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Detection of *Theileria equi* infection in ixodid ticks of equines using nested polymerase chain reaction from Punjab, India

DEEPAK SUMBRIA[1], L D SINGLA[2], AMRITA SHARMA[3] and M S BAL[4]

Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab 141 004 India

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ABSTRACT

The aim of this study was to perceive *Theileria equi* DNA in ticks by nested PCR and to list the risk factors associated with infection in equines from Punjab. A total of 84 ticks were obtained from 464 screened animals. The local tick variety was identified and verified on the basis of characters revealed in scanning electron microscopy. Amplicons of 665 bp obtained by nPCR specific for 18S rRNA of *T. equi* were detected in 13.1% of ticks, with higher risk of infection in western than in eastern Punjab. Incidence of parasite was higher in *Hyalomma anatolicum anatolicum* as compared to *Rhipicephalus* (*Boophilus*) *microplus*, particularly the female ticks. Phylogenetic analyses indicated closed homology with strain from Brazil. This is the first study of *T. equi* incidence in ixodid ticks in Punjab which is the most important vector for the spread of the infection to healthy equine in endemic areas.

Key words: Equines, *Hyalomma anatolicum anatolicum,* Nested-PCR, Scanning electron microscopy, *Theileria equi*

Prevalence of equine piroplasmosis (EP), the worldwide distributed vector borne disease of equines, synchronizes with the existence of the ixodid tick-vector. Out of the two major causative agents of EP, *Theileria equi* is more virulent and tends to cause fulminating parasitaemia than *Babesia caballi* (Soulsby 2005). *T. equi* is endemic in India; out of 10 ixodid tick species, *H. a. anatolicum* have been shown to transmit *T. equi* (Sharma *et al.* 1982). The status of EP in ixodid tick had not been evaluated in Punjab, India yet. So, this study aimed to use nested polymerase chain reaction (nPCR), for the detection of *T. equi* parasite on ticks DNA samples collected from equines distributed in different district of Punjab, India.

MATERIALS AND METHODS

Ethical aspects: The study has the approval (IAEC/2014/46-73) of the ethics committee for animal experiments duly constituted by the Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, India. Tick samples were collected in a scientific manner, so as to avoid any accidental injury to the equines as well as to the mouth parts of ticks. A prior consent of the equines keepers was also sought.

Study areas: The province of Punjab covers a total – area of 50,362 square kilometers between 29"30'N to 32″32′N latitude and 73″55′E to 76″50′E longitudes having about 34 thousand horses and ponies (Sumbria *et al.* 2014). During blood collection (from 464 equine to see the status of EP), animal were thoroughly examined for ecto-parasite and only 84 ticks were collected from 27 equines (Table 1). Moreover, an epidemiological questionnaire was also maintained to calculate the risk factors associated with EP transmission and owners response to it.

Identification of ticks: The species and sex of the collected ticks was identified by studying the characteristics microscopically as per the identification keys (Estrada-Pena *et al.* 2004). After proper identification, all the ticks were kept individually in absolute ethanol before being treated for further process.

Scanning electron microscopy (*SEM*)*:* As there is no report regarding the characteristic feature of Indian isolate of tick affecting equine population, SEM of collected tick was done in Electron Microscopy and Nanoscience Lab at Punjab Agricultural University (PAU), Ludhiana, Punjab, India as per standard protocol.

DNA extraction and PCR amplification: All the collected ticks were kept in the laboratory for 30 days (29°C and 85% relative humidity) to digest the blood meal so as to rule out the probability of residue DNA from blood meal (Anbalagan *et al.* 2014) and then washed in three sterile water baths followed by one absolute ethanol bath, and at last air dried and collected in sterile microtubes (Halosa *et al.* 2004). DNA extraction from tick was performed using the DNeasy Blood and Tissue (Qiagen, Hilden, Germany) as per manufacturer's protocol.

Present address: [1]Post-Doctoral Research Associate (deepak_sumbria@yahoo.com), University of Tennessee, United States. [2]Professor and Head of Department (ldsingla@gmail.com), [4]Assistant Professor (bal_epi@rediffmail.com), Department of Veterinary Parasitology, College of Veterinary Sciences. [3]PhD Scholar (amrita14887@gmail.com), University of Georgia, United States.

Table 1. Zone and district wise tick prevalence and *Theileria equi* prevalence in ticks in Punjab.

Zone	District	Animal screened (%)	Animal infested	Ticks collected	Tick positive for *T. equi* (%)
SMZ	Hoshiarpur	38	0	0	0
	Pathankot	17	0	0	0
UZ	Nawanshahr	22	4 (18.18)	23	5 (21.73)
CPZ	Mohali	31	0	0	0
	Amritsar	29	0	0	0
	Tarn taran	25	0	0	0
	Jalandhar	22	2 (9.09)	6	0
	Ludhiana	64	0	0	0
WZ	Patiala	73	0	0	0
	Moga	30	0	0	0
	Bathinda	17	0	0	0
	Muktsar	33	5 (15.15)	20	3 (15)
WPZ	Ferozepur	37	8 (21.62)	21	1 (4.76)
	Fazilka	26	8 (30.76)	14	2 (14.28)
	Total	464	27 (5.82)	84	11 (13.09)

SMZ, Sub mountain zone; UZ, undulating zone; CPZ, central plain zone; WPZ, western plain zone; WZ, western zone.

DNA was finally extracted in 100 µl of elution buffer and was kept at –20°C until further use. Nested PCR was performed as per Sumbria *et al.* (2016), the amplified PCR products were electrophoresed on 1.5% agarose and visualized under UV Transilluminator for 665 bp band (Fig. 1). A non template control, i.e. control having no DNA amplicons was also used to rule out any contamination of the products.

Analysis of nucleotide sequence: Amplicons from nested PCR product targeting 18S rRNA gene specifically were custom sequenced from Xcelris Genomics, Ahmedabad, India. The nucleotide sequences were then subjected to BLASTn analysis, the homologous sequences belonging to different strains were retrieved from database and were used to make phlylogetic tree (Sumbria *et al.* 2015a). The final sequences were submitted to NCBI database and the accession numbers was obtained (GeneBank Accession No LC010333).

Statistical analysis: The prevalence of *T. equi* with respect to various risk factors (age, species, sex, deworming/ vaccination, management and other pets on farm etc) was statistically analyzed employing Pearson's chi-square test at P≤0.05. Analysis of risk factor was done on WinEpiscope software v0.1.

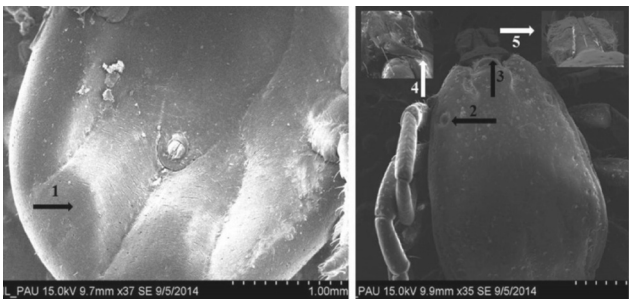

Fig. 1. *Rhipicephalus* (*Boophilus*) *microplus.* Body had finger marking (1), eyes were present (2) and basis capituli was hexagonal and broad with short palpi and hypostome (3), Coxa 1 was bifid [ventral side] (4) and head with measurements (5).

RESULTS AND DISCUSSION

The species and sex of the ticks was identified based on the characteristic morphological features by compound microscopy followed by scanning electron microscopy. In SEM, *R.*(*B*). *microplus* ticks revealed basic character as per the literature (Kang and Jang 1985, Soulsby 2005) (Fig. 1). Spiracle plate was oval in shape and situated posterolaterally to coxa 4, two different types of setae (short and long) were present on body and coxa respectively and festoons and anal grove was absent (not in image). In *H. a. anatolicum,* also the characters were as per the literature (Kang and Jang 1985, Soulsby 2005) (Fig. 2). Scanning electron microscopy seems to be a better option as specimens are always seen in three dimensions along with all its structural measurements (Kang and Jang 1985). The scarcity of literature related to

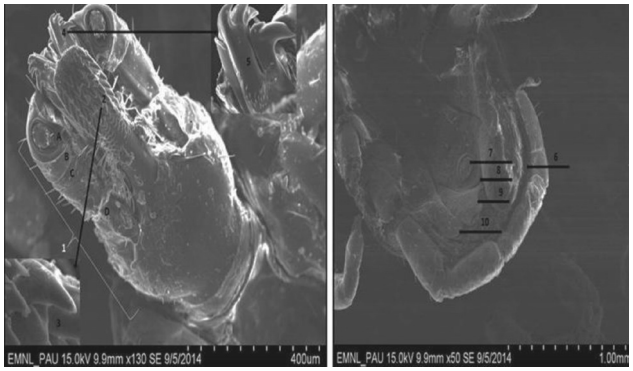

Fig. 2. *Hyalomma anatolicum anatolicum.* Capitulum with hypostome and palpi were long (1), hypostome with inverted teeth (2,3), chelicerae (4,5), four segments of palpi (A,B,C,D), festoon (6), anus (7), anal groove (8), adanal and accessory adanal shields (9,10).

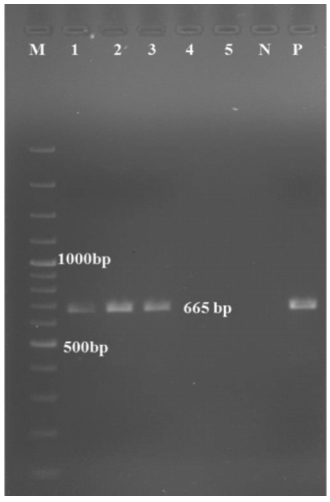

Fig. 3. Agarose gel electrophoresis (1.5%) showing amplified DNA of 665 bp for *T. equi*. Lane M, 100 bp plus DNA ladder; lane P, positive control; lane N, negative control; lanes 1–5, tested field ticks samples.

Table 2. Univariate analysis of risks associated with *Theileria equi* prevalence in ticks of Punjab

Factor	Variable		Total ticks	Ticks positive	%	Confidence interval (CI)	χ^2-square	Degree of freedom (Df)	P value	Odds ratio	Confidence interval (CI)
Area	Eastern	SMZ-UZ	23	1	4.34	–2.84–11.54	3.70	2	NS	0.265	0.01–1.96
	Central	CPZ	6	0	0	0–0				0	0–6.75
	Western	WPZ-WZ	55	10	18.18	9.39–26.97				6.22	0.74–136.94
Temperature: Humidity	Highest: Lowest:	WPZ-WZ	55	10	18.18	9.39–26.97	3.70	2	NS	6.22	0.74–136.94
	Moderate: Moderate	CPZ	6	0	0	0–0				0	0–6.75
	Lowest: Highest	SMZ-UZ	23	1	4.35	–2.84–11.54				0.265	0.01–1.96
Vector	Species	*H. a. anatolicum*	68	10	14.70	7.45–21.96	0.81	1	NS	2.58	0.29–58.19
		R. (*B*). *microplus*	16	1	6.25	–3.97–16.47				0.387	0.02–3.39
	Sex	Male	55	4	7.27	1.35–13.19	4.75	1	0.05	0.246	0.05–1.07
		Female	29	7	24.13	10.70–37.56				4.057	0.93–18.72
	Hyalomma anatolicum anatolicum	Male	48	4	8.33	1.59–15.07	5.28	1	0.05	0.212	0.07–1.02
		Female	20	6	30	12.68–47.32				4.714	0.98–14.14
	Rhipicephalus (*Boophilus*) *microplus*	Male	7	0	0	0–0	0.83	1	NS	0	0–25.24
		Female	9	1	11.11	–6.59–28.81				∞	0.04-∞
Host	Age	Less than 2 year	18	5	27.78	9.94–45.61	4.34	1	0.05	3.056	0.87–10.09
		More than 2 year	66	6	9.09	3.11–15.07				0.327	0.09–1.15
	Species	Horse	64	9	14.06	6.72–21.41	0.22	1	NS	1.473	0.26–10.91
		Donkey/Mule	20	2	10	–1.34–21.34				0.679	0.09–3.89
	Sex	Male	17	1	5.88	–3.76–15.53	0.97	1	NS	0.394	0.02–2.56
		Female	67	10	14.93	7.56–22.28				2.53	0.39–52.83
	Infection	Blood film Positive	22	4	18.18	4.28–32.07	0.03	1	NS	1.74	0.375–7.802
		Blood film negative	62	7	11.29	4.49–18.08				0.573	0.128–2.669
Farm practices	Management	Organized	12	1	8.33	–5.15–21.81	0.28	1	NS	0.564	0.03–5.15
		Unorganized	72	10	13.88	7.00–20.77				1.77	0.19–40.71
	Deworming	Yes	9	1	11.11	–6.59–28.81	0.04	1	NS	0.813	0.03–7.84
		No	75	10	13.33	6.69–19.96				1.231	0.13–29.02
	Pets on farm	Yes	60	11	18.33	9.89–26.77	5.06	1	0.05	∞	0.98-∞
		No	24	0	0	0–0				0	0–1.05

SMZ-UZ, Sub mountain-undulating zone; CPZ, central plain zone; WPZ-WZ, western plain zone-western zone.

equines revealing any previous study targeting tick population responsible for theileriosis in Punjab state has restricted any comparison on the prevalence of ixodid tick vectors in the region.

Molecular assay for detection of *T. equi* displayed high fidelity 665 bp long amplicons with no non specific or non target amplifications (Fig. 3). Phylogenetic analyses were conducted in MEGA 6; there were a total of 606 positions in the final dataset (Fig. 4). The cladogram indicated closed homology of local strain (GeneBank Accession No LC010333) with *T. equi* strain from Brazil (KJ573371). The nBLAST analysis revealed 100% identity of the 665 bp sequence obtained using BeqF/BeqR primers in the present study with *T. equi* strain from Brazil (KJ573373.1). The sequence showed significant difference from *T. equi* strain obtained from the horse of South Korea (HM229407) (P=0.032), giraffe of Kenya (JQ928908) (P=0.039) and *T. sergenti* from Japan (AB016074) (P=0.001).

Highest prevalence of ticks infected with *T. equi* was recorded from western Punjab (Table 2), as in India, EP is mainly transmitted by *H. a. anatolicum* and these tick are found more often in western part of Punjab due to relatively higher temperature and lower rainfall. All these conditions favour the development of *H. a. anatolicum* (Haque *et al.* 2011). A significantly higher incidence of *T. equi* infection was reported in female tick population similar to the findings of Sangwan *et al.* (1989). Incidence of parasite was higher in *H. a. anatolicum* as compared to *R* (*B*). *microplus*, particularly the female ticks. *H. a. anatolicum* was established as the chief vector responsible for spread of infection among equine population (Kumar *et al.* 2007). As only one *R* (*B*). *microplus* tick was detected positive for

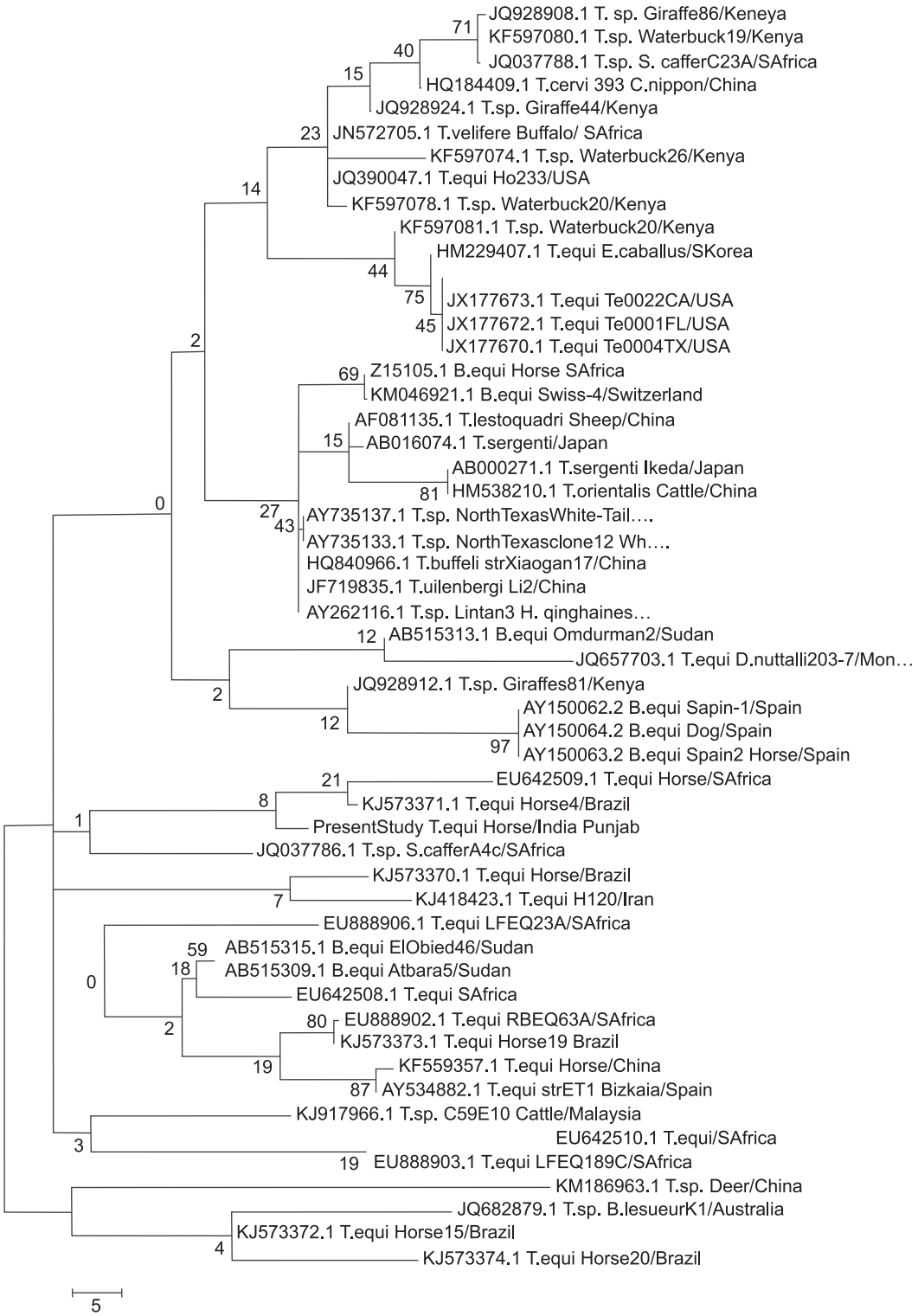

Fig. 4. Phylogenetic tree of *Theileria equi.* This tree was constructed by Neighbour-Joining method with MEGA 6 program. Numbers shown at branch nodes indicate bootstrap values. This phylogenetic analysis showed high genetic diversity of 18S rRNA gene among different strains of *T. equi.* Consensus sequences obtained in this study are indicated as ‘‘Present Study”.

T. equi, its role as a vector for transfer of EP remains inconclusive due to limited data. Many times ticks get some haemoprotozoan while feeding on blood meal of infected carrier, so finding protozoan in engorged ticks must always be interpreted with great caution along with detail study covering all its aspect (egg, larva, nymph, adults stages as well as mode of transmission), as positive results may be due to the remnants of imbibed blood meals containing protozoan DNA (Adamska and Skotarczak 2017). Furthermore some study support the development of *T.* (*B*). *equi* in adult female *R.* (*B*). *microplus* (Battsetseg *et al.* 2002). On the other hand, in our study more number *H. a.*

anatolicum were positive for *T. equi* thus supporting the fact that *H. a. anatolicum* has potential to transmit EP in India (Kumar *et al.* 2007).

Ticks obtained from young equids possessed significantly higher chances of parasite infection. It may be due to different management practices. Moreover, maternal antibodies can persist for only 3–6 months and after that passive immunity vanishes so chance of EP infection increases and animal may become lifelong carrier (Santos *et al.* 2011). Statistically, no significant variation was seen in the prevalence of *T. equi* among tick vectors in relation the species, sex of the host equid. Among the various farm management factors taken into consideration, the presence of pets on the farms has infinite odds of parasitic infection in the tick vectors.

T. equi infection in ticks collected from horses was higher as compared to donkeys/mules (Hussain *et al.* 2014). In our study, ticks were collected from only two donkeys/mules so to clearly access their role a further detail study having large sample size should be conducted, these results were in concordance with some previous study (Sumbria *et al.* 2017).

Ticks collected from equine females were in higher number as compared to male equine, as also reported by Baldani *et al.* (2010) and Manna *et al.* (2018). The higher infection encountered in ticks recovered from the female as compared to male equines could also be due to higher number of tick samples collected from female equines resulting in increased probability of infected ticks (Ebrahimi *et al*. 2018). Tick collected from equines without deworming/vaccination schedule had higher prevalence of *T. equi,* and it may be corroborated with the fact that in India equines are generally immunized against equine influenza virus and tetanus, but not against EP (non availability of vaccine). These diseases produce immunosuppressive effect in non-vaccinated equids, thus may increases the chance of infection. All these finding were similar to Garcia-Bocanegra *et al*. (2013). In unorganized farm, due to unhygienic feeding, non-grooming practices and use of equines for cart purpose, the incidence of direct contact with tick increases as compared to organized farm (Sumbria *et al.* 2015b, 2017, 2018). Equines kept with other pets showed high prevalence of tick as well as EP, similar results were shown by Garcia-Bocanegra *et al.* (2013).

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