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The occurrence of pathogens in *Rhipicephalus microplus* ticks from cattle in Madagascar

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ABSTRACT: *Rhipicephalus microplus* is one of the most important ectoparasites of cattle in tropical and subtropical regions. In ticks collected from cattle the pathogens *Babesia bovis, Anaplasma* spp. and *Ehrlichia* spp. can be detected. Here, we report the first detection of the pathogen *Anaplasma phagocytophilum* in Madagascar from ticks infesting cattle. Furthermore, we report for the first time *Anaplasma ovis, Ehrlichia canis*, *Ehrlichia ewingii* and *Ehrlichia muris* in both *R. microplus* and in Madagascar. We show no correlation between the detection of *B. bovis*, *Ehrlichia* spp. and *Anaplasma* spp. DNA within the same tick. Previous reports have demonstrated strong interactions between *A. marginale*, *A. centrale* and *A. ovis* in the same tick, as well between these pathogens and *A. phagocytophilum*. A strong correlation also existed between the occurrences of *Ehrlichia* species within the same tick. Our findings suggest that *R. microplus* ticks are potential vectors and reservoirs of many tick-borne diseases of cattle.

Keywords: African ticks; *Anaplasma*; *Babesia*; *Borrelia*; *Ehrlichia*; zebu; *Boophilus*; southern cattle tick

Ticks represent a serious problem worldwide among livestock and cause huge losses by transmitting diseases. Studies of ways to combat or prevent infection transmitted by ticks have been most frequently conducted in America, Australia and Europe. An even more significant problem exists in African countries, but because of other more urgent challenges, tick-borne diseases have not been extensively studied on this continent.

Approximately 40 species of ticks can affect the health of domestic animals in Africa. Tick-associated diseases cause much suffering in animals as well as economic losses. They continue to be a major impediment to improving the livestock industry, and Africa is particularly affected because of its abundance of tick species and the variety of diseases that they cause (Walker et al. 2003).

One of the most important ectoparasites of cattle in tropical and subtropical regions is *Rhipicephalus microplus* (Canestrini 1887) (Estrada-Pena et al. 2006a; Klafke et al. 2006). Initially, this organism was found in India and Indonesia as a parasite of Asian species of cattle (Labruna et al. 2009). To date, it has been reported from Mexico, Central and South America, Africa, Madagascar, Australia, and Taiwan (Jones et al. 1972; Estrada-Pena et al. 2006b; Olwoch et al. 2007). The spread of this tick species has intensified to affect European breeds of cattle in tropical regions. Notably, some races of the species *Bos indicus* lack an immune response against ticks and tick-associated pathogens (Frisch 1999). Unfortunately, depending on the geographic location, there are morphological and genetic differences that affect the efficacy of tick control efforts (Labruna et al. 2009). *R. microplus* exhibits rapid adaptation to a new environment (Chevillon et al. 2007) and resistance to acaricid insecticides, since their continual use has led to the evolution of insecticide resistance (Sutherst and Comins 1979; Frisch 1999).

R. microplus is primarily a dangerous parasite of cattle, but it can also be found in horses, sheep,

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goats and dogs (Jones et al. 1972; Uilenberg et al. 1979). In Brazil, it can also be found on capybara, deer, and coati (Figueiredo et al. 1999). In Brazil, 80% of the cattle population is affected, resulting in huge economic losses (Grisi et al. 2002). Australia is also actively struggling with this problem. Through many years of research, it has been demonstrated that this parasite mainly affects cattle of the species *Bos taurus*, whereas *Bos indicus* attacks may be as low as 10% of that species (Jonsson 2006). The effects of these tick attacks result in an economic impact for herd owners because of weight loss and reduced milk production in cattle, as well as anaemia and the transmission of various pathogens (Peter et al. 2005; Barros-Battesti et al. 2006). *R. microplus*, also known as the "southern cattle tick", is the main vector of bovine babesiosis (caused by *Babesia bigemina* and *Babesia bovis*) and anaplasmosis (*Anaplasma marginale*).

In addition to the global economic importance of ticks to the livestock industry, ticks also have a large impact on public health, primarily because of Lyme borreliosis, as well as other zoonotic tick-borne illnesses (Figueiredo et al. 1999). Tick-borne diseases of viral origin, which are characterised by encephalitis and hemorrhagic fevers, cause the greatest morbidity and mortality in man. In Madagascar, the most widely characterised tick species is *Amblyomma variegatum*, while less research has been carried out on *R. microplus*.

We hypothesise that ticks are a major problem for cattle in Madagascar because they transmit a number of pathogens.

This study aimed to identify the pathogens of *R. microplus* on cattle in Madagascar.

MATERIAL AND METHODS

Study area and species. The ticks used in this study were collected from July to August in 2014 in pastures near the cities of Ankofafa (22°20'S 47°29'E) and Antsirabe (19°52'S 47°02'E) in the Vakinankaratra region, Madagascar. This is a typical agricultural area located in the centre of a milk production region on the island. The cattle belonged to one of three breeds: Holstein Friesian, Norwegian Red, and a local breed (*Bos indicus*). Ticks were sampled directly from cattle using tweezers, after which they were placed into an Eppendorf test tube that contained 70% ethanol. From each animal sampled, all ticks were collected. Some cattle breeders used Amitraz on the skin or Ivermectin injection to control tick infestations.

DNA isolation. Ticks were removed from ethanol storage and crushed. DNA isolation was carried out using a Genomic Mini kit (A&A Biotechnology, Poland) according to the manufacturer's instructions.

Polymerase chain reaction. PCR reactions were carried out to test for the presence of the following four pathogens: *Babesia bovis*, *Borellia* spp., *Anaplasma* spp. and *Ehrlichia* spp. The amplifications were carried out in a MJ Research PTC-200 DNA Engine (BioRad, USA).

Each PCR reaction was carried out in a 25 µl reaction volume which contained 12.5 µl DreamTaq Green PCR Master Mix (ThermoFisher Scientific, USA), 0.6 µl of 10µM stocks of each primer (DNA Sequencing and Synthesis Service of the Institute of Biochemistry and Biophysics, Polish Academy of Sciences in Warsaw, Poland), 3 µl of matrix DNA and 8.3 µl nuclease-free water.

PCR for *Babesia bovis* was carried out using the primers Bo5'-CTTGCGGCGATTTGGC-3' and BoR5'-CGTGAAGGAGCGGTGTAGAG-3', which can amplify a product of 408 bp from the nucleotide sequence of the internal transcribed spacers (ITSs) (Liu et al. 2014). Amplification conditions were as follows: initial denaturation at 96 °C for 5 min, followed by 35 cycles of denaturation at 92 °C for 1 min, annealing at 56 °C for 1 min, extension at 72 °C for 1 min, and then a final extension at 72 °C for 1 min (Liu et al. 2014).

Detection of *Borrelia* spp. was carried out using the primers FlaF_5'-TGTGATATCC-TTTTAAAGAGACAAATGG-3' and FlaR_5'-TAAGCAATGACAATACATATTGAGG-3', which amplify a product of 1284 bp for sequencing the flagellin *flaB* gene (Schwan et al. 2012). Reactions were performed under the following conditions: initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 56 °C for 45 s, extension at 72 °C for 3 min, followed by a final extension at 72 °C for 5 min.

To identify *Anaplasma*/*Ehrlichia* spp., the following primers were used: EHR16SD (5-GGTACCYA-CAGAAGAAGTCC-3') and EHR16SR (5'-TAGCA-CTCATCGTTTACAGC-3'), which amplify a product of 345 bp from the 16S rRNA gene (Maia et al. 2014). Reactions were performed under the following conditions: initial denaturation at 95 °C for 3 min, followed by 35 cycles with denaturation at

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94 °C for 45 s, annealing at 55 °C for 45 s, extension at 72 °C for 90 s, and a final extension at 72 °C for 5 min.

PCR products were subjected to electrophoresis in 2% agarose gels for the detection of *B. bovis* and *Anaplasma*/*Ehrlichia* spp. and in 1% agarose gels for *Borrelia* spp.; gels were stained with ethidium bromide and visualised under ultraviolet light.

DNA sequencing of PCR products. Reaction products that contained an amplified fragment were purified using a GenElute PCR Clean-Up Kit (Sigma, Germany) and were then sequenced at the DNA Sequencing and Synthesis Service of the IBB, PAS in Warsaw, Poland. DNA sequencing was performed on both strands using the same primers employed for PCR.

The resulting sequences were then subjected to Basic Local Alignment Search Tool analysis to determine similarities with those sequences available in the GenBank database hosted by the National Institutes of Health, Bethesda, MD, USA.

Statistical analysis. Statistical analyses were performed using SPSS for Windows (SPSS Inc., Chicago, IL, USA); all tests were two-tailed.

RESULTS

During this study, 187 ticks were found in 34 of 75 (45.3%, 95% CI: 33.8–56.9) cattle that were examined. Twenty six individual cattle were of the *Bos indicus* (zebu) breed and 49 were *B. taurus* (domestic cattle). Mean cattle age was 4.0 ± 2.1 years, and mass was 318.5 ± 132.0 kg. In 17 of these individuals zebu ticks were found (13 in males and four in females). The presence of ticks was also determined in 17 individuals of domestic cattle (nine in males and eight in females). The mean intensity in infected cattle was 5.5 (95% CI: 2.68–8.32) ticks per animal (max. 36). There was a significant association between the number of ticks and the sex of cattle, since male cattle harboured more ticks (Tau b Kendall correlation, $\tau_b = -0.364$; $P < 0.001$, Figure 1), and between the number of ticks and cattle mass (Spearman correlation, $r = -0.236$, $n = 75$, $P < 0.05$) but no significant association was found between tick numbers and cattle age ($r = -0.112$, $n = 63$, $P > 0.05$). A total of 104 ticks from 23 cattle were successfully obtained for further analyses. The sex of ticks was strongly female-biased (chi-square test with Yates' correction, $\chi^2 = 34.78$, df = 1, $P < 0.001$; Table 1). All ticks were identified as *R. microplus*. Pathogen DNA was detected in 35 of the 104 ticks (33.7%, 95% CI: 24.4–42.9) that were

Table 1. Sex and age of *Rhipicephalus microplus* ticks collected from cattle in Madagascar

	Adult	Nymph	Total
Female	75	19	94
Male	12	0	12
Total	87	19	106

Table 2. Pathogens identified in *Rhipicephalus microplus* ticks from cattle in Madagascar. See the Material and Methods section for detailed information about the procedures used

	Infected	Uninfected	%	95% CI	Cattle	%
All pathogens	35	69	33.7	24.4–42.9	12	52.2
Babesia bovis	18	86	17.3	9.9–24.7	12	52.2
Anaplasmataceae	24	80	23.1	14.8–31.3	8	34.8
Anaplasma	13	91	12.5	6.0–19.0	2	8.7
A. marginale	13	91	12.5	6.0–19.0	2	8.7
A. centrale	13	91	12.5	6.0–19.0	2	8.7
A. ovis	13	91	12.5	6.0–19.0	2	8.7
A. phagocytophilum	6	98	5.8	1.2–10.3	1	4.3
Ehrlichia	3	98	2.9	0.4–6.2	1	4.3
E. canis	3	101	2.9	0.4–6.2	1	4.3
E. ewingii	2	102	1.9	0.8–4.6	1	4.3
E. muris	1	103	1.0	0.9–2.9	1	4.3
unidentified Anaplasmataceae	8	96	7.7	2.5–12.9	7	30.4
Borrelia spp.	0	104	0.0	–	0	0.0

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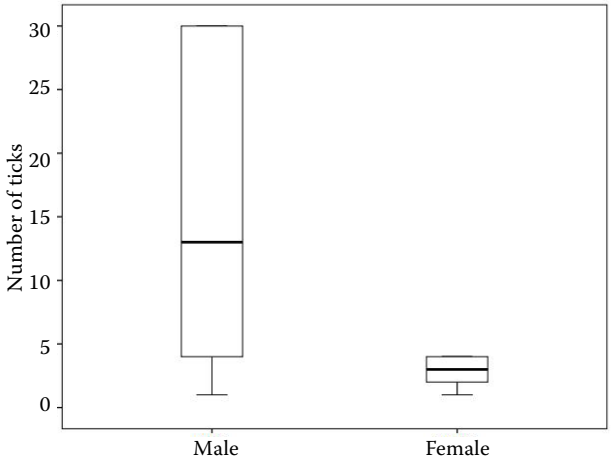

Figure 1. Boxplots showing the relationship between the number of *Rhipicephalus microplus* ticks on infected cattle and the sex of cattle (22 males, 12 females)

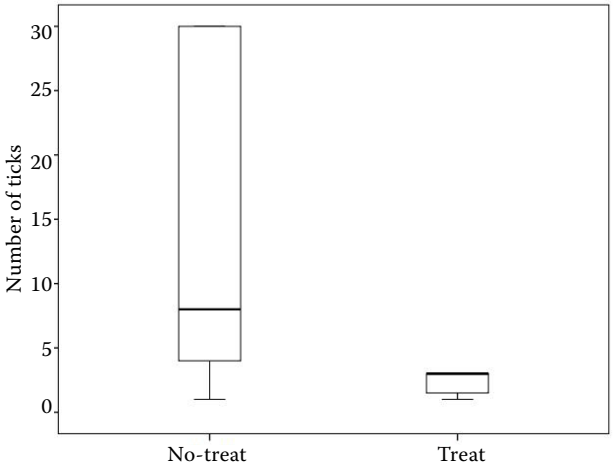

Figure 2. Boxplot showing the number of *Rhipicephalus microplus* ticks on cattle treated or not with acaricides (45 and 30, respectively)

taken from 12 cattle (Table 2). Ticks from the cattle were found to harbour *Babesia bovis*, *Anaplasma marginale*, *A. centrale*, *A. ovis*, *A. phagocytophilum, Ehrlichia canis, E. ewingii* and *E. muris* pathogens. No *Borrelia* spp. pathogens were detected. The number of ticks and the number of ticks that were PCR-positive for pathogens per animal correlated with each other (τ_b = 0.425, n = 23, P < 0.05; Figure 3).

However, there were no significant Kendall correlations between the detection of *B. bovis, Ehrlichia* spp. and *Anaplasma* spp. DNA in the same tick (all P > 0.05; Table 3). We did detect a significant positive correlation between *A. marginale*, *A. centrale* and *A. ovis* (all in the same ticks), as well as between these species and *A. phagocytophilum* (Tau b Kendall correlation, τ_b = 0.655, n = 104, P < 0.001). There was also a significant positive correlation between the occurrence of *E. canis, E. ewingii* and *E. muris* within the same tick (Table 4). We found significantly fewer ticks on cattle where insecticide treatment was used (Mann-Whitney U-test, Z = –4.48, P < 0.001; Figure 2).

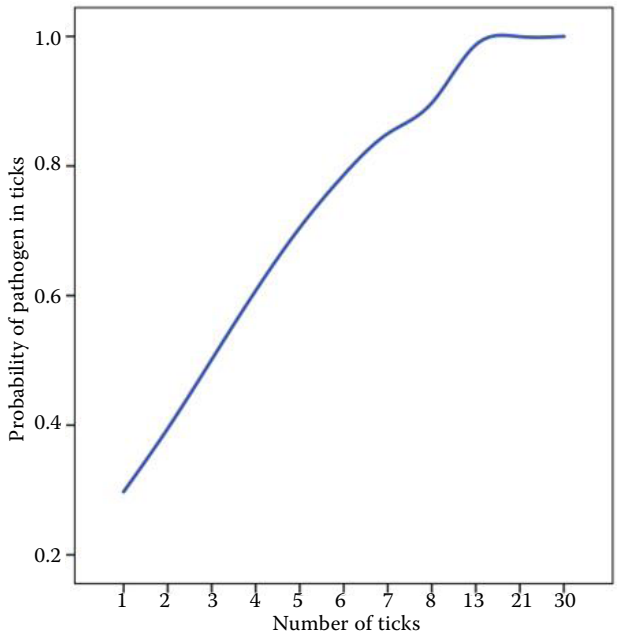

Figure 3. Probability of finding ticks infected with a pathogen plotted against the number of ticks on an individual host cattle

Table 3. Co-infection with *Babesia bovis* and Anaplasmataceae pathogens in *Rhipicephalus microplus* ticks (n = 104)

		B. bovis	*Anaplasma* spp.
Anaplasma spp.	τ_b	0.058	
	P	0.559	
Ehrlichia spp.	τ_b	0.073	–0.065
	P	0.459	0.509

Table 4. Co-infection of *Ehrlichia* spp. pathogens in *Rhipicephalus microplus* ticks (n = 104)

		E. canis	*E. ewingii*
E. ewingii	τ_b	0.812	
	P	0.001	
E. muris	τ_b	0.572	0.704
	P	0.001	0.001

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DISCUSSION

During this study, 75 cattle were assessed for ticks. On almost half of these cattle (34 individuals), at least one tick was found. When cattle harboured ticks, the mean intensity of tick presence was found to be 5.5. Overall, we found a significant association between the number of ticks and the sex and mass of cattle, with more parasites found on males, whereas we detected no association between parasites and the age of cattle. In many cattle species, males have more ticks than females (Seifert 1971; Martinez et al. 2006; Dudek et al. 2016), likely because of differences in hormone levels. This may also simply be due to the weight of the animals; those that are bigger capture more ticks, and males are generally larger than females. This can also be due to differences in behaviour. With *R. microplus*, it is the animals that are in the front of the herd that are most infested (they come into contact with packets of larvae on vegetation before others). Studies in rats have shown that high testosterone concentrations enhance the locomotion of animals (resulting in a higher probability of encountering ticks), whilst also decreasing the activity of the immune system and thereby prolonging and increasing the rate of infection (Hughes and Randolph 2001). All ticks that we collected belonged to the species *R. microplus*. Previous studies have shown that this tick species is common on cattle and is a vector of tick-borne pathogens (Uilenberg et al. 1979; Estrada-Pena et al. 2006a; Barre and Uilenberg 2010). In the present study, the tick-borne pathogens *B. bovis* and members of the Anaplasmataceae were detected in 34% of the ticks that we examined (Table 2). *B. bovis* DNA was detected in 18 ticks (17%). We note that *B. bovis* is a frequent cattle pathogen in tropical countries that can be transmitted by *R. microplus* (Coetzer and Tustin 2004; Bastos et al. 2010).

During this study, Anaplasmataceae bacterial DNA was detected in 24 ticks (23%). DNA sequencing revealed that 13 and three ticks, respectively, were infected by *Anaplasma* spp. and *Ehrlichia* spp. (Table 2). *Anaplasma* spp. bacteria are frequent pathogens transmitted by *R. microplus* (Aguirre et al. 1994; Estrada-Pena et al. 2006b; Barre and Uilenberg 2010). In the present study, we detected four species of this class of pathogens: *A. marginale*, *A. centrale, A. ovis* and *A. phagocytophilum*. In Madagascar, *A. marginale* is commonly found in *R. microplus*, which feeds on cattle as its main host (Uilenberg et al. 1979). Our report represents the first detection of *A. ovis* in *R. microplus*, as well as the first report of this pathogen in Madagascar. The common vectors of *A. ovis* are ticks of the genus *Dermacentor* (Friedhoff 1997). Although the ticks in the present study were collected from cattle, this pathogen generally occurs in goats and sheep since cattle have innate resistance to it (Nakamura et al. 1993). The pathogen *A. phagocytophilum* is rarely identified in *R. microplus* and is better known to be transmitted by ticks of the genus *Ixodes* (Ekner et al. 2011; Zhang et al. 2012a). This represents the first report of this pathogen on the island of Madagascar. This pathogen has a great impact on sheep production, can predispose animals to other bacterial and viral infections (Larsen et al. 1994), and can also cause cattle anaplasmosis (Zhang et al. 2012b). Furthermore, *A. phagocytophilum* can infect humans, causing granulocytic anaplasmosis disease (Chen et al. 1994).

In the ticks that we collected, DNA of the genus *Ehrlichia* was detected for *E. canis*, *E. ewingii* and *E. muris*, and was present in 2.9% of all ticks. This represents the first case of the occurrence of these pathogens in Madagascar and in the tick *R. microplus*. Only *E. ruminantium* had been previously recorded in Madagascar (Provost and Bezuidenhout 1987). The detection of *Ehrlichia* spp. in *R. microplus* has been rarely reported and its hosts are usually dogs (Zweygarth et al. 2013). The first two reports came from China in 1999 (Pan et al. 1999) and from Tibet in 2002 (Wen et al. 2002). A subsequent report describing isolation from a group of the cattle in Thailand was published in 2003 (Parola et al. 2003). *E. canis* can be transmitted by the dog ticks *R. sanguineus* (Groves et al. 1975; Lewis et al. 1977). Among *Ehrlichia* spp., cattle are most often affected by *E. ruminantium*. This pathogen causes a disease known as heartwater or cowdriosis (Allsopp 2010). This bacterium is a threat, particularly for ruminants, as it can cause economic losses, but there are no authenticated reports that this organism can cause disease in non-ruminants or humans (Allsopp et al. 2005).

Ticks can be simultaneously infected with two or more micro-organisms (Goldstein et al. 2001; Ginsberg 2008; Pesquera et al. 2015), but the relationship between them in a tick can differ. Such microbes can manifest antagonistic, positive, or neutral interactions with each other (Ginsberg

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2008). Interpretation of such results can be difficult because many factors, other than simple interactions between micro-organisms, can affect the number of co-infections. Additionally, the incidence of tick-borne pathogens in nature can be affected by many factors such as the microclimate, vegetation, and density of ticks (Cumming 2002; Randolph 2004; Gray et al. 2009). The present study showed no correlation between the detection of *B. bovis*, *Ehrlichia* spp. and *Anaplasma* spp. DNA within the same tick (Table 3). These findings may suggest the absence of an interaction between these bacteria. Nevertheless, Lee and Chae (2010) found that *Anaplasma* spp. and *Ehrlichia* spp. can jointly infect individual ticks. Moreover, that study detected a high rate of interaction between *A. marginale*, *A. centrale* and *A. ovis* within the same tick (all pathogens were found on the same tick), as well as between those pathogens and *A. phagocytophilum*. Recent reports have shown that *A. marginale*, *A. centrale* and *A. phagocytophilum* can coexist in certain regions, with concurrent infections occurring in ruminants and ticks (de la Fuente et al. 2005). A strong correlation also exists between the occurrences of *Ehrlichia* species within the same tick (Table 4). An understanding of co-infections is very important for improving the health of animals, especially for the proper diagnosis and prevention of tick-borne diseases. However, hosts infected by several different pathogens can exhibit different disease symptoms (Berggoetz et al. 2014). It is important to understand how bacteria can coexist within the same tick because this represents an essential condition for the occurrence of co-transmission from tick to host (Alekseev et al. 2004). Quantitative studies with migrating *R. microplus* ticks under known natural conditions will be needed to better understand the importance of this tick species in the epidemiology of anaplasmosis and babesiosis. Furthermore, our research has established that the use of acaricides can affect the prevalence of ticks (Figure 2). This is an important issue because a higher prevalence of ticks can increase the probability of infection (Figure 3). This study has shown that the sampling of over a dozen of ticks is enough to reach near 100% probability of pathogen occurrence.

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