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DETECTION OF CRIMEAN-CONGO HAEMORRHAGIC FEVER VIRUS (CCHFV) IN TICKS COLLECTED FROM LIVESTOCK IN GHANA

BY

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THIS THESIS IS SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF MPHIL MOLECULAR CELL BIOLOGY OF INFECTIOUS DISEASES DEGREE

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DECLARATION

I, Charlotte Adwoa Addae hereby declare that this thesis is the outcome of my own research project under the supervision of Dr. Osbourne Quaye of the Department of Biochemistry, Cell and Molecular Biology, University of Ghana and Dr. Shirley Nimo-Paintsil, United States Naval Medical Research Unit No. 3, Ghana Detachment, Accra, Ghana. To the best of my knowledge, this thesis has not been presented for the award of any degree or published elsewhere. Any mention of other authors' works has been duly acknowledged and properly referenced.

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ABSTRACT

Crimean-Congo haemorrhagic fever virus (CCHFV) is an arbovirus which belongs to the Crimean-Congo haemorrhagic fever serogroup. It belongs to the viral family, Nairoviridae and genus, *Orthonairovirus*. Crimean-Congo haemorrhagic fever serogroup and Nairobi sheep disease serogroup both fall under this genus. Viruses from these two serogroups are pathogenic to humans and animals respectively, and therefore have a significant economic impact. Tick species of three genera are known to transmit these viruses; *Rhipicephalus*, *Amblyomma*, and *Hyalomma*. This study focused on screening field-collected ticks for the presence of CCHFV. Ticks were collected from dogs, sheep, cattle and goats in seven sites within three regions of Ghana; Greater Accra, Northern and Upper East. A total of 1,813 ticks were collected and morphologically identified using the African Ixodidae identification keys. Ticks were pooled (by species, gender, the site collected, and animal host), homogenized, nucleic acid extracted and screened for CCHFV. Seven of the pools were positive for CCHFV and were further analyzed using United States Army Medical Research Institute of Infectious Diseases (USAMRIID) next-generation RNA Access protocol. Sequencing performed on all seven pools failed to confirm the presence of CCHFV however, the resulting data from an *Amblyomma variegatum* pool (from Michel camp-Greater Accra) showed whole genome sequence of Dugbe virus. Phylogenetic analysis of the complete sequence of the L and S segments of the genome using maximum likelihood tree algorithm showed a close relationship (bootstrap value of 99%) with the Dugbe strain previously found in Ghana. However, there was also a close relationship with the reference Dugbe virus strains from Kenya and Nigeria with a bootstrap value of 99%.

The findings from this surveillance study demonstrate the circulation of Dugbe virus in Ghana. Therefore, the need to further investigate to detect the virus prevalence and risk of human and veterinary infections in Accra and the country as a whole.

DEDICATION

This thesis is dedicated to my family; Adepa and William for always being there.

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LIST OF ABBREVIATIONS AND ACRONYMS

Act D - Actinomycin D

AVE – Elution buffer

AVL – Lysis buffer

AW1 – Wash buffer 1

AW2 – Wash buffer 2

BLAST – Basic Local Alignment Search Tool

CCHFV - Crimean-Congo Haemorrhagic Fever Virus

CDC – Center for Disease Control

cDNA – complementary Deoxyribonucleic acid

CT3 - Capture Target Buffer 3

DD - Decimal Degrees

DMS - Degrees Minutes and Seconds

dTTP - deoxythymidine triphosphate

dUTP - deoxyuridine triphosphate

EE1 - Enrichment Elution Buffer 1 (EE1)

ELISA - Enzyme-linked immunosorbent assay

EPF - Elute Prime Fragment

EWS - Enrichment Wash Solution

FSA - First Strand Synthesis Act D

G_C – Glycoprotein C

GenBank – Genome bank

G_N – Glycoprotein N

GPS - Global Positioning System

HT1 – High target 1

ICAM-1 – intercellular adhesion molecule 1

IgG – Immunoglobulin G

IL-6 – Interleukin 6

IL-8 – Interleukin 8

IL-10 – Interleukin 10

IgM – Immunoglobulin M

L segment – Large segment

MEGA6 – Molecular Evolutionary Genetics Analysis 6

mRNA - Messenger RNA

NAMRU3 – Navy medical research unit 3

NGS - Next generation sequencing

NSDV - Nairobi Sheep Disease Virus

PCR – Polymerase chain reaction

qPCR – quantitative (real time) Polymerase Chain Reaction

RdRp - RNA dependent RNA polymerase

RNA – Ribonucleic acid

ROX - 6-Carboxyl-X-Rhodamine (dye)

rpm – runs per minute

RSB - Resuspension Buffer

RT – Reverse Transcriptase

S segment – Small segment

TNF-a – Tumour Necrosis Factor-alpha

USAMRIID – United States Army Medical Research Institute of Infectious Diseases

VCAM – Vascular Cell Adhesion Molecule 1

VHF - Viral Haemorrhagic Fever

WHO - World Health Organization

CHAPTER ONE

1.0 INTRODUCTION

The virus that causes Crimean-Congo haemorrhagic fever (CCHF) was first detected in Crimea (1944) (Grashchenkov, 1945) and Congo (1956) (Hoogstraal, 1979). The virus was named after these two places where it was first discovered.

The CCHF virus belongs to the viral family, Nairoviridae and genus *Orthonairovirus*. It is in the same CCHF serogroup with Hazara, Tofla and Artashat viruses. Crimean-Congo haemorrhagic fever virus (CCHFV) is a negative-sense enveloped virus with a single-stranded ribonucleic acid (RNA) genome which is divided into three segments; small (S), medium (M) and large (L) segments (Bente *et al*., 2013). The S segment is the smallest RNA segment, and it codes for nucleoproteins that encapsulate the three RNA segments of the virus. The M segment is the second largest RNA segment in the virus. This RNA segment codes for the glycoproteins G_N and G_C found on the surface of the virus. These glycoproteins bind to host cell surface receptors leading to the invasion of the host cell by the virus. The L (large) segment codes for RNA-dependent RNA polymerase enzyme, the virus uses this enzyme to synthesize its RNA genome (Bente *et al*., 2013).

The CCHF virus has been found in arthropods such as ixodid ticks *Rhipicephalus spp*. and *Hyalomma spp.* (Keshtkar-Jahromi *et al*., 2013). Household pets, wild animals, and farm animals serve as reservoirs and hosts for viral amplification in which case the virus has been shown to have very low pathogenicity (Al-Abri *et al*., 2017). The virus circulates between the parasitic ticks and animals when virus-infected ticks feed on the animals and transfer the virus into the bloodstream of the animals or take up the virus from infected animals (Bray, 2005). Infected parasitic female ticks are also known to pass on the virus transovarially. Infected male ticks also pass it on sexually to uninfected female ticks. Also, an infected tick

feeding at the same location as other ticks can pass on the virus through its saliva to other ticks (Walker *et al*., 2014).

Tick bites can cause human CCHFV infection or exposure to infected blood from infected animals or persons. Another way by which a person can be infected with the virus is from hospitals or health centers when there is an outbreak (Leblebicioglu, 2010). Veterinarians, animal farm workers, abattoir workers and people living in households with livestock are all at risk of CCHFV infection due to their constant exposure to animals or preferred tick hosts (CDC, 2014). The incubation period after exposure to the virus for humans is typically between five to six days. However, up to thirteen days have been reported (CDC, 2014). Like other viral haemorrhagic fever (VHF) viruses, CCHFV causes sporadic outbreaks with symptoms such as headache, severe fever, back pains, vomiting, and joint pains (Ergönül, 2006). Even though it falls under the class of haemorrhagic fevers, infected people do not necessarily bleed through their mouths, nostrils, and other orifices. Instead, in severe cases, patients often develop large patterns of ecchymoses resulting from internal organ bleeding which may eventually lead to death (Ergönül, 2008).

There is no cure for the disease; symptoms are managed as they appear. Therefore, it is essential that people who live in areas where the vectors are present or people who work with and handle animals take the necessary precautions to prevent an infection (Gunes *et al*., 2009). Veterinarians, abattoir workers, and farm workers are advised to wear the proper clothing to protect them from any tick bites. When coming into contact with bodily fluids from the animals, they are encouraged to take extra care and wear the necessary personal protection equipment for safety (Fajs *et al*., 2014).

Detection of the virus is done using Real-time reverse transcriptase polymerase chain reaction (real-time RT-PCR) to detect the small (S) RNA segment of the viral genome. The Enzyme-linked immunosorbent assay (ELISA) is also another technique for identifying viral

antigens or host antibodies to the viral antigens (CDC, 2014). However, if an individual dies due to viral infection, immuno-histochemical staining can be used to identify the virus antigens in the person's tissues fixed in formalin (Vanhomwegen *et al*., 2012).

The CCHF virus is found in hot and semiarid areas such as sub-Saharan Africa and some regions in the Middle East and Europe. Current global distribution models predict a relatively high occurrence of the virus in West Africa (Messina *et al*., 2015) due to the abundance of the vector in the sub-region (Walker *et al*., 2014). Typically, CCHFV infections are not seasonal but somewhat sporadic, and so they occur at any given time. In the temperate regions, CCHFV human infections are known to occur during the summer when the weather is favourable for the vector to move about, feed, and reproduce (Bente *et al*., 2013).

According to Al-Abri *et al*., (2017), due to the spread of CCHFV infected vectors by migratory birds and livestock trade across various countries, CCHFV is gradually spreading and should, therefore, be of significant concern to the World Health Organization (WHO). Also, ecological models indicate that temperature rise and rainfall reduction is gradually increasing the habitats for the viral vector (Estrada-Pena and Venzal, 2007). An increase in the vector habitat means more ticks will emerge over time resulting in the spread of the virus among animals (Williams *et al*., 1972) which may eventually increase the risk of the spread of the virus to human populations. It is therefore vital that policies be put in place for prevention and control of the virus infection. Sporadic outbreaks of the disease and distribution of likely infected vectors by migratory birds or exported livestock have led to the question of the transmission of strains specific to one region of the globe being found in other areas of the world (Estrada-Pena and Venzal, 2007). Phylogenetic analysis of CCHFV strains shows the presence of West African strains in China (Papa *et al*., 2002). Middle Eastern and European strains have also been found in Africa (Burt and Swanepoel, 2005).

1.1 JUSTIFICATION

Even though CCHFV case-fatalities of up to about 30% have been reported in some West African countries (Bente *et al*., 2013; Sang *et al*., 2011), there is little epidemiological information on the disease in Ghana. Previously collected tick samples from farm animals in Ghana have tested positive for CCHFV (Akuffo *et al*., 2016). Also, CCHFV antibodies have been detected in animal handlers and animal farmers in areas where there has been no case of CCHFV infection. This information is another area of concern about the incidence and prevalence of the virus (Akuffo *et al*., 2016).

There is, therefore, the need to characterize the strains of the virus that are found in the country to be better informed and prepared for any eventual future outbreaks. The information can also influence the development of various testing platforms for diagnosis that will be used to detect CCHFV isolates found in Ghana. Early detection will help curb the threats posed by the virus.

1.2 HYPOTHESIS

The CCHFV and other orthonairoviruses are prevalent in Ghana, but these viruses may have been imported from other endemic regions in Africa.

1.3 AIM

To detect and characterize CCHFVs and other orthonairoviruses in field-collected ticks.

1.4 SPECIFIC OBJECTIVES

1. To determine the different species of ticks collected from livestock in Ghana.

2. To determine the presence of CCHFV using real-time Reverse Transcriptase Polymerase Chain Reaction (real-time RT-PCR).

3. To determine the phylogenetic relationships between the various strains of orthonairoviruses circulating in Ghana.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 HISTORY OF CRIMEAN-CONGO HAEMORRHAGIC FEVER VIRUS AND DUGBE VIRUS

Crimean-Congo Haemorrhagic Fever Virus (CCHFV) causes Crimean-Congo haemorrhagic fever (CCHF). It was first discovered in Crimea after a group of Russian soldiers got sick with an unknown disease in 1944 (Grashchenkov, 1945). Years later in 1956, a disease with similar symptoms was detected in a group of people in the Congo (Hoogstraal, 1979). The virus was therefore named after the two places it was discovered. Since its first discovery, the disease has been reported in various countries and has a fatality rate of 3 – 30% (Sang *et al*., 2011).

Dugbe virus was first isolated at the Virus research laboratory, faculty of medicine, University of Ibadan, Nigeria. It was first detected in *Amblyomma variegatum* ticks and has since been detected in livestock, mosquitoes, and humans. The virus was named after the town it was discovered in, Dugbe in Ibadan Nigeria (David-West, 1974).

2.2 ENDEMIC REGIONS

The CCHFV is found in hot and semiarid areas such as sub-Saharan Africa and some regions in the Middle East and Europe (Messina *et al*., 2015). Due to the epidemic potential and high fatality rate, CCHF is a threat to public health (WHO, 2013). Some of the regions that are endemic for CCHF are considered as conflict zones with the potential of military or civil conflicts. Historically, more casualties of war are caused by arthropod-borne diseases than the enemy (Tucker, 2009). Besides the potential negative impact on public health or the

population during wars, there is also the concern that CCHFV can be used as a biological weapon (Mertens *et al*., 2013).

Current global distribution models predict a relatively high potential for the occurrence of CCHF in West Africa (Messina *et al*., 2015). Previous prediction modeling techniques for CCHFV concentrated on environmental factors such as temperature and precipitation, but current modelling also incorporates land cover type. Land cover type is defined as the observed physical cover of the earth surface. This consists of the vegetation and man-made features of a geographical location (Comber *et al*., 2005). Land cover type is critical to identifying ecological niches for wild or domestic animals that would serve as potential hosts.

As of 2014, the United States Centre for Disease Control (CDC) reported that 47 countries are endemic for CCHF (CDC, 2014). In Africa, CCHF has been recorded in over 30 countries (Morikawa *et al*., 2007). It has been documented that CCHF case-fatalities of up to approximately 30% or more have been reported in the various endemic African countries. According to current CCHF distribution maps (Figure 2.1), it is uncertain if CCHF is prevalent in Ghana, Sierra Leone, Togo, Liberia and Cote d'Ivoire (Leblebicioglu, 2010).

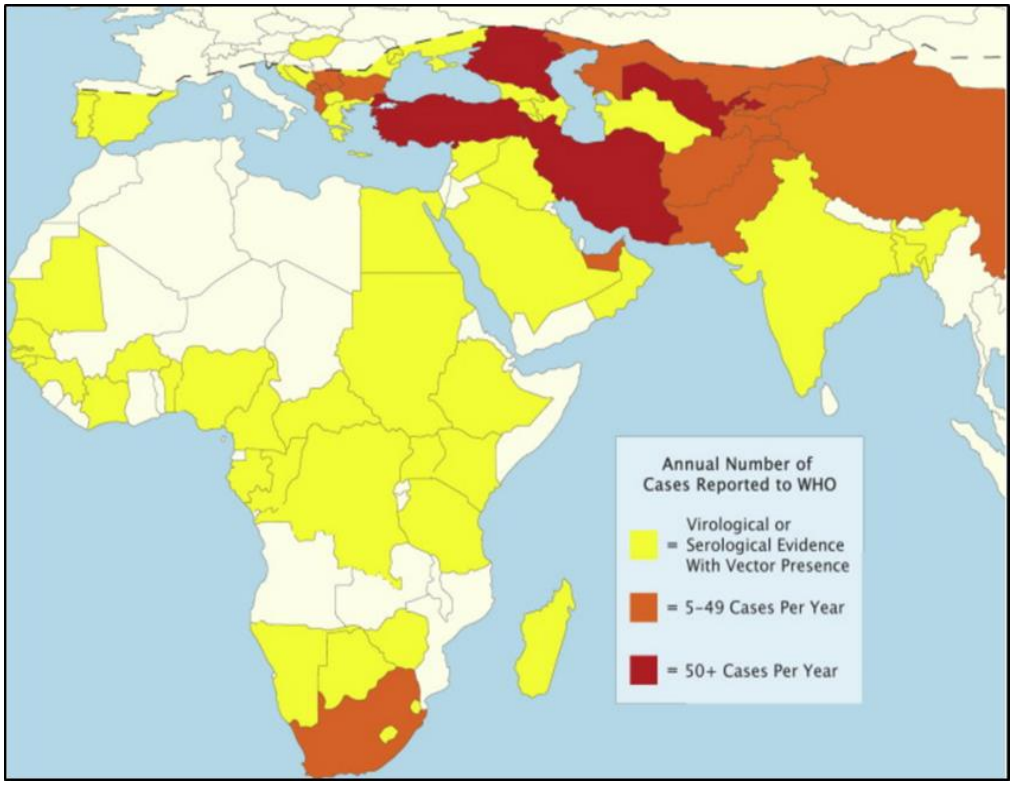

Figure 2.1: Annual global distribution of CCHFV. In regions marked yellow, there is the presence of the CCHFV vector and also virological or serological evidence indicating the presence of CCHFV. Areas marked orange record 5-49 CCHF cases per year. Areas marked red record 50 or more cases per year (Bente *et al.*, 2013).

Dugbe virus just like CCHFV is distributed in regions where its vector is found. So far it has been detected in East Africa (Kenya and Uganda), West Africa (Ghana and Nigeria), Central Africa and Asia (India, Sri Lanka, and China) (Coates, 1990). Serological evidence also suggests the presence of the virus in Mozambique, Botswana (Health, 2016).

Variant strains of Dugbe virus have been detected in East Africa, (Kenya), Central Africa and India. In Central Africa, the virus was detected in an infected laboratory worker (Digoutte, 1971). A strain of the virus known as the Nairobi sheep disease virus has also been identified in Uganda and Kenya (East Africa) from ticks ((Tukei, 1970, Coates, 1990). In

India, Ganjam virus which is also another strain of the virus has been detected (Coates, 1990).

2.2.1 Global distribution of CCHFV

The CCHF virus has been shown to have a long distance geographical genetic linkage from country to country and continent to continent. The global occurrence of CCHFV has been predicted using niche modelling with the goal of providing critical information to assist in prevention (Messina *et al*., 2015). The modelling does not take genetic relationships into account even though that may explain possible virus migration. In recent serological studies in Africa, CCHFV was first documented in Mozambique (Muianga *et al*., 2017).

Over 1000 serum samples from cows across Mali were tested with CCHFV IgG ELISA that yielded 66% CCHFV positive samples (Maiga *et al*., 2017). When the positive samples were compared by region, there was a direct correlation between high densities of the cattle with a higher concentration of positives. In Sudan, there was a high prevalence of CCHFV found in sampled camels. Although CCHFV has been reported in Sudan previously, it has not been well characterized (Suliman *et al*., 2017).

Ghana, Mozambique, Mali, and Sudan have little information on the prevalence of CCHFV, and no sequence information has been published regarding the virus to date. Ticks sampled from farm animals in Ghana tested positive for CCHFV (Akuffo *et al*., 2016), but no sequencing data was obtained from those samples.

In 1999, different clusters of CCHFV was detected in Iran; this initiated intense research to identify the genetic diversity of CCHFV in the country (Keshtkar-Jahromi *et al*., 2013). According to Mild *et al*., (2010), the first CCHFV isolate collected in Iran in 1979 shared its genotype with an African strain. This initial strain was in the same clade as those from Mauritania, South Africa, and Senegal.

In 2003, the initial case of CCHF in human was recorded in 38 patients in Nouakchott, Mauritania (Nabeth *et al*., 2004). Utilizing basic local alignment search tool (BLAST), the S-segment was identified as the CCHFV from Mauritania from 1988. The initial 2003 outbreak is thought to have started by a young woman who had slaughtered an infected goat. Tick and animal sampling conducted suggest that the CCHFV responsible for this outbreak originated from a different region of the country and may have been imported through animal trade.

Previous phylogenetic analyses of the S-segment of the virus have demonstrated that viruses sharing the same genotype can be spread over distances (Mild *et al*., 2010). Besides the same genotype circulating in West Africa and Iran; the same genotype found in the Middle East was also found in Madagascar (Burt and Swanepoel, 2005). Different genotypes can circulate in one country and share separate genetic linkages to other countries; the S-segment from South Africa, Burkina Faso, Nigeria, Namibia, Senegal, and Mauritania have been shown to be in the same genotype. A CCHFV was found in *Hyalomma* spp. from camels and cattle in Kenya (Sang *et al*., 2011). Although there were only two other reports of CCHFV in Kenya before these ticks being collected, these samples were not sequenced. A previous study comparing the global diversity of CCHFV phylogenetic relationships does not list any East African countries but did demonstrate multiple RNA segment re-assortment events (Deyde *et al*., 2006). A CCHFV positive human blood sample collected in 1956 from the Democratic Republic of the Congo shared a genetic relationship with CCHFV samples in a tick in 1969 and one goat in 1972 from Senegal on the M-segment. However, the S segment and L segment of the Congo strain were closely related to European strains (Leblebicioglu, 2010).

2.3 TRANSMISSION AND ENDEMIC CYCLE OF CCHFV AND DUGBE VIRUS

According to the WHO (2013) and CDC (2014), CCHFV can infect humans when they come in contact with blood from an infected human or animal. Humans can also be infected with CCHFV through bites from infected ixodid ticks. Household pets, wild and farm animals serve as reservoirs and amplifying hosts for the virus since it has very low pathogenicity in animals (Leblebicioglu, 2010). There is a high prevalence of CCHFV in wild birds in endemic areas, but like other animals, these birds show resistance to the virus. This was demonstrated in South Africa where a CCHF outbreak occurred at an ostrich abattoir; the ostriches did not show any symptoms of infection (WHO, 2013).

Dugbe virus is also transmitted through tick bites. Infected adult ticks can transmit the virus for up to 2 years. Dugbe virus cannot be transmitted among animals through contact with an infected animal. However, humans can get infected when they come in contact with an infected human or animal (Health, 2016).

Generally, wild and domestic animals are infected through the bite of an ixodid tick (CDC, 2014). Although CCHFV and Dugbe virus only remain in the animal's bloodstream for approximately a week, the presence of multiple vectors taking blood meals from the infected animal keeps the viruses in circulation (CDC, 2014).

Animal to human transmission mostly occurs among individuals that come in contact with blood or tissues of infected animals, such as veterinarians or slaughterhouse employees. Akuffo and his colleagues reported that abattoir workers in the Ashanti region tested positive for CCHF virus antibodies, this may be because of exposure to the virus during animal handling and slaughtering (Akuffo *et al.*, 2016). Transmission of viruses from one human to the other can occur, through direct contact with infected body fluids or organs. Infection can also occur through contaminated medical equipment or needles (CDC, 2014).

Reservoirs of the viruses; wild animals and livestock, can assist in keeping them endemic in a region (Wilson *et al*., 1991). The tick vector also serves to maintain these because they are transmitted in the vector population transovarially and transtadially. It is not common for humans to be infected directly from a tick bite, but it is possible (Hoogstraal, 1979). To assist in the migration of CCHFV and Dugbe virus, it has been shown that migrating birds can carry virus positive ticks to other geographical regions (Palomar *et al*., 2013). Ticks on these birds can drop off from the birds and get onto other animals which may eventually get infected with any of the viruses. This explains why viruses of the same or similar strain found in one country have been found in other countries as well (Palomar *et al*., 2013). Livestock animals transported from one region to the other may also carry infected ticks with them to their destination (Williams *et al*., 1972).

2.4 DISEASE PATHOGENESIS OF CCHFV AND DUGBE VIRUS

In 1945, Grashchenkov's work described CCHF disease pathogenesis as blood circulatory disturbances in the patient's organs mainly in the capillaries and small blood vessels. The actual disease pathogenesis is not very clear, but a study was done on lab mice infected with Ebola haemorrhagic virus which has many characteristics that are the same as as CCHFV. This study suggested that the host immune response induces a change in the host's vascular functions; induction of proinflammatory cytokines, platelet degranulation and aggregation, leukocyte adhesion and activation of the intrinsic coagulation cascade (Mahanty and Bray, 2004; Schnittler and Feldmann, 2003). Further studies have also shown elevated levels of intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) with the secretion of high levels of interleukin 6 (IL-6) and interleukin 8 (IL-8) in CCHF patients. Also, tumour necrosis factor-alpha (TNF-a) is associated with critical cases since IL-6 and IL-10 have been detected in critical and fatal cases (Saksida *et al*., 2010).

Studies have found that CCHFV replicates in monocyte-derived dendritic cells causing proinflammatory cytokines to be released (Connolly-Andersen *et al*., 2009). Nitric oxide and natural killer cells have been implicated in host immune response mechanisms to the virus (Yilmaz *et al*., 2008; Simon *et al*., 2006).

Dugbe virus is a neuro-invasive virus (Boyd, 2008). The virus first infects cells of the major organs of the host; liver and lung (Coates, 1990). It then targets neuronal cells in the brain and eventually spreads to other cells in the brain. It is known that the type1 IFN system plays a role in host immune response, but the exact mechanism of action and types of proteins involved in this process is not clearly understood (Health, 2016). Young animals are most susceptible to the virus and have a higher mortality rate of 30% – 40% than adult animals (Coates, 1990).

2.5 SIGNS AND SYMPTOMS OF CCHFV AND DUGBE VIRUS INFECTION

The incubation period after exposure to CCHFV in humans depends on how the virus was acquired (Hoogstraal, 1979). The incubation period after viral transmission through tick bite is between one to five days. After contact with infected blood, incubation period is typically between five to seven days, however, up to thirteen days have been reported (CDC, 2014). Like other viral haemorrhagic fevers (VHFs), CCHFV has been shown to cause sporadic outbreaks with flu-like symptoms such as severe fever, headache, red eyes, vomiting, back and joint pains. In severe cases, patients often develop large patterns of ecchymoses, which can lead to death (Leblebicioglu, 2010). Patients may also die from multiple organ failure as the disease progresses with no medical intervention (Bente *et al*., 2010). Haemorrhage rarely occurs, but when they do, they may occur at injection sites, severe nose bleeds and severe bruising occurs as the illness progresses (CDC, 2014).

The incubation period for Dugbe virus infection in humans may range from one to seven days. In animals, it may vary from one to five days. The virus has been known to cause mild fever and, in some cases, thrombocytopenia in people (Moore *et al*., 1975) but it is more pathogenic in animals (Crabtree *et al*., 2009). The virus causes Nairobi sheep disease in animals, with symptoms such as diarrhoea accompanied with blood and mucus, difficulty in breathing, conjunctivitis, and discharge of bloody mucus from the nose (Coates and Sweet, 1990). Young animals infected with the virus may die within 12 hours of onset of fever (Health, 2016). Pregnant female animals may have still-births. Loss of livestock due to this disease can become a financial burden to farmers (Crabtree *et al*., 2009).

2.6 DIAGNOSIS OF CCHFV AND DUGBE VIRUS INFECTION

Due to the colour of the typical West African's skin, ecchymoses from CCHFV infection can sometimes go unnoticed as a symptom. When a key symptom like ecchymoses (Bente *et al*., 2013), is not noticed, the patient may not be appropriately diagnosed and their condition could become fatal. Misdiagnosis is also a significant factor of high mortality in CCHF virus-infected patients and Dugbe virus-infected animals. Real-time RT-PCR and ELISA are techniques that are used in diagnosis (Vanhomwegen *et al*., 2012), but these types of diagnostic techniques may not be available in the rural areas of Ghana. Real-time RT-PCR detects the small RNA segment of the viral genome whereas ELISA detects the presence of viral antigen or host antibodies to the viral antigens (CDC, 2014). Another technique which can be used in identifying the viruses is Immuno-histochemical staining. This technique detects the presence of viral antigens in tissues fixed in formalin. This is typically done post-mortem (Vanhomwegen *et al*., 2012).

2.7 TREATMENT

Symptoms of the disease are like other febrile illnesses. They initially present like flu, often a patient who presents such symptoms may be mis-treated for a more common fever illness such as malaria, especially in Africa (CDC, 2014). The primary form of treatment for CCHF is supportive care; symptoms are treated as they manifest (WHO, 2013). Patients who lose a lot of blood are given blood transfusion. Body fluids that are lost can be replaced by intravenous fluids administered to the patient (Ergönül, 2008).

For about two decades, Ribavirin, a drug used to treat hepatitis C and respiratory virus infections has been used to treat CCHFV infected patients. The drug inhibits CCHFV replication in patients (Bergeron *et al*., 2010). Ribavirin is a guanosine or adenosine analog depending on its rotation. The carboxamide group attached to molecule gives it this unique feature (Graci and Cameron, 2006). This analog binds to either cytosine or uracil. This causes mutation in the viral RNA and viral proteins which are eventually fatal to the virus (Ortega-Prieto *et al*., 2013). The drug has shown success in treating patients in South Africa (van de Wal *et al*., 1985), Iran and Turkey (Jabbari *et al*., 2006; Smego *et al*., 2004; Midilli *et al*., 2007) and Pakistan (Fisher-Hoch *et al*., 1992). Ribavirin is believed to be more effective if administered in the early stage of illness (Mardani *et al*., 2003; Alavi-Naini *et al*., 2006).

In Bulgaria, immune globulin therapy was used to treat CCHF patients (Papa *et al*., 2004; Vassilev *et al*., 1991; Christova *et al*., 2009). This therapy involves the intramuscular injection of anti-CCHF immunoglobulin into the patient. In South Africa (van Eeden *et al*., 1985b) and Turkey (Kubar *et al*., 2011) hyperimmune serum developed from CCHF survivors' blood has also been used to treat patients.

In Dugbe virus infected humans, symptoms are treated as they appear. Adult animals may survive the disease if supportive treatment, improved sanitation and quality feed are given (Health, 2016). There are no known drugs for treating Nairobi sheep disease.

2.8 CRIMEAN-CONGO HAEMORRHAGIC FEVER VIRUS

The CCHFV is an arbovirus (viruses transmitted by arthropods) from the family Nairoviridae and genus *Orthonairovirus* (Adams *et al*., 2017). Nairoviruses can be distinguished by their large segments (Morikawa *et al*., 2007). Within the *Orthonairovirus* genus, there are seven serogroups. Viruses in the various serogroups are found or associated with different animals; Hughes, Dera Ghazi Khan and Sakhalin serogroups - birds, Crimean-Congo Haemorrhagic Fever and Nairobi Sheep Disease (NSD) serogroups - birds, rodents, ungulates and humans, Thiafora and Qalyub serogroups - shrews and rodents (Adams *et al*., 2017).

Viruses within CCHF serogroup and NSDV serogroups are of significant concern because they are pathogenic in humans and animals, respectively. The CCHF serogroup is made up of Hazara, Tofla Artashat and CCCHF viruses. These viruses are pathogenic in humans but not animals even though they can be detected in animals (Adams *et al*., 2017). Nairobi sheep disease, Dugbe and Kupe viruses which fall within the NSD serogroup cause abortions in infected pregnant female livestock. This, therefore, causes a major economic burden on animal farmers (Coates and Sweet, 1990). These viruses are also geographically distributed as CCHFV since they share the same vector.

Crimean-Congo haemorrhagic fever virus is spherical and is made up of a lipid envelope and three RNA segments covered with nucleocapsid proteins. The envelop is made up of two lipid layers and contains glycoprotein spikes (Morikawa *et al*., 2007). The single-stranded RNA genome is negative-sense and is divided into three segments namely, small,

medium and large segments (Kinsella *et al*., 2004). The small (S) segment (1,700–2,100 nucleotides) encodes nucleocapsid proteins that encapsulate the three RNA segments. The medium (M) segment which is 4,400–6,300 nucleotides long encodes glycoproteins G_C and G_N. These glycoproteins interact with host cell surface receptors during host cell invasion. The 11,000–14, 000 nucleotides long large (L) segment encodes RNA-dependent RNA polymerase which is required for RNA synthesis, Figure 2.2 shows the various components of CCHFV (Bente *et al*., 2013).

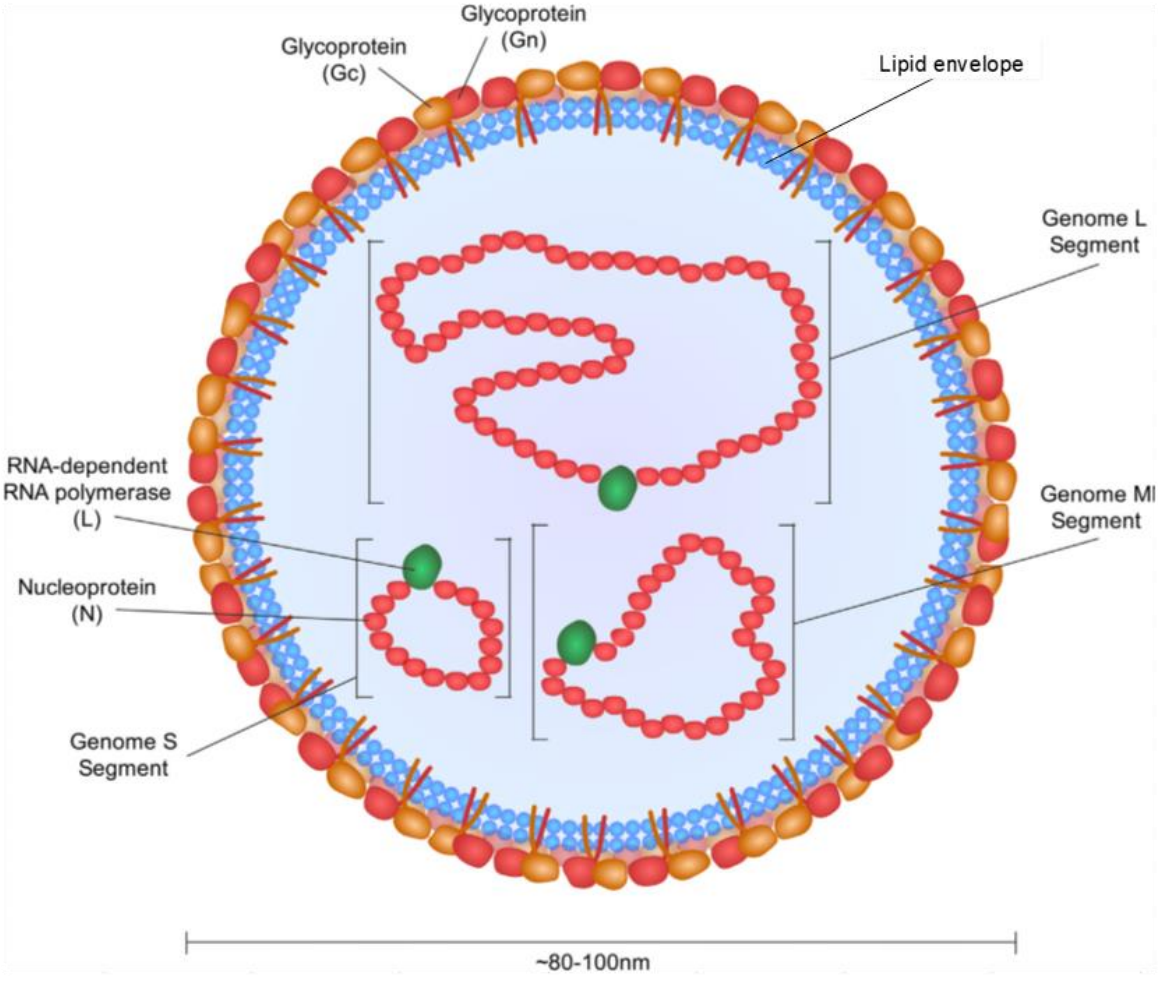

Figure 2.2: A diagram of CCHFV showing its various components (Bente *et al*., 2013).

2.9 HOST CELL INVASION AND REPLICATION

The virus targets macrophages, hepatocytes, and endothelial cells when it infects a host (Simon *et al*., 2009). Once a human host is infected by direct contact to CCHFV, the virus interacts with host cell surface receptors using the glycoprotein spikes on the surface of the viral envelop. The virus enters the cell through clathrin-dependent receptor-mediated endocytosis (Bente *et al*., 2013). The viral lipid envelope fuses with the endosomal membrane of the host cell resulting in the RNA genome being transferred into the host cell cytoplasm. The nucleocapsid is dissociated and complementary RNA is made from the viral negative sense RNA. This complementary RNA is used to produce more negative sense RNA for new viral particles to be made (Simon *et al*., 2009).

Messenger RNA (mRNA) is transcribed from the viral negative sense RNA and translated to essential proteins for the assembly of new viral particles. Complementary RNA and mRNA are generated by RNA-dependent RNA-polymerase (RdRp) found as part of the viral genome (Simon *et al*., 2009). New viral RNA-dependent RNA-polymerase, viral RNA and capsid proteins assemble to form new nucleocapsids. Viral mRNAs are translated into precursor proteins in the endoplasmic reticulum and further processed into glycoproteins in the Golgi body. Finally, new particles bud out of the cell through exocytosis as shown in Figure 2.3 (Bente *et al*., 2013).

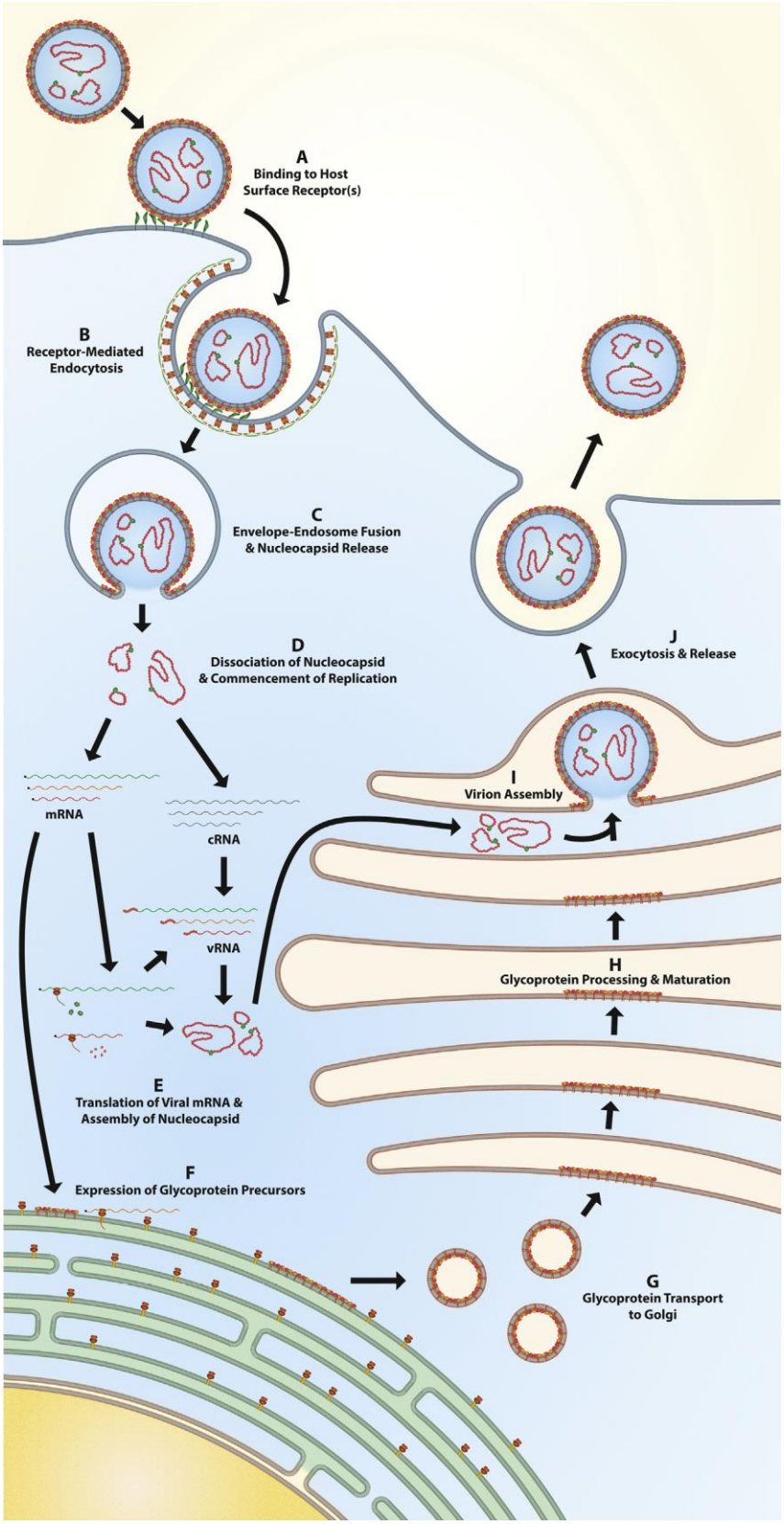

Figure 2.3: Life cycle of CCHFV inside a host cell (Bente *et al*., 2013). Virions bind to host cell surface receptors (A) and through clathrin-dependent, receptor-mediated endocytosis, they enter the host cell (B). nucleocapsids are released into the cytosol after viral envelope and endosomal membranes fuse (C).

Nucleocapsids are dissociated (D) leading to the generation of mRNA and cRNA by RdRp. New viral proteins are assembled from translated mRNA. The cRNA is used as template for new viral RNA (vRNA) genome (E). Viral glycoproteins are translated in the endoplasmic reticulum (F) and are transported to the Golgi complex (G) for further processing (H). Matured glycoproteins are then used to form new virions (I). These new virions are transported to the plasma membrane and released (J).

2.10 DUGBE VIRUS

Dugbe virus has the same structural and genomic features as CCHFV and other viruses of the Nairovirus family (Marriott and Nuttall, 1996). Its negative sense RNA genome encodes glycoproteins, RNA dependent RNA polymerase and nucleocapsid (Bente *et al*., 2013). Genetically, Dugbe virus is different from CCHFV through its RNA genome nucleotide composition (Clerex-Van *et al*., 1982). This variability in nucleotide composition results in the translation of different viral proteins unique to each virus. Genetic diversity among viruses in the Nairovirus family is as a result of genome recombination and reassortment in the tick vector (Marriott and Nuttall, 1996). This virus also uses the glycoproteins spikes on its envelope to invade host cells just like CCHFV (Figure 2.2) and also replicates in the same manner (Figure 2.3).

Dugbe virus is mostly found in Central and East Africa but was first detected in Nairobi, Kenya. It has since been detected in other countries in Asia, other regions of Africa and the Middle East (Hotez and Kamath, 2009; David-West and Porterfield, 1974). Like CCHFV, the vector for Dugbe virus transmission is the ixodid tick. Ticks of the genera; *Rhipicephalus*, *Amblyomma*, *Haemaphysalis* and *Hyalomma* (Honig *et al*., 2004) are implicated in its transmission.

2.11 VECTOR OF CCHFV AND DUGBE VIRUS

The primary vector for CCHFV and Dugbe virus includes many species of ixodid ticks. There are 866 identified species of ticks in the world (Madder *et al*., 2013b). Ticks belong to the phylum Arthropoda just as insects but are in the class Arachnida with mites and spiders. Ticks and mites share the same order; Acari, but there is a suborder of Ixodida that ticks are generally classified. Within the order Ixodida there are two families for ticks: Argasidae for soft ticks and Ixodidae for hard ticks. The dominant vector species for CCHFV and Dugbe virus tend to be Ixodidae (Walker *et al*., 2014).

Crimean-Congo haemorrhagic fever virus has been detected in Ixodidae or hard ticks *Hyalomma spp*. and *Rhipicephalus spp*. (Bente *et al*., 2013). The genus *Rhipicephalus* contains 80 species; they are easily identified by their hexagonal shaped capituli from the dorsal angle. The genus *Hyalomma* are Old World ticks that are comprised of 32 species. However, there have been 25 tick species that have been previously reported as vectors of CCHFV (Hoogstraal, 1979). Tick species implicated as vectors of CCHFV in different regions of the world can vary, in Southern and West Africa; *Hyalomma rufipes, Hyalomma marginatum* and *Hyalomma turanicum*, in Madagascar; *Boophilus microplus*, in China and Uzbekistan; *Hyalomma asiaticum*, in Tajikisstan; *Dermacentor niveus*, in Pakistan; *Hyalomma anatolicum*, in Russia Balkan; *Hyalomma marginatum marginaum*, in Turkey; *Hyalomma marginatum marginaum, Rhipicephalus bursa*, in Greece; *Rhipicephalus bursa* (Morikawa *et al*., 2007).

Dugbe virus, on the other hand, has been detected in mostly *Rhipicephalus, Amblyomma, Haemaphysalis* and *Hyalomma* ticks (Hotez and Kamath, 2009). In Ghana, the virus was detected in *Amblyomma variegatum*, however, in Kenya and Central Africa, the virus has been detected in *Rhipicephalus appendiculatus*. Dugbe virus has also been detected

in China from *Haemaphysalis longicornis.* The main vector for Dugbe virus in Asia (Sri Lanka and India) is the *Haemaphysalis spp.* (Health, 2016).

These ticks are known to parasitize a variety of small and medium-sized wild mammals and are a major ectoparasite of livestock. Ticks are associated with a variety of hosts, which include birds, snakes, lizards, mammals and turtle species (Walker *et al*., 2014).

2.11.1 Distribution of ticks

The geographical distribution of CCHFV and Dugbe virus is a direct representation of the vector abundance (Morikawa *et al*., 2007). Ticks maintain the virus in endemic areas through transovarial transmission of CCHFV and Dugbe virus; this explains the high rate of infected adult ticks (Garrison *et al*., 2013). Dependant on the species of tick, a single female can oviposit a few hundred to over 20,000 eggs in one large batch (Walker *et al*., 2014). Females lay their eggs during the rainy season when the soil is moist.

Various tick species are distributed across warmer climates around the world. This is because ticks require habitats that have three essential elements; high humidity, warm temperature and viable hosts (Sonenshine, 2018). Since ticks do not drink water, they require an environment that has enough moisture in the atmosphere that their bodies can absorb. High temperatures are required for easy movement in their environment when questing for hosts. Viable hosts are needed for easy access to blood, the host's body should also make it easy for the ticks to firmly anchor onto it (Walker *et al*., 2014). The rain forest climate around central Africa and coastal regions and the savanna climate of the northern parts of Africa fit this criteria and are suitable places for ticks to survive (Walker *et al*., 2014). The climate differences across the continent also influences tick distribution as certain tick species may require more or less of these essential elements. Ticks found in locations that have viable hosts but moderately low humidity and temperatures have adapted to these habitats. Ticks can move to different locations geographically due to the mobility of their hosts. An example is

the detection of CCHFV infected ticks on migratory birds from northern Spain that were identified in Morocco (Palomar *et al*., 2013).

Ghana has a good climate across its six ecological zones (Figure 2.4) which makes it suitable for ticks to survive. There are five genera of ticks distributed across Ghana; *Rhipicephalus*, *Amblyomma*, *Haemaphysalis*, *Hyalomma*, and *Ixodes* (Ntiamoa-Baidu *et al*., 2004). Four genera within these five genera; *Rhipicephalus*, *Amblyomma*, *Haemaphysalis*, and *Hyalomma* have been implicated in CCHFV and Dugbe virus transmission.

Figure 2.4: The ecological zones of Ghana shown in different shades of purple; Sudan Savannah, Guinea Savannah, Forest Savannah Transition, Semi-Decideous Rainforest, High Rainforest and Coastal Savannah. show the various towns. UW – Upper West region, UE – Upper East region, NR – Northern region, BA – Brong Ahafo region, ASH – Ashanti region, ER – Eastern region, VR – Volta region, GA – Greater Accra region, CR – Central region, WR – Western region (Ntiamoa-Baidu *et al*., 2004).

2.11.2 Tick life cycle

There are four stages in a tick's life cycle: egg, larvae (looks like the adult but has six legs), nymph (looks like the adult but smaller), and adult. Apart from the egg, all stages of the tick are parasitic. When a tick hatches from an egg into a six-legged larva, it undergoes at least one or more nymph instars until it is an adult (Vesco *et al*., 2011). Depending on the species, ticks can feed on one or multiple hosts to acquire a blood meal. Ixodid ticks essentially feed on one, two or three hosts during their lifetime whereas argasids and other species of ticks feed on multiple hosts. Female ticks come off their host to lay eggs on the ground. Unlike ixodid ticks which lay eggs once in their lifetime and die afterward, argasid ticks lay eggs intermittently (Estrada-Peña and Salman, 2013). The one-host tick will undergo all its developmental stages on the same host. It remains on the same host while it moults to the next stage of its lifecycle. One-host female ticks drop off their host to lay eggs on the ground. Two-host ticks spend their immature life stages (larva and nymph stages) on one host and then drop off that host onto another host at their adult stage. Three-host ticks feed on three separate hosts at the three life stages; larva, nymph, and adult (Walker *et al*., 2014).

2.11.3 Hunting and feeding behaviour of ticks

Ticks will quest for a potential host in vegetation with their odour detecting sensory organ located on the dorsal surface of the tarsus found on their legs called Haller's organ (Williams *et al*., 1972). The Haller's organ is on all stages of ticks' life cycle. Ticks detect hosts through their odour, heat, and visual cues. They gain access to the host by crawling on to the body of the host when the animal brushes the leaf blade or object they are hanging (Walker *et al*., 2014).

Male and female ticks are obligate parasites and must take blood meals for spermatogenesis and oogenesis, respectively. The tick penetrates the host's skin with its mouthparts made up of the palps, chelicerae, and the hypostome. The chelicerae are used to

cut through the host's dermis (Figure 2.5). Once attached to a host, a tick can feed for 3 to 7 days (Walker *et al*., 2014). To initiate a blood meal, a tick inserts its hypostome into the host tissue to assist in anchoring the tick to the host by its curved teeth. During feeding, the tick secretes a cement-like substance in its saliva which reinforces its attachment to the host. The saliva also contains antihistamines and anticoagulants that decrease the host immune response and promote blood flow (Francischetti *et al*., 2010). In the process of feeding, the tick continuously alternates between injecting its salvia into and sucking blood from the host. If the tick is infected with a tick-borne pathogen, it will infect the host via the saliva (de la Fuente *et al*., 2017). A fully engorged tick will fall off its host after feeding.

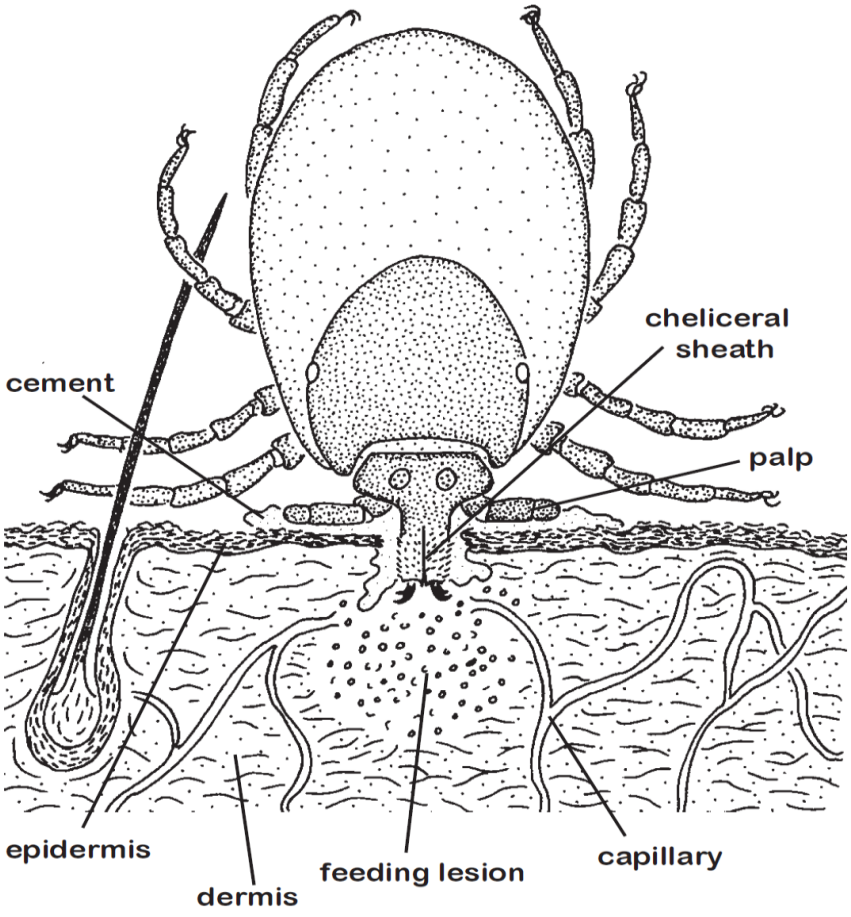

Figure 2.5: An ixodid tick feeding on its host (Walker et al., 2014). The tick penetrates the host's skin with its chelicerae. The palp and a cement-like secretion reinforce its attachment to the host. the exchange of saliva from the tick and blood from the host may result in the transfer of a virus from one to the other.

2.11.4 Life cycle of CCHFV and Dugbe virus in tick vectors

Infected adult female ixodid ticks will transovarially pass on the virus to their offspring when they lay eggs. Six-legged larvae hatch from the eggs and climb onto their hosts, blood feed and moult into nymphs (Walker *et al*., 2014). These nymphs also blood feed and moult into adult male or female ticks. At each blood feeding stage, the arthropod will infect its host with the virus. In the case where more than one tick feed at the same location on the host at the same time, the virus can be transmitted to the uninfected ticks when they pick up saliva of an infected tick. Uninfected ticks can also pick up the virus from infected hosts (CDC, 2014).

2.12 METHODS OF TICK SURVEILLANCE

Vector surveillance consists of methodical monitoring of medically important arthropods that are associated with the transmission of pathogens (Madder *et al*., 2013b). Surveillance can assist in understanding vector ecology: vector abundance and distribution, species diversity, seasonality of targeted vector. Vector surveillance is also essential to detect medically important arthropod species (de la Fuente *et al*., 2017). This could provide critical information if a potential disease-causing vector was not previously detected in the area. Continuous vector population monitoring and surveillance can also be helpful to determine the efficacy of control strategies by adding insecticide resistance assays (Madder *et al*., 2013a). A systematic surveillance approach that incorporates laboratory assays can be used to evaluate the risk or presence of vector-borne pathogens. Vectors collected from sentinel surveillance sites can be used as bio-indicators to assist in early or real-time detection of pathogens and evaluate infected vector abundance that can serve as possible predictor for an epidemic event (Estrada-Pena, 1999.).

Ticks are collected from harbouring vegetation and from host species such as goats, cattle, and dogs (Bryson *et al*., 2000). Collecting Global Positioning System (GPS) and climate data during collection can associate the point of occurrence with the geographical location to evaluate the abundance of ticks seasonally for a single or more host species. Collecting free-living adult and immature ticks can be done by tick flagging or dragging (Sprickett *et al*., 1991). Using flannel strips of cloth attached to a long wooden bar, a person can collect ticks by pulling the bar and flannel strips by a string or twine harness for approximately 250 m over vegetation areas. After reaching a set distance, the flannel strips are flipped to remove attached ticks that were questing in the vegetation (Ginsberg and Ewing, 1989**).** Attached ticks can also be removed with forceps and stored in vials of 70% ethanol. Upon reaching the lab, ticks are stored appropriately based on the assay they will be used for. Ticks that will be used for viral detection studies or RNA studies are stored in RNAlater right from the point of collection. This is to preserve the viral RNA in them (Madder *et al*., 2013a).

Tick drags can be conducted multiple times as needed in woodland and grassland. Tick drags are not done over wet or early morning dew grass. When wet, the flannel strips decrease in their efficacy (Sprickett *et al*., 1991). Adult ticks are also collected by hand from vegetation. Sampling ticks from vegetation and tick drag methods are typically used to collect host questing exophilic ticks. Other free-living tick sampling methods can be conducted using vacuum systems in nest and burrows of host animals as well as carbon-dioxide or lure baited traps (Norval *et al*., 1989). Ticks collection from the live host can also be performed using forceps to remove them. Collecting ticks from the host will result in collecting mostly engorged blood fed ticks, therefore, care must be taken to not crush them (Koffi *et al*., 2012). Passive surveillance is another method of tick surveillance where

residents in an area voluntarily submit ticks they find to a lab or health center (Koffi *et al*., 2012).

2.13 TICK IDENTIFICATION

2.13.1 Morphological identification

In tick identification, the most important features to determine genus are length of mouthparts, the presence of eyes, conscutum (ornate or inornate), leg colour, existence or nonexistence of festoons, and existence or nonexistence of anal plates (Walker *et al*., 2014). For this reason, it is important that ticks collected with forceps be handled with care, so they do not loose vital body parts needed for accurate morphological identification. For example; characteristics of the genus *Amblyomma* include: very long mouthparts with an elongated second segment of palps; the conscutum and scutum ornate; the presence of eyes; the presence of festoons; on males the adanal plates are absent or reduced; the legs are banded (Walker *et al*., 2014).

The genus *Rhipicephalus*, which was previously named *Boophilus*, are identified by: very short mouthparts with sclerotized palp segments II and III; the conscutum may be sclerotized and have a dark pattern of caeca that can be visualized from above; eyes are inconspicuous but present; festoons are absent; on males adenal plates are well defined, males may also have caudal processes (Madder *et al*., 2013a).

The *Hyalomma* genus are identified by their long mouthparts with the second segment of palps that is elongated; the scutum are pale to dark brown; there are also convex eyes; festoons are visible; the adenal and accessory anal plates are sub-anal and present on males; legs are banded and coxae of first pair of legs long prominent posteriorly directed spurs (Walker *et al*., 2014).

Another indicator of identification is the geographical location of ticks (Madder *et al*., 2013a). Certain species of ticks are restricted to certain locations geographically, this helps to narrow down the possible genus or species of tick being identified.

2.13.2 Molecular identification

Molecular identification of ticks can also be done. This identification method is more accurate than morphological identification. In this type of method, only a small body part of the tick is needed for DNA extraction (Adama *et al*., 2017). Primers specific for different species are used to determine the species of an unknown tick (Wodecka *et al*., 2010). This method can also be used with the morphological identification method. In that, when the genus of a tick has been determined morphologically, molecular method can be used to determine the species. In recent times, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has used to identify ticks based on protein analysis of the ticks (Adama *et al*., 2017).

Sequencing has in recent times been used to identify ticks. Even though it is expensive as compared to the other methods mentioned above, it is a sure way of acquiring the accurate information of a detected tick species (Wilhelmsson *et al.*, 2010). Ribosomal subunits such as 12S, 16S or 18S genes have been established as a reliable tool for ixodid tick identification. Sequences from these genes are compared to online databases to help identify the tick specimen (Adama *et al*., 2017).

2.14 DETECTION OF CCHFV AND DUGBE VIRUS IN VECTORS AND HOSTS

Genetic diversity across CCHFV and Dugbe virus strains is quite extensive within the three RNA segments, L, M and S segment (Whitehouse, 2004; Hewson *et al*., 2004). This diversity therefore requires a detection assay that will detect a broad range of strains (Deyde *et al*., 2006). Due to the similarity of CCHF symptoms to other febrile illnesses, misdiagnosis is very common (Vanhomwegen *et al*., 2012). Clinical observation, patient information and history are some of the useful indicators of human diagnosis of CCHF (Whitehouse, 2004). These diagnosis methods are not conclusive and are not helpful in distinguishing the specific haemorrhagic fever (Drosten *et al*., 2003). In the case of Dugbe virus, it is quite difficult to diagnose infected livestock with the virus. Cell culture, ELISA and real-time RT-PCR are the tools used in CCHFV and Dugbe virus detection and infection diagnosis (Burt *et al*., 1994).

In most cases, ELISA is used in serological analysis; this is where antibodies for CCHFV and Dugbe virus, IgM and IgG, are found in infected animal or human blood samples. Antigens from the viruses are exposed to isolated viral antibodies from the blood samples (Sas *et al*., 2018). Detection of IgG and IgM is limited until after about seven days from the disease onset. At this point, a person infected with CCHFV may die since the disease may become fatal at this point (Kalvatchev and Christova, 2008). These antibodies are also rarely detectable after death (Shayan *et al*., 2015). There is also the issue of cross reactivity with antigens of viruses from the same family of Nairoviruses (Spengler *et al*., 2016). Cell culture is the way of propagating the virus by injecting into tick cell lines (Ferraris *et al*., 2015, Bell-Sakyi *et al*., 2012).

In real-time RT-PCR detection method, primers that are specific to the S segment of the virus are used (Atkinson *et al*., 2012). The use of primer sequences that target conserved regions on the viral S segment is important. This is because other viruses in the Nairoviridae family have similar RNA genome (Khurshida *et al.*, 2015). Consensus sequence of the virus

isolates from GenBank were used to design the primers that were used in this study (Garrison *et al*., 2007). Positive results from the real time PCR show cycle threshold (C_t) values of less than 40 (Afonina *et al*., 2002).

2.15 SEQUENCING AND CHARACTERISATION OF CCHFV AND DUGBE VIRUS

DNA and RNA sequencing involve determining the composition and order of nucleotides of a DNA fragment (Sengupta and Cookson, 2010, Victoria *et al*., 2008). Several techniques have been developed for this process; Sanger sequencing, Pyro sequencing and next generation sequencing. Whereas DNA sequencing involves the sequencing of nucleotides in a genome, RNA sequencing shows the portion of the genome that has been actively expressed in the cell. RNA sequencing also gives information about the functions of the cell by determining the types of genes that are transcribed in the cell (Djikeng *et al*., 2009).

In the case of RNA viruses, RNA sequencing does not necessarily tell the story of the transcriptome but also gives an insight into the genetic makeup of the virus (Sengupta and Cookson, 2010). Viruses have diverse genomes and this diversity aids in proper identification of viruses (Edwards and Rohwer, 2005). In recent times, viruses are mainly identified using sequencing methods (Willner *et al*., 2009; Djikeng *et al*., 2009; Riesenfeld *et al*., 2004; Victoria *et al*., 2008; Edwards and Rohwer, 2005), either using Sanger sequencing or next generation sequencing (NGS).

Prior to sequencing a viral genome, the sample containing the virus may or may not go through a lot of sample/library preparation. The aim is to minimize sample contamination as much as possible. However, some samples may contain small amount of viral nucleic acids and therefore they have to be enriched. Depending on the sequencing method, viral nucleic acid content and presence of carrier RNA or other contaminants in sample, some samples

may go through stringent manipulation steps before sequencing (Li *et al*., 2015). Sanger sequencing is preferred when only a target sequence is required, whereas NGS can sequence multiple sequence targets simultaneously. Also, there is more accuracy with NGS (Sengupta and Cookson, 2010).

Sequenced data are mostly used for phylogenetic analysis this analysis involves the construction of phylogenetic trees that show evolutionary relationship between sequenced data and already known sequences in online databases. This evolutionary relationship is based on the similarities or differences of their genetic makeup. Bioinformatics tools are used for phylogenetic analysis (Ciccarelli *et al*., 2006). This analysis has been used over the years to acquire knowledge on the relationships among the viruses detected globally.

Previous phylogenetic analysis of CCHFV has been generated using S-segment and M-segment. However, there are few CCHFV sequences in GenBank utilizing all three segments. In recent times, phylogenetic analysis is focused on the S and L segments of the virus. This is because, these two segments are not prone to mutations as much as the M segment. The M segment mutates to produce glycoproteins that will evade host immune defence mechanisms (Bente *et al*., 2013).

The CCHFV demonstrates the greatest degree of diversity when compared to other arboviruses with divergence of 20, 22 and 31% among the virus isolates compared in Bente *et al*., (2013). The CCHFV has shown ancestral linkages in specific and distant geographical regions. Previous phylogenetic trees have shown that there is a genetic relationship between the S-segment of CCHFVs circulating in Congo and Uganda while the S-segment in Senegal falls into its own separate clade. There is also evidence showing a linkage between the S-segment circulating in Mauritania with those in Uganda and South Africa (Anagnostou and Papa, 2009).

The S-segment of viruses in Sudan, South Africa and Nigeria is shown to have direct genetic relationship. However, high bootstrap values have been shown to demonstrate a close genetic relationship between Nigerian and South African M-segment strains. This has also been documented in Turkey and several African countries. The Turkey-kelkit06 CCHFV strain was found in patient blood samples collected in 2006 in a CCHFV endemic area of Turkey (Ozdarendeli *et al*., 2010). Phylogenetic tree created on the complete genome sequence of Turkey-kelkit06 strain demonstrated a close relationship to both European and African strains of the virus. Phylogenetic analysis of two CCHFV strains found in the Xinjiang Province, China, revealed three subtypes of the virus with one cluster containing known sequences from West African strains (Nigeria) (Papa *et al*., 2002).

CHAPTER THREE

3.0 METHODOLOGY

3.1 CHEMICALS AND REAGENTS

The reagents for the homogenisation and RNA extraction which included QIAamp Viral RNA Kit were obtained from QIAGEN, Valencia, CA. Superscript CCHFV master mix and reverse transcriptase Platinum Taq Mix were obtained from the immunodiagnostics and biologics department of the United States Army Medical Research Institute, (USAMRIID), Fort Dettrick, MD. Reagents used in the library preparation for next generation sequencing were obtained from Illumina Inc, San Diego CA. Standard chemicals and consumables were obtained from various commercial sources.

3.2 ETHICAL CONSIDERATIONS

Although live animals were included in the study, animals have been raised as livestock, not as research animals. Therefore, review by the Institutional Animal Care and Use Committee was not required. Informed verbal consent was obtained from the livestock owners, herdsmen and farm managers. Protocols for tick collection were developed as part of a larger study under the GS-115 project. Institutional review board (IRB) approval was sort from the Noguchi Memorial Institute for Medical Research (NMIMR).

3.3 STUDY SITES

Ticks were collected from seven study sites within two ecological zones in Ghana (guinea savannah and coastal savannah (Figure 3. 1). The seven study sites were, Navrongo in the Upper East region; 6th Battalion Infantry (Kamina Barracks), Air Borne Force (Barwah Barracks) and Airforce Base all in Tamale in the Northern region; 5th Battalion Infantry

(Burma camp), 1st Battalion Infantry (Michel camp) and Army Recruit Training School (Shai hills) in the Greater Accra region.

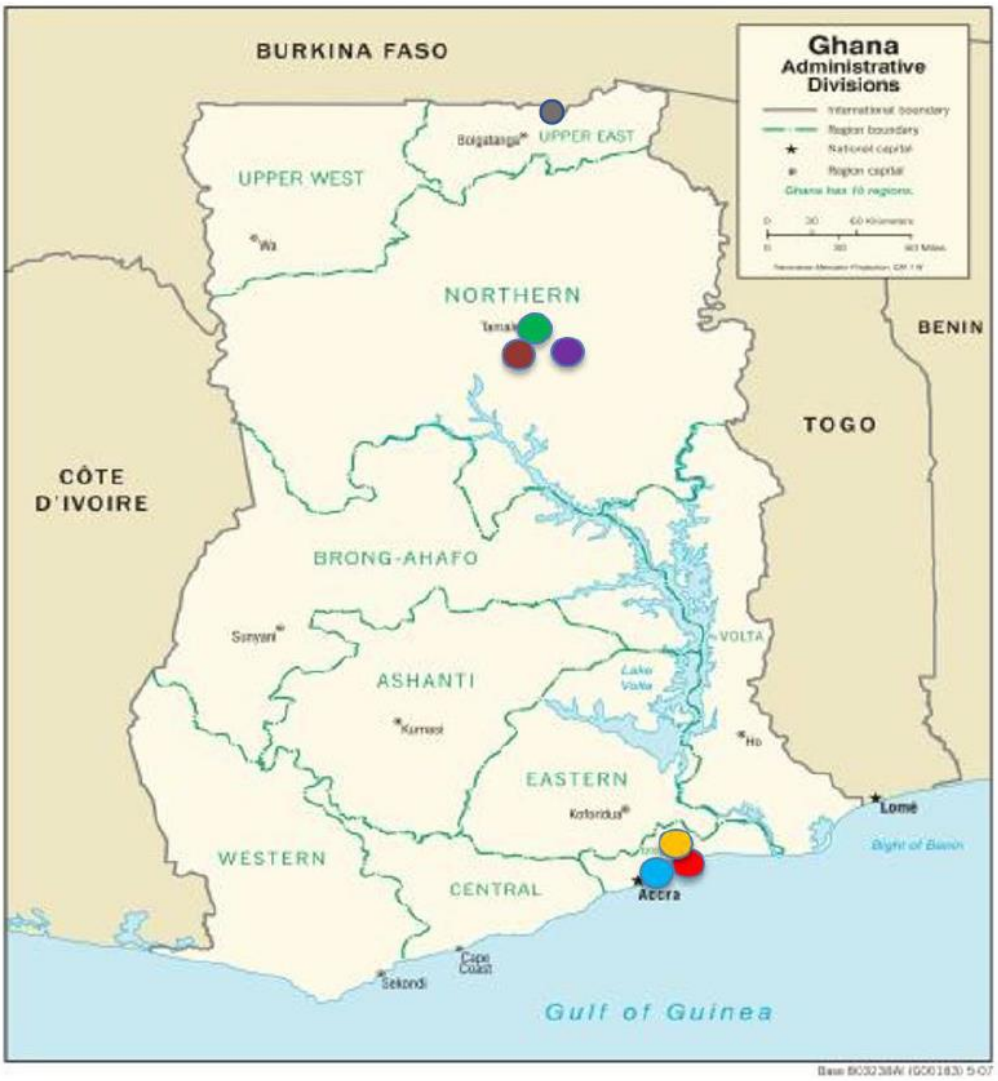

Figure 3. 1: Map of Ghana showing the various study sites. Navrongo (grey), Airforce Base, Tamale (green), 6th Battalion Infantry, Tamale brown (brown) Air Borne Force, Tamale (purple), Army Recruit Training School, Shai hills (yellow), 1st Battalion Infantry, Michel camp (red) and 5th Battalion Infantry, Burma camp (blue).

At the Accra sites, ticks were collected monthly from December 2017 to March 2018. Ticks from the Northern and Upper East regions were collected twice in August 2017 and twice in March 2018. All the collection sites except Navrongo are Ghana Armed Forces military camps. These military camps have kraals where cattle are kept and sent out for grazing daily. Cattle are treated every other month with fipronil, a type of acaricide to rid them of pests especially ticks. On the other hand, the sheep, goats and dogs included in the study are kept in households.

3.4 TICK COLLECTION, SORTING AND IDENTIFICATION

Ticks were collected from sheep, cattle, goats and dogs and transported in RNAlater to the Naval Medical Research Unit No. 3 (NAMRU3) laboratory of NMIMR for further analysis. The collected ticks were sorted and identified morphologically using the African Ixodidae identification keys (Walker *et al*., 2014). This involved identification based on unique features such as the adenal plates, mouthparts and patterns on scotum on the tick. Identified ticks were pooled based on species, gender, developmental stage, and animal source. A pool either consisted of three males or two females. Some pools comprised of either two males or one female depending on the size of the ticks. Pooled samples were kept at -80 °C until further analysis.

3.5 RNA EXTRACTION FROM TICKS

According to the reference protocol, (Crowder *et al*., 2010), each tick pool was transferred into clean 1.5 mL tubes containing 0.15 gm of 0.1 mm beads and 0.75 gm of 2.0 mm beads. A volume of 560 µL lysis buffer (Buffer AVL), was added to the tubes. The tick pools were homogenized for 2 minutes in the Mini-Beadbeater-96 and later centrifuged at 8000 rpm for 2 minutes. Clear supernatant was transferred into clean 1.5 mL microcentrifuge tubes. The tubes were incubated at room temperature for 10 minutes and briefly centrifuged to remove drops from the lid. To each of the samples, 560 µL 96% ethanol was added and mixed using a vortex. The tubes were again briefly centrifuged to remove drops from inside the lid. RNA was extracted from the crushed ticks following the QIAamp Viral RNA Kit (Qiagen, Valencia, CA). QIAamp Mini spin columns were placed in a 2 mL collection tube and 600 µL of supernatant was carefully transferred into them without wetting the rim. With the caps tightly closed, the tubes were centrifuged at 8000 rpm for 1 minute. The QIAamp Mini spin columns were placed into clean 2 mL collection tubes and the tube containing the filtrate was discarded. The QIAamp Mini spin columns in new collection tubes were again centrifuged at 8000 rpm for 1 minute, filtrate discarded and the QIAamp Mini spin columns placed into clean 2 mL collection tubes.

A volume of 500 µL wash buffer 1 (Buffer AW1) was added to the QIAamp mini column and centrifuged for 8000 rpm for 1 minute. This buffer washed the extract by removing proteins and other biomolecules aside RNA. The filtrate was discarded and the QIAamp mini column placed in a clean 2 mL collection tube. A volume of 500 µL wash buffer 2 (Buffer AW2) was added to the QIAamp mini column and centrifuged for 14000 rpm for 3 minutes. The filtrate was discarded and the QIAamp mini column placed in a clean 2 mL collection tube. The QIAamp mini column was placed in a clean 1.5 mL microcentrifuge tube and the old collection tube containing the filtrate was discarded. A

volume of 80 µL elution buffer (Buffer AVE) was carefully added to the QIAamp mini column and left to incubate at room temperature for 1 minute. It was then centrifuged at 8000 rpm for 1 minute. The 1.5 mL microcentrifuge was removed, cap closed and stored at -80 ^{o}C.

3.6 VIRUS DETECTION, REAL-TIME REVERSE TRANSCRIPTION PCR

The virus was detected from the extracted RNA following the protocol by Crowder *et al*., (2010). Master mix containing, 14.6 µL Superscript CCHFV master mix, 0.4 µl reverse transcriptase/Platinum Taq enzyme mix was added to each well in a 96 well plate. Five microliters RNA extract was added to each well and the plate sealed with a micro seal B adhesive. The real-time reverse transcription reaction and amplification of the viral RNA S segment was set up on the Applied Biosystems 7300 Real-Time PCR System. The cycling conditions included an initial melting temperature at 50 °C for 15 minutes followed by a cycle at 95 °C for 5 minutes and 45 cycles of amplification (5 seconds at 95 °C, 30 seconds at 60 °C). A final 30 second extension step at 40 °C. The superscript master mix consisted of 2X reaction mix, 1.0 mM forward primer and reverse primer and MGB probe (0.2 mM) and nuclease free water. The set of primers had less diversity; the forward primer which is made up of 20 nucleotides binds to nucleotide region from nucleotide 649 (5` GGA VTG GTG VAG GGA RTT TG 3`), and the reverse primer which is also 17 nucleotides long, (5` CAG GGT GGR TTG AAR GC 3`), binds to nucleotide region from nucleotide 705 on the S segment. They also, accounted for a significant amount of diversity within the CCHFV strains by the presence of the nonspecific letters (ambiguous nucleotides) included in the primer sequences (Koehler *et al*., 2017). The probe for the reaction was 6FAM-CAARGGCAARTACATMAT.

The results were then analysed. Pools that had cycle threshold (C_t) values of less than 29 were strong positives, an indicator of high amount amplified RNA content. Pools with C_t

values from 30 to 37 had moderate amount of amplified RNA. Pools with C_t values from 38 to 40 had very small amount of amplified RNA. Pools within all categories were identified as positive.

3.7 SAMPLE PREPARATION FOR NEXT GENERATION SEQUENCING

To further characterise the isolated CCHFV genome, next generation sequencing was done using the USAMRIID optimised RNA-Access protocol.

3.7.1 RNA Fragmentation

RNA quality was evaluated using nanodrop and fragmented for cDNA synthesis. C6/36 cells were added to samples with lower RNA concentration. Prior to addition of cells to the test samples, the cells were fragmented for 7 minutes. This was done by adding 0.5 µL of Elute Prime Fragment Mix (EPF mix) to 0.5 µL of cells RNA (C6/36) at 94 ^{o}C. The RNA was diluted with nuclease-free water to a final volume of 8.5 µL in a 96-well plate. Elute Prime Fragment High Mix of volume, 8.5 µL was added to the RNA. The contents in the plate was sealed and mixed thoroughly by shaking on a microplate shaker continuously at 1600 rpm for 20 seconds. The sealed plate was placed on the pre-programmed thermal cycler and incubated at 94 ^{o}C for 0-2 minutes to fragment and prime the RNA with random hexamers in the EPF mix. Prior to the incubation, the thermocycler was preheated to 100 ^{o}C. Degraded samples were not incubated, but rather moved immediately to the next stage which was to Synthesize First Strand cDNA

3.7.2 First strand cDNA synthesis

The adhesive seal was removed from the plate and 50 µL of SuperScript IV was added to 450 µL of thawed First Strand Synthesis Act D Mix tube. The mixture was mixed by gently swirling the tube and centrifuged briefly. First Strand Synthesis Mix Act D and

SuperScript IV mix of 8 µL was added to each well, the plate was sealed with a Micro Seal B and mixed thoroughly by shaking the plate on a microplate shaker continuously at 1600 rpm for 20 seconds. The sealed plate was incubated in a pre-programmed thermal cycler with the following program: pre-heat lid option set to 100 ºC, 25 ºC for 10 minutes, 42 ºC for 15 minutes, 70 ºC for 15 minutes, hold at 4 ºC. The next procedure was commenced immediately after the thermocycler reached 4 ºC.

3.7.3 Second strand cDNA synthesis

To each well, 5 µL resuspension buffer was added, 20 µL of thawed Second Strand Marking Master Mix was also added after the tube had been centrifuged. The plate was sealed with a Micro-Seal B and its contents thoroughly mixed by shaking it on a microplate shaker continuously at 1600 rpm for 20 seconds. The plate was incubated on a pre-heated thermal cycler at 16 ºC for 1 hour. The seal was removed, and the plate was left to cool for 2 minutes.

3.7.4 Sample clean-up

The tube containing the thawed AMPure XP beads was thoroughly mixed using a vortex to evenly disperse them. An amount of 90 µL of the beads were added to each well in the plate. The plate was sealed and its contents, thoroughly mixed by shaking on a micro plate shaker at 1800 rpm for 2 minutes. The plate was incubated at room temperature for 5 minutes and later moved onto a magnetic stand for 2 minutes. An amount of 135 µL of clear supernatant was discarded removed from each well. With the plate still on the stand, 200 µL of freshly prepared 80% ethanol was added to each well gently without disturbing the beads. The plate was left to stand for 30 seconds after which the 80% ethanol was discarded. Ethanol was added one more time and discarded as indicated earlier. Using a 10 µL multichannel pipette, remaining ethanol was removed from each well without disturbing the beads.

The plate was left to stand at room temperature for 5 minutes to dry on the magnetic stand. The plate was removed from the magnetic stand and 17.5 µL resuspension buffer was added to each well. The plate was sealed and shaken on the micro plate shaker at 1800 rpm for 2 minutes. The plate was incubated at room temperature for 2 minutes and centrifuged at 280 g for 1 minute. The seal was removed and the plate placed on the magnetic stand for 1 minute. A volume of 15 µL of clear supernatant was transferred from each well of the plate to a new plate.

3.7.5 3'-ends adenylation

A volume of 2.5 µL resuspension buffer was added to each well of the plate. Thawed A-Tailing Mix tube was centrifuged at 600 g for 5 seconds and 12.5 µL added to each well of the plate. Contents in the sealed plate were thoroughly mixed by shaking the plate on a microplate shaker at 1800 rpm for 2 minutes. The plate was centrifuged at 280 g for 1 minute. The plate was incubated in a thermocycler at 37 ^{o}C for 30 minutes. The plate was removed immediately and placed in another thermocycler to incubate at 70 ^{o}C for 5 minutes. The plate was immediately removed from the thermocycler after incubation and placed on ice for 1 minute.

3.7.6 Ligation of adapters

TruSeq dual index (TruSeq RNA Adapter Index) was diluted at a ratio of 1:1 with nuclease free water, 2.5 µL each of resuspension buffer and Ligation Mix was added to each well after which 2.5 µL of the diluted TruSeq RNA Adapter Index was also added. The plate was sealed with Micro-Seal 'B' and its contents thoroughly mixed by shaking on a microplate shaker at 1800 rpm for 2 minutes. The plate was centrifuged at 280 g for 1 minute and incubated in a thermocycler at 30 ^{o}C for 10 minutes.

After removing the adhesive seal covering the plate, 5 µL of Stop Ligation Buffer was added to each well to inactivate the ligation mix. The contents in the plate were thoroughly mixed by shaking on a microplate shaker at 1800 rpm for 2 minutes after the plate had been sealed. The plate was centrifuged at 280 g for 1 minute.

3.7.7 Sample clean-up

The tube containing the thawed AMPure XP beads was thoroughly mixed using a vortex to evenly disperse them. To each well in the plate, 42 µL of the beads were added, the plate was sealed. The contents were thoroughly mixed by shaking on a micro plate shaker at 1800 rpm for 2 minutes. The plate was incubated at room temperature for 5 minutes and moved onto a magnetic stand for 2 minutes. An amount of 79.5 µL of the supernatant was removed from each well and discarded. With the plate still on the stand, 200 µL of freshly prepared 80% ethanol was added to each well gently without disturbing the beads. The plate was left to stand for 30 seconds after which the 80% ethanol was removed and discarded. Ethanol was added one more time and discarded as indicated above. Using a 10 µL multichannel pipette, remaining ethanol was removed from each well without disturbing the beads.

The plate was left to dry at room temperature for 5 minutes on the magnetic stand. The plate was removed from the magnetic stand and 52.5 µL resuspension buffer added to each well. The plate was sealed and shaken on the micro plate shaker at 1800 rpm for 2 minutes. The plate incubated at room temperature for 2 minutes, centrifuged at 280 g for 1 minute. The seal was removed and the plate placed on the magnetic stand for 1 minute. A clear supernatant of 50 µL was transferred from each well of the plate to a new plate.

To each well in the plate, 50 µL of thawed and well mixed AMPure XP beads were added. The plate was sealed and its contents, thoroughly mixed by shaking on a micro plate

shaker at 1800 rpm for 2 minutes. The plate was incubated at room temperature for 5 minutes, moved onto a magnetic stand for 2 minutes. An amount of 95 µL of the supernatant was removed from each well and discarded. With the plate still on the stand, 200 µL of freshly prepared 80% ethanol was added to each well gently without disturbing the beads. The plate was left to stand for 30 seconds after which the ethanol was removed and discarded. Ethanol was added one more time and removed as indicated above. Using a 10 µL multichannel pipette, remaining ethanol was removed from each well without disturbing the beads.

The plate was left to dry on the magnetic stand at room temperature for 5 minutes. The plate was removed from the magnetic stand and 22.5 µL resuspension buffer was added to each well. It was sealed and shaken on the micro plate shaker at 1800 rpm for 2 minutes. The plate was incubated at room temperature for 2 minutes and centrifuged at 280 g for 1 minute. The seal was removed, and the plate placed on the magnetic stand for 1minute. A clear supernatant of 20 µL was transferred from each well of the plate to a new plate.

3.7.8 First PCR amplification

To each well in the PCR plate, 5 µL and 25 µL of thawed PCR Primer Cocktail and PCR Master Mix were added respectively. The plate was sealed and shaken on the micro plate shaker at 1600 rpm for 20 seconds. The plate was incubated at room temperature for 2 minutes, centrifuged at 280 g for 1 minute. The plate was placed in a thermocycler and amplification was done under the following conditions. The pre-heat lid option was selected and set to 100 ^{o}C, 98 ^{o}C for 30 seconds, 20 cycles of: (98 ^{o}C for 10 seconds, 60 ^{o}C for 30 seconds), 72 ^{o}C for 30 seconds, 72 ^{o}C for 5 minutes and hold at 4 ^{o}C.

3.7.9 Sample clean-up

To each well in the plate, 50 µL of the thawed and well mixed AMPure XP beads were added. The plate was sealed and its contents, thoroughly mixed by shaking on a micro plate shaker at 1800 rpm for 2 minutes. It was then incubated at room temperature for 5 minutes moved onto a magnetic stand for 2 minutes. An amount of 95 µL of the supernatant was removed from each well and discarded. With the plate still on the stand, 200 µL of freshly prepared 80% ethanol was added to each well gently without disturbing the beads. The plate was left to stand for 30 seconds after which the 80% ethanol was removed and discarded. Ethanol was added one more time and removed as indicated above. Using a 10 µL multichannel pipette, remaining ethanol was removed from each well without disturbing the beads.

The plate was left to stand at room temperature for 5 minutes to dry on the magnetic stand. It was removed from the magnetic stand and 17.5 µL resuspension buffer was added to each well. The plate was sealed and shaken on the micro plate shaker at 1800 rpm for 2 minutes. It was then incubated at room temperature for 2 minutes, centrifuged at 280 g for 1 minute. The seal was removed, and the plate placed on the magnetic stand for 1 minute. A clear supernatant of 15 µL was transferred from each well of the plate to a new plate.

3.7.10 Library validation

The concentration of the DNA library was measured in nanogram using a Qubit. DNA libraries with standard concentrations were first measured and the concentration of the DNA libraries of the samples were measured against those of the standards.

3.7.11 First hybridization

The volume was calculated to a final concentration of 200 ng in a final volume of 11.25 µL. The maximum volume required for a sample was 11.25 µL. Therefore, for samples

with volumes less than 11.25 µL, the volume was brought up to 11.25 µL with nuclease-free water. Also, samples whose final concentration could not reach 200 ng, their initial concentrations were used with a volume 11.25 µL was used. The reagents were added to each well in the 96 well plate in the following order to make a total volume of 25 µL. Library sample (DNA) - 11.25 µL, resuspended Capture Target Buffer 3 (CT3) - 12.50 µL and Capture Oligos 1X Stock - 1.25 µL. The plate was sealed with a Micro-seal 'B' adhesive seal and its contents thoroughly mixed on a microplate shaker at 1200 rpm for 1 minute and centrifuged at 280 g for 1 minute. The sealed plate was placed on a pre-programmed thermal cycler under the following conditions: Preheat lid at 100 ^{o}C, 10 minutes at 95 ^{o}C, 18 cycles of 1-minute incubation, starting at 94 ^{o}C, then decreasing 2 ^{o}C per cycle, 95 minutes at 58 ^{o}C.

3.7.12 First capture

The plate was centrifuged at 280 g for 1 minute. To each well in the plate, 62.5 µL of well mixed Streptavidin Magnetic Beads were added. Contents in the sealed plate were thoroughly mixed on a microplate shaker at 1200 rpm for 5 minutes. The plate was left to stand at room temperature for 25 minutes. The plate was centrifuged at 280 g for 1 minute, the adhesive tape removed, and the plate placed on the magnetic stand for 1 minute at room temperature. The supernatant was carefully removed and discarded from each well in the plate without disturbing the beads. The plate was removed from the magnetic stand.

3.7.13 First Wash

To each well in the plate, 50 µL of thoroughly mixed Enrichment Wash Solution was added. The entire contents in each well was gently pipetted up and down three times to ensure complete resuspension of the sample. The plate was sealed and its contents thoroughly mixed on a microplate shaker at 1800 rpm for 4 minutes. The sealed plate was incubated at 50 ^{o}C for 20 minutes in a thermocycler. The adhesive tape was removed, and the plate placed on the magnetic stand for 2 minutes at room temperature. Clear supernatant was carefully

removed and discarded from each well of the plate without disturbing the beads. The plate was removed from the magnetic stand. This process was repeated one more time to make a total of two washes.

3.7.14 First Elution

To make an elution pre-mix, the reagents were added to a new 1.5 mL microcentrifuge tube in the following order 7.12 µL Enrichment Elution Buffer 1 (EE1), 0.38 µL 2 M NaOH to make a total volume of 7.5 µL. Six microliters of elution pre-mix was added to each well. The plate was sealed and its contents thoroughly mixed on a microplate shaker at 1800 rpm for 2 minutes. The sealed plate was left to stand at room temperature for 2 minutes. The plate was centrifuged at 280 g for 1 minute. The adhesive tape was removed and the plate placed on the magnetic stand for 2 minutes at room temperature. Clear supernatant of about 5.25 µL was transferred from well into a new plate. The plate was removed from the magnetic stand. To neutralize the elution, 1 µL Elute Target Buffer 2 was added to each well of the new plate containing the samples. The plate was sealed and its contents thoroughly mixed on a microplate shaker at 1200 rpm for 1 minute. The plate was centrifuged at 280 g for 1 minute.

3.7.15 Second hybridization

Resuspension buffer of volume, 5 µL, 12.5 µL Capture Target Buffer 3 (CT3) and 1.25 µL Capture Oligos 1 X Stock were added to each well in the 96 well plate. The plate was sealed with a Micro-seal ‘B’ adhesive seal and its contents thoroughly mixed on a microplate shaker at 1200 rpm for 1 minute and centrifuged at 280 g for 1 minute. The sealed plate was placed on a pre-programmed thermal cycler under the following conditions: Preheat lid at 100 ^{o}C, 10 min at 95 ^{o}C, 18 cycles of 1-minute incubations, starting at 94 ^{o}C, then decreasing 2 ^{o}C per cycle, 95 minutes at 58 ^{o}C.

A second capture, second wash, and second elution were done following the same steps as the first capture, wash and elution procedures.

3.7.16 Capture sample clean-up

To each well of the plate, 11.25 µL of well-mixed AMPure XP beads was added. The plate was sealed with a Micro-seal 'B' adhesive seal, its contents thoroughly mixed by shaking it on a microplate shaker at 1800 rpm for 1 minute. It was then incubated at room temperature for 5 minutes and placed on the magnetic stand for 2 minutes. All the supernatant was removed from each well and discarded. With the plate on the magnetic stand, 200 µL freshly made 80% ethanol was slowly added to each well without disturbing the beads. The plate was left to stand at room temperature for 30 seconds. The 80% ethanol was carefully removed from each well and discarded. The 80% ethanol was added to the wells and removed again as stated above. This made up two washes. Using a 10 µL multichannel pipette, remaining ethanol was removed from each well without disturbing the beads. The plate was left on the magnetic stand to stand at room temperature for 5 minutes to dry. The plate was removed from the magnetic stand and 7 µL resuspension buffer was added to each well. The plate was sealed with a Micro-seal 'B' adhesive seal and its contents thoroughly mixed on a microplate shaker at 1800 rpm for 2 minutes. The plate was left to stand at room temperature for 2 minutes. The plate was centrifuged at 280 g for 1 minute, seal removed and left on the magnetic stand for 2 minutes at room temperature. Clear supernatant of 6.25 µL was carefully removed from each well without disturbing the beads and transferred into a new plate.

3.7.17 Second PCR amplification

In this process, the DNA library was enriched through PCR amplification for sequencing. To each well in the plate, 1.25 µL PCR Primer Cocktail and 5 µL Enhanced PCR Mix were added in that order. The plate was sealed with a Micro-seal 'B' adhesive seal and

its contents thoroughly mixed on a microplate shaker at 1200 rpm for 1 minute and centrifuged at 280 g for 1 minute. The seal was removed, and the plate placed on a pre-programmed thermal cycler under the following conditions: Preheat lid at 100 ºC, 98 ºC for 30 seconds, 17 cycles of: (98 ºC for 10 seconds, 60 ºC for 30 seconds, 72 ºC for 30 seconds), 72 ºC for 5 minutes. Hold at 10 ºC.

3.7.18 Second PCR clean-up

This process used AMPure XP beads to purify the enriched library and remove unwanted products. The same procedure used in the first PCR clean-up (section 3.7.19) was used here with a few changes to the volumes of AMPure XP beads (22.5 µL), and Resuspension buffer (11 µL). At the end of the clean-up, 10 µL clear supernatant was carefully removed from each well without disturbing the beads and transferred into a new plate.

3.7.19 Quality assessment of Library

To assess the quality of the enriched library, 1µL of the library was loaded on Agilent High Sensitivity DNA Chip. The size of the library was checked for distribution of DNA fragments with a size range from approximately 200 bp -1kb. All samples were normalized to 2 nm. Concentrations of the samples were measured in pg/µl using the bioanalyzer.

3.7.20 Library qPCR

To ensure that an accurate concentration of the samples was loaded for sequencing, each library was also quantified by quantitative PCR using the KAPA SYBER FAST Universal qPCR kit (Illumina) (KAPA Biosystems, KK4824). For a 10 µL reaction volume, 1.8 µL nuclease-free water, 6 µL 2X KAPA SYBR FAST qPCR Master Mix with primers, 2 µL diluted 1:1000/1:5000 pooled libraries and 0.2 µL 50X ROX Low were added to each

well on 1 cycle initial denaturation at 95 °C for 3 minutes, 40 cycles denaturation at 95 °C for 1-3 seconds and annealing/extension/data acquisition at 60 °C for 30 seconds

3.7.21 Normalization and pooling

All samples were normalized to the same concentration of 1 nm to have an equal representation of each sample. Sample concentrations were changed from pico-mole since that was the concentration of samples from qPCR to nano-mole and calculated as described below. Sample concentrations were normalized with the required volume of resuspension buffer.

Calculation with the dilution factor: (sample concentration) (dilution factor: 1000)/1000

(2.99 pmol) (1000 dilution factor)/1000 = 2.99 nm

In a 1.5 mL microfuge tube, the total volume of resuspension buffer needed to normalize each sample to 1 nm was added and the corresponding volume for the individual samples was added. The tube was briefly vortexed and centrifuged. This tube was labelled as tube 1.

3.7.22 MiSeq loading preparation

PhiX (product of bacteriophage Phi X174) was diluted from 10 nM to 2 nM PhiX using resuspension buffer with volumes, 1 µL 10 nM PhiX + 9 µL resuspension buffer. The tube was labelled 2; it was briefly vortexed and centrifuged. Using resuspension buffer, 2 M NaOH was diluted to 0.2 M NaOH using the volumes 2 µL of 2 M NaOH and 18 µL resuspension buffer. The tube was labelled as 3 and briefly vortexed and centrifuged.

In a new 1.5 mL tube labelled 4, 2 µL of diluted PhiX from tube 2 and 18 µL pool library from tube 1 was added, the content of the tube was mixed using a vortex and centrifuged. To tube 4, 20 µL of 0.2 M NaOH was added. The content of the tube was mixed using a vortex, centrifuged and incubated at room temperature for 5 minutes. To the same

tube labelled 4, 20 µL resuspension buffer was added. Also, 940 µL pre-chilled HT1 buffer was added to make up 1000 µL. The content of the tube was mixed using a vortex, centrifuged and kept on ice. To dilute the library to a final concentration of 20 pM, 400 µL pre-chilled HT1 was added to 600 µL of library from tube 4 into a new tube labelled 5. The diluted library from tube 5 was loaded into the sample compartment of the thawed MiSeq cartridge. The cartridge was loaded onto the MiSeq desktop sequencer (Illumina).

3.8 STATISTICAL ANALYSIS

For categorical variables, chi-square was used to determine if there was any statistical association between the various sites. The statistical significance level was set at p-value <0.05 with a degree of freedom of 2. Bar charts were used to describe the tick species distribution and animal sources of ticks across the seven study sites. Bar chart was also used to compare the number of ticks collected from the two ecological zones. Tables were used to describe tick species distribution, tick sex and animal source of ticks between the two ecological zones.

The infection rate of the virus in pools of different species was detected using PoolScreen 2.0. Software Version 2.0.1 (Katholi and Unnasch, 2006). This calculation was done at 95% confidence interval and it included the pool size used, the number of pools studied and the number of negative pools.

CHAPTER FOUR

4.0 RESULTS

4.1 TICK COLLECTION

A total of 1,813 ticks were collected and grouped into 813 pools. Ticks collected from the Guinea savannah ecological zone made up 70% of the total number whereas, ticks collected from the Coastal savannah made up 30%. The difference in the proportion of ticks from the two ecological zones was statistically significant (P<0.01), this difference shows that there is an association between tick abundance and ecological zones Figure 4. 1 gives an overview of the total number of tick distribution across the two ecological zones.

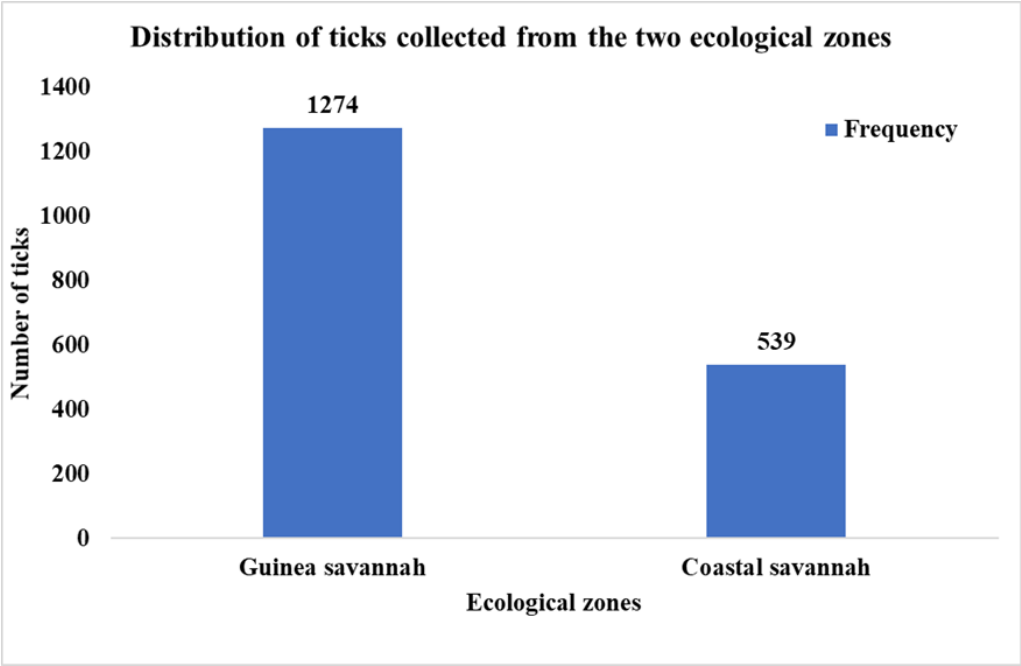

Figure 4. 1: Distribution of ticks from the two ecological zones, blue bars show the number of ticks collected from the ecological zones. Comparison of tick distribution using Chi square at a p value of <0.05.

Male ticks accounted for 72% of the total number of ticks collected whereas females recorded 28% (Table 4. 1). Across the seven study sites, more male ticks were collected than female ticks. The highest number of males and females were collected from the Tamale Airborne force barracks (16%) and Navrongo (10%) respectively. Michel camp (3%) and Kamina barracks (1%) recorded the lowest number of males and females respectively. Since both male and female ticks can transmit both CCHFV and Dugbe virus, the difference in the number of male and female ticks collected is not statistically significant in CCHFV infection rate. The number of male ticks collected was statistically significant ($p<0.01$).

Table 4. 1: Distribution of tick sex across the two ecological zones

Vector sex	Guinea savannah	Coastal savannah	Total
Female	298	211	509
	23.39	39.15	28.08
Male	976	328	1,304
	76.61	60.85	71.92
Total	1274	539	1,813
	70.27	29.73	100

At 95% confidence interval and p value of less than 1, there was a significant difference in the tick species collected from each ecological zone. *Amblyomma variegatum* represented about 66% of the tick species collected from all the ecological zones making it the highest number of tick species collected and *Rhipicephalus boophilus*, the least number of tick species collected. Ticks of the *Rhipicephalus* genus, represented about 24% of total

number of ticks collected. Most of the ticks in this genus were in the *sanguineus* species. The genus *Hyalomma* made up about 10% of the total ticks collected making them the least number of ticks collected (Table 4. 2).

Table 4. 2: Distribution of tick species across the ecological zones.

Morphological ID	Guinea savannah	Coastal savannah	Total
Amblyomma variegatum	751	449	1,200
	58.95	83.3	66.19
Hyalomma rufipes	12	40	52
	0.94	7.42	2.87
Hyalomma truncatum	126	1	127
	9.89	0.19	7
Rhipicephalus sanguineus	372	41	413
	29.2	7.61	22.78
Rhipicephalus boophilus	0	1	1
	0	0.19	0.06
Rhipicephalus evertsi	13	7	20
	1.02	1.3	1.1
Total	1274	539	1813
	70.27	29.73	100

From all the seven study sites, *Amblyomma variegatum* was the highest number of tick species collected except for Navrongo where 95.14% of the ticks collected were of the *Rhipicephalus* genus. Ticks from the *Amblyomma variegatum* species and the *Hyalomma* genus were detected in all seven study sites whereas the *Rhipicephalus* genus was detected in only four study sites namely, Navrongo, Burma camp, Michel camp and Shai hills (Figure 4. 2 and Figure 4. 3). The difference in the proportion of the various species of ticks from the seven sites was statistically very significant (P<0.01). This is an indication that there is an association between the study sites/location and the tick species. This also correlates to the ecological zones and tick species.

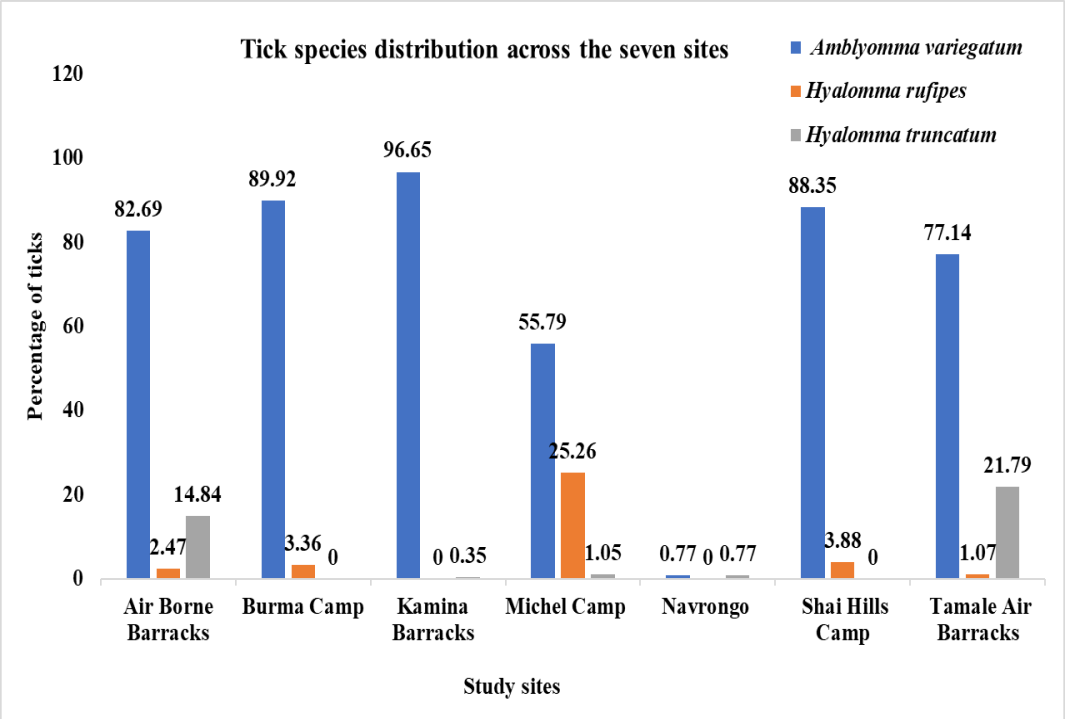

Figure 4. 2: Tick species distribution across the seven study sites. *Amblyomma variegatum*, *Hyalomma rufipes*, H*yalomma truncatum*. Comparison of tick species distribution using Chi square at a p value of <0.05.

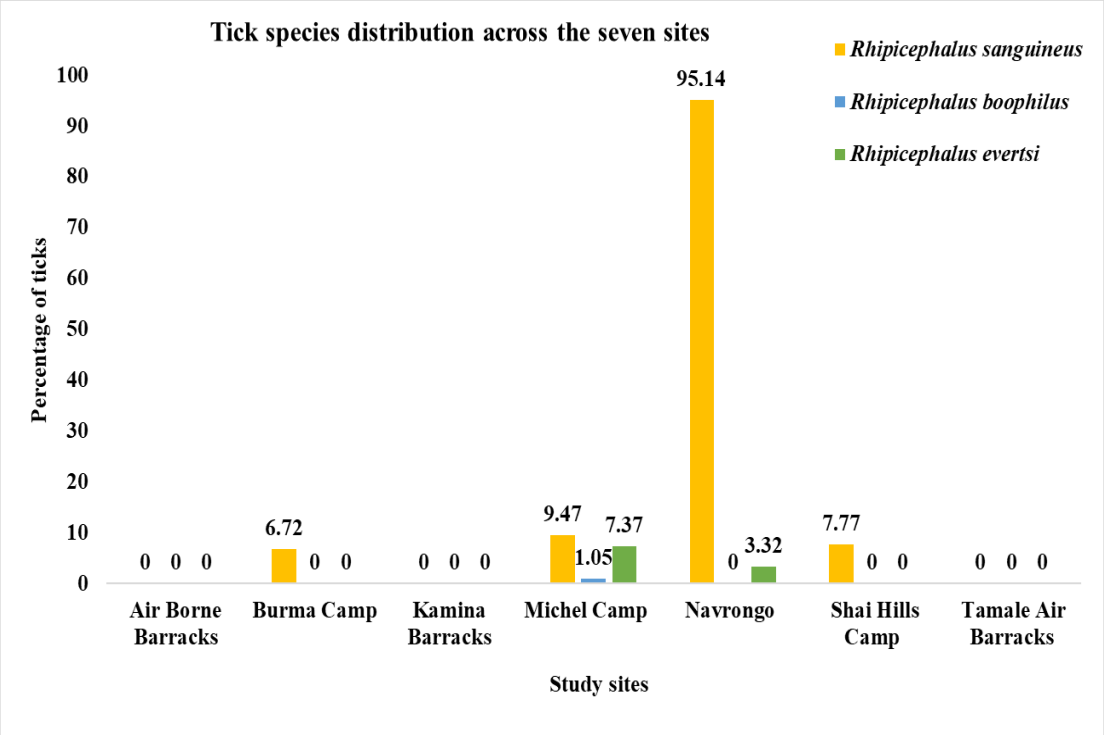

Figure 4. 3: Tick species distribution across the seven study sites. *Rhipicephalus* sanguineus, *Rhipicephalus boophilus*, *Rhipicephalus* evertsi. Comparison of tick species distribution using Chi square at a p value of <0.05.

The animal sources of ticks in this study were sheep, cattle, dogs and goats. In all, cattle were the highest source of ticks (82%), dogs were the second highest (17%), and the least number of ticks collected were from goats and sheep, 0.4% and 0.6%, respectively Table 4. 3. Ticks were collected from only cattle in all the six military sites; however, in Navrongo, ticks were collected from all four animal sources (Figure 4. 4). The highest number of ticks collected in Navrongo were from dogs (80%) and this made up 17% of the total number of ticks collected. Navrongo also recorded a much lower number of ticks from cattle (n=60).

Table 4. 3: Distribution of animal sources of ticks across the two ecological zones.

Animal source	Guinea savannah	Coastal savannah	Total
Cattle	943	539	1,482
	74.02	100	81.74
Dog	314	0	314
	24.65	0	17.32
Goat	7	0	7
	0.55	0	0.39
Sheep	10	0	10
	0.78	0	0.55
Total	1274	539	1,813
	70.27	29.73	100

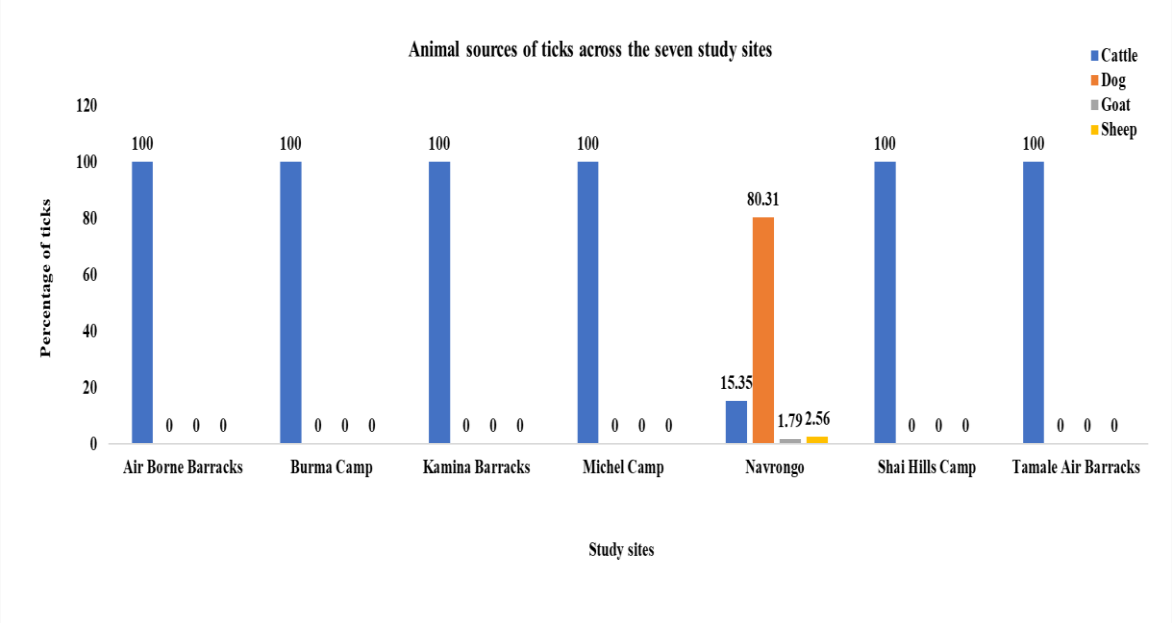

Figure 4. 4: Distribution of animal source of ticks from the seven study sites. Cattle, Dog, Goat, Sheep.

Comparison of tick hosts using Chi square at 95% confidence interval.

4.2 CCHFV DETECTION

Of the total number of ticks collected (1,813) ~1% (n=15) tested positive for CCHFV. Seven pools (0.9%) of the 813 pools tested positive for the presence of the CCHFV. Table 4. 4 shows the positive pools, the site from which they were collected, animal source, tick species and vector sex. The cycle threshold (Ct) values for the 7 positive pools ranged from strong positives to weak positives are indicated in Figure 4. 5 and Figure 4. *6* below.

Table 4. 4: Details of CCHFV positive pools

	Region	Study site	Host	Tick species	Vector sex	Pool size
1.	**Greater**	Shai hills	Cattle	*Hyalomma rufipes*	Female	1
2**	**Accra**	Michel camp	Cattle	*Amblyomma variegatum*	Male	2
3.		Airforce base	Cattle	*Amblyomma variegatum*	Male	2
4.	**Northern**	Airforce base	Cattle	*Amblyomma variegatum*	Male	3
5.		Navrongo	Sheep	*Rhiphicephalus sanguineus*	Male	2
6.	**Upper East**	Navrongo	Dog	*Rhiphicephalus sanguineus*	Male	2
7.	**Region**	Navrongo	Dog	*Rhiphicephalus sanguineus*	Male	3

** Even though sample two initially tested positive for CCHFV, in a second assay which had more specific CCHFV primers showed ct values that gave an indication of low CCHFV RNA content. Also, full genome sequence showed reads of Dugbe virus sequence. This could mean that the pool was coinfected.

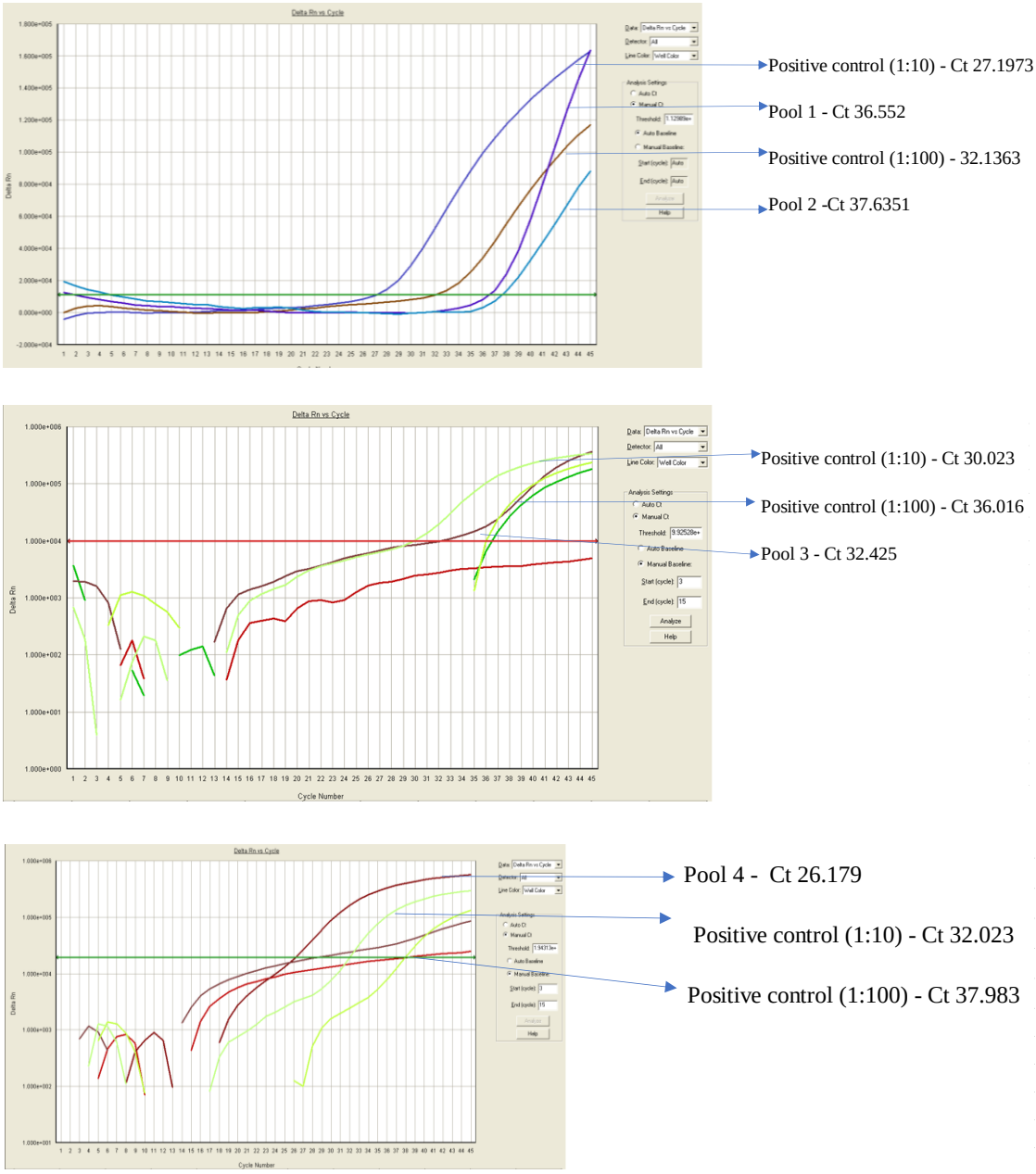

Figure 4. 5: Positive curves for pools 1, 2, 3 and 4 from the real-time RT-PCR. CCHFV detection assay targeting the S segment of genome (Koehler *et al*, 2017).

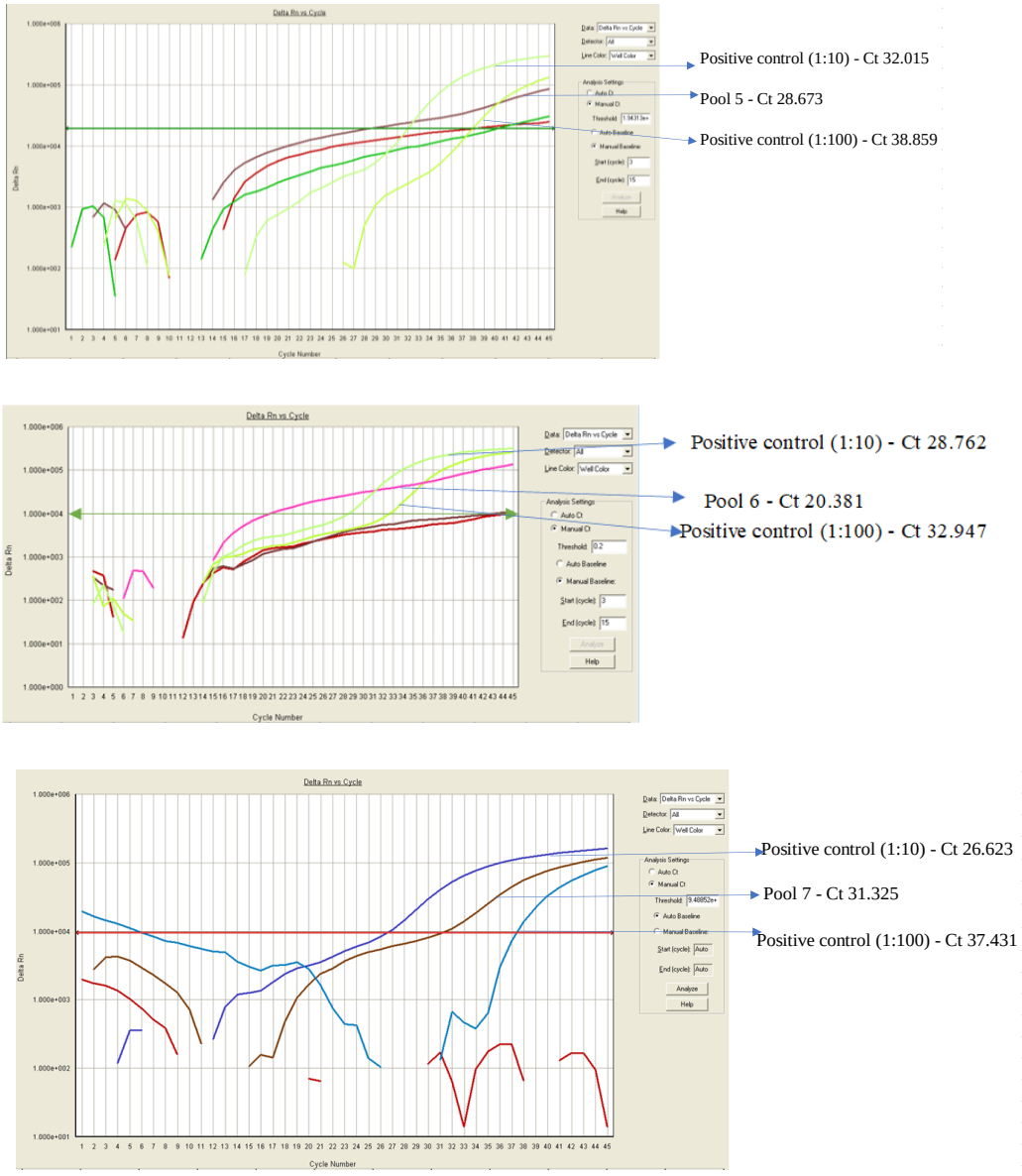

Figure 4. 6: Positive curves for pools 5, 6 and 7 from the real-time RT-PCR. CCHFV detection assay targeting the S segment of genome (Koehler *et al*, 2017).

4.3 CCHFV INFECTION RATE

Hyalomma rufipes recorded the highest rate of infection among the three species that tested positive for CCHFV. Even though *Rhipicephalus sanguineus* and *Amblyomma variegatum* were the highest number of CCHFV infected ticks, they had the lower infection rates of 0.70% and 0.25%, respectively (Table 4. 5).

Table 4. 5: Prevalence of CCHFV among different tick species collected from Greater Accra, Northern and Upper East region

Tick species	Male (%)	Female (%)	Overall infection rate (%)
Amblyomma variegatum	0.30 (0.06[a]-0.88[b])	0	0.25 (0.05[a]-0.72[b])
Hyalomma rufipes	0	4.17 (0.13[a]-19.68[b])	1.87 (0.06[a]-9.34[b])
Rhipicephalus sanguineus	1.55 (0.30[a]-4.45[b])	0	0.70 (0.14[a]-2.02[b])

[a, b] Minimum and maximum likelihood of infection respectively

4.4 NEXT GENERATION SEQUENCING AND PHYLOGENETIC ANALYSIS

To further confirm and identify the isolated viral genome as that of CCHFV, next generation sequencing was done using the USAMRIID RNA-Access protocol. This protocol aimed at targeting unknown RNA viral genome with a variety of viral probes. Analysis of the sequencing data from the PCR positive CCHFV pools showed reads from one pool (sample 2) aligning to the full genome sequence of each segment of Dugbe virus as shown in Figure 4. 7 and Figure 4. *8*. No reads specific for CCHFV or Dugbe virus were detected in the remaining six PCR-positive pools. The six pools tested positive for CCHFV after a second run. The detection of Dugbe virus genome in one of the pools confirmed a theory of CCHFV and Dugbe virus coinfection.

Sequence alignment using muscle and phylogenetic analysis were done on the sequenced data using MEGA6 software. The L and S segments of the Dugbe virus was phylogenetically analysed with Dugbe virus strains and reference strains from the

Orthonairovirus genus and Nairoviridae family. Using maximum likelihood tree with bootstrap value of 1000, the L and S segments of the detected Dugbe virus had closer relationships with Dugbe virus strains from Kenya and Nigeria.

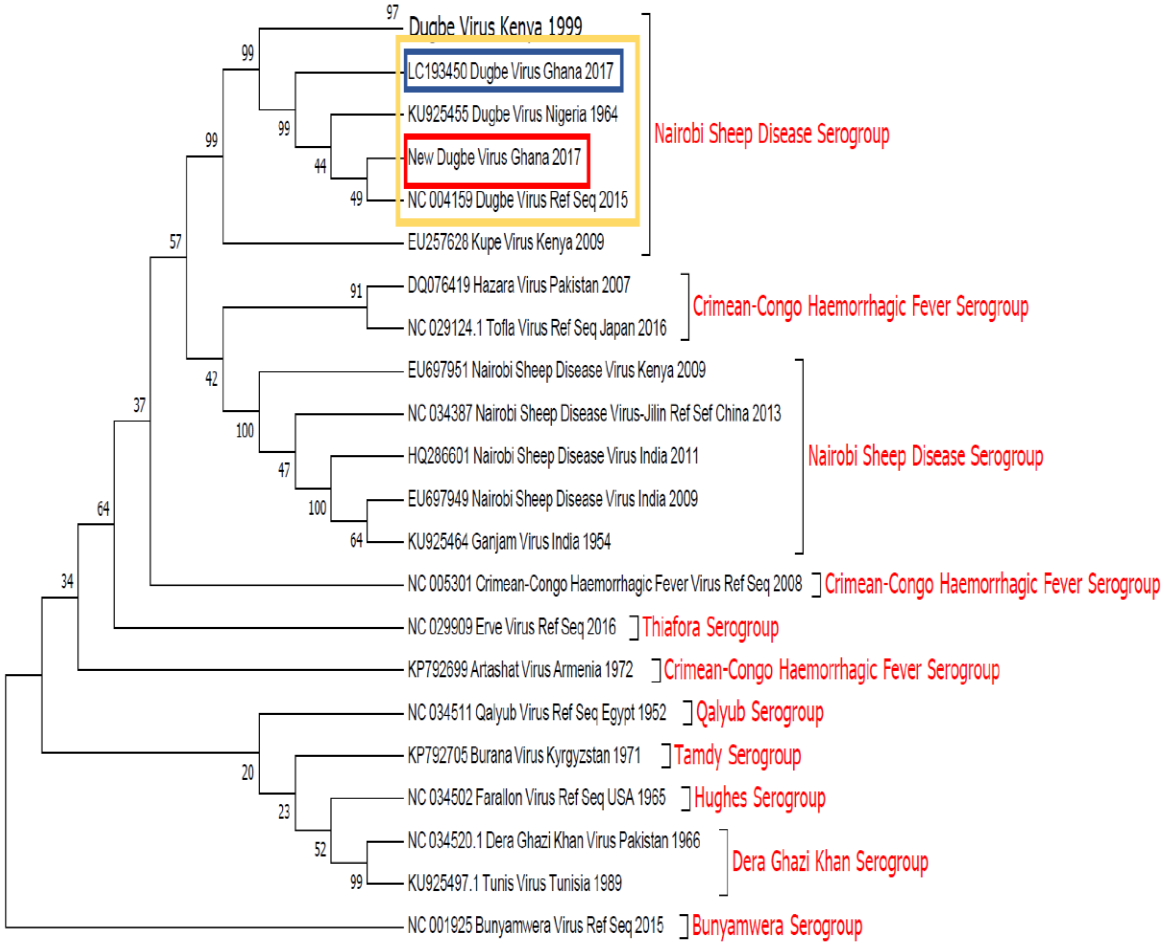

Figure 4. 7: Phylogenetic tree illustrating the relationship between the L segments of the new Dugbe virus detected in Ghana, other Dugbe virus strains and other viruses in the Nairoviridae family. The tree was obtained using MEGA 6 software from MUSCLE sequence alignment. The new Dugbe virus detected in the red box. The first virus detected in Pokuase blue box. Bunyamwera virus is the root of the tree. Strains that are closely related are highlighted in the yellow box.

Figure 4. 8: Phylogenetic tree illustrating the relationship between the S segments of new Dugbe virus detected in Ghana, other Dugbe virus strains and other viruses in the Nairoviridae family. The tree was obtained using MEGA 6 software from MUSCLE sequence alignment. The new Dugbe virus detected in red box. Bunyamwera virus is the root of the tree. Strains that are closely related are highlighted in the yellow box.

CHAPTER FIVE

5.0 DISCUSSION

It is well documented that CCHFV distribution is directly proportional to the abundance or presence of the tick vector (Sang *et al*., 2011). However, there is an uncertainty of the actual global distribution of CCHFV. There is little information in literature that can map out the true distribution of CCHF, possibly due to a lack of cases reported. This project aimed to acquire a better understanding of CCHFV circulating in Ghana and assess its genetic lineage. The detection of CCHFV in Ghana, 2016 contributed in filling in a knowledge gap since CCHF was previously listed as undetermined in literature (Bente *et al*., 2013). The field collected ticks that tested positive for CCHFV with real-time RT-PCR in this surveillance effort contributed more data points that agreed with the findings in Akuffo *et al*., (2016).

5.1 ECOLOGICAL ZONES AND TICK DISTRIBUTION

During this study, three tick genera were detected in the country across the two ecological zones sampled. The ecological zones sampled were; coastal savanna which covers the Greater Accra region and guinea savanna which covers the Northern and parts of the Upper East regions. The coastal savanna records an annual rainfall of 600 mm. The guinea savanna records an annual rainfall of 1000 mm.

Previously reported by Ntiamoa-Baidu *et al*., (2004); five genera of ticks are distributed across the ecological zones of Ghana. The five genera are, *Amblyomma*, *Ixodes, Haemaphysalis, Hyalomma,* and *Rhipicephalus*. The high abundance of *Amblyomma* and *Rhipicephalus* genera found in both ecological zones supports what is already described by Ntiamoa-Baidu *et al*., 2004. The study also indicated that the most commonly found tick genera in the guinea savannah zone; *Rhipicephalus, Amblyomma, Ixodes, Hyalomma.*

Rhipicephalus, *Amblyomma*, *Haemaphysalis*, and *Ixodes* genera are commonly found in the coastal savannah. Again, *Haemaphysalis* and *Ixodes* were not found in the guinea savannah zone. The high abundance of *Amblyomma variegatum* in all seven study sites is an indication of how widely distributed this species is across the two ecological zones.

In the rainy season, the soil is moist and provides a suitable environment for female ticks to oviposit. Environmental conditions such as high temperatures that occur in the dry season provide ideal conditions to promote transitions from larval stages to adult and provides optimal breeding habitat. This was one of the reasons ticks were collected in the dry season (December) to the beginning of the rainy season (March) in Accra. Collections in the Northern and Upper East regions were done from August before the second rainy season through the dry season in December to March the following year, right before the rains began, to capitalize on the high abundance of the tick population. The guinea savannah zone experiences longer dry season than rainy season, whereas the coastal savannah experiences longer rainy seasons than dry seasons. The seasonality difference in the guinea savannah and coastal savannah ecological zones of the country may be one of the major factors why more ticks were collected from the Northern and Upper East regions than the Greater Accra region.

The species from *Amblyomma*, *Hyalomma*, and *Rhipicephalus* genera have the highest impact on human and animal health. These three genera are among the five genera previously documented as CCHFV vectors and implicated for CCHFV transmission. This study confirms what is reported in Sang *et al*., (2011); that the presence or abundance of these vectors in Ghana could mean that the virus is also present. This could also lead to the assertion that the CCHFV vector can also be carrying other disease-causing pathogens that can affect both humans and animals. If the vector burden is too high, it could cause major economic loss to a nation by affecting human and animal health and productivity (Rajput *et al*., 2006; Vesco *et al*., 2011).

5.2 VECTOR HOST

At the six military sites, ticks were not found on household animals or livestock (sheep, goats, dogs). Most of the collected ticks were from cattle because most of the other smaller and household animals screened for ticks did not have any ectoparasites. The absence of ticks on the animals was because they do not graze or sleep outside of the household compounds. Owners explained that the animals are always kept in confined spaces and they do not wonder around to pick up ectoparasites. Also, animals are routinely treated with acaricides to prevent ticks, fleas and other pests. Veterinarians from the Ghana Army that were assisting with the tick collections routinely visit various households in the military barracks to examine and treat animals. These routine check-ups help maintain healthy livestock. Acaricide treatment of cattle in the military kraals were frequent (twice in a month) in the Greater Accra region, but once a month in the Northern region, this could explain why more ticks were sampled from the Northern region than the Greater Accra region.

In the more rural part of Navrongo, cattle and dogs were the highest source of ticks than any other animal source. For the security of the cattle herds, dogs join the herd when they are grazing from one area to another. Since the dogs are roaming in the same areas as the cattle, they are exposed to the same potential ectoparasites that the cattle encounter. This could most likely be the reason the Navrongo dogs had ticks. Livestock in Navrongo were mostly owned by civilians and therefore not regularly treated for ectoparasites and a veterinarian may not be called unless an animal is sick. Cattle in the Navrongo area were also not kept in kraals like on the military camps. The military kraals tend to be far away from households, but in Navrongo the common practice is to have cattle either staked in a field or allowed to graze freely during the day and then kept in pens that are directly adjacent to the households of the cattle caretakers or owners at night. These practices in Navrongo do not only increase the exposure of the cattle to ticks, but also increase the potential risk of tick-

borne illnesses to the families and other household animals since they all sleep in close proximity (Camitas *et al*., 1990).

This project focused on domestic animals that were in close proximity to humans. However, based on the finding of this surveillance effort the hope is that a further investigation is conducted that will include the screening of wild animals.

5.3 CCHFV DETECTION

Sample 2 may have been co infected with CCHFV and Dugbe virus. There may have been a higher amount of Dugbe virus genomic material in the pool that may have resulted in the amplification of only Dugbe virus during the sequencing process. This may explain why the sample tested positive for CCHFV after repeated tests. The similarity in genome sequences of orthonairoviruses may have resulted in the first assays reading as positive. This could be attributed by the fact that Dugbe virus and CCHFV belong to the same genus, *Orthonairovirus* (Bente *et al*., 2013). The set of primers used in the detection assay reduced the possibility of false positives due to the similarities between the two viruses. The benefit of the false positives was the incidental detection of Dugbe virus in sample 2. The remaining six pools not showing any reads from the sequencing may have resulted from the low viral load within the ticks.

In library preparation for next generation sequencing, nucleic acid concentrations were detected for all positive samples. For samples with low nucleic acid concentration, their concentrations were enriched with C6/36 mosquito cells. This was to help increase the nucleic acid content after PCR amplification. The DNA library was validated at different stages of the process to ensure that there was ample concentration of DNA in the library and to also ensure that the DNA has not been fragmented to smaller fragments. During the entire

sample preparation process, all data analyzed showed that the library was not compromised. Also, the PhiX control added to the library pool provided 100% diversity because, it contains 25% each of AGT and C. PhiX was also used in troubleshooting problems with cluster generation to ensure that the library preparation was correct. These procedures were to increase the confidence that all the samples were properly sequenced (Illumina, 2017). Having both confidence in the library preparation and the modified real-time RT-PCR CCHFV assays it is speculated that the positive CCHFV ticks could not be sequenced because there simply was not enough virus material present in the ticks to sequence but was enough to detect.

The non-detection of reads from the CCHFV positive samples could be attributed to viral RNA degradation or fragmentation (smaller fragments) making it impossible for the sequencer to pick up reads. Although high concentrations of nucleic acids were detected during the various library validation stages in the protocol, the concentrations recorded may not have been from the CCHFV genome but rather some other nucleic acid from the ticks (Giorgi *et al*., 2013). The RNA extract may have degraded due to power fluctuations. Also, viral detection assays were done a week after RNA had been extracted. Sample 2 may have been coinfected with CCHFV and Dugbe virus, but only Dugbe virus genome was detected for the same reason as the other samples.

5.4 CCHFV INFECTION RATE AND VECTOR DISTRIBUTION

The favourable weather conditions and presence of the tick species in the country (Ntiamoa-Baidu *et al*., 2004) is a cause of concern for public health and safety (Chinikar *et al*., 2012; Shayan *et al*., 2015). The global distribution of CCHFV is closely similar to the global distribution of ixodid ticks especially *Hyalomma* ticks (Ergönül, 2006). The high

infection rate (1.87%) recorded by *Hyalomma rufipes* in this study which is similar to 2% infection rate in Kenya, supports what is known in literature that *Hyalomma* ticks have a high infection rate (Sang *et al*., 2011). Even though *Amblyomma* and *Rhipicephalus* genera have also been implicated in CCHFV distribution, (Messina *et al*., 2015; Akuffo *et al*., 2016), *Hyalomma rufipes* stands as the tick species with the highest potential of infecting both humans and animals (Bente *et al*., 2013). This surveillance effort detected CCHFV in ~1% of 1,813 field collected ticks across two ecological zones demonstrating that there is a dispersion of the virus across the guinea savannah and coastal savannah zones.

Comparing the infection rates of CCHFV in both males and females from *Amblyomma variegatum, Rhipicephalus sanguineus* and *Hyalomma rufipes*, reflects that even though *Amblyomma variegatum* species were the highest number of ticks; their potential to cause infection is less as compared to *Hyalomma rufipes.* A contributing factor is that species with high sample size or a larger pool size, require more positive pools in order to have a high infection rate. Species with smaller pool size as a result of a small sample size do not require a high number of positives to have a high infection rate. This would conclude that, even for a small number, their potential to cause infection is high, and so a person stands the risk of infection when exposed to fewer numbers of *Hyalomma rufipes* bites. In a larger population, a person will have to be exposed to a relatively larger number of tick bites from *Amblyomma variegatum, Rhipicephalus sanguineus* before he/she can be infected.

These CCHFV infected ticks may not have contracted the virus from the animals they were collected from, but from other animals that co inhabit with them in their households. This may also explain why positive pools were from sheep. Since Navrongo is close to the Ghana-Burkina Faso border, which is an entry for livestock, infected ticks may have been transported from Burkina Faso into Navrongo through the cattle trade routes. Based on the CCHFV enzootic and epizootic-epidemic life cycle, other ticks in Ghana may be maintaining

the virus after infection. To address the possibility of tick-borne diseases in livestock and for public health concern, livestock being transported into Ghana through the various border towns should be screened for infectious diseases such as CCHF. Also, a program should be implemented to examine animals for the presence of ticks with a system to analyze any ticks collected for the presence of infectious pathogens such as CCHFV could serve as a bio-indicator for the early detection of potential health risk.

5.5 PHYLOGENETIC ANALYSIS OF DUGBE VIRUS

Dugbe virus is in the same Nairovirus family and *Orthonairovirus* genus with CCHFV (Sang *et al*., 2011). The virus belongs to the Nairobi sheep disease serogroup (Crabtree *et al*., 2009). After its first detection in *Amblyomma variegatum* ticks in Nigeria in 1964, it has been detected in other African countries; Chad, Egypt, Cameroon, Kenya, Uganda, Central African Republic, Ethiopia, Senegal and Sudan (Hoogstraal, 1979; Darwish *et al*., 1976). This study provided the first full Dugbe virus genome sequenced in Ghana. A recent study in Ghana detected Dugbe virus in *Amblyomma variegatum* ticks in Accra (Kobayashi *et al*., 2017). The L segment, which was the only sequenced segment of the virus genome from that study showed a close relationship to the Dugbe virus reference strain isolated from Kenya.

Phylogenetic analysis in this study was based on the large and small segments of the virus RNA. This is because, the medium segment encodes glycoproteins that are used by the virus to identify host cell surface proteins and also attach to these cell surfaces (Connolly-Andersen *et al*., 2009). These proteins are therefore a target for host immune defence mechanisms and must therefore be modified from time to time to evade recognition by the

host immune cells (Garrison *et al*., 2013). Due to this frequent modification, it is not always used in phylogenetic analysis.

Phylogenetic trees for the two viral sequences were rooted with Bunyamwera virus of the Nairovirus family and Bunyamwera serogroup (Gerrard *et al*., 2004). The trees were rooted with this virus because it is seen as the prototype for other viruses in this family (Odhiambo *et al*., 2016). Maximum likelihood trees with 1000 bootstrap replications were derived as shown in Figure 4. 7 and Figure 4. *8* . The complete L sequence of the virus provided in this study reflects a close relationship (99%) with the Dugbe strain (L segment) found in Ghana by Kobayashi's team (LC193450). The L segment is also found in the same clade as LC193450, Nigeria Dugbe virus strain and Kenya Dugbe virus strain (reference Dugbe virus strain). The S segment is closer to Dugbe strain from Nigeria, AF434163, which is in the same clade with Dugbe virus strain from Senegal, Kenya, and Nigeria. More importantly, all two sequences, (L and S segments) reported a close relationship with the Dugbe virus reference strains from Kenya and Nigeria.

Based on the close relationship of the two segments with other East and West African strains, we may conclude that the virus may have originated from any of these countries. Also, the L segment of the new detected virus was in the same clade as the strain detected earlier in Ghana by Kobayashi and his team, (LC193450). Since both strains were detected in Ghana, we may conclude they both could have originated from the same strain.

5.6 LIMITATIONS

The results of the study are not a full representation of the country because the middle belt was not covered. Pesticide treatment of the livestock especially in the study sites in the Greater Accra region affected the true distribution of ticks collected there. The un-specificity of the primers used in the CCHFV detection assay affected the results therefore much emphasis must be placed on the primer design.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATION

6.1 CONCLUSION

This study shows that CCHFV and Dugbe viruses are present in the country. Furthermore, this is the first time a full Dugbe virus genome has been sequenced in Ghana, and this study gave information on the genetic linkage of the Dugbe virus detected in Ghana to the other strains detected in Africa and other regions of the world. The detected Dugbe virus may have originated from the same virus from which LC193450 was sequenced. Both viruses have a close relationship with Kenya and Nigeria Dugbe virus strains. Additionally, CCHFV infection rate in *Hyalomma rufipes* is higher than the infection rate of other tick species in the Upper East, Northern and Greater Accra regions of Ghana.

6.2 RECOMMENDATION

Further investigation into the prevalence of Dugbe virus and CCHFV in Ghana needs to be done. Serum of livestock from sites where positive pools were detected have to be tested for CCHFV and Dugbe virus. Also, serum from the animal farmers and herdsmen have to be tested for CCHFV and Dugbe virus. The study has to be broadened to include other households that are not on military barracks and also parts of the middle belt of the country especially Kumasi where CCHFV antibodies have been detected in abattoir workers.

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APPENDICES

APPENDIX 1

Global positioning system coordinates for the seven study sites

Regions	Study sites	GPS Coordinates		
		Type	Latitude	Longitude
Greater Accra- Accra	Army Recruit Training School, Shai Hills	DD	5.87948	0.032874
		DMS	N 5° 52' 46.128"	E 0° 1' 58.346"
	5BN, Burma Camp	DD	5.603544	0.155419
		DMS	N 5°36'12.8"	W 0°09'19.5"
	1BN, Michel Camp	DD	5.732687	-0.041898
		DMS	N 5° 43' 57.673'	W 0° 2' 30.832"
Northern Region (Tamale)	Air Borne Force, Tamale	DD	9.542479	-0.856517
		DMS	N 9° 32' 32.924'	W 0° 51' 23.461"
	Air Force Base, Tamale	DD	9.546874	-0.855683
		DMS	N 9° 32' 48.746"	W 0° 51' 20.458"
	6BN, Kamina Barracks-Tamale	DD	9.46842	-0.85396
		DMS	N 9° 28' 6.312"	W 0° 51' 14.256"
Upper East Region	Navrongo	DD	10.97986559	-0.940994779
		DMS	N 10° 53' 5"	W 1° 05' 25"

APPENDIX 2

Total number of tick species and tick sex collected from animals from the three regions

Tick species	Animal source	Northern region		Upper East region		Greater Accra region		Total
		Male	Female	Male	Female	Male	Female	
Amblyomma variegatum	Cattle	677	71	0	1	303	146	**1198**
	Dog	0	0	2	0	0	0	**2**
Total		**677**	**71**	**2**	**1**	**303**	**146**	**1200**
Hyalomma rufipes	Cattle	**8**	**4**	**0**	**0**	**20**	**20**	**52**
Hyalomma truncatum	Cattle	**86**	**37**	**0**	**3**	**1**	**0**	**127**
Rhipicephalus sanguineus	Cattle	0	0	25	29	4	37	**95**
	Dog	0	0	167	145	0	0	**312**
	Goat	0	0	0	6	0	0	**6**
Total		**0**	**0**	**192**	**180**	**4**	**37**	**413**
Rhipicephalus evertsi	Cattle	0	0	2	0	0	7	**9**
	Sheep	0	0	8	2	0	0	**10**
	Goat	0	0	1	0	0	0	**1**
Total		**0**	**0**	**11**	**2**	**0**	**7**	**20**
Rhipicephalus boophilus	Cattle	**0**	**0**	**0**	**0**	**1**	**0**	**1**
Overall Total		**771**	**112**	**205**	**186**	**329**	**210**	**1,813**

APPENDIX 3

Total number of ticks collected from animals from the seven study sites in the three regions

Region	Study site	Host				Total
		Cattle	Dog	Goat	Sheep	
Northern region	Air borne force	364	0	0	0	**364**
	Kamina Barracks	239	0	0	0	**239**
	Air force base	280	0	0	0	**280**
Northern region Total		**883**	**0**	**0**	**0**	**883**
Upper East region	Navrongo	**60**	**314**	**7**	**10**	**391**
Greater Accra region	Burma camp	238	0	0	0	**238**
	Michel camp	95	0	0	0	**95**
	Shai hills	206	0	0	0	**206**
Greater Accra region Total		**539**	**0**	**0**	**0**	**539**
Over all total		**1,482**	**314**	**7**	**10**	**1,813**

APPENDIX 4

Total number of tick sex collected from the seven study sites in the three regions

Region	Study site	Male	Female	Total
Northern region	**Air borne force**	301	63	**364**
	Kamina Barracks	223	16	**239**
	Air force base	244	36	**280**
Northern region Total		**768**	**115**	**883**
Upper East region	**Navrongo**	**208**	**183**	**391**
Greater Accra region	**Burma camp**	132	106	**238**
	Michel camp	60	35	**95**
	Shai hills	136	70	**206**
Greater Accra region Total		**328**	**211**	**539**
Overall Total		**1,304**	**509**	**1,813**

APPENDIX 5.1

Total number of tick species from seven study sites in the three regions

Region	Study site	*Amblyomma variegatum*	*Hyalomma rufipes*	*Hyalomma truncatum*	Total
Northern region	Air borne force	301	9	54	**364**
	Kamina Barracks	231	0	8	**239**
	Air force base	216	3	61	**280**
Northern region Total		**748**	**12**	**123**	**883**
Upper East region	Navrongo	**3**	**0**	**3**	**6**
Greater Accra region	Burma camp	214	8	0	**222**
	Michel camp	53	24	1	**78**
	Shai hills	182	8	0	**190**
Greater Accra region Total		**449**	**40**	**1**	**490**
Overall Total		**1,200**	**52**	**127**	**1,379**

APPENDIX 5.2

Total number of tick species from seven study sites in the three regions

Region	Study site	*Rhipicephalus sanguineus*	*Rhipicephalus evertsi*	*Rhipicephalus boophilus*	Total
Northern region	**Air borne force**	0	0	0	**0**
	Kamina Barracks	0	0	0	**0**
	Air force base	0	0	0	**0**
Northern region Total		**0**	**0**	**0**	**0**
Upper East region	**Navrongo**	**372**	**13**	**0**	**385**
Greater Accra region	**Burma camp**	16	0	0	**16**
	Michel camp	9	7	1	**17**
	Shai hills	16	0	0	**16**
Greater Accra region Total		**41**	**7**	**1**	**49**
Total		**413**	**20**	**1**	**434**