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Ticks (Acari: Ixodidae) infesting cattle in coastal Kenya harbor a diverse array of tick-borne pathogens

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ABSTRACT

Ticks and the microbes they transmit have emerged in sub-Saharan Africa as a major threat to veterinary and public health. Although progress has been made in detecting and identifying tick-borne pathogens (TBPs) across vast agroecologies of Kenya, comprehensive information on tick species infesting cattle and their associated pathogens in coastal Kenya needs to be updated and expanded. Ticks infesting extensively grazed zebu cattle in 14 villages were sampled and identified based on morphology and molecular methods and tested for the presence of bacterial and protozoan TBPs using PCR with high-resolution melting analysis and gene sequencing. In total, 3,213 adult ticks were collected and identified as *Rhipicephalus appendiculatus* (15.8%), *R. evertsi* (12.8%), *R. microplus* (11.3%), *R. pulchellus* (0.1%), *Amblyomma gemma* (24.1%), *A. variegatum* (35.1%), *Hyalomma rufipes* (0.6%), and *H. albiparmatum* (0.2%). Ticks were infected with *Rickettsia africae, Ehrlichia ruminantium, E. minasensis, Theileria velifera* and *T. parva. Coxiella* sp. endosymbionts were detected in the *Rhipicephalus* and *Amblyomma* ticks. Co-infections with two and three different pathogens were identified in 6.9% ($n = 95/1382$) and 0.1% ($n = 2/1382$) of single tick samples, respectively, with the most common co-infection being *R. africae* and *E. ruminantium* (7.2%, CI: 4.6 – 10.6). All samples were negative for *Coxiella burnetii, Anaplasma* spp. and *Babesia* spp. Our study provides an overview of tick and tick-borne microbial diversities in coastal Kenya.

1. Introduction

Ticks (Acari: Ixodidae) are obligate blood-feeding ectoparasites that transmit a broad range of bacterial, protozoan and viral pathogens to humans and animals (de la Fuente et al., 2008). Ixodid ticks commonly infesting livestock in sub-Saharan Africa (SSA) pose enormous constraints on cattle health and productivity by acting as vectors of the etiological agents of East Coast fever (ECF), heartwater, anaplasmosis, and babesiosis (Walker et al., 2003). Besides acting as vectors, tick parasitism causes severe economic losses in the livestock sector due to weight loss, anemia, and damage to the udder, skin, and hide (Jonsson, 2006; Walker et al., 2003).

In recent decades, the geographic range of ticks has been expanded in SSA, primarily due to climate change, habitat modification, transboundary animal trade and the increased movement of animals (Githaka et al., 2021; Madder et al., 2011). These changes may potentially lead to a shift in the epidemiology of tick-borne diseases (TBDs) as tick-borne pathogens (TBPs) may spread to new areas where they were previously inexistent and thus represent a potential threat to animal health (Ouedraogo et al., 2021a, 2021b). For example, previously unrecognized or emerging TBPs were recently reported in Kenya, including *Anaplasma phagocytophilum* (Mwamuye et al., 2017), *Ehrlichia minasensis* (Chiuya et al., 2021; Peter et al., 2020), *Ehrlichia chaffeensis* (Mwamuye et al., 2017) and *Candidatus* Rickettsia moyalensis (Kimita et al., 2016). These reports highlight the need for regular updating of the data on the distribution of tick species and TBPs in various geographical settings.

Available information on tick species infesting cattle and their occurrence and diversity in coastal Kenya is outdated and limited. The existing data on tick species was published over two decades ago and was based solely on phenotypic characteristics (Zulu et al., 1998). Therefore, there is a need to generate new accurate data on tick diversity, abundance and phylogenetic relationships using molecular approaches (Lv et al., 2014).

The traditional extensive system of cattle production in coastal

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Kenya favors the convergence of herds, mainly at grazing and watering points. This may increase the likelihood of high tick infestations among the herds and hence the risk of TBP transmission. Further, some cattle owners in the region move with their cattle during the dry season in search of water and pastures for their animals. This uncontrolled transboundary cattle movement could significantly spread ticks and TBPs to new areas (Ouedraogo et al., 2021b). Therefore, there is a need for active surveillance of ticks and TBPs to regularly update information on the presence, distribution, abundance, and prevalence of ticks and TBPs. Accordingly, we aimed to i) investigate the species composition of ticks infesting cattle and their infestation prevalence in the coastal region of Kenya, and ii) examine the prevalence and diversity of TBPs belonging to *Rickettsia* spp., *Theileria* spp., *Ehrlichia* spp., *Anaplasma* spp., *Babesia* spp. and *Coxiella burnetii* in the collected tick specimens using PCR with high-resolution melting analysis.

2. Materials and methods

2.1. Study area

The study was conducted in Kayafungo Ward (Kilifi County) and Kinango Ward (Kwale County) in coastal Kenya (Fig. 1). Coastal Kenya is hot and dry from January to March and relatively cool from June to August. The annual temperatures range from 23 to 34 °C, while the average relative humidity is 60–80%. The predominant livestock kept in the region includes cattle, goats, and chickens. More details on the climate of coastal Kenya are provided in a previous study (Mwangangi et al., 2013).

2.2. Study design

The present study was conducted in 14 village clusters as a baseline survey of a more extensive operational research project that aimed to improve food and nutritional security through integrated control of tsetse and tick-borne livestock diseases (ICTLD). The two administrative wards were selected purposively based on their potential for livestock production in the study area, accessibility and the difference in access to veterinary extension services. The final listing of village clusters was made based on the cooperation of farmers and logistical feasibility (accessibility by vehicle, security, distance). The ticks were collected in two field-sampling trips, in December 2019, coinciding with the short rains, and in May 2021, at the onset of the long rains period. Attempts were made to collect samples from the same herds in both periods. However, some herds were lost during the second sampling due to the mortality or relocation of herds, and therefore alternative herds in the same villages were included. Herd selection was made based on their location by village and the willingness of the farmers to participate in the study.

2.3. Tick collection, morphological identification and pooling

Cattle were examined for tick infestation in the following predilection sites: head, ears, neck, dewlap, belly, back, legs, udder in the case of females and testes in males, perineum region and tail. Tick-infested animals were restrained and all visible live-attached ticks were removed using blunt steel forceps. Ticks were stored in 2-ml cryovial tubes labeled with a unique sample ID, comprising the sampling site, host ID, predilection site and sampling date. Categorical data on the age, sex and breed of each cattle were recorded on predesigned forms. The collected ticks were frozen in liquid nitrogen and transported to the Martin Lüscher Emerging Infectious Disease (ML-EID) laboratory at the International center of Insect Physiology and Ecology (ICIPE) in Nairobi, where they were stored at −80 °C before species identification and pathogen screening.

Before morphological identification, the tick samples were disinfected by immersion in 70% ethanol solution for five minutes, with

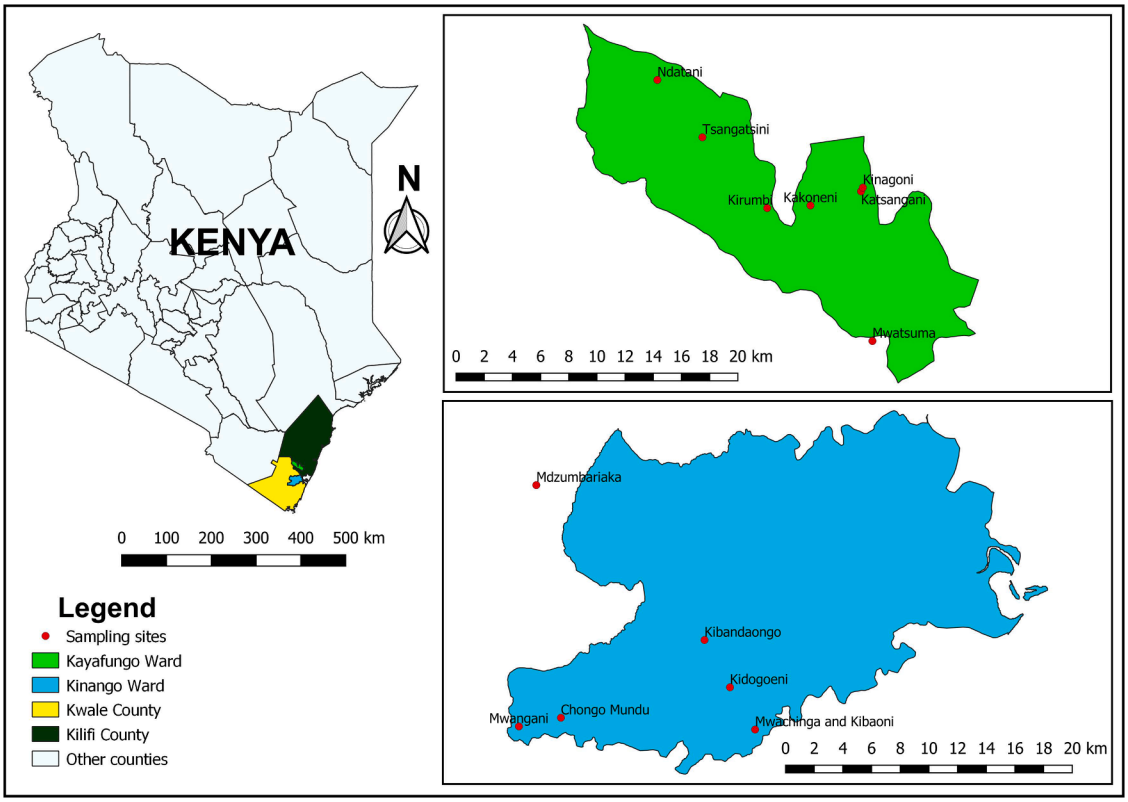

Fig. 1. Map of Kayafungo and Kinango Wards in coastal Kenya showing the villages where the tick sampling took place. The map was prepared using common licensed shape files in QGIS software version 3.10 (QGIS Development Team, 2020).

occasional vortex mixing, rinsed twice with deionized water, and dried on filter paper. Ticks were then identified by developmental stage, species and sex based on published morphological descriptions (Walker et al., 2003) under a stereomicroscope (ZEISS Stemi 2000-C, Oberkochen, Germany). Representative tick species of either sex were photographed using a microscope-mounted Axio-cam ERc 5 s digital camera (Zeiss). The identified ticks were sorted by sex, species and sampling site and then processed individually or in a pool of 2–5 ticks in 1.5-ml Eppendorf tubes. During extraction, partially and fully engorged ticks were discarded to reduce vertebrate host DNA.

2.4. DNA extraction

The ticks were mechanically crushed with 750 mg of 2.0-mm yttria-stabilized zirconium oxide (zirconia/yttria) beads (Glen Mills, Clifton NJ) using a Mini-Beadbeater-16 (BioSpec, Bartlesville, OK) twice for one minute. Genomic DNA was extracted from the homogenates using a previously described method (Oundo et al., 2020). The quality and quantity of extracted DNA samples were measured using a Nanodrop ND-2000 instrument (Thermo Fischer Scientific, UK). The DNA concentration was then adjusted to 50 ng/µl for all samples. The remaining stock of DNA was stored at −80 °C, while diluted DNA extracts were stored at −20 °C until further use.

2.5. Molecular identification of ticks

Molecular identification was carried out on two to four randomly selected ticks of each species. The PCR assays targeting the tick 16S ribosomal DNA (rDNA), cytochrome *c* oxidase subunit I (COI) and internal transcribed spacer 2 (ITS2) were carried out using a SimpliAmp™ Thermal Cycler (Applied Biosystems, Foster City, CA, USA) as previously described (Oundo et al., 2020). The PCR products were gel-purified using QIAquick® Gel Extraction Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol and then sent to Macrogen Inc. (The Netherlands) for sequencing in both directions.

2.6. Molecular detection of protozoan and bacterial pathogens

The genomic DNA of ticks was screened by PCR with high-resolution melting (PCR-HRM) analyses for infection with *Rickettsia, Theileria, Babesia, Ehrlichia* and *Anaplasma* species. The PCR-HRM assays were conducted on a Magnetic Induction Cycler (MIC) machine (BioMolecular Systems, Australia) as previously described (Oundo et al., 2020). Positive controls containing genomic target DNA of *Rickettsia africae, Anaplasma bovis, Ehrlichia ruminantium* and *Theileria parva* and a negative control without a DNA template were included in each respective amplification run. Amplicons with unique HRM melt curves were purified for sequencing.

To re-confirm the identity of rickettsial pathogens, DNA from the samples that were positive for *Rickettsia* spp. using PCR-HRM primers were re-amplified using primers targeting rickettsial citrate synthase (*gltA*) gene, outer membrane protein A (*ompA*) gene, outer membrane protein B (*ompB*) gene, and cell surface antigen (*sca4*) gene as previously described (Mwamuye et al., 2017; Sekeyova et al., 2001). Additionally, samples positive for *Ehrlichia* spp. were further re-amplified using primers targeting the heat shock protein (*groEL*) gene (Bell and Patel, 2005). All PCR reactions were carried out using SimpliAmp™ Thermal Cycler (Applied Biosystems, Foster City, CA, USA). The PCR amplicons were electrophoresed, gel-purified and sequenced as described previously.

2.7. Sequence analyses

Generated raw sequences from ticks and positive pathogen samples were edited and aligned using the MAFFT plugin (Katoh and Standley, 2013) in Geneious software version 11.1.5 (https://www.geneious.com) (Kearse et al., 2012). To confirm the identity of each species, the sequences were compared with those available in the GenBank database using the BLASTn tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.8. Statistical analysis

Raw data were entered into Microsoft® Excel 2016 and verified for missing observations and erroneous entries. Statistical analysis was performed using R software version 4.1.3 (R Core Team, 2022). Given that the two wards (i.e., Kinango and Kayafungo) are autonomous administrative units with different access to veterinary extension services, we analyzed the tick infestation prevalence at the administrative ward level. Further, we only considered the first survey data for analysis of infestation prevalence and intensity and excluded the second survey due to insufficient data to perform the analysis. The tick infestation prevalence at the animal level was calculated as the number of cattle infested with ≥ 1 tick out of the total number of cattle examined. For the herd prevalence, a herd was considered positive if at least one animal was infested with ticks. The 95% confidence intervals (95% CIs) for the infestation prevalence were estimated using the *'binom'* package (Dorai-Raj, 2014) with the exact-Clopper-Pearson interval method. The mean infestation intensity was calculated as the total number of ticks divided by the number of infested cattle. The tick infestation prevalence in the different administrative wards, sex and age groups was compared using the Chi-square test. The infestation intensity in cattle in different villages and administrative wards was compared using the Kruskal-Wallis test and the Mann-Whitney-Wilcoxon test with continuity correction, respectively. The effects of host traits (i.e., sex and age) on tick infestation were assessed using the Mann-Whitney-Wilcoxon test and the Kruskal-Wallis test, and the multiple pair-wise comparisons among age groups were done using the Tukey and Kramer (Nemenyi) test. The breed category was excluded from the analysis due to insufficient data to perform the statistical analysis.

The infection rate in tick pools was calculated using the minimum infection rate (MIR) method, with 95% CIs for unequal pool sizes, using the PooledInfRate v4.0 Excel add-in (Biggerstaff, 2009). The MIR was expressed per 100 ticks. For co-infection analyses, pools with multiple ticks were removed from the dataset as we could not confirm true co-infections in these samples. The 95% CIs for the prevalence of observed co-infection was calculated using the exact-Clopper-Pearson interval method from the R package *'binom'*. The expected coinfection prevalence was calculated by multiplying the infection rates of each of the pathogens and then multiplying by 100 (Zembsch et al., 2021). Correlation among pathogens and between pathogens and endosymbionts in single tick samples were analyzed using Spearman's rank correlation. Differences were considered statistically significant at p-values ≤ 0.05.

2.9. Ethics statement

Before sampling ticks, the cattle owners were verbally informed about the goals of the project and the sampling protocol. All owners gave their verbal informed consent to collect ticks from their animals. The study protocol was approved by the Institutional Animal Care and Use Committee of *icipe* (IACUC, Reference No. Oundo-icipeACUC-Mar2020), and the Pwani University Ethics Review (approval number ERC/EXT/002/2020). Further approval was sought from the Kenyan National Commission for Science, Technology and Innovation (NACOSTI/P/21/6726). This study did not involve endangered or protected species.

3. Results

3.1. Tick species composition

A total of 3213 adult ixodid ticks (including 2157 males and 1056 females) were collected from 333 cattle in 14 villages in coastal Kenya.

They belonged to three tick genera i.e., *Amblyomma, Hyalomma, Rhipicephalus* including *Boophilus* subgenus. *Amblyomma variegatum* (n = 1129, 35.1%) was the most abundant species, followed by *A. gemma* (n = 773, 24.1%), *R. appendiculatus* (n = 508, 15.8%), *R. evertsi* (n = 412, 12.8%), *R.* (*Bo*) spp. (n = 360, 11.2%), *H. rufipes* (n = 18, 0.6%), *H. albiparmatum* (n = 7, 0.2%) and *R. pulchellus* (n = 6, 0.2%) (Table A.1).

Sequence analysis of 16S rDNA and ITS2 sequences showed that molecular identification was consistent with morphological identification (Table A.2). The CO1 marker yielded amplicons only for *R. pulchellus* and *H. rufipes* ticks. All the collected *Rhipicephalus* (*Boophilus*) spp. were either semi-engorged or fully engorged females and thus could not be morphologically differentiated any further than the subgenus level. Analysis of ITS2 and 16S rRNA gene sequences of these species showed that they were closest to *R. microplus* sequences with 99.2–100% nucleotide sequence identities. Hence, these ticks were designated as *R. microplus*.

3.2. Tick infestation prevalence

Of the 1522 cattle examined in the first survey, 333 (21.9%, CI: 19.8 - 24.1) were infested (Table 1). Based on the number of ticks per animal, 270 cattle were infested with 1–9 ticks, while 63 cattle were infested with 10–18 ticks. There was no statistically significant difference between the proportions of male and female cattle infested with ticks (χ^2 = 0.0029851, df = 1, p = 0.96). However, the tick infestation prevalence significantly varied among the different age groups, highest in adults and lowest in calves. The tick infestation prevalence was also significantly different among the administrative wards, being highest in Kayafungo ward in Kilifi County compared to Kinango ward in Kwale County (Table 1).

3.3. Tick infestation intensity

A total of 333 cattle were infested with 2109 ticks (mean infestation intensity of 6.3 ticks), with the number of ticks per cattle ranging from 1 to 18 (Table A.3). The tick infestation intensity was significantly different across the study villages (H = 71.76, df = 11, $p < 0.001$) and the administrative wards (W = 17,834, $p < 0.001$). The infestation intensity was also significantly different among the different age groups of cattle (H = 17.213, df = 2, p = 0.0002), with the pairwise comparison showing that calves and adults and calves and juveniles were significantly different ($p < 0.0001$). The infestation intensity was not statistically significant between male and female cattle (W = 14,510, p = 0.1344).

Table 1
Effect of host characteristics and administrative ward on tick infestation prevalence in cattle from coastal Kenya.

Variable	Category	Number of observed cattle[a]	Infestation prevalence	
			No. of infested cattle	*P*-value
Animal sex	Male	603 (39.6%)	131 (21.7%)	χ^2 = 0.0029851, df = 1, p = 0.96
	Female	919 (60.4%)	202 (22.0%)	
Age	Calves	117 (7.7%)	14 (12.0%)	χ^2 = 7.5314, df = 2, p = 0.02*
	Juvenile	434 (28.5%)	95 (21.9%)	
	Adults	971 (63.8%)	224 (23.1%)	
Administrative ward	Kinango	799 (52.5%)	156 (19.5%)	χ^2 = 5.1701, df = 1, p = 0.03*
	Kayafungo	723 (47.5%)	177 (24.5%)	

[a] total number of observed cattle is 1522; Calves (<6 months), Juvenile (6–24 months) and Adults (>24 months); *statistically significant ($P \leq 0.05$).

3.4. Prevalence of tick-borne pathogens

A total of 1382 single ticks, and 682 tick pools representing 1831 ticks, were screened for *Rickettsia* spp., *Theileria* spp., *Ehrlichia* spp., *C. burnetii, Babesia* spp. and *Anaplasma* spp. infections. We detected *Rickettsia* spp., *Theileria* spp. and *Ehrlichia* spp. pathogen DNA, while none of the samples was positive for *Anaplasma* spp., *Babesia* spp., or *C. burnetii.* The prevalence of infection in individual ticks and the MIRs of pooled ticks are summarized in Tables 2a and b, respectively.

The prevalence of *Rickettsia* spp. infection in single ticks was observed to be 78.1%, while the MIR of tick pools was 23.4%. The 16S rDNA rickettsial gene sequencing confirmed *R. africae* in all positive samples, showing 100% identity with *R. africae* isolate from Uganda (Table A.4). Additional amplification of the *ompA, ompB, gltA* and *Sca4* gene fragments in the *Rickettsia*-positive samples also showed maximum identities (99.8–100%) with *R. africae* as validated species. The prevalence of infection in single ticks was highest in *A. variegatum* and lowest in *R. microplus*, while the MIR was highest in *A. variegatum* and lowest in *R. evertsi*.

Interestingly, 10.6% of single ticks and 54/1831 tick pools were positive in the 16S rRNA *Rickettsia* PCR but were negative for additional PCR amplifications targeting the *Rickettsia ompA, ompB, gltA* and *sca4* genes. Sequencing of these PCR products revealed the presence of *Coxiella* sp. endosymbionts (Table A.4). Subsequent amplification of these samples with *C. burnetii*-specific primers also yielded no amplification.

Theileria spp. DNA was identified in 1.4% of individual ticks and in six tick pools. Based on the 18S rRNA gene sequences, *T. parva* was detected in four of the *R. appendiculatus* pools with 100% identity to *T. parva* isolate from Kenya. *Theileria velifera* 18S rRNA sequences sharing 100% identity with *T. velifera* isolate from Saudi Arabia were observed in 19 single ticks and two-tick pools (Table A.4).

DNA of *Ehrlichia* spp. was detected in 86 single ticks and in 18 tick pools. Sequencing of the 16S rDNA gene revealed the presence of *E. ruminantium* in 80 single ticks and none in the pooled ticks, with the sequence showing 100% identity to *E. ruminantium* isolated from *Amblyomma hebraeum* in South Africa (Table A.4). On the other hand, *E. minasensis* 16S rDNA sequences were detected in six single ticks and 18 tick pools, and the sequences were 100% identical to the sequence of *E. minasensis* isolated from *R. microplus* from Brazil and Egypt. The identity of *E. minasensis* species was further confirmed by re-amplification of the groEL gene, which also showed 100% identity with *E. minasensis* sequences detected in cattle from Australia.

3.5. Pathogen co-infections and associations

For co-infections, we analyzed a subset of ticks limited to samples with a single tick. Out of the 1382 single ticks, 1138 (82.3%) ticks were infected with one TBP, while mixed infections with two and three different pathogens were observed in 6.9% (n = 95/1382) and 0.1% (n = 2/1382) of single tick samples, respectively. The most common mixed infections were with *R. africae* and *E. ruminantium* (Table 3). Co-infection with *R. africae* and *E. ruminantium* was highest in *A. gemma* ticks with a prevalence of 7.2% (95% CI: 4.6 – 10.6), the same as the expected prevalence (7.2%).

Analysis of associations among pathogens and between pathogens and *Coxiella* sp. endosymbionts in single tick samples revealed a significant negative correlation between *R. africae* infection and *Coxiella* sp. endosymbionts (r = −0.64, p = 0.0133). All other combinations of pathogens were tested for their associations but showed no significant correlations (Fig. 2).

4. Discussion

This survey was conducted to assess the species diversity of ixodid ticks infesting cattle, their infestation levels and the associated TBPs in

Table 2a
Prevalence of pathogens and *Coxiella* sp. endosymbiont identified in single ticks collected from cattle sampled in coastal Kenya.

Tick species	Tested single ticks	*Ehrlichia minasensis*		*Ehrlichia ruminantium*		*Rickettsia africae*		*Theileria velifera*		*Theileria parva*		*Coxiella* sp. endosymbiont	
		+ ticks	% prevalence	+ ticks	% prevalence	+ ticks	%prevalence	+ ticks	prevalence	+ ticks	% prevalence	+ ticks	% prevalence
Rhipicephalus appendiculatus	4	0	0	0	0	2	50%	0	0	0	0	0	0
Rhipicephalus pulchellus	3	0	0	0	0	1	33.3	0	0	0	0	0	0
Rhipicephalus evertsi	27	0	0	0	0	9	33.3	0	0	0	0	12	44.4
Rhipicephalus microplus	175	4	2.3	1	0.6	55	31.4	0	0	0	0	54	30.9
Amblyomma gemma	321	2	0.6	27	8.4	273	85.0	2	0.6	0	0	26	8.1
Amblyomma variegatum	835	0	0	52	6.2	734	87.9	17	2.0	0	0	54	6.5
Hyalomma rufipes	12	0	0	0	0	5	41.2	0	0	0	0	0	0
Hyalomma albiparmatum	5	0	0	0	0	0	0	0	0	0	0	0	0
Overall	**1382**	**6**	**0.4%**	**80**	**5.8%**	**1079**	**78.1%**	**19**	**1.4%**	**0**	**0**	**146**	**10.6%**

+ ticks - Positive single ticks.

Table 2b
Minimum infection rate (MIR) of pathogens and *Coxiella* sp. endosymbiont identified in pools (2–5 ticks/pool) of tick species collected from cattle sampled in coastal Kenya.

Tick species	Number of ticks	Number of pools	*Ehrlichia minasensis*		*Ehrlichia ruminantium*		*Rickettsia africae*		*Theileria velifera*		*Theileria parva*		*Coxiella* sp. endosymbiont	
			+ pools	MIR per 100 ticks (95% CI)	+ pools	MIR per 100 ticks (95% CI)	+ pools	MIR per 100 ticks (95% CI)	+ pools	MIR per 100 ticks (95% CI)	+ pools	MIR per 100 ticks (95% CI)	+ pools	MIR per 100 ticks (95% CI)
Rhipicephalus appendiculatus	504	112	6	1.2 (0.2 – 2.1)	0	0	64	12.7 (9.8 – 15.6)	0	0	4	0.8 (0.0 – 1.6)	12	2.4 (1.1 – 3.7)
Rhipicephalus evertsi	385	112	4	1.0 (0.0 – 2.1)	0	0	34	8.8 (6.0 – 11.7)	0	0	0	0	8	2.1 (0.7 – 3.5)
Rhipicephalus microplus	188	81	6	3.2 (0.7 – 5.7)	0	0	31	16.5 (11.2 – 21.8)	0	0	0	0	29	15.4 (10.3 – 20.6)
Amblyomma gemma	452	226	2	0.4 (0.0 – 1.1)	0	0	217	48.0 (43.4 – 52.6)	1	0.2 (0.0 – 0.7)	0	0	1	0.2 (0.0 – 0.7)
Amblyomma variegatum	294	147	0	0	0	0	81	27.6 (22.4 – 32.7)	1	0.3 (0.0 – 1.0)	0	0	4	1.4 (0.0 – 2.7)
Hyalomma rufipes	6	3	0	0	0	0	1	16.7 (0.0 – 46.5)	0	0	0	0	0	0
Hyalomma albiparmatum	2	1	0	0	0	0	0	0	0	0	0	0	0	0
Overall	**1831**	**682**	**18**	**1.0 (0.5 – 1.4)**	**0**	**0**	**428**	**23.4** (21.4 – 25.3)	**2**	**0.1** (0.0 – 0.3)	**4**	**0.2** (0.0 – 0.4)	**54**	**3.0** (2.2 – 3.7)

+ Pools - Positive tick pools; **MIR –** minimum infection rate**; CI -** confidence Intervals.

Table 3
Prevalence of tick-borne pathogen co-infections in single ticks from coastal Kenya.

Co-infection	Tick species	Ticks analyzed	Positive ticks (%)	Observed prevalence % (95% CI)	Expected prevalence %
Double co-infections					
R. africae + *E. minasensis*	*A. gemma*	321	2	0.6 (0.1 – 2.2)	0.5
	R. microplus	175	4	2.3 (0.6 – 5.7)	0.7
R. africae + *E. ruminantium*	*A. gemma*	321	23	7.2 (4.6 – 10.6)	7.2
	A. variegatum	835	47	5.6 (4.2 – 7.4)	5.5
R. africae + *T. velifera*	*A. gemma*	321	2	0.6 (0.1 – 2.2)	0.5
	A. variegatum	835	15	1.8 (1.0 – 2.9)	1.8
E. ruminantium + *Coxiella* sp. endosymbiont	*A. gemma*	321	2	0.6 (0.1 – 2.2)	0.7
	A. variegatum	835	4	0.5 (0.1 – 1.2)	0.4
E. ruminantium + *T. velifera*	*A. variegatum*	835	2	0.2 (0.0 – 0.8)	0.1
T. velifera + *Coxiella* sp. endosymbiont	*A. variegatum*	835	1	0.1 (0.0 – 0.7)	0.1
Triple co-infections					
R. africae + *E. ruminantium* + *T. velifera*	*A. variegatum*	835	2	0.2 (0.0 – 0.8)	0.1

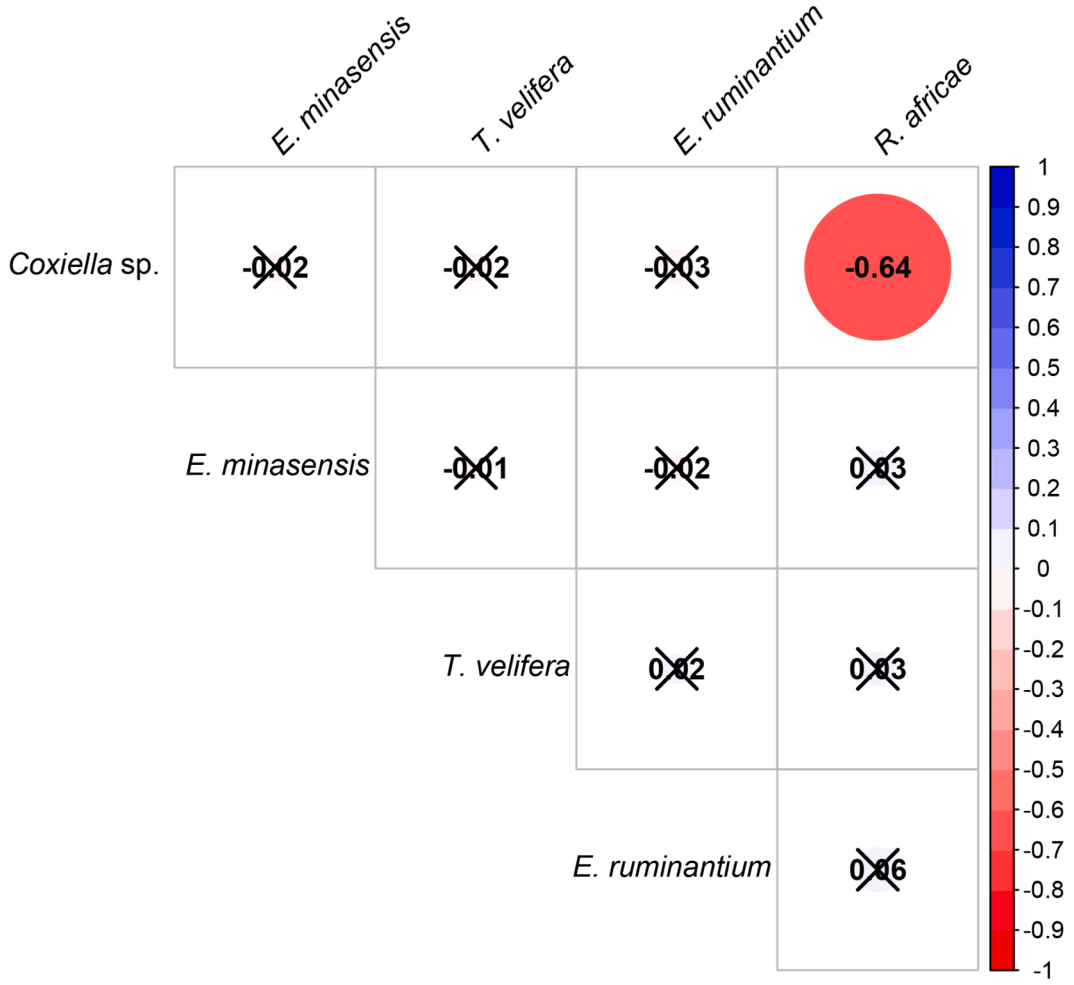

Fig. 2. Correlogram showing the association between tick-borne pathogens detected in single tick samples from coastal Kenya. In the right side of the correlogram, the legend color shows the correlation coefficients and the corresponding colors. Positive correlations are displayed in blue and negative correlations in red color. Color intensity and the size of the circle are proportional to the correlation coefficients. The numbers inside are correlation coefficients. The correlation matrix is reordered according to the correlation coefficient using "hclust" method.

the coastal region of Kenya. We report the presence of eight tick species belonging to *Rhipicephalus* (four species), *Amblyomma* (two species), and *Hyalomma* (two species) that are infesting cattle in this region. We also provide molecular evidence showing that ticks in this region harbor a diverse array of microorganisms.

Based on morphological and genetic criteria, ticks were classified as *R. appendiculatus, R. evertsi, R. microplus, R. pulchellus, A. gemma, A. variegatum, H. rufipes* and *H. albiparmatum.* Except for *H. albiparmatum*, all these tick species have been reported to parasitize cattle in Kenya (Kariuki et al., 2012; Zulu et al., 1998). *Hyalomma albiparmatum* is a rare species that occurs only in southern Kenya and northern Tanzania (Walker et al., 2003). *Rhipicephalus decoloratus*, previously described in cattle in low numbers in some areas of coastal Kenya (Zulu et al., 1998) was not observed in our samples. However, the presence of the invasive

Asian blue tick *R. microplus* in our study corroborates earlier findings, which reported this species in coastal Kenya (Kanduma et al., 2020; Zulu et al., 1998).

In the present study, the tick infestation was significantly higher in Kayafungo Ward in Kilifi County compared to Kinango Ward in Kwale County ($\chi^2 = 5.1701$, df = 1, $p = 0.03$). This could be partially explained by the presence of functional cattle dips in Mwachinga and Kibaoni villages of Kinango Ward in Kwale County. We also observed a significantly lower prevalence of tick infestation in calves than in juveniles and adults ($\chi^2 = 7.5314$, df = 2, $p = 0.02$). The lower tick infestations recorded in calves could be due to the husbandry practice of maintaining calves together close to the homesteads, separated from the adult cattle, resulting in lower tick exposure.

We report the presence of *E. minasensis* in four tick species with varying infection rates, namely *R. appendiculatus, R. evertsi, R. microplus* and *A. gemma*. This pathogen was previously reported in cattle in Kenya (Chiuya et al., 2021; Peter et al., 2020). The repeated detection in Kenya warrants further studies on their epidemiological implications for livestock health and productivity in the region. This is because *E. minasensis* has been experimentally demonstrated to cause clinical ehrlichiosis in cattle, a disease characterized by fever, lethargy, depression, thrombocytopenia, anemia, leukopenia and morulae in peripheral blood monocytes (Aguiar et al., 2014).

Rickettsia africae is the etiologic agent of African tick-bite fever (ATBF) in humans and is transmitted by *A. hebraeum* and *A. variegatum* ticks (Parola et al., 2013). We observed *R. africae* in *A. variegatum* with a prevalence of 87.9%. This high rate of *R. africae* in *A. variegatum* suggests that the risk for human infections is likely underestimated. The disease has previously been reported in international travelers returning from rural SSA, with an estimated annual incidence of 4 - 5.3% (Jensenius et al., 2003).

East Coast fever (ECF) caused by the protozoan parasite *T. parva* and transmitted by *R. appendiculatus* is the most economically important tick-borne disease of cattle in eastern, central and southern Africa, often leading to a loss in productivity and cases of mortality (Nene et al., 2016). In this study, *T. parva* was observed in *R. appendiculatus*, confirming the link between *R. appendiculatus* ticks and the epidemiology of ECF in SSA. The apparent presence of *T. parva* in its biological vector highlights the persistent risk of ECF to cattle, especially the exotic breeds, and thus the need to intensify tick control programs in this region.

Theileria velifera is non-pathogenic in cattle and is transmitted by *Amblyomma* ticks (Lawrence and Williamson, 2004). In the present study, *T. velifera* was detected in *A. gemma* and *A. variegatum* ticks and thus corroborates earlier studies that reported a close association between the distribution of *T. velifera* and *Amblyomma* ticks. Although *T. velifera* does not have any significant economic importance, its presence could complicate the specific diagnosis of the pathogenic *T. parva* in cattle and buffalo (Chaisi et al., 2013).

Coxiella sp. endosymbionts have previously been detected with varying prevalence in several tick genera, including *Rhipicephalus, Hyalomma, Ixodes, Amblyomma, Haemaphysalis* and *Dermacentor* (Oundo et al., 2020; Papa et al., 2017). This study also reports a varying prevalence of *Coxiella* sp. endosymbionts in *R. microplus, A. variegatum, R. appendiculatus, R. evertsi* and *A. gemma*. Thus, our findings add to the growing evidence of the widespread occurrence of *Coxiella* sp. endosymbionts across various tick species and geographical regions (Duron et al., 2015).

In this study, we observed that the infection frequency of *Coxiella* sp. endosymbionts was negatively correlated with the frequency of *R. africae* infection in *Amblyomma* ticks, and at no instance did we find concomitant co-infection between the pathogenic *R. africae* and *Coxiella* spp. symbionts. A similar observation has been reported in *Rhipicephalus sanguineus* sensu lato, which was dominantly infected by either *Rickettsia* spp. or *Coxiella* spp. symbionts, but never both at the same abundance (René-Martellet et al., 2017). Thus, our finding suggests that infection with this *Coxiella* sp. symbionts may affect the colonization of *R. africae* in *Amblyomma* tick species and therefore warrants further mechanistic investigations to elucidate their interactions and their role in vector competence.

Ehrlichia ruminantium is the causative agent of heartwater disease in domestic ruminants (sheep, goats and cattle) and it is transmitted by *Amblyomma* ticks, mainly *A. hebraeum* in southern Africa and *A. variegatum* in the rest of SSA, the Caribbean and the Indian Ocean islands (Allsopp, 2010). We detected *E. ruminantium* DNA in *A. gemma* and *A. variegatum*, confirming the strong link between the distribution of *Amblyomma* ticks and heartwater disease in SSA (Allsopp, 2010). The detection of *E. ruminantium* in *R. microplus* in this study is not completely surprising since a recent study has reported the potential of *R. microplus* to transmit *E. ruminantium* in West Africa (Biguezoton et al., 2016). The presence of *E. ruminantium* in *Amblyomma* and *R. microplus* ticks in the study area suggests that the risk for heartwater infections in cattle is underestimated.

We observed co-infections in 7.0% of the analyzed single ticks and that ticks could be infected with up to three different pathogen species. The most frequent pathogen combination observed in this study was *R. africae* and *E. ruminantium*, suggesting the possibility of ticks vectoring multiple pathogens in this region. It is worth noting that co-infections of multiple pathogens can alter typical disease symptoms or enhance disease severity, thus resulting in diagnostic and treatment challenges (Diuk-Wasser et al., 2016; Moutailler et al., 2016). Therefore, it is important to continue assessing the range and frequency of co-infections occurring naturally in ticks.

Due to the nature of the cross-sectional design, this work had limited ability to investigate the typical seasonal fluctuations in tick densities, and the influence of animal movement on tick dispersal and the challenges it poses in correlating infection and infestation risk factors. As such, this study provides only one snapshot of tick diversity and infestation prevalence, as well as TBPs prevalence in ticks. Additionally, the mere presence of pathogen DNA in the collected ticks does not necessarily mean that they are biological vectors, as a tick can test positive for a pathogen if it ingests infected blood without necessarily transmitting it to a susceptible animal host during its next blood meal. Therefore, the results of the present study should be interpreted with caution. Future studies should aim to cover a broader geographical area, allowing for a more comprehensive understanding of TBDs and the development of effective control strategies.

5. Conclusions

Our study provides contemporary evidence that multiple TBPs of zoonotic and veterinary importance are harbored by the bovine tick population in coastal Kenya. The observed co-infections in ticks represent a risk of acquiring multiple infections as a consequence of a single tick bite. Further studies are needed to elucidate the functional roles of *Coxiella* sp. endosymbionts in pathogen colonization and transmission in ticks. More active surveillance will help to detect the spread and potential risk of *E. minasensis* infection for the bovine population throughout coastal Kenya.

CRediT authorship contribution statement

Joseph Wang'ang'a Oundo: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. **Shewit Kalayou:** Conceptualization, Methodology, Project administration, Supervision, Writing – review & editing. **Quirine ten Bosch:** Writing – review & editing. **Jandouwe Villinger:** Writing – review & editing. **Constantianus J.M. Koenraadt:** Writing – review & editing. **Daniel Masiga:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare they have no competing interests.

Data availability

Data will be made available on request.

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Availability of data and materials

The data supporting the conclusions of this article are included within the article and its additional file.

Consent for publication

Not applicable

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Supplementary materials

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