DETECTION OF CRIMEAN-CONGO HAEMORRHAGIC FEVER VIRUS AND *RICKETTSIA* spp. IN TICKS AND DISEASE EXPOSURE OF LIVESTOCK HANDLERS IN THE GREATER ACCRA AND UPPER EAST REGIONS OF GHANA

BY

RONALD ESSAH BENTIL

(10459351)

This thesis is submitted to the University of Ghana, Legon in partial fulfilment of the requirement for the award of **MPHIL in APPLIED PARASITOLOGY Degree**

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DECLARATION

I do hereby declare that this thesis is my own work, undertaken under the supervision of the names listed below and has not been previously submitted either partially or wholly for the award of a degree in any University. References to the works of other investigators have been duly acknowledged.

28th October 2022

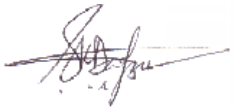
Ronald Essah Bentil — Date

(Student)

28th October 2022

Daniel Oduro (PhD) — Date

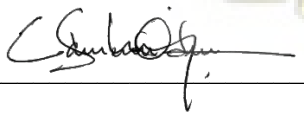
(Supervisor)



28th October 2022

Samuel Dadzie (PhD) — Date

(Co-Supervisor)



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Shirley C. Nimo-Paintsil (PhD) — Date

(Co-Supervisor)

DEDICATION

This work is dedicated to God almighty, my parents (Mr. Evans Korsah Bentil and Mrs. Mercy Welhemina Bentil) and sister (Joanita Abokoma Bentil). I can never thank you enough for everything you have done for me.

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ABSTRACT

Ticks are important blood-sucking arthropod vectors. They are known to spread a wide range of diseases that cause severe and life-threatening illnesses in humans and animals all over the world through feeding. In population-dense and trade-dominant areas such as the Greater Accra and Upper East Regions, the risk of zoonotic infections may be on the rise. This study, therefore, sought to identify the circulating tick species, assess the pathogens they carry and the risk of exposure of primary animal handlers.

A total of 705 ticks were collected from cattle (n=188) and horses (n=11). Three tick genera (*Hyalomma, Amblyomma* and *Rhipicephalus*) were observed in the study with the predominant species being *Hyalomma rufipes* (n=290, 41.13%), followed by *Amblyomma variegatum* (n=157, 22.27%) and the least, *Rhipicephalus sanguineus* (n=1, 0.14%). It was also observed that the preferred point of attachment of all identified ticks was predominantly the Anal region (n=469, 68.37%) with the least site being the Abdominal region (n=5, 0.73%) for cattle except for Horses that had the least site being the chest (n=1, 5.26%).

Out of the 705 tick samples, it was observed that in the Upper East Region, higher percentage of ticks collected (62.67%) were infected with *Rickettsia* as compared to ticks from Greater Accra (42.13%). Similarly, slightly more ticks were infected with *Rickettsia africae* in the Upper East Region (91.8%) than Greater Accra (91.38%). In addition, although *Amblyomma variegatum* was the second most prevalent species identified, the highest *Rickettsia* and *Rickettsia africae* infection rates were recorded in this species. The highest CCHFV infection rates were observed in *Hyalomma rufipes*.

Serology carried out for a total of 120 human sera samples, for *Rickettsia* using an in-house ELISA revealed that 27.50% had been exposed to Spotted Fever Group (SFG) and 4.17% to

Typhus Group (TG) and none exposed to the Scrub Typhus group (STG). Furthermore, preliminary analysis for antibodies to CCHFV IgG was detected in 42.5% of the human serum (n=120) samples pending confirmatory Plaque Reduction Neutralization Test (PRNT).

This study further reports the first whole-genome sequencing of CCHFV in tick species within Ghana with sequence analysis revealing genotype III (Africa III) may be circulating in the Upper East Region of Ghana. This suggests the possible importation of the CCHFV virus into the country through trade and thus puts livestock and humans who may have primary contact with livestock at risk of infection of these diseases.

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LIST OF ABBREVIATIONS

CCHFV	Crimean Congo Haemorrhagic Fever Virus
TBDs	Tick-borne diseases
SSA	Sub-Saharan Africa
SAT	Saliva-activated transmission
CDC	Centers for Disease Control and Prevention
SFG	Spotted Fever Group
TG	Typhus Group
STG	Scrub Typhus Group
RMSF	Rocky Mountain spotted fever
MSF	Mediterranean Spotted Fever
TIBOLA	Tick-Borne Lymphoadenopathy
USSR	Union of Soviet Socialist Republics
RNA	Ribonucleic Acid
SSTs	Serum separator tubes
PCR	Polymerase Chain Reaction
RT-PCR	Real-time PCR
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with 0.02% Tween-20

PBST-SK	Phosphate buffered saline with 0.02% Tween-20 and 5% skim milk
eBLT	enrichment Bead-Linked Transposomes
TB1	tagmentation buffer
ST2	stop tagment buffer
TWB	tagment wash buffer
EPM	Enhanced PCR Mix
RSB	Resuspension Buffer
EHB 2	Enrichment Hybridization Buffer 2
SMB	Streptavidin Magnetic Beads
EEW	Enhanced Enrichment wash
EE1	Enrichment Elution Buffer 1
ET2	elute target buffer
PPC	PCR primer cocktail
GLMM	Generalized Linear Mixed Model

CHAPTER ONE

GENERAL INTRODUCTION

1.1 BACKGROUND

Ticks are obligate hematophagous Acari that parasitize mammals, birds and reptiles and occasionally bite humans (Parola, 2004). Ticks are important disease-causing arthropod (De La Fuente *et al*., 2008; Liu & Bonnet, 2014) vectors known to spread a wide range of diseases such as protozoa, bacteria, and viruses which are known to cause severe and life-threatening illnesses in humans and animals all over the world (Dantas-Torres *et al*., 2012; Rosenberg *et al.*, 2018). In warm and humid climates, they are known to be livestock's most important ectoparasites and are responsible for significant livestock economic losses (Kasaija *et al.*, 2021). Ticks are responsible for the majority of losses due to their potential to spread diseases of various aetiologies.

These ectoparasites can have an impact on animal production and health, either directly through their bites or indirectly by the infectious agents they transfer. (Jaime *et al*., 2018). Feeding on their hosts involves the tick biting and pushing the hypostoma into the host's skin, causing injury to the epidermis and blood vessel rupture. Tick bites cause skin damage comprised of oedema, irritation, infiltration of cells, inflammation or hypersensitivity and a large amount of blood loss (Jaime *et al*., 2018). The animals are predisposed to reduce their food absorption and lose weight as a result of these injuries. Tick infestations can result in anaemia as a result of blood loss, a decrease in the quality of the hides, and severe dermatitis. (Jaime *et al*., 2018; Rajput *et al*., 2006).

Further, tick feeding stresses the host and reduces its immunological response, lowering productivity and resulting in meat and milk output losses. (L'hostis & Seegers, 2002; Leal *et al.*, 2020; Peter *et al*., 2005). These morbidities can lead to death in some situations; with the carcass's poor appearance diminishing its marketability. (Jaime *et al.*, 2018). Examples of diseases in livestock include bovine babesiosis and anaplasmosis, east coast fever and cowdriosis (Blood & Radostits, 1989; Dinkisa, 2018; Marcelino *et al*., 2012).

Ticks are the second most prevalent vectors of human infectious disease pathogens, after mosquitoes. (De La Fuente *et al*., 2008; Lawrie *et al*., 2004) and as pathogen vectors for animals (De La Fuente *et al.*, 2008). Infection of ticks with microorganisms occur when they feed on hosts (either human or animal) that are infected and in turn further introduce these microorganisms into another host on a next blood meal (Jongejan & Uilenberg, 2004; Šimo et al., 2017). Ticks are thus known to play a major role when it comes to zoonotic disease transmission which contributes to high disease and death rates. Human tick-borne diseases zoonoses have been detected in both wild and domestic animals (Baneth, 2014).

Globally, there have been a considerable increase in tick-borne diseases of epidemiologic importance (Rochlin & Toledo, 2020). These diseases include those caused by bacteria; *Rickettsia*, Lyme disease, Rocky Mountain spotted fever, Q-fever and by viruses; Crimean-Congo Haemorrhagic fever (CCHF), Tick-borne encephalitis, and Powassan encephalitis. Other tick borne infections includes those caused by protozoans; Babesiosis (Sambri *et al.*, 2004).

Although these vectors are ubiquitous, they are diverse and more prevalent in the world's tropical and subtropical areas (Rehman *et al*., 2017). Ticks are divided into three groups, two of which are veterinary and medically important—*Ixodidae* (hard ticks) and *Argasidae* (soft ticks) (Rajput *et al*., 2006). The family *Ixodidae*, which encompasses most tick species, are

characterized by their pestiferous nature and the highest rate of transmitting diseases and disease-causing agents among the three (Leal *et al*., 2020). Ixodidae ticks comprise thirteen genera, seven of which are veterinary and medically important species. These are *Amblyomma,* subgenus *Rhipicephalus (Boophilus)*, *Rhipicephalus, Haemaphysalis, Hyalomma, Dermacento*r and *Ixodes* (Wondimu & Bayu, 2021). The genera Hyalomma, Boophilus, Rhipicephalus, and Amblyomma are the most economically important ixodid ticks in tropical areas. (Rajput *et al*., 2006).

Among the different tick-borne illnesses, CCHF was included on both the United States National Institutes of Allergy and Infectious Diseases priority A list as threat to national security and public health (Geddes, 2021) and the World Health Organization's priority list for research and development (World Health Organization, 2021). CCHF is endemic throughout Africa and has a 40% case fatality rate (Shahhosseini *et al*., 2021). Depending on how the virus was contracted, the incubation period ranges between 1 to 13 days. Initial symptoms are minor, but they gradually get worse with time (Kaya *et al*., 2011). Although an effective vaccine is not yet available, Ribavirin (an antiviral medication) has been used in therapy and has been shown to reduce mortality (Soares-Weiser *et al*., 2010).

Rickettsioses on the other hand are focal; with war, famine, overcrowding, and unhygienic circumstances being the main drivers of epidemics. (Wallace *et al*., 2002). This suggests that, in addition to some cases documented in advanced countries, it is an issue in underdeveloped and developing nations (Wallace *et al*., 2002). In humans, incubation can last up to two weeks with typical symptoms appearing as rashes that later evolve and progress into other manifestations (McCollough, 2018). With a fatality rate of 10 to 40 %, mortality has been found to rise with age and rarely deadly in children under the age of 10 (Woods, 2013). Despite the fact that some antibiotics have been effective at treating the condition, there is currently no licensed vaccination for use, which adds to the issue (McCollough, 2018).

In general, there are two types of detection methods: direct and indirect (Springer *et al*., 2021). In the former (direct), patient samples are used for the direct detection of diseases carried by ticks using techniques like microscopy and culture. Microscopy is less sensitive, despite being quick and inexpensive. This method cannot be utilized for all tick-borne infections since it depends on the parasitaemia level and makes it difficult to distinguish between various species (Ord & Lobo, 2015). Additionally, culturing typically takes a long period, is difficult, requires a lot of work, and requires specific biosafety conditions. (Eldin *et al*., 2019). As a result, nucleic acid amplifications using molecular techniques are the most recommended since they have higher sensitivity, produce results quickly, and improve diagnostic effectiveness (Korber *et al*., 2017). On the other hand, indirect detection procedures include serological testing techniques based on immunological targets and markers (Portillo *et al*., 2017).

The difficulty in diagnosis is a characteristic similarity between the two above-mentioned tick-borne diseases (Stewart & Stewart, 2021; Tezer & Polat, 2015). Serologic assays and polymerase chain reaction (PCR) are two recommended diagnostic procedures that are efficient (Stewart & Stewart, 2021; Tezer & Polat, 2015). However, in resource-constrained environments, the accessibility, price, and skill required to perform these assays continue to be a problem (Walker, 2007). As a result, incorrectly administering unsuitable medication and misdiagnosis may occur, which may lead to the persistence of diseases in the general population.

Several approaches have been made to control ticks, the primary of these is chemical control with acaricides. However, this has proven futile because ticks have developed resistance against a range of acaricides. In addition, besides being expensive, these chemicals are toxic and leave residues in animal products which are in turn harmful to humans upon consumption. It has, however, become necessary that control methods that are cost-effective, environmentally friendly and pose a minimal adverse effect on the health of livestock and

human beings be implemented. Therefore this calls for Integrated Tick Management involving novel approaches of pest control, active surveillance and improved testing to mitigate the effect of the problem posed (George *et al*., 2004; Nath *et al*., 2018).

1.2 PROBLEM STATEMENT

In several countries across Sub-Saharan Africa, epidemiological data on tick-borne diseases are scarce, and their prevalence and distribution are largely less studied. The difficulty in identifying, managing tick populations, and treating infections of tick-borne pathogens makes efforts to combat these emerging diseases futile (Kobayashi *et al*., 2017; Madison-Antenucci *et al*., 2020). In addition, similarities in the symptoms of tick-borne infections with other febrile illnesses, often leads to misdiagnosis and, an eventual increase in prevalence through transmission (Madison-Antenucci *et al*., 2020).

Even though there are significant advances in research and diagnostic techniques such as molecular or immunological techniques, which could aid in the diagnosis of tick-borne diseases, these advancements remain in highly resourced facilities. Underdiagnosis still remain in limited resource facilities, making the extent of the problem difficult to determine, despite these improvements. The identification and selection of effective care and control methods include knowledge of the diseases that can be spread by ticks in particular locations. To deal with the problem, the capacity to detect, explain, and acknowledge the expanding public health hazard must evolve as new tick-transmitted illnesses are identified and appear in new geographic areas (Madison-Antenucci *et al*., 2020).

Epidemiology and ecology of tick-borne diseases are influenced by dynamic interactions between biotic and abiotic components. Further research has confirmed that pathogens of

zoonotic nature as well as their vectors live in separate environments, giving rise to the idea of nidality in the natural world, or landscape epidemiology of infectious diseases. The distribution and number of ticks, as well as the development, revival, and geographic spread of tick-borne illnesses, are all influenced by the following factors: tick and tick-borne pathogen demography, micro and macro climate variations, travel, human behaviour, politics, land use and modification of habitats (for farming, housing, and entertainment purposes), population growth, economy and migration. (Baneth, 2014; Dantas-Torres, 2015). Tick-borne diseases are becoming increasingly important, and people are becoming more aware of their effects (Wikel, 2018).

Furthermore, since the last release of the list of ticks in Ghana and assessment of the distribution across various vegetation zones (Ntiamoa-Baidu, Carr-Saunders, Matthews, Preston, & Walker, 2004), it is uncertain if these tick species exist, have undergone adaptations, or new species have been introduced. Climate change, which has a significant impact on arthropod disease vectors and their capacity to spread diseases (Bett *et al*., 2019), as well as trading of livestock (Nimo-Paintsil *et al*., 2022) may be contributing factors. Additionally, given the incidence of disease outbreaks spread by ticks in neighbouring countries (Ehounoud *et al*., 2016; Nordstrand *et al*., 2007; Ouedraogo *et al*., 2021), and past research that has revealed the presence of certain tick-borne diseases in Ghana (Akuffo *et al*., 2016; Kobayashi *et al*., 2017; Sothmann *et al*., 2017), it is crucial that the impact of such events be evaluated in order to be able to promptly alleviate the repercussions.

That said, due to the indiscriminate hematophagous arthropods, and can transmit infections when they feed (Gondard *et al*., 2017), livestock and domestic animals are at risk of infestation. In turn, this creates a health concern to people who take care of these animals and come into contact with them. As a result, there is a high chance of exposure should the ticks be harbouring any pathogens.

1.3 JUSTIFICATION

Many countries in Sub-Saharan Africa lack epidemiological data on infections caused by ticks, and their incidence and spread remaining mostly unknown (Kobayashi *et al.*, 2017). Similarly, in the few studies that have been performed in Ghana, some gaps have been identified that need further investigation. For example, findings from work done in Kumasi by Clarke *et al.* (2014) revealed the existence of tick-borne pathogens in ticks collected from dogs as well as the seroprevalence of these pathogens upon assaying sera collected from the domestic animals. In a study conducted by Akuffo *et al*., (2016), ticks were collected off slaughtered animals and blood drawn from animal handlers at the Kumasi abattoir. Study findings showed that some ticks carried the Crimean Congo Haemorrhagic fever Virus as well as detecting human exposure to the virus among the slaughterhouse workers. Another study conducted by Sothmann *et al*. (2017), further revealed *Rickettsia felis* infection of some febrile children in Ghana upon screening their blood samples. However, the source of exposure/ infection was not ascertained by the authors in this case. In addition, serological assays performed on blood samples collected from livestock in the Volta Region, Johnson *et al*. (2019) revealed the seroprevalence of Q-fever in their findings.

Furthermore, although taxonomically reliable information on ticks in Ghana is limited, previous work done within the same areas and surrounding areas indicate the presence of diverse tick species infesting domestic ruminants and wild mammals within different vegetation zones in Ghana (Walker & Koney, 1999; Ntiamoa-Baidu *et al*., 2004).

The above information is an indication that ticks identified in the country can host some tick-borne pathogens and thus humans who especially cater for or live near livestock are likely to be exposed. These studies, however, were localized to study sites and thus do not represent a

consistent occurrence of the situation in Ghana. Due to the extensive agricultural activities in the study areas, there will likely be more human activity, which will favour the spread of zoonoses and a rise in exposure to ticks and tick-borne pathogens in these expanding urban environments. It is therefore critical, to determine the prevalence of various tick species infesting livestock, as well as the risk burden they pose to their owners. This national surveillance system is essential for filling gaps in the data set by providing information on the prevalence of tick-borne pathogens and the level of exposure to them across the country.

1.4 GENERAL OBJECTIVE

The purpose of the study was to determine the presence of tick-borne pathogens in ticks and pathogen exposure among livestock handlers within selected study sites.

1.4.1 SPECIFIC OBJECTIVES

1. Identify tick species sampled from livestock within the selected ecozones.
2. Determine the prevalence of tick-borne pathogens in ticks within the selected ecozones.
3. Assess previous human exposure to CCHFV and *Rickettsia* through seroprevalence in livestock handlers at selected ecozones
4. Determine by genomic analysis whether tick-borne pathogens are localised or emerging new pathogens

CHAPTER TWO

LITERATURE REVIEW

2.1 Overview of ticks and their impact

Hematophagous ectoparasites are efficient vectors of diseases. Ticks fall within this category, derive nutrition through blood-feeding on vertebrates and are of medical and veterinary importance (Jongejan & Uilenberg, 2004; De la Fuente *et al.*, 2017). Ticks have been implicated as the most important vectors of pathogens that cause disease in animals (both domestic and wild), and are responsible for more than 100,000 cases of illness in humans worldwide (Jose de La Fuente et al., 2008; Yu *et al.*, 2015). They are involved in the transmission of a several pathogenic microorganisms such as *Theileria* spp., *Babesia* spp., Rickettsiae, Borrelia spp., Powassan, Crimean Congo Hemorrhagic fever and Dugbe viruses, compared to any other arthropod vector group (De La Fuente *et al.*, 2008). As transmission of tick-borne infections to humans and the associated morbidity and mortality pose significant public health concerns, transmission in livestock is an additional major challenge in animal production especially in tropical and subtropical regions of the world (De La Fuente *et al.*, 2008; Dantas-Torres *et al.*, 2012).

In developing countries, tick-borne diseases have the greatest influence on the lives of resource-scarce animal husbandry communities (Jongejan & Uilenberg, 2004; Minjauw & McLeod, 2003; Perry *et al.*, 2002). This is certainly relevant in parts of Sub-Saharan Africa, Asia, and Latin America, where animal products are in high demand. (Thornton, 2010). Ticks and tick-borne illnesses have co-evolved with a variety of wild animal hosts, which often coexist and serve as reservoir hosts for ticks and tick-borne pathogens in cattle, pets, and humans. (Cançado *et al.*, 2013). Domestic livestock may become infected upon contact with

ticks that have fallen off these wild hosts, either because humans moved animals into tick-infested areas, or because humans moved tick-infested cattle into previously uninfested areas.

Most ticks have a preference for feeding on certain groups of wild animals, with some even being host-specific. Consequently, the number of species pertinent to domestic animals and/or humans is limited. Relatively, few species of ticks have successfully adapted to livestock or feed on a human subject, and these have developed into efficient vectors of a range of pathogenic microorganisms, while virtually all human tick-borne diseases are zoonoses (De la Fuente *et al.*, 2017; De La Fuente *et al.*, 2008). A tick species is considered a vector for a particular pathogen only if it feeds on an infectious vertebrate host; can acquire the pathogen during the blood meal; maintains the pathogen through one or more life stages; and further transmit to another host on the next blood meal. (De la Fuente *et al*., 2017; Kahl *et al*., 2009)

2.2 Tick Systematics and Biology

There are about 898 recognized tick species distributed among three families, namely *Argasidae* (soft-bodied ticks), comprising about 194 species; *Ixodidae* (hard-bodied ticks) comprising about 703 species; and monotypic *Nuttalliellidae*, made up of 1 species (Guglielmone, 2010; Estrada-Peña & De la Fuente, 2014). However, only *Argasidae* and *Ixodidae* are considered to be of veterinary and medical importance (Tahir *et al*., 2020). The family *Ixodidae* (hard ticks), which encompasses most tick species (about 80%), are pestiferous and have the highest rate of transmitting diseases and disease-causing agents among the three families (Leal *et al*., 2020). The vectorial capacity of Ixodid ticks is linked to their long-term co-evolution with the pathogens they transmit, their longevity, their high reproductive potential, the wide range of host feeding preferences of several species, and their ability to take in a substantial quantity of blood over a relatively long period (De la Fuente *et al.*, 2017).

2.2.1 Ticks of medical and veterinary importance

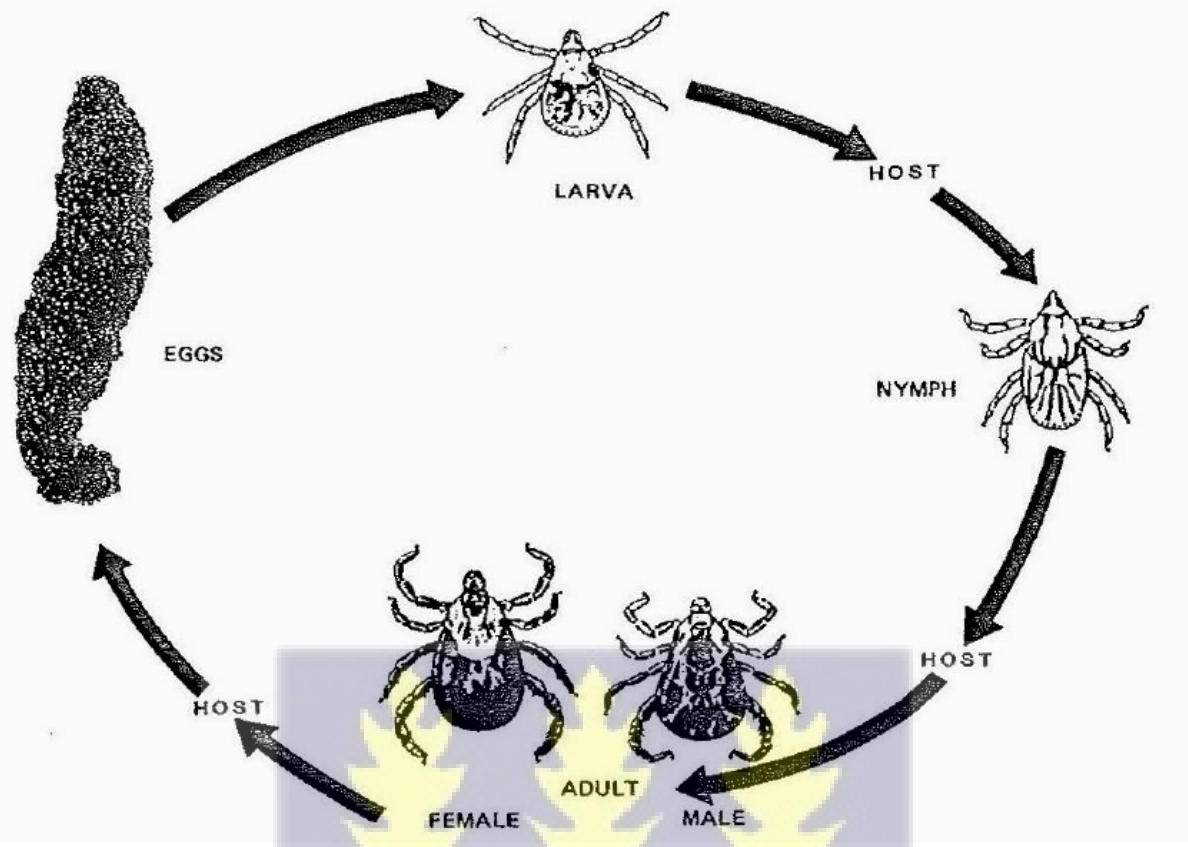

Figure 1: Life Cycle of ticks of the Ixodidae family (hard ticks)

Source: (Varela-Stokes et al., 2009)

Four stages exist in the life cycle of an ixodid tick, namely egg, larva, nymph and adult. Unlike in argasid ticks where mating occurs off the host in the vegetation, ixodid ticks mate while on their host (Horak *et al*., 2002). All post-embryonic stages of the tick require a blood meal to survive. Engorged females drop to the ground and lay eggs in the soil, after which larvae hatch from the batches of eggs after about four weeks and take about five days to fully engorge with blood. Nymphs may also take up to about twenty days to fully engorge with blood. Upon engorgement with blood, ticks detach from the host and fall onto the ground. Although male ticks feed, they do not engorge to become as large as the females would, which is attributed to the limitation in the quantity of blood imbibed due to the presence of the scutum that covers

the dorsum. The females, however, have smaller scutum thus become prominently distended when engorged (Cupp, 1991; Nyangiwe *et al.*, 2018).

Depending on the number of host animals they attach to during their life cycle, Ixodid ticks can have one, two, or three hosts. (Walker *et al.*, 2003). The larvae and nymphs feed once to engorgement after which moulting occurs. For single-host ticks, moulting occurs two times on the same host, from the larval stage to nymph stage and then to the adult stage (that is larval, nymph and adult stages remain and feed on the same host and drop off only just before laying eggs) *(*Walker *et al.*, 2003; Latif & Walker, 2004; Nyangiwe *et al.*, 2018). Moulting happens only once on a host for two-host ticks, from larvae to nymph. After the engorged nymph falls off and moults from the host, the adult seeks out a new host, which may not necessarily be of the same species as the first. (that is, the larval and nymph stages feed on the same host, and adult stage on another) (Walker *et al.*, 2003; Latif & Walker, 2004; Nyangiwe *et al.*, 2018). For three-host ticks, however, moulting does not occur on the host. The larva that is engorged falls off and moults into a nymph, which further engorges on a second host animal. It falls off a second time to moult into the adult stage, which then attaches to a third host animal (Walker *et al.*, 2003; Latif & Walker, 2004; Nyangiwe *et al.*, 2018). Adult Ixodidae normally carry out mating on the host, after which the female feeds till it becomes engorged, falls off, lays a huge number of eggs (about 1500 to 5000 eggs and dies (while males remain on the host for months) (Jongejan & Uilenberg, 2004). One-host ticks lay relatively fewer eggs than three-host ticks, as the latter are exposed to more risk in the process of finding a new host at each stage in their life cycle (Jongejan & Uilenberg, 2004). Two-host ticks on the other hand are at less risk than species that have three hosts but more than one-host ticks, and also have egg batches that are intermediate with regards to size.

The most important genera of hard-bodied ticks are *Ixodes, Haemaphysalis*, *Dermacentor*, *Hyalomma*, *Rhipicephalus Amblyomma*, and *Boophilus*. However, owing to the close

evolutionary and phylogenetic relationship between those of the genus *Boophilus* to the genus *Rhipicephalus*, the former (*Boophilus*) *is used* as a subgenus of the latter (*Rhipicephalus*) (Barker & Murrell, 2002; Horak *et al.*, 2002; Jongejan & Uilenberg, 2004).

About 129 species of *Amblyomma* ticks exist. Characteristic of these ticks are mouthparts that are long and scuta that are beautifully coloured and ornamented. Although they have eyes, most species do not have them contained in sockets. *Amblyomma* species have three hosts ticks in their life cycle and are common in (sub)tropical regions where they infest diverse mammalian hosts as well as reptiles and amphibians (Spickler, 2003). Birds may get infested with some undeveloped stages of some tick species and this contributes significantly to how the ticks are distributed. On the African continent, *A. variegatum* is the regarded as a vital species because it has the widest distribution in Tropical Sub-Saharan Africa and is well-suited to domestic livestock. (Nabarro *et al*., 2020; Saari *et al*., 2019). Another important pest of livestock is *A. hebraeum*, which is found in Africa's south-eastern region (Jongejan *et al.*, 2020). In addition to its prevalence, *A. variegatum* was introduced into the Caribbean region with cattle from West Africa, as far back as the 18th or 19th century. *A. variegatum* is thus, the only *Amblyomma* of African origin to have established itself successfully beyond Africa (Camus & Barré, 1990).

Boophilus, which are one-host ticks finish their developmental cycles on the host, growing from an unfed larva to an engorged female. *Boophilus* ticks have mouthparts that are short and could cause destruction to skins as the preferred feeding sites are often of good leather potential (Jongejan & Uilenberg, 2004; Mkwanazi *et al*., 2021). *B. microplus* is the most important species which originates from the South-East of Asia but has increased significantly throughout the tropics in East and Southern Africa, Australia and South and Central America (Hoogstraal, 1956; Spickler, 2003). Till this day, other species such as *B. geigyi* and *B. decoloratus* continue to be restricted to Africa and are typically found on ruminants within West and Central Africa (Estrada-Peña *et al.*, 2006).

The genus *Dermacentor* have three hosts in their life cycle (Földvári *et al.*, 2016) and comprises 33 species. Characteristic of them are the presence of eyes, ornate scuta and palps that are short (Cupp, 1991; Walker *et al*., 2003; Jongejan & Uilenberg, 2004). The distribution of *Dermacentor* is focal (Földvári *et al*., 2016). Several species such as *D. reticulatus D. marginatus* parasitize farm and other domestic animals in Europe (Rubel *et al*., 2016) and Asia (Fawaz Dabaja *et al*., 2017; Hosseini-Chegeni, 2019). In North America, species of the genus *Dermacentor* are among the ticks that infest livestock. However, *Dermacentor* ticks do not have adverse effects on livestock in Africa (Földvári *et al.*, 2016) although some species have been identified in the wild (Horak *et al*., 2017; Walker *et al*., 2003).

About 168 species fall under the genus *Haemaphysalis*. Small ticks with short mouthparts are characteristic of ticks within this genus. Some species have lateral extending posterolateral angles on the second segment of the palps, which makes them have a triangular appearance and distinguishes them from other tick genera (Marchiondo & Endris, 2019). In these three-host ticks, eyes are absent (Cupp, 1991). They are found on livestock in Asia, Europe and Australia (Rubel *et al*., 2018). In parts of Southern Asia for example, *H. bispinosa* is found on cattle (Brahma *et al*., 2013), while *H. longicornis*, which is an East Asian species found on cattle and other domestic animals, is now identified in Australia (Marendy *et al*., 2020). In Europe however, the most common on ruminants is *H. punctate* (Hornok *et al.*, 2015).

Hyalomma species are ticks ranging in size from medium to large that have long mouthparts and eyes found in sockets. They infest wild and domestic mammals as well as birds and are abundant in semi-arid climate zones (Sajid *et al*., 2018). This genus there are 30 species in all, the majority of which carry out their life cycle on three hosts. Their ability to undergo either a two-host (e.g., *H. truncatum and rufipes*) or a three-host (e.g., *H. dromedarii*) cycle is host dependent, while others such as *H. scupense* undergo a one-host cycle (Spengler *et al.,* 2016). Adult *Hyalomma* ticks actively move from their resting sites to pursue hosts when they

approach contrasting most other ixodid ticks, that rest on the vegetation in anticipation of a host (Jongejan & Uilenberg, 2004).

Being the genus of hard ticks with the largest number of 241 species, Ixodes have no eyes and a scutum that has no ornamentation. Their life cycle occurs on three hosts and many species inhabit nests or holes (Walker *et al.*, 2003). Although commonly scattered among shrubs, fewer species infest larger animals (Schwarz *et al.*, 2009). *I. persulcatus* and *I. ricinus* are the common species in Asia and Europe (Rumer *et al.*, 2011), whereas the in North America, *I. scapularis* is prevalent (Ginsberg *et al*., 2021). However, the unselective feeding attitude of *I. persulcatus* and *I. Ricinus* on a wide range of hosts makes them important vectors of varied zoonotic tick-borne diseases (Aliota *et al*., 2014; Hyesung Yang & Han, 2018).

Having a three-host life cycle, *Rhipicephalus* genus has 70 species made up of ticks of average sizes with palps that are short and broad and commonly inornate. They have eyes and festoons are present (Dantas-Torres, 2010). In Africa, *Rhipicephalus* are common on mammals (Kanduma et al., 2020). They may either be three-host or two-host ticks (e.g. *R. evertsi*) (Walker *et al*., 2000). in Southern and Eastern Africa, the brown ear tick, *Rhipicephalus appendiculatus*, is the most prevalent rhipicephalid tick (Jongejan & Uilenberg, 2004), infecting both domestic and wild ruminants with a preference for the ears (Walker *et al.*, 2000).

2.3 Tick Behaviour

2.3.1 Host seeking

Ticks spend about 90% of their life cycle off-host. (Needham & Teel, 1991; Leal *et al.*, 2020). Although oviposition, egg hatching, metamorphosis, and moulting between the stages often occur off-host in the environment, these activities and the survival of the many stages within the life cycle require nutrition from a blood meal. (Leal *et al.*, 2020). As a result, being able to

quickly locate and attach themselves to a host is critical for survival. In finding a host, ticks employ questing behaviour. Generally, two strategies are employed by ticks for this, namely the ambush and hunter strategies. The ambush strategy involves ticks (typically of the genera *Rhipicephalus, Haemaphysalis and Ixodes*) climbing to the top of rocks or resting on blades of vegetation and extending their front legs in anticipation of a host. The hunter strategy however involves the tick (typically of the genera *Amblyomma* and *Hyalomma*) actively moving to pursue a potential host (Parola & Raoult, 2001; Leal *et al.*, 2020).

Ticks perceive certain cues in recognizing their hosts and determine whether to attach for feeding or to fall off and resume seeking another host. These include radiant heat, odours, vibrations or visual images. Odours are major stimuli perceived such as butyric and lactic acids, produced during perspiration and from other fluids from the body. Although breath of humans also stimulates a response, it is not as strong as that of cattle. In addition, carbon dioxide in the breath of the animal and ammonia in urine and other animal wastes are some essential attractants released by hosts. Subtle surges in radiant heat stimulate ticks and act synergistically with host odours. Hunter ticks also rely on visual cues in distinguishing between shapes that are dark against the sky's background. In that, they respond to shadows, via the extending their legs to enhance interaction. Attachment of tick to host will not proceed unless adequate stimuli are perceived in a specific order (Osterkamp *et al*., 1999; Randolph, 2013).

2.3.2. Feeding

The majority of tick species inhabit forests, savannahs, grasslands and scrublands, and survive long periods until a suitable host to feed on is found. Upon strategically positioning themselves (questing) and successfully identifying and climbing onto a host, the ticks assess suitable sites of attachment and hold on firmly to the host, boring into the skin with their mouthparts which favours the transmission of pathogens they may be carrying from a previous blood meal from

another host (Richter *et al*., 2013). Ticks are pool feeders who inject saliva through feeding and finish engorgement through the same channel in a sporadic sequence of salivating and sucking (Bonnet *et al*., 2018). During feeding, ticks remain attached to the host. Earlier during attachment, mouthparts some ticks are cemented to the host's skin, releasing them when most bodily fluids and erythrocyte meals have completely been taken up (Sonenshine & Roe, 2013). Salivary glands are thus essential in tick feeding and transmission of pathogens (Tahir *et al.*, 2020). In that, before a transmission, saliva plays a role in reactivating and/or replicating the pathogen from the infected tick to a naïve host (Ebel & Kramer, 2004).

2.3.3 Disease transmission

Ticks pose several debilitating threats on their hosts and these are attributed to their bites. These range from toxicosis and paralysis to irritation and allergic reactions. Some tick species commonly found attached to humans have been implicated as competent vectors of viral, bacterial and protozoan pathogens (Estrada-Peña & Jongejan, 1999). Molecules within the saliva are exploited by infectious agents to promote their transmission hence the term Saliva-activated transmission (SAT). At the site of attachment of the tick on the skin, the SAT factor is among the immunomodulatory proteins that help maintain prolonged feeding of ticks, thus fuelling pathogen transmission (Nuttall, 2019; Randolph *et al.*, 1996).

2.3.4 Tick-borne Zoonoses

Ixodid ticks serve as vectors of some pathogens that cause diseases transferrable from animals to humans. An example is species of the bacterial genus *Rickettsia*, which typically is the African tick typhus agent *R. africae* in sub-Saharan Africa, *and* transmitted by the tropical bont tick, *Amblyomma variegatum*. On another hand, particularly *Hyalomma* spp is known to transmit the virus that causes Crimean-Congo haemorrhagic fever in humans.

2.3.4.1 Rickettsial Diseases

Rickettsia are small gram-negative bacilli that completely replicate inside of eukaryotic cells. Organisms within this genus are prevalent worldwide and are usually harboured by arthropod vectors that suck blood such as ticks, mites, lice and fleas. Despite the problem posed by *Rickettsia* globally, not much attention is given to it with limited resources allocated to face the challenge. This could be attributed to the fact that they present symptoms similar to acute febrile infections which are endemic to (sub)tropical regions (Parola *et al*., 2013; Fang *et al*., 2017; Blanton, 2019) and thus result in individuals usually being misdiagnosed. In addition, the absence of rapid point of care tests make it difficult to detect acute infections, hence healthcare providers usually resort to employing serological methods to ascertain past exposure (Blanton, 2019). *Rickettsia* pathogens thus persist and are emerging and remerging throughout the world (Fang *et al.*, 2017). According to the CDC, *Rickettsia* are divided into spotted fever group (SFG), typhus group (TG), scrub typhus group (STG) and other Rickettsioses (Centers for Diseases Control and Prevention, 2020). Transmission of Rickettsiae occurs through the skin of a person. Following inoculation into the skin, dendritic cells phagocytose pathogens, after which transportation to lymph nodes takes place. Replication occurs and organisms enter the bloodstream and further infect the endothelium of the microcirculation. This results in damage and increased vascular permeability which manifests as rashes and in severe cases interstitial pneumonia, acute kidney injury, meningoencephalitis, multiorgan failure and even death (Blanton, 2019).

2.3.4.2 Spotted Fever Group (SFG)

SFG Rickettsiae infect a wide range of tick species worldwide and thus have a wide geographic distribution (Parola *et al*., 2013). Although ticks are vectors, they may also double as reservoir hosts by passing pathogens from one stage to the next in the life cycle as well as from infected

females to eggs. The most pathogenic species of Rickettsial diseases, *Rickettsia rickettsii* falls within this group. Less pathogenic species such as *R. africae*, which causes African tick-bite fever also belongs in this class (Blanton, 2019; Parola *et al.*, 2013)

Others, identified and described based on their geographical prevalence include Rocky Mountain spotted fever (RMSF), that is caused by R. *Rickettsii*, *R. parkeri* and *Candidatus R. philippi* pathogens, is a disease of the Americas. Mediterranean spotted fever (MSF), that is also caused by *R. conorii*, exists predominantly in Asia, Africa and Europe. Tick-borne lymphoadenopathy (TIBOLA), also within this class is caused by *R. slovaca* and *R. raoultii* in Europe and Asia (Socolovschi *et al*., 2009)

2.3.4.3 Typhus Group (TG)

Vector-borne bacteria that cause typhus group rickettsiosis are *Rickettsia typhi* and *R. prowazekii*. The former has been implicated to cause the less severe endemic murine typhus in tropical coastal regions and transmitted by fleas. The latter however which causes severe epidemic typhus in temperate and tropical regions is transmitted by body lice (Rauch *et al.*, 2018).

2.3.4.4 Scrub Typhus Group (STG)

This rickettsiosis is confined to the Asia Pacific region specifically distributed in the Tsutsugamushi triangle - the soviet union to the north, China, Philippines and tropical Australia to the south , Japan to the east and India, Pakistan through Afghanistan in the west (Chakraborty & Sarma, 2017). The pathogen implicated in causing the disease is *Orientia tsutsugamushi*, transmitted by chigger mites (Chakraborty & Sarma, 2017).

2.3.4.5 Other Rickettsioses

In the United States, ehrlichiosis and anaplasmosis are the commonly reported tick-borne infections with *Ehrlichia chaffeensis* and *Anaplasma phagocytophilum* being implicated in infections respectively. In certain parts of Europe, Africa, Asia and South America, cases with various *Ehrlichia* and *Anaplasma* spp. have been recorded. In Europe and Asia for example, *Neoehrlichia mikurensis* is the causative tick-borne pathogen. *Neorickettsia sennetsu*, responsible for causing Sennetsu fever has also been reported in Japan and Malaysia and this is usually contacted via the consumption of raw fish infected with neorickettsia-infected flukes (Centers for Diseases Control and Prevention, 2020).

2.3.4.6 Crimean Congo Haemorrhagic Fever (CCHFV)

This is described as one of the very severe zoonotic viral diseases of humans, characterized by fever and haemorrhage and often fatal (Ergönül, 2006; Belobo *et al*., 2021). The virus was first identified in Crimea, Russia in 1944 and later found to be identical to the Congo virus identified in Congo in 1967, hence its current name and has since been detected in more than 50 counties across Asia, Europe and Africa (Ergönül, 2006; Belobo *et al.*, 2021).

It is tick-borne disease caused by CCHFV of the Orthonairovirus genus within the Nairoviridae family and the Bunyavirales order (Adams *et al.*, 2017). Transmission is facilitated by *Hyalomma* ticks of the family Ixodidae, which spread the virus to humans as well as animals (both domestic and in the wild) (Gargili *et al.*, 2017). Humans get infected via tick bites, contact with individuals carrying the virus in the early stages of infection or upon contact with viraemic livestock's blood or tissues (Ergönül, 2006). Geographically, CCHFV is widely distributed and causes severe human disease with a high mortality rate, thus making it an important human pathogen (Ergönül, 2006). The virus spreads through ticks to animals and migratory birds, and genetic analysis can be used to determine its geographical distribution. Africa, Southeast

Europe, and Asia are the most common locations for CCHF (Cajimat *et al.*, 2017; Okely *et al.*, 2020; Wölfel *et al.*, 2007).

2.4 Crimean Congo Haemorrhagic Fever Virus (CCHFV) Genome

The CCHFV is sphere-shaped about 80–100 nm in diameter, a lipid envelope of 5–7 nm thickness and glycoprotein spikes that are 8–10 nm in length (Wahid *et al.*, 2019). The CCHFV has a tripartite RNA genome (small [S], medium [M], and large [L] segments) with high genetic diversity and also has a negative-sense. The major nucleocapsid protein is encoded by the small portion of the genome (S, 1.7 kb), the amino acids' precursor (M ~ 1700) is encoded by the medium portion (M, 5.3 kb), which results in the assembly of two envelope glycoproteins, Gn (37-kDa) and Gc (75-kDa), whereas an RNA-dependent RNA polymerase (450 kDa- L-RdRp) is encoded by the large portion (L, 12.1 kb) (Cajimat *et al.*, 2017; Papa, 2019; Voorhees *et al.*, 2018; Wahid *et al.*, 2019). At the nucleotide level, the S segment which is the most conserved has sequences that can be grouped into six lineages (Chamberlain *et al.*, 2005). Every genetic lineage has been associated with geographic regions in the Middle East, Africa, Europe and Asia where *Hyalomma* spp. ticks are existent (Hoogstraal, 1979). In Africa, genotypes I, II, and III have been described; whereas in Asia genotype IV is described and in Europe, genotypes V and VI (Deyde *et al.*, 2006; Monsalve Arteaga *et al.*, 2021). CCHF has a wide ecological distribution. In Asia (India, Pakistan, Tajikistan, Kazakhstan, and Russia), the Middle East (Iran and Afghanistan), Europe (Turkey, Bulgaria, Greece, Albania, and Kosovo), and more than 30 African republics (including Sudan, Mauritania, Kenya, Senegal, and South Africa), minor outbreaks of Crimean-Congo haemorrhagic fever virus (CCHFV) have been reported since 2000 (Farhadpour *et al.*, 2016).

2.5 Strategies for tick control and Tick-borne Disease (TBD) prevention

Several strategies to prevent and control the abundance of ticks, the prevalence of pathogens they carry and the risk of humans being exposed to these pathogens have been outlined in many instances. Broadly, the strategies can be classified into adopting personal protective measures (which includes protective clothing, use of repellents, etc), managing ticks to reduce their contact with humans (through the application of chemical acaricides on domestic animals and the environment), and lowering the transmission of tick-borne diseases via reducing the prevalence of pathogens being carried (such as landscape and habitat modification, use of biological control agents) (Stafford *et al.*, 2017). However, due to the persistence of tick and tick-borne diseases, despite the various efforts made to alleviate these challenges, it is evident that no one method is effective at completely tackling the problem. It is thus important that Integrated Tick Management programs be employed. This includes surveillance, identification, routine disease testing, landscape management, and targeted control of adult ticks (De La Fuente *et al.*, 2015).

CHAPTER THREE

METHODOLOGY

3.1 Study sites

Samples were collected from January to March 2020. Sampling was carried out within environs in Greater Accra and Upper East Region, which fall within the Coastal savannah and Sudan Savannah ecological zones respectively in Ghana (Figure 1). These locations also serve as study sites for a through a collaboration between the Ghana Armed forces, the Navrongo Health Research Centre (NHRC) and the US Naval Medical Research Unit 3 (US NAMRU-3). The selected sites included military-owned kraals at Burma camp (5 Infantry Battalion and 3 Mounted Squadron), Michel camp (1 Infantry Battalion) and Asutuare within Greater Accra; and Abattoir, Cow market and Nakong within Navrongo.

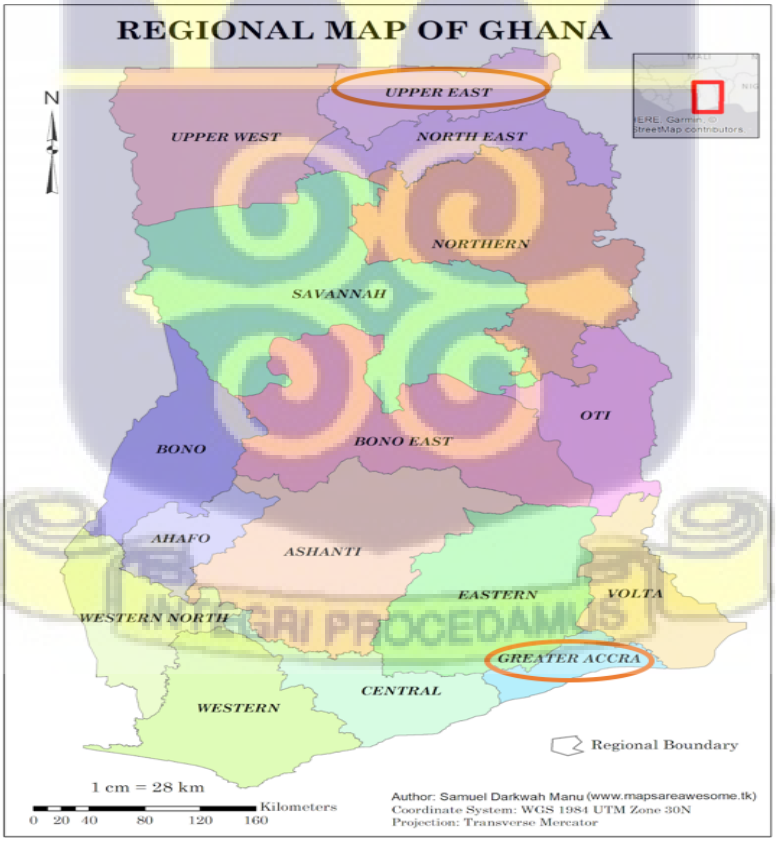

Figure 2: Regional Map of Ghana showing study sites.

3.2 Ethical clearance

Ethical clearance for the study was obtained from the Noguchi Memorial Institute for Medical Research (NMIMR) (CPN 110/15-16), Ghana Armed Forces (GAF) (IPN: 093/2017), Ghana Health Service (GHS) (GHS ERC 004/03/19) and the Naval Medical Research Centre (NMRC) (NAMRU3.2016.0010) Institutional Review Boards.

3.3 Study Population

Animal Handlers which included livestock rearers and Abattoir workers who resided or worked within a 10-kilometre periphery of the study sites were invited to participate in the study.

3.4 Sample collection

3.4.1 Sampling from Animals

3.4.1.1 Inclusion/ Exclusion of Animals

There was no justification for the exclusion of livestock from being sampled. The study did not experiment with animals but only involved collecting ticks off the animals for infection studies. At each sampling site, ticks were removed from a convenience sample of animals for tick identification and further molecular testing.

3.4.1.2 Tick collection from Animals

Ticks were hand-picked either directly or using blunt-tipped forceps. Before tick collection, animals were restrained by the owner or handler to provide easy access. Caution was taken not to twist or jerk the tick to ensure mouthparts did not break off or remain in the skin. Having done these, the tick was pulled up straight until all parts of it were totally out of the animal's skin (Figure 2). The ticks were then placed into labelled tubes containing RNA later and further transported to the AFI laboratory at NMIMR for morphological identification.

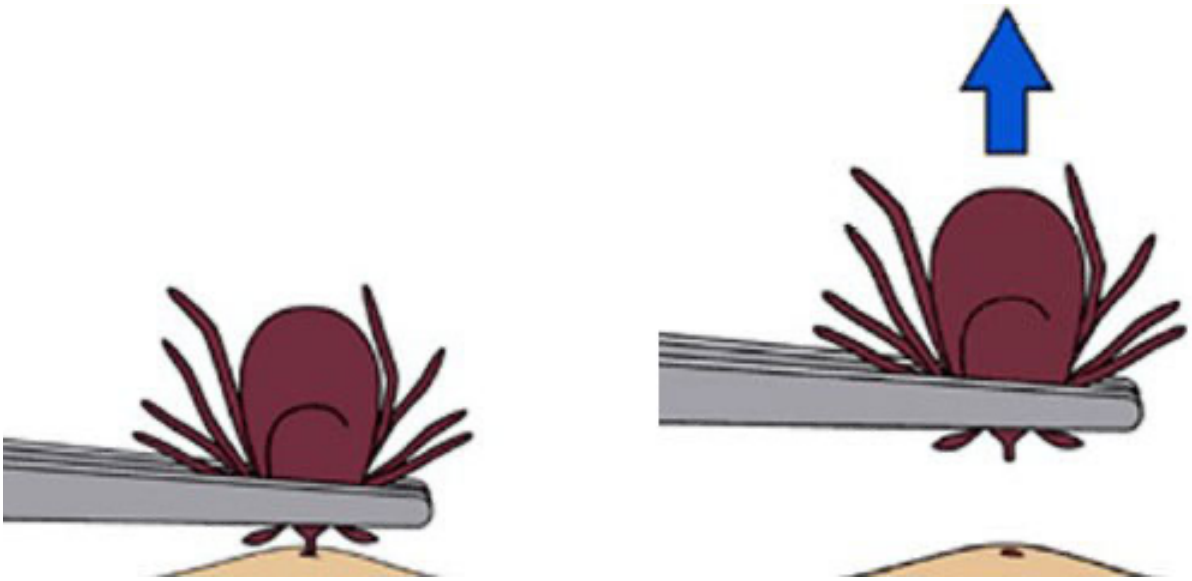

Figure 3: Image demonstrating how ticks were taken off livestock

Source: (Centers for Disease Control and Prevention, 2021)

3.4.2 Sampling from Humans

3.4.2.1 Inclusion criteria for human sampling

Primary animal handlers above 18 years and who were willing to participate and provide informed consent were recruited for the study.

3.4.2.2 Exclusion criteria for human sampling

Individuals who did not have primary contact with animals, under 18 years and were unwilling to participate and provide informed consent were excluded.

3.4.2.3 Community entry

For Navrongo, which was a non-military site, permission was sought from the town elders and local chiefs who had jurisdiction over the areas. For military sites within Greater Accra however, approval was sought from command. Before initiation of the study, the target population were assembled in groups based on the area of their activities. The study was then explained (background, purpose, procedures, time and place for enrolment) to the groups. Participants were then individually approached and invited to enrol in the study.

3.4.2.4 Participant eligibility and informed consent procedure

Participants were first screened for eligibility. Upon meeting the criteria, the procedure was clearly explained to the participants, allowing them to ask questions and to ensure they were all comfortable about the procedure. Further, unique identification numbers were assigned to each enrolled participant as this information was necessary to enable linking to laboratory specimens. Afterwards, informed consent was obtained from participants in duplicate as per protocol before blood draw and a copy of informed consent was given to the enrolled participant.

3.4.2.5 Blood Collection from participants

Serum separator tubes (SSTs) were labelled with the unique participant study ID number and date after which a tourniquet was applied proximal to the site of venepuncture upon identifying a good-sized vein to ensure engorgement of the vein with blood. The site of venepuncture was cleaned with an alcohol swab and a 10 ml syringe with the appropriate needle prepared and inserted into the vein. About 10 ml of blood was gently drawn into the syringe after a blood flashback in the bevel of the syringe. Immediately, a cotton swab was used to apply direct pressure over the puncture site to stem any bleeding. The blood was then transferred from the syringe into SST by directly puncturing the tube in the centre and gently injecting blood into

the empty tube. Thereafter, the tube was inverted about 10 times to ensure mixing of the sample with the anticoagulant inside the tube. The SSTs were then stored in a cool box with ice packs while waiting for blood to clot. Band-aids were then affixed to sites of venepuncture to ensure bleeding stops after which the specimen logbook with study number and details of patients was completed.

3.4.2.5.1 Blood Sample Processing

Blood tubes (SSTs) were centrifuged for 10 minutes at 1,000–2,000 x g while ensuring the tubes were properly balanced inside of the centrifuge and handled very carefully so as not to disturb the separation of layers. Using a sterile pipette, 1 ml serum aliquots for each sample were transferred into labelled cryovials. Sera and blood clots were stored in a -80°C mobile freezer for transport to the AFI laboratory at Noguchi for analyses.

3.5 Laboratory Testing

3.5.1 Morphological Identification of ticks

Ticks were removed from RNA later onto a clean petri dish and observed under a Motic SMZB series dissecting microscope. The various parts (mouthparts, ventral plates, eyes, scutum, etc) were observed as outlined in the identification keys by Walker *et al.*, (2003) and ticks grouped into the various genera and species. Pictures of identified ticks within the various genera and species were taken. Following this, purification of nucleic acid was carried out.

3.5.2 Extraction of total nucleic acid from ticks

Extraction of total nucleic acid from each tick was carried out using QIAamp Viral RNA Mini Kit (250). Following the protocol of the manufacturer, 0.5g and 0.75g of 0.1mm and 2mm beads respectively were added to clean 1.5 ml microcentrifuge tubes. Single ticks were placed in each tube and 560µl of prepared Buffer AVL were added. Tubes were then centrifuged at

8000rpm for 2minutes to homogenize the ticks. Four hundred microlitres (400uL) of the homogenate was transferred into clean 1.5 ml microcentrifuge tubes after which incubation was done at room temperature (between 15-25°C) for 10 minutes. Furthermore, centrifugation of the tubes was briefly done to remove drops from inside the lid and 560 µl of absolute ethanol (96-100%) added and vortexed for 15 seconds. To remove drops of the mixture from inside the caps, the tubes were briefly centrifuged.

A volume of 600 µl of the solution was carefully pipetted from the previous step into the QIAamp Mini spin column without wetting the rim and centrifugation further done at 8000 rpm for 1 minute. After discarding the filtrate, the QIAamp Mini spin column was placed into a clean 2ml collection tube. Further, this step was repeated.

Following this, Buffer AW1(500 µl) was added to the spin column, and centrifuged at 8000 rpm for 1 minute. The QIAamp Mini spin column was placed into a clean 2ml collection tube and the filtrate was discarded. After carefully opening the QIAamp Mini spin column, 500 µl of Buffer AW2 was added and centrifugated at maximum speed (14,000 rpm) for 3 minutes. The spin column was then placed in a new 2ml collection tube. An 80 ul volume of elution buffer AVE was carefully added to the membrane of the spin column and incubated at room temperature for a minute. The nucleic acid was finally eluted by centrifuging the column at 8000 rpm for 1 minute. At -20°C, the eluate containing the total nucleic acid was stored at.

3.5.3 Molecular detection of tick-borne pathogens

3.5.3.1 Real-time PCR (RT-PCR) for detection of CCHFV

In screening for CCHFV, in-house reagents from United States Army Medical Research Institute of Infectious Diseases (USAMRIID) were used with primers targeting the S segment of the CCHFV genome to screen the nucleic acid extracted from the ticks (Atkinson et al., 2012)

The total volume of the reaction was 20 µl, comprising 14.6 µl of prepared master mix, 0.4 µl of platinum Taq and 5 µl of the template (nucleic acid extract). Each reaction was ran with a positive control, with nuclease-free water as the negative control, simultaneously. The PCR was performed on the Applied Biosystems (USA) 7300 Real-time PCR system with cycling conditions as follows: 1 cycle of the first hold for 15 minutes at 50°C, 1 cycle of the second hold for 5 minutes at 95°C, 45 cycles of the third hold for 1 second at 94°C, 30 seconds at 55°C (fluorescence read and data acquisition), for 5 seconds at 68°C and 1 cycle of the fourth hold at for 30 seconds at 40° C. No passive reference was chosen, FAM was selected as reporter and TAMRA as the quencher.

3.5.3.2 Real-time PCR (RT-PCR) for detection of *Rickettsia* species

For the genus *Rickettsia* (R17b), in-house reagents from the Vector-borne Diseases division of the Naval Medical Research Center (NMRC) were used with primers that targeting the 17kDa surface protein to screen the nucleic acid extracted from the ticks for the pathogen (Jiang *et al*., 2004).

Each reaction had a total volume of 20 µl, comprising 14.8 µl of prepared master mix, 0.2 µl of platinum Taq and 5 µl of the template (nucleic acid extract). Each reaction was ran with a positive control, with nuclease-free water as the negative control, simultaneously. The PCR

was performed on the Applied Biosystems (USA) 7300 Real-time PCR system with cycling conditions as follows: 1 cycle of the first hold for 2 minutes at 95°C, 45 cycles of the second hold at for 5 seconds at 95°C and for 30 seconds at 60°C (fluorescence read and data acquisition) and 1 cycle of the third hold for 30 seconds at 40°C. No passive reference was chosen, FAM was selected as a reporter and no quencher was selected.

3.5.3.3 Realtime PCR (RT-PCR) for detection of *Rickettsia africae*

A species-specific assay was chosen due to the incidence and spread of spotted fever Rickettsiae on the African continent to test samples that were positive for the *Rickettsia* genus, for *Rickettsia africae* (Maina *et al.*, 2014). Each reaction had a total volume of 25 µl, comprising 12.5 µl of 2X TaqMan Universal PCR master mix (Applied Biosystems, USA), 5 µl of nuclease-free water, 1.25 µl of forward and reverse primers, 1 µl of the probe, 1 µl of $MgCl_2$ and 3µl of the template (nucleic acid extract). Each reaction was ran with a positive control, with nuclease-free water as the negative control, simultaneously. The PCR was performed on the Applied Biosystems (USA) 7300 Real-time PCR system with cycling conditions as follows: 1 cycle of the first hold for 10 minutes at 95°C, 45 cycles of the second hold for 15 seconds at 95°C and for 1 minute at 60°C (fluorescence read and data acquisition stage). ROX was chosen as the passive reference, FAM as a reporter and no quencher selected.

3.5.4 Serological analysis for antibodies against tick-borne pathogens

3.5.4.1 Magpix Immunoassay for detection of CCHFV antigen

In a biological safety hood, 2 µl patient samples were diluted in 198 µl PBST-SK (Phosphate buffered saline with 0.02% Tween-20 and 5% skimmed milk) in a 1:100 ratio. Negative controls were then diluted in PBST-SK in a 1:10 ratio (450 µl PBST-SK added to a negative control aliquot tube). Positive control was also diluted in a 1:2 ratio in PBST-SK (100 µl PBST-SK was added to one positive control aliquot tube). The capture bead mix was vortexed for 10

seconds and diluted in PBST (Phosphate buffered saline with 0.02% Tween-20) in a ratio of 1:250, (ensuring beads are protected from light before and after dilution. Into the desired wells of a white 96 well plate 50 µl was added to the wells. Sealing of the plate was done and plate further placed on the Luminex Plate Magnet (ensuring that the connection was secure with the two metal arms latched on the sides of the plate), covered with foil and the beads allowed to collect at the bottom for 60 seconds. After, the Luminex plate magnet was grasped (while still latched to the plate) and the buffer was discarded into a sink with a downward motion. Following this, the negative and positive controls, as well as the samples, were added to the plate and the sample locations recorded. The plate was then sealed and put onto a plate shaker to shake for an hour at 400 rpm (making sure the plate was covered with foil during this step). After this, the plate was removed from the shaker, plate sealer removed, placed on the Luminex plate magnet and covered with foil. The liquid was discarded in the sink while the magnet was still latched to the plate as above. Washing of the beads were done by adding 100 µl of PBST to the wells of the plate after which the plate was put onto the magnet, sealed, covered with a foil and then buffer discarded after a minute. This step was carried out two additional times to make a total of three washes. Furthermore, the Human anti-IgG-PE was diluted in PBST-SK (1:100) after which 50 µl was added to the wells. The plate was then sealed, covered with a foil and placed on the plate shaker for an hour. After, taking the plate off the shaker, and placing on the Luminex magnet for a minute, the liquid was discarded, leaving the beads bound to the bottom of the plate. Three washes were then done by adding 100 µl PBST to the well, incubating on the Luminex magnet for a minute and buffer discarded. After the final wash, 100 µl of PBST was added to the wells and then proceeded to read the plate on the Luminex Magpix. A sample was called positive if the signal to noise (S/N) ratio was greater than 10 (i.e., $S/N > 10$)

3.5.4.2 ELISA Assay to detect Group-specific antibodies against Rickettsiae

One-half of 96 well microtiter plates were coated with 100 µl *Rickettsia* STG (Karp = 1:1000; Kato = 1:1000 and Gilliam = 1:1500), SFG (1:2500) and TG (1:2000) antigens diluted in PBS. For the other half of the plates, however, 100 µl of PBS only was added with no antigen. Plate sealers were used to seal the plates, which were subsequently wrapped in aluminium foil and incubated for a minimum of 48 hours at 4°C. Furthermore, wash buffer was used to wash the coated plates three times (0.1% Tween in 20 in PBS). After that, 200 µl of blocking buffer (5 percent skim milk [Difco] in wash buffer) was added to each well to block the plates. Plates were covered in aluminium foil and incubated for 1 hour at room temperature, after which wash buffer was used three times. After diluting the patient serum in blocking buffer (1:100), 100 µl was pipetted into allotted wells for each sample on both the antigen and PBS half of the plate. Washing was done three times with wash buffer after an hour of incubation at room temperature. 100 µl of peroxidase-conjugated goat antihuman IgG (Kirkegaard & Perry) was added to each well after diluting it in blocking buffer (1:2000) and incubating it at room temperature for 1 hour. After that, the plates were washed three times in wash buffer. 100 µl of ABTS (Kirkegaard & Perry) substrate was mixed in a 1:1 ratio and added to each well of the plate, which was then incubated in the dark for 30 minutes. Plates were then scanned at 405 nm (minus a reference value of 650 nm) with ODs from antigen-free wells used to subtract background absorbance. If the net optical density (NET OD) values were more than or equal to 1.0 (i.e., NET OD 1.0), the sample was considered positive.

3.5.5 Whole Genome Sequencing (WGS) for CCHFV PCR positives

Whole Genome Sequencing was carried out to determine the entire sequence of the pathogen's genome after which genome assembly and phylogeny was carried out.

3.5.5.1 RNA PICO 6000 assay (for quantification and assessing quality of RNA for NGS library preparation)

The RNA ladder was stretched down and heat-denatured for 2 minutes at 70°C, after which the vial was placed on ice to cool. A total of 90 µl of RNase-free water were added and well mixed. The appropriate amount for normal daily use was then prepared in approved 0.5 mL RNase-free vials and stored at -70°C. Before use, all reagents were brought to room temperature for 30 minutes. To begin, 550 µl of RNA 6000 Pico gel matrix was deposited in the top receptacle of a spin filter, which was centrifuged at 4000 rpm for 10 minutes. After that, 65 µl of filtered gel was aliquoted into the provided 0.5 ml RNase-free microcentrifuge tubes. Aliquots were kept at 4°C for a month and then used.

The RNA 6000 Pico dye concentrate was briefly vortexed, span down, and 1 µl pipetted into a vial containing the prepared 65 µl aliquoted filtered gel. The tube was capped, thoroughly vortexed and visually inspected to ensure gel and dye were properly mixed after which centrifugation was done at 14000 rpm for 10 minutes. The dye concentrate was then stored in the dark at 4°C to ensure that it was shielded from light.

A new RNA chip was removed from its sealed package and placed on the chip priming station after allowing the gel-dye mix to equilibrate to room temperature for 30 minutes. In addition, 9 µl of the gel-dye mix was pipetted and placed into the bottom of the indicated well (G). The chip priming station was then closed after a 30-second timer was set, ensuring that the plunger was positioned at 1 ml. The syringe plunger was then pushed down until it was caught by the clip. The plunger was visually inspected until it moved to at least the 0.3 ml mark after 30 seconds, and then carefully pushed back to the 1 ml position using the clip release mechanism. The chip priming station was next opened, and 9.0 µl of the gel-dye mix was added to the two remaining wells (G). Pipetting 9 µl of RNA 6000 Pico conditioning solution into the indicated

well CS and 5 µl of RNA 6000 Pico marker into the well with a ladder symbol and each of the 11 sample wells was done.

In addition, 1 l of diluted RNA 6000 Pico ladder (made as indicated above) was pipetted into the ladder symbol-marked well. The samples were then placed in the sample wells (1 l of each sample). On an IKA vortex mixer, the chip was vortexed for 60 seconds at 2400 rpm. The chip was carefully inserted into the receptacle of the Agilent 2100 Bioanalyzer instrument, the proper assay was chosen from the assay menu, and the chip run began.

3.5.5.2 First-strand synthesis

The fragmentation and priming reaction mix were made on ice in a nuclease-free tube using 5 µl of input RNA, 4 µl of NEBNext First Strand Synthesis Reaction Buffer, and 1 µl of Random Primers for a total of 10 µl per reaction while ensuring thorough mixing. The samples were then placed in a thermocycler and heated to 94 degrees Celsius. The incubation time differed depending on whether the RNA was intact or partially degraded, as determined by the bioanalyzer (RIN >7 (intact RNA) = 15 minutes; RIN 2-6 (partially degraded RNA) =7-8 minutes). Following this, the tubes were immediately placed on the ice.

The first-strand synthesis reaction mix was then put together on ice in a nuclease-free tube with 10 µl fragmented and primed RNA, 8 µl NEBNext Strand Specificity Reagent, and 2 µl NEBNext First-Strand Synthesis Enzyme Mix to form a total of 20 µl per reaction while assuring complete mixing. The reaction mixture was then incubated in a preheated thermocycler under the following cycling conditions: Step 1: 25°C for 10 minutes, 42°C for 15 minutes, 70°C for 15 minutes, step 3: 70°C for 15 minutes, and step 4: 4°C hold.

3.5.5.3 Second strand synthesis

The second strand cDNA synthesis reaction was made on ice in a nuclease-free tube with the 20 l first-strand synthesis product, 8 µl NEBNext Second Strand Synthesis Reaction Buffer, 4 µl NEBNext Second Strand Synthesis Enzyme Mix, and 48 µl nuclease-free water to make a total of 80 µl per reaction while ensuring thorough mixing. The thermocycler was used to incubate the samples for 1 hour at 16°C with the heated lid set to a temperature of 40°C. After that, the NEBNext Sample Purification Beads were vortexed to resuspend them, and 144 µl of the second strand synthesis product (80 µl) was added. This was followed by thorough vortexing and a 5-minute incubation period at room temperature. The tube was briefly centrifuged before being put on a magnet to separate the beads from the supernatant. The supernatant was eliminated when the solution became clear, taking care not to disturb the beads (which are bound to DNA). 200 µl of freshly made 80 % ethanol was added while the magnetic stand was still in use, and the supernatant was discarded after 30 seconds of incubation at room temperature. After two washes, the beads were air-dried for 5 minutes with the tube remaining on the rack. After that, the tube was taken from the magnetic stand, and the DNA target was eluted from the beads using 53 µl 0.1X TE buffer, which was thoroughly mixed. Brief spinning was performed, followed by an incubation period at room temperature. The tube was placed back on the magnetic stand, and 50 µl of supernatant was transferred to a clean nuclease-free PCR tube when the solution became clear.

3.5.5.4 Qubit quantification of cleaned pre-enriched cDNA

Standards and samples were placed in separate assay tubes. 190 µl of Qubit working solution was pipetted into the tube for standards, and 10 µl of standards (1 and 2) were pipetted into separate tubes. In separate tubes for each sample, 198 µl of Qubit working solution and 2 µl of samples were added to the tubes containing the cDNA samples. All mixtures were briefly

vortexed and allowed to incubate at room temperature before the concentrations were measured (standards first, then samples) using the Qubit (Invitrogen life technologies).

3.5.5.5 Tagmentation of Genomic DNA

A 30 µ volume of DNA was poured into the wells of a 96-well PCR plate. 20 µl tagmentation master mix containing 11.5 µl enrichment Bead-Linked Transposomes (eBLT) and 11.5 µl tagmentation buffer 1 (TB1) was added to each sample, mixed completely, sealed, and shaken for 1 minute at 1600 rpm. The plate was placed on a thermal cycler, with the preheat lid set to 100°C and the reaction volume set to 50 µl, incubation at 55°C for 5 minutes and a hold at 10°C. After allowing the plate to cool for 2 minutes, 10 µl of stop tagment buffer 2 (ST2) was added to the tagmentation reaction, sealed, and shaken for 1 minute at 1600 rpm. After that, the plate was placed on a magnetic stand for 3 minutes to allow the liquid to clear.

After that, 60 µl of the supernatant was removed and discarded before washing. The plate was removed from the magnetic stand, and 100 µl of tagment wash buffer (TWB) was added directly onto the beads and stirred thoroughly until they were fully resuspended. The plate was re-positioned on the magnetic stand until the liquid was clear, and 100 µl of supernatant was discarded. For a total of two washes, the wash process was repeated.

The plate was further taken off the magnetic stand after removing and discarding 100 µl of the supernatant. After, 40 µl of PCR master mix, consisting of 23 µl of Enhanced PCR Mix (EPM) and 23 µl of Nuclease-free water was added and mixed thoroughly till beads were fully resuspended. The index adapter plate was then prepared and briefly centrifuged, after which 10 µl of pre-paired index 1 (i7) and index 2 (i5) distinct index adapters were added to each sample in the wells. This was thoroughly mixed, the plate was sealed, and the preheat lid option was selected and set on a thermal cycler to 100°C, and volume set to 50 µl. Cycling conditions for the run were as follows: 72°C for 3 minutes for step 1, 98°C for 3 minutes for step 2, 12

cycles of 98°C for 20 seconds, 60°C for 30 seconds and 72°C for 1 minute for step 3, 72°C for 3 minutes for step 4 and a final hold at 10°C.

The plate was then placed on the magnetic stand till liquid was clear and 45 µl of each sample was transferred to a new plate. Further, ensuring AMPure XP beads were brought to room temperature, 88 µl was added, followed by the addition of 77 µl of nuclease-free water. This was thoroughly combined and allowed to incubate for 5 minutes at room temperature. After that, the plate was placed on the magnetic stand until the liquid was clear, and 200 µl supernatant from each sample was transferred to a fresh plate. In addition, 20 µl of AMPure XP beads were pipetted into the wells containing the supernatant, vigorously mixed, and incubated for 5 minutes at room temperature. While on the magnet, the supernatant was removed and disposed without harming the beads.

After that, two washes were done as follows: with the plate on the magnet, 200 µl of freshly produced 80 % ethanol was added without mixing, followed by incubation at room temperature for 30 seconds and removal and discarding the supernatant. After draining any remaining ethanol, the plate was dried for 5 minutes on the magnetic stand. The plate was taken off the magnetic stand and 17 µl of resuspension buffer (RSB) was added to the beads in each well, which were then incubated at room temperature for 2 minutes before being placed back on the magnetic stand for 2 minutes. For library enrichment, 15 l of each sample's supernatant was transferred to wells of a fresh plate.

3.5.5.6 Library enrichment

Into the wells of a new plate, 9 µl of each sample was transferred, 12 µl of new hybridization buffer 1 (NHB 1), 1 µl of probes, 2.5 µl of enrichment hybridization buffer 2 (EHB 2) were added, making a total of 25 µl reaction volume. The plate was then placed on a thermocycler programmed as follows: denaturation at 95°C for 5 minutes for step 1, 18 cycles of ramping

down from 94°C to 58°C (decreasing 2°C every cycle) for 1 minute for step 2, hybridization at 58°C for 90 minutes for step 3 and a temperature hold at 58°C for step 5.

The plate was removed from the thermocycler at the end of the run, 62.5 µl of streptavidin magnetic beads (SMB) were added, the plate was sealed and shaken for 4 minutes at 1200 rpm, then incubated at 58°C for 15 minutes.

After that, the plate was removed from the thermocycler and placed on a magnetic stand for two minutes. 88 l of the supernatant was collected and discarded once the liquid had become clear. The plate was removed from the magnet and washed three times, first with 50 l of pre-warmed enhanced enrichment wash (EEW), then shaking at 1600 rpm for four minutes, incubating at 58°C for five minutes, and then returning to the magnet to collect and discard the supernatant. After the washes, 50 µl of pre-warmed EEW was added to each well and shaking was done at 1600 rpm for 4 minutes, incubated at 58°C for 5 minutes and then placed on the magnet after which 50 µl of supernatant was removed and discarded.

Thereafter, the plate was taken off the magnet, and 5.75 µl from elution master mix comprising 7.125 µl enrichment elution buffer 1 (EE1) and 0.375 µl sodium hydroxide was added to each well, shaking was done at 1800 rpm for 2 minutes and incubated at room temperature for 2 minutes. The plate was placed on the magnetic stand, and 5.25 µl of supernatant was transferred to a new plate, after which 1 µl of elute target buffer (ET2) was added and thoroughly mixed.

The enriched library was then amplified by mixing 1.25 µl of PCR primer cocktail (PPC) with 5 µl of enhanced PCR mix (EPM) in a thermocycler set to the following: 95°C for 5 minutes for step 1, 12 cycles of 98°C for 10 seconds, 60°C for 30 seconds and 72°C for 1 minute for step 2, 72°C for 5 minutes for step 3 and a temperature hold at 4°C.

After the amplification, 22.5 µl of AMPure XP beads were added to samples in each well, thorough mixing was done, shaking at 1800 rpm for 2 minutes was carried out and further incubated at room temperature for 5 minutes. Further, the plate was placed on the magnetic stand for 2 minutes, and 35 µl of the supernatant was discarded. While on the magnet, 200 µl of freshly prepared 80% ethanol was added to each well and discarded after 30 seconds. After two washes, the plate was kept at room temperature for 5 minutes to evaporate any remaining ethanol. After that, the plate was removed from the magnet and 32 µl of RSB was added to resuspend it, followed by 2 minutes of shaking at 1800 rpm and 2 minutes of incubation at room temperature. After 2 minutes on the magnet, 30 µl of the supernatant (clean enriched library) was transferred to the wells of a new plate.

3.5.5.7 DNA high sensitivity assay (for Quantification of DNA library)

Before use, all reagents were brought to room temperature for 30 minutes. To begin, 15 µl of High Sensitivity dye concentrate was pipetted into the High Sensitivity DNA gel matrix vial after brief vortexing. The dye concentrate was stored again at 4 °C in the dark.

The tube was capped, thoroughly vortexed and visually inspected to ensure gel and dye were properly mixed. The gel-dye mix was completely transferred to the top receptacle of a spin filter, and the tube was centrifuged at 6000 rpm for 15 minutes at room temperature.

After allowing the gel-dye mix to equilibrate to room temperature for 30 minutes, a new High Sensitivity DNA chip was taken out of its sealed bag. 9 µl of the gel-dye mix was pipetted and dispensed into the bottom of the well which was marked (G). The chip priming station was then closed after a 60-second timer had been set and the plunger had been set at 1 ml. The syringe plunger was pushed down until the clip caught it. The plunger was visually inspected until it moved to at least the 0.3 ml mark after 60 seconds, and then carefully pulled back to

the 1 ml point using the clip release mechanism. The chip priming station was then opened, and each of the additional wells labelled with a 9.0 l gel-dye mix was added (G).

Further, 5 µl of the DNA marker was pipetted into the well with a ladder symbol and each of the 11 sample wells. After, 1 µl of the ladder was pipetted into the well with the ladder symbol. The samples (cleaned enriched library) were then put to the sample wells (1 µl of each sample). On an IKA vortex mixer, the chip was vortexed for 60 seconds at 2400 rpm. The chip was carefully inserted into the receptacle of the Agilent 2100 Bioanalyzer instrument, the proper assay was chosen from the assay menu, and the chip run began. For use, the average library size (bp) and the Molarity (nM) for each library were recorded.

3.5.5.8 Library qPCR

Using the concentrations from the high sensitivity bioanalyzer, qPCR was conducted to further quantify libraries to obtain proper concentrations that will help in library loading. The libraries were first diluted 1:1000 so that they were within the range of the qPCR standards (20-0.0002 pm). The total volume of each reaction was 10 µl consisting of 1.8 µl of nuclease-free water, 2X KAPA SYBR FAST qPCR Master Mix with primers, 0.2 µl of 50X ROX High and 2 µl of the template (diluted library). The samples were run in duplicates. Six standards (1-6) of concentrations 20 pM, 2 pM, 0.2 pM, 0.02 pM, 0.002 pM and 0.0002 pM, as well as nuclease-free water as the negative control, were run simultaneously with each reaction. The PCR was run on a 7300 Real-time PCR system (Applied Biosystems, USA) with cycling conditions as follows: 1 cycle of the first hold at 95°C for 5 minutes and 35 cycles of the second hold at 95°C for 30 seconds and 60°C for 45 minutes (fluorescence read and data acquisition stage). ROX was chosen as the passive reference, SYBR as reporter and ROX as the quencher.

3.5.5.9 Library Pooling for loading

Upon obtaining concentrations from the qPCR run, the average concentrations from the qPCR run were computed together with fragment sizes from high sensitivity bioanalyzer run per sample. To ensure an equal representation of each sample, all samples were normalized to the same 2 nm concentration. Since the concentration of samples from qPCR was pico-mole, the sample concentrations were converted to nano-mole. With the needed volume of resuspension buffer, sample concentrations were standardized. The total amount of resuspension buffer required to normalize each sample to 2 nm was added to a 1.5 mL microcentrifuge tube, along with the equivalent volume for each sample. The tube was then vortexed and centrifuged.

3.5.5.10 Pool qPCR

A confirmatory qPCR was done to further confirm the concentration of the pool of 2 nM.

An aliquot of the pool was first diluted to 1:1000 so that they were within the range of the qPCR standards (20-0.0002 pm). The total volume of each reaction was 10 µl consisting of 1.8 µl of nuclease-free water, 2X KAPA SYBR FAST qPCR Master Mix with primers, 0.2 µl of 50X ROX High and 2 µl of the template (diluted pooled library). The samples were run in duplicates. Six standards (1-6) of concentrations 20 pM, 2 pM, 0.2 pM, 0.02 pM, 0.002 pM and 0.0002 pM, as well as nuclease-free water as the negative control, were run simultaneously with each reaction. The PCR was run on a 7300 Real-time PCR system (Applied Biosystems, USA) with cycling conditions as follows: 1 cycle of the first hold at 95°C for 5 minutes and 35 cycles of the second hold at 95°C for 30 seconds and 60°C for 45 minutes (fluorescence read and data acquisition stage). ROX was chosen as the passive reference, SYBR as reporter and ROX as the quencher.

3.5.5.11 Loading the Illumina iSeq sequencer

Dilution of PhiX (product of bacteriophage Phi X174) was done from 10 nM to the same concentration as the pool by the addition of resuspension buffer. This was briefly vortexed and centrifuged after which 2% PhiX in a total pool volume of 10 µl was made by adding PhiX to the pool. The pool with 2% PhiX was further diluted to 100 pM and then to 60 pM. Finally, 20 µl of the diluted pool with 2% PhiX was loaded into the sample compartment of the thawed iSeq v3 cartridge which was then loaded onto the Illumina iSeq 100 sequencer.

3.6 Data Analysis

Descriptive statistics such as frequency, percentage and bar graphs were used to describe the data. Association between infection with human demographic characteristics, occupational exposure, clinical signs and symptoms was determined using Chi-square or Fisher's exact test where necessary. Generalized Linear Mixed Model (GLMM) with Negative binomial distribution was fitted for Tick count (dependent variable) with animal characteristics, gender of the animal, age of animal, country of origin of animal, vaccination status and body part where ticks were sampled. Multiple comparison test between the levels of exposure variables was performed using Tukey contrasts with single-step P-value adjustment.

Statistical analysis was done using STATA version 4.1.0. Infection rates in the tick species were estimated using Version 2.0.1 January 2002 of the PoolScreen 2.0. Software. This was done at a 95 % confidence interval (Katholi & Unnasch, 2006). The phylogeny was constructed in MEGA X and visualised in FigTree.

CHAPTER 4

RESULTS

4.1 Distribution of Tick species

A total of 705 ticks comprising 345 females and 360 males were collected from cattle (n=188) and horses (n=11). Three tick genera (*Hyalomma, Amblyomma* and *Rhipicephalus*) were observed in the study. The predominant tick species was *Hyalomma rufipes* (n=290, 41.13%), followed by *Amblyomma variegatum* (n=157, 22.27%) with the least being *Rhipicephalus sanguineus* (n=1, 0.14%) (Figure 3).

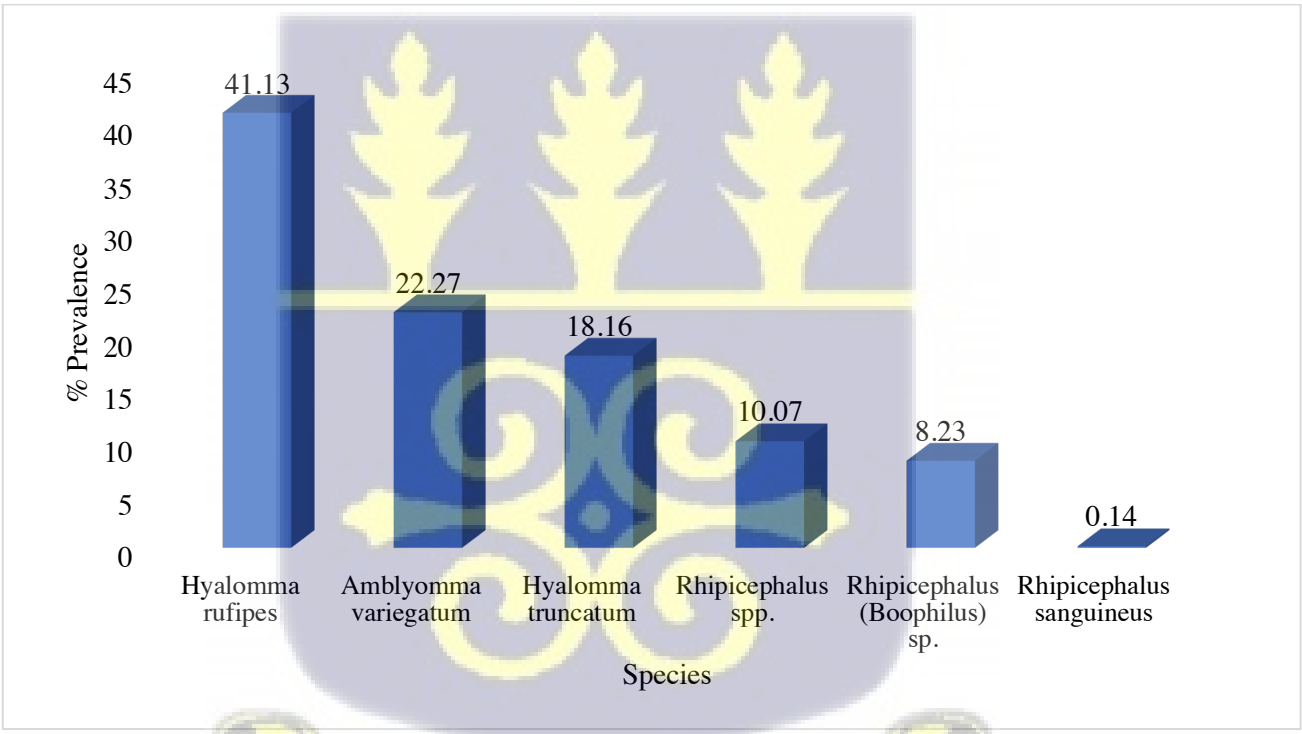

Figure 4: Distribution of tick species from the study sites

4.2 Preferred part of tick attachment on the animal host

From the 705 ticks collected, it was generally observed that the preferred points of attachment on the animals sampled were Anal (n=480, 68.08%), Udder/Scrotum (n=101, 14.33%), Chest (n=91, 12.91%), Leg/Thigh (n=16, 2.27%), Head/Neck (n=12, 1.70%) and Abdomen (n=5, 0.71%) (Table 1).

The preferred point of attachment on cattle was found to be predominantly Anal (n=469, 68.37%) with the least site being the Abdomen (n=5, 0.73%). The predominant species *Hyalomma rufipes* was mostly attached to the Anal region (n=258, 95.20%). The second most occurring species *Amblyomma variegatum* was found to be attached mostly to the Anal (n=57, 36.30%) followed by the Chest (n=47, 29.94%) and Udder/Scrotum (n=46, 29.30%). Furthermore, *Hyalomma truncatum* had a preference for Anal (n=73, 57.03%) followed by Udder/Scrotum (n=31, 24.22%) and Chest n=22, 17.19%).

From 19 ticks collected from Horses, the attachment sites were observed to be Anal (n=11, 57.90%), Udder/Scrotum (n=7, 36.84%) and Chest (n=1, 5.26%). Only *Hyalomma rufipes* however, were identified to infest the sampled horses.

Table 1: Occurrence of tick species sampled from the various body parts of livestock

		Site of attachment on Host						Total
Tick species	Host	Udder/Scrotum (%)	Anal (%)	Chest (%)	Abdomen (%)	Head/Neck (%)	Leg/Thigh (%)	
Hyalomma rufipes	Cattle	5 (1.84)	258 (95.20)	1 (0.37)	1 (0.37)	4 (1.48)	2 (0.74)	271
	Horse	7 (36.84)	11 (57.90)	1 (5.26)	0	0	0	19
Amblyomma variegatum	Cattle	46 (29.30)	57 (36.30)	47 (29.94)	0	4 (2.55)	3 (1.91)	157
Hyalomma truncatum	Cattle	31 (24.22)	73 (57.03)	22 (17.19)	0	1 (0.78)	1 (0.78)	128
Rhipicephalus spp.	Cattle	10 (14.08)	51 (71.83)	6 (8.45)	2 (2.82)	1 (1.41)	1 (1.41)	71
Rhipicephalus (*Boophilus*) sp.	Cattle	2 (3.45)	29 (50)	14 (24.13)	2 (3.45)	2 (3.45)	9 (15.52)	58
Rhipicephalus sanguineus	Cattle	0	1 (100)	0	0	0	0	1
Total		101 (14.33)	480 (68.08)	91 (12.91)	5 (0.71)	12 (1.70)	16 (2.27)	705

Table 2: Risk factor analysis of Ticks burden of livestock using Generalized mixed model

	Estimate	SE	z	p-value
Gender of Animal				
Male vs. Female	0.06749	0.2564	0.263	0.792
Age of Animal				
>11years vs ≤3years	0.5309	0.2238	2.372	0.0438 *
4-10years vs ≤3years	0.3309	0.1253	2.641	0.0209 *
4-10years vs >11years	-0.2	0.219	-0.913	0.623
Country of origin				
Ghana vs Burkina Faso	-0.05489	0.29258	-0.188	0.9974
Mali vs Burkina Faso	-1.43174	0.81457	-1.758	0.2715
South Africa vs Burkina Faso	-1.07669	0.37863	-2.844	0.0199 *
Mali vs Ghana	-1.37685	0.84228	-1.635	0.3348
South Africa vs Ghana	-1.0218	0.42183	-2.422	0.0645
South Africa vs Mali	0.35505	0.83157	0.427	0.9713
Vaccination status				
Yes vs No	0.06749	0.2564	0.263	0.792
Animal Body Part				
Anal vs Abdomen	0.6779	0.3084	2.198	0.2095
Armpit vs Abdomen	-0.1101	0.3881	-0.284	0.9997
Chest vs Abdomen	0.294	0.3236	0.909	0.9346
Ear vs Abdomen	-0.2413	0.4496	-0.537	0.9935
Groin vs Abdomen	0.1331	0.3246	0.41	0.9982
Armpit vs Anal	-0.788	0.2698	-2.921	0.0339 *
Chest vs Anal	-0.3839	0.1382	-2.777	0.0510.
Ear vs Anal	-0.9192	0.3867	-2.377	0.1418
Groin vs Anal	-0.5448	0.1313	-4.148	<0.001 ***
Chest vs Armpit	0.4041	0.2844	1.421	0.6811
Ear vs Armpit	-0.1312	0.4645	-0.283	0.9997
Groin vs Armpit	0.2432	0.2876	0.846	0.9513
Ear vs Chest	-0.5353	0.4053	-1.321	0.7445
Groin vs Chest	-0.1609	0.1702	-0.945	0.9233
Groin vs Ear	0.3744	0.4019	0.932	0.9276

The asterisk (*) in the p-value column of the table is an indication of statistical significance (p<0.0

4.3 Prevalence of *Rickettsia* in tick species

It was observed that in the Upper East Region, more ticks collected (62.67%) were infected with *Rickettsia* as compared to ticks from Greater Accra (42.13%) (Figure 4). Furthermore, slightly more ticks were infected with *Rickettsia africae* in the Upper East Region (91.8%) than Greater Accra (91.38%) (Figure 5).

Even though *Amblyomma variegatum* was the second most prevalent species identified, the highest *Rickettsia* infection rate of 80.89% (95% CI, 73.25-87.23) was recorded in this species. This was followed by an infection rate of 63.45% (95% CI, 56.98-69.60) in *Hyalomma rufipes* (most prevalent species) and the least infection rate of 5.63% (95% CI, 1.46-13.86) in *Rhipicephalus* spp. (Table 3).

Furthermore, *Amblyomma variegatum* had a *Rickettsia africae* infection rate of 98.43% (95% CI, 94.58-99.81), followed by an infection rate of 94.02% (95% CI, 89.30-97.16) in *Hyalomma rufipes* (Table 3).

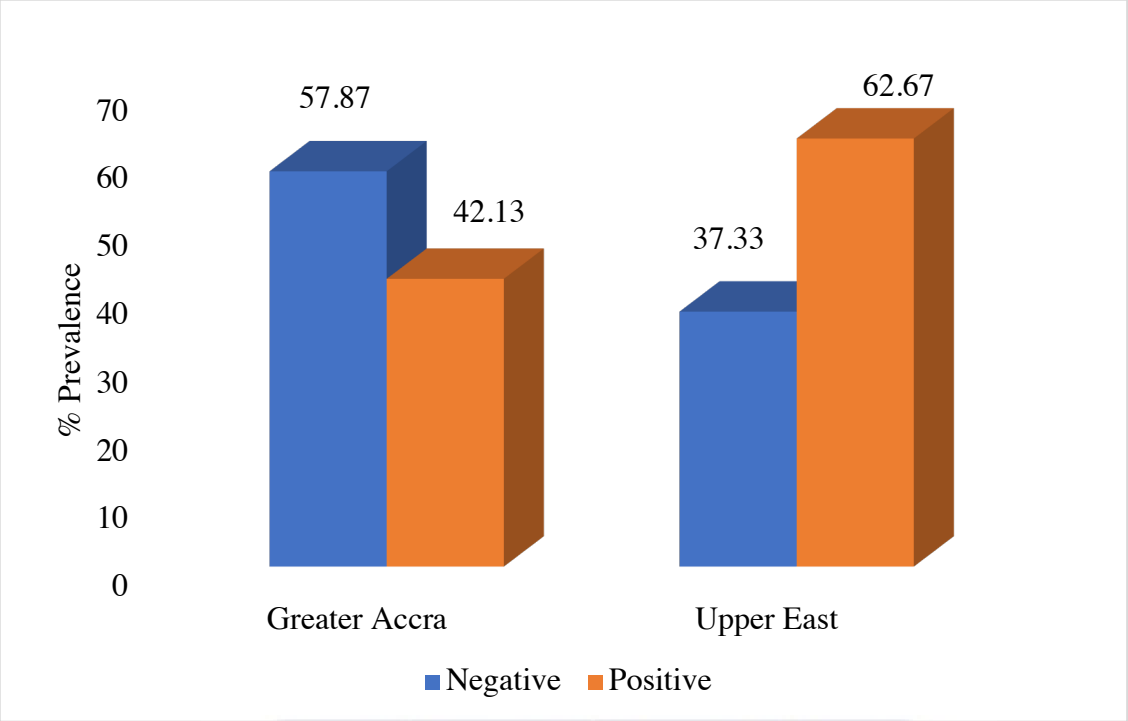

Figure 5: *Rickettsia* detected in tick samples

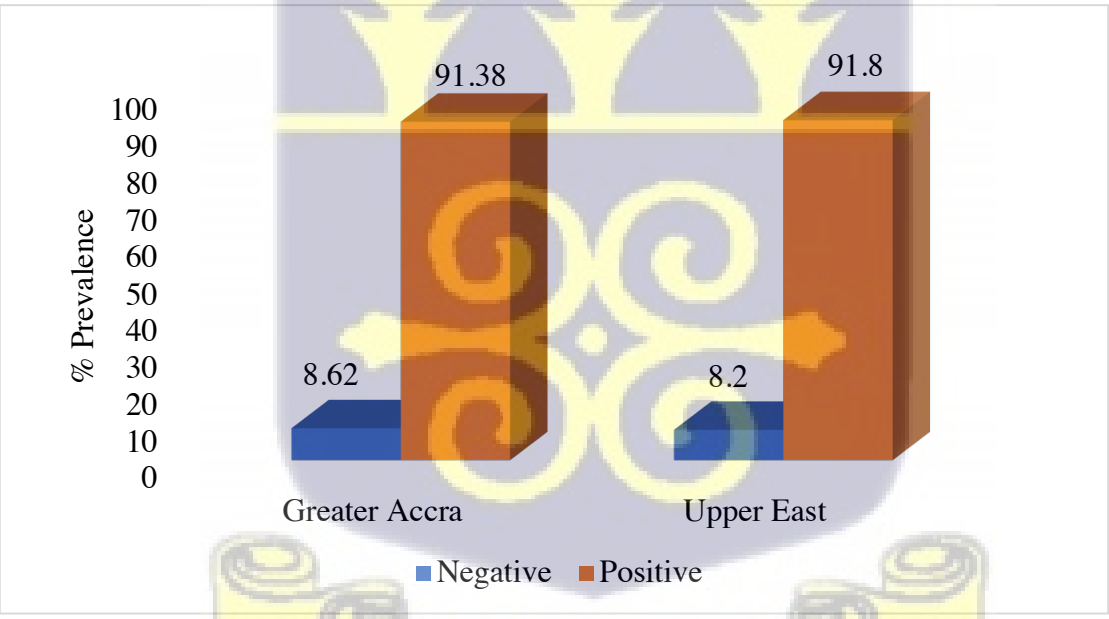

Figure 6: *Rickettsia africae* detected in tick samples

Table 3: Prevalence of *Rickettsia* and *Rickettsia africae* in sampled tick species

Tick Species	*Rickettsia*-positive			*R. africae*	
	No. of individual ticks tested	No. positive	Prevalence (95%CI)	No. positive	Prevalence
Amblyomma variegatum	157	127	80.89 (73.25-87.23)	125	98.43 (94.58-99.81)
Hyalomma rufipes	290	184	63.45 (56.98-69.60)	173	94.02 (89.30-97.16)
Hyalomma truncatum	128	33	25.78 (17.82-35.00)	26	78.79 (60.52-91.50)
Rhipicephalus (*Boophilus*) sp.	58	9	15.52 (6.93-27.97)	2	22.22 (2.92-58.86)
Rhipicephalus spp.	71	4	5.63 (1.46-13.86)	1	25.0 (0.88-77.74)
Rhipicephalus sanguineus	1	0	0	0	0
Total	**705**	**357**		**327**	

4.4 Prevalence of CCHFV in tick species

Out of the 705 tick samples, CCHFV infection rates of 0.78% (95% CI, 0.02-3.96), 0.69% (95% CI, 0.08-2.4) and 0.64% (95% CI, 0.02-3.24) were recorded in *Hyalomma truncatum*, *Hyalomma rufipes* and *Amblyomma variegatum* respectively (Table 4). No infection was detected in the *Rhipicephalus* species. All the positive CCHFV ticks were collected from the Upper East Region with none from the Greater Accra Region.

Table 4: Prevalence of CCHFV in sampled tick species

Tick Species	*CCHFV*		
	No. of individual ticks tested	No. positive	Prevalence (95%CI)
Amblyomma variegatum	157	1	0.64 (0.02-3.24)
Hyalomma rufipes	290	2	0.69 (0.08-2.4)
Hyalomma truncatum	128	1	0.78 (0.02-3.96)
Rhipicephalus (*Boophilus*) sp.	58	0	0
Rhipicephalus spp.	71	0	0
Rhipicephalus sanguineus	1	0	0
Total	**705**	**4**	

4.5 Whole-genome sequencing and Phylogeny

Nucleic acid extracts that tested positive by PCR for CCHFV were further taken through whole-genome sequencing using Next Generation Sequencing approach. A total of 4 samples were sequenced, out of which a strain (T-826_GHA/2021) was recovered from one (Figure 7)

G

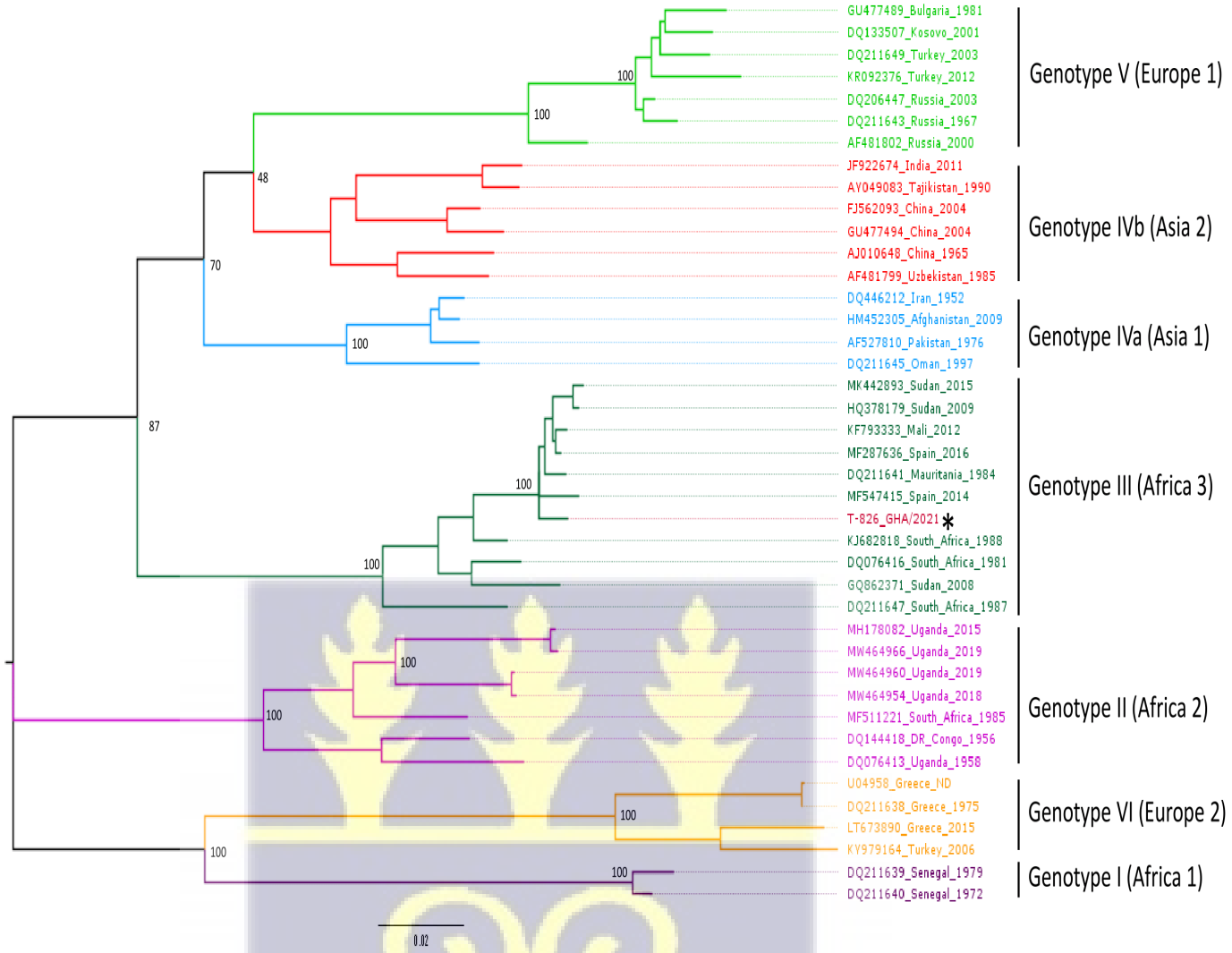

Figure 7: Maximum likelihood phylogeny of CCHF viruses from different geographical origins based on the complete coding sequence of the S segment.

Strains are labelled with their NCBI accession numbers, country of origin and date of collection or 'ND' for samples with no dates. Node numbers represent bootstrap values and scale represents the number of nucleotide substitutions per site. The strain from the current study is denoted with an asterisk '*'. The phylogeny was tested with 1000 bootstrap replicates.

4.6 Seroprevalence of *Rickettsia* and CCHFV in Human blood samples

A total of 120 human sera samples were analysed for *Rickettsia* exposure using an in-house ELISA. Sixty (60) were from Greater Accra and the other 60 from Upper East Region. Generally (n=120), it was observed that 33 (27.50%) were IgG positive for Spotted Fever Group (SFG) and 5 (4.17%) IgG positive for Typhus Group (TG) (Figure 8).

With sera samples from Greater Accra (n=60), it was observed that SFG IgG positives were 21 (35%) whereas TG IgG positives were 4 (6.67%). Sera samples from the Upper East Region (n=60) showed 12 (20%) positives for SFG IgG whereas TG IgG was 1 (1.67%) positive.

Using the Magpix assay, CCHFV IgG was detected in the 120 human sera. It was observed that samples were IgG positive for the following targets; CCHFV Gn (n=38, 31.67%), CCHFV NP (n=20, 16.67%) and CCHFV NP & CCHFV Gn (n=8, 6.67%) (Figure 9).

Participants in the study were administered a questionnaire to determine association of demographic factors (Table 5), risk factors (Table 6) and clinical symptoms (Table 7) with exposure to CCHFV and *Rickettsia*. Only cutting the throat (p=0.025) of an animal was significantly associated with exposure to CCHFV (Table 6). On another hand, the presence of Rash (p=0.01) on an individual was significantly associated with exposure to *Rickettsia*.

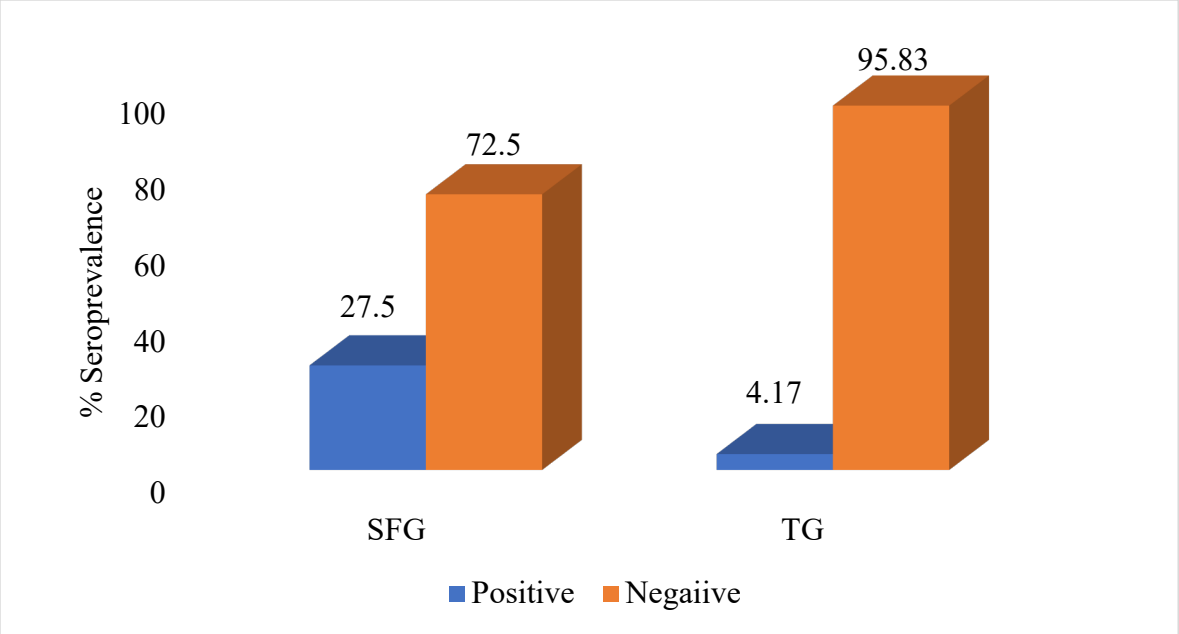

Figure 8: Seroprevalence of *Rickettsia* in human samples

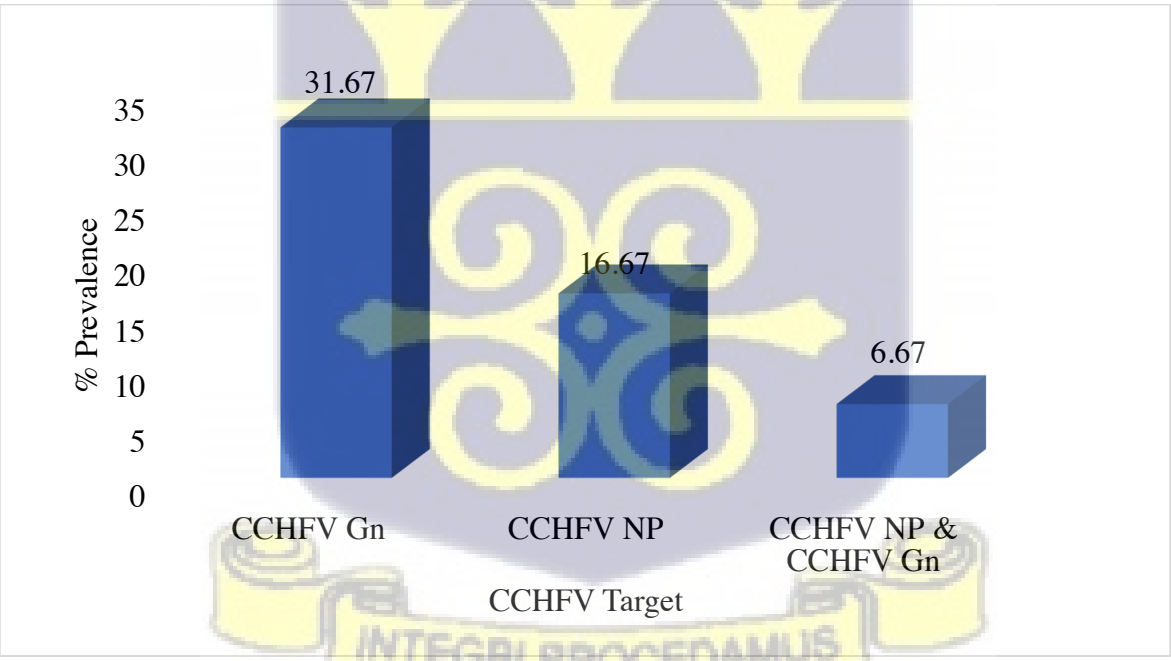

Figure 9: Seroprevalence of CCHFV in human samples

Table 5: Demographic factors associated with exposure to CCHFV and *Rickettsia*

	Total N(%)	**CCHF +ve n(%)**	**CCHF -ve n(%)**	***p-value***	**Rick +ve n(%)**	**Rick -ve n(%)**	***p-value***
Gender							
Male	105 (87.50)	45 (42.46)	60 (57.14)	0.582	30 (28.57)	75 (71.43)	0.758
Female	15 (12.50%)	5 (33.33)	10 (66.67)		3 (20.00)	12 (80.00)	
Age group							
18-25	13 (10.83)	6 (46.15)	7 (73.85)		2 (15.38)	11 (84.62)	
26-35	34 (28.33)	13 (38.24)	21 (61.76)		8 (23.53)	26 (76.47)	
36-45	29 (24.17)	14 (48.28)	15 (51.72)	0.920	7 (24.14)	22 (75.86)	0.401
46-55	29 (24.17)	11 (37.93)	18 (62.07)		9 (31.03)	20 (68.97)	
56 and above	15 (12.50)	6 (40.00)	9 (60.00)		7 (46.67)	8 (53.33)	

Table 6: Risk factors associated with exposure to CCHFV and *Rickettsia*

	Total N(%)	**CCHF +ve n(%)**	**CCHF -ve n(%)**	***p-value***	**Rick +ve n(%)**	**Rick -ve n(%)**	***p-value***
Years cared for animals							
Less than a year	3 (2.50)	1 (33.33)	2 (66.67)		0	3 (100)	
1 to 10	38 (31.67)	13 (34.21)	25 (65.79)	0.530	11 (28.95)	27 (71.05)	0.785
>11	79 (65.83)	36 (45.57)	43 (54.43)		22 (27.85)	57 (72.15)	
Live animal-cattle							
Yes	88 (73.33)	39 (44.32)	49 (55.68)	0.404	25 (28.41)	63 (71.59)	0.819
No	32 (26.67)	11 (34.38)	21 (65.63)		8 (25.00)	24 (75.00)	
Animal part-cattle							
Yes	24 (20.00)	9 (37.50)	15 (762.50)	0.817	6 (25.00)	18 (75.00)	1.000
No	96 (80.00)	41 (42.71)	55 (57.29)		27 (28.13)	69 (71.88)	
Live animal-sheep							
Yes	52 (43.33)	21 (40.38)	31 (59.62)	0.853	14 (26.92)	38 (73.08)	1.000
No	68 (56.67)	29 (42.65)	39 (57.35)		19 (27.94)	49 (72.06)	
Animal part-sheep							
Yes	22 (18.33)	10 (45.45)	12 (54.55)	0.812	6 (27.27)	16 (72.73)	1.000
No	98 (81.67)	40 (40.82)	58 (59.18)		27 (27.55)	71 (72.45)	
Live animal-goat							

Yes	55 (45.83)	20 (36.36)	35 (63.64)	0.353	15 (27.27)	40 (72.73)	1.000
No	65 (54.17)	30 (46.15)	35 (53.85)		18 (27.69)	47 (72.31)	
Animal part-goat							
Yes	21 (17.50)	11 (52.38)	10 (47.62)	0.332	6 (28.57)	15 (71.43)	1.000
No	99 (82.50)	39 (39.99)	60 (60.61)		27 (27.27)	72 (72.73)	
Live animal-pigs							
Yes	18 (15.00)	5 (27.78)	13. (72.22)	0.300	4 (22.22)	14 (77.78)	0.777
No	102 (85.00)	45 (44.12)	57 (55.88)		29 (28.43)	73 (71.57)	
Animal part-pigs							
Yes	5 (4.17)	2 (40.00)	3 (60.00)	1.000	1 (20.00)	4 (80.00)	1.000
No	115 (95.83)	48 (41.74)	67 (58.26)		32 (27.83)	83 (72.17)	
Cared for Live animals							
Yes	118 (98.33)	49 (41.53)	69 (58.47)	1.000	33 (27.97)	85 (72.03)	1.000
No	2 (1.67)	1 (50.00)	1 (50.00)		0	2 (100)	
Cut animal throats							
Yes	48 (40.00)	14 (29.17)	34 (70.83)	0.025*	14 (29.17)	34 (70.83)	0.835
No	72 (60.00)	36 (50.00)	36 (50.00)		19 (26.39)	53 (73.61)	
Skinned animals							
Yes	38 (31.67)	15 (39.47)	23 (60.53)	0.843	11 (28.95)	27 (71.05)	0.829
No	82 (68.33)	35 (42.68)	47 (57.32)		22 (26.83)	60 (73.17)	

Butchered carcasses							
Yes	31 (25.83)	9 (29.03)	22 (70.97)	0.138	9 (29.03)	22 (70.97)	0.819
No	89 (74.17)	41 (46.07)	48 (53.93)		24 (26.97)	65 (73.03)	
Collected animal blood							
Yes	33 (27.50)	12 (36.36)	21 (63.64)	0.537	10 (30.30)	23 (69.70)	0.655
No	87 (72.50)	38 (43.68)	49 (56.32)		23 (26.44)	64 (73.56)	
Handles animal parts							
Yes	63 (52.50)	23 (36.51)	40 (63.49)	0.258	19 (30.16)	4 (69.84)	0.543
No	57 (47.50)	27 (47.37)	30 (52.63)		14 (24.56)	43 (75.44)	
Care for animals outside of work							
Yes	72 (60.00)	27 (37.50)	45 (62.50)	0.265	18 (25.00)	54 (75.00)	0.533
No	48 (40.00)	23 (47.92)	25 (52.08)		15 (31.25)	33 (68.75)	

The Asterisk (*) in the p-value column of the table is an indication of statistical significance ($p<0.05$)

Table 7: Clinical symptoms associated with exposure to CCHFV and *Rickettsia*

	Total N(%)	**CCHF +ve n(%)**	**CCHF -ve n(%)**	***p-value***	**Rick +ve n(%)**	**Rick -ve n(%)**	***p-value***
Headache							
Yes	69 (57.50)	30 (43.48)	39 (56.52)	0.710	22 (31.88)	47 (68.12)	0.224
No	51 (42.50)	20 (39.22)	31 (60.78)		11 (21.57)	40 (78.43)	
Fever							
Yes	52 (43.33)	25 (48.08)	27 (51.92)	0.263	17 (32.69)	35 (67.31)	0.306
No	68 (56.67)	25 (36.76)	43 (63.24)		16 (23.53)	52 (76.47)	
Malaise							
Yes	63 (52.50)	28 (44.44)	35 (55.56)	0.580	19 (30.16)	44 (69.84)	0.543
No	57 (47.50)	22 (38.60)	35 (61.40		14 (24.56)	43 (75.44)	
Chills							
Yes	31 (25.83)	17 (54.84)	14 (45.16)	0.095	11 (35.48)	20 (64.52)	0.253
No	89 (74.17)	33 (37.08)	56 (62.92)		22 (24.72)	67 (75.28)	
Cough							
Yes	33 (27.50)	14 (42.42)	19 (57.58)	1.000	12 (36.36)	21 (63.64)	0.252
No	87 (72.50)	36 (41.38)	51 (58.62)		21 (24.14)	66 (75.86)	
Sore throat							
Yes	20 (16.67)	5 (25.00)	15 (75.00)	0.136	6 (30.00)	14 (70.00)	0.788

No	100 (83.33)	45 (45.00)	55 (55.00)		27 (27.00)	73 (73.00)	
Rash							
Yes	11 (9.24)	6 (54.55)	5 (45.45)	0.357	7 (63.64)	4 (36.36)	0.010*
No	108 (90.76)	43 (39.81)	65 (60.19)		26 (24.07)	82 (75.93)	
Back pain							
Yes	55 (45.83)	22 (40.00)	33 (60.00)	0.853	12 (21.82)	43 (78.18)	0.224
No	65 (54.17)	28 (43.08)	37 (56.92)		21 (32.31)	44 (67.69)	
Joint pain							
Yes	73 (60.83)	34 (46.58)	39 (53.42)	0.19	21 (28.77)	52 (71.23)	0.835
No	47 (39.17)	16 (34.04)	31 (65.96)		12 (25.53)	35 (74.47)	
Abdominal pain							
Yes	34 (28.33)	13 (38.24)	21 (61.76)	0.685	11 (32.35)	23 (67.65)	0.499
No	86 (71.67)	37 (43.02)	49 (56.98)		22 (25.58)	64 (74.42)	
Nausea							
Yes	22 (18.33)	12 (54.55)	10 (45.45)	0.232	8 (36.36)	14 (63.64)	0.303
No	98 (81.67)	38 (38.78)	60 (61.22)		25 (25.51)	73 (74.49)	
Vomiting							
Yes	11 (9.17)	6 (54.55)	5 (45.45)	0.523	4 (36.36)	7 (63.64)	0.492
No	109 (90.83)	44 (40.37)	65 (59.63)		29 (26.61)	80 (73.39)	

Diarrhoea							
Yes	12 (10.00)	3 (25.00)	9 (75.00)	0.355	2 (16.67)	10 (83.33)	0.508
No	108 (90.00)	47 (43.52)	61 (56.48)		31 (28.70)	77 (71.30)	
Weight loss							
Yes	31 (25.83)	17 (54.84)	14 (45.16)	0.095	12 (38.71)	19 (61.29)	0.160
No	89 (74.17)	33 (37.08)	56 (62.92)		21 (23.60)	68 (76.40)	
Admitted in hospital							
Yes	19 (15.83)	8 (42.11)	11 (57.89)	1.000	6 (31.58)	13 (68.42)	0.780
No	101 (84.17)	42 (41.58)	59 (58.42)		27 (26.73)	74 (73.27)	

The Asterisk (*) in the p-value column of the table is an indication of statistical significance ($p<0.05$)

CHAPTER 5

DISCUSSION

Ticks carry the widest range of infectious pathogens of any arthropod disease vectors, and they are the most important arthropod vectors of infections to humans and domestic animals worldwide (Colwell *et al*., 2011; Jongejan and Uilenberg 2004; Pfäffle *et al.* 2013). It has been shown that weather and environment have a considerable impact on arthropod disease vectors and their ability to transmit disease-causing pathogens (Githeko *et al*., 2000). Thus, under favourable conditions, ticks can effectively spread pathogens even to non-endemic regions.

5.1 Distribution of tick species

In this study, ticks of the genera *Amblyomma*, *Hyalomma* and *Rhipicephalus* were found to infest the animals sampled. Previous studies have suggested the predominance of *Amblyomma variegatum* in Ghana (Bell-Sakyi *et al.*, 1996; Walker & Koney, 1999). However, this study identified *Hyalomma rufipes* to be abundant on the animals sampled. *Hyalomma* is a known vector of CCHFV (Gargili *et al*., 2017) hence an increased population would mean an increased risk of pathogen transmission to present animals as well as humans nearby. Irrespective of the numbers of *Amblyomma variegatum*, these species greatly affect animal health (Stachurski, 2000) and transmit pathogens such as *Rickettsia africae* that cause African tick bite fever (Tomassone *et al*., 2018). Furthermore, *Rhipicephalus* species can also harbour and transmit pathogens including *Rickettsia* (Wikswo *et al.*, 2007). It is obvious that the identified tick species from the study areas are of veterinary and public health importance hence there is a need to control the tick populations to prevent disease spread and reduce the risk of zoonotic infections.

Generally, it was discovered in this study that majority of the ticks preferred the anal part of the animals. Further, a much higher percentage of *Hyalomma rufipes,* as compared to the other

tick species were found to reside at the anal region of the animals. This could be because most tick species look for a suitable attachment location on the animals where they can feed on the most blood without disturbance (Ogden *et al.*, 1998). Furthermore, tick attachment site selection is influenced by host body part features such as skin thickness, humidity, blood circulation, and de-ticking by grooming behaviour (Ogden *et al.*, 1998). Thus, the Anal region of the animals was most suitable for blood-feeding as seen in this study. From the findings, control efforts such as the use of acaricides should focus on the Anal, Udder/Scrotum and Chest regions of the animals to effectively control tick populations and in the process reduce the spread of tick-borne zoonotic pathogens.

5.2 Tick-borne pathogens in sampled ticks

Amblyomma variegatum has been identified as a significant vector for the transmission of rickettsial and viral infections (Akuffo *et al.*, 2016; Kelly *et al.*, 2010). Furthermore, *Amblyomma variegatum* is one of the most common and widespread ticks in tropical livestock, and it is a source of veterinary and public health concerns in Africa (Bournez *et al.*, 2015; Stachurski *et al.*, 2010). In this study, the highest *Rickettsia* infection rate of 80.89% was recorded in this species. This is in contrast with studies in Nigeria (Reye *et al.*, 2012) and Senegal (Sambou *et al.*, 2014) which identified *Rickettsia* infections in ticks to be 21.5% and 5.8% respectively. Further analysis showed that *Amblyomma variegatum* were highly infected with *R. africae* (98.43%). A. variegatum was previously assumed to be the only tick vector for rickettsial DNA in Sub-Saharan Africa(Parola *et al.*, 2013). However, recent research has found rickettsial DNA in a variety of tick species (Chitanga *et al.*, 2021; Koka *et al.*, 2017; Mahlobo-Shwabede *et al.*, 2021) and this work confirms this. Previous studies in Ghana have identified the presence of *R. aeschlimannii* in ticks (unpublished data) and *R. felis* in febrile children (Sothmann *et al.*, 2017). It's worth noting that tick-borne spotted fever is the second

most prevalent cause of fever in travellers returning from Africa (Mediannikov *et al*., 2010). The presence and high occurrence of *R. africae* in the studied ticks suggests that African tick-bite fever could spread to humans, necessitating the establishment and maintenance of efficient control activities.

Ticks of the genus *Hyalomma* are considered to be the major vectors of CCHFV (Spengler & Estrada-Peña, 2018). However, in ticks of other genera that co-occur with *Hyalomma*, CCHFV transmission has been proven under laboratory settings (Spengler & Estrada-Peña, 2018). In this study, ticks of the genera *Hyalomma* and *Amblyomma* were identified to harbour CCHFV which can be compared to an earlier study in Ghana that resulted in the detection of the virus in both *Hyalomma excavatum* and *Amblyomma variegatum* ticks collected off slaughtered animals from the Kumasi abattoir (Akuffo *et al*., 2016). Livestock such as cattle, sheep and goats serve as amplifying hosts for the virus (Chisholm *et al*., 2012). With the high dependence on livestock in Ghana and the presence of suitable tick species, CCHFV is of great veterinary and public health importance. In this investigation, finding CCHFV in tick vectors places emphasis on the need for enhanced surveillance in communities frequently exposed to infected ticks, as well as blood and tissues of affected livestock.

5.3 Phylogeny

The strain recovered in the study T-826_GHA/2021 belongs to the third genotype (Africa 3) and clustered with other strains from Sudan (2008, 2009, 2015), Mali (2012), Spain (2014, 2016), Mauritania (1984) and South Africa (1981, 1987, 1988) with accession numbers GQ862371, HQ378179, MK442893, KF793333, MF547415, MF287636, DQ211641, DQ076416, DQ211647 and KJ682818 respectively (Figure 6). Phylogenetic analysis was based on complete open reading frames (ORF) of the S- segment of the CCHFV genome which is the most conserved at the nucleotide level (Ramírez de Arellano *et al*., 2017) and plays a

role in the encapsulation of viral RNA by forming ribonucleoprotein complexes (Han & Rayner, 2011; Kalkan-Yazıcı *et al*., 2021; Zivcec *et al*., 2015) and is important in assessing the topology of viruses (Deyde *et al*., 2006).

The strain recovered from this study shared 98.9% nucleotide identity with DQ211641_Mauritania_1984 and MF287636_Spain_2016 which suggests possible importation of the virus into the country. The movement of livestock infected with the CCHF virus (or uninfected livestock carrying ticks infected with the virus) via trade may be associated with some of the movement of virus genetic lineages within regions (Deyde *et al*., 2006). For instance, there is considerable movement of livestock into the country primarily from the border regions in the northern part of the country from Burkina Faso. Furthermore, migratory animals and birds have been implicated to be carriers of virus-infected ticks (Lindeborg *et al.*, 2012). Though certain studies have reported birds are not easily infected with CCHFV, ostriches and several ground-feeding birds in West Africa have been proven to be susceptible to infection, and are capable of moving infected ticks attached to them without becoming infected themselves (Lindeborg *et al*., 2012).

Synonymous and non-synonymous mutations were observed upon analysis. These differ in such a way that the former which involves no alteration in the amino acid sequences and are therefore 'silent' whereas the latter involves alteration of amino acid sequences of a protein via nucleotide mutations (Choudhuri, 2014). A total of 26 nucleotide substitutions classified as synonymous mutations were shared between the Ghana, Mauritania and Spain strains (i.e., DQ211641Mauritania_1984, MF287636_Spain_2016 and T-826_GHA/2021). However, protein level analysis revealed that at position 124, there was a change from Alanine to Serine resulting from substitution from guanine in Mauritania and Spain strains to thymine in the Ghana strain. At position 240, there was a change from Lysine to Arginine resulting from substitution from adenine in the Mauritania strain to guanine in the Ghana strain. Further, at

position 350, there was a change from Threonine to Serine resulting from substitution from cytosine in the Mauritania strain to guanine in the Ghana strain. In addition, at position 386, there was a change from Glycine to Serine resulting from substitution from guanine in Spain strain to adenine in the Ghana strain. These non-synonymous mutations occurred from nucleotide substitutions which resulted in changes in amino acid sequences. Although some studies suggest nucleotide differences can be an extrapolative factor in CCHF disease (Say Coskun & Asik, 2019), further functional characterization, however, would be required to ascertain the implications of these changes.

5.4 Seroprevalence of CCHFV and Rickettsia

Human CCHFV infections can cause severe haemorrhagic signs and have a high mortality rate of up to 30% (Whitehouse, 2004). CCHFV seroprevalence studies provide valuable information on the epidemiology of the diseases including risk factors and hot spots which ultimately help limit the spread of infections in many countries (Fajs *et al.*, 2014; Lwande *et al.*, 2012). As a result, CCHFV surveillance in human and vector populations allows researchers to track the risk of disease with potentially severe consequences in people (Drosten *et al.*, 2002). Even though the overall seroprevalence of CCHFV was 41.67%, it can be compared to a previous study in Ghana which found 5.7% of abattoir workers exposed to CCHFV (Akuffo *et al.*, 2016) and a study in Kenya that observed the seroprevalence of CCHFV to be 19% (Lwande *et al.*, 2012). Furthermore, it was observed that cutting the throat of an animal significantly increases the risk of CCHFV infection which shared similar assertions with findings by Gunes *et al.* (2009) suggesting exposure to blood and tissues of viraemic animals during slaughter as a possible source of infection. As such a way of preventing infections would be to wear protective gloves and exercise caution to avoid cuts whiles slaughtering an animal. This study highlights the risk of CCHFV infections within

Ghana, especially individuals in close contact with animals. Thus, it is important to educate owners of animals on the importance of adhering to personal protection from zoonotic infection.

The spotted fever group (SFG) and the typhus fever group (TG) are the two disease-associated groups of *Rickettsia* species (Salmon-Mulanovich *et al.*, 2019). The Spotted Fever Group Rickettsiae includes about 20 *Rickettsia* species (*R. rickettsii*, *R. conorii*, *R. africae*, and others), all of which are spread by ticks (Wood & Artsob, 2012). It is important to note that *R. felis* which is part of SFG is found in rat fleas of the genus *Xenopsylla* (Dieme *et al.*, 2015). *R. prowazekii* and *R. typhi*, the organisms responsible for epidemic and murine typhus, respectively, are members of the TG. *Rickettsia typhi* is most commonly seen in rodents and is spread by the rat flea *Xenopsylla cheopis* (Raoult & Roux, 1997). Humans become infected when their skin, respiratory system, or conjunctivae become contaminated with infected flea faeces (Philippe Parola & Raoult, 2006). *Rickettsia* species are found all over the world, but little is known about their epidemiology and health effects in Africa, with most serological studies focusing on IgG seroprevalence in Cameroon (Ndip *et al.*, 2004), South Africa (Berrian *et al.*, 2019; Simpson *et al.*, 2018), Kenya (Maina *et al.*, 2016; Thiga *et al.*, 2015), Djibouti (Horton *et al.*, 2016), Zimbabwe (Kelly & Mason, 1991), Tunisia (Kaabia *et al.*, 2006), Angola (Dupont *et al.*, 1995), Ivory Coast (Botros *et al.*, 1989), and Egypt (Botros *et al.*, 1989).

Rickettsioses are rarely evaluated in patients with undifferentiated febrile infections, and identification is difficult without confirmatory laboratory tests due to overlapping symptoms with other endemic diseases such as malaria, yellow fever and dengue fever (Barradas *et al.*, 2020). Rickettsioses commonly appear 1 to 2 weeks after being exposed to the agent with fever, malaise, headache, rash, nausea, and vomiting as the most common symptoms (Salmon-Mulanovich *et al.*, 2019). In this study, it was observed that more individuals were exposed to the SFG as compared to the TG. This suggests the circulation of different *Rickettsia* strains

since a previous study has reported the exposure to *R. felis* in febrile children (Sothmann *et al*., 2017) whereas this study confirms the presence of *R. africae* in sampled ticks. In addition, having rash was found to be significantly associated with exposure to *Rickettsia* in the study areas. With the overall *Rickettsia* exposure of 27.5% in the sampled humans in this study, there is a need to educate animal handlers on the risk of zoonotic pathogen exposure and enforce preventive measures.

5.5 Limitations

Since ticks were not sampled from all ecological zones, the study's findings do not represent the entire country. Also, pesticides used to treat the animals at the study sites may have influenced the true distribution of ticks collected. The tick samples were not sequenced to identify all the present *Rickettsia* strains. Furthermore, blood samples were not taken from livestock hence the difficulty in assessing livestock-tick-human transmission of pathogens to establish zoonosis.

5.6 Conclusions

In this study, *Hyalomma rufipes* was found to be the dominant tick species. However, a high infection rate of *R. africae* was detected in sampled *Amblyomma variegatum*. Nonetheless, it has been discovered that a variety of tick species can carry and transmit Rickettsia in Ghana. This study further reports the first whole-genome sequencing of CCHFV in tick species within Ghana obtained by analysing a small fragment in the S segment. Sequence analysis further corroborating findings, indicates that CCHFV from genotype III (Africa III) may be circulating in the Upper East Region of Ghana. Inhabitants within the study sites were also found to be exposed to both *Rickettsia* and CCHFV. This suggests a continuous increase in the risk of zoonotic tick-borne infections since livestock rearing is an integral component of major communities in Ghana. It is therefore imperative that nationwide surveillance is conducted to

establish the burden of tick-borne pathogens in livestock and human populations to create effective control and preventive strategies.

5.7 Recommendations

It will be essential to sample rodent populations at the study sites to determine the risk of pathogen transmission to inhabitants since some *Rickettsia* species identified in Ghana can be transmitted through fleas on rodents. Further, it would be beneficial to sample the blood of livestock from which ticks are picked to be able to establish the linkage between the livestock, ticks and human transmission. In addition, there is a need to conduct surveillance studies that examine the different *Rickettsia* strains circulating in Ghana to establish effective control strategies. Also, sampling from other ecological zones would have been ideal in assessing the risk of transmission of tick-borne diseases within the country.

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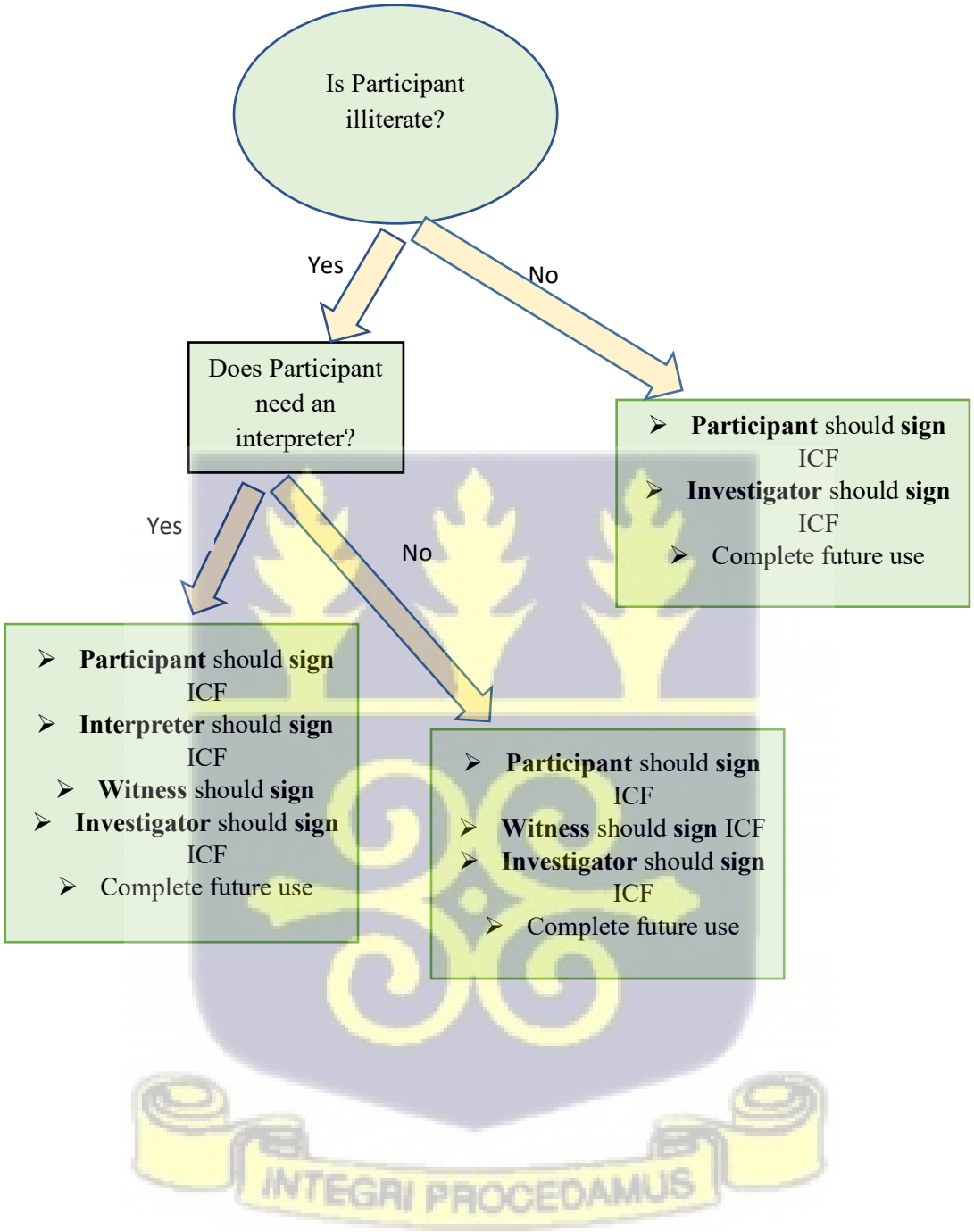
APPENDICES

Appendix I: Flow chart for consenting Eligible Participants



Appendix II: Participant Questionnaire

Ecological, Environmental, and Human Surveillance for
Tick-Borne Infections in Ghana

QUESTIONNAIRE

STUDY LABEL

attach label here

Did patient consent? ☐ **Yes** ☐ **No**

Did patient consent to future use of specimens? ☐ **Yes** ☐ **No**

Initials of interviewer ☐☐☐ **Questionnaire Date** ☐☐ – ☐☐ – ☐☐☐☐ (D D – M M – Y Y Y Y)

DEMOGRAPHIC DATA

1. Date of birth ☐☐ (D D) ☐☐☐ (M M) ☐☐☐☐ (Y Y Y Y) If date of birth unknown, estimate age in years ☐☐

2. Sex ☐ Male ☐ Female

3. Region ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

4. Village ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

OCCUPATIONAL EXPOSURE DATA

5. How long have you cared for live animals? ☐☐ years ☐☐ months

If greater than twelve months, skip to question 6.

5.1. Prior to caring for animals, what work did you do? ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

5.2. Did you have contact with animals in that job? ☐ Yes ☐ No ☐ Unknown

6. Which of these animals have you cared for in the last year?

	Live animals	Animal parts	None	Unknown
Cattle	☐	☐	☐	☐
Sheep	☐	☐	☐	☐
Goats	☐	☐	☐	☐
Other, please specify: ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐	☐	☐	☐	☐

	Live animals	Animal parts	None	Unknown
Pigs	☐	☐	☐	☐
Unknown	☐	☐	☐	☐

If all responses are 'None' or 'Unknown', please explain to the individual that he is ineligible for study.

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7. Which of the following have you been involved in the last three months?

Yes	No	Unknown		Yes	No	Unknown	
☐	☐	☐	Cared for live animals	☐	☐	☐	Collected animal blood
☐	☐	☐	Cut animals' throats	☐	☐	☐	Handled animal parts
☐	☐	☐	Skinned animals	☐	☐	☐	Cleaned up
☐	☐	☐	Butchered carcasses				

Other, please specify: ☐

8. Do you own or care for animals outside of work? ☐ Yes ☐ No ☐ Unknown

If 'No' or 'Unknown', skip to question 10.

9. Which animals do you own or care for outside of work?

Yes	No	Unknown		Yes	No	Unknown	
☐	☐	☐	Cattle	☐	☐	☐	Pigeons
☐	☐	☐	Sheep	☐	☐	☐	Bats
☐	☐	☐	Goats	☐	☐	☐	Other birds
☐	☐	☐	Pigs				
☐	☐	☐	Chickens	Other: ☐			

10. Have you been exposed to animal abortus in the last month? ☐ Yes ☐ No ☐ Unknown

CLINICAL SIGNS AND SYMPTOMS

11. Which of the following clinical signs and symptoms have you had in the last thirty days?

Yes	No	Unknown		Yes	No	Unknown	
☐	☐	☐	Headache	☐	☐	☐	Back pain
☐	☐	☐	Fever	☐	☐	☐	Joint pain
☐	☐	☐	Malaise	☐	☐	☐	Abdominal pain
☐	☐	☐	Chills	☐	☐	☐	Nausea
☐	☐	☐	Cough	☐	☐	☐	Vomiting
☐	☐	☐	Sore throat	☐	☐	☐	Diarrhea
☐	☐	☐	Rash	☐	☐	☐	Weight loss
☐	☐	☐	Other, please specify: ☐				

12. Have you been admitted to the hospital in the past year? ☐ Yes ☐ No ☐ Unknown

12.1. If 'Yes', for how long? ☐☐☐ days

12.2. What was the final diagnosis? ☐

SAMPLE COLLECTION

13. Was a blood sample collected for this study? ☐ Yes ☐ No ☐ Unk ☐ Refused

If 'No', explain why no sample was collected in comments.

Comments ☐

☐

Initials of interviewer ☐☐☐

Appendix III: Checklist for consenting

Title of Study: Ecological,Environmental, and Human Surveillance for Tick-Borne Infection in Ghana **LIST**	**CHECK**	**Remarks**
Study ID :		
1. Has a unique ID been assigned to the ICF?	Yes☐ ☐ No	
2.Has **future use** been indicated?	Yes☐ ☐ No	
3.Has **Participant** Signed future use of Sample?	Yes☐ ☐ No	
4. Has language in which participant was consented been indicated?	Yes☐ ☐ No	
5.Has **Participant** indicated Name or Initials?	Yes☐ ☐ No	
6.Has **Participant** indicated date of consenting?	Yes☐ ☐ No	
7.Has **Participant** Signed? If Yes, and **Witness** has not signed ICF, **Skip to Q22 to Q25**	Yes☐ ☐ No	
8.Has **Participant** Thumb printed ICF?	Yes☐ ☐ No	
9. If **Participant** Thumprinted, has **Witness** indicated the language consenting was done?	Yes☐ ☐ No	
10. Is the language indicated by the **Witness** the same as the Language the **Participant** was consented?	Yes☐ ☐ No	
11.Has Witness indicated name on ICF?	Yes☐ ☐ No	
12. Has **Witness** Signed the form?	Yes☐ ☐ No	
13. Has Witness indicated the date consenting was done?	Yes☐ ☐ No	
14. Is the date indicated by the **Participant and witness** the same ?	Yes☐ ☐ No	
15. Is the date indicated by the **witness** and **investigator** the same ?	Yes☐ ☐ No	
For only those who need interpreter,Answer Q16 to Q25, otherwise skip to Q22 to Q25		
16. Has an **interpreter** signed the ICF?	Yes☐ ☐ No	
17. Is the language of consenting indicated by the **interpreter** the same as the **Witness and Participant**?	Yes☐ ☐ No	
18.Has name of the **Interpreter** been indicated on ICF?	Yes☐ ☐ No	
19.Has **interpreter** indicated the date the consenting was done?	Yes☐ ☐ No	
20. Is the date of consenting recorded by the **interpreter** the same as that of the **Witness**, **Participant** and **Investigator** ?	Yes☐ ☐ No	
21. Has the interpreter indicated the contact details?	Yes☐ ☐ No	
22.Has investigator indicated name of the ICF?	Yes☐ ☐ No	
23.Has investigator signed the ICF?	Yes☐ ☐ No	
24. Has the investigator indicated date ICF was signed?	Yes☐ ☐ No	
25. Is the date indicated by the **Participant** and **investigator** the same ?	Yes☐ ☐ No	
Reviewer's Name: **Signature**:	**Date**:	

Appendix IV: Participant screening form

Title of Study:Ecological,Environmental, and Human Surveillance for Tick-Borne Infections in Ghana **SCREENING FORM**	
1. Age _____	
2.Do you handle animals	Yes☐ ☐ No
3.Type of work	Animal handler☐ Abattoir worker☐
4. Eligibility Status	Eligible ☐ Not Eligible☐
NB: If Particpant is ≥**18years** and **handle animals** then **Participant is Eligible**	

Appendix V: Animal vector questionnaire form

Ecological, environmental, and human Surveillance for tick-borne infections in Ghana

Animal ID #: ☐☐☐☐☐☐☐☐☐☐

Date: ____/____/_____

DEMOGRAHIC DATA

1. Sex ☐ Male ☐ Female
2. Age ☐☐☐
3. Species
   - ☐ Cattle
   - ☐ Sheep
   - ☐ Goats
   - ☐ Other, please specify _____
4. Country of origin
   - ☐ Burkina Faso
   - ☐ Republic of Togo
   - ☐ Chad
   - ☐ Cote D'ivoire
   - ☐ Ghana
   - ☐ Other, please specify _____
5. Region of origin _____
6. Has the animal been vaccinated? ☐ Yes ☐ No ☐ Unknown
7. Vaccine name _____

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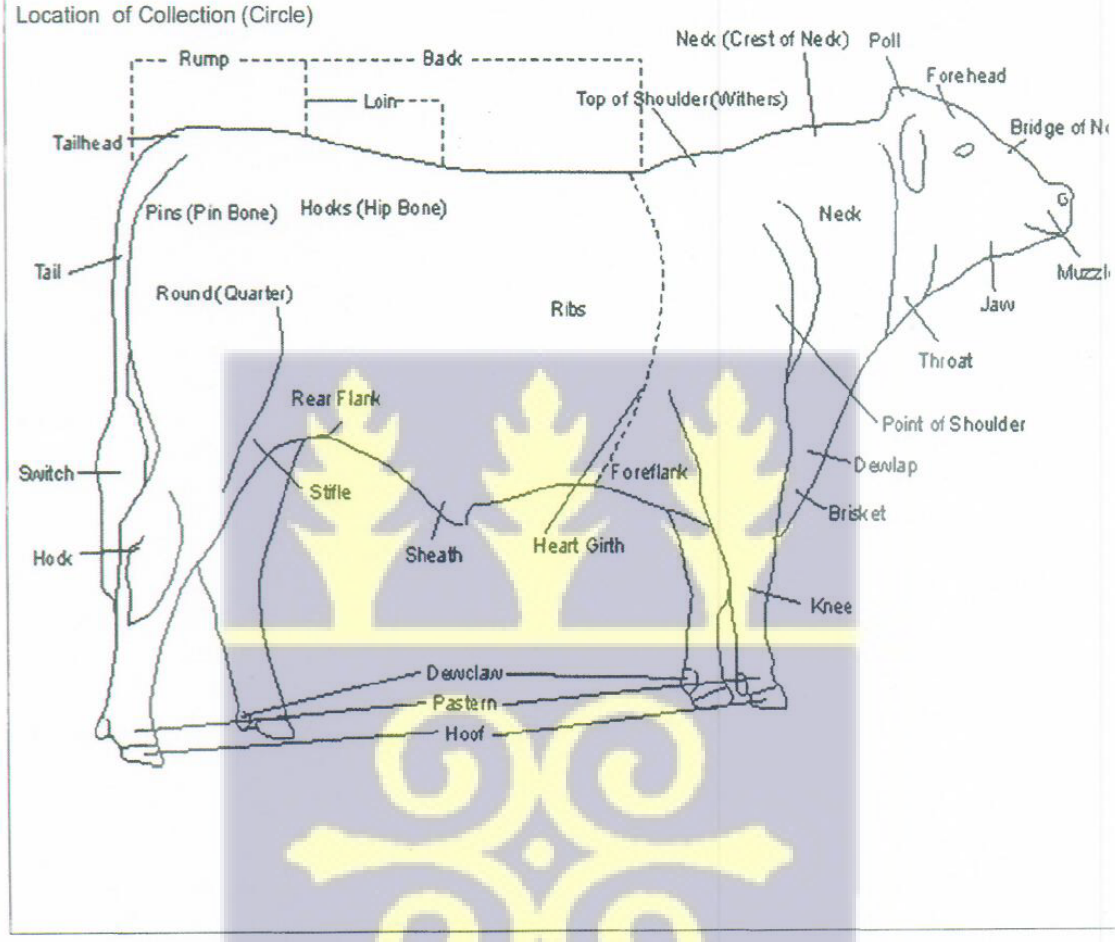
Appendix VI: Animal vector recording sheet

Date	
Sample ID	
Site name	
Animal Type	1. Cow 2. Dog 3. Sheep 4. Pig 5. Goat 6. Other ( )
Temp (°C)	
GPS Location	

Location of Collection (Circle)



Appendix VII: Oligonucleotide primer sequences for Real-time PCR

Name	Gene targeted	Primers &Probes	Sequence	Reference
Rick 17b	17KDa	R17k128F	GGGCGGTATGAAYAAACAAG	(Jiang et al., 2004)
		R17k238R	CCTACACCTACTCCVACAAG	
		R17k202TaqP	FAM-CCGAATTGAGAACCAAGTAATGC-TAMRA	
R. africae	*OmpB*	Raf1797F	TTGGAGCTAATAATAAAACTCTTGGAC	(Maina et al., 2014)
		Raf1915R	GAATTGTACTGCACCGTTATTTCC	
		Raf1879P	FAM-CGCGATGTTAATAGCAACATCACCGCCACTATCGCG-BHQ	
CCHFV	S-segment	CCHF S1 F	TCTCAAAGAAACACGTGCC	(Atkinson et al., 2012)
		CCHF S122 R	CCTTTTTGAACTCTTCAAACC	
		CCHF P	FAM-ACTCAAGGKAACACTGTGGGCGTAAG-BHQ1	