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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 × *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× 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.