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Prevalence of *Theileria equi* and *Babesia caballi* Infections in Horses in South Gujarat, India

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ABSTRACT

Background: Equine piroplasmosis (EP) is a tick-borne disease of horses caused by the intraerytrocytic protozoan parasites. The infected animals remain carriers of these blood parasites for long periods and spread the disease. The introduction of carrier animals into areas where competent tick vectors are prevalent can lead to an epizootic spread of the disease.

Methods: Total 295 blood smears from diseased and healthy horses were examined and 295 serum samples were analyses by cELISA for the presence of antibodies against *T. equi* and *B. caballi* whereas 90 DNA samples from seropositive horses were screened by PCR for presence of parasite's DNA.

Result: In present study, 1.35% horses were found positive for *T. equi* by means of blood smear examination. Using c-ELISA, it was found that 03 (1.02%) horses had antibodies against *B. caballi* and 182 (61.69%) against *T. equi*, while none of the sample showed mixed reactions. Ninety (90) seropositivehorses screened for *T. equi* and *B. caballi* by PCR method, out of which, only Nine (09) horses werefound positive indicating an overall prevalence rate of *T. equi* was 10.00% by PCR. None ofthe horses found positive for *B. caballi* through blood smear examination and PCR method.

Key words: *Babesia caballi,* cELISA, Horse, PCR, *Theileria equi.*

INTRODUCTION

Equine piroplasmosis (EP) is a tick-borne disease caused by *Babesia caballi* and *Theileria equi* that affects horses, mules, donkeys and zebras. Both parasites are transmitted by ticks of genera *Dermacentor, Rhipicehalus* and *Hyalomma*. The disease is reported from many parts of India (Sanjeev *et al.,* 2020). In 2004, the OIE approved the competitive Enzyme Linked Immunosorbent Assay (cELISA) for detection of antibodies against *T. equi* and *B. caballi* and as a specified test for global horse activity (OIE, 2014).

The prevalence rates of the *T. equi* were earlier reported by many workers in Gujarat and adjoining states using blood smear examinations and PCR (Sumbria *et al.,* 2016; Vidhyalakshmi *et al.,* 2018; Bharai *et al.,* 2020; Sanjeev *et al.,* 2020). However, a different level of sero-prevalence of antibodies against *T. equi* was documented from various states of India (Khurana *et al.,* 2014; Dahiya *et al.,* 2018; Bhojani *et al*., 2021). There is no previous serological study focusing on the occurrence of *T. equi* infection in horses of south Gujarat in India, therefore,–present study was conducted to determine the prevalence of *T. equi* and *B. caballi* in horses by blood smears and to identify the presence of *T. equi* antibodies in the serum of horses with cELISA.

MATERIALS AND METHODS

Sample collection

The present study was conducted from March, 2016 to March, 2020. Blood samples from horses were collected using vacutainer tubes with or without anticoagulant. The samples were transported under refrigeration to the laboratory and blood smear were prepared. Samples were finally stored at -20°C for molecular analysis.

Serological detection of *T. equi* and *B. caballi* by cELISA

The blood was collected from jugular vein of horses into a serum clot activators. The blood samples were centrifuged at 3000-3500 rpm for 15-30 minutes. The serum was separated and stored at -20°C until performing cELISA. The stored serum samples were assessed for the presence of antibodies to *T. equi* and *B. caballi* using a commercial cELISA test kit (VMRD, Inc., Pullman, USA) following the manufacturer's instructions. The optical density (OD) of the controls and samples were measured at 630 nm wave length

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Table 1: Reaction components for VetPCR™as per VMRD kit.

Kit components	Samples	Positive control	Negative control	Internal control
T. equi/B. caballi premixture	5.5 µL	5.5 µL	5.5 µL	
PCR internal control				5.5 µL
DNase/RNase free water	6 µl	6 µl	6 µl	6 µl
DNA isolated from the sample	2 µl			2 µl
T. equi/B. caballi PCR positive control		2 µl		
PCR negative control			2 µl	
Mineral oil solution	11 µl	11 µl	11 µl	11 µl

using an automatic microplate reader (Cyberlab, R01, USA) and the percentage of inhibition (%) was calculated as follows:

$$I\ (\%) = \frac{100-(\text{Sample OD} \times 100)}{(\text{Mean OD of three negative controls})}$$

Serum samples with ≥40% inhibition were considered positive and samples with <40% inhibition were considered negative as per the manufacturer guidelines.

Molecular detection of *T. equi* by PCR

Total 90 blood samples were selected randomly from the horses showed seropositivity. The DNA was extracted from blood samples as per the protocol outlined in the user manual. VetPCR™ detection Kit (VMRD, Inc., Pullman, USA) was used to detect the DNA of *T. equi* and *B. caballi*.

The products obtained were subjected to electrophoresis (100 volts, 45 minutes) in agarose gels at 2% in TBE with GelRed. Samples that showed amplificationproducts with a size of 499 bp (*T. equi*) were considered as positive (Fig 1). Reaction mixture was prepared for sample, positive control, negative control and internal control by combining the reagents as shown in Table 1 and PCR cycling protocol in Table 2.

Statistical analysis

The data were subjected to statistical analysis using chi-square test and R-software version 3.6.3 as per the method described by Snedecor and Cochran (1994).

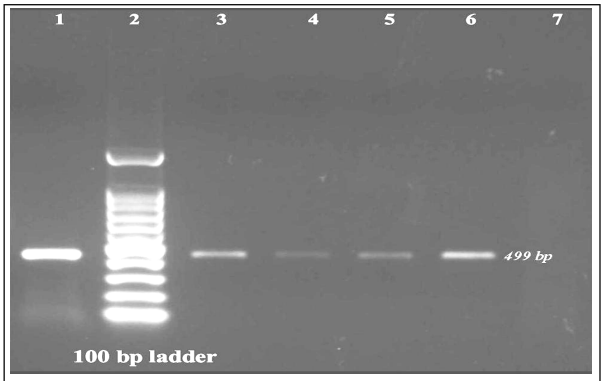
RESULTS AND DISCUSSION

Microscopic examination of blood smears

Blood smears were shown to have a low sensitivity (1.35%) to detect *Babesia* and *Theileria* parasites, although it is a simple, inexpensive and useful tool to diagnose the disease in the field. Identification of equine piroplasmosis in carrier animals by means of blood smear examination is difficult, inaccurate and not practical, on large-scale; serological methods are preferred (OIE, 2014). Prevalence studies conducted in other areas of India reported different levels of positivity as 4.17% from Punjab (Deepak *et al.,* 2016), 5.05% from Gujarat (Vidhyalakshmi *et al.,* 2018), 5.71% from Republic of Guinea and India (Diallo *et al.,* 2018), 6.97% from Mathura, U.P. (Sanjeev *et al.,* 2020), 63.41% from Junagadh, Gujarat (Bharai *et al.,* 2020) and only one horse found positive out of 151 blood smears for *T. equi* using blood smear examination (Bhojani *et al.,* 2021).

Table 2: PCR protocol followed for *Theileria equi* DNA amplification.

Steps	Temperature and time	Cycle
Initial denaturation	94°C for 2 minutes	1 cycle
Denaturation	94°C for 30 Second	30 cycles
Annealing	55°C for 30 Second	
Extension	72°C for 30 Second	
Final extension	72°C for 5 minutes	1 cycle



Lane-1: Positive control, Lane-2: DNA ladder, Lane-3 to 6: Tested field samples and Lane-7: Negative control.

Fig 1: Agarose gel electrophoresis showing amplified DNA of *Theileria equi* (499 bp).

cELISA for detection of *T. equi* and *B. caballi*

In the present research, the overall prevalence of equine piroplasmosis was 62.71% (185/295) by cELISA, out of which 61.69% (182/295) for *T. equi* and 1.02% (03/295) for *B. caballi* (Table 3). Similarly, very law 0.55% (01/182) prevalence of *B. caballi* was reported using cELISA in Gujarat by Vidhyalakshmi (2015) whereas Khurana *et al.* (2014) carried out sero-surveillance of *T. equi* on 291 samples collected from various states of India and overall prevalence of *T. equi* was 24.66%. Bhojani *et al.* (2021) carried out a cross-sectional study on the seroprevalence of *T. equi* using cELISA in 151 horses from Bikaner, Rajasthan state, out of which 75/151 (49.66%) were found positive for *T. equi.* The high prevalence of piroplasmosis might be due to the carrier animals responsible for the maintenance of the infection. Warm humid environment favours the tick vectors and high incidence of disease (Patel *et al.,* 2013). The highest positive sample for *T. equi* by cELISA

Table 3: Prevalence rate of *T. equi* and *B. caballi* in horses.

Species	Blood smears (N=295)	cELISA (N=295)	PCR (N=90)	P value
Theileria equi	04 (1.35%)	182 (61.69%)	09 (10.00%)	< 0.00001
Babesia caballi	00	03 (1.02%)	00	
Overall	04 (1.35%)	185 (62.71%)	09 (10.00%)	< 0.00001

in the present study could be due to presence of antibodies of particular parasites in animal's body. These findings are suggestive and supportive that animals might have come in contact with the parasite and developed antibodies approximately ten days after post-infection. Antibodies against *B. caballi* usually last about four years and in the case of *T. equi*, developed antibodies remain for life as reported by De Waal (2004).

PCR for detection of *T. equi* and *B. caballi*

Out of 90 seropositive horses screened for *T. equi* by PCR, 9 horses were found positive indicating an overall prevalence rate of 10.00% by PCR while none of the samples showed the presence of DNA for *B. caballi* (Table 3). It was in agreement with that of Vidhyalakshmi *et al.* (2018) who reported 11.52% horses positive for *T. equi* from Gujarat State by PCR method. In contrarily to our study, 33.33% prevalence of *Theileria* species in equines had been reported by (Deepak *et al.,* 2016) from Punjab state. Whereas, 48.14% for *B. caballi* and 29.63% prevalence rates for *T. equi* were reported from Saurashtra region of Gujarat by Bharai (2018). Comparable findings were accounted in area of Mathura (Uttar Pradesh) and boarder areas of Rajasthan by Sanjeev *et al.* (2020) who recorded 10.46% prevalence rate of *T. equi* by PCR. Similarly, Diallo *et al.* (2018) carried out molecular analysis of *T. equi* from various agro-climatic zones of Punjab and reported overall 8.57% of equid samples positive for *T. equi* by primary PCR. Moreover, high prevalence of *T. equi* through PCR in other parts of world had been reported as 27.7% by Ebrahimi *et al.* (2018) from Iran, 66.0% by Montes Cortesa *et al.* (2019) from Spain and 50.00% by Sharon *et al.* (2020) from Israel.

CONCLUSION

cELISA is useful as an efficient diagnostic assay to determined *T. equi* and *B. caballi* antibodies from carrier horses. PCR was more useful diagnostic assay for the detection of *T. equi* than blood smear examination. Further research is needed to compare different diagnostic techniques in different regions, to identify the specific piroplasm and to produce geographic distribution maps of equine piroplasms in Gujarat and elsewhere for better understanding the epidemiology of equine piroplasmosis.

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