

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

Appendix

Genotyping by DNA Amplification and Sequencing

Peripheral blood mononuclear cells collected from acute-phase blood samples of patients with scrub typhus were purified using a QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. Nested PCR was performed. Primers 34 (forward, 5'-TCA AGC TTA TTG CTA GTG CAA TGT CTGC-3'; the 56-kDa gene based on the Gilliam strain) and 55 (5'-AGG GAT CCC TGC TGT GCT TGC TGCG-3') were used in the first PCR. Nested PCR primers 10 (5'-GAT CAA GCT TCC TCA GCC TAC TAT AAT GCC-3') and 11 (5'-CTA GGG ATC CCG ACA GAT GCA CTA TTA GGC-3') were used in the second PCR amplification to generate a 483-bp fragment. Nested PCR was performed as described previously by Kim et al. (1). The amplified PCR products were confirmed using 1.2% agarose gel electrophoresis, purified with a QIAquick gel extraction kit (QIAGEN) and sent to COSMO Genetech (Seoul, South Korea) for sequencing.

Identification of 56-kDa Gene by DNA Amplification and Sequencing Using Bile-Derived DNA

Total genomic DNA was extracted from the bile using a QIAamp DNA Mini Kit (QIAGEN), as previously described (2,3). The 56-kDa gene of *O. tsutsugamushi* was amplified using nested PCR, as previously described (4). Briefly, initial rounds of amplifications were performed using touchdown PCR, and products from the initial amplification were used as the template in the second PCR reaction to generate an 83-bp fragment. Nested PCR was performed using Solg 2X Multiplex PCR Smart mix (Solgent, Daejeon, South Korea). Levels of the 56-kDa gene were normalized using the human *Gapdh* (*hGapdh*) gene. Information on the primers used in this study is summarized in the Appendix Table. The final PCR products were visualized with a chemiDoc XRS+ system (Bio-Rad,

Hercules, CA, USA) (Appendix Figure 1) and sent to COSMO Genetech for sequencing. The sequencing results were aligned with the original sequence of the 56-kDa gene using Clustal Omega, which revealed the 56-kDa gene in bile-derived DNA (Appendix Figure 2).

Transmission Election Microscopy (TEM)

For ultrastructural analysis of ultrathin sections, collected human bile was pelleted via centrifugation and resuspended in 0.1% glutaraldehyde solution and 4% paraformaldehyde, 3.5% sucrose in phosphate buffer (0.1 M, pH 7.4) for 2 h at 4°C. The samples were washed in phosphate buffer, and then post-fixed in 1% osmium tetroxide (Electron Microscopy Sciences, Hatfield, PA, USA) in phosphate buffer (0.1 M, pH 7.4) for 2 h at 4°C. Samples were dehydrated in a graded ethanol series, followed by propylene oxide, and then progressively infiltrated with a 2:1, 1:1, and 1:2 mixture of propylene oxide and Epon 812 resin (Electron Microscopy Sciences). Samples finally embedded in 100% Epon 812 resin and polymerized at 70°C for 24 h. Ultrathin plastic sections (80-nm thick) were cut at room temperature using a Leica EM UC6 ultramicrotome (Leica Microsystems GmbH, Wetzlar, Germany) and collected on 200-mesh carbon-coated grids. The grids were post-stained with 2% aqueous uranyl acetate and 1% lead citrate at room temperature for 15 and 5 min, respectively. A FEI Tecnai G2 Spirit Twin 120 KV TEM (FEI Company, Hillsboro, OR, USA) was used for TEM analysis (4,5)

References

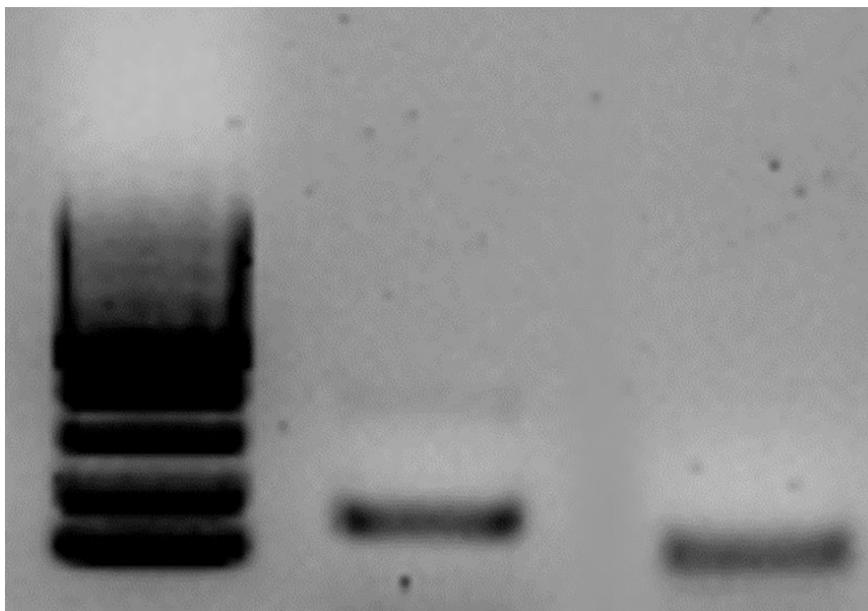
1. Lee YM, Kim DM, Lee SH, Jang MS, Neupane GP. Phylogenetic analysis of the 56 kDa protein genes of *Orientia tsutsugamushi* in southwest area of Korea. Am J Trop Med Hyg. 2011;84:250–4. [PubMed https://doi.org/10.4269/ajtmh.2011.09-0601](https://doi.org/10.4269/ajtmh.2011.09-0601)
2. Neri V, Margiotta M, de Francesco V, Ambrosi A, Valle ND, Fersini A, et al. DNA sequences and proteic antigens of *H. pylori* in cholecystic bile and tissue of patients with gallstones. Aliment Pharmacol Ther. 2005;22:715–20. [PubMed https://doi.org/10.1111/j.1365-2036.2005.02644.x](https://doi.org/10.1111/j.1365-2036.2005.02644.x)
3. Lee JW, Lee DH, Lee JI, Jeong S, Kwon KS, Kim HG, et al. Identification of *Helicobacter pylori* in gallstone, bile, and other hepatobiliary tissues of patients with cholecystitis. Gut Liver. 2010;4:60–7. [PubMed https://doi.org/10.5009/gnl.2010.4.1.60](https://doi.org/10.5009/gnl.2010.4.1.60)
4. 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. [PubMed https://doi.org/10.1038/s41598-018-35775-9](https://doi.org/10.1038/s41598-018-35775-9)

5. Goldsmith CS, Miller SE. Modern uses of electron microscopy for detection of viruses. Clin Microbiol Rev. 2009;22:552–63. [PubMed https://doi.org/10.1128/CMR.00027-09](https://doi.org/10.1128/CMR.00027-09)

Appendix Table. List of primers used in the study

Primer name	Sequence
c56k F	5'-AGCTGATCGTGACTTTGGGATT-3'
c56k R	5'-AGCATTTGATAATGCAGCAAGACC-3'
rt56k F	5'-CCTAACATACCTCAGGCGCA-3'
rt56k R	5'-AACCAAGCGATCCTAGCTGC-3'
Human GAPDH F	5'-CGGGAAACTGTGGCGTGATG-3'
Human GAPDH R	5'-ATGACCTTGCCACAGCCTT-3'

*F, forward; R, reverse.



Appendix Figure 1. PCR results in bile-derived DNA of *Orientia tsutsugamushi*. Each lane indicates the ladder and the amplification product obtained using primer pairs for *hGapdh* and *56 kDa*, respectively

CLUSTAL O(1.2.4) multiple sequence alignment

```

PCR_product      CCTAACATACCTCAGGCGCAAGCACAAGCGGCGCGCCTCAGCTAATATGATGAGCACGT      60
56kDa            CCTAACATACCTCAGGCGCAAGCGCAAGCTGCACAGCCTCCGCTTAATGATCAGAAGCGT      60
*****          *****  ** * *****  *** *          ***

PCR_product      GCTGCAGCTAGGATCGCTTGGTTA      84
56kDa            GCTGCAGCTAGGATCGCTTGGTT-      83
*****

```

Appendix Figure 2. Sequence alignment for *Orientia tsutsugamushi*.