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# PCR detection of *Ehrlichia ruminantium* and *Babesia bigemina* in cattle from Kwara State, Nigeria: unexpected absence of infection

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**Abstract**
Ticks and tick-borne diseases (TBDs) continue to pose an insidious and ever-present threat to livestock and livelihoods across the globe. Two of the most significant TBDs of cattle in Africa are heartwater and babesioisis, caused by *Ehrlichia ruminantium* and *Babesia bigemina* respectively. Both pathogens are endemic in Nigeria. However, to date, little data has been published regarding the number of cattle infected. In this study, blood samples were collected from cattle of the Kwara State, north-central Nigeria. Probe-based quantitative PCR (qPCR) and semi-nested PCR were used to investigate the presence of both pathogens, respectively. Our study found all samples ($n = 157$) to be surprisingly negative for both *B. bigemina* and *E. ruminantium*. These results contribute new information on the current burden of these two pathogens in Kwara State and may be helpful in informing more effective targeting of control strategies in Nigeria.

**Keywords** *Ehrlichia ruminantium* · *Babesia bigemina* · Tick-borne diseases · Semi-nested PCR · qPCR · Nigeria

## Introduction

Two of the most significant tick-borne diseases (TBDs) of cattle in Africa are heartwater and babesiosis (Jongejan and Uilenberg 2004). Heartwater is a lethal bacterial disease, caused by the intracellular rickettsia *Ehrlichia ruminantium*, affecting ruminants and wild animals throughout sub Saharan Africa (OIE 2009). Transmission is largely thought to occur by ticks of the genus *Amblyoma* (Provost and Bezuidenhout 1987). The clinical signs include sudden fever, anorexia, respiratory failure and sudden death with mortality rates as high as 80% (Camus et al. 1996). Native cattle breeds living in endemic areas display varying levels of resistance and susceptibility; however, highly productive (European) breeds and their crosses suffer high morbidity and mortality rates (Camus et al. 1996). Bovine babesiosis is caused by a protozoan of the genus *Babesia* that parasitises host erythrocytes. It is estimated that over half a billion cattle may be at risk of

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infection by bovine babesiosis worldwide (Kim et al. 2007). One of the most important *Babesia* species reported in Africa is *B. bigemina* (Young et al. 1988), with infected animals displaying common clinical signs of fever, inappetence, jaundice, weight loss, haemoglobinemia and haemoglobinuria (Young et al. 1988; Susan and Asa 1998; Bock et al. 2004). Transmission in cattle occurs principally by ticks of the genus *Rhipicephalus* (OIE 2013). To date, few studies have investigated the prevalence of *B. bigemina* and *E. ruminantium* in Nigeria. Of those that have, only a small number have employed molecular methods, largely relying instead on serological techniques with the disadvantage of antigenic cross-reactions (Akinboade and Dipeolu 1984; Ajayi and Dipeolu 1986; Ogunsusi 1989; Kelly et al. 1994; Papadopoulos et al. 1996; Savadye et al. 1998). The aim of this study was to provide more information regarding the presence of *B. bigemina* and *E. ruminantium* in cattle blood samples obtained from a region of Kwara State, Nigeria, using probe-based quantitative real-time PCR (qPCR) and a semi-nested PCR. Our results will contribute toward improving disease control strategies, enabling the development of sustainable and productive livestock farming systems, ensuring food security and ultimately supporting economic growth.

## Materials and methods

### Study population

A total of 253 bovine blood samples collected from 11 cattle-producing villages across the Edu Local Government Area (LGA) in Kwara State, Nigeria, were included in the study (Table 1). The animals were randomly selected and clinically healthy at sampling. The procedures for sample selection, collection, extraction and storage can be found in Elelu et al. (2016). From the 253 samples collected, 96 were excluded from the current study due to a lack of sufficient biological material.

**Table 1** Villages across the Edu Local Government Area (LGA) in Kwara State, Nigeria, which were included in the study and their geographic locations (decimal degrees)

| Village | Latitude | Longitude |
|---|---|---|
| Bacita | 9.03716 | 4.57834 |
| Belle | 9.10084 | 4.56429 |
| Bokungi | 8.50345 | 5.01229 |
| Fanagun | 9.08517 | 4.57767 |
| Fedudangi | 8.51867 | 5.02557 |
| Gonandogo | 9.04780 | 4.57341 |
| Mokwagi | 9.03135 | 4.55922 |
| Ndabata | 8.57171 | 5.09441 |
| Ndachewoye | 9.04550 | 4.54668 |
| Tshonga farm | 8.57522 | 5.08022 |
| Yelwa | 9.08366 | 4.56211 |

### Pathogen detection and data analysis

A PCR that amplifies a 437-bp fragment of the *GAPDH* gene was carried out to confirm DNA integrity and the absence of PCR inhibitors as previously described (Birkenheuer et al. 2003). All the samples used in this study were positive for the correct amplicon. A probe-based quantitative qPCR assay with a sensitivity of 100% was used to detect and amplify a fragment of the *B. bigemina 18S* rRNA gene as previously described (Kim et al. 2007). Briefly, 20 μl reactions were prepared with 10 μl of SensiFAST™ Probe Low-Rox mix (Bioline, London, UK), 100 nM of probe (StabVida, Lisbon, Portugal), 400 nM of reverse and forward primers (StabVida), 2 μl of DNA template and nuclease-free water up to the final volume. Triplicate reactions were set up for each sample and were loaded into 96-well plates (VWR, Pennsylvania, USA). Thermal cycling conditions were as follows: one stage at 95 °C for 10 min, followed by 45 cycles of 95 °C for 20 s and 55 °C for 1 min. An Applied Biosystems 7500 Fast thermal cycler was used (ThermoFisher Scientific, Waltham, MA). Positive controls were prepared with the *B. bigemina* Israel strain, and negative (no template) controls with nuclease-free water. PCR efficiency was determined using sequence-specific fivefold serial dilution standard curves prepared with DNA from the positive control.

A semi-nested hot-start PCR with a sensitivity of 90% was also conducted to determine the presence of *B. bigemina* as previously reported by Martins et al. (2008), to amplify 614 bp and 275 bp fragments of a target region of the aspartic proteinase *babesipsin* gene. PCR reactions were prepared with 5 μl of Supreme NZYTaq 2X Green Master Mix (NZYTech, Lisbon, Portugal), 0.5 μM of primers BigBAF1 and BigBAF2, 2 μl of nuclease-free water and 2 μl of DNA template. Negative controls were prepared with no template and positive controls with *B. bigemina* DNA. Samples were loaded into 96-well plates (VWR) and the thermal cycling conditions used were the same as previously described by Martins et al. (2008) in a Bioer Gene Pro thermocycler (Hangzhou Bioer Technology Co., Ltd., China). PCR products from the first reaction were used for a second reaction using the primers BigBAF1 and BigBAR2 and under the same conditions as above. PCR products were separated on a 1.2% agarose (NZYTech) gel (*w*/*v*) in 0.5× TBE buffer (20 mM Tris, 20 mM boric acid, 0.5 mM EDTA, pH 7.2) stained with SybrSafe™ (Invitrogen, CA, USA) and examined by UV transillumination. The NZYDNA Ladder VIII DNA molecular weight marker (NZYTech) was used to confirm the size of the products.

The presence of *E. ruminantium* was investigated with a probe-based qPCR as described by Steyn et al. (2008) to

amplify a fragment containing two overlapping genes, *rnc* and *ctaG*, within the *PCS20* gene region. This assay has sensitivity to detect *PCS20* down to a dilution of $10^{-8}$ (7 PCS20 copies/μl) (Steyn et al. 2008). Briefly, 10 μl reactions were prepared with 5 μl of Grisp Xpert Fast Probe mix (Grisp, Porto, Portugal), 0.5 μM of each primer (StabVida), 0.4 μM of probe (StabVida), 1 μl of DNA template and nuclease-free water up to the final volume. Negative controls were prepared without DNA template, and positive controls with DNA extracted from *E. ruminantium*–infected tick cells. Triplicate reactions for each sample were loaded into 96-well plates (VWR) and run in a CFX Connect TM® Real-Time PCR Detection System with the thermal cycling conditions described previously (Bio-Rad, CA, USA). A standard curve was constructed with fivefold serial dilutions of the positive control DNA to determine reaction efficiency. Real-time PCR data was analysed using Bio-Rad CFX manager software version 3.1 (Bio-Rad) and the Applied Biosystems 7500 Fast 2.0.6 Software (ThermoFisher Scientific). Based on the standard curves, the threshold detection levels at each quantification cycle were set at 19.00 and 0.012 for *E. ruminantium* and *B. bigemina*, respectively. Samples requiring more than 36 quantification cycles (Cq) for detection above these thresholds were considered negative for the presence of pathogen. Confidence intervals (CI) for the proportion of samples which were found positive were calculated using the Wilson score (Brown et al. 2001).

## Results and discussion

A total of 157 blood samples collected from White Fulani (97.5%) and Jersey (2.5%) bovines of 11 cattle-producing villages in Edu LGA were tested for presence of *E. ruminantium* and *B. bigemina*. A probe-based qPCR targeting a fragment of the *18S rRNA* gene was conducted to screen the presence of *B. bigemina* in the blood samples. No samples were found to be positive according to the defined threshold. A few studies have reported that in this region, the frequency of *B. bigemina* should be higher than that found in this study (Abdullahi et al. 2014; Olabode et al. 2014; Lorusso et al. 2016). In view of this, a semi-nested hot-start PCR targeting the putative aspartic proteinase *babesipsin* gene of *B. bigemina* was performed to confirm the result. Again all samples were found to be negative. Our finding of no positive animals does not agree with the reported data on *B. bigemina* prevalence across Africa, which is recorded to be as high as 90% in cattle (Martins et al. 2008). The detection of pathogens in ticks frequently does not correlate directly with their presence in their hosts; indeed, a low prevalence in ticks could be associated with a very high prevalence in cattle (Tay et al. 2014). Ogo et al. (2012) analysed 218 ticks collected in north-central Nigeria, a similar geographic area to this study, and reported the presence of *B. bigemina* in 1.3% of the specimens. A possible explanation for low detection rates in ticks and hosts is the existence of a second parasite co-infection (Purnell et al. 1977; Friedhoff 1990; Kocan 1995; Zintl et al. 2003). Elelu et al. (2016) sampled the same individuals included in this study and found 75.9% to be positive for *Anaplasma marginale*. Within the definitive host, it has been suggested, through experimental infection of splenectomised calves, that concurrent infection with *Anaplasma* is able to supress *Babesia* colonisation (Purnell et al. 1977; Zintl et al. 2003). However, further studies of the interaction mechanisms would be beneficial, and certainly, a higher number of samples would give a more accurate idea about the presence of *B. bigemina* in this area of Nigeria.

*Ehrlichia ruminantium* infection has been reported to affect 61% of the cattle in West Africa (Koney et al. 2004) and is considered to be endemic to Nigeria (Leeflang 1977; Leeflang and Llemobade 1977). Despite these reports of high morbidity, all the samples in this study were found to be negative for the pathogen. A similarly low occurrence of 1.1% was reported by Lorusso et al. (2016) in 704 indigenous cattle tested in the Plateau State, Nigeria. Findings of low prevalence are often attributed to the biology of *E. ruminantium* infection. Initial disease is associated with a febrile period during which high concentrations of microorganisms are found in the blood. Animals that survive often become asymptomatic carriers, releasing *E. ruminantium* just periodically into the circulation (Andrew and Norval 1989). The resulting low levels of pathogen in host blood samples has proven problematic for pathogen detection, and thus carrier animals can appear negative when tested. Although the qPCR protocol used in this study has been reported to be highly sensitive and to identify carriers in the field (Steyn et al. 2008), it is possible that circulating *E. ruminantium* levels in carrier animals included in this study were too low for detection. A different explanation is that reported prevalence of *E. ruminantium* is artificially high. Serological techniques have previously been popular for its detection but are now known to lack accuracy (Kelly et al. 1994; Papadopoulos et al. 1996; Savadye et al. 1998).

## Conclusion

The incidence of tick-borne diseases is increasing globally, challenging our approach toward diagnosis, treatment and control options. Heartwater and babesiosis are generally considered to be endemic and two of the most important diseases that affect cattle in Nigeria, not only directly affecting animal health negatively, but also impacting on the local economy. Our study has shown no detection of *B. bigemina* nor *E. ruminantium* in any of the blood samples tested. These results disagree with the current accepted presence of these pathogens within Nigeria. As such, further investigations with an

increased number of samples and over a wider geographic area within central Nigeria are warranted to confirm these findings. Better understanding of ticks and TBDs circulating amongst Nigerian cattle will be vital to improve animal health and consequently livelihoods of local populations.

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## Compliance with ethical standards

**Ethical approval** All applicable international, national and institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

**Conflict of interest** The authors declare that they have no competing interests.

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