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Original Article

Utility of next-generation sequencing for the etiological diagnosis of *Orientia tsutsugamushi* infection

Nannan Xu [a], Lintao Sai [a], Gang Wang [a,*], Gregory A. Dasch [b], Marina E. Eremeeva [c,*]

[a] Department of Infectious Disease of Qilu Hospital, Cheeloo College of Medicine, Shandong University, Jinan 250012, China
[b] Rickettsia Unlimited LLC., Stone Mountain, GA 30087, USA
[c] Jiann-Ping Hsu College of Public Health, Georgia Southern University, Statesboro, GA 30458, USA

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ABSTRACT

Background: Scrub typhus, an acute febrile disease caused by *Orientia tsutsugamushi,* is transmitted to humans through infected chigger mites. We present a case of scrub typhus in a previously healthy man from Shandong Province diagnosed using next-generation sequencing (NGS) and PCR and review recent literature on NGS for scrub typhus diagnosis.

Methods: NGS was utilized for testing whole blood collected on admission. Confirmatory testing was done by detecting IgM and IgG antibodies to *Orientia* in acute and convalescent sera by ELISA. *Orientia* 47-kDa protein gene TaqMan and standard PCR of the 56-kDa protein gene and Sanger sequencing were performed on eschar scab DNA.

Results: The NGS diagnosis was confirmed by 47-kDa protein gene TaqMan and sequencing of a fragment of the *O. tsutsugamushi* 56-kDa protein gene from the eschar scab. Analysis of this sequence and the NGS data indicated *O. tsutsugamushi* strain Cheeloo2020 is a novel genotype. Mapping of the NGS data against the *O. tsutsugamushi* Gilliam strain genome sequence identified 304 reads with high similarity.

Conclusions: NGS is not only useful for multiplex diagnosis of scrub typhus, but also provides insight into the genetic diversity of *O. tsutsugamushi*. The common failure to submit sequences to databases makes it difficult to determine the minimal quantity and quality of NGS data being used for the positive identification of *Orientia* DNA in clinical specimens.

1. Introduction

Scrub typhus is an acute febrile disease caused by *Orientia tsutsugamushi* [1,2], which is transmitted to humans through infected chigger mites. Most scrub typhus cases occur in Southeast Asia and the Pacific Rim including China, where this disease presents as two different types with distinct clinico-epidemiological features [2,3]. Scrub typhus has also been detected outside of the tsutsugamushi triangle, increasing the need for enhanced surveillance of this disease [1,2]. Summer-type scrub typhus is transmitted by *Leptotrombidium deliensis* mites in 11 provinces south of 31 °N and is caused by more virulent *Orientia* serotypes, including Gilliam, Karp, and Kato [3]. Autumn-winter type scrub typhus has been formally recognized since 1986 in the southern part of Shandong Province and northern Jiangsu Province; since then, it has been reported in many areas of northern China [3]. In these parts of the country, scrub typhus is transmitted by *Leptotrombidium scutellare*, and the disease occurs from September through December with the peak occurrence in October [3,4]. Genetic typing of *O. tsutsugamushi* has detected genetically diverse strains circulating in humans, rodents, and mites in Shandong Province, with many strains being closely related to Kawasaki-type *Orientia*, which is thought to cause mild forms of scrub

Abbreviations: NGS, next-generation sequencing; mNGS, metagenomic next-generation sequencing; kDa, kilodalton; DNA, Deoxyribonucleic acid; BLAST, Basic Local Alignment Search Tool; UPGMA, Unweighted Pair Group Method with Arithmetic Mean.

* Corresponding author.
E-mail addresses: wginfection@email.sdu.edu.cn (G. Wang), meremeeva@georgiasouthern.edu (M.E. Eremeeva).

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typhus [5–11]. Studies focused on wildlife thought to be involved in the ecology of *Orientia* also detected DNA of the STA-07-type of *O. tsutsugamushi* and Shimokoshi-NS Fuji-related genotypes [8,12].

While human cases of the autumn-winter type reported in the literature are mostly due to Kawasaki-type *Orientia* [13], co-infection with *O. tsutsugamushi* genotypes, Kawasaki-type and Sdu-2-type, has also been reported [9]. The delay in diagnosis and inadequate antibiotic therapy of scrub typhus are known risk factors associated with severe outcomes and poor prognosis [14]. Scrub typhus may also be misdiagnosed as hemorrhagic fever with renal syndrome because of similar clinical manifestations and shared seasonality and spatial occurrence in Shandong and other endemic areas [15]. Furthermore, scrub typhus may manifest at onset with several atypical features, thus complicating proper patient management and progression of this infection. Therefore, access to prompt and accurate laboratory diagnosis is key for optimal patient management and timely recovery.

Next-generation sequencing (NGS) technologies have been used increasingly for molecular diagnosis of infectious diseases, including scrub typhus [16–18]. Access to and implementation of NGS technology in the clinical diagnostic pipeline provides opportunities for expediting diagnosis of scrub typhus with atypical clinical manifestations [19–23], particularly in neonates and co-infections [15,24,25].

We present here a case of scrub typhus in a previously healthy man from Shandong Province diagnosed using NGS and PCR. We also review the utility and current limitations of NGS technology for clinical practice and patient management of scrub typhus.

2. Case description and laboratory data

A 65-year-old man without any underlying conditions was admitted to the hospital in November 2020 with a 7-day history of febrile illness. At onset, he experienced a sudden fever rising to 39.5 °C, a headache, fatigue and myalgia, and self-treated with generic antipyretics with no improvement; thus, hospital evaluation was suggested by his family. The patient lived in an urban area of Licheng District, Jinan City, Shandong Province, and reported mountain climbing in the province 2 weeks before disease onset.

On admission to the hospital, physical evaluation revealed a body temperature of 39 °C, a heart rate of 95 beats per minute, a respiratory rate of 22 breaths per minute, and a blood pressure of 124/76 mm Hg. An oval black lesion of typical eschar appearance was found on the patient's left upper arm, and enlarged lymph nodes were palpated (Fig. 1). The remainder of the physical examination was normal. Laboratory testing revealed increased procalcitonin (1.620 ng/mL), C-reactive protein (68 mg/L), liver transaminase (ALT 405 IU/L, AST 291 IU/L), lactate dehydrogenase (325 U/L), and DD-i (13.84 μg/mL). The platelet count was relatively low (108 × 10^9 per L), but other routine blood parameters, such as coagulation function and creatinine levels, were normal (Table 1). Pharynx swabs tested negative for viral nucleic acids including influenza virus, parainfluenza virus, respiratory syncytial virus, adenovirus, rhinovirus, metapneumovirus, and coronavirus using multiplex quantitative PCR. A test for severe fever associated with thrombocytopenia syndrome virus was also negative, as deter-

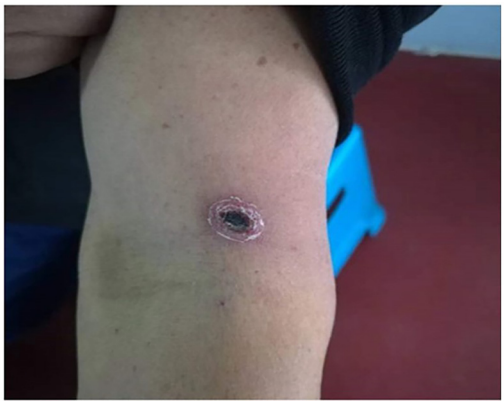

Fig. 1. Eschar that developed on the upper left arm of the patient.

Table 1
Laboratory findings from the blood of the scrub typhus patient from Shandong Province, China.

Variable	Reference range	Patient's test results at hospital admission[1]
Blood routine		
Leucocytes (× 10^9 per L)	3.5–9.5	7.7
Neutrophils (× 10^9 per L)	1.8–6.3	5.11
Lymphocytes (× 10^9 per L)	1.1–3.2	2.56
platelets (× 10^9 per l)	125–350	108 ↓
hemoglobin (g/l)	130–175	172
Infection-related biomarkers		
Procalcitonin (ng/mL)	<0.1	1.620 ↑
C-reactive protein (mg/L)	0–10	68 ↑
Erythrocyte sedimentation rate (mm/h)	0–15	52 ↑
Blood biochemistry		
Alanine aminotransferase (U/L)	21–72	405 ↑
Aspartate aminotransferase (U/L)	17–59	291 ↑
Albumin (g/L)	35–50	37
Total bilirubin (μmol/L)	3–22	19
Alkaline phosphatase (U/L)	38–126	154 ↑
Blood urea nitrogen (mmol/L)	3.2–7.1	5.2
Serum creatinine (μmol/L)	58–133	69
γ-Glutamyl transferase (U/L)	15–73	184 ↑
Lactate dehydrogenase (U/L)	120–230	325 ↑
Creatine kinase (U/L)	38–174	42
Coagulation series		
APTT (s)	26.0–42.0	24.5 ↓
PT (s)	8.80–13.80	11.10
FIB (g/L)	2.0–4.0	3.85
DD-i (μg/mL)	<0.50	13.84 ↑

Note:
[1] Symbol next to the value indicates the corresponding deviation from the reference range, i.e., lower (↓) or higher (↑).Abbreviations: APTT, activated partial thromboplastin time; PT, prothrombin time; FIB, fibrinogen DD-i: D-Dimer.

mined using the SFTSV Real Time RT–PCR Kit (Liferiver, Shanghai, China). An OX19 Weil-Felix test (Ningbo Tianrun Biopharmaceutical Co., Ltd, Ningbo, China) was negative. Scrub typhus was suspected and oral doxycycline (0.1 g q 12 h) was prescribed.

Immediately before antibiotic administration, informed consent was obtained, and EDTA blood was collected and submitted to the CapitalBio MedLab (Beijing, China) for metagenomic NGS (mNGS) analysis. Two days later, preliminary review of the mNGS data identified 152 sequence reads corresponding to *O. tsutsugamushi*. Conventional bacterial cultures of blood samples were negative. Doxycycline therapy was continued as first prescribed.

The patient's body temperature returned to normal after 72 h of doxycycline therapy. The main symptoms including headache, fatigue, and myalgia were alleviated. Both the procalcitonin and C-reactive protein levels gradually returned to their corresponding normal ranges. The patient was discharged after 6 days of hospitalization and continued oral doxycycline for another day. The entire treatment course spanned 7 days. The patient returned to the clinic on day 22 after onset for a follow-up visit. A convalescent-phase blood sample and a scab specimen from the spontaneously desquamated eschar were collected from the recovered patient.

3. Confirmatory laboratory testing

Acute (7 d after onset) and convalescent (22 d after onset) sera were tested for both IgM and IgG type antibodies to *Orientia* by ELISA at a 1:100 dilution according to the manufacturer's protocol (InBios International, Inc., Seattle, WA, USA). No antibody to *Orientia* was detected in the acute sera (OD 0.237 and 0.126 for IgM and IgG type antibody, respectively), and convalescent sera tested positive for IgM antibody (OD 1.250) and IgG antibody (OD 0.668). These results were consistent with a recent acute scrub typhus infection.

Total DNA was extracted from the eschar scab using the QIAamp® DNA Mini Kit (QIAGEN, Mannheim, Germany) and eluted using 50 μL of AE buffer according to the manufacturer's instructions. Extracted DNA was tested by *Orientia* 47-kDa protein gene TaqMan using primers and probes listed in Table S1, according to a previously published protocol [26]. To complete identification, a standard PCR assay targeting a 372-bp fragment of the 56-kDa protein gene [27] and Sanger sequencing were performed. The nucleotide sequence of the 56-kDa protein gene was further analyzed using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and multiple sequence alignment comparison against all reference strains of *Orientia* for which largely complete whole-genome sequences were available. Phylogenetic analysis was performed using the UPGMA (Tamura–Nei model) method and bootstrap testing on 100 replicates with Geneious Prime 22.1 (Dotmatics, Boston, MA, USA). The 56-kDa protein gene nucleotide sequence generated was submitted to the NCBI GenBank database under accession number ON733250.

The nucleotide sequence of the 56-kDa protein gene fragment derived from the patient's sample, designated Cheeloo2020, had most similarity to the homologous fragment of *O. tsutsugamushi* Shandong-XDM2 (DQ514320), from which it differed by only two nucleotides (single-nucleotide polymorphisms). When compared with 39 other well-characterized isolates of *O. tsutsugamushi*, which are known to be biologically, genetically, and serologically diverse, the nucleotide sequence of Cheeloo2020 DNA belonged to a unique genetic lineage (Fig. 2). To further confirm that the Cheeloo2020 strain 56-kDa protein gene fragment was indeed from a different type, this fragment was compared with the homologous region extracted bioinformatically from complete 56-kDa protein gene sequences. The sequences from robust sets of strains from various locations in Laos [28], Cambodia [29,30], and Taiwan, China [31] also all diverged from the sequence of this Cheeloo2020 fragment (Supplemental materials: Fig. S1– S3). The use of complete 56-kDa protein gene sequences that fully translate provides evidence that Cheeloo2020 is a different strain type. Unfortunately, only this gene of the Shandong XDM2 isolate is available and no Cheeloo2020 NGS reads mapped to the 56-kDa protein gene.

Subsequent analysis of the NGS sequence file using Bowtie2 (Geneious Prime 22.1, Dotmatics) identified 304 reads of 100 bp to 250 bp in length that mapped to regions in the whole-genome sequence of the Gilliam strain of *O. tsutsugamushi* (OtsuGilliamLS398551.1 [32]) (Fig. 3 and Table S2). Cheeloo2020 NGS read sequences mapped to regions along the entire length of the *O. tsutsugamushi* Gilliam chromosome with relatively few independent reads mapping to the same region (2–5 reads). Using NCBI whole-genome blastn analysis to *O. tsutsugamushi*, these reads exhibited variation in the specific strain with the greatest similarity. This strongly suggested that the Cheeloo2020 isolate was not closely related to any one strain and may thus represent a novel strain type. NGS reads that were not identical to *O. tsutsugamushi* Gilliam genome sequences contained either differences in the length of polynucleotide runs or other sequencing errors. These mismatches are also probably due in part to comparison with unrelated strains. Consistent with this idea is that some of the 100% matches were in conserved regions for many of the strain sequences, as shown in Fig. 4, with the S4 Peptidase read from Cheeloo2020 showing a perfect match with DNA from nine isolates. This provided further evidence that the patient was indeed infected with *O. tsutsugamushi*.

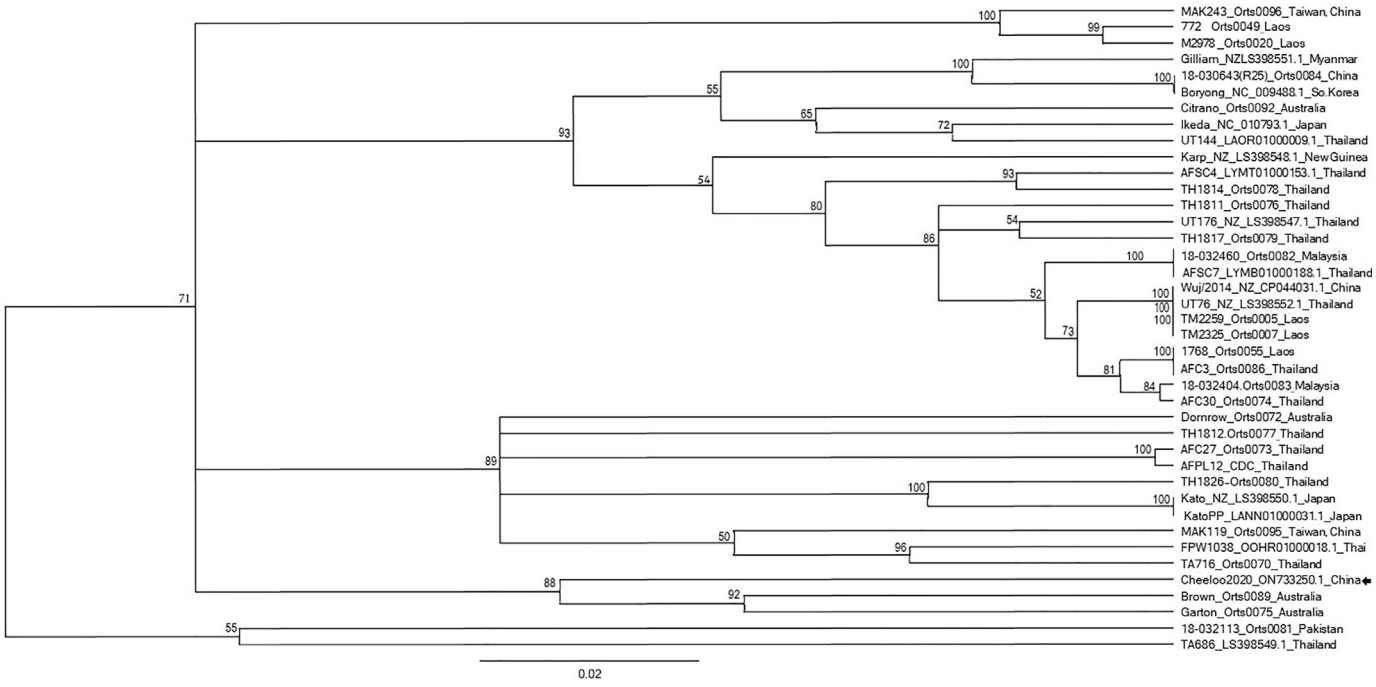

Fig. 2. Comparative analysis of the 377-bp 56-kDa protein gene fragment sequence and the genetic relationship of *O. tsutsugamushi* Cheeloo2020 to this region obtained from other diverse *O. tsutsugamushi* isolates with nearly complete genome sequences. Homologous sequences were aligned using MUSCLE, and phylogenetic analysis was conducted using UPMGA (Tamura–Nei model). The names of the strains and their origin are indicated as per the NCBI listing and the original publications; individual sequences used for this alignment are included in the supplemental materials. The position of the Cheeloo2020 sequence is indicated by an arrow.

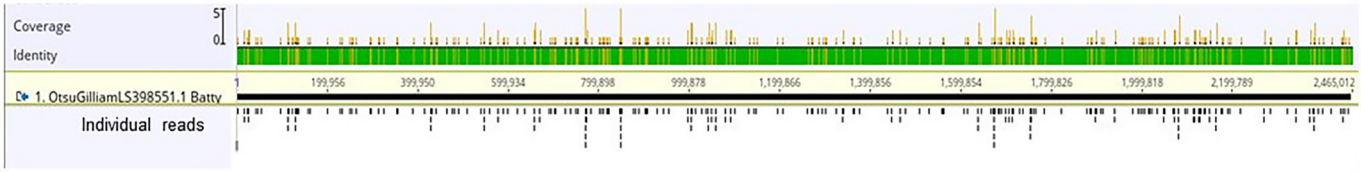

Fig. 3. Mapping by Bowtie2 Geneious Prime BLAST of 304 NGS reads generated from a patient sample to the *O. tsutsugamushi* Gilliam genome (OtsuGilliamLS398551.1).

4. Discussion and review of NGS approaches to diagnose scrub typhus

NGS was first widely applied to assess the diversity of the microbiome [33]. It was generally accomplished by selective PCR amplification of ribosomal RNA or DNA present in bacteria and did not result in host ribosomal RNA amplification. In this regard, it resembled the widespread use of nested PCR or quantitative PCR (e.g., TaqMan) assays in being relatively simple to analyze the data by comparison of the ribosomal 16S rDNA sequences with those present in deep microbial databases. This approach was widely used in ecological surveys and for detecting pathogens and commensal (symbiotic bacteria) in arthropod vectors [34]. In the case of clinical samples such as blood, the main problems arose from inadvertent or concomitant contamination of the sample with non-pathogenic organisms; reagents of bacterial origin had to be scrupulously prepared to avoid this source of signal.

The desire for an equally sensitive method that did not require a specific primer set(s) while providing more robust and discriminatory signals than could be obtained with 16S rDNA (which is often conserved at the genus level) led to mNGS methods where the target DNA or RNA molecule (in the form of cDNA) was amplified strictly as nucleic acid [35]. Unfortunately, this meant that all DNA or RNA associated with the non-target microorganisms would also be amplified and sequenced. Consequently, this drawback put a premium on methods for bioinformatically sorting all the signals obtained from the sample into categories of host, target pathogen, and other bacteria in the sample [36]. Eventually, target enrichment methods based on hybridization to a defined target overcame much of the background signal but with the disadvantage of not being able to detect unknown agents [37].

The use of mNGS sequencing to detect *O. tsutsugamushi* has been consistently applied since 2016, but hybridization enrichment of this agent prior to sequencing has not been incorporated to date. A review of published papers reporting the detection of *O. tsutsugamushi* (Table S3) revealed a wide variety of samples, most notably those obtained from patients where the diagnosis of scrub typhus

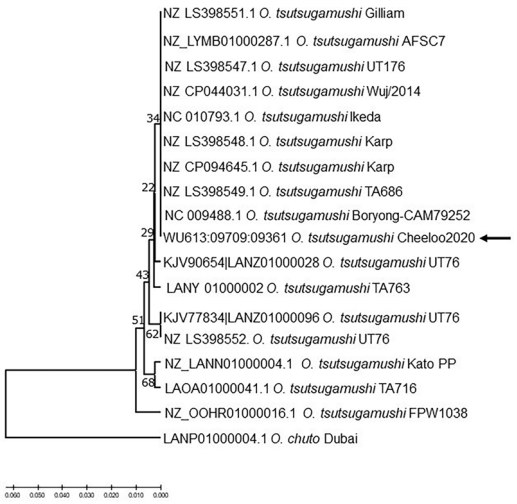

Fig. 4. Identification of *Orientia tsutsugamushi* Cheeloo2020 S49-type peptidase gene read nu613:09709–09361 using blastn and sequence alignment to other isolates of *O. tsutsugamushi*. The evolutionary history was inferred using the UPGMA method in MEGAX ; the optimal tree is shown. The percentage of replicate trees in which the associated taxa clustered together in the 500 replicate bootstrap tests is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method and are in the units of the number of base differences per site. This analysis involved 18 nucleotide sequences and a total of 221 positions in the final dataset; all ambiguous positions were removed for each sequence pair using the pairwise deletion option.

was not a given [38–45]. In most, but not all cases, the positive mNGS signal was confirmed by standard clinical criteria (eschar and fever with potential mite exposure), seroconversion, or PCR assays targeting the 47-kDa or 56-kDa antigen genes [22,24,25,40,41,46–48]. Our results in the current study fit this description. A remarkable finding from these papers was that despite using a variety of nucleic extraction, amplification, library construction, and sequencing (machine, read length and depth of coverage) methodologies, many samples contained enough *Orientia* DNA that sufficient positive reads could be detected to confirm the diagnosis of scrub typhus.

Several authors have discussed the steps needed to standardize diagnosis in clinical settings based on mNGS approaches [17,36,49,50]. Based on these analyses and our own experience, we recommend the following to increase the likelihood of successful diagnosis. The most important step is obtaining an appropriate sample before antibiotic therapy is initiated. In general, and if present, the eschar or an attached chigger are preferable to a late-stage blood sample. The nucleic acid in the sample should be promptly extracted. To date, no studies have compared the preparation of cDNA to direct DNA recovery. Illumina sequencing technology is widely employed, particularly using the MiSeq platform, as this low-cost instrument has become widely available. Although the quality of the sequence data is high, the read length is not as long as with other methods such as the Ion Torrent PGM or even sequencing the DNA directly on the Oxford Nanopore instrument. It is unclear whether competing methodologies will eventually gain penetrance into the market. We are unaware of efforts to directly compare sequence data recovered (quality, amount, length) from the same *Orientia* sample using different methodologies. Finally, the bioinformatic extraction and use of sequence data varies substantially between investigators [19,40–42,45,47,48], possibly as a function of the predilections of the bioinformaticians handling the data. We have attempted to illustrate some uses for moderately robust data (in the low median of data that are reported). Regrettably, in many papers the authors provide little information regarding their final data other than a map to a particular genome (as we show in Fig. 3) and many fail to submit to standard repositories of such information. If the initial raw data are submitted to a repository, it can serve as a source of samples for efforts to improve recovery of specific agent reads. It also can permit others to examine those reads for their specificity. Shandong strains of *O. tsutsugamushi* most frequently are typed as Kawasaki based on 56-kDa protein gene sequencing, but no genome sequence for a standard strain is currently available. Such a reference genome would have been used in the current study, rather than the Gilliam genome sequence, for sequence mapping and analysis (Fig. 3 and Table S2). This may be completed by others in the future once a Kawasaki strain genome becomes available since our raw data have been deposited in the NCBI SRA database.

5. Conclusion

NGS enables the rapid diagnosis of scrub typhus by detecting multiple regions of the genome of *O. tsutsugamushi*. Depending on the quantity and quality of the generated sequences, NGS can permit characterization of *O. tsutsugamushi* even to the strain level. We demonstrated this herein by identification of the novel genotype Cheeloo2020. Further work is needed to propagate this isolate type. Optimal use of NGS for routine diagnosis of scrub typhus requires consensus on the appropriate methodology and its standardization. Fulfilling these objectives depends on the availability of all NGS sequences in the public domain; this should be a requirement for all studies published in peer-reviewed literature.

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Author contributions

N.N.X.: concept and study design, sample collection and processing, data analysis, writing the draft and approval of the final manuscript. L.T.S.: sample collection

and processing, data analysis and interpretation, editing of the manuscript. G.W.: concept and study design, sample collection and processing, data analysis, project coordination and supervision, writing of the draft and editing of the final manuscript. G.A.D.: data analysis and interpretation, writing and editing of the final manuscript. M.E.E.: data analysis and interpretation, writing and editing of the final manuscript.

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Declaration of competing interest

G.W. and M.E.E. are Editorial Board Members for the *Infectious Medicine* journal but were not involved in the editorial review or the decision to publish this article. All authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data available statement

The NGS sequencing data are deposited at the National Center for Biotechnology Information Sequence Read Archive (accession no. B2026709). The nucleotide sequence of the 56 kDa protein gene fragment of *Orientia tsutsugamushi* generated as part of this study was submitted to the GenBank database under accession number ON733250.

Ethics statement

The study was reviewed and approved by Shandong University Qilu Hospital Human Research Protection Committee (IRB # KYLL-2019-268, IRB # KYLL-202008-058).

Informed consent

Patient signed consent form.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.imj.2024.100116.

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