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Detection of *Theileria equi* and *Babesia caballi* infections in Venezuelan horses using Competitive-Inhibition ELISA and PCR

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ABSTRACT

The focus of this study was the detection of equine piroplasmosis in Distrito Capital, Miranda, Aragua, Guárico and Apure States from Venezuela, using two methods: Competitive-Inhibition ELISA and multiplex PCR and the analysis of the possible differences in occurrence in relation to the primary purpose of the horses, which is related to varied degrees of exposure to tick. Antibody levels to *Babesia caballi* and *Theileria equi* were assessed in 694 equine serum samples using Competitive-Inhibition ELISA, while PCR assays were performed in 136 horses, using two sets of oligonucleotides to establish the presence of *T. equi*, *B. caballi* or both. The overall seroprevalence of equine piroplasmosis was 50.2%, antibodies to *B. caballi* were found in 161 horses (23.2%), whereas 97 (14.0%) were seropositive to *T. equi* and 90 (13.0%) were positives to both parasites (mixed infections). PCR determinations ($n = 136$) showed a prevalence of 66.2%, distributed in 84 (61.8% positives) for *T. equi* and, 6 (4.4%) were positive to both parasites. The cELISA showed higher levels of prevalence of *B. caballi* and mixed infections, as compared to the PCR method. This discrepancy can be explained by the different parameters that are evaluated by each technique, PCR detect the parasite itself, while cELISA detects antibodies to the parasite. By PCR, the highest prevalence was found in Apure state, where 92.3% of the samples were positive to *T. equi* infections. In this locality, free grazing animals are used for livestock management. This high prevalence may be linked to the tick species present in that area. More epidemiological studies will be necessary to assess the epidemiological status of equine piroplasmosis in Venezuela.

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1. Introduction

Equine piroplasmosis (EP) caused by *Theileria equi* and *Babesia caballi* is a tick-borne hemoprotozoan disease affecting horses, mules, donkeys, and zebras distributed in most tropical and subtropical areas of the world, as well as in some temperate zones (Schein, 1988; Kappmeyer et al., 2012). The symptoms of clinical disease are fever, anemia, icterus and in some cases, death. *T. equi*, which is diagnosed more frequently than *B. caballi*, causes a persistent infection for which effective drug therapy or vaccination are not available. Infected animals may remain carriers of these blood parasites for long periods and will act as sources of infection for tick vectors. Control of equidae piroplasmosis

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is principally directed at continuous monitoring of the vector and tick control (Seo et al., 2011). Chemotherapy, using certain aromatic diamidines, is used in conjunction with tick control and also facilitates the international relocation of horses and others equidae (Rashid et al., 2008).

In recent years, breeding of thoroughbred race horses and other equidae have become important in international trade. Such trade has focused attention on the global spread of certain infectious and communicable animal diseases including equine piroplasmosis (EP). It is not possible to differentiate between *T. equi* and *B. caballi* infections based on clinical signs. In these animals, chronic cases usually present clinical signs such as mild inappetence, poor performance and a drop in body mass, the disease can easily be confused with other conditions.

EP can be diagnosed by microscopic examination, which enables the detection of parasites from stained blood films. However, this method is not very sensitive and does not permit diagnosis of mixed infections or in cases of low parasitemia. Serological assays, such as indirect inmunofluorescent antibody test (IFAT), complement fixation test (CFT), immunochromatographic test (ICT) and enzyme-linked immunoabsorbent assay (ELISA) are routinely used for the detection of EP (Kim et al., 2008). However, there are limitations with serological assays due antibody detection limits and cross-reactivity (Knowles et al., 1991; Bruning et al., 1997; Kappmeyer et al., 1999).

The polymerase chain reaction (PCR) method has been applied for the detection of EP (Conrad et al., 1992; Fahrimal et al., 1992; Figueroa et al., 1993; Calder et al., 1996; Bashiruddin et al., 1999). The sensitivity of the PCR method for detecting parasites for equine piroplasmosis has been shown to be higher than that of microscopic detection methods (Alhassan et al., 2005; Bashiruddin et al., 1999; Nicolaiewsky et al., 2001; Rampersad et al., 2003). Diagnosis by PCR has been found sensitive enough to detect parasite DNA from 2.5 μl blood sample with a parasitemia of 0.000001% (Alhassan et al., 2007; Xuan et al., 2001). In our study, a multiplex PCR was carried out, using two sets of primers, the first pair to determine both parasites in one reaction and the second, to define the species of the equine piroplasm, as described by Alhassan et al. (2005).

A high seroprevalence of *T. equi* and *B. caballi* has been reported in some countries of South America, as in Brazil, with prevalence from 75 to 91% (Heim et al., 2007; Heuchert et al., 1999; Xuan et al., 2001), and Trinidad with 82.8% of prevalence (Asgarali et al., 2007).

Previous, studies on EP prevalence in Venezuela have been performed by Gómez et al. (1972), Oliveira and García (2001), Vera et al. (2006), and Castellanos et al. (2010) using direct diagnostic or other diagnostic tests such as blood smears, FC and IFAT to assess the incidence in race or farm horses. Recently, (Mujica et al. (2011) analyzed EP seroprevalence using the cELISA commercial kit with 360 samples from Lara State (Venezuela) and correlated it with parameters such as sex and age of animals. To date, the information on the epidemiology of equine piroplasmosis in Venezuela is poorly studied and new methods currently used have not been employed.

The goal of this study was therefore to determine the presence of *T. equi* and *B. caballi* infections in the equine population, classified by the type of horse purpose, in different states from Venezuela, that represent different agro-ecological areas. Also, we used competitive-Inhibition ELISA and PCR methods and, compared the results obtained by the two methodologies.

2. Materials and methods

2.1. Study area and sample collection

Samples were collected from six different agro ecological areas in Venezuela zones. They were classified by different horse purposes that corresponded to varied degrees of exposure to ticks. Type A, Animals confined to paddocks with high care including thoroughbred horses; collected in Distrito Capital (1), Miranda State (2), Aragua State (3) and Carabobo State (4). Type B: horses partially confined and fed with grass and special food; collected in Guárico State (5) Type C: free grazing animals used to work with cattle, collected in Apure State (6) (Fig. 1). A total of 694 horses were sampled and distributed as follows: Type A, 523; Type B, 118 and Type C, 53. The horse in groups A and B had very low and low degree of exposure to ticks, respectively and tick-control was implemented. The types C horses were fully exposed to the thick vectors and no control measures were in place.

Whole blood was collected from horses by jugular venipuncture using Vacutainer tubes®. Tubes with EDTA were employed for DNA extraction and tubes without anticoagulant were used to separate sample sera, which were then stored at −20 °C until use.

2.2. ELISA

Sera were tested for the presence of antibodies to *T. equi* and *B. caballi* using the commercially available cELISA kits as described by the manufacturer (VMRD Inc., Pullman, WA, USA). The cELISA kit developed for the detection of antibody to *T. equi*, employs a monoclonal antibody (MAb 36/133.97) to recombinant EMA-1, while the *B. caballi*-specific cELISA is based on a monoclonal antibody to recombinant RAP-1. The cut-off values for positives infections was 40% of inhibition for both tests, as indicated by the manufacturer (VMRD Inc., Pullman, WA, USA).

2.3. DNA extraction and PCR amplification

Total genomic DNA was extracted from 300 μl of blood samples using the Wizard® Genomic DNA Purification kit (Promega, USA). The purified DNA was used as a template for subsequent PCR amplifications. A multiplexed PCR method for the simultaneous detection and differentiation of *T. equi* and *B. caballi* was employed (Alhassan et al., 2005). The universal primers amplify a fragment of the 18S rRNA gene from both parasites, while primers Equi-R amplify *T. equi* and primers Cab-R amplify *B. caballi* specifically.

PCR assays were performed in 25 μl of a mixture (20 mM Tris–HCl [pH 8.4], 50 mM KCl, and 2 mM $MgCl_2$) containing 2 μl of the template DNA, 5 pmol of each of the two set of primers, 0.2 mM dNTP mixture, and 1 U of Platinum®