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A comparison of the presence of *Theileria ovis* by PCR amplification of their SSU rRNA gene in small ruminants from two provinces of Pakistan

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ABSTRACT

Objective: To compare the presence of *Theileria ovis* in small ruminants from two provinces of Pakistan and to determine the risk factors associated with the spread of theileriosis. **Methods:** In present study, a total of 210 blood samples were collected from sheep ($n=99$) and goats ($n=111$) from 5 sampling sites in Punjab (Dera Ghazi Khan, Layyah, Multan and Rahim Yar Khan districts) and Khyber Pukhtoon Khwa (district Kohat) provinces, in Pakistan, from randomly selected herds. Data on the characteristics of the animals (species, gender, age, tick presence or absence, prior treatment for babesiosis) and the herd (location, size, species of animals, dogs associated with the herds, tick burden of dogs associated with the herds) was collected through questionnaires. **Results:** Twelve blood samples (6% of total), 11 from district Kohat, produced the 520 base pairs DNA fragment specific for small subunit ribosomal RNA (ssu rRNA) gene of *Theileria ovis*, by PCR amplification, of which 11 were sheep and 1 was goat indicating that sheep are more significantly ($P=0.001$) prone to this parasite. On the other hand parasite was detected only in 2 out of 210 samples (1%) by blood smear screening confirming PCR as the reliable detection tool. **Conclusions:** PCR is more sensitive and reliable diagnostic tool for detection of *Theileria* sp. as compared to blood smear screening. Incidence of *Theileria ovis* is very high in Khyber Pukhtoon Khwa as compared to Punjab province. It was also observed that presence of ticks on animals was the only significant risk factor associated with the theileriosis in small ruminants.

1. Introduction

The economy of Khyber Pukhtoon Khwa and Punjab provinces of Pakistan heavily relies on live stock[1]. 30–35 million rural population is engaged in livestock raising which helps them to derive 30–40 percent of their income from it. These facts are evidence that livestock is playing an important role in Pakistan's rural economy[2]. Piroplasm are transmitted through the ticks to their hosts and thus tick infestation in ruminants is one of the major constrain to livestock industry and ultimately affects the economic

performance[3]. In Pakistan *Ixodid* sp. ticks are involved in the transmission of various piroplasms to small and large ruminants causing various diseases including theileriosis resulting in economic losses[4,5].

Theileria sp. has very pronounced effect on live stock production, including small ruminants (sheep and goat), in tropical and subtropical areas of the world as they cause serious damage to animals by producing diseases like anemia, which ultimately leads to death, and results in production losses, i.e., production of less meat, milk and offspring by infected animal as compared to healthy one[3,6].

Ovine theileriosis is widely distributed in areas where sheep and goats are reared. Fever, enlargement of spleen and liver, damage to kidney and lungs are the general sign of theileriosis. *Theileria lestoquardi*, *Theileria* sp. (China) and *Theileria ovis* (*T. ovis*) are the pathogenic species for sheep and goats and among them *T. ovis* is considered

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as less pathogenic or benign because of long evolutionary relationship between parasite and the host[7]. Nevertheless, clinical disease may occur in stressful situations related to translocation of animals or/and when a host is debilitated by other parasitic organisms or malnutrition[8,9].

In acute cases, small-ruminant theileriosis can be diagnosed by microscopic examination of Giemsa-stained thin blood smears and by clinical symptoms[10]. In some cases, recovered animals frequently sustain sub clinical infections, which are microscopically undetectable[11]. They can be considered as a source of infection for the potential vector causing natural transmission of the disease. Serological methods are frequently employed in determining sub clinical infections. However, serology for detecting carrier state lack specificity and sensitivity, especially for infection status[3]. Therefore, DNA amplification methods, which are more sensitive and specific than other conventional methods may facilitate and be used as a powerful tool for the diagnosis of theileriosis[3,12–16].

The aim of the present study was to establish and optimize a specific, reliable and sensitive molecular tool, the polymerase chain reaction (PCR), for the detection of *T. ovis* in blood of small ruminants and to compare the results with the conventional method of blood film screening for parasitic detection. The objective of this pilot study is to provide and compare baseline data regarding the presence of *T. ovis* and risk factors involved in the spread of theileriosis in four districts of southern Punjab and Kohat district of Khyber Pukhtoon Khaw province.

2. Materials and methods

2.1. Samples and data collection

Blood samples were collected from 210 clinically healthy small ruminants (99 sheep and 111 goats) from 5 sampling sites in Punjab (Dera Ghazi Khan, Layyah, Multan and Rahim Yar Khan districts) and Khyber Pukhtoon Khwa (district Kohat) provinces, in Pakistan. Blood of 10% animals from each herd was sampled from jugular vein and immediately preserved in 10 mL Eppendorf tubes by adding 400 μ L of 0.5 M ethylene diamine tetraacetic acid (EDTA). Data was collected for each animal including species, gender, age, presence or absence of ticks, and prior treatment for theileriosis, and for the herd including location, size, species of animals, dogs associated with the herds and presence or absence of ticks on dogs associated with the herds was collected through questionnaires completed by the investigators on the spot during sample collection in order to calculate the risk factors involved in the spread of theileriosis. All the experiments were approved by the Research and Ethic Committee of Bahauddin Zakariya University Multan, Pakistan.

2.2. Blood film screening

Blood smears were prepared, fixed with methanol, stained with Giemsa and microscopically observed for the detection of *Theileria* sp. in blood.

2.3. DNA isolation

Inorganic method of DNA extraction was used following Shahnawaz *et al*[3]. The quality of the DNA extract in regard to purity and integrity was assessed with optical density counts at 260/280 nm and submerged gel electrophoresis.

2.3. Oligonucleotide design and PCR amplification

A pair of oligonucleotide primer was used to amplify the 520 bp region of small sub unit rRNA (ssu rRNA) gene sequence of *T. ovis* following method of Durrani *et al*[10]. The nucleotide sequence of the primer-pair was: (TSsr 170F) 5' TCGAGACCTTCGGGT 3' and (TSsr 670R) 5' TCCGGACATTGTAACAAA 3'. The PCR was performed in a total reaction volume of 50 μ L containing 5 μ L of 10 \times PCR buffer (100 mM Tris-HCl pH 9.5, 500 mM KCl, 1% Triton X-100), 250 μ M of each of the four deoxynucleotide triphosphates, 2 U Taq DNA polymerase (Vivantis, UK) and 10 pg of each primer. Five microlitres of the DNA suspension was used as template in the PCR. Remaining volume was adjusted with double distilled water. PCR amplification was obtained in a thermocycler (Bio Rad, USA) after 35 cycles. Thermo profile consisting of an initial denaturing step of 3 minutes at 96 $^{\circ}$ C was followed by 5 cycles: denaturing step of 30 sec at 94 $^{\circ}$ C, an annealing step of 30 sec at 56 $^{\circ}$ C and an extension step of 1 minute at 72 $^{\circ}$ C. These 5 cycles were followed by 30 cycles. Each cycle consisted of denaturing step of 1 minute at 94 $^{\circ}$ C, an annealing step of 1 minute at 54 $^{\circ}$ C and an extension step of 1 minute at 72 $^{\circ}$ C. The PCR program was ended with a final extension step of 7 minutes at 72 $^{\circ}$ C. Distilled water and *T. ovis* DNA (previously isolated from naturally infected sheep) was used in each test as negative and positive controls, respectively. Ten μ L of the amplification products were visualized on 2% agarose gel stained with ethidium bromide and observed under UV illumination.

2.4. Statistical analysis

For statistical purposes animals were grouped into two age categories: less than 1-year and more than 1-year-old. Herds were divided into two size categories: herds composed of 1–15 and herds with more than 15 animals. Also, herds were divided according to their composition into three categories: herds with sheep only, herds with goat only and herds containing both sheep and goats. The presence or absence of ticks on sheep, goats and dogs associated with the herds was also noted.

Association between the presence (positive and negative

blood samples) of *T. ovis* and the various parameters, *i.e.* herd location, herd size, species, gender and age of animal, herd composition, presence or absence of ticks on sheep and goats, presence and absence of ticks on dogs associated with herds was assessed by contingency table analysis using the Fisher's exact test (for 2×2 tables). Statistical package Mini Tab (Version 16) was used for statistical analysis.

3. Results

Table 1

The total number of samples collected (N) from the sampling sites. Prevalence of *T. ovis* is given in parenthesis.

District	N	<i>T. ovis</i> positive (%)	<i>T. ovis</i> negative (%)
Kohat	92	10 (12%) ^a	82 (88%) ^a
Dera Ghazi Khan	39	0 (0%) ^b	39 (100%) ^b
Multan	35	2 (6%) ^c	33 (94%) ^c
Rahim Yar Khan	25	0 (0%) ^d	25 (100%) ^d
Layyah	19	0 (0%) ^c	19 (100%) ^c
Total animals (sheep and goat)	210	12 (6%)	198 (94%)

Different superscript letter– The prevalence of *T. ovis* was significantly different between various sampling sites ($P < 0.05$).

Table 2

Association between presence of parasite in sheep and goat and the studied parameters describing animal characters.

Animal type	Parameters	No. of samples	<i>T. ovis</i> positive (%)	<i>T. ovis</i> negative (%)	
Sheep and goats (combined)	Sex	Male	81	5 (6%)	76 (94%)
		Female	129	4 (10%)	37 (90%)
	Age	Upto 1 year	75	3 (4%)	72 (96%)
		More than 1 year	135	9 (7%)	126 (93%)
	Ticks	Present	73	8 (11%)*	65 (89%)*
		Absent	137	4 (3%)	133 (97%)
Sheep	Sex	Male	49	5 (10%)	44 (90%)
		Female	50	6 (12%)	44 (88%)
	Age	Upto 1 year	30	2 (07%)	28 (93%)
		More than 1 year	69	9 (13%)	60 (87%)
	Ticks	Present	33	7 (21%)*	26 (69%)*
		Absent	66	4 (6%)	62 (94%)
Goat	Sex	Male	32	0 (0%)	32 (100%)
		Female	79	1 (1%)	78 (99%)
	Age	Upto 1 year	45	1 (2%)	44 (98%)
		More than 1 year	66	0 (0%)	66 (100%)
	Ticks	Present	40	1 (3%)	39 (97%)

* $P < 0.05$ when compared within each parameter.

Table 3

Association between presence of parasite in sheep and goat and the studied parameters describing animal and herd characters.

Parameters	No. of samples	<i>T. ovis</i> positive (%)	<i>T. ovis</i> negative (%)	
Size of herd	1–15	127	7 (6%)	120 (84%)
	More than 15	83	5 (6%)	78 (84%)
Herd composition	Goat only	39	1 (3%)	38 (97%)
	Sheep only	30	6 (20%)*	24 (80%)*
	Goat and sheep	141	5 (4%)	136 (96%)
Association of dog with herd	Dog absent	115	5 (4%)	110 (96%)
	Dog present	95	7 (7%)	88 (93%)
Ticks on dog	Absent	125	5 (4%)	120 (96%)
	Present	85	7 (8%)	78 (92%)

*** $P < 0.001$, when compared with goat herds and herds composed of both sheep and goats.

Sampling sites, total number of samples collected and parasite positive sample along with prevalence details are mentioned in Table 1. Results of PCR amplification revealed that 12 blood samples (6% of total), 11 from Kohat and 1 from Multan district, produced the 520 base pairs DNA fragment specific for small subunit Ribosomal RNA (ssu rRNA) gene of *T. ovis*. 11 of the *T. ovis* positive were sheep and 1 was goats indicating that sheep are more significantly ($P < 0.001$) prone to this parasite than goats.

When two parasite detection techniques, blood film screening and PCR, were compared, it was found that blood film screening is a non specific and non reliable tool as only 2 out of 210 samples (1%) were found *Theileria* sp. positive as compared to 12 parasite positive samples detected by PCR amplification.

Table 2 presents the presence of *T. ovis* in sheep and goats in relation to the parameters describing the characteristics of the animals. Statistical analysis of the characteristics of animals revealed that small ruminants having ticks present on their bodies (8/12) were more prone to the parasite and this association was statistically significant ($P < 0.05$). Presence of tick had also strong association with theileriosis in sheep ($P < 0.05$). The characteristics of herds as possible risk factors for the spread of theileriosis are mentioned in Table 3. It is also obvious from the data that the sheep herds were more infected with *T. ovis* ($P < 0.001$) as compared to goat herds and herds composed of both sheep and goats indicating that *T. ovis* infestation is more common in sheep.

4. Discussion

In the present study, we have reported the incidence of *T. ovis*, detected by PCR amplification of ssu rRNA gene, in blood samples of small ruminants collected from 5 districts in two provinces of Pakistan, along with the risk factors involved in the spread of theileriosis. We have observed that 6% of the sampled animals were positive for *T. ovis*. Incidence of this parasite is comparatively low in the study areas with comparatively higher parasite prevalence in Khyber Pukhtoon Khwa province than Punjab. Durrani et al^[10] has recently conducted a similar study in small ruminants of Punjab province in Lahore district and has reported 27% prevalence of *T. ovis* which is very high as compared to our findings in 4 districts of southern Punjab indicating that climatic conditions and geographical distribution of animals affects the prevalence of parasites^[14]. A similar study in sheep from eastern Turkey has indicated 54% prevalence of piroplasms (*Babesia* and *Theileria* sp.^[14]) while Li et al^[17] has also reported 78% prevalence of *T. ovis* in Xinjiang province of China. Incidence of *T. ovis* was very high in sheep as compared to goats as parasite was detected in 11% of the sheep samples confirming the findings of Altay et al^[13] that sheep are more prone to this parasite.

We observed that the prevalence of ovine theileriosis was higher in animals having ticks present (11%) on them indicating a positive correlation between the incidence of the disease and the presence of vector ticks. Similar trend was observed in case of sheep, the most parasite affected species during the present studies, where 21% of the sheep having ticks present on them were *T. ovis* blood positive. These observations are in agreement with the previous observations that ticks are the vectors for the transmission of theileriosis^[8,18].

It was observed that sheep were more infected by *T. ovis* than goats as 11 infected animals were sheep and the incidence was higher in Khyber Pukhtoon Khwa as 9 of the 11 infected sheep belonged to district Kohat. There was no association of gender or age, in sheep, with parasitic presence. Interestingly, the only infected goat was females, having tick present on her body and was younger than one year indicating that younger female goats have less immunity against this parasite and ticks are playing role in the transmission of parasite but these results did not reached statistical significance.

Composition of herd was also associated with the presence of *T. ovis* in small ruminants as herds composed of sheep only had higher parasitic prevalence than herds composed of goat only or having both sheep and goats kept together. This association was statistically highly significant ($P < 0.001$).

Several studies documented that PCR is more specific and sensitive than conventional techniques for the detection of carrier ruminants having *Theileria* sp. present in blood without any apparent signs of theileriosis^[3,19,20–29]. We had similar experience as the presence of *T. ovis* detected through PCR was 6% (N=12) as compared to 1% (N=2) parasitic detection by microscopic examination of Giemsa-stained blood films. Furthermore, these 2 blood samples were also found to be parasite positive by PCR. Only microscopic examination of PCR positive samples would have declared them parasite free. A similar comparison was made by Durrani et al^[10] in Lahore (Pakistan) and found 27% prevalence for *T. ovis* in sheep and goat by PCR as compared to 18% prevalence detected by blood smear examination.

There are only few report on the prevalence of theileriosis in small ruminants of Punjab but to our knowledge this is the first report describing the survey on ovine theileriosis in small ruminants through PCR amplification in Kohat district (Pakistan). Presence of *T. ovis* indicates that other species of this genus might be existing as well. In the present study, all 210 small ruminants were raised locally indicating that the theileriosis is endemic in this region. Poor hygienic conditions and poverty especially in small towns and villages could be a contributing factor for prevalence of this parasite in this region. In many cases, veterinarians are not available for the help and guidance of livestock owners. By generating the public awareness regarding the risk factors, the prevalence of piroplasms can be significantly decreased resulting in better health and output of sheep and goats.

Conflict of interest statement

We declare that we have no conflict of interest.

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