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Zoonotic importance of rodents and their vectors in relation to perpetuation of scrub typhus in population

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Abstract
Rodents are the natural reservoir and maintenance hosts for scrub typhus. In order to know the prevalence of scrub typhus; a total of 160 rodent samples (80 blood and 80 tissue samples) and 18 vector pools were processed. Of the 80 rodents captured based on external morphological features five different species *viz*; *Rattus bandicot* (30), *Rattus Norvegicus* (21), *Mus musculus* (05), *Rattus rattus* (15) and nine shrews (*Suncus murinus*) were recorded. Further DNA was extracted from rodent and vector samples and amplified by employing 56kDa and 47 kDa conventional and nested PCR. Of the 160 analysed rodent samples; no single sample was reported positive for 56kDa gene whereas; two blood and one tissue sample was reported positive for 47kDa gene. None of vectors were reported positive by 56kDa or 47kDa PCR. The geographical distribution of scrub typhus vectors and reservoir hosts are important aspects for understanding the epidemiology as well as the potential impacts of the disease on human health.

Keywords: Scrub typhus, rodents, nested PCR, vectors

Introduction
Scrub typhus is a zoonotic illness caused by *Orientia tsutsugamushi*. Transmission of *Orientia tsutsugamushi* to the rodent or human host occurs through a bite of larval mites (chiggers) during feeding, primarily of the genus *Leptotrombidium* and family *Trombiculidae*. Transovarial transmission is essential for maintenance of infection in nature; thus, the mite serves as both vector and reservoir [1]. Rodents of the family *Muridae* (rats and mice) are common hosts and may support *O. tsutsugamushi* and are also key hosts of chiggers [2, 3]. Rodents are thus crucial for the survival of chiggers and play important role in the transmission of scrub typhus [4]. Geographically specific foci of scrub typhus are thus determined by the distribution of vector mites and their rodent hosts and by interactions of mites and rodents with humans [5]. *O. tsutsugamushi* is also recorded in other trombiculid species [6] but there is still a need of partisan evidence to confirm their vectorial role in disease transmission.

Scrub typhus is considered as a re-emerging infectious disease in India [7]. Recently, outbreaks of scrub typhus have been reported all over India[8]. Approximately one million cases of scrub typhus occur each year and more than a billion people are at risk worldwide [9]. Seasonal occurrence of scrub typhus varies with climate change in different geographical regions, it occurs more frequently during the cooler months [10]. Apart from human other small terrestrial mammals such as rodents (rats, mice and ground squirrels), insectivores and tree-shrews have been reported as natural or maintenance hosts of *Leptotrombidium* and are infected by the bacterium. However; very less information is available on the occurrence of *O. tsutsugamushi*, in them [11].

Considering the paucity of studies related to key role of different rodents and other trombiculid species in maintenance and perpetuation of *O. tsutsugamushi*, the present study was carried out to look in to the profusion and distribution of trombiculid mite vectors on rodents trapped from endemic areas recorded with human cases of scrub typhus in and around Nagpur city, their identification and probable role as vector for *O. tsutsugamushi.*

Materials and Methods

Collection of rodents

Rodents were collected using wire cage traps. The traps were baited with eatables smeared with butter and laid in evening or early morning at various sites near human dwellings. A total of 80 rodents were trapped from different locations in and around Nagpur region (Table1).

Table 1: Details of rodent collection

Place of collection	Number of rodent	Type of samples			
		Blood		Spleen	
		Monsoon	Post monsoon	Monsoon	Post monsoon
Nagpur railway station	24	09	15	09	15
Gittikhadan, Nagpur	04	-	04	-	04
Gorewada, Nagpur	02	-	02	-	02
Telangkhedi, Nagpur	09	02	07	02	07
Surendragarh, Nagpur	06	03	03	03	03
Maharjbhag, Nagpur	07	02	05	02	05
TVCC, Nagpur Veterinary College, Nagpur.	06	02	04	02	04
Mahal, Nagpur	06	06		06	
Nagpur Veterinary College Nagpur	14	02	12	02	12
Wadi, Nagpur	02	02	-	02	
Total	80	28	52	28	52

Dissection and sample collection from rodents

Collected rodents were anaesthetized and species was identified by observing their morphological characteristics which resulted in to five different species *viz*; *Rattus bandicot* (30), *Rattus norvegicus* (21), *Mus musculus* (05) *Rattus rattus* (15) and nine shrews (*Suncus murinus*). After dissection blood and spleen samples were collected and transferred aseptically for further processing [12]. The proposed study was approved and consent was obtained from the institute ethics committee.

Vector collection and identification

External parasites like chigger mites/adult mites, ticks, fleas and lice were collected by reverse combing of rodents fur over a white paper or tin pan. The snout, ears, limbs and axillary region of individual rodents were combed and vectors were collected and preserved in 70% alcohol until further processing [13]. A total of 74 vectors (40 mites, seven flea and 27 lice) were collected from rodents (table 2). All samples were transported to Department of Veterinary Parasitology, Nagpur Veterinary College, Nagpur for identification and preparation of vector database. The ectoparasites *viz*; mites and fleas were screened by transferring them into 70% alcohol followed by mounting in Hoyers media and identification using phase contrast microscope.

Table 2: Details of ectoparasite collection

Sr.no	Ectoparasites		Number	No. of pools	Number of ectoparasites per pool
1.	Mites	*Myobiusmusculi*	25	Five pools	Four to five mites/pool
		Radfordiaensifera	07	Two pools	Three to four mites / pool
		Ornithonyssusbacoti	05	Two pools	Two to three mites / pool
		Unidentified mite	03	One pool	Three mites / pool
2.	Lice	*Hoplopleuraacanthopus*	23	Five pools	Four to five louse / pool
		Polyplexspinulosa	04	One pool	Four louse / pool
3.	Flea	*Xenopsylla spp.*	07	Two pool	Three to four fleas / pool

Isolation of DNA from rodent's blood and spleen tissue

Blood and spleen samples were processed for DNA extraction as stated in Martins method [15], 400µl of blood or 5-10 mg tissue from rodents in 1.5 ml of eppendorf tube was added with 1 ml of RBC lysis buffer. The contents were mixed properly and centrifuged at 10000 rpm for 3 minutes at 25 °C. The supernatant was discarded and clear pellet was re-suspended in 50-80 µl of KTT-20 buffer containing 100µl of proteinase-K per ml. After through mixing tubes were incubated at 56 °C for 2 hours and stored at -20 °C followed by Nanodrop analysis. The recovered contents were used as DNA template for molecular study.

DNA isolation from ectoparasites

DNA was isolated from ectoparasites using DNeasy Blood and Tissue Kit, as per manufacturer's protocol. Whole DNA was extracted from mites, louse and fleas (in pools) and stored at -20 °C.

PCR amplification

Conventional PCR by targeting 56kDa gene

The PCR analysis by targeting 56 kDa gene was performed using primers *Otsu* F and *Otsu* R with amplification size of 410-bp (table 3). The reaction mixture (25µl) was prepared containing 12.5µl of 2X Go Taq Green master mix (Promega), 1.5µl of template and 1µl each of *Otsu* F and *Otsu* R primers{10 pmol (IDT))}. After through vortexing the reaction mixture was run in a thermal cycler (Applied Biosystems, USA). The PCR conditions consisted of denaturation at 94 ºC for 5 minute, followed by 40 cycles, each consisting of denaturation at 94ºC for 1 minute, annealing at 60 ºC for 1 minute and extension at 72 ºC for 1 minute. The final extension step was carried out for 10 minute at 72 ºC [16].

Table 3: Details of primers for PCR targeting 56kDa type specific antigen (OMP) gene

Target Gene	Primer	Primer sequence	Product size
56kDa type specific antigen (OMP) gene	Otsu F	5'-AATTGCTAGTGCAATGTCTG-3'	410bp
	Otsu R	5'-GGCATTATAGTAGGCTGAG-3'	
	P34	5'-TCAAGCTTATTGCTAGAT CTG C-3'	1003bp
	P55	5'-AGGGATCCCTGCTGCTGTGCTTGCTGCG-3'	
	P10	5'-GATCAAGCTTCCTCAGCCTACTATAATGCC-3'	483bp
	P11	5'-CTAGGGATCCCGACAGATGCACTATTAGGC-3'	

Nested PCR by targeting 56kDa gene

A 56kDa nested PCR was used to minimize the contamination during amplification and to improve the sensitivity of conventional PCR. Nested PCR was performed using two sets of primers (table 3). The external nested PCR was performed using P34 and P55 primers with the amplicon size of 1003bp. The reaction mixture (20µl) was prepared containing 10µl of 2X Master Mix (Promega), 1µl of P34- (10 pmol, eurofins), 1µl of P55- (10 pmol) (eurofins), 6µl of Nucleus free water and 2µl of DNA template. PCR reaction was carried out with initial denaturation at 94 ºC for 5 minutes followed by denaturation at 94 ºC for 30 seconds, annealing at 61 ºC for 1 minute and extension at 72 ºC for 2 minute. The internal nested PCR was prepared by using external PCR product as template and P10 and P11 (table 3) as primers with amplification size of 483bp. PCR reaction was carried out in thermal cycler with cycling condition consisted of initial denaturation at 94 ºC for 10 minutes followed by denaturation at 94 ºC for 30 seconds, annealing at 64 ºC for 30 seconds and extension at 72 ºC for one minute. [17].

Nested PCR targeting 47kDa gene

The 47kDa nested polymerase chain reaction, was carried using two sets of primers *viz*; *Otsu* F P555 and *Otsu* R P771 with amplification size of 238bp (table 4). The PCR reaction was carried out with initial denaturation at 94ºC for 5 minutes followed by denaturation at 94 ºC for 30 seconds, annealing at 51 ºC for 1 minute and extension at 72 ºC for 1 minute. The second round of nested PCR was performed using the first product as template DNA, 1.5µl and 1µl of each primer *Otsu* F P630 and *Otsu* R P747 and 9.0µl of nucleus free water with 12.5µl of 2X Master Mix (Promega). The PCR conditions consisted of denaturation at 94 ºC for 10 minutes, followed by 35 cycles, each consisting of denaturation at 94 ºC for 30 seconds, annealing at 60 ºC for 1 minute, extension at 72 ºC for 30 seconds, with an expected amplicon size of 118-bp [18].

Table 4: Details of primers and probe for PCR targeting 47kDa high temperature requirement (OMP) gene

Target Gene	Primer	Primer Sequence	Product Size
47kDa High temperature requirement A (OMP) gene	OtsuFP630	5'-AACTGATTTTATTCAACTAATGCTGCT-3'	118bp
	OtsuRP747	5'-TATGCCTGAGTAAGATACRTGAATRGAATT-3'	
	OtsuFP555	5'-TCCTTTCGGTTTAAGAGGAACA-3'	238bp
	OtsuRP771	5'-GCATTCAACTGCTTCAAGTACA-3'	

Results

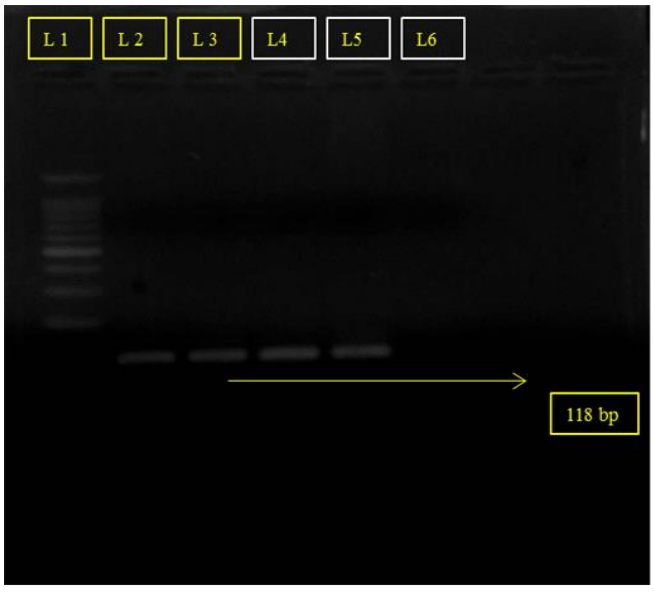
Prevalence of scrub typhus in rodent population

By targeting 56kDa gene

The analysis of all 160 samples (80 blood samples and 80 tissue samples) by employing conventional PCR targeting 56kDa outer membrane protein gene revealed that as per the area and season of collection no any rodent was found positive for scrub typhus. The present findings of zero prevalence are on lower side than 18% and 9.8% positivity for *O. tsutsugamushi* in rodents (collected from Nagpur region) by 56kDa conventional PCR [18, 20]. The negativity of results may be attributable to need of high concentration of DNA during conventional PCR and also less sensitivity and specificity of the technique as reported by Murai [19]. The variation in the present results may be credited to the endemicity of the disease in those regions as compared to the region from which rats have been trapped for study during present investigation.

For rapid diagnosis of scrub typhus, nested PCR has been widely used to improve the sensitivity of conventional PCR. Analysis of all the rodents tissue and blood samples employing 56 kDa external and internal nested PCR reported all samples negative for scrub typhus. The present findings are lower than the recorded prevalence of 12.60%, 34.25%, 12% and 22.22% in rodents by nested PCR [20-23].

Analysis of all 160 rodent samples by employing external PCR targeting 47kDa high temperature requirement A (OMP) gene for *Orientia tsutsugamushi* revealed three samples positive registering an overall prevalence of scrub typhus to the tune of 1.875% (3/160). Of which in concern with season 5.17% of prevalence was observed during post monsoon season whereas; no prevalence was recorded during monsoon season. The present observations 5.17% prevalence are higher than zero prevalence of scrub typhus by employing 47kDa high temperature requirement gene A in rodents recorded by Akhunji [20] but the present findings are lower than 8% and 0% recorded prevalence of scrub typhus in rodents [19, 24].



Prevalence of scrub typhus in arthropods

The scrub typhus is a *rickettsial* zoonotic and chigger-born disease caused by *Orientia tsutsugamushi*. It is transmitted by *Leptotrombidium spp*. and other tropical rat mites. Various arthropods were collected from rodent's fur after anesthetizing them. Total 18 pools (10 mite pools, six lice pools and two flea pools) were processed for conventional and nested PCR. Analysis of all DNA samples with nested as well as conventional PCR revealed all samples negative for presence of *Orientia tsutsugamushi*. The present study findings are also comparable with the work done by earlier researchers who studied mite infestation in rodent population but failed to detect *Orientia tsutsugamushi* by PCR. [2, 24, 25]

Conclusion

The ectoparasites like *Myobius musculi*, *adfordiaensifera* mites, X*enopsylla* spp. fleas and *Hoplopleura acanthopus and polyplexspinulosa* louse has no public health importance in transmission of scrub typhus. Moreover; the study suggested that nested PCR targeting 47kDa high temperature requirement gene A has more sensitivity than nested PCR targeting 56kDa outer membrane protein gene. The study also suggests that, to discern the epidemiology of scrub typhus infection the reservoir hosts and vectors had important role thus the vectors and rodents should be collected throughout the year to understand the scenario of scrub typhus.

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