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Original article

Molecular evidence of the reservoir competence of water buffalo (*Bubalus bubalis*) for *Anaplasma marginale* in Cuba

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ABSTRACT

Water buffalo (*Bubalus bubalis*) is a potential reservoir for *Anaplasma marginale* in livestock ecosystems of tropical countries. However, their participation in the epidemiological process of bovine anaplasmosis in endemic areas remains unclear. In the present study, the reservoir competence of water buffalo for *A. marginale* was explored by focusing on the analysis of rickettsemia levels in carrier animals, and the genetic characterization of *A. marginale* strains from cattle and buffalo. Eight groups of cattle and water buffaloes were randomly selected from cohabiting herds in four livestock ecosystems of Cuba, together with two control groups from unrelated cattle and buffalo herds. A total of 180 adult animals (88 water buffalo and 92 cattle) were sampled. Rickettsemia in carrier animals was determined by quantitative real-time PCR. The rickettsemia (parasitemia) levels in cattle were higher than in buffaloes, however the rickettsemia in buffalo may be enough to infect *R. microplus* ticks. The genetic diversity of *A. marginale* was assessed by strain characterization and phylogenetic analysis of 27 *msp1α* gene sequences. The results showed genetic similarity among strains from cattle and water buffalo, suggesting the occurrence of cross-species transmission.

1. Introduction

Anaplasma marginale (Rickettsiales: Anaplasmataceae) is the etiological agent of bovine anaplasmosis, a hemolytic disease that affects both dairy and beef industries, representing a major constraint to cattle production in tropical and subtropical regions of the world (OIE, 2015). Cattle of all breeds and ages can be infected by *A. marginale*; however, the severity of the disease depends on age, nutritional status, and management (Aubry and Geale, 2011). The Giemsa-stained blood smear is a conventional diagnosis method in clinically infected animals. However, carrier animals have low levels of rickettsemia ranging between 10^3 and 10^7 infected erythrocytes/ml blood (IE/ml) (Brown and Barbet, 2016). Such levels are undetectable by blood smear, posing a challenge to the direct diagnosis that currently requires PCR assays. Notably, carrier animals are the main infection source for competent vectors in endemics areas (Aubry and Geale, 2011).

Although cattle are the natural hosts for *A. marginale*, this rickettsia is a multi-host pathogen that can infect several ruminant species, including water buffalo (*Bubalus bubalis*) and other wild animals such as American bison (*Bison bison*), white-tailed deer (*Odocoileus virginianus*), black-tailed deer (*O. hemionus culumbianus*), mule deer (*O. h. hemionus*) and other cervids (Kocan et al., 2010), being even found in non-ruminants (Guillemi et al., 2016). These species only have been regarded as carrier host of *A. marginale* (Kuttler, 1984; Kocan et al., 2010). However, there are no substantiating field studies that demonstrate the transmission of *A. marginale* between cattle and wild ruminants, which might be incidental hosts, unable to maintain a transmit the pathogen (reservoir competence) (Aubry and Geale, 2011; Kocan et al., 2015).

The reservoir of a multi-host pathogen can be one or more epidemiologically connected populations in which the pathogen persists and from which it is transmitted to the target population (Haydon et al., 2002). Under this approach, cattle and other species could form the

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reservoir of *A. marginale* in a given endemic region, depending on the reservoir competence of such species. For vector-borne pathogens, four factors determine the reservoir competence of a host: 1) the probability that a host acquires the infection when bitten by an infected vector; 2) the ability of the pathogen to magnify and persist in the host; 3) the probability that a vector acquires the pathogen from an infected host (host infectiousness); 4) the host ability to sustain vector populations (LoGiudice et al., 2003; Cronin et al., 2010).

The water buffaloes are robust and easily adapt to the tropical conditions, which are characterized by poor pastures and high temperature and humidity. Further, water buffaloes are more resistant to many infectious and parasitic diseases, constituting an alternative to cattle livestock. Consequently, this species is in expansion in Latin America and the Caribbean countries (FAO, 2014). In this region, it is common that buffalo and cattle cohabit in the same livestock ecosystems, which turns buffaloes into potential reservoirs of *A. marginale*. Yet, to our knowledge, there is no scientific evidence on the occurrence of cross-species transmission. In addition, a few studies have analyzed the infection prevalence of *A. marginale* in buffalo herds, and these studies generally found low prevalence rates, even in endemic areas (Khan et al., 2004; Rajput et al., 2005; Silva et al., 2014a).

Recently, a molecular survey carried out in Brazil by Silva et al. (2014b), using the gene *msp1α* as genetic marker, found strains of *A. marginale* infecting buffaloes which were genetically related with strains previously reported from cattle in nearby areas. However, despite the clear suggestions of cross-transmission, this finding was reached in a randomized study that did not include any bovine population, and epidemiological links (i.e., spatial distance, common exposure to vectors, cattle movements and within-herd contacts) between cattle and buffalo herds were not considered. Consequently, new studies on the subject should deepen the significance and patterns of such epidemiological relationships. In this regard, the genetic and/or antigenic characterization of strains in different host populations is currently the most powerful tool to identify components in the reservoir structure of a certain pathogen (Haydon et al., 2002; Viana et al., 2014).

MSP1 is a heterodimer composed of two structurally unrelated proteins: MSP1a which is encoded by a single gene *msp1α*, and MSP1b which is encoded by members of the *msp1β* multigene family (Camacho-Nuez et al., 2000). The *msp1β* is a sensitive and specific target for detection of *A. marginale*, which has been used for diagnostics in nested PCR (nPCR) (Molad et al., 2006) and quantitative real-time PCR (qPCR) assays (Carelli et al., 2007; Decaro et al., 2008). Of these two PCR approaches, qPCR was shown to be the most appropriate assay for detection of *A. marginale* in blood samples from cattle (Chaisi et al., 2017). The gene *msp1α* is associated with tick transmission fitness and plasticity to infect multiple host species, evolving under immune selection pressure, making it a good predictor of the genetic diversity in a restricted geographical area (de la Fuente et al., 2007a; Estrada-Peña et al., 2009). The strains of *A. marginale* can be identified by differences in the molecular weight of MSP1a because of the variable number of 20–31 amino acid serine-rich tandem repeats (TR) located in the N-terminal region of the protein (Cabezas-Cruz et al., 2013).

In Cuba, the bovine anaplasmosis had a high incidence and mortality in adult cattle in the 80s (Corona et al., 2005). Subsequently, the epidemiological situation evolved to endemic stability characterized by annual average rates of 200 outbreaks and 0.03% of incidence during the last 25 years (LNP, 2014). Concurrently, the bubaline species was introduced and widespread throughout the country during this period, occupying farms generally close to cattle herds (Mitat, 2009). Preliminary studies in the Western regions found buffaloes infested by *R. microplus* (Obregón et al., 2010), and also infected by *A. marginale* (Corona et al., 2012). To determine the contribution of water buffalo to the epidemiological process of anaplasmosis will contribute to the surveillance program of this disease in Cuba.

This work aims to explore the reservoir competence of water buffaloes for *A. marginale* in endemic areas from Cuba. Particularly, we addressed two questions: the host competence, through the analysis of rickettsemia levels in carrier animals, and the occurrence of cross-species transmission, in cattle and water buffalo herds cohabiting in contiguous grazing areas.

2. Materials and methods

2.1. Study site and sample collection

A cohort study was conducted in livestock areas of Habana and Mayabeque provinces, Cuba. The climate of the region is tropical, seasonally humid, with an annual average of temperature between 22 and 28 °C, and a relative humidity of 80% (INSMET, 2016). In these provinces, *R. microplus* is the only tick diagnosed in cattle, which is maintained throughout the year with population increases during the dry season (December–March) (LNP, 2014). Mechanical vectors as stable flies (*Stomoxys calcitrans*) and some tabanid species (Diptera: Tabanidae) may be present, however they are of little importance in the transmission of *A. marginale* in the region (Alonso et al., 1992), although there are no in-depth studies on this topic in Cuba.

Four farms were randomly selected. In these farms, both cattle and water buffalo herds coexist together in contiguous grazing areas infested with ticks. The geographical areas of sample collection were divided in “ecosystems” and nominated as “I”, “II, “III” and “IV” (Fig. 1). Herds with only cattle or only buffaloes were included as control groups (Fig. 1) and they belonged to farms 5 km away from the herds in ecosystems “I”, “II, “III” and “IV” as well as any other population of cattle or buffaloes respectively. In each ecosystem, 20 cattle and 20 buffaloes were randomly selected, however, in “II” only nine and 11 samples of each species were included, respectively. In total, the animals sampled were 180 (88 buffalo and 92 cattle). Only adult animals ($\geq$ 2 years) were included in this study. During the study period, there were no clinical cases of anaplasmosis or animal introductions in any of the selected herds.

The blood samples were obtained by puncturing the jugular vein, collected in 4 mL vacutainer tubes containing K2 EDTA (BD Vacutainer), and kept frozen at −80 °C in cryovials until processing. DNA was extracted from 200 µL of thawed blood using DNeasy® Blood and Tissue DNA Purification Kit (Qiagen, USA). The DNA samples were examined (concentration and purity) using a Nanodrop Spectrophotometer 1000 v.3.5 (Thermo Fisher Scientific, USA) and stored at −20 °C.

2.2. Molecular detection and quantification

The TaqMan-based real-time PCR assay based on the amplification of a 95 bp sequence from *msp1β* gene was used, as described by Carelli et al. (2007) with modifications. Briefly, the qPCR reactions were carried out in 10 µL containing 5 µL of (2×) TaqMan® Gene Expression Master Mix (Qiagen, USA), 0.5 µL of forward and reverse primers (10 µM), 0.2 µL of TaqMan probe (10 µM), 1 µL of DNA template and nuclease-free water (Qiagen, USA). The amplifications were performed in a CFX96 Thermal Cycler (BioRad, USA), starting in 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. All samples were tested in duplicate, and no-template reactions (NTC) were included in each trial as contamination control.

The quantification of the copy number of DNA target sequence (CN) was performed using the IDT pSMART plasmids (Integrated DNA Technologies, USA) containing the target DNA sequence. Ten-fold serial dilutions from 2×10^7 CN μL^{-1} to 2×10^0 CN μL^{-1} were made to obtain a standard curve. The copy number in the standard dilutions (CN μL^{-1}) was estimated according to the formula: CN = Conc. (g μL^{-1}) x N_A / MW (g mol^{-1}), where: N_A -Avogadro constant (6.022×10^{23} copy mol^{-1}), MW- molecular weight of the nucleotide pair (660) multiplied by the plasmid size (bp).