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# Prevalence of theileriosis in donkeys (*Equus asinus*) Based on parasitological and molecular technique

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**Abstract**
Equine theileriosis, being an OIE listed disease mainly requires frequent disease screening and monitoring. The aim of the present study was to monitor the prevalence of theileriosis in donkeys by using both parasitological method and molecular diagnostic technique based on Equine Merozoite Antigen-5 and 6. In the present study, samples from 216 donkeys were collected randomly. Positive samples detected by using Giemsa stained blood smear technique (GSBT) were used as the positive control samples for screening randomly collected blood samples by using Polymerase Chain Reaction technique (PCR) based on Equine Merozoite Antigen-5 and 6. Out of 216 donkeys samples screened for *Theileria equi* DNA, 67 were positive indicating an overall prevalence rate of 31.02% by universal PCR whereas GSBT revealed only 8.80% prevalence. This study emphasized the usefulness of universal PCR technique as an efficient diagnostic tool for determination of *T. equi* in field samples rather than Giemsa stained blood smear technique.

**Keywords:** Theileriosis, donkeys, *Equus asinus*, parasitological, molecular technique

## Introduction
Since the beginning of time, equines have made a significant contribution to the advancement of the human species, and India is blessed with valuable equine genetic material. According to the 2019 census of animals, India has roughly 0.54 million equines. The donkey, mule, horse, and pony populations, which make up the majority of equids, support rural and semi-urban communities. There are three registered breeds of donkeys, including Spiti, Halari, and Kachchhi. Therefore, it is crucial to protect and conserve horse and donkey breeds both in-situ and ex-situ, especially in light of their declining numbers (Devi A., 2017) [5].

The total population of donkeys in India is 1.24 lakhs in 2019, which was decreased by 61.2% over the previous census (3.19 lakhs). Amongst India, the major donkey population is in the states of Rajasthan (20,935), Uttar Pradesh (12,590), Maharashtra (13,345), Bihar (10,622), and Gujarat (11,286). Gujarat ranked at the 5$^{th}$ position and contributed 8.1% in India. 50% donkey population lies in districts (Anand, Kheda, and Panchmahal) of middle Gujarat, and remaining 50% population is sporadically spread in different districts of Gujarat (Anonymous, 2019) [3]

Interest in the welfare and diseases of donkeys is constantly increasing in several countries, mostly due to the recent rediscovery of donkey milk as an alternative food source for milk-intolerant children. Clinical research on donkeys needs to be in continual development since they have different reactions compared to donkeys in many conditions (resistance to pain in case of colic), including infectious diseases, making it more difficult to recognize the symptoms normally observed in horses.

Equine theileriosis caused by *Theileria equi* is an OIE listed tick transmitted hemoprotozoan disease with worldwide socio-economic impact on equine industry.

*Theileria equi* (Previously known as *Babesia equi*, later reclassified as *Theileria equi* (Ibrahim *et al*., 2011; Mehlhorn and Schein, 1998) [6, 8] is the causative agent of equine theileriosis which occurs in tropical and subtropical regions of the world. Being an endemic disease in Asia, Southern Europe, Latin America and Africa (Ristic, 1988) [9], long-term monitoring of

the prevalence of infection is very important in these areas. The disease has been reported by more than 20 countries of the world and the prevalence study results are found to be varying depending upon the study design, sample size and diagnostic techniques used. Equine piroplasmosis is among the listed diseases of the World Organization for Animal health (OIE), notifiable within 72 hours. Long-term monitoring of the prevalence of infection therefore is very important in areas where the disease has been reported, given the international importance of the disease and the potential impact on the horse industry. So knowledge of the prevalence of infection is essential to set up efficient control measures. Equine theileriosis is a challenge as the parasitemia often remains very low and the infected horses may act as the lifelong nucleus for spreading the disease through vector ticks, thereafter the outbreak or re-emergence of the disease (Wise *et al*., 2013) [11]. The latently infected horses may exhibit poor performance following physical, immunological or mental stress and may predispose horses to the clinical manifestation of the disease and death. But the clinical signs of equine theileriosis are variable and non-specific making the diagnosis difficult (Chhabra *et al*., 2012) [4]. So it is important to identify infected and non-infected horses based on more sensitive and specific diagnostic tool, thus to prevent the spread of the disease among the susceptible equine population by appropriate preventive and control measures. The aim of the present study was PCR based unravelling of prevalence of equine theileriosis by targeting the equine merozoite antigen (EMA-5 and EMA-6) which may contribute well to the future diagnostic as well as therapeutic approaches in cases of equine theileriosis. In the present study, Polymerase chain reaction as well as microscopic methods was compared in which PCR, being molecular diagnostic technique determines the actual presence of parasitic DNA for confirmation of horses as a latent carrier of *T. equi* infection.

Piroplasmosis in donkeys has been recognized as a serious problem of major economic importance, since the affected animals manifest loss of appetite and decreased working capacity. Donkeys usually show an asymptomatic form of the disease, with lower *T. equi* parasitemia when compared to infection in donkeys. Donkeys usually remain asymptomatic carriers with positive antibody titres throughout life. The present work is aimed at detecting infection of donkeys to *Theileria equi* using molecular approach and at recording the hemato-biochemical alterations in affected donkeys.

## Materials and Methods

### Sample Collection

A total of 216 blood samples from donkeys were collected via jugular vein using anti-coagulant ($K_2$EDTA) and without anticoagulant sterile vacutainers tubes. The sex, age and breed of the donkeys were also recorded during this study. The study was carried out for a period of nine months and blood samples were collected for blood smear examination as well as for PCR. The blood samples were collected as per the standard procedure without any stress or harm to the donkeys.

### Examination of Blood smears

Thin blood smears were prepared from blood samples in EDTA tubes. The smears were air dried, fixed in absolute methanol for 10 minutes, stained with 10% Giemsa stain for 40 minutes, washed with distilled water and allowed to dry. The stained blood smears were examined under the high power and oil immersion lens (100x) of the microscope.

### DNA extraction

The whole genomic DNA was extracted from the blood samples of donkeys by using commercial DNA Extraction kit (QIAamp-DNA extraction blood mini kit, Quigen, USA) according to the manufacturer's instructions. The obtained DNA was stored at -20 °C until further use.

### Primers

DNA of *Theileria equi* was amplified by using PCR where the primer sequences used were Forward (EMA-5) 5'-TCGACTTCCAGTTGGAGTCC-3' and Reverse (EMA-6) 5'-AGCTCGACCCACTTATCAC-3' (Vidhyalakshmi *et al*., 2015) [10] yielding a 268bp product.

### PCR protocol

All PCR reactions were performed in 25 µl of reaction mixture consisting of 12.5 µl Master mix, 2 µl of each forward and reverse primer, 5.5 µl of Millique water and 5 µl of extracted DNA. PCR reactions also included a negative control, consisting of the 19.5 µl of reaction mix and 5.5 µl of Millique water. The amplification conditions for *T. equi* included 40 cycles with enzyme activation at 95 °C for 10 min, denaturation at 94 °C for 1 min, primer annealing at 60 °C for 1 min and amplification at 72 °C for 1 min followed by final extension at 72 °C for 5 min. The final PCR products were subjected to electrophoresis in a 1.5% agarose gel with TBE buffer and DNA bands were visualized by using UV transilluminator or Gel documentation System. Positive samples for *T. equi* detected by using Giemsa stained blood smear technique were used as positive control.

### Agarose gel electrophoresis

Preparation of 2% agarose gel was done by taking 1 gm of agarose powder into a 250 ml flask and added 50ml of 0.5x TBE buffer. The solution was heated in a microwave until agarose was completely dissolved. Gel casting tray was used for casting and appropriate number of comb was placed in gel tray and added 2.5 µl of ethidium bromide to the gel. Gel was poured into tray and allowed to cool for 15-30 minutes at room temperature. Later the comb was removed from gel tray and placed in electrophoresis chamber and covered with TBE buffer. Thereafter 1 µl of 1oading dye was added to a 6 µl PCR product and mixed well and loaded. About 2-3 µl of standard (Ladder) or 3 µl positive control was loaded in another well and electrophoresis was done at 80 V for 30 minutes.

Finally DNA bands were visualized using UV transilluminator or Gel documentation System. For molecular detection, *Theileria equi* oligo-primers specifically amplify at 268 bp, 18s rRNA gene of *Theileria equi* (Vidhyalakshmi *et al*., 2015) [10].

## Results and Discussion

### Parasitological method

Examination of the stained blood smears revealed few parasitized red blood cells with *T. equi* and shown in Figure 1. Out of 216 blood smears examined, 19 were found to be positive indicating a prevalence rate of 8.80% indicating a low sensitivity when compared to PCR.

### Polymerase chain reaction

Out of 216 donkey samples screened for *T. equi* DNA by using universal PCR technique and gel electrophoresis, 67 were found to be positive indicating an overall prevalence rate of 31.02%. Out of 67 donkeys positive for *T. equi* as detected

by PCR, only 8 donkeys have shown varying clinical symptoms whereas rest of 59 donkeys positive for theileriosis were found to be apparently healthy. DNA extracted from 216 blood samples were amplified and shown in Figure 2. The present study revealed low sensitivity of Giemsa stained blood smear examination technique to detect low level of parasitemia or subclinical or carrier status when compared to the PCR technique therefore supporting observations of other studies. (Abedi *et al*., 2015; Kurra H. M. & Nageib B. R., 2017; Ahedor *et al*., 2022) [1, 7, 2]. Abedi *et al*., (2015) [1] observations consists of prevalence of *T. equi* as 3.77% positive by Giemsa stained blood smear examination technique, whereas 50.94% by PCR technique (n=106). Kurra H. M. & Nageib B. R., (2017) [7] shows the prevalence of *T. equi* as 14%(7/50) positive by Giemsa stained blood smear examination technique, whereas 38% (19/50) by PCR. Ahedor *et al*., (2022) [2] observations consists of prevalence of *T. equi* as 57.7% (64/111) positive by Giemsa stained blood smear examination technique, whereas 85.6% (95/111) by PCR technique (n=106).

In the present study, PCR technique is used to detect *T. equi* DNA based on Equine merozite antigen-5 and 6 and found to be more sensitive than microscopic methods.

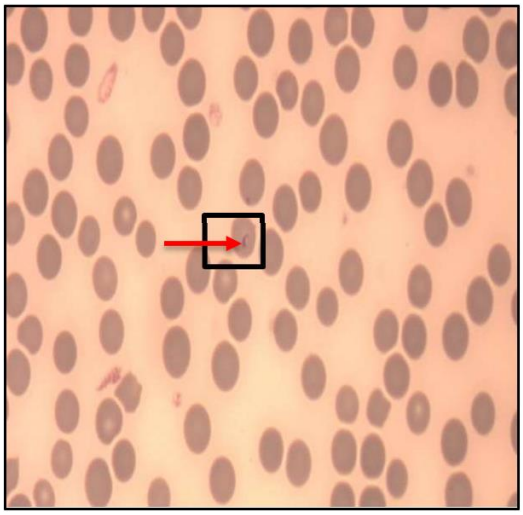

**Fig 1:** Piroplasm of Theileria (red arrow) (Giemsa stain; 100 x)


**Fig 2:** Agarose Gel Image of *Theileria equi* showing amplification at 268 bp

In conclusions, the present study revealed the prevalence of theileriosis among the donkey population based on EMA-5 and EMA-6 antigen of *T. equi* by using universal molecular technique and the same is found to be more dependable diagnostic tool than microscopic method as the former detects circulating parasitic DNA. Thus present study revealed the efficiency of molecular technique for monitoring Theileriosis among donkeys. Therefore universal PCR technique may be helpful to develop better strategies to minimise the likelihood of clinical cases or outbreaks of theileriosis in donkeys of endemic areas under the circumstances of existing challenge in complete elimination of theileriosis. Priority should be given for the development and validation of highly sensitive, simple and specific diagnostic tool to distinguish infected and noninfected donkeys.

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