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Risk Factors and Human coronaviruses Associated with Upper Respiratory Tract Infections in Three Rural Areas of Ghana

By

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DOCTOR OF PHILOSOPHY

School of Medical Sciences College of Health Sciences

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DECLARATION

I hereby declare that this submission is my own work towards the PhD and that to the best of my knowledge, it contains no material previously published by another person nor material which has been accepted for the award of any other degree of the University, except where due acknowledgement has been made in text.

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Student Name	Signature	Date

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Supervisor's Name	Signature	Date

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Head of Dept.'s Name	Signature	Date

DEDICATION

This work is dedicated to my wife, Dorcas Owusu and my mother, Alice Tweneboah.

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ABBREVIATIONS

AFGMK	African green monkey kidney cells,
ALRTI	Acute lower respiratory tract infection
ARI	Acute respiratory infections
BCoV	Bovine coronavirus
BFMK	Buffalo green monkey kidney.
BHQ1	Black Hole Quencher 1
CCoV	Canine coronavirus
COPD	Chronic obstructive pulmonary disease
DFA	Direct fluorescent test
E	envelope protein
ECMO	Extracorporeal membrane oxygenation
ECoV	Equine coronavirus
EIA	Enzyme immunosorbent assay
ELISA	Enzyme linked immunosorbent test
FAM	6-carboxyfluorescein,
FCoV	Feline coronavirus
FIG	Figure
H/HA/HN	Haemaglutinin
HCoV	Human coronaviruses
HE	Haemaglutinin Esterase
HEV	Human enteroviruses
HI/HAI	Haemagglutinin inhibition
HPIV	Human parainfluenza virus

HRV	Human Rhinoviruses
HS	Heparin Sulphate
IBV	avian infectious bronchitis virus
ICTV	International Committee on Taxonomy of Viruses (ICTV)
IFA:	Immunofluorescence assay
JHS	Junior High School
KCCR	Kumasi Centre for Collaborative Research in Tropical Medicine
LPR	Lipoprotein receptor
LRTI	Lower respiratory tract infection
M	membrane protein
MDCK	Madin-Darby canine kidney
MERS	Middle east respiratory syndrome
MHV	Murine hepatitis virus (MHV)
Mv1Lu	mink lung epithelial cell line
N	Nucleocapsid protein
NA	Neuramidase
NAT	Nucleic Acid Test
NCR	Non-Coding Region
NT	Neutralization Test
PCoV	Pheasant coronavirus
PCR	Polymerase chain reaction
PEDV	Porcine epidemic diarrhea virus
PHCoV	Porcine haemagglutinating encephalomyelitis virus

PIV	Parainfluenza virus
RBD	receptor binding domain
RCoV	Rat coronavirus
REP	Replicase protein
RhMK	Rhesus monkey kidney
RNA	Ribonucleic acid
ROC	Receiver operating characteristic
RSV:	Respiratory syncytial virus
RT-PCR	Real-Time Reverse Transcriptase Polymerase Chain Reaction
SARS:	Severe acute respiratory syndrome
SHS	Senior High School
TCoV	Turkey coronavirus
TGEV	Transmissible gastroenteritis virus
U.S.A	United States of America
UTR	Unstranslated region
VNT	Virus Neutralization Tests

ABSTRACT

Acute respiratory tract infections (ARI) are the leading cause of morbidity and mortality in developing countries, especially in Africa. In spite of its importance, information on the viral aetiology and risk factors associated with ARI are limited in Ghana. Even though human coronaviruses (HCoVs) are known to be associated with respiratory disease outbreaks and severe infections in some developed countries, their epidemiological role is understudied in many African countries including Ghana. It is therefore not known whether HCoVs are pathogenic viruses associated with ARI or only exist as normal commensals of the upper respiratory tract. The aim of this study was to find the association between HCoVs and ARIs, describe the seromolecular epidemiology of HCoVs and identify the risk factors associated with upper respiratory tract infection.

An unmatched case control study was conducted in Buoyem, Forikrom and Kwamang communities of Ghana. Subjects were interviewed on various sociodemographic factors and hygienic practices using structured questionnaires. Nasal/Nasopharyngeal swabs were taken from older children and adults, and tested for Middle East respiratory syndrome coronavirus (MERS-CoV), HCoV-229E,
HCoV-OC43, HCoV-NL63 and HCoV-HKU1 using Reverse Transcriptase RealTime Polymerase Chain Reaction.

A total of 1272 subjects were recruited comprising of 662 (52%) controls and 610 (48%) cases. Risk factors associated with upper respiratory tract infections were school attendance to the level of Senior High and tertiary education, and being a health worker. Out of 322 subset of cases interviewed, 212 (66%) covered their nose with handkerchiefs when they sneezed, 52 (16%) covered with their hands upon sneezing

and 79 (25%) sneezed in the open. Self-administered drugs such as herbs (2%), analgesics (25%) and antibiotics (16%) were used to manage upper respiratory tract infections.

Out of the 1,272 subjects recruited, nasal swabs were taken from 1,213. Of the 1,213, 150 (12.4%) subjects were positive for one or more viruses. Of these, single virus detections occurred in 146 subjects (12.0%) and multiple detections occurred in 4 (0.3%). Compared with control subjects, infections with HCoV-229E (OR = 5.15, 95% CI = 2.24 – 11.78), HCoV-OC43 (OR = 6.16, 95% CI = 1.77 – 21.65) and combine HCoVs (OR = 2.36, 95% CI = 1.5 = 3.72) were associated with upper respiratory tract infections. Significant median virus concentration difference was observed for only HCoV- NL63 (cases: 2.41 x 10^6 copies per PCR reaction; IQR = 1.96 x 10^4 - 2.3 x 10^6 vrs controls: 1876.5 copies per PCR reaction; IQR =387.2 – 8.6 x 10^4, P=0.003) and the clinically relevant cut-off viral concentration was determined to be 7,510 copies per PCR reaction.

HCoVs were found to be seasonally dependent with high proportions identified in the harmattan season (54/215, 25.1%) compared to the wet (80/516, 15.5%) seasons. The most frequent viruses detected in the harmattan and wet seasons were HCoV229E and HCoV-NL63 respectively. HCoV-OC43 and HCoV-HKU1 were almost distributed equally throughout the year.

Sequencing of the partial spike region was successful for 53 out of 146 samples (36.3%). Of the 53, 12 (22.6%) were HCoV-OC43, 14 (26.4%) were HCoV-NL63, 24 (45.3%) were HCoV-229E and 3 (5.7%) were HCoV-HKU1. A comparison of the obtained sequences resulted in no differences to sequences already published in

GenBank.

This study has identified risk factors of URTI and also demonstrated that HCoVs could play significant role in causing upper respiratory tract infections among adults and older children in rural arrears of Ghana. This information could be useful to policy makers, public health practitioners and other stakeholders in Ghana.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background to the study

Acute respiratory tract infections (ARI) are the leading cause of morbidity and mortality among young children and adults in developing countries (Denny and Loda, 1986; Williams et al., 2002). A review by Gessner et al (2011) showed that the highest countries mostly affected by ARIs still remain in Africa (Gessner, 2011).

Majority of ARI are known to be of viral origin with the predominant viruses being Respiratory Syncytial Virus (RSV), Influenza virus, Rhinoviruses, Parainfluenza viruses, Human Metapneumovirus and Human coronaviruses (HCoVs) (Arden, K.E. et al., 2006; Smuts et al., 2008; Venter et al., 2011; Xiao et al., 2012). The role of HCoVs in causing respiratory diseases was however thought to be mild until the outbreak of severe acute respiratory syndrome (SARS) coronavirus infection which resulted in a mortality rate of approximately 10% (Drosten, C. et al., 2003a; Ksiazek et al., 2003). Since this worldwide outbreak, there has been increasing research in finding epidemiologic association between HCoVs and respiratory diseases in population and community based studies. Of interest to the global community has also been to determine the serological prevalence of this virus in community based studies. These broad based studies at the population level has been made feasible by the rapid upsurge in advanced molecular and serological techniques which can identify the subtypes and novel strains of HCoVs within short turnaround time (Corman et al., 2012).

HCoVs are enveloped ribonucleic acid (RNA) viruses which belong to the family *Coronaviridae* and subfamily *Coronavirinae*. Members of the subfamily have positive strand RNA with genome size ranging from 27-33 kilobases (kb). Currently six HCoVs have been described namely HCoV-OC43, HCoV-229E, severe acute respiratory syndrome -associated coronaviruses (SARS CoV), HCoV-NL63, HCoV-HKU1 and the recent Middle East respiratory syndrome virus (MERS-CoV) (Drosten, C. et al., 2013; Gaunt et al., 2010).

Certain populations in developed countries have reported HCoV-OC43 and HCoV-229E to be responsible for up to 30% of upper respiratory tract infections (Graat et al., 2003; Holmes, 2001) whereas others have identified these viruses along with HCoV-NL63 and HCoV-HKU1 to be more prevalent in severe respiratory tract infections of immunocompromised individuals, institutional elderly subjects and infants (El-Sahly et al., 2000; Falsey et al., 1997; Vabret, Astrid et al., 2008; Vabret, A. et al., 2003; van Elden et al., 2004). Severe infections and deaths have been reported in individuals with underlying diseases as in the recent case of MERS-CoV (Chan, J. F. et al., 2012; van Boheemen et al., 2012; Zaki et al., 2012). Other studies have however not found significant association of respiratory tract infections with HCoVs thus raising questions about its aetiology in causing respiratory illness (Dare et al., 2007; Prill et al., 2012; van Gageldonk-Lafeber et al., 2005). Currently there has been debate as to whether HCoVs could be pathogenic viruses associated with respiratory illness or nonpathogenic viruses as part of the normal human microbial flora (McIntosh, K., 2012). Even though this debate

is believed to be more relevant to children, the case of older children and adults still needs to be addressed in other geographical settings.

In spite of the immense research on HCoVs in developed countries, not much is known about their contribution to respiratory tract infections in sub-Saharan Africa. Apart from South Africa (Venter, et al., 2011), most research done on HCoVs did not include healthy human subjects as controls (Njouom et al., 2012; Smuts, et al., 2008; Venter, et al., 2011). It is therefore not clear whether the detection of HCoVs can be associated with respiratory tract infections in Africa. This knowledge gap has not been adequately tackled for long probably due to the lack of modern diagnostic molecular assays in many African countries.

To date, few studies have reported the detection of 3- 10% of four HCoVs (HCoV-229E, HCoV-OC43, HCoV-NL63 and HCoV-HKU1) with the predominant being HCoV-NL63 (Njouom, et al., 2012; Smuts, et al., 2008; Venter, et al., 2011) . However these studies were limited to hospital based subjects and the epidemiologic association with respiratory illness was not well described. There is also lack of information on the serological prevalence of HCoVs in sub-Saharan Africa. Furthermore there is insignificant research on the contribution of HCoVs as well as their specific genotypic strains to the burden of respiratory tract infections in rural areas of Africa. Rural regions are considered as the focus of morbidity and mortality ascribed to general conditions such as malaria or bacterial infections due to the poor hygienic practices and the lack of quality healthcare delivery system. Therefore the determination of aetiologies of diseases in rural

areas is considered paramount in informing policies on preventive and control interventions aimed at reducing disease burden in Africa.

This study is therefore necessary as it will provide information on the risk factors of viral associated upper respiratory tract infections, the association of HCoVs to upper respiratory tract infections, the serological prevalence of HCoVs and the HCoV genotypic strains circulating in remote rural areas of Ghana.

1.2 Problem statement/ Rationale for study

The extrapolated incidence of upper respiratory tract infections (URTI) in Ghana was reported to be 17, 481 cases per 100, 000 per year (Krumkamp et al., 2012). Although these infections could be considered mild, the associated mortality tends to be high because of the wide diversity of viruses involved, and the possibility of secondary bacterial infections especially in children. One approach that can be used to reduce transmission of ARI is to intervene in the risk factors and practices contributing to respiratory infection. There is however limited information of these factors (especially in rural areas) to guide policy makers in implementing preventive and control interventions. This information is therefore necessary and could be used to develop national policies on reducing URTIs.

The burden of HCoV associated respiratory illness and its contribution to morbidity and mortality cannot be underestimated in developing countries including Ghana. In spite of some reports that HCoVs could be associated with acute respiratory illness, their presence in both symptomatic and asymptomatic subjects makes it challenging in associating virus infections with clinical illness. Viruses that may be considered as pathogenic in developed

countries could just be part of the human microbial flora in other geographical settings (e.g. Africa) possibly because of immunological differences and environmental exposures. Moreover, there is also lack of information on the cut-off viral concentration at which clinical symptoms correlate with successful laboratory diagnosis.

It is therefore necessary that epidemiological associations using properly designed case control studies are used to define the clinical relevance and concentrations of HCoVs within defined geographical settings such as Ghana. Of interest will also be to identify the proportion of subjects with past history of exposure to HCoVs through serological techniques. Such findings will aid clinicians and public health practitioners to apply appropriate interventions to populations at risk.

1.3 Study aim

The aim of this study is to determine the epidemiological association of HCoVs to upper respiratory tract infections.

1.4 Main Objective

To identify the risk factors of upper respiratory tract infections and describe the seroepidemiology of HCoVs.

1.5 Specific objectives

1. To identify the possible risk factors that are associated with exposure to upper respiratory tract infections.
2. To determine the seroprevalence of HCoVs among subjects
3. To determine whether HCoVs are associated with upper respiratory tract

infections.

4. To determine the clinically relevant cut off viral concentration that could predict an exposure to upper respiratory tract infection due to HCoVs.
5. To determine if HCoVs in Ghana are comparable to global circulating strains.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 General introduction to HCoVs

HCoVs are recognized as pathogens mostly associated with both upper and lower respiratory tract infections worldwide (Njouom, et al., 2012; Tyrrell, DAJ and Bynoe, 1965; van der Hoek, 2007). Even though infections with HCoVs were believed to be mild, the outbreak of human coronavirus related severe acute respiratory syndrome in China prompted scientists about the potential virulence of this pathogen (Drosten, Christian et al., 2003b; Ksiazek, et al., 2003). The threat posed by coronaviruses is believed to lie in its ability to cause zoonotic infections through crossing inter- species barriers and undergoing multiple recombinations to evade the immune system. This characteristic of HCoVs was reported in China where Horseshoe bats were implicated as the likely reservoir of all coronaviruses (Lau et al., 2005). In Ghana and other African countries, coronaviruses related to the HCoV-229E and SARS-like coronaviruses have been identified in Hipposiderous bats. There is the possibility of recombination with human strains and initiating outbreaks (Pfefferle et al., 2009; Tao et al., 2012; Tong et al., 2009).

2.2 History and classification

Coronaviruses (CoVs) generally possess about 100 nm diameter enveloped particles with 20 nm long surface projections which are rounded and that is believed to give it a

'corona' appearance (Sturman L . S et al., 1980).

The first description of coronaviruses was in 1931 when respiratory tract diseases in birds was associated with an agent called avian infectious bronchitis virus (Almeida et al., 1968; Schalk AF and Hawn MC, 1931). This discovery was later followed by the isolation of human CoVs in 1965 from a school boy with common cold (Tyrrell, DAJ and Bynoe, 1965). The medium from these cultures were intranasally inoculated in human volunteers and they produced symptoms of common cold in significant proportions (Tyrrell, DA and Bynoe, 1966).

Around the same time Tyrell and Bynoe (Tyrrell, DA and Bynoe, 1966) did their study, Hamre and Procknow (Hamre and Procknow, 1966) also isolated a virus from five medical students with symptoms of upper respiratory tract infections. These two human viruses were named CoV 229E. Two years after these discoveries, McIntosh et al.,(McIntosh, K et al., 1967) reported the recovery of multiple strains of human respiratory agents with similar in-vitro characteristics to that isolated by Tyrrell and Bynoe (Tyrrell, DA and Bynoe, 1966). His viruses were termed "OC43" to describe their origin as being from organ cultures. Virologists in subsequent years identified viruses with similar characteristics in rats, mice, chicken, turkeys, dogs, pigs and other animals (Haring J and Pearlman S, 2001).

As reports of coronavirus discovery increased, the world body for viral classification (International committee for Taxonomy of Viruses) recognized and published its first report on the *Coronaviridae* in 1975 (Tyrreli DAJ et al., 1975). The possession of RNA genome, replication in the cytoplasm and maturation through the endoplasmic reticulum were added as the short list of the inclusion criteria in the *Coronaviridae* family. Further virological studies uncovered viruses

of the Torovirus and Arterivirus genera and these were made part of the *Coronaviridae* family (Cavanagh D and Horzinek MC, 1993). Human and animal coronaviruses were segregated into 3 broad groups based on their antigenic and genetic makeup. Group I and II contained viruses pathogenic for mammals and group III contained viruses that infect birds (Cavanagh, D, 1997; Ejuanes J et al.,

2000). Group I was made up of Feline coronavirus (FCoV), Canine coronavirus (CCoV), Transmissible gastroenteritis virus (TGEV) Porcine epidemic diarrhea virus (PEDV) and Human coronavirus 229E (HCoV-229E) ; Group II includes Equine coronavirus (ECoV), Rat coronavirus (RCoV), Porcine haemagglutinating encephalomyelitis virus (PHCoV), Human coronavirus OC43 (HCoV-OC43), Bovine coronavirus (BCoV) and Murine hepatitis virus (MHV); Group III was made up of avian infectious bronchitis virus (IBV) and Turkey coronavirus (TCoV) and Pheasant coronavirus (PCoV) (Ejuanes J, et al., 2000; Hegyi A et al., 2002; Siddell S.G, 1995; Stephensen C.B et al., 1999).

With the advent of genome molecular techniques and phylogenetic analysis (Cavanagh D et al., 1994; Gonzalez J.M et al., 2003), HCoVs designated as causes of severe acute respiratory syndrome (SARS) were identified in a large outbreak of respiratory infections in China, Europe, South American and other countries (Drosten, Christian, et al., 2003b; Ksiazek, et al., 2003).

Approximately 8098 individuals were infected resulting in 774 fatalities mainly due to severe acute respiratory syndrome (Centers For Disease Control and Prevention; World Health Organisation, 2004). Series of investigations traced the source of these infections to palm civets and raccoon dogs sold at retail markets in the Guangdong province of China

(Chen et al., 2011; Guan et al., 2003). Further serological investigations in the animal handlers revealed the detection of SARS-related HCoV antibodies thus suggesting interspecies transmission of the virus. Bats were later identified as the natural reservoirs (Li, W. et al., 2005a).

Just as the SARS-related HCoVs seemed to disappear, another human coronavirus described as HCoV-NL63 was isolated in Amsterdam from a seven month old child with symptoms of respiratory tract infection (Pyrc, K. et al., 2007; van der Hoek et al., 2004). This virus unlike the SARS-related human coronavirus caused mild respiratory symptoms and sometimes severe lower respiratory tract infections especially in immunocompromised individuals. Even though the natural reservoir of this virus was believed to be humans, evidence of its nucleotide similarity with bat coronaviruses and its ability to replicate in bat cell lines suggest it could be of zoonotic origin (Huynh et al., 2012).

The discovery of HCoV-NL63 was followed by the identification of HCoV-HKU1 from a 70 year old man with pneumonia who had returned from China to Hong Kong in 2005 (Woo, P. C. Y. et al., 2005b). The clinical features were however similar to HCoV-NL63 and less severe than the SARS-related HCoVs. Currently MERS-CoV is the new respiratory virus which has already been reported to have caused the death of immunocompromised elderly individuals in the Middle East (Chan, J. F., et al., 2012; van Boheemen, et al., 2012; Zaki, et al., 2012). The virus has also been found to be closely associated with bat coronaviruses (Annan et al., 2013; Drosten, C., et al., 2013).

The changing dynamism of HCoVs in the human and animal population has caused the review of the subfamilies and families of HCoVs. The international committee of

taxonomy of viruses ratified these changes and published them in 2009 (Carstens E.B, 2009). Currently family *Coronaviridae* is part of the Nidovirales order that also includes two other families *Arteriviridae (*equine arteri virus, Lactate dehydrogenase-elevating virus, Porcine reproductive and respiratory syndrome virus, simian hemorrhagic fever virus*)* and *Roniviriadae* (Gill- associated virus) (Cowley JA et al., 2000; den Boon et al., 1991). The unique features of the order Nidoviridae are: gene expression of the 3' end of the RNA through transcription of multiple subgenomic mRNAs; expression of the replicase proteins through ribosomal frameshifting; presence of a membrane protein and a multiplespanning of an integral membrane protein (Ejuanes J, et al., 2000).

Coronaviridae has now been assigned two subfamilies; *Coronavirinae* and *Torovirinae*. The continuous search for the reservoirs of coronaviruses after the SARS-CoV outbreak has led to the discovery of about 89 unclassified species of coronaviruses (DHHS, 2011). **Table 2.0.1** illustrates the genus and the species of *Coronavirinae* subfamily.

Table 2.0.1: Coronaviruses classified under the subfamily of Coronavirinae

Genus	Species

Alphacoronavirus	*HCoV-NL63,Porcine epidemic diarrhea virus* *HCoV-229E,Alphacoronavirus1* *Rhinolophus bat coronavirus HKU2* *Scotophilus bat coronavirus 512,* *Miniopterus bat coronavirus 1, Miniopterus bat coronavirus HKU8* *Miniopterus bat coronavirus HKU8*
Betacoronavirus	*HCoV-HKU 1, HCoV-OC43,Betacoronavirus 1,* *Murine coronavirus* *Rousettus bat coronavirus HKU9* *Tylonycteris bat coronavirus HKU4, Pipistrellus bat coronavirus HKU5* *Severe acute respiratory syndrome-related coronavirus* MERS-CoV
Deltacoronavirus	*Bulbul coronavirus HKU11, Munia coronavirus HKU13* *Thrush coronavirus HKU12*
Gammacoronavirus	*Avian coronavirus, Beluga whale coronavirus SW1*

Virus classification adapted from the international committee on taxonomy of viruses (International committee on taxonomy of viruses, 2013)

2.3 Structure of coronaviruses

Coronaviruses are the largest contiguous RNA virus genomes in nature (Cavanagh, D, 1997). They are positive stranded and embedded in helical nucleocapsids (Cavanagh, D, 1997). The helical nucleocapsid coronaviruses is believed to make them distinct since most positive stranded RNA genomes have icosahedral nucleocapsids. coronaviruses have typical average diameters of 80–120 nm, but extreme sizes as small as 50 nm and as large

as 200 nm have been reported in (McIntosh, K, 1974; Oshiro, 1973). All coronavirus virion particles contain four to five structural proteins and several nonstructural proteins. The structural proteins are spike proteins (S; old terms: E2 or gpI80), nucleocapsid protein (N), the membrane protein (M), small envelope glycoprotein (E) and Hemagglutinin acetylesterase glycoprotein (HE) (**Figure 2.0.1**).

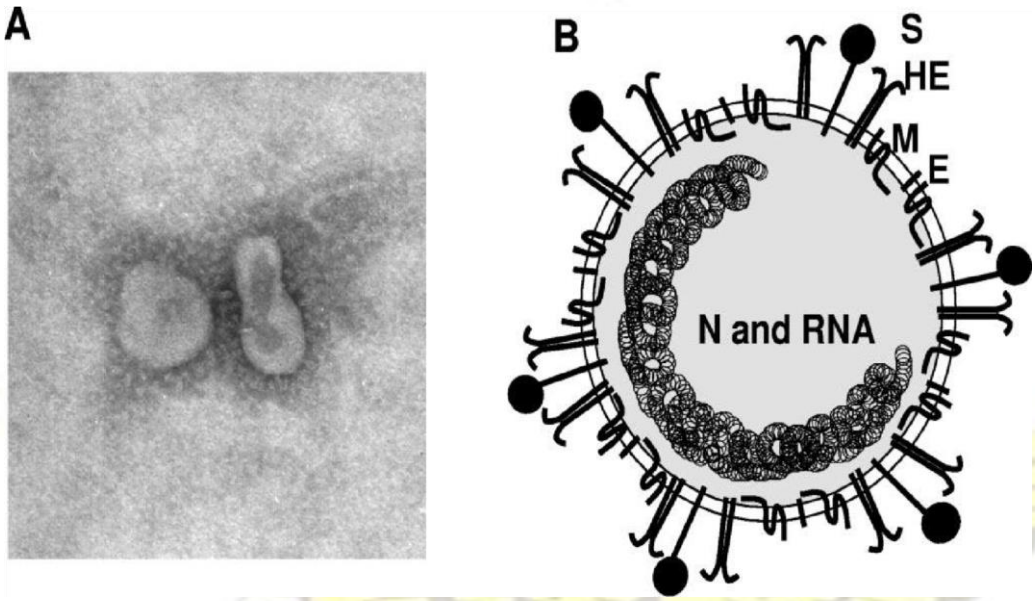


Figure 2.0.1: The Coronavirus virion

A represents the Electron micrograph of Coronavirus. **B** represents the Schematic virion of Coronavirus. Adapted from Weiss and Navas-Martin (Weiss and Navas-Martin, 2005).

Coronavirus particles contain an internal helical RNA-protein nucleocapsid surrounded by an envelope containing viral glycoproteins. Nucleocapsid (N) protein is a phosphoprotein that is complexed with genome RNA. Spike glycoprotein (S) forms large glycosylated peplomers that are characteristic of coronaviruses.The transmembrane protein (M), is highly hydrophobic and spans across the membrane. The membranespanning protein, is a minor component of the membrane. Some

Betacoronaviruses express another glycoprotein, hemagglutinin-esterase (HE), which forms smaller spikes on virions.

2.4 Genome organization

The genera of coronaviruses are generally arranged in the order shown in **Figure 2.0.2.**

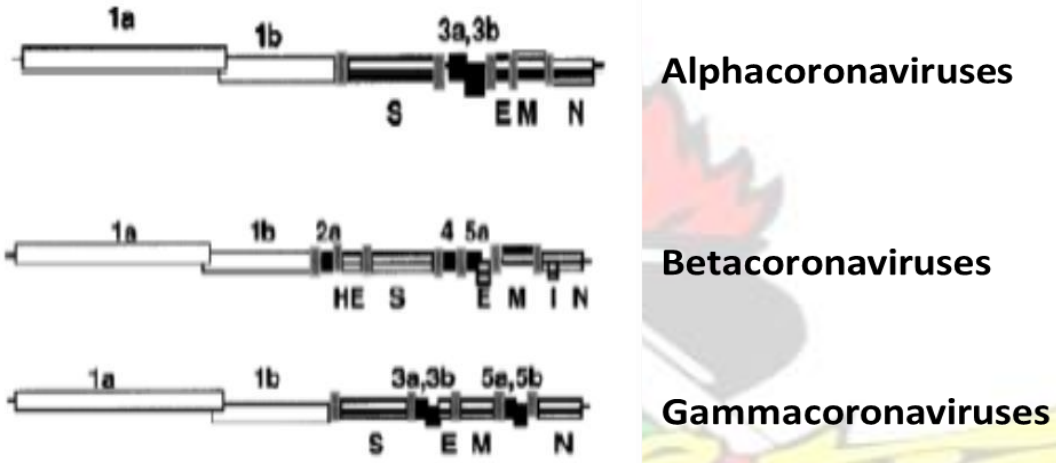


Figure 2.0.2: Genomes of Coronavirus groups.

The open reading frame (ORFs 1a, 1b) is shown by open bars; structural genes S (spike protein), E (envelope protein), M (membrane protein), N (Nucleocapsid protein), and HE (Haemagglutinin Esterase) are depicted with striped bars; nonstructural genes (black bars) are variable in number and location in the coronavirus genome among the different viral groups. Small open reading frames (ORFs) are depicted in solid bars.

The 5' end coronavirus a short sequence leader sequence and the sequence is followed by two open reading frames (ORFs). The two open reading frames encode for a replicase polyprotein (rep). The ORFs are also followed by sequences that encode for the structural proteins and accessory proteins of coronaviruses. The accessary proteins tend to differ

depending on the species of coronavirus. The five HCoVs species are arranged in the order shown below (McBride and Fielding, 2012; O'Connor J and Brian, 1999) :

HCoV-229E [rep] - [S] - 4a, 4b - [E] - [M] - [N],

HCoV-NL63 [rep] - [S] - 3 - [E] - [M] - [N],

HCoV-OC43 [rep] - 2a - 2b (HE) - [S] - 5(12.9k) - [E] - [M] - [N]-7b (I),

HCoV-HKU1 [rep] - 2(HE) - [S] - 4 - [E] - [M] - [N]-7b (I) and

SARS-CoV [rep] - [S] – 3a-3b - [E] - [M] - 6 - 7a-7b-8a-8b [N] 9b (I).

The accessory proteins have been shown to be incorporated in coronavirus particles however their exact role in enhancing virulence is still being debated (Pewe et al., 2005; Varshney et al., 2012). The accessory proteins were initially thought to be non-structural but some have been shown to be part of the structural components of the virion. Examples include the HE proteins of *Betacoronaviruses* and the SARS-CoV 3a protein (Fischer et al., 1997; Freundt et al., 2010; McBride and Fielding, 2012).

One very important structural protein in coronaviruses is the spike protein. This protein has been reported to be essential for the attachment of the virus to host cell receptor and fusion of the virus envelope with the host cell membrane. It also serves as the major antigen that elicits an immune response to neutralising antibodies in humans. The structure of the spike protein is illustrated in **Figure 2.0.3.**

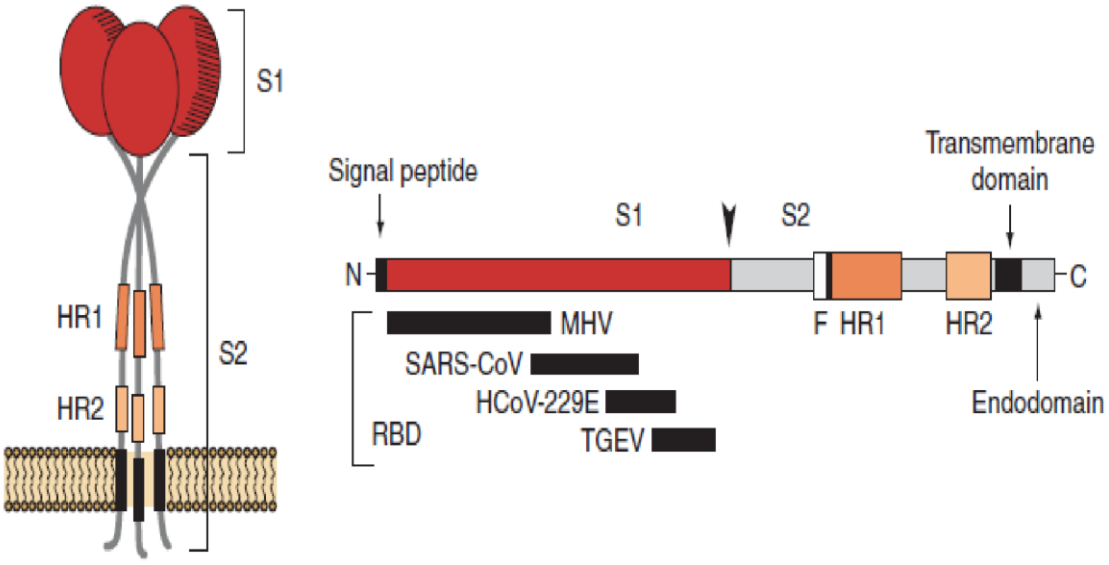


Figure 2.0.3: Schematic representation of the spike protein of HCoVs

The left diagram describes the structure of the spike proteins with a trimer assembled globular head. The globular head (S1) is the receptor binding domain (RBD) and the fusion domain is S2. The right diagram describes the linear map of the spike structure with the corresponding colours showing the location of the RBDs for three coronaviruses. HR1 and HR2 are heptad repeat domains 1 and 2 which mediate conformation changes that is needed to present the fusion peptide (F) to cellular membranes. Adapted from S.C Baker (Baker, 2008).

The S1 proteins are reported to be specific in the attachment to host cells. This characteristic feature has been demonstrated by the manipulation of receptors in different coronavirus species. Expression of specific HCoV receptors on non-permissive cells has rendered them susceptible to different species (Li, W. et al., 2004; Li, W. et al., 2003; Mossel et al., 2005). Similarly the swapping of the ectodomains of the S protein has been shown to modify the *in-vitro* host cell species specificity of murine hepatitis virus (MHV) to that of Feline infectious peritonitis virus (FIPV) (Kuo et al., 2000).

These experiments thus provide evidence of the uniqueness of the spike protein of coronaviruses.

2.5 Receptors of HCoVs

HCoV-229E binds to host species with human aminopeptidase N receptors (Yeager et al., 1992), HCoV-NL63 and SARS-CoV bind to human angiotensin-converting enzyme 2 (Hofmann et al., 2005; Li, W et al., 2005b) located in the respiratory (lungs) and gastrointestinal tracts and MERS-CoV is also reported to bind to dipeptidyl peptidase 4 (Raj et al., 2013). Similarly HCoV-OC43 and other *Betacoronaviruses* also bind to the sialic acid *N*-acetyl-9-*O*-acetylneuraminic acid (Schwegmann-Wessels and Herrler, 2006).

Coronavirus binds to its host cell receptor using the S1 portion and then through transformation changes, it fuses with the host cell membrane. After fusion, the S1 portion breaks away and rearrangement of S2 brings together heptad repeated domains 1(HR1) and heptad repeated domains 2 (HR2) to form a bundle thus enhancing the introduction of the RNA into the cytoplasm.

The membrane and envelope proteins are used for the efficient assembly of coronavirus particles whereas the nucleocapsid protein (N) binds to the RNA to assemble the ribonucleoprotein complexes.

2.6 Replication of coronaviruses

Replication and transcription of coronaviruses takes place in the cytoplasm of infected cells. The process of replication is described in **Figure 2.0.4 and 2.0.5.**

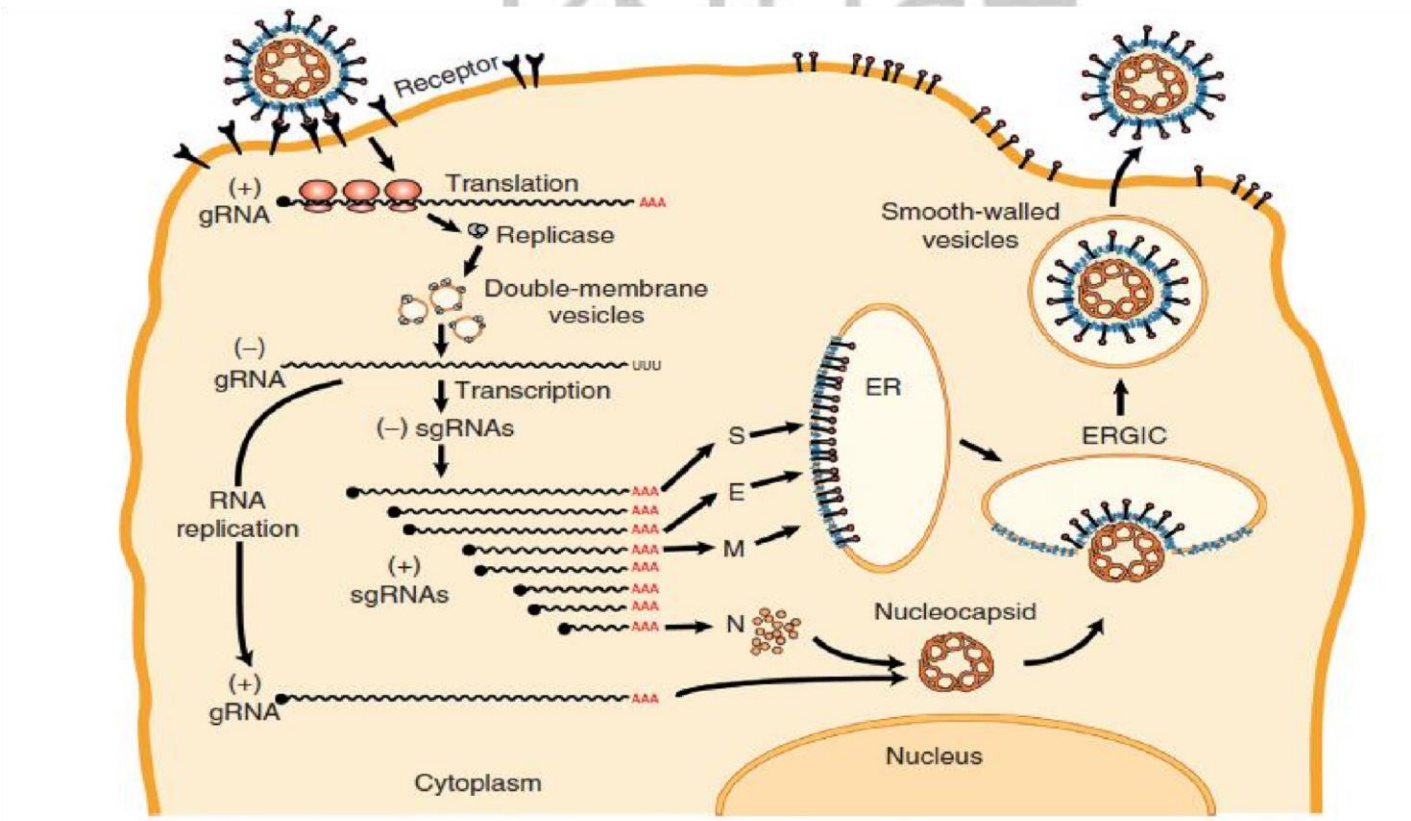


Figure 2.0.4: Replication cycle of coronaviruses

The spike glycoprotein on the virus particle interacts with host cell receptors to mediate fusion of the virus and host cell membranes resulting in the release of the positive-strand RNA genome into the cytoplasm. The 5' end of the open reading frames (ORF1a and ORF1b) is translated from the genomic RNA to generate the replicase polyprotein. The replicase polyprotein is processed by viral proteases into 16 non-structural proteins which assemble with membranes to generate doublemembrane vesicles (DMVs) where RNA synthesis takes place. A nested set of 3' end subgenomic (sg) RNAs is generated by a discontinuous transcription. The subgenomic mRNA are translated to generate the viral structural and accessory proteins. Viral genomic RNA is replicated and associates with nucleocapsid protein and viral structural proteins in the endoplasmic reticulum-Golgi intermediate compartment (ERGIC), where virus particles bud into vesicles before transport and release from the cell. Adapted from Baker in encyclopaedia of virology (Baker, 2008).

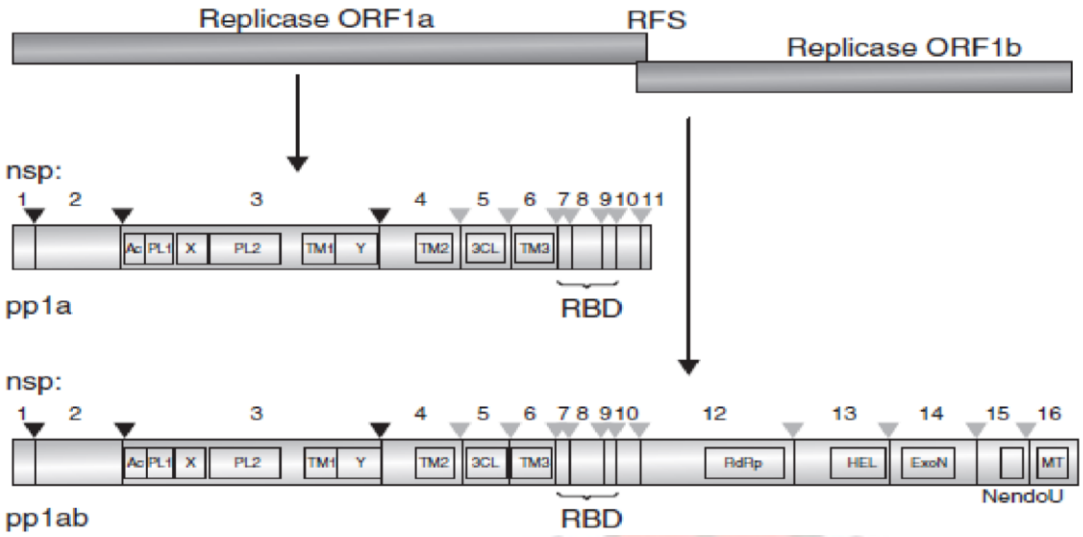


Figure 2.0.5: Organization of the coronavirus replicase gene products

Translation of the coronavirus replicase open reading frame (ORF) 1a and ORF 1b sequences results in polyprotein (pp) 1a and pp1ab. PP1 ab synthesis is dependent on the extension of pp1a, following a programmed –ribosomal frameshift (RFS) event. The two polyproteins are proteolytically cleaved into 10 (pp1a; nsp1–11) and 16 (pp1ab; nsp1–16) non-structural products by papain-like proteinases. The black and gray triangles show the sites where the proteinases cleave. The nsp11 product of pp1a is produced as a result of the ribosomes terminating at the ORF 1a translational termination codon, a –1 frameshift results in the generation of nsp12, part of the pp1ab replicase gene product. Various domains have been identified within some of the replicase products: "Ac" is a conserved acidic domain; X = ADP-ribose 1′-phosphatase (ADRP) domain; PL (papain-like proteinases) 1 and PL2 the two papain-like proteinases; Y is a conserved domain; TM (transmembrane) 1, TM2, and TM3 are conserved putative transmembrane domains; 3CL = 3CL pro domain; RdRp, RNA-dependent RNA polymerase domain; HEL, helicase domain; ExoN, exonuclease domain; NendoU; uridylatespecific endoribonuclease domain; MT, 2′-O-ribose-methyltransferase domain. nsp's 7–9 contain RNA-binding domains (RBDs). Adapted from Cavanagh in encyclopaedia of virology (Cavanagh, D. and Britton, 2008).

Once the spike proteins attach to the host cell receptor, fusion mediated by the spike proteins with endosomal membranes enables the release of viral genomic RNA into the cytoplasm. The RNA then acts as a messenger RNA and the open reading frame (ORF) 1a and 1b are translated into polyprotein 1a and 1ab using the host ribosomes (**Figure 2.0.4**). The polyproteins are then cleaved by ORF1a-encoded proteases to become part of the replicase complexes needed for the synthesis of negative sense copies of the genomic RNA or subgenomic messenger RNA (mRNAs) (Baric and Yount, 2000; Sethna et al., 1989).

These replicase proteins produced from cleavage of the polyproteins pulls host cell membranes to generate double membrane vesicles. These membrane vesicles have been shown to be the site of coronavirus RNA synthesis and also mediate the replication of positive-strand RNA. The synthesis of the subgenomic mRNAs leads to the production of structural proteins which enables assembling of the synthesized genomic RNA resulting in the release of new viruses. In some cases, a fraction of the structural proteins that is not assembled into virions is transported into the plasma membranes where it could cause the fusion of infected and uninfected cells leading to the formation of large, multinucleate syncytia.

The release of new viruses is believed to start 3-4 hours after initial infection. Single cells infected by two related coronaviruses may swap the polymerase between two RNA templates leading to genetic recombinations that result in chimeric RNA. Such chimeric RNAs may give rise to novel viruses with the ability to infect other cells and in some cases become more virulent.

2.7 Epidemiology and clinical presentation of HCoVs

2.7.1 Molecular epidemiology

HCoV-NL63, HCoV-229E, HCoV-OC43 and HCoV-HKU1 are the major types that are circulating worldwide among the human populations and cause approximately 10% of all upper and lower respiratory tract illnesses (Gerna et al., 2007; Regamey et al., 2008; Vabret, Astrid, et al., 2008). Another type of coronavirus which is not very common worldwide is SARS-CoV (Drosten, Christian, et al., 2003b). This virus was associated with aggressive respiratory syndrome and reported to cause over 800 deaths in 2003 (Berger et al., 2004). Currently the outbreak of a novel MERS-CoV has been reported in the Middle East and is gradually spreading across the globe (Chan, J. F., et al., 2012; Zaki, et al., 2012). The virus is associated with atypical pneumonia and acute respiratory distress especially among immunocompromised subjects (Drosten, C., et al., 2013). Guery et al (Guery et al., 2013)., reported two adult infected cases of MERS-CoV infections from Dubai and France in which clinical presentations of fever, chills and myalgia were described in both patients at the onset of illness. Respiratory symptoms rapidly became predominant as the disease advanced with acute respiratory failure leading to mechanical ventilation and extracorporeal membrane oxygenation (ECMO) (Guery, et al., 2013). Both patients developed acute renal failure in latter stages of the disease.

In children, infections with HCoV-NL63, HCoV-229E, HCoV-OC43 and HCoV-HKU1 are reported to be associated with acute respiratory tract illness, pneumonia and croup that eventually may lead to hospitalization (van der Hoek, 2007). Adult infections are generally thought to be mild and often present as upper respiratory tract infections however other

reports have indicated their presence in patients with chronic pulmonary obstructive disease (Gorse et al., 2009). All four types of HCoVs have however been reported in all age groups with high frequencies in children within the age group of 7-12 months (Gaunt, et al., 2010). HCoV-OC43 tends to be higher in males compared to other types of HCoVs (Gaunt, et al., 2010). Among the *Betacoronaviruses*, HKU1 tends to occur at low levels (about 2% in adult populations) compared to HCoV-OC43 (Woo, P.
C. et al., 2005a). In a study by Gaunt et al., (Gaunt, et al., 2010) which investigated 11,661 respiratory samples, HCoV-OC43 was identified as the commonest virus followed by HCoV-NL63, HCoV-HKU1 and then HCoV-229E.

Generally, symptoms manifested by patients with HCoV-NL63 infections range from fever, pharyngitis, bronchitis, croup and rhinorrhea (Arden, K. E. et al., 2005; Bastien et al., 2005; Fouchier et al., 2004). In Canada, HCoV-NL63 was reported to be commonly associated with fever, sore throat and cough (Bastien, et al., 2005) while epidemiologic association with Kawasaki disease was also reported in England (Esper et al., 2005). However, a study in Taiwan did not find any association of HCoV-NL63 with Kawasaki disease (Chang et al., 2006).

There have been few studies on the occurrence of HCoVs in sub-Saharan Africa. In Cameroon, HCoVs have been reported to constitute 5.3% of outpatients presenting with influenza-like illness (Njouom, et al., 2012) while South Africa has reported a prevalence of 4.4% (Venter, et al., 2011). The study in South Africa noted the association of HCoV-NL63 with severe pneumonia and croup in a premature infant patient exposed to HIV. Similarly HCoV-OC43 was detected in patients with apnea attacks and lower respiratory

tract infections. The numbers of HCoVs detected were however low so epidemiologic association using healthy subjects as controls were not properly described. In Senegal, out of 55 positive viruses detected in 82 episodes of acute respiratory illness, HCoV constituted 10.9%. The positive HCoVs were made up of 3.7% HCoV-229E and 7.2% HCoV-NL63 (Niang et al., 2010). No cases of HCoVHKU1 and HCoV-OC43 were detected. In Madagascar, only HCoV-OC43 infection was reported among patients with influenza-like illness and this was found to be 6.1% (Razanajatovo et al., 2011). No study has yet reported a well-balanced epidemiologic association of HCoVs with upper or lower respiratory tract infections in sub-Saharan Africa.

Even though HCoVs have been reported to be associated with infections as described previously, they could also be found as normal commensals in the respiratory tract thus making is difficult to associate them with clinical illness. The question of whether HCoVs could be associated with upper respiratory tract infection has been reported to vary in different geographical locations. In the Netherlands, Van Elden et al. (van Elden, et al., 2004) identified 11% of HCoV-229E and HCoV-OC43 among children with acute respiratory illness compared to 1% in control groups thus providing evidence of their pathogenicity. Among adults, Graat et al. (Graat, et al., 2003) similarly found significant occurrence of HCoVs among sick elderly people in the Netherlands compared to asymptomatic subjects. In the United States of America (U.S.A), McIntosh et al. (McIntosh, K. et al., 1970) identified HCoV-OC43 and HCoV-229E in 34 (8%) of 417 children with acute respiratory illness and in 1 (8%) of 13 control children using serologic assays thus corroborating the findings by Van Elden (van Elden, et al., 2004). On the contrary,

other case-control studies among all age groups and children in Thailand and the U.S.A did not find association of HCoVs with clinical illness (Dare, et al., 2007; Prill, et al., 2012).

2.7.2 Serological prevalence of HCoVs

In terms of serological prevalence, most adults have higher levels antibodies to HCoVNL63, HCoV-229E, and HCoV-OC43 compared to HCoV-HKU1 (Hofmann, et al., 2005; Severance et al., 2008). The prevalence of HCoVs reported however differ based on the portion of the genome used for targeting the antibodies. Studies that have used nucleocapsid based assays have reported over 90% of HCoVs prevalence for HCoVNL63, HCoV-OC43, HCoV-229E and 59.2% for HCoV-HKU1 (Severance, et al., 2008). Based on the spike protein, Chan et al. (Chan, C. M. et al., 2009) has reported up to 21.6% prevalence of HCoV-HKU1 in the age groups 31 to 40 years. Similarly Hofmann reported that children less than 2 years do not carry neutralizing antibodies against the spike proteins from HCoV-229E and HCoV-NL63. However, children older than 1.5 years could carry some levels of neutralizing antibodies against HCoV-NL63 and HCoV-229E (Hofmann, et al., 2005). The higher detection of IgG antibodies based on the nucleocapsid is believed to occur based on its highly conserved region compared to the spike protein (Peiris et al., 2003). Rota et al.(Rota et al., 2003) reported that position 125 to 132 amino acid homology exist in the nucleocapsid terminal portion of HCoV-OC43 and therefore if immunogenic, would induce cross-reactivity antibodies against other coronaviruses.

2.8 Evolutionary relationship and genetic variability of HCoVs

Phylogenetic analyses of the various structural proteins of HCoVs have resulted in grouping and regrouping the viruses over the years. Through phylogenetic analyses, the

evolutionary relationship among the strains of HCoVs could be determined in different geographical areas. Phylogenetic tree construction of HCoVs have been done based on full or partial sequences of the spike gene, envelope gene, nucleocapsid and some nonstructural proteins (Dominguez et al., 2012; Vabret, A. et al., 2006). Other studies have also sequenced various portions of the HCoV-229E polymerase gene (Stephensen C.B, et al., 1999) , HCoV-OC43 spike gene (Vijgen et al., 2005) and HCoV-HKU1 nucleocapsid gene (Lee et al., 2013).

The nucleotide similarities are compared using maximum likelihoods, evolutionary distance and maximum parsimony (Tamura et al., 2011). Reports on phylogenetic analysis of HCoV-NL63 in Australia, Japan, Canada, France, Belgium and the Netherlands indicate the virus has circulated as a mixture of variants without specific geographical segregation (van der Hoek et al., 2006). Dominguez et al., (Dominguez, et al., 2012) on the other hand noted some unique strains of HCoV-NL63 (based on spike gene) which were seasonally dependent and more restricted to the United States of America (U.S.A).

In Africa information on HCoV sequences are limited. A study in South Africa has reported HCoV-NL63 to be clustered in two clades (A and B) based on sequence information from ORF 1a (Smuts, et al., 2008). In Ghana, Hayes et al. (Hays and Myint, 1998) reported of genetic variability in the first 1000 base pairs of the spike gene of HCoV-229E compared to isolates from the U.S.A and United Kingdom (U.K). His information is however limited since he only tested one patient. The findings however corroborate other studies which have similarly reported high frequency of recombination in the 5' half of coronaviruses (Banner et al., 1990; Dominguez, et al., 2012).

Phylogenetic relationship of coronaviruses among humans, animals and avian strains has shown the possibility of recombination which have led to generation of new strains. These new strains are able to adapt to human host and ecological niches, sometimes causing major zoonotic outbreaks (Woo, P. C. et al., 2006). Reasons such as the infidelity of RNA-dependent RNA polymerase (Duffy et al., 2008; Jenkins et al., 2002), distinctive random template switching during RNA replication (Lai, 1992; Pasternak et al., 2006) and the large nature of their genome (27-32kb) have been attributed to this unique recombination. Woo et al (Woo, P. C., et al., 2009)., described the evolutionary relationship among various HCoV species using sequences from the RNA dependent RNA polymerase (**Figure 2.0.6**). In Ghana, Annan et al (Annan, et al., 2013)., reported similarity between bat coronaviruses and the MERS-CoV (previously known as EMC/2012).

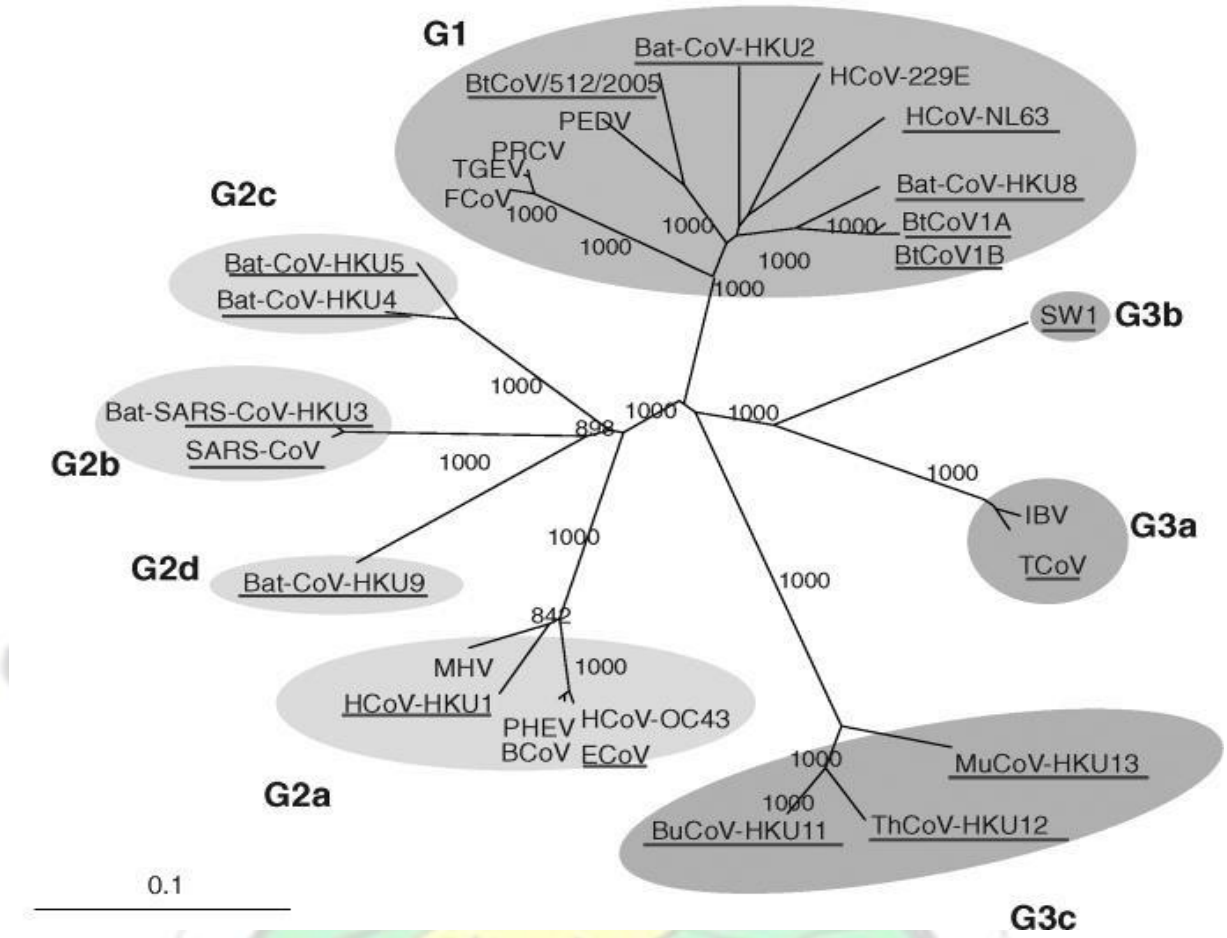


Figure 2. 0.6: Phylogenetic analysis of the RNA-dependent RNA polymerase

Trees were constructed using neighbour joining method at a bootstrap of 1000. G1 – G3; represent HCoV groups with subgroups denoted by a, b and c. The scale bars indicate the estimated number of substitutions per 10 amino acids. The viruses are defined as follows PED V; porcine epidemic diarrhea virus, TGEV; porcine transmissible gastroenteritis FCoV; feline coronavirus, PRCV, MHV; mouse hepatitis virus, BCoV; bovine Coronavirus, PHEV; porcine hemagglutinating encephalomyelitis virus, ECoV; equine coronavirus, IBV, infectious bronchitis virus, TCoV; turkey coronavirus, SW1; beluga whale coronavirus, BuCoV -HKU11; Bulbul coronavirus HKU11, ThCoV-HKU12; Thrush coronavirus HKU12; MuCoV-HKU13, Munia coronavirus HKU13. Adapted from Woo et al (Woo, P. C. et al., 2009).

2.9 Seasonality of HCoVs

The seasonality of HCoVs has been described to differ from year to year in tropical and temperate countries. In temperate countries such as the Netherlands, all four HCoVs circulate annually with high detection frequencies in winter months but low or no detections in summer seasons (Gaunt, et al., 2010).

In China, the seasonal distribution of HCoVs from 2005 to 2009 was reported to spike every two years particularly in the months of October 2005, June 2007, and April 2009 (Ren et al., 2011). HCoV-229E was detected mainly in October 2005 and 2007. HCoVNL63 occurred frequently during the period from August-December 2005-2007 (Ren, et al., 2011). In South Africa, high occurrence of HCoV-OC43 has been reported in the months of January, July and August of 2007 (Venter, et al., 2011). In the previous year (2006) however, no distinct seasonal pattern was observed (Venter, et al., 2011).

2.10 Laboratory diagnosis

Identification of HCoVs using conventional culture techniques and serology are generally uncommon due to the difficulty in growing the viruses in animal and human tissues. The discovery of HCoVs was however done using these techniques (Hamre and Procknow, 1966; McIntosh, K, et al., 1967). Mclntosh et al.,(McIntosh, K., et al., 1970; McIntosh, K. et al., 1978) in the 1970's, demonstrated the use of immunofluorescence assays to detect HCoV-229E and HCoV-OC43 in symptomatic human volunteers who had received HCoV inocula.

Other studies have also described the presence of HCoV-NL63 and HKU1 using viral culture techniques such as immortalized cells and human ciliated airway cell cultures (Donaldson et

al., 2008; Herzog et al., 2008; Pyrc, Krzysztof et al., 2010; Schildgen et al., 2006). Serologic methods using pseudotype viruses have also been used by Hoffman et al (Hofmann, et al., 2005)where they reported specific neutralizing antibodies to the nucleocapsid proteins of HCoV-229E and HCoV-NL63. The difficulty in culturing HCoVs and the lengthy periods associated with cultures have limited the use of serologic and culture techniques for viral diagnosis.

Currently PCR techniques are commonly used to detect all four major types of HCoVs including novel strains (Corman, et al., 2012; Gaunt, et al., 2010; Poon et al., 2003; Yam et al., 2005). RT-PCR assays designed as both multiplex and singleplex have been described by various authors (Dare, et al., 2007; Gaunt, et al., 2010). RT-PCR systems commonly described are based on the use of Taqman probes labeled at the 5' end and 3' end of short and specific oligonucleotides. The use of specific and sensitive probes allow for accurate detection of all four major species of HCoVs including novel strains such as MERS-CoV and SARS-CoV (Corman, et al., 2012; Wang, M. et al., 2005). One major advantage of RT-PCR is its ability to quantify virus loads in clinical samples thus aiding in the management of patients. RT-PCRs however cannot be used to identify coronaviruses of unknown sequences. New and undiscovered coronaviruses are best identified using pan-coronaviruses assays. Pan-coronavirus assays require the use of reverse transcriptase polymerase chain reactions that target homologous or consensus regions within the genera of coronaviruses and then followed by phylogenetic analysis (Annan, et al., 2013).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study area

The study was performed in three rural areas: Buoyem, Kwamang and Oforikrom communities (**Figure 3.0.1**). Buoyem and Oforikrom are located in the Techiman municipality of the Brong Ahafo Region of Ghana. The Techiman Municipality is among the twenty-two administrative districts in the region (Techiman Municipal Health Directorate, 2010). It shares common boundaries with the Wenchi District to the northwest and Nkoranza district to the south-east. The municipality has a total land surface of 669.7 square kilometers with climate and vegetation that promotes the production of food.

Kwamang community is in the Sekyere Central District of the Ashanti Region. The community is part of 5 sub-districts of Sekyere Central and forms one third of the total land size of the district. The three communities are predominantly agricultural populations and they mostly rely on small scale farming as means of livelihood. The study areas were chosen purposively as part of a broader research that is investigating the prevalence of coronaviruses among bats in the area. Previous studies have indicated that *Hipposiderous* bats in the community bat caves have about 9.6% prevalence of coronaviruses (Pfefferle, et al., 2009). The present study was carried out from September 2011 to August, 2013.

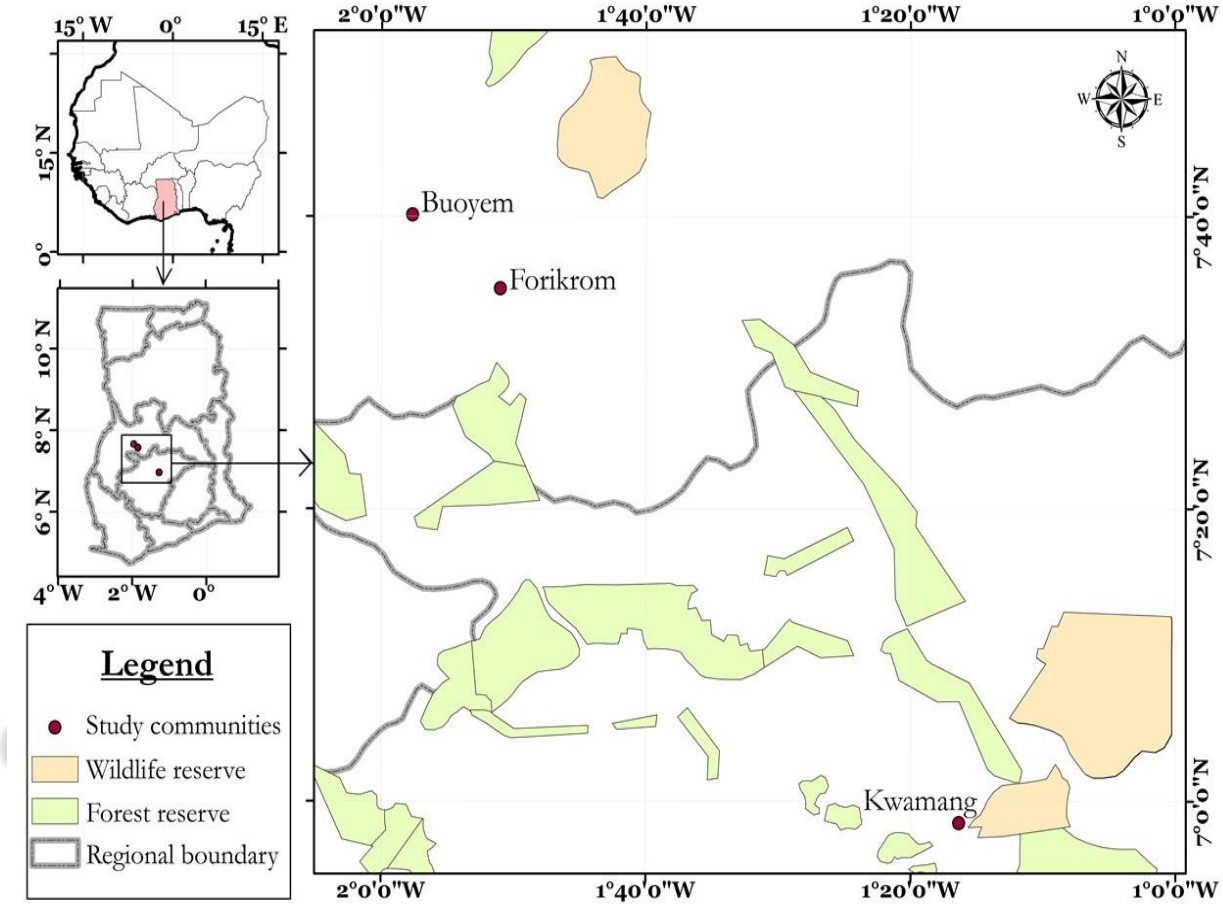


Figure 3.0.1: Geographical location of study areas in Ghana

3.2 Population characteristics

The population of Techiman municipality for 2010 was estimated to be 223,503 projected from the 2000 population census with growth rate of 2.5% (Techiman Municipal Health Directorate, 2010). Extrapolating from the total population, Forikrom and Buoyem have 16,178 and 9,549 individuals respectively (Techiman Municipal

Health Directorate, 2010). The municipal has a population density of 318 people per square kilometer with ethnic diversities including Akans, Ewes, Dagarba, Dagombas, Mamprusi, Frafra etc. Buoyem and Forikrom recorded high cases of malaria among

expectant mothers in 2010. Upper respiratory tract infections ranked second among the top ten causes of morbidity in the municipality and pneumonia accounted for 5.8% of deaths (Techiman Municipal Health Directorate, 2010).

The population of Sekyere Central District is 100,138 and that of Kwamang is 28,038. Acute respiratory infections in Kwamang also ranked second among the top ten causes of morbidity in 2011 (Sekyere Central District Assembly, 2010). The three communities have almost equal gender distribution. Forty five percent (45%), 56% and 59% of Kwamang, Oforikrom and Buoyem populations fall between 15 and 64 years respectively.

3.3 Study design

The study was designed in two parts: unmatched community based case-control study and cross-sectional study. The cross-sectional study was nested in the case control study. The case-control study was designed to identify risk factors of respiratory tract infections and also determine whether HCoVs are associated with upper respiratory tract infections whereas the cross-sectional study aimed at finding the seroprevalence of HCoVs. Recruitment of case control subjects was done on seasonal basis with major focus on the dry and wet seasons. This recruitment pattern was used because of the reported seasonality of HCoVs in literature (Gaunt, et al., 2010; Venter, et al., 2011). Recruitments were made in the pre-dry season (September, October, November), dry season (December, January, February), wet season (May, June, July) and pre-wet season (March, April). Recruitment was not done in parallel manner in each community. Cases were recruited in each season

alongside controls. Recruitment of subjects enrolled in the cross-sectional study was done in the pre-dry season. Subjects were recruited at random.

3.4 Study participants

3.4.1 Inclusion criteria

Cases: A subject was selected as a **case** if he or she had symptoms of upper respiratory illness i.e. sudden onset of any of the following: cough, sneezing, runny nose and nasal congestion. All subjects were above 10 years of age. All cases were identified by study health professionals and researchers.

Controls: A subject was selected as control if he or she did not have any symptom of upper respiratory illness as defined above for at least 8 days prior to recruitment. All controls were also above 10 years.

Cross-sectional study subjects: Subjects were enrolled into the cross-sectional study irrespective of whether they presented with symptoms of respiratory illness or not. All subjects were recruited from households and they were above 10 years of age.

3.4.2 Exclusion criteria

Subjects were excluded if they were less than 10 years, had history of epistaxis or refused clinical samples to be collected.

3.5 Sample size calculation

Case Control Study: The Sample size for the case control study was determined based on a similar study done in South Africa (Venter, et al., 2011). A known RT-PCR tested HCoV (HCoV-229E+HCoV-OC43+HCoV-HKU1+HCoV-NL63) prevalence of 4.5%

among patients with URTI and 0.01% among controls (adjusted upwards by 1% due to the small number of subject enrolled as controls) were used for calculation. MERS-CoV was not taken into consideration because it had just emerged in the Middle East therefore population based prevalence were not available. The formula used for calculating the sample size is shown below:

- Let "n" represent the number of cases or controls needed for the study (based on a 1:1 case-control design)

$$n \text{ (each group)} = \frac{(P_0Q_0+P_1Q_1) * (Z_{1-\alpha/2}+Z_{1-\beta})^2}{(P_1-P_0)^2}$$

P_0= Prevalence of HCoVs in control group = 1%

P_1= Prevalence of HCoVs in cases = 4.5%

$Q_0 = 1-P_0 = 1- 0.01 = 0.99$

$Q_1 = 1-P_1 = 1- 0.045 = 0.96$

$Z_{1-\alpha/2}$ = 1.96 = value of standard normal distribution corresponding to a significance level of 0.05 for a two sided test.

$Z_{1-\beta}$ = 0.84 = value of the standard normal distribution corresponding to level of power of 80%.

Substituting the above figures in the formula, "n" will be 339 in each group. Thus a sample size of 339 cases and 339 controls will be needed to detect a significant difference between cases and controls at a power of 80%

Cross-sectional study: The sample size for the cross-sectional study was determined based on an estimated HCoV serum IgG antibody prevalence of 94% in the U.S.A (Gorse et al., 2010; Severance, et al., 2008), a marginal error of 5%, and design effect of

2. The formula for calculation is shown below:

- Let "n" represent the number of subjects needed for the cross-sectional study

$$n = \frac{(Z_{1-\alpha/2})^2 * P * Q * e}{}$$

$(d)^2$

P = Estimated seroprevalence of HCoVs = 0.94

Q = 1 – P = 0.06 d

= estimated marginal error = 0.05 e

= estimated design effect = 2

$Z_{1-\alpha/2}$ = 1.96 = value of standard normal distribution corresponding to a significance level of 0.05 for a two sided test.

Substituting the above figures in the formula, "n" will be 174

3.6 Ethical approval and collaborating institutions

The study protocol was approved by the Committee for Human Research, Publications and Ethics (CHRPE) of KATH and School of Medical Sciences, KNUST, Kumasi, Ashanti Region, Ghana. Written informed consent was obtained for data and sample collection for all subjects. Thumb prints were obtained from subjects who could not provide written informed consent. For subjects less than 18 years of age, informed consent was obtained from parents or guardians and assent was obtained from the minors.

Collaborators for this study were the Department of Clinical Microbiology (KNUST), Kumasi Centre for Collaborative Research in Tropical Medicine (KCCR) and the Department of Virology, University of Bonn Medical Centre (Bonn, Germany). The study was funded by the German Research Foundation (DFG).

3.7 Field training

Field workers for this study were recruited and trained on the standard process of conducting interviews and to also understand the culture or traditions of the rural areas.

Nurses from the Komfo Anokye Teaching Hospital were also recruited to assist with nasopharyngeal sampling in the community.

3.8 Recruitment methods

3.8.1 Community entry

Prior to initiation of the study, focus group discussions were organized in each of the communities to inform them about the study rational. The focus groups were made of individuals from diverse ethnic groups, adult males and females, school boys and girls, opinion leaders, chiefs, healthcare practitioners, religious leaders and other stake holders in the community. The groups were educated on the importance of the study to the community and the need for them to support the study team in their work. They were also informed that a number of houses would be marked by the study team and such activity should also be supported. The entire protocol of the study was explained to them and all questions and doubts they had about the study were adequately addressed.

3.8.2 Selection of subjects

Subjects enrolled were selected in a cluster sampling design. In Buoyem, the community was divided into four quadrants (Q1, Q2, Q3, Q4) and clusters of house compounds were marked using satellite images (**Figure 3.0.2**). Houses close to social centres were identified in each cluster and subjects were enrolled from every other household using systematic random sampling design. Once subjects became familiar with the study team, radio announcements were made in all communities and eligible subjects were recruited at designated social centres. In Kwamang and Oforikrom communities, satellite images could not be retrieved so major roads that divide it into four quadrants were identified and

social centres at each quadrant of the roads were selected at random. Every other adjacent house starting from the identified social centre was marked and selected.

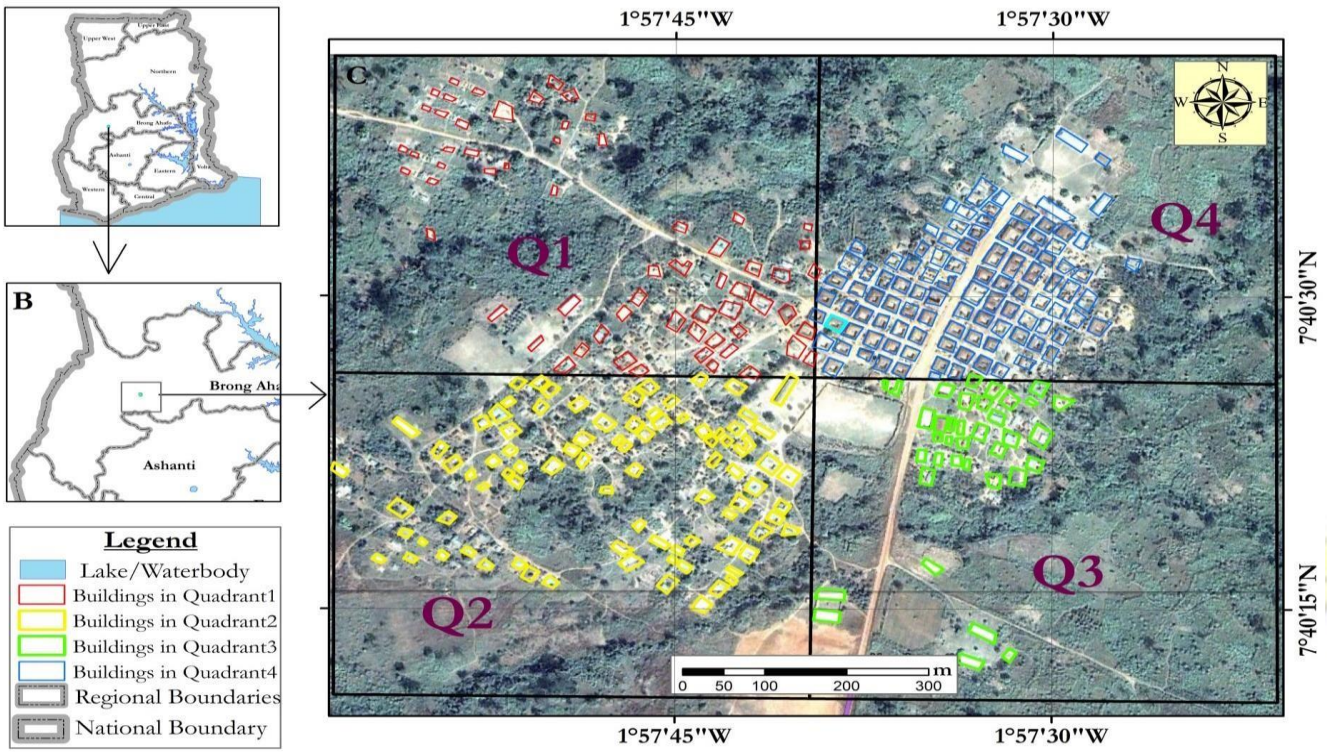


Figure 3.0.2: Cluster sampling from four quadrants in Buoyem community

The rectangular areas marked in each quadrant represent house compounds located in the Buoyem community. The houses were selected at random using randomized numbering.

3.9 Data collection techniques and tools

Data collection tools included administering structured questionnaires to study participants. The questionnaires were written in English and interpreted to study participants in their local dialects with the assistance of research assistants. Subjects were interviewed on some demographic parameters such as age, gender, occupation, religion, level of education, religious affiliation and type of accommodation.

Questionnaires were pre-tested before implementation on the field.

3.10 Clinical sampling of subjects

Nasopharyngeal or nasal swab specimens were taken with nasopharyngeal flocked swab (Copan, Italy) from subjects who consented to the study. The samples were taken by healthcare personnel on the research team. Swab samples were taken from all subjects enrolled in the case control and cross-sectional study. For the cross-sectional study subjects, 5 ml of blood samples were taken together with nasal/nasopharyngeal swabs. The nasal/nasopharyngeal sample was taken by gently inserting the swab up the nostril towards the pharynx until resistance was felt and was then rotated 3 times to obtain epithelial cells. Blood samples were spun at high speed and serum were aliquoted into 5ml cryotubes and transported in liquid nitrogen to the laboratory. The swabs were stored in RNAlater (Qiagen, Hilden, Germany). **Figure 3.0.3** describes the flow chart of sampling.

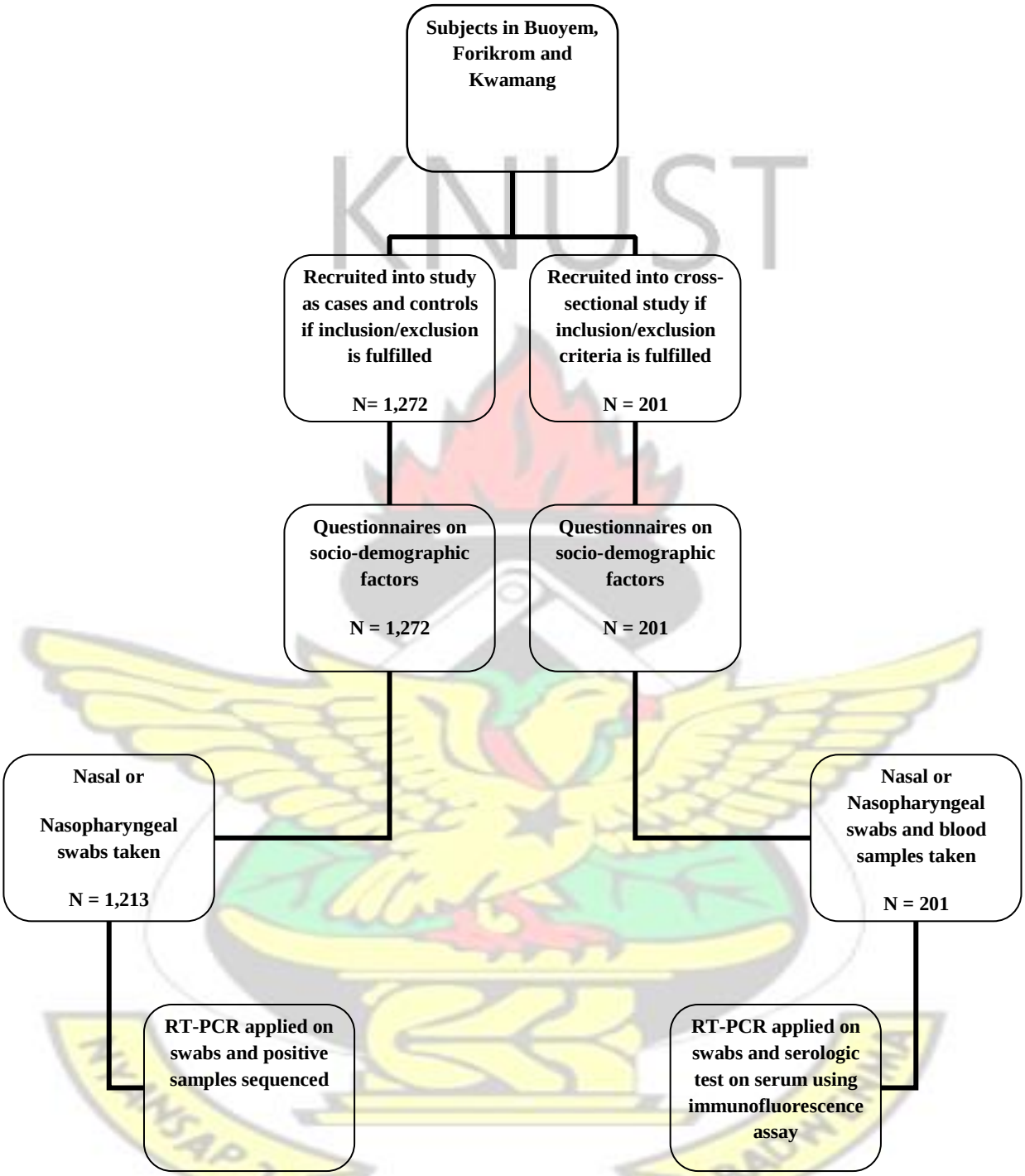


Figure 3.0.3: Flow chart of overall study design

3.11 Laboratory methods

3.11.1 Laboratory training

Before commencement of this study, a 3-month laboratory training was undertaken by the author of this study at the virology laboratory of Prof. Dr. Christian Drosten at the Department of Virology, University of Bonn Medical Centre (Bonn, Germany). Training on the use of molecular techniques for RT-PCR testing, preparation of in-vitro transcripts and DNA/RNA were undertaken. The techniques learnt were applied at the Kumasi Centre for Collaborative Research in Tropical Medicine where RNA extraction and preliminary testing of nasal swabs were done.

A further six month training was undertaken at the Department of Virology, University of Bonn Medical Centre (Bonn, Germany).where serologic techniques and advanced molecular techniques in primer design and phylogenetic analysis were learnt and applied on study samples.

3.11.2 Testing of serum samples for HCoVs using Immunofluoresence Assay

Serum samples collected from subjects enrolled in the cross-sectional study were tested for the presence of IgG antibodies to three HCoVs; HCoV-229E, HCoV-OC43 and HCoV-NL63 using immunofluorescence assay. The assay principle is based on the binding of HCoV antibodies to the spike protein of the respective viruses. The spike proteins of the individual viruses were expressed by transfecting African green monkey kidney (VeroB4) cells with plasmids (pCG1). Visualization of this binding is then made by applying commercially available anti-human antibodies labeled with fluorescein dyes.

Testing and preparation of the spot slides for detection of antibodies to the HCoVs was done in the following stages:

Stage 1: Antigen preparation: The spot slides used for this test were prepared in-house with the assistance of the serology team of the Bonn Institute of Virology. VeroB4 cells transfected with pcG1 plasmids (in-house, Bonn) expressing the corresponding CoV spike proteins were used. To prepare the plasmids, DNA products that encode the spike region of HCoVs were generated using conventional PCR on samples positive for the respective viruses (HCoV-229E, HCoV-OC43, HCoV-NL63) using specific primers (**Table 3.0.1**). The DNA products were ligated into pcG1 vector (In-house, Bonn) and then transfected into Vero cells using FuGENE transfection reagents (Promega, USA) overnight at 37C. The cells were harvested after one day and then resuspended in Dulbecco's Modified Eagle Medium (DMEM) (PAA Laboratories, Germany) in 10% Fetal Calf Serum (FCS). Aliquots of cells were pelleted at 300xg for 5 minutes and then washed twice with 1 ml phosphate buffered saline (PBS). The pellet suspension were examined with an inverted microscope (Zeiss, Axio, Germany) and cells were counted using Neubauer counting chamber. Pelleting and washing was repeated again and the density of cells was adjusted to a final cell density of 10^7 cells per ml. Fifty microliters (50 µl) of cell pellets were then spotted on 12 well slides by dispensing and immediately aspirating. Two seconds interval was allowed between spotting of each well and slides were dried overnight at room temperature. The slides were then fixed and permeabilized using ice cold acetone/methanol (1:1) and then stored at 4°C after drying for twenty minutes.

Table 3.0.1: Primer sequences for generating HCoV spike region

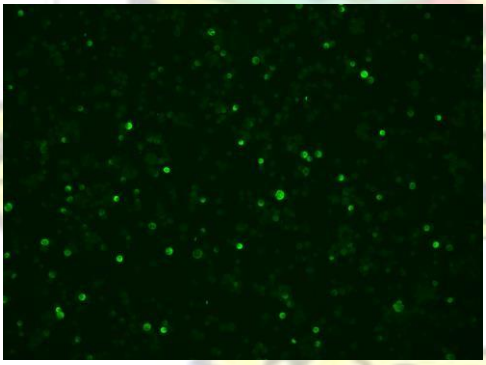
HCoV-229E-S-Bam HI-KZK-F	TACGGATCCGCCACCATGTTTGTTTTGCTTGTTGCATATG
HCoV-229E-SflagC-Xba I-R	AGC TCT AGA TTA CTT GTC ATC GTC ATC CTT GTA ATC GCC TCC CTG TAT GTG GAT CTT TTC AAC GT
NL63-SflagN-Bam HI-F*	TACGGATCCGCCACCATGgattacaaggatgacgatgacaagggaggc AAACTTTTCTTGATTTTGCTTGTTTTG
NL63-S-Sal I-R	AGC GTC GAC TTA TTG AAC GTG GAC CTT TTC AAA T
OC43-SflagN-Bam HI-F*	TACGGATCCGCCACCATGgattacaaggatgacgatgacaagggaggc TTTTTGATACTTTTAATTTCCTTACCAAC
OC43-S-Sal I-R	AGC GTC GAC TTA GTC GTC ATG TGA AGT TTT GAT T

* Small letters in sequence denotes the region where the flag proteins are expressed. Flag proteins allow for controlling spike proteins that will be expressed.
* Red letters are the restriction sites targeted by restriction enzymes

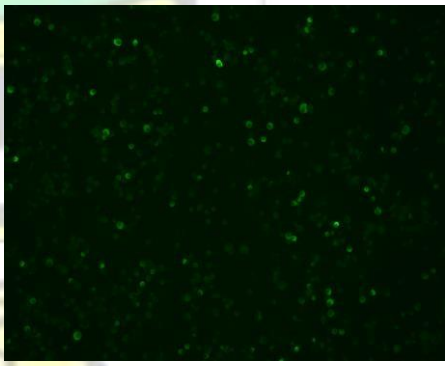
Stage 2: Staining of control samples: To test the performance of the immunofluorescence assay, serum samples of three patients infected with HCoV-NL63, HCoV-OC43 and HCoV-229E were diluted in Euroimmun buffer (Baker, U.S.A) at dilutions of 1:40, 1:80, 1:160, 1:320, 1:640 and 1:1280. Similarly, the serum samples of four rabbits (two not immunized and two immunized) were also diluted with Euroimmun buffer at a dilution of 1:40 for each serum. The immunized rabbits were used as positive controls for HCoV-229E, HCoV-OC43 and HCoV-NL63.

Twenty five (25µl) of all diluted sera were applied to each well on the glass slide and incubated for 1 hour in humid box at 37ºC. Afterwards, slides were washed three times with phosphate buffered saline in 0.1% tween (PBS-T) for 5 minutes and 25µl of secondary antibodies were applied and incubated for another 30 minutes. The secondary antibodies were goat-anti human Cy2 (Dianova, Germany) diluted to 1:400 in albuminazid and donkey anti-rabbit Cy2 (Dianova, Germany) diluted to 1:200. Slides were afterwards washed for three times at 5 minutes interval with PBS-T. Cotton tipped swabs were used to drain off excess liquid outside the stained areas and a drop of mounting medium (DAPI Prolong, Invitrogen, Germany) was applied to the stained wells. The slides were kept in a dark cool environment for 24 hours prior to examination with immunofluorescence microscope.

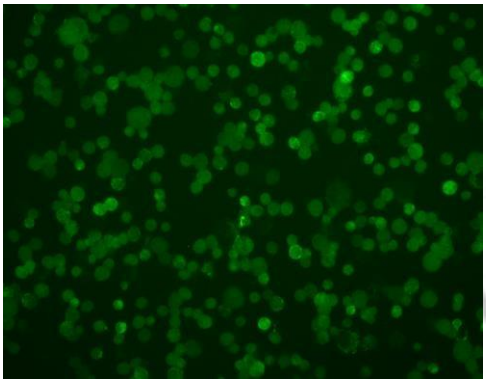
Examination of stained slides: Slides were examined with an immunofluorescent microscope (Carl Zeiss, Germany) under x10 and then confirmed with x20. Pictures were taken with AxioVision Rel 4.8 software **Figure 3.0.4** shows examples of slide images of the assay controls.



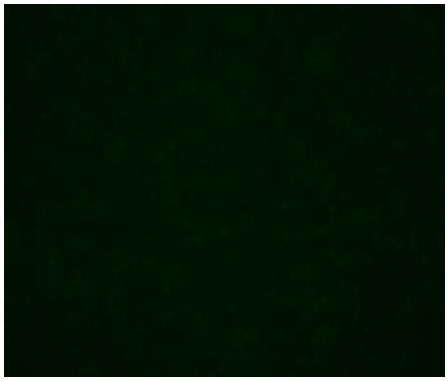
A. Positive control: HCoV OC43



B. Positive control: HCoV NL63



A. Positive control: HCoV 229e



A. Negative control

.

Figure 3.0.4: HCoV controls examined with x10 objective lens.

A, B and C show the images of positive controls (1:40 dilution) stained for immunofluorescence antibodies against patients infected with HCoV -OC43, rabbit infected HCoV-NL63 and patient infected HCoV-229E respectively. D is an image of a negative control from rabbit not immunized against HCoVs.

3.11.3 Testing of human respiratory swabs using RT-PCR

3.11.3.1 Viral RNA Extraction

Viral RNA was extracted from the samples using the spin protocol of the QIAamp Viral RNA Mini kit (Qiagen, Hilden, Germany) and following the manufacturer's procedures (Qiagen, 2005). RNA extraction was done in four (4) steps namely; Lysis, Binding, Washing and Elution.

Lysis Step: This step ensured that viral particles were lysed to release the viral RNA. Briefly, 560 µl of prepared buffer AVL-containing carrier RNA was pipetted into 1.5ml eppendorf tube. One hundred and forty microliters (140 µl) of the clinical sample was added and the mixture was vortexed for 15 seconds and incubated at room temperature for 10 minutes.

Binding Step: The released viral RNA was bound to a membrane spin column in this step. Five hundred and sixty (560) µl of ethanol (96-100%) was added to the resultant mixture. The mixture was pulse-vortexed for 15 seconds and centrifuged at 8000 revolution per minute (r.p.m) for 1 minute. Six hundred and thirty microliters (630 µl) of the mixture was then transferred into a QIAamp mini column seated in a 2 ml collection tube. The mixture was spun for 8000 r.p.m and the filtrate was discarded. The mini column was then placed into another 2 ml collection tube. The rest of the sample was reloaded into the QIAamp mini column and the centrifugation step was repeated until all the samples were loaded unto the QIAamp mini column.

Washing Step: This step was done to wash and purify the viral RNA which had bound to the QIAamp mini column. Two buffers were used; AW1 and AW2. Five hundred microliters (500 µl) of buffer AW1 was added to the QIAamp mini column (containing viral RNA loaded samples) and spun for 8000 r.p.m. The filtrate was discarded and the QIAamp mini column was refilled with buffer AW2. This was spun at 14000 r.p.m for 3 minutes and repeated for another 2 minutes to completely empty the left over buffer.

Elution Step: This step was done to elute the viral RNA bound to the QIAamp mini column. Briefly, the QIAamp mini column was placed in a new 1.5 ml eppendorf tube and 100 µl of buffer AVE pre-warmed at 80°C was added. This was spun at 8000 r.p.m

for 1 minute and the eluted RNA was stored at -20°C until they were tested for respiratory viruses using real time polymerase chain reaction PCR.

3.11.3.2 Preparation of in-vitro transcripts for evaluation of RT-PCRs for HCoVs

Prior to testing the study samples, RNA transcribed in-vitro transcripts were prepared from PCR products for each of the five HCoVs; HCoV-229E, HCoV-OC43, HCoVNL63, HCoV-HKU1 and MERS-CoV. The process of in-vitro transcripts preparation was done as follows:

Step 1: **PCR product generation**: PCR products of HCoV-OC43, HCoV-229E, NL63 and HKU1 were generated from multiplex RT-PCR performed on known clinical samples positive for the respective viruses using assays targeting unique regions of the viral genome. In the case of MERS-CoV, gel reverse transcription polymerase chain reaction was performed on known positive clinical samples. A 25 µl reaction mixture for MERS-CoV testing was made up of 3.1 µl of RNase free water, 0.4 µl of 50mM MgSO4, 12.5 µl of 2x reaction mix (Invitrogen,CA, U.S.A), 1 µl of 1 mg/ml of Bovine serum albumin (BSA), 1 µl of 10 µM each of forward and reverse primers, 1 µl of superscript III Onestep RT-PCR with platinum Taq enzyme mix (Invitrogen) and 5 µl of template RNA. Reaction conditions were initial reverse transcription at 55°C for 20 minutes, Taq DNA polymerase activation at 94°C for 3 minutes followed by 45 cycles of denaturation at 15 seconds for 94°C and annealing at 58°C for 30 seconds.

Multiplex RT-PCR testing of 25 µl reaction of HCoV-NL63 and HCoV-HKU1 was done using 5 µl of template RNA, 1 µl of 10mM dNTP mix (Qiagen, Germany), 1 µl each 10 µM of forward primer and reverse primers of HKU1, 1.5 µl each of forward and reverse

primers of HCoV-NL63, 0.5 µl each of NL63 probe and HKU1 probe, 7 µl of RNase free water, 5 µl of OneStep 5x buffer (Qiagen) and 1 µl of Enzyme Mix (Qiagen, Germany). HCoV-229E and HCoV-OC43 were similarly tested using a 25 µl reaction of 1 µl of 10mM dNTP Mix (Qiagen), 1 µl of each forward (10 µM) and reverse primers (10 µM) of each virus, 0.5 µl of each virus probe (10 µM), 5 µl of Onestep 5x buffer (Qiagen, Germany), 8 µl of RNase free water, 1 µl of Enzyme mix (Qiagen, Germany) and 5 µl of template RNA. The reaction conditions of the multiplex PCR's were the same as that of the MERS-CoV assay.

The target regions and primers used in generating the PCR products are described in the **Figure 3.0.5** and **Table 3.0.2** respectively below.

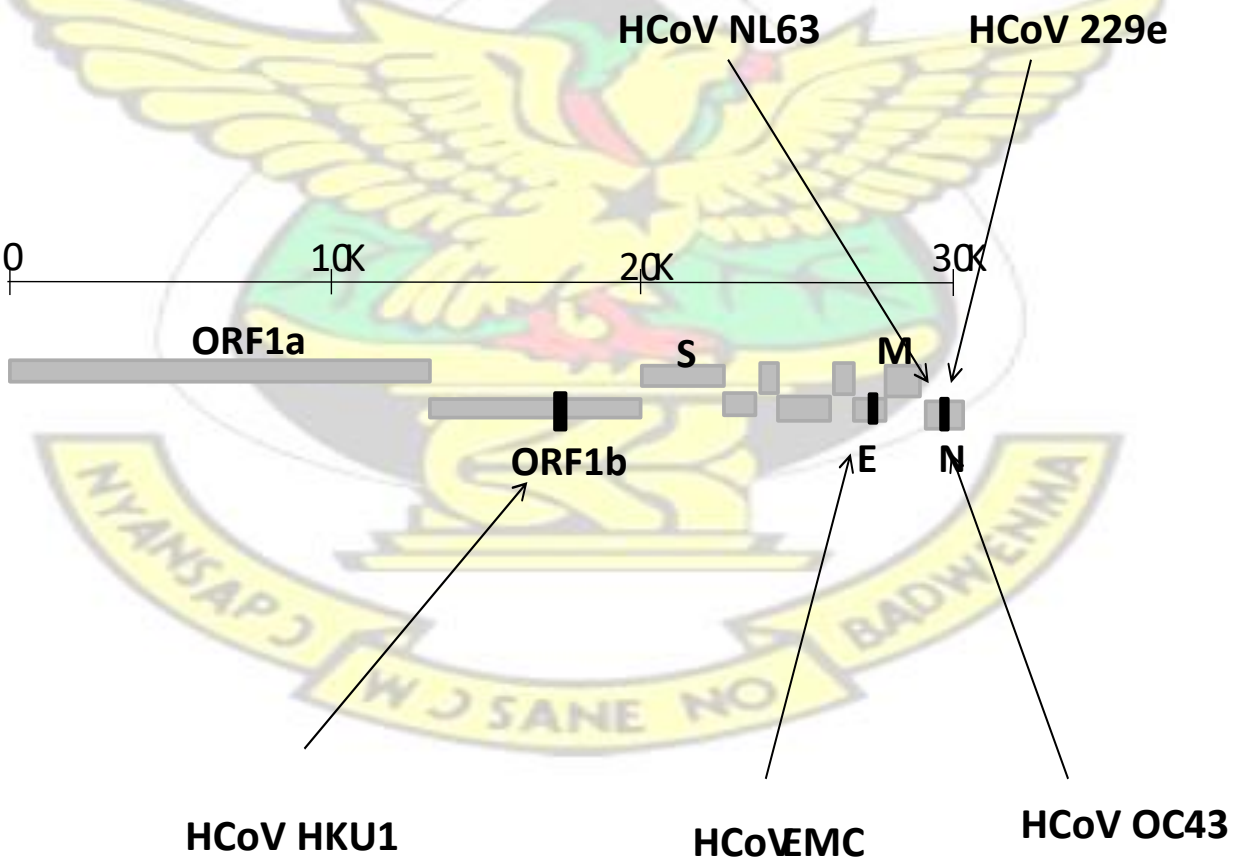


Figure 3.0.5 Genome regions targeted by assays.

The regions targeted by the assay are the black filled areas. The open reading frame 1b (ORF1b) is targeted by primers specific to HKU1, envelope (E) encoding protein is targeted by HCoVs EMC (also known as MERS) and nucleoprotein (N) is targeted by primers specific to unique regions of HCoV-229E, NL63 and OC43. S represents the spike protein and M is the membrane protein. The scale at the top (0-30K) shows the approximate base pairs of the segments of the genomes.

Table 3.0.2: Primer sequences for generating in-vivo transcripts

Virus Type	Forward Primers	Reverse Primer	Probe	Target Region	Reference
	5'----------> 3'	5'----------> 3'			
HCoV-229E	CAGTCAAATGGGCTGATGCA	AAAGGGCTATAAAGAGAATAAGGTATTCT	JOE-CCCTGACGACCACGTT GTGGTTCA-[a]BHQ1	Nucleoprotein	(Dare, et al., 2007)
HCoV-OC43	CGATGAGGCTATTCCGACTAGGT	CCTTCCTGAGCCTTCAATATAGTAACC	[b]FAM-TCCGCCTGGCACGGTA CTCCCT-BHQ1	Necleoprotein	(Dare, et al., 2007)
HCoVNL63	GACCAAAGCACTGAATAACATTTTCC	ACCTAATAAGCCTCTTTCTCAACCC	FAMATGTTATTCAGTGCTTTGGTCCTCGTGATBHQ1	Necleoprotein	(Dare, et al., 2007)

HCoV-HKU1	CCTTGCGAATGAATGTGCT	TTGCATCACCACTGCTAGTACCAC	JOE-TGTGTGGCGGTTGCTATTATGTTAAGCCTGBHQ1	Replicase 1b	(Dare, et al., 2007)
MERS-CoV	CTTCTCATGGTATGGTCCCTGT	AAGCCATACACACCAAGAGTGT	N/A	Envelope	(Corman, et al., 2012)

[a] FAM: 6-carboxyfluorescein, [b]BHQ1: Black Hole Quencher 1

Step 2: Overhang of deoxy-Adenosine Triphosphates (dATPs): Overhang PCR was performed on DNA products from step 1 in order to add dATPs at the 3' ends of the PCR products. This step is necessary for the DNA products to be inserted into the vector and was done to rule out any possibility of loss of dATPs. A 20µl of PCR reaction was done by taking 2µl of x10 PCR buffer, 1µl of $MgCl_2$ (50mM), 1µl of dATPs, 0.1µl of Platinum Taq Polymerase enzyme and 15.9µl of PCR products. PCR was performed at 95ºC for 3 minutes denaturation, 72ºC for 15 minutes elongation and final cooling at 4ºC for 1 minute.

Step 3: **Ligation of DNA product into vector:** DNA products generated from step 2 were ligated into a vector (pCR® 4-TOPO) using Invitrogen TOPO TA Cloning Kit

(Invitrogen,CA, USA). The ligation was done by pipetting 4µl of PCR product, 1µl of salt (1.2 M NaCl+0.06 M $MgCl_2$) and 1µl of the TOPO® vector into a 1.5ml eppendorf tube. The mixture was kept at room temperature for 30 minutes. The mixture was again put on ice for 2 minutes after which it was put back at room temperature.

Step 4: Transforming of ligated products into TOP10 *E.coli cells*: TOP10 *E.coli* was unfrozen unto dry ice. Two (2µl) of ligated PCR product (from step 2) was added to One Shot® Chemically Competent *E.Coli* and mixed gently. Caution was taken not to pipette the mixture up and down to avoid the reaction being interrupted. The mixture was incubated for 5 to 30 minutes on ice and heat-shocked by placing it immediately at 42º C without shaking. The tubes were immediately transferred unto dry ice and 250µl of S.O.C medium (Invitrogen, CA, USA) was added and kept at 37ºC on a shaker.

Afterwards, 75µl and 120µl of the mixture were streaked on two pre-warmed LB Kanamycin selective plates and incubated overnight at 37ºC. The Kanamycin agar plates were used so as to enhance the selection of only *E.coli* cells that have picked the vector. This is because the vector has resistant genes for Kanamycin and would therefore allow the growth of only *E.coli* cells that have acquired it.

Step 5: Clone screening: The plates were checked for growth and M13 PCR was performed on each of the colonies by using 2.5µl of x10 PCR buffer, 0.5µl of 12.5mM dNTPs, 1µl of 50mM of $MgCl_2$, 0.5µl each of 0.1µg/µl of M13 forward primer and reverse primers, 0.1µl of platinum Taq and 19.4 µl of RNase free water. The cycling conditions of the PCR was 95ºC for 6 minutes and 30 cycles of 95ºC for 15 seconds, 58ºC for 15

seconds and 72ºC for 25 seconds. The cooling step was 1 minute. The products of the PCR were run on 2% agarose gel and the size was confirmed to be 160 bp.

Step 6: Selection of plasmid products and purification: Two clear bands were noted from the gel PCR and the corresponding colonies were selected. Colonies were inoculated into 1.5ml of Luria Broth (LB) (containing Kanamycin) in a 2ml tube. The broth was inoculated at 37ºC in a shaker overnight after which the plasmid products were purified using QIAamp Spin-miniprep kit (Qiagen, Germany). The purification was done according to the manufacturer's protocol. The final elution of the pure plasmid was done with 50µl of elution buffer (EB) medium. The purified plasmids were diluted serially from 10^{-1} to 10^{-10} in carrier RNA free water. The carrier RNA enhanced the stabilization of the plasmids products and was used at a concentration of 10µg/ml. To increase copies of the PCR product inserted and also reduce plasmid background, M13 gel PCR was performed on the serially diluted plasmids (10^{-3} to 10^{-10}) using 5µl of x10 PCR buffer, 1µl of 12.5mM dNTPs, 2µl of 50mM $Mgcl_2$, 1µl of 0.1µg/µl of M13 forward and reverse primers and 0.2µl of Platinum Taq enzyme. The PCR conditions were similar to step 5 except that the cycling was increased to 50. The PCR product generated was further screened to check for the correct insertion by using M13 forward primer and a virus specific (eg HCoV-229E) reverse primer. The lowest dilution which was visibly positive (**Figure 3.0.6**) was selected for RNA transcript synthesis.

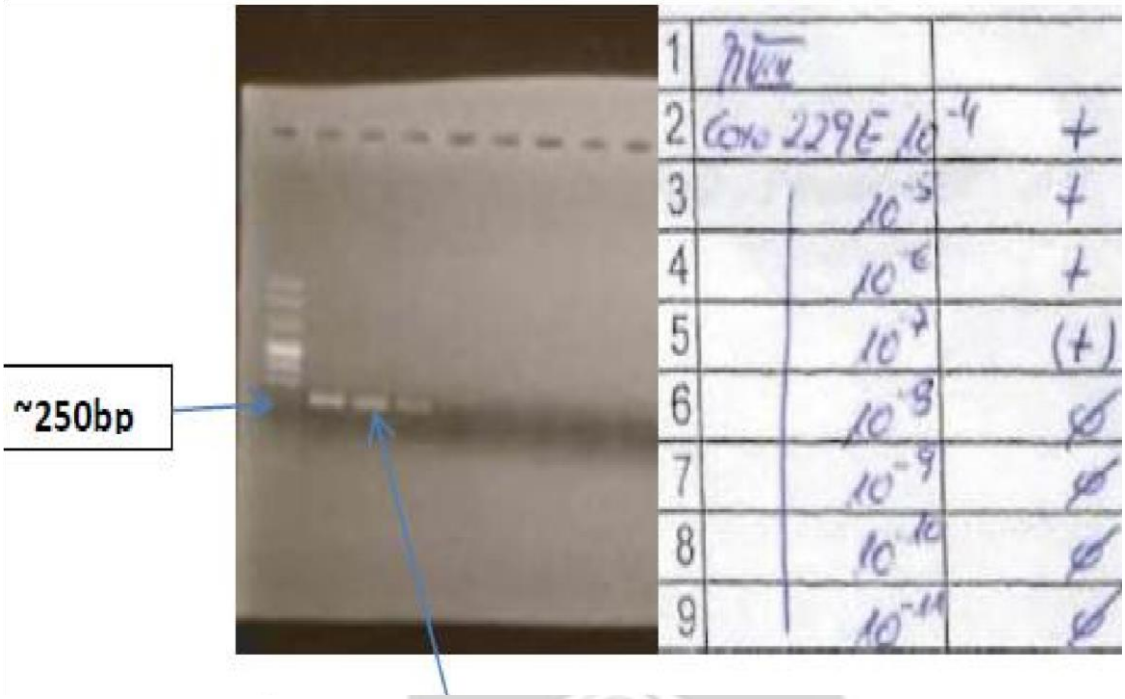


Figure 3. 0.6: Gel Picture of HCoV-229E plasmid product The band size is approximately 250 base pairs.

Step 7: Synthesis of RNA transcripts: RNA synthesis was performed using the Ambion Megascripts T3 kit (Invitrogen, CA, USA). Two microliters (2µl) of ATP (adenosine triphosphate), CTP (cytidine triphosphate, GTP (guanosine triphosphate) and TTP (thymidine triphosphate) were each pipetted into 0.2 ml eppendorf tube. Two microliters (2µl) of 10x reaction buffer, 2µl of enzyme mix (RNA polymerase specific for SP6, T7 and T3 promoter sites) and 4µl of the purified plasmid products (10^{-8}) were added to the mixture to make up a total reaction volume of 20µl. The mixture was incubated at 37ºC for four hours after gentle mixing. One microliter (1µl) of turbo Dnase was added and the mixture was again incubated at 37ºC. The mixture was purified using RNeasy kit

(Qiagen, Germany) by using 21µl of the invitro-transcript and following the manufacturer's protocol. The final elution was done in 50µl of RNA free water. The concentration of the in-vitro-transcripts was measured in micrograms per microliters using Nanodrop 2000 Spectrophotometer (Thermoscientific, Germany). The final concentration was calculated using the formula:

$$\text{Viral copies of in-vitro transcript/µl} = \frac{\text{Conc. (Transcript)} \times \text{Avogadro's constant}}{\text{Mwt (P+V)} \times \text{Mwt (Bp)}}$$

Conc. (Transcript): Concentration of in-vitro transcript determined from Nanadrop

Mwt (P+V) : Molecular weight of PCR product (P) + Vector (V)

Mwt (Bp) : Molecular weight per base pair of RNA = 340 g/mol

3.11.3.3 Determining the sensitivities of HCoV assays

The sensitivities of the assays were assessed by determining its minimum detection limit. The sensitivities were determined in two stages:

Stage 1: **Selecting the appropriate in-vitro transcript dilution**. Tenfold serial dilutions of the in-vitro transcripts prepared were made and Real-time reverse transcription PCR was performed on the transcripts using primers specific to each virus. The primers used were the same as in **Table 3.0.2** above with the exception of MERSCoV which has the forward primer as GCAACGCGCGATTCAGTT and reverse primer of

GCCTCTACACGGGACCCATA. The probe was labelled with 6-carboxyfluorescein [FAM]) CTCTTCACATAATCGC CCCG- AGCTCG -6-carboxy-N,N,N, N´-tetramethylrhodamine [TAMRA]). From the PCR results, the diluted transcripts with cycling thresholds of 30 were selected for each virus. The threshold value was chosen so as to avoid possible contamination of the clinical samples and laboratory reagents.

Stage 2: Determining the sensitivity of HCoV assays. The selected in-vitro transcripts from stage 1 were further diluted serially in tenfolds and RT-PCR was performed on all samples using the protocols described in **Tables 3.0.3 to 3.0.5.**

Table 3.0.3: Master mixture protocol for testing MERS-CoV

	25µl		
Master Mixture:	**single rxn, µl**	**x 10 (µl)**	**Cycler:**
H_20 (RNase free)	2.6	26	
MgSO4 (50mM)	0.4	4	55ºC 20'
2x Reaction mix*	12.5	125	94ºC 3'
BSA (1mg/ml)	1	10	
Forward primer (10µM)	1	10	94ºC 15" x45
Reverse primer (10µM)	1	10	58ºC 30"
Probe (10µM)	0.5	5	
SS111/Taq EnzymeMix*	1	10	40ºC 30"
Total	20		
Template RNA	5		' = minutes; "=seconds

* Invitrogen: Superscript111 Onestep RT-PCR System with Platinum Taq DNA Polymerase **Table 3.0.4: Master mixture for testing HCoV-NL63 and HKU1**

	25µl		
Master Mixture:	**single rxn, µl**	**x 20 (µl)**	**Cycler:**
H_20 (RN ase free)	7	140	
OneStep 5x buffer, Qiagen	5	100	55ºC 20'
dNTP Mix, Qiagen OneStep kit	1	20	94ºC 3'
NL63 Forward primer (10µM)	1.5	30	
NL63 Reverse primer (10µM)	1.5	30	94ºC 15" x 45
NL63 Probe (10µM)	0.5	10	58ºC 30"
HKU1 Forward primer (10µM)	1.0	20	
HKU1 Reverse primer (10µM)	1.0	20	
HKU1 Probe (10µM)	0.5	10	

Enzyme Mix, Qiagen OneStep	1	20	
Total	20		
Template RNA	5		' = minutes; "=seconds

* Qiagen: Qiagen OneStep RT-PCR Kit.

Table 3.0.5: Master mixture for testing of HCoV-OC43 and HCoV-229E

	25µl			
Master Mixture:	**single rxn, µl**	**x 20 (µl)**	**Cycler:**	
H_20 (RNase free)	8	140		
OneStep 5x buffer, Qiagen	5	100	55ºC	20' dNTP Mix,
Qiagen OneStep kit	1	20	94ºC 3'	
HCoV-229E Forward primer (10µM)	1	30		
HCoV-229E Reverse primer (10µM)	1	30	94ºC	15" x 45 HCoV-229E Probe (10µM)
	0.5	10	58ºC 30"	
OC43 Forward primer (10µM)	1.0	20		
OC43 Reverse primer (10µM)	1.0	20		
OC43 Probe (10µM)	0.5	10		
Enzyme Mix, Qiagen OneStep	1	20		

Total	20	
Template RNA	5	' = minutes; "=seconds

* Qiagen: Qiagen OneStep RT-PCR Kit.

To perform the PCR, five (5) microliters of all serially diluted samples were pipetted into Lightcycler 480 multiwell plate (Roche Diagnostics, GmbH) seated in 96 well cooling block. The cooling block was used so as to slow the activity of the reverse transcriptase enzyme contained in the master mix. Twenty microliters (20µl) of the master mixture was added to the samples in the multiwall plate and centrifuged at low speed to concentrate the mixture. The plate was placed in a light cycler version 480 (Roche Diagnostics, GmbH) which had been programmed according to the conditions in the cycling protocol. The detection limits of all viruses (defined as the minimum viral copy number which yields amplification) were determined from the RT-PCR results to be one (1) copy per PCR reaction for MERS-CoV, 1.8 copies per PCR reaction for HCoV-HKU1, 1.7 copies per PCR reaction for HCoV-NL63, 212 copies per PCR reaction for HCoV-OC43 and 209.5 copies per PCR reaction for HCoV-229E.

External standard curves were generated from the PCR results using the Absolute/2nd derivative maximum based on which unknown concentration of viruses in clinical samples were extrapolated. **Figure 3.0.7 and 3.0.8** show an example of a standard and amplification curved for MERS-CoV respectively.

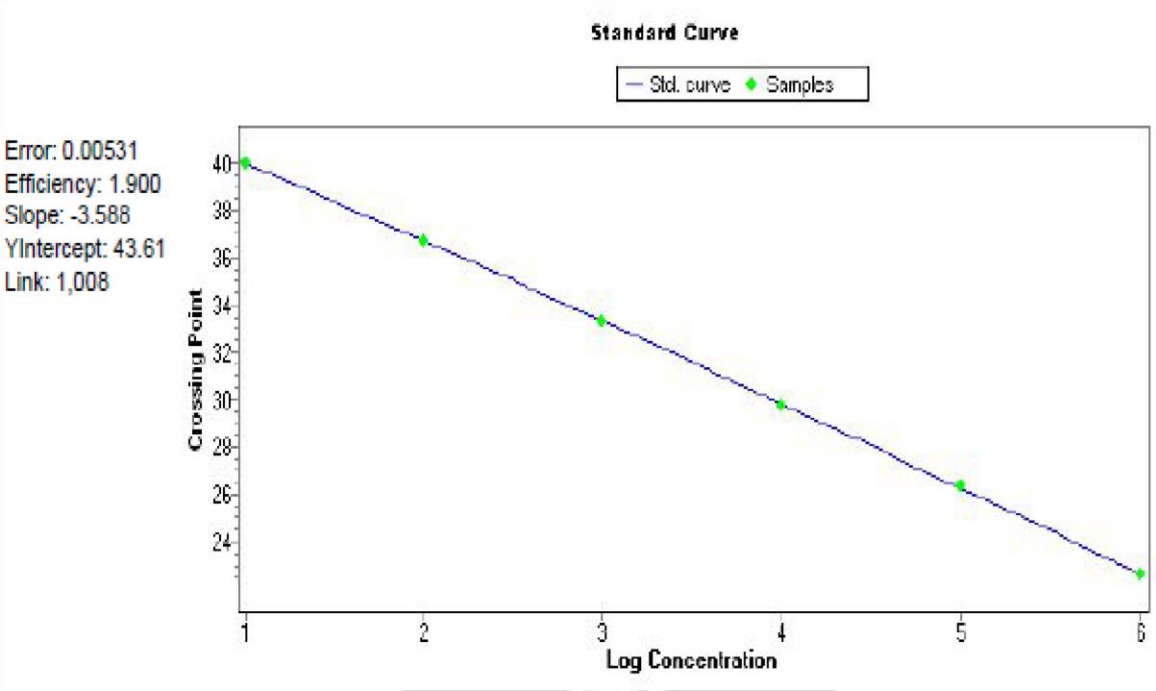


Figure 3.0.7: Standard curve of MERS-CoV

The standard curve was generated from performing RT-PCR on serial dilutions of MERS-CoV in-vitro transcripts. The logarithm concentrations of the serial dilutions show high correlation with cycling thresholds with a minimum error of 0.00535 and PCR amplification efficiency of 1.9.

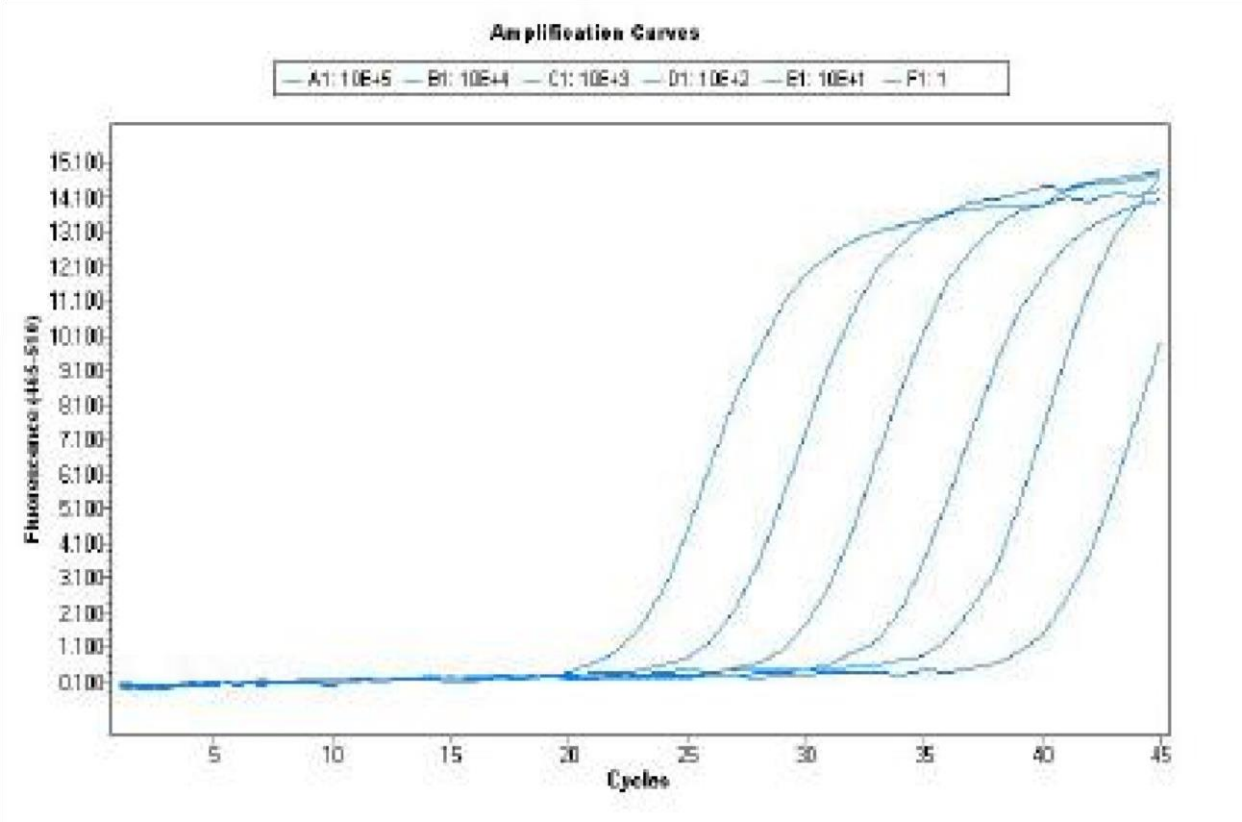


Figure 3. 0.8: Amplification curves of MERS-CoV serial dilutions

In-vitro transcripts were serially diluted from 10^5 copies per PCR reaction down to 1 copy per reaction. Curve shows equidistant amplification with the minimum detection limit occurring at cycle 40.

3.11.3.4 Clinical sample testing using RT-PCR

Once the sensitivities and precision of the HCoV assays were established, testing of clinical samples were initiated. All RNA extracts from clinical samples were thawed and

pooled in fives and tens for cases and control subjects respectively. Pools were labeled and screened for all viruses using the protocol described for each virus in **Table 3.0.3 to 3.0.5.** The calculation of the master mixture was varied depending on the number of samples that were tested in each run. Two in-vitro transcripts (10^2 copies per PCR reaction and 10^3 copies per PCR reaction) were included in each run for each virus to serve as controls for checking the reproducibility of each HCoV assay. RNA free water was used a negative control for each run. All positive results were quantified in copies per PCR reaction using the stored external standard curves generated from transcripts.

3.11.4 Checking the interassay variation of RT-PCR assays

At the end of the PCR runs for all samples, the reliability of the results generated was evaluated by determining the reproducibility of each HCoV assay. To do this, cycling thresholds (Cts) of the in-vitro transcripts included in each batch of samples run were analyzed and the coefficients of variation as well as the standard error of the mean were determined (**Table 3.0.6)**.

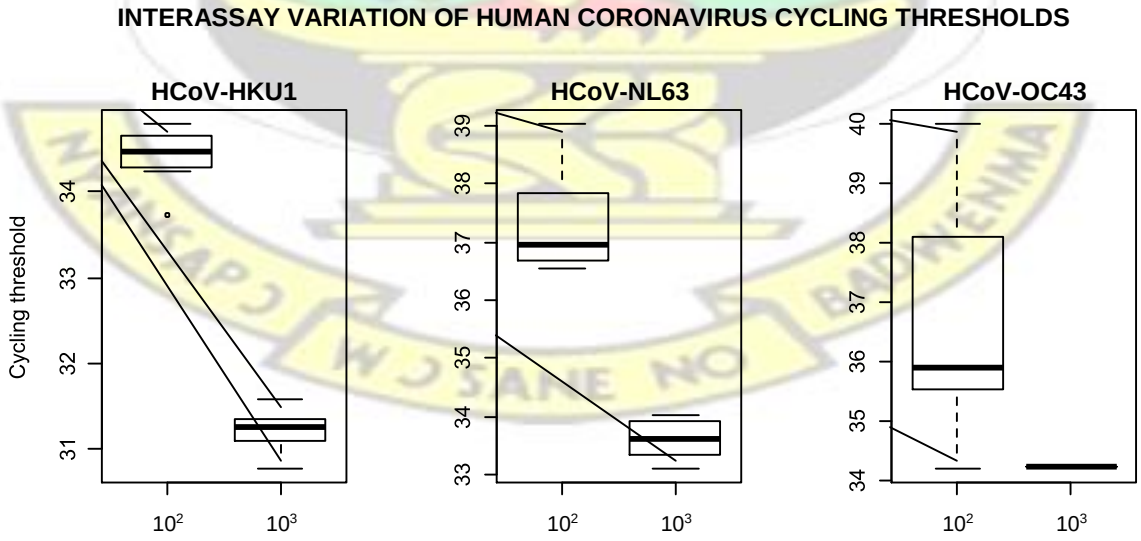
Table 3.0.6: Interassay variation of HCoV cycling thresholds

Virus	Concentration	No. of Replicates	mean Ct	SD	CV (%)	Error of mean (SE)
HKU1	1.00E+03	8	31.27	0.24	0.77	0.08
HKU1	1.00E+02	8	34.42	0.34	0.97	0.13
NL63	1.00E+03	8	33.61	0.34	1.01	0.12
NL63	1.00E+02	8	36.95	0.87	2.33	0.31

OC43	1.00E+03	8	35.06	0.6	1.71	0.21
OC43	1.00E+02	8	38.21	1.6	3.8	0.57
EMC	1.00E+02	8	33.85	0.94	2.76	0.33
EMC	1.00E+01	8	37.48	0.84	2.23	0.29
HCoV-229E	1.00E+03	8	36.31	1.12	2.7	0.39
HCoV-229E	1.00E+02	8	38.88	1.22	2.3	0.43

The results indicated the HCoV assays were reproducible and therefore very reliable in quantifying viral concentrations. To further determine the ability of the HCoV assays to quantify viral concentration with a difference of 10 copies per PCR reaction, box plots were constructed using the cycling thresholds of the in-vitro transcripts included in each batch of PCR run (**Figure 3.0.9**).

The results indicated the HCoV assays could accurately identify a median concentration difference of 10 copies per PCR reaction (equivalent to 3.3 Cts). The only slight variation difference was with HCoV-OC43 which was found to be due to the RT-PCR light cyclers used.



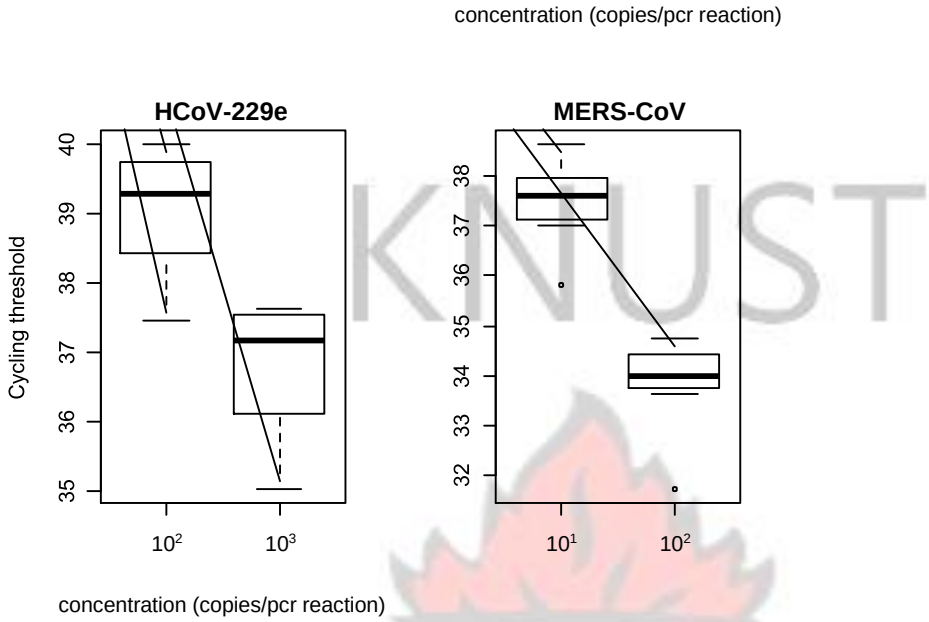


Figure 3.0.9: Median variation of HCoV cycling thresholds.

The x-axis represents the concentrations of the individual viruses and the y-axis represents the cycling thresholds. Variation in the cycling thresholds was within normal ranges for HCoV-229E, HCoV-NL63 and HCoV-HKU1. HCoV-OC43 was slightly wide and means concentration might differ marginally in individual samples

3.11.5 Sequencing of HCoV spike region using heminested PCR

A heminested PCR was designed to target the first 500 base pairs of the spike region (S) for each HCoV species (refer **Figure 3.0.5**). Primers were designed by aligning the spike reference sequences of HCoV-229E, HCoV-OC43, HCoV-NL63 and HCoV-HKU1. All obtained sequences were compared to GenBank via the BLAST Algorithm and were aligned together with reference sequences from the gene bank. A phylogenetic tree was determined by using the neighbor-joining method with a nucleotide acid percentage distance substitution model and the complete deletion option in MEGA5 (www.megasoftware.net).

The designed primers and protocols used for the heminested PCR are presented in **Table**

3.0.7 and Table 3.0.8 respectively.

Table 3.0.7: Primers for heminested PCR of the spike region of HCoVs

Nl63Spike-R: GTG TGG TGA CAT TCA CAG TAA CG Nl63Spike-F: GAG TTT GAT TAA GAG TGG TAG GT NL63Spike-Fnest:TAA GAG TGG TAG GTT GTT GTT AC
HKU1Spike-F: TTG CCT ACA ACA TTA GCT GTT A HKU1Spike-Fnest: CAA CAT TAG CTG TTA TAG GTG AT HKU1Spike-R: CCA CGT TCT TGA TAA AAA TGA AAA TAC
OC43Spike-F: ACTAGGCTGCATGATGCTTAGA OC43Spike-Fnest: GCATGATGCTTAGACCATAATCT OC43Spike-R: CAC ATA TTA TAC TGG CAA ACA GA
HCoV-229ESpike-F: GTG CTT AGT CTT GTT AGG AGT GG HCoV-229ESpike-Fnest: GTA AGT TGC TTG TAA GGG GTA ATG HCoV-229ESpike-R: TCA CGA ACT GTC TTA GGT AGT GC

Primers designated F and R were used in the first round while Fnest and R (same) were in the second round.

Table 3.0.8: Protocol for Heminested PCR of the spike region of HCoVs

		Cycle			
Round 1	25µl/reaction		55^{o}C	15'	52^{o}C for HKU1
	x1				
	Master mix 1: µl	95^{o}	3'		

H_2O	3.1	x N	94°C	15''					
2x Rxn Mix	12.5				60°C*	15''	10x		
$MgSO_4$ (50mM)	0.4		72°C	40''					
BSA (1mg/ml	1								
FWD_1(10µM)	1		95°C	15''					
REV (10µM)	1				56°C	15''	40x	HKU1	54°C for
IVT Onestep Enz Mix									
			(SSIII)	1			72°C	40''	
	20								
Template RNA	5		72°C	1'					

* Touch Down -0.5°C

Round 2									
Master mix 2:									
H_2O	37.3	x N	94°C	3'					
10x PCR Buffer	5								
dNTP Mix (10mM)	1		94°C	15''					
$MgCl_2$ (50mM)	2.5				50°C	15''	45x		
FWD_2nested (10µM)	2		72°C	40''					
REV (10µM)	2								
			Platinum Taq DNA Pol	0.2				72°C	2' at 4°C
	49								
RD 1 Product	1								

N= estimated number of samples

FWD_1 = round 1 forward primer, FWD_2 = round 2 forward primer, REV = reverse primer. (') represents seconds and ('') represents minutes.

Prior to using the primers, serial dilutions of highly positive samples were performed for each virus to determine the sensitivity and performance of each assay. **Figure 3.0.10** shows an example of serial dilution of two highly positive samples of HCoV-NL63.

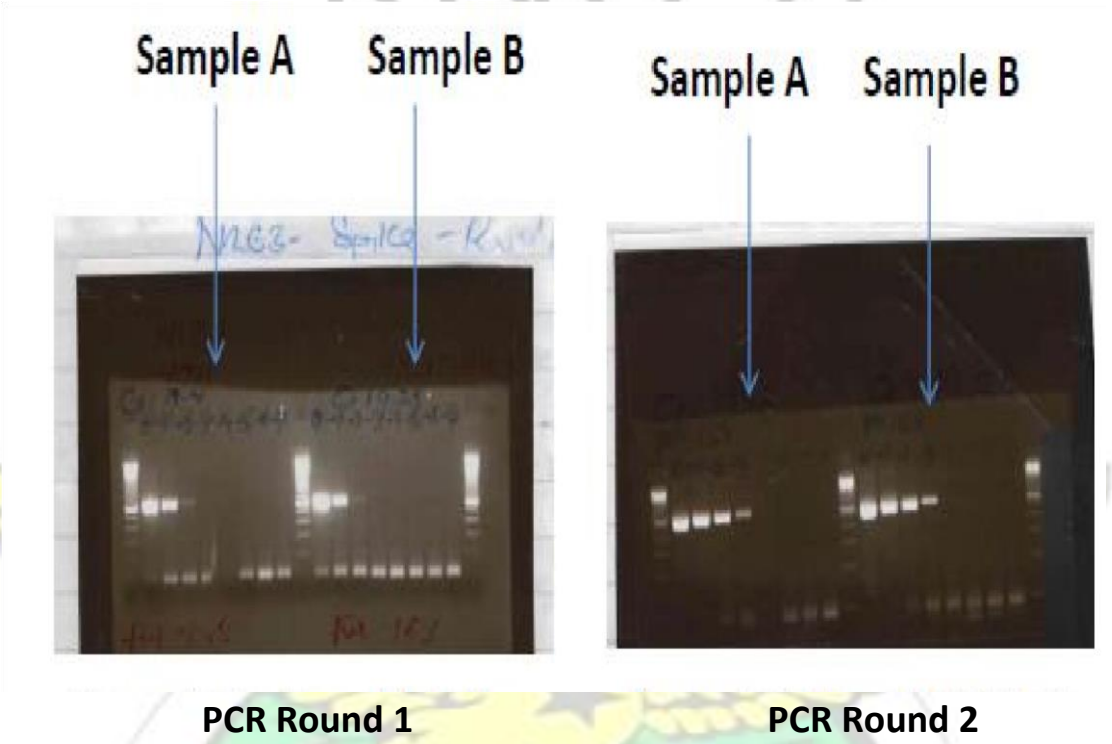


Figure 3.0.10 First and second round PCR products of HCoV-NL63 samples The ladder used in the gel picture is 100 bp. The base pair of HCoV is about 400. Gel picture for round 2 PCR shows almost two fold increase in product formation.

3.11.6 Purification of DNA products for sequencing

The DNA products generated from round 2 heminested PCR were purified using Seqlab purification reagents (Seqlab, Germany). The principle of the purification method is based

on the binding of DNA products to spin column membranes using purification reagents and then eluting the DNA into eppendorf tubes. The process was done by pipetting the respective DNA products of each virus into 500 µl of seqlab binding buffer. The mixture was vortexed for few seconds and then centrifuged at quick spin.

The mixture was then transferred into spin columns seated in 2ml tube. It was spun at 1000 r.p.m and the filtrate was discarded. The spin column was transferred into 1.5ml labeled eppendorf tube and the membrane bound DNA product was eluted using 30µl of seqlab elution solution.

A master mixture for the eluted DNA products of the respective viruses was then prepared using 2µl of the DNA product, 1µl each of forward (round 2) or reverse primers and 4µl of RNase Free Water. The mixture was sent to seqlab where sequencing of the DNA was done using the Sanger method.

3.12 Data management and analysis

All field data obtained from the communities were recorded using EPI INFO version 5 (CDC). Data were imported into Microsoft® Excel and analysis was performed using R statistical software (R Development Core Team, 2008) after adding laboratory data. Categorical variables and their association with respiratory agents were analysed using the Fischer's exact test or Chi square test where necessary. Continuous variables were expressed as medians with their inter-quartile ranges (IQR). A non-parametric K-sample test on the equality of medians was used to evaluate the differences in the medians of the various subgroups of the continuous variables.

Using logistic regression, bivariate analysis was performed to estimate the odds ratios (OR) and their 95% CI. To determine the independent risk factors of upper respiratory tract infections, socio-demographic variables that were significant at probability value (p) < 0.1 from the bivariate analysis were entered into an unconditional multiple logistic regression model. A forward and backward stepwise approach was used for selection of significant variables while adjusting for all variables in the model. All variables from the model were expressed as the adjusted odd ratios (OR) and 95% confidence interval (CI). Risk factors were plotted using "plotOdds" package in R.

The association between HCoVs and upper respiratory tract infections was determined by comparing the proportion of HCoV infections between cases and controls at the univariate level. The true association of HCoVs with upper respiratory tract infection was further assessed by fitting five unconditional logistic regression models controlling for age group, age as a continuous variable and study communities. Differences between cases and controls were assessed before and after excluding viral loads of less than 100 copies per PCR reaction. The cut off viral loads were applied to allow for a comparative analysis of cases and controls at equivalent PCR sensitivity. Results were expressed as adjusted odd ratios and 95% confidence interval (CI). A two-sided p-value of less than 0.05 was considered significant.

CHAPTER FOUR

4.0 RESULTS

4.1 Study population

A total of 1272 subjects were recruited for the study period, 540 (42.5%) in 2011 and 732 (57.5%) in 2012. Recruitment was done in all three communities as follows: Buoyem 412 (32.4%), Forikrom 361 (28.4%) and Kwamang 499 (39.2%). **Figure 4.0.1** describes the seasonal sampling distribution of subjects recruited**.** Overall**,** 662 (52%) subjects were enrolled as controls and 610 (48%) as cases.

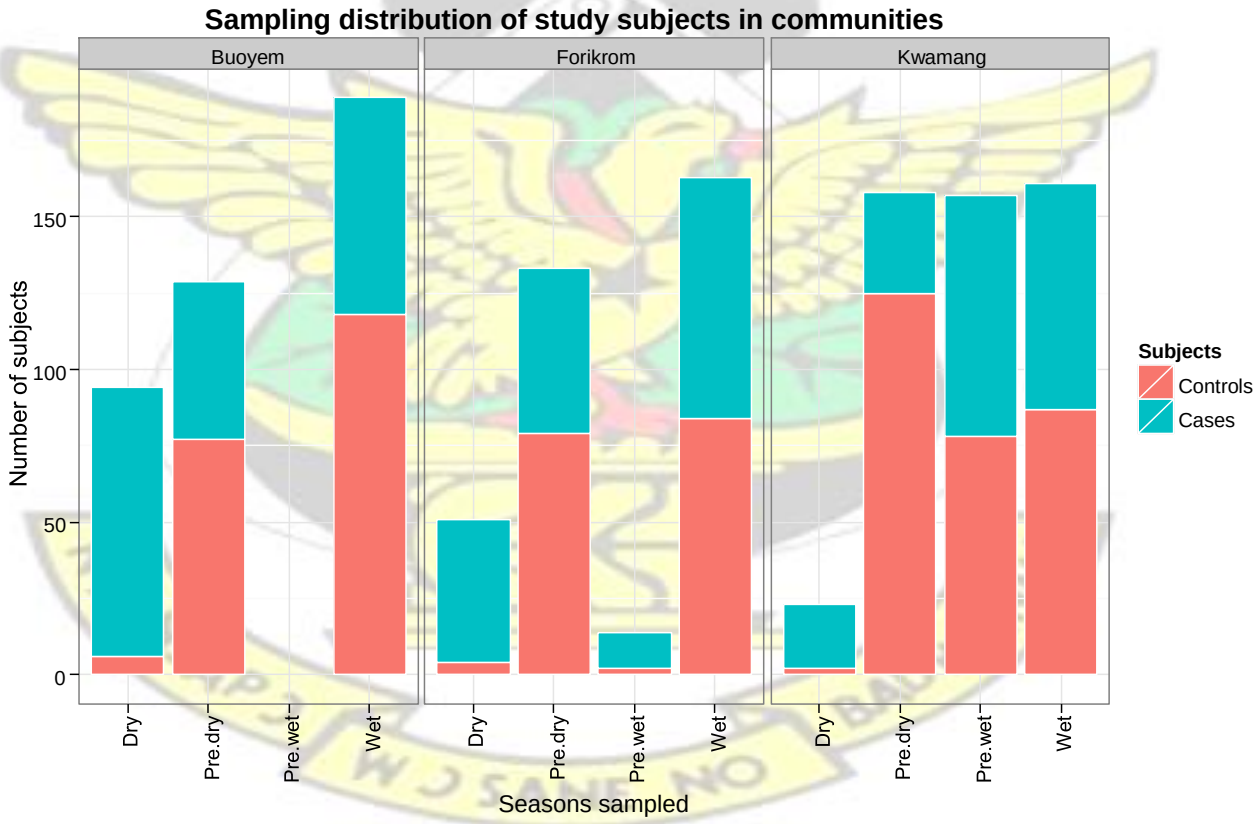


Figure 4.0.1: Sampling distribution of study subjects in communities

4.2 Socio-demographic characteristics of cases and controls

The age of all study participants ranged from between 22 and 53 years, with a median age of 37 and mean age of 39.8. The cases were younger than the controls and the difference was statistically significant (P=0.004). The age was subsequently adjusted in all analysis of the association between upper respiratory tract infection and all sociodemographic variables (**Table 4.0.1**). There was no difference in gender distribution between cases and controls. The number of persons occupying the room in which the study persons lived was not a predictor of having URTI (p-value = 0.472). The educational levels of subjects who attended junior high school (JHS) were comparable between cases and controls (OR = 0.82, 95% CI = 0.62 – 1.07). Subjects who attended senior high school (SHS) and tertiary school had higher odds of exposure to URTI compared to those who did not attend those schools. For subjects who attended primary education, the odds of exposure to URTI were lower compared to those who did not attend those schools. Most of the study subjects were in the informal sector and mainly engaged in self-owned occupations. The most common occupation was farming (49.5% controls and 42.1% cases) and the proportion was similar for cases and controls (OR= 0.84, 95% CI = 0.65 – 1.08). Students were the next highest groups enrolled (16.2% controls and 22.5% cases) and the proportion was slightly higher for cases than controls (p-value = 0.09). Among the students, the proportion of subjects who were cases (83;

13.6%) was higher compared to controls (35; 5.3%).

Table 4.0.1: Baseline demographic characteristics associated with URTI

	Control Group	Case Group	Crude	Age Adjusted	P - value
Total	n (%)	n (%)	Odd's Ratio (95% CI)	Odd Ratio (95% CI)	
Number per room					0.464
1-4 n (%)	447 (89.4)	452 (87.8)	Ref		
> 4 n (%)	53 (10.6)	63 (12.2)	1.17 (0.79-1.73)	1.16 (0.78 - 1.71)	
Age Group					**#0.010**
10 - 19 n (%)	110 (16.6)	146 (24.2)	Ref.		
20-29 n (%)	106 (16.0)	103 (17.1)	0.73 (0.51 - 1.06)	0.67 (0.44 - 1.01)	
30-39 n (%)	99 (15.0)	93 (15.4)	0.71 (0.48 - 1.03)	0.57 (0.32 - 1.01)	
40-49 n (%)	116 (17.5)	85 (14.1)	0.55 (0.38 - 0.8)	0.39 (0.18 - 0.86)	
50-59 n (%)	109 (16.5)	78 (12.9)	0.54 (0.37 - 0.78)	0.34 (0.13 - 0.93)	
60-110 n (%)	122 (18.4)	98 (16.3)	0.6 (0.42 - 0.87)	0.31 (0.07 - 1.28)	
Gender Male n (%)	287 (43.4)	266 (43.6)	0.97 (0.78 - 1.21)	0.96 (0.77 - 1.2)	0.95
Highest level of education					
Primary education n (%)	101 (15.3)	68 (11.1)	0.71 (0.51 - 0.98)	0.70 (0.50 - 0.97)	**0.03**
JHS education n (%)	174 (26.3)	152 (24.9)	0.93 (0.72 - 1.20)	0.82 (0.62 - 1.07)	0.133
SHS education n (%)	170 (25.7)	219 (35.9)	1.65 (1.30 - 2.10)	1.59 (1.25 - 2.03)	**< 0.001**
Tertiary education n (%)	14 (2.1)	31 (5.1)	2.42 (1.27 - 4.62)	2.36 (1.24 - 4.49)	**0.007**
Religious affiliation					
Christians n (%)	613 (92.6)	560 (91.8)	0.95 (0.62 - 1.43)	0.9 (0.7 - 1.62)	0.627
Muslims n (%)	17 (2.6)	19 (3.1)	1.17 (0.6 - 2.29)	1.09 (0.55 - 2.14)	0.804
Traditionalists n (%)	3 (0.5)	6 (1.0)	2.21 (0.55 - 8.86)	2.36 (0.58 - 9.54)	0.213
Other religions n (%)	27 (4.1)	20 (3.3)	0.72 (0.39 - 1.33)	0.81 (0.44 - 1.50)	0.507
Occupation					
Health workers n (%)	2 (0.3)	13 (2.1)	6.7 (1.49 - 30.04)	6.08 (1.35 - 27.34)	**0.005**
Farmers n (%)	328 (49.5)	257 (42.1)	0.75 (0.6 - 0.93)	0.84 (0.65 - 1.08)	0.17
Teachers n (%)	18 (2.70)	14 (2.30)	0.85 (0.42 - 1.73)	0.86 (0.42 - 1.74)	0.666

Traders n (%)	96 (14.5)	70 (11.5)	0.76 (0.55, 1.06)	0.76 (0.55 - 1.06)	0.11
Students n (%)	107 (16.2)	137 (22.5)	1.52 (1.15 - 2.02)	1.34 (0.95 - 1.88)	**0.09**
Other occupation n (%)	112 (16.9)	91 (14.9)	0.89 (0.55 - 1.41)	0.89 (0.56 - 1.42)	0.615

JHS: Junior high School, SHS: Senior high School. Other occupation: hairdressers, masons, carpenters, food vendors. # = Unadjusted P-value

Among all the occupational groups, health workers had the highest odds of exposure to URTI compared to other workers (OR = 6.08, 95% CI = 1.35 – 27.34). Hairdressers, masons, carpenters, food vendors, hunters and tour guides classified as other occupations, represented 14.9% cases and 16.9% controls.

4.3 Multivariate analysis

To determine the socio-demographic variables that could best predict exposure to URTI, a logistic regression model allowing for adjustment of all factors associated with URTI at p-value ≤ 0.1 was performed. The socio-demographic variables selected were age group, health workers, primary education, SHS education and students. The study communities were also adjusted in the model. From the analysis, being a health worker and having SHS or tertiary school education were identified as the independent risk factors of URTI (**Figure 4.0.2**). Subjects with age groups from 20-29 years and those from 40 to 59 years were more likely to be protected from URTI.

4.4 Management of URTI

Three hundred and twenty two (322) out of the 610 cases were interviewed on how they managed the symptoms of URTI. Fifty two (16%) of those interviewed covered their nose with their hands any time they sneezed, two hundred and twelve (66%) covered their nose with handkerchiefs and 79 (25%) did not cover their nose with their hands or handkerchiefs when they sneezed. Similarly, 51 (16%) of subjects used selfadministered

antibiotics to treat their symptoms, 5 (2%) used herbs and 80 (25%) used analgesics (commonly paracetamol)

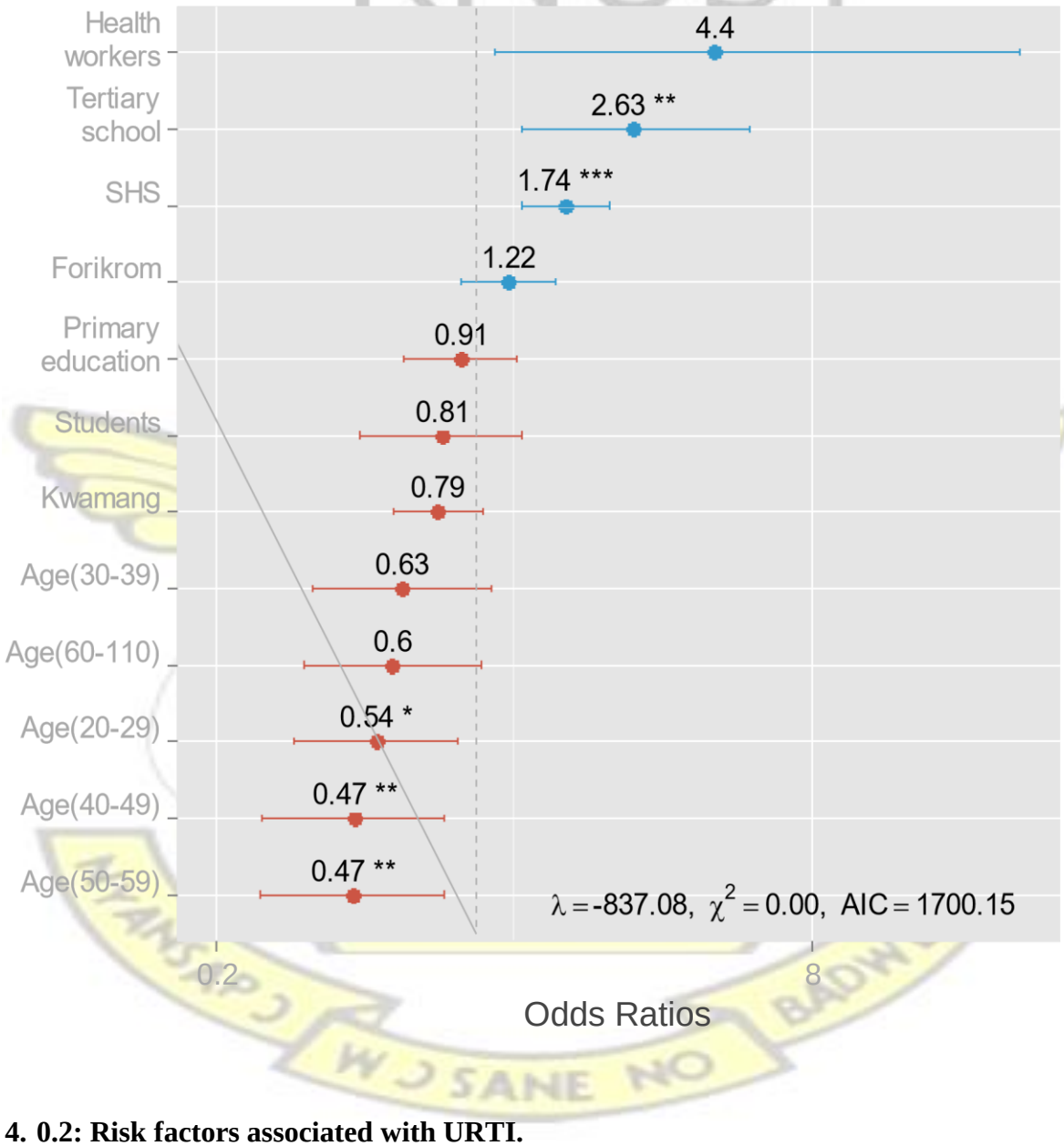


Figure 4. 0.2: Risk factors associated with URTI.

X-axis represents the odd's ratios and the y-axis represents socio-demographic variables. Bars with blue colours denote variables wit h odd's ratio greater than 1 and red denote variables with odd ratio less than

1. The p-values (generated from likelihood ratio tests) for significant Odd's ratios are described as follows: * = p <0.05, ** = p < 0.01 and *** = p < 0.001.

4.5 Sero-prevalence and factors associated with HCoVs

A total of 201 out of the 1,272 subjects were enrolled in the serological study. Subjects were tested for IgG antibodies to three HCoVs namely; HCoV-NL63, HCoV-OC43 and HCoV-229E. Of the 201 subjects, 97 (48.3%) were positive for all viruses. **Figure 4.0.3** shows examples of patient slides positive for the three viruses.

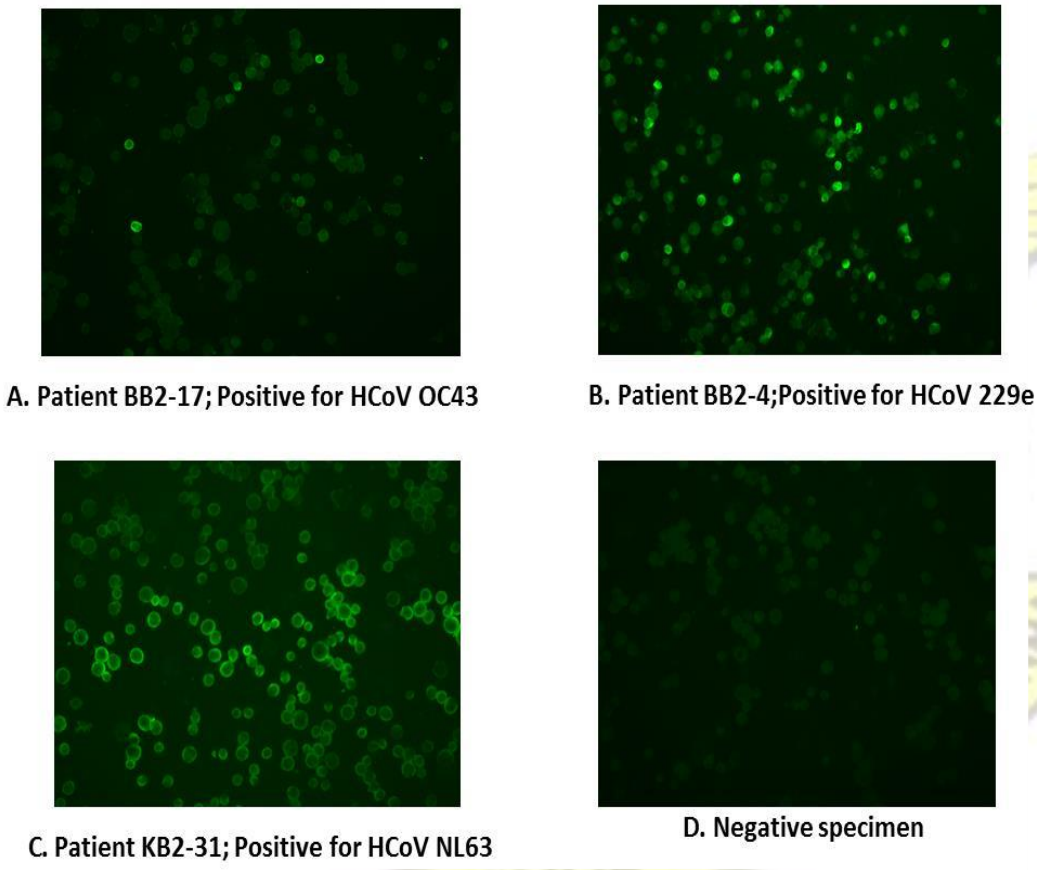


Figure 4. 0.3: HCoV samples examined with x20 objective.

A, B and C show examples of positive images (1:40 dilution) stained for

immunofluorescence antibodies against patients infected with HCoV-OC43, HCoVHCoV-229E and HCoV-NL63 respectively. D is an image of a patient negative for all three viruses.

The most prevalent virus was HCoV-229E (23%; 95% CI: 17.2 - 29.3), followed by HCoV-OC43 (17%; 95% CI: 12.4 – 23.4), then HCoV-NL63 (8%, 95% CI: 4.6 – 12.6). Association between some demographic factors and sero-positive HCoVs were determined as shown in **Table 4.0.2**. Of all positive HCoV-NL63 subjects, those in Kwamang had the highest sero-prevalence (68.8%). In contrast, HCoV-229E (41.3%) and HCoV-OC43 (45.7%) were much higher in Forikrom compared to the other study areas. There was however no statistical difference between living in any of the study areas and being positive for HCoVs. The gender distribution for all three viruses was also similar. The median ages of those positive for HCoV-OC43 (47 years, IQR = 33 – 52.5) and HCoV-229E (40 year, IQR = 27 – 54) were higher than negative subjects. The age difference for HCoV-NL63 subjects were similar (p = 0.994). A comparison of the blood group types between subjects positive for HCoVs and those negative showed no significant statistical difference (p = 0.163).

4.6 HCoVs identified using RT-PCR

Nasal swabs were taken and tested for 1,213 study participants. The HCoVs identified were HCoV-229E, HCoV-OC43, HCoV-NL63 and HCoV-HKU1. No case of MERSCoV was identified. The overall occurrence of HCoVs identified in all study subjects was 150 (12.4%). Of these, single viral detections occurred in 146 (12.0%) and multiple detections

occurred in 4 subjects (0.3%). Three subjects (0.2%) had multiple infections of OC43 and HCoV-229E and 1 had NL63 and HCoV-229E infections

Table 4.0.2: Factors associated with sero-positive HCoVs

	HCoV-NL63			HCoV-OC43			HCoV-229E		
	Negative n = 185	Positive n = 16	P-value	Negative n = 166	Positive n = 35	P-value	Negative n = 155	Positive n = 46	P-value
Community			0.08			0.113			0.238
Buoyem n (%)	49 (26.5)	3 (18.8)		46 (27.7)	6 (17.1)		41 (26.5)	11 (23.9)	
Forikrom n (%)	61 (33)	2 (12.5)		47 (28.3)	16 (45.7)		44 (28.4)	19 (41.3)	
Kwamang n (%)	75 (40.5)	11 (68.8)		73 (44)	13 (37.1)		70 (45.2)	16 (34.8)	
Age median (IQR)	35 (21.5-52	33 (19-50)	0.994	30 (19-46.2)	47 (33-52.5)	**0.005**	30 (19-47.5)	40 (27-54)	**0.014**
Gender n (%)			0.386			0.195			0.447
Females n (%)	105 (58.3)	7 (43.8)		97 (59.5)	15 (45.5)		89 (58.9)	23 (51.1)	
Males n (%)	75 (41.7)	9 (56.2)		66 (40.5)	18 (54.5)		62 (41.1)	22 (48.9)	
Blood Group			0.65			0.711			0.163
A Rh "D" Negative n (%)	1 (0.6)	0 (0)		1 (0.7)	0 (0)		0 (0)	1 (2.3)	
A Rh "D" Positive n (%)	31 (19)	3 (21.4)		25 (17.1)	9 (0)		27 (20.3)	7 (15.9)	
B Rh "D" Positive n (%)	53 (32.5)	5 (35.7)		48 (32.9)	10 (32.3)		47 (35.3)	11 (25)	
B Rh "D" Negative n (%)	1 (0.6)	0 (0)		1 (0.7)	0 (0)		1 (0.8)	0 (0)	
AB Rh "D" Positive n (%)	3 (1.8)	1 (7.1)		4 (2.7)	0 (0)		4 (3)	0 (0)	
AB Rh "D" Negative n (%)	0	0		0	0		0	0	
O Rh "D" Positive n (%)	68 (41.7)	5 (35.7)		62 (42.5)	11 (35.5)		51 (38.3)	22 (50)	
O Rh "D" Negative n (%)	6 (3.7)	0 (0)		5 (3.4)	1 (3.2)		3 (2.3)	3 (6.8)	

The most prevalent virus identified was HCoV-NL63 (82, 6.8%) followed by HCoV-229E (41, 3.4%), HCoV-OC43 (18, 1.5%) then HCoV-HKU1 (5, 0.4%) (**Table 4.0.3**). **Table 4.0.3:** Viruses identified in study subjects

	Frequency	Percentage of total samples
Neither viruses	1063	87.6
HCoV-229E	41	3.4
HCoV-OC43	18	1.5
HCoV-NL63	82	6.8
HCoV-HKU1	5	0.4
HCoV-229E and OC43	3	0.2
HCoV-NL63 and 229E	1	0.1
Total	1213	100

4.7 Demographic variables associated with HCoVs in cases and controls

The demographic variables associated with the occurrence of HCoVs were determined for cases and controls. For this analysis, the occupation of subjects was classified into one group with more social contact (occupations with high social interactions) and a second group with less social contact (occupations with low social interactions). Occupations with high social contact include health workers, dressmakers, students, teachers, hairdressers, traders, drivers and food vendors. The second group comprised farmers, hunters, traditional authorities and carpenters. As shown among control groups in **Table 4.0.4**, there was no association between any of the variables and HCoVs except for accommodation. Subjects who lived in hostels had higher chances of colonization with HCoVs compared to those who lived in compound houses.

Table 4.0.4: Factors associated with HCoVs in control subjects

	229E	NL63	OC43	P value
Total	n = 9	n = 53	n = 3	
Occupation classification				0.499
High social contact n (%)	4 (44.4)	17 (32.1)	0 (0)	
Low social contact n (%)	5 (55.6)	36 (67.9)	3 (100)	
Accomodation				0.018
Hostel n (%)	9 (100)	29 (54.7)	2 (66.7)	
Compound n(%)	0 (0)	24 (45.3)	1 (33.3)	
Highest Level of Education				
Primary n (%)	4 (44.4)	9 (17)	0 (0)	0.165
JHS n (%)	1 (11.1)	11 (20.8)	1 (33.3)	0.558
SHS n (%)	2 (22.2)	18 (34)	1 (33.3)	0.866
Tertiary n (%)	1 (11.1)	0 (0)	0 (0)	0.185
Religion				
Christians n (%)	9 (100)	43 (81.1)	3 (100)	0.465
Muslims n (%)	0 (0)	2 (3.8)	0 (0)	1
Traditional n (%)	0 (0)	1 (1.9)	0 (0)	1

Among case groups, HCoVs were found to be associated with occupation and senior high school attendance (**Table 4.0.5**). The proportion of low social contact occupations with HCoV-HKU1 (100%), HCoV- NL63 (69%) and HCoV-OC43 (60%) infections were higher compared to the low social contact group.

Table 4.0.5: Factors associated with HCoVs in case subjects

	229E	HKU1	NL63	OC43	P value
Total	n = 32	n = 5	n = 29	n = 15	
Occupation classification					**0.02**
High social contact n (%)	22 (68.8)	0 (0)	9 (31)	6 (40)	
Low social contact n (%)	10 (31.2)	5 (100)	20 (69)	9 (60)	
Accomodation					0.281
Hostel n (%)	22 (68.8)	5 (100)	16 (55.2)	10 (66.7)	
Compound n (%)	10 (31.2)	0 (0)	13 (44.8)	5 (33.3)	
Highest level of education					
Primary n (%)	5 (15.6)	1 (20)	2 (6.9)	0 (0)	0.267
JHS n (%)	4 (12.5)	1 (20)	12 (41.4)	3 (20)	0.065
SHS n (%)	19 (59.4)	1 (20)	8 (27.6)	8 (53.3)	**0.044**
Tertiary n (%)	1 (3.1)	1 (20)	0 (0)	0 (0)	0.15
Religion					
Christians n (%)	30 (93.8)	5 (100)	24 (82.8)	12 (80)	0.41
Muslims n (%)	1 (3.1)	0 (0)	1 (3.4)	1 (6.7)	0.831
Traditional n (%)	0 (0)	0 (0)	1 (3.4)	1 (6.7)	0.412

4.8 Qualitative virus comparison in cases and controls

As shown in **Figure 4.0.4,** a comparison of the age distribution of the different viruses detected in case and control groups revealed HCoV-229E to be more frequent in cases with age below 20 years whereas HCoV-NL63 was common in the middle age group. Among control subjects, HCoV-NL63 was found to occur almost equally in all ages. Statistically significant difference was not found ($p = 0.43$) comparing the proportion of combined HCoVs detections in all subjects with ages from 10 to 40 years (54.1%; 84/146) and those above 40 years (61%; 42.1).

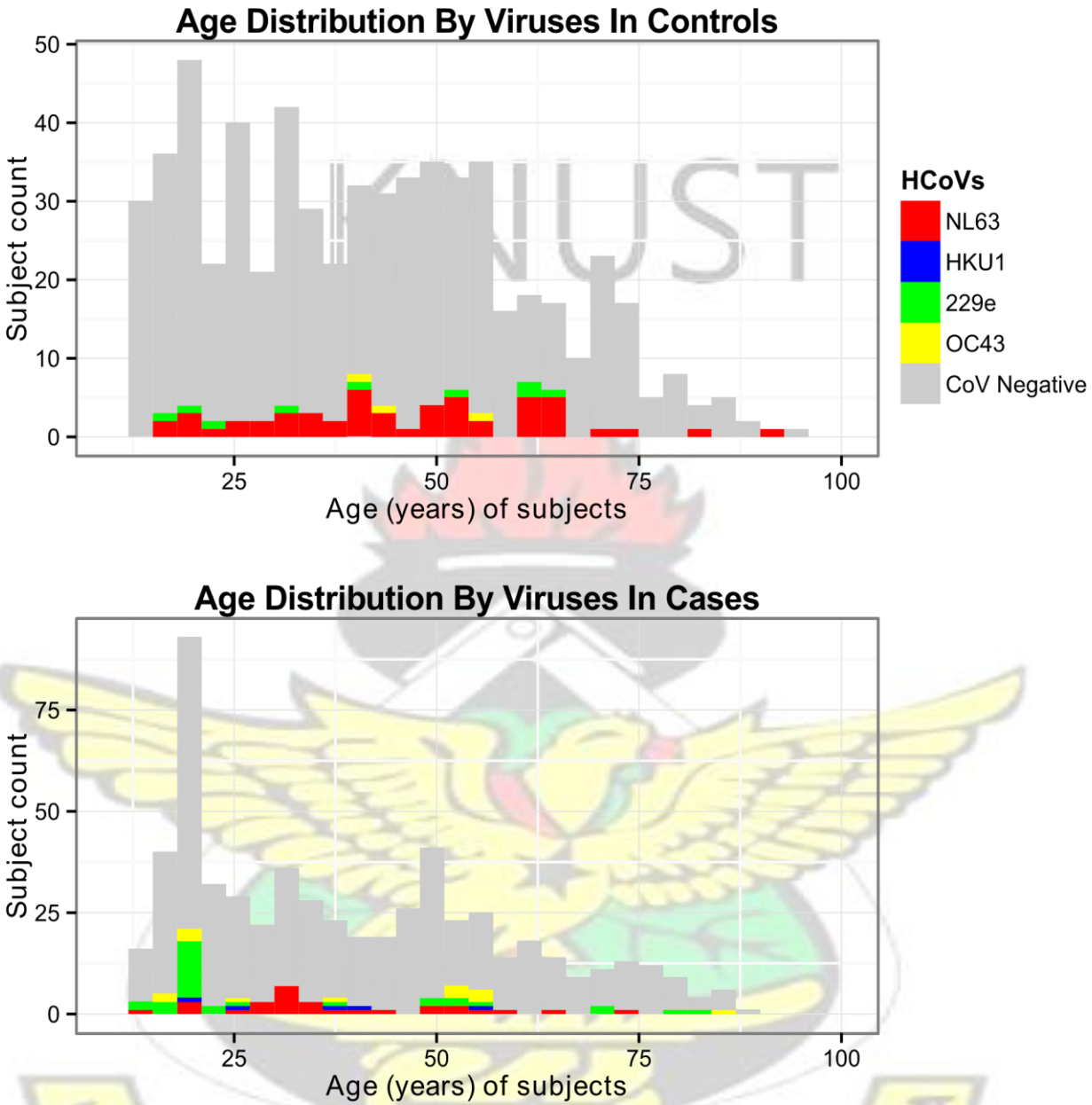


Figure 4.0.4: Different age groups of cases and controls infected by HCoVs.
The x-axis represents the age groups in years and y-axis shows the number of subjects

Of the 146 single virus infections, 65 (44.5%) occurred in controls and 81 (55.5%) was detected in cases. The difference was not statistically significant (p=0.11). The most

prevalent virus among cases and controls were HCoV-229E (36, 6.1%) and HCoVNL63 (53, 8.5%) respectively (**Table 4.0.6)**. HCoV-229E and HCoV-OC43 were significantly higher among cases compared to controls whereas HCoV-NL63 was more common in control subjects. There was no significant difference in the results between cases and controls when HCoV coinfections were excluded from the analysis.

Table 4.0.6: Viruses detected in cases and controls

	Control groups	Case groups	Total	p-value
Viruses	n = 620	n = 593	1213	
HCoV-229E n (%)	9 (1.5)	36 (6.1)	45 (3.7)	**0.001**
HCoV-HKU1 n (%)	0 (0)	5 (0.8)	5 (0.4)	0.065
HCoV-NL63 n (%)	53 (8.5)	30 (5.1)	83 (6.8)	< **0.022**
HCoV-OC43 n (%)	3 (0.5)	18 (3)	21 (1.7)	**0.001**
Overall HCoV n (%)	65 (10.5)	81 (13.7)	146 (12)	0.107

For further comparison of virus detections, viral loads of 100 or less copies per PCR reaction were excluded from the analysis, because of the different sensitivities of the used PCR assays. The revised data is presented in **Table 4.0.7**

The proportion of HCoV-NL63 was similar in both cases and controls after the cut off viral loads. The detection of overall HCoVs in subjects was also higher among cases compared to control subjects (p = 0.001).

Table 4.0.7: Viruses detected in cases and controls after cut off

Viruses	Control groups n = 620	Case groups n = 593	Total 1213	p-value
HCoV-229E n (%)	7 (1.1)	35 (5.9)	42 (3.5)	**0.001**
HCoV-HKU1 n (%)	0 (0)	3 (0.5)	3 (0.2)	0.23
HCoV-NL63 n (%)	20 (3.2)	20 (3.4)	40 (3.3)	1
HCoV-OC43 n (%)	3 (0.5)	17(2.9)	20 (1.6)	**0.002**
Overall HCoV n (%)	30 (4.8)	68 (11.5)	98 (8.1)	**0.001**

To determine the likelihood of exposure to the overall HCoVs, a bivariate logistic regression was used (**Figure 4.0.5**). The results showed that case groups had 2.55 odds of exposure to HCoVs compared to control groups.

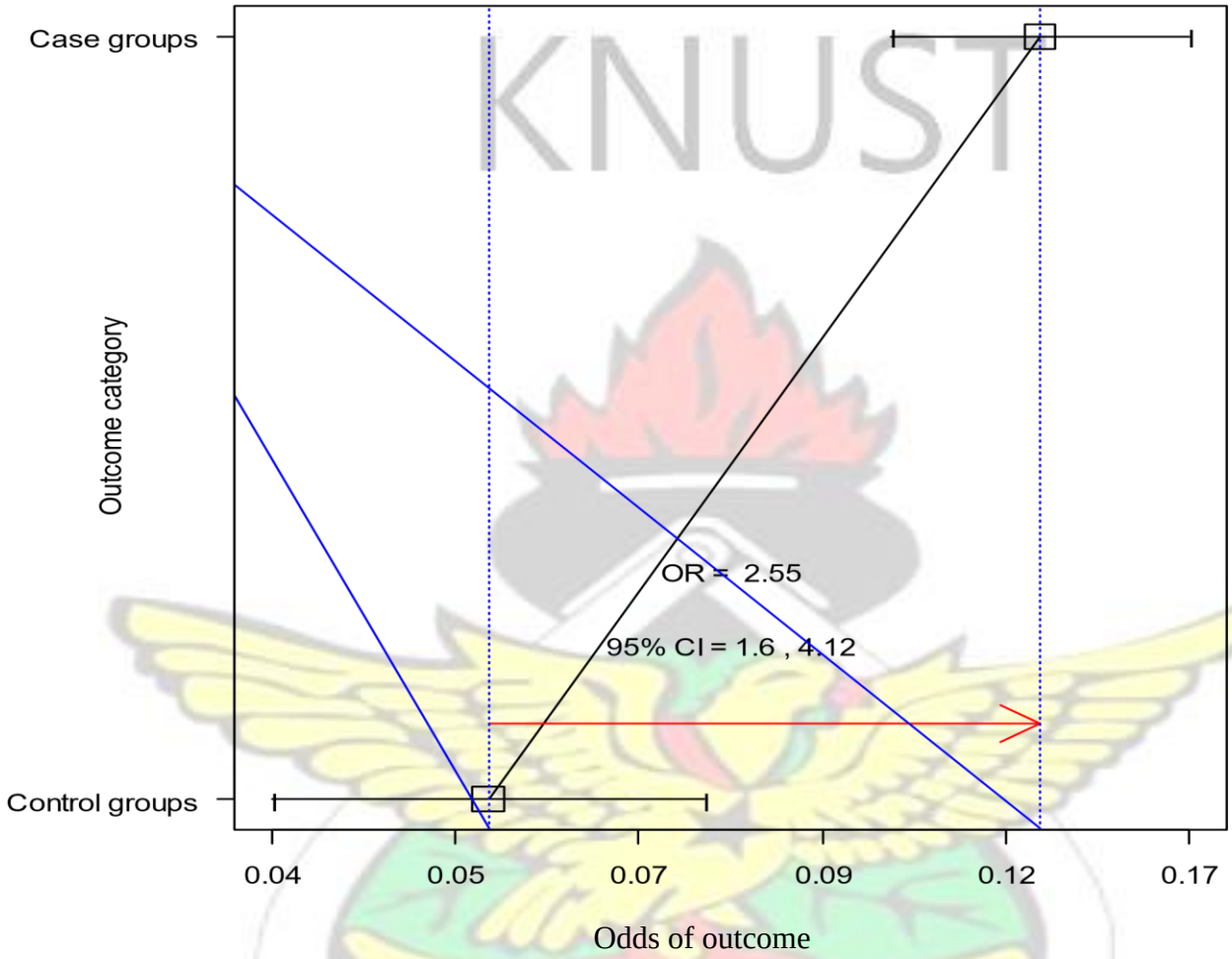


Figure 4.0.5: Unadjusted Odds of exposure to HCoVs.

The x-axis represents the odds of control and case groups. Y-axis represents the outcome category (cases and controls)

4.9 Adjusted model comparing HCoV detections between cases and controls

An adjusted logistic regression model was used to compare HCoV detections between cases and controls to find out if combined or single infections of human coronavirus could be associated with exposure to upper respiratory tract infection. The model was adjusted

for age (continuous variable), age group and study area. From the results, the odds ratio of exposure to HCoV-229E, HCoV-OC43 and HCoV-NL63 were 5.13 (95% CI = 2.24 – 11.78), 6.19 (95% CI = 1.77 – 21.65) and 0.96 (95% CI = 0.5 – 1.83) respectively (**Table 4.0.8**). The odd of exposure to the overall HCoV was 2.36 (1.5 – 3.72). Apart from HCoV-NL63, being positive for HCoV-229E, HCoV-OC43 and HCoV-HKU1 were significantly associated with upper respiratory tract infection.

Table 4. 0.8: Adjusted Odds of exposure to HCoVs

Virus	Adjusted odd ratio	lower 95% CI	upper 95% CI	P (LR-test)
Combine HCoVs	2.36	1.50	3.72	**0.001**
HCoV-229E	5.13	2.24	11.78	**0.001**
HCoV-OC43	6.19	1.77	21.65	**0.001**
HCoV-NL63	0.96	0.50	1.83	0.90
HCoV-HKU1	N/A	N/A	N/A	**0.04**

Odd ratios were determined from unconditional logistic regression model controlling for age group, age (continuous) and study area. NA: means odd's values could not be determined because of the small numbers of HCoV-HKU1 detected.

4.10 Quantitative virus comparison in cases and controls

The concentrations of HCoVs identified in this study were determined using external standard curves generated from quantified in-vitro transcripts. The cut off viral loads were

used for analysing the median virus concentration to allow for comparison at similar PCR sensitivities. The virus load of HCoV-229E showed no significant difference in cases (27400 copies per PCR reaction; IQR = 637- 2.4 x 10^7) compared to controls (197000 copies per PCR reaction; IQR = 4110 - 1.28 x 10^6) (**Figure 4.0.6).**

The trend was similar respectively for cases and controls with HCoV-OC43 detections (115000 copies per PCR reaction; IQR = 412 – 7.5 x 10^5 vrs 5480 copies per PCR reactions; IQR = 3270 – 3.47 x 10^7). HCoV-NL63 median concentration was however significantly higher (p=0.003) in cases (2.41 x 10^6 copies per PCR reaction; IQR = 1.96 x 10^4 - 2.3 x 10^6) compared to controls (1876.5 copies per PCR reaction; IQR =387.2 – 8.6 x 10^4). HCoV-HKU1 was only detected in cases groups and not in control subjects.

Variation of Virus Concentration by Cases and Controls

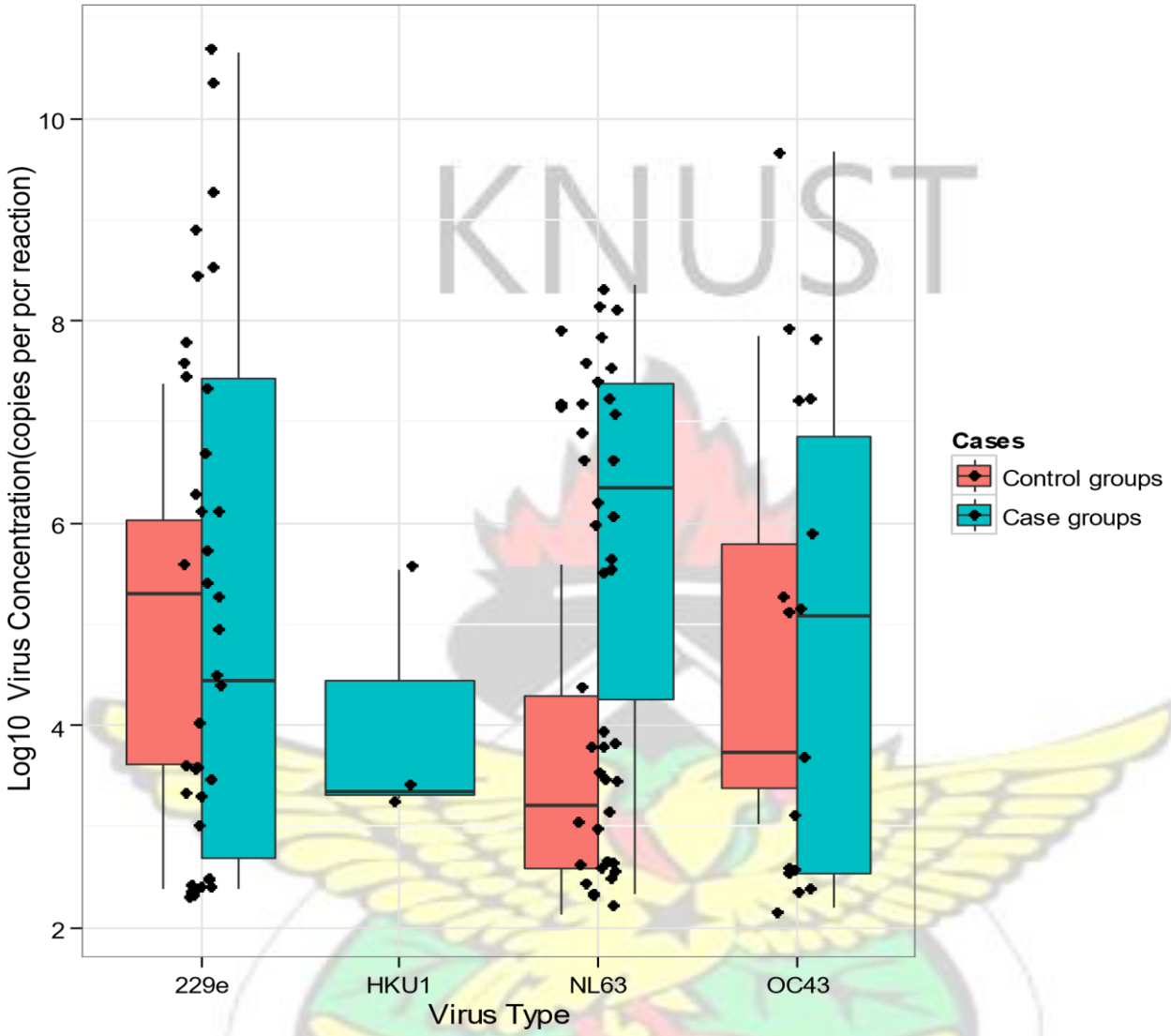


Figure 4.0.6: Variation of HCoV concentration.

The black dots scattered within the box plots represent jittered data points of the different viruses. X -axis shows the HCoV type and y-axis shows the log10 of the virus concentration. Only HCoV-NL63 virus concentration were significantly different between cases and controls

Due to the difference of HCoV-NL63 detections, the virus load of cases and controls were further analysed at the community level (**Figure 4.0.7).** The viral concentrations for

subjects in Buoyem were similar (p = 0.21) for cases (3.8×10^5 copies per PCR reaction; IQR = 3350- 9.26×10^6) and controls (5540 copies per PCR reaction; IQR = 304 – 6520). The trend was the same for subjects in Kwamang (1.36×10^7 copies per PCR reaction cases vrs 1.39×10^7 copies per PCR reaction, P = 1). Subjects in Forikrom however had higher (P = 0.005) HCoV-NL63 concentration among cases (3.34×10^6 copies per PCR reaction; IQR = $1.02 \times 10^6 – 2.25 \times 10^7$) compared to controls (906 copies per PCR reaction; IQR = 405 – 6465).

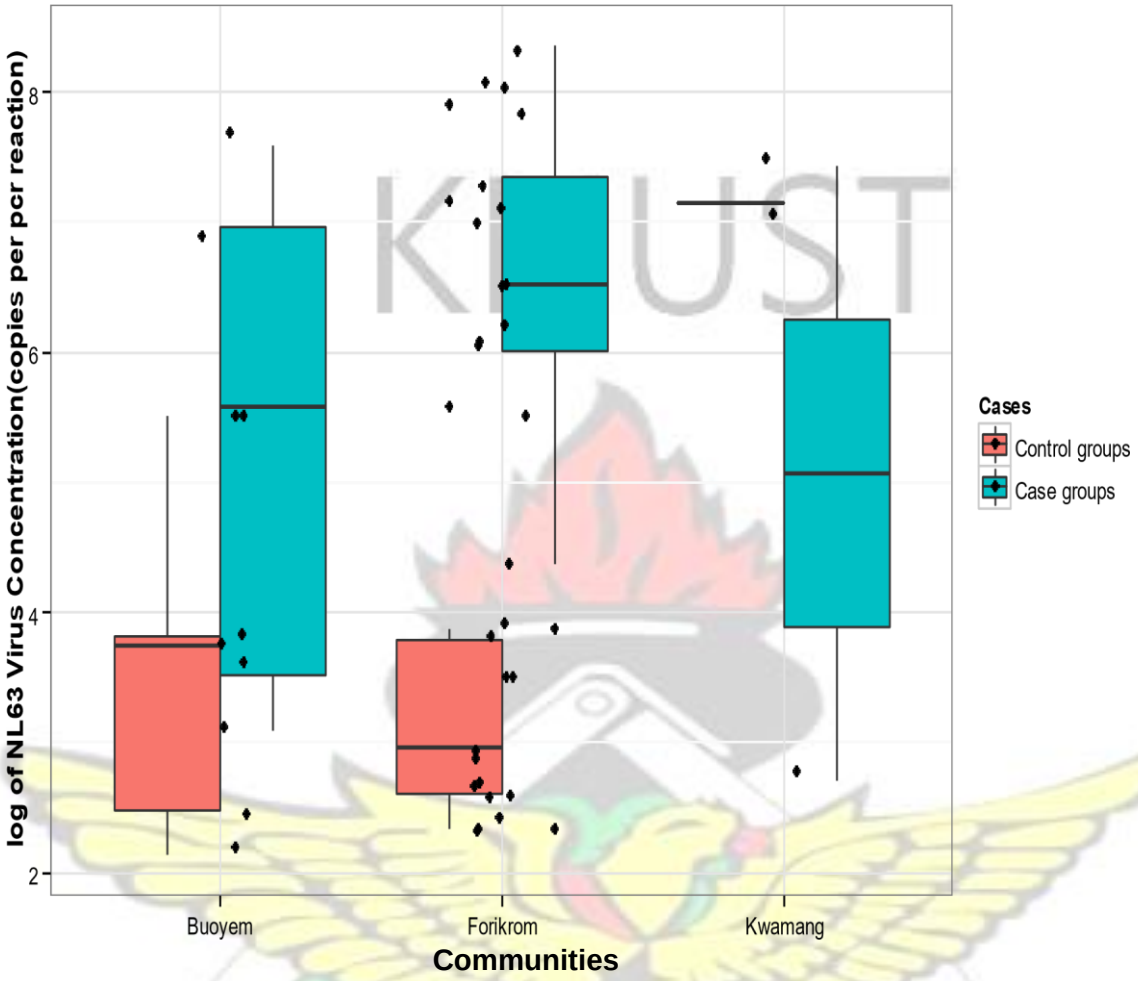


Figure 4.0.7: Variation of HCoV-NL63 at study areas

X-axis shows the study areas and y-axis shows the log10 of the NL63 viral concentration. Black dots represent jittered data points. Significant difference occurred for only subjects in Forikrom community.

Determination of clinically relevant cut off viral concentration

To assess the effect of viral concentration values on the sensitivity and specificity for diagnosing upper respiratory tract infection, a receiver operating characteristic (ROC) analysis was performed for HCoV-NL63. HCoV-NL63 was the only virus used for this analysis because of the observed significant difference between virus concentrations in cases and controls. The ROC analysis showed the clinically relevant viral concentration to be 7,510 copies per PCR reaction (**Figure 4.0.8**).

The sensitivity of diagnosing upper respiratory tract infection at such concentration was between a 95% confidence interval of 53.3% to 90.6%. The specificity was more than 90%. Viral concentrations lower than 7,510 copies per PCR reaction lost correlation to symptoms of URTI at low specificity. Similarly viral concentrations higher than 7,510 copies per PCR reaction also lost correlation to symptoms of URTI at low sensitivity. An area under the curve (AUC) value of 71.6% describes a reliable accuracy for using the cut off viral concentration for diagnosis.

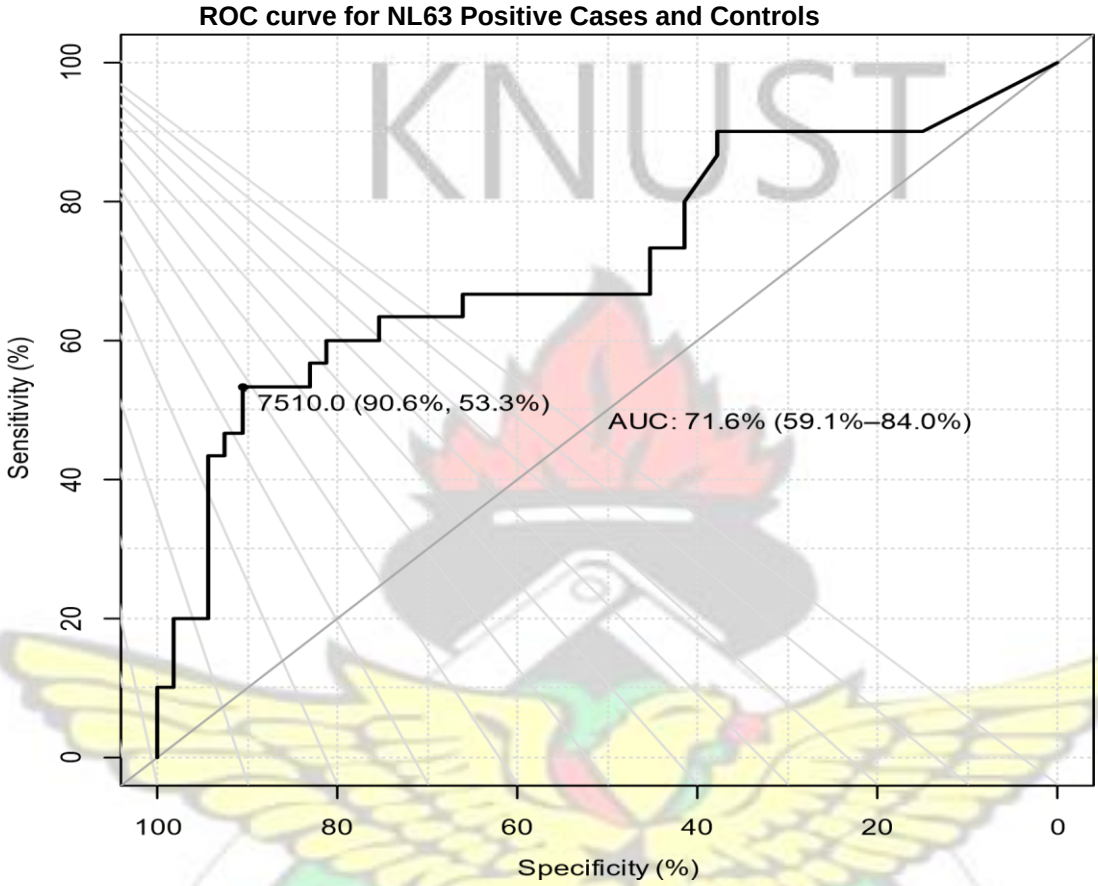
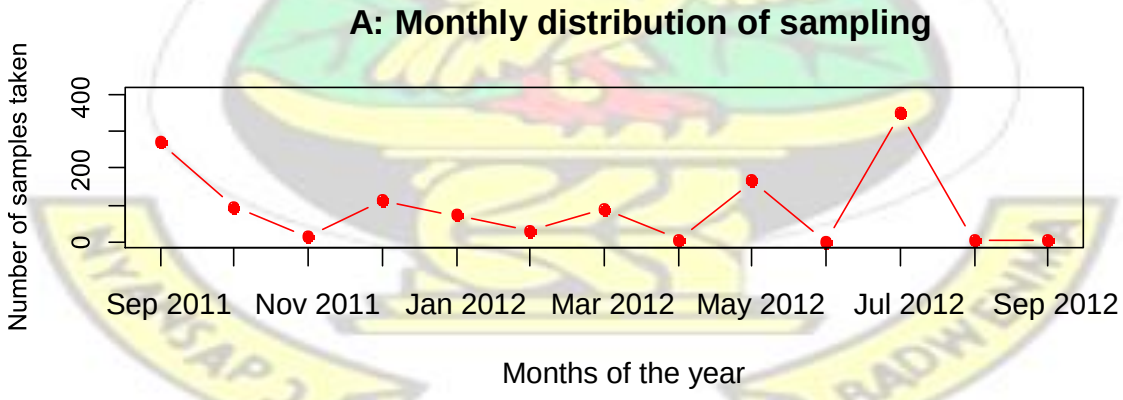


Figure 4.0.8: ROC curve for HCoV NL63 positives

The curve shows the relationship between sensitivity and specificity of cases and controls that were positive for HCoV NL63. X-axis represents the specificity and y-axis represents the sensitivity. AUC represents area under the c urve. Values in brackets are 95% confidence interval values.

4.11 Seasonal distribution of HCoVs

The present study also investigated the seasonal variation of HCoVs in the study communities. High proportions of HCoVs were identified in the harmattan season (54/215, 25.1%) compared to the wet (80/516, 15.5%) seasons (p=0.003). The high occurrence of HCoVs in the harmattan season was also significant compared to detections in the pre-harmattan season (1.3%, p <0.01) and the interim season (7.2%, p < 0.0004). The most frequent viruses detected in the harmattan and wet seasons were HCoV-229E and HCoV-NL63 respectively. HCoV-OC43 and HCoV-HKU1 were almost distributed equally throughout the year (**Figure 4.0.9**).



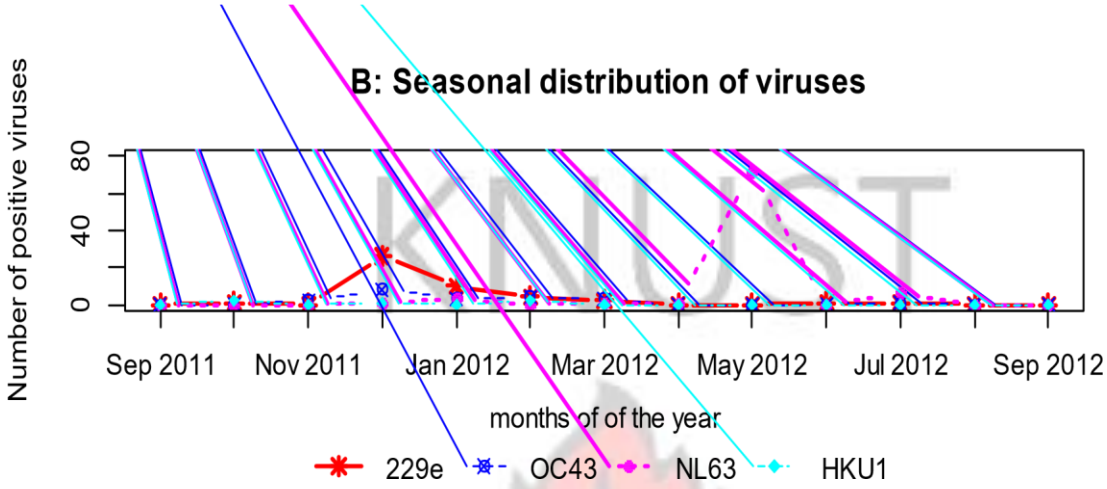


Figure 4.0.9 : Seasonal variation of HCoVs.
Months on the X-axis are labelled in a consecutive order. Those omitted hence represent Oct 2011, Dec 2011, Feb 2012, April 2012, June 2012 and August 2012 respectively. The y-axis represents the number of positive subjects.

The seasonal variation was further described in the study communities to find out the similarity and differences in virus circulation (**Figure 4.0.10**).

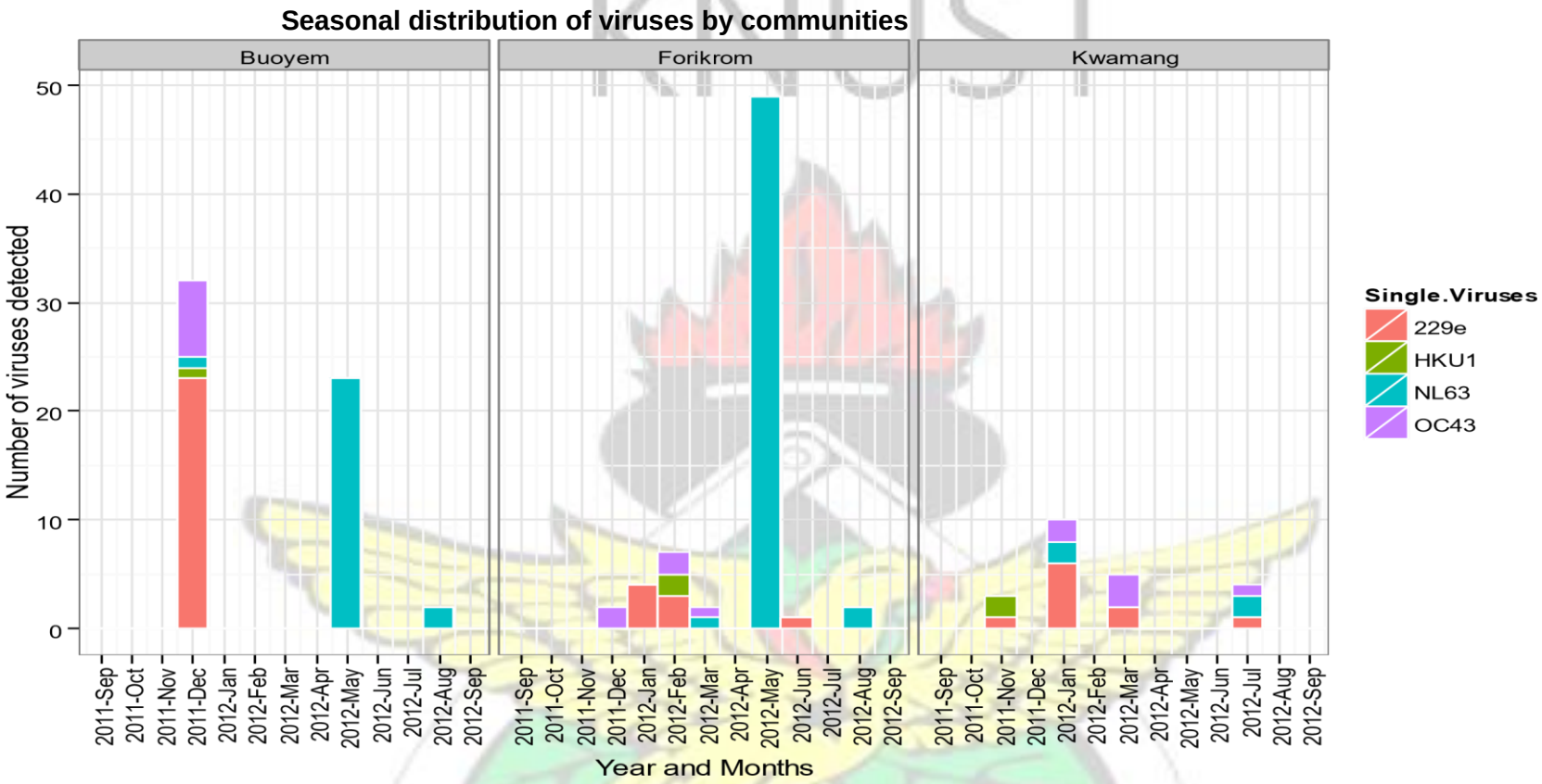


Figure 4.0.10: Seasonal variation of HCoVs in study areas Months on the X-axis are labelled in a consecutive order. The y-axis represents the number of positive subjects.

The detection of HCoV-NL63 in the wet seasons (particularly May) was more predominant in Forikrom and Buoyem compared to Kwamang whereas HCoV OC43 and HCoV-229E were much common in Buoyem during the dry season.

4.12 Sequencing and phylogenetic Analysis

Sequencing of the partial spike region was successful for 53 out of 146 samples (36.3%). Of the 53, 12 (22.6%) were HCoV-OC43, 14 (26.4%) were HCoV-NL63, 24 (45.3%) were HCoV-229E and 3 (5.7%) were HCoV-HKU1. **Figure 4.0.11 to 4.0.14** show the phylogenetic tree constructed for all four HCoV species.

Sequences obtained for HCoV-229E (**Figure 4.0.11**) were almost similar to those already published in GenBank. Small nucleotide differences were observed for isolates from Buoyem/93, Kwamang/48, Forikrom/90, Buoyem/38 and Buoyem/92 compared to the others. These differences were however insignificant to be considered as separate clusters of species.

Similarly, sequences obtained from HCoV-OC43 were comparable to those already published in the GenBank (**Figure 4.0.12**). Within the Ghana isolates, insignificant nucleotide diversities were observed for isolates Kwamang/Case 164 and isolates Forikrom/case/75.

HCoV-NL63 sequences obtained from this study were also similar to those already published in the GenBank (**Figure 4.0.13**). Sequences from isolates Forikrom/Control/120 and Forikrom/Cases/144 showed minor differences compared to other Ghana sequences. This difference was however insignificant to be considered as a separate HCoV species.

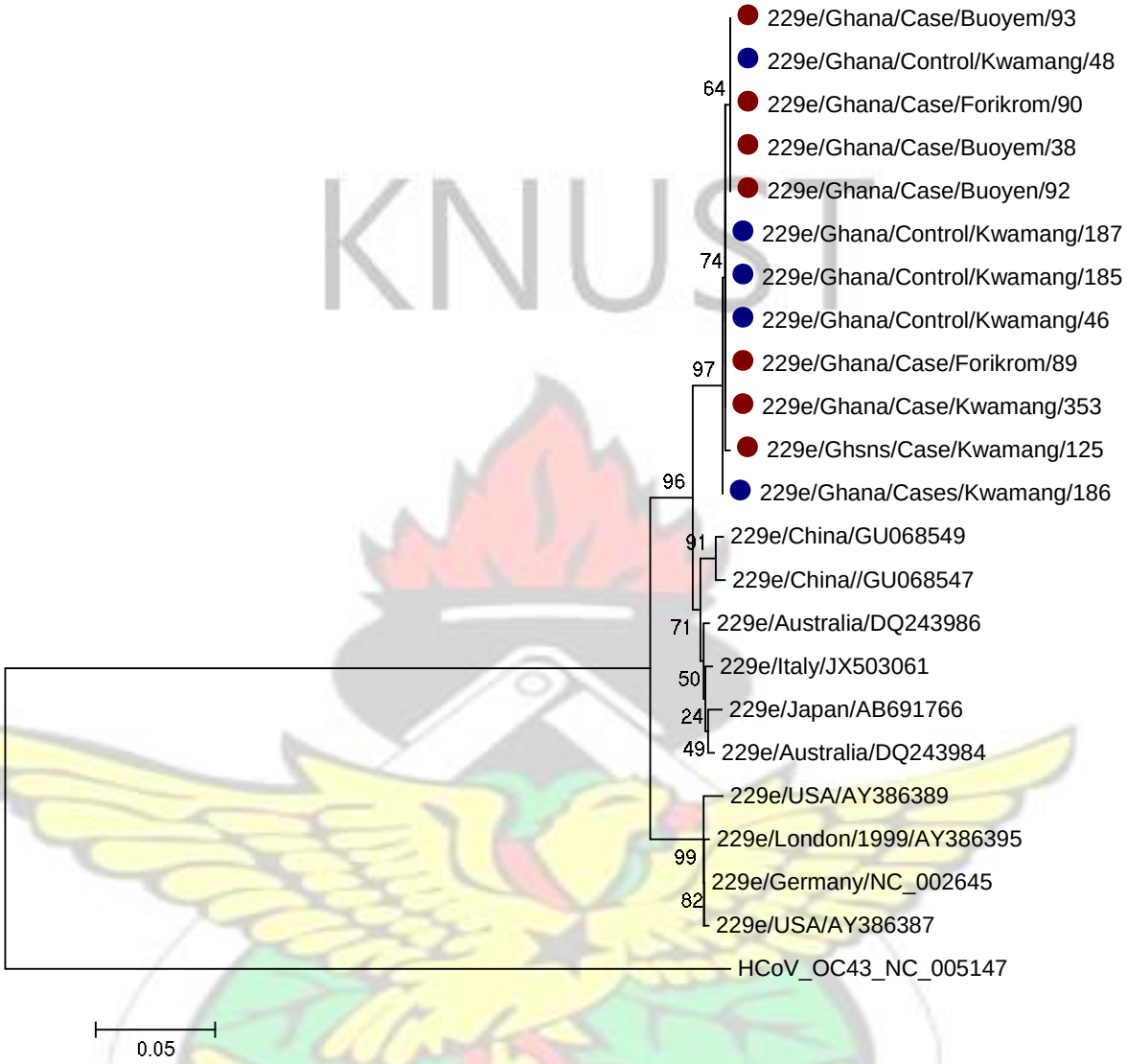


Figure 4.0.11: Phylogenetic tree of HCoV-229E

Coloured dots represent isolates obtained from Ghana with their corresponding patient ID numbers. Red dots were isolates from case groups and blue dots were isolates from control subjects. HCoV-OC43 reference sequence was used as the out-group.

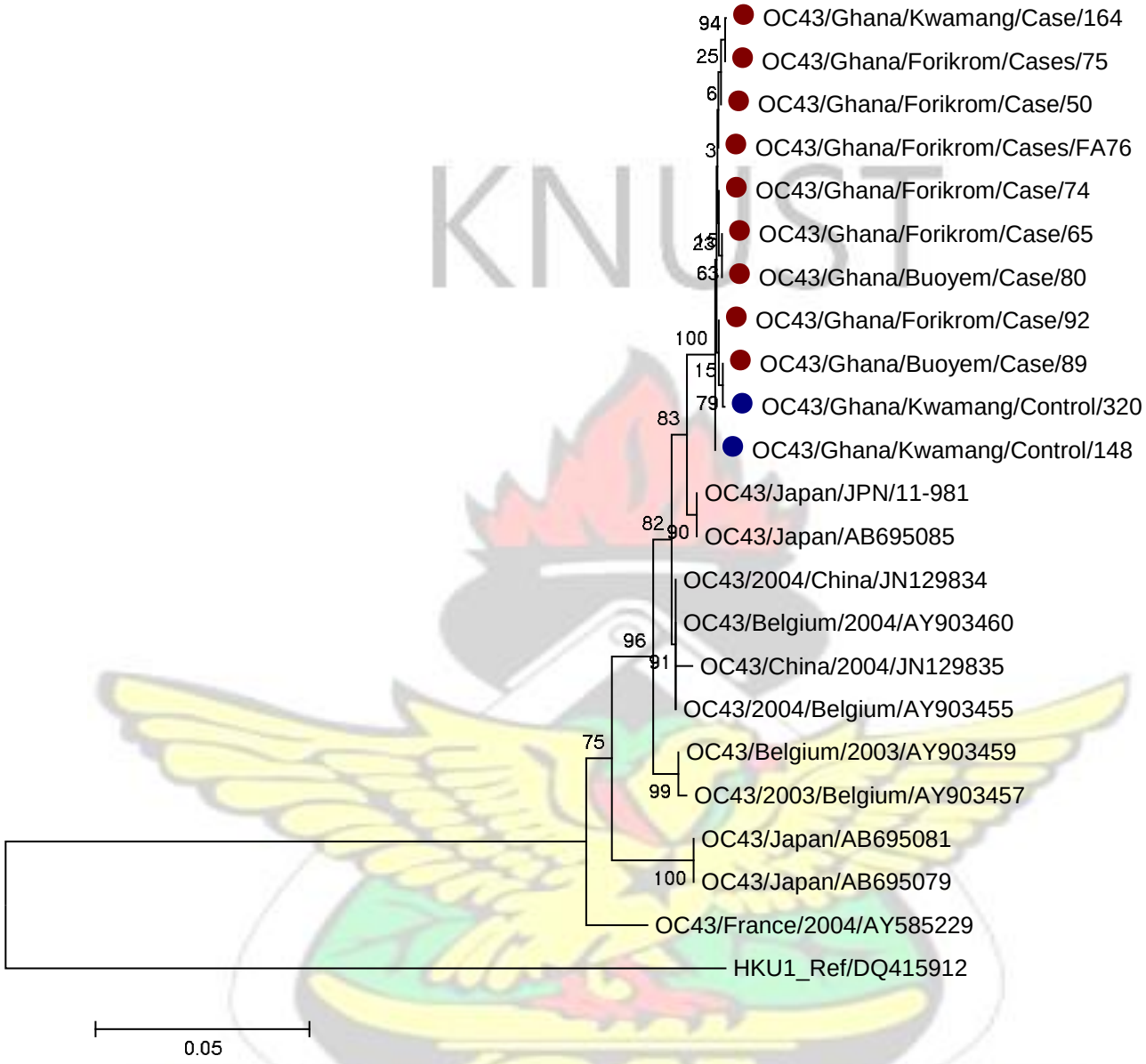


Figure 4.0.12: Phylogenetic tree of HCoV-OC43

Coloured dots represent isolates obtained from Ghana with their corresponding patient ID numbers. Red dots were isolates from case groups and blue dots were isolates from control subjects. HCoV-HKU1 reference sequence was used as the out-group.

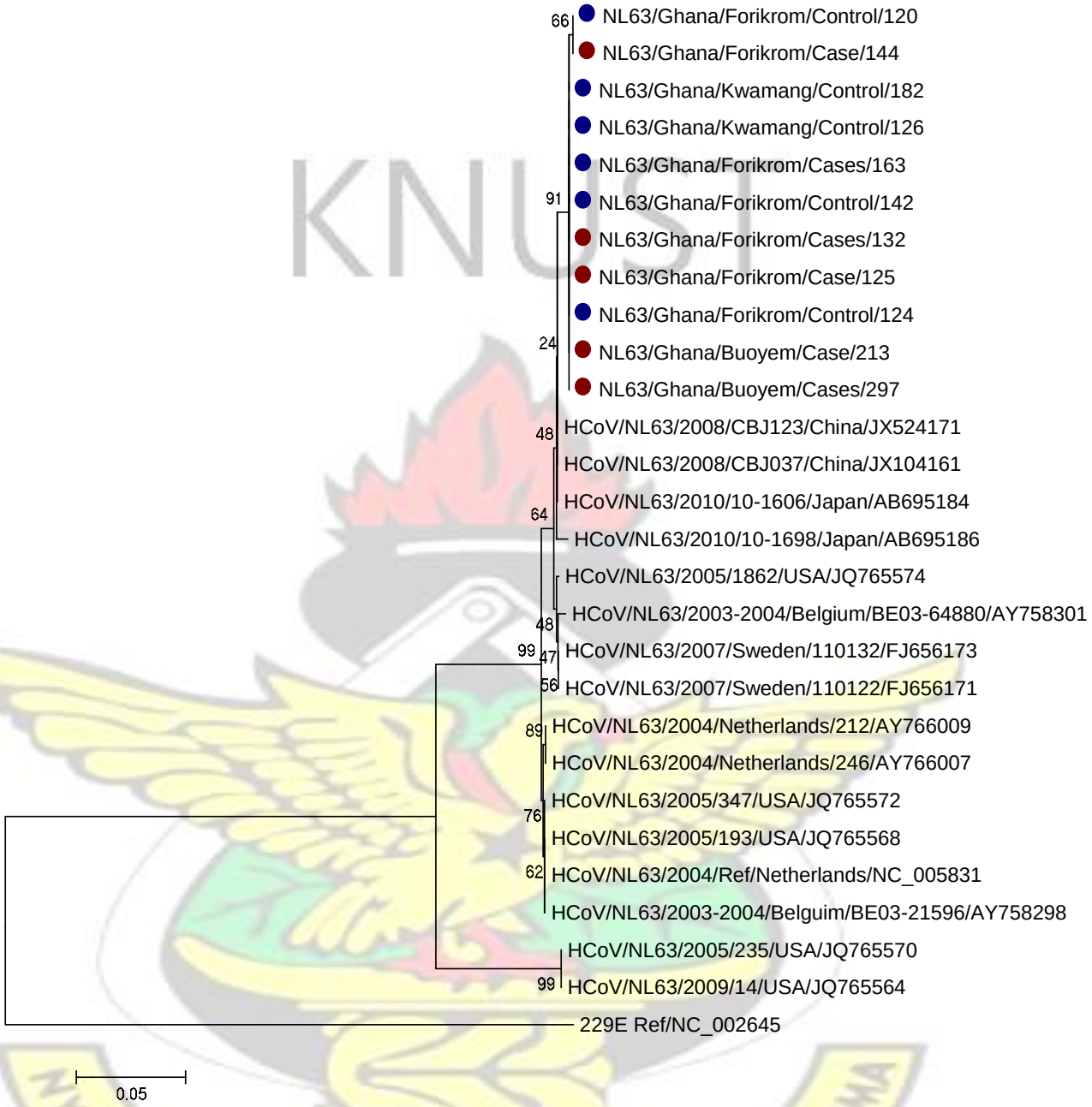


Figure 4.0.13: Phylogenetic tree of HCoV-NL63.

Coloured dots represent isolates obtained from Ghana with their corresponding patient ID numbers. Red dots were isolates from case groups and blue dots were isolates from control subjects. HCoV-229E reference sequence was used as the outgroup.

Sequences from HCoV-HKU1 were also similar to already published isolated from the GenBank (**Figure 4.0.14**). Ghana isolates were obtained from only case subjects and these were similar.

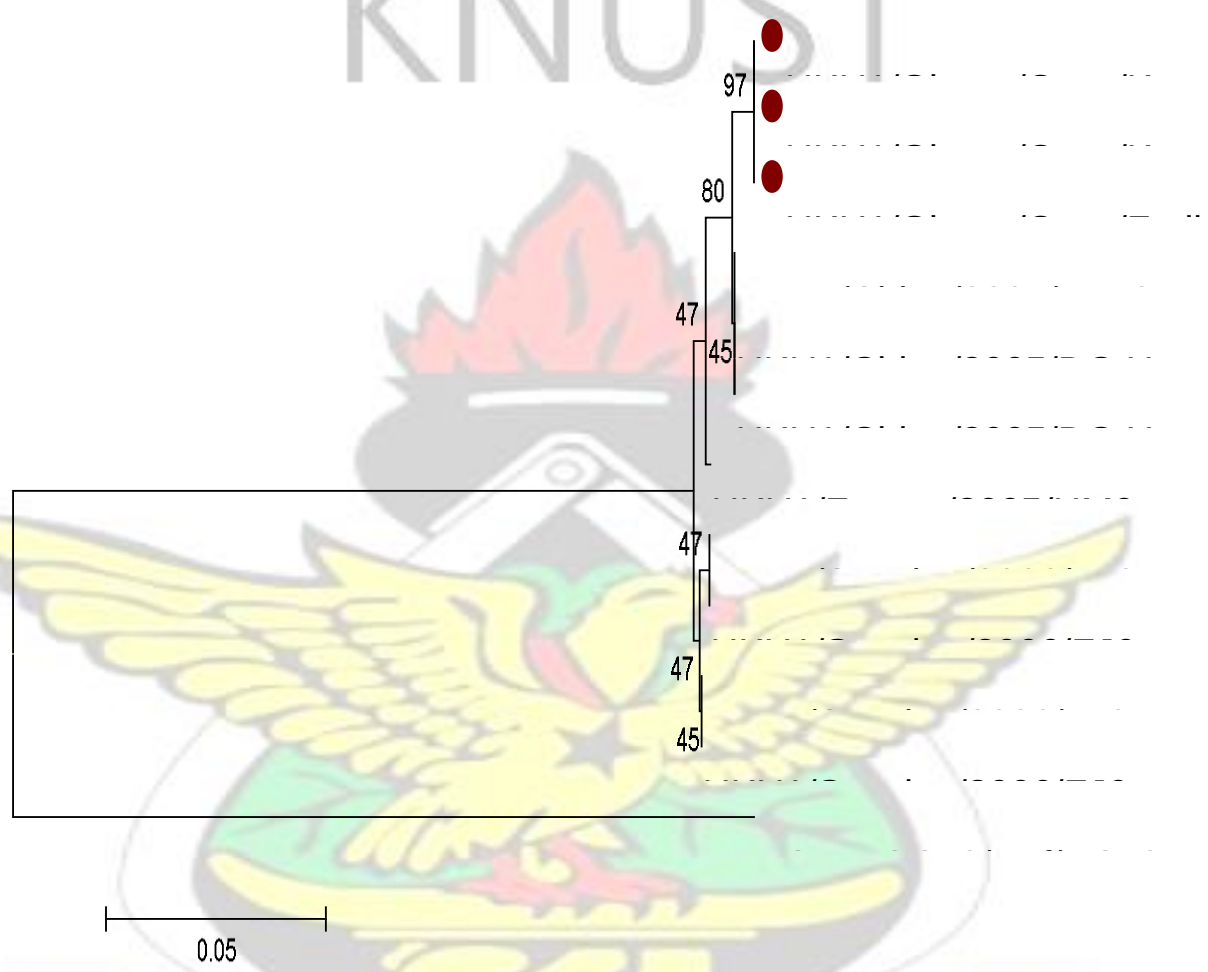


Figure 4.0.14: Phylogenetic tree of HCoV-HKU1.
Coloured dots represent isolates obtained from Ghana with their corresponding patient ID numbers. Red dots were isolates from case groups and blue dots were isolates from control subjects. HCoV-OC43 reference sequence was used as the out-group.

CHAPTER FIVE

5.0 DISCUSSION

5.1 Risk factors and behavioural practices associated with URTI

Acute respiratory infections are recognized as major causes of morbidity and mortality in developing countries. Despite their importance, the epidemiology of ARI is poorly studied and unappreciated. The studies available have mainly concentrated on young children and most were done in the early 1990's (Afari, 1991; Bale, 1990; Selwyn, 1990). The role of adults (especially from rural areas of Africa) in spreading respiratory infection has not been fully investigated. Even though children tend to suffer most from ARI, adults are the primary route of disease transmission and hence the focus of disease prevention and controls.

The present study identified attendance at levels of education (SHS and tertiary education), and being a health worker as independent risk factors associated with URTI in rural areas of Ghana. These findings are similar to other studies which also identified these classes of individuals as more susceptible to ARI (Kar-Purkayastha et al., 2009; Sun et al., 2011). The risk associated with SHS level could be explained by the educational status of these subjects. Most of the subjects enrolled in the SHS category were students (13.6% cases vrs 5.3%) living in crowded boarding houses. It would therefore be expected that such students might have interacted with sick individuals thereby increasing their chances of exposure to URTI. Similar observation was reported in China where overcrowding and poor ventilation were noted as risk factors for transmission of respiratory tract infections (Sun, et al., 2011). In Israel, Tuberculosis associated

respiratory illness was also reported among students living in boarding houses (Stein-Zamir et al., 2006). Subjects with the highest educational levels of tertiary had higher risk of exposure to URTI possibly because these subjects were more likely to enroll their wards in school compared to less educated subjects. Such subjects could therefore have interacted with these wards and thus exposing them to URTI. This possible occurrence was similarly reported from England where a student was identified as the index case for the transmission of pandemic H1N1 associated respiratory illness into the community (Kar-Purkayastha, et al., 2009).

The present study also noted that elderly subjects were more protected from URTI compared to younger ones. This is likely to be expected because older age groups might have multiple IgG antibody responses to most respiratory viruses as a result of multiple exposures to these viruses as one ages. Younger age groups on the other hand may now be acquiring antibodies against some of the respiratory respiratory hence their susceptibility to these viral agents may be higher. Another reason could be that the younger age groups who were mainly students interacted frequently compared to the elderly, thus increasing their chances of exposure to URTI. Other studies have however documented high occurrence of respiratory illness in adults, mostly from institutional elderly homes (Graat, et al., 2003). This phenomenon might be due to the frequent interactions among individuals in institutional homes. Older adults in our study were living in their private homes and therefore less social interaction was likely to occur.

The present study also found out how case groups managed the symptoms of URTI. Case groups generally did not use hygienic ways to manage the symptoms of URTI.

Most of the subjects used handkerchiefs to cover their nose when they sneezed (66%). Others used their hands to cover their nose (16%) while the rest did not cover their nose (25%). These findings are not surprising as another study in a rural area of Bangladesh has similarly reported this practice (Nasreen et al., 2010). Nasreen et al, (2010) reported that 81% of 1122 households' participants coughed or sneezed into the air, 11% coughed into their hands and 7% coughed into their clothing. These unhygienic practices are highly favourable for the transmission of viruses associated with respiratory tract infections. Even though the U.S Centres for Disease Control and Prevention recommends covering the nose with tissue after sneezing or sneezing into the upper sleeves (where tissue is not available) (Centers For Disease Control and Prevention,

2010), the guidelines are not adhered to in many developing countries including Ghana. The reasons may be due to the lack of public awareness about these hygienic practices. This reason could explain the high incidence of respiratory tract infections reported in Ghana (Krumkamp, et al., 2012). There is therefore the need for national policy guidelines to educate individuals on the good practices that will curb the spread of URTI.

This study also found that 16% (51/322) of subjects used self-administered antibiotics to treat URTI, 5 (2%) used herbs and 80 (25%) used analgesics (commonly paracetamol). The use of antibiotics and herbs for managing URTI has been similarly reported in other countries (Palla et al., 2012; Panagakou et al., 2012). In Ghana, Denno et al. (Denno et al., 1994) similarly reported the misuse of antibiotics and herbs by mothers of children with URTI. The lack of access to quality healthcare delivery system and public awareness about the management of URTI might have encouraged these practices in the rural areas. The misuse of these antibiotics could contribute to the development of resistant strains of

bacterial pathogens as reported by others (Ndip et al., 2008). The need for a national approach to address these challenges is therefore necessary in order to reduce the burden of URTI.

5.2 Sero-prevalence of HCoVs

The seroprevalence of HCoVs has not been studied widely in the field of virology. Data on the seroprevalence and factors associated is limited in Africa. The present study identified HCoV-229E as the most prevalent virus, followed by HCoV-OC43 then HCoV-HKU1. Other studies from developed countries have also reported the detection of IgG antibodies to human coronaviruses in their study subjects (Dijkman et al., 2008; McIntosh, K., et al., 1970; Severance, et al., 2008). The prevalences of HCoVs identified in the above studies were however higher compared to the present study. Severance et al., (Severance, et al., 2008) reported over 90% prevalence for each of HCoV-229E, HCoV-OC43 and HCoV-NL63 among U.S.A metropolitan population. Dijkman et al.(Dijkman, et al., 2008) similarly reported over 60% prevalences each for HCoV-229E and HCoV-NL63. The difference in the sero-prevalences could be due to the type of assays used for testing these viruses. The assays used by Dijkman and Severance were based on the nucleocapsid protein and these are known to have conserved regions that could elicit cross-reactivity (Peiris, et al., 2003). Other studies based on the use of spike proteins and whole viruses have reported HCoV prevalences of between 3 and 22% and thus similar to the findings of this study (Chan, C. M., et al., 2009; Kaye et al., 1971;

McIntosh, K., et al., 1970).

Demographic variable analysis revealed age to be associated with HCoV-OC43 and HCoV-229E exposure. The median ages were higher for subjects sero-positive for HCoV-

229E and HCoV-OC43 compared to negative subjects. This was to be expected because older subjects might have had prior exposure to HCoV antigens thereby aiding in the development of IgG antibodies. Callow et al., (Callow, 1985) reported humoral immune protection against human coronaviruses.

Some ABO blood groups are believed to act as receptors for viral transmission. Viruses such as Chikungunya and hepatitis B are reported to be associated with blood groups rhesus positive AB and A (Kumar et al., 2010; Wang, D. S. et al., 2012). Individuals with blood group O were also reported to be protected from SARS-CoV infection (Cheng et al., 2005). Guillon et al., explained that naturally occurring antibodies (Anti-

A) in group O individuals could block the spike proteins of the SARS-CoVs from infecting the epithelial cells lining the small intestines (Guillon et al., 2008). The present study did not find any association between the ABO blood groups and human coronavirus infection. This could be due to the low numbers of study subjects involved in the serology study. Future cross-sectional studies with large numbers are recommended to elucidate these findings.

5.3 Qualitative comparison of HCoVs among cases and control

Data on the molecular epidemiology of HCoVs is scarce in developing countries especially sub-Saharan Africa. The few published data were mostly from hospitalized subjects under five years and without the inclusion of control subjects. The present study resulted in the detection of 146 (12.4% of tested specimens) HCoVs occurring in both cases and controls. A comparison of viruses in cases and controls showed higher detections of HCoV-229E and HCoV-OC43 among cases compared to controls. These

findings are similar to previous reports that identified HCoV-229E and HCoV-OC43 as being commonly associated with upper respiratory tract infections (McIntosh, Kenneth et al., 1974; van Elden, et al., 2004). These associations did not change significantly when viral loads of less than 100 copies per PCR reaction were excluded from the analysis. Contrary to the findings of this study, Dare et al., (Dare, et al., 2007) reported no association between HCoV-229E or HCoV-OC43 to upper respiratory tract infections in Thailand. It is possible that infection with HCoV-229E and HCoV-OC43 might be dependent on geographical location of subjects, and that in the case of Ghana, these viruses are relevant.

HCoV-NL63 on the other hand was found to occur almost equally between cases and controls (**Table 4.0.8**). This observation was previously reported among young children (Prill, et al., 2012) and could possibly suggest the virus may have insignificant role in causing ARI among adults and older children. However, the findings have to be interpreted with caution because some control subjects positive for HCoV-NL63 might have subclinical infections at the time of recruitment. This scenario cannot be ruled out, especially because HCoV-NL63 has generally been associated with mild respiratory infection. Another explanation for the lack of association between HCoV-NL63 and URTI might be the persistent or residual RNA fragments in the respiratory tract of adults. This phenomenon of RNA persistence has been generally described for other respiratory viruses (Herberhold et al., 2009; Jartti et al., 2004; Sato et al., 2009).

The present study did not identify the novel MERS-CoV. The reason could be that the virus is localized in some geographical areas (e.g Middle East) and perhaps has not spread to Ghana.

5.4 Quantitative comparison of viruses in cases and controls

The use of quantitative PCRs has the advantage of determining the viral loads or concentration of infectious agents in clinical samples. This assessment could aid the interpretation of positive results and further promote clinical management of patients. The present study recorded no statistically significant difference between cases and controls for HCoV-229E and HCoV-OC43 except for HCoV-NL63. The median viral load for HCoV-NL63 in case groups was higher than control subjects. This could mean viral loads may not be appropriate for interpreting positive results for HCoV-229E and HCoV-OC43 but useful in the case of HCoV-NL63 infection. Jansen et al.,(Jansen et al., 2011) similarly reported higher median viral loads of HCoVs in older children with ARI compared to asymptomatic subjects. His study did not however determine the difference in viral loads for the various subspecies of HCoVs.

Whenever there is a difference in viral concentration for viruses that overlap in cases and controls (as shown for HCoV-NL63), ROC curves are mostly used to define cut off values that would aid in the clinical interpretation of patients results (Jansen, et al., 2011). ROC curves demonstrate the effect of different cut off values on the sensitivity and specificity of diagnostic tests or assays (Fan et al., 2006). The present study identified HCoV-NL63 viral copies of 7,510 copies per PCR reaction as the clinically relevant cut-off viral concentration that could be associated with HCoV-NL63 URTIs.

Clinicians could therefore be informed that HCoV-NL63 viral concentrations of more than or equal to 7,510 copies per PCR reaction could be the cause of the presenting illness while doubts concerning the aetiology of infections could remain for loads less than 7,510

copies per PCR reaction. Using ROC curves, Jansen et al.(Jansen, et al., 2011) determined the clinically relevant cut-off of human rhinoviruses to be $10^{4.5}$ copies/ml but did not analyse this for the various HCoV species.

5.5 Seasonality of HCoVs

In temperate countries, the seasonal variations of HCoVs are discernible with most cases occurring in winter (Dare, et al., 2007; Prill, et al., 2012; Smuts, et al., 2008). In China, detection of HCoV-OC43 is reported to increase in summer whereas HCoV-229E and HCoV-NL63 occurs mainly in autumn (Cui et al., 2011; Ren, et al., 2011). Other tropical countries like Thailand however have peak detections of HCoV-OC43 in winter whereas HCoV-NL63 occurred frequently in autumn (Dare, et al., 2007). In sub-Saharan African countries with unique seasonal patterns such as harmattan and wet seasons, the circulation of HCoVs tend to be different. The present study recorded high detections of HCoVs in the harmattan and the wet seasons compared to the other seasons. HCoV229E occurred more commonly in the harmattan season whereas HCoV-NL63 was detected frequently in the wet season. Possible explanations for this include seasonal variations in host immune status to infection (Cannell et al., 2006) and changes in humidity which increase viral survival in the environment (Shaman and Kohn, 2009). In the harmattan season for instance humidity is extremely low with heavy amount of dust that could injure the respiratory system thus exposing individuals to infection (Adefolalu, 1984). The results of the present study is comparable to a study in Senegal that equally identified HCoVs in October (rainy season) (Niang, et al., 2010). Additional investigation is needed to define

the seasonality of HCoVs especially in sub-Saharan African countries with tropical climates.

5.6 Phylogenetic Analysis of HCoVs

A comparison of the first 500 base pairs of the spike region; the region with most variations in the CoV genome (Dominguez, et al., 2012), did not show major difference in the strains from this study compared to reference strains from the GenBank. Variations were also not observed in strains from the different communities as well as between cases and controls. The findings of this study contrasts with reports from HCoV sequences in South Africa (Smuts, et al., 2008) and previous study in Ghana (Hays and Myint, 1998). The reason for the lack of distinct nucleotide difference in the present study could be due to the limited (I year) period of sampling. Dominguez et al., (Dominguez, et al., 2012) identified unique strains of HCoVs in the third year of clinical sampling and suggested the circulation of novel strains could be dependent on annual variation. The isolates from South Africa were also collected over a two year period (Smuts, et al., 2008). This could mean novel strains may have been identified in the present study if additional samplings were done(Hays and Myint, 1998). The previous study in Ghana was done in only one subject and could be biased in describing the genetic variation of HCoV sequences. The similarity of viral sequences between case and control subjects could mean the same viruses that colonized individuals were also responsible for infections.

CHAPTER SIX

6.1 CONCLUSION, LIMITATIONS AND RECOMMENDATIONS

6.2 Conclusions

One way of reducing the transmission of ARI in resource poor settings is to intervene in the risk factors and cultural practices that could predispose individuals to infection. Information about these factors are however limited especially in rural areas of Africa. This study identified that being in SHS, having tertiary education and being a health worker as independent risk factors associated with URTI in rural areas of Ghana. Unhygienic practices such as sneezing into handkerchiefs or in the open were also described in the rural areas. The risk factors and unhygienic practices provide baseline data that could be used to influence public health policies targeted at reducing transmission of URTI in Ghana.

Viral associated respiratory tract infections have long been recognised as major causes of morbidity and mortality in many developing countries. Even though HCoVs have been implicated in 10% of viral associated ARI, their presence in asymptomatic subjects raises doubts about its pathogenicity. The present study identified HCoV-229E and HCoV-OC43 as the pathogens associated with URTI in older children and adults in Ghana. The study further identified HCoV-NL63 viral concentration of $\geq$ 7,510 copies per PCR reaction as clinically relevant to URTIs. The PCR results of the HCoVs were further corroborated by the serologic surveillance which also identified high IgG detection of HCoV-229E and HCoV-OC43. HCoV-OC43 and HCoV-229E could therefore be said to be more relevant to ARIs in Ghana.

The genetic variability of HCoVs has been a subject of great interest to researchers in the field of virology. This interest is fuelled by earlier reports about the propensity of HCoVs to recombine with animal strains and cause outbreaks and pandemics worldwide. It is therefore necessary that sequences of HCoVs are always described to understand their molecular evolution in Ghana. The present study reported similarity of all HCoVs strains to the ones already published in the GenBank. This emphasizes that the strains in Ghana have not yet undergone recombination with animal reservoirs.

The present study again described HCoVs to be seasonally dependent with HCoV-NL63 mostly skewed to the early wet season (May) whereas HCoV-229E and HCoVOC43 occurred mostly in the dry season. The seasonal distribution could provide some preliminary information about the appropriate seasons to be used for public health campaigns against these infections.

6.3 Limitations

1. This study might have overestimated viral detections in control subjects probably due to shedding of HCoVs following earlier symptomatic infections. The ideal approach would have been to recruit controls that had no symptoms of respiratory infections for at least one month. However the enrolment of such subjects during sampling will be difficult, as subjects may not be able to document or recall the occurrence of symptoms after prolonged periods.

2. The present study did not test and rule out other viral and bacterial causes of URTI before logistic regression models were applied to evaluate disease association. This was not done due to financial constraint.

3. The one year period of sampling for this study was not enough for assessing HCoV seasonality. Moreover, sampling was not done throughout the year as expected. Seasons tend to vary from year to year so possibly a three to five year seasonal distribution of viruses could have provided a strong evidence for circulation of the viruses. The lack of unique HCoVs sequence could also have been due to the limited period of sampling. Extensive sampling was not done due to limited financial resources.

6.4 Recommendations

1. Future studies could explore the possibility of selecting control subjects without symptoms of URTI for more than 1 month. In addition, such control subjects may have to be followed up for more than 1 month to exclude those who would develop symptoms of URTIs from the analysis.

2. Future studies may also test and exclude all human respiratory viruses and bacterial agents in the logistic regression analysis. This will further strengthen the association of diseases with HCoVs.

3. The use of longitudinal studies in both infant and adults will also be beneficial in associating HCoV with causality.

4. Future serologic evaluation of antibodies should include assays for identifying both IgM and IgG antibodies. This will enable assessment of current state of infection among study subjects.

5. Studies that will involve description of seasonality of HCoVs should be done for more than a year.

REFERENCES

Adefolalu, D. O. (1984). On bioclimatological aspects of Harmattan dust haze in Nigeria. *Archives for meteorology, geophysics, and bioclimatology, Series B, 33*(4), 387-404.

Afari, E. A. (1991). "Acute Respiratory Infections in Children under Five in Two Rural Communities in Southern Ghana.". *Japanese Journal of Tropical Medicine and Hygiene., 19*, 275-280.

Almeida, J., Berry DM, Cunning CH, Hamre D, Hofstad MS, Mallucci L, McIntosh K, and Tyrrell DAJ. (1968). Coronaviruses. *Nature, 220*, 650.

Annan, A., Baldwin, H. J., Corman, V. M., Klose, S. M., Owusu, M., Nkrumah, E. E., Badu, E. K., Anti, P., Agbenyega, O., Meyer, B., Oppong, S., Sarkodie, Y. A., Kalko, E. K., Lina, P. H., Godlevska, E. V., Reusken, C., Seebens, A., GlozaRausch, F., Vallo, P., Tschapka, M., Drosten, C., and Drexler, J. F. (2013). Human Betacoronavirus 2c EMC/2012-related Viruses in Bats, Ghana and Europe. *Emerg Infect Dis, 19*(3), 456-459.

Arden, K. E., McErlean, P., Nissen, M. D., Sloots, T. P., and Mackay, I. M. (2006). Frequent detection of human rhinoviruses, paramyxoviruses, coronaviruses, and bocavirus during acute respiratory tract infections. *Journal of medical virology, 78*(9), 1232-1240.

Arden, K. E., Nissen, M. D., Sloots, T. P., and Mackay, I. M. (2005). New human coronavirus, HCoV-NL63, associated with severe lower respiratory tract disease in Australia. *J Med Virol, 75*(3), 455-462.

Baker, S. C. (2008). Coronaviruses: Molecular Biology. In B. W. J. Mahy, and M. H. V.

v. Regenmortel (Eds.), *Encyclopedia of Virology* (pp. 554-562). Oxford: Academic Press.

Bale, J. R. (1990). Creation of a research program to determine the etiology and epidemiology of acute respiratory tract infection among children in developing countries. *Rev Infect Dis, 12 Suppl 8*, S861-866.

Banner, L. R., Keck, J. G., and Lai, M. M. (1990). A clustering of RNA recombination sites adjacent to a hypervariable region of the peplomer gene of murine coronavirus. *Virology, 175*(2), 548-555.

Baric, R. S., and Yount, B. (2000). Subgenomic negative-strand RNA function during mouse hepatitis virus infection. *J Virol, 74*(9), 4039-4046.

Bastien, N., Anderson, K., Hart, L., Van Caeseele, P., Brandt, K., Milley, D., Hatchette, T., Weiss, E. C., and Li, Y. (2005). Human coronavirus NL63 infection in Canada. *J Infect Dis, 191*(4), 503-506.

Berger, A., Drosten, C., Doerr, H. W., Stürmer, M., and Preiser, W. (2004). Severe acute respiratory syndrome (SARS)--paradigm of an emerging viral infection. *Journal of Clinical Virology, 29*(1), 13-22.

Callow, K. A. (1985). Effect of specific humoral immunity and some non-specific factors on resistance of volunteers to respiratory coronavirus infection. *J Hyg (Lond), 95*(1), 173-189.

Cannell, J. J., Vieth, R., Umhau, J. C., Holick, M. F., Grant, W. B., Madronich, S., Garland, C. F., and Giovannucci, E. (2006). Epidemic influenza and vitamin D. *Epidemiol Infect, 134*(6), 1129-1140.

Carstens E.B. (2009). Ratification vote on taxonomic proposals to the International

Committee on Taxonomy of Viruses. *Archives of virology*, *155*, 133–146.

Cavanagh, D. (1997). Nidovirales: a new order comprising Coronaviridae and Arteriviridae. *Archives of virology*, *142*(3), 629-633.

Cavanagh D, Brien DA, Holmes K . V, Horzinek MC, Launde H, and W.J.M, S. (1994). Revision of the taxonomy of the Coronavirus, Torovirus and Arterivirus genera. *Archives of virology*, *135*, 228-237.

Cavanagh D, and Horzinek MC. (1993). Genus Torovirus assigned to the Coronaviridae. *Archives of virology*, *128*, 395-396.

Cavanagh, D., and Britton, P. (2008). Coronaviruses: General Features. In B. W. J. Mahy, and M. H. V. v. Regenmortel (Eds.), *Encyclopedia of Virology* (pp. 549554). Oxford: Academic Press.

Centers For Disease Control and Prevention. (2010). Cover your Cough. Retrieved 03/07/2013, 2013, from http://www.cdc.gov/flu/protect/covercough.htm

Centers For Disease Control and Prevention. (2013). Severe acute respiratory syndrome. Retrieved 01/08/2013, 2013, from www.cdc.gov/ncidod/sars/index.htm

Chan, C. M., Tse, H., Wong, S. S., Woo, P. C., Lau, S. K., Chen, L., Zheng, B. J., Huang, J. D., and Yuen, K. Y. (2009). Examination of seroprevalence of coronavirus HKU1 infection with S protein-based ELISA and neutralization assay against viral spike pseudotyped virus. *J Clin Virol*, *45*(1), 54-60.

Chan, J. F., Li, K. S., To, K. K., Cheng, V. C., Chen, H., and Yuen, K. Y. (2012). Is the discovery of the novel human betacoronavirus 2c EMC/2012 (HCoV-EMC) the beginning of another SARS-like pandemic? *J Infect*, *65*(6), 477-489.

Chang, L. Y., Chiang, B. L., Kao, C. L., Wu, M. H., Chen, P. J., Berkhout, B., Yang, H. C., and Huang, L. M. (2006). Lack of association between infection with a novel human coronavirus (HCoV), HCoV-NH, and Kawasaki disease in Taiwan. *J Infect Dis, 193*(2), 283-286.

Chen, H., Guan, Y., and Fan, X. (2011). Animal reservoirs for SARS-like coronavirus in southern China. *Hong Kong Med J, 17 Suppl 6*, 36-40.

Cheng, Y., Cheng, G., Chui, C. H., Lau, F. Y., Chan, P. K., Ng, M. H., Sung, J. J., and Wong, R. S. (2005). ABO blood group and susceptibility to severe acute respiratory syndrome. *JAMA, 293*(12), 1450-1451.

Corman, V. M., Eckerle, I., Bleicker, T., Zaki, A., Landt, O., Eschbach-Bludau, M., van Boheemen, S., Gopal, R., Ballhause, M., Bestebroer, T. M., Muth, D., Muller, M. A., Drexler, J. F., Zambon, M., Osterhaus, A. D., Fouchier, R. M., and Drosten, C. (2012). Detection of a novel human coronavirus by real-time reversetranscription polymerase chain reaction. *Euro Surveill, 17*(39).

Cowley JA, Dimmock CM, Spaan KM, and Walker PJ. (2000). Gill-associated virus of Penaeus monodon prawns: an invertebrate virus with ORF1a and ORF1b genes related to arteri and coronavirus. . *Journal of General Virology, 81*, 1473-1484.

Cui, L. J., Zhang, C., Zhang, T., Lu, R. J., Xie, Z. D., Zhang, L. L., Liu, C. Y., Zhou, W. M., Ruan, L., Ma, X. J., and Tan, W. J. (2011). Human Coronaviruses HCoVNL63 and HCoV-HKU1 in Hospitalized Children with Acute Respiratory Infections in Beijing, China. *Adv Virol, 2011*, 129134.

Dare, R. K., Fry, A. M., Chittaganpitch, M., Sawanpanyalert, P., Olsen, S. J., and

Erdman, D. D. (2007). Human coronavirus infections in rural Thailand: a comprehensive study using real-time reverse-transcription polymerase chain reaction assays. *J Infect Dis, 196*(9), 1321-1328.

den Boon, J. A., Snijder, E. J., Chirnside, E., De Vries, A., Horzinek, M., and Spaan, W. J. M. (1991). Equine arteritis is not a togavirus but belongs to the coronaviruslike superfamily. *Journal of virology, 65*, 2910-2920.

Denno, D. M., Bentsi-Enchill, A., Mock, C. N., and Adelson, J. W. (1994). Maternal knowledge, attitude and practices regarding childhood acute respiratory infections in Kumasi, Ghana. *Ann Trop Paediatr, 14*(4), 293-301.

Denny, F. W., and Loda, F. A. (1986). Acute respiratory infections are the leading cause of death in children in developing countries. *The American journal of tropical medicine and hygiene, 35*(1), 1-2.

DHHS, N. I. o. A. a. I. D. N. (2011). The Virus Pathogen Database and Analysis Resource (ViPR). Retrieved Febuary 14 2011, from

Dijkman, R., Jebbink, M. F., El Idrissi, N. B., Pyrc, K., Muller, M. A., Kuijpers, T. W., Zaaijer, H. L., and van der Hoek, L. (2008). Human coronavirus NL63 and 229E seroconversion in children. *J Clin Microbiol, 46*(7), 2368-2373.

Dominguez, S. R., Sims, G. E., Wentworth, D. E., Halpin, R. A., Robinson, C. C., Town, C. D., and Holmes, K. V. (2012). Genomic analysis of 16 Colorado human NL63 coronaviruses identifies a new genotype, high sequence diversity in the N-terminal domain of the spike gene and evidence of recombination. *J Gen Virol, 93*(Pt 11), 2387-2398.

Donaldson, E. F., Yount, B., Sims, A. C., Burkett, S., Pickles, R. J., and Baric, R. S.

(2008). Systematic Assembly of a Full-Length Infectious Clone of Human Coronavirus NL63. *Journal of virology, 82*(23), 11948-11957.

Drosten, C., Günther, S., Preiser, W., Van Der Werf, S., Brodt, H. R., Becker, S., Rabenau, H., Panning, M., Kolesnikova, L., and Fouchier, R. A. M. (2003a). Identification of a novel coronavirus in patients with severe acute respiratory syndrome. *New England Journal of Medicine, 348*(20), 1967-1976.

Drosten, C., Preiser, W., Günther, S., Schmitz, H., and Doerr, H. W. (2003b). Severe acute respiratory syndrome: identification of the etiological agent. *Trends in Molecular Medicine, 9*(8), 325-327.

Drosten, C., Seilmaier, M., Corman, V. M., Hartmann, W., Scheible, G., Sack, S., Guggemos, W., Kallies, R., Muth, D., Junglen, S., Muller, M. A., Haas, W., Guberina, H., Rohnisch, T., Schmid-Wendtner, M., Aldabbagh, S., Dittmer, U., Gold, H., Graf, P., Bonin, F., Rambaut, A., and Wendtner, C. M. (2013). Clinical features and virological analysis of a case of Middle East respiratory syndrome coronavirus infection. *Lancet Infect Dis*.

Duffy, S., Shackelton, L. A., and Holmes, E. C. (2008). Rates of evolutionary change in viruses: patterns and determinants. *Nat Rev Genet, 9*(4), 267-276.

Ejuanes J, Brian D, Cavanagh D, Holmes K . V, Lai Michael C, Laud H, Masters P, Rottier P, Siddell S, Spaan W.J.M, Taguchi F, and Talbot Pierre. (2000). *Coronaviridae*. In van Regenmortel M.H.V, Fauquet C.M, Bishop D.H.L, Carsten E.B, Estes M.K, Lemon S.M, McGeoch D.J, Maniloff J, Mayo M.A, Pringle C.R, and W. RB (Eds.), *Classification and nomenclature of viruses* (pp. 827-834). San Diego: Academic Press.

El-Sahly, H. M., Atmar, R. L., Glezen, W. P., and Greenberg, S. B. (2000). Spectrum of clinical illness in hospitalized patients with "common cold" virus infections. *Clin Infect Dis, 31*(1), 96-100.

Esper, F., Shapiro, E. D., Weibel, C., Ferguson, D., Landry, M. L., and Kahn, J. S. (2005). Association between a novel human coronavirus and Kawasaki disease. *J Infect Dis, 191*(4), 499-502.

Falsey, A. R., McCann, R. M., Hall, W. J., Criddle, M. M., Formica, M. A., Wycoff, D., and Kolassa, J. E. (1997). The "common cold" in frail older persons: impact of rhinovirus and coronavirus in a senior daycare center. *J Am Geriatr Soc, 45*(6), 706-711.

Fan, J., Upadhye, S., and Worster, A. (2006). Understanding receiver operating characteristic (ROC) curves. *CJEM, 8*(1), 19-20.

Fischer, F., Peng, D., Hingley, S. T., Weiss, S. R., and Masters, P. S. (1997). The internal open reading frame within the nucleocapsid gene of mouse hepatitis virus encodes a structural protein that is not essential for viral replication. *J Virol, 71*(2), 996-1003.

Fouchier, R. A., Hartwig, N. G., Bestebroer, T. M., Niemeyer, B., de Jong, J. C., Simon, J. H., and Osterhaus, A. D. (2004). A previously undescribed coronavirus associated with respiratory disease in humans. *Proc Natl Acad Sci U S A, 101*(16), 6212-6216.

Freundt, E. C., Yu, L., Goldsmith, C. S., Welsh, S., Cheng, A., Yount, B., Liu, W., Frieman, M. B., Buchholz, U. J., Screaton, G. R., Lippincott-Schwartz, J., Zaki, S. R., Xu, X. N., Baric, R. S., Subbarao, K., and Lenardo, M. J. (2010). The open

reading frame 3a protein of severe acute respiratory syndrome-associated coronavirus promotes membrane rearrangement and cell death. *J Virol, 84*(2), 1097-1109.

Gaunt, E. R., Hardie, A., Claas, E. C., Simmonds, P., and Templeton, K. E. (2010). Epidemiology and clinical presentations of the four human coronaviruses 229E, HKU1, NL63, and OC43 detected over 3 years using a novel multiplex real-time PCR method. *J Clin Microbiol, 48*(8), 2940-2947.

Gerna, G., Percivalle, E., Sarasini, A., Campanini, G., Piralla, A., Rovida, F., Genini, E., Marchi, A., and Baldanti, F. (2007). Human respiratory coronavirus HKU1 versus other coronavirus infections in Italian hospitalised patients. *Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology, 38*(3), 244-250.

Gessner, B. D. (2011). Acute lower respiratory infection in the developing world. *Expert Review of Respiratory Medicine, 5*(4), 459-463.

Gonzalez J.M, Gomez-Puertas P, Cavanagh D, Gorbalenya, A. E., and Enjuanes, L. (2003). A comparative sequence analysis to revise the current taxonomy of the family *Coronaviridae*. *Archives of virology, 148*, 2207-2235.

Gorse, G. J., O'Connor, T. Z., Hall, S. L., Vitale, J. N., and Nichol, K. L. (2009). Human coronavirus and acute respiratory illness in older adults with chronic obstructive pulmonary disease. *J Infect Dis, 199*(6), 847-857.

Gorse, G. J., Patel, G. B., Vitale, J. N., and O'Connor, T. Z. (2010). Prevalence of antibodies to four human coronaviruses is lower in nasal secretions than in serum. *Clin Vaccine Immunol, 17*(12), 1875-1880.

Graat, J. M., Schouten, E. G., Heijnen, M. L., Kok, F. J., Pallast, E. G., de Greeff, S. C., and Dorigo-Zetsma, J. W. (2003). A prospective, community-based study on virologic assessment among elderly people with and without symptoms of acute respiratory infection. *J Clin Epidemiol, 56*(12), 1218-1223.

Guan, Y., Zheng, B. J., He, Y. Q., Liu, X. L., Zhuang, Z. X., Cheung, C. L., Luo, S. W., Li, P. H., Zhang, L. J., Guan, Y. J., Butt, K. M., Wong, K. L., Chan, K. W., Lim, W., Shortridge, K. F., Yuen, K. Y., Peiris, J. S., and Poon, L. L. (2003). Isolation and characterization of viruses related to the SARS coronavirus from animals in southern China. *Science, 302*(5643), 276-278.

Guery, B., Poissy, J., el Mansouf, L., Sejourne, C., Ettahar, N., Lemaire, X., Vuotto, F., Goffard, A., Behillil, S., Enouf, V., Caro, V., Mailles, A., Che, D., Manuguerra, J. C., Mathieu, D., Fontanet, A., and van der Werf, S. (2013). Clinical features and viral diagnosis of two cases of infection with Middle East Respiratory Syndrome coronavirus: a report of nosocomial transmission. *Lancet, 381*(9885), 2265-2272.

Guillon, P., Clement, M., Sebille, V., Rivain, J. G., Chou, C. F., Ruvoen-Clouet, N., and Le Pendu, J. (2008). Inhibition of the interaction between the SARS-CoV spike protein and its cellular receptor by anti-histo-blood group antibodies. *Glycobiology, 18*(12), 1085-1093.

Hamre, D., and Procknow, J. (1966). A new virus isolated from the human respiratory tract. *Proc Soc Exp Biol Med, 121*, 190-193.

Haring J, and Pearlman S. (2001). Mouse hepatitis virus. *Current Opin Microbiology, 4,* 462-466.

Hays, J. P., and Myint, S. H. (1998). PCR sequencing of the spike genes of geographically and chronologically distinct human coronaviruses 229E. *J Virol Methods, 75*(2), 179-193.

Hegyi A, Friebe A, and Gorbalenya A.E. (2002). Mutational analysis of the active center of coronavirus 3C-like proteases. *Journal of General Virology, 83*, 581-593.

Herberhold, S., Eis-Hubinger, A. M., and Panning, M. (2009). Frequent detection of respiratory viruses by real-time PCR in adenoid samples from asymptomatic children. *J Clin Microbiol, 47*(8), 2682-2683.

Herzog, P., Drosten, C., and Muller, M. (2008). Plaque assay for human coronavirus NL63 using human colon carcinoma cells. *Virology Journal, 5*(1), 138.

Hofmann, H., Pyrc, K., van der Hoek, L., Geier, M., Berkhout, B., and Pohlmann, S. (2005). Human coronavirus NL63 employs the severe acute respiratory syndrome coronavirus receptor for cellular entry. *Proc Natl Acad Sci U S A, 102*(22), 7988-7993.

Holmes, K. V. (2001). Coronavirus. In D. L. Knipe, and P. Howley (Eds.), *Fields virology* (4 ed., Vol. 1, pp. 1187-1204). Philadelphia: Lippincott Williams & Wilkins.

Huynh, J., Li, S., Yount, B., Smith, A., Sturges, L., Olsen, J. C., Nagel, J., Johnson, J. B., Agnihothram, S., Gates, J. E., Frieman, M. B., Baric, R. S., and Donaldson, E. F. (2012). Evidence supporting a zoonotic origin of human coronavirus strain NL63. *J Virol, 86*(23), 12816-12825.

International committee on taxonomy of viruses. (2013). Virus taxonomy list 2012. Retrieved 17-03-2013, 2013, from http://ictvonline.org/virusTaxonomy.asp?version=2012

Jansen, R. R., Wieringa, J., Koekkoek, S. M., Visser, C. E., Pajkrt, D., Molenkamp, R., de Jong, M. D., and Schinkel, J. (2011). Frequent Detection of Respiratory Viruses without Symptoms: Toward Defining Clinically Relevant Cutoff Values. *Journal of clinical microbiology, 49*(7), 2631-2636.

Jartti, T., Lehtinen, P., Vuorinen, T., Koskenvuo, M., and Ruuskanen, O. (2004). Persistence of rhinovirus and enterovirus RNA after acute respiratory illness in children. *J Med Virol, 72*(4), 695-699.

Jenkins, G. M., Rambaut, A., Pybus, O. G., and Holmes, E. C. (2002). Rates of molecular evolution in RNA viruses: a quantitative phylogenetic analysis. *J Mol Evol, 54*(2), 156-165.

Kar-Purkayastha, I., Ingram, C., Maguire, H., and Roche, A. (2009). The importance of school and social activities in the transmission of influenza A(H1N1)v: England, April - June 2009. *Euro Surveill, 14*(33).

Kaye, H. S., Marsh, H. B., and Dowdle, W. R. (1971). Seroepidemiologic survey of coronavirus (strain OC 43) related infections in a children's population. *Am J Epidemiol, 94*(1), 43-49.

Krumkamp, R., Schwarz, N. G., Sarpong, N., Loag, W., Zeeb, H., Adu-Sarkodie, Y., and May, J. (2012). Extrapolating respiratory tract infection incidences to a rural area of Ghana using a probability model for hospital attendance. *Int J Infect Dis, 16*(6), e429-435.

Ksiazek, T. G., Erdman, D., Goldsmith, C. S., Zaki, S. R., Peret, T., Emery, S., Tong, S., Urbani, C., Comer, J. A., Lim, W., Rollin, P. E., Dowell, S. F., Ling, A. E., Humphrey, C. D., Shieh, W. J., Guarner, J., Paddock, C. D., Rota, P., Fields, B.,

DeRisi, J., Yang, J. Y., Cox, N., Hughes, J. M., LeDuc, J. W., Bellini, W. J., and Anderson, L. J. (2003). A novel coronavirus associated with severe acute respiratory syndrome. *N Engl J Med, 348*(20), 1953-1966.

Kumar, N. C., Nadimpalli, M., Vardhan, V. R., and Gopal, S. D. (2010). Association of ABO blood groups with Chikungunya virus. *Virol J, 7*, 140.

Kuo, L., Godeke, G. J., Raamsman, M. J., Masters, P. S., and Rottier, P. J. (2000). Retargeting of coronavirus by substitution of the spike glycoprotein ectodomain: crossing the host cell species barrier. *J Virol, 74*(3), 1393-1406.

Lai, M. M. (1992). RNA recombination in animal and plant viruses. *Microbiol Rev, 56*(1), 61-79.

Lau, S., Woo, P., Li, K., Huang, Y., Tsoi, H., Wong, B., Wong, S., Leung, S., Chan, K., and Yuen, K. (2005). Severe acute respiratory syndrome coronavirus-like virus in Chinese horseshoe bats. *Proceedings of the National Academy of Sciences, 102*(39), 14040.

Lee, W. J., Chung, Y. S., Yoon, H. S., Kang, C., and Kim, K. (2013). Prevalence and molecular epidemiology of human coronavirus HKU1 in patients with acute respiratory illness. *J Med Virol, 85*(2), 309-314.

Li, W., Greenough, T. C., Moore, M. J., Vasilieva, N., Somasundaran, M., Sullivan, J. L., Farzan, M., and Choe, H. (2004). Efficient replication of severe acute respiratory syndrome coronavirus in mouse cells is limited by murine angiotensin-converting enzyme 2. *J Virol, 78*(20), 11429-11433.

Li, W., Moore, M. J., Vasilieva, N., Sui, J., Wong, S. K., Berne, M. A., Somasundaran,

M., Sullivan, J. L., Luzuriaga, K., Greenough, T. C., Choe, H., and Farzan, M. (2003). Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. *Nature*, *426*(6965), 450-454.

Li, W., Shi, Z., Yu, M., Ren, W., Smith, C., Epstein, J. H., Wang, H., Crameri, G., Hu, Z., Zhang, H., Zhang, J., McEachern, J., Field, H., Daszak, P., Eaton, B. T., Zhang, S., and Wang, L. F. (2005a). Bats are natural reservoirs of SARS-like coronaviruses. *Science, 310*(5748), 676-679.

Li, W., Zhang, C., Sui, J., Kuhn, J., Moore, M., Luo, S., Wong, S., Huang, I., Xu, K., and Vasilieva, N. (2005b). Receptor and viral determinants of SARS-coronavirus adaptation to human ACE2. *The EMBO Journal, 24*(8), 1634-1643.

McBride, R., and Fielding, B. C. (2012). The role of severe acute respiratory syndrome (SARS)-coronavirus accessory proteins in virus pathogenesis. *Viruses, 4*(11), 2902-2923.

McIntosh, K. (1974). Coronaviruses.A comparative review. *Currunt Topical Microbiology Imunology, 63*, 85–129.

McIntosh, K. (2012). Proving etiologic relationships to disease: the particular problem of human coronaviruses. *Pediatr Infect Dis J, 31*(3), 241-242.

McIntosh, K., Chao, R. K., Krause, H. E., Wasil, R., Mocega, H. E., and Mufson, M. A. (1974). Coronavirus Infection in Acute Lower Respiratory Tract Disease of Infants. *Journal of Infectious Diseases, 130*(5), 502-507.

McIntosh, K., Dees, J., Becker, W., Kapikian, A., and Chanock, R. (1967). Recovery in tracheal organ cultures of novel viruses from patients with respiratory disease. *Proc Natl Acad Sci USA, 57*, 933-940.

McIntosh, K., Kapikian, A. Z., Turner, H. C., Hartley, J. W., Parrott, R. H., and Chanock, R. M. (1970). Seroepidemiologic studies of coronavirus infection in adults and children. *Am J Epidemiol, 91*(6), 585-592.

McIntosh, K., McQuillin, J., Reed, S. E., and Gardner, P. S. (1978). Diagnosis of human coronavirus infection by immunofluorescence: method and application to respiratory disease in hospitalized children. *J Med Virol, 2*(4), 341-346.

Mossel, E. C., Huang, C., Narayanan, K., Makino, S., Tesh, R. B., and Peters, C. J. (2005). Exogenous ACE2 expression allows refractory cell lines to support severe acute respiratory syndrome coronavirus replication. *J Virol, 79*(6), 38463850.

Nasreen, S., Azziz-Baumgartner, E., Gurley, E. S., Winch, P. J., Unicomb, L., Sharker, M. A., Southern, D., and Luby, S. P. (2010). Prevalent high-risk respiratory hygiene practices in urban and rural Bangladesh. *Trop Med Int Health, 15*(6), 762-771.

Navas-Martín, S., and Weiss, S. R. (2004). Coronavirus replication and pathogenesis: Implications for the recent outbreak of severe acute respiratory syndrome (SARS), and the challenge for vaccine development. *Journal of Neurovirology, 10*(2), 75-85.

Ndip, R. N., Ntiege, E. A., Ndip, L. M., Nkwelang, G., Akoachere, J. F., and Akenji, T. N. (2008). Antimicrobial resistance of bacterial agents of the upper respiratory tract of school children in Buea, Cameroon. *J Health Popul Nutr, 26*(4), 397-404.

Niang, M., Diop, O., Sarr, F., Goudiaby, D., Malou-Sompy, H., Ndiaye, K., Vabret, A., and Baril, L. (2010). Viral etiology of respiratory infections in children under 5 years old living in tropical rural areas of Senegal: The EVIRA project. *Journal of Medical Virology, 82*(5), 866-872.

Njouom, R., Yekwa, E. L., Cappy, P., Vabret, A., Boisier, P., and Rousset, D. (2012). Viral etiology of influenza-like illnesses in Cameroon, January-December 2009. *J Infect Dis, 206 Suppl 1*, S29-35.

O'Connor J, B., and Brian, D. A. (1999). The major product of porcine transmissible gastroenteritis coronavirus gene 3b is an integral membrane glycoprotein of 31 kDa. *Virology, 256*(1), 152-161.

Oshiro, L. (1973). Ultrastructure of Animal Viruses and Bacteriophages: An Atlas. In A. J. Dalton, and F. Haguenau (Eds.), *Coronaviruses* (pp. 331–343). New York: Academic Press.

Palla, A. H., Khan, R. A., Gilani, A. H., and Marra, F. (2012). Over prescription of antibiotics for adult pharyngitis is prevalent in developing countries but can be reduced using McIsaac modification of Centor scores: a cross-sectional study. *BMC Pulm Med, 12*, 70.

Panagakou, S. G., Papaevangelou, V., Chadjipanayis, A., Syrogiannopoulos, G. A., Theodoridou, M., and Hadjichristodoulou, C. S. (2012). Risk factors of antibiotic misuse for upper respiratory tract infections in children: results from a crosssectional knowledge-attitude-practice study in Greece. *ISRN Pediatr, 2012*, 685302.

Pasternak, A. O., Spaan, W. J., and Snijder, E. J. (2006). Nidovirus transcription: how to make sense...? *J Gen Virol, 87*(Pt 6), 1403-1421.

Peiris, J. S., Lai, S. T., Poon, L. L., Guan, Y., Yam, L. Y., Lim, W., Nicholls, J., Yee, W.

K., Yan, W. W., Cheung, M. T., Cheng, V. C., Chan, K. H., Tsang, D. N., Yung, R. W., Ng, T. K., and Yuen, K. Y. (2003). Coronavirus as a possible cause of severe acute respiratory syndrome. *Lancet, 361*(9366), 1319-1325.

Pewe, L., Zhou, H., Netland, J., Tangudu, C., Olivares, H., Shi, L., Look, D., Gallagher, T., and Perlman, S. (2005). A severe acute respiratory syndrome-associated coronavirus-specific protein enhances virulence of an attenuated murine coronavirus. *J Virol*, *79*(17), 11335-11342.

Pfefferle, S., Oppong, S., Drexler, J., Gloza-Rausch, F., Ipsen, A., Seebens, A., Müller, M., Annan, A., Vallo, P., and Adu-Sarkodie, Y. (2009). Distant relatives of severe acute respiratory syndrome coronavirus and close relatives of human coronavirus 229E in bats, Ghana.

Poon, L. L. M., Chan, K. H., Wong, O. K., Yam, W. C., Yuen, K. Y., Guan, Y., Lo, Y. M. D., and Peiris, J. S. M. (2003). Early diagnosis of SARS Coronavirus infection by real time RT-PCR. *Journal of Clinical Virology*, *28*(3), 233-238.

Prill, M. M., Iwane, M. K., Edwards, K. M., Williams, J. V., Weinberg, G. A., Staat, M. A., Willby, M. J., Talbot, H. K., Hall, C. B., Szilagyi, P. G., Griffin, M. R., Curns, A. T., Erdman, D. D., and NETWORK, F. T. N. V. S. (2012). Human Coronavirus in Young Children Hospitalized for Acute Respiratory Illness and Asymptomatic Controls. *The Pediatric Infectious Disease Journal, Publish Ahead of Print*, 10.1097/INF.1090b1013e31823e31807fe.

Pyrc, K., Berkhout, B., and van der Hoek, L. (2007). Identification of new human coronaviruses. *Expert Rev Anti Infect Ther, 5*(2), 245-253.

Pyrc, K., Sims, A. C., Dijkman, R., Jebbink, M., Long, C., Deming, D., Donaldson, E.,

Vabret, A., Baric, R., van der Hoek, L., and Pickles, R. (2010). Culturing the Unculturable: Human Coronavirus HKU1 Infects, Replicates, and Produces Progeny Virions in Human Ciliated Airway Epithelial Cell Cultures. *Journal of virology, 84*(21), 11255-11263.

Qiagen. (2005). Protocol: Purification of Viral RNA(spin Protocol), *Qiagen Viral RNA Mini Handbook* (2nd ed., pp. 23-25). Hilden, Germany.

R Development Core Team. (2008). A language and environment for statistical computing. R Foundation for Statistical Computing. from Vienna, Austria: http://www.R-project.org

Raj, V. S., Mou, H., Smits, S. L., Dekkers, D. H., Muller, M. A., Dijkman, R., Muth, D., Demmers, J. A., Zaki, A., Fouchier, R. A., Thiel, V., Drosten, C., Rottier, P. J., Osterhaus, A. D., Bosch, B. J., and Haagmans, B. L. (2013). Dipeptidyl peptidase 4 is a functional receptor for the emerging human coronavirus-EMC. *Nature, 495*(7440), 251-254.

Razanajatovo, N. H., Richard, V., Hoffmann, J., Reynes, J. M., Razafitrimo, G. M., Randremanana, R. V., and Heraud, J. M. (2011). Viral etiology of influenza-like illnesses in Antananarivo, Madagascar, July 2008 to June 2009. *PLoS One, 6*(3), e17579.

Regamey, N., Kaiser, L., Roiha, H. L., Deffernez, C., Kuehni, C. E., Latzin, P., Aebi, C., Frey, U., and Group, o. B. o. t. S. P. R. R. (2008). Viral Etiology of Acute Respiratory Infections With Cough in Infancy: A Community-Based Birth Cohort Study. *The Pediatric Infectious Disease Journal, 27*(2), 100-105 110.1097/INF.1090b1013e31815922c31815928.

Ren, L., Gonzalez, R., Xu, J., Xiao, Y., Li, Y., Zhou, H., Li, J., Yang, Q., Zhang, J., Chen, L., Wang, W., Vernet, G., Paranhos-Baccala, G., Wang, Z., and Wang, J. (2011). Prevalence of human coronaviruses in adults with acute respiratory tract infections in Beijing, China. *J Med Virol, 83*(2), 291-297.

Rota, P. A., Oberste, M. S., Monroe, S. S., Nix, W. A., Campagnoli, R., Icenogle, J. P., Penaranda, S., Bankamp, B., Maher, K., Chen, M. H., Tong, S. X., Tamin, A., Lowe, L., Frace, M., Derisi, J. L., Chen, Q., Wang, D. E., dman, D. D., Peret, T. C. T., Burns, C., Ksiazek, T. G., Rollin, P. E., Sanchez, A., Liffick, S., Holloway, B., Limor, J., McCaustland, K., Olsen-Rasmussen, M., Fouchier, R., Gunther, S., Osterhaus, A. D. M. E., Drosten, C., Pallansch, M. A., Anderson, L. J., and Bellini, W. J. (2003). Characterization of a novel coronavirus associated with severe acute respiratory syndrome. *Science, 300*, 1394–1399.

Sato, M., Li, H., Ikizler, M. R., Werkhaven, J. A., Williams, J. V., Chappell, J. D., Tang, Y. W., and Wright, P. F. (2009). Detection of viruses in human adenoid tissues by use of multiplex PCR. *J Clin Microbiol, 47*(3), 771-773.

Schalk AF, and Hawn MC. (1931). An apparently new respiratory disease of baby chicks. *Journal of American Verterinary Medical Association, 78,* 413-422.

Schildgen, O., Jebbink, M. F., de Vries, M., Pyrc, K., Dijkman, R., Simon, A., Müller, A., Kupfer, B., and van der Hoek, L. (2006). Identification of cell lines permissive for human coronavirus NL63. *Journal of Virological Methods, 138*(1–2), 207-210.

Schwegmann-Wessels, C., and Herrler, G. (2006). Sialic acids as receptor determinants for coronaviruses. *Glycoconj J, 23*(1-2), 51-58.

Sekyere Central District Assembly. (2010). Sekyere Central District. Retrieved 15-07-

2013, 2013, from sekyerecentral.ghanadistricts.gov.gh

Selwyn, B. J. (1990). The epidemiology of acute respiratory tract infection in young children: comparison of findings from several developing countries. Coordinated Data Group of BOSTID Researchers. *Rev Infect Dis, 12 Suppl 8*, S870-888.

Sethna, P. B., Hung, S. L., and Brian, D. A. (1989). Coronavirus subgenomic minusstrand RNAs and the potential for mRNA replicons. *Proc Natl Acad Sci U S A, 86*(14), 5626-5630.

Severance, E. G., Bossis, I., Dickerson, F. B., Stallings, C. R., Origoni, A. E., Sullens, A., Yolken, R. H., and Viscidi, R. P. (2008). Development of a nucleocapsidbased human coronavirus immunoassay and estimates of individuals exposed to coronavirus in a U.S. metropolitan population. *Clin Vaccine Immunol, 15*(12), 1805-1810.

Shaman, J., and Kohn, M. (2009). Absolute humidity modulates influenza survival, transmission, and seasonality. *Proc Natl Acad Sci U S A, 106*(9), 3243-3248.

Siddell S.G. (1995). The *Coronaviridae*: an introduction. In S. S.G (Ed.), *The Coronaviridae* (pp. 1-10). New York: Plenum Press.

Smuts, H., Workman, L., and Zar, H. J. (2008). Role of human metapneumovirus, human coronavirus NL63 and human bocavirus in infants and young children with acute wheezing. *J Med Virol, 80*(5), 906-912.

Stein-Zamir, C., Volovik, I., Rishpon, S., Atamna, A., Lavy, A., and Weiler-Ravell, D. (2006). Tuberculosis outbreak among students in a boarding school. *Eur Respir J, 28*(5), 986-991.

Stephensen C.B, Casebolt D.B, and Gangopadhyay N.N. (1999). Phylogenetic analysis of a conserved region of the polymerase gene from 11 coronavirus and development of a consensus polymerase chian reaction assay. *Virus Research, 60*, 181-189.

Sturman L . S, Holmes K . V, and J, B. (1980). Isolation of coronavirus envelope glycoproteins and interaction with the viral nucleocapsid. *Journal of Virology, 33*, 449-462.

Sun, Y., Wang, Z., Zhang, Y., and Sundell, J. (2011). In China, students in crowded dormitories with a low ventilation rate have more common colds: evidence for airborne transmission. *PLoS One, 6*(11), e27140.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol, 28*(10), 2731-2739.

Tao, Y., Tang, K., Shi, M., Conrardy, C., Li, K. S., Lau, S. K., Anderson, L. J., and Tong, S. (2012). Genomic characterization of seven distinct bat coronaviruses in Kenya. *Virus Res, 167*(1), 67-73.

Techiman Municipal Health Directorate. (2010). *Annual Report*. Techiman, Brong Ahafo Region.

Tong, S., Conrardy, C., Ruone, S., Kuzmin, I. V., Guo, X., Tao, Y., Niezgoda, M., Haynes, L., Agwanda, B., Breiman, R. F., Anderson, L. J., and Rupprecht, C. E. (2009). Detection of novel SARS-like and other coronaviruses in bats from Kenya. *Emerg Infect Dis, 15*(3), 482-485.

Tyrreli DAJ, Almeida JD, Cunningham CH, Dowdle WR, Hofstad MS, McKintosh K,

Tajima M, Zakstelskays LY, Easterday BC, Kapikian A, and Bingham RW. (1975). Coronaviridae. *Intervirology, 5*, 76-82.

Tyrrell, D., and Bynoe, M. (1965). Cultivation of a novel type of common-cold virus in organ cultures. *British Medical Journal, 1*(5448), 1467-1470.

Tyrrell, D., and Bynoe, M. (1966). Cultivation of viruses from a high proportion of patients with colds. *Lancet, 1*, 76-77.

Vabret, A., Dina, J., Gouarin, S., Petitjean, J., Corbet, S., and Freymuth, F. (2006). Detection of the new human coronavirus HKU1: a report of 6 cases. *Clin Infect Dis, 42*(5), 634-639.

Vabret, A., Dina, J., Gouarin, S., Petitjean, J., Tripey, V., Brouard, J., and Freymuth, F. (2008). Human (non-severe acute respiratory syndrome) coronavirus infections in hospitalised children in France. *Journal of Paediatrics and Child Health, 44*(4), 176-181.

Vabret, A., Mourez, T., Gouarin, S., Petitjean, J., and Freymuth, F. (2003). An outbreak of coronavirus OC43 respiratory infection in Normandy, France. *Clin Infect Dis, 36*(8), 985-989.

van Boheemen, S., de Graaf, M., Lauber, C., Bestebroer, T. M., Raj, V. S., Zaki, A. M., Osterhaus, A. D., Haagmans, B. L., Gorbalenya, A. E., Snijder, E. J., and Fouchier, R. A. (2012). Genomic characterization of a newly discovered coronavirus associated with acute respiratory distress syndrome in humans. *MBio, 3*(6). van der Hoek, L. (2007). Human coronaviruses: what do they cause? *Antivir Ther, 12*(4 Pt B), 651-658.

van der Hoek, L., Pyrc, K., and Berkhout, B. (2006). Human coronavirus NL63, a new respiratory virus. *FEMS Microbiol Rev, 30*(5), 760-773.

van der Hoek, L., Pyrc, K., Jebbink, M. F., Vermeulen-Oost, W., Berkhout, R. J., Wolthers, K. C., Wertheim-van Dillen, P. M., Kaandorp, J., Spaargaren, J., and Berkhout, B. (2004). Identification of a new human coronavirus. *Nat Med, 10*(4), 368-373.

van Elden, L. J., van Loon, A. M., van Alphen, F., Hendriksen, K. A., Hoepelman, A. I., van Kraaij, M. G., Oosterheert, J. J., Schipper, P., Schuurman, R., and Nijhuis, M. (2004). Frequent detection of human coronaviruses in clinical specimens from patients with respiratory tract infection by use of a novel real-time reversetranscriptase polymerase chain reaction. *J Infect Dis, 189*(4), 652-657.

van Gageldonk-Lafeber, A. B., Heijnen, M. L., Bartelds, A. I., Peters, M. F., van der Plas, S. M., and Wilbrink, B. (2005). A case-control study of acute respiratory tract infection in general practice patients in The Netherlands. *Clin Infect Dis, 41*(4), 490-497.

Varshney, B., Agnihothram, S., Tan, Y. J., Baric, R., and Lal, S. K. (2012). SARS coronavirus 3b accessory protein modulates transcriptional activity of RUNX1b. *PLoS One, 7*(1), e29542.

Venter, M., Lassauniere, R., Kresfelder, T. L., Westerberg, Y., and Visser, A. (2011). Contribution of common and recently described respiratory viruses to annual hospitalizations in children in South Africa. *J Med Virol, 83*(8), 1458-1468.

Vijgen, L., Keyaerts, E., Lemey, P., Moes, E., Li, S., Vandamme, A. M., and Van Ranst,

M. (2005). Circulation of genetically distinct contemporary human coronavirus OC43 strains. *Virology*, *337*(1), 85-92.

Wang, D. S., Chen, D. L., Ren, C., Wang, Z. Q., Qiu, M. Z., Luo, H. Y., Zhang, D. S., Wang, F. H., Li, Y. H., and Xu, R. H. (2012). ABO blood group, hepatitis B viral infection and risk of pancreatic cancer. *Int J Cancer*, *131*(2), 461-468.

Wang, M., Jing, H. Q., Xu, H. F., Jiang, X. G., Kan, B., Liu, Q. Y., Wan, K. L., Cui, B. Y., Zheng, H., Cui, Z. G., Yan, M. Y., Liang, W. L., Wang, H. X., Qi, X. B., Li, Z. J., Li, M. C., Chen, K., Zhang, E. M., Zhang, S. Y., Hai, R., Yu, D. Z., and Xu, J. G. (2005). [Surveillance on severe acute respiratory syndrome associated coronavirus in animals at a live animal market of Guangzhou in 2004]. *Zhonghua Liu Xing Bing Xue Za Zhi*, *26*(2), 84-87.

Weiss, S. R., and Navas-Martin, S. (2005). Coronavirus Pathogenesis and the Emerging Pathogen Severe Acute Respiratory Syndrome Coronavirus. *Microbiol. Mol. Biol. Rev.*, *69*(4), 635-664.

Williams, B. G., Gouws, E., Boschi-Pinto, C., Bryce, J., and Dye, C. (2002). Estimates of world-wide distribution of child deaths from acute respiratory infections. *The Lancet infectious diseases*, *2*(1), 25-32.

Woo, P. C., Lau, S. K., Chu, C. M., Chan, K. H., Tsoi, H. W., Huang, Y., Wong, B. H., Poon, R. W., Cai, J. J., Luk, W. K., Poon, L. L., Wong, S. S., Guan, Y., Peiris, J. S., and Yuen, K. Y. (2005a). Characterization and complete genome sequence of a novel coronavirus, coronavirus HKU1, from patients with pneumonia. *J Virol*, *79*(2), 884-895.

Woo, P. C., Lau, S. K., Huang, Y., and Yuen, K. Y. (2009). Coronavirus diversity, phylogeny and interspecies jumping. *Exp Biol Med (Maywood), 234*(10), 11171127.

Woo, P. C., Lau, S. K., and Yuen, K. Y. (2006). Infectious diseases emerging from Chinese wet-markets: zoonotic origins of severe respiratory viral infections. *Curr Opin Infect Dis, 19*(5), 401-407.

Woo, P. C. Y., Lau, S. K. P., Chu, C.-m., Chan, K.-h., Tsoi, H.-w., Huang, Y., Wong, B. H. L., Poon, R. W. S., Cai, J. J., Luk, W.-k., Poon, L. L. M., Wong, S. S. Y., Guan, Y., Peiris, J. S. M., and Yuen, K.-y. (2005b). Characterization and Complete Genome Sequence of a Novel Coronavirus, Coronavirus HKU1, from Patients with Pneumonia. *Journal of virology, 79*(2), 884-895.

World Health Organisation. (2004). Summary of probable SARS cases with onset of illness from November 1, 2002 to July 31, 2003.,

Xiao, N. G., Zhang, B., Duan, Z. J., Xie, Z. P., Zhou, Q. H., Zhong, L. L., Gao, H. C., Ding, X. F., Zeng, S. Z., Huang, H., and Hou, Y. D. (2012). [Viral etiology of 1165 hospitalized children with acute lower respiratory tract infection]. *Zhongguo Dang Dai Er Ke Za Zhi, 14*(1), 28-32.

Yam, W. C., Chan, K. H., Chow, K. H., Poon, L. L. M., Lam, H. Y., Yuen, K. Y., Seto, W. H., and Peiris, J. S. M. (2005). Clinical evaluation of real-time PCR assays for rapid diagnosis of SARS coronavirus during outbreak and post-epidemic periods. *Journal of Clinical Virology, 33*(1), 19-24.

Yeager, C. L., Ashmun, R. A., Williams, R. K., Cardellichio, C. B., Shapiro, L. H., Look, A. T., and Holmes, K. V. (1992). Human aminopeptidase N is a receptor for human coronavirus 229E. *Nature, 357*(6377), 420-422.

Zaki, A. M., van Boheemen, S., Bestebroer, T. M., Osterhaus, A. D., and Fouchier, R. A. (2012). Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. *N Engl J Med, 367*(19), 1814-1820.

APPENDIX

1: Study questionnaire

Community: Buoyem ☐ Kwamang ☐ Forikrom ☐

House Number if cohort study: Date of sampling:

Demographic data

1 Respondent Code No: ……. 2 Name : ……..

3 Age : …….(yrs) 4 Gender: Male ☐ Female ☐

5 What is your educational background? Not Educated ☐ ☐ Educated ☐ ☐

6 What is the highest level of your education? Primary JHS SHS ☐ Tertiary ☐ Other

7 Which religion do you belong to? Christian ☐ Muslim ☐ Traditional ☐ Other…….

8 Do you have any occupation? Yes ☐ No ☐

9 How many?

10 What kind of occupation are you engaged in? Farming ☐ Hunting ☐ Trading ☐

Other…….

11 Which is your primary/secondary occupation?.....

12 What is your major source of income? Student ☐ Self-employed ☐ Regular

Employment ☐ Unemployed ☐

6 What kind of house do you stay? Compound ☐ Private ☐ Other……

7 What numbers of you most often live together and share the same food source? ……

8 How many people are in your house 4 ☐ 6 ☐ 10 ☐ Other…..

9 How many people do you stay in room with ? 2 ☐ 3 ☐ 4 ☐ Other…..

Clinical Data

10i) Do you have symptoms of common cold? Yes ☐ No ☐ ii) What symptoms are you experiencing ? Cough ☐ Runny nose ☐ Nasa

l Congestion

Headache ☐ Feverish

Other……

iii) How long have you experienced these symptoms? 2 days ☐ 4days ☐ 6 days Other…..

iv) How do you think you contracted the 'cold'? Through Somebody who was infected ☐

After visiting the farm ☐ After visiting bat caves ☐

Other: ……..

v) Have you been managing it? Yes ☐ No

☐

vi) How have you been managing it?Use of antibiotics ☐ Traditional herbs ☐ Paracetmol☐

Others.....

vii) Do you practice any of the following while sneezing or coughing?

Covering the nose with hands ☐

Covering the nose with handkerchief ☐

Sneezing into the air ☐

Sneezing into your sleeves ☐

Other.....

11. Have you been vaccinated against influenza(pH1N1)? Yes ☐ No ☐

2. Ethical approval form

KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY
COLLEGE OF HEALTH SCIENCES
SCHOOL OF MEDICAL SCIENCES
COMMITTEE ON HUMAN RESEARCH PUBLICATION AND ETHICS

Our Ref: CHRPE49/09 May 22, 2009

Prof Yaw Adu-Sarkodie
Department of Clinical Microbiology
SMS, KNUST
Kumasi

Dear Sir,

LETTER OF APPROVAL
Protocol Titled: ***Virus Biology, Host Ecology and Human Behaviour as Determinants for Viral Zoonosis***

Your submission to the Committee on Human Research, Publications and Ethics on the above named protocol refers.

The Committee has considered the ethical merit of your submission and approved the protocol.

The Committee wishes to state however, that samples and data gathered for the study should be used for study purposes only. It is recommended that permission should be sought from the committee if any amendment to the protocol or use, other than submitted, is made of your research data.

The Committee would expect a periodic report on your study, annually or at close of the project, whichever one comes first. It should also be informed of any publications arising from the study.

Many thanks for your application.

Yours faithfully,

Prof. Sir JW Acheampong, MD, FWACP
Chairman

Private Mail Bag, University Post Office, Kumasi, Ghana. Phone: 233-51-60303, Fax 233-51-60302
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3. Consent form

Statement of person obtaining informed consent:

I have fully explained this research to _____ and have given sufficient information, including that about risks and benefits, to enable the prospective participant make an informed decision to or not to participate.

DATE: _____ SIGNATURE: _____

NAME: _____

Statement of person giving consent:
I have read the information on this study/research or have had it translated into a language I understand. I have also talked it over with the interviewer to my satisfaction. I understand that my participation is voluntary (optional). I know enough about the purpose, methods, risks and benefits of the research study to judge that I want to take part in it. I understand that I may freely stop being part of this study at any time. I agree to take part in the study.

NAME OF PARTICIPANT: _____

DATE: _____ SIGNATURE/THUMB PRINT: _____

WITNESS' SIGNATURE (if participant could be non-literate): _____

WITNESS' NAME: _____

4. Participant Information Leaflet

Title of Research: Virus biology and zoonoosis among individuals living in bat communities

Name(s) and affiliation(s) of researcher(s) of applicant(s): The study is being conducted by the School of Medical Sciences, KNUST. Researchers are Mr Owusu Michael and Prof Yaw Adu-Sarkodie.

Purpose(s) of research: To investigate possible respiratory viruses and bat viruses among individuals living in bat communities.

Procedure of the research, what shall be required of each participant and approximate total number of participants that would be involved in the research:

Nasopharyngeal specimens and blood will be taken from participants. The swab will gently be inserted up the nostril towards the pharynx until resistance is felt and then rotated 3 times to obtain epithelial cells. 5ml of blood specimens will also be taken using sterile procedures.

Risk(s): There are no major risks in this study. Mild pain may be experienced by participants after their blood has been taken.

Benefit(s): The project will inform us of viruses that are associated with common cold among adults. You may not get direct benefits now but the information will help policy makers in finding appropriate antidote to viral causes of respiratory illness.

Confidentiality: All information collected in this study will be treated with confidentiality. Your name will be recorded so we could help you seek medical attention after the laboratory analysis.

Contacts: If you have any question concerning this study, please do not hesitate to contact

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