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Molecular detection of pathogens in ticks infesting cattle in Nampula province, Mozambique

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Abstract Ticks are ectoparasites that can act as vectors of a large number of pathogens in wild and domestic animals, pets, and occasionally humans. The global threat of emerging or re-emerging tick-borne diseases supports the need for research focused in the zoonotic transmission, especially in countries like Mozambique where rural populations are in close contact with domestic animals. The present study aims to: (1) identify tick species infesting cattle from Monapo and Nacala Porto, districts of Nampula province, Mozambique; and (2) investigate the presence of pathogens in the collected ticks. A total of 646 ticks were collected from cattle and morphologically identified as *Amblyomma variegatum*, *Rhipicephalus microplus*, and *R. evertsi evertsi*. For convenience, 72 *A. variegatum* and 15 *R. microplus* from Monapo, and 30 *A. variegatum* from Nacala Porto were screened for the presence of the selected pathogens: *Rickettsia* spp. (*A. variegatum*), and *Babesia/Theileria* spp. and *Anaplasma/Ehrlichia* spp. (*R. microplus*). *Rickettsia africae* was detected in four of the 72 *A. variegatum* collected in Monapo (5.6%). Additionally, one *R. microplus* tick

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(6.7%) was positive for *Theileria velifera*, one positive for *Colpodella* spp., one positive for *Candidatus* Midichloria mitochondrii, and another one positive for *Anaplasma ovis.* Using the present approach, no microorganisms were detected in tick samples from Nacala Porto. These findings expand our knowledge about the repertoire of tick-borne microorganisms in ticks in Nampula province, Mozambique.

Keywords Mozambique · Cattle ticks · Zoonotic pathogens · PCR

Introduction

Ticks are the most significant vectors of diseases in animals with medical and veterinary importance since they can act as vectors for many pathogens that cause disease in wild and domestic animals and pets (Jongejan and Uilenberg 2004; Dennis and Piesman 2005; de la Fuente et al. 2008). The greatest impact of tick-borne diseases (TBDs) is focused on domestic animals, particularly in cattle in which every year severe economic losses are reported (Jonsson 2006; Grisi et al. 2014). Humans, especially individuals who perform activities related to the field or livestock farming, or whose homes are close to farming properties are more susceptible to tick bites (Ramos et al. 2014; Otranto et al. 2014). Some of the tick-transmitted pathogens are of exceptional importance because of the high morbidity and mortality (in both humans and animals), long-lasting or permanent neuropsychiatric sequelae and impact on animal production (Mediannikov and Fenollar 2014; Baneth 2014).

In Mozambique, more than half of the population depends on agriculture and cattle production (Rocha et al. 1991). Like in other African countries, farmers face an immense challenge in keeping cattle productive, as a range of endemic parasitic and vector-borne diseases affect their livestock (Eisler et al. 2007) and are at higher risk to contracting a TBD.

African tick-bite fever is an important emerging infectious disease caused by *Rickettsia africae* that belong to the spotted fever group. This rickettsia is transmitted by ticks of the *Amblyomma* genus, of which, *A. variegatum* and *A. hebraeum* are recognised as principal vectors (Jensenius et al. 2003). Despite being a very common disease in Africa, African tick bite fever reports in the native population are scarce since it is in general a mild disease. However, in tourists, the number of reported cases is high probably due to the aggressive host-seeking behaviour of the vector ticks (Kelly et al. 1996; Oostvogel et al. 2007; Roch et al. 2008; Jensenius et al. 2009; Althaus et al. 2010; Parola et al. 2013; Delord et al. 2014). In Mozambique, both species have been reported (Bournez et al. 2015) but there is no reference to *R. africae* prevalence in either ticks or cattle. In the case of TBD affecting cattle, anaplasmosis and babesiosis continue to be the most serious problem in animal production (Martins et al. 2008; Suarez and Noah 2011). Bovine anaplasmosis is mainly caused by the rickettsia *Anaplasma marginale*, which can be transmitted by approximately twenty tick species (including *R. microplus*), and by bloodsucking diptera (Elelu et al. 2016; Domingos et al. 2013; Kocan et al. 2004). At least some areas around Maputo province can be considered endemic for bovine babesiosis (Martins et al. 2010) which is caused by the protozoa *Babesia bovis* and *Babesia bigemina*, and the "cattle tick" *R. microplus* plays an important role in the transmission of this disease (Bock et al. 2004).

The present study was designed with two objectives: first, considering that Nampula livestock are of great importance to the economy, the study aims to elucidate about the tick species occurring in cattle in two districts of Nampula province, Mozambique. Moreover,

since the extent of zoonotic transmission of tick-borne pathogens in Mozambique remains poorly understood, and that humans are in constant contact with animals, this study also intends to investigate the presence of some pathogenic agents the collect ticks.

Materials and methods

Study area

A brief description of Mozambique: the country covers an area of 799,380 km^2 between 11° and 27°S latitude, and ranges in altitude from 1600 m to sea level, with an extensive (2800 km) coastline along the Indian Ocean. Three major rivers cross the country—the Zambezi, the Sare and the Limpopo. The valleys and flood plains of the Zambezi, Limpopo and Incomati rivers provide fertile soils where most of the country's cattle are found. The present study was conducted from June to August 2015 on private farms in the Nacala Porto (14°33′45″S; 40°41′07″ E) and Monapo (14°55′01″; 40°41′07″E), districts of Nampula province, the third largest city in Mozambique (Fig. 1). The distance between these two districts is approximately 72 km.

Fig. 1 Location map of the study area

Tick collection

Feeding adult ticks were manually collected from mixed breed bovines by searching different body areas that are preferred by these ectoparasites (e.g. ear, dewlap, shoulder, belly, groin udder and perineum). Collection of ticks from the hosts was performed after obtaining permission from the owners and care was taken to minimize the animal's discomfort during the procedure. Ticks were stored in properly labelled 1.5 mL Eppendorf microtubes containing 70% ethyl alcohol and transported to Eduardo Mondlane University. The ticks were then separated by stage/gender and identified to the species level according to the morphological keys of Walker et al. (2003), under a stereomicroscope, and stored at −20 °C until DNA extraction.

DNA extraction

Tick DNA extraction was conducted at the Biotechnology Center, Eduardo Mondlane University (CB-UEM), Maputo, Mozambique. The samples were defrosted, ticks were washed in sterile phosphate-buffered saline (PBS) solution, cut into small pieces with sterile scissors and then placed into sterile 1.5 mL microtubes. The extractions were performed using the Qiagen Kit QIAamp DNA Tissue Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer instructions. DNA was eluted in 200 µl of elution buffer and samples were stored at −20 °C for downstream applications.

Molecular detection of pathogens

Conventional PCR was used to amplify *Anaplasma* spp. and *Ehrlichia* spp. with the primers EHR16SF/EHR16SR that aims to amplify a 345 bp fragment of the 16S rRNA gene of bacteria within the family Anaplasmataceae, including the genera *Anaplasma*, *Ehrlichia*, *Neorickettsia* and *Wolbachia*. For detection of piroplasms a touch-down PCR was carried out using the primer set PiroA/B targeting a 408 bp fragment from the 18S rDNA gene of *Babesia* spp. and *Theileria* spp. PCRs were performed in 25 µL reactions with Supreme NZYTaq 2× Green Master Mix (NZYTech, Lisbon, Portugal), 1 µM primers and 5 µl of template DNA. Water was used as negative control; DNA from reference strains was used as positive controls: *E. canis* Jabotical strain and *B. bigemina* Israel strain. Amplifications were performed in a T100 thermal cycler (Biorad, Hercules, CA, USA) with the cycling conditions stated in Table 1.

Regarding the detection of *Rickettsia* spp., the set of primers CS-78 and CS-323 were used to amplify a 401 bp fragment of the citrate synthase gene (gltA), previously reported as adequate for detection of *Rickettsia* spp. (Labruna et al. 2004). Reactions were performed in a total volume of 25 µL containing 100 ng of DNA, 10 µM of each primer, 1U Taq DNA polymerase, 2.5 mM MgCl2, and 200 µM of dNTPS (Promega, Mannheim, Germany). After amplifications, PCR products were separated by electrophoresis in a 1.5% agarose gel, stained with SYBRSafe (Invitrogen, CA, USA) and examined in a transilluminator model TFX-20-M (6 × 15 W). A 100 bp DNA ladder (Qiagen, Hilden, Germany) was used as a standard molecular weight. Samples that were found to be positive for *Rickettsia* spp. were subjected to a second PCR round with the primers Rr190.70p and Rr190.701n that are specific for *R. africae* and target a 631 bp fragment of the ompA gene (Fournier et al. 1998). Amplifications were carried out in a Biometra thermocycler

Table 1 Nucleotide sequences of specific primers, PCR conditions and the targeted size for pathogens transmitted by ticks

Pathogen	Gene	Primer	Primer sequence (5′-3′)	PCR condition	Size (Bp)	Positive control	References
Rickettsia spp.	*gltA*	CS78-F	F: GCAAGTATCGGTGAGGATGTAAT	95 °C for 3 min, 40 cycles at 95 °C for 15 s, 48 °C for 30 s	401	*R. conorii*	Venzal et al. (2012)
		CS323-R	R: GCTTCCTTAAAATTCAATAAATCAGGAT	72 °C for 30 s and final extension (f. ext.) at 72 °C for 7 min			
	ompA	Rr190 k.71p	F: TGGCCAATATTTCTCCAAAA	95 °C for 3 min, 40 cycles at 95 °C for 20 s, 46 °C for 30 s	650	*R. conorii*	Venzal et al. (2012)
		Rr190 k.720n	R: TGCATTTGTATTACCTATTGT	63 °C for 60 s and f. ext. at 72 °C for 7 min			
Ehrlichia spp.	16S rRNA	EHR 16SF	F: GGTACCYACAGAAGAAGTCC	95 °C for 3 min, 40 cycles at 95 °C for 30 s, 62 °C for 30 s	345	*E. canis*	Parola et al. (2000)
		EHR 16SR	R: TAGCACTCATCGTTTACAGC	72 °C for 1 min and f. ext. at 72 °C for 5 min			
Babesia spp.	18S rRNA	Piro-A	F: AATACCCAATCCTGACACAGGG	95 °C for 2 min, 40 cycles at 95 °C for 30 s, touchdown from 64 to 52 °C	400	*B. bovis*	Armstrong et al. (1998)
		Piro-B	R: TTAAATACGAATGCCCCCAAC	for 30 s, 72 °C for 35 s and f. ext. at 72 °C for 5 min			

(Biometra, Göttingen, Germany). PCR cycling conditions and specific primers are summarized in Table 1.

In all cases, positive amplicons were purified using the NZYGelpure kit (NZYTech) and sent for Sanger sequencing at StabVida (Lisbon, Portugal). Obtained sequences were analysed using Geneious v.7.1 software (www.geneious.com) and Basic Local Alignment Search Tool (BLAST) used for comparison with sequences deposited in the National Centre for Biotechnology Information (NCBI) nucleotide database (http://blast.ncbi.nlm.nih.gov/Blast).

Results

Ticks

In total 646 ticks were collected in this study, of which 382 (59%) specimens were from Monapo and 264 (41%) were from Nacala Porto. From these, in Monapo, 252 (66.0%) were identified as *A. variegatum* and 130 (34.0%) as *R. microplus* ticks. From the ticks collected in Nacala Porto 51 (19.3%) were identified as *A. variegatum*, 209 (79.2%) as *R. microplus*, and four (1.5%) as *R. evertsi evertsi*. For DNA extraction specimens weighting 25–40 mg were selected to be included in this study, as recommended in the protocol provided by the manufacturer of the DNA extraction kit. For convenience, some *A. variegatum* ticks were randomly selected from those collected in Monapo (72 specimens, 28.5%) and in Nacala Porto (30 specimens, 58.8%) and screened for the presence of *R. africae* while 15 (11.5%) *R. microplus* ticks, collected in Monapo, were tested for the presence of bacteria of the family Anaplasmataceae and for the presence of the protozoa *Babesia*/*Theileria*.

Molecular detection of pathogens

The results of prevalence of tick borne pathogens obtained in this study are shown in Table 2. All obtained amplicons were analysed, trimmed in order to evaluate clean sequences and deposited in GenBank. *R. africae* was detected using a fragment of *ompA* gene in four (5.6%) of the 72 *A. variegatum* ticks from Monapo. The sequences of PCR-derived DNA demonstrated 98.9–100% of similarity with the sequence of this pathogen under the accession number KX063619. All the tested samples of *A. variegatum* coming from Nacala Porto were negative by PCR for the *gltA* gene.

Among the 15 *R. microplus* from Monapo, one tick (6.7%) was found to harbour *Theileria velifera*, showing 98% similarity with the sequence of the pathogen under the accession number KU206307. Interestingly, one amplicon obtained from *R. microplus* (6.7%) demonstrated 96% similarity with the sequence under the accession number KP213195 identified as an alveolate organism, with a query coverage of only 62%. The second hit showed a query coverage of 89% and a 86% similarity to *Colpodella* sp. (accession number AY142075) thus suggesting that the amplified sequence should be accounted as *Colpodella* sp.

One (6.7%) *R. microplus* was positive for *Anaplasma ovis* showing 97.3% homology with the previously reported sequence of this pathogen under the accession number JQ917877 and another one (6.7%) demonstrated 99% similarity with the sequence of *Candidatus* Midichloria mitochondrii (accession number KU5559921).

Table 2 Prevalence of microorganisms in ticks collected from cattle in Monapo and Nacala Porto districts, Nampula, Mozambique

District	Species	Specimens	Gene	Positive samples	Microorganism DNA detected	Prevalence	Accession number	Percentage identify by BLAST
Monapo	*A. variegatum*	72	*gltA*	5	not applied (na)	na	na	na
			ompA	4	*Rickettsia africae*	4.5%	MF190574-7	98.9—100% (KX063619)
			18S rDNA		*Theileria velifera*	6.7%	KY888169	99% (KJ941106)
	R. microplus	15			*Colpodella* spp.	6.7%	KY914473	91.6% (KU159286)
			16S rDNA	2	*Candidatus* Midichloria mitochondrii	6.7%	MF176957	99% (EU315779)
					Anaplasma ovis	6.7%	MF176956	97.3% (JQ917877)
Nacala Porto	*A. variegatum*	30	gltA	0	na	na	na	na
			ompA	0	na	na	na	na

Discussion

Tick-borne rickettsial diseases (e.g. African tick- bite fever and anaplasmosis) pose important threats to human health throughout the world. Other tick-borne pathogens such as *Babesia* sp., despite capable of infecting humans, have their greatest impact in animal health. Knowledge on the dynamics of the circulation of tick-borne pathogens is essential to assess infection risks on both animals and humans in different geographical areas. In countries such as Mozambique, this information is even more critical since the majority of the population depend and live close to grazing animals being more susceptible to zoonoses (Rocha et al. 1991; Molyneux et al. 2011). In this study, ticks, collected from cattle of farms in the Mozambican providence of Nampula were firstly identified and afterwards, screened for some tick-borne pathogens.

The identification of ticks found parasitizing cattle resulted in the recognition of only three ixodid tick species: *A. variegatum*, *R. microplus* and *R. evertsi evertsi* which have all been previously identified in Mozambique (De Matos et al. 2009; Horak et al. 2009; Bournez et al. 2015). In Nacala Porto 66.0% of the collected ticks were identified as *A. variegatum* and 34.0% as *R. microplus*. Regarding the Monapo district, an inversion of proportions was observed, with 19.3% of *A. variegatum* ticks recovered and 79.2% of *R. microplus*. Also here, about 1.5% of the ticks infesting cattle were accounted has *R. evertsi evertsi*. A 2009 study conducted in Maputo, Mozambique, shows similar prevalence's to this latter result whereas *R. microplus* was found to be the main tick infesting cattle (De Matos et al. 2009). Contrary to this report, herein, within the *Amblyomma* genus only *A. variegatum* ticks were identified, which can be explained by sexual competition with *A. hebraeum* a closely related tick. These species are widely distributed in Mozambique but co-occurrences of both species is not frequent explaining the absence of *A. hebraeum* in our collection (Santos Dias 1993; Bournez et al. 2015).

It has been established that the abundant and aggressive *A. variegatum* tick is the main vector of *R. africae* (Socolovschi et al. 2009; Mediannikov et al. 2010). In combination with the high rates of *R. africae*, transovarial and trans-stadial transmission (Socolovschi et al. 2009) makes *A. variegatum* a major threat in the dissemination of African tick bite fever. Bearing this in mind, 102 *A. variegatum* ticks were screened by PCR for the presence of *R. africae*. Amplification of the ompA gene from *R. africae* was detected in four samples all in the district of Monapo. Using the same approach, this pathogen has been reported in many sub-Saharan African countries with prevalence rates of 67% in Uganda, 62% in Nigeria (Lorusso et al. 2013), 75% in Cameroon (Ndip et al. 2004), 34% in Mali and Niger and 7.6% in Burundi (Parola et al. 2001). Our results, showing a global prevalence of 3.9% and 5.6% in Monapo, are closer to the survey performed in Burundi, which is the country that is least distant from Mozambique. Our findings extend knowledge about the geographic range and dynamics of this pathogen in *A. variegatum* ticks in Africa.

Rhipicephalus microplus ticks are the major concern in cattle industry and are responsible for the transmission of several pathogens, in particular *Anaplasma* spp. and *Babesia* spp. responsible for the most important livestock diseases worldwide (Suarez and Noah 2011). Although not frequent, *R. microplus* ticks can bite humans (Guglielmone et al. 1991; Estrada-Peña and Jongejan 1999; Liyanaarachchi et al. 2015; Lamattina and Nava 2016) thus, information regarding the circulation of pathogens within hosts (bovine and tick) can be useful for preventing the transmission of tick-borne diseases.

A small survey in 15 *R. microplus* collected from Monapo was perfomed using two broad range PCRs that can detect bacteria within the family Anaplasmataceae or *Babesia* sp./*Theileria* sp. From these, one sample (6.7%) was positive for *T. velifera*. According to Capinera (2008), this apicomplexan protozoan parasite is found throughout the south of the Sahara. This species is considered to have low pathogenicity in cattle, thus having little economic importance (Bell-Sakyi et al. 2004). Kubelová et al. (2012) reported the presence of this pathogen in 14% of bovines in Angola and Lorusso et al. (2016) found this pathogen in 52.4% of bovines in Nigeria. According to Wernecke et al. (1979) and Capinera (2008), *A. variegatum, A. hebraeum*, and *A. lepidum* are vectors of *T. velifera* in South Africa and possibly also in Mozambique. The presence of *T. velifera* in a *R. microplus* suggests that this pathogen may be circulating in the cattle blood, however, more experiments such as real-life transmission experiments are necessary to determinate if *R. microplus* could also act as a vector of this protozoan to bovines.

Interestingly, the DNA amplicon sequenced from one (6.7%) *R. microplus* was found to have high homology to *Colpodella* sp. a genus that is closely related to Apicomplexa. These organisms are not common in nature, and few are known to be associated with humans, animals, and arthropods (Leander and Keeling 2003). Cong et al. (2012) described the case of an apparent erythrocyte infection in a human in China, in which the organism's DNA sequence was shown to be related to colpodellids. Our finding does not provide evidence, that *Colpodella* sp. could infect ticks and vertebrates because the presence of this pathogen in cattle was not screened in this experiment. Moreover, we cannot discard the possibility that the amplified DNA may have resulted from an environmental contaminant (although this pathogen is not commonly found in nature). Thus, more studies are needed to confirm the occurrence of *Colpodella* sp. in vertebrates and ticks.

Regarding the family Anaplasmataceae, two positive samples were obtained. The first one showed 99% homology with the previously reported sequence of *Candidatus* Midichloria mitochondrii, an alphaproteobacterium endosymbiont of ticks. It has already been identified in the tick genera *Ixodes*, *Amblyomma, Dermacentor, Haemaphysalis, Hyalomma*, and *Rhipicephalus* (Epis et al. 2008; Dergousoff and Chilton 2011). The importance of this bacterium in ticks is attested by records that it has a prevalence of 100% in the female ovary and salivary glands, eggs, and immature *I. ricinus*, while in males, the prevalence of this microorganism did not exceed 44% (Beninati et al. 2004; Lo et al. 2006). Di Venere et al. (2015) emphasized that investigations on its potential role as an infectious agent have not provided adequate answers concerning the possible link to pathological effects.

The other positive sample showed 97.3% homology with the previously reported sequence of *A. ovis.* This intraerythocytic bacterium is transmitted primarily by *R. bursa* and infects sheep, goats, deer, and wild ruminants (Friedhoff 1997; Ferrolho et al. 2016). In small ruminants, infection is usually subclinical, but occasionally, it can be severe, with symptoms such as anemia, hemoglobinuria, and fever (Hornok et al. 2007). Chochlakis et al. (2010) reported a case of a positive blood sample from a woman from Cyprus showing 100% homology with *A. ovis* and other *Anaplasma* spp. However, transmission of *A. ovis* to humans is unclear.

Conclusion

Our findings expand the knowledge about the repertoire of tick-borne microorganisms that are present in ticks in Nampula, Mozambique and highlight the risk of human infection with pathogens, mainly *R. africae*. Information about the prevalence of infection in ticks is

essential to draw a strategic framework for research and develop novel control methodologies, which are necessary to restrain the ongoing and new threats posed by tick-borne diseases.

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Author contributions AM and VM participated in tick collections, tick identification, conducted DNA extraction and performed *R. africae* PCR screening. GS and SA performed the PCR for *Babesia/Theileria* and *Anaplasmataceae* family. GS, SA, and AD analysed data and wrote the article. LN, EM and AD designed and supervised the study. All authors edited and approved the final manuscript.

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