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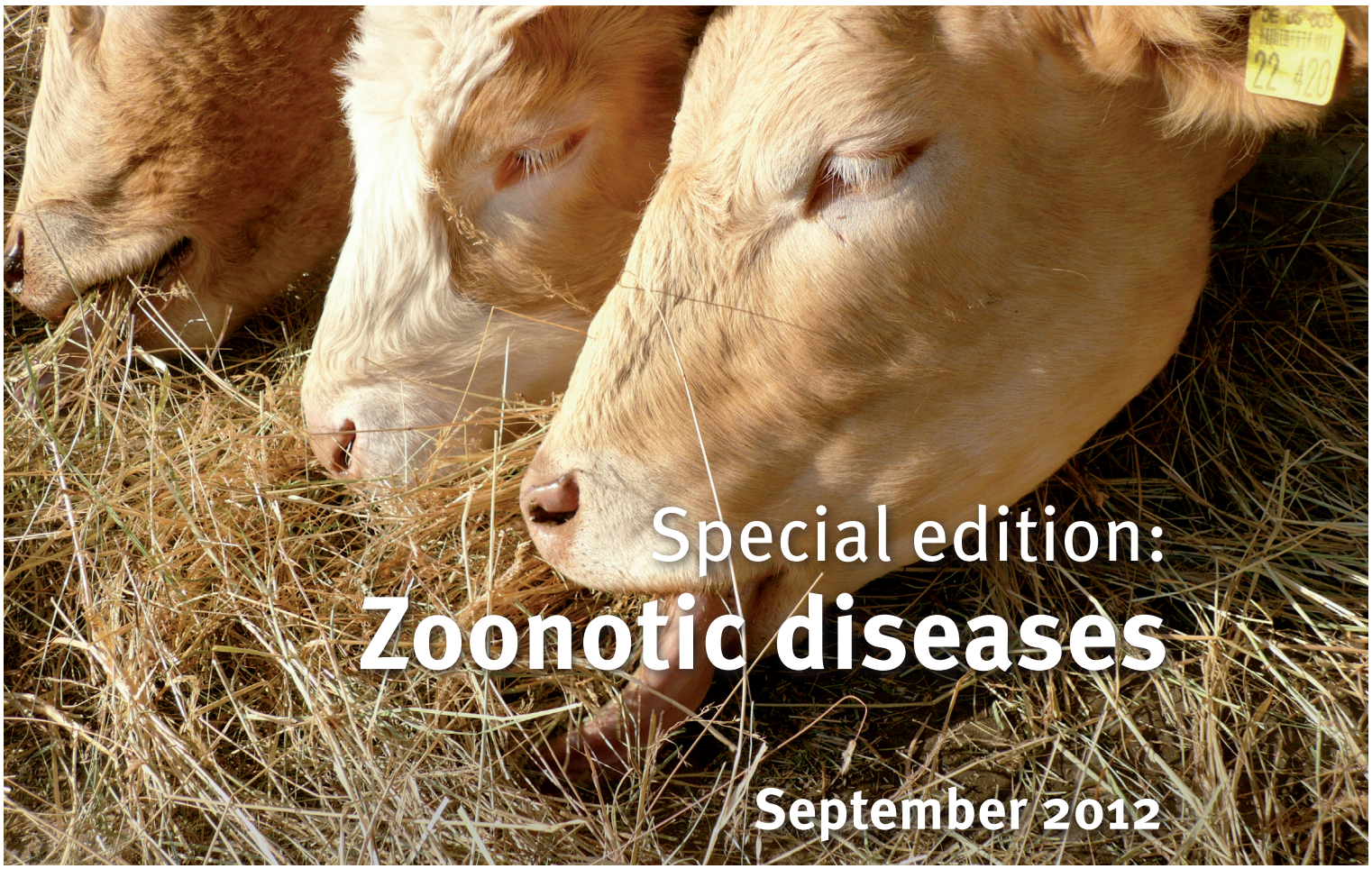

Special edition:

Zoonotic diseases

September 2012

- In this issue we present a collection of outbreak and case reports on anthrax, brucellosis, echinococcosis, leprospirosis, psittacosis, rabies, Q fever, *Salmonella* Paratyphi B and tularaemia.

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Three probable cases of cutaneous anthrax in autonomous province of Vojvodina, Serbia, June 2011

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Three probable cases of cutaneous anthrax were reported in June 2011 in the eastern part of the Autonomous Province of Vojvodina, Serbia. All cases were involved in slaughtering of a heifer that died and was suspected to have had anthrax. In the same village, anthrax was confirmed in other animals.

Introduction

Anthrax is an acute bacterial infection caused by the aerobic, spore-forming, Gram-positive organism *Bacillus anthracis*, found throughout the world. It is primarily an animal disease that occurs in wild and domestic livestock (such as cattle, sheep and goats) and rarely affects humans under normal circumstances. Humans can acquire anthrax by exposure to infected animals, animal products or spores in the soil and depending on the mode of transmission can develop one of four distinct clinical forms: cutaneous, respiratory, gastrointestinal and oropharyngeal [1].

The cutaneous form of disease is involved in 95% of human cases but the diagnosis can be very difficult in atypical presentations and in non-endemic regions when rarely encountered in clinical practice. Any delay in treatment, especially in systemic anthrax, may have fatal consequences, illustrated by case reports [2-4].

Anthrax spores and bacilli are present in the soil in some countries in Europe and continue to cause disease in animals and, occasionally, humans. In the middle and northern latitudes of Europe, anthrax in animals is either absent or found only in sporadic cases, while it remains relatively common in Turkey, Greece, the Balkan countries, Italy, Spain and the Russian Federation [5]. Given the proximity of the neighbouring countries in which human cases of confirmed anthrax have recently been found (Bosnia and Herzegovina, Bulgaria, Croatia and Romania) and uncontrolled transport of cattle across the border is very possible that there would be new cases of disease [6-9].

In Serbia anthrax occurs sporadically in animals and rarely in humans. During last five years, only five human cases were reported: four in 2008 and one in 2009 [10]. Human anthrax has been well known in the Autonomous Province of Vojvodina (APV) before 1988, a region of flat land in the north of Serbia where animal husbandry is one of the primary occupations. According to the Institute of Public Health of Vojvodina, human anthrax occurred after the Second World War with an incidence of 0.05 to 8.59 per 100,000 population [11]. The last case of human anthrax that occurred in the APV before 2011 was reported in 1988 [12].

We describe here cases of anthrax that occurred in humans and animals in Bocar, APV, in June 2011. Further cases among animals were also registered in early November 2011 in eastern Serbia, near the city of Pirot, close to the border of Bulgaria.

Anthrax in animals

In a household in the village of Bocar (Household A) in the north-eastern part of the APV, one heifer died on 2 June 2011. This death was not recognised at the time as caused by anthrax and was not reported to the veterinary authorities. The heifer was butchered in another household (Household B) on the next day.

A routine investigation of the unexplained death of the heifer took place four days later, on 6 June 2011 by the veterinary institute and veterinary centres of the two districts Zrenjanin and Kikinda, as Bocar is situated on the border of these districts. The veterinary inspection detected another two sick animals (horse and heifer) in Household A. Samples were taken from the ear muscles of those two animals. The presence of *B. anthracis* was established on 7 June by deep isolation agar and Ascoli precipitin test [13].

In Household B, a goat died on 19 June. The presence of *B. anthracis* was confirmed in a tissue sample. In another village Novo Milosevo, about 10 kilometres away from Bocar, a sick cow was reported on 17 June, which subsequently died on 22 June, with microbiologically confirmed anthrax.

Clinically ill animals have fever, difficulty in breathing, with bloody discharges from natural openings in the heifer. The animals died within two or three days after beginning of symptoms. Common to all ill animals was that they were pastured in areas recently covered with high groundwater. The dead animals had not been vaccinated against anthrax.

Human cases of anthrax

From 5 to 9 June 2011, cutaneous anthrax was diagnosed in three workers who were in contact with the first dead heifer during slaughter, and had not had any contact to the other sick animals. They were classified as probable cases of anthrax based on epidemiological and clinical data. Skin manifestation occurred on the patients' hands after the incubation period of one to two days [13], followed by high fever, without any other symptoms. The infection started as a pruritic papula. In two days the papula enlarged and formed an ulcer, about 2 cm in diameter, with typical black central crust. There was oedema of the ulcer and surrounding skin. The skin lesions were painless. The patients were treated with antibiotic therapy 15 days at home and recovered. No laboratory diagnostic tests were done on the human patients.

A further five persons who had been exposed to dead animals were followed up for 24 days during the incubation period, but no clinical signs and symptoms appeared. No human deaths were reported in the area in that period which that could have been due to undiagnosed anthrax. Meat from infected animals was not consumed because the carcasses were destroyed after appropriate transport. The meat from the first slaughtered heifer was not available for investigation and may have been used to feed pets.

Control measures

After the outbreak was reported following the death of the second animal, the veterinary authorities ordered control measures:

- a ban on releasing animals from the infected pastures for the duration of the outbreak and the risk of spread (six weeks),
- a ban on the slaughter of sick animals,
- prohibition of the use of milk and dairy products, or meat, skin and other products, as well as the sale of animals with clinical manifestation of illness or suspected to infection,
- vaccination of cattle, sheep, goats and Equidae,
- prohibition of butchering of dead and sick animals,
- disinfection of places where a dead animal had been kept.

The population of affected areas was encouraged to report all animal deaths to the veterinary authorities, to treat carcasses of dead animals only in accordance with the instructions of experts, to limit the number of persons present in contaminated yards, to use protective equipment in contact with the animals and to disinfect it after use, and to contact a medical service immediately in case they developed fever or skin changes. Educational material about anthrax was distributed to households in affected areas.

Discussion

Currently, very few cases of anthrax occur in developed countries [14], but in developing countries anthrax continues to be an important infectious disease. The incidence of infection can be reduced dramatically by the vaccination of animals at high risk, along with improvements in industrial hygiene [15]. In Serbia routine vaccination of livestock against anthrax is not implemented regularly but is carried out only in areas where this bacterium is present in the soil and nature.

The three probable human cases of anthrax in Bocar we describe here could be a result of feeding cattle with hay and grass on pastures which had high ground water following heavy rains during spring. Several studies have shown that meteorological factors such as shifts between rainy and dry periods can contribute to the migration of anthrax spores on the surface of pasture land and contamination by anthrax spores [16-20]. Under adverse environmental conditions, e.g. after release into soil from dead or dying animals, the vegetative bacilli die but endospores survive. The spores are remarkably resistant to a range of adverse environmental conditions such as temperature, desiccation, pH, chemicals or irradiation, which makes decontamination difficult.

As anthrax spores can persist for a long time in the environment, decontamination of the ground and vaccination of animals are important public health measures to prevent further cases in animals and humans and they should be continued even after several anthrax-free years. Existing programmes of prevention and control of both animal and human anthrax need to be evaluated and the surveillance system enhanced, including the development of laboratory diagnostic capacities for human anthrax in Serbia. The enhanced surveillance system requires close collaboration between services for the prevention and control of human and animal diseases, and prompt reaction of both services after reports of possible cases of anthrax.

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Fatal anthrax infection in a heroin user from southern Germany, June 2012

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Blood cultures from a heroin user who died in June 2012, a few hours after hospital admission, due to acute septic disease, revealed the presence of *Bacillus anthracis*. This report describes the extended diagnosis by MALDI-TOF and real-time PCR and rapid confirmation of the anthrax infection through reference laboratories. Physicians and diagnostic laboratories were informed and alerted efficiently through the reporting channels of German public health institutions, which is essential for the prevention of further cases.

In early June 2012, a case of anthrax infection was identified in an injecting drug user in Germany. Anthrax wasn't suspected initially and the patient died on the day of hospital admission. Two days later anthrax was confirmed and the relevant authorities were informed. This report underlines the importance of considering anthrax as a possible diagnosis in injecting heroin users presenting with fever or sepsis at emergency rooms and of the rapid management of such cases.

Clinical case description

In early June 2012 an injecting drug user in their 50s presented at the emergency department of a hospital in the south of Germany, with a two-day history of worsening swelling and reddening at an injection site, nausea and dyspnoea. The patient had been on oral substitution therapy for two years. Moreover, a history of chronic hepatitis C infection with liver cirrhosis was reported. In the next hours after admission to hospital, the patient developed respiratory failure and was transferred to the intensive care unit (ICU) where they were ventilated mechanically. An elevated white blood cell count (15.9 cells/nL), anaemia (haemoglobin 10.4 g/dL), thrombocytopenia (38 cells/nL), elevated procalcitonin (1.05 ng/mL) and hypokalaemia (2.5 mmol/L) were observed. Elevated liver enzymes, lowered coagulation parameters and extremely high levels of D-dimers (>36,364 ng/mL) were pointing to multi-organ failure. Blood and urine cultures were sent to the Institute of Medical Microbiology and Hygiene, University of Regensburg. The patient's condition worsened and they died on the day of admission due to a septic shock with multi-organ failure and massive disseminated bleeding. At the time, there was no clinical suspicion of anthrax.

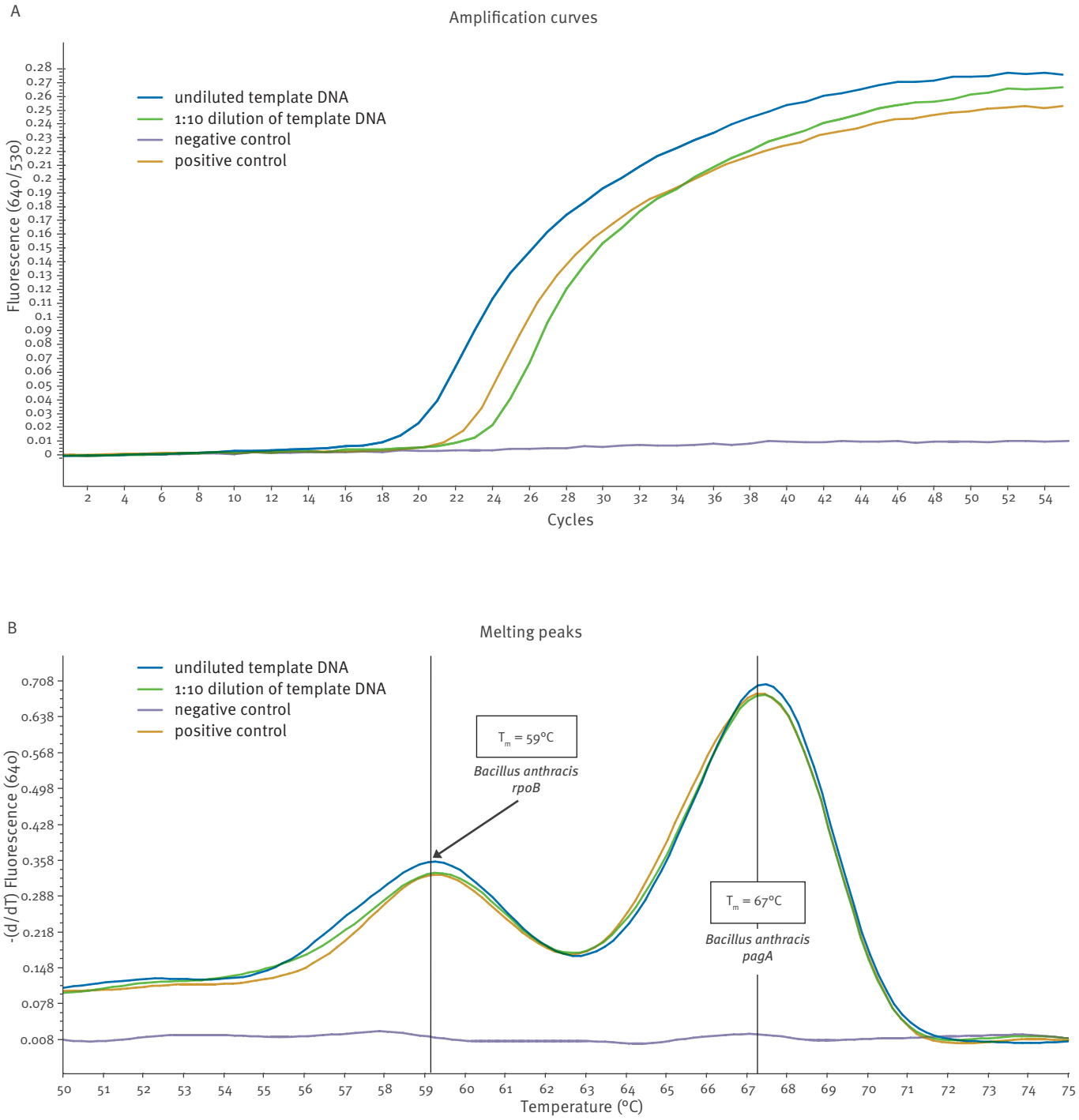
Laboratory analysis

Blood cultures (Becton Dickinson, Heidelberg, Germany) turned positive after 53 minutes of incubation. Gram-stained microscopy showed non-branching Gram-positive bacilli growing in chains. Subcultures presented typical growth of aerobic spore-forming bacilli without haemolysis. Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) identification revealed *Bacillus cereus* (Bruker Daltonics, Bremen, Germany). The patient's history led to reanalysis with the Bruker 'SR Database' that contains so-called security-relevant organisms, which correctly identified *B. anthracis*.

An initial set of molecular diagnostic tests was performed for confirmation at the Institute of Medical Microbiology and Hygiene, University of Regensburg. Briefly, a loopful of cells was suspended in 500 µL of detergent buffer. The buffer consisted of TE (pH 7.5) containing 0.5% Triton X-100 and 0.25% Tween 20. The suspension was heated at 95°C for 30 minutes with occasional shaking, sonicated for 1 minute, and heated again at 95°C for 30 minutes. After 10 minutes centrifugation at 11,000 × g, the supernatant was passed through a 0.2 µm sterile filtration membrane. Extracting genomic DNA from a boiled bacterial culture proved to be reproducible, easy-to-perform and rapid before [1]. The DNA was directly used as template for a series of real-time PCR assays performed with the Light Cycler PCR system (Roche Diagnostics, Mannheim,

Figure

Real-time PCR amplification plots (A) and melting curve analysis (B), anthrax infection, Germany, June 2012



T_m: melting temperature.

LightMix *Bacillus anthracis* PCR Kit (testing 5 µl aliquots of the original and 1:10 diluted template DNA preparations, respectively).

Germany). The real-time assays included an in-house protocol for pan-bacterial 16S rDNA amplification and the LightMix kit *B. anthracis* (Cat. No: 40-0252-16, TIB Molbiol, Berlin, Germany). The kit is designed to detect the *pagA* gene as marker for the plasmid pXO1 and a *B. anthracis*-specific segment of the bacterial *rpoB* gene for species identification, using hybridisation probes. Early crossing points (around cycle 18) and the specific melting points of the respective target genes *pagA* and *rpoB* were observed in the melting curve analysis, indicating the presence of *B. anthracis* carrying at least the virulence plasmid pXO1 (Figure).

To substantiate the initial test results, an aliquot of the DNA preparation was sent to the Bundeswehr Institute of Microbiology in Munich. The initial PCR results were confirmed and extended using PCR assays for the *capC* gene (marker for the second virulence plasmid pXO2) and an additional chromosomal marker highly specific for *B. anthracis* (dhp61) [2]. First results of molecular genotyping of the strain showed close relationship to strains from a large anthrax outbreak among IDUs in Scotland [3].

Control measures

The District Health Office was informed about the suspected case of human *B. anthracis* infection immediately after obtaining the PCR results. Their experts got involved in the management of the case in close contact with the diagnostic institutions, the police authorities and the Task Force Infectiology of the Bavarian Health and Food Safety Authority (LGL). Health officials considered contaminated heroin or cutting agents mixed with the heroin as possible source of the infection. Further investigations by the German police authorities were initiated immediately.

The competent public health authorities at national level were informed immediately about the confirmation of *B. anthracis*. The information on the occurrence of the case was distributed to the public health authorities in all 16 German federal states, at international level through the Early Warning and Response System (EWRS) of the European Commission and via ProMED-mail [4]and according to the International Health Regulations (IHR). In Bavaria, the medical associations were informed. Substance abuse counselling agencies were contacted nationally and at European level through the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) in order to spread the information among drug users. Additional information and materials were published by the public health institutes on their websites.

Identification of the second case

Two weeks after the first case was admitted to hospital, a second case of anthrax was identified in an IDU from the same region as the first case. The patient is stable under antibiotic therapy after surgical debridement [5]. The raised level of awareness created with the first case lead to a much faster workflow in the laboratory analysis in the second case. *B. anthracis* was confirmed three hours after blood cultures turned positive.

Discussion

Injectional anthrax has first been reported 1988 as fourth route of infection besides cutaneous, gastrointestinal and inhalational anthrax infections [6]. The first anthrax case related to injecting drug use was described 2000 from Norway [7]. There were no subsequent reports of injectional anthrax until 10 December 2009 when anthrax was identified in blood cultures from two injecting drug users from Glasgow, Scotland [8]. In the following months an increasing number of cases were identified [9]. By the end of the outbreak in December 2010, there were 47 confirmed cases of injectional anthrax (including 13 deaths), 35 probable cases (including one death) and 37 possible cases in Scotland and five cases including four deaths in England [3]. There were two confirmed cases in Germany related to this outbreak, including one fatal case [10]. The favoured outbreak hypothesis assumed that heroin had been in contact with goat skin contaminated with anthrax spores during transportation to Scotland [3]. Risk factors for infection were longer injection history, receiving opioid substitution therapy, and alcohol consumption [11]. All cases of injectional anthrax reported so far including the case presented here were not associated with the typical black eschar seen in patients with cutaneous anthrax [12].

Box 1

Timeline of events, fatal case of anthrax infection, Germany, June 2012

Day 1	• Patient admitted to the hospital
	• Blood cultures sent to the laboratory
	• Patient dies due to septic shock
	• Blood cultures positive with Gram-positive bacilli (late afternoon)
Day 2	• Growth of *Bacillus* spp. on subcultures
	• MALDI-TOF: *Bacillus cereus*
	• Discussions on anthrax suspicion
	• Different PCRs and 16S sequencing over night
Day 3	• *B. anthracis* confirmed by PCR
	• Information of local health authorities
	• Involvement of regional and national health and police authorities
	• DNA sent to Bundeswehr Institute of Microbiology
Day 4	• *B. anthracis* confirmed using further PCRs
	• Robert Koch Institute promotes further information at national and international level

MALDI-TOF MS: matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry.

Because *B. anthracis* is seen very rarely in Germany and other developed countries, laboratory staff and clinicians should raise their attention when Gram-positive bacilli growing in chains are detected in clinical specimens (Box 2).

B. anthracis cannot be reliably distinguished from *B. cereus* by growth characteristics, bacterial cell morphology or biochemical methods. The applicability of MALDI-TOF-MS for the identification of *B. anthracis* was demonstrated elsewhere [13]. Because of safety regulations, *B. anthracis* and other potential bioterroristic agents are not included in the manufacturer's (Bruker Daltonics) database. As in our case, the isolate is classified as *B. cereus* with the standard databases. Using a special database, containing the missing spectra, *B. anthracis* is identified correctly. The manufacturer discourages the standard use of the *B. anthracis* spectra due to misidentification of members of the *B. cereus* group. Consequently, the result 'B. cereus' in combination with a patient's history of injecting drug use should lead to further diagnostic steps. To differentiate between *B. anthracis* and non-anthracis Bacillus species harbouring anthrax-specific virulence plasmids, PCR targeting a chromosomal marker should be performed in addition to PCR assays covering the virulence plasmids pXO1 and pXO2. Non-pathogenic *B. anthracis* strains not containing plasmids can be identified using this combination as well [2, 14].

Box 2

Recommendations and lessons learnt from the fatal case of anthrax infection, Germany, June 2012

- When growth of *Bacillus cereus* sensu lato is identified by the MALDI species typing database, a sound anamnesis of the underlying clinical case should be performed.
- Suspicious cultures should be transferred to a biosafety level 3 environment and, whenever possible, a spectrum of validated molecular tests should be kept in stock for level 3 pathogens (especially anthrax).
- An agreed case definition and protocol for alerting the authorities should be available and known to all microbiologists and clinicians.
- Appropriate reporting channels should be maintained and exercised by the public health authorities to prevent that similar (or parallel) cases remain undetected.
- Confirmatory PCR testing in a specialised laboratory should be immediately requested. Diagnostic laboratories should know such specialised laboratories in their vicinity for support and check the logistics of sample transport in a situation of emergency (ideally before they encounter their first uncommon strain).
- Clinicians and microbiologists should be trained on a regular basis in the identification of anthrax and other rare infectious diseases that are highly pathogenic.

MALDI: Matrix-assisted laser desorption/ionisation.

Conclusions

Health professionals and diagnostic laboratories should consider anthrax as a possible diagnosis in injecting heroin users presenting with fever or sepsis at the emergency room. The observed re-emergence of drug-related anthrax in Germany supports the hypothesis that heroin may provide a continuing entry route of *B. anthracis* into western Europe.

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Cutaneous infection caused by *Bacillus anthracis* in Larissa, Thessaly, Central Greece, July 2012

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In July 2012, a confirmed case of cutaneous anthrax infection in a stockbreeder in the prefecture of Larissa, Thessaly, Central Greece was reported. The investigation revealed five related deaths in animals (two dogs and three sheep). Control measures have been taken immediately in order to prevent further spread in humans and animals.

On 7 July 2012, a stockbreeder in his early 60s was admitted to the Department of Medicine, University Hospital of Larissa, Greece with high fever up to 39.5 °C accompanied by rigors, malaise and generalised weakness that had been present for the previous six hours. The patient reported the appearance of three pruritic papular lesions on the left forearm five days earlier. He further reported that he had slaughtered and flayed a sheep six days before admission to hospital.

Case description

Upon hospital admission, the patient was febrile, his vital signs were normal, and during the physical examination three painless ulcers on the left forearm with surrounding vesicles and oedema, covered by black eschars were observed (Figure). The left axillary lymph nodes were significantly swollen. No other signs or symptoms were found during the physical examination.

Laboratory results on the day of hospital admission revealed elevation of acute phase response markers (white blood cells: 17,100/µL (range: 4,000-10,000/µL), neutrophils: 14,600/µL (range: 2,400-6,000/µl), C-reactive protein: 3.5 mg/dL (range: 0.5 mg/dL)). A working diagnosis of cutaneous anthrax was established on the basis of the patient's place of residence and typical clinical presentation. Therefore, intravenous treatment with penicillin (24 million units per day) was started immediately [1]. After 10 days of hospitalisation, he was discharged in good health with clinical and laboratory results indicating complete recovery. Although the possibility of inhalation exposure in this case was very unlikely, the precise conditions of the direct contact that took place during flaying are not known. Therefore, upon discharge from hospital, the patient received amoxicillin (oral dose of 1,500 mg per day) for an additional 45 days as a post-exposure prophylaxis against the potential development of anthrax pneumonitis.

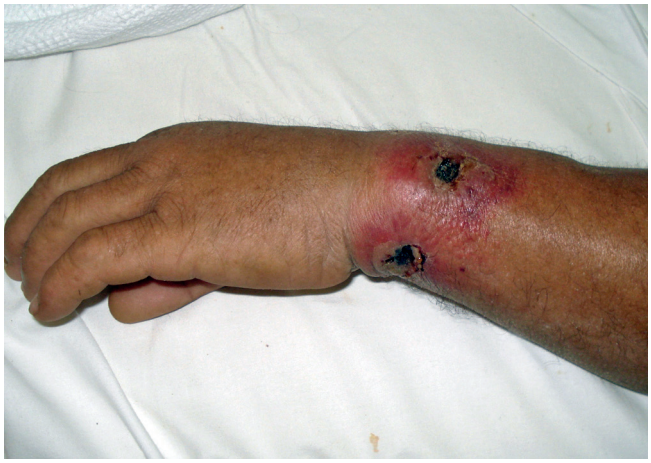
Laboratory investigation

On 8 July, one day after hospitalisation of the patient, biological samples (smears from pustules) were sent to the Department of Microbiology at the Medical School of the University of Thessaly. Microscopic examination of the smears showed the presence of Gram-positive rods, typical for *Bacillus anthracis*. However, bacterial cultures remained negative; this finding could be explained by the fact that, at the time the samples were taken, the patient was already under penicillin treatment at a high dose.

Blood samples were obtained by the local veterinarian from two more sheep that have died in the same herd after 7 July. These two were also inspected and microscopic examination revealed the presence of

Figure

Skin lesions due to cutaneous anthrax infection, Larissa, Thessaly, Central Greece, July 2012



Gram-positive rods and bacterial cultures grown for 24 h on 5% blood agar produced grey-white colonies. Preliminary identification was performed using conventional methodology. Briefly, haemolysis detection and motility testing was performed as described previously, using 5% horse blood and trypticase soy broth (Bioprepare, BioPa Kerateas, Greece) [2]. Capsular testing was performed using nutrient agar plates supplemented with 0.7% $NaHCO_3$ (Bioprepare), incubated in 5% CO_2 for 24 h, followed by McFadyen methylene blue staining. Genus and species confirmation, as well as detection of the two *B. anthracis* plasmids, pXO1 and pXO2, responsible for the species' pathogenicity, was performed using SYBR Green real-time PCR and the primer pairs BA813F/R, PAG67/68 and CAP57/58, as well as the Genesig *Bacillus anthracis* Real Time PCR kit (PrimerDesign Ltd, Southampton, UK), which is based on TaqMan chemistry [3].

The microorganism isolated from the sheep was identified as *B. anthracis* and carried the two pathogenic plasmids pXO1 and pXO2; the pXO1 plasmid contains the *lef*, *cya* and *pag* genes, which encode the lethal factor, oedema factor and protective antigen, respectively, while the pXO2 plasmid contains the *cap* gene, which encodes the capsule [3].

Epidemiological investigation

The stockbreeder was contaminated after having handled the slaughtered sheep due to direct contact with the infected animal. He had flayed the animal together with his wife and then fed two dogs with the contaminated meat. These dogs died during the next day. After 36 hours, the specific anthrax cutaneous lesions appeared on the exposed area of the stockbreeder's skin. Since 7 July, two more sheep have died in the same herd. No other death occurred in this or other herd in the same village (Tsabournia).

It can be assumed that the stockbreeder's wife was also exposed to the spores of the infected animal. However, she did not present any signs or symptoms of infection and is now under post-exposure prophylactic treatment.

Control measures

The stockbreeder's wife hasn't developed any symptoms during the maximum incubation period of 15 days, but is currently receiving post-exposure prophylaxis. The residents of the village (Tsabournia) have been informed about this case in order to recognise early clinical symptoms of anthrax and they were advised to seek medical treatment immediately if anthrax was suspected. The local health centre and general practitioners are aware of this need for careful monitoring. Special directions have been given to the stockbreeders of Tsabournia regarding the use of protective equipment. The local Veterinary Authority has taken measures for the correct disposal of animal carcasses, including disinfection of contaminated material and decontamination of the environment. Mass vaccination of 7,000 animals is currently in progress.

Background information

Anthrax is an acute infectious disease caused by a large, spore-forming, toxin-producing bacterium *B. anthracis* [4]. It is the oldest known zoonosis with worldwide distribution and has been known to man for hundreds of years, mostly as an animal disease, typically in agricultural areas [4,5]. The disease is endemic in many countries of the world, particularly in tropical and sub-tropical areas, such as southern Europe, Asia, Africa, North and South America, and Australia [6,7]. It commonly occurs in well defined endemic areas where environmental conditions are particularly favourable for the survival of the spores. In Europe, there is a definite declining trend: The number of reported human cases remained at around 25 cases per year during a ten-year period (1995–2004), and has since decreased even more (2005: 10 cases, 2006: 16 cases, 2007: five cases, 2008: three cases, 2009: 14 cases) [8-12]. In the last four years, several reports of anthrax infections in heroin drug users have been reported in European countries [13-15].

Until 1979, Greece, particularly the northern part of the country, was considered as an enzootic zone for anthrax [6]. Although the number of animal outbreaks between 1970 and 1979 had declined to almost a quarter of that of the previous decade (1960–1969), there were 300 outbreaks a year, mostly involving sheep. During this period, there were 8,475 sheep and 1,675 bovine losses in 3,669 separate outbreaks. During the same period, 482 human anthrax cases occurred in the country and all patients were from rural areas [6]. The highest incidences were observed in the prefectures of Aetoloakarnania, Evros, Ioannina, Larissa, Rodopy and Thessaloniki [6]. Since then, strict control measures have eliminated the disease and only sporadic cases in animals and humans have been reported. According to the epidemiological reports from the European Centre for Disease Prevention and Control (ECDC), only 38 confirmed human cases of anthrax were reported between 1994 and 2010 [8-12]. However, it should be stated that although anthrax is included in the notifiable diseases and every suspected case should be reported to the Hellenic Center for Disease Control and Prevention (HCDCP), there is some degree of underreporting and the low number of reported cases does not allow general conclusions regarding the accurate incidence trend.

Thessaly is a rural region located in Central Greece and includes four prefectures (Karditsa, Larissa, Magnesia, Trikala). The estimated number of goats and sheep in this region is above 2 million. The large majority of them (more than 1 million goats and sheep) are farmed in Larissa prefecture. According to the records of the local Veterinary Authority of Larissa, three outbreaks of anthrax have been reported in Larissa in the past 35 years (in 1978, in 2000, and in 2006) (unpublished

data). All of them occurred in herds kept in two villages (Livadi and Tsabournia) situated at a distance of 35 km from each other in the area of Elassona, Larissa prefecture. Approximately 90 animals were affected in total, and the outbreaks were contained after correct disposal of animal carcasses and vaccination of exposed animals. According to the epidemiological data of the Veterinary Authority, no case of anthrax in animals or humans has ever been declared in the other three prefectures of Thessaly.

In 1978, anthrax infection had been confirmed in animals of three different herds in Tsabournia. However, no human infection has been reported. Vaccination and appropriate control measures have been taken; since then until the incident described here no other anthrax case in animals or in humans has been reported.

Conclusions

From a public health point of view, anthrax is important for Europe as well as for other regions. Infections still occur in Greece and clinicians should be aware of the disease and of the need for immediate management and reporting to the HCDCP [16].

In the management of the case described above, the level of post-prophylactic treatment may be seen as unusual according to the World Health Organization (WHO) recommendations (no post–prophylactic treatment required in a patient previously treated by intravenous penicillin) [1]. Here, post-exposure prophylaxis was nevertheless recommended after hospital discharge because the precise conditions of direct contact which took place during flaying were not clearly known [17].

Early recognition of this suspected human case and reporting to the local authorities without delay have led to the prevention of further spread of the disease both in humans and animals.

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Re-emergence of brucellosis in cattle in France and risk for human health

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A case of human brucellosis was diagnosed in France in January 2012. The investigation demonstrated that the case had been contaminated by raw milk cheese from a neighbouring dairy farm. As France has been officially free of bovine brucellosis since 2005, veterinary investigations are being conducted to determine the origin of the infection and avoid its spread among other herds. Hypotheses about the source of this infection are discussed.

In January 2012, a human case of brucellosis was diagnosed by blood culture in a district of the French Alps. The isolated strain was identified as *Brucella melitensis* biovar 3. The patient had presented in late November 2011 with non-specific symptoms that had been ongoing since that date. Usual at-risk exposures were investigated: recent or ancient travel in an endemic/enzootic country, consumption of raw milk or raw milk products imported from an enzootic country, professional or accidental exposure to *Brucella* strains in a laboratory, direct contact with animals, etc. As the patient had not had such an exposure at any point before, the case was considered to be an autochthonous case of acute brucellosis of undetermined origin.

In April 2012, brucellosis was confirmed in a dairy cow in a herd of the same district of the French Alps. The seropositive cow had aborted in late January, and a strain of *Brucella melitensis* biovar 3 was isolated from the milk sampled from the animal. The animal belonged to a herd 21 dairy cows, and no other animal in the herd presented with symptoms suggesting brucellosis or showed any serological reaction. Approximately 20 kg of Reblochon cheese (soft raw milk cheese) are usually produced daily on the affected farm.

Brucellosis surveillance in France

France has been officially free of brucellosis in cattle since 2005, and the last outbreak of brucellosis in sheep and goats was reported in 2003. In order to detect and prevent any re-emergence of the disease, annual screening using Rose Bengale test or complement fixation test is carried out in all cattle, sheep and goat farms producing raw milk as well as in all cattle herds, and every one to three years in small ruminant, according to EU regulations [1-4]. Moreover, abortion in ruminants is mandatorily notifiable and the investigation of abortion includes examination for brucellosis.

Human brucellosis in France is mandatorily notifiable. The National Reference Centre (NRC) determines the characteristics of *Brucella* strains isolated from patients [5,6]. Serological suspicions also have to be confirmed by the NRC, as the low specificity of available tests can be responsible for false-positive results. The confirmation is carried out using a combination of in-house tests including Rose Bengale test, immunoassay, complement fixation test, and specific detection of antibodies against *Yersinia enterocolitica*.

Veterinary investigation

All animals were tested serologically (Rose Bengale test, complement fixation test and indirect enzyme linked immunosorbent assay) before slaughter in April [5]. Following French regulations, all animals in the infected herd were immediately slaughtered, and three pairs of lymph nodes (retro-pharyngeal, retro-mammary and internal iliac) were sampled from all animals for *Brucella* culture [5] and PCR [7]. All animals were seronegative with the exception of the index animal which showed a very strong reaction in all three tests. However, *Brucella* was isolated from a second animal in the herd, and PCR-positive results were obtained for

four further animals, in addition to the index animal and the second cow with an isolation of *Brucella*.

Following the confirmation of brucellosis in the cow, a trace-back investigation was implemented by the veterinary services to determine the origin of the contamination of the herd. The animals of the infected herd had not taken part in a transhumance nor did they graze with other herds on the same pastures. Other neighbouring farms as well as farms that had traded animals with the infected farm in the year before the outbreak were investigated. All tested negative in serology [5].

A trace-forward investigation was also carried out to determine the places of distribution of cheese produced at the affected farm since the abortion of the cow.

Reblochon cheese is a raw milk soft cheese, requiring a maturation period of three weeks to one month. The cheese from the affected farm had been commercialised after the abortion in seven districts. Cheese was sold directly at the farm, and as whole pieces or in parts in supermarkets. Cheese produced by the affected farm had not been exported to other countries but might have been bought by foreign tourists during their winter holidays in several ski resorts in the area. For this reason, the European rapid alert system for food and feed (RASFF) was informed.

Human investigations

After the identification of the first bovine case, the human case was interviewed again to investigate any direct or indirect epidemiological link with the infected herd. During the second interview, it became clear that the patient and their family had visited the infected farm in autumn 2011, although it was not possible to determine the exact date. During this visit, the family had bought *Tome Blanche* cheese, a fresh cheese obtained during the first step of Reblochon production. The four family members had shared the *Tome Blanche* on the same day, but the index case was the only one who later presented with symptoms. The other three family members were serologically investigated in May 2012 and only one presented with a positive high titre in agglutination (1,600). The farm reported no other visitors during that period, apart from neighbours.

Microbiological investigations

The strain isolated from the human case and from the two cows both belonged to *Brucella melitensis* biovar 3. The strains had the same genotype as determined by multilocus variable number tandem repeat analysis (MLVA) [8].

Control measures

All cheese pieces produced by the affected farm and still within the shelf life were withdrawn from retailers. In addition, a recall of already sold products was carried out via a national press release by the cheese producer and by posters in the sale points. Medical doctors in the concerned districts were informed by the regional health authorities. Consumers of these products were advised to seek medical attention should they present symptoms consistent with brucellosis.

The release of cheese from the affected farm was immediately stopped. The movements of animals from other herds that had epidemiological links with the infected herd (those that were geographically close to the infected herd, or had been bought from the infected herd) have been restricted until the end of the investigation. Furthermore, raw cheese products from farms with epidemiological links to the infected farm were put on sale only after negative bacteriological tests results had been obtained.

Reinforcement of human surveillance

Notification of human brucellosis is mandatory in France. All notified human cases in France have to be confirmed by the national reference laboratory. From 2002 to 2011, 219 human cases were confirmed in France. Among them, 183 (84%) were patients infected through the consumption of raw milk products or direct contact with animals in (or from) countries with enzootic brucellosis, 14 (6%) were laboratory workers infected through the handling of *Brucella* strains, 17 (8%) were relapses in people with past infection, while the origin of contamination could not be determined for five patients (2%) [9].

Because the investigation of the origin of the human case diagnosed in January 2012 had been inconclusive, it was decided to reinforce the surveillance immediately. Since January 2012, all notified suspected cases have been interviewed with a trawling questionnaire before the diagnosis was confirmed. Since April 2012, any epidemiological link with the infected herd has been systematically investigated. No other related human cases have been identified so far.

Discussion

At this time, several hypotheses can be proposed to explain the re-emergence of brucellosis in cattle in France. One explanation is contact with an infected cattle or small ruminant. Knowing that the affected herd had not received any imported animals, it needs to be investigated whether animals had been introduced in one of the herds that sold animals to the affected farm or whether the affected herd had been in contact with animals of neighbouring farms. Another hypothesis would be a contamination of cattle by wildlife. Some chamois (*Rupicapra rupicapra*) were found infected with *B. melitensis* biovar 3 in 1988 in the Alps, and some of these animals may have become chronically infected and not display symptoms [10]. However, no infected chamois has been identified in the last 10 years, despite several serological surveys (Garin-Bastuji, personal communication, July 2012). *B. melitensis* biovar 3 is the most common biovar isolated in ruminants worldwide, and therefore the identification of this biovar in a district like the French Alps

with many different ruminant species cannot contribute to a more precise hypothesis.

The veterinary investigations are still ongoing to determine the origin of the contamination of the herd, to investigate the possible spread of the infection to other herds and to take control measures to avoid the infection of new herds and consequently the occurrence of additional human cases.

However, the absence of infected animals in the herds that are epidemiologically linked with the infected herd, and the absence of other autochthonous human cases argue in favour of a single outbreak and a limited episode. The index animal on the farm was born from a dam that itself was born in 1999 before the last outbreak in the area and died in 2006. The lifetime of the mother of the index infected animal is therefore consistent with the hypothesis of a congenital case of bovine brucellosis [11].

In addition to the investigations already carried out, all herds coming back from transhumance in the concerned district will be serologically screened during the fall. Serological tests lack specificity but they have a good sensitivity and are of good value to detect recent or active infections. The index animal had an active infection demonstrated by *Brucella* excretion in milk. This animal displayed a high level of antibodies in relation with the active although possibly chronic infection. During the early investigation, a *Brucella* strain and *Brucella* DNA were detected in ganglions of seronegative animals, demonstrating chronic latent infections, with no antibodies. Strengthened surveillance of human and animal brucellosis will be maintained until the end of the investigations.

The surveillance of human brucellosis in non-endemic countries is complicated by the lack of specificity of serological tests [12-16]. In our experience, all available tests still may cross-react with other bacteria (mainly *Y. enterocolitica*, but not only), and can also give false positive results in patients presenting with immune disorders. In countries with low prevalence and incidence of the disease, this low specificity contributes to the low positive predictive value of serology. A positive diagnosis has important consequences for the patients (long antimicrobial therapy with possible adverse effects and ecological consequences on intestinal bacteria), and for the dairy animals (culling of the entire herd in our country). It is therefore important to obtain as much evidence as possible to confirm a serological diagnosis.

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Investigations and actions taken during 2011 due to the first finding of *Echinococcus multilocularis* in Sweden

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Echinococcus multilocularis is a parasite that can cause alveolar echinococcosis disease. After the first positive finding of *E. multilocularis* in Sweden in 2011, a consulting group with representatives from relevant authorities was summoned. In this group, all relevant information was shared, strategies for information dissemination and any actions to be taken due to the finding of *E. multilocularis* were discussed and decided. The present paper describes the actions taken during 2011 and the results thereof, including surveillance in animals, risk assessment for humans to become infected and recommendations given to the public. Further discussion about whether the parasite was introduced, and if so, how, as well as possible future development of the infection in animals and humans in Sweden and future actions are included.

Introduction

Alveolar echinococcosis (AE) is a disease in humans caused by the larval stage of the tapeworm *Echinococcus multilocularis* (EM). It is considered to be the most serious parasitic disease in humans in Europe [1].The parasite develops with a tumour-like growth almost exclusively in the liver and the disease is characterised by a long incubation period, between five and 15 years, followed by a subsequent chronic course [2]. Although a serious disease, in Europe, the reported prevalence in humans is low, up to 1.4 per 100,000 population [2]. During the last decades, the known range of the parasite in Europe has extended and, although data is not comprehensive, it is assumed that the parasite is present over most of Europe with the exception of the British Isles and the Mediterranean region [1]. It is however unclear whether this extension corresponds to its true range or whether it reflects previous absence of surveillance [1]. In Sweden, Norway and Finland, surveillance in animals from 2000 to 2009 had shown that in 2009, using a design prevalence of 1%, these countries were most probably free from the parasite [3]. However, in February 2011, EM was identified in a red fox (***Vulpes vulpes***) in Lanneröd, Sweden for the first time [4]. The fox was shot within the routine surveillance programme in 2010. After this finding, a consulting group, lead by the National Board of Health and Welfare (SoS), was summoned. The group consisted of representatives of the Swedish Board of Agriculture (JV), the Swedish Institute for Communicable Disease Control (SMI), the National Food Agency (NFA), National Veterinary Institute (SVA), the Swedish Work Environment Authority and the relevant county medical- and county veterinary officers. Regular teleconferences were usually held every 1–2 weeks, during which information concerning EM and the situation in the country was shared, and strategies for information dissemination and actions to be taken were discussed and decided.

The aim of the present paper is to describe the actions taken due to this finding and the results thereof, i.e. surveillance in animals, risk assessment for humans to become infected and recommendations given to the public. Further discussion about whether the parasite was introduced, and if so, how, as well as possible future development of the infection and future actions are included.

Methods

Surveillance in animals

Immediately after the finding of EM, increased surveillance in foxes was started [4]. Hunters were requested to submit foxes primarily from southern Sweden because it was considered that EM was most probably introduced in this area. The aim was to analyse 3,000 foxes with segmental sedimentation and counting technique (SSCT) [5], thereby detecting a prevalence

of 0.1% on country basis. Furthermore, faecal samples from hunting dogs (n=119) in the four municipalities around Lanneröd were examined at SVA by egg flotation [6] and an in-house real-time polymerase chain reaction (PCR). A non-random sampling of potential intermediate hosts was also started in an area within a 50-km radius surrounding Lanneröd. During March–April, 2011, a total of 236 rodents were collected, mainly ***Arvicola amphibius*** followed by ***Myodes glareolus***, ***Microtus agrestis***, ***Apodemus sylvaticus***, and ***Apodemus flavicollis***. The rodents were autopsied and liver or other organs with lesions (n=72) were tested by an in-house PCR. As extensive sampling of rodents is probably needed to identify the intermediate host species in an area with very low prevalence of EM, sampling of rodents continues.

Risk assessment

By 3 March 2011, the Swedish government gave a mandate to JV and SoS to, in cooperation with relevant authorities and organisations, clarify necessary actions to protect public health as a consequence of the finding of EM. Within the government mandate, a qualitative risk assessment about the probability of humans becoming infected with EM was performed in the spring of 2011 by SMI and NFA.

Recommendations and public health measures

To ensure that relevant and harmonised information concerning what was known as well as what was not known was given to the public, this issue was continuously discussed in the consulting group. Furthermore, optimal ways of dissemination of this information was also investigated.

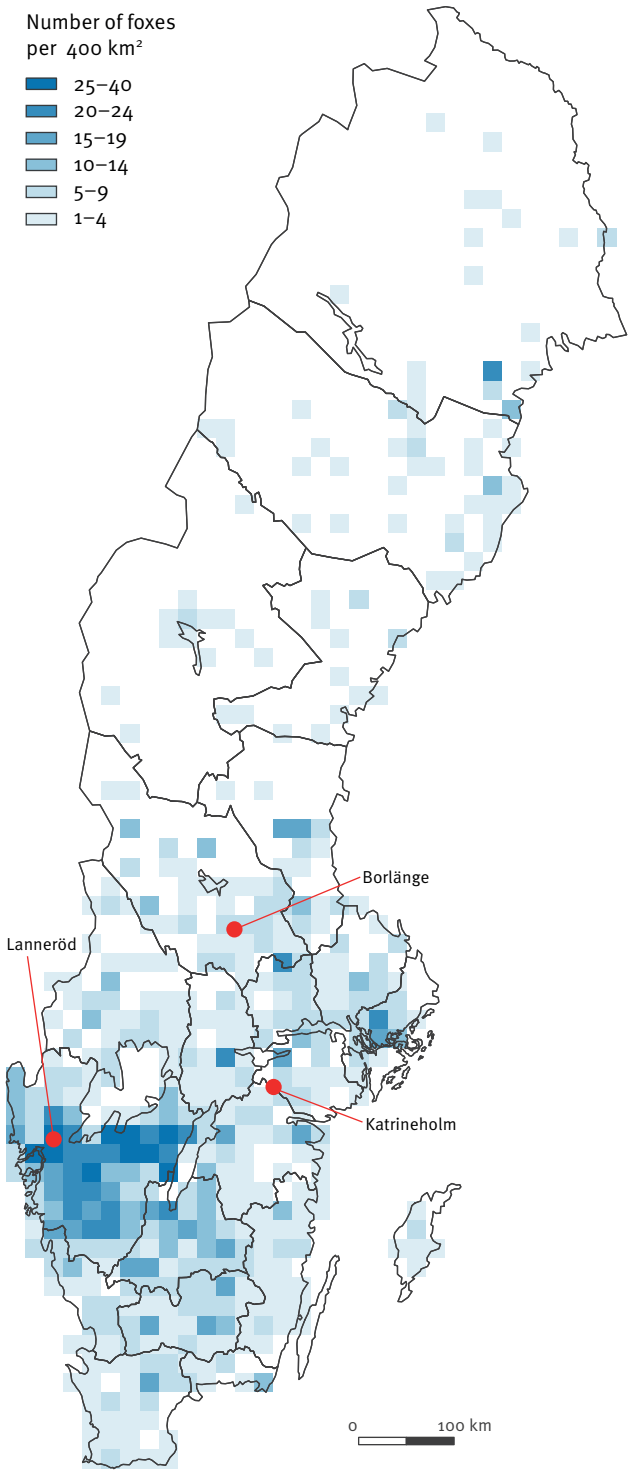
Results

Surveillance in animals

Shortly after the first fox testing positive for EM was found, the prevalence of EM in foxes seemed to be very low in Sweden, probably well below 1%. Surveillance of red foxes during 2000–2009 (n=2,962) had yielded negative results [3] and after the first positive finding, several hundred foxes, shot within the increased surveillance, were analysed with no further animals testing positive. Analysis of all faecal samples from hunting dogs in the four municipalities around Lanneröd did not yield any positive results and none of the rodents tested within the 50 km radius surrounding Lanneröd were found positive. The question was raised whether it could be possible to control and even eradicate EM. It was considered most probable that EM had been introduced to Sweden in recent years by infected dogs [4] and therefore the spread of EM could be geographically restricted. Besides Rebun Island, Japan, where EM was eradicated [7], the parasite had previously only been successfully controlled in geographically limited areas. However, based on advice from international experts and literature research, it was concluded that it might be possible to eradicate EM. A preliminary cost–benefit

Figure 1

Geographical distribution of all georeferenced foxes shot and analysed for *Echinococcus multilocularis*, Sweden, January–June 2011 (n=2,900)



The three locations where the three *Echinococcus multilocularis* positive foxes were respectively shot are indicated on the map. Foxes were georeferenced with the coordinate system RT90.

analysis showed that if eradication was possible, benefits would exceed the costs [8].

By 31 March 2011, when a total of 1,140 foxes (shot in 2011) had been analysed for EM, a second infected fox was found. This fox was shot in the Lanneröd region at the same location and by the same hunter as the first infected fox (shot in 2010). This finding confirmed the presence of the infection in this region but did not change the interpretation of the situation. By 27 April, when 1,758 foxes had been analysed, a third case was found nearby Katrineholm, more than 200 km northeast of Lanneröd. Although the probability that EM was spread to other parts of Sweden increased, investigation into ways to eradicate EM continued and the deworming recommendation was extended to include dogs at risk in this area as well. However, by the end of May, when 2,525 foxes had been analysed, a fourth infected fox was found outside Borlänge about 200 and 300 km respectively north of the previous findings (Figure 1). Thus it was concluded that EM was probably not restricted to only the few known infected areas in Sweden and that eradication was not feasible. By the end of June, the increased surveillance of foxes was completed and had resulted in the finding of a total of three positives of 2,985 analysed foxes (0.1%). The geographical distribution of foxes with georeferences (n=2,900) is illustrated in Figure 1.

Risk assessment

Humans become infected by ingesting eggs from the parasite and several modes of transmission are plausible, such as consuming contaminated food or water, inhaling eggs from contaminated environments or by letting contaminated hands or objects come in contact with the mouth. However, due to the long incubation period and the low incidence of AE there is little evidence in the literature to help discriminate the relevance of the different modes.

Evidence for direct food transmission is the observation that monkeys and pigs became infected by consumption of grass probably contaminated with fox faeces [9]. One epidemiological study identified consumption of unwashed strawberries as well as chewing on grass as risk factors, but not picking berries, eating unwashed herbs or vegetables [10]. In another study, consumption of strawberries, mushrooms, blueberries, herbs, parsley or cranberries were not identified as risk factors [11]. In contrast, using well water rather than tap water [12] or using water from certain lakes [2], was identified as a risk factor.

The results of the literature search were similarly inconsistent for risk factors regarding farming, gardening and hunting [11-13]. Many risk factors regarding environmental exposure are hard to separate from the consumption of food. One of the studies related two-thirds of the cases to farming or similar activities, probably reflecting contact with a contaminated environment [10]. The only garden activity more common among cases than controls was growing (not consuming) leaf or root vegetables, supposedly due to the amount and intensity of care required for annual compared to perennial plants [10].

Interaction with animals, regarding the risk of humans getting infected, has been investigated and inconsistent results have been presented. Two of five case–control studies identified dog ownership as a risk factor for acquiring AE [10,14], especially if the dog was left unattended in the garden or if it was killing game, whereas in the three remaining studies dog ownership was not found to be a significant risk factor [11-13]. The two studies on cat ownership as a potential risk factor, both found an association between being an AE case and owning a cat [10,11]. However, in one study the risk was small and much smaller compared to owning a dog [10].

A correlation between the prevalence in foxes and in humans has been found. However, although the prevalence in fox populations in some countries is high, the reported number of cases in humans is relatively low [15,16]. This may indicate that the actual risk of becoming infected is not only linked to exposure to the pathogen, but also to individual susceptibility, perhaps because of immunological differences [17].

In conclusion, risk factors most often identified in epidemiological studies are associated with living, working or other activities in rural environments, which makes it difficult to distinguish between environmental, food, soil, and other routes of transmission. With the evidence available, contact with contaminated environment, is considered to be an important risk factor and farmers, hunters and dog owners, whose dogs eat rodents were considered to be the group at highest risk.

Due to the current low prevalence in foxes and since no cases of AE have been reported in Sweden, the competent authorities concluded that the risk to humans in Sweden of developing AE was considered to be small. It was estimated that about one person among the nine million Swedes would be infected and develop AE every fifth year. Moreover, if the probability of infection in humans were to become similar to Switzerland this figure could increase to 20–30 cases yearly. As the prevalence of EM in the fox population could change over time, it was considered important to repeatedly monitor the fox population to be able to assess a possible increase of EM prevalence, and the risk that this may pose to humans

Recommendations and public health measures

Initially, recommendations to prevent human infection were kept general, but emphasised the importance of proper hand hygiene after contact with free running pets in risk areas. After finalisation of the risk assessment, it was concluded that the importance of food and drinking water for the transmission of AE to

humans could not be assessed and that there were no documented risk-reducing effects of washing vegetables and berries. Based on these knowledge gaps and the low number of reported AE cases even in areas in mainland Europe where the prevalence of EM in foxes is high, and taking the benefits of outdoor activities including harvesting and consuming berries and vegetables into consideration, the NFA and SMI concluded that it was not appropriate to issue any specific recommendations about EM and food. However, consumers were informed that good hygienic practices when handling food apply also with regard to EM. To consumers who do not accept any risk, information was given that boiling food is the only effective way to inactivate EM. Recommendations were communicated by authorities via the internet and also by a common information site (www.krisinformation.se).

After the first positive finding of EM in a Swedish fox in Lanneröd, JV issued recommendations that dogs at risk, i.e. dogs that could catch rodents, in the four surrounding municipalities should be dewormed monthly. Later, when another fox tested positive near Katrineholm, the deworming recommendation was extended to also include dogs at risk in this area. However when results of the surveillance indicated that EM was endemic at a very low prevalence in Sweden, recommendations to dog owners in the country were withdrawn. For worried dog owners, whose dogs eat rodents, deworming the dogs monthly was nevertheless suggested to prevent infection.

For the particular case of pet dogs entering the country from abroad, it was decided that dog owners should be informed, that dogs coming from endemic regions of mainland Europe need be dewormed before entry in Sweden. It is important to highlight that the risk of dogs becoming infected is greater in many European countries where the prevalence of EM is much higher compared to Sweden. In Sweden the prevalence in foxes appears so far to be very low, about 0.1%, but in certain areas in Europe 50% of foxes or more may be infected [2]. Deworming will reduce the risk not only for the individual dog owners, but also prevent introduction to areas where EM may not yet be present.

It was concluded that should the prevalence of the EM within the Swedish fox population remain very low, no further recommendations to the public would be given. Monitoring the fox population, however, was considered important to be able to reassess information campaigns to the public if an increase of EM would be observed. In addition, increased monitoring was considered necessary as the geographical spread of EM as well as the prevalence in different areas is not well known. There is also a need for more information on the fox population density in different areas of Sweden and how the population changes over time. Of special interest are urban foxes as they, due to closer contact with people, are considered to pose a greater risk. It was therefore concluded that increased and repeated monitoring of EM in foxes as well as monitoring of the fox population is needed.

If high population densities of urban foxes with a high prevalence of EM were found in Sweden, this would increase the risk to humans. Because control strategies applied locally, such as deworming dogs and baiting strategies for foxes can reduce this risk [16,18] it was concluded that an action plan should be prepared in case such high risk areas were found in Sweden. The action plan should also clarify how relevant information is provided to the public and groups most at risk.

Finally it was concluded that there is a need for research. More knowledge about the epidemiology of EM in Sweden is also needed, such as which intermediate hosts are involved in the life cycle of EM and what the present and expected future distribution and prevalence of EM in the country may be. More knowledge is needed on risk factors for developing AE as well as what can be done to prevent infection.

Discussion

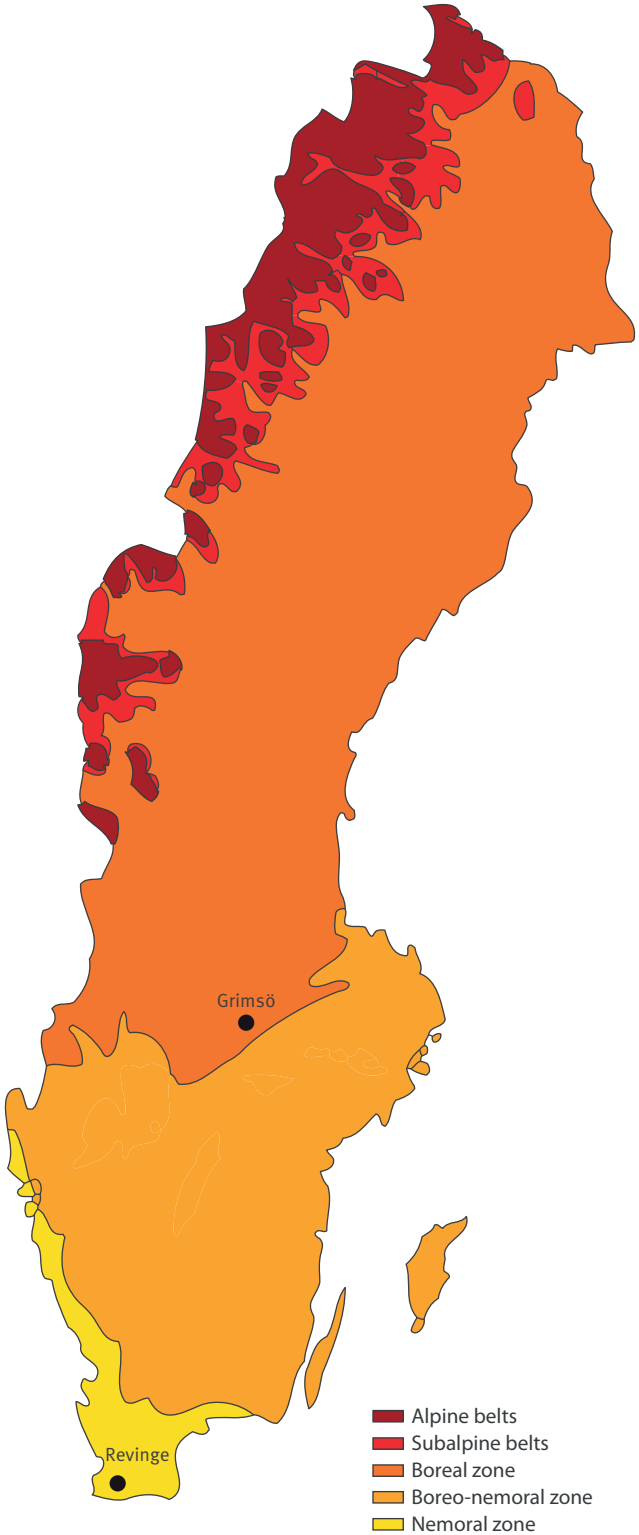
It is not known when EM was introduced to the Scandinavian Peninsula. However, if introduction was recent, unlawful admission of dogs from mainland Europe is the most probable explanation. Risk assessments have shown that without a very high compliance with import requirements, introduction of dogs from endemic areas constitutes a risk of introduction of EM [19,20]. Compliance with import requirements has decreased and the number of imported dogs has increased substantially in Sweden since 1994 (personal communication, Maria Cedersmyg, January 2012). Prior to 1994, all dogs were dewormed in quarantine prior to entry to Sweden. In 1994, for dogs from certain European countries, this was replaced by a requirement that a veterinary deworming certificate should be shown at the border. Furthermore, in 1995, border control was restricted as Sweden joined the European Union (EU), thereby prohibiting routine control of deworming certificates of dogs.

Another possible explanation for the present findings of EM in foxes is that the parasite has been endemic for a long time but escaped detection due to limited surveillance. According to the negative binomial distribution and assuming a test with 100% sensitivity, 3,000 foxes have to be analysed to have a 95% probability of detecting EM given a prevalence of 0.1%. In the routine surveillance in Sweden, started in the year 2000, more than 2,900 samples were analysed before the first case was detected. This highlights that extensive surveillance is needed to detect a low prevalence of EM. Introduction by foxes from Finland was considered unlikely as, despite intensive surveillance [3], the parasite has not been found in this country.

The present and future spread of EM in Sweden is unknown. The epidemiology of EM depends on the fox population density as well as the interaction with

Figure 2

Vegetational zonation in Sweden, 1999



Locations where fox population densities have been estimated, Grimsö and Revinge, are shown. Vegetation data is reproduced with permission from Acta Phytogeographica Suecica [35].

intermediate hosts. For non-urban mainland Europe fox population densities have been reported to be 0.5–3 foxes/km² [21-25]. In Sweden, the corresponding figures (during the 1970s) were 0.8 (Revinge, nemoral zone) and 0.2–0.4 foxes/km² (Grimsö, southern boreal zone) [26,27] (Figure 2). During the 1980s an epizootic of sarcoptic mange struck the Swedish fox population and the density of foxes declined considerably especially in southern Sweden [28]. However, the population recovered to the levels of the 1970s in the early 1990s, and monitoring has not revealed any dramatic change after this recovery [29,30]. The fox population density varies, from relatively high and stable in the nemoral and boreonemoral zones (south) to a lower density with a much higher degree of fluctuation in the boreal zone (north) [26,27,31,32] and the fluctuations in the north follow those of vole populations [33]. The three areas where EM has been found have suitable fox habitat characterised by a mixture of forest and agricultural land. It is concluded that although the fox population density in Sweden is lower compared to mainland Europe, it is sufficient to maintain the lifecycle of EM. Perhaps besides northern Sweden, where the decreased fox density during the lowest phase of the population fluctuation may be too low for EM to prevail, there is no reason to believe that EM could not be established in the rest of Sweden. In urban areas, the fox populations in mainland Europe have been reported to be high and may exceed 10 foxes/km² [34] and these fox populations play an important role in the transmission of human AE [18]. However, although foxes are present in cities also in Sweden, information on the urban fox population densities are lacking.

Furthermore, it is not known which intermediate host species are involved in the life cycle of EM in Sweden. Based on previous knowledge on EM prevalence among intermediate host species [36-38], known and expected food preference by the red fox in Sweden and Norway [39], and the occurrence of different small rodents in the identified EM-infected areas in Sweden, the most likely intermediate host candidates should be ***Arvicola amphibius, Microtus agrestis and Myodes glareolus***; all common and distributed throughout Sweden [40]. ***Microtus arvalis***, one of the principal intermediate hosts in mainland Europe does not occur in Sweden.

It was concluded that the risk of developing AE in Sweden is low. However, it might be argued that the risk of being infected by EM could be higher in Sweden than in other countries with similar prevalence. One reason is the unique legislation on Right of Public Access to land, which gives the public right to roam freely in the countryside. Outdoor activities such as hiking, camping and berry- and mushroom picking are long standing traditions in Sweden. Hunting is a widespread activity that adds to the number of people in close contact with nature. Still, there is a lack of scientific studies comparing behaviour in different countries, making it not possible to assess whether the risk is higher in Sweden due to particular behaviours, such

as outdoor activities. Another reason for the risk being hypothetically higher in Sweden is that EM was only recently detected, so there is no tradition of how to minimise risk of exposure. It has not been shown that information will reduce the risk, but there are studies reporting differences between countries in Europe in terms of knowledge and perception of the risk of AE [41]. In some other countries in Europe, where EM is endemic, there are recommendations to rinse and/or cook berries and vegetables before eating them and to wash the hands thoroughly after contact with soil or vegetation, to avoid being infected with EM. For dog and cat owners there are recommendations to regularly deworm the pets in case they roam outdoors and eat wild rodents.

After concluding that eradication was not possible, the only preventive action taken by the authorities was issuing recommendations. However, due to lack of knowledge, the recommendations given were quite general. In this situation, there was a requirement from the general public and especially from hunters to at least try to prevent further spread of EM. The question was raised whether increased fox hunting could be beneficial. However, because hunting may increase the immigration rate and lower the age distribution of the fox population [26], hunting may increase the spread of EM especially if the prevalence of EM is higher in adjacent areas. Hunting may also increase the EM biomass if the proportion of young foxes increases as, apart from one recent study in Lithuania [42], the worm burden has been reported to be higher in younger foxes [43,44]. A hunting pressure high enough to influence spring density of reproducing animals is probably seldom attained. It was concluded that intensified hunting in infected areas and especially in hot-spots may be beneficial however, increased fox hunting in areas where EM has not been found is not recommended.

According to the authorities, more knowledge about the prevalence of EM in different areas is needed. Although an extensive surveillance was performed after the first finding, there is a need for additional sampling especially in areas where the sampling intensity was lower. Furthermore, there is a need for long term monitoring to follow any future changes in prevalence. It is also important to extend the current monitoring of the population density of small rodents [45,46] and to also involve the south of Sweden. At present there is no suitable method for large scale surveillance of EM. Until now surveillance in Sweden has been based on foxes shot by hunters. The latter foxes were analysed with coproantigen enzyme-linked immunosorbent assay (ELISA) [47] or in-house PCR, and after the first positive finding with SSCT [5]. However, collection of foxes shot by hunters is cumbersome, costly and associated with a risk of exposure to EM. Sampling of fox faeces is expected to lower the costs and also the risk of exposure but none of these are considered suitable for large scale surveillance. However, earlier modeling results have indicated that, depending on the expected prevalence of EM infections in wild boars and the sensitivity of the test, surveillance of EM-lesions or antibodies in wild boars could be used to monitor EM in areas with a dense wild boar population [3]. Investigations are ongoing to evaluate whether surveillance in wild boars could be appropriate for the southern half of Sweden where 57,300 wild boars were shot during the hunting season 2010/11 [48].

Finally, the need for more research was identified by the authorities. Most important, more knowledge about risk factors for becoming infected with EM is needed so that relevant recommendations can be given to minimise risk of infection. Risk factor studies using diagnostic tools such as serology may have the potential to improve knowledge about risk for exposure to EM. The most important knowledge gaps identified in the risk assessment of transmission of EM via food were the importance of the risk of consumption of berries, fruits and other vegetables and how much the risk can be reduced by careful washing/rinsing of berries and vegetables. There is also a need for a cost effective surveillance that could be implemented on a large scale to estimate the level of contamination in different geographical regions and also assess future trends. Furthermore, from a Swedish point of view, there is a need for scientific studies comparing human behaviour in different countries, so it can be investigated whether the Swedish Right of Public Access to land (allowing people to roam freely in the country side and for example pick berries and mushrooms) and the present use of it, affects the risk of becoming infected by EM. Finally, there is a need to increase our understanding of the epidemiology of the disease in Sweden by efforts such as increased surveillance to identify the intermediate host species for EM.

Conclusions

The present risk to humans of becoming infected with EM and developing AE is considered to be small. It is most probable that EM is already spread within Sweden. Increased surveillance is needed to enhance knowledge about present and future prevalence of EM. An action plan will be developed to handle a potential future increased risk for humans, if the prevalence of EM increases. There is a need for more research about the epidemiology and surveillance of EM.

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Severe leptospirosis in a Dutch traveller returning from the Dominican Republic, October 2011

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In October 2011, a case of leptospirosis was identified in a Dutch traveller returning from the Dominican Republic to the Netherlands. The 51-year-old man had aspired muddy water in the Chavón river on 29 September. Twenty days later he presented with fever, nausea, vomiting, diarrhoea, arthralgia, headache, conjunctival suffusion and icterus. *Leptospira* serovar Icterohaemorrhagiae or Australis infection was confirmed ten days later by laboratory testing.

We report on a patient diagnosed with leptospirosis following travel to the Dominican Republic. Only a few cases of leptospirosis have been described among travellers to the Dominican Republic [1]. This case serves as a reminder for physicians to consider leptospirosis in the differential diagnosis of febrile patients returning from the Dominican Republic.

Case report

At the end of September 2011, a 51-year-old Dutch male spent 14 days at a tourist resort in Punta Cana, Dominican Republic. During his stay he made several excursions, among which one was a swimming excursion to the Chavón river near the village Altos de Chavón. While swinging from a vine, he fell in the river. His travel companions covered his body and face with mud from the river bank, which caused the patient to aspire muddy water. Twenty days after this incident, when back in the Netherlands, he presented with fever, nausea, vomiting, diarrhoea, arthralgia and headache at the outpatient department of the Havenziekenhuis in Rotterdam. On physical examination conjunctival suffusion and icterus was noted. Laboratory results showed raised C-reactive protein (280 mg/L, norm: 0-10 mg/L), thrombocytopaenia ($44x10^9$/L norm: 150-400$x10^9$/L) and total bilirubin (104 µmol/L, norm: 0-17 µmol/L) without a marked increase in liver transaminases, and signs of renal dysfunction (creatinine 268 µmol/L, norm: 65-115 µmol/L). After admission, the clinical condition of the patient deteriorated with hypotension, progressive kidney failure and anuria for which he was admitted to the Intensive Care Unit. Because there had been typical exposure to mud, twenty days prior to the clinical manifestations, the working diagnosis was septicaemia due to leptospirosis.

The diagnosis was confirmed by the demonstration of specific agglutinating antibodies against *Leptospira* spp in a microscopic agglutination test (MAT), titer 1:320, and specific immunoglobulin M (IgM) antibodies (ELISA › 1:160) in a second sample taken 10 days after presentation. Interestingly, even though serology was negative in the serum sample taken on admission, a real-time PCR was positive [2,3]. The causative serovar was identified by the MAT as probably belonging either to the *Leptospira* serovar Icterohaemorrhagiae or Australis [4]. Other potential diseases such as malaria and dengue, were excluded. Blood cultures taken on admission remained negative.
He was treated with ceftriaxone intravenously and doxycycline orally. The patient's condition improved following intensive fluid resuscitation and infusion of vasopressors. His renal function had recovered completely after seven days and after 10 days, the patient left the hospital.

His fellow travellers remained asymptomatic throughout this period.

Background

Leptospirosis is a worldwide zoonotic infection with a much greater incidence in tropical regions [5,6]. An increasing number of imported cases of leptospirosis following international travel are being published [7]. High risk areas include India, Sri-Lanka, Thailand, Vietnam, Malaysia, China, Seychelles, the Caribbean, Brazil and the Pacific Islands. Leptospirosis is now considered an emerging disease in travellers [8]. Human infection results from exposure to infected urine from carrier mammals, either directly or via contamination of soil or water. Leptospirosis in travellers is usually associated with recreational activities that involve contact with freshwater, soil and animals such as jungle trekking and kayaking [9].

Conclusions

Statistics published by the Epidemiology Department of the Dominican Ministry of Public Health show that from January until mid-March 2012 there were 211 suspected cases of leptospirosis [10]. In 2011, there were a total of 891 suspected cases of leptospirosis in the Dominican Republic, a clear decrease compared with 2010 when there were 1,270 suspected cases [10]. As outbreaks often occur following natural disasters such as earthquakes, weather conditions as rainstorms and ensuing floods could have an impact on the incidence of leptospirosis in the Dominican Republic [11].

Physicians taking care of travellers returning ill with fever should consider leptospirosis a differential diagnosis in those who have travelled to areas where *Leptospira* spp are endemic and those who participated in high-risk activities. Given the potentially fatal course of severe leptospirosis, pre-emptive antibiotic treatment for leptospirosis should be considered without delay in febrile travellers returning from endemic regions, who have been exposed to freshwater and soil or have had skin contact with animals [12–15]. Travellers who plan to engage in water activities should be advised about preventive measures such as wearing protective clothing and shoes, and to cover up abrasions.

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Psittacosis outbreak in Tayside, Scotland, December 2011 to February 2012

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A Tayside outbreak of psittacosis December 2011–February 2012 involved three confirmed and one probable cases. Confirmed cases were indistinguishable by sequencing of polymerase chain reaction (PCR) products. The epidemiological pattern suggested person-to-person spread as illness onset dates were consistent with the incubation period and no single common exposure could explain the infections. In particular the only common exposure for a healthcare worker case is overlap in place and time with the symptomatic index case.

Outbreak description

During February 2012, Tayside's Health Protection Team was notified of five cases of pneumonia. These illnesses affected four family members and one healthcare worker (HCW) who had tended the index case. Four of these developed severe symptoms, two requiring intensive care unit (ICU) admission. These four had complement fixation tests (CFT) suggesting infection with a *Chlamydophila* species. Although speciation was not possible at this stage, the time interval of one to 22 days between the symptom onset of consecutive cases, suggested person-to-person spread. An outbreak of *Chlamydophila pneumoniae* infection therefore seemed likely. Pending identification, the outbreak response proceeded on this basis. By mid-February *C. psittaci* was confirmed by polymerase chain reaction (PCR).

Background

Psittacosis is a systemic infectious disease caused by *Chlamydophila psittaci*. Usual features include fever, malaise, unproductive cough, headache and atypical pneumonia. The incubation period is one to four weeks [1]. Since its first description in 1879 [2], epidemics occurred during the next century. Where identified, the source of such outbreaks and infections was zoonotic, and predominantly avian but not necessarily psittacine. For example, large outbreaks occurred among poultry workers [3]. Subsequently, these have become rare, as avicultural hygiene has intensified. In Scotland, up to 10 sporadic cases per year were notified (no outbreaks) in the past 10 years (Table) [4]. We have found no case described in the literature where person-to-person spread has accounted for cases of psittacosis, although person-to-person transmission has evidently been suggested but not proven [5].

Outbreak investigation and results

During a series of outbreak management team (OMT) meetings, results were assessed and further investigation directed. Awareness raising among Tayside medical practitioners aimed to increase case ascertainment. The investigation progressed on three fronts: epidemiological, microbiological and environmental.

TABLE

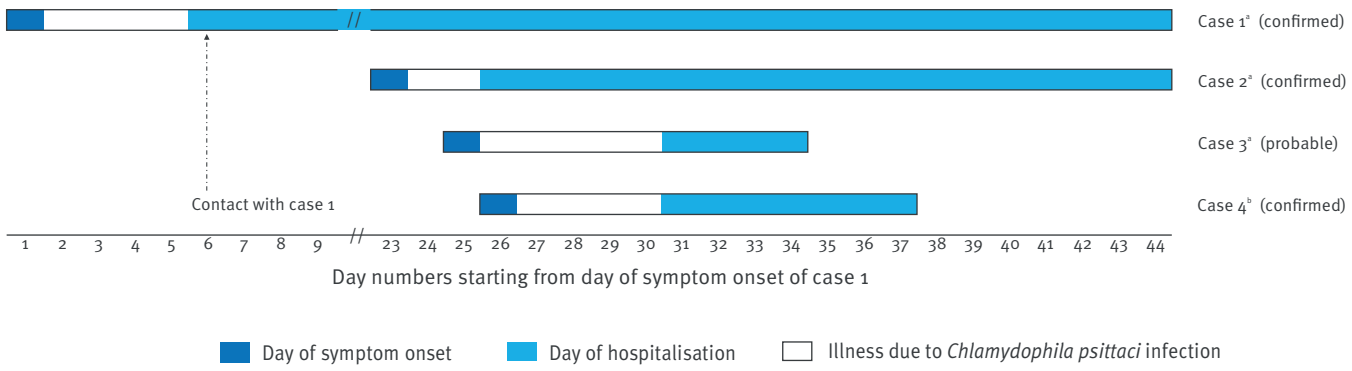
Total number of cases of *Chlamydophila psittaci* infections notified annually, Scotland, 2001–2011 (n=27)

Year	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011
Number of cases	2	10	1	4	0	0	1	1	2	5	1

Source: Health Protection Scotland (HPS) (Lynda Browning, personal communication, 23 May 2012) [4].

Figure

Time of symptom onset and clinical course of probable and confirmed cases, psittacosis outbreak in Tayside, Scotland, December 2011–February 2012 (n=4)



[a] Cases 1, 2 and 3 were part of an extended family and had extensive and frequent contact with eachother.
[b] Case 4, a healthcare worker, had contact with case 1 on the sixth day of case 1's illness, as indicated by an arrow.

Epidemiological investigation

A modified Centers for Disease Control and Prevention (CDC) case definition [6] was agreed. To be considered, cases must have compatible clinical illness. All notified cases were interviewed about their illness, contacts and relevant possible exposures. Confirmed cases had either *Chlamydophila* species detected in respiratory secretions (by culture or PCR) or a fourfold or greater increase in antibody (IgG or IgM) to *Chlamydophila* species (to a reciprocal titre of 32 between paired acute- and convalescent-phase serum specimens taken at least two weeks apart) by CFT. Cases which were epidemiologically linked to a confirmed case were considered probable, given an antibody (IgG or IgM) titre of 256 or greater, and possible given one of 32 to 128 (all by CFT in a serum specimen taken after symptom onset).

Applying this, by 22 February 2012, the outbreak involved three confirmed, one probable and two possible cases, with the index case having had onset of illness in late December 2011. The figure describes the time of onset and clinical course for confirmed and probable cases. These comprised three female and one male with an age range of 41 to 65 years.
A further two possible cases were identified: a family member with mild respiratory illness and an unrelated patient from the same ICU as the index case.

Microbiological investigation

Initial investigations used CFT performed according to standard methods using antigen obtained from Launch Diagnostics, Longfield, Kent, United Kingdom (UK) [7]. The CFT antigen is a chlamydia group specific antigen. The test detects total complement fixing antibody: both IgG and IgM.

Real-time PCR was performed using in house assay on respiratory samples which were initially used for investigations for respiratory viruses. The screen for *Chlamydophila* species was an assay targeted to 16S ribosomal sequences. Any positive sample was further investigated by specific real-time PCR to *C. psittaci* or *C. pneumoniae* targeting a different region of the 16S ribosomal sequence. This enabled determination of which *Chlamydophila* species was involved in a case.

Of the confirmed cases, two showed a rising CFT titre, one a static raised titre. All were PCR positive. Sequence analysis of the outer membrane protein A (ompA) gene showed 100% similarity between these *C. psittaci* strains. The probable case had a static CFT titre above 256 and was PCR negative. Possible cases had static titres of 64 to128 and were PCR negative.

Environmental investigation

Extensive cartographical and field searches were made for possible avian sources of infection. These were directed by information gleaned from interviews with cases. Workplaces and residences of cases were plotted on an Ordnance Survey map. Cases 2 and 3 lived together a kilometre from case 1. Case 4 resided a further ten kilometres west. Although not within any of the cases' respective place of residence, two pigeon coops and a cage of small birds were found in the neighbourhood of where cases 1, 2 and 3 lived. None were within 500 m of case 1, but as these could be considered a plausible source, faecal samples were taken for PCR analysis.

The index case's pet dog was reported to have rolled in the remains of a dead bird in December. Also, this

case's workplace was reported to be affected by a large number of gulls. Searches in both areas revealed insufficient sample material. On veterinary recommendation (included in the OMT), a PCR analysis of a pooled canine faecal sample was done, using an unpublished method, developed at the UK Animal Health and Veterinary Laboratories Agency, Weybridge. This PCR detects the presence of *C. psittaci* and *C. abortus* and was negative.

No environmental source of any *Chlamydophila* species was revealed by environmental investigations. This is not unusual [8].

Control measures

Since the source of the infection was thought to be a pathogen which was not readily transmissible from person-to-person, standard infection control measures were recommended for those HCWs and other people in contact with cases.

Discussion and conclusion

The main issue in this outbreak is the picture of person-to-person spread. The authors can find no description of this in psittacosis. Incubation ranging from one to four weeks implies up to 21 days between shortest and longest. The longest gap between onset of confirmed cases was 25 days. While the cases amongst the extended family might be explained by a putative persistent source to which family members were sequentially exposed (e.g. a geographical, not temporal, point source), case 4 (the HCW) cannot.

Since cases 1 to 3 were members of an extended family and had extensive and frequent contact with each other (especially over the winter holiday season) it was not possible to retrospectively identify particularly significant 'mutual exposure events'. However, shared exposures between case 4 and the others were sought. The only spatial-temporal overlap was with case 1 and occurred during the admission of case 1 to the ward where case 4 worked. Case 4's duties included personal care (not invasive procedures). Conceivably, case 4 may have been exposed while caring for case 1 who required intensive medical support and investigation. Since it was not possible to explore direct contact between the two cases, it is uncertain what such exposure might be.

It is difficult to explain all cases in this outbreak by exposure to a common non-human source. While inconclusive, features consistent with person-person spread are demonstrated. In our view, clinicians and public health specialists should therefore keep an open mind to the possibility of person to person spread of psittacosis despite the received opinion that this generally does not occur.

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Fatal case of imported human rabies in Amadora, Portugal, August 2011

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We report on a case of imported human rabies in Portugal, in July 2011 in a woman who presented initially complaining of back pain, without relating exposure to animal bites. She had travelled from Portugal to Bissau, Guinea-Bissau, in April where she had been bitten by a dog on 1 May. She was diagnosed with rabies on 26 July and died two weeks later in spite of being treated following the Milwaukee protocol.

Case report

On 19 July 2011, a 41-year-old woman, born in Guinea-Bissau and a resident of Amadora, Portugal, consulted the emergency department of the local hospital with lower back pain radiating to the left leg. She did not relate having been exposed to animal bites and was not asked about animal exposure or travel history. She was discharged with symptomatic therapy. As the hypothesis of rabies was not initially suspected she was not vaccinated. Five days later, on 24 July, she returned to the emergency department presenting new symptoms: anorexia, hydrophobia, aggressiveness and agitation. She was neurologically evaluated and diagnosed with an encephalitic syndrome and peripheral polyneuropathy, with the working diagnosis of rabies encephalitis. The same day, she was transferred to the intensive care unit (ICU) of the hospital Pulido Valente, Lisbon.

On 25 July, biological samples were taken (a skin biopsy, a cerebrospinal fluid (CSF) sample and three saliva samples) and sent to the World Health Organization (WHO) Collaborating Centre for Reference and Research on Rabies, Institut Pasteur, France, for diagnosis. Based on clinical symptoms, treatment following the Milwaukee protocol was initiated [1]. This protocol was applied for the first time in 2004, when a teenager survived clinical rabies caused by the bite of a bat, following supportive intensive care and the use of an anti-excitatory strategy that included general anaesthesia, antiviral drugs and neuroprotection, with amantadine, ketamine, midazolam and ribavirin [1]. An amended version of the protocol (V3.1) [2] was used to treat the patient described (amantadine, ketamine, midazolam, nimodipine and valproic acid).

One day later, on 26 July, the rabies diagnosis was confirmed by reverse transcription polymerase chain reaction, RT–PCR, [3] on the three saliva samples. The CSF and the skin biopsy remained negative. On 29 July, the typing results indicated that the causal virus was a lyssavirus of the rabies virus species belonging to lineage Africa 2 group B, which usually circulates in Senegal, Guinea-Bissau and Sierra Leone [4]. Despite treatment with an adapted version of the updated Milwaukee protocol including invasive mechanical ventilation and heavy sedation, the patient's condition progressively worsened and finally died 15 days after diagnosis.

Case history obtained through relatives, revealed that the patient had travelled to Bissau, capital of Guinea-Bissau on 22 April. In Bissau on 1 May, she was bitten by a dog in the lower left limb and the dog was shot on the same day, a common measure in a country where animal rabies is enzootic. No tests were carried out on the dog to confirm rabies. The patient went to the local health authorities of Bissau to report having been bitten by a dog and as vaccine was not available in the country she was not vaccinated. She returned to Portugal on 28 May without any symptoms and had not initiated any vaccination schedule. While in Portugal, she developed the first symptoms.

Public health measures

On 26 July, the local public health department in Amadora received information of the suspicion of a case of human rabies from the Hospital Pulido Valente in Lisbon. The health authority in Amadora notified the Directorate-General of Health in Lisbon and on the same day, interviewed the patient's family. It was possible to identify her contacts among relatives and health professionals. Risk assessments were carried out for those who might have been in contact with the case. Human infection usually occurs following

a transdermal bite or scratch by an infected animal. Transmission may also occur when infectious material, usually saliva, comes into direct contact with the victim's mucosa or with fresh skin wounds.

Six individuals were identified for treatment with post-exposure prophylaxis (PEP) which consisted of four intramuscular doses of rabies vaccine, 1 ml, with two doses on day 0, followed by one dose each on day seven and 21. Although scientific evidence for human-to-human transmission is limited to few cases worldwide [5,6], it was decided to also give PEP to the husband, considering the sexual intercourse during the communicability period. Furthermore, five health professionals from the Lisbon central hospital, who performed or helped with invasive procedures, were vaccinated following specific indications from the Directorate-General of Health. Fast identification of all the persons who had been in contact with the patient was done through efficient cooperation between the hospitals, local public health authorities and the Directorate-General of Health. All parties communicated with each other and supported the epidemiological investigation in a coordinated way in order to allow for rapid application of public health measures. The case was reported through the European Union Early Warning and Response System (EWRS) and the focal point of the World Health Organization International Health Regulations (IHR) in Guinea-Bissau was contacted.

New guidelines for epidemiological inquiries as well as for vaccination and prophylaxis were developed by the Portuguese Directorate-General of Health following the event.

Epidemiological background

Rabies is a viral zoonosis largely distributed worldwide. The natural reservoirs are mainly dogs (canine rabies represents 99% of the source of infection for humans [7]), foxes, raccoon dogs, skunks and bats. However, a large number of other mammals can be infected and can act as vectors. In Europe, the main epidemiologic cycle of rabies in sylvatic terrestrial non-flying animals is maintained by the red fox (*Vulpes vulpes*) and the raccoon dog (*Nyctereutes procyonoides*). Large vaccination campaigns of foxes were implemented in numerous western and central European countries. However, fox rabies is still present in the eastern and in some southern parts of Europe, such as Croatia, Serbia and Slovenia [8,9]. Bat rabies has also been diagnosed in numerous European countries, with reports of transmission to humans. There have been three confirmed deaths since 1985 [10]. Spill-over infections from bat rabies to terrestrial mammals [10] is still a threat, thereby maintaining its potential to infect humans.

The main risk of canine rabies resides in the translocation of unvaccinated animals originating from countries bordering the east and south of Europe [11,12]. From 2008 to 2011, at least three reports have described the importation of rabid dogs from Africa to Europe [13-15]. One of the reports concerned an infected dog imported from Morocco, identified in France, that travelled to Portugal and Spain and which may have infected susceptible dogs [14]. Between January 2000 and January 2009, there were 13 reports of cases of imported human rabies in Europe [9]. The 2010 European Centre for Disease Prevention and Control (ECDC) annual epidemiological report describes one case of human rabies in the European Union (EU), a woman in a rural area of Romania that had been bitten by a fox [16]. The annual average number of cases of human rabies in the EU has been limited to one in the last years. This single case would seem to confirm that trend [16].

Conclusion

Rabies is a zoonotic disease fatal in humans which can be prevented either through vaccination or if adequate measures are applied after exposure [17] Portugal is a country free of rabies since 1960 [18] and the probability of an autochthonous case is virtually inexistent. However, the possibility of imported cases, especially from the Portuguese-speaking African countries (mainly Angola and Guinea-Bissau where rabies is an epizootic) exists, mainly because of the influx of migrants to Portugal and to other parts of Europe [11,12]. This report, and a recent report about an imported rabid puppy [19], confirms the need for vigilance with regard to human and animal rabies.

The handling of the case described is an example of efficient coordination between the local public health authorities, the hospital, the Portuguese Directorate-General of Health and the collaboration with an international laboratory, the Institut Pasteur in Paris. There was constant and rapid exchange of information between these entities to confirm the case and to identify the exposed individuals. The case is another example of the failure of the Milwaukee protocol applied to rabid patients [20,21].

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Fatal case of human rabies imported to Italy from India highlights the importance of adequate post-exposure prophylaxis, October 2011

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In October 2011, an Indian man resident in Italy was admitted to a hospital in Mantua, Italy with symptoms of acute encephalitis. Due to a recent history of bite by a suspected rabid dog in India, where he had received incomplete post-exposure treatment, rabies was suspected. The patient died after 22 days of intensive care treatment and rabies was confirmed post mortem. This report stresses the need of appropriate post-exposure prophylaxis in rabies-endemic countries.

Case report

An Indian man in his 40s, who had been resident in Italy for 10 years, was admitted to a public hospital in Mantua, Italy, on 23 October 2011, with fever (40.4 °C), malaise, headache, diplopia, unilateral ptosis (left eye), whole body paraesthesia, ataxia, myalgia and flaccid paresis of the arms, especially of the left one. His behaviour appeared abnormal, with signs of anxiety and agitation. While undergoing clinical evaluation and tests, he developed ventricular tachycardia and acute respiratory distress and was therefore intubated, sedated and put under assisted mechanical ventilation.

The patient reported an extensive biting on his left arm and right leg by a dog showing marked aggressiveness, on 28 September 2011 while he was in a suburban area of the city of Manpur, north-east India, visiting relatives and friends. One month after the bite and at the time of hospital admission in Italy, the lesions had become purulent. Immediately after the accident, he had received post-exposure prophylaxis (PEP) in India, consisting of four vaccine injections (on day 0, 3, 6 and 14) with a locally-produced purified duck embryo vaccine against rabies. However, rabies immunoglobulin was not administered. On 17 October, he left from India to Germany, where he visited his sister living in Hamburg. During his stay in Hamburg, until 23 October, he started to experience a generalised weakness.

On the first day of hospital admission, a lumbar puncture was performed and revealed a white blood cell count of 25/µl (normal value: ‹4/µl), 70% lymphocytes, 20% neutrophils and 10% monocytes, absence of red blood cells, glucose of 86 mg/dl (normal range: 40–70 mg/dl) and protein 97 mg/dl (normal range: 15–60 mg/dl). Complete blood count and routine chemistries revealed a slight increase of leucocytes (11.34; normal: 4.4–11.0 x10^3µl) and moderate hyperglycaemia (125; normal range: 75–100 mg/dl). Progressive metabolic acidosis was also revealed as the blood pH value had decreased from 7.429 to 7.074 within six hours.

Computed tomography (CT) of the head and thoracic radiography performed on the day of hospital admission were normal. The CT was repeated four days later and revealed substantial alteration of the basal nuclei (particularly in the left hemisphere), the thalamus and the cerebral peduncles.

Symptoms and findings from the cerebrospinal fluid (CSF) tests and from the CT were highly indicative of a viral encephalopathy. Due to the clinical findings and to the exposure history, rabies was immediately suspected and diagnostic samples (saliva, skin biopsy, CSF and blood serum) were submitted to the National Reference Laboratory for Rabies at the World Organisation for Animal Health (OIE) Collaborating Centre for Diseases at the Animal-Human Interface, Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe) in Legnaro, Padua (Italy), on 25 October. In the meantime, CSF was tested for the presence of the following bacterial and viral pathogens, either using molecular methods or antigen agglutination: meningococcus, group B streptococcus, *Haemophilus*, pneumococcus, enteroviruses (poliovirus 1-3, Coxsackie A 2-12, 15-18, 20, 21 and 24, Coxsackie B 1-16, echovirus 1-9, 11-15, 17-21, 24-27, 29-33, enterovirus 68-71) JC

polyomavirus, herpes virus simplex 1 and 2, varicella-zoster virus. The presence of specific herpes virus 6 and 8, Epstein-Barr virus and cytomegalovirus DNA, as well as the presence of specific anti-echovirus antibodies were also investigated in the blood. Following all these investigations, the results were negative.

Serological tests performed on both blood serum and CSF at IZSVe gave positive results for specific anti-rabies IgG but results for IgM were unclear, due to the weak fluorescent signal obtained. Viral RNA or viral antigens were not detected in the skin biopsy and saliva specimens (Table).

However, the presence of specific rabies antibodies in the CSF was consistent with the initial suspicion of rabies. A second panel of samples were collected on 27 October and submitted to IZSVe for serological confirmation and viral detection. Tests on the second panel at IZSVe confirmed the previous findings. On 28 October, the patient developed severe coma (Glasgow Coma Scale 3) and was maintained alive by intensive care treatment and mechanical ventilation. The sample panels were sent to the World Health Organization (WHO) Collaborating Centre for Reference and Research on Rabies at the Centers for Disease Control and Prevention (CDC), Atlanta (USA) that confirmed the absence of viral RNA and antigen and the presence of specific IgG and IgM.

Neither rabies vaccine nor immunoglobulin was administered during the hospitalisation. The patient died on 14 November 2011 in hospital.

Post mortem, the entire central nervous system (CNS) was collected and tested for the presence of the virus. Fluorescent antibody testing performed on different portions of the CNS revealed the presence of viral antigen in all regions, and particularly in the cerebellum and thalamus and, to a lesser extent, in the medulla oblongata, the corpus callosum, the hippocampus and in the brain cortex. A similar pattern was revealed by immunohistochemistry on formalin fixed paraffin embedded tissues (Figure 1).

One step RT-PCR and sequencing analysis were performed as previously described [1] on brain tissues and the obtained viral sequences (GenBank accession number JQ845907) were aligned and compared with 92 sequences representative of rabies viruses available

TABLE

Laboratory diagnosis of rabies performed at Istituto Zooprofilattico Sperimentale delle Venezie on samples submitted ante mortem and post mortem, rabies case, Italy, October and December 2011

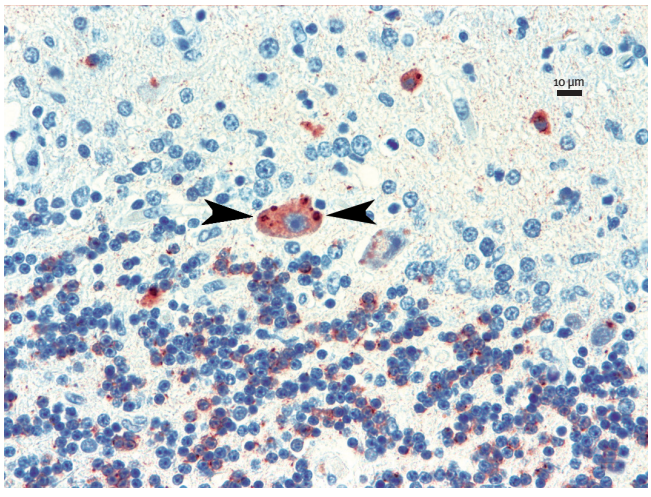
Sample	Method	Result
Samples submitted on 25 October 2011		
Skin	FAT	Negative
Skin	RT-PCR	Negative
Saliva	RT-PCR	Negative
CSF	IFA test for IgG	Positive
CSF	IFA test for IgM[a]	Positive
Blood serum	IFA test for IgG	Positive
Blood serum	IFA test for IgM[a]	Positive
Samples submitted on 27 October 2011		
Skin	FAT	Negative
Skin	RT-PCR	Negative
Saliva	RT-PCR	Negative
Saliva	RT-PCR	Negative
Saliva	RT-PCR	Negative
Saliva	RT-PCR	Negative
CSF	IFA test for IgG	Positive
CSF	IFA test for IgM[a]	Positive
Blood serum	IFA test for IgG	Positive
Blood serum	IFA test for IgM[a]	Positive
Samples submitted on 7 December 2011		
CNS	FAT	Positive
CNS	RT-PCR	Positive

CNS: central nervous system; CSF: cerebrospinal fluid; FAT: fluorescent antibody test; IFA: immunofluorescent-antibody.

[a] Serological tests were positive for specific anti-rabies IgG but unclear for IgM both in blood serum and CSF. Results obtained from samples submitted ante mortem were confirmed by further investigation at the Centers for Disease Control and Prevention (Atlanta, USA).

FIGURE 1

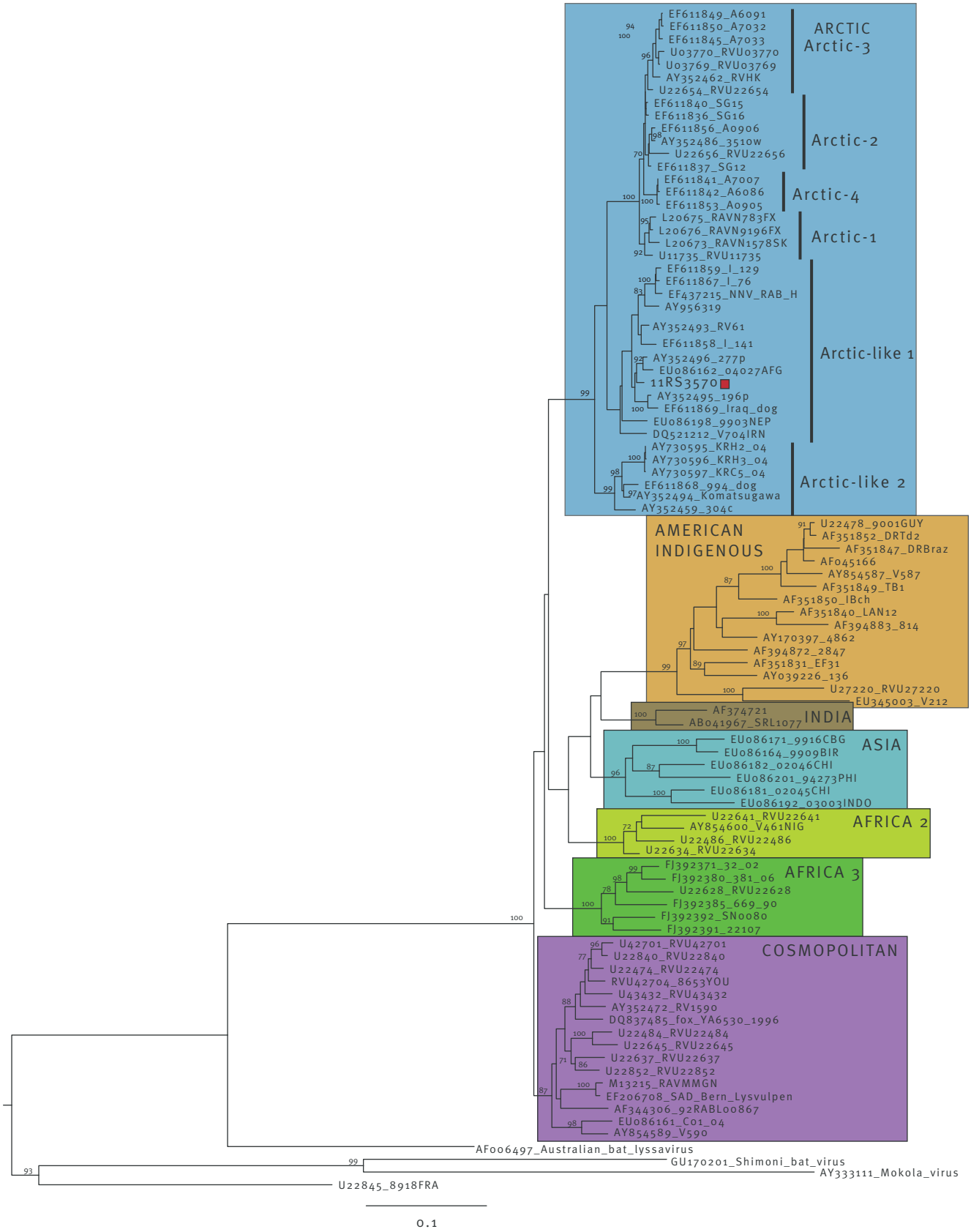
Fine granular staining and Negri bodies within the cytoplasm of a Purkinije cell in cerebellum positive for rabies viral antigen, rabies case, Italy, 2011



Fine positive staining is present also in the granular cell layer. Immunohistochemistry, EnVision FLEX/HRP, diaminobenzidine (DAB) as chromogen and hematoxylin counterstain.

Figure 2

Maximum likelihood phylogenetic tree[a] estimated for the partial N gene sequence of the imported human rabies case (11RS3570[b]) from India to Italy, October 2011



[a] Using PhyML version 3.0.

[b] GenBank accession number JQ845907.

The red square indicates the isolated strain.

A bootstrap re-sampling process (1,000 replications) employing the neighbour-joining method was used to assess the robustness of individual nodes of the phylogeny. Bootstrap values are indicated as numbers at the nodes.

in GenBank. The phylogenetic analysis confirmed that the virus causing the infection belonged to the Arctic-like 1 lineage of the rabies virus (RABV) circulating in southern Asia, northern India and the Middle East [2] (Figure 2).

Risk assessment for contacts

A risk assessment was carried out for health professionals who might have been in contact with the case. Although human-to-human transmission has never been documented in a healthcare setting, transmission of rabies virus could occur if open wounds or mucus membranes were contaminated with infected saliva or neural tissue. In the case described here, hospital staff had adhered to standard infection control procedures and did not require the administration of PEP.

The sister of the patient living in Hamburg was contacted and, following a risk assessment, she undertook PEP.

Conclusions

Laboratory diagnosis of rabies ante mortem is generally based on the detection of the viral antigen or RNA in a skin biopsy from the neck base, or from saliva and by detecting specific rabies antibodies in serum and CSF. However, viral antigen and RNA are rarely detected intra vitam because of low viral replication in peripheral nerves and intermittent excretion in saliva. Detection of specific rabies antibodies in serum samples can be a result of previous vaccine administration or of exposure to any lyssavirus, and thus, cannot be considered alone as confirmatory diagnostic tool. In this case, ante mortem laboratory diagnosis was complicated by the administration of post-exposure vaccine, which inevitably yields the production of specific antibodies. However, the detection of specific immunoglobulins in CSF, IgG and particularly IgM, was strongly indicative of rabies, if combined with anamnestic and clinical data. Diagnosis was performed post-mortem and was conclusive of fatal rabies. A summary of this case was reported through ProMED-mail on 6 February 2012 [3].

This is the 23rd case of imported human rabies in the European Union (EU) in the last 20 years (since 1992 [4,5]), and the fourth in Italy since 1975. The most recent infection in the EU was reported in August 2011 in a woman who was bitten by a dog in Guinea Bissau three months before developing symptoms while in Portugal [5]. In Italy, the most recent cases were imported from Asia, specifically from India and Nepal [6-8]. According to WHO data, the Indian subcontinent is affected by a high number of human deaths caused by rabies, most of them following the bite of a domestic dog (from about 1.7 to 3.3 per 100,000 population and more than 20,000 deaths per year) [9,10]. Efforts in raising public awareness and improving medical infrastructures are being carried out in several rabies-endemic countries including India [10], and it is also essential to ensure that the full range of products for PEP is available for residents and travellers.

Travellers should be informed of the risks before travelling in an area endemic for rabies. Pre-travel advice and further decision to apply preventive vaccination are based on several factors including: a risk assessment based on the duration of stay, the likelihood of engagement in risky activities, the age of the traveller, the rabies endemicity and access to appropriate medical care in the country of destination. However, information on the latter two is generally poorly available for endemic countries [11]. In the case described here, the patient likely lacked of pre-travel consultation, nevertheless he sought and underwent immediate PEP in India. Unfortunately, PEP was incomplete as rabies immunoglobulin was not administered. This was likely the cause of spread to the CNS, which resulted in the patient's death. In most cases, appropriate PEP is successful and can prevent infection and death of the patient. However, a recent publication reviewing the management of PEP in injured travellers indicates that vaccine and immunoglobulin are often unavailable or improperly administered abroad [11], as the case presented herein may confirm.

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Rabid puppy-dog imported into the Netherlands from Morocco via Spain, February 2012

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In February 2012 a rabid puppy dog was imported into Amsterdam, the Netherlands from Morocco via Spain. In a joint action between the Netherlands' Food and Consumer Product Safety Authority, the Public Health Service of Amsterdam and the Centre for Infectious Disease Control all exposed human and animal contacts were traced and, when necessary, provided with post-exposure prophylaxis. During the importation, the international legislations with respect to vaccination requirements were not fully obeyed by veterinarians and custom services.

On 28 January 2012, a Dutch couple residing in Morocco obtained an eight-week-old puppy at a parking lot. They took the dog to a local veterinarian who micro-chipped the dog and issued a certificate of good health, yet no vaccinations were given. On 4 February 2012 the couple travelled by car and ferry from Morocco to Spain. At a veterinary clinic they acquired a European pet passport. On 11 February they returned to the Netherlands by air. Although the dog was cuddled by three Spanish customs officers at Malaga Airport, the dog passport was not examined by customs in Spain, nor in the Netherlands. Upon arrival the couple immediately introduced the puppy to friends and family. It showed normal behaviour at the time, yet became increasingly hostile over the following days. On 14 February, the owners contacted the veterinary practice after they had been bitten by the dog. The puppy was assumed to suffer from 'puppy stress' caused by the new environment and was given sedative medication. In the morning of 15 February the dog's behaviour became uncontrollable. When they realised that the puppy originated from Morocco, the veterinarians contacted the Netherlands Food and Consumer Product Safety Authority (NVWA). As clinical signs indicated rabies, the NVWA advised to euthanise the dog for investigation. Rapid post-mortem rabies diagnostics were performed by the Central Veterinary Institute (CVI). On the evening of 15 February rabies (classical rabies virus, genotype I) was confirmed.

After the notification on 15 February, the NVWA, the Public Health Service of Amsterdam (PHS Amsterdam) and the Centre for Infectious Disease Control (CIb/RIVM) initiated a joint action to identify and trace all humans and animals with possible exposure to the dog's saliva in order to provide post-exposure prophylaxis and assess the risk to the general population. The dog was considered to be infectious to others during the two weeks prior to the day of onset of symptoms and until its death (28 January through 15 February).

Contact tracing

The owners were interviewed about their travel history since the date they acquired the puppy on 28 January. Throughout their journey, they had constantly supervised the puppy, and no unobserved exposure had taken place. In Morocco, no contacts were identified except for the local veterinarian. During the journey to Spain no other people or animals were in contact with the dog. In Spain, the couple stayed with two Dutch friends, visited a Spanish friend and a veterinary clinic, and stayed in four different hotels in two different towns. Apart from the three custom officers, the dog was stroked by an unknown person at a restaurant and one at Malaga airport. During the flight to Amsterdam, the dog was kept in a basket on the owners' lap, and no contacts were reported. At Amsterdam Airport they were collected by car by two friends and their dog. On 11 and 12 February they met with numerous family, friends and their children at four private locations. In one location, two cats were present. The remaining days they mostly stayed at home, except for the last visit to the veterinary clinic. A total of 43 contacts (including the two owners) residing in the Netherlands

were identified among family, friends and the animal clinic. On several occasions, unidentified people in the street were petting the dog.

Public health action in the Netherlands

Upon notification the PHS physician on call immediately arranged post-exposure treatment for the owners (rabies vaccination with human diploid cell rabies vaccine (HDCV) and human rabies immunoglobulin (HRIG) at the emergency department of the Academic Medical Centre (AMC). On the same evening most known contacts were informed by telephone. Within 24 hours their risk for transmission was assessed, and according to national and international guidelines post-exposure prophylaxis was recommended (Table) depending on the type of contact and category of exposure [1,2]. As it is known that children's recollection of exposure might be unreliable, all nine children were considered as having had a category III exposure. Casual petting on the street was categorised as category I exposure. No treatment was deemed necessary for these contacts.

As the investigations revealed no risk of rabies transmission to the general population, warning messages to alert the public were deemed unnecessary. Instead, an informative joint press statement by the PHS and NVWA was issued on 16 February describing the incident.

International public health action

The CIb/RIVM issued an EWRS (Early Warning and Response System) message to inform the Member States of the European Union about this incident.

Bilateral contact was established with Spain in order to facilitate contact tracing there. In Spain three known contacts were informed directly by the PHS. The couple's Spanish friend, considered to have category I exposure, had been previously vaccinated against rabies. Their Dutch friends, a category II and a category III contact, received treatment at a local hospital in Spain. As HRIG was not available locally, they returned to Amsterdam so that the category III contact could receive HRIG the following day. The contact details of the Spanish veterinarian and a picture of the dog were provided to the Spanish EWRS contact point. Unfortunately, it was not possible to obtain additional information on the other contacts who stroked the puppy, nor on how many contacts were traced or vaccinated in Spain overall.

The CIb/RIVM established a bilateral contact with their counterpart in Morocco, providing them with the contact details of the veterinarian that had seen the dog prior to its departure. We have as yet no information on the actions taken there.

Veterinary action

The investigation revealed only few exposed animals. One dog and two cats were traced within 24 hours. The dog (imported from Greece in 2010 and vaccinated against rabies) received a booster vaccination. The two cats received vaccination on 15 February and quarantine was indicated. As a suitable quarantine place was not available, it was decided to euthanise both cats.

Conclusions

This is the first case of rabies (caused by the classical rabies virus) in domestic and/or wild animals in the Netherlands since 1988.The accidental import of a rabid puppy led to a resource-intensive and costly public health response. A total of 48 known contacts in three different countries needed to be traced, of whom 45 required post-exposure treatment. Including the imported dog, three animals were euthanised.

The owners tried to import the dog in a legal way, yet the international legislations were not followed properly by the consulted veterinarians in Morocco and Spain and customs in Spain and the Netherlands. In hindsight, the European dog passport was incorrectly issued by a Spanish veterinarian as, according to the EU legislation, dogs/animals from outside the European Union should be vaccinated for rabies and kept in quarantine for three months upon arrival [3,4]. Customs at three locations upon arrival and leaving in Spain and arrival in the Netherlands failed to check the vaccination status of the dog.

The NVWA is evaluating this course of events. Lessons learnt from the evaluation should be communicated internationally to urge veterinarians and customs departments to follow international legislation appropriately.

Veterinarians and customs officials across Europe should be aware of the risk of rabies importation by animals from within and outside Europe. Particular attention should be given to puppies under the age of three months, which must be vaccinated against rabies and consequently cannot be imported into Europe [3].

Illegal importation of animals from rabies-endemic countries outside the European Union probably occurs

Table

People exposed to the rabid dog and treated by PHS Amsterdam and/or AMC, the Netherlands, February 2012 (n=43)

Exposure category[a]	Treatment given	Number of exposed people
Category I	Not indicated	1
Category II	Vaccination	21
Category III	Vaccination and HRIG	21

HRIG: human rabies immunoglobulin; PHS Amsterdam: Public Health Service of Amsterdam; AMC: Academic Medical Centre.

[a] Category I: touching animals, licks on intact skin; Category II: nibbling of uncovered skin, minor scratches or abrasions without bleeding; Category III: transdermal bites or scratches, (saliva from) licks on broken skin or on mucous membrane.

frequently. France reported nine illegally imported rabid puppies and dogs over the last ten years, of which seven were imported from Morocco [5,6]. Therefore the public should be made aware of the risks involved in bringing home a living souvenir, and of the rules and regulations governing such an action.

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Letter to the editor: Rabid puppy-dog imported into the Netherlands from Morocco via Spain, February 2012

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To the editor: We read with interest the article by van Rijckevorsel et al. on a rabid puppy-dog imported into the Netherlands from Morocco via Spain, recently published in *Eurosurveillance* [1]. We would like to complete the information on this event with actions taken by the Spanish health authorities and lessons learnt.

On 16 February 2012, Thursday afternoon, the Coordinating Centre for Health Alerts and Emergencies at the Ministry of Health in Spain received a selective message via the Early Warning and Response System (EWRS) from the Dutch health authorities, communicating the laboratory confirmation of rabies in a puppy-dog from Morocco that had been imported into the Netherlands via Spain. The dog was transported by car from Morocco to Spain by a Dutch couple who stayed in Spain for a week before departure by plane to the Netherlands. Dutch authorities informed that a risk exposure had been identified in at least three persons living in Spain (Contacts 1, 2 and 3). These persons had already been informed by the Public Health Service Amsterdam with the advice to seek medical care for post-exposure prophylaxis.

Upon reception of this message, immediate public health action was initiated in Spain:

A request for more information such as the name of the hotels where the couple had stayed in Spain, dates, itinerary and contact details of the contacts living in Spain was made in order to complete contact tracing and start prophylaxis. Information on the couple's itinerary, hotels and restaurants visited, and on human and animal contacts of the dog was obtained from different sources the following day, after active request from the Spanish authorities.

Information available at that time was sent to the Spanish Alerts' Network (consisting of public health professionals at national and regional level and other sectors involved in detection and response) and an alert concerning this event was issued to the regional public health authorities who alerted their regional health services and started an active search for possible contacts at risk in hotels and places visited by the Dutch couple, once this information was available on the evening of 17 February. Health centres and veterinary services serving the area concerned were also contacted to make sure that no people seeking medical attention for dog bites or any incident with a dog had been reported. As a result of these actions, no further human or animal contacts were identified in addition to the first three human contacts identified by Dutch authorities.

Contact details of contacts living in Spain were at no time accessible for the Spanish authorities because of Dutch national laws which do not allow the disclosure of personal data. This delayed public health action in Spain and caused unnecessary difficulties. For instance, as instructed by phone by the Dutch authorities, Contacts 1 and 2 sought medical care on 16 February, before the Spanish authorities were informed of this event. This caused confusion in the healthcare centre as in mainland and insular Spain there has not been any rabies in terrestrial animals since 1975. Following current protocols, Contacts 1 and 2 were asked to provide a written proof of their exposure history, while adequate healthcare and follow-up were organised the same day. Post-exposure prophylaxis (first dose of vaccine) was given after they presented email documentation from the Dutch National Institute for Public Health and the Environment mentioning the laboratory confirmation of rabies in the puppy. Human rabies immunoglobulin was available on the morning of 17 February but both contacts failed to show and left the country that evening without informing Spanish health authorities.

Contact 3 could not be followed until they contacted Spanish health authorities several days later. Despite being informed of the exposure risk and offered

prophylaxis following current protocols, this contact refused to take it.

A summary of the control measures taken in Spain was posted on the EWRS site on 23 February 2012.

An internal evaluation of this event has shown the need to reinforce the appropriate control at customs and following of European Union (EU) legislation on non-commercial movement of pet animals [2]. We also think that the public should be made aware through travel advice of the risks and their responsibility when bringing back animals from abroad [1].

Lessons learnt also include difficulties in accessing personal information within the EU despite efforts made by the European commission and the EU Member States, as well as the need to respect official channels for communication with contacts living in another Member State. Public health activities to be carried out in a given country should be managed by the health authorities of that country who are responsible for risk management in their territory and know the current protocols and response mechanisms in place. The use of channels other that those established in each country can create dysfunction for all actors involved in the response, including a deficient attention to the exposed or affected population.

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Authors' reply: Rabid puppy-dog imported into the Netherlands from Morocco via Spain, February 2012

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To the editor: The additional information from the Spanish Health Authorities is much appreciated. In their letter the Spanish Health Authorities express their concerns about the informal channels used by the Amsterdam Public Health Service in tracing three persons living in Spain (Contacts 1, 2 and 3). In their opinion access to the personal details of these contacts was denied because of Dutch national laws prohibiting such disclosure of information. However, Dutch national laws do allow Public Health Services to reveal personal data to third parties, but only with the approval of the involved contact, or when contacts cannot otherwise be reached. Also, in case of direct health emergency this law can be overruled.

The Amsterdam Public Health Service regrets the confusion caused in this case by not using the official channels in the process of contact investigation. However, in this case the owners of the rabid dog had contacted their three personal friends before the official channels could be informed. Upon realising these friends were likely at risk (Category II and III exposure), the Amsterdam Public Health Service considered it right at that time to advise them persons to consult a doctor as soon as possible for post-exposure prophylaxis. Rabies is a devastating infectious disease which only can be prevented by timely post-exposure prophylaxis. Identifying contacts is therefore of utmost importance and needs immediate public health action. As described in the rapid communication, the Centre for Infectious Disease Control at the National Institute for Public Health and the Environment (CIb/RIVM) informed their Spanish counterparts at the earliest convenience with as much detailed information as was available at that time.

The lesson learnt from this case is that clear communication between all parties involved is needed for a successful response to public health threats which require instant actions.

Q fever outbreak in the village of Noćaj, Srem county, Vojvodina province, Serbia, January to February 2012

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From 27 January to 10 February 2012, a total of 43 cases of Q fever were notified in the village of Noćaj, Srem county, Autonomous Province of Vojvodina, Republic of Serbia. Q fever was laboratory confirmed in 37 notified cases. Alhough, the outbreak is considered over, the outbreak investigation is still ongoing in order to identify aetiologic factors relevant for this outbreak.

On 27 January 2012 after 10 patients were hospitalised with atypical pneumonia, an outbreak of Q fever was discovered in Srem county, Vojvodina province, Serbia. Laboratory testing of some of the first patients for pathogens such as *Coxiella burnetii, Chlamydia pneumoniae*, *Mycoplasma pneumoniae*, influenza A and B, parainfluenza, and respiratory syncytial virus had all resulted negative, except for *C. burnetii*.

Between 27 January and 10 February, 2012, 43 cases of Q fever were reported. The majority of patients (n=41) were residents of Noćaj, a village with 2,120 inhabitants located in the vicinity of the city of Sremska Mitovica (Srem county) near the border between Serbia and Bosnia and Herzegovina. The attack rate in this period was 2%.

Hereby we describe the preliminary results of the ongoing outbreak investigations started on 30 January 2012, by the Center for Disease Control and Prevention of the Institute of Public Health, Sremska Mitrovica. The investigation was assisted by the World Health Organization (WHO), Regional Office for Europe.

Epidemiological investigation

Specific notification criteria and case definitions adapted to the current situation were applied. A probable case of Q fever, according to the European Union criteria, which are used in Serbia [1], was not relevant in this investigation because the source of the current outbreak was not yet identified, and no epidemiological link could be established.

A 'clinical case' was defined as having acute fever and one or more of the following: rigors, severe retrobulbar headache, acute hepatitis, pneumonia, or elevated liver enzymes' levels with onset of illness between 20 January and 10 February, and no other likely cause for illness in a patient who either lived or visited Noćaj in the period from 1 to 20 January, 2012. The risk period for exposure was estimated considering an average incubation period of 20 days [2] and time distribution of cases.

A 'clinical case' who had not been serologically tested was defined as a possible case of Q fever.

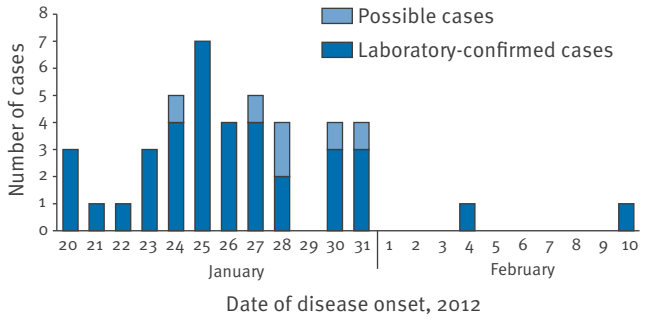
A laboratory-confirmed case of acute Q fever was defined as a 'clinical case' with serologic evidence of a positive IgM and/or IgG antibody result to phase II antigen *C. burnetii*, by enzyme-linked immunosorbent assay (ELISA). The results were interpreted in line with the manufacturer's guidance as follows: ‹9, negative; 9–11, equivocal; ›11, positive (ELISA, NovaLisa). Paired sera samples tested at least two weeks appart were taken for four patients for whom the result of the first sera tests were equivocal or negative (two sera samples were positive after the second test).

All sera samples were tested in the Reference Laboratory for Q fever, Institute of Public Health, Zrenjanin, Serbia. Of 43 notified cases, 37 were laboratory confirmed and the rest were classified as possible cases. All laboratory-confirmed cases were classified as acute Q fever cases. All cases of acute Q fever with known preconditions for chronic disease were reffered for laboratory follow-up in periods of three, six and twelve months after onset of illness, in order to detect

the development of chronic Q fever [3]. The majority of cases (n=41) reported illness onset between 20 January and 1 February 2012 (Figure).

Figure

Cases of Q fever by date of symptom onset, Noćaj, Sremska Mitrovica, Serbia, 20 January–10 February 2012 (n=43)



The male to female ratio of cases was 3.6:1. The mean age ± standard deviation was 35.65±14.3 years with the age distribution of cases ranging from 14 to 75 years. Data about the age and sex of cases rates are shown in Table 1.

Thirty-six of 43 registered cases were diagnosed with atypical pneumonia by chest X-ray. Sixteen of them were hospitalised. All patients had good outcomes without sequelae. The clinical features of Q fever in this outbreak are presented in Table 2.

During the epidemiological investigation in the village households, all present family members were interviewed about symptoms of Q fever and possible preconditions for chronic Q fever. Efforts were made to conduct laboratory testing, in order to detect recent Q fever infection in asymptomatic people, with known preconditions for chronic Q fever or at risk for complications, like pregnant women (n=10) and newborns (n=2), people with heart valvular diseases (n=1) or immunosupression (n=2). Also exposed healthcare workers (n=9) were tested. The testing was done by ELISA in order to detect a *C. burnetii* specific antibody response (IgG or IgM phase II), as previously described. By 26 March, eight additional cases of asymptomatic Q fever were discovered including three pregnant women, four exposed healthcare workers and one child with undefined symptoms. They were all refered to infectious disease specialists for review.

In exploratory interviews taken between 30 January and 16 February, 28 of 43 patients denied direct contact with livestock, although most of them own livestock in their households. In Serbia, reporting on aborted pregnancy in domestic animals is mandatory and requires a standard number of tests including tests for *C. burnetii*. However in the previous few months, local farmers and veterinary services in Noćaj had not observed such cases.

Only two patients in the current outbreak were not residents of Noćaj. They visited their relatives in Noćaj for a few hours each on different days (8 and 16 January). The time of the visits to Noćaj is compatibile with the incubation period and onset of disease in these particular patients. Overall 30 of 43 patients mentioned that they had visited a football tournament in the village school sport hall, from 4 to 7 January, 2012.

Environmental investigation and results

As the large number of cases in a small area was suggestive of a point source, smear samples were taken from heating ventilators, seats and the floor of the sport hall. DNA extraction from swabs was performed using QIAamp DNA Mini Kit (Qiagen, Germany) in the Veterinary Specialized Institute Kraljevo, Serbia. Two polymerase chain reaction (PCR) protocols were used for molecular detection of *C. burnetii*: The Real-Time PCR protocol published by Klee et al. [4] and the PCR protocol published by Berri et al. [5] The PCR assays for *C. burnetii* were all negative.

The Veterinary Scientific Institute, Novi Sad, conducted an epizootiologic investigation in the households of patients and their neighbours by order of the Republic Veterinary Inspectorate. Of 207 tested sheeps, goats and cattle, only one seropositive sheep in the village was found. Although seropositive, the vaginal swab sample of this seropositive sheep analysed by PCR was negative. Interestingly, this seropositive sheep was detected in a particular household in which two of seven human cases were registered during an outbreak of Q fever in Noćaj in 2009.

Epidemiological situation in Serbia

Q fever, a zoonosis distributed worldwide, was recognised as a specific disease in 1937 [6], and is caused by

Table 1

Cases of Q fever, by age group and sex, Noćaj, Sremska Mitovica, Serbia, 20 January–10 February 2012 (n=43)

Cases	Age groups in years							Total number of cases
	‹15	15–24	25–34	35–44	45–54	55–64	›65	
Number of male cases	1	7	12	5	7	2	0	34
Number of female cases	0	1	4	1	1	0	2	9
Total number of cases	**1**	**8**	**16**	**6**	**8**	**2**	**2**	**43**

C. burnetii. A wide range of animals serves as a natural reservoir for the pathogen [7]. Inhaling aerosols that are contaminated by *C. burnetii* is the most frequent route of transmission in large human outbreaks [8,9]. Q fever outbreaks are regulary reported thoughout Europe as well as in other parts of the world [10].

In Serbia, Q fever is a notifiable disease since 1966. Notification of Q fever is made on the basis of clinical diagnosis, epidemiological link and laboratory confirmation. During the last 14 years notification is based on European Union case-definition criteria in the absence of criteria adopted at the national level [1].

The Autonomous Province of Vojvodina, (Northern Province of Serbia) including Srem county is considered as an endemic region for Q fever. The latest seroepidemiological investigation of Q fever, which was conducted in 1985 and included 5,599 persons (representing 0.5% of the adult population of Vojvodina aged between 19 and 59 years), revealed a seroprevalence of *C. burnetii* antibodies of 9.3% [11]. In the period from 2002 to 2011, the incidence rate of Q fever in Vojvodina varied between 0.1–2.3 per 100,000 population. The incidence rate in Srem county varied between 0 and 2.1 per 100,000 population, with two outbreaks reported in 2009 [12] and 2011 (unpublished data). In the 2009 outbreak, seven human cases were notified in the village of Noćaj. Considering the high rate of mild cases and non-specific symptoms of Q fever [10], it is estimated that the actual incidences might be higher than presented above.

Table 2

Clinical features in cases of Q fever, Noćaj, Sremska Mitovica, Serbia, 20 January–10 February 2012 (n=43)

Clinical features	Number of cases
Fever (≥38°C)	35
Headache	27
Chills, shivers	22
Pneumonia	20
Muscle ache	19
Cough	13
Discomfort	4

Outbreak control measures

The Center for Disease Control and Prevention of the Institute of Public Health Sremska Mitrovica proposed to the management of the Noćaj elementary school to improve hygiene and proceed to a desinfection of the sport hall, and these measures were applied by order of the Provincial Sanitary Inspectorate.

General practitioners in the area and the nearest healthcare centre in Bosnia and Herzegovina were informed about the outbreak in order to make sure that any new arising cases of Q fever would be notified. All authorised institutions were informed, including the WHO, Regional Office for Europe following obligations included in the International Health Regulations (IHR) [13].

Efficient data sharing with the veterinary services was ensured in order to identify potential source(s) of the outbreak and to conduct veterinary control measures. Livestock trading, slaughter and use of unpasteurised milk and products from unpasteurised milk were temporarely prohibited in the investigated households until the serology results of tested animals were obtained.

Exclusion of blood donors (rather than screening) from the affected region was done. Health promotion campaigns to educate citizens on how to prevent possible Q fever infection took place in the village in the form of interviews, lectures and the delivering of information leaflets. Appropriate hygiene practices when dealing with livestock by-products of birth and manure and safe procedures for clothing and footwear were the key messages in the health education campaign for farmers. People at high risk for severe Q fever infection or complications were advised not to visit or stay in the livestock holding areas or barns.

In order to investigate potential factors for airborne spread of the bacteria, official meteorological data were analysed. Epidemiological reports were updated and published on the website of the Institute of Public Health of Sremska Mitrovica providing authoritative and accurate informations regarding the outbreak and reducing fear and panic in the village and area.

In order to prevent hospital acquired *C. burnetii* infections among healthcare workers and patients, the commission for the prevention of hospital infections in the general hospital Sremska Mitrovica proposed implementation of enhanced standard precautionary measures, such as monitoring compliance with hand hygiene, the use of gloves for contact with blood or body fluids, excretions and secretions, as well as anticipating the need for use of personal protective equipment (gowns, masks) according to the patient condition and type of procedure.

Discussion and conclusions

Considering the unusual high rate of hospitalisations and atypical pneumonia in this outbreak, we can assume that the number of cases is far higher than reported. The predominance of male sex among patients is not surprising, because the infection may be asymptomatic in 60% of Q fever infections, especially among women and children aged 15 years and younger [14-16].

Although a single animal source can cause many human Q fever cases [17], compared to 2009, the larger geographic area in which cases occurred in 2012 may indicate a multiple sources or possible airborne spread of *C. burnetii*. The low annual number of cases

of Q fever in Noćaj registered during past few decades was due to direct contact with animal placenta and/or birth products. The sudden and unusual acute presentation of the large outbreak in the current situation, required the consideration of other routes of Q fever infection. Although many cases in the village of Noćaj had attended the same football tournament in a school sport hall, the environmental investigation yielded negative results. Moreover, there were no registered cases of Q fever among residents of other villages who attended the tournament, nor among school children/staff where the tournament took place, which argues against the school sporthall as being the source of the outbreak. Until now, no common exposure has been identified among patients who did not attend the football tournament.

The data obtained from the epidemiological investigation were not indicative of a foodborne route of infection. The presumable route of infection in this outbreak is airborne by inhalation of contaminated dust and aerosol in the period around the orthodox Christmas. During January the weather in Noćaj was unusually dry and windy so the conditions to transmit *C. burnetii* were present. The heavy snowfall during February possibly reduced the further spread of this outbreak and limited its duration. We cannot rule out other possible causes via direct contact with livestock or by other possible exposures. Epidemiological investigation of infection sources and routes of transmission is ongoing. With this report, we would like to inform of this outbreak and raise awareness in neighbouring countries.

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Salmonella Paratyphi B var Java infections associated with exposure to turtles in Bizkaia, Spain, September 2010 to October 2011

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Between September 2010 and October 2011, the Unit of Epidemiology in the Department of Public Health in Bizkaia, Spain identified eight cases of *Salmonella* Paratyphi B var Java infection and three cases of infection with its possible monophasic variant 4,5,12:b:- dT+. Six cases reported contact with turtles and *S.* Java was isolated from three of these turtles' habitats. The isolates from the patients and their respective turtles were indistinguishable by pulsed-field gel electrophoresis (PFGE). Although other reptiles can also carry *Salmonella*, turtles pose a special risk, as they are commonly kept as pets for children. This emphasizes the need to give recommendations regarding ownership and handling of aquatic turtles and other reptiles. As parents are often not aware of the risk of infection associated with the presence of turtles in the household, it would be appropriate to inform potential buyers at points of sale about the risk of infection and measures they can take to minimise this risk.

Introduction

Salmonella infections are predominantly acquired through the consumption of contaminated food, but contact with animals may also be an important source of infection [1]. Reptiles are frequent carriers of *Salmonella* in their intestinal tract [2], they usually show no signs of illness and shed the bacteria in their faeces, contaminating the water and any surface in contact with them [3-6].

Several *Salmonella* serotypes have been found in reptile-associated salmonellosis, including *Salmonella* Java, *S.* Poona, *S.* Pomona, *S.* Marina, *S.* Stanley, *S.* Litchfield, *S.* Newport and the most common serotypes, *S.* Typhimurium and *S.* Enteritidis [2-7].

Although other reptiles can also carry *Salmonella*, turtles pose a special risk, as they are commonly kept as pets for children.
S. Paratyphi B infections can cause enteric fever (paratyphoid fever) or gastroenteritis. In some cases, serious complications can occur (septicaemia, meningitis), especially in young children and immunocompromised patients [7].

S. Paratyphi B var Java shares the same somatic and flagellar antigens as *S.* Paratyphi B, but uses d-tartrate as a carbon source. This variant appears to be less virulent, causing infections characterised by watery diarrhoea, abdominal pain and fever, although infection can also be invasive. In sporadic cases and outbreaks, infection with *S.* Java has been associated with consumption of contaminated food, including salads, goat's milk cheese and poultry and with contact with reptiles and tropical fish aquariums [8-11].

The Epidemiology Unit of the Department of Public Health in Bizkaia (a territory of the Basque Country, in the north of Spain, with a population of nearly 2,150,000 inhabitants) identified, between September 2010 and October 2011, 14 cases of *S.* Paratyphi B infection (incidence rate: 0.65/100,000 inhabitants). In Spain, the most common *Salmonella* serotypes are Enteritidis and Typhimurium. *S.* Paratyphi B biovar Java represented 2.1%, 1.4% and 1.7% of the *Salmonella* strains isolated from humans and serotyped at the National Reference Laboratory for *Salmonella* in 2009, 2010 and 2011 respectively. As *S.* Java is an unusual serotype, an investigation was initiated to identify the risk factors.

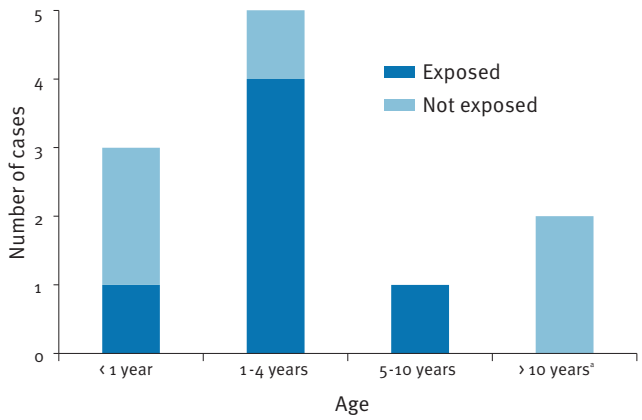
Methods

A case was defined as a patient, resident in Bizkaia, who had an isolate of *S.* Paratyphi B var Java between September 2010 and October 2011.

Adult cases and the parents of the affected children were contacted by telephone and questioned using a standard questionnaire about potential risk factors, such as other cases of gastroenteritis in their environment, travel, consumption of suspected food items and animal exposure. Where contact with turtles was

FIGURE 1

Cases of *Salmonella* Java and its possible monophasic variant by age group and exposure to turtles, Spain, September 2010–October 2011 (n=11)



[a] Two adults in their mid-20s and early 60s.

reported, a water sample was collected from the turtle's aquarium or terrarium for *Salmonella* testing. Another water sample was taken from the turtle tank at the shop where one of the turtles was bought, for laboratory analysis. The detection of *Salmonella* in the water samples was performed using enzyme-linked fluorescence assay (ELFA) method (bioMérieux´s VIDAS) and by culture (ISO 19250 Water quality-detection of *Salmonella* spp.).

Isolates from patients and environmental samples which were positive for *S.* Paratyphi B were submitted for confirmation to the reference laboratory, National Centre for Microbiology, Carlos III Institute of Health, Madrid, Spain. The strains were typed using phenotypic (lead acetate method) and molecular methods to detect the tartrate reaction [12]. Susceptibility to antimicrobials was tested by the disc diffusion method according to Clinical and Laboratory Standards Institute (CLSI) guidelines [13]. The panel included the following antimicrobials: ampicillin, cefalotin, cefotaxime, amoxicillin/clavulanic acid, tetracycline, streptomycin, kanamycin, gentamicin, nalidixic acid, ciprofloxacin, chloramphenicol, trimethoprim/sulfamethoxazole and a sulphonamide compound (sulfadiazine, sulfathiazole and sulfamerazine sodium). The XbaI-PFGE patterns of strains were compared according to the PulseNet protocol [14].

Three of the 14 cases, identified as *S.* Paratyphi B, were excluded from the investigation because they were not *S.* Java or its variant.

Results

Out of the 14 strains of *S.* Paratyhi B studied, eight were identified as S Paratyphi B variant Java (*S.* Java), three as possible monophasic variants of *S.* Java (S. 4,5,12:b:-), and three as *S.* Paratyphi B sensu stricto. The last three, which came from a family outbreak involving three siblings, produced different clinical manifestations, and were excluded from this description.

The 11 patients from whom *S.* Java or its possible monophasic variant was isolated were not related to each other, and developed a mild disease, with symptoms of gastroenteritis.

Except for two adults in their mid-20s and early 60s, all cases were children aged between three months and

TABLE

Description of cases and laboratory results, *Salmonella* Paratyphi B var Java infections, Spain, September 2010–October 2011 (n=11)

Case	Age group (years)	Turtle exposure	Pacient		Turtle's water	
			Serotype	PFGE	Result (Serotype)	PFGE
1	5–10	yes	Salmonella Java	Type 1	negative[a]	NA
2	1–4	yes	S. Java	Type 1	negative[a]	NA
3	›10[b]	no	S. Java	Type 1	NA	NA
4	1–4	yes	S. Java	Type 1	S. Java	Type 1
5	‹1	no	4,5,12:b:-	Type 2	NA	NA
6	1–4	no	S. Java	Type 2	NA	NA
7	‹1	yes	S. Java	Type 2	S. Java	Type 2
8	5–10	yes	S. Java	Type 2	S. Java	Type 2
9	‹1	no	4,5,12:b:-	Type 3	NA	NA
10	1–4	yes	4,5,12:b:-	Type 3	negative[a]	NA
11	›10[b]	no	4,5,12:b:-	Type 3	NA	NA

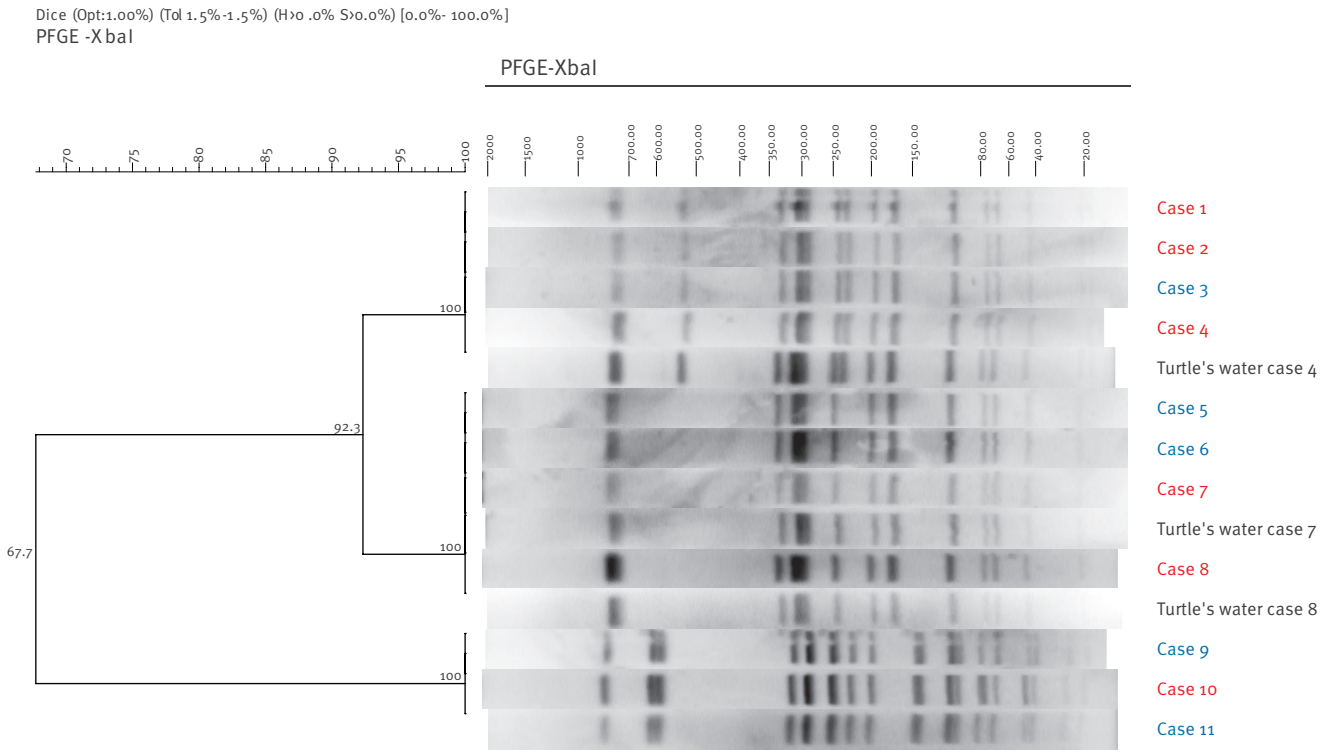
NA: not applicable; PFGE: pulsed-field gel electrophoresis.

[a] These samples were taken with a delay of five to 13 months after the infection.

[b] Adults in their mid-20s and early 60s.

Figure 2

PFGE profiles of cases, *Salmonella* Paratyphi B var Java infections, Spain, September 2010–October 2011



PFGE: pulsed-field gel electrophoresis.
Cases in red: exposed to turtles.
Cases in blue: not exposed turtles.

10 years. Six of the cases among children were male and three were female).

During the interviews, the only common factor found to constitute a risk according to the literature was having been in contact with aquatic turtles during the days before illness onset in six of the nine children, either at home (four cases), or at a relative's house (one case) or at school (one case).

The laboratory results show three different PFGE profiles, which we call type 1, 2 and 3 (Table, Figure 2). All strains were fully susceptible to all antimicrobials tested.

Three of the six samples of turtle's water yielded *Salmonella* Java, with the same PFGE patterns as the bacteria isolated from the children who had contact with them. Two of them were type 2, and the other was type 1. The three negative results came from samples collected more than five months after the infection.

The turtles were purchased at different shops and the supplier or suppliers could not be identified.

The PFGE patterns of isolates of patients with and without turtle exposure were indistinguishable, although the source of infection could not be found. All PFGE profiles were compared with those deposited at the PulseNet network and no match was found.

The water sample taken from the shop where the turtle of case 8 had been bought yielded *Salmonella* serogroup C. This turtle belonged to the subspecies *Trachemys scripta scripta*. The species of the other turtles are not known.

Discussion

Although we lacked a control group, the epidemiological and laboratory findings from our investigation indicate that turtles were the most likely source of infection with *S.* Paratyphi B var Java or its possible monophasic variant in this cluster of cases. Although any *Salmonella* serotype may be carried and transmitted by turtles, *S.* Java has been particularly associated with these reptiles [4].

For the first time, a possible monophasic variant of *S.* Java associated with reptile contact is described.

This is the second time we find an association between contact with turtles and *Salmonella* infection. In 2008, following an increase in *S.* Typhimurium infections in our region, a case-control study was performed, which estimated the odds of infection to be 1.62 times higher if the case had been exposed to turtles (95% confidence interval (CI): 0.68–3.89). In this study, 67/145 (46.2%) of cases were children aged between one and four years and 24/138 (17.4%) of cases reported contact with turtles. The association between reptile exposure and *Salmonella* infection has been described in several countries [2-7,15-18].

Most cases of turtle-associated salmonellosis occur in young children, who are in the most susceptible age spectrum, probably because they usually have a closer contact with these pets, and play with the aquarium water, which is a good medium for the growth of *Salmonella*. Moreover, their hygiene practices tend to be worse than those of adults [2,15]. In addition, parents are often not aware of the risk of infection associated with the presence of turtles in the household.

Not all the cases in this cluster reported exposure to turtles. However, direct contact is not necessary for infection; environmental contamination and symptomatic or asymptomatic patients represent possible sources of infection that may have gone unnoticed. As *Salmonella* bacteria survive in the environment for a long time [2,5], indirect transmission can play an important role.

Three of the six samples of turtle's water tested negative. However, *Salmonella* shedding can be intermittent and increase in response to stress like crowding, living in an environment with inadequate temperature, humidity or cleanliness, transportation, a change of habitat or excessive handling. A negative result doesn't rule out the possibility of intermittent water contamination [2,5]. For this same reason, a mixed infection in the water of the shop where *Salmonella* serogroup C was found is possible.

In the United States of America (USA), the association between contact with small turtles and *Salmonella* infection lead, in 1975, to a ban on the sale and distribution of turtles under 10.2 cm in carapace length, except for scientific or educational purposes. As a consequence, an important reduction in the number of *Salmonella* infections was observed in the following years, especially among children [2-5]. Since then, many sporadic turtle-associated salmonellosis cases have been detected.

In recent years, there has been an increase in the number of reptiles kept as pets, as well as in the number of infections linked to contact with reptiles, including more common serotypes, such as Typhimurium [2-4]. Currently, an estimated 6% of *Salmonella* infections in the USA are caused by direct or indirect contact with reptiles [4]. In February 2012, the Centers for Disease Control and Prevention (CDC) reported 132 cases of *S.* Paratyphi B var. L (+) tartrate + infection between 5 August 2010 and 26 September 2011. The median age of the patients in this outbreak was six years and of the 56 patients interviewed, 36 reported turtle exposure [19].

In Europe, *Salmonella* infection cases attributed to direct or indirect contact with reptiles have also been described, although the number is likely to be underestimated, as in many cases the source of infection is unknown [17]. In Sweden for instance, between 1990 and 2000, 339 reptile-associated *Salmonella* infections were reported, accounting for approximately 5% of all reported cases [5]. In this country, from 1970 to 1994, a certificate was required for the import of reptiles, stating that the animals were free of *Salmonella*, and the commercial distribution of turtles with a carapace length less than 10.2 cm was banned. When import regulations ceased, an increase in the number of cases was observed between 1996 and 1997. After a public education campaign launched in 1997, the number of cases decreased again [20].

Attempts to eliminate *Salmonella* from turtles by antibiotic treatment have not been successful, as the animals readily become reinfected from the environment, food or other turtles and can result in the development of antibiotic resistance. As *Salmonella* shedding may be intermittent and related to stress, it is difficult to determine whether turtles are free of bacteria [2]. For this reason, the way to prevent transmission is to avoid contact of susceptible persons with turtles and to follow strict hygiene practices to minimise the risk of infection.

In the US, apart from the restrictions on the sale of small turtles, there are recommendations published by CDC for preventing reptile-associated salmonellosis, which include washing hands after handling reptiles and keeping reptiles away from food and food preparation areas [21].

Conclusions and recommendations

In conclusion, there is a risk of *Salmonella* infection linked to contact with turtles, which emphasises the need to give recommendations regarding ownership and handling of aquatic turtles and other reptiles kept as pets by young children. These recommendations can also apply to immunocompromised persons. It would also be appropriate to give information to potential buyers at points of sale about the risk of *Salmonella* infection and measures that can be taken to minimise this risk.

A report of this outbreak with the following recommendations was sent to the public health authorities and the Department of Agriculture in Bizkaia so that preventive measures can be taken.

Recommendations given for preventing *Salmonella* infection from turtles included:

- washing hands with water and soap immediately after handling turtles (or other reptiles);
- cleaning and disinfecting surfaces that have been in contact with the animal;
- not using the kitchen to wash the aquarium/terrarium (if the bathroom is used, this should be disinfected after use);
- avoiding contact of the turtle with food (turtles should not live in the kitchen or roam freely in the house);
- avoiding contact of especially susceptible people (children under five years, pregnant women, patients with cancer or undergoing chemotherapy treatment, transplanted patients, persons with diabetes, hepatic conditions or other immunocompromised persons) with turtles and any object that has been in contact with them.

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Surveillance of tularaemia in Kosovo*, 2001 to 2010

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Tularaemia, caused by *Francisella tularensis*, had not been registered in Kosovo* before an outbreak in 1999 and 2000. A national surveillance system has been implemented in Kosovo* since 2000 to monitor a number of diseases, including tularaemia. Antibody detection in human sera was used for laboratory diagnosis of tularaemia and *F. tularensis* lipopolysaccharide antigen was used as a marker of infection. The purpose of this study is to describe the incidence of tularaemia in Kosovo* after the 1999–00 outbreak. In 2001 and 2002, a second outbreak occurred, with 327 serologically confirmed cases. From 2001 to 2010, 25–327 cases were registered per year, giving a mean annual incidence of 5.2 per 100,000 population. The most likely sources of infection were contaminated drinking water and food. The dominant clinical manifestations were the glandular (79%) and ulcero-glandular (21%) forms. By 2010, the disease had spread throughout Kosovo*. Presumably as a result of war and subsequent environmental disruption, mass population displacement and breakdown of sanitation and hygiene, the two major outbreaks of tularaemia resulted in the establishment of an active endemic area of tularaemia in Kosovo*.

Introduction

The causative agent of tularaemia, a relatively rare zoonotic disease, is *Francisella tularensis*. The bacterium is widely distributed in the northern hemisphere and is found in Europe [1]. Most countries in central and southern Europe reported single cases over the past decades. The disease is a more serious public health problem in Balkan countries and has also been reported in Turkey [2-4].

The cells of this Gram-negative, non-motile, capsule-forming, facultative intracellular bacterium are pleomorphic, typically appearing as short rods or coccoid forms [5]. Two subspecies of *F. tularensis* cause tularaemia in man. Biovar A1 of the subspecies *tularensis* (or type A) is the most virulent type of *Francisella* bacteria and can be associated with lethal pulmonary infections in humans [6]. Subspecies *holarctica* (or type B) is assumed to be less virulent. *F. tularensis* ssp. *tularensis* has been isolated almost exclusively in North America, whereas *F. tularensis* ssp. *holarctica* could be found in the entire northern hemisphere [5]. Interestingly, a second biovar of *F. tularensis* ssp. *tularensis*, A2, has a lower morbidity and mortality in humans than ssp. *holarctica* [6].

Depending on the site of entry of the pathogen, tularaemia can occur as ulcero-glandular and glandular forms, as well as oculo-glandular, oropharyngeal, respiratory or typhoidal [5]. Humans acquire the bacterium through contact with infected animals and/or vectors, by inhaling contaminated dust or aerosols, or by consumption of contaminated food or water. Human-to-human transmission is unlikely [5].

Francisella is capable of infecting a large number of animal species such as hares, rabbits, mice, lemmings and even fish [5]. Birds are known to be carriers, but probably do not develop the disease themselves [5]. Various vectors could play a role in transmission of *F. tularensis* from animals to humans: in Scandinavia, mosquitoes probably play a major role, while in North America, ticks are considered to be the most important vector [5].

F. tularensis is able to survive in the environment under cool and humid conditions, probably for weeks, and has been found in water and soil [7]. The mechanisms for survival of the bacteria are not yet well understood. Protozoa and nutrient conditions could play a role in protecting the bacteria [8-10].

History shows that tularaemia outbreaks are associated with poor hygienic conditions, especially in war and post-war situations, accompanied by a large increase in rodent populations and subsequent mass death of these animals [1,11-13]. Natural outbreaks occur in various endemic areas and can involve several hundred patients [1]. *F. tularensis* has been listed as a potential biowarfare agent [13,14].

Tularaemia had not been recorded in Kosovo* until an outbreak of the disease in 1999–2000 [13,15]. An intensive case investigation was carried out during the first outbreak [15]: specific antibodies against *F. tularensis* were detected in 247 serum samples from 912 suspected cases. Kosovo*, in south-east Europe, covers an

area of 10,908 km2, with a population of approximately 2.1 million inhabitants and population density of 192 per km2. It proclaimed its independence in 2008.

In January 2000, the national Institute of Public Health in Pristina implemented a surveillance system for 20 communicable diseases, based on syndromic approaches and clinical diagnoses. Timely reporting of disease syndromes from the different municipalities (administrative regions) allows a number of the most important infectious diseases in Kosovo* to be monitored simultaneously.

This study aims to provide a follow-up on the incidence of tularaemia in Kosovo* after the first outbreak in 1999–00 until 2010.

Methods

Case definition

A suspected case of tularaemia was defined as a person with fever and enlargement of cervical lymph nodes. The following indicators could also be present: skin ulcers and perspiration; weakness, body pain and headache; and throat pain and ingestion problems. Confirmed cases were individuals with the above clinical picture in whom laboratory confirmation of the infection was obtained.

To ensure that all individuals with the tularaemia were identified, the case definition was rather broad. The clinical manifestation of *F. tularensis* infection can be quite similar to that of tuberculosis, brucellosis or mumps – all of these diseases have a relatively high prevalence and incidence in Kosovo* (data not shown). Therefore, laboratory conformation by detecting specific antibodies or the pathogen itself is required for the final diagnosis of tularaemia.

Collection of epidemiological data

All outpatient clinics and medical centres are obliged to fill in official reporting forms every week to record aggregated and individual data on the 20 specified communicable diseases, including tularaemia. The forms are sent to the regional Institute of Public Health, which subsequently passes them on to the national institute. Double reporting is prevented by checking the personal data of reported cases. At the national institute, the data are entered into a central database, to be regularly analysed by means of Epi-Info software.

Diagnostic sera were obtained by local physicians from suspected cases and sent for analysis to the national Institute of Public Health. The sera were analysed for specific antibodies to *F. tularensis*, routinely using a microagglutination assay [16]. Positive sera and additional sera from persons suspected to be infected with *F. tularensis* were checked by a highly specific enzyme-linked immunosorbent assay (ELISA) and western blot analysis, as described below.

Microagglutination

The live vaccine strain of *F. tularensis* biovar *holarctica* (ATCC 29648) was grown for 2–3 days at 37 °C in a 5% CO2 humid atmosphere on heart-cysteine-blood agar and harvested into sterile distilled water or isotonic sodium chloride. Bacterial concentrations were adjusted photometrically at 580 nm to an optical density of 1.0. The suspension was inactivated with paraformaldehyde and prepared as agglutination antigen as described elsewhere [16]. The assay was adjusted by the optimal antigen concentration and evaluated on the basis of a titre of 1:16 or higher being considered positive.

Antibody detection in human sera by ELISA and western blot

The ELISA was used for screening and western blot for confirmation. Both have been described elsewhere [16-18] and were used with some modifications. Briefly, a 96-well microtitre plate (Polysorb, Nunc, Germany) was coated with purified lipopolysaccharide (LPS) from the live vaccine strain as antigen. Bound human antibodies to *F. tularensis* were detected by polyvalent goat anti-human IgA-IgM-IgG horseradish peroxidase-conjugated secondary antibody (dianova, Germany) and subsequent substrate reaction. For the western blot, the soluble fraction of formalin-inactivated live vaccine strain was separated using sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride (PVDF) membranes (ImmobilonP, Millipore, United States). Using polyvalent horseradish peroxidase-conjugated secondary antibodies, the typical LPS ladder revealed the presence of specific anti-*F. tularensis* antibodies. The final results were given after confirmation of the ELISA results by western blot: 'positive' denoted strong bands, 'negative' – almost no bands and 'borderline' – weak but clearly visible bands).

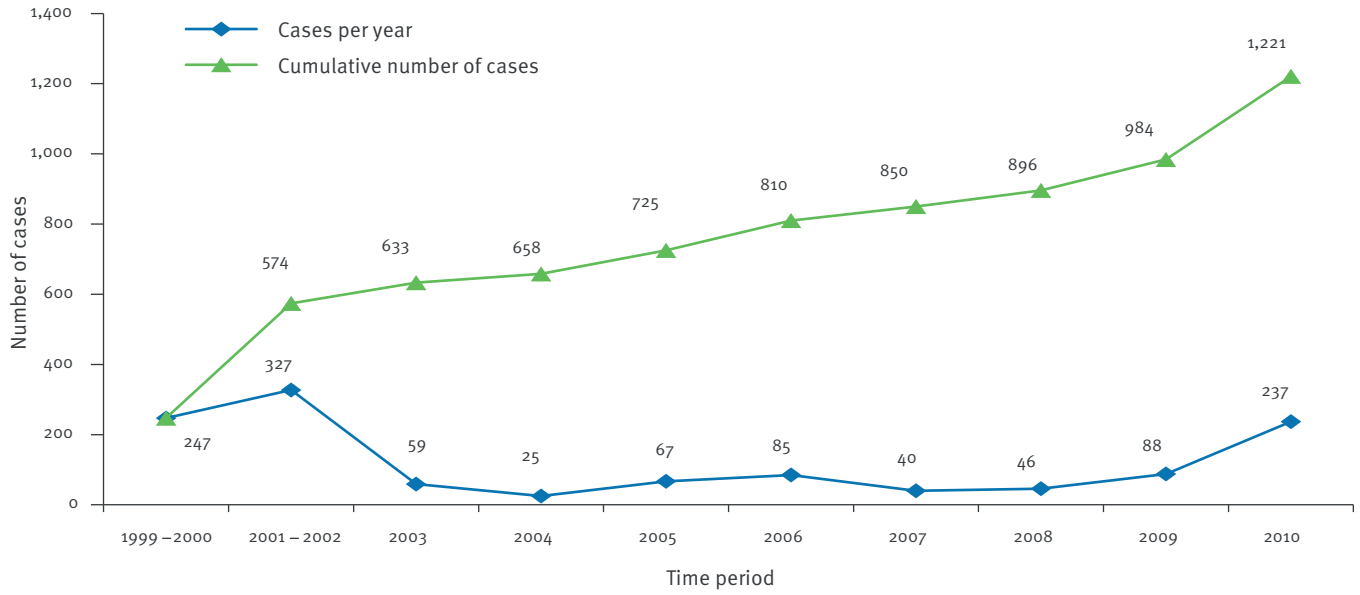
Samples for antigen detection and capture ELISA

Environmental samples (faeces from rabbits and mice, water samples), tissue samples (spleen, liver) from dead mice, rats and rabbits, as well as clinical samples from serologically confirmed tularaemia patients were analysed using a capture ELISA. It was essentially performed as described previously, using the *F. tularensis* LPS-specific murine monoclonal antibody 11/1/6 as capture antibody bound to the solid phase [19-21].

Faeces from pathogen-free inbred and outbred mice and rabbits were kindly provided by the National Research Centre for Environment and Health (GSF) in Munich, Germany, which were used as negative controls. Samples were homogenised and pre-treated with LPS-extraction buffer containing chenodeoxycholic acid in phosphate buffered saline/ethylenediaminetetraacetic acid (PBS/EDTA). Large particles were allowed to sediment for 5 minutes and the supernatant was analysed for the presence of *F. tularensis* LPS using the cELISA.

Figure 1

Reported confirmed cases of tularaemia, Kosovo*, 1999–2010 (n=1,221)



* This designation is without prejudice to positions on status, and is in line with United Nations Security Council Resolution 1244/99 and the International Court of Justice Opinion on the Kosovo declaration of independence.

Results

Tularaemia outbreak in 2001 and 2002

After the tularaemia outbreak in 1999–00, a second outbreak occurred from November 2001 to June 2002, which was investigated by a Kosovar/German team from the Bundeswehr Institute of Microbiology, Munich, Germany. This outbreak has not been previously described in the scientific literature. During this period, 1,168 serum samples from suspected cases were tested; 327 cases laboratory confirmed by ELISA and western blot (Figure 1). Although the second outbreak started in other parts of Kosovo* (east and south-east), the affected areas generally overlapped those of the previous outbreak.

The epidemiology of the second outbreak was quite similar to that of the first [15]. It can be assumed that the main reason for the spread of the disease seemed to be again the bad sanitary conditions, especially in rural areas of Kosovo*. As described for the first outbreak [15], housewives (37%) and farmers (27%) were the most affected occupational groups. Similarly, cases were mainly female (60%) and the age group 20–40 years (52%) were also most affected.

It was characteristic of both outbreaks that people in affected regions reported an enormous increase in the rodent population, especially field, forest and domestic mice before the outbreak among humans.

The investigation of animal and environmental specimens by the capture ELISA, which is highly specific for *F. tularensis* [19], showed that the antigen was detected mainly in mouse and hare faeces (Table). During the outbreaks, faeces of small rodents were regularly found by the investigation teams in products stored in food stores of affected households and showed the most striking positive results in antigen detection of *F. tularensis*. During the first and second outbreaks, 145 and 220 samples were collected respectively from similar sources, of which 10 and 22, respectively, were positive. We could not detect *F. tularensis* antigen in a very limited number of available clinical specimens: throat swabs (n=18), pus and wound secretions (n=4). At that time, a more sensitive polymerase chain reaction (PCR) was not available.

As in the first outbreak, the predominant manifestation of the disease during the second was oropharyngeal. The main route of transmission leading to this oropharyngeal form was probably ingestion of contaminated food or water [15]. More than 90% of the patients (305/327) had as the leading symptom enlarged cervical lymph nodes, whereas the other patients had enlarged lymph nodes in other locations such the axilla or inguinal region. This clinical manifestation of oropharyngeal tularaemia was dominant throughout the study period.

Table

Francisella tularensis antigen detection in animal and environmental specimens during tularaemia outbreaks in Kosovo*, 1999–2002 (n=365)

Sample type	Source	1999–00		2001–02		Total without controls	
		Number of samples	Number positive	Number of samples	Number positive	Number of samples	Number positive
Faeces	Mice	55	7	58	9	**113**	**16**
	Control: pathogen-free mice	NT	NT	100	0	–	–
	Hares	NT	NT	104	12	**104**	**12**
	Control: pathogen-free rabbits	NT	NT	57	0	–	–
Animal tissue	Mice, rats, hares	63	3	35	1	**98**	**4**
	Control: pathogen-free mice	NT	NT	25	0	–	–
Water	Wells, ponds	27	0	23	0	**50**	**0**
Total without controls	–	**145**	**10**	**220**	**22**	**365**	**32**

NT: not tested.

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Surveillance of tularaemia from 2001 to 2010

After the first outbreak, the surveillance system revealed the presence of cases every year (Figure 1). From 2003 to 2010, the annual number of tularaemia cases was between 25 and 237, with a mean annual incidence of 3.9 ±3.2 standard deviation (SD) per 100,000 population. During this period, a total of 647 cases were reported.

In 2010, more than 200 tularaemia cases were registered. The reason for this high number of cases is unknown: no specific source of infection nor a specific outbreak scenario could be identified. The cases were distributed equally throughout Kosovo* and throughout the year. This could indicate a high epizootic and zoonotic activity in that year, for some unknown reason.

Housewives and farmers were the most affected occupational groups, representing about 33% (n=216) and 24% (n=153) of cases, respectively. This is also reflected in the sex distribution of all cases during this period: 57% (n=372) of cases were female and 43% male (n=275) (Figure 2). Most cases (n=309) were in the age group 20–40 years, of whom 61% (n=188) were female and 39% (n=121) male. In addition, quite a high proportion, about 20% (n=128) of children and teenagers (aged under 20 years) were infected.

Since 2001, the clinical manifestation in the 974 patients was oropharyngeal or glandular tularaemia: about 93% (n=906) of the cases had unilaterally enlarged cervical lymph nodes or swollen axillar or inguinal lymph nodes; 7% (n=67) had the ulcero-glandular form.

Since the first outbreak, tularaemia has spread to other parts of Kosovo*. As a result, all municipalities participating in the surveillance system have reported human tularaemia cases (Figure 3). The three municipalities marked in grey in Figure 3B were not participating in the surveillance system.

Discussion

By mid-1999, more than 10 years of political crisis and warfare in Kosovo* had resulted in environmental disruption, mass population displacement and a breakdown of sanitation and hygiene [15]. Many essential public health functions, such as disease surveillance and outbreak response, had collapsed [15]. It

Figure 2

Age and sex distribution of confirmed tularaemia cases, Kosovo*, 1999–2010 (n=1,221)

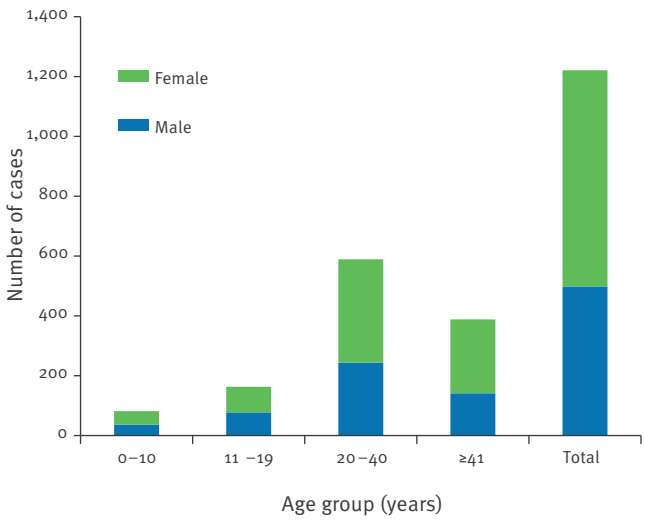

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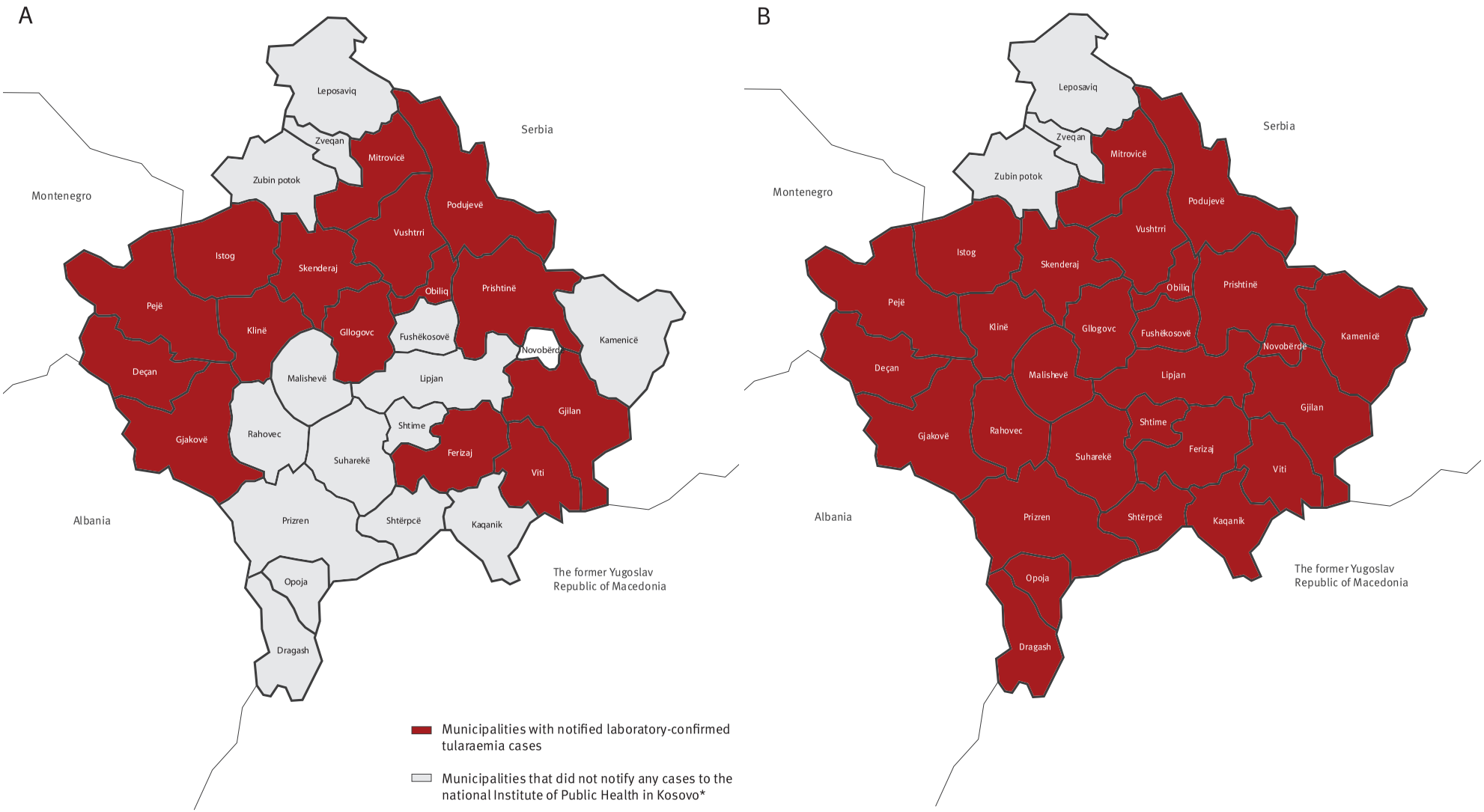
Figure 3

Distribution of confirmed tularaemia cases, Kosovo*, Panel A: 1999–2000 (n=247), Panel B: 1999–2010 (n=1,221)



The three municipalities marked in grey in Panel B were not participating in the surveillance system.

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is suspected that tularaemia had been present in the region during and/or after the Second World War, but there are no official data about the disease in Kosovo* during that time. Although the disease was notifiable in the former Yugoslavia, local representatives stated that no cases of tularaemia had been detected before the war in Kosovo* in 1998 to 1999 [13].

The number of laboratory-confirmed cases of tularaemia in the first outbreak in 1999–00 was unexpectedly high, given that Kosovo* had been considered non-endemic for the disease at that time. An even larger number of cases was seen during the second outbreak in 2001–02. The circumstances of a typical post-war situation in the autumn of 1999 were probably responsible for the outbreaks: people left their homes and did not harvest the fields, which led to an oversupply of food for rodents [13]. Consequently, an unusually large increase in the rodent population was observed until January 2000. It is known that an increased density of the rodent population can facilitate the spread of zoonotic infectious pathogens including *F. tularensis* among animals and induce an epizootic spread to man [22]. We found *F. tularensis* antigen in a relatively high percentage of samples from collected rodents and hares. However, for further identification of the infectious source and characterisation of the causative agent, attempts should still be made to obtain isolates of *F. tularensis* from the samples collected between 1999 and 2002.

As almost all the tularaemia patients during 2001 to 2010, as in the first outbreak [15], had the oropharyngeal form with fever and a unilateral cervical lymph node enlargement as the main symptoms, obviously the main route of infection was alimentary ingestion of *F. tularensis*. It was rather surprising that during the first outbreak, cases were spread over a large part of Kosovo*. It can be speculated that either the pathogen had already been present in these regions in spite of not having been observed or it was spread by human and animal migration as a consequence of the war. It was assumed by the national Institute of Public Health and the outbreak investigation team at that time that an emerging or re-emerging endemic region with periodic outbreaks of tularaemia might develop in Kosovo*. In fact, the data for 2003 to 2010 indicate a continuous activity of tularaemia after the initial outbreaks.

The clinical manifestation in 2010 was similar to that during the outbreaks in 1999–2002, which suggests that the routes of transmission have remained the same. In comparison, in other parts of the world, the ulcero-glandular form of tularaemia is primarily detected, which can arise due to direct exposure of the patients' skin to infected animals, carcasses, water or other materials, or to arthropod vectors [1,5]. Climate change is believed to influence the spread of vectors and therefore of tularaemia [23]. Obviously alimentary ingestion of the pathogen has been the major route of infection in Kosovo*. Given the situation, ingestion of contaminated food and water arising from the poor hygiene conditions seem to be the most likely risk factors for the infection.

Interestingly, the mean incidence of tularaemia in Kosovo* from 2001 to 2010 (5.2±4.6 SD per 100,000 population) is comparable to that in Sweden (3.2±2.08 SD per 100,000 population, calculated for the same time period from data from the Swedish Institute for Communicable Disease Control [24]), which is known to be endemic for tularaemia, and about 100 times higher than that in Germany (0.013±0.012 SD per 100,000 population; calculated for the same time period from SurvStat data from the Robert Koch Institute in Germany [25]). Tularaemia in Germany is less evident than in some other countries, but little is known about the epizootic activity of the disease in Germany. Thus, low numbers of reported human cases in Germany may not reflect the actual prevalence of the pathogen in nature and the potential risk of epidemics [26].

In 2010, Kosovo* represented an emergent endemic region for tularaemia. The reasons for this development are not fully understood. More recent data are in the process of being evaluated at the national Institute of Public Health in Pristina. Further surveillance of this disease is important in order to detect possible outbreaks in a timely manner and to take adequate measures to prevent the further spread of the disease. The main reason for ongoing activity of the disease seems to be the still poor sanitary conditions, especially in rural areas of Kosovo*. In addition, animal control and surveillance, including that of rodents, should be carried out to prevent further outbreaks. Further field investigation is required to obtain *Francisella* isolates for clarification of the subspecies prevalent in Kosovo* and to further identify reservoirs and routes of transmission of the pathogen. Additional resources are required to manage this serious health problem, although other infectious diseases may have an even higher impact on public health in Kosovo*.

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