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Molecular survey of *Coxiella burnetii* in wildlife and ticks at wildlife–livestock interfaces in Kenya

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Abstract *Coxiella burnetii* is the causative agent of Q fever, a zoonotic disease of public health importance. The role of wildlife and their ticks in the epidemiology of *C. burnetii* in Kenya is unknown. This study analysed the occurrence and prevalence of the pathogen in wildlife and their ticks at two unique wildlife–livestock interfaces of Laikipia and Maasai Mara National Reserve (MMNR) with the aim to determine the potential risk of transmission to livestock and humans. Blood from 79 and 73 animals in Laikipia and MMNR,

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respectively, and 756 and 95 ixodid ticks in each of the areas, respectively, was analysed. Ticks were pooled before analyses into 137 and 29 samples in Laikipia and MMNR, respectively, of one to eight non-engorged ticks according to species and animal host. Real-time PCR amplifying the repetitive insertion element IS*1111a* of the transposase gene was used to detect *C. burnetii* DNA. Although none of the animals and ticks from MMNR tested positive, ticks from Laikipia had an overall pooled prevalence of 2.92% resulting in a maximum-likelihood estimate of prevalence of 0.54%, 95% CI 0.17–1.24. Ticks positive for *C. burnetii* DNA belonged to the genus *Rhipicephalus* at a pooled prevalence of 2.96% (maximum-likelihood estimate of prevalence of 0.54%, 95% CI 0.17–1.26). These ticks were *Rhipicephalus appendiculatus, R. pulchellus* and *R. evertsi* at pooled prevalence of 3.77, 3.03 and 2.04%, respectively. The presence of *C. burnetii* in ticks suggests circulation of the pathogen in Laikipia and demonstrates they may play a potential role in the epidemiology of Q fever in this ecosystem. The findings warrant further studies to understand the presence of *C. burnetii* in domestic animals and their ticks within both study areas.

Keywords Q fever · *Coxiella burnetii* · Kenya · Wildlife

Introduction

Coxiella burnetii is a highly infectious obligate intracellular bacterium and the causative agent of Q fever, a zoonotic disease of great public health importance worldwide (Porter et al. 2011). The bacterium is a potential bioterrorism agent because of its low infectious dose, ease of dispersal through air and ability to cause substantial morbidity in an exposed population (Jones et al. 2006). The spore-like formation of *C. burnetii* also makes it a very successful pathogen that can survive for a prolonged period in the environment (Marrie 2009).

Q fever has multiple transmission modes, which include inhalation, contact with infected body fluids and consumption of infected animal products such as unpasteurised milk (Marrie 2009). Ticks are important reservoirs and potential vectors of *C. burnetii* (Maurin and Raoult 1999) transmitting infection between mammals (wild and domestic) during a blood meal (Mediannikov et al. 2010). Although human infection through tick bites is rare (Porter et al. 2011), it is possible (McQuiston et al. 2002). Over 40 species of ticks are naturally infected (Porter et al. 2011). In infected ticks, *C. burnetii* multiplies in the middle gut cells and the bacterium is excreted in faeces, saliva and coxal fluids thus playing an important role in its epidemiology by contaminating the environment (Maurin and Raoult 1999). The most important hosts of *C. burnetii* in nature are small rodents but infection also occurs in a wide range of wild and domestic animals (Gardon et al. 2001; Barandika et al. 2007; Kersh et al. 2012). Domestic ruminants primarily cattle, sheep and goats are the main sources of human infection (Porter et al. 2011). Infected wildlife may be important in infecting domestic animals particularly where the source of infection is unknown (Marrie 2009).

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Most Q fever infected animals remain asymptomatic but the presence of *C. burnetii* can be traced in blood, lungs, spleen and liver in the acute phase of infection, which often becomes chronic with persistent shedding of the bacterium in faeces, urine and sometimes milk (Maurin and Raoult 1999). The female uterus and mammary glands are the primary sites of chronic infection and shedding of *C. burnetii* into the environment occurs mainly during parturition and spontaneous abortion (Maurin and Raoult 1999). Infection in animals is mostly associated with various reproductive problems including abortions, stillbirths, delivery of weak offsprings, post-partum metritis and infertility (Marrie 2009; Porter et al. 2011). In humans, the disease can be acute, self-limiting and characterised by non-specific symptoms such as fever, headache, vomiting, pneumonia and hepatitis, or a chronic form characterised by endocarditis, hepatitis, osteomyelitis or endovascular infection (Marrie 2009; Porter et al. 2011) and sometimes spontaneous abortions in pregnant women (Porter et al. 2011).

The epidemiology of *C. burnetii* in Africa including Kenya is poorly understood (Vanderburg et al. 2014), a factor attributable to low level of awareness and inadequate diagnostic resources (Ari et al. 2011; Brah et al. 2015). Recently, a study reported four infected people from a group of 50 travellers (8%) in the Maasai Mara (Potasman et al. 2000). Another study in western Kenya showed prevalence of antibodies among humans to be 30.9 and 18.2–32% in sheep, cattle and goats (Knobel et al. 2013). This study also reported PCR detection of *C. burnetii* DNA in 2.1–4.7% of cattle, goats and sheep, and in 2.5–50% of ticks collected from cattle and domestic dogs. DePuy et al. (2014) reported sero-prevalence of 3–46% in cattle, sheep, goats and camels in different locations in Laikipia while Wardrop et al. (2016) reported sero-prevalence of 10.5% in cattle and 2.5% in humans in western Kenya. Despite the close contact between humans, livestock and wildlife in many parts of Kenya (Wambwa 2003; Wambuguh 2007), a factor that favours emergence and re-emergence of diseases (Jones et al. 2008) thus highlighting the need for a holistic approach to surveillance and detection of diseases, there are no reports of the potential role wildlife and their ticks could be playing in *C. burnetii* epidemiology. Wild animals often have high tick infestation and can be important reservoirs of many tick-borne pathogens including *C. burnetii*, transmittable across different species at wildlife–livestock interfaces, where wildlife and livestock share habitats and other resources.

This study aimed to investigate the occurrence and prevalence of *C. burnetii* in wildlife and ticks in Laikipia County and Maasai Mara National Reserve (MMNR), two regions of Kenya with huge numbers of wildlife with large diversity of species and unique wildlife–livestock interfaces.

Materials and methods

Study areas and sample collection

Blood and tick samples were collected from wildlife species in Laikipia County and MMNR between February 2014 and October 2015. Laikipia County covers an area approximately 9500 km^2 and is located in the central region of Kenya to the north-west of Mt. Kenya between latitudes 0°53′N and 0°16′S and longitudes 36°11′ and 37°23′E. It is one of the most important areas for biodiversity in Kenya with much of it occupied by large privately or community owned ranches populated by livestock and sharing the land with free ranging wildlife (Wambuguh 2007). The MMNR covers approximately 1510 km^2

between latitudes 1°13′ and 1°45′S and longitudes 34°45′ and 35°25′E within Narok County in south-western Kenya along the border with Tanzania and is a contiguous ecosystem with the Serengeti national park in Tanzania. It is one of the most important protected wildlife areas in Kenya accounting for about 25% of Kenya's wildlife (Reid et al. 2003). It forms part of the greater Mara ecosystem, which also includes the Mara Triangle and numerous group ranches. This is an open ecosystem without fences and free ranging wildlife share resources with livestock (Reid et al. 2003). The main human populations in both study areas are pastoralists whose livelihoods are dependent on livestock including cattle, goats and sheep. Other forms of land uses include agriculture, commercial ranching, wildlife conservation and ecotourism. The two study areas have large wildlife populations, which share habitats and other resources with humans and domestic animals providing a likely interface for disease transmission.

Sampling of wildlife species was highly dependent on presence and abundance throughout. Lack of information on population sizes, movements and distribution of wildlife at both localities favoured a randomised sampling protocol and therefore easily available species were targeted. The most common wildlife species with high tendency to interact with livestock were buffalo (*Syncerus caffer*), zebra (*Equus burchellii*), Grant's gazelle (*Nanger granti*), common waterbuck (*Kobus ellipsiprymnus ellipsiprymnus*), impala (*Aepyceros melampus*), Topi (*Damaliscus lunatus jimela*), Coke's hartebeest (*Alcelaphus buselaphus*) and wildebeest (*Connochaetes taurinus*). Sampling sites were selected based on high coexistence of livestock and wildlife and accessibility to enable animal darting. In Laikipia, these were Ol Pejeta conservancy, ADC Mutara ranch and Mpala ranch, all of which practice livestock ranching, wildlife conservation and ecotourism, and Kiamariga locality, a community area where free ranging wildlife interacts with livestock (Fig. 1). In MMNR, the sampled sites where at different locations inside the national reserve where neighbouring community group ranches livestock interact freely with wildlife (Fig. 2).

Animals were immobilised following protocols recommended by McKenzie (1993) with etorphine hydrochloride (M99®, Verico, UK) and azaperone tartarate (Kyron Laboratories, S. Africa) delivered remotely from a vehicle by projectile darts using a Dan-Inject® darting rifle (Dan-Inject APS, Denmark). Blood (30 ml) was collected by jugular venipuncture into EDTA coated tubes and split into four aliquots. Each aliquot was labelled with information identifying the sample number, date, location and animal species and stored in frozen in liquid nitrogen (−196 °C) until required for processing. Animals' bodies were examined completely for presence of ticks and all adult non-engorged ticks representing the different genera were collected. These were placed in 1.5 ml vials, which were then labelled with information identifying the date, location and animal host and stored in liquid nitrogen (−196 °C) and transported to the laboratory. Ticks were identified to the species level using standard taxonomic keys as described by Walker et al. (2003). Ticks identified as same species from the same animal were pooled together (one to eight ticks) and preserved at −70 °C. Pooling of ticks for molecular detection of pathogens is common practice (Knobel et al. 2013; Kamani et al. 2013; Aktas 2014; Barghash et al. 2016) and subsequent infection rates of individual ticks are calculated from pooled prevalence (Aktas 2014). The method is reliable and useful in resource scarce settings (Speybroeck et al. 2012).

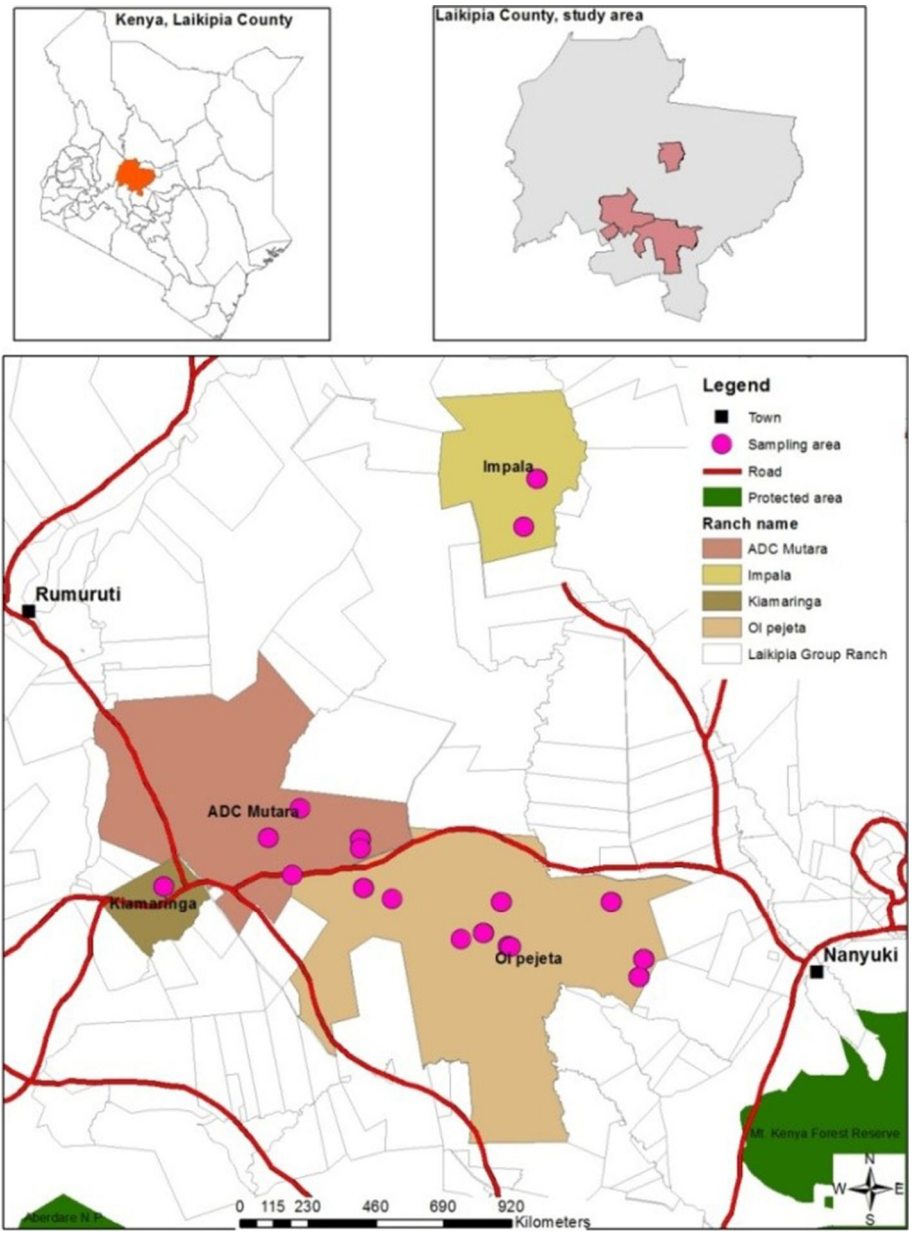

Fig. 1 Sampling sites in Laikipia

DNA extraction

Genomic DNA was extracted from preserved blood samples after thawing for one hour using the DNeasy® Blood and Tissue Kits (QIAGEN, Hilden, Germany) following the manufacturer's instructions. To extract DNA from the ticks, each pool sample was crushed

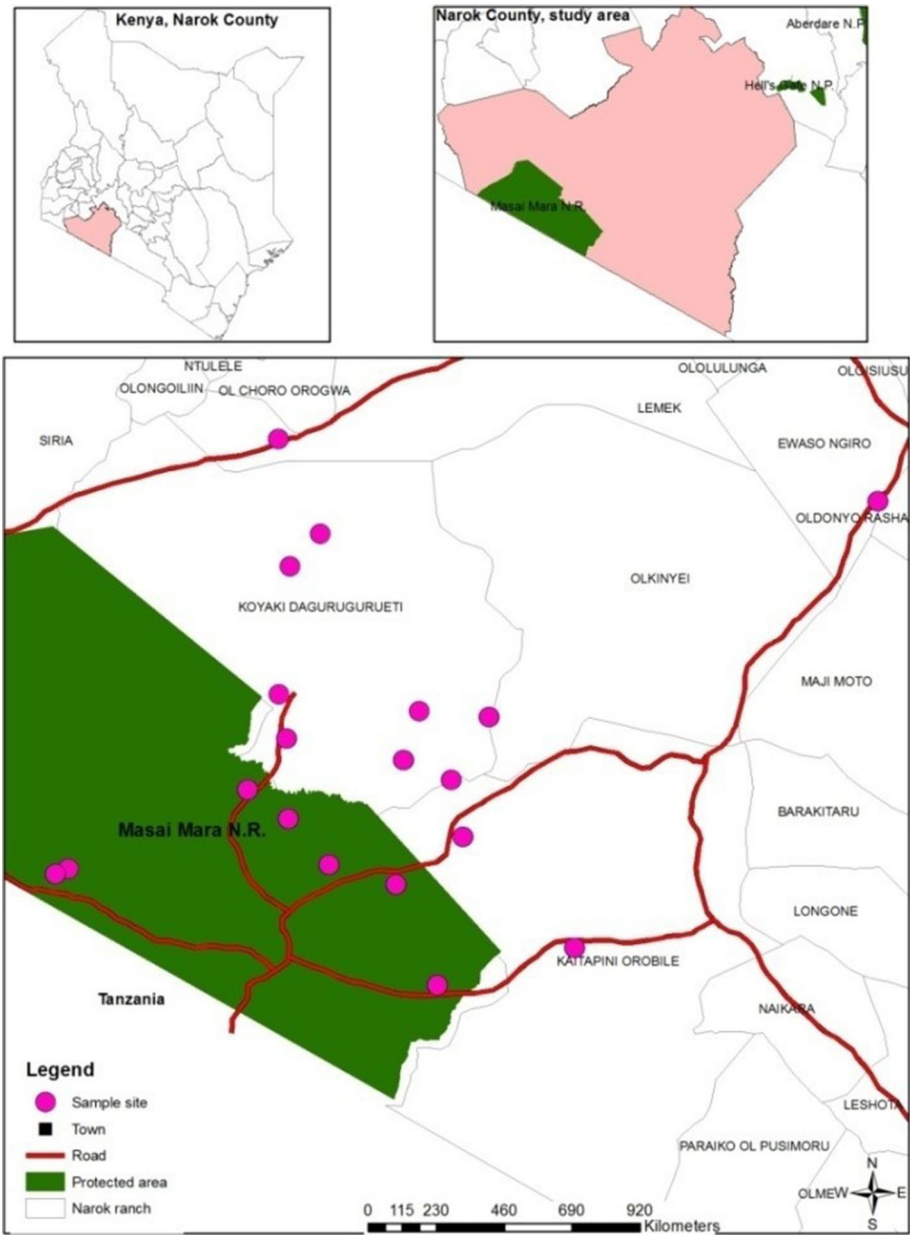

Fig. 2 Sampling sites in Maasai Mara National Reserve

and homogenised using a BioSpec Mini-Bead Beater 16® (BioSpec Products, Bartlesville, UK) in screw-cap tubes containing Yttria-stabilised zirconium oxide beads (Glen Mills, Clifton, NJ, USA) and 500 µl of homogenisation media (2% L-glutamine and 15% Fetal Bovine Serum). The homogenates were then short-centrifuged at maximum speed at 4 °C in an Eppendorf 5417R® bench-top centrifuge (Eppendorf Nordic, Horsholm, Denmark).

DNA was extracted from 200 µl of the homogenate using the MagNa 96 Pure DNA® and Viral NA Small Volume Kit® (Roche Diagnostics, Sussex, UK) in a MagNa Pure 96® automatic extractor (Roche Diagnostics). Extracted DNA quality was evaluated using the agarose gel electrophoresis protocol in which an aliquot of the extracted DNA was run on 1.2% agarose gel. Extracted DNA was stored at −80 °C until required for analysis.

Coxiella burnetii PCR

Extracted blood and tick DNA were tested for *C. burnetii* using real-time PCR targeting the repetitive insertion element IS*1111a* of the transposase gene using primers 5′-GCTCCTCCACACGCTTCCAT-3′ and 5′-GGTTCAACTTGTGTGGAATTGATGAGT-3′ (Tokarz et al. 2009). The total reaction volume was 10 µl, and each reaction mixture contained 5 µl of 5X HOT FIREPol® EvaGreen® HRM mix (no ROX) (Solis BioDyne, Estonia), 0.5 µl of each primer and 1 µl of DNA template in UlraPure® DNase/RNase-Free PCR-grade water (Thermo Fisher Scientific, USA). PCR assays were performed under the following conditions: enzyme activation at 95 °C for 15 min, 43 cycles of denaturation at 94 °C for 20 s, annealing at 65–50 °C (the temperature was decreased 1 °C between consecutive cycles) for 25–50 s and an extension at 72 °C for 5–30 s, holding at 72 °C for 3 min for final elongation and a 1 min final hold at 45 °C for complete annealing. The PCR cycling was followed by high resolution melt (HRM) analysis that involved gradual increase of temperature from 75 to 90 °C in 0.1 °C increments with fluorescence acquisition at intervals of 2 s. The PCR-HRM analyses were performed with Qiagen's Rotor Gene Q thermo-cycler (QIAGEN) operating on Rotor-Gene Q series software V2.1.0 (SABioSciences, USA). The IS*1111* plasmid pBluescript® SH6E (Stratagene, USA) was used as positive control while UlraPure® DNase/RNase-Free PCR-grade water (Thermo Fisher Scientific) was used as negative control.

DNA sequencing and analysis

Positive PCR products were purified using QIAquick® purification kit (QIAGEN) following the manufacturer's instructions in order to recover DNA for sequencing free of left over primers, excess deoxynucleotides and buffer salts. Sequencing was done by direct cycle sequencing on both strands of purified positive DNA products using the ABI PRISM BigDye Terminator V3.1 cycle sequencing kit and analyzed on an ABI310 DNA analyser (Applied Biosystems, USA). The same primers as those used for the PCR amplifications were used for both forward and reverse sequencing reactions. Reverse and forward trace files were assembled using Geneious version 8.1.6 sequence analyses software (Kearse et al. 2012; http://www.geneious.com). Consensus nucleotide sequences minus the primers were used to query the GenBank database and the highest similarity was identified by Basic Local Alignment Search Tool (BLASTN) available from the National Center for Biotechnology Information (Bethesda, MD, USA).

Data analysis

Data were managed using SAS statistical software and Microsoft Excel to analyse *C. burnetii* DNA prevalence according to independent variables such as host species (wildlife or tick pool) for each study area. Estimation of true prevalence of infection of individual ticks in a pool was analysed based on the number of individuals in each pool and the test

result for that pool (Cowling et al. 1999). The frequentist method for multiple variable pool sizes was used to compute maximum-likelihood estimates of true prevalence and 95% confidence intervals (CI) using EpiTools epidemiological calculator, an open-source software available at http://www.ausvet.com.au/ (Cameron 1999) under the assumption of perfect (100%) sensitivity and specificity for the PCR tests (Williams and Moffitt 2001).

Results

A total of 79 and 73 animals of different species were sampled in Laikipia County and MMNR, respectively, during six separate expeditions, between February 2014 and October 2015 (Table 1). None of these animals from both study areas tested positive for *C. burnetii* DNA. A total of 851 non-engorged adult ixodid ticks consisting of 756 in Laikipia County and 95 in MMNR were collected from wildlife hosts (Table 1). Tick taxonomical identification matched four genera: *Rhipicephalus*, *Hyalomma*, *Dermacentor* and *Amblyomma*. They were pooled according to species and the animal host into 137 and 29 pool samples in Laikipia and MMNR, respectively, of 1–8 ticks. Pool samples of the genus *Rhipicephalus* were the most abundant in both study areas (Table 2). At the species level, the numbers of pool samples are shown in Table 3.

The pooled prevalence was used to estimate the maximum-likelihood true prevalence of infection for individual ticks. Overall pooled prevalence in Laikipia was four out of 137 pool samples (2.92%) resulting in an overall maximum-likelihood estimated true prevalence of 0.54% (95% CI 0.17–1.24). Only tick pool samples of the genus *Rhipicephalus* tested positive at a pooled prevalence of four out of 135 pool samples (2.96%) resulting in a maximum-likelihood estimate of true prevalence of 0.54% (95% CI 0.17–1.26) in this

Table 1 Number of animals and ticks sampled in Laikipia and MMNR

Animal species	No. of animals	No. of ticks	No. of tick pools
Laikipia			
African (Cape) buffalo (*Syncerus caffer*)	31	330	61
Burchell's (common) zebra (*Equus burchellii*)	39	384	67
Grant's gazelle (*Nanger granti*)	7	25	4
Common waterbuck (*Kobus ellipsiprymnus ellipsiprymnus*)	2	8	3
Eastern black rhinoceros (*Diceros bicornis michaeli*)[a]	–	9	2
Total	79	756	137
MMNR			
Burchell's (common) zebra (*Equus burchellii*)	21	16	4
Impala (*Aepyceros melampus*)	2	6	2
Blue (common) wildebeest (*Connochaetes taurinus*)	35	58	16
Topi (*Damaliscus lunatus jimela*)	10	10	5
Coke's Hartebeest (*Alcelaphus buselaphus*)	5	5	2
Total	73	95	29

[a] Ticks were sampled opportunistically during an autopsy

Table 2 Genus of ticks and percentage positive for *C. burnetii* DNA

Tick Genus	No. of pools	No. (%) of positive pools	No. of ticks in positive pools	Animal hosts of positive ticks	% positive[a]
Laikipia					
Rhipicephalus	135	4 (2.96)	1, 7, 8, 8	Buffalo, Zebras	0.54
Dermacentor	1	0			0
Amblyomma	1	0			0
Total	137	4 (2.92)			0.54
MMNR					
Rhipicephalus	24	0			0
Hyalomma	4	0			0
Amblyomma	1	0			0
Total	29	0			0

[a] Maximum-likelihood estimate of true prevalence

Table 3 Species of ticks and percentage positive for *C. burnetii* DNA

Tick Species	No. of pools	No. (%) of positive pools	No. of ticks in positive pools	Animal hosts of positive pools	% positive[a]
Laikipia					
R. appendiculatus	53	2 (3.77)	1, 8	Buffalo, Zebra	0.67
R. pulchellus	33	1 (3.03)	8	Zebra	0.64
R. evertsi	49	1 (2.04)	7	Zebra	0.0029
D. rhinocerinus	1	0			0
A. gemma	1	0			0
Total	137	4 (2.92)			0.54
MMNR					
R. appendiculatus	10	0			0
R. evertsi	12	0			0
R. pulchellus	2	0			0
H. dromedary	1	0			0
H. albiparmatum	2	0			0
H. truncatum	1	0			0
A. variegatum	1	0			0
Total	29	0			0

R Rhipicephalus, *H Hyalomma*, *D Dermacentor*, *A Amblyomma*

[a] Maximum-likelihood estimate of true prevalence

genus of ticks (Table 2). When the tick-pool samples were classified according to species, infection was detected in *Rhipicephalus appendiculatus* (from a buffalo and zebra), *R. pulchellus* (from a zebra) and *R. evertsi* (from a zebra) at pooled prevalence of 3.77, 3.03 and 2.04%, respectively (Table 3). These ticks were collected at Ol Pejeta conservancy in Laikipia. None of the tick-pool samples in MMNR tested positive for *C. burnetii* DNA.

The alignment of the sequences of the detected *C. burnetii* isolates resulted in a 127 base pair consensus sequence, recovering one only haplotype (Genbank accession: KU994893). Blast searches revealed that sequences were 100% similar to those already existing in GenBank (see Supplementary material).

Discussion

The finding of an overall prevalence of *C. burnetii* DNA of 2.92% in Laikipia is similar to the 2.5% found in ticks from western Kenya (Knobel et al. 2013) but almost half than the pooled prevalence collected from domestic animals in nearby Ethiopia (Kumsa et al. 2015). The detection in *R. pulchellus* corroborates previous sampling from domestic animals in Ethiopia, but at a higher prevalence of 25% (Kumsa et al. 2015). Similar to the findings here reported, Knobel et al. (2013) also detected *C. burnetii* DNA in *Rhipicephalus appendiculatus* ticks collected from domestic dogs but at a higher pooled prevalence of 11.1%. The detection of *C. burnetii* DNA in *R. evertsi* ticks in this study has never been reported and suggests that this species is its potential reservoir and vector. Although *C. burnetii* DNA was not detected in *A. variegatum* ticks, Knobel et al. (2013) reported a pooled prevalence of 2.5 and 20% in this species collected from cattle and dogs, respectively, in western Kenya, while Kumsa et al. (2015) reported a pooled prevalence of 3.2% in the same species collected from domestic animals in Ethiopia and a pooled prevalence of 28.6% in *A. gemma* ticks. Mediannikov et al. (2010) also reported *C. burnetii* DNA in 6.8 and 31.3% of *H. truncatum* ticks collected from domestic animals in Senegal. To the best of our knowledge there are no available reports on *C. burnetti* DNA in *D. rhinocerinus, H. dromedary* and *H. albiparmatum* ticks.

The molecular methods employed in this study for the detection of *C. burnetii* DNA in ticks and wild ungulates have been previously used in different wild and domestic animals. Studies in Spain on wild and domestic small mammals (Barandika et al. 2007) and in Turkey on cattle (Kirkan et al. 2008) using similar methods reported low prevalence of 0.8 and 4.3%, respectively. In Kenya, Knobel et al. (2013) used PCR methods to detect *C. burnetii* DNA in the western part of Kenya and reported prevalence of 2.1% in cattle, 2.3% in goats, 4.7% in sheep and 50% (3 out of 6) in small ruminants following parturition. Although there are also several reports of sero-prevalence of Q fever in domestic animals in Kenya, this study failed to detect the disease in wildlife species. For instance, DePuy et al. (2014) reported sero-prevalence of 3–4% in cattle, 13–20% in sheep, 31–40% in goats and 5–46% in camels across five ranches in Laikipia. In western Kenya, Knobel et al. (2013) reported sero-prevalence of 28.3% in cattle, 32.0% in goats and 18.2% in sheep and Wardrop et al. (2016) reported 10.5% sero-prevalence in cattle in the western part of Kenya. Outside Kenya, sero-prevalence has also been documented in a wide range of wild mammals and birds including black bears, fallow deer, Cameroun goat and sheep, mouflons and some marine mammals (Gardon et al. 2001; McQuiston et al. 2002; Barandika et al. 2007; Kersh et al., 2012). The variation in the findings of this study and previous reports may be attributed to differences in the animal species sampled as well as by the geographical locations, which might affect disease prevalence. Additionally, some of the previous studies used serology to detect antibodies against *C. burnetii* whereas this study used a highly sensitive and specific molecular method to amplify the repetitive insertion element *IS1111a* of the transposase gene that has been validated before (Mediannikov et al. 2010; Roest et al. 2011) and the findings were confirmed by positive and negative controls.

Non detection of *C. burnetii* DNA in MMNR ticks may be attributed to differences in geographic locations which can affect disease prevalence and tick species and abundance. More acaricide treatments in MMNR than in Laikipia cattle, known to reduce tick abundance in wildlife (Keesing et al. 2013), may have also contributed to the fewer ticks collected at MMNR.

All hosts tested negative for *C. burnetti* DNA, including those from which ticks tested positive for *C. burnettii*. Although PCR diagnosis of Q fever is very effective in acute infections, detection of *C. burnetii* DNA can be difficult to trace as the infection becomes established and the serological response develops (Fournier and Raoult 2003; Schneeberger et al. 2010; Wielders et al. 2013). Acute infection is usually characterized by a 7–15 days delay in antibody response, at this time PCR testing for *C. burnetti* DNA is most effective (Wielders et al. 2013). As the infection progresses, immunoglobulin (Ig) M and IgG antibodies are expressed against phase II antigens in acute infection and phase I antigens in established infection (Schneeberger et al. 2010). IgG antibodies are a major PCR inhibitor of PCR assays for diagnosis of microbial infections (Al-Soud et al. 2000), consequently, false negatives are likely to occur in hosts with high expression of antibodies (Wielders et al. 2013), which might have been the case in this study.

Conclusion

This study confirms, by molecular methods, the presence of *C. burnetii* in ticks collected from wild ungulates (i.e. buffalo and zebras) from Laikipia region of Kenya. This finding suggests the circulation of this microorganism within this ecosystem. The three species of ticks positive for *C. burnetii* DNA (i.e. *Rhipicephalus evertsi, R. pulchellus* and *R. appendiculatus*) are widely distributed in eastern Africa and hosts include a wide range of domestic and wild ungulates (Walker et al. 2003) implying that there is a likelihood of transmission by wildlife ticks to livestock. The lack of detection from the animals sampled from the same area despite ticks testing positive, hints to the presence of false negatives by PCR inhibitors by antibody expression. The non-detection of the pathogen in animals and ticks from MMNR possibly suggest non-circulation of the pathogen in wildlife and their ticks within this ecosystem. Overall, the findings of the study warrant further molecular-based studies to better understand the presence of *C. burnetii* in domestic animals and their ticks within both study areas.

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Contributions The authors contributed equally in the study conception and design, data collection and analysis and preparation of the manuscript.

Compliance with ethical standards

Conflict of interest The authors report no conflicts of interest in this work.

Ethical approval The Research Authorisation Committee of KWS, the government agency responsible for wildlife conservation and management, approved the study (Approval Ref: KWS/BRM/5001). Animals were immobilised following protocols recommended by McKenzie (1993) by experienced personnel to

ensure a humane exercise as much as possible following applicable KWS guidelines on wildlife veterinary practice (2006).

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