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Hellen Ayilo Akengo

h.akengo@cgiar.org

International Livestock Research Institute

James Mutisya

Kenya Medical Research Institute

Francis Musili Mulwa

Kenya Medical Research Institute

Betty Chelangat

Kenya Medical Research Institute

Hellen Koka

Kenya Medical Research Institute

Edith Chepkorir

Kenya Medical Research Institute

Solomon Langat

Kenya Medical Research Institute

Joel Lutomiah

Jomo Kenyatta University of Agriculture and Technology

Eugine Mukhaye

Kenya Medical Research Institute

Richard Nyamota

Kenya Medical Research Institute

James Miser Akoko

International Livestock Research Institute

Dana Mitzel

United States Department of Agriculture

William Wilson

United States Department of Agriculture

Steven Ger Nyanjom

United States Department of Agriculture

Bernard Bett

International Livestock Research Institute

Hussein Molu Abkallo

International Livestock Research Institute

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Arbovirus surveillance and bloodmeal analysis in mosquito species from Northern Kenya

Authors

Hellen Ayilo Akengo[1,2,*], James Mutisya[3], Francis Musili Mulwa[3], Betty Chelangat[3], Hellen Koka[3], Edith Chepkorir[3], Solomon Langat[3], Joel Lutomiah[3], Eugine Mukhaye[2], Richard Nyamota[5], James M. Akoko[1], Dana N. Mitzel[4], William C. Wilson[4], Steven G. Nyanjom[2], Bernard Bett[1], Hussein M. Abkallo[1,*]

Authors affiliations

1 Health Program, International Livestock Research Institute (ILRI), P.O. Box 30709, Nairobi 00100, Kenya

2 Jomo Kenyatta University of Agriculture and Technology (JKUAT), P.O. Box 62000, Nairobi, 00200, Kenya

3 Kenya Medical Research Institute (KEMRI), P.O. Box 54840, Nairobi, 00200, Kenya.

4 United States Department of Agriculture, Foreign Arthropod-Borne Animal Diseases Research Unit, Agricultural Research Service, Manhattan, Kansas, USA.

5 Kenya Medical Research Institute (KEMRI)-Wellcome Trust Research Programme (KWTRP), P.O Box 230, Kilifi, 80108, Kenya

* Corresponding authors: H.Akengo@cgiar.org and H.Abkallo@cgiar.org

Abstract

Zoonotic viruses transmitted by mosquitoes pose serious health and economic risks globally, and can cause febrile illness, hemorrhagic fever, or even death in animals and humans. As globalization and climate variability alter mosquito bionomics, it is important to conduct periodic surveillance to characterize the prevalent mosquito species, types of viruses they carry and their feeding patterns. Northern Kenya has a history of hemorrhagic fever outbreaks and periodically experiences extreme climate events, yet it is rarely targeted for mosquito and arbovirus surveillance.

To investigate the diversity of arboviruses that were prevalent in the area, we conducted a longitudinal survey and collected mosquitoes from Kinna and Gafarsa wards in Isiolo County. The mosquitoes were collected using CO_2-baited CDC light traps, morphologically identified, grouped into 1,170 pools, then screened for arboviruses using multiplex PCR combined with high-resolution melting (PCR-HRM) analysis. For blood-engorged mosquitoes, blood meal analysis was performed using *cytochrome b* and 16S rRNA vertebrate mitochondrial gene markers.

Among the most abundant mosquito species morphologically identified were *Culex univittatus* (10,949), *Culex annurlioris* (2,516), *Anopheles gambiae* (2,272), and *Aedes tricholabis* (485). Of the 1,170 mosquito pools screened, PCR-HRM detected multiple arboviruses across diverse mosquito species, with infection rates estimated using MLE to account for pooled sampling. Bunyamwera virus was the most abundant accruing in 27 mosquito species, with the highest infection rates in *Anopheles funestus* (MLE= 134.9). RVFV was the least detected with notable infection rates in *Anopheles squamous* (MLE= 44.6). Positive pools were subsequently confirmed

by conventional PCR and Sanger sequencing. Blood meal analysis identified mosquito feeding preference across wildlife, livestock, and humans, reflecting the complex vector-host interactions that facilitate arbovirus transmission cycles.

This study demonstrated that mosquitoes in the area were infected with a wide range of zoonotic arboviruses. Periodic mosquito sampling and screening should therefore be conducted in the region under the existing One Health surveillance systems to generate data that can be used to track the evolution of vectorial capacity of the local mosquito populations with climate change.

Keywords: Multiplex PCR-HRM analysis, arboviruses, blood meal source identification.

1. BACKGROUND

Arboviral diseases transmitted by mosquitoes are an emerging public and animal health concern. Vectors like *Aedes*, *Culex*, and *Anopheles* mosquito species transmit the viruses during a blood meal (1). *Aedes* and *Culex* mosquito species have been implicated in the transmission of chikungunya virus (CHIKV), dengue virus (DENV), and Zika virus (2), which are re-emerging globally (3) driven by landscape anthropization and climate change which alter the abundance and diversity of the vectors (4) . The sub-Saharan Africa remains a hotspot for arbovirus infections like CHIKV and DENV (5) because of increased interactions between human, animal, and mosquito vectors (6). Sindbis, Bunyamwera, and Thogoto virus are some of the arboviruses that have been detected in mosquito pools from Kenya (7). Isiolo County, which is located in the northern part of Kenya, has experienced repeated outbreaks of hemorrhagic fever viruses (HFV) such as RVFV (8,9) and yellow fever virus (10), which mainly affect animals and humans (11). The County has a diverse ecosystem which presents ideal conditions for arbovirus transmission, with ecological

features like wildlife, seasonal water bodies, livestock, and pastoralism, promoting vector proliferation and favoring vector-host interactions (12).

Mosquito surveillance is key in early detection of virus circulation before outbreaks can occur. It involves routine collection, identification, and laboratory screening of mosquitoes for the presence of arboviruses (13). It includes techniques like molecular surveillance to detect viral RNA (14), mapping at risk areas to identify drivers of transmission (15), and genomic epidemiology to monitor viral evolution (16). Mosquito surveillance can be strengthened through an integrated One Health approach that includes blood meal analysis to identify preferred hosts (17), and collecting environmental and climate data to understand mosquito habitats and their seasonal distribution (18). Identifying mosquito blood meal sources is also important in mapping the risk of arbovirus transmission dynamics, potential arbovirus reservoirs, and host feeding preference (19). Mitochondrial cytochrome oxidase subunit I (COI) gene sequencing has been previously used to identify mosquito blood meal sources, but it is expensive when screening many samples (20). An alternative is to use high-resolution melting (HRM) of Polymerase chain reaction (PCR) products of mitochondrial *cytochrome b* and 16S rRNA genes as it is cheaper and has been successfully used to detect vertebrate bloodmeal sources in mosquitoes (21).

Molecular diagnostic tools like PCR and metagenomics are preferred in the diagnosis of arboviruses because they are highly sensitive and specific hence used for the detection of emerging and circulating pathogens. Even though metagenomics is powerful, it is costly, requires specialized expertise, and relies on good lab infrastructure, making it unsuitable for routine diagnosis and fast outbreak response (22). Real Time PCR, which is also known as quantitative PCR (qPCR) has also been used to detect arboviruses due to its accuracy and faster turnaround time (23). Lately,

multiplex qPCR followed by HRM analysis has been effective in detecting and differentiating arbovirus genera and species from mosquito pools. This method enables detection of co-infections and virus diversity in a single reaction (7,24).

This study employed the multiplex PCR-HRM because of its ability to detect and differentiate multiple targets in a single reaction tube (25) with high specificity, sensitivity, and reproducibility (26), making it a reliable tool for detecting both individual arboviruses and coinfections in mosquito pools. A minimal sample volume of 1 µl was used for arbovirus detection in mosquito pools without compromising the required sensitivity. Kinna and Gafarsa were selected because of the presence of key risk factors like wildlife-livestock-human interface, seasonal mosquito breeding habitats, and the recent outbreaks of RVFV (27), and yellow fever virus (28).

2. METHODS

2.1 Sampling area

Mosquitoes were collected in Kinna and Gafarsa wards of Isiolo County (Figure 1), a region prone to hemorrhagic fever virus (HFV) outbreaks, including yellow fever (29), and RVFV (30), among other historically recorded outbreaks (10,31). The two wards were also selected due to their proximity to wildlife reserves, and presence of dense forests that support mosquito breeding. Kinna borders national parks and game reserves that harbour wildlife species like monkeys, ruminants, rodents, and birds, which may serve as sylvatic reservoirs. Gafarsa lies along the Ewaso Nyiro river which has riverine forests, ideal for mosquito breeding (32). Both areas are inhabited by the pastoralists who rear camel, cattle, sheep, and goats, (33) which serve as hosts for some of the

zoonotic viruses. Isiolo County has got bimodal rainfall (February to May, October to December) (34) and high humidity of 75.3% (35), which support mosquito proliferation.

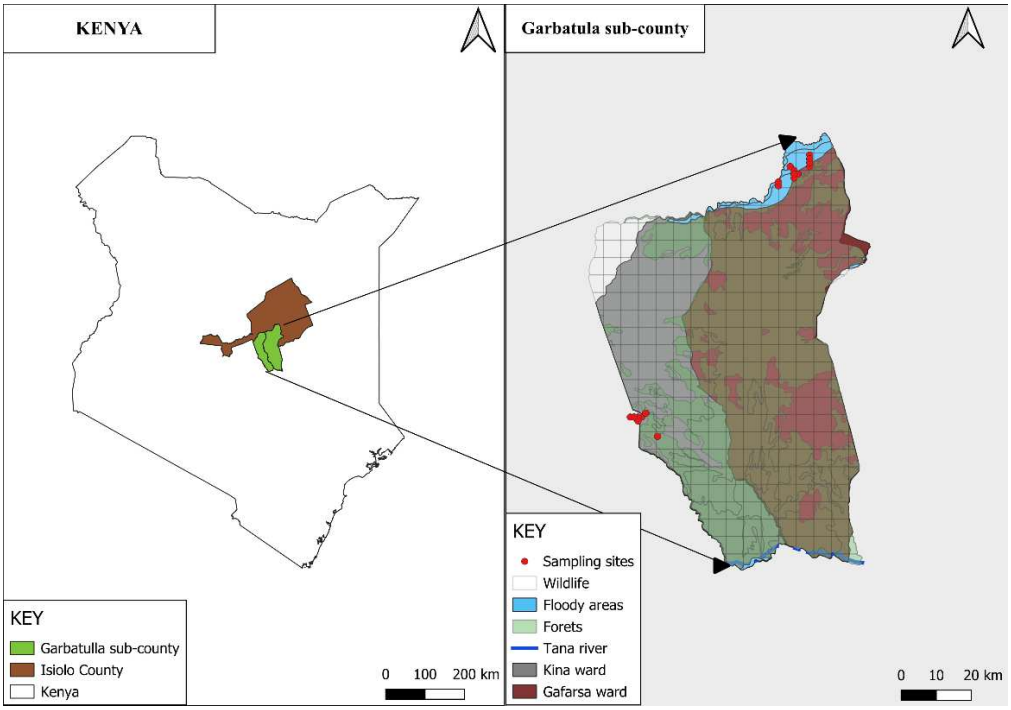


Figure 1: A map of Isiolo County showing mosquito sampling sites in Kinna and Gaffarsa wards within Garbatulla Sub-County. This map was created in QGIS v3.34, using shapefiles obtained from GADM (GADM, 2024) and Humanitarian Data Exchange (HDX, 2024).

2.2 Mosquito sampling

This was a longitudinal study that involved sampling of mosquitoes in selected months of 2023 (January, March, April, and June to December) and 2024 (January). The sites selected for sampling in Kinna were Jamia Mosque, Kinna Meru, Malkagiro, and Shambole, and in Gafarsa were Gafarsa Town, Malka Mansa, Malkagiro, Muchuro, Muchuro Manyatta, and Muchuro grazing area (Figure 1).

Mosquitoes were trapped using carbon dioxide-baited CDC light traps in the evening at 1800 hrs and collected the following morning at 0600 hrs. Triethylamine was used to anesthetize all the captured insects for 10 minutes (8). Mosquitoes were then separated from other insects and transferred into 15-ml falcon tubes, then transported in liquid nitrogen to KEMRI headquarters, in Nairobi, Kenya. They were morphologically identified to species level using standard taxonomic keys following the Edwards's key book (36) then pooled by collection area, site, date, species, and sex. Each pool contained up to 25 mosquitoes sharing similarities in grouping characteristics and were stored in 1.5-ml Eppendorf tubes at −80°C. A total 1,170 pools were obtained: 316 from Kinna and 854 from Gafarsa, totaling to 21,535 mosquitoes. Blood-fed mosquitoes were separated and stored individually in 1.5 ml Eppendorf tubes at −20°C.

2.3 Sample processing

2.3.1 Mosquito homogenization

Mosquito pools were homogenized using a mini bead beater (BioSpec Products Inc, Bartlesville, OK 74005 USA) with the aid of a copper bead (BB-caliber airgun shot). To each pool, 1 ml of homogenization media (Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS), Penicillin-Streptomycin antibiotics), and a 4.5-mm copper bead were added to facilitate mosquito tissue disruption. An OMNI Bead Ruptor 96+ (Revvity, Inc) was used for homogenization at 30Hz for 5 min to ensure thorough lysis. The mosquito homogenates were centrifuged at 4°C for 10 min at 16,115 ×g. A volume of 700 µl of the resultant supernatant was transferred into a sterile cryovial, and the remaining pellet retained in the original tube. Both the supernatant and pellet fractions were stored at −80°C until further analysis.

Blood-fed mosquitoes were processed individually. The engorged abdomens were dissected using sterile scalpels and homogenized in 600 µl 1× phosphate buffer saline (PBS) with a 5-mm copper bead using a TissueLyser II (Qiagen) at a frequency of 30 Hz for 6 min. The homogenates were centrifuged at 25°C for 10 min at 16,115 ×g. Both the supernatant and pellet fractions were stored at −20°C awaiting further analysis. The supernatants were used for nucleic acid extraction while the pellets were stored as backup samples for possible future extractions.

2.3.2 Nucleic acid extraction

RNA was extracted from the supernatant of mosquito pool homogenates, including those from individual blood-fed mosquitoes, using the TANBead Nucleic Acid Extraction Kit following the manufacturer's instructions (TAN Bead, OptiPure Viral Auto Plate, Germany). Extraction was performed on a Maelstrom^{TM} 9600 automated instrument (Taiwan Advanced Nanotech). Genomic DNA was extracted from the supernatant of individually homogenized blood-fed mosquitoes using the DNeasy Blood and Tissue Kit (Qiagen, Hannover, Germany) according to the manufacturer's instructions. Both RNA and DNA were eluted in 60 µl; RNA was stored at −80°C while DNA at −20°C.

2.3.3 Molecular identification of arboviruses

Molecular screening for arboviruses was done on 1170 mosquito pools and 61 individual blood-fed mosquitoes, reflecting all the blood-fed mosquitoes captured. Complementary DNA (cDNA) was synthesized from extracted RNA using the LunaScript® Rt SuperMix Kit (New England BioLabs, Ipswich, MA) according to the manufacturer's instructions. Published primers were used at a final equimolar concentration of 500 nM in a multiplex PCR reaction to screen for Flaviviruses, Alphaviruses, Phleboviruses, and Bunyamwera group viruses Table S1. The reaction

was set up as previously described (7). We used a HRM-capable thermocycler, the 48-well rotor model of Mic qPCR cycler (Biomolecular systems, Australia). The multiplex PCR cycling conditions are provided in Table S2. Viral amplicons were subjected to HRM analysis as previously described (7). Standard controls used were RVFV, Bunyamwera, Ngari, chikungunya, *Alphavirus*, West Nile, and dengue virus, while non-template control was nuclease free water. Viral amplicons were identified by comparing sample melt curves and temperatures to known standard controls and clustered by melting profiles to confirm identity. The HRM normalization range was set between 76°C and 88°C based on the controls melting profiles. Singleplex PCR-HRM was also used to confirm positive results from the multiplex PCR-HRM.

PCR-HRM positive samples were further validated by conventional PCR using genus-specific primers for Bunyamwera group, *Alphavirus*, *Phlebovirus*, and *Flavivirus*, as previously described (37,38). Conventional PCRs were performed in 25-µl reaction volumes containing 2 µl of cDNA, 12.5 µl of One*Taq*® Quick-Load® 2× Master Mix with Standard Buffer (New England Biolabs), and 10 µM of each primer (Table S3). Amplicons were electrophoresed on 2% agarose gels to confirm product size. Amplicons were purified using the PureLink™ Quick PCR Purification Kit (Invitrogen, Thermo Fisher Scientific) and shipped to Macrogen Europe (Netherlands) for Sanger sequencing.

2.3.4 Blood-meal identification

Blood meal sources were identified from blood-fed mosquitoes using PCR-HRM targeting the *cyt b* and 16S rRNA genes as previously described (24). DNA from mosquito legs was used as the negative control, while DNA from humans, domestic animals (sheep, cattle, goat, camel, pig, and chicken), and wildlife species (rodent, giraffe, eland, impala, topi, zebra, warthog, waterbuck, and

179 buffalo) were used as positive controls. PCR-HRM was carried out in 10 µl reactions containing
180 5 µM of each primer, 5 µl 2× MyTaq^{TM} HS master mix (Meridian Bioscience), 50 µM Syto-9 dye
181 (Thermo Fisher Scientific) and 1µl of DNA template. The thermal cycling conditions for *cyt b*
182 involved initial denaturation at 95°C for 1 min; 35 cycles of 95°C for 15 sec, 58°C for 20 sec, 72°C
183 for 10 sec followed by a final extension step at 72°C for 5 min. Cycling conditions for 16S rRNA
184 were similar, with an annealing temperature of 56°C for 20 sec. After PCR, HRM was done by
185 melting the amplicons from 65°C to 90°C in 0.1°C increments every 2 sec using Mic qPCR
186 Thermocycler (Bio Molecular Systems). Changes in fluorescence against temperature (dF/dT)
187 were analyzed using Mic PCR software v2.12.7 (Bio Molecular Systems) with normalization range
188 between 78.0°C and 88.0°C. Standard controls were used to validate the protocol for PCR-HRM
189 for sensitivity and accuracy. Mosquito bloodmeal sources were determined by comparing sample
190 melt curves and temperatures to those of reference species. Representative 16S rRNA results were
191 confirmed with *cyt b* data to identify the vertebrate host.

192 **2.4 Data analysis**

193 PCR-HRM results were analyzed in Mic PCR software v2.12.7, with clustering based on the curve
194 shape and Tm. Positive samples confirmed by conventional PCR were sequenced. The resulting
195 Sanger sequences were visually inspected, trimmed and edited in BioEdit v7.2 (Hall, 1999), and
196 consensus sequences generated from forward and reverse reads for each sample. Consensus
197 sequences were queried against NCBI GenBank database (39) using default Blast parameters to
198 infer identity based on hits with e-value $<10^{-40}$, the cut-off ensured reliability and reduced chances
199 of false matches. Sample sequences were aligned with matching GenBank reference sequences
200 using Mega 11 software (40). Phylogenetic relationships were inferred using the Maximum

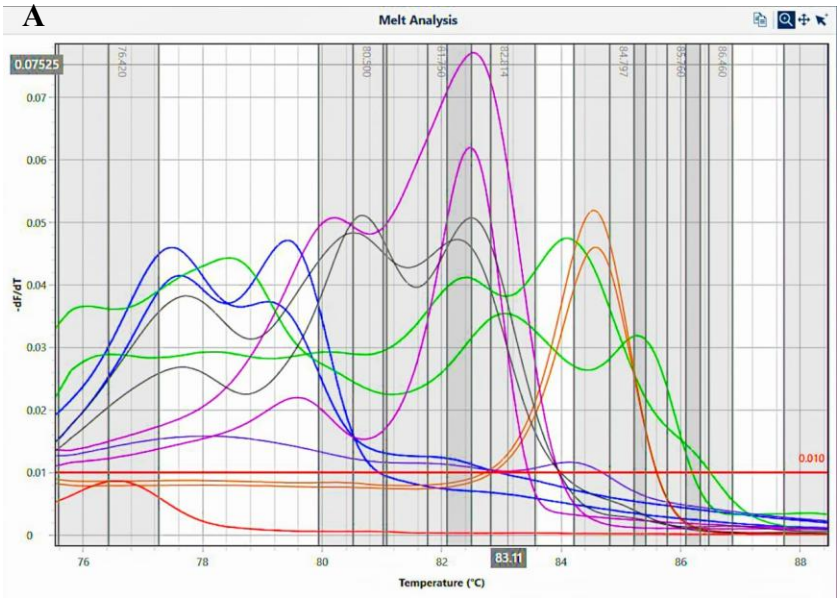
Likelihood method with the Kimura 2-parameter model for RVFV and Tamura-Nei 1993 model with Gamma distribution for flaviviruses. Nearest-Neighbor-Interchange (NNI) heuristic, and 1,000 bootstrap replicates were applied and the resulting trees visualized using Figtree v1.4.4 (41).

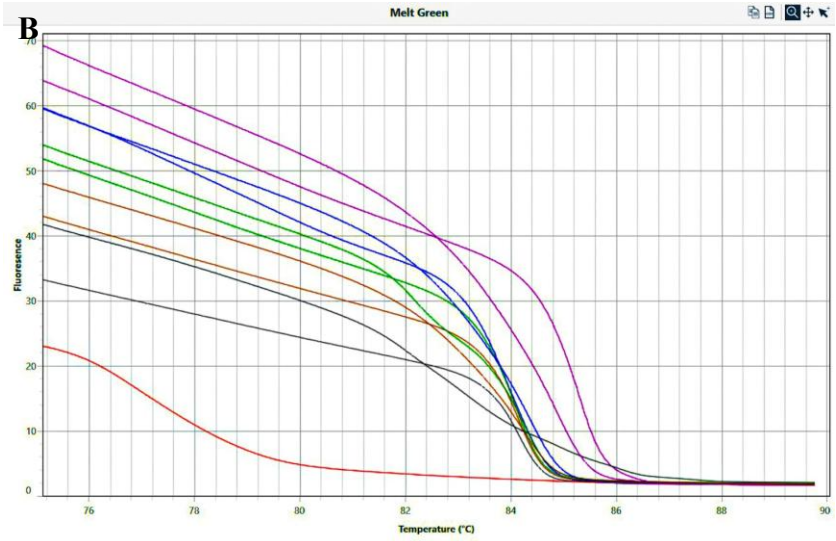
All datasets were organized, cleaned and compiled in Microsoft Excel where the counts of mosquito pools, and proportions of positive pools were computed. Relevant datasets were then exported as comma-separated values (CSV) files for further analysis. Inferential statistics and data visualization which involved generation of graphs illustrating arbovirus detection and blood meal host distribution, were generated using R statistical software v4.4.1 (R Core Team, 2024).

3. RESULTS

3.1 Molecular detection of Viruses.

The multiplex PCR-HRM assay detected and differentiated arboviruses from 1,170 mosquito homogenate pools and 61 blood-fed mosquitoes. Arbovirus identity was inferred by comparing sample HRM profiles and melt curves to reference controls, considering matches within ±1°C in melting temperature (Tm) as the same species (Figure S4). Distinct HRM profiles were observed for different virus species (Figure 2). Pools containing multiple virus species (Bunyamwera and CHIKV; Sindbis and Bagaza; Bunyamwera and RVFV; Dengue and Ngari; RVFV and CHIKV) were also identified based on their mixed melting profiles (Figure 3).





-Chikungunya -Bunyamwera -Ngari -Flavivirus -RVFV -Negative control

Figure 2: Unique profiles of the multiplex PCR-HRM arbovirus screen. The distinct melt rates of the different arboviruses are plotted in (A) as change in fluorescence with increasing temperature (dF/dT), and their normalized HRM profiles represented as percent fluorescence are plotted in (B).

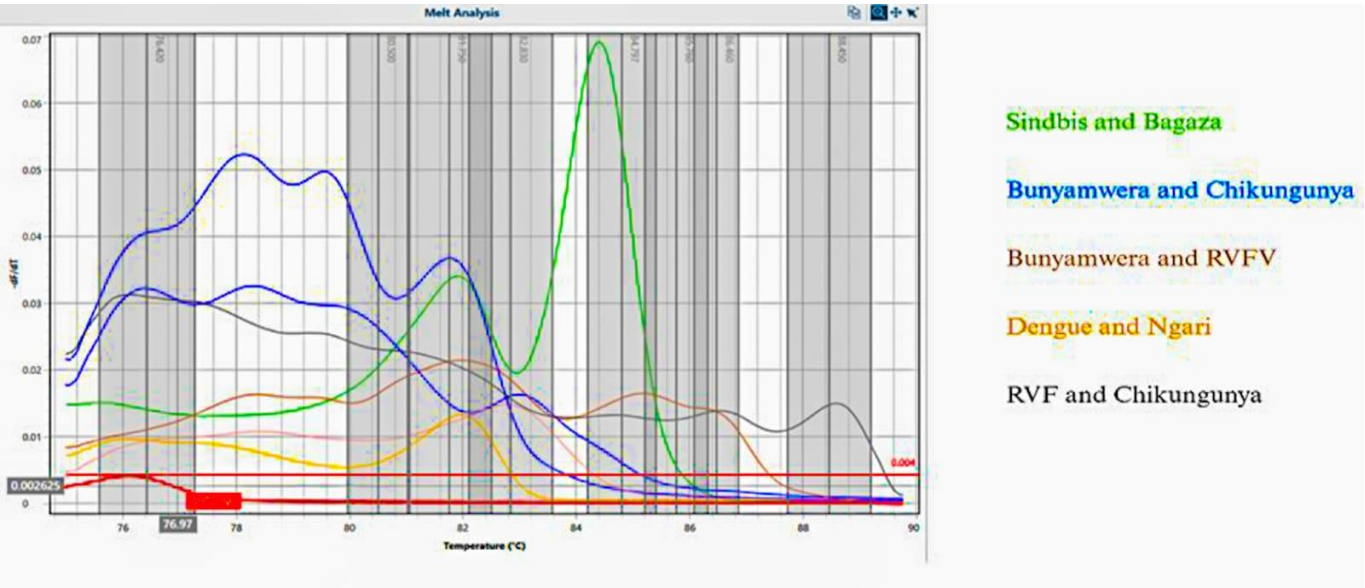
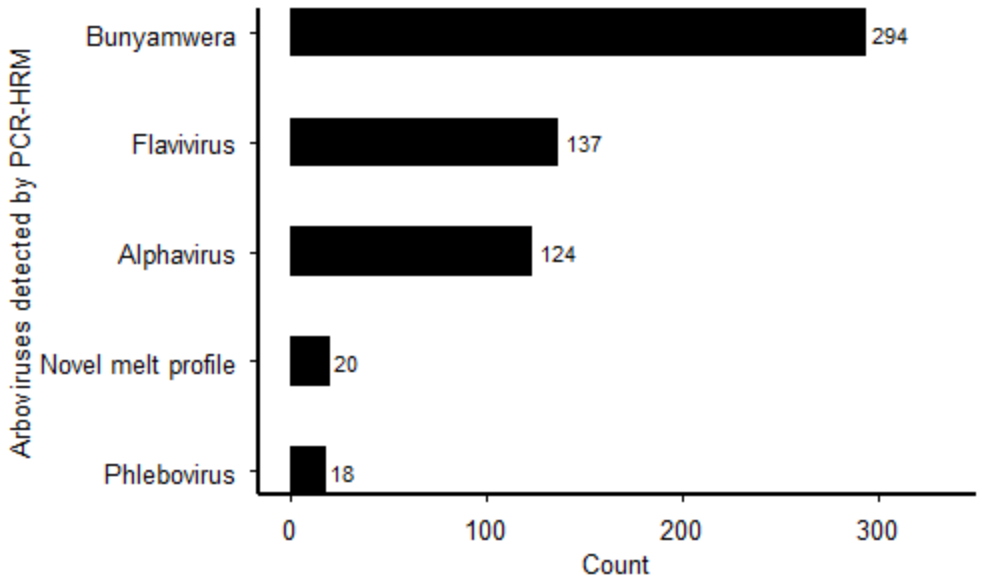


Figure 3: Melt curves showing mixed infections of arbovirus.

Out of the 1,170 mosquito pools screened for arboviruses, 573 (49.0%) were positive for known arboviruses, and 20 (1.7%) produced novel melt profiles. Among the positive mosquito pools detected, Bunyamwera virus was the most frequent (in 294 pools), followed by flaviviruses (137 pools), alphaviruses (124 pools), phleboviruses (18 pools), and 20 pools showed novel melt profiles not matching any reference viruses (Figure 4).



231

232 **Figure 4:** Histogram showing the distribution of positive arboviruses from the 573 pools screened
233 using multiplex PCR-HRM.

234 In PCR-HRM screening, the infection rates were estimated using the maximum likelihood estimate
235 (MLE) which considers the varying pool sizes and the number of positive pools, to estimate virus
236 prevalence in mosquito populations. The MLE value represents the most probable infection rate
237 per 1,000 mosquitoes from the pooled samples. Bunyamwera virus was the most abundant virus
238 detected, occurring in 27 different mosquito species. It was frequently detected in 29 *Culex*
239 *univittatus* (MLE = 13.1), 26 *Anopheles funestus* (MLE= 134.9), 25 *Anopheles gambiae* (MLE =
240 44.2), and 13 *Aedes tricholabis* (MLE = 76.3). Ngari virus was detected in 19 different mosquito
241 species with high occurrence in 60 *Culex univittatus* (MLE = 28.1). The CHIKV was detected in
242 20 different mosquito species, mostly abundant in 27 *Anopheles gambiae* (MLE = 55.7), and 25
243 *Culex univittatus* (MLE = 11.2). Rift Valley fever virus was detected in 11 pools, with detections
244 in *Culex pipiens* (MLE = 1.9), *Aedes mcintoshi* (MLE = 19.1), *Aedes sudanensis* (MLE = 23.6),

Anopheles squamosus (MLE = 44.6), *Anopheles gambiae* (MLE = 5.5), *Aedes aegypti* (MLE = 4.5), *Aedes mettalicus* (MLE = 19.1), *Aedes tricholabis* (MLE = 4.9), *Culex annurlioris* (MLE = 5.2), and *Anopheles species* (MLE = 29.3). Flaviviruses were identified in highest numbers in 58 *Culex univittatus* (MLE = 27.1), and 14 *Culex pipiens* (MLE = 28.3). The distribution of arboviruses in the different mosquito species, as detected by PCR-HRM screening prior to conventional PCR and sequence confirmation was per Table S5.

Conventional PCR performed to confirm positive PCR-HRM results from the 593 pools revealed different amplicon sizes i.e. 250 bp for Bunyamwera viruses using genus-specific primers, 310 bp for alphaviruses using *Alphavirus* semi-nested primers, 668 bp for Rift Valley fever virus (a Phlebovirus) using RVF nested primers, and 1,100 bp for flaviviruses using *Flavivirus* nested primers. Confirmation of arboviruses by conventional PCR showed that Bunyamwera viruses (130 pools) were dominant, flaviviruses (23 pools) and Rift Valley fever virus (11 pools) were less common, and alphaviruses were rare, detected in only three pools (Figure 5).

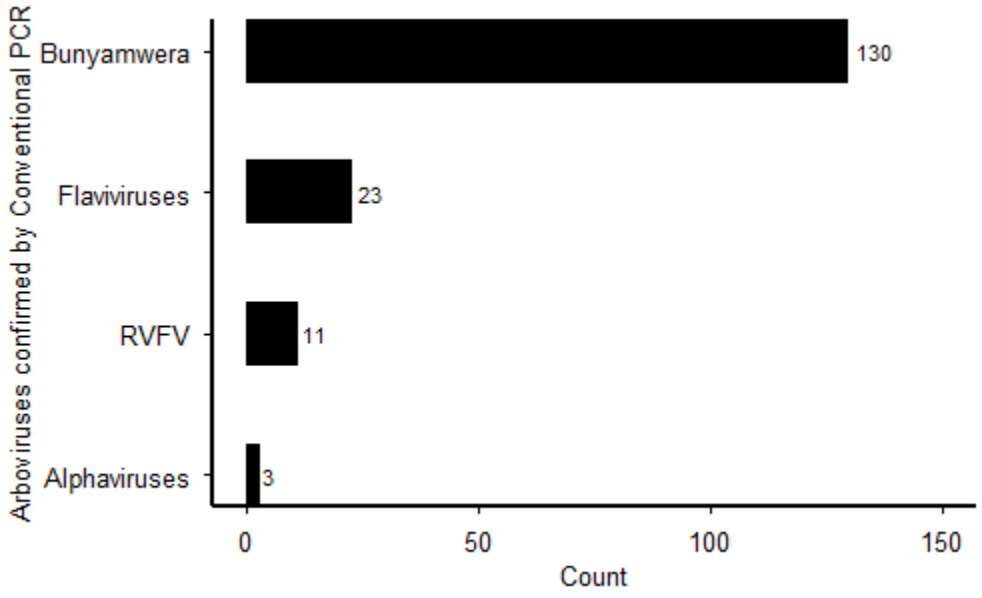


Figure 5: The numbers of confirmed arboviruses in conventional PCR using genus-specific primers. The confirmed arboviruses were initially positive in the multiplex PCR-HRM.

Sanger sequencing confirmed 14 pools as Bunyamwera, eight pools as *Orthobunyavirus* (unresolved species), 19 pools as Ngari virus, 11 pools as RVFV, 6 pools as West Nile, 1 pool as Usutu virus, and two pools as Sindbis virus. The detection included insect-specific flavivirus and a coinfection of Sindbis and Bagaza virus (Table 1). The 20 mosquito pools that produced novel melt profiles were re-screened by conventional PCR using all the genus specific primers, but no bands were generated.

Table 1: Summary of arboviruses confirmed by Sanger sequencing and their closest GenBank matches.

Arbovirus Group	Virus Name	Related GenBank sequences	Matching Pools (n/N)
Orthobunyavirus	Bunyamwera virus	MZ773507.1, NC_001927.1, KC512397.1	14/73
Orthobunyavirus	Orthobunyavirus	MT731757.1, MW314029.1, OQ790103.1	8/73
Orthobunyavirus	Ngari virus	ON755200.1, KJ716848.1, MT929319.1	19/30
Phlebovirus	Rift Valley fever virus	OP146109.1, OM519314.1, ON856671.1	11/18

Arbovirus Group	Virus Name	Related GenBank sequences	Matching Pools (n/N)
Flavivirus	West Nile virus	PP212883.1, KY711338.1, ON813210.1, MW383507.1, OZ201856.1	6/23
Flavivirus	Usutu virus	OR141547.3	1/23
Flavivirus	Culex flavivirus	GQ165808.1, MT019260.1	2/23
Flavivirus	Anopheles flavivirus	MG372058.1	1/23
Flavivirus	Donggang virus (novel in Kenya)	MZ357899.1	1/23
Flavivirus	Kamiti River virus	AY149004.1	1/23
Flavivirus	Bagaza virus (novel in Kenya)	MW672101.1	8/23
Flavivirus	Berkedji virus (novel in Kenya)	LC494740.1	1/23
Flavivirus	Cell fusing agent	LC496857.1	1/23
Alphavirus	Sindbis virus	OK644075.1	1/3
Coinfection	Sindbis & Bagaza virus	OK644705.1 & MW672101.1	1/3

Phylogenetic analysis of the RVFV (Figure 6) and flavivirus (Figure S6) sequences showed that our isolates clustered into distinct clades together with reference strains within and outside Kenya, suggesting local circulation and genetic relatedness with strains reported globally.

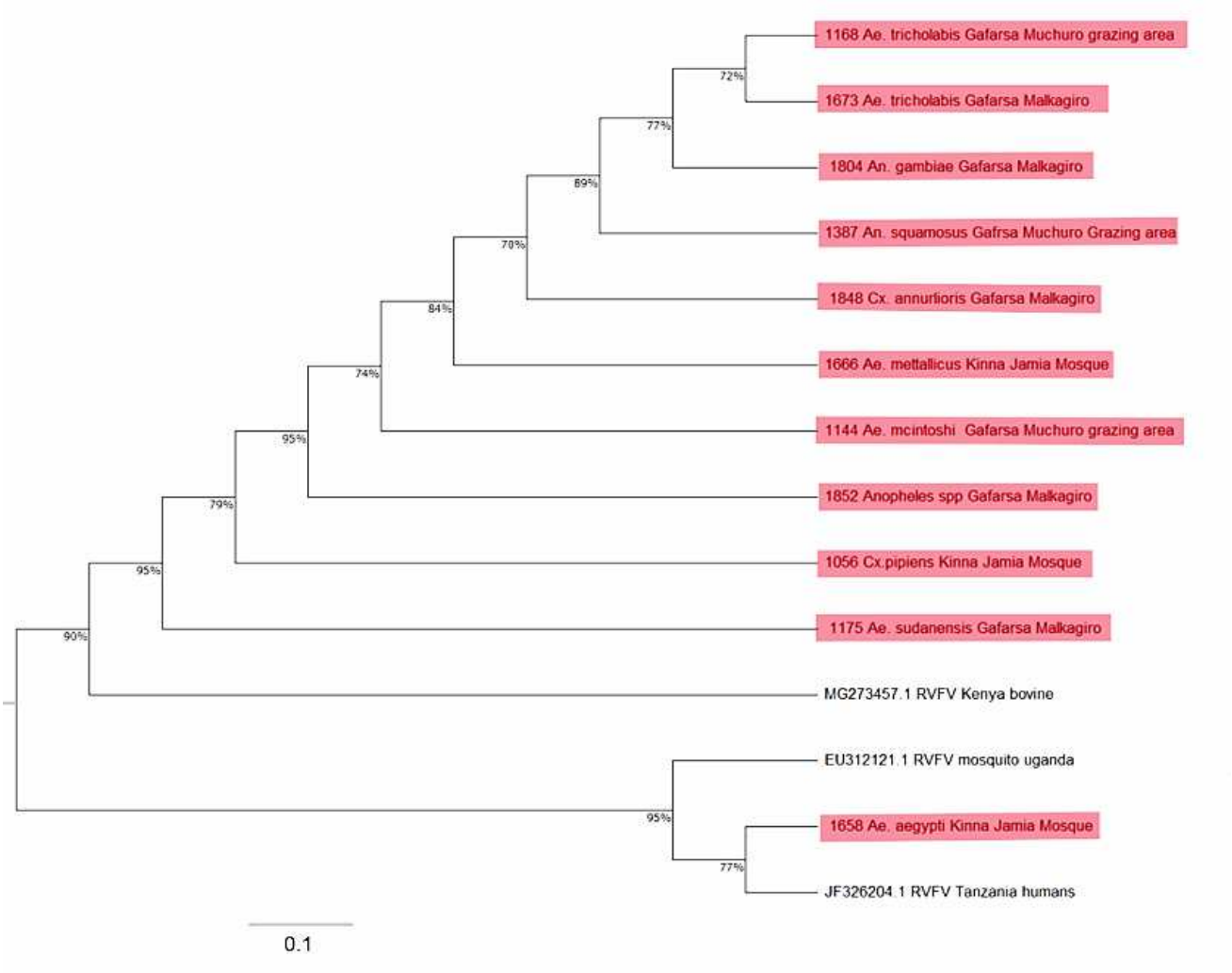


Figure 6. Phylogenetic tree inferred from RVFV NSs nucleotide sequences. The phylogenetic analysis includes 500-700-bp fragments of 11 sample sequences (highlighted in red) and three reference sequences from GenBank (not highlighted) with their accession number, source, and the country they were isolated from. Bootstrap values at the nodes are of agreement among 1000 replicates. The tree forms two clusters with the branch scale (0.1) representing substitution per site. Our RVFV sequences cluster into two clades, one sharing ancestry with a Kenyan strain isolated from bovine, and the other clade sharing ancestry with East African strains from Uganda and Tanzania.

3.2 Blood Meal Source Identification

A total of 61 blood-fed mosquitoes were analyzed for vertebrate blood meal sources using PCR-HRM targeting the 16S rRNA and *cyt b* gene regions. HRM profiles generated from 16S rRNA were validated against those from *cyt b.* Distinct HRM profiles from both 16S rRNA and *cyt b* (Figure S7). Vector-host interactions showed that sampled mosquitoes fed on a variety of hosts in the Kinna and Gaffarsa (Table S8).

Culex pipiens (23) was the predominant blood fed mosquito species in Kinna while *Aedes tricholabis* (9) was the predominant one in Gafarsa. A total of 13 different blood-fed mosquito species were identified from the two sampling areas. The highest numbers of vertebrate hosts were humans, detected in *Culex pipiens* (17), *Aedes tricholabis* (4), *Culex univittatus* (2), and *An. maculipalpis* and *An. funestus* (3 each). Livestock blood meal was also detected in significant numbers from cattle, goats, sheep, and camels. *Aedes tricholabis* showed a diverse host range, from goats, sheep, cattle, and humans, while *Culex pipiens* and *Culex univittatus* fed on humans, cattle, and goats. Wildlife blood meals were less frequent but included buffalo, waterbuck and rodent (Figure S9).

Arbovirus screening by HRM from the 61 blood fed mosquitoes revealed several positive profiles. Dengue virus was detected in camel, chicken, and human. Chikungunya in cattle, chicken, goat, and humans, and Bunyamwera, Ngari, and RVFV in human (Figure S10). However, confirmation of HRM-positives using genus specific conventional PCR primers did not yield detectable amplicons, likely due to low viral load since the mosquitoes were processed individually and not pooled. Multiplex PCR-HRM positive viruses for blood fed mosquitoes were hence confirmed by

singleplex PCR-HRM. Arboviruses identified by PCR-HRM from the blood-fed mosquitoes are shown in Table 2.

The vector-host-virus association were as follows; from humans, *Aedes aegypti* harbored CHIKV, Ngari virus, and RVFV, *Culex pipiens* and *Culex univittatus* harbored CHIKV and dengue virus, *Anopheles funestus* transmitted CHIKV, and *An. maculipalpis* carried Bunyamwera virus. From livestock, with *Culex univittatus* harbored CHIKV from cattle, and *Aedes aegypti* harbored Ngari virus from sheep. Wild hosts such as buffalo, rodents, and waterbuck were identified as blood meal sources, but no viruses were detected from them.

Table 2: Vector-host-virus association from the captured blood fed mosquitoes.

Mosquito Species	Host	Arbovirus detected
Anopheles funestus	Buffalo	None
Aedes mcintoshi	Camel	None
Anopheles squamosus	Camel	None
Culex pipiens	Camel	None
Culex univittatus	Camel	None
Culex pipiens	Cattle	None
Culex univittatus	Cattle	CHIKV
Aedes aegypti	Goat	None
Anopheles funestus	Goat	CHIKV
Aedes mcintoshi	Pig	None

Mosquito Species	Host	Arbovirus detected
Culex pipiens	Rodent	None
Aedes tricholabis	Waterbuck	None
Aedes tricholabis	Sheep	None
Anopheles gambiae	Sheep	None
Aedes aegypti	Sheep	Ngari
Aedes aegypti	Human	CHIKV, Ngari, RVFV
Anopheles funestus	Human	CHIKV
Culex pipiens	Human	CHIKV, Dengue
Culex univittatus	Human	CHIKV, Dengue
Anopheles maculipalpis	Human	Bunyamwera
Aedes tricholabis	Human	Dengue

4.0 DISCUSSION

Our findings show that Isiolo County, a region with history of significant hemorrhagic fever viruses outbreaks in northern Kenya, harbors a diverse range of arboviruses in circulation. The detection of both zoonotic and insect-specific arboviruses from different mosquito species in these areas illustrates the complex viral ecosystem and potential ecological conditions that favor the maintenance of these arboviruses. The broad feeding patterns and arboviruses detected from the fed mosquitoes illustrate how interconnected the mosquito vectors, and vertebrate hosts are and how they contribute to sustaining arbovirus transmission.

We detected both insect-specific and zoonotic viruses across four mosquito-borne arbovirus genera: Bunyamwera and Ngari virus (*Orthobunyavirus*), Sindbis (*Alphavirus*), RVFV (*Phlebovirus*), and West Nile, Usutu and Berkedji (*Flavivirus*). A coinfection of Sindbis and Bagaza viruses was detected in *Culex annurlioris*, the natural vector of Sindbis virus (42). Bagaza virus, a mosquito-borne *Flavivirus*, share overlapping ecological niches and vectors with Sindbis virus (43). Although a coinfection of the two viruses has not been previously reported in individual mosquitoes, it is theoretically possible. The coinfection was detected in a mosquito pool and not a single mosquito. These arboviruses were identified from mosquito pools collected in areas where livestock graze in wildlife zones during the rainy season. This association between livestock and wildlife highlights the potential for livestock-wildlife interactions in transmission of many of the arboviruses detected, with occasional spillage to humans.

Bunyamwera virus (BUNV) was detected and confirmed in multiple mosquito species, including *Anopheles funestus*, *Anopheles gambiae*, *Anopheles maculpalpis*, *Culex univittatus*, *Aedes tricholabis*, *Aedes egypti*, and *Culex cinereus*, indicating a broad vector competency. *Aedes* mosquito are recognized as the primary vectors for BUNV, supporting both vertical and environmental transmission (44). Natural infections of BUNV in mosquitoes have been reported in several of these mosquito species, consistent with our findings (45,46). Ngari virus was detected in *Anopheles coustani*, *Culex univittatus*, *Culex cinereus*, *Culex pipiens*, *Aedes simpsoni*, *Aedes aegypti*, *Aedes sudanensis*, and *Mansonia africana*. Ngari virus is a natural reassortant of Bunyamwera and Batai virus and primarily vectored by *Aedes, Anopheles,* and *Culex* mosquitoes (47). Their isolation from similar mosquito species has been reported previously (48,49), corroborating our findings. West Nile virus (WNV), a neuroinvasive flavivirus in animals and humans, is transmitted and bridged from birds to humans by *Culex univittatus* mosquitoes (50) .

342 In our study, West Nile virus was identified in *Aedes mettalicus*, and *Culex univittatus*. However,
343 transmission of West Nile by *Aedes mettalicus* remains undocumented but could suggest *Aedes*
344 *mettalicus* as a mechanical vector for WNV or emergence of *Aedes mettalicus* as a novel vector
345 for WNV. We therefore recommend comprehensive transmission and vector competence studies
346 to confirm this finding.

347 The primary vectors for RVFV are the floodwater *Aedes* species such as *Aedes mcintoshi*, *Aedes*
348 *ochraceus*, *Aedes tricholabis* and *Aedes sudanensis* which were well distributed in our study areas.
349 These vectors maintain the virus in their drought resistant eggs. The
350 *Culex, Anopheles* and *Mansonia* species are secondary vectors for RVFV (8). This pattern
351 supports our findings of RVFV across multiple *Culex* species and in *Ae. tricholabis*. Phylogenetic
352 analysis of the RVFV sequences from Kinna and Gafarsa formed clusters with high bootstrap
353 values indicating genetic similarity among the isolates with some divergence across the two
354 locations. Our sample sequences clustered with reference isolates from Kenya, Uganda, and
355 Tanzania (51,52), indicating a local circulation of strains from East Africa in Isiolo County. This
356 clustering also suggests an ongoing transmission of established arbovirus lineages within East
357 Africa and reflects a shared ecology that supports vector-host interaction in the region, hence the
358 importance of coordinated surveillance and control measures across East Africa.

359 Bunyamwera virus was identified in two male mosquitoes: *Anopheles species* and *Aedes aegypti*,
360 while Ngari virus was identified in a male *Aedes aegypti*, suggesting the possibility of vertical
361 transmission and its role in maintaining the virus in the mosquito population (46) or *Aedes aegypti*
362 acting as mechanical vectors for Ngari virus. Vertical transmission of BUNV has been documented
363 in *Anopheles gambiae, Culex univittatus,* and *Aedes aegypti* (46). However, to our knowledge, this

364 is the first documentation of Ngari virus vertical transmission in *Aedes aegypti.* The seasonal
365 occurrence of arboviruses in this study aligns with the rainfall patterns and mosquito population
366 dynamics in Isiolo County (31,53). Ngari and Bunyamwera virus, which circulates in livestock
367 and mosquitoes during or after the short rainy seasons (44,54) were detected in December, January
368 and June aligning with Isiolo's rainy seasons. West Nile virus often detected during the warm
369 months following rainy seasons (55), was detected in December and January. Sindbis virus usually
370 detected in Kenya during and after the rainy periods (56) was detected from mosquitoes captured
371 in June following the long rains in Isiolo county.

372 Aedes mosquitoes are capable of vertical arbovirus transmission via their drought resistant eggs
373 laid in flood plain areas. The eggs survive long dry seasons and hatch when flooding occurs
374 resulting in the mass emergence of infected adult mosquitoes (57). We did not directly assess
375 vertical transmission, however, West Nile virus, Bunyamwera, *Orthobunyavirus*, Ngari virus,
376 RVFV, and Kamiti River virus were identified from mosquitoes pooled from the flood areas of
377 Gafarsa along the Ewaso Nyiro river.

378 One of our West Nile virus isolates from *Culex univittatus* clustered closely with a lineage 1 WNV
379 from Hungary, another fell as an outlier suggesting independent evolutionary divergence from the
380 other strains. The other WNV isolates clustered together indicating local circulation of genetically
381 similar strains. We detected Usutu virus, an emerging zoonotic flavivirus, from *Culex univittatus*.
382 Usutu virus was first isolated in South Africa from *Culex neavei* and later spread to several
383 European countries (58). Its infection in humans are mostly asymptomatic to mild symptoms (59).
384 We also detected Bagaza virus, which has never been isolated in Kenya before, from 8 mosquito
385 pools. Bagaza sequences clustered tightly indicating a recent introduction and an active local

transmission. It is transmitted by *Culex tritaeniorhynchus* and *Aedes aegypti* and has only been isolated from birds (60). There is no confirmed human isolation of Bagaza virus in humans with the only report limited to serological presence in encephalitis patients from India (61). These findings emphasize the need for further investigation on the potential of Bagaza virus infections to humans.

Blood meal analysis revealed diverse host feeding patterns. *Aedes* mosquitoes, which are daytime biters and strongly anthropophilic but also feed on domestic animals (62), were also found to have fed on humans, goats, sheep, camel and waterbuck. *Culex* mosquitoes, primarily nocturnal ornithophilic feeders but sometimes feed on humans and other mammals as opportunistic feeders (63), were found to have fed on camel, cattle, chicken, rodent, and humans. *Anopheles* mosquitoes are nocturnal and prefer feeding on humans, cattle (64) and other mammal including buffalos (65) and camel (66), were found to have fed on buffalo, goat, sheep, camel and humans in our study.

Ngari and Chikungunya were detected in mosquitoes that had fed on livestock and humans indicated the role of domestic animals in facilitating arbovirus transmission between vectors and humans as amplification hosts. Ngari virus was detected in *Aedes aegypti* that fed on sheep, consistent with a previous report implicating small ruminants in its transmission (54). Dengue and RVFV were identified in mosquitoes that fed exclusively on humans suggestive of an urban transmission cycle. Humans and livestock were the dominant blood source linked to virus positive mosquitoes, emphasizing their key roles in sustaining transmission of arbovirus in Isiolo County. The main vector for CHIKV is *Aedes aegypti* (67), its detection in *Culex univittatus*, *Anopheles funestus*, *Anopheles funestus*, *Culex pipiens*, and Culex univittatus, suggests that these mosquito species could act as incidental carriers or potential secondary vectors. Ngari virus transmission by

a number of *Aedes* spp mosquitoes highlights its broad vector range (49).Detection in *Aedes aegypti* in our study could suggest spillover of the virus during feeding. *Culex pipiens*, *Culex univittatus*, *Aedes tricholabis* could have also acquired Dengue virus through spillover when feeding. Despite the detection by PCR-HRM, the failure to amplify them using conventional PCR reflects low viral load due to individual pooling of the mosquitoes.

5.0 CONCLUSIONS

This study provides important insight into the circulation of multiple medically significant arboviruses and blood feeding patterns of mosquito species in Isiolo County, northern Kenya. Detection of these viruses in various mosquito vectors underscores the complex arbovirus transmission dynamics in this semi-arid region. Human blood meals dominated among the fed mosquitoes, indicating frequent human-vector interactions and heightened risk of arbovirus spillovers to human populations. The detection of both wildlife and livestock blood meal suggests the existence of both domestic and sylvatic transmission cycles. The use of PCR-HRM in this study proved to be a cheaper, rapid, and sensitive tool for preliminary arbovirus differentiation and affirms its utility in early detection and outbreak preparedness even in resource limited settings. Our findings underscore the need to adopt integrated vector management and One Health surveillance approaches in Isiolo County and similar ecologies where human, livestock, and wildlife interactions foster the emergence and sustained circulation of zoonotic arboviruses.

LIST OF ABBREVIATIONS

ILRI: International Livestock Research Institute

JKUAT: Jomo Kenyatta University of Agriculture and Technology

KEMRI: Kenya Medical Research Institute

USA: United States of America

KWTRP: KEMRI Wellcome Trust Research Programme

CDC: Centers for Disease Control and Prevention

PCR: Polymerase Chain Reaction

PCR-HRM: Polymerase Chain Reaction–High Resolution Melting

CHIKV: Chikungunya virus

DENV: Dengue virus

RVFV: Rift Valley fever virus

HFV: Hemorrhagic fever virus

RNA: Ribonucleic Acid

COI: Cytochrome c oxidase subunit I

NGS: Next Generation Sequencing

RT-PCR: Reverse Transcription Polymerase Chain Reaction

WHO: World Health Organization

QGIS: Quantum Geographic Information System

GADM: Global Administrative Areas (Database)

HOX: Humanitarian Data Exchange

MEM: Minimum Essential Medium

FBS: Fetal Bovine Serum

PBS: Phosphate Buffered Saline

DNA: Deoxyribonucleic Acid

NCBI: National Center for Biotechnology Information

NNI: Nearest Neighbor Interchange

MLE: Maximum Likelihood Estimate

BUNV: Bunyamwera virus

WNV: West Nile virus

DECLARATION

Ethics approval and consent to participate

This study was conducted under an approved co-infection project with ethical clearance from the Institutional Research Ethics Committee (IREC) (Ref: ILRI-IREC2020-07/6), and authorization from the National Commission for Science, Technology and Innovation (NACOSTI) (Ref No: 218346). The project was also reviewed by the Defense Threat Reduction Agency (DTRA). Approval from the ILRI Institutional Biosafety Committee (IBC) was also updated prior to commencement of field activities to ensure compliance with biosafety and biosecurity requirements. All field and laboratory procedures were undertaken in accordance with the approved protocols and relevant national and institutional guidelines.

Consent to Participate declaration

Not applicable

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

HAA: Investigation; Formal analysis; Data Curation; Writing – Original Draft.

HMA: Conceptualization; Funding acquisition; Supervision; Project administration; Writing – Original Draft, Review & Editing; Methodology.

BB: Conceptualization; Funding acquisition; Supervision; Project administration.

SGN: Supervision; Writing – Review & Editing.

JM, FMM, BC, HK, EC, SL, JMA, JL: Investigation; Resources; Writing – Review & Editing.

EM: Formal analysis.

RN: Methodology; Investigation.

DNM, WCW: Project administration; Funding acquisition; Writing – Review & Editing.

All authors read and approved the final manuscript.

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