Old Agents and Novel Variants of Tick-borne Microorganisms From Angola, 2017

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Research Article

Keywords: Ticks, tick-borne microorganisms, zoonotic agents, Rickettsia, Anaplasmataceae, Coxiella, Borrelia, Spiroplasma, Babesia, Angola

Posted Date: December 22nd, 2021

DOI: https://doi.org/10.21203/rs.3.rs-1153383/v1

Abstract

The study of microorganisms from ticks collected in cattle from Angola is reported herein, demonstrating the circulation of the pathogen *R. aeschlimannii* and potential novel tick-borne microorganisms with unknown pathogenicity belonging to *Ehrlichia*, *Spiroplasma*, *Coxiella*, *Babesia* and *Francisella* spp. and corroborating the presence of *Rickettsia africae* and *Babesia bigemina*.

Introduction

The COVID-19 pandemic and epidemics like EBOLA, Lassa fever, Zika virus disease, Nipah virus infection, avian influenza, etc. have strengthened the importance of One Health to prevent spillovers. Human and animal health and the environment are interconnected, and factors such as globalization, climate change, changes in land uses, population growth, etc. could trigger new zoonotic outbreaks [1]. Early detection and knowledge of potential zoonotic agents, including vector-borne microorganisms, are relevant to implement containment measures and prevent related infectious diseases. Thus, surveillance systems of vectors and their microorganisms are required.

Zoonotic agents, often underdiagnosed due to lack of diagnostic resources, are a known major cause of disease in Sub-Saharan Africa, and studies have raised the need of improving protocols for fever of unknown origin (FUO) management [2]. Tick-borne relapsing fever, rickettsiosis and babesiosis have been reported from southern Africa [2–3], but tick-borne diseases from Angola are hardly known. Angolan livestock population is increasing (https://www.fao.org/faostat/en/ # data/QCL), mainly based on cattle production, and the expansion of livestock industry is linked to the incidence of zoonosis [4]. Therefore, we report the study of selected microorganisms in ticks from Angolan cattle.

Materials And Methods

Ticks were collected from cattle in a slaughterhouse of Cubal (Benguela Province, Angola) from 1-8 July 2017, and preserved in ethanol 70%. Specimens were classified using a taxonomic key [5]. Selected individuals (at least two specimens from each morphologically classified species and those doubtful according to morphological features) were genetically characterized by PCR of mitochondrial genes (Additional file: Table S) using individual DNAs from legs subjected to ammonium extraction [6]. Furthermore, tick halves were pooled (1-9 specimens) according to species and developmental stages. DNA from pools was extracted using DNeasy Blood & Tissue kit (Qiagen), following manufacturer's recommendations with overnight lysis. Mitochondrial 16S rRNA PCRs were performed as controls of pool extractions (Additional file: Table S). Bacteria (*Rickettsia*, Anaplasmataceae, *Borrelia*, *Coxiella* and *Spiroplasma*) and protozoa (*Theileria* and *Babesia*) were screened using specific PCR assays. Pan-bacterial 16S rRNA PCR was also performed (Additional file: Table S).

Nucleotide sequences were analyzed, compared with those available in NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and submitted to GenBank, when different. Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) was used for multiple sequence alignment. Phylogenetic analyses were conducted with MEGA X (http://www.megasoftware.net) using maximum likelihood method including all sites. Confidence values for individual branches of resulting trees were determined by bootstrap analysis (500 replicates).

Results

A total of 124 ticks (five nymphs, 28 males and 91 females) were collected and morphologically classified as six *Amblyomma variegatum*, six *Hyalomma truncatum*, 107 *Rhipicephalus decoloratus* and five *Rhipicephalus* spp. Whenever performed, genetic characterization confirmed morphological identification, and also allowed to identify three *Rhipicephalus duttoni* and one *Rhipicephalus evertsi mimeticus* (Tables 1-2) among those *Rhipicephalus* spp.

Table 1
Comparison (% identity) of the studied Angolan tick mitochondrial amplicons with available GenBank sequences.

Tick species	% identity (bp)-GenBank accession No. (No. of analysed amplicons)		
	16S RNA	12S RNA	COI
A. variegatum	99.0 (404/408)-L34312 (3)	99.4 (339/341)-HQ856466 (3)	99.3 (560/564)-MK648415 (1)
H. truncatum	99.8 (401/403)-LC634545 (2)	100 (341/341)-AF150031 (2)	99.2-99.4 (617/622-670/674)-KY457529 (2)
R. decoloratus	99.5-99.8 (399-400/401)- KY457525 (4)	99.7 (343/344)-NC_052828 (4)	99.4-99.1 (616/620-652/658)-NC_052828 (3)
R. evertsi mimeticus	99.7 (370/371)-MF425975 (1)	100 (318/318)- AF031862 (1)	NA
R. duttoni	99.7 (352/353)-MW080164 (3)	98.7 (310/314)-MF425966 (1)	NA
Rhipicephalus sp.	97.0 (393/405)-LC634554[†] (1)	98.2 (333/339)-KY457542[1] (1)	NA

bp: base pairs; *A.*: *Amblyomma; H.*: *Hyalomma; R.*: *Rhipicephalus*; NA: Not amplified; [†] *Rhipicephalus simus*

Table 2

Microorganisms amplified in this study. Data show the species names and the highest identity with public sequences (%; GenBank accession number) followed by the number of pools in which they have been detected and, in brackets, the number of ticks from each pool.

Microorganisms	Target gene	*Amblyomma variegatum* (2: 5N, 1M)†	*Hyalomma truncatum* (3: 1M, 5F)†	*Rhipicephalus decoloratus* (16: 24M, 83F)†	*Rhipicephalus duttoni* (2: 1M, 2F)†	*Rhipicephalus evertsi mimeticus* (1: 1F)†	*Rhipicephalus* sp. (1: 1M)†
Rickettsia spp.	*ompA*	*R. africae* (100;CP001612) 2(5N, 1M)	*R. aeschlimannii* (100;HQ335157) 3(1M, 5F)	*R. africae* (100;CP001612) 1(9M)	-	-	-
Anaplasma/ Neoehrlichia/ Ehrlichia spp.	*groESL*	-	-	*Ehrlichia* spp. (100;MW054557) 6(44F)	-	-	-
Ehrlichia spp.	*gltA*	NP	NP	*Ehrlichia* spp. (96.9-97.0; KX987353)‡ 6(44F)	NP	NP	NP
	16S rRNA§	NP	NP	*Ehrlichia* sp. (99.9;AF497581) 1(9F)¶	NP	NP	NP
Borrelia spp.	*flaB*	-	-	-	-	-	-
	glpQ	-	-	-	-	-	-
Coxiella burnetii	*IS1111*	-	-	-	-	-	-
Coxiella/ Francisella spp.	*rpoB*	*Coxiella* spp. (98.9-99.2; KP985305) 2(5N, 1M)	SNC	*Coxiella* spp. (100;KP985329) 16 (24M,38F)	*Coxiella* sp. (95.9; KP985337) 2 (1M, 2F)	*Coxiella* sp. (99.1;KP985331) 1 (1F)	*Coxiella* sp. (97.8;KP985337) 1 (1M)
	groEL	*Coxiella* spp. (99.5; KP985486) 2 (5N, 1M)	*Francisella* sp. (96.8;CP013022, CP012505)†† 1 (4F)	*Coxiella* spp. (100;KP985510) 16 (24M,38H)	*Coxiella* sp. (97.3;KY678195) 2 (1M, 2F)	*Coxiella* sp. (98.2;KY678195) 1 (1F)	*Coxiella* sp. (98.3;CP011126) 1 (1M)
	16S rRNA§	NP	*Francisella* sp. (99.6;AB001522) 1 (4F)‡‡	*Coxiella* sp. (99.4;JQ480818) 1(5H)¶	NP	NP	NP
Spiroplasma spp.	*rpoB*	-	-	*Spiroplasma* spp. (99.4;KP967687)§§ 3 (24M)	-	-	-
	16S rRNA	NP	NP	*Spiroplasma* spp. (98.7-100; KP967685)¶¶ 3 (24M)	NP	NP	NP
Theileria spp./ *Babesia* spp.	18S rRNA	*Babesia* spp. (91.4;AB734390) 2 (5N, 1M)	-	*B. bigemina* (100;KF606863) 2 (10F)	-	-	-

Microorganisms	Target gene	*Amblyomma variegatum* (2: 5N, 1M)†	*Hyalomma truncatum* (3: 1M, 5F)†	*Rhipicephalus decoloratus* (16: 24M, 83F)†	*Rhipicephalus duttoni* (2: 1M, 2F)†	*Rhipicephalus evertsi mimeticus* (1: 1F)†	*Rhipicephalus* sp. (1: 1M)†
Babesia spp.	ITS 1	*Babesia* spp. (70.9; LK391709) 2(5N, 1M)	NP	*B. bigemina* (98.8-100; EF458251)¶¶ 2 (10H)	NP	NP	NP
	ITS 2	*Babesia* spp. (74.7; EF186914) 2 (5N, 1M)	NP	*B. bigemina* (99.5;EF458266) 2 (10F)	NP	NP	NP

†Numbers in brackets indicate (number of pools: number of ticks and developmental stage); ‡Two genetic variants were identified; §Pan-bacterial PCR assay; ¶PCR assay performed to four samples but, because this is a pan-bacterial PCR assay (Additional file:Table S), the bacterium was only amplified from one sample; ††With 87,6% and 65% query cover, it reached 98.2% and 98.7% identity with *Francisella* sp. detected in soft and hard ticks, respectively (MW287617 and KY678032); ‡‡With 92% query cover, it reached 99.8% identity with *Francisella* sp. amplified from *Hyalomma truncatum* (JF290387); §§with 42% query cover, the sequences are identical to available *Spiroplasma* sequences from *Rhipicephalus decoloratus* (MK267083-4) but also to those detected in other *Rhipicephalus* and *Ixodes* species (MK267073-7,MK267082,MK267085); ¶¶Nucleotide sequences show several ambiguous bases; N: nymphs; M: males; F: females; SNC: Sequences not conclusive; NP: Not performed.

Twenty-five pools (two *A. variegatum*, three *H. truncatum*, 16 *R. decoloratus*, two *R. duttoni*, one *R. evertsi mimeticus*, and one *Rhipicephalus* sp.) were screened for microorganisms.

Rickettsia spp. was found in 6/25 pools. According to *ompA*, *Rickettsia africae* was detected in two *A. variegatum* and one *R. decoloratus* pools; and *R. aeschlimannii*, in three *H. truncatum* pools (Table 2). *Ehrlichia* spp. was found in 6/25 pools of female *R. decoloratus*. Analysis of *groESL*, *gltA* and 16S rRNA amplicons revealed the highest identities with unclassified *Ehrlichia* (Table 2, Figure), and showed less than 93.5%, 87.6% and 99.2% identity, respectively, with validated species. Other Anaplasmataceae, *Borrelia* spp. (relapsing fever or Lyme groups) or *Coxiella burnetii* were not detected. Nevertheless, *Coxiella* spp. were found in all but *H. truncatum* pools. For *H. truncatum*, *rpoB* sequences showed inconclusive data, whereas g*roEL* and universal 16S rRNA sequences showed the highest similarity (<97% and 99.6%, respectively) with *Francisella* sp. in one pool. This 16S rRNA amplicon showed 99.8% identity (92% query cover) with *Francisella* endosymbiont of *H. truncatum* JF290387 (Table 2). For the remaining tick species, different *Coxiella* genotypes were found. All but two were identical or closely related to public sequences. Genotypes detected in *R. duttoni* and *Rhipicephalus* sp. did not reach >98.3% identity with *Coxiella* (Table 2, Figure). *Spiroplasma* sp. was amplified from three *R. decoloratus* male pools (Table 2). According to *rpoB*, it was closely related to *Spiroplasma ixodetis* and related strains of hard ticks (Figure).

Babesia bigemina was identified in two *R. decoloratus* female pools, and *Babesia* sp. was detected in two *A. variegatum* pools, according to 18S rRNA, ITS-1 and ITS-2 analysis (Table 2, Figure).

Novel sequences of this study were deposited on GenBank under accession numbers: OK481091-OK481100; OK481107-OK481113; OK491113-OK491116; OK482869-OK482874; OK514711-OK514725.

Discussion

This study reports the detection of well-known pathogens: *R. africae*, *R. aeschlimannii* and *B. bigemina*, and scarce characterised *Ehrlichia*, *Coxiella*, *Francisella*, *Spiroplasma* and *Babesia* species with unknown pathogenicity in ticks from cattle in Angola.

Our results corroborate the circulation of *R. africae* and demonstrate the circulation of *R. aeschlimannii* in Angola. Although *R. aeschlimannii* human infection had been reported from South Africa and *H. truncatum* had been suggested as vector [7–8], this pathogen had not been previously found in Angola. African tick-bite fever is endemic in Sub-Saharan Africa but no cases from Angola have been notified [2–3]. This study confirms the recent detection of *R. africae* in *A. variegatum* (recognized vector) [9], suggesting that cases could be misdiagnosed. The presence of *R. africae* in *R. decoloratus* is known but their role as vector should be investigated [3, 10]. Moreover, our finding in fed ticks could be due to blood meal or cofeeding.

Only six *Ehrlichia* species are currently recognized and all but one cause ehrlichiosis [11], a disease with human cases reported from southern Africa [3]. Moreover, '*Candidatus'* have been proposed and *Ehrlichia* genotypes have been partially characterized. Further studies are needed to determine their taxonomic status and pathogenic potential. Herein, a novel *Ehrlichia* genotype has been detected in six *R. decoloratus* pools.

Tick diet based on blood is unbalanced, and endosymbionts (e.g. *Coxiella*-like, *Francisella*-like…) provide essential nutrients for ticks [12]. Although virulence genes identified in pathogenic related species, *C. burnetii* and *Francisella tularensis*, could be absent or non-functional in symbionts, *Coxiella*-like has been considered pathogen [13]. Herein, *Coxiella*-like was detected in all but *H. truncatum* pools, and isolates were identical or closely related to those

previously amplified in the corresponding tick species, except potential novel *Coxiella* genotypes of *R. duttoni* and *Rhipicephalus* sp. *Francisella* sp. was detected in 1/3 *H. truncatum* pools, showing a sequence genetically related with a *Francisella* sp. endosymbiont amplicon of this species.

Spiroplasma spp. have been found in several hard tick species, and the role of this genus as pathogen has been suggested [14]. Herein, *Spiroplasma* sp. closely related to *S. ixodetis* was detected in 3/16 *R. decoloratus* pools. *Spiroplasma* sp. was previously detected in this species according to a short *rpoB* sequence (Table 2), and this study provides a wider genetic identification.

Babesia bigemina, responsible for babesiosis, is prevalent in Angolan cattle [15]. Our study demonstrates its presence in *R. decoloratus* (competent vector) in Angola. Moreover, a potential novel *Babesia* species is circulating in Angolan *A. variegatum*.

These results should be considered to elaborate protocols for FUO patients' management in Angola. Surveillance of ticks and tick-borne microorganisms is needed to evaluate the risk of tick-borne diseases in Angola.

Declarations

Acknowledgments

Partial results of this study were presented at XXIII National Congress SEIMC (Madrid-Spain, 2019).

Funding

This work has been partially funded by European Regional Development Funds (FEDER).

Competing interests

The authors have no competing interests to declare.

Ethics approval and consent to participate

Not application

Authors' contributions

Palomar AM: Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Writing - original draft, Writing - review & editing. **Molina I:** Conceptualization, Resources, Formal analysis, Writing - review & editing. **Bocanegra C:** Conceptualization, Investigation, Writing - review & editing. **Portillo A:** Resources, Methodology, Formal analysis, Writing - review & editing. **Salvador F:** Conceptualization, Investigation, Supervision, Writing - review & editing. **Moreno M:** Investigation, Writing - review & editing. **Oteo JAO:** Conceptualization, Resources, Formal analysis, Funding acquisition, Writing - original draft, Writing - review & editing.

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Figures

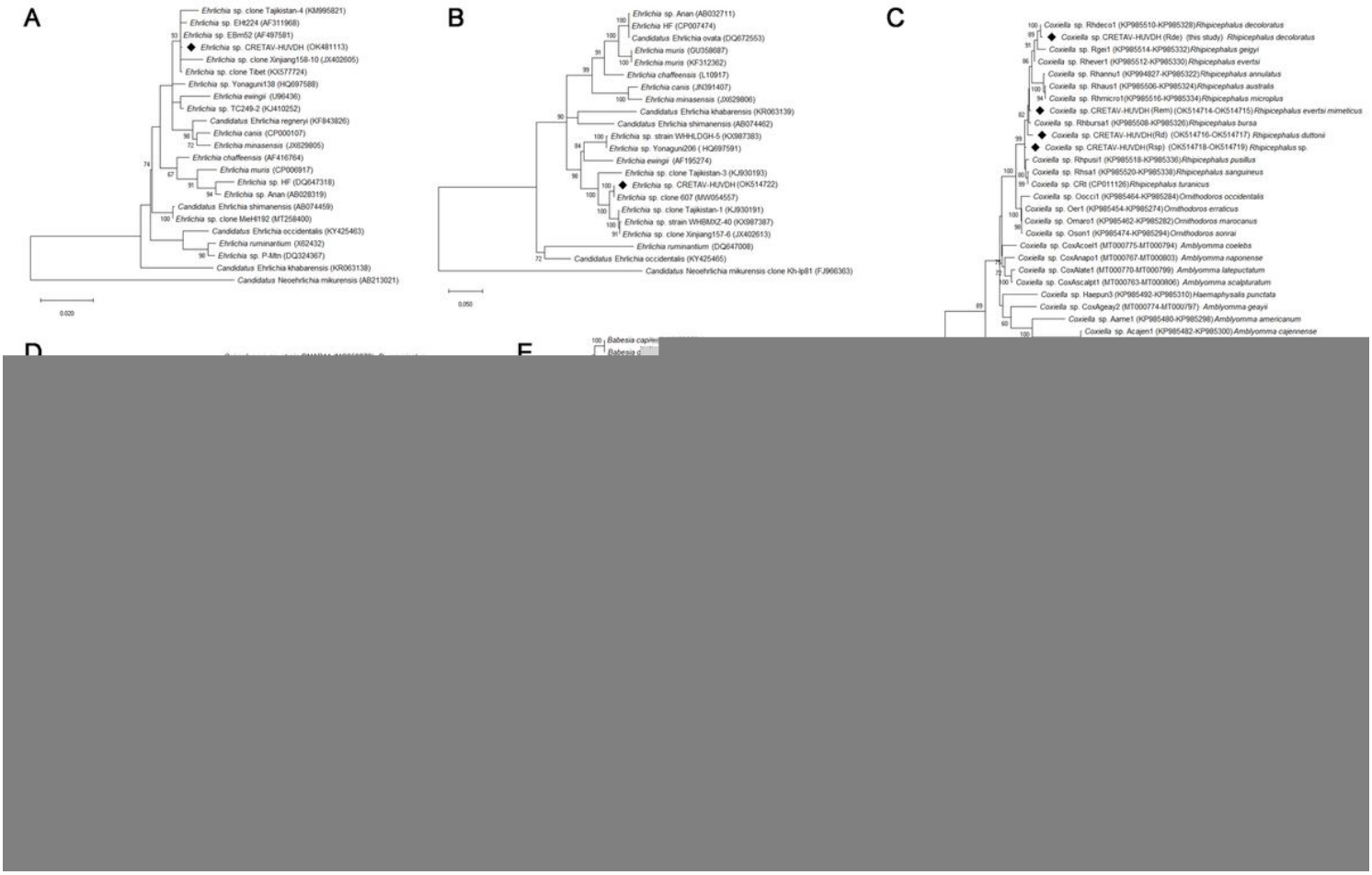

Figure 1

Phylogenetic analysis of the microorganisms detected in this study from ticks collected from cattle in Angola (marked with diamond). The maximum likelihood trees were obtained using the General Time Reversible model, a discrete Gamma-distribution and a proportion of invariable sites (GTR+G+I), nucleotide substitution selected according to the Akaike information criterion implemented in Mega X. The trees are drawn to scale, with branch lengths measured in the number of substitutions per site. Numbers (>60%) shown at the nodes correspond to bootstrapped percentages (for 500 repetitions). The GenBank accession numbers of the sequences used in these analyses are shown in brackets. **A.** *Ehrlichia* phylogeny was based on 23 partial 16S rRNA gene sequences with a total of 1,373 positions in the final dataset. *Candidatus* Neoehrlichia mikuresis was used as an outgroup. **B.** *Ehrlichia* phylogeny was based on 22 partial *groESL* gene sequences with a total of 1,232 positions in the final dataset. *Candidatus* Neoehrlichia mikuresis was used as an outgroup. **C.** *Coxiella*-like phylogeny was based on 51 partial *rpoB* and *groEL* concatenated sequences with a total of 1,055 positions in the final dataset. *Rickettsiella* sp. was used as an outgroup. **D.** Phylogeny of *Spiroplasma* spp. found in ticks based on 18 partial *rpoB* sequences with a total of 588 positions in the final dataset. **E.** Phylogeny of *Babesia* species based on 18S rRNA analysis. The analysis involved 40 nucleotide sequences and a total of 481 positions in the final dataset. *Plasmodium falciparium* was used as outgroup.

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