One Health approach to vector biology and epidemiology of arboviruses, *Rickettsia*, and protozoa in smallholder livestock systems in western Kenya

by

Tatenda Chiuya

Submitted in partial fulfilment of the requirements for the degree

Doctor of Philosophy

(Zoology)

Department of Zoology and Entomology

Faculty of Natural and Agricultural Sciences

University of Pretoria

Supervisors:

Prof A.D.S Bastos, Dr D.K Masiga, Dr J. Villinger, and Prof E.M Fèvre

Declaration

I, Tatenda Chiuya, hereby declare that: '*One Health approach to vector biology and epidemiology of arboviruses, Rickettsia, and protozoa in smallholder livestock systems in western Kenya*' is my own work, and has not been submitted for examination at any university, and that all the sources I quoted are referenced.

Signed: 

Date: 11 December 2020

Disclaimer

This thesis contains a series of chapters each answering a specific research objective and each chapter has been published or formatted for submission to specific peer-reviewed scientific journals. Therefore, there might be some repetitions in some sections, differences in citation styles and duplication of references.

Summary

Background: Vector-borne pathogens (VBP) present a serious threat to both animal and human health globally due to their zoonotic potential. '*One Health*' epidemio-surveillance systems that integrate human, animal, and arthropod vector facets of VBP transmission enhance detection and can give rise to informed effective multi-sectoral vector-borne disease control options. The most important vectors are ticks and mosquitoes, which have a cosmopolitan distribution due to their ecological plasticity. Collectively, they transmit viral, bacterial, and protozoal pathogens in sub-Saharan Africa. The distributional ranges of VBP are expected to expand due to increased global travel and trade in goods and livestock. Arboviruses, such as Rift Valley fever (RVF), chikungunya, dengue, and Crimean-Congo haemorrhagic fever (CCHF), are endemic in East Africa with regular occurrence of outbreaks, while *Rickettsia africae* causes African tick bite fever in travellers with few clinical cases reported in local populations. Furthermore, the co-occurrence of these causes of febrile illness and malaria complicates the diagnosis of fevers in hospitals. Correspondingly, *Theileria parva* and *Anaplasma marginale* are serious constraints to cattle production in the region. The Lake Victoria basin that encompasses the East African community countries of Kenya, Uganda, and Tanzania has, an ecology that is conducive for the endemic proliferation of mosquitoes and ticks, high livestock density under subsistence production system and is an important trade corridor for the East African community. These factors are likely to enhance circulation of zoonotic VBPs in the area. Therefore, this surveillance project in western Kenya sought to establish the most important species of ticks and mosquitoes transmitting zoonotic pathogens among local livestock and human populations. It also sought to determine if livestock harbour these pathogens as asymptomatic carriers for human infection, in which they may cause zoonotic febrile illness.

Methods: I sampled mosquitoes and ticks at hospitals, livestock markets (LMs), and slaughterhouses (SHs) in Kenya's Busia, Bungoma and Kakamega counties. Ticks and blood were collected from livestock at LMs and SHs between the year 2017 and 2019. Mosquitoes were trapped using CDC light and BG sentinel traps at selected hospitals and LMs during the short and long rainy seasons of 2018/2019. Additionally, febrile patients were recruited at hospitals for blood-sample collection during the same period. Vectors were identified to their lowest taxa using identification keys and molecular markers. DNA and RNA were extracted from samples and subjected to PCR, high-resolution melting analysis and representatives sequenced to identify pathogens in mosquitoes, ticks, and blood samples from humans and cattle. In addition, blood meal analyses were performed and human and cattle demographic data were collected and analysed by logistic regression to identify risk factors for pathogen infection.

Results: I collected 486 ticks, lice, and fleas from livestock at LMs and SHs wherein the most abundant tick species were *Rhipicephalus decoloratus* (35%) in 108 pools and *Amblyomma variegatum* (30%) in 99 pools, while *Ctenocephalides felis* fleas and *Haematopinus suis* lice were also present. Overall, pools of *Rh. decoloratus* ticks were infected with *A. marginale* (estimated true prevalence: 2.44%), *Anaplasma ovis* (1.21%), *Anaplasma platys* (3.05%), *Babesia bigemina* (0.61%), *Hepatozoon canis* (0.61%), *R. africae* (1.83%), *Theileria mutans* (7.32%) and *Theileria taurotragi* (1.21%). *Amblyomma variegatum* tick pools were infected with *B. bigemina* (0.77%), *Babesia caballi* (6.28%), *R. africae* (78.95%), *T. mutans* (0.76%) and *Theileria velifera* (1.53%). I also detected CCHF virus in two pools of *Rh. decoloratus* (0.61%) and *Rhipicephalus* sp. (1.45%) ticks. No pathogen DNA was detected in *C. felis*, while a single pool of *H. suis* was positive for *R. africae* (3.71%). Concomitantly, 6,848 mosquitoes corresponding to 21 species were trapped with the most abundant being *Aedes aegypti* (59 %) and *Culex pipiens* (40 %). The majority of the blood-fed mosquitoes were *Cx. pipiens* with

humans, chickens, and sparrows (*Passer* sp.) being identified from blood meal analyses. One *Culex poicilipes* pool was positive for sindbis virus, 30 pools of *Ae. aegypti* had cell fusing agent virus (CFAV; infection rate (IR) = 1.27%**,** 95% CI = 0.87%-1.78%), 11 pools of *Ae. aegypti* had Aedes flavivirus (AeFV; IR = 0.43%**,** 95% CI = 0.23%-0.74%), and seven pools of *Cx. pipiens* had Culex flavivirus (CxFV; IR = 0.23%, 95% CI = 0.1%-0.45%). None of the 422 blood samples collected from cattle at LMs and SHs were positive for arboviruses or *Rickettsia*. However, *Anaplasma* spp. (19.67 %), *Theileria* spp. (12.32 %), *Ehrlichia* spp. (6.64 %), and *Babesia* spp. (0.24 %) were detected in 39% of cattle samples. The most prevalent species were *T. velifera* (7.35 %), *A. marginale* (4.98 %), *Theileria mutans* (3.08 %), although *A. platys* (2.84 %), *T. parva* (1.60%) and *B. bigemina* (0.24 %) were also detected. Cattle breed and tick infestation were risk factors for *Anaplasma* spp. and *Ehrlichia* spp. infection. Exotic breeds of cattle had significantly higher infection rates with *Ehrlichia* spp. (OR: 2.39, 95 % CI: 0.98-5.63, $p = 0.049$) and *A. marginale* (OR: 4.50, 95 % CI: 1.75-11.91, $p = 0.002$) compared to local breeds. Tick infestation was a significant predictor for *Anaplasma* spp. (OR: 2.01, 95 % CI: 1.27-3.51, $p = 0.004$) and *Ehrlichia* spp. (OR: 2.83, 95 % CI: 1.22-7.38, $p = 0.021$) infection. None of the 336 febrile patients recruited into the study were positive for arboviruses or *Rickettsia*; however, 18.5% were positive for *Plasmodium falciparum*. Patients living in Busia (OR: 5.478; 95% CI: 2.509-13.055; $p = 0.000$) and Bungoma counties (OR: 3.027; 95 % CI: 1.358-7.316; $p = 0.009$) had 5.5 and 3 times higher odds of being PCR-positive for malaria, respectively, compared to those living in Kakamega County. Patients from a household in which the female household head/spouse did not receive any level of formal education had higher odds of being infected (OR: 4.446, CI: 1.402-14.044, $p = 0.010$).

Relevance/Conclusions: The presence of CCHF and sindbis viruses in ticks and mosquitoes, respectively, suggest risk of transmission to humans and livestock. Crimean-Congo haemorrhagic fever virus is harboured asymptomatically in cattle and causes serious

haemorrhagic fever in humans, while sindbis causes febrile illness and arthralgia also in humans. The relatively high infection rates of insect-specific flaviviruses (ISFs) in the sampled mosquitoes in a region where few arbovirus outbreaks have been reported points to the possibility that ISFs may be acting as a natural control mechanism preventing transmission of the pathogenic arboviruses. East Coast fever and anaplasmosis caused by *T. parva* and *A. marginale*, respectively, are major constraints to cattle production. Both were detected in this study. Correspondingly, risk factor analysis showed that cattle breed improvement in the study region by introducing exotic breeds can be hampered by tick-borne pathogens. Accordingly, the livestock markets can be utilised as control points for ticks using acaricides and other biological control mechanisms. Despite not finding CCHF virus, sindbis virus, and *R. africae* in livestock and human blood as detected in the vectors, clinicians should be made aware of their existential risk and include them in the differential diagnostics of febrile illnesses. A combination of IgM serology and RT-PCR could also improve detection sensitivity given the fragility of viruses in field samples. Only a small percentage of fevers in the recruited patients could be attributed to malaria (*P. falciparum*), suggesting undetected circulation of zoonotic VBPs or other pathogens causing fever not investigated in this study. Expectedly the malaria prevalence was not uniformly distributed due to the ecological differences in the three counties. Female household heads/spouses are in charge of households and most likely to impart their knowledge on mosquito control to other members of the households, highlighting gender aspects in malaria control. This knowledge can also be extrapolated to the control of arbovirus-transmitting mosquitoes. *Anaplasma platys* and genetically-related strains found in high prevalence in cattle blood have zoonotic potential even though they are yet to be detected in febrile patients in East Africa. Further studies are therefore needed to confirm their ability to cause disease in humans. In spite of the high infection rate of *Am. variegatum* ticks with *R. africae*, this zoonotic pathogen was not detected in cattle or humans, indicating either low

transmissibility or low/diminishing titers in infected vertebrates. This surveillance study generated data that can inform vector control strategies, raise clinical awareness, and serve as a baseline for further studies on vector-borne diseases of livestock and humans, including zoonoses.

Keywords: Kenya, Cattle, *Anaplasma*, *Rickettsia*, *Theileria parva*, *Rhipicephalus*, Crimean-Congo haemorrhagic fever, Livestock market, Hospital, Slaughterhouse, Human, Tick, Mosquito, Surveillance

Publications and manuscripts from this research work

- **Chiuya**, **T**., Masiga, D.K., Falzon, L.C., Bastos, A.D.S., Fèvre, E.M. and Villinger, J. (2020), Tick-borne pathogens, including Crimean-Congo haemorrhagic fever virus, at livestock markets and slaughterhouses in western Kenya. *Transboundary and Emerging Diseases.* https://doi.org/10.1111/tbed.13911

- **Chiuya**, **T**., Masiga, D.K., Falzon, L.C., Bastos, A.D.S., Fèvre, E.M. and Villinger, J. A survey of mosquito-borne and insect specific viruses in hospitals and livestock markets in western Kenya. *Under review. PLOS One*

- **Chiuya**, **T**., Villinger, J., Masiga, D.K., Ondifu, D.O., Wambua, L., Bastos, A.D.S., Fèvre, E.M. and Falzon, L.C. Molecular prevalence of tick-borne pathogens in cattle and risk factors in western Kenya. *In preparation for submission. BMC Veterinary Research*

- **Chiuya**, **T**., Falzon, L.C., Bastos, A.D.S., Fèvre, E.M., Villinger, J. and Masiga, D.K. Aetiology of non-malarial febrile illness in western Kenya: Insights from hospital surveys. *In preparation for submission. Malaria Journal*

Dedication

This thesis is dedicated to the Chiuya family. To my mum, dad, siblings; Dennis and Kudzai, your unwavering support and belief towards my achievement of this goal is forever cherished.

Acknowledgements

I am grateful to my supervisors Dr. Daniel Masiga, Dr. Jandouwe Villinger, Prof. Eric Fèvre and Prof. Armanda Bastos for their guidance, criticism, analysis and support towards the implementation of this study. I also want to thank the Zoonoses in Livestock in Kenya (ZooLinK) team in Busia led by Dr. Laura C. Falzon for collection of tick and blood samples in the field. I also want to acknowledge them for introducing me to the relevant stakeholders in the study site which was important for setting up the mosquito trapping and interviews with key hospital personnel. I also want to thank Dr. Lilian Wambua (ZooLinK) for her criticism and support towards this study as well as her hard work in managing the ZooLinK samples logistics. In that regard I want to acknowledge the efforts of Lydiah Mueni, Lynn Kirwa and Christian Odinga (ZooLinK). I also want to thank Dr. Christine Mosoti and Lilian Achola of the ZooLinK project for organizing the logistics of my field work in Busia and Nairobi. I am grateful to the German Academic Exchange Service (DAAD) through the African Region Postgraduate Programme in Insect Science (ARPPIS) under the Capacity Building & Institutional Development (CB&ID) unit of the International Centre of Insect Physiology and Ecology (*icipe*) for granting me this scholarship. I am also grateful to the University of Pretoria for awarding me a postgraduate bursary. My gratitude also goes to all the financiers of the various projects at *icipe* and the International Livestock Research Institute (ILRI) that made this work possible. My sincere thanks go to Dr Robert Skilton, Vivian A. Atieno, Esther Ndung'u and Margaret Ochanda of *icipe* CB&ID for their support throughout the course of my studies. I am also grateful for the support from Dr Bester T. Mudereri (*GIS* and remote sensing unit), Dr. Sheilah B. Agha, Daniel O. Ouso, Kevin K. Ogola, Edwin O. Ogola, Louis A. Okwaro, Dickens O. Ondifu and all my colleagues at the ML-EID lab. I am also grateful to all of my ARPIS 2017 colleagues for walking the PhD journey with me.

Funding

This study received financial support from the following organizations and agencies: The ZooLinK project which was supported by the Biotechnology and Biological Sciences Research Council, the Department for International Development, the Economic & Social Research Council, the Medical Research Council, the Natural Environment Research Council and the Defence Science & Technology Laboratory, under the Zoonoses and Emerging Livestock Systems (ZELS) programme (grant reference BB/L019019/1). It also received support from the CGIAR Research Program on Agriculture for Nutrition and Health (A4NH), led by the International Food Policy Research Institute (IFPRI) and in that regard I also acknowledge the CGIAR Fund Donors (http://www.cgiar.org/funders/). The study also received support from the European Union's Integrated Biological Control Applied Research Program (EU-IBCARP) (grant number: DCI-FOOD/2014/346-739), the ANTI-VeC Pump-Priming Award (AV/PP12) sub awarded by the University of Glasgow from UK government Global Challenges Research Fund (GCRF) Networks in Vector Borne Disease Research funds. The study also received financial support from the International Centre of Insect Physiology and Ecology (*icipe*) institutional funding from UK's Foreign Commonwealth and Development Office (FCDO); Swedish International Development Cooperation Agency (Sida); the Swiss Agency for Development and Cooperation (SDC); Federal Democratic Republic of Ethiopia; and the Kenyan Government. I was supported by a German Academic Exchange Service (DAAD) through the *icipe's* African Region Postgraduate Programme in Insect Science (ARPPIS)-DAAD scholarship and a University of Pretoria postgraduate bursary.

Table of contents

List of figures

List of tables

Chapter 1

General introduction and objectives

1.1 General introduction

Vector-borne zoonotic pathogens are a threat to public health and a constraint to livestock production globally. The chief vectors involved in their transmission are mosquitoes and ticks (Colwell et al., 2011; Dantas-Torres et al., 2012; Jongejan, 2004). Mosquitoes are vectors of several arboviruses of global importance and have recently also been implicated in the transmission of *Rickettsia* (Dieme et al., 2015). Ticks transmit important livestock diseases such as East Coast fever and anaplasmosis (Abanda et al., 2019). They are also reservoirs of some important zoonotic arboviruses and bacteria (Dantas-Torres et al., 2012). Recently, there has been a global resurgence of outbreaks of arboviruses, as well as tick-borne bacteria for which the vectorial range and pathogenicity is still vague (Doudier et al., 2010; Wilder-Smith et al., 2017). In most instances, livestock act as carriers/reservoirs and therefore rarely suffer clinical disease in the vector-livestock/wildlife-human triad of vector-borne zoonotic diseases.

Arboviruses cause undifferentiated febrile sickness which affect the normal lifestyle and production of people, especially in developing countries (Labeaud et al., 2011). Complications such as microcephaly in Zika virus infections can also arise (Heymann et al., 2016). Yellow fever can result in kidney and liver damage with mortalities of up to 50% being reported in some cases (Monath, 2015), while chikungunya-induced fever is associated with chronic arthralgia (Wilder-Smith et al., 2017). Dengue haemorrhagic syndrome and/or dengue shock syndrome are potentially fatal complications of the mild form of the disease (Guha-Sapir, 2005). In contrast, Crimean-Congo haemorrhagic fever (CCHF) virus produces severe disease with mortality of up to 30% in humans (Ergonul, 2012). Human rickettsiosis, ehrlichiosis, and anaplasmosis also present with the same clinical signs, however the geographical range of the latter two has mostly been limited to America and Europe (Doudier et al., 2010).

Vaccines for arboviruses other than Rift Valley fever (livestock) (Epin et al., 2010), dengue (humans, limited use) (Cortes da Silveira et al., 2019) and yellow fever (Monath, 2015), are not currently available. There is a lack of specific treatment protocols for arboviral and other vector-borne zoonotic infections and diagnosis is a challenge because the presenting clinical signs are not pathognomonic. These limitations are pronounced in most health facilities in Africa, where zoonotic vector-borne pathogens are endemic leading to many of the infections being misdiagnosed as malaria or non-specific febrile illnesses (Kagira et al., 2011). This has resulted in the understating of incidence rates and burden of other causes of fever, especially in malaria-endemic regions (Crump et al., 2013; Kipanga et al., 2014; Waggoner et al., 2017).

East Africa is endemic for several arboviruses and in Kenya several outbreaks have occurred (Konongoi et al., 2018; Munyua et al., 2010; Nguku et al., 2010; Obonyo et al., 2018; Sergon et al., 2008). Kenya is also endemic for malaria (Noor et al., 2016) and several tick-borne diseases, some such as East Coast fever which are a major impediment to livestock production (Moumouni et al., 2015; Wesonga et al., 2010). Past surveillance studies in livestock, humans, mosquitoes and ticks have generated substantial data on the incidence and ecology of vector-borne zoonotic pathogens (Grossi-Soyster et al., 2017; Mease et al., 2011; Mutai et al., 2013; Ochieng et al., 2015) and the vector species that are likely to sustain transmission during inter-epidemic and/or epidemic periods (Crabtree et al., 2009; Koka et al., 2017; Lutomiah et al., 2013, 2014; Maina et al., 2014; Mwamuye et al., 2017; Oundo et al., 2020; Sang et al., 2010; Omondi et al., 2017).

The close interaction of humans, livestock, and disease vectors is important in the transmission dynamics of vector-borne zoonoses. The main drivers of this interaction are urbanisation, rural to urban migration, global human travel and animal trade (Ahmed et al., 2019; Colwell et al., 2011; Fèvre et al., 2006; Kilpatrick, 2012; Morse, 1995). Several factors make the western Kenya region a potential hotspot for circulation of vector-borne zoonotic disease. Firstly, it is

on the border with Uganda which is an important corridor facilitating transport and trade in the East African Community (EAC). Secondly in addition to the movement of goods, there is a lot of human and livestock movement across the border (Nyaruaba et al., 2019). Thirdly, the East Africa Lake Victoria basin ecology is capable of supporting the proliferation of disease vectors (Fèvre et al., 2017). Therefore, a rising human population, rapid urbanisation, increased intensification and market-based livestock production systems may all serve to amplify the transmission risk of vector-borne zoonoses with livestock as reservoirs/carriers in this region (Fèvre et al., 2017).

Therefore, a '*One Health'* surveillance approach of vector-borne zoonoses is important for integrating vector surveillance with prevalence studies in humans and livestock. The surveillance of vectors measures the risk of disease outbreaks and identifies novel pathogens and/or pathogen-vector interactions. The level of animal and subsequent human exposure serves to determine potential disease reservoirs and the disease burden on society, respectively. This information is important for disease outbreak forecasting, preparedness, and instigation of control measures.

Due to the unavailability of vaccines and limited treatment options highlighted previously, the control of vector-borne zoonotic diseases usually targets vectors and their ecology. Control of vector-borne zoonoses generally involves preventing the vector from biting or coming into contact with humans and livestock and/or interfering with its intrinsic ability to transmit the pathogen. Traditionally, insecticide-treated bed nets (ITNs) and indoor and outdoor residual insecticide spraying have been used widely against mosquitoes. Such efforts have been more successful in the control of malaria vectors and have been widely deployed in malaria endemic areas such as in western Kenya (Steketee, 2010). However, elimination of mosquito habitats, use of repellents and window screens also serve to reduce biting rates and concurrently reduces transmission of arboviruses.

Transmission-blocking technologies that takes advantage of the superinfection exclusion capabilities of *Wolbachia* and insect-specific flaviviruses (ISFs) are being deployed in some developed countries (O'Neill et al., 2018), but not in Africa. Modification of the mosquito microbiome by *Wolbachia* interferes with their ability to transmit aborviruses (Schultz et al., 2018), whereas ISFs are thought to upregulate the antiviral response of mosquitoes, inhibiting superinfection by pathogenic viruses (Pepin et al., 2008). However, other studies have shown contrasting results with some ISFs showing no effect against the transmission of pathogenic arboviruses (Kent et al., 2010), paving the way for further conclusive studies to be done. Other novel strategies such as genetically engineered mosquitoes that make use of RNA interference (RNAi) pathways are being explored to suppress competent vector populations (Kean et al., 2015). Livestock is the main host for ticks and therefore an important control target via the application of acaricides, which subsequently reduces the incidence of tick-borne diseases and reduces the risk of infective tick bites in humans. Tick vaccines based on the midgut recombinant proteins are also commercially available however these are mostly effective against *Rhipicephalus* spp. and have been not been widely used in Africa (Merino et al., 2013).

Acaricides and insecticides are expensive, select for resistant vectors and have deleterious effects on the environment (Merino et al., 2013). Biological methods therefore offer an alternative and as such the use of entomopathogenic fungi has been explored with some success against ticks and mosquitoes (Akutse et al., 2020; Kean et al., 2015). An integrated vector management (IVM) approach has therefore been advocated which encompasses different control methods targeting different stages of the pathogen and involves both humans, animals, and the environment in a *One Health* approach (WHO, 2004). Overall, the success of control methods for vector-borne zoonotic diseases depends on the integration of chemical and biological control methods, collaboration between government, communities, and private stakeholders, public awareness and evidence-based instigation of measures. Therefore,

accurate information on vector-borne zoonotic disease epidemiology in a specific area is needed before control measures are deployed (WHO, 2004).

Lack of methodologies suited for large-scale concurrent vector, livestock, and human surveillance of vector-borne pathogens (VBPs) has hampered vector-borne zoonoses surveillance in developing countries. However, economical, high-throughput molecular tools such as PCR coupled with high-resolution melting analysis (HRM) can be used for identification of vectors, and their blood-meals, and pathogen screening in a variety of biological samples (Ajamma et al., 2016; Mwamuye et al., 2017; Omondi et al., 2015; Villinger et al., 2016). With the availability of these molecular tools, I carried out surveillance for vector-borne zoonoses in collaboration with the International Livestock Research Institute's (ILRI) Zoonoses in Livestock in Kenya (ZooLinK) project in western Kenya.

1.2 Main objective

To describe the epidemiology of vector-borne zoonotic pathogens in smallholder livestock production systems in western Kenya.

1.2.1 Specific objectives

1. To identify zoonotic arboviruses, *Rickettsia,* and protozoan parasites in ticks collected from livestock at slaughterhouses and livestock markets
2. To detect pathogenic and insect-specific viruses in mosquitoes sampled at hospitals and livestock markets
3. To assess the role of cattle as carriers of arboviruses, *Rickettsia,* and protozoa
4. To determine the incidence of arboviruses, *Rickettsia* and *Plasmodium* spp. in patients with acute undifferentiated fever at selected hospitals in western Kenya

1.3 References

Abanda, B., Paguem, A., Abdoulmoumini, M., Kingsley, M.T., Renz, A., Eisenbarth, A. (2019). Molecular identification and prevalence of tick-borne pathogens in zebu and taurine cattle in North Cameroon. *Parasites and Vectors*, *12*(1), 448. https://doi.org/10.1186/s13071-019-3699-x

Adjou Moumouni, P.F., Aboge, G.,T.,Masatani, T., Cao, S., Kamyingkird, K., Jirapattharasate, C., Zhou, M., Wang, G., Liu, M., Iguchi, A., Vudriko, P., Ybanez, A., Inokuma, H., Shirafuji-Umemiya, R., Suzuki, H., & Xuan, X. (2015). Molecular detection and characterization of *Babesia bovis*, *Babesia bigemina*, *Theileria* species and *Anaplasma marginale* isolated from cattle in Kenya. *Parasites and Vectors*. *8*. doi.10.1186/s13071-015-1106-9.

Ahmed, S., Dávila, J. D., Allen, A., Haklay, M. M., Tacoli, C., & Fèvre, E. M. (2019). Does urbanization make emergence of zoonosis more likely? Evidence, myths and gaps. *Environment and urbanization*, *31*(2), 443–460. https://doi.org/10.1177/0956247819866124

Ajamma, Y. U., Villinger, J., Omondi, D., Salifu, D., Onchuru, T. O., Njoroge, L., Muigai, A. W., & Masiga, D. K. (2016). Composition and genetic diversity of mosquitoes (Diptera: Culicidae) on islands and mainland shores of Kenya's Lakes Victoria and Baringo. *Journal of medical entomology*, *53*(6), 1348–1363. https://doi.org/10.1093/jme/tjw102

Akutse, K.S., Subramanian, S., Maniania, N.K & Dubois, T. (2020). Biopesticide Research and product development in Africa for sustainable agriculture and food security – Experiences from the International Centre of Insect Physiology and Ecology (icipe). *Frontiers in Sustainable Food Systems*. *4*:563016. doi: 10.3389/fsufs.2020.563016

Colwell, D. D., Dantas-Torres, F., & Otranto, D. (2011). Vector-borne parasitic zoonoses: emerging scenarios and new perspectives. *Veterinary Parasitology*, *182*(1), 14–21. https://doi.org/10.1016/j.vetpar.2011.07.012

Crabtree, M., Sang, R., Lutomiah, J., Richardson, J., & Miller, B. (2009). Arbovirus surveillance of mosquitoes collected at sites of active Rift Valley fever virus transmission: Kenya, 2006-2007. *Journal of medical entomology*, *46*(4), 961–964. https://doi.org/10.1603/033.046.0431

Crump, J. A., Morrissey, A. B., Nicholson, W. L., Massung, R. F., Stoddard, R. A., Galloway, R. L., Ooi, E. E., Maro, V. P., Saganda, W., Kinabo, G. D., Muiruri, C., & Bartlett, J. A. (2013). Etiology of severe non-malaria febrile illness in Northern Tanzania: a prospective cohort study. *PLoS neglected tropical diseases*, *7*(7), e2324. https://doi.org/10.1371/journal.pntd.0002324

Dantas-Torres, F., Chomel, B. B., & Otranto, D. (2012). Ticks and tick-borne diseases: a One Health perspective. *Trends in Parasitology*, *28*(10), 437–446. https://doi.org/10.1016/j.pt.2012.07.003

Dieme, C., Bechah, Y., Socolovschi, C., Audoly, G., Berenger, J. M., Faye, O., Raoult, D., & Parola, P. (2015). Transmission potential of *Rickettsia felis* infection by *Anopheles gambiae* mosquitoes. *Proceedings of the National Academy of Sciences of the United*

States of America, *112*(26), 8088–8093. https://doi.org/10.1073/pnas.1413835112

Doudier, B., Olano, J., Parola, P., & Brouqui, P. (2010). Factors contributing to emergence of *Ehrlichia* and *Anaplasma* spp. as human pathogens. *Veterinary Parasitology*, *167*(2-4), 149–154. https://doi.org/10.1016/j.vetpar.2009.09.016

Ergonul O. (2012). Crimean-Congo hemorrhagic fever virus: new outbreaks, new discoveries. *Current opinion in virology*, *2*(2), 215–220. https://doi.org/10.1016/j.coviro.2012.03.001

Fèvre, E. M., Bronsvoort, B. M., Hamilton, K. A., & Cleaveland, S. (2006). Animal movements and the spread of infectious diseases. *Trends in microbiology*, *14*(3), 125–131. https://doi.org/10.1016/j.tim.2006.01.004

Fèvre, E., De Glanville, W., Thomas, L., Cook, E., Kariuki, S., & Wamae, C.N. (2017). An integrated study of human and animal infectious disease in the Lake Victoria crescent small-holder crop-livestock production system. Kenya. *BMC Infectious Diseases*, *17*, 10.1186/s12879-017-2559-6.

Grossi-Soyster, E. N., Cook, E., de Glanville, W. A., Thomas, L. F., Krystosik, A. R., Lee, J., Wamae, C. N., Kariuki, S., Fèvre, E. M., & LaBeaud, A. D. (2017). Serological and spatial analysis of alphavirus and flavivirus prevalence and risk factors in a rural community in western Kenya. *PLoS neglected tropical diseases*, *11*(10), e0005998. https://doi.org/10.1371/journal.pntd.0005998

Guha-Sapir, D., & Schimmer, B. (2005). Dengue fever: new paradigms for a changing epidemiology. *Emerging themes in epidemiology*, *2*(1), 1. https://doi.org/10.1186/1742-7622-2-1

Heymann, D. L., Hodgson, A., Sall, A. A., Freedman, D. O., Staples, J. E., Althabe, F., Baruah, K., Mahmud, G., Kandun, N., Vasconcelos, P. F., Bino, S., & Menon, K. U. (2016). Zika virus and microcephaly: why is this situation a PHEIC?. *Lancet (London, England)*, *387*(10020), 719–721. https://doi.org/10.1016/S0140-6736(16)00320-2

Jongejan, F., & Uilenberg, G. (2004). The global importance of ticks. *Parasitology*, *129 Suppl*, S3–S14. https://doi.org/10.1017/s0031182004005967

Kagira, J. M., Maina, N., Njenga, J., Karanja, S. M., Karori, S. M., & Ngotho, J. M. (2011). Prevalence and types of coinfections in sleeping sickness patients in kenya (2000/2009). *Journal of tropical medicine*, *2011*, 248914. https://doi.org/10.1155/2011/248914

Kean, J., Rainey, S. M., McFarlane, M., Donald, C. L., Schnettler, E., Kohl, A., & Pondeville, E. (2015). Fighting arbovirus transmission: Natural and engineered control of vector competence in *Aedes* mosquitoes. *Insects*, *6*(1), 236–278. https://doi.org/10.3390/insects6010236

Kent, R. J., Crabtree, M. B., & Miller, B. R. (2010). Transmission of West Nile virus by *Culex quinquefasciatus* say infected with *Culex* flavivirus Izabal. *PLoS neglected tropical diseases*, *4*(5), e671. https://doi.org/10.1371/journal.pntd.0000671

Kilpatrick, A. M., & Randolph, S. E. (2012). Drivers, dynamics, and control of emerging vector-borne zoonotic diseases. *Lancet (London, England)*, *380*(9857), 1946–1955.

https://doi.org/10.1016/S0140-6736(12)61151-9

Kipanga, P. N., Omondi, D., Mireji, P. O., Sawa, P., Masiga, D. K., & Villinger, J. (2014). High-resolution melting analysis reveals low *Plasmodium* parasitaemia infections among microscopically negative febrile patients in western Kenya. *Malaria journal*, *13*, 429. https://doi.org/10.1186/1475-2875-13-429

Koka, H., Sang, R., Kutima, H.L., & Musila, L. (2017). The detection of spotted fever group *Rickettsia* DNA in tick samples from pastoral communities in Kenya, *Journal of Medical Entomology*, *54*(3), 774–780. https://doi.org/10.1093/jme/tjw238

Konongoi, S. L., Nyunja, A., Ofula, V., Owaka, S., Koka, H., Koskei, E., Eyase, F., Langat, D., Mancuso, J., Lutomiah, J., & Sang, R. (2018). Human and entomologic investigations of chikungunya outbreak in Mandera, Northeastern Kenya, 2016. *PloS one*, *13*(10), e0205058. https://doi.org/10.1371

Labeaud, A. D., Bashir, F., & King, C. H. (2011). Measuring the burden of arboviral diseases: the spectrum of morbidity and mortality from four prevalent infections. *Population health metrics*, *9*(1), 1. https://doi.org/10.1186/1478-7954-9-1

Lutomiah, J., Bast, J., Clark, J., Richardson, J., Yalwala, S., Oullo, D., Mutisya, J., Mulwa, F., Musila, L., Khamadi, S., Schnabel, D., Wurapa, E., & Sang, R. (2013). Abundance, diversity, and distribution of mosquito vectors in selected ecological regions of Kenya: public health implications. *Journal of vector ecology : Journal of the Society for Vector Ecology*, *38*(1), 134–142. https://doi.org/10.1111/j.1948-7134.2013.12019.x

Lutomiah, J., Omondi, D., Masiga, D., Mutai, C., Mireji, P. O., Ongus, J., Linthicum, K. J., & Sang, R. (2014). Blood meal analysis and virus detection in blood-fed mosquitoes collected during the 2006-2007 Rift Valley fever outbreak in Kenya. *Vector borne and zoonotic diseases (Larchmont, N.Y.)*, *14*(9), 656–664. https://doi.org/10.1089/vbz.2013.1564

Maina, A. N., Jiang, J., Omulo, S. A., Cutler, S. J., Ade, F., Ogola, E., Feikin, D. R., Njenga, M. K., Cleaveland, S., Mpoke, S., Ng'ang'a, Z., Breiman, R. F., Knobel, D. L., & Richards, A. L. (2014). High prevalence of *Rickettsia africae* variants in *Amblyomma variegatum* ticks from domestic mammals in rural western Kenya: implications for human health. *Vector borne and zoonotic diseases (Larchmont, N.Y.)*, *14*(10), 693–702. https://doi.org/10.1089/vbz.2014.1578

Mease, L. E., Coldren, R. L., Musila, L. A., Prosser, T., Ogolla, F., Ofula, V. O., Schoepp, R. J., Rossi, C. A., & Adungo, N. (2011). Seroprevalence and distribution of arboviral infections among rural Kenyan adults: a cross-sectional study. *Virology journal*, *8*, 371. https://doi.org/10.1186/1743-422X-8-371

Merino, O., Alberdi, P., Pérez de la Lastra, J. M., & de la Fuente, J. (2013). Tick vaccines and the control of tick-borne pathogens. *Frontiers in cellular and infection microbiology*, *3*, 30. https://doi.org/10.3389/fcimb.2013.00030

Monath, T. P., & Vasconcelos, P. F. (2015). Yellow fever. *Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology*, *64*, 160–173. https://doi.org/10.1016/j.jcv.2014.08.030

Morse S. S. (1995). Factors in the emergence of infectious diseases. *Emerging infectious*

diseases, *1*(1), 7–15. https://doi.org/10.3201/eid0101.950102

Munyua, P., Murithi, R. M., Wainwright, S., Githinji, J., Hightower, A., Mutonga, D., Macharia, J., Ithondeka, P. M., Musaa, J., Breiman, R. F., Bloland, P., & Njenga, M. K. (2010). Rift Valley fever outbreak in livestock in Kenya, 2006-2007. *The American journal of tropical medicine and hygiene*, *83*(2 Suppl), 58–64. https://doi.org/10.4269/ajtmh.2010.09-0292

Mutai, B. K., Wainaina, J. M., Magiri, C. G., Nganga, J. K., Ithondeka, P. M., Njagi, O. N., Jiang, J., Richards, A. L., & Waitumbi, J. N. (2013). Zoonotic surveillance for rickettsiae in domestic animals in Kenya. *Vector borne and zoonotic diseases (Larchmont, N.Y.)*, *13*(6), 360–366. https://doi.org/10.1089/vbz.2012.0977

Mwamuye, M. M., Kariuki, E., Omondi, D., Kabii, J., Odongo, D., Masiga, D., & Villinger, J. (2017). Novel *Rickettsia* and emergent tick-borne pathogens: A molecular survey of ticks and tick-borne pathogens in Shimba Hills National Reserve, Kenya. *Ticks and tick-borne diseases*, *8*(2), 208–218. https://doi.org/10.1016/j.ttbdis.2016.09.002

Nguku, P. M., Sharif, S. K., Mutonga, D., Amwayi, S., Omolo, J., Mohammed, O., Farnon, E. C., Gould, L. H., Lederman, E., Rao, C., Sang, R., Schnabel, D., Feikin, D. R., Hightower, A., Njenga, M. K., & Breiman, R. F. (2010). An investigation of a major outbreak of Rift Valley fever in Kenya: 2006-2007. *The American journal of tropical medicine and hygiene*, *83*(2 Suppl), 5–13. https://doi.org/10.4269/ajtmh.2010.09-0288

Noor, A., Macharia, P., Ouma, P., Oloo, S., Maina, J., Gogo, E., Kyalo, D., Olweny, L., Kabaria, C., Kinyoki, D., Snow, R., Erondu, N., Schellenberg, D., Kiptui, R., Njagi, K., Wamari, A., Mbuli, C., Omar, A., & Ejersa, Waqo. (2016). The epidemiology and control profile of malaria in Kenya: reviewing the evidence to guide the future vector control. doi: 10.13140/RG.2.2.32068.12167

Nyaruaba, R., Mwaliko, C., Mwau, M., Mousa, S., & Wei, H. (2019). Arboviruses in the East African Community partner states: a review of medically important mosquito-borne Arboviruses. *Pathogens and global health*, *113*(5), 209–228. https://doi.org/10.1080/20477724.2019.1678939

Obonyo, M., Fidhow, A., & Ofula, V. (2018). Investigation of laboratory confirmed dengue outbreak in north-eastern Kenya, 2011. *PloS one*, *13*(6), e0198556. https://doi.org/10.1371/journal.pone.0198556

Ochieng, C., Ahenda, P., Vittor, A. Y., Nyoka, R., Gikunju, S., Wachira, C., Waiboci, L., Umuro, M., Kim, A. A., Nderitu, L., Juma, B., Montgomery, J. M., Breiman, R. F., & Fields, B. (2015). Seroprevalence of infections with dengue, Rift Valley Fever and chikungunya viruses in Kenya, 2007. *PloS one*, *10*(7), e0132645. https://doi.org/10.1371/journal.pone.0132645

Omondi, D., Masiga, D. K., Ajamma, Y. U., Fielding, B. C., Njoroge, L., & Villinger, J. (2015). Unraveling host-vector-arbovirus interactions by two-gene high resolution melting mosquito bloodmeal analysis in a Kenyan wildlife-livestock interface. *PloS one*, *10*(7), e0134375. https://doi.org/10.1371/journal.pone.0134375

Omondi, D., Masiga, D. K., Fielding, B. C., Kariuki, E., Ajamma, Y. U., Mwamuye, M. M., Ouso, D. O., & Villinger, J. (2017). 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. *Frontiers in veterinary science*, *4*, 73. https://doi.org/10.3389/fvets.2017.00073

O'Neill, S. L., Ryan, P. A., Turley, A. P., Wilson, G., Retzki, K., Iturbe-Ormaetxe, I., Dong, Y., Kenny, N., Paton, C. J., Ritchie, S. A., Brown-Kenyon, J., Stanford, D., Wittmeier, N., Jewell, N. P., Tanamas, S. K., Anders, K. L., & Simmons, C. P. (2019). Scaled deployment of *Wolbachia* to protect the community from dengue and other *Aedes* transmitted arboviruses. *Gates open research*, *2*, 36. https://doi.org/10.12688/gatesopenres.12844.3

Oundo, J. W., Villinger, J., Jeneby, M., Ong'amo, G., Otiende, M. Y., Makhulu, E. E., Musa, A. A., Ouso, D. O., & Wambua, L. (2020). Pathogens, endosymbionts, and blood-meal sources of host-seeking ticks in the fast-changing Maasai Mara wildlife ecosystem. *PloS one*, *15*(8), e0228366. https://doi.org/10.1371/journal.pone.0228366

Pepin, K. M., Lambeth, K., & Hanley, K. A. (2008). Asymmetric competitive suppression between strains of dengue virus. *BMC Microbiology*, *8*, 1–10. https://doi.org/10.1186/1471-2180-8-28

Sang, R., Kioko, E., Lutomiah, J., Warigia, M., Ochieng, C., O'Guinn, M., Lee, J. S., Koka, H., Godsey, M., Hoel, D., Hanafi, H., Miller, B., Schnabel, D., Breiman, R. F., & Richardson, J. (2010). Rift Valley fever virus epidemic in Kenya, 2006/2007: the entomologic investigations. *The American journal of tropical medicine and hygiene*, *83*(2 Suppl), 28–37. https://doi.org/10.4269/ajtmh.2010.09-0319

Schultz, M. J., Tan, A. L., Gray, C. N., Isern, S., Michael, S. F., Frydman, H. M., & Connor, J. H. (2018). *Wolbachia* wStri blocks Zika virus growth at two independent stages of viral replication. *mBio*, *9*(3), e00738-18. https://doi.org/10.1128/mBio.00738-18

Sergon, K., Njuguna, C., Kalani, R., Ofula, V., Onyango, C., Konongoi, L. S., Bedno, S., Burke, H., Dumilla, A. M., Konde, J., Njenga, M. K., Sang, R., & Breiman, R. F. (2008). Seroprevalence of chikungunya virus (CHIKV) infection on Lamu Island, Kenya, October 2004. *The American journal of tropical medicine and hygiene*, *78*(2), 333–337.

Steketee, R. W., & Campbell, C. C. (2010). Impact of national malaria control scale-up programmes in Africa: magnitude and attribution of effects. *Malaria journal*, *9*, 299. https://doi.org/10.1186/1475-2875-9-299

Villinger, J., Mbaya, M. K., Ouso, D., Kipanga, P. N., Lutomiah, J., & Masiga, D. K. (2017). Arbovirus and insect-specific virus discovery in Kenya by novel six genera multiplex high-resolution melting analysis. *Molecular ecology resources*, *17*(3), 466–480. https://doi.org/10.1111/1755-0998.12584

Waggoner, J., Brichard, J., Mutuku, F., Ndenga, B., Heath, C. J., Mohamed-Hadley, A., Sahoo, M. K., Vulule, J., Lefterova, M., Banaei, N., Mukoko, D., Pinsky, B. A., & LaBeaud, A. D. (2017). Malaria and chikungunya detected using molecular diagnostics among febrile Kenyan children. *Open forum infectious diseases*, *4*(3), ofx110. https://doi.org/10.1093/ofid/ofx110

Wesonga, F.D., Kitala, P.M., Gathuma, J.M., Njenga, M.J. & Ngumi, P.N.. (2010). An assessment of tick-borne diseases constraints to livestock production in a smallholder livestock production system in Machakos District, Kenya. Livestock Research for

Rural Development. *22*(6).

WHO. Global Strategic Framework For Integrated Vector Management. WHO/CDS/CPE/PVC/2004.10. Geneva: World Health Organization. (2004). http://whqlibdoc.who.int/hq/2004/WHO_CDS_CPE_PVC_2004_10.pdf.

Wilder-Smith, A., Gubler, D. J., Weaver, S. C., Monath, T. P., Heymann, D. L., & Scott, T. W. (2017). Epidemic arboviral diseases: priorities for research and public health. *The Lancet. Infectious diseases*, *17*(3), e101–e106. https://doi.org/10.1016/S1473-3099(16)30518-7

Chapter 2

Tick-borne pathogens, including Crimean-Congo haemorrhagic fever virus, at livestock markets and slaughterhouses in western Kenya

Tatenda Chiuya[1, 4], Daniel K. Masiga[1], Laura C. Falzon[2, 3], Armanda D.S. Bastos[4], Eric M. Fèvre[2, 3], Jandouwe Villinger[1*]

1. International Centre of Insect Physiology and Ecology (icipe), P.O Box 30772-00100, Nairobi, Kenya
2.Institute of Infection, Veterinary and Ecological Sciences, University of Liverpool, Leahurst Campus, Chester High Road, Neston, CH64 7TE, United Kingdom
3. International Livestock Research Institute, Old Naivasha Road, PO Box 30709, 00100 Nairobi, Kenya
4. Department of Zoology and Entomology, University of Pretoria, Pretoria, South Africa

This chapter has been published in part as:

Chiuya, T., Masiga, D.K., Falzon, L.C., Bastos, A.D., Fèvre, E.M. and Villinger, J. (2020), Tick-borne pathogens, including Crimean-Congo haemorrhagic fever virus, at livestock markets and slaughterhouses in western Kenya. *Transboundary and Emerging Diseases.* https://doi.org/10.1111/tbed.13911

2.1 Abstract

Vectors of emerging infectious diseases have expanded their distributional ranges in recent decades due to increased global travel, trade connectivity, and climate change. Transboundary range shifts, arising from the continuous movement of humans and livestock across borders, are of particular disease control concern. Several tick-borne diseases are known to circulate between eastern Uganda and the western counties of Kenya, with one fatal case of Crimean-Congo haemorrhagic fever (CCHF) reported in 2000 in western Kenya. Recent reports of CCHF in Uganda has highlighted the risk of cross-border disease translocation and the importance of establishing inter-epidemic, early warning systems to detect possible outbreaks. I therefore carried out surveillance of tick-borne zoonotic pathogens at livestock markets and slaughterhouses in three counties of western Kenya that neighbour Uganda. Ticks and other ectoparasites were collected from livestock and identified using morphological keys. The two most frequently sampled tick species were *Rhipicephalus decoloratus* (35%) and *Amblyomma variegatum* (30%). *Ctenocephalides felis* fleas and *Haematopinus suis* lice were also present. In total 486 ticks, lice, and fleas were screened for pathogen presence using established molecular workflows incorporating high-resolution melting analysis and identified through PCR-sequencing and phylogenetic analyses. I detected CCHF virus in *Rh. decoloratus* and *Rhipicephalus* sp. cattle ticks and 82 of 96 pools of *Am. variegatum* were positive for *Rickettsia africae*. Apicomplexan protozoa and bacteria of veterinary importance, such as *Theileria parva*, *Babesia bigemina*, and *Anaplasma marginale*, were primarily detected in rhipicephaline ticks. My findings show the presence of several pathogens of public health and veterinary importance in ticks from livestock at livestock markets and slaughterhouses in western Kenya. Confirmation of CCHF virus, a *Nairovirus* that causes haemorrhagic fever with a high case fatality rate in humans highlights the risk of under-diagnosed zoonotic diseases and calls for the continuous surveillance and development of preventative measures.

Keywords: Emerging infectious disease, Zoonoses, *Nairovirus*, *Rickettsia*, *Rhipicephalus*, East Africa.

2.2 Introduction

Ticks are vectors of viruses, bacteria, and protozoa of economic and public health importance (de la Fuente et al., 2008). Babesiosis, theileriosis, and anaplasmosis cause major livestock production losses in Kenya (Adjou Moumouni et al., 2015; Gachohi et al., 2012; Latib et al., 1995; Norval et al., 1984), while rickettsiosis constitutes a serious emerging public health threat globally (Brown, 2016; Fournier et al., 2017; Jensenius et al., 2017; Maina et al., 2017; Ndip., et al., 2004; Parola et al., 2013; Rutherford et al., 2004). In addition to *Rickettsia*, tick-borne bacteria such as *Ehrlichia* and *Anaplasma*, and protozoa such as *Babesia* have been shown to infect humans in Europe and America (Doudier et al., 2010). Ticks also transmit nairoviruses, most of which cause a mild non-pathognomonic febrile illness in humans, but some, such as Crimean-Congo haemorrhagic fever (CCHF) and Dugbe viruses, can cause severe systemic illness and mortality, affirming the importance of ticks in the transmission of viral haemorrhagic fevers (Papa et al., 2017). In livestock, Nairobi sheep disease virus, also a *Nairovirus*, is a constant threat to sheep production in East Africa (Baron, 2015).

While the vectorial capacity of ticks is established, the role of lice and fleas in the epidemiology of vector-borne zoonoses is rarely investigated. *Rickettsia typhi* and *Rickettsia felis*, both endemic in East Africa, are transmitted by *Xenopsylla cheopis* and *Ctenocephalides felis* fleas, respectively. However, these *Rickettsia* species have been detected in several other flea species in addition to the chief vectors (Luce-fedrow et al., 2015). Louse infestations result in severe pruritic mange in livestock, leading to production losses (Hornok et al., 2010), and epidemic typhus, caused by *Rickettsia prowazekii*, in humans, especially in overcrowded and poor social settings (Raoult & Roux, 1997).

With travel and trade thought to be major drivers of emerging pathogen spread (Kilpatrick, 2012), the movement of livestock and people among East African countries could enhance the

circulation of emerging pathogens, especially given the high arboviral activity across the region (Mossel et al., 2017; Nyaruaba et al., 2019). Smallholder livestock production in East Africa is associated with livestock movement across provincial and national borders to livestock markets (LMs) in peri-urban areas (Fèvre et al., 2005) and animals in this sector have been found to be heavily infested by ticks (Sang et al., 2006). Livestock movement is instrumental in the introduction of infective foci in naïve areas where they can then be disseminated by capable vectors (Fèvre et al.,2006). Livestock movements in Kenya have been implicated in past and recent Rift Valley fever (RVF) outbreaks (Baba et al. 2016; Munyua et al., 2010; WHO, 2018).

Outbreaks of CCHF (Dunster et al., 2002) and RVF (WHO, 2018) have been reported before in western Kenya, and presence of antibodies to chikungunya, yellow fever, West Nile, and RVF viruses (Cook et al., 2017; Inziani et al., 2020; Mease et al., 2011; Nyaruaba et al., 2019) in the human population shows undetected circulation. While reports on the occurrence of zoonotic vector-borne bacteria are scant, the high prevalence of malaria in western Kenya may result in under-investigation of other causes of febrile illnesses. Ticks, fleas, and lice may be both vectors and reservoirs of most pathogens they transmit, making them an important component in the transmission dynamics of vector-borne zoonoses (Raoult & Roux, 1997). Elsewhere in Kenya and East Africa, the occurrence of bacterial pathogens of zoonotic and veterinary potential in ticks and fleas has been reported. Tick and flea-borne spotted fever group (SFG) rickettsiosis agents (*Rickettsia africae*, *Rickettsia conorii*, *Rickettsia aeschlimanii*, *R. felis*, and *Rickettsia asembonensis* sp. nov.) have been detected in Kenya (Maina et al., 2014, 2019; Mwamuye et al., 2017; Macaluso et al., 2003) and East Africa (Kumsa et al., 2015; Nakao et al., 2013; Nakayima et al., 2014). A broad spectrum of bacteria and protozoa of veterinary and public health importance have also been detected, including *Theileria parva*, *Ehrlichia ruminantium*, *Ehrlichia chaffeensis*, *Anaplasma marginale*, *Anaplasma phagocytophilum*, and *Anaplasma platys* (Oundo et al., 2020; Ringo et al., 2018; Omondi et

al., 2017; Mwamuye et al., 2017; Teshale et al., 2015). *Rhipicephalus*, *Hyalomma* and *Amblyomma* ticks collected from livestock in north-eastern Kenya were previously shown to be infected with CCHF, Bunyamwera, Dugbe, Ndumu, Semliki forest, Thogoto, Ngari, Dhori, and West Nile viruses (Lwande et al., 2013; Sang et al., 2011, 2006). These viruses are endemic in East Africa (Nyaruaba et al., 2019) and some, such as Semliki Forest, Wesselsbron, Ngari, and Bunyamwera viruses, have only been detected in mosquitoes (Ajamma et al., 2018; Villinger et al., 2017; Lwande et al., 2013). In most instances, ticks with arboviruses were collected from cattle at LMs and abattoirs, confirming the importance of these facilities for epidemiological investigations of these viruses.

Active surveillance for zoonotic pathogens and their vectors generates information on their presence and prevalence and can identify novel vector-pathogen associations. Such information can facilitate early detection and quantification of pathogen burdens and thus is important for planning control strategies to reduce spill-over infection from livestock to humans. Most of the diseases are characterised by non-specific febrile illness, which can be easily confused with other fever-causing agents. Awareness of their presence improves clinical referral and diagnosis.

To investigate the risk posed by the movement of arthropod vector infested animals via LMs in the Lake Victoria basin of East Africa, I collected ticks, fleas, and lice from livestock at both LMs and slaughterhouses (SHs). I employed high-throughput molecular techniques coupled with Sanger sequencing to rapidly detect pathogens of zoonotic and veterinary importance in these arthropods.

2.3 Materials and methods

2.3.1 Study site

The study was carried out in three neighbouring counties, viz. Busia, Bungoma, and Kakamega, in western Kenya (Figure 2.1). This region, part of which shares borders with Uganda, is representative of the larger Lake Victoria basin ecosystem in East Africa. Mixed crop and livestock production is the major farming system in this area characterised by a high livestock and human population. Recently, this production system is rapidly becoming more market-oriented rather than just for subsistence. This evolution is likely to impact on the emergence of pathogens shared between humans and animals in the region (Fèvre et al., 2017).

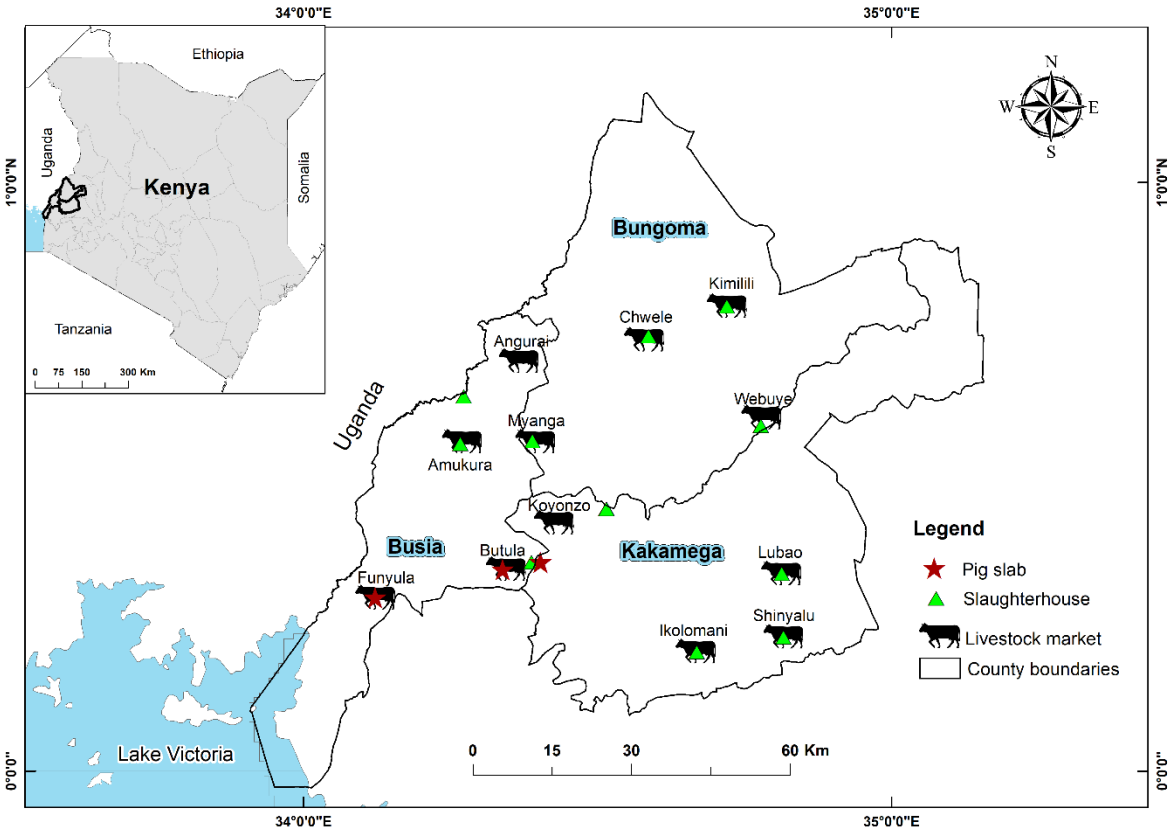


Figure 2.1: Map of the three neighbouring counties of Busia, Bungoma, and Kakamega showing the livestock markets and slaughterhouses from which arthropod samples were collected.

2.3.2 Study design and sample collection

The study design and sampling are detailed elsewhere (Falzon et al., 2019). Briefly, four LMs and neighbouring SHs were selected in each county (Figure 2.1), where each LM was closely associated with a cattle or pig SH. At each LM, 10 animals (six cattle, three goats, and one sheep) were selected via systematic random sampling. An attempt was made to select six cattle, three goats, and one sheep during each visit, though the number of animals sampled did not always follow the above ratio as it was occasionally challenging to get consent from owners of small ruminants. Signed consent was sought from the animal owners or traders accompanying sampled animals, and a short questionnaire was administered to capture demographic and animal ownership details. Animals were then physically restrained and, after a general clinical examination, blood was drawn by a qualified veterinarian from the jugular vein using a vacutainer. Nasal swabs and faecal samples were also collected. Any external parasites present on the hide of the selected animals were removed with gloved hands and placed into falcon tubes containing 70% ethanol. At cattle and pig SHs, a similar procedure was followed. In addition to ticks, lice and fleas were collected if present on sampled animals. Sample bottles and blood tubes were barcoded and transported to the field lab in Busia in a cool box with ice packs. Arthropods were stored at -40°C at the International Livestock Research Institute (ILRI) Department of Veterinary Services lab in Busia before being shipped on dry ice to the Martin Lüscher Emerging Infectious Disease (ML-EID) laboratory at the International Centre of Insect Physiology and Ecology (*icipe*) where they were stored at -80°C.

2.3.3 Morphological identification of ticks, lice, and fleas

Ticks, lice, and fleas were morphologically identified to species level using a stereomicroscope (Zeiss, Oberkochen, Germany) with the aid of identification keys (Walker et al., 2004; Pratt, 1973). Excessively engorged tick specimens were excluded from the analysis. Representative specimens were photographed using an Axio-cam ERc 5s digital camera (Zeiss) mounted on a

stereomicroscope. Ticks, lice, and fleas were pooled (1-3) according to developmental stage, sex, species, and host from which they were sampled.

2.3.4 Nucleic extraction from arthropods and selected livestock blood samples

Arthropod pools were homogenised before nucleic acid extraction. Each pool was put in a 1.5-ml Eppendorf tube with pre-weighed scoops of 750 mg of 2.0-mm and 150 mg of 0.1-mm yttria stabilized zirconium oxide (zirconia/yttria) beads (Biospec, USA), in which they were mechanically disrupted using a Mini-Beadbeater-16 (BioSpec, Bartlesville, OK) for 60-90 seconds. Phosphate buffered saline (PBS) (360 µl) was added to each tube, vortexed, and 210 µl of the resulting homogenate was transferred to a 96-well specimen processing cartridge. DNA and RNA were extracted using a MagNA 96 DNA and Viral NA Small Volume Kit (Roche Applied Science, Penzberg, Germany) in a MagNA Pure 96 robot (Roche Molecular Systems, California, USA). A sindbis virus culture isolate and PBS were included in each run as positive and negative extraction control respectively. DNA and RNA were eluted in 50 µl of RNAse-free water.

Animal blood samples associated with arthropod pools identified as positive for *R. africae* and CCHF virus were selected for pathogen screening. Nucleic acids from blood samples were extracted using the magnetic bead-based High Prep Viral DNA/RNA kit (MagBio Genomics, Gaithersburg, USA). First, 200 µl of blood was added to 1.5-µl Eppendorf tubes containing 528 µl of a lysis master-mix consisting of VDR lysis buffer, isopropanol, and carrier RNA, and vortexed. Then 10 µl of proteinase K and 10 µl of MAG-S1 magnetic beads were added and mixed into solution by inversion. The subsequent steps were carried out as per manufacturer's instructions.

2.3.5 Molecular identification of ticks

Molecular identification was performed on 15 single ticks for which morphological identification to species level was equivocal. I amplified three target genes: the internal

transcribed spacer-2 (ITS2), cytochrome oxidase 1 (CO1), and 16S ribosomal (r)RNA (Table A.1). The PCRs were performed in a SimpliAmp PCR Thermal Cycler (Applied Biosystems, Singapore) in 10-µl reactions that consisted of 2 µl of 5x HOT FIREPol® Blend Master Mix (Solis BioDyne, Estonia), 2 µl of template, and 0.5 µl of 10 µM primer. PCR grade water was included as a negative control on each run and cycling conditions were as previously described (Mwamuye et al., 2017), with the exception that the final extension step for the three fragments was seven minutes. Amplicons of the correct size were visualised alongside Quick-Load® 100-bp DNA Ladder (Biolabs, UK) by electrophoresis on 1.6 % ethidium bromide-stained agarose gels under UV light. Bi-directional sequencing of amplicons purified by Exo 1-rSAP combination (Biolabs, UK) was done at Macrogen (Netherlands). Sequence chromatograms were inspected, edited, and aligned using Geneious Prime version 2019.0.4 software (Biomatters, New Zealand). The resulting sequence contigs were used in nucleotide BLAST searches (Altschup et al., 1990) against the GenBank nr database to identify matches.

2.3.6 Molecular detection of arboviral, bacterial and protozoal pathogens

Detection of arboviruses

A previously described multiplex reverse transcription (RT)-PCR-HRM test was initially utilised for the detection of arboviruses within the *Flavivirus, Alphavirus, Nairovirus, Phlebovirus, Orthobunyavirus*, and *Thogotovirus* genera (Villinger et al., 2017) (Table A.1). This was preceded by cDNA synthesis using the High Capacity cDNA Reverse Transcription (RT) kit (Applied Biosystems, Lithuania) in a 20-µl reaction mixture that contained 10 µl nucleic acid extract, 1 U/µl RNase inhibitor, 100 mM dNTPs, 1X RT buffer, 2.5 U/µl reverse transcriptase enzyme and 600µM non-ribosomal random hexa-nucleotide primers (Endoh et al., 2005). The PCRs were carried out in a SimpliAmp thermocycler (Applied Biosystems, Singapore) using previously described thermal cycling conditions (Ajamma et al., 2018). The 10-µl reaction mixture for the multiplex PCR-HRM contained 1 µl cDNA template, 5 µl of 2x

MyTaq HS Mix (Bioline, UK) and 1 µl of 50 µM SYTO-9 (Life Technologies, USA). Multiplex PCR-HRM reactions were performed in a Rotor-Gene Q real-time PCR thermocycler (Qiagen, Hilden, Germany) using touchdown thermal cycling conditions described in detail elsewhere (Villinger et al., 2017). Each run included cDNA of the sindbis virus as a positive control and no-template extraction controls and molecular grade water as PCR negative controls. HRM profiles were visualised with Rotor-Gene Q Series software 2.1.0. All positives were separately re-run using primer mixes for each of alphaviruses, flaviviruses, and nairoviruses and the same conditions for the multiplex PCR-HRM runs (Villinger et al., 2017) (Table A.1). I purified all specific PCR products with an Exo 1-rSAP combination (Biolabs, UK) and sent them for bidirectional sequencing at Macrogen (Netherlands). Larger fragments using a conventional PCR assay that targets the *Nairovirus* L-polymerase gene (Table A.1) were also amplified, purified, and sequenced as previously described (Honig et al., 2004).

Detection of bacterial and protozoan pathogens

Tick, louse, flea, and livestock-blood samples were also screened for bacteria and protozoa using a combination of PCR-HRM and conventional PCR. Previously developed primers that target the 16S rRNA gene of *Anaplasma* (Mwamuye et al., 2017), *Ehrlichia* (Mwamuye et al., 2017), and *Rickettsia* (Nijhof et al., 2007), as well as primers that target the 18S rRNA gene of *Babesia* and *Theileria* protozoa (Georges et al., 2001), were used for initial screening (Table A.1). Ten-microliter reactions that consisted of 2 µl template, 2 µl 5X HOT FIREPol® EvaGreen HRM Mix (Solis BioDyne, Estonia), and 0.5 µl of each primer at 10 µM concentrations. Cycling was carried out in a Rotor-Gene Q real-time PCR thermocycler (Qiagen, Hilden, Germany) as described before (Mwamuye et al., 2017). Positive controls for *Anaplasma* (*A. marginale*) and *Rickettsia* (*R. africae*) (previously detected in *icipe*'s ML-EID lab from *Amblyomma* spp. ticks) were included in the runs. Resultant HRM profiles were

visually inspected with Rotor-Gene Q Series software 2.1.0 and PCR products with unique HRM profiles were purified using an Exo 1-rSAP combination (Biolabs, UK) and sequenced at Macrogen (Netherlands).

Positive *Ehrlichia* and *Anaplasma* samples were further amplified with a semi-nested PCR to generate a longer fragment of the 16S rRNA gene (1030 bp) by combining the Anaplasmataceae-specific forward primer, EHR16SD (Parola et al., 2001) with universal reverse primers pH1522 (Edwards et al., 1989) and pH1492 (Reysenbach et al., 1992) for first and second round amplification, respectively (Supplementary Table 1). Primary amplifications were performed using a hot-start activation step of 95°C for 15 min followed by 1 cycle of 95°C for 20 s, 63°C for 30 s, and 72°C for 90 s, 2 cycles of 95°C for 20 s, 62°C for 30 s, and 72°C for 90 s, 2 cycles of 95°C for 20 s, 61°C for 30 s, and 72°C for 90 s, followed with 35 cycles of 95°C for 20 s, 60°C for 30 s, and 72°C for 80 s, and a final extension at 72°C for 10 min. The secondary 20-µl amplification reactions utilised 2 µl of PCR products from primary reactions as templates. The cycling profile consisted of: 95°C for 15 min; 3 cycles of 95°C for 20 s, 61°C for 30 s, and 72°C for 90 s; 37 cycles of 95°C for 20 s, 60°C for 30 s, and 72°C for 80 s, and a final extension at 72°C for 10 min. To minimise the risk of contamination I set up the second reaction in a PCR cabinet and opened only one tube at a time. Products were visualised after gel electrophoresis to confirm the presence of the expected product at 1030-bp. For *Rickettsia*, all samples with positive HRM profiles were further amplified with Rick-*ompB* primers (Roux & Raoult, 2000) specific for a 856-bp region of the outer membrane protein B gene of all *Rickettsia* species (Table A.1). Positive samples were prepared for sequencing using the QuickClean II Gel Extraction Kit (GenScript, New Jersey, USA) and submitted to Macrogen (Netherlands) for bidirectional sequencing.

2.3.7 Phylogenetic analysis

Sequence editing was done in Geneious Prime version 2019.0.4 software (Biomatters, New Zealand). Homologous sequences of reference and sequence entries closely related with each of the individual sequences generated in this study were identified through BLAST nucleotide searches against the GenBank nr database (Altschup et al., 1990). Each of the datasets compiled in this manner were aligned and the terminal regions corresponding to the primer sequences were removed prior to phylogenetic analysis. Maximum likelihood phylogenies were inferred for each gene using PhyML version 3.0., employing the Akaike information criterion for automatic selection of the appropriate model of sequence evolution (Guindon et al., 2010). Trees were visualised and edited in Figtree 1.4 (Rambaut, 2014).

2.3.8 Estimation of individual-level pathogen prevalences from pooled samples

Individual-level prevalences of pathogens detected in pooled samples were estimated by a maximum likelihood approach in a frequentist model. True prevalence estimates within vector populations assumed 100% sensitivity and specificity of pooled-sample results and took into account the number of individuals in each pool tested (Cowling et al. 1999; Williams & Moffitt, 2001). The computations were performed online using Epitools an epidemiological calculator accessed from https://epitools.ausvet.com.au/ppvariablepoolsize (Sergeant, 2009).

2.4 Results

2.4.1 Vector collection and diversity at LMs and SHs

A total of 456 ticks (434 adults and 22 nymphs), 28 lice (*Haematopinus suis*), and two fleas (*Ct. felis*) collected from cattle, goats, sheep, and pigs at LMs and SHs were analysed in this study. Over 80% of the vectors collected at LMs and SHs came from cattle (Table A.2). This was partially due to the fact that 60% of the animals sampled at each of these locations were cattle, which were generally more tick-infested than goats, sheep, or pigs. The lice were primarily collected from pigs at SHs and the fleas were collected from cattle.

Representative specimens of *Rhipicephalus evertsi* (one adult), *Rhipicephalus appendiculatus* (one adult, one nymph), *Amblyomma gemma* (one adult), *Amblyomma variegatum* (one adult, one nymph), *Haemaphysalis* sp. (one adult), *Rhipicephalus decoloratus* (one adult), and *Rhipicephalus* sp. (six adults, one nymph), identified morphologically (Figure A.1), were selected for molecular tick identification (Table 2.1). Molecular identifications concurred with morphological identifications for *Rh. appendiculatus* (T16), *Rh. decoloratus* (T134), and *Am. variegatum* (T199). However, I resolved a tick specimen (T105) that I morphologically identified as *Rh. decoloratus* to be *Rhipicephalus microplus* based on its 16S rRNA sequence homology. The ITS2 sequence of an *Am. gemma* (T222) had highest homology with *Amblyomma hebraeum*, as there were no other *Am. gemma* ITS2 reference in the GenBank database. Seven out of nine specimens of *Rhipicephalus*, *Haemaphysalis*, and *Amblyomma* spp. unresolved by morphology alone were identified based on sequence homologies of at least two of the markers. The most common tick species were *Rh. decoloratus* (35%) and *Am. variegatum* (30%).

Table 2.1: Comparison of molecular and morphological identification of ticks

Sample identification	Morphological identification†	16S rRNA (% homology, GenBank accession)	ITS2 (% homology, GenBank accession)	CO1 (% homology, GenBank accession)	Consensus identification† (GenBank accessions)
T15	*Rhipicephalus* sp.	*Rh. decoloratus* (100, EU918193)	*Boophilus decoloratus* (96.7, U97716)	-	*Rh. decoloratus* (16S: MN266914; ITS2: MN266918)
T16	*Rh. appendiculatus*	*Rh. appendiculatus* (99.35, KC503257)	*Rh. appendiculatus* (99.8, KX276951)	*Rh. appendiculatus* (100, KC503257)	*Rh. appendiculatus* (16S: MN266911; ITS2: MN266949; CO1: MN294736)
T34	*Rhipicephalus* sp.	*Rh. microplus* (99.2, MH513311)	*Rh. microplus* (99.6, KC503265)	*Rh. microplus* (100, KY678120)	*Rh. microplus* (16S: MN264523; ITS2: MN266952; CO1: MN294738)
T50	*Rhipicephalus* sp.	*Rh. microplus* (99.3, KY020993)	*Rh. microplus* (99.6, MG721035)	*Rh. microplus* (100, KY678120)	*Rh. microplus* (16S: MN264524; ITS2: MN266953; CO1: MN294739)
T62	*Rhipicephalus* sp.	*Rh. decoloratus* (100, EU918193)	*Boophilus decoloratus* (96.7, U97716)	-	*Rh. decoloratus* (16S: MN266915; ITS2: MN266919)
T63	*Rhipicephalus* sp. nymph	*Rh. appendiculatus* (99.35, KC503257)	*Rh. appendiculatus* (99.35, KC503257)	*Rh. appendiculatus* (99.9, KC503257)	*Rh. appendiculatus* (16S: MN266912; ITS2: MN266950; CO1: MN294737)
T105	*Rh. decoloratus*	*Rh. microplus* (99.1, MH513311)	-	-	*Rh. microplus* (16S: MN264525)
T134	*Rh. decoloratus*	*Rh. decoloratus* (100, EU918193)	*Boophilus decoloratus* (96.7, U97716)	-	*Rh. decoloratus* (16S: MN266916; ITS2: MN266921)
T192	*Haemaphysalis* sp.	*Ha. elliptica* (95.6, HM068961)	*Ha. erinacei* (88, KU364288)	*Ha. erinacei* (99.3, KU880573)	*Haemaphysalis* sp. (16S: MN264214; ITS2: MN266944;CO1: MN294735)
T199	*Am. variegatum*	*Am. variegatum* (99.3, L34312)	*Am. variegatum* (100, HQ856803)	-	*Am. variegatum* (16S: MN266929; ITS2: MN401349)
T218	*Rhipicephalus* sp. nymph	*Rh. appendiculatus* (99.51, KC503257)	*Rh. appendiculatus* (99.73, KY457500)	-	*Rh. appendiculatus* (16S: MN266913; ITS2: MN266951)
T222	*Am. gemma*	-	*Am. hebraeum* (99.65, KY457490)	-	*Am. gemma* (ITS2: MN401350)
T311	*Amblyomma* sp. nymph	*Am. variegatum* (99.3, L34312)	*Am. variegatum* (100, HQ856803)	-	*Am. variegatum* (16S: MN266930; ITS2: MN401351)
T321	*Rhipicephalus* sp.	*Rh. simus* (96.28, KJ613641)	-	-	*Rhipicephalus* sp. (16S: MN266945)

2.4.2 Pathogens detected

I detected *Anaplasma* and *Rickettsia* bacteria, *Babesia*, *Theileria*, *Hepatozoon* protozoa, and CCHF virus (Figure 2.2) in ticks and lice collected from 13 LMs and 13 SHs across the three sampled counties (Table A.3).

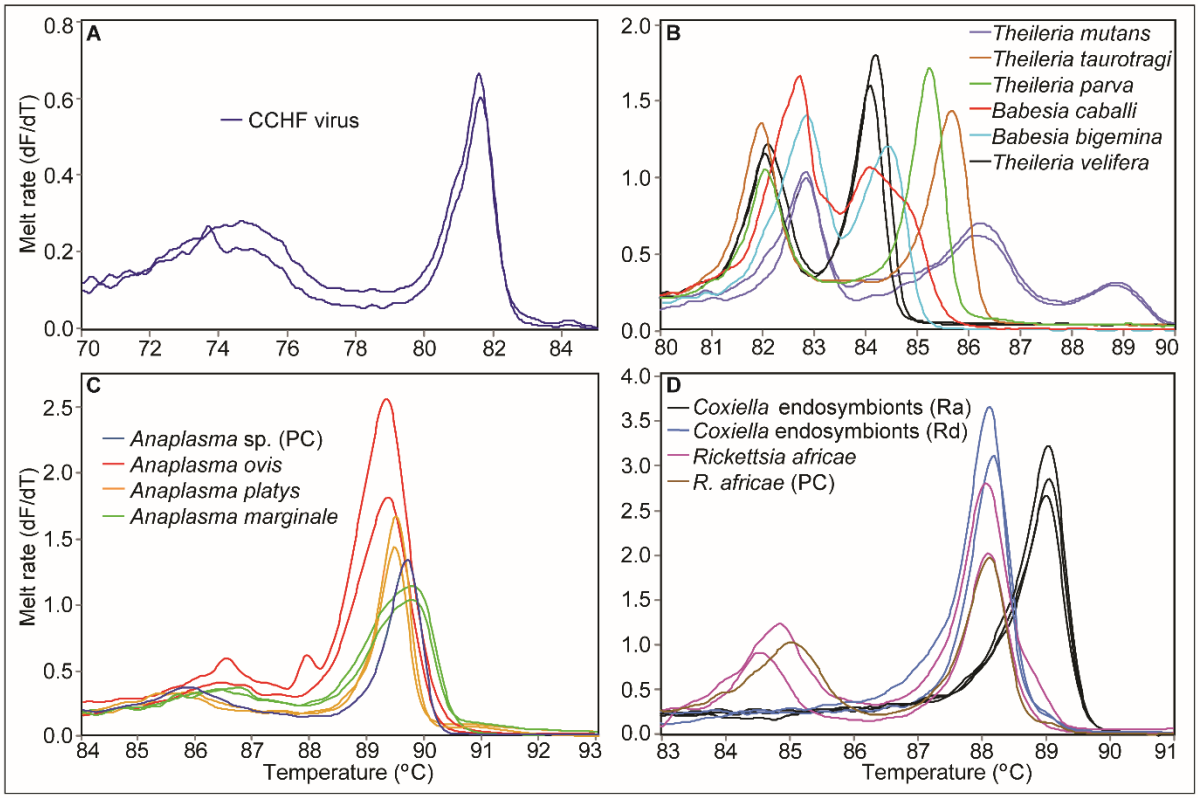


Figure 2.2: Tick-borne pathogen melt rate profiles.
(A) CCHF virus RdRp amplicons, (B) *Theileria/Babesia* 18S rRNA amplicons, (C) *Anaplasma* 16S rRNA amplicons, and (D) *Rickettsia/Coxiella* 16S rRNA amplicons. PC: positive control. Ra: *Rh. appendiculatus*; Rd: *Rh. decoloratus*.

Out of the 333 pools tested, one *Rh. decoloratus* and one *Rhipicephalus* sp. were positive for CCHF virus (deposited GenBank accessions MN267048, MN267049; 0.62% estimated true prevalence). These ticks were removed from cattle at two SHs. The CCHF virus isolates identified fell into the genotype II clade, which includes isolates from Uganda and the Democratic Republic of Congo (DRC) (Figure 2.3). Their nucleotide sequence identity was highest (98.6%) to the Nakiwogo (GenBank accession KX013483) strain isolated from Uganda (Simpson et al., 1967).

Eighty-two out of 96 pools of *Am. variegatum*, three pools of *Rh. decoloratus*, four pools of *Rhipicephalus* sp., one pool of *Rh. appendiculatus*, one pool of *Am. gemma*, and one pool of *H. suis* were positive for *R. africae* (deposited GenBank accessions MN294740-MN294749) (Table 2.2). These *R. africae*-positive ectoparasites were removed from cattle, sheep, goats and pigs. Two of the *R. africae* sequences from this study were identical to those previously detected in *Am. variegatum* ticks in Asembo in Kenya (93.8 %; GenBank accession KF660534) and another to a strain detected in an African tick bite fever patient in Tanzania (96.8%; unpublished; GenBank accession KU721071). *Rickettsia africae* variants in this study were characterised by base substitutions in several positions and possessed a four-base insertion that is absent from most Kenyan isolates (Figure 2.4).

I detected *A. platys* and genetically-related strains (deposited GenBank accessions MN266939-MN266941) in five pools of *Rh. decoloratus*, two pools of *Rhipicephalus* sp., and three pools of *Rh. appendiculatus*, all obtained from cattle (Table A.3). These all shared 100% identity with an *A. platys* strain from China using the 16S rRNA gene (GenBank accession MH762081). *Anaplasma marginale* (deposited GenBank accessions MN266931-MN266935) was detected in four pools of *Rh. decoloratus* and two pools of *Rhipicephalus* sp. These were 99.9-100% related to *A. marginale* strains in GenBank; KU58603, KX987330 (both from China) and KU686794 (Uganda) using the 16S rRNA. *Anaplasma ovis* (deposited GenBank accessions MN266936-MN266938) was detected in two pools of *Rh. decoloratus*, three pools of *Rhipicephalus* sp. and one pool of *Rh. evertsi* from goats and cattle. These all shared 99.9-100% identity with an *A. ovis* strain from China using the 16S rRNA gene (GenBank accession MG869525).

Table 2.2: Vector-borne pathogens detected in pools of ticks and lice from livestock markets and slaughterhouses

Pathogen	***Rhipicephalus* spp.**	*Rh. decoloratus*	*Rh. appendiculatus*	*Rh. evertsi*	*Rhipicephalus* sp.	***Amblyomma* spp.**	*Am. variegatum*	*Am. gemma*	*H. suis*	**Total**
Total pools	215‡	108	33	18	54	99	96	3	17	333‡
A. marginale	6 (1.88 %)	4 (2.44%)†	-	-	2 (2.90%)	-	-	-	-	6 (1.24%)
A. ovis	6 (1.88%)	2 (1.21%)	-	1 (4.17%)	3 (4.35%)	-	-	-	-	6 (1.24%)
A. platys	10 (3.15%)	5 (3.05%)	3 (5.51%)	-	2 (2.92%)	-	-	-	-	10 (2.07%)
B. bigemina	2 (0.63%)	1 (0.61%)	1 (1.84%)	-	-	1 (0.75%)	1 (0.77%)	-	-	3 (0.62%)
B. caballi	-	-	-	-	-	8 (6.14%)	8 (6.28%)	-	-	8 (1.66%)
H. canis	1 (0.31%)	1 (0.61%)	-	-	-	-	-	-	-	1 (0.21%)
R. africae	8 (2.52%)	3 (1.83%)	1 (1.82%)	-	4 (5.89%)	83 (77.45%)	82 (78.95%)	1 (33.33%)	1 (3.71%)	92 (19.85%)
T. mutans	18 (5.64%)	12 (7.32%)	-	-	6 (8.83%)	1 (0.75%)	1 (0.76%)	-	-	19 (3.93%)
T. parva	1 (0.31%)	-	-	-	1 (1.45%)	-	-	-	-	1 (0.21%)
T. taurotragi	6 (1.88%)	2 (1.21%)	1 (1.80%)	-	3 (4.38%)	-	-	-	-	6 (1.24%)
T. velifera	1 (0.31%)	-	-	-	1 (1.45%)	2 (1.49%)	2 (1.53%)	-	-	3 (0.62%)
CCHF virus	2 (0.62%)	1 (0.61%)	-	-	1 (1.45%)	-	-	-	-	2 (0.41%)

†Estimated individual-level prevalence percentages (in brackets) were calculated based on the size of each pool tested
‡These totals also include *Rh. microplus*, *Haemaphysalis* sp., and *Ct. felis* pools that were not positive for any pathogens

Only one *Rhipicephalus* sp. tick pool was positive for *T. parva* (GenBank accession MN294730), which shared 100% identity with a *T. parva* strain from Zambia (GenBank accession MG952926) using the 18S rRNA gene. (Table 2.2). Twelve out of 108 pools of *Rh. decoloratus* were positive for *Theileria mutans* (deposited GenBank accessions MN294725-MN294729) (96.9-100% identity to *T. mutans* strains: GenBank accessions; KJ941104, AF078815, KU206320 across the 18S rRNA gene region), while two pools were positive for *Theileria taurotragi* (deposited GenBank accessions MN294731-MN294732) (100% identity with 18S rRNA gene of a *T. taurotragi* strain: GenBank accession L19082 from South Africa). In *Rhipicephalus* sp., six pools were positive for *T. mutans*, three for *T. taurotragi*, and one for *Theileria velifera* (deposited GenBank accessions MN294733-MN294734) (100% identity with 18S rRNA gene of a *T. velifera* strain: GenBank accession MH424329 from Guinea). *Theileria mutans* was also detected in one *Rh. appendiculatus* and one *A. variegatum* pool. All *Theileria* spp. positive ticks were removed from cattle (Table A.3). I detected *Babesia caballi* (deposited GenBank accessions MN294721-MN294723) (99.3% identity with 18S rRNA gene of a *B. caballi* strain: GenBank accession MH424325 from Guinea) exclusively in eight *Am. variegatum* tick pools. Single pools each of *Rh. decoloratus*, *Rh. appendiculatus*, and *Am. variegatum* were positive for *Babesia bigemina* (deposited GenBank accession MN294720) (100% identity with 18S rRNA gene of a *B. bigemina* strain: GenBank accession MH356483 from Iraq). One pool of *Rh. decoloratus* was positive for *Hepatozoon canis* (deposited GenBank accession MN294724) (100% identity with 18S rRNA gene of a *H. canis* strain: GenBank accession MK673850 from France). The phylogenetic relationships of the apicomplexan parasite sequences identified in this study with homologous pathogen sequences are shown in Figure 2.5.

In addition to these pathogens, I detected *Coxiella* endosymbionts (deposited GenBank accessions MN262071-MN262076, MN266922-MN266928, MN266946-MN266948), which

are phylogenetically close to, but distinct from, *Coxiella burnetii*, the pathogen responsible for Q fever, in all tick genera except *Haemaphysalis*. The *Coxiella* endosymbionts characterised in this study fell into the group B and C clades of previously detected *Coxiella* endosymbionts of ticks (Figure 2.6).

No DNA/RNA of the pathogens evaluated in this study was detected in the flea specimens. All of the 33 selected associated livestock blood samples were negative for *R. africae* and CCHF virus. Thirty-one of these blood samples were from animals (28 cattle and three pigs) from which *R. africae* positive *Am. variegatum* ticks were collected, while the other two were from the cattle from which the two CCHF virus-positive *Rhipicephalus* spp. were obtained.

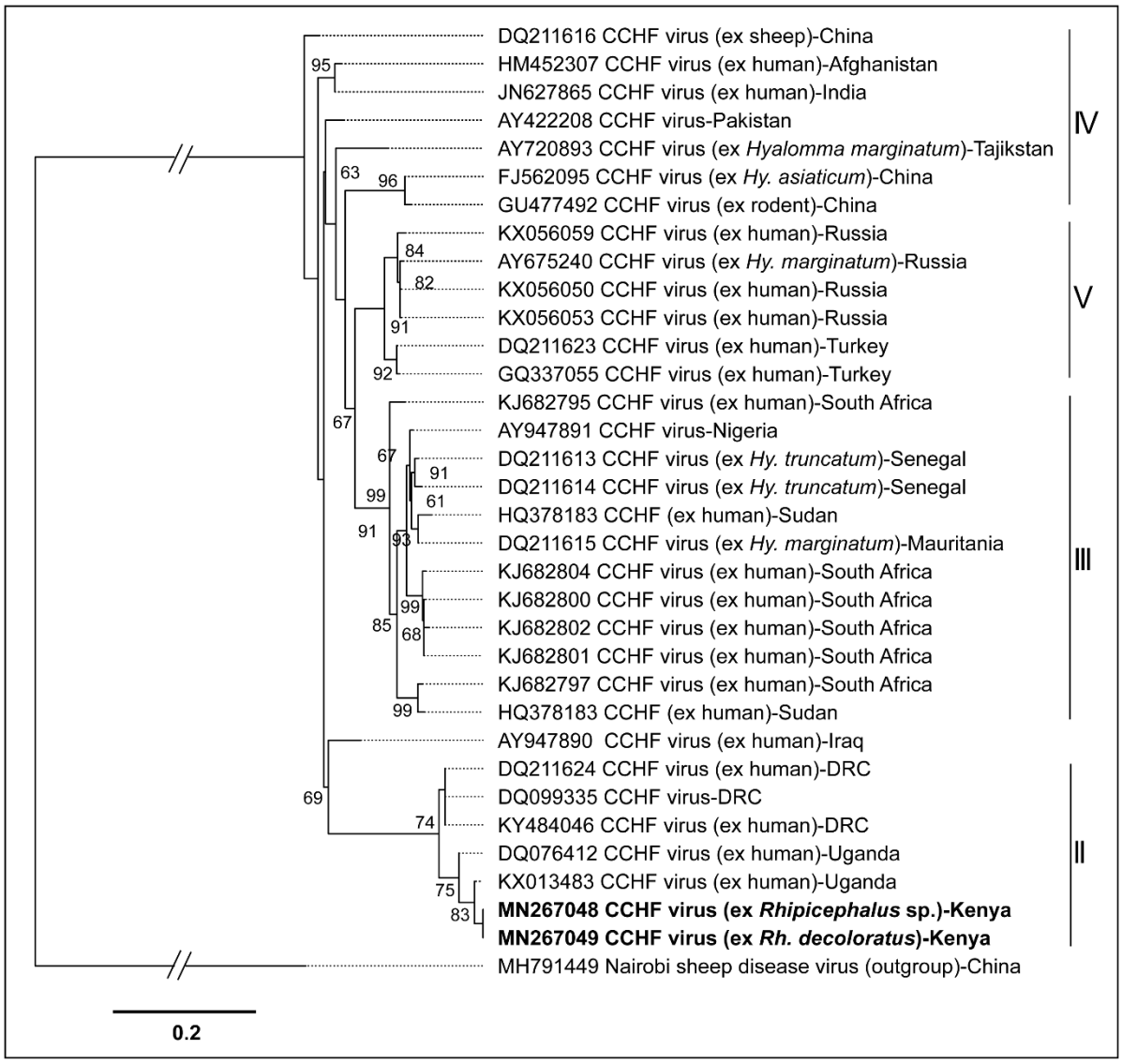


Figure 2.3: Maximum likelihood phylogeny of Crimean-Congo haemorrhagic fever virus strains inferred from 34 aligned 434-nt segments of the L-segment (RdRp gene).
Bootstrap values show percentage agreement from 1,000 replicates. The gaps to the Nairobi sheep disease outgroup represent 0.8 substitutions per site. The sequences from this study are in bold and fall into African genotype II as indicated by the vertical bars.

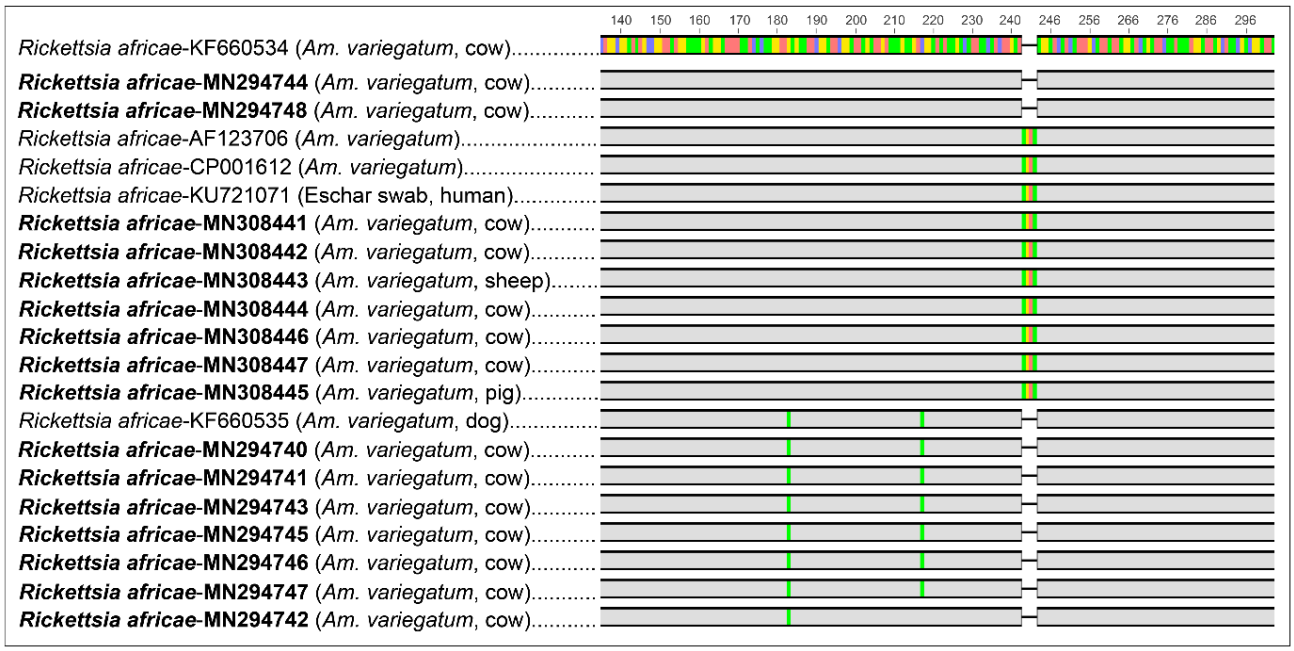


Figure 2.4: *Rickettsia africae* partial *ompB* gene sequences obtained from this study with GenBank reference sequences

Accession numbers of sequences from this study are in bold. Note the deletion mutation of a 4-base pair motif and several base substitutions in the sequences. Pink = Adenine; Blue = Cytosine; Green = Thymine; Yellow = Guanine; Grey = consensus with *R. africae* reference sequence**.**

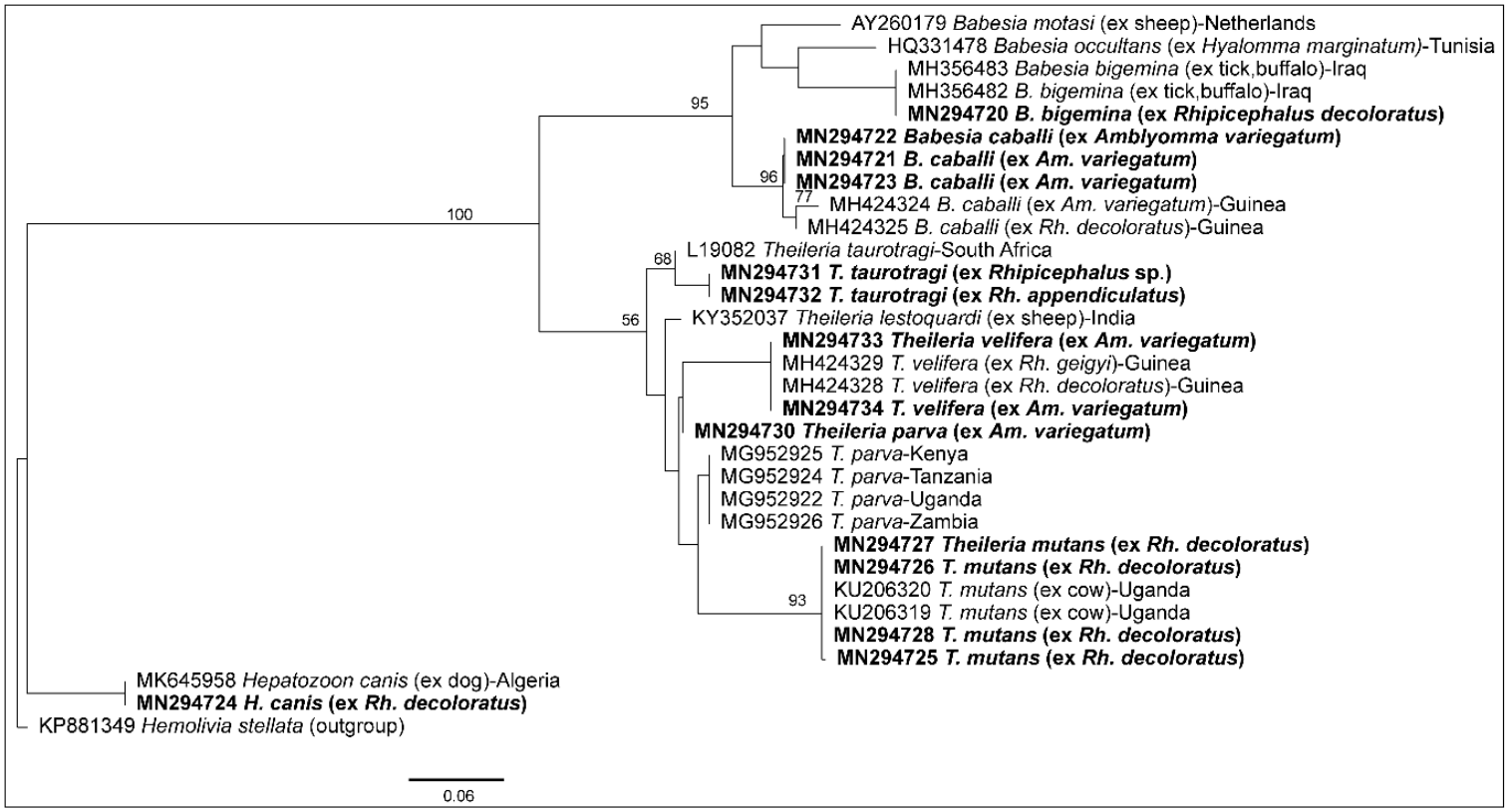


Figure 2.5: Maximum likelihood phylogeny of apicomplexan protozoa inferred from 32 aligned 502-nt segments of the 18S rRNA gene.
Sequences from this study are in bold. Bootstrap values indicate percentage conformity from 1,000 replicates.

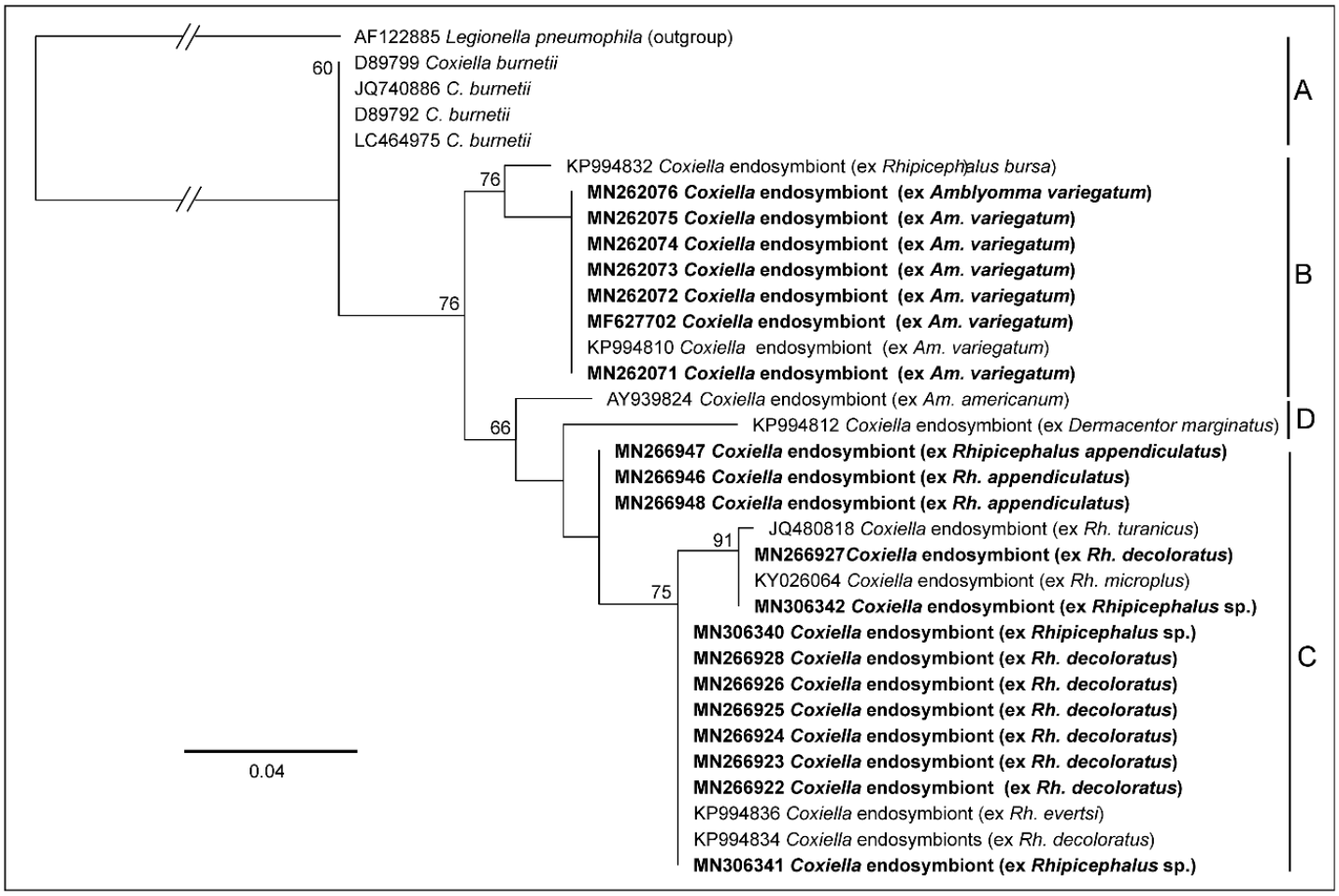


Figure 2.6: Maximum likelihood phylogeny of tick-associated *Coxiella* endosymbionts inferred from 33 aligned 279-nt segments of the 16S rRNA gene.
Sequences from this study are in bold and bootstrap values show percentage agreement from 1,000 replicates. The gaps to the *L. pneumophila* outgroup represent 0.12 substitutions per site. Sequences from this study and those from GenBank fall into three genotypes: **A** = *Coxiella burnetii* **B** = *Coxiella* endosymbionts of *Amblyomma* spp. ticks; **C** = *Coxiella* endosymbionts of *Rhipicephalus* spp. ticks; **D** = *Coxiella* endosymbionts of *Dermacentor* and *Amblyomma* spp. ticks.

2.5 Discussion

2.5.1 CCHF detection in ticks

I detected CCHF virus in ticks removed from cattle destined for slaughter at two SHs. This is the first description of CCHF virus in *Rh. decoloratus* ticks in Kenya, with previous studies reporting detection only in hyalommid ticks from the north-eastern region (Sang et al., 2011). This implies that besides *Hyalomma* spp. other tick species may support the local transmission of the virus. As the infected *Rhipicephalus* spp. ticks in this study were blood-fed and collected from livestock, I also tested the blood of the livestock from which they came from for CCHF virus, but they were negative. Association between infected ticks and seropositivity is common; however, ticks can also be found on seronegative animals and *vice-versa* (Spengler et al., 2016). Domestic animals, especially sheep, have been shown to be asymptomatic carriers of the virus (Spengler et al., 2016), acting as reservoirs of infection (*via* ticks) to humans, who suffer significant morbidity (Ergönül, 2006). While *Hyalomma* ticks are the natural vector and reservoir of CCHF virus, other tick genera such as *Rhipicephalus*, have been found infected with the virus (Fakoorziba et al., 2015; Hoogstraal, 1979). *Rhipicephalus* spp. have also transmitted CCHF virus in laboratory settings and are believed to support the circulation of the virus in natural settings (Balinandi et al., 2018; Ergönül, 2006). Therefore, *Rhipicephalus* spp. ticks may support transmission of the virus in areas where *Hyalomma* spp. are absent. However, confirmation of this requires comprehensive competency studies, and an understanding of the landscape epidemiology of this virus and its transmission is in its early stages.

In Africa, there are three distinct clades of CCHF virus and the close phylogenetic relationship between my isolates and the Nakiwogo strain isolated in Uganda is not surprising (Ergönül, 2006; Lukashev et al., 2016) given the geographical proximity of my study site to Uganda and the extensive trade in live animals between the two countries. This finding supports the circulation of a single strain of virus between the two countries, which may be facilitated

through cross-border movement of infected livestock. At-risk groups for CCHF virus infection include farmers, veterinarians, abattoir and health-care workers (Cook et al., 2017; Ergönül, 2006). CCHF outbreaks have not been reported in Kenya since the year 2000 when a fatal case occurred in western Kenya. However, Lwande et al. (2012) found a 23% human seroprevalence of IgG antibodies to CCHF virus in north eastern Kenya. Infection has been reported after skin contact with livestock, blood spatters during slaughtering, tick bites, and when health care workers take care of haemorrhaging patients (Ergönül, 2006). These findings therefore highlight the potential for human exposure to CCHF virus at these and other LMs and SHs, and at public health facilities, and emphasise the need for routine surveillance for this pathogen and adopting a *One Health* approach. Other LM/SH-based surveillance studies in Kenya have described the occurrence of other arboviruses in ticks, which indicates the importance of ticks in their epidemiology (Lwande et al., 2013; Sang et al., 2011, 2006). While most of these studies targeted pastoralist regions, my findings demonstrate that the risk of human exposure to tick-borne arboviruses is also present in tropical small-holder systems in East Africa.

While *Hyalomma* spp. ticks are the chief vectors of CCHF virus, other species may also be important in transmission ecologies due to co-feeding transmission even in the absence of viraemia in the host. An infected tick may transmit a virus to a non-infected co-feeding tick without the host having detectable virus in its blood (Kazimírová et al., 2017). Such non-viraemic transmission is presumed to contribute to amplification of CCHF virus in nature because the virus can be transmitted among ticks even without detectable viraemia in the host (Bente et al., 2013).

2.5.2 *Rickettsia africae* in ticks and lice

I demonstrated a high estimated true prevalence (78.95%) of *R. africae*, the cause of African tick bite fever (ATBF, also known as African tick typhus) in humans, in *Am. variegatum* ticks collected mostly from cattle. Ever since the first description in Kenya of *R. africae* in

Amblyomma ticks from the Maasai Mara region (Macaluso et al., 2003), high prevalence in *Amblyomma* ticks have been reported at SHs in Mombasa and Nairobi (Mutai et al., 2013), Siaya County, which borders Busia County (Maina et al., 2014), pastoralists in north eastern Kenya (Koka et al., 2017), the Shimba Hills National Reserve (Mwamuye et al., 2017), Baringo County (Omondi et al., 2017), and the Maasai Mara National Reserve (Oundo et al., 2020). *Rickettsia africae* has similarly been reported in *Amblyomma* ticks from Cameroon (Ndip et al., 2004), Zimbabwe (Beati et al., 1995), Senegal (Kelly et al., 2010), and the Central African Republic (CAR) (Dupont et al., 1995). I also detected *R. africae* at much lower prevalence in rhipicephaline ticks and, for the first time I am aware of it, in *H. suis* lice. While *Rickettsia* spp. have been detected in *Haematopinus eurysternus* lice before (Hornok et al., 2010), the epidemiological implications of this novel finding on the transmission of *Rickettsia* can be investigated by carrying out competence studies. Large scale collection and screening of *H. suis* for *Rickettsia* can then be carried out to ascertain the role of this species in transmission in local settings as there is a paucity of studies that have surveyed rickettsiae in lice in Africa. Admittedly, the detected *R. africae* could have been from the remnants of a blood-meal during co-feeding with *Am. variegatum* ticks, which were also infesting the pigs in this study.

The finding that all 34 livestock blood samples, from which the *R. africae*-positive ticks were obtained, were negative for the pathogen reinforces the notion that *Amblyomma* ticks are the major reservoir of the pathogen, but also suggests a low transmissibility to livestock; however, to ascertain this, competence studies need to be carried out.

In travel medicine, ATBF, which is characterised by headaches, inoculation eschar, rash, and myalgia (Jensenius et al., 2003), is believed to be only second to malaria as the aetiology of fever in travellers to sub-Saharan Africa (SSA). Most acute cases occur in tourists and foreign travellers with some fatal cases being reported (Rutherford et al., 2004). Although seroprevalence is usually high in native populations, acute clinical cases are few (Kelly et al.,

1991; Ndip et al., 2004). This may be due to exposure at an early age leading to only mild clinical cases that are ignored, poor visibility of inoculation eschars on pigmented skin, and lack of diagnostic capacity at most hospitals (Jensenius et al., 2003). Alternatively, some *R. africae* may be more virulent than others. In this study, I found *R. africae* variants that have been previously reported (Kimita et al.,2016; Macaluso et al., 2003; Maina et al., 2014). The differences found in the nucleotide composition of the *omp* B gene, which codes for the most immuno-dominant surface cell antigen of *Rickettsia,* could possibly affect the virulence of *R. africae* variants. Surface cell antigens are involved in cellular adhesion of *Rickettsia* and subsequent entry into cells (Blanc et al., 2003). The hypothesis that variants with an intact *omp* B gene are less virulent than those with the deletion (Maina et al., 2014) may explain the absence of acute ATBF cases in Kenya, despite the high seroprevalence. This is supported by the evidence that genome reduction may lead to increased virulence in *Rickettsia* (Fournier et al., 2009). However, it remains to be seen if some of these variants can be detected in febrile patients in my study area. Clearly there is a need for studies that focus on the public health aspect of this pathogen in endemic areas.

2.5.3 *Theileria, Babesia,* and *Anaplasma* spp. in ticks

I detected *A. marginale*, the cause of gall sickness, *B. bigemina,* which causes redwater, and *T. parva,* which causes East Coast fever in (*Rh. decoloratus* and *Rhipicephalus* sp.) 1.88%, (*Rh. decoloratus* and *Rh. appendiculatus*) 0.63%, and (*Rhipicephalus* sp.) 0.31% (estimated true prevalences) respectively. These three diseases are major impediments to livestock production in Kenya and SSA, causing severe loss of production in affected animals (Wesonga et al., 2010; Woolhouse et al., 2015). Recently *T. parva* was found more frequently in *Rh. appendiculatus* (15.7% of tick pools) sampled in the Maasai Mara National Reserve, where no *Babesia* spp. were detected (Oundo et al., 2020). The absence of *T. parva* in animal blood samples in this study may be partly explained by its biology, as for most of its life cycle it occurs in the

lymphoid system and it only multiplies in RBC for completion of its life cycle (Mans et al., 2015). Accordingly, I found higher prevalence in ticks of the mildly pathogenic *Theileria* spp., *T. taurotragi*, *T. velifera*, and *T. mutans* than reported by Njiiri *et al.* (2015) in calves in Busia, Kenya, and by Lorusso *et al.* (2016) in Nigerian cattle. Nonetheless, these species can also cause theileriosis in immuno-compromised animals. I also detected *Anaplasma platys*, in several pools of *Rhipicephalus* ticks from cattle. However, the *A. platys* sequences I detected could not distinguished between canine strains or other genetically-related strains. In future studies, genetic markers like the *groEL* gene, which are more discriminative than the 16S rRNA gene, should be used. This pathogenic bacterium has been reported in other studies in ticks and blood from livestock (Omondi et al., 2017; Said et al., 2017; Lorusso et al., 2016) and recent evidence suggests that *A. platys* and related strains may infect humans, posing a risk in cases of opportunistic tick bites (Arraga-Alvarado et al., 2014; Breitschwerdt et al., 2014; Maggi et al., 2013). Sheep and other small ruminants are considered the reservoirs and main hosts of *A. ovis*, however, its detection in ticks from cattle is not surprising as the main vectors; *Rhipicephalus evertsi* and other *Rhipicephalus* spp., also feed on cattle (Dahmani et al., 2019).

2.5.4 *Coxiella* endosymbionts of ticks

As with recent studies by Mwamuye *et al.* (2017) and Oundo *et al.* (2020), I also obtained *Coxiella* endosymbiont sequences from *Rickettsia* 16S rRNA primer amplicons. Previous studies have shown that these endosymbionts provide additional essential nutrients and reproductive fitness to ticks. Their elimination with antibiotic treatment was shown to negatively impact the fitness of the lone star tick *Amblyomma americanum* (Zhong et al., 2007). The phylogenetic co-divergence between the different tick species and their *Coxiella* endosymbionts shows the high specificity of these endosymbionts to their tick hosts. Four phylogenetic clades (A-D) have been described for tick-associated *Coxiella* endosymbionts and those from this study clustered into B and C clades. Group B consists of *Coxiella*-

endosymbionts of *Amblyomma* and *Ornithodoros*, while group C consists of rhipicephaline endosymbionts (Duron et al., 2015). These endosymbionts are non-pathogenic. However, it is important to note that there is evidence that *C. burnetii* evolved from these tick endosymbionts (Duron et al., 2015).

2.6 Conclusions

An array of protozoa, bacteria and viruses were identified in vectors collected from domestic animals at LMs and SHs. Significantly, the host animals were either being traded to destinations that were different from their origin or taken to slaughter, carrying infected vectors. These findings show how the animal trade can be the driver for new foci of infection in new areas, with risks to both domestic animal and human populations. Furthermore, their presence at SHs exposes abattoir workers, meat inspectors, butchers, and consumers to diseases like CCHF. The zoonotic pathogens detected here cause febrile illness that can be clinically difficult to differentiate from malaria or other non-specific fevers (Crump et al., 2013). Indeed, a large majority of non-malarial febrile cases are never properly diagnosed. Therefore, evidence of their possible circulation and risk for human infection warrants their inclusion, if not routinely due to limitations in clinical differential diagnostics, at least in routine prospective surveys in hospitals receiving febrile patients.

2.7 Acknowledgements

I gratefully acknowledge the Zoonoses in Livestock in Kenya (ZooLinK) team for collection of tick samples and Dr. Lillian Wambua (ZooLinK) for shipment of ticks to Nairobi from Busia and access to blood sample biobank and the database containing the metadata of the samples. I also acknowledge the technical contribution of Daniel O. Ouso for robotic total nucleic acid extraction, Kevin K. Ogola, Edwin K. Ogola, and the rest of the Martin Lüscher Emerging

Infectious Diseases (ML-EID) laboratory team, to this work. I also acknowledge Bester Mudereri of *icipe* GIS and Remote Sensing unit for assistance in producing the study site map.

2.8 Ethics statement

This study was nested within the Zoonoses in Livestock in Kenya (ZooLinK) project. Tick, louse, flea and blood samples were collected from cattle, goats, sheep, and pigs at LMs or presented for slaughter at SHs and approved by the International Livestock Research Institute Institutional Animal Care and Use Committee (ref IACUC-RC 2017-04). Data from human owners of livestock was collected after approval by the International Livestock Research Institute (ILRI) Institutional Research Ethics Committee (ref ILRI-IREC 2017-08/2). Both committees are licensed by the National Commission for Science, Technology and Innovation (NACOSTI) in Kenya.

2.9 References

Adjou Moumouni, P. F., Aboge, G. O., Terkawi, M. A., Masatani, T., Cao, S…Xuan, X. (2015). Molecular detection and characterization of *Babesia bovis*, *Babesia bigemina*, *Theileria* species and *Anaplasma marginale* isolated from cattle in Kenya. Parasites & Vectors, 8, 496. *doi*:10.1186/s13071-015-1106-9

Ajamma, Y. U., Onchuru, T. O., Ouso, D. O., Omondi, D., Masiga, D. K., & Villinger, J. (2018). Vertical transmission of naturally occurring Bunyamwera and insect-specific flavivirus infections in mosquitoes from islands and mainland shores of Lakes Victoria and Baringo in Kenya. PLoS Neglected Tropical Diseases, 12(11). *doi*: 10.1371/journal.pntd.0006949.

Altschul, S. F., Gish, W., Miller, W., Myers, E.W., & Lipman D.J. (1990). Basic Local Alignment Search Tool. Journal of Molecular Biology, 215, 403-410. *doi*: 10.1016/S0022-2836(05)80360-2

Arraga-alvarado, C. M., Qurollo, B. A., Parra, O. C., Berrueta, M. A., Hegarty, B. C., & Breitschwerdt, E. B. (2014). Case report: Molecular evidence of *Anaplasma platys* infection in two women from Venezuela. American Journal of Tropical Medicine and Hygiene, 91, 1161–1165. *doi*:10.4269/ajtmh.14-0372.

Baba, M., Masiga, D. K., Sang, R., & Villinger, J. (2016). Has Rift Valley fever virus evolved with increasing severity in human populations in East Africa? Emerging Microbes and Infections, 5(6), e58. *doi*:10.1038/emi.2016.57

Balinandi, S., Patel, K., Ojwang, J., Kyondo, J., Mulei, S., Tumusiime, A… Shoemaker, T.R (2018). Investigation of an isolated case of human Crimean-Congo hemorrhagic fever in Central Uganda. International Journal of Infectious Diseases, 68, 88-93. *doi*:10.1016/j.ijid.2018.01.013

Baron, M. D., & Holzer, B. (2015). Nairobi sheep disease virus/Ganjam virus. Scientific and Technical Review of the Office International des Epizooties, 34, 411-417. *doi*: 10.20506/rst.34.2.2367

Beati, L., Kelly, P. J., Matthewman, L. A., Mason, P. R., & Raoult, D. (1995). Prevalence of *Rickettsia*-like organisms and spotted fever group rickettsiae in ticks (Acari: Ixodidae) from Zimbabwe. Journal of Medical Entomology, 32, 787-792. *doi*: 10.1093/jmedent/32.6.787

Ben Said, M., Belkahia, H., El Mabrouk, N., Saidani, M., Alberti, A., Zobba, R…Messadi L (2017). *Anaplasma platys*-like strains in ruminants from Tunisia. Infection, Genetics and Evolution, 49, 226–233. *doi*:10.1016/j.meegid.2017.01.023

Bente, D. A., Forester, N. L., Watts, D. M., Mcauley, A. J., Whitehouse, C. A., & Bray, M. (2013). Crimean-Congo hemorrhagic fever: history, epidemiology, pathogenesis, clinical syndrome and genetic diversity. Antiviral Research, 100(1), 159-89. *doi*:10.1016/j.antiviral.2013.07.006

Blanc, G., Ngwamidiba, M., Ogata, H., Fournier, P., Claverie, J., & Raoult, D. (2003). Molecular evolution of *Rickettsia* aurface antigens: Evidence of positive selection. Molecular Biology and Evolution, 22,(10), 2073-83. *doi*:10.1093/molbev/msi199

Breitschwerdt, E. B., Hegarty, B. C., Qurollo, B. A., Saito, T. B., Maggi, R. G., & Blanton, L. S. (2014). Intravascular persistence of *Anaplasma platys*, *Ehrlichia chaffeensis*, and

Ehrlichia ewingii DNA in the blood of a dog and two family members. Parasites & Vectors, 7, 298. *doi*: 10.1186/1756-3305-7-298

Brown, L. D., & Macaluso, K. R. (2016). *Rickettsia felis*, an emerging flea-borne rickettsiosis. Current Tropical Medicine Reports, 3, 27–39. *doi*:10.1007/s40475-016-0070-6

Cook, E.A.J., Grossi-soyster, E. N., De Glanville, W. A., Thomas, L. F., Kariuki, S., Bronsvoort, B.M…Fevre, E.M. (2017). The sero-epidemiology of Rift Valley fever in people in the Lake Victoria Basin of western Kenya. PLoS Neglected Tropical Diseases, 11(7). *doi*:10.1371/journal.pntd.0005731

Cook, E. A. J., De Glanville, W. A., Thomas, L. F., Kariuki, S., Bronsvoort, B. M. de C., & Fèvre, E. M. (2017). Working conditions and public health risks in slaughterhouses in western Kenya. BMC Public Health, 17(1), 1–12. *doi*:10.1186/s12889-016-3923-y

Cowling, D. W., Gardner, I. A., & Johnson, W. O. (1999). Comparison of methods for estimation of individual-level prevalence based on pooled samples. Preventive Veterinary Medicine, 39(3), 211–225. *doi*:10.1016/s0167-5877(98)00131-7

Crump, J. A., Morrissey, A. B., Nicholson, W. L., Massung, R. F., Stoddard, R. A., Galloway, R. L…Bartlett, J.A.(2013). Etiology of severe non-malaria febrile illness in northern Tanzania: A prospective cohort study. PLoS Neglected Tropical Diseases, 7(7),e2324. *doi*:10.1371/journal.pntd.0002324

Dahmani, M., Davoust, B., Sambou, M., Bassene, H., Scandola, P., Ameur, T., Raoult, D Fenollar, F., & Mediannikov, O. (2019) Molecular investigation and phylogeny of species of the Anaplasmataceae infecting animals and ticks in Senegal. Parasites and Vectors 12, 495. *doi*.org/10.1186/s13071-019-3742-y

de la Fuente, J., Kocan, K. M., Almazán, C., & Blouin, E. F. (2008). Targeting the tick-pathogen interface for novel control strategies. Frontiers in Bioscience, 1, 6947-6956. *doi*:10.1093/cid/cir155

Doudier, B., Olano, J., Parola, P., & Brouqui, P. (2010). Factors contributing to emergence of *Ehrlichia* and *Anaplasma spp*. as human pathogens. Veterinary Parasitology, 167, 149–154. *doi*:10.1016/j.vetpar.2009.09.016

Dunster, L., Dunster, M., Ofula, V., Beti, D., Kazooba-Voskamp, F., Burt, F., … DeCock, K. M. (2002). First documentation of human Crimean-Congo hemorrhagic fever, Kenya. Emerging Infectious Diseases, 8(9), 1005–1006. *doi*:10.3201/eid0809.010510

Dupont, H. T., Brouqui, P., Faugere, B., & Raoult, D. (1995). Prevalence of antibodies to *Coxiella burnetii*, *Rickettsia conorii*, and *Rickettsia typhi* in seven African countries. Clinical Infectious Diseases, 21, 1126 - 33. *doi*: 10.1093/clinids/21.5.1126

Duron, O., Noël, V., Mccoy, K. D., Bonazzi, M., Sidi-Boumedine, K., Morel, O…Chevillon, C. (2015). The recent evolution of a maternally-inherited endosymbiont of ticks led to the emergence of the Q fever pathogen, *Coxiella burnetii*. PLoS Pathogens, 11, 1–23. *doi*:10.1371/journal.ppat.1004892

Edwards, E., Rogall, T., Blocker, H., Emde, M. & Bottger, E.C. (1989).Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. Nucleic Acids Research, 17, 7843–7853. *doi*: 10.1093/nar/17.19.7843

Endoh, D., Mizutani, T., Kirisawa, R., Maki, Y., Saito, H., Kon, Y., Morikawa, S., & Hayashi, M. (2005). Species-independent detection of RNA virus by representational

difference analysis using non-ribosomal hexanucleotides for reverse transcription. Nucleic Acids Research, 33(6), e65. *doi*:10.1093/nar/gni064

Ergönül, Ö. (2006). Crimean-Congo haemorrhagic fever. Lancet Infectious Diseases, 6, 203–14. *doi*: 10.1016/S1473-3099(06)70435-2

Fakoorziba, M. R., Naddaf-Sani, A. A., Moemenbellah-Fard, M. D., Azizi, K., Ahmadnia, S., & Chinikar, S. (2015). First phylogenetic analysis of a Crimean-Congo hemorrhagic fever virus genome in naturally infected *Rhipicephalus appendiculatus* ticks (Acari: Ixodidae). Archives of Virology, 160(5), 1197–1209. *doi*:10.1007/s00705-015-2379-1

Falzon, L. C., Alumasa, L., Amanya, F., Kang, E., Kariuki, S., Momanyi, K… Fèvre, E.M. (2019). One Health in action: Operational aspects of an integrated surveillance system for zoonoses in western Kenya. Frontiers in Veterinary Science, 6, 1–13. *doi*:10.3389/fvets.2019.00252

Fèvre, E. M., Picozzi, K., Fyfe, J., Waiswa, C., Odiit, M., Coleman, P. G., & Welburn, S. C. (2005). A burgeoning epidemic of sleeping sickness in Uganda. Lancet, 366(9487), 745–747. *doi*:10.1016/S0140-6736(05)67179-6

Fèvre, Eric M., Bronsvoort, B. M. D. C., Hamilton, K. A., & Cleaveland, S. (2006). Animal movements and the spread of infectious diseases. Trends in Microbiology, 14(3), 125–131. *doi*:10.1016/j.tim.2006.01.004

Fournier, P. E., El Karkouri, K., Leroy, Q., Robert, C., Giumelli, B., Renesto, P…Raoult, D. (2009). Analysis of the *Rickettsia africae* genome reveals that virulence acquisition in Rickettsia species may be explained by genome reduction. BMC Genomics, 10, 1–15. *doi*:10.1186/1471-2164-10-166

Fournier, P., Roux, V., Caumes, E., Donzel, M., & Raoult, D. (2017). Outbreak of *Rickettsia africae* infections in participants of an adventure race in South Africa. Clinical Infectious Diseases, 27, 316–23. *doi*: 10.1086/514664

Gachohi, J., Skilton, R., Hansen, F., Ngumi, P., & Kitala, P. (2012). Epidemiology of East Coast fever (*Theileria parva* infection) in Kenya : past , present and the future, Parasites and Vectors, 5,194. *doi*: 10.1186/1756-3305-5-194

Georges, K., Loria, G. R., Riili, S., Greco, A., Caracappa, S., Jongejan, F., & Sparagano, O. (2001). Detection of haemoparasites in cattle by reverse line blot hybridisation with a note on the distribution of ticks in Sicily.Veterinary Parasitology, 99, 273–286. *doi*: 10.1016/s0304-4017(01)00488-5

Guindon S., Dufayard J.F., Lefort V., Anisimova M., Hordijk W., Gascuel O., (2010). "New Algorithms and methods to estimate maximum-likelihood phylogenies: Assessing the performance of PhyML 3.0." Systematic Biology, 59, 307-21. *doi*: 10.1093/sysbio/syq010

Honig, J. E., Osborne, J. C., & Nichol, S. T. (2004). The high genetic variation of viruses of the genus *Nairovirus* reflects the diversity of their predominant tick hosts. Virology, 318, 10–16. *doi*:10.1016/j.virol.2003.09.021

Hoogstraal, H. (1979). The epidemiology of tick-borne Crimean-Congo Hemorrhagic fever in Asia, Europe, and Africa. Journal of Medical Entomology, 15 (4), 307-417

Hornok, S., Hofmann-Lehmann, R., de Mera, I. G., Meli, M. L., Elek, V., Hajtós, I., Répási, A., Gönczi, E., Tánczos, B., Farkas, R., Lutz, H., & de la Fuente, J. (2010). Survey on blood-sucking lice (Phthiraptera: Anoplura) of ruminants and pigs with molecular detection of *Anaplasma* and *Rickettsia* spp. Veterinary parasitology, 174(3-4), 355–358. *doi*:10.1016/j.vetpar.2010.09.003

Inziani, M., Adungo, F., Awando, J., Kihoro, R., Inoue, S., Morita, K., … Mwau, M. (2020). Seroprevalence of yellow fever, dengue, West Nile and chikungunya viruses in children in Teso South Sub-County, western Kenya. International Journal of Infectious Diseases, 91, 104–110. *doi*:10.1016/j.ijid.2019.11.004

Jensenius, M., Fournier, P., Kelly, P., Myrvang, B., & Raoult, D. (2003). African tick bite fever. The Lancet, 3, 557–564. *doi*: 10.1016/s1473-3099(03)00739-4

Jensenius, M., Fournier, P., & Raoult, D. (2017). Rickettsioses and the international traveler. Clinical Infectious Diseases, 39, 1493–9. *doi*: 10.1016/j.ijid.2003.06.004

Kazimírová, M., Thangamani, S., & Bartíková, P. (2017). Tick-borne viruses and biological processes at the tick-host-virus interface. Frontiers in Cellular and Infection Microbiology, 73, 39. *doi*:10.3389/fcimb.2017.00339

Kelly, P. J., Lucas, H., Eremeeva, M. E., Dirks, K. G., Rolain, J. M., Yowell, C…Raoult, D. (2010). *Rickettsia africae*, western Africa. Emerging Infectious Diseases, 16(3), 571-573 *doi*:10.3201/eid1603.090346

Kelly, P. J., Raoult, J. D., & Mason, P. R. (1991). Isolation of spotted fever group *Rickettsia* from triturated ticks using a modification of the centrifugation-shell vial technique. Transactions of the Royal Society of Tropical medicine and Hygiene, 85, 397-398. *doi*: 10.1016/0035-9203(91)90303-g

Kilpatrick, A. M., & Randolph, S. E. (2012). Drivers, dynamics, and control of emerging vector-borne zoonotic diseases. The Lancet, 380, 1946–1955. *doi*:10.1016/S0140-6736(12)61151-9

Kimita, G., Mutai, B., Nyanjom, S. G., Wamunyokoli, F., & Waitumbi, J. (2016). Phylogenetic variants of *Rickettsia africae*, and incidental identification of "*Candidatus* Rickettsia moyalensis" in Kenya. PLoS Neglected Tropical Diseases, 10, 1–14. *doi*:10.1371/journal.pntd.0004788

Koka, H., Sang, R., Kutima, H. L., Musila, L., & Macaluso, K. (2017). The detection of spotted fever group *Rickettsia* DNA in tick samples from pastoral communities in Kenya. Journal of Medical Entomology, 54, 774–780. *doi*:10.1093/jme/tjw238

Kumsa, B., Socolovschi, C., Raoult, D., & Parola, P. (2015). Spotted fever group rickettsiae in ixodid ticks in Oromia, Ethiopia. Ticks and Tick-Borne Diseases, 6(1), 8–15. *doi*:10.1016/j.ttbdis.2014.08.001

Latib, A. A., Rowlandsbj, G. J., Punyuaa, D. K., & Hassana, S. M. (1995). An epidemiological study of tick-borne diseases and their effects on productivity of zebu cattle under traditional management on Rusinga Island, western Kenya. Preventive Veterinary Medicine, 22, 169–181. *doi*: 10.1016/0167-5877(94)00408-B

Lorusso, V., Wijnveld, M., Majekodunmi, A. O., Dongkum, C., Fajinmi, A., Dogo, A. G…Picozzi1, K. (2016). Tick-borne pathogens of zoonotic and veterinary importance in Nigerian cattle. Parasites & Vectors, 9, 217. *doi*:10.1186/s13071-016-1504-7.

Luce-fedrow, A., Maina, A. N., Otiang, E., Ade, F., Omulo, S., & Ogola, E. (2015). Isolation of *Candidatus* Rickettsia asemboensis from *Ctenocephalides* fleas. International Journal of Systematic and Evolutionary Microbiology, 66, 4512–4517 *doi*:10.1089/vbz.2014.1744

Lukashev, A. N., Klimentov, A. S., Smirnova, S. E., Dzagurova, K., Drexler, J. F., & Gmyl, A. P. (2016). Phylogeography of Crimean Congo Hemorrhagic fever virus. PLoS ONE, 11, 1–14. *doi*:10.1371/journal.pone.0166744

Lutomiah, J., Musila, L., Makio, A., Ochieng, C., Koka, H., Chepkorir, E…Sang, R. (2014). Ticks and tick-borne viruses from livestock hosts in arid and semiarid regions of the eastern and northeastern parts of Kenya. Journal of Medical Entomology, 51, 269–277. *doi*:10.1603/ME13039

Lwande, O.W., Irura, Z., Tigoi, C., Chepkorir, E., Orindi, B., Musila, L., Venter, M., Fischer, A., Sang, R. (2012). Seroprevalence of Crimean Congo hemorrhagic fever virus in Ijara District, Kenya. Vector-Borne and Zoonotic Diseases, 12, 727-432. *doi*:10.1089/vbz.2011.0914

Lwande, O. W., Lutomiah, J., Obanda, V., Gakuya, F., Mutisya, J., Mulwa…Sang, R. (2013) Isolation of tick and mosquito-borne arboviruses from ticks sampled from livestock and wild animal hosts in Ijara District, Kenya. Vector-Borne and Zoonotic Diseases, 13, 637–642. *doi*:10.1089/vbz.2012.1190

Macaluso, K. R., Davis, J. O. N., Alam, U., Korman, A. M. Y., Rutherford, J. S., Rosenberg, R., & Azad, A. F. (2003). Spotted fever group rickettsiae in ticks from the Masai Mara region of Kenya. American Journal of Tropical Medicine and Hygiene, 68, 551–553. *doi*:10.4269/ajtmh.2003.68.551

Maggi, R. G., Mascarelli, P. E., Havenga, L. N., Naidoo, V., & Breitschwerdt, E. B. (2013). Co-infection with *Anaplasma platys*, *Bartonella henselae* and *Candidatus* Mycoplasma haematoparvum in a veterinarian. Parasites and Vectors, 6, 103. *doi*:10.1186/1756-3305-6-103

Maina, A. N., Jiang, J., Omulo, S. A., Cutler, S. J., Ade, F., Ogola, E…Richards, A. (2014). High prevalence of *Rickettsia africae* variants in *Amblyomma variegatum* ticks from domestic mammals in rural western Kenya: implications for human health. Vector-Borne and Zoonotic Diseases, 14, 693–702. *doi*:10.1089/vbz.2014.1578.

Maina, A. N., Klein, T. A., Kim, H., Chong, S., Yang, Y., Mullins, K…Richards, A. (2017). Molecular characterization of novel mosquito-borne *Rickettsia* spp. from mosquitoes collected at the Demilitarized Zone of the Republic of Korea. PLoS ONE, 12(11), e0188327, *doi*:10.1371/journal.pone.0188327

Maina, A. N., Luce-fedrow, A., Omulo, S., Hang, J., Chan, T., Ade, F…Richards, A. (2019). Isolation and characterization of a novel *Rickettsia* species (*Rickettsia asembonensis* sp. nov.) obtained from cat fleas (*Ctenocephalides felis*). International Journal of Systematic and Evolutionary Microbiology 66, 4512–4517. *doi*:10.1099/ijsem.0.001382.

Mans, B. J., Pienaar, R., & Latif, A. A. (2015). A review of *Theileria* diagnostics and epidemiology. International Journal for Parasitology: Parasites and Wildlife, 4(1), 104–118. *doi*:10.1016/j.ijppaw.2014.12.006

Mease, L. E., Coldren, R. L., Musila, L. A., Prosser, T., Ogolla, F., Ofula, V. O…Adungo, A. (2011). Seroprevalence and distribution of arboviral infections among rural Kenyan adults: A cross-sectional study. Virology Journal, 8, 371. *doi*:10.1186/1743-422X-8-371.

Mossel, E. C., Crabtree, M. B., Mutebi, J., Lutwama, J. J., Erin, M., Powers, A. M., & Miller, B. R. (2017). Arboviruses isolated from mosquitoes collected in Uganda, 2008–2012. Journal of Medical Entomology, 54, 1403–1409. *doi*:10.1093/jme/tjx120

Munyua, P., Murithi, R. M., Wainwright, S., Githinji, J., Hightower, A., Mutonga, D…Njenga, M.K. (2010). Rift Valley fever outbreak in livestock in Kenya, 2006 – 2007. American Journal of Tropical Medicine and Hygiene, 83, 58–64.

doi:10.4269/ajtmh.2010.09-0292

Mutai, B. K., Wainaina, J. M., Magiri, C. G., Nganga, J. K., Ithondeka, P. M., Njagi, O. N…Waitumbi, J.N. (2013). Zoonotic surveillance for rickettsiae in domestic animals in Kenya. Vector-Borne and Zoonotic Diseases, 13, 360–366. *doi*:10.1089/vbz.2012.0977

Mwamuye, M. M., Kariuki, E., Omondi, D., Kabii, J., Odongo, D., Masiga, D., & Villinger, J. (2017). Novel *Rickettsia* and emergent tick-borne pathogens: A molecular survey of ticks and tick-borne pathogens in Shimba Hills National Reserve, Kenya. Ticks and Tick-borne Diseases, 8, 208–218. *doi*:10.1016/j.ttbdis.2016.09.002

Nabeth, P., Cheikh, D. O., Lo, B., Faye, O., Ould, I., & Vall, M. (2004). Crimean-Congo hemorrhagic fever, Mauritania. Emerging Infectious Diseases, 10, 2143–2149. *doi*: 10.3201/eid1012.040535

Nakao, R., Qiu, Y., Igarashi, M., Magona, J. W., Zhou, L., Ito, K., & Sugimoto, C. (2013). High prevalence of spotted fever group rickettsiae in *Amblyomma variegatum* from Uganda and their identification using sizes of intergenic spacers. Ticks and Tick-Borne Diseases, 4(6), 506–512. *doi*:10.1016/j.ttbdis.2013.07.001

Nakayima, J., Magona, J. W., & Sugimoto, C. (2014). Molecular detection of tick-borne pathogens in ticks from Uganda. Research, 1:767. *doi*:10.13070/rs.en.1.767

Ndip, L. M., Bouyer, D. H., Travassos, A. P. A., Rosa, D., Titanji, V. P. K., Tesh, R. B., & Walker, D. H. (2004). Acute spotted fever rickettsiosis among febrile patients, Cameroon. Emerging Infectious Diseases, 10, 3–8. *doi*: 10.3201/eid1003.020713

Ndip, L. M., Fokam, E. B., Bouyer, D. H., Ndip, R. N., & Titanji, V. P. K. (2004). Detection of *Rickettsia africae* in patients and ticks along the coastal region of Cameroon. American Journal of Tropical Medicine and Hygiene, 71, 363–366.

Nijhof, A. R. D. M., Bodaan, C., Postigo, M., Nieuwenhuijs, H., Opsteegh, M., Franssen, L…Jongejan, F.(2007). Ticks and Associated Pathogens Collected from Domestic Animals in the Netherlands. Vector-Borne and Zoonotic Diseases, 7(4), 585-95. *doi*:10.1089/vbz.2007.0130

Njiiri, N. E., Bronsvoort, B. M. de C., Collins, N. E., Steyn, H. C., Troskie, M., Vorster, I…Toye, P.(2015). The epidemiology of tick-borne haemoparasites as determined by the reverse line blot hybridization assay in an intensively studied cohort of calves in western Kenya. Veterinary Parasitology, 210, 69–76. *doi*:10.1016/j.vetpar.2015.02.020

Norval, R. A. I., Fivaz, B. H., Lawrence, J. A., & Brown, A. F. (1984). Epidemiology of tick-borne diseases of cattle in Zimbabwe. Tropical Animal Health and Production, 16, 63-70. *doi*: 10.1007/bf02239846

Nyaruaba, R., Mwaliko, C., Mwau, M., Mousa, S., & Wei, H. (2019). Arboviruses in the East African Community partner states: a review of medically important mosquito-borne Arboviruses. Pathogens and Global Health, 113(5), 209–228. *doi*:10.1080/20477724.2019.1678939

Omondi, D., Masiga, D.K., Fielding, B.C., Kariuki, E., Ajamma, Y.U., Mwamuye, M.M., Ouso, D.O. & Villinger, J. (2017). 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. Frontiers in Veterinary Science, 4, 73. *doi*:10.3389/fvets.2017.00073

Oundo, J.W., Villinger, J., Jeneby, M., Ong'amo, G., Otiende M.Y., Makhulu, E.E., Musa, A.A., Ouso, D.O., & Wambua, L. (2020). Pathogens, endosymbionts, and blood-meal

sources of host-seeking ticks in the fast-changing Maasai Mara wildlife ecosystem. PLOS ONE, 15(8), e0228366. *doi*.org/10.1371

Papa, A., Tsergouli, K., Tsioka, K., & Mirazimi, A. (2017). Crimean-Congo hemorrhagic fever: Tick-host-virus interactions. Frontiers in Cellular and Infection Microbiology, 7, 213. *doi*:10.3389/fcimb.2017.00213

Parola, P., Inokuma, H., Camicas, J., Brouqui, P., & Raoult, D. (2001). Detection and identification of spotted fever group rickettsiae and ehrlichiae in African ticks. Emerging Infectious Diseases, 7(6), 1014-7. *doi*: 10.3201/eid0706.010616

Parola, P., Paddock, C. D., Socolovschi, C., Labruna, M. B., Mediannikov, O., & Kernif, T. (2013). Update on tick-borne rickettsioses around the world: a geographic approach. Clinical Microbiology Reviews, 26, 657–702. *doi*:10.1128/CMR.00032-13

Pratt, H. D & Wiseman, J.S. (1962) Fleas of public health importance and their control: Training guide - insect control series. Public Health Service Publication, 772.

Rambaut, A. (2014). FigTree v1.4.2, A Graphical Viewer of Phylogenetic Trees. Available from http://tree.bio.ed.ac.uk/software/figtree/

Raoult, D., & Roux, V. (1997). Rickettsioses as paradigms of new or emerging infectious diseases. Clinical microbiology reviews, 10(4), 694–719.*doi*:10.1128/CMR.10.4.694-719.1997

Reysenbach, A., Giver, L. J., Wickham, G. S., & Pace, N. R. (1992). Differential amplification of rRNA genes by polymerase chain reaction. Applied and Environmental Microbiology, 58, 3417–3418.

Ringo, A. E., Adjou Moumouni, P. F., Lee, S. H., Liu, M., Khamis, Y. H., Gao, Y., … Xuan, X. (2018). Molecular detection and characterization of tick-borne protozoan and rickettsial pathogens isolated from cattle on Pemba Island, Tanzania. Ticks and Tick-Borne Diseases, 9(6), 1437–1445. *doi*:10.1016/j.ttbdis.2018.06.014

Roux, V., & Raoult, D. (2000). Phylogenetic analysis of members of the genus *Rickettsia* using the gene encoding the outer membrane protein rOmpB (*ompB*). International Journal of Systematic and Evolutionary Microbiology, 50, 1449–1455. *doi*: 10.1099/00207713-50-4-1449

Rutherford, J. S., Macaluso, K. R., Smith, N., Zaki, S. R., Paddock, C. D., Davis, J…Rosenberg, R. (2004). Fatal spotted fever. Emerging Infectious Diseases, 10, 910–913. *doi*: 10.3201/eid1005.030537

Sang, R., Lutomiah, J., Koka, H., Makio, A., Chepkorir, E., Ochieng, C…Schnabel, D.(2011). Crimean-congo hemorrhagic fever virus in hyalommid ticks, northeastern Kenya. Emerging Infectious Diseases, 17, 1502–1505. *doi*:10.3201/eid1708.102064

Sang, R., Onyango, C., Gachoya, J., Mabinda, E., Konongoi, S., Ofula, V…Miller, B.(2006). Tickborne arbovirus surveillance in market livestock, Nairobi, Kenya. Emerging Infectious Diseases, 12, 1074–1080. *doi*:10.3201/eid1207.060253

Sergeant, E. S. G. (2018). Epitools Epidemiological Calculators. Ausvet. Available at: http://epitools.ausvet.com.au.

Simpson, D. I. H., Knight E.M, Courtois. G, Williams M. C, Weinbren M.P, Kibukamusoke J.W. (1967). Congo virus: a hitherto undescribed virus occurring in Africa. Part I. Human isolations-clinical notes. East African Medical Journal, 44, 87–92.

Spengler, J. R., Bergeron, É., & Rollin, P. E. (2016). Seroepidemiological studies of

Crimean-Congo hemorrhagic fever virus in domestic and wild animals. PLoS Neglected Tropical Diseases, 10, 1–28. *doi*:10.1371/journal.pntd.0004210

Teshale, S., Geysen, D., Ameni, G., Asfaw, Y., & Berkvens, D. (2015). Improved molecular detection of *Ehrlichia* and *Anaplasma* species applied to *Amblyomma* ticks collected from cattle and sheep in Ethiopia. Ticks and Tick-Borne Diseases, 6(1), 1–7. *doi*:10.1016/j.ttbdis.2014.04.023

Villinger, J., Mbaya, M. K., Ouso, D., Kipanga, P. N., Lutomiah,m J., Masiga, D.K. (2017). Arbovirus and insect-specific virus discovery in Kenya by novel six genera multiplex high-resolution melting analysis. Molecular Ecology Resources, 17(3), 466-480. *doi*:10.1111/1755-0998.12584

Walker, A. R., Bouattour, A., Camicas, J-L., Estrada-Peña, A., Horak I.G., Latif A.A…Preston, P.M. (2004). Ticks of domestic animals in the Mediterranean region: a guide to identification of species. University of Zaragoza, Zaragoza, Spain.

Wesonga, F. D., Kitala, P. M., Gathuma, J. M., Njenga, M. J., & Ngumi, P. N. (2010). An assessment of tick-borne diseases constraints to livestock production in a smallholder livestock production system in Machakos District, Kenya. Livestock Research for Rural Development, 22(6), 111.

Williams, C. J., Moffitt, C. M. (2001). A critique of methods of sampling and reporting pathogens in populations of fish. Journal of Aquatic Animal Health, 13(4), 300-309. *doi*: 10.1577/1548-8667(2001)013<0300:ACOMOS>2.0.CO;2

Woolhouse, M. E. J., Thumbi, S. M., Jennings, A., Chase-Topping, M., Callaby, R., Kiara, H., … Toye, P. G. (2015). Co-infections determine patterns of mortality in a population exposed to parasite infection. Science Advances, 1(2). *doi*:10.1126/sciadv.1400026

WHO (2018). Weekly bulletin on outbreaks and other emergencies. Week 26: June 2018. World Health Organisation. https://www.afro.who.int/health-topics/disease-outbreaks/outbreaks-and-other-emergencies-updates

Zhong, J., Jasinskas, A., & Barbour, A. G. (2007). Antibiotic treatment of the tick vector *Amblyomma americanum* reduced reproductive fitness. PLoS ONE, 2(5), e405. *doi*:10.1371/journal.pone.0000405

Chapter 3

A survey of mosquito-borne and insect-specific viruses in hospitals and livestock markets in western Kenya

Tatenda Chiuya[1,2*], Daniel K. Masiga[1], Laura C. Falzon[3,4], Armanda D.S. Bastos[2], Eric M. Fèvre[3,4], Jandouwe Villinger[1]

1. International Centre of Insect Physiology and Ecology (icipe), P.O Box 30772-00100, Nairobi, Kenya.
2. Department of Zoology and Entomology, University of Pretoria, Private Bag 20, Pretoria 0028, South Africa
3. Institute of Infection, Veterinary and Ecological Sciences, University of Liverpool, Leahurst Campus, Chester High Road, Neston, CH64 7TE, United Kingdom
4. International Livestock Research Institute, Old Naivasha Road, PO Box 30709, 00100 Nairobi, Kenya

This chapter has been submitted in part to *PLOS One* and is under review.

3.1 Abstract

Aedes aegypti and *Culex pipiens* mosquitoes are prolific vectors of arboviruses that affect the health of humans and animals. Increased globalization has facilitated the worldwide dissemination of these mosquitoes and the viruses they transmit. To assess disease risk, I determined the frequency of pathogenic arboviruses in western Kenyan counties bordering an area of high arboviral activity. In addition, insect-specific flaviviruses (ISFs), known to impair pathogenic arbovirus transmission, were also evaluated. I trapped mosquitoes in the short and long rainy seasons in 2018 and 2019 at livestock markets and hospitals. Mosquitoes were screened for dengue, chikungunya and other human pathogenic arboviruses, ISFs, and their blood-meal origins using high-resolution melting analysis of (RT-)PCR products. Of 6,848 mosquitoes collected, 89% were trapped during the long rainy season, with *A. aegypti* (59%) and *Cx. pipiens* (40%) being the most abundant. Most blood-fed mosquitoes were *Cx. pipiens* with blood-meals identified as being of human, chicken, and sparrow (*Passer* sp.) origin. I did not detect dengue or chikungunya viruses. However, one *Culex poicilipes* female was positive for sindbis virus, 30 pools of *Ae. aegypti* had cell fusing agent virus (CFAV; infection rate (IR) = 1.27%, 95% CI = 0.87%-1.78%); 11 pools of *Ae. aegypti* had Aedes flavivirus (AeFV; IR = 0.43%, 95% CI = 0.23%-0.74%); and seven pools of *Cx. pipiens* (IR = 0.23%, 95% CI = 0.1%-0.45%) and one pool of *Culex annulioris* had Culex flavivirus. The presence of sindbis virus in a single mosquito and infection of mosquitoes with ISFs may explain the limited arboviral outbreaks in the region given their transmission-blocking potential.

Keywords: *Aedes aegypti*, *Culex pipiens*, sindbis virus, surveillance, insect-specific flavivirus.

3.2 Introduction

Mosquitoes of the genera *Culex* and *Aedes* are the major vectors of arboviruses, bridging the transmission of viruses from the sylvatic world to urban settings [1]. *Culex pipiens* complex mosquitoes transmit West Nile and sindbis viruses. West Nile virus, first documented in Uganda [2], causes self-limiting febrile illness, which in rare cases proceeds to a fatal meningoencephalitis, while sindbis virus causes a febrile illness associated with chronic arthritis in humans [1]. Passerine birds are the reservoir and amplifying hosts for both viruses, while mammals, when infected, are considered dead-end hosts [3–5]. *Aedes aegypti* transmits dengue, yellow fever, chikungunya, Zika, and Rift Valley fever viruses, which are endemic in East Africa, including Kenya [6].

The ability of these viruses to cause worldwide epidemics is of increasing concern due to intensified globalization and travel [7–9]. Globally, vaccines against arboviruses are either not available or have limited use, and treatment is usually palliative [7, 10]. In developing countries, inadequate diagnostic capacity for these viruses is an additional challenge, especially in areas where other causes of febrile disease, like malaria, are present [11].

Arboviral disease control is more likely to be successful when the vector species present, and their competence, is known. The use of bed nets and residual insecticide spraying have been successful in reducing malaria transmission, but are less effective in reducing arbovirus transmission due to differences in the feeding and resting behaviour of anopheline and culicine mosquitoes [12]. The use of insect-specific flaviviruses (ISFs) that naturally infect *Aedes* and *Culex* mosquitoes as potential regulators against infection with pathogenic arboviruses via superinfection exclusion mechanisms has been suggested [13]. Replication of ISFs in co-infected cells is believed to be more efficient, thereby competitively suppressing the proliferation of pathogenic arboviruses [14].

Many *Aedes* and *Culex* mosquitoes are adapted to a domestic life cycle, breeding in man-made habitats and biting people indoors and outdoors. Some of their breeding sites include open septic tanks, bushy/grassy places, discarded tyres/cars, jars, drums, and any other open water containers [1,15]. Studies in East Africa have demonstrated the presence of several arboviruses of public health importance [6,16], but the links between human and livestock infections have not been explored. Therefore, in this study, I surveyed selected hospitals and livestock markets (LMs) in western Kenya for the presence of mosquito-borne viruses. Some of the markets where ticks were collected from livestock in Chapter 2 were selected for mosquito trapping. Specifically, I investigated mosquito diversity and abundance associated with these settings, host-feeding preferences and the frequency of arboviruses and ISFs within the mosquitoes. Additionally, I described the implementation of mosquito control strategies at these sites.

3.3 Materials and methods

3.3.1 Sampling sites selection

The sampling sites were in the three western Kenyan counties of Bungoma, Busia, and Kakamega, which border Uganda. This region occurs within the wider Lake Victoria basin of East Africa whose ecology is likely to support an abundant mosquito population. The selection of sampling sites is described elsewhere [17]. Briefly, 12 LMs and neighbouring hospitals, four in each of the three counties, were selected for an integrated surveillance program. The selection of the LMs was based on the size and catchment area, whereas selection of the hospitals was based on the number of outpatients and the type of hospital. Specifically, both public (Referral and sub-County) and private (Missionary) hospitals were included. Finally, logistical factors such as the distance to the field laboratory in Busia, were also taken into consideration.

For this survey, six hospitals (a public and a Missionary hospital in each county) were originally selected for mosquito sampling. Factors impacting mosquito habitat, resting, and feeding behavior, such as hospital size and in/outpatient number, were considered in the selection process. Similarly, availability of mosquito habitat, resting places, proximity to human dwellings, and security for setting up mosquito traps were considered when selecting LMs in each county.

A pilot study was conducted in the short rainy season from 17 October 2018 to 7 December 2018 at six hospitals (Lugulu Missionary, Bungoma Referral, Busia Referral, Butula Missionary, Matungu sub-County, and Mukumu Missionary) and four LMs (Lubao, Angurai, Kimilili, and Chwele). In Kakamega county Matungu sub-County hospital replaced the Kakamega Referral hospital due to on-site consent challenges. Sampling in the long rainy season was done from 9 May to 26 June 2019 when mosquito habitat and density were expected to be high. The same six hospitals were sampled during the long rainy season; however, due to poor mosquito catches and logistical challenges at some LMs during the short rainy season (pilot), only Lubao LM and an additional LM in Funyula were sampled. Figure 3.1 shows the locations of all the hospitals and LMs where mosquitoes were sampled in the short and long rainy seasons.

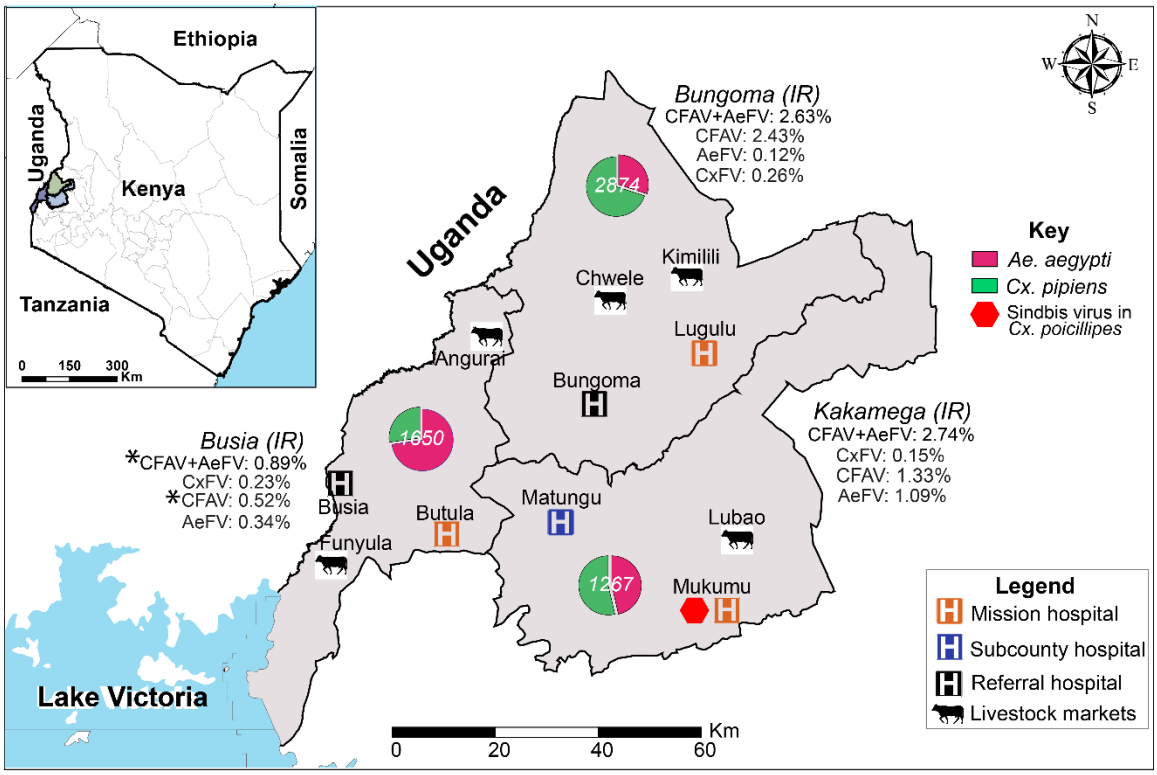


Figure 3.1: Location of mosquito traps in the three counties in western Kenya.
Pie charts of the relative abundance of *Ae. aegypti* and *Cx. pipiens* and their infection rates (IR) with cell fusing agent virus (CFAV)/Aedes flavivirus (AeFV) and Culex flavivirus (CxFV), respectively, are shown for each county. Infection rates that were significantly lower in Busia than in the other two counties are indicated by asterisks.

3.3.2 Questionnaire on mosquito control at hospitals

Concurrently with the mosquito collection at hospitals during the long rainy season, a short questionnaire was administered to capture information about the methods implemented to control the breeding of mosquitoes and prevent them from biting patients and personnel at hospitals (Figure A.3). I administered the questionnaire at all sites to the public health officers, medical superintendents, or hospital administrators. I also recorded direct observations on the presence of the above-mentioned mosquito habitats.

3.3.3 Mosquito trapping and schedule

During the short rainy season (pilot), seven CDC light (John W. Hock Company, Gainesville, USA) and seven BG sentinel traps with a lure (Biogents, Regensburg, Germany) were set for one night and one day, respectively, at each site. All traps were baited with dry ice delivered from insulated dry ice containers. CDC light traps were set and run from 6:30 pm to 6:30 am the next day, while BG sentinel traps were run from 6:30 am to 6:15 pm. In the long rainy season, seven CDC light traps and seven BG sentinel traps were set for three consecutive nights and days, respectively, at the six hospitals. At the LMs, the CDC and BG traps were set for two consecutive nights and days, respectively.

Traps in hospital settings were placed away from direct wind, foot traffic, and artificial lighting. Preferred locations for CDC traps were disused pit latrines, dilapidated buildings, broken-down vehicles, and uncovered septic tanks. In some instances, and following consultation with the hospital staff, traps were set in patient wards and consultation rooms. BG sentinel traps were placed in grassy or bushy locations of the hospital, away from direct sunlight and wind gusts. At LMs, CDC light traps were hung around the perimeter of the market and close to any surrounding homesteads, while BG sentinel traps were placed in grassy shaded places around the markets. Traps were set for a cumulative 231 trap days and 223 trap nights; these included 63 trap days and nights during the pilot phase conducted during the short rainy season, and 168 trap days and 160 trap nights in the long rainy season.

This study was nested within the Zoonoses in Livestock in Kenya (ZooLinK) project. The trapping of mosquitoes and interviews at hospitals were carried out under the approval of the ILRI Institutional Research Ethics Committee (IREC) under protocol number ILRI-IREC2017-08/2. The IREC is licensed by the National Commission for Science, Technology and Innovation (NACOSTI) in Kenya. At each of the selected hospitals sampling was carried out with permission from the medical superintendent or administrative officer. In Kakamega

County, Matungu sub-County Hospital replaced the planned sampling site at Kakamega Referral Hospital due to logistical and consent challenges. At LMs, the chairpersons of the two markets were informed of the planned exercise before sampling began.

3.3.4 Storage of mosquitoes and identification

Mosquitoes were collected alive in the evening and early morning. They were anaesthetized with ethyl acetate, sorted to remove non-target insects and stored in cryovials in a nitrogen tank. They were shipped to the Martin Lüscher Emerging Infectious Disease (ML-EID) Laboratory at the International Centre of Insect Physiology and Ecology (*icipe*) in Nairobi and identified morphologically on chilled blocks under a stereomicroscope with the aid of identification keys [18,19]. Mosquitoes were grouped in pools of up to 25 mosquitoes per pool, according to the site, trap type, date of collection, sex, and species. Blood-fed mosquitoes were placed individually in Eppendorf tubes for blood-meal determination.

3.3.5 Nucleic acid extraction

Mosquito pools and individual mosquito abdomens were homogenized by mechanical disruption in 1.5-ml micro-centrifuge tubes with ten 2.0-mm zirconia/yttria stabilised zirconium oxide beads (Biospec, USA) using a Mini Bead Beater 16 (BioSpec, Bartlesville, USA) for 45-70 seconds. For blood-fed mosquitoes, the head and thorax were removed from the rest of the body using sterile 10-µl pipette tips before processing. After homogenization, 410 µl of PBS was added to each micro-centrifuge tube containing either a mosquito pool, or an engorged abdomen. The magnetic-based Magbio HighPrep™ Viral DNA/RNA Kit (Gaithersburg, USA) was used for rapid isolation of total nucleic acids. Initially, 200 µl of the homogenized sample was mixed with 528 µl of a lysis master-mix, 10 µl magnetic beads, and 10 µl proteinase K before proceeding with the rest of the protocol as per manufacturer' instructions. Total nucleic acid was eluted in 100 µl elution buffer. Dengue serotype 2 and

sindbis viruses cultured on Vero cell lines in a previous study were included as positive extraction controls in each extraction run [20].

After nucleic acid extraction, 15 µl of the total RNA was subjected to cDNA synthesis using a High Capacity cDNA Reverse Transcription (RT) Kit (Life Technologies, USA). The 30-µl reaction mixtures contained 1X RT buffer, 4 mM dNTPs, 600 µM random hexamers [21], 2.5 U/µl reverse transcriptase enzyme, and 1U/µl RNAse inhibitor.

3.3.6 Blood-meal analysis

Blood-meal analysis was carried out on each individually extracted blood-fed mosquito to determine the vertebrate host of the blood meal, using cytochrome *b* (cyt *b*) and 16S rRNA markers [22]. Total nucleic acid (1 µl) from each blood-fed mosquito was used as template in 10-µl PCRs containing 2 µl of 5X HOT FIREPol® EvaGreen® qPCR Mix (Solis BioDyne, Estonia) and 10 pmoles of each forward/reverse primer. Thermo-cycling and high-resolution melting (HRM) analysis were carried out in a Rotor-Gene Q real-time PCR thermo-cycler (Qiagen, Hilden Germany) as previously described [23]. DNA extracted from human, cattle, sheep, goat, pig, camel, and chicken samples served as positive controls in each of the runs. Rotor-Gene Q software 2.1.0 was used to select representative amplicons for post-PCR purification (Exo 1-rSAP combination, Biolabs, UK) and sequencing at Macrogen (The Netherlands).

3.3.7 Molecular detection of viruses

Mosquito pools were screened for six arbovirus genera (*Flavivirus*, *Alphavirus*, *Phlebovirus*, *Orthobunyavirus*, *Nairovirus*, and *Thogotovirus*) using a multiplex PCR that uses degenerate primers coupled with end-reaction high resolution melting analysis (PCR-HRM) [20]. The most important viruses targeted in these genera were dengue, yellow fever, chikungunya, West Nile, Rift Valley fever and sindbis viruses. Each 10-µl reaction mixture contained 5 µl of 2X

MyTaq master-mix (Bioline, UK), 50 mM Syto-9 dye (Life Technologies, Carlsbad, USA), and a degenerate primer mix (Table 3.1). Cycling and HRM analysis was done in a Rotor-Gene Q real-time PCR thermo-cycler (Qiagen, Hilden Germany) using conditions described by Villinger et al. [20]. Dengue virus serotype 2 and sindbis virus cDNA served as *Flavivirus* and *Alphavirus* positive controls, respectively, and molecular grade PCR water as the negative control.

All positive samples from the multiplex PCR-HRM, identified by visual inspection of the HRM profiles on the Rotor-Gene Q software 2.1.0, were selected for genus-specific (single-plex) amplification using the conditions described above. Representative positive samples from the single-plex runs were selected and prepared for sequencing using the Exo 1-rSAP combination (Biolabs, UK). Bi-directional sequencing was outsourced to Macrogen (The Netherlands). Sequence chromatograms were inspected, edited, and aligned using Geneious Prime version 2019.0.4 software (Biomatters, New Zealand). The resulting sequence contigs were used in nucleotide BLAST search.

To generate a longer 900 bp fragment for flaviviruses, positive samples were re-amplified using nested conventional PCR targeting the non-structural protein 5 (NS5) gene [24] (Table 3.1). The 20-µl primary reaction mix contained 4 µl 5X HOT FIREPol® EvaGreen® qPCR Mix (Solis BioDyne, Estonia), 10 pmoles of each primer and 1 µl of the template. For the nested amplifications, 1 µl of the first-round PCR product was used as template. Thermal cycling conditions for first and second round PCR comprised an initial hot start step of 95°C for 15 minutes followed by denaturation at 94°C for 60 secs, annealing for 40 secs, and extension at 72°C for 2 min, with a final extension at 72°C for 5 min. Annealing temperature and cycle number for first and second round PCR were 54°C and 40 cycles, and 60°C and 35 cycles, respectively. The DNA (no-RT controls) of all samples positive for flaviviruses were screened

using the same methods described above to rule out non-specific amplification of integrated viral elements in the mosquito genome.

For alphaviruses, to generate a longer 320-nt fragment, I used a conventional, hemi-nested PCR targeting the non-specific protein 1 (NS1) gene with previously described primers [25]. The 10-µl first-round reaction mixtures contained 2 µl 5X HOT FIREPol® EvaGreen® qPCR Mix (Solis BioDyne, Estonia), 10 pmoles of each primer and 1 µl of the template. In the second round of amplification, 1.25 µl of the product was used as a template in a 20 µl mixture. The cycling conditions were as follows: An initial hot start step of 95°C for 15 minutes followed by 45 cycles of 94°C for 20 secs, 50°C for 30 secs, and 72°C for 30 secs, and a final extension at 72°C for 5 min. The same conditions were used for the second round except that the annealing was at 48°C. Positive controls were included in each run as above. Amplicons were visualized by agarose gel electrophoresis. Positives were then purified for sequencing, resulting sequences edited and then identity confirmed as described above.

Table 3.1: Primer details of all the primers used in this study

Target gene	Primer name	Primer sequence (5' – 3')	Product size (bp)	References
Vertebrate 16S	Vert 16S F	GAGAAGACCCTRTGGARCTT	250	[23]
	Vert 16S R	CGCTGTTATCCCTAGGGTA		
Vertebrate cyt *b*	Cytb F	CCCCTCAGAATGATATTTGTCCTCA	310	[26]
	Cytb R	CATCCAACATCTCAGCATGATGAAA		
***Alphavirus* NS4**	Vir 2052 F	TGGCGCTATGATGAAATCTGGAATGTT	150	[27]
	Vir 2052 R	TACGATGTTGTCGTCGCCGATGAA		
***Flavivirus* NS5**	Flavi JV2a F	AGYMGHGCCATHTGGTWCATGTGG	150	[20]
	Flavi JV2b F	AGCCGYGCCATHTGGTATATGTGG		
	Flavi JV2c F	AGYCGMGCAATHTGGTACATGTGG		
	Flavi JV2d F	AGTAGAGCTATATGGTACATGTGG		
	Flavi JV2a R	GTRTCCCADCCDGCDGTRTCATC		
	Flavi JV2b R	GTRTCCCAKCCWGCTGTGTCGTC		
***Flavivirus* NS5**	1NS5F	GCATCTAYAWCAYNATGGG	930	[24]
	1NS5R	CCANACNYNRTTCCANAC		
	2NS5F	GCNATNTGGTWYATGTGG		
	2NS5R	CATRTCTTCNGTNGTCATCC		
***Alphavirus* NS1**	m2w	YAGAGCDTTTTCGCAYSTRGCHW	320	[25]
	m2w2	TGYCCNVTGMDNWSYVCNGARGAYCC		
	cm3w	ACATRAANKGNGTNGTRTCRAANCCDAYCC		

3.3.8 Phylogenetic analysis, calculation of infection rates and statistical analysis

A maximum likelihood phylogeny of the detected *Flavivirus* NS5 gene sequences was constructed with PHyML v. 3.0 [28]. The appropriate model of phylogeny was automatically selected using the Akaike information criterion (AIC) [29]. The phylogenetic trees were viewed in FigTree v.1.4.2 [30]. To estimate the infection rates (IRs), maximal likelihood estimates were calculated using the PooledInfRate, version 4.0 Microsoft Excel® Add-In, and expressed per 100 (%) mosquitoes tested [31]. Logistic regression analysis was done in R® version 3.5.3 to test the association between sampling sites, mosquito sex, and season (predictor variables), and a mosquito pool testing positive for ISFs (outcome variable). The odds ratios (OR), 95% confidence intervals (CI), and *p*-values were computed, and a *p*-value of 0.05 or less was considered statistically significant.

3.4 Results

3.4.1 Mosquito abundance and species diversity

A total of 6,848 mosquitoes separated into 545 pools (≤25 individuals/pool) were collected (Table 3.2). The 2019 long rainy season collections accounted for 89.08% (n = 6,100) of the total catch, while the 2018 short rainy season (pilot) made up the remainder (10.92%; n = 748). The collection comprised 21 mosquito species from three genera (Table 3.2), inclusive of 38 blood-fed specimens which were processed individually. The most abundant mosquitoes were from the genus *Culex,* contributing 59.49% (n = 4,074) of the total catch, followed by 39.66% (n = 2,716) *Aedes* and 0.85% (n = 58) *Anopheles* mosquitoes. The most abundant species were *Cx. pipiens* (n = 3,130) and *Ae. aegypti* (n = 2,661), translating to 45.71% and 38.86% of the total catch, respectively. A total of 6,539 mosquitoes were collected from the selected six hospitals, 631 and 5,908 during the short and long rainy seasons, respectively. Among these, the mosquito abundance was highest at Lugulu Missionary (n = 2,170), followed by Busia Referral (n = 1,212), Bungoma Referral (n = 1,065), Mukumu Missionary (n = 775), Matungu sub-County (n = 675), and Butula Missionary (n = 642) hospitals (Table 3.2).

BG sentinel collections during the day consisted mostly of *Aedes* spp., of which *Ae. aegypti* was the dominant species, comprising 38.29% (n = 2,622) of the total catch; very few specimens of the other six *Aedes* spp. were collected (Table 3.2). CDC light trap collections were dominated by *Cx. pipiens*, which accounted for 45.02% (n = 3,083) of the total catch. There was also a significant number (n = 777) of *Cx. zombaensis.* Overall, 309 mosquitoes were collected at LMs: 117 during the short rainy season (pilot) and 192 during the long rainy season. *Aedes aegypti* (33.33%; n = 103) was the most abundant species collected, followed by *Cx. pipiens* (27.18%; n = 84) at LMs (Table 3.2).

Of particular note were the higher night catches of *Cx. pipiens* mosquitoes in the vicinity of several sewage tanks that were not covered at Lugulu Missionary (n = 1,300) and Bungoma Referral (n = 702) hospitals, compared to the other four sites where these habitats were absent. More *Ae. aegypti* mosquitoes were also collected during the day at Busia Referral (n = 837) and Lugulu Missionary (n = 630), where there was a combination of huge piles of disused vehicle tyres and tall grasses, compared to the other sites where such features were absent. Dilapidated houses, broken down vehicles, bushes, and grassy areas were present at all of the hospital sites (Figure A.2).

Table 3.2: Summary table of mosquitoes caught during the long and short rainy season in western Kenya

	Busia RH	Butula MH	Bungoma RH	Lugulu MH	Mukumu MH	Matungu SCH	Lubao LM	Funyula LM	Angurai LM	Chwele LM	Kimilili LM
Long rainy season (May - June 2019)											
Aedes aegypti	762	240	157	582	307	119	30	44	-	-	-
Aedes africanus	-	9	-	2	-	-	-	-	-	-	-
Aedes hirsutus	1	1	-	-	-	-	-	-	-	-	-
Aedes mcintoshi	-	-	-	-	-	1	-	8	-	-	-
Aedes metallicus	-	-	-	-	4	3	5	-	-	-	-
Aedes simpsoni	-	-	-	-	3	2	-	-	-	-	-
Aedes tricholabis	-	-	-	2	-	-	-	-	-	-	-
Anopheles coustani	-	-	-	-	-	-	3	-	-	-	-
Anopheles funestus	-	1	-	-	-	1	7	-	-	-	-
Anopheles gambiae	1	16	3	-	1	14	3	-	-	-	-
Culex annulioris	-	5	-	2	10	1	7	-	-	-	-
Culex cinerellus	-	-	-	-	-	-	5	-	-	-	-
Culex cinereus	-	11	1	-	2	3	-	-	-	-	-
Culex pipiens	255	129	646	1273	269	307	63	1	-	-	-
Culex poicilipes	-	-	-	-	1	-	-	-	-	-	-
Culex rubinotus	-	5	-	-	-	-	-	-	-	-	-
Culex tigripes	1	-	-	18	-	-	1	-	-	-	-
Culex univittatus	1	5	-	-	-	-	3	-	-	-	-
Culex vansomereni	-	3	1	8	2	5	2	-	-	-	-
Culex zombaensis	114	27	119	202	85	165	10	-	-	-	-
	Short rainy season (October - December 2018)										
Aedes aegypti	75	84	79	48	62	43	27	-		1	1
Aedes metallicus	-	1	-	-	-	-	-	-	-	-	-
Aedes sp.	-	-	-	-	-	-	11	-		2	
Anopheles funestus	-	-	-	-	-	-	1	-	-	-	-
Anopheles gambiae	-	-	-	-	-	-	5	-	-	-	1
Anopheles squamosus	-	-	-	-	-	-	1	-	-	-	-
Culex annulioris	-	-	-	-	-	-	9	-	-	-	-
Culex cinerellus	-	5	-	-	-	1	30	-	-	-	-
Culex pipiens	2	58	56	27	14	10	16	-	-	3	1
Culex sp.	-	-	-	-	-	-	-	-	4	-	-
Culex vansomereni	-	2	0	2	7	-	4	-	-	-	-
Culex zombaensis	-	40	3	4	8	-	-	-	-	-	-

3.4.2 Blood-meal analysis

Of the 38 blood-fed individual mosquitoes, 35 were *Cx. pipiens*, two were *Ae. aegypti*, and one was *An. gambiae.* The blood-meals were from five vertebrate species: human, cattle, dog, chicken, and sparrow (Table 3.3). Two blood-meals (from one *An. gambiae* and one *Cx. pipiens*) could not be resolved by amplification with either cyt *b* or 16S rRNA markers (Table 3). The melt rate profiles of the samples and the positive controls are shown in Figure 3.2. Non-amplification in one of the markers was resolved by amplification with the other marker. Most of the blood-fed mosquitoes were caught at Matungu sub-County hospital (Table 3.3).

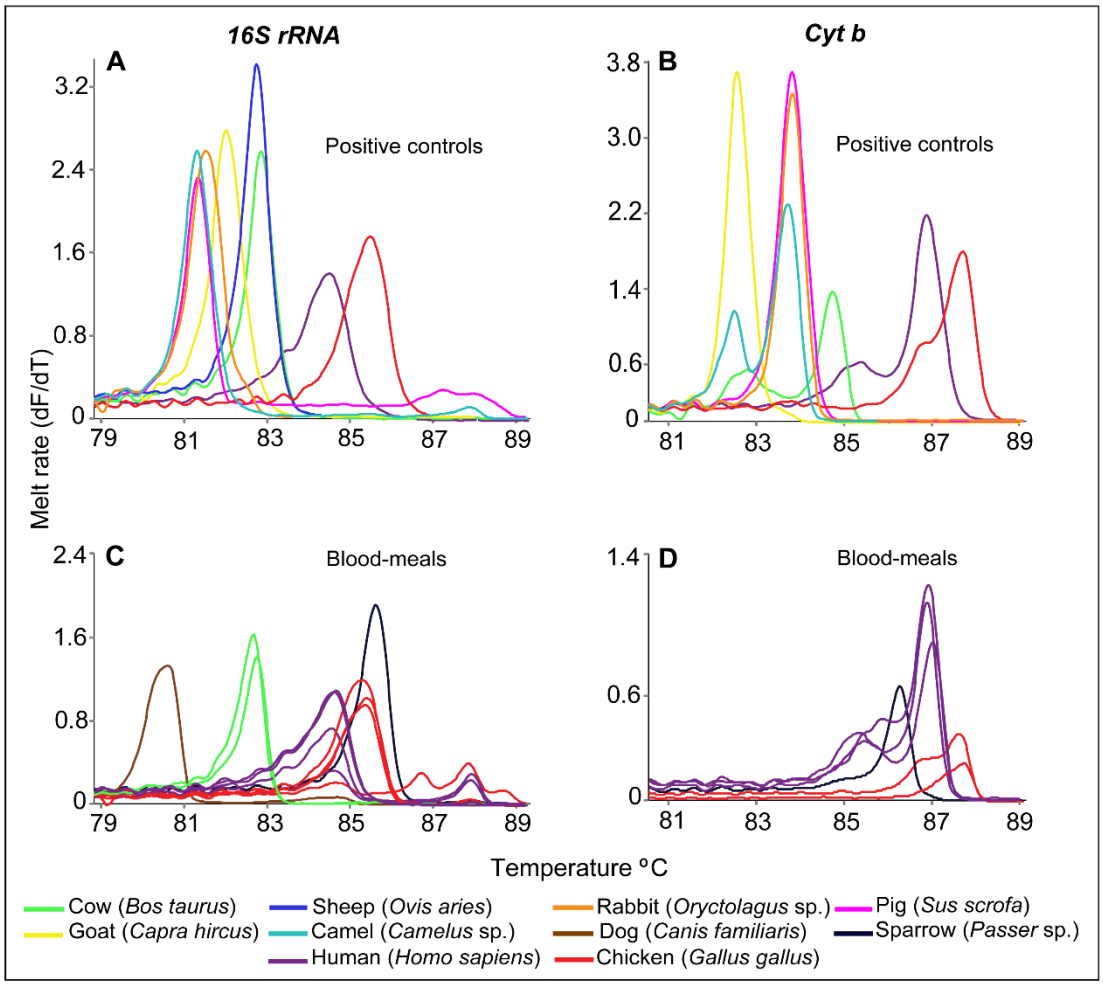


Figure 3.2: Melt rate profiles of resolved blood-meal sources from mosquitoes sampled at selected hospitals in Busia, Bungoma, and Kakamega counties

Table 3.3: Number of blood-meal sources of mosquitoes sampled at hospitals in Busia, Bungoma and Kakamega counties

Sampling site†	Species	Total	Human	Cattle	Dog	Chicken	Sparrow	‡ND
Bungoma RH	*Culex pipiens*	7	5	0	1	1	0	0
Busia RH	*Culex pipiens*	7	7	0	0	0	0	0
	Aedes aegypti	1	1	0	0	0	0	0
Butula MH	*Culex pipiens*	2	1	0	0	1	0	0
Lugulu MH	*Culex pipiens*	2	0	1	0	0	1	0
Matungu	*Anopheles gambiae*	1	0	0	0	0	0	1
SCH	*Culex pipiens*	12	10	0	0	1	0	1
Mukumu MH	*Culex pipiens*	5	1	0	0	4	0	0
	Aedes aegypti	1	0	1	0	0	0	0

†RH = referral hospital; MH = missionary hospital; SCH = sub-County hospital
‡ND: not determined by the two markers

3.4.3 Viruses detected

While the mosquito pools analyzed were negative for most of the human pathogenic arboviruses endemic in Kenya, a single *Culex poicilipes* female sampled in Mukumu Missionary hospital (Kakamega County) was positive for sindbis virus (deposited GenBank accession MT019267). The NS1 sequence of this sindbis virus showed highest similarity (98.5%) with a sindbis strain detected in a mosquito in Uganda (MK045248).

A total of 49 mosquito pools were positive for ISFs, among which 30 pools were positive for cell fusing agent virus (CFAV), 11 for Aedes flavivirus (AeFV), and eight for Culex flavivirus (CxFV) (Figure 3.3). Nucleotide sequence identities of the NS5 gene region ranged from 98.3-100% for CFAV, 98.6-99.6% for AeFV, and 98.2-99.9% for CxFV when comparing strains characterized in this study with those in the Genbank database. None of the ISF-positive samples amplified using DNA (no-RT controls). Culex flavivirus positive mosquito pools were all comprised of *Cx. pipiens* mosquitoes, except for one *Culex annulioris*. All 38 fed specimens were negative for both ISFs and pathogenic arboviruses.

The overall maximum likelihood estimates of IRs for sampled *Ae. aegypti* with ISFs were 1.27% (95% CI = 0.87%-1.78%) for CFAV infection, and 0.43% (95% CI = 0.23%-0.74%) for

AeFV. The overall IR estimate for *Cx. pipiens* with CxFV was 0.23% (95% CI = 0.1%-0.45%) (Table A.4). The odds of *Ae. aegypti* testing positive for ISFs (AeFV and CFAV) were significantly higher in Bungoma (OR = 2.53, 95% CI = 1.18-5.72, p = 0.02) and Kakamega (OR = 2.70, 95% CI = 1.18-6.36; p = 0.02) compared to Busia (Figure 3.1, Table 3.4). For CFAV alone, the odds for *Ae. aegypti* to be infected were also higher in Bungoma (OR = 3.99, 95% CI = 1.65-11.10, p = 0.004) than in Busia, while there was no significant difference between sites in Kakamega and Busia. The odds of *Ae. aegypti* being infected with AeFV, and *Cx. pipiens* with CxFV, were not significantly different in the three counties (Table 3.4). Both female and male pools of *Ae. aegypti* were positive for CFAV and AeFV, but only female *Culex* were positive for CxFV. However, the odds of *Ae. aegypti* being positive for ISFs (CFAV and AeFV) were not significantly different between the two sexes (Table 3.4). The odds for ISFs (CFAV and AeFV) infection of *Ae. aegypti* were not significantly different between the rainy seasons. Logistic regression analysis for CxFV in *Cx. pipiens* for seasonality and sex was not performed due to insufficient data.

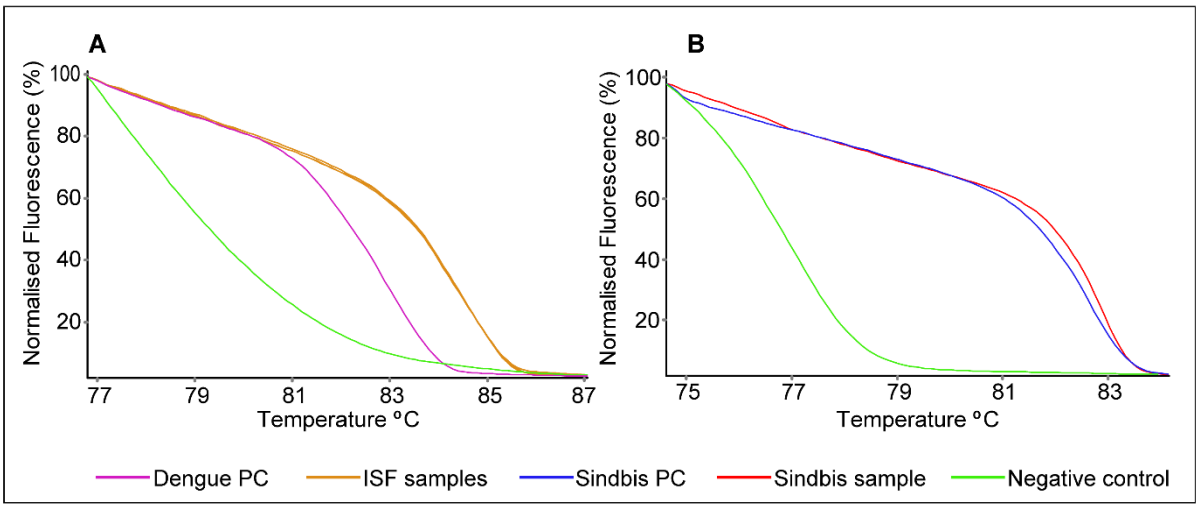


Figure 3.3: High resolution melting profiles of ISF-positive (A) and sindbis virus positive (B) mosquito pools.

Table 3.4: Logistic regression model with County, mosquito sex and season as independent variables and odds of being infected with ISFs†.

Variable	Category	Infection rate (95% CI)	Odds ratio (95% CI)	*p*-value
CFAV+AeFV				
Site	Bungoma	2.63 (1.62-4.10)	2.53 (1.18-5.72)	*0.02*
	Kakamega	2.74 (1.56-4.58)	2.70 (1.18-6.36)	*0.02*
	Busia	0.89 (0.46-1.59)	Reference	
Sex	Female	2.04 (1.37-2.95)	1.30 (0.69-2.52)	0.43
	Male	1.50 (0.88-2.41)	Reference	
Season	Long rainy	1.71 (1.21-2.37)	0.77 (0.37-1.80)	0.51
	Short rainy	2.29 (1.07-4.42)	Reference	
CFAV				
Site	Bungoma	2.43 (1.48-3.83)	3.99 (1.65-11.10)	*0.004*
	Kakamega	1.33 (0.59-2.64)	2.41 (0.80-7.51)	0.12
	Busia	0.52 (0.21-1.08)	Reference	
Sex	Female	1.33 (0.82-2.06)	1.12 (0.54-2.40)	0.77
	Male	1.17 (0.64-1.99)	Reference	
Season	Long rainy	1.14 (0.75-1.68)	0.61 (0.27-1.55)	0.26
	Short rainy	1.67 (0.44-2.89)	Reference	
AeFV				
Site	Bungoma	0.12 (0.007-0.56)	0.35 (0.02-2.35)	0.34
	Kakamega	1.09 (0.46-2.25)	3.10 (0.88-12.15)	0.08
	Busia	0.34 (0.11-0.81)	Reference	
CxFV				
Site	Bungoma	0.26 (0.095-0.57)	1.11 (0.18-21.28)	0.93
	Kakamega	0.15 (0.009-0.72)	0.65 (0.03-16.59)	0.77
	Busia	0.23 (0.01-1.10)	Reference	

† CFAV = Cell fusing agent virus; AeFV = Aedes flavivirus; CxFV = Culex flavivirus; *P*-values of 0.05 or less are shown in italics

The CFAV NS5 (deposited GenBank accessions MT019229-MT019258) gene sequences clustered according to county, with those from Busia being closely related to CFAV NS5 gene sequences detected previously in Busia (77% bootstrap support; GenBank accession

KP792624). Aedes flavivirus NS5 gene sequences from this study were related to those from Homa Bay (GenBank accession MK015648) [32] and Mombasa (GenBank accession LC348555) [33], the latter county being distant from the western region where my study was based (Figure 3.4). One of the CxFV NS5 sequences from Kakamega (deposited GenBank accession MT019266) clustered with two CxFV sequences from Taiwan (88% bootstrap support; GenBank accessions JX897905; JX897906). Three other CxFV sequences from Kakamega (deposited GenBank accession MT019264), Bungoma (deposited GenBank accession MT019261), and Busia (deposited GenBank accession MT019263) were closely related to a strain from Uganda (77% bootstrap support; GenBank accession GQ165808) [34] and some previously found in Busia (GenBank accessions LC388536; LC3885345) [33]. All ISFs sequences clustered together according to the mosquito species from which they were detected.

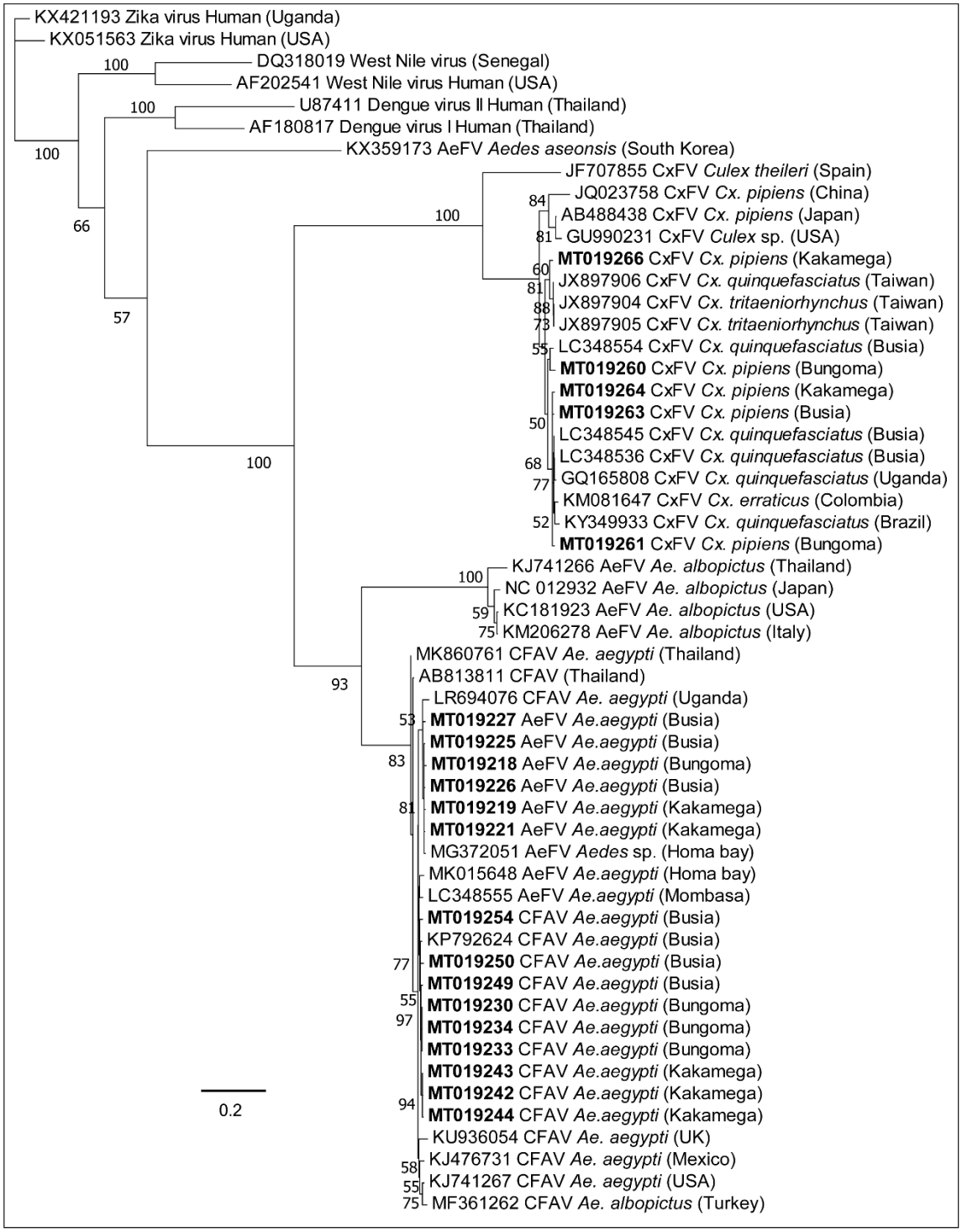


Figure 3.4: Maximum likelihood phylogeny of flaviviruses inferred from 36 aligned 900-nt segments of the NS5 gene.
Taxon names comprise of the GenBank accession number, isolation source and country of origin, with sequences characterised in this study being indicated in bold. Bootstrap values show percentage agreement from 1,000 replicates.

3.4.4 Implementation of mosquito control methods at hospitals

At all six participating hospitals, a public health officer, medical superintendent or administration officer provided information on the measures they implement to prevent mosquitoes from proliferating and from biting patients, visitors, and staff. The use of insecticide-treated nets provided by the Public Health Department of the Ministry of Health was reported and also observed in patient wards at all the six hospitals. Three of the hospitals reported that outdoor and indoor residual insecticide spraying, also done by the Public Health Department of the Ministry of Health, had ceased 2-10 years prior to the study. The other three hospitals were still undertaking residual insecticide spraying inside patient wards and staff quarters every four to eight months with Icon® insecticide as the main insecticide used. Only one of the hospitals reported that they provided topical repellant to patients, who reportedly preferred not applying them because of the odour. Only two of the hospitals had installed window screens and in one of these, the process was still ongoing. At all the institutions, grass cutting and clearing bushes were highlighted as an important tool in the control of mosquitoes. Only one institution highlighted the importance of draining water puddles, disposal of hospital waste, and rubbish, to control mosquito breeding.

3.5 Discussions and conclusions

These findings, which highlight the risk for arbovirus infection in western Kenya, can assist public health institutions to implement informed mosquito control measures. Mosquitoes were trapped mostly outdoors, therefore fewer *Anopheles* spp. were collected for both day and night trapping, in comparison to the more abundant *Cx. pipiens* and *Ae. aegypti*. The common malaria vectors in the study region, *Anopheles gambiae* sensu stricto, are known to be highly arthropophilic and endophilic [12]. This is in comparison to *Cx. pipiens*, which is both endophilic and exophilic but mostly ornithorphilic [35], and *Ae. aegypti*, which is exophilic

and anthropophilic [36]. These factors might account for the low number of blood-fed mosquitoes.

This is the first report of analysis of pathogens in mosquitoes trapped at hospitals and LMs in Kenya. Most similar studies focus on homesteads [33], peri-domestic sites [37], and/or human-wildlife interfaces [38]. The hospitals were generally representative of urban settings, which tend to achieve high mosquito catches and are important for assessing urban transmission of arboviruses. Mosquitoes captured in hospital settings can be considered as '*flying syringes*' that sample patients visiting hospitals for pathogens may not naturally be transmitted by mosquitoes [39]. However, whilst this sampling of urban or hospital settings cannot be used to trace sylvatic to urban spillover, which happens at wildlife/forest-human interfaces, trapping at LMs can be used to assess zoo-prophylactic/potentiation effects on mosquito abundance, which I did not manage to do in this study due to logistical challenges. However, the open nature of the LMs corresponds to fewer habitat and resting sites for mosquitoes, thereby limiting catches. Furthermore, the poor security at the open markets leaves trapping equipment susceptible to theft.

As expected, the mosquito abundance and species diversity in this study coincided with the presence of favourable habitats. *Aedes aegypti* mosquitoes are known to be highly anthropophilic and their larvae breed where there are tall grasses and artificial water stagnation [40]. For *Cx. pipiens*, open septic tanks and pit latrines are favourable habitats for their breeding [41,42]. Therefore, proper management of these habitats in urban settings will go a long way in preventing arboviral transmission and outbreaks.

The detection of sindbis virus illustrates the risk of arbovirus transmission even in regions where outbreaks have not been reported. Sindbis virus, first isolated from mosquitoes in Egypt [43], circulates between birds and *Culex* spp. mosquitoes, with humans acting as dead-end

hosts [44]. The virus causes rash, febrile illness, myalgia, and arthralgia. In Kenya, two acute cases of sindbis virus were detected in febrile patients from Mfangano islands of Lake Victoria following inoculation on Vero cell lines and RT-PCR [45], and seroprevalence studies in other Kenyan regions have confirmed exposure in local populations [46,47]. Clinical cases may be masked by malaria and other causes of fever in Kenya, and may therefore go unnoticed due to lack of awareness and inadequate diagnostic capabilities in health institutions [11,48,49].

Birds are known to be amplifying hosts of West Nile and sindbis viruses; hence, the detection of a sparrow (*Passer* sp.) blood meal in a *Cx. pipiens* mosquito suggests risk of transmission of sindbis virus to humans [3,50]. In Kenya, sindbis virus was identified in several *Culex* and *Aedes* mosquitoes [16, 23,51]. It is endemic in northern Europe and South Africa [3] where outbreaks are common. Several *Culex* spp. are vectors of the virus in different parts of the world, with *Culex univittatus* and *Cx. pipiens/Culex torrentium* thought to be the main vectors [52,53]. Increased IRs in the chief vectors in northern Europe have been found to be a predictor of sindbis related rash and arthralgia outbreaks in humans [44].

The detection of ISFs is not likely to warrant public health concern since they have not been shown to infect or grow in vertebrate cells [54]. Previous studies in Kenya have detected CFAV, AeFV, and CxFV in mosquitoes mostly around the lake shores [20,32,33]. In contrast, this study reports significantly higher odds of *Ae. aegypti* infection by ISFs in Bungoma and Kakamega, which are more inland compared to Busia, which is closer to Lake Victoria. The local ecology has been shown to have a pronounced impact on mosquito viral infection status [55]. Higher CxFV infection has, for example, been reported in sites with dense housing, compared to urban open spaces in Chicago, USA [56]. Busia County is closer to the shores of Lake Victoria with a distinct ecology from that of Bungoma and Kakamega. While the probability of being infected with ISFs was not significantly different between male and female mosquitoes, most previous studies suggest higher infection rates in females, though these

results may be biased due to low numbers of male mosquitoes collected, and in some cases males are not processed for viral detection [33]. The detection of ISFs in male mosquitoes emphasises the occurrence of vertical and venereal transmission of ISFs [32,57]. Seasonality did not seem to have an effect on ISF positivity in this study, which could be due to the similarity of environmental variables during the short and long rainy seasons. Therefore, in the future it would be important to sample also during the dry season. In Houston, USA, virus-infected mosquito pools were detected only in the cooler months, compared to the warmer months, showing an effect of seasonality [58].

There has been growing interest in the possibility of using ISFs to interfere with the acquisition and transmission of pathogenic arboviruses [57]. Studies have shown that West Nile virus growth rate was lower in cell cultures co-infected with CxFV, compared to those not co-infected, and mosquito dissemination rates were lower in persistently-infected *Cx. pipiens* colonies, compared to mosquitoes not infected with CxFV [59]. Other studies have shown *in vitro* interference by CFAV on Zika virus growth [60]. High sequence similarity between my viral sequences and those from Uganda is not surprising as my study site closely borders Uganda and cross-border translocation of these viruses can easily occur. The clustering of ISF sequences by mosquito host species shows that they are relatively conserved within mosquito species across geographical divides.

Due to logistical challenges, the number of trap days and nights were not uniform across the two rainy seasons (short and long) and the sampling sites (hospitals and LMs), making statistical comparisons of mosquito abundance difficult. A more extensive sampling exercise over several seasons may lead to the detection of more pathogenic arboviruses as the IR in mosquitoes is usually very low during inter-epidemic periods [61]. It is also important to determine *Stegmoyia* indices in future sampling studies as they are important in monitoring arbovirus vector abundance. While the transmission-blocking potential of ISFs has been

studied in the lab, it is important for future studies to compare the IRs of ISFs in my study site to those from arboviral endemic areas, such as the coast and north-eastern Kenya, to assess this effect in a field setting. This study confirms the presence of both pathogenic arboviruses and ISFs in mosquitoes from western Kenya and calls for further evaluation of the role of ISFs in the epidemiology of arboviruses.

3.6 Acknowledgements

I acknowledge the assistance rendered by Francis Mulwa of *icipe* in the identification of mosquitoes. I acknowledge the following members of the ZooLinK project: Dr. Lilian Wambua for technical assistance during mosquito trapping, Dr. Kelvin Momanyi, Lorren Alumasa and Fredrick Amanya for the initial identification of field sites for collection of mosquitoes. I also acknowledge the assistance of Dr. Sheilah Agha for planning of mosquito trapping schedule. I also acknowledge Bester Mudereri of *icipe* GIS and Remote Sensing Unit for assistance in producing the study site map. I also acknowledge Jane Njaramba and Barack Omondi from ML-EID for providing positive controls for the blood-meal analysis

3.8 References

[1] Brugman VA, Hernández-Triana LM, Medlock JM, Fooks AR, Carpenter S, Johnson N. The role of *Culex pipiens* L. (Diptera: Culicidae) in virus transmission in Europe. Int. J. Environ. Res. Public Health. 2018; 15(2): 389. https://doi.org/10.3390/ijerph15020389.

[2] Smithburn KC, Hughes TP, Burke AW, Paul JH. A neurotropic virus isolated from the blood of a native of Uganda. Am. J. Trop. Med. 1940; s1-20(4): 471-492. https://doi.org/10.4269/ajtmh.1940.s1-20.471.

[3] Adouchief S, Smura T, Sane J, Vapalahti O. Sindbis virus as a human pathogen-epidemiology, clinical picture and pathogenesis. Rev. Med. Virol. 2016; 26(4): 221-241. https://doi.org/10.1002/rmv.1876.

[4] Glaser A. West Nile virus and north America : An unfolding story. Rev. Sci. Tech. 2004; 23(2): 557–568. https://doi.org/10.20506/rst.23.2.1504.

[5] Nyamwaya D, Wang'ondu V, Amimo J, Michuki G, Ogugo M, Ontiri E, et al. Detection of West Nile virus in wild birds in Tana river and Garissa Counties, Kenya. BMC Infect. Dis. 2016; 16(1): 696. https://doi.org/10.1186/s12879-016-2019-8.

[6] Nyaruaba R, Mwaliko C, Mwau M, Mousa S, Wei H. Arboviruses in the east African Community partner states: A review of medically important mosquito-borne arboviruses. Pathog. Glob. Health. 2019; 113(5): 209–228. https://doi.org/10.1080/20477724.2019.1678939.

[7] Nedjadi T, El-kafrawy S, Sohrab SS, Desprès P, Damanhouri G, Azhar E. Tackling dengue fever: Current status and challenges. Virol. J. 2015; 12: 212. https://doi.org/10.1186/s12985-015-0444-8.

[8] Njenga MK, Nderitu L, Ledermann JP, Ndirangu A, Logue CH, Kelly CHL, et al. Tracking epidemic Chikungunya virus into the Indian ocean from east Africa. J. Gen. Virol. 2008; 89(11): 2754–2760. https://doi.org/10.1099/vir.0.2008/005413-0.

[9] Song BH, Yun SI, Woolley M, Lee YM. Zika virus: History, epidemiology, transmission, and clinical presentation. J. Neuroimmunol. 2017; 308: 50–64. https://doi.org/10.1016/j.jneuroim.2017.03.001.

[10] Caragata EP, Rocha MN, Pereira TN, Mansur SB, Dutra HLC, Moreira LA. Pathogen blocking in *Wolbachia*-infected *Aedes aegypti* is not affected by Zika and dengue virus co-infection. PLoS Negl. Trop. Dis. 2019; 13(5): e0007443. https://doi.org/10.1371/journal.pntd.0007443.

[11] Crump JA, Morrissey AB, Nicholson WL, Massung RF, Stoddard RA, Galloway RL, et al. Etiology of severe non-malaria febrile illness in Northern Tanzania: A prospective cohort Study. PLoS Negl. Trop. Dis. 2013; 7(7): e2324. https://doi.org/10.1371/journal.pntd.0002324.

[12] Iwashita H, Dida GO, Sonye GO, Sunahara T, Futami K, Njenga SM, et al. Push by a net, pull by a cow: can zooprophylaxis enhance the impact of insecticide treated bed nets on malaria control? Parasit. Vectors. 2014; 7: 52. https://doi.org/10.1186/1756-3305-7-52.

[13] Patterson EI, Villinger J, Muthoni JN, Dobel-Ober L, Hughes GL. Exploiting insect-specific viruses as a novel strategy to control vector-borne disease. Curr. Opin. Insect Sci. 2020; 39: 50–56. https://doi.org/10.1016/j.cois.2020.02.005.

[14] Hall-Mendelin S, McLean BJ, Bielefeldt-Ohmann H, Hobson-Peters J, Hall RA, Van Den Hurk AF. The insect-specific Palm Creek virus modulates West Nile virus infection in and transmission by Australian mosquitoes. Parasit. Vectors. 2016; 9(1): 414. https://doi.org/10.1186/s13071-016-1683-2.

[15] Powell JR, Tabachnick WJ. History of domestication and spread of *Aedes aegypti* - A review. Mem. Inst. Oswaldo Cruz. 2013; 108(suppl. 1): 11–17. https://doi.org/10.1590/0074-0276130395.

[16] Mossel EC, Crabtree MB, Mutebi JP, Lutwama JJ, Borland EM, Powers AM, et al. Arboviruses isolated from mosquitoes collected in Uganda, 2008–2012. J. Med. Entomol. 2017; 54(5): 1403–1409. https://doi.org/10.1093/jme/tjx120.

[17] Falzon LC, Alumasa L, Amanya F, Kang'ethe E, Kariuki S, Momanyi K, et al. One Health in action: Operational aspects of an integrated surveillance system for zoonoses in western Kenya. Front. Vet. Sci., 2019; 6: 252. https://doi.org/10.3389/fvets.2019.00252.

[18] Edwards FW. Mosquitoes of the Ethiopian region III-culicine adults and pupae. London: British Museum (Natural History); 1941

[19] Huang YM, Ward RH. A pictorial key for the identification of the mosquitoes associated with yellow fever in Africa. Mosq. Syst. 1981; 13(2): 138-149.

[20] Villinger J, Mbaya MK, Ouso D, Kipanga PN, Lutomiah J, Masiga DK. Arbovirus and insect-specific virus discovery in Kenya by novel six genera multiplex high-resolution melting analysis. Mol Ecol Resour. 2017; 17(3): 466-480. https://doi.org/10.1111/1755-0998.12584.

[21] Endoh D, Mizutani T, Kirisawa R, Maki Y, Saito H, Kon Y, et al. Species-independent detection of RNA virus by representational difference analysis using non-ribosomal hexanucleotides for reverse transcription. Nucleic Acids Res. 2005; 33 (6):e65. https://doi.org/10.1093/nar/gni064.

[22] Ouso DO, Otiende MY, Jeneby MM, Oundo JW, Bargul JL, Miller SE, et al. Three-gene PCR and high-resolution melting analysis for differentiating vertebrate species mitochondrial DNA for biodiversity research and complementing forensic surveillance. Sci. Rep. 2020; 10(1): 4741. https://doi.org/10.1038/s41598-020-61600-3.

[23] Omondi D, Masiga DK, Ajamma YU, Fielding BC, Njoroge L, Villinger J. Unraveling host-vector-arbovirus interactions by two-gene high resolution melting mosquito bloodmeal analysis in a Kenyan wildlife-livestock interface. PLoS One. 2015; 10(7): e0134375. https://doi.org/10.1371/journal.pone.0134375.

[24] Vázquez A, Sánchez-Seco MP, Palacios G, Molero F, Reyes N, Ruiz S, et al. Novel flaviviruses detected in different species of mosquitoes in Spain. Vector Borne Zoonotic Dis. 2012; 1 (3): 223–229. https://doi.org/10.1089/vbz.2011.0687.

[25] Pfeffer M, Proebster B, Kinney RM, Kaaden OR. Genus-specific detection of alphaviruses by a semi-nested reverse transcription-polymerase chain reaction. Am. J.

Trop. Med. Hyg. 1997; 57(6): 709–718. https://doi.org/10.4269/ajtmh.1997.57.709.

[26] Boakye DA, Tang J, Truc P, Merriweather A, Unnasch TR. Identification of bloodmeals in haematophagous diptera by cytochrome B heteroduplex analysis. Med. Vet. Entomol. 1999; 13 (3): 282-287. https://doi.org/10.1046/j.1365-2915.1999.00193.x.

[27] Eshoo MW, Whitehouse CA, Zoll ST, Massire C, Pennella TD, Blyn LB, et al. Direct broad-range detection of alphaviruses in mosquito extracts. Virology. 2007; 368(2): 286-295. https://doi:10.1016/j.virol.2007.06.016.

[28] Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O. New algorithms and methods to estimate maximum-likelihood phylogenies: Assessing the performance of PhyML 3.0. Syst. Biol. 2010; 59(3): 307–321. https://doi.org/10.1093/sysbio/syq010.

[29] Lefort V, Longueville JE, Gascuel O. SMS: Smart model selection in PhyML. Mol. Biol. Evol. 2017; 34(9): 2422–2424. https://doi.org/10.1093/molbev/msx149.

[30] Rambaut A. FigTree v1.4.2, a graphical viewer of phylogenetic trees. Edinburgh: University of Edinburgh. Available from http://tree.bio.ed.ac.uk/software/figtree/ 2014.

[31] Biggerstaff BJ. PooledInfRate, version 3.0: a Microsoft excel add-in to compute prevalence estimates from pooled samples. Centers for Disease Control and Prevention; 2006

[32] Ajamma YU, Onchuru TO, Ouso DO, Omondi D, Masiga DK, Villinger J. Vertical transmission of naturally occurring Bunyamwera and insect-specific flavivirus infections in mosquitoes from islands and mainland shores of lakes Victoria and Baringo in Kenya. PLoS Negl. Trop. Dis. 2018; 12(11): e0006949. https://doi.org/10.1371/journal.pntd.0006949.

[33] Iwashita H, Higa Y, Futami K, Lutiali PA, Njenga SM, Nabeshima T, et al. Mosquito arbovirus survey in selected areas of Kenya: Detection of insect-specific virus. Trop. Med. Health. 2018; 46(1): 19. https://doi.org/10.1186/s41182-018-0095-8.

[34] Cook S, Moureau G, Harbach RE, Mukwaya L, Goodger K, Ssenfuka F, et al. Isolation of a novel species of flavivirus and a new strain of Culex flavivirus (Flaviviridae) from a natural mosquito population in Uganda. J Gen Virol. 2009; 90(11): 2669–2678. https://doi.org/10.1099/vir.0.014183-0.

[35] Gomes B, Sousa CA, Vicente JL, Pinho L, Calderón I, Arez E, et al. Feeding patterns of molestus and pipiens forms of *Culex pipiens* (Diptera: Culicidae) in a region of high hybridization. Parasit. Vectors. 2013; 6: 93. https://doi.org/10.1186/1756-3305-6-93.

[36] Ndenga BA, Mutuku FM, Ngugi HN, Mbakaya JO, Aswani P, Musunzaji PS, et al. Characteristics of *Aedes aegypti* adult mosquitoes in rural and urban areas of western and coastal Kenya. PLoS One. 2017; 12(12): e0189971. https://doi.org/10.1371/journal.pone.0189971.

[37] Chepkorir E, Venter M, Lutomiah J, Mulwa F, Arum S, Tchouassi DP, et al. The occurrence, diversity and blood feeding patterns of potential vectors of dengue and yellow fever in Kacheliba, west Pokot County, Kenya. Acta Trop. 2018; 50-57. https://doi.org/10.1016/j.actatropica.2018.07.008.

[38] Musa AA, Muturi MW, Musyoki AM, Ouso DO, Oundo JW, Makhulu EE, et al. Arboviruses and blood meal sources in zoophilic mosquitoes at wildlife-human interfaces in Kenya. Vector Borne Zoonotic Dis. 2020; 20(6): 444-453. https://doi.org/10.1089/vbz.2019.2563.

[39] Fauver JR, Gendernalik A, Weger-Lucarelli J, Grubaugh ND, Brackney DE, Foy BD, et al. The use of xenosurveillance to detect human bacteria, parasites, and viruses in mosquito bloodmeals. Am. J. Trop. Med. Hyg. 2017; 97(2): 324–329. https://doi.org/10.4269/ajtmh.17-0063.

[40] Trpis MA, Hausermannt W. Dispersal and other population parameters of *Aedes aegypti* in an African village and their possible significance in epidemiology of vector-borne diseases. Am. J. Trop. Med. Hyg. 1986; 35(6): 1263-79. https://doi.org/10.4269/ajtmh.1986.35.1263.

[41] Service MW. Mosquito ecology: Field sampling methods. 2nd ed. Liverpool: Elsevier Science Publishers Ltd; 1993

[42] Shaman J, Day JF, Komar N. Hydrologic conditions describe West Nile virus risk in Colorado. Int. J. Environ. Res. Public Health. 2010; 7(2): 494–508. https://doi.org/10.3390/ijerph7020494.

[43] Ling J, Smura T, Lundström JO, Pettersson JH-O, Sironen T, Vapalahti O, et al. Introduction and dispersal of Sindbis virus from central Africa to Europe. J. Virol. 2019; 93(16): e00620-19. https://doi.org/10.1128/JVI.00620-19.

[44] Lundström JO, Hesson JC, Schäfer ML, Östman Ö, Semmler T, Bekaert M, et al. Sindbis virus polyarthritis outbreak signalled by virus prevalence in the mosquito vectors. PLoS Negl. Trop. Dis. 2019; 13(8): e0007702. https://doi.org/10.1371/journal.pntd.0007702.

[45] Omondi DO. Bionomics of vector-borne diseases in sites adjacent to lakes Victoria and Baringo in Kenya. PhD thesis, University of the Western Cape. 2016. Available from: http://etd.uwc.ac.za/xmlui/handle/11394/5338.

[46] Ofula VO, Oundo J, Irura Z, Chepkorir E, Tigoi C, Ongus J, et al. Evidence of presence of antibodies against selected arboviruses in Ijara and Marigat districts, Kenya. Int. J. Infect. Dis. 2016; 45(1): 188-189. https://doi.org/10.1016/j.ijid.2016.02.438.

[47] Tigoi C, Lwande O, Orindi B, Irura Z, Ongus J, Sang R. Seroepidemiology of selected arboviruses in febrile patients visiting selected health facilities in the lake/river basin areas of lake Baringo, lake Naivasha, and Tana river, Kenya. Vector Borne Zoonotic Dis. 2015; 15(2): 124-32. https://doi.org/10.1089/vbz.2014.1686.

[48] Hooft AM, Ripp K, Ndenga B, Mutuku F, Vu D, Baltzell K, et al. Principles, practices and knowledge of clinicians when assessing febrile children: A qualitative study in Kenya. Malar. J. 2017; 16(1): 381. https://doi.org/10.1186/s12936-017-2021-7.

[49] Nyaoke BA, Mureithi MW, Beynon C. Factors associated with treatment type of non-malarial febrile illnesses in under-fives at Kenyatta National Hospital in Nairobi, Kenya. PLoS One. 2019; 14(6): e0217980. https://doi: 10.1371/journal.pone.0217980.

[50] Nemeth N, Young G, Ndaluka C, Ohmann HB, Komar N, Bowen R. Persistent West Nile virus infection in the house sparrow (*Passer domesticus*). Arch. Virol. 2009;

154(5): 783-9. https://doi.org/10.1007/s00705-009-0369-x.

[51] Ochieng C, Lutomiah J, Makio A, Koka H, Chepkorir E, Yalwala S, et al. Mosquito-borne arbovirus surveillance at selected sites in diverse ecological zones of Kenya; 2007 – 2012. Virol. J. 2013; 10: 140. https://doi.org/10.1186/1743-422X-10-140.

[52] Sane J, Kurkela S, Putkuri N, Huhtamo E, Vaheri A. Complete coding sequence and molecular epidemiological analysis of Sindbis virus isolates from mosquitoes and humans, Finland. J. Gen. Virol. 2012; 93(9): 1984-1990. https://doi.org/10.1099/vir.0.042853-0.

[53] Storm N, Weyer J, Markotter W, Leman PA, Kemp A, Nel LH, et al. Phylogeny of Sindbis virus isolates from South Africa. South Afr. J. Epidemiol. Infect. 2015; 28(4): 207-214. https://doi.org/10.1080/10158782.2013.11441552.

[54] Öhlund P, Lundén H, Lie A. Insect-specific virus evolution and potential effects on vector competence. Virus Genes. 2019; 55(2): 127–137. https://doi.org/10.1007/s11262-018-01629-9.

[55] Grisenti M, Vázquez A, Herrero L, Cuevas L, Perez-Pastrana E, Arnoldi D, et al. Wide detection of Aedes flavivirus in north-eastern Italy – a European hotspot of emerging mosquito-borne diseases. J. Gen. Virol. 2015; 96(2): 420–430. https://doi.org/10.1099/vir.0.069625-0.

[56] Newman CM, Cerutti F, Anderson TK, Hamer GL, Walker ED, Kitron UD, et al. Culex flavivirus and West Nile virus mosquito co-infection and positive ecological association in Chicago, United States. Vector Borne Zoonotic Dis. 2011; 11(8): 1099-1105. https://doi.org/10.1089/vbz.2010.0144.

[57] Bolling BG, Weaver SC, Tesh RB, Vasilakis N. Insect-specific virus discovery: significance for the arbovirus community. Viruses. 2015; 7(9): 4911-28. https://doi.org/10.3390/v7092851.

[58] Kim DY, Guzman H, Bueno Jr R, Dennett JA, Auguste AJ, Carrington CVF, et al. Characterization of Culex flavivirus (Flaviviridae) strains isolated from mosquitoes in the United States and Trinidad. Virology. 2009; 386(1): 154–159. https://doi: 10.1016/j.virol.2008.12.034.

[59] Bolling BG, Olea-popelka FJ, Eisen L, Moore CG, Blair CD. Transmission dynamics of an insect-specific flavivirus in a naturally infected *Culex* pipiens laboratory colony and effects of co-infection on vector competence for West Nile virus. Virology. 2012; 427(2): 90–97. https://doi.org/10.1016/j.virol.2012.02.016.

[60] Schultz MJ, Frydman HM, Connor JH. Dual insect specific virus infection limits arbovirus replication in *Aedes* mosquito cells. Virology. 2018; 518: 406-413. https://doi.org/10.1016/j.virol.2018.03.022.

[61] Gu W, Unnasch TR, Katholi CR, Lampman R, Novak RJ. Fundamental issues in mosquito surveillance for arboviral transmission. Trans. R. Soc. Trop. Med. Hyg. 2008; 102(8): 817-22. https://doi.org/10.1016/j.trstmh.2008.03.019.

Chapter 4

Molecular prevalence of tick-borne pathogens in cattle and risk factors in western Kenya.

Tatenda Chiuya[1, 4], Jandouwe Villinger[1], Daniel K. Masiga[1], Dickens O. Ondifu[1], Lilian Wambua[4], Armanda D.S. Bastos[4], Eric M. Fèvre[2, 3], Laura C. Falzon[2, 3]

1. International Centre of Insect Physiology and Ecology (icipe), P.O Box 30772-00100, Nairobi, Kenya
2. Institute of Infection, Veterinary and Ecological Sciences, University of Liverpool, Leahurst Campus, Chester High Road, Neston, CH64 7TE, United Kingdom
3. International Livestock Research Institute, Old Naivasha Road, PO Box 30709, 00100 Nairobi, Kenya
4. Department of Zoology and Entomology, University of Pretoria, Pretoria, South Africa

4.1 Abstract

Tick-borne pathogens (TBPs) are a major constraint to livestock production worldwide, especially in sub-Saharan Africa. Their association with human disease is also increasing signaling their zoonotic importance. It is therefore crucial to investigate TBP prevalence in local cattle populations and the risk factors associated with their spread. I set out to identify tick infestations and TBP infections and relate them to breed, age, and body-condition in cattle in western Kenya, where smallholder livestock production is important for subsistence and market-driven income. I selected blood samples collected from cattle at livestock markets and slaughterhouses between May 2017 and January 2019 and identified TBP infections using molecular methods. Out of the 422 cattle, 38.86% were infected with at least one TBP, while 8.76% had co-infections with two species. The most prevalent genera were *Anaplasma* spp. (19.67 %), followed by *Theileria* (12.32 %), *Ehrlichia* (6.64 %), and *Babesia* (0.24 %) spp. The TBP species encountered most frequently were *Theileria velifera* (7.35 %), *Anaplasma marginale* (4.98 %), *Theileria mutans* (3.08 %), and *Anaplasma platys* (2.84 %), as well as *Theileria parva* (1.60 %) and *Babesia bigemina* (0.24 %). *Ehrlichia ruminantium*, *Rickettsia* spp., or arboviruses were not detected in cattle. Exotic breeds of cattle had higher odds of being infected with *Ehrlichia* spp. (OR: 2.39, 95 % CI: 0.98-5.63, $p = 0.049$) and *A. marginale* (OR: 4.50, 95 % CI: 1.75-11.91, $p = 0.002$) compared to local breeds. Tick infestation was a significant predictor for infections with *Anaplasma* spp. (OR: 2.01, 95 % CI: 1.27-3.51, $p = 0.004$) and *Ehrlichia* spp. (OR: 2.83, 95 % CI: 1.22-7.38, $p = 0.021$). This study reports important data on TBP prevalence and corresponding risk factors, which can guide control measures.

***Keywords*:** Livestock markets, Slaughterhouse, Co-infection, *Anaplasma*, *Ehrlichia*, Western Kenya

4.2 Introduction

Ticks and tick-borne pathogens (TBPs) are constraints to livestock production, causing huge losses in the industry in Africa [1]. Furthermore, several tick-transmitted pathogens are capable of infecting humans and thus pose a public health threat [2]. The major tick vectors in sub-Saharan (SSA) are in the genera *Amblyomma, Rhipicephalus,* and *Hyalomma* collectively transmitting *Babesia, Theileria, Anaplasma, Ehrlichia,* and *Rickettsia* pathogens [3]. Ticks also transmit *Nairoviruses* such as Crimean-Congo haemorrhagic fever (CCHF) and Nairobi sheep disease viruses [4]. In Kenya, the commonly reported tick-borne diseases (TBDs) constraining livestock production include East Coast fever (*Theileria parva*: *Rhipicephalus appendiculatus*), babesiosis (*Babesia bigemina*: *Rhipicephalus decoloratus*), anaplasmosis (*Anaplasma marginale*: *Rhipicephalus decoloratus*), and heartwater (*Ehrlichia ruminantium*: *Amblyomma variegatum*) [5–8]. Other benign *Theileria* spp. such as *Theileria taurotragi*, *Theileria mutans,* and *Theileria velifera* are also highly prevalent [9]. Due to their endemic stability in the predominantly indigenous cattle herds in Kenya, most of these TBDs are clinically inapparent. However, complicating conditions of pregnancy, pathogen co-infections, and the introduction of exotic breeds into these endemic regions can lead to loss of weight, declines in milk production and mortalities [10].

Tick-borne CCHF virus does not cause clinical disease in livestock. However, cattle act as carriers for the infection of ticks (*Hyalomma*) and humans [11]. A case of CCHF was recorded in Kenya in 2000 [12], and I recently detected the CCHF virus in *Rh. decoloratus* ticks from cattle at slaughterhouses in western Kenya [Chapter 2; 13]. Several CCHF outbreaks have been reported in neighbouring Uganda between August 2017 and January 2019, with associated human fatalities [14–19]. *Rickettsia africae,* chiefly vectored by *Am. variegatum* ticks, is common in SSA as the cause of African tick bite fever (ATBF), mostly in tourists and travellers [20] with only a few cases being reported in indigenous people [21-22]. Surveillance for

Rickettsia in domestic livestock has shown a higher prevalence of spotted fever group (SFG) *Rickettsia*, notably *R. africae*, in cattle, sheep, and their ticks [Chapter 2; 13,23–25]. The presence of large hosts such as cattle is important for the survival of *Am. variegatum*, the major vector of most *Rickettsia* spp. [26].

The incidence of human babesiosis (*Babesia divergens* and *Babesia microti*), ehrlichiosis (*Ehrlichia chaffeensis*), and anaplasmosis (*Anaplasma phagocytophilum*) is high in Europe and north America [1,27-28], but there have been few reports in Africa. Additionally, recent reports of *Anaplasma platys*, *Anaplasma ovis*, and *Anaplasma capra* in humans, suggests that they may also be of zoonotic importance [29–31]. The afore-mentioned *Babesia, Ehrlichia* and *Anaplasma* spp. with zoonotic potential are found in cattle with apparent [32] or inapparent clinical manifestations [33,34].

The estimated 17 million cattle in different production systems in Kenya represent an important source of livelihood and cultural and social value [35]. In western Kenya alone, there are 843,608 and 219,904 indigenous and exotic cattle, respectively [36]. Only a handful of reports have focused on the prevalence and epidemiology of TBDs constraining livestock production and the presence of zoonotic TBPs as a measure of risk for human infection in Kenya. These studies reported high seroprevalence of TBDs and associated risk factors in smallholder livestock production systems in the western Kenya highlands [7] and Machakos County [5,37–38]. In Lambwe Valley of western Kenya at a wildlife-livestock interface, a high animal-level prevalence of TBPs [39] was reported, while emerging *Anaplasma* and *Ehrlichia* spp. were found in dairy cows in peri-urban Nairobi [34].

Rising human population and increased demand for human habitation have resulted in people and their livestock living more closely, facilitating the transmission of zoonotic diseases [40,41]. The shift to intensive and market-inclined smallholder livestock production systems

being witnessed in East Africa is likely to exacerbate the situation [42]. The livestock markets and slaughterhouses located in peri-urban areas are a conduit for the movement of livestock across internal and country borders in East Africa [43]. Given that animal trade and migration are key factors in the spread of diseases into new uninfected areas [44] these facilities may be important in the epidemiology of TBDs. In Chapter 2, I detected a wide range of pathogens (viral, bacterial and protozoal) in ticks collected from livestock at livestock markets (LMs) and slaughterhouses (SHs) in western Kenya. Therefore, as a follow up I determined the incidence of veterinary and medically important TBPs in cattle at these LMS and SHs, using molecular methods. I also assessed the risk factors that are associated with the spread of TBPs in cattle and the presence of co-infections, which complicate diagnosis and prognosis of TBDs.

4.3 Materials and methods

4.3.1 Study site and livestock sampling

The study was based in Busia County on the border with Uganda extending to the neighboring counties of Bungoma and Kakamega counties. The site lies in the East Africa Lake Victoria basin where there is an abundance of livestock in smallholder production systems. From a sampling frame of all the LMs and SHs in these three counties, 4-6 LMs and SHs were selected for sampling in each county (Figure 4.1). The selection was dependant on the number of animals coming to LMs/SHs and accessibility from Busia town where the field lab was located. The sampling frame and final site selection are described elsewhere [45]. This study was approved by the International Livestock Research Institute (ILRI) Institutional Animal Care and Use Committee (ref IACUC-RC2017-04). Data from human owners of livestock was collected after approval by the ILRI Institutional Research Ethics Committee (ref ILRI-IREC2017-08/2). Both committees are licensed by the National Commission for Science, Technology and Innovation (NACOSTI) in Kenya. Informed and signed consent was obtained from the owner/custodians of the sampled animals. Sampling was also facilitated by market

chairpersons and handlers. Animal sampling was performed as described by Falzon et al., 2019 [45]. Blood samples were transported to the ILRI field lab in Busia in a cooler box on ice packs and then shipped to the Martin Lüscher Emerging Infectious Disease (ML-EID) laboratory at the International Centre of Insect Physiology and Ecology (*icipe*) on dry ice where they were stored at -80 ^{O}C. The blood samples analysed in this study were collected from seven LMs and seven SHs however, my selection of blood samples was focused on cattle from Kakamega county where I detected the CCHF virus and also all those cattle from which ticks positive for TBPs were detected in Chapter 2.

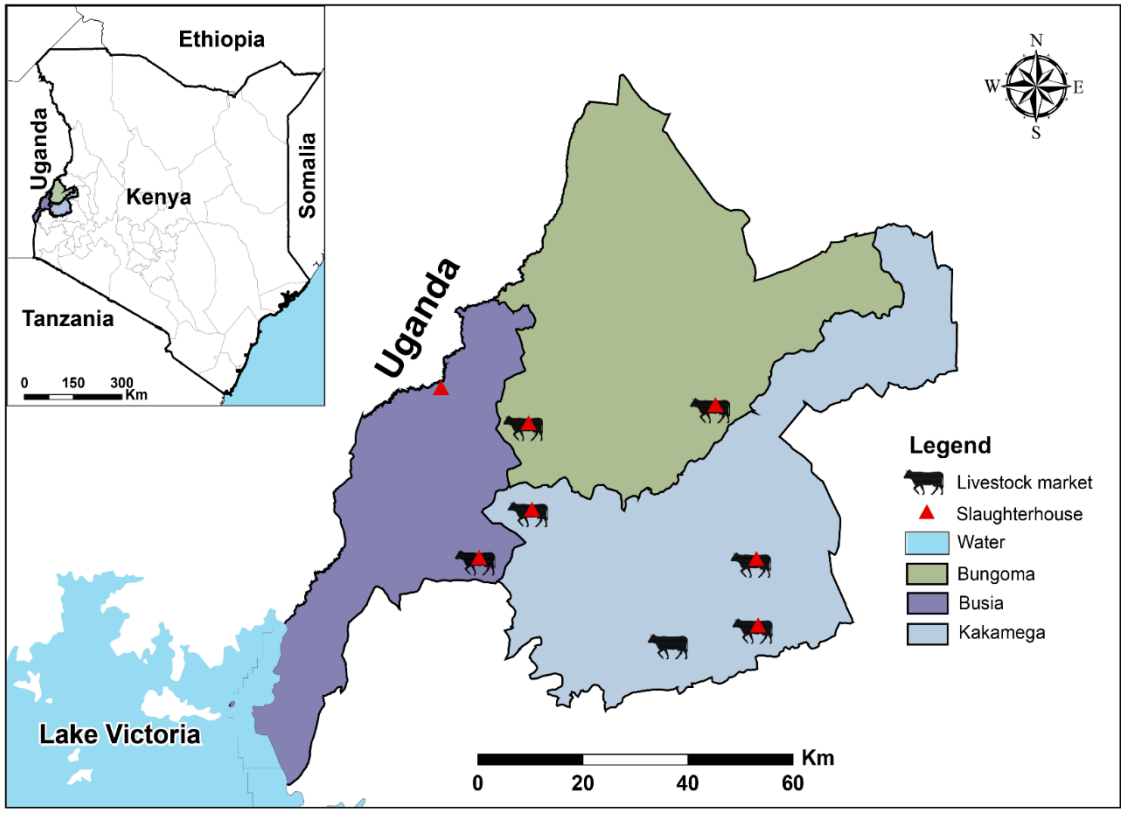


Figure 4.1: Map of western Kenya showing the three neighbouring counties and the slaughterhouses and livestock markets from which blood samples were collected from cattle

4.3.2 Nucleic acid extraction

DNA and RNA were extracted from blood using the TRIzol™ reagent standard protocol (Thermofisher, USA). Dengue serotype 2 and sindbis virus cultured on Vero cell lines in a previous study were included in each RNA extraction run [46]. After nucleic acid extraction, 5 µl of the total RNA was subjected to cDNA synthesis using a High Capacity cDNA Reverse Transcription (RT) kit (Life Technologies, USA). The 10-µl reaction mixtures contained 1X RT buffer, 4 mM dNTPs, 600 µM random hexamers [47], 2.5U/µl reverse transcriptase enzyme, and 1U/µl RNAse inhibitor.

4.3.3 Molecular detection of arboviral, bacterial and protozoan pathogens

Detection of arboviruses

An established multiplex PCR-high resolution melting analysis (PCR-HRM) was used to screen blood samples for arboviruses [46]. The reaction mixture contained 1 µl of cDNA template, 5 µl of 2x MyTaq HS Mix (Bioline, UK), 1 µl of 50 µM SYTO-9 (Life Technologies, USA) and degenerate primer mix (Table 4.1). The amplicons were subjected to high-resolution melting after thermo-cycling. The cycling conditions are outlined elsewhere [46]. End-reaction melting profiles were visually inspected and all samples with melt peaks representing specific amplification were selected for the next stage of amplification after preliminary identification. In the second stage, all samples that were positive in the first round screening were tested with a different set of primers using conventional PCR, to generate longer fragments (Figure 4.2).

Detection of bacterial and protozoan pathogens

A combination of available PCR-HRM and conventional PCR methods and primers were used to detect the 16S rRNA gene of *Rickettsia, Anaplasma, Ehrlichia* spp. and the 18S rRNA gene of *Theileria,* and *Babesia* spp. Ten-microliter reactions that consisted of 2 µl template, 2 µl 5X HOT FIREPol® EvaGreen HRM Mix (Solis BioDyne, Estonia) and 0.5 µl of each primer at

10 pmoles were constituted for the PCR-HRM. Positive controls of *Anaplasma*, *Rickettsia*, *Theileria*, and *Babesia* spp. previously detected in *icipe*'s ML-EID lab were included in the runs. A second stage amplification of positive samples utilised different primers to generate larger PCR products, where possible (Table 4.1 and Figure 4.2).

In the second stage *Ehrlichia* spp. and *Anaplasma* spp. positive samples were amplified with a semi-nested conventional PCR using the following conditions: for primary amplification, a hot-start activation step of 95°C of 15 min was followed by 1 cycle of 95°C for 20 s, 63°C for 30 s, and 72°C for 90 s, 2 cycles of 95°C for 20 s, 62°C for 30 s, and 72°C for 90 s, 2 cycles of 95°C for 20 s, 61°C for 30 s, and 72°C for 90 s, followed with 35 cycles of 95°C for 20 s, 60°C for 30 s, and 72°C for 80 s, and a final extension at 72°C for 10 min. The secondary amplification utilised 2 µl PCR products from the primary reaction in a 20 µl reaction. The cycling profile consisted of 95°C for 15 min; 3 cycles of 95°C for 20 s, 61°C for 30 s, and 72°C for 90 s; 37 cycles of 95°C for 20 s, 60°C for 30 s, and 72°C for 80 s, and a final extension at 72°C for 10 min. All amplicons were analysed on a 1.5% agarose by gel electrophoresis before purification and sequencing (Figure 4.2).

4.3.4 Phylogenetic analysis

Sequences were inspected and edited in Geneious prime version 2019.0.4 software (Biomatters, New Zealand). Sequence contigs were then used in BLAST nucleotide searches against the GenBank nr [48]. Closely-related sequences and reference strains identified in this manner were added to the dataset. Sequence alignments were built using MAFFT and phylogenies were constructed in PhyML version 3.0 using the maximum-likelihood analysis over 1000 replicates. Automatic model selection was based on the Akaike Information Criterion (AIC) [49]. Trees were viewed in Figtree 1.4 [50].

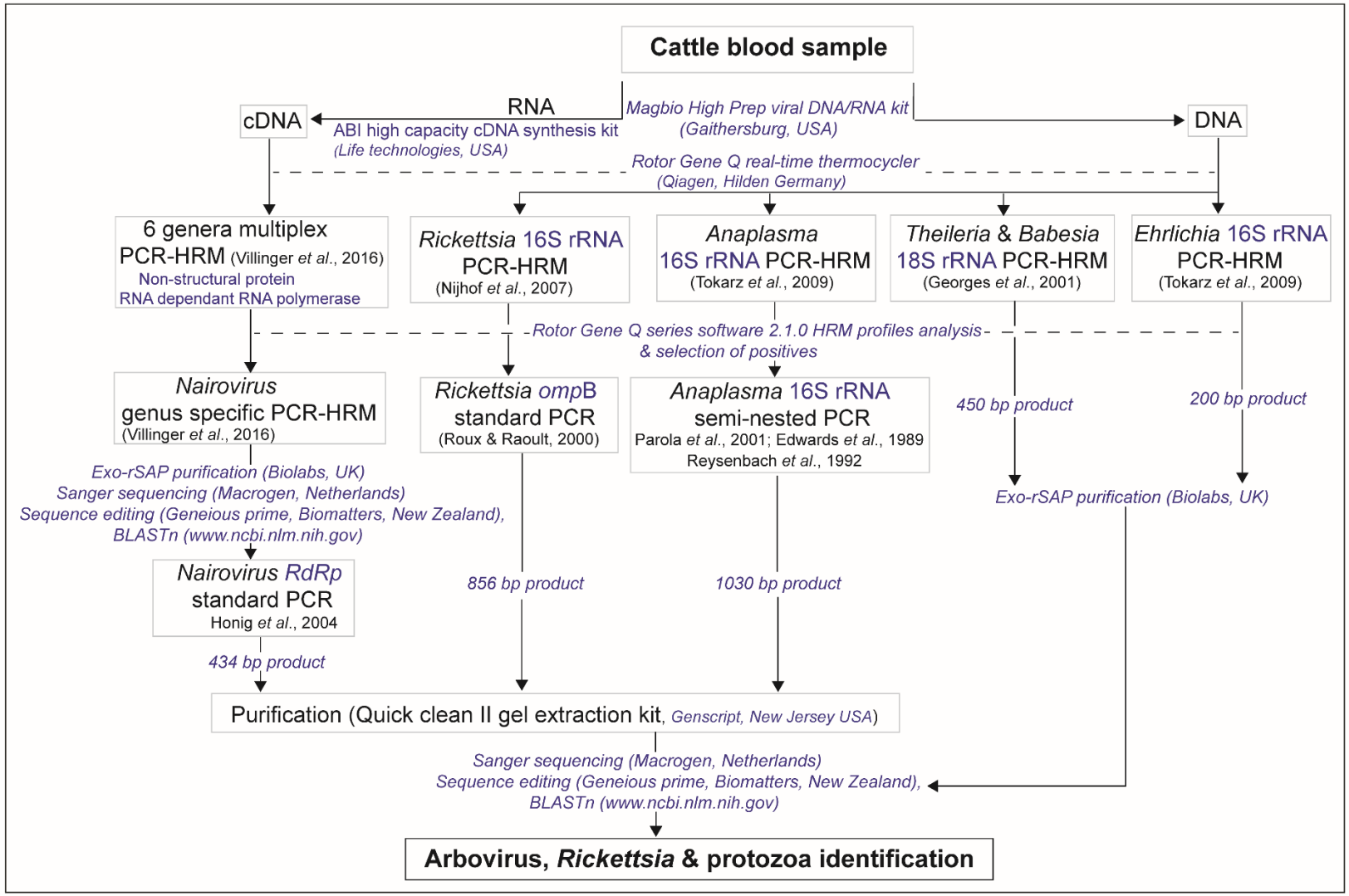


Figure 4.2: A schematic diagram showing the processing of cattle blood samples from DNA/RNA extraction up to the identification of tick-borne pathogens

Table 4.1: Primers that were used for the detection of arboviruses, *Rickettsia* spp. and protozoa in cattle blood

Target gene	Primer name	Primer sequence (5' – 3')	Product size (bp)	Reference
***Phlebovirus* NP (S-segment)**	Phlebo JV3a F	AGTTTGCTTATCAAGGGTTTGATGC	150	[46]
	Phlebo JV3b F	GAGTTTGCTTATCAAGGGTTTGACC		
	Phlebo JV3 R	CCGGCAAAGCTGGGGTGCAT		
***Nairovirus* RdRp (L-segment)**	Nairo L 1a F	TCTCAAAGATATCAATCCCCCCITTACCC	150	[46]
	Nairo L 1b F	TCTCAAAGACATCAATCCCCCTTWTCCC		
	Nairo L 1a R	CTATRCTGTGRTAGAAGCAGTTCCCATC		
	Nairo L 1b R	GCAATACTATGATAAAAACAATTMCCATCAC		
	Nairo L 1c R	CAATGCTGTGRTARAARCAGTTGCCATC		
	Nairo L 1d R	GCAATGCTATGGTAGAAACAGTTTCCATC		
***Alphavirus* NS4**	Vir 2052 F	TGGCGCTATGATGAAATCTGGAATGTT	150	[51]
	Vir 2052 R	TACGATGTTGTCGTCGCCGATGAA		
***Flavivirus* NS5**	Flavi JV2a F	AGYMGHGCCATHTGGTWCATGTGG	150	[46]
	Flavi JV2b F	AGCCGYGCCATHTGGTATATGTGG		
	Flavi JV2c F	AGYCGMGCAATHTGGTACATGTGG		
	Flavi JV2d F	AGTAGAGCTATATGGTACATGTGG		
	Flavi JV2a R	GTRTCCCADCCDGCDGTRTCATC		
	Flavi JV2b R	GTRTCCCAKCCWGCTGTGTCGTC		
***Orthobunyavirus* NP (s-segment)**	Bunyagroup F	CTGCTAACACCAGCAGTACTTTTGAC	210	[52]
	Bunyagroup R	TGGAGGGTAAGACCATCGTCAGGAACTG		
Dhori virus NP	Dhori F	CGAGGAAGAGCAAAGGAAAG		[46]
	Dhori R	GTGCGCCCCTCTGGGGTTT		
Thogoto virus (M-segment)	Thogoto S6 F	GATGACAGYCCTTCTGCAGTGGTGT		[46]
	Thogoto S6 R	RACTTTRTTGCTGACGTTCTTGAGGAC		
***Rickettsia* 16S rRNA**	Rick-F	GAACGCTATCGGTATGCTTAACACA	364	[53]
	Rick-R	CATCACTCACTCGGTATTGCTGGA		
***Theileria* and Babesia 18S rRNA**	RLB-F	GAGGTAGTGACAAGAAATAACAATA	450	[54]
	RLB-R	TCTTCGATCCCCTAACTTTC		
***Anaplasma* 16S rRNA**	*Anaplasma*JV F	CGGTGGAGCATGTGGTTTAATTC	300	[55]
	*Anaplasma*JV R	CGRCGTTGCAACCTATTGTAGTC		
***Ehrlichia* 16S rRNA**	*Ehrlichia* 16S F	CGTAAAGGGCACGTAGGTGGACTA	200	[56]
	Ehrlichia 16S R	CACCTCAGTGTCAGTATCGAACCA		
***Anaplasma/Ehrlichia* 16S rRNA**	EHR16SD	GGTACCYACAGAAGAAGTCC	1090	[57–59]
	pH1522	AAGGAGGTGATCCAGCCGCA		
	pH1492	GGCTACCTTGTTACGACTT		

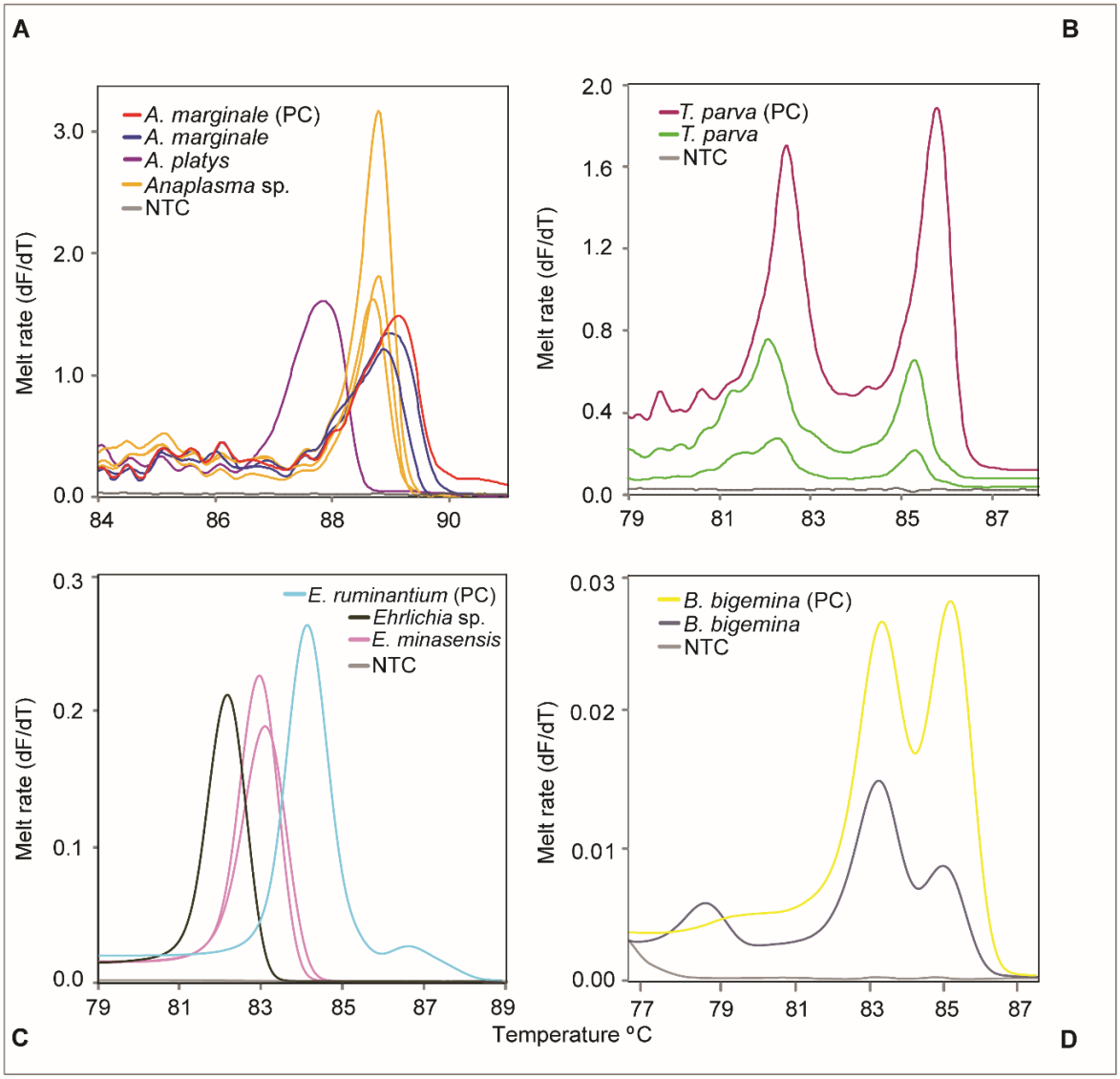
4.3.5 Statistical analysis

Logistic regression in R® version 3.5.3 was performed using the variables listed in Table 4.3 as explanatory variables and the PCR-based positivity of cattle to *Anaplasma* spp., *Ehrlichia* spp., *Theileria* spp. and *A. marginale* as the response variables. Risk factor analysis was only performed for *A. marginale* due to the lack of sufficient data of other TBPs. I also considered the economic importance of *A. marginale* in cattle production compared to other pathogens. The association of these variables with TBPs acquisition was determined by calculating odds ratios, confidence intervals, and *P*-values. A *P*-value of ≤ 0.05 was considered statistically significant.

4.4 Results

4.4.1 Diversity of tick-borne pathogens detected by PCR-HRM

Out of the 422 cattle blood samples analyzed, *Anaplasma* spp., *Babesia* spp., *Ehrlichia* spp., and *Theileria* spp. were detected by PCR-HRM. The identifications were based on the distinct HRM profiles and confirmatory sequencing of representative PCR amplicons as shown in Figure 4.3.



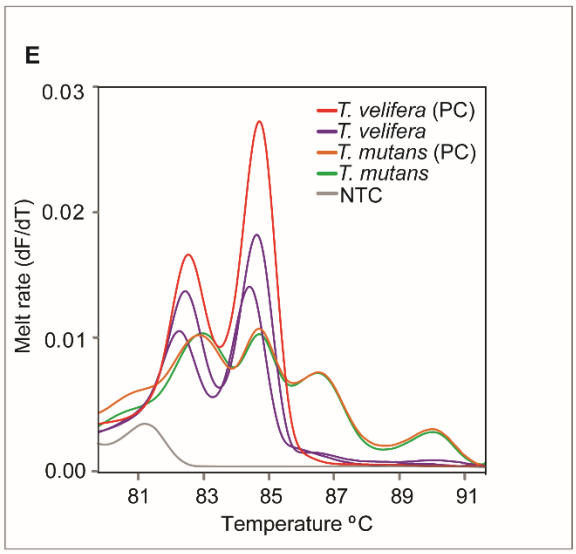


Figure 4.3: Melt rate profiles of representative samples of tick-borne pathogens detected in cattle.
(A) *Anaplasma* spp. 16S rRNA, (B) *T. parva* 18S rRNA, (C) *Ehrlichia* spp. 16S rRNA, (D) *B. bigemina* 18S rRNA and (E) non-transforming *Theileria* spp. Positive controls are indicated by 'PC'. No template controls are shown by 'NTC'

4.4.2 Prevalence of TBP in cattle based on PCR-HRM

Overall, 164 cattle out of 422 (38.86%) were infected with at least one TBP. Detailed information on the prevalence and infection status of the cattle is shown in Table 4.2 and Figure 4.4. Single infections were detected in 21.32% (90/422) of the cattle and an additional 8.76% (37/422) had two co-infecting species of the TBPs screened for. The most frequent double infection was a combination of *T. velifera* and *Anaplasma* sp. The most prevalent genera were *Anaplasma* spp. (19.67%) followed by *Theileria* (12.32%), *Ehrlichia* (6.64%), and *Babesia* (0.24%). Kakamega County had the highest prevalence for most of the pathogens; however, sample size was higher due to the prior detection of CCHF virus-positive ticks [Chapter 2; 13]. At species level of each TBP detected prevalences were highest for *T. velifera* (7.35%), *A. marginale* (4.98%) and *T. mutans* (3.08%), with minor occurrences of other pathogens (Table 4.2). In this chapter I did not detect *E. ruminantium*, *Rickettsia* spp. or any arboviruses such as CCHF.

Table 4.2: Overall prevalence of tick-borne pathogens detected in cattle from western Kenya

Tick-borne pathogen	**Percent prevalence by County**			
	Busia	Bungoma	Kakamega	**Total**
***Anaplasma* spp**.	**11.76 (6/51)**	**17.17 (17/99)**	**22.06 (60/272)**	**19.67 (83/422)**
A. centrale	0 (0/51)	0 (0/99)	1.84 (5/272)	1.18 (5/422)
A. marginale	0 (0/51)	5.05 (5/99)	5.88 (16/272)	4.98 (21/422)
A. platys	3.92 (2/51)	2.02 (2/99)	2.94 (8/272)	2.84 (12/422)
Anaplasma sp.	7.84 (4/51)	10.10 (10/99)	11.40 (31/272)	10.66 (45/422)
***Babesia* spp**.	**0 (0/51)**	**0 (0/99)**	**0.36 (1/272)**	**0.24 (1/422)**
B. bigemina	0 (0/51)	0 (0/99)	0.36 (1/272)	0.24 (1/422)
***Ehrlichia* spp**.	**11.56 (6/51)**	**5.05 (5/99)**	**6.25 (17/272)**	**6.64 (28/422)**
E. minasensis	0 (0/51)	1.01 (1/99)	0.37 (1/272)	0.47 (2/422)
Ehrlichia sp.	11.56 (6/51)	4.04 (4/99)	5.88 (16/272)	6.16 (26/422)
***Theileria* spp.**	**9.80 (5/51)**	**10.10 (10/99)**	**13.60 (37/272)**	**12.32 (52/422)**
T. mutans	3.92 (2/51)	4.04 (4/99)	2.57 (7/272)	3.08 (13/422)
T. parva	0 (0/51)	0 (0/99)	2.57 (7/272)	1.66 (7/422)
T. taurotragi	0 (0/51)	0 (0/99)	0.37 (1/272)	0.24 (1/422)
T. velifera	5.88 (3/51)	6.06 (6/99)	8.09 (22/272)	7.35 (31/422)

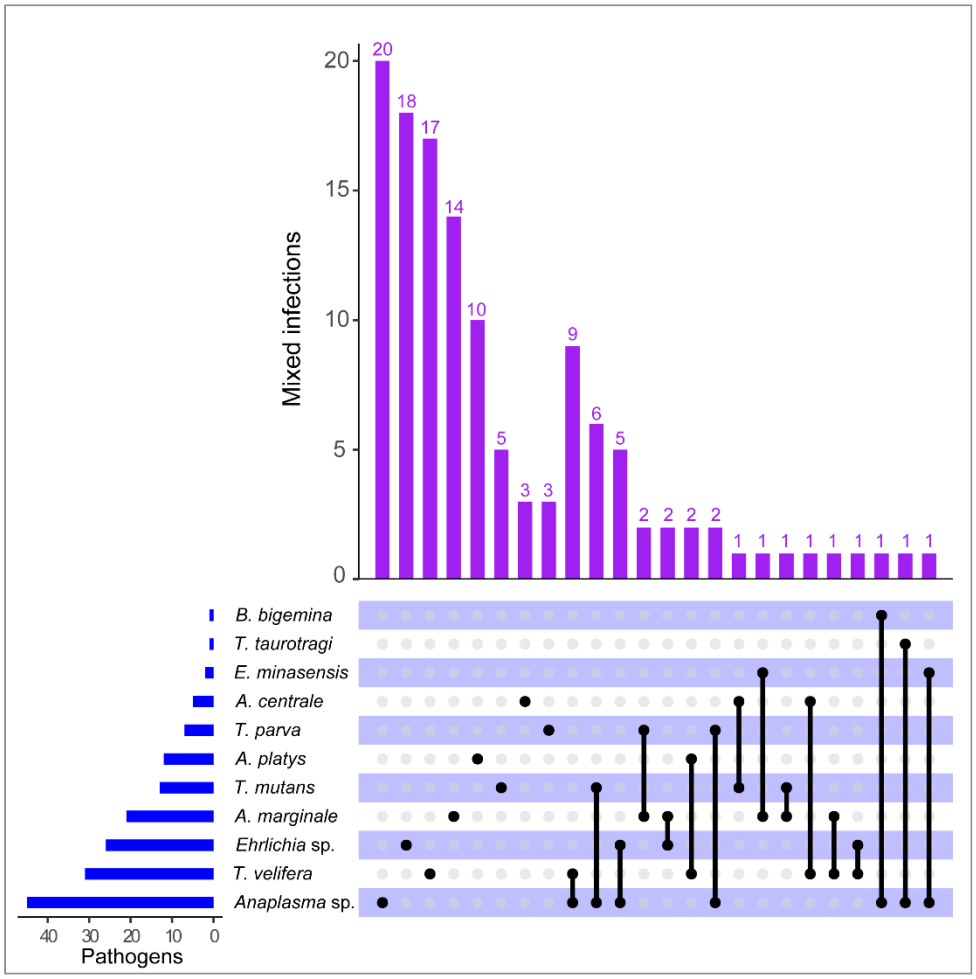


Figure 4.4: UpSetR plot showing the frequency of single and double infections of tick-borne pathogens detected in cattle from western Kenya.
The blue bar plot on the left shows the total number of pathogens of each species detected while the matrix shows single (black dots) and double infections (black dots connected by black lines) whose frequency is depicted by the purple bar plot.

The phylogeny of representative sequences of pathogens identified in this study is shown in Figures 4.5 and 4.6. *Ehrlichia minasensis* detected in this study (deposited GenBank accession MT672517-MT672518) clustered with *E. minasensis* identified in cattle from Kenya (63 % bootstrap support; GenBank accession MT163429) [34] and in *Rhipicephalus microplus* ticks from Brazil (57% bootstrap support; GenBank accession NR_148800) [60]. *Anaplasma marginale* strains (deposited GenBank accession MT459306) were closely related to those from Uganda (77 % bootstrap support; GenBank accession KU686794) [61] while the

Anaplasma centrale strains (deposited GenBank accession MT459303-MT459304) were closely related to strains detected in China (GenBank accession MF289480) [62]. *Anaplasma* spp. (deposited GenBank accession MT459326-MT459329; MT459319) that could not be resolved to species level even after amplification of a longer 1030-nt fragment were closely related to *A. platys* (GenBank accession JQ396431) [63]; (GenBank accession MH762081) [64]; (GenBank accession MN266942) [Chapter 2;13], *Candidatus* Anaplasma camelii (GenBank accession MN266943) [Chapter 2; 13] and *Anaplasma* sp. (GenBank accession MF576175) [65]; (GenBank accession KP006405) [66] from different parts of the world.

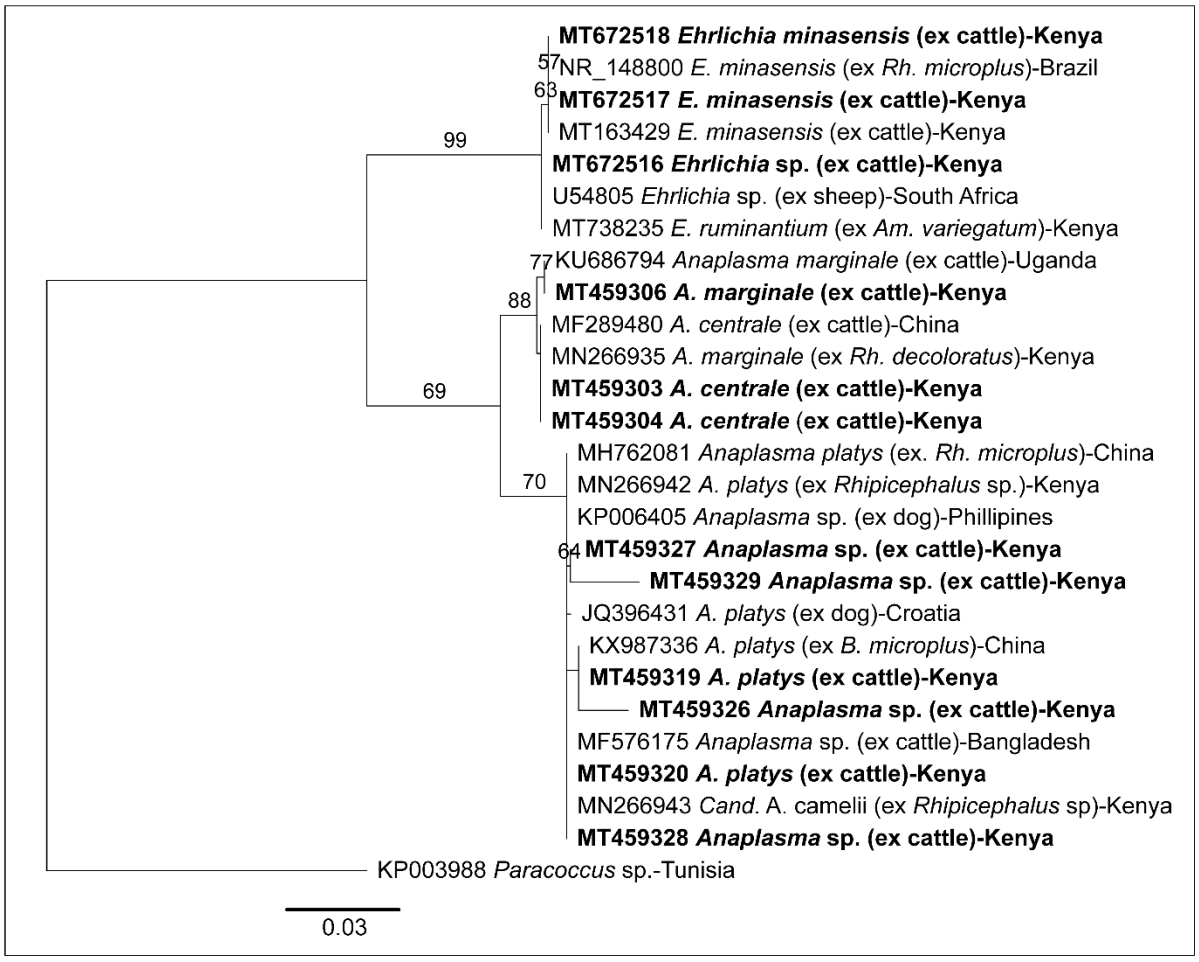


Figure 4. 5: Maximum-likelihood phylogeny inferred from 26 aligned 200-900-nt 16S rRNA partial sequences of Anaplasmataceae detected in cattle.
Numbers at the nodes indicate bootstrap support and the scale bar represents 0.03 substitutions per site.

Theileria parva strains (deposited GenBank accession MT49411) were closely related to strains previously detected in South Africa (60 % bootstrap support; GenBank accession MK792971) [67], Zambia (72 % bootstrap support; GenBank accession MG952926) [68] and Kenya (72 % bootstrap support; GenBank MH929322) [23]; (60 % bootstrap support; GenBank accession MN294730) [Chapter 2; 13]. *Theileria velifera* strains (deposited GenBank accession MT459436-MT459437) fell into the same clade with those detected in Kenya (66 % bootstrap support; GenBank accession MN853560) [39]; (66 % bootstrap support; GenBank accession MN294734) [Chapter 2; 13]; South Africa (66 % bootstrap support; GenBank MK792966) [67] and Mozambique (66 % bootstrap support; GenBank accession FJ869897) [69]. *Theileria mutans* strains (deposited GenBank accession MT704609; MT704611) were closely related to those detected in Uganda (70 % bootstrap support; GenBank KU206320) [70], Kenya (70 % bootstrap support; GenBank accession MN853552) [39]; (55 % bootstrap support; GenBank accession MN294729) [Chapter; 13] and South Africa (79 % bootstrap support; GenBank accession MK792976) [67]. *Babesia bigemina* strains (deposited GenBank accession MT459333) were closer to those detected in Kenya (GenBank accession MN294720) [Chapter 2; 13], India (99 % bootstrap support; GenBank accession MT322431) and Uganda strains (GenBank accession KU206297) [70]. All strains of TBPs detected in this study were phylogenetically close to strains of the same pathogens I detected in ticks removed from the same animals in Chapter 2 [13] (Figure 4.5 and 4.6).

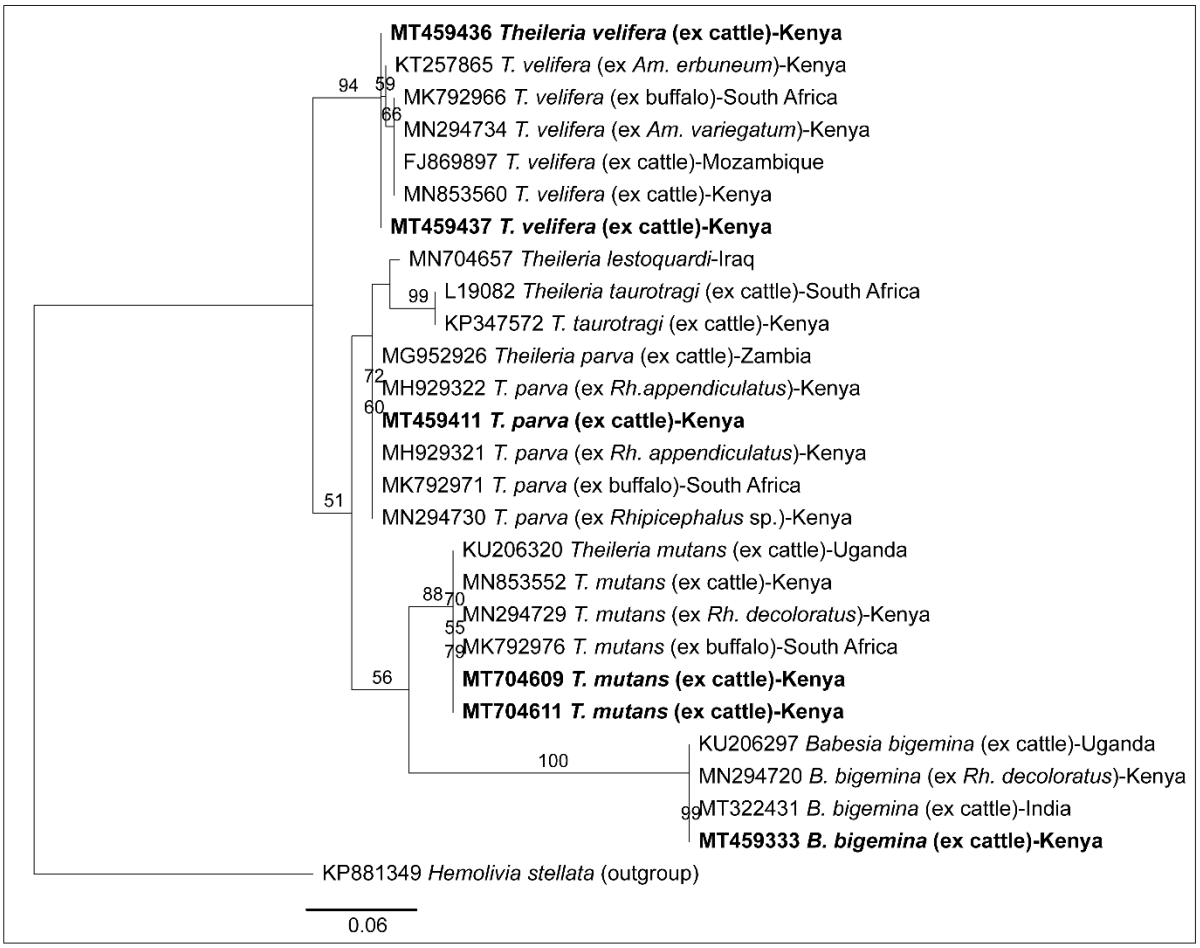


Figure 4. 6: Maximum-likelihood phylogeny inferred from 26 aligned 450-nt 18S rRNA partial sequences of *Theileria* and *Babesia* spp. detected in cattle.
Numbers at the nodes indicate bootstrap support and the scale bar represents 0.06 substitutions per site.

4.4.3 Risk factor analysis

Exotic breeds of cattle had higher odds of being infected with *Ehrlichia* spp. (OR: 2.39, 95 % CI: 0.98-5.63, p = 0.049), *A. marginale* (OR: 4.50, 95 % CI: 1.75-11.91, p = 0.002) and less with *Theileria* spp. (OR: 0.20, 95 % CI: 0.05-0.58, p = 0.009) compared to local breeds and cross-bred cattle. Cattle infested with ticks had higher odds of being infected with *Anaplasma* spp. (OR: 2.01, 95 % CI: 1.27-3.51, p = 0.004) and *Ehrlichia* spp. (OR: 2.83, 95 % CI: 1.22-7.38, p = 0.021) than those which were tick-free. (Table 4.3).

Table 4.3: Descriptive statistics and logistic regression analysis of risk factors associated with occurrence of tick-borne pathogens in cattle from western Kenya.

Variables	Categories	Prevalence %	Odds ratio (95 % CI)	*P*-value
Anaplasma spp.				
†Breed	Exotic	18/90 (20)	1.04 (0.56-1.88)	0.9
	Cross	6/31 (19.4)	0.92 (0.32-2.26)	0.859
	Local	59/301 (19.6)	Ref	
Gender	Male	42/207 (20.3)	1.01 (0.67-1.80)	0.707
	Female	41/217 (18.9)	Ref	
Age	≥ 12 months	78/379 (20.6)	1.9 (0.81-6.00)	0.172
	< 12 months	5/43 (11.6)	Ref	
BCS	1-2.5	56/289 (19.4)	0.99 (0.59-1.71)	0.975
	3-5	27/133 (20.3)	Ref	
‡Ticks	Present	56/225 (24.9)	2.01 (1.27-3.51)	*0.004*
	Absent	27/197 (13.7)	Ref	
Ehrlichia spp.				
†Breed	Exotic	10/90 (11.1)	2.39 (0.98-5.63)	*0.049*
	Cross	3/31 (9.7)	1.78 (0.39-6.06)	0.397
	Local	15/301 (5)	Ref	
Gender	Male	15/207 (7.2)	1.13 (0.51-2.52)	0.764
	Female	13/215 (6)	Ref	
Age	≥ 12 months	26/379 (6.9)	1.58 (0.43-10.19)	0.552
	< 12 months	2/43 (4.7)	Ref	
BCS	1-2.5	21/289 (7.3)	1.26 (0.56-3.38)	0.616
	3-5	7/133 (5.3)	Ref	
‡Ticks	Present	21/225 (9.3)	2.83 (1.22-7.38)	*0.021*
	Absent	7/197 (3.6)	Ref	
Theileria spp.				
†Breed	Exotic	3/90 (3.3)	0.20 (0.05-0.58)	*0.009*
	Cross	4/31 (12.9)	0.94 (0.27-2.61)	0.918
	Local	45/301 (15)	Ref	
Gender	Male	25/207 (12.1)	1.00 (0.55-1.82)	0.996
	Female	27/215 (12.6)	Ref	
Age	≥ 12 months	46/379 (12.1)	0.75 (0.30-2.13)	0.553
	< 12 months	6/43 (14)	Ref	
BCS	1-2.5	31/289 (10.7)	0.65 (0.35-1.24)	0.185
	3-5	21/133 (15.8)	Ref	
‡Ticks	Present	28/225 (12.4)	1.00 (0.55-1.82)	0.999
	Absent	26/197 (13.2)	Ref	
A. marginale				
†Breed	Exotic	11/90 (12.2)	4.50 (1.75-11.91)	*0.002*
	Cross	1/31 (3.2)	0.92 (0.048-5.30)	0.935
	Local	9/301 (3)	Ref	
Gender	Male	11/207 (5.3)	1.02 (0.407-2.59)	0.961
	Female	10/215 (4.7)	Ref	
Age	≥ 12 months	21/379 (5.5)	6.84e+06 (3.72e-15-3.86e+124)	0.987
	< 12 months	0/43 (0)	Ref	
BCS	1-2.5	16/289 (5.5)	1.40 (0.51-4.48)	0.536
	3-5	5/133 (3.8)	Ref	
‡Ticks	Present	14/225 (6.2)	1.92 (0.76 -5.27)	0.18
	Absent	7/197 (3.6)	Ref	

†local breeds consisted of indigenous and zebu cattle, cross-bred cattle were shorthorn x zebu while exotic breeds were other imported breeds

‡highlights whether ticks were present or not on the animal by visual inspection of predilection sites

P-values less than 0.05 are shown in italics

4.5 Discussion

I detected the majority of TBPs of economic importance in livestock production such as *T. parva*, *B. bigemina* and *A. marginale* in Kenya. Notably these same TBPs were detected in ticks collected from the same animals in Chapter 2 [13]. The general TBP prevalence in this study was less than described before from Kenya, in two specific dairy farms in Kajiado and Machakos counties [5], in calves from western Kenya [9], in smallholder livestock systems from western Kenya highlands [7], Machakos County [37,38] and at a wildlife-livestock interface [39]. A probable explanation for this consistent variation between my study and previous ones is that some of the latter were carried out on predominantly dairy cattle breeds and farming systems that are more likely to be more susceptible to TBPs. Moreover, these studies used serology rather than PCR for determining positivity to TBPs which is likely to generate higher prevalence than PCR since it measures historical exposure rather than active infection. Antibodies to *T. parva* were reported to persist in cattle for about six months after initial production [71]. Higher prevalence of TBPs is also expected in cattle at wildlife-livestock interfaces due to spill-over from wildlife such as buffalo which are the reservoirs of most of the TBPs [72].

In Uganda, a higher prevalence of TBPs was reported in transhumant livestock in the Karamoja region [70] while lower *T. parva* infection rates comparable to my study were reported in Tororo district in eastern Uganda which borders western Kenya [73]. Expectedly in smallholder livestock systems such as in western Kenya there is variable immunity to East Coast fever and the mortality ranges from 3- 20% however the disease causes higher mortality rates of between 40 and 80 % in pastoral systems [6,74]. Poor tick control strategies and veterinary consultation behaviour have been attributed to the high incidence of TBPs in pastoralist communities [75]. The prevalence of TBPs was also lower than that described in Ethiopia [76] and Cameroon [3], but comparable to that reported in China [77]. In this study the low prevalence of *B. bigemina*

and *A. marginale*, the major causes of bovine babesiosis and anaplasmosis respectively in Kenya was consistent with other studies done in the same region [7,9]. Conversely higher infection rates been reported in other ecological zones which are likely to be more suited to survival of *Rh. decoloratus* the major vector of these pathogens in Kenya [5]. Additionally, the ecology has been shown to influence parasite development in the tick as higher temperatures are thought to retard or even eliminate the infective stages of *B. bigemina* in *Rhipicephalus* ticks [78].

There was a higher prevalence of the mildly pathogenic non-transforming *Theileria* spp. compared to *T. parva* infections synonymous with reports from Kenya [9], Uganda [70], and Ethiopia [76]. Expectedly, in Chapter 2 the begnin *Theileria* species were also more prevalent in the screened ticks. These species are vital in reducing morbidity and mortality due to the pathogenic *T. parva* in indigenous co-infected cattle [79]. I also report the occurrence of co-infections in some of the positive samples which is expected as I detected multiple TBP infections in individual ticks collected from the same cattle sampled in this study [Chapter 2; 13]. Co-infection with benign-pathogenic species is desirable as outlined before however co-infection with pathogenic species only may hinder the host from mounting an effective immune response [80]. Indeed, the mildly pathogenic *Theileria* spp. have dominated co-infections in previous studies [5,9,39,81].

I detected several *A. platys*-like organisms, which are principally canine pathogens causing cyclic thrombocytopenia in dogs, however, they have recently been described infecting humans and causing clinical disease [29,31,82]. There has also been widespread detection of *A. platys* in apparently healthy cattle [39,77,83] which I speculate that the purported vector, *Rhipicephalus sanguineus* may be feeding on both dogs and cattle where they co-exist like in my study region. This is further supported by the fact that the *A. platys* like organisms from cattle and dogs in this study were phylogenetically close. However, the fact that in Chapter 2

this organism was detected in *Rh. decoloratus* and *Rh. appendiculatus* suggests that other tick species beside *Rh. sanguineus* may drive its transmission.

I also report the occurrence of the recently described *E. minasensis* [84] in cattle blood which has been shown to cause bovine ehrlichiosis in Brazil [85]. In this study the positive cattle did not show overt disease likewise in Kenya it was detected in apparently healthy dairy cows highlighting the need for more studies to determine its association with clinical disease in the country [34].

Exotic cattle breeds were more infected with *Ehrlichia* spp. and *A. marginale*. Generally, indigenous breeds of cattle such as Zebu and other local breeds are less predisposed to TBPs than exotic breeds due to their innate resistance and constant exposure to TBP infected tick bites which regularly primes their immune system. This infection pressure ensures that new-born calves get exposed to the pathogen early before their maternal acquired immunity wanes [37]. Comparatively, the immune system of exotic breeds will be naïve, hence they suffer more of the adverse effects of TBPs.

Tick infestation was significantly associated with occurrence of *Anaplasma* spp. and *Ehrlichia* spp., which is expected since ticks are the vectors driving the transmission therefore their epidemiology closely mirrors that of TBPs [86]. The stability of endemicity in a given region depends on the suitability of the ecology for the survival of ticks in that area [37]. In Chapter 2, I demonstrated that there was a diversity of tick species removed from the same animals screened in this study, therefore this association is most likely to be pronounced for *Anaplasma* spp. and *Ehrlichia* spp. because they are vectored by several tick species and even mechanically, unlike *T. mutans* and *T. velifera*, which are vectored mainly by *Amblyomma* spp. Other risk factors that I investigated such as gender, body condition score, and age were not significant predictors of TBP infection however for *Anaplasma* spp. there was twice as much risk of infection in cattle older than 12 months compared to younger animals. Generally, older

cattle are more susceptible to TBDs due to the existence of pre-immunity in calves [87]. The effect could have been more pronounced for *Anaplasma* spp. than any other TBP in my study because they were the most prevalent. This age-dependant effect has been reported before in other studies [3,37].

In this study I did not detect *E. ruminantium*, *Rickettsia* spp., or any arboviruses such as CCHF. *Ehrlichia ruminantium* the cause of heartwater in ruminants is found mostly in endothelial cells causing vasculitis and has very few stages circulating in the blood system at a given time. This means due to its low presence in peripheral blood it is less likely to be picked up by ticks and other mechanical vectors hence low-transmissibility [88,89]. Recent studies have not detected this pathogen in cattle [39] and low detection of *Ehrlichia* spp. as I encountered in ticks in Chapter 2 has been reported in previous studies in Kenya [13,55,90]. The absence of *Rickettsia* spp., especially *R. africae*, is surprising because I previously detected a high prevalence of the pathogen in *Am. variegatum* ticks removed from the same animals [13; Chapter 2]. However, a similar recent study in Kenya also did not report any *Rickettsia* spp. from cattle [39]. While cattle are known to asymptomatically harbour CCHF virus its absence in cattle from which positive ticks were collected in Chapter 2 is possible as outlined in section 2.5.1.

4.6 Conclusions

I detected a high prevalence of TBPs of economic and potential zoonotic importance in cattle at LMs/SHs. The veterinary or zoonotic importance of the recently described *E. minasensis* needs further investigation in the local context. The practice of LMs is important in subsistence livestock production but it can also pose a risk of translocation of apparently healthy but infected cattle to other areas. With this information, the Department of Veterinary Services should emphasise the importance of tick-control and regulate cattle movement trade at these points of livestock concentration.

4.7 Acknowledgements

I acknowledge the Zoonoses in Livestock in Kenya (ZooLinK) team for collection of blood samples. I also acknowledge the assistance rendered by Samuel Njoroge, Lydiah Mueni, Lynn Kirwa and Christian Odinga in retrieving the relevant samples from the ZooLinK bio-bank and associated metadata. I also acknowledge Bester Mudereri of *icipe* GIS and Remote Sensing unit for assistance in producing the study site map.

4.8 References

[1] Jongejan F, Uilenberg G. The global importance of ticks. Parasitology. 2004; 129 (S3-S14). https://doi.org/10.1017/s0031182004005967

[2] Dantas-Torres F, Chomel BB, Otranto D. Ticks and tick-borne diseases: A One Health perspective. Trends Parasitol. 2012; 28(10): 437–46. doi: 10.1016/j.pt.2012.07.003

[3] Abanda B, Paguem A, Abdoulmoumini M, Kingsley MT, Renz A, Eisenbarth A. Molecular identification and prevalence of tick-borne pathogens in zebu and taurine cattle in North Cameroon. Parasit Vectors. 2019; 12(1): 1–13. https://doi.org/10.1186/s13071-019-3699-x

[4] Ergönül Ö. Crimean-Congo haemorrhagic fever. Lancet Infect Dis. 2006; 6(4): 203-214. doi:10.1016/S1473-3099(06)70435-2

[5] Adjou Moumouni PF, Aboge GO, Terkawi MA, Masatani T, Cao S, Kamyingkird K, et al. Molecular detection and characterization of *Babesia bovis, Babesia bigemina, Theileria* species and *Anaplasma marginale* isolated from cattle in Kenya. Parasit Vectors. 2015;8(1):1–14. https://doi.org/10.1186/s13071-015-1106-9

[6] Gachohi J, Skilton R, Hansen F, Ngumi P, Kitala P. Epidemiology of East Coast fever (*Theileria parva* infection) in Kenya : past , present and the future. Parasit Vectors. 2012; 1–13. doi:10.1186/1756-3305-5-194

[7] Okuthe OS, Buyu GE. Prevalence and incidence of tick-borne diseases in smallholder farming systems in the western-Kenya highlands. Vet Parasitol. 2006; 141(3–4): 307-12. doi:10.1016/j.vetpar.2006.05.016

[8] Wesonga FD, Kitala PM, Gathuma JM, Njenga MJ, Ngumi PN. An assessment of tick-borne diseases constraints to livestock production in a smallholder livestock production system in Machakos District, Kenya. Livest Res Rural Dev. 2010; 22(6).

[9] Njiiri NE, Bronsvoort BM de C, Collins NE, Steyn HC, Troskie M, Vorster I, et al. The epidemiology of tick-borne haemoparasites as determined by the reverse line blot hybridization assay in an intensively studied cohort of calves in western Kenya. Vet Parasitol. 2015; 210(1–2): 69–76. doi: 10.1016/j.vetpar.2015.02.020

[10] Lorusso V, Wijnveld M, Majekodunmi AO, Dongkum C, Fajinmi A, Dogo AG, et al. Tick-borne pathogens of zoonotic and veterinary importance in Nigerian cattle. Parasit Vectors. 2016; 9(1): 217. https://doi.org/10.1186/s13071-016-1504-7

[11] Spengler JR, Bergeron É, Rollin PE. Seroepidemiological Studies of Crimean-Congo Hemorrhagic Fever Virus in Domestic and Wild Animals. PLoS Negl Trop Dis. 2016; 10(1): e0004210. Available from: http://doi.org/10.1371/journal.pntd.0004210

[12] Dunster L, Dunster M, Ofula V, Beti D, Kazooba-Voskamp F, Burt F, et al. First documentation of human Crimean-Congo hemorrhagic fever, Kenya. Emerg Infect Dis. 2002;8(9):1005–6. doi: 10.3201/eid0809.010510

[13] Chiuya T, Masiga D, Falzon L, Bastos A, Fevre E, Villinger J. Tick-borne pathogens , including Crimean-Congo haemorrhagic fever virus , at livestock markets and slaughterhouses in western Kenya. 2020; **in press**. https://doi.org/10.1111/tbed.13911

[14] Mbonye A. Crimean-Congo haemorrhagic fever - Uganda: (QO, NK), UN Office for the Coordination of Humanitarian Affairs (OCHA), ReliefWeb, Monitor-Uganda

report. ProMED mail. 2017; Available from: https://promedmail.org/promed-posts/

[15] Ainebyoona E. Crimean-Congo haemorrhagic fever - Africa: Uganda (MZ), Daily Monitor. ProMED. 2019; Available from: https://promedmail.org/promed-posts/

[16] Kiwanuka IK. Crimean-Congo haemorrhagic fever - East Africa: Uganda (LW), URN (Uganda Radio Network). ProMED. 2018; Available from: https://promedmail.org/promed-posts/

[17] Kopp K. Crimean-Congo haemorrhagic fever - East Africa (04): Uganda (MD), One Health Pathogenomics Consulting. ProMED. 2018; Available from: https://promedmail.org/promed-posts/

[18] Kizito S, Okello PE, Kwesiga B, Nyakarahuka L, Balinandi S, Mulei S, et al. Notes from the Field: Crimean-Congo Hemorrhagic Fever Outbreak - Central Uganda, August-September 2017. MMWR Morb Mortal Wkly Rep. 2018; 67(22): 646-647. doi: 10.15585/mmwr.mm6722a6

[19] Mugahi R. Crimean-Congo haemorrhagic fever - East Africa (10): Uganda (BR), Daily Monitor. ProMED. 2018; Available from: https://promedmail.org/promed-posts/

[20] Jensenius M, Davis X, Von Sonnenburg F, Schwartz E, Keystone JS, Leder K, et al. Multicenter GeoSentinel analysis of rickettsial diseases in international travelers, 1996-2008. Emerg Infect Dis. 2009; 15(11): 1791–8. doi: 10.3201/eid1511.090677

[21] Kelly P. African tick-bite fever: a new spotted fever group rickettsiosis under an old name. Lancet. 1992 ; 340(8825): 982-3. doi: 10.1016/0140-6736(92)92878-j

[22] Ndip LM, Bouyer DH, Travassos APA, Rosa D, Titanji VPK, Tesh RB, et al. Acute Spotted Fever Rickettsiosis among Febrile Patients , Cameroon. Emerg Infect Dis. 2004; 10(3): 432-7. doi: 10.3201/eid1003.020713

[23] Oundo JW, Villinger J, Jeneby M, Ong'amo G, Otiende MY, Makhulu EE, et al. Pathogens, endosymbionts, and blood-meal sources of host-seeking ticks in the fast-changing Maasai Mara wildlife ecosystem. PLoS One. 2020;15(8):e0228366. doi: 10.1371/journal.pone.0228366

[24] Eisawi NM, Hassan DA, Hussien MO, Musa AB, El Hussein ARM. Seroprevalence of spotted fever group (SFG) rickettsiae infection in domestic ruminants in Khartoum State, Sudan. Vet Med Sci. 2017; 3(2): 91-98. doi: 10.1002/vms3.59

[25] Mutai BK, Wainaina JM, Magiri CG, Nganga JK, Ithondeka PM, Njagi ON, et al. Zoonotic surveillance for rickettsiae in domestic animals in Kenya. Vector Borne Zoonotic Dis. 2013; 13(6): 360-6. doi: 10.1089/vbz.2012.0977

[26] Ndip LM, Biswas HH, Nfonsam LE, Lebreton M, Ndip RN, Bissong MA, et al. Risk factors for African tick-bite fever in rural central Africa. Am J Trop Med Hyg. 2011; 84(4): 608-13. doi: 10.4269/ajtmh.2011.10-0191.

[27] Ismail N, Bloch KC, McBride JW. Human ehrlichiosis and anaplasmosis. Clin Lab Med. 2010; 30(1): 261-92. doi: 10.1016/j.cll.2009.10.004.

[28] Doudier B, Olano J, Parola P, Brouqui P. Factors contributing to emergence of *Ehrlichia* and *Anaplasma* spp. as human pathogens. Vet Parasitol. 2010; 167(2-4): 149-54. doi: 10.1016/j.vetpar.2009.09.016.

[29] Breitschwerdt EB, Hegarty BC, Qurollo BA, Saito TB, Maggi RG, Blanton LS, et al. Intravascular persistence of *Anaplasma platys*, *Ehrlichia chaffeensis*, and *Ehrlichia ewingii* DNA in the blood of a dog and two family members. Parasit Vectors. 2014; 7:298. doi: 10.1186/1756-3305-7-298

[30] Chochlakis D, Ioannou I, Sharif L, Kokkini S, Hristophi N, Dimitriou T, et al. Prevalence of *Anaplasma* sp. in goats and sheep in Cyprus. Vector-Borne Zoonotic Dis. 2009; 9(5): 457–63. doi. 10.1089/vbz.2008.0019

[31] Maggi RG, Mascarelli PE, Havenga LN, Naidoo V, Breitschwerdt EB. Co-infection with *Anaplasma platys*, *Bartonella henselae* and *Candidatus* Mycoplasma haematoparvum in a veterinarian. Parasit Vectors. 2013; 6:103. doi: 10.1186/1756-3305-6-103

[32] Johnson N, Johnson N, Paul Phipps L, McFadzean H, Barlow AM. An outbreak of bovine babesiosis in February, 2019, triggered by above average winter temperatures in southern England and co-infection with *Babesia divergens* and *Anaplasma phagocytophilum*. Parasit Vectors. 2020; 13(1):1–5. https://doi.org/10.1186/s13071-020-04174-3

[33] Koh FX, Panchadcharam C, Sitam FT, Tay ST. Molecular investigation of *Anaplasma* spp. in domestic and wildlife animals in Peninsular Malaysia. Vet Parasitol Reg Stud Reports. 2018; 13: 141-147. doi: 10.1016/j.vprsr.2018.05.006

[34] Peter SG, Aboge GO, Kariuki HW, Kanduma EG, Gakuya DW, Maingi N, et al. Molecular prevalence of emerging *Anaplasma* and *Ehrlichia* pathogens in apparently healthy dairy cattle in peri-urban Nairobi, Kenya. BMC Vet Res. 2020; 16(1): 364. doi: 10.1186/s12917-020-02584-0

[35] Onono JO, Wieland B, Rushton J. Productivity in different cattle production systems in Kenya. Trop Anim Health Prod. 2013; 45(2): 423-30. doi: 10.1007/s11250-012-0233-y

[36] Kenya National Bureau of Statistics. Livestock population. 2016; Available from: https://www.knbs.or.ke/?p=167

[37] Wesonga FD, Gachohi JM, Kitala PM, Gathuma JM, Njenga MJ. Seroprevalence of *Anaplasma marginale* and *Babesia bigemina* infections and associated risk factors in Machakos County, Kenya. Trop Anim Health Prod. 2017; 49(2): 265–72. http://doi.org/10.1007/s11250-016-1187-2

[38] Wesonga FD, Gachohi JM, Kitala PM, Gathuma JM, Njenga MJ. *Theileria parva* infection seroprevalence and associated risk factors in cattle in Machakos County, Kenya. Trop Anim Health Prod. 2015; 47(1):93-101. doi: 10.1007/s11250-014-0690-6

[39] Okal MN, Odhiambo BK, Otieno P, Bargul JL. *Anaplasma* and *Theileria* Pathogens in Cattle of Lambwe Valley, Kenya : A Case for Pro-Active Surveillance in the Wildlife-Livestock Interface. Microorganisms. 2020; 8(11): 1830. https://doi.org/10.3390/microorganisms8111830

[40] Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis. 1995; 1(1):7-15. doi: 10.3201/eid0101.950102

[41] Ahmed S, Dávila JD, Allen A, Haklay M, Tacoli C, Fèvre EM. Does urbanization make emergence of zoonosis more likely? Evidence, myths and gaps. Environ Urban.

2019; 31(2): 443–60. https://doi.org/10.1177/0956247819866124

[42] Fèvre EM, Glanville WA De, Thomas LF, Cook EAJ, Kariuki S, Wamae CN. An integrated study of human and animal infectious disease in the Lake Victoria crescent small-holder crop-livestock production system, Kenya. BMC Infect Dis. 2019; 17: 457. https://doi.org/10.1186/s12879-017-2559-6

[43] Nyaruaba R, Mwaliko C, Mwau M, Mousa S, Wei H. Arboviruses in the east African Community partner states: A review of medically important mosquito-borne arboviruses. Pathog. Glob. Health. 2019; 113(5): 209–228. https://doi.org/10.1080/20477724.2019.1678939.

[44] Fèvre EM, Bronsvoort BM, Hamilton KA, Cleaveland S. Animal movements and the spread of infectious diseases. Trends Microbiol. 2006; 14(3): 125-31. doi: 10.1016/j.tim.2006.01.004.

[45] Falzon LC, Alumasa L, Amanya F, Kang'ethe E, Kariuki S, Momanyi K, et al. One Health in action: Operational aspects of an integrated surveillance system for zoonoses in western Kenya. Front. Vet. Sci. 2019; 6: 252. https://doi.org/10.3389/fvets.2019.00252.

[46] Villinger J, Mbaya MK, Ouso D, Kipanga PN, Lutomiah J, Masiga DK. Arbovirus and insect-specific virus discovery in Kenya by novel six genera multiplex high-resolution melting analysis. Mol Ecol Resour. 2017; 17(3): 466-480. https://doi.org/10.1111/1755-0998.12584.

[47] Endoh D, Mizutani T, Kirisawa R, Maki Y, Saito H, Kon Y, et al. Species-independent detection of RNA virus by representational difference analysis using non-ribosomal hexanucleotides for reverse transcription. Nucleic Acids Res. 2005; 33 (6):e65. https://doi.org/10.1093/nar/gni064.

[48] Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990; 215(3): 403-10. doi: 10.1016/S0022-2836(05)80360-2

[49] Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O. New algorithms and methods to estimate maximum-likelihood phylogenies: Assessing the performance of PhyML 3.0. Syst. Biol. 2010; 59(3): 307–321. https://doi.org/10.1093/sysbio/syq010.

[50] Rambaut A. FigTree v1.4.2, a graphical viewer of phylogenetic trees. Edinburgh: University of Edinburgh. Available from http://tree.bio.ed.ac.uk/software/figtree/ 2014

[51] Eshoo MW, Whitehouse CA, Zoll ST, Massire C, Pennella TD, Blyn LB, et al. Direct broad-range detection of alphaviruses in mosquito extracts. Virology. 2007; 368(2): 286-295. https://doi:10.1016/j.virol.2007.06.016.

[52] Lambert AJ, Lanciotti RS. Consensus amplification and novel multiplex sequencing method for S segment species identification of 47 viruses of the Orthobunyavirus, Phlebovirus, and Nairovirus genera of the family Bunyaviridae. J Clin Microbiol. 2009; 47(8): 2398-404. doi: 10.1128/JCM.00182-09

[53] Nijhof AM, Bodaan C, Postigo M, Nieuwenhuijs H, Opsteegh M, Franssen L, et al. Ticks and associated pathogens collected from domestic animals in the Netherlands. Vector Borne Zoonotic Dis. 2007; 7(4): 585-95. doi: 10.1089/vbz.2007.0130.

[54] Georges K, Loria GR, Riili S, Greco A, Caracappa S, Jongejan F, et al. Detection of

haemoparasites in cattle by reverse line blot hybridisation with a note on the distribution of ticks in Sicily. Vet Parasitol. 2001; 99(4): 273-86. doi: 10.1016/s0304-4017(01)00488-5.

[55] Mwamuye MM, Kariuki E, Omondi D, Kabii J, Odongo D, Masiga D, et al. Ticks and Tick-borne Diseases Novel *Rickettsia* and emergent tick-borne pathogens : A molecular survey of ticks and tick-borne pathogens in Shimba Hills National. 2017; 8: 208–18. doi. 10.1016/j.ttbdis.2016.09.002

[56] Tokarz R, Kapoor V, Samuel JE, Bouyer DH, Briese T, Lipkin WI. Detection of tick-borne pathogens by masstag polymerase chain reaction. Vector-Borne Zoonotic Dis. 2009; 9(2): 147–51. doi. 10.1089/vbz.2008.0088

[57] Parola P, Roux V, Camicas JL, Baradji I, Brouqui P, Raoult D. Detection of ehrlichiae in African ticks by polymerase chain reaction. Trans R Soc Trop Med Hyg. 2000; 94(6): 707–8. doi: 10.1016/s0035-9203(00)90243-8.

[58] Reysenbach AL, Giver LJ, Wickham GS, Pace NR. Differential amplification of rRNA genes by polymerase chain reaction. Appl Environ Microbiol. 1992; 58(10): 3417–8.

[59] Edwards U, Rogall T, Blöcker H, Emde M, Böttger EC. Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. Nucleic Acids Res. 1989; 17(19): 7843-53. doi: 10.1093/nar/17.19.7843.

[60] Cabezas-Cruz A, Zweygarth E, Vancová M, Broniszewska M, Grubhoffer L, Passos LMF, et al. *Ehrlichia minasensis* sp. nov., isolated from the tick *Rhipicephalus microplus*. Int J Syst Evol Microbiol. 2016; 66(3): 1426–30. doi: 10.1099/ijsem.0.000895

[61] Byaruhanga C, Nicola E. Collins D, Knobel, Zamantungwa T.H. Khumalo MEC, Oosthuizen MC. Molecular detection and phylogenetic analysis of *Anaplasma marginale* and *Anaplasma centrale* amongst transhumant cattle in north-eastern Uganda. Ticks Tick Borne Dis. 2018; 9(3): 580–8. doi: 10.1016/j.ttbdis.2018.01.012.

[62] Zhou Z, Wu Y, Chen Y, Wang Z, Hu S, Zhou R, et al. Molecular and serological prevalence of *Toxoplasma gondii* and *Anaplasma* spp. infection in goats from Chongqing Municipality, China. Parasite. 2018; 25: 20. doi: 10.1051/parasite/2018024.

[63] Dyachenko V, Pantchev N, Balzer H, Meyersen A, Straubinger RK. First case of *Anaplasma platys* infection in a dog from Croatia. Parasit Vectors. (2012); 5: 49 https://doi.org/10.1186/1756-3305-5-49

[64] Guo WP, Zhang B, Wang YH, Xu G, Wang X, Ni X, et al. Molecular identification and characterization of *Anaplasma capra* and *Anaplasma platys*-like in *Rhipicephalus microplus* in Ankang, Northwest China. BMC Infect Dis. 2019; 9(1):434. doi: 10.1186/s12879-019-4075-3

[65] Roy BC, Kraucken J, Ahmed JS, Majumder J, Baumann MP, Clausen PH, et al. Molecular identification of tick-borne pathogens infecting cattle in Mymensingh district of Bangladesh reveals emerging species of *Anaplasma* and *Babesia*. Transbound Emerg Dis. 2018; 65(2): e231-e242. doi: 10.1111/tbed.12745

[66] Ybañez AP, Ybañez RH, Yokoyama N, Inokuma H. Multiple infections of *Anaplasma platys* variants in Philippine dogs. Vet World. 2016; 9(12): 1456-1460. doi:

10.14202/vetworld.2016.1456-1460.

[67] Glidden CK, Koehler AV, Hall RS, Saeed MA, Coppo M, Beechler BR, et al. Elucidating cryptic dynamics of *Theileria* communities in African buffalo using a high-throughput sequencing informatics approach. Ecol Evol. 2019; 10(1): 70-80. doi: 10.1002/ece3.5758

[68] Knowles DP, Kappmeyer LS, Haney D, Herndon DR, Fry LM, Munro JB, et al. Discovery of a novel species , *Theileria haneyi* n .sp ., infective to equids , highlights exceptional genomic diversity within the genus *Theileria* : implications for apicomplexan parasite surveillance. Int J Parasitol. 2018; 48 (9–10):679–90. https://doi.org/10.1016/j.ijpara.2018.03.010

[69] Martins TM, Neves L, Pedro OC, Fafetine JM, Do Rosário VE, Domingos A. Molecular detection of *Babesia* spp. and other haemoparasitic infections of cattle in Maputo Province, Mozambique. Parasitology. 2010; 137(6): 939-46. doi: 10.1017/S003118200999196X

[70] Byaruhanga C, Collins NE, Knobel D, Chaisi ME, Vorster I, Steyn HC, et al. Molecular investigation of tick-borne haemoparasite infections among transhumant zebu cattle in Karamoja Region, Uganda. Vet Parasitol Reg Stud Reports. 2016; 3-4: 27–35. http://doi.org/10.1016/j.vprsr.2016.06.004

[71] Katende J, Morzaria S, Toye P, Skilton R, Nene V, Nkonge C, et al. An enzyme-linked immunosorbent assay for detection of *Theileria parva* antibodies in cattle using a recombinant polymorphic immunodominant molecule. Parasitol Res. 1998 ; 84(5): 408-16. doi: 10.1007/s004360050419

[72] Eygelaar D, Jori F, Mokopasetso M, Sibeko KP, Collins NE, Vorster I, et al. Tick-borne haemoparasites in African buffalo (*Syncerus caffer*) from two wildlife areas in Northern Botswana. Parasit Vectors. 2015; 8: 26. doi: 10.1186/s13071-014-0627-y

[73] Muhanguzi D, Picozzi K, Hatendorf J, Thrusfield M, Welburn SC, Kabasa JD, et al. Prevalence and spatial distribution of *Theileria parva* in cattle under crop-livestock farming systems in Tororo District, Eastern Uganda. Parasit Vectors. 2014; 7:91. doi: 10.1186/1756-3305-7-91

[74] Homewood K, Trench P, Randall S, Lynen G, Bishop B. Livestock health and socio-economic impacts of a veterinary intervention in Maasailand: Infection-and-treatment vaccine against East Coast fever. Agric Syst. 2006; 89(2–3): 248–71.

[75] Byaruhanga C, Oosthuizen MC, Collins NE, Knobel D. Using participatory epidemiology to investigate management options and relative importance of tick-borne diseases amongst transhumant zebu cattle in Karamoja Region, Uganda. Prev Vet Med. 2015; 122(3): 287-97. doi: 10.1016/j.prevetmed.2015.10.011

[76] Hailemariam Z, Krücken J, Baumann M, Ahmed JS, Clausen PH, Nijhof AM. Molecular detection of tick-borne pathogens in cattle from Southwestern Ethiopia. PLoS One. 2017; 12(11): e0188248. doi: 10.1371/journal.pone.0188248

[77] Zhou Z, Li K, Sun Y, Shi J, Li H, Chen Y, et al. Molecular epidemiology and risk factors of *Anaplasma* spp., *Babesia* spp. and *Theileria* spp. infection in cattle in Chongqing, China. PLoS One. 2019; 14(7): e0215585. http://doi.org/10.1371/journal.pone.0215585

[78] Kocan KM. Targeting ticks for control of selected hemoparasitic diseases of cattle. Vet Parasitol. 1995; 57(1-3): 121-51. doi: 10.1016/0304-4017(94)03116-e

[79] Woolhouse MEJ, Thumbi SM, Jennings A, Chase-Topping M, Callaby R, Kiara H, et al. Co-infections determine patterns of mortality in a population exposed to parasite infection. Sci Adv. 2015; 1(2): e1400026. 10.1126/sciadv.1400026

[80] Vannier E, Gewurz BE, Krause PJ. Human babesiosis. Infect Dis Clin North Am. 2008; 22(3): 469-88, viii-ix. doi: 10.1016/j.idc.2008.03.010

[81] Ringo AE, Adjou Moumouni PF, Lee SH, Liu M, Khamis YH, Gao Y, et al. Molecular detection and characterization of tick-borne protozoan and rickettsial pathogens isolated from cattle on Pemba Island, Tanzania. Ticks Tick Borne Dis. 2018; 9(6): 1437-45. doi: 10.1016/j.ttbdis.2018.06.014.

[82] Arraga-alvarado CM, Qurollo BA, Parra OC, Berrueta MA, Hegarty BC, Breitschwerdt EB. Case Report : Molecular Evidence of *Anaplasma platys* Infection in Two Women from Venezuela. Am J Trop Med Hyg. 2014; 91(6): 1161-5. doi: 10.4269/ajtmh.14-0372

[83] Ben Said M, Belkahia H, El Mabrouk N, Saidani M, Alberti A, Zobba R, et al. *Anaplasma platys*-like strains in ruminants from Tunisia. Infect Genet Evol. 2017; 49: 226-233. doi: 10.1016/j.meegid.2017.01.023

[84] Cruz AC, Zweygarth E, Ribeiro MFB, Da Silveira JAG, De La Fuente J, Grubhoffer L, et al. New species of *Ehrlichia* isolated from *Rhipicephalus (Boophilus) microplus* shows an ortholog of the *E. canis* major immunogenic glycoprotein gp36 with a new sequence of tandem repeats. Parasit Vectors. 2012; 5: 291. doi: 10.1186/1756-3305-5-291

[85] de Aguiar DM, Junior JPA, Nakazato L, Bard E, Aguilar-Bultet L, Vorimore F, et al. Isolation and characterization of a novel pathogenic strain of *Ehrlichia minasensis*. Microorganisms. 2019; 7(11): 528. doi: 10.3390/microorganisms7110528

[86] Magona JW, Walubengo J, Olaho-Mukani W, Jonsson NN, Welburn SW, Eisler MC. Spatial variation of tick abundance and seroconversion rates of indigenous cattle to *Anaplasma marginale, Babesia bigemina* and *Theileria parva* infections in Uganda. Exp Appl Acarol. 2011; 55(2): 203-13. doi: 10.1007/s10493-011-9456-2

[87] Tabor AE. Anaplasmosis. MSD Veterinary Manual (2015). Available at: https://www.msdvetmanual.com/circulatory-system/blood-parasites/anaplasmosis

[88] Andrew HR, Norval RA. The carrier status of sheep, cattle and African buffalo recovered from heartwater. Vet Parasitol. 1989; 34(3): 261-6. doi: 10.1016/0304-4017(89)90056-3

[89] Lorusso V, Wijnveld M, Majekodunmi AO, Dongkum C, Fajinmi A, Dogo AG, et al. Tick-borne pathogens of zoonotic and veterinary importance in Nigerian cattle. Parasit Vectors. 2016; 9(1): 1–13. http://doi.org/10.1186/s13071-016-1504-7

[90] Omondi D, Masiga DK, Fielding BC, Kariuki E, Ajamma YU, Mwamuye MM, et al. 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. Front. Vet. Sci. 2017; 4: 73. *doi*:10.3389/fvets.2017.00073

Chapter 5

Aetiology of non-malarial febrile illness in western Kenya: Insights from hospital surveys

Tatenda Chiuya[1, 4], Laura C. Falzon[2, 3], Armanda D.S. Bastos[4], Eric M. Fèvre[2, 3], Jandouwe Villinger[1]
Daniel K. Masiga[1]

1. International Centre of Insect Physiology and Ecology (icipe), P.O Box 30772-00100, Nairobi, Kenya
2. Institute of Infection, Veterinary and Ecological Sciences, University of Liverpool, Leahurst Campus, Chester High Road, Neston, CH64 7TE, United Kingdom
3. International Livestock Research Institute, Old Naivasha Road, PO Box 30709, 00100 Nairobi, Kenya
4. Department of Zoology and Entomology, University of Pretoria, Pretoria, South Africa

5.1 Abstract

Malaria is the common diagnosis for fever of unknown origin in Africa resulting in the under-diagnosis of other causes of fever such as arboviruses and *Rickettsia*. While the latter two may not be significant causes of mortality in malaria-endemic areas, they affect the daily life and performance of affected individuals. It is therefore important to have a clear picture of the cause of fevers to institute correct diagnoses at hospitals and improve patient outcomes. I selected bio-banked blood samples collected from febrile patients at selected hospitals in the malaria-endemic counties of Busia, Bungoma, and Kakamega and screened them for arboviruses, *Rickettsia* spp., and *Plasmodium* spp. using high-throughput real-time PCR techniques. I used logistic regression to test the correlation of demographic and socio-economic independent variables with malaria positivity. Out of the 336 blood samples from febrile patients recruited 18.5% were positive for *Plasmodium falciparum* and 0.02% for *Plasmodium malariae.* One patient had a dual *P. falciparum*/*P. malariae* infection. None of the patients were positive for arboviruses or *Rickettsia* species. Patients living in Busia (OR: 5.478; 95 % CI: 2.509-13.055; $p = 0.000$) and Bungoma counties (OR: 3.027; 95 % CI: 1.358-7.316; $p = 0.009$) had 5.5 and 3 times higher malaria infection rates, respectively, compared to those living in Kakamega County. Patients coming from a household where the female head/spouse did not have a formal education had a four times higher risk (OR: 4.446; CI: 1.402-14.044; $p = 0.010$) of contracting malaria in comparison to those who came from a household where the female head/spouse had some level of formal education. The reported malaria prevalence is in line with previous studies, while the absence of acute arboviruses and *Rickettsia* cases calls for further investigation as serological studies have indicated their circulation in the area. Other zoonotic febrile illnesses previously reported to be prevalent in this region such as brucellosis and leptospirosis should also be evaluated.

***Keywords*:** *Plasmodium*, Fever, Diagnosis, Prevalence, Socio-economic factors, Kenya

5.2 Introduction

Viral and bacterial zoonotic diseases usually cause a mild non-pathognomonic febrile illness in humans which in severe cases can proceed to a range of complications such as encephalitis, haemorrhagic disorder, hepatitis, musculoskeletal impairment, and death [1]. The impact of the milder form of these diseases on human health has been under-appreciated, however, studies show that they have a substantial impact on the daily performance of affected individuals in terms of disability-adjusted life years (DALYS) [2]. At least 100 arboviruses are known to cause human disease [3], while about twenty *Rickettsia* spp. are documented to do so [4]. Traditionally arboviruses and *Rickettsia* are not considered as significant causes of mortality and morbidity, especially in malaria-endemic resource-poor communities. Thus, funds allocated for their study and surveillance are limited [5], potentially leading to misdiagnosis and poor assessment of their cumulative impact on community health [6,7].

In Kenya and the whole East African region, the most important arboviruses affecting humans are Rift Valley fever (RVF), chikungunya, and dengue viruses [8]. Diverse tick-borne *Rickettsia* spp. have been widely reported [9–11], however, there is limited surveillance in the human population [12]. Few acute cases of rickettsioses have been reported in indigenous African populations [13,14], but are widely reported in travellers and expatriates visiting endemic areas in Africa. Spotted fever group rickettsioses are only second to malaria as the aetiology of illness in travellers to sub-Saharan Africa (SSA) [15–17]. Some previous studies show that SFG rickettsioses and arboviral illness contribute to non-malaria febrile illness in patients visiting hospitals [18–20]. Elsewhere in Africa concurrent malaria and arboviral infections have also been documented [21,22].

Busia County, neighbouring with Bungoma and Kakamega counties, is situated at the border of Uganda and Kenya within the Lake Victoria basin of East Africa. The movement of animals,

people and goods across the borders in this region can result in the propagation of the viruses to new areas [8,23]. In this region, previous studies have reported seropositivity against chikungunya [24], alphaviruses, flaviviruses [25,26], phleboviruses (RVF) [27] and rickettsiae [12]. A single fatal human case of Crimean-Congo haemorrhagic fever (CCHF) was also reported in this region [28]. In terms of malaria endemicity, Busia County is defined as a lake-endemic transmission zone, while Bungoma and Kakamega counties have both lake-endemic and highland epidemic zones [29].

Great strides have been taken in Kenya to study arboviral diseases during outbreaks in the known hotspots such as the northern and coastal areas. However, less investigative efforts have been taken during inter-epidemic periods, in those areas where clinical cases have been detected and have the potential to support circulation and outbreaks. In Chapter 2 and 3, I detected a range of fever causing zoonotic pathogens such as CCHF virus, sindbis virus and *Rickettsia africae* in ticks and mosquitoes collected at livestock markets and hospitals. Therefore, I undertook this cross-sectional study in western Kenya on febrile patient blood samples to determine the occurrence of acute cases of arboviral illness and rickettsioses in relation to malaria prevalence.

5.3 Materials and methods

5.3.1 Study area

This study was carried out in three neighbouring counties of Busia, Bungoma, and Kakamega (Figure 5.1). The inhabitants of this region are mostly of the Luhya tribe. Busia County is closest to Lake Victoria among the three counties and directly borders Uganda. Mt Elgon in Bungoma County is the highest point in the province while the Kakamega rainforest is found in Kakamega County. The economic activity in the area is centered around sugar farming in Bungoma and Kakamega County while in Busia fishing is the major economic activity. Common to all the three counties is mixed subsistence farming characterised by intensification,

diversification, and close integration of crops and livestock production. The Busia-Uganda border is one of the busiest in East Africa with traffic transiting to Rwanda, Burundi, DRC, and South Sudan. A huge number of informal traders cross the border daily. It is most likely that Busia County Referral hospital also receives patients from Uganda and Kenya. The human population has been estimated at 89.3681 in Busia, 1.671 million in Bungoma, and 1.868 million in Kakamega county as of the 2019 population census [30].

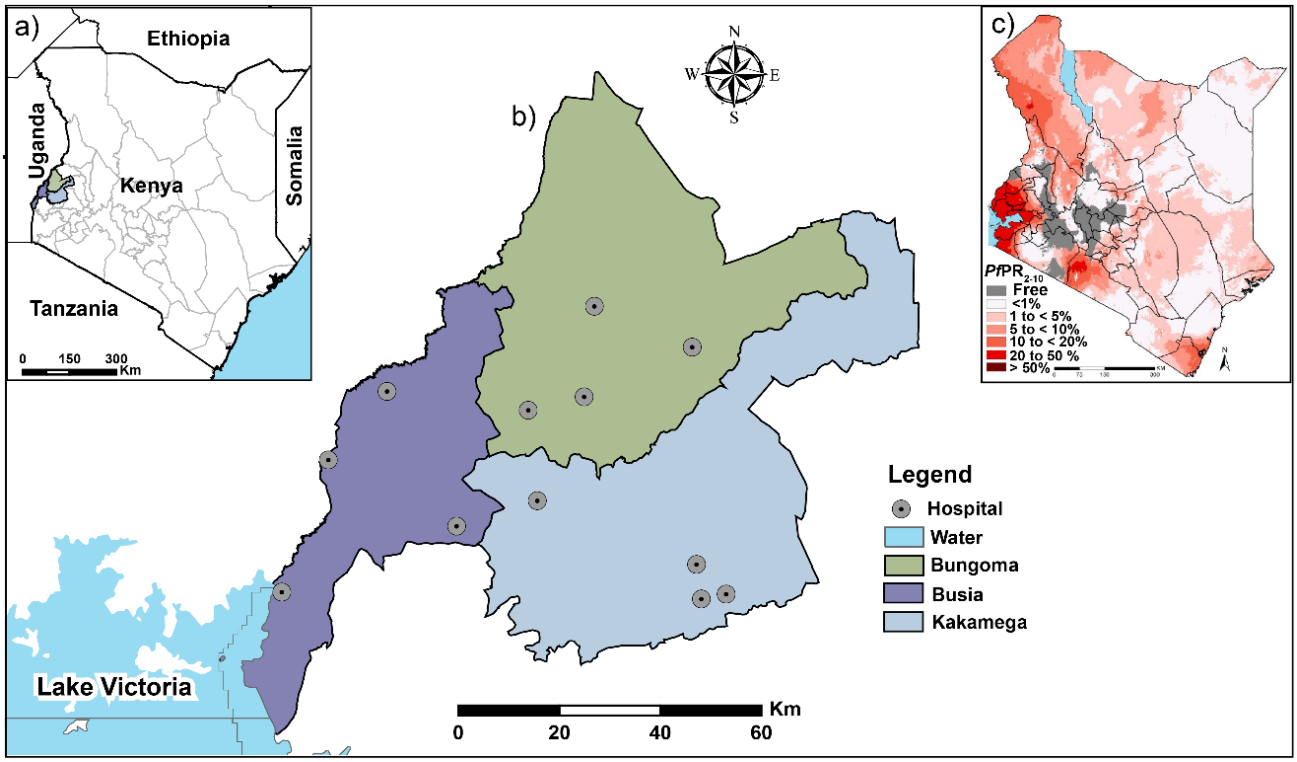


Figure 5.1: Map showing the study site in western Kenya.
(a) the highlighted three counties of Kenya where the study was conducted, (b) the distribution of the hospitals from which patients were recruited and (c) malaria risk grading in Kenya based on the *Plasmodium falciparum* parasite prevalence data in children aged 2-10 years ($PfPR_{2-10}$) [29].

5.3.2 Study design and sampling procedure

The blood samples used in this study were collected by the Zoonoses in Livestock and Humans (ZooLinK) project described in detail elsewhere [31]. The study aimed to pilot and develop a *One Health* based surveillance system for fifteen zoonotic diseases in the area for better

diagnostics and awareness. Samples and data from patients were collected after approval by the International Livestock Research Institute (ILRI) Institutional Research Ethics Committee (ref ILRI-IREC2017-08/2) licensed by the National Commission for Science, Technology and Innovation (NACOSTI) in Kenya.

Briefly a sampling frame of 24 hospitals in the three counties was created from which a total of 12 (4 per County) were selected for sampling. In each county a Referral hospital, a Missionary hospital, and a sub-County hospital were included in the study. Each of the selected hospitals was sampled at least 20 times from 2017 to 2019. At the hospitals two project clinical officers liaised with the hospital staff to ensure smooth recruitment of study participants. Up to 10 participants were recruited at each hospital per visit. The inclusion criterion was patients presenting with febrile illness suggestive of a zoonotic infection (Figure 5.2).

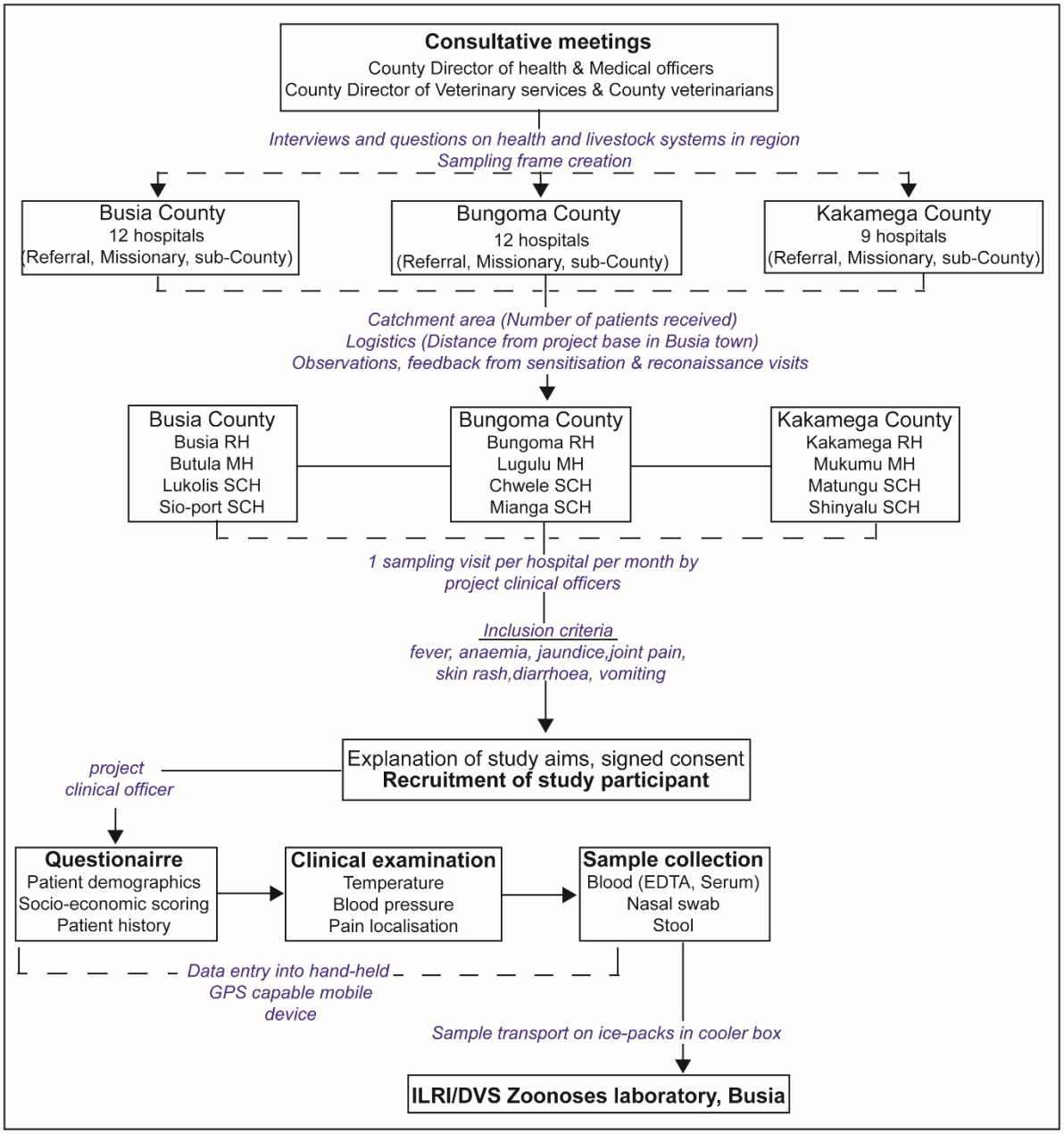


Figure 5.2: Graphical illustration of the framework for sampling at hospitals in western Kenya.
Based on Falzon et al., 2019 [31]

5.3.3 Nucleic acid extraction

Blood samples were retrieved from the ZooLinK bio-bank and thawed on ice before proceeding with nucleic acid extraction. I purposively selected a total of 336 blood samples that were collected between 2017 and 2018, the period in which I detected the Crimean-Congo haemorrhagic virus in a previous analysis on ticks from livestock [9; Chapter 2]. The blood

samples were collected at the same hospitals where sindbis and insect specific flaviviruses infected mosquitoes were trapped in Chapter 3. I used the magnetic-based High Prep Viral DNA/RNA kit (Magbio Genomics Gaithersburg, USA), which can isolate total nucleic acid from whole blood. Initially 200 µl of blood was added to 528 µl of a lysis master-mix consisting of VDR lysis buffer, isopropanol, and carrier RNA. After vortexing, 10 µl of proteinase K and 10µl of MAG-S1 magnetic beads were added and mixed into solution by vortexing. The subsequent wash steps to separate protein and cellular debris from nucleic acid bound to magnetic particles were carried out as per manufacturer's instructions. I synthesized cDNA in 10-µl reactions using the High Capacity cDNA Reverse Transcription Kit (Life technologies Carlsbad, USA) as per manufacturer's instructions, supplementing the random primers with 600 µM non-ribosomal random hexanucleotide primers previously described for maximum yield [32].

5.3.4 Detection of arbovirus in febrile patients

To detect arbovirus infections in febrile patients I applied a multiplex touchdown PCR-high resolution melting analyses (PCR-HRM) described by Villinger et al. 2017 [33] and Ajamma et al. 2018 [34] as an initial screening test to select positive samples for further identification. The individual primers in the multiplex reaction were optimised to detect flavi-, nairo-, phlebo-, alpha-, dhori-, thogoto-, and bunya-viruses. The reactions were carried out in a Rotor-Gene Q HRM thermo-cycler (Qiagen Hilden, Germany). The HRM products were purified using an Exo 1-rSAP combination (Biolabs, UK) and preliminarily identified after sequencing (Macrogen, Netherlands). To supplement the relatively short fragments generated by the PCR-HRM I then amplified all the positive samples using standard PCR to get longer fragments for definitive identification and phylogenetic analysis. Previously described primer pairs were optimised and used for further discrimination of flaviviruses [35], alphaviruses [36] and

nairoviruses [37]. The primer information and the cycling conditions are shown below (Table 5.1).

Table 5.1: Arboviral primers for PCR and their thermo-cycling conditions

Target gene	Primer name	Final product size	PCR step	1° amplification			2° amplification		
				Temp	Time	Cycles	Temp	Time	Cycles
Flavivirus NS5 gene nested PCR [35]	1NS5F† 1NS5Re† 2NSF5F‡ 2NSF5Re‡	1019 bp	Initial hold	95°C	15 mins	-	95°C	15 mins	-
			Denaturation	94°C	1 min	40	94°C	1 min	35
			Annealing	54°C	40 sec		60°C	40 sec	
			Extension	72°C	2 mins		72°C	2 mins	
			Final extension	72°C	5 mins	1	72°C	5 mins	1
			Final hold	4°C	∞	-	4°C	∞	-
Alphavirus NS1 gene hemi-nested PCR [36]	M2W† cM3W†‡ M2W2‡	310 bp	Initial hold	95°C	15 mins	-	95°C	15 mins	-
			Denaturation	94°C	20 sec	45	94°C	20 sec	45
			Annealing	50°C	30 sec		48°C	30 sec	
			Extension	72°C	30 sec		72°C	30 sec	
			Final extension	72°C	5 mins	1	72°C	5 mins	1
			Final hold	4°C	∞	-	4°C	∞	-
Nairovirus RdRp gene standard PCR [37]	6942F 7385R	420 bp	Initial hold	95°C	15 mins	-	NA		
			Denaturation	94°C	30 sec	45			
			Annealing	52°C	30 sec				
			Extension	72°C	30 sec				
			Final extension	72°C	5 mins	1			
			Final hold	4°C	∞	-			

†first round primer
‡second round primer
†‡primer for both first and second round

5.3.5 Detection of *Rickettsia* and *Plasmodium* spp.

To detect the presence of *Rickettsia* I screened patients' blood with PCR-HRM using Rick-F1 and Rick-R2 primers that target the *Rickettsia* 16S rRNA region [38]. The PCR mixture contained 2 µl of 5X HOT FIREPol EvaGreen HRM mix (Solis BioDyne, Estonia), 10 pmol. of each forward and reverse primer, 2 µl of template DNA and topped up with molecular grade water. *Rickettsia africae* DNA amplified and sequenced in previous analysis in my lab was used as a positive control in each run [11]. Thermocycling and high-resolution melting (HRM) analysis were carried out in a Rotor-Gene Q HRM thermo-cycler (Qiagen Hilden, Germany) using previously described conditions [39].

I screened patients' blood for malaria-causing *Plasmodium* spp. using two sets of primers. Initially, I used a primer pair ncMS-F/ncMS-R to amplify a non-coding mitochondrial region (large subunit rRNA fragment E) of all *Plasmodium* spp. [40] using PCR-HRM [41]. The reaction mixture was made up of 2 µl of 5x HOT FIREPol EvaGreen HRM mix (Solis BioDyne, Estonia), 10 pmol of each forward and reverse primer and 1 µl of template DNA. Thermal cycling was carried out in a Rotor-Gene Q HRM thermo-cycler (Qiagen Hilden, Germany) and conditions were set as follows: initial denaturation at 95°C for 15 min, followed by 40 cycles of denaturation at 95°C for 20 secs, annealing at 61°C for 15 sec and extension at 72°C for 20 sec. There were five post-cycling hold steps, at 72°C for 7 mins, 95°C for 15 sec., 68°C for 1 min., 80°C for 15 sec. and 60°C for 15 sec. The 190-bp amplicons were melted from 75°C to 90°C with 0.1°C increments. *Plasmodium falciparum* DNA amplified and sequenced previously was included as a positive control in all the runs [42]. All positive samples were selected by analysing melt and normalised profiles on Rotor-Gene Q software 2.1.0.

Positive samples were further amplified using *cox* 1 primers targeting a 540-bp region of the cytochrome oxidase 1 gene of *Plasmodium* spp. [43]. The 15-µl reaction PCR mix contained 7.5 µl of 2x MyTaq HS Mix (Bioline, UK), 10 pmol of each forward and reverse primer, 2 µl of the template and topped up with molecular grade water. Thermo-cycling was carried out in a SimpliAmp thermo-cycler (Applied biosystems, Singapore) with the following set of conditions: initial denaturation at 95°C for 3 mins followed by 35 cycles of denaturation at 95°C for 20 secs., annealing at 59°C for 30 secs and extension at 72 °C for 30 secs. Successful amplification was confirmed by agarose gel electrophoresis against a molecular weight marker (Quick-Load® 100-bp DNA Ladder (Biolabs, UK).

Representative amplicons were purified by an Exo 1-rSAP combination (Biolabs, UK) and sequenced at (Macrogen, Netherlands). Sequences were edited and cleaned using Geneious Prime version 2019.0.4 software (Biomatters, New Zealand). To confirm identity and

relationship with previously described *Plasmodium* spp. BLAST nucleotide searches were performed against the GenBank nr database (Figure 5.3 outlines the sample processing in the lab).

5.3.6 Phylogenetic analysis of *Plasmodium* spp.

Sequences were inspected and edited in Geneious prime version 2019.0.4 software (Biomatters, New Zealand). Sequence contigs were then used in BLAST nucleotide searches against the GenBank nr [44]. Closely-related sequences and reference strains identified in this manner were added to the dataset. Sequence alignments were built using MAFFT and phylogenies were constructed in PhyML version 3.0 using the maximum-likelihood analysis over 1000 replicates. Automatic model selection was based on the Akaike Information Criterion (AIC) [45]. Trees were viewed in Figtree 1.4 [46].

5.3.7 Statistical analysis

All the patient meta-data associated with the blood samples analysed in this study were stored in a database accessible only to relevant project staff. Irrelevant specific patients and contact details were masked. Barcodes and new lab generated sample identities were used during the analysis. All the statistical analyses were done in R® version 3.5.3. Initially I calculated the proportions of the participants in each county and the independent variable categories and generated an exact 95 % confidence interval (Clopper-Pearson) [47]. Malaria prevalence was calculated in each of the independent variable categories. All the demographic independent variables (Table 5.2) were fitted in a multiple logistic regression model after testing them for multi-collinearity with a positive/negative *P. falciparum* PCR as the response variable. The final model selection was based on employing the Akaike's Information Criterion in both directions [48].

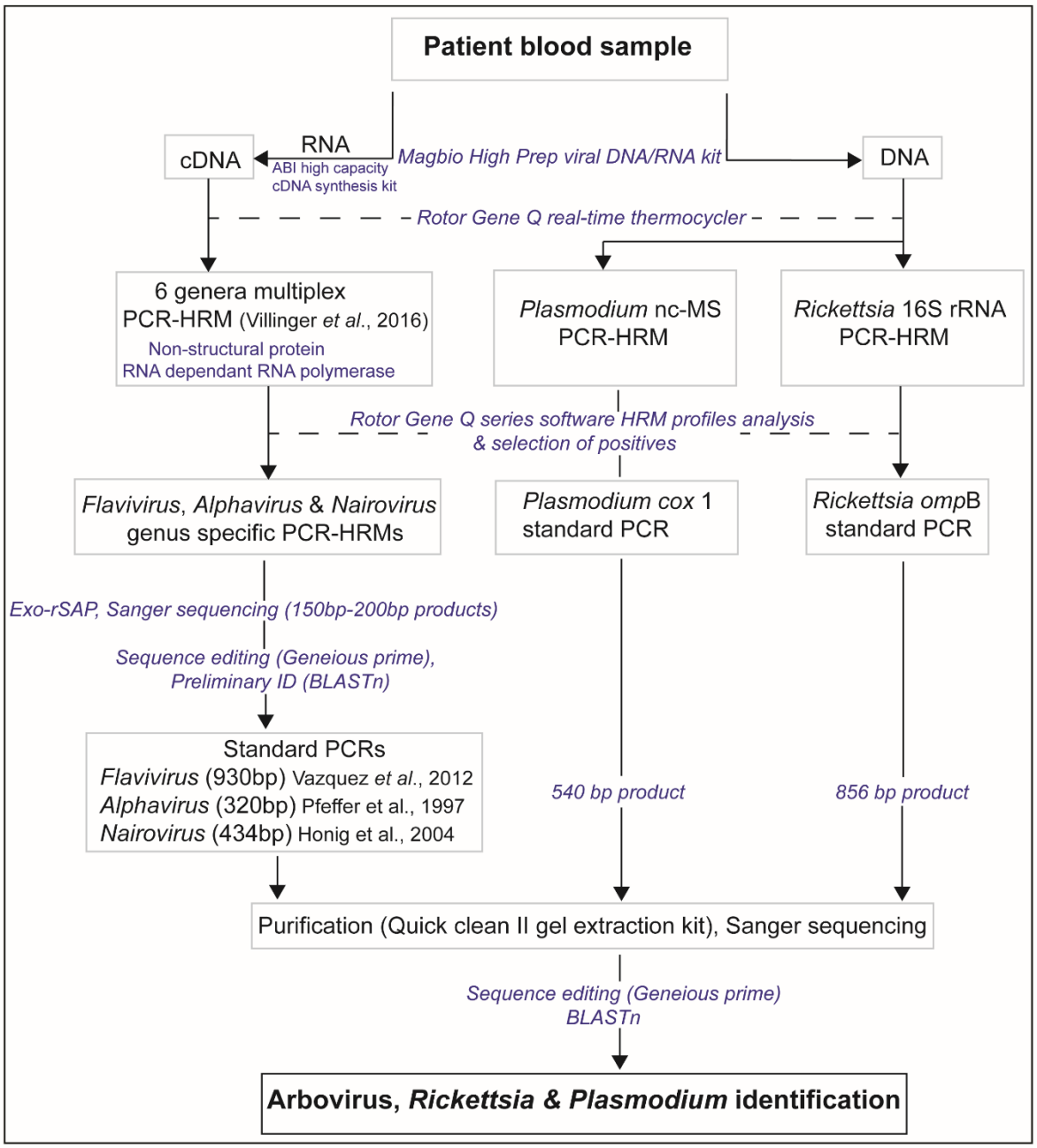


Figure 5.3: Graphical illustration of the sample processing and molecular analyses workflow

5.4 Results

5.4.1 Socio-demographic characteristics of study participants

Table 5.2 shows the socio-demographic profile of the 336 patients who participated and whose blood samples were analysed in this study. Overall 37 % of the participants were from Bungoma County, 35% from Kakamega, and 28% from Busia County. Twenty-five percent of the participants who presented to the hospitals and were recruited into the study were males compared to about 74% females which was also the case at the County level. The age range of the participants was from 6 to 88 years, most were teenagers (27%) and those aged 50 years and above (22.8%) which was also the case in each of the three counties under study. Farming was the most common occupation while students contributed an equal proportion of the study participants overall and at the county level. Ninety-two percent of the participants reported coming from a household that owned livestock in the form of goats, sheep, chicken, pig, and cattle. About 96% of the participants reported having at least one mosquito bed-net at home. Only 5.7 % (19/336) of the participants came from a household where the female head/spouse had not received any level of formal education while 29.5 % (99/336) came from a household where the female head/spouse had gone up to form 4 and beyond.

5.4.2 *Plasmodium* spp., arbovirus, and *Rickettsia* detection

I detected by high resolution melting analysis, *P. falciparum* (deposited GenBank accessions: MT430947-MT430948), and *Plasmodium malariae* (deposited GenBank accession: MT430946) infections in febrile patients presenting to selected hospitals in the counties under study. They were a total of six patients infected with *P. malariae* and one patient with *P. falciparum*/*P. malariae* dual infection (Figure 5.4). All the febrile patients' blood samples tested negative for selected arboviruses such as chikungunya, sindbis, and Crimean-Congo haemorrhage fever. They were also negative for *Rickettsia* such as *R. africae* and *Rickettsia felis*.

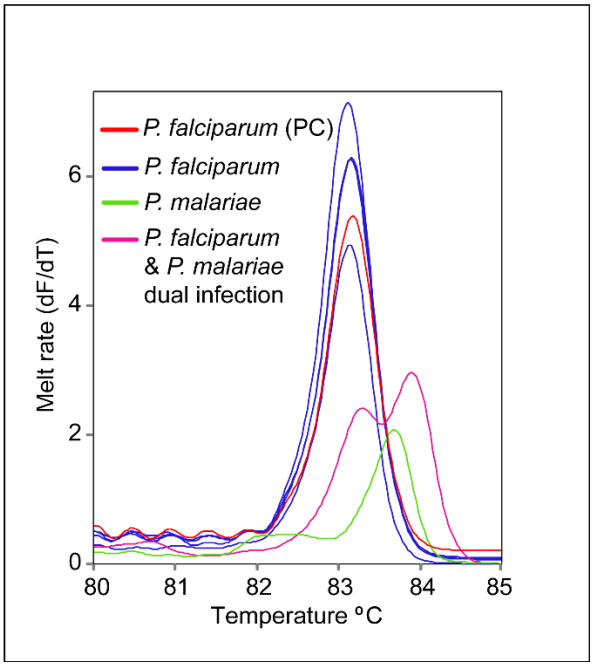


Figure 5.4: Melt rate profiles of representative samples positive for *Plasmodium* spp. *Plasmodium falciparum*, *P. malariae* and *P. falciparum*/*P. malariae* mixed infections based on amplification of the non-coding mitochondrial gene (large subunit rRNA fragment E)

The phylogeny of representative sequences of *P. falciparum* and *P. malariae* detected in this study with previously described strains is shown in Fig 5.5. My *P. falciparum* strains (deposited GenBank accession MT430947-MT430948) shared 99.6% and 100% identity with previously described strains from Thailand (GenBank accession NC_037526) [49] and Kenya (GenBank accession MH547440) [50], respectively. The *P. malariae* strain from this study (deposited GenBank accession M430946) shared 99.6% identity with a strain from Cameroon (GenBank accession MF693433) [51].

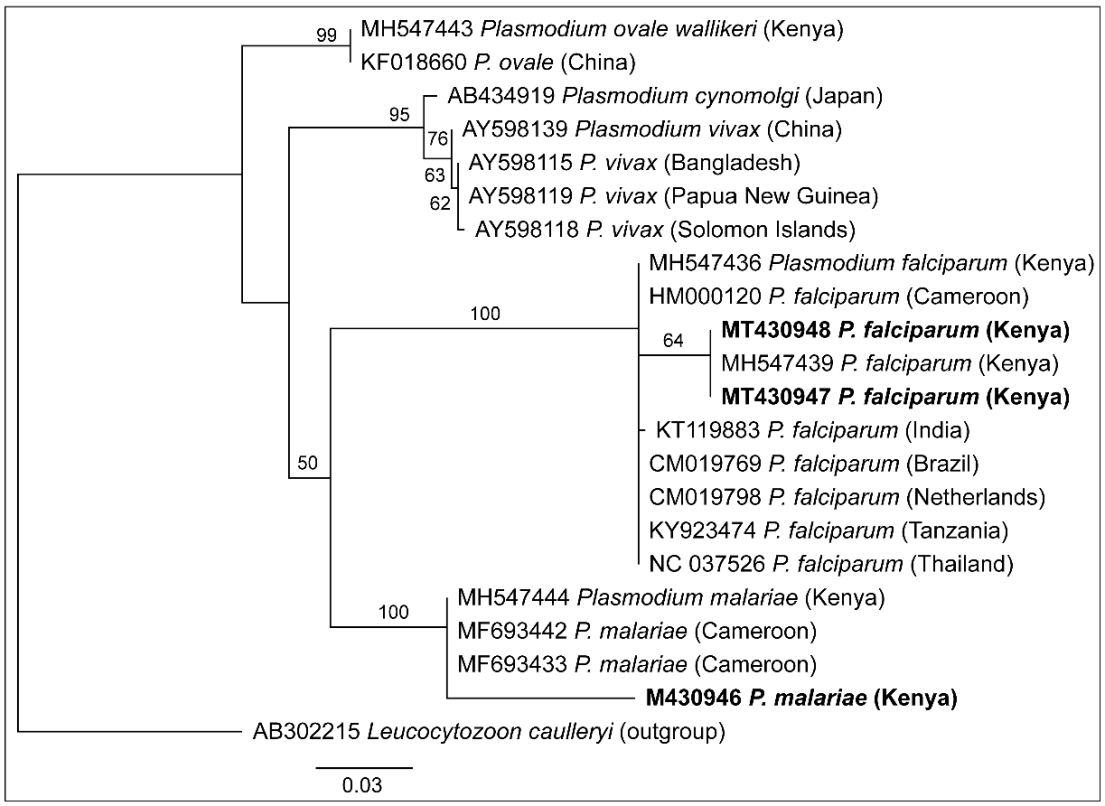


Figure 5.5: Maximum likelihood phylogeny of *Plasmodium* spp. inferred from 22 aligned 500-nt segments of the *cox* 1 gene.
Taxon names comprise of the GenBank accession number and country of origin, with sequences characterised in this study being indicated in bold. Bootstrap values show percentage agreement from 1,000 replicates.

Table 5.2: Socio-demographics of febrile patients recruited into the study at hospitals in three different counties

Characteristic	Busia County		Bungoma County		Kakamega County		Combined	
	N	%	N	%	N	%	N	%
Total	**95**	**100**	**124**	**100**	**117**	**100**	**336**	**100**
Sex								
Male	23	24.2	34	27.4	28	24	85	25.3
Female	71	74.7	88	71	89	76	248	73.8
Missing	1	1.1	2	1.6	0	0	3	0.9
Age (years)								
0-9	4	4.2	0	0	4	3.4	8	2.4
10-19	33	34.7	33	27	25	21.4	91	27.1
20-29	18	19	17	13.7	30	25.6	65	19.3
30-39	10	10.5	19	15	18	15.4	47	14
40-49	12	12.6	20	16.1	13	11.1	45	13.4
50+	17	17.9	33	26.6	27	23.1	77	22.9
Missing	1	1.1	2	1.6	0	0	3	0.9
Occupation								
Farmer	28	29.5	44	35.5	35	29.9	107	31.8
Trader	13	13.7	15	12.1	19	16.2	47	14
Student	37	38.9	37	29.8	31	26.5	105	31.3
Other†	6	6.3	20	16.1	16	13.7	42	12.5
Unemployed	11	11.6	8	6.5	16	13.7	35	10.4
Floor-type								
Mud/wood	47	49.5	49	39.5	54	46.2	150	44.6
Cement/tiles	48	50.5	75	60.5	63	53.8	186	55.4
Livestock ownership								
YES	86	90.5	112	90.3	112	95.7	310	92.3
NO	9	9.5	12	9.7	5	4.3	26	7.7
Mosquito nets								
NO	3	3.2	5	4	3	2.6	11	3.3
1	44	46.3	59	47.6	42	35.9	145	43.1
≥ 2	48	50.5	60	48.4	72	61.5	180	53.6
Education level‡								
None	9	9.5	3	2.4	7	6	19	5.7
Pr. 1-7	24	25.3	22	17.7	27	23.1	73	21.7
Pr. 8 & Form 1-3	39	41	58	46.8	48	41	145	43.1
Form 4 & above	23	24.2	41	33.1	35	29.9	99	29.5

†Other category for occupation includes those who worked as teacher, police, tailor, security guard, rider, driver, house help and shopkeeper.

‡Education level of female head of household/spouse: Pr.=Primary School, characterised as: grade 1-7 (ISCED level 1), grade 8 (ISCED level 1) and Form 1-3 (ISCED level 2) and Form 4 and above (ISCED level 3-8). United Nations Educational, Scientific and Cultural Organization (UNESCO) International Standard Classification of Education (ISCED) levels are shown in brackets [52].

5.4.3 Malaria prevalence and multivariable analysis

The overall malaria prevalence in the febrile patients that were recruited in this study at hospitals in the three neighbouring counties was 18.5% (62/336) (CI: 14.4-23) as determined by a positive PCR result for *P. falciparum.* Malaria prevalence was highest in Busia (OR: 5.478; 95 % CI: 2.509-13.055; $p = 0.000$) and in decreasing order of prevalence Bungoma (OR: 3.027; 95 % CI: 1.358-7.316; $p = 0.009$) and Kakamega counties respectively (Table 5.3 & 5.4). These estimated odds ratio signified a 5 and 3 times higher risk of contracting malaria in the patients residing in the counties of Busia and Bungoma respectively in comparison to those from Kakamega County. Overall, more females were recruited into the study in proportion to male participants and hence the malaria prevalence was also reported higher for females however this difference in prevalence was not statistically significant. They were proportionally more participants aged between 10 and 19 years and those above 50 years compared to the other age group and subsequently these two categories had the highest malaria prevalence. Most of the participants were farmers followed by those who reported as being students however the malaria prevalence was higher in the latter group. None of the two independent variables gender and occupation was significantly associated with malaria positivity.

Table 5.3: Malaria prevalence in each of the independent variable categories relative to their proportion in the study population

Independent variable	Proportion	Malaria prevalence	95 % CI	
n = 336	*%*	*%*	*lower*	*upper*
County				
Busia	(95/336) 28	(30/336) 8.9	6.1	12.5
Bungoma	(124/336) 37	(23/336) 6.8	4.4	10
Kakamega	(117/336) 35	(9/336) 2.7	1.2	5
Sex				
Male	(87/336) 25.9	(18/336) 5.4	3.2	8.3
Female	(249/336) 74.1	(44/336) 13.1	9.7	17.2
Age				
0-9	(12/336) 3.6	(5/336) 1.5	0.3	3
10-19	(90/336) 26.8	(21/336) 6.3	3.9	9.4
20-29	(65/336) 19.3	(11/336) 3.3	1.6	5.8
30-39	(47/336) 14	(5/336) 1.5	0.5	3.4
40-49	(45/336) 13.4	(7/336) 2.1	0.8	4.2
50+	(77/336) 22.9	(13/336) 3.9	2.1	6.5
Occupation				
Farmer	(106/336) 31.5	(18/336) 5.4	3.2	8.3
Trader	(47/336) 14	(4/336) 1.2	0.3	3
Student	(105/336) 31.3	(26/336) 7.7	5.1	11.1
Other	(42/336) 12.5	(7/336) 2.1	0.8	4.2
Unemployed	(36/336) 10.7	(7/336) 2.1	0.8	4.2
Floor type				
Mud/wood	(185/336) 55.1	(31/336) 9.2	6.4	12.8
Cement/tile	(151/336) 44.9	(31/336) 9.2	6.4	12.8
Livestock ownership				
YES	(309/336) 91.9	(56/336) 16.7	12.8	21.1
NO	(27/336) 8.1	(6/336) 1.8	0.7	3.8
Mosquito nets				
NO	(12/336) 3.6	(5/336) 1.5	0.5	3.4
1	(144/336) 42.8	(26/336) 7.7	5.1	11.1
≥2	(180/336) 53.6	(31/336) 9.2	6.4	12.8
Education level				
None	(20/336) 5.9	(8/336) 2.4	0.1	4.6
Primary 1-7	(73/336) 21.7	(14/336) 4.2	2.3	6.9
Primary 8 & Form 1-3	(144/336) 42.9	(27/336) 8	5.4	11.5
Form 4 & Above	(99/336) 29.5	(13/336) 3.9	2.1	6.5

† Education level of female head of household/spouse

Being a student however predicted twice the risk for malaria positivity than the other occupations in the full model (OR: 1.947, 95 % CI: 0.454-8.421; $p = 0.368$). The participants were equally divided into those who reported living in a house with a mud floor and those living in a house with a cement/tile floor with an equal prevalence of malaria in both groups. At least 90% of the participants had livestock in the household and only 3% did not have a mosquito net. Not having a mosquito net, floor type and livestock ownership were not predictors of malaria positivity. However, having no bed net predicted twice as much risk as having one/at least two bed nets for malaria positivity in the full model (OR: 1.992, 95% CI: 0.361-9.756, $p = 0.403$). Patients coming from a household where the female head/spouse did not have any formal education were four times more likely to be positive for *P. falciparum* (OR: 4.446, 95% CI: 1.402-14.044, $p = 0.010$) in comparison to those from a household where the female head/spouse had some level of formal education (Table 5.4).

Table 5.4: The logistic regression analysis of malaria (*Plasmodium falciparum* PCR-positive) and risk factors in patients presenting to hospitals in the three counties of Kakamega, Bungoma, and Busia (n = 336).
P-values less than 0.05 are shown in italics

Variable	***P. falciparum* positive**		
	OR	**95 % CI**	***P*-value**
County			
Kakamega	Reference	-	-
Bungoma	3.027	1.358-7.316	*0.009*
Busia	5.478	2.509-13.055	*0.000*
Education level†			
Form 4 & above	Reference	-	-
Primary 8 & Form 3	1.478	0.720-3.161	0.297
Primary 1-7	1.510	0.643-3.570	0.341
None	4.446	1.402-14.044	*0.010*

†Education level of female head of household/spouse

5.5 Discussion

Higher prevalence of malaria in Busia and Bungoma compared to Kakamega county concurs with past reports of heterogeneity in transmission patterns in western Kenya with higher transmission up to 27% around the Lake Victoria shores in Busia compared to other areas [53]. This region is classified as having both stable and epidemic malaria transmission. Stable transmission is characterised by hyper endemic transmission rates while epidemic transmission occurs in less prevalent zones where the population has less immunity [29]. Unsurprisingly, the *Plasmodium* spp. detected here were phylogenetically closely related to geographically closer strains.

The impact of education on malaria prevalence has been reported before and knowledgeable household heads are more likely to possess knowledge on the transmission dynamics of malaria and impart this know-how about malaria prevention on other members of the household [54]. In this study I sought to assess the impact of the education level of the female spouse or female head of the house on malaria positivity. This is because female spouses/heads of households are more hands-on in taking care of other family members in the household than male spouses. They are therefore more likely to be responsible for the imparting of knowledge and behavioural change in the face of malaria challenges [55]. Education has also been reported to influence malaria morbidity and mortality [56,57].

The other variables in my study were not significantly associated with malaria positivity. Bed net possession was high among the participants however I did not assess its correct and consistent use. The bed net/malaria prevalence association is masked in some studies because some patients get infective bites before retiring to bed or early morning due to the changing ecology and behaviour of the vector [58], improper use of the bed nets [59] and aging of the nets themselves [60]. Age is usually an important predictor in higher transmission settings in infants under the age of 5 years [61]. However, in low transmission settings the risk will also

extend into adulthood [62,63]. The effect of gender has not been established with previous studies showing conflicting findings. Women were more likely to be infected than males in Kisumu [64]. Certain socio-cultural activities expose both women and men to infective mosquito bites albeit in different ways [65].

The effect of livestock at households on malaria transmission is dynamic with the consensus that it can zoo-potentiate [66–68] when close or inside human dwellings but can also act as zoo-prophylaxis when placed a distance away from households [69]. In this study the proximity of livestock to the homesteads was not assessed. Floor-type was used as a proxy to determine the socio-economic status of the household from which the individual came from. Higher socio-economic status is usually associated with improvement in the housing conditions and less predisposition to malaria [70,71]. However, this association is prone to being confounding given that most of the proxies for socio-economic status are in essence more directly related to malaria exposure and risk [72].

I attempted to determine acute arboviral and rickettsial infections in relation to malaria infection as has been reported before in western Kenya in Kisumu [20], Homa bay [73], Asembo [12] and in Tanzania [19]. However, inter-epidemic circulation of arboviruses is very low and it remains a challenge to detect clinical/acute cases [20,74]. This is also reflected in the low infection rates of the vector mosquito species from the same region where I found only one pool of *Culex* mosquitoes that was positive for sindbis virus in combination with a comparatively high infection rate with insect-specific flaviviruses [Chapter 3] that are thought to block superinfection of vectors with pathogenic viruses [75]. On the other hand, *Plasmodium* spp./chikungunya co-infection has been shown to cause interferon gamma induced suppression of viraemia and joint pathology caused by arthralgic alphaviruses [76]. This is plausible in this study given that this is a malaria-endemic region where most people are likely to be asymptomatic carriers [77] and the most sero-prevalent arbovirus there is chikungunya [24].

The absence of CCHF virus detected in Chapter 2 in these patients is also related to the reasons mentioned for mosquito-borne arboviruses above.

Due to low detection, previous studies in the same region have tried to characterise the amount of febrile illness due to arboviruses by serology [24,26,78]. A shortcoming is that serology shows past exposure but is not an indicator of clinical disease. While I have described high infection rates in *Amblyomma variegatum* ticks by *R. africae* previously sampled as part of this broader study [Chapter 2], absence in febrile patients, in this chapter, is suggestive of low transmissibility of the pathogen to humans and livestock.

While I did not detect arboviruses or *Rickettsia* in this study its importance in raising clinical awareness in hospitals to other probable causes of febrile illnesses besides malaria cannot be ignored. Associating our findings with the demographics and socio-economic status of the participants enables us to make inferences on factors that might be keeping outbreaks in check, given the conducive environment of the study site.

5.6 Limitations and conclusions

The first limitation was the use of archived blood samples for this study which might have affected the recovery and detection of arboviruses in the blood but not the detection of *Plasmodium* parasites by PCR. To counter the occurrence of false negatives internal PCR controls that amplify host messenger RNA can be developed and used in future viral analysis. Secondly, the malaria positivity described in this study was PCR-based but not related to the final clinical diagnosis made by the clinical staff at the hospital after the collection of blood samples from the patients. Combining the use of IgM serology and RT-PCR may be able to generate more informative conclusions on the occurrence of arboviral disease and rickettsioses. Serology could not be performed in this case due to serum sample constraints. This is on the backdrop of the reported circulation of arboviruses and *Rickettsia* by several serological studies

in this region. While my main focus was on arboviruses, *Rickettsia* and malaria, future studies should be expanded to include other zoonotic pathogens that cause non-pathognomonic fever such as *Brucella abortus*, *Coxiella burnetii*, *Bartonella henselae* and *Leptospira* spp. There are previous serological reports of brucellosis, Q-fever and leptospirosis in this region. Livestock presence in the participants' households is a variable of interest as reports on zoo-potentiation and zoo-prophylaxis are still conflicting. It is also an important factor in the control of arbovirus transmitting mosquitoes which are more zoophilic compared to malaria vectors. Distinguishing the proximity of the livestock to households is therefore of paramount importance in future studies.

5.7 Acknowledgements

I acknowledge the Zoonoses in Livestock in Kenya (ZooLinK) team for the collection of blood samples used in this analysis. I also acknowledge Bester Mudereri of *icipe* GIS and Remote Sensing unit for assistance in producing the study site map.

5.8 References

[1] Hollidge BS, González-Scarano F, Soldan SS. Arboviral encephalitides: transmission, emergence, and pathogenesis. J Neuroimmune Pharmacol. 2010;5(3): 428-42. doi: 10.1007/s11481-010-9234-7

[2] Labeaud AD, Bashir F, King CH. Measuring the burden of arboviral diseases: the spectrum of morbidity and mortality from four prevalent infections. Popul Health Metr. 2011; 9(1): 1. doi: 10.1186/1478-7954-9-1

[3] Pfeffer M, Dobler G. Emergence of zoonotic arboviruses by animal trade and migration. Parasit Vectors. 2010; 3(1): 35. doi: 10.1186/1756-3305-3-35.

[4] Richards AL, Jiang J, Omulo S, Dare R, Abdirahman K, Ali A, et al. Human Infection with *Rickettsia felis*, Kenya. Emerg Infect Dis. 2010; 16(7): 1081-6. doi: 10.3201/eid1607.091885

[5] Wilder-Smith A, Gubler DJ, Weaver SC, Monath TP, Heymann DL, Scott TW. Epidemic arboviral diseases: priorities for research and public health. Lancet Infect Dis. 2017; 17(3):e101-6. http://doi.org/10.1016/S1473-3099(16)30518-7

[6] Hooft AM, Ripp K, Ndenga B, Mutuku F, Vu D, Baltzell K, et al. Principles, practices and knowledge of clinicians when assessing febrile children: A qualitative study in Kenya. Malar. J. 2017; 16(1): 381. https://doi.org/10.1186/s12936-017-2021-7.

[7] Nyaoke BA, Mureithi MW, Beynon C. Factors associated with treatment type of non-malarial febrile illnesses in under-fives at Kenyatta National Hospital in Nairobi, Kenya. PLoS One. 2019; 14(6): e0217980. https://doi: 10.1371/journal.pone.0217980

[8] Nyaruaba R, Mwaliko C, Mwau M, Mousa S, Wei H. Arboviruses in the east African Community partner states: A review of medically important mosquito-borne arboviruses. Pathog. Glob. Health. 2019; 113(5): 209–228. https://doi.org/10.1080/20477724.2019.1678939.

[9] Chiuya T, Masiga D, Falzon L, Bastos A, Fevre E, Villinger J. Tick-borne pathogens , including Crimean-Congo haemorrhagic fever virus , at livestock markets and slaughterhouses in western Kenya. 2020; **in press**. https://doi.org/10.1111/tbed.13911

[10] Koka H, Sang R, Kutima HL, Musila L, Macaluso K. The detection of spotted fever group *Rickettsia* DNA in tick samples from pastoral communities in Kenya. J Med Entomol. 2017;54(3):774–80. https://doi.org/10.1093/jme/tjw238

[11] Oundo JW, Villinger J, Jeneby M, Ong'amo G, Otiende MY, Makhulu EE, et al. Pathogens, endosymbionts, and blood-meal sources of host-seeking ticks in the fast-changing Maasai Mara wildlife ecosystem. PLoS One. 2020;15(8):e0228366. doi: 10.1371/journal.pone.0228366

[12] Maina AN, Knobel DL, Jiang J, Halliday J, Feikin DR, Cleaveland S, et al. *Rickettsia felis* infection in febrile patients, western Kenya, 2007-2010. Emerg Infect Dis. 2012; 18(2): 328-31. doi: 10.3201/eid1802.111372.

[13] Kelly P. African tick-bite fever: a new spotted fever group rickettsiosis under an old name. Lancet. 1992 ; 340(8825): 982-3. doi: 10.1016/0140-6736(92)92878-j

[14] Ndip LM, Bouyer DH, Travassos APA, Rosa D, Titanji VPK, Tesh RB, et al. Acute

Spotted Fever Rickettsiosis among Febrile Patients , Cameroon. Emerg Infect Dis. 2004; 10(3): 432-7. doi: 10.3201/eid1003.020713

[15] Althaus F, Greub G, Raoult D, Genton B. African tick-bite fever: a new entity in the differential diagnosis of multiple eschars in travelers. Description of five cases imported from South Africa to Switzerland. Int J Infect Dis. 2010;14 Suppl 3:e274-6. doi: 10.1016/j.ijid.2009.11.021

[16] Jensenius M, Davis X, Von Sonnenburg F, Schwartz E, Keystone JS, Leder K, et al. Multicenter GeoSentinel analysis of rickettsial diseases in international travelers, 1996-2008. Emerg Infect Dis. 2009; 15(11): 1791-8. doi: 10.3201/eid1511.090677

[17] Parola P, Paddock CD, Raoult D. Tick-borne rickettsioses around the world: emerging diseases challenging old concepts. Clin Microbiol Rev. 2005;18(4): 719-56. doi: 10.1128/CMR.18.4.719-756.2005

[18] Chipwaza B, Mugasa JP, Selemani M, Amuri M, Mosha F, Ngatunga SD, et al. Dengue and Chikungunya Fever among Viral Diseases in Outpatient Febrile Children in Kilosa District Hospital, Tanzania. PLoS Negl Trop Dis. 2014; 8(11): e3335. doi: 10.1371/journal.pntd.0003335

[19] Crump JA, Morrissey AB, Nicholson WL, Massung RF, Stoddard RA, Galloway RL, et al. Etiology of severe non-malaria febrile illness in Northern Tanzania: A prospective cohort Study. PLoS Negl. Trop. Dis. 2013; 7(7): e2324. https://doi.org/10.1371/journal.pntd.0002324.

[20] Waggoner J, Brichard J, Mutuku F, Ndenga B, Heath CJ, Mohamed-Hadley A, et al. Malaria and Chikungunya Detected Using Molecular Diagnostics Among Febrile Kenyan Children. Open Forum Infect Dis. 2017; 4(3): ofx110. doi: 10.1093/ofid/ofx110

[21] Ayorinde AF, Oyeyiga AM, Nosegbe NO, Folarin OA. A survey of malaria and some arboviral infections among suspected febrile patients visiting a health centre in Simawa, Ogun State, Nigeria. J Infect Public Health. 2016; 9(1):52-9. doi: 10.1016/j.jiph.2015.06.009

[22] Sow A, Loucoubar C, Diallo D, Faye O, Ndiaye Y, Senghor CS, et al. Concurrent malaria and arbovirus infections in Kedougou, southeastern Senegal. Malar J. 2016; 15: 47. doi: 10.1186/s12936-016-1100-5

[23] Fèvre EM, Bronsvoort BM, Hamilton KA, Cleaveland S. Animal movements and the spread of infectious diseases. Trends Microbiol. 2006; 14(3): 125-31. doi: 10.1016/j.tim.2006.01.004.

[24] Mease LE, Coldren RL, Musila LA, Prosser T, Ogolla F, Ofula VO, et al. Seroprevalence and distribution of arboviral infections among rural Kenyan adults: A cross-sectional study. Virol. J. 2011; 8(1): 371. https://doi: 10.1186/1743-422X-8-371

[25] Grossi-Soyster EN, Cook EAJ, de Glanville WA, Thomas LF, Krystosik AR, Lee J, et al. Serological and spatial analysis of alphavirus and flavivirus prevalence and risk factors in a rural community in western Kenya. PLoS Negl Trop Dis. 2017; 11(10): e0005998. doi: 10.1371/journal.pntd.0005998

[26] Inziani M, Adungo F, Awando J, Kihoro R, Inoue S, Morita K, et al. Seroprevalence of yellow fever, dengue, West Nile and chikungunya viruses in children in Teso south

sub-County, western Kenya. Int. J. Infect. Dis. 2020; 91: 104–110. https://doi: 10.1016/j.ijid.2019.11.004

[27] Anne E, Cook J, Grossi-soyster EN, De WA, Thomas LF, Kariuki S, et al. The sero-epidemiology of Rift Valley fever in people in the Lake Victoria Basin of western Kenya. 2017; 11(7): e0005731. doi: 10.1371/journal.pntd.0005731

[28] Dunster L, Dunster M, Ofula V, Beti D, Kazooba-Voskamp F, Burt F, et al. First documentation of human Crimean-Congo hemorrhagic fever, Kenya. Emerg Infect Dis. 2002;8(9):1005–6. doi: 10.3201/eid0809.010510

[29] Ministry of Health. The epidemiology and control profile of malaria in Kenya: reviewing the evidence to guide the future vector control. National Malaria Control Programme, Ministry of Health. 2016.

[30] KNBS. Kenya Population and Housing Census Volume II: Distribution of Population by Administrative Units. Kenya Population and Housing Census. 2019; 2: 251. Available from: http://www.knbs.or.ke

[31] Falzon LC, Alumasa L, Amanya F, Kang'ethe E, Kariuki S, Momanyi K, et al. One Health in action: Operational aspects of an integrated surveillance system for zoonoses in western Kenya. Front. Vet. Sci., 2019; 6: 252. https://doi.org/10.3389/fvets.2019.00252.

[32] Endoh D, Mizutani T, Kirisawa R, Maki Y, Saito H, Kon Y, et al. Species-independent detection of RNA virus by representational difference analysis using non-ribosomal hexanucleotides for reverse transcription. Nucleic Acids Res. 2005; 33 (6):e65. https://doi.org/10.1093/nar/gni064.

[33] Villinger J, Mbaya MK, Ouso D, Kipanga PN, Lutomiah J, Masiga DK. Arbovirus and insect-specific virus discovery in Kenya by novel six genera multiplex high-resolution melting analysis. Mol Ecol Resour. 2017; 17(3): 466-480. https://doi.org/10.1111/1755-0998.12584.

[34] Ajamma YU, Onchuru TO, Ouso DO, Omondi D, Masiga DK, Villinger J. Vertical transmission of naturally occurring Bunyamwera and insect-specific flavivirus infections in mosquitoes from islands and mainland shores of lakes Victoria and Baringo in Kenya. PLoS Negl. Trop. Dis. 2018; 12(11): e0006949. https://doi.org/10.1371/journal.pntd.0006949.

[35] Vázquez A, Sánchez-Seco MP, Palacios G, Molero F, Reyes N, Ruiz S, et al. Novel flaviviruses detected in different species of mosquitoes in Spain. Vector Borne Zoonotic Dis. 2012; 1 (3): 223–229. https://doi.org/10.1089/vbz.2011.0687.

[36] Pfeffer M, Proebster B, Kinney RM, Kaaden OR. Genus-specific detection of alphaviruses by a semi-nested reverse transcription-polymerase chain reaction. Am. J. Trop. Med. Hyg. 1997; 57(6): 709–718. https://doi.org/10.4269/ajtmh.1997.57.709.

[37] Honig JE, Osborne JC, Nichol ST. The high genetic variation of viruses of the genus Nairovirus reflects the diversity of their predominant tick hosts. Virology. 2004; 318(1): 10-6. doi: 10.1016/j.virol.2003.09.021.

[38] Nijhof AM, Bodaan C, Postigo M, Nieuwenhuijs H, Opsteegh M, Franssen L, Jebbink F, Jongejan F. Ticks and associated pathogens collected from domestic animals in the Netherlands. Vector Borne Zoonotic Dis. 2007; 7(4): 585-95. doi:

10.1089/vbz.2007.0130

[39] Mwamuye MM, Kariuki E, Omondi D, Kabii J, Odongo D, Masiga D, et al. Ticks and Tick-borne Diseases Novel Rickettsia and emergent tick-borne pathogens : A molecular survey of ticks and tick-borne pathogens in Shimba Hills National. 2017; 8: 208–18. doi. 10.1016/j.ttbdis.2016.09.002

[40] Bourgeois N, Boutet A, Bousquet P, Basset D, Charachon S, Lachaud L, et al. Comparison of three real-time PCR methods with blood smears and rapid diagnostic test in Plasmodium sp . infection. Eur Soc Clin Infect Dis. 2009; 16(8): 1305–11. http://doi.org/10.1111/j.1469-0691.2009.02933.x

[41] Chaumeau V, Andolina C, Fustec B, Ndam NT, Brengues C, Herder S, et al. Comparison of the Performances of Five Primer Sets for the Detection and Quantification of *Plasmodium* in Anopheline Vectors by Real-Time PCR. PLoS One. 2016; 11(7): e0159160. doi: 10.1371/journal.pone.0159160

[42] Ogola EO, Fillinger U, Ondiba IM, Villinger J, Masiga DK, Torto B, et al. Insights into malaria transmission among Anopheles funestus mosquitoes , Kenya. Parasit Vectors. 2018; 1–10. https://doi.org/10.1186/s13071-018-3171-3

[43] Echeverry DF, Deason NA, Makuru V, Davidson J, Xiao H, Niedbalski J, et al. Fast and robust single PCR for *Plasmodium* sporozoite detection in mosquitoes using the cytochrome oxidase I gene. Malar J. 2017;16(1):1–8. https://doi.org/10.1186/s12936-017-1881-1

[44] Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990; 215(3): 403-10. doi: 10.1016/S0022-2836(05)80360-2

[45] Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O. New algorithms and methods to estimate maximum-likelihood phylogenies: Assessing the performance of PhyML 3.0. Syst. Biol. 2010; 59(3): 307–321. https://doi.org/10.1093/sysbio/syq010.

[46] Rambaut A. FigTree v1.4.2, a graphical viewer of phylogenetic trees. Edinburgh: University of Edinburgh. Available from http://tree.bio.ed.ac.uk/software/figtree/ 2014

[47] Puza B, O'neill T. Generalised Clopper-Pearson confidence intervals for the binomial proportion. J Stat Comput Simul. 2006;76(6):489-508. https://doi.org/10.1080/10629360500107527

[48] Snipes M, Taylor DC. Model selection and Akaike Information Criteria: An example from wine ratings and prices. Wine Econ Policy. 2014; 3(1): 3–9. http://dx.doi.org/10.1016/j.wep.2014.03.001

[49] Conway DJ, Fanello C, Lloyd JM, Al-Joubori BM, Baloch AH, Somanath SD, et al. Origin of *Plasmodium falciparum* malaria is traced by mitochondrial DNA. Mol Biochem Parasitol. 2000; 111(1):163-71. doi: 10.1016/s0166-6851(00)00313-3.

[50] Ogola EO, Fillinger U, Ondiba IM, Villinger J, Masiga DK, Torto B, et al. Insights into malaria transmission among *Anopheles funestus* mosquitoes, Kenya. Parasit Vectors. 2018; 11(1):577. doi: 10.1186/s13071-018-3171-3.

[51] Loy DE, Rubel MA, Avitto AN, Liu W, Li Y, Learn GH, et al. Investigating zoonotic infection barriers to ape *Plasmodium* parasites using faecal DNA analysis. Int J Parasitol. 2018; 48(7): 531-542. doi: 10.1016/j.ijpara.2017.12.002.

[52] UNESCO. International Standard Classification of Education (ISCED) 2011. 2012. Available at: http://uis.unesco.org/en/topic/international-standard-classification-education-isced

[53] Bashir IM, Nyakoe N, Van Der Sande M. Targeting remaining pockets of malaria transmission in Kenya to hasten progress towards national elimination goals: An assessment of prevalence and risk factors in children from the Lake endemic region. Malar J. 2019; 18(1): 1–10. https://doi.org/10.1186/s12936-019-2876-x

[54] Sichande M, Michelo C, Halwindi H, Miller J. Education attainment of head of households associated with insecticide-treated net utilization among five to nineteen-year old individuals: Evidence from the malaria indicator survey 2010 in Zambia. Malar J. 2014; 13:378. doi: 10.1186/1475-2875-13-378

[55] Ghahremani L, Azizi M, Moemenbellah-Fard MD, Ghaem H. Malaria preventive behaviors among housewives in suburbs of Bandar-Abbas City, south of Iran: interventional design based on PRECEDE model. Pathog Glob Health. 2019; 113(1): 32–8. https://doi.org/10.1080/20477724.2019.1583847

[56] Chitunhu S, Musenge E. Spatial and socio-economic effects on malaria morbidity in children under 5 years in Malawi in 2012. Spat Spatiotemporal Epidemiol. 2016; 16: 21-33. doi: 10.1016/j.sste.2015.11.001

[57] Ma C, Claude KM, Kibendelwa ZT, Brooks H, Zheng X, Hawkes M. Is maternal education a social vaccine for childhood malaria infection? A cross-sectional study from war-torn Democratic Republic of Congo. Pathog Glob Health. 2017; 111(2): 98–106. http://dx.doi.org/10.1080/20477724.2017.1288971

[58] Van Bortel W, Trung HD, Hoi LX, Van Ham N, Van Chut N, Luu ND, et al. Malaria transmission and vector behaviour in a forested malaria focus in central Vietnam and the implications for vector control. Malar J. 2010; 9:373. doi: 10.1186/1475-2875-9-373

[59] Guerra M, De Sousa B, Ndong-Mabale N, Berzosa P, Arez AP. Malaria determining risk factors at the household level in two rural villages of mainland Equatorial Guinea. Malar J. 2018; 17(1): 203. doi: 10.1186/s12936-018-2354-x

[60] Andronescu LR, Buchwald AG, Coalson JE, Cohee L, Bauleni A, Walldorf JA, et al. Net age, but not integrity, may be associated with decreased protection against *Plasmodium falciparum* infection in southern Malawi. Malar J. 2019; 18(1):1–7. https://doi.org/10.1186/s12936-019-2930-8

[61] WHO. WHO Malaria report. 2020; Available from: https://www.who.int/news-room/fact-sheets/detail/malaria

[62] O'Meara WP, Mwangi TW, Williams TN, McKenzie FE, Snow RW, Marsh K. Relationship between exposure, clinical malaria, and age in an area of changing transmission intensity. Am J Trop Med Hyg. 2008; 79(2): 185-91

[63] Kapesa A, Kweka EJ, Atieli H, Afrane YA, Kamugisha E, Lee MC, et al. The current malaria morbidity and mortality in different transmission settings in Western Kenya.

PLoS One. 2018; 13(8): e0202031. doi: 10.1371/journal.pone.0202031

[64] Jenkins R, Omollo R, Ongecha M, Sifuna P, Othieno C, Ongeri L, et al. Prevalence of malaria parasites in adults and its determinants in malaria endemic area of Kisumu County, Kenya. Malar J. 2015; 14:263. doi: 10.1186/s12936-015-0781-5

[65] UNDP. United Nations development programme: Discussion Paper Gender and Malaria. 2015; Available from: https://www.undp.org/content/undp/en/home/librarypage/hiv-aids/gender--hiv-and-health-discussion-papers.html

[66] Hasyim H, Dhimal M, Bauer J, Montag D, Groneberg DA, Kuch U, et al. Does livestock protect from malaria or facilitate malaria prevalence? A cross-sectional study in endemic rural areas of Indonesia. Malar J. 2018; 17(1): 1–11. https://doi.org/10.1186/s12936-018-2447-6

[67] Iwashita H, Dida GO, Sonye GO, Sunahara T, Futami K, Njenga SM, et al. Push by a net, pull by a cow: can zooprophylaxis enhance the impact of insecticide treated bed nets on malaria control? Parasit Vectors. 2014; 7: 52. https://doi.org/10.1186/1756-3305-7-52.

[68] Mayagaya VS, Nkwengulila G, Lyimo IN, Kihonda J, Mtambala H. The impact of livestock on the abundance, resting behaviour and sporozoite rate of malaria vectors in southern Tanzania. 2015; 14. https://doi.org/10.1186/s12936-014-0536-8

[69] Franco AO, Gomes MGM, Rowland M, Coleman PG, Davies CR. Controlling malaria using livestock-based interventions: a one health approach. PLoS One. 2014; 9(7): e101699. doi: 10.1371/journal.pone.0101699

[70] Ayele DG, Zewotir TT, Mwambi HG. Prevalence and risk factors of malaria in Ethiopia. Malar J. 2012; 11: 195. doi: 10.1186/1475-2875-11-195

[71] Kateera F, Mens PF, Hakizimana E, Ingabire CM, Muragijemariya L, Karinda P, et al. Malaria parasite carriage and risk determinants in a rural population: A malariometric survey in Rwanda. Malar J. 2015; 14(1):1–11. https://doi.org/10.1186/s12936-014-0534-x

[72] Worrall E, Basu S, Hanson K. Is malaria a disease of poverty? A review of the literature. Trop Med Int Health. 2005; 10(10): 1047-59. doi: 10.1111/j.1365-3156.2005.01476.x

[73] Omondi DO. Bionomics of vector-borne diseases in sites adjacent to lakes Victoria and Baringo in Kenya. PhD thesis, University of the Western Cape. 2016. Available from: http://etd.uwc.ac.za/xmlui/handle/11394/5338.

[74] Gu W, Lampman R, Novak RJ. Problems in estimating mosquito infection rates using minimum infection rate. J Med Entomol. 2003; 40(5): 595-6. doi: 10.1603/0022-2585-40.5.595

[75] Bolling BG, Olea-popelka FJ, Eisen L, Moore CG, Blair CD. Transmission dynamics of an insect-specific flavivirus in a naturally infected *Culex pipiens* laboratory colony and effects of co-infection on vector competence for West Nile virus. Virology. 2012; 427(2): 90–97. https://doi.org/10.1016/j.virol.2012.02.016.

[76] Teo TH, Lum FM, Ghaffar K, Chan YH, Amrun SN, Tan JJL, et al. *Plasmodium* co-infection protects against chikungunya virus-induced pathologies. Nat Commun. 2018;

9(1). http://doi.org/10.1038/s41467-018-06227-9

[77] Touray AO, Mobegi VA, Wamunyokoli F, Herren JK. Diversity and Multiplicity of *P. falciparum* infections among asymptomatic school children in Mbita, Western Kenya. Sci Rep. 2020; 10(1): 1–8. http://doi.org/10.1038/s41598-020-62819-w

[78] Kwallah A, Inoue S, Thairu-muigai AW, Kuttoh N, Morita K, Mwau M. Seroprevalence of Yellow Fever Virus in Selected Health Facilities in Western Kenya from 2010 to 2012. Jpn J Infect Dis. 2015; 68(3): 230-4. doi: 10.7883/yoken.JJID.2014.288

Chapter 6

General conclusion and recomendations

Viruses, bacteria and protozoa of zoonotic and/or veterinary significance were detected in ticks and mosquitoes in the first two chapters of this study confirming their importance as disease vectors. The detection of Crimean-Congo haemorrhagic fever (CCHF) virus in a *Rhipicephalus decoloratus* tick, which is not the known vector of the virus, underscores the risk of infective tick bites to humans and the need to follow up such entomological surveys with vector competence studies. This is because *Hyalomma* spp. are the known vectors of CCHF worldwide but it is important to determine the specific vectors for these pathogens in a specific setting so as to set up targeted control measures. In addition, Chapter 2 reinforces the fact that ticks are the chief vectors of protozoa and bacteria of veterinary significance such as *Theileria parva*, *Babesia bigemina* and *Anaplasma marginale*. While several other tick species and lice were infected with *Rickettsia africae*, its high prevalence in *Amblyomma variegatum* ticks affirms them as reservoirs of the pathogen. On the other hand, this high prevalence should be taken with caution as a recent unpublished study detected *R. africae* gene elements integrated in the genome of *Am. variegatum* ticks. Future studies should therefore anticipate this possible source of false positive PCR results. Consequently, the control of ticks by acaricide dipping and spraying is recommended. However, excessive and incorrect use of acaricides may actually upset endemic stability and select for resistant ticks. The use of biological tick control methods such as entomopathogenic fungi is therefore a promising alternative. Tick control also protects humans from getting infective tick bites. Overall, tick control will reduce the translocation of infected ticks across different regions *via* livestock markets and slaughterhouses. I screened a limited number of fleas and lice for pathogens as they were not the focus of this study, however, these are important vectors of zoonoses and should also be the focus of future large scale epidemiological studies.

The abundance of *Aedes aegypti* and *Culex pipiens* mosquitoes at hospitals relative to the occurrence of their favourable habitats shows how the urban ecology can be easily altered, enhancing mosquito breeding, resting, and biting activity. Therefore, communities should be encouraged to eliminate these habitats to control the proliferation of mosquitoes. Other measures such residual spraying and distribution of insecticide-treated bed nets need to be reactivated and supported by the relevant stakeholders especially in resource-poor arbovirus and malaria endemic areas. While the detection of sindbis virus in *Culex* spp. mosquitoes indicates risk of transmission of the virus to humans, the infection of mosquitoes by insect-specific flaviviruses (ISFs) may modulate the transmission of pathogenic arboviruses. Transmission blocking strategies are promising in the control of vector-borne zoonotic diseases, however their large-scale deployment is hampered by the high costs and current lack of conclusive evidence on their effectiveness. Future studies should, therefore, compare the prevalence of ISFs in mosquitoes from this region and other arbovirus outbreak-prone areas such as the coast and north-eastern parts of the country. This builds on the knowledge that has been generated by *in vitro* and *in vivo* laboratory studies on the interaction of pathogenic arboviruses and ISFs. The interaction of these ISFs and viral elements integrated into mosquito genomes also needs further investigation in natural mosquito populations. The mosquito trapping itself can be done over several seasons to improve the sample size and subsequently the chances of detecting arboviruses and assess the effect of seasonality on mosquito infection with arboviruses.

The detection of protozoa and bacteria in ticks and cattle shows the high prevalence and transmissibility of these pathogens from ticks. It also asserts the importance of pathogens like *T. parva, A. marginale* and *B. bigemina* in cattle health and production. It is surprising that in spite of the high prevalence of *R. africae* in *Am. variegatum* ticks it was undetected in the blood collected from the same animals. In future competence studies should be done to ascertain the

vectorial capacity of ticks for this pathogen in the local context. These competence studies should also compare the virulence of *R. africae* variants as several studies have now reported their presence in *Am. variegatum* ticks. Furthermore, it shows that cattle only serve to harbour the tick not the pathogen in the epidemiology of rickettsioses. Whereas cattle are known to be carriers of CCHF, failure to detect it in blood by PCR could be due to insignificant viraemia which can be circumvented by using serology which measures a prolonged antibody response. From the results of my study and previous ones, livestock is less likely to be carriers/reservoirs of mosquito-borne viruses.

Similarly, due to the low-level circulation of arboviruses during inter-epidemic periods detection in febrile patients may be difficult with PCR-based methods on blood especially after several freeze-thaw cycles. However, combining it with IgM serology may give a clearer picture of recent exposure. Developing arbovirus screening assays that incorporate an endogenous control, will be valuable for excluding the possibility of false-negative PCR results. While the study focused on hospitals for human blood sample collection it is important in future to do targeted sampling on slaughterhouse workers and butchers who represent the high-risk group for contracting CCHF virus, using a combination of PCR and serological screening. The use of personal protective equipment by these high-risk groups should be encouraged as to protect against infection with pathogens like CCHF virus. As outlined in Chapter 5 there are other zoonotic causes of fever such as brucellosis, Q-fever and leptospirosis that have been reported previously in western Kenya. These could explain some of the fever in my study participants and they should be investigated in future studies. It is also noteworthy that the transmission of the above-mentioned bacterial zoonoses is equally linked to contact with ticks and livestock especially during slaughter. This underlines the importance of LMs and SHs in the transmission dynamics of zoonotic diseases. The close phylogenetic relationship

of pathogens detected in this study and those reported in previous studies from Uganda suggests pathogen translocation across the border most likely due to live animal movements.

The surveillance carried out in this study can be expanded to include more zoonotic pathogens that cause febrile illness so as to collect reliable baseline data on their occurrence. This prior knowledge raises awareness and is important for correct diagnosis of fevers in health centres, distinguishing them from malaria which is still the top priority in sub-Saharan Africa. This distinction is important in instituting the proper treatment protocols and improves patient prognosis. In addition, the *One health* approach builds collaborations between the relevant stakeholders in health related research resulting in effective control of zoonoses.

Appendix

Table A.1: Details of primers used in the molecular identification of ticks and screening for vector-borne pathogens

Primer name	Target gene	Primer sequence (5' – 3')	Product size (bp)	Citation
Lep F	Tick CO1	ATTCAACCAATCATAAAGATATTGG	658	Hebert et al., 2004
Lep R		TAAACTTCTGGATGTCCAAAAAATCA		
ITS2 F	Tick ITS2	CGAGACTTGGTGTGAATTGCA	920-1850	Chitimia et al., 2009
ITS2 R		TCCCATACACCACATTTCCCG		
F-RMI16S	Tick 16S rRNA	AATTGCTGTAGTATTTTGAC	450	Brahma et al., 2014
R-RMI16S		TCTGAACTCAGATCAAGTAG		
Phlebo JV3a F	*Phlebovirus* NP	AGTTTGCTTATCAAGGGTTTGATGC		Villinger et al., 2017
Phlebo JV3b F		GAGTTTGCTTATCAAGGGTTTGACC	150	
Phlebo JV3 R		CCGGCAAAGCTGGGGTGCAT		
Nairo L 1a F	*Nairovirus* RdRp	TCTCAAAGATATCAATCCCCCCITTACCC		Villinger et al., 2017
Nairo L 1b F		TCTCAAAGACATCAATCCCCCTTWTCCC		
Nairo L 1a R		CTATRCTGTGRTAGAAGCAGTTCCCATC		
Nairo L 1b R		GCAATACTATGATAAAAACAATTMCCATCAC	150	
Nairo L 1c R		CAATGCTGTGRTARAARCAGTTGCCATC		
Nairo L 1d R		GCAATGCTATGGTAGAAACAGTTTCCATC		
Nairo L 1e R		CRAKGCTGTGGTAAAAGCAGTTRCCATC		
Bunyagroup F	*Orthobunyavirus* NP	CTGCTAACACCAGCAGTACTTTTGAC	210	Lambert and Lanciotti, 2009
Bunyagroup R		TGGAGGGTAAGACCATCGTCAGGAACTG		
Vir 2052 F	*Alphavirus* NS4	TGGCGCTATGATGAAATCTGGAATGTT		Eshoo et al., 2007
Vir 2052 R		TACGATGTTGTCGTCGCCGATGAA	150	
Flavi JV2a F	*Flavivirus* NS5	AGYMGHGCCATHTGGTWCATGTGG		Villinger et al., 2017
Flavi JV2b F		AGCCGYGCCATHTGGTATATGTGG		
Flavi JV2c F		AGYCGMGCAATHTGGTACATGTGG	150	

Flavi JV2d F		AGTAGAGCTATATGGTACATGTGG		
Flavi JV2a R		GTRTCCCADCCDGCDGTRTCATC		
Flavi JV2b R		GTRTCCCAKCCWGCTGTGTCGTC		
Thogoto S6 F	*Thogoto* virus M6	GATGACAGYCCTTCTGCAGTGGTGT		Villinger et al., 2017
Thogoto S6 R		RACTTTRTTGCTGACGTTCTTGAGGAC		
Dhori F	*Dhori* virus	CGAGGAAGAGCAAAGGAAAG		Villinger et al., 2017
Dhori R	NP6	GTGCGCCCCTCTGGGGTTT		
Nairo Honig 6942	*Nairovirus* NS1	ATGATTGCIAAYAGIAAYTTYAA	434	Honig et al., 2004
Nairo Honig 7385		ACAGCARTGIATIGGICCCCAYTT		
AnaplasmaJV F	*Anaplasma* 16R rRNA	CGGTGGAGCATGTGGTTTAATTC	300	Mwamuye et al., 2017
AnaplasmaJV R		CGRCGTTGCAACCTATTGTAGTC		
EhrlichiaJV F	*Ehrlichia* 16S rRNA	GCAACCCTCATCCTTAGTTACCA	300	Mwamuye et al., 2017
EhrlichiaJV R		TGTTACGACTTCACCCTAGTCAC		
EHR16SD	*Anaplasma*16S rRNA	GGTACCYACAGAAGAAGTCC	1090	Parola et al., 2000 Edwards et al., 1989
Ehr-pH1522		AAGGAGGTGATCCAGCCGCA		Reysenbach et al., 1992
EHR16SD		GGTACCYACAGAAGAAGTCC		
Ehr-pH1492		GGCTACCTTGTTACGACTT	1030	
Rick-F	*Rickettsia* 16S rRNA	GAACGCTATCGGTATGCTTAACACA	364	Nijhof et al., 2007
Rick-R		CATCACTCACTCGGTATTGCTGGA		
Rick ompB 120–2788	*Rickettsia* ompB	AAACAATAATCAAGGTACTGT	856	Roux and Raoult, 2000
Rick ompB 120–3599		TACTTCCGGTTACAGCAAAGT		
RLB-F	*Theileria/Babesia* 18S rRNA	GAGGTAGTGACAAGAAATAACAATA	450	Georges et al., 2001
RLB-R		TCTTCGATCCCCTAACTTTC		

Table A.2: Numbers of vertebrate species from which vectors were collected in the different livestock markets and slaughterhouses in western Kenya between 6/04/2017 and 7/06/2018

				No. of hosts sampled from			
Study area	**Vector species**	**Number (N)**	**No. Pools**	**Cattle**	**Goat**	**Sheep**	**Pig**
Amerikwa	*Rhipicephalus* sp.	4	2	1			
Amukura	*Am. variegatum*	7	6	6			
	Rhipicephalus sp.	1	1	1			
	Rh. decoloratus	1	1	1			
	Rh. evertsi	3	1	1			
	Rh. appendiculatus	6	3	2			
Angurai	*Rh. decoloratus*	11	5	2			
	Rh. appendiculatus	9	4	2			
Bumala	*Haemaphysalis* sp.	1	1	1			
Butula	*Am. variegatum*	27	16	11			
	Rh. decoloratus	8	5	3			
	Rhipicephalus sp.	2	2	2			
	Amblyomma sp.	1	1		1		
	H. suis	5	2				2
Chwele	*Am. variegatum*	5	3	3			
	A. gemma	3	3	1			
	Rh. decoloratus	2	1	1			
	Rh. evertsi	1	1	1			
Funyula	*Am. variegatum*	11	7	3			3
	Rh. appendiculatus	6	4	2			1
	Rh. appendiculatus nymph	1	1	1			
	Rhipicephalus sp.	2	2	2			
	Rh. evertsi	3	1		1		
	H. suis	6	4				4
Harambe	*Am. variegatum*	2	2	2			
	Rh. decoloratus	2	1	1			
	Rh. microplus	2	1	1			
	Rhipicephalus sp.	1	1	1			
Ikolomani	*Am. variegatum*	7	5	3		1	
	Rh. decoloratus	8	6	5		1	
	Rh. microplus	4	1	1			
	Rh. evertsi	4	2		1		
	Rh. appendiculatus	4	2	2			
	Rhipicephalus sp.	7	4	4			
	H. suis	6	4				4
Kimilili	*Am. variegatum*	6	4	3			
	Rh. decoloratus	41	24	16			
	Rh. appendiculatus	1	1	1			

	Rh. evertsi	3	3	2	1		
	Rhipicephalus sp.	23	18	12	1	1	
Koyonzo	*Am. variegatum*	9	7	4			
	Rh. decoloratus	3	3	3			
	Rh. evertsi	1	1	1			
Lubao	*Am. variegatum*	3	3	1			1
	Amblyomma sp.	1	1	1			
	Rh. decoloratus	29	20	13			
	Rh. evertsi	3	2	2			
	Rhipicephalus sp.	8	8	5			
	H. suis	5	4				4
	Rh. appendiculatus	1	1	1			
Malaba	*Am. variegatum*	13	10	6			
	Rh. decoloratus	3	2	1	1		
	Rh. evertsi	1	1	1			
	Rh. appendiculatus	2	2	2			
Myanga	*Am. variegatum*	22	15	11			
	Amblyomma sp.	1	1	1			
	Rh. decoloratus	9	6	5			
	Rh. appendiculatus	18	11	8			
	Rh. evertsi	5	5	3			
Shinyalu	*Am. variegatum*	8	8	7			
	Rh. decoloratus	31	22	15			
	Rh. appendiculatus	4	2	2			
	Amblyomma sp.	2	2	2			
	H. suis	6	3				3
	Rhipicephalus sp.	10	9	5	1		
Webuye	*Am. variegatum*	1	1	1			
	Rh. decoloratus	14	10	7			
	Rhipicephalus sp.	10	6	4		1	

Table A.3: Occurrence of vector-borne pathogens isolated from ticks and lice from livestock markets and slaughterhouses

Pathogen detected	Vector species	LM/SH	Vertebrate species	GenBank accessions
Anaplasma platys	*Rhipicephalus decoloratus, Rhipicephalus* sp.*, Rh. appendiculatus*	Lubao, Kimilili, Funyula, Shinyalu, Butula, Angurai, Myanga, Webuye	Cattle	MN266939-MN266941
A. ovis	*Rhipicephalus* sp.*, Rh. evertsi, Rh. decoloratus*	Shinyalu, Ikolomani, Kimilili, Webuye, Amukura	Goat, cattle	MN266936-MN266938
A. marginale	*Rh. decoloratus, Rhipicephalus* sp.	Kimilili, Shinyalu, Lubao	Cattle	MN266931-MN266935
Rickettsia africae	*Amblyomma variegatum, Am. gemma, Rh. appendiculatus, Haematopinus suis, Rh. decoloratus, Rhipicephalus* sp.	Myanga, Ikolomani, Amukura, Malaba, Funyula, Lubao, Shinyalu, Butula, Chwele, Kimilili, Koyonzo, Angurai, Amerikwa, Harambe	Cattle, pig, goat, sheep	MN294740-MN294749
Babesia caballi	*Am. variegatum*	Myanga, Malaba, Ikolomani, Koyonzo, Funyula, Butula	Cattle, sheep, pig	MN294721-MN294723
B. bigemina	*Rh. decoloratus, Am. variegatum, Rh. appendiculatus*	Angurai, Amukura	Cattle	MN294720
Hepatozoon canis	*Rh. decoloratus*	Kimilili	Cattle	MN294724
Theileria mutans	*Rh. decoloratus, Rhipicephalus* sp*. Am. variegatum, Rh. appendiculatus*	Shinyalu, Kimilili, Lubao, Butula, Koyonzo, Myanga, Webuye, Ikolomani	Cattle	MN294725-MN294729
T. parva	*Rhipicephalus* sp.	Funyula	Cattle	MN294730
T. taurotragi	*Rh. decoloratus, Rhipicephalus* sp.*, Rh. appendiculatus*	Kimilili, Lubao, Angurai	Cattle	MN294731-MN294732
T. velifera	*Am. variegatum, Rhipicephalus* sp.	Kimilili, Shinyalu, Lubao	Cattle	MN294733-MN294734
CCHF virus	*Rh. decoloratus, Rhipicephalus* sp.	Shinyalu, Lubao	Cattle	MN267048-MN267049
Coxiella **endosymbionts**	All tick species except *Haemaphysalis* sp.	All LM/SH	All species	MN262071-MN262076; MN266946-MN266948; MN266922-MN266928

Table A.4: Details of mosquito pools positive for insect-specific flaviviruses

Site	Season	Species	#Mosquitoes	No. pools	CFAV (IR %; 95% CI)	AeFV (IR %; 95% CI)	CxFV (IR %; 95% CI)
Bungoma	County overall	***Ae. aegypti***	868	53	17 (2.43); 1.48-3.83	1 (0.12); 0.007-0.56	-
	short rain	*season total*	129	13	2 (1.67); 0.31-5	-	-
		females	65	8	1 (1.62); 0.096-8.63	-	-
		males	64	5	1 (1.46); 0.096-7.33	-	-
	long rain	*season total*	739	40	15 (2.55); 1.51-4.14	1 (0.14); 0.008-0.66	-
		females	329	18	7 (2.54); 1.17-5.05	1 (0.31); 0.02-1.5	-
		males	410	22	8 (2.46); 1.16-4.79	-	-
Busia	County overall	***Ae. aegypti***	1205	68	6 (0.52); 0.21-1.08	4 (0.34); 0.11-0.81	-
	short rain	*season total*	159	14	4 (3.20); 1.02-8.20	-	-
		females	120	10	3 (3.15); 0.82-9.28	-	-
		males	39	4	1 (2.71); 0.16-15.08	-	-
	long rain	*season total*	1046	54	2 (0.19); 0.04-0.63	4 (0.39); 0.13-0.94	-
		females	598	32	2 (0.34); 0.06-1.12	49 (6.97); 0.23-1.67	-
		males	371	18	-	-	-
Kakamega	County overall	***Ae. aegypti***	588	37	7 (1.33); 0.59-2.64	6 (1.09); 0.46-2.25	
	short rain	*season total*	132	10	1 (0.75); 0.05-3.72	1 (0.78); 0.05-3.93	-
		females	68	5	1 (1.44); 0.09-7.42	-	-
		males	64	5	-	1 (1.65); 0.098-9.09	-
	long rain	*season total*	456	27	6 (1.49); 0.62-3.11	5 (1.16); 0.44-2.54	-
		females	345	18	4 (1.29); 0.43-3.16	3 (0.91); 0.25-2.45	-
		males	111	9	2 (1.92); 0.36-6.51	2 (1.80); 0.36-5.84	-
***Ae. aegypti* total**			2661	158	30 (1.27); 0.87-1.78	11 (0.43); 0.23-0.74	-
Bungoma	County overall	***Cx. pipiens***	2006	108	-	-	5(0.26); 0.095-0.57
	short rain	season total	87	8	-	-	1(1.16); 0.07-6.17
		females	78	4	-	-	1(1.26); 0.08-6.92
		males	9	4	-	-	-
	long rain	season total	1919	100	-	-	4(0.21); (0.07-0.51)
		females	1780	88	-	-	4(0.23); 0.08-0.55
		males	139	12		-	-
Busia	County overall	***Cx. pipiens***	445	45			1(0.23); 0.01-1.10
	short rain	season total	60	8	-	-	-
		females	40	4	-	-	-
		males	20	4	-	-	-
	long rain	season total	385	37	-	-	1(0.26); 0.02-1.28

		females	348	28	-	-	1(0.29); 0.02-1.42
		males	37	9	-	-	-
Kakamega	County overall	***Cx. pipiens***	679	63			1(0.15); 0.009-0.72
	short rain	season total	40	10	-	-	-
		females	35	7	-	-	-
		males	5	3	-	-	-
	long rain	season total	639	53	-	-	1(0.16); 0.009-0.76
		females	592	44	-	-	1(0.17); 0.01-0.82
		males	47	9	-	-	-
***Cx. pipiens* total**			3130	216			7(0.23); 0.10-0.45

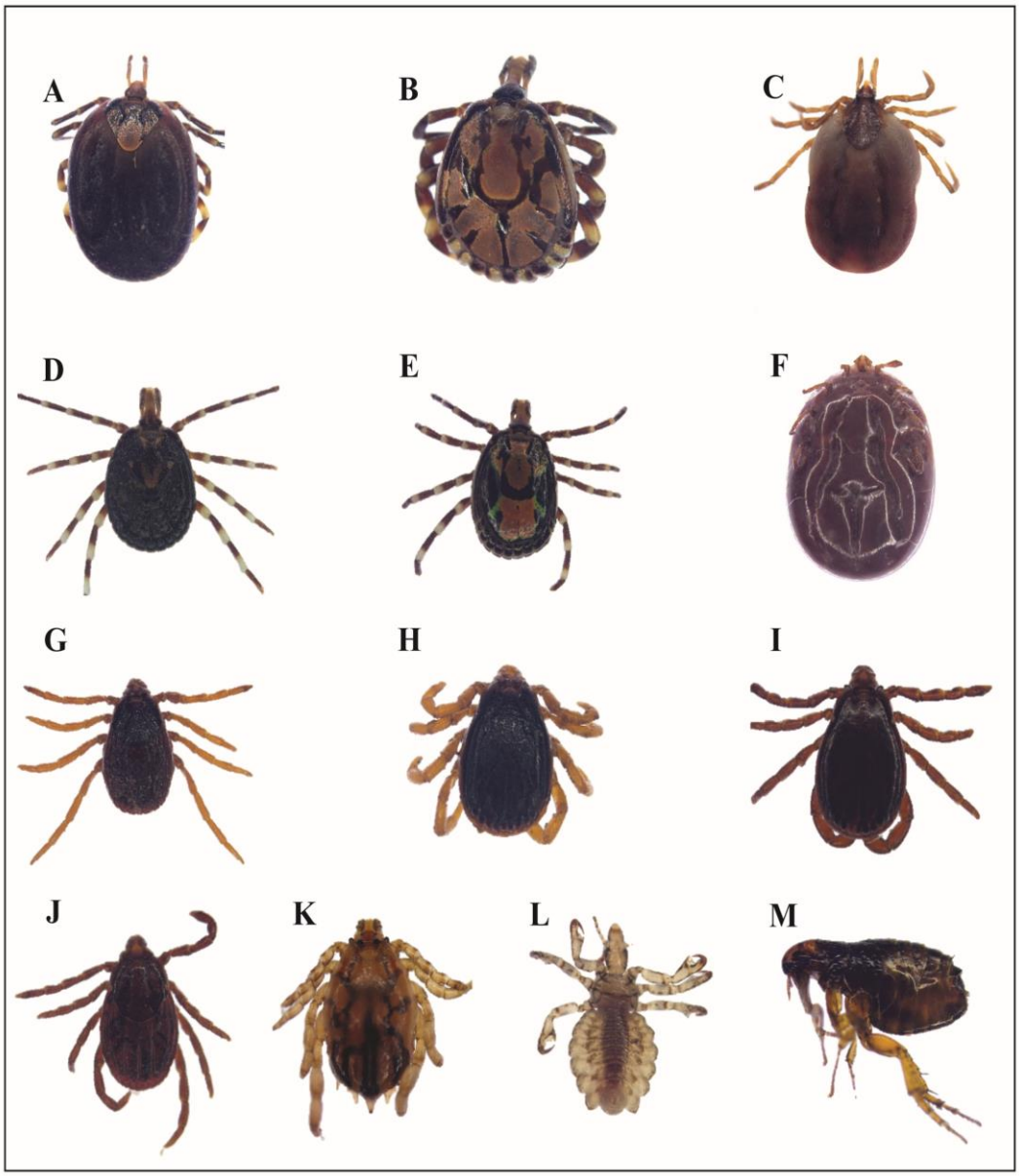


Figure A. 1*:* Photographs of representative specimens of vectors collected from livestock at livestock markets and slaughterhouses
A. *Am. gemma female;* **B**. *Am. gemma male;* **C**. *Amblyomma sp. nymph;* **D**. *Am variegatum female;* **E**. *Am variegatum male;* **F**. *Haemaphysalis sp.;* **G**. *Rhipicephalus evertsi female and male;* **H**. *Rh. evertsi male;* **I**. *Rh. appendiculatus male;* **J**. *Rh. appendiculatus female;* **K**. *Rh. decoloratus;* **L**. *Haematopinus suis;* **M**. *Ctenocephalides felis.*

Figure A.2: Some of the observed favorable mosquito breeding and resting places at the six hospitals investigated

Mosquito control questionnaire

Hospital……... **Date**……

Name of interviewee…… **Position at hospital**…….

1. Do you implement any mosquito control methods at this hospital?

……

2. If **YES** can you explain what you do?

Control activity	**YES/NO**	**Comments (frequency, seasonality, type of insecticide [indoor/outdoor], ITN or not,**
Bed nets		
Insecticide spray		
Topical repellants		
Window screens		
Grass cutting and bush clearing		
Draining of water puddles		
Disposal of rubbish and hospital waste		
Covering disused pit latrines and sewage manholes		

1

Other……

Comments……..

……

……

……

……

……

3. Observations:

Observation	Pres/Abs	Comments
Disused pit latrines		
Open sewage manholes		
Disused dilapidated buildings		
Piled up rubbish/waste		
Broken down vehicles		

Disused tyres		
Broken down equipment		
Water puddles		
Tall grass and bushes		

Figure A.3: Sample of the questionnaire used to collect information about the methods implemented to control mosquitoes