Evidence of *Orientia* spp. Endemicity among Severe Infectious Disease Cohorts, Uganda

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At 3 severe infection cohort sites in Uganda, *Orientia* seropositivity was common. We identified 4 seroconversion cases and 1 PCR-positive case. These results provide serologic and molecular support for *Orientia* spp. circulating in sub-Saharan Africa, possibly expanding its endemic range. *Orientia* infections could cause severe illness and hospitalizations in this region.

Scrub typhus is a leading cause of nonmalarial febrile illness in Southeast Asia (*1*). Scrub typhus is caused by miteborne *Orentia tsutsugamushi* infections, which until recently were thought to be limited to South and Southeast Asia. Molecular identification of different *Orientia* species in clinical cases from Chile (*2*) and the United Arab Emirates (*3*) has suggested a broader epidemiology. *Orientia* spp. were found in mites in Kenya (*4*), and descriptions of *Orientia* seroconversion in patients from sub-Saharan Africa have slowly accrued, suggesting the possibility of *Orientia* spp. transmission in Africa (*5*). We used archived samples collected in 2 severe infection prospective cohorts in western, central, and northwest Uganda to assess *Orientia* endemicity in the country.

The Study

Using archived samples, we measured serial *Orientia* immunofluorescence assay (IFA) IgG titers and performed reflex *Orientia* spp. reverse transcription PCR (RT-PCR). Samples were collected as part of 2 severe infection prospective cohorts and had undergone broad microbiologic testing. In both cohorts, adult patients ≥18 years of age who fulfilled acute febrile illness (AFI; hospitals in Mubende and Arua, Uganda) or sepsis-specific (hospital in Fort Portal, Uganda) eligibility criteria were evaluated for enrollment at admission in the outpatient or emergency department, or on medical wards (Appendix, https://wwwnc.cdc.gov/EID/article/30/7/23-1040-App1.pdf) (*6*). Matched acute and convalescent serum samples were available from 269 of 310 participants enrolled in the sepsis cohort and 67 of 132 participants in the AFI cohort.

In brief, across both prospective cohorts, study teams collected demographic and symptom information, examination findings, and laboratory data on standardized forms during hospitalization and at 1 month after enrollment. Clinical tests were routinely performed, including complete blood counts and chemistries. Microbiologic testing included blood culture with antimicrobial sensitivity testing, HIV testing, malaria smears, and rapid diagnostic tests, as previously described (*6*) (Appendix).

We performed IgG IFAs by using *Orientia tsutsugamushi* Karp strain antigen slides (BIOCELL Diagnostics Inc., https://biocelldx.com). Baseline (acute) and 1-month follow-up (convalescent) serum samples were screened at a titer of 1:64 and titrated up to 1:65,000. We considered a sample seropositive at a threshold titer of ≥128. We performed IgG IFAs by using commercial slides to evaluate for cross-reactivity

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to spotted fever group rickettsia (SFGR), *Rickettsia conorii* Molish 7 strain, typhus group rickettsia (TGR), and *Rickettsia typhi* Wilmington strain (BIOCELL Diagnostics, Inc.). We performed a Kruskal-Wallis test to evaluate for differences between *Orientia* IFA IgG titers between those with and without available matched samples. We used a titer of 32 to calculate -fold increase if the screen was negative at a titer of 1:64. We had a blind second reader review ≤5% of each batch.

Because no prior estimates of *Orientia* seroprevalence were available for Uganda, we used stringent criteria to define probable cases (Appendix Figure 1). To evaluate the specificity of IFA results, we used a subset of high titer samples to corroborate evidence of antibody binding by using a dot blot, Western blot, and Gilliam strain IFA (Appendix Methods, Figure 2). To optimize sensitivity for RT-PCR, we targeted mRNA and rRNA from serum from both cohorts (*7*), whole blood from the AFI cohort, or buffy coat from the sepsis cohort. We used QIAamp RNA Mini Kit (QIAGEN, https://www.qiagen.com) to extract RNA. We performed RT-PCR targeting *Orientia* spp. 16S rRNA, Orien16S and *rrs* by using previously published methods (*3*,*8*), and mRNA from *Orientia* spp. 56-kDa antigen gene, SFGR OmpA (*sca0*) gene, and TGR kDa (*9*) outer membrane protein gene. We only called positives that were in duplicate.

We found that 33.9% (148/436) of acute samples and 38.4% (129/336) of convalescent samples were seropositive (≥128) for *Orientia* spp. Among acute samples, 25.5% (111/436) were positive at ≥256 titer and 19.0% (85/436) were positive at ≥512 (Figure 1). We observed no difference in acute IFA titers between patients with and without a convalescent blood samples (p = 0.33). Among samples with a positive 1:64 titer screen, the median acute titer was 128 (up to 8,192; interquartile range [IQR] 64–512) and median convalescent titer was 256 (up to 4,096; IQR 64–1,024). Seropositivity was highest (acute, 38.7% [120/310]; convalescent, 41.6% [112/269]) in Fort Portal, but was also high in Arua (acute, 26.5% [9/34]; convalescent, 30.0% [6/20]) and Mubende (acute, 20.7% [19/92]; convalescent, 23.4% [11/47]). The overall geometric mean titers were 90.8 (95% CI 80.2–102.8) for acute samples and 100.3 (95% CI 86.1–116.9) for convalescent samples.

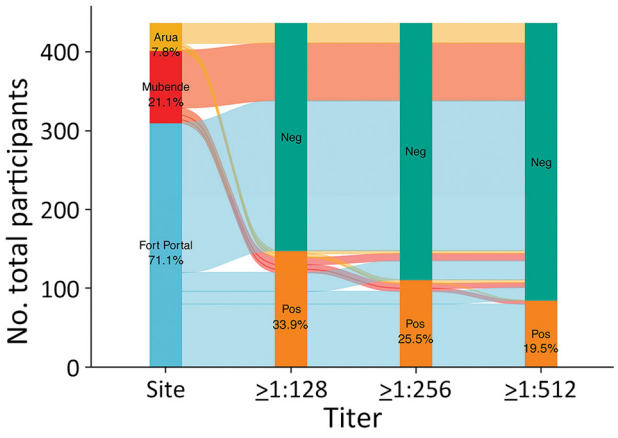

Figure 1. Alluvial diagram of serology from acute serum samples used in a study of *Orientia* genus endemicity among severe infectious disease cohorts, Uganda. The diagram represents *Orientia* spp.–positive immunofluorescent assay IgG at ≥128, ≥256, and ≥512 from 3 sites in Uganda. Colored lines indicate total participants from each site with positive or negative serology at 3 different titer cutoffs. Neg, negative; pos, positive.

Four participants met our case definition for *Orientia* spp. seroconversion (Table 1). Participants meeting the case definition were 24–56 years of age; 3 were female and 1 was male, and 3 had HIV (Table 2). Leukocyte counts ranged from 5–10 × 10^3 cells/μL, platelet counts were 56–220 × 10^3 cells/μL, and aspartate transaminase was 21–136 U/L. Three patients survived, but a 34-year-old woman with HIV in whom a papular rash developed died of unknown causes 8 months after follow-up. Three participants with seroconversion had negative malaria smears, blood cultures, and rapid antigen and molecular diagnostic tests for nonrickettsial pathogens (Table 2).

We used molecular methods to confirm *Orientia* spp. infection. The acute serum sample from participant D was repeatedly *rrs*-positive with RT-PCR (mean

Table 1. Rickettsia IgG results from participants with *Orientia spp.* seroconversion in a study of *Orientia* genus endemicity among severe infectious disease cohorts, Uganda*

	Days after illness onset†		*Orientia* spp.–positive titer‡			Spotted fever group titer			Typhus group titer		
Participant	Acute	Conv.	Fold change	Acute	Conv.	Fold change	Acute	Conv.	Fold change	Acute	Conv.
Mubende											
A	7	34	4	256	1,024	1	32	32	1	32	32
Fort Portal											
B	2	32	8	64	512	1	32	32	1	32	32
C	14	36	4	128	512	1	32	32	1	32	32
D	1	38	4	128	512	1	32	32	1	32	32

*Conv., convalescent; IFA, immunofluorescent assay.
†Sample collection after illness onset.
‡Karp strain IFA. Gilliam strain IFA seroconversion also observed in Fort Portal participant D (acute titers 1:512 and convalescent titers 1:2,048) but not among participants A-C.

Table 2. Clinical characteristics of participants with *Orientia spp.* seroconversion in a study of *Orientia* genus endemicity among severe infectious disease cohorts, Uganda*

Characteristics	Patient identification			
	A	B	C	D
Age, y/sex	24/M	34/F	23/F	56/F
Occupation	Mine worker	Business or trade	Fuel attendant	Farmer
Rash, type	Y, pustular and eschar	Y, papular	N	N
Clinical laboratory parameters				
WBC, x 10^3 cells/μL	7	10	5	8
Platelet count, x 10^3 cells/μL	56	220	128	177
AST, U/L	21	62	136	26
Microbiologic results†				
HIV (CD4)	+ (603)	+ (NA)	+ (NA)	–
Malaria smear	+, 126 parasites/μL	–	–	–
TB				
PCR	NA	NA	NA	–
Urine LAM	+	–	–	NA
Clinical diagnosis	TB	Urinary tract infection	Unidentified	Abdominal source
Inpatient treatment	ACT	CIP, CTX, MTZ	CTX, cefixime	CIP, MTZ
Outcome	Survived	Died, 8.2 mo.	Survived	Survived

*ACT, artemisinin-based combination therapy; AST, aspartate transaminase; CIP, ciprofloxacin; CTX, ceftriaxone; LAM, lipoarabinomman; MTZ, metronidazole; NA, not available; NG, no growth; TB, tuberculosis; WBC, white blood cells; –, negative; +, positive.
†All had negative blood cultures and negative multiplex PCR results.

cycle threshold 34.1, SD 0.4) and was confirmed by Sanger sequencing of the amplicon. A BLAST analysis (https://blast.ncbi.nlm.nih.gov) of a 96-bp sequenced fragment of the amplicon revealed 96%–100% homology with *Orientia* spp., and a single polymorphism aligned with *Candidatus* O. chuto (Figure 2). RT-PCR was negative using other primers for *Orientia* spp. (Orien16S 56-kDa) targets, SFGR (*sca0* [*ompA*] targets, and TGR (17-kDa antigen gene) targets.

Conclusions

We identified *Orientia* seroconversion among 4 participants hospitalized with severe infection in sub-Saharan Africa. We demonstrated that *Orientia* seropositivity was common among patients admitted for severe infection at 3 hospitals in Uganda. Our findings of highly prevalent seropositivity at 3 sites, identification of seroconversion, and molecular confirmation of a case with otherwise negative broad microbiologic testing support *Orientia* circulation and raise suspicion for infections extending to East Africa.

Prior clinical evidence of suspected scrub typhus in Africa relied on case reports of returning travelers with *Orientia* seroconversion (*5*). In addition to seroconversion identified in this study, seroconversions were observed in a pediatric cohort in Kenya (3.6%; n = 10) (*10*), and in 1 case among 49 abattoir workers in Djibouti (*11*). Our well-characterized multisite results supplement the limited literature suggesting *Orientia* spp. infections in sub-Saharan Africa.

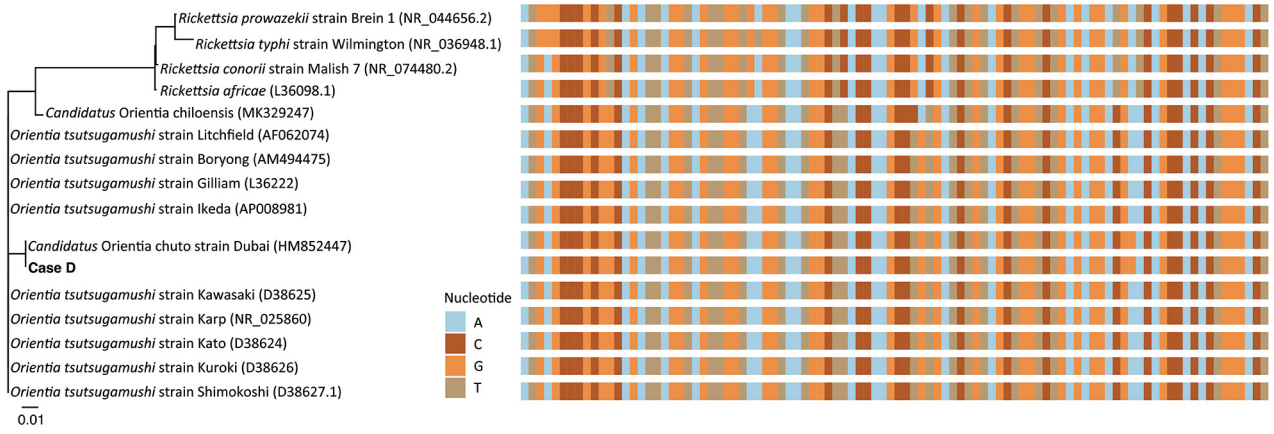

Figure 2. Phylogenetic tree (left) and aligned sequences (right) of *Orientia* spp. and locally endemic *Rickettsia* spp. in a study of *Orientia* genus endemicity among severe infectious disease cohorts, Uganda. We compared the 16S rRNA gene with an *Orientia* infection (case D) in Uganda. We aligned the 96-bp amplicon region and created the tree by using the neighbor-joining algorithm in R (The R Foundation for Statistical Computing, https://www.r-project.org). GenBank accession numbers of reference sequences are in parentheses. A single polymorphism aligned with *Candidatus* O. chuto, possibly differentiating case D from other *Orientia* spp. Scale bar indicates nucleotide substitutions per site.

In addition to prior suggestive evidence, our results build on a shift in understanding of worldwide *Orientia* spp. clinical infections. SFGR and TGR test results were negative in our cohorts, decreasing the likelihood of cross-reactivity. Despite IFA being the preferred method for rickettsial diagnosis, intrinsic interobserver variability limitations exist (*12*); we aimed to reduce those limitations through our reading approach and seroconversion criteria. Although we were able to confirm an infection by using real-time RT-PCR, sequence results were limited to a small fragment of the abundant 16S rRNA. The clinical relevance requires further confirmation with *Orientia* culture growth and extended genome sequencing. Because we relied on convalescent serology, we might have missed early fatal cases, which could skew our results toward less severe illness. Research efforts are needed to characterize the circulating species, incidence, pathogenic potential, and clinical relevance of *Orientia* infections in East Africa.

In summary, our findings suggest *Orientia* spp. circulation within the human–environment interface in Uganda and suggest novel *Orientia* infections within severe infection cohorts in Uganda. After excluding common causes of infections, our findings provide evidence of locally acquired *Orientia* infections among adults in sub-Saharan Africa.

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This study was conducted in compliance with the Declaration of Helsinki and Good Clinical Practice Guidelines. All participants signed written informed consent prior to study procedures. The investigators have adhered to the policies for protection of human subjects as prescribed in 45 CFR 46. Parent acute febrile illness cohort: The study and informed consent process were reviewed and approved by the Joint Clinical Research Centre (JCRC) Research Ethics Committee (JC1518) and the Uganda National Council for Science and Technology (UNCST), HS 371ES, and Johns Hopkins University School of Medicine Internal Review Board (IRB no. 00176961). Parent sepsis cohort: This protocol and informed consent were approved by the US Army Medical Research and Development Command Institutional Review (approval no. M-10573) and Makerere University School of Public Health (IRB no. 490). *Secondary use protocol:* this laboratory work was reviewed and received an exempt determination by Uniformed Services University (IRB no. DBS.2020.174).

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Appendix

Supplemental Methods

Acute Febrile Illness Cohort

As previously described (*1*), in the acute febrile illness cohort, patients presenting to Mubende and Arua Regional Referral Hospitals (RRHs) with a measured or reported temperature ≥38.0°C occurring within the past 48 hours or a clinical history consistent with fever within 48 hours of presentation were enrolled from August 2019 to August 2020 (*1*). Participants with COVID-19 diagnoses were in separate designated areas and were not enrolled. Exclusion criteria included being hospitalized for ≥72 hours, receipt of antibiotics, previous participation in the study, being a prisoner, and being a psychiatric patient. The two tertiary care hospitals were chosen as study sites because both sites are in regions with different ecology and serve different populations. Mubende RRH (bed capacity 173) is located in central Uganda and serves a district population of over 688,000 people (*2*). Arua RRH (bed capacity 323) is located in the more arid northwest Uganda and serves over 785,000 people in the districts.

Sepsis Cohort

Briefly, in the sepsis cohort, 301 patients ≥18 years of age with suspected infection and ≥2 modified systemic inflammatory response syndrome (SIRS) criteria (temperature <36°C or >38°C, heart rate ≥90 beats per minute, or respiratory rate ≥20 breaths per minute) were enrolled at Fort Portal Regional Referral Hospital in Fort Portal, Uganda from October 2017 to November 2021 (P.W. Blair et al., unpub. data, https://doi.org/10.1101/2023.09.14.23295526). Patients presenting at the outpatient department, emergency department, or medical wards were evaluated

for enrollment. Patients were not eligible if they were deemed too ill to participate with an imminently terminal comorbidity or if they presented with severe anemia (hemoglobin <7 g/dL). Due to inflammation biomarker objectives, participants were excluded with known immunocompromising conditions, including drug induced immunosuppression, anatomic or functional asplenia, recent chemotherapy, pregnancy and <6 weeks postpartum females, but participants with HIV were eligible. FPRRH serves as a health facility to eight districts in western Uganda.

Sepsis and AFI Cohort

If there was clinical suspicion, PCR testing for tuberculosis was performed using expectorated sputum (Xpert MTB/RIF Ultra, Cepheid, Sunnyvale, CA, USA) and participants with HIV had a urine lipoarabinomman (LAM test). Whole blood was run on the FilmArray Global Fever panel for 19 non-rickettsial pathogen targets (*3*).

Seroconversion Criteria

To validate the findings of seropositivity, we also read Gilliam strain IFA slides (BIOCELL Diagnostics Inc., Baltimore, MD, USA) for a portion (n = 50), including those that seroconverted and had a >1:256 titer (n = 13) and also 37 (12%) randomly selected samples. We used seroconversion criteria to decrease the risk of a false Karp IFA seroconversion. First, we included seroconversions defined as a $\geq$4-fold increase in titer from acute to convalescent sera resulting in a convalescent titer of at least 512, excluding n = 4 at 256 (the upper interquartile range [IQR] of the *Orientia* IgG titers). Second, while serum cross-reactivity with spotted fever group rickettsia (SFGR) or typhus group rickettsia (TGR) has not been widely reported (*4*), we excluded participants with convalescent SFGR (n = 7) or TGR (n = 2) IFA IgG titers that were greater than or equal to *Orientia spp.* (SFGR *Rickettsia conorii* Molish 7 strain, TGR *Rickettsia typhi* Wilmington strain; BIOCELL Diagnostics Inc, Baltimore, MD, USA). Third, we excluded those with severe symptoms present for >14 days at time of enrollment (n = 2).

Western Blot and Dot Blot

For the Western blot and dot blot, we used the TSA 56-kDa *Orientia* protein (*5*) to determine existence of *Orientia* spp. antibodies from the serum sample. The recombinant protein was received frozen after preparation using the referenced procedures (*5*) but was dissolved in 8 M urea. The protein was dialyzed to remove urea using Amicon Ultra 0.5 mL centrifugal filter

units (Merck), following established procedures. Centrifugation occurred at 1,000 to 2,000 g for ten minutes and was followed by a wash step with phosphate-buffered saline (PBS, pH 7.4) and an additional centrifugation. The dialyzed protein was carefully collected from the filter units and stored in PBS. Positive controls included *Orientia tsutsugamushi* IgG–positive serum and a healthy unexposed human serum negative control.

For the Western blot, recombinant TSA56 protein was prepared in two concentrations, 2 µg and 5 µg, in NuPAGE LDS Sample Buffer and NuPAGE Sample Reducing Agent (Invitrogen, Thermo Fisher Scientific). The samples underwent denaturation at 70°C for 10 minutes using a heat block (Thermo Fisher Scientific) and were loaded onto a NuPAGE 10% Bis-Tris gel (Invitrogen, Thermo Fisher Scientific). Electrophoresis was conducted at 200V. The proteins were transferred to a membrane using the Trans-Blot Turbo Transfer System (Bio-Rad). Transfer efficiency was assessed by Ponceau S staining (Sigma-Aldrich). The membrane was blocked with 5% non-fat dry milk in PBST (PBS + 0.1% Tween-20) and then incubated with human serum samples diluted 1:100,000, including a positive control serum containing scrub typhus IgG and a negative control of normal human serum. The membrane was treated with HRP-conjugated anti-human IgG (Invitrogen), diluted to 1:2,000. Subsequently, to assess protein purity, the TSA56 protein weight was estimated using ImageJ software (*6*). After $\log_{10}$ transformation to fit a standard curve, the weight of the TSA56 protein was estimated to be 59.8 kDa and, accordingly, within range of anticipated measurement error of 56 kDa.

For the dot plot, we used a nitrocellulose membrane. we prepared a nitrocellulose membrane (Bio-Rad) and applied TSA56 recombinant protein (*2*) and bovine serum albumin (BSA), the latter serving as a negative control to assess antibody specificity. To ensure non-specific binding was identified, *Rickettsia parkeri* (Rp) protein was used as an unrelated antigen. The assay incorporated positive controls consisting of scrub typhus IgG serum and negative controls of normal human serum. Blocking was conducted using a buffer of 5% non-fat dry milk in PBST (PBS + 0.1% Tween-20) to prevent non-specific binding. The secondary antibody used was HRP-conjugated anti-human IgG (Invitrogen). 1 µg of TSA56 protein, BSA and Rp were spotted onto the membrane, followed by air drying. Serum samples were diluted ranging from 1:100 to 1:100,000 in blocking buffer and incubated on the membrane followed by a 1-hour room temperature incubation. Signal was detected for both the dot plot and the western blot using iBright FL1000 (Thermo Fisher Scientific).

After identification of a reproducible positive RT-PCR result, the *rrs* RNA PCR amplicon from case D was sequenced in both forward and reverse directions. The flanking forward and reverse sequences had high confidence electropherograms to include sequences in both directions for a total high confidence sequence of 96 bp with 46 bp of overlap in both directions. To evaluate the association with the 96-bp sequence from case D with other *Orientia* spp., we performed a phylogenetic analysis of the 96-bp sequence using the Neighbor-Join (NJ) algorithm method using the Biostrings and ape package and figures created using the ggplot2 and ggtree packages in R.

Supplementary Results

We identified evidence of antibody binding against the TSA protein with 6 high titer archived serum samples using a dot blot and with the Western blot in a high titer serum sample. To investigate and validate the findings of seropositivity, we read IFA slides from those that seroconverted with a >1:256 titer (n = 13 including 9 with higher SFG or TG titers excluded from the primary clinical description) and also randomly selected an additional 30 to determine IFA titers. We found that the majority (9 of 13 convalescent samples) of those that seroconverted with the Karp strain also had a Gilliam strain IFA titer of >1:256. The median Gilliam IFA titers among the samples with seroconversion and Karp titers >1:256 was 512 (IQR 32–2,048; range 32–8,192). The Fort Portal participant D with molecularly confirmed *Orientia* spp. infection had an acute serum titer of 1:512 and convalescent serum titer 1:8,192 (16-fold titer change) with Gilliam strain IFA. Among those randomly selected, 2 of 8 acute samples and 1 of 7 convalescent samples with titers >1:256 also had Gilliam strain titers >1:256.

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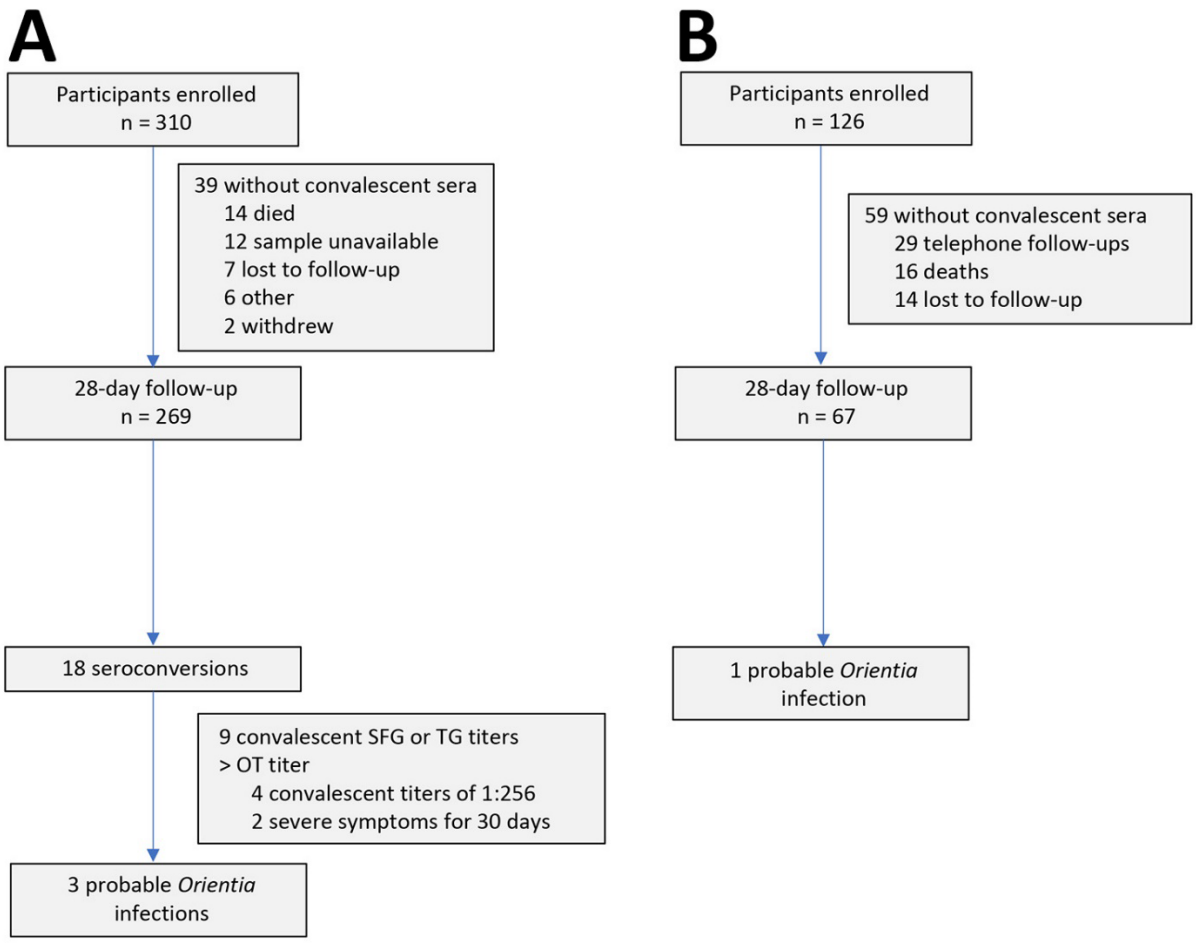
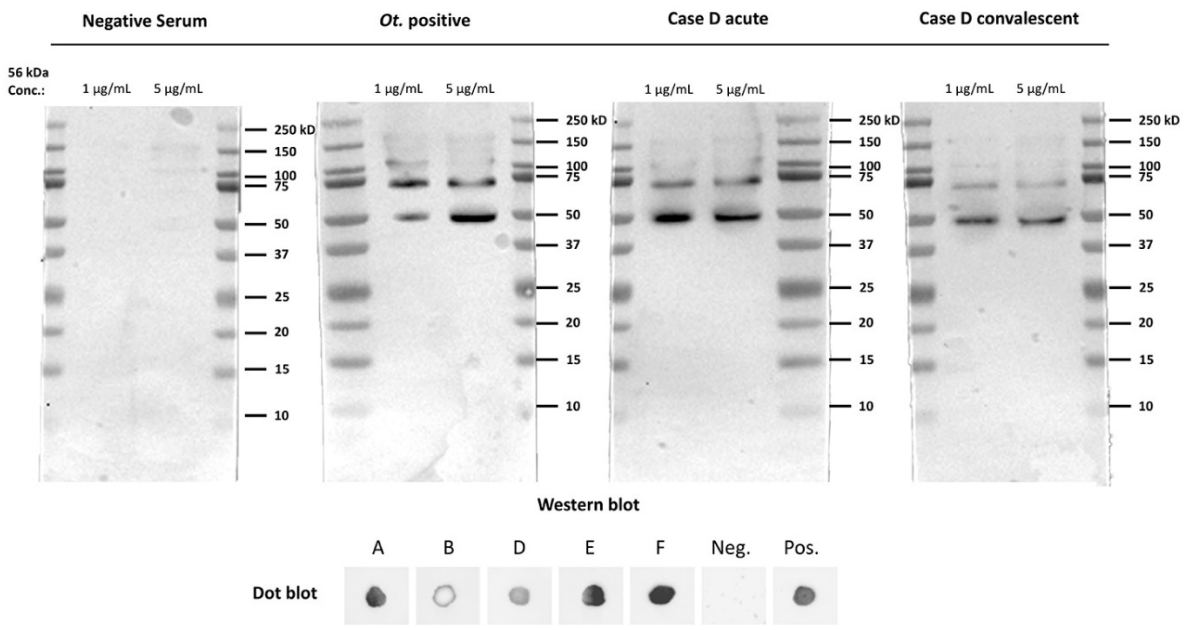


Appendix Figure 1. Flow diagram of patient enrollment and samples used from sepsis (Fort Portal) and acute febrile illness (Arua and Mubende) cohorts, Uganda.



Appendix Figure 2. Western blot identified evidence of a positive band at 56-kDa at two protein concentrations and case D's acute and convalescent serum samples matched those of a positive serologic *Orientia tstutugamushi* control. This was absent in an unexposed healthy serum control. Primary antibodies were diluted at 1:100,000. The dot blot showed evidence of antibody binding with convalescent sera (diluted at 1:1,000) from IFA seroconversion cases A, B, D, and high IFA IgG titer cases E and F. Neg, healthy control serum; Pos, scrub typhus IgG serum positive control.