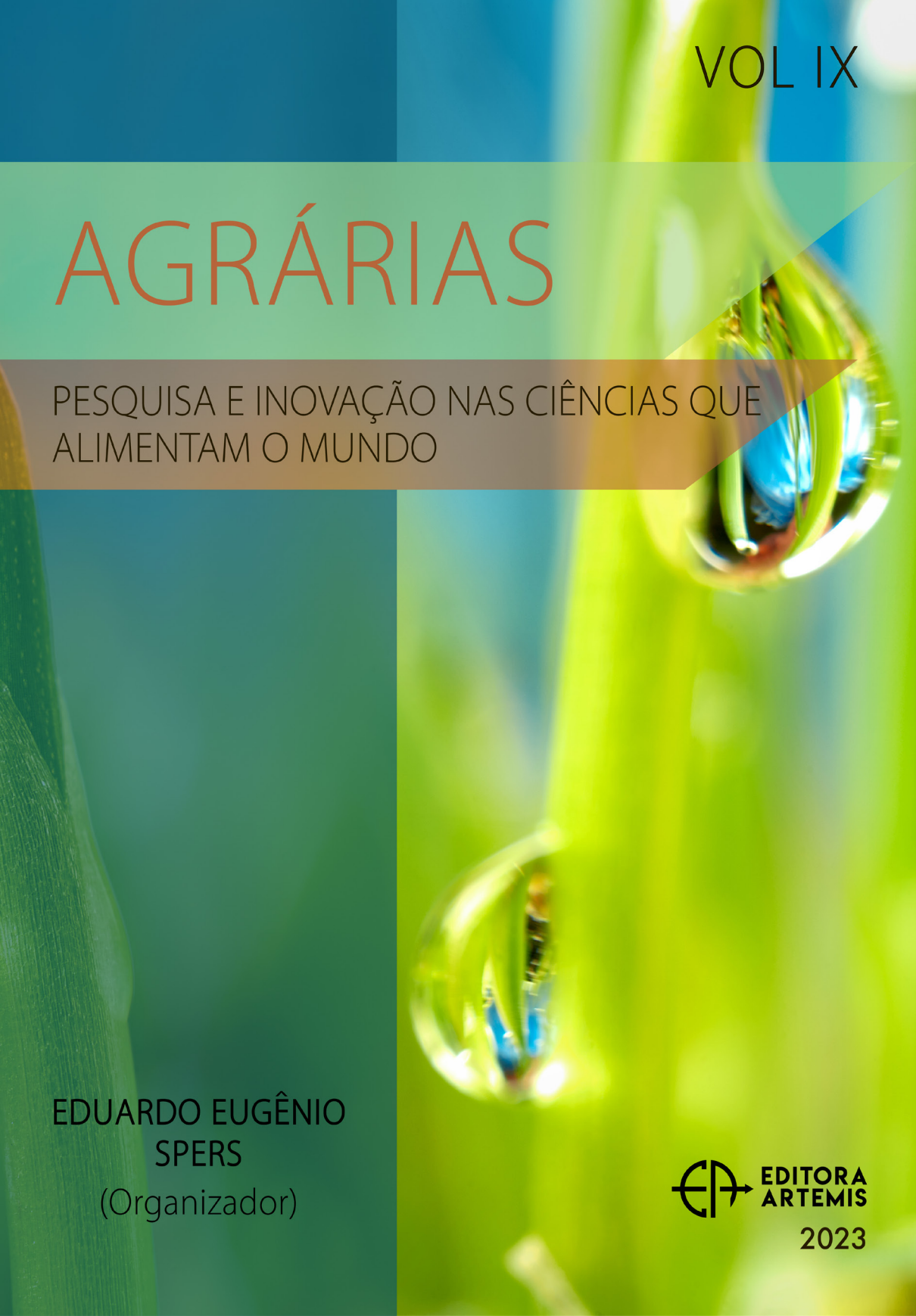
VOL IX

AGRÁRIAS

PESQUISA E INOVAÇÃO NAS CIÊNCIAS QUE ALIMENTAM O MUNDO



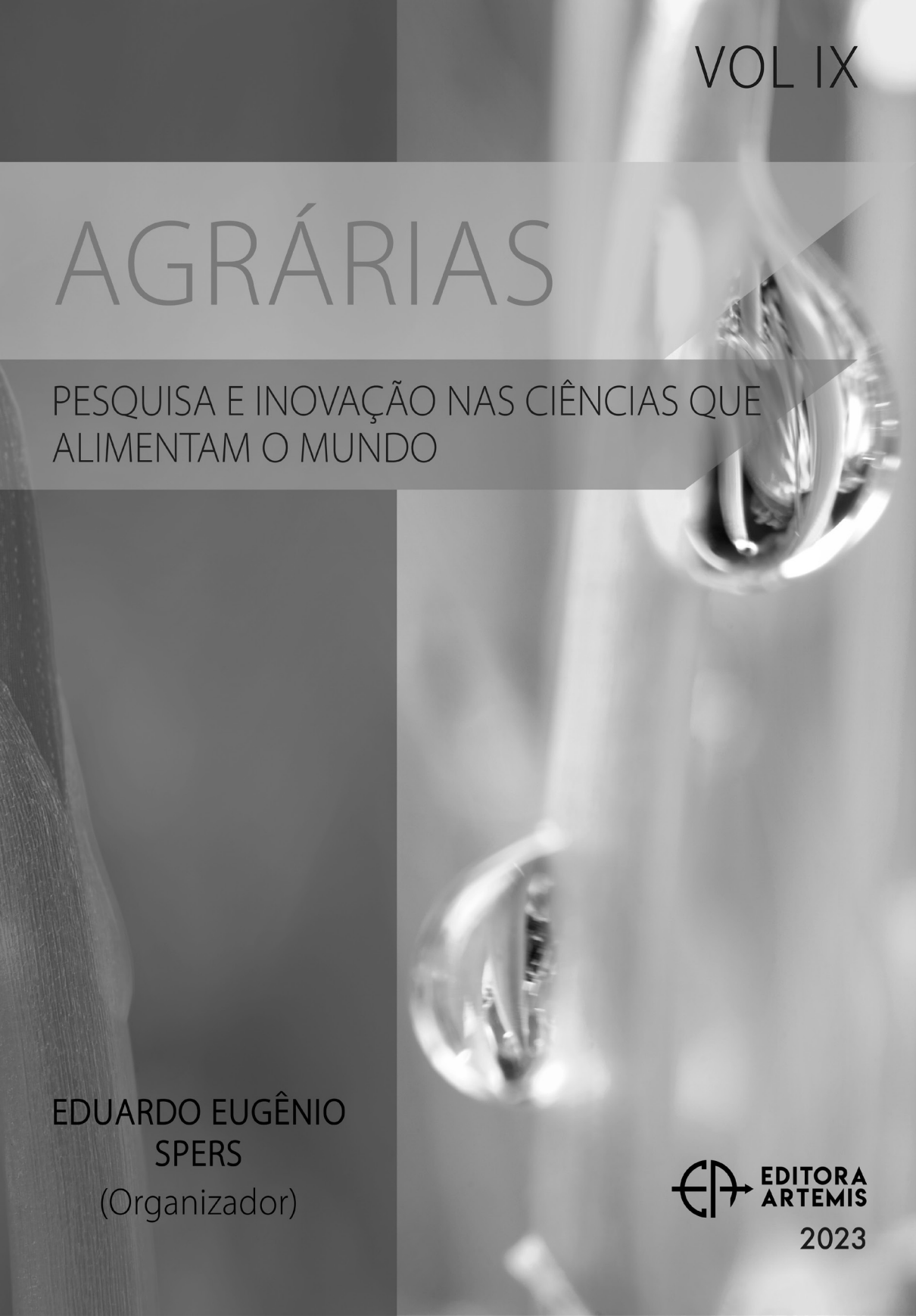
EDUARDO EUGÊNIO SPERS
(Organizador)

EDITORA ARTEMIS
2023

VOL IX

AGRÁRIAS

PESQUISA E INOVAÇÃO NAS CIÊNCIAS QUE ALIMENTAM O MUNDO



EDUARDO EUGÊNIO SPERS
(Organizador)

EDITORA ARTEMIS
2023

Editora Chefe	Profª Drª Antonella Carvalho de Oliveira
Editora Executiva	M.ª Viviane Carvalho Mocellin
Direção de Arte	M.ª Bruna Bejarano
Diagramação	Elisangela Abreu
Organizador	Prof. Dr. Eduardo Eugênio Spers
Imagem da Capa	Shutterstock
Bibliotecário	Maurício Amormino Júnior – CRB6/2422

EDITORA ARTEMIS
2023

Editora Artemis
Curitiba-PR Brasil
www.editoraartemis.com.br
e-mail:publicar@editoraartemis.com.br

Editora Artemis
Curitiba-PR Brasil
www.editoraartemis.com.br
e-mail:publicar@editoraartemis.com.br

Dados Internacionais de Catalogação na Publicação (CIP)
(eDOC BRASIL, Belo Horizonte/MG)

A277 Agrárias [livro eletrônico] : pesquisa e inovação nas ciências que alimentam o mundo IX / Organizador Eduardo Eugênio Spers. – Curitiba, PR: Artemis, 2023.

Formato: PDF
Requisitos de sistema: Adobe Acrobat Reader
Modo de acesso: World Wide Web
Inclui bibliografia
Edição bilíngue
ISBN 978-65-87396-79-8
DOI 10.37572/EdArt_260223798

1. Ciências agrárias – Pesquisa. 2. Agronegócio. 3. Sustentabilidade. I. Spers, Eduardo Eugênio.

CDD 630

Elaborado por Maurício Amormino Júnior – CRB6/2422

EDITORA ARTEMIS
2023

Editora Artemis
Curitiba-PR Brasil
www.editoraartemis.com.br
e-mail:publicar@editoraartemis.com.br

APRESENTAÇÃO

As Ciências Agrárias são um campo de estudo multidisciplinar por excelência, e um dos mais profícuos em termos de pesquisas e aprimoramento técnico. A demanda mundial por alimentos e a crescente degradação ambiental impulsionam a busca constante por soluções sustentáveis de produção e por medidas visando à preservação e recuperação dos recursos naturais.

A obra **Agrárias: Pesquisa e Inovação nas Ciências que Alimentam o Mundo** compila pesquisas atuais e extremamente relevantes, apresentadas em linguagem científica de fácil entendimento. Na coletânea, o leitor encontrará textos que tratam dos sistemas produtivos em seus diversos aspectos, além de estudos que exploram diferentes perspectivas ou abordagens sobre a planta, o meio ambiente, o animal, o homem e a sociedade no ambiente rural.

É uma obra que fornece dados, informações e resultados de pesquisas tanto para pesquisadores e atuantes nas diversas áreas das Ciências Agrárias, como para o leitor que tenha a curiosidade de entender e expandir seus conhecimentos.

Este Volume IX traz 16 trabalhos de estudiosos de diversos países, divididos em dois eixos temáticos: *Eficiência e tecnologia na produção agrícola* e *Meio ambiente e produtividade agrícola.*

Desejo a todos uma proveitosa leitura!

Eduardo Eugênio Spers

SUMÁRIO

EFICIÊNCIA E TECNOLOGIA NA PRODUÇÃO AGRÍCOLA

CAPÍTULO 16

USE OF A PCR-RFLP MOLECULAR TEST FOR THE DIFFERENTIATION OF *Babesia bovis* AND *Babesia bigemina* IN THE DIAGNOSIS OF BOVINE BABESIOSIS

Data de submissão: 20/12/2022
Data de aceite: 05/01/2023

Carmen Rojas Martínez
Centro Nacional de Investigación Disciplinaria en Salud Animal e Inocuidad, INIFAP
Jiutepec, Morelos, México
ORCID: 0000-0001-9177-5634

José Juan Lira Amaya
Centro Nacional de Investigación Disciplinaria en Salud Animal e Inocuidad INIFAP
Jiutepec, Morelos, México
ORCID: 0000-0003-4909-8769

Jesús Antonio Álvarez Martínez
Centro Nacional de Investigación Disciplinaria en Salud Animal e Inocuidad, INIFAP
Jiutepec, Morelos, México

Diego Jesús Polanco Martínez
Centro Nacional de Investigación Disciplinaria en Salud Animal e Inocuidad, INIFAP
Jiutepec, Morelos, México
ORCID: 0000-0002-9239-5788

Julio Vicente Figueroa Millán[1]
Centro Nacional de Investigación Disciplinaria en Salud Animal e Inocuidad, INIFAP
Jiutepec, Morelos, México
ORCID: 0000-0003-4731-3592

Rebeca Montserrat Santamaría Espinosa
Centro Nacional de Investigación Disciplinaria en Salud Animal e Inocuidad, INIFAP
Jiutepec, Morelos, México
ORCID: 0000-0002-1153-6455

Grecia Martínez García
Centro Nacional de Investigación Disciplinaria en Salud Animal e Inocuidad, INIFAP
Jiutepec, Morelos, México
ORCID: 0000-0002-5795-5567

ABSTRACT: This study aimed to evaluate the diagnostic capacity of a PCR test to detect *Babesia* spp, based on genus-specific oligonucleotides that amplify a portion of the small subunit ribosomal RNA gene (18S rDNA), as well as on the species determination by digestion of the amplification product with restriction enzymes (PCR-RFLP). *Babesia* spp DNA extraction was obtained from infected erythrocytes using a commercially available kit. DNA amplification was performed with

[1] Corresponding autor Julio V. Figueroa Millán. CENID-Salud Animal e Inocuidad, INIFAP. Km 11.5 Carr. Cuernavaca-Cuautla, Jiutepec, Morelos, 62550.

a PCR master mix using as primers oligonucleotides targeting a variable region of the 18S rDNA gene. The amplified PCR products were digested with the restriction enzymes (RE) *Hinc* II, *ScrF* I, *Msp* I, *Acu* I, *Nla* III and *Box* I. The amplified and RE-digested PCR products were visualized by electrophoresis on 3% agarose gels. The PCR test allowed the amplification of a DNA fragment of ≈ 400 bp. Amplicons digested with RE *ScrF* I and *Msp* I showed fragments of 250 and 150 bp in samples containing *B. bovis* DNA, while amplicons of samples infected with *B. bigemina* were not digested. Amplicons digested with RE *Acu* I and *Box* I showed fragments of 290 and 110 bp in samples infected with *B. bigemina*, while in samples infected with *B. bovis* the 400 bp amplicon was not digested. Therefore, the PCR-RFLP test could be utilized to differentiate the *Babesia* species that infect bovine erythrocytes according to the pattern result obtained after RE digestion. In addition, amplicons digested with RE *Hinc* II, fragments of 330 and 65 bp are produced in the samples infected with *B. bovis*, but not in the amplicons obtained in samples infected with *B. bigemina*. Thus, the PCR-RFLP test targeting the 18S rDNA gene may be a useful diagnostic tool to confirm the bovine babesiosis infection status and species discrimination in the acute phase of the disease.
KEYWORDS: *Babesia bovis*, *Babesia bigemina*, PCR-RFLP.

UTILIZAÇÃO DE UM TESTE MOLECULAR PCR-RFLP PARA A DIFERENCIAÇÃO DE *Babesia bovis* E *Babesia bigemina* NO DIAGNÓSTICO DE BABESIOSE BOVINA

RESUMO: Este estudo teve como objetivo avaliar a capacidade diagnóstica de um teste de PCR para detecção de *Babesia* spp, baseado em oligonucleotídeos gênero-específicos que amplificam uma porção da pequena subunidade do gene do RNA ribossômico (18S rDNA), bem como na determinação da espécie por digestão do produto de amplificação com enzimas de restrição (PCR-RFLP). A extração de DNA de *Babesia* spp foi obtida de eritrócitos infectados usando um kit comercialmente disponível. A amplificação do DNA foi realizada com uma master mix de PCR usando como primers oligonucleotídeos direcionados a uma região variável do gene 18S rDNA. Os produtos de PCR amplificados foram digeridos com as enzimas de restrição (ER) *Hinc* II, *ScrF* I, *Msp* I, *Acu* I, *Nla* III e *Box* I. Os produtos de PCR amplificados e digeridos com ER foram visualizados por eletroforese em géis de agarose a 3%. O teste de PCR permitiu a amplificação de um fragmento de DNA de ≈ 400 pb. Amplicons digeridos com ER *ScrF* I e *Msp* I apresentaram fragmentos de 250 e 150 pb em amostras contendo DNA de *B. bovis*, enquanto amplicons de amostras infectadas com *B. bigemina* não foram digeridos. Os amplicons digeridos com ER *Acu* I e *Box* I apresentaram fragmentos de 290 e 110 pb nas amostras infectadas com *B. bigemina*, enquanto nas amostras infectadas com *B. bovis* o amplicon de 400 pb não foi digerido. Portanto, o teste de PCR-RFLP poderia ser utilizado para diferenciar as espécies de *Babesia* que infectam eritrócitos bovinos de acordo com o resultado do padrão obtido após a digestão de RE. Além disso, amplicons digeridos com ER *Hinc* II, fragmentos de 330 e 65 pb são produzidos nas amostras infectadas com *B. bovis*, mas não nos amplicons obtidos em amostras infectadas com *B. bigemina*. Assim, o teste de PCR-RFLP visando o gene 18S rDNA pode ser uma ferramenta diagnóstica útil para confirmar o estado de infecção da babesiose bovina e a discriminação de espécies na fase aguda da doença.
PALAVRAS-CHAVE: *Babesia bovis*. *Babesia bigemina*. PCR-RFLP.

1 INTRODUCTION

Among the diseases that cause the greatest economic losses in livestock in the world are those transmitted by ticks and that considerably affect productive species, such as cattle. Bovine babesiosis is a disease caused by the intraerythrocytic parasites *Babesia bovis* and *Babesia bigemina* (Bock et al, 2004). In Mexico, the disease is transmitted by the tick *Rhipicephalus (Boophilus) microplus* and *Rhipicephalus (Boophilus) annulatus*, and is characterized by causing high morbidity and mortality, with the presence of fever, hemolytic anemia, hemoglobinuria, jaundice and, frequently, death (Figueroa & Álvarez, 2003; Bock et al, 2004; Álvarez et al, 2020). In the affected herds there is a temporary reduction in reproduction and abortions after the first third of gestation (Morilla González, 1981; Bock et al, 2004; OIE, 2021).

Cattle farming in Mexico amounts to more than 32 million heads (SIAP, 2022) of which 70% are raised in tropical and subtropical regions, regions of high incidence of the tick vector (Navarrete, et al., 2002). Diagnosis of the disease can be made by using direct and indirect techniques. One of the most widely used indirect methods in Mexico is the indirect fluorescence antibody test (IFAT), which is characterized by the detection of circulating antibodies in blood and with an apparently high diagnostic sensitivity and specificity (≥90%). However, the IFAT has the disadvantage of being subjective and not being able to discriminate between the infecting species, given the cross-reaction observed in sera from animals where both parasites coexist (OIE, 2021). Direct methods include those that directly detect the presence of the pathogen: The most common is the preparation of smears and the observation of intraerythrocytic parasites in slides stained with Giemsa stain.

In veterinary clinical practice, laboratory tests are required to confirm the clinical diagnosis and to differentiate the species of parasites that infect sick animals suffering from acute babesiosis due to *Babesia bovis* or *B. bigemina*. Currently available conventional diagnostic methods, such as microscopic examination of blood smears stained with Romanoswski derivatives, have some limitations due to low analytical sensitivity (particularly for *B. bovis*), requirement of a trained microscopist to identify and differentiate species involved, and low performance in terms of the number of samples analyzed in a workday (Martínez-García et al 2021; Momčilović et al, 2019).

Diagnostic tests based on the Polymerase Chain Reaction (PCR) have high analytical sensitivity and specificity and are likely to provide high performance. Molecular procedures have been developed that allow detecting, with greater analytical sensitivity, the presence of parasites and the species identification species. These include nucleic acid probes and the Polymerase Chain Reaction (PCR) test (Figueroa and Álvarez, 2003;

Figueroa et al, 1996). In most of these methods, however, one PCR test per species is required and sometimes two PCR tests per species if DNA re-amplification is performed with the nested PCR method (Martínez et al, 2021; Kumar et al, 2022).

The objective of this work was aimed at evaluating the diagnostic capacity of a PCR test for *Babesia* spp, based on genus-specific oligonucleotides that amplify a portion of the ribosomal DNA gene of any *Babesia* species, as well as on the determination of the species by digestion of the amplification product with restriction enzymes (PCR-RFLP).

2 MATERIALS AND METHODS

In order to fine-tune the PCR-RFLP test, red blood cells infected with *B. bovis* and *B. bigemina* were obtained from strains established in *in vitro* culture (*Álvarez* et al 2020). Additionally, whole blood was obtained from animals infected with 9 different *B. bovis* isolates (Genis et al, 2009; Pérez et al, 2010), and 9 different *B. bigemina* isolates including one isolate with mixed infection (Figueroa et al, 2013). In addition, 10 engorged female ticks that fed on calves experimentally infected with *B. bovis* and *B. bigemina* and positive to the tick hemolymph test (Castañeda et al, 2012) were included in the study.

The extraction of the genomic DNA of *Babesia* spp was carried out by means of the column purification procedure using a commercial kit. For the PCR test, we proceeded to amplify the variable portion of the gene that codes for the 18S small subunit ribosomal RNA (ssrRNA), using the oligonucleotides PIRO-A and PIRO-B (Olmeda et al 1997; Carret et al, 1999) which can be used to amplify different species of the Piroplasmids *Babesia* (Carret, 1999) and *Theileria* (Kumar et al, 2022). PCR reactions were performed in 0.2 ml PCR tubes, with a final volume of 25 µl, to which 12.5 µl of Master mix (Taq polymerase, dNTPs, Magnesium Chloride), 5.5 µl of nuclease-free water, 5 µl of purified DNA (containing 100 ng), and 2 µl of a mixture of the sense oligonucleotides PIRO-A (5'-AATACCCAATCCTGACACAGGG-3') and anti-sense PIRO-B (5'-TTAAATACGAATGCCCCCAAC-3') (Carret et al, 1999). The amplification reaction was carried out in a thermal cycler, under the following cycling protocol: initial denaturation at 94°C for 5 minutes one cycle, followed by 35 cycles of denaturation at 94°C for 1 minute, alignment at 55°C for 1 minute, extension at 72°C for 1 minute, final extension at 72°C for 5 minutes and storage at 4°C until removal of the PCR tubes for sample analysis. The product amplified by PCR with the oligonucleotides PIRO-A and PIRO-B was digested with the restriction enzymes *Hinc* II, *ScrF* I, *Msp* I, *Acu* I, *Nla* III and *Box* I, according to the supplier's specifications. Based on a bioinformatic analysis performed with the ribosomal DNA sequence of each *Babesia* species, a recognition site for each of these enzymes was identified in the variable part of the parasite's rDNA gene. For example, theoretically, the restriction enzyme *Box* I (recognition sequence

5'-GACNN↓NNGTC-3') would cut only the amplicon corresponding to *Babesia bigemina* ribosomal DNA, while the enzyme *Msp I* (recognition site 5' -C↓CGG-3') would cut only the amplicon corresponding to *Babesia bovis* ribosomal DNA. According to the amount of amplified DNA needed for each restriction enzyme digestion, the final reaction volume was 31 µl, where 10 µl of PCR product, 18 µl of nuclease-free water, 2 µl of 10x tango buffer, 1 µl of enzyme *Hinc* II, *ScrF* I, *Msp* I, *Acu* I, *Nla* III or *Box* I. Digestion with these enzymes was carried out in a water bath at a temperature of 37°C and for 16 hours. PCR products and restriction enzyme digestion derivatives were visualized by horizontal electrophoresis on 2-3% agarose gels in 1X TAE buffer and stained with 1.5 µl of ethidium bromide (10 mg/ml). Molecular markers of 1 Kb or 100 bp were used to discern and estimate the size of amplicons and enzymatic digestion products. For this, 24 µl of reaction were placed in each well of the product obtained by PCR and subjected to electrophoresis at 85 volts with running buffer (TAE 1X) and the gel was visualized in a transilluminator with ultraviolet light. In addition, RE *Msp* I and *Box* I were selected and tested in PCR amplicons obtained from samples containg *B. bovis*, *B. bigemina* and a mixed infection of *B. bovis*/*B. bigemina*.

3 RESULTS

In order to implement a confirmatory diagnostic test for bovine babesiosis, a procedure consisting of *Babesia* spp DNA amplification by Polymerase Chain Reaction (PCR) and restriction enzyme fragment length polymorphism analysis (RFLP) was developed. The PCR assay amplifies a fragment of ≈400 base pairs (bp) in *B. bovis* and *B. bigemina* derived from in vitro culture. If the obtained amplicon is digested with the RE *ScrF* I and *Msp* I, 2 fragments of ≈250 and 150 bp in length (RFLP) are obtained only in samples containing *Babesia bovis*, while in samples infected with *B. bigemina* the amplicon is not digested. If the amplicon is digested with RE *Box* I and *Acu* I, 2 fragments of ≈290 and 110 bp are obtained in samples infected with *B. bigemina*, while in samples infected with *B. bovis* the 400 bp amplicon is not digested, thus differentiating the infecting species by means of the restriction pattern (PCR-RFLP). In addition, in the amplicons digested with RE *Hinc* II, fragments of 330 bp and 65 bp are produced (difficult to resolve and identify the latter in the gel) in the samples infected with *B. bovis*, but not in the amplicons obtained in samples infected with *B. bigemina*. However, the RE *Nla* III digests the amplicon of the 2 *Babesia* species, generating fragments of approximately 250 bp and a 75 bp doublet in *B. bovis*, while in the sample containing *B. bigemina* a 300 bp fragment and one of 100 bp are produced after digestion. Figures 1 and 2 show a representative result of the amplicons obtained in the PCR test and subject to enzymatic digestion (RFLP) in the *Babesia* samples derived from in vitro culture.

Figure 1. PCR-RFLP analysis of products amplified with PIRO-A/PIRO-B oligonucleotides and digested with restriction enzymes. Lane identification: 100 bp marker (M); PCR product digested with Restriction Enzyme *ScrF* I (1); *Hinc* II (2); *Msp* I (3); *Box I* (4); *Nla* III (5); *Accu* I (6); Not digested (N).

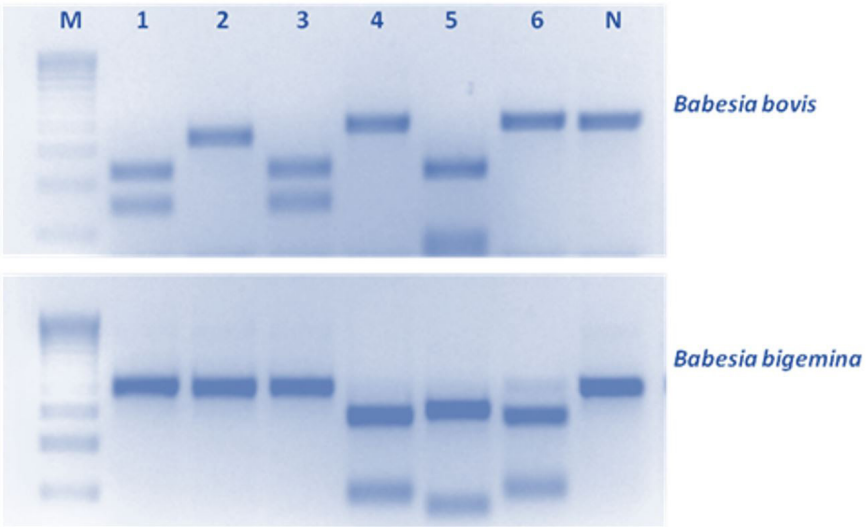
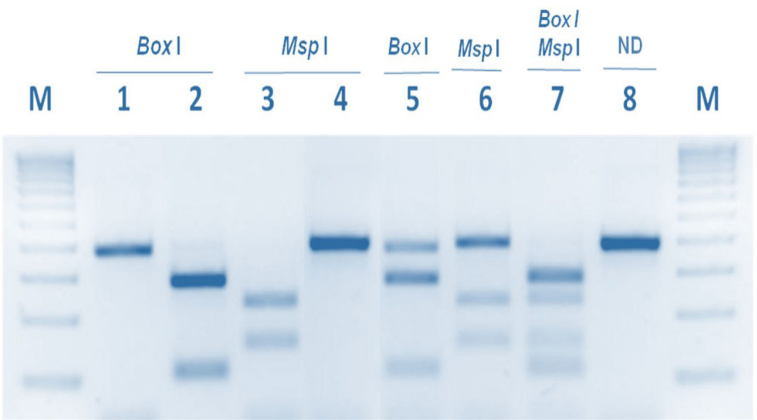


Figure 2. PCR-RFLP analysis of products amplified with PIRO-A/PIRO-B oligonucleotides and digested with restriction enzymes. Lane identification: M) Molecular marker, 100 bp; 1 and 3) *B. bovis*; 2 and 4) *B. bigemina*; 5-8) Mixed *B. bovis*/*B. bigemina*; ND) Not digested with RE.

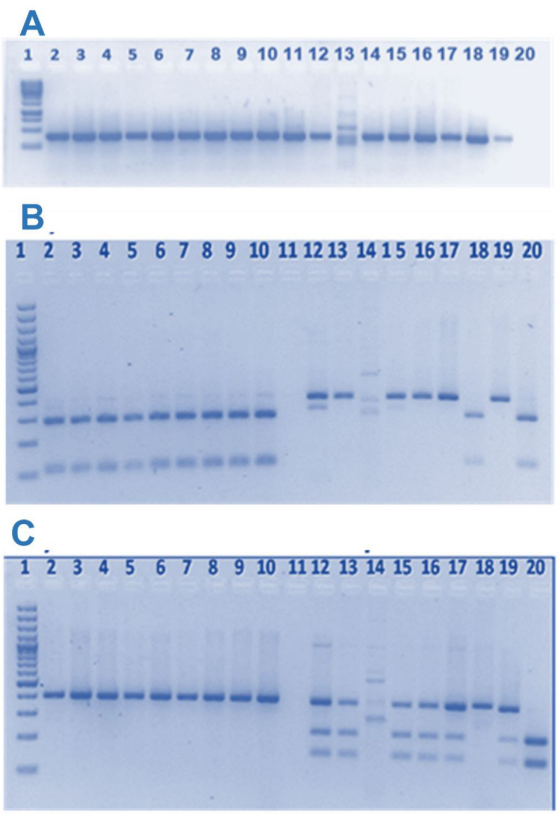


The amplicons obtained were analyzed by PCR-RFLP to verify the specificity of the test with the use of restriction enzymes for each of the *Babesia* species analyzed in this work (Figure 2). The amplicons corresponding to *B. bigemina* were fully digested, obtaining the expected 290 bp and 110 bp fragments in *B. bigemina* samples, wheter single infection or mixed infection (Figure 2, lanes 2 and 5). On the other hand, samples digested with the enzyme *Msp* I, corresponding to *B. bovis*, were also fully digested, obtaining the two fragments of interest with expected sizes of 250 bp and 150 bp (Figure 2, lanes 3 and 6). In lane 1 we can observe undigested DNA that served as a control to identify those

samples that could possibly be undigested. In addition, to rule out posible non-specificities of the RE used in the study, the RE were inverted to confirm their specificity, ensuring that both enzymes are specific to the amplicon for each species. Thus, *B. bigemina* samples that were digested with *Box* I were also digested with RE *Msp* I and *B. bovis* was digested with RE Box I, visualizing only the 400 bp fragments in each case (Figure 2, lanes 1 and 4), unless the amplicon was from a mixed sample containg both *Babesia* sp (Fig 2, lanes 5 and 6). Additionally, two digestion controls were added to confirm that the specific enzymatic reaction was taking place, one where the mixed *Babesia* sp sample was double-digested with RE Box I/Msp I, obtaining the four DNA fragments of the expected size, ie, 290 bp, 250 bp, 150 bp and and 110 bp (Figure 2 lane 7), and the undigested control (Figure 2. Lane 8).

The PCR test with the PIRO A/B oligonucleotides managed to amplify a fragment of approximately 400 bp in all the DNA samples obtained from the 18 different *Babesia* spp. isolates. The PCR-RFLP test implemented then allows the identification of erythrocytes infected with either *Babesia bovis* or *B. bigemina* (Figure 3A).

Figure 3. PCR-RFLP analysis of products amplified with PIRO-A/PIRO-B oligonucleotides and digested with restriction enzymes. Lane identification: 1) 100 bp marker; Lanes 2-9: Isolates classified as *B. bovis*; Lanes 12-20: Isolates classified as *B. bigemina*. Panel A: PCR amplification results with oligonucleotides PIRO A/B); Panel B: PCR-RFLP result with amplicons digested with RE *Box* I; Panel C: PCR-RFLP result with amplicons digested with RE *Msp* I.



The PCR test amplifies a fragment of approximately 400 bp in *B. bovis* and *B. bigemina* from the different geographic isolates. Figure 2 shows the representative result of the amplicons obtained in the PCR assay when amplicons are subject to enzymatic digestion (RFLP) in samples derived from 18 different geographic isolates (9 classified microscopically as *B. bovis* and 9 classified as *B. bigemina*). If the obtained amplicon is digested with RE *Box* I, as expected, 2 fragments of ≈290 and 110 bp are obtained in the samples infected only with *B. bigemina*, while in the samples infected with *B. bovis* the 400 bp amplicon is not digested, unless the sample was co-infected (Figure 3B, lanes 18 and 20). If the obtained amplicon is digested with the RE *Msp* I, fragments of 400 bp, ≈250 and 150 bp in length (RFLP) are obtained in samples containing only *B. bovis* (denoting partial digestion in most of the samples classified as *B. bovis*), while in samples infected with *B. bigemina* the amplicon is not digested, thus differentiating the infecting species by means of the restriction pattern (PCR-RFLP). In addition, if the PCR-derived amplicons from a sample co-infected with *B. bovis* and *B. bigemina* are digested with RE *Box* I, bands of 290 and 110 bp are obtained, while in the sample digested with RE *Msp* I fragments of 250 and 150 bp are observed, differentiating the species present in the samples co-infected with *B. bovis* and *B. bigemina* (Figure 3C, lane 20). The PCR test performed on the 10 *Rh microplus* tick samples processed allowed the 10 specimens to be identified with amplicons of the expected size (400 bp). However, only in 6 specimens could an intense band be visualized (not shown). Enzymatic digestion with RE *Box* I and *Msp* I of the amplicons obtained allowed us to identify a pattern corresponding to *B. bigemina* in 5 of the ticks and a pattern corresponding to *B. bovis* in 3 ticks, with a mixed infection in 2 ticks (not shown).

4 DISCUSSION AND CONCLUSIONS

It was possible to implement the PCR test to detect *Babesia* spp in infected erythrocytes from in vitro culture and in isolates propagated in experimental animals, through the use of generic PIRO-A and PIRO-B oligonucleotides. It was shown that the PCR test for *Babesia* sp DNA amplification using oligonucleotides that align the gene that codes for 18S ribosomal RNA, can detect an infection due to the presence of *Babesia bigemina* and/or *B. bovis* in experimentally inoculated animals.

It was possible to implement the PCR test to detect *Babesia* spp in DNA extracted from erythrocytes infected with 18 different geographic isolates of *B. bigemina* and *B. bovis*. By using generic oligonucleotides that align to the gene that codes for 18S ribosomal RNA, the PCR test can detect the presence of *B. bigemina* and/or *B. bovis* in

field samples, thus demonstrating the conservation of the 18S ribosomal DNA gene in *Babesia* species in Mexico.

Previous work used DNA purified from in vitro cultured parasites as template (Carret, 1999; Carret et al, 1999), obtaining an amplicon of the expected size but without being able to differentiate the species: *B. bovis* or *B. bigemina*, given that no enzymatic digestion process was carried out. Several studies have been reported in different countries based on the use of PCR in conjunction with the RFLP technique to detect and differentiate pathogens of economic importance, which mainly affect the livestock industry, as in many cases those Pathogens may cause the death of the infected animal. In a study carried out in 200, it was possible to implement the enzymatic digestion of a product of rDNA amplification with primers F: 5'-GAG TAA ATT AGA GTG TTC CAA GCA-3' and R: 5'-CGG AAT TAA CAA GAC AAA TC-3', which amplify a portion of the 18S subunit of approximately 564 bp and using the PCR-RFLP assay with the enzyme *Kpn* I, the detection of *Theileria annulata* was achieved in field conditions where mixed infections with *B. bigemina* and *T. evansi* are very common (Ravindran et al 2007a). With these primers it was possible to amplify *B. bigemina* and *T. annulata* rDNA, both ~564 bp fragments, in contrast to *T. evansi* rDNA and bovine DNA which were not amplified. At the same time, the use of other RE such as *Mbo* I, *Hue* III, *Alu* I, *Hinf* I and *Taq* I was ruled out as a potential marker for the differentiation of *Theileria* and *Babesia* species, resulting in the RE Kpn I as the only restriction marker for differentiation in mixed infections, since it produces a double digestion pattern only for *T. annulata* of 466 bp and 98 bp. Another study carried out the same year was able to establish a diagnosis to differentiate mixed infections caused by B. bigemina and Theileria annulata in calves by using restriction enzymes in PCR amplification products using the same set of oligonucleotides described in the previous experiment (Ravindran et al, 2007b). In this study RE Cfr131 was capable of differentiating between both species, obtaining different digestion patterns between species, finding a triple digestion pattern for *B. bigemina*, obtaining sizes of 393bp, 101bp and 66bp while for *T. annulata* a double digestion pattern of 463 bp and 101 bp.

A PCR_RFLP assay has also been designed for the molecular identification of *Theileria* spp. with the use of the oligonucleotides FThBab 5′-GCATTCGTATTTAACTGTCAGAGG-3′ and RThBab 5′- GATAAGGTTCACAAAACTTCCCTAG-3′ based on the amplification of a region of the 18S rDNA gene of ~861bp used to make a differentiation between *T. ovis T. lestoquardi* and *T. annulata* species, by using the RE *Hinc* II and *Vsp* I (Jalali et al, 2014).

With the implementation and use of the PCR-RFLP assay, the aim is to increase the specificity and reduce the difficulty in making confirmatory and differential diagnoses, as traditional methods used, such as microscopy and serological methods, might have

high variability since the diagnosis depends on factors such as the analyst's interpretation, morphological similarity between species, and cross-reaction, just to name a few (Delespaux et al, 2003; Solano-Gallego et al, 2008; Karimi et al, 2012).

In the present work, the presence of *Babesia* spp. in most of the isolates analyzed was accomplished and, in turn, it was possible to differentiate between species using the PCR-RFLP assay, with the oligonucleotides PIRO A/PIRO B, and RE *Box* I, and *Msp* I.

The PCR-RFLP assay used as a technique for direct detection can be accurate and reliable, when compared to the light microscopy examination of thin blood smears. However, it is necessary to evaluate a large number of positive samples from truly infected animals in order to determine their analytical sensitivity and diagnostic specificity, as compared to the conventional microscopic test (Figueroa et al, 1996). However, it can be argued that it is in terms of the specificity of the tests and the number of samples that can be processed in a workday by an analyst, where clear differences between the two diagnostic tests can be established. For example, a microscopist can easily analyze 30 thin blood smears observed in a workday if the operator spends approximately 10-15 minutes microscopically analyzing blood smears (Figueroa et al 1996; Bock et al, 2004). However, to achieve this, it is necessary to have a person with experience in the detection and speciation of blood parasites. On the other hand, the PCR test, although it is a high-cost technique due to the reagents it uses, it has the great advantage that in a 8-hour workday, and given the (semi)automation of the DNA amplification procedure, it can provide better performance in terms of having a greater number of samples processed in a workday. Additional experiments carried out with the PCR-RFLP-based test, allowed the identification of *Babesia* infections in animals experimentally and naturally infected with *B. bovis* and/or *B. bigemina*, as well as in *Rhipicephalus microplus* ticks that fed on these animals. Thus, the PCR-RFLP assay tested in Mexico, allows a differentiation of *B. bovis* and/or *B. bigemina* with the use of RE *Box* I and *Msp* I for digestion of an amplicon obtained by PCR. The assay can be implemented to monitor infection by *Babesia* spp primarily in the acute phase of the disease, in such a way that the prepatent and/or patent period of infection in premunized and/or naturally infected animals can be estimated.

A more sensitive and species-specific confirmatory diagnostic test, such as the PCR-RFLP test, is instrumental for the detection and differentiation of *B. bovis* and *B. bigemina*, since it allows the analysis of a greater number of samples in less time and facilitates the establishment of a more effective and timely medical intervention for the treatment of bovine babesiosis. The PCR-RFLP test can be applied in any región or country where there are laboratories with basic equipment for the PCR test, where a more accurate diagnosis of babesiosis is required. Even though the PCR-RFLP test is indicated

for the analysis of DNA extracted from the blood of animals in the acute phase of the disease, additional experiments need to be carried out for its implementation in tissues (brain, kidney, liver) obtained from recently dead animals with signs compatible with the disease. The test may also be used in the detection of mixed infection in cattle, as well as in the identification and differentiation of *Babesia* spp in the tick vector *Rh. microplus* and/or *Rh. annulatus*.

BIBLIOGRAPHIC REFERENCES

Álvarez-Martínez JA., Figueroa-Millán JV. 2007. Cultivo in vitro de *Babesia bovis* y *Babesia bigemina* y su aplicación para la producción de vacuna. *Ciencia Veterinaria,* 10: 137-153.

Alvarez JA, Rojas C, Figueroa JV. 2020. An overview of current knowledge on in vitro Babesia cultivation for production of live attenuated vaccines for bovine babesiosis in Mexico. Front Vet Sci. 26; 7:364.

Bock R., Jackson J., de vos A. and Jorgensen W. 2004. Babesiosis of Cattle. *Parasitology*. 129, S247-S269.

Carret CM. 1999. *Babesia canis: caracterisation d´antigenes parasitaires solubles potentiellement impliques dans l´immunoprotection induite chez le chien et analyse molecularire du polymorphisme genetique des sous especies.* Tesis de doctorado. Facultad de Farmacia, Universidad de Montpellier I. Montpellier, Francia.

Carret C, Walas F, Carcy B, Grande N, Précigout É, Moubri K, Schetters TP, Gorenflot A. 1999. *Babesia canis canis*, *Babesia canis vogeli*, *Babesia canis rossi*: Differentiation of the three subspecies by a Restriction Fragment Length Polymorphism analysis on amplified small subunit ribosomal RNA genes. *J. Eukaryotic Microbiol.* 46(3): 298-303.

Castañeda-Arriola RO, Rojas-Martínez C, Figueroa-Millán JV, Martínez-Ibáñez F, Álvarez-Martínez JA. 2012. Uso de PCR anidada para determinación de infección por *Babesia* spp en garrapatas *Boophilus (Rhipicephalus) microplus*. 1er Congreso Latinoamericano de Acarología 2012. Sociedad Mexicana de Entomología A.C., Primera Ed 2012. p. 176-180.

Delespaux V, Ayral F, Geysen D, Geerts S. 2003. PCR-RFLP using ssu-rDNA amplification: applicability for the diagnosis of mixed infections with different *Trypanosome* species in cattle. *Veterinary Parasitology* 117, 185-193.

Figueroa-Millán JV, Álvarez-Martínez JA. 2003. Investigaciones sobre la aplicación de técnicas moleculares en el diagnóstico y control de la babesiosis bovina. Ciencia Veterinaria, 9: 75-103.

Figueroa JV, Alvarez JA, Canto GJ, Ramos JA, Mosqueda JJ, Buening GM. 1996. Comparative sensitivity of two tests for the diagnosis of multiple hemoparasite infection of cattle. Ann. N. Y. Acad. Sci. 23: 117-127.

Figueroa-Millán JV, Pérez-Sosa J, Vargas-Uriostegui P, Rojas-Martinez C, Álvarez-Martinez JA. 2013. Clonación y secuenciación de rap-1α a partir de aislados mexicanos de Babesia bigemina. XXXVII Congreso Nacional de Buiatría, 1-3 de agosto, 2013. Acapulco, Gro. Mexico. pp. 618-624.

Genis AD, Perez J, Mosqueda JJ, Alvarez A, Camacho M, Muñoz MDL. Rojas C, Figueroa JV. 2009. Using *msa-2b* as a molecular marker for genotyping Mexican isolates of *Babesia bovis*. Infect. Genet. Evol. 2009, 9, 1102–1107.

Jalali SM, Khaki Z, Kazemi B, Rahbari S, Shayan P, Bandehpour M, Yasini SP. 2014. Molecular detection and identification of *Theileria* species by PCR-RFLP method in sheep from Ahvaz, Southern Iran. Iranian Journal of Parasitology. 9(1), 99-106.

Karimi A, Behnam A, Vafafar A, Ghorbani A, Mard M. 2012. Simultaneous detection and differentiation of ovine piroplasms in *Hyaloma anatolicum* using PCR-RFLP. World Applied Sciences Journal. 20, (8): 1092-1097.

Kumar B, Maharana BR, Thakre B, Brahmbhatt NN, Joseph JP. 2022. 18S rRNA gene-based piroplasmid PCR: An assay for rapid and precise molecular screening of *Theileria* and *Babesia* species in animals. Acta Parasitol. 67(4): 1697-1707.

Martínez-García G, Santamaría-Espinosa RM, Lira-Amaya JJ, Figueroa JV. (2021). Challenges in tick-borne pathogen detection: The case for *Babesia* spp. identification in the tick vector. *Pathogens* 10, no. 2: 92.

Momčilović S, Cantacessi C, Arsić-Arsenijević V, Otranto D, Tasić-Otašević S. 2019. Rapid diagnosis of parasitic diseases: current scenario and future needs. Clin Microbiol Infect. 25(3):290-309.

Morilla-González A. 1981. Inmunología de la babesiosis. *Ciencia Veterinaria,* 3: 240-268.

Navarrete I, Serrano FJ, Reina D. 2002. Parásitos hemáticos. En: M. Cordero del Campillo & R. A. Rojas Vazquez, edits. *Parasitologia Veterinaria.* España: Mc Graw Hill, pp. 283-294.

OIE World Organization for Animal Health (WOAH). Bovine Babesiosis; OIE WOAH Terrestrial Manual: Paris, France, 2021; Chapter 3.4.2; Available online: https://www.woah.org/fileadmin/Home/eng/Health_standards/tahm/3.04.02_BABESIOSIS.pdf (accessed on 28 June 2022).

Olmeda AS, Armstrong PM, Rosenthal BM, Valladares B, del Castillo A, de Armas F, Miguelez M, González A, Rodríguez Rodríguez JA, Spielman A, Telford SR 3rd. 1997. A subtropical case of human babesiosis. Acta Trop. 30; 67(3):229-234.

Pérez J, Perez JJ, Vargas P, Álvarez JA, Rojas C, Figueroa JV. 2010. Sequence conservation of 12D3 gene in Mexican isolates of *B. bovis*. Transb. Emerg. Dis. 57: 57-60.

Ravindran R, Saravanan BC, Rao JR, Mishra AK, Bansal GC, Ray D. 2007a. A PCR-RFLP method for specific detection of *Theileria annulata*. *Journal of Applied Animal Research*. 32:1, 111-112.

Ravindran R, Saravanan BC, Rao JR, Mishra AK, Bansal GC, Ray D. 2007b. A PCR-RFLP method for the simultaneous detection of Babesia bigemina and Theileria annulata infections in cattle. Current Science 93 (12): 1840-1843.

SIAP. Población Ganadera|Servicio de Información Agroalimentaria y Pesquera|Gobierno|gob.mx. Available online: www.gob.mx (accessed on 30 June 2022).

Solano-Gallego L, Trotta M, Carli E, Carcy B, Caldin M, Furlanello T. 2008. *Babesia canis canis* and *Babesia canis vogeli* clinicopathological findings and DNA detection by means of PCR-RFLP in blood from Italian dogs suspected of tick-borne disease. *Veterinary Parasitology* 157, 211–221.

SOBRE O ORGANIZADOR

EDUARDO EUGENIO SPERS realizou pós-doutorado na Wageningen University (WUR), Holanda, e especialização no IGIA, França. Possui doutorado em Administração pela Universidade de São Paulo (USP). Foi Professor do Programa de Mestrado e Doutorado em Administração e do Mestrado Profissional em Comportamento do Consumidor da ESPM. Líder do tema Teoria, Epistemologia e Métodos de Pesquisa em Marketing na Associação Nacional de Pós-Graduação e Pesquisa em Administração (ANPAD). Participou de diversos projetos de consultoria e pesquisa coordenados pelo PENSA e Markestrat. É Professor Titular no Departamento de Economia, Administração e Sociologia, docente do Mestrado em Administração e Coordenador do Grupo de Extensão MarkEsalq no campus da USP/Esalq. Proferiu palestras em diversos eventos acadêmicos e profissionais, com diversos artigos publicados em periódicos nacionais e internacionais, livros e capítulos de livros sobre agronegócios, com foco no marketing e no comportamento do produtor rural e do consumidor de alimentos.

ÍNDICE REMISSIVO

A

B

C

D