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Original Article

Microscopic and Molecular Identification of *Trypanosoma lewisi* among *Rattus rattus* and *Rattus norvegicus* from The District Kasur, Punjab, Pakistan

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Abstract

Background: We investigated the prevalence of *Trypanosoma lewisi* in Black rats (*Rattus rattus*) and Brown rats (*R. norvegicus*) using both microscopic and molecular detection methods, along with the analysis of associated risk factors.

Methods: A total of 178 rodents were trapped in Kasur district between November 2023 and November 2024, with epidemiological data and geographical coordinates recorded. Rodents were identified, euthanized and blood samples were collected. *T. lewisi* was confirmed through microscopy, PCR, sequencing and phylogenetic analysis. A GIS map was generated using ArcGIS 10.5.1 to illustrate geographical distribution.

Results: A total of 178 blood samples were examined by microscopically and by PCR. PCR confirmed to be either microscopically positive or negative samples. Overall prevalence of *T. lewisi* was 10(5.62%) and the findings were consistent across both diagnostic methods. At the species level, *T. lewisi* prevalence was higher in *R. rattus* (7.59%) compared to *R. norvegicus* (4.04%). Gender and the presence of ectoparasites were identified as potential risk factors.

Conclusion: These findings confirm the presence of *T. lewisi* in black and brown rats and serve as a baseline for further surveillance and control strategies.

Introduction

Rodents, with over 2,200 identified species, are widespread, synanthropic mammals that act as natural reservoirs for numerous pathogens, including parasites, bacteria, fungi and viruses (1). They play a significant role in the transmission of zoonotic diseases, posing serious public health risks (2). They can also transmit a variety of pathogens, at least 85 through direct and indirect ways, acting as definite and intermediate hosts for

ectoparasites (3). Given their role as hosts for ectoparasites and the increasing risk of disease spillover especially under the One Health framework, there is a critical need to manage rodent populations, particularly in the context of human-animal interactions and climate change (4).

Trypanosomes are unicellular and flagellar protozoan parasites of Trypanosomatidae family and the *Trypanosoma* genus (5). Based on vector to host transmission, *Trypanosoma* is divided into two groups: Salivarian and Stercorarian groups (6). The most severe diseases caused by *Trypanosoma* spp. are American Chaga disease and African Sleeping Sickness. The Chagas disease is caused by *T. cruzi* (7). African sleeping sickness, commonly known as Human African trypanosomiasis, is caused by two subspecies of *T. brucei*; *T brucei gambiensis* and *T. brucei rhodesiense* (8). In addition to affecting humans, this infection also impacts animal health, causing diseases such as Surra and Nanga in horses (9).

Rattus. rattus and *Rattus. norvegicus* serve as hosts for *T. lewisi* (1), which is transmitted among rodents by fleas like *Xenopsylla cheopis* and *Nosopsyllus fasciatus* through contact with and ingestion of infected fleas (10). Globally, at least 43 documented *T.* spp. infect 125 d rodent species (11). *T. lewisi* was previously considered a host-specific and non-zoonotic threat to humans. However, fatal cases reported in Asia and Africa revealed that it could infect humans as opportunistic pathogen, causing atypical human trypanosomiasis. Nevertheless, in most rodents, *T. lewisi* is thought to be non-pathogenic (12, 13). In humans, *T. lewisi* infection can cause non-specific symptoms such as severe convulsions, protracted fever and also appetite loss (14).

This study is the first to report *T. lewisi* detection in rodents from Kasur, Punjab, Pakistan via microscopic and molecular analysis, while similar studies have been reported in various countries, including Uganda (11), Southeast Asia (15), Southern China (16), Malaysia (17), Thailand (18) and Indonesia (19). The present study aimed to determine the presence of *T. lewisi* in *R. rattus* and *R. norvegicus* in Kasur district, through microscopic and molecular identification techniques. Additionally, we also aimed to explore the prevalence of infection along with associated risk factors for *T. lewisi* as causative agent.

Methods

Study area

This research was conducted between November 2023 and November 2024 in Kasur district, Punjab Province, Pakistan. Kasur, located at approximately 31°05'N and 74°31'E, covers an area of about 3995 km^2. It lies 55 km away from Lahore along the Lahore-Ferozepur Road. Kasur was formerly a part of the Lahore district but is now an independent district comprising four tehsils: Kasur, Chunian, Pattoki, and Kot Radha Kishan (20).

Ethical approval

The animal experiments were approved by the Institutional Ethical Review Committee, University of Veterinary and Animal Sciences, Lahore, Pakistan (Approval number DR/260, Dated 26-05-2022) and conducted in accordance with ethical guidelines. Rodent capture was permitted by the Wildlife and Parks department, Punjab, Pakistan (Approval No. 5004/DG(W&P) Mgt-3(51)/2022, Letter No 826, Dated 08-02-2022).

Rodent trapping and epidemiological data collection

Due to a lack of data on the *Rattus* genus in the study area. To address this gap, a multistage stratified random sampling technique was used to determine the total number of sampling sites. The Sampling sites were calculated based on a previous prevalence of *T. lewisi* of 6.2% (21), using Sample Size formula $N= (Z\alpha)^2P(1-P)/d^2$ (22**)**, where Zα is 1.96 for 95% Cl and d is 5% or 0.05. The total num-

ber of sampling sites formed the basis of the stratification. The Kasur district was considered the main group, while the tehsils served as subgroups. The probability proportional to size technique was used to randomly select groups and subgroups (23). Based on the sample size calculation, 178 rodents were captured from the study area.

Rodents were captured using Sherman traps, baited with peanuts, butter, apples and oily bread to attract the rodents (24). Field data such as tehsil, species, capture sites, urbanicity, gender, age, body condition, presence of ectoparasites and presence of livestock were recorded on a structured proforma. GPS coordinates of each sampling point were collected using a mobile application (Version 4.4.25) (Micheal Schollmeyer, Seattle, WA, USA) (25).

Rodent identification

The captured rodents were transferred to the Epidemiology and Molecular Laboratory (One Health Research Group), Department of Wildlife and Ecology, University of Veterinary and Animal Sciences and chemically anesthetized for blood collection and identified based on morphological characteristics (26).

Blood collection and preservation

Blood was collected from the tail vein of anesthetized rodents with minimal pain and stress, following CERoPath protocols (26), using sterilized syringe and transferred into EDTA tubes for further analysis (27).

Thin blood smear and staining

To detect *T. lewisi*, a thin blood smear was prepared and stained with 10% Giemsa stain. After staining, the slide was examined under a microscope at 100x magnification (18). Blood smears were analyzed for the presence of selected protozoan parasites and identified based on their morphological characteristics as described by (17).

DNA extraction

DAN was extracted from both microscopic positive and negative samples using a QIAamp DNA extraction kit (Qiagen, Germany; Cat. No. 51306), following the manufacturer's instructions. Extracted DNA was stored at -20 °C in labeled 1.5 ml Eppendorf tubes for PCR analysis (19).

Molecular analysis

A specific primer set, TRYP1S (5-CGTCCCTGCCATTTGTACACAC-3) and TRYP1R (5-GGAAGCCAAGTCATCCATCG-3) was used to amplify a 623bp ITS1 fragment. PCR was carried out as stated earlier (19).

Phylogenetic analysis

PCR products were sequenced by a commercial facility (1st BASE Pte Ltd., Singapore) and analyzed using BLAST and BioEdit 7.2. Sequence alignment was performed using ClustalW algorithm in MEGAX. and the Maximum Likelihood method was used to create the phylogenetic tree (29). All sequences were submitted to GenBank under accession numbers (PQ008833, PQ008834, PQ031065).

Development of GIS (Geographical Information System) Map

A GIS map was developed using ArcGIS software 10.5.1 (Micheal Schollmeyer, Seattle, WA, USA) to examine the geographical distribution of *T. lewisi,* employing the inverse Distance Weighting (IDW) method to analyze the spatial patterns (25).

Statistical analysis

The field data were analyzed using the statistical software SPSS (Version 21; IBM Armonk, New York, USA). Descriptive statistics summarized frequency of species along with associated risk factors. The Chi-Square was applied to evaluate the association between categorical variables and outcomes (30). Binary logistic regression was performed to evaluate the association between risk factors

and the *T. lewisi* prevalence with 95% confidence intervals (CIs) and odd ratios (ORs) with a significance level of $P<0.05$ (25).

Results

A total of 178 rodents were captured from Kasur. Based on external morphological characteristics, these rodents belonged to the two common species of the genus *Rattus*; *R. rattus* and *R. norvegicus*. A descriptive comparison of these two species showed the percentage of each species regarding various risk factors. A higher percentage of both species, Black rats n=29;(36.7%) and Brown rats n=39;(39.4%), were trapped in Kasur tehsil compared to the other tehsils. Details of both species across various risk factors are described in Table 1.

Table 1: Number and frequency of *R. rattus* and *R. norvegicus* regarding various risk factors

Variables	Factors	*R. rattus* (n=79)		*R. norvegicus* (n=99)	
		No. of Captured	Frequency %	No. of Captured	Frequency %
Tehsils	Kasur	29	36.7	39	39.4
	Chunian	25	31.6	27	27.3
	Kot Radha Kishan	10	12.7	10	10.1
	Pattoki	15	19.0	23	23.2
Capturing sites	Human settlements	28	35.4	23	23.2
	Livestock sheds	30	38.0	39	39.4
	Godowns	11	13.9	25	25.3
	Shops	10	12.7	12	12.1
Urbanicity	Rural	59	74.7	59	59.6
	Urban	20	25.3	40	40.0
Gender	Females	54	68.4	69	69.7
	Males	25	31.6	30	30.3
Age(yr)	Adults	49	62.0	56	56.6
	Youngs	30	38.0	43	43.4
Health status	Healthy	40	50.6	46	46.5
	Medium	26	32.9	35	35.4
	Weak	13	16.5	18	18.2
Season	Summer	42	53.2	59	59.6
	Winter	37	46.8	40	40.4
Presence of Ectoparasites	Yes	19	24.1	26	26.3
	No	60	75.9	73	73.7
Presence of livestock	Yes	42	53.2	43	43.4
	No	37	46.8	56	56.6

Out of 178 rodent blood samples examined, 10(5.62%) were found positive for *T. lewisi* as determined by both microscopy and PCR that indicating complete agreement between the two diagnostic methods (Table 2). All samples, whether positive or negative by microscopy were also confirmed by PCR analysis. Interestingly, no additional positive cases were detected by PCR and only microscopically positive samples were also positive according to PCR analysis of *T. lewisi*.

Table 2: Comparative detection of *T. lewisi* by microscopy and PCR

Case No.	Microscopy Result	PCR Result
16	Positive (+)	Positive (+)
32	Positive (+)	Positive (+)
52	Positive (+)	Positive (+)
82	Positive (+)	Positive (+)
92	Positive (+)	Positive (+)
105	Positive (+)	Positive (+)
124	Positive (+)	Positive (+)
135	Positive (+)	Positive (+)
154	Positive (+)	Positive (+)
168	Positive (+)	Positive (+)

Species-wise black rats showed a higher positive rate (7.59%) compared to brown rats (4.04%; $P>0.05$). The highest infection rate was observed in Kasur tehsil (7.35%; $P>0.05$), followed by other tehsils. Among the capturing sites, rodents trapped from human settlements had higher prevalence (9.80%; $P>0.05$). A slightly higher prevalence was observed in rodents that were trapped in rural areas (5.93%; $P>0.05$). Male rats were significantly more likely to be positive than female rats ($P<0.05$; 11.964). Additionally, the prevalence was significantly higher in adult rats ($P<0.05$; 8.57%). Detailed results are presented in Table 3.

Microscopic examination confirmed *T. lewisi* based on morphological characteristics including a slender body, tadpole-like shape, pointed posterior end, oval kinetoplast, elongated nucleus, undulating membrane and single flagellum (Fig. 1). The observed mature trypomastigote's appearance was consistent with the description of *T. lewisi*. All positive and negative samples were further confirmed by PCR, with a 623bp bend of *T. lewisi* ITS1 gene observed on a 1.5% agarose gel (Fig. 2).

Table 3: Prevalence of *T. lewisi* and associated risk factors based on Chi-square test

Variables	Factors	Examines samples	*T. lewisi*		Statistical analysis	
			No. of positive sample	Prevalence (%)	*P*-Value	Chi-Square
Species	Black rat	79	6	7.59	0.306	1.047
	Brown rat	99	4	4.04		
Tehsils	Kasur	68	5	7.35	0.869	0.717
	Chunian	52	2	3.85		
	Kot Radha Kishan	20	1	5.00		
	Pattoki	38	2	5.26		
Capturing sites	Human settlements	51	5	9.80	0.361	3.205
	Livestock sheds	69	3	4.35		
	Godowns	36	2	5.56		
	Shops	22	0	0		
Urbanicity	Urban	60	3	5.00	0.798	0.065
	Rural	118	7	5.93		
Gender	Females	123	2	1.63	**0.001**	11.964
	Males	55	8	14.55		
Age	Adults	105	9	8.57	**0.040**	4.212
	Youngs	73	1	1.37		
Health status	Healthy	86	6	6.98	0.708	0.690
	Medium	61	3	4.92		
	Weak	31	1	3.23		
Season	Summer	101	9	8.91	**0.029**	4.775
	Winter	77	1	1.30		
Presence of Ectoparasite	Yes	45	8	15.56	**0.000**	16.794
	No	133	2	2.26		
Presence of livestock	Yes	85	6	7.06	0.425	0.637
	No	93	4	4.30		

Gender and presence of ectoparasites were identified as potential risk factors for *T. lewisi* through binary logistic regression test. However, their associations were statistically significant ($P<0.05$) (Table 4).

Table 4: Final binary logistic regression model for *T. lewisi* along with associated risk factors

Variables	Factors	Multiple Logistic Regression			*P*-value
		Odd Ratio	95% Confidence interval		
			Lower value	Upper value	
Gender	Female	0.131	0.025	0.699	0.017
	Male	Ref.			
Age	Adult	4.309	0.465	39.917	0.198
	Young	Ref.			
Season	Summer	3.725	0.403	34.417	0.247
	Winter	Ref.			
Presence of Ectoparasites	Yes	9.068	1.704	48.248	0.010
	No	Ref.			

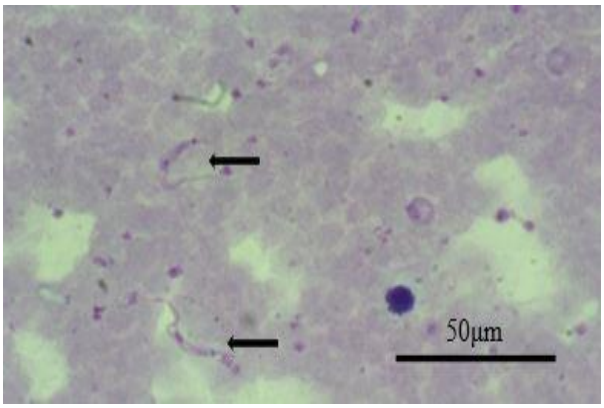

Fig. 1: Microscopic view of a thin blood smear from an infected rat showing the presence of *T. lewisi*. Black arrows indicate adult trypomastigotes. Giemsa staining; 100X magnification; 50µm scale bar

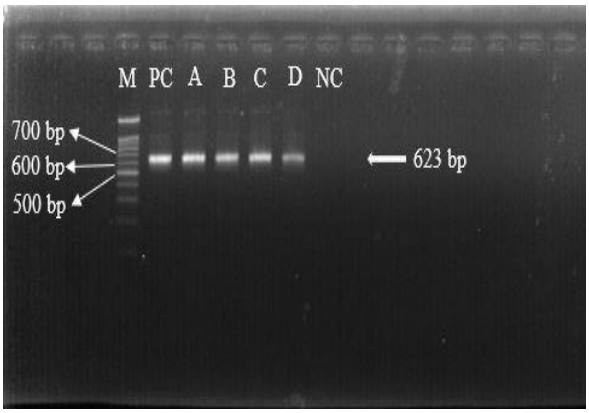

Fig. 2: PCR result for *T. lewisi* targeting gene ITS1 (623bp). (M=100bp DNA ladder; PC=positive control; Lanes A-D=positive DNA samples; NC=negative control)

The phylogenetic tree showed that *T. lewisi* sequences from this study clustered with previously reported *Trypanosoma* spp. based on ITS1 gene data, using *T. rabinowitschae* (accession no. AY491765) as an outgroup and 1,000 bootstrap replicates (Fig. 3). Spatial analysis using the IDW method in ArcGIS highlighted infected rodent locations (red dots) across the four tehsils, with all capture sites marked (white dots) (Fig. 4).

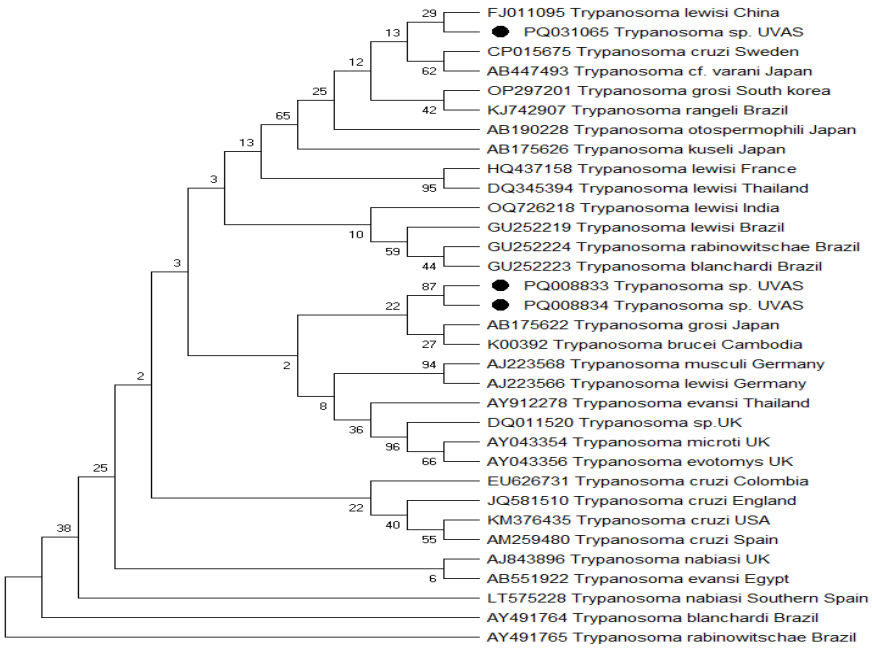

Fig. 3: Maximum likelihood (ML) phylogenetic analysis of *T. lewisi* based on ITS1 gene sequences. Black dots indicate the positive samples obtained in the present study

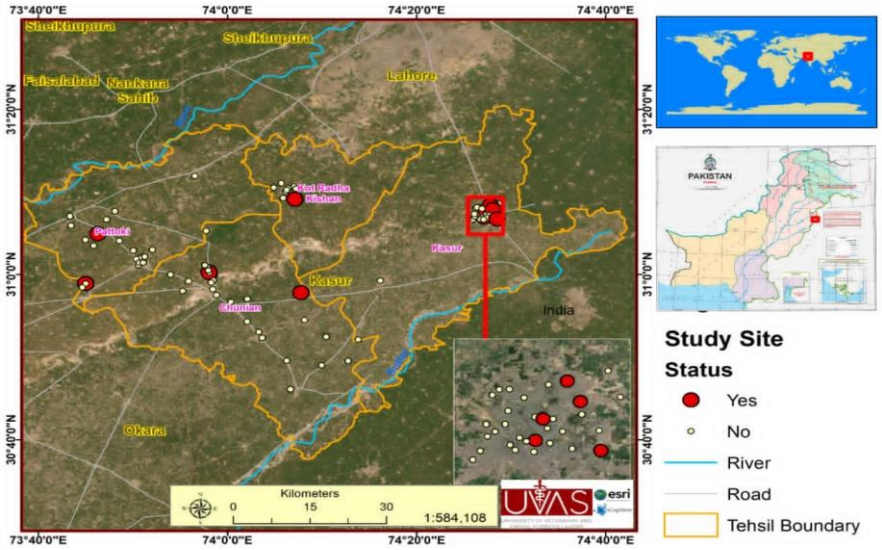

Fig. 4: Spatial distribution of T. lewisi in rodents from district Kasur, Punjab, Pakistan. Red dots show the positive samples in this study

Discussion

Rodents play a key role as definitive hosts for many zoonotic diseases, transmitting around 60 such diseases globally (31,32). About 10% of the rodent population are carriers or reservoirs of pathogens affecting human health, including blood-borne protozoan parasites like trypanosomes (33,34). Out of 178 rodents trapped from four tehsils of Kasur, ten tested positive through both microscopic examination and PCR. In this study, the overall prevalence of *T. lewisi* was found to be 5.62%. According to previous literature, this study is the first to report *T. lewisi* in rodent blood samples from Kasur district, unlike previous research in Pakistan, which primarily focused on trypanosome infection in livestock. Interestingly, Neighboring countries have reported varying prevalence rates: 1.5% in Malaysia (35), 7.7% in Sulawesi (36), 21% in Thailand (37), 31.1% in Venezuela (38) and 71% in Niger (39). However, Variations in reported prevalence across studies may result from differences in environmental conditions, methodologies, vector presence, rodent species, sample sizes, and parasite developmental stages (40).

We found a 7.59% prevalence of *T. lewisi* in *R. rattus*, lower than the 27.8% reported in Senegal (41). In this study, a higher prevalence (9.80%) was noted in rodents from human settlements, likely due to close human-rodent contact, food availability, and synanthropic behavior. Similar findings from Western Uganda and Southeast Asia highlight public health concerns linked to rodent-borne trypanosome infections in domestic environments (11, 42). Our study showed a relatively high prevalence of *T. lewisi* (5.93%) in rodents captured in rural areas. A previous investigation conducted in urban areas of New Orleans documented an overall prevalence of 11% for this parasite (43). Unsanitary rural practices, like improper waste disposal, provide food for commensal rodents, increasing their population and infection rates. Rural areas pose a higher risk than urban settings due to larger, older, and more widespread rodent colonies (44).

This study reported a higher prevalence of infection (14.55%) in male rodents. In Saudi Arabia, a higher prevalence was reported in male rats (73.8%) compared to female rats (26.2%) (45). Similarly, More *T.* infections

were recorded in male rats (24.9%) than in female rats (16%) in Brazil, which was attributed to ecological and behavioural factors (46). The increased prevalence in males is likely due to their territorial behavior and wider range, which heightens exposure to flea infestations and the risk of *Trypanosoma* transmission (47).

The present study revealed a higher prevalence of infection in adult rodents (8.57%) compared to young rodents (1.37%). In contrast to the present investigation, a study in Brazil reported a higher positive rate in young rodents (29.3%) and a lower rate in adults (8.8%) (46). These differences may stem from variations in host species, geography, and sample characteristics. Adults may have higher infection rates due to greater environmental exposure, potential vertical or sexual transmission, while young rodents, confined to nests, have limited pathogen contact (48). In our study, a higher prevalence was reported among rodents that were captured during the summer season (8.91%) and those infected with ectoparasites (15.56%). In Brazil, the summer or rainy season was associated with a significantly higher infestation prevalence (48.1%) in rats, indicating a link between climatic conditions and flea population size (46). During the summer, rodents have a higher risk of infection due to increased rodent activity, feeding behavior, and abundance of host or vector population (49).

Conclusion

These findings confirm the presence of *T. lewisi* in *R. rattus* (7.59%) and *R. norvegicus* (4.04%) through both microscopic and molecular analysis. Although preliminary, this study offers valuable insights that can support future epidemiological investigations on this parasite. There is a pressing need to implement rodent control strategies, promote hygienic practices and develop education programs aimed at controlling this infection in Pakistan.

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Conflict of interest

The authors declare no competing interests.

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