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RESEARCH ARTICLE
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# Detection of natural prevalence and infection of ixodid ticks with *Theileria equi* in hilly equines of Palam valley (India)

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## Abstract

**Aim:** The aim was to study the prevalence of tick infestation in equines of Palam valley and specific detection of *Theileria equi* infection in tick samples with nested polymerase chain reaction (PCR) based assay.

**Materials and Methods:** In order to study the prevalence of ixodid tick population in hilly equines and their potential role in the transmission of *T. equi*, a total of 74 ticks were collected from apparently healthy equines, which were then processed and identified by classical parasitological technique. The molecular techniques (nested PCR) were applied for identification of infection of *T. equi*.

**Results:** The ticks (n=74) collected from apparently healthy equines belonged mainly to three different species, of which 42 (56.75%) were *Rhipicephalus microplus*, whereas 16 (21.62%) were of *Hyalomma* species and 16 (21.62%) were of *Hemaphysalis* species. A total of 21 (30%) ticks were recovered from male and 53 (75.7%) from female equines. Adult equines harbored 94.6% (n=70) when compared to 5.4% (n=4) harbored by young ones. On nested PCR amplification an amplicon of 665 bp size specific for *T. equi* was detected in 6.75% (5/74) ticks (in 7.5% ticks recovered from a female and 4.7% from male equines).

**Conclusion:** Nested PCR assay resulted in significantly higher efficacy of detection of the parasite in ticks. These results clearly demonstrate the presence of equine theileriosis in hilly northern state of the country and potential roles of ticks (*R. microplus*, *Haemaphysalis* and *Hyalomma* species) in its transmission.

**Keywords:** equines, Himachal Pradesh, nested polymerase chain reaction, *Theileria equi*

## Introduction

The hilly equines, especially ponies and donkeys, play a pivotal role in the lives of the people of the hilly terrains as being used as draught and pack animals [1]. Equine piroplasmosis is important tick borne disease condition caused by large form of *Babesia* species i.e. *Babesia caballi* and a *Theileria* species i.e. *Theileria equi* [2]. The hazardous disease entity is ubiquitous in distribution [3] and is responsible for morbidity and mortality of horses worldwide. The disease entity rendered by these haemoprotozoans is either peracute, acute, subacute and chronic depending upon the inoculums of the infective dose and course of the disease followed. The peracute forms are observed in the foals following the *in-utero* infection [4].

The chronic disease condition prevails in endemic areas, where the affected equines are apparently healthy without overt signs of the disease condition, but overexertion and excessive stress leads to subclinical manifestation [5].



The diagnosis of the disease condition relies on serological and molecular techniques associated with clinical signs [6]. Classical parasitological technique involving microscopic examination is only possible in clinically ill equines as parasitemia is often very low to detect in latent and chronic cases [7]. For epidemiological cases involving large areas, serological diagnosis plays a vital role. Various serological tests employed till date for epidemiological studies involve complement fixation test, indirect fluorescence antibody test and enzyme-linked immunosorbent assay [8-10]. However, molecular methods to diagnose the disease condition are more specific and reliable, especially for the detection of carrier animals [11]. Various polymerase chain reaction (PCR) based detection methods targeting ribosomal 18S ribosomal ribonucleic acid (rRNA) sequence, equine merozoites antigen-1 gene, rhoptry-associated protein 1, and 16S-rRNA have been developed and employed in a recent past [12-15].

The aim of the present study was to determine the prevalence of ixodid tick population in hilly equines and their correlation with the blood samples examination of the animals to determine their potential role in the transmission of *T. equi*.

## Materials and Methods

### Ethical approval

Prior consent was taken from the owners of the equines for tick collection. Complete care and measures were taken to avoid any accidental injury to the equine while collecting the ticks.

### Field study area

The study was carried out in Palam valley, Kangra district of northern state of India i.e. Himachal Pradesh from 1st August 2013 to 31st December 2013. The district covers an area of 5739 $km^2$. The temperature and annual rainfall of the area is varying and is approximately 2-30°C (range) and 1000 mm, respectively. The hilly breed of equine is reared by the residents as draught and pack animals. The age and sex of the sampling population was recorded.

### Sampling of the ticks

A total of 74 apparently healthy equines were selected, which were not exhibiting signs of any disease condition but harbouring ticks. The ticks were recovered and preserved in 70% alcohol for morphological and molecular studies.

### Identification of ticks

The species of the collected ticks was identified by studying the characteristics microscopically as per Hoogstraal [16] and Estrada-Pena *et al.* [17].

### Deoxyribonucleic acid (DNA) extraction and PCR

DNA was extracted from ticks by using the Qiagen DNeasy Blood and Tissue kits (Qiagen, Hilden, Germany) as per manufacturer's protocol. The DNA was finally extracted in 100 µl of elution buffer and was kept at −20°C until further use. A nested PCR was conducted using the protocol of Rampersad *et al.* [15]. One microliter of DNA sample from all 74 ticks was subjected to primary PCR (BeqF1 5′-ttcgttgactgcgcttggcg- 3′ and BeqR1 5′-ctaagaagcggaaatgaaa- 3′) and amplicons thus obtained were subjected to nested PCR (BeqF 5′-catcgttgcggcttggttgg- 3′ and BeqR 5′-ccaagtctcacaccctattt- 3′). The PCR cycle conditions for all primers were 94°C for 15 s, 57°C for 30 s, 72°C for 60 s for 30 cycles followed by an extension at 72°C for 5 min. The reactions were performed in the presence of 1.5 mM $MgCl_2$. The presence and size of amplified DNA in PCR reactions was determined by gel electrophoresis on a 1.0% gel. The results were considered as positive if the amplicons obtained were of expected molecular weight i.e. 665 bp. A non-template control i.e. control having no DNA amplicon was also used to rule out any contamination of the products.

### Statistical analysis

Data were analyzed using Win Episcope 2.0 software (College of Medicine and Veterinary Medicine, University of Edinburgh, Scotland) for evaluation of risk factors with 95% confidence interval (CI).

## Results

Of 74 ticks collected 42 (56.75%) (95% CI=0.4472-0.6823%) were *Rhipicephalus microplus*, whereas 16 (21.62%) (95% CI=0.1289-0.3272%) were of *Hyalomma* species and 16 (21.62%) (95% CI=0.1289-0.3272%) were of *Haemaphysalis* species. Sex-wise division revealed females equines (71.62%) (95% CI=0.5995-0.8150%) to be heavily infested by ticks as compared to their male counterparts (28.38%) (95% CI=0.1850-0.4005%). The adult equines also showed more tick infestation (94.6%) (95% CI=0.8673-0.9851%) than young ones (5.4%) (95% CI=0.0149-0.1327%). The details are depicted in Table-1.

On nested PCR amplification an amplicon of 665 bp size specific for *T. equi* was observed in 6.75% (5/74) ticks (95% CI=0.0223-0.1507%) (Figure-1, Table-2). All the five infected ticks were recovered from five different horses belonging to different areas. Three horses among these five horses also showed presence of *T. equi* in blood samples through nested PCR. Only 4.7% (1/21) (95% CI=0.0012-0.2382%) tick population recovered from apparently healthy male equines showed presence of *T. equi* infection, whereas 7.5% (3/53) (95% CI=0.0118-0.1566%) ticks recovered from female equines revealed the presence of infection. The ticks recovered from young equines showed less infection (2.5%) (95% CI=0.0063-0.8059%) of *T. equi* when compared to the adults (5.7%) (95% CI=0.0158-0.1399%). However, values of relative risk were higher in case of the ticks recovered from male equines as compared to female equines and in case of young animals when compared to adults.

## Discussion

The maximum number of ticks was recovered from equine adult females as compared to adult males and young animals, which could be due

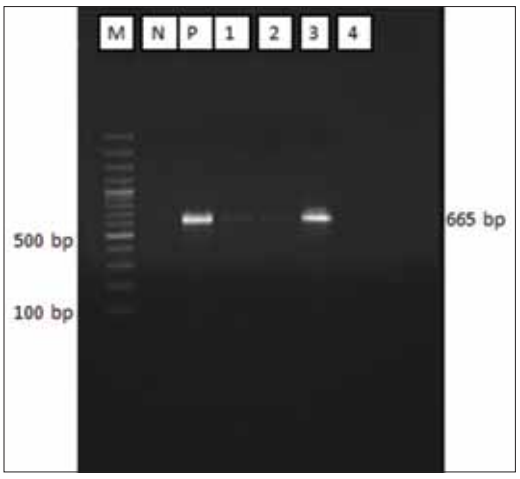


**Figure-1:** Agarose gel electrophoresis (1.0%) showing amplicons of 665 bp for *Theileria equi*. Lane M: 100 bp deoxyribonucleic acid (DNA) ladder/marker; Lane N: Non-template control; Lane P: Positive control; Lane 1-4: Tick DNA amplicons.

**Table-1:** Prevalence of ticks targeting age and sex of equines.

| Equines | Species of ticks | | | | |
|---|---|---|---|---|---|
| | ***Rhipicephalus microplus* (%)** | ***Hemaphysalis* species (%)** | ***Hyalomma* species (%)** | **Total (%)** | **Relative risk (95% CI)** |
| Sex | | | | | |
| Male | 9 (12.16) | 7 (9.45) | 5 (6.75) | 21 (28.38) | 43 (2.653-696.99) |
| Female | 33 (44.59) | 9 (12.16) | 11 (14.86) | 53 (71.62)* | 107 (6.729-1701.31) |
| Total | 42 (56.75)* | 16 (21.62) | 16 (21.62) | 74 | |
| Relative risk (95% CI) | 85 (5.328-1356.05) | 33 (2.0162-540.117) | 33 (2.0162-540.117) | | |
| Age | | | | | |
| Young (<2 years) | 3 (4.05) | 0 | 1 (1.35) | 4 (5.4) | 9 (0.493-164.258) |
| Adult (more than 2 years) | 39 (52.07) | 16 (21.62) | 15 (20.27) | 70 (94.6)* | 141 (8.896-2234.91) |
| Total | 42 (56.75)* | 16 (21.62) | 16 (21.62) | 74 | |
| Relative risk (95% CI) | 85 (5.328-1356.05) | 33 (2.0162-540.117) | 33 (2.0162-540.117) | | |

*Factor associated with relatively higher risk, CI=Confidence interval, *Rhipicephalus microplus=R. microplus*

**Table-2:** PCR amplification targeting *T. equi* infection in ixodid ticks.

| Equines | PCR targets | | |
|---|---|---|---|
| | Ticks | | **Relative risk (95% confidence interval) (%)** |
| | **Total** | **Positive (%)** | |
| Sex | | | |
| Male | 21 | 1 (4.7)* | 10.2273 (0.431-242.32) |
| Female | 53 | 3 (7.5) | 9.7222 (0.512-184.39) |
| Total | 74 | 5 (6.75) | 10.3125 (0.580-183.33) |
| Age | | | |
| Young (<2 years) | 4 | 1 (2.5)* | 45 (2.089-969.36) |
| Adult (more than 2 years) | 70 | 4 (5.7) | 9.5070 (0.521-173.43) |
| Total | 74 | 5 (6.75) | 10.3125 (0.580-183.33) |

*Factor associated with relatively higher risk, PCR=Polymerase chain reaction, *Theileria equi=T. equi*

to partial selection of the female equines over their male counterparts due to their significant reproductive and draught potential [18,19]. The conditions in hilly areas are less favorable for the development of ticks [1], thus the prevalence observed in the present study could be attributed to trade and immigration of equines to the study area from the different plain zones of the country, contributing to addition of ticks and the diseases, transmitted by these vectors to the native hilly equine population. The scarcity of literature revealing any previous study targeting tick population in Palam valley has restricted any comparison about the prevalence of ixodid tick vectors.

As in case of PCR, especially nested PCR there are chances of contamination resulting in false positive results due to contaminating DNA [15]. Thus, at every instance a non-template (without amplicons) control was used to establish the authenticity of the positive results, indicating that they had not been generated as a result of contaminating DNA [20,21].

The presence of positive cases in apparently healthy equines in this part of India also raises concern about the availability and prevalence of the parasite, which could be attributed to the introduction of the parasite along with the ticks and horses brought to these hilly areas from the other parts of India. The large number of clinically healthy animals testing positive suggest that disease may have precipitated from a sub-clinical infection and has resulted from certain adverse factors such as strenuous exercise [22-24]. The comparatively higher infection encountered in ticks recovered from the female when compared to male equines could be due to higher number of tick samples collected from female equines resulting in increased probability of infected ticks recovery. The values of the relative risk were high in case of the ticks recovered from male equines as compared to female equines and from young equines as compared to adults, which could be due to lesser number of ticks retrieved from male and young equines when compared to their counterparts and hence probability of infected ticks and relative risk of infection in these animals increases. Thus, ticks play as an important role as vectors of fatally infective diseases to healthy animals [25].

## Conclusion

Ticks play an important role as vector of fatal diseases of equines. The prevalence study targeting tick population can help in formulating the control measures against them and the suspected diseases transmitted by them. Based on the results of the present

study, nested PCR could be assumed to be a useful tool for the detection of hemoprotozoans i.e. *T. equi* especially in case of apparently healthy animals, which could act as carriers or possess latent infection.

### Authors' Contribution

PK prepared the study outline and carried out collection of samples. ADM carried out classical parasitological work related to identification of ticks and P performed the molecular work. All the authors were involved in review of literature and manuscript preparation. All authors read and approved the final manuscript.

### Competing Interests

The authors declare that they have no competing interests.

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