Mixed genotypes of *Orientia tsutsugamushi* in conserved genes and a single immune-dominant *tsa56* genotype discovered from a patient with scrub typhus in Hainan Island, China: a case report

Chuanning Tang1,2,3,4†, Liyuan Zhang1,5†, Yi Huang1,2,3,4†, Wenhui Mai6†, Liying Xue1,2,4, Gaoyu Wang1,2,3,4, Shu Wen7, Ruoyuan Peng1,2,3,4, Kunliang Wu1,5, Xiuying Tian1,2,4, Hua Pei5, Jiang Du1,2,3,4, Kwok-Yung Yuen4,8,9, Jasper Fuk-Woo Chan7,8†, Yongguo Du1,5† and Feifei Yin1,2,3,4*†

Abstract

**Background:** *Orientia tsutsugamushi* (*O. tsutsugamushi*), an obligate intracellular bacterium, is transmitted to humans through infected larval trombiculid mite bites, causing scrub typhus. Mixed genotypes of *O. tsutsugamushi* in canonical conserved genes were reported in 8–25% of blood samples from patients. Yet, there are few clinical descriptions of these mixed *O. tsutsugamushi*-infected patients.

**Case presentation:** We report a patient with scrub typhus complicated with pulmonary involvement and hepatic dysfunction, who carried mixed genotypes of the conserved genes but had a single immune-dominant 56-kDa type-specific antigen (*tsa56*) genotype. The patient was successfully recovered by doxycycline treatment.

**Conclusions:** In this reported case, both patient’s eschar and blood samples have repeatedly shown the same results, i.e., no variants were discovered in *tsa56* gene that bears multiple hypervariable regions. Whereas the selected highly conserved genes were identified with up to 32 variants in a 2700 base-pair concatenated sequence. The prevalence, disease severity and mechanism of these single-*tsa56*-genotype mixed infections remain to be investigated on a large scale with more cases.

**Keywords:** Scrub typhus, *Orientia tsutsugamushi*, Mixed-infections, *tsa56*, Multi-locus sequence typing

© The Author(s) 2022. Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated in a credit line to the data.
of co-infections with mixed tsa56 genotypes have been reported in patients [3–7]. These findings have suggested a rather distinct inconsistency in the discovery rates of mixed genotypes O. tsutsugamushi infections between the conserved genes and the 56-kDa type-specific antigen (tsa56) gene of O. tsutsugamushi. Consequently, this inconsistency limits our understanding of O. tsutsugamushi co-infections. In addition, clinical characteristics of O. tsutsugamushi mixed-infections of conserved genes have not been described in details. Here, we report a patient with scrub typhus who was infected by an unreported mixed-infection, i.e., mixed genotypes being discovered in the highly conserved genes’ region, including several novel genotypes, but only being shown one tsa56 genotype of the O. tsutsugamushi strains. Both patient’s eschar and blood samples have shown the same results, suggesting consistency of evaluations on the discovery and the O. tsutsugamushi strains originated from the carrying host.

Case presentation
A sixty-year-old male farmer, residing on the northern coast of Hainan Island, was admitted to the Second Affiliated Hospital of Hainan Medical University on December 8, 2018. He presented with a high fever (38.4–40.0 °C), chills, headache, myalgia, and cough for six days. A typical 5 × 5 mm eschar was observed on the lateral side of the left waist (Fig. 1A). Laboratory examination showed normal white blood cell level (9.6 × 10⁹ /L) and elevated levels of lactate dehydrogenase (266 U/L), procalcitonin (0.46 ng/ml), C-reactive protein (100.23 mg/L), alanine aminotransferase (60 U/L), gamma-glutamyl transferase (238 U/L), and alkaline phosphatase (168 U/L). A CT scan showed multilobar interstitial infiltrates and a small left pleural effusion. Rapid immunochromatographic scan showed multilobar interstitial infiltrates and a small right pleural effusion.

Patient’s DNA samples were isolated from both venous blood and eschar swabs collected on the day of his admission. Nested polymerase chain reaction (PCR) was then performed to amplify the hypervariable regions of the tsa56 gene using primers described previously [8]. Primers 34 and 55 were used in the first round of PCR with 2 μl DNA template and primers 10 and 11 were used in the second PCR amplification to generate a 408–453 base-pair fragment with 2 μl of the product from the first round as the template. A high-fidelity DNA polymerase (KOD One PCR Master Mix, TOYOBO) was used in all PCR steps in this report. The thermal cycling conditions for both PCRs were 98 °C for 5 min, followed by 30 cycles of 98 °C for 10 s, 58 °C for 5 s, 68 °C for 5 s, and a final elongation step at 68 °C for 1 min. The nested PCR products were sequenced via Sanger sequencing. Its results revealed that the target sequences of blood and eschar samples were identical (GenBank accession no. MZ856313). Sequencing results were also found to be related to the reference O. tsutsugamushi Karp genotype (GenBank accession no. LS398548.1) with 99.33% identity at the nucleotide level, and were completely identical to a Karp genotype strain reported in Taiwan (GenBank accession no. MW464199.1). To avoid the sequence variability of the tsa56 gene affecting primer annealing in some strains, such as some strains of Kato, Shimokoshi and TA686, another pair of primers (5’-AATGTCGTTGGC3’-3’) and (5’-ATAGCGCACCACTACACTTGCC-3’) were designed to detect nearly full-length tsa56 coding DNA sequence (CDS) region, based on total 325 sequences of tsa56 gene collected from the National Center for Biotechnology Information [9, 10] The PCR products were cloned into pLB vectors and the colonies of each sample were randomly selected for Sanger sequencing. The sequencing results have confirmed that there is indeed only one tsa56 genotype (GenBank accession No. ON568725) via the results of 35 colonies from the blood and 36 colonies from the eschar samples.

Sequencing results were also found to be related to the reference O. tsutsugamushi Karp genotype (GenBank accession no. LS398548.1) with 99.33% identity at the nucleotide level, and were completely identical to a Karp genotype strain reported in Taiwan (GenBank accession no. MW464199.1). To avoid the sequence variability of the tsa56 gene affecting primer annealing in some strains, such as some strains of Kato, Shimokoshi and TA686, another pair of primers (5’-AATGTCGTTGGC3’-3’) and (5’-ATAGCGCACCACTACACTTGCC-3’) were designed to detect nearly full-length tsa56 coding DNA sequence (CDS) region, based on total 325 sequences of tsa56 gene collected from the National Center for Biotechnology Information [9, 10] The PCR products were cloned into pLB vectors and the colonies of each sample were randomly selected for Sanger sequencing. The sequencing results have confirmed that there is indeed only one tsa56 genotype (GenBank accession No. ON568725) via the results of 35 colonies from the blood and 36 colonies from the eschar samples.

Similar to the tsa56 gene, seven canonical highly conserved genes, as previously selected and reported in the PubMLST database (https://pubmlst.org/), were also subjected to nested PCR amplification, followed by Sanger sequencing [3, 10]. Different from the single tsa56 genotype, the sequencing results from both the blood and eschar samples exhibited several double peaks at multiple sites in five of the seven conserved genes (gpsA, mdh, nrdB, ppdk, and sucD) (Fig. 1B–D), which suggests the patient could be co-infected by at least two different O. tsutsugamushi strains. To confirm this discovery, the nested PCR products of gpsA, mdh, nrdB and sucD were also cloned into the pLB vectors. Thirty to forty colonies of each gene were randomly selected for Sanger sequencing. The sequencing results have showed that it is indeed a co-infection. Both the gpsA and mdh gene fragments had two different genotypes in the original nested PCR samples (Fig. 1C, D). And both nrdB and sucD gene fragments had three genotypes, including a major genotype, a minor genotype and another minor genotype mutated from the major genotype (Additional file 1: Fig. S1). There is no significant difference observed in the ratio of the mixed genotypes between the blood and eschar samples, suggesting the evaluations are consistent and the discoveries are very reliable.
**Fig. 1** Mixed genotypes of *O. tsutsugamushi* identified from both eschar and blood samples of the patient. 

A. A crater-shaped eschar (5 × 5 mm) was discovered on the left waist of the patient. 

B. Five of the seven conserved genes showing mixed genotypes of *O. tsutsugamushi* in the patient’s eschar and blood samples. 

C. The results of direct sequencing of PCR amplicon and colony verification of the *gpsA* and *mdh* genes. The logo presents the nested PCR fragments of *gpsA* and *mdh* genes collected in the pubMLST database. Mixed sites are indicated by filled arrows. These mixed sites are either synonymous (light blue letters) or missense (red letters). Two novel SNP sites in the *mdh* gene are also indicated by the unfilled line. These sites are all verified by sequencing of 30–40 clones for each gene and each sample.
Furthermore, the patient’s PCR sequencing results of the conserved genes were compared with all current publicly available typing sequences of 225 O. tsutsugamushi isolates from South Korea, Laos, Thailand, Japan, Myanmar, and New Guinea in the PubMLST database [10]. Our results have shown that there were three new single nucleotide polymorphism (SNP) sites discovered that were not reported in any previous sequence types, including two in the mdh gene and one in nrdB gene. Moreover, we discovered eight new sequence types of alleles that were new combinations of previously reported SNP sites, including one for the gpsA gene, one for the nuoF gene, two for the mdh gene, two for the nrdB gene and two for the sucD gene.

Discussion and conclusions

The mixed-genotype infection was possibly caused by bites of several chiggers infected with different genotypes of O. tsutsugamushi or bites of a single chigger infected with multiple genotypes. Because only one eschar was found in the patient and the ratio of mixed genotypes of conserved genes was consistent between the eschar and blood samples, the mixed-genotype infection in this patient was possibly caused by bites of a single chigger infected with multiple O. tsutsugamushi genotypes. Interestingly, mixed genotypes of O. tsutsugamushi in conserved genes have been reported in approximately 25% of patient blood samples in Thailand [3]. In contrast, although a mixed-genotype of the tsa56 gene was found in 17.9% of 28 chiggers [11], few mixed infections have been reported in patients [5–7]. A possible explanation of this observable difference could be response to the reservoir host immune selection pressure. Some genotypes of conserved genes originally in the extincted strain were probably preserved due to high frequency recombination events occurring in O. tsutsugamushi.

This mixed-infected patient was successfully recovered by the doxycycline treatment. Yet, the correlation between the single-tsa56-genotype mixed infections and disease severity remains unclear and would be evaluated at a large scale with more cases. Moreover, the ratio of the mixed genotypes of conserved genes was consistent between the eschar and blood samples, suggesting the discovery is very reliable and, meanwhile, eschar samples also can be utilized to detect minor genotypes in O. tsutsugamushi infections.

Acknowledgements

The authors wish to thank the patient for participating in this study.

Author contributions

Contributed to conception and design: FY, YD, JF-WC, and K-YY. Contributed to acquisition of data: CT, LZ, YH, WM, and LX. Contributed to analyses of data: CT, LZ, GW, SW, RP, and KW. Contributed to interpretation of data: XT, HP, and JD. Drafting the work: FY, YD, JF-WC, CT, LZ, YH, WM, and LX. Revising the paper for important intellectual content: all authors. Final approval of the version submitted: all authors. Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved: all authors. All authors read and approved the final manuscript.

Funding

This study was partly supported by the Hainan Provincial Natural Science Foundation of China (Grant No. 820RC628, 820QN267, B19QN360, B19WS142 and 2019RC218), the Education Department of Hainan Province (Grant No. Hrky2021–40), the National Natural Science Foundation of China (Grant No. 82060378, 81860367), the Open Foundation of Key Laboratory of Tropical Translational Medicine of Ministry of Education, Hainan Medical University (Grant No. 2021TTM005, HYP201919 and JBG202105), the Major Science and Technology Program of Hainan Province (ZDKJ202003), the research project of Hainan Academy Innovation Platform (YSPTZX202004), the Hainan talent development project (SR200003), and donations from the Lee-Wan Keung Charity Foundation Limited, Marina Man-Wai Lee, and the Hong Kong Hainan Commercial Association South China Microbiology Research Fund. The funding sources had no role in the study design, data collection, analysis, interpretation, or writing of the report.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12879-022-07682-y.

Additional file 1: Figure S1. Mixed genotypes of O. tsutsugamushi identified from both eschar and blood samples of the patient. A and B The results of direct sequencing of PCR amplicon and colony verification of nrdB and sucD genes. These sites are all verified by sequencing of 30–40 clones for each gene and each sample. C The results of direct sequencing of PCR amplicon of ppdk gene. The logo presents the nested PCR fragments of nrdB, sucD, and ppdk genes collected in the pubMLST database. Mixed sites are indicated by filled arrows. These mixed sites are either synonymous (light blue letters) or missense (red letters).
Availability of data and materials
All sequences analyzed during this study are available from the NCBI database (accession numbers MZ856313, ON568725-ON568737).

Declarations

Ethics approval and consent to participate
This study was approved by the Ethics Committee of Hainan Medical University.

Consent for publications
Written informed consent was obtained from the patient for the publication of this Case Report.

Competing interests
The authors declare that no competing interests.

Author details
1. Key Laboratory of Tropical Translational Medicine of Ministry of Education, Hainan Medical University, Haikou 571199, China. 2. The University of Hong Kong Joint Laboratory of Tropical Infectious Diseases, Hainan Medical University, Haikou, Hainan, China. 3. Department of Pathogen Biology, Hainan Medical University, Haikou, Hainan, China. 4. Academician Workstation of the Hainan Province, Hainan Medical University, Haikou 571199, Hainan, China. 5. Department of Infectious Disease, The Second Affiliated Hospital of Hainan Medical University, Haikou, China. 6. Haikou Maternal and Child Health Hospital, Haikou 571199, Haian, China. 7. Clinical Laboratory, People’s Hospital of Qiongzhong Li Miao Autonomous County, Qiongzhong, Hainan, China. 8. State Key Laboratory of Emerging Infectious Diseases, Carol Yu Center for Infection, Department of Microbiology, Li Ka Shing, Faculty of Medicine, The University of Hong Kong, Pokfulam, Hong Kong Special Administrative Region, China. 9. Department of Clinical Microbiology and Infection Control, The University of Hong Kong-Shenzhen Hospital, Shenzhen, Guangdong, China.

References
1. Izzard L, Fuller A, Blacksell SD, Richards AL, Aukkanit N, Nguyen C, Jiang J, Fenwick S, Day NP, et al. Isolation of a novel Orientia species (O. chuto sp. Nov) from a patient infected in Dubai. J Clin Microbiol. 2010;48(12):4404–9.
2. Weitzel T, Ditrich S, Lopez J, Phuklia W, Martinez-Valdebenito C, Velasquez K, Blacksell SD, Paris DH, Abarca K. Endemic scrub typhus in South America. N Engl J Med. 2016;375(10):954–61.
3. Sonthayanon P, Peacock SJ, Chierakul W, Wuthiekanun V, Blacksell SD, Holden MT, Bentley SD, Feil EJ, Day NP. High rates of homologous recombination in the mite endosymbiont and opportunistic human pathogen Orientia tsutsugamushi. PLoS Negl Trop Dis. 2010;4(7):e752.
4. Phetsouvanh R, Sonthayanon P, Pukrittayakamee S, Paris DH, Newton PN, Feil EJ, Day NP. The diversity and geographical structure of Orientia tsutsugamushi strains from scrub typhus patients in Laos. PLoS Negl Trop Dis. 2015;9(8):e0004024.
5. Le-Viet N, Phan DT, Le-Viet N, Trinh S, To M, Raoldt D, Parola P. Dual genotype Orientia tsutsugamushi infection in patient with rash and Eschar, Vietnam, 2016. Emerg Infect Dis. 2018;24(8):1520–3.
6. Yoo JR, Heo ST, Kang JH, Park D, Kim J, Bae JH, Woo JJ, Kim J, Lee KH. Mixed infection with severe fever with thrombocytopenia syndrome virus and two genotypes of scrub typhus in a patient, South Korea, 2017. Am J Trop Med Hyg. 2018;99(2):287–90.
7. Zhang M, Zhao ZT, Wang XJ, Li Z, Ding L, Ding SJ, Yang LP. Mixed scrub typhus genotype. Shandong, China, 2011. Emerg Infect Dis. 2014;20(3):484–5.
8. Kim DM, Kim HL, Park CY, Yang TY, Lee JH, Yang JT, Shim SK, Lee SH. Clinical usefulness of eschar polymerase chain reaction for the diagnosis of scrub typhus: a prospective study. Clin Infect Dis. 2006;43(10):1296–300.
9. Kim M, Ha NY, Min CK, Kim HI, Yen NTH, Lee KH, Oh I, Kang JS, Choi MS, Kim IS, et al. Diversification of Orientia tsutsugamushi genotypes by intragenic recombination and their potential expansion in endemic areas. Plos Neglect Trop Dis. 2017. https://doi.org/10.1371/journal.pntd.0005408.
10. Jolley KA, Bray JE, Maiden MCJ. Open-access bacterial population genomics: BIGSdb software, the PubMLST.org website and their applications. Wellcome Open Res. 2018;3:124.
11. Takahampunya R, Korkusol A, Promsathaporn S, Tippayachai B, Leeptrakat S, Richards AL, Davidson SA. Heterogeneity of Orientia tsutsugamushi genotypes in field-collected trombiculid mites from wild-caught small mammals in Thailand. Plos Neglect Trop Dis. 2018. https://doi.org/10.1371/journal.pntd.0006632.
12. Fleshman A, Mullins K, Sahl J, Hepp C, Nieto N, Wiggins K, Hornstra H, Kelly D, Chan TC, Phetsouvanh R, et al. Comparative pan-genomic analyses of Orientia tsutsugamushi reveal an exceptional model of bacterial evolution driving genomic diversity. Microb Genomics. 2018. https://doi.org/10.1099/mgen.0.000199.

Publisher’s Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.