Proceedings of the Pakistan Academy of Sciences: B Life and Environmental Sciences 61(1): 29-34 (2024)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(61-1)800

Pakistan Academy of Sciences

Research Article

PCR-based Detection and Prevalence of *Theileria* Species in Sheep from Quetta District, Balochistan

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Abstract: Theileriosis is a serious hemoparasitic illness that severely limits the production of small ruminants. The current study aims for early and accurate detection of theileriosis using polymerase chain reaction for treatment as conventional techniques are not specific. Blood samples were collected from sheep between July and October of the year 2020. A total of 100 samples were taken, 69 samples were from tick-infested sheep exhibiting illness signs, while 31 samples were from tick-free sheep. To detect *Theileria* species in sheep, DNA was extracted, and the PCR method was used. The prevalence of *T. lestoquardi* and *T. ovis* was 22.47% and 64.04%, respectively, with a 13.48% frequency of mixed infection with both species. *T. ovis* was detected in around 80% of tick-infested sheep, indicating a significant association ($P < 0.01$) with ticks. *T. lestoquardi* was found in 28.99% of tick-infested sheep, indicating a significant connection ($P < 0.001$) with ticks. The incidence of *T. ovis* was significantly related to the summer season but *T. lestoquardi* was non-significantly related to the summer season ($P > 0.05$). A high prevalence of Theileriosis is found in Quetta. The parameters studied were strongly correlated to the infection except for *T. lestoquardi* with the summer season. Early detection with the help of polymerase chain reaction can accelerate treatment and reduce transmission to increase livestock production in Pakistan.

Keywords: *Theileria* Species, Sheep, Quetta, Balochistan, Pakistan.

1. INTRODUCTION

Livestock is the backbone of Pakistan's economy, accounting for around 61.89% of agriculture and 14.04% of GDP. The national herd population of sheep is 31.9 million [1]. Balochistan contains 34.8 million hectares of land, of which only 4 percent is arable, and the rest is employed to feed small ruminants [2]. In Balochistan 80% of the population is rural and 3/4th of them are associated with livestock somehow. Livestock in Balochistan generates almost 50% of agriculture and 10% of the overall provincial GDP. The herd population of sheep in Balochistan is 15.85 million which is 52% of the national herd [3]. However, numerous unsanitary conditions and parasite infestations have an unfavorable impact on the country's animal sector, where Theileriosis is a prominent parasite infestation that has proven lethal in animals [4].

Pakistan's tropical and subtropical climate facilitates Theileriosis by providing optimum conditions for ticks to infest Bovidae. Insufficient disease management in Pakistan also increases the risk of Theileriosis [5]. Theileriosis occurs due to the members of the genus *Theileria* [6] which is associated to the phylum Apicomplexa and the order Piroplasmorida [7]. *Theileria lestoquardi* (*T. lestoquardi*) which is responsible for malignant ovine theileriosis (MOT) and *Theileria ovis* (*T. ovis*) which is responsible for benign ovine theileriosis, are considered the causative agents for ovine theileriosis in Pakistan. MOT is accompanied by pale and yellow mucous membranes, enlargement of superficial lymph nodes, diarrhea or constipation, listlessness, emaciation, and high fever; whereas benign ovine theileriosis has symptoms of weight loss, fever, reduced production, and ultimately death of infected animals [8]. The sporozoan protozoa "*Theileria*" is transmitted by ixodid ticks mainly by Hyalomma species in the case of *T. lestoquardi* and *T. ovis* [9]. This parasite has three different stages

Received: December 2022; Revised: August 2023; Accepted: March 2024
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in the life cycle, i.e., sporogony, merogony, and gametogony [10]. The parasites belonging to the *Theileria* genus have a digenetic life cycle. *Theileria* species and strains are either classified pathogenic as they have a schizont-associated "transforming" leukocyte stage or benign to mildly pathogenic as they have a schizont-associated "non-transforming" leukocyte stage. The disease occurs in benign cases due to piroplasm-evoked acute hemolytic anemia. *T. lestoquardi* is pathogenic whereas *T. ovis* is a benign species of sheep and goats [11]. *Theileria* can be treated with buparvaquone with high efficacy when used in the early stages of the disease. Imidocarb and oxytetracyclines are also found to be effective but only in the initial stage [12]. As the treatment is effective only in the initial stages, it is necessary to detect it early.

2. MATERIALS AND METHODS

A total of 100 sheep were examined at random from various herds in Quetta city, including seemingly healthy sheep with ticks on their bodies and animals with significant signs of Theileriosis. All individuals' ear veins and jugular veins were sampled for 3ml of blood, then processed for DNA extraction using Grimberg's non-organic approach [13]. The present investigation utilised two sets of oligonucleotide primers. The first set [fwd. 5′-GTGCCGCAAGTGAGTCA-3′ and rev. 5′-GGACTGATGAGAAGACGATGAG-3′] was used to amplify the 785 bp sequence of *T. lestoquardi* and a second set [fwd. 5′-TCGAGACCTTCGGGT-3′ and rev. 5′-TCCGGACATTGTAAAACAAA-3′ was used to amplify the 520 bp sequence of *T. ovis, T. lestoquardi and T. ovis* sequences were amplified in a thermal cycler (Prime thermal cycler: SPRIMEG/02).

For *T. lestoquardi* the PCR was performed in a total volume of 25 µl reaction mixture containing genomic DNA (250 ng), PCR buffer (1X), $MgCl_2$ (2.5 mM), primers (20 pmol), dNTPs (0.16 mM), and Taq polymerase (2.5 U). The PCR was started with a 3-minute denaturation at 94 °C, followed by 40 cycles of denaturation at 94 °C for 30 seconds, annealing at 60 °C for 30 seconds, and extension at 72 °C for 45 seconds. Following this, a final 5-minute step at 72 °C was taken. PCR products were visualized on 1.5% ethidium bromide-stained agarose gel. The amplicon size for *T. lestoquardi* was 785 bp.

For *T. ovis* the PCR was performed in a total volume of 25 µl reaction mixture containing genomic DNA (250 ng), PCR buffer (1X), $MgCl_2$ (2.5 mM), primers (20 pmol), dNTPs (250 mM), and Taq polymerase (2 U). The PCR was started with a 3-minute denaturation at 94 °C, followed by 30 cycles of denaturation at 94 °C for 1 minute, annealing at 56 °C for 1 minute, and extension at 72 °C for 1 minute. A 5-minute final stage followed this at 72 °C. PCR products were observed on a 1% ethidium bromide-stained agarose gel. The amplicon size for *T. ovis* was 520 bp.

2.1. Statistical Analysis

The Chi-square (χ^2) test was used to examine the relationship between the detection of *T. lestoquardi and T. ovis* and the presence or absence of ticks on sheep, as well as the influence of season.

3. RESULTS AND DISCUSSION

The *Theileria* spp. was found in 89 sheep of which the isolated infection of *T. lestoquardi* and *T. ovis* was recorded to be present in 20 (22.47%) and 57 (64.04%) of the infected sheep, respectively. However, 12 (13.48%) of infected sheep exhibited mixed infection, i.e., infested by both the species. 80% of animals were negative for isolated *T. lestoquardi,* 43% of animals were negative for isolated *T. ovis*, and 88% of animals were found to be negative for mixed infection (Table 1).

Several indicators related to the prevalence and transmission of Theileriosis were also calculated in this investigation. The study examined the correlation between the infection rate and variables such as the presence or absence of ticks on the sheep, as well as the months during which the investigation was conducted. The study months showed a statistically non-significant (P = 0.089) correlation with *T. lestoquardi,* whereas tick

Table 1. PCR results for *Theileria* spp.

***Theilaria* spp. Infection**	**Total N=100**	
	Positive (%) n=89(89%)	**Negative (%) n=11 (11%)**
Isolated *T.lestoquardi*	20 (22.47%)	80 (80%)
Isolated *T.Ovis*	56(64.04%)	43 (43%)
Mixed (*T.lestoquardi + T.Ovis)*	12(13.48%)	88 (88%)

presence revealed a statistically very significant (P = 0.0008) correlation with *T. lestoquardi*. *T. ovis* prevalence exhibited a statistically very significant (P = 0.00001) connection between ticks and study months (Table 2).

The *T. lestoquardi* was identified by a specific band size of 785 bp obtained on 1.5% ethidium bromide-stained agarose gel. The absence of the 785 bp band indicated the parasite-negative samples (Figure 1). Whereas *T. ovis* was identified by a specific fragment of band size 520 bp unique to it. The result was obtained on 1% ethidium bromide-stained agarose gel. The absence of the 520 bp band indicated the parasite-negative samples (Figure 2). Microscopic tests cannot distinguish between *Theileria* species since the piroplasms have the same appearance, making the researcher's job more difficult if discovered in mixed infections. It is critical to distinguish these parasites accurately to comprehend their epidemiology. As a result, using PCR to detect distinct species of *Theileria* in carrier animals has shown to be a powerful technique for epidemiologically investigating *Theileria* infection. PCR amplification is a more sensitive and dependable method compared to microscopy when it comes to assessing carrier animals with low levels of parasitemia [14, 15]. Since light microscopy has a lower capacity to detect parasitemia levels than PCR, it may be verified that light microscopy cannot distinguish carrier animals.

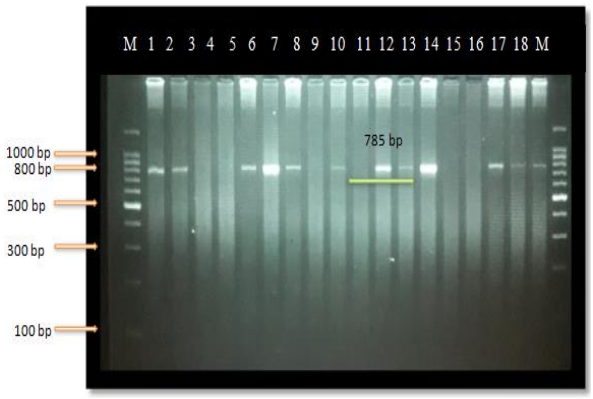

Fig. 1. 1.5% Agarose gel stained with ethidium bromide displaying 785 bp bands of PCR products for the infection of *T. lestoquardi*.

Lane M: 100 bp DNA sequence marker;
Lane 1: Positive control for *T. lestoquardi* DNA;
Lanes 2, 5, 6, 7, 9, 11,12, 13, 16, 17, 18: positive samples *T. lestoquardi* DNA;
Lane 3: (Distilled water) Negative control;
Lane 4, 8, 10, 14, 15: Parasite-free blood samples.

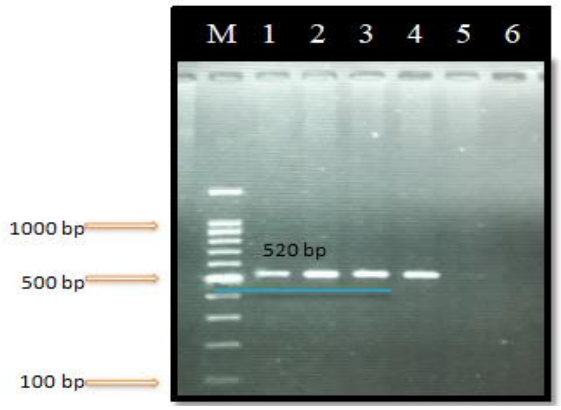

Fig. 2. 1% Agarose gel stained with ethidium bromide displaying 520 bp bands of PCR products for the infection of *T. ovis*.

Lane M: 100-bp DNA marker;
Lane 1: *Theileria ovis* positive control;
Lanes 2, 3, 4: Parasite-positive blood sample;
Lane 5: Parasite-negative blood sample;
Lane 6: Negative control (distilled water).

In sheep, the current study found a higher prevalence of *T. ovis* (64.04%) than *T. lestoquardi* (22.47%), along with mixed infection of both species (13.48%). The frequency of theileriosis in sheep is 89% which is much higher than the findings of Riaz and Tasawar [8], who observed 39.31% (57/145) of sheep in Multan infected with *Theileria* spp. However, the findings are consistent with the frequency of infections observed in infected sheep. They found that 63.16% (36/57) of infected sheep had *T. ovis* infection, 22.81% (13/57) of infected sheep had *T. lestoquardi* infection, and 14.04% (8/57) of infected sheep had mixed infection. But the molecular survey conducted in different regions of Multan in 2013 [16] found that 41.7% (65/156) of sheep had *Theileria* while *T. ovis* was found in 24.6% (16/65), *T. lestoquardi* was found in 57% (37/65) and mixed infection was found in 20% (12/65) of sheep. Tick infestation was also found to have significant relation with the infection in their study. A similar study by Durrani *et al*. [17] in district Lahore, found theileriosis in 70/200 (35%) of sheep while *T. ovis* was identified in 79% (55/70) and *T. lestoquardi* was identified in 21% (15/70) of samples.

In Balochistan, the only large-scale study on theileriosis in small ruminants was conducted between June 2012 to May 2013 in the Northern highlands and Suleiman mountain range of Balochistan comprising 2200 sheep. A significant relation was found between the summer season and

the prevalence of infection. 22.82% of sheep were positive for theileriosis, *T. lestoquardi* accounted for 73.80% (338/458), and the remaining infections 26.20% (120/458) were caused by *T. ovis* [18]. The results contradicted our study this might be due to variations in sample size, herd conditions, and region. In the present study, *T. lestoquardi* accounted for 22.47% (20/89) and *T. ovis* was recorded to be present in 64.04% (57/89) of infected sheep respectively.

Heidarpour *et al*. [19] identified 56% (56/100) of sheep positive for *Theileria* spp. in East and South-East Iran; where 12.5% (7/56) were positive for *T. ovis* and 87.5% (49/56) were positive for *T. lestoquardi*. Similarly, Yaghfoori *et al*. [20.] found 76% (76/100) of sheep infected with *Theileria* spp. in the Fasa Province of Iran. A higher prevalence of 56.58% (43/76) of *T. ovis* was found in infected sheep as compared to *T. lestoquardi* 3.95% (3/76) in sheep. Meanwhile, 39.47% (30/76) presented mixed infection. The findings of Zaeemi *et al*. [10] in the western half of Iran found 32.8% (82/250) of sheep positive for *Theileria,* where 54.8% (45/82) and 40.2% (33/82) were positive for *T. lestoquardi* and *T. ovis*, respectively. Mixed infection was detected in 4.8% (4/82) cases. The first molecular data on *Theileria* infection of sheep in eastern Turkey found 41.2% (90/218) of sheep positive for *Theileria* spp. but *T. lestoquardi* was not detected [21]. Altay *et al*. [22] reported that 58.79% (398/677) of sheep suffering from theileriosis caused by *T. ovis* and *T. lestoquardi* were absent. Altay *et al*. [23] found only 28.90% (37/128) of sheep suffering from theileriosis but *T. ovis* had significantly a higher prevalence of 94.59% (35/37) compared to other *Theileria* species, and the absence of *T. lestoquardi* and mixed infection was observed.

The prevalence of *Theileria* infection is determined by several variables, including tick density and environmental conditions (seasons of the year). From July to August, our study detected a high prevalence of Theileriosis triggered by *T. lestoquardi* and *T. ovis* (24.5% and 73.5%, respectively), as compared to September to October (12.7% and 38.2%, respectively). *T. lestoquardi* showed a non-significant relation between its prevalence and study seasons but a significant association was shown by *T. ovis*. Based on our research, (28.99%) of *T. lestoquardi*-positive sheep were infested with ticks, and (79.7%) of *T. ovis*-positive sheep had ticks. Both species showed a statistically significant association indicating that ticks are involved in parasite spread. Hosseini *et al*. [24] conducted a study in western Iran and their findings proposed that tick infestation is higher during the summer season (June - August) and so is the *Theileria* infection; these findings suggested that higher tick infestation increases the rate of theileriosis. Muhammad *et al*. [25] identified June as the peak season for bovine disease outbreaks in the Faisalabad region of Pakistan because the extreme temperature of the climate promotes tick development and multiplication, making ticks more energetic and thus more likely to transmit Theileriosis. Hegab *et al*. [26], also identified June as the peak month of *Theileria* infection in Egypt but no significant relation was observed between season and infection rate. In the southern

Table 2. *Theileria* spp. prevalence in accordance with investigated parameters.

Parameters		**Ticks**		**Study Months**	
		Absent	**Present**	**July - August**	**September - October**
Sample Count		n=31	n=69	n=53	n=47
T. lestoquardi	(+ve)	0 (0%)	20 (28.99%)	14 (24.5%)	6 (12.7%)
	(-ve)	31 (100%)	49 (71%)	39 (75.4%)	41 (87.2%)
Chi-square		11.23		2.90	
P-value		0.0008^{***}		0.089^{ns}	
T. ovis	(+ve)	2 (6.45%)	55 (79.7%)	39 (73.5%)	18 (38.2%)
	(-ve)	29 (93.5%)	14 (20.2%)	14 (26.4%)	29 (61.7%)
Chi-square		46.84		12.66	
P-value		0.00001^{***}		0.00001^{***}	

Punjab area of Pakistan, Saeed *et al*. [27] found that 0% of tick-absent sheep had *Theileria* caused by *T. lestoquardi*. Iqbal *et al*. [28] discovered a statistically significant positive connection ($p = 0.03$) between the presence of vector ticks and the occurrence of the disease in the collected samples of goats and sheep.

4. CONCLUSIONS

A high prevalence of Theileriosis is found in Quetta. The parameters studied were strongly correlated to the infection except for *T. lestoquard*i with the summer season. Early detection with the help of polymerase chain reaction can accelerate treatment and reduce transmission to increase livestock production in Pakistan.

5. CONFLICT OF INTEREST

The authors declare no conflict of interest.

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