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Short communication

Serological and molecular detection of *Theileria equi* and *Babesia caballi* in Philippine horses

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ABSTRACT

Theileria equi and *Babesia caballi* are tick-borne protozoan parasites that can cause anemia in horses. In the Philippines, serological detection of these parasites has only been reported in the Northern area (Luzon). In this study, 105 horses from Cebu and Bohol, Philippines were tested using peripheral blood smear examination (PBSE), immunochromatographic test (ICT) strips, and PCR. Clinical history, presenting clinical signs and complete blood count were obtained. Results revealed that although all horses were negative using PBSE, 23 (21.9%) were positive (12 for *T. equi*, and 11 for *B. caballi*) using ICT. PCR revealed 26 and 2 horses positive for *T. equi* and *B. caballi*, respectively. All positive horses showed no clinical signs. Partial DNA sequences of representative amplicons were found 100% identical to GenBank registered *T. equi* and *B. caballi* sequences. Statistical analyses revealed that location was found associated with *T. equi* PCR positivity and *B. caballi* seropositivity. This study documents the first serological detection of *T. equi* and *B. caballi* in horses in the southern area of the Philippines, and their first molecular detection and characterization in the country.

1. Introduction

Equine theileriosis and babesiosis are caused by the apicomplexan *Theileria equi* and *Babesia caballi*, respectively. These parasites target the erythrocytes and are transmitted by ticks from the genera *Rhipicephalus*, *Dermacentor*, and *Hyalomma* (Friedhoff, 1988; Hirata et al., 2003; Xuan et al., 2001; Ikadai et al., 1999; Soulsby, 1982). Concurrent infections of both parasites are known to occur (Soulsby, 1982). The disease is characterized by anemia, icterus, jaundice, edema, gastro-enteritis, bronchopneumonia, and abortions, which may lead to eventual loss of animal condition (Friedhoff, 1988; Kuttler, 1988). Infected animals may remain parasite carriers for long periods and act as sources of infection for other ticks (OIE, 2013). Recovery is possible, but recovered animals may become life-long carriers (Del Valle et al., 2012).

Several methods to detect *T. equi* and *B. caballi* include peripheral blood smear examination (PBSE), enzyme-linked immunosorbent assay (ELISA), polymerase chain reaction (PCR), complement fixation test (CFT), indirect fluorescent antibody test (IFAT) and immunochromatographic test (ICT). Evaluation of the peripheral blood smear is the cheapest method, but is unreliable especially if the parasitemia is very low (Ybañez et al., 2013a,b) or if the worker's level of competence or familiarity with the morphological features of different species is low (Homer et al., 2000; Levine, 1971). ELISA is more sensitive and specific than CFT and IFAT but is time consuming and labor intensive (Weiland and Reiter, 1988; Verdida et al., 2005; James, 1988; Hirata et al., 2000) while ICT has been used in the diagnosis of acute and latent babesiosis in horses (Verdida et al., 2005; Mohebali et al., 2004; Huang et al., 2003; Huang et al., 2004; Huang et al., 2006). From the aforementioned methods, PCR is considered the most reliable in detecting the pathogens.

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Reports of equine theileriosis and babesiosis in the Philippines have been limited to the Northern area (Luzon) (Cruz-Flores et al., 2010). These were reported using ICT and peripheral blood smear. To the author's knowledge, there have been no reports yet regarding the occurrence of these diseases among horses in the southern area of the country, including Cebu and Bohol. This study utilized peripheral blood smear examination (PBSE), immunochromatographic tests (ICT) and PCR in assessing the presence of the parasites.

2. Materials and methods

2.1. Horse blood samples and DNA extraction

A total of 105 horses (47 females and 58 males; 3 months-25 years old) from 10 different locations in Cebu and Bohol, Philippines were sampled. Blood was aseptically collected from the jugular vein using a sterile 5 ml syringe and was divided into three aliquots: 2 ml in EDTA tube for complete blood count (CBC) analysis and peripheral blood smear examination, 2 ml in EDTA tube for DNA extraction, and 1 ml in plain tube for serum extraction for ICT. Samples were transported in a cooler packed with ice until further processing. Aliquots for DNA extraction were stored at −20 °C until the procedure. DNA extraction was performed as previously described (Ybañez et al., 2012). Extracted DNA was stored at −20 °C until further use.

2.2. PBSE

Using the Wright's stain, triplicates of thin wedge blood smears were prepared from each sample on clean glass slides and labeled accordingly. Smears were examined for the presence of *Theileria* or *Babesia* parasite under oil immersion.

2.3. ICT

Separate strips containing recombinant truncated *T. equi* merozoite antigen 2 (rEMA-2t) and recombinant *B. caballi* 48-kDa rhoptry protein (rBc48) were used for the detection of anti-horse *Theileria* and *Babesia* spp. antibodies, respectively (Huang et al., 2006). ICT strips were stored in 4–8 °C, while the negative and positive serum samples were stored in −20 °C until further use. The result was considered positive if either purple, red, or pink coloration in the control and test line is seen, and negative if coloration is observed only in the control line. The absence of color change or reactivity in both the test and control lines was considered invalid (Cruz-Flores et al., 2010).

2.4. PCR

For *T. equi* PCR based on the *T. equi* merozoite antigen 1 (EMA-1) gene (with a final target of 430 bp), the outer primer pair EMA-5 (5′-TCGACTTCCAGTTGGAGTCC-3′)/EMA-6 (5′-AGCTCGACCCACTTATCACC-3′) and inner primer pair EMA-7 (5′- ATTGACCACGTCACCATCGA-3′)/EMA-8 (5′-GTCCTTCTTGAGAACGAGGT-3′) were used (Battsetseg et al., 2002). For *B. caballi* PCR based on the rhoptry-associated protein 1 (RAP-1) gene (with a final target of 218 bp), outer primers BC48F1 (5′-ACGAATTCCCACAACAGCCGTGTT-3′)/BC48R3 (5′-ACGAATTCGTAAAGCGTGGCCATG-3′) and inner primers BC48F11 (5′-GGGCGACGTGACTAAGACCTTATT- 3′)/BC48R31 (5′-GTTCTCAATGTCAGTAGCATCCGC-3′) were used (Ikadai et al., 1999). Amplification conditions were performed as described previously (Battsetseg et al., 2002). Final volumes were modified to 10 μL and 25 μL for the first and second round PCRs, respectively (Ybañez et al., 2013a,b). The negative control used was double distilled water. DNA of the same species was used as positive control for each specific PCR. Amplicons were viewed under UV light illumination using 1.5% agarose gel after electrophoresis.

2.5. Sequencing, sequence comparison and phylogenetic analysis

PCR amplicons (two for each parasite) were randomly selected and purified using QIAquick® Gel Extraction Kit (Qiagen, Germany). DNA sequencing of the purified amplicons was performed as described previously (Ybañez et al., 2013a,b). Briefly, direct sequencing utilized the inner primers used in the PCR assays. DNA sequences were determined using an ABI PRISM 3100 genetic analyzer (Applied Biosystems, USA). Sequences were manually trimmed to include only the sequence of interest (including the primer sequence). Percent identities were computed using an EMBOSS pairwise alignment hosted by the European Bio-informatics Institute (http://www.ebi.ac.uk/Tools/emboss/align/index.html). Aligned DNA sequences were compared to the existing database using the Basic Local Alignment Sequence Test (BLAST) hosted by the National Center for Biotechnology Information, USA. Phylogenetic analysis by neighbor-joining using maximum composite likelihood was employed using MEGA 5 (Tamura et al., 2011).

2.6. Data processing and statistical analysis

Information on the profile, CBC and PCR results were processed and encoded in Microsoft Excel. The file was then imported to IBM SPSS version 23. Data was analyzed using Mann-whitney, Chi square and likelihood ratio tests. The level of significance was set at 5%.

2.7. Ethical considerations

The procedures performed in this study were guided by the principles of animal welfare, Animal Welfare Act (RA 8485) and Administrative Order 45 of the Bureau of Animal Industry of the Philippines. The study was also reviewed by the Institutional Animal Care and Use Committee of the University of the Visayas, Philippines.

3. Results and discussion

PBSE results were negative, but ICT revealed that 23 (21.9%) were positive (12 for *T. equi*, and 11 for *B. caballi*) using ICT (Table 1). A negative PBSE but positive ICT result suggests an infection that is not in the acute stage, where parasites are detectable in the blood smear. Animals that survive the acute infection become carriers, which can be diagnosed serologically (Young, 1988; Huang et al., 2003; Hirata et al., 2000). Positive animals in this study had no apparent clinical signs, suggesting the carrier or "post-carrier" state. A similar observation was reported by Rampersad et al. (2003) where apparently healthy animals were found negative with PBSE. There were no mixed infections observed in this study. However, using PBSE alone to aid diagnosis of suspected animals may not sufficient as this technqiue has low sensitivity (Ybañez et al., 2017).

PCR results showed 23 and 2 horses positive for *T. equi* and *B. caballi*, respectively (Table 1). For *T. equi*, more horses were detected using PCR than ICT. It is possible that the proteins currently used in the ICT is not highly reactive to Philippine strains, which prompts for further studies to characterize the pathogen found in the country. The

Table 1
T. equi and *B. caballi* ICT and PCR results of horses from Cebu and Bohol, Philippines (n = 105).

Location	*T. equi*				*B. caballi*			
	ICT		PCR		ICT		PCR	
	Positive	%	Positive	%	Positive	%	Positive	%
Cebu	9	8.6	17	16.2	8	7.6	1	1.0
Bohol	3	2.9	6	5.7	3	2.9	1	1.0
Total	12	11.5	23	21.9	11	10.5	2	2.0

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