Molecular and serological detection of *Babesia bovis*- and *Babesia bigemina*-infection in bovines and water buffaloes raised jointly in an endemic field

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ABSTRACT

Babesia bovis and *Babesia bigemina* are causative agents of bovine babesiosis, a tick-borne disease of cattle in tropical and subtropical regions. *Babesia* spp. infection adversely affects cattle health and can be fatal resulting in considerable economic loss worldwide. Under endemic stability conditions, herds contain high numbers of chronically infected, asymptomatic carrier animals, in which no parasitemia is detected by microscopic blood smear examination. In addition to bovines, also water buffaloes are infected by both *Babesia* spp. commonly leading to a subclinical infection. The infection rate (by nPCR) and herd exposure (by IFAT) of bovines and water buffaloes reared under similar field conditions in an area of endemic stability were determined and compared. In order to optimize direct parasite detection, highly sensitive nPCR assays were developed and applied, allowing the detection of as little as 0.1 fg DNA of each *Babesia* pathogen. Significantly lower percentages ($p < 0.001$) of seropositive water buffaloes compared to bovines were observed for *B. bovis* (71.4% *vs.* 98%) and *B. bigemina* (85% *vs.* 100%). Interestingly, in comparison, differences noticed between water buffaloes and bovines were considerably larger with direct parasite detection by nPCR (16.2% *vs.* 82.3% and 24% *vs.* 94.1% for *B. bovis* and *B. bigemina*, respectively).

As expected, bovines subjected to monthly acaricide applications exhibited a significant lower infection rate as determined by nPCR than bovines not subjected to these measures (*B. bovis* 33.3% *vs.* 90.7%, $p < 0.001$; *B. bigemina* 80% *vs.* 96.5%, $p < 0.001$, for treated *vs.* untreated animals). Interestingly no differences between these groups were observed with respect to seropositivity, suggesting similar rates of parasite exposure (*B. bovis* 100% *vs.* 97.7%, $p < 0.001$; *B. bigemina* 100% *vs.* 100%, $p < 0.001$). Importantly, a significantly higher number of water buffaloes as determined by nPCR were infected when reared jointly with bovines not subjected to tick control than when reared jointly with bovines subjected to tick control (*B. bovis* 31.6% *vs.* 9.5%, $p < 0.01$; *B. bigemina* 42.1% *vs.* 9.5%, $p < 0.01$, for water buffaloes reared with untreated *vs.* treated bovines) and/or when reared without bovines (*B. bovis* 31.6% *vs.* 11.6%, $p < 0.01$; *B. bigemina* 42.1% *vs.* 20%, $p < 0.01$). An accumulation of seropositivity and a decline of infection rates were observed in older animals, while differences observed with regard to gender may warrant further investigation. In summary, our findings suggest that water buffaloes are much more capable to limit or eliminate *Babesia* infection, possibly due to a more capable immune defense. Furthermore, an increased *Babesia* spp. parasite reservoir of bovines seems to increase the infection rate of water buffaloes when both are reared on the same pasture.

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1. Introduction

Bovine babesiosis is the economically most important vector-transmitted disease of cattle in tropical and subtropical regions worldwide (Bock et al., 2004). The causative agents are tick-transmitted intraerythrocytic apicomplexan protozoans of the genus *Babesia*, among which *B. bovis* and *B. bigemina* are the most prevalent (Schnittger et al., 2012). Clinical disease caused by *B. bigemina* infection is characterized by fever, hemoglobinuria, and acute anemia. In contrast, *B. bovis* infections can take a more severe and often fatal course, since in addition to high fever and hemoglobinuria, it also involves nervous symptoms, like incoordination, abnormal salivation, teeth grinding, and lethargy (Bock et al., 2004; Rodriguez et al., 2013; Florin-Christensen et al., 2014).

Traditionally, parasite detection by microscopic examination of blood smears is used for bovine babesiosis diagnosis during the acute phase. After recovery from clinical disease, at the onset of the chronic phase of infection, parasitemia drops to an extremely low level in asymptomatic carrier animals and is commonly undetectable by microscopic diagnostics. In contrast, PCR-based diagnostics has the potential to detect directly the parasite with high specificity and sensitivity (Mosqueda et al., 2007, 2012). Notwithstanding, it has been demonstrated that parasites of carrier animals often escape detection even from highly sensitive PCR-based diagnostic methods, posing a challenge to further optimize nPCR assay design and format in order to maximize sensitivity (Calder et al., 1996; Gubbels et al., 1999; Oliveira-Sequeira et al., 2005; Martins et al., 2008).

Alternatively, serological diagnostic methods, such as indirect immunofluorescent antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA), can be applied to detect parasite-specific antibodies (Mosqueda et al., 2012). However, presence of antibodies as a sign of previous parasite exposure does not necessarily correspond with the current infectious status as antibody titers may decline with time. Further drawbacks of serology methods are that they cannot be applied to young calves due to possible interference with colostrum antibodies and specificity is inferior to molecular diagnostics as cross-reaction is frequently observed (Passos et al., 1998).

Since water buffaloes are robust and easily adapt to poor pasture and floodable lands, they often constitute an attractive alternative to cattle livestock. Commonly, water buffaloes are raised together with bovines on the same pastures and are not included in sanitation campaigns (da Silva et al., 2014). Recently, it has been demonstrated that the *Babesia* spp. vector tick *Rhipicephalus microplus* can complete its life cycle on water buffaloes, although reaching a considerably lower infestation rate than in bovines (Benitez et al., 2012). Molecular and serological assays have demonstrated that buffaloes can be infected by *B. bovis* and *B. bigemina*, albeit, clinical disease is not observed even under conditions of endemic instability and low herd immunity (Ferreri et al., 2008; Terkawi et al., 2011). Based on this observation, it has been hypothesized that buffaloes constitute an important reservoir of parasite infection, potentially increasing bovine babesiosis of cattle and impeding efforts of vector tick and pathogen eradication.

In the context of this study, we have designed a nPCR assay for specific and highly sensitive *B. bovis* and *B. bigemina* detection. This novel molecular tool, as well as a serological assay, was applied to assess and compare the infection rate and herd exposure to *B. bovis* and *B. bigemina* of bovines and water buffaloes raised in the same fields under a situation of endemic stability. Three different contrasting epidemiological situations were compared: (i) bovines and buffaloes raised jointly without tick control, (ii) bovines and buffaloes raised jointly and only bovines, but not buffaloes, have been subjected to tick control, and (iii) exclusively buffaloes were raised without tick control. Comparison of contrasting epidemiological field situations, including animal age and gender, allowed to provide a first insight into the interrelated infection dynamics between bovines and water buffaloes.

2. Materials and methods

2.1. Parasite sampling and genomic DNA extraction

A total of 203 bovines and 154 water buffaloes were sampled. They belonged to three different farms, located in the county of Papaloapan, Veracruz state, México: La Granja (173 bovines, 38 water buffaloes), San Agustin (30 bovines, 21 water buffaloes), and El Bufalo (95 water buffaloes). Sampling was carried out from December 2011 to July 2012. None of the sampled animals showed signs of clinical disease. Bovines of San Agustin farm were subjected to tick control by monthly dipping in amitraz (Taktic®), according to the manufacturer instructions, while La Granja bovines as well as water buffaloes from the three farms had no acaricide treatment.

Bovine blood was sampled from the coccygeal vein and water buffalo blood, from the yugular vein. Two types of blood samples were obtained from each animal, one with citrate as anticoagulant and one without anticoagulants. The first were used for isolation of genomic DNA, following the procedure of Bartlett and Stirling (2003). The latter were centrifuged at $1000 \times g$ for 15 min, and the sera harvested and stored at $-20\,°C$ until further use. In addition, genomic DNA was isolated from blood of a bovine experimentally infected with *B. bigemina*, strain S1A, and from *in vitro* cultured *B. bovis*, strain T2Bo (Aguirre et al., 1989; Hines et al., 1992). The DNA concentration was adjusted to 500 pg/μl and stored at $-20\,°C$ for subsequent PCR and nPCR sensitivity assays.

2.2. PCR and nPCR

Amplification by PCR and nPCR was carried out with newly designed primer pairs specifically binding the apocytochrome b genes (*CYTb*) of *B. bovis* (accession no: AF053002, external primer pair: oBb_mit_F/oBb_mit_R, internal primer pair: iBb_mit_F/iBb_mit_R) and of *B. bigemina* (accession no: AF109354, external primer pair: oBig_mit_F/oBbig_mit_R, internal primer pair: iBbig_mit_F/iBbig_mit_R) (Table 1). Genomic DNA samples of *Anaplasma marginale, B. bigemina* or *B. bovis* were used as templates to test possible cross reactivity of the novel primer pairs. As a sensitivity control, well-described species-specific primers for the detection of *B. bigemina* and *B. bovis* were used and the PCR and nPCR reactions done as previously reported (Figueroa et al., 1993).

The first and second PCR amplification reactions were carried out in $1\times$ Dream Taq buffer (Thermo Scientific, Waltham, Massachusetts, USA), containing 2 mM $MgCl_2$, 200 μM of dNTP, 0.5 μM of forward and reverse primers, and 0.3 U Dream Taq DNA Polymerase. Nine μl genomic DNA were added as template to the first PCR reaction, and 2 μl of the generated amplicon were used as a template for the second PCR amplification. For nPCR sensitivity assays, ten-fold serial dilutions of DNA (1 ng to 10^{-11} ng) were used as template in the initial PCR round. The final volume of reactions was 12.5 μl.

Thermocycling conditions of PCR amplifications started with an initial denaturation for 3 min at $95\,°C$ after which 30 cycles were carried out consisting of denaturation for 30 s at $95\,°C$, an annealing step according to the used primer pairs as indicated in Table 1, and an extension reaction for 30 s at $72\,°C$. A subsequent final extension reaction consisted of 7 min at $72\,°C$. Amplicons were subjected to electrophoresis on a 1.8% agarose gel, stained with ethidium bromide and visualized by UV light.

Table 1
Primer sets used for *Babesia bovis* and *Babesia bigemina* DNA detection.

Hemoparasite	Assay	Primer name	Primer sequence	Amplicon size (bp)
B. bovis	PCR	oBb_mit_F	TGAACAAAGCAGGTATCATAGG	260
		oBb_mit_R	CCAAGGAGATTGTGATAATTCA	
	nPCR	iBb_mit_F	TCCACGATCTGTGATACGTCA	195
		iBb_mit_R	CAAATCCTTTGCAAACTCCAA	
B. bigemina	PCR	oBbig_mit_F	TCCAACACCAAATCCTCCTA	394
		oBbig_mit_R	CGTGGGTTTCGTTTTTGTAT	
	nPCR	iBbig_mit_F	AAGAGATACCATATCAGGGAACCA	250
		iBbig_mit_F	TTGGGCACTTCGTTATTTCC	

2.3. Immunofluorescence antibody test (IFAT)

Serum samples were tested by IFAT for the presence of specific antibodies against *B. bigemina* and *B. bovis*, respectively. Slides were coated with blood from splenectomized bovines experimentally infected with field strains of *B. bigemina* or *B. bovis*, and fixed with absolute acetone (Boonchit et al., 2006). Before application, all serum test samples and appropriate positive and negative control samples were 1:80 diluted in PBS, pH 7.0 containing 4% (v/v) fetal bovine serum. After incubation with fluorescein-conjugated goat anti-bovine IgG (ThermoFisher Scientific, USA), slides were inspected under oil immersion at $1000\times$ magnification using a fluorescent microscope (Primo Star, Zeiss, NY, USA).

2.4. Cloning and sequencing of amplicons

PCR products from three independent amplifications were each ligated into pJET1.2/blunt Cloning Vector (Thermo Scientific, Waltham, Massachusetts, USA). The ligation reaction was subsequently used to transform *Escherichia coli* TOP 10-competent cells. After plating, recombinant clones were selected and cultured overnight, plasmids were purified using ADN Puri Prep-P kit (Inbio Highway, Tandil, Argentina), and inserts were bi-directionally sequenced (Macrogen, Korea).

2.5. Statistical analysis

Chi-square and exact Fisher tests were used to analyze the frequency of *B. bigemina* and *B. bovis*- infection in bovines and water buffaloes with respect to farm, age, and gender. All analyses were made utilizing STATA, Data Analysis and Statistical Software (StataCorp, 2015; Texas, USA).

3. Results and discussion

3.1. Sensitivity and specificity of a nPCR assay for the detection of B. bovis and B. bigemina

The sensitivity of novel designed *CYTb*-targeting nPCR primer pairs to detect *B. bovis* and *B. bigemina* was tested in comparison with the well-characterized nPCR assay reported by Figueroa et al. (1993). Ten-fold dilutions ranging from 1 to 10^{-11} ng genomic DNA of *B. bovis* and *B. bigemina* were subjected to both nPCR protocols. Amplification products of the expected sizes were observed after the first and second PCR reactions (Figs. 1 and 2, Table 1). The detection limit of *B. bovis CYTb* specific primers was determined at 10 pg in the first amplification round while nPCR allowed to detect as little as 0.1 fg (10^{-10} ng) (Figs. 1 B and 2 B). Fig. 2 demonstrates a 100 times higher sensitivity of *CYTb* primers as compared to those described by Figueroa et al. (1993) that target the *rap-1* gene (Figs. 1 A and 2 A). In addition, this new method was also 100 times more sensitive than a recently reported nPCR assay that targets a membrane protein-encoding gene (AbouLaila et al., 2010). Similar detection limits for the first and second rounds were

found for *B. bigemina CYTb* nPCR (Figs. 1 D and 2 D), demonstrating an at least 1000 times higher sensitivity than the nPCR that targets a specific *Spe*I–*Ava*I restriction fragment, using the primers described by Figueroa et al. (1992, 1993),) (Figs. 1 C and 2 C). Notably, using the same *CYTb* target gene, a similar sensitivity of *B. bovis* and *B. bigemina* detection as displayed by the presented nPCR has been also demonstrated in a real-time PCR format (Criado-Fornelio et al., 2009). Importantly, as the genomic DNA used for the sensitivity assay of *B. bigemina* was extracted from whole blood, it contains significant amounts of host DNA diluting the parasite DNA. This indicates that even lower amounts than 0.1 fg of *B. bigemina* DNA can be detected with the method here described. The *CYTb* gene has also been successfully used for the molecular detection of the related piroplasmid *Theileria annulata* (Bilgiç et al., 2010, 2013).

The *B. bigemina* nPCR assay produced, in addition to the expected fragment, a secondary band of the same size as that generated in the first amplification round. Sequencing of this fragment and that generated in the first PCR reaction confirmed their sequence identity, which corresponded to the *CYTb* target gene. The secondary amplicon faded out in nPCR reactions when higher genomic DNA template dilutions were used. These observations strongly suggest that the secondary fragment is generated during the second nPCR round by carry-over residues of first round primers.

The specificity of *CYTb* primer pairs selected for *B. bovis* and *B. bigemina* detection was tested in the first PCR and nPCR reactions using DNA templates of bovine hemoparasites that are endemic in the study region. PCR and nPCR assays for *B. bovis* showed no cross-reactivity with *B. bigemina* and *vice versa*. Additionally, no amplification was obtained in any of these assays when genomic DNA of *A. marginale* was used as template (Figs. 1 and 2). Specificity of both nPCR assays could be further corroborated in the subsequently described screening of field samples as amplification fragments of the expected sizes were always obtained.

3.2. Molecular and serological detection of B. bovis and B. bigemina in bovines and water buffaloes from a tick-endemic area of Mexico

The presence of *B. bovis* and *B. bigemina* DNA in 203 bovine and 154 water buffalo blood samples from Veracruz, Mexico, was investigated using the developed *CYTb* nPCR assays, and results were compared to the presence of specific antibodies by IFAT. By nPCR, 82.3% and 94.1% of the bovines were positive for *B. bovis* and *B. bigemina*, respectively. In comparison, when IFAT was applied, 98% and 100% of the bovines tested were seropositive for *B. bovis* and *B. bigemina*, respectively. In comparison, only 16.2% and 24% of the water buffaloes tested nPCR-positive, while 71.4% and 85% tested IFAT-positive for *B. bovis* and *B. bigemina*, respectively (Fig. 3, Table 2).

The high detection rates of *B. bovis* (82.3%) and *B. bigemina* (94.1%) infections observed in bovines when using the *CYTb* nPCR assays corroborate their high sensitivity. Earlier reports based on other nPCR formats have described that *Babesia* spp. may often escape nPCR detection due to low parasitemias in carrier animals
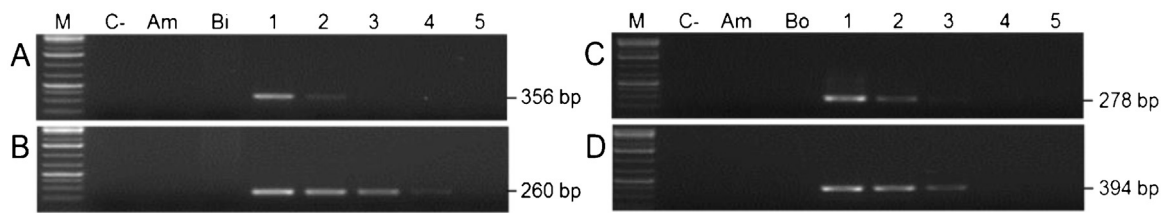
Fig. 1. Determination of the sensitivity limits of direct PCR detection of *Babesia bovis* and *Babesia bigemina* DNA. Different amounts of *B. bovis* or *B. bigemina* DNA were used as template for nested PCR amplification using the first round primer sets described by Figueroa et al. (1993) (A, C) or *CYTb*-targeting primers (B, D). Lanes 1–5 correspond to the following quantities of *B. bovis* (A, B) or *B. bigemina* (C, D) DNA: 1, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} ng, respectively. C-: no DNA template; Am, Bo and Bi: *Anaplasma marginale*, *B. bovis*, and *B. bigemina* DNA templates were used as negative controls; M: DNA ladder. The size of the generated bands is indicated.
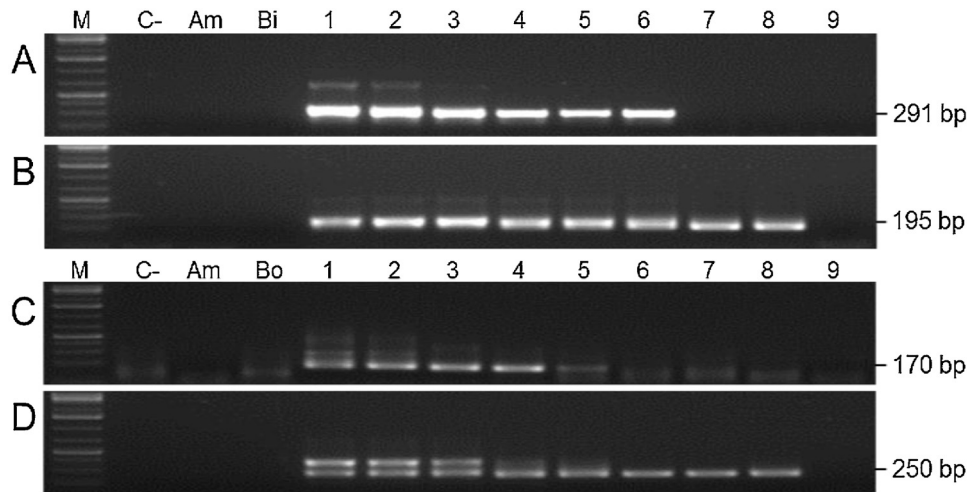
Fig. 2. Determination of the sensitivity limits of nested PCR detection of *Babesia bovis* and *Babesia bigemina* DNA. The amplification products of the first PCR round, shown in Fig. 1, for *B. bovis* (A, B) and *B. bigemina* (C, D) were used as templates for nPCR assays, using the second round primer sets described by Figueroa et al. (1993) (A, C) or those targeting *CYTb* genes (B, D). Lanes 1–9 correspond to template DNA amounts in the first PCR round of 1, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} ng, respectively. C-: no DNA template; Am, Bo and Bi: *A. marginale*, *B. bovis*, and *B. bigemina* amplification products from the first round were used as negative controls; M: DNA ladder. The size of generated bands is indicated.

Table 2
Serological and molecular detection of *Babesia bovis* and *Babesia bigemina* in bovines and water buffaloes.

	Bovines (n = 203)				Buffaloes (n = 154)			
	IFAT[a] (%)		nPCR[b] (%)		IFAT[a] (%)		nPCR[b] (%)	
			(+)	(−)			(+)	(−)
B. bovis	(+)	98.0	80.3	17.7	(+)	71.4	12.3	59.1
	(−)	2.0	2.0	0.0	(−)	28.6	3.9	24.7
			82.3	17.7			16.2	83.8
B. bigemina	(+)	100.0	94.1	5.9	(+)	85.1	18.8	66.2
	(−)	0.0	0.0	0.0	(−)	14.9	5.2	9.7
			94.1	5.9			24.0	75.9
Mixed infection	(+)	98.0	77.3	20.6	(+)	60.4	3.2	57.1
	(−)	2.0	2.0	0.0	(−)	39.6	37.7	1.9
			79.3	20.6			40.9	59.1

[a] Percentage of seropositive (+) or seronegative (−) animal.
[b] Percentage of IFAT-positive and IFAT-negative animals, respectively, that tested positive and negative by nPCR.

(Calder et al., 1996; Gubbels et al., 1999). The newly presented nPCR assays may thus significantly reduce or eliminate false negative test results as compared to previously reported assays.

As sampled bovines and buffaloes were kept under similar field conditions and in some cases, jointly bred, the observed differences may be most likely attributed to the following. On one hand, the significantly higher number of bovines testing positive for *Babesia* spp.-specific antibodies as compared to water buffaloes suggests higher infection rates in the first. Possibly the thicker hide of buffaloes and their regular habit of mud wallowing protect them from tick bites, thus diminishing transmission with subsequent parasite exposure. Accordingly, lower tick loads are normally found in

field water buffaloes as compared to bovines (results not shown; Ferreri et al., 2008). On the other hand, the highly significant lower number of buffaloes with positive parasite detection by nPCR as compared to bovines (16.2% *vs.* 82.3%, $p < 0.001$ for *B. bovis*; and 24% *vs.* 94.1%, $p < 0.001$ for *B. bigemina*, respectively) could also suggest that buffaloes have comparatively more competent immune defense mechanisms that can limit or even eliminate *Babesia* spp. infections. This notion is underscored by the observed extreme difference in the numbers of *B. bovis* and *B. bigemina* mixed infections in water buffaloes as compared to bovines (3.2% *vs.* 77%, respectively), exemplifying that both parasite species rarely succeed to establish jointly in the former (Table 2). Lower infection rates in
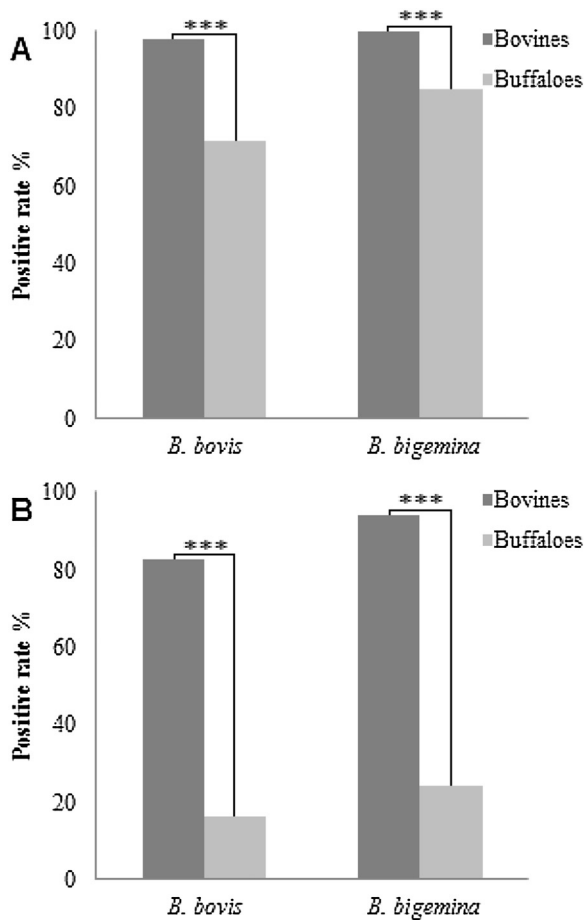
Fig. 3. Comparison of the serological and molecular detection of *Babesia bovis* and *Babesia bigemina* in bovines and water buffaloes from Veracruz, Mexico. Percentages of positive detection in bovines ($n = 203$) and water buffaloes ($n = 154$) by IFAT (A) and *CYTb*-nPCR (B) are shown (***$p < 0.001$).

buffaloes compared to bovines as assessed by serologic and molecular tools have been also reported in a recent study carried out in Egypt in an area of enzootic instability (Mahmoud et al., 2015).

Clinical cases of *B. bovis* and/or *B. bigemina* infection of water buffaloes have not been observed in this or other studies (Ferreri et al., 2008; Mahmoud et al., 2015). Acute babesiosis episodes reported in the past for water buffaloes have been shown to be due to *Babesia orientalis*-infection, but have been previously mistakenly attributed to *B. bovis* (Liu et al., 1997; Yao et al., 1997; Liu et al., 2005; Ferreri et al., 2008).

3.3. Influence of co-breeding of bovines and water buffaloes in the detection of B. bovis and B. bigemina infections

All three farms included in this study are subject to similar climatic conditions allowing for a continuous tick presence throughout the year, which results in a scenario of endemic stability for *Babesia* spp. infections. In the farms La Granja (bovines, $n = 173$; water buffaloes, $n = 38$) and San Agustin (bovines, $n = 30$; water buffaloes, $n = 21$) both species are reared jointly, while in El Búfalo exclusively water buffaloes are kept. Monthly tick control was applied to bovines at San Agustin, but not at La Granja, which allowed studying whether this measure resulted in significantly different *Babesia* spp. positive rates, as determined by molecular (nPCR) or serological (IFAT) detection. Furthermore, the epidemiological situation allowed testing the hypothesis on whether a high

infection rate of bovines jointly reared with water buffaloes results in a significantly increased parasite detection in the latter.

Virtually all bovines of La Granja and San Agustin farms tested seropositive to *B. bovis* (97.7% and 100%, respectively) and *B. bigemina* (100% in both farms), demonstrating an endemic situation for both parasite species. In contrast, nPCR results showed a highly significant larger proportion of bovines infected with *B. bovis* and *B. bigemina* in La Granja, as compared to San Agustin (90.7% *vs.* 33.3%, $p < 0.001$, for *B. bovis*, and 96.5% *vs.* 80%, $p < 0.01$, for *B. bigemina*) (Fig. 4, Suppl. Table 1). As gender distribution in both bovine herds was similar ($p > 0.22$) this factor cannot explain these differences. Most likely the observed difference has to be attributed to the regular acaricide application to cattle in San Agustin as compared to La Granja farm, where animals are kept untreated. However, at least in the case of *B. bovis*, the significantly increased number of older animals (>36 months) in San Agustin might have also contributed to this difference as this age group seems to be less susceptible to infections by this parasite (see Section 3.4). Importantly, acaricide treatment of animals in San Agustin did not result in a lower detection rate of parasite-specific antibodies by IFAT, yet in a highly significantly reduced nPCR-positive rate of animals.

In water buffaloes originating from La Granja, San Agustin, and El Búfalo farms, IFAT-detection rates were not significantly different for *B. bovis* (68.4%, 71.4%, and 72.6%, $p = 0,9$) and significantly different for *B. bigemina* (89.5%, 100%, and 80% for *B. bigemina*, $p \leq 0.02$, respectively). In contrast, significantly increased proportions of *B. bovis* and *B. bigemina* nPCR-positive buffaloes were detected in La Granja, as compared to San Agustin and El Búfalo (*B. bovis* 31.6% *vs.* 9.5% and 11.6%, $p < 0.01$; *B. bigemina* 42.1% *vs.* 9.5% and 20%, $p < 0.01$) (Fig. 4, Suppl. Table 1).

Since age distribution among water buffaloes was similar in the three analyzed herds ($p > 0.87$), differences in positivity percentages are not associated to an age factor. On the other hand, although the number of male buffaloes was larger in La Granja as compared to the other two farms, gender was found not to be associated with a different susceptibility to infection, as will be described in Section 3.4. Therefore, the significantly increased *Babesia* spp. infection of La Granja water buffaloes might be explained by the presence of a bovine population with high infection rates for both parasites, as compared to San Agustin, and also to El Búfalo where bovines are absent. Interestingly, several reports have suggested that water buffaloes, which are usually not included in sanitation campaigns, may represent an additional parasite reservoir leading to increased incidence of bovine babesiosis of cattle when raised in the same field (Bock et al., 2004; Ferreri et al., 2008; Benitez et al., 2012; da Silva et al., 2013). In contrast, this study suggests an opposite scenario, *i.e.*, that an increased parasite reservoir in bovines not subjected to acaricide treatment might be leading to a higher incidence of parasite exposure and subsequent infection in water buffaloes.

3.4. Influence of age and gender in the detection of B. bovis and B. bigemina in bovines and water buffaloes

Detection rates of *B. bovis* and *B. bigemina* as determined by nPCR and IFAT in three different groups <12 months, 13–36 months, and >36 months of age, as well as male and female gender distributions were compared for bovines and buffaloes.

Serological diagnosis of *B. bovis* was significantly lower in the bovine <12 months old group than in animals of the two older age groups (94.8% *vs.* 100% and 100%, $p < 0.05$). These differences were not observed for *B. bigemina*, for which all three age groups tested 100% IFAT-positive. Interestingly, water buffaloes showed a more pronounced stepwise and significant increase of IFAT-positive animals from the youngest to the oldest age group for *B. bovis* (53.3%, 68.7%, and 81.7%, $p < 0.05$), as well as for *B. bigemina* (66.7%, 81.2%, and 96.7%, $p < 0.001$) (Fig. 5, Suppl. Table 2). This
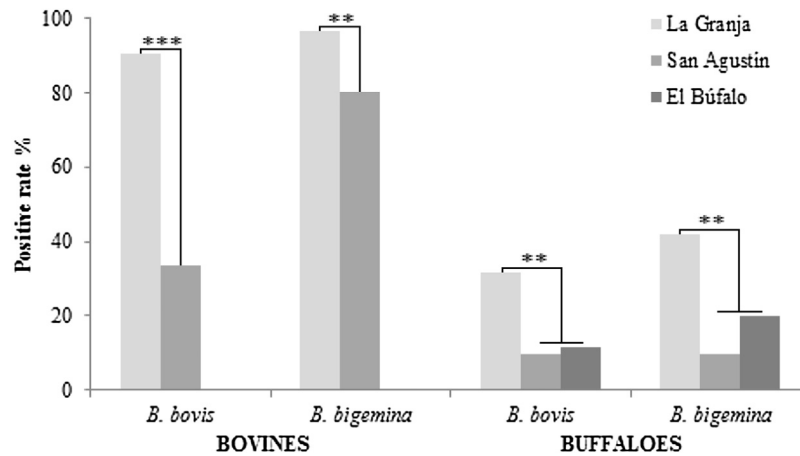
Fig. 4. Molecular detection of *B. bovis* and *B. bigemina* in bovines and water buffaloes in three different farms of Veracruz, Mexico. Highly significant differences between La Granja and San Agustin farms in the case of bovines (**$p < 0.01$, ***$p < 0.001$), or between La Granja *vs.* San Agustin, and El Búfalo farms were found (**$p < 0.01$, ***$p < 0.001$).
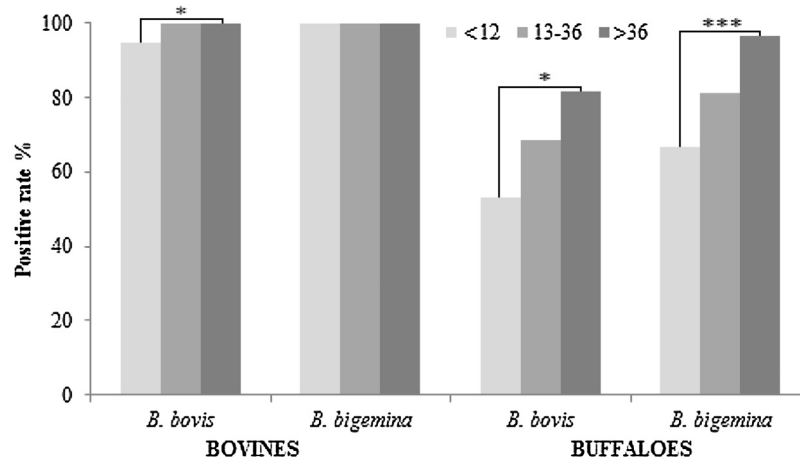
Fig. 5. Serological detection of *B. bovis* and *B. bigemina* in bovines and water buffaloes of age groups <12 months, 12–36 months, and >36 months. Significant (*$p < 0.05$) and highly significant (***$p < 0.001$) differences within each group are indicated by asterisks.

observation strongly suggests an accumulative parasite exposure with advancing age of buffaloes due to continuous year-round tick transmission. This effect cannot be appreciated in bovines as all (in the case of *B. bigemina*) or nearly all (in the case of *B. bovis*) animals are already positive in the youngest group.

In contrast to the data obtained by IFAT, direct nPCR detection of *B. bovis* decreased significantly in bovines between the youngest and the two older age groups (71.0% *vs.* 87.7% and 88.3%, $p \leq 0.05$) while for *B. bigemina*, a non-significant decrease between the youngest and the remaining two older age groups (98.7% *vs.* 91.2% and 91.3%, $p = 0.06$) was noted (Suppl. Table 2). Also for buffaloes, a non-significant decrease of the rate of infection of *B. bigemina* (30.0%, 26.6%, 18.3%, $p = 0.3$) was associated with an increasing age of the three groups, but this was not observed for *B. bovis*. Thus, in contrast to serological data suggesting that parasite exposure continuously accumulates from the youngest toward the oldest age group in bovines and water buffaloes, a reciprocal distribution pattern of *B. bovis* and *B. bigemina* infection was observed for bovines and also for *B. bigemina* infection of buffaloes when direct parasite detection was implemented. However, for *B. bovis*-infection of buffaloes, as a sole exception, this reciprocal distribution pattern was not observed. The data may suggest that immune defense mechanisms of older animals are more effectively limiting parasite infection.

With respect to gender, exclusively a significantly increased IFAT detection in male bovines ($p < 0.05$) and nPCR detection in female bovines ($p < 0.05$) were observed for *B. bovis* (data not shown). However, as only 11.0% of the studied buffalo (17 of 154) and 20.2% of the studied bovine samples (41 of 203) were of male gender, these observations need to be further corroborated in future studies using samples of a more balanced gender distribution.

In summary, we developed a highly sensitive and specific nPCR assay format for detection of *B. bovis* and *B. bigemina* in bovines and water buffaloes. The utility of these assays was demonstrated in a subsequent field study comparing bovines and water buffaloes raised jointly under similar epidemiological conditions. For the first time, evidence is presented that water buffaloes show higher *B. bovis* and *B. bigemina* parasite infection rates when raised with highly infected bovines, in contrast to those raised alone or together with acaricide treated bovines.

Conflict of interest

None declared.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetpar.2015.12.030.

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