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Nested PCR Detection of *Theileria equi* Infection and Frequency in Horses Imported into Mexico

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Abstract: The purpose of this study was to assess the frequency of *Theileria equi* in horses imported into Mexico. During different stages of quarantine, 348 blood samples were taken from clinically healthy horses imported into Mexico from 2011 to 2013. Nested PCR (nPCR) of the Merozoite Antigen-1 (*EMA-1*) gene was performed for pathogen detection. In total, 93 horses tested positive for *T. equi* resulting in a 26.72% frequency with a 95% Confidence Interval (CI) of 22.07-31.37%. This is the first molecular diagnostics study to identify *T. equi*-positive horses imported into Mexico these results highlight the importance of nPCR analysis for *T. equi* in clinically healthy imported horses.

Key words: *Theileria equi*, horse, nPCR, Mexico, blood samples

INTRODUCTION

Theileria equi (*T. equi*) is a hemoparasitic protozoan responsible for equine piroplasmosis (Mehlhorn and Schein, 1998). This pathogen causes a disease in equids (horses, donkeys, mules and zebras) that is characterised by fever, anaemia, jaundice, haemoglobinuria and occasionally death (Friedhoff *et al*., 1990; Bhoora *et al*., 2010). Animals that survive the acute infection are carriers for life and thus become parasite reservoirs for ticks, the usual vectors of the disease (Phipps, 1996; Hailat *et al*., 1997). Placental transmission from pregnant mares to their foetuses has been documented. This transmission can result in an abortion, a stillbirth or in the birth of weak and anaemic foal (Phipps and Otter, 2004; Allsopp *et al*., 2007; Georges *et al*., 2011; Chhabra *et al*., 2012).

Equine Piroplasmosis (EP) has a worldwide distribution and is usually endemic in most tropical and sub-tropical regions, although regions with more temperate weather can also be affected (Dewaal, 1992; Bruning, 1996).

The international horse trade is limited by equine piroplasmosis, thus, it has an economic impact (Friedhoff *et al*., 1990). Horse importation statutes require several serological analyses such as Complement Fixation Test (CFT) testing, Indirect Fluorescent Antibody Test (IFAT) and Enzyme-Linked Immunosorbent Assay (ELISA) (Wise *et al*., 2013). In some disease-free countries (USA, Canada, Japan, Australia and New Zealand) the goal of serological testing is to identify seropositive animals whose migration is restricted (Bose *et al*., 1995; Wise *et al*., 2013).

EP is a disease for which it is obligatory to report (OIE, 2013), however not all countries report their cases of identified animals to the OIE which makes it difficult to determine the precise current distribution of the infection (Wise *et al*., 2013).

There have been a few published studies regarding the prevalence of equine piroplasmosis in Mexico (Cantu-Martinez *et al*., 2012). Routine examinations used by health authorities in Mexico are mainly based on CFT. Different studies have shown that serological techniques such as CFT lack sensitivity in the diagnosis of equine

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piroplasmosis, therefore this test is insufficient as a diagnostic tool for imported horses (Holman *et al*., 1997; Butler *et al*., 2008; Ogunremi *et al*., 2008). Moreover, other studies have shown that PCR is a highly sensitive and specific test for the detection of *T. equi*, even in chronically infected horses (Bashiruddin *et al*., 1999; Nicolaiewsky *et al*., 2001; Farah *et al*., 2002; Rampersad *et al*., 2003; Ribeiro *et al*., 2013). The purpose of this study was to use nested PCR to determine the frequency of *T. equi* in horses imported into Mexico.

MATERIALS AND METHODS

Animals and samples: Between January 2011 and August 2013, 348 blood samples were collected from clinically healthy horses imported into Mexico at different stages of official quarantine. The clinical history was obtained for each animal and those animals showing any clinical symptomatology (e.g., fever, pale mucous membranes) were excluded from the study. In the first week of arrival the horses, blood samples for molecular analysis were then obtained by puncturing the jugular vein with a vacutainer needle. Samples were collected in vacuum-sealed sterile tubes containing 1.5 mg mL^{-1} EDTA and stored at 5°C for <24 h.

DNA extraction: Samples were centrifuged at 2500 rpm for 10 min to separate the plasma from the cellular fraction and 500 μL of erythrocytes was taken and mixed with 900 μL of Buffer A (0.32 M sucrose, 10 mM Tris-HCl, 5 mM $MgCl_2$, 1% Triton X-100). Samples were then centrifuged at 13,000 rpm for 2 min and the supernatant was decanted. This process was repeated until only a white cellular pellet remained. Afterward, samples were incubated for 1 h at 50°C with Proteinase K (8 mg mL^{-1}) in Buffer D (50 mM KCl, 10 mM Tris-HCl, 2.5 mM $MgCl_2$, 0.45% NP-4O, 0.45% Tween-20). The Proteinase K was heat inactivated at 90°C for 10 min (Ayala-Valdovinos *et al*., 2007).

Primary and nested primers were designed based on the Merozoite Antigen-1 (EMA-1) genetic sequence of *Theileria equi* (GenBank Accession No. L13784) (Battsetseg *et al*., 2001).

Gradient PCR: A gradient PCR was performed using the primers for EMA-1 (GenBank Accession Number L13784.1) as previously reported (Battsetseg *et al*., 2001). The primer sequences for the primary PCR were EMA5 5'-TCGACTTCCAGTTGGAGTCC-3' for the forward primer and EMA6 R 5'-AGCTCGACCCACTTATCAC-3' for the reverse primer. For the nested PCR (nPCR), the primers used were EMA7 5'-ATTGACCACGTCACCATCGA-3' for the forward primer and EMA8 R 5'-GTCCTTCTTGAGA ACGAGGT-3' for the reverse primer. The gradient PCR was performed following a standard protocol: 3 μL of *T. equi* DNA as a positive control, 2 μL of 10X PCR buffer (Thermo Scientific), 20 mM $MgCl_2$, 1 μL of 10 mM dNTP mix (Thermo Scientific), 1.0 U of DreamTaq DNA polymerase (Thermo Scientific), 20 pmol of each primer and ddH_2O to a 20 μL final volume. The thermocycler (TC-5000, Techne®) program was as follows: 40 denaturation cycles consisting of 94°C for 1 min, 45 sec at the Tm determined by a PCR gradient experiment and 72°C for 1 min.

The resulting amplicons were analysed by TBE agarose gel electrophoresis and stained with ethidium bromide or GelRed®. DNA bands were detected and photo documented in a Gel Logic 200 (Kodak®).

Theileria equi detection by nPCR: The Tm for the primary and nested PCR (nPCR) was selected based on the previous gradient PCR experiment results. The following PCR experiments were performed in a TC-3000 (Techne®) following the same reaction parameters as described above. The thermocycler program was as follows: The 95°C for 5 min followed by 40 cycles of 94°C for 30 sec, 62.7°C for 30 sec and 72°C for 1 min and a final extension of 72°C for 5 min. *T. equi*-positive samples amplified a DNA fragment of 259 bp during the primary PCR. For the nPCR, 3 μL of the primary PCR product was used under the same reaction parameters, except the Tm was set to 57.5°C. *T. equi*-positive samples showed an amplicon of 218 bp.

RESULTS AND DISCUSSION

The optimal alignment temperature for the EMA-1 primers used to detect *T. equi* in this study was determined using a temperature gradient (Techne® thermocycler). Figure 1 shows the amplicons obtained from *T. equi* DNA template in a 2% agarose gel that were used to determine the optimal alignment temperature of the primary PCR primers (forward EMA5 and reverse EMA6). Based on this experiment, it was determined that 62.7°C would be the Tm chosen for these primers.

Likewise, Fig. 2 shows the electrophoresis analysis of the nPCR products amplified from *T. equi* DNA in a 3% agarose gel using the primers forward EMA7 and reverse EMA8. Based on these data, 57.5°C was chosen as the Tm for this primer set.

Both primary and nested PCR enabled the amplification of 259 and 218 bp DNA fragments, respectively, from *T. equi* EMA-1. The presence and size

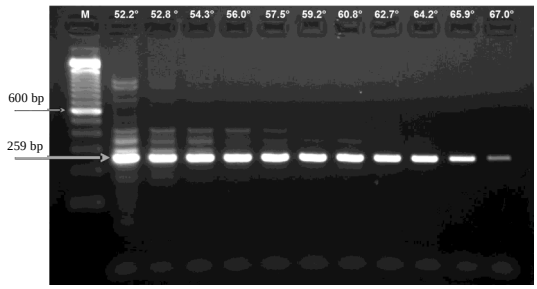

Fig. 1: Primer Tm (EMA5 and EMA6) optimisation by gradient PCR using *T. equi* DNA as the template. M: Molecular weight marker (100 bp, Life Technologies); wells 2-12: DNA amplicons produced at different temperatures along the gradient (°C)

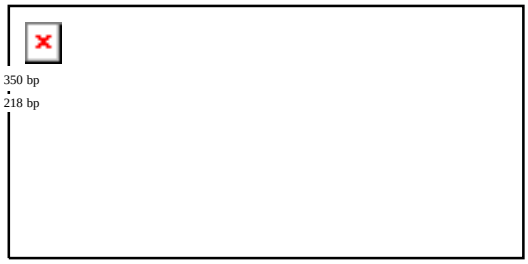

Fig. 2: Primer Tm (EMA7 and EMA8) optimisation by gradient PCR using *T. equi* DNA as the template. M: Molecular weight marker (50 bp, Life Technologies); wells 2-12: temperature gradient (°C)

of the obtained amplicons was determined in a 3% agarose gel stained with ethidium bromide, thus allowing *T. equi* diagnosis (presence or absence) in the blood samples obtained from 348 horses imported into Mexico (Fig. 3a and b).

Due to the sensitivity of PCR and the goal of avoiding any false-negative outcomes due to DNA contamination, every test from this study also included negative controls. The negative controls all came out negative, indicating that the nPCR-positive samples were not cross-contaminated.

Nested PCR using the primers to detect EMA-1 for the diagnosis of equine piroplasmosis caused by *T. equi* allowed the detection of this pathogen's DNA in 93 horses (26.72%) from 348 blood samples from horses imported into Mexico (Table 1). The calculated frequency of 26.72±4.65% allowed us to estimate a CI ($p<0.05$) with lower and upper limits of 22.07 and 31.37%, respectively for the frequency of *T. equi* in horses tested.

It has been shown that nPCR is more efficient in detecting *T. equi* in clinically healthy horses compared

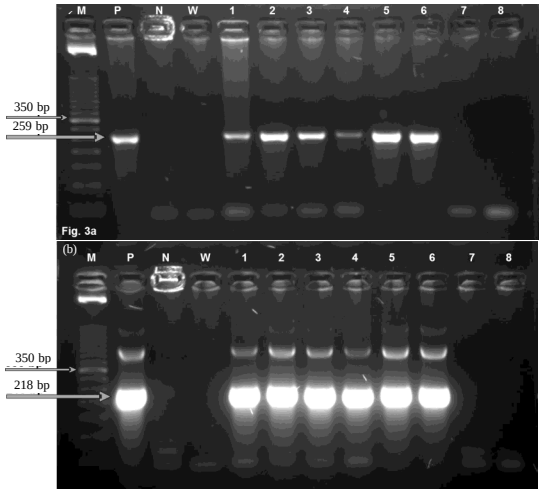

Fig. 3: a, b) Agarose gel electrophoresis of PCR and nPCR amplicons. M: Molecular weight marker (50 bp, Life Technologies); P: *T. equi* Positive control; N: *T. equi* Negative control; W: PCR control; wells 1-6: *T. equi*-positive horse samples; 7 and 8: *T. equi*-negative horse samples

Table 1: Theileria equi frequency in horses imported into Mexico as determined by nPCR analysis

	Negative		Positive		
Pathogen	n	%	n	%	Total animals
Theileria equi	255	73.28	93	26.72	348

Frequency: 26.72±4.65% (95% CI: 22.07-31.37%)

with primary PCR (Rampersad *et al*., 2003). Because the main objective of this study was to use nPCR to assess the frequency of *T. equi* in horses imported into Mexico, every primary PCR amplicon was used as a template in the nPCR analysis where in all of the nPCR amplicons were analyzed by electrophoresis.

With respect to horse importation, Complement Fixation Test (CFT) Methods have been used as one of the official tests for the detection of antibodies against the hemoparasites *Babesia caballi* and *Theileria equi* (Suzuki *et al*., 1996; Friedhoff and Soule, 1996). However, because CFT can also provide false-negative results the U.S. Department of Agriculture (USDA) replaced this assay with a competitive ELISA procedure (cELISA) in 2005 to increase the sensitivity of piroplasmosis infection detection, especially in horses within chronic infections (Hall *et al*., 2013).

Routine examinations employed by Mexican sanitary authorities are based on CFT. However, CFT-negative samples have been shown to be positive by PCR, reflecting the ability of this method to detect hemoparasites in chronically infected imported horses (Butler *et al*., 2008).

The considerable number of *T. equi* carriers (93/348 = 26.72%) identified by nPCR in the analysed horses imported into Mexico was surprising. There is likely a high rate of CFT false-negative results when tests are performed by Mexican sanitary authorities, possibly in part because some of these horses are pharmacologically treated before being imported into Mexico. Therefore, a more sensitive test is recommended in horses originating from countries where piroplasmosis is endemic.

Likewise, the interval between the initial infection by *T. equi* and when the first symptoms become apparent is 12-14 days (Mehlhorn and Schein, 1998) therefore, the sampling stage is of utmost importance to detect parasites in the circulation. It is possible that some of the samples used in this study were false negatives as determined by the official serological tests used in Mexico (CFT and IFAT) that were performed during quarantine, thereby allowing the entrance of these horses as negative carriers. It was not possible to obtain the results of the official tests to provide a comparison with the results obtained from the nPCR analysis but perhaps some of these same samples belong to animals that tested positive by nPCR because it has been shown that nPCR can detect the presence of parasites in recently infected animals before any antibodies can be detected by serological tests (Jaffer *et al*., 2010).

Different reports are available concerning the prevalence of *T. equi* in the horse populations of several EP endemic countries. For instance on the American continent, the following seroprevalence values have been reported: Brazil, 91.00% (Heim *et al*., 2007); Venezuela, 85.83% (Mujica *et al*., 2011); Trinidad, 52.70% (Asgarali *et al*., 2007) and Mexico, 45.20% (Cantu-Martinez *et al*., 2012). Most reported studies concerning the prevalence of *T. equi* have been performed in geographically different horse populations within the same country. However, it is equally important to determine the hemoparasite's frequency in each country's imported horses. Sigg *et al*. (2010) reported that 35 of 459 horses (7.62%) imported into Switzerland, tested positive for *T. equi* when analysed by IFAT.

CONCLUSION

This is the first molecular diagnostic study of the detection of *T. equi* in horses imported into Mexico these results highlight the importance of nPCR analysis in the diagnosis of equine piroplasmosis in clinically healthy horses. Based on this study, the importance of EP diagnosis by nPCR is evident. Tight regulation of the health of horses imported into Mexico that will eventually be exported to other countries diminishes the infection and propagation rate of *T. equi* into non-endemic countries such as the USA through vector species such as ticks.

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