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Prevalence of Ovine *Theileria* Species in District Lahore, Pakistan

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Abstract.- A total of 200 whole blood samples along with thin blood samples smears and 100 samples of ticks from 20 flocks of sheep from different localities of district Lahore with a history of tick infestation , relapse of fever and anemia were screened for the prevalence of *Theileria* species during spring and summer seasons in 2007. On microscopic examination 44/200 (22%) samples were positive for *Theileria*, while 70/200 (35%) blood samples were found positive for *Theileria* species by PCR, out of which 79% were positive for *T. ovis* and 21% for *T. lestoquardi.* The clinical signs were recorded in 30% (60/200) sheep. Out of total of 100 tick samples the prevalence of *Hyalomma* was highest (45%) followed by *Rhipicephalus* (41%) and *Boophilus* (14%).The prevalence of *T. ovis* was 65.8% (27/41) as compared to 66.6% (30/45) for *T. lestoquardi* in *Rhipicephalus* and *Hyalomma* ticks, respectively.

Key word: *Theileria*, Pakistan, ovine.

INTRODUCTION

Of the species infecting small domestic ruminants, *T. lestoquardi* (syn. *T. hirci*) is regarded as the only pathogenic one (Morel and Uilenberg, 1981). This parasite often occurs in mixed infections with other parasites. *T. lestoquardi* being reported from south-eastern Europe, northern Africa, the Near and Middle East and India (Levine, 1985). The other species, *Theileria ovis*, *Theileria recondita* and *Theileria separata* cause subclinical infection in small ruminants (Alani and Herbert, 1988). Recently, *T. ovis* has been reported from sheep in different countries (Altay *et al*., 2005). However, not much is known about the epidemiology of ovine theileriosis caused by *T. ovis*, particularly tick vector competency (Aktas *et al*., 2005).

Generally, the diagnosis of small ruminant piroplasmosis is based on the morphological examination of blood smears and clinical symptoms (Kirvar *et al*., 1998). These methods are reliable for the detection of acute cases but have limited value for chronic cases, where only low numbers of piroplasms exist. PCR techniques allow the detection of piroplasms at low parasitaemias, discrimination among *Babesia* and *Theileria* species, and that of *T. annulata* from other non-pathogenic *Theileria* species. PCR assays have been developed and used for the detection of *Theileria annulata* in *Hyalomma* species (Kirvar *et al*., 2000), and *Babesia bovis* and *Babesia bigemina* in *Boophylus microplus* (Oliveira-Sequeira *et al.*, 2005), and *B. caballi* and *B. equi* in *Dermacentor nuttalli* (Battsetseg *et al.*, 2001). Likewise specific PCR assay was developed for *T. ovis* in sheep by Altay *et al.* (2005), whereas its vector was established by Aktas *et al*. (2005). The 18S rRNA gene analysis has been successfully applied to identify several previously unknown *Theileria* as well as *Babesia* parasites (Gubbels *et al.,* 2002). In the present study, ovine *Theileria* parasite species were detected by PCR in district Lahore, Pakistan in sheep as well as in tick vector to better understand the disease in sheep in Pakistan.

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MATERIALS AND METHODS

Collection of samples

This study was carried out in order to investigate the prevalence of *Theileria* species in sheep in district Lahore during spring and summer season in year 2007. A total of 200 whole blood in EDTA coated vacutainers and thin blood smears were collected from 20 flocks in different locations in district Lahore with a history of tick infestation , relapse of fever and anemia. Clinical signs were also recorded. One hundred samples of ticks were were collected manually from the head, ears, axilae,

abdomen and genital regions in labeled bottles. Adult ticks were morphologically examined under the stereomicroscope and identified according to Estrada-Pena *et al.* (2004). The identified ticks were dissected in 0.85% saline using a stereomicroscope, and salivary glands were transferred to micro centrifuge tubes and frozen at −20°C until used for extraction of DNA. The forceps used in dissections were immersed for 5 s each in 5 M HCl followed by 5 M NaOH to remove any contaminating DNA as described by Aktas *et al.* (2005). Uninfected tick salivary glands required for a negative control in the PCR, were obtained by dissecting unfed adult *Hyalomma* and *Rhipicephalus* ticks.

Microscopic examination

Thin blood smears were fixed in methanol for 10 min and stained in Giemsa diluted at 5% with buffer solution for 30 min and examined, at 1000× magnification for the presence of *Theileria* piroplasms (Durrani *et al*., 2005).

PCR amplification

Total DNA was extracted from the blood samples with the help of DNA Extraction Kit (PUREGENE ® USA, GENTRA) according to prescribed method, whereas from salivary glands the extraction of DNA was done according to the Aktas *et al*. (2005). Each individual salivary gland was added to 250 µl of lysis mixture (0.32 M sucrose, 0.01 M Tris, 0.005 M $MgCl_2$, 1% Triton X-100, pH 7.5) and the mixture was centrifuged at 11,000 × *g* for 1 min. The pellet was washed two times with 250 µl lysis mixture. The supernatants was carefully discarded and the pellets was resuspended in 240 µl of TEN-SDS (0.4 M NaCl, 10 mM Tris–HCl, 2 mM EDTA, 0.2% SDS) and 10 µl proteinase K (25.000 µg/ml). The mixture was incubated at 56°C for 4 h and then 150 µl 5 M NaCl was added to each tube and vortexed for 30 s, followed by centrifugation at 14,000 rpm for 5 min. The supernatant was transferred to another 1.5 ml microcenrifuge tube. Afterward, standard phenol–chloroform (25:24:1) extraction procedure was adopted. DNA was precipitated with Na-acetate and resuspended in 25 µl distilled water. Five microlitres was used in PCR. The PCR was performed in a total reaction volume of 50 µl containing 5 µl of 10× PCR buffer (100 mM Tris–HCl pH 9, 500 mM KCl, 1% Triton X-100), 250 µM of each of the four deoxynucleotide triphosphates, 2 U Taq DNA polymerase (Promega, Madison, WI, USA) and 10 pg of each primer as described in Table I. Five microlitres of the DNA suspension was used as template in the PCR. The reaction mixture was overlaid with 100 µl mineral oil and amplification was carried out in a no hot-lid minicycler (MJ Research, USA). Cycling conditions for *T.ovis* were 96°C for 3 min followed by 40 cycles each of 94°C for 30s, 60°C for 30 s and 72°C for 2 min with a final extension step of 72°C for 10 min. PCR products were visualized by UV transillumination in a 1.5% agarose gel following electrophoresis and staining with ethidium bromide (Aktas *et al.,* 2005). Cycling conditions for *T. lestoquardi* were 94°C for 3 min, followed by 40 cycles at 94°C for 1 min, 60°C for 1 min and 72°C for 1 min with a final extension step of 72°C for 5 min. The amplification was carried out with 30 cycles for *Theileria* spp. Each cycle involved denaturation for 1 min at 94°C, annealing for 1 min at 56°C and extention for 1 min at 72°C. (Aktas *et al.,* 2005). For *Theileria* specific primers amplification was done with the help of thermal cycler set for 30 cycles. Annealing temperature was set at three conditions *i.e*. 60°C for 30 seconds while rest of the conditions were same as described for *T. ovis / T. lestoquardi.* Analysis of amplified product by electrophoresis

Table I.- *Theileria* specific, *T. ovis* specific and *T. lestoquardi* primers used in the present study.

Theileria Specific: 1098 bp fragment of the small subunit ribosomal RNA (ssu rRNA) gene (Allsopp *et al*., 1993):
989 F;5′-AGTTTCTGACCTATCAG-3′
990 R; 5′-TTGCCTTAAACTTCCTTG-3′

***T. ovis*.** 520 bp fragment of the small subunit ribosomal RNA (ssu rRNA) gene (Altay *et al*., 2005):
TSsr 170F; 5′-TCGAGACCTTCGGGT-3′,
TSsr 670R; 5′-TCCGGACATTGTAAAACAAA-3′

T. lestoquardi. 785 bp to amplify the gene coding for the 30 kDa *T. lestoquardi* merozoite surface antigene (Kirvar *et al*., 1998):
5′-GTGCCGCAAGTGAGTCA-3′
5′-GGACTGATGAGAAGACGATGAG-3′

was done with a 1% agarose gel (Durrani *et al.,* 2005).

Statistical analysis

Fischer's exact test was used to evaluate the differences between diagnostic tests; $P < 0.05$ was accepted to be statistically significant.

RESULTS

Clinical signs observed

Sheep were monitored for clinical signs of infection The signs recorded were elevated body temperature over 41.8°C in sheep, emaciation, reduced appetite, laboured breathing, coughing, conjunctival and nasal discharge ,hyperplasia of palpable lymph nodes, paleness of mucus membranes. A total of 60 sheep out of 200 samples showed the clinical manifestations.

Microscopic examination

In the microscopic examination of blood smears, the parasitemia ranged an average of 0.055%. Piroplasms, detected inside the red blood cells were oval and ring forms. Of the 200 blood smears examined, 44 (22%) were positive for these piroplasms. In four blood smears low numbers of piroplasms compatible with *Babesia* sp. were observed. Abnormalities in erythrocytes including anisocytosis, poikilocytosis and basophilic strippling were evident.

Polymerase chain reaction

In the examination of DNA extracted from 200 blood samples, amplification with the molecular length 1098 bp was obtained in 70/200 (35%) while 520 base pair was obtained in 79% (55/70) and 785bp was obtained in 21% (15/70) samples. All samples positive in the microscopic examination of thin blood smears, were also positive with PCR test. The sensitivity of the nested PCR, which was assessed showed that one infected cell in 10 sheep erythrocytes, equivalent to a blood parasitemia of 0.00001%, could be detected. This is more sensitive than light microscopy. The primer pairs described in this study will be useful for epidemiological studies on ovine theileriosis and for discrimination between *T. lestoquardi* and *T. ovis* infections in sheep (Aktas *et al.,* 2005). The difference between microscopic examination and the PCR results was statistically significant ($p < 0.05$).

Prevalence of Theileria *in ticks*

The tick genera identified from sheep were *Hyalomma* (45%), *Rhipicephalus* (41%) and *Boophilus* (14%). Forty one of *Rhipicephalus* were examined by PCR using *T. ovis*-specific primers. An expected 520 bp fragment of *T. ovis* DNA was amplified in 27 (65.8%) out of 41 samples. The expected 520 bp fragment was also generated from *T. ovis* positive control DNA obtained from infected sheep blood, but no product was amplified from *Hyalomma* negative control in PCR. Forty five of *Hyalomma* were examined by PCR using *T. lestoquardi*-specific primers. An expected 785 bp fragment of *T. lestoquardi* DNA was amplified in 30 (66.6%) out of 45 samples. The expected 785 bp fragment was also generated from *T. lestoquardi* positive control DNA obtained from infected sheep blood, but no product was amplified from *Rhipicephalus* negative control in PCR.

DISCUSSION

Ruminants with clinical and sub clinical theileriosis are sources for tick infection since they carry piroplasms. *Theileria* species are transmitted by ixodid ticks. Therefore, detection and discrimination of these parasites in their definitive host are crucial for understanding the epidemiology of the diseases (Aktas *et al*., 2005). During the present study the advantage of PCR over the conventional methods of detecting hosts infected with *Theileria* species as being more sensitive and specific than parasite detection by conventional methods is in accordance with Aktas *et al*. (2005).

Data on clinical disease caused by *Theileria* species in sheep are also in accordance with Almeria *et al*. (2001). The molecular study revealed that *T. ovis* is widespread in sheep and specifically found in the region where the tick samples were collected as described by Altay *et al*. (2005). The prevalence of *T. ovis* in sheep was higher than *T. lestoquardi*. Domestic ruminants are often exposed to a large number of tick species upon the distribution of vectors in the environment. According to Sayin and

Dumanli (1982) in a tick survey carried out in eastern Turkey, the majority of the ticks found on sheep and goats have been identified as *R. bursa* while during the present study *Hyalomma, Rhipicephalus* and *Boophilus* were identified.

Amplification of parasite DNA in ticks using specific PCR has been applied to *T. annulata* (Kirvar *et al*., 2000), *T. lestoquardi* (Kirvar *et al.*, 1998), *B. bovis* and *B. bigemina* (Oliveira-Sequeira *et al*., 2005), *B. caballi* and *B. equi* (Battsetseg *et al*., 2001). *T. ovis* (Aktas *et al*., 2005). In the present study, *T. ovis* was detected by PCR in 27 of 41 (65.8%) *Rhipicephalus* while *T. lestoquardi* was detected by PCR in 30 of 45(66.6%) Hyalomma collected from naturally infested sheep in district Lahore. The result suggests that *Rhipicephalus* and *Hyalomma* may play an important role as a vector of the parasite.

Primers used in the present study were previously designed and determined to be specific for *T. ovis and T. lestoquardi.* A 520 bp fragment of ssu rRNA gene of *T. ovis* and 785 bp of *T. lestoquardi* can be amplified from blood samples obtained from carrier sheep and from infected ticks. Which is in accordance with Altay *et al.* (2005).

In conclusion, the results presented in this study have demonstrated the presence of *T. ovis* in *Rhipicephalus* and *T. lestoquardi* in *Hyalomma* collected from naturally infested sheep. Therefore, both tick genera may play an important role in the field as a natural vector of *Theileria* species.

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