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Original Article

Microscopic and Molecular Detection of *Theileria (Babesia) Equi* Infection in Equids of Kurdistan Province, Iran

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Abstract

Background: Equine piroplasmosis (EP) is the cause of persistent tick-borne infection with no symptoms, but the most important problem of EP is due to the persistent carrier state. Carrier animals to *Babesia* (*Theileria*) *equi* (Laveran 1901) and *B. caballi* (Nuttall, 1910) infestation could be identified by extremely sensitive PCR-based method. The purpose of this study was to identify the causative agents of equine piroplasmosis based on molecular and microscopic assays in equids from Kurdistan Province, Iran.

Methods: Thirty one horse and mule blood samples were used with history of living in Kurdistan Province of Iran. The blood specimens were utilized for *T. equi* and *B. caballi* DNA identification by PCR and Giemsa stained smears for microscopic observation.

Results: The results clearly showed the presence of *B.* (*Theileria*) *equi* DNA in 30 of 31 blood samples (96.77%), but the microscopic examination revealed the 3 of 31 positive *Babesia* like organisms in the red blood cells (9.67%).

Conclusion: The obtained results demonstrated the presence of hidden *B.* (*Theileria*) *equi* infection in horses with previous habitance in Kurdistan Province of Iran. The carrier animals became a main source of infection and can transmit the disease. Therefore, hidden infection might be considered as a health threatening and limiting factor in animals used in therapeutic antisera research and production centers.

Introduction

Equine piroplasmosis (EP) is endemic, prevalent and a parasitic disease of all equids, caused by *Theileria equi* and/or *Babesia caballi* that are distributed in most parts of the world (1). The cause of Equine theileriosis is a blood protozoan *T. equi* (Laveran 1901). The majority of infected animals showing no clinical symptoms, but they are important because of their role as a source of infection for tick-borne transmission (2, 3).

No pathognomonic symptoms exist for Equine piroplasmosis, thus the EP should be differentiated from other hemolytic types showing fever, anemia and jaundice. Since the most animals in endemic areas carry the infection, and are asymptomatic, therefore, the paraclinical diagnosis was carried out by examining blood smears, serum samples and DNA extracted from blood samples for detection and identification of *T. equi* infection (1).

T. equi and *B. caballi* have been reported from North-West, South-West and North-East of Iran by using clinical diagnosis, microscopic examination of blood smears and PCR (4-9). Moreover, the vector ticks for *T. equi* "*Hyalomma marginatum marginatum, H. anatolicum excavatum, Rhipicephalus bursa, H. marginatum turanicum,* and *H. anatolicum anatolicum*" have been identified in two large regions of North and West of Iran (8, 10, 11). *T. equi* could be transmitted by *Dermacentor* spp. (*D. reticulatus* and *D. marginatus*) (12, 13). Although there are a few reports on prevalence of *B.* (*Theileria*) *equi* in Iran, but the protozoa might be extensively distributed in Iran. However, these studies generally utilized clinical findings, microscopic examination of blood smears, serological assay and molecular methods.

Principally, the genus of *Equus* have been trained and used in army, farming, transportation, hobby, and sport. Moreover, equids remain the animal of choice for therapeutic antivenoms and antitoxin production in applied immunology.

In Razi institute, the equid are subjected to immunisation department for producing antisera. It is necessary to utilize healthy equid to ensure pure, consistent and compatible antiserum manufacturing. Therefore, the health monitoring of applied horses and mules is an essential part of this immunisation procedure. This descriptive study aimed to inspect of EP infectious agents by PCR in horses and mules inhabit in Piranshahr, Kurdistan Province in West of Iran.

Materials and Methods

Animals

Thirty one newcomer equids (24 horses and 7 mules) were kept under the care and control in Razi institute quarantine area in 2014. The horses were workhorse and Kurdish-breed, between 5 and 7 years of age, with previous living in Piranshahr, Kurdistan Province, Iran. The ethical approval has been obtained from Ethics and Animal Welfare Committee of Razi Vaccine and Serum Research Institute.

Blood samples

Blood samples were obtained individually from all animals by jugular vein puncture and placed under vacuum in sterile tubes containing ethylenediamine tetraacetic acid (EDTA) anticoagulant. Blood specimens from horses and mules were subjected for genomic DNA isolation.

Microscopic examination

Giemsa-stained blood smears were air-dried, fixed in absolute methanol, and stained with Giemsa, prior to examination at ×1000 magnification using a bright-field microscope (Nikon). A total of 100 fields were observed, and the number of red blood cells (RBC) infected with *B. (Theileria) equi* recorded.

DNA isolation

Genomic DNA was extracted from individual blood samples using proteinase K method

(14). Briefly, the cell pellets were incubated in cell lysis solution containing proteinase K. Then, proteins were precipitated and genomic DNA was recovered in cold pure ethanol, washed in 70% ethanol, dried and resuspended in dH_2O (14).

PCR

The detection of *B.* (*Theileria*) *equi* DNA was performed by using specific PCR assays to amplify the 18S rRNA gene sequence. The assay used the BallF (5' gta att cca gct cca ata g) and BallR (5' aaa gtc cct cta aga agc) primer pair to amplify both *B.* (*Theileria*) *equi* the 788 bp and *B. caballi* the 759 bp fragment of 18S rRNA gene sequence (15), BeqF (5' cat cgt tgc ggc ttg gtt gg) and BeqR (5' cca agt ctc aca ccc tat tt) primers were applied to amplify *B.* (*Theileria*) *equi* (16). Amplification used cycles of 94 °C for 3 min; 35 cycles of 94 °C for 30 sec, 54 °C for 45 sec, and 72 °C for 1 min; final extension at 72 °C for 5 min; and holding at 4 °C by Techgene thermal cycler (Techne, Cambridge, United Kingdom). The PCR products were electrophoresed on 1.5% agarose gel and visualized through in-gel staining using RedGel (Biotium, Inc. Hayward, CA) and visualized by UV Transillumination (Uvidoc, Gel Documentation System, Cambridge, UK).

PCR product sequencing

The amplicons were examined for expected size and finally for nucleic acid sequencing. The PCR product was cleaned up and sent for direct sequencing in both directions (Bioneer, S. Korea).

Statistical analysis

The differences in results between these two methods were compared using paired Student's *t*-test and the differences were considered statistically significant if the p values were less than 0.05 (Microsoft Office Excel 2007).

Results

The microscopic examination for 31 Giemsa-stained blood smears revealed the three positive samples (red blood cells that are infected with *Babesia* / *Theileria* parasites) out of 31 samples (9.67%) (Fig. 1).

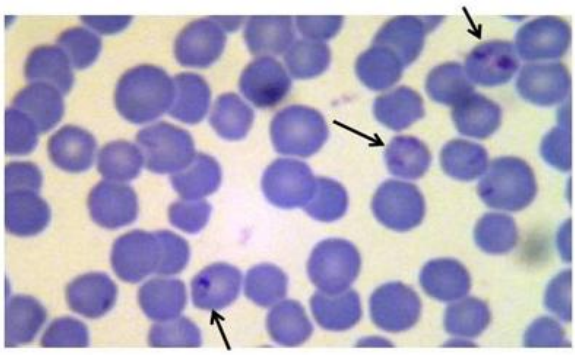

Fig. 1: Blood smear stained with Giemsa, illustrating several red blood cells infected with *Theileria equi* (showed by arrows). 1000X magnification was used for microscopic inspection

The PCR test showed the isolated DNAs were successfully amplified specifically by using *Equine* piroplasmosis primers. The outcomes of amplification demonstrated 30 positive out of 31 samples for *B. (Theileria) equi* (96.77%) but no amplification for *B. caballi* (Fig. 2).

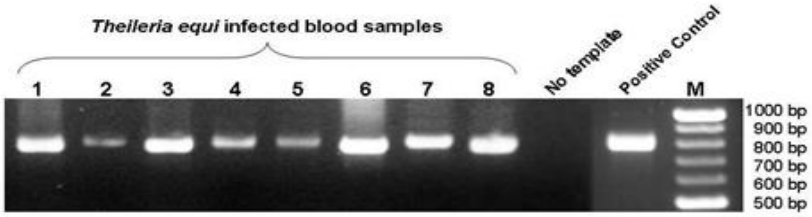

Fig. 2: Gel agarose electrophoresis of PCR targeting *Theileria equi* 18S rRNA. 1.5% agorose gel showing 18S rRNA amplicons from 8 *T. equi* infected blood samples. Negative and positive controls as well as molecular size markers are indicated on the right

The comparison of results between two considered methods, microscopic inspection and PCR assay by statistical analysis showed a significant differences between two applied tests (P<0.001).

The PCR product of Kurdistan isolate was sequenced (697 nucleotides length) and the data were analyzed using "Blast" online program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The "blast" study demonstrated 96-100% identity with *T. equi* 18S rRNA gene sequences in GenBank. Then, the sequence of "*T. equi* Kurdistan of Iran isolate" was submitted to GenBank and has been deposited under GenBank accession no. KJ418423.

Discussion

Equine piroplasmosis (EP) is a tick-borne disease of equine, vastly distributed in the world (1, 2). Moreover, most infected animals showing no clinical features and develop into asymptomatic carriers that can potentially spread the infection (2, 3). As there is no adequate epidemiological data for Equine piroplasmiosis in Iran, this study was designed to detect and identify the causative agent (s) of Equine piroplasmosis in provided equids for immunisation department of Razi Institute for producing therapeutic antisera.

The number of 31 healthy horses and mules were available from Piranshahr in Kurdistan Province of Iran a border city close to Iraq. High incidence of *Babesia* (*Theileria*) *equi* infection was determined by molecular PCR assay (96.77%), but the microscopic observation showed that only 9.67% of samples were positive for *B.* (*Theileria*) *equi* infection. The intresting finding was the lack of positive sample for *Babesia caballi*. This finding is agreed with previous studies in North East and in South West of Iran who demostrated the *T. equi* was the only causautive agent for EP in these two provinces (8, 9).

The comparison of two methods showed there was a significat difference between two applied techniques and the PCR was more sensitive than the microscopic inspection (P < 0.001). Nevertheless, microscopic examination remains one of the most important techniques for laboratory confirmation of haemoprotozoan parasites because it is a simple method that is well-known to the majority of laboratory technicians.

There are only *few* reports for Equine piroplasmosis in Iran (4-9), while the *H. anatolicum anatolicum* tick vector for EP was widely distributed all over the country (8, 10, 11).

The prevalence of EP was determined, microscopically, 6.25% and 2.80% were infected by *T. equi* and *B. caballi*, respectively, but the PCR revealed the 10.83% and 5.83% for *T. equi* and *B. caballi* infection, respectively in Urmia, west Azerbaijan Province of Iran (7).

In North East of Iran, *T. equi* infection was microscopically detected in 5% of the blood smears with low parasitemia, while a multiplex PCR for *T. equi* and *B. caballi* DNA showed no *B. caballi* infections, but *T. equi* DNA was detected in 45% of the samples (8). The prevalence of *T. equi* was reported 28.5% in the healthy Persian Arab horses of the Khuzestan Province of Iran (9).

As previously mentioned, EP is an endemic tick borne disease in most areas of the world (1, 2), and the majority of infected equine appear clinically normal, but they remain infected and develop into lifelong carriers of the infection (1-3). Equine piroplasmosis was eradicated from the US in 1988, after 25 years of actions for eradication program. This is the cause of removing the EP positive animals for sports competitions or export into a number of countries believed free of EP (17, 18).

Although, the manufacturing of antisera in chronically infected equid does not affected significantly, but the performance of the immunised horses and mules are related to the good and healthy circulatory system of the incubated animals. Therefore, it is strongly recommended to benefit of the healthy and good condition of equid for producing antise-

ra in such centers as Razi Vaccine and Serum Research Institute.

Conclusion

According to the prevalence of tick vector and some reports based on clinical signs, microscopic inspection and now molecular assay for EP in different parts of Iran as well as a report from Iraq (19), it is crucial to monitor the equids for *B.* (*Theileria*) spp. by the mentioned techniques and further required control plan and eradication program.

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