Abundance & distribution of trombiculid mites & *Orientia tsutsugamushi*, the vectors & pathogen of scrub typhus in rodents & shrews collected from Puducherry & Tamil Nadu, India

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Background & objectives: Human cases of scrub typhus are reported every year from Puducherry and adjoining areas in southern India. However, information on the presence of causative agent, *Orientia tsutsugamushi*, and its vectors is lacking. Hence, the objective of the study was to find out the vector as well as pathogen distribution in rodents and shrews present in the scrub typhus-reported areas in southern India.

Methods: Trombiculid mites were collected by combing rats and shrews collected using Sherman traps and identified to species level following standard taxonomical keys. The serum samples of the animals were used for Weil–Felix test and the clots containing blood cells were used for DNA extraction and polymerase chain reaction (PCR).

Results: A total of 181 animals comprising four rodent species and one shrew species were collected from 12 villages. High proportion of chiggers was collected from the shrew, *Suncus murinus* (79.1%) and *Rattus rattus* (47.6%). A total of 10,491 trombiculid mites belonging to nine species were collected. *Leptotrombidium deliense*, the known vector of scrub typhus pathogen, was the predominant species (71.0%) and the chigger (*L. deliense*) index was 41.1 per animal. Of the 50 animals screened for the pathogen, 28 showed agglutination against OX-K in Weil–Felix test indicating the presence of antibodies against *O. tsutsugamushi,* the causative agent of scrub typhus. PCR carried out with the DNA extracted from blood samples of two of the animals were positive for GroEl gene of *O. tsutsugamushi*.

Interpretation & conclusions: *L. deliense* index was well above the critical limit of chigger load, indicating that all the villages were receptive for high risk of transmission of scrub typhus to human. Pathogen positivity was higher among animals collected from villages recorded for higher chigger indices due to active transmission between the chigger mites and reservoir host animals. The results are suggestive of routine vector/pathogen surveillance at hot spots to initiate timely preventive measures.

Scrub typhus is an acute, febrile disease caused by infection with *Orientia tsutsugamushi* (Family: *Rickettsiaceae*). It is an obligate intracellular Gram-negative bacterium, transmitted among small animals and to humans by some species of larval trombiculid mites (chiggers). Mites carry the bacterium from larval stages to adults and to their progenies through transtadial and transovarial transmission. Small animals such as rodents and shrews act as natural or maintenance hosts[1]. Approximately one million cases of scrub typhus occur each year and more than a billion people are at risk worldwide[2]. Mortality rates in untreated patients range from 0 to 30 per cent depending on the geographical region. It is endemic in the Asia-Pacific region, known as the 'tsutsugamushi triangle'[3,4].

Scrub typhus is considered as a re-emerging infectious disease in India[5]. Recently, outbreaks of scrub typhus have been reported all over India including southern States[6,7]. It has emerged as an important cause of febrile illness in Puducherry and adjoining areas of Tamil Nadu, and confirmed cases have been reported every year[8]. However, no information is available on the occurrence of the causative agent of the disease, *O. tsutsugamushi*, on the host animals such as rodents and shrews and its vectors. Therefore, a preliminary study was undertaken to examine the abundance and distribution of trombiculid mite vectors through collection and identification of mites from the animals trapped from areas reported for human cases of scrub typhus.

Material & Methods

The study area was Puducherry district of the Union Territory of Puducherry and adjoining areas of Tamil Nadu, India. The district has plain land with dry and evergreen species of vegetation typical of tropical regions. Puducherry experiences tropical maritime climate with moderate variation in temperature and rainfall. The mean maximum temperature is 38.2°C and mean minimum temperature is 24°C. The average annual rainfall is about 126 cm and almost 68 per cent of it occurs from October to December.

The survey was carried out in 12 villages selected randomly from areas reported with human cases of scrub typhus. The data on human cases of scrub typhus were obtained from Puducherry Institute of Medical Sciences (PIMS), Puducherry. The diagnosis of scrub typhus was done by detection of the pathogen through immunoglobulin M enzyme-linked immunosorbent assay (ELISA), Weil–Felix test and nested polymerase chain reaction (PCR). Of the 12 villages, nine were from Puducherry district and three from adjoining Viluppuram district of Tamil Nadu. Month-wise collection of trombiculid mites was done in all the selected villages from November 2013 to October 2014.

Trapping and identification of rodents and shrews: Rodents and shrews were trapped using Sherman traps of the size of 3" × 3" × 10" (W × H × L), designed for live capture of rats. In each of the selected village, traps were set outdoors (peri-domestic areas) in pre-selected sites with scrubby vegetation and rodent burrows. Fifteen to 25 traps were placed in each of the villages selected on each day of sampling. The traps were baited with fried coconut and peanut butter. The traps were placed one hour before sun set (1700 h) and retrieved the next day morning (0600 h). The captured animals were anaesthetized and identified through morphological features[9].

This study was conducted in the ICMR - Vector Control Research Centre, Puducherry. The study was approved by the institutional animal ethics committee.

Collection of ectoparasites and identification: The ectoparasites including chigger (larval) mites were collected by combing the animals against the fur over a white enamel tray. The snout, ears, limbs and axillary regions of individual animals were combed and the ectoparasites were preserved in 70 per cent ethanol until they were mounted on slides. Mites were mounted in Hoyer's medium[10], examined under microscope and identified up to species level, following standard taxonomical keys[11]. Other ectoparasites collected were mounted in Hoyer's medium, examined under microscope and identified using the standard taxonomical keys[12].

Detection of rickettsial pathogens in animals through serological assay and polymerase chain reaction (PCR): After collection of ectoparasites, blood samples were collected from the animals. A total of 50 rodents and shrews, selected randomly from four of the study areas, were used. The numbers of different species used depended on their availability from these places. From each animal, 1 ml of blood sample was taken through heart puncture without anticoagulant and serum separated. The serum samples were used for Weil–Felix test[13] and the clots containing blood cells were used for DNA extraction and PCR. Weil–Felix test for antibody titre was done against three antigens, namely, OX-19 (*Rickettsia typhi*), OX-2

(*Rickettsia conorii*) and OX-K (*O. tsutsugamushi*)[14] (Progen, Tulip diagnostics, Goa). The serum samples were diluted in physiological saline to get doubling dilutions of 1:10-1:160. An equal amount of the respective antigen was added to get final dilutions of 1:20-1:320. Tubes were incubated overnight at 37°C and the readings were taken. Matt formation in the tube due to antigen and antibody reaction was taken as positive. Button formation due to the absence of the reaction was counted as negative. Samples reacted with OX-19 antigen were considered to be positive for antibodies against murine typhus. Samples reacted with OX-2 antigen were attributed to be positive for antibodies against tick typhus and those reacted with OX-K antigen were considered to be positive for antibodies against scrub typhus. Against all the above antigens, matt formation at 1:80 dilution was taken as positive for the respective antibodies[15].

Extraction of DNA from rat blood samples was done using GenElute Blood Genomic DNA kit (Sigma-Aldrich, USA). The extracted DNA was used as templates for amplification using primers. All the samples were processed for amplification of three different genes, namely, 56 kDa, GroEl and 16s rRNA of *O. tsutsugamushi* either through conventional or nested PCR. Detection of gene encoding 56 kDa, which amplifies 483 bp segments, was done through nested PCR, following the method described by Saisongkorh *et al*[16]. Primers used for the reaction were as follows: F'-5′- TCAAGCTTATTGCTGAGTG CAATGTCTGC-3'; R'-5'-AGGGATCCCTGCTGCTGTGCTTGCTGCG-3' for the first round and F'- 5′-GATCAAGCTTCTC AGCCTACTATAATGCCC-3'; R'-5'-CTAGGGATCC CGACAGAGCACTATTAGGC-3' for the second round. The PCR amplification mixture contained Green Master Mix (Promega, Madison, USA), each of 10 pmol of forward and reverse primers, and 1 μl of extracted DNA in a final volume of 25 μl. The cycling conditions were 95°C for 10 sec, 57°C for 30 sec and 72°C for 1 min, which was repeated 30 times, in a thermocycler (Eppendorf, Germany). The presence of diagnostic amplicons were visualized on a gel documentation system (GelDoc-It Imaging System, UVP, California, USA) after electrophoresis on 1.5 per cent agarose gel containing 0.5 μg/ml ethidium bromide.

The method involved in the detection of 16s rRNA through the amplification of a 220 bp segment was as described by Sonthayanon *et al*[17]. The primers used were as follows: F- 5'-CGAATTAATGCTGAGTTTGCTTAG-3';

R-5'-CTCTCAGACCAGCTAGAGATCACA-3'. The reaction mixture contained Green Master Mix, each of 10 pmol forward and reverse primers, and 4 μl of the DNA in a final volume of 30 μl. The thermal conditions were 35 cycles of 95°C for 1 min, 61°C for 1 min and 72°C for 30 sec in an Eppendorf thermocycler.

The primers used for GroEl gene PCR were as follows: F-5'-TTGCTGATGATGTAGACGGA-3'; R-5'-TGTTCACAACGAGAATTAACTT-3' for amplification of 300 bp segment. Primers were designed from the conserved regions of GroEl gene sequences obtained from NCBI (*https://www.ncbi.nlm. nih.gov/*) using Primer 3 software (*http://simgene.com/ Primer3*) and manufactured by Eurofins, Bangalore. The PCR reaction mixture contained Green Master Mix, 10 pmol each of forward and reverse primers, and 4 μl of the DNA in a final volume of 30 μl. Samples which showed amplification were sequenced using the respective forward primer and BigDye terminator (Applied Biosystems, USA). Sequences were obtained in an ABI automated Genetic analyzer 3130XL (Applied Biosystems, USA), edited using BioEdit 7.0.0 version (Ibis Biosciences, Carlsbad, USA), and analyzed using BLAST (*https://blast.ncbi.nlm.nih.gov/ Blast.cgi*).

Statistical analysis: The trap positivity rate and chigger infestation index (average number of chiggers per animal) were estimated. The difference in the chigger indices between villages/animal reservoirs was tested using one-way analysis of variance. The relationship between monthly chigger indices and incidence of human cases of scrub typhus was analyzed using Poisson regression analysis. The statistical analysis was carried out using the STATA SE 9.0 version, Stata Corp., Texas, USA.

Results

Village-wise number of traps placed, number of animals trapped and ectoparasites retrieved are given in Table I. During the study period, a total of 181 rodents and shrews were collected from 1422 traps set in the 12 villages. The trap positivity rate ranged from 3.6 to 21.8 per cent in different villages, and the overall trap positivity rate was 12.7 per cent.

The animals trapped during the survey belonged to two orders, namely, *Rodentia* with four species and *Soricomorpha* with one species. The species of rodents were *Rattus rattus* (44.8%), *Bandicota bengalensis* (11.0%), *Tatera indica* (0.55%) and *Mus musculus* (0.55%). The species belonging to *Soricomorpha*

Table I. Number of rodents and ectoparasites collected from the study villages of Puducherry

Area/Villages	Number of traps set	Number of rodents collected	Trap positivity (%)	Total ectoparasites collected
Bahour	105	11	10.5	645
Bommayapalayam	103	17	16.5	358
Chinnakalapet	110	16	13.3	524
Kadaperikuppam	117	10	8.5	1661
Keel Kozhuvari	134	23	17.2	1142
Mangalam	122	13	10.7	895
Kozhuvari	100	13	13.0	769
Periyakalapet	124	27	21.8	966
Pillaichavady	131	18	13.7	1152
Pudupattu	119	13	10.9	675
Thimmanayakanpalayam	136	16	11.8	1940
Thirubuvanai	111	4	3.6	627
Total	1422	181	12.7	11,354

Table II. Species diversity of trombiculid mites collected in different study villages of Puducherry

Species	Area/Villages												
	Bahour	Bommayapalayam	Chinnakalapet	Kadaperikuppam	Keel Kozhuvari	Mangalam	Kozhuvari	Periyakalapet	Pillaichavady	Pudupattu	Thimmanayakanpalayam	Thirubuvanai	Total
L. deliense	408 (65.0)	307 (92.2)	432 (97.7)	696 (43.1)	411 (54.4)	631 (72.6)	248 (36.7)	883 (97.4)	1094 (96.6)	512 (79.0)	1468 (76.4)	354 (62.7)	7444 (71.0)
L. insigne	68	6	0	834	172	144	346	0	8	104	211	61	1964 (18.7)
Schoengastiella sp.	139	1	1	64	5	86	18	16	24	18	65	139	576 (5.5)
T. hypodermata	9	6	1	11	0	3	24	5	6	6	111	8	190 (1.8)
Microtrombicula sp.	0	2	0	5	43	4	28	0	0	4	40	3	129 (1.2)
Helenicula sp.	0	2	0	0	79	0	0	0	0	1	0	0	82 (0.8)
Schoengastia sp.	1	2	7	2	44	1	12	0	0	1	2	0	72 (0.7)
Walchia sp.	3	7	1	2	0	0	0	3	0	2	15	0	33 (0.3)
Schoutedenichia sp.	0	0	0	0	1	0	0	0	0	0	0	0	1 (0.01)
Total	628	333	442	1614	715	869	676	907	1132	648	1912	565	10,491

Figures in parentheses denote per cent. *T. hypodermata*, *Trombicula hypodermata*; *L. insigne*, *Leptotrombidium insigne*

collected was *Suncus murinus* (43.1%). Among these animals, 89 per cent were positive for ectoparasites such as mites, ticks, lice and/or fleas, and the total number collected was 11,354. The overall ectoparasite index was 62.7 per animal. More importantly, 83 per cent of the trapped animals were positive for trombiculid mites and 92.4 per cent of the total ectoparasite fauna sampled were trombiculid mites.

Species diversity and spatial distribution of trombiculid mites: Species diversity of trombiculid mites collected from different study villages is given in Table II. A total of 10,491 trombiculid mites belonging to nine

Table III. Infestation of mites per rodent (chigger index)					
Rodent species	Number of rats collected	Number of mites collected	Number of mites per rodent	Number of *L. deliense* collected	Number of *L. deliense* per rodent
B. bengalensis	20	439	22.0	332	16.6
R. rattus	81	3868	47.6	2599	32.1
S. murinus	78	6173	79.1	4508	57.8
T. indica	1	11	11.0	5	5.0
M. musculus	1	0	0	0	0
Total	181	10491	58.0	7444	41.1

B. bengalensis, Bandicota bengalensis; *R. rattus, Rattus rattus*; *S. murinus, Suncus murinus*; *T. indica, Tatera indica*;
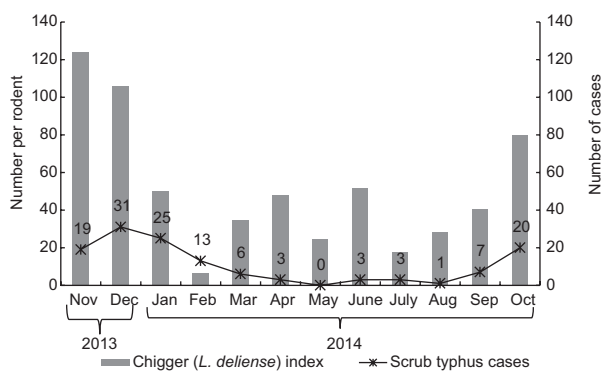*M. musculus, Mus musculus*; *L. deliense, Leptotrombidium deliense*

Fig. 1. Month-wise reported cases of scrub typhus and estimated chigger (*Leptotrombidium deliense*) index.

species were retrieved from the trapped animals and *Leptotrombidium deliense* was the predominant species (71.0%) followed by *L. insigne* (18.7%) and *Schoengastiella* sp. (5.5%). The other trombiculid mite species collected were *Trombicula hypodermata, Microtrombicula* sp., *Helenicula* sp., *Schoengastia* sp., *Walchia* sp. and *Schoutedenichia* sp. (Table II). Village-wise analysis showed predominance of *L. deliense* in 10 out of 12 villages. The percentage of *L. deliense* ranged from 36.7 to 97.7 per cent in different villages surveyed.

Chigger infestation index: Majority of the trombiculid mites (58.8%) was collected from the known index animal of scrub typhus, *i.e. S. murinus* followed by *R. rattus* (36.9%), *B. bengalensis* (4.2%) and *T. indica* (0.10%). The overall chigger (all trombiculid mites) index was the highest of 79.1 for *S. murinus*, and it was, respectively, 47.6, 22.0 and 11.0 for *R. rattus*, *B. bengalensis* and *T. indica* (Table III). The chigger index for *S. murinus* was significantly higher than that of *R. rattus* and *B. bengalensis* ($P<0.05$). The *L. deliense* index was also the highest for *S. murinus* (57.8) as compared to *R. rattus* (32.1), *B. bengalensis* (16.6) and *T. indica* (5.0) ($P<0.01$).

The chigger (all trombiculid mites) index estimated from different villages ranged from 19.5 to 161.4 and the overall index was 58.0 per animal. The index of the known vector mite, *L. deliense,* ranged from 17.9 to 91.8 in different villages surveyed, and the overall index was 41.1 per animal. The index for *Schoengastiella* sp., the suspected vector of scrub typhus pathogen, ranged from 0.06 to 34.8 in different villages and the overall index was 3.2 per animal.

Seasonal distribution of scrub typhus cases and chigger mites: The data on human cases of scrub typhus reported during the study period (November 2013-October 2014) were obtained from PIMS, Puducherry, and analyzed. Month-wise reported human cases of scrub typhus and estimated chigger indices are presented in Fig. 1. Scrub typhus cases were reported in all the months, except in May. Most of the cases occurred in the relatively cooler months (October-January) and the peak was in December. Very few cases were recorded from April to August. Month-wise analysis of chigger index showed that it was higher during the cooler months (October-December) and was at its peak in November. The number of human cases of scrub typhus reported in different months showed a significant association with that of the chigger index ($P<0.05$).

Ectoparasites other than trombiculid mites: A total of 863 ectoparasites, other than trombiculid mites, were retrieved from the animals. Of these, the fur mite, *Demodex* sp. (Order: Trombidiformes) formed 56.0 per cent of the total other ectoparasites collected. The tropical rat mite *Ornithonyssus bacoti* (Order: Mesostigmata) was the other species of mite collected (0.58%). Brown dog ticks *Rhipicephalus sanguineus* (29.2%) and spined rat lice *Polyplax spinulosa* (13.3%) were also retrieved from the trapped animals. Flea species *Xenopsylla cheopis* formed 0.9 per cent of the

| Table IV. Rodent serum samples positive for agglutination against OX-K/OX-19/OX-2 antigens in Weil–Felix test					
Rodent species	Number tested	Number of positive for agglutination against OX-K (scrub typhus) Titre=1:80/1:160	Number of positive for agglutination against OX-19/OX-2 (murine/tick typhus) Titre=1:80/1:160	Number of positive for agglutination against OX-19/OX-2/OX-K (murine/tick/scrub typhus) Titre=1:80/1:160	Number of positive for PCR (GroEl gene)
B. bengalensis	6	4	2	1	0
R. rattus	28	14	8	4	1
S. murinus	16	10	8	6	1
Total	50	28	18	11	2
B. bengalensis, *Bandicota bengalensis*; *R. rattus*, *Rattus rattus*; *S. murinus*, *Suncus murinus*; PCR, polymerase chain reaction					
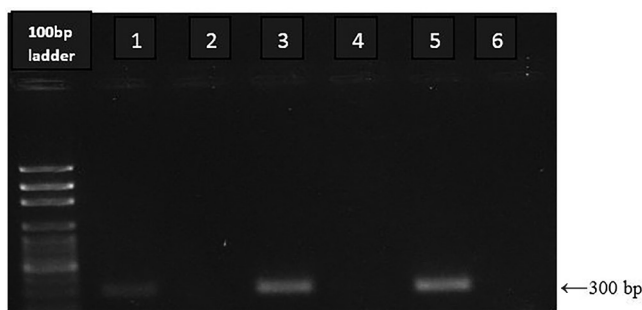
Fig. 2. Agarose gel showing polymerase chain reaction product of GroEl gene of *Orientia tsutsugamushi* blood samples of animals [Lane 1: Positive control; Lane 6: Negative control; Lanes 2 & 4: animal samples (Negative); Lane 3: animal sample no. 23 (Positive); Lane 5: animal sample no. 46 (Positive)].

total other ectoparasites collected and the overall flea index was 0.04 per animal.

Prevalence of rickettsial pathogens in animals: Of the 50 blood samples collected from rodents and shrews, 28 showed agglutination against OX-K in Weil–Felix test indicating the presence of antibodies against *O. tsutsugamushi*, the causative agent of scrub typhus (Table IV). Among the 28 rodent serum samples, 20 showed matt formation at 1:80 and eight showed matt formation at 1:160. *R. rattus* showed the highest number of positivity followed by *S. murinus* and *B. bengalensis*. Apart from this, 18 of the animals screened were positive for antibodies of murine/tick typhus pathogen. Among those positive for *O. tsutsugamushi* antibodies, 11 samples reacted for the antibodies of murine/tick typhus pathogen.

PCR carried out with the blood samples for detection of *O. tsutsugamushi* showed that of the 50 rats tested, two were positive for GroEl gene (Fig. 2). Subsequently, the amplified 300 bp product was sequenced and confirmed as that of *O. tsutsugamushi*. These two samples were positive for antibodies also.

PCR for 16s RNA (220 bp) and 56 kDa (483 bp) did not detect the respective genes.

Number of small animals with antibodies against scrub typhus pathogen was more in Periyakalapet (10 of 24), Bommayapalayam (11 of 16) and Pillaichavady (all the six rodents) villages, which showed higher chigger indices. The two animals which were PCR positive for GroEl gene were collected from Bommayapalayam, the area with highest number of seropositivity for scrub typhus in animals as well as one of the areas with higher chigger index.

Discussion

Scrub typhus has been reported as a re-emerging disease in Puducherry and adjoining areas of Tamil Nadu in India[7]. However, information on the vector mites involved or natural existence of the causative rickettsial pathogen in small animals was lacking. A total of 181 animals were collected using rat traps and the trap positivity rate was significantly greater than that reported for the scrub typhus-affected areas of Himachal Pradesh and Kolkata[18,19]. However, it was lower than that observed in West Bengal, Meghalaya and Kerala[3,20,21] which could be due to the different ecological situations.

L. deliense was the most abundant species among the nine species of trombiculid mites collected during the present study. This species was present at substantial number in all villages surveyed. Being the main vector of the scrub typhus pathogen, *O. tsutsugamushi*, such predominance of *L. deliense*, has been reported during the disease outbreaks in many places in India[18] as well as in Northern Thailand[22].

L. deliense is the established vector of scrub typhus in most of the endemic countries, including India. A total of 204 trombiculid mite species have been recorded from different ecological settings in India. Of these, four species, namely, *L. deliense, Leptotrombidium*

dihumerale, Leptotrombidium subintermedium and *Schoengastiella ligula*, have been implicated as vectors of scrub typhus[11,23]. However, most of the outbreaks have been attributed only to the abundance of *L. deliense* as the vector species[3]. In the present study, pertaining to the recent outbreak of scrub typhus in Puducherry, *L. deliense* was the most prevalent and abundant vector species. Apart from this, *Schoengastiella* sp., and *R. sanguineus*, the incriminated tick vector of Indian tick typhus pathogen[24] have also been documented.

The chigger index recorded in the present study was significantly higher than that reported in other eco-epidemiological settings such as Meghalaya[20], Kerala[21] and Kolkata[19]. The highest chigger index was observed with *S. murinus*. This species is emerged as the most preferred host for the vector mites present in the study villages. Previous study in Darjeeling[3] also reported higher chigger index for *S. murinus*.

Seasonal occurrence of scrub typhus varies with climate change in different geographical regions, and in the southern part of India, it occurs more frequently during the cooler months (October-January)[5]. This seasonal difference could be the reason for the reported higher number of cases during the cooler months in Puducherry and it coincided with the peak prevalence of trombiculid mites. As a result, a significant association was observed between the monthly index of *L. deliense* and the number of human cases of scrub typhus.

Although occurrence of scrub typhus pathogen in small animals has been reported from many countries such as Malaysia and Thailand[25,26], no reports are available on its presence in animals considered as natural hosts of this rickettsial pathogen in India. In the present study, more than 50 per cent of animals tested were positive for antibodies against the scrub typhus pathogen, indicating its high prevalence in the villages of Puducherry. Three species of animals tested were positive for antibodies against the pathogen and PCR showed presence of *O. tsutsugamushi* in two animals (*R. rattus* and *S. murinus)*. Antibody positivity was higher among animals collected from villages recorded for higher chigger indices, indicating active transmission between the chigger mites and reservoir/maintenance animals.

Only a few animals were positive for murine-borne (endemic typhus) and tick-borne (epidemic typhus) rickettsials, indicating the risk of their transmission to human. Endemic and epidemic typhus caused by different species of rickettsial pathogens have already been reported from many places in India[27,28].

The present study had a few limitations. Rodents were examined for rickettsial antibodies by the non-specific Weil–Felix test. However, to confirm *O. tsutsugamushi* infection, rodent samples were subjected to PCR and two of the samples were positive for GroEl gene of *O. tsutsugamushi*. These two samples were positive in Weil–Felix test also. Most of the 12 villages were along the East Coast road. A larger area comprising other parts of Puducherry district and surrounding districts of Tamil Nadu could have identified more scrub typhus foci and 'mite islands'.

Scrub typhus has been reported from diverse ecological settings such as mountainous regions, rainforests, semi-arid deserts, sea shores, river banks and terrain undergoing secondary growth[29]. The terrain features of Puducherry with secondary scrub and bushy vegetation growth and tropical maritime climate are congenial for the survival of rodents and shrews as well as trombiculid mite vectors. The *L. deliense* index was well above the critical level of chigger load, *i.e.* 0.69 per animal[30] in all the villages surveyed. The presence of scrub typhus pathogen in the reservoir/maintenance animals along with abundance of the vector population above the critical level, as observed in this preliminary study, was indicative of the facts that these villages were at the risk of transmission of scrub typhus. However, an extensive study is required to identify the ecological hot spots for routine and regular small animal and mite surveillance, which will help initiate timely preventive measures.

Conflicts of Interest: None.

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