Isolation of *Orientia tsutsugamushi* from Bat-Associated Chiggers, Japan

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We isolated *Orientia tsutsugamushi* bacteria from bat-associated *Whartonia prima* chiggers collected from *Hipposideros turpis* bats on Yonaguni Island, Japan. Serial passage, microscopic examination, growth kinetics, and multilocus sequence analysis confirmed bacterial propagation and identity. Our findings highlight an overlooked bat–chigger interface with potential relevance to broader ecologic surveillance of scrub typhus.

Scrub typhus, or tsutsugamushi disease, is a vectorborne zoonosis caused by *Orientia tsutsugamushi* bacteria. *O. tsutsugamushi* is transmitted by larval trombiculid mites, or chiggers, and is primarily maintained within chigger populations through transovarial and transstadial transmission (*1*). Ecologic studies of scrub typhus have largely focused on rodent-associated *Leptotrombidium* chiggers, which are the principal vectors in many endemic areas (*1*). In Japan, where ≈300–500 scrub typhus cases are reported annually (*2*), the ecologic diversity of *O. tsutsugamushi* in wildlife-associated chiggers remains poorly understood.

Recent molecular detection of *O. tsutsugamushi* DNA in bats from the China–Myanmar border raised questions about the roles of bats and their ectoparasites in *Orientia* ecology (*3*). Bat-associated chiggers are diverse and widely distributed (*4*), but their involvement in *Orientia* ecology remains limited. We report the isolation and propagation of *O. tsutsugamushi* from *Whartonia prima* chiggers collected on Yonaguni Island, Japan, from *Hipposideros turpis*, a cave-dwelling insectivorous bat endemic to the Yaeyama Islands (*5*).

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The Study

We collected larval chiggers from *H. turpis* bats on Yonaguni Island in September 2024 and 2025. Bat capture and handling were approved by the Ministry of the Environment, Tokyo, Japan (permission nos. 2402141 and 2502282), and complied with relevant institutional and national guidelines. We examined a total of 80 *H. turpis* bats, yielding 212 chiggers: 31 in 2024 and 181 in 2025. We identified all chigger mites morphologically as *W. prima* by using taxonomic descriptions and keys (*6*,*7*). We used the chigger mites collected in 2024 for molecular screening. Of the 181 chigger mites we collected in 2025, we used 37 mites for molecular screening, 129 mites for isolation attempts, and 4 mites for scanning electron microscopy; we excluded 11 mites because of poor specimen condition.

We tested 68 chigger mites individually for *O. tsutsugamushi* by using real-time PCR targeting the 16S rRNA gene (*8*) and nested PCR targeting the 56-kDa type-specific antigen (TSA56) gene (*9*). We sequenced amplicons from all nested PCR-positive chigger mite samples, revealing 2 partial TSA56 genotypes. We amplified full-length *O. tsutsugamushi* TSA56 from 10 mite samples representing both partial genotypes by using previously described primers (Appendix, https://wwwnc.cdc.gov/EID/article/32/12/26-1156-App1.pdf) (*10*). Of the 68 chigger mites tested, 64 (94%, 95% CI 86%–98%) were positive for *O. tsutsugamushi* by real-time PCR, and 55 (81%, 95% CI 70%–89%) were positive for *O. tsutsugamushi* by nested PCR. We obtained full-length TSA56 sequences from 2 of the 10 selected chigger mite samples; both clustered within the Karp-related lineage (Figure 1, panel A).

For isolation, we pooled the 129 *W. prima* chiggers collected in 2025 by individual bat host. Each pool consisted of 2–5 chiggers from the same bat and was homogenized in sucrose–phosphate–glutamate buffer. We inoculated the homogenates onto L929 cells

maintained in Leibovitz's L-15 medium supplemented with 2% fetal bovine serum without antimicrobials. We passaged the cultures every 2 weeks and monitored *O. tsutsugamushi* DNA by using real-time PCR at each passage. We maintained uninfected L929 cell controls in parallel that remained negative for *O. tsutsugamushi*. We discarded contaminated cultures immediately. Of 28 chigger pools, 4 pools yielded *O. tsutsugamushi* isolates: Donan 18-3R (3 chiggers), Donan 17-2L (2 chiggers), Donan 14-1L (5 chiggers), and Donan 14-2R (5 chiggers). Three cultures remained negative, and the remainder were excluded because of contamination. We maintained all 4 isolates through 4 passages and stored fourth-passage infected cell suspensions in sucrose–phosphate–glutamate buffer.

We generated full-length TSA56 gene sequences for 2 *O. tsutsugamushi* isolates, Donan 18-3R and Donan 17-2L, and compared them with sequences obtained directly from selected PCR-positive *W. prima* chiggers (GenBank accession nos. LC896643, LC896644, LC939506, and LC939507) (Figure 1, panel A). Partial TSA56 sequences from the remaining *O. tsutsugamushi* isolates, Donan 14-1L and Donan 14-2R (accession nos. LC939530 and LC939531), were identical to the corresponding regions of the full-length sequences from Donan 18-3R and Donan 17-2L. We performed multilocus sequence analysis (MLSA) on the basis of 11 housekeeping genes for 10 chigger samples selected for full-length TSA56 analysis and 2 *O. tsutsugamushi* isolates, Donan 18-3R and Donan 17-2L, by using primers described previously (*11*) (accession nos. LC897744–55 and LC939508–29). The concatenated MLSA sequences of Donan 18-3R and Donan 17-2L were identical. The *O. tsutsugamushi*

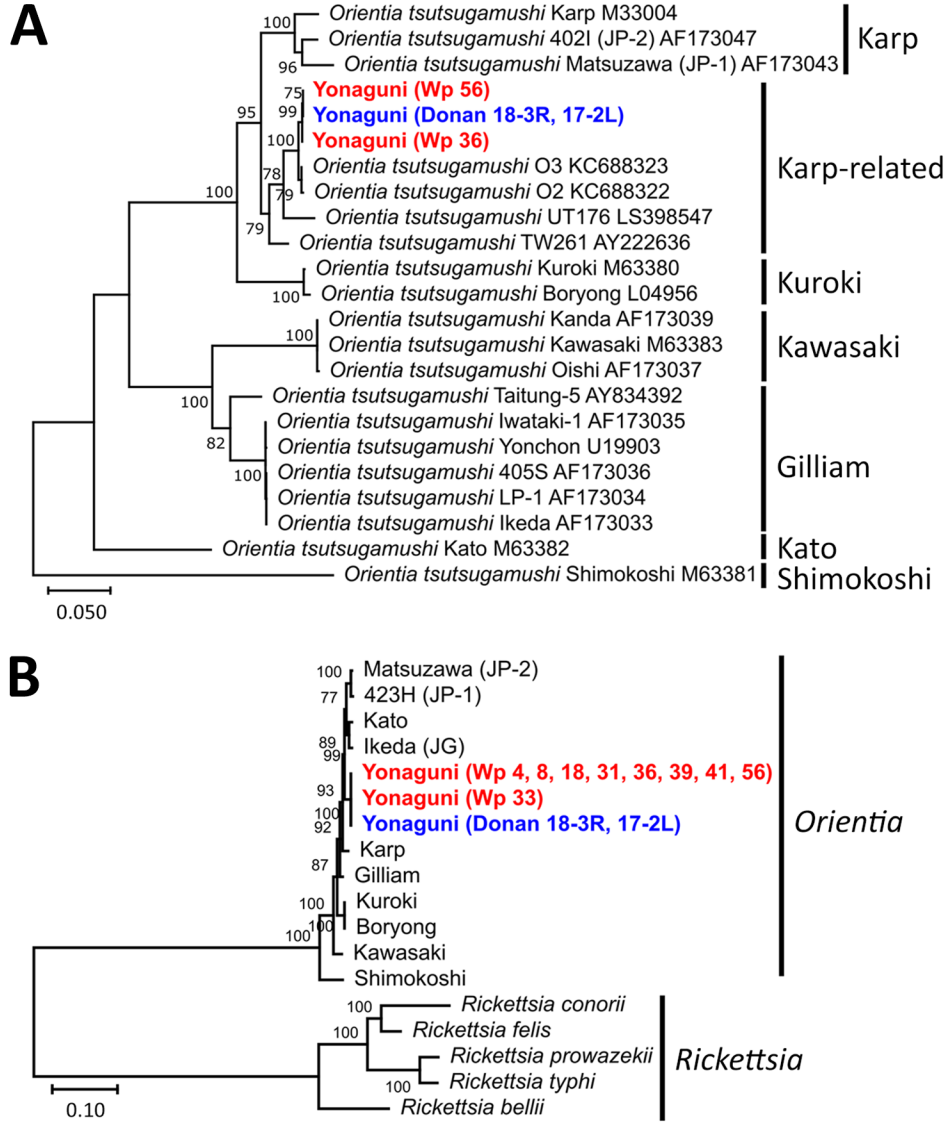


Figure 1. Phylogenetic analyses of *Orientia tsutsugamushi* detected and isolated from bat-associated *Whartonia prima* chiggers, Yonaguni Island, Japan. A) Maximum-likelihood tree on the basis of full-length 56-kDa type-specific antigen gene sequences from 2 *O. tsutsugamushi* isolates, Donan 18-3R and Donan 17-2L, and selected PCR-positive chigger specimens. B) Maximum-likelihood tree on the basis of concatenated sequences of 11 housekeeping genes from 2 *O. tsutsugamushi* isolates, Donan 18-3R and Donan 17-2L, and selected PCR-positive chigger specimens. Bootstrap values were calculated from 1,000 replicates. Blue indicates isolates obtained in this study and red indicates sequences obtained directly from selected PCR-positive *W. prima* chiggers. Scale bars indicate the number of nucleotide substitutions per site. JG, Japanese Gilliam; JP, Japanese Karp; Wp, prefix used for DNA sample identifiers from *Whartonia prima* chiggers.

isolate sequences and sequences obtained directly from PCR-positive chigger samples clustered within the *O. tsutsugamushi* clade in MLSA, supporting their identification as *O. tsutsugamushi* (Figure 1, panel B).

To characterize a representative isolate, we inoculated fourth-passage Donan 18-3R onto fresh L929 cells. We identified numerous punctate, basophilic structures in the cytoplasm outside the nucleus from Giemsa-stained cells 5 days postinoculation (dpi), a pattern consistent with intracellular *O. tsutsugamushi* (Figure 2). For growth analysis, we inoculated fourth-passage Donan 18-3R onto L929 cells in 24-well plates. We extracted DNA daily from 1 well and calculated bacterial copy numbers by using quantitative PCR, which we independently performed 3 times. After an initial decrease at 1 dpi, mean ±SD bacterial loads increased from 4.19 ±0.21 $\log_{10}$ copies/well at 1 dpi to 6.93 ±0.59 $\log_{10}$ copies/well at 8 dpi, demonstrating growth of Donan 18-3R in L929 cells (Figure 3).

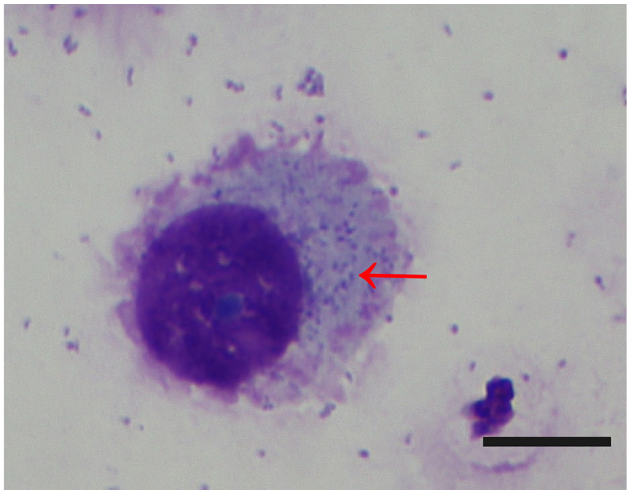


Figure 2. Microscopy of *Orientia tsutsugamushi* isolate Donan 18-3R detected and isolated from bat-associated *Whartonia prima* chiggers, Yonaguni Island, Japan. Giemsa-stained L929 cells 5 days after inoculation with fourth-passage Donan 18-3R, shows punctate, basophilic structures in the cytoplasm outside the nucleus, consistent with intracellular *O. tsutsugamushi*; the red arrow indicates representative intracellular bacteria. Oil immersion; scale bar represents 10 μm.

Conclusions

We provided evidence that bat-associated *W. prima* chiggers can harbor viable *O. tsutsugamushi*, demonstrated by isolation, serial propagation, microscopic examination, and growth kinetics. The *O. tsutsugamushi* isolates clustered phylogenetically within the Karp-related lineage.

Although vector competence, vertebrate host involvement, and human exposure were not demonstrated, isolation of viable *O. tsutsugamushi* from bat-associated chiggers demonstrates bacterial viability, which cannot be inferred from molecular detection alone. In the Miyako Islands of the southern Ryukyu Archipelago, Japan, *Leptotrombidium deliense* mites are implicated in the transmission of *O. tsutsugamushi* causing human scrub typhus (*12*). A Karp-related strain was reported from a human scrub typhus case on Ikema Island in the Miyako Islands, Japan, although it was not predominant among genotyped cases (*12*). In contrast, *O. tsutsugamushi* was not reported previously in the literature from animals on Yonaguni Island or elsewhere in the Yaeyama Islands, Japan, and no human scrub typhus cases have been reported from that region to our knowledge. We identified a previously unrecognized ecologic setting for *O. tsutsugamushi* in the southern Ryukyu Archipelago, Japan, suggesting more diverse regional maintenance.

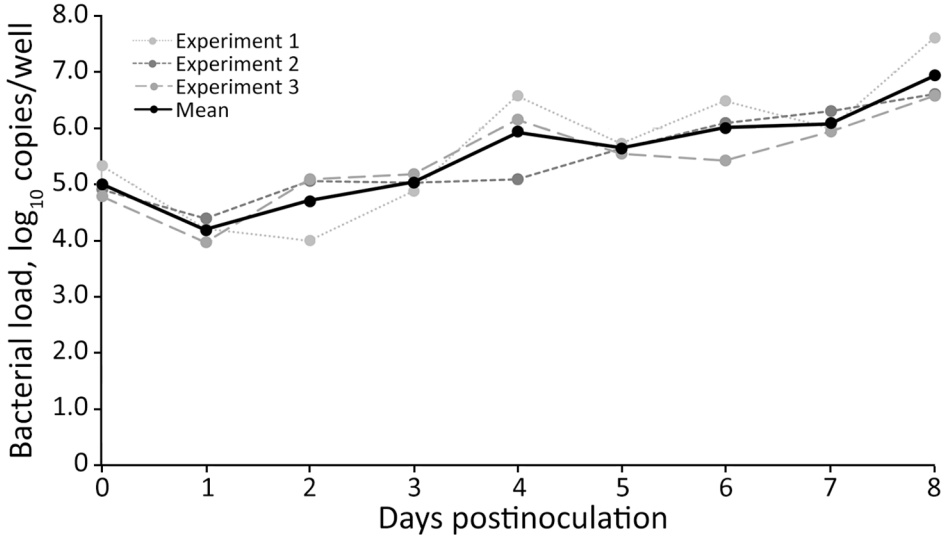


Figure 3. Growth kinetics of *Orientia tsutsugamushi* isolate Donan 18-3R detected and isolated from bat-associated *Whartonia prima* chiggers, Yonaguni Island, Japan. Growth kinetics were assessed by quantitative PCR and expressed as $\log_{10}$ bacterial copies/well after inoculation of fourth-passage bacteria onto L929 cells in 24-well plates. Three independent growth experiments were performed.

W. prima chiggers have been reported from several bat species across East and Southeast Asia (*4*,*13*). However, *W. prima* chigger strict host specificity has not been established, and knowledge of this species' role in *O. tsutsugamushi* transmission to vertebrates remains limited. The broad geographic distribution of *W. prima* chiggers suggests similar bat-associated chigger systems might occur elsewhere. Together with recent detection of *O. tsutsugamushi* DNA in bats from the China–Myanmar border (*3*), our findings raise the possibility that bat–ectoparasite interfaces represent an underrecognized component of *Orientia* ecology. Additional studies are needed to clarify bacterial localization in chiggers, vector competence, vertebrate host involvement, human exposure, and public health relevance. Because the *W. prima* chigger larvae examined in this study were collected from bats, detection and preferably isolation of *O. tsutsugamushi* from unfed *W. prima* larvae will be needed to confirm transovarial transmission. Our findings expand the known ecologic settings of viable *O. tsutsugamushi* and highlight bat–chigger interfaces as an underrecognized component of *Orientia* ecology.

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An AI tool (ChatGPT-5.5; OpenAI, https://openai.com) was used solely to assist with language polishing. The authors reviewed and edited the AI-assisted text and take full responsibility for the final content.

About the Author

Dr. Qiu is an associate professor at Kitasato University, Towada, Japan. His research focuses on the diversity and maintenance mechanisms of vector-borne bacterial pathogens, including *Borrelia*, *Orientia*, and *Rickettsia*, in natural ecosystems.

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