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SHORT COMMUNICATION

A comparative study of serological tests and PCR for the diagnosis of equine piroplasmosis

Osman Jaffer · Faysal Abdishakur · Fatima Hakimuddin · Anie Riya · Ulrich Wernery · Rolf K. Schuster

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Abstract A total of 105 serum samples from endurance horses from different stables in Dubai were examined for the presence of antibodies against *Theileria equi* and *Babesia caballi* using immunofluorescence antibody test (IFAT) and competitive enzyme-linked immunosorbent assay (cELISA). A TaqMan real-time polymerase chain reaction (PCR) was used to detect DNA of piroplasms in specimens of clotted blood or EDTA blood samples of the same animals. Out of the 105 serum samples, the IFAT detected antibodies against *T. equi* in 35 (33.3%) cases while the cELISA gave 34 (32.4%) positive results. Eleven (10.5%) of the 105 sera were positive in the *B. caballi* IFAT while an additional five (4.8%) other specimens were diagnosed positive using the cELISA. The serological results showed that 13 (12.4%) horses had antibodies against both *T. equi* and *B. caballi*. The TaqMan real-time PCR detected DNA of piroplams in 33 (31.4%) samples while serological methods found antibodies in 38 (36.2%) horses.

Introduction

Equine piroplasmosis is one of the most important tickborne diseases of horses. The causative agents, the haemoprotozoans *Theileria equi* and *Babesia caballi* are present mainly in tropical and subtropical areas of Africa, America and Asia (Friedhoff 1982). The presence of these parasites was also detected in Japan (Ikadai et al. 2002), China (Xuan et al. 2002) and in Mongolia (Battsetseg et al. 2001; Boldbaatar et al. 2005; Rüegg et al. 2006, 2007). According to the OIE (2009), 27 countries reported cases of equine piroplasmosis in 2008. Amongst them were Israel, Jordan and Qatar.

The host spectrum of these parasites includes equids (horse, donkey, mule and zebra). Although there are some major differences in the life cycle both parasites were placed in the genus *Babesia* until Mehlhorn and Schein (1998) transferred *Babesia equi* into the genus *Theileria*. These authors demonstrated that *T. equi* like other members of the *Theileria* genus have a preerythrocytic development within lymphatic cells of their vertebrate host before the merozoites enter red blood cells and form the so-called Maltese cross. Unlike *Babesia* species *T. equi* can only be transmitted within one tick generation. A transovarial transmission like in the case of *Babesia* species does not occur.

Altogether, 21 species of ixodid ticks of the genera *Boophilus, Dermacentor, Hyalomma* and *Rhipicephalus* are listed as vectors for equine piroplasms. Four species are able to transmit *T. equi*, eight are vectors for *B. caballi* and nine for both species (APHIS 2008).

The aim of this study was to compare the efficacy of two serological methods and to test a TaqMan real-time polymerase chain reaction (PCR) for the diagnosis of equine piroplasmosis with samples from spontaneously infected horses.

Materials and methods

Serology

For serological examination, blood serum samples were taken from 105 horses based at endurance stables in Dubai, UAE. The horses did not show clinical symptoms of piroplasmosis but had bad performance at the races.

O. Jaffer · F. Abdishakur · F. Hakimuddin · A. Riya · U. Wernery · R. K. Schuster (✉)
Central Veterinary Research Laboratory,
PO Box 597, Dubai, UAE
e-mail: moniezia@zedat.fu-berlin.de

Immunofluorescence antibody test

Commercially available parasite coated slides (Labor, Dr. Boese, GmbH) were brought to room temperature. Double serum dilutions of all samples were prepared, starting at 1:20 up to 1:160. Dilutions were performed with phosphate buffered saline (PBS). A 100 µl of each sample was pipetted onto the slides and incubated in a moist chamber at 37°C for 30 min. The slides were then washed twice with PBS, rinsed with distilled water, dried and 5 µl of 1:30 diluted FITC labelled affinipure rabbit anti-horse IgG serum (Jackson Immunoresearch Labor, USA) was added onto the slides and incubated. The slides were washed again with PBS, covered with buffered glycerin and a coverslip. The slides were then examined under ultraviolet light in a fluorescent microscope at a magnification of 480 (12×40). In all experiments, reference negative and positive controls were included (*T. equi* and *B.caballi*).

Competitive enzyme-linked immunoabsorbent assay

The competitive enzyme-linked immunoabsorbent assay (cELISA) to detect antibodies to *T. equi* and *B. caballi* was carried out using commercially available kits distributed by VMRD, Inc. following the USDA standard protocol codes 501B.20 and 501A.20, respectively. Briefly, 50 µl of diluted control and 105 equine serum samples were pipetted into the wells of the microplates coated with *T. equi* and *B. caballi* antigens, respectively, and incubated for 30 min at room temperature. Plates were then washed and the primary antibody was added to each of the wells in the plate. The plates were incubated for an additional 30 min and washed after which the secondary antibody conjugated with horse radish peroxidase was added. After incubating the plates for 30 min, 50 µl of substrate solution was added to each well. The plates were again incubated for 15 min, and the reaction was stopped by adding stop solution and immediately read on a plate reader (Tecan, Sunrise, USA) at a wavelength of 620 nm. A strong colour development indicated little or no inhibition of primary monoclonal antibody binding and therefore the absence of *T. equi*/*B. caballi* antibodies in the sample.

Table 1 Comparison of the performance of IFAT and cELISA for the diagnosis of *Theileria equi* infection in horses

IFAT	cELISA		
	Positive	Negative	Amount
Positive	34	1	35 (33.3%)
Negative	0	70	70
Amount	34 (32.45)	71	105

Table 2 Comparison of the performance of IFAT and cELISA for the diagnosis of *Babesia caballi* infection in horses

IFAT	cELISA		
	Positive	Negative	Amount
Positive	11	0	11 (10.5%)
Negative	5	89	94
Amount	16 (15.2%)	89	105

DNA extraction for PCR

A total of 105 samples, 80 of which were ethylenediaminetetraacetic acid (EDTA) blood samples and 25 were clotted blood samples and were collected from the same horses at the time of serum collection. One millilitre of each of the EDTA blood samples was extracted using Magnpure according to the manufacturer's protocol (Roche, Germany), whereas 50 mg of the clotted bloods were extracted using phenol-chloroform extraction method according to Sambrook and Russel (2001).

Real-time PCR primers and TaqMan probes

For the real-time PCR assay, a forward primer (BEC-F1), a reverse primer (BEC-R1) and a TaqMan probe (BEC probe) were designed using primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) to specifically amplify overlapping region of 18S rRNA of both *T. equi* and *B. caballi* (GeneBank accession no., EU888903, EU888904). Briefly, the two primers BEC-F1 (5′ ACC AGG TCC AGA CAG AG 3′) and BEC-R1 (5′ AGG TTA AGG TCT CGT TCG 3′) amplifies an 120 bp DNA fragment in the target region, and the fluorescent TaqMan probe BEC probe (5′ TTG ATT CTT TGG GTG GTG GT 3′) was labelled at the 5′ end with FAM reporter dye and at the 3′ end with TAMRA as a quencher dye. The primers and the probe were obtained from Metabion, Germany. The real-time PCR assay was done as follows: 2–5 µl of the extracted DNA template from 105 equines blood, 15 µl of TaqMan universal PCR master mix (Roche), 500 nM of each primer and 300 nM of

Table 3 Serologically diagnosed *T. equi* and *B. caballi* mixed and mono-infections in endurance horses in Dubai

B. caballi	*T. equi*		
	Positive	Negative	Amount
Positive	13	3	16 (15.2%)
Negative	22	67	89
Amount	35 (33.3%)	70	105

Table 4 Detection of equine piroplasmosis using serological methods and TaqMan real-time PCR in horse samples

Serology	PCR		
	Positive	Negative	Amount
Positive	30	8	38 (36.2%)
Negative	3	64	67
Amount	33 (31.4%)	72	105

the TaqMan probe was prepared in a total volume of 20 µl. The real-time PCR was performed in a 32 capillary light cycler 2 (Roche, Germany) under the following conditions: 10 min at 95°C hot start and then 45 cycles of 20 s at 95°C, 30 s at 55°C and 30 s at 72°C.

Results

The serological examination of serum samples of 105 horses showed that the animals had antibodies to both *T. equi* and *B. caballi*. In the case of *T. equi*, immunofluorescence antibody test (IFAT) and cELISA gave similar results. With both tests 34 (32.4%) animals were recognised positive for *T. equi* antibodies and only one serum that was negative in the cELISA reacted positive in the IFAT (Table 1). With *B. caballi*, the cELISA was more effective. This test found five serum samples positive that showed negative results in the IFAT bringing the total number of seroreagents to 16 (15.2%; Table 2).

Altogether 13 (12.4%) horses had antibodies to both *T. equi* and *B. caballi* and 70 (66.7%) sera were negative for piroplasms (Table 3).

The PCR recognised piroplasm DNA in 30 (28.6%) out of the 38 horses with antibodies while in three animals with a negative antibody response, the PCR gave positive results (Table 4).

Discussion

Equine piroplasmosis is of great importance because of the international movement of horses in connection with equine sport competitions, and some countries restrict the entrance of horses that are serologically positive for piroplasma species. For this reason, specific and sensitive tests to detect piroplasma infections are needed.

The clinical picture of piroplasmosis is variable and often non-specific and gives only a hint for further investigation. Fever, icterus, anorexia and depression are the most frequent symptoms and low erythrocyte counts, low haemoglobin, low platelets and high billirubin are the main laboratory findings in infected horses (Zobba et al. 2008).

A direct detection of the piroplasms in the erythrocytes in a stained blood smear is possible during the acute phase of the infection. Later, during the latent phase, the parasitaemia becomes too low to detect positive cases. The carrier status of horses can be proven by in vitro cultivation (Holman et al. 1993, 1997; Zweygarth et al. 1995, 1999, 2002).

Already in the early 1990s, molecular methods to detect DNA of *T. equi* and *B. caballi* in horse blood (Posnett and Ambrosio 1991; Posnett et al. 1991) were introduced. A comparison of in vitro cultivation and molecular methods like loop-mediated isothermal amplification and PCR showed that the latter are more sensitive (Alhassan et al. 2007).

Serological tests are the method of choice to detect latent infections. The development and pros and cons of such tests for equine piroplasmosis are described in detail by Brüning (1996). The complement fixation test for the detection of piroplasma antibodies in horse serum samples developed by (Hirato et al. 1945) was used worldwide in the past but is now replaced by IFAT and ELISAs.

The horses in our case did not show clinical symptoms except for poor race performance. The serological testing however revealed that 38 out of 105 horses had antibodies

Table 5 Serological (IFAT) testing of imported endurance horses for equine piroplasmosis between 2003 and 2008

Year	Number of horses tested	Serologically positive for			
		T equi		*B caballi*	
		In %	95% C.I.	In %	95% C.I.
2003	166	6.0	3.30; 10.01	1.2	0.14; 3.75
2004	191	39.8	32.79; 47.11	10.5	6.51; 15.71
2005	289	16.3	20.67; 31.05	9.0	5.96; 12.91
2006	202	34.7	28.11; 41.66	6.9	3.84; 11.36
2007	232	34.5	28.38; 40.99	14.2	9.99; 19.40
2008	313	25.6	20.81; 3,077	9.0	5.28; 13.41

to equine piroplasms. Sixteen horses (15%) were tested positive for *B. caballi* and 35 (33%) showed antibodies to *T. equi*.

Although the Middle East is considered to be endemic for equine piroplasmosis, there is very little reliable information about the prevalence of this disease. In the Sultanate of Oman, a first tick fever outbreak was diagnosed in 1974 and out of 56 examined horses by IFAT, 53 and 38 reacted positively for *T. equi* and *B. caballi*, respectively Donelli et al. 1980). Cases of clinical theileriosis in horses in Jordan and Iran were published but prevalence data of the disease were not given (Hailat et al. 1997; Seifi et al 2000). In a seroprevalence study on *T. equi* carried out in Israel (Shkap et al. 1998), IFAT and cELISA detected antibodies in 29.4% and 33.7% of the samples, respectively, while in our study, the performance of IFAT and cELISA for this parasite was similar.

In our study, both serological tests and PCR revealed 30 (28.6%) horses positive for equine piroplasms. Eight further horses (7.6%) were reconised positive only by serology. The reason for this is that the antibodies can be demonstrated yet when the piroplasms have been eliminated. In three other horses (2.9%) found positive only with PCR, antibodies may have not yet developed indicating a fresh infection. In a field study carried out in Mongolia, 78.8% and 65.7% out of 499 horses had IFAT Antibodies against *T. equi* and *B. caballi*, respectively, while DNA of these piroplasms were found only in 66.5% and 19.1% of the samples, respectively (Rügg et al. 2007).

According to annual reports of the Central Veterinary Research Laboratory Dubai antibodies against *T. equi* and *B. caballi* over the past years were found in 6.0% to 38.9% and 1.2% to 14.2% of imported endurance horses, respectively (Table 5). The prevalence of *T. equi* was always higher compared with that of *B. caballi*. This was also the case in feral donkeys in the UAE where antibodies against *T. equi* and *B. caballi* were present in 9.7% and 1.6% of the sera, respectively (Turnbull et al. 2002).

There are only two species of hard ticks, *Hyalomma dromedarii* and *Rhipicephalus sanguineus* present in the UAE. Although both of them are named in the vector list for equine piroplasms (APHIS 2008), tick infestation of horses in Dubai has rarely been observed.

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