Our biobank has a few limitations. First, we enrolled a convenience sample of children depending on the availability of study staff. However, in this study, the proportion of children with Lyme disease did not differ between enrolled and unenrolled but eligible patients. Second, some children with early or early-disseminated Lyme disease might have had false negative serologic results. However, we conducted follow-up to identify children who had initially negative 2-tier Lyme serologic results but tested positive within 30 days of enrollment. Finally, because our network includes only 8 enrollment sites, we were unable to include all regions to which Lyme disease is endemic.

Acknowledgments

We would like to thank patients and families who agreed to participate in the study.

This study received funding from Boston Children's Hospital and Global Lyme Alliance.

Interested collaborators should contact Pedi Lyme Net to discuss potential collaborations.

About the Author

Dr. Nigrovic is an associate professor of Pediatrics and Emergency Medicine at Harvard Medical School. Her research interests include the diagnosis of Lyme disease in children.

References

1. Hinckley AF, Connally NP, Meek JI, Johnson BJ, Kemperman MM, Feldman KA, et al. Lyme disease testing by large commercial laboratories in the United States. Clin Infect Dis. 2014;59:676–81. https://doi.org/10.1093/cid/ciu397
2. Wormser G-PP, Dattwyler R-JJ, Shapiro E-DD, Halperin JJ, Steere AC, Klempner MS, et al. The clinical assessment, treatment, and prevention of Lyme disease, human granulocytic anaplasmosis, and babesiosis: clinical practice guidelines by the Infectious Diseases Society of America. Clin Infect Dis. 2006;43:1089–134. https://doi.org/10.1086/508667
3. Branda JA, Body BA, Boyle J, Branson BM, Dattwyler RJ, Fikrig E, et al. Advances in serodiagnostic testing for Lyme disease are at hand. Clin Infect Dis. 2018;66:1133–9. https://doi.org/10.1093/cid/cix943
4. Garro A, Bennett J, Balamuth F, Levas MN, Neville D, Branda JC, et al.; Pedi Lyme Net. Positive 2-tiered Lyme disease serology is uncommon in asymptomatic children living in endemic areas of the United States. Pediatr Infect Dis J. 2019;38:e105–7. https://doi.org/10.1097/INF.0000000000002157
5. Molins CR, Sexton C, Young JW, Ashton LV, Pappert R, Beard CB, et al. Collection and characterization of samples for establishment of a serum repository for Lyme disease diagnostic test development and evaluation. J Clin Microbiol. 2014;52:3755–3762. https://doi.org/10.1128/JCM.01409-14
6. Horn EJ, Dempsey G, Schotthoefer AM, Prisco UL, McArdle M, Gervasi SS, et al. The Lyme Disease Biobank: characterization of 550 patient and control samples from the East Coast and Upper Midwest of the United States. J Clin Microbiol. 2020;58:1–12.
7. Boston Children's Hospital. Pedi Lyme Net. 2020 [cited 2020 Aug 3]. http://www.childrenshospital.org/research/centers-departmental-programs/Pedi-Lyme-Net
8. Centers for Disease Control and Prevention. Recommendations for test performance and interpretation from the Second National Conference on Serologic Diagnosis of Lyme Disease. MMWR Morb Mortal Wkly Rep. 1995;44:590–1.
9. Nowakowski J, Schwartz I, Liveris D, Wang G, Aguero-Rosenfeld ME, Girao G, et al.; Lyme Disease Study Group. Laboratory diagnostic techniques for patients with early Lyme disease associated with erythema migrans: a comparison of different techniques. Clin Infect Dis. 2001;33:2023–7. https://doi.org/10.1086/324490
10. Lantos PM, Lipsett SC, Nigrovic LE. False positive Lyme disease IgM immunoblots in children. J Pediatr. 2016;174:267–269.e1. https://doi.org/10.1016/j.jpeds.2016.04.004

Address for correspondence: Lise Nigrovic, Division of Emergency Medicine, Boston Children's Hospital, 300 Longwood Avenue, Boston, MA 02115, USA; email: lise.nigrovic@childrens.harvard.edu

Transmission Electron Microscopy Confirmation of *Orientia tsutsugamushi* in Human Bile

Yujeong Lee,[1] Seung Il Kim,[1] Yoon-sun Yi, Hayoung Lee, Joo-Hee Hwang, Edmond Changkyun Park, Sangmi Jun,[2] Chang-Seop Lee[2]

Author affiliations: Korea Basic Science Institute, Cheongju, South Korea (Y. Lee, S.I. Kim, Y.-s. Yi, H. Lee, E.C. Park, S. Jun); Korea Research Institute of Chemical Technology, Daejeon, South Korea (Y. Lee, S.I. Kim, E.C. Park, S. Jun); University of Science and Technology, Daejeon (S.I. Kim, H. Lee, E.C. Park); Jeonbuk National University, Jeonju, South Korea (J.-H. Hwang, C.-S. Lee); Jeonbuk National University Hospital, Jeonju (J.-H. Hwang, C.-S. Lee)

[1]These authors contributed equally to this article.

[2]These authors were co-principal investigators.

Scrub typhus, the third most frequently reported infectious disease in South Korea, causes serious public health problems. In 2019, we collected a bile specimen from a patient with scrub typhus through percutaneous transhepatic gallbladder drainage and performed transmission electron microscopy to confirm the ultrastructure of *Orientia tsutsugamushi*.

Orientia tsutsugamushi is a gram-negative obligately intracellular coccobacillus and a causative pathogen of scrub typhus, which is transmitted to humans by bites from trombiculid (chigger) mites (*1*). Scrub typhus is a prevalent acute febrile disease that mainly occurs in the Asia-Pacific region, infecting ≈1 million persons worldwide each year. In South Korea, infections have increased rapidly since 2014 because of climate change, increased outdoor activities, and numbers of elderly farmers (*2*). The most typical clinical manifestation of scrub typhus is an eschar at the site of the bite (Figure, panel A); symptoms include fever, headache, muscle pain, nausea, and vomiting (*3*). Without proper diagnosis and antimicrobial drug treatment, severe illness with multiple organ system involvement can occur; the death rate is ≈10% (*4*). Immunohistochemical staining for *O. tsutsugamushi* antigens have revealed extensive endothelial cell infection in the heart, lung, kidney, pancreas, skin, and brain (*5*). Bacteria also have been detected in cardiac muscle cells and in macrophages in the liver and spleen (*5*,*6*).

In humans, the liver secretes ≈1 L of bile daily into the intestinal tract. However, little information is available about the presence of gram-negative bacteria in bile (*7*). Pathogenic microorganisms must endure potential impediments, such as variations in pH, low oxygen levels, nutrient limitation, and elevated osmolarity, to survive in this harsh environment (*7*). We collected bile from a patient with scrub typhus in South Korea (Figure, panel B) and visualized the ultrastructure of *O. tsutsugamushi* in the clinical sample using transmission electron microscopy.

In 2019, a 68-year-old woman reported fever, drowsy mental state, abdominal pain, and reduced oral intake. These symptoms had begun 7 days earlier. Her vital signs were blood pressure 100/60 mm Hg and body temperature 38.9°C. Laboratory analysis revealed a leukocyte count 10,350/mL (reference range 4,800–10,800/mL), platelet count 45,000/mL (reference 130,000–450,000/mL), serum creatinine 0.4 mg/dL (reference 0.7–1.7 mg/dL), aspartate aminotransferase 31 IU/L (reference 12–33 IU/L), alanine aminotransferase 41 IU/L (reference 5–35 IU/L), total bilirubin 1.72 mg/dL (reference 0.2–1.2 mg/dL), and C-reactive protein 196.86 mg/L (reference <5 mg/L). Abdominal computed tomography scan detected acute cholecystitis, and percutaneous transhepatic gallbladder drainage was performed. The presence of acute cholecystitis in scrub typhus cases is rare (5 [1.1%] instances of 442 cases) (*8*). *O. tsutsugamushi* can

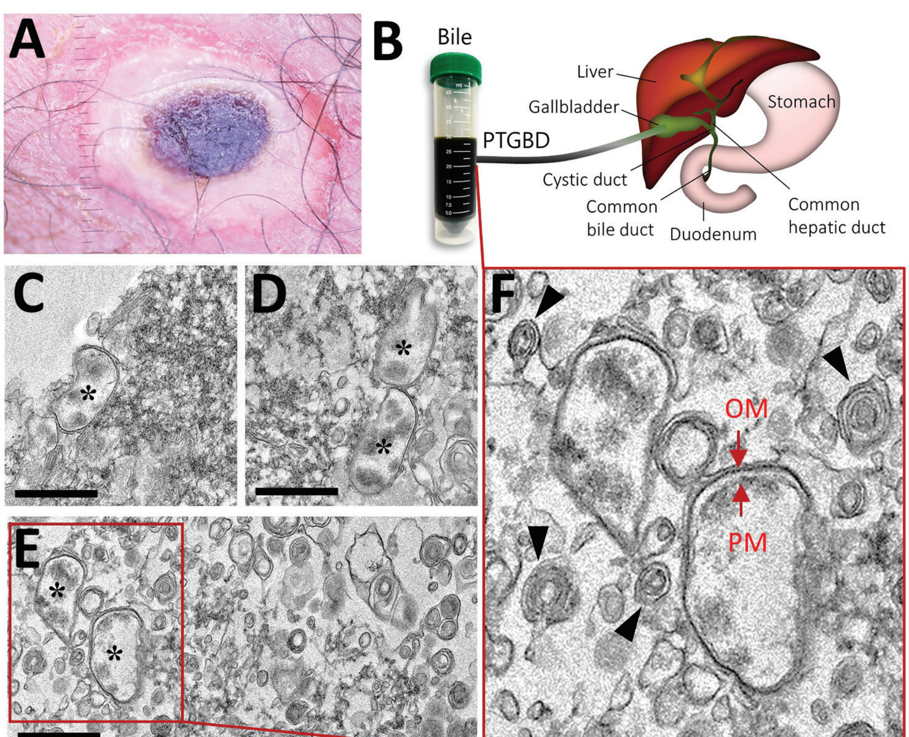

Figure. Findings from a 68-year-old woman with scrub typhus, South Korea, 2019. A) Eschar in the right inguinal area. B) Human bile collected through percutaneous transhepatic gallbladder drainage in the gallbladder of a patient affected with scrub typhus. C–F) Transmission electron microscopy images of *Orientia tsutsugamushi* in the bile. Bacteria (black asterisks); outer membrane (OM) and plasma membrane (PM) (red arrows); multilamellar body (black arrowheads); Scale bars indicate 1 μm.

also cause liver injury in some patients, but the patient reported here did not have any such signs (*9*). We confirmed scrub typhus using indirect immunofluorescence assay (IgG 5,120) and nested PCR selective for the 56-kDa gene of *O. tsutsugamushi* (Appendix, https://wwwnc.cdc.gov/EID/article/26/12/20-2188-App1.pdf). The *O. tsutsugamushi* identified belonged to the Boryong strain (the most common strain in South Korea). We also detected an eschar in the right inguinal area (Figure, panel A). The patient completely recovered after doxycycline treatment.

A drainage tube was placed in the patient's gallbladder, and the bile was directly discharged and collected through the tube (Figure, panel B). We tested the bile specimen for pathogens using nested quantitative reverse transcription PCR and DNA sequencing to detect a specific *O. tsutsugamushi* gene encoding a 56-kDa protein (Appendix) (*10*). After chemical fixation, the sample was embedded in 100% Epon 812 resin and ultrathin (≈80-nm thick) sections were stained with 2% uranyl acetate and 1% lead citrate (Appendix) (*10*). This sample preparation method might not preserve the ultrastructure of live bacteria, but structural features of the bacteria can be clearly observed. The ultrastructural details were acquired using transmission electron microscopy at 120 kV. Despite the presence of a wide variety of components, we detected *O. tsutsugamushi* in the bile (asterisks in Figure, panels C–E). The bacteria showed a coccobacillus shape and were 0.5–0.7-μm in diameter and 1.2–2.5-μm long, all typical features of *O. tsutsugamushi* (*5*,*10*). The bacterial cytoplasm was surrounded by an outer membrane, an internal plasma membrane, and a peptidoglycan layer (Figure, panel F). Moreover, the periplasmic space appeared as an electron-lucent gap between the 2 membranes. We also observed a thicker outer leaflet of the cell wall membrane, which is a typical and diagnostic sign of *Orientia* (*5*). We also detected multilamellar bodies, which are cholesterol-carrying particles, in the bile sample (black arrowheads in Figure, panel F).

Previously, human scrub typhus disease was studied using a mouse model mimicking the disease and examining clinical samples postmortem (*5*,*6*). However, the host cell of *O. tsutsugamushi* in humans has not been completely defined. In this study, we confirmed detection of *O. tsutsugamushi* in human bile, an environment in which bacterial survival is challenging. This observation (i.e., the presence of *O. tsutsugamushi* in human bile) might be useful for diagnosing scrub typhus in patients who do not show clear eschars or skin rash, broadening the potential routes for diagnosing the disease.

This work was supported by the Basic Science Research Programs (NRF-2015R1D1A1A01060251 and 2018R1D-1A3B07049557) of the National Research Foundation of Korea, which is funded by the Ministry of Education; the Korea Basic Science Institute grant (C030221), and National Research Council of Science & Technology grant by the Korea government (CRC-16-01-KRICT).

About the Authors

Ms. Y. Lee is a researcher at the Korea Basic Science Institute. Her primary interests are electron microscopy of infectious viral and bacterial diseases. Dr. Kim is a senior researcher at the Korea Basic Science Institute. His research interests are in the proteomics of infectious diseases.

References

1. Liu YX, Cao WC, Gao Y, Zhang JL, Yang ZQ, Zhao ZT, et al. *Orientia tsutsugamushi* in eschars from scrub typhus patients. Emerg Infect Dis. 2006;12:1109–12. https://doi.org/10.3201/eid1207.050827
2. Kim DM. Clinical features and diagnosis of scrub typhus. Infect Chemother. 2009;41:315–22. https://doi.org/10.3947/ic.2009.41.6.315
3. Lee CS, Hwang JH. Images in clinical medicine. Scrub syphus. N Engl J Med. 2015;373:2455. https://doi.org/10.1056/NEJMicm1503639
4. VieBrock L, Evans SM, Beyer AR, Larson CL, Beare PA, Ge H, et al. *Orientia tsutsugamushi* ankyrin repeat-containing protein family members are type 1 secretion system substrates that traffic to the host cell endoplasmic reticulum. Front Cell Infect Microbiol. 2015;4:186. https://doi.org/10.3389/fcimb.2014.00186
5. Moron CG, Popov VL, Feng HM, Wear D, Walker DH. Identification of the target cells of *Orientia tsutsugamushi* in human cases of scrub typhus. Mod Pathol. 2001;14:752–9. https://doi.org/10.1038/modpathol.3880385
6. Shelite TR, Saito TB, Mendell NL, Gong B, Xu G, Soong L, et al. Hematogenously disseminated *Orientia tsutsugamushi*–infected murine model of scrub typhus [corrected]. Erratum in: PLoS Negl Trop Dis. 2014;8:e3175. PLoS Negl Trop Dis. 2014;8:e2966. https://doi.org/10.1371/journal.pntd.0002966
7. Begley M, Gahan CG, Hill C. The interaction between bacteria and bile. FEMS Microbiol Rev. 2005;29:625–51. https://doi.org/10.1016/j.femsre.2004.09.003
8. Lee H, Ji M, Hwang JH, Lee JY, Lee JH, Chung KM, et al. Acute cholecystitis in patients with scrub typhus. J Korean Med Sci. 2015;30:1698–700. https://doi.org/10.3346/jkms.2015.30.11.1698
9. Chung JH, Lim SC, Yun NR, Shin SH, Kim CM, Kim DM. Scrub typhus hepatitis c onfirmed by immunohistochemical staining. World J Gastroenterol. 2012;18:5138–41. https://doi.org/10.3748/wjg.v18.i36.5138
10. Ro HJ, Lee H, Park EC, Lee CS, Il Kim S, Jun S. Ultrastructural visualization of *Orientia tsutsugamushi* in biopsied eschars and monocytes from scrub typhus patients in South Korea. Sci Rep. 2018;8:17373. https://doi.org/10.1038/s41598-018-35775-9

Address for correspondence: Sangmi Jun, Center for Research Equipment, Korea Basic Science Institute, 162 Yeongudanji-ro, Ochang-eup, Cheongwon-gu, Cheongju-si, Chungcheongbuk-do, 28119, South Korea; email: smjun@kbsi.re.kr