Molecular Detection of Tick-Borne Pathogen Diversities in Ticks from Livestock and Reptiles along the Shores and Adjacent Islands of Lake Victoria and Lake Baringo, Kenya

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Although diverse tick-borne pathogens (TBPs) are endemic to East Africa, with recognized impact on human and livestock health, their diversity and specific interactions with tick and vertebrate host species remain poorly understood in the region. In particular, the role of reptiles in TBP epidemiology remains unknown, despite having been implicated with TBPs of livestock among exported tortoises and lizards. Understanding TBP ecologies, and the potential role of common reptiles, is critical for the development of targeted transmission control strategies for these neglected tropical disease agents.

During the wet months (April–May; October–December) of 2012–2013, we surveyed TBP diversity among 4,126 ticks parasitizing livestock and reptiles at homesteads along the shores and islands of Lake Baringo and Lake Victoria in Kenya, regions endemic to diverse neglected tick-borne diseases. After morphological identification of 13 distinct Rhipicephalus, Amblyomma, and Hyalomma tick species, ticks were pooled (≤8 individuals) by species, host, sampling site, and collection date into 585 tick pools. By supplementing previously established molecular assays for TBP detection with high-resolution melting analysis of PCR products before sequencing, we identified high frequencies of potential disease agents of ehrlichiosis (12.48% Ehrlichia ruminantium, 9.06% Ehrlichia canis), anaplasmosis (6.32% Anaplasma ovis, 14.36% Anaplasma platys, and 3.08% Anaplasma bovis), and rickettsiosis (6.15% Rickettsia africae, 2.22% Rickettsia aeschlimannii, 4.27% Rickettsia rhipicephali, and 4.95% Rickettsia spp.), as well as Paracoccus sp. and apicomplexan hemoparasites (0.51% Theileria sp., 2.56% Hepatozoon fitzsimonsi, and 1.37% Babesia caballi) among tick pools. Notably, we identified E. ruminantium in both Amblyomma and Rhipicephalus pools of ticks sampled from livestock in both study areas as well as in Amblyomma falsomarmoreum (66.7%) and Amblyomma nuttalli (100%) sampled from tortoises and Amblyomma sparsum (83.6%) sampled in both cattle and tortoises at Lake Baringo. Similarly, we identified E. canis in rhipicephaline ticks sampled from livestock and dogs in both regions and Amblyomma latum (75%) sampled from monitor lizards at Lake Victoria. These novel tick–host–pathogen interactions have implications on the risk of disease transmission...
INTRODUCTION

Tick-borne pathogens (TBPs) are responsible for some of the most serious emerging infectious diseases facing sub-Saharan Africa (SSA) and the rest of the world today (1, 2). In Kenya, TBPs (including viral diseases—arboviruses) like Crimean Congo hemorrhagic fever (CCHF), Dugbe, Kupe, and Hazara, as well as hemoparasites that cause babesiosis, theileriosis, and rickettsiosis, are major impediments to livestock productivity and public health (3–6).

Baringo and Homa Bay counties of Kenya are both characterized by unique land-lake biogeographies with fluctuating fresh water levels of Lakes Baringo and Victoria, respectively. These ecological habitats form shallow lagoons with abundant aquatic and terrestrial biodiversity that include diverse vertebrate host species and disease vectors (7). The interaction of domestic animals (mainly livestock, dogs, chickens, and cats), migratory birds, humans, and wildlife has the potential to facilitate the spread of zoonotic pathogens, including TBPs. Wildlife can act as both sources and maintenance hosts for TBPs (8) that can also be transmitted to livestock and humans (9, 10), causing significant morbidity and mortality (11). For example, East Coast fever (ECF), caused by Theileria parva, originates from African buffalo and circulates in cattle, which often succumb to the disease (12, 13).

Recent studies have implicated reptiles as potential reservoirs involved in TBP transmission cycles. Ehrlichia ruminantium, considered a pathogen of ruminants responsible for heartwater disease, has been reported among Amblyomma sparsum (toroise tick) sampled from leopard tortoises imported into the United States from Zambia (14). Furthermore, diverse Borrelia, Rickettsia, Ehrlichia, Anaplasma, and Babesia species have been identified in lizard species in Portugal (15), Australia (16), and the Netherlands (17), as well as in diverse reptiles imported into Japan (18). These studies suggest a possible role of reptiles in the epidemiology of diverse tick-borne diseases, including heartwater, which has not been investigated in African endemic settings.

Increased TBP sharing between wildlife and livestock species may result from human-induced interactions (19, 20). Nomadic and pastoralist lifestyles lead to direct and indirect contact that facilitate exposure and sharing of previously isolated pathogens (21, 22). Wildlife translocations to new habitats have also resulted in outbreaks and mortality among naïve inhabitants, as was the case in cattle when T. parva-infected buffalo was translocated to the Highveld of Zimbabwe (23).

In Baringo County, the burden of tick-borne diseases remains largely unknown. The pastoralist communities that inhabit Baringo County plains keep large herds of livestock that are parasitized by an abundant diversity of vector tick species with pathogens that inflict significant economic losses on already drought impoverished populations (24). Interestingly, tick-laden to humans and domestic animals and highlight the complexity of TBP ecologies, which may include reptiles as reservoir species, in sub-Saharan Africa.

Keywords: tick-borne diseases, Ehrlichia, Anaplasma, Rickettsia, Babesia, Hepatozoon, Theileria, Kenya

MATERIALS AND METHODS

Study Locality

A TBP survey was conducted in 2012–2013 along the shores and adjacent islands of Lake Baringo and Lake Victoria in Kenya.

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Baringo County is located in the Great Rift Valley, 250 km northwest of Nairobi and covers ~8,655 km² in area. It has an average rainfall of 700 mm and altitude of 700 m above the sea level with average temperatures of 28°C (36). Three indigenous agro-pastoralist communities (Pokot, Tugen, and Njemps) inhabit Baringo County. They rely on livestock (mostly goats as well as cattle, sheep, and donkey) and irrigated crop production along the Perkerra, Molo, and Kerio rivers.

Homa Bay County lies within the Kenyan part of the Lake Victoria basin and covers ~3,155 km² in area. It has an a bimodal rainfall ranging between 250 and 1,650 mm per annum and an altitude of 970 m above the sea level with an average temperature range of 17.1 to 34.8°C (37). The “long rains” peak in April and the “short rains” in October. Most inhabitants belong to the Luo and Suba ethnic groups whose main socioeconomic activities are fishing and small-scale mixed farming, which includes keeping of livestock (cattle, sheep, and goats).

**Sampling**

Ethical clearance for the study was obtained from the Kenya Medical Research Institute ethics review committee (Approval Ref: non-SSC Protocol #310) and sampling from wildlife was approved by the Kenya Wildlife Service Biodiversity Research and Monitoring committee (Permit Ref: KWS/BRM/5001). Informed oral consent was obtained from village elders on the study activities and from household heads before inclusion of their livestock in the study. Written consent could not be used due to low literacy levels and language barriers that required translation into local languages (Luo and Suba) among most of the community elders and livestock owners, hence oral consent was adopted to all for standardization. The Kenya Wildlife Services, Kenya's Directorate of Veterinary Services and Ministry of Health, were consulted before the study was initiated, and supervised oral consent and sampling. The oral consent was not documented since tick collection presented minimal risk to the livestock and involved no protocol for which written documentation is normally required. Before sampling, all animals were restrained manually in order to allow for tick collection. To minimize risks to livestock, animal sampling was carried out in a manner that addressed all pertinent animal welfare issues.

Homesteads were surveyed for livestock and reptiles parasitized with ticks in 2012 and 2013 during the wet months of April–May and October–December in both study areas close to human habitation. Those that had recently treated their animals with acaricides were excluded. Ticks were opportunistically sampled and pooled from goats (117), cattle (76), sheep (54), poultry houses (17), and dogs (15) found in selected homesteads, as well as tortoises (*Stigmochelys pardalis*) (18) and monitor lizards (*Varanus niloticus*) (4) that were common to homesteads in Baringo and Homa Bay counties, respectively. Up to 30 ticks were sampled per animal within each homestead and fully engorged ticks were not collected to minimize contamination from vertebrate host nucleic acids during extraction. Animals were manually restrained before plucking of live ticks from their bodies. Crocodiles were excluded because of high risk they pose.

Sampling was conducted in Baringo and Homa Bay counties alongside previously described mosquito sampling efforts (38–40) (Figure 1). In Baringo, we sampled in and adjacent to (i) Ruko Wildlife Conservancy, where livestock and humans live in close proximity to wildlife, (ii) Logumgum, a transmission hot spot for the 2006/2007 RVF outbreak (41), (iii) Kokwa, and (iv) Kampi ya Samaki, where locals keep relatively large numbers of livestock (≥20). In Homa Bay County, Rusinga and Mbita areas were chosen due to previous studies in the area that detected the presence of *T. parva* antigens (42) and their association with reduced productivity of Zebu cattle under traditional management (43). Mfangano Island and Ngodhe offer sanctuary to a wide

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**FIGURE 1** | Map of Kenya showing tick sampling areas in Kenya. Most of the sampling points were in rural villages where mosquito sampling has previously been described.
diversity of wild birds that could be important in introductions of ticks to these areas.

After collection, ticks were frozen in liquid nitrogen and transported to the Martin Lüscher Emerging Infectious Diseases Laboratory at the International Centre of Insect Physiology and Ecology in Nairobi, where they were identified morphologically and sorted on a chilled surface (paper towels over −80°C icepacks) under a dissecting microscope (Leica Microsystems, Buffalo Groove, Illinois). Ticks were identified morphologically to species level based on taxonomic keys of the genera *Rhipicephalus* (44), *Amblyomma* (45), *Hyalomma*, and *Argas* (46), developed by Walker and colleagues (47). Tick sorting entailed removal of highly engorged samples to reduce vertebrate host nucleic acids during extraction and pooling into groups of one to eight individuals by species, host, sampling site, and collection date.

**Molecular Identification of TBPs**

Tick pools were homogenized for 25 s in 1.5 ml screw-cap tubes filled with zirconia/ytrria-stabilized zirconium oxide beads (750 mg of 2.0 mm diameter and 150 mg of 0.1 mm diameter) (Glen Mills, Clifton, NJ, USA) (48), 650 µl of phosphate-buffered saline using a Mini-Beadbeater-16 (BioSpec, Bartlesville, OK, USA). Total nucleic acids were extracted from these homogenates in an automated MagNa Pure 96 extraction system (Roche Diagnostics, Risch-Rothkreuz, Switzerland) using the small volume DNA/viral RNA kits (Roche Diagnostics). TBPs were detected and characterized by PCR followed by high-resolution melting (HRM) analyses. All gene fragments were amplified in an HRM capable RotorGene Q thermo cycler (QIAGEN, Hannover, Germany) to a final volume of 10 µl using HOT FIREPol EvaGreen HRM Mix (Solis BioDyne, Tartu, Estonia) and primers listed in Table 1. For identification of *Ehrlichia* and *Anaplasma*, we utilized previously described primers (49) of a short 16S rRNA amplicon (< 200 bp) and unresolved samples were subsequently analyzed by amplification and sequencing of longer (> 300bp) 16S rRNA fragments using the newly designed primer pairs, “*Anaplasma* long 16S rRNA Fwd/Rev” and “*Ehrlichia* long 16S rRNA Fwd/Rev” (Table 1). For *Rickettsia*, we utilized the rpmE-tRNA\textsuperscript{Met} intergenic spacer typing (50). The apicomplexan hemoparasites, *Theileria*, *Babesia* and *Hepatozoon*, were amplified and resolved using previously described primers specific to the 18S ribosomal gene (Table 1) (51).

The thermal cycling conditions used for amplification were as follows: initial denaturation at 95°C for 15 min, followed by 10 cycles of 94°C for 20 s, step-down annealing from 63.5°C decreasing by 1°C per cycle for 25 s, and primer extension at 72°C for 30 s; then 25 cycles of denaturation at 94°C for 25 s, annealing at 50.5 for 20 s, and extension at 72°C for 30 s, followed by a final extension at 72°C for 7 min. Following PCR, HRM profiles of amplicons were obtained through gradual increase in temperature from 75 to 90°C at 0.1°C/2 s increments. Changes in fluorescence with time (dF/dT) were plotted against changes in temperature (°C). Positive samples/amplicons were detected by observation of HRM curves and peaks. To identify the specific pathogen sequences associated with each unique HRM profiles, representative samples with single peaks for each of the profiles were purified with ExoSAP-IT PCR Product Cleanup kit (Affymetrix, Santa Clara, CA, USA) to remove unincorporated dNTPs and PCR primers before sequencing, which was outsourced from Macrogen (South Korea).

**Phylogenetic Analysis**

The returned sequences were edited and aligned, using the MAFFT (52) plugin in Geneious software version 8.1.4 (created by Biomatters) (53), with closely related sequences revealed by querying the GenBank nr database using the Basic Local Alignment Search Tool (54). Study sequences that were >200 bp were submitted to GenBank. From multiple alignments, some pathogens could not be fully characterized to species and were classified based on their genus. After visualizing neighboring phylogenetic trees (55) of the alignments, constructed within Geneious software, we constructed comparable maximum likelihood phylogenetic trees of the alignments, using PHYML v. 3.0 (56). The phylogenies employed the Akaike information criterion for automatic model selection and tree topologies were estimated using nearest neighbor interchange improvements over 1,000 bootstrap replicates. Phylogenetic trees were depicted using FIGTREE (57).

| Pathogen/gene target | Primer pair | Amplicons size (bp) | Reference sequence | Primer coordinates | Citation |
|----------------------|-------------|---------------------|--------------------|--------------------|---------|
| *Anaplasma* short 16S rRNA | Fwd: GGGCGATGTAGGGCGGTTCGCTG<br>Rev: TCAGCGTCACTGACCGGAACCA | ~200 | KJ410254 | 491–510<br>675–656 | Tokarz et al. (49) |
| *Anaplasma* long 16S rRNA | Fwd: CGTGAGGAGCATGTTTTAATTC<br>Rev: CGRCGGTGCAACCTATGTCGTC | ~330 | 853–875<br>1,183–1,161 | Mwamuye et al. (32) |
| *Ehrlichia* short 16S rRNA | Fwd: CGTAAAGGGCACGTAGGTGGACTA<br>Rev: CACCTCGTGTGAGTCTGACCA | ~200 | NR_074155 | 507–530<br>701–678 | Tokarz et al. (49) |
| *Ehrlichia* long 16S rRNA | Fwd: GGAACCTCCTCGTATTGGAACCA<br>Rev: TGGATCAGCCCTCACAGGTTACAC | ~400 | 1,045–1,067<br>1,439–1,417 | Mwamuye et al. (32) |
| *Rickettsia* ompB | For: GTGACGTTTCAAGGCGACCA<br>Rev: ATACAAAGGTGCTATGGAAGTGG | ~200 | CP001612 | 1,020,786–1,020,813<br>1,020,984–1,020,961 | Tokarz et al. (49) |
| *Rickettsia* rpmE/tRNA\textsuperscript{Met} | For: TCAGGTTAGGGCGTGACT<br>Rev: TTCGCGGATTTGAGTACATC | 175–402 | 130,181–130,206<br>130,522–130,503 | Zhu et al. (50) |
| *Theileria/Babesia* 18S rRNA | Fwd: GAGGAGTGGGAGCAAGAAAATAAAATAAAT<br>Rev: TCTCTGAGCCCTTCACTTTC | ~500 | HG684067 | 330–354<br>832–813 | Gubbels et al. (51) |
Tick-borne pathogen gene fragments with distinct HRM profiles (Figure 2) and representative sequences sharing ≥96% identity with a recognized TBP species, or ≥90% with a TBP genus in GenBank (Table 4), were detected in tick pools sampled in Baringo and Homa Bay counties (Table 5) from diverse individual vertebrate hosts (Table 6). In Baringo County, we detected sequences of agents of livestock or canine ehrlichiosis (E. ruminantium, Ehrlichia canis, and Ehrlichia sp.) (Figure 2A), anaplasmosis (Anaplasma ovis, Anaplasma platys, and A. bovis) (Figure 2B), rickettsiosis (Rickettsia aesculapianii, Rickettsia

**RESULTS**

**Tick Species Sampled**

A total of 585 tick pools comprised of 4,126 ticks of 14 species were collected and analyzed from both study areas. We sampled more ticks (80.47%) in Baringo County (3,320 ticks in 456 tick pools) (Table 2), which had higher numbers of livestock per household (>20), than in Homa Bay County (806 ticks in 129 tick pools) (Table 3), which had fewer (<5) livestock per household, from diverse vertebrate hosts. In both study areas, most ticks were from goats, which were more heavily parasitized than the other animals sampled (Tables 2 and 3).

In Baringo County, 12 species were sampled from goats, sheep, cattle, poultry houses, dogs, and free ranging tortoises (Table 2), 11 of which were hard ticks (Family; Ixodidae) and one of which was a soft tick species (Argas persicus, 7.04%) sampled from poultry houses in Kampi ya Samaki. *Rhipicephalus pravus* (44.88%) sampled from domestic ruminants and dogs was the most frequent tick species identified, while *Amblyomma falsomarmoreum* (1.23%) and *Amblyomma nuttalli* (0.66%) sampled from free ranging tortoises at Kampi ya Samaki were the least frequent tick species identified.

In Homa Bay County, eight hard tick species were sampled from goats, sheep, cattle, dogs, and monitor lizards (Table 3). *Rhipicephalus pravus* (34.49%), sampled from livestock and dogs, was the most frequent species identified, while *Hyalomma truncatum* (2.23%) sampled from livestock was least frequent. *Rhipicephalus appendiculatus* (3.59%), a known vector for ECF, and *Amblyomma* (Aponomma) *latum* (3.34%), sampled from monitor lizards, were common only on Rusinga Island.

**TBPs Identified**

Livestock TBPs in Reptile Ticks
TABLE 3 | Ticks sampled from different host species in four study areas of Homa Bay County.

| Study Area | Tick species | Pools | N (%) | Cattle (%) | Goats (%) | Sheep (%) | Dogs (%) | Monitor lizards (%) |
|------------|--------------|-------|-------|------------|-----------|-----------|----------|---------------------|
| Ngothe     | Rh. pulchellus | 9     | 61 (7.56) | 24 (2.97) | 23 (2.85) | 14 (1.73) |          |                     |
|            | Rh. evertsi evertsi | 8     | 45 (5.58) | 3 (0.37) | 30 (2.72) | 4 (0.49) | 8 (0.23) |                     |
|            | Am. variegatum | 4     | 18 (2.23) | 9 (1.11) | 4 (0.49) | 5 (0.62) |          |                     |
|            | Hy. truncatum | 3     | 12 (1.48) | 9 (1.11) | 3 (0.37) |          |          |                     |
| Mbita      | Rh. pravus | 13    | 83 (10.29) | 13 (1.61) | 38 (4.71) | 28 (2.47) | 4 (0.49) |                     |
|            | Am. gemma | 9     | 67 (8.31) | 51 (6.32) | 12 (1.48) | 4 (0.49) |          |                     |
|            | Rh. evertsi evertsi | 7     | 40 (4.96) | 11 (1.36) | 18 (2.23) | 6 (0.74) | 5 (0.62) |                     |
|            | Am. Variegatum | 5     | 32 (3.97) | 16 (1.98) | 6 (0.74) | 10 (1.24) |          |                     |
|            | Rh. pulchellus | 1     | 6 (0.74) | 6 (0.74) |          |          |          |                     |
| Mtangano   | Rh. pravus | 14    | 94 (11.66) | 18 (2.23) | 37 (4.59) | 27 (2.35) | 12 (1.48) |                     |
|            | Am. variegatum | 6     | 45 (5.58) | 25 (3.1) | 14 (1.73) | 6 (0.74) |          |                     |
|            | Rh. pulchellus | 8     | 41 (5.08) | 9 (1.11) | 19 (2.35) | 13 (1.61) |          |                     |
|            | Rh. evertsi evertsi | 5     | 26 (3.22) | 5 (0.62) | 14 (1.73) | 4 (0.49) | 3 (0.37) |                     |
| Rusinga    | Rh. pravus | 14    | 101 (12.53) | 15 (1.86) | 57 (7.07) | 15 (1.86) | 14 (1.73) |                     |
|            | Am. variegatum | 6     | 45 (5.58) | 25 (3.1) | 14 (1.73) | 6 (0.74) |          |                     |
|            | Rh. pulchellus | 8     | 41 (5.08) | 9 (1.11) | 19 (2.35) | 13 (1.61) |          |                     |
|            | Rh. evertsi evertsi | 5     | 26 (3.22) | 5 (0.62) | 14 (1.73) | 4 (0.49) | 3 (0.37) |                     |
|            | Rh. appendiculatus | 5     | 29 (3.59) | 25 (3.1) | 4 (0.49) |          |          |                     |
|            | Am. istum | 4     | 27 (3.34) |          |          |          |          |                     |
|            | Rh. evertsi evertsi | 6     | 23 (2.85) | 4 (0.49) | 11 (1.36) | 2 (0.24) | 6 (0.74) |                     |
|            | Hy. truncatum | 1     | 6 (0.74) | 6 (0.74) |          |          |          |                     |
| Total      |            | 129   | 806 (96.66) | 261 (32.38) | 307 (38.08) | 152 (18.85) | 53 (6.57) | 27 (3.34) |

Percentages are out of the total number of ticks sampled. N, Number of ticks sampled.

FIGURE 2 | Melting rate profiles of tick-borne pathogens in field collected tick samples. PCR amplicon melt rates are represented as change in fluorescence with increasing temperature (df/dT) of (A) Ehrlichia spp. and Paracoccus sp. 16S rRNA, (B) Anaplasma 16S rRNA, (C) Rickettsia rpmE/rRNA\textsuperscript{αα}, and (D) apicomplexan hemoparasite 18S rRNA gene segments.
TABLE 4 | Nucleic acid sequence identities of tick-borne pathogens detected in Baringo and Homa Bay counties.

| Pathogen detected | Tick species | Study areas | Reference GenBank Accessions | Locus | Sequence length, bp (GenBank Accession) |
|-------------------|--------------|-------------|-----------------------------|-------|----------------------------------------|
| Anaplasma bovis   | Am. gemma, Am. variegatum, Rh. evertsi evertsi, and Rh. rhipicephali | Logumgum and Ruko | U03775 99 (1e−135) Short 16S 185 |       |
|                   | Hy. truncatum | 99 (1e−163) Long 16S 320 (KT266580) |
|                   | Hy. rufipes, Rh. praetextatus | 99 (1e−153) Short 16S 185 |

| A. ovis | Am. variegatum, Rh. pulchellus, Am. gemma, and Rh. appendiculatus | Logumgum, Kampi Ya Samaki, and Rusinga | KJ410245 99 (1e−135) Short 16S 185 | 328 (KT266581) |
|         |                     | 100 (8e−170) Long 16S |

| A. platys | Rh. evertsi, Rh. pulchellus, and Rh. pravus | Ngothe, Mfangano, Kampi ya Samaki, and Kokwa | LC018183 100 (5e−162) Short 16S 185 |

| Ehrlichia ruminantium | Am. gemma, Am. variegatum, Am. sparsum, and Am. evertsi evertsi | Ruko and Logumgum | NR_074155 99 (1e−94) Short 16S 194 |

| Ehrlichia ruminantium | Ruko and Kokwa Island | 100 (0.0) Long 16S 384 (KT266592) |

| Ehrlichia ruminantium | R. pravus, Rh. evertsi evertsi, Rh. pulchellus, and Rh. rhipicephali | Logumgum, Rusinga, and Mbita | CP000107 100 (3e−96) 100 (0.0) Short 16S 194 |

| Rickettsia africana | Am. gemma, Am. variegatum, Am. sparsum, and Am. evertsi evertsi | Kampi Ya Samaki, Ruko, and Logumgum | CP001612 100 (3e−143) tRNA 555 (KT266591) |

| Paracoccus sp. | Am. variegatum, Am. gemma, Am. sparsum, and Am. falsoomarmoreum | Logumgum | KP003988 97 (6e−88) Short 16S 151 |

| Rickettsia sp. | Am. variegatum, Am. gemma, Am. falsoomarmoreum | Ruko | AF414399, JX042805 98 (3e−96) Short 16S 194 |

| Rickettsia sp. | Rh. evertsi evertsi, Rh. pulchellus, and Rh. praetextatus | Ruko and Kokwa Island | KJ410245 99 (1e−135) Short 16S 185 |

| Rickettsia sp. | Rh. evertsi evertsi, Rh. pulchellus, and Rh. praetextatus | Ruko | U03776 99 (6e−93) Short 16S 194 |

| Rickettsia sp. | R. aeschlimanni | Logumgum, Rusinga, and Mbita | CP0013133 99 (2e−176) tRNA 344 (KT266584) |

| Rickettsia sp. | R. aeschlimanni | Kampi Ya Samaki, Ruko, and Logumgum | HQ335165 100 (1e−178) tRNA 344 (KT266585) |

| Rickettsia sp. | R. rhopappalbus | Logumgum, Rusinga, and Mbita | CP001612 100 (3e−143) tRNA 555 (KT266591) |

| Rickettsia sp. | R. rhopappalbus | Kampi Ya Samaki, Ruko, and Logumgum | CP0013133 99 (2e−171) tRNA 344 (KT266585) |

| Rickettsia sp. | R. rhopappalbus | Ruk | AP011532 94 (7e−115) tRNA 280 (KT266590) |

| Rickettsia sp. | R. rhopappalbus | Ruko and Kokwa Island | CP0013133 99 (2e−171) tRNA 343 (KT266589) |

| Rickettsia sp. | R. rhopappalbus | Ruko | AP011532 94 (7e−115) tRNA 280 (KT266590) |

| Babesia caballi | Rh. pulchellus | Logumgum, Rusinga, and Mbita | CP0013133 99 (2e−171) tRNA 343 (KT266589) |

| Babesia caballi | Rh. pulchellus | Ruko | AP011532 94 (7e−115) tRNA 280 (KT266590) |

| Babesia caballi | Rh. pulchellus | Ruko | AP011532 94 (7e−115) tRNA 280 (KT266590) |

| Babesia caballi | Rh. pulchellus | Ruko | AP011532 94 (7e−115) tRNA 280 (KT266590) |

| Theileria sp. | Rh. evertsi evertsi | Logumgum and Ruko | AF245279 90 (3e−140) 18S 438 (KT266582) |

| Theileria sp. | Rh. evertsi evertsi | Logumgum and Ruko | KR069084 100 (0.0) 18S 438 (KT266582) |

rhipicephali, Rickettsia africana, and Rickettsia spp.) (Figure 2C), as well as Babesia caballi, Hepatozoon fitzsimonsi, Theileria sp. (Figure 2D), and Paracoccus sp. (Figure 2A; Table 5). However, in Homa Bay County, we only detected E. ruminantium, E. canis, A. ovis, and A. platys (5). The maximum likelihood phylogenies of all sequences obtained among previously characterized, closely related TBPs are represented in Figures 3–5.

Among Ehrlichia sequences identified, E. ruminantium sequences from Baringo shared 99% nucleotide sequence identity with GenBank accession NR_074155 (59), whereas those from Homa Bay County were more similar (99% identity) to an E. (Cowdria) ruminantium sequence (GenBank accession U03776) (60) (Table 4). In both sampling areas, these were detected in Am. variegatum, Amblyomma gemma, and Rhipicephalus evertsi evertsi tick pools (Table 5) from livestock and dogs (Tables 2, 3 and 6). Surprisingly, E. ruminantium was also detected in Am. falsoomarmoreum (four pools) and Am. nuttalli (three pools) ticks (Table 5) sampled from tortoises (Tables 2 and 6) and, more importantly, Am. sparsum ticks (Table 5) sampled from both tortoises (five pools) and cattle (two pools) in Logumgum and Kampi ya Samaki, respectively, in Baringo County (Tables 2 and 6). Ehrlichia canis sequences (submitted GenBank accession KT266591) (Table 4) were detected in Rh. pravus, Rh. evertsi evertsi, and Rhipicephalus pulchellus tick pools (Table 5) sampled from livestock and domestic dogs of Logumgum in Baringo County (Tables 2 and 6). Rh. pulchellus ticks (Table 5) sampled from cattle in Mbita (Tables 3 and 6), and notably, Am. (aponomma) latum ticks (Table 5) parasitizing monitor lizards in Rusinga Island (Tables 3 and 6) of Homa Bay County. Ehrlichia sp. sequences (submitted GenBank accession KT266592) sharing 98–100% identity with Ehrlichia spp. isolates from Tibet and Xinjiang, China (GenBank accesses AF414399, JX042805) (61) (Table 4) were detected in Am. gemma (7 pools), Am. variegatum (3 pools), Rh. evertsi evertsi (13 pools), Rh. pulchellus (9 pools),
Table 5 | Tick-borne pathogens (TBPs) isolated from tick pools collected in Baringo and Homa Bay counties.

| Region           | Amblyomma variegatum | Anaplasma platys | Anaplasma ovis | Babesia caballi | Ehrlichia ruminantium | Hyalomma rufipes | Rickettsia aeschlimannii | Rickettsia africae | Rickettsia conorii | Rhipicephalus appendiculatus | Rickettsia sp. | TICPA84 |
|------------------|----------------------|------------------|----------------|----------------|----------------------|------------------|------------------------|-------------------|-----------------|-------------------------------|---------------|---------|
| Baringo County   | 6 (30.62%)           | 5 (26.32%)       | 6 (30.62%)     | 3 (15.79%)     | 11 (57.89%)          | 6 (30.62%)       | 3 (15.79%)              | 11 (57.89%)        | 5 (26.32%)     | 3 (15.79%)                     | 6 (30.62%)   |         |
| Homa Bay County  | 5 (26.32%)           | 4 (21.05%)       | 6 (30.62%)     | 3 (15.79%)     | 10 (52.63%)          | 5 (26.32%)       | 4 (21.05%)              | 10 (52.63%)        | 5 (26.32%)     | 4 (21.05%)                     | 5 (26.32%)   |         |

We also amplified 195 bp Paracoccus sp. sequences using the “Ehrlichia short 16S rRNA” primers (Table 4) in Am. variegatum (one pool), Am. sparsum (nine pools), Am. folsomarmororum (two pools), and Am. gemma (eight pools) (Table 5) ticks sampled from livestock and tortoises (Tables 2 and 6). There were instances in which we detected both Paracoccus sp. and E. ruminantium within single tick pools as illustrated by double HRM peaks (Figure 2A). Six pools of Am. sparsum and four of Am. variegatum had both E. ruminantium and Paracoccus sp., while three pools of Am. variegatum had a mixed infection of Ehrlichia sp. and Paracoccus sp. (Figure 2A).

The A. ovis sequences (submitted GenBank accession KT266581) (Table 4) were detected in Am. variegatum (22 pools), Am. gemma (7 pools), and Rh. pulchellus (5 pools) ticks (Table 5) parasitizing livestock in Logumugum and Kampi ya Samaki of Baringo County (Tables 2 and 6) and in Rh. appendiculatus (3 pools) (Table 5) parasitizing goats and cattle in Rusinga Island of Homa Bay County (Tables 3 and 6). Anaplasma platys sequences (Table 4) were detected from Rhipicephalus tick pools, including Rh. evertsi evertsi (15 pools), Rh. pravus (45 pools), and Rh. pulchellus (4 pools) (Tables 5 and 6) sampled from domesticated dogs in Ruko Conservancy, Kampi ya Samaki, Ngodhe, Mbita, Mfangano, and Rusinga Island study areas (Tables 2, 3, and 6). Anaplasma bovis sequences (submitted GenBank accession KT266580) (Table 4) were detected in 18 pools of Amblyomma ticks. Hyalomma and Rhipicephalus ticks (Table 5) sampled from livestock of Logumugum and Ruko study areas (Tables 2 and 6).

The A. ovis sequences (submitted GenBank accession KT266585) (Table 4) were detected in H. truncatum (6 pools), H. rufipes (5 pools), and Rh. pulchellus (2 pools) ticks (Table 5) sampled from livestock in Logumugum, Kampi ya Samaki, and Kokwa Island (Tables 2 and 6). Rickettsia rhipicephali sequences (submitted GenBank accession KT266586) (Table 4) were amplified in Rh. evertsi evertsi (4 pools) and Rh. pulchellus (21 pools) ticks (Table 5) sampled from goats, sheep, cattle, and dogs at Kampi ya Samaki, Logumugum, and Ruko Conservancy of Baringo County (Tables 2 and 6). Rickettsia aeschlimannii sequences (submitted GenBank accession KT266590) (Table 4) were detected in Am. variegatum (14 pools), Am. variegatum (16 pools), Am. sparsum (3 pools), and Rh. evertsi evertsi (3 pools) (Table 5) parasitizing livestock in Kampi ya Samaki, Logumugum, and Ruko Conservancy of Baringo County (Tables 2 and 6). We also obtained three novel Rickettsia spp. sequences from Baringo County tick pools. The Rickettsia sp. BR62 (submitted GenBank accession KT266587) and BR33 (submitted GenBank accession KT266589) sequences (Table 4) were detected in Rh. pulchellus (12 pools) and Rh. evertsi evertsi (9 pools) ticks (Table 5) sampled from goats in Ruko Conservancy (Tables 2 and 6). Rickettsia sp. TICPA84 sequences (submitted GenBank accession KT266588) were detected in Am. gemma (six pools) from Logumugum and Rh. evertsi evertsi (three pools) from livestock and dogs (Tables 5 and 6) in Kokwa Island (Table 2).

Among apicomplexan hemoparasites, we detected B. caballi sequences (submitted GenBank accession KT266583) (Table 4).
TABLE 6 | Vertebrate hosts from which ticks with tick-borne pathogens (TBPs) were isolated in the two study regions.

| TBP | Cattle (%) | Goat (%) | Sheep (%) | Dog (%) | Tortoise | Monitor lizard (%) |
|-----|------------|----------|-----------|---------|----------|-------------------|
| **Baringo County** | | | | | | |
| N (vertebrate hosts) | 45 | 87 | 33 | 9 | 18 | 0 |
| Anaplasma bovis | 8 (17.8) | 6 (6.8) | 3 (9.1) | | | |
| A. ovis | 7 (15.6) | 5 (5.7) | 10 (30.3) | | | |
| A. platys | 17 (19.5) | | | 9 (100) | | |
| Ehrlichia ruminantium | 17 (37.8) | 14 (16.1) | 3 (9.1) | | 12 (66.7) | |
| E. canis | 15 (17.2) | | | 9 (100) | | |
| Ehrlichia sp. | 11 (24.4) | 6 (6.9) | 6 (18.2) | | 2 (13.3) | |
| Paracoccus sp. | 5 (11.1) | 8 (9.2) | 3 (9.1) | | 3 (16.7) | |
| Rickettsia africae | 17 (37.8) | 6 (6.8) | 11 (33.3) | | | |
| R. rhipicephali | 8 (17.7) | 4 (3.4) | 5 (15.2) | | 2 (22.2) | |
| R. aeschlimanni | 5 (11.1) | 2 (2.3) | 6 (18.2) | | | |
| Rickettsia sp. BR62 | | 5 (5.7) | | | | |
| Rickettsia sp. TICPA84 | 3 (6.7) | 2 (2.3) | 5 (15.2) | | 2 (22.2) | |
| Rickettsia sp. BR33 | | | | | | |
| Babesia caballi | 8 (17.7) | | | | | |
| Theileria sp. | 3 (6.7) | | | | | |
| Hepatozoon fitzsimonsi | | | | 14 (77.8) | | |

**Homa Bay County**

| N (vertebrate hosts) | 31 | 30 | 21 | 7 | 0 | 4 |
| A. ovis | 1 (3.2) | 1 (3.3) | 1 (4.8) | | | |
| A. platys | 4 (12.9) | 2 (6.6) | 3 (14.3) | 4 (57.1) | | |
| E. (Cowdria) ruminantium | 9 (29.0) | 4 (13.3) | | | | |
| E. canis | 1 (3.2) | | | 4 (57.1) | | 4 (100) |

Likely novel vertebrate host-TBP associations are highlighted in bold. N, number of vertebrate hosts.
Percentages are out of the number of specific vertebrate host species sampled.

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**FIGURE 3** | Maximum likelihood phylogenetic analysis of 16S rRNA gene fragments of *Anaplasma*, *Ehrlichia*, and *Paracoccus* sequences identified with related sequences. GenBank accession numbers, species identifications, isolates, and country of origin are indicated for each 16S rRNA gene sequence. Sequences from this study are in bold with tick and vertebrate host species associated with the study isolates indicated in brackets. Bootstrap values at the major nodes are of percentage agreement among 1,000 replicates. The branch length scale represents substitutions per site.
Fig Ure 4 | Maximum likelihood phylogenetic analysis of rpmE/16S rRNA gene fragments of *Rickettsia* sequences identified with related sequences. GenBank accession numbers, species identifications, isolates, and country of origin are indicated for each 16S rRNA gene sequence. Sequences from this study are in bold with tick and vertebrate host species associated with the study isolates indicated in brackets. Bootstrap values at the major nodes are of percentage agreement among 1,000 replicates. The branch length scale represents substitutions per site. The gaps indicated in the branches to the *Bacillus subtilis* outgroup represent 1.2 substitutions per site.

Fig Ure 5 | Maximum likelihood phylogenetic analysis of 18S rRNA gene fragments of *Apicomplexa* (*Hepatozoon, Babesia, and Theileria*) sequences identified with related sequences. GenBank accession numbers, species identifications, isolates, and country of origin are indicated for each 16S rRNA gene sequence. Sequences from this study are in bold with tick and vertebrate host species associated with the study isolates indicated in brackets. Bootstrap values at the major nodes are of percentage agreement among 1,000 replicates. The branch length scale represents substitutions per site.
in *Rh. pulchellus* (eight pools) ticks (*Table 5*) parasitizing cattle in Kampi ya Samaki, Baringo County (*Tables 2* and *6*). *Hepatozoon Fitzsimonsii* sequences (submitted GenBank accession KT266582) (*Table 4*) were amplified in *Am. sparsum* (nine pools) and *Am. falsomarameorum* (five pools) ticks (*Table 5*) sampled from free ranging tortoises in Baringo County (*Tables 2* and *6*). *Theileria* sp. sequences (submitted GenBank accession KT266584) (*Table 4*) were also amplified in *Rh. evertsi evertsi* (three pools) ticks (*Table 5*) parasitizing cattle at Logumgum in Baringo County (*Tables 2* and *6*).

**DISCUSSION**

We sampled 13 tick species that are potential vectors of ehrlichiosis, anaplasmosis, rickettsiosis, theileriosis, and babesiosis from livestock, poultry houses, and reptiles in domestic surroundings of Baringo and Homa Bay Counties. Most tick species were taken from multiple host taxa, except for *Hy. truncatum* that was restricted to poultry, *Ar. persicus* that was restricted to poultry, *Am. latum* that was restricted to monitor lizards, and *Am. nuttalli* and *Am. felsomarameorum* that were both restricted to tortoises. Overall, we identified 14 TBP s, most from multiple tick species and predominantly from Baringo County samples. These included novel *Ehrlichia* sp., *Rickettsia* sp., *Paracoccus* sp., and *Theileria* sp. sequences that warrant further investigations into their potential pathogenicity. Most interestingly, *Am. sparsum* ticks infected with *E. ruminantium*, the causative agent of heartwater ehrlichiosis, were sampled from both tortoises and cattle. The complex pathogen–tick–host relationships presented here are important to public health in mitigating TBP transmission and possible associated disease outbreaks in these foci, as well as other areas of Kenya, with wider geographical implications.

Heartwater ehrlichiosis is an important rickettsial disease of wildlife and livestock ruminants in SSA, with considerable economic impact (62). Unlike past studies, which found *E. ruminantium* to be specific to *Amblyomma* tick species (63, 64), we also isolated the pathogen from *Rh. evertsi evertsi* sampled from livestock in both study locations, though we cannot rule out that this may have come from livestock blood meals. *Amblyomma variegatum* Fabricius 1794 is the most common and widely distributed *Amblyomma* tick species of livestock in SSA and is a very important vector of the *E. ruminantium* in cattle (65). *Amblyomma gemma* Döñitz 1909 sampled from livestock in both study areas was once thought to be less important as a disease vector, but has since been linked with the transmission of a number of pathogens (65, 66). Laboratory studies have also shown that *Am. gemma* can transmit *E. ruminantium* from infected African buffalo to sheep (67). In this study, we detected *E. ruminantium* in 58.97 and 35.29% of *Am. variegatum* tick pools and in 68.42 and 66.67% of *Am. gemma* tick pools sampled in Baringo and Homa Bay Counties, respectively, suggesting very high infection rates. However, due to the pooled approach used to identify TBP s, these infection percentages do not provide a true estimate of infection rates.

Most importantly, *E. ruminantium* was also detected in *Am. sparsum* samples from both cattle and tortoises in Baringo as well as in *Am. felsomarameorum* and *Am. nuttalli* found exclusively on tortoises. The occurrence of *E. ruminantium* has previously been reported among *Am. sparsum* that were sampled from tortoises imported into the United States from Zambia (14). *Amblyomma nutalli* and *Am. felsomarameorum* have both been associated with tortoises before (68, 69), but no report that we are aware of has implicated them with harboring or transmitting of agents of heartwater ehrlichiosis. Our findings suggest that *E. ruminantium* may be potentially transmitted between cattle and tortoises by *Am. sparsum* ticks and within tortoise populations by *Am. nutalli* and *Am. felsomarameorum* ticks. Similarly, we found *E. canis*, the causative agent of canine ehrlichiosis, in *Am. latum* ticks taken from monitor lizards in Homa Bay County. These pathogen–tick–host associations suggest complex transmission dynamics in the epidemiology of heartwater in Baringo County and canine ehrlichiosis in Homa Bay County that potentially involve reptilian reservoir hosts that are rarely considered in epidemiological studies of these pathogens.

From domestic ruminants and dogs, *Rh. pravus* Döñitz 1910 was the most frequent tick parasite sampled in Baringo and Homa Bay Counties, followed by *Rh. evertsi evertsi* and *Rh. pulchellus*. Each of these three tick species were found parasitizing dogs, with pools infected with canine anaplasmosis (*A. platys*) and ehrlichiosis (*E. canis*), the latter only in Baringo. *Rhipicephalus pravus* is considered the most common tick species among domestic and wildlife animals in Kenya (70–72), but has rarely been implicated in pathogen transmission. These findings potentially incriminate *Rh. pravus* in the transmission of both *A. platys* and *E. canis*. Further studies to determine its vector competence will be critical to understanding its role in TBP transmission.

We detected *A. bovis* in multiple species of each of the tick genera (*Rhipicephalus*, *Amblyomma*, and *Hyalomma*) parasitizing livestock in Baringo County, but none in Homa Bay. The highest rates of *A. bovis* detection occurred in *Rh. praetextatus* Gerstäcker 1873, a three-host tick species that was sampled from cattle and goats in Baringo County. *Rhipicephalus praetextatus* from Ngorongoro crater of Tanzania has been implicated in the transmission of *A. marginale*, the principal agent of bovine anaplasmosis (73). Similarly, we found *A. ovis* in both *Rhipicephalus* and *Amblyomma* ticks in Baringo, but only in *Rh. appendiculatus* sampled from livestock in Homa Bay County. In contrast, in Baringo County, where no *Rh. appendiculatus* were sampled, *A. ovis* occurred most frequently in *Am. variegatum*. These *Anaplasma* infections are rarely associated with clinical symptoms in livestock, but may affect livestock health synergistically during coinfections with other livestock disease agents (74, 75) in Baringo County.

Sequences of spotted fever group (SFG) *Rickettsiae* (*R. africae*, *R. aethiopienensis*, and *R. rhipicephali*) and three novel *Rickettsia* spp. were identified in tick (*Rh. evertsi evertsi* and *Rh. pulchellus*) samples from livestock and dogs only in Baringo County; no *Rickettsia* were identified in Homa Bay County, despite the fact that in neighboring Siaya County, a high prevalence of *R. africae*, the etiological agent of African tick bite fever, was previously identified among *Am. variegatum* parasitizing domestic ruminants (27). In this study, *Rickettsia africae* was particularly prevalent in *Am. gemma* (73.68%) and *Am. variegatum* (41.02%) tick pools, but was also found in *Rh. evertsi evertsi* (4.67%) and
Rh. pulchellus (5.17%) tick pools sampled from livestock. We detected R. aeschlimannii predominantly in Hyalomma tick pools (Hy. truncatum and Hy. marginatum rufipes) (55.0%) and incidentally in Rh. pulchellus tick pools (3.44%). In Hy. truncatum tick pools that were sampled from cattle, sheep, and goats, we detected both A. bovis and R. aeschlimannii. Separate pools of Hy. marginatum rufipes parasitizing livestock were also positive for A. bovis and R. aeschlimannii. *Rickettsia aeschlimannii* has previously been isolated from Hy. truncatum parasitizing camels in the Kano area of Nigeria (76). Though SFG rickettsiosis caused by this TBP has been rarely reported in SSA, it has been reported in humans in Algeria (77) and has also been found in *Am. variegatum* ticks in western Kenya (27). The likely role of *Hyalomma* ticks as reservoirs for *R. aeschlimannii* (78) makes these ticks of particular importance in the epidemiology of SFG rickettsiosis in East Africa and warrants further attention. *Rickettsia rhipechphalus*, as well two of the novel *Rickettsia* spp. (BR62, BR33), were confined to rhipicephaline (*Rh. pulchellus* and *Rh. eversti eversti*) ticks. A third *Rickettsia* sp. (TICPA84) was found in *Am. gemma* (31.57%) and *Rh. eversti eversti* (4.76%) tick pools from livestock and dogs. While these findings confirm higher prevalences of *Rickettsia* spp. in specific *Amblyomma* and *Hyalomma* species, their occurrence in *Rhipicephalus* ticks, perhaps opportunistic, should be considered in the transmission ecology of these TBPs.

From livestock parasitizing ticks in Baringo, we only detected one novel *Theileria* sp. sequence in *Rh. eversti eversti* ticks and *B. caballi* in *Rh. pulchellus* ticks from cattle, despite the fact that diverse *Theileria* (79) and *Babesia* (6) species antigens have been found in livestock surveillance studies in different regions in Kenya. Nonetheless, 29 (0.7% of sampled ticks) *Rh. appendiculatus* Neumann 1901, a vector of *T. parva* (80), were sampled from livestock of Homa Bay County, but not in Baringo. This was contrary to a previous study done over two decades previously, which found *Rh. appendiculatus* to be highly prevalent among Zebu cattle grazing along the shores of Lake Victoria in Rusinga Island (25). This change may be an indication of tick control efforts, most likely with acaricides, or difference in sampling period. Although we detected *A. ovis* in three *Rh. appendiculatus* tick pools, we did not find *T. parva*, indicating a possible absence of *T. parva* or extremely low prevalence within our study sites.

Additionally, we found both *H. Fitzsimonsi* and *Paracoccus* sp. bacteria in *Am. falsomarmoreum* and *Am. sparsum* ticks from tortoises in Baringo. In South Africa, tortoises have recently been documented to harbor concurrent parasitic infection with *H. Fitzsimonsi* (81). *Paracoccus* sp. bacteria, also identified in pools of *Am. variegatum* and *Am. gemma* ticks sampled from livestock in Baringo County, was first reported in a population of *Am. cajennense* from South America in 2012, but it is still unknown if *Paracoccus* infection in ticks is a group of pathogenic rhodobacteraceae or simply plays a role in tick physiology (82). Nonetheless, our findings demonstrate that primers targeting specific *Ehrlichia* 16S rRNA gene fragment could also be used for detection of *Paracoccus* species.

While only one to three TBPs were identified in most tick species, none were identified in *Ar. persicus* sampled from poultry houses, and more than five TBPs were identified in *Rh. eversti eversti*, *Rh. Pulchellus*, and *Am. gemma* tick pools, all of which have been widely implicated in TBP transmission (65, 66, 83–85). Although all 28 pools of *Ar. persicus* were negative for TBPs, its large population in poultry houses and frequent blood feeding behavior has been linked with nuisance, severe anemia, paralysis, and toxicosis in poultry (86). Furthermore, more TBPs were isolated from Baringo County ticks, suggesting more complex and higher transmission rates in the region, where higher numbers of livestock per household (>20) are kept, compared to the fewer (<5) livestock that are kept by Homa Bay County households.

This study reports the presence, possible circulation, and putative transmission sources of TBPs that are etiological agents of ehrlichiosis, anaplasmosis and rickettsiosis, and hemoparasites of importance to livestock health in the study areas. Among these, we found surprisingly high infection rates of *E. ruminantium* and *E. canis*, agents of livestock and canine ehrlichiosis, respectively, in ticks sampled from reptilian hosts, suggesting their likely role as reservoir species in the epidemiology of these TBPs. Proper and rapid diagnoses that include analyses of these TBPs in livestock and their proximal wildlife species are important in mitigating disease burden and possible outbreaks in these areas and the rest of SSA.

**ETHICS STATEMENT**

This study was carried out in accordance with the recommendations of the Kenya Medical Research Institute (KEMRI), Wildlife Service (KWS), Directorate of Veterinary Services and Ministry of Health. The protocol was approved by the KEMRI ethics review committee (Approval Ref: non-SSC Protocol #310) and the KWS Biodiversity Research and Monitoring committee (Permit Ref: KWS/BRM/5001).

**AUTHOR CONTRIBUTIONS**

DO, DM, and JV designed the study. DO, EK, and YA conducted the fieldwork. DO, MM, and DOO conducted the laboratory work. DO and JV conducted the analysis and drafted the manuscript; BM, MM, and DM contributed to the interpretation of the data. All the authors contributed to the manuscript editing and approved the final manuscript.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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