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Detection of *Orientia tsutsugamushi* in Novel Trombiculid Mite Species in Northern Tamil Nadu, India: Use of Targeting the Multicopy *traD* Gene

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Abstract

Detection of *Orientia tsutsugamushi* DNA in a trombiculid mite chigger species suggests that it might be a potential vector of scrub typhus in an endemic area. Over a period of 20 mo, 85 rats were trapped, 57 had chiggers that were identified by standard morphometric techniques. The chigger pools were assessed by performing PCR assays targeting fragments of the single-copy genes 56 kDa type-specific antigen gene (TSA56) by nested PCR and the 47 kDa (htrA) quantitative real-time PCR (qPCR). The novel *traD* SYBR green assay that detects a multicopy gene was also performed. In total, 27 chigger pools were positive by *traD* qPCR, of which only 7 were positive by 47 kDa qPCR and in 3 of these, 56 kDa gene was amplified by nested PCR. *Orientia tsutsugamushi*-specific DNA was detected in *Ascoschoengastia* spp., *Schoengastiella ligula*, *Leptotrombidium rajasthanense*, *Leptotrombidium deliense*, and *Leptotrombidium jayawickremei* chigger pools. Therefore, they could be potential vectors of scrub typhus in Southern India. The three 56 kDa sequences belonged to TA716 genotype and Kato genogroup. Further studies are needed to confirm these chigger species as scrub typhus vectors in Northern Tamil Nadu.

Key words: scrub typhus, *Ascoschoengastia*, *Leptotrombidium*, *Schoengastiella*, *traD*

Scrub typhus, a vector-borne acute febrile illness, caused by *Orientia tsutsugamushi* has reemerged in India in the 21st century and is being reported from all Indian states (Tilak and Kunte 2019). The vectors and the reservoir of infection are the chiggers (larvae) of the trombiculid mite (Luce-Fedrow et al. 2018, Elliott et al. 2019, Acosta-Jamett et al. 2020). The trombiculid chigger mites are common ectoparasites of rodents (Acosta-Jamett et al. 2020), and disease is common in 'mite islands' (Elliott et al. 2019, Acosta-Jamett et al. 2020). The common vectors implicated worldwide belong to the genus *Leptotrombidium* (Acariformes: Trombiculidae) of these the most common are *Leptotrombidium pallidum* (Nagayo), *Leptotrombidium deliense* (Walsh), *Leptotrombidium scutellare* (Nagayo), and *Leptotrombidium akamushi* (Brumpt) (Wulandhari et al. 2021). The other mite species implicated by molecular techniques include *Ascoschoengastia indica* (Hirst) (Elliott et al. 2019, Binh et al. 2020), *Guntheria cassiope* (Womersley), *Odontacarus* sp. (Ewing), *Eutrombicula wichmanni* (Oudemans), *Microtrombicula chamlongi* (Nadchatram and Kethley) (Elliott et al. 2019), and *Neotrombicula japonica* (Tanaka, Kaiwa, Teramura & Kagaya) (Lee et al. 2011, Seo et al. 2021), *Gahrliepia* sp. (Oudemans), *Lorillatum* sp. (Nadchatram), and *Blankaartia* spp. (Oudemans) (Takhampunya et al. 2019) (Acariformes: Trombiculidae). In addition, *Euschoengastia koreaensis* (Jameson and Toshioka) (Acariformes: Trombiculidae)

has been confirmed as a scrub typhus vector in South Korea (Lee et al. 2011, Choi et al. 2018). The nontrombiculid species have been reported as vectors of *O. tsutsugamushi* and include *Ixodes* spp. (Zhang et al. 2013), *Haemophysalis*, Koch (Acari: Ixodida) ticks, *Echinolaelaps echidninus* (Berlese), and *Laelaps turkestanicus* (Lange) (Acariformes: Laelapidae) (Elliott et al. 2019).

Molecular assays used to confirm the presence of *O. tsutsugamushi* in chiggers include 56 kDa nested PCR (Zhang et al. 2013, Kuo et al. 2015, Choi et al. 2018, Takhampunya et al. 2019, Park et al. 2020), 47 kDa quantitative real-time PCR (qPCR; Takhampunya et al. 2019), and 16S rRNA (Chaisiri et al. 2019, Binh et al. 2020). Different genotypes of *O. tsutsugamushi* have been identified in chiggers in various countries and reflect the genotypes identified in human infections in those areas. In China, Shimokoshi and Kawasaki have been reported (Zhang et al. 2013), while in Thailand, Kato, Karp, TA763, and Gilliam have been reported (Takhampunya et al. 2019). In Korea, the Boryong strain is predominant (Park et al. 2020), while in Taiwan it is commonly Kato, Karp, and Kawasaki (Kuo et al. 2015). In India, the Kato and Karp genotypes are the commonly reported strains in human samples (Varghese et al. 2015, Bhate et al. 2017, Bora et al. 2018, Kumar et al. 2019, Devaraju et al. 2020).

Outside the 'tsutsugamushi triangle', *Microtrombicula* spp. (Ewing) and *Neotrombicula* spp. (Hirst) (Acariformes: Trombiculidae) infesting *Mastomys natalensis* (Smith) (Rodentia: Muridae) have been demonstrated to harbor *Candidatus* Orientia chuto by 47 kDa nested PCR in Kenya (Masakhwe et al. 2018). In Chiloe Island, Chile, *Herpetacarus* spp. Vercammen-Grandjean (Acariformes: Trombiculdae) infesting *Abrothrix olivacea* (Waterhouse), *Abrothrix sanborni* (Osgood), and *Geoxus valdivianus* (Philippi) (Rodentia: Cricetidae) have been confirmed to carry *Candidatus* Orientia chiloensis, a proposed new species (Acosta-Jamett et al. 2020). In the Asia-Pacific region, vertebrate hosts for trombiculid mites infected with *O. tsutsugamushi* include the common rodents: *Rattus norveigicus* (Berkenhout), *Bandicota indica* (Bechstein), and *Mus musculus* (Linnaeus) (Elliott et al. 2019). Other than these, *Apodemeus agrarius* (Pallas) (Rodentia: Muridae) and *Myodes regulus* (Thomas) (Rodentia: Cricetidae) (Park et al. 2020) from Korea, *Rattus losea* (Swinhoe) and *Rattus tanezumi* (Temminck) (Rodentia: Muridae) (Kuo et al. 2015) from Taiwan, *Rattus argentiventer* (Robinson & Kloss) and *Rattus norveigicus* (Rodentia: Muridae) from South Vietnam (Binh et al. 2020) have also been confirmed to carry *Orientia tsutsugamushi* positive chiggers.

In India, the predominant rodent species infested with chiggers include *S. murinus* (Linnaeus) (Eulipotyphla: Soricidae) (Tilak et al. 2011, Sadanandane et al. 2018, Candasamy et al. 2016, Devaraju et al. 2020), *R. rattus* (Tilak et al. 2011, Candasamy et al. 2016, Bhate et al. 2017, Akhunji et al. 2019, Devaraju et al. 2020), *R. norvegicus*, and *B. bengalensis* (Gray) (Rodentia: Muridae) (Bhate et al. 2017, Akhunji et al. 2019). The common chigger species infesting these rodents in India include *L. deliense* (Candasamy et al. 2016, Khan et al. 2016, Sadanandane et al. 2018, Rose et al. 2019, Devaraju et al. 2020), *L. fulmentum* (Vercammen-Grandjean and Langston), *L. burmense* (Ewing) (Tilak et al. 2011), *L. insigne* (Fernandes) (Candasamy et al. 2016, Rose et al. 2019, Devaraju et al. 2020), *Schoengastiella ligula* (Radford) (Acariformes: Trombiculidae) (Tilak et al. 2011, Candasamy et al. 2016, Rose et al. 2019), and *Ornithonyssus bacoti* (Hirst) (Mesostigmata: Macronyssidae) (Bhate et al. 2017, Akhunji et al. 2019). In India, *O. tsutsugamushi* has been detected by PCR in *L. deliense* (Sadanandane et al. 2018, Devaraju et al. 2020) and *O. bacoti* (Bhate et al. 2017, Akhunji et al. 2019), a mesotigmatid mite (Bhuyan and Nath 2015).

Molecular assays like PCR (conventional, nested, and real-time) have targeted single-copy genes like 16 S rRNA, GroEl, 56 kDa, and the 47 kDa gene (Prakash 2017). Though qPCR assays for scrub typhus are considered equivalent or even superior to the nested PCR assays, they have detection limits that need a minimum of 10 copies per reaction for consistent results (Tantibhedhyangkul et al. 2017). The qPCR assays for *O. tsutsugamushi* detection in the clinical laboratory is limited to the very low copy number found in the blood (sensitivity around 50–70%). However, there is no limitation when utilizing the proper clinical specimens of choice, that is eschars (biopsies and swabs) and rash (biopsies), which demonstrate 100% sensitivity even after proper antibiotic treatment for up to 2 wk (Chao et al. 2019). It has been demonstrated that amplifying the multiple copy gene *traD* by qPCR (SYBR Green) is a very sensitive method to detect *O. tsutsugamushi* DNA in a footpad infection mouse model (Keller et al. 2014). This has been further verified by Chao et al. (2019), who detected the *traD* gene in all seven replicates at a genome concentration of 0.4/µl. In this study, we performed the *traD* qPCR along with the 47 kDa qPCR and the 56 kDa nested PCRs for detection of *O. tsutsugamushi* in chigger mites.

Materials and Methods

The study was conducted over a period from 24 August 2018 to 10 February 2020 after ethics approval from the Institutional Review Board (IRB Min No. 9370 dated 27 October 2016). Rodent trapping was performed in rural and suburban localities (Hosur and Vellore, Tamil Nadu, India) where scrub typhus cases were identified earlier (Refer Suppl Fig. 1 [online only] for details). After obtaining informed consent from the house owner, rodent trapping was performed. GPS locations of areas where rodents were trapped were noted. Rodents were trapped using locally sourced Wonder Traps (G. Srinivasa Traders, Salem, Tamil Nadu, India). Coriander leaves, fried fish, and roasted coconut were used as rodent bait. Rodents were identified using morphometric characteristics (Agrawal 2000). Chigger mites were gathered from rodents after were euthanized using chloroform (Sigma–Aldrich, Bangalore, India) as described previously (Park et al. 2016). The chiggers were identified using the standard key for identification of Indian *Trombiculidae* (Fernandes and Kulkarni 2003). Identification features included shape of the scutum, specialized leg setae, palpal chaetotaxy, and chelicerae as described previously (Kuo et al. 2015, Kumlert et al. 2018). Confirmation of the genus and identification to species level (wherever possible) was based on published keys (Fernandes and Kulkarni 2003). About 10–20% chiggers were identified per rodent (data not shown).

For DNA extraction, chiggers were pooled by rodent host (20 chiggers/pool) and trapping date. Where pools had various species, it is highly likely such pools also contained these species. Chigger DNA was extracted using a tissue extraction protocol (QIAamp DNA Mini Kit, Qiagen, Hilden, Germany). The chigger pools were homogenized in 200 µl ATL buffer, treated with 20 µl Proteinase K and overnight incubation at 56°C to ensure digestion. The subsequent steps were carried out according to the manufacturer's instructions. The final elute (50 µl) was stored at –20°C pending PCR amplification. qPCR to detect the 118 bp of the 47 kDa gene was performed as per the Jiang protocol performed as per the Jiang protocol (Jiang et al. 2004, Masakhwe et al. 2018). The SYBR green assay amplifying the multicopy gene *traD* (176 bp segment) was performed as described (Keller et al. 2014, Chao et al. 2019). The 56 kDa gene target was amplified using two different nested PCR assays, Furuya protocol (Lee et al. 2011) and the Horinouchi protocol (Masakhwe et al. 2018). All PCR assays were performed in 50 µl

volumes containing 5-µl template using 25-µl QIAGEN Multiplex PCR Mix (Qiagen, Hilden, Germany) for amplifying the respective targets.

The nested PCRs for amplifying the 56 kDa gene were performed on a Veriti Thermal Cycler (Applied Biosystems, Foster City, CA). The cycling parameters for the initial amplification for the 56 kDa Furuya PCR protocol included an initial denaturation (94°C for 15 min) and 35 cycles of denaturation, annealing, and extension of 60 s each at 94, 57, and 72°C with a final extension time of 72°C for 7 min. The Horinouchi protocol had similar cycling parameters with the only exception being that the annealing temperature was 55°C. The same cycling parameters were used for the second round (nested) PCR for both protocols. The PCR products were subjected to 2% (w/v) agarose gel electrophoresis containing ethidium bromide. The gel was visualized using a gel documentation system (GelDoc XR, BioRad Inc, Hercules, CA, USA). The PCR products were purified using QIAquick DNA purification kit (Qiagen, Hilden, Germany). Sanger sequencing was performed using the Big Dye Terminator Cycle Sequencing Kit v 3.1 (Applied Biosystems) in an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). Phylogenetic analysis to determine genotype was carried out using Seaview ver4 (Gouy et al. 2010).

The qPCR was performed on a 7500 Fast Dx Real-Time PCR machine (Applied Biosystems). After an initial denaturation of 5 min at 95°C, 45 cycles each of annealing at 95°C for 15 s and 60°C for 60 s was carried for amplifying the 118 bp segment of the 47 kDa gene. The *traD* SYBR green assay used the same parameters except that number of cycles was 40. A Ct value ≤ 38 cycles was considered as positive for the 47 kDa qPCR. For the *traD* SYBR green assay, melt curve analysis was performed, and a T_m of 82–83°C was considered as probable amplification (Chao et al. 2019). This was confirmed by demonstrating a band of 176 bp by gel electrophoresis. Sanger sequencing as described above and BLAST analysis were performed to confirm the identity.

Results

Of 85 rats trapped, 57 had chiggers (24 from Hosur and 33 from Vellore), of these only 3 were *Rattus norveigicus*, while the remaining 54 were *Rattus rattus*. The *traD* qPCR was positive in 27 chigger pools by melt curve analysis and agarose gel electrophoresis. *Orientia tsutsugamushi* DNA was detected by *traD* qPCR in chigger pools comprising *Ascoschoengastia* spp., *L. deliense*, *L. jayawickremei*, *S. ligula*, and *L. rajasthanense*. Of the 27 chigger pools positive by *traD*, 18 were from Hosur, while the remaining 9 were from Vellore. The 47 kDa qPCR and 56 kDa nested PCR (Horinouchi protocol) were positive in seven and three chigger pools (refer Table 1 for details). The chigger pools positive by 47 kDa and 56 kDa gene were from chiggers collected in Hosur, while none were positive by these two assays from chiggers collected in Vellore. The Furuya protocol was negative in all the 57 chigger pools tested. The details of the rodents captured, chigger species identified by morphometry, and *O. tsutsugamushi* PCR results of pools tested are given in Table 2.

Four chigger traD amplicons were sequenced and showed 95–100% homology (BLASTn analysis) with *O. tsutsugamushi* details as given in Table 3. All the three *Ascoschoengastia* pools positive by 56 kDa nested PCR (nPCR; Horinouchi protocol) were sequenced. These three sequences (Hosur TNHB, Hosur AN, and Hosur 19/78) have been submitted to the GenBank with accession numbers MW604716–MW604718. A homology ranging from 99.66 to 99.83% with *O. tsutsugamushi* sequences (MH003839, MH922787, MH003838, and MH003841) was obtained by Nucleotide BLAST (NCBI, Bethesda,

Table 1. Chigger species identified in this study with *Orientia tsutsugamushi* positivity

Chigger species	Number of pools tested (*n*[a])	*TraD* Pos (%)	47 kDa Pos (%)	56 kDa Pos (%)
Aschoschoengastia spp.	22	18 (81.8)	7 (31.8)	3 (13.6)
L. deliense	8	2 (25)	0 (0)	0 (0)
L. jayawickremei	9	2 (22.2)	0 (0)	0 (0)
L. rajasthanense	6	1 (16.7)	0 (0)	0 (0)
L. scutellare	5	0 (0)	0 (0)	0 (0)
Aschoschoengastia spp. and *L rajasthanense*	2	0 (0)	0 (0)	0 (0)
Leptotrombidium. spp. and *Schoengastiella* spp.	2	2 (100)	0 (0)	0 (0)
Schoengastiella ligula	2	2 (100)	0 (0)	0 (0)
Walchia hayashi	1	0 (0)	0 (0)	0 (0)
Total pools tested	57	27 (47.4)	7 (12.3)	3 (5.3)

[a]Chigger pools tested (20 chiggers per pool).

Table 2. Details of vector survey along with results of molecular assays

ID	Rodent	Date	Area	47 kDa	*TraD*	56 kDa	Chigger species identified
C1	*R. rattus*	15 Mar. 2018	Hosur	0	1	0	*Aschoschoengastia* spp.
C2	*R. rattus*	19 Apr. 2018	Hosur	1	1	1	*Aschoschoengastia* spp.
C3	*R. rattus*	10 May 2018	Hosur	1	1	1	*Aschoschoengastia* spp.
C 4	*R. rattus*	26 May 2018	Hosur	0	1	0	*Aschoschoengastia* spp.
C 5	*R. rattus*	6 June 2018	Hosur	0	1	0	*Aschoschoengastia* spp.
C6	*R. rattus*	9 June 2019	Hosur	0	0	0	*Aschoschoengastia* spp.
C7	*R. rattus*	9 June 2019	Hosur	0	0	0	*Aschoschoengastia* spp.
C8	*R. rattus*	26 July 2019	Hosur	0	0	0	*Aschoschoengastia* spp. and *L rajasthanense*
C9	*R. rattus*	10 Oct. 2019	Hosur	0	0	0	*Aschoschoengastia* spp.
C10	*R. rattus*	10 Oct. 2019	Hosur	0	0	0	*Aschoschoengastia* spp.
C11	*R. rattus*	22 Oct. 2019	Hosur	0	0	0	*Aschoschoengastia* spp. and *L rajasthanense*
C12	*R. rattus*	04 June 2020	Hosur	0	1	0	*Aschoschoengastia* spp.

Table 2. Continued

ID	Rodent	Date	Area	47 kDa	*TraD*	56 kDa	Chigger species identified
C13	*R. rattus*	10 June 2020	Hosur	0	1	0	*Aschoschoengastia* spp.
C14	*R. rattus*	11 June 2020	Hosur	1	1	0	*Aschoschoengastia* spp.
C15	*R. rattus*	12 June 2020	Hosur	0	1	0	*Aschoschoengastia* spp.
C16	*R. rattus*	13 June 2020	Hosur	1	1	1	*Aschoschoengastia* spp.
C17	*R. rattus*	14 June 2020	Hosur	0	1	0	*Aschoschoengastia* spp.
C18	*R. rattus*	15 June 2020	Hosur	1	1	0	*Aschoschoengastia* spp.
C19	*R. rattus*	15 June 2020	Hosur	0	1	0	*Aschoschoengastia* spp.
C20	*R. rattus*	16 June 2020	Hosur	0	1	0	*Aschoschoengastia* spp.
C21	*R. rattus*	17 June 2020	Hosur	0	1	0	*Aschoschoengastia* spp.
C22	*R. rattus*	18 June 2020	Hosur	1	1	0	*Aschoschoengastia* spp.
C23	*R. rattus*	19 June 2020	Hosur	1	1	0	*Aschoschoengastia* spp.
C24	*R. norveigicus*	20 June 2020	Hosur	0	1	0	*Aschoschoengastia* spp.
C25	*R. rattus*	2 Sept. 2018	Vellore	0	0	0	*L. rajasthanense*
C26	*R. rattus*	28 Sept. 2018	Vellore	0	1	0	*L. deliense*
C27	*R. rattus*	28 Sept. 2018	Vellore	0	0	0	*L. deliense*
C28	*R. rattus*	23 Oct. 2018	Vellore	0	1	0	*L. deliense*
C29	*R. rattus*	23 Oct. 2018	Vellore	0	0	0	*L. deliense*
ID	Rodent	Date	Area	47 kDa	TraD	56 kDa	Chigger species identified
C30	*R. rattus*	24 Oct. 2018	Vellore	0	0	0	*L. rajasthanense*
C31	*R. rattus*	30 Nov. 2018	Vellore	0	0	0	*L. scutellare*
C32	*R. rattus*	4 Dec. 2018	Vellore	0	0	0	*L. scutellare*
C33	*R. rattus*	4 Dec. 2018	Vellore	0	0	0	*Walchia hayashi*
C34	*R. rattus*	17 Dec. 2018	Vellore	0	1	0	*L. rajasthanense*
C35	*R. rattus*	17 Dec. 2018	Vellore	0	0	0	*L. rajasthanense*
C36	*R. rattus*	17 Dec. 2018	Vellore	0	0	0	*L. rajasthanense*
C37	*R. rattus*	22 Dec. 2018	Vellore	0	0	0	*L. jayawickremei*
C38	*R. rattus*	22 Dec. 2018	Vellore	0	0	0	*L. jayawickremei*
C39	*R. rattus*	26 Dec. 2018	Vellore	0	0	0	*L. jayawickremei*
C40	*R. norveigicus*	5 Jan. 2019	Vellore	0	0	0	*L. jayawickremei*
C41	*R. rattus*	5 Jan. 2019	Vellore	0	0	0	*L. jayawickremei*
C42	*R. rattus*	7 Jan. 2019	Vellore	0	1	0	*L. jayawickremei*
C43	*R. rattus*	7 Jan. 2019	Vellore	0	0	0	*L. jayawickremei*
C44	*R. rattus*	8 Jan. 2019	Vellore	0	0	0	*L. jayawickremei*
C45	*R. rattus*	24 Jan. 2019	Vellore	0	0	0	*L. scutellare*
C46	*R. rattus*	29 Jan. 2019	Vellore	0	0	0	*L. deliense*
C47	*R. rattus*	29 Jan. 2019	Vellore	0	0	0	*L. deliense*
C48	*R. rattus*	29 Jan. 2019	Vellore	0	0	0	*L. deliense*
C49	*R. norveigicus*	30 Jan. 2019	Vellore	0	0	0	*L. deliense*
C50	*R. rattus*	5 Feb. 2019	Vellore	0	0	0	*L. scutellare*
C51	*R. rattus*	7 Feb. 2019	Vellore	0	0	0	*L. scutellare*
C52	*R. rattus*	24 Nov. 2019	Vellore	0	1	0	*L. jayawickremei*
C53	*R. rattus*	10 Feb. 2020	Vellore	0	1	0	*Schoengastiella ligula*
C54	*R. rattus*	10 Feb. 2020	Vellore	0	1	0	*Leptotrombidium*. sp. and *Schoengastiella* sp.
C55	*R. rattus*	10 Feb. 2020	Vellore	0	1	0	*Leptotrombidium*. sp. and *Schoengastiella* sp.
C56	*R. rattus*	10 Feb. 2020	Vellore	0	1	0	*Schoengastiella ligula*
C57	*R. rattus*	10 Feb. 2020	Vellore	0	0	0	*L rajasthanense*

Table 3. Homology of *traD* amplicons with *O. tsutsugamushi* strains (by BLAST)

Accession no. (strain)	Chigger ID			
	C4 (176 bp)	C13 (168 bp)	C20 (176 bp)	C28 (160 bp)
LS398549 (TA686)	100%	96.08%	99.43%	98.30%
AP008981 (Ikeda)	99.43%	97.06%	98.86%	97.73%
LS398550 (Kato)	99.43%	96.08%	98.86%	97.73%
LS398551 (Gilliam)	99.43%	96.08%	98.86%	97.73%
LS398552 (UT76)	99.43%	96.08%	98.86%	97.73%
LS398548 (Karp)	99.43%	96.00%	98.86%	97.73%
CP044031 (Wuj/2014)	98.86%	96.00%	98.29%	97.16%
LS398447 (UT176)	98.29%	96.08%	97.71%	97.16%
AM494475 (Boryong)	97.14%	95.10%	96.57%	95.45%

MD) analysis of the three sequences. Phylogenetic analysis (Seaview Ver4) performed by constructing a maximum likelihood tree showed the three sequences are clustering with Kato genogroup and TA716 genotype (Fig. 1). Type sequences used for assigning genotypes and genogroups to the three 56 kDa partial gene sequences (MW604716–18) amplified in the Hosur chiggers (*Ascoschoengastia* spp.) are as described previously (Kim et al. 2017, Long et al. 2020). Bootstrap values were calculated using the Seaview ver4 (Gouy et al. 2010) and annotated using the iTOL software (Letunic and Bork 2019). The accession numbers, genotype, genogroup, and length (in bp) of 56 kDa sequences of *O. tsutsugamushi* used for constructing and assessing the phylogeny of the three Hosur sequences are shown in Table 4.

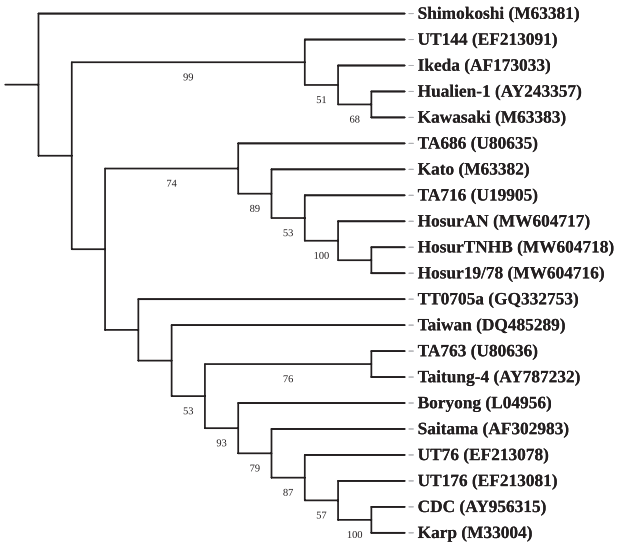

Fig. 1. Phylogenetic analysis of the 56 kDa gene obtained in this study (MW604716–MW604718) with representative sequences from the GenBank.

Discussion

Scrub typhus, a chigger (mite) borne disease, though underdiagnosed has emerged as a serious public health threat in the recent years (Long et al. 2020). It has also been documented to exist in areas such as South America, Middle East, sub-Saharan Africa and the traditional 'tsutsugamushi' triangle (Chao et al. 2019, Chaisiri et al. 2019, Acosta-Jamett et al. 2020). Various chigger species have been demonstrated to be vectors and reservoirs of this infection. Traditionally, 56 kDa gene, 47 kDa, and 16 S rRNA targets have been amplified to confirm the presence of *O. tsutsugamushi* in the vectors (Chaisiri et al. 2019, Elliott et al. 2019). Sequence analysis of the amplified complete or partial gene sequence of 56 kDa gene has been the traditional method for determining *O. tsutsugamushi* genotypes (Shim et al. 2009, Lin et al. 2011, Zhang et al. 2013, Luce-Fedrow et al. 2018).

The multicopy *traD* gene can have copies ranging from 32 to 1,000 gene copies in each *O. tsutsugamushi* genome. In contrast, the 47 kDa is a single-copy gene, i.e., there will be one copy of 47 kDa gene in one *O. tsutsugamushi* genome (Chao et al. 2019). In this study, we found that the *traD* SYBR Green PCR was more likely to be positive (detected 27), while 47 kDa was positive in only 7. This is in concordance with the findings of the other two research groups who have used this assay (Keller et al. 2014, Chao et al. 2019). Further validation of this finding requires studies using well-defined samples to determine the sensitivity and specificity of this assay. *Orientia tsutsugamushi* was detected in *Aschoschoengastia* spp., *L. deliense*, *L. jayawickremei*, *S. ligula*, and *L. rajasthanense* by *traD*. If 47 kDa qPCR and 56 kDa nPCR were used, only *Aschoschoengastia* spp. would have been detected. The *traD* gene, which has multiple copies per genome, seems to be a better target for detection of *O. tsutsugamushi* DNA in chiggers in contrast to the single-copy genes (47 and 56 kDa as observed by Keller et al. 2014 and Chao et al. 2019). The multicopy gene *traD* was the only assay detecting *O. tsutsugamushi* in *L. deliense*, *L. jayawickremei*, *L. rajasthanense*, and *S. ligula*. This suggests that these vectors have low copy numbers of the scrub typhus pathogen. Furthermore, PCRs targeting single-gene copies of *O. tsutsugamushi*

Table 4. Details of sequences used for assessing phylogeny of Hosur 56 kDa sequences

Strain ID	Accession no.	Genotype	Genogroup	Sequence length
HosurTNHB (C2)*	MW604718	Kato	Kato	592
HosurAN (C3)*	MW604717	Kato	Kato	598
Hosur19/78 (C16)*	MW604716	Kato	Kato	601
Karp USA	M33004	Karp_C	Karp	2,280
CDC Karp	AY956315	Karp	Karp	1,884
Shimokoshi	M63381	Shimokoshi	Shimokoshi	2,303
Kato Japan	M63382	Kato_B	Kato	2,271
Kawasaki	M63383	Kawasaki	Gilliam	2,260
TA716	U19905	Kato_A	Kato	1,575
UT176	EF213081	Karp B	Karp	1,602
UT76	EF213078	Karp A	Karp	1,541
Boryong	L04956	Boryong	Karp	1,944
Saitama	AF302983	Saitama	Karp	1,454
UT144	EF213091	JG_C	Gilliam	1,596
Hualien-1	AY243357	JG_B	Gilliam	1,842
Ikeda	AF173033	JG_A	Gilliam	1,424
Taiwan CDC	DQ485289	Gilliam	Gilliam	1,860
TT0705a	GQ332753	TD	Gilliam	1,593
Taitung-4	AY787232	TA763_B	TA763	1,889
TA763	U80636	TA763_A	TA763	1,581
TA686	U80635	TA686	TA686	1,599

*Our 56 kDa sequences integrated with Chigger pool ID.

being positive for *Ascoschoengastia* spp. suggest that this mite species probably carries a heavier burden of the scrub typhus pathogen. It can be probably hypothesized that *Ascoschoengastia* species is a better host for *O. tsutsugamushi*. Further prospective studies and experiments are needed to confirm or refute this hypothesis.

In this study, we detected *O. tsutsugamushi* DNA in *Ascoschoengastia* spp., *L. jayawickremei*, and *L. rajasthanense* for the first time in India. *Ascoschoengastia indica* has been reported in Thailand (Wulandhari et al. 2021) Vietnam (Binh et al. 2020), and Malaysia Paramasvaran et al. 2009), *Ascoschoengastia audyi* in Malaysia (Elliott et al. 2019, Stekolnikov 2021), while Takhampunya et al. (2019) reported *Ascoschoengastia* spp. like us as have Ernieenor et al. (2021) from Malaysia recently.

Leptotrombidium deliense has been reported as the most common vector of scrub typhus globally (Tilak and Kunte 2019) and in India from Puducherry (Devaraju et al. 2020) and Gorakhpur (Sadanandane et al. 2018). *Schoengastiella ligula* has been identified as infesting rodents in Darjeeling (Tilak et al. 2011), Puducherry (Candasamy et al. 2016), and Vellore (Rose et al. 2019) and was considered a possible vector, but this was considered controversial (Acosta-Jamett et al. 2020). Detection of the *traD* gene in two chigger pools of *S. ligula* suggests that this could be another potential vector of scrub typhus in Vellore. Though many members of the *Leptotrombidium* genus like *L. deliense* have been reported as vectors (Elliott et al. 2019, Tilak and Kunte 2019), this is the first report identifying *L. jayawickremei*, and *L. rajasthanense* as potential scrub typhus vectors.

We observed only two species of rodents infested with chiggers, the vast majority being *Rattus rattus*, while only three were *Rattus norveigicus*. This is because we set rodent traps around human dwellings, and these species are highly synanthropic (Puckett et al. 2020). Phylogenetic analysis of the 56 kDa gene sequence suggests that the three 56 kDa sequences analyzed belong to the TA716 genotype and Kato genogroup. This is also the predominant strain of *O. tsutsugamushi* observed in human cases in this region (Varghese et al. 2015).

Conclusion

In comparison with the 47 kDa qPCR assay, the *traD* SYBR green assay detected *O. tsutsugamushi* DNA more frequently. Our results suggest that *Ascoschoengastia* spp., *L. jayawickremei*, *L. rajasthanense*, *L. deliense*, and *S. ligula* can harbor *O. tsutsugamushi* in this area (North Tamil Nadu). Furthermore, prospective surveys focusing on vector capacity of these mites for scrub typhus are needed to definitively confirm these mite (chigger) species as scrub typhus vectors.

Supplementary Data

Supplementary data are available at *Journal of Medical Entomology* online.

Suppl Fig. 1. Areas where rodent trapping was performed.

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References Cited

Agrawal, V. C. 2000. Taxonomic studies on Indian Muridae and Hystricidae: Mammalia, Rodentia. Records of the Zoological Survey of India. Zoological Survey of India, Calcutta, India.

Acosta-Jamett, G., C. Martínez-Valdebenito, E. Beltrami, M. C. Silva-de La Fuente, J. Jiang, A. L. Richards, T. Weitzel, and K. Abarca. 2020. Identification of trombiculid mites (*Acari: Trombiculidae*) on rodents from Chiloé Island and molecular evidence of infection with *Orientia* species. PLoS Negl. Trop. Dis. 14: e0007619.

Akhunji, B., R. Bhate, N. Pansare, S. P. Chaudhari, W. Khan, N. V. Kurkure, S. W. Kolte, and S. B. Barbuddhe. 2019. Distribution of *Orientia tsutsugamushi* in rodents and mites collected from Central India. Environ. Monit. Assess. 191: 82.

Bhate, R., N. Pansare, S. P. Chaudhari, S. B. Barbuddhe, V. K. Choudhary, N. V. Kurkure, and S. W. Kolte. 2017. Prevalence and phylogenetic analysis of *Orientia tsutsugamushi* in Rodents and Mites from Central India. Vector Borne Zoonotic Dis. 17: 749–754.

Bhuyan, P. J., and A. J. Nath. 2015. Record of tropical rat mite, *Ornithonyssus bacoti* (Acari: Mesostigmata: Macronyssidae) from domestic and peridomestic rodents (*Rattus rattus*) in Nilgiris, Tamil Nadu, India. J. Arthropod. Borne. Dis. 10: 98–101.

Binh, M. D., S. C. Truong, D. L. Thanh, L. C. Ba, N. L. Van, and B. D. Nhu. 2020. Identification of trombiculid chigger mites collected on rodents from Southern Vietnam and molecular detection of *Rickettsiaceae* Pathogen. Korean J. Parasitol. 58: 445–450.

Bora, T., S. A. Khan, L. Jampa, and B. Laskar. 2018. Genetic diversity of *Orientia tsutsugamushi* strains circulating in Northeast India. Trans. R. Soc. Trop. Med. Hyg. 112: 22–30.

Candasamy, S., E. Ayyanar, K. Paily, P. A. Karthikeyan, A. Sundararajan, and J. Purushothaman. 2016. Abundance & distribution of trombiculid mites & *Orientia tsutsugamushi*, the vectors & pathogen of scrub typhus in rodents & shrews collected from Puducherry & Tamil Nadu, India. Indian J. Med. Res. 144: 893–900.

Chaisiri, K., A. C. Gill, A. A. Stekolnikov, S. Hinjoy, J. W. McGarry, A. C. Darby, S. Morand, and B. L. Makepeace. 2019. Ecological and microbiological diversity of chigger mites, including vectors of scrub typhus, on small mammals across stratified habitats in Thailand. Anim. Microbiome. 1: 18.

Chao, C.-C., T. Belinskaya, Z. Zhang, L. Jiang, and W.-M. Ching. 2019. Assessment of a sensitive qPCR assay targeting a multiple-copy gene to detect *Orientia tsutsugamushi* DNA. Trop. Med. Infect. Dis. 4: 113.

Choi, Y. J., I. Y. Lee, H. J. Song, J. Kim, H. J. Park, D. Song, and W. J. Jang. 2018. Geographical distribution of *Orientia tsutsugamushi* strains in chiggers from three provinces in Korea. Microbiol. Immunol. 62: 547–553.

Devaraju, P., B. Arumugam, I. Mohan, M. Paraman, M. Ashokkumar, G. Kasinathan, and J. Purushothaman. 2020. Evidence of natural infection of *Orientia tsutsugamushi* in vectors and animal hosts—risk of scrub typhus transmission to humans in Puducherry, South India. Indian J. Public Health 64: 27–31.

Elliott, I., I. Pearson, P. Dahal, N. V. Thomas, T. Roberts, and P. N. Newton. 2019. Scrub typhus ecology: a systematic review of *Orientia* in vectors and hosts. Parasit. Vectors 12: 513.

Ernieenor, F. C. L., M. J. NorJaiza, A. Fadillah, J. Canedy, and A. Mariana. 2021. Screening and genotyping of *Orientia tsutsugamushi* from field-collected on-host chiggers (*Acari: Prostigmata*) recovered from a positive scrub typhus locality in Kelantan, Malaysia. Exp. Appl. Acarol. 84: 171–182.

Fernandes, S., and Kulkarni, S. M. 2003. Studies on the trombiculid mite fauna of India. Records of the Zoological Survey of India. The Survey, Kolkata, India.

Gouy, M., S. Guindon, and O. Gascuel. 2010. SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. Mol. Biol. Evol. 27: 221–224.

Jiang, J., T. C. Chan, J. J. Temenak, G. A. Dasch, W. M. Ching, and A. L. Richards. 2004. Development of a quantitative real-time polymerase chain reaction assay specific for *Orientia tsutsugamushi*. Am. J. Trop. Med. Hyg. 70: 351–356.

Keller, C. A., M. Hauptmann, J. Kolbaum, M. Gharaibeh, M. Neumann, M. Glatzel, and B. Fleischer. 2014. Dissemination of *Orientia tsutsugamushi* and inflammatory responses in a murine model of scrub typhus. PLoS Negl. Trop. Dis. 8: e3064.

Khan, S. A., T. Bora, S. Chattopadhyay, J. Jiang, A. L. Richards, and P. Dutta. 2016. Seroepidemiology of rickettsial infections in Northeast India. Trans. R. Soc. Trop. Med. Hyg. 110: 487–494.

Kumar, A., M. Biswal, K. Zaman, N. Sharma, V. Suri, and A. Bhalla. 2019. Genetic diversity of *Orientia tsutsugamushi* strains from patients in north India. Int. J. Infect. Dis. 84: 131–135.

Kumlert, R., K. Chaisiri, T. Anantatat, A. A. Stekolnikov, S. Morand, A. Prasartvit, B. L. Makepeace, S. Sungvornyothin, and D. H. Paris. 2018. Autofluorescence microscopy for paired-matched morphological and molecular identification of individual chigger mites (Acari: Trombiculidae), the vectors of scrub typhus. PLoS One 13: e0193163.

Kuo, C. C., P. L. Lee, C. H. Chen, and H. C. Wang. 2015. Surveillance of potential hosts and vectors of scrub typhus in Taiwan. Parasit. Vectors 8: 611.

Lee, H. I., S. K. Shim, B. G. Song, E. N. Choi, K. J. Hwang, M. Y. Park, C. Park, and E. H. Shin. 2011. Detection of *Orientia tsutsugamushi*, the causative agent of scrub typhus, in a novel mite species, *Eushoengastia koreaensis*, in Korea. Vector Borne Zoonotic Dis. 11: 209–214.

Letunic, I., and P. Bork. 2019. Interactive Tree Of Life (iTOL) v4: recent updates and new developments. Nucl. Acids Res. 47(W1): W256–W259. doi:10.1093/nar/gkz239

Lin, P. R., H. P. Tsai, P. Y. Tsui, M. H. Weng, M. D. Kuo, H. C. Lin, K. C. Chen, D. D. Ji, D. M. Chu, and W. T. Liu. 2011. Genetic typing, based on the 56-kilodalton type-specific antigen gene, of *Orientia tsutsugamushi* strains isolated from chiggers collected from wild-caught rodents in Taiwan. Appl. Environ. Microbiol. 77: 3398–3405.

Long, J., Y. Wei, X. Tao, P. He, J. Xu, X. Wu, W. Zhu, K. Chen, and Z. Yang. 2020. Representative genotyping, recombination and evolutionary dynamics analysis of TSA56 gene segment of *Orientia tsutsugamushi*. Front. Cell. Infect. Microbiol. 10: 383.

Luce-Fedrow, A., M. L. Lehman, D. J. Kelly, K. Mullins, A. N. Maina, R. L. Stewart, H. Ge, H. S. John, J. Jiang, and A. L. Richards. 2018. A review of scrub typhus (*Orientia tsutsugamushi* and related organisms): then, now, and tomorrow. Trop. Med. Infect. Dis. 3: 8.

Masakhwe, C., P. Linsuwanon, G. Kimita, B. Mutai, S. Leepitakrat, S. Yalwala, D. Abuom, N. Auysawasi, T. Gilbreath, E. Wanja, and J. Waitumbi. 2018. Identification and characterization of *Orientia chuto* in Trombiculid chigger mites collected from wild rodents in Kenya. J. Clin. Microbiol. 56: e01124-18.

Paramasvaran, S., R. A. Sani, L. Hassan, M. Krishnasamy, J. Jeffery, P. Oothuman, I. Salleh, K. H. Lim, M. G. Sumarni, and R. L. Santhana. 2009. Ectoparasite fauna of rodents and shrews from four habitats in Kuala Lumpur and the states of Selangor and Negeri Sembilan, Malaysia and its public health significance. Trop. Biomed. 26: 303–311.

Park, J. W., J. K. Chung, S. H. Kim, S. J. Cho, Y. D. Ha, S. H. Jung, H. J. Park, H. J. Song, J. Y. Lee, D. M. Kim, et al. 2016. Seroepidemiological survey of zoonotic diseases in small mammals with PCR detection of *Orientia tsutsugamushi* in chiggers, Gwangju, Korea. Korean J. Parasitol. 54: 307–313.

Park, J. W., D. S. Yu, G. S. Lee, J. J. Seo, J. K. Chung, and J. I. Lee. 2020. Epidemiological characteristics of rodents and chiggers with *Orientia Tsutsugamushi* in the Republic of Korea. Korean J. Parasitol. 58: 559–564.

Prakash, J. A. J. 2017. Scrub typhus: risks, diagnostic issues, and management challenges. Res. Rep. Trop. Med. 8: 73–83.

Puckett, E. E., D. Orton, and J. Munshi-South. 2020. Commensal rats and humans: integrating rodent phylogeography and zooarchaeology to highlight connections between human societies. Bioessays 42: e1900160.

Rose, W., G. Kang, V. P. Verghese, S. Candassamy, P. Samuel, J. J. A. Prakash, and J. Muliyil. 2019. Risk factors for acquisition of scrub typhus in children admitted to a tertiary centre and its surrounding districts in South India: a case control study. BMC Infect. Dis. 19: 665.

Sadanandane, C., P. Jambulingam, K. P. Paily, N. P. Kumar, A. Elango, K. A. Mary, S. Agatheswaran, T. Sankari, and B. B. Mishra. 2018. Occurrence of *Orientia tsutsugamushi*, the etiological agent of scrub typhus in animal hosts and mite vectors in areas reporting human cases of acute encephalitis syndrome in the Gorakhpur Region of Uttar Pradesh, India. Vector Borne Zoonotic Dis. 18: 539–547.

Seo M. G., B. G. Song, T. K. Kim, B. E. Noh, H. S. Lee, W. G. Lee, and H. I. Lee. 2021. Nationwide incidence of chigger mite populations and molecular detection of *Orientia tsutsugamushi* in the Republic of Korea, 2020. Microorganisms. 9: 1563.

Shim, S. K., E. N. Choi, K. O. Yu, H. J. Park, C. M. Kim, K. H. Lee, J. K. Park, P. H. Park, M. H. Yoon, S. H. Park, et al. 2009. Characterisation of *Orientia tsutsugamushi* genotypes from wild rodents and chigger mites in Korea. Clin. Microbiol. Infect. 15 (Suppl 2): 311–312.

Stekolnikov, A. A. 2021. A checklist of chigger mites (Acariformes: Trombiculidae) of Southeast Asia. Zootaxa 4913: 1–163. doi:10.11646/zootaxa.4913.1.1

Takhampunya, R., A. Korkusol, C. Pongpichit, K. Yodin, A. Rungrojn, N. Chanarat, S. Promsathaporn, T. Monkanna, S. Thaloengsok, B. Tippayachai, et al. 2019. Metagenomic approach to characterizing disease epidemiology in a disease-endemic environment in Northern Thailand. Front. Microbiol. 10: 319.

Tantibhedhyangkul, W., E. Wongsawat, S. Silpasakorn, D. Waywa, N. Saenyasiri, J. Suesuay, W. Thipmontree, and Y. Suputtamongkol. 2017. Use of multiplex real-time PCR to diagnose scrub typhus. J. Clin. Microbiol. 55: 1377–1387.

Tilak, R., and R. Kunte. 2019. Scrub typhus strikes back: are we ready? Med. J. Armed Forces India 75: 8–17.

Tilak, R., R. Kunwar, U. B. Wankhade, and V. W. Tilak. 2011. Emergence of *Schoengastiella ligula* as the vector of scrub typhus outbreak in Darjeeling: has *Leptotrombidium deliense* been replaced? Indian J. Public Health 55: 92–99.

Varghese, G. M., J. Janardhanan, S. K. Mahajan, D. Tariang, P. Trowbridge, J. A. Prakash, T. David, S. Sathendra, and O. C. Abraham. 2015. Molecular epidemiology and genetic diversity of *Orientia tsutsugamushi* from patients with scrub typhus in 3 regions of India. Emerg. Infect. Dis. 21: 64–69.

Wulandhari S. A., Y. Paladsing, W. Saesim, V. Charoennitiwat, P. Sonthayanon, R. Kumlert, S. Morand, S. Sumruayphol, and K. Chaisiri. 2021. High prevalence and low diversity of chigger infestation in small mammals found in Bangkok Metropolitan parks. Med. Vet. Entomol. doi:10.1111/mve.12531

Zhang, M., Z. T. Zhao, H. L. Yang, A. H. Zhang, X. Q. Xu, X. P. Meng, H. Y. Zhang, X. J. Wang, Z. Li, S. J. Ding, et al. 2013. Molecular epidemiology of *Orientia tsutsugamushi* in chiggers and ticks from domestic rodents in Shandong, northern China. Parasit. Vectors 6: 312.