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Research Article

# Molecular Prevalence and Haematology of Tropical Theileriosis in Cholistani Cattle from Nomadic Herds of the Cholistan Desert, Pakistan

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## Abstract

This is the first report on tropical theileriosis in Cholistani cattle, with the aim of 1) assessing the reliability of PCR as a tool for diagnosis of the early/carrier state; 2) determining the prevalence of theileriosis; and 3) comparing haematological profiles of parasite-positive and parasite-negative cattle. A total of 264 cattle (142 female and 122 male; 127 adult and 137 young) were examined for tropical theileriosis through clinical examination, stained smear screening, and polymerase chain recation. No cattle showed clinical signs of the disease. Of the diagnostic tests, PCR was more sensitive for detection of the early/carrier state of theileriosis (19.3%) compared to stained thin blood smear examination (1.9%). Female (24.6%) and young animals (23.4%) showed higher prevalence than males and adults, but not significant (P>0.05). Prevalence of the disease (51.6%) was significantly higher (P<0.05) in summer. Haematological indices were not significantly different in parasite-positive compared to parasite-negative cattle, except for total protein and creatinine which were significantly higher in infected animals. The study revealed a substantial prevalence of tropical theileriosis in Cholistani cattle. Nevertheless, their adaptation to the climate and their potential for tick and disease resistance may reflect in the absence of clinical signs and in normal haematological indices.

***Keywords:*** *Theileria annulata, Cattle, PCR, Pakistan*

# Pakistan'ın Cholistan Çölünde Başıboş Dolaşan Cholistan Sığırlarında Tropikal Theileriosisin Prevalansı ve Kan Değerleri

## Özet

Cholistan sığırlarında tropical theileriosis ile ilgili ilk rapor olan bu çalışma ile; 1 teşhis yöntemi olarak taşıyıcı hayvanlarda PCR metodunun güvenirliliğinin belirlenmesi; theileriosisin prevalansının tespit edilmesi; parazit pozitif ve negative bireylerde kan parametrelerinin karşılaştırılması hedeflenmiştir. Toplam 264 sığır klinik, mikroskopik ve PCR ile tropical theileriosis yönünden muayene edilmiştir. Sığırların hiç birinde klinik bulgu gözlenmemiştir. PCR (%19.3), subklinik enfeksiyonların belirlenmesinde mikroskopik bakıya (%1.9) göre oldukça duyarlı bulunmuştur. Hastalığın yaygınlığı dişi (24.6%) ve genç hayvanlarda (%23.4) erkek ve erişkinlere göre daha yüksek bulunmuş, ancak bu farklılığın istatistiksel olarak anlamlı olmadığı (P>0.05) görülmüştür. Hastalığın, yaz aylarında (%51.6) daha yüksek olduğu tespit edilmiştir. *T. annulata* yönünden pozitif ve negative hayvanların kan değerlerinde total protein ve creatinine hariç (bu değerler enfekte hanvanlarda yüksek bulunmuştur) bir farklılık gözlenmemiştir. Bu çalışma Cholistan sığırlarında hastalığın prevalansının yüksek olduğunu ortaya koymuştur. Muayene edilen hayvanların hiç birinde klinik bulguların gözlenmemesi, kan değerlerinde değişikliğin olmaması, bu sığırların kenelere ve hastalığa karşı dirençlerini yansıtmaktadır.

***Anahtar sözcükler:*** *Theileria annulata, Sığır, PCR, Pakistan*

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# INTRODUCTION

Pakistan has 15 indigenous breeds of cattle belonging to zebu (one-humped) breed *(Bos indicus)*, comprising 43% of the cattle population [1]. Cholistani cattle are considered to be ancestral to the Sahiwal and are thermotolerant, and tick-resistant [2]. Tropical theileriosis, caused by *Theileria annulata*, is an important tick-borne disease of cattle in tropical and sub-tropical regions [3-6]. The disease is transmitted by the tick *Hyalomma* [7,8]. Research has been conducted in Pakistan on various aspects of this disease in Sahiwal and crossbred cattle [6], as well as in sheep and goats [9]. It is a serious constraint to cattle production in endemic areas, causing lethal infections in exotic cattle and considerable mortality in indigenous as well as in crossbred stock [10]. Factors including Pakistan's location in a warm climate and extensive uncontrolled crossbreeding programmes have rendered it an endemic area for theileriosis [6]. Exotic cattle and their crossbreds are highly susceptible, while indigenous cattle are relatively resistant to tropical theileriosis [11].

The objectives of the study were to determine the prevalence of *T. annulata* in Cholistani cattle from Pakistan reared under desert nomadic conditions, to assess PCR as a tool for diagnosis of the carrier state of the parasite, and to compare the haematological profiles of infected and uninfected cattle.

# MATERIAL and METHODS

### *Geo-location and Study Animals*

The study was conducted from February 2013 to January 2014 in the Cholistan desert of Pakistan. Location and climatic conditions of the area have been described elsewhere [12]. This area is an extension of the Great Indian Desert, which includes the Thar Desert in Sindh Province, Pakistan and the Rajhsatan desert in India. It is located 30 km from the city of Bahawalpur, Punjab, Pakistan and covers an area of 26.000 km², from latitude 27°42´to 29°45´ North and longitude 69°52´to 75°24´ East [12]. The climate is arid subtropical continental with low/sporadic rainfall, high temperature, low relative humidity, high rate of evaporation, and strong summer winds. It is the driest and hottest area of Pakistan, with summer spanning May through October. Randomly selected artificial and natural reservoirs and ponds, called Tobas [12,13] in the desert were visited to collect the samples during the study. Two-hundred-sixty-four Cholistani cattle (142 females and 122 males; 127 adult and 137 young) were examined in a survey approved by the Ethical Review Committee for the Use of Animals, under the administrative control of the Office of Research, Innovation, and Commercialization of Bahauddin Zakariya University. Written consent was obtained from the Cholistani pastoralists involved in our study.

### *Blood and Tick Sampling*

Animals were examined for clinical signs (fever, enlargement of superficial lymph nodes, anemia, salivation and drop in milk production) and tick infestation. Approximately 7 ml of blood was collected from the jugular vein under appropriate restraint and stored as two aliquots: clotted for harvesting serum and un-clotted (0.5 M EDTA) for DNA extraction and haematological analysis. The body of each animal was inspected by palpation for the presence of tick infestation, primarily on the ears, perineum, scrotum, udder, tail base, and along the nape of the neck. The ticks were manually removed and placed in 25 mL containers with perforated caps containing a small strip of filter or paper towel. The ticks were identified according to the standard taxonomic keys using a stereomicroscope.

### *Microscopic Examination*

Thin blood smears were prepared, labelled, air dried, and transported to the Molecular Biology Laboratory of Bahauddin Zakariya University. The smears were fixed in absolute methanol for 5 min, stained with 10% Giemsa stain for 30 min, and examined under oil immersion (×1.000) for the presence of parasites. In each smear, 20 fields (minimum 5.000 erythrocytes) were screened for the presence of intra-erythrocytic *Theileria* piroplasms.

### *DNA Extraction and PCR Amplification*

DNA was extracted by an inorganic method [14]. PCR amplification was carried out through an optimized method described by Shahnawaz et al.[15] in which the 30 kDa merozoite surface antigen of *T. annulata* was amplified using a set of oligonucleotide primers. The forward [N516 (5'- GTAACCTTTAAAAACGT-3')] and the reverse [N517 (5'-GTTACGAACATGGGTTT-3')] primers were as described by d'Oliveira et al.[16]. A final reaction volume of 25 µL was used for the PCR. Each reaction contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl, 0.1% Triton X-100, 200 µM (each) deoxynucleotide triphosphate, 2.5 U of Taq DNA polymerase (Merck, USA), 20 pMol of primers, and 5 µL of extracted DNA sample. Positive control DNA from *T. annulata* (previously detected by PCR from a naturally infected cow) and sterilized de-ionized water (without DNA) were run with each PCR amplification as positive and negative controls, respectively.

The sensitivity, specificity, positive predictive and negative predictive values for for blood smear examination and for PCR were determined through an online calculator available at the web link (http://www.wikihow.com/Calculate-Sensitivity,-Specificity,-Positive-Predictive-Value,-and-Negative-Predictive-Value).

### *Haematological Analysis*

An automated Haematology Analyzer (Sysmex K21, Kobe, Japan) was used for determination of haematological

indices including haemoglobin (Hb), packed cell volume (PCV), total erythrocyte count (TEC), and total leukocyte count (TLC). Blood smears stained with Wright's stain were simultaneously prepared for differential leukocytic count (DLC). Mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC) were calculated. The haematology analyzer was designed for human application; hence, before analysis of samples, it was validated against blood samples from 100 dogs and 100 cows as well as with manual reference methods (cynmethaemoglobin photometry, haematocrit analysis, and haemocytometry) [17]. Total protein (TP), creatinine, alanine transaminase (ALT), aspartate transaminase (AST), and triglycerides (TGs) were determined by using APEL PD-303S spectrophotometer (Japan) and diagnostic kits (Spinreact, Spain), following the manufacturer's instructions.

### *Statistical Analysis*

The data were classified according to sex, age, and season. Animals were categorized as adolescent (≥ 24 months) or adult (<24 months). Seasons were designated as temperate spring (February through April), hot dry summer (May through July), hot humid summer (August through October), and cool dry winter (November through January). Results for sex and age are presented as odds ratio with 95% confidence intervals, and seasonal fluctuation was assessed through Mantel-Haenszel $c^2$ test using Minitab v. 16. Significance was considered at $P<0.05$. The difference in haematological profile of parasite-positive and parasite-negative animals was calculated through an un-paired t-test. Predictive values were determined through sensitivity and specificity of smear examination and PCR results.

## RESULTS

A total of 200 ticks feding on cattle were collected. Seventy-eight of the 264 (29.5%) examined cattle carried at least one tick. The mean rate of infestation of cattle was 2.6, with the number of ticks per animal ranging from 1 to 40. Taxonomic identification revealed that all the ticks were belonged to the species *Hyalomma anatolicum*.

None of the cattle exhibited clinical signs of tropical theileriosis. Prevalence, as detected through blood smear examination and PCR, was 1.9 (5/264; CI 0.25-3.53) and 19.3% (51/264; CI 14.5-24.1) respectively *(Table 1)*. Microscopic findings were confirmed by PCR positive signals for *Theileria annulata*. All PCR positive samples produced the 721 bp fragment specific for *T. annulata*. Sensitivity, specificity, positive predictive, and negative predictive values for blood smear examination were 8.9, 45.1, 1.8, and 80.6%, respectively. Similar values for PCR were 91.0, 54.8, 19.3, and 98.1%, respectively.

Prevalence of tropical theileriosis in females was higher, but not significantly ($P>0.05$), than in males at 24.6% (35/142; CI 17.5-31.7) vs. 13.1% (16/122; CI 7.1-19.1). A similar non-significant ($P>0.05$) difference in prevalence was found between adolescent and adult cattle at 23.4% (32/137; CI 16.3-30.4) vs. 15% (19/127; CI 8.8-21.2). Significant differences in prevalence of the disease were found among seasons ($P<0.05$) *(Table 1)*. Highest prevalence was in hot dry summer at 51.6% (33/64; CI 39.2-63.8), followed by that in cold dry winter (14.5%; CI 5.7-23.3), temperate spring (7.9%; CI 1.8-13.9) and hot humid summer (4.8%; CI 0.5-10.2) *(Table 1)*.

Haematological parameters of parasite-positive and

***Table 1.*** *Association between the presence of Theileria annulata infection in Cholistani cattle detected by PCR and the studied parameters (sex, age, season)*
***Tablo 1.*** *Sığırlarda PCR ile tespit edilmiş Theileria annulata enfeksiyonu ile bazı parametreler (cinsiyet, yaş, mevsim) arasındaki ilişki*

| Parameters | No.of Sample | Positive no. of Cattle (PCR) | Odds Ratio/*P*-value* |
|---|---|---|---|
| **Gender** | | | |
| Female | 142 | 35 (24.6%; CI 17.5-31.7)** | 2.17 [reciprocal =0.46] |
| Male | 122 | 16 (13.1%; CI 7.1-19.1) | |
| Total | 264 | 51 (19.3%; CI 14.5-24.1) | |
| **Age** | | | |
| Adult | 127 | 19 (15%; CI 8.8-21.2) | 0.58 [reciprocal =1.73] |
| Young | 137 | 32 (23.4%; CI 16.3-30.4) | |
| **Season** | | | |
| Temperate spring | 76 | 6 (7.9%; CI 1.8-13.9) | Mantel-Haenszel $X^2$ $P$=0.392 |
| Hot Dry Summer | 64 | 33 (51.6%; CI 39.2-63.8) | |
| Hot Humid Summer | 62 | 3 (4.8%; CI 0.5-10.2) | |
| Cold Dry Winter | 62 | 9 (14.5%; CI 5.7-23.3) | |

** Odds Ratio/P-value analyses show the PCR results, ** 95% CI: Confidence Interval*

parasite-negative cattle showed no significant differences, except for total protein and creatinine, which were significantly higher in infected animals *(Table 2)*.

## DISCUSSION

This is the first study on Cholistani cattle directed towards assessment of PCR as a reliable diagnostic tool for carrier state of theileriosis, deducing its prevalence and assessing effect of the disease on haematological indices. Absence of clinical signs of theileriosis, despite being infested with ticks, may be indicative of innate resistance of Cholistani cattle as previously reported for zebu cattle [12,13,18]. Haematological parameters showed a consistent pattern throughout the seasons, without showing variation with respect to level of weather-induced stress [2,12]. The conventional diagnostic tools for theileriosis and babesiosis are being replaced by modern, sensitive, and specific molecular diagnostic methods such as PCR and PCR-based reverse line blotting [15,19-26]. In the present study, the prevalence of *T. annulata* found was significantly higher with PCR as compared to that with blood smear examination. This is in accord with earlier reports that stained smear blood examination cannot detect all sub-clinical or chronic infections, because parasitemia is often extremely low and may be missed [4,27]. Several reports have clearly demonstrated that PCR is a more sensitive and specific test than the conventional thin blood smear [28-30] and is reliable for detecting early or carrier infections.

The prevalence of *T. annulata* is higher in exotic and cross-bred cattle than in locally-adapted zebu breeds [6,31]. The overall prevalence in Cholistani cattle in the present study as detected through PCR was lower than the 24% reported for Holstein-Friesian at Pattoki region of Pakistan [32]. A prevalence of 33% has been reported from Pakistan for cross-bred cattle through blood smear examination [6]. A prevalence of 23% has been reported in Sahiwal cattle in Pakistan using PCR [28]. An absence of clinical signs of the disease and its lower prevalence in Cholistani cattle in the present study may indicate that they are resistant to the disease.

Higher prevalence of *T. annulata* found in females is similar to previous reports [29,33]. There is only one report, from Egypt, of higher prevalence of *T. annulata* in males than in females [34]. This difference can be attributed to ecological/geographical factors and differences in housing systems. The Cholistani nomadic pastoralists are moving in search of food and water. This livestock production system could be a feature responsible for the difference between males and females, since both male and female cattle are equally exposed to tick infestation [12].

Young animals showed a higher prevalence than did adults, consistent with other reports [6,15]. Innate immunity in calves is not developed enough to combat *T. annulata* [35].

A higher prevalence of *T. annulata* in hot dry summer is in line with various reports [6,15]. High ambient temperature in this season provides an environment conducive to growth and multiplication of ticks and ultimately increases the transmission of theileriosis [6].

In this study, no haematological index showed difference in positive cattle compared to non-infected cattle. This is in contrast to most previous reports that document significantly lower TEC, Hb, and PCV values in theileriosis-affected cattle [36,37]. These alterations have been attributed to parasitaemia-induced-anaemia and immune-mediated erythrophagocytosis [37]. The lack of difference in haematological indices of *T. annulata* infection Cholistani cattle in this study may indicate an innate potential to maintain haematological parameters at consistent levels

***Table 2.*** *Haematological profile of parasite-positive and parasite-negative blood samples from Cholistani cattle*
***Tablo 2.*** *Cholistan sığırlarından elde edilen parazit pozitif ve negatif kan örneklerinde hematolojik profil*

| Parameters | Positive (n = 51) | Negative (n = 213) | *P*-value |
|---|---|---|---|
| Total Leukocyte Count ($10^{12}$/L) | 9.8±0.3 | 10.5±0.52 | 0.2 (NS) |
| Total Erythrocyte Count ($10^{6}$/L) | 7.5±0.2 | 7.5±0.1 | 0.8 (NS) |
| Haemoglobin (g/dL) | 10.7±0.2 | 11.1±0.1 | 0.1 (NS) |
| Packed Cell Volume (%) | 36.0±1.7 | 35.8±0.6 | 0.9 (NS) |
| Mean Corpuscular Volume (fL) | 48.0±1.7 | 48.3±0.7 | 0.8 (NS) |
| Mean Corpuscular Haemoglobin (pg) | 15.2±0.7 | 15.3±0.2 | 0.8 (NS) |
| Mean Corpuscular Haemoglobin Concentration (g/dL) | 31.1±0.7 | 32.1±0.3 | 0.1 (NS) |
| Total Protein (g/dL) | 6.9±0.1 | 7.3±0.9 | 0.03* |
| Creatinine (mg/dL) | 1.5±0.5 | 1.3±0.02 | 0.01* |
| Alanine Transaminase (U/L) | 28.6±1.5 | 28.8±0.5 | 0.8 (NS) |
| Aspartate Transaminase (U/L) | 58.8±2.7 | 57.1±1.2 | 0.5 (NS) |
| Triglycerides (mg/dL) | 24.5±0.6 | 26.0±0.5 | 0.06 (NS) |

*Values are mean ±SEM, NS: Non significant, * P<0.05*

regardless of stressors, as reported by Farooq et al.[2]. Among biochemical factors, total protein was significantly decreased in infected cattle. This is in agreement with previous reports of decreased serum proteins in affected cattle. This decrease has been attributed to effects on lymph nodes in the diseased animals, resulting in extra-vascular proteinaceous fluid in body cavities [38].

The present study revealed a substantial prevalence of theilerial infection in Cholistani cattle reared by the nomads of Cholistan. However, their adaptation to the climate and their potential for disease resistance may evident from absence of clinical signs and un-affected haematological indices as observed in present study. This could indicate the state of endemic stability, which needs further research. Uncontrolled cross-breeding and mixed farming being carried out by the nomads of Cholistan desert, Pakistan might be a source of infection-transfer from this endemically stable population to other livestock. Future research needs to be directed towards the assessment of blood biomarkers in Cholistani cattle that may be associated with *T. annulata* resistance.

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