Biotecnología Aplicada

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REPORT

Molecular detection and identification of tick-borne pathogens in *Equus caballus* and ticks from western Cuba

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ABSTRACT

Babesia caballi, *Theileria equi* and several species of rickettsias are agents of vector-borne diseases that affect equines. The objective of this study was to detect infections by *B. caballi* and *T. equi* in horses and to identify rickettsias in horses and ticks in the western region of Cuba. Two nPCR assays were developed and standardized for the detection of *B. caballi* and *T. equi*. Blood samples from horses and ticks were collected. Identification by blood smear and molecular detection and identification of *B. caballi*, *T. equi* and *Rickettsia* spp. were carried out. Intraerythrocytic formations compatible with *B. caballi* and *T. equi* were observed. The nPCR showed that 25 % of the samples were positive for *B. caballi*, 73 % for *T. equi* and 20 % showed coinfection. The results were confirmed with the partial sequencing of the genes *bc48* (*B. caballi*) and *ema-1* (*T. equi*). The sequencing of the 18S rRNA gene of *T. equi* demonstrated the presence of at least two genotypes of *T. equi* isolates in Cuba. The real time qPCR assay and sequencing revealed the presence of *Rickettsia amblyommatis* in *A. mixtum* and *Rickettsia felis* in *D. nitens*. Conclusions: These results constitute the first piece of molecular evidence of *B. caballi* and *T. equi* in horses and the first report of *R. felis* in *D. nitens* in Cuba, which broadens the knowledge about the distribution of pathogens and the spectrum of potential vectors contributing to the strengthening of management and control programs.

Keywords: Babesia caballi, *Theileria equi*, *Rickettsia* spp., *Dermacentor nitens*, *Amblyomma mixtum*

RESUMEN

Detección e identificación molecular de patógenos transmitidos por garrapatas en *Equus caballus* y garrapatas del occidente de Cuba. *Babesia caballi*, *Theileria equi* y varias especies de rickettsias son agentes de enfermedades transmitidas por vectores que afectan a los equinos. El objetivo del presente estudio fue detectar infecciones por *B. caballi* y *T. equi* en caballos e identificar rickettsias en caballos y garrapatas en la región occidental de Cuba. Se estandarizaron 2 ensayos de nPCR para la detección de *B. caballi* y *T. equi*. Se colectaron muestras de sangre de caballos, y garrapatas. Se realizó identificación por frotis sanguíneo y detección e identificación molecular de *B. caballi*, *T. equi* y *Rickettsia* sp. Se observaron formaciones intraeritrocíticas compatibles con *B. caballi* y *T. equi*. El nPCR mostró que el 25 % de las muestras fueron positivas para *B. caballi*, 73 % para *T. equi* y 20 % mostraron coinfección. Los resultados se confirmaron con la secuenciación parcial de los genes *bc48* (*B. caballi*) y *ema-1* (*T. equi*). La secuenciación del gen 18S de ARNr de *T. equi* demostró la presencia de al menos 2 genotipos de aislados de *T. equi* en Cuba. El ensayo de PCR en tiempo real y la secuenciación revelaron la presencia de *Rickettsia amblyommatis* en *A. mixtum* y *Rickettsia felis* en *D. nitens*. Como conclusiones estos resultados constituyen la primera evidencia molecular de *B. caballi* y *T. equi* en equinos y el primer reporte de *R. felis* en *D. nitens* en Cuba, lo que amplía el conocimiento sobre la distribución de patógenos y el espectro de vectores potenciales contribuyendo al fortalecimiento de los programas de manejo y control.

Palabras clave: Babesia caballi, *Theileria equi*, *Rickettsia* sp., *Dermacentor nitens*, *Amblyomma mixtum*

How to cite (Vancouver style):
Díaz-Sánchez AA, Corona-González B, Chilton NB, Lobo-Rivero E, Vega-Cañizares E, Yrurzun-Estrada C, *et al*. Molecular detection and identification of tick-borne pathogens in *Equus caballus* and ticks from western Cuba. Biotecnol Apl. 2022; 39(2):2501-5.

Introduction

Equine piroplasmosis is considered the most important vector-borne disease that affects equines in tropical, subtropical and temperate regions. It is an acute, subacute or chronic intraerythrocytic disease produced by the haematozoa *Babesia caballi* and *Theileria equi*, which is transmitted by ticks of the *Amblyomma*, *Dermacentor*, *Rhipicephalus* and *Hyalomma* genera [1]. Previous studies in Cuba based on the blood smears and serological tests described the presence of these piroplasms in horse herds [2].

Assays based on nested polymerase chain reaction (nPCR) have been used up to now to detect *B. caballi* and *T. equi*, that are characterized by their high sensitivity and analytical specificity. They enable the

1. Scoles GA, Ueti MW. Vector ecology of equine piroplasmosis. Annu Rev Entomol. 2015;60:561-80.

2. Salabarria FF, Godshaev A, Ferrer J, Jimenez T, Villalba G, Jorge J. *Babesia equi* and *Babesia caballi* in zebras. Morphological and serological diagnosis. Rev Cubana Cienc Vet. 1982;12:171-6.

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detection of these hemoparasites in equines with low levels of parasitemia and carrier vectors [3-6].

Members of the *Rickettsia* genera are obligate intracellular Gram-negative bacteria that require eukaryotic cells for their replication. They are transmitted by hematophagous arthropods to humans and animals. In many parts of the world, the tick-borne rickettsiosis represent emerging or reemerging diseases. Two species have been reported in Cuba, *Rickettsia amblyommatis* and *Rickettsia felis*, in ticks (*Amblyomma mixtum*) and in blood samples of dogs, respectively [7, 8].

Although horses in Cuba are frequently exposed to ticks (*A. mixtum* and *Dermacentor nitens*) [7], there are no surveillance studies to determine the prevalence of rickettsia or to examine their possible functions as reservoirs for zoonotic pathogens, and as sentinels for public health. The aim of this study was to detect infections by *B. caballi* and *T. equi* in horses, and to identify *Rickettsia* in horses and ticks within the western region of Cuba.

Materials and methods

Hematologic alterations in horses infected with *B. caballi* and *T. equi*

We analyzed 58 blood samples from clinically healthy horses (17 from Havana and 41 from the province of Mayabeque) [9]. The diagnosis of *B. caballi* and *T. equi* were made through blood smears and the hematologic examination of the red and white blood cells. We identified each hemoparasite based on the morphologic and biometric parameters described in the literature, such as their form, localization and size [10]. The animals were individually inspected and subjected to physical examination, and determined the presence of ticks. Ticks collected were preserved in 70 % ethanol and for their taxonomic identification we used the keys published by Barros-Battesti *et al*. [11]. It was determined the mean, maximum and minimum values of the hematocrit, hemoglobin, total erythrocytes, mean corpuscular volume, mean corpuscular hemoglobin concentration and total leukocytes in the studied animals. To detect the association between sex, age, presence of ticks, contact with cattle and hematocrit lower than 0.32 L/L, with the presence of *B. caballi* and *T. equi*, 2 × 2 contingency tables were used. The nonparametric U Mann-Whitney test was run to identify the differences of hematocrit values between positive and negative animals to *B. caballi* and *T. equi*. Data analysis was done using the Epidat 3.1 [12] and InfoStat [13] software with a 95 % confidence interval (CI). Significant differences were considered as $p < 0.05$.

Development, standardization and evaluation of the nPCR assays for the detection of *B. caballi* and *T. equi*

For the diagnosis of *B. caballi* and *T. equi* we used the primers described by Battsetseg *et al*. [3], which amplify 2 DNA fragments within the *bc48* and ema-1 genes, respectively. In all nPCR assays, for both stages (PCR1 and PCR2), the amplification reactions were carried out in a final volume of 25 µL, containing 1× GoTaq® Green Master Mix (Promega, Madison, USA) and 0.8 µM of each primer. In PCR1 were used 2 µL of DNA and in PCR2 1 µL of the product of PCR1. The reactions took place in an Eppendorf Mastercycler gradient 96 thermocycler (Eppendorf AG, Hamburg, Germany).

The results of the nPCR were applied in 2 % agarose gels (Sigma-Aldrich, St. Louis, MI, USA) in the TBE 0.5 × buffer, and they were stained with ethidium bromide (0.5 µg/mL). Bands were visualized through an ultraviolet light using a macro vue trans-illuminator (Pharmacia Biotech Inc., USA). In all cases, the 100 bp molecular weight marker was used (Promega, Madison, USA).

The main critical parameters were optimized at each assays steps (PCR1 and PCR2), performing PCR2 from the product of PCR1. Each experiment was made in duplicate. It was determined the optimum temperature for primers' alignment, the optimum concentration of primers, and the analytical specificity and sensitivity.

Molecular detection of *B. caballi* and *T. equi* using nPCR

A simple random method was used for sampling [14]. The population studied was located in six municipalities of western Cuba, corresponding to the provinces of Mayabeque (San José de las Lajas, Jaruco and Madruga), Artemisa (Artemisa), Havana (Boyeros) and Matanzas (Unión de Reyes).

Blood samples were collected from 100 horses by jugular vein piercing with 25 mm × 0.8 mm (21G) hypodermic needles and blood was extracted in 4-mL vacuum tubes (BD Vacutainer®) containing 7.2 mg of K_2EDTA as anticoagulant. No animal showed any clinical signs of the hemolytic syndrome during sampling. DNA was extracted from samples using the DNeasy Blood and Tissue DNA Purification Kit (QIAGEN®, USA), according to the manufacturer's instructions. DNA quality and concentration were determined with the Colibri Microvolume Nanodrop Spectrophotometer (Titertek-Berthold, Germany).

B. caballi and *T. equi* were diagnosed using the steps of each standardized nPCR assay of the study. PCR products were visualized as abovementioned.

The nPCR results were confirmed by sequencing three amplimeres of the *bc48* (*B. caballi*) gene and three of the *ema-1* (*T. equi*) gene. Furthermore, three samples positive to *T. equi* were selected to amplify the 18S rRNA gene (~ 1800 bp) with primers reported by Liu *et al*. [15]. The PCR products were purified using the QIAquick® PCR Purification Kit (QIAGEN®, USA), and they were sequenced by the Sanger method with the ABI Prism BigDye™ Terminator Cycle Sequencing kit (Applied Biosystems®, USA), in an automatic ABI 3730 DNA Analyzer sequencer (Applied Biosystems®, USA).

The sequences obtained were assembled, edited and analyzed using the Molecular Evolutionary Genetics Analysis 7 (MEGA7) software [16]. The BLASTn algorithm [17] was used to evaluate the identity percentage of consensus sequences generated with the *bc48*, *ema-1* and 18S rRNA genes' nucleotide representative sequences, available at the GenBank database. Sequences obtained in this study were deposited at GenBank database: *B. caballi bc48* (Accession numbers KY111763, KY111764 and

3. Battsetseg B, Xuan X, Ikadai H, Bautista JLR, Byambaa B, Boldbaatar D, *et al*. Detection of *Babesia caballi* and *Babesia equi* in *Dermacentor nuttalli* adult ticks. Int J Parasitol. 2001;31:384-6.

4. Rampersad J, Cesar E, Campbell MD, Samlal M, Ammons D. A field evaluation of PCR for the routine detection of *Babesia equi* in horses. Vet Parasitol. 2003;114:81-7.

5. Baptista C, Lopes MS, Tavares AC, Rojer H, Kappmeyer L, Mendonca D, *et al*. Diagnosis of *Theileria equi* infections in horses in the Azores using cELISA and nested PCR. Ticks Tick Borne Dis. 2013;4:242-5.

6. Ayala Valdovinos MA, Lemus Flores C, Galindo García J, Banuelos Pineda J, Rodríguez Carpena JG, Sanchez Chipres D, *et al*. Diagnosis and prevalence of *Theileria equi* horses in western Mexico by nested PCR. Parasitol Int. 2016;66:821-4.

7. Noda AA, Rodríguez I, Miranda J, Mattar S, Cabezas Cruz A. First report of spotted fever group Rickettsia in Cuba. Ticks Tick Borne Dis. 2016;7:1057-8.

8. Corona BG, Díaz Sánchez AA, Meli ML, Cañizares EV, Arias LR, Dorta YL, *et al*. Occurrence of tickborne pathogens in stray dogs from Havana, Cuba. Acta Biomedica Scientifica. 2018;3:158-9.

9. Díaz Sánchez AA, Fonseca Rodríguez O, Castillo Domínguez S, López Dorta Y, Lobo Rivero E, Corona González B, *et al*. Hematological alterations found in horses (*Equus caballus*) infected with *Babesia caballi* and *Theileria equi*. Rev Salud Anim. 2018;40:1. ISSN: 2224-4667.

10. OIE TM. Equine piroplasmosis. NB: Version adopted by the World Assembly of Delegates of the OIE. 2014.

11. Barros Battesti DM, Hernández MR, Famadas KM, Onofrio VC, Beati L, Guglielmone AA. The ixodid ticks (Acari: Ixodidae) of Cuba. Syst Appl Acarol. 2009;14(2):101-28.

12. Hervada VX, Santiago PM, Vázquez FE, Castillo SC, Loyola EE, Silva ALC. Epidat 3.0 programa para análisis epidemiológico de datos tabulados. Rev Esp Salud Públ. 2004;78(2):277-80.

13. Di Rienzo JA, Casanoves F, Balzarini MG, Gonzalez L, Tablada M, Robledo CW. InfoStat, versión 24-03-2011 [programa de cómputo]. Córdoba, Argentina: Universidad Nacional de Córdoba; 2011 [cited 2020 Oct 16]. Available from: http://www.infostat.com.ar

14. Díaz Sánchez AA, Sandes M, Yrurzun C, Vega Cañizares E, Castillo Domínguez S, Cabezas Cruz A, *et al*. First molecular evidence of *Babesia caballi* and *Theileria equi* infections in horses in Cuba. Parasitol Res. 2018;117:3109-18.

15. Liu Q, Zhao JL, Zhou YQ, Liu EY, Yao BA, Fu Y. Study on some molecular characterization of *Babesia orientalis*. Vet Parasitol. 2005;130:191-8.

16. Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Mol Biol Evol. 2016;33:1870-4.

17. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990;215:403-10.

KY111765), *T. equi ema-1* (KY111766) and *T. equi* 18S rRNA (KY111760, KY111761 and KY111762).

The sequences analyzed were aligned according to the equivalent isolate sequences using the ClustalW algorithm [18]. The maximum likelihood (ML) method was chosen to construct the phylogenetic tree, with the best possible topology, and the genetic distance matrices for the aligned sequences were calculated using the Kimura two-parameter model [19]. The MEGA7 software was used for the graphical representation and phylogenetic tree editing.

Detection and molecular identification of *Rickettsia* in horses and ticks collected from horses in western Cuba

Blood samples were collected from the jugular vein of 164 clinically healthy horses from 6 rural areas: Aguacate, El Perú, Tapaste, San Antonio de las Vegas, Santa Bárbara, Zaragoza and 34 samples from an urban area: San José de las Lajas; a total of 200 horses (126 females and 74 males) were included [20]. Their ages ranged from 5 months to 18 years old, according to their teeth and the information offered by their owners. Up to 209 partially engorged ticks were collected from 11 horses at San José de las Lajas and 68 horses from rural areas. Ticks were morphologically identified using the taxonomic key of Nava et al. [21]. The identity of the *A. mixtum* species was confirmed by sequencing the mitochondrial rRNA 12S and rRNA 16S genes.

DNA was extracted from each horse blood sample and tick, using the Wizard® Genomic DNA Purification kit (Promega, Madison, WI, USA). The presence of *Rickettsia* was detected by real time TaqMan® PCR assay targeting the 17-kDa rickettsial antigen gene (*htrA*) [22]. Samples with a Ct below 40 were considered positive for *Rickettsia*.

Rickettsia species were identified by sequencing the amplicon produced from a nPCR targeting a 394 bp segment of the 17-kDa rickettsial antigen gene [23]. The nPCR was run on all tick samples that were positive for RT-qPCR. Eight ampliconswere purified, five from *A. mixtum* (three females and two males) and three from *D. nitens* (three females), were further subjected to automate DNA sequencing with the primers used for amplification.

Results and discussion

The mean value of all hematological parameters studied was in the reference range described for the equine species. Intraerythrocytic forms were observed, compatible with *B. caballi* and *T. equi* in 3/58 (5.2 %) and 8/58 (13.8 %) of the animals, respectively, of which 6/11 (10.3 %) showed hematocrit values lower than 0.32 L/L. Statistical analyses demonstrated significant differences when evaluating the relationship between the hematocrit values and the samples positive to *T. equi*. On the other hand, the presence of these infections is similarly distributed between animals of different sexes, in the two age groups studied, and it was not favored by the presence of ticks, or by the contact with cattle. However, the animals infected with *T. equi* showed an association with the presence of anemia, in contrast with the animals infected with *B. caballi*.

From these results, we can conclude that in the region studied, the infections with *B. caballi* and *T. equi* are found in horses without any sign of equine piroplasmosis, which may act as a potential source of infection of these hemoparasites in susceptible equine herds.

With the verification of the nPCR assays, it was confirmed that these techniques are feasible for their use under our laboratory conditions, where the bands can be visualized corresponding to the expected sizes of the stages of PCR1 and PCR2, consistent to the description by Battsetseg *et al*. [3]. Other authors, such as Kang *et al*. [24] and Braga *et al*. [25] obtained similar results when performing these assays.

The optimum alignment temperature was set to 56 ºC for all steps, considering the intensity of the amplified bands and the absence of unspecific amplifications. From the results obtained, the 0.4 and 0.2 µM concentrations were selected for the first step of the nPCR assays for *B. caballi* and *T. equi*, respectively; for the second step, 0.2 µM was selected for each primer in both nPCR assays.

The final conditions for temperature and amplification time were arranged as follows for both, *B. caballi* and *T. equi*: one cycle at 96 ºC/4 min, followed by 40 cycles at 94 ºC/1 min, 56 ºC/2 min, 72 ºC/2 min and one final extension of 72 ºC/5 min. The amplification conditions for the PCR2 were similar to those described above, but during 30 cycles. Each assay proved to be specific for the hemoparasite analyzed on evaluating the analytical specificity, in agreement with the results by Battsetseg *et al*. [3].

The standardized nPCR assays showed the sensitive and specific detection of these hemoparasites. Therefore, they can be regarded as powerful diagnostic tools that may be used in the programs for the management and control of these hemoparasite infestations.

As a result of the molecular diagnosis of *B. caballi* and *T. equi* with the use of two nPCR assays in blood samples from 100 animals, 78 (78 %; 95 % CI; 68.5-85.7 %) were positive to the presence of at least 1 of the hematozoa in the study. Of these, five (5 %; 95 % CI; 2.2 %-11.2 %) were infected only with *B. caballi*, 53 (53 %; 95 % CI; 43.3 %-62.5 %) with *T. equi* and 20 (20 %; 95 % CI; 13.4 % -28.9 %) were coinfected. Noteworthy, no animal showed clinical symptoms of the hemolytic syndrome at sampling. This led us to believe that these animals were asymptomatic carriers of *B. caballi* and *T. equi*, and that they may act as a reservoir for the infection of vector ticks that participate in the natural transmission of these hemoparasites.

Even when we are not dealing with an epidemiologic study, the distribution of positive samples leads us to believe that *T. equi* infections may be distributed in different herds of the region studied, considering the number of animals that were positive in the nPCR. Similar results have been described in studies made in Brazil, Spain, Portugal, Italy, Iran, Mongolia and India, where most of the equine herds are carriers of *B. caballi* and *T. equi*. [26-28].

The identification of *B. caballi* and *T. equi* was confirmed by sequencing of fragments of the *bc48* and *ema-1* genes, respectively, from three randomly-

18. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, *et al*. Clustal W and Clustal X version 2.0. Bioinformatics. 2007;23:2947-8.

19. Tamura K, Nei M. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol Biol Evol. 1993;10:512-26.

20. Díaz Sánchez AA, Chilton NB, Roblejo Arias L, Fonseca Rodríguez O, Marrero Perera R, Diyes ChP, *et al*. Molecular detection and identification of spotted fever group rickettsiae in ticks collected from horses in Cuba. Med Vet Entomol. 2020;35(2):207-12.

21. Nava S, Venzal JM, González Acuña D, Martins TF, Guglielmone AA. Chapter 2 - Genera and Species of Ixodidae. In: Nava S, Venzal JM, González-Acuña D, Martins TF, Guglielmone AA, editors. Ticks of the Southern Cone of America. London: Academic Press. 2017. p. 25-267.

22. Jiang J, Stromdah, EY, Richards AL. Detection of *Rickettsia parkeri* and *Candidatus* Rickettsia andeanae in *Amblyomma maculatum* Gulf Coast ticks collected from humans in the United States. Vector-Borne Zoonotic Dis. 2012;12:175-82.

23. Labruna MB, Whitworth T, Horta MC, Bouyer DH, McBride JW, Pinter A, *et al*. *Rickettsia* species infecting *Amblyomma cooperi* ticks from an area in the state of São Paulo, Brazil, where Brazilian spotted fever is endemic. J Clin Microbiol. 2004;42:90-8.

24. Kang SW, Doan HT, Choe SE, Noh JH, Yoo MS, Reddy KE, *et al*. Molecular investigation of tick-borne pathogens in ticks from grazing cattle in Korea. Parasitol Int. 2013;62:276-82.

25. Braga M, Costa FN, Gomes DRM, Xavier DR, Andre MR, Goncalves LR, *et al*. Genetic diversity of piroplasmids species in equids from island of Sao Luis, northeastern Brazil. Rev Bras Parasitol Vet. 2017;26:331-9.

26. Munkhjargal T, Sivakumar T, Battsetseg B, Nyamjargal T, Aboulaila M, Purevtseren B, *et al*. Prevalence and genetic diversity of equine piroplasms in Tov province, Mongolia. Infec Genet Evol. 2013;16:178-85.

27. Peckle M, Pires MS, Dos Santos TM, Roier EC, da Silva CB, Vilela JA, *et al*. Molecular epidemiology of *Theileria equi* in horses and their association with possible tick vectors in the state of Rio de Janeiro, Brazil. Parasitol Res. 2013;112:2017-25.

28. Sumbria D, Moudgil AD, Singla LD. Equine Piroplasmosis: Current status. Veterinaria. 2014;1:9-14.

29. Bhoora R, Franssen L, Closthuizen MC, Guthrie AJ, Zweygarth E, Penzhorn BL, *et al*. Sequence heterogeneity in the 18S rRNA gene within *Theileria equi* and Babesia caballi from horses in South Africa. Vet Parasitol. 2009;159:112-20.

30. Qablan MA, Obornik M, Petrzelkova KJ, Sloboda M, Shudiefat MF, Horin P, *et al*. Infections by *Babesia caballi* and *Theileria equi* in Jordanian equids: epidemiology and genetic diversity. Parasitol. 2013;140:1096-103.

31. Chitimia-Dobler L, Schaper S, Mansfeld P, Gonschorrek J, Bröker M, Nava S. Detection of *Amblyomma mixtum* (Acari: Ixodidae) in Germany on a human traveler returning from Cuba. J Med Entomol. 2020;57:962-4.

selected samples positive to each hemoparasite. This proved that all sequences obtained for *B. caballi* corresponded with the *bc48* gene, which showed 96 to 100 % identity with the sequences reported in Brazil, United States, Egypt, Philippines and Mongolia. In the case of *T. equi* we also confirmed that all sequences obtained corresponded to the *ema-1* gene, and they showed 99 to 100 % identity with sequences from South Africa, Brazil, the United States, Israel, India, Japan and Thailand.

The sequences of the 18S rRNA gene of *T. equi* proved to have 96 to 100 % identity with the sequences of *T. equi* previously deposited at GenBank. The phylogenetic analysis clustered them in clades that corresponded to genotypes A and C, previously described for this agent [29]. The sequence located in clade A showed 99 % identity with sequences from South Africa, Brazil, the United States and India. At the same time, the 2 sequences located in clade C showed 99 % identity with the sequences of South Africa, Brazil and Mexico.

In our study, we identified at least two genotypes of *T. equi* (A and C), in the equine herds of western Cuba, demonstrating genetic diversity among the existing isolates, which is well known even within the herds located in the same geographic region. These results were previously reported in South Africa, the United States, Jordan, Sudan, Mongolia and Turkey [26, 30].

The results obtained show the first molecular evidence of *B. caballi* and *T. equi* infecting horses in Cuba, which was confirmed using gene-sequencing analysis. The positive animals are a reservoir for the infection of other susceptible equines and of vector ticks, which must be considered in the strategy for the management and control programs of these hemoparasites in the country.

In *Rickettsia* identification studies, the ticks collected were 14 *A. mixtum* (4 females and 10 males) and 195 *D. nitens* (58 females and 53 males and 84 nymphs). The nymphs of *D. nitens* were grouped in 19 groups of 2-5 ticks each, collected from the same host.

We detected rickettsial DNA in 29 (20 %) of the 144 tick samples (3 females and 6 males of *A. mixtum*, 12 females and 6 males of *D. nitens*, and 2 sample groups of nymphs of *D. nitens*). The prevalence of *Rickettsia* in *A. mixtum* adults (64 %) was significantly greater ($p < 0.001$) than in adults of *D. nitens* (16 %). The nine adults of *A. mixtum* that were PCR positive for *Rickettsia* were collected from nine horses, and the 18 adults and two groups of nymphs of *D. nitens* that were PCR positive, were collected from 16 horses. Five horses had parasites from both tick species. Of these, three had *A. mixtum* adults, the other two had *A. mixtum* and *D. nitens*, which were all PCR-positive for *Rickettsia*. All horses with tick parasites that were PCR-positive for *Rickettsia* came from rural areas of the Mayabeque province, while the ticks collected in urban areas were all PCR negative.

The nucleotide sequences obtained for the rickettsial 17-kDa gene from the five *A. mixtum* (GenBank Accession Numbers MN885528-MN885532) were identical. The results of the BLASTn analysis revealed that these five sequences were 100 % identical to the sequences of the 17-kDa gene of *R. amblyommatis* from *Amblyomma* spp. in Mexico, Argentina and Brazil, and horses in Brazil. They were also 99 % similar to the DNA sequences of the GAT-30V reference strain of *R. amblyommatis*, and other isolates of this bacterium of *Amblyomma* spp. from Argentina and the United States.

In this work, the detection of *R. amblyommatis* in *A. mixtum* adults of the Mayabeque province was consistent with the findings of Noda *et al*.[7]. They reported a high prevalence of this bacterium in *A. mixtum* as a parasite, in horses and dogs in the Artemisa province. Recently, *R. amblyommatis* was detected in a nymph of *A. mixtum* extracted from a German tourist that returned to his country after a visit to the Mayabeque province [31]. It is not clear whether *R. amblyommatis* is a human pathogen. Despite, the sera of three to six patients that were assumed to have contracted Rocky Mountains Spotted Fever (RMSF) in North Carolina (USA) in 2005, showed seroconversion with a four-fold or more in IgG titers against *R. amblyommatis*, and not to *R. rickettsia* [32], the agent producing RMSF. In 2006, a woman from North Carolina had a rash located within the area where she had been bitten by an Amblyomma americanum tick infected with 'Ca. *R. amblyommii*' [33], showing that *R. amblyommatis* may represent a potential risk for human health.

The nucleotide sequences (394 bp) obtained for the rickettsial 17-kDa gene from three *D. nitens* adults (GenBank Accession Numbers MN885533-MN885535) were 100 % identical to the sequences of the 17-kDa gene from a reference strain of *R. felis* (URRWXCal2/California 2) and other isolates of *R. felis* from Brazil, the United States, Mexico and China.

The presence of *R. felis* in nymphs and adults of *D. nitens* suggests that horses may be a potential reservoir for vertebrates of this bacterium in Cuba. In contrast to tick samples, however, all blood samples from the 200 horses were PCR-negative for *Rickettsia*. This does not exclude the possibility of rickettsial infection in these horses, because the molecular detection in the blood has a relatively low sensitivity after the bacteria start infecting endothelial cells [34], which occurs after the first few days or weeks of an infection [35].

Horses may be a potential reservoir for *R. felis*, if it can be transmitted by other tick species such as *Rhipicephalus sanguineus*, with three hosts and parasiting dogs and humans [36], and sometimes horses [37]. Although no human cases of rickettsial diseases have been reported in Cuba, more studies are required to investigate the potential threat of the infection by *R. felis* in humans and other vertebrates in our country.

In summary, these results represent the first report of *R. felis* in *D. nitens* in Cuba, thus enhancing the knowledge on the spectrum of potential vectors, and the distribution of the pathogens that they can spread in the western part of Cuba.

Conclusions

We now have nPCR assays for the sensitive and specific detection of *B. caballi* and *T. equi*, which may contribute to the strengthening of the management and control programs for these hemoparasites. For the first time in Cuba, we have detected and molecularly identified *B. caballi* and *T. equi* in horses. The phylogenetic analysis

32. Apperson CS, Engber B, Nicholson WL, Mead DG, Engel J, Yabsley MJ, *et al*. Tick-borne diseases in North Carolina: Is "*Rickettsia amblyommii*" a possible cause of rickettsiosis reported as Rocky Mountain spotted fever? Vector-Borne Zoonotic Dis. 2008;8:597-606.

33. Billeter SA, Blanton HL, Little SE, Levy MG, Breitschwerdt EB. Detection of "*Rickettsia amblyommii*" in association with a tick bite rash. Vector-Borne Zoonotic Dis. 2007;7:607-10.

34. Walker DH, Valbuena GA, Olano JP. Pathogenic mechanisms of diseases caused by *Rickettsia*. Ann New York Acad Sci. 2003;990:1-11.

35. Znazen A, Sellami H, Elleuch E, Hattab Z, Ben Sassi L, Khrouf F, *et al*. Comparison of two quantitative real time PCR assays for *Rickettsia* detection in patients from Tunisia. PLOS Negl Trop Dis. 2015;9:e0003487.

36. Dantas-Torres F. Biology and ecology of the brown dog tick, *Rhipicephalus sanguineus*. Parasit Vectors. 2010;3:26.

37. Medrano Bugarini RA, Figueroa Millán JV, Rivera Chavira BE, Lira Amaya JJ, Rodríguez Alarcón CA, Beristain Ruiz DM, *et al*. Detection of *Theileria equi*, *Babesia caballi*, and *Anaplasma phagocytophilum* DNA in soft ticks and horses at Ciudad Juarez, Mexico. Southwest Entomol. 2019; 44:647-58.

of the complete sequences of the 18S rRNA gene of *T. equi* demonstrated the presence of at least two different genotypes of this hemoparasite in horses of the western region, thus confirming their genetic diversity, within equine herds from the same geographical area. It is the first time that *R. felis* is reported in the *D. nitens* tick in Cuba. With the results obtained, we enhance the knowledge on the array of potential vectors and the distribution of pathogens that they can spread in western Cuba.

Acknowledgements

The authors wish to acknowledge Chulantha Prasanga Diyes, Matthew E. M. Yunik, Yanet López Dorta, Marcelina Santos Ordaz, Enrique Pérez Pérez, Cristian Díaz Corona, Fulgencio Pupo Batista, Oscar Fernández Martínez, Armando Perfecto Pérez, Marcio Díaz Ravelo, Kelvin Estrada Porras, Evelio Castellón Madruga, Nelson Albelo Chávez and Eleuterio Hernández Hernández for their cooperation in this study.

Adrian A Díaz Sánchez received a scholarship from the Emerging Leaders in the Americas Program (ELAP), through the International Scholarships Program from Global Affairs Canada. This project was partially funded through a Research Council Scholarship in Engineering and Natural Sciences of Canada to Professor Neil B Chilton.

Conflicts of interest statement

The authors declare that there are no conflicts of interest.

Published translated from: Díaz-Sánchez AA, Corona-González B, Chilton NB, Lobo-Rivero E, Vega-Cañizares E, Yrurzun-Estrada C, *et al*. Detección e identificación molecular de patógenos transmitidos por garrapatas en *Equus caballus* y garrapatas del occidente de Cuba. An Acad Cienc Cuba. 2022;12(1). Available from: http://www.revistaccuba.cu/index.php/revacc/article/view/1125

Received in July, 2021.
Accepted in November, 2021.