	Tolagnaro	Yes	Yes	Yes
406-08	Bovine	Fetal liver	2008 Feb 20	Miarinarivo	Yes	Yes	Yes
413-08	Bovine	Fetal liver	2008 Feb 20	Antsirabe II	Yes	Yes	Yes
427-08	Human	Serum	2008 Feb 22	Anjozorobe	Yes	Yes	Yes
428-08	Human	Serum	2008 Feb 15	Anjozorobe	Yes	Yes	Yes
435-08	Bovine	Serum	2008 Feb 22	Anjozorobe	Yes	No	Yes
437-08	Bovine	Serum	2008 Feb 22	Anjozorobe	Yes	No	No
448-08	Human	Serum	2008 Feb 26	Anjozorobe	Yes	Yes	Yes
449-08	Human	Serum	2008 Feb 26	Anjozorobe	Yes	No	Yes
619-08	Human	Serum	2008 Mar 9	Ankazobe	Yes	Yes	Yes
682-08	Human	Serum	2008 Mar 12	Ambatondrazaka	Yes	Yes	Yes
683-08	Human	Serum	2008 Mar 12	Ambatondrazaka	Yes	Yes	Yes
684-08	Human	Serum	2008 Mar 12	Ambatondrazaka	Yes	Yes	Yes
693-08	Human	Serum	2008 Mar 11	Amparafaravola	Yes	Yes	Yes
776-08	Human	Serum	2008 Mar 14	Ambatondrazaka	Yes	Yes	Yes
779-08	Human	Serum	2008 Mar 16	Amparafaravola	Yes	No	Yes
845-08	Human	Serum	2008 Mar 23	Manjakandriana	Yes	Yes	Yes
846-08	Human	Serum	2008 Mar 23	Manjakandriana	Yes	Yes	Yes
847-08	Human	Serum	2008 Mar 23	Manjakandriana	Yes	Yes	Yes
848-08	Human	Serum	2008 Mar 23	Manjakandriana	Yes	Yes	Yes
849-08	Human	Serum	2008 Mar 23	Manjakandriana	Yes	Yes	Yes
850-08	Human	Serum	2008 Mar 23	Manjakandriana	Yes	Yes	No
851-08	Human	Serum	2008 Mar 23	Manjakandriana	Yes	Yes	Yes
852-08	Human	Serum	2008 Mar 23	Manjakandriana	Yes	Yes	Yes
853-08	Human	Serum	2008 Mar 23	Manjakandriana	Yes	Yes	Yes
854-08	Human	Serum	2008 Mar 23	Manjakandriana	Yes	Yes	Yes
855-08	Bovine	Serum	2008 Mar 24	Manjakandriana	Yes	Yes	Yes
856-08	Bovine	Serum	2008 Mar 24	Manjakandriana	Yes	No	Yes
857-08	Bovine	Serum	2008 Mar 24	Manjakandriana	Yes	No	No
859-08	Bovine	Serum	2008 Mar 24	Manjakandriana	Yes	No	Yes
863-09	Human	Serum	2008 Mar 25	Antananarivo	Yes	Yes	Yes
878-09	Human	Serum	2008 Mar 26	Amparafaravola	Yes	Yes	Yes
879-08	Human	Serum	2008 Mar 26	Manjakandriana	Yes	Yes	Yes
889-08	Human	Serum	2008 Mar 26	Manjakandriana	Yes	Yes	No
892-09	Human	Serum	2008 Mar 26	Manjakandriana	Yes	Yes	Yes
895-09	Human	Serum	2008 Mar 26	Manjakandriana	Yes	Yes	Yes
897-08	Human	Serum	2008 Mar 26	Manjakandriana	Yes	Yes	Yes
1464-08	Human	Serum	2008 Mar 28	Tolagnaro	Yes	Yes	Yes
1585-08	Caprine	Serum	2008 Apr 13	Antsiranana I	Yes	Yes	Yes
1586-08	Human	Serum	2008 Apr 15	Antavy Atsimo	Yes	Yes	Yes
1627-08	Bovine	Serum	2008 Apr 18	Ambalavao	Yes	Yes	Yes
1695-08	Ovine	Serum	2008 Apr 20	Antsiranana II	Yes	Yes	Yes
1730-08	Bovine	Spleen	2008 Apr 25	Fianarantsoa I	Yes	Yes	Yes
2032-08	Human	Serum	2008 May 25	Amparafaravola	Yes	Yes	Yes
2033-08	Human	Serum	2008 Apr 29	Amparafaravola	Yes	Yes	Yes
2034-08	Human	Serum	2008 Apr 23	Amparafaravola	Yes	Yes	Yes
6510-08	Bovine	Liver	2008 Dec 1	Fianarantsoa I	Yes	Not done	Not done
6546-08	Bovine	Liver	2008 Nov 28	Fianarantsoa I	Yes	Not done	Not done
6547-08	Bovine	Liver	2008 Nov 29	Fianarantsoa II	Yes	Not done	Not done
6660-08	Human	Serum	2008 Dec 12	Fianarantsoa I	Yes	Not done	Not done

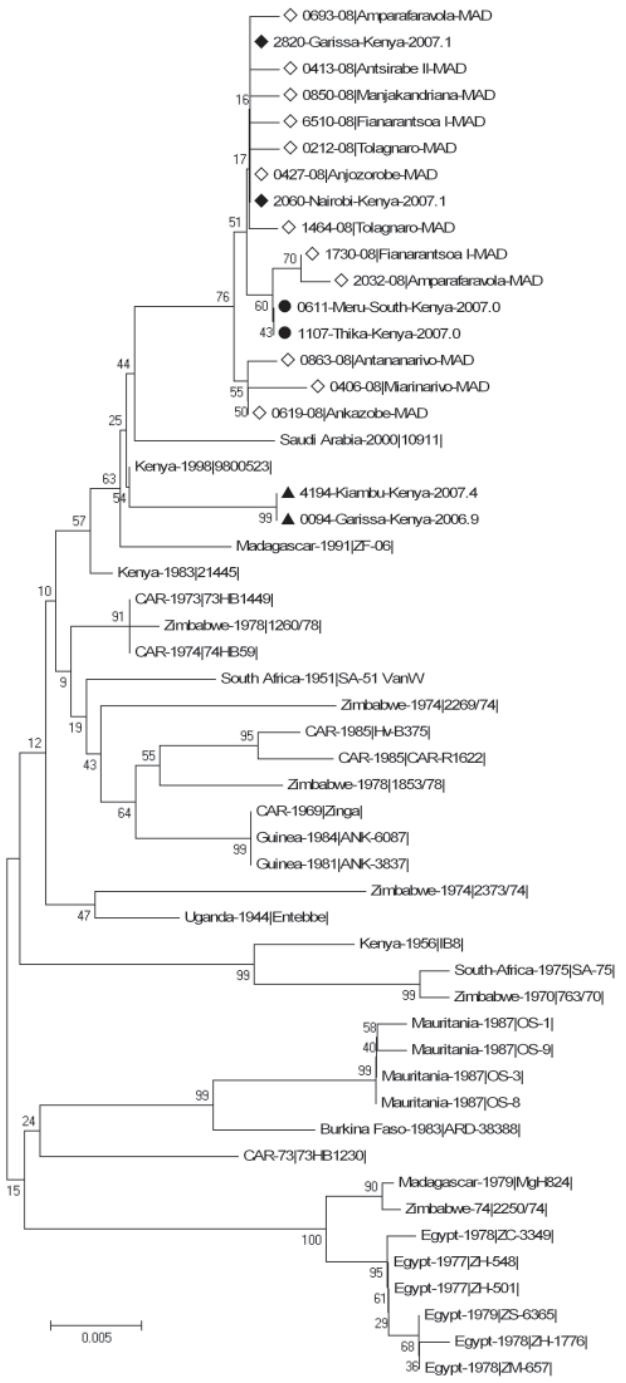


Figure 2. Phylogenetic tree based on the entire small sequences from 33 Rift Valley fever virus strains described by Bird et al. (*16*), from 6 sequences representative of the Kenya 1, 1a, and 2 lineages described by Bird et al. (*18*), and from 1 Madagascar strain circulating in 1991 and 12 Madagascar strains circulating in 2008. Boostrap percentages (from 1,000 resamplings) are indicated at each node. ◆ indicates sequences from the 2006–2007 Kenya-1 lineage, ● indicates sequences from the 2006–2007 Kenya-1a lineage, ▲ indicates sequences from the 2006–2007 Kenya 2 lineage, and ◇ indicates the 2008 Madagascar sequences. Scale bar indicates nucleotide substitutions per site.

disease occurring mainly during the rainy season. Transfer of information from the central administration to the health centers and back was challenging during this period in this low-income country. This lack of recorded information prevented us from describing the outbreak in more detail and estimating the extent of human disease. However, the extent of the disease could be established through a retrospective study in the outbreak area, comparing the crude death rate observed during the outbreak period with an expected death rate computed from data for previous years. The effect on livestock is even more difficult to quantify because of the lack of animal population data and the limited number of specimens submitted for diagnosis.

The addition of the Madagascar RVFV 2008 strains to the Kenya-1 2006–2007 lineage raises the question of this lineage's introduction from Kenya. We detected IgG against RVF in serum from 18 of 24 goats sampled by the end of June 2008 in Toliara II district (no. 602) on the southeast coast where abortions were reported in early 2007 (J.-M.R., unpub. data). RVFV was probably circulating in this area while it was circulating in Kenya, and perhaps it was introduced from Kenya at that time. Official records document exportation (but no importation) of ruminants from Madagascar to the Comoros archipelago; subsequent exportation to the African continent is possible (L.T.R., pers. comm.). However, illegal importation cannot be excluded. The question of introduction remains unanswered. More complete phylogenetic studies, and full sequences of Madagascar 2008 isolates, are needed to detect the circulation of $\geq$1 lineages during the 2008 outbreak and to get a better understanding of the movements and evolution of Madagascar and Kenya isolates.

The nationwide cross-sectional serosurvey supplemented the information obtained during the outbreak. The serologic observations suggest that the virus has probably circulated in the past in most districts and more recently, in 2008–2009, at least in all regions of the country. The sample collection from persons at risk started 3 months and ended 12 months after the detection of the last case of the 2008 outbreak. Despite not having data on duration of IgM against RVFV in humans, we suspect that IgM may have already disappeared in some of the serum samples tested and that the area of recent RVFV circulation is indeed larger than the one we studied. The lack of evidence of virus circulation in some adjoining districts from arid southern Madagascar may be also explained by our small sample size from some of them (online Appendix Table). However, the serologic investigation conducted among cattle sampled after the 2008 RVF outbreak indicated that the virus has circulated in the following districts: Midongy-Atsimo (no. 318), Vangaindrano (no. 320), Ampanihy (no. 605), Sakaraha (no. 620), Betioky (no. 612), and Toliara-I. (no. 601) (Elisabeth Jeanmaire, unpub. data) and reduced

the area of contiguous districts where the virus circulation was not detected to the following 6 districts: Iakora (no. 311), Befotaka (no. 307), Amboasary-Atsimo (no. 603), Ambovombe-Androy (no. 604), Tsihombe (no. 621), and Bekily (no. 607).

Recent circulation of RVFV in the country was extensive. The detection of the same haplotype from serum sampled at the same period, from the 2 first reported outbreak places 500 km apart may be explained by the large-scale movement of cattle within the country. Observed movements of cattle from rural areas to provincial capitals and between provincial capitals and Antananarivo, could explain the rapid spread of the virus. However, we do not know where the outbreak started; thus, reemergence of RVFV from different places cannot be ruled out. We found the results of some unpublished studies reporting the movement of ruminants in some areas, but a comprehensive study of these movements is needed for a better understanding of the epidemiology of the disease and to organize its surveillance and control.

The geographic distribution of RVF encompasses all 4 ecozones of Madagascar (www.nationalgeographic.com/wildworld/terrestrial.html). This finding suggests that mosquito transmission may occur in all of them. Extensive entomologic studies conducted out in the 1980s in Madagascar have shown that some species described as vectors on the African continent were present in some or all 4 ecozones (*19*). This finding implies that cycles of transmission involving different species may occur in Madagascar. Until now, little information on RVFV vectors in Madagascar has been available (*19*). Thousands of mosquitoes were collected in the highlands during the 2008–2009 outbreaks. The results of the virus detection are still pending and will contribute to the knowledge of the RVFV vectors in Madagascar.

The model used to predict at risk RVF situation has been efficient in the Horn of Africa (*3*,*23*). When this model was applied to Madagascar, the area where probable and confirmed cases were reported was not predicted to be at risk (*24*), suggesting that the model needs to be adjusted for Madagascar. This last point and the questions raised above underline the need for research studies and surveillance on RVF in Madagascar to better predict, declare, and respond to RVF outbreaks.

Acknowledgments

We are grateful to Pierre Formenty, Stéphane de la Rocque, Antoine Talarmin, and Roland Robinson Andrianirina for encouraging and facilitating this study. We also thank Stuart Nichol, Serena Carroll, and Craig Manning for helpful comments on the manuscript, Amadou A. Sall for providing mouse immune ascetic fluids against arboviruses; and Manfred Weidmann for providing RVFV transcript RNA.

This study was supported in part by funds raised by the World Health Organization and the Food and Agriculture Organization of the United Nations through the Central Emergency Response Fund of the United Nations.

Dr Andriamandimby is a medical doctor. As head of the National Reference Center for arbovirus and virus responsible for hemorrhagic fever in Madagascar, she is involved with surveillance, training, and research on arboviruses and hemorrhagic fever viruses.

References

1. Swanepoel R, Coetzer JAW. Rift Valley fever. In: Coetzer JAW, Thomson GR, Tustin RC, editors. Infectious diseases of livestock. Oxford (UK): Oxford University Press; 2004. p. 1037–70.
2. Davies FG, Linthicum KJ, James AD. Rainfall and epizootic Rift Valley fever. Bull World Health Organ. 1985;63:941–3.
3. Anyamba A, Chretien JP, Small J, Tucker CJ, Formenty PB, Richardson JH, et al. Prediction of a Rift Valley fever outbreak. Proc Natl Acad Sci U S A. 2009;106:955–9. DOI: 10.1073/pnas.0806490106
4. Mathiot C, Ribot JJ, Clerc Y, Coulanges P, Rasolofonirina N. Rift valley fever and Zinga virus: a pathogenic arbovirus in man and animal new for Madagascar. Arch Inst Pasteur Madagascar. 1984;51:125–33.
5. Morvan J, Saluzzo JF, Fontenille D, Rollin PE, Coulanges P. Rift Valley fever on the east coast of Madagascar. Res Virol. 1991;142:475–82. DOI: 10.1016/0923-2516(91)90070-J
6. Morvan J, Fontenille D, Saluzzo JF, Coulanges P. Possible Rift Valley fever outbreak in man and cattle in Madagascar. Trans R Soc Trop Med Hyg. 1991;85:108. DOI: 10.1016/0035-9203(91)90178-2
7. Morvan J, Rollin PE, Laventure S, Rakotoarivony I, Roux J. Rift Valley fever epizootic in the central highlands of Madagascar. Res Virol. 1992;143:407–15. DOI: 10.1016/S0923-2516(06)80134-2
8. Morvan J, Lesbordes JL, Rollin PE, Mouden JC, Roux J. First fatal human case of Rift Valley fever in Madagascar. Trans R Soc Trop Med Hyg. 1992;86:320. DOI: 10.1016/0035-9203(92)90329-B
9. World Health Organization. Outbreaks of Rift Valley fever in Kenya, Somalia and United Republic of Tanzania, December 2006–April 2007. Wkly Epidemiol Rec. 2007;82:169–78.
10. Madani TA, Al-Mazrou YY, Al-Jeffri MH, Mishkhas AA, Al-Rabeah AM, Turkistani AM, et al. Rift Valley fever epidemic in Saudi Arabia: epidemiological, clinical, and laboratory characteristics. Clin Infect Dis. 2003;37:1084–92. DOI: 10.1086/378747
11. Reynes JM, Laurent A, Deubel V, Telliam E, Moreau JP. The first epidemic of dengue hemorrhagic fever in French Guiana. Am J Trop Med Hyg. 1994;51:545–53.
12. Sall AA, Thonnon J, Sene OK, Fall A, Ndiaye M, Baudez B, et al. Single-tube and nested reverse transcriptase–polymerase chain reaction for detection of Rift Valley fever virus in human and animal sera. J Virol Methods. 2001;91:85–92. DOI: 10.1016/S0166-0934-(00)00252-4
13. Weidmann M, Sanchez-Seco MP, Sall AA, Ly PO, Thiongane Y, Lô MM, et al. Rapid detection of important human pathogenic phleboviruses. J Clin Virol. 2008;41:138–42. DOI: 10.1016/j.jcv.2007.10.001
14. Sall AA, Zanotto PM, Sene OK, Zeller HG, Digoutte JP, Thiongane Y, et al. Genetic reassortment of Rift Valley fever virus in nature. J Virol. 1999;73:8196–200.
15. Rozen S, Skaletsky H. Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol. 2000;132:365–86.

16. Bird BH, Khristova ML, Rollin PE, Ksiazek TG, Nichol ST. Complete genome analysis of 33 ecologically and biologically diverse Rift Valley fever virus strains reveals widespread virus movement and low genetic diversity due to recent common ancestry. J Virol. 2007;81:2805–16. DOI: 10.1128/JVI.02095-06
17. Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol. 2007;24:1596–9. DOI: 10.1093/molbev/msm092
18. Bird BH, Githinji JW, Macharia JM, Kasiiti JL, Muriithi RM, Gacheru SG, et al. Multiple virus lineages sharing recent common ancestry were associated with a large Rift Valley fever outbreak among livestock in Kenya during 2006–2007. J Virol. 2008;82:11152–66. DOI: 10.1128/JVI.01519-08
19. Fontenille D. Arbovirus transmission cycles in Madagascar. Arch Inst Pasteur Madagascar. 1989;55:1–317.
20. Morvan J, Fontenille D, Digoutte JP, Coulanges P. The Wesselsbron virus, a new arbovirus for Madagascar [in French]. Arch Inst Pasteur Madagascar. 1990;57:183–92.
21. Morvan JM, Digoutte JP, Marsan P, Roux JF. Ilesha virus: a new aetiological agent of haemorrhagic fever in Madagascar. Trans R Soc Trop Med Hyg. 1994;88:205. DOI: 10.1016/0035-9203-(94)90297-6
22. Ratsitorahina M, Harisoa J, Ratovonjato J, Biacabe S, Reynes JM, Zeller H, et al. Outbreak of dengue and chikungunya fevers, Toamasina, Madagascar, 2006. Emerg Infect Dis. 2008;14:1135–7. DOI: 10.3201/eid1407.071521
23. Food and Agriculture Organization. Possible RVF activity in the Horn of Africa. Empres watch. November 2006 [cited 2009 Jun 10]. ftp://ftp.fao.org/docrep/fao/011/aj218e/aj218e00.pdf
24. Food and Agriculture Organization. Rift Valley fever outbreaks in Madagascar and potential risks to neighbouring countries. Empres watch. April 2008 [cited 2009 Jun 10]. ftp://ftp.fao.org/docrep/fao/011/aj213e/aj213e00.pdf

Address for correspondence: Jean-Marc Reynes, Service de Virology, Centre Pasteur du Cameroun, rue Dunant, BP 1274, Yaounde, Cameroon; email: reynes@pasteur-yaounde.org

Invasive Aspergillosis after Pandemic (H1N1) 2009

Asma Lat, Nahid Bhadelia, Benjamin Miko, E. Yoko Furuya, and George R. Thompson III

We report 2 patients with invasive aspergillosis after infection with pandemic (H1N1) 2009. Influenza viruses are known to cause immunologic defects and impair ciliary clearance. These defects, combined with high-dose corticosteroids prescribed during influenza-associated adult respiratory distress syndrome, may be novel risk factors predisposing otherwise immunocompetent patients to invasive aspergillosis.

Invasive aspergillosis has emerged as a major cause of life-threatening infections in immunocompromised patients. Patients with prolonged neutropenia, inherited immunodeficiency, or those receiving immunosuppressive agents are at risk for infection (*1*). Recent evidence has identified other populations at risk for invasive aspergillosis, including those with chronic obstructive pulmonary disease (COPD) and nontransplant patients in intensive care units (ICUs) (*2*,*3*). We recently treated 2 immunocompetent patients who had invasive aspergillosis after infection with pandemic (H1N1) 2009 and are aware of similar cases at other centers (*4*).

Influenza strains have been reported to cause cell-mediated defects, disruption of normal ciliary clearance (*5*), and leukopenia (*6*). These abnormalities and use of high-dose corticosteroids during treatment for influenza-associated acute respiratory distress syndrome (ARDS) may form a unique group of risk factors for invasive aspergillosis.

The Patients

Patient 1 was a 28-year-old man (college student) with no unusual medical history (body mass index 18 kg/m^2) who was hospitalized after having shortness of breath for 1–1.5 weeks. Upon admission, he required intubation for respiratory failure. A chest radiograph showed multilobar pneumonia. His condition was complicated by pneumothoraces and bronchopleural fistula formation secondary to barotrauma during mechanical ventilation. A nasopharyngeal influenza swab specimen obtained at admission was positive for influenza A by PCR and was confirmed as pandemic (H1N1) 2009 by the New York State Health Department. Results of bronchoscopic evaluation for copathogens were negative. The patient was not treated with antivirus medication during his hospitalization because the diagnosis was made outside the optimal treatment time frame.

He received high-dose methylprednisolone (1 mg/kg/day) for ARDS for 28 days and later underwent bronchoscopy because of poor clinical improvement. Necrosis of the airway wall and cartilage, with extensive hyphae, was

Author affiliations: New York–Presbyterian Hospital, New York, New York, USA (A. Lat, N. Bhadelia, B. Miko, E. Yoko Furuya); and University of California, Davis, Sacramento, California, USA (G.R. Thompson III)

DOI: 10.3201/eid1606.100165

MEDSCAPE CME ACTIVITY

Medscape, LLC is pleased to provide online continuing medical education (CME) for this journal article, allowing clinicians the opportunity to earn CME credit. This activity has been planned and implemented in accordance with the Essential Areas and policies of the Accreditation Council for Continuing Medical Education through the joint sponsorship of Medscape, LLC and Emerging Infectious Diseases. Medscape, LLC is accredited by the ACCME to provide continuing medical education for physicians. Medscape, LLC designates this educational activity for a maximum of *0.25 AMA PRA Category 1 Credits™*. Physicians should only claim credit commensurate with the extent of their participation in the activity. All other clinicians completing this activity will be issued a certificate of participation. To participate in this journal CME activity: (1) review the learning objectives and author disclosures; (2) study the education content; (3) take the post-test and/or complete the evaluation at **www.medscapecme.com/journal/eid**; (4) view/print certificate.

Learning Objectives

Upon completion of this activity, participants will be able to:

- Describe diagnostic and management strategies for invasive aspergillosis following influenza infection.
- Identify historical outcomes of influenza-associated invasive aspergillosis.

Editor

Thomas J. Gryczan, Copyeditor, Emerging Infectious Diseases. *Disclosure: Thomas J. Gryczan, has disclosed no relevant financial relationships.*

CME Author

Charles P. Vega, MD, Associate Professor; Residency Director, Department of Family Medicine, University of California, Irvine, California, USA. *Disclosure: Charles P. Vega, MD, has disclosed no relevant financial relationships.*

Authors

Disclosures: ***Asma Lat, PharmD; Nahid Bhadelia, MD; Benjamin Miko, MD;*** *and* ***E. Yoko Furuya, MD, MSc****, have disclosed no relevant financial relationships.* ***George R. Thompson III, MD,*** *has disclosed the following relevant financial relationships: served as an advisor or consultant for Basilea Pharmaceutica Ltd.; served as a speaker or member of a speakers bureau for Merck & Co., Inc.; received grants for clinical research from Pfizer Inc.*

found and tissue cultures showed *Aspergillus fumigatus*. Intravenous voriconazole was initiated (6 mg/kg every 12 h [2 doses], then 4 mg/kg every 12 h for 24 days), but his clinical condition deteriorated; micafungin (100 mg/d) was added and continued for 14 days until he was transferred to another hospital on day 52.

On admission, tests showed lymphopenia (700 cells/mm^3) and renal and respiratory failure. *A. fumigatus* was isolated from sputum, bronchoscopy, and pleural fluid cultures. A computed tomographic scan of the chest showed multifocal pneumonia. He was initially treated with amphotericin B lipid complex, cefepime, metronidazole, tobramycin, and vancomycin. The patient became hemodynamically unstable and required multiple vasopressors. On day 70, he had cardiopulmonary arrest and died. An autopsy was not performed.

Patient 2 was a 51-year-old man (office worker) with no unusual medical history (body mass index 24.5 kg/m^2) who was hospitalized for fatigue and fever (≤104°F). A chest radiograph showed bilateral infiltrates. Laboratory tests showed a leukocyte count of 1,500 cells/mm^3 and a thrombocyte count of 65,000 cells/mm^3. A bone marrow biopsy specimen was negative for malignancy. A computed tomographic scan of the chest showed diffuse alveolar consolidation. Broad-spectrum antimicrobial drugs (vancomycin, aztreonam, azithromycin, and fluconazole) were given but the patient's respiratory status rapidly deteriorated and he was intubated on hospital day 2. Bronchoscopy showed alveolar hemorrhage, and he underwent video-assisted thoracoscopy and right lung wedge resection.

Virus cultures were positive for influenza A and verified as pandemic (H1N1) 2009 by the New York State Health Department. Antiviral treatment was not started because the diagnosis was made >48 hours after the onset of symptoms. A lung biopsy specimen showed widespread alveolar hemorrhage without fungal elements. Development of ARDS prompted administration of methylprednisolone (1 mg/kg/day for 3 days); his fever was quickly reduced. Once the methylprednisolone dose was tapered, fever recurred. Bronchoscopy on day 12 showed spontaneous bleeding from the right middle lobe and multiple blood clots. Pathologic examination identified fungal hyphae and *A. fumigatus* grew in culture. Treatment with intravenous voriconazole (6 mg/kg every 12 h [2 doses], followed by 4 mg/kg every 12 h) was initiated and continued for 3 days until he was transferred to another hospital on day 16.

At the new hospital, broad-spectrum antimicrobial therapy (linezolid, cefepime, tobramycin, oseltamivir, and voriconazole) was initiated. The patient showed decompensation over the next 72 h; his family withdrew care on day 21, and the patient died later that day. An autopsy showed severe pulmonary congestion, hemorrhage, and acute necrotizing bronchopneumonia. Several fungal abscesses consistent with *Aspergillus* spp. were identified in the lung, thyroid gland, and liver.

Conclusions

The number of patients at risk for invasive aspergillosis continues to increase. Recently, patients with COPD who are receiving long-term corticosteroids and immunocompetent ICU patients have been identified as nontraditional hosts at risk for invasive aspergillosis. Mortality rates in these groups are high, ≈95% in COPD patients (*2*) and 80% in ICU patients (*3*). However, infection with influenza and other respiratory viruses may pose a similar risk for invasive aspergillosis. Despite these high mortality rates, this association remains largely unnoticed (*7–10*). Thus, *Aspergillus* spp. observed in bronchoscopically obtained cultures from ICU patients diagnosed with pandemic (H1N1) 2009 may be overlooked as a contaminant despite their potential to cause invasive disease.

Infection with influenza virus is known to cause cell-mediated defects, disruption of normal ciliary clearance after infection (*5*), and leukopenia (*6*). These symptoms may predispose patients for invasive fungal disease. Additionally, ARDS (*11*) and immunodysregulation (*12*) may develop in patients with pandemic (H1N1) 2009. Severe structural lung disease apparent in this syndrome may also impair ciliary clearance, further predisposing these patients to invasive infections. Data suggest a potential benefit of corticosteroids in treating ARDS patients (*13*), but potentially life-saving, high-dose corticosteroids, combined with structural and immunologic abnormalities observed in patients with pandemic (H1N1) 2009, may predispose patients to invasive aspergillosis. Development of this disease after influenza may be a rare complication. However, infection with pandemic (H1N1) 2009 is widespread, thus placing many patients at risk for invasive aspergillosis.

Previous studies of influenza-associated aspergillosis have reported mortality rates of 100% (*7–10*). However, most of these reports predate routine use of noninvasive markers of invasive appergillosis or availability of voriconazole. Previous reports were published before the availability of oseltamivir, and specific antivirus therapy has been shown to decrease the incidence of influenza-associated complications (*14*,*15*). Early treatment with oseltamivir may have prevented complications seen in our patients.

Although intubated ICU patients commonly undergo bronchosopy, lack of a positive culture or direct smear result does not rule out a diagnosis of invasive aspergillosis (*3*). Moreover, although radiographic imaging may suggest aspergillosis, invasive diagnostic tests may be impractical when patients are hemodynamically unstable or have severe hypotoxicity, thrombocytopenia, or advanced coagulation deficits (*1*). Before serum testing for galactomannan, these patients would have satisfied criteria only for possible

invasive aspergillosis and appropriate treatment could have been withheld. However, assays for detection of serum galactomannan or 1–3-β-D glucan and compatible imaging studies can aid in the diagnosis of probable invasive aspergillosis and thus the initiation of appropriate antifungal therapy. With increased awareness of invasive aspergillosis in nontraditional hosts, high mortality rates in patients with this disease can be avoided.

In conclusion, we report 2 patients with invasive aspergillosis after infection with pandemic (H1N1) 2009. Development of ARDS, structural lung disease, high doses of corticosteroids, and T-cell defects during infection with influenza viruses may be responsible for an emerging group of patients at high risk for invasive aspergillosis. Early diagnostic and treatment strategies should be used for these patients, and multicenter studies are needed to better define incidence and outcomes.

Dr Lat is a clinical manager of infectious diseases in the Department of Pharmacy at New York–Presbyterian Hospital, Columbia University Medical Center. Her research interests are antifungal therapy and fungal diagnostics.

References

1. Walsh TJ, Anaissie EJ, Denning DW, Herbrecht R, Kontoyiannis DP, Marr KA, et al. Treatment of aspergillosis: clinical practice guidelines of the Infectious Diseases Society of America. Clin Infect Dis. 2008;46:327–60. DOI: 10.1086/525258
2. Bulpa P, Dive A, Sibille Y. Invasive pulmonary aspergillosis in patients with chronic obstructive pulmonary disease. Eur Respir J. 2007;30:782–800. DOI: 10.1183/09031936.00062206
3. Meersseman W, Vandecasteele SJ, Wilmer A, Verbeken E, Peetermans WE, Van Wijngaerden E. Invasive aspergillosis in critically ill patients without malignancy. Am J Respir Crit Care Med. 2004;170:621–5. DOI: 10.1164/rccm.200401-093OC
4. Chen TK, Babbitt CJ, Maggi C, Abbosaida A, Randhawa I, Walsh TJ. Pulmonary aspergillosis following H1N1 influenza A infection in pediatric patients. In: Programs and Abstracts of the 20th Annual Focus on Fungal Infections. New Orleans, LA: 2010 Mar 3–5. Alpharetta (GA): Imedex, LLC. Poster P-0026.
5. Lewis DE, Gilbert BE, Knight V. Influenza virus infection induces functional alterations in peripheral blood lymphocytes. J Immunol. 1986;137:3777–81.
6. Sedger LM, Hou S, Osvath SR, Glaccum MB, Peschon JJ, van Rooijen N, et al. Bone marrow B cell apoptosis during in vivo influenza virus infection requires TNF-alpha and lymphotoxin-alpha. J Immunol. 2002;169:6193–201.
7. Lewis M, Kallenbach J, Ruff P, Zaltzman M, Abramowitz J, Zwi S. Invasive pulmonary aspergillosis complicating influenza A pneumonia in a previously healthy patient. Chest. 1985;87:691–3. DOI: 10.1378/chest.87.5.691
8. Fischer JJ, Walker DH. Invasive pulmonary aspergillosis associated with influenza. JAMA. 1979;241:1493–4. DOI: 10.1001/jama.241.14.1493
9. Clancy CJ, Nguyen MH. Acute community-acquired pneumonia due to *Aspergillus* in presumably immunocompetent hosts: clues for recognition of a rare but fatal disease. Chest. 1998;114:629–34. DOI: 10.1378/chest.114.2.629
10. Boots RJ, Paterson DL, Allworth AM, Faoagali JL. Successful treatment of post-influenza pseudomembranous necrotising bronchial aspergillosis with liposomal amphotericin, inhaled amphotericin B, gamma interferon and GM-CSF. Thorax. 1999;54:1047–9. DOI: 10.1136/thx.54.11.1047
11. Australia and New Zealand Extracorporeal Membrane Oxygenation (ANZ ECMO) Influenza Investigators, Davies A, Jones D, Bailey M, Beca J, Bellomo R, Blackwell N, et al. Extracorporealv membrane oxygenation for 2009 influenza A (H1N1) acute respiratory distress syndrome. JAMA. 2009;302:1888–95. DOI: 10.1001/jama.2009.1535
12. To KK, Hung IF, Li IW, Lee KL, Koo CK, Yan WW, et al. Delayed clearance of viral load and marked cytokine activation in severe cases of pandemic H1N1 2009 influenza virus infection. Clin Infect Dis. 2010;50:850–9. DOI: 10.1086/650581
13. Steinberg KP, Hudson LD, Goodman RB, Hough CL, Lanken PN, Hyzy R, et al. Efficacy and safety of corticosteroids for persistent acute respiratory distress syndrome. N Engl J Med. 2006;354:1671–84. DOI: 10.1056/NEJMoa051693
14. Louie JK, Acosta M, Winter K, Jean C, Gavali S, Schechter R, et al. Factors associated with death or hospitalization due to pandemic 2009 influenza A(H1N1) infection in California. JAMA. 2009;302:1896–902. DOI: 10.1001/jama.2009.1583
15. Dutkowski R. Oseltamivir in seasonal influenza: cumulative experience in low- and high-risk patients. J Antimicrob Chemother. 2010;65(Suppl 2):ii11–24. DOI: 10.1093/jac/dkq012

Address for correspondence: George R. Thompson III, Department of Internal Medicine, Division of Infectious Diseases, University of California, Davis, 4150 V St, Ste 3400, Sacramento, CA 95817, USA; email: george.thompson@ucdmc.ucdavis.edu

Causes of Infection after Earthquake, China, 2008

Yue Wang, Peng Hao, Bo Lu, Hua Yu, Wenfang Huang, Hongliang Hou, and Kerong Dai

To determine which organisms most commonly cause infection after natural disasters, we cultured specimens from injured earthquake survivors in Wenchuan, China, 2008. Of 123 cultures, 46 (59%) grew only 1 type of pathogenic bacteria. Smear was more effective than culture for early diagnosis of gas gangrene. Early diagnosis and treatment of wounds are crucial.

On May 12, 2008, an 8.0-magnitude earthquake occurred in Wenchuan, People's Republic of China. For many survivors who were hospitalized, wound or other generalized infections developed. To learn more about relevant factors and therapeutic solutions for infections in earthquake survivors, we analyzed the characteristics and antimicrobial drug susceptibility of organisms cultured from wounds, blood, and sputum of persons admitted to Sichuan Provincial People's Hospital after the earthquake. This hospital, 1 of the biggest public hospitals in the earthquake zone, admitted 2,131 survivors, including 856 patients who had been transferred from other hospitals after preliminary treatment; 2,105 recovered and were discharged by July 31, 2008. Thus, this hospital's patients may be representative of all survivors of the Wenchuan earthquake.

The Study

Earthquake sequelae were as follows: among survivors, 1,970 (92.4%) had limb or spinal trauma, 108 had head injuries, and 40 had thoracic injuries. Among the 78 patients in whom testing of wound secretion, sputum, or blood samples yielded positive results, 65 (83.3%) had fractures, including 38 with open limb fractures and 29 with multiple fractures; 35 (44.9%) had crush injuries; 15 (19.2%) had renal dysfunction; 30 (38.5%) had pulmonary injury or recurrent or aggravation of existing pulmonary disease; and 19 (24.4%) had gas gangrene. We included in our study those patients who had been injured in the earthquake and admitted to Sichuan Provincial People's Hospital from May 12 through July 31, 2008, and who had any of the following conditions: increased wound secretion after debridement, indicating possible infection; continuous fever >38.5°C and pulmonary signs and symptoms, indicating possible pulmonary infection; continuous fever that could not be explained by wound or specific organ infection or fever with suspicion of bacteremia; and clinical signs indicating special infections such as gas gangrene.

To identify bacteria and fungi, we cultured 571 specimens of wound secretions, sputum, or blood from 123 patients (74 male and 49 female); results were positive for 78 patients. Of these 78 patients, 46 were male and 32 were female, average age was 49 years (range 9–95 years), and 13 were <20 and 31 were >60 years of age. From these 78 patients, 19 had specimens collected from 1 location, 28 from 2 locations, 14 from 3 locations, and 17 from ≥4 locations. We also conducted anaerobic culturing of wounds with suspected gas gangrene.

An average of 3 cultures were performed for each patient. All cultures for patients who had multiple-collection or multiple-location specimens that grew consistent bacterial strains were considered as 1 culture. Bacteriologic and antimicrobial drug susceptibility testing was performed by using a VITEK-32 automatic microbial analyzer or an ATB automatic microbial analyzer (both from bioMérieux, Durham, NC, USA). Drugs tested were cefepime, ceftazidime, aztreonam, ciprofloxacin, gentamicin, imipenem, piperacillin/tazobactam, sulfamethoxazole/trimethoprim, and amikacin.

Of the 571 specimens, positive culture results were obtained from 169 (59.5%) of 284 wound secretions, 120 (61.2%) of 196 sputum samples, and 22 (24.2%) of 91 blood samples (Table 1). Culture results indicated that 46 (59.0%) patients were infected with 1 type of pathogenic bacteria and 31 were infected with >1 type (21 [26.9%] with 2 types, 7 [9.0%] with 3 types, and 4 [5.1%] with ≥4 types). Of those infected with >1 type of bacteria, 15 (46.9%) were >60 years of age. The predominant bacteria causing infection were *Acinetobacter baumannii*, *Escherichia coli*, and *Staphylococcus aureus*. Antimicrobial drug susceptibility testing results for the 5 most common among the 180 strains of isolated gram-negative bacilli are shown in Table 2.

To detect suspected cases of gas gangrene, we harvested 53 smear specimens, of which 22 produced gram-positive thick bacilli (18 with capsules and 4 without). Further culturing after a period of isolation and treatment of the patients showed the pathogen for 4 patients to be a non–gas-producing bacillus. Of these 22 patients, 3 did not have gas gangrene, but the remaining 19 did, confirmed by culture and clinical signs (accuracy rate 86.4%). Culture results for another 10 patients showed other bacteria (intermediate-size gram-positive bacilli).

Author affiliations: Sichuan Academy of Medical Sciences and Sichuan Provincial People's Hospital, Chengdu, Sichuan, People's Republic of China (Y. Wang, P. Hao, B. Lu, H. Yu, W. Huang); Ninth People's Hospital, Shanghai, People's Republic of China (H. Hou, K. Dai); and Shanghai Jiaotong University School of Medicine, Shanghai (H. Hou, K. Dai)

DOI: 10.3201/eid1606.091523

Table 1. Pathogenic bacteria isolated from persons injured during earthquake, Wenchuan, China, 2008

	Bacteria, no. (%)*											
Source	*A.b.*	*E.c.*	*S.a.*	*K.p.*	Enteric bacilli	*P.a.*	*C.a.*	CN staph	*S.m.*	*S.s.*	Others	Total
Wound	42 (24.9)	26 (15.4)	20 (11.8)	16 (9.5)	16 (9.5)	13 (7.7)	0	0	0	0	36 (21.3)	169
Sputum	29 (24.2)	6 (5.0)	12 (10.0)	6 (5.0)	12 (10.0)	5 (4.2)	18 (15.0)	6 (5.0)	0	0	26 (21.6)	120
Blood	3 (13.6)	4 (18.2)	6 (27.3)	0	2 (9.1)	0	1 (4.5)	0	2 (9.1)	2 (9.1)	2 (9.1)	22

**A.b., Acinetobacter baumanii*; *E.c., Escherichia coli; S.a., Staphylococcus aureus; K.p., Klebsiella pneumoniae; P.a., Pseudomonas aeruginosa; C.a., Candida albicans;* CN staph, coagulase-negative staphylococci*; S.m., Serratia marcescens; S.s.,Staphylococcus saprophyticus.*

Risk for nosocomial infection might have been increased because many patients had been treated with antimicrobial drugs at other hospitals before being transferred to Sichuan Provincial People's Hospital. Instead of genotype testing (because resources were limited), we used the resistance spectra of bacteria to differentiate nosocomial from primary infection. *A. baumannii* and *E. coli* are gram-negative bacilli that exist widely in the environment, mainly in water and soil, the main site for development of antimicrobial drug resistance (*1*,*2*). Because *A. baumannii* were strongly resistant and because resistance spectra for several strains of *A. baumannii* were nearly identical, we concluded that any infections with this pathogen were probably obtained during hospitalization. In contrast, because the resistance spectra of *E. coli* and enteric bacilli varied substantially, we concluded that infections with these bacteria were most likely obtained before hospitalization. Average susceptibility of several gram-negative bacilli (except *A. baumannii*) to imipenem was >90%; susceptibility to aminoglycosides, cefepime, and ceftazidime was also high.

Among the 49 strains of staphylococcal bacteria isolated (38 strains of *S. aureus* and 11 strains of other coagulase-negative staphylococci), 39 (79.6%) strains were methicillin resistant. Susceptibility test results (no. susceptible/% susceptibility) were as follows: 49/100% for vancomycin and linezolid; 47/95.9% for quinupristin/dalfopristin; 36/73.5% for sulfamethoxazole/trimethoprim; 25/51.0% for chloramphenicol and rifampicin; 14/28.6% for clindamycin; 13/26.5 for gentamicin; 11/22.4% for erythromycin, moxifloxacin, and ampicillin/sulbactam sodium; and 10/20.4% for cefazolin and gatifloxacin.

For fungi, 19 strains were detected. All were 100% susceptible to amphotericin B, 5-fluorouracil, fluconazole, voriconazole, and itraconazole.

Conclusions

More attention should be paid to early diagnosis and treatment of multiple infections and special infections in survivors of natural disasters. One such infection, gas gangrene, is an acute, severe, life-threatening disease for which early diagnosis is critical. For diagnosis of gas gangrene, smear examination of wound secretions is a valuable diagnostic tool and is more effective than culture for early diagnosis. No nosocomial spread of gas gangrene occurred during treatment.

Acknowledgment

We thank Katharine O'Moore-Klopf for providing English-language editorial assistance.

Dr Wang is director of the Department of Orthopaedics, Sichuan Provincial People's Hospital and Sichuan Academy of Medical Sciences. His research interests include traumatology and emergency medicine.

References

1. Kim SH, Wei CI, Tzou YM, An H. Multidrug-resistant *Klebsiella pneumoniae* isolated from farm environments and retail products in Oklahoma. J Food Prot. 2005;68:2022–9.
2. Burgos JM, Ellington BA, Varela MF. Presence of multidrug-resistant enteric bacteria in dairy farm topsoil. J Dairy Sci. 2005;88:1391–8.

Address for correpondence: Kerong Dai, Department of Orthopaedics, Ninth People's Hospital, Shanghai Jiaotong University School of Medicine, 639 Zhizaoju Rd, Shanghai 200011, People's Republic of China; email: krdai@163.com

Table 2. Antimicrobial susceptibility of 180 strains of gram-negative bacilli isolated from persons injured during earthquake, Wenchuan, China, 2008

		No. (%) specimens susceptible, by antimicrobial drug*								
Organism	No.	AMK	AZT	CAZ	CIP	FEP	GM	IMP	SMZ	TZP
A. baumannii	74	16 (21.6)	0	5 (6.8)	7 (9.5)	15 (20.1)	0	26 (35.1)	10 (13.5)	12 (16.2)
Escherichia coli	36	36 (100)	13 (36.1)	13 (36.1)	0	11 (30.6)	10 (27.8)	35 (97.2)	11 (30.6)	28 (77.8)
Enteric bacilli	30	15 (50.0)	10 (33.3)	10 (33.3)	8 (26.7)	13 (43.3)	13 (43.3	30 (100)	8 (26.7)	10 (33.3
Klebsiella pneumoniae	22	20 (90.9)	4 (18.2)	7 (31.8)	0	7 (31.8)	4 (18.2)	22 (100)	4 (18.2)	7 (31.8)
P. aeruginosa	18	18 (100)	4 (22.2)	11 (61.1)	12 (66.7)	11 (61.1)	15 (83.3)	17 (94.4)	0	15 (83.3)
Total	180	105 (58.3)	31 (17.2)	46 (25.6)	27 (15.0)	57 (31.7)	42 (23.3)	130 (72.2)	33 (18.3)	72 (40.0)

*Semisusceptible results were considered resistant. AMK, amikacin; AZT, aztreonam; CAZ, ceftazidime; CIP, ciprofloxacin; FEP, cefepime; GM, gentamicin; IMP, imipenem; SMZ,sulfamethoxazole/trimethoprim; TZP, tazobactam/piperacillin; *A. baumannii, Acinetobacter baumannii; P. aeruginosa, Pseudomonas aeruginosa.*

Vaccinia Virus Infection in Monkeys, Brazilian Amazon

Jônatas S. Abrahão, André T. Silva-Fernandes, Larissa S. Lima, Rafael K. Campos, Maria I.M.C. Guedes, Marcela M.G. Cota, Felipe L. Assis, Iara A. Borges, Milton F. Souza-Júnior, Zélia I.P. Lobato, Cláudio A. Bonjardim, Paulo C.P. Ferreira, Giliane S. Trindade, and Erna G. Kroon

To detect orthopoxvirus in the Brazilian Amazon, we conducted a serosurvey of 344 wild animals. Neutralizing antibodies against orthopoxvirus were detected by plaque-reduction neutralizing tests in 84 serum samples. Amplicons from 6 monkey samples were sequenced. These amplicons identified vaccinia virus genetically similar to strains from bovine vaccinia outbreaks in Brazil.

In Brazil, several exanthematic vaccinia virus (VACV) outbreaks affecting dairy cattle and rural workers have been reported since 1999 (*1*,*2*). VACV, the prototype of the genus *Orthopoxvirus*, shows serologic cross-reactivity with other Orthopoxvirus species and was used during the smallpox eradication campaign (*3*). Bovine vaccinia causes economic losses and affects public health services in Brazil (*4*). VACV reservoirs and the role of wildlife in outbreaks remain unidentified. Although some data indicate that VACV strains circulate in rodents in forests in Brazil (*5*,*6*), there is no evidence of VACV infection in other wild mammals.

To detect orthopoxviruses in the Brazilian Amazon, we conducted a serosurvey of wild animals in this region. We detected antibodies against orthopoxviruses in 4 mammalian species. Using molecular methods, we confirmed exposure of monkeys to VACV. Although our findings are uncertain in the context of bovine vaccinia outbreaks, we provide new biologic and epidemiologic information about VACV.

The Study

During February 2001–September 2002, we captured 344 wild mammals in an overflow area in a fauna-rescue program during construction of a hydroelectric plant in Lajeado and Ipueiras counties (9°44′58″S, 48°21′23″W) in Tocantins State, Brazil. During this program, 269 capuchin monkeys (*Cebus apella*), 27 black-howling monkeys (*Allouata caraya*), 12 coatis (*Nasua nasua*), 20 agoutis (*Dasyprocta* sp.), 2 opossums (*Didelphis albiventris*), 5 armadillos (*Euphractus sexcinctus*), 5 collared anteaters (*Tamandua tetradactila*), and 4 gray foxes (*Cerdocyon thous*) were captured.

All animals were captured in a sylvatic area and did not have contact with humans and dairy cattle. In field-screening laboratories, animals were sedated, serum samples were collected, and veterinary evaluations were made. Animals were then released in areas selected during environmental conservation programs. Until 2002, bovine vaccinia had been restricted to southeastern Brazil, ≥1,400 km from the study area (*7*).

Serum samples were inactivated by heating at 56°C for 30 min, and an orthopoxvirus plaque-reduction neutralizing test (PRNT) was performed. PRNT was used rather than ELISA because secondary antibodies required for an ELISA for all analyzed species were unavailable. Inactivated samples were diluted 1:20×–1:1,640× in minimal essential medium and tested in Vero cells by using the VACV-Western Reserve strain in the PRNT as described (*8*). Human samples positive for antibodies to orthopoxvirus obtained during bovine vaccinia outbreaks were used as positive controls (*9*); samples negative for these antibodies were used as negative controls (*10*). Serum titer was defined as the highest dilution that inhibited ≥50% of viral plaques compared with negative controls. Orthopoxvirus PRNT specificity (97.4%) and sensitivity (93.5%) were confirmed by using receiver-operating characteristic analysis, which compared results of PRNT, ELISA, and clinical symptoms during bovine vaccinia outbreaks (*9*,*10*).

PRNT showed a high prevalence of seropositive monkeys (Table). Of 269 *C. apella* samples, 68 (25.3%) had antibodies to orthopoxvirus. Of 27 *A. caraya* samples, 13 (48.1%) had antibodies to orthopoxvirus. Seropositivity was detected in 2 (16.6%) coatis and 1 (5.0%) agouti. Antibodies to orthopoxvirus were not detected in any other species tested. Of 344 animals studied, 84 (24.4%) had antibodies to orthopoxvirus (Table). In samples with high neutralizing antibody titers, 55.95% (47) had titers of 80–320. Only 5 (6.0%) PRNT-positive samples had titers <40 (Table).

Given the serologic cross-reactivity of orthopoxvirus (*3*), positive samples could indicate any of ≥9 virus species, although it is well established that VACV is endemic to Brazil, and infections with other orthopoxviruses are geographically restricted to other continents and have not been identified in Brazil. Therefore, we performed a molecular investigation to identify orthopoxviruses associated with orthopoxvirus sylvatic circulation. Serologic and molecular

Author affiliation: Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil

DOI: 10.3201/eid1606.091187

Table. Seroprevalence of vaccinia virus in monkeys, Brazilian Amazon*

Species	No. serum samples tested	PRNT titer, no. positive					Total no. (%)	No. (%) PCR positive
		20	40	80	160	320		
Cebus apella	269	2	24	36	4	2	68 (25.3)	11 (4.1)
Allouata caraya	27	3	5	5	0	0	13 (48.1)	7 (25.9)
Nasua nasua	12	0	2	0	0	0	2 (16.6)	0
Dasyprocta sp.	20	0	1	0	0	0	1 (5.0)	0
Didelphis albiventris	2	0	0	0	0	0	0	0
Euphractus sexcinctus	5	0	0	0	0	0	0	0
Tamandua tetradactila	5	0	0	0	0	0	0	0
Cerdocyon thous	4	0	0	0	0	0	0	0
Total	344	5	32	41	4	2	84 (24.4)	18 (5.2)

*PRNT, plaque-reduction neutralization test.

tests were performed in a blinded fashion and in triplicate. On the basis of previous studies that detected orthopoxvirus DNA in serum of infected hosts (*9*,*11*,*12*), a semi-nested PCR was used to amplify the highly conserved orthopoxvirus vaccinia growth factor (*vgf*) gene (J.S. Abrahão et al., unpub.data) from mammal serum samples. Human VACV DNA–positive and DNA–negative serum samples obtained during bovine vaccinia outbreaks (*9*) were used as positive and negative controls, respectively. Field and laboratory clinical samples were processed separately to avoid cross-contamination.

Eighteen of 344 serum samples were positive in PCR assays (11 from *C. apella* and 7 from *A. caraya*; all were PRNT positive). Six of the 18 *vgf* PCR-positive samples were chosen for sequencing and analysis of *vgf* (4 from *C. apella* and 2 from *A. caraya*). In addition, using primers described by Ropp et al. (*14*), we amplified the hemagglutinin (*ha*) gene from 2 samples (1 from *C. apella* and 1 from *A. caraya*; both were *vgf* positive). The *vgf* and *ha* PCR products were cloned into the pGEMT-easy vector (Promega, Madison, WI, USA). Three clones from distinct PCR amplicons of each sample were sequenced in both orientations by using M13 universal primers and the Mega-BACE-sequencer (GE Healthcare, Little Chalfont, UK).

Optimal alignment of the highly conserved *vgf* gene with ClustalW (www.ncbi.nlm.nih.gov/pmc/articles/PMC308517) and MEGA version 3.1 (www.megasoftware.net) showed 100% identity among all nucleotide and amino acid sequences for monkey serum (online Appendix Figure, panel A, www.cdc.gov/EID/content/16/6/976-appF.htm). When compared with nucleotide sequences available in GenBank, *vgf* sequences were highly similar (98%–100% identity) to the homologous gene from other VACV strains and showed 100% identity. The *ha* sequences for *C. apella* and *A. caraya* showed a signature deletion (online Appendix Figure, panel B) also present in sequences of other VACV isolates from Brazil. These *ha* sequences showed 99.6% identity at the nucleotide level and 99.7% identity at the amino acid level (736 nt of the *ha* gene were analyzed).

Phylogenetic trees of the *vgf* (Figure, panel A) or *ha* (Figure, panel B) genes were constructed by using the neighbor-joining method, 1,000 bootstrap replicates, and the Tamura 3-parameter model (MEGA version 3.1). These sylvatic VACV sequences clustered with several VACVs isolated during several bovine vaccinia outbreaks. The *vgf* and *ha* sequences from monkey samples were deposited in GenBank (accession nos. VACV-TO *vgf* GQ465372 and GQ465373 and *ha* GU322359 and GU322360). Orthopoxvirus DNA was not detected in any other species tested.

Conclusions

Although VACV strains have been isolated from rodents in forests in Brazil (*5*,*6*) (the nearest location, Belém, is 750 km from the study area), we detected VACV in wildlife in the Brazilian Amazon 3 years after reports of exanthematic outbreaks of bovine vaccinia and 40 years after isolation of VACV from forests. Our data provide evidence of high prevalence of orthopoxviruses among capuchin and black-howling monkeys in the Brazilian Amazon. The relationship between infected monkeys and emergence of VACV in rural regions of Brazil is unknown. However, transmission of VACV in northeastern Brazil has been reported, and outbreaks have been reported in Mato Grosso, Pernambuco (www.amep.org.br/pox.doc), Maranhão (E.G. Kroon et al., unpub. data), and Tocantins (*13*), which are in or adjacent to the Brazilian Amazon. Some of these viruses may be related to those isolated in this study because some VACV isolates have the same signature deletion in the *ha* gene as VACV-TO.

Anthropogenic disturbance of the Amazon ecosystem and increases in agricultural and livestock areas increase contact between wildlife and rural populations (*15*). However, the effect of VACV in environments in Brazil that contain wild animals has not been studied. Clinical data for pox lesions in animals tested were not well documented by veterinarians in the study area. Ecologic and public health studies should be designed to evaluate risks for infection with VACV during wildlife conservation efforts and determine whether surveillance systems can predict bovine vac-

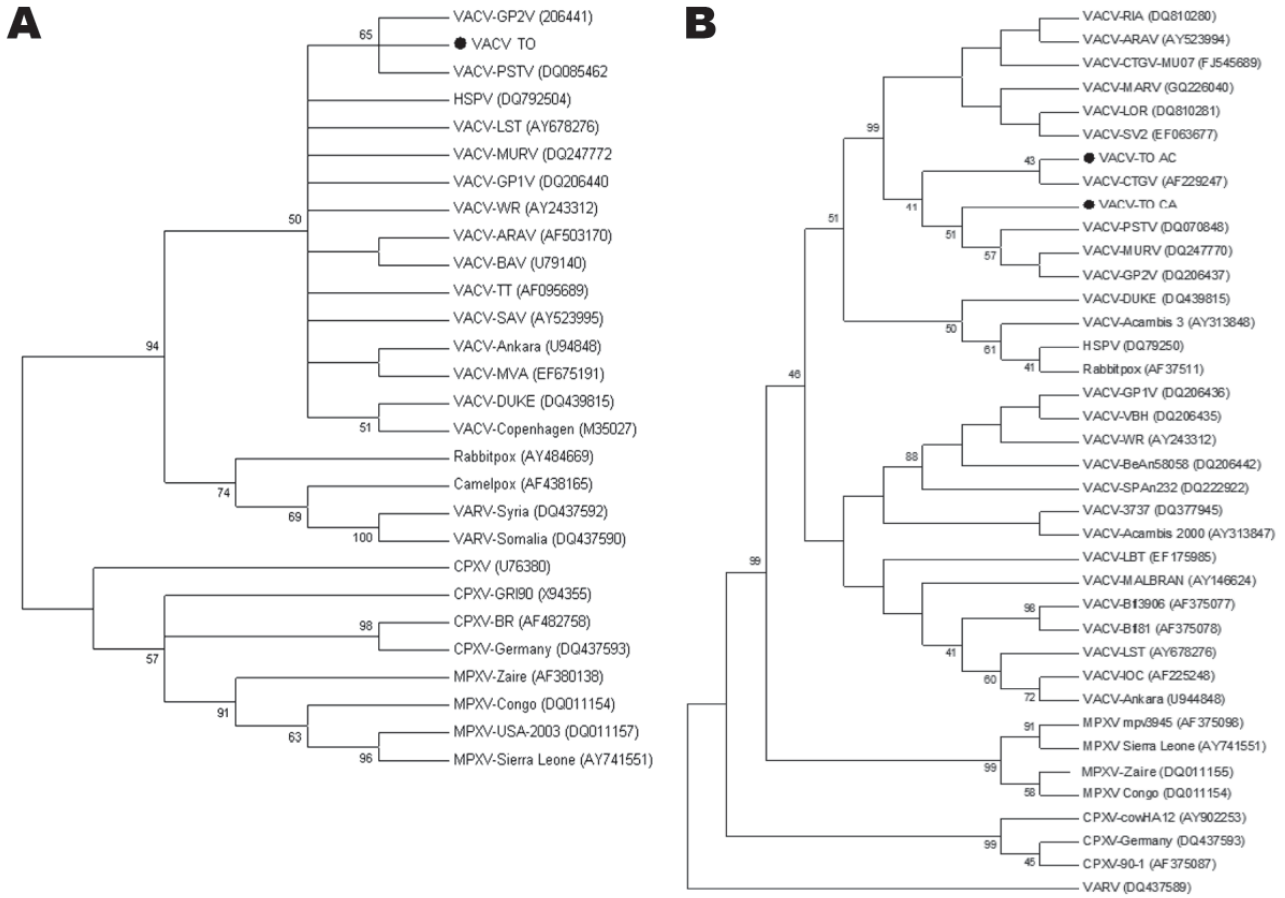


Figure. Consensus bootstrap phylogenetic trees based on nucleotide sequences of orthopoxvirus vaccinia growth factor (*vgf*) (A) and hemagglutinin (*ha*) (B) genes. Trees were constructed with *ha* or *vgf* sequences by using the neighbor-joining method with 1,000 bootstrap replicates and the Tamura 3-parameter model in MEGA version 3.1 software (www.megasoftware.net). Bootstrap values >40% are shown. Nucleotide sequences were obtained from GenBank. Black dots indicate vaccinia virus (VACV) obtained from *Cebus apella* (VACV-TO CA) and *Allouata caraya* (VACV AC). All *vgf* sequences obtained from monkey serum samples showed 100% and are represented as a unique sequence in the *vgf* tree (VACV TO). HSPV, horsepoxvirus; VARV, variola virus; CPXV, cowpoxvirus; MPXV, monkeypoxvirus.

cinia outbreaks by monitoring VACV infection in monkeys and other wild animals.

Acknowledgments

We thank João Rodrigues dos Santos, Ângela Sana Lopes, Ilda Gama, and their colleagues for excellent technical support.

This study was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Fundação de Amparo à Pesquisa do Estado de Minas Gerais, and Ministério da Agricultura, Pecuária e Abastecimento. J.S.A. was supported by a fellowship from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior. E.G.K., C.A.B., G.S.T., and P.C.P.F. are researchers of Conselho Nacional de Desenvolvimento Científico e Tecnológico.

Mr Abrahão is a biologist and doctoral candidate at the Laboratório de Vírus, Microbiology Department, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil. His research interests focus on monitoring, controlling, and preventing emerging infectious diseases.

References

1. de Souza Trindade G, da Fonseca FG, Marques JT, Nogueira ML, Mendes LC, Borges AS, et al. Araçatuba virus: a vaccinialike virus associated with infection in humans and cattle. Emerg Infect Dis. 2003;9:155–60.
2. Leite JA, Drumond BP, Trindade GS, Lobato ZI, da Fonseca FG, dos Santos JR, et al. Passatempo virus, a vaccinia virus strain, Brazil. Emerg Infect Dis. 2005;11:1935–8.
3. Damon IK. Poxviruses. In: Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman B, et al., editors. Fields Virology. Vol II. 5th ed. Philadelphia: Lippincott Williams and Wilkins; 2007. p. 2947–75.

4. Lobato ZI, Trindade GS, Frois MC, Ribeiro EB, Dias GR, Teixeira BM, et al. Outbreak of exantemal disease caused by vaccinia virus in human and cattle in Zona da Mata Region, Minas Gerais. Arquivo Brasiliero Medicina Veterinária Zootecnia. 2005;57:423–9.
5. de Lopes S, Lacerda JP, Fonseca IE, Castro DP, Forattini OP, Rabello EX. Cotia virus: a new agent isolated from sentinel mice in São Paulo, Brazil. Am J Trop Med Hyg. 1965;14:156–7.
6. Fonseca FG, Lanna MC, Campos MA, Kitajima EW, Peres JN, Golgher RR, et al. Morphological and molecular characterization of the poxvirus BeAn 58058. Arch Virol. 1998;143:1171–86. DOI: 10.1007/s007050050365
7. Damaso CR, Esposito JJ, Condit RC, Moussatche N. An emergent poxvirus from humans and cattle in Rio de Janeiro State: Cantagalo virus may derive from Brazilian smallpox vaccine. Virology. 2000;277:439–49. DOI: 10.1006/viro.2000.0603
8. Office International des Epizooties. West Nile fever in the United States of America in horses. In: Manual of standard techniques. Paris: The Office; 1999. p. 150–1.
9. Silva-Fernandes AT, Travassos CE, Ferreira JM, Abrahão JS, Rocha ES, Viana-Ferreira F, et al. Natural human infections with vaccinia virus during bovine vaccinia outbreaks. J Clin Virol. 2009;44:308–13. DOI: 10.1016/j.jcv.2009.01.007
10. Madureira M. Study of the bovine vaccine outbreaks in Minas Gerais State [PhD dissertation]. Belo Horizonte (Brazil): Escola de Veterinária, Universidade Federal de Minas Gerais; 2009.
11. Cohen JI, Hohman P, Preuss JC, Li L, Fischer SH, Fedorko DP. Detection of vaccinia virus DNA, but not infectious virus, in the blood of smallpox vaccine recipients. Vaccine. 2007;25:4571–4. DOI: 10.1016/j.vaccine.2007.03.044
12. Savona MR, Dela Cruz WP, Jones MS, Thornton JA, Xia D, Hadfield TL, et al. Detection of vaccinia DNA in the blood following smallpox vaccination. JAMA. 2006;295:1898–900. DOI: 10.1001/jama.295.16.1898
13. Medaglia ML, Pessoa LC, Sales ER, Freitas TR, Damaso CR. Spread of Cantagalo virus to northern Brazil. Emerg Infect Dis. 2009;15:1142–3. DOI: 10.3201/eid1507.081702
14. Ropp SL, Jin Q, Knight JC, Massung RF, Esposito JJ. PCR strategy for identification and differentiation of small pox and other orthopoxviruses. J Clin Microbiol. 1995;33:2069–76.
15. Soares-Filho BS, Nepstad DC, Curran LM, Cerqueira GC, Garcia RA, Ramos CA, et al. Modelling conservation in the Amazon basin. Nature. 2006;440:520–3. DOI: 10.1038/nature04389

Address for correspondence: Erna G. Kroon, Laboratório de Vírus, Departamento de Microbiologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6627, Caixa Postal 486, CEP 31270-901, Belo Horizonte, Minas Gerais, Brazil; email: kroone@icb.ufmg.br

Novel Norovirus in Dogs with Diarrhea

João Rodrigo Mesquita, Leslie Barclay, Maria São José Nascimento, and Jan Vinjé

To identify the prevalence and genetic variability of noroviruses in dogs, we tested fecal samples by using reverse transcription–PCR. We found canine norovirus in 40% and 9% of dogs with and without diarrhea, respectively. The virus was genetically unrelated to other noroviruses and constitutes a tentative new genogroup.

Human noroviruses (NoVs) are the most frequent cause of epidemic and sporadic acute gastroenteritis worldwide among humans of all ages (*1*,*2*). The virus is transmitted through ingestion of contaminated food or water or from person to person through the fecal–oral route. The close genetic relatedness of swine NoV with human NoVs of genogroup (G) II suggests the potential for transfer from animals to humans (*3*–*5*). In addition, recent findings of viruses genetically related to human NoVs, as well as to animal NoV sequences in pigs and calves, have raised concerns about the possible emergence of recombinant viruses (*4*).

NoVs are genetically heterogeneous viruses that belong to the family *Caliciviridae*. The viral capsid encloses a single-stranded, positive-sense RNA genome of 7.3–7.7 kb that is organized in 3 open reading frames (ORFs), of which ORF1 encodes a polyprotein that is proteolytically cleaved into 6 nonstructural proteins, including RNA-dependent RNA polymerase (RdRp), helicase, and protease (*1*). ORF2 and ORF3 encode major (viral protein [VP] 1) and minor (VP2) capsid proteins. The GLPSG and YGDD motifs of the RdRp protein are conserved among all members of the family *Caliciviridae* (*6*). The genus *Norovirus* currently comprises 5 genogroups, designated GI–GV, which can be grouped into at least 32 genetic clusters (*2*,*7*). Only viruses from GI, GII, and GIV have been associated with human disease. Recently, the finding of a novel GIV norovirus in a young dog (*5*) indicated that pets can be infected with NoVs. To identify the prevalence and genetic variability of NoVs in dogs, we tested fecal specimens from dogs with and without diarrhea.

Author affiliations: University of Porto, Porto, Portugal (J.R. Mesquita, M.S.J. Nascimento); Polytechnic Institute of Viseu, Viseu, Portugal (J. R. Mesquita); and Centers for Disease Control and Prevention, Atlanta, Georgia, USA (L. Barclay, J. Vinjé)

DOI: 10.3201/eid1606.091861

The Study

A total of 105 fecal samples from dogs in municipal dog shelters, veterinary clinics, and pet shops from 3 districts (Porto, Viseu, and Guarda) in Portugal were collected during December 2007–November 2008. Veterinarians evaluated the dogs for diarrhea at the time of the visit. The fecal panel consisted of 63 samples from dogs with diarrhea and 42 samples from dogs with formed, normal brown feces (i.e., controls). All samples were kept at –20°C until processed. Fecal suspensions (10%) were made in phosphate-buffered saline pH 7.2, and solids were removed by centrifugation at 8,000 × *g* for 5 min. Nucleic acid was extracted by using the QIAamp viral RNA mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions and tested for the presence of NoV RNA by 2 broadly reactive NoV conventional reverse transcription–PCR (RT-PCR) assays (*8*,*9*), selective for a partial RdRp region of the genome, by using One-Step RT-PCR kit (QIAGEN) and 37°C as annealing temperature.

Two (2%) of the 105 fecal samples tested positive for NoV with primer pair JV12y/13i, whereas no RT-PCR products were found using the p289-p290 primer pair. The 2 JV12y/13i sequences were identical and contained the GLPSG amino acid motif characteristic of viral RNA polymerases.

We designed specific canine norovirus oligonucleotide primers JV102 (5′-TGG GAT TCA ACA CAG CAG AG-3′) and JV103 (5′-TGC GCA ATA GAG TTG ACC TG-3′) and retested all 105 samples. Fecal samples from 25 (40%) of the 63 dogs with diarrhea and 4 (9%) of the 42 controls tested positive for a new canine NoV (Viseu strain) (Table). An ≈3.3-kb fragment from the RdRp region in ORF1 to the poly-A tail, including the complete ORF2 and ORF3 genes, was generated by long-template RT-PCR by using previously described methods (*10*). Gel-purified PCR products were cloned and sequenced by primer walking, and a 3,357-nt sequence including partial ORF1, full-length ORF2 and ORF3, and the 3′ end noncoding region from the Viseu strain was obtained. The consensus sequence from 5 different clones was submitted to GenBank and assigned accession no. GQ443611. All 105 nucleic acid extracts also were tested by RT-PCR for the recently reported canine NoV strain (*5*), canine coronavirus (CCV) (*11*), and by PCR for canine parvovirus (CPV-2) combining oligonucleotide primers for the detection of types 2a and 2b (*12*). None of the samples tested positive for the reported GIV.2 canine NoV strain (GIV.2/170/07/ITA). A total of 32 (51%) samples from dogs with diarrhea tested positive for CCV, whereas 2 (5%) of the control samples were positive. In addition, 58 (55%) samples tested positive for CPV-2, of which 18 also were positive for the novel canine NoV (Table). Univariate analysis (SPSS version 17.0, SPSS Inc., Chicago, IL, USA) demonstrated that the

Table. Detection of canine norovirus, parvovirus, and coronavirus in 105 fecal samples from dogs with and without diarrhea, Porto, Viseu, and Guarda, Portugal, December 2007–November 2008*

Diarrhea	Canine norovirus,† no. (%) dogs	Canine parvovirus 2,‡ no. (%) dogs	Canine coronavirus,§ no. (%) dogs	Negative, no. (%) dogs	Total no. dogs
Yes	25 (40)	36 (57)	32 (51)	11 (17)	63
No	4 (9)	22 (35)	2 (5)	18 (43)	42

*Detection by reverse transcription–PCR.
†Odds ratio (OR) 6.25, 95% confidence interval (CI) 1.87–26.65; p = 0.0007 (univariate analysis).
‡OR 20.8, 95% CI 5.06–83.92; p<0.0001 (univariate analysis).
§OR 1.21, 95% CI 0.51–2.86; p = 0.63 (univariate analysis).

novel canine NoV and CCV were significantly associated with diarrhea (Table).

Conclusions

We detected novel norovirus sequences in 40% of samples from dogs with diarrhea and 9% of specimens from dogs without diarrhea. All canine norovirus RdRp sequences had a high nucleotide sequence identity (range 98%–100%) and differed 19%–22% from recently reported canine norovirus strains (*5*,*13*) (Figure, panel A).

In the complete VP1, the Viseu strain was most closely related to canine NoV strain Bari/91/07/IT (63.2% amino acid identity) and human strain Chiba/040502/2004/JP (55.1% identity) (Figure, panel B). Therefore, we tentatively classifed the Viseu strain, together with the Bari and Chiba strains, as a novel genogroup (GVI). To elucidate the potential pathogenic enteric role of the new canine NoV, we also tested all fecal specimens for CPV-2 and CCV, which are well-established enteric pathogens of dogs. Our results show that the novel canine NoV and CCV, but not CPV-2, were significantly associated with diarrhea.

For several reasons, our findings should be interpreted with caution. Viral shedding from dogs without diarrhea could represent asymptomatic infection, a resolution stage of the disease, or detection of CPV-2 vaccine virus. In addition, other canine enteric pathogens for which we did not test could bias the results. Further research that includes electron microscopy or infectivity studies is needed to confirm the existence of this new canine NoV.

The new virus sequences were detected in dogs from several dog owners throughout central Portugal and from a dog shelter in Viseu. Because Portuguese law requires offi-

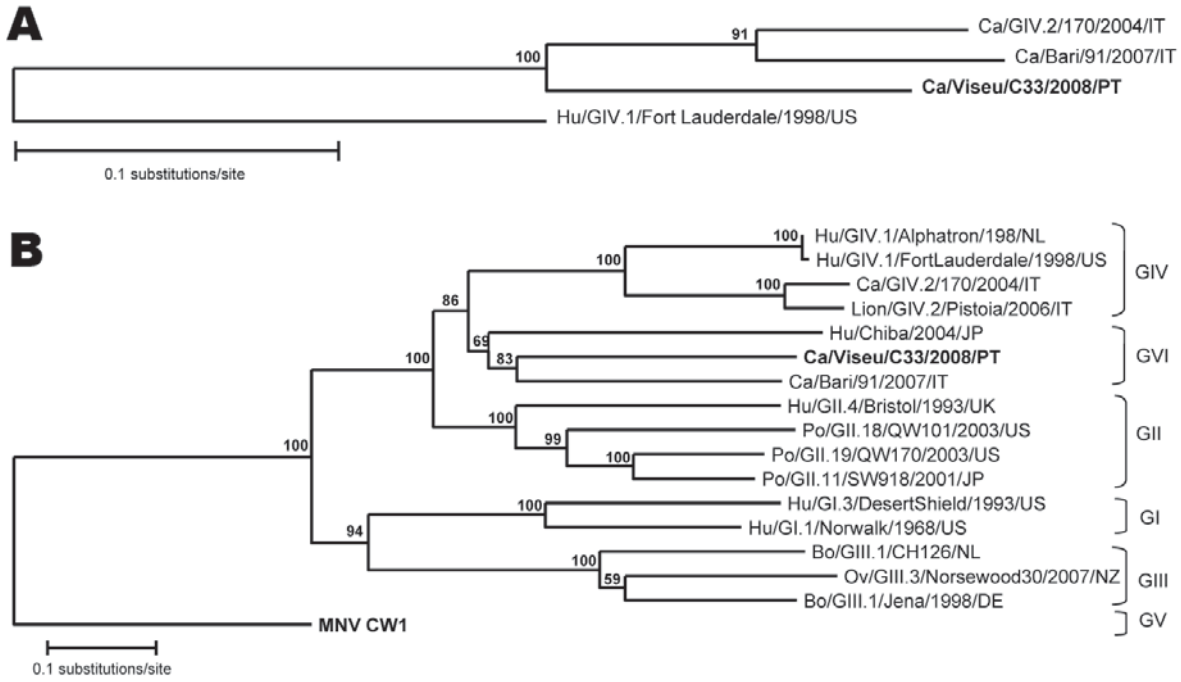


Figure. Phylogenetic trees of A) a 206-nt region of the RNA-dependent polymerase gene of 1 human genogroup (G) IV strain (Hu/GIV.1/FortLauderdale/1998/US), 2 recently published canine noroviruses (GIV.2/170/2004/IT, Bari/91/2007/IT) (*5*,*13*), and the novel canine Viseu strain reported in the study (**boldface**); and B) full-length amino acid sequence of viral protein (VP) 1 of norovirus strains of GI–GV detected in animals and human strains Hu/GI.1/Norwalk/1968/US, Hu/GI.3/DesertShield/1993/US, and Hu/GII.4/Bristol/1993/UK. The Viseu strain (**boldface**) forms tentatively a novel genogroup (GVI) with strains Ca/Bari/91/2007/IT and Hu/Chiba/2004/JP. Phylogenetic analysis was performed by using TreeCon software with Jukes and Cantor correction with bootstrap analysis (n = 1,000), and the tree topology was inferred by using neighbor-joining. Ca, canine; Hu, human; Po, porcine; Bo, bovine; Ov, ovine; Mu, murine.

cial shelters to provide sanctuary for unwanted animals and keep them as adoptable pets for a certain period, humans potentially could be exposed to canine NoV that could increase the possibility for the emergence of canine/human NoV recombinants and increase the likelihood of zoonotic transmission. Additional serologic studies are needed to determine the level of exposure to canine NoVs in dogs and humans similar to previous studies on bovine NoV (*14*). Zoonotic transmission between dogs and humans is not new, and the close and often intimate interactions between these 2 species have been suggested as a major disease risk for humans (*15*). Only a few studies on NoV infections in dogs have been reported (*5*,*13*). Experimental infections in gnotobiotic dogs could ultimately provide essential data for describing the prevalence of canine NoVs in dogs and their causal relationship with diarrhea.

Acknowledgments

We thank Jennifer Cannon and Nicole Gregoricus for technical support and Veronica Costantini for statistical analysis.

The study was partly supported by grant SFRH/BD/45407/2008 to J.R.M. from Fundação para a Ciência e a Tecnologia.

Dr Mesquita is a veterinary assistant at the Agrarian Superior School of Viseu and a PhD fellow at the Faculty of Pharmacy, University of Porto. His research interests include the epidemiology of enteric viruses in animals and humans.

References

1. Glass RI, Parashar UD, Estes MK. Norovirus gastroenteritis. N Engl J Med. 2009;361:1776–85. DOI: 10.1056/NEJMra0804575
2. Patel MM, Hall AJ, Vinjé J, Parashar UD. Noroviruses: a comprehensive review. J Clin Virol. 2009;44:1–8. DOI: 10.1016/j.jcv.2008.10.009
3. van der Poel WH, Vinjé J, van der Heide R, Herrera MI, Vivo A, Koopmans MP. Norwalk-like calicivirus genes in farm animals. Emerg Infect Dis. 2000;6:36–41.
4. Mattison K, Shukla A, Cook A, Pollari F, Friendship R, Kelton D, et al. Human noroviruses in swine and cattle. Emerg Infect Dis. 2007;13:1184–8.
5. Martella V, Lorusso E, Decaro N, Elia G, Radogna A, D'Abramo M, et al. Detection and molecular characterization of a canine norovirus. Emerg Infect Dis. 2008;14:1306–8. DOI: 10.3201/eid1408.080062
6. Farkas T, Sestak K, Wei C, Jiang X. Characterization of a rhesus monkey calicivirus representing a new genus of *Caliciviridae*. J Virol. 2008;82:5408–16. DOI: 10.1128/JVI.00070-08
7. Martella V, Campolo M, Lorusso E, Cavicchio P, Camero M, Bellacicco A, et al. Norovirus in captive lion cub (*Panthera leo*). Emerg Infect Dis. 2007;13:1071–3.
8. Jiang X, Huang PW, Zhong WM, Farkas T, Cubitt DW, Matson DO. Design and evaluation of a primer pair that detects both Norwalk- and Sapporo-like caliciviruses by RT-PCR. J Virol Methods. 1999;83:145–54. DOI: 10.1016/S0166-0934(99)00114-7
9. Vennema H, Bruin E, Koopmans M. Rational optimization of generic primers used for Norwalk-like virus detection by reverse transcriptase polymerase chain reaction. J Clin Virol. 2002;25:233–5. DOI: 10.1016/S1386-6532(02)00126-9
10. Vinjé J, Koopmans MPG. Simultaneous detection and genotyping of "Norwalk-like viruses" by oligonucleotide array in a reverse line blot hybridization format. J Clin Microbiol. 2000;38:2595–601.
11. Pratelli A, Tempesta M, Greco G, Martella V, Buonavoglia C. Development of a nested PCR assay for the detection of canine coronavirus. J Virol Methods. 1999;80:11–5. DOI: 10.1016/S0166-0934(99)00017-8
12. Pereira CA, Monezi TA, Mehnert DU, D'Angelo M, Durigon EL. Molecular characterization of canine parvovirus in Brazil by polymerase chain reaction assay. Vet Microbiol. 2000;75:127–33. DOI: 10.1016/S0378-1135(00)00214-5
13. Martella V, Decaro N, Lorusso E, Radogna A, Moschidou P, Amorisco F. Genetic heterogeneity and recombination in canine noroviruses. J Virol. 2009;83:11391–6. DOI: 10.1128/JVI.01385-09
14. Widdowson MA, Rockx B, Schepp R, van der Poel WH, Vinje J, van Duynhoven YT, et al. Detection of serum antibodies to bovine norovirus in veterinarians and the general population in the Netherlands. J Med Virol. 2005;76:119–28. DOI: 10.1002/jmv.20333
15. Grøndalen J, Sævik B, Sørum H. Companion animals as reservoir for zoonotic diseases. European Journal of Companion Animal Practice. 2008;18:213–22.

Address for correspondence: Jan Vinjé, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop G04, Atlanta, GA 30333, USA; email: jvinje@cdc.gov

Pulsed-field Gel Electrophoresis for *Salmonella* Infection Surveillance, Texas, USA, 2007

Stephen G. Long, Herbert L. DuPont, Linda Gaul, Raouf R. Arafat, Beatrice J. Selwyn, Joan Rogers, and Eric Casey

To identify sources of transmission for area clusters, in 2007 the Houston Department of Health and Human Services conducted an 8-month study of enhanced surveillance of *Salmonella* infection. Protocol included patient interviews and linking the results of interviews to clusters of pulsed-field gel electrophoresis patterns detected by the local PulseNet laboratory.

To detect *Salmonella* clusters, public health laboratories perform pulsed-field gel electrophoresis (PFGE) that provides a PFGE pattern, or DNA fingerprint. If the PFGE patterns of isolates from ≥2 persons are indistinguishable, the responsible bacteria may be related to a common source (*1*–*3*). PulseNet is a network of public health laboratories coordinated by the Centers for Disease Control and Prevention (CDC), in which bacteria that cause foodborne diseases, including *Salmonella* isolates, are analyzed by using PFGE. This network provides the means to rapidly compare PFGE patterns from isolates submitted in different geographic areas. State and local laboratories upload PFGE patterns to the national CDC PulseNet database. Indistinguishable patterns at the national level might represent a large multistate outbreak (*4*–*6*).

As a city health department located in the state of Texas, the Houston Department of Health and Human Services (HDHHS) investigates all local *Salmonella* cases to detect outbreaks and vehicles of transmission. The HDHHS laboratory has been certified as a PulseNet laboratory since 2001 and serves residents of Houston (≈2.1 million persons) and adjacent counties.

Author affiliations: Houston Department of Health and Human Services, Houston, Texas, USA (S.G. Long, R.R. Arafat, J. Rogers); University of Texas School of Public Health, Houston (H.L. DuPont, B.J. Selwyn); St. Luke's Episcopal Hospital, Houston (H.L. DuPont); Baylor College of Medicine, Houston (H.L. DuPont); and Texas Department of State Health Services, Austin, Texas, USA (L. Gaul, E. Casey)

DOI: 10.3201/edi1606.091276

Because PFGE patterns obtained by a local health department may appear to be sporadic or unrelated to a more generalized process (*2*), local public health practitioners may gain a larger perspective by receiving notification of state and national clusters (*4*,*5*). During 2002–2005, before this study was conducted but during a time HDHHS was in routine communication with PulseNet, most local PFGE patterns were not recognized as linked to statewide or nationwide clusters.

In this study, HDHHS sought to determine more rigorously the utility of PFGE in local surveillance (as opposed to national surveillance) in detecting area clusters and vehicles of transmission. Another goal was to determine how local PFGE patterns and clusters are associated with larger-scale clusters. The study was approved by the Committee for the Protection of Human Subjects, University of Texas Health Science Center.

The Study

During an 8-month period, May 1 through December 31, 2007, HDHHS received 145 *Salmonella* case reports in which patients resided in Houston. The HDHHS laboratory performed PFGE for 106 (73%) isolates from the Houston case-patients. The laboratory performed PFGE for all isolates it received. The remaining 39 Houston cases had been reported by providers that did not forward the isolate to HDHHS. The HDHHS laboratory used a standardized PulseNet *Salmonella* protocol for PFGE and compared PFGE patterns for these isolates by using Bionumerics 4.0 software (Applied Maths, Sint-Martens-Latem, Belgium). Using a hypothesis-generating questionnaire, immediately upon receiving the case the first author interviewed 96 (91%) of the 106 case-patients with an assigned PFGE pattern. Follow-up was not feasible for the remaining 10 case-patients. Table 1 provides the demographic characteristics of the 106 case-patients. The HDHHS laboratory posted the PFGE patterns weekly to HDHHS epidemiologists, who then further investigated the clusters attempting to identify common sources.

Epidemiologists considered a group of *Salmonella* cases to be a cluster if 1) PFGE patterns of all isolates were indistinguishable; and 2) specimens were collected each within 90 days of at least 1 other case. A more inclusive 90-day interval was used, rather than the 60-day interval used by PulseNet, because the number of cases in a local PFGE cluster is typically small. A case that was not in a cluster was considered a singlet case.

Analysis of 106 *Salmonella* isolates from Houston residents yielded 74 distinctive PFGE patterns, of which 66 were forwarded to the Texas Department of State Health Services (DSHS) for comparison with the DSHS laboratory's database and to further identify clusters. Eight singlet patterns were not further analyzed because of lack of

Table 1. Cases of *Salmonella* infection reported to HDHHS, incidence rates, and PFGE results, May 1, 2007–December 31, 2007*

Case-patient characteristic	No. cases reported to HDHHS (%), n = 145	Incidence rate,† n = 145	No. (%) case-patients assigned a PFGE pattern, n = 106	No. (%) case-patients assigned a PFGE pattern and interviewed, n = 96
Sex				
M	65 (44.8)	10.0	50 (47.2)	45 (46.9)
F	80 (55.2)	12.3	56 (52.8)	51 (53.1)
Age, y				
<1	27 (18.6)	119.3	21 (19.8)	19 (19.8)
1–4	40 (27.6)	47.3	31 (29.2)	27 (28.1)
5–19	21 (14.5)	7.3	11 (10.4)	11 (11.5)
20–34	9 (6.2)	2.6	7 (6.6)	7 (7.3)
35–54	20 (13.8)	5.5	15 (14.2)	13 (13.5)
55–74	16 (11.0)	10.4	13 (12.3)	11 (11.5)
≥75	12 (8.3)	25.4	8 (7.5)	8 (8.3)
Race/ethnicity				
White				
Non-Hispanic	37 (25.5)	9.2	27 (25.5)	25 (26.0)
Hispanic	70 (48.3)	14.4	45 (42.4)	43 (44.8)
Black	23 (15.9)	7.1	20 (18.9)	17 (17.7)
Asian	9 (6.2)	13.1	9 (8.5)	9 (9.4)
Unknown	6 (4.1)	‡	5 (4.7)	2 (2.1)
Total	145	11.1	106	96

*HDHHS, Houston Department of Health and Human Services; PFGE, pulsed-field gel electrophoresis.
†Rate was calculated as number of cases/100,000 population/year, based on the 8-month study period.
‡Rate was not calculable.

staff in the laboratories. The DSHS returned a list of state ID numbers and county of residence for case-patients with matching isolate PFGE patterns, and HDHHS and DSHS epidemiologists conferred about the data.

Of the 106 *Salmonella* cases with identified PFGE patterns, 42 assembled into 10 clusters, with 2–13 cases per cluster. PFGE patterns for 8 of these clusters matched patterns in the DSHS statewide database, and patterns of 5 clusters matched those in other states obtained during the same period (Table 2).

HDHHS identified a likely exposure for 3 local PFGE clusters (Table 2). The first cluster, *S. enterica* serovar Braenderup JBPX01.0516 (PulseNet nomenclature), included 2 Houston case-patients and 5 case-patients residing in adjoining counties. Two persons reported travel to Matamoros, Mexico, before getting sick. Four isolates in the DSHS database had this PFGE pattern, of which 3 had been obtained from case-patients who resided in Brownsville, Texas, near Matamoros. HDHHS posted the PFGE pattern on PulseNet Listserve, and the Ohio Department of

Table 2. Ten *Salmonella* pulsed-field gel electrophoresis clusters among residents of Houston, Texas, USA, and 2 Houston singlet cases linked by PFGE to national outbreaks, detected May 1, 2007–December 31, 2007*

Serotype	PFGE pattern, *Xba*I†	No. cases in Houston	No. other cases in DSHS database	Associated national outbreak	Common exposure or other link
Braenderup	JBPX01.0516	2	4	PulseNet outbreak 0708HUJBP-1c	Traveled or resided in southern Texas
Corvallis	SCVX01.0014	2	0	–	Unknown
Enteritidis	JEGX01.0004	13	Numerous	PulseNet outbreak 0801PAJEG-1	Egg consumption
Enteritidis	JEGX01.0005	6	25	–	Unknown
Infantis	JFXX01.0022	3	5	–	Unknown
Infantis	JFXX01.0041	5	1	–	Unknown
Paratyphi b var. java	JKXX01.0014	4	2	PulseNet outbreak 0710NCJKX-1c (*7*)	Contact with miniature turtles
Typhimurium	JPXX01.0276	2	0	–	Unknown
Typhimurium	JPXX01.0621	3	3	PulseNet outbreak 0801ORJPX-1c	Unknown
Typhimurium	JPXX01.0006	2	3	Possible bovine outbreak (multistate) 0708MLJPX-1c	Unknown
Typhimurium	JPXX01.1037	1	0	PulseNet outbreak 0704WIWWS-1c	Packaged vegetable product‡
Typhimurium	JPXX01.1354	1	1	PulseNet outbreak 0703MLJPX-2c	Contact with hamsters‡

*PFGE, pulsed-field gel electrophoresis; DSHS, Texas Department of State Health Services.
†PulseNet nomenclature.
‡The case was linked by PFGE to a PulseNet cluster, but the patient denied having been exposed to the hypothesized epidemiologic link.

Health responded with information regarding a concurrent outbreak of the same strain in a church group whose members became ill while visiting southern Texas. *S. enterica* serovar Enteritidis JEGX01.0004, one of the most common patterns in the HDHHS and DSHS PFGE databases, was noted by HDHHS to be occurring at above expected levels in December 2007. The Pennsylvania Department of Health posted outbreak clusters in Pennsylvania with the same strain, associated with the consumption of improperly cooked eggs. Nine of the 13 (69%) Houston case-patients reported eating eggs during the week before illness onset. In 2 Houston households, persons became sick after eating eggs purchased in farmers' markets. The North Carolina Division of Public Health linked a third PFGE cluster, *S. enterica* serovar Paratyphi B var. Java, JKXX01.0014, to miniature turtles (*7*). Two of 4 Houston patients and another patient in Victoria, Texas, reported having contact with miniature turtles.

During the 8-month study, the HDHHS laboratory also sent patterns for 56 (87%) of the 64 singlet isolates to DSHS, which coupled 11 (20%) of these with more cases in their statewide database. Isolates from 2 Houston singlet cases had patterns matching 2 concurrent multistate outbreak patterns. An isolate of *S. enterica* serovar Typhimurium JPXX01.1037 matched a PulseNet PFGE cluster pattern attributed to a nationally distributed packaged vegetable product. The other isolate, *S. enterica* serovar Typhimurium JPXX01.1354, matched a pattern linked to an outbreak investigated by Wisconsin Division of Public Health in which case-patients were exposed to hamsters. For these singlets, HDHHS was unable to confirm an epidemiologic link between the Houston case and the national outbreak (Table 2).

Conclusions

Using PFGE patterns, HDHHS discerned vehicles of transmission for local clusters. Such findings could enable a local health department to intervene to address outbreaks currently in progress. Even small clusters are strong indicators because the actual number of cases in an outbreak is typically vastly larger.

Consistent cooperation between HDHHS and DSHS epidemiologists enabled them to see Houston PFGE patterns in a context of statewide and national patterns and clusters. A Houston PFGE pattern that was part of a local cluster was quite likely to match a DSHS (statewide) or CDC (national) pattern. This finding is in contrast to results for 56 singlet patterns; only 11 were found to match patterns of cases outside the local area.

Analysis of PFGE clustering assisted this surveillance system in detecting outbreaks successfully. Findings on PulseNet helped HDHHS epidemiologists identify sources of bacteria in local clusters. HDHHS conducted prompt interviews of 91% of the Houston patients. Of course, a 100% follow-up would have been better, but this study demonstrates the successes that are possible through routine surveillance by a local health department, given its resources. In an ideal situation, a PulseNet-certified laboratory performs local surveillance in sustained close cooperation with epidemiologists who conduct timely investigations based on laboratory findings.

Acknowledgments

We gratefully acknowledge the considerable support of microbiologists at the Houston Department of Health and Human Services and Texas Department of State Health Services.

The costs of the PFGE analyses and epidemiologic investigations were supported in part by funding through the Epidemiology and Laboratory Capacity Cooperative Funding Agreement with CDC.

Dr Long is an epidemiologist with the Houston Department of Health and Human Services. He specializes in the surveillance of foodborne diseases.

References

1. Bender JB, Hedberg CW, Boxrud DJ, Besser JM, Wicklund JH, Smith KE, et al. Use of molecular subtyping in surveillance for *Salmonella* enterica subtype Typhimurium. N Engl J Med. 2001;344:189–95. DOI: 10.1056/NEJM200101183440305
2. Tauxe RV. Molecular subtyping and the transformation of public health. Foodborne Pathog Dis. 2006;3:4–8. DOI: 10.1089/fpd.2006.3.4
3. Barrett TJ, Gerner-Smidt P, Swaminathan B. Interpretation of pulsed-field gel electrophoresis patterns in foodborne disease investigation and surveillance. Foodborne Pathog Dis. 2006;3:20–31. DOI: 10.1089/fpd.2006.3.20
4. Swaminathan B, Barrett TJ, Hunter SB, Tauxe RV. CDC PulseNet Task Force. PulseNet: The molecular subtyping network for foodborne bacterial disease surveillance, United States. Emerg Infect Dis. 2001;7:382–9.
5. Gerner-Smidt P, Hise K, Kincaid J, Hunter S, Rolando S, Hyytiä-Trees E, Ribot EM, Swaminathan B, CDC Pulsenet Taskforce. PulseNet USA: a five-year update. Foodborne Pathog Dis. 2006;3:9–19. DOI: 10.1089/fpd.2006.3.9
6. Hedberg CW, Besser JM. Commentary: cluster evaluation, PulseNet, and public health practice. Foodborne Pathog Dis. 2006;3:32–5. DOI: 10.1089/fpd.2006.3.32
7. Centers for Disease Control and Prevention. Multistate outbreak of human *Salmonella* infections associated with exposure to turtles—United States, 2007–2008. MMWR Morb Mortal Wkly Rep. 2008;57:69–72.

Address for correspondence: Stephen G. Long, Bureau of Epidemiology, 4th Floor, Houston Department of Health and Human Services, 8000 N Stadium Dr, Houston, TX 77054, USA; email: stephen.long@cityofhouston.net

Novel Betaherpesvirus in Bats

Shumpei Watanabe,[1] Ken Maeda,[1] Kazuo Suzuki, Naoya Ueda, Koichiro Iha, Satoshi Taniguchi, Hiroshi Shimoda, Kentaro Kato, Yasuhiro Yoshikawa, Shigeru Morikawa, Ichiro Kurane, Hiroomi Akashi, and Tetsuya Mizutani

Because bats are associated with emerging zoonoses, identification and characterization of novel viruses from bats is needed. Using a modified rapid determination system for viral RNA/DNA sequences, we identified a novel bat betaherpesvirus 2 not detected by herpesvirus consensus PCR. This modified system is useful for detecting unknown viruses.

Since the 1990s, bats have been associated with several emerging zoonotic agents, including Hendra, Nipah, Ebola, lyssa, and severe acute respiratory syndrome coronavirus-like viruses (*1*). Bats seem to have great potential as reservoirs for emerging viruses. Therefore, to understand the role of bats as a host species, identification and characterization of novel viruses from bats is needed. For virus isolation, we have been attempting to establish primary cell cultures from various bats (*2*,*3*). Using a rapid determination system for viral RNA sequences (RDV), we discovered a novel adenovirus and gammaherpesvirus in bats (*2*,*4*). This system, which we simplified to a less laborious one (*5*), is useful for detecting viruses, regardless of virus species (*6*).

The Study

During June–August, 2008, with the permission of the governor of Wakayama Prefecture, Japan, we caught 8 insectivorous vespertilionid bats, *Miniopterus fuliginosus*, and used their spleens and kidneys to establish primary cell cultures. During passage of the primary spleen adherent cells, cytopathic effect (cell death) was noted at third passage. The collected supernatant was injected into fresh primary kidney cells and caused apparent cytopathic effect at first passage.

Author affiliations: The University of Tokyo, Tokyo, Japan (S. Watanabe, N. Ueda, K. Iha, S. Taniguchi, K. Kato, Y. Yoshikawa, H. Akashi); Yamaguchi University, Yamaguchi, Japan (K. Maeda, H. Shimoda); Hikiiwa Park Center, Wakayama, Japan (K. Suzuki); and National Institute of Infectious Diseases, Tokyo (S. Morikawa, I. Kurane, T. Mizutani)

DOI: 10.3201/eid1606.091567

Before using the RDV method, we had attempted to detect herpesvirus by nested PCR with the consensus primer sets DFA, ILK, KG1, TGV, and IYG, which were designed according to the consensus-degenerate hybrid oligonucleotide primers program (*7*). These consensus degenerate primers are effective for detecting many herpesviruses from vertebrate hosts. However, in this study they failed to detect any herpesviruses.

We then attempted to detect herpesvirus by using RDV version 3.1, our modification from version 3.0 (*5*). The adapters and primers for construction of the second cDNA library in RDV version 3.1 were newly designed and replaced those used in RDV version 3.0 (online Technical Appendix 1, www.cdc.gov/EID/content/16/6/986-Techapp1.pdf). Both adapters have sticky-end structures digested with *Sau*3AI or *Hpy*CH4 IV. RDV version 3.1 can determine an unknown viral cDNA fragment with 64 primer pairs, which we used for constructing the second cDNA library.

With RDV version 3.1, we obtained 4 unknown cDNA fragments, which had no matches in a BLASTn (www.ncbi.nlm.nih.gov/blast/Blast.cgi) search. In a BLASTx search, 1 cDNA fragment (deduced sequence of 29 aa) was homologous to the glycoprotein B (gB) amino acid sequence of the tupaiid herpesvirus 1 (TuHV-1) (79% identity), which belongs to subfamily *Betaherpesvirinae*. We designed new consensus-degenerate hybrid oligonucleotide primers (http://blocks.fhcrc.org/codehop.html) selective for the betaherpesvirus gB and DNA polymerase (DPOL) genes, and we determined the complete gB sequence and the partial DPOL sequence of the isolated virus (5,029 bp, DNA Data Bank of Japan accession no. AB517983). BLAST search indicated that the complete gB sequence was novel and most similar to that of TuHV-1 (59% aa sequence identity) (online Appendix Figure, www.cdc.gov/EID/content/16/6/986-appF.htm). We named the isolated virus bat betaherpesvirus 2 (BatBHV-2).

We constructed a phylogenetic tree by using the neighbor-joining method with the gB amino acid sequence and the available sequences of known herpesviruses (Figure). The phylogenetic tree based on betaherpesvirus gB genes showed that BatBHV-2 is most closely related to TuHV-1 and caviid herpesvirus 2 (guinea pig cytomegalovirus). The subfamily *Betaherpesvirinae* consists of the genera *Cytomegalovirus*, *Muromegalovirus,* and *Roseolovirus*. TuHV-2 and caviid herpesvirus 2 are species unassigned to any genus in the subfamily *Betaherpesvirinae*.

In May 2009, we collected, again with permission, another 50 bats belonging to 1 species, *M. fuliginosus,* from the same location for an epizootologic study (online Technical Appendix 2, www.cdc.gov/EID/content/16/6/986-Techapp2.pdf). Spleens and blood were collected from all

[1]These authors contributed equally to this article.

bats, and other organs (liver, kidney, lung, brain, intestine, trachea, and urinary bladder) were collected from 10 bats. Nested PCR was performed by using specific primers selective for the DPOL gene of BatBHV-2, and PCR products were subjected to direct sequencing. Viral nucleotide sequences were obtained from 4 of the 50 spleen samples. Each nucleotide sequence showed complete identity to the partial DPOL sequence of the BatBHV-2. Other organs and serum collected from 2 of the bats were also tested by nested PCR, and viral DNA was detected in the liver, kidneys, and lungs of both bats.

Conclusions

Although PCRs with consensus primers effectively detect known and unknown viruses, they failed to detect BatBHV-2, possibly because of minor mismatches between the sequences of BatBHV-2 and the primer sets (TGV, IYG, and KG1). The variety of virus sequences and gene mutations often prevents successful amplification of virus genes. RDV, however, can detect viral cDNA fragments independent of virus species and thus is useful as a first-choice tool for identifying emerging known and unknown viruses in animals and humans.

BLAST search showed that the complete gB sequence of the isolated virus was novel and most similar to that of TuHV-1. Recently, bats have been described as hosts for herpesviruses in several countries in Europe, America, Africa, and Asia (*4*,*9*,*10*). Wibbelt et al. reported that the partial DPOL sequence (175 bp) of a betaherpesvirus, bat betaherpesvirus 1 (BatBHV-1), was obtained from several insectivorous bat species (*10*). Although the length of the BatBHV-1 sequence was short, similarity between BatBHV-1 and BatBHV-2 was relatively high (58%). BatBHV-1 is most similar to TuHV-1(61%). These findings suggest that BatBHV-2 is a different species than BatBHV-1.

Our epizootologic study found relatively high (8%) prevalence of BatBHV-2 in insectivorous bats. Although the virus genome was detected in a few parenchymal organs by nested PCR, no amplification was possible for serum, intestine, or urinary bladder samples, which may exclude apparent virus shedding by the bats. In addition, all 50 bats collected appeared clinically healthy. To understand the life cycle of this virus, the possibility of a latent infection in these insectivorous bats must be explored.

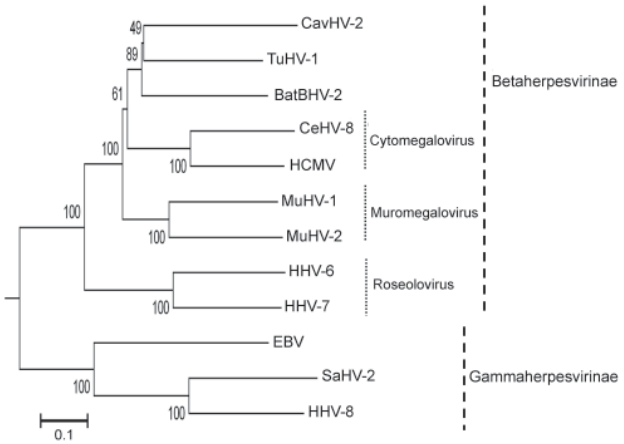


Figure. Phylogenetic tree based on the deduced amino acid sequences of complete glycoprotein B. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The tree was rooted to herpes simplex virus type 1 (X14112). The evolutionary distances were computed by using the Poisson correction method and are in units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the dataset. The final dataset included a total of 698 positions. Phylogenetic analyses were conducted in MEGA4 (*8*). The herpesviruses used for comparison and their accession numbers are as follows: Epstein-Barr virus 1 (EBV, NC_007605), caviid herpesvirus (CavHV-2, FJ355434); mouse cytomegalovirus (MuHV-1, NC_004065), human cytomegalovirus (HCMV, X17403), human herpesvirus 6 (HHV-6, AF157706), human herpesvirus 7 (HHV-7, AF037218), human herpesvirus 8 (HHV-8, AF148805), rat cytomegalovirus (MuHV-2, NC_002512), cercopithecine herpesvirus 8 (CeHV-8, AY186194), saimiriine herpesvirus 2 (SaHV-2, NC_001350), and tupaiid herpesvirus 1 (TuHV-1, AF281817). Scale bar indicates evolutionary distance.

Acknowledgments

We thank Momoko Ogata and Miho Nishimura for their assistance.

This study was supported in part by grants from the Japan Society for the Promotion of Science; the Ministry of Health, Labor, and Welfare; and the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

Mr Watanabe is a PhD student in the Department of Veterinary Microbiology, Graduate School of Agricultural and Life Sciences, The University of Tokyo. His research interests include the epidemiology and pathogenic mechanisms of emerging viruses from bats.

References

1. Wong S, Lau S, Woo P, Yuen KY. Bats as a continuing source of emerging infections in humans. Rev Med Virol. 2007;17:67–91. DOI: 10.1002/rmv.520
2. Maeda K, Hondo E, Terakawa J, Kiso Y, Nakaichi N, Endoh D, et al. Isolation of novel adenovirus from fruit bat (*Pteropus dasymallus yayeyamae*). Emerg Infect Dis. 2008;14:347–9. DOI: 10.3201/eid1402.070932
3. Omatsu T, Watanabe S, Akashi H, Yoshikawa Y. Biological characters of bats in relation to natural reservoir of emerging viruses. Comp Immunol Microbiol Infect Dis. 2007;30:357–74. DOI: 10.1016/j.cimid.2007.05.006
4. Watanabe S, Ueda N, Iha K, Masangkay JS, Fujii H, Alviola P, et al. Detection of a new bat gammaherpesvirus in the Philippines. Virus Genes. 2009;39:90–3. DOI: 10.1007/s11262-009-0368-8

5. Watanabe S, Mizutani T, Sakai K, Kato K, Tohya Y, Fukushi S, et al. Ligation-mediated amplification for effective rapid determination of viral RNA sequences (RDV). J Clin Virol. 2008;43:56–9. DOI: 10.1016/j.jcv.2008.05.004
6. Mizutani T, Endoh D, Okamoto M, Shirato K, Shimizu H, Arita M, et al. Rapid genome sequencing of RNA viruses. Emerg Infect Dis. 2007;13:322–4. DOI: 10.3201/eid1302.061032
7. VanDevanter DR, Warrener P, Bennett L, Schultz ER, Coulter S, Garber RL, et al. Detection and analysis of diverse herpesviral species by consensus primer PCR. J Clin Microbiol. 1996;34:1666–71.
8. Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol. 2007;24:1596–9. DOI: 10.1093/molbev/msm092
9. Razafindratsimandresy R, Jeanmaire EM, Counor D, Vasconcelos PF, Sall AA, Reynes JM. Partial molecular characterization of alphaherpesviruses isolated from tropical bats. J Gen Virol. 2009;90:44–7. DOI: 10.1099/vir.0.006825-0
10. Wibbelt G, Kurth A, Yasmum N, Bannert M, Nagel S, Nitsche A, et al. Discovery of herpesviruses in bats. J Gen Virol. 2007;88:2651–5. DOI: 10.1099/vir.0.83045-0

Address for correspondence: Tetsuya Mizutani, Department of Virology 1, National Institute of Infectious Diseases, Gakuen 4-7-1, Musashimurayama, Tokyo 208-0011, Japan; email: tmizutan@nih.go.jp

Dengue Virus 3 Genotype I in *Aedes aegypti* Mosquitoes and Eggs, Brazil, 2005–2006

Ana P.P. Vilela, Leandra B. Figueiredo, João R. dos Santos, Álvaro E. Eiras, Cláudio A. Bonjardim, Paulo C.P. Ferreira, and Erna G. Kroon

Dengue virus type 3 genotype I was detected in Brazil during epidemics in 2002–2004. To confirm this finding, we identified this virus genotype in naturally infected field-caught *Aedes aegypti* mosquitoes and eggs. Results showed usefulness of virus investigations in vectors as a component of active epidemiologic surveillance.

Dengue virus (DENV) is a member of the family *Flaviviridae* and a positive-sense RNA virus. Epidemics caused by the 4 DENV serotypes have emerged as major public health problems in tropical and subtropical regions over the past 20 years (*1*). DENV is transmitted to humans by the bite of an infected mosquito. Female *Aedes aegypti* mosquitoes are the main vector involved in the urban transmission cycle of the virus. *Ae*. *aegypti* is a tropical mosquito that lays its eggs on the walls of containers commonly found in and around homes (*1*). Female mosquitoes remain infectious for their entire lives and have the potential to transmit virus during each human feeding.

Mosquitoes and larvae may be infected by vertical transmission and maintain the virus in nature (*2*). Spread of the mosquito vector and virus has led to a resurgence of dengue fever epidemics and the emergence of dengue hemorrhagic fever (DHF) (*3*). No dengue vaccine is currently available, and dengue control relies solely on vector control. For successful epidemiologic investigations, identification and typing of DENV from field-caught mosquitoes and eggs are needed.

The current epidemiology of dengue in Minas Gerais state, Brazil, is characterized by cocirculation of DENV-1, DENV-2 and DENV-3 serotypes (state and metropolitan health departments, unpub. data). DENV-3 serotype was detected in 2002 and during 2005–2006; this was the most common serotype detected in Minas Gerais in those periods (*4*,*5*). Previous work in our laboratory identified DENV-3 genotype I, which was associated with dengue fever and DHF in Minas Gerais (*6*).

In this study, we confirm circulation of DENV-3 genotype I in naturally infected field-caught *Ae*. *aegypti* mosquitoes and eggs. We show the useful role of virus investigations in mosquitoes and eggs for monitoring DENV circulation.

The Study

Traps designed to catch mosquitoes and eggs were installed in an urban residential area in the northwestern borough of Belo Horizonte, Minas Gerais, Brazil. Since 1998, Belo Horizonte has had a high concentration of dengue cases and high rates of vector infestation. Mosquitoes were obtained during 8 weeks (during October 2005–May 2006). Adult mosquitoes were collected by using 2 capture traps (Figure 1, panels A and B): MosquiTRAP version 2.0 (M trap; Ecovec Ltd., Belo Horizonte, Brazil) (*7*) and the BG-Sentinel trap (BG trap; Biogents, Regensburg, Germany) (*8*). Eggs were collected with an ovitrap (Figure 1, panel C) and hatched into larvae (*9*).

An area of 20 blocks was selected for the survey. In each block, 3 representative houses were selected, and each house received 1 type of trap. All traps were installed outdoors in a shaded area that was also protected from rain. Within each block, traps were rotated so that each house had only 1 trap type for a maximum of 1 week. Mosquitoes collected were identified by sex, trap, and epidemiologic week. Eggs collected were counted, identified by epidemiologic week, and hatched into larvae. All samples were stored at –70°C until analyzed (Table 1).

Pools of ≤30 mosquitoes and ≤50 larvae were triturated on ice, macerated in 300 μL of Leibowitz L15 medium (GIBCO-BRL, Gaithersburg, MD, USA), and centrifuged at 2,000 × *g* for 5 min. RNA from each pool was extracted according to a modified protocol (*10*) and used as template in a reverse transcription–PCR as described (*11*). To amplify the virus genome, a seminested PCR was conducted with a forward primer (5′-CGA GAA ACC GCG TGT CAA C-3′) designed to amplify a 434-nt region that contains the capsid–premembrane (C-prM) gene and a reverse primer as described (*11*). The PCR products were then used for sequencing (MegaBACE sequencer; GE Healthcare, Little Chalfont, UK). Sequences and inferred amino acid sequences were aligned with other available DENV-3 sequences.

Alignments were used to construct midpoint-rooted phylogenetic trees by using the neighbor-joining method. The Tamura-Nei statistical model was implemented by using MEGA4.1 software (Arizona State University, Tem-

Author affiliations: Universidade Federal de Minas Gerais, Belo Horizonte, Brazil

DOI: 10.3201/eid1606.091000

Figure 1. MosquiTRAP version 2.0 (Ecovec Ltd., Belo Horizonte, Brazil) (A), BG-Sentinel trap (Biogents, Regensburg, Germany) (B), and an ovitrap (C) used for obtaining mosquitoes in the northwestern borough of Belo Horizonte, Minas Gerais, Brazil.

pe, AZ, USA) with 1,000 bootstrap replicates. These sequences have been deposited in GenBank (accession nos. GQ330909–11 and GU588695–8).

A total of 237 adult *A. aegypti* mosquitoes (137 females and 100 males) were tested in 25 pools, and 5,573 larvae were tested in 101 pools (Table 1). Fifteen mosquito pools contained only females, and 10 contained only males. Fifteen pools (8 containing females and 7 containing males) were obtained in BG traps, and 10 pools (7 containing females and 3 containing males) were obtained in M traps. Viral RNA was detected in 4 of the 25 *A. aegypti* mosquito pools analyzed. Two positive pools were obtained in M traps, and 2 positive pools were obtained in BG traps. Viral RNA was detected in 1 of 101 larvae pools tested (Table 1). Minimum infection rates were 16.9% for adult mosquitoes and 0.18% for larvae (Table 2). The higher minimum infection rates for mosquitoes could be explained by the lower number of analyzed specimens.

These data also confirm vertical transmission by detection of DENV-3 in male mosquitoes hatched from eggs. C-prM sequences obtained showed a high degree of similarity to isolates from the Philippines and the People's Republic of China and recent isolates from Minas Gerais and Rondônia, Brazil (*6*,*12*). Sequence homology values ranged from 98.8% to 99.8%. Phylogenetic analysis confirmed that all 4 samples obtained from adult mosquitoes (female mosquito BH-14/2005, male mosquito BH-1/2006, female mosquito BH-15/2006, and female mosquito BH-17/2006) and larvae (BH-11/2006) were grouped in a well-supported distinct cluster of genotype I (Figure 2).

Table 1. *Aedes aegypti* mosquitoes and larvae obtained by using 2 traps and analyzed by PCR, Belo Horizonte, Minas Gerais, Brazil, October 2005–May 2006*

	BG-Sentinel trap†				MosquiTRAP version 2.0‡				Ovitrap	
	Mosquitoes		Pools		Mosquitoes		Pools		Larvae	Pools
Date	F	M	F	M	F	M	F	M		
2005										
Oct	–	–	–	–	3	2	1	1	483	7
Nov	1	–	1	–	–	–	–	–	198	4
Dec	23	21	2	2	30	1	2§	1	760	9
2006										
Jan	–	–	–	–	–	–	–	–	665	13§
Feb	12	13	1	1	8	–	1	–	1,076	22
Mar	16	36	2§	2§	3	–	1§	–	754	14
Apr	14	11	1	1	12	1	1	1	1,004	20
May	11	15	1	1	4	–	1	–	1,298	25
Total	77	96	8	7	60	4	7	3	5,573	101

*–, not available.
†Biogents, Regensburg, Germany.
‡Ecovec Ltd., Belo Horizonte, Brazil.
§One sample was positive for dengue virus type 3.

Table 2. MIR for dengue virus type 3 in *Aedes aegypti* mosquitoes and larvae, Belo Horizonte, Minas Gerais, Brazil, October 2005–May 2006*

Stage	Positive pools/ analyzed pools	No. specimens analyzed	MIR, %
Male adult	1/10	100	10.0
Female adult	3/15	137	21.9
Both	4/25	237	16.9
Larvae	1/101	5,573	0.18

*MIR, minimum infection rate.

Conclusions

Our results suggest that the DENV-3 strains circulating in Minas Gerais, Brazil, in 2005 and 2006 may share a common origin. We identified (*6*) co-circulation of 2 DENV-3 genotypes (I and III) in Brazil (*6*,*12*). DENV-3 isolates detected in Rio de Janeiro, Brazil, during the 2001 and 2002 outbreaks and DENV-3 detected in Latin America were assigned to genotype III, which has been associated with DHF outbreaks (*13*). DENV-3 genotype I identified in outbreaks during 2002–2004 in Minas Gerais (*6*) also showed associations with DHF. This genotype was also identified in Colombia (*14*) and French Guiana (*14*). Sequences showed high similarity to isolates from Southeast Asia and the South Pacific islands and have not been previously reported in South America.

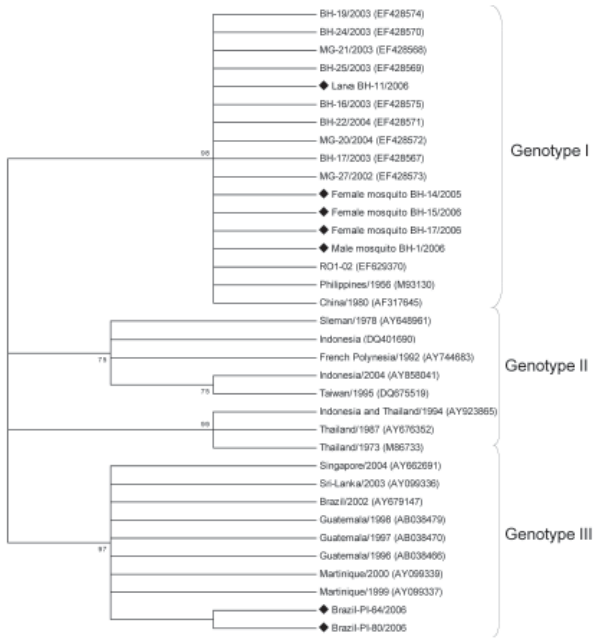


Figure 2. Phylogenetic tree of dengue type 3 serotypes and sequences from *Aedes aegypti* mosquitoes and larvae obtained in Belo Horizonte, Minas Gerais, Brazil. The tree is based on a 434-nt sequence of the capsid–premembrane gene and was generated by using neighbor-joining analysis with the Tamura-Nei model in MEGA4.1 software (Arizona State University, Tempe, AZ, USA). Numbers to the left of the nodes are bootstrap values (1,000 replicates) in support of the grouping to the right. Numbers in parentheses are GenBank accession numbers. Diamonds indicate viruses sequenced in this study.

Detection of DENV-3 genotype I in sylvatic animals (*15*) may support the hypothesis of a sylvatic origin for this genotype in South America. Our analysis of C-prM gene sequences from mosquitoes naturally infected with DENV-3 confirmed circulation of genotype I in Minas Gerais. Additionally, our results indicated that dengue virus sequences in mosquitoes and larvae are highly similar to sequences in DENV-3 isolates from patients who are spatially and temporally related. Although vertical transmission has not been routinely determined (*2*), our findings also confirmed that vertical transmission of DENV that may be a major factor in virus prevalence and survival in nature.

Acknowledgments

We thank Angela Lopes, Ilda Gama, and Andreza Carvalho for secretarial and technical assistance; and Fabrício Santos for assistance with sequencing.

This study was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico; Coordenação de Aperfeiçoamento de Pessoal de Nível Superior; Departamento de Ciência, Tecnologia da Secretaria de Ciência, Tecnologia e Insumos Estratégicos/Departamento de Ciência e Tecnologia–Ministério da Saúde; and Fundação de Amparo à Pesquisa do Estado de Minas Gerais, Brazil. C.A.B, P.C.P.F., and E.G.K are recipients of research fellowships from Conselho Nacional de Desenvolvimento Científico e Tecnológico.

Ms Vilela is a biologist and PhD candidate at the Laboratório de Virus, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil. Her research interests are molecular epidemiology and dengue.

References

1. Gubler DJ. Dengue and dengue hemorrhagic fever. Clin Microbiol Rev. 1998;11:480–96.
2. Günther J, Martínez-Muñoz JP, Pérez-Ishiwara DG, Salas-Benito J. Evidence of vertical transmission of dengue virus in two endemic localities in the State of Oaxaca, Mexico. Intervirology. 2007;50:347–52. DOI: 10.1159/000107272
3. Cologna R, Armstrong PM, Rico-Hesse R. Selection for virulent dengue viruses occurs in humans and mosquitoes. J Virol. 2005;79:853–9. DOI: 10.1128/JVI.79.2.853-859.2005
4. Secretaria de Vigilância em Saúde/Ministério da Stério da Saúde. Informe epidemiológico da dengue, boletim semana epidemiológica; 2009. p. 1–30 [cited 2009 Dec 13]. http://portal.saude.gov.br/portal/arquivos/pdf/boletim_semana_1_a_30_revisado_03_11.pdf
5. Corrêa PR, Franca E, Bogutchi TF. *Aedes aegypti* infestation and occurrence of dengue in the city of Belo Horizonte, Brazil. Rev Saude Publica. 2005;39:33–40. DOI: 10.1590/S0034-89102005000100005
6. Barcelos Figueiredo L, Cecílio AB, Ferreira GP, Drumond BP, De Oliveira JG, Bonjardim CA, et al. Dengue virus 3 genotype I associated with dengue fever and dengue hemorrhagic fever, Brazil. Emerg Infect Dis. 2008;14:314–6. DOI: 10.3201/eid1402.070278

7. Fávaro EA, Mondini A, Dibo MR, Barbosa AA, Eiras AE, Neto FC. Assessment of entomological indicators of *Aedes aegypti* (L.) from adult and egg collections in São Paulo, Brazil. J Vector Ecol. 2008;33:8–16. DOI: 10.3376/1081-1710-(2008)33[8:AOEIOA]2.0.CO;2
8. Krockel U, Rose A, Eiras AE, Geier M. New tools for surveillance of adult yellow fever mosquitoes: comparision of trap catches with human landing rates in an urban environment. J Am Mosq Control Assoc. 2006;22:229–38. DOI: 10.2987/8756-971-X(2006)22[229:NTFSOA]2.0.CO;2
9. Gama RA, Eiras AE, Resende MC. Effect of lethal ovitrap on the longevity of females of *Aedes aegypti* (Diptera: Culicidae). Rev Soc Bras Med Trop. 2007;40:640–2.
10. Boom R, Sol CJ, Salimans MM, Jansen CL, Wertherim-Van Dillen PM, van der Noordaa J. Rapid and simple method for purification of nucleic acids. J Clin Microbiol. 1990;28:495–503.
11. Lanciotti RS, Calisher CH, Gubler DJ, Chang GJ, Vorndam AV. Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase polymerase chain reaction. J Clin Microbiol. 1992;30:545–51.
12. Nogueira MB, Stella V, Bordignon J, Batista WC, Borba L, Silva LH, et al. Evidence for the circulation of dengue vírus type 3 genotypes III and V in northern region of Brazil during 2002–2004 epidemics. Mem Inst Oswaldo Cruz. 2008;103:483–8. DOI: 10.1590/S0074-02762008000500013
13. Nogueira RM, Schatzmayr HG, de Filippis AM, dos Santos FB, da Cunha RV, Coelho JO, et al. Dengue virus type 3, Brazil, 2002. Emerg Infect Dis. 2005;11:1376–81.
14. Usme-Ciro JA, Mendez JA, Tenorio A, Rey GJ, Domingo C, Gallego-Gomes JC. Simultaneous circulation of genotypes I and III of dengue virus 3 in Colombia. Virol J. 2008;5:101. DOI: 10.1186/1743-422X-5-101
15. De Thoisy B, Lacoste V, Germain A, Muñoz-Jórdan V, Colón C, Mauffrey JF, et al. Dengue infection in neotropical forest mammals. Vector Borne Zoonotic Dis. 2009;9:157–69. DOI: 10.1089/vbz.2007.0280

Address for correspondence: Erna G. Kroon, Laboratório de Vírus, Departamento de Microbiologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6627, Caixa Postal 486, CEP 31270-901, Belo Horizonte, Minas Gerais, Brazil; email: kroone@icb.ufmg.br

Pneumovirus in Dogs with Acute Respiratory Disease

Randall W. Renshaw, Nancy C. Zylich, Melissa A. Laverack, Amy L. Glaser, and Edward J. Dubovi

To determine which respiratory viruses circulate among confined dogs, we analyzed nasal and pharyngeal swab specimens from shelter dogs with acute respiratory disease. An unknown virus was isolated. Monoclonal antibody testing indicated that it was probably a pneumovirus. PCR and sequence analysis indicated that it was closely related to murine pneumovirus.

Domestic dogs housed in close confinement, as in kennels or animal shelters, are often involved in outbreaks of acute respiratory disease (*1*,*2*). To determine which viruses are associated with these outbreaks, we studied 200 dogs in 2 animal shelters in the northeastern United States during 2008–2009.

The Study

Nasal and pharyngeal swab specimens were collected from the dogs, and swab eluate extracts were prepared. Pooled extracts were added to monolayer cultures of canine A72 cells (American Type Culture Collection, CRL-1542). After 3 passages in culture, cells in some of the flasks showed subtle cytopathic changes. After continued passage, small foci of rounded cells developed, and rapid and progressive cell death throughout the flask ensued in a pattern uncharacteristic of the viruses commonly isolated from dogs. Testing these cultures with a panel of diagnostic reagents specific for common canine respiratory agents failed to identify a known virus. Immunofluorescence assays (IFAs) ultimately detected 13 positive cultures over a 4-month period when a monoclonal antibody (MAb) pool against human respiratory syncytial virus (RSV) (VP-R151, Vector Laboratories, Burlingame, CA, USA) was used. We have commonly used this antibody preparation to detect bovine RSV. The staining pattern included filamentous membrane-bound and free-floating virions and cytoplasmic inclusions, typical of the pattern in RSV-infected cells.

After obtaining IFA results, we attempted to amplify a fragment of the nucleocapsid gene (N) from the virus by using PCR primers designed on the basis of an alignment of human, bovine, and ovine RSV sequences. This attempt was unsuccessful (data not shown). Stocks of the individual MAbs in the anti-RSV pool and their specificities were obtained from the manufacturer and used for IFA (Figure). MAb 2G122 (P protein–specific) stained primarily inclusions and gave a relatively uniform membrane-associated signal. Staining with MAb 5H5N (M2 protein–specific) illuminated virions and inclusions. No fluorescence was noted with MAbs 1C3 (N protein–specific) or 5A6 (F protein–specific). All 4 individual MAbs recognized bovine RSV by IFA (data not shown). Recognition of the canine virus by only 2 of 4 MAbs and the inability to amplify a conserved region of the RSV genome suggested that it was related to, but unlikely to be, RSV.

To elucidate sequence information from 1 of the 13 isolates, we used a consensus-degenerate hybrid oligonucleotide primer algorithm to design degenerate PCR primers based on highly conserved amino acid sequences within multiple sequence alignments of all viruses in the subfamily *Pneumovirinae* (*3*). Specific regions within the L (polymerase) and N genes were targeted (Table). Sequencing of the reaction products and BLAST (www.ncbi.nlm.nih.gov/blast/Blast.cgi) analysis showed the virus to be closely related to murine pneumovirus (MPV), traditionally known as pneumonia virus of mice. Two L-gene PCR products were found to be 96%–97% identical to MPV, and an N gene fragment was 96% identical (Table).

Discussion and Conclusions

MPV is 1 of only 3 virus species classified in the family *Paramyxoviridae*, subfamily *Pneumovirinae,* genus

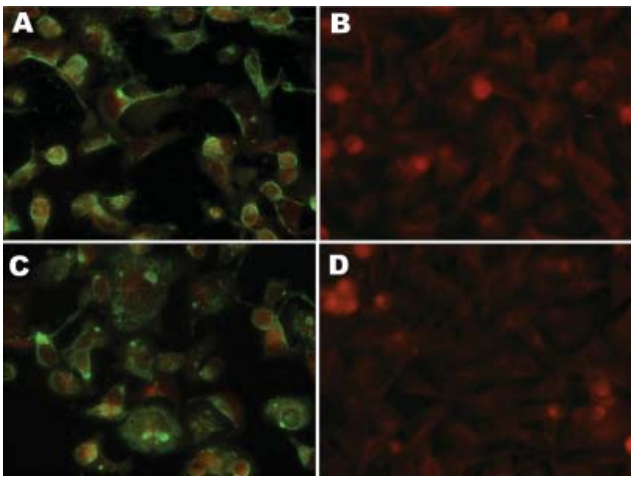


Figure. Slides showing immunofluorescence of A72 cells with human respiratory syncytial virus monoclonal antibodies (MAbs). A) MAb 2G122 on infected cells. B) MAb 2G122 on uninfected cells. C) MAb 5H5N on infected cells. D) MAb 5H5N on uninfected cells. Primary MAb stocks were used as obtained from the manufacturer at a dilution of 1:100. The red background is produced by counterstaining with Evans blue dye. Original magnification ×200.

Author affiliation: Cornell University, Ithaca, New York, USA

DOI: 10.3201/eid1606.091778

Table. Partial gene locations of virus isolated from dogs with respiratory disease and relatedness to other pneumoviruses*

Region sequenced†	PCR primers (5′ → 3′) and aa target sequences in pneumoviruses	% Identity to MPV, nt, aa†	% Identity to HRSV, nt, aa‡	% Identity to HMPV, nt, aa§
N gene,¶ nt 852–1182 (331 bp)	P1R: ggaactcgggggcgaayttytccat target: MEKFAPEFH N276F: tccgtgcaggccgaratggarcarg target: SVQAEMEQV	96.4, 96.3	68.5, 70.1	56.5, 54.1
L gene, no. 1,¶ nt 1143–1452 (310 bp)	L428F: ccggatcttcggccayccnatggt target: RIFGHPMV L538R: ttcttaggaggggagatggcyttrtcrtt target: NDKAISPPKN	95.8, 97.1	62.1, 55.3	58.9, 47.6
L gene, no. 2,¶ nt 1962–2511 (550 bp)	L698F: catcaccgacctgtccaagttyaaycargc target: ITDLSKFNQA L894R: ttgaagtcgtccaggatggtrttdatcca target: WINTILDDFK	96.7, 99.5	68.4, 71.6	66.4, 68.3

*MPV, murine pneumovirus; nt, nucleotide; aa, amino acid; HRSV, human respiratory syncytial virus; HMPV, human metapneumovirus; N, nucleocapsid; R, reverse primer; F, forward primer L, polymerase.
†Based on comparison with MPV strain J3666, GenBank accession no. NC_006579.
‡Based on comparison with HRSV, GenBank accession no. NC_001803.
§Based on comparison with HMPV, GenBank accession no. NC_004148.
¶The N gene fragment is entered under GenBank accession no. GU247050; L gene fragments nos. 1 and 2 are entered under GenBank accession no. GU247051.

Pneumovirus. Human RSV is the type species and is closely related to bovine RSV; MPV is more distantly related. For example, the N protein nucleotide sequences of human and bovine RSV are ≈94% identical to each other but only 60% identical to those of MPV. Only 2 strains of MPV, J3666 and Strain 15, have been fully sequenced, and they are 99.7% identical at the nucleotide level.

The nucleotide identity between virus isolated from the dogs and MPV was consistently >95% throughout the gene regions examined. Because the degenerate primers used were designed to cover regions that are generally highly conserved, regions of the genome that are typically more variable in other pneumoviruses may show greater differences between the canine pneumovirus and MPV. Of the conserved regions sequenced, amino acid identities were 70% (N), 55% (L fragment no. 1), and 72% (L fragment no. 2) when aligned with the same regions of human RSV, so finding cross-reactivity between human RSV MAbs and the newly isolated virus is not completely unexpected. Serologic cross-reactivity between RSV and MPV has been previously observed. Gimenez et al. (*4*) did not find recognition of MPV when they used 2 anti–human RSV MAbs, but they did observe recognition of MPV N protein when they used mouse anti–human RSV serum in immunoblot assays. Ling and Pringle (*5*) showed cross-reactivity of N proteins with polyclonal serum and cross-reactivity with a MAb that recognized P in immunoblot assays. Although the MAbs used in this study had been extensively tested for reactivity against a panel of 13 viruses, including several members of the family *Paramyxoviridae* (*6*,*7*), they were probably not tested for cross-reactivity against MPV because it is not a known human pathogen. The finding that reagents used to identify human RSV showed strong recognition of a closely related but distinct virus highlights the need for caution when interpreting research studies and conducting diagnostic evaluations.

The isolation of a previously unknown virus from dogs does not imply disease causation. However, comparison with MPV leads to speculation that the virus isolated in this study may have pathogenic potential. MPV is commonly known to infect laboratory rodent colonies, and serologic evidence points to infection of several wild rodent species. However, little is known about MPV epidemiology, such as whether MPV has multiple natural hosts or whether closely related viruses are circulating in other species. Neutralizing antibodies to MPV in other mammalian species, including humans, were first reported in the 1940s (*8*,*9*), and more recently, high prevalence of neutralizing antibodies but low (3%–4%) association with clinical disease in humans has been reported (*10*). Natural infection of rodents may be subclinical or latent. Sequelae to experimental infection in laboratory mice can vary from asymptomatic to severe disease with high morbidity and mortality rates. Pathogenic strains, including J3666 and Strain 15, can produce severe pneumonia and death in 6–10 days after mouse inoculation with a low dose (*11*,*12*). The pathogenicity or lack thereof may depend on the virus and on the mouse strain (*13*).

Questions remain as to whether this newly isolated virus commonly infects dogs and, if so, why it has not been previously isolated. Perhaps the strain that was circulating in these particular animal shelters is more easily isolated in culture. Or, because the initial cytopathic changes observed with these isolates were subtle, they could easily have been missed. Outbreaks of acute respiratory disease in dogs often involve multiple pathogens. As anticipated, other viruses, primarily canine influenza and parainfluenza viruses, were isolated during the study, often from the same animals that carried the pneumovirus. Work is ongoing to further

determine pneumovirus prevalence among dogs and its involvement in acute respiratory disease of dogs.

Acknowledgments

We thank Rebecca Harman, Jacqueline Nattrass, and Carol Nelson for their excellent technical assistance with diagnostic specimens.

This work was supported by departmental funding from the Animal Health Diagnostic Center at Cornell University.

Dr Renshaw is a molecular virologist at the Animal Health Diagnostic Center at Cornell University. His interests include identification, characterization, and development of diagnostic detection methods for viruses that infect domestic and wild animal species.

References

1. Buonavoglia C, Martella V. Canine respiratory viruses. Vet Res. 2007;38:355–73. DOI: 10.1051/vetres:2006058
2. Mochizuki M, Yachi A, Ohshima T, Ohuchi A, Ishida T. Etiologic study of upper respiratory infections of household dogs. J Vet Med Sci. 2008;70:563–9. DOI: 10.1292/jvms.70.563
3. Rose TM, Henikoff JG, Henikoff S. CODEHOP (COnsensus-DEgenerate hybrid oligonucleotide primer) PCR primer design. Nucleic Acids Res. 2003;31:3763–6. DOI: 10.1093/nar/gkg524
4. Gimenez HB, Cash P, Melvin WT. Monoclonal antibodies to human respiratory syncytial virus and their use in comparison of different virus isolates. J Gen Virol. 1984;65:963–71. DOI: 10.1099/0022-1317-65-5-963
5. Ling R, Pringle CR. Polypeptides of pneumonia virus of mice. I. Immunological cross-reactions and post-translational modifications. J Gen Virol. 1989;70:1427–40. DOI: 10.1099/0022-1317-70-6-1427
6. Routledge EG, McQuillin J, Samson AC, Toms GL. The development of monoclonal antibodies to respiratory syncytial virus and their use in diagnosis by indirect immunofluorescence. J Med Virol. 1985;15:305–20. DOI: 10.1002/jmv.1890150311
7. Wright C, Oliver KC, Fenwick FI, Smith NM, Toms GL. A monoclonal antibody pool for routine immunohistochemical detection of human respiratory syncytial virus antigens in formalin-fixed, paraffin-embedded tissue. J Pathol. 1997;182:238–44. DOI: 10.1002/(SICI)1096-9896(199706)182:2<238::AID-PATH822>3.0.CO;2-7
8. Horsfall FL, Hahn RG. A latent virus in normal mice capable of producing pneumonia in its natural host. J Exp Med. 1940;71:391–408. DOI: 10.1084/jem.71.3.391
9. Horsfall FL, Curnen EC. Studies on pneumonia virus of mice (PVM): I. Immunological evidence of latent infection with the virus in numerous mammalian species. J Exp Med. 1946;83:43–64. DOI: 10.1084/jem.83.1.43
10. Pringle CR, Eglin RP. Murine pneumonia virus: seroepidemiological evidence of widespread human infection. J Gen Virol. 1986;67:975–82. DOI: 10.1099/0022-1317-67-6-975
11. Cook PM, Eglin RP, Easton AJ. Pathogenesis of pneumovirus infections in mice: detection of pneumonia virus of mice and human respiratory syncytial virus mRNA in lungs of infected mice by in situ hybridization. J Gen Virol. 1998;79:2411–7.
12. Krempl CD, Collins PL. Reevaluation of the virulence of prototypic strain 15 of pneumonia virus of mice. J Virol. 2004;78:13362–5. DOI: 10.1128/JVI.78.23.13362-13365.2004
13. Rosenberg HF, Domachowske JB. Pneumonia virus of mice: severe respiratory infection in a natural host. Immunol Lett. 2008;118:6–12. DOI: 10.1016/j.imlet.2008.03.013

Address for correspondence: Edward J. Dubovi, Animal Health Diagnostic Center, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853, USA; email: ejd5@cornell.edu

Rhinovirus C and Respiratory Exacerbations in Children with Cystic Fibrosis

Marina B. de Almeida, Rodrigo M. Zerbinati, Adriana F. Tateno, Cristina M. Oliveira, Renata M. Romão, Joaquim C. Rodrigues, Cláudio S. Pannuti, and Luiz Vicente F. da Silva Filho

To investigate a possible role for human rhinovirus C in respiratory exacerbations of children with cystic fibrosis, we conducted microbiologic testing on respiratory specimens from 103 such patients in São Paulo, Brazil, during 2006–2007. A significant association was found between the presence of human rhinovirus C and respiratory exacerbations.

Cystic fibrosis (CF) is an autosomal inherited disease characterized by recurrent and chronic respiratory infections that ultimately lead to the need for a lung transplant early in life or to death (*1*). The role of bacterial infections in CF is well established, and most treatments focus on eradication or suppression of bacterial infections (mainly those caused by *Pseudomonas aeruginosa*) (*1*).

Respiratory viruses such as respiratory syncytial virus (RSV) and influenza also seem to cause early damage or increase the risk for respiratory exacerbations (*2*,*3*) in these patients. However, the role of newly described respiratory viruses is not well known. Infection of these patients with human rhinovirus (HRV), a member of the family *Picornaviridae*, has been described. Although some studies have suggested a substantial pathogenic role for these viruses (*3*), controversy still exists (*4*).

Recently a new clade of human rhinovirus, named rhinovirus C (*5*), was identified through molecular methods. This new clade has been found throughout the world (*6*), and some studies have attributed severe respiratory infections in children to this agent (*7*,*8*). We investigated whether this agent played a role in the respiratory exacerbations of children and adolescents with CF who attended the Instituto da Crinaça, Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo.

Author affiliations: Hospital das Clínicas da Faculdade de Medicina de Universidade de São Paulo, São Paulo, Brazil (M.B. de Almeida, J.C. Rodrigues, L.V.F. da Silva Filho); and Universidade de São Paulo, São Paulo (R.M. Zerbinati, A.F. Tateno, C.M. Oliveira, R.M. Romão, C.S. Pannuti, L.V.F. da Silva Filho)

DOI: 10.3201/eid1606.100063

The Study

A total of 103 CF patients (49 girls, 54 boys; median age 8.9 years; age range 3.8 months–17.8 years) were enrolled in the study from September 6, 2006 through September 4, 2007. Nasopharyngeal aspirates or nasal mucus specimens for viral investigation, as well as sputum or oropharyngeal samples for microbiology culture, were collected during scheduled visits or unscheduled visits on 408 occasions, with a median ± SD of 4 ± 1.74 visits per patient (range 1–9 visits).

Clinical and lung function data were obtained at all visits. Exacerbation of respiratory disease was defined as the presence of ≥2 of the following signs or symptoms: fever, increase in the amount of secretion or cough intensity, change in sputum's color, worsening of dyspnea, loss of appetite, a decrease of forced expiratory volume in 1 s ≥10%, and weight loss.

Total nucleic acids were extracted from nasopharyngeal samples by using a QIAmp Viral RNA Mini Kit (QIAGEN, Hamburg, Germany), according to manufacturer's instructions. Reverse transcription was conducted with High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) by using 20 μL of the previously extracted RNA. Respiratory viruses were identified by individual reverse transcription–PCRs or PCRs selective for RSV; influenza viruses A and B; human parainfluenza viruses 1, 2, and 3; human coronavirus; human metapneumovirus; adenovirus; human bocavirus; picornavirus; and the β-actin gene (*9*). For picornavirus, we used the primer pair OL26–OL27, which include a portion of the 5′ noncoding region (NCR) common to all picornaviruses, in the same conditions previously described (*10*). After sequence amplification, products were examined by capillary electrophoresis in an automated DNA sequencer (MegaBace, General Electric Healthcare–Amersham Biosciences, Little Chalfont, UK), and results were visualized through the MegaBace FragmentProfiler software, which discriminates fragment sizes and fluorescent intensities.

Samples in which picornavirus cDNA had been identified were submitted to a TaqMan-based real-time PCR protocol (*11*) to identify HRV and enterovirus. Only samples in which rhinovirus had been identified by real-time PCR were submitted to 5′ NCR sequencing with an ABI 377 automated sequencer (Applied Biosystems), and results were submitted to the GenBank database, accession nos. GU933027–GU933118. Sequencing of the 5′ NCR region has been shown to accurately discriminate among subtypes of rhinovirus, including genotypes C and A2 (*12*). All sequencing chromatograms obtained were edited manually to obtain contiguous fragments (contigs), by using Sequence

Table 1. Respiratory viruses and clinical status of 103 children with cystic fibrosis , Brazil, 2006–2007

Virus	No. samples collected during routine visits, n = 266	No. samples collected during respiratory exacerbations, n = 142	Total no. (%) samples
Rhinovirus	91	48	139 (34.1)
Enterovirus	13	11	24 (5.9)
Human bocavirus	14	9	23 (5.6)
Human coronavirus	13	6	19 (4.7)
Respiratory syncytial virus	7	8	15 (3.7)
Influenza A	1	3	4 (1.0)
Human metapneumovirus	1	2	3 (0.7)
Influenza B	1	0	1 (0.2)
Parainfluenza 1	0	1	1 (0.2)
Parainfluenza 2	0	1	1 (0.2)
Parainfluenza 3	0	1	1 (0.2)
Adenovirus	0	1	1 (0.2)

Navigator software (Applied Biosystems). All sequences were screened at the National Center for Biotechnology Information website by using the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). HRV genotype was confirmed by phylogenetic analysis as described below. Sequences generated and a set of reference strains representative of HRV genotypes, available in the GenBank database, were aligned by using ClustalW (www.ebi.ac.uk/Tools/clustalw2). Minor manual adjustments were made to improve the alignment with BioEdit software (www.mbio.ncsu.edu/BioEdit/BioEdit.html). The sequence of echovirus 11 was used as the outgroup. Phylogenetic analysis was performed with PAUP* version 4b10 (http://paup.csit.fsu.edu). Neighbor-joining and maximum-likelihood trees were constructed on the basis of appropriate nucleotide substitution models determined by Modeltest v3.7 (University of Vigo, Vigo, Spain). Bootstrapping was assessed by using 1,000 replicates. Trees were visualized by using the TreeView program (www.taxonomy.zoology.gla.ac.uk/rod/treeview.html).

To account for correlations among samples from the same patient, we used binomial generalized linear models to identify the virologic variables associated with the main endpoints (respiratory exacerbation and hospital admission). Results were presented as odds ratios (ORs) and 95% confidence intervals.

At least 1 respiratory virus was identified in 203 (49.8%) of 408 samples; rhinovirus was the main identified agent (139 samples, 34.1%). The results of virus identification, in relation to clinical status, are shown in Table 1. Co-infections were found in only 26 samples (6.4%); rhinovirus was the most frequent agent.

Sequencing was performed in 93 of 139 samples that were positive for rhinovirus and showed a predominance of genotype A (36 samples; 38.7%) (Figure 1). Rhinovirus subtypes A2 and C were identified in 14 samples each. Isolates in 3 samples were identified as HRV87, coxsackievirus, and echovirus. Therefore, 5 isolates were enteroviruses, which indicates that the TaqMan–based real-time PCR platform misidentified them. Sequencing was not successful in 1 sample. A maximum-likelihood phylogenetic tree of our samples and reference HRV sequences was constructed (Figure 2).

Patients were examined during acute exacerbation of respiratory disease on 142 occasions, and patients required hospital admission on 31 occasions. The identification of respiratory viruses was not associated with pulmonary exacerbations. Because rhinovirus was the main agent identified among stable patients, we verified the effects of respiratory viruses, excluding rhinovirus from the analysis. A significant association with respiratory exacerbation was found (OR 1.195, p = 0.010) (Table 2).

In contrast, when looking at rhinovirus subtypes, we noticed that identification of rhinovirus subtypes A2 or C was also significantly associated with respiratory exacerbations (OR 1.213) (Table 2). Identification of influenza A was the only variable associated with an increase in the risk for hospital admission (OR 1.988, 95% confidence interval 1.238–3.194, p = 0.004).

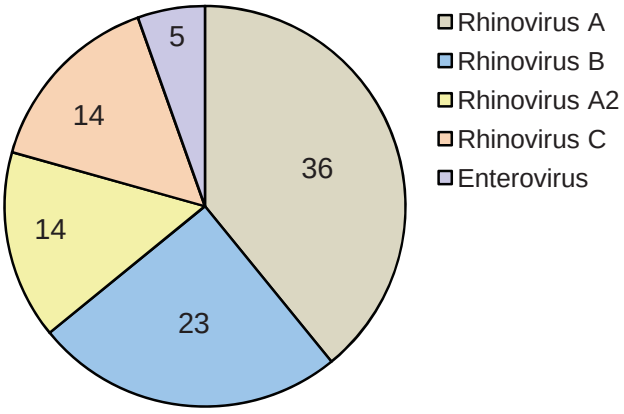


Figure 1. Results of 5′ noncoding region sequencing of 93 samples with identification of human rhinovirus by real-time reverse transcription–PCR, obtained from samples from 103 children with cystic fibrosis, Brazil, 2006–2007.

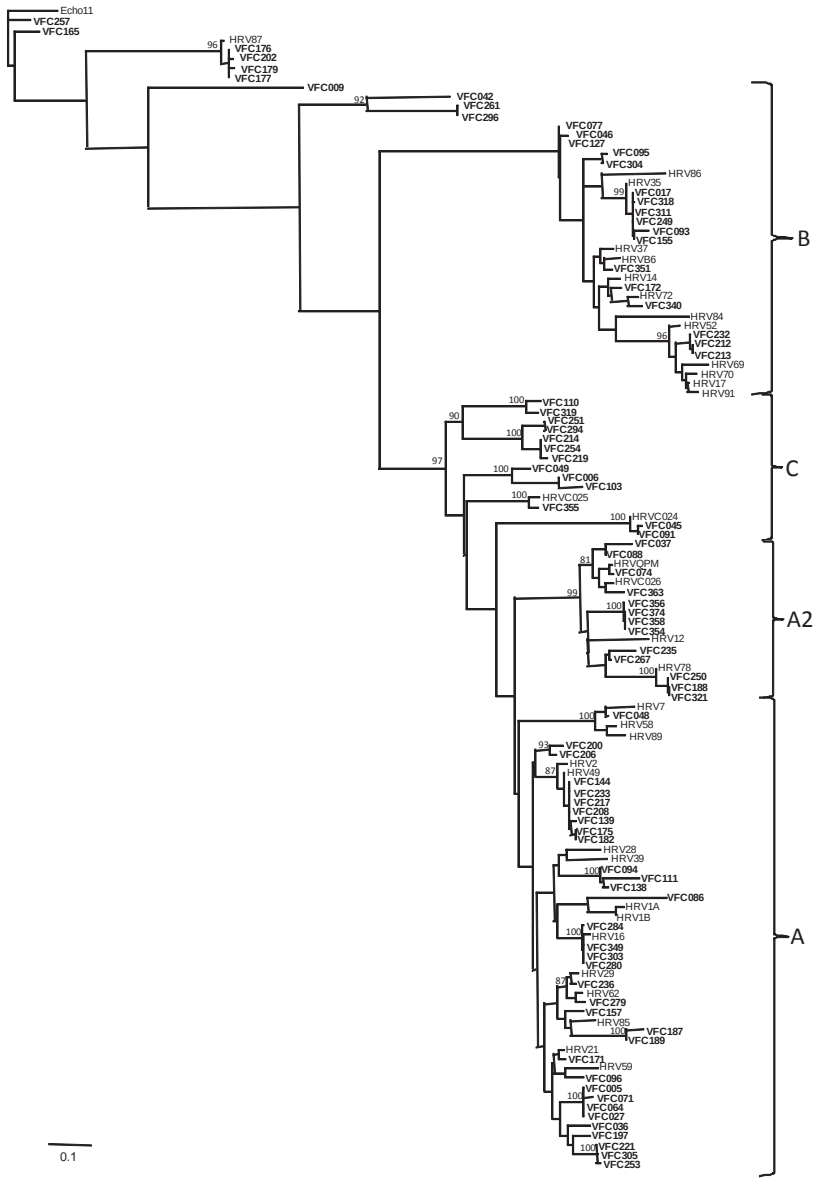


Figure 2. Representative maximum-likelihood phylogenetic tree of a partial 5′ noncoding region of human rhinovirus generated with general time reversible substitution model, including gamma distribution shape parameter. Reference human rhinovirus (HRV) genotypes were obtained from the GenBank database. Echovirus 11 was defined as the outgroup. Virus isolates obtained in this study are indicated by **boldface** and are labeled VFC. Bootstrap values >70% in the key branches are depicted. Scale bar indicates nucleotide substitutions per site.

Conclusions

These new HRV genotypes were initially described in samples from patients with influenza-like illnesses (*13*). Recently, evidence has been increasing for the involvement of HRV-C in severe respiratory conditions such as bronchiolitis in infants (*7*) and in exacerbation of asthma (*14*). However, these studies that attempted to clarify the pathogenicity of these new species did not include control groups or nonsymptomatic persons.

In the study reported here, we obtained samples from patients during routine visits and exacerbations, which enabled us to identify a distinct role of different HRV subtypes. Our findings, however, cannot be extrapolated to infants with CF, which were underrepresented in our study.

Table 2. Virologic test results and respiratory exacerbation outcome for 103 children with cystic fibrosis, Brazil, 2006–2007*

Virus	OR (95% CI)	p value
Any respiratory virus	1.063 (0.979–1.154)	0.144
Rhinovirus	1.020 (0.931–1.117)	0.666
Any respiratory virus except rhinoviruses	1.195 (1.043–1.369)	0.010
Rhinovirus A2 or C (excluding samples not sequenced)	1.213 (1.024–1.436)	0.025

*Estimates from binomial, generalized linear models. OR, odds ratio; CI, confidence interval.

These infants may be at greater risk of HRV-C respiratory infections as they are for with RSV infections (*2*).

Previous studies of respiratory virus infections in CF patients provided conflicting results on the potential effect of rhinovirus. Smyth et al. (*15*), who evaluated respiratory exacerbations in CF patients, identified rhinovirus as the leading agent, with exacerbations following a more benign course but resulting in greater use of intravenous antimicrobial drugs (*15*). Olesen et al. (*4*) studied 75 CF patients for 1 year and used PCR to investigate the presence of 7 different viruses in sputum or laryngeal aspirates. The group reported that viral infections did not reduce lung function or increase respiratory symptoms. Rhinovirus was by far the leading agent identified throughout their study (*4*). A more recent study by Wat et al. (*3*), in which they used real-time nucleic acid sequence–based amplification in conjunction with molecular markers to investigate the presence of 9 respiratory viruses in children with CF, described an association of viral infections with respiratory exacerbations, particularly those caused by influenza A, influenza B, and rhinovirus (*3*).

We report infections by the novel rhinovirus subtypes A2 and C in CF patients. A significant association was found between the presence of these agents and respiratory exacerbations. Our findings indicate the need for further investigation of HRV-C in CF patients.

This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo, grant 05/01625-8.

Dr de Almeida is a pediatric pulmonologist at Instituto da Criança Hospital das Clínicas da Faculdade de Medicina de Universidade de São Paulo and a PhD candidate at the University of São Paulo. Her research interests are primarily related to respiratory diseases in children.

References

1. Ratjen F, Doring G. Cystic fibrosis. Lancet. 2003;361:681–9. DOI: 10.1016/S0140-6736(03)12567-6
2. Armstrong D, Grimwood K, Carlin JB, Carzino R, Hull J, Olinsky A, et al. Severe viral respiratory infections in infants with cystic fibrosis. Pediatr Pulmonol. 1998;26:371–9. DOI: 10.1002/(SICI)1099-0496(199812)26:6<371::AID-PPUL1>3.0.CO;2-N
3. Wat D, Gelder C, Hibbitts S, Cafferty F, Bowler I, Pierrepoint M, et al. The role of respiratory viruses in cystic fibrosis. J Cyst Fibros. 2008;7:320–8. Epub 2008 Feb 6. DOI: 10.1016/j.jcf.2007.12.002
4. Olesen HV, Nielsen LP, Schiotz PO. Viral and atypical bacterial infections in the outpatient pediatric cystic fibrosis clinic. Pediatr Pulmonol. 2006;41:1197–204. DOI: 10.1002/ppul.20517
5. McErlean P, Shackelton LA, Andrews E, Webster DR, Lambert SB, Nissen MD, et al. Distinguishing molecular features and clinical characteristics of a putative new rhinovirus species, human rhinovirus C (HRV C). PLoS One. 2008;3:e1847.
6. Briese T, Renwick N, Venter M, Jarman RG, Ghosh D, Kondgen S, et al. Global distribution of novel rhinovirus genotype. Emerg Infect Dis. 2008;14:944–7. DOI: 10.3201/eid1406.080271
7. Lau SK, Yip CC, Tsoi HW, Lee RA, So LY, Lau YL, et al. Clinical features and complete genome characterization of a distinct human rhinovirus (HRV) genetic cluster, probably representing a previously undetected HRV species, HRV-C, associated with acute respiratory illness in children. J Clin Microbiol. 2007;45:3655–64. DOI: 10.1128/JCM.01254-07
8. Wisdom A, Leitch EC, Gaunt E, Harvala H, Simmonds P. Screening respiratory samples for detection of human rhinoviruses (HRVs) and enteroviruses: comprehensive VP4–VP2 typing reveals high incidence and genetic diversity of HRV species C. J Clin Microbiol. 2009;47:3958–67. DOI: 10.1128/JCM.00993-09
9. da Silva Filho LVF, de Almeida MB, Flores TR, Tomikawa S, Wickbold D, Tateno AF, et al. Clinical and functional impact of acute viral respiratory infections in cystic fibrosis. Pediatr Pulmonol. 2008;43:344.
10. Papadopoulos NG, Sanderson G, Hunter J, Johnston SL. Rhinoviruses replicate effectively at lower airway temperatures. J Med Virol. 1999;58:100–4. DOI: 10.1002/(SICI)1096-9071(199905)58:1<100::AID-JMV16>3.0.CO;2-D
11. Deffernez C, Wunderli W, Thomas Y, Yerly S, Perrin L, Kaiser L. Amplicon sequencing and improved detection of human rhinovirus in respiratory samples. J Clin Microbiol. 2004;42:3212–8. DOI: 10.1128/JCM.42.7.3212-3218.2004
12. Kiang D, Kalra I, Yagi S, Louie JK, Boushey H, Boothby J, et al. Assay for 5′ noncoding region analysis of all human rhinovirus prototype strains. J Clin Microbiol. 2008;46:3736–45. DOI: 10.1128/JCM.00674-08
13. Lamson D, Renwick N, Kapoor V, Liu Z, Palacios G, Ju J, et al. MassTag polymerase-chain-reaction detection of respiratory pathogens, including a new rhinovirus genotype, that caused influenza-like illness in New York State during 2004–2005. J Infect Dis. 2006;194:1398–402. DOI: 10.1086/508551
14. Khetsuriani N, Lu X, Teague WG, Kazerouni N, Anderson LJ, Erdman DD. Novel human rhinoviruses and exacerbation of asthma in children. Emerg Infect Dis. 2008;14:1793–6. DOI: 10.3201/eid1411.080386
15. Smyth AR, Smyth RL, Tong CY, Hart CA, Heaf DP. Effect of respiratory virus infections including rhinovirus on clinical status in cystic fibrosis. Arch Dis Child. 1995;73:117–20. DOI: 10.1136/adc.73.2.117

Address for correspondence: Luiz Vicente Ferreira da Silva Filho, Rua Gregório Paes de Almeida, 1148 Vila Madalena, CEP 05450-001, São Paulo, SP, Brazil; email: vicres@terra.com.br

Xenotropic Murine Leukemia Virus–related Gammaretrovirus in Respiratory Tract

Nicole Fischer, Claudia Schulz, Kristin Stieler, Oliver Hohn, Christoph Lange, Christian Drosten, and Martin Aepfelbacher

Xenotropic murine leukemia virus–related gammaretrovirus (XMRV) has been recently associated with prostate cancer and chronic fatigue syndrome. To identify nucleic acid sequences, we examined respiratory secretions by using PCR. XMRV-specific sequences were detected in 2%–3% of samples from 168 immunocompetent carriers and ≈10% of samples from 161 immunocompromised patients.

Xenotropic murine leukemia virus–related gammaretrovirus (XMRV) was originally discovered in tissue from patients with familial prostate cancer homozygous for a missense mutation in the RNase L gene, R462Q (*1*). Detection of viral nucleic acid in tissue sections of cancerous prostate glands and cloning of the viral integration sites confirmed XMRV as a bona fide human infection with a murine leukemia virus–related retrovirus (*1*). Whether XMRV is actively involved in prostate cancer tumorigenesis or whether it is just a bystander virus (*2*,*3*) remains unclear.

On the basis of its close homology (up to 94% nt identity) to endogenous and exogenous full-length sequences from *Mus musculus* mice (*1*), XMRV most likely originated in mice, although they are probably not the current reservoir of infection (*4*). Recent findings of XMRV sequences in up to 67% of peripheral blood mononuclear cells (PBMCs) of patients with chronic fatigue syndrome and in 3.4% of PBMCs of healthy controls raise the question whether XMRV could be a blood-borne pathogen (*5*). However, the finding of XMRV in PBMCs from patients with chronic fatigue syndrome is controversial because multiple studies in Europe have failed to detect XMRV (*6*–*8*). Similarly, frequency of XMRV in prostate cancer samples ranges from 0 to 23%, depending on geographic restriction of the virus or, more likely, diagnostic techniques used (PCR, quantitative PCR, immunohistochemistry) (*1*–*3*,*9*,*10*). Indirect evidence has suggested sexual transmission (*9*). Questions remain about worldwide distribution, host range, transmission routes, and organ tropism of the virus. To begin to answer some of them, we looked for XMRV in respiratory samples from 267 patients with respiratory tract infection (RTI) and 62 healthy persons.

Author affiliations: University Medical Center Hamburg-Eppendorf, Hamburg, Germany (N. Fischer, C. Schulz, K. Stieler, M. Aepfelbacher); Robert Koch-Institute, Berlin, Germany (O. Hohn); Leibniz-Center for Medicine and Biosciences, Borstel, Germany (C. Lange); and University of Bonn Medical Centre, Bonn, Germany (C. Drosten)

DOI: 10.3201/eid1606.100066

The Study

During 2006–2009, the 267 samples were collected from 3 groups of patients (Table). Group 1 comprised patients who had traveled from Asia to Germany; location of their permanent residency was unknown. Groups 2 and 3 and the control group comprised only persons from northern Germany. From group 1, a total of 75 sputum and nasal swab specimens were collected from patients who had unconnected cases of RTI and who had recently traveled by air (*11*). From group 2, a total of 31 bronchoalveolar lavage (BAL) samples were collected from patients with chronic obstructive pulmonary disease (defined by a forced expiratory volume in 1 second/forced vital capacity <70% and forced expiratory volume in 1 second <80% of the predicted value) who had signs of RTI. From group 3, a total of 161 BAL and tracheal secretion samples were collected from patients with severe RTI and immunosuppression as a result of solid organ or bone marrow transplantation. From the control group, throat swabs were collected from 52 healthy persons and BAL samples were collected from 10 healthy volunteers who had no signs of RTI and no known underlying disease.

All samples were analyzed by culture for pathogenic bacteria and fungi and by PCR for rhinoviruses, adenoviruses, enteroviruses, influenza viruses A and B, parainfluenza viruses 1–3, respiratory syncytial virus, cytomegalovirus, Epstein-Barr virus, and human metapneumovirus. All samples were tested in duplicates obtained by individual RNA extractions. XMRV RNA was reverse transcribed from total RNA, after which nested PCR or real-time PCR were conducted as recently described (*1*,*12*). No serum samples were available from these patients to confirm the results by serologic testing.

For group 1, XMRV-specific sequences were detected with relatively low frequency (2.3%). For group 2, XMRV-specific sequences were amplified in 1 BAL sample, which was also positive for *Staphylococcus aureus* by routine culture methods. For group 3, XMRV-specific sequences were detected at a frequency of 9.9%, which was significantly higher than that for the healthy control group (3.2%) at the 90% confidence level but not at the 95% level ($p = 0.078$, 1 sample *t*-test). Of 16 group

Table. Detection of XMRV in respiratory tract secretions from 329 persons*

Group	Patient median age, y	Underlying disease	Sample	XMRV+
1 (75 patients with RTI)	42	None	Sputum, nasal swab	3/75 (2.3%)
2 (31 patients with RTI)	60	COPD	BAL	1/31 (3.2%)
3 (161 patients with RTI)	32	Immunosuppression after SOT or BMT	BAL, TS	16/161 (9.9%)
Control (62 persons with no RTI)	35	None	BAL, throat swab	2/62 (3.2%)

*XMRV, xenotropic murine leukemia virus; +, positive for XMRV–specific sequences by PCR; RTI, respiratory tract infection; COPD, chronic obstructive pulmonary disease; BAL, bronchoalveolar lavage; SOT, solid organ transplantation; BMT, bone marrow transplantation; TS, tracheal secretion.

3 samples, 10 showed no signs of co-infection. The remaining 6 samples showed co-infection with rhinovirus or adenovirus (1 sample each); *S. aureus* (3 samples); or mixed infection with pathogenic fungi, *Candida albicans* and *Asperigillus fumigatus* (1 sample).

All samples that were positive for XMRV by gag-nested PCR, together with a set of those that were negative for XMRV, were retested by real-time PCR. Results showed low XMRV RNA concentrations, 10^3 –10^4/mL of specimen.

To confirm the validity of XMRV detection, a subset of 6 specimens (3 XMRV positive and 3 XMRV negative) were tested by using an alternative PCR assay for viral RNA (*3*) and a C-Type RT Activity Kit (Cavidi, Uppsala, Sweden) for type C reverse-transcription activity. XMRV sequences from alternative targets in the gag and env regions were confirmed in 2 of the 3 XMRV-positive samples but in none of the controls. One XMRV-positive BAL specimen showed an 8-fold increase above background of specific type C retroviral reverse-transcriptase activity, suggesting presence of active type C retrovirus within this sample. This assay is substantially less sensitive than reverse transcription–PCR.

All XMRV gag sequences (390-bp fragment) were 98%–99% identical to previously published XMRV sequences from persons with prostate cancer (*1,2*). Phylogenetic analysis showed close clustering (Figure).

Conclusions

XMRV, originally identified in RNase L–deficient patients with familial prostate cancer, has gained interest since recent work showed its protein expression in as many as 23% of prostate cancer cases (*10*) and XMRV-specific sequences were detected in PBMCs of 67% patients with chronic fatigue syndrome (*5*). These results, however, could not be confirmed by others (*6–8*). Both studies also detected XMRV protein or sequences in their control cohorts with frequencies of 6% and 4%, respectively.

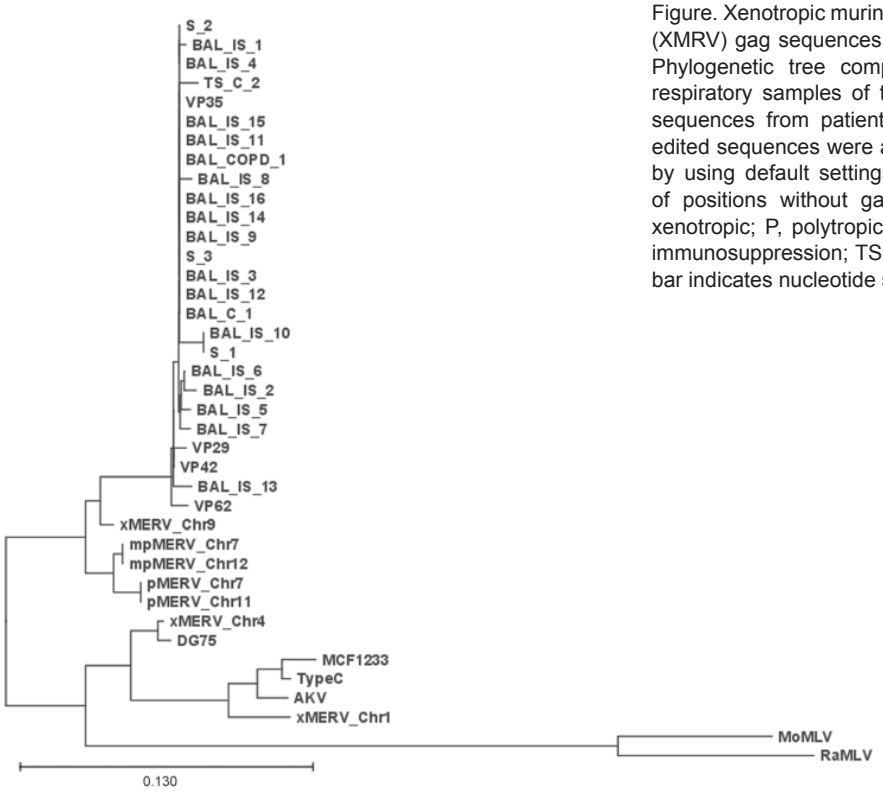


Figure. Xenotropic murine leukemia virus–related gammaretrovirus (XMRV) gag sequences derived from respiratory tract secretions. Phylogenetic tree comparing the 390-nt gag fragment of all respiratory samples of this study with recently published XMRV sequences from patients with familial prostate cancer (*1*). The edited sequences were aligned with ClustalX version 1.82 (*13,14*) by using default settings. The tree was generated on the basis of positions without gaps only. Sequences are labeled as X, xenotropic; P, polytropic; mP, modified polytropic; S, sputum, IS, immunosuppression; TS, tracheal secretion; and C, control. Scale bar indicates nucleotide substitutions per position.

Among the most pressing information gaps with regard to XMRV is its preferred route of transmission. Detection of XMRV in PBMCs and plasma of patients with chronic fatigue syndrome raises the possibility of blood-borne transmission; sexual transmission has also been hypothesized on the basis of indirect evidence (*5*,*9*). We detected XMRV in respiratory secretions of immunocompetent patients with and without RTI at a frequency of ≈3.2%, which is in good concordance with the recently reported prevalence in the general population of up to 4% (*5*). Frequency of XMRV detection in group 1 patients (2.25%) was comparable to that of human metapneumovirus and rhinovirus within this group and considerably less frequent than that of parainfluenzavirus (15.5%) or influenza A virus (7.6%) detection (*11*).

Our findings indicate that XMRV or virus-infected cells might be carried in and transmitted by the respiratory tract. Attempts to isolate infectious virus from XMRV sequence–positive respiratory samples failed, possibly because of inadequate storage of samples before virus culturing attempts or relatively low copy numbers of the virus within the samples. Thus, whether the respiratory tract serves as a putative transmission route for XMRV cannot be determined at this time. The observed increase in prevalence among immunosuppressed patients with RTI suggests that XMRV might be reactivated in absence of an efficient antiviral defense. Together with earlier observations on increased XMRV replication in RNase L–deficient cells (*1*,*12*), this finding implies that the immune system plays a role in controlling XMRV replication. It remains unknown whether immunosuppression predisposes a patient to secrete infectious XMRV from the respiratory tract or whether presence of virus might be meaningless for epidemiology in a way similar to HIV-1 (*15*). Future studies should address whether the respiratory tract might serve as a source of XMRV infection or whether immunosuppression might cause an increased risk for primary infection.

This study was supported by the Werner Otto Stiftung grant no. 4/69 to N.F. The study was approved by the ethics committee at the board of physicians of the Free and Hanseatic City of Hamburg (No.WF-005/09).

Dr Fischer works as a group leader at the Institute for Medical Microbiology and Virology at the University Medical Center Hamburg-Eppendorf. Her main research interests are emerging viruses, in particular the gammaretrovirus XMRV.

References

1. Urisman A, Molinaro RJ, Fischer N, Plummer SJ, Casey G, Klein EA, et al. Identification of a novel gammaretrovirus in prostate tumors of patients homozygous for R462Q RNASEL variant. PLoS Pathog. 2006;2:e25. DOI: 10.1371/journal.ppat.0020025
2. Fischer N, Hellwinkel O, Schulz C, Chun FK, Huland H, Aepfelbacher M, et al. Prevalence of human gammaretrovirus XMRV in sporadic prostate cancer. J Clin Virol. 2008;43:277–83. DOI: 10.1016/j.jcv.2008.04.016
3. Hohn O, Krause H, Barbarotto P, Niederstadt L, Beimforde N, Denner J, et al. Lack of evidence for xenotropic murine leukemia virus–related virus (XMRV) in German prostate cancer patients. Retrovirology. 2009;6:92. DOI: 10.1186/1742-4690-6-65
4. Stieler KSC, Lavanya M, Aepfelbacher M, Stocking C, Fischer N. Host range and cellular tropism of the human exogenous gammaretrovirus XMRV. Virology, 2010:399:23–30.
5. Lombardi VC, Ruscetti FW, Das Gupta J, Pfost MA, Hagen KS, Peterson DL et al. Detection of an infectious retrovirus, XMRV, in blood cells of patients with chronic fatigue syndrome. Science, 2009;326:530–1.
6. Erlwein O, Kaye S, McClure MO, Weber J, Wills G, Collier D, et al. Failure to detect the novel retrovirus XMRV in chronic fatigue syndrome. PLoS One. 2010;5:e8519.
7. Groom HC, Boucherit VC, Makinson K, Randal E, Baptista S, Hagan S, et al. Absence of xenotropic murine leukaemia virus–related virus in UK patients with chronic fatigue syndrome. Retrovirology. 2010;7:10.
8. van Kuppeveld FJ, de Jong AS, Lanke KH, Verhaegh GW, Melchers WJ, Swanink CM, et al. Prevalence of xenotropic murine leukaemia virus–related virus in patients with chronic fatigue syndrome in the Netherlands: retrospective analysis of samples from an established cohort. BMJ. 2010;340:c1018.
9. Hong S, Klein EA, Das Gupta J, Hanke K, Weight CJ, Nguyen C,et al. Fibrils of prostatic acid phosphatase fragments boost infections with XMRV (xenotropic murine leukemia virus–related virus), a human retrovirus associated with prostate cancer. J Virol. 2009;83:6995–7003. DOI: 10.1128/JVI.00268-09
10. Schlaberg R, Choe D, Brown KR, Thaker HM, Singh IR. XMRV is present in malignant prostatic epithelium and is associated with prostate cancer, especially high-grade tumors. Proc Natl Acad Sci U S A. 2009;106:16351–6. DOI: 10.1073/pnas.0906922106
11. Luna LK, Panning M, Grywna K, Pfefferle S, Drosten C. Spectrum of viruses and atypical bacteria in intercontinental air travelers with symptoms of acute respiratory infection. J Infect Dis. 2007;195:675–9. DOI: 10.1086/511432
12. Dong B, , Kim S, Hong S, Das Gupta J, Malathi K, Klein EA, et al. An infectious retrovirus susceptible to an IFN antiviral pathway from human prostate tumors. Proc Natl Acad Sci U S A. 2007;104:1655–60. DOI: 10.1073/pnas.0610291104
13. Jeanmougin F, Thompson JD, Gouy M, Higgins DG, Gibson TJ. Multiple sequence alignment with Clustal X. Trends Biochem Sci. 1998;23:403–5. DOI: 10.1016/S0968-0004(98)01285-7
14. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 1997;25:4876–82. DOI: 10.1093/nar/25.24.4876
15. Wood KL, Chaiyarit P, Day RB, Wang Y, Schnizlein-Bick CT, Gregory RL et al. Measurements of HIV viral loads from different levels of the respiratory tract. Chest. 2003;124:536–42. DOI: 10.1378/chest.124.2.536

Address for correspondence: Nicole Fischer, Institute for Medical Microbiology and Virology, University Medical Center Hamburg-Eppendorf, Martinistrasse 52, 20246 Hamburg, Germany; email: nfischer@uke.de

Variations in Human Herpesvirus Type 8 Seroprevalence in Native Americans, South America

Vanda A.U.F. Souza, Francisco M. Salzano, Maria Luiza Petzl-Erler, Maria Claudia Nascimento, Philippe Mayaud, Jaila Dias Borges, and Claudio S. Pannuti

To determine the epidemiology of human herpesvirus type 8 (HHV-8) among non-Amazonian native populations, we conducted a cross-sectional study in Brazil, Bolivia, and Paraquay. Our data show striking ethnic and geographic variations in the distribution of HHV-8 seroprevalences in Amazonian (77%) and non-Amazonian native populations (range 0%–83%).

Human herpesvirus type 8 (HHV-8) is the etiologic agent of all forms of Kaposi sarcoma, primary effusion lymphoma, and certain lymphoproliferative diseases. HHV-8 seroprevalence is low (<5%) in northern Europe and North America, where HIV-seropositive homosexual men represent the highest risk group. HHV-8 infection is endemic in eastern and central Africa, with seroprevalences >50% in some adult populations (*1*). However, the highest HHV-8 seroprevalences worldwide (>80% in adults) have been reported in Native Americans from the Amazon region of Brazil (*2*–*5*), French Guiana (*6*), and Ecuador (*7*). In HHV-8–endemic areas in Africa and the Amazon, the sharp and linear increases of HHV-8 seroprevalence in children before puberty, with only modest increases later in life (*3*,*5*), and the association of HHV-8 seropositivity in children with having at least 1 first-degree relative who is seropositive (*8*) suggest nonsexual transmission of HHV-8 within families, probably through saliva (*9*). Indeed, this hypothesis is supported by the finding that, in Native Americans, nearly one fourth of HHV-8–seropositive persons, and ≤40% of children, shed HHV-8 DNA in their saliva (*5*). A particular feature of the epidemiology of HHV-8 in Native Americans is that infection is caused by a recently discovered distinct and unique HHV-8 strain (subtype E) (*2*,*5*,*7*). Previous research has also shown that HHV-8 seroprevalence is 10-fold lower in nonnative populations living in similar conditions in adjacent remote geographic areas, which suggests that HHV-8 infection in Native Americans may be associated with specific risk factors or behaviors but not with environmental factors (*5*). Little is known, however, of the epidemiology of HHV-8 among other Native American populations. We conducted a cross-sectional study to investigate whether HHV-8 was also endemic among Native American populations living outside Amazonia.

Author affiliations: Universidade de São Paulo, São Paulo, Brazil (V.A.U.F. Souza, C.S. Pannuti, M.C. Nascimento, J.D. Borges); Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil (F.M. Salzano); Universidade Federal do Paraná, Curitiba, Brazil (M.L. Petzl-Erler); and London School of Hygiene and Tropical Medicine, London, UK (M.C. Nascimento, P. Mayaud).

DOI: 10.3201/eid1606.090961

The Study

Serum samples from the Wai Wai, a Native American tribe living in the Mapuera village in the area of the Trombetas, a tributary of the Amazon River, in Pará State, Brazil, were collected during May–June 2007. In addition, we obtained archived serum samples (collected during studies conducted during1972–1988 and frozen at –20°C) from 5 non-Amazonian Native American populations (*10*–*13*). The following non-Amazonian ethnic groups were evaluated: 1) the Xikrin, Gorotire, and Kuben-Kran-Kegn, who live in a transition region between the Amazonian forest and the savannah of central Brazil; 2) the Krahó, from a savannah region of central Brazil; 3) the Ayoreo, from 3 different localities of the Chaco region of Bolivia and Paraguay; 4) the Kaingang, from Ivaí and Rio das Cobras in southern Brazil; and 5) the Guarani, from Rio das Cobras, in southern Brazil (Figure). The genetic and serologic studies were approved by the Brazilian National Ethics Commission (CONEP Resolutions. 123/98 and 005/2003).

Research aims and methods of the previous studies were diverse, but all enrolled participants (range 1–70 years of age) were enlisted without any previous selection from those who agreed to voluntarily participate in the study and convened at a specific place in the village to provide serum samples. Informed consent procedures were done individually, mostly verbally, with the assistance of local indigenous health agents. Consent for children <15 years of age was given by a parent or guardian (*10*–*13*). Antibodies to HHV-8 latency-associated nuclear (LANA) and lytic antigens were determined by immunofluorescence assays (IFA) at a 1:40 starting dilution, using the body cavity–based lymphoma (BCBL) 1 cell line. Punctuate nuclear staining in noninduced BCBL-1 cells was considered positive for LANA antibodies. The viral lytic cycle was induced by incubating BCBL-1 cells with 20 ng/mL of 12-O-tetradecanoylphorbol-13–acetate (TPA) (Sigma, St. Louis, MO, USA) for 96 hours. Entire cell fluorescence in ≈20% of TPA-treated cells was considered positive for antibodies to the lytic-phase antigens (*5*). Overall HHV-8 seroposi-

Figure. Locations and respective human herpesvirus type 8 seroprevalence rates (%) of Native American populations studied, South America.

tivity was defined as positivity by any of these IFAs, but results are also presented by assay type. Chi-square statistics were used to estimate the associations between HHV-8 seropositivity and sex and age groups.

The prevalence of HHV-8 antibodies by any assay ranged from 0% to 83% in the various groups (Table 1). HHV-8 seroprevalence did not differ between men and boys (222/350, 63%) and women and girls (265/410, 64%) ($p = 0.7$). We found an overall significant trend for increasing prevalence with age (84/150, 56%) in children 0–12 years of age, 63% (282/450) in young adults 13–40 years of age, and 76% (121/160) in adults >40 years (p trend<0.001), although these results mostly reflected age seroprevalence patterns in the largest group (Wai Wai). The highest seroprevalences were observed in the populations living in the Amazon (77%) and in the transitional geographic area between the Amazon forest and the savannah of central Brazil (50%–83%). The Ayoreo from Bolivia and Paraguay had an intermediate seroprevalence (18%–45%), and the lowest seroprevalence was observed among Native Americans living in southern Brazil (Guarani and Caingang, 0%–14%), following a north-south gradient.

Conclusions

Our data show striking geographic and ethnic variations in the distribution of HHV-8 seroprevalences among South American native populations. Previous reports have shown that most Amazonian Native American groups have high HHV-8 seroprevalence, but substantial between-group and sometimes within-group differences have been observed in previous studies (Table 2). These differences could be explained, to some extent, by the different techniques that have been used to detect HHV-8 antibodies. No clearly defined acceptable standard test exists to determine HHV-8 seropositivity, and different assays have shown different levels of accuracy, including intra-assay variations when the same test is performed in different laboratories (*14*). Therefore, comparing HHV-8 seroprevalence in different settings has limitations. In this study, the same assays (anti-LANA and anti-lytic IFAs) were performed on all samples from all populations in 1 laboratory, and ma-

Table 1. Prevalence of HHV-8 antibodies in Amazonian and non-Amazonian Native American populations, by assay type*

Population, locality†	No. persons tested	No. (%) persons HHV-8 seropositive			
		By IFA-LANA or IFA-lytic	By IFA-LANA	By IFA-lytic	By IFA-LANA and IFA-lytic
Wai-Wai, Mapuera, Brazil	530	397 (75)	372 (70)	204 (38)	177 (33)
Xikrin, Caeteté, Brazil	30	15 (50)	13 (43)	6 (20)	4 (13)
Gorotire, PI Gorotire, Brazil	30	20 (67)	20 (67)	7 (23)	7 (23)
Kuben-Kran-Kegn, Nilo Peçanha, Brazil	30	25 (83)	25 (83)	4 (13)	4 (13)
Krahó, Cachoeira, Brazil	20	12 (60)	10 (50)	10 (50)	7 (35)
Ayoreo,Tobité, Bolivia	18	5 (28)	5 (28)	2 (11)	2 (11)
Ayoreo, Pozo Verde, Bolivia	17	3 (18)	2 (12)	1 (6)	0 (0)
Ayoreo, M Auxiliadora, Paraguay	20	9 (45)	9 (45)	7 (35)	7 (35)
Kaingang, Ivaí, Brazil	30	0	0	0	0
Kaingang, Rio das Cobras, Brazil	26	0	0	0	0
Guarani, Rio das Cobras, Brazil	29	4 (14)	4 (14)	2 (7)	2 (7)

*HHV-8, human herpesvirus type 8; IFA, immunofluorescence assay; LANA, latency-associated nuclear antigen.
†Listed in a north–south gradient (Figure).

Table 2. Previous human herpesvirus type 8 seroprevalence studies in Amazonian Native American populations*

Population†	No. positive/no. tested (%)	HHV-8 assay used‡	Reference
Wai Wai, Brazil	275/339 (81)	IFA-LANA, IFA-Lytic	(*5*)
Zoé, Brazil	18/49 (37)	EIA-ORF59/0RF65/K8.1	(*4*)
Emerillon, French Guiana	12/78 (15)	IFA-Lytic (ABI test)	(*6*)
Palikur, French Guiana	22/167 (13)	IFA-Lytic (ABI test)	(*6*)
Wayana, French Guiana	41/144 (28)	IFA-Lytic (ABI test)	(*6*)
wayampi, French Guiana	93/302 (31)	IFA-Lytic (ABI test)	(*6*)
Wayampi, Brazil	85/123 (69)	IFA-LANA, IFA-Lytic	(*3*)
Wayampi, Brazil	28/33 (85)	IFA-LANA, EIA w-virus	(*2*)
Tiriyó, Brazil	381/664 (57)	IFA-LANA, IFA-Lytic	(*3*)
Tiriyó, Brazil	30/35 (86)	IFA-LANA, EIA w-virus	(*2*)
Tiriyó, Brazil	24/56 (43)	EIA-ORF59/0RF65/K8.1	(*4*)
Huaroni, Ecuador	38/38 (100)	IFA-LANA, EIA-K8.1, EIA-ORF73	(*7*)
Siona, Ecuador	10/41 (24)	IFA-LANA, EIA-K8.1, EIA-ORF73	(*7*)
Apalaí, Brazil	8/44 (18)	IFA-LANA, EIA w-virus	(*2*)
Arara-Laranja, Brazil	18/92 (20)	EIA-ORF59/0RF65/K8.1	(*4*)
Arara-Laranjal, Brazil	0/11 (0)	IFA-LANA, EIA w-virus	(*2*)
Kararao, Brazil	6/24 (25)	EIA-ORF59/0RF65/K8.1	(*4*)
Asurini-Koatinemo, Brazil	7/10 (70)	IFA-LANA, EIA w-virus	(*2*)
Munduruku, Brazil	36/40 (90)	IFA-LANA, EIA w-virus	(*2*)
Araweté, Brazil	12/17 (71)	IFA-LANA, EIA w-virus	(*2*)
Parakanã, Brazil	27/30 (90)	IFA-LANA, EIA w-virus	(*2*)
Xikrin, Brazil	20/34 (59)	IFA-LANA, EIA w-virus	(*2*)
Karitiana, Brazil	12/25 (48)	IFA-LANA, EIA w-virus	(*2*)
Suruí, Brazil	15/17 (88)	IFA-LANA, EIA w-virus	(*2*)
Mekranoti, Brazil	34/52 (65)	IFA-LANA, EIA w-virus	(*2*)
Cinta Larga, Brazil	1/14 (7)	IFA-LANA, EIA w-virus	(*2*)

*ABI, Advanced Biotechnologies Inc (Columbia, MD, USA); EIA, enzyme immunoassay; IFA, immunofluorescence assay; LANA, latency-associated nuclear antigen; ORF, open reading frame; w-virus, whole-virus assay.
†Listed in north-south gradient (Figure).
‡Only series with ≥10 persons are presented.

jor variations in the distribution of HHV-8 seroprevalence were observed. The low HHV-8 seroprevalence among the Guarani (14%) and its absence among 56 Kaingang persons, both populations living in southern Brazil, contrasts with the high seroprevalences in populations living in central Brazil and Amazonia.

Although this study lacks the power to assess statistically significant differences in HHV-8 seroprevalences by geographic region, the variation in the distribution of HHV-8 is intriguing. Current consensus suggests that the Bering Strait land bridge was prehistorically the only entry point to the American landmass and that human migration occurred not earlier than 15,000 years ago, using the Pacific Coast route, with subsequent spread to the Amazon and the rest of South America. The presence of the Guarani and Kaingang at their present locations is thought to have occurred comparatively recently, ≈1,000–2,000 years ago (*15*). This history suggests that the differences in HHV-8 seroprevalence between these groups and others from the Amazon may be caused by more recent events.

Phylogenetic analysis of HHV-8 strains obtained from Native Americans living in different regions and from different groups could contribute to establishing the period when HHV-8 was introduced in Amazonian and non-Amazonian native populations. However, the use of archived serum samples in the present series precluded the exploration of genetic characteristics of HHV-8 strains in the non-Amazonian groups. Another limitation concerns the recruitment of study participants; thus, selection bias cannot be excluded. As previously reported (*5*,*7*), and, in contrast to other populations, all Native American groups exhibited a higher prevalence of antibodies against LANA than against lytic antigen, although the significance of this finding cannot be determined in this study. Larger prospective population-based studies and detailed historical, epidemiologic, and genetic investigations are needed to explore the substantial epidemiologic differences in HHV-8 infection found between Amazonian and non-Amazonian Native American populations.

Acknowledgments

For assistance in arranging for blood donations by tribal members and/or help in the field work, we thank the following colleagues: Shinai Arriel Gregório, Fernando Pagés, Ricardo Moreno, Andres A. Pérez Diez, James V. Neel, and Francis L. Black.

Financial support was received from Conselho Nacional de Desenvolvimento Científico e Tecnológico–CNPq (contract 305258/2006-0) and Wellcome Trust (contract 075454/8/04/2).

Dr Souza is an assistant professor at the University of São Paulo Medical School in the Department of Infectious Diseases and Parasites and a research virologist at the São Paulo Institute of Tropical Medicine, University of São Paulo. Her work involves the epidemiologic and virologic aspects of human viral diseases.

References

1. Boshoff C, Weiss RA. Epidemiology and pathogenesis of Kaposi's sarcoma–associated herpesvirus. Philos Trans R Soc Lond B Biol Sci. 2001;356:517–34. DOI: 10.1098/rstb.2000.0778
2. Biggar RJ, Whitby D, Marshall V, Linhares AC, Black F. Human herpesvirus 8 in Brazilian Amerindians: a hyperendemic population with a new subtype. J Infect Dis. 2000;181:1562–8. DOI: 10.1086/315456
3. Cunha AM, Caterino-de-Araujo A, Costa SC, Santos-Fortuna E, Boa-Sorte NC, Goncalves MS, et al. Increasing seroprevalence of human herpesvirus 8 (HHV-8) with age confirms HHV-8 endemicity in Amazon Amerindians from Brazil. J Gen Virol. 2005;86:2433–7. DOI: 10.1099/vir.0.81087-0
4. Ishak Mde O, Martins RN, Machado PRL, Souza LBB, Machado LFA, Azevedo VN, et al. High diversity of HHV-8 molecular subtypes in the Amazon region of Brazil: evidence of an ancient human infection. J Med Virol. 2007;79:1537–44. DOI: 10.1002/jmv.20995
5. de Souza VA, Sumita LM, Nascimento MC, Oliveira J, Mascheretti M, Quiroga M, et al. Human herpesvirus-8 infection and oral shedding in Amerindian and non-Amerindian populations in the Brazilian Amazon region. J Infect Dis. 2007;196:844–52. DOI: 10.1086/520549
6. Kazanji M, Dussart P, Duprez R, Tortevoye P, Pouliquen J-F, Vandekerkhove J, et al. Serological and molecular evidence that human herpesvirus 8 is endemic among Amerindians in French Guiana. J Infect Dis. 2005;192:1525–9. DOI: 10.1086/491744
7. Whitby D, Marshall VA, Bagni RK, Wang CD, Gamache CJ, Guzman JR, et al. Genotypic characterization of Kaposi's sarcoma-associated herpesvirus in asymptomatic infected subjects from isolated populations. J Gen Virol. 2004;85:155–63. DOI: 10.1099/vir.0.19465-0
8. Plancoulaine S, Abel L, van Beveren M, Tregouet DA, Joubert M, Tortevoye P, et al. Human herpesvirus 8 transmission from mother to child and between siblings in an endemic population. Lancet. 2000;356:1062–5. DOI: 10.1016/S0140-6736(00)02729-X
9. Dedicoat M, Newton R, Alkharsah KR, Sheldon J, Szabados I, Ndlovu B, et al. Mother-to-child transmission of human herpesvirus-8 in South Africa. J Infect Dis. 2004;190:1068–75. DOI: 10.1086/423326
10. Salzano FM, Gershowitz H, Junqueira PC, Woodal JP, Black FL, Hierholzer W. Blood groups and H-Le[a] salivary secretion of Brazilian Cayapo Indians. Am J Phys Anthropol. 1972;36:417–25. DOI: 10.1002/ajpa.1330360313
11. Salzano FM, Neel JV, Gershowitz H, Migliazza EC. Intra and intertribal genetic variation within a linguistic group: the Ge-speaking Indians of Brazil. Am J Phys Anthropol. 1977;47:337–47. DOI: 10.1002/ajpa.1330470214
12. Tsuneto LT, Probst CM, Hutz MH, Salzano FM, Rodriguez-Delfin LA, Zago MA, et al. HLA class II diversity in seven Amerindian populations. Clues about the origins of the Aché. Tissue Antigens. 2003;62:512–26. DOI: 10.1046/j.1399-0039.2003.00139.x
13. Dornelles CL, Battilana J, Fagundes NJR, Freitas LB, Bonatto SL, Salzano FM. Mitochondrial DNA and Alu insertions in a genetically peculiar population: the Ayoreo Indians of Bolivia and Paraguay. Am J Hum Biol. 2004;16:479–88. DOI: 10.1002/ajhb.20038
14. Nascimento MC, Souza VA, Sumita LM, Freire W, Munoz F, Kim J, et al. Comparative study of Kaposi's sarcoma-associated herpesvirus serological assays using clinically and serologically defined reference standards and latent class analysis. J Clin Microbiol. 2007;45:715–20. DOI: 10.1128/JCM.01264-06
15. Marrero AR, Silva-Junior WA, Bravi CM, Hutz MH, Petzl-Erler ML, Ruiz-Linares A, et al. Demographic and evolutionary trajectories of the Guarani and Kaingang natives of Brazil. Am J Phys Anthropol. 2007;132:301–10. DOI: 10.1002/ajpa.20515

Address for correspondence: Claudio S. Pannuti, Laboratório de Virologia–Instituto de Medicina Tropical de São Paulo, Universidade de São Paulo, Av Eneas de Carvalho Aguiar, 470 – CEP 05403-00, São Paulo, SP, Brazil; email: cpannuti@usp.br

Genetic Evidence for a Tacaribe Serocomplex Virus, Mexico

Catherine C. Inizan, Maria N. B. Cajimat, Mary Louise Milazzo, Artemio Barragán-Gomez, Robert D. Bradley, and Charles F. Fulhorst

We isolated arenavirus RNA from white-toothed woodrats (*Neotoma leucodon*) captured in a region of Mexico in which woodrats are food for humans. Analyses of nucleotide and amino acid sequence data indicated that the woodrats were infected with a novel Tacaribe serocomplex virus, proposed name Real de Catorce virus.

The Tacaribe serocomplex (family *Arenaviridae*, genus *Arenavirus*) comprises 7 North American viruses (Table 1) and 15 South American viruses (*9*). The South American viruses include Guanarito virus, 4 other agents of hemorrhagic fever, and Pirital virus. Members of the rodent family *Cricetidae* are the principal hosts of the Tacaribe serocomplex viruses for which natural host relationships have been well characterized. For example, the southern plains woodrat (*Neotoma micropus*) in southern Texas is the principal host of Catarina virus (*4*), and the hispid cotton rat (*Sigmodon hispidus*) in southern Florida is the principal host of Tamiami virus (*6*).

A recent study found antibody to a Tacaribe serocomplex virus in white-toothed woodrats (*N. leucodon*) captured in Mexico (M.L. Milazzo, unpub. data). We report the determination of the nucleotide sequence of a 3352-nt fragment of the small (S) genomic segment of arenavirus AV H0030026 from RNA isolated from a white-toothed woodrat captured in 2005 in northeastern Mexico.

The Study

Rodents were captured in a rural area 22.8 km north of the town of Real de Catorce in the municipality of Catorce, San Luis Potosí, Mexico (Figure 1). A total of 400 live-capture traps were set each night on 2 consecutive nights in the first week of August 2005. Each rodent caught was assigned a unique identification number. Samples of whole blood, samples of kidney and other solid tissues, and the skins and skeletons of the rodents were deposited into the Museum of Texas Tech University.

The blood samples were tested by ELISA (*10*) for antibody (immunoglobulin [Ig] G) to Whitewater Arroyo virus strain AV 9310135 (*7*). Samples of spleen and kidney from white-toothed woodrats TK133448 and TK133451, 7 other white-toothed woodrats, 2 antibody-positive Nelson's pocket mice (*Chaetodipus nelsoni*), and an antibody-positive Merriam's kangaroo rat (*Dipodomys merriami*) were tested for arenavirus by cultivation in monolayers of Vero E6 cells (*11*). Samples of kidney from the antibody-positive rodents were tested for arenavirus RNA by using heminested PCR assays. The first-strand cDNA for the PCR was synthesized by using SuperScript II Reverse Transcriptase (Invitrogen Life Technologies, Inc., Carlsbad, CA, USA) in conjunction with oligont 19C-cons (*2*). The nucleotide sequence alignments were analyzed by using MRBAYES 3.1.2 (*12*) in the computer software package PAUP*, version 4.0b10 (http://paup.csit.fsu.edu). (Details of the assays for IgG, arenavirus, and arenavirus RNA and the analyses of the amino acid sequence data and nucleotide sequence data are available from C.F.F.)

Nine white-toothed woodrats, 3 northern grasshopper mice (*Onychomys leucogaster*), 19 deer mice (*Peromyscus* spp.), 11 harvest mice (*Reithrodontomys* spp.), 13 Nelson's pocket mice, 45 Merriam's kangaroo rats, and 16 other kangaroo rats (*Dipodomys* spp.) were captured during 800 trap-nights; overall trap success rate was 116/800 (14.5%). IgG against Whitewater Arroyo virus was found in 2 (22.2%) of the white-toothed woodrats, 2 (15.4%) of the Nelson's pocket mice, 1 (2.2%) of the Merriam's kangaroo rats, and none of the 49 other rodents. Arenavirus

Table 1. Natural hosts and geographic distribution of the North American Tacaribe serocomplex viruses

Virus*	Natural host(s)	State
Bear Canyon	Large-eared woodrat (*Neotoma macrotis*), California mouse (*Peromyscus californicus*)	California (*1,2*)
Big Brushy Tank	White-throated woodrat (*N. albigula*)	Arizona (*3*)
Catarina	Southern plains woodrat (*N. micropus*)	Texas (*4*)
Skinner Tank	Mexican woodrat (*N. mexicana*)	Arizona (*5*)
Tamiami	Hispid cotton rat (*Sigmodon hispidus*)	Florida (*6*)
Tonto Creek	White-throated woodrat (*N. albigula*)	Arizona (*3*)
Whitewater Arroyo	White-throated woodrat (*N. albigula*)	New Mexico (*7*)

*Arenaviruses antigenically and phylogenetically related to Whitewater Arroyo virus have been isolated from Mexican woodrats (*N. mexicana*) captured in New Mexico, a Mexican woodrat and bushy-tailed woodrat (*N. cinerea*) captured in Utah, and woodrats (*Neotoma* spp.) captured in Oklahoma (*8*).

Author affiliations: University of Texas Medical Branch, Galveston, Texas, USA (C.C. Inizan, M.N.B. Cajimat, M.L. Milazzo, C.F. Fulhorst); Universidad Autónoma de Nuevo León, San Nicolas de los Garza, México (A. Barragán-Gomez); and Texas Tech University, Lubbock, Texas, USA (R.D. Bradley)

DOI: 10.3201/eid1606.091648

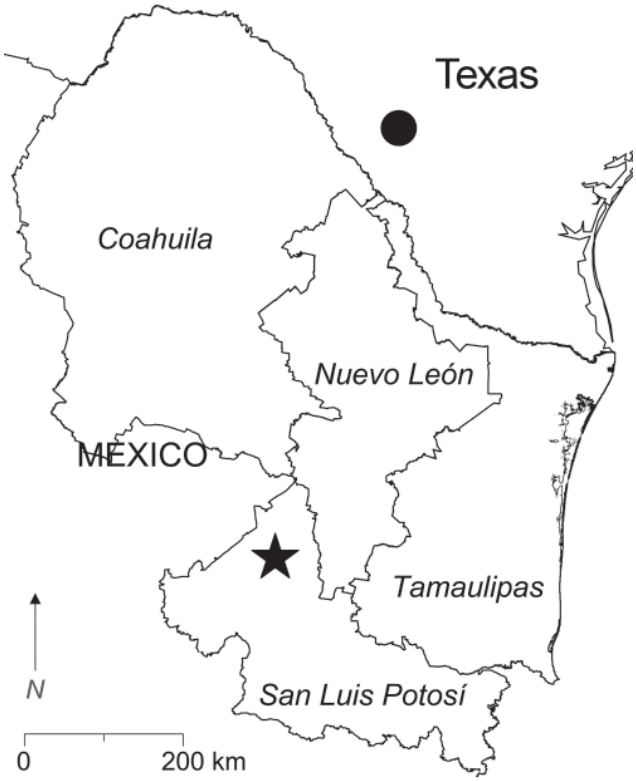


Figure 1. Southern Texas and 4 states in northeastern Mexico. The filled circle in southern Texas indicates the locality in which Catarina virus is enzootic. The star in San Luis Potosí indicates the location of the study site (23°49′5″N, 100°49′54″W). Antibody (immunoglobulin G) to Whitewater Arroyo virus previously was found in white-toothed woodrats (*Neotoma leucodon*), a Mexican woodrat (*N. mexicana*), and deer mice (*Peromyscus* spp.) captured in Nuevo León; white-throated woodrats (*N. albigula*) and white-toothed woodrats captured in San Luis Potosí; and deer mice (*Peromyscus* spp.) and a southern plains woodrat (*N. micropus*) captured in Tamaulipas (M.L. Milazzo, unpub. data).

was isolated from none of the 12 animals included in the virus assays.

The sequence of the 3,352-nt fragment of the S genomic segment of arenavirus AV H0030026 (GenBank accession no. GQ903697) was determined from RNA isolated from white-toothed woodrat TK133448. The 3,352-nt fragment included the complete glycoprotein precursor (GPC) gene and complete nucleocapsid (N) protein gene of AV H0030026. Nonidentities between the amino acid sequences of the GPC and N protein of AV H0030026 and the amino acid sequences of the GPC and N proteins of the 10 other North American viruses included in the analyses of amino acid sequence data ranged from 28.3% to 35.2% and from 11.6% to 21.0%, respectively (Table 2). The results of the Bayesian analyses of complete GPC gene sequences (Figure 2, panel A) and complete N protein gene sequences (Figure 2, panel B) indicated that AV H0030026 represents a unique phylogenetic lineage but failed to resolve the relationship of AV H0030026 to the other North American viruses analyzed.

The nucleotide sequence of a 687-nt fragment of the S genomic segment of arenavirus AV H0030028 was determined from RNA isolated from a kidney of white-toothed woodrat TK133451. The sequence of this fragment (GenBank accession no. GU137350) was 99.9% identical to the sequence of the homologous region of the N protein gene AV H0030026. Results of assays for arenavirus RNA in the kidneys of the antibody-positive Nelson's pocket mice and the antibody-positive kangaroo rat were negative.

Conclusions

Tacaribe serocomplex viruses have been isolated from white-throated woodrats (*N. albigula*), large-eared woodrats (*N. macrotis*), Mexican woodrats (*N. mexicana*), southern plains woodrats (*N. micropus*), and a bushy-tailed woodrat (*N. cinerea*) captured in the western United States

Table 2. Nonidentities among the predicted amino acid sequences of the glycoprotein precursors and among the predicted amino acid sequences of the nucleocapsid proteins of the North American arenaviruses*

	% Sequence nonidentity							
Virus†	AV H0030026	BBTV	BCNV	CTNV	SKTV	TAMV	TTCV	WWAV
AV H0030026		28.6	35.2	31.3	28.3	33.8	29.2	32.0–33.8
BBTV	12.3		33.8	27.5	26.8	35.4	26.4	30.6–33.7
BCNV	18.0	17.6		34.9	34.0	33.3	34.8	37.4–39.1
CTNV	11.6	10.1	17.8		29.4	36.1	30.0	32.4–34.2
SKTV	13.0	11.0	16.9	11.4		33.3	19.8	30.2–33.3
TAMV	21.0	19.4	21.4	18.5	19.0		35.2	38.0–40.6
TTCV	12.5	11.0	17.1	10.5	9.4	18.9		30.8–33.1
WWAV	13.7–15.5	10.7–13.0	18.3–20.5	12.6–14.6	13.9–15.5	19.2–20.8	13.0–14.1	

*Numbers above and below the diagonal are the nonidentities among the glycoprotein precursors and nucleocapsid proteins, respectively.
†BBTV, Big Brushy Tank virus strain AV D0390174 (GenBank accession no. EF619035); BCNV, Bear Canyon virus strain AV A0070039 (AY924391); CTNV, Catarina virus strain AV A0400135 (DQ865244); SKTV, Skinner Tank virus strain AV D1000090 (EU123328); TAMV, Tamiami virus strain W 10777 (AF512828); TTCV, Tonto Creek virus strain AV D0150144 (EF619033); WWAV, Whitewater Arroyo virus strains AV 9310135 (AF228063), AV 96010024 (EU123331), AV 96010151 (EU123330), and AV D1240007 (EU123329). The results of a previous study indicated that AV 96010024, AV 96010151, and AV D1240007 are strains of WWAV or strains of arenaviruses that are phylogenetically closely related to WWAV (*5*). Nonidentities among the amino acid sequences of the glycoprotein precursors and among the amino acid sequences of the nucleocapsid proteins of AV 9310135, AV 96010024, AV 96010151, and AV D1240007 in this study ranged from 16.0% to 25.8% and 7.3% to 10.3%, respectively.

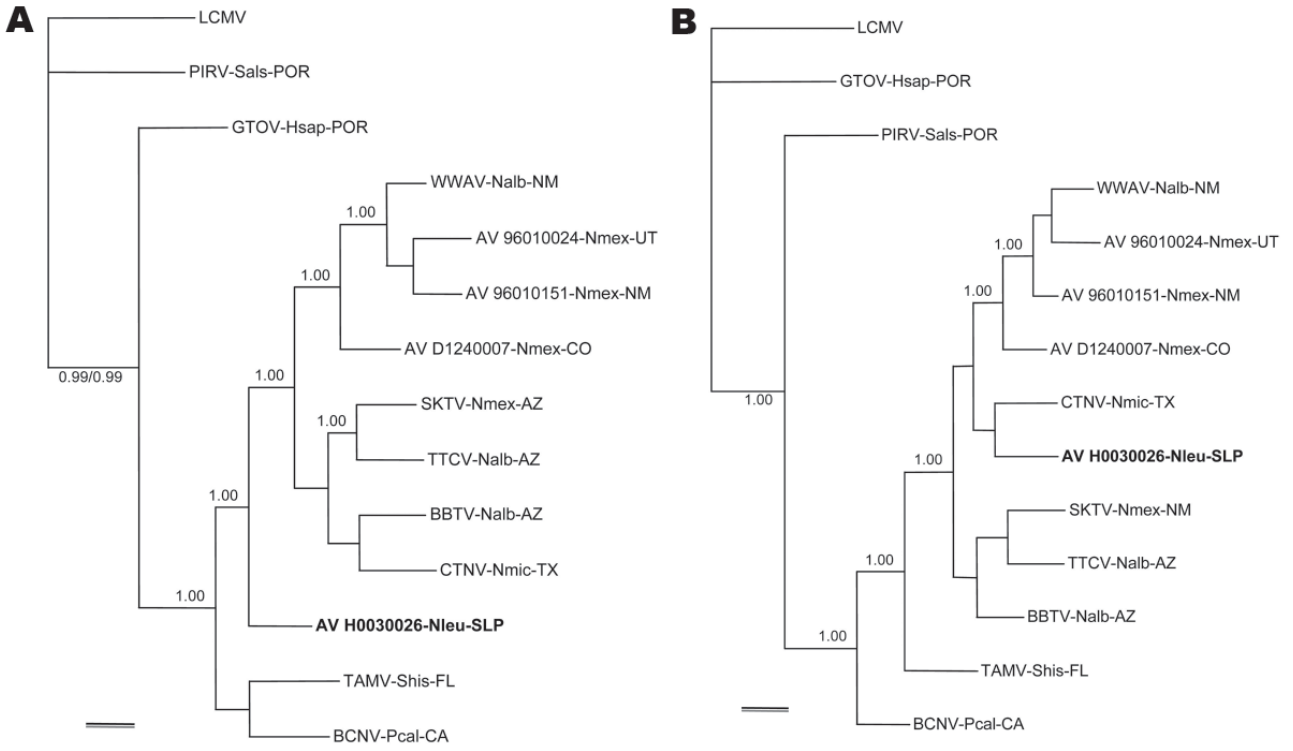


Figure 2. Phylogenetic relationships among 11 North American arenaviruses based on Bayesian analyses of A) glycoprotein precursor gene sequences and B) nucleocapsid protein gene sequences. The number(s) at the nodes are clade probability values, a single 1.00 indicates that the clade probability values for both analyses were 1.00, and clade probability values <0.93 were not included in the phylograms. The branch labels include (in the following order) virus, host species, and state. BBTV, Big Brushy Tank virus strain AV D0390174 (GenBank accession No. EF619035); BCNV, Bear Canyon virus strain AV A0070039 (AY924391); CTNV, Catarina virus strain AV A0400135 (DQ865244); SKTV, Skinner Tank virus strain AV D1000090 (EU123328); TAMV, Tamiami virus strain W 10777 (AF512828); TTCV, Tonto Creek virus strain AV D0150144 (EF619033); WWAV, Whitewater Arroyo virus strain AV 9310135 (AF228063); arenaviruses AV 96010024 (EU123331), AV 96010151 (EU123330), and AV D1240007 (EU123329); GTOV, Guanarito virus strain INH-95551 (AY129247); PIRV, Pirital virus strain VAV-488 (AF485262); LCMV, lymphocytic choriomeningitis virus strain WE (M22138). AZ, Arizona; CA, California; CO, Colorado; FL, Florida; NM, New Mexico; SLP, San Luis Potosí; TX, Texas; UT, Utah; POR, Portuguesa (Venezuela). Nalb, *Neotoma albigula* (white-throated woodrat); Nleu, *N. leucodon* (white-toothed woodrat); Nmex, *N. mexicana* (Mexican woodrat); Nmic, *N. micropus* (southern plains woodrat); Pcal, *Peromyscus californicus* (California mouse); Sals, *Sigmodon alstoni* (Alston's cotton rat); Shis, *S. hispidus* (hispid cotton rat). Pirital virus and GTOV are South American Tacaribe serocomplex viruses and were selected to represent South American lineages A and B, respectively. The LCMV strain WE is a member of the lymphocytic choriomeningitis-Lassa (Old World) serocomplex and was included in the analyses to enable inference of the ancestral node among the North American arenaviruses. Scale bars indicate 0.1 substitutions per site.

(*2*–*5*,*7*,*8*), and antibody to Whitewater Arroyo virus has been found in woodrats captured in San Luis Potosí (M.L. Milazzo, unpub. data). Our results indicate that an arenavirus is naturally associated with the white-toothed woodrat (*N. leucodon*) and that Tacaribe serocomplex viruses are enzootic to Mexico.

The results of the Bayesian analyses indicated that AV H0030026 is phylogenetically distinct from other North American arenaviruses. Sequence nonidentities between the GPC of AV H0030026 and the GPC of the other North America arenaviruses (Table 2) were substantively greater than sequence nonidentities between the GPC of strains of phylogenetically closely related South American arenavirus species (*3*). Furthermore, sequence nonidentities between the N protein of AV H0030026 and the N proteins of the other North American arenaviruses (Table 2) were comparable to the amino acid sequence nonidentities between the N proteins of strains of phylogenetically closely related South American arenavirus species (*3*). Thus, AV H0030026 may represent a novel species (proposed name Real de Catorce) in the family *Arenaviridae*, genus *Arenavirus* (*9*).

Nelson's pocket mouse (*C. nelsoni*) and Merriam's kangaroo rat (*D. merriami*) are members of the rodent family Heteromyidae. No accounts of arenavirus infections in these or other heteromyid rodents have been published. The anti-arenavirus antibody in the pocket mice and kangaroo rat in this study may be a consequence of infection with an

arenavirus other than Real de Catorce virus. Alternatively, the results of the ELISA on these rodents are inaccurate (i.e., falsely positive).

Five Tacaribe serocomplex viruses are known to naturally cause severe febrile disease in humans: GTOV, Junín, Machupo, Sabiá, and Chapare viruses. The diseases caused by these viruses range from sporadic cases to small outbreaks to hyperendemic episodes. Humans usually become infected with arenaviruses by inhalation of virus in aerosolized droplets of secretions or excretions from infected rodents. Other means of infection may include ingestion of infected rodents (*13*).

Human consumption of woodrats is common in rural regions in the highlands of Mexico. For example, white-throated woodrats are consumed by humans in the Potosí-Zacatecan Mexican Plateau (*14*), and Mexican woodrats (*N. mexicana*), Mexican deer mice (*Peromyscus mexicanus*), and other cricetid rodents are consumed by the Tzeltzal Indians in Chiapas (*15*). Future studies on arenaviruses native to North America should include assessments of whether humans who consume woodrats or live or work in close association with woodrats are infected by the Tacaribe serocomplex viruses associated with these rodents.

Acknowledgments

Ildefonso Fernández-Salas facilitated the work at the study site. Ryan Chambers, Nevin Durish, Michelle Knapp, Vicki Swier, and J. Delton Hanson assisted with the collection of rodents. J. Delton Hanson also provided the map in Figure 1. Mary J. Wilson assisted with development of the assays for arenavirus RNA in rodent TK133448.

Financial support for this study came from National Institutes of Health grants AI-41435 and AI-67947. Patricia Repik and Christina Cassetti facilitated the grant support for this study.

Ms Inizan is a master's degree student in physiopathology at the École Normale Supérieure de Cachan, Cachan, France. Her research interests include early events in the immune response in infectious diseases of humans and other vertebrate animals.

References

1. Fulhorst CF, Bennett SG, Milazzo ML, Murray HL Jr, Webb JP Jr, Cajimat MNB, et al. Bear Canyon virus: an arenavirus naturally associated with *Peromyscus californicus* (California mouse). Emerg Infect Dis. 2002;8:717–21.
2. Cajimat MNB, Milazzo ML, Hess BD, Rood MP, Fulhorst CF. Principal host relationships and evolutionary history of the North American arenaviruses. Virology. 2007;367:235–43. DOI: 10.1016/j.virol.2007.05.031
3. Milazzo ML, Cajimat MNB, Haynie ML, Abbott KD, Bradley RD, Fulhorst CF. Diversity among Tacaribe serocomplex viruses (family *Arenaviridae*) naturally associated with the white-throated woodrat (*Neotoma albigula*) in the southwestern United States. Vector Borne Zoonotic Dis. 2008;8:523–40. DOI: 10.1089/vbz.2007.0239
4. Cajimat MNB, Milazzo ML, Bradley RD, Fulhorst CF. *Catarina virus*, an arenaviral species principally associated with *Neotoma micropus* (southern plains woodrat) in Texas. Am J Trop Med Hyg. 2007;77:732–6.
5. Cajimat MNB, Milazzo ML, Borchert JN, Abbott KD, Bradley RD, Fulhorst CF. Diversity among Tacaribe serocomplex viruses (family *Arenaviridae*) naturally associated with the Mexican woodrat (*Neotoma mexicana*). Virus Res. 2008;133:211–7. DOI: 10.1016/j.virusres.2008.01.005
6. Calisher CH, Tzianabos T, Lord RD, Coleman PH. Tamiami virus, a new member of the Tacaribe group. Am J Trop Med Hyg. 1970;19:520–6.
7. Fulhorst CF, Bowen MD, Ksiazek TG, Rollin PE, Nichol ST, Kosoy MY, et al. Isolation and characterization of Whitewater Arroyo virus, a novel North American arenavirus. Virology. 1996;224:114–20. DOI: 10.1006/viro.1996.0512
8. Fulhorst CF, Charrel RN, Weaver SC, Ksiazek TG, Bradley RD, Milazzo ML, et al. Geographical range and genetic diversity of Whitewater Arroyo virus (family *Arenaviridae*) in the southwestern United States. Emerg Infect Dis. 2001;7:403–7.
9. Salvato MS, Clegg JCS, Buchmeier MJ, Charrel RN, Gonzalez J-P, Lukashevich IS, et al. Family *Arenaviridae*. In: Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA, editors. Virus taxonomy: eighth report of the International Committee on Taxonomy of Viruses. Amsterdam: Elsevier Academic Press; 2005:725–33.
10. Bennett SG, Milazzo ML, Webb JP Jr, Fulhorst CF. Arenavirus antibody in rodents indigenous to coastal southern California. Am J Trop Med Hyg. 2000;62:626–30.
11. Fulhorst CF, Milazzo ML, Bradley RD, Peppers LL. Experimental infection of *Neotoma albigula* (Muridae) with Whitewater Arroyo virus (*Arenaviridae*). Am J Trop Med Hyg. 2001;65:147–51.
12. Huelsenbeck JP, Ronquist F. MRBAYES: Bayesian inference for phylogenetic trees. Bioinformatics. 2001;17:754–5.
13. Montali RJ, Scanga CA, Pernikoff D, Wessner DR, Ward R, Holmes KV. A common-source outbreak of callitrichid hepatitis in captive tamarins and marmosets. J Infect Dis. 1993;167:946–50.
14. Márquez Olivas M. Características reproductivas de la rata magueyera (*Neotoma albigula leucodon*) en cautiverio. Acta Zool Mex. 2002;86:139–44.
15. Barragán F, Retana OG, Naranjo EJ. The rodent trade of Tzeltzal Indians of Oxchuc, Chiapas, Mexico. Hum Ecol. 2007;36:769–73. DOI: 10.1007/s10745-007-9116-7

Address for correspondence: Charles F. Fulhorst, Department of Pathology, University of Texas Medical Branch, 301 University Blvd, Galveston, TX 77555-0609, USA; email: cfulhors@utmb.edu

Pandemic (H1N1) 2009, Shanghai, China

Yinzhong Shen and Hongzhou Lu

To understand the clinical and epidemiologic characteristics of pandemic (H1N1) 2009 virus infection, we retrospectively reviewed medical records of 237 patients with laboratory-confirmed cases reported in Shanghai, China, during May–July 2009. Surveillance activities effectively contained the outbreak and provided useful epidemiologic data for future strategies.

In early April 2009, human infections caused by pandemic (H1N1) 2009 virus were identified in the United States (*1*) and Mexico (*2*). The virus then spread rapidly around the world. The People's Republic of China reported its first case of pandemic (H1N1) 2009 on May 10, 2009. As of July 31, a total of 2,090 confirmed cases had been reported in mainland China. Cases were detected in 25 provinces and municipalities; the largest numbers of cases were found in Guangdong Province, Beijing, and Shanghai. To understand the clinical and epidemiologic characteristics of infected patients, we reviewed medical records of 237 patients with laboratory-confirmed cases reported in Shanghai during May–July 2009.

The Study

On April 30, 2009, guidelines for surveillance, reporting, diagnosis, and treatment of pandemic (H1N1) 2009 were published by the Ministry of Health of the People's Republic of China (revised May 9) (*3*). On the basis of these guidelines, the Shanghai Bureau of Health issued a working document for prevention and control of pandemic (H1N1) 2009 in Shanghai.

Briefly, ill persons with a temperature ≥37.5°C were asked to visit fever clinics in local general hospitals. A suspected case of pandemic (H1N1) 2009 was defined as 1) an influenza-like illness (fever ≥37.5°C with ≥1 signs or symptoms, including sore throat, cough, runny nose, nasal congestion) in a person who had traveled to a country where >1 case had been confirmed in the past 7 days or 2) clinical symptoms or signs of influenza-like illness in a person epidemiologically linked to a patient with confirmed or suspected infection identified in the previous 7 days. A confirmed case was defined as laboratory confirmation of infection by PCR performed on a nasopharyngeal swab specimen at the Shanghai Center for Disease Control and Prevention (Shanghai CDC). All suspected cases were required to be reported to the Shanghai CDC within 24 hours after diagnosis.

Nasopharyngeal swabs obtained from patients with suspected cases were sent to the Shanghai CDC for detection of virus. Virus RNA was extracted and tested for all influenza types and specific subtypes by using a series of PCRs specific for matrix gene sequences of influenza A and B viruses. All patients with PCR results positive for pandemic (H1N1) 2009 virus were admitted to the Shanghai Public Health Clinical Center (SPHCC). We analyzed the clinical and epidemiologic features of confirmed cases reported in Shanghai during May 24–July 31, 2009.

In China, a national active surveillance system was established preemptively for recent travelers to areas affected by pandemic (H1N1) 2009. Thermal scanners were installed at all airports to detect fevers in travelers. Health questionnaires were administrated to travelers, and on the basis of answers to these questionnaires, all asymptomatic contacts of patients with suspected and confirmed pandemic (H1N1) 2009 were quarantined for 7 days. Symptomatic persons from an affected area were asked to visit fever clinics for confirmation of infection. Ambulances transported persons with suspected pandemic (H1N1) 2009 from airports to hospitals for screening. Health advisories encouraged travelers in whom influenza-like symptoms developed after arrival in Shanghai to seek medical care.

Shanghai reported its first case of pandemic (H1N1) 2009 on May 24, 2009, in a traveler returning from Australia. As of July 31, 2009, SPHCC had identified 237 confirmed cases: 5 in May, 112 in June, and 120 in July (Figure 1). The maximum number of confirmed cases reported per date of onset (14) occurred on June 28 and July 7. Epidemiologic investigations suggested that the outbreak peaked in Shanghai in late June and early July.

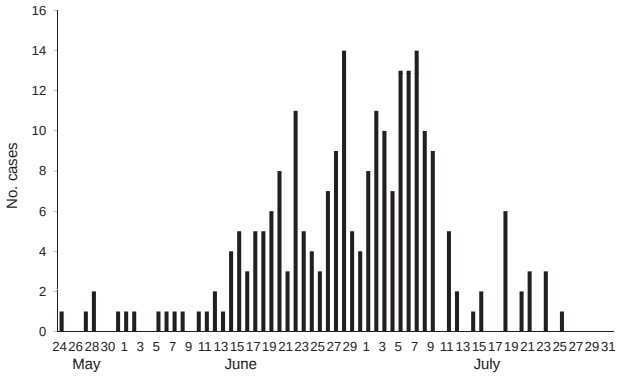


Figure 1. Daily number of laboratory-confirmed cases of pandemic (H1N1) 2009 virus infection, Shanghai, China, May 24–July 31, 2009.

Author affiliation: Shanghai Public Health Clinical Center, Shanghai, People's Republic of China

DOI: 10.3201/eid1606.090991

Of the 237 case-patients, 129 (54.4%) were male. Median age of confirmed case-patients was 24 years (range 2–75 years). Eighty-two percent of case-patients were 10–39 years of age (Figure 2).

Patients were from 15 countries; 64.6% of patients were Chinese citizens, 10.1% were Australian citizens, 5.9% were American citizens, and 5.9% were Indonesian citizens. Among the 237 cases, 230 (97.0%) were identified as imported (i.e., confirmed case in a person with recent travel outside mainland China who had arrived in China during the surveillance period and had illness onset within 7 days after arrival). A total of 115 of these persons were from Australia; 36 from the United States; 18 from Canada; 11 from Indonesia; 10 from Singapore; 8 from the Philippines; 7 from the United Kingdom; 6 from Hong Kong; 4 each from Thailand, Taiwan, and South Korea; 2 each from Argentina and New Zealand; and 1 each from Japan, Mexico, and Italy. Seven (3.0%) patients (1 in June and 6 in July) contracted pandemic (H1N1) 2009 while in Shanghai and showed a clear epidemiologic link to a person with imported pandemic (H1N1) 2009. Of 230 imported cases, 124 (53.9%) were identified in airports upon arrival. No secondary cases occurred among hospital staff at SPHCC.

The most commonly reported symptoms were fever or history of fever (94.9%), dry cough (51.5%), sore throat (32.9%), runny nose (19.8%), and productive cough (18.1%) (Table). Only 2 case-patients (a 58-year-old man and a 49-year-old woman) had diarrhea (0.8%). One case-patient was a pregnant (13 weeks) Australian woman (31 years of age) in whom fever (≥37.8°C) developed on the second day after she arrived from Sydney. Seventeen patients had underlying medical conditions: asthma (3 patients), obesity (3), allergic rhinitis (2), lymphoma (1), essential hypertension (5), hypothyroidism and hepatitis B (1), and gastric ulcer (2).

Of the 237 case-patients, 236 (99.6%) received oseltamivir for 5 days; the pregnant woman refused antiviral therapy. Of 236 patients treated, 186 (78.5%) received oseltamivir within 48 hours after illness onset. Median time between onset of symptoms and start of oseltamivir treatment was 2 days (range 1–5 days). All patients had a mild illness. As of July 31, all patients had recovered and were discharged from the hospital. Mean ± SD length of hospital stay was 4.9 ± 1.7 days (range 3–9 days). No severe cases or deaths were reported. No patients were reported to have complications or to require intubation or oxygen.

Conclusions

Our investigation indicates that the outbreak in Shanghai evolved similarly to outbreaks in other regions (*4*,*5*). The epidemiologic pattern in Shanghai did not differ from that in the Americas and Europe (*6*–*8*). Pandemic (H1N1) 2009 virus preferentially infects younger age groups. Most patients had mild symptoms and fully recovered within 1 week.

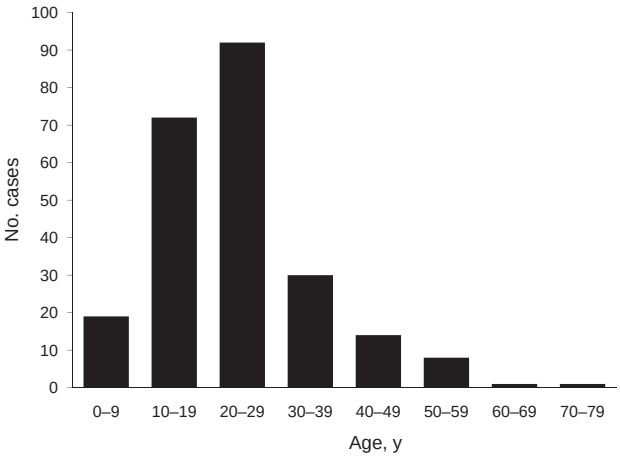


Figure 2. Number of laboratory-confirmed cases of pandemic (H1N1) 2009 virus infection, by age group, Shanghai, China, May 24–July 31, 2009.

Clinical responses were favorable among these 237 case-patients, including 17 with underlying disease. Patients in this series seemed to benefit from early antiviral therapy. Early use of oseltamivir may have prevented complications.

Our study indicates that surveillance activities in Shanghai identified a substantial number of cases of pandemic (H1N1) 2009 among travelers early in the outbreak. The early response strategy in Shanghai has been containment; many case-patients were identified quickly after they arrived in China. Our data show that containment may have had a useful role in the initial phase of the outbreak. In Shanghai, patients with influenza were hospitalized for isolation purposes. Because the number of influenza cases has increased in Shanghai, the quarantine policy has been changed; since August 1, 2009, hospitalization for isolation purposes has not been mandated.

Table. Signs and symptoms in 237 patients with pandemic (H1N1) 2009 virus infection, Shanghai, China, May–July, 2009

Sign or symptom	No. (%)
≥1 symptoms	237 (100)
Fever or history of fever	225 (94.9)
Dry cough	122 (51.5)
Sore throat	78 (32.9)
Runny nose	47 (19.8)
Productive cough	43 (18.1)
Headache	14 (5.9)
Muscle pain	11 (4.6)
Altered consciousness	1 (0.4)
Diarrhea	2 (0.8)
Joint pain	2 (0.8)

Cases of pandemic (H1N1) 2009 in Shanghai during May–July 2009 were identified rapidly and treated with oseltamivir, resulting in mild illness and absence of deaths. As the pandemic evolves, continued investigation is needed to describe its epidemiologic and clinical characteristics.

Acknowledgment

We thank the Department of Emergency Response, Shanghai Public Health Clinical Center, for providing data.

This study was supported by the Science and Technology Commission of Shanghai Municipality (09DZ1906604).

Dr Shen is an attending physician at Shanghai Public Health Clinical Center, Fudan University, Shanghai, China. His research interests include invasive fungal infections, AIDS, and tuberculosis.

Dr Lu is a professor at Shanghai Public Health Clinical Center, Fudan University, Shanghai, China. His research interests include AIDS, central nervous system infections, and tuberculosis.

References

1. Centers for Disease Control and Prevention. Swine influenza A (H1N1) infection in two children—southern California, March–April 2009. MMWR Morb Mortal Wkly Rep. 2009;58:400–2.
2. Centers for Disease Control and Prevention. Outbreak of swine-origin influenza A (H1N1) virus infection—Mexico, March–April 2009. MMWR Morb Mortal Wkly Rep. 2009;58:467–70.
3. Ministry of Health of China. Guidelines for diagnosis and treatment, 2009 May 9 [cited 2009 Aug 11]. http://www.moh.gov.cn/publicfiles/business/htmlfiles/mohyzs/s3585/200905/40478.htm
4. Kelly H, Grant K. Interim analysis of pandemic influenza (H1N1) 2009 in Australia: surveillance trends, age of infection and effectiveness of seasonal vaccination. Euro Surveill. 2009;14 pii:19288.
5. Munayco CV, Gomez J, Laguna-Torres VA, Arrasco J, Kochel TJ, Fiestas V, et al. Epidemiological and transmissibility analysis of influenza A (H1N1)v in a southern hemisphere setting: Peru. Euro Surveill. 2009;14 pii:19299.
6. ECDC Working Group on Influenza A (H1N1)v. Collaborators (8). Preliminary analysis of influenza A(H1N1)v individual and aggregated case reports from EU and EFTA countries. Euro Surveill. 2009;14 pii:19238.
7. Centers for Disease Control and Prevention. Update: novel influenza A (H1N1) virus infection—Mexico, March–May, 2009. MMWR Morb Mortal Wkly Rep. 2009;58:585–9.
8. Perez-Padilla R, de la Rosa-Zamboni D, Ponce de Leon S, Hernandez M, Quiñones-Falconi F, Bautista E, et al. Pneumonia and respiratory failure from swine-origin influenza A (H1N1) in Mexico. N Engl J Med. 2009;361:680–9. DOI: 10.1056/NEJMoa0904252

Address for correspondence: Hongzhou Lu, Department of Infectious Diseases, Shanghai Public Health Clinical Center, Fudan University, Shanghai, People's Republic of China; email: luhongzhou@fudan.edu.cn

Transfer of Carbapenem-Resistant Plasmid from *Klebsiella pneumoniae* ST258 to *Escherichia coli* in Patient

Moran G. Goren, Yehuda Carmeli, Mitchell J. Schwaber, Inna Chmelnitsky, Vered Schechner, and Shiri Navon-Venezia

Klebsiella pneumoniae carbapenemase (KPC) 3–producing *Escherichia coli* was isolated from a carrier of KPC-3–producing *K. pneumoniae*. The KPC-3 plasmid was identical in isolates of both species. The patient's gut flora contained a carbapenem-susceptible *E. coli* strain isogenic with the KPC-3–producing isolate, which suggests horizontal interspecies plasmid transfer.

Over the past 2 years, the extremely drug-resistant *Klebsiella pneumoniae* carbapenemase (KPC)–producing *K. pneumoniae* sequence type 258 (KpnST258) has emerged as an important nosocomial pathogen worldwide. It has spread in the United States and in various countries in Europe and Asia (*1*–*3*). The high level of antimicrobial drug resistance in this bacterium is conferred by a plasmid-encoded KPC, which confers resistance to all cephalosporins, monobactams, and carbapenems (*4*). Infection with carbapenem-resistant *K. pneumoniae* is associated with an increased proportion of deaths compared to carbapenem-susceptible *K. pneumoniae* (*5*). Although *Klebsiella* with plasmid-mediated carbapenem resistance is a major risk to hospitalized patients, spread of these resistance plasmids into *Escherichia coli* poses an even greater public health threat because resistant *E. coli* may become part of the normal gut flora and thereby become a notable source of infections among sick and the healthy persons in healthcare settings and in the community (*6*).

In 2008, a carbapenem-nonsusceptible *E. coli*–producing KPC-3 isolate (Eco2) was identified in Tel Aviv Sourasky Medical Center in Israel. Until this case, carbapenem resistance in *E. coli* at the hospital was related exclusively to KPC-2 production (*7*). KPC production in *E. coli* remains rare worldwide, even in areas where KPC-producing *K. pneumoniae* isolates are identified. We aimed to investigate the origin of KPC-3 in this *E. coli* isolate and to explore a possible molecular and epidemiologic link between the presence of bla_{KPC-3} in this species and in the KpnST258 strain prevalent in our hospital.

Author affiliations: Tel Aviv Sourasky Medical Center, Tel Aviv, Israel (M.G. Goren, Y. Carmeli, I. Chmelnitsky, V. Schechner, S. Navon-Venezia); and National Center for Infection Control, Tel Aviv (Y. Carmeli, M.J. Schwaber, S. Navon-Venezia)

DOI: 10.3201/eid1606.091671

The Study

In April 2008 a carbapenem-nonsusceptible *E. coli* strain, marked as Eco2, was recovered from the gall bladder drainage of a 91-year-old man with dialysis-dependent end-stage renal disease, congestive heart failure, anemia, and peptic ulcer disease. A month earlier, the patient had been hospitalized with sepsis that developed after an infected heel wound had required amputation of the left leg below the knee. The patient was treated with ertapenem, metronidazole, colistin, and vancomycin. Acute cholecystitis developed, and the patient underwent cholecystostomy. During his hospital stay, the patient underwent screening for carriage of carbapenem-resistant *Enterobacteriacae* (CRE) as part of a routine infection control program aimed at limiting the spread of CRE. Two rectal swabs were collected 1 week apart. The first swab specimen was negative for CRE by culture, and the second swab specimen showed a carbapenem-resistant *K. pneumoniae* strain (marked Kpn1), which was PCR positive for bla_{KPC}. One month after the patient's admission, a carbapenem-nonsusceptible *E. coli* (Eco2) was isolated from drainage at the cholecystectomy site, which prompted this study.

Microbiologic and molecular investigations (pulsed-field gel electrophoresis [PFGE], DNA isolation, isoelectric focusing analysis [IEF], PCR detection of resistance genes, plasmid isolation, transformation, and Southern analysis) were performed as described (*2*,*4*,*7*). The carbapenem-nonsusceptible *E. coli* isolated from the clinical specimen (Eco2) was initially identified by Vitek-2 (bioMérieux, Marcy-l'Etoile, France) as resistant to imipenem (MIC>16 mg/L). Further antimicrobial-drug susceptibility testing of the strain by using agar dilution and Etest (AB Biodisk, Solna, Sweden) showed MICs in the resistant range for ceftriaxone and aztreonam; in the intermediate range for ceftazidime and piperacillin/tazobactam; and in the susceptible range for cefepime, ertapenem, meropenem, imipenem, aminoglycosides, quinolones, tigecycline, and colistin (online Appendix Table, www.cdc.gov/EID/content/16/6/1014-appT.htm). IEF identified 2 β-lactamases with isoelectric pH values of 5.4 and 6.7, corresponding to those of TEM-type and KPC. β-lactamase gene PCR screening and sequencing indicated the presence of bla_{TEM-1} and bla_{KPC-3}. Results of screening for other β-lactamase genes were negative.

Transformation of plasmids purified from Eco2 into an *E. coli* DH10B recipient strain (Eco2-T) indicated transfer of a single plasmid that encoded these bla_{TEM-1} and bla_{KPC-3} (Figure 1, panel A), and increased the MICs of the recipient strains to broad-spectrum cephalosporins and carbapenems (online Appendix Table). PFGE identified the genetic similarity between the colonizing *Klebsiella* (Kpn1) and a representative KpnST258 (isolate Kpn557 described previously [*4*]) (Figure 2). Susceptibility testing of Kpn1 reflected the extremely drug-resistant phenotype characteristic of isolates belonging to this clone (*12*) (online Appendix Table).

We compared plasmids of Kpn1 and Eco2. Kpn1 carried 4 different plasmids, whereas Eco2 carried 1 plasmid that correlated with the 105-kb plasmid of Kpn1. Experiments to transform Eco2 and Kpn1 plasmids into an *E. coli* DH10B recipient, followed by selection on plates containing 100 μg/mL ampicillin and screening for bla_{KPC}-positive colonies, showed that DH10B was transformed with the105-kb KPC-3–encoding plasmid (Figure 1, panel A). This plasmid correlated in size with that of pKpQIL, the KPC-3-encoding plasmid of *Klebsiella* ST258 in Israel (*13*). Plasmid DNA restriction fragment length polymorphism showed that band patterns of the 2 KPC-3–encoding plasmids of Kpn1 and Eco2 were highly similar (98% similarity) (Figure 1, panel B), and Southern analysis with a bla_{KPC-3} probe showed the same hybridization pattern (Figure 1, panel C).

We aimed to determine whether the patient's gut was colonized with a carbapenem-susceptible *E. coli* strain, which would ultimately serve as the in vivo recipient of the bla_{KPC-3}-encoding plasmid. Thus, the first broth culture prepared (obtained before the patient was colonized with KPC-3–producing *E. coli*) was processed. Aliquots (0.1 mL) were directly plated onto a MacConkey agar plate (Hy-Labs, Rehovot, Israel). *E. coli* colonies isolated from the plate were restreaked onto a MacConkey agar plate, yielding an *E. coli* strain 7364 (Eco1) that was susceptible to all antimicrobial drugs tested (online Appendix Table). PFGE DNA fingerprinting showed that Eco1 was 100% identical to the KPC-3-producing clinical strain Eco2, isolated from the clinical specimen (Figure 2). Plasmid analysis of this strain, however, proved that it lacked plasmid pKpQIL (results not shown).

Elements belonging to KPC transposon Tn*4401*, including *tnpA, tnpR, ISKpn6,* and *ISKpn7* (*14*), were identified by PCR and sequencing on both KPC-3–encoding plasmids originating from Kpn1 and Eco2. These genetic determinants were absent in the susceptible Eco1. These data suggest that Eco1 has acquired pKpQIL from Kpn1 in the patient's gut, leading to the formation of Eco2. Although acquisition of the plasmid increased MICs for imipenem, meropenem, and ertapenem considerably, it did not confer full resistance (online Appendix Table) presumably due to copy number of the plasmid or the expression level

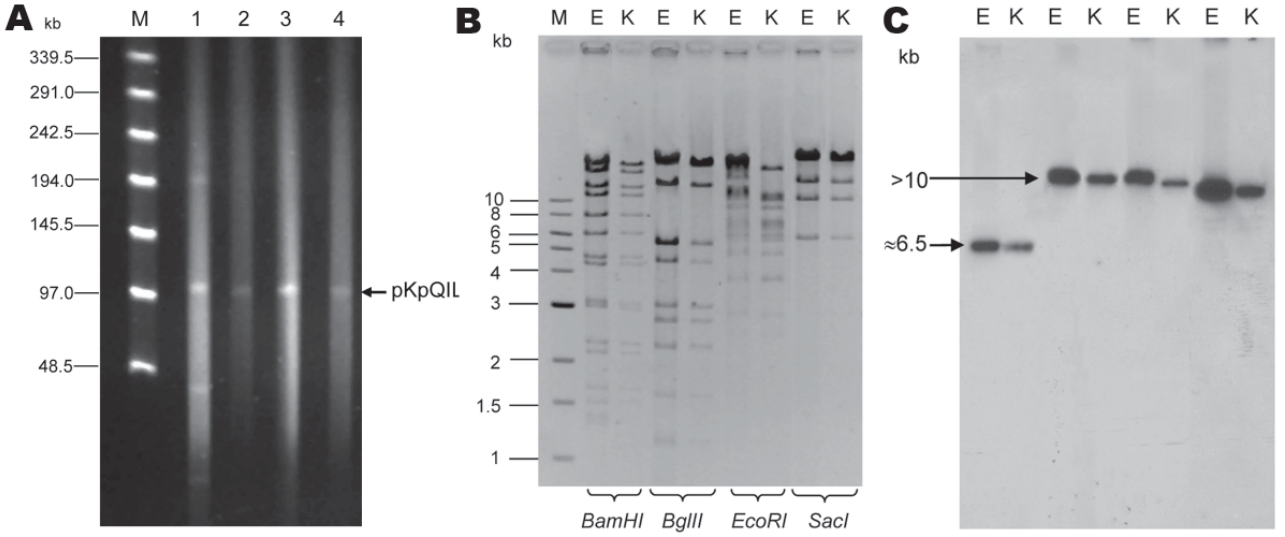


Figure 1. A) Analysis of *Klebsiella pneumoniae* carbapenemase (KPC)–encoding plasmids in isolates Kpn1 (1), Eco2 (3), Kpn1-T (2), and Eco2-T (4), Israel, 2008. Plasmid size estimation was performed by digestion of DNA with S1 nuclease (20 U; Promega, Madison, WI, USA) followed by pulsed-field gel electrophoresis (PFGE) with the CHEF-DR III apparatus (Bio-Rad Laboratories, Inc., Hercules, CA, USA), as described (*8*–*11*). Lambda ladder PFG marker (New England Biolabs, Beverly, MA, USA) was used as a molecular size marker (lane M). B) Restriction fragment length polymorphism of the KPC-3–encoding plasmid from Kpn1-T (K) and Eco2-T (E). Plasmid DNA was digested with *Bam*HI, *Bgl*II, *Eco*RI, and *Sac*I endonucleases (New England Biolabs) and underwent PFGE on a 1% agarose gel. The level of similarity between restriction patterns was calculated by using GelcomparII software version 5 (Applied Maths, Kortrigk, Belgium). Lane M, 1-kb DNA ladder (New England Biolab). C) Southern blot analysis of plasmid DNA hybridized with bla_{KPC-3}-labeled probe. Plasmid restriction products were transferred to a Hybond N^+ membrane (Amersham Biosciences, Little Chalfont, United Kingdom), cross-linked with UV light, and hybridized with a bla_{KPC-3}-labeled probe (892-bp product of bla_{KPC-3}).

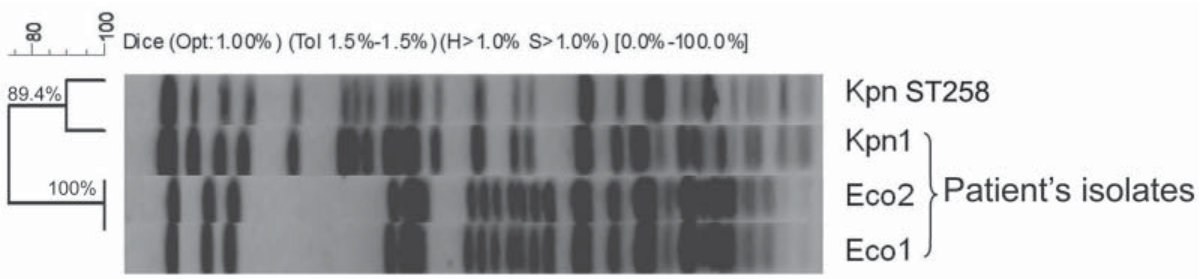


Figure 2. Pulsed-field gel electrophoresis demonstrating genetic relatedness of study isolates Eco2, Eco1, and Kpn1, and a representative *Klebsiella pneumoniae* isolate of the epidemic clone, Kpn ST258, Israel, 2008. Bacterial DNA was prepared and cleaved with 20U *Spe*I endonuclease (New England Biolabs, Beverly, MA, USA), followed by electrophoresis in a CHEF-DR III apparatus (Bio-Rad Laboratories, Inc., Hercules, CA, USA), as described (*4*). The macrorestriction patterns of the isolates were compared according to the Dice similarity index (1.5 % tolerance interval) by using GelcomparII software (Applied Maths, Kortrigk, Belgium).

of bla_{KPC-3} in *E. coli*. Curing of pKpQIL from Eco2 was performed by sequential transfers at an elevated temperature (42°C). The cured strain, which lacked the KPC-encoding plasmid, showed full susceptibility to all antimicrobial drugs tested, similar to the Eco1 strain isolated from the patient's gut flora.

The patient received a combination of 4 antimicrobial agents concomitantly (ertapenem, metronidazole, colistin, and vancomycin) during the period in which Eco1 acquired in vivo the plasmid pKpQIL, thereby becoming Eco2. We believe that the selection pressure imposed by these antimicrobial agents contributed to the sequence of events that led to plasmid transfer. We hypothesize that interspecies conjugation and antimicrobial pressure led to the preferential selection of Eco2, rather than Eco1, as a determinant of infection in this patient.

Interspecies KPC transfer can presumably occur through the dissemination of mobile genetic elements as has been described for transfer of the *mec*A gene between strains of *Staphylococcus aureus* (*15*). bla_{KPC} may spread through transfer by virtue of its location on the Tn*4401* transposon (*14*), or by dissemination of the intact KPC-encoding plasmid, likely through natural conjugation. Multiple attempts to mimic the natural transfer of pKpQIL, the KPC-3-encoding plasmid from Kpn1 into Eco1 by using conjugation experiments were not successful. The isolation of an isogenic, antimicrobial drug–susceptible *E. coli* clone enabled us to decipher the natural order of the interspecies genetic transfer event

Conclusions

With increasing global spread of KPC-producing *K. pneumoniae* ST258, the likelihood increases of interspecies transfer of drug-resistance determinants into a highly fit *E. coli* clone. Such an event may have severe public health consequences, leading to elimination of any effective antimicrobial drug treatment against the most common human bacterial pathogens.

This work was performed in partial fulfillment of the requirements for the MS degree of M.G.G., Sackler Faculty of Medicine, Tel Aviv University, Israel

Mrs Goren is a researcher and master's degree candidate in the Molecular Epidemiology and Antibiotic Resistance Laboratory in Tel Aviv Medical Center. Her primary research focus is characterization of the molecular epidemiology of carbapenem resistance among *Escherichia coli* clinical isolates.

References

1. Yigit H, Queenan AM, Anderson GJ, Domenech-Sanchez A, Biddle JW, Steward CD, et al. Novel carbapenem-hydrolyzing beta-lactamase, KPC-1, from a carbapenem-resistant strain of *Klebsiella pneumoniae*. Antimicrob Agents Chemother. 2001;45:1151–61. DOI: 10.1128/AAC.45.4.1151-1161.2001
2. Kitchel B, Rasheed JK, Patel JB, Srinivasan A, Navon-Venezia S, Carmeli Y, et al. Molecular epidemiology of KPC-producing *Klebsiella pneumoniae* isolates in the United States: clonal expansion of multilocus sequence type 258. Antimicrob Agents Chemother. 2009;53:3365–70. DOI: 10.1128/AAC.00126-09
3. Woodford N, Zhang J, Warner M, Kaufmann ME, Matos J, Macdonald A, et al. Arrival of *Klebsiella pneumoniae* producing KPC carbapenemase in the United Kingdom. J Antimicrob Chemother. 2008;62:1261–4. DOI: 10.1093/jac/dkn396
4. Leavitt A, Navon-Venezia S, Chmelnitsky I, Schwaber MJ, Carmeli Y. Emergence of KPC-2 and KPC-3 in carbapenem-resistant *Klebsiella pneumoniae* strains in an Israeli hospital. Antimicrob Agents Chemother. 2007;51:3026–9. DOI: 10.1128/AAC.00299-07
5. Schwaber MJ, Klarfeld-Lidji S, Navon-Venezia S, Schwartz D, Leavitt A, Carmeli Y. Predictors of carbapenem-resistant *Klebsiella pneumoniae* acquisition among hospitalized adults and effect of acquisition on mortality. Antimicrob Agents Chemother. 2008;52:1028–33.Medline DOI: 10.1128/AAC.01020-07
6. Schwaber MJ, Carmeli Y. Carbapenem-resistant Enterobacteriaceae: a potential threat. JAMA. 2008;300:2911–3.Medline DOI: 10.1001/jama.2008.896
7. Navon-Venezia S, Chmelnitsky I, Leavitt A, Schwaber MJ, Schwartz D, Carmeli Y. Plasmid-mediated imipenem-hydrolyzing enzyme KPC-2 among multiple carbapenem-resistant *Escherichia coli* clones in Israel. Antimicrob Agents Chemother. 2006;50:3098–101. DOI: 10.1128/AAC.00438-06

8. Noller AC, McEllistrem MC, Stine OC, Morris JG Jr, Boxrud DJ, Dixon B, et al. Multilocus sequence typing reveals a lack of diversity among *Escherichia coli* O157:H7 isolates that are distinct by pulsed-field gel electrophoresis. J Clin Microbiol. 2003;41:675–9.
9. Schlesinger J, Navon-Venezia S, Chmelnitsky I, Hammer-Münz O, Leavitt A, Gold HS, et al. Extended-spectrum beta-lactamases among *Enterobacter* isolates obtained in Tel Aviv, Israel. Antimicrob Agents Chemother. 2005;49:1150–6. DOI: 10.1128/AAC.49.3.1150-1156.2005
10. Barton BM, Harding GP, Zuccarelli AJ. A general method for detecting and sizing large plasmids. Anal Biochem. 1995;226:235–40. DOI: 10.1006/abio.1995.1220
11. Li L, Lim CK. A novel large plasmid carrying multiple beta-lactam resistance genes isolated from a *Klebsiella pneumoniae* strain. J Appl Microbiol. 2000;88:1038–48. DOI: 10.1046/j.1365-2672.2000.01069.x
12. Navon-Venezia S, Leavitt A, Schwaber MJ, Rasheed JK, Srinivasan A, Patel JB, et al. First report on a hyperepidemic clone of KPC-3-producing *Klebsiella pneumoniae* in Israel genetically related to a strain causing outbreaks in the United States. Antimicrob Agents Chemother. 2009;53:818–20. DOI: 10.1128/AAC.00987-08
13. Leavitt A, Chmelnitsky I, Ofek I, Carmeli Y, Navon-Venezia S. Plasmid pKpQIL harboring KPC-3 and TEM-1 renders carbapenem resistance in extremely drug resistant epidemic *Klebsiella pneumoniae.* J Antimicrob Chemother. 2009. In press.
14. Naas T, Cuzon G, Villegas MV, Lartigue MF, Quinn JP, Nordmann P. Genetic structures at the origin of acquisition of the beta-lactamase bla_{KPC} gene. Antimicrob Agents Chemother. 2008;52:1257–63. DOI: 10.1128/AAC.01451-07
15. Wielders CL, Vriens MR, Brisse S, de Graaf-Miltenburg LA, Troelstra A, Fleer A, et al. Evidence for in-vivo transfer of mecA DNA between strains of *Staphylococcus aureus.* Lancet. 2001;357:1674–5. DOI: 10.1016/S0140-6736(00)04832-7

Address for correspondence: Shiri Navon-Venezia, Molecular Epidemiology and Antimicrobial Resistance Laboratory, Division of Epidemiology, Tel Aviv Medical Center, 6 Weizman St, Tel Aviv 64239, Israel; email: shiri_nv@tasmc.health.gov.il

Enterovirus Genotype EV-104 in Humans, Italy, 2008–2009

Antonio Piralla, Francesca Rovida, Fausto Baldanti, and Giuseppe Gerna

In an epidemiologic investigation of respiratory infections in Italy, October 2008–September 2009, we tested samples from patients for respiratory viruses. Human enterovirus genotype EV-104 (identified in Switzerland) was found in 3 immunocompromised and 2 immunocompetent patients. EV-104 is closely related to human rhinoviruses; thus, both types of viruses should be sought in respiratory syndromes.

Human rhinoviruses (HRVs) and enteroviruses (HEVs) have been grouped within the same genus (*Enterovirus*) because of their identical genomic organization and high sequence homology (*1*). However, HRVs infect primarily the respiratory tract, whereas HEVs infect primarily the gastrointestinal tract, from which they can spread to distant sites, such as the central nervous system or myocardial tissue. In addition, HRVs and HEVs differ in several in vitro properties, such as cell tropism, optimal growth temperature, and low pH sensitivity. Notwithstanding these different characteristics, some HEVs possess a respiratory tract tropism similar to that of HRVs; they infect both infants and adults and cause infections of the upper and lower respiratory tracts. Several HEV genotypes, including enterovirus 68; coxsackieviruses (CVs) A9, A21, B2, and B4; and echoviruses 9 and 11, have reportedly been recovered from respiratory secretions or from tissues of patients with bronchitis, bronchiolitis, or pneumonia (*2*–*6*). In an extended epidemiologic study of HEV respiratory infections in children in France in 2008, respiratory syndromes were the second most common HEV-induced pathologic condition after meningitis (31% vs. 44%); HEV caused infections of the lower respiratory tract in 43 (54%) of 79 respiratory infections (*7*).

In Switzerland during 2004–2007, a new enterovirus genotype, EV-104, was reported in association with respiratory signs and symptoms and acute otitis media in 8 children from various regions of the country (*8*). In the past, this virus probably escaped detection because cell cultures lacked sensitivity or because appropriate molecular methods were not used. In addition, only a small percentage of detected HEVs are actually typed. EV-104 belongs to the HEV-C species, and its closest serotypes are CV-A19, CV-A22, and CV-A1 (*9*).

The Study

During an epidemiologic survey conducted from October 1, 2008, through September 30, 2009, of viral infections of the respiratory tract, in which we collected respiratory samples from all patients admitted to our University Hospital, Fondazione Istituto Di Ricovero e Cura a Carattere Scientifico (IRCCS) Policlinico San Matteo, we detected 5 strains of the new EV-104 genotype. The 5 Pavia (Pav) strains were neither temporally nor epidemiologically related to each other and were recovered from 2 immunocompetent patients (Pav-2 and Pav-4) and 3 immunocompromised patients (Pav-1, Pav-3, and Pav-5) (Table).

All respiratory secretion samples (1,500) were routinely tested for respiratory viruses (*10*). In addition, they were tested by real-time reverse transcription–PCR (RT-PCR) for amplification and quantification of both HEVs (*11*) and HRVs (*12*). Primers and probes used for HRV and HEV detection in this study were the following: HRV-forward 5′-CPXGCCZGCGTGGC-3′, HRV-reverse 5′-GAAACACGGACACCCAAAGTA-3′, HRV-probe 5′-TCCTCCGGCCCCTGAATGYGGC-3′, HEV-forward 5′-CCTCCGGCCCCTGA-3′, HEV-reverse 5′-GATTGTCACCATAAGCAGCC-3′, and HEV-probe 5′-CGGAACCGACTACTTTGGGT-3′. However, the HEV assay (*11*) does exhibit several mismatches with EV-104 target.

Using these 2 assays, we detected 5 strains from 5 patients; these strains were amplified to a comparable degree. When these 5 strains were tentatively amplified in the viral protein (VP) 1 region (*13*), no amplicon was obtained, as reported by others (*8*). Amplification products were obtained when typing was attempted by amplifying the VP4/VP2 region (*14*), which yielded a sequence resembling HEV-C genotypes but did not match any of the sequences published in GenBank. Upon request, the new EV-104 strain (*9*) sequence was received from the researchers in Switzerland (GenBank accession no. EU840733). Comparison of the sequences from the strains from Switzerland and the Pav strains showed that the 5 new Pav strains belonged to genotype EV-104 within the HEV-C species (GenBank accession nos. Pav-5, GU722097; Pav-4, GU722098; Pav-3, GU722099; Pav-2, GU722100; Pav-1, GU722101).

From the 5 new Pav strains, a 637-nt fragment was obtained. In detail, 181 nt were within the 5′ noncoding region, 207 in VP4, and 249 in VP2. The nucleotide identity within the 5 Pav strains was in the range of 97.9%–99.3%, and the identity with the reference strain from Switzerland was

Author affiliation: Fondazione Istituto Di Ricovero e Cura a Carattere Scientifico Policlinico, San Matteo, Pavia, Italy

DOI: 10.3201/eid1606.091533

Table. Characteristics of 5 patients with HEV genotype EV-104 infection, Pavia, Italy, 2009*

Patient no.	Sample no.	Age, y/sex	Hospital department, date of admission	Respiratory secretion Ct value		Clinical symptoms	Underlying disease	Virus identified/ coinfecting virus (Ct)
				HEV	HRV			
Pav-1	NPA/9210	60/M	Hematology outpatient, Apr	29.67	Undetected	Fever, cough, rhinorrhea	HSCT (AML)	EV-104
Pav-2	NPA/9570	62/M	Infectious Diseases outpatient, Apr	23.06	23.05	Chronic rhinopharyngitis	None	EV-104
Pav-3	NPA/11228	7/F	Pediatric Oncohematology, May	33.64	Undetected	Fever, rhinorrhea, conjunctivitis	AML (chemotherapy)	EV-104
Pav-4	NPA/11230	37/F	Infectious Diseases outpatient, May	32.60	Undetected	Chronic rhinopharyngitis	None	EV-104
Pav-5	NPA/13174	2/M	Pediatric Oncohematology, Jun	23.78	31.61	Cough, rhinorrhea, diarrhea, wheezing	HSCT (AML)	EV-104/RSV (18.53)

*HEV, human enterovirus; Ct, cycle threshold; HRV, human rhinovirus; NPA, nasopharyngeal aspirate; HSCT, hematopoietic stem cell transplant; AML, acute myelocytic leukemia; RSV, respiratory syncytial virus.

95.4%–97.0%. Amino acid identity among the Pav strains was 100% for 4 strains; Pav-5 strain showed a 2-aa difference, and the amino acid identity for all Pav strains with the strain found in Switzerland was 98.1%–99.4%. Within the HEV-C species, the closest genotypes are CV-A19, CV-A1, and CV-A22 with a nucleotide identity of 63%, 59%, and 62% and an amino acid identity of 81%, 80%, and 81% with the Pav strains, respectively (Figure 1).

In the 2 immunocompetent persons, EV-104 was associated with episodes of chronic rhinopharyngitis, whereas 3 immunocompromised patients exhibited symptoms of acute respiratory tract infection (Table). However, in the patient infected with Pav-5, a hematopoietic stem cell transplant recipient, at the beginning of a 4-month follow-up after the transplant, respiratory syncytial virus was detected in association with EV-104 and was likely responsible for the acute respiratory symptoms (Figure 2) in the immediate posttransplant period. Subsequently, respiratory syncytial virus disappeared, while EV-104 remained at the same stable level (cycle threshold 24–29) during the entire follow-up period. In the last month of the follow-up period, EV-104 was not associated with any clinical or respiratory symptoms. Because we did not grow the virus in cell cultures, we cannot exclude that our real-time RT-PCR assay may have detected only RNA left over from the EV-104 infection. Even in the patient infected with Pav-5, we could not detect any mutation in several samples examined during the 4 months of follow-up.

Conclusions

The 5 EV-104 strains from Italy, along with the reference strain from Switzerland, form a separate clade within the HEV-C species, which includes 3 polioviruses, several CV-A genotypes, EV-99, and EV-102 (Figure 1). The new HEV genotype confirms the close relationship between HRVs and HEVs, and these findings suggest that both species of viruses should be sought in respiratory syndromes (*8*). Although the EV-104 strains detected in Switzerland were in children from different regions and covered a period of 4 years (*8*), our 5 strains were detected during a short period of the same year and in the same geographic area; the patients, however, had presumably not been in contact with one another. However, population-based studies are needed to infer the actual prevalence of EV-104 infections.

In the patients reported here, only the upper respiratory tract was involved in the EV-104 infection, whereas in the study in Switzerland, otitis media and pneumonia were also

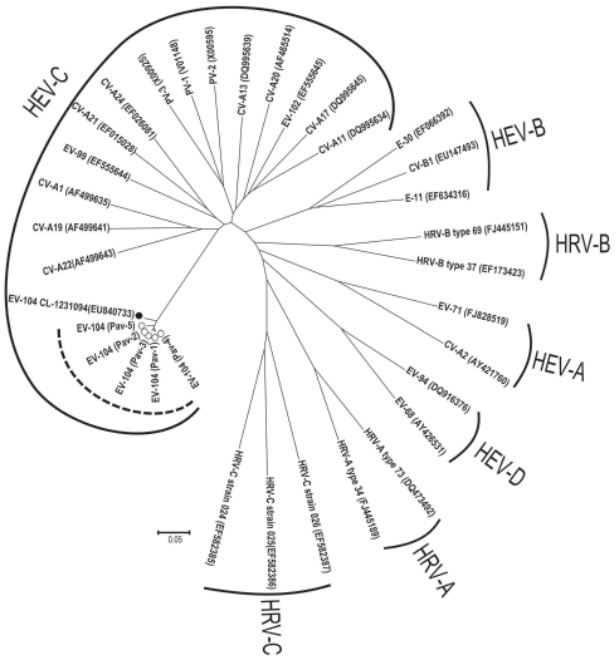


Figure 1. Phylogenetic analysis of the viral protein (VP) 4/VP2 region of the 5 enterovirus 104 (EV-104) strains belonging to the human enterovirus C (HEV-C) species (delimited by circular dotted line), along with the reference strain from Switzerland (GenBank accession no. EU840733). Prototype strains are also reported for the different HEV and human rhinovirus (HRV) species. CV, coxsackievirus; E, echovirus; PV, poliovirus. Scale bar indicates nucleotide substitutions per position.

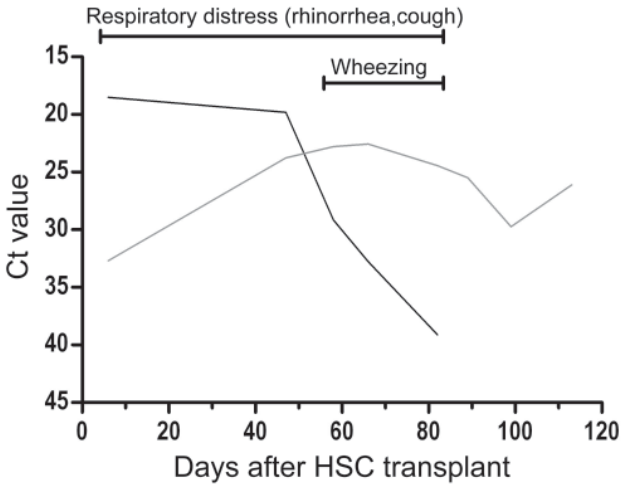


Figure 2. Virologic and clinical follow-up of immunocompromised patient infected with Pavia strain (Pav-5) showing the kinetics of enterovirus 104 (EV-104) and respiratory syncytial virus (RSV) viral loads, along with respiratory symptoms. Starting on day 90 after transplantation, the patient's clinical symptoms began to disappear in the presence of a substantially unchanged EV-104 viral load in respiratory secretions. Ct, cycle threshold; HSC, hematopoietic stem cells.

reported. Obviously, the range of EV-104 pathogenicity will have to be defined in an extended clinical and epidemiologic survey. However, virus detection in patient Pav-5 in the absence of clinical symptoms indicates a potential nonpathogenic role for this virus, as is already known for HEVs, HRVs, and other respiratory viruses. In contrast, the sustained persistence of EV-104 in the respiratory tract of patient Pav-5 indicates that, at least in immunocompromised patients, virus can be shed for a long period, as has been shown for other respiratory viruses in this patient population (*15*). We found also that the 2 immunocompetent persons (Pav-2 and Pav-4) had a chronic respiratory infection; however, because of limited sampling, the persistence of EV-104 was not shown.

From a methodologic standpoint, we believe it is reasonable to conclude that, although only VP1 has been fully validated in multiple laboratories as the optimal typing region, other genome regions that code for capsid proteins may be amplified to enable typing of known or even unknown HEV genotypes. This method could facilitate the detection of new virus genotypes.

Acknowledgments

We thank all the technical staff of the Servizio di Virologia for handling the specimens and conducting the assays. We are also indebted to Daniela Sartori for preparing the manuscript and Laurene Kelly for revising the English.

This work was partially supported by the Ministero della Salute, Ricerca Corrente (grants 80221 and 80622).

Dr Piralla is a fellow at the Servizio di Virologia, Fondazione IRCCS Policlinico, San Matteo, Italy. His primary interest is sequencing respiratory virus genes.

References

1. Tapparel C, Junier T, Gerlach D, Cordey S, Van Belle S, Perrin L, et al. New complete genome sequences of human rhinoviruses shed light on their phylogeny and genomic features. BMC Genomics. 2007;8:224.. DOI: 10.1186/1471-2164-8-224
2. Andreoletti L, Lesay M, Dewilde A, Lambert V, Wattré P. Differential detection of rhinoviruses and enteroviruses RNA sequences associated with classical immunofluorescence assay detection of respiratory virus antigens in nasopharyngeal swabs from infants with bronchiolitis. J Med Virol. 2000;61:341–6. DOI: 10.1002/1096-9071(200007)61:3<341::AID-JMV10>3.0.CO;2-0
3. Oberste MS, Maher K, Schnurr D, Flemister MR, Lovchik JC, Peters H, et al. Enterovirus 68 is associated with respiratory illness and shares biological features with both the enteroviruses and the rhinoviruses. J Gen Virol. 2004;85:2577–84. DOI: 10.1099/vir.0.79925-0
4. Jartti T, Lehtinen P, Vuorinen T, Österback R, van den Hoogen B, Osterhaus AD, et al. Respiratory picornaviruses and respiratory syncytial virus as causative agents of acute respiratory wheezing in children. Emerg Infect Dis. 2004;10:1095–101.
5. Schiff GM, Sherwood JR. Clinical activity of pleconaril in an experimentally induced coxsackievirus A21 respiratory infection. J Infect Dis. 2000;181:20–6. DOI: 10.1086/315176
6. Johnson KM, Bloom HH, Mufson A, Chanock RM. Acute respiratory disease associated with Coxsackie A-21 virus infection. I. Incidence in military personnel: observations in a recruit population. JAMA. 1962;179:112–9.
7. Jacques J, Moret H, Minette D, Lévêque N, Jovenin N, Deslée G, et al. Epidemiological, molecular, and clinical features of enterovirus respiratory infections in French children between 1999 and 2005. J Clin Microbiol. 2008;46:206–13. DOI: 10.1128/JCM.01414-07
8. Tapparel C, Junier T, Gerlach D, Van Belle S, Turin L, Cordey S, et al. New respiratory enterovirus and recombinant rhinoviruses among circulating picornaviruses. Emerg Infect Dis. 2009;15:719–26. DOI: 10.3201/eid1505.081286
9. Brown B, Oberste MS, Maher K, Pallansch MA. Complete genomic sequencing shows that polioviruses and members of human enterovirus species C are closely related in the noncapsid coding region. J Virol. 2003;77:8973–84. DOI: 10.1128/JVI.77.16.8973-8984.2003
10. Piralla A, Rovida F, Campanini G, Rognoni V, Marchi A, Locatelli F, et al. Clinical severity and molecular typing of human rhinovirus C strains during a fall outbreak affecting hospitalized patients. J Clin Virol. 2009;45:311–7. DOI: 10.1016/j.jcv.2009.04.016
11. van Doornum GJ, Schutten M, Voermans J, Guldemeester GJ, Niesters HG. Development and implementation of real-time nucleic acid amplification for the detection of enterovirus infections in comparison to rapid culture of various clinical specimens. J Med Virol. 2007;79:1868–76. DOI: 10.1002/jmv.21031
12. Lu X, Holloway B, Dare RK, Kuypers J, Yagi S, Williams JV, et al. Real-time reverse transcription–PCR assay for comprehensive detection of human rhinoviruses. J Clin Microbiol. 2008;46:533–9. DOI: 10.1128/JCM.01739-07
13. Nix WA, Oberste MS, Pallansch MA. Sensitive, seminested PCR amplification of VP1 sequences for direct identification of all enterovirus serotypes from original clinical specimens. J Clin Microbiol. 2006;44:2698–704. DOI: 10.1128/JCM.00542-06

14. Savolainen C, Blomqvist S, Mulders MN, Hovi T. Genetic clustering of all 102 human rhinovirus prototype strains: serotype 87 is close to human enterovirus 70. J Gen Virol. 2002;83:333–40.
15. Piralla A, Percivalle E, Di Cesare-Merlone A, Locatelli F, Gerna G. Multicluster nosocomial outbreak of parainfluenza virus type 3 infection in a pediatric oncohematology unit: a phylogenetic study. Haematologica. 2009;94:833–9. DOI: 10.3324/haematol.2008.003319

Address for correspondence: Giuseppe Gerna, Laboratori Sperimentali di Ricerca, Area Trapiantologica, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy; email: g.gerna@smatteo.pv.it

Whence Feral Vaccinia?

Richard C. Condit

When the World Health Organization declared smallpox eradicated in 1979, smallpox vaccination was discontinued worldwide. Although cessation of smallpox vaccination is well justified, given the risks associated with complications from the vaccine, lack of vaccination nevertheless creates a growing population of persons now susceptible to infection by a few poxviruses previously covered by the smallpox vaccine. These include the orthopoxviruses monkeypox; cowpox; and, ironically, vaccinia, the virus used for smallpox vaccination. Although few persons die from these infections, they are nevertheless a public health nuisance and expense. Thus, understanding the epidemiology of these viruses is in the interest of public health.

The most common vaccine-preventable poxvirus infections in humans are cowpox and monkeypox (*1*). Both are zoonoses; the natural host for each seems to be rodents, and transmission occurs through close contact. Monkeypox virus occurs in western and central Africa, causes a disseminated infection in humans, can be transmitted among humans at a low rate, and is associated with a 1%–10% (depending on the virus strain) case-fatality rate. Cowpox virus is relatively common in Europe and Asia, causes a limited exanthema, and does not often cause death of previously healthy persons.

Vaccinia, the virus used in the live smallpox vaccine, was originally isolated in the late 18th century from persons with illness that clinically resembled cowpox. However, modern genomics have shown that the vaccinia virus strains used for smallpox control in the 20th and 21st centuries are genetically distinct from cowpox viruses currently circulating. In fact, with 2 notable exceptions, vaccinia virus is not found in nature. However, vaccinia infection has been documented in India and Brazil (*2*). In India, some strains of buffalopox, transmitted to humans through buffaloes, appear to be vaccinia. Likewise, in Brazil, reports of a cowpox-like disease, caused by a vaccinia virus and transmitted from cattle to humans, have increased substantially since the first report in 2000. In both outbreaks (India and Brazil), evidence suggests that the original source of the virus was the smallpox vaccine virus that has been introduced into the wild—or feral vaccinia, as it has sometimes been called. The reservoirs for these viruses in the wild are not well understood.

In this issue of Emerging Infectious Diseases, Abrahão et al. (*3*) describe a serosurvey for orthopoxvirus among 344 wild animals from the Brazilian Amazonia ecosystem. The animals, 296 monkeys and a variety of other mammals, were captured by a fauna-rescue program during the construction of a hydroelectric plant in Tocantins State, Brazil, far removed from other human activity. Of these animals, 84 (24%), predominantly monkeys, were seropositive for orthopoxvirus. Furthermore, 18 serum samples were positive for orthopoxvirus DNA according to PCR. From these 18 positive samples, sequencing of 6 isolates revealed the vaccinia strain commonly associated with vaccinia outbreaks among cattle and humans in Brazil.

These finding suggest a remarkably high incidence of vaccinia infection in the Brazilian wilderness and in a host, namely monkeys, not normally considered as an active reservoir for orthopoxviruses. Although Abrahão et al. do not specifically identify monkeys as a primary reservoir for vaccinia virus and do not address the mode of transmission of the virus among these animals, the results suggest a substantial repository of vaccinia virus in the Brazilian wilderness. Especially given the broad host range of vaccinia, these findings warrant a substantial effort to characterize further the circulation of vaccinia virus in this region. If the results described in the article by Abrahão et al. (*3*) can be confirmed and expanded by other laboratories, they would have major implications for public health.

Dr Condit is a professor in the Department of Molecular Genetics and Microbiology at the University of Florida. His research interests are the fundamental mechanisms of poxvirus transcription and assembly.

References

1. Damon IK. Poxviruses. In: Knipe DM , Howley PM, editors, Fields virology. Philadelphia: Lippincott Williams & Wilkins; 2007. p. 2947–75.
2. Moussatché N, Damaso CR, McFadden G. When good vaccines go wild: feral *Orthopoxvirus* in developing countries and beyond. Journal of Infections in Developing Countries. 2008;2:156–73.
3. Abrahão JS, Silva-Fernandes AT, Lima LS, Campos RK, Guedes MIMC, Cota MMG, et al. Vaccinia virus infection in monkeys, Brazilian Amazon. Emerg Infect Dis. 2010;16:976–9.

Author affiliation: University of Florida, Gainesville, Florida, USA

DOI: 10.3201/eid1606.100315

Address for correspondence: Richard C. Condit, Department of Molecular Genetics and Microbiology, PO Box 100266, University of Florida, Gainesville, FL 32610, USA: email: condit@mgm.ufl.edu

The Wages of Original Antigenic Sin

David M. Morens, Donald S. Burke, and Scott B. Halstead

"The deliberate sin of the first man is the cause of original sin"

—[Saint] Augustine of Hippo, Algerian Christian theologian (354 AD–430 AD), De nuptiis et concupiscientia [On Marriage and Concupiscence], II, xxvi, 43

What epidemiologist Thomas Francis, Jr. (1900–1969) was thinking when pondering certain inexplicable serologic data from a 1946 influenza vaccine trial may never be known. Whether in religious reverence for the beauty of science or impish delight fueled by the martini breaks of which he was so fond, Francis coined the term "original antigenic sin" to describe a curious new immunologic phenomenon. Elsewhere in this issue, Adalja and Henderson propose that original antigenic sin has altered the population age–specific incidence of infection and disease caused by influenza A pandemic (H1N1) 2009 virus and that public health responses must account for the disruption (*1*). What is original antigenic sin, what is its immunologic basis, and into what sort of trouble is it getting us?

Discovery of influenza viruses in the early 1930s ignited a search to understand the epidemiology of pandemic/endemic influenza. Serologic data showed that decendents of the 1918 pandemic influenza virus were still circulating and were changing antigenically (we would now say drifting and undergoing intrasubtypic reassortment); that contemporary human and swine viruses were closely related; and that over a lifetime of repeated exposures, different human birth cohorts were acquiring fundamentally different influenza infection experiences. The surprise appearance in 1946 of a new and antigenically different influenza A virus (designated influenza A′ and recently shown to be a subtype H1N1 intrasubtypic reassortant) provided Francis a unique opportunity. College students participating in a 1946 trial of the old 1946 virus vaccine were infected in March 1947 with the new A′ virus. Surprisingly, these students developed low serologic titers to the new infecting virus and higher seroconverting titers to old viruses with which they previously had been infected. Moreover, recent recipients of the old virus vaccine had the highest seroconverting titers of all to the old—but not to the new—virus (*2*,*3*).

Absorption studies, in which various viruses were used to selectively remove serum antibodies, suggested that repeat exposures to dominant antigens of first-infecting viruses, when seen later as lesser or secondary antigens on subsequently infecting viruses, somehow reinforced antibody responses to the original strains at the apparent expense of responses to newer strains (*4*). Francis announced "the doctrine of original antigenic sin" (*5*,*6*): "[t]he antibody-forming mechanisms appear to be oriented by the initial infections of childhood so that exposures later in life to antigenically related strains result in a progressive reinforcement of the primary antibody" (*3*). Later studies by many investigators showed original antigenic sin to be a general phenomenon associated with numerous related/sequentially infecting virus strains that contain multiple external epitopes of varying cross-specificity (i.e., ability to elicit cross-reactive antibody), including antigenically drifting viruses such as influenza A, and the more stable flaviviruses, which circulate concurrently as multiple distinct viruses, virus serotypes, and virus strains (*7*,*8*).

Original antigenic sin seems to be most pronounced when sequential viruses are of intermediate antigenic relatedness; when they are antigenically complex; and when sequential exposure intervals are long, consistent with ongoing selection and expansion of lymphocyte clones that have increasing antibody avidity at key cross-reactive epitopes (*7*–*10*) and possibly with epitope competition between naïve and antigen-specific B cells (*8*). A phenomenon analogous to original antigenic sin also has been described with cytotoxic T lymphocytes (*11*). Although conclusive evidence in humans is lacking, original antigenic sin recently has come under scrutiny as a possible cause of viral immune escape, enhanced disease severity, decreased efficacy of influenza vaccines (*8*,*12*–*14*), and increased incidence of influenza in 2009 after vaccination with a related virus in 2008–2009 (*15*). On a positive note, original antigenic sin has also been linked to vaccine-induction of heterosubtypic neutralizing antibodies (*16*).

Adalja and Henderson note that the apparently lower incidence and severity of disease in older persons during the 2009–10 influenza pandemic probably reflects immunity to previously circulating influenza (H1N1) subtypes (*1*).

Author affiliations: National Institutes of Health, Bethesda, Maryland, USA, and Associate Editor, Emerging Infectious Diseases (D.M. Morens); University of Pittsburgh Graduate School of Public Health, Pittsburgh, Pennsylvania, USA, and former Editorial Board member, Emerging Infectious Diseases (D.S. Burke); and Uniformed Services University of the Health Sciences, Bethesda, and Editorial Board, Emerging Infectious Diseases (S.B. Halstead)

DOI: 10.3201/eid1606.100453

Reichert et al. also attribute this age structure to original antigenic sin but emphasize the importance of exposures to the changing hemagglutinin glycosylation patterns of earlier influenza (H1N1) subtypes (e.g., those circulating before and after 1948) on a background of relatively conserved T-cell epitopes (*14*). However, the possibility that the age structure of pandemic (H1N1) 2009 infection is due simply to single or repeated exposures to different or differentially exposed hemagglutinin epitopes has not been ruled out. Useful information bearing on these questions might be gained by comparing antibody levels, antibody reactivities, and the original antigenic sin phenomenon in serum samples from the various age cohorts that had early exposures to markedly different (or to no) influenza (H1N1) serotypes, e.g., persons born before 1918; during 1918–1927, 1928–1946, 1947–1956, and 1957–1976; and after 1976. Of related interest are the 2009 influenza experiences of the ≈25.6 million persons living in America vaccinated with the 1976 Hsw1N1 vaccine (*17*), including 2.5 million born during 1957–1975, when influenza (H1N1) viruses did not circulate

The current pandemic provides the challenge to public health responses that Adjala and Henderson describe, as well as an opportunity to extend the efforts of Francis to better understand the complicated epidemiology of influenza. Is original antigenic sin really a sin from which our immune systems need to be saved? Or is it an epidemiologic blessing in disguise? We have much more to learn. As St. Augustine wrote (Confessiones, 8, 7): "Lord make me chaste—but not yet."

Dr Morens is an epidemiologist and historian who serves as senior advisor to the director, National Institute of Allergy and Infectious Diseases. His interests include infectious disease pathogenesis, determinants of disease emergence, and the history of infectious diseases.

References

1. Adalja AA, Henderson DA. Original antigenic sin and pandemic (H1N1) 2009 [letter]. Emerg Infect Dis. 2010;16:1028–9.
2. Francis T, Salk JE, Quilligan JJ. Experience with vaccination against influenza in the spring of 1947. Am J Public Health. 1947;37:1013–6. DOI: 10.2105/AJPH.37.8.1013
3. Davenport FM, Hennessy AV, Francis T. Epidemiologic and immunologic significance of age distribution of antibody to antigenic variants of influenza virus. J Exp Med. 1953;98:641–55. DOI: 10.1084/jem.98.6.641
4. Jensen KE, Davenport FM, Hennessy AV, Francis T. Characterization of influenza antibodies by serum absorption. J Exp Med. 1956;104:199–209.DOI: 10.1084/jem.104.2.199
5. Francis T. The current status of the control of influenza. Ann Intern Med. 1955;43:534–8. Medline
6. Francis T. On the doctrine of original antigenic sin. Proc Am Philos Soc. 1960;104:572–8.
7. Halstead SB, Rojanasuphot S, Sangkawibha N. Original antigenic sin in dengue. Am J Trop Med Hyg. 1983;32:154–6.
8. Kim JH, Skountzou I, Compans R, Jacob J. Original antigenic sin responses to influenza viruses. J Immunol. 2009;183:3924–301. DOI: 10.4049/jimmunol.0901495
9. Fazekas de St. Groth S. Webster RG. Disquisitions of original antigenic sin. I. Evidence in man. J Exp Med. 1966;124:331–45. DOI: 10.1084/jem.124.3.331
10. Virelizier J-L, Allison AC, Schild GC. Antibody responses to antigenic determinants of influenza virus hemagglutinin. II. Original antigenic sin: a bone marrow–derived lymphocyte memory phenomenon modulated by thymus-derived lymphocytes. J Exp Med. 1974;140:1571–8. DOI: 10.1084/jem.140.6.1571
11. Klenerman P, Zinkernagel RM. Original antigenic sin impairs cytotoxic T lymphocyte responses to viruses bearing variant epitopes. Nature. 1998;394:482–5. DOI: 10.1038/28860
12. Powers DC, Belshe RB. Vaccine-induced antibodies to heterologous influenza A H1N1 viruses: effects of aging and "original antigenic sin." J Infect Dis. 1994;169:1125–9.
13. Gulati U, Kumari K, Wu W, Keitel WA, Air GM. Amount and avidity of serum antibodies against native glycoproteins and denatured virus after repeated influenza whole-virus vaccination. Vaccine. 2005;23:1414–25. DOI: 10.1016/j.vaccine.2004.08.053
14. Reichert T, Chowell G, Nishiura H, Christiensen RA, McCullers JA. Does glycosylation as a modifier of original antigenic sin explain the case age distribution and unusual toxicity in pandemic novel H1N1 influenza? BMC Infect Dis. 2010;10:5. DOI: 10.1186/1471-2334-10-5
15. Skowronski DM, De Serres G, Crowcroft NS, Janjua NZ, Boulianne N, Hottes TS, et al.; Canadian SAVOIR Team. Association between the 2008–09 seasonal influenza vaccine and pandemic H1N1 illness during Spring–Summer 2009: four observational studies from Canada. PLoS Med. 2010;7:e1000258.
16. Corti D, Suguitan AL Jr, Pinna D, Silacci C, Fernandez-Rodriguez BM, Vanzetta F, et al. Heterosubtypic neutralizing antibodies are produced by individuals immunized with a seasonal influenza vaccine. J Clin Investig. 2010;120:1663–73.
17. Kash JC, Li Q, Dugan VG, Jagger BW, Hrabal RJ, Memoli MJ, et al. Prior infection with classical swine H1N1 influenza viruses is associated with protective immunity to the 2009 pandemic H1N1 virus. Influenza Other Respir Viruses. 2010;4:121–7. DOI: 10.1111/j.1750-2659.2010.00132.x

Address for correspondence: David M. Morens, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bldg 31, Rm 7A-03, 31 Center Dr, Bethesda, MD 20892, USA; email: dm270q@nih.gov

Photo Quiz

Who is this man?

Here is a clue: He died in a self-experiment that showed that verruga peruana and Oroya fever are etiologically related.

Who is he?

A) Albert Barton

B) Daniel Alcides Carrión

C) Max Kuczynski-Godard

D) Ernest Odriozola

E) Garcilasco de la Vega

Decide first. Then turn the page.

Daniel Alcides Carrión

Myron G. Schultz

This is a photograph of Daniel Alcides Carrión (1858–1885). Carrión was a medical student in Lima Peru. He died from an experiment that he performed on himself when he was 26 years old. Carrión's experiment showed that 2 strikingly different diseases endemic to Peru, verruga peruana and Oroya fever, are etiologically related to each other. In the light of modern knowledge, we now know that these 2 syndromes, 1 benign, the other pernicious, are both caused by the hemotrophic bacterium, *Bartonella bacilliformis*. Verruga peruana and Oroya fever are now called Carrión disease. Carrión's motives for his self-experiment were initially misunderstood by his contemporaries, and subsequently by some medical historians. Nonetheless, his self-sacrifice has been honored by Peruvians for the past century, and he is the foremost hero of Peruvian medicine. The day of his death, October 5, is celebrated yearly as the "Dia de la Medicina Peruana," and the Peruvian National University in Cerro de Pasco carries his name.

From pre-Inca times, through the Spanish Conquest, to the "Guano Age" of the 19th century and up to the modern period, Carrión disease has played a role in the life of Peru. It is distributed in the Andean cordillera in certain mountain valleys that are positioned at right angles to the prevailing wind. It is in this special environment that the diminutive sandfly vectors, of which *Lutzomyia verrucarum* (formerly *Phlebotomus verrucarum*) is the most important species, can survive.

Verruga peruana, which is usually a benign condition, is clinically manifested as multiple eruptions of disfiguring, hemangioma-like tumors. Oroya fever, in contrast, is a disease of fever and anemia, with a high case-fatality rate. The disease acquired its name from the epidemic in 1871 that struck the workers on the Oroya railroad line at Cocachacra, 40 miles from Lima and 5,000 feet above sea level. It was commonly said that "every tie in the railroad had cost a life." Although this disease became known as Oroya fever, it occurred at a considerable distance from the city of Oroya and it has never been contracted there.

Author affiliation: Centers for Disease Control and Prevention, Atlanta, Georgia, USA

DOI: 10.3201/eid1606.091937

When Daniel Carrión undertook his experiment, he was in his sixth year of training at the Facultad de Medicina in Lima. To qualify for his medical degree he was required to prepare an original thesis, and he had devoted himself with increasing vigor during the preceding 3 years to a study of the epidemiology and clinical manifestations of verruga peruana. It was a work in which he invested his emotions as well as his intellect. As a young boy, he had made frequent trips with his uncle through the Peruvian mountains, going to and from school in Lima to his home in Cerro de Pasco, a mining town at the height of the Andes. He saw people with verrugas during these trips, which made a deep impression on him. He later told a classmate that through his research he hoped "to make an important contribution to aching humanity."

As his studies progressed, Carrión became concerned with the difficulty in diagnosing verruga peruana before the eruption appeared. He was vaguely aware that the appearance of the verrugas was preceded by fever and anemia, but his chief concern was the confusion between the prodromal phase of verruga and other febrile disorders, particularly malaria. Carrión thought that if verruga peruana could be recognized early enough, more effective treatment could be applied by the local practitioners. Gradually, Carrión came to the conclusion that the most effective way to study the incubation period and symptoms of verruga peruana would be to inoculate himself. As the months went by, his determination to do this became more intense. He often spoke of his plan to his friends and professors, and they repeatedly tried to dissuade him. When the Academia Libre de Medicine set up a prize competition on the subject of verruga peruana, Carrión could no longer be stopped.

On the morning of August 27, 1885, Carrión was in the Nuestra Señora de las Mercedes ward of the Dos de Mayo Hospital in Lima. In bed no. 5 was Carmen Paredes, a 14-year-old boy with a verruga on his right eyebrow. The boy was anxious to leave the hospital, and Carrión became concerned that he would miss the chance to inoculate himself before his forthcoming vacation period. Using a lancet, Carrion tried to inoculate his arm with blood taken from the verruga. He succeeded with the assistance of Dr Chavez, a young ward physician.

Although Carrión kept a diary of his clinical illness, he did not record his thoughts until the first symptoms appeared, 21 days after the inoculation. On September 17, Carrión felt discomfort and pain in his left ankle. Two days later, fever began. He also experienced chills, abdominal cramps, and pain in all the bones and joints in his body. He was unable to eat anything, and he commented in his diary that his thirst was devastating. His urine became dark red and scanty, and his skin took on an icteric tint. By September 26, he had become too weak to maintain his diary. This task was taken up by his classmates who were keeping vigil at his bedside. They were amazed at how quickly anemia had developed. At the same time, a systolic murmur developed and grew in intensity. On October 1, fasciculations appeared in his arm muscles. Carrión's health was failing, but he was thinking clearly enough to say to his friends: "Up to today, I thought I was only in the invasive stage of the verruga as a consequence of my inoculation, that is, in the period of anemia that precedes the eruption. But now I am deeply convinced that I am suffering from the fever that killed our friend, Orihuela. Therefore, this is the evident proof that Oroya fever and the verruga have the same origin, as Dr. Alarco once said."

This remarkable insight expressed the essence of Carrión's experiment. He had not, as is often said, set out to prove the single cause of verruga peruana and Oroya fever. He had merely intended to study the onset of verruga peruana; yet, when a completely different disease developed, he was able, despite his grave state, to grasp the full meaning of his experiment.

On the evening of October 3, Carrión was completely delirious and rambled on about the different opinions that existed on the pathology of verruga peruana. On October 5, thirty-nine days after the inoculation, he fell into a coma. Most of what he uttered was incomprehensible, but his last words were heard clearly by one of his friends. He said "Enrique, c'est fini [it is finished in French]." At 11:30 PM he died.

Scientific understanding of the disease that killed Daniel Carrión has grown substantially in the 125 years since his death. The etiologic agent, vector, laboratory manipulation of the organism, treatment, and prevention of Carrión disease have all been elucidated. Carrión disease is recognized as 2 different clinical manifestations of human bartonellosis due to *Bartonella bacilliformis* infection. In recent years, bartonellosis has also been recognized as a group of emerging zoonotic infectious diseases comprised of at least a dozen species of the genus *Bartonella*, which can cause cat-scratch fever, trench fever, bacillary angiomatosis, and peliosis hepatitis.

Advances in medical science come about through the collective work of hundreds of thousands of investigators. Only on rare occasions do investigators dare to experiment on themselves and seldom do these experiments end fatally. Daniel Carrión is a martyr whose courage should be remembered by current and future generations. In his short life as an unknown medical student, he was able to fulfill his wish to "make an important contribution to aching humanity." Not only did he demonstrate the unitary etiology of verruga peruana and Oroya fever, he also revealed the phenomenon that diverse diseases can be produced by a single pathogen.

Dr Schultz is a senior medical officer in the Global Disease Detection Operations Center at the Centers for Disease Control and Prevention (CDC). Formerly, he was director, Parasitic Diseases Division, and worked in the Epidemiology Program Office at CDC. He has published articles on medical history in numerous medical journals and writes the text for CDC's annual contest, Great Moments in Public Health.

Suggested Reading

1. Altman LK. Who goes first? New York: Random House; 1987. p. 1–5.
2. Chomel BB, Boulouis H-J, Maruyama S, Breitschwerdt EB. *Bartonella* spp. in pets and effect on human health. Emerg Infect Dis. 2006;12:389–94.
3. Leonard J. Daniel Carrión and Carrión's disease. Bull Pan Am Health Organ. 1991;25:258–66.

Address for correspondence: Myron G. Schultz, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop D69, Atlanta, GA 30333, USA; email: mgs1@cdc.gov

Original Antigenic Sin and Pandemic (H1N1) 2009

To the Editor: While pandemic (H1N1) 2009 was in its earliest stages, age distribution data indicated surprisingly few cases among persons >65 years of age. The initial assumption was that few persons >65 years of age had yet to be exposed. However, as more data became available from Mexico, Australia, and the United States, the age distribution pattern persisted (*1*).

This observation raised the question about whether older persons were protected from infection with an influenza virus A (H1N1) strain acquired many years ago. Indeed, data from the Centers for Disease Control and Prevention showed that approximately two thirds of older persons have evidence of immunity to pandemic (H1N1) 2009 virus. In 1960, Thomas Francis proposed the hypothesis of original antigenic sin, a phenomenon whereby a person who as a child was first exposed to a specific influenza virus A would, throughout life, mount an immune response to the virus of childhood, even when exposed to other antigenically dissimilar influenza viruses. In effect, the original antibody response generated by the immune system against a specific influenza viral strain was hypothesized to have colored all future responses to influenza (*2*).

Serologic responses of humans and other mammals have supported this theory. A new hemagglutinin (HA) subtype emerged in 1918 that was responsible for the pandemic that year. Through 1956, the strain evolved, accumulating mutations. In an era before influenza viruses were subtyped was performed, the original 1918 influenza virus A (H1N1) was dubbed a swine strain, whereas the virus of the 1930s was known as influenza A. However, the amount of drift accrued by 1947 was enough to render the seasonal vaccine of the time ineffective, and the new drifted virus strain was named A′. Throughout the period, the virus continued to be the subtype H1N1, as it is now designated.

In 1956, Davenport and Hennessy examined the antibody responses of 3 different age cohorts, each of which received different monovalent influenza vaccines prepared with vaccine strains circulating at different earlier periods (*3*) (Table). Prevaccination serum samples confirmed the presence of antibodies specific to the influenza virus that circulated during each respective cohort's childhood.

Each of the 3 monovalent vaccines was administered to a group from each age cohort. Vaccination directed toward influenza strains distinct from the virus of childhood not only resulted in development of immunity to the vaccine strain but also boosted the immune response to the virus strain that circulated during each person's childhood, i.e., original antigenic sin was apparent in each age cohort. Several other studies with humans, ferrets, rats, and rabbits yielded similar results (*4*,5).

Evidence from more recent studies largely supported the veracity of original antigenic sin. In a 1976 study, persons were vaccinated with a virus that circulated in 1973, an antigenically drifted variant of the 1968 influenza virus A (H3N2), and the response was assessed. As in earlier studies, examination of the antibodies generated indicated that the vaccine-induced antibodies were not only to the 1973 variant it contained but also to the virus that had circulated earlier. As the hypothesis postulates, the vaccine-induced antibodies to the 1968 strain were more numerous than those to the actual vaccine strain (*6*). Results from a 1984 experiment that used cell cultures with donor lymphocytes were similar (*7*). A 1994 study found that current vaccine strains induced antibodies to the influenza virus circulating during the childhood of persons in each age cohort (*8*). An additional study, published in 2009, confirmed the presence of antigenic sin in mice and showed a greater tendency for live-virus vaccines to produce the phenomenon (*9*).

One recent study is at variance with the others. It showed that monoclonal antibodies generated through vaccination were highly specific to the current vaccine strain rather than to influenza strains that had circulated in the past (*10*).

At the advent of the 2009 pandemic, fears of a severe pandemic were rampant. However, any prior immunity that was present in the population would dampen the impact of the virus. Early reports confirmed that the virus was less common in groups of older adults. Vaccine recommendations for certain age groups were developed according to that pattern of illness.

Because influenza virus A (H1N1) circulated continually after 1918 until 1957, most persons born before 1957 had been infected primarily with subtype H1N1. According to the theory of original antigenic sin, these persons may have partial protection from severe disease from infection with the new influenza virus A (H1N1), i.e., pandemic (H1N1) 2009. Supporting this hypothesis is the paucity of infections in Mexico from persons now in their 50s and 60s and few reports in the United States or Australia of cases in this age group (*1*). This fact should inform policy decisions and merits further immunologic consideration.

Table. Influenza strains dominant for specific age cohorts from 1956 study*

Age cohort, y	Influenza strain
4–10 (born 1946–1952)	A′
17–28 (born 1928–1939)	A
>30 (born <1926)	Swine

*Adapted from (*3*).

Influenza surge planning is premised on a high incidence of illness among elderly persons, but if the current pattern of illness continues, healthcare facilities also should prepare to treat younger persons who may constitute the bulk of cases. Additionally, studies of persons born during 1957–1968 should be conducted to quantify antibody levels to pandemic (H1N1) 2009 virus, focusing on the degree of preexisting immunity that may have existed and was boosted by prior encounters with subtype H1N1 viruses

Amesh A. Adalja and D.A. Henderson

Author affiliations: University of Pittsburgh Medical Center, Baltimore, Maryland, USA

DOI: 10.3201/eid1606.091563

References

1. Centers for Disease Control and Prevention. Update: novel influenza A (H1N1) virus infections—worldwide, May 6, 2009. MMWR Morb Mortal Wkly Rep. 2009;58:453–8.
2. Francis T. On the doctrine of original antigenic sin. Proc Am Philos Soc. 1960;104:572–8.
3. Davenport FM, Hennessy AV. A serologic recapitulation of past experiences with influenza A; antibody response to monovalent vaccine. J Exp Med. 1956;104:85–97. DOI: 10.1084/jem.104.1.85
4. Fazekas de St Groth B, Webster RG. Disquisitions on original antigenic sin. II. Proof in lower creatures. J Exp Med. 1966;121:347–61. DOI: 10.1084/jem.124.3.347
5. Fazekas de St Groth B, Webster RG. Disquisitions on original antigenic sin. I. Evidence in man. J Exp Med. 1966;121:331–45. DOI: 10.1084/jem.124.3.331
6. Webster RG, Kasel JA, Couch RB, Laver WG. Influenza virus subunit vaccines. II. Immunogenicity and original antigenic sin in humans. J Infect Dis. 1976;134:48–58.
7. Yarchoan R, Nelson DL. Specificity of in vitro anti-influenza virus antibody production by human lymphocytes: analysis of original antigenic sin by limiting dilution cultures. J Immunol. 1984;132:928–35.
8. Powers DC, Belshe RB. Vaccine-induced antibodies to heterologous influenza A H1N1 viruses: effects of again and "original antigenic sin." J Infect Dis. 1994;169:1125–9.
9. Kim JH, Skountzou I, Compans R, Jacob J. Original antigenic sin responses to influenza viruses. J Immunol. 2009;183:3294–301. DOI: 10.4049/jimmunol.0900398
10. Wrammert J, Smith K, Miller J. Rapid cloning of high-affinity human monoclonal antibodies against influenza virus. Nature. 2008;453:667–72. DOI: 10.1038/nature06890

Address for correspondence: Amesh A. Adalja, Division of Infectious Diseases, University of Pittsburgh Medical Center, 3601 5th Ave, Ste 300, Pittsburgh, PA 15212, USA; email: ameshaa@aol.com

Swine Influenza A Vaccines, Pandemic (H1N1) 2009 Virus, and Cross-Reactivity

To the Editor: Since its first emergence in the human population in spring 2009 (*1*–*3*) infections with pandemic (H1N1) 2009 virus have been reported in pigs, turkeys, and some carnivore species (*4*,*5*). The pandemic (H1N1) 2009 virus can be experimentally transmitted between pigs (*6*). The reported transmissibility of the virus raises the question as to whether authorized swine influenza vaccine strains may be cross-reactive to pandemic (H1N1) 2009 virus. Kyriakis et al. (*7*) investigated the cross-reactivity of 66 pig serum samples from different infection and vaccination trials and reported cross-reactions between the avian-like H1N1 viruses circulating in the European pig population (avH1N1) and the classical swine H1N1 viruses (cH1N1) with pandemic (H1N1) 2009 virus by hemagglutination inhibition assay.

To investigate this cross-reactivity in more detail, a neutralization test was applied in the study we report here. A serial dilution of serum samples was prepared ($\log_4$). All virus strains were adjusted to 100 fifty-percent tissue culture infectious doses. This working dilution of virus was mixed with serum dilutions and incubated 1 hour at 37°C. Madin-Darby bovine kidney monolayers were infected with the neutralization mixtures. After 48 hours of incubation, cells were fixed with acetone (4°C–8°C) and investigated by indirect immunofluorescent assay. Finally, the 50% neutralization titer was calculated.

Hyperimmune serum samples were established by using a 4-fold vaccination of pigs with antigens of H1N1 vaccine strains (A/New Jersey/8/1976, A/sw/Netherlands/25/1980, A/sw/IDT/Re230/1992, A/sw/Haselünne/IDT2617/2003), and a strain of pandemic (H1N1) 2009 virus (A/Hamburg/7/2009) by using Freund adjuvant. Blood samples were taken 14 days after last immunization. A vaccine containing the pandemic (H1N1) 2009 virus was produced. Swine influenza vaccines available in central Europe and the newly produced vaccine containing pandemic (H1N1) 2009 virus (A/Hamburg/7/2009) were administered to pigs (2-fold vaccination with 1–2 mL of the vaccine 21–28 days apart intramuscularly). Blood was withdrawn 7 days after second administration.

In addition, an experimental aerosol infection was conducted by using the parental strain of the most recent avH1N1 strain contained in a European swine influenza vaccine (A/sw/Haselünne/IDT2617/2003). Blood samples were taken 10 days after infection.

The investigation of the hyperimmune serum samples detected neutralizing activity between the pandemic (H1N1) 2009 virus and European avH1N1 vaccine strains (A/sw/Netherlands/25/1980, A/sw/IDT/Re230/1992, A/sw/Haselünne/IDT2617/2003), as well as with the cH1N1 strain A/New Jersey/8/1976

(Fort Dix reassortant). The hyperimmune serum established against pandemic (H1N1) 2009 virus also showed cross-reactivity with European avH1N1 virus. The reactions against several strains of the pandemic virus were similar, reflecting high titers against pandemic (H1N1) 2009 virus but also cross-reactions with hyperimmune serum samples of all swine influenza A virus H1N1 vaccine strains (online Appendix Table, www.cdc.gov/EID/content/16/6/1029-appT.htm).

The bivalent vaccines induced high titers of neutralizing antibodies against avH1N1 virus and human-like H3N2 virus (huH3N2). Only a low number of pigs reacted with H1N2 virus whereas the trivalent vaccine induced high neutralizing activity in serum samples of all vaccinated pigs. The vaccines induced neutralizing antibodies against pandemic (H1N1) 2009 virus. The titers were lower in comparison to those obtained for avH1N1 and not all pigs responded. The reactions were best for the vaccines containing mineral oil. Pigs vaccinated with the trivalent vaccine with carbomer adjuvant showed almost no antibodies against pandemic (H1N1) 2009 virus, although the vaccine strain reacted well in hyperimmunization tests.

A vaccine batch of the trivalent vaccine was produced that contained mineral oil instead of carbomer. All pigs vaccinated with the trivalent vaccine with mineral oil had antibodies against the pandemic (H1N1) 2009 virus (data not shown). At the same time, efficacy trials with all authorized vaccines were conducted (*8*; T.W. Vahlenkamp, pers. comm.) in which all vaccines including the trivalent vaccine with carbomer adjuvant showed a comparable level of protection (limited period of viral shedding). Mineral oil adjuvants can induce severe distress in pig herds due to their limited safety. Despite cross-reactivity between avH1N1 and cH1N1 with pandemic (H1N1) 2009 virus, the highest degree of cross-neutralization was achieved by the vaccine containing pandemic (H1N1) virus strain.

Proof of cross-reactivity was also reflected in the infection trial. Pigs infected with avH1N1 responded to avH1N1 as well as to pandemic (H1N1) 2009 virus. All results were additionally confirmed by hemagglutination inhibition assay (data not shown).

Furthermore, 1,559 pig serum samples from 195 German pig herds collected from mid-June through mid-September 2009 were tested in routine diagnostics by hemagglutination inhibition assay. All reflected almost similar results for avH1N1 and the pandemic (H1N1) 2009 virus (seroprevalences for individual pigs were pandemic [H1N1] 2009 virus 52%, avH1N1 53%, huH1N2 28%, and huH3N2 52%; for pig herds pandemic [H1N1] 2009 virus 46%, avH1N1 46%, huH1N2 24%, and huH3N2 45%). These results suggest cross-reactivity between porcine H1N1 viruses and pandemic (H1N1) 2009 virus. Despite this cross-reactivity, a vaccine consisting of pandemic (H1N1) 2009 virus is superior in terms of efficacy in comparison with vaccines already authorized in Europe.

Acknowledgments

We thank Brunhilde Schweiger for providing the human influenza virus strains.

This work was supported by a grant of the German Bundesministerium für Bildung und Forschung (01KI07142 and 01KI07143) awarded to R.Z. and R.D.

Ralf Dürrwald, Andi Krumbholz, Sigrid Baumgarte, Michael Schlegel, Thomas W. Vahlenkamp, Hans-Joachim Selbitz, Peter Wutzler, and Roland Zell

Author affiliations: IDT Biologika GmbH, Dessau Rosslau, Germany (R. Dürrwald, M. Schlegel, H.-J. Selbitz); Universitätsklinikum Jena, Jena, Germany (A. Krumbholz, P. Wutzler, R. Zell); Institut für Umwelt und Hygiene, Hamburg, Germany (S. Baumgarte); and Friedrich-Loeffler-Institut, Insel Riems, Germany (T.W. Vahlenkamp)

DOI: 10.3201/eid1606.100138

References

1. Garten RJ, Davis CT, Russell CA, Shu B, Lindstrom S, Balish A, et al. Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. Science. 2009;325:197–201. DOI: 10.1126/science.1176225
2. Smith GJ, Vijaykrishna D, Bahl J, Lycett SJ, Worobey M, Pybus OG, et al. Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. Nature. 2009;459:1122–5. DOI: 10.1038/nature08182
3. Itoh Y, Shinya K, Kiso M, Watanabe T, Sakoda Y, Hatta M, et al. In vitro and in vivo characterization of new swine-origin H1N1 influenza viruses. Nature. 2009;460:1021–5.
4. Influenza pandemic (H1N1), animal. Latest information on influenza A (H1N1) [cited 2010 Jan 31]. http://www.promedmail.org
5. Hofshagen M, Gjerset B, Er C, Tarpai A, Brun E, Dannevig B, et al. Pandemic influenza A(H1N1)v: Human to pig transmission in Norway? Euro Surveill. 2009;14:pii=19406.
6. Brookes SM, Irvine RM, Nunez A, Clifford D, Essen S, Brown IH, et al. Influenza A (H1N1) infection in pigs. Vet Rec. 2009;164:760–1.
7. Kyriakis CS, Olsen CW, Carman S, Brown IH, Brookes SM, Van Doorsselaere J, et al. Serologic cross-reactivity with pandemic (H1N1) 2009 virus in pigs, Europe. Emerg Infect Dis. 2010;16:96–9.
8. Lange E, Kalthoff D, Blohm U, Teifke JP, Breithaupt A, Maresch C, et al. Pathogenesis and transmission of the novel swine-origin influenza virus A/H1N1 after experimental infection of pigs. J Gen Virol. 2009;90:2119–23. DOI: 10.1099/vir.0.014480-0

Address for correspondence: Ralf Dürrwald, IDT Biologika GmbH, Am Pharmapark, 06861 Dessau-Rosslau, Germany; email: ralf.duerrwald@idt-biologika.de

Human Anaplasmosis and *Anaplasma ovis* Variant

To the Editor: Anaplasmosis is a disease caused by bacteria of the genus *Anaplasma*. *A. marginale*, *A. centrale*, *A. phagocytophilum*, *A. ovis*, *A. bovis*, and *A. platys* are obligate intracellular bacteria that infect vertebrate and invertebrate host cells. *A. ovis*, which is transmitted primarily by *Rhipicephalus bursa* ticks, is an intraerythrocytic rickettsial pathogen of sheep, goats, and wild ruminants (*1*).

Anaplasma spp. infections in humans have been reported in Cyprus (*2*,*3*). We report infection of a human with a strain of *Anaplasma* sp. other than *A. phagocytophilum*, which was detected by PCR amplification of anaplasmatic 16S rRNA, major surface protein 4 (*msp4*), and heat shock protein 60 (*gro*EL) genes.

A 27-year-old woman was admitted to the pathology clinic of a hospital in Famagusta, Cyprus on May 14, 2007, with an 11-day history of fever (≤39.5°C) after a tick bite. Before admission, the patient was treated with cefixime (400 mg/d for 3 days) and cefradine (2 g/d for 2 days) without abatement of the fever. Physical examination showed hepatosplenomegaly and an enlarged lymph node.

Initial laboratory examinations showed moderate anemia (hemoglobin 11.5 g/dL), thrombocytopenia (95,000 thrombocytes/mm^3), increased levels of transaminases (aspartate aminotransferase 178 U/L, alanine aminotransferase 313 U/L, γ-glutamyl transferase 79 U/L, lactate dehydrogenase 698 U/L), an increased level of C-reactive protein (10.4 mg/L), and an increased erythrocyte sedimentation rate (80 mm/h). Blood samples were obtained from the patient at the time of admission and 7 days and 3 months later. Results of blood and urine cultures were negative for bacteria. A chest radiograph, computed tomography of the abdomen, and an echocardiograph of the heart showed unremarkable results. Blood samples were negative for antibodies against cytomegalovirus, Epstein-Barr virus, hepatitis, HIV, mycoplasma, coxackie virus, adenovirus, parvovirus, *Coxiella burnetii*, *R. conorii*, and *R. typhi*, and for rheumatoid factors. A lymph node biopsy specimen was negative for infiltration and malignancy. After treatment with doxycycline (200 mg/day for 11 days), ceftriaxone (2 g/day for 5 days), and imipenem/cilastatin (1,500 mg/day for 1 day), the patient recovered and was discharged 17 days after hospitalization.

Three serum samples from the patient were tested in Crete, Greece, for immunoglobulin (Ig) G and IgM against *A. phagocytophilum* antigen by using an immunofluorescent antibody assay (Focus Diagnostics, Cypress, CA, USA). Serologic analysis showed IgG titers of 0, 0, and 128 and IgM titers of 20, 20, and 20 against *A. phagocytophilum* in the 3 serum samples, respectively.

Because the blood samples were transported frozen, detection of morulae was not possible. DNA was extracted by using the QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany). PCR amplifications (MyCycler DNA thermal cycler; Bio-Rad, Hercules, CA, USA) were conducted for the anaplasmatic 16S rRNA gene; *A. marginale*, *A. centrale*, and *A. ovis* heat shock protein 60 (*groEL)* genes; and *A. marginale*, *A. centrale*, and *A. ovis* major surface protein 4 (*msp4*) genes (*4*,*5*). DNA from previous studies in Cyprus (*4*,*5*) was used as a positive control. Double-distilled water was used as a negative control.

PCR amplicons were purified by using the QIAquick Spin PCR Product Purification Kit (QIAGEN) and sequenced on a 4200 double-beam automated sequencer (LI-Cor, Inc., Lincoln, NE, USA). Sequences were processed by using ClustalW2 software (www.ebi.ac.uk/Tools/clustalw2/index.html) and the GenBank/European Molecular Biology database library (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic trees were constructed by using MEGA 4 software (www.megasoftware.net).

The first blood sample was positive for *A. ovis* by PCR; the other 2 were negative. A 16S rRNA gene sequence (EU448141) from the positive sample showed 100% similarity with other *Anaplasma* spp. sequences (*A. marginale*, *A. centrale*, *A. ovis*) in GenBank. *Anaplasma* sp. *groEL* and *msp4* genes showed a 1,650-bp sequence (FJ477840, corresponding to 748 of 1,650 bp) and an 852-bp sequence (FJ460443) for these genes, respectively. Phylogenetic trees (Figure) were constructed by using *A. ovis* strains detected in sheep and goats in Cyprus (*5*).

Fever is common in cases of human infection with *A. phagocytophilum* (*6*). We also detected thrombocytopenia and elevated levels of transaminases. However, hepatosplenomegaly, lymphadenopathy, and anemia are not common in persons infected with *A. phagocytophilum*.

Immunofluorescent antibody analysis showed weak antibody titers against *A. phagocytophilum*. Serologic cross-reactivity of *Anaplasma* spp. is caused by conservation of major surface protein sequences (*7*).

A. phagocytophilum infection usually resolves after treatment with doxycyline for 4 days (*8*). The patient reported here was treated with doxycycline for 11 days. However, 1 case is not sufficient to form conclusions on severity and duration of illness.

In a study conducted in Cyprus, *Anaplasma* sp. was identified in birds (*9*). Because birds may be carriers of zoonotic pathogens, infection of humans with these pathogens may occur. However, transmission of *A. ovis* to humans is unclear. The role of *R. bursa*, a common tick species in sheep and goats in Cyprus (D.

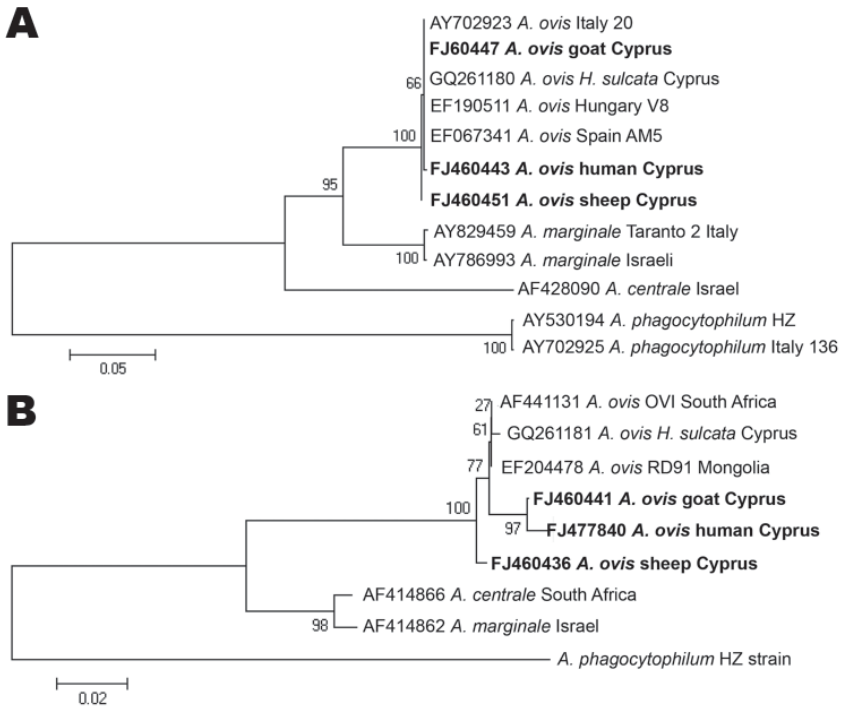


Figure. Evolutionary trees based on major surface protein 4 (A) and heat shock protein 60 (B) genes sequences of *Anaplasma phagocytophilum*, *A. marginale*, and *A. ovis*. Evolutionary history was inferred by using the neighbor-joining method. *H. sulcata*; *Haemaphysalis sulcata*. A) Optimal tree (branch length = 0.87919908) is shown. Percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown. B) Optimal tree (branch length = 0.34047351) is shown. Percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown. Trees are drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Evolutionary distances were computed by using the Kimura 2-parameter method. Strains detected in Cyprus are indicated in **boldface**. Scale bars indicate number of base substitutions per site.

Chochlakis, unpub. data), as a vector of other pathogens for humans has been proposed (*10*). Whether these pathogens include *A. ovis* is unknown. Thus, laboratory testing of human blood samples should include universal primers against all *Anaplasma* spp. to avoid missing cases such as the one we report.

Dimosthenis Chochlakis, Ioannis Ioannou, Yannis Tselentis, and Anna Psaroulaki

Author affiliations: University of Crete, Heraklion, Greece (D. Chochlakis, Y. Tselentis, A. Psaroulaki); and Veterinary Services, Nicosia, Cyprus (I. Ioannou)

DOI: 10.3201/eid1606.090175

References

1. de la Fuente J, Atkinson MW, Naranjo V, Fernandez de Mera IG, Mangold AJ, Keating KA, et al. Sequence analysis of the *msp4* gene of *Anaplasma ovis* strains. Vet Microbiol. 2007;119:375–81. DOI: 10.1016/j.vetmic.2006.09.011
2. Chochlakis D, Koliou M, Ioannou I, Tselentis Y, Psaroulaki A. Kawasaki disease and *Anaplasma* sp. infection of an infant in Cyprus. Int J Infect Dis. 2009;13:e71–3. DOI: 10.1016/j.ijid.2008.08.001
3. Psaroulaki A, Koliou M, Chochlakis D, Ioannou I, Mazeri S, Tselentis Y. *Anaplasma phagocytophilum* infection in a child. Pediatr Infect Dis J. 2008;27:664–6. DOI: 10.1097/INF.0b013e31816a0606
4. Chochlakis D, Ioannou I, Sharif L, Kokkini S, Hristophi N, Dimitriou T, et al. Prevalence of *Anaplasma* sp. in goats and sheep in Cyprus. Vector Borne Zoonotic Dis. 2009;9:457–63. DOI: 10.1089/vbz.2008.0019
5. Psaroulaki A, Chochlakis D, Sandalakis V, Vranakis I, Ioannou I, Tselentis Y. Phylogentic analysis of *Anaplasma ovis* strains isolated from sheep and goats using *groEL* and *mps4* genes. Vet Microbiol. 2009;138:394–400. DOI: 10.1016/j.vetmic.2009.04.018
6. Dumler JS, Choi KS, Garcia-Garcia JC, Barat NS, Scorpio DG, Garyu JW, et al. Human granulocytic anaplasmosis and *Anaplasma phagocytophilum*. Emerg Infect Dis. 2005;11:1828–34.
7. Palmer GH, Abbott JR, French DM, McElwain TF. Persistence of *Anaplasma ovis* infection and conservation of the *msp-2* and *msp-3* multigene families within the genus *Anaplasma*. Infect Immun. 1998;66:6035–9.
8. Bakken JS, Dumler JS. Clinical diagnosis and treatment of human granulocytotropic anaplasmosis. Ann N Y Acad Sci. 2006;1078:236–47. DOI: 10.1196/annals.1374.042
9. Ioannou I, Chochlakis D, Kasinis N, Anayiotos P, Lyssandrou A, Papadopoulos B, et al. Carriage of *Rickettsia* spp., *Coxiella burnetii* and *Anaplasma* spp. by endemic and migratory wild birds and their ectoparasites in Cyprus. Clin Microbiol Infect. 2009 Mar 11. [Epub ahead of print].
10. Merino FJ, Nebreda T, Serrano JL, Fernández-Soto P, Encinas A, Pérez-Sánchez R. Tick species and tick-borne infections identified in population from a rural area of Spain. Epidemiol Infect. 2005;133:943–9. DOI: 10.1017/S0950268805004061

Address for correspondence: Anna Psaroulaki, Laboratory of Clinical Bacteriology, Parasitology, Zoonoses and Geographical Medicine (World Health Organization Collaborating Center), Medical School, University of Crete, Heraklion, Greece; email: annapsa@med.uoc.gr

Diagnostic Difficulties with *Plasmodium knowlesi* Infection in Humans

To the Editor: Studies conducted in Malaysia have raised questions about *Plasmodium knowlesi* as the fifth human pathogenic malaria parasite (*1*,*2*); additional cases of *P. knowlesi* malaria have subsequently been reported from other Asian countries (*3*–*5*). Microscopic diagnosis is hindered because *P. knowlesi* morphologically resembles *P. falciparum* or *P. malariae*, depending on blood stage (*6*). Singh et al. has designed a nested PCR assay for identification of *P. knowlesi* infections (*1*).

As part of an ongoing research project focusing on characterizing genes from malaria isolates in Indonesia (E. Sulistyanisih, unpub. data), during December 2008–February 2009, blood samples from 22 gold miners with uncomplicated malaria were collected in South Kalimantan Province in Indonesia. Ring forms typical for *P. falciparum* were seen during microscopy. DNA was extracted and species were identified by nested PCR by using *Plasmodium* genus- and species-specific primers derived from the small subunit RNA gene described elsewhere (*1*). PCR products were directly sequenced and verified by 2 independent amplifications of the same DNA sample. PCR using *P. knowlesi*–specific primers yielded a 153-bp product in samples from 4 of the 22 malaria cases. Sequencing showed perfect matching with the recently published *P. knowlesi* S-type from Malaysian Borneo for 1 of the 4 samples. The other sequences were repeatedly consistent with the small subunit RNA gene of sporozoite *P. vivax* (S-type), and random blasting (http://blast.ncbi.nlm.nih.gov) showed higher homology (93%–100%) with various *P. vivax* strains than with different *P. knowlesi* (<84%) or other *Plasmodium* strains. The vivax-specific PCR showed the expected bands in each case, and sequencing confirmed *P. vivax* A-type DNA that matched perfectly with a strain from Thailand. Of the miners with malaria, 3 case-patients were coinfected with *P. falciparum*. All 22 samples from the case-patients were negative for *P. malariae*. One case-patient (P 15) infected with *P. knowlesi* (4,000 parasite ring forms/μL) had a mixed infection with *P. vivax* and was successfully treated with chloroquine-primaquine (Table).

The results of this study indicate the geographic distribution of natural *P. knowlesi* human infections includes Indonesian Borneo, although this detection is no surprise because many *P. knowlesi* isolates are found in Malaysian Borneo *(1,2).* However, the diagnosis would have been unrecognized without molecular techniques, and even those techniques posed a problem.

The species-specific nested PCR assay repeatedly showed bands of 153 bp, indicating 4 *P. knowlesi* cases, but sequencing confirmed *P. knowlesi* in only 1 sample. There was no indication of contamination of the samples tested by PCR, and the other 18 samples and the negative control remained negative for *P. knowlesi*. All 3 samples showed molecularly confirmed mixed infections with *P. falciparum* and *P. vivax* in the case-patients. As *P. vivax* was only molecularly detected, low parasitemia was assumed.

The reverse primer sequence (pmkr 9) is found in *P. vivax* S-type strains and other *Plasmodium* spp., especially those related to *P. vivax*, thus, amplification from this site should be theoretically possible. The forward primer pmk 8, on the other hand, seemed to be highly specific.

One *Plasmodium* strain (GenBank accession no. DQ660817) found in orangutans in Kalimantan, Indonesia, and classified as *P. vivax,* seemed to be more likely to bind to pmk 8 (*7*). However, this classification was recently disproved by Singh and Divis (*8*), and the parasite was categorized as probably being *P. pitheci* or *P. silvaticum*, where human infections are not described. Other primate malaria parasites, such as *P. hylobati*, *P. inui*, *P. cynomolgi*, *P. simium*, *P. fieldi*, and *P. simiovale*, showed better binding sites for pmk 8 than *P. vivax* S- or A-strains. Regarding the theory of *P. vivax* originating in macaques in Southeast Asia and the close relationship to other primate malaria parasites (*9*), one might imagine that *P. vivax* strains in Indonesia differ slightly from the strains described so far. A *P. vivax* isolate from Indonesia, recently sequenced in cooperation with the University of Heidelberg (GenBank accession no. GU233452), showed 2 point mutations; the patient had been in Flores, Bali, and Lembata. However, the 3 *P. vivax* samples presented no mutations at the pmk 8 binding sites. Notably, pmk 8 and pmkr 9 seem always to amplify the S-type and the rVIV 1

Table. Profile of *Plasmodium knowlesi*–positive patients, South Kalimantan Province, Indonesia, December 2008–February 2009*

Patient no.	Age, y	Microscopy-based diagnosis	PCR-based diagnosis	*P. knowlesi*–specific PCR for quality of 153-bp band	Sequence analysis of 153-bp sequence
8	35	*P. falciparum*	*P. falciparum, P. vivax, P. knowlesi*	Strong	*P. vivax*
9	41	*P. falciparum*	*P. falciparum, P. vivax, P. knowlesi*	Strong	*P. vivax*
14	54	*P. falciparum*	*P. falciparum, P. vivax, P. knowlesi*	Weak	*P. vivax*
15	16	*P. falciparum*	*P. knowlesi, P. vivax*	Weak	*P. knowlesi* (GU233448)†

*All patients were men who received a diagnosis of uncomplicated malaria.
†GenBank accession number.

and rVIV 2 primers, the A-type DNA, respectively. The genus-specific DNA amplified both types at random.

Some colleagues have experienced similar difficulties with the primers pmk 8 and pmkr 9 in samples from Vietnam (*5*); 2 of 5 samples gave false positive results for *P. knowlesi*. Unfortunately, their report did not mention which species was actually amplified (*5*).

Until recently, we had no satisfying explanation for the 3 assumed false-positive results. Then, in 2009, Imwong et al. reported that these *P. knowlesi* primers stochastically cross-react with *P. vivax* genomic DNA. No polymorphisms alleviating the binding of pmk8 were found; however, a new PCR for *P. knowlesi* was introduced (*10*).

Given the large distribution of the vector and the natural host of *P. knowlesi* in Southeast Asia, it is likely that *P. knowlesi* will be found in other parts of Indonesia. As microscopic and molecular diagnosis of this parasite seems difficult, the underestimation of its distribution and clinical relevance can be assumed.

Acknowledgments

We thank Janet Cox-Singh and colleagues for providing the *P. knowlesi*–positive control (*P. knowlesi* S-strain).

Erma Sulistyaningsih, Loeki Enggar Fitri, Thomas Löscher, and Nicole Berens-Riha

Author affiliations: University of Jember, Jember, Indonesia (E. Sulistyaningsih); Brawijaya University, Malang, Indonesia (L.E. Fitr); and Universität München, Munich, Germany (E. Sulistyaningsih, T. Löscher; N. Berens-Riha)

DOI: 10.3201/eid1606.100022

References

1. Singh B, Kim Sung L, Matusop A, Radhakrishnan A, Shamsul SS, Cox-Singh J, et al. A large focus of naturally acquired *Plasmodium knowlesi* infections in human beings. Lancet. 2004;363:10017–24.
2. White NJ. *Plasmodium knowlesi*: the fifth human malaria parasite. Clin Infect Dis. 2008;46:172–3. DOI: 10.1086/524889
3. Jongwutiwes S, Putaporntip C, Iwasaki T, Sata T, Kanbara H. Naturally acquired *Plasmodium knowlesi* malaria in human, Thailand. Emerg Infect Dis. 2004;10:2211–3.
4. Luchavez J, Espino F, Curameng P, Espina R, Bell D, Chiodini P, et al. Human infections with *Plasmodium knowlesi*, the Philippines. Emerg Infect Dis. 2008;14:811–3. DOI: 10.3201/eid1405.071407
5. Van den Eede P, Van NH, Van Overmeir C, Vythilingam I, Duc TN, Hung LX, et al. Human *Plasmodium knowlesi* infections in young children in central Vietnam. Malar J. 2009;8:249–54. DOI: 10.1186/1475-2875-8-249
6. Cox-Singh J, Singh B. Knowlesi malaria: newly emergent and of public health importance? Trends Parasitol. 2008;24:406–10. DOI: 10.1016/j.pt.2008.06.001
7. Reid MJ, Ursic R, Cooper D, Nazzari H, Griffiths M, Galdikas BM, et al. Transmission of human and macaque *Plasmodium* spp. to ex-captive orangutans in Kalimantan, Indonesia. Emerg Infect Dis. 2006;12:1902–8.
8. Singh B, Divis PCS. Orangutans not infected with *Plasmodium vivax* or *P. cynomolgi*, Indonesia. Emerg Infect Dis. 2009;15:1657–8.
9. Escalante AA, Cornejo OE, Freeland DE, Poe AC, Durrego E, Collins WE, et al. A monkey's tale: the origin of *Plasmodium vivax* as a human malaria parasite. Proc Natl Acad Sci U S A. 2005;102:1980–5. DOI: 10.1073/pnas.0409652102
10. Imwong M, Tanomsing N, Pukrittayakamee S, Day NPJ, White NJ, Snounou G. Spurious amplification of *Plasmodium vivax* small subunit RNA gene using the primers currently used to detect *P. knowlesi.* J Clin Microbiol. 2009;47:4173–5. Epub 2009 Oct 7. DOI: 10.1128/JCM.00811-09

Address for correspondence: Nicole Berens-Riha, Leopoldstrasse 5, 80802 Munich, Germany; email: berens@lrz.uni-muenchen.de

Toscana Virus Infection Imported from Elba into Switzerland

To the Editor: Toscana virus (TOSV) is a serotype of *Sandfly fever Naples virus* (SFNV) within the family *Bunyaviridae* and the genus *Phlebovirus*. TOSV is transmitted to humans by sandflies (*Phlebotomus* spp.) and is a prominent cause of aseptic meningitis in Mediterranean countries (*1*). In Italy, for populations living in rural areas and persons engaging in outdoor activities, the highest risk for acquiring TOSV is from August through October (*1*). TOSV infections should therefore be considered in travelers returning from the Mediterranean area who have fever and signs of meningitis. Laboratory diagnosis of TOSV infections is often limited to the detection of immunoglobulin (Ig) M and IgG because of the short period of viremia and the low amount of virus in the cerebrospinal fluid (CSF) during the acute phase (*2*). We report a reverse transcription–PCR (RT-PCR)–confirmed TOSV infection acquired on the island of Elba that was then imported into Switzerland.

A 17-year-old man was referred to Basel University Medical Clinic, Liestal, Switzerland, in August 2009 with headache, recurrent episodes of vomiting, photophobia and phonophobia, and an elevated temperature of 38.1°C. The patient had returned to Switzerland from a vacation on the island of Elba, Italy, 14 days before. He recalled that he had received multiple insect bites on the beach. Cardiopulmonary and neurologic examination showed tachycardia and nuchal rigidity. Results of a complete blood count and liver and kidney function tests showed no abnormalities. CSF analysis showed lymphocytic pleocytosis (47 cells/μL), and aseptic meningitis of viral origin was suspected. Empirical treatment with acyclovir (2.3 g/day) was started for the first 48 hours. The results of a

PCR for herpesviruses were negative in the CSF sample, and serologic testing showed no evidence of acute infection with herpesviruses. CSF, urine, and blood cultures showed negative results for fungi and bacteria, including mycobacteria. The patient did not show signs of immune deficiency.

Serum and CSF samples were sent to the Bernhard-Nocht-Institute for Tropical Medicine in Germany for SFNV diagnostics. Results of immunofluorescent assays for TOSV and SFNV were positive with IgM titers of 1,280 and 160, respectively (cut-off 20) and IgG titers of 5,120 and 640, respectively (cut-off 20). Real-time RT-PCRs for detection of TOSV and SFNV were performed using the CSF sample according to a recently published protocol (*3*). A positive result was obtained for TOSV, and the PCR result was confirmed by sequencing the PCR product. Phylogenetic analysis demonstrated that the TOSV from Elba clustered with the TOSV A lineage (Figure). Attempts to isolate TOSV from the CSF sample in cell culture failed. The patient was afebrile on the second day of hospitalization, headache vanished on the third day, and he was discharged on day 5 restitutio ad integrum (fully recovered).

This report demonstrates the presence of TOSV on the island of Elba by molecular detection and typing. This finding is in agreement with previous serologic reports on imported TOSV infections from this area into central Europe (*5*,*6*). However, because of serologic cross-reactivity, serologic tests are usually not able to clearly discriminate between TOSV and other SFNV infections (*2*).

Real-time RT-PCR is the most appropriate tool for the differentiation of TOSV from other SFNV infections and enables molecular typing of amplified sequences. The Bayesian phlyogenetic tree calculated with the short PCR fragment (111 bp, GenBank accession no. GU270841) of the nucleocapsid coding sequence (Figure) shows the same topology of the main clades when compared with trees obtained with the complete N coding sequence (*7*,*8*). The assignment of the TOSV from Elba to lineage A (Figure) is consistent with results of previous studies, demonstrating that this is the dominant genotype of TOSV in mainland Italy and the island of Sardinia (*8*).

The presence of TOSV A on the island of Elba is a major public health issue for the local population and for the >2 million tourists that visit Elba every year (*9*). Given the incidence of TOSV infections in other surrounding Mediterranean countries, one could assume that the virus is present in other islands of the Mediterranean, posing a public health problem for the resident population and tourists alike. Molecular and serologic surveillance studies in Mediterranean countries could identify potential high risk areas for TOSV infections to help prevent exposure of local residents and tourists to the virus. Moreover, the risk of transfusion-associated transmission of arboviruses in European countries should be addressed.

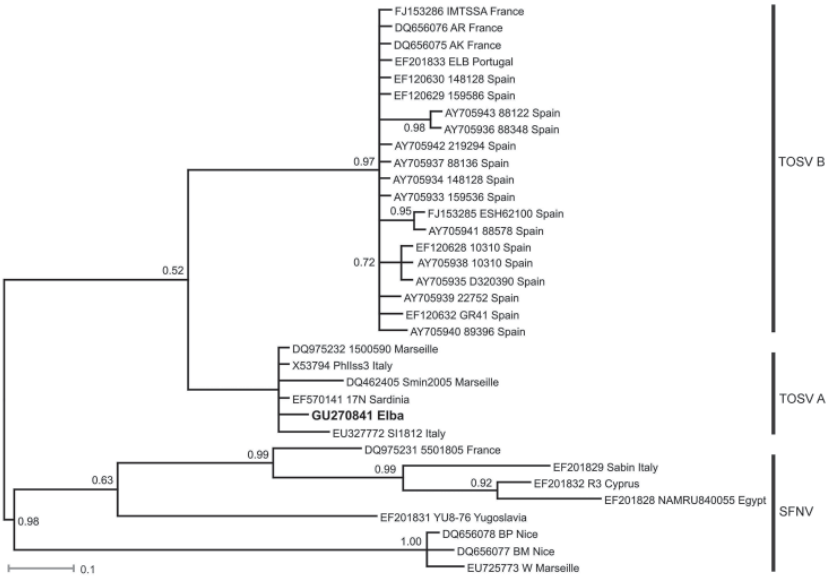


Figure. Bayesian phylogenetic tree of Toscana virus (TOSV) and *Sandfly fever Naples virus* (SFNV) strains. For each sequence used, GenBank accession number, strain designation, and strain origin are shown. Phylognetic analysis was performed by using MrBayes 3.0 program (*4*) with a general time reversible substitution model. Substitution rates were assumed to follow a gamma plus invariants distribution. Three heated chains and a single cold chain were used in all Markov Chain Monte Carlo analyses, which were run for 1,000,000 generations, sampling 1 tree every 100 generations. Trees obtained before convergent and stable likelihood values were discarded (i.e., a 2,500 tree burn-in). Four independent runs, each started from different, randomly chosen trees, were performed to assess convergence. Posterior probabilities for nodes were assembled from all post burn-in trees (i.e., 30,004 trees per analysis). Posterior probabilities are shown on each node. Scale bar indicates nucleotide substitutions per site. The newly described TOSV sequence from Elba is shown in **boldface**.

Acknowledgments

We thank Rebecca Stanway and Petra Herzog for critical reading of the manuscript.

Martin Gabriel, Christiane Resch, Stephan Günther, and Jonas Schmidt-Chanasit

Author affiliations: Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany (M. Gabriel, S. Günther, J. Schmidt-Chanasit); and Basel University Medical Clinic, Liestal, Switzerland (C. Resch)

DOI: 10.3201/eid1606.091763

References

1. Charrel RN, Gallian P, Navarro-Mari JM, Nicoletti L, Papa A, Sánchez-Seco MP, et al. Emergence of Toscana virus in Europe. Emerg Infect Dis. 2005;11:1657–63.
2. Dionisio D, Esperti F, Vivarelli A, Valassina M. Epidemiological, clinical and laboratory aspects of sandfly fever. Curr Opin Infect Dis. 2003;16:383–8. DOI: 10.1097/00001432-200310000-00003
3. Weidmann M, Sanchez-Seco MP, Sall AA, Ly PO, Thiongane Y, Lô MM, et al. Rapid detection of important human pathogenic Phleboviruses. J Clin Virol. 2008;41:138–42.
4. Ronquist F, Huelsenbeck JP. Mrbayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics. 2003; 19:1572–4. DOI: 10.1093/bioinformatics/btg180
5. Dobler G, Treib J, Haass A, Frösner G, Woesner R, Schimrigk K. Toscana virus infection in German travellers returning from the Mediterranean. Infection. 1997;25:325. DOI: 10.1007/BF01720413
6. Sonderegger B, Hachler H, Dobler G, Frei M. Imported aseptic meningitis due to Toscana virus acquired on the island of Elba, Italy, August 2008. Euro Surveill. 2009;14:pii=19079.
7. Charrel RN, Moureau G, Temmam S, Izri A, Marty P, Parola P, et al. Massilia virus, a novel Phlebovirus (Bunyaviridae) isolated from sandflies in the Mediterranean. Vector Borne Zoonotic Dis. 2009;9:519–30. DOI: 10.1089/vbz.2008.0131
8. Collao X, Palacios G, Sanbonmatsu-Gámez S, Pérez-Ruiz M, Negredo AI, Navarro-Marí JM, et al. Genetic diversity of Toscana virus. Emerg Infect Dis. 2009;15:574–7. DOI: 10.3201/eid1504.081111
9. Towards regional economic development 2006/2010. Tourism and innovations objectives and instruments [in Italian]. 2006 [cited 2010 Apr 1]. http://www.provincia.livorno.it/economia/turismo/forumturismo2006/FormatLivorno4aprile2006.pdf

Address for correspondence: Jonas Schmidt-Chanasit, Department of Virology, Clinical Virology LaboratoryBernhard Nocht Institute for Tropical Medicine, Bernhard Nocht Strasse 74, D-20359 Hamburg, Germany; email: jonassi@gmx.de

Imported Mollusks and Dissemination of Human Enteric Viruses

To the Editor: The globalization of food production and trade has increased the potential risk for infectious foodborne diseases. Hepatitis A virus (HAV) and norovirus (NoV) constitute the most important foodborne pathogens of humans in terms of numbers of outbreaks and persons affected in industrialized countries (*1*,*2*). In these countries, improvement of health conditions and development of specific vaccines are changing the epidemiologic pattern of diseases such as hepatitis A, decreasing their prevalence and increasing the susceptibility of the unvaccinated adult population (*1*). In recent years, numerous cases of gastroenteritis caused by NoV and hepatitis A linked to imported shellfish have been reported (*2*–*5*). In Spain, 2 notable hepatitis A outbreaks associated with clams (*Donax* sp.) imported from Peru occurred in 1999 and 2008. In both situations, the Spanish Ministry of Health activated the National System of Epidemiologic Surveillance and the European Community Rapid Alert System for Foodstuffs. The implicated shellfish batches were immobilized or removed, and all the shellfish from Peru were banned from the European Union (*6*). We present further evidence that imported shellfish from developing countries, where these pathogens are endemic, can be a vehicle for viral gastroenteritis and HAV infections in areas where they are not endemic.

Fifty mollusk samples imported into Spain during September 2006–March 2009 were analyzed for NoV genotype I (GI) and GII, HAV and astrovirus (AsV). Countries of origin were Morocco, Peru, Vietnam, and South Korea (Table). The species studied were clams (*Callista chione,* n = 25; *Transanella pannosa*, n = 6; *Meretrix lyrata,* n = 3; and *Donax* sp., n = 5), oysters (*Crassostrea angulata,* n = 1), cockles (*Cerastoderma edule,* n = 1), and razor clams (*Solen marginatus,* n = 1 and *Ensis* sp., n = 8). Digestive tissue was dissected from duplicated samples (10–20 individual mollusks) and homogenized with 0.1% peptone water (pH 7.4), centrifuged at 1,000 × g for 5 min, and supernatant recovered. RNA was extracted by using both Total Quick RNA extraction Cells and Tissue kit (Talent, Trieste, Italy) and Nucleospin RNA Virus Kit (Macherey-Nagel, Düren, Germany).

NoV and HAV were detected by real-time reverse transcription–PCR (RT-PCR) by using the Platinum Quantitative RT-PCR Thermoscript 1-step system (Invitrogen, Carlsbad, CA, USA) (25 μL final volume) with 5 μL of template RNA, and primers, probes, and conditions as described (*7*). A sample that displayed a cycle threshold value ≤41 was considered positive. AsV was detected by standard RT-PCR (*7*), coupled with hybridization by using specific biotin-labeled probes with the commercial Kit Hybridowell universal (Argene, Varilhes, France).

Negative and specific positive controls for HAV, NoV, and AsV were introduced in each run. Real-time RT-PCR included appropriate external controls in each analysis to avoid underestimation of viral load. A mutant, nonvirulent, infective strain of mengovirus (vMC_0) (10^3 PFU) was used as control for extraction. To calculate the real-time RT-PCR efficiencies, external viral RNA (HAV, 10^3 copies) or synthetic DNA (NoV, 10^5 copies) controls for the respective virus were co-amplified with each template viral RNA as described (*8*). The number of RNA viral genome copies per gram of digestive tissue (RNA copies/g digestive tissue) was estimated by using standard curves generated from RNA transcripts and synthetic DNA (*8*) and corrected with the extraction and real-time RT-PCR efficiencies.

Table. Viral detection and quantification in imported mollusk samples*

Country of origin (no. samples)	HAV positive†		Norovirus positive				Astrovirus positive, no. (%)¶
			GI‡		GII§		
	No. (%)	QR	No. (%)	QR	No. (%)	QR	
Morocco (34)			5 (15)	3.5×10^4–1.1×10^7	2 (6)	1.4×10^5–8.9×10^5	7 (21)
Peru (13)	1 (8)	4.7×10^3	4 (31)	2×10^5–1.8×10^7	2 (15)	1×10^5–1.4×10^6	1 (8)
Vietnam (2)	1 (50)	4.4×10^7	2 (100)	3.3×10^6–7.7×10^7			1 (50)
South Korea (1)			1 (100)	1.2×10^6			–
Total (50)	2 (4)	10^3–10^7	12 (24)	10^4–10^7	4 (8)	10^5–10^6	9 (18)

*HAV, hepatitis A virus; GI, genotype I; QR, quantification range (RNA copies/g digestive tissue).
†Obtained from *Donax* sp. and *Meretrix lyrata.*
‡Obtained from *Donax* sp., *Callista chione*, *Transanella pannosa*, *M. lyrata*, and *Ensis* sp.
§Obtained from *Donax* sp., *T. pannosa*, and *Ensis* sp.
¶Obtained from *C. chione, T. pannosa, M. lyrata*, and *Ensis* sp.

Twenty (40%) of 50 samples were contaminated by ≥1 virus (Table), although all the mollusk imports complied with the current sanitary standards. NoV GI was most prevalent, detected in 24% of samples, followed by AsV (18%), NoV GII (8%), and HAV (4%). One sample showed a low extraction efficiency (<1%), yielding all samples high real-time RT-PCR efficiencies (>10%) (*9*).

Six samples (30% of positive samples) were positive for >1 virus. Thus, 2 samples from Morocco showed contamination with NoV GI and AsV. From Peru, 1 sample was contaminated with both genogroups of NoV and another with NoV GI, NoV GII, and AsV. Samples from Vietnam (n = 2) were contaminated with HAV–NoV GI and NoV GI–AsV. Co-infection with different viruses or multiple virus strains could lead to more severe symptoms and the occurence of 2 episodes of the same or different diseases and also be a way to facilitate emergence of new recombinant strains (*10*).

Contamination levels for NoV GI ranged from 3.5×10^4 to 7.7×10^7 RNA copies/g digestive tissue; for NoV GII, from 1.03×10^5 to 8.9×10^5 RNA copies/g digestive tissue; and for HAV, from 4.7×10^3 to 4.4×10^7 RNA copies/g digestive tissue (Table). For HAV, these values are in the same range or even higher than in the coquina clams from Peru implicated in the outbreak in Spain in 2008, which is noteworthy because the attack rate for different batches of shellfish is dose dependent (*6*).

Determining the association of a viral infection with a particular contaminated product is often complicated, and the epidemiologic investigations necessary for finding this association are time consuming and allow the virus to spread before the problem is recognized. Furthermore, there are analytical difficulties in detecting and quantifying virus in shellfish samples and in monitoring them; other problems include ascertaining the representativeness of the sample (*2*,*6*) and the high cost of applying the technique in areas with extensive mollusk production.

The inadequacy of the bacterial indicators makes it necessary to develop new prevention strategies, based on microbial risk assessment, to ensure the sanitary quality of shellfish, both in production areas and in international trade operations. Implementing these methods and providing training to laboratories in developing countries are essential to achieving these objectives.

David Polo, M. Luz Vilariño, Carmen F. Manso, and Jesús L. Romalde

Authors affiliations: Universidad de Santiago de Compostela. Santiago de Compostela, Spain

DOI: 10.3201/eid1606.091748

References

1. Koopmans M, Duizer E. Foodborne viruses: an emerging problem. Int J Food Microbiol. 2004;90:23–41. DOI: 10.1016/S0168-1605(03)00169-7
2. Webby RJ, Carville KS, Kirk MD, Greening G, Ratcliff RM, Crerar SK, et al. Internationally distributed frozen oyster meat causing multiple outbreaks of norovirus infection in Australia. Clin Infect Dis. 2007;44:1026–31. DOI: 10.1086/512807
3. Romalde JL, Torrado I, Ribao C, Barja JL. Global market: shellfish imports as a source of reemerging food-borne hepatitis A virus infections in Spain. Int Microbiol. 2001;4:223–6. DOI: 10.1007/s10123-001-0041-0
4. Liko J, Keene WE. Use of templates to identify source of norovirus outbreak. Emerg Infect Dis. 2009;15:839–40. DOI: 10.3201/eid1505.081625
5. Simmons G, Garbutt C, Hewitt J, Greening G. A New Zealand outbreak of norovirus gastroenteritis linked to the consumption of imported raw Korean oysters. N Z Med J. 2007;120:U2773.
6. Pintó RM, Costafreda MI, Bosch A. Risk assessment in shellfish-borne outbreaks of hepatitis A. Appl Environ Microbiol. 2009;75:7350–5. DOI: 10.1128/AEM.01177-09
7. Vilariño ML, Le Guyader FS, Polo D, Schaeffer J, Kröl J, Romalde JL. Assesment of human enteric viruses in cultured and wild bivalve molluscs. Int Microbiol. 2009;12:145–51.
8. Costafreda MI, Bosch A, Pintó RM. Development, evaluation and standarization of a real time TaqMan RT-PCR assay for quantification of hepatitis A virus in clinical and shellfish samples. Appl Environ Microbiol. 2006;72:3846–55. DOI: 10.1128/AEM.02660-05

9. da Silva AK, Le Saux JC, Parnaudeau S, Pommepuy M, Elimelech M, Le Guyader FS. Evaluation of removal of noroviruses during wastewater treatament, using real time RT-PCR: different behaviors of genogroups I and II. Appl Environ Microbiol. 2007;73:7891–7. DOI: 10.1128/AEM.01428-07
10. Lees D. Virus and bivalve shellfish. Int J Food Microbiol. 2000;59:81–116. DOI: 10.1016/S0168-1605(00)00248-8

Address for correspondence: Jesús L. Romalde, Departamento de Microbiología y Parasitología, Centro de Investigaciones Biológicas (CIBUS)–Facultad de Biología, Universidad Santiago de Compostela, 15782, Santiago de Compostela, Spain; email: jesus.romalde@usc.es

Atypical Chikungunya Virus Infections in Immunocompromised Patients

To the Editor: Chikungunya fever was first described in Tanganyika (now Tanzania) in 1952 and is now emerging in Southeast Asia. Chikungunya virus (CHIKV) infection, a self-limiting febrile illness, shares similarities with dengue fever such as headache and myalgia. Additionally, patients with CHIKV infection typically have arthralgia, arthritis, and tenosynovitis (*1*). Although usually benign, CHIKV infection may on rare occasions lead to neurologic and hepatic manifestations with high illness and mortality rates (*2*). We report 2 immunocompromised patients with CHIKV infection associated with peritonitis, encephalitis, and secondary bacterial infections.

Patient A, a 66-year-old Singaporean-Chinese man, had a history of chronic renal disease secondary to obstructive uropathy. His baseline creatinine level was 300–400 μmol/L. For 3 years, he had ingested traditional Chinese medicine, which we suspect was contaminated by steroids because he appeared cushingoid. An outbreak of CHIKV infection was reported at his workplace. He was admitted to National University Hospital, Singapore, in July 2008 with abdominal pain, vomiting, and fever of 1 day. He had no joint symptoms. Clinically, he had systemic inflammatory response syndrome complicated by acute-on-chronic renal failure. His creatinine level was elevated at 921 μmol/L on admission. A complete blood count showed leukocytosis (19.24×10^9 cells/L) with neutrophilia and thrombocytopenia (62×10^9 cells/L). Initial blood and urine cultures and serologic results were negative for dengue virus, but serum reverse transcription–PCR (RT-PCR) and indirect immunofluorescent assay for immunoglobulin G (IgG) (Euroimmun Medizinische Labordiagnostika, Lubeck, Germany) and IgM (CTK Biotech, Inc, San Diego, CA, USA) were positive for CHIKV (*3*,*4*). Computed tomographic scans of the abdomen showed dilated small bowel loops.

An urgent laparotomy did not show bowel perforation, but peritoneal cultures yielded *Klebsiella pneumoniae*, *Escherichia coli,* and *Candida glabrata*, and RT-PCR from the concentrated peritoneal fluid was positive for CHIKV (*3*). He was administered appropriate antimicrobial drugs. He required repeat laparotomies because of elevated intraabdominal pressure. He subsequently received broad spectrum antimicrobial drugs to treat secondary intraabdominal infections caused by *P. aeruginosa* and *Enterococcus faecalis*.

Ventilator-associated pneumonia also developed. Despite maximal support and prolonged antimicrobial therapy, this patient died after 5 months of hospitalization.

Patient B, a 45-year-old Malaysian–Chinese man with diabetes mellitus, had undergone a cadaveric liver transplant in 2001 for hepatitis B liver cirrhosis. He was receiving immunosuppressants (azathioprine and prednisolone). He was admitted in August 2008 after experiencing fever, headache, and abdominal bloating for 3 days. He had no neurologic symptoms. Acute self-limiting febrile illnesses with arthritis had occurred in his hometown; CHIKV infections were suspected.

Results of his examination on admission were normal, except for bilateral enlarged cervical lymph nodes. Chest radiograph results were unremarkable. He had mild transaminitis (alanine aminotransferase 173 U/L, aspartate aminotransferase 170 U/L), elevated C-reactive protein (107 mg/L), and thrombocytopenia (120×10^9 cells/L) without leukocytosis. Results of comprehensive serum and urine microbial studies were negative for posttransplant infections. Results of serum RT-PCR were negative for CHIKV, but IgG and IgM tests were positive for CHIKV.

Brain magnetic resonance imaging was performed because of the patient's persistent severe headache and transient drowsiness. It showed several nonspecific areas of enhancement, which suggested encephalitis, given the clinical scenario (Figure). However, a lumbar puncture was not performed, and hence, whether the patient's cerebrospinal fluid contained CHIKV could not be determined. Bilateral frontoparietal white matter lesions with restricted diffusion has been suggested as an early sign of viral encephalitis (*5*). However, a retrospective series demonstrated that, in CHIKV encephalitis, abnormalities on magnetic resonance imaging were uncommon, and no pathognomonic features were found (*6*).

Hospital-acquired pneumonia also developed and was treated with broad-spectrum antimicrobial drugs. Bron-

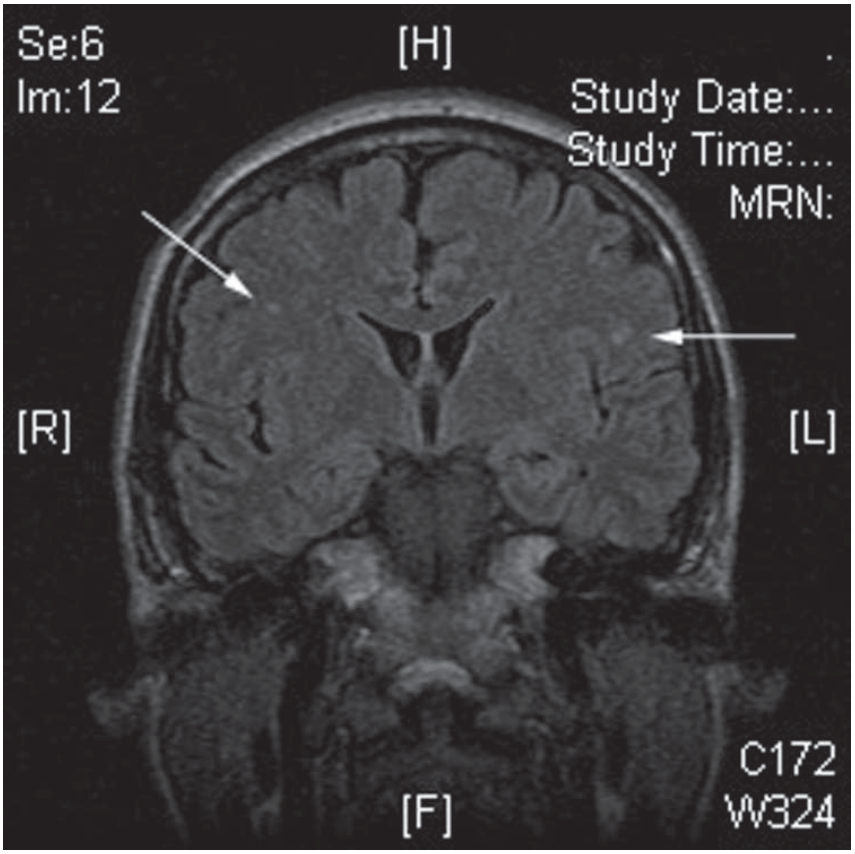


Figure. Magnetic resonance imaging of the brain of patient B, showing several nonspecific areas of enhancement (arrows), which suggests encephalitis, given the clinical scenario.

choscopic cultures were negative for CHIKV. The patient responded well to antimicrobial drugs, and his mental status was normal on discharge. He possibly had encephalitis associated with CHIKV infection, complicated by secondary hospital acquired pneumonia.

In this case, CHIKV was detected in peritoneal fluid, but because of the positive bacterial cultures, we are not confident about its causative role in Patient A's peritonitis. Although a series reported that 6 patients with CHIKV infection had perforated jejunal diverticula while receiving long-term nonsteroidal antiinflammatory drugs and steroids (*7*), the perforations were likely secondary to prolonged steroid use rather than CHIKV infection. In addition, both immunocompromised patients in our study had their CHIKV infections secondarily complicated by nosocomial infections. We note that other viral infections have been associated with bacterial translocation and secondary nosocomial infections (*8*). Whether these infections were linked to CHIKV infection or to the underlying chronic immunosuppressed state is unclear.

Both of our patients did not have the joint manifestations that are characteristic of CHIKV infection (*9*). More prospective studies are required to determine the full spectrum of clinical features of CHIKV infection in immunocompromised patients. Recently identified biomarkers may predict patients at risk for complications but we were unable to study them in our patients (*10*). Although most cases of CHIKV infection are self-limiting, clinicians should be alert to atypical presentations and severe complications in immunosuppressed patients.

Adrian C.L. Kee, Samantha Yang, and Paul Tambyah

Author affliation: National University Hospital, Singapore

DOI: 10.3201/eid1606.091115

References

1. Simon F, Parola P, Grandadam M, Fourcade S, Oliver M, Brouqui P, et al. Chikungunya infection: an emerging rheumatism among travelers returned from Indian Ocean islands. Report of 47 cases. Medicine (Baltimore). 2007;86:123–37. DOI: 10.1097/MD/0b013e31806010a5
2. Lemant J, Boisson V, Winer A, Thibault L, André H, Tixier F, et al. Serious acute chikungunya virus infection requiring intensive care during the Reunion Island outbreak in 2005–2006. Crit Care Med. 2008;36:2536–41. DOI: 10.1097/CCM.0b013e318183f2d2
3. Leo YS, Chow AL, Tan LK, Lye DC, Lin L, Ng LC. Chikungunya outbreak, Singapore, 2008. Emerg Infect Dis. 2009;15:836–7. DOI: 10.3201/eid1505.081390
4. Thein S, La Linn M, Aaskov J, Aung MM, Aye M, Zaw A, et al. Development of a simple indirect enzyme-linked immunosorbent assay for the detection of immunoglobulin M antibody in serum from patients following an outbreak of chikungunya virus infection in Yangon, Myanmar. Trans R Soc Trop Med Hyg. 1992;86:438–42. DOI: 10.1016/0035-9203(92)90260-J
5. Ganesan K, Diwan A, Shankar SK, Desai SB, Sainani GS, Katrak SM. Chikungunya encephalomyeloradiculitis: report of 2 cases with neuroimaging and 1 case with autopsy findings. AJNR Am J Neuroradiol. 2008;29:1636–7. DOI: 10.3174/ajnr.A1133
6. Robin S, Ramful D, Le Seach F, Jaffar-Bandjee MC, Rigou G, Alessandri JL. Neurologic manifestations of pediatric chikungunya infection. J Child Neurol. 2008;23:1028–35. DOI: 10.1177/0883073808314151
7. Palanivelu C, Rangarajan M, Rajapandian S, Maheshkumaar GS, Madankumar MV. Perforation of jejunal diverticula in steroids and nonsteroidal anti-inflammatory drug abusers: a case series. World J Surg. 2008;32:1420–4. DOI: 10.1007/s00268-008-9469-0
8. Lahiri M, Fisher D, Tambyah PA. Dengue mortality: reassessing the risks in transition countries. Trans R Soc Trop Med Hyg. 2008;102:1011–6. DOI: 10.1016/j.trstmh.2008.06.005

9. Parola P, de Lamballerie X, Jourdan J, Rovery C, Vaillant V, Minodier P, et al. Novel chikungunya virus variant in travelers returning from Indian Ocean islands. Emerg Infect Dis. 2006;12:1493–9.
10. Ng LF, Chow A, Sun YJ, Kwek DJ, Lim PL, Dimatatac F, et al. IL-1beta, IL-6, and RANTES as biomarkers of chikungunya severity. PLoS One. 2009;4:e4261. DOI: 10.1371/journal.pone.0004261

Address for correspondence: Paul Tambyah, Department of Medicine, National University Hospital, 5 Lower Kent Ridge Rd, Singapore 119074; email: paul_anantharajah_tambyah@nuhs.edu.sg

Lassa Fever, Nigeria, 2005–2008

To the Editor: Lassa fever affects ≈100,000 persons per year in West Africa (*1*). The disease is caused by Lassa virus, an arenavirus, and is associated with bleeding and organ failure. The case-fatality rate in hospitalized patients is 10%–20%. The reservoir of the virus is multimammate mice (*Mastomys natalensis*). Investigations in the 1970s and 1980s pointed to the existence of 3 disease-endemic zones within Nigeria: the northeastern region around Lassa, the central region around Jos, and the southern region around Onitsha (*2*,*3*). The current epidemiologic situation is less clear because no surveillance system is in place.

In 2003 and 2004, we conducted a hospital-based survey in Irrua, which demonstrated ongoing transmission of the virus in Edo State, Nigeria (*4*). Since then, laboratory capacity at the University of Lagos for diagnosing Lassa fever has been improved and used for small-scale passive surveillance in other parts of the country. Public health officials or hospital staff reported suspected cases. Blood samples were sent to Lagos, or staff from Lagos collected samples on site. Confirmatory testing, sequencing, and virus isolation were performed at the Bernhard Nocht Institute for Tropical Medicine in Hamburg, Germany. Primary testing was done by reverse transcription–PCR (RT-PCR) that targeted the glycoprotein (GP) gene (*5*,*6*). An RT-PCR that targeted the large (L) gene was used as a secondary test (*7*), and PCR products were sequenced. Serologic testing for Lassa virus–specific immunoglobulin (Ig) G and IgM was performed by immunofluorescent antibody test using Vero cells infected with Lassa virus. Virus isolation with Vero cells was conducted in the BioSafety Level 4 laboratory in Hamburg.

From 2005 through 2008, 10 cases of Lassa fever were confirmed by virus detection (cases 3–10) or implicated by epidemiologic investigation and serologic testing (cases 1 and 2) (online Appendix Table, www.cdc.gov/EID/content/16/6/1040-appT.htm). Case-patients 1–4 were involved in a nosocomial outbreak that occurred in February 2005 at the Ebonyi State University Teaching Hospital (EBSUTH) in Abakaliki. Retrospective investigation suggests the following transmission chain. The presumed index case-patient was a male nurse living in Onitsha, who became ill on January 21, 2005, and traveled ≈200 km to EBSUTH for better medical treatment. The detection of Lassa virus–specific IgM during his convalescent phase indicates that he had Lassa fever. The second case-patient was a female nurse who had contact with the index case-patient on February 4. She was admitted on February 7 and died 6 days later. Her clinical features were compatible with Lassa fever, but laboratory confirmation is lacking because specimens were not collected. Two additional case-patients among hospital staff (case-patients 3 and 4) were seen on February 21; each had had contact with case-patient 2. Case-patient 3 took care of case-patient 2 and slept in the same room with her for 4 days. Lassa fever was confirmed in case-patients 3 and 4 by RT-PCR as well as by IgM and IgG seroconversion in the surviving patient (case-patient 3). Case-patient 4, a pregnant nurse, had a spontaneous abortion and died on day 9 of hospitalization. Sequencing the GP and L gene PCR fragments showed that case-patients 3 and 4 were infected with the same virus strain (100% identity). In March and April 2005, blood was collected from 50 hospital staff members (including those who had had contact with the case-patients) and screened for Lassa virus–specific IgM and IgG. No positive blood samples were found, which indicated that no additional staff members were involved in the outbreak.

Case-patients 5 and 6 were admitted to EBSUTH in 2008 on January 17 and March 5, respectively. Both were medical doctors, one at a local hospital and the other at EBSUTH, and both died. Encephalopathy with generalized seizures and loss of consciousness preceded death in both cases. The source of infection is unknown, although it is likely that they became infected while they treated patients without knowing they had Lassa fever. In agreement with the epidemiology, the viruses from the 2 patients were similar, though not identical (89% and 87% identity in the GP and L genes, respectively).

Cases 7 to 10 occurred in Abuja and Jos from December 2007 through March 2008. Healthcare workers appeared not to be involved, and no molecular epidemiologic evidence indicated that transmission occurred among the 3 case-patients from Jos (94–97% and 90–94% identity in the GP and L genes, respectively).

In conjunction with our previous report (*4*), the cases presented here demonstrate current Lassa fever activity in the states of Edo, Ebonyi, Federal Capital Territory, and Plateau. These findings correspond to early re-

ports on Lassa fever in southern and central parts of Nigeria. That healthcare workers are still at as high a risk of contracting and dying from the disease as they were 20 years ago (*8*) is alarming.

A key to solving this problem would be the establishment of diagnostic facilities that can provide rapid molecular testing at referral centers in the disease-endemic zones. This testing would facilitate appropriate case and contact management, including early treatment and postexposure prophylaxis with ribavirin, and eventually raise awareness that Lassa fever should be considered in every severe febrile illness in these regions.

The study was supported by a grant from the Alexander von Humboldt Foundation to S.A.O., grant I/82 191 from the Volkswagen Foundation, and grant 228292 (European Virus Archive) from the European Community. The Department of Virology of the Bernhard Nocht Institute is a World Health Organization Collaborating Centre for Arbovirus and Haemorrhagic Fever Reference and Research (DEU-000115).

Deborah U. Ehichioya, Meike Hass, Stephan Ölschläger, Beate Becker-Ziaja, Christian O. Onyebuchi Chukwu, Jide Coker, Abdulsalam Nasidi, Osi-Ogbu Ogugua, Stephan Günther, and Sunday A. Omilabu

Author affiliations: University of Lagos, Lagos, Nigeria (D.U. Ehichioya, S.A. Omilabu); Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany (M. Hass, S. Ölschläger, B. Becker-Ziaja, S. Günther); Ebonyi State University Teaching Hospital, Abakaliki, Nigeria (C.O. Onyebuchi Chukwu); Federal Ministry of Health, Abuja, Nigeria (J. Coker, A. Nasidi); and National Hospital, Abuja (O.-O. Ogogbu)

DOI: 10.3201/eid1606.100080

References

1. McCormick JB, Webb PA, Krebs JW, Johnson KM, Smith ES. A prospective study of the epidemiology and ecology of Lassa fever. J Infect Dis. 1987;155:437–44.
2. Fichet-Calvet E, Rogers DJ. Risk maps of Lassa fever in West Africa. PLoS Negl Trop Dis. 2009;3:e388. DOI: 10.1371/journal.pntd.0000388
3. Bowen MD, Rollin PE, Ksiazek TG, Hustad HL, Bausch DG, Demby AH, et al. Genetic diversity among Lassa virus strains. J Virol. 2000;74:6992–7004. DOI: 10.1128/JVI.74.15.6992-7004.2000
4. Omilabu SA, Badaru SO, Okokhere P, Asogun D, Drosten C, Emmerich P, et al. Lassa fever, Nigeria, 2003 and 2004. Emerg Infect Dis. 2005;11:1642–4.
5. Demby AH, Chamberlain J, Brown DW, Clegg CS. Early diagnosis of Lassa fever by reverse transcription–PCR. J Clin Microbiol. 1994;32:2898–903.
6. Ölschläger S, Lelke M, Emmerich P, Panning M, Drosten C, Hass M, et al. Improved detection of Lassa virus by reverse transcription PCR targeting the 5′ region of S RNA. J Clin Microbiol. 2010. Epub 2010 Mar 29.
7. Vieth S, Drosten C, Lenz O, Vincent M, Omilabu S, Hass M, et al. RT-PCR assay for detection of Lassa virus and related Old World arenaviruses targeting the L gene. Trans R Soc Trop Med Hyg. 2007;101:1253–64. DOI: 10.1016/j.trstmh.2005.03.018
8. Fisher-Hoch SP, Tomori O, Nasidi A, Perez-Oronoz GI, Fakile Y, Hutwagner L, et al. Review of cases of nosocomial Lassa fever in Nigeria: the high price of poor medical practice. BMJ. 1995;311:857–9.

Address for correspondence: Stephan Günther, Department of Virology, Bernhard Nocht Institute for Tropical Medicine, Bernhard-Nocht-Str 74, 20359 Hamburg, Germany; email: guenther@bni.uni-hamburg.de

Laboratory Diagnosis of Lassa Fever, Liberia

To the Editor: Lassa fever is endemic in West Africa, with ≤300,000 Lassa virus (LASV) infections occurring annually (*1*). Persons on humanitarian missions and peacekeeping forces in regions comprising Sierra Leone and Liberia are at risk for Lassa fever (*2*–*4*). Reliable laboratory diagnosis, particularly in acute cases, is crucial for triage, implementation of barrier nursing, and contact tracing, as well as for initiation of treatment with ribavirin. Reverse transcription–PCR (RT-PCR) is routinely used for confirmation of cases, but few proven assay formulations are available, and these have not been evaluated on larger cohorts of patients (*5*).

We summarize our experiences from testing 184 patients from Liberia with suspected cases of Lassa fever with the most widely used LASV-specific RT-PCR assay (*6*). Patients were suspected of having Lassa fever on clinical grounds by physicians of the United Nations peacekeeping troops and other international relief organizations. Patients included local citizens as well as members of the mentioned organizations. EDTA-plasma samples or serum specimens packed on ice were sent to our laboratory in Hamburg, Germany, by international airfreight, taking 4–7 days for shipment. Information on clinical signs and symptoms or outcome was generally not available.

Conventional RT-PCR specific for the glycoprotein precursor gene was conducted as described (*7*). RT-PCR results positive for LASV was seen in 35 (19%) of 184 patients. Median time between onset of symptoms and sampling was 7 days. Median time from reception of samples to final RT-PCR or culture results was 1 day and 4 days, respectively.

Although samples were usually thawed upon reception, all samples positive by RT-PCR were also positive by cell culture. Three additional samples were positive by culture but repeatedly negative by RT-PCR in serum. PCR inhibition had been excluded in these samples by testing duplicates of the same samples spiked with LASV RNA. The associated culture supernatants tested positive by the same RT-PCR, as well as immunofluorescent antibody microscopy. A 522-bp fragment of the glycoprotein precursor gene spanning the entire RT-PCR fragment (334 nt) was amplified for sequence analysis with primers S36 (*6*) and LVS526 (5′-AAAATCGCAGC TCATTGCCTCATA-3′).

In each of the 3 individual isolates, several mismatched nucleotides at the binding site of the antisense primer S80 (*6*) were observed. To obtain a clearer picture of the relevance of sequence variability, we randomly selected and sequenced 9 additional samples positive by RT-PCR samples described in this study (Table).

Mismatched nucleotides were observed with all 12 strains. Up to 3 nucleotide mismatches apparently did not prevent amplification, whereas 4 positions appeared to be a critical threshold for PCR failure (nevertheless, 2 samples with 4 and 7 mismatches did amplify). Primer S80 (*7*) was modified with respect to mismatches, and RT-PCR was repeated on the 3 plasma samples that initially were negative by RT-PCR. As expected, they now were positive.

For an exact determination of virus RNA concentrations in the 12 samples, 12 individual probe-based real-time RT-PCRs were designed upon determined virus sequences (Table). Mean RNA concentration in all samples was 8.13×10^4 copies/mL. Notably, samples that had initially tested false negative showed significantly lower mean virus RNA concentrations than the overall mean (5.8×10^3 cop/mL; $p<0.05$ by *t* test). The limit of detection of the screening RT-PCR was 2,500 copies/mL for a perfectly matched template (*7*), making it clear that there was not a huge buffer (reserve) in sensitivity for the screening assay.

A total of 17 (9.2%) of 184 samples displayed immunoglobulin (Ig) M or IgG antibodies to LASV, or both types of antibodies. LASV-specific antibodies were detectable in only 11 (29%) of 38 cell culture–positive samples, and 6 samples yielded LASV-specific antibodies with no concomitant positive result by cell culture (Table). Four of these displayed IgG only, indicating previous rather than acute infection. The 3 false-negative RT-PCR samples were negative for IgM and IgG.

Our study underscores the utility and shortcomings of RT-PCR diagnostics for Lassa fever. Although RT-PCR is an appropriate diagnostic tool, it may fail to amplify strains even with limited sequence deviations, as already cautioned in early presentations of methods (*6*). Failure to amplify divergent LASV strains has been observed (*2*,*8*), and considerable sequence diversity in LASVs has been noted even within relatively constricted geographic areas (*9*). Current oligonucleotide binding sites are not conserved enough for diagnostic application without continuous assessment and revision of primer sequences. Cell culture remains the diagnostic standard for LASV (*10*), but this technique remains unavailable in many Lassa fever–endemic areas of West Africa. If virus isolation is not feasible, diagnostics should include RT-PCR, combined with enzyme immunoassay antigen detection (which may be less susceptible to false negatives due to strain variation [*10*]), or at least 2 complementary LASV RT-PCR assays. Serologic testing for IgM and IgG antibodies by enzyme immunoassay or immunofluorescent antibody assay should also be performed. Although not currently feasible in West Africa, external quality control

Table. Laboratory results and primer sequences on 12 patients with Lassa fever, Liberia*†

Strain designation‡	Deviations from sequence (5′ → 3′)‡ TGCACAAAGAACAACAGTCATCATTATAT	Original primers (*6*)	Modified primers	Cell culture	Log_{10} RNA copies/mL	Anti-LASV IgM titer‡	Anti-LASV IgG titer‡
129/05	---T----A-----T-----C--------	–	+	+	3.20	Neg	<10
UN133	---T----A-----T-----C--------	–	+	+	3.99	Neg	<10
121/1580	--T-----A----------C--C-----	–	+	+	4.14	Neg	<10
127/05	--------A--------------------	+	+	+	6.22	Neg	<10
4094/05	--------A-----------------C--	+	+	+	4.53	Neg	<10
Lib3800	--T-----------------C--------	+	+	+	4.90	160	5,120
Lib88	--T-----------------C—-C-----	+	+	+	7.49	40	20
Lib90	--T-----------------C—-C-----	+	+	+	5.17	Neg	<10
295/06	--T-----------------C—-C-----	+	+	+	5.41	80	640
383/06	-------------------C--C--C--	+	+	+	4.38	40	<10
120/06	--------A--------C--C-----C--	+	+	+	4.50	Neg	<10
174/06	----------TG-T--C--C--C--C--	+	+	+	5.02	320	640

*LASV, Lassa fever virus; Ig, immunoglobulin.
†Primers sequences are compared to that published by Demby et al. (*6*) (reverse primer binding sites). Virus isolation was done on Vero cells in a BioSafety level 4 laboratory. Serologic testing for anti-LASV IgM and IgG was performed by using the indirect immunofluorescent assay.
‡From reference strain AY628203, position 3092–3064 = reverse primer binding site in (*6*); note that the forward primer is not analyzed because it is located in the conserved stem-loop structure of LASV small segment RNA.

should include virus culture. Physicians should be aware of the limitations of laboratory diagnostic assays for LASV.

Financial support was provided to the diagnostic facilities of the Bernhard Nocht Institute by the German Ministry of Health under a contract supporting the National Reference Centre for Imported Infections.

Marcus Panning, Petra Emmerich, Stephan Ölschläger, Sergiusz Bojenko, Lamine Koivogui, Arthur Marx, Peter Clement Lugala, Stephan Günther, Daniel G. Bausch, and Christian Drosten

Author affiliations: Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany (M. Panning, P. Emmerich, S. Ölschläger, S. Günther, C. Drosten); United Nations Mission in Sierra Leone, Freetown, Sierra Leone (S. Bojenko); Program on Hemorrhagic Fevers, Conakry, Guinea (L. Koivogui); World Health Organization, Geneva, Switzerland (A. Marx); World Health Organization, Monrovia, Liberia (P.C. Lugala); and Tulane University Health Sciences Center, New Orleans, Louisiana, USA (D.G. Bausch)

DOI: 10.3201/eid1606.100040

References

1. McCormick JB, Webb PA, Krebs JW, Johnson KM, Smith ES. A prospective study of the epidemiology and ecology of Lassa fever. J Infect Dis. 1987;155:437–44.
2. Ter Meulen J, Koulemou K, Wittekindt T, Windisch K, Strigl S, Conde S, et al. Detection of Lassa virus antinucleoprotein immunoglobulin G (IgG) and IgM antibodies by a simple recombinant immunoblot assay for field use. J Clin Microbiol. 1998;36:3143–8.
3. Fair J, Jentes E, Inapogui A, Kourouma K, Goba A, Bah A, et al. Lassa virus–infected rodents in refugee camps in Guinea: a looming threat to public health in a politically unstable region. Vector Borne Zoonotic Dis. 2007;7:167–71. DOI: 10.1089/vbz.2006.0581
4. Khan SH, Goba A, Chu M, Roth C, Healing T, Marx A, et al. New opportunities for field research on the pathogenesis and treatment of Lassa fever. Antiviral Res. 2008;78:103–15. DOI: 10.1016/j.antiviral.2007.11.003
5. Drosten C, Kummerer BM, Schmitz H, Gunther S. Molecular diagnostics of viral hemorrhagic fevers. Antiviral Res. 2003;57:61–87. DOI: 10.1016/S0166-3542(02)00201-2
6. Demby AH, Chamberlain J, Brown DW, Clegg CS. Early diagnosis of Lassa fever by reverse transcription–PCR. J Clin Microbiol. 1994;32:2898–903.
7. Drosten C, Gottig S, Schilling S, Asper M, Panning M, Schmitz H, et al. Rapid detection and quantification of RNA of Ebola and Marburg viruses, Lassa virus, Crimean-Congo hemorrhagic fever virus, Rift Valley fever virus, dengue virus, and yellow fever virus by real-time reverse transcription–PCR. J Clin Microbiol. 2002;40:2323–30. DOI: 10.1128/JCM.40.7.2323-2330.2002
8. Trappier SG, Conaty AL, Farrar BB, Auperin DD, McCormick JB, Fisher-Hoch SP. Evaluation of the polymerase chain reaction for diagnosis of Lassa virus infection. Am J Trop Med Hyg. 1993;49:214–21.
9. Bowen MD, Rollin PE, Ksiazek TG, Hustad HL, Bausch DG, Demby AH, et al. Genetic diversity among Lassa virus strains. J Virol. 2000;74:6992–7004. DOI: 10.1128/JVI.74.15.6992-7004.2000
10. Bausch DG, Rollin PE, Demby AH, Coulibaly M, Kanu J, Conteh AS, et al. Diagnosis and clinical virology of Lassa fever as evaluated by enzyme-linked immunosorbent assay, indirect fluorescent-antibody test, and virus isolation. J Clin Microbiol. 2000;38:2670–7.

Address for correspondence: Christian Drosten, Institute of Virology, University of Bonn Medical Centre, Sigmund Freud-Str. 25, 53127 Bonn, Germany; email: drosten@virology-bonn.de

Pandemic (H1N1) 2009 in Skunks, Canada

To the Editor: In March 2009, a novel influenza virus A (H1N1) emerged in Mexico, and, because of widespread human-to-human transmission, a global pandemic was declared in June 2009 (*1*). Although most cases have involved humans, pandemic (H1N1) 2009 has sporadically infected swine and turkeys and has also been reported in a small number of pet ferrets, cats, and captive cheetahs, and in a dog (*2*). Many of these animals were cared for by persons who experienced influenza-like illness and the owner of 1 cat who died had confirmed pandemic (H1N1) 2009 respiratory disease before the cat became ill, which suggests probable human-to animal-transmission of the virus (*2*).

During mid-December 2009–mid-January 2010, eight striped skunks (*Mephitis mephitis*) died on a mink farm near Vancouver, British Columbia, Canada. On January 12, 2010, two of the skunks were brought to the Animal Health Centre in Abbotsford, British Columbia, for postmortem examination. One skunk exhibited purulent nasal exudates. In both skunks, investigators observed splenomegaly and severe pneumonia, characterized by heavy, dark red to purple, lung lobes involving >70% of the lung field. Microscopic examination showed moderate rhinitis and severe bronchopneumonia with intralesional bacteria, areas of interstitial pneumonia, and occasional nematode larvae. Also observed were splenic extramedullary hematopoiesis, plasmacytosis of both lymph nodes and spleen, and mild plasmacytic glomerulonephritis with proteinuria.

Routine bacteriologic culture of lung showed heavy growth of *Streptococcus dysgaslactiae* subsp. *equisimilis*, *Staphylococcus aureus*, and *Hafnia alvei*. That death was caused

by uncomplicated mixed bacterial bronchopneumonia in 2 (and possibly up to 8) adult skunks over a 6-week period was considered unlikely. The presence of lungworm was considered incidental. However, the areas of interstitial pneumonia suggested that a primary viral pathogen was likely.

Molecular testing was conducted initially on fresh lung, liver, kidney, and spleen for canine distemper virus and, subsequently, for influenza A virus. The splenic and nodal plasmacytosis and plasmacytic glomerulonephritis also prompted testing for Aleutian disease virus (ADV). Organ samples were negative for canine distemper virus and positive for ADV.

Detection of influenza A virus nucleoprotein and matrix genes and hemagglutinin and neuraminidase typing was performed with real-time reverse transcription–PCR. Organ samples were positive for pandemic (H1N1) 2009, which was confirmed by sequence analysis of DNA fragments obtained in the hemagglutinin, neuraminidase, and matrix gene testing.

Primary viral interstitial pneumonia is frequently complicated by opportunistic bacterial bronchopneumonia and influenza virus A infection has been shown to predispose to pulmonary bacterial toxicity (*3*). Thus, we concluded that primary pandemic (H1N1) 2009 interstitial pneumonia had predisposed the 2 skunks to mixed bacterial bronchopneumonia and death. The skunks were also infected with ADV, presumably as a result of viral shedding by the minks, which are known to be ADV carriers. Striped skunks can be experimentally infected with ADV, and antibodies to ADV have been detected in wild skunks (*4*). Although ADV does not cause pneumonia (*4*), co-infection with ADV and influenza A virus is associated with higher mortality rates in minks with respiratory disease (*5*). Thus, ADV co-infection may have contributed to the severity of the pneumonia and the death of the skunks.

The source of the pandemic (H1N1) 2009 virus is unclear. Nasal discharge was also observed in many of the minks, which suggests that they had a respiratory viral infection. However, no diagnostic workup was undertaken. Although severe outbreaks of interstitial pneumonia on mink farms can occur (*6*), most natural influenza A virus infections in minks are either mild or asymptomatic (*5*). Thus, the minks may also have been infected with pandemic (H1N1) 2009. Many of the pandemic (H1N1) 2009 infections reported in animals are believed to have been the result of exposure to infected humans (*2*). Workers on the mink farm did not experience influenza-like illness. However, humans with asymptomatic pandemic (H1N1) 2009 infection may have transmitted it to the mink. Because the skunks visited the mink farm daily, transmission of pandemic (H1N1) 2009 from humans to minks to skunks is a possibility.

In view of the detection of pandemic (H1N1) 2009 virus in 2 striped skunks with fatal pneumonia, this species should now be regarded as a potential source of influenza A virus. Wild animals participate in the transmission of influenza A viruses between species, and the presence of wildlife on farms is known to be a risk factor for infection of poultry (*7*). Similar to raccoons, skunks express both α2,3 and α2,6 sialic acid receptors for avian and human influenza viruses in the respiratory tract (M. Shrenzel, San Diego Zoo, pers. comm.), which is believed to create the opportunity for mixed influenza infections with potential for genetic reassortment (*8*). Skunks, like raccoons, are highly mobile animals with large home ranges in rural and urban areas, which provides numerous opportunities for influenza A virus exposure and transmission to poultry, livestock, pets, and, ultimately, humans. The inclusion of striped skunks in wildlife influenza surveillance programs may be warranted.

Acknowledgments

We thank Sandra Etheridge, Joanne Taylor, and Erin Zabek for their assistance and technical expertise; and Helen Schwantje, John Robinson, and Victoria Bowes for their valuable contribution to the case workup.

Ann P. Britton, Ken R. Sojonky, Andrea P. Scouras, and Julie J. Bidulka

Author affiliation: Animal Health Centre, British Columbia Ministry of Agriculture and Lands, Abbotsford, British Columbia, Canada

DOI: 10.3201/eid1606.100352

References

1. World Health Organization. Programmes and projects, media centre, statements 2009 [cited 2010 Feb 11]. http://www.who.int/mediacentre/news/statements/2009/h1n1_pandemic_phase6_20090611/en/
2. American Veterinary Medical Association. Public health, influenza, 2009 H1N1 flu virus outbreak, February 8, 2010 [cited 2010 Feb 22]. http://www.avma.org/public_health/influenza/new_virus/
3. Jakeman KJ, Rushton DI, Smith H, Sweet C. Exacerbation of bacterial toxicity to infant ferrets by influenza virus: possible role in sudden infant death syndrome. J Infect Dis. 1991;163:35–40.
4. Kenyon AJ, Kenyon BJ, Hahn EC. Protides of the mustelidae: immunoresponse of mustelids to Aleutian mink disease virus. Am J Vet Res. 1978;39:1011–5.
5. Gagnon CA, Spearman G, Hamel A, Godson DL, Fortin A, Fortin G, et al. Characterization of a Canadian mink H3N2 influenza A virus isolate genetically related to triple reassortant swine influenza virus. J Clin Microbiol. 2009;47:796–9. DOI: 10.1128/JCM.01228-08
6. Englund L, Klingeborn B, Mejerland T. Avian influenza A virus causing an outbreak of contagious interstitial pneumonia in mink. Acta Vet Scand. 1986;27:497–504.
7. Vandalen KK, Shriner SA, Sullivan HJ, Root JJ, Franklin AB. Monitoring exposure to avian influenza viruses in wild mammals. Mammal Rev. 2009:1–11 [cited 2010 Feb 11]. http://www.aphis.usda.gov/wildlife_damage/nwrc/publications/09pubs/vandalen091.pdf

8. Hall JS, Bentler KT, Landolt G, Elmore SA, Minnis RB, Campbell TA, et al. Influenza infection in wild raccoons. Emerg Infect Dis. 2008;14:1842–8. DOI: 10.3201/eid1412.071371

Address for correspondence: Ann P. Britton, Animal Health Centre, British Columbia Ministry of Agriculture and Lands, 1767 Angus Campbell Rd, Abbotsford, British Columbia V3G 2M3, Canada; email: ann.p.britton@gov.bc.ca

Community-acquired Oseltamivir-Resistant Pandemic (H1N1) 2009 in Child, Israel

To the Editor: During the spring of 2009, a pandemic influenza A (H1N1) virus emerged and spread globally. Initial testing of the virus found it susceptible to neuraminidase inhibitors and resistant to adamantanes (*1*,*2*). As of March 5, 2010, only 264 cases of oseltamivir-resistant pandemic (H1N1) 2009 infection had been reported to the World Health Organization, but the number of cases has been steadily increasing (*2*). These viruses were carrying the H275Y mutation, which conferred resistance to oseltamivir (*2*). Most of the reported cases were in immunocompromised patients who had prolonged viral shedding or in patients who had received oseltamivir prophylaxis or treatment (*1–4*). We describe an otherwise healthy 2-year-old boy with oseltamivir-resistant pandemic (H1N1) 2009 infection and a traumatic lung contusion, complicated by acute respiratory distress syndrome (ARDS). He had not received prior chemoprophylaxis or treatment with oseltamivir.

In November 2009, a healthy 2-year-old boy was admitted to the pediatric intensive care unit at the Western Galilee Hospital in Nahariya, Israel, after he had been hit by a car. One day before the accident, he had exhibited fever and cough (for which he was treated with acetaminophen). His 4-year-old brother had recovered recently from an influenza-like illness without antiviral treatment. The other household contacts were his parents, who did not have a respiratory illness.

On admission, small, bilateral lung contusions, right pneumothorax, and liver lacerations were shown on computed tomographic scan. The patient was treated with a chest tube for drainage, supplemental oxygen, and oseltamivir from hospital day 1 (30 mg 2 ×/day; child's body weight = 13 kg) and was placed in droplet isolation. Respiratory swab specimens, obtained on hospital day 1, were sent to the Israel Central Virology Laboratory (ICVL) and found to be positive for pandemic (H1N1) 2009 by real-time reverse transcription–PCR (RT-PCR). On hospital day 3, the child was intubated because of worsening respiratory distress and hypoxemia, and he required a second chest tube drain. His chest film showed bilateral pulmonary infiltrates. His condition was then treated with nitric oxide, dopamine, and milrinone for ARDS and failure of the right side of the heart. The dosage of oseltamivir was doubled on hospital day 4 because of gastric residuals. Antimicrobial drug therapy with vancomycin and piperacillin-tazobactam was added because sepsis and secondary bacterial lung infection were suspected. Because of the severity of his symptoms and persistence of fever, additional lower and upper airway specimens were sent to ICVL on hospital days 5 and 10; they were positive for pandemic (H1N1) 2009.

After these results were received, oseltamivir resistance was suspected, and his respiratory specimens were also checked by ICVL. A mixture of both wild-type and mutant pandemic (H1N1) 2009 was found in the specimens from hospital days 1, 5, and 10 by an in-house q-RT-PCR assay designed to detect the H275Y mutation (*4*,*5*). Further testing by sequence analysis of the neuraminidase gene showed a mixed population of wild-type and mutant pandemic (H1N1) 2009; the mutant virus was carrying the histidine-to-tyrosine substitution at position 275, which conferred the quantitative RT-PCR result and the H275Y phenotype of oseltamivir-resistant pandemic (H1N1) 2009. By the time these laboratory results were known, the patient's respiratory condition was improving without changing the oseltamivir therapy. Cultures of blood and endotracheal specimens were sterile and antimicrobial drug therapy was stopped. On hospital day 15, he was extubated, oseltamivir therapy was ended, and he was weaned off oxygen a few days later. The respiratory specimen on hospital day 20 was negative for pandemic (H1N1) 2009. No secondary influenza cases were detected among healthcare personnel or patients in the unit.

In Israel, oseltamivir resistance has been detected by ICVL in 6 cases (*5*). The fact that our patient had oseltamivir-resistant pandemic (H1N1) 2009 without a previous oseltamivir exposure is surprising because almost all cases of oseltamivir-resistance have been associated with previous oseltamivir prophylaxis or therapy and with prolonged viral shedding (which is often combined with oseltamivir therapy) in immunocompromised patients (*1–5)*. Our patient did not attend daycare and his parents had not been ill recently. Therefore, he likely was infected by his older brother who probably had pandemic (H1N1) 2009 but was neither diagnosed nor treated with antiviral medications. This theory suggests that oseltamivir-resistant viruses circulate in the community with the potential to be transmitted between persons.

Lung contusions and pandemic (H1N1) 2009 can cause ARDS (*6*,*7*). We do not know the relative role of each in causing the ARDS that our patient had, but the severity of clinical symptoms, although the lung injury was judged to be only of moderate magnitude, suggests that influenza played a major role in the development of his acute lung disease. The infection with oseltamivir-resistant virus, for which he probably did not receive effective therapy, likely had an effect on the duration and severity of his course.

Although our patient had a favorable outcome, the possibility of widespread resistance, similar to the phenomenon observed with seasonal influenza in the 2008–2009 season, is alarming and should be monitored. The suspicion of resistance should be based upon compatible clinical scenario, such as continuation of symptoms in spite of antiviral therapy (even in patients who are not immunocompromised), combined with early performance of resistance assays. Early and rapid detection of oseltamivir resistance and a change of antiviral treatment (if feasible) might benefit the patient.

Zeev Zonis, Dan Engelhard, Musa Hindiyeh, Daniela Ram, Michal Mandelboim, Ella Mendelson, and Daniel Glikman

Author affiliations: Western Galilee Hospital, Nahariya, Israel (Z. Zonis, D. Glikman); Hadassah University Hospital, Jerusalem, Israel (D. Engelhard); Israel Ministry of Health, Public Health Services and Chaim Sheba Medical Center, Tel-Hashomer, Israel (M. Hindiyeh, D. Ram, M. Mandelboim, E. Mendelson)

DOI: 10.3201/eid1606.091875

References

1. Oseltamivir-resistant 2009 pandemic influenza (H1N1) virus infection in two summer campers receiving prophylaxis—North Carolina, 2009. MMWR Morb Mortal Wkly Rep. 2009;58:969–72.
2. Pandemic (H1N1) 2009 update 90, weekly update on oseltamivir resistance to pandemic influenza A (H1N1) 2009 viruses. Geneva: World Health Organization; 2010 [cited 2010 Mar 5]. http://www.who.int/csr/disease/swineflu/oseltamivirresistant20100305.pdf
3. Centers for Disease Control and Prevention. Oseltamivir-resistant novel influenza A (H1N1) virus infection in two immunosuppressed patients—Seattle, Washington, 2009. MMWR Morb Mortal Wkly Rep. 2009;58:893–6.
4. Glikman D, Zonis Z, Hindiyeh M, Ram D, Mandelboim M, Mendelson E, et al. Development of oseltamivir resistance during oseltamivir therapy in child with severe 2009 H1N1 influenza. Pediatr Infect Dis J. 2010;29:385–6. DOI: 10.1097/INF.0b013e3181cd9524
5. Hindiyeh M, Ram D, Mandelboim M, Meningher T, Hirsh S, Robinov J, et al. Rapid detection of influenza A pandemic (H1N1) 2009 virus neuraminidase resistance mutation H275Y by real-time RT-PCR. J Clin Microbiol. 2010 Mar 10; [Epub ahead of print].
6. Schroeder JE, Weiss YG, Mosheiff R. The current state in evaluation and treatment of ARdS and SIRS. Injury. 2009;40(Suppl 4):s82–9. DOI: 10.1016/j.injury.2009.10.041
7. Australia and New Zealand Extracorporeal Membrane Oxygenation (ANZ ECMO) Influenza Investigators, Davies A, Jones D, Bailey M, Beca J, Bellomo R, Blackwell N, et al. Extracorporeal membrane oxygenation for 2009 influenza A (H1N1) acute respiratory distress syndrome. JAMA. 2009;302:1888–95. DOI: 10.1001/jama.2009.1535

Address for correspondence: Daniel Glikman, Infectious Diseases Unit, Western Galilee Hospital, PO Box 21, Nahariya, 22100, Israel; email: danny.glikman@naharia.health.gov.il

Human Infection with Lymphocytic Choriomeningitis Virus

To the Editor: I read with great interest the article regarding lymphocytic choriomeningitis virus (LCMV) meningitis in a New York City resident (*1*). The authors' conclusion that there is a need to ascertain the true incidence of LCMV infection is worthy of underscoring. Nearly 15 years ago, in this same journal, we described congenital LCMV as an unrecognized teratogen and recommended further "research to define the frequency of LCMV" (*2*). Five years later, we reiterated that recommendation when reporting acquired LCMV meningoencephalitis in an adolescent from Tucson, Arizona (*3*). Despite, or because of, the lack of prospective studies, the fact that this author has accrued data regarding >60 congenitally infected infants from all geographic areas in the United States during the past 15 years reinforces the concept that LCMV is a neglected pathogen whose time for more extensive study has indeed come.

Leslie L. Barton

Author affiliation: University of Arizona, Tucson, Arizona, USA

DOI: 10.3201/eid1606.100250

References

1. Asnis DS, Muana O, Kim DG, Garcia M, Rollin PE, Slavinski S. Lymphocytic choriomeningitis virus meningitis, New York, NY, USA, 2009. Emerg Infect Dis. 2010;16:328–30.
2. Barton LL, Peters CJ, Ksiazek TG. Congenital lymphocytic choriomeningitis virus: an unrecognized teratogenic pathogen. Emerg Infect Dis. 1995;1:152–3. DOI: 10.3201/eid0104.950410
3. Barton LL, Hyndman NJ. Lymphocytic choriomeningitis virus: reemerging central nervous system pathogen. Pediatrics. 2000;105:e35–6. DOI: 10.1542/peds.105.3.e35

Address for correspondence: Leslie L. Barton, University of Arizona, College of Medicine, 2335 East Seneca St, Tucson, AZ 85719; USA: email: llb@peds.arizona.edu

Increasing Incidence of Nontuberculous Mycobacteria, Taiwan, 2000–2008

To the Editor: Lai et al. (*1*) reported an increase in the number of nontuberculous mycobacteria (NTM) isolates and patients with pulmonary NTM diseases after implementation of the BACTEC system (Becton Dickinson, Sparks, MD, USA) late in 2001. These authors also reported that the increase was mainly in persons infected with *Mycobacterium avium* complex (MAC) and *M. abscessus*. They stated that diseases caused by NTM were defined according to current diagnosis criteria published in 2007 (*2*). This finding suggests that Lai et al. were able to review the clinical and radiologic information for all patients.

We wonder whether they were also able to identify and exclude people with NTM colonization, i.e., persons with positive cultures for NTM who did not meet the American Thoracic Society disease criteria. It would have been interesting to know the trend in colonized persons. In a previous study from British Columbia (*3*), we found an increase in the number of NTM isolates mostly in persons with MAC colonization. This finding coincided with implementation of a new laboratory technique in 2000, which suggested that the new technology is more sensitive in detecting MAC. In contrast with the findings of Lai et al., our study from British Columbia showed that the incidence in patients treated for NTM pulmonary disease (the group used as a surrogate of NTM disease) has been decreasing over time, which is reassuring.

Eduardo Hernández-Garduño and R. Kevin Elwood

Author affiliation: British Columbia Centre for Disease Control, Vancouver, British Columbia, Canada

DOI: 10.3201/eid1606.100228

References

1. Lai CC, Tan CK, Chou CH, Hsu HL, Liao CH, Huang YT, et al. Increasing incidence of nontuberculous mycobacteria, Taiwan, 2000–2008. Emerg Infect Dis. 2010;16:294–6.
2. Griffith DE, Aksamit T, Brow-Elliott BA, Catanzaro A, Daley C, Gordin F, et al. An official ATS/IDSA statement: diagnosis, treatment, and prevention of nontuberculous mycobacterial diseases. Am J Respir Crit Care Med. 2007;175:367–416 10.1164/rccm.200604-571. DOI: 10.1164/rccm.200604-571ST
3. Hernández-Garduño E, Rodrigues M, Elwood RK. The incidence of pulmonary non-tuberculous mycobacteria in British Columbia, Canada. Int J Tuberc Lung Dis. 2009;13:1086–93.

Address for correspondence: Eduardo Hernández-Garduño, Department of Tuberculosis Control, British Columbia Centre for Disease Control, 1063-655 W 12th Ave, Vancouver, British Columbia V5Z 4R4, Canada; email: eduardo.hernandez@bccdc.ca

In Response: We thank Hernández-Garduño and Elwood for drawing attention to the issue of nontuberculous mycobacteria (NTM) colonization (*1*), which was not described in our study (*2*). Among 4,786 patients with NTM isolates treated at our hospital during 2000–2008, colonization was found in 76.9% (3,681), and only 23.1% (1,105) had NTM diseases according to the criteria of the American Thoracic Society and Infectious Diseases Society of America (*3*).

Annual proportions of NTM isolates causing colonizations ranged from 29.2% in 2001 to 19.8% in 2007. During the study period, annual incidences of NTM colonization and disease increased from 6.6/100,000 inpatients and 2.7/100,000 outpatients in 2000 to 34.5/100,000 inpatients and 10.2/100,000 outpatients in 2008. *Mycobacterium avium* complex (MAC) was the most prevalent species, colonizing 1,282 (34.8%) of 3,681 patients. Annual proportions of MAC isolates causing colonization ranged from 20.0% in 2000 to 12.6% in 2006. Annual incidence of MAC colonization increased from 1.9/100,000 inpatients in 2000 to 12.3/100,000 inpatients in 2008; incidence of MAC disease also increased from 0.5/100,000 inpatients in 2000 to 2.1/100,000 inpatients in 2008. *M. abscessus*, the second most common species in our study (*2*), caused colonization and disease in 669 and 155 patients, respectively. Annual incidence of *M. abscessus* colonization and infection also increased from 1.49/100,000 inpatients and 0.3/100,000 outpatients in 2000 to 7.0/100,000 inpatients and 1.9/100,000 outpatients in 2008.

Our study and a previous study in British Columbia (*4*) suggest that improvement in diagnostic methods would detect increased incidence of NTM, especially of MAC; most isolates identified in these studies were associated with colonization. We also demonstrated a gradual increase in the incidence of all NTM, MAC, and *M. abscessus* over time in Taiwan, which may be attributable to increasing vigilance and awareness of these bacteria as human pathogens and the increased population of immunocompromised patients. Thus, clinicians should consider diagnosing NTM diseases with sensitive and advanced laboratory methods because of the increasing population of patients at risk.

Chih-Cheng Lai, Che-Kim Tan, Chien-Hong Chou, Hsaio-Leng Hsu, Chun-Hsing Liao, Yu-Tsung Huang, Pan-Chyr Yang, Kwen-Tay Luh, and Po-Ren Hsueh

Author affiliations: Cardinal-Tien Hospital, Taipei, Taiwan (C.-C. Lai); Chi-Mei Medical Center, Tainan, Taiwan (C.-K. Tan); National Taiwan University College of Medicine, Taipei (C.-H. Chou, H.-L. Hsu, Y.-T. Huang, P.-C. Yang, K.-T. Luh, P.-R. Hsueh); and Far Eastern Memorial Hospital, Taipei (C.-H. Liao)

DOI: 10.3201/eid1606.100329

References

1. Hernández-Garduño E, Elwood RK. Increasing incidence of nontuberculous mycobacteria, Taiwan, 2000–2008. Emerg Infect Dis. 2010;16:1047.
2. Lai C-C, Tan C-K, Chou C-H, Hsu H-L, Liao C-H, Huang Y-T, et al. Increasing incidence of nontuberculous mycobacteria, Taiwan, 2000–2008. Emerg Infect Dis. 2010;16:294–6.
3. Griffith DE, Aksamit T, Brow-Elliott BA, Catanzaro A, Daley C, Gordin F, et al. An official ATS/IDSA statement: diagnosis, treatment, and prevention of nontuberculous mycobacterial diseases. Am J Respir Crit Care Med. 2007;175:367–416. DOI: 10.1164/rccm.200604-571ST
4. Hernández-Garduño E, Rodrigues M, Elwood RK. The incidence of pulmonary nontuberculous mycobacteria in British Columbia, Canada. Int J Tuberc Lung Dis. 2009;13:1086–93.

Address for correspondence: Po-Ren Hsueh, Departments of Laboratory Medicine and Internal Medicine, National Taiwan University Hospital, No. 7, Chung-Shan South Rd, Taipei 100, Taiwan; email: hsporen@ntu.edu.tw

Food Reservoir for *Escherichia coli* Causing Urinary Tract Infections

To the Editor: We read with interest the article by Vincent et al. that compared *Escherichia coli* isolates from 3 sources (human urinary tract infections [UTIs], retail meat, and restaurant/ready-to-eat foods) by multiple molecular typing methods (*1*). This study has to be considered in the context of the larger debate about the possible animal origin of *E. coli* isolates that cause extraintestinal infections in humans (*2*–*5*), and the same authors (Vincent et al.) have declared, in the introduction, that their efforts were directed toward investigating the hypothesis that retail chicken is the main reservoir for extraintestinal *E. coli*.

We strongly appreciate the amount of the experimental data and some interesting findings, but we are not totally convinced of the authors' conclusions, particularly the assumption that the study strongly supports the preliminary hypothesis. First, the observation that only a low proportion (73/844, 8.6%) of the *E. coli* isolates analyzed belonged to clonal groups (defined as ≥2 *E. coli* isolates that had indistinguishable multilocus variable number tandem repeats and enterobacterial repetitive intergenic consensus 2 patterns), including members from >1 source, suggests an overall high degree of genetic heterogeneity among isolates from different sources. Second, looking at the single isolates within clonal groups reported in Table 2, twelve (2.9%) of the 417 isolates from retail meat shared multilocus variable number tandem repeats, enterobacterial repetitive intergenic consensus 2, and multilocus sequence types with some human UTI isolates; however, only 1 isolate (strain EC01DT06–1737–01) was also found to be indistinguishable from a human isolate (strain MSHS 161) by pulsed-field gel electrophoresis , indicating that identical genotypes (between isolates from retail meat and human infections) were observed only once.

Although we agree that the finding of a partial overlap between multilocus sequence types of isolates from retail meat and from human UTI isolates is noteworthy (especially recovery of an ST131 isolate of avian origin), the emphasis posed for the role of food transmission in the dissemination of the *E. coli* strains that cause community-acquired UTIs, in our opinion, does not seem strongly supported by the experimental data. Nevertheless, the topic is relevant, and we would highlight the importance of further research on this issue.

Maria Giufrè, Caterina Graziani, Marisa Accogli, and Marina Cerquetti

Author affiliations: Istituto Superiore di Sanità, Rome, Italy

DOI: 10.3201/eid1606.100158

References

1. Vincent C, Boerlin P, Daignault D, Dozois CM, Dutil L, Galanakis C, et al. Food reservoir for *Escherichia coli* causing urinary tract infections. Emerg Infect Dis. 2010;16:88–95.
2. Collignon P, Angulo FJ. Fluoroquinolone-resistant *Escherichia coli*: food for thought. J Infect Dis. 2006;194:8–10.
3. Graziani C, Luzzi I, Corrò M, Tomei F, Parisi G, Giufrè M, et al. Phylogenetic background and virulence genotype of ciprofloxacin-susceptible and resistant *Escherichia coli* strains of human and avian origin. J Infect Dis. 2009;199:1209–17. DOI: 10.1086/597423
4. Johnson JR, Kuskowski MA, Menard M, Gajewski A, Xercavins M, Garau J. Similarity between human and chicken *Escherichia coli* isolates in relation to ciprofloxacin resistance status. J Infect Dis. 2006;194:71–8.
5. Johnson JR, Sannes MR, Croy C, Johnston B, Clabots C, Kuskowski MA, et al. Antimicrobial drug–resistant *Escherichia coli* from humans and poultry products, Minnesota and Wisconsin, 2002–2004. Emerg Infect Dis. 2007;13:838–46.

Address for correspondence: Marina Cerquetti, Department of Infectious Parasitic and Immune-mediated Diseases, Istituto Superiore di Sanità, Viale Regina Elena, 299, 00161 Roma, Italy; email: marina.cerquetti@iss.it

In Response: Giufrè et al. (*1*) responded to our recent article about the possibility of a food reservoir, specifically in retail chicken meat, for *Escherichia coli* causing human extraintestinal infections (*2*). They are not convinced by the data of "strong support" for the hypothesis that retail chicken meat could be a reservoir for these *E. coli* organisms and indicate that the observed proportion of total clonal group members (8.6%) is low and heterogeneity is significant among the isolates tested.

We assembled the 844 study isolates from 3 sources (human, retail meat, and restaurant-ready-to-eat foods) in 2 provinces during a 3-year period. Given the ecologic design of the study, the fact that 72 isolates actually were related across these sources is surprising and compelling. Furthermore, we identified a retail chicken meat isolate and human urinary tract infection isolate that were indistinguishable by pulsed-field gel electrophoresis (PFGE), again a surprising result given the study design. PFGE remains the standard for *E. coli* genotyping because of its discriminatory power; these results were also confirmed by PFGE by using a second enzyme. This group was identified as containing *E. coli* O25:H4–ST131, a clonal group that appears to cause extraintestinal disease worldwide.

Our study is among the first to extensively genotype *E. coli* isolates from these sources. Hence, the amount of genetic diversity expected in these *E. coli* organisms is unclear. This diversity is the primary issue raised by Giufrè et al.: how much genetic relatedness would be expected in a comparison of *E. coli* isolates from these sources? Although our study was fairly modest in size and was limited by the study design, we observed evidence supporting our primary hypothesis that retail chicken meat may be a reservoir for *E. coli* causing extraintestinal infections in humans. More studies certainly will help resolve the debate.

Amee R. Manges

Author affiliation: McGill University, Montreal, Quebec, Canada

DOI: 10.3201/eid1606.100407

References

1. Giufrè M, Graziani C, Accogli M, Cerquetti M. Food reservoir for *Escherichia coli* causing urinary tract infections [letter]. Emerg Infect Dis. 2010;16:1048–9.
2. Vincent C, Boerlin P, Daignault D, Dozois CM, Dutil L, Galanakis C, et al. Food reservoir for *Escherichia coli* causing urinary tract infections. Emerg Infect Dis. 2010;16:88–95. DOI: 10.3201/eid1601.091118

Address for correspondence: Amee R. Manges, Epidemiology, Biostatistics and Occupational Health, McGill University, 1020 Pine Ave, W, 36B, Montreal, Quebec H3A 1A2, Canada; email: amee.manges@mcgill.ca

etymologia

Lassa [laĺ sə] ***virus***

This virus was named after the town of Lassa at the southern end of Lake Chad in northeastern Nigeria, where the first known patient, a nurse in a mission hospital, had lived and worked when she contracted this infection in 1969. The virus was discovered as part of a plan to identify unknown viruses from Africa by collecting serum specimens from patients with fevers of unknown origin. Lassa virus, transmitted by field rats, is endemic in West Africa, where it causes up to 300,000 infections and 5,000 deaths each year.

Source: Frame JD, Baldwin JM Jr, Gocke DJ, Troup JM. Lassa fever, a new virus disease of man from West Africa. I. Clinical description and pathological findings. Am J Trop Med Hyg. 1970;19:670–6; Mahy BW. The dictionary of virology, 4th ed. Burlington (MA): Elsevier; 2009.

Human-Animal Medicine: Clinical Approaches to Zoonoses, Toxicants and Other Shared Health Risks

Peter M. Rabinowitz and Lisa A. Conti

Saunders, Maryland Heights, MD, USA, 2009
ISBN: 10-1416068376
Pages: 432; Price: US $99.95

Hooray, finally a book emerges about the human–animal interface that addresses both perspectives equitably and seamlessly. Peter Rabinowitz, a physician, and Lisa Conti, a veterinarian, effectively present material that is thorough, balanced, and of great relevance for practitioners of all varieties of medicine.

More than half the pages comprise reports on each of 35 zoonoses. At the beginning of each report are key points divided into professional categories—public health professionals, human health clinicians, and veterinary clinicians—ensuring relevance for multiple readers. In addition, 55 pages deal with toxicoses, including environmental, gaseous, poisonous plants, herbicides/pesticides, and envenomations. Clinical signs, symptoms, species comparisons, treatment and prevention for these toxicoses are all spelled out clearly.

This inclusive approach, with its plentiful and accurate technical information, might be enough to justify purchasing the book for the shelf of any human or veterinary medical practice, but it is the additional 175 pages that set this volume apart from all others on the subject. A lengthy introductory chapter discusses the general concept of one medicine and why that concept does not mean one practitioner but rather integration of practitioners from multiple sectors. The chapter describes the serious legal and ethical considerations associated with professionally crossing the human–animal interface. The occupational health of animal workers is covered in detail and includes not only zoonotic agents that immediately come to mind, but also allergens, use of live vaccines, noise, anesthetic gases, and the psychosocial impacts of such issues as euthanasia. Animal-assisted therapy is covered in detail. The book includes a particularly useful section on immunocompromised persons and their exposure to animals and another one on animal bites. There is even a segment on travel, including concerns about wild animal contact, as well as disease hazards for pets that travel. These additional chapters make parts of this book relevant for a much wider audience that could potentially include policy makers, regulators, students, and academicians.

Throughout, the book is graphically pleasing, with text broken regularly by subheadings, tables, pen-and-ink drawings, algorithms, photographs, and intriguing side bars. Some of the side bars are case studies, with interesting scenarios and quick tips for promoting health.

Corrie Brown

Author affiliation: University of Georgia, Athens, Georgia, USA

DOI: 10.3201/eid1606.100367

Address for correspondence: Corrie Brown, College of Veterinary Medicine, D.W. Brooks Dr, University of Georgia, Athens, GA 30602-7388, USA; email: corbrown@uga.edu

Who's in Charge? Leadership during Epidemics, Bioterror Attacks, and Other Public Health Crises

Laura H. Kahn

Praeger Security International, Santa Barbara, CA, USA, 2009
ISBN 978-0275994853
Pages: 236; Price: US $49.95

Dr Laura Kahn has produced a useful book that provides a brief historical background on public health and terrorism, followed by interesting examples of leadership during outbreaks and events that escalated to public health crises. The roles of astute clinicians, public health professionals, appointed public health leaders, and elected officials are described by the players themselves. These insights provide important perspectives and are fascinating reading, but each event includes the voices of only a few of many participants. This omission may leave the reader hungry for a wider variety of viewpoints.

Kahn takes the reader through a thought-provoking overview of the complexity of leadership and some early milestones in public health. Kahn makes it clear that politics, economics, communications, and interpersonal relations are as central to today's public health crises as they were in the past.

Persuasive examples support Kahn's main thesis that political leadership is critical during a public health crisis, whether the crisis results from natural causes or from bioterrorism. Kahn says, "Questions about leaders and leadership have intrigued scholars in both Western and Eastern civilizations for centuries. Plato, Confucius, and Machiavelli all speculated about leaders… and the qualities of leadership." Kahn concludes that 1) informed, engaged, and prepared elected officials are essential to effective

response; 2) because crisis response decisions inevitably will be made in the absence of perfect information, leaders require judgment and common sense; 3) elected and appointed leaders must be effective; and 4) dual leadership during a crisis can cause confusion.

The author provides a convincing case for her conclusions with lively examples and first-hand accounts and offers several concrete suggestions to prepare elected officials for leadership roles. The same compelling case is not made for Kahn's assertion of a "legal conundrum when dealing with the bioterrorism attack." She suggests that the Centers for Disease Control and Prevention (CDC) should lead the public health response to such episodes but alleges that legal and organizational impediments hinder CDC from fulfilling that lead role.

Unquestionably, CDC must and does play a lead role during large-scale, multistate public health events. The legal and organizational impediments to fulfilling that role are not obvious to this reviewer, especially given CDC's success in addressing many such crises. Kahn may be referring to impediments within the federal structure and chain of command. However, current law specifies the roles of CDC and the departments of Health and Human Services, and Homeland Security. CDC has ample legal authority to supplement its technical and scientific leadership during an emergency, especially when state and local capacities are outstripped.

Kahn suggests federalizing and centralizing the national response system through changes in the legal framework and organizational structures of the public health system, arguing that if CDC were organized for response as the Environmental Protection Agency or the Federal Bureau of Investigation is, delays, leadership confusion, and communication issues would be resolved. She identifies some leadership problems but fails to acknowledge the strong collegial relationship between state public health authorities and CDC that has produced innumerable successful responses to crises. Restructuring the traditional relationship between states and the federal government seems unnecessary.

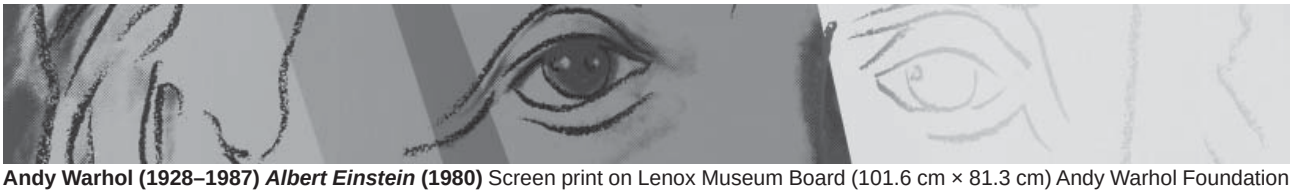
This problem does not overshadow an otherwise informative and engrossing book. In an era of emerging infectious diseases, bioterrorism, and large-scale natural disasters, we will continue to have to address the types of events Dr. Kahn describes. Those involved in responding to such events would benefit from studying the lessons of the past to better manage future emergencies.

Patrick J. McConnon

Author affiliation: Council of State and Territorial Epidemiologists, Atlanta, Georgia, USA

DOI: 10.3201/eid1606.100345

Address for correspondence: Patrick J. McConnon, Council of State and Territorial Epidemiologists, 2872 Woodcock Blvd, Suite 303, Atlanta, GA 30341, USA; email: pmcconnon@cste.org



Andy Warhol (1928–1987) *Albert Einstein* (1980) Screen print on Lenox Museum Board (101.6 cm × 81.3 cm) Andy Warhol Foundation for the Visual Arts/ARS, New York. Ronald Feldman Fine Arts, New York, New York, USA

The Unbearable Lightness of Being[1]

Polyxeni Potter

"I'd prefer to remain a mystery. I never like to give my background and, anyway, I make it all up different every time I'm asked," Andy Warhol said. "It's not just that it's part of my image not to tell anything, it's just that I forget what I said the day before, and I have to make it all up over again." The man described as "a serious artist whose posture was unseriousness" called himself "a deeply superficial person." He frustrated those who wanted to know more about him: "Just look at the surface of my paintings and films and me, and there I am. There's nothing behind it."

Though he claimed to "come from nowhere," he was born Andrew Warhola in Pittsburgh, Pennsylvania, to Czech immigrant parents. He lost his father early and grew up in a modest household during the Depression, shy and frail and plagued by childhood diseases, among them Sydenham chorea, a movement disorder that occurs with rheumatic fever, which interfered with his early formal education. As he lay bedridden for months, he listened to the radio; discovered magazines, which he read avidly; and started to draw, entering his artistic career in tune with the popular culture of his generation.

His talent was recognized early. He attended the then Carnegie Institute of Technology in his hometown, to study pictorial design and soon moved to New York to work as commercial illustrator. His first assignment for Glamour magazine was titled "Success Is a Job in New York." He found such success quickly with other magazines. He designed window displays for major retailers and promotional materials for record labels. Much of this work, which received many accolades, relied on photographs and other existing images, a process he would continue to use throughout his life.

The 1960s saw Warhol's debut in the fine arts, his Pop paintings, depicting celebrities, dollar bills, and supermarket products. Pop Art, a movement of the 1950s and 1960s, trampled tradition by embracing mass-produced elements of popular culture. Drawn from advertising, comic books, and the mass media and examined in a different light, these mundane elements were transformed into icons of contemplation—product labels and all. The controversial images were created by mechanical techniques of reproduction and established new boundaries for originality and aesthetic legibility. "What about your transformation from being a commercial artist to a real artist?" Warhol was asked in an interview. "I'm still a commercial artist," he retorted. "I was always a commercial artist."

Warhol's *Campbell's Soup Cans* in 1962, along with his depictions of cartons used for shipping and his sculptures *Brillo Soap Box*, started him on the way to fame. Unlike other Pop artists, who injected their works with irony or parody, he treated them with detachment, emphasizing, even idealizing, mass production. When asked why he painted cans of soup, he said, "I used to drink it. I used to have the same lunch every day for 20 years, I guess, the same thing over and over again." As for the dollar bills, he explained, "A teacher once told me to paint what I like."

He opened a studio, dubbed the Factory, and surrounded himself with artists, writers, musicians, and the avant-garde. There he perfected the two-layer screen printing technique for his portraits. Also called silk screening, this technique generated a precise image that could mass produce prints—a most appropriate goal considering the duplicative nature of his subjects, who existed not only in life but also on film, in magazines, newspapers, and billboards. Clips from these sources were transferred photographically to the silk or other fine mesh. The canvas was painted, completely or in part, and the screened image, which was slightly larger or smaller than the canvas, was printed on top of the painted designs, allowing some variation in each image.

At the Factory, Warhol created sculptures, published a magazine, and managed an electronic rock band called the Velvet Underground. "Your work as an artist has always been so varied, like Leonardo. You're a painter, a filmmaker, a publisher …. Do you think that's what an artist is?" He was once asked. "No," he said, "I think an artist is anybody

Author affiliation: Centers for Disease Control and Prevention, Atlanta, Georgia, USA

DOI: 10.3201/eid1606.000000

[1]Novel by Czech author Milan Kundera.

who does something well, like if you cook well." At the time of his death he was host of an MTV cable program called "Andy Warhol's Fifteen Minutes—a reference to his famous comment that in the future everyone will be world famous for 15 minutes. In the 1960s, 1970s, and 1980s, he produced more than 1,000 portraits, among them, of Marilyn Monroe, Nelson Rockefeller, Elvis Presley, Jackie Kennedy, Brigitte Bardot, and Mao Zedong.

Warhol, always cognizant of the cinematic and telegraphic nature of life, became interested in death and created portraits of people grieving, deceased public figures, the electric chair, car wrecks, race riots. This obsession with death ironically collided with an attempt on his own life inside his studio when a disgruntled disciple shot him and he nearly died. "Before I was shot, I always thought that I was more half-there than all-there. I always suspected that I was watching TV instead of living life. People sometimes say that the way things happen in movies is unreal, but actually it's the way things happen in life that's unreal. The movies make emotions look so strong and real, whereas when things really do happen to you, it's like watching television—you don't feel anything."

The philosophical issues surrounding life and death permeated Warhol's work. "Isn't life a series of images that change as they repeat themselves?" he said about image repetition in series and within individual works. When he painted soup cans or Coca-Cola bottles, he filled the canvas with them in every direction, suggesting that the line-up did not end at the edge. He insisted that all images are portraits. When he painted a portrait, it was a series.

Repetition, present at many levels in Warhol's work, is not unique in the history of art. He was aware of its use by Giorgio de Chirico (1888–1978): "He repeated the same images over and over again …. What he repeated regularly, year after year, I repeat the same day in the same painting. All my images are the same, but very different at the same time." Warhol also cited Edvard Munch: "We see with different eyes at different times. We see things one way in the morning and another in the evening, and the way we view things also depends on the mood we are in. That is why one subject can be seen in so many ways and that is what makes art so interesting."

In *Ten Portraits of Jews of the 20th Century*, the artist used archival photos over patches of color. He outlined the features in graphite, enlarged the source image on paper, and transferred both onto acetate transparencies. Against convention, the prints preceded the paintings by several months. The same silk screens were used for multiple prints and paintings, further challenging the divide between painted original and printed reproduction. One of the 10 portraits was *Albert Einstein*, on this month's cover. Like the others, this is less a portrait and more an icon; the icon of intelligence.

Further repetition and simulacra exist beyond these portraits and other Warhol works. His paintings are a sequence of recurrences already preceded by many and to be followed by many, like the eternal paths encountered by Friedrich Nietzsche's famed Zarathustra on the mountainside, embodying the philosopher's discussion of eternal recurrence: the universe and all in it have already occurred and will recur *ad infinitum*, "the most burdensome thought." Yet each icon reflects the moment of its creation and is original. Thus the artist challenges the nature of seriality as described in antiquity and in the work of Nietzsche and others, who attributed meaning to life by suggesting it was cyclical—the snake bites its tail. The artist was closer to those who disagreed, among them novelist Milan Kundera, who considered the "unbearable" possibility of no recurrence at all. At death, each person, each copy is gone.

Warhol understood how the media shape our view of the world by appropriating and projecting icons, a process he adopted and used to great advantage. Along with another media favorite, repetition, this "deeply superficial" artist captured and held up for us to see, as in a mirror, the media's fragmented version of reality. His technique fit perfectly the portrait of Einstein, whose expansive vision also allowed more than one version of reality.

With characteristic unseriousness, Warhol proposed a more contemporary view of eternal recurrence: originality in duplication. This approach has a parallel in nature, which in this issue of Emerging Infectious Diseases relates to virus replication. A virus inserts itself into the cellular machinery, which produces and reassembles new viral particles—an age-old serial activity that will continue to recur. Like Warhol's portraits, the replication is not always completely true, so some copies are not identical. The next virus, even though a replica, is not the same. The defining differences can be beneficial for virus replication and survival and may favor the emergence of infectious disease.

Bibliography

1. Andy Warhol interview [cited 2010 Apr 9]. http://www.warholstars.org/warhol1n/last.html
2. Bockris V. The life and death of Andy Warhol. New York: Bantam Books; 1989.
3. Ganis WV. Andy Warhol's iconophilia [cited 2010 Apr 9]. http://www.rochester.edu/in_visible_culture/issue3/ganis.htm
4. Stiles K, Selz PH, eds. Theories and documents of contemporary art: a sourcebook of artists' writings. Berkeley (CA): University of California Press; 1996.
5. Warhol A. Andy Warhol: in his own words. Wrenn M, editor. New York: Omnibus Press; 1991.

Address for correspondence: Polyxeni Potter, EID Journal, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop D61, Atlanta, GA 30333, USA; email: PMP1@cdc.gov

EMERGING INFECTIOUS DISEASES

Upcoming Issue

Persistence of Highly Pathogenic Avian Influenza Viruses in Natural Ecosystems

Extensive Drug Resistance in Tuberculosis and Malaria

Oseltamivir-Resistant Pandemic (H1N1) 2009 in Hospitalized Adults, Australia, 2009

Zoonotic Transmission of Avian Influenza Virus (H5N1), Egypt, 2006–2009

Ebola Hemorrhagic Fever Outbreak and New Virus Strain, Uganda, 2007–2008

High Diversity and Common Ancestry of Lymphocytic Choriomeningitis Virus

Deforestation and Malaria, Mâncio Lima County, Brazil

Population Structure of East African Relapsing Fever

Human Infections with *Rickettsia felis*, Kenya

Stepwise Evolution of Pandemic Cholera

Postexposure Treatment of Marburg Virus Infection

Human Parechovirus Infections in Monkeys with Diarrhea

Detection of Lassa Virus, Mali

Dogs, But Not Cats, as Sentinels for Japanese Encephalitis Virus Infection

Rickettsia felis as Cause of Uneruptive Fever, Rural Senegal

Novel Human Parvovirus 4 Genotype 3 in Infants, Ghana

Novel Genotypes of Influenza Viruses H1 and H3 in Pigs, China

Complete list of articles in the July issue at
http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

June 7–8, 2010
Research Advances in Malaria: Biology of Mosquito Vectors
Johns Hopkins Bloomberg School of Public Health
Baltimore, MD, USA
http://malaria.jhsph.edu/events/2010/vector_biology/index.html

June 8–11, 2010
Asia Infectious Diseases Forum 2010 (Part of World Vaccine Congress Asia 2010)
Grand Hyatt, Singapore
http://www.terrapinn.com/2010/infectious

July 11–14, 2010
International Conference on Emerging Infectious Diseases 2010
Hyatt Regency Atlanta
Atlanta, GA, USA
http://www.iceid.org

August 28–September 1, 2010
Infectious Disease 2010 Board Review Course – 15th Annual Comprehensive Review for Board Preparation
Hyatt Regency Crystal City
Arlington, VA, USA
http://www.IDBoardReview.com

November 11–13, 2010
European Scientific Conference on Applied Infectious Disease Epidemiology (ESCAIDE)
Lisbon, Portugal
http://www.escaide.eu

Announcements

To submit an announcement, send an email message to EIDEditor (eideditor@cdc.gov). In 50–150 words, describe timely events of interest to our readers. Include the date of the event, the location, the sponsoring organization(s), and a website that readers may visit or a telephone number or email address that readers may contact for more information.

Announcements may be posted on the journal Web page only, depending on the event date.

EID online
www.cdc.gov/eid

Earning Medscape CME Credit

To obtain credit, you should first read the journal article. After reading the article, you should be able to answer the following, related, multiple-choice questions. To complete the questions and earn continuing medical education (CME) credit, please go to **www.medscapecme.com/journal/eid**. Credit cannot be obtained for tests completed on paper, although you may use the worksheet below to keep a record of your answers. You must be a registered user on Medscape.com. If you are not registered on Medscape.com, please click on the New Users: Free Registration link on the left hand side of the website to register. Only one answer is correct for each question. Once you successfully answer all post-test questions you will be able to view and/or print your certificate. For questions regarding the content of this activity, contact the accredited provider, CME@medscape.net. For technical assistance, contact CME@webmd.net. American Medical Association's Physician's Recognition Award (AMA PRA) credits are accepted in the US as evidence of participation in CME activities. For further information on this award, please refer to http://www.ama-assn.org/ama/pub/category/2922.html. The AMA has determined that physicians not licensed in the US who participate in this CME activity are eligible for *AMA PRA Category 1 Credits*™. Through agreements that the AMA has made with agencies in some countries, AMA PRA credit is acceptable as evidence of participation in CME activities. If you are not licensed in the US and want to obtain an AMA PRA CME credit, please complete the questions online, print the certificate and present it to your national medical association.

Article Title

Invasive Aspergillosis after Pandemic (H1N1) 2009

Medscape CME Questions

1. Mr. Washington is a 35-year-old patient with a 2-day history of high fever, malaise, and cough. His son was diagnosed with infection with H1N1 influenza last week. None of the family was vaccinated against H1N1 influenza. He receives supportive care only for his symptoms. However, he presents to the emergency department the following week. He has experienced significant shortness of breath, and a chest x-ray reveals bilateral infiltrates. Mr. Washington is started on antibiotics but decompensates and develops ARDS.

Which of the following diagnostic strategies in this case is most appropriate if invasive aspergillosis (IA) is suspected?

A. The potential influenza infection does not influence the risk for IA in this immunocompetent patient, and further assessment for IA is not indicated
B. Bronchoscopy and culture for aspergillosis
C. Bronchoscopy with direct smear for aspergillosis
D. Serum galactomannan assay

2. Mr. Washington is diagnosed with IA. On the basis of this activity, which of the following strategies should be considered?

A. Aggressive early treatment with antiviral medications
B. Rapid diagnosis of fungal infection after the initial bronchoscopy
C. Treatment with corticosteroids
D. Initial treatment with voriconazole

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

EMERGING INFECTIOUS DISEASES®

May 2010

Respiratory Infections

The National Museum of Fine Arts, Stockholm, Sweden

Emerging Infectious Diseases is a peer-reviewed journal established expressly to promote the recognition of new and reemerging infectious diseases around the world and improve the understanding of factors involved in disease emergence, prevention, and elimination.

The journal is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, social sciences, and other disciplines. Manuscripts in all categories should explain the contents in public health terms. For information on manuscript categories and suitability of proposed articles see below and visit www.cdc.gov/eid/ncidod/ EID/instruct.htm.

Emerging Infectious Diseases is published in English. To expedite publication, we post articles online ahead of print. Partial translations of the journal are available in Japanese (print only), Chinese, French, and Spanish (www.cdc. gov/ncidod/EID/trans.htm).

Instructions to Authors

Manuscript Preparation. For word processing, use MS Word. List the following information in this order: title page, article summary line, keywords, abstract, text, acknowledgments, biographical sketch, references, tables, figure legends, appendixes, and figures. Each figure should be in a separate file.

Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and email address). Include separate word counts for abstract and text.

Keywords. Include up to 10 keywords; use terms listed in Medical Subject Headings Index Medicus.

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

References. Follow Uniform Requirements (www.icmje.org/index.html). Do not use endnotes for references. Place reference numbers in parentheses, not superscripts. Number citations in order of appearance (including in text, figures, and tables). Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text. Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title. List the first six authors followed by "et al." Do not cite references in the abstract.

Tables. Provide tables within the manuscript file, not as separate files. Use the MS Word table tool, no columns, tabs, spaces, or other programs. Footnote any use of boldface. Tables should be no wider than 17 cm. Condense or divide larger tables. Extensive tables may be made available online only.

Figures. Provide figures as separate files, not embedded in MS Word. Use Arial font for text content. Place keys within figure area. Provide footnotes and other information (e.g., source/copyright data, explanation of boldface) in figure legend. Submit figures with text content in native, editable, PC file formats (e.g., MS Excel/PowerPoint). Submit image files (e.g., electromicrographs) without text content as high-resolution (300 dpi/ppi minimum) TIFF or JPG files. Submit separate files for multiple figure panels (e.g., A, B, C). EPS files are admissible but should be saved with fonts embedded (not converted to lines). No PNG or BMP files are admissible. For additional guidance, contact fue7@cdc.gov or 404-639-1250.

Manuscript Submission. Include a cover letter indicating the proposed category of the article (e.g., Research, Dispatch) and verifying that the final manuscript has been seen and approved by all authors. Complete provided Authors Checklist. To submit a manuscript, access Manuscript Central from the Emerging Infectious Diseases web page (www.cdc.gov/eid).

Types of Articles

Perspectives. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch. Articles in this section should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may also address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Synopses. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Research Studies. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary, and a brief biographical sketch. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be no more than 1,200 words and need not be divided into sections. If subheadings are used, they should be general, e.g., "The Study" and "Conclusions." Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed 2); tables (not to exceed 2); and a brief biographical sketch. Dispatches are updates on infectious disease trends and research. The articles include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but no figures or tables.

Another Dimension. Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit.

Letters. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 figure or table and should not be divided into sections. All letters should contain material not previously published and include a word count.

Books, Other Media. Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Name, publisher, number of pages, other pertinent details should be included.

Announcements. We welcome brief announcements (50–150 words) of timely events of interest to our readers. (Announcements may be posted online only, depending on the event date.)

Conference Summaries. Summaries of emerging infectious disease conference activities are published online only. Summaries, which should contain 500–1,000 words, should focus on content rather than process and may provide illustrations, references, and links to full reports of conference activities.