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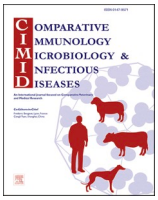

A cross sectional study on molecular prevalence of *Orientia tsutsugamushi* in household rat population of South India

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ABSTRACT

This study aimed to assess the molecular prevalence of mite-borne zoonotic pathogen *O. tsutsugamushi* in household rats of South India through nested polymerase chain reaction amplification of *O. tsutsugamushi* 47-kDa *htrA* gene and to determine the most suitable sample type for screening of *O. tsutsugamushi* in rats. Out of 85 rats trapped in Tamil Nadu, Karnataka, and Puducherry regions, 47 rats were found positive for the *O. tsutsugamushi* genome with prevalence of 55.29 %. Among different sample types screened, faecal samples exhibited the highest positivity rate, followed by liver, spleen, kidney, and blood samples. Agreement between faecal and spleen samples of rats for the presence of *O. tsutsugamushi* was the highest. Principal component analysis revealed a positive correlation between the spleen, liver, and faeces and a negative correlation between blood and faeces for the presence of *O. tsutsugamushi* genome. These findings underscore the varied distribution of *O. tsutsugamushi* among different samples and indicate that the faecal and liver samples of rats are an ideal choice of samples for epidemiological studies. This is the first study to report a high level of presence of *O. tsutsugamushi* in faecal samples of rats.

1. Introduction

Scrub typhus is a mite-borne rickettsial disease of human beings documented in various regions, particularly in the Asia-Pacific region [1]. The disease is transmitted to humans through the bite of infected trombiculid larval mites, commonly known as chiggers, which are more prevalent in agriculture and natural habitats than urban habitats [2,3]. Chigger mites attach to reservoir hosts such as rodents (rats, mice, and squirrels), insectivores and shrews, and feed on them. Humans living in proximity to these reservoir hosts are at higher risk of getting scrub typhus and it is a potentially fatal acute febrile illness [4]. If left untreated approximately 6 % of human cases end up in death and 1.5 % of death reported in human cases underwent treatment. Reports of encephalitis syndrome, multiple organ failure and high miscarriage rates in human beings were associated with scrub typhus [5]. The average Disability-Adjusted Life Years (DALYs) associated with scrub typhus was 9 years and higher in females than in males [6]. Scrub typhus was found to be common in low to middle-income countries with significant socio-economic impact. In South India, the median cost of hospital admission due to scrub typhus from 2013 to 2018 was found to be ₹ 37,026 [7].

Clinical manifestations of scrub typhus in humans often mimic other febrile illnesses, making diagnosis and timely intervention a considerable challenge [8]. Fever for more than 5 days, escher, regional lymphadenopathy, and rash are hallmark clinical signs of scrub typhus in humans. Scrub typhus is highly endemic to the "tsutsugamushi

Abbreviations: PCR, polymerase chain reaction; KDa, Kilo daltons; EDTA, Ethylenediaminetetraacetic acid; DNA, Deoxyribonucleic acid; qPCR, Quantitative real-time PCR; PCA, Principal Component Analysis; PC, Principal Component; RRNA, ribosomal ribonucleic acid; OMP, Outer Membrane Protein.

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triangle" and covers areas expanding from far eastern Russia in the north, to Pakistan in the west, Australia in the south, and Japan in the east [9], but also emerging in non-endemic areas such as Europe, Chile, Middle East, and Africa [10]. The distribution of scrub typhus lies in the latitude range of 60° N to 60° S, and longitude range of 105° W to 180° E [11]. There were several reports of scrub typhus cases in human beings in different Indian states, including Rajasthan [12], Tamil Nadu [13], Himachal Pradesh, Meghalaya [14] and Andhra Pradesh [15]. Case prevalence of scrub typhus infection in human beings was 1.1 %, 4.5 %, 5.5 %, 7.4 %, and 31.5 % in Western India, Eastern India, Southern India, North-Eastern India, and Northern India, respectively. The fatality rate was reported to be around 7 % in India with a significant increase to 40 % with the involvement of multiple organs [16]. Risk factors were mainly associated with the high distribution and density of trombiculid mites, resting on grass fields, harvesting vegetables, and herding cattle [17]. Availability of infected rodent population, climate conditions such as rainfall and human activities such as trekking and camping contribute to transmission and higher incidence of scrub typhus in human beings [18].

Various rodents such as *Apodemus agrarius* [19], *R. rattus mindanensis*, *R. flavipectus*, *R. losea* [20], *R. rattus*, and *Bandicota indica* [21] were found to be the reservoir of *O. tsutsugamushi* in regions of Korea, Taiwan and Thailand. Several published reports on seroprevalence and molecular prevalence of *O. tsutsugamushi* in rodents including rats were available in India and abroad [4,19–38]. The reported seroprevalence of *O. tsutsugamushi* in rodents in different Indian states was 0–57 % based on the Weil-Felix test [26,35–37]. In Thailand, 18.3 % prevalence in different rodents was reported based on indirect ELISA that detects specific antibodies against *O. tsutsugamushi* [27]. In Korea, a 17.4 % prevalence of *O. tsutsugamushi* in rodents was identified through indirect immunofluorescence assay [38]. The molecular prevalence of *O. tsutsugamushi* in rodents was between 0–16.9 % based on 56 kDa gene-based assays, 1.87–12.59 % based on 47 kDa *htrA* gene-based assays, and 4–19.53 % based on *GroEL* gene-based molecular assays in different regions of India such as Maharashtra [34], Maharashtra and Chhattisgarh [22,23], Puducherry [36], Tamil Nadu and Puducherry [26], and Uttar Pradesh [37]. The molecular prevalence of *O. tsutsugamushi* in rodents in Thailand, China and Korea was 2.3 % based on *16S rRNA* gene-based assay [4], 23.2 % and 6.5 % based on 56 kDa gene-based assays, respectively [24,25]. Overall, the percentage prevalence based on serological assays was higher than the molecular assays.

Previously, diagnosis of scrub typhus was performed using the Weil-Felix test despite being less specific and sensitive in comparison with serological and molecular detection techniques [39]. Molecular screening of *O. tsutsugamushi* in rodents was carried out by either using nested polymerase chain reaction (PCR) targeting 56 kDa type-specific antigen gene or 47 kDa membrane protease high-temperature requirement A (*htrA*) gene [22] or using Real-Time PCR targeting 47 kDa membrane protease, *htrA* gene [32,39]. The 47 kDa *htrA* protein, also known as the outer membrane protein belongs to the family of serine proteases which is a highly conserved region in different strains of *O. tsutsugamushi* [40]. Nested PCR showed higher specificity and sensitivity compared to conventional PCR amplifying the target genes [1,34]. The 56 kDa gene was widely used for the diagnosis of scrub typhus due to its group and type-specific epitopes. In comparison to the 47-kDa gene, there was no significant variation in the results of conventional and nested PCR targeting 56-kDa gene [1], however, 47 kDa *htrA* gene-based qPCR assay exhibited higher accuracy [32].

The majority of the epidemiological studies used blood or spleen [4,23,24] as a preferred sample for molecular detection of the *O. tsutsugamushi* genome in different types of rodents [26,28,29]. Tissue samples such as liver, and kidney were also used as target sample types in some studies [22,30–32]. No reports have been found involving the screening of rat faecal samples for the presence of *O. tsutsugamushi* in India.

Seasonal occurrence of scrub typhus has been reported majorly across the world in areas such as Japan, Korea, China, and Taiwan [41–45]. Studies from different regions of India and Nepal concluded that the prevalence of *O. tsutsugamushi* in rodents (*Rattus rattus, Rattus norvegicus, Bandicota bengalensis, Mus musculus* and *Tatera indica*) and scrub typhus cases in humans were higher in monsoon and post-monsoon season indicating an increase in the population of chigger mites in rats and the outdoor environment [23,37,46–52]. In the present study, samples from household rats of various regions of South India were collected as they are synanthropic and pose a great zoonotic disease threat to human beings. This cross-sectional study aimed to assess the prevalence of *O. tsutsugamushi* in household rats in the South Indian regions of Tamil Nadu, Karnataka, and Puducherry, their reservoir potential and to assess the most suitable sample type for screening *O. tsutsugamushi* in rats through nested PCR.

2. Materials and methods

2.1. Sampling plan

Sample size was determined by using online tool – Epitools (https://epitools.ausvet.com.au/prevalencess) to estimate the true prevalence of *O. tsutsugamushi* in household rats in Tamil Nadu, Karnataka, and Puducherry. The assumed prevalence of *O. tsutsugamushi* in rodent population was taken as 20 % [37]. Using inputs such as assumed true prevalence of 0.2, sensitivity of 0.95, specificity of 0.95, desired precision of 0.1 and confidence of 0.95, overall sample size of 84 was estimated. For seasonal prevalence, samples collected over a period of time in different seasons were used for analysis.

2.2. Blood collection in household rats

Household rats were randomly trapped using humane traps by the residents from different locations of Tamil Nadu, Puducherry, and Karnataka on a request basis, which were then transported to the laboratory. After reaching the laboratory, the rats were anaesthetised using isoflurane [53] and blood was collected by cardiac puncture [54] followed by euthanasia with carbon dioxide in a desiccator. Whole blood (~ 2 ml) was collected in an EDTA tube and stored at 4 °C until DNA isolation.

2.3. Organ and faecal samples collection in household rats

After the euthanization of the rat, the tissue samples (liver, spleen, heart, lung, and kidney) were collected in 1.5 ml sterile Eppendorf tubes and rinsed in sterile phosphate-buffered saline (PBS) to remove excess blood. Faecal samples were collected directly from the large intestine of the rat in 1.5 ml sterile Eppendorf tubes without contamination of body fluids and blood of euthanized rat. Sterile microspoon spatula were used to collect faeces from large intestine directly. The entire procedure was carried out inside the biosafety cabinet class II (Biobase, China). Organ samples were collected from all the euthanized rats, however faecal samples could not be collected in all rats due to non-availability of the fresh faeces inside the large intestine as trapped rats defecated the entire faecal contents into the traps. Though faeces were found inside the traps, they were soiled with urine and other organic materials hence not considered for screening in this study. The collected samples were kept at − 20 °C until DNA isolation [55].

2.4. DNA isolation from rat blood, tissue, and faecal samples

DNA extraction from rat blood samples was performed using a QIAamp Blood DNA kit (Qiagen, Hilden, Germany) following the protocol provided in the kit. DNA extraction from rat liver, spleen, heart, lung, and kidney was carried out using a QIA-amp Blood & Tissue DNA isolation kit (Qiagen, Hilden, Germany). DNA extraction from rat faecal

samples was carried out using a QIA-amp faecal DNA extraction kit (Qiagen, Hilden, Germany). DNA extraction from all the biological samples was done fresh on the day of collection. Using a NanoDrop Lite spectrophotometer (Thermo Fisher Scientific, USA) the quantity and quality of the DNA was assessed. The isolated DNA samples were labelled, and stored at − 20 °C until further analysis. Further, all the DNA samples isolated from blood, organs and faeces were subjected to nested PCR assay targeting the *16S rRNA* mitochondrial gene of rat [56]. Samples that failed to amplify the partial *16S rRNA* mitochondrial gene of rat was not considered for the study indicating its poor quality.

2.5. Nested PCR assay targeting O. tsutsugamushi specific 47-kDa htrA (OMP) gene

DNA isolated from rat blood, tissue and faecal samples was used as a template in the nested PCR assay. The nested PCR assay was performed as described previously [1] with slight modification and listed in Table I. The primer pairs used for the first and second rounds of nested PCR assay are OtsuFP555, OtsuRP771 and OtsuFP630, OtsuRP747 respectively. The primary PCR set-up was carried out using the DNA extracted from rat blood, liver, spleen, kidney, and faeces. The PCR mixture in a final volume of 20 µl contained 10 µl Taq DNA Polymerase 2 × Master Mix RED - 1.5 mM $MgCl_2$ (Amplicon, Denmark), 1 µl forward (10 pmol) and reverse primers (10 pmol) each, 1–2 µl of DNA template (~ 60 ng/µl) and nuclease-free water adjusted based on template volume to the final volume. A positive *O. tsutsugamushi* DNA sample was provided by CMC Hospital, Vellore, Tamil Nadu [14]. Faecal sample collected from rat tested negative for *O. tsutsugamushi* genome in blood and tissues was used as negative control, after testing different concentrations of same faecal DNA sample for non-amplification of *O. tsutsugamushi* genome. For primary PCR, initial denaturation was performed at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 51 °C for 1 min, and extension at 72 °C for 1 min, and a final elongation step at 72 °C was done for 7 min using a C1000 thermal cycler (Bio-Rad, USA). Primary PCR was performed to amplify 238 bp fragment of *O. tsutsugamushi* 47 kDa *htrA* gene. The secondary PCR was carried out by using the primary PCR amplified DNA as a template. For Secondary PCR, initial denaturation was performed at 94 °C for 10 min followed by 35 cycles at 94 °C for 30 s, 57°C for 1 min, and 72 °C for 1 min, and a final elongation at 72 °C for 7 min. The second round PCR primers was designed to amplify a 118-bp fragment of 47 kDa *htrA* gene of *O. tsutsugamushi*. The PCR products were resolved by electrophoresis on a 1.5 % agarose gel, stained with Midori Green Advance DNA stain (NIPPON Genetics, Europe) and observed under an ultraviolet trans-illuminator (Gel Doc XR+, Bio-Rad, USA). The sample was designated as positive when the 118 bp specific DNA band was visualised on agarose gel. The amplified nested PCR product was purified and subjected to Sanger sequencing by using forward and reverse primers. The contigs were assembled using SeqMan II software (Version 5.0, DNAStar Inc., Madison, WI, USA) and submitted in GenBank. The primary PCR product 238 bp was gel purified, quantified and 10-fold serial diluted, and nested PCR was performed to determine the limit of detection.

Table I
Primers and PCR product size details for the amplification of partial 47 kDa *htrA* gene of *Orientia tsutsugamushi* used in this study [1].

Primer	Sequence	Product size (bp)
OtsuFP555	5′-TCCTTTCGGTTTAAGAGGAACA-3′	238
OtsuRP771	5′-GCATTCAACTGCTTCAAGTACA-3′	
OtsuFP630	5′-AACTGATTTTATTCAACTAATGCTGCT-3′	118
OtsuRP747	5′-TATGCCTGAGTAAGATACRTGAATRGAATT-3′	

2.6. Phylogenetic analysis

A maximum likelihood phylogenetic tree was constructed along with the global *O. tsutsugamushi* sequences available in GenBank using the Jukes-Cantor nucleotide substitution model built within MEGA11 (version 11.0.10) software [57]. The relatedness of the *O. tsutsugamushi* sequence from this study was compared with that of 14 partial 47 kDa *htrA* gene sequences from different countries (Accession nos.: OP413427.1, LS398550.1, OR513503.1, HM595490.1, OL770348.1, CP094645.1, KY594257.1, HM595492.1, MH561914.1, HM595491.1, HM156060.1, MG844360.1, and NM002775.1). Using MEGA11 software, the multiple sequences were aligned with the ClustalW algorithm. The reliability of the phylogenetic analysis was evaluated using the 1000 bootstrap replicates.

2.7. Kappa statistics to determine the agreement between the distributions of O. tsutsugamushi in different sample types collected from the same rat

Kappa statistics (Inter-rater reliability) was done on PCR test results from different sample types obtained from the same household rats to find the agreement in the distribution of *O. tsutsugamushi* organism between different sample types (Blood, Spleen, Liver, Kidney, and Faeces). Two different sample types of the same household rats were compared using the kappa coefficient which is an appropriate measurement of reliability. Variation in the number of sample types compared in kappa statistics was due to differing numbers of sample types collected from the trapped rats. Kappa statistics were calculated using GraphPad Prism Version 10.20.0 software.

2.8. Principal component analysis and Venn diagram

Principal Component Analysis (PCA) was done using the data collected from 22 household rats from which all types of biological samples such as blood, spleen, liver, kidney, and faecal samples were collected. Data sets of positive (1) and negative (0) values of five different principal components – Faeces, Blood, Spleen, Liver and Kidney of 22 household rats were entered into GraphPad Prism Version 10.20.0 software. Data were analysed and visualised by using the centre method, while principal components were selected based on their eigenvalues and percentage of total explained variance (90 %).

Venn diagram-based data analysis of the distribution of *O. tsutsugamushi* between different sample types was done. Venn diagram analysis was performed using the same sample sizes and sample types used in kappa statistics analysis. The total number of positives and negatives between each specific sample type was used for the Venn diagram. Venn diagram was created by using the online tool CANVA (https://www.canva.com/).

2.9. Ethics statement

The Institutional Animal Ethics Committee (IAEC), Tamil Nadu Veterinary and Animal Sciences University, Chennai approved the study (No. 793/DFBS/IAEC/2023) to trap household rats for the screening of *O. tsutsugamushi* in its blood, tissues, and faeces.

3. Results

3.1. Molecular prevalence of O. tsutsugamushi in household rats

A total of 85 household rats were captured over nineteen months from August 2022 to February 2024. Among the 85 household rats trapped, a total of 221 samples, which includes blood (N = 54), spleen (N = 48), liver (N = 50), kidney (N = 33), heart (N = 7), lung (N = 7), faecal (N = 22) were obtained. A rat was designated positive for *O. tsutsugamushi* if at least any one of its sample types tested positive for the 118 bp amplicon (partial 47 kDa *htrA* gene) in nested PCR (Fig. 1).

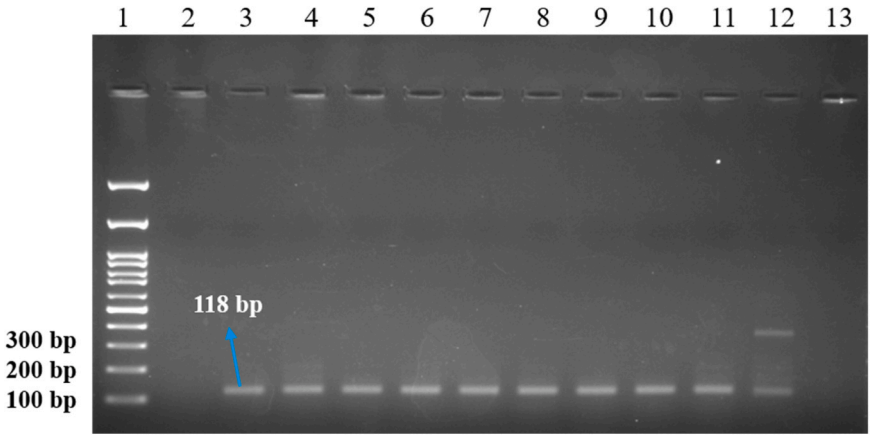

Fig. 1. Agarose gel (1.5 %) image showing nested PCR amplification of 118 bp partial *htrA* gene of *Orientia tsutsugamushi* from household rat faecal DNA. **Lane 1: 100 bp DNA marker (Bio-helix, Taiwan), Lane 2: Negative rat faecal DNA sample, Lane 3–11: Positive rat faecal DNA samples, Lane 12: Positive *Orientia tsutsugamushi* genomic DNA and Lane 13: No template control. Lane 12 consists of two bands, one of the bands (238 bp) is the result of amplicon carryover from primary PCR and 118 bp is the specific DNA fragment.**

Out of 85 rats, 47 were tested positive for *O. tsutsugamushi* on screening blood, tissue, and faecal samples. In Tamil Nadu, Karnataka, and Puducherry, 41, 4 and 2 household rats tested positive for *O. tsutsugamushi* genome, respectively by screening rat blood, and tissue samples (Table II) with an overall prevalence of 55.29 %. The percentage prevalence *O. tsutsugamushi* was tested higher in Tamil Nadu at 75.93 % followed by Karnataka at 30.76 % and Puducherry at 11.11 %. Rat faecal samples were only collected and tested in Tamil Nadu with a positive percentage of 54.55. The amplified PCR product of 118 bp was gel purified, sequenced, assembled, and confirmed as *O. tsutsugamushi*. The DNA sequence of 47 kDa *htrA* gene fragment amplified from rat spleen was submitted in GenBank (Accession no.: OR683703). The resulting sequence was matched with the *O. tsutsugamushi* 47 kDa protein gene sequence (Accession no.: MH561913.1) with 99 % similarity using nucleotide BLAST. The limit of detection was determined as 100 aM for the nested PCR assay.

3.2. Phylogenetic analysis

The partial 47 kDa gene sequence of *O. tsutsugamushi* (Accession no.: OR683703) of a rat spleen isolate from Tamil Nadu showed sequence similarity of 96.83–100 % with *O. tsutsugamushi* isolates from different countries such as Thailand, Nepal, Malaysia, China, Taiwan, Chile, Laos, USA, United Kingdom, Japan, and Australia (Supplementary Fig. 1). Highest sequence similarity of 100 % was observed between Tamil Nadu isolate and isolate from Malaysia, China, India, Nepal, and Thailand, while lowest similarity of 96.83 % was observed in sequence from Australia. Overall, the global sequences have a sequence similarity of 96.03–100 % in this region of *htrA*. No region- or species-specific clustering was observed in the phylogenetic tree (Fig. 2).

Table II
Prevalence of *Orientia tsutsugamushi* in household rat population in South India based on nested PCR amplification of partial 47 kDa *htrA* gene from rat blood and tissue samples.

Location/ State	Number of rats positive for *O. tsutsugamushi* by screening blood, tissue, and faecal samples	Percentage prevalence of *O. tsutsugamushi* (%)	Total percentage prevalence
Tamil Nadu (N = 54)	41	75.93	**55.29 % (N = 85)**
Karnataka (N = 13)	4*	30.76	
Pondicherry (N = 18)	2*	11.11	

* No faecal samples were collected.

3.3. Molecular prevalence of O. tsutsugamushi in different rat sample types

Among the 221-rat blood and tissue samples tested, a total of 90 samples were found to be positive for *O. tsutsugamushi*, while 131 yielded negative results. Overall, 40.72 % of the samples tested positive for *O. tsutsugamushi*, indicating a substantial level of presence of *O. tsutsugamushi* in the blood, faeces, and different organs of the rat. The results of nested PCR performed on rat blood, tissue, and faecal DNA samples of rats offer valuable insights into the distribution of *O. tsutsugamushi* in blood and various organs of rats (Table III). Notably, the rat faecal samples exhibited the highest level of presence of *O. tsutsugamushi* genome (54.55 %), followed by rat liver samples at 50.00 % and spleen samples at 45.83 %. Rat kidney and blood samples showed a 33.33 % and 33.33 % positivity rate, respectively. Also, heart and lung samples had a 14.29 % positivity rate; however, this result is based on a smaller number of samples (N = 7 each).

3.4. Seasonal prevalence O. tsutsugamushi in household rats

All 85 rats collected were classified into seasonal samples based on the month of collection. Among the 54 rats collected in Tamil Nadu, 11 rats were collected in the summer season (March–May), 12 rats in the monsoon 1 season (June–September), 23 rats in the monsoon 2 season (October–December), and 8 rats in post monsoon/winter season (January–February). In Karnataka, all 13 rats were collected in the monsoon 2 season, while in Puducherry all 18 rats were collected in the summer season. In Tamil Nadu, the monsoon 1 season has the highest prevalence of *O. tsutsugamushi* at 91.66 % (N = 12) followed by post-monsoon season with 87.5 % (N = 8), monsoon 2 season with 86.96 % (N = 23) and summer season with 36.36 % (N = 11). In Karnataka, 30.77 % (N = 13) of prevalence was observed in the monsoon 2 season while in Puducherry, 20.69 % (N = 18) of prevalence was observed in the summer season. The seasonal prevalence of *O. tsutsugamushi* in household rats in each location is given in Table IV.

3.5. Agreement analysis on the distribution of O. tsutsugamushi genome between rat blood, tissue, and faecal samples

In this study, we conducted an inter-rater agreement analysis using Kappa statistics to assess the agreement and distribution of *O. tsutsugamushi* between five distinct rat biological sample types: blood,

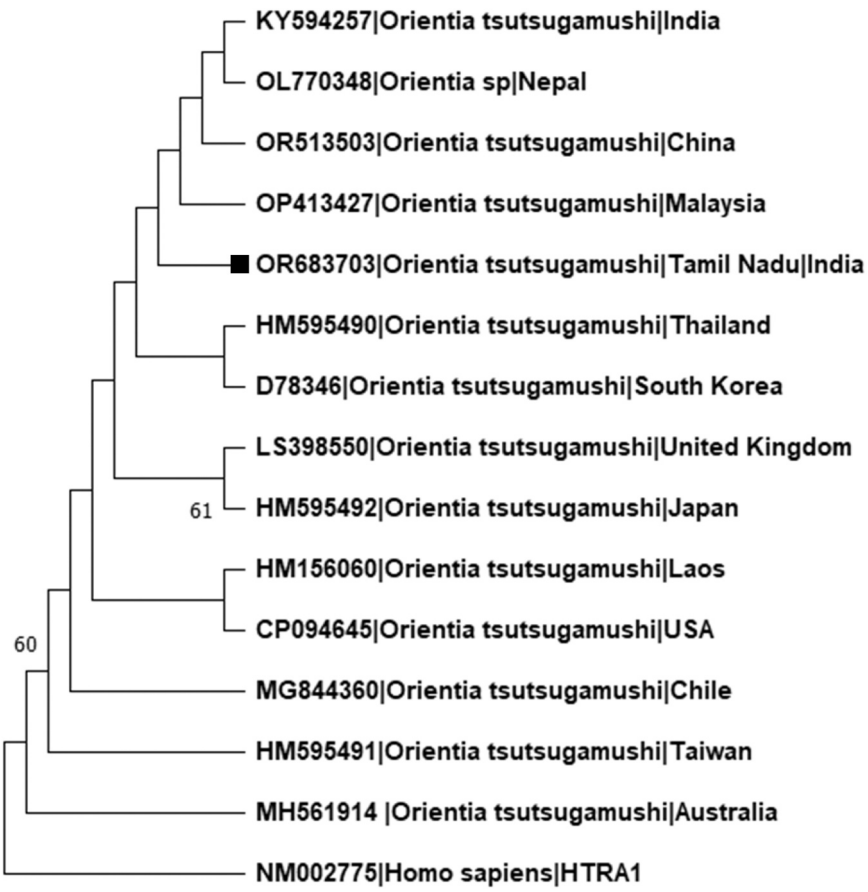

Fig. 2. Phylogeny of the partial 47 kDa *htrA Orientia tsutsugamushi* gene. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test 1000 replicates are shown next to the branches. The tree is rooted using the antigenically homologous human *htrA*1 gene (NM002775.1). The sequence marked with ■ was sequenced in this study.

Table III
Rat sample wise percentage positivity and distribution of *Orientia tsutsugamushi* in blood, tissue, and faecal samples of household rat population in South India.

Sample types	Number of samples tested	Positive	Negative	Percentage positive
Faeces	22	12	10	54.55
Liver	50	25	25	50.00
Spleen	48	22	26	45.83
Kidney	33	11	22	33.33
Blood	54	18	36	33.33
Heart	7	1	6	14.29
Lungs	7	1	6	14.29
Total	221*	90	131	40.72

* Not all the biological samples were collected from all the trapped rats.

spleen, liver, kidney, and faeces. Individual household rats with all five distinct sample types were only used for analysis. The results of the Kappa agreement coefficients are presented in Table V. The Kappa coefficients ranged from − 0.01 to 0.27, signifying varying degrees of agreement. The comparison between rat spleen and faecal showed a fair agreement with a Kappa coefficient of 0.27, which indicates fair possibility of the presence of *O. tsutsugamushi* between rat spleen and faeces. The agreement between liver and faecal samples exhibited a Kappa coefficient of 0.24, signifying slight agreement. However, the comparison between blood and faecal, kidney and faecal, and kidney and spleen, kidney and liver samples resulted in a negative Kappa coefficient value, indicating no agreement for the presence of *O. tsutsugamushi* between them.

Table IV
Seasonal prevalence *Orientia tsutsugamushi* in household rats across South India based on nested PCR amplification of partial 47 kDa *htrA* gene from rat blood and tissue samples.

Location	Summer (March–May)			Monsoon 1 (Southwest monsoon, June–September)			Monsoon 2 (Northeast monsoon, October–December)			Post monsoon (Winter, January–February)		
	No. of rats collected	No. of rats positive	Percentage prevalence	No. of rats collected	No. of rats positive	Percentage prevalence	No. of rats collected	No. of rats positive	Percentage prevalence	No. of rats collected	No. of rats positive	Percentage prevalence
Tamil Nadu	**11**	**4**	**36.36**	**12**	**11**	**91.66**	**23**	**19**	**86.96**	**8**	**7**	**87.5**
Karnataka	NA	NA	NA	NA	NA	NA	**13**	**4**	**30.77**	NA	NA	NA
Puducherry	**18**	**2**	**11.11**	NA	NA	NA	NA	NA	NA	NA	NA	NA
Total	29	6	**20.69**	12	11	**91.66**	36	23	**66.67**	8	7	**87.5**

Table V
Kappa statistics analysis on the distribution of *Orientia tsutsugamushi* genome between rat blood, tissue, and faecal samples.

Paired sample type comparison	Number of rats	Kappa value	Standard error
Spleen vs. faeces	22	0.27	0.20
Liver vs. faeces	22	0.24	0.20
Blood vs. liver	44	0.08	0.13
Kidney vs. liver	28	0.09	0.17
Liver vs. spleen	42	0.05	0.15
Blood vs. spleen	42	0.02	0.14
Blood vs. kidney	29	-0.05	0.18
Kidney vs. spleen	28	-0.01	0.18
Kidney vs. faeces	22	-0.02	0.18
Blood vs. faeces	22	-0.26	0.18

3.6. Principal component analysis and Venn diagram

The PCA biplot explained the variance in the distribution of *O. tsutsugamushi* across 5 sample types**.** The PC1 and PC2 components have a variance of 34.48 % and 22.00 % respectively with a cumulative variance of 56.48 % (Fig. 3). All five PCs explain about 90 % total variance explaining the distribution of *O. tsutsugamushi* in different samples types in rat samples. Faecal and spleen samples strongly influence PC2, while kidney and blood samples have more influence on PC1. Spleen and liver were positively correlated for the presence of the *O. tsutsugamushi* genome since they are clustered together. Faecal, liver and spleen samples form a small angle, the variables are positively correlated for the presence of *O. tsutsugamushi.* Faecal and blood samples form a large angle and they are negatively correlated for the presence of *O. tsutsugamushi* and are indirectly proportional to each other. Spleen and kidney, liver and kidney appear to be clustered closely forming an acute angle indicating poor correlation between them. Loadings of faecal and kidney samples as well as kidney and blood samples showed wider angle suggesting these samples are not likely to be correlated for the presence of *O. tsutsugamushi.* Correlation for the presence of *O. tsutsugamushi* genome can also be seen between faecal, spleen and liver samples which agreed with the kappa values between faecal, spleen and liver. Kidney and faeces were poorly correlated since it forms an obtuse angle. The correlation between two different samples such as spleen and faecal, spleen and liver, and liver and faecal were 0.27, 0.27 and 0.24 respectively indicating fair correlation (Table VI). The negative correlation could be found between blood and faecal, and blood and liver sample types. No correlation could be found between blood and

Table VI
PCA correlation matrix for distribution of *Orientia tsutsugamushi* genome between rat blood, tissue, and faecal samples.

	Blood	Spleen	Kidney	Liver	Faecal
Blood	1	0.000	0.019	-0.113	-0.320
Spleen	0.000	1	0.097	0.277	0.277
Kidney	0.019	0.097	1	0.171	-0.027
Liver	-0.113	0.277	0.171	1	0.247
Faecal	-0.320	0.277	-0.027	0.247	1

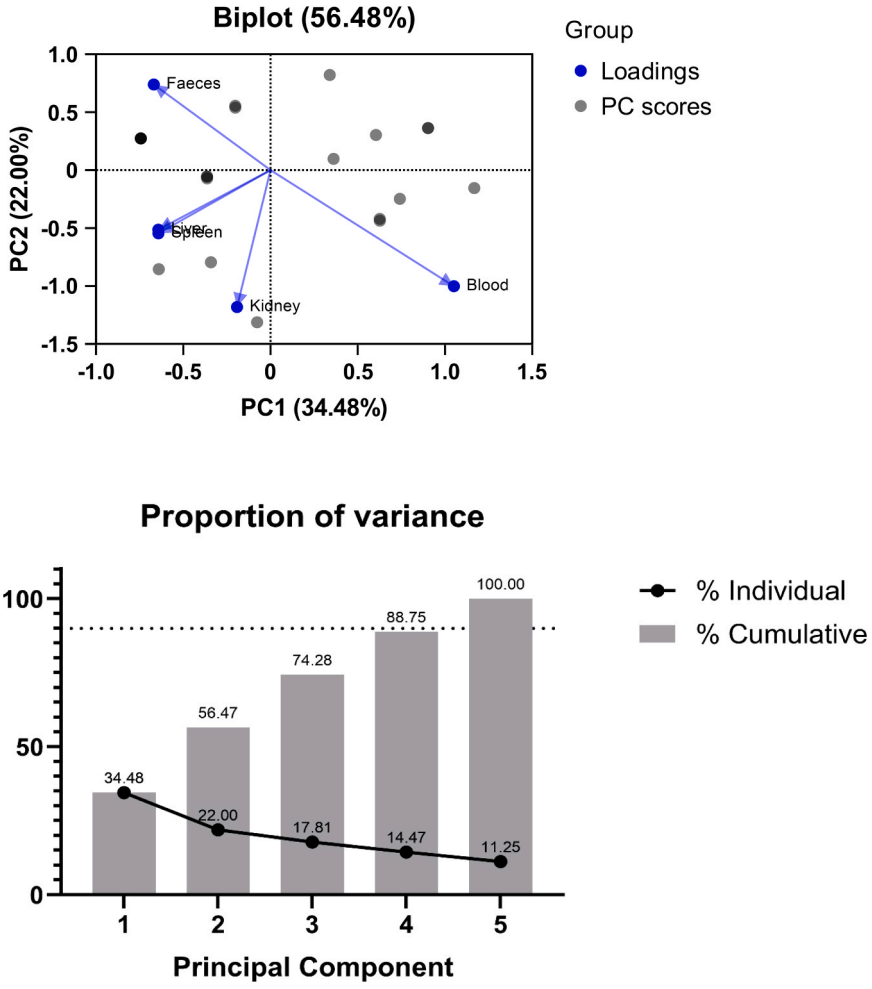

Fig. 3. The PCA biplot of the first two principal components of a data set of different sample types showing distribution of *Orientia tsutsugamushi* in 22 individual household rat and scree plot explaining proportion of variance of different principal components with their individual and cumulative variance.

spleen, kidney and faecal, and kidney and blood.

Venn diagram analysis was performed on different sample types of individual household rats (Fig. 4). Among 22 rats screened 6 rats showed the presence of *O. tsutsugamushi* in faeces, liver, and spleen and 9 rats showed the presence of *O. tsutsugamushi* in both faeces and spleen and both faeces and liver, respectively. However, out of 22 rats screened only one rat showed presence of *O. tsutsugamushi* in faeces, kidneys, and blood. Among 42 rats screened 4 rats showed presence of *O. tsutsugamushi* in blood, liver, and spleen and 13 rats showed presence of *O. tsutsugamushi* in both liver and spleen. Out of 28 rats screened 3 rats showed the presence of *O. tsutsugamushi* in the kidney, liver, and spleen while 7 rats showed the presence of *O. tsutsugamushi* in both the kidney and liver and 5 rats showed the presence of *O. tsutsugamushi* in both kidney and spleen.

4. Discussion

Household rats are synanthropic animals well-adapted to the human environment and thrive in closer proximity to the human population which, increases the risk of humans getting exposed to the scrub typhus vector infesting the synanthropic rodents. This cross-sectional study focused on identifying the prevalence of *O. tsutsugamushi* in household rats and to better understand their reservoir potential. In our study, the observed prevalence of *O. tsutsugamushi* 55.29 % in household rats is significantly higher than previously published prevalence rates of 1.87–12.59 % based on 47 kDa *htrA* gene, 0–16.9 % based on 56 kDa gene-based assays, and 4–19.53 % based on *GroEL* gene-based molecular assays reported from different regions of India [23,24,26,34,36,37]. Prevalence is also higher when compared to other countries such as Thailand (2.3 %), China (23.2 %), and Korea (6.5 %) [4,24,25]. The high prevalence could be due to the use of nested PCR assay which is highly specific and sensitive [32,34]. The molecular assays to screen the rodents for *O. tsutsugamushi* were based on 47 kDa, 56 kDa, *16S rRNA* and *GroEL* genes of *O. tsutsugamushi* and most of the assays were based on nested PCR suggesting a low abundance of *O. tsutsugamushi* genome in the reservoir host. A higher positivity percentage suggests the high risk of transmission of scrub typhus in South India. All the *O. tsutsugamushi* positive rats were healthy and active when captured suggesting that they act as reservoir host and appear to be not suffering from the disease. The prevalence of *O. tsutsugamushi* in household rat populations contributes to the enzootic maintenance of scrub typhus in South India which could experience regular cycles of infection, with household rats playing a crucial role in maintaining the pathogen. Phylogenetic analysis of *O. tsutsugamushi* positive rat spleen showed high similarity with that of partial 47 kDa gene from *O. tsutsugamushi* submitted from different regions. However, this analysis is based on a small fragment (126 bp) of the *O. tsutsugamushi* 47 kDa gene and the *htrA* region may not be an ideal candidate for studying diversity in *O. tsutsugamushi*.

A higher percentage of prevalence *O. tsutsugamushi* in household rats of Tamil Nadu could be due to the collection of greater sample numbers in monsoon and post-monsoon seasons compared to other states. These findings indicate that the prevalence of *O. tsutsugamushi* in rats was higher in the monsoon 1 season followed closely by post-monsoon and monsoon 2 seasons, while the summer season showed less prevalence. However, sample size estimation was done only for finding the overall prevalence not for seasonal prevalence analysis. The monsoon season, characterised by increased rainfall and higher humidity, is commonly associated with the peak prevalence of scrub typhus in some regions. The moist environment during the monsoon creates favourable

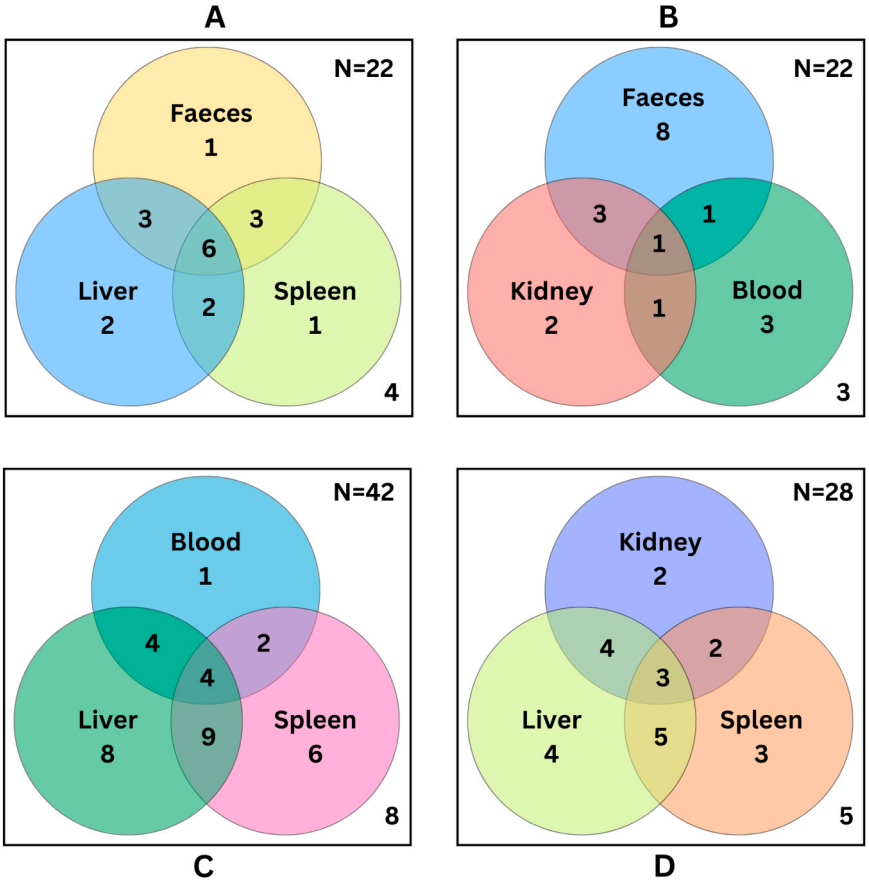

Fig. 4. Venn diagram showing distribution of *Orientia tsutsugamushi* genome in different sample types of individual household rats. **A)** Faeces, liver and spleen, **B)** Faeces, kidney and blood, **C)** Blood, liver and spleen, **D)** Kidney, liver and spleen.

conditions for the survival of chigger mites [58]. They were found to be more active during periods of higher humidity. The larvae of these mites, which are responsible for transmitting the bacterium to humans, are particularly abundant in vegetation and grassy areas. Hence, increased mite activity during specific seasons contributes to the higher prevalence of scrub typhus. A study from Korea also concluded that a higher population of chigger mites found in the October to December months correlates with a higher number of human cases [42]. The percentage prevalence of *O. tsutsugamushi* in household rats was high in winter and monsoon seasons and low in summer seasons which is similar to the findings in humans [23,48,49,51,52]. In contradiction, a study from China reported a percentage prevalence of 2.39 % in summer and 4.59 % in autumn [43] which correlates with another study from northern China, where the rate of chigger infestation on domestic rodents was higher in summer, autumn, indicating spring and winter with a low chance of *O. tsutsugamushi* [44]. In Taiwan, the number of cases increased significantly in higher temperature and rainfall seasons [45].

This study is the first report showing the presence of *O. tsutsugamushi* in rat faecal samples. The primary mode of transmission for *O. tsutsugamushi* is through the bites of infected mites, however, the epidemiological significance and transmission biology for the presence of *O. tsutsugamushi* in rat faeces needs to be explored further. In our study among the samples analyzed, a notable observation was that a single rat tested positive for *O. tsutsugamushi* genome exclusively in the faecal sample. This observation raises the possibility that the pathogen excretion through faces may have occurred through cannibalism of deceased rats carrying *O. tsutsugamushi* or by consuming infected chigger larvae present in the environment, hence has been considered negative and not been included in prevalence data but has been added in kappa statistics analysis and principal component analysis.

Kappa statistics analysis revealed varying levels of agreement for the presence of *O. tsutsugamushi* among the different sample types, ranging from no agreement to slight agreement. The agreement between the spleen, liver and faecal for the presence of *O. tsutsugamushi* was high in household rats indicating the distribution pattern of *O. tsutsugamushi* in rat samples. There is variation in the number of sample types since not all samples were taken from each rat. Principal component analysis of 22 household rats revealed the positive correlation between spleen, liver, and faecal samples for the presence of *O. tsutsugamushi* which agrees well with kappa statistics. A positive correlation between spleen and liver samples revealed that testing either spleen or liver will yield the same results and only one sample type is sufficient for testing. A negative correlation between blood and faecal samples for the presence of *O. tsutsugamushi* indicates that if blood is tested positive for the presence of *O. tsutsugamushi* genome then it is less likely to be present in faecal and vice versa.

Venn diagram analysis also highlighted that a greater number of samples had the shared distribution of *O. tsutsugamushi* among the spleen, liver and faecal and a smaller number of samples had the shared distribution of *O. tsutsugamushi* between faeces, kidney and blood. This suggests a potential interaction between these regions regarding the distribution of *O. tsutsugamushi* in rats. Rat spleen and stomach are well connected through arteries which in turn transfer the organism to the intestine and the liver excretes the organism through bile secretion to the intestine which could explain the high agreement for the co-presence of *O. tsutsugamushi* genome between the spleen, liver and faecal samples found in this study. However, there is a possibility of *O. tsutsugamushi* rapidly multiplying in intestinal epithelial cells as rat intestinal samples were reported to be an ideal sample for screening *O. tsutsugamushi* [32]. These findings highlight that a rat faecal sample could be a better sample of choice for molecular screening *O. tsutsugamushi* in rats which in turn avoids animal ethical issues and makes the sampling easier. However, Ritu et al. [32] recommended the intestine, lungs along with blood samples for the zoonotic surveillance of scrub typhus infection. Further investigations are warranted to explore the factors influencing the distribution of *O. tsutsugamushi* in rat blood, tissue and faecal and to find the epidemiological significance of faecal excretion of *O. tsutsugamushi* in rat reservoir host.

5. Conclusion

Prevalence and distribution of *O. tsutsugamushi* in different sample types of household rats in South India shedding light on their significant role as reservoir hosts for scrub typhus. Our findings revealed a high prevalence rate of *O. tsutsugamushi* in household rats in South India, suggesting an elevated risk of transmission in this region. Notably, the prevalence varied across seasons, with higher prevalence rate was observed during monsoon and winter seasons, coinciding with increased chigger activity and favourable environmental conditions. Additionally, we observed a positive correlation for the presence of *O. tsutsugamushi* in spleen, liver, and faecal samples of household rats which revealed unique pattern of distribution of this organism. The identification of *O. tsutsugamushi* genome in rat faecal samples warrants further investigation into the disease transmission and epidemiological significance of this finding. Our study also underscores the use of faecal samples as an ethical alternative for *O. tsutsugamushi* detection in household rats. Overall, this study contributes valuable insights into the epidemiology, seasonal prevalence, and distribution pattern of *O. tsutsugamushi* in different organs of household rats and the potential role of household rats as reservoir host for scrub typhus in South India.

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CRediT authorship contribution statement

John A J Prakash: Writing – review & editing. **Panneer Devaraju:** Writing – review & editing. **Aravindh Babu R P:** Writing – original draft, Funding acquisition, Conceptualization. **Soundararajan C:** Writing – review & editing. **Tirumurugaan KG:** Writing – review & editing, Conceptualization. **Gokula kannan R:** Writing – original draft, Software, Methodology, Investigation. **Dharman M:** Writing – original draft, Software, Methodology, Investigation. **Purushothaman S:** Writing – original draft, Software, Methodology, Investigation. **Azhahianambi Palavesam:** Writing – original draft, Visualization, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The 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.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.cimid.2024.102212.

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