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High co-infection rates of *Babesia bovis*, *Babesia bigemina*, and *Anaplasma marginale* in water buffalo in Western Cuba

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Abstract
Water buffalo is important livestock in several countries in the Latin American and Caribbean regions. This buffalo species can be infected by tick-borne hemoparasites and remains a carrier of these pathogens which represent a risk of infection for more susceptible species like cattle. Therefore, studies on the epidemiology of tick-borne hemoparasites in buffaloes are required. In this study, the prevalence of *Babesia bovis*, *Babesia bigemina*, and *Anaplasma marginale* were determined in water buffalo herds of western Cuba. To this aim, a cross-sectional study covering farms with large buffalo populations in the region was performed. Eight buffalo herds were randomly selected, and blood samples were collected from 328 animals, including 63 calves (3–14 months), 75 young animals (3–5 years), and 190 adult animals (> 5 years). Species-specific nested PCR and indirect ELISA assays were used to determine the molecular and serological prevalences of each hemoparasite, respectively. The molecular and serological prevalence was greater than 50% for the three hemoparasites. Differences were found in infection prevalence among buffalo herds, suggesting that local epidemiological factors may influence infection risk. Animals of all age groups were infected, with a higher molecular prevalence of *B. bigemina* and *A. marginale* in young buffalo and calves, respectively, while a stepwise increase in seroprevalence of *B. bovis* and *B. bigemina* from calves to adult buffaloes was found. The co-infection by the three pathogens was found in 12% of animals, and when analyzed by pair, the co-infections of *B. bovis* and *B. bigemina*, *B. bigemina* and *A. marginale*, and *B. bovis* and *A. marginale* were found in 20%, 24%, and 26%, respectively, underlying the positive interaction between these pathogens infecting buffaloes. These results provide evidence that tick-borne pathogen infections can be widespread among water buffalo populations in tropical livestock ecosystems. Further studies should evaluate whether these pathogens affect the health status and productive performance of water buffalo and infection risk of these pathogens in cattle cohabiting with buffalo.

Keywords Water buffalo · Tick-borne pathogens · Prevalence · Co-infections · nPCR · iELISA

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Introduction

Tick-borne diseases (TBDs) pose a major constraint to livestock production in tropical and subtropical regions, with considerable economic losses (Marcelino et al. 2012). In Latin America and the Caribbean countries, the most important tick-borne pathogens are the apicomplexan protozoa *Babesia bovis* and *Babesia bigemina*, and the rickettsia *Anaplasma marginale* (Alonso et al. 1992; Guglielmone 1995; Gondard et al. 2017). These are intracellular pathogens that infect the erythrocytes of the vertebrate host (Suarez and Noh 2011) and, in South America, are mainly transmitted by *Rhipicephalus microplus* (Nari 1995). Babesiosis and anaplasmosis are endemic in this region, forming a complex of diseases also known as cattle tick fever (CTF) (Suarez and Noh 2011). Notably, these pathogens are frequently found co-infecting cattle in endemic areas (Guglielmone 1995; Jirapattharasate et al. 2017). Hofmann-Lehmann et al. (2004) demonstrated the presence of *A. marginale*, *A. phagocytophilum*, *Babesia* spp., *Theileria*, and haemotrophic *Mycoplasma* spp. in a Swiss dairy herd and hypothesized that *A. marginale* was the most important cause of anemia, but co-infections with other agents may aggravate disease development.

Although cattle are the natural host of *B. bovis*, *B. bigemina*, and *A. marginale*, water buffalo (*Bubalus bubalis*) and several wild ruminants can also be infected by these hemoparasites (Chauvin et al. 2009; Kocan et al. 2010). Currently, water buffalo are the focus of scientific research in South America due to the economic importance of this animal in the region (García et al. 2012; FAO 2014). Buffalo and cattle herds frequently coexist in grassland areas, posing a potential risk of cross-species transmission of pathogens. Recent reports showed that cattle were infected with *A. marginale* strains that were also identified in water buffalo in Brazil (Silva et al. 2013, 2014b), a phenomenon also observed in Cuba (Obregón et al. 2018). Despite the fact that buffaloes are more resistant to many infectious and parasitic diseases than cattle, these animals are also infested by ticks (Obregón et al. 2010; Corrêa et al. 2012; Abbasi et al. 2017; Rehman et al. 2017), and they can sustain the complete life cycle of *R. microplus* (Benitez et al. 2012).

Epidemiological surveys conducted in Argentina (Ferreri et al. 2008), Brazil (Silva et al. 2013; Néo et al. 2016; Silveira et al. 2016), and Mexico (Romero-Salas et al. 2016) found buffaloes infected with *Babesia* spp. Additionally, in Mexico, it was found that the infection rate was higher in buffaloes when raised together with infected cattle (Romero-Salas et al. 2016). Likewise, *A. marginale* infection was detected in buffalos in Brazil, either living with cattle or in distant areas. Besides, the prevalences were higher in areas lacking sanitary control programs (Silva et al. 2014c). Evidence suggests that buffaloes constitute an important reservoir of vector-borne pathogens throughout the Latin American region

(Ferreri et al. 2008; Silva et al. 2014a; Romero-Salas et al. 2016; Benitez et al. 2018), and the inclusion of this species in surveillance programs of CTF was suggested (Ferreri et al. 2008; Silva et al. 2014b; Obregón et al. 2018).

Interestingly, water buffalos are resistant to clinical babesiosis and anaplasmosis, even under endemic instability conditions (Terkawi et al. 2011; Mahmmod 2014; Sivakumar et al. 2014; Mahmoud et al. 2015; Benitez et al. 2018). Buffaloes carrying *Babesia* spp. and *A. marginale* have low parasitemia levels (Silva et al. 2014a; Obregón et al. 2016), usually undetectable by Giemsa stained blood smear examination (Corona et al. 2012; Mahmoud et al. 2015; Néo et al. 2016; Benitez et al. 2018). This fact imposes a challenge for direct diagnosis, including molecular techniques which must have a high analytical sensitivity to ensure detection of the pathogens (Néo et al. 2016; Romero-Salas et al. 2016). The combination of molecular and serological assays, such as nested PCR and ELISA, is an effective approach in the epidemiological study of CTF pathogens infecting buffaloes (Ferreri et al. 2008; Terkawi et al. 2011; Silva et al. 2013; Li et al. 2014; Mahmoud et al. 2015; Romero-Salas et al. 2016).

In Cuba, high incidence and mortality of adult cattle due to CTF was reported during the 1980s (Corona et al. 2005). Subsequently, the epidemiological situation progressed to endemic stability as result of an integrated control system of *Boophilus microplus* ticks (Valle et al. 2004), wherein low infection rates are maintained and no clinical disease is observed (LNP. National Laboratory of Parasitology 2014). Water buffaloes were introduced in Cuba in 1989 (Mitat 2009), and there are currently over 60,000 animals spread all over the country, usually in grazing areas adjacent to cattle herds (CENCOP. National Livestock Registration Center 2015). This work aimed to determine the molecular and serological prevalences of *B. bovis*, *B. bigemina*, and *A. marginale* as well as co-infections with these pathogens in water buffalo herds from the western region of Cuba. Consequently, this information will contribute to the understanding and prevention of CTF diseases in endemic areas with the presence of water buffalo herds.

Materials and methods

Study site and sample size determination

A cross-sectional study was conducted in livestock areas in the western provinces of Cuba, covering the regions with the largest buffalo populations, according to the National Center for Livestock Control (CENCOP 2015). The climate of the region is tropical, seasonally humid, with temperature and relative humidity of 24 °C and 75%, respectively. The tick *R. microplus* occurred throughout the year (LNP 2014). Buffaloes in the region are crossbred animals, resulting from

the indiscriminate crossbreeding between buffalypso and carabao. Regarding the production systems, buffalo calves remain with their dams only for 10 days, and thereafter, they are kept in separate groups. Calves usually have no access to wallowing areas (García et al. 2012).

One-stage cluster sampling was applied to determine the number of buffalo herds for the study. The sample size (CI.95%) was calculated according to Thrusfield (2006), by the formula: $g = 1.96^2 [nV_c + P_e (1-P_e)]/nd^2$, where g = number of clusters (buffalo herds), n = predicted number of animals per, V = between-cluster variance (5%), P_e = expected prevalence, and d = desired absolute precision (5%). Afterward, the g value was adjusted from the number of buffalo herds on the region (200 herds, CENCOP 2015) by the formula: $g_{aj} = G \times g/G + g$, where G = number of herds.

The P_e and V values were fixed at 30% and 5%, respectively, according to results of other studies in Latin American countries (Ferreri et al. 2008; Silva et al. 2013; Silva et al. 2014b; Romero-Salas et al. 2016). Finally, eight buffalo herds from the major farms of the region were sampled and nominated from "I" to "VIII" (Fig. 1). All animals on each herd were sampled, totalizing 328 individuals, of which 63 were calves, 75 young animals, and 190 adult animals. All of them were clinically healthy.

Sample collection

Two blood samples were collected in EDTA-treated Vacutainer and anticoagulant-free tubes, respectively, by puncturing the jugular vein of each buffalo. DNA was extracted from 300 μL of EDTA-blood samples by using a Wizard® Genomic DNA Purification Kit (Promega, USA) according to the manufacturer's instructions. The DNA samples were examined for concentration and purity with the Nanodrop spectrophotometer 1000 v.3.5 (Thermo Fisher Scientific, USA). Blood samples without EDTA were incubated at room temperature to allow clotting formation, afterward centrifuged at 3000 rpm for 15 min, and then sera were collected. Both DNA and sera samples were stored at − 20 °C until further use.

Molecular diagnosis

The nested PCR (nPCR) assays targeting the 18S rRNA gene from *B. bigemina* and *B. bovis* were used as described by
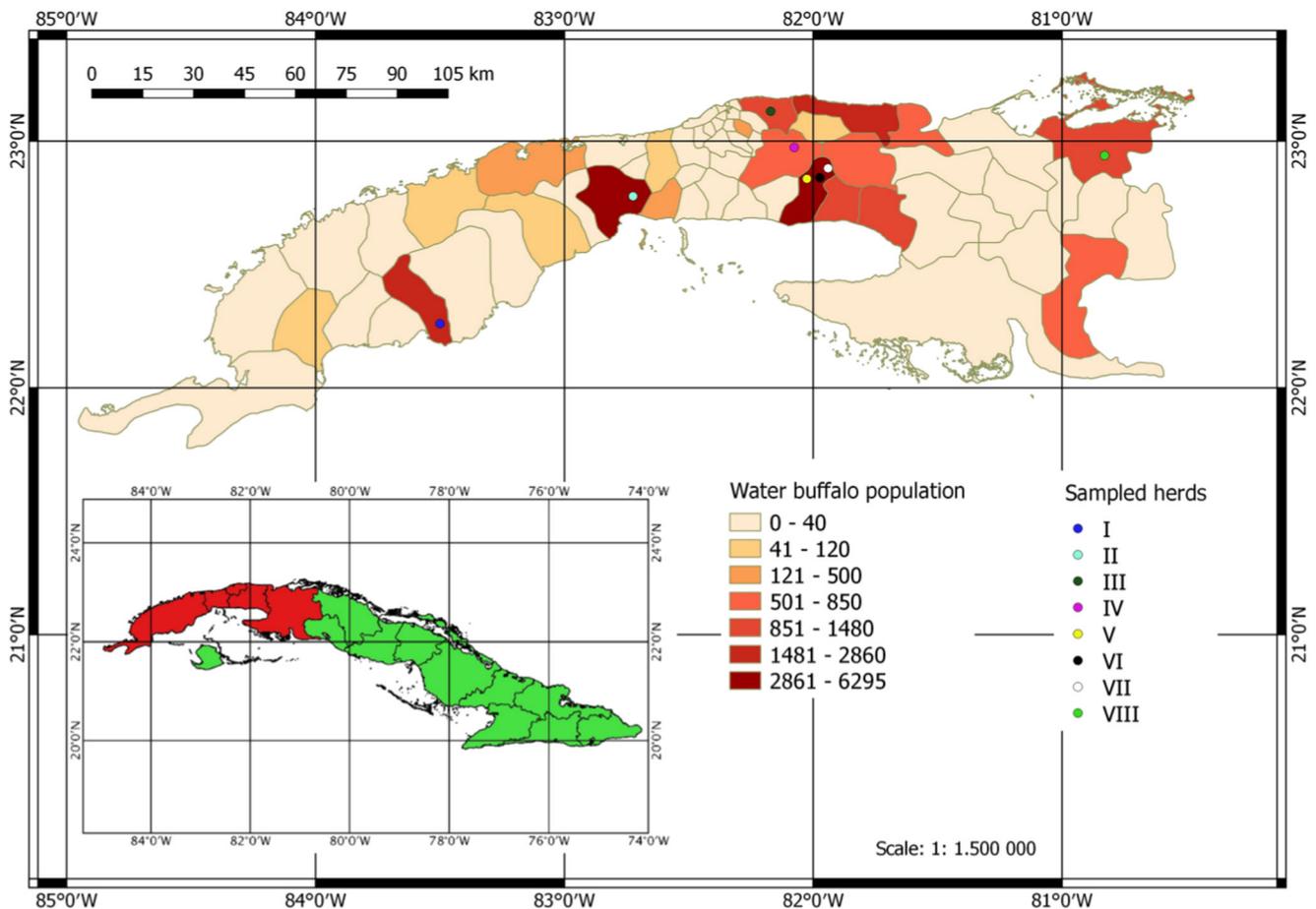

Fig. 1 Geographical location of the sampled buffalo herds (farm). The location of the sampled herds is shown in relation to the water buffalo population in western Cuba (CENCOP 2015), distributed across the municipalities

Guerrero et al. ([2007](#)). For this purpose, the primer Kb_{16} (forward): 5′-CATCAGCTTGACGGTAGGG-3′ and Kb_{17} (reverse): 5′-GTCCTTGGCAAATGCTTTC-3′ were used in a first step (PCR), and after, an nPCR reaction was performed using the primers Kb_{18} (f): 5′GATGTACAACCTCACCAGAGTACC-3′and Kb_{19}(r): 5′-CAACAAAATAGAACCAAGGTCCTAC-3′ to detect *B. bigemina*, and the primer set Kb_{24}(f): 5′-GGGGGCGACCTTCAC-3′ and Kb_{25}(r): 5′-CTCAATTATACAGGCGAAAC-3′ detecting *B. bovis*. The primary PCR reactions were carried out in 25 µL containing 12.5 µL of 2× Jump Start RED TaqReady Mix (Sigma-Aldrich, USA), 1 µL of forward and reverse primers (10 µM), 2 µL of DNA template, and nuclease-free water (Qiagen, USA). The same PCR buffer was used in the nested PCR reactions, using 1 µL of primary PCR product as a template.

The optimal thermocycling parameters were experimentally determined for all PCR protocols in a Mastercycler Gradient thermocycler (Eppendorf, USA). The thermocycling of the primary PCR was started with a cycle at 95 °C for 2 min, followed by 39 cycles at 95 °C for 60 s, 66 °C for 45 s and 72 °C for 45 s, and also included a final extension step at 72 °C for 7 min. The nPCR assays were performed under the same conditions, except the annealing step which was at 62 °C and 64 °C for 45 s for *B. bovis* and *B. bigemina*, respectively.

A nested PCR assay targeting the *msp5* gene of *A. marginale* was used as previously reported by (Torioni de Echaide et al. [1998](#)), by using the primers Ef: 5′-GCATAGCCTCCGCGTCTTTC-3′ and Er: 5′-TCCTCGCCTTGCCCCTCAGA-3′ in the first round, and the primer forward If: 5′-TCCTCGCCTTGCCCCTCAGA-3′ and Er in the nPCR step. The PCR reactions were performed in 25 µL, containing 2.5 µL of 10× PCR buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.30; Invitrogen, USA), 2.5 µL (25 mM) of $MgCl_2$ (Invitrogen, USA), 0.5 µL (10 mM) of each dNTP (Invitrogen, USA), 0.2 µL (0.2 U µL^{-1}) of *Taq* DNA polymerase (Invitrogen, USA), 1.0 µL (10 µM) of each primer, 2 µl of template DNA, and nuclease-free water. The nPCR reactions were performed using 1 µL of PCR product as a template and the same reaction buffer. The same thermocycling conditions were used for both PCR steps, included an initial cycle at 95 °C for 3 min, 35 cycles at 95 °C for 30 s, 60 °C for 45 s, and 72 °C for 30 s.

Genomic DNA from isolate ¨Rio Grande¨ of *B. bovis*, ¨Jaboticabal¨ form *B. bigemina*, and ¨Havana¨ from *A. marginale* were used as positive controls. These isolates were maintained over time, through cryopreservation in 10% dimethyl sulfoxide (DMSO) and systematic inoculations in splenectomized calves as originally described by Machado et al. ([1994](#)) and Corona et al. ([2004](#)), respectively. The infected blood samples were washed in phosphate-buffered saline (PBS) and the buffy coat removed (leucocyte free), then DNA was extracted from infected erythrocytes (iRBC) by using a Wizard® Genomic DNA Purification Kit (Promega, USA).

No-template reactions (NTC) were included in each trial as contamination control. The lower limit of detection (LOD) of each nPCR assay was experimentally determined. For this purpose, ten-fold serial dilutions (10 to 10^{-8} ng) of DNA from positive controls were used as a template in the initial PCR step. All nPCR products were visualized in a 2% agarose gel (Sigma, USA) containing ethidium bromide (0.015%) in 1× TBE buffer (89 mM Tris–HCl, 89 mM boric acid and 2 mM EDTA, pH 8.4).

Serological diagnosis

Purified antigens for each hemoparasite were used in indirect ELISA (iELISA) test. Antigens of merozoite rhoptries of *B. bigemina* and *B. bovis* were produced as described by (Machado et al. [1993](#), [1994](#)). The antigens for *A. marginale* were extracted from initial bodies, isolated from iRBC from a splenectomized calf experimentally infected (at the peak of parasitemia ~ 60%) as described by Silva et al. ([2014c](#)). The blood samples (300 ml) were diluted 1:2 in PBS and centrifuged at 1500×*g* for 20 min at 4 °C. The initial bodies were disrupted by ammonium chloride lysis as described by Machado et al. ([1994](#)). The parasite membranes were isolated by sucrose density gradient centrifugation as described by Hindahl et al. ([1986](#)). The selected bands were collected individually in HEPES buffer (Sigma-Aldrich, USA) and centrifuged at 177000×*g* for 1 h. The protein titration of each band was performed by the bicinchoninic acid method, using a BCA Reagents Kit (Sigma-Aldrich, USA).

The iELISA assays were originally described by Machado et al. ([1997](#)) and used for *B. bovis*, *B. bigemina*, and *A. marginale* in the standardized conditions described by Trindade et al. ([2010](#)), and Silva et al. ([2014b](#)), respectively. The analytical sensitivity and specificity of these assays is 98% and 89% for *Babesia* spp. and 92% and 80% for *A. marginale* respectively. Briefly, 100 µl of antigen (10 ng µL^{-1}) in a sodium bicarbonate-carbonated buffer (0.05 M, pH 9.6) was added to microtiter plates (Nunclon™ Surface, Denmark) and incubated overnight at 4 °C, blocked for 1 h at 37 °C with carbonate-bicarbonate buffer (pH 9.6) and 5% skim milk powder (Sigma-Aldrich, USA) and washed with PBS-Tween 20 buffer (saline phosphate, pH 7.2, 5% Tween 20). Then, 100 µL of sample serum, diluted in PBS-Tween 20 (1: 400), and 5% normal rabbit serum were added in duplicate and incubated at 37 °C for 90 min. After, 100 µL of the alkaline phosphatase-labeled anti-Rabbit IgG (A-0668; Sigma-Aldrich, USA), diluted 1: 10,000 in PBS-Tween 20 with 5% skim milk powder, was added and incubated for 90 min. The substrate pNPP (p-nitrophenyl phosphate) diluted 1 mg mL^{-1} in diethanolamine buffer (pH 9.8) was added, and the plates incubated for 50 min at room temperature. Then, the

reaction was stopped by the addition of 25 μL of sodium hydroxide (3.2 M).

The absorbance was measured at 405 nm using Microplate Reader MRXTC Plus (Dynex Technologies, UK.). The ELISA data were determined by the mean optical densities at 405 nm (OD_{405}). The discriminant absorbance value (cutoff) was established as being two and a half times the mean OD_{405} value of the negative control sera. A reference sample panel of sera was used, including ten serum samples from newborn water buffaloes, which were considered negative controls, and five positive sera of each hemoparasite from adult buffaloes.

Statistical analysis

The molecular and serological prevalences of each pathogen were determined, and calculated the 95% confidence interval for an estimated population of 26,000 buffaloes in the region (CENCOP 2015). The analyses were performed on ProMESA WebSite v1.62 (http://www.promesa.co.nz). The χ^2 test was used to evaluate differences (CI.95%) of each infection rate in between herds; afterward, the Pearson (r) correlation coefficient (CI.95%) was used to assess the association between the molecular and serological prevalence values. The analyses were performed using the statistical software package Statgraphics Centurion v. 16.1.03 (StatPoint technologies, VA).

The association between animal age and the infection prevalence of each pathogen was analyzed. For this purpose, three age groups were considered: (1) buffalo calves (3–14 months), (2) young buffaloes (3–5 years), and (3) adult buffaloes (> 5 years). Due to the breeding system, no animals between 1.5 to 3 years were found. Accordingly, the relative risk and the significance levels of such associations were determined. The analyses were executed using the statistical platform online "VassarStats" (http://www.vassarstats.net).

The frequency of co-infection was explored by pairs of hemoparasites (all possible combinations), analyzing molecular (nPCR) prevalence values. In addition, the conditional probability of co-infection occurrence (expected) was calculated, as multiplying the molecular prevalence of a single infection from each hemoparasite into the pair (i.e., B. bovis × B. bigemina). The differences between observed and expected co-infections prevalence were analyzed using chi-square for a one-dimensional "goodness of fit" test (CI.95%). In this analysis, the chi-square value is equal to the sum of the squares of the standardized residuals (z), and z calculated as $z = $ (observed-expected)/sqrt [expected]. The analyses were performed on the VassarStats website (http://www.vassarstats.net).

Multiple correspondence analysis (MCA) was used to analyze the associations between variable categories: (1) infections (single and co-infection) occurrence and (2) origin of the animals (farm and age). The inertia values were calculated by "Burt matrix" method, and the inertia values determined by decomposition of χ^2. The analyses were performed using the statistical software package Statgraphics Centurion v. 16.1.03 (StatPoint technologies Inc., Warrenton, VA).

Results

Cut off values of nPCR and iELISA assays

The analytical sensitivity of all nPCR protocols was tested, specifically determining their LOD through the analysis of ten-fold dilutions of DNA template. Amplicons of the expected size, 262pb, 217pb, and 345pb, were observed after nPCR reactions using specific primers for B. bigemina, B. bovis, and A. marginale, respectively (Supplementary Fig SF1). The nPCR assays of B. bovis and B. bigemina were able to detect the concentrations of DNA template until 10 fg (10^{-5} ng), while the nPCR for A. marginale was even more sensitive, detecting until 1 fg (10^{-6} ng) of DNA template.

In the iELISA assays, the mean OD_{405} values of the negative control sera were 0.107 ± 0.013 for B. bovis, 0.092 ± 0.012 for B. bigemina, and 0.106 ± 0.015 for A. marginale. Consequently, the cutoff points (OD_{405}) for B. bovis, B. bigemina, and A. marginale ELISA were ≥ 0.268, ≥ 0.230, and ≥ 0.265, respectively.

Molecular and serological detection of Babesia spp. and A. marginale in water buffaloes

The results of the serological and molecular diagnosis are presented in Table 1, in a cross-tabulation arrangement for each hemoparasite. Several seropositive animals resulted in no infected by nPCR and, similarly, many infected animals were seronegative. For example, of 232 B. bovis-seropositive buffaloes, only 52% (120/232) were nPCR-positive, and consequently, only 78% (120/156) of nPCR-positive animals

Table 1 Cross-tabulation of results from molecular (nPCR) and serological (iELISA) detection of B. bovis, B. bigemina, and A. marginale in water buffaloes in western Cuba

nPCR	iELISA								
	B. bovis			B. bigemina			A. marginale		
	(+)	(−)	Total	(+)	(−)	Total	(+)	(−)	Total
(+)	120	36	156	94	63	157	127	132	259
(−)	112	60	172	106	65	171	65	4	69
Total	232	96	328	200	128	328	192	136	328

The data indicate the number of animals in each situation, in relation to the sampled population ($n = 328$)

were seropositive. A similar pattern was observed for *B. bigemina* and *A. marginale*.

The molecular and serological prevalences in the buffalo herds are shown in Fig. 2. A high prevalence was recorded for the three tick-borne pathogens under study, with molecular prevalence of 46.6% (95% CI 41.2–51.9), 47.9% (95% CI 42.5–53.2), and 54.6% (95% CI 51.2–61.9) for *B. bovis*, *B. bigemina*, and *A. marginale*, respectively. No significant difference ($\chi^2 = 4.99$; $p = 0.08$) was observed between prevalence values for each pathogen. However, the highest value of seroprevalence was registered for *B. bovis* 70.7% (95% CI 65.8–75.6) which was significantly different ($\chi^2 = 11.72$; $p = 0.00$) to *B. bigemina* 61% (95% CI 55.7–66.2) and *A. marginale* 58.5% (95% CI: 53.2–63.8).

Infected buffaloes were found in all the buffalo herds sampled (Fig. 2). No significant difference ($\chi^2 = 12.7$; $p = 0.12$) was found on the molecular prevalence of *B. bovis* across the eight buffalo herds. Similarly, there was no difference ($\chi^2 = 15.4$; $p = 0.05$) in the molecular prevalence values of *B. bigemina* infection. However, the differences were significant in the molecular prevalence of *A. marginale* ($\chi^2 = 35.2$; $p < 0.00$), as well as in the seroprevalence of *B. bovis* ($\chi^2 = 52.0$; $p < 0.00$), *B. bigemina* ($\chi^2 = 39.7$; $p < 0.00$) and *A. marginale* ($\chi^2 = 29.5$; $p < 0.00$). No correlation was found between molecular and serological prevalences values for any of the three pathogens (*B. bovis*: $r = 0.43$; $p = 0.28$, *B. bigemina*: $r = 0.05$; $p = 0.52$, and *A. marginale*: $r = 0.18$; $p = 0.25$).

Influence of age on infection prevalence of *Babesia* spp. and *A. marginale* in buffaloes

The nPCR-positive results showed variations when comparing buffaloes of different ages (calves, young, and adult buffaloes). However, different trends were observed for each hemoparasite on the association between infection prevalence and age of host (Fig. 3a). No differences were observed in the prevalence of *B. bovis* among calves (52.4%) and young buffalos (42.7%) ($\chi^2 = 0.94$; $p = 0.33$; RR = 1.22) and regarding adult buffaloes (47.9%) ($\chi^2 = 0.22$; $p = 0.63$; RR = 1.09). In the case of *B. bigemina*, the infection prevalence in young buffaloes was 64.0%, which was significantly higher ($\chi^2 = 10.45$; $p = 0.00$; RR = 1.83) than in calves (34.9%) and adults animals (45.8%) ($\chi^2 = 6.43$; $p = 0.01$; RR = 1.39), respectively. On the other hand, the group of calves presented higher ($\chi^2 = 3.41$; $p = 0.06$; RR = 1.39) infection prevalence of *A. marginale* (71.4%) regarding young buffalos (54.7%) and increased risk of infection ($\chi^2 = 8.76$; $p = 0.00$; RR = 1.45) than aged buffaloes (48.9%).

In seroprevalence, a pronounced stepwise and significant increase of iELISA-positive animals from the youngest to the oldest age group (38.1%, 68.0%, 82.6%) for *B. bovis* ($\chi^2 = 36.07$; $p = 0.00$) and likewise (41.3%, 56.0%, 69.5%) for

B. bigemina ($\chi^2 = 18.22$; $p = 0.00$) (Fig. 3b), suggesting a cumulative effect on humoral immunity in buffalo throughout age increase, possibly associated with the increased chance of encounter (exposure) with *Babesia* spp. In contrast, no defined pattern was observed in seroprevalence of *A. marginale* among age groups, with a non-significant ($\chi^2 = 2.37$; $p = 0.30$) reduction in the group of young buffaloes (60.3%, 48.0%, 62.1%).

Prevalence of co-infections of tick-borne pathogens in water buffaloes

Co-infections by the three hemoparasites were found in all buffalo herds when analyzed by pairs of pathogens. The co-infection rates (nPCR) were 20%, 24%, and 26% for *B. bovis* and *B. bigemina*, *B. bigemina* and *A. marginale*, and *B. bovis* and *A. marginale*, respectively. Furthermore, 12% of buffaloes were co-infected by the three pathogens. When compared with the expected prevalence (calculated as a product of the individual prevalence of each parasite on a pair) we observed no significant differences between these values for co-infection by *B. bovis* and *B. bigemina*, as well as for *B. bigemina* and *A. marginale* (Table 2). However, significant differences were found between the observed and expected prevalence of co-infection by *B. bovis* and *A. marginale* and in the co-infection *B. bovis* and *B. bigemina* and *A. marginale*. Interestingly, around 50% of doubly infected animals presented triple infection, suggesting a synergistic interaction of CTF pathogens underlying co-infection occurrence.

When analyzing the relationship between infections and co-infections occurrence and the origin of animals in a multiple correspondence analyses (MCA), we confirmed independence on the distribution of single infections among farms and also regarding age groups, although a tendency to associate between *B. bovis* and *A. marginale* infection in calves was observed as well as infection by *B. bigemina* in young animals (Fig. 4). In this case, the first 2 dimensions in MCA analysis explained 55.8% of the total variability (inertia), and the variables with the most significant contribution to the total variability were the occurrence of co-infections, firstly the triple infection (inertia = 0.09) and then the co-infection (by two pathogens) of *B. bovis* and *B. bigemina* (0.07), *B. bigemina* and *A. marginale* (0.06), *B. bovis* and *A. marginale* (0.06), respectively. A strong association was observed on the single infection occurrence by the three pathogens that clustered together on the main dimension of the MCA maps (Fig. 4), and a similar trend was observed between all the co-infections by pairs of hemoparasites. Therefore, this indicated a pattern of co-prevalence of these three pathogens in studied buffalo herds, even to parasitizing the same host.

Fig. 2 Molecular (nPCR) and serological (iELISA) prevalence of *B. bovis* (**a**), *B. bigemina* (**b**), and *A. marginale* (**c**) in different water buffalo herds of the western regions of Cuba. The asterisk indicates those prevalence values that differ significantly ($p \leq 0.05$) from the global mean in each dataset (within the set of values of molecular prevalence or seroprevalence respectively)
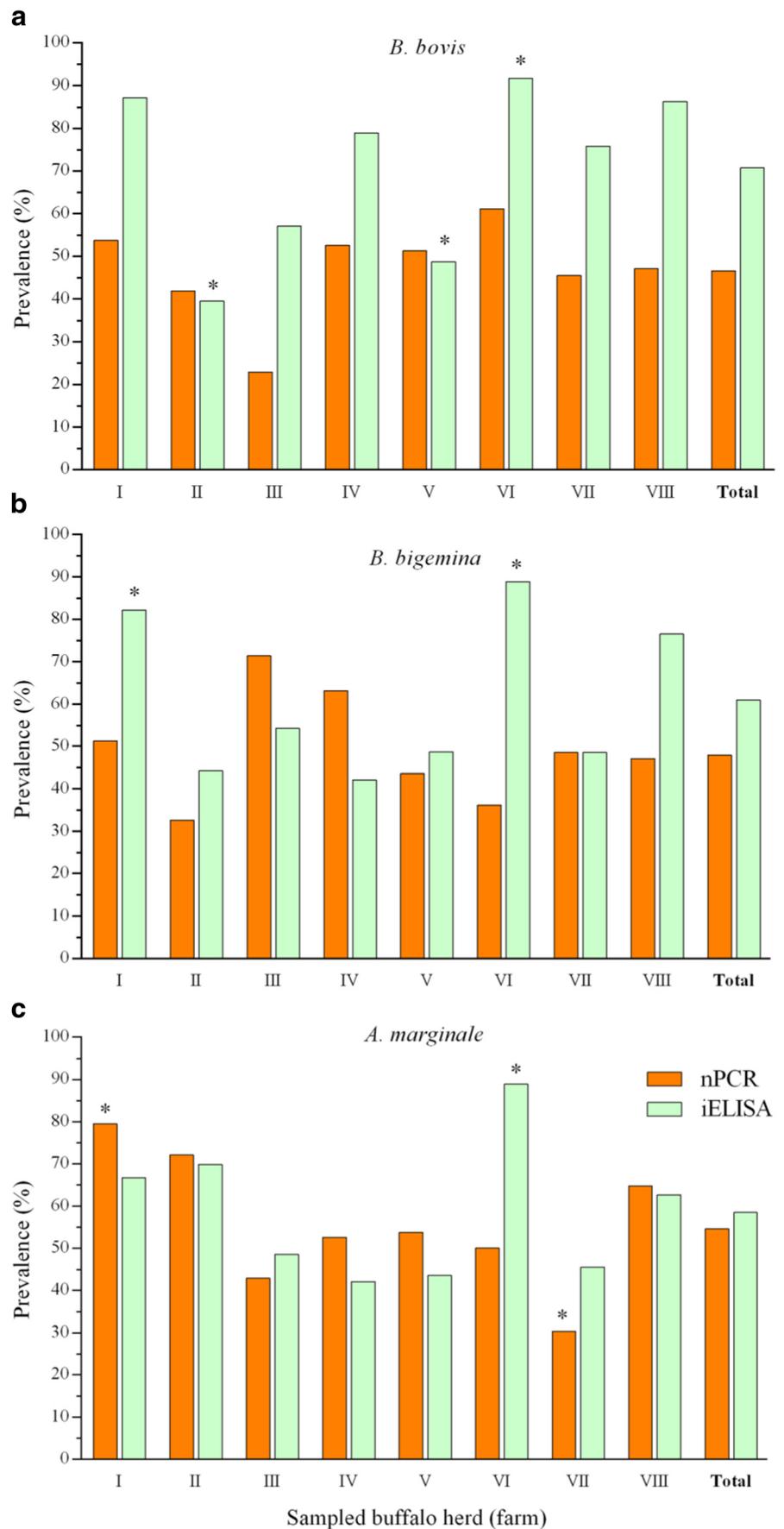
Discussion

Previous studies suggest low parasitemia and sera-antibody levels of infections by *Babesia* spp. and *A. marginale* in water buffalos (Terkawi et al. 2011; Ibrahim et al. 2013; Silva et al. 2014c; Obregón et al. 2016). Consequently, a combination of nPCR and ELISA is a powerful approach for determining the prevalence of these pathogens in cross-sectional surveys on buffalo herds (Terkawi et al. 2011; Silva et al. 2013; Mahmoud et al. 2015; Romero-Salas et al. 2016). In this work we did not intend to evaluate the analytical performance of the nPCR and iELISA tests used; all the assays were selected according to references and were suitable for this study. The results showed that 45% ((112 + 36)/328) of studied buffaloes were only detected as positive by iELISA or by nPCR, respectively (Table 1), as well as 51% and 60% for *B. bigemina* and *A. marginale*, respectively.

The mismatch between the results of serological and molecular diagnoses underscores that nPCR and ELISA assays detect different analytes that display an antipodal time response after infection (Ferreri et al. 2008; Mahmoud et al. 2015), i.e., Benitez et al. (2018), accompanied four buffaloes and five bovines experimentally inoculated with *B. bovis* of which only one buffalo was seropositive at 60 days post infection (dpi), while all bovines were seropositive at 19 dpi. On the other hand, sera antibodies can be detectable for a long period in the infected host (Terkawi et al. 2011), even after parasite clearance as observed by Mahmoud et al. (2015), who did not find buffaloes nPCR-positives for *B. bigemina* among 10 seropositive animals. In addition, water buffalo seem to

Fig. 3 Comparison of molecular prevalence (nPCR) of *B. bovis*, *B. bigemina*, and *A. marginale* in water buffaloes of different age groups: calves (3–14 months), young (3–5 years), and adults (> 5 years). The group of buffalo calves was used as a reference. RR: relative risk. The asterisks indicate statistical significance of the association between age and infection prevalence
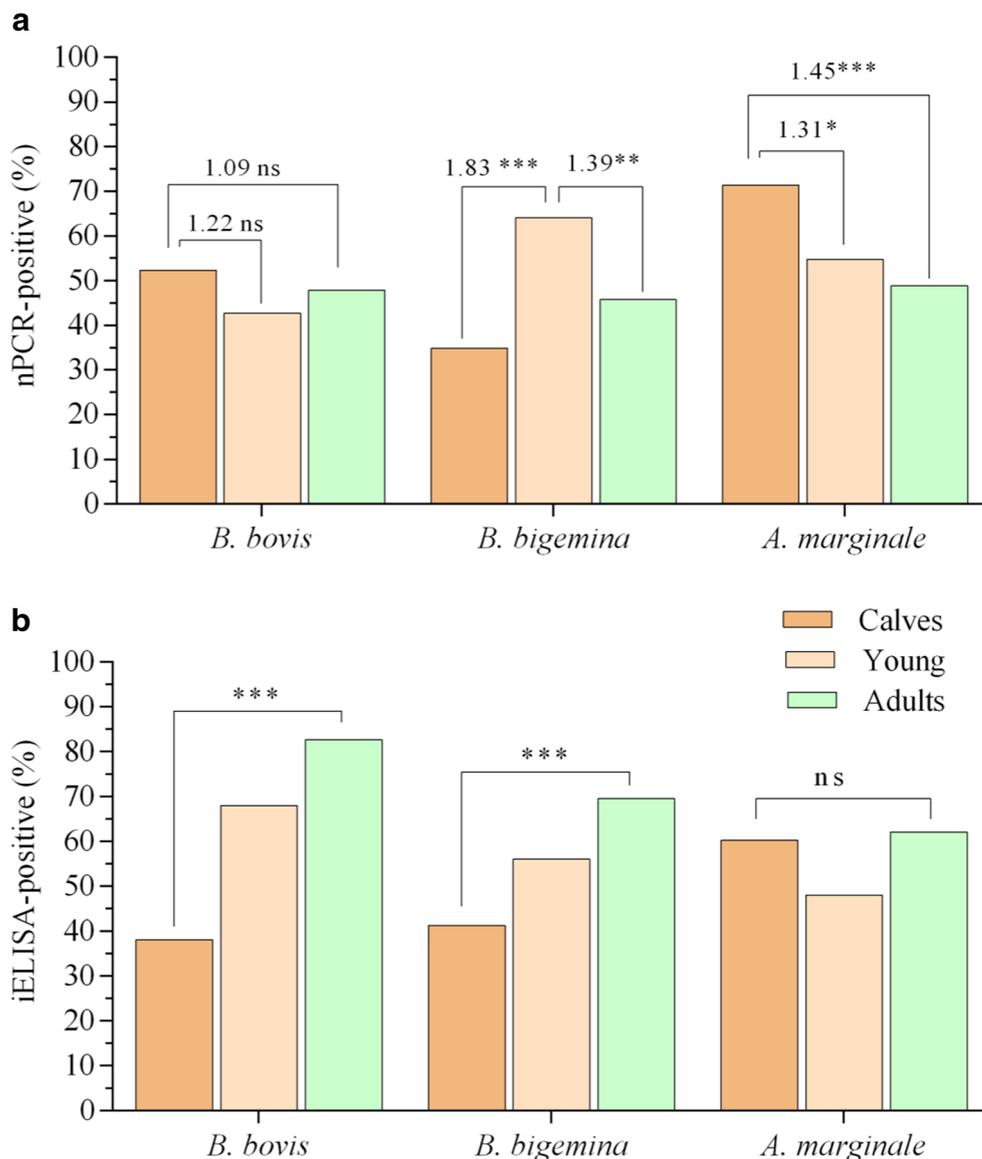
Table 2 Molecular prevalence (nPCR) of co-infections of hemoparasites in buffalo herds

Buffalo herds	n	B. bov / B. big (%)			B. big / A. mar (%)			B. bov / A. mar (%)			B.bo / B.bi / A. ma (%)		
		Obs.	Exp.	z	Obs.	Exp.	z	Obs.	Exp.	z	Obs.	Exp.	z
I	39	26	20	+0.7	41	28	+1.1	41	28	+1.9	23	13	+1.7
II	43	21	26	−0.6	21	30	−1.1	28	14	+2.4	14	11	+0.4
III	35	15	38	−2.1	24	34	−0.9	18	28	−1	12	19	−1.1
IV	19	21	24	−0.2	32	17	+1.5	26	13	+2.1	11	7	+0.5
V	39	26	14	+1.8	26	24	+0.2	33	34	−0.1	15	11	+0.8
VI	36	19	27	−0.8	17	36	−1.9	33	38	−0.4	8	19	−1.4
VII	66	18	23	−0.7	11*	18	−1.4	12*	29	−2.5	3	11	−1.9
VIII	51	20	35	−1.7	31	40	−0.4	25	51*	−2.5	14	26	−1.7
Total	328	20	25	−1.5	26	27	−0.9	24	26	−1.0	12	13	−1.1
Chi-square	–	$\chi^2 = 13.3$; $p = 0.06$			$\chi^2 = 12.0$; $p = 0.09$			$\chi^2 = 24.1$; $p < 0.01$			$\chi^2 = 14.5$; $p = 0.04$		

Obs.: observed prevalence; *Exp.*: expected prevalence; *z*: standardized residual, positive sign when observed>expected and a negative sign when observed<expected. *Indicates values that are significantly different within the same column ($p < 0.05$)

possess a potent innate immune response able to neutralize the parasitemia of CTF pathogens (Mahmmod 2014; Benitez et al. 2018; Obregón et al. 2018). Therefore, the suitability of combining nPCR and ELISA test allows detecting current and previous infections (Terkawi et al. 2011), even with high analytical sensitivity that is crucial for diagnosis at the sub-clinical phase (Mahmoud et al. 2015).

There are few studies on the infection prevalence of *A. marginale* and *Babesia* spp. in water buffalo. However, the prevalence of *B. bovis* and *B. bigemina* appears to differ among tropical regions, depending on the respective continents. For instance, low molecular prevalence (< 11%) of *B. bovis* and *B. bigemina* was found in South Asian countries like Thailand (Terkawi et al. 2011), Sri Lanka (Sivakumar et al. 2014), and South China (He et al. 2011). A similar trend was observed in Egypt (Northern Africa), with molecular prevalence of 4% and 7% for *B. bovis* and *B. bigemina*,

respectively (Ibrahim et al. 2013), or 3% and 0% according to Mahmoud et al. (2015). In contrast, in Brazil, Argentina, and Mexico, the molecular prevalence was close to 20% for each *Babesia* spp. and high seroprevalence was also recorded (Ferreri et al. 2008; Silva et al. 2013; Romero-Salas et al. 2016), reaching, for example, 70% in Mexico (Romero-Salas et al. 2016). The fluctuation of prevalence across geographical regions could be due to differences in tick distribution, micro-climate pattern, breeds, farm management, and the sampling conditions (Ibrahim et al. 2013; Silva et al. 2013).

We could hypothesize that the highest prevalence of *Babesia* spp. in water buffalo in South America is a consequence of cattle and buffalo herds cohabiting in endemic areas, wherein both cattle and buffaloes are infested by the same populations of tick *R. microplus* (Ferreri et al. 2008). The situation may be similar for *A. marginale*. In fact, Silva et al. (2014b) found a greater prevalence of *A. marginale* in

Fig. 4 Multiple correspondences map. The outcome from multiple correspondence analyses (MCA) between the infection prevalence (nPCR) of *B. bovis*, *B. bigemina*, and *A. marginale* (single infection and co-infection) and the origin (farm and age group) of buffaloes under study. The correspondence in co-infection occurrence is highlighted, as well as the correspondence between single infections and age groups of calves and young buffalos
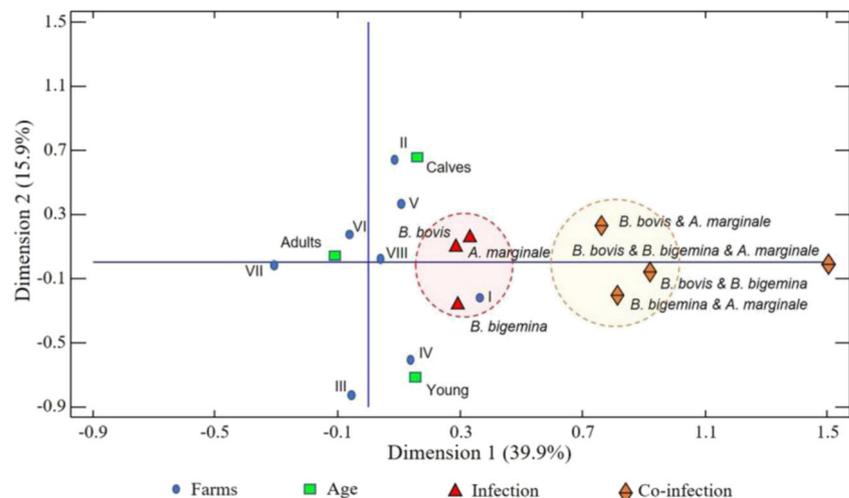
regions of Brazil where cattle and buffalo commingled. Hence, our result supports the idea that tick-borne pathogen infection in buffaloes is influenced by local epidemiological factors including the presence of cattle in nearby regions and the common infestation by *R. microplus* (Terkawi et al. 2011; Ibrahim et al. 2013; Silva et al. 2014c; Romero-Salas et al. 2016). Therefore, we consider that buffalo herds should be included in the surveillance programs of the CTF diseases in those regions where both livestock species co-occur.

Differences in molecular prevalence based on age concurred with the results of Terkawi et al. (2011), who also found a higher prevalence of *B. bovis* and *B. bigemina* in younger animals than in aged. However, as these authors combined iELISA and nPCR prevalence data, it is difficult to compare our results with theirs. Instead, Ibrahim et al. (2013) in Egypt and Romero-Salas et al. (2016) in Mexico showed that molecular and serological prevalence exhibit different patterns across age. We did not observe significant differences on the molecular prevalence of *B. bovis* in aged buffaloes compared to younger animals and calves respectively (Fig. 3a), which is in agreement with Ibrahim et al. (2013) who did no observed differences in the molecular prevalence of *B. bovis* between aged and younger buffaloes. However, it does not match with the results of Romero-Salas et al. (2016) who found prevalence values of 88.3%, 87.7%, and 71.0% in the aged, younger, and calves respectively. Differences in the sample scaling strategy on these studies limit the recognition of patterns of association between age and infection prevalence. Further studies may include an improved age stratification, considering immuno-physiological parameters.

A clear trend was observed on the molecular prevalence of *A. marginale* that was higher in calves and decreased in younger and adult buffaloes, respectively, although we do not find any relevant reference on this aspect. However, our result on the molecular prevalence of *B. bigemina* (higher prevalence in younger animals, Fig. 3a) was different from Romero-Salas et al. (2016), which did not find significant differences between groups of calves, younger, and adults (30.0%, 26.6%, 18.3%, *p* = 0.3), respectively. On the other hand, the seroprevalence of *B. bovis* and *B. bigemina* was in increasing between groups of calves, young, and adult buffaloes, respectively (Fig. 3b). A similar trend was observed by Romero-Salas et al. (2016), and this could be explained by a cumulative humoral immunity in response to parasite exposure that increases the chance that older animals will be found seropositive (Romero-Salas et al. 2016).

Since tick infestation rate is a key factor on the dynamics of CTF (Aubry and Geale 2011; Jonsson et al. 2012), the higher infection prevalence of CTF in buffalo calves seems to be linked with their ability to sustain tick

populations. While adult buffaloes are resistant to *R. microplus* infestation (Nithikathkul et al. 2002; Corrêa et al. 2012), calves with a thinner skin seem to be more susceptible to tick infestation and, therefore, at increased risk for exposure to tick-borne pathogens. This hypothesis is supported by a previous study reporting 28% tick infestation in adult buffaloes (having two adult ticks per animal) compared to 95% tick infestation in buffalo calves (20 adult females per animal) (Obregón et al. 2010). Nevertheless, the results of this work, as well as others mentioned above, did not find a higher prevalence of *Babesia* spp. in buffalo calves; hence, more aspects of vector-pathogen-host interaction must be evaluated in further studies (i.e., the inoculation rate of pathogens, the occurrence of persistent infection of *B. bovis* in buffaloes, etc.)

To the best of our knowledge, this is the first report of a high prevalence of *Babesia* spp. and *A. marginale* co-infections in buffaloes. These results agree with the coexistence of these pathogens in most endemic areas, where co-infections are common and likely to be the cause of comorbidities in cattle (Alonso et al. 1992; Guglielmone 1995; Ochirkhuu et al. 2015). Two different patterns of co-infections by CTF pathogens were observed in this study: (i) co-infections following the "law of conditional probability" where prevalence estimates for individual pathogens serve as predictors of co-infection rates (i.e., *B. bovis* and *B. bigemina* and *B. bigemina* and *A. marginale*) and (ii) co-infections that did not follow the "law of conditional probability" (i.e., *B. bovis* and *A. marginale* and triple co-infections). The explanation of the first type of co-infection is straightforward; the higher prevalence of individual pathogens will increase the probability of finding more than one pathogen in the same host. However, the second type of co-infection is more complex and may be influenced by other factors related to vector-pathogen-host interactions. For example, during a co-infection, pathogens can interact between them and with host-related symbionts for utilization of host resources or through modulation of host immunity (Graham 2008).

On the other hands, differences in co-infection rates of *B. bovis* and *B. bigemina* were found between buffaloes and bovines (3.2% vs. 77%) by Romero-Salas et al. (2016), who attributed it to the strong immune competence of buffalo hosts limiting *Babesia* spp. infections. Thus, further studies should be carried out focusing on the epidemiological dynamics of the CTF infectious process, and on the parasite-host-vector interaction in water buffalo, taking into account that the risk for pathogen dispersal, establishment, and colonization is determined by the suitability of the host, the transmission rates between hosts, and the strength of pathogen propagule

pressure (Diuk-Wasser et al. 2016). In such an analysis, it should also be considered that the emergence of a pathogen often involves multiple interactions (positive and negative) on several ecological scales (i.e., host, herds, regions).

Conclusion

Infection prevalence of *B. bovis*, *B. bigemina*, and *A. marginale* above 50% in water buffalo in the western region of Cuba is strong evidence that the bubaline species participate in the epidemiological process of these tick-borne diseases. Differences in infection prevalence among buffalo herd suggest the influence of local epidemiological factors even within the same endemic region. These pathogens are affecting calves, young, and aged buffaloes, although different trends are observed in molecular and serological prevalence; thus, no risk groups were found according to age although the calves were the most infected by *A. marginale*. High co-infection rates of *Babesia* spp. and *A. marginale* were found in buffalo; thus, there is a likely positive interaction between these pathogens underlying the infection of buffalo hosts. However, there is little scientific information on the infection dynamics of CTF pathogens in buffaloes, which requires thorough studies, considering the economic importance of tick-borne pathogens in the Latin American region and the imminent expansion of the buffalo species in several countries.

Compliance with ethical standards

The procedures involving animals in this work were according to the principles established by The International Guiding Principles for Biomedical Research Involving Animals (2012). Consequently, the committee on ethics and animal welfare at CENSA approved the experimental design of this research. The Frontier Veterinary Service of the Institute of Veterinary Medicine under the Ministry of Agriculture of the Republic of Cuba authorized the export of the DNA samples used in this work, under the Zoosanitary Export Certification number R.S.1522010.

Conflicts of interest The authors declare that they have no conflict of interest.

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