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Diverse gene targets for *Trypanosoma* detection in equines – a comparative analysis

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ABSTRACT

Zoonotic parasitic diseases, particularly those caused by *Trypanosoma* spp., pose a significant global health challenge, resulting in substantial economic losses. The infection caused by this parasite in equines is complex, affecting weight gain and the working capacity of animal. The present study was designed to estimate the occurrence of *Trypanosoma* in equines from the Rahim Yar Khan District, Southern Punjab, Pakistan, by amplifying the variable surface glycoprotein gene (RoTat 1.2 VSG gene) and the nuclear gene (18S rRNA) of the parasite. To ascertain the prevalence of *Trypanosoma*, a total of 384 blood samples, determined by calculation of the sample size formula, were collected from equines, i.e., horses, mules and donkeys. Microscopic detection of the parasite was performed using the microhematocrit method. For molecular detection and characterization of *Trypanosoma evansi*, DNA was extracted from blood samples using commercially available kits, and PCR was performed. Data on the associated risk factors were collected through a pre-designed questionnaire. The overall prevalence of *Trypanosoma* was 7.12 and 21.44% using microscopic and molecular techniques, respectively. Regarding the associated risk factors, the infection was found to be significantly more prevalent in females compared to male animals, in younger animals with low body weight compared to older animals with high body weight, and in donkeys compared to horses and mules. Among other factors, the prevalence of *Trypanosoma* was significantly higher in pregnant females, non-dewormed, symptomatic, and emaciated animals. The phylogenetic grouping and sequence analysis revealed that the sequences of both genes appeared in the *T. evansi* clade, reported in various hosts in different countries. In conclusion, the nuclear gene, that is, the 18S rRNA gene was found to be more suitable to detect *Trypanosoma* compared to the VSG gene, that is, the RoTat 1.2 VSG gene.

Key words: *Trypanosoma*; equine; Punjab; epidemiology; 18S rRNA; RoTat 1.2 VSG

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Introduction

Zoonotic parasitic diseases are neglected tropical diseases that cause considerable global health issues in humans, and huge economic losses in animals. The spectrum of these diseases varies greatly in developing and low-income countries compared to developed countries (PISARSKI, 2019). Livestock serves as the main transmission source for zoonotic infection to humans via various products, that is, milk, meat, and other products (SACK et al., 2020). A systematic review identified 45 different pathogens that can be transmitted to humans from cattle (MCDANIEL et al., 2014). The risk of zoonotic disease transmission from horses is generally overlooked. According to a survey by Canadian Public Health Professionals, every 6th individual believed that horses have no impact on public health (SNEDEKER et al., 2013). The majority of the population living in developing countries, including Pakistan, have close interaction with animals, as their economy greatly relies on livestock for their needs. In this respect, they have a greater chance of being exposed to zoonotic infections (TORGERSON and MACPHERSON, 2011).

Regarding zoonotic transmission, vectors play a significant role in the transmission of parasites. Moreover, the emergence and re-emergence of these zoonotic parasites are closely related to the vector population. Vector-borne zoonotic diseases have increased at an exponential rate in recent times. According to one report, 22% of all the emerging infectious diseases are vector-borne (VOROU et al., 2007). Many of these vectors are blood-sucking insects, feeding on the infected host (humans and animals). According to the Center for Disease Control, the number of tick-borne diseases has almost doubled in the United States in recent years (ROSENBERG et al., 2018). Sandflies, Tsetse flies and Triatoma bugs are well-known blood-sucking insects that transmit *Trypanosoma* and *Leishmania* spp. to humans and animals (COLWELL et al., 2011).

Livestock performance is strongly affected by ecto- and endoparasites, and infectious and, to a lesser extent, non-infectious diseases (ASHRAF et al., 2014; SAZMAND and JOACHIM, 2017; JABEEN et al., 2022; KANDEEL and AL-MUBARAK, 2022; QAMAR et al., 2022; YASEIN et al., 2022; DE BARROS MOURA et al., 2024). *Trypanosoma* is usually host-specific, but cross-contamination is possible due to common vectors, such as the tsetse fly (*Glossina* spp.) and kissing bug (*Triatominae*). Livestock trypanosomes include *Trypanosoma (T.) evansi*, *T. brucei*, *T. equiperdum* and *T. equinum*, causing a variety of diseases in equines (Surra, Mal de Cedras and Hip paralysis) leading to huge economic losses by reducing the productivity of the animals. *T. evansi* can be mechanically transmitted through tsetse flies, *Stomoxys*, *Tabanus*, *Haematopota*, *Crysops* and *Lyperosia* (AREGAWI et al., 2019; HALDER and GHOSH, 2019). Another study also reported the occurrence of this parasite in the equine population in Pakistan (KHAN et al., 2018).

For identification of this parasite, multiple diagnostic methods have been used. Apart from microscopy, the presence of parasites is further clarified through various concentration techniques, including the microhematocrit centrifugation method (BÜSCHER et al., 2019). For detection at species level, molecular detection such as PCR is necessary. Molecular methods have more sensitivity, specificity and rapidity than conventional methods, providing improved diagnosis and genetic characterization of field isolates (MHADHBI and SASSI, 2020; ASLAM et al., 2023). The sensitivity of molecular techniques is associated with targeting the selected sequence of parasites, using species or genus-specific primers and their number of copies in the target genome (ELHAIG and SALLAM, 2018). Numerous researchers have used PCR technique to detect *Trypanosoma* in equines by amplifying multiple genes, such as the TBR, ITS-1 and RoTat 1.2 VSG genes (SUDAN et al., 2017; ALANAZI et al., 2018; ELHAIG and SALLAM, 2018). The protozoan parasite selected is often considered a neglected pathogen in equines in Pakistan. Moreover, very limited information on the molecular epidemiology of *Trypanosoma* in equines from Southern Punjab, Pakistan, has been published so far, indicating an urgent need to develop surveillance of vector-borne

protozoan parasites at a molecular level. Therefore, the present research was conducted to ascertain the epidemiology of *Trypanosoma* in equines in Rahim Yar Khan District, Southern Punjab, Pakistan.

Materials and methods

Study area. The present study was performed in Rahim Yar Khan District, Southern Punjab, Pakistan (Fig. 1). The selection of the study area was based on the hot and humid conditions of the region, which may favor the emergence of vector populations transmitting this parasite in various hosts. Moreover, there is a significant equine population residing in the area which may become infected by this parasite, leading to reduced working capacity.

On the basis of sample size calculation through the statistical formula taking a 95% confidence interval, an expected prevalence of 50%, and a

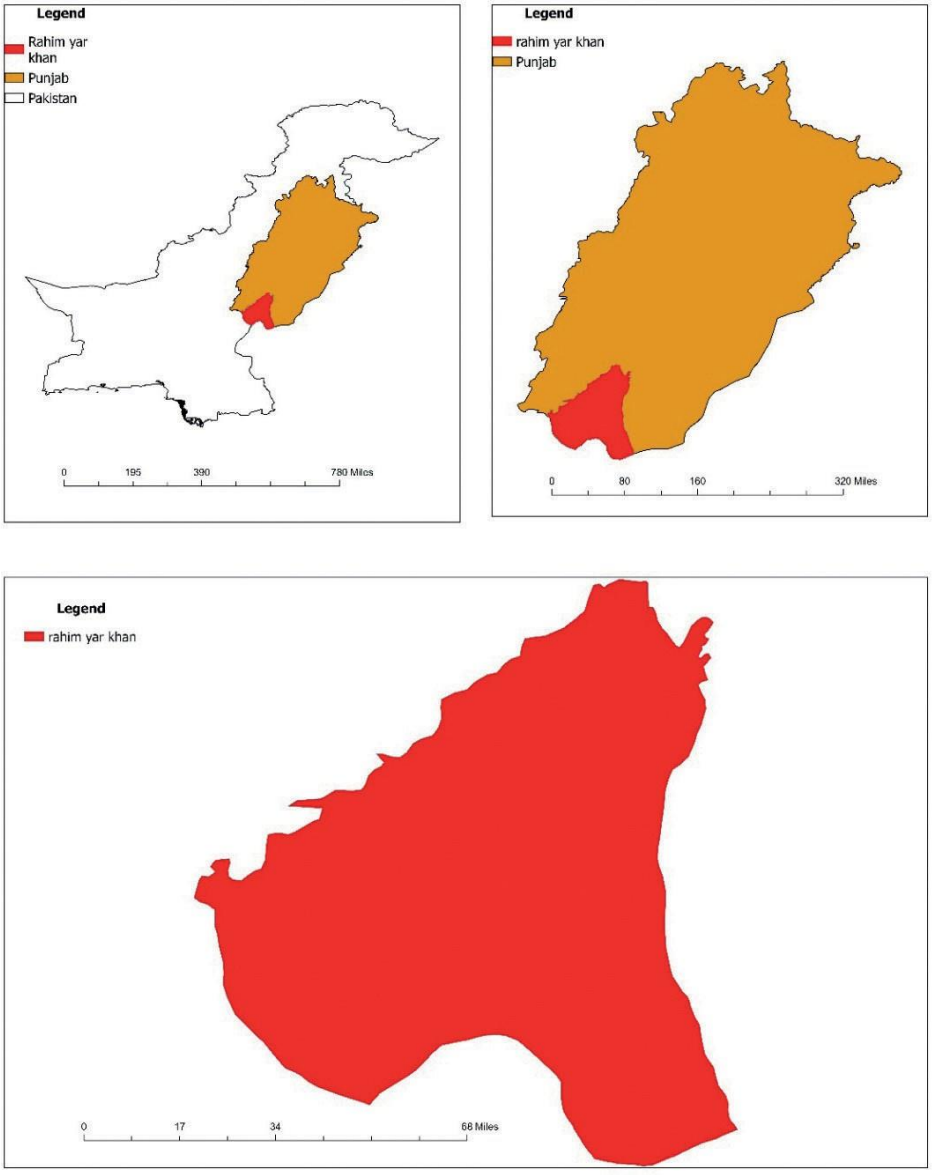

Fig. 1. Map of Rahim Yar Khan District, Southern Punjab, Pakistan

Sample size estimation and collection

desired precision of 5% (TURUSFIELD, 2018), a total of 384 blood samples from equines (horses, donkeys and mules) from the selected district were collected through the simple random sampling and proportionate allocation method. These blood samples were taken from the jugular vein of the equines into EDTA-coated vacutainers using sterile syringes (10 ml). Further processing of the samples was performed at the Epidemiology Laboratory of the Department of Parasitology, University of Agriculture, Faisalabad.

Microscopic examination. The microhematocrit method was used for detection of *Trypanosoma*. For detection, the samples were placed into capillary tubes with the help of a Pasteur pipette, followed by sealing and centrifugation at 11000 to 12000 rpm for 4 to 5 minutes. The plasma layer was discarded, and the parasite was examined from the RBC layer (TEJEDOR-JUNCO et al., 2011).

DNA extraction and molecular detection. DNA extraction from the blood samples was done using a WizPrep™ gDNA Mini Kit for blood (Wiz Bio Solutions). The procedure for DNA extraction was followed according to the manufacturer's instructions. For molecular identification, the nuclear gene, that is, the 18S rRNA gene, and the variant surface glycoprotein gene, that is, the RoTat 1.2 VSG gene of *Trypanosoma*, were amplified by PCR using genus-specific primers. The 18S rRNA gene primers (newly designed) used for the amplification were; TF: GCCCAAAATCTCACCTTGCG and TR: CGGGAATATCCTCAGCACG and the RoTat 1.2 VSG gene primers were; TF: GCCACCACGGCGAAAGAC and TR: TAATCAGTGTGGTGTGC (NGAIRA et al., 2004).

PCR was performed in a 20 µl reaction mixture containing 10 µl 2X master mixture, 1 µl of forward primer, 1 µl of reverse primer, 5 µl of DNA sample, and 3 µl of distill water, totaling 20 µl in the reaction mixture. The reaction conditions used for PCR were: initial denaturation at 94°C for 5 minutes, followed by 35 cycles of short denaturation at 94°C for 30 seconds, annealing (52°C for 18S rRNA and 46°C for RoTat 1.2 VSG) for 30 seconds, extension at 72°C for 1 minute, and final extension at 72°C for 7 minutes. The amplified products were separated using gel electrophoresis on a 1.5% agarose gel. Bands were visualized using the ChemiDoc™ imaging system of Bio-Rad. Subsequently, the specific bands were isolated from the gel using a commercially available kit (WizPrep™ Gel/PCR Purification Mini Kit; Wiz Bio Solutions) following the manufacturer's instructions.

Phylogenetic and sequence analysis. The amplicons were sequenced in a single direction by the LAB GENETIX® sequencer. All the possible sequences obtained after amplification of the selected genes of the parasite were uploaded onto the NCBI, followed by BLAST analysis and downloading the related sequences. Clustal X software was used to perform the multiple sequence alignment (HALL et al., 1999). Editing and gap filling of these aligned sequences was done using BioEdit software, followed by the construction of a phylogenetic tree. The neighbor-joining method was used in PAUP* v4b10 for construction of the phylogenetic tree. The evolutionary divergence and percentage of nucleotide identity of the obtained sequences were calculated from the aligned sequences (RONQUIST and HUELSENBECK, 2003).

Questionnaire-based survey. Information regarding the influence of possible risk factors on the occurrence of this parasite in equines was collected through a pre-designed questionnaire. The questionnaire was refined for the addition or deletion of factors, on the basis of interviews with the owners, and personal observations (TURUSFIELD, 2018).

Statistical analysis. The risk factors linked with the occurrence of *Trypanosoma* were statistically analyzed using multiple logistic regression and odds ratio. The analysis was performed using SAS version 9.4 at a 0.5% level of significance (SAS Institute, Cary, NC).

Results

Microscopic results of Trypanosoma spp. *Trypanosoma* spp. was identified on the basis of its long slender shape, with a free flagellum outside the blood cells (Fig. 2). The overall microscopic prevalence noted for *Trypanosoma* from Rahim Yar Khan District was 8.07% (31/384). The highest prevalence of *Trypanosoma* was observed in tehsil Sadiq Abad (10.31%) followed in decreasing order

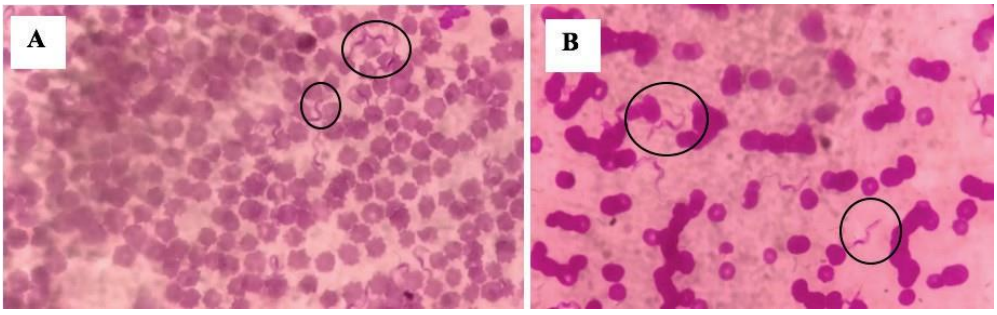

Fig. 2. Microscopic images showing *Trypanosoma* (A & B) in blood smears from equines (Giemsa stain, 100x)

by: tehsil Rahim Yar Khan (8.33%), Khanpur (7.95%), and Liaquatpur (5.49%). Further details regarding the number of samples, odds ratio, confidence intervals and p-values are given in Table 1.

Molecular (PCR) results of Trypanosoma spp. For molecular analysis, two genes were targeted to check the prevalence of *Trypanosoma evansi* from Rahim Yar Khan District, Southern Punjab, including the 18S rRNA gene (437 bp) and the RoTat

Table 1. Overall microscopic prevalence of *Trypanosoma* spp. in Rahim Yar Khan District

Tehsils	Total sample	Positive	CI	Odds ratio	P-value
Rahim Yar Khan	108	9 (8.33)	0.2064-1.9813	0.6395	0.4384
Sadiq Abad	97	10 (10.31)	0.1660-1.5412	0.5058	0.2305
Liaquatpur	91	5 (5.49)	–	–	–
Khanpur	88	7 (7.95)	0.2053-2.2050	0.6728	0.5128
Total	**384**	**31 (8.07)**	–	–	–

Table 2. Overall prevalence of *Trypanosoma* spp. in Rahim Yar Khan District using PCR by amplifying the 18S rRNA gene and the RoTat 1.2 VSG gene

Tehsils	Total samples	18S rRNA gene				RoTat 1.2 VSG gene			
		Positive sample n (%)	CI	Odds ratio	P-value	Positive sample n (%)	CI	Odds ratio	P-value
Rahim Yar Khan	108	25 (23.15)	0.4136-1.6201	0.8186	0.5656	25 (23.15)	0.3821 – 1.5225	0.7627	0.4424
Sadiq Abad	97	2 (27 83)	0.3237- 1.2626	0.6393	0.1976	26 (26.80)	0.3138 – 1.2540	0.6273	0.1870
Liaquatpur	91	1 (19 78)	–	–	–	17 (18.68)	–	–	–
Khanpur	88	2 (22 72)	0.4091- 1.7178	0.8384	0.6300	18 (20.45)	0.4267 – 1.8707	0.8934	0.7650
Overall	384	9 (23 44)	–	–	–	86 (22.39)	–	–	–

1.2 VSG gene (530 bp). The PCR results showed that the ITS gene of *Trypanosoma* was successfully amplified in 90 samples, while 86 samples were found positive for *Trypanosoma* by the RoTat 1.2 VSG gene. Regarding the district wise prevalence of the parasite in equines, similar to microscopic analysis, amplification of both the genes showed the highest prevalence of *Trypanosoma* in Sadiq Abad tehsil (administrative area) followed by Rahim Yar Khan, Khanpur and Liaquatpur tehsils. Further details regarding prevalence (percentages), the number of samples, odds ratio, confidence intervals and p-values are given in Table 2.

Associated risk factors. The molecular prevalence observed for *Trypanosoma evansi* by amplifying the 18S rRNA gene from Rahim Yar Khan District was 23.44%. Table 3 shows the varied prevalence of *Trypanosoma evansi* between different levels of risk factors that influence the occurrence of the parasite in the study area. In relation to sex, a higher prevalence was found in female animals (62/222: 27.93%) compared to male animals (28/162: 17.28%). In relation to age, a higher prevalence was found in animals of 1-2 years of age (27/98; 27.55%) followed by animals more than 15 years of age (40/154; 25.97%) and animals aged between 3 and 15 years (23/132;

Table 3. Targeted risk factors associated with the prevalence of *Trypanosoma* spp. (18S rRNA gene)

Determinant	Category	No. of samples	Positive samples n (%)	Confidence interval	Odds ratio	P-value
Gender	Male	162	28 (17.28)	–	–	–
	Female	222	62 (27.93)	0.3265-0.8907	0.5392	0.0159
Age	1-2	98	27 (27.55)	0.2951-1.0433	0.5549	0.0675
	3-15	132	23 (17.42)	–	–	–
	>15	154	40 (25.97)	0.3380-1.0700	0.6014	0.0837
Body weight	150-350	195	50 (25.64)	0.4254-1.4863	0.7952	0.4726
	350-500	112	25 (22.32)	0.4753-1.9157	0.9542	0.8951
	>500	77	15 (19.48)	–	–	–
Species	Horse	134	28 (20.89)	0.3968-1.7836	0.8413	0.6521
	Mule	66	12 (18.18)	–	–	–
	Donkey	184	50 (27.17)	0.2943-1.2051	0.5956	0.1495
Physiological status	Pregnant	63	20 (31.75)	0.1679-0.6941	0.3413	0.0030
	Lactating	146	20 (13.70)	–	–	–
	Dry	175	50 (28.57)	0.2234-0.7049	0.3968	0.0016
Medication history	Dewormed	160	25 (15.62)	–	–	–
	Non-dewormed	224	65 (29.02)	0.2706-0.7583	0.4530	0.0026
Health status	Symptomatic	122	37 (30.33)	0.3570-0.9507	0.5826	0.0306
	Non-symptomatic	262	53 (20.23)	–	–	–
Body condition	Good	155	33 (21.29)	–	–	–
	Moderate	134	30 (22.39)	0.5360-1.6406	0.9377	0.8217
	Emaciated	95	27 (28.48)	0.3781-1.2275	0.6812	0.2013
Season	Spring	174	35 (20.11)	–	–	–
	Summer	210	55 (26.19)	0.4383-1.1487	0.7096	0.1628

17.42%). In relation to body weight, the infection was found to be more prevalent in animals with body weight between 150-350 kg (50/195; 25.64%) followed by animals with body weight of 350-500 kg (25/112; 22.32%), and animals with body weight more than 500 kg (15/77; 19.48%). In relation to species, a higher prevalence was found in donkeys (50/184; 27.17%) followed by horses (28/134; 20.89%) and then mules (12/66; 18.18%). Regarding physiological status, there was a higher prevalence of infection in pregnant animals (20/63; 31.75%) followed by dry (50/175; 28.27%) and lactating animals (20/146; 13.70%). In relation to medication history, a higher prevalence was found in non-dewormed animals (65/224; 29.02%) compared to dewormed animals (25/160; 15.62%). With respect to health status, a higher prevalence was found in symptomatic animals (37/122; 30.33%) compared to non-symptomatic animals (53/262; 20.23%). In relation to body condition, the infection was found to have a higher prevalence in animals with emaciated body condition (27/95; 28.48%), followed by moderate (30/134; 22.39%) and good body condition (33/155; 21.29%). Similarly, regarding season, the infection was found to be more prevalent in the summer season (55/210; 26.19%) compared to the spring season (35/174; 20.11%).

Table 4. Targeted risk factors associated with the prevalence of *Trypanosoma* spp. (RoTat 1.2 VSG gene)

Determinant	Category	No. of samples	Positive samples n (%)	Confidence interval	Odds ratio	P-value
Gender	Male	162	28 (17.28)	–	–	–
	Female	222	58 (26.13)	0.3564-0.9795	0.5908	0.0413
Age	1-2	98	25 (25.51)	0.3442-1.2232	0.6489	0.1812
	3-15	132	24 (18.18)	–	–	–
	>15	154	37 (24.03)	0.3948-1.2506	0.7027	0.2303
Body weight	150-350	195	47 (24.10)	0.3967-1.4630	0.7618	0.4139
	350-500	112	24 (21.43)	0.4308-1.8268	0.8871	0.7451
	>500	77	15 (19.48)	–	–	–
Species	Horse	134	30 (22.38)	0.3654-1.6241	0.7704	0.4930
	Mule	66	12 (18.18)	–	–	–
	Donkey	184	44 (23.91)	0.3472-1.4401	0.7071	0.3395
Physiological status	Pregnant	63	18 (28.57)	0.1928-0.8169	0.3968	0.0121
	Lactating	146	20 (13.69)	–	–	–
	Dry	175	48 (27.42)	0.2359-0.7478	0.4200	0.0032
Medication history	Dewormed	160	27 (16.87)	–	–	–
	Non-dewormed	224	59 (26.34)	0.3411-0.9449	0.5677	0.0294
Health status	Symptomatic	122	34 (27.86)	0.3892-1.0554	0.6409	0.0804
	Non-symptomatic	262	52 (19.85)	–	–	–
Body condition	Good	155	32 (20.64)	–	–	–
	Moderate	134	31 (23.13)	0.4942-1.5118	0.8644	0.6094
	Emaciated	95	23 (24.21)	0.4427-1.4984	0.8144	0.5093
Season	Spring	174	33 (18.96)	–	–	–
	Summer	210	53 (25.23)	0.4245-1.1323	0.6933	0.1433

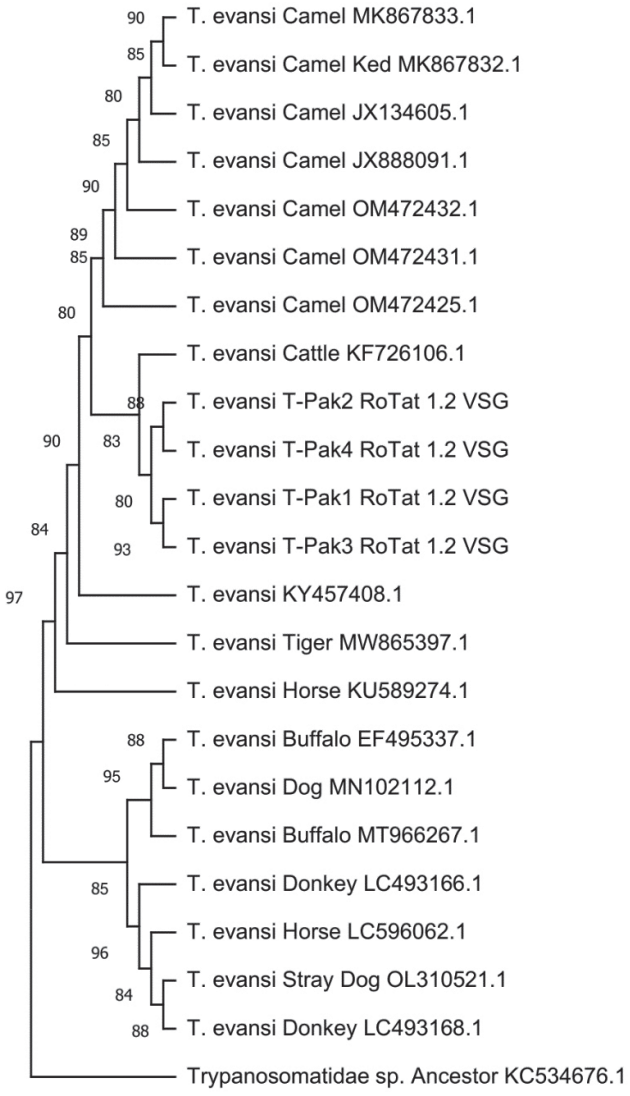

Fig. 3. Neighbor joining tree showing the phylogenetic relationships among the obtained sequences of *T. evansi* (RoTat 1.2 VSG gene) and closely related sequences

The molecular prevalence observed for *Trypanosoma evansi* by amplifying the RoTat 1.2 VSG gene from Rahim Yar Khan District was 22.39%. Like the 18S rRNA gene, a similar trend in the prevalence of *Trypanosoma evansi* in relation to the different associated risk factors was seen (Table 4).

Sequence analysis and phylogeny of Trypanosoma spp.

RoTat 1.2 VSG gene. A total of four samples were randomly selected out of 86 samples for sequencing using forward and reverse primers. All the selected sequences showed high similarity with each other and were included in the same clade. The obtained sequences showed 98.7% similarity with *T. evansi* isolated from equines from Uttar Pardesh, India (KY457408.1), 98.23% similarity with *T. evansi* isolated from cattle from Egypt (KF726106.1), 98.23% similarity with *T. evansi* isolated from camels from Kenya, and 98.06% similarity with the *T. evansi* isolated from camels from Nigeria (OM472432.1). To explore genetic diversity, a phylogenetic tree was

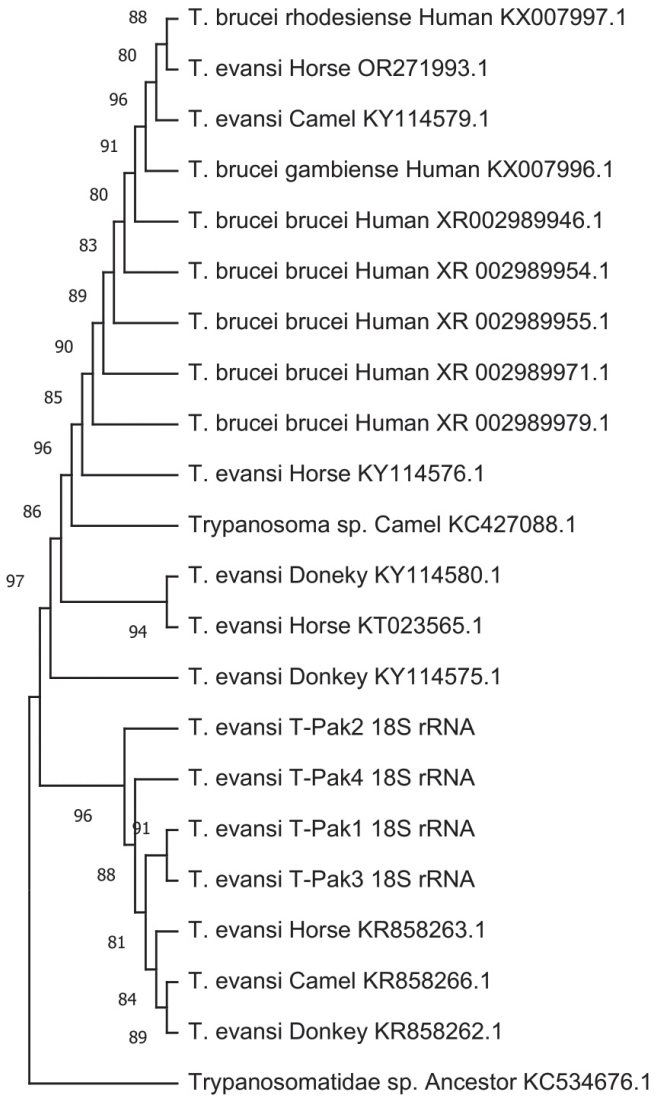

Fig. 4. Neighbor joining tree showing the phylogenetic relationships among the obtained sequences of *T. evansi* (18S rRNA) and closely related sequences

constructed using the neighbor joining method. Among the subject sequences, all sequences appeared in the same clade. All sequences were ascendant to the sequence of *T. evansi* from equines in India (KY457408.1), and *T. evansi* from tigers in Pakistan (MW865397.1). All sequences were descendent to the sequence of *T. evansi* from cattle in Egypt (KF726106.1) and *T. evansi* from camels in Nigeria (OM472425.1). *Paratrypanosoma confusum* (KC534676.1) was used as the outgroup/ancestor (Fig. 3).

18S rRNA gene. A total of four samples were randomly selected out of 90 samples for sequencing by the Sanger sequencing method by using forward and reverse primers. All the selected sequences showed high similarity with each other and were included in the same clade. The obtained sequences showed 99.51% similarity with *T. evansi* isolated from horses from Hisar, Haryana, India (KR858263.1), 99.26% similarity with *T. evansi* isolated from donkeys from Junagarh, Gujrat, India (KR858362.1), 99.51% similarity

with *T. evansi* isolated from camels from Bikaner, Rajhastan, India (KR858266.1), and 99.51% similarity with *T. equiperdum* isolated from horses from South Africa (KY609968.1). To explore genetic diversity, a phylogenetic tree was constructed using the neighbor joining method. All the subject sequences appeared in the same clade. The obtained sequences were ascendant to the sequence of *T. evansi* from horses in India (KR858263.1) and *T. evansi* from camels in India (KR858266.1). However, the subject sequences were descendent to the sequence of *T. evansi* from donkeys in India (KY114575.1) and *T. evansi* from horses in India (KT023565.1). *Paratrypanosoma confusum* (KC534676.1) was used as the outgroup/ancestor (Fig. 4).

Comparative sequence analysis of two genes. Comparative percent identity analysis between the two gene sequences of the isolates here showed that the RoTat 1.2 VSG gene sequences had high percent similarity compared to those of the 18S rRNA sequences, indicating that the region amplified by the 18S rRNA genus specific primers is more diverse than that of the RoTat 1.2 VSG gene (Table 5). Moreover, the PCR results revealed that the 18S rRNA gene is more sensitive in detecting *Trypanosoma* compared to the RoTat 1.2 VSG gene (Table 5). Altogether, it can be concluded that 18S rRNA is more suitable for detection of *Trypanosoma* in equines.

Discussion

Zoonotic parasitic infections are a growing concern for developing countries such as Pakistan and are generally overlooked, causing huge economic losses in terms of animals, in addition to their zoonotic transmission to humans (PISARSKI, 2019). The transmission of these diseases from horses is also a serious issue that needs to be addressed, as indicated by the Canadian Public Health Professionals (SNEDEKER et al., 2013). The emergence and re-emergence of these zoonotic parasites are closely related to the vector population. Multiple studies also showed an exponential increase in emerging infectious diseases transmitted by vectors in the past (VOROU et al., 2007; JONES et al., 2008). Among these, Tsetse flies and Triatoma bugs are well-known blood-sucking insects that transmit various species of *Trypanosoma* to humans and animals (COLWELL et al., 2011). The important species of *Trypanosoma* in equines include *Trypanosoma equiperdum*, *Trypanosoma equinum* and *Trypanosoma evansi*, causing mal de cedras (hip dysplasia) and surra disease, characterized by edematous swelling on the genitalia and ventral abdomen, and hind quarter paralysis (MARTINS SANTOS et al., 2018; HALDER and GHOSH, 2019).

A total of 384 blood samples were collected from the Rahim Yar Khan District, Southern Punjab, Pakistan. After microscopic examination of the

Table 5. Percent identity between gene sequences of isolates

18S rRNA gene				
Sequence	***T. evansi* T-Pak2**	***T. evansi* T-Pak4**	***T. evansi* T-Pak1**	***T. evansi* T-Pak3**
T. evansi T-Pak2	–			
T. evansi T-Pak4	97.8	–		
T. evansi T-Pak1	95.6	94.4	–	
T. evansi T-Pak3	97	96.3	95.5	–
RoTat 1.2 VSG gene				
Sequence	***T. evansi* T-Pak2**	***T. evansi* T-Pak4**	***T. evansi* T-Pak1**	***T. evansi* T-Pak3**
T. evansi T-Pak2	–			
T. evansi T-Pak4	99.7	–		
T. evansi T-Pak1	98.7	98.9	–	
T. evansi T-Pak3	97.9	98.1	98.5	–

collected samples using the microhematocrit method, DNA was extracted from the whole blood. Sequence and phylogenetic analysis were performed by amplifying multiple genes for species characterization. From 384 blood samples of equines examined, 8.07% of the samples were found positive for *Trypanosoma* through microscopy. Regarding molecular analysis, 23.44% of the samples were found positive for *Trypanosoma* by amplifying the 18S rRNA gene and 22.39% of samples by amplifying the RoTat 1.2 VSG gene. Previously, many scientists detected this parasite from equine blood using simple microscopic techniques, serological techniques, and molecular techniques (ESCOBAR et al., 2020; GAZZONIS et al., 2020; GUMMERY et al., 2020; GAZZONIS et al., 2022; SUGANUMA et al., 2022; VERNEY et al., 2022; JAVANSHIR et al., 2023).

The higher infection rate found by amplifying various genes of *Trypanosoma* (22.39-23.44%) compared to microscopy (8.07%) indicated that animals were infected with low-level parasitemia that could not be detected by microscopic techniques. The results of the current study coincide with the findings of VOURCHAKBÉ et al. (2020). They also found higher infection rates (35.3%) in horses and donkeys through PCR compared to the capillary tube centrifugation test (12.6%) from three active sleeping sickness foci in the south of Chad. In the current study, the infection of trypanosomiasis was found higher in female animals (27.93%) compared to male animals (17.28%) by amplifying multiple genes (18S rRNA and RoTat 1.2 VSG). Similar findings were reported by AGINA et al. (2021). They amplified the ITS1 gene to identify the infection of equine trypanosomiasis in Nigerian horses. The infection was found slightly higher among female animals compared to male animals. Similarly, YAMAZAKI et al. (2022) also detected a higher level of parasitemia in female horses (31.78%) compared to male horses (27.90%) using TBR primers from Paraguay. In general, the higher infection rate among female animals is generally attributed to immune system deficiencies during pregnancy. Moreover, the hormonal differences between the sexes may also exaggerate vector feeding behavior in male and female animals.

In relation to age, the infection of trypanosomiasis was found highest in animals younger than 2 years of age (25.51-27.55%) followed by animals more than 15 years (24.03-25.97%), and animals 3 to 15 years of age (17.42-18.18%), respectively. Alternatively, EREQAT et al. (2020) reported a higher level of infection among older animals compared to young animals. The higher infection in younger animals might be due to the presence of low immunity and higher chances of secondary infections. In the present study, the trypanosomiasis infection was found higher in animals with a body weight of 150-350kg (24.10-25.64%) followed by animals with a body weight of 350-500kg (21.43-22.32%), and then animals with a body weight more than 500kg (19.48%). In relation to species, the trypanosomiasis infection was found to have higher prevalence in donkeys (23.91-27.17%), followed by horses (20.89-22.38%) and then mules (18.18%). Similar findings were reported by VOURCHAKBÉ et al. (2020) from horses and donkeys in the south of Chad. They reported a higher prevalence of trypanosomiasis in donkeys (39.3%) compared to horses (30.5%) through PCR. Alternatively, the findings of ALANAZI et al. (2018) contradict the findings of current study. They reported a higher infection rate in horses (3.3%) compared to donkeys (2.8%) by amplifying the universal ITS gene through PCR in Riyadh province, Saudi Arabia. However, there was no significant effect of age or sex reported in relation to the occurrence of disease. In general, horses are more susceptible to acquiring infection compared to donkeys. The reason behind the high prevalence of the disease in donkeys in the current study is unknown but it may be due to the density and behavior of the biting flies.

In the current study, trypanosomiasis infection was found higher in pregnant animals (28.57-31.75%) compared to dry (27.42-28.57%) and lactating animals (13.69-13.70%). The findings of the current study coincided with the results of SINGH et al. (2021). They reported a slightly higher prevalence of trypanosomiasis in pregnant animals (26.47%) compared to non-pregnant animals (26%) in equines reared in the northern areas of India. The higher infection rate in pregnant

animals is attributed to the immunocompromised state of animals during pregnancy.

In relation to health status, a marked difference was reported between animals showing disease symptoms (27.86-30.33%) and those showing the asymptomatic disease (19.85-20.23%). Similar findings were reported by SINGH et al. (2021) and BIRAL et al. (2021). They reported significantly higher infection among animals showing clinical disease symptoms than those showing no disease symptoms. The presence of subclinical infection in the case of trypanosomiasis in equines is rare, and most animals show various disease symptoms during infection. Moreover, in the current study, a higher prevalence of infection with trypanosomiasis was reported in animals with emaciated body condition (24.21-28.48%), followed by moderate (22.39-23.13%), and then good body condition (20.64-21.29%) respectively. The results regarding body condition score coincide with the findings of RAFTERY et al. (2020). They also observed a higher infection rate in animals with emaciated body condition compared to animals with good body condition. In the present study, a higher prevalence of infection was found during the summer season (25.23-26.19%) compared to the spring (18.96-20.11%). However, GOLOMBIESKI et al. (2023) reported a higher prevalence of trypanosomiasis in equines during the fall compared to the summer and spring seasons in various locations in Brazil. The marked difference between the occurrence of infection in Pakistan and other countries may be due to the different climatic conditions and ultimately vector population responsible for transmission of disease.

In the phylogenetic analysis, regarding the RoTat 1.2 VSG gene, all sequences were ascendant to the sequence of *T. evansi* from equines in India (KY457408.1) and *T. evansi* from cattle in Egypt (KF726106.1). Similarly, for the 18S rRNA gene, the sequences were ascendant to the sequence of *T. evansi* from horses in India (KR858263.1) and *T. evansi* from camel in India (KR858266.1).

For detection and molecular characterization of *Trypanosoma* species in various hosts, various genes of *Trypanosoma* have been used in various studies, such as 18S rRNA, ITS, RoTat 1.2 VSG (SUDAN et al., 2017; ALANAZI et al., 2018; ELHAIG and SALLAM, 2018). In the present study, two genes, 18S rRNA and RoTat 1.2 VSG, were used to find suitable markers for detection and molecular characterization of *Trypanosoma* species in equines. The results showed a higher prevalence of *T. evansi* in equines by amplifying the 18S rRNA gene, followed by the RoTat 1.2 VSG gene. This difference may be attributed to the lack of expression of the RoTat 1.2 VSG gene in some strains of *T. evansi* results in negative outcomes. Moreover, the comparative percent analysis between the two genes indicated that the region of the 18S rRNA gene amplified by the current primers is more diverse compared to that of the RoTat 1.2 VSG gene. The findings of the current study coincided with the results of SALIM et al. (2011). They also found a higher prevalence of *T. evansi* in camels by amplifying another nuclear gene (i.e. ITS-1 gene) compared to using the RoTat 1.2 VSG gene.

Conclusions

In conclusion, *Trypanosoma* is prevalent in all species of the equine population in the study area, including horses, donkeys and mules. Various risk factors influence the prevalence of *Trypanosoma* in equines in the study area. Of the two genes amplified in the present study, 18S rRNA was found to be more suitable for detection and differentiation of *Trypanosoma* species. Further research is suggested in the form of a comparative analysis of different genes and their different regions using different primer pairs to find more suitable markers for detection and differentiation of *Trypanosoma* in hosts.

Authorship contribution statement

We hereby confirm that all the authors (MASM, MKK, RZA, MS) made contributions regarding the conception and design of the study, acquisition of data, analysis and interpretation of data, drafting the article and revising it critically for important intellectual content.

Declaration of competing interest

The authors declare no conflict of interest.

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MUGHAL, M. A. S., M. K. KHAN, R. Z. ABBAS, M. SAQIB: Različiti ciljni geni za otkrivanje tripanosoma u kopitara – komparativna analiza. Vet. arhiv 95, 157-172, 2025.

SAŽETAK

Zoonotske parazitske bolesti, osobito one uzrokovane parazitom *Trypanosoma* spp., znatan su zdravstveni izazov u svijetu i čest uzrok velikih ekonomskih šteta. Invazija koju ovaj parazit uzrokuje u kopitara složena je između ostaloga i zbog utjecaja na tjelesnu masu životinja odnosno njihovu radnu sposobnost. Istraživanje je provedeno kako bi se ustanovila pojavnost tripanosoma u kopitara iz regije Rahim Yar Khan, Punjab u Pakistanu. Provedeno je umnožavanje promjenljivog glikoproteinskog gena *VSG* (RoTat 1.2 VSG gen) i nukleusnoga gena (18S rRNA) parazita. Kako bi se ustanovila prevalencija tripanosoma prema formuli za izračun veličine uzorka, prikupljeno je ukupno 384 uzoraka krvi, od kopitara - konja, magaraca i mula. Mikroskopska detekcija parazita provedena je metodom mikrohematokrita. Za molekularnu detekciju i karakterizaciju parazita *Trypanosoma evansi*, DNA je ekstrahirana iz uzoraka krvi upotrebom komercijalnog kita te je proveden PCR. Podaci o rizičnim faktorima za tripanosomijazu prikupljeni su putem prethodno sastavljenog upitnika. Mikroskopijom je utvrđena ukupna prevalencija tripanosoma od 7,12%, a molekularnim metodama prevalencija od 21,44%. S obzirom na rizične čimbenike, utvrđena je veća prevalencija invazije u ženki u usporedbi s mužjacima, zatim veća prevalencija u mlađih životinja s manjom tjelesnom masom u usporedbi sa starijim jedinkama s većom tjelesnom masom te veća prevalencija u magaraca u usporedbi s konjima i mulama. Što se tiče ostalih čimbenika, prevalencija tripanosoma bila je znakovito veća u gravidnih ženki, onih koje nisu bile tretirane protiv parazita te u simptomatskih i mršavijih jedinki. Filogenetsko grupiranje i sekvencijska analiza pokazali su da se sekvencije obaju gena pojavljuju kod *T. evansi*, o kojoj postoje izvješća u različitim zemljama i različitim domaćinima. Zaključno, utvrđeno je da je nukleusni gen, odnosno gen 18S rRNA mnogo prikladniji u otkrivanju tripanosoma u usporedbi s genom VSG, odnosno genom RoTat 1.2 VSG.

Ključne riječi: *Trypanosoma;* konj; Punjab; epidemiologija; 18S rRNA; RoTat 1.2 VSG