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Employment of the 18s rRNA screening PCR technique in the detection of Equine Piroplasmosis, in horses of sports and military operations, of the Brazilian Army

[*Emprego da técnica de PCR para triagem da região do genoma 18s rRNA, detecção de Piroplasmose em equinos de desporto e operações militares do Exército Brasileiro*]

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ABSTRACT

The present work had the objective of detecting the occurrence of Equine Piroplasmosis in horses housed in the 3rd Guards Cavalry Regiment (GCR) – Brazilian Army (BA) - Porto Alegre, RS-Brazil, as well as to demonstrate the proactivity of PCR (Polymerase Chain Reaction) technique, aiming at the judicious use of the resources involved in the training and employment of Equines in the Brazilian Army. Fifty horses of the 3rd GCR - Porto Alegre - RS, which are employed for Sport, Military Ceremonial, Law and Order Guarantee Operations (LOGO), were evaluated by means of the 18s r RNA screening with PCR technique, thirty eight horses with Babesia Caballi and Theileria Equi were detected, which corresponds to an incidence of 76% of the horses effective analyzed at the time. In this way, it can be verified that the Military activity have its "performance and effectiveness" factors threatened in case the health of the principal of his means employed, that is the horse, is compromised. The PCR technique then offers a reliable and feasible tool for the detection of Equine Piroplasmosis in BA horses.

Keywords: Horses, Babesia Caballi, Detection, PCR, LOGO, GCR, BA

RESUMO

O presente trabalho teve como objetivo detectar a ocorrência de Piroplasmose equina em cavalos alojados no 3º Regimento de Cavalaria de Guarda (RCG) – Exército Brasileiro (EB) – Porto Alegre, RS, Brasil, bem como demonstrar a forma proativa do método da PCR (reação em cadeia de polimerase), objetivando o uso criterioso dos recursos envolvidos no treinamento e emprego de equinos no Exército Brasileiro. Foram avaliados 50 cavalos da 3ª GCR-Porto Alegre, RS, empregados nas modalidades de: esporte, cerimonial militar e operações de garantia da lei e da ordem (GLO), por meio da triagem da região do genoma 18S rRNA mediante a aplicação do método da PCR. Foram positivas as amostras de 38 equinos para Babesia caballi e Theileria Equi, *o que corresponde a uma incidência de 76% dos cavalos efetivos analisados na época. Dessa forma, verifica-se que as atividades militares tem seus fatores de "desempenho e efetividade" ameaçados no caso da saúde do principal de seus meios empregados, o Cavalo, estar comprometida. A técnica de PCR, então, oferece uma ferramenta confiável e viável para a detecção de* Piroplasmose *em equinos do EB.*

Palavras-chave: Cavalos, Babesia Caballi, *Detecção, EB.*

INTRODUCTION

Equine Piroplasmosis is a tick-transmitted intra erythrocytic parasitic disease of horses, the small piroplasm of horses (Bruning, 1996), long known as *Babesia Equi*, is already commonly designated associated with fever, hemolytic anemia, and haemoglobinuria (De Tarso; Botteon, 2005), loss of athletic performance and can lead to death, caused by either *Babesia Caballi or Theileria Equi* (Fonseca, 2012).

B. Caballi and *T. Equi*, are presented in temperate as well as in tropical regions. Fourteen species of ixodid ticks of the genera

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Dermacentor, Hyalomma, and Rhipicephalus have been identified worldwide as vectors of either T. Equi or B. Caballi. (Garci *et al*., 2004).

Equine piroplasmosis diagnostics can be serological tests such as: an enzyme-linked immunosorbent assay (Santos *et al*., 2009), an indirect fluorescent antibody test (Mullan, 2016) immune chromatographic tests, or alternatively on identification of the agent by microscopic examination or molecular tools (Nagore *et al*., 2004). Many PCR methods have been described recently (Weiland *et al.*, 1986), including single round and multiplex PCR, to allow simultaneous identification of both *B. Caballi and T.equi* (Alhassan *et al*., 2005), PCR - reverse line blot hybridization (Butler et al, 2008), and real-time PCR(Bhoora,2010).

The Brazilian Army has about 1,876 horses distributed in almost all Brazilian territory (Brazil, 2017), in several military several Cavalry Regiment Units(1º, 2º, 3º); employees in LOGO, participation in Military Ceremonial such as Parades, Guard and Escorts; Patrolling in Military Organizations and in the Fields of Instruction; Military Training in official and squad Training Schools, Military Academy of Agulhas Negras (AMAN), Army Sergeants School (EsSA) , Army Riding School (EsEQEx), and the Rincão- São Borja Stud - RS (Campos, 2015).

Piroplasmosis causes numerous economic losses (Ferreira *et al*., 2016) with the loss of performance of horses (Oliveira, 1918), being a variable of failure, with a zoonosis which is a threat to the health of the human component used in military activities indeed may be transmitted to another animals (Fritz, 2010).
The sensitivity of the PCR assay is higher than that of a classical microscopic examination (Jaffer et al, 2010).

The aim of this study was the application of the18s r RNA screening with PCR method, in addition to its economic viability, sensitivity and selectivity, and in the future can be

recommended for use in the Military Units of the BA, for the diagnosis of Equine Piroplasmosis.

MATERIALS AND METHODS

This study has been approved by the Ethics Committee in Animal Experimentation and Animal Welfare at the Universidade Federal de São Carlos, (protocol number CEUA 8586190816/16), S ão Paulo State, Brazil.

Blood samples of 50 horses housed in the 3rd. GCR located in the city of Porto Alegre, RS, were evaluated among these equine sports, ceremonial, and employees in the LOGO operations, collected by veterinarians from the veterinary section of the regiment in the month of December 2016.

Blood samples were submitted to the extraction protocol from blood, using Brazol®-LGC protocol of extraction of genomic DNA.

The 100µL of blood was added to a 1.5ml tube already containing 200µL of Brazol (LCG Biotechnology, São Paulo, Brazil).

After the solution was homogenized for 2 minutes, 50µL of chloroform and the homogenized solution were added. The sample was then centrifuged at 13,000g for 15 minutes. After this step, the supernatant (aqueous phase) was transferred to a new labeled tube and 200µL of Isopropanol cooled at 4°C was added thereto. The solution was centrifuged at 13,000g for 20 minutes. The supernatant was then discarded and 800µL of absolute ethanol PA was added. The material was centrifuged at 13,000g for 10 minutes and the supernatant discarded. The precipitate was washed with 500µL of 70% ethanol and centrifuged at 13,000g for 10 minutes. The supernatant was discarded and after drying the precipitate was suspended in 30µL of ultra-pure autoclaved water or T.E. (10mM Tris pH 8.0, 1mM EDTA pH 8.0) and stored in a freezer at -20°C.

The nucleotide sequences of the primers used in this study to the PCR amplifications of 18s r RNA gen, (Table 1) were performed according to previously published procedures (Spolido*rio e*t al., 2011).

Table 1. Primers table used to detect *Babesia Caballi and Theileria Equi* in Horses of Brazilian Army

Babesia spp. *Theileria* spp.	BAB 143-167 BAB 694-667	CCGTGCTAATTGTAGGGCTAATACA GCTTGAAACACTCTARTTTTCTCAAAG	Size 551bp	Spolidorio *et al*., 2011

For the PCR amplification reactions, a Taq DNA Polymerase enzyme was used in reactions with a final volume of 15µL. For this volume, the reaction was prepared with 1U of enzyme; 1X buffer solution; 0.2mM of the phosphate deoxy ribonucleotide solution (dNTPs) and 10pmol of each primer. 1µL of the DNA extracted from each sample was used as template DNA; Reagents and their respective volumes used for each amplification reaction (Table 2), were performed according to previously published procedures (Spolidorio *et al*., 2011) and the thermal cycle (Table 3). An additional reaction was performed as a negative control in which the volume relative to the template DNA was replaced by 1µL of sterile water.

The amplification products (Figure 1.) were then subjected to electrophoresis in agarose gel 2% buffered solution containing 40mM Tris-acetate pH 8.0; 1mm EDTA (TAE buffer solution) for 30min at 110V. For viewing through the traviolet (UV) region, was used the DNA intercalating etílio bromide.

Table 2. Reagents and their respective volumes used for each amplification reaction of *Babesia Caballi and Theileria Equi*

Reagents	1 Reaction(µl)
Buffered (10X)	1.5
Mg Cl (50 mM)	0.45
DNTPs (1,25mM)	2.4
Primer F (10 p Mol)	1.0
Primer R (10 p Mol)	1.0
Enzyme Taq Polymerase	0.2
DNA Template	1.0
H_2O	7.45

Table 3. Thermal cycle

Temperatures (°C)	Time (min)
94	5
94	30 seg.-40x
56	30 seg.-40x
72	30 seg.-40x
72	10
4	Hold

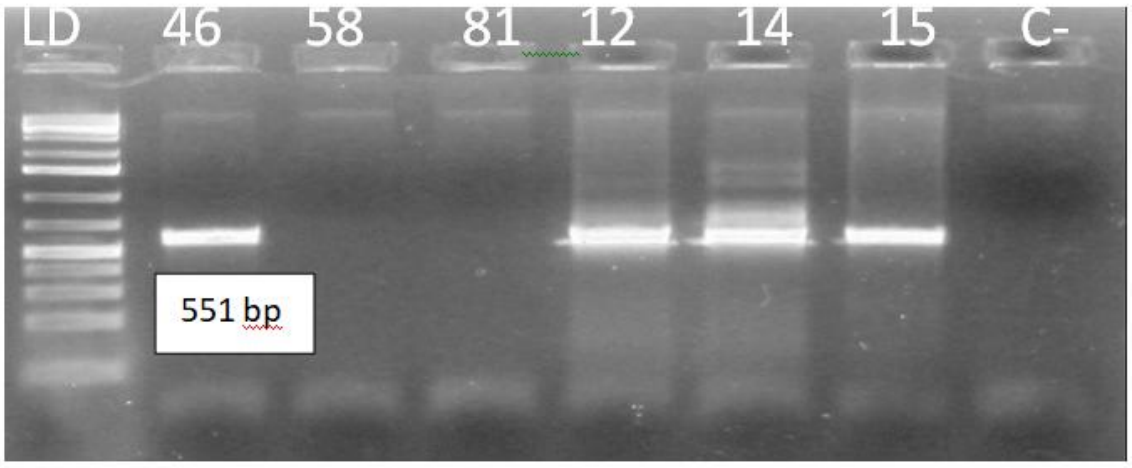

Figure 1. PCR products: LD - Ladder-; Positive probes: 46, 12, 14, 15; Negative Probes; 58, 81; C- - Negative control, characterizing the reaction for the specific band of 551 base pairs, expected amplification for positives of Babesia Caballi and Theileria Equi.

RESULTS AND DISCUSSION

The PCR products were submitted to genetic sequencing (GENOTYPING - LABORATORIO DE BIOTECNOLOGIA LTDA-Botucatu-SP-Brazil) using Big Dye terminator v3.1 Cycle Sequencing kit (thermofisher), ethanol / EDTA / sodium acetate precipitation, according to the manufacturer's instructions in automatic sequencer (ABI PRISM-3500 Genetic Analyzer). The sequences obtained were edited in the CHROMA software (http://technelysium.com.au/wp/Chromas/), and similarity levels were analyzed through the Basic Local Alignment Search Tool (BLAST) program in order to verify the identity with other corresponding sequences available in Gen Bank.

The 38 positive samples showed 100% similarity to the samples with the *Babesia Caballi* sequence (Gen Bank AB734392), and with *Theileria Equi* sequence available on Gen Bank (JX049129).

To our knowledge, this paper is the first to report on the identification of both *T. Equi and B. Caballi*, with the aim to perform the employment of the horse sources, inside an Operational headquarter, through the PCR techniques application to this goal.

CONCLUSION

The PCR technique was shown to be an effective tool for the detection and prevention of *Babesia Equi and Theileria Equi*. The economic feasibility of applying animal health screening in BA is favorable, since the costs are low, also the time of the results and their effectiveness were proven in this study. Based on the results of the present study, it is economically feasible to apply animal health, the application of the PCR method for the detection of equine piroplasmosis, in addition to its economic viability, sensitivity and selectivity, could in the future be recommended for Prevention and improvement of Equine Health in the Brazilian Army. None of the authors of this paper have a financial or personal relationship, with other people or organization that could inappropriately influence or bias the content of the paper.

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