Evaluation of PCR and multiplex PCR in relation to nested PCR for diagnosing *Theileria equi*¹

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Conventional PCR (PCR-Teq) for diagnosing *Theileria equi* and multiplex PCR (M/PCR-Teq-Bc) for diagnosing *T. equi* and *Babesia caballi* were comparatively evaluated with nested PCR (N/PCR-Teq) for diagnosing equine piroplasmosis. In DNA sensitivity determinations, in multiple dilutions of equine blood that had tested positive for *T. equi*, PCR-Teq and N/PCR-Teq detected hemoparasite DNA in the larger dilutions (1:128), but did not differ significantly from the M/PCR-Teq-Bc (1:64). In analyses on equine serum tested by ELISA, there was high agreement between this serological test and PCR-Teq (k = 0.780) and moderate agreement with N/PCR-Teq (k = 0.562) and M/PCR-Teq-Bc (k = 0.488). PCR-Teq found a higher frequency of *T. equi* both in extensively and intensively reared horses, but this was not significant in relation to N/PCR-Teq (P>0.05), and both PCRs indicated that there was an endemic situation regarding *T. equi* in the population of horses of this sample. PCR-Teq was only significantly different from M/PCR-Teq-Bc (P<0.05). PCR-Teq presented high sensitivity and specificity, comparable to N/PCR-Teq, but with the advantage of higher speed in obtaining results and lower costs and risks of laboratory contamination. This accredits PCR-Teq for epidemiological studies and for determinations on affected horses.

INDEX TERMS: *Theileria equi*, PCR, multiplex PCR, horses.

INTRODUCTION

Equine piroplasmosis is caused by the protozoa *Babesia caballi* and *Theileria equi*. The geographical distribution of these protozoa is directly related to the presence of vector ticks, among which the genera *Anocentor*, *Hyalomma* and *Rhipicephalus* (especially the subgenus *Boophilus*) stand out. These are found in tropical and subtropical zones (Uielsen 2006). Equine piroplasmosis causes losses through morbidity, mortality, treatment expenditure and restriction of international trade, because of the risk of introducing...
followed by 40 cycles of 94°C for 1 minute, with a final extension of 72°C for 1 minute and 5 seconds. The nested PCR for detection of *T. equi* (N/PCR-Teq) was performed in accordance with the technique described by Nikolaiewsky et al. (2001).

The products from the three PCRs were stained with Sybr Gold (Invitrogen) solution and subjected to a horizontal electrophoresis run on 1.5% agarose gel in Tris-borate EDTA (TBE) buffer, together with the 100 bp molecular weight marker (Invitrogen). The results were viewed in a transilluminator and were documented by photographing them (Biometra).

The *T. equi* and *B. caballi* amplicons were purified from the agarose gel using a commercial kit (PureLink™ Quick Gel Extraction Kit; Invitrogen), and were cloned and sequenced in a capillary automated sequencer (ABI 3100; Applied Biosystems). The clone sequences of the gene 18SrRNA of these microorganisms that are filed in GenBank, using the BLASTX algorithm, from the NCBI website (http://www.ncbi.nlm.nih.gov). The clone sequences of *T. equi* were also compared with each of these separately, using the MEGALIGN software (Lasergene/DNASTAR Inc.).

**Sensitivity and specificity analysis**

The specificities and sensitivities of PCR-Teq, M/PCR-Teq-Bc and N/PCR-Teq were analyzed using DNA extracted from red blood cell concentrates obtained from 49 horses that had been reared in stables in the municipality of Bagé, Rio Grande do Sul. These horses were serologically either negative or positive in the ELISA test with recombinant antigen EMA-1 that had been developed in the Federal University of Pelotas by Dr. Sergio da Silva (personal information). The relationship between the results from the ELISA test and from the PCRs was determined using the kappa test.

Additionally, to evaluate the sensitivity of the three PCRs, the equine DNA that had been proven positive for *T. equi*, with a concentration of 0.328 μg/ml that had been determined using fluorometry (Qubit® - Invitrogen), was multiply diluted from 1:2 to 1:512.

**Epidemiological study**

Blood samples from 118 horses in the state of Bahia were used to evaluate the performance of the three molecular tests in epidemiological studies. Among these 118 horses, 62 were reared in an intensive system, with the transmitter tick under control, came from breeding stables owned by the military police. The remaining 56 horses came from commercial farms, without tick control (extensive rearing system). In this evaluation, the PCR results were analyzed using the Kruskal-Wallis test. For classification purposes, ticks were gathered from all the 15 horses that presented infestation at the time of drawing blood.

**RESULTS AND DISCUSSION**

The PCR-Teq and M/PCR-Teq-Bc reactions produced amplicons of 283 bp (Fig.1), and sequencing of the products from these reactions found 100% identity with the corresponding sequences of *Theileria equi* (AY50063.2; EU 888902.1; EU888906.1) that exist in GenBank.

The relative sensitivity results from PCR-Teq, M/PCR-Teq-Bc and N/PCR-Teq, with multiple DNA dilutions on horses that were positive for *T. equi*, are shown in Table 1. PCR-Teq and N/PCR-Teq presented identical sensitivity that was greater (1:128) than for M/PCR-Teq-Bc (1:64), thus demonstrating that PCR-Teq has high efficiency in relation to other conventional
Evaluation of PCR and multiplex PCR in relation to nested PCR for diagnosing Theileria equi

PCR protocols described in the literature. These other PCR protocols present sensitivity that is about half that of nested PCR, as seen in diagnosing other hemoparasites (Aboulaila et al. 2009) and T. equi (Rampersad et al. 2003). From the results presented by Baldani et al. (2008), it is evident that nested PCR has higher sensitivity: in their study, nested PCR identified 100% of the horses affected by T. equi, while conventional PCR on the same samples only presented negative results. The greater capacity of nested PCR for identifying naturally infected horses is due to the fact that this technique detects smaller-sized parasitemia (6x10^-6 % to 8x10^-6 %) than does conventional PCR (only down to 8x10^-5 %) (Bashiruddin et al. 1999, Nicolaikvsky et al. 2001, Baldani et al. 2008). So far, only real-time PCR has been described as having sensitivity greater than or equal to nested PCR (Kim et al. 2007).

From analysis of the PCRTeq, M/PCRTeq-Bc and N/PCRTeq results in relation to the findings from the ELISA test, it was found that there was substantial agreement between ELISA and PCRTeq, while N/PCRTeq and M/PCR-Teq presented moderate agreement with ELISA (Table 2). This suggests that PCRTeq has a high capacity to detect affected individuals. Because of the high sensitivity, the agreement between this type of PCR and ELISA could even be bigger, considering that there may be false positive results, due to persistency of antibodies in horses that are no longer affected. In a study in Mongolia, Ruegg et al. (2007) examined 510 horses using indirect immunofluorescence (IFI) techniques and PCR for T. equi, and obtained positive results in 78.8% and 66.5% respectively. Jaffer et al. (2009) also found that larger number of horses reacted to the serological test (36.2%) than to PCR (31.4%).

The three PCRs presented possible false positive reactions in relation to the ELISA results, and these occurrences did not differ significantly between the three tests (Table 2). Considering that the seronegative results can be attributed to failure of the test to detect recent infection when the level of antibodies is still low (Nantes & Zappa 2008), it is possible that the results obtained from the PCRs are actually positive. This would be a factor favoring PCR for diagnosing T. equi infection early on.

The specificity of the PCRs for T. equi used in this study was evaluated using two equine samples that were parasitologically positive for B. caballi, and both of them presented a negative result (data not shown).

PCRTeq was capable of identifying a larger number of horses reared in both extensive and intensive systems that tested positive for T. equi, although this difference was only significant in relation to M/PCR-Teq-Bc (Table 3). These results corroborate previous comparative evaluations of sensitivity and specificity, in which PCRTeq showed identical performance to N/PCRTeq in the statistical analysis.

With regard to husbandry type among the horses examined, all three PCRs detected a larger number of positive reactions in relation to the findings from the ELISA test, it was found that there was substantial agreement between ELISA and PCRTeq, while N/PCRTeq and M/PCR-Teq presented moderate agreement with ELISA (Table 2). This suggests that PCRTeq has a high capacity to detect affected individuals. Because of the high sensitivity, the agreement between this type of PCR and ELISA could even be bigger, considering that there may be false positive results, due to persistency of antibodies in horses that are no longer affected. In a study in Mongolia, Ruegg et al. (2007) examined 510 horses using indirect immunofluorescence (IFI) techniques and PCR for T. equi, and obtained positive results in 78.8% and 66.5% respectively. Jaffer et al. (2009) also found that larger number of horses reacted to the serological test (36.2%) than to PCR (31.4%).

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Table 1. Sensitivity of conventional PCR (PCRTeq), multiplex PCR (M/PCRTeq-Bc) and nested PCR (N/PCRTeq) for diagnosing Theileria equi, determined by means of multiple DNA dilutions on equine samples with proven infection by this hemoparasite.

<table>
<thead>
<tr>
<th>Types of PCR</th>
<th>DNA dilutions</th>
<th>PCRTeq</th>
<th>M/PCRTeq-Bc</th>
<th>N/PCRTeq</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1:4</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1:8</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1:16</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1:32</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1:64</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1:128</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1:256</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1:512</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 2. Number and percentage of positive and negative reactions in conventional PCR (PCRTeq), multiplex PCR (M/PCRTeq-Bc) and nested PCR (N/PCRTeq) for diagnosing Theileria equi in DNA extracted from serologically positive equine blood that had been tested by means of ELISA.

<table>
<thead>
<tr>
<th>ELISA</th>
<th>Positive</th>
<th>Negative</th>
<th>Positive</th>
<th>Negative</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>16 (84.2%)</td>
<td>3 (15.8%)</td>
<td>8 (42.1%)</td>
<td>11 (57.9%)</td>
<td>12 (62.3%)</td>
<td>7 (36.8%)</td>
</tr>
<tr>
<td></td>
<td>2 (6.7%)</td>
<td>28 (93.3%)</td>
<td>1 (3.3%)</td>
<td>29 (96.7%)</td>
<td>3 (10.0%)</td>
<td>27 (90.0%)</td>
</tr>
</tbody>
</table>

(a) k = 0.784; (b) k = 0.488; (c) k = 0.562.
animals in the extensive system than in the intensive system. Only the latter rearing system includes tick control (Table 3). A similar result was described previously by Botteon et al. (2002), in a comparative study using the IFI technique that investigated the frequency of infection by *T. equi* among 121 horses that were subjected to different rearing systems. In that study, the authors registered positive results in 89.58% of the animals in an extensive system; 87.89% in a semi-confined system and only 45.24% in a confined system. These results reflect the lowest rate of inoculation of this hemoparasite in animals reared in an intensive system, due to the lower infestation of vector ticks.

The tick sample from the infested horses that were evaluated in this study identified 14 specimens of the genus *Anocentor* and only one of *Amblyomma cajennense*. Predominance of the genus *Anocentor* and high frequency of *T. equi* were also reported in the state of Rio de Janeiro (Pereira et al. 2004, Costa-Pereira et al. 2005, 2007), although some authors believe that these ticks are not capable of transmitting *T. equi* (Stiller & Coan 1995). The main vector for this hemoparasite in Brazil is the *Rhipicephalus B. microplus* (Heuchert et al. 1999, Batteseg et al. 2002a), especially in cases of pasture sharing with cattle (Ribeiro et al. 1999, Labruna et al. 2001).

Comparisons between epidemiological situations of endemism present a certain degree of subjectivity, due to various factors that affect *T. equi* transmission, such as the animal management system; tick species and populations; and climatic conditions and seasonal variations. The results from the epidemiological evaluation, using PCRTest, M/PCRTest and M/PCRTest-Bc (Table 3), characterize an endemic situation comparable to what was observed in surveys on horses in Minas Gerais, Goiás, São Paulo and Mato Grosso do Sul, using PCR techniques (Heim et al. 2007, Kim et al. 2007). In other countries in which *T. equi* is endemic, frequencies similar to those observed in this study have been found, also using PCR (Rüegg et al. 2007, Jaffer et al. 2009). Based on the presented data, we conclude that PCRTest, which is similar to N/PCRTest, presents high sensitivity and specificity, which makes it appropriate for epidemiological studies and detection of affected individuals, and it also presents advantages in terms of speed of execution, lower cost and reduced risk of contamination.

**Table 3. Results from conventional PCR (PCRTest), multiplex PCR (M/PCRTest-Bc) and nested PCR (N/PCRTest) for diagnosing *Theileria equi* in horses in extensive and intensive rearing systems in the state of Bahia**

<table>
<thead>
<tr>
<th>Rearing system</th>
<th>PCRTest</th>
<th>M/PCRTest-Bc</th>
<th>N/PCRTest</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extensive</td>
<td><em>84.6%</em> (44/52)</td>
<td><em>67.3%</em> (35/52)</td>
<td>82.7% (43/52)</td>
</tr>
<tr>
<td>Intensive</td>
<td><em>51.6%</em> (32/62)</td>
<td><em>29.1%</em> (18/62)</td>
<td>43.6% (27/62)</td>
</tr>
</tbody>
</table>

* PCRTest - M/PCRTest-Bc (p<0.05); PCRTest - N/PCRTest (p<0.05); N/PCRTest - M/PCRTest (p<0.05).

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REFERENCES


