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Prevalence of *Theileria equi* among Horses Based on Parasitological and Universal Molecular Technique

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A B S T R A C T

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 horses by using both parasitological method and molecular diagnostic technique based on Equine Merozoite Antigen-5 and 6. In the present study, samples from 248 horses 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 248 horse samples screened for *Theileria equi* DNA, 30 were positive indicating an overall prevalence rate of 12.10% by universal PCR whereas GSBT revealed only 5.05% 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.

Introduction

Equine theileriosis caused by *Theileria equi* is an OIE listed tick transmitted hemoprotozoan disease with worldwide socio-economic impact on horse industry. *Theileria equi* (Previously known as *Babesia equi,* later reclassified as *Theileria equi* (Ibrahim *et al.,* 2011; Mehlhorn and Schein, 1998) 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), 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 the 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). 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). 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.

Materials and Methods

Sample collection

A total of 248 working as well as racing horses of all ages, regardless of sex and breed were included in the study for screening theileriosis by using PCR technique. The study was carried out for a period of nine months and blood samples were collected for Giemsa stained blood smear examination as well as for extraction of DNA of *T. equi* by using universal polymerase chain reaction. Consent from the owners of the horses was obtained for sample collection and the blood samples were collected as per the standard procedure without any stress or harm to the horses.

Giemsa stained blood smear examination

Giemsa staining technique was used for microscopical detection of *T.equi* in blood smears. Thin blood smears of 248 horses, prepared by using freshly collected blood samples were allowed to dry and fixed in methanol for 1 minute. Giemsa stain was flooded on these slides for 30-35minute followed by washing under the running tap water and dried for examination under oil immersion microscopy.

Extraction of genomic DNA of *T. equi* from whole blood

Blood samples collected from 248 horses into sterile K_3 EDTA vials were stored at -20^0 C until used for DNA extraction. Genomic DNA of *T. equi* was extracted from whole blood samples by using commercially available QIAamp-DNA extraction blood mini kit (CATALOG NO.51106). Spin protocol was used for the purification of the genomic DNA from whole blood by using a microcentrifuge DNA extraction was carried out according to the instruction manual of QUIAamp DNA blood mini kit (Quiagen, Germany).

Quantification of DNA

The DNA was quantified by using nanodrop spectrophotometer (Eppendorf Thermofisher) and stored at -20^0 C until use for PCR.

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' (Battsetseg *et al.,* 2001) yielding a 268bp product.

PCR protocol

All PCR reactions were performed in 19 µlof reaction mixture consisting of 10 µl Master mix, 2 µl of each forward and reverse primer, 2.5 µl of Millique water and 6 µl of extracted DNA.PCR reactions also included a negative control, consisting of the 19 µl of reaction mix and 6 µl of Millique water. The amplification conditions for *T. equi* included 40 cycles with enzyme activation at 95^0 C for 10 min, denaturation at 94^0 C for 1 min, primer annealing at 60^0 C for 1 min and amplification at 72^0 C for 1 min followed by final extension at 72^0 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.

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 248 blood smears examined, only 5 were found to be positive indicating a prevalence rate of 2.02% indicating a low sensitivity when compared to PCR.

Polymerase chain reaction

Out of 248 horse samples screened for *T. equi* DNA by using Universal PCR technique and gel electrophoresis, 30 were found to be positive indicating an overall prevalence rate of 12.10%. Out of 30 horses positive for *T. equi* as detected by PCR based on EMA-5 and EMA-6, only 13 horses have shown varying clinical symptoms whereas rest of 17 horses positive for theileriosis were found to be apparently healthy. DNA extracted from 248 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 (Bahrami *et al.,*

2014; Moatloang *et al.,* 2008; Rampersad *et al.,* 2003). Rampersad *et al.,* (2003) observations consists of seroprevalence of *T. equi* as 9.5% positive by Giemsa stained blood smear examination technique, whereas 15.24% by PCR technique (Rampersad *et al.,* 2003). Bahrami *et al.,* (2014) shows the seroprevalence of *T. equi* as 4.76% positive by Giemsa stained blood smear examination technique, whereas 22.86% by PCR (Bahrami *et al.,* 2014).

Fig.1 Giemsa stained blood smear positive for *T. equi*

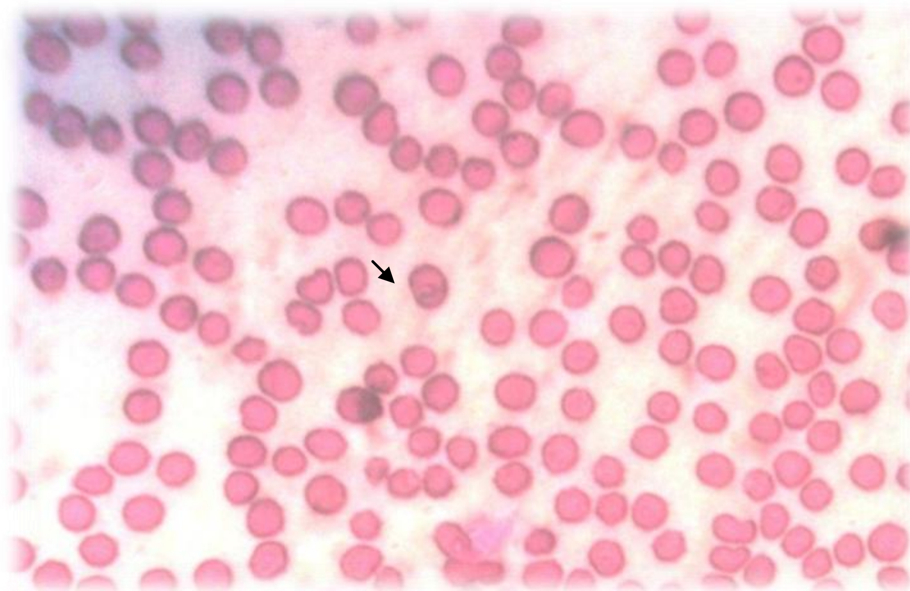
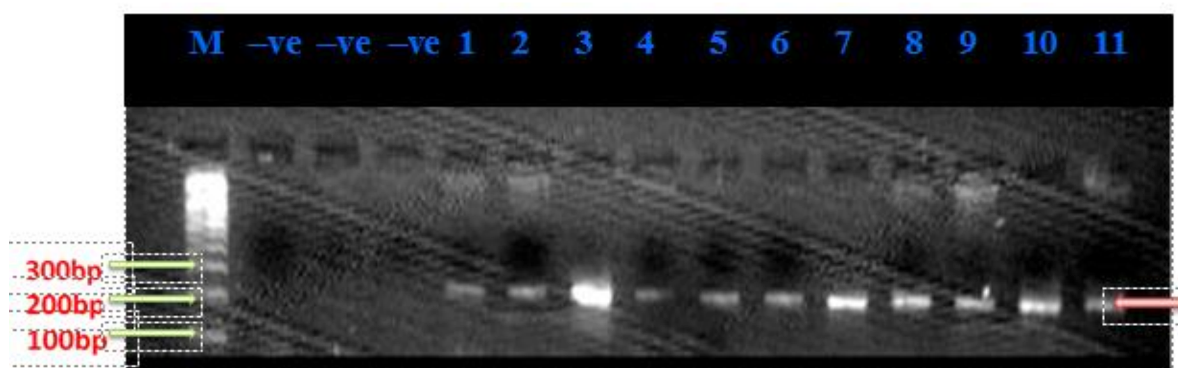


Fig.2 Agar gel electrophoresis revealing PCR product of 268 bp positive for *T. equi*



LANE M: Molecular weight ladder
LANE –VE: Negative controls
LANE-1 to 11: PCR product of field samples positive for *T. equi*

Even though, molecular and serological techniques give more dependable tools for diagnosis of equine piroplasmosis (Salim *et al.,* 2008), the previous reports shows higher prevalence rate (Khurana *et al.,* 2014) based on the fact that most of the serological techniques provide the prevalence status depending on the circulating antibodies, not

necessarily the antigen, which may be detected even in horses with a past history of positivity for *T. equi* infection (Bruning, 1996). 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 (Bruning, 1996; Ibrahim *et al.,* 2011; Malekifard *et al.,* 2014) and specific than serological methods (Ibrahim *et al.,* 2011; Tenter and Friedhoff, 1986).

In conclusions, the present study revealed the prevalence of theileriosis among the horse 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 horses. Therefore universal PCR technique may be helpful to develop better strategies to minimise the likelihood of clinical cases or outbreaks of theileriosis in horses 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 non-infected horses.

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