HUMAN CORONAVIRUSES IN ACUTE RESPIRATORY INFECTIONS IN GHANA

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DECLARATION

Declaration by candidate

I hereby declare that this is the product of my own research undertaken under supervision towards the award of the master of philosophy degree in microbiology in the Department of Microbiology, University of Ghana Medical School, College of Health Sciences. This work has neither been presented in whole nor in part for another degree elsewhere.

Signature…….. Date……/……../……..

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Declaration by supervisors

We hereby declare that the practical work and presentation of this thesis were supervised in accordance with guidelines on supervision of thesis laid down by the University of Ghana.

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DEDICATION

I dedicate this work to God Almighty who has seen me through it all. I also dedicate this work to my mom, Madam Emelia Armah, Mr. C. Q. Parbie, Mr. Eric Parbie, Mrs. Ama Klinger and Mrs. Hannah Ofoe for their invaluable support towards my academic career.

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LIST OF ABBREVIATIONS AND ACRONYMS

3CLpro	Chymotrypsinlike protease 3
ACE2	Angiotensin Converting Enzyme 2
AFLP	Amplified Fragment Length Polymorphism
APN	Aminopeptidase-N
ARDS	Acute Respiratory Distress Syndrome
ARI	Acute Respiratory Infection
BCoV	Bovine Coronavirus
BLAST	Basic Local Alignment Search Tool
CEACAM1	Carcinoembryonic Cell Adhesion Molecule 1a
CoV	Coronavirus
CPE	Cytopathic Effect
DNA	Deoxyribonucleic Acid
DPP4	Dipeptidyl Peptidase 4
E	Envelope protein
ELISA	Enzyme-Linked Immunosorbent Assay
ERGIC	Endoplasmic Reticulum-Golgi Intermediate Compartment
FIPV	Feline Infectious Peritonitis Virus
FRET	Fluorescent Resonance Energy Transfer
HCoV	Human Coronavirus
IBV	Infectious Bronchitis Virus
ILI	Influenza-Like Illness

Kbs	Kilobases
kDa	KiloDaltons
M	Membrane glycoprotein
MERS-CoV	Middle East Respiratory Syndrome Coronavirus
MHV	Mouse Hepatitis Virus
Mpro	Main protease
N	Nucleocapsid protein
NIC	National Influenza Centre
NP	Nasopharyngeal
nsp	non-structural protein
OP	Oropharyngeal
ORF	Open Reading Frame
PDEV	Porcine Diarrhoea Virus
PLpro	Papain-Like protease
RER	Rough Endoplasmic Reticulum
RNA	Ribonucleic Acid
rRT-PCR	real-time Reverse Transcription Polymerase Chain Reaction
RTC	Replicase-Transcriptase Complex
RTI	Respiratory Infection
S	Spike glycoprotein
SARI	Severe Acute Respiratory Illness
SARS-CoV	Severe Acute Respiratory Syndrome Coronavirus

Sg	Sub-genomic
TGEV	Transmissible Gastroenteritis Virus
TRS	Transcription Regulatory Sequence
URTI	Upper Respiratory Tract Infection
VIDISCA	Virus discovery cDNA- ALFP
WHO	World Health Organization

ABSTRACT

Background:

Acute respiratory infections (ARI) remain a leading cause of morbidity, mortality, and economic loss globally. Until recently, human coronaviruses (HCoVs) have been mainly associated with mild upper respiratory tract infections. The 2003 outbreak of severe acute respiratory syndrome coronavirus (SARS-CoV) and cases of Middle East respiratory syndrome coronavirus (MERS-CoV) since 2012, illustrate the potential of coronaviruses to cause severe disease. Thus, it was necessary to examine cases of acute respiratory infections in Ghana for the presence of human coronaviruses.

Aim:

This study investigated the presence of human coronaviruses in recent acute respiratory infections in Ghana.

Method:

Oropharyngeal and nasopharyngeal (OP/NP) swabs from patients with acute respiratory illness received at the National Influenza Centre, Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana, from January 2013 to March 2014 were screened for HCoV-229E, HCoV-OC43, HCoV-NL63, HCoV-HKU1 as well as MERS-CoV using real-time reverse transcriptase polymerase chain reaction (rRT-PCR). Also NP/OP swabs collected from returning Hajj 2013 pilgrims with symptoms including cough, sore throat, running nose, headache and fever were screened for MERS-CoV by rRT-PCR. The human coronaviruses identified were then sequenced and subjected to phylogenetic analysis.

Results:

Human CoVs were detected in 7(3.5%) out of 200 ARI samples tested. Human CoV-HKU1 was detected in 3 (1.5 %), 229E in 2 (1 %), OC43 in 1 (0.5 %), and NL63 in 1 (0.5 %). No co-infection of HCoV types occurred. Detection of HCoVs was more in patients >5 years (85.7% of positives) than in children less than 5 years (14.3% of positives). Also HCoVs were detected more frequently in out-patients (5%) than in hospitalised patients (2%). None of the respiratory specimens tested were positive for MERS-CoV.

Conclusion:

This study has shown that HCoVs are uncommonly associated with ARI in Ghana, though clinical symptoms of cases cannot be exclusively attributed to HCoV infection. No evidence of introduction of MERS-CoV was detected. The study has further enhanced surveillance of respiratory illness in Ghana to guide appropriate public health response for an epidemic of a new HCoV.

CHAPTER ONE

1.1 Introduction

Acute respiratory infection (ARI) is a major public health problem and recognized as a leading cause of illness worldwide (Yousif. 2006). These illnesses are classified as upper respiratory tract infections (URTIs) or lower respiratory tract infections (LRTIs). Viruses have been shown to represent a considerable proportion of the pathogens associated with URTIs (Dasaraju *et al.,* 1996). Upper Respiratory Tract Infection is the second leading cause of out-patient visits to health facilities in Ghana after malaria (Ghana Health Service, 2010). Common viral agents that have been associated with ARI include Rhinoviruses, Respiratory Syncytial Viruses, Parainfluenza, Influenza Viruses, Human Metapneumoviruses, Adenoviruses and Coronaviruses (Kesson *et al.,* 2007, Hegele *et al.,* 2010). The burden of viral respiratory tract infections has been studied extensively in children worldwide (Berman. 1991, Campbell, 1995; Mulholland 1999, Williams *et al.,* 2002, Black *et al.,* 2003, Henrickson *et al.,* 2004, Pierangeli *et al.,* 2007, Nair *et al.,* 2010,). However, viral respiratory infections are not limited to children. Human coronaviruses have been reported to be involved in a wide spectrum of clinical presentation in upper and lower respiratory tract illnesses (van der Hoek. 2007, Han. *et al.,* 2007, van Elden *et al.,* 2004, Arden *et al.,* 2005). They have also been reported to contribute significantly to ARI (Fouchier *et al.,* 2005; van der Hoek *et al.,* 2005). Human Coronavirus (HCoV) OC43 and HCoV-229E have been known since the 1960s and chiefly cause mild respiratory disease (Saif, 2004). In 2003 outbreak of severe acute respiratory syndrome (SARS) leading to about 850 deaths was caused by a novel coronavirus known as the SARS coronavirus (SARS-CoV) (Drosten *et al.* (2003). Soon after this, HCoV types NL63 and HKU1, were

detected in respiratory tract specimens of patients with lower respiratory tract illness (Esper *et al.*, 2005, Fouchier *et al.*, 2004, Van der Hoek *et al.*, 2004, Woo *et al.*, 2005). Previously, HCoVs, with the exception of SARS-CoV, had been mainly associated with mild upper respiratory tract infections (Monto *et al.*, 1974). However, in young children, the elderly and immunocompromised patients, these viruses were known to cause severe respiratory tract disease that required hospitalization (Krzysztof *et al.*, 2007).

Genetic variability in coronaviruses is facilitated by the infidelity of the RNA dependent RNA polymerase in combination with the high frequency of RNA recombination and the unusually large genomes for RNA viruses (Woo *et al.*, 2007). These factors have also facilitated the emergence of viruses with new traits that adapt to new hosts and ecologic niches, sometimes causing zoonotic events (Zaki *et al.*, 2012).

In September 2012, the World Health Organization (WHO) notified health authorities of cases of severe HCoV infection caused by a novel virus type in the Middle East. This disease first detected in April 2012, involved severe acute respiratory failure and also affected the kidneys, causing acute renal failure (Bermingham *et al.*, 2012, Pebody *et al.*, 2012). A novel beta coronavirus was isolated from one of two patients in Jeddah, Saudi Arabia, and termed HCoV-EMC (also named HCoV-EMC/2012) (Zaki *et al.*, 2012, Corman *et al.*, 2012). The virus has since been renamed Middle East Respiratory Syndrome Coronavirus (MERS-CoV) by the Coronavirus Study Group of the International Committee on Taxonomy of Viruses (De Groot *et al.*, 2013). Since April 2012, several countries in the Middle East have been affected, including Jordan, Kuwait, Oman, Qatar, Saudi Arabia, the United Arab Emirates, and Yemen. Furthermore, countries outside the Arabian Peninsula including France, Germany, Greece, Italy, United Kingdom, Egypt,

Tunisia, Malaysia, Philippines and United States of America have reported cases; all of which first acquired the infection in the Middle East (Bermingham *et al.,* 2012, Pebody et al. 2012, Memish *et al.,* 2012, WHO. Global Alert and Response 2014). There are speculations that dromedary camels serve as potential reservoirs for human transmission (Reusken *et al.,* 2013, Alagaili *et al.,* 2014). However, questions that remain unanswered about this new coronavirus include whether infections spread from camels to humans or vice versa, or via another host to both species and the mode of human-to-human transmission. Public health authorities around the world have been alerted about the need to increase monitoring of respiratory illnesses and are collaborating to learn more about the new coronavirus and the disease in humans ("WHO, Interim surveillance recommendations for human infection with Middle East respiratory syndrome coronavirus," 2014)

The SARS-CoV is no longer circulating in humans since the last human case reported in July 2003 (van der Hoek 2007; www.who.int/mediacentre/news/releases/2003).

1.2 Problem Statement

Coronaviruses have in the past been mainly associated with mild respiratory tract infections. For this reason, the circulation of HCoVs was not routinely monitored and attempts to develop vaccines or drugs had been limited (Principi *et al.,* 2010). The 2003 outbreak of the SARS-CoV illustrated the epidemic potential of coronaviruses to threaten global health (Ksiazek *et al.,* 2003). The concern is not far-fetched in the wake of the recent emergence of MERS-CoV with 699 laboratory-confirmed cases of infection including 209 deaths as at June 11, 2014 (WHO 2014). The National Influenza Centre (NIC) at the Noguchi Memorial Institute for Medical Research, University of Ghana,

Legon, in collaboration with the Ghana Health Service (GHS), WHO, the US Centers for Disease Control and Prevention (CDC) and the US Naval Medical Research Unit 3 (NAMRU-3) have been monitoring influenza virus activity in Influenza-like Illness (ILI) and severe acute respiratory illness (SARI) in humans since 2007. Currently, respiratory samples received from ILI and SARI patients are only screened for Influenza A and B viruses at the NIC. The presence of other respiratory viruses among these ILI/SARI patients had not been examined. Annually, Ghanaian pilgrims undertake the Hajj in the Kingdom of Saudi Arabia and the reports of the MERS-CoV outbreak sparked fears that returning pilgrims could serve to introduce the virus to Ghana. Thus, the contribution of HCoVs to ARI in Ghana has not been fully assessed.

1.3 Rationale

In the last 10 years, a number of novel human viral respiratory pathogens including HCoVs have been identified, leading to heightened alertness and the development of appropriate control measures. Human coronaviruses are known to cause a spectrum of symptoms including fever, sore throat, cough, and breathing difficulties that may be confused with influenza. Between January 2013 and December 2013, the NIC detected influenza viruses in 10% of 2627 of the ILI and SARI samples received. This leaves a larger proportion of respiratory samples with unexplained aetiology for respiratory virus involvement. The emergence of SARS-CoV and MERS-CoV has necessitated the need to re-examine the extent of coronaviruses involvement in severe respiratory illness. The spread of respiratory infections are facilitated by modern travel trends as has been reported with MERS-CoV in the cases confirmed outside the Middle East including United Kingdom, France, Italy, Germany, Spain and Tunisia (www.who.int. 2013). There was a potential risk for the

introduction of MERS-CoV into Ghana since approximately 10,400 Ghanaian Muslims undertook the Hajj pilgrimage in the Kingdom of Saudi Arabia (KSA) between October 2012 and November, 2013.

It was therefore necessary to retrospectively screen recent respiratory samples received at the NIC for HCoVs to determine and describe their association with ARIs. Any such circulating HCoVs was to be characterized to provide surveillance baseline data on human coronaviruses circulating in Ghana.

1.4 Aim and Objectives

Aim:

To investigate the involvement of human coronaviruses in acute respiratory infections in Ghana

Specific objectives:

- Identify strains of human coronaviruses in ARI cases by molecular methods.
- Detect the presence of MERS-CoV ARI in Ghana.
- Determine phylogenetic relationships of HCoV identified.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Taxonomy and Classification:

Coronaviruses (CoVs) are enveloped viruses with a positive-sense, single-stranded RNA genome belonging to the family *Coronaviridae* and sub-family Coronavirinae that infect a wide host range, including mammalian and avian species (Woo *et al.,* 2009. King *et al.,* 2012). The family *coronaviridae* belong to the order *Nidovirales* together with the families *Arteriviridae*, *Mesoniviridae*, *Roniviridae* with a common feature of 3' co-terminal nested set of sub-genomic viral mRNAs that are produced during infection (Antoine *et al.,* 1997. King *et al.,* 2012). Initially, the genus had been subdivided into three main groups based on serological relationships which was subsequently supported by gene sequencing (Gonzalez *et al.,* 2003). Currently, these have been reclassified into four genera designated Alphacoronavirus (former groups 1a and 1b), Betacoronavirus (groups 2a to 2d), Gammacoronavirus (group 3), and as recently suggested, Deltacoronavirus (Woo *et al.,* 2012).

Coronaviruses (CoVs) infect a variety of livestock, poultry, and human companion animals, in which they can cause serious and often fatal respiratory, enteric, cardiovascular, and neurological diseases (Holmes *et al.,* 1996). The alphacoronavirus group include HCoV-229E, HCoV-NL63, and some bat and Porcine coronaviruses. The Betacoronavirus include HCoV-OC43, HCoV-HKU1 (lineage A), SARS-CoV (lineage B), MERS-CoV (linage C) some bat and murine coronaviruses (Woo *et al.,* 2009, de Groot *et al.,* 2013). The gamma and delta-coronavirus groups are mainly made up of avian species. Figure 1 summarises the classification of common coronaviruses (de Groot *et al.,* 2013).

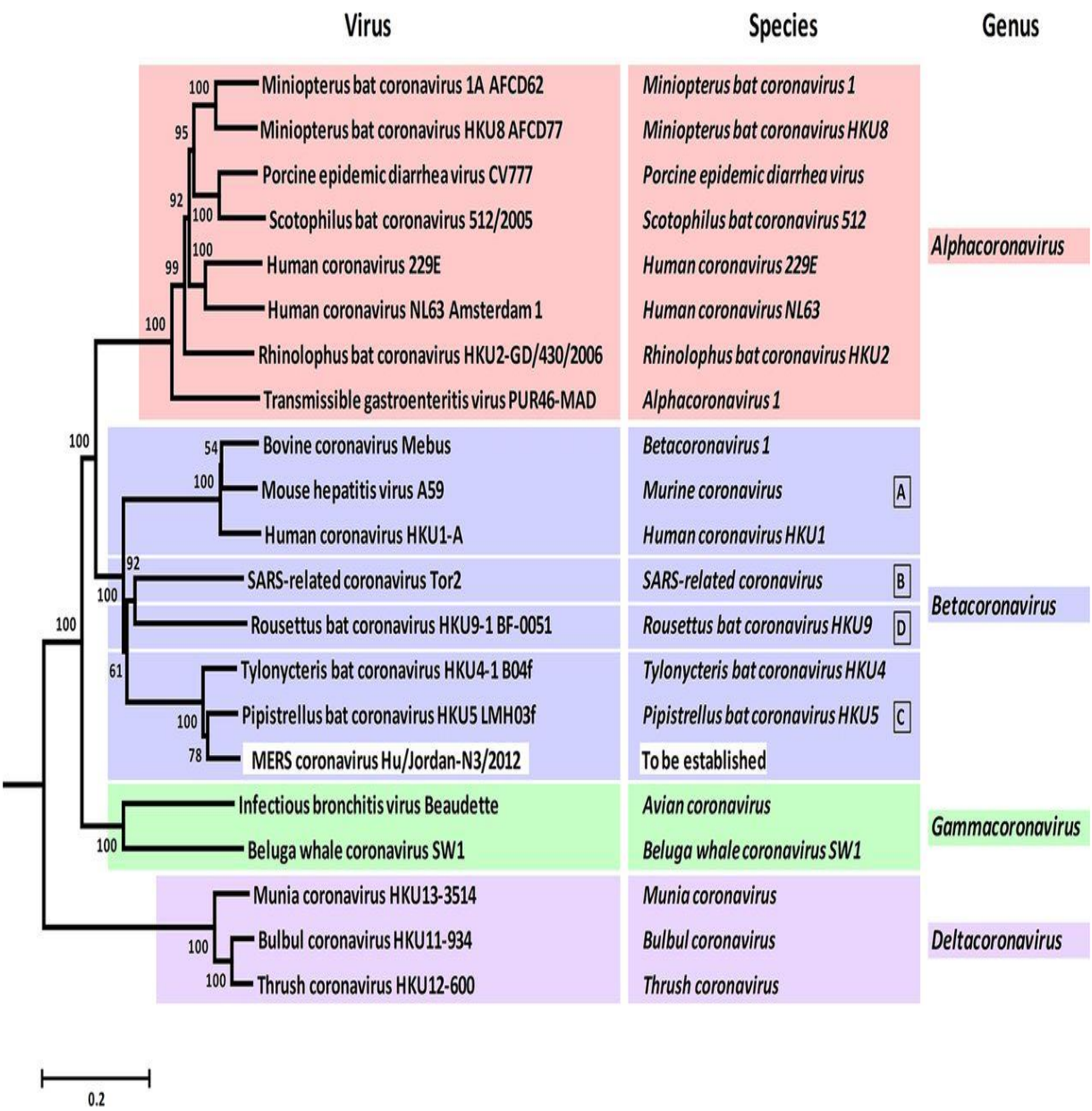

Figure 1. Phylogenetic relationships among members of the subfamily Coronavirinae and taxonomic position of MERS-CoV. A Neighbor-Joining tree generated from amino acid sequence alignments in conserved domains in the replicase polyprotein 1ab of 20 representative coronaviruses. The tree shows the four main monophyletic clusters, corresponding to genera Alpha-, Beta- Gamma- and Deltacoronavirus (color-coded), and the position of MERS-CoV. Also indicated are betacoronavirus lineages A through D (corresponding to former CoV subgroups 2A through D). de Groot *et al.*, 2013

2.2 VIRUS STRUCTURE AND MORPHOLOGY:

The virion is a spherical and sometimes pleomorphic enveloped particle typically ranging in size from 70nm to 120nm in diameter, containing approximately 26-32 kb RNA genome size which is associated with the N phosphoprotein to form a helical nucleocapsid (Perlman and Netland, 2009). It is characterized by its crown-like or halo-shaped appearance under the electron microscopic (Herold *et al.*, 1993, Lai *et al.*, 1994, Thiel *et al.*, 2003. Gonzalez *et al.*, 2003). Coronaviruses have four structural proteins in common, namely: long spike (S) glycoprotein, envelope (E) protein, transmembrane (M) glycoprotein, and nucleocapsid (N) protein (Woo *et al.*, 2005, Graham *et al.*, 2013) (Figure 2). A shorter spike-like glycoprotein called hemagglutinin-esterase (HE) is present on some members of the betacoronavirus subgroup.

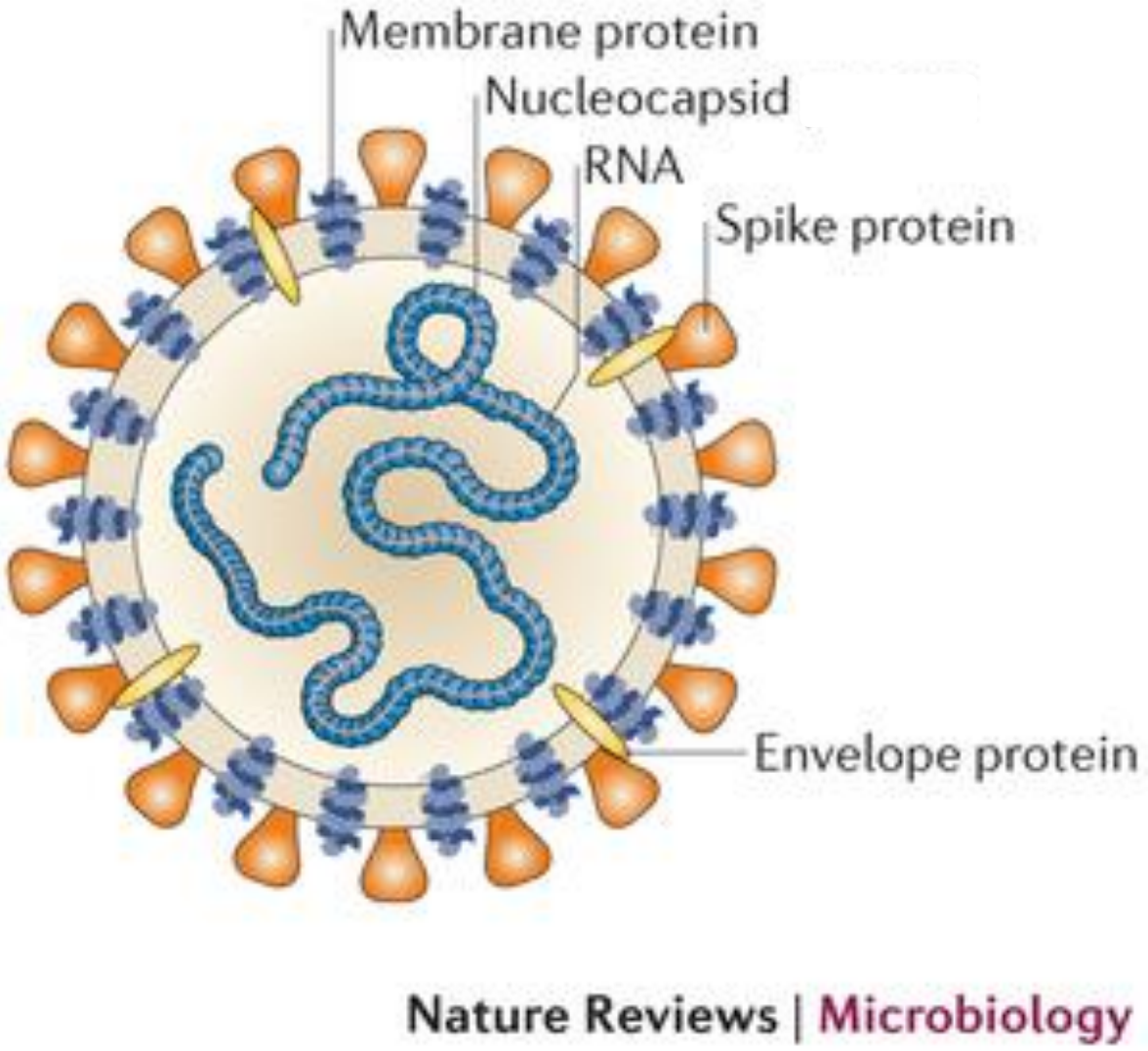

Figure 2: The structure of a typical coronavirus. Coronaviruses have four structural proteins in common, namely: spike (S) glycoprotein, envelope (E) protein, transmembrane (M) glycoprotein, and nucleocapsid (N). The virus particles range from 70 to 120 nm in diameter and are surrounded by characteristic spike-shaped (S) glycoproteins giving it the crown-like appearance. It contains approximately 26-32 kb RNA associated with the N phosphoprotein to form a helical nucleocapsid. Source: Graham *et al.*, (2013)

Spike (S) glycoprotein:

The spike glycoprotein is a large heavily glycosylated surface protein with molecular weight of about 150 to180kd (1150–1450 amino acids, 20nm) which forms the petal-shaped spikes on the surface of the virion. It can be divided from the N- to C- terminus into three domains including a large external domain, a transmembrane domain and a short carboxyl terminal cytoplasmic domain (Weiss *et al.*, 2005, Du *et al.*, 2009). The external domain is further divided into 2 sub-domains including a more variable S1 and a mainly conserved S2. The S protein plays a central role as a multifunctional protein in the biology and pathogens in coronaviruses including interaction with and entry into the target cell. It induces protective immune responses as well the as fusion of the viral envelope to host cell membrane and cell-cell fusion (Gallagher *et al.*, 2001, Holmes *et al.*, 1986, Lai *et al.*, 1997).

Envelope (E) protein:

The envelope protein is a small envelope protein of ~9 to 12kDa (100 amino acids), present in very small amounts in virions. It is a highly hydrophobic protein that spans the lipid bilayer twice such that the N and C termini are both present in the virus lumen (Maeda *et al.*, 2001). Together with the M protein, the E protein is required for budding of virus from infected cells (Vennema *et al.*, 1996), and also appears also to play a role in induction of apoptosis (Yang *et al.*, 2005, An *et al.*, 1991). It has also been suggested that E protein induces membrane curvature (Kuo *et al.*, 2003) and possesses ion channel activity, which is required for optimal virus replication (Wilson *et al.*, 2004, Madan *et al.*, 2005).

Membrane (M) protein:

The membrane protein is an integral and most abundant glycoprotein of ~26-kDa (250 amino acids). It spans the membrane three times and has a short N-terminal ectodomain and a cytoplasmic tail. The M protein has been found to play a role in virus assembly (Kuo *et al.*, 2002, Haan *et al.*, 1999), as well as involved in viral RNA packaging into nucleocapsid (Narayanan *et al.*, 2002).

Nucleocapsid (N) protein:

The nucleocapsid is a helical phosphorylated protein of ~50 to 60-kDa (500 amino acids) which interacts with the viral genomic RNA to form viral nucleocapsid. The N protein is known to interact with M, and is believed to play a role in viral RNA synthesis (Kuo *et al.*, 2002, Sims *et al.*, 2000, Almazan *et al.* 2004).

Hemagglutinin –esterase (HE) protein:

The HE protein is found on virions of some members of betacoronavirus subgroup A. It is a shorter spike-like protein (425 amino acids) (klausegger *et al.*, 1999, Zhang *et al.*, 1992, Popova *et al.*, 2002). Though the function of the HE protein is not well understood, it has been shown to bind 9-O-acetylated neuraminic acid residues and has acetylesterase activity (Vlaska *et al.*, 1988), suggesting its involvement in either virus entry or release from infected cells. However some studies have indicated that the HE protein is not essential for viral replication (Gagneten *et al.*, 1995, Popova *et al.*, 2002).

2.3 GENOME STRUCTURE AND ORGANIZATION

Coronaviruses possess a positive-stranded RNA genome, the largest of approximately 26 to 32 kb amongst RNA viruses (Perlman and Netland, 2009). The viral genome can function as mRNA and is infectious when purified (Lai *et al.,* 1997). Coronaviruses share a conserved organization of their RNA genomes (Figure 3). Two-thirds of the 5' end of the genome carries the replicase-transcriptase gene. This portion contains 2 partially overlapping open reading frames (ORFs) 1a and 1b. The replicase-transcriptase gene is translated into two large polypeptides (pp1a measuring approximately 450 kDa and pp1ab measuring approximately 750 kDa) via a frame shifting mechanism involving a hairpin-pseudoknot structure formed by the genomic RNA (Lee *et al.,* 1991, Ziebuhr *et al.,* 2005, Namy *et al.,* 2006). The 3' end also has a 200 to 500 nucleotide untranslated region (UTR) which is followed by a poly (A) tail. One-third of the 3' end contains the ORFs coding for the structural proteins S, E, M and N as well as HE for some sub-groups. There are other accessory protein genes interspersed among the structural protein genes but vary among species in number (1 to 8 genes) and position. A common transcription regulatory sequence (TRS), previously known as intergenic sequences, which is also essential for the formation of subgenomic mRNAs is located upstream of the 5' end of each ORF on the viral genome. The sequence at the 5' end of the genome (~65 to 98 nucleotides), up to the first TRS, is called the leader sequence (Pasternak *et al.,* 2006). A 200 to 400 nucleotide untranslated region (UTR) follows the leader sequence. Each subgenomic mRNA is translated monocistronically to yield only a single protein encoded by the ORF located immediately downstream of the TRS sequence (Lai *et al.* 1983, Spann *et al.,* 1983). Other reports have shown that some subgenomic mRNAs are multicistronic, where translation of other ORF depends on an internal ribosomal entry site (Thiel *et al.,* 1994). The ORF1ab is

translated as a polyprotein that is cleaved by two proteases encoded in the 5' region of the 1ab protein gene. These are a papain-like protease (PLpro) which is encoded by the non-structural protein 3 (nsp3) gene and a chymotrypsinlike protease (3CLpro) with a predicted serine-like protease activity, encoded by nsp5 gene (Rota *et al.,* 2003, Ziebuh *et al.,* 2004). The 3CLpro processes the majority of cleavage sites between the protein domains of the 1ab polyprotein (Ziebuhr *et al.,* 2005).

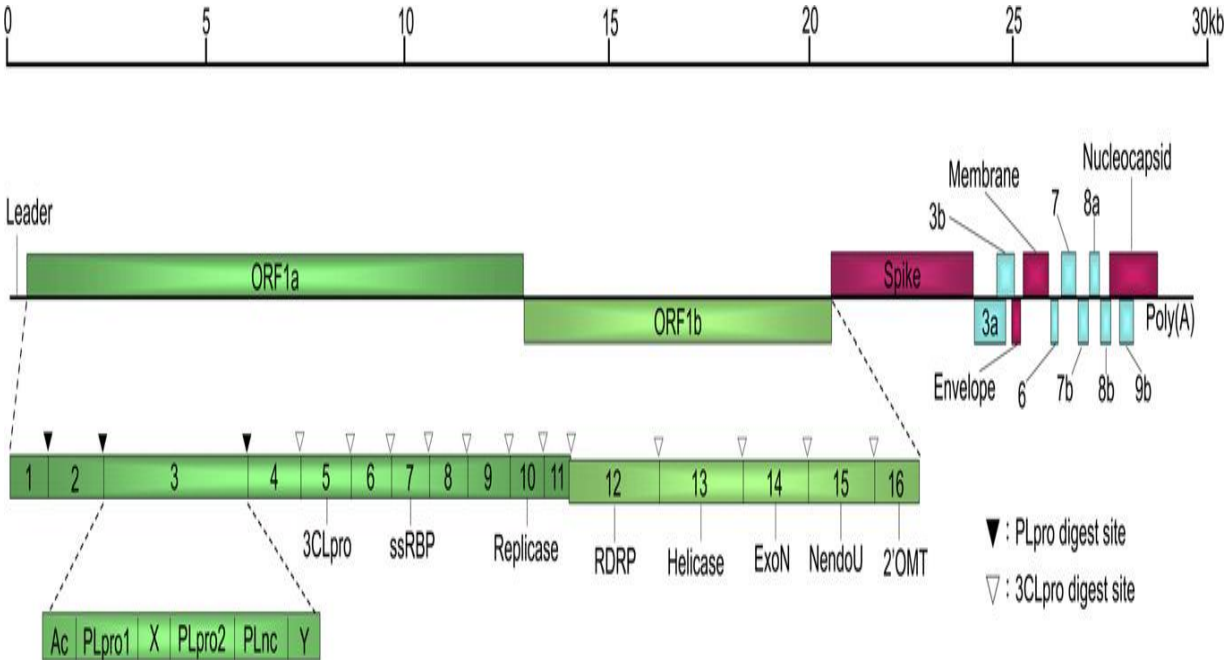

Figure 3. Coronavirus genome. ORF1a and 1b are located at the 5'-terminal 2/3 gene of the coronavirus and encode two polyproteins, namely pp1a (w450 kDa) and pp1ab (w750 kDa). The protein domains of the replicase polyprotein are indicated by nonstructural protein numbers (1 to 16). By confirmed or predicted functions: PLpro1 and PLpro2, papain-like proteases; X, domain encoding predicted adenosine diphosphate-ribose 1"-phosphatase activity (ADRP); 3CLpro, 3C-like protease; ssRBP, single-stranded RNA binding protein; RdRp, putative RNA-dependent RNA polymerase; Hel, helicase; ExoN, putative 3'-to-5' exonuclease; NendoU, putative uridylate-specific endoribonuclease; 2'-O-MT, methyltransferase. 1/3 of the 3' end contains the ORFs coding for structural proteins (S, E, M, N etc) translated from subgenomic mRNA species. Source:Li and Lin (2013).

2.4 PHYLOGENETIC ANALYSIS AND RECOMBINATION

The increased interest in coronaviruses after the SARS epidemic with associated increased in genomic data has enabled meaningful bioinformatics analyses on the Coronaviridae family (Woo *et al.,* 2010). By gene sequence and phylogenetic analysis, coronaviruses are classified into four genera designated Alphacoronavirus, Betacoronavirus, Gammacoronavirus), and Deltacoronavirus (Woo et al., 2012). Bat coronaviruses dominate the Alphacoronavirus and Betacoronavirus genera and bird coronaviruses dominate the Gammacoronavirus and Deltacoronavirus genera. Hence, bats and birds are considered an excellent gene pools for coronaviruses in these four genera (Woo et. al., 2009). Phylogenetic analysis for coronaviruses have mostly been performed using the S, N, chymotrypsin-like protease and Pol helicase genes (Woo *et al.,* 2010). Coronaviruses exhibit a high frequency of homologous RNA recombination as a result of their unique random template switching during RNA replication (Pasternak et. al., 2006, Weiss and Navas-Martin, 2005). Such have resulted in the Generation of coronavirus species or different genotypes within a coronavirus species may result from such recombination mechanisms (Woo *et al.,* 2010). For instance, a feline CoV type II has been generated by double recombination between feline CoV type I and Canine CoV. (Herrewegh et. al., 1998) Also, recombination has resulted in the generation of three genotypes, A, B and C, of HCoV-HKU1 as revealed by phylogenetic analysis using different parts of the 22 complete genomes (Woo et. al., 2006). Furthermore, phylogenetic analysis based on neighbour-joining method have showen three distinct clusters (A, B, C) and the emergence of a fourth group D of HCoV-OC43 strains circulating in Beijing, China (Lau et. al., 2011).

2.5 VIRUS LIFE CYCLE (REPLICATION)

After receptor interaction and fusion of viral and plasma membranes mediated by the S protein, replication starts with the translation of the replicase polyprotein. The genomic RNA serves as the template to synthesize sub-genomic mRNAs as well as full-length genomic RNA. Proteins are translated from each sub-genomic mRNA after which new virions are assembled by budding into intracellular membranes and released through vesicles by the cell secretory mechanisms. The major steps of coronavirus replication are summarized in Figure 4 (Weiss and Navas-Martin, 2005).

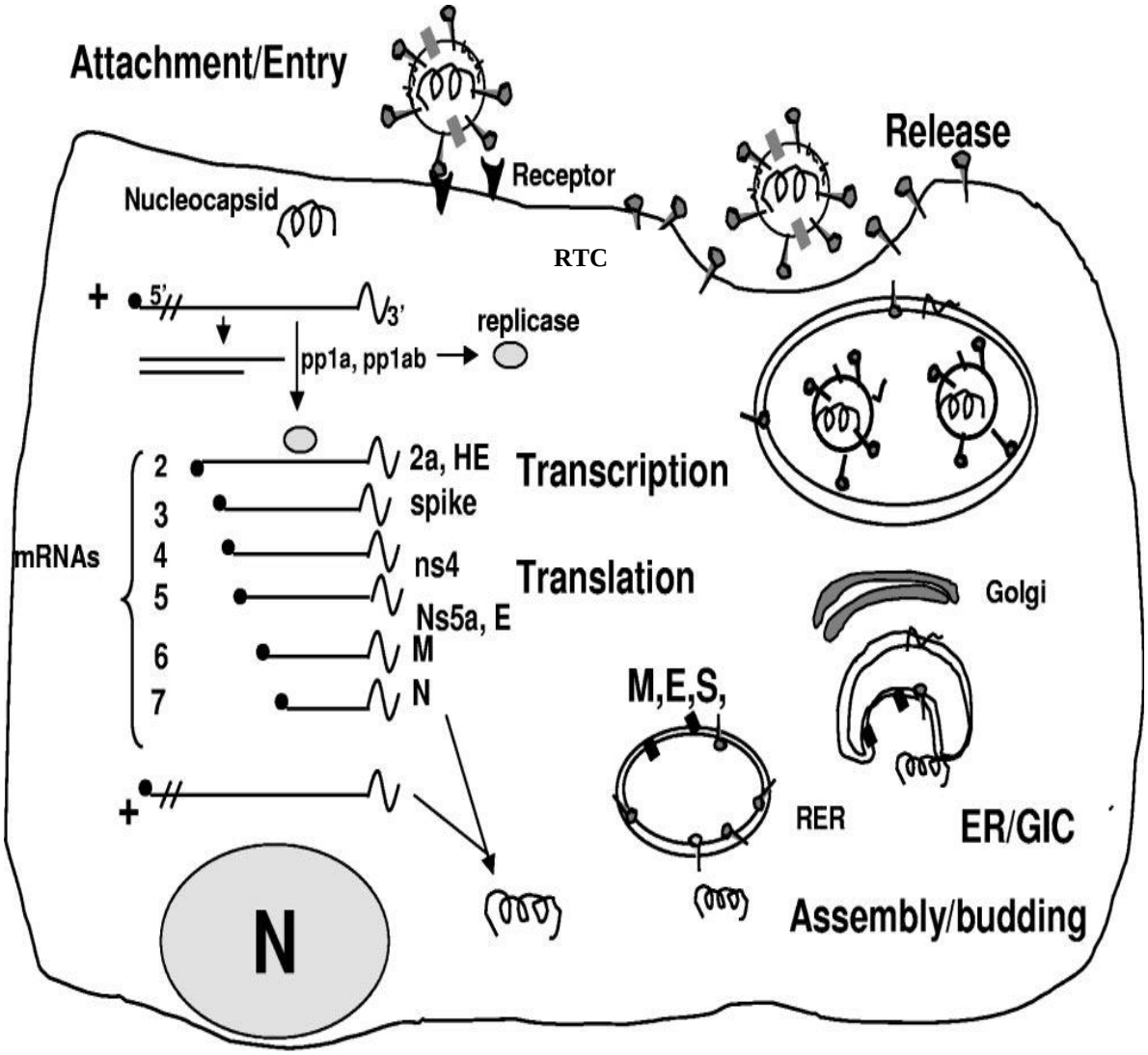

Figure 4. Model of coronavirus replication. After receptor interaction and fusion of viral and plasma membranes, expression of coronaviruses starts with translation of two polyproteins, pp1a and pp1ab, which undergo co-translational proteolytic processing into the proteins that form the replicase-transcriptase complex (RTC). The RTC is used to transcribe a 3'coterminal set of nested subgenomic mRNAs, as well as genomic RNA, that have a common 5' leader sequence derived from the 5' end of the genome. Proteins are translated from the 5' end of each mRNA. New virions are assembled by budding into intracellular membranes and released through vesicles by the cell secretory mechanisms. The entire cycle of coronavirus replication occurs in the cytoplasm. RER, rough endoplasmic reticulum; ER/GIC, endoplasmic reticulum/Golgi intermediate compartment. Source: Weiss and Navas-Martin, 2005

Attachment

The first step in the viral replication cycle is the attachment to host-specific cellular receptors mediated by the spike protein. The receptor-binding domain of the S protein is poorly conserved among coronaviruses and, as a result, host receptor usage varies between viral genera and species (Weiss *et al.*, 2005, Perlman and Netland, 2009). Different receptors identified for several coronaviruses include Aminopeptidase-N (APN), angiotensin-converting enzyme 2 (ACE2), carcinoembryonic cell adhesion molecule 1a (CEACAM1), and recently dipeptidyl peptidase 4 (DPP4) (Tan *et al.*, 2002, Weiss *et al.*, 2005, Perlman and Netland, 2009, Graham *et al.*, 2010) (Table 1). Several alpha-coronaviruses, including HCoV-229E use APN for attachement to adhere to host cells (Lai *et al.*, 1997. Severe Acute Respiratory Syndrome CoV and HCoV-NL63 binds to ACE2 (Li *et al.*, 2003, Li *et al.*, 2007). The HCoV-OC43 binds to 9-O-acetylated sialic acid moieties (Lai *et al.*, 1997). Also MERS-CoV targets the cellular receptor DPP4 on host cell membrane (Raj *et al.*, 2013).

Table 1. Representative (Alpha and Beta) coronavirus species and their receptors

Virus	Group	Receptor
HCoV-229E	Alpha	aminopeptidase N (APN)
Feline infectious peritonitis virus (FIPV)	Alpha	APN
Feline enteric coronavirus (FeCoV)	Alpha	APN
Feline coronavirus (FCoV)	Alpha	APN
Porcine respiratory coronavirus (PRCoV)	Alpha	APN
Porcine transmissible gastroenteritis virus (TGEV)	Alpha	APN
Canine coronavirus (CCoV)	Alpha	APN
HCoV-NL63	Alpha	Angiotensin-converting enzyme 2 (ACE2)
Murine hepatitis virus (MHV)	Beta	Carcinoembryonic antigen adhesion molecule 1 (CEACAM1a)
SARS-CoV	Beta	ACE2
MERS-CoV	Beta	dipeptidyl peptidase 4 (DPP4)
HCoV-OC43	Beta	9-O-acetylated sialic acid

(Adapted from Perlman and Netland, 2009)

Penetration and uncoating:

Coronavirus entry into the target cell is facilitated either by direct fusion with the plasma membrane or by endocytosis and subsequent fusion to the endosomal membrane (Lai *et al.*, 1997, Gallagher *et al.*, 2001, Krzysztof *et al.*, 2007). Upon attachment, subsequent conformational changes in the S protein facilitate fusion between the viral envelope and the host cell membrane (Zelus *et al.*, 2003, Wong *et al.*, 2004, Li *et al.*, 2006). Studies have shown that S protein cleavage is required for viral and host cell membrane fusion. For some CoVs like the Mouse Hepatitis Virus, cleavage occurs during budding whereas in others like the SARS-CoV it occurs on cell entry (Matsuyama *et al.*, 2005). The viral nucleocapsid is released into the cytoplasm of host cell where RNA is uncoated for

translation and transcription. There is evidence of the association of dephosphorylation as well as ubiquitination of certain factors in the uncoating of nucleocapsid though the process is not well understood (Mohandas *et al.,* 1991, Yu *et al.*, 2005).

Replication of genome:

After release of viral RNA in the cytoplasm, the virus initially expresses its own replicase-transcriptase complex (RTC) encoded by ORF1a and b (Sawicki, 2009, Weiss and Navas-Martin, 2005). There are two distinguishable activities of the RTC, namely, the replicase and transcriptase activities. The replicase synthesizes a full-length genomic minus-strand template and subgenomic minus-strand templates which serves as a template for the transcriptase to transcribe these to full-length positive-strand genomic RNA and subgenomic mRNA (Weiss and Navas-Martin, 2005).

Transcription of viral mRNA:

In addition to the full-length genomic RNA serving as mRNA, multiple overlapping 3'-coterminal nested subgenomic mRNAs also serve as mRNAs. Each mRNA has a common (75- to 78-nucleotide) leader sequence at its 5' end which is derived from the 5' end of genome RNA (Shieh *et al.,* 1987, Lai *et al.* 1983). Though the mechanism for synthesis of overlapping 3'-coterminal subgenomic mRNAs is not completely understood, existing evidence (Brian *et al.,* 1994; Makino *et al.,* 1991) favour a unique discontinuous transcription model (Sawicki and Sawicki, 1995). The model proposes that the replicase pauses after copying the internal TRS during replication, and then moves with the nascent subgenomic minus strand, which has an anti-TRS at its 3'end, to the TRS at the end of the leader where it serves to prime and resume elongation before terminating and completing

the synthesis of a minus-strand template. The product is subgenomic minus-strand templates all having the same 3' end as the genomic minus-strand templates. The subgenomic minus-strand templates is then used as a template for transcribing subgenomic mRNA (Sawicki and Sawicki, 1995).

Translation:

Coronaviruses, like typical plus-stranded RNA viruses do not package a RNA dependent RNA polymerase in their virions. Most of the enzymatic functions of the non-structural proteins are associated with viral RNA synthesis though some might have relevance in the cellular processes (Sawicki, 2009).

Translation begins in ORF1a and may continue in ORF1b via a frame shifting mechanism into a polyproteins pp1a and pp1b which undergo co- or posttranslational proteolytic processing by inherent proteases, PLpro and 3CLpro (sometimes referred to as Mpro or main protease), embedded in the polyprotein (Ziebuhr *et al.*, 2005, Ziebuhr *et al.*, 2001). The pp1a and pp1b are known to be the first and only proteins synthesized from the incoming viral genome before synthesis of viral RNA is initiated. The remaining viral proteins are translated from subgenomic mRNAs (Sawicki and Sawicki, 1995). Most viral proteins are translated from individual mRNAs, generally from the 5'-most (a) ORF by a cap dependent scanning mechanism (Zhang, 2001). In some cases ORFs other than the one closest to the 5' end may be translated as shown in the translation of the E protein by ORF5b for MHV (Jendrach *et al.*, 1999, Thiel and Siddell, 1994) and ORF3c for Infectious Bronchitis Virus (IBV) (Liu and Inglis, 1992). This is mediated by internal ribosomal entry sites preceding these genes, allowing a cap independent translation mechanism (Zhang, 2001, Hussain *et al.*, 2005).

Processing and intracellular transport:

The primary gene product of ORF1a and 1b, pp1a and pp1ab, are processed by inherent proteases PLpro, 3CLpro and transported to a site around the peri-nuclear regions in the cell where they are co-localised with N protein and newly synthesized viral RNA on a double membranous structure that are derived from rough endoplasmic reticulum (RER) (Brockway *et al.*, 2003, Gosert *et al.*, 2002).

The S protein, after co-translational glycolylation with N-linked glycans are inserted into the RER. The S protein is then exported to the endoplasmic reticulum (ER), after forming trimmers (Delmas and Laude 1990), where it interacts with M and E proteins via C-terminal regions of the S protein (Godeke *et al.*, 2000, Opstelten *et al.*, 1995, de Haan *et al.*, 1999). Some coronaviruses, group 2 and 3 CoVs, have their S protein cleaved into S1 and S2 subunits during its transport through the Golgi complex (Lai *et al.*, 1997). Some S proteins after synthesis, are also transported to the plasma membrane which is responsible for cell fusion (Luo and Weiss, 1998).

The M protein, after synthesis on the RER is inserted into the ER membrane (de Haan and Rottier. 2005). It is mainly localised in the budding compartment with a small portion in the Golgi. It has been shown to accumulate in the Golgi in homomultimeric complexes (de Haan *et al.*, 1998, de Haan *et al.*, 2000). The E protein is localised on the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) (Opstelten *et al.*, 1995), and N protein is associated with cellular membrane (Sims et al., 2000)

Assembly and release

The major location of coronavirus particle formation is the Golgi complex, although early and late in infection budding can also occur in the ERGIC and RER respectively (Salanueva *et al.,* 1999). Budding is believed to occur at the ERGIC (Klumperman *et al.,* 1994), but the RER becomes the site of virus maturation as the infection proceeds (Tooze *et al.,* 1984). The budding process is triggered by interaction between M and N proteins (Kuo *et al.,* 2002). Also, the S protein interacts with the transmembrane region of the M protein during assembly (de Haan *et al.,* 199). The virus particles are transported through Golgi compartments to secretory vesicles, for subsequent release of virions at the cell surface. Virions accumulate small walled vesicles which fuse with the plasma membrane through exocytosis to release virus. Virus release is restricted to certain areas of the cells as shown in SARS-CoV released preferentially at the apical membrane (Tseng *et al.,* 2005), while MHV is released preferentially at the basolateral surface (Rossen *et al.,* 1995).

2.6 PATHOGENESIS:

The *Coronaviridae* exhibit a broad host range, infecting many mammalian and avian species and causing upper respiratory tract, gastrointestinal tract, hepatic, and central nervous system diseases of varying clinical severity (Wunschmann *et al.,* 2002, Loa *et al.,* 2002, Pedersen *et al.,* 2008, Gallagher *et al.,* 2001, Van der Hoek et al. 2010). This has been attributed to variations in the receptor binding domain of the S glycoprotein among the coronaviruses (Weiss *et al.,* 2005, Perlman and Netland, 2009). It is well known that animal coronaviruses such as FIPV, IBV, TGEV, bovine coronavirus (BCoV) could cause life-threatening disease though the severe disease capabilities of human coronaviruses were only recognized after the SARS epidemic (Perlman and Netland, 2009). In both SARS-

CoV and MERS-CoV pathogenesis, the elderly seem to be more vulnerable particularly in the presence of co-morbidities such as diabetes, cardiac disease, hypertension and renal disease (Drosten *et al.*, 2003, Ksiazek *et al.*, 2003, Assiri *et al.*, 2013, Drosten *et al.*, 2013), The other human coronaviruses, namely HCoV-229E, HCoV-NL63, HCoV-OC43, and HCoV-HKU1, predominantly cause acute self-limited upper respiratory tract infections such as fever, cough and rhinorrhoea. They are however known to occasionally cause significant lower respiratory tract infections mainly in the elderly, children and immunocompromised populations (Chiu *et al.*, 2005, Lau *et al.*, 2006, Esper *et al.*, 2006, Vabret *et al.*, 2006, Lai *et al.*, 2007, Perlman and Netland, 2009). Evidence of infection of the gastrointestinal tract, liver, kidney and brain, among other tissues suggests SARS-CoV also causes systemic disease (Gu *et al.*, 2005). Unlike HCoVs 229E and OC43 where inoculation of healthy volunteers in 1960s helped describe the spectrum of disease caused, it has been difficult to fulfil Koch's postulate for recent HCoVs.

It is generally accepted that the host response is responsible for many of the disease manifestations in infections caused by coronaviruses (Dandekar and Perlman, 2005, Bergmann *et al.*, 2006). It is proposed that the kinetics of virus clearance is delayed perhaps because double stranded RNA, a potent stimulator of the innate immune system, is buried in a double-membrane vesicle. Subsequent robust T and B cell and cytokine and/or chemokine (pro-inflammatory) responses results in immunopathological disease that occurs during the process of virus clearance as seen in the lungs of SARS-CoV-infected humans (Dandekar and Perlman, 2005, Gu and Korteweg, 2007, Chen and Subbarao, 2007).

Coronaviruses have evolved mechanisms to block or evade such antiviral responses such as that triggered by type I interferon pathway (Zhong *et al.,* 2012, Perlman and Netland, 2009). Some immune evasion strategies have been attributed to protein functions such as nsp1, nsp3, N protein as well as the SARS-CoV accessory proteins ORF6 and ORF3b which have been shown to prevent interferon induction and signalling (Cheung *et al.,* 2005, Ratia *et al.,* 2006, Frieman *et al.,* 2007, Zhou *et al.,* 2012).

2.7 EPIDEMIOLOGY OF HCoVs

HCoVs excluding SARS and MERS-CoVs circulate worldwide and the prevalence of the different species differ by year and geographical location (Lau *et al.,* 2006, Chiu *et al.,* 2005, Dare *et al.*, 2007, Greenberg 2011). Several studies have reported HCoVs infections as mainly presenting clinical signs of fever, cough, sore throat and headache (Vabret *et al.,* 2003, Greenberg 2011, Lu et al. 2012). HCoVs have been detected across all age groups, but described more in children and the elderly (Lu *et al.,* 2012). The prevalence of the different HCoVs appears to vary by year and location as reported by several studies (Table 2). A prevalence of 6.3% (Kuypers et al., 2007) and 5.6% (Dominguez *et al.,* 2009) both in the USA, 2.1% in Thailand (Dare *et al.,* 2007) , 10% in France (Vabret *et al.,* 2008), 2.1% in Hong Kong (Lau *et al.,* 2006), 6.4% (Gerna *et al.,* 2006) and 3.8%(Esposito *et al.,* 2006) both in Italy, 5.4% in Switzerland (Garbino et al., 2006), 11.5% in Brazil (Cabeça *et al.,* 2013b), 16% in China (Lu *et al.,* 2012), 8.15% in the United Kingdom (Gaunt *et al.* 2010). Human coronaviruses have shown a winter seasonality in the temperate regions (van der Hoek 2007, Chiu *et al.,* 2005, Bastien *et al.,* 2005, Lau *et al.,* 2006, van der Hoek 2007 *et al.,* 2005, Vabret *et al.,* 2008, Gaunt *et al.* 2010).

In humans, the transmission of coronaviruses between an infected individual and others can occur via respiratory secretions. This can happen either directly through droplets from coughing or sneezing, or indirectly through touching contaminated objects or surfaces as well as close contact, such as touching or shaking hands. The major modes of transmission of HCoVs is from person-to-person and by droplet infection (Li and Lin 2013).

Table 2. Table showing human coronavirus types detected in different countries and populations by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Reference	Country	Population type	Prevalence rates (%)				
			HKU1	NL63	OC43	229E	All four
Woo *et. al.*, 2006	Hong Kong	All age groups	0.3	0.4	1.3	0.1	2.1
Chiu *et al.*, 2005	Hong Kong	children	***not investigated***	2.6	1.5	0.3	4.4
Dare *et al.*, 2007	Thailand	All age groups	0.6	0.4	1.9	0.5	3.5
Kuypers et al., 2007	Washington, USA	children	2.7	1.1	1.8	0.7	6.3
Dominguez *et al.*, 2009	Colarado, USA	children	0.1	2.2	2	0.7	5.0
Cabeça *et al.*, 2013	Brazil	All age groups	0.4	3.9	2.1	1.3	7.7
Lu *et al.*, 2012	China	adults	1.6	1.1	4.3	9.8	16.0
Vabret *et al.*, 2008	France	children	3.8	3.3	2.7	0.2	10.0
Gaunt *et al.* 2010	UK	All age groups	0.5	0.6	1	0.3	2.4

2.8.0 HUMAN CORONAVIRUSES

The first human coronavirus to be isolated, called B814, was cultured from a boy with a typical cold in organ culture prepared from tracheal cells of a human embryo (Bradburne *et*

al., 1967). After, several coronaviruses including 229E, OC16, OC37, OC38, OC43, OC44, and OC48 were described (Hamre and Procknow. 1966, McIntosh *et al.,* 1967, Bradburne *et al.,* 1972). All the above mentioned HCoVs have been lost to follow up with the exception of 229E and OC43 as these 2 strains are easy to culture (van der Hoek 2007). Currently, members of the subfamily *Coronavirinae* that infect humans include the respiratory coronaviruses HCoV-OC43, HCoV-229E, HCoV-NL63, HCoV-HKU1, SARS-CoV, and MERS-CoV.

2.8.1 HCoV-229E

Human CoV-229E was first described in 1966 from the respiratory tract of medical students with colds (Hamre and Procknow. 1966). This virus was the cause of common cold in healthy adult volunteers who were inoculated with HCoV-229E culture in England (Bradburne *et al.,* 1967). Some studies have detected this virus less frequently than the other HCoVs (Lau *et al.,* 2006, Chiu *et al.,* 2005). Human CoV-229E was proven to be serologically different from HCoV-OC43 when antibodies from HCoV-OC43 infected individuals did not neutralize HCoV-229E (McIntosh *et al.,* 1969). The HCoV-229E has been recognized as an important cause of nosocomial respiratory viral infections in high-risk infants (Sizun *et al.,* 2001, Gagneur *et al.,* 2002). Human CoV-229E have been associated with hospital acquired lower respiratory tract infection in elderly people (Falsey *et al.,* 1997) and approximately 30% of common colds in humans (Myint, 1995).

2.8.2 HCoV-OC43

This virus previously referred to as B814, was first isolated in a patient showing typical symptoms of common cold (Tyrrell and Bynoe 1965). In addition to its established role in minor upper respiratory tract infections, there is evidence of its association with lower

respiratory disease including croup, bronchiolitis and pneumonia especially in those coinfected with other respiratory viruses (McIntosh *et al.,* 1974, Chiu *et al.*, 2005, Lau *et al.*, 2006, Vabret *et al.*, 2003, Jean *et al.,* 2013). Human CoV-OC43 has been reported to cause nosocomial related viral respiratory infection in neonates (Gagneur *et al.,* 2002, Falsey *et al.*, 1997).

2.8.3 HCoV-NL63

Human CoV-NL63, an alphaCoV, was first identified in a 7-month-old child with bronchiolitis in the Netherlands in 2004 by a cDNA Amplified Fragment Length Polymorphism (AFLP-VIDISCA) method (Van der Hoek *et al.*, 2004). That same year, the virus was isolated from a nasal swab from an 8 month old baby suffering from pheumonia (Fouchier *et al.,* 2004). A year later, HCoV-NL63 was detected in children less than 5 years old (Esper *et al*., 2005). Since its isolation, HCoV-NL63 has been shown to have a global distribution with detection in various populations (Chiu *et al.,* 2005, Vabret *et al.,* 2005, Kaiser *et al.,* 2005, Ebihara *et al.,* 2005, Moes *et al.,* 2005). Though some studies have reported frequent detection in children under 5 years of age (Chiu *et al.,* 2005, Han. *et al.,* 2007), HCoV-NL63 was detected in all age groups (Dare *et al.,* 2007). Some other studies have shown that infection with HCoV-NL63 can present as asthma exacerbation, croup, high fever and febrile seizures in children (Chiu *et al.,* 2005, Choi *et al.,* 2006).

2.8.4 HCoV-HKU1

The discovery of HCoV-HKU1 was reported in a 71 year old man with chronic pulmonary disease in Hong Kong (Woo *et al.* 2005). A high sero-prevalence (59.2%) of HKU1 was obtained in adults (Severance *et al.,* 2008), suggesting a high level of previous exposure.

Human CoV-HKU1 have been reported as the most common HCoV detected in children (Kuypers *et al.,* 2007). However, a recent study reported that HKU1 predominated in adults (Lepiller *et al.,* 2013). Many studies have suggested HCoV-HKU1 infection may aggravate the conditions of persons with an underlying disease, thus requiring hospitalization (Lau *et al.*, 2006, Esper *et al.*, 2006, Vabret *et al.*, 2006, Gorse *et al.*, 2009)

2.8.5 SARS-CoV

Severe Acute Respiratory Syndrome CoV caused a fatal severe acute respiratory lung disease in humans throughout parts of Asia, North America, and Europe during 2002 and 2003 (Drosten *et al.*, 2003; Ksiazek *et al.,* 2003, Lee *et al.,* 2003). It has been reported that the SARS-CoV probably originated from a wild animal reservoir, most likely bats (Lau et al., 2005; Li et al., 2005), Himalayan palm civets and raccoon dogs (Shi an Hu, 2008, Li *et al.,* 2006 Guan *et al.,* 2003). This virus has been shown to employ strategies to limit host antiviral mechanisms by evading interferon (IFN) responses (Cheung *et al.,* 2005). SARS was mainly characterized clinically, by symptoms of fever, dyspnea, lymphopenia, severe lower respiratory tract illness but several patients also had gastrointestinal complaints (Leung *et al.,* 2003, Peiris *et al.,* 2003). The SARS epidemic was officially controlled by July 2003 and since then no human cases have been reported (van der Hoek 2007; www.who.int/mediacentre/news/releases/2003).

2.8.6 MERS-CoV

In June 2012, the first case of a novel human coronavirus, subsequently termed Middle East respiratory syndrome coronavirus (MERS-CoV), was identified in a patient with acute pneumonia and renal failure in Jeddah, Kingdom of Saudi Arabia in the Middle East (Zaki

et al., 2012). Since then, in accordance with the provisions of the 2005 International Health Regulations, the WHO have been notified of 837 cases including at least 291 related deaths as of July 23, 2014 (WHO 2014). The virus causes a spectrum of illness that includes asymptomatic infection, mild illness (Omrani *et al.*, 2013) and life-threatening severe disease. All MERS-CoV infected individuals primarily manifest respiratory illness, although a number of secondary complications have also been reported, including acute renal failure, multi-organ failure, acute respiratory distress syndrome (ARDS), and consumptive coagulopathy. Sever disease due to MERS-CoV has mostly been observed in individuals with underlying medical comorbidities (Bermingham *et al.*, 2012, Memish *et al.*, 2013). In addition, many patients have also reported gastrointestinal symptoms, including diarrhoea.

Genetic sequencing indicates that the MERS-CoV is a betacoronavirus closely related to two bat coronaviruses (HKU4 and HKU5) (Figure 1), but distinct from other human coronaviruses, including SARS-CoV (Zaki *et al.*, 2012, van Boheemen *et al.*, 2012, de Groot *et al.*, 2013). The MERS-CoV is the first known lineage C betacoronavirus associated with human infection (Woo *et al.*, 2007). Studies have postulated the emergence of MERS-CoV represents another series of interspecies transmission events in coronaviruses, from bats to possibly other animals and then to humans, a scenario similar to the SARS epidemic (Woo *et al.*, 2012b). A recent study showed that a number of different bat species in Ghana and Europe are infected with coronaviruses, which also share close homologies with MERS-CoV (Annan *et al.*, 2013). Other studies have reported the presence of neutralizing antibodies to MERS-CoV in dromedary camels, suggesting the circulation of MERS-CoV or a closely related virus in these camels (Reusken *et al.*, 2013,

Perera *et al.*,2013, Chu *et al.*,2014, Alagaili *et al.,* 2014, Meyer *et al.,* 2014, Reusken *et al.,* 2014). This has led to speculations that dromedary camels are potential reservoirs for human transmission (Alagaili *et al.,* 2014). However, information available is insufficient to determine whether infections spread from camels to humans or vice versa, or via another host to both species.

Other coronaviruses infecting humans include human enteric coronavirus, which is closely related to HCoV-OC43 and is associated with necrotizing enterocolitis and gastroenteritis (Gerna *et al.,* 1985. Resta *et al.,* 1985).

2.9 LABORATORY DIAGNOSIS OF CORONAVIRUS INFECTION

Coronavirus infections, like other viral infections, have been detected by cell culture, electron microscopy, serological assays, antigen detection assays and molecular assays (Chan *et al.,* 2004, Lai *et al.,* 2007, van Elden *et al.,* 2004). Electron microscopy has been used in conjunction with virus isolation techniques to diagnose many coronavirus infections. Isolation of human coronavirus from infected individuals has often been unsuccessful though those from other mammals and birds are readily isolated from infected tissues (Lai *et al.,* 2007). Human coronavirus OC43 and 229E have been isolated using human rhabdomyosarcoma (RD) cells (Schmidt *et al.,* 1971). Fetal rhesus kidney cells and African green monkey kidney cells (Vero) have been used to isolate SARS-CoV (Drosten *et al.,* 2003, Ksiazek *et al.,* 2003), whereas HCoV-NL63 have been successfully isolated in tertiary monkey kidney, Vero and rhesus monkey kidney (LLC-MK2) cells (Fouchier *et al.,* 2004). Virus isolation in cell culture has long been the gold standard for diagnosis of viruses. Growth of viruses in cell culture is usually detected by visualizing morphological changes in the cells known as cytopathic effect (CPE). Limitations of cell culture include

production of relatively inconspicuous CPE, and the fact that it is time-consuming and less sensitive. These limitations were reduced by the development of the shell vial culture method (Gleaves *et al.,* 1984). The method involves centrifugation of the specimen onto the cell culture monolayer and incubation for 1–2 days, followed by fluorescent antibody staining of the cell culture, regardless of whether CPE is visible (Gleaves *et al.,* 1984). While the relative importance of viral isolation as a diagnostic method has changed due to faster methods for direct detection of virus, it still remains necessary because it provides a viable isolate that can be used for further characterization.

Serological assays used in detecting coronavirus infections include Immunofluorescence assay (IFA), enzyme immunoassay (EIA), compliment fixation, hemaglutination inhibition (Mcintosh *et al.*, 1970, Kaye *et al.*, 1971, Kraaijeveld *et al.,* 1980, Chan *et al.*, 2004).

The development of molecular tools such the polymerase chain reaction (PCR) (Mullis *et al.,* 1987) has made possible, the diagnosis of viral infection through sensitive detection of specific viral nucleic acids (Storch, 2000). Diagnosis of HCoV infections in the past had been hampered, in part, by the difficulty to propagate in cell cultures, and the time consuming serological testing limiting usefulness for clinical management. However, in recent times, nucleic acid detection by molecular methods such as RT-PCR and nested RT-PCR has gained popularity (van Elden *et al.*, 2004, Myint *et al.,* 1994, Vabret *et al.,* 2001). These methods have been shown to be highly sensitive and specific for the detection of HCoV in different populations of patients (Cabeça *et al.*, 2013, Woo *et al.*, 2012, Van der Hoek *et al.*, 2004, Falsey *et al.,* 2002, Arbour *et al.,* 2000, Pitkaranta *et al.,* 1998, Nicholson *et al.,* 1997). More recently, real-time RT-PCR assays which allows rapid and specific detection of HCoVs in patients with various presentations of respiratory infections have been developed (Dare *et al.,* 2007, Krzysztof *et al.,* 2007, Cabeça *et al.,* 2013,

Corman *et al.,* 2012). It has also increased the detection of HCoVs, thus providing a tool for large-scale epidemiological studies to further clarify the role coronavirus infection plays in respiratory infections in humans (van Elden *et al.,* 2004).

2.9.1 Principle of Real - Time Reverse Transcriptase Polymerase Chain Reaction (rRT-PCR):

The polymerase chain reaction (PCR) is one of the most powerful technologies in molecular biology (Bustin, 2004). Theoretically, PCR amplifies DNA exponentially, using sequence specific oligonucleotides, heat stable DNA polymerase, and thermal cycling to double the number of target molecules with each amplification cycle. The PCR process can be summarized in 3 steps including a high temperature (95°C) incubation which is used to separate double-stranded DNA into single strands and loosen secondary structure in single-stranded DNA, primer annealing at 50 – 70°C, and extension at 72 – 78°C (Bustin, 2004, Kubista *et al.*, 2006, "Real-Time PCR Handbook", 2012). In traditional (endpoint) PCR, detection and quantification of the amplified sequence are performed at the end of the reaction after the last PCR cycle, and involve post-PCR analysis such as gel electrophoresis and image analysis (Invitorgen Corperation Carlsbad Carlifonia 2008, "Real-Time PCR Handbook", 2012). When the start material for PCR is RNA, it first needs to be copied to cDNA in a reverse transcription step (RT) using a reverse transcriptase. The introduction of real-time PCR (Higuchi *et al.* 1992) have resolved some limitations of the Traditional PCR. The Real-time PCR assay has the advantage of combining the RT step and PCR in one step termed One-step RT-PCR, simplifying reaction setup and reducing the possibility of contamination (Invitorgen Corperation Carlsbad Carlifonia 2008, "Real-Time PCR Handbook" , 2012.). In real-time PCR, the

amount of DNA is measured after each cycle via fluorescent dyes or probes that yield increasing fluorescent signal in direct proportion to the number of PCR amplicons generated (Kubista *et al.,* 2006, "Real-Time PCR Handbook" , 2012.). The change in fluorescence over the course of the reaction is measured by an instrument that combines thermal cycling with fluorescent dye scanning capability. Fluorescent reporters used in real-time PCR include double-stranded DNA binding dyes, or dye molecules attached to PCR primers or probes that hybridize with PCR product during amplification. Of the several existing real-time fluorescent PCR chemistries, the most widely used are the 5' nuclease assay and SYBR® Green dye-based assays ("Real-Time PCR Handbook" , 2012.). The TaqMan® Assay which is the most well-known 5' nuclease assay (Holland *et al.*, 1991), uses a fluorogenic probe to enable the detection of a specific PCR product as it accumulates during PCR. The 5' nuclease assay is named for the 5' nuclease activity associated with Taq DNA polymerase. There are two key elements in the 5' nuclease assay, namely the ability of the 5' domain to degrade DNA bound to the template, downstream of DNA synthesis, and also the phenomenon called Fluorescent Resonance Energy Transfer (FRET) where emissions of a fluorescent dye can be strongly reduced by the presence of another dye, often called the quencher, in close proximity contamination (Invitorgen Corperation Carlsbad Carlifonia 2008, "Real-Time PCR Handbook" , 2012.). If the target sequence is present, the probe anneals downstream from one of the primer sites and is cleaved by the 5´ nuclease activity of Taq DNA polymerase as this primer is extended. The cleavage of the probe separates the reporter dye from the quencher dye, increasing the reporter dye signal. The additional cleavage of reporter dye molecules from their respective probes at each cycle, results in an increase in fluorescence intensity proportional to the amount of amplicon produced. The fluorescence activity is plotted

against the cycle number during the course of the reaction. The point at which the fluorescence passes from insignificant levels to clearly distinguishable levels is called the threshold cycle (Ct). The Ct is the cycle number at which the fluorescent signal of the reaction crosses the threshold. It is a relative measure of the concentration of target in the PCR reaction (Invitorgen Corperation Carlsbad Carlifonia 2008, "Real-Time PCR Handbook" , 2012.).

2.9.2 Principle of procedures used during DNA sequencing

Gel electrophoresis of post PCR product works under the principle that negatively charged DNA fragments in an electric field will migrate through agarose gels with a mobility that is inversely proportional to the their molecular weight towards the positive electrode (Applied Biosystems, 2009). Ethidium bromide (a fluorescent dye) when added to PCR product binds to the DNA to enable DNA fragment visualization under ultraviolet light. There are several methods for purifying PCR products including Column purification, ethanol precipitation, gel purification, enzymatic purification (Applied Biosystems, 2009). Purification of post PCR product with the QIAquick PCR purification kit (Qiagen, Hilden, Germany) is an example of a column purification method. It works under the principle of DNA being adsorbed to a silica membrane spin-column in the presence of high concentrations of chaotropic salt at optimum pH (95% adsorption at pH≤7.5) while contaminants pass through the column. Impurities are efficiently washed away, and the pure DNA is eluted with Tris buffer (QIAGEN GmbH 2006).

Sanger et al. (1974) used the principles of DNA replication in the development of the process now known as Sanger dideoxy sequencing. This process takes advantage of the ability of DNA polymerase to incorporate nucleotide base analogues that lack the 3′-

hydroxyl group essential in phosphodiester bond formation resulting in the formation of extension products of various lengths terminated with dideoxynucleotides at the 3′ end (Applied Biosystems, 2009). A refinement of Sanger dideoxy sequencing method is the fluorescence-based cycle sequencing system by Applied Biosystems. This improved method has the advantages of being robust, easy to perform, and effective for sequencing PCR Products. The cycle sequencing procedures incorporate fluorescent dye labels using dye-labeled dideoxynucleotide triphosphates (dye terminators). Each of the four dideoxynucleotide terminators are tagged with a different fluorescent dye (Applied Biosystems, 2009).

Methods for purifying post sequencing products vary according to user preference and the cycle sequencing chemistry employed. They include BigDye® XTerminator Purification Kit, ethanol precipitation, spin columns, size-exclusion membranes, and magnetic beads methods (Applied Biosystems, 2009). The Agencourt CleanSEQ sequencing reaction clean-up system (Agencourt Bioscience, USA) is a magnetic bead adsorption/elution method purification. It works under the principle that magnetic particles in an optimized binding buffer will selectively capture sequencing extension products while unincorporated dyes, nucleotides, salts and contaminants are removed using a simple washing procedure. And finally eluting the purified sequencing product from the paramagnetic beads (Agencourt Bioscience Corporation: Protocol 000600v031).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

Ghana lies in the coastal belt of West Africa, bordered by Togo on the East, Cote D'Ivoire on the West, Burkina Faso on the North and the Atlantic Ocean on the south. The country occupies an area of 238,533 sq km (92,098 sq miles) [Government of Ghana official portal]. The country is divided into 10 administrative regions; Greater Accra, Ashanti, Brong-Ahafo, Central, Eastern, Northern, Upper East, Upper West, Volta and Western regions. The country has a warm, humid climate with an annual mean temperature between 26°C and 29°C. The principal feature of rainfall in Ghana is its seasonal character and its variability from year to year. In the Northern part, the rainy season which starts from April to October and a dry season from November to March are 2 main seasons. In the South however there are 2 rainy seasons with monthly rainfall totals reaching their highest values in May or June and in October and also 2 dry seasons. The first one in August and the second one from December through to January. January is a dry month throughout the country, usually referred to as the hamattan ("Ghana Meteorological Agency, Climatology" 2014).

3.2 Study Population and Sample size

Ghana's population was approximately 24.7 million in 2010 (Ghana Statistical Service, 2010). According to the national analytical report of the 2010 census, Ghana's population has a youthful structure (58.3% ≤ 24 years), with a broad base consisting of large numbers of children and a conical top of a small number of elderly persons. More than half of

Ghana's population live in urban areas, where urban is defined as population of 5,000 and more. The sex ratio is determined as 95.2 males to 100 females.

The National Influenza Centre in monitoring influenza like illness employs the following case definitions for patients with ARI from sentinel sites across the country.

1. Influenza-like Illness (ILI): An acute respiratory infection with the following symptoms (onset within the last 10 days):

- History of fever or measured fever of ≥37.5°C (Axillary);
- And any one of the following respiratory symptoms; cough, sore throat, rhinorrhoea, chest pain, difficulty in breathing, wheezing

2. Severe Acute Respiratory Illness (SARI): An acute respiratory infection that meets ILI and requires hospitalization.

Sentinel sites for influenza surveillance have been established at various health facilities situated in all 10 regions of the country and mandated to submit at the first 5 oropharengeal (OP) and/or nasopharengeal (NP) samples weekly for an respiratory illness that meet the case definitions above. For the period of January 2013 to March 2014, the NIC received 2627 respiratory samples.

The sample size was calculated using a formula accessed from OpenEpi (Version 3): a web-based epidemiologic and statistical calculator for public health (Sullivan *et al.*, 2009; http://www.openepi.com/v37/SampleSize/SSPropor.htm)

Sample size $n = [DEFF*Np(1-p)]/ [(d^2/Z^2_{1-\alpha/2}*(N-1)+p*(1-p)]$

A prevalence (P) of 11.5% detection of human coronaviruses in respiratory specimens from ARI (Cabeça *et al.* 2013b) was adapted in calculation of sample size using a 5% confidence limit (d) as percentage of 100, a standard (Z) score of 1.96 at 95% confidence level. Design effect (DEFF) was set at 1 for a random sampling technique.

The minimum sample size calculated was 157 which was rounded up to 200.

3.3 Study design and Sample selection

This was a cross sectional study involving archived respiratory specimens stored at -80^{0}C at the NIC. Sample selection was carried out to ensure an even distribution within January 2013 - March 2014 and also to coincide with period of MERS-CoV outbreak reports in the Middle East. One hundred (100) samples each were selected from ILI and SARI cases. Samples from ILI cases were selected by a random sampling technique using Microsoft Excel version 2013. Briefly, samples that had tested negative for influenza A and B viruses were stratified according to age groups (≤1, 2-4, 5-14, 15-49, 50-64, ≥65), adapted from recommendations for Global Epidemiological Surveillance Standards for Influenza (World Health Organization 2013). Samples were then randomly selected from each group in representative proportion to the respective age group numbers in the total samples for the period of interest (January 2013 to March 2014). For SARI, 100 samples that were received from November 2013 to March 2014 were used in this study. Samples from ILI cases were oropharyngeal swabs in viral transport medium (VTM), whereas SARI samples were combined NP and OP swabs in VTM. All ILI and SARI samples were screened for

HCoV-229E, OC43, NL63, and HKU1. Specimen from SARI cases were further screened for MESR-CoV.

This study also included specimens from returning pilgrims from the 2013 Hajj with respiratory symptoms. As part of a medical screening program for the pilgrims, the NIC was mandated by the Ghana Health Service to screen returning Hajj pilgrims with respiratory symptoms for respiratory viruses at the Hajj camp established at the Kotoka International Airport in November 2013. Combined Nasopharyngeal (NP) and oropharyngeal (OP) specimens were obtained from returning pilgrims with symptoms of respiratory infection including cough, sore throat, running nose, head ache and fever and transported in a cold box to the laboratory for analysis. Out of over 5400 returning pilgrims, respiratory samples were obtained from a total of 518 pilgrims who were symptomatic and analysed.

3.4 Ribonucleic acid extraction

Viral RNA was extracted using the QIAamp® Viral RNA Mini Kit (Qiagen, Hilden, Germany). Extraction was performed according to manufacturer's recommendations (QIAGEN GmbH 1999). Initially, VTM containing swabs were homogenised by pulse vortexing. After that, 560 µl of lysis buffer containing carrier RNA was added to 140 µl of each sample, mixed by pulse vortexing and incubated at room temperature for 10 minutes. This was to ensure lysis under highly denaturing conditions to inactivate RNases as well as ensure isolation of intact viral RNA. Also, carrier RNA added to lysis buffer improves the binding of viral RNA to the QIAamp silica-gel-based membrane in the spin column. Molecular grade absolute ethanol (560 µl) was added to adjust buffering conditions and

optimize binding of the RNA to the QIAamp membrane. Six hundred and thirty microlitres of the mixture was transferred to the QIAmp spin column and centrifuged (eppendorf centrifuge 5415D) at 6000g for 1 minute. The filtrate was discarded, the collection tube was changed and the remaining 630 µl of the mixture was transferred into the spin column and spun. The filtrate was again discarded, the collection tube changed and 500µl of washing buffers (AW1 and AW2) were added sequentially to ensure complete removal of any residual contaminants without affecting RNA binding. The AW1 wash was done by centrifugation at 6000g for 1 minute while that with AW2 was done at maximum speed (16,000g) for 3 minutes. To eliminate any chance of possible Buffer AW2 carryover, the collection tube was changed and the spin column centrifuged for an extra 1 minute. Bound viral RNA was then eluted by adding 60µl of RNase/DNase-free elution buffer containing 0.04% sodium azide to prevent microbial growth and subsequent contamination with RNases to the spin column and spinning in a microcentfuge at 8000 rpm for 1 minute. The elution buffer containing extracted RNA was stored at -80^{0}C until use.

Human Specimen Control (HSC) provided by the Influenza Reagent Resource (IRR), CDC, was subjected to nucleic acid extraction procedures just as clinical specimens. This served as a negative control to validate nucleic extraction procedure and reagent integrity.

3.5.0 Real - Time Reverse Transcriptase Polymerase Chain Reaction (rRT-PCR) for detection of Coronaviruses

3.5.1 Real-time RT-PCR protocol for HCoVs 229E, OC43, NL63, HKU1

Real-time RT-PCR for the four HCoV-229E, OC43, NL63 and HKU1 was conducted according to standardized protocols as described in rRT-PCR assays for non-influenza

respiratory viruses by the Centres for Disease Control and Prevention (CDC) in Atlanta, USA (Centre for Disease Control and Prevention n.d.). Reaction mixtures were prepared using the AgPath-ID™ One Step RT-PCR Kit (Applied Biosystems). Samples were tested in a 25µl reaction volumes containing 5µl of RNA, 12.5µl of 2X RT-PCR Buffer (Ag-Path-ID™), 1µl of reverse transcriptase/Taq mix (25X RT-PCR Enzyme Mix, Ag-Path-IDTM), 0.5µl of each type specific primer and probe set. Primers and probes were synthesized by a commercial supplier (Eurofins MWG Operon, Huntsville, AL, USA) based on previously published sequences (Table 2) (Dare *et al.*, 2007, van Elden *et al.*, 2004). Amplification was performed on the Applied Biosystems® 7300 Real-Time PCR instrument (Life Technologies, USA). Cycling conditions for all primer/probe sets consisted of a reverse transcriptase step of 45°C for 10 minutes, followed by a *Taq* polymerase activation step of 95°C for 10 minutes and then 45 cycles of 95°C for 15 seconds (denaturing) and 55°C for 1 minute (annealing and extension step). Data was collected at the 55°C for 1 minute (annealing and extension) step.

As recommended in molecular laboratory settings, a unidirectional workflow technique was employed to prevent contamination and ensure integrity of all laboratory testing. This was achieved by working from the reagent (master mix) preparation area through to the amplification area. Also integrity of reagents were ensured by segregating reagents and materials for master mix from those for sample manipulations. No template control (NTC), negative template control and positive template controls were included in all PCR tests performed to monitor integrity of PCR reagents and technique. For NTC no template was added to the master mix. Negative control constituted RNA extracts known to be negative for coronavirus RNA. Positive controls for coronaviruses were obtained from the Influenza

Reagent Resource (IRR), CDC. Each sample was also tested for the human ribonuclease P gene serving as an internal positive control for human nucleic acid.

RNA extraction procedure was considered valid when HSC tested positive for only RNP but negative for every other target. A PCR test was considered valid when all NTC and NC tested negative for all coronavirus targets while all PCs tested positive for respective targets. When all controls have yielded appropriate results, a sample was considered positive by rRT-PCR for a target when detected at a Ct value ≤37 and showed an exponential curve with logarithmic, linear, and plateau phases.

3.5.2. Real-time RT-PCR protocol for MERS-CoV

An rRT-PCR screening assay developed for the upstream of E gene (upE assay) was used for MERS-CoV detection (Corman *et al.,* 2012). A 25µl reaction was set up containing 5µl of RNA, 12.5µl of 2X RT-PCR Buffer (Ag-Path-IDTM), 1µl of reverse transcriptase/Taq mix (25X RT-PCR enzyme mix, Ag-Path-IDTM), 400 nM concentrations of each primer as well as 200nM of probe. Primers and probes were synthesized by a commercial supplier (Eurofins MWG Operon, Huntsville, AL, USA) based on previously described sequences (Table 2) (Corman *et al.,* 2012). Thermal cycling was performed on the Applied Biosystems® 7300 Real-Time PCR instrument (Life Technologies) at 55°C for 20 minutes, followed by 94°C for 3 minutes and then 45 cycles of 94°C for 15 seconds, 58°C for 30 seconds. Data was collected at the 58°C for 30 seconds step. Controls were included in the all tests as described in section 3.6.1 above. Positive controls for MERS-CoV UpE assay were obtained from the Institute of Virology, University of Bonn Medical Centre, Germany.

RNA extraction procedure was considered valid when HSC tested positive for only RNP but negative for every other target. A PCR test was considered valid when all NTC and NC tested negative for all targets while all PCs tested positive for respective targets. When all controls had yielded appropriate results, a sample was considered positive by rRT-PCR for a target when detected at a Ct value ≤37 and showed an exponential curve with logarithmic, linear, and plateau phases.

3.6.0 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) and Cycle sequencing for HCoV positive cases

3.6.1 Reverse Transcriptase Polymerase Chain Reaction

Samples that tested positive by rRT-PCR for HCoV were selected and amplified by conventional PCR using the Qiagen One-Step RT-PCR kit (Qiagen, Hilden, Germany). Phylogenetic analysis for coronaviruses have mostly been performed using the S, N due to their conserved nature and appreciable sequence length (Woo *et al.*, 2010). Human CoV HKU1 positives were amplified in the replicase 1b gene while the others in the N gene with type specific primers (Table 2). This was performed by preparing a 25µl reaction containing 5µl of 5X RT-PCR Buffer (Contains 12.5 mM $MgCl_2$), 0.5µl of dNTP Mix), 0.5µl each of forward and reverse primers, 1µl of enzyme mix and 5µl of RNA template. Thermal cycling for all primer/probe sets were performed on the Applied Biosystems® 2720 thermal cycler. Cycling conditions consisted of a reverse transcriptase step of 45°C for 10 min, followed by a HotStar*Taq*® DNA Polymerase activation step of 95°C for 10 min and then 45 cycles of 95°C for 15 sec (denaturing step) and 55°C for 1 min (annealing step) and an extension step of 72°C for 30 sec.

3.6.2 Gel electrophoreses to analyse amplified PCR products

Five microlitres each of the PCR products were subjected to gel electrophoreses on 2% agarose gel 1XTAE (Tris-acetate-EDTA) buffer (Life technologies, Ambion®, USA) with ethidium bromide (Life technologies, Invitrogen™, USA) staining. After which they were visualized under ultraviolet transillumination using the KODAK Gel Logic 100 Imaging system.

3.6.3 Purification of PCR products

Products were then purified using QIAquick PCR purification kit (Qiagen, Hilden, Germany). Procedures were performed according to manufacturer's recommendations (QIAGEN GmbH 2006). Briefly, 100 µl of buffer PBI was added to 20 µl of PCR product and mixed by vortexing. The mixture was then transferred to a spin column and centrifuged for 1minute at 17,900 x g. The columns were washed by adding 0.75 ml buffer PE and centrifuging for 1minute at 17,900 x g. DNA was eluted with 30 µl buffer EB (10 mM Tris·Cl, pH 8.5) by centrifugation at 17,900 x g for 1 minute.

3.6.4 Cycle sequencing of PCR products

Cycle sequencing was performed using Big Dye terminator V3.1 Cycle Sequencing kit (Applied Biosystems, California, USA) with specific forward/reverse primer set for each coronavirus type (Table 2). A 10µl reaction mixture consisting of 2µl each of 5X BigDye Sequencing Buffer, Big Dye Termisnator Mix, 2µM primer, nuclease-free water and purified PCR product was used for cycle sequencing. The cycling conditions were 94^{O}C

for 2 minutes, 25 cycles of 94^{O}C for 30 seconds, 50^{O}C for 15 seconds and 60^{O}C for 4 minutes.

3.6.5 Purification of sequencing product

Purification of sequencing product was performed with the Agencourt CleanSEQ sequencing reaction clean-up system (Agencourt Bioscience, USA). Procedures were performed according to manufacturer's recommendations (Agencourt Bioscience Corporation: Protocol 000600v031 n.d.). Briefly, 5µl of magnetic particles (Agencourt CleanSEQ) were added to the cycle-sequenced products (10 µl) and 420µl of 85% ethanol was added to the mixture. The mixture was placed in on a magnetic plate (Agencourt SPRIPlate 96R ring magnetic plate) at room temperature for 3 minutes. After that, the liquid portion of the mixture was carefully taken out and discarded leaving the DNA bound to the beads on the wall of the tube. With the tube still on the magnetic plate, 100 µl of 85% ethanol was added to the tube and kept for 3 minutes. Again the liquid was carefully taken off leaving the DNA bound to the beads on the wall. The tube was left on the magnetic plate for 10 minutes for the ethanol to completely dry off. After, 40µl of nuclease-free water (Life technologies, Ambion®, USA) was then added and the tube taken off the magnetic plate for 2 minutes to ensure the complete suspension of beads in the water and the elution of DNA. The tube was placed back on the magnetic plate and purified DNA transferred into a 96 well optical plate (Applied Biosystems, USA) to be loaded onto the 3130 Genetic Analyzer (Applied Biosystems, USA).

3.6.6 Analyses of sequence data

Purified sequences were analysed on the ABI 3130 genetic analyzer Applied Biosystems, USA) to generate raw sequence data. Nucleotide sequences for each primer were edited and assembled with Bioedit (Hall. 2004). Sequence contiqs were then multiple aligned with reference and representative coronavirus sequences after which they were exported for phylogenetic analysis with Molecular Evolutionary Genetic Analysis software v. 5.0 (Tamura *et al.* 2011). Analysis was performed separately for samples amplified in the N gene (HCoV-229E, OC43, NL63) and samples amplified in the replicase gene. Reference sequences used for the phylogenetic analysis were obtained from the GenBank after nucleotide Basic Local Alignment Search Tool (blastn). Reference and representative sequences used include JN129834, JN129835, NC_005147, AY461522, KF530068, KF530078, KF530088, KF530098, AY903460, DQ846901, JQ765574, AB695182, JPN/11-119, DQ462771, DQ445912, NC_005831, KF530109, KF530112, DQ243939, DQ243946, DQ243952, DQ243962, NC_006577, KF686346. These included those with highest identity (lowest expected, E) value together with other common type HCoV strains. A phylogenetic tree was constructed by neighbour joining method.

3.7 Data analyses

Data was entered in Microsoft Excel version 2013 and imported into Statistical Package for the Social Sciences (SPSS; IBM Corporation, Armonk, NY) for descriptive statistical analysis.

3.8 Ethics

The study was approved by the Ethical and Protocol Review Committee of the University of Ghana Medical School, College of Health Sciences; Protocol identification number (MS-Et/M.9-P3.1/2013-2014)

Table 3. Human coronavirus real-time reverse-transcription polymerase chain reaction (RT-PCR) panel primer and probe sequences (Dare *et al.*, 2007, van Elden *et al.*, 2004)

HCoV type	*primer/probe*	*Final concentration (nmol/L)*	*Gene target*	*Sequence (5' > 3')*
229E	Forward	750	Nucleoprotein	CAGTCAAATGGGCTGATGCA
	Reverse	500		AAAGGGCTATAAAGAGAATAAGGTATTCT
	Probe*	50		CCCTGACGACCACGTTGTGGTTCA
NL63	Forward	250	Nucleoprotein	GACCAAAGCACTGAATAACATTTTCC
	Reverse	250		ACCTAATAAGCCTCTTTCTCAACCC
	Probe**	50		AACACGCT“T”CCAACGAGGTTTCTTCAACTGAG
OC43	Forward	500	Nucleoprotein	CGATGAGGCTATTCCGACTAGGT
	Reverse	750		CCTTCCTGAGCCTTCAATATAGTAACC
	Probe*	50		TCCGCCTGGCACGGTACTCCCT
HKU1	Forward	100	Replicase 1b	CCTTGCGAATGAATGTGCT
	Reverse	750		TTGCATCACCACTGCTAGTACCAC
	Probe*	50		TGTGTGGCGGTTGCTATTATGTTAAGCCTG
MERS-CoV	Forward	400	Upstream of Envelope	GCAACGCGCGATTCAGTT
	Reverse	400		GCCTCTACACGGGACCCATA
	Probe#	200		CTCTTCACATAATCGCCCCGAGCTCG

*** Labelled at the 5’ end with reporter fluorophore (6-carboxy- fluorescein, FAM) and terminally quenched at the 3’ end with Black Hole Quencher–1.**

**** labelled at the 5’ end with FAM, internally quenched with Black Hole Quencher–1 (indicated by “T”), and labelled at the 3’ end with a phosphate.**

 # labelled at the 5’ end with FAM, and terminally quenched at the 3’ end with tetramethylrhodamine (TAMRA)

CHAPTER FOUR

4.0 RESULTS

4.1.0 Archived respiratory specimens from ARI cases

4.1.1 Demographic Characteristics

A total of 200 archived respiratory specimen (OP and/or NP) were obtained from the NIC between January 2013 and March 2014 (Figure 5). The mean age was 20 years within a range of 1 month to 95 years, of which 113 (56.5%) were males with an average age of 19 years and 87 (43.5%) were females with an average age of 23 years (Table 4). There were more samples (36.5%) from patients in the 15-49 age group than any other (Figure 6). Specimens were from 9 out of 10 regions in Ghana.

4.1.2 Clinical Characteristics

All patients manifested fever with at least one respiratory symptom. The most common respiratory symptom was cough (89%) with each of the other symptoms reported in less than 50% of samples tested as shown in Figure 7. Headache was the least reported symptom (6%). Four samples were from patients with pre-existing medical conditions of asthma (1) and chronic heart disease (3). Majority (76.5%) of samples were collected within 7 days of illness onset.

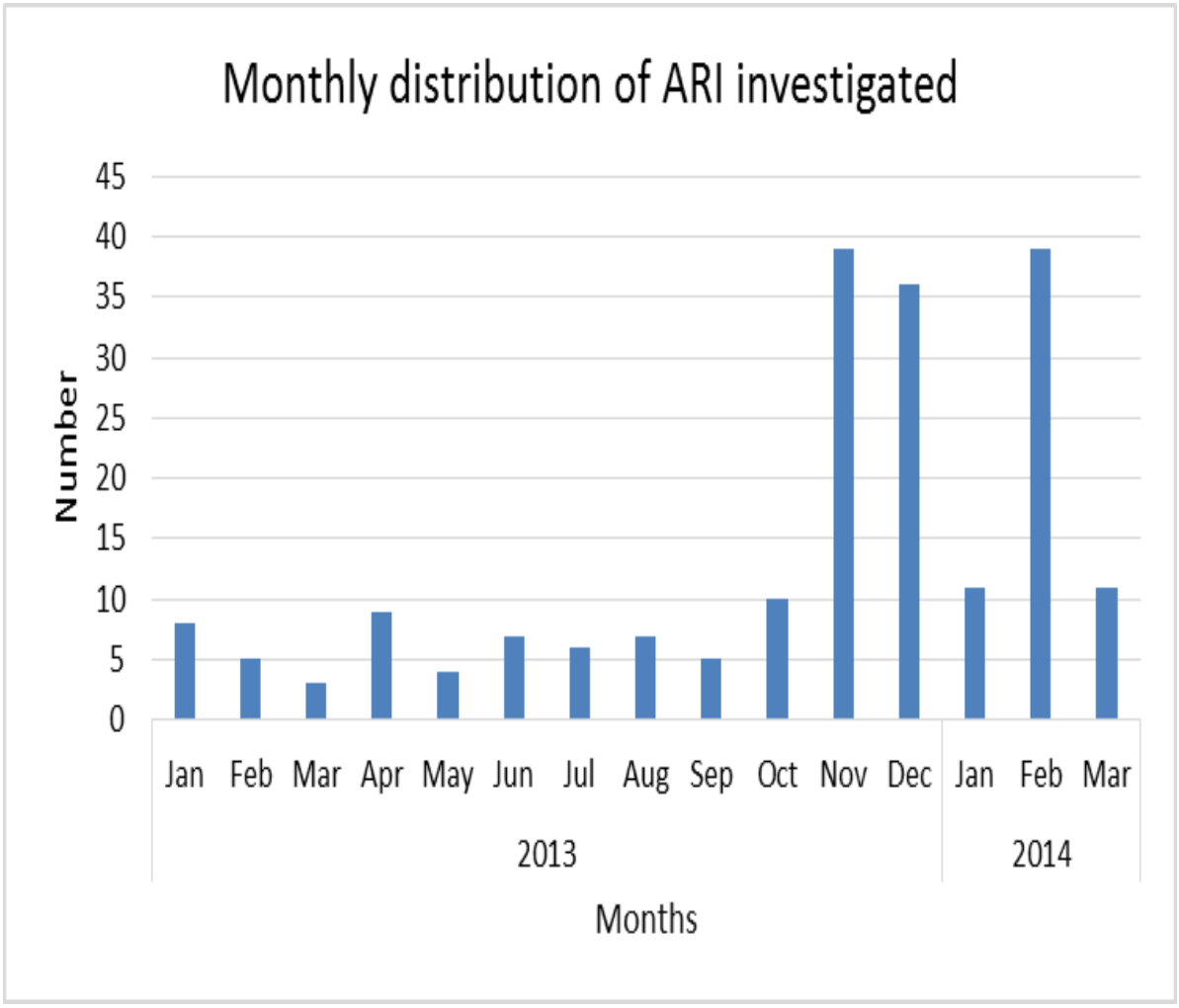

Figure 5. Monthly distribution of ARI cases investigated. Samples were selected from archived samples of patients with ARI received from January 2013 to March 2014.

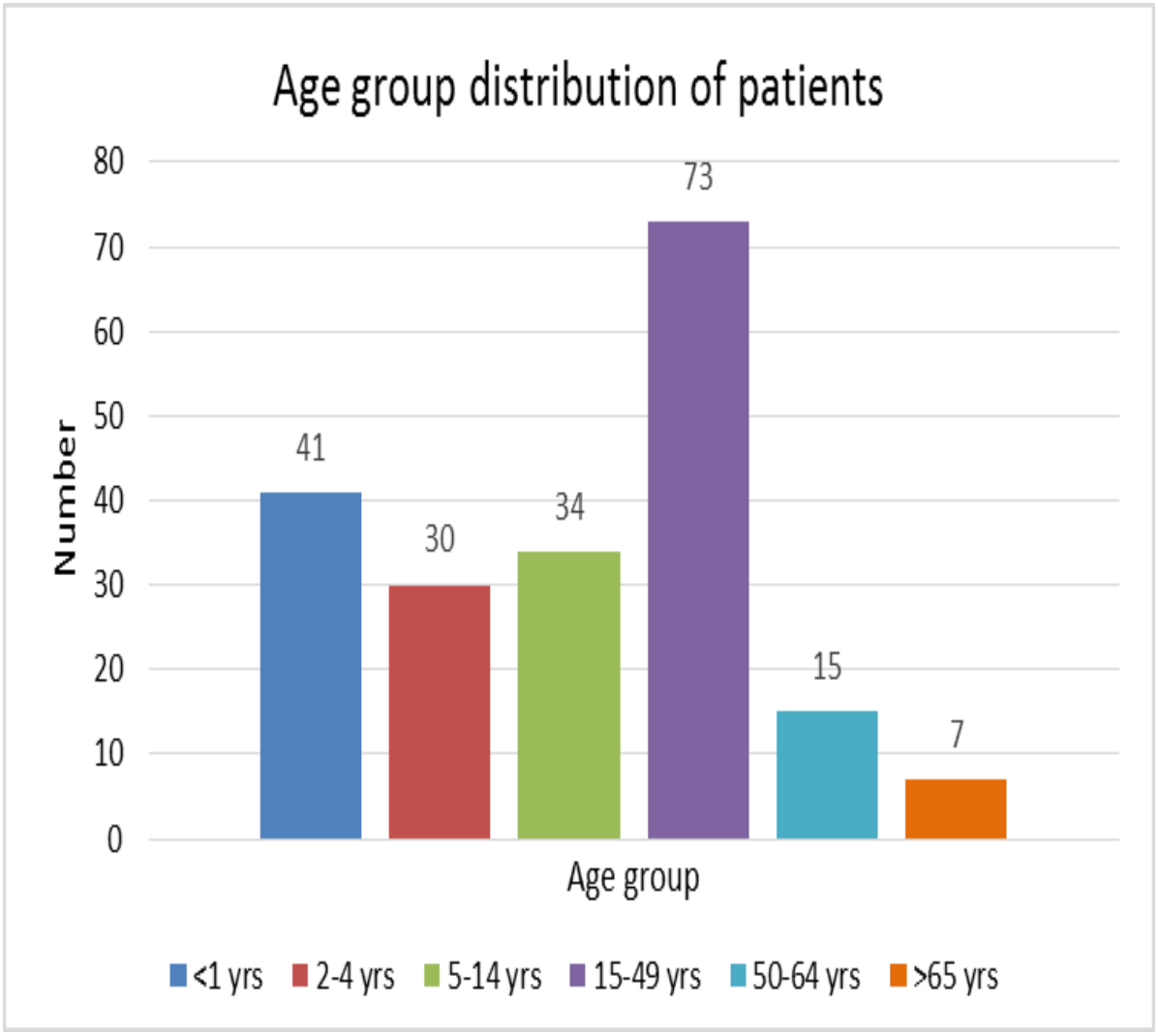

Figure 6. Age distribution of ARI cases investigated. Samples were selected from archived samples of patients with ARI received from January 2013 to March 2014.

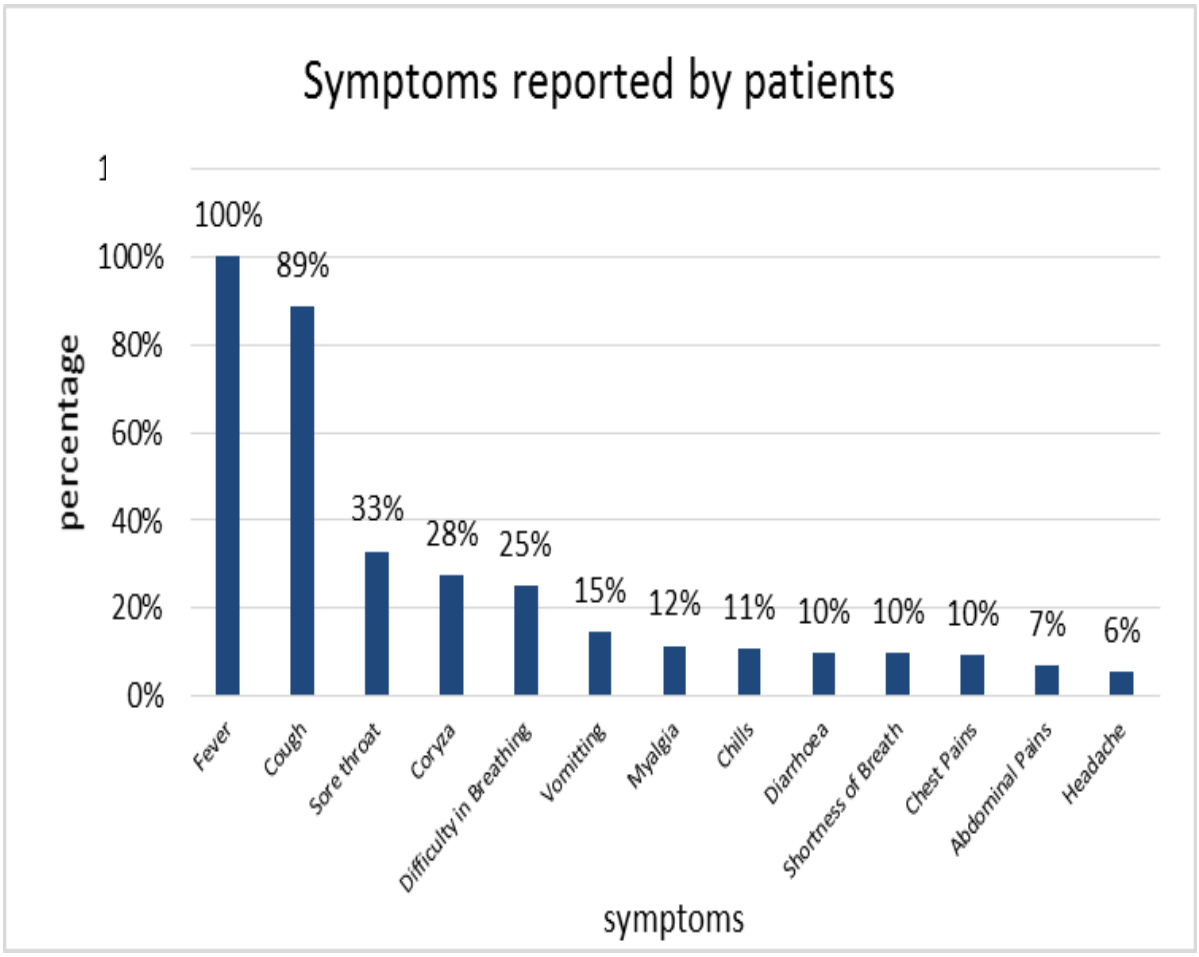

Figure 7. Graph showing the frequency of clinical symptoms reported for samples tested. Cough was the most common respiratory symptom and headache being the least recorded.

4.1.3 Detection of HCoV by rRT-PCR

Four types of HCoVs were detected in 7 (3.5%) ARI cases. These were HKU1 in (3), 229E in (2), OC43 in (1), and NL63 in (1). No co-infection of HCoV types was detected. Out of the 7 positive cases, HCoVs were detected more frequently (6) in specimens taken within the first 7 days of onset of illness as compared with specimen taken in >7 days (1). Detection of HCoVs was more in patients >5 years (85.7%) than in children less than 5 years (14.3%). Also HCoVs were detected more frequently in ILI cases (5) than in SARI cases (2). All four HCoVs types identified in this study were detected in ILI cases while only 2 HCoV types were found in SARI cases (Table 6).

There was no detection of MERS-CoV in archived respiratory specimens with SARI.

4.1.4 Clinical Characteristics of Patients with HCoVs Infection

Human CoV infections were almost equal in males (43%) and females (57%) (Table 4). All HCoV positive samples were patients with URTI. Of these, 2 of them were hospitalized (SARI) cases and 5 were out-patients. Cough and sore throat were the most common symptoms in these patients. There were no pre-existing medical conditions in positive cases (Table 6). The median age of HCoV-infected patients was 35 years (range, 5 months to 57 years).

Table 4. Demographic characteristics of archived ARI samples screened for HCoVs

Variable		Characteristic	Number of Cases N=200 (%)	HCoV Pos n=7 (%)
Case Syndrome		ILI	100(50)	5 (71.4)
		SARI	100(50)	2(28.6)
Region	Northern Zone	NORTHERN	24(12)	1(14.3)
		UPPER EAST	1(0.5)	1(14.3)
		ASHANTI	1(0.5)	0(0)
		BRONG AHAFO	8(4)	0(0)
	Southern Zone	VOLTA	12(6)	0(0)
		EASTERN	6(3)	0(0)
		CENTRAL	2(1)	0(0)
		GREATER ACCRA	128(64)	4 (57.1)
		WESTERN	18(9)	1(14.3)
Zones		SOUTHERN	166(83)	5(71.4)
		NORTHERN	34(17)	2(28.6)
Sex		FEMALE	87(43.5)	4 (57.1)
		MALE	113(56.5)	3(42.9)
Age Grp		0-1 yrs	41(21)	1(14.3)
		2-4 yrs	30(15)	0(0)
		5-14 yrs	34(17)	0(0)
		15-49 yrs	73(37)	4(57.1)
		50-64 yrs	15(8)	2(28.6)
		65+ yrs	7(4)	0(0)

Table 5. Clinical characteristics of archived ARI samples screened for HCoVs

Variable	Characteristic	Number of Cases N=200 (%)	HCoV Pos n=7 (%)
Day Difference*	0-7 days	153 (76.5)	6(85.7)
	> 7 days	39(19.5)	1(14.3)
	Unknown	8(4)	0(0)
Pre-existing condition	Asthma	1(0.5)	0(0)
	Chronic Heart Disease	3(1.5)	0(0)
	No condition	197(98)	7(100)
Symptoms	Fever	200(100)	7(100)
	Cough	177(88.5)	5 (71.4)
	Sore throat	66(33)	4 (57.1)
	Coryza	55(27.5)	1(14.2)
	Myalgia	23(11.5)	0(0)
	Headache	11(5.5)	1(14.3)
	Difficulty in Breathing	50(25)	2(28.6)
	Chills	22(11)	0(0)
	Diarrhoea	20(10)	0(0)
	Abdominal Pains	14(7)	0(0)
	Vomitting	29(14.5)	0(0)
	Chest Pains	19(9.5)	0(0)
	Shortness of Breath	20(10)	0(0)

Duration from day of symptoms onset to day of sample collection

Table 6. Details of ARI cases that were positive for HCoVs

ID	Region	Age	Sex	Symptoms aside fever	Sample type	Sample Date	HCoV Type
ILI-014	Greater Accra	51 years	F	Cough	OP	13-Mar-2013	229E
ILI-042	Western	15 years	F	Cough, Sore throat	OP	11-Jun-2013	NL63
ILI-060	Upper East	22 years	F	Breathing difficulty	OP	18-Oct-2013	HKU1
ILI-088	Greater Accra	35 years	M	Sore throat	OP	14-Feb-2014	HKU1
ILI-090	Greater Accra	45 years	M	Cough, Sore throat, Head ache	OP	20-Feb-2014	OC43
SARI-019	Greater Accra	5 months	M	Cough, Coryza	OP/NP	19-Nov-2013	229E
SARI-084	Northern	57 years	F	Cough, Sore throat, Headache, Breathing difficulty	OP/NP	17-Feb-2014	HKU1

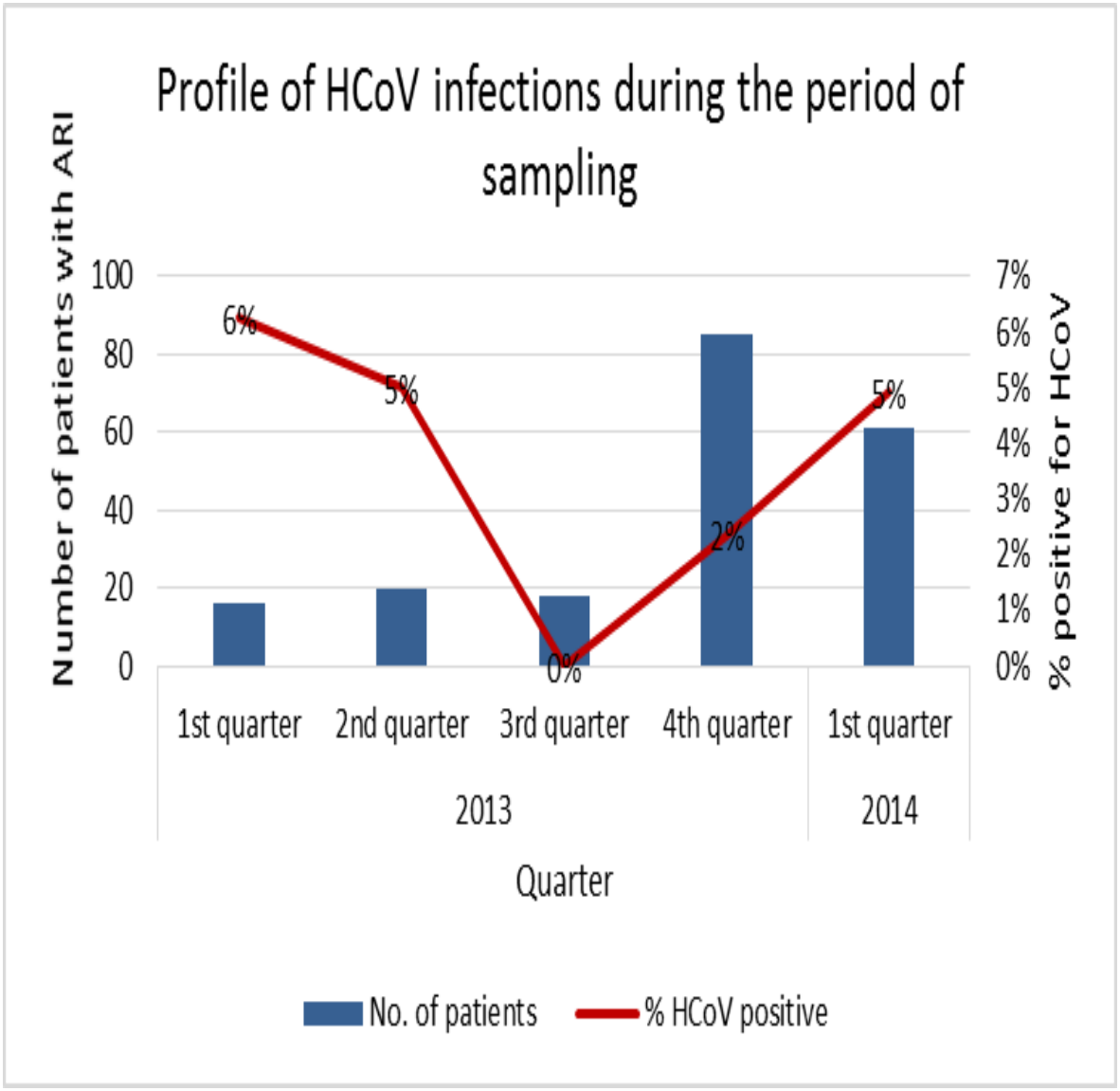

Figure 8. Graph showing profile of HCoV infection in ARI cases by quarter. Detection rate (% positive) was compared between quarters

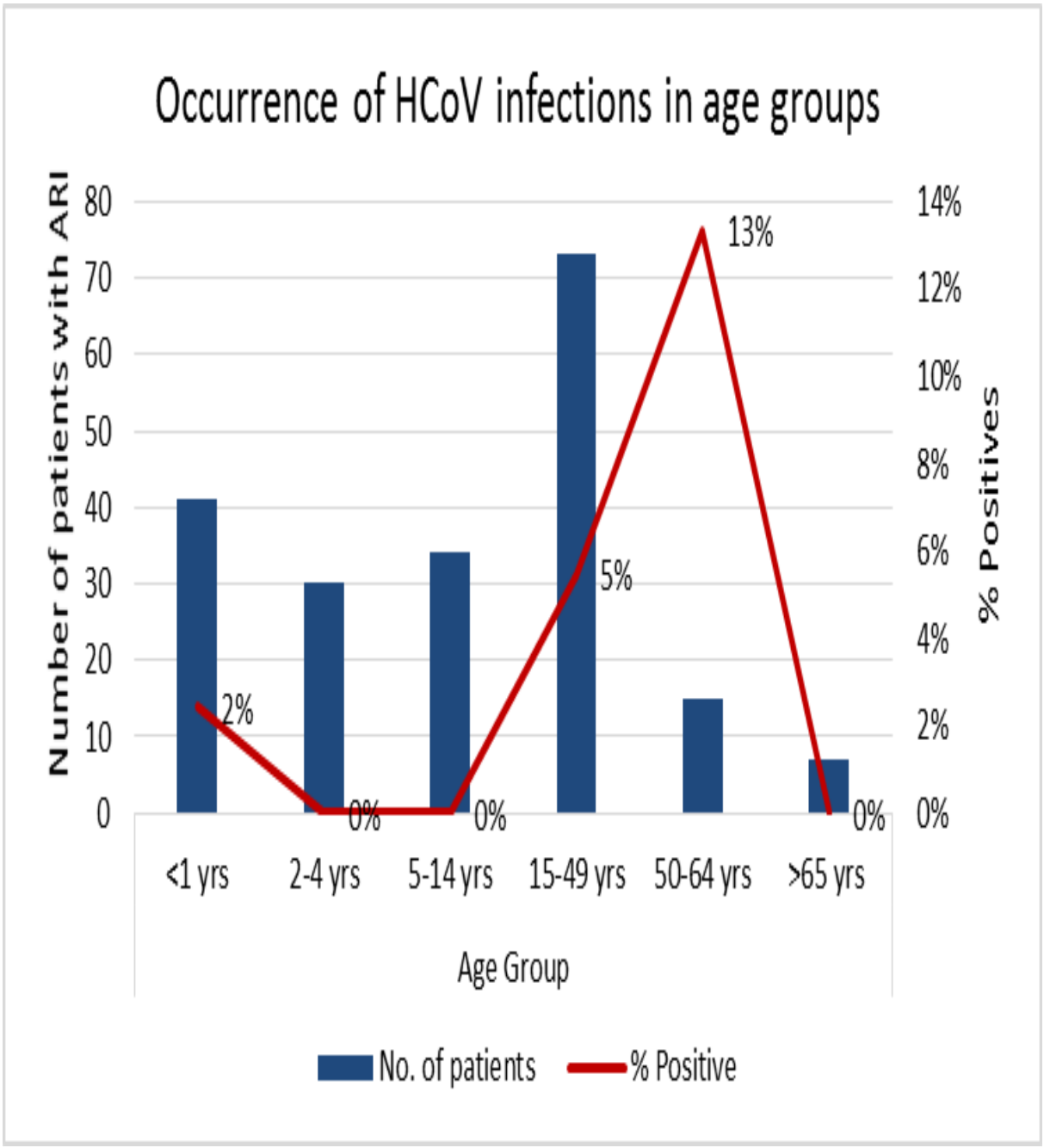

Figure 9. Graph showing occurrence of HCoV infections in the different age groups of ARIs investigated. Detection rate (% positive) was compared amongst age groups.

4.2.0 Hajj samples

4.2.1 Demographic Characteristics

Out of about 5400 pilgrims who participated in the Hajj in Kingdom of Saudi Arabia, a total of 518 combined OP/NP swab specimens were obtained from symptomatic pilgrims who returned to Ghana, at the Kotoka International Airport, Accra. Symptomatic pilgrims enrolled were from all the 10 regions in the country with the largest group of 29% coming from the Greater Accra Region of Ghana. Females accounted for 63% of the total pilgrims. The mean age of the symptomatic pilgrims enrolled was 56 years within a range of 18 – 90 years. About half (52%) of pilgrims were above 55 years (Table 7).

4.2.2 Clinical Characteristics

All enrolled pilgrims manifested at least one respiratory symptom. The most dominant symptoms were cough and sore throat, seen in 90% of the enrolled pilgrims. Twenty four per cent reported having fever (subjective fever) prior to their arrival. A proportion (7%) declared having pre-existing medical conditions which included pregnancy, diabetes, Asthma, hypertension, hepatitis B and tuberculosis (Table 7).

None of the samples were found to be positive for MERS-CoV by rRT-PCR.

Table 7. Demographic and clinical characteristics of symptomatic Hajj Pilgrims screened for MERS-CoV

Variable	Characteristic	Cases of ILI no.(%); N=518
Sex	Male	191 (36.9)
	Female	327 (63.1)
Age Groups	18 – 24	2 (0.4)
	25 – 34	26 (5.0)
	35 – 55	201 (38.8)
	> 55	271 (52.3)
	Not stated	18 (3.5)
Symptoms	Cough	467 (90.2)
	Sore throat	467 (90.2)
	Myalgia	31 (6.0)
	Breathing Difficulty	199 (38.4)
	Headache	43 (8.3)
Pre-existing medical conditions	Pregnant	5 (1.5)
	Diabetic	4 (0.8)
	Asthma	2 (0.4)
	Hypertensive	22 (4.2)
	Hepatitis B	1 (0.2)
	Tuberculosis	1 (0.2)

4.3 Phylogenetic Analysis to classify HCoVs detected

For the seven HCoVs detected, phylogenetic analysis for the N gene with selected sequences of HCoVs with highest identity (lowest expected (E) value) and some reference strains from the GenBank showed that two samples clustered with HCoV 229E and one sample each clustered with OC43 and NL63 respectively (Figure 10). Phylogenetic analysis of the replicase gene also showed three samples clustering with HKU1 human coronaviruses (Figure 11).

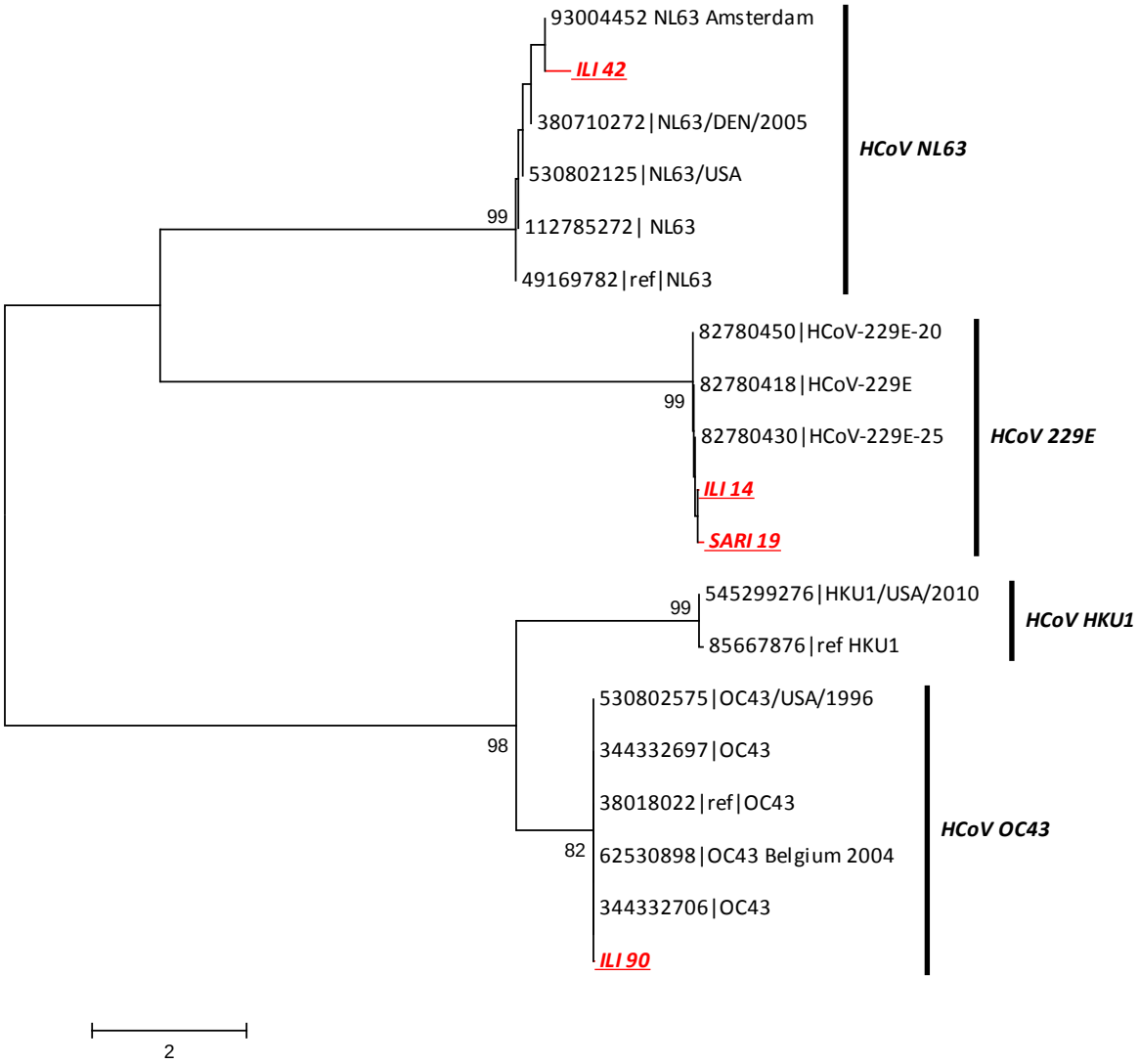

Figure 10. Neighbour-joining phylogenetic tree of N gene sequences. This is based on the multiple alignment of positive samples identified in Ghana, with selected sequences of HCoVs with highest identity (lowest expected (E) value) and some reference strains from the GenBank. Local HCoV strains are identified in red font. Bootstrap values >70% are shown.

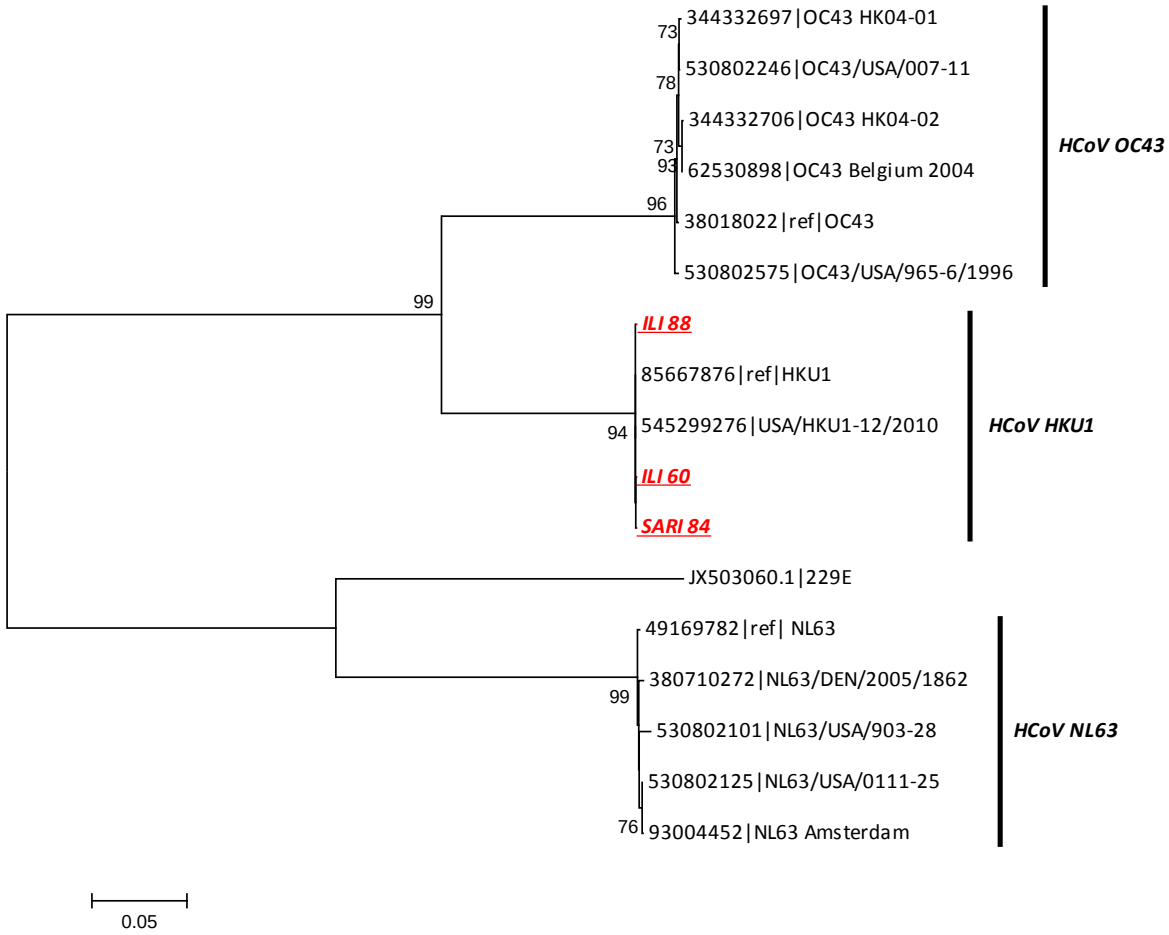

Figure 11. Neighbor-joining phylogenetic tree of relpicase gene sequences. This is based on the multiple alignment of positive samples identified in Ghana, with selected sequences of HCoVs with highest identity (lowest expected (E) value) and some reference strains from the GenBank. Local HCoV strains are identified in red font. Bootstrap values >70% are shown.

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSION AND RECOMMENDATION

5.1.0 DISCUSSION

5.1.1 Identification of HCoV in ARI

The prevalence of HCoV infections in respiratory specimens reported in literature range from 2% to 16% mainly due to differences in study areas and studied populations (Gerna *et al.*, 2006, Lau *et al.*, 2006, Esposito *et al.*, 2006, Garbino et al., 2006, Kuypers et al., 2007, Dare *et al.*, 2007, Vabret *et al.*, 2008, Dominguez *et al.*, 2009, Gaunt *et al.* 2010, Lu *et al.*, 2012, Lepiller *et al.*, 2013, Cabeça *et al.*, 2013a, Cabeça *et al.*, 2013b). In this study, HCoV was detected in 3.5% of archived respiratory specimens from patients with ARI in Ghana which is consistent with the range in literature.

Association of HCoV infections with seasonality have been published in several studies, though there are variations in regional and annual circulation of different HCoV types (van der Hoek 2007, Chiu *et al.*, 2005, Bastien *et al.*, 2005, Lau *et al.*, 2006, van der Hoek 2007 *et al.*, 2005, Vabret *et al.*, 2008, Gaunt *et al.* 2010). These studies have reported HCoV showing winter seasonlity in temperate regions. For this study, though there is not enough evidence to describe seasonality of HCoV in relation to climate in the northern and Southern parts of Ghana ("Ghana Meteorological Agency, Climatology" 2014), data from this study interestingly reveals a circulating trend of HCoVs in Ghana (Figure 8). Detection frequency peaked in the first and second quarters with no detection in the third quarter.

As reflected in the proportion of samples investigated in this study (Table 4), the Greater Accra region has more sentinel sites submitting specimens from ARI patients, 128 out of 200 (64%). Hence, the region had the majority of HCoV cases, 4 out of 7 (57%). Also, the high population density observed in the Greater Accra region (Ghana Statistical Service (2010) may be an important influence to this occurrence as respiratory infections are easily transmitted in populated communities.

All 100 samples from SARI cases had been tested for influenza virus infection out of which only 25 had also been tested for other respiratory viruses by the NIC (data not shown). Similar to findings in children in Washington (Kuypers *et al.*, 2007) where HCoVs were frequently co-detected with human respiratory syncytial viruses, co-infection of HCoV with RSV was detected in one SARI case in this study. This finding though, not providing strong evidence to describe co-infection of HCoV with other respiratory viruses in Ghana is in accord with previous studies reporting HCoV co-infection with other respiratory viruses (Glezen *et al.*, 2000, Dominguez *et al.*, 2009, Esposito *et al.*, 2006, Yu *et al.*, 2012, Gaunt *et al.* 2010, Lu *et al.*, 2012, Jevšnik *et al.*, 2012, Cabeça *et al.*, 2013a, Lepiller *et al.*, 2013). To better describe this phenomenon, all samples must be tested for other viral agents too.

A higher rate of HCoV detection was reported among adults in the 50- to 59-year range (Cabeça *et al.*, 2013a). Similarly, this study detected HCoVs more frequently among the adult age groups (Figure 9), though HCoV is considered one of the most common respiratory viruses associated with respiratory tract infections in children (Dominguez *et al.*, 2009, Esposito *et al.*, 2006, Talbot *et al.*, 2009, Kuypers *et al.*, 2007). The highest number of HCoV positive cases was recorded for the 50-64 years age group (Figure 9).

Consistent with studies in France and Slovenia (Vabret *et al.*, 2008, Jevšnik *et al.*, 2012), HCoV-HKU1 could be the predominant circulating type of HCoV in Ghana. Studies have suggested that the distributions of HCoV types vary between age groups (Lepiller *et al.*, 2013, Dijkman *et al.*, 2012). Human CoV-229E and HKU1 were observed to have predominated in adults (Lepiller *et al.*, 2013). This may explain the higher detection rate observed in adults than children in this study as HKU1 followed by 229E were the two most dominant types detected (Table 6).

As shown in Table 4, HCoVs were detected more frequently in ILI cases (2.5%) than in SARI case (1%). This correlates with the circulation of all four HCoV types detected in this study in ILI cases (Table 6). The low detection rate in SARI cases may suggest the involvement of other respiratory pathogens resulting in the patients requiring hospitalization. Also since most SARI cases may involve lower respiratory tract infections and pneumonia, OP/NP swabs may not be the appropriate specimens to describing the aetiology of these illnesses. The low HCoV detection rate in the hospitalized patients is similar to reports by in Brazil (Cabeça *et al.*, 2013a; Cabeça *et al.*, 2013b). Also it is similar to unpublished reports from the NIC where the detection of influenza viruses were lower in SARI cases.

A study in France reported URTI as the most common clinical presentation during HCoV infections (Lepiller *et al.*, 2013). In this study, all HCoV positive samples were from patients with URTI which confirms the contribution of HCoV as a major viral agent in URTI. Clinical signs reported in HCoV associated ARI included fever, cough, sore throat and headache, which is consistent with other studies (Makela *et al.*, 1998, Vabret *et al.*, 2003, Greenberg 2011, Lu *et al.*, 2012, Lepiller *et al.*, 2013).

5.1.2 Presence of MERS-CoV ARI in Ghana

Negative findings for MERS-CoV in archived respiratory specimen from hospitalized patients with ARI is indicative that the virus has not been circulating in Ghana. These samples were collected from November 2013 to March 2014, a period that coincided with the period of increase in number of cases of MERS-CoV reported to the WHO. The objective of screening for MERS-CoV in SARI samples fulfils the WHO recommendations for retrospective testing of respiratory specimens from patients with unexplained respiratory disease ("WHO Guidelines for Investigation of Cases of Human Infection with Middle East Respiratory Syndrome Coronavirus" 2013). There are serologic evidence suggesting the circulation of MERS-CoV or MERS-like CoV in dromedary camels well before March 2012 in Nigeria and other countries including Eastern and Northern African countries (Reusken *et al.,* 2014, Alagaili *et al.,* 2014, Meyer *et al.,* 2014). However, these cannot establish circulation of MER-CoV in humans in West Africa in the absence of serological evidence as well as viral detection in humans, of which the negative findings in this study in Ghana is consistent with.

Negative findings for MERS-CoV from pilgrims with respiratory symptoms suggests that MERS-CoV was not widely circulating during the 2013 Hajj though it's high incidence in the Middle East. This support reports from a similar study that showed a lack of nasal carriage of MERS-CoV among Hajj pilgrims (Gautret et al. 2013, Gautret et al. 2014, Memish *et al.,* 2014). The absence of MERS-CoV from these pilgrims with respiratory symptoms was a relief as it had been feared that returning Hajj pilgrims would pose a considerable risk to introduce this new virus into Ghana. Though there was no detection of MERS-CoV in respiratory specimens from pilgrims with respiratory symptoms, the NIC

detected influenza viruses in 29 (5.6%) of these pilgrims, suggesting it may play a role in respiratory tract infections among the Hajj pilgrims. The WHO recommends lower respiratory specimens such as sputum, endotracheal aspirate or bronchoalveolar lavage for molecular diagnosis of MERS-CoV (" WHO, Laboratory Testing for Novel Coronavirus Interim Recommendations" September, 2013). However, the use of combined OP/NP swabs in this study was in accordance with methods used in detecting the virus (Assiri *et al.*, 2013). So far, several studies have provided valuable information about MERS-CoV infections. However, some questions still remains unanswered about this new virus. Further investigations which can be achieved by continued surveillance for acute respiratory infections are required to better understand the transmission patterns of this virus.

5.1.3 Characterization of HCoVs circulating in Ghana

Phylogenetic analysis for N gene sequences showed 2 samples clustering with HCoV 229E, 3 clustering with HKU1 and 1 sample each clustering with NL63 and OC43. This corresponds to and confirms results obtained from the rRT-PCR. Phylogenetic analysis was performed separately for samples amplified in the N gene (HCoV-229E, OC43, NL63) and samples amplified in the replicase gene. Three genotypes of NL63 (Dominguez *et al.*, 2012) and HKU1 (Woo *et al.*, 2006) have been identified in phylogenetic full length genome analysis. However, partial gene sequence obtained for N gene of HCoV-NL63 and replicase 1b gene of HKU1 did not enable genotyping of these HCoVs identified in this study.

5.2 LIMITATIONS

The selection of ILI samples that were negative for influenza infections may have underestimated the prevalence of coronaviruses and excluded the detection of co-infections with influenza viruses. Also the study did not allow association of described clinical symptoms with only HCoV infections as infections by other respiratory pathogens were not ruled out. The profile of HCoV could not be fully described as this requires a systematic collection of samples over a long period of time.

5.3 CONCLUSION

This study has shown that HCoVs are uncommonly associated with ARI in Ghana, though clinical symptoms of cases cannot be exclusively attributed to HCoV infection. The results from this study provides some insight into the epidemiology and clinical knowledge of human coronavirus infections in the Ghanaian population. And also providing evidence of associating HCoVs with ARI. Negative findings for MERS-CoV in archived respiratory specimen from hospitalized patients with ARI and in pilgrims with respiratory symptoms indicates that the virus may not be circulating in Ghana and not widely circulating during the 2013 Hajj though it had a high incidence in the Middle East. Phylogenetic analyses have shown that HCoVs detected in this study cluster with their respective reference strains.

5.4 RECOMMENDATIONS

The NIC's platform for influenza virus surveillance could be used to monitor HCoVs as well as other respiratory viruses. In addition, a more comprehensive study with systematic sample collection over a longer period to establish seasonal pattern of HCoVs should be

carried out. Low detection of HCoV in hospitalized patients with ARI indicates, other pathogens may be implicated in the illness. There is therefore the need to investigate the role of these other respiratory pathogens in ARI. Surveillance for MERS-CoV should continue and be enhanced. Since the WHO anticipates additional cases of MERS-CoV infection from the Middle East, and the likelihood of increased exported cases to other countries by travellers or pilgrims.

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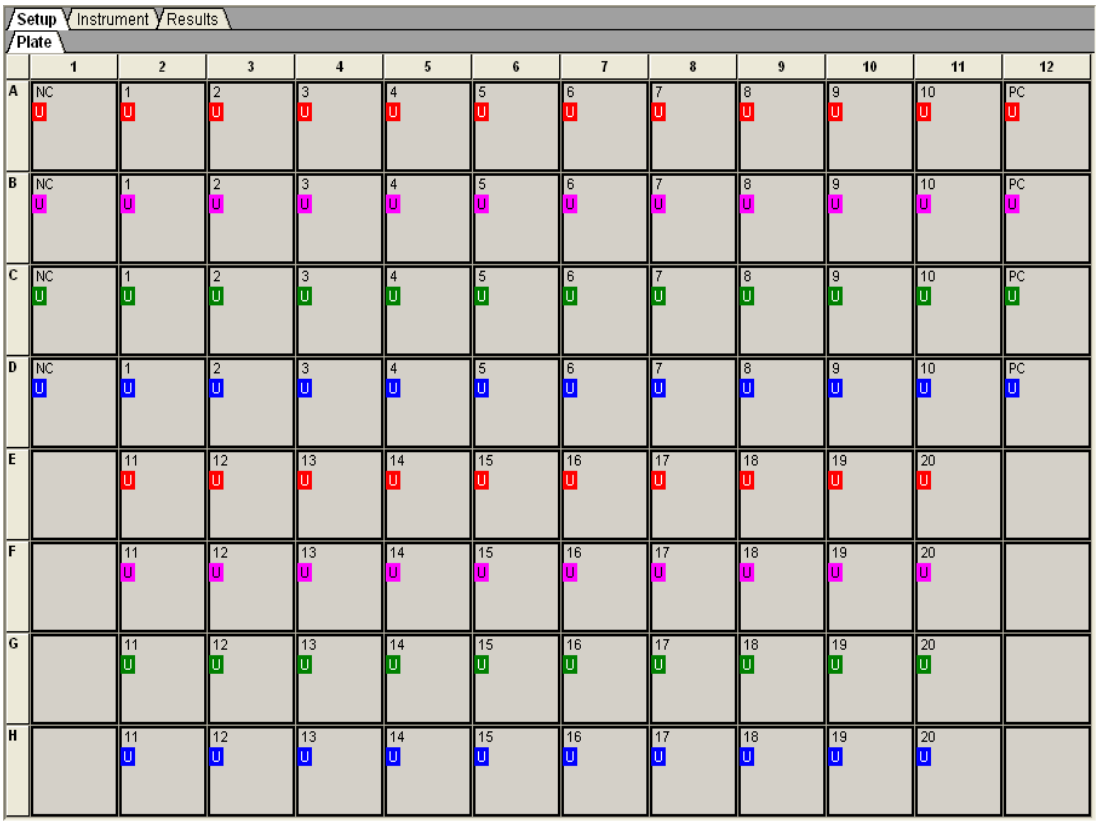
APPENDICES

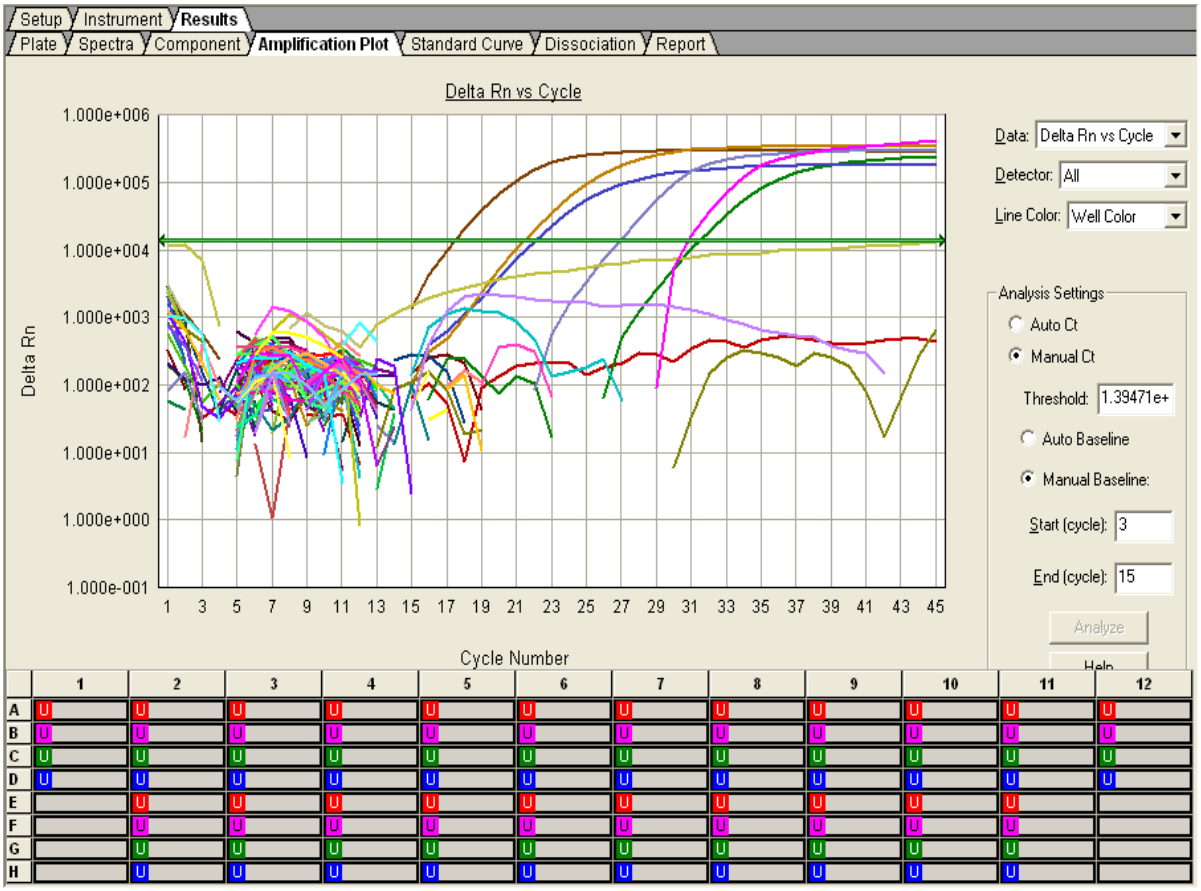
Appendix A: Screen shot of rRT-PCR plate set up for HCoV; 229E, OC43, NL63, HKU1

Plate set up for testing some (20) samples with test controls. For this plate, each specimen was tested for 229E (red), OC43 (violet), NL63 (green) and HKU1 (blue).



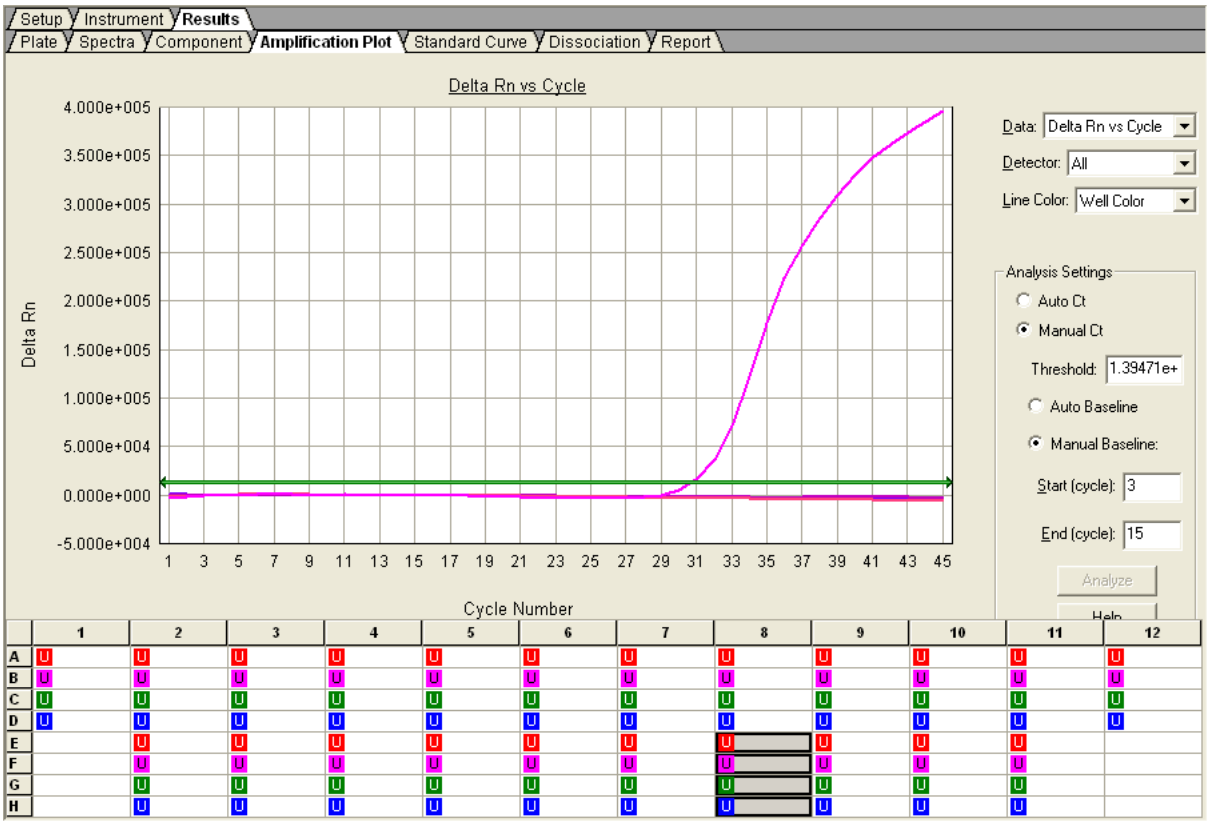
Appendix B: Screen shot of amplification plot of rRT-PCR for HCoV; 229E, OC43, NL63, HKU1

Amplification plot showing results for test performed for 20 samples with controls. Four positive controls (for 229E, OC43, NL63, HKU1) and two samples showing positive results (exponential curve) for amplification.



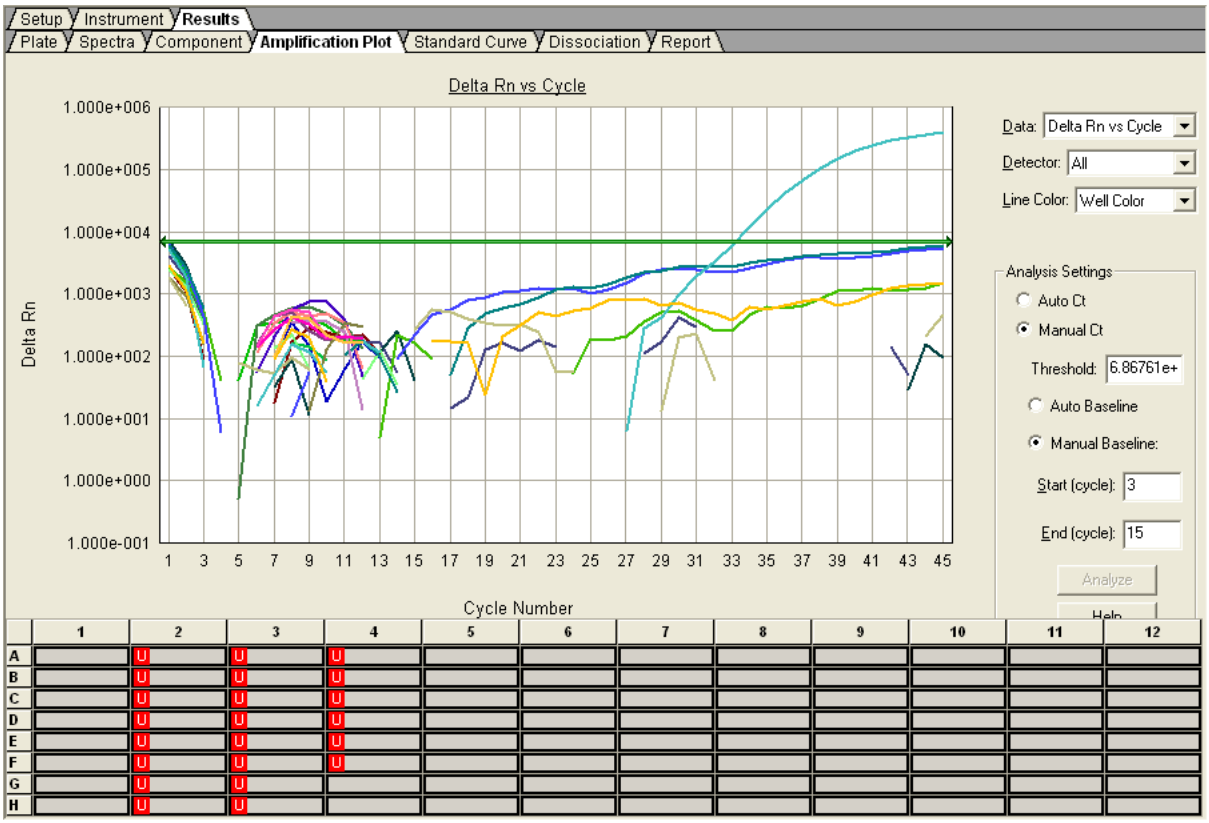
Appendix C: Screen shot of linear amplification plot of rRT-PCR for a positive sample

Linear amplification plot for a sample (highlighted) being tested for four targets; 229E, OC43, NL63, HKU1. This sample shows a typical exponential (positive) curve for one of the four targets.



Appendix D: Screen shot of amplification plot of rRT-PCR for MERS-CoV

Amplification plot for some samples tested with UpE assay for MERS-CoV. All samples tested negative by the UpE real-time assay. Postive control onbatained from the Institute of Virology, University of Bonn Medical Centre, Germany, yielding appropriate results (exponential curve with logarithmic, linear, and plateau phases).



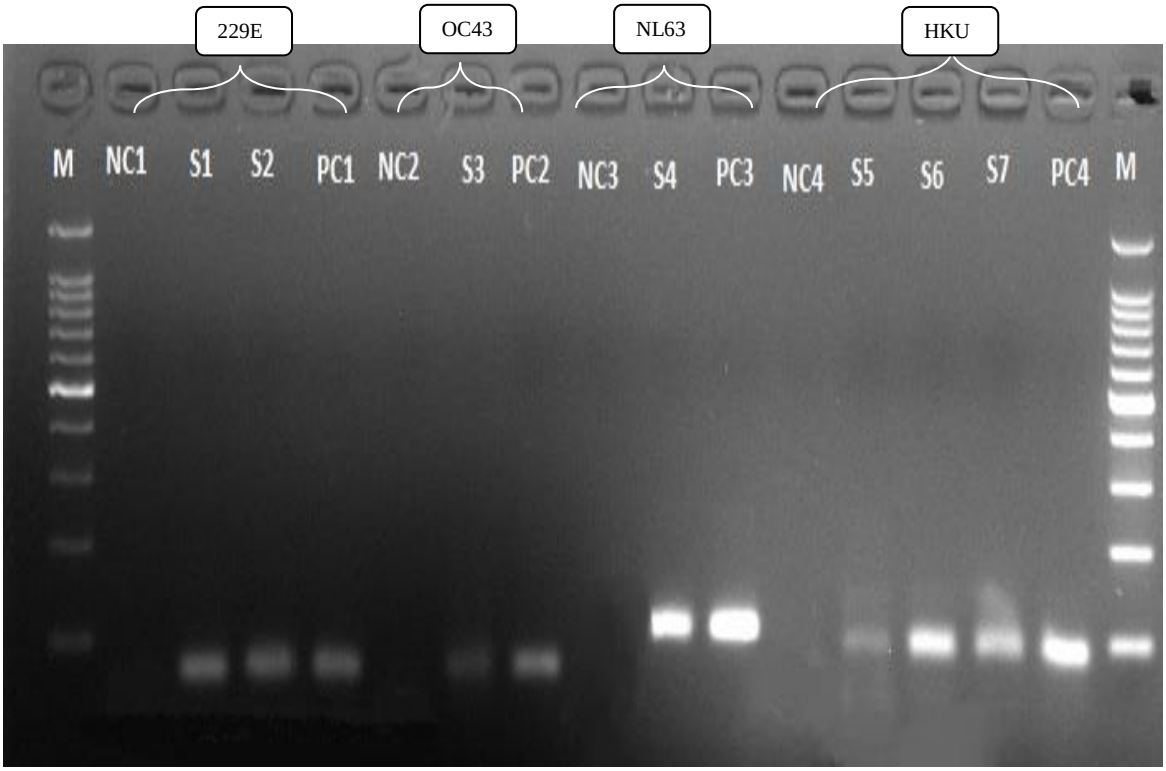
Appendix E: Table showing results (Ct values) for positive cases

Table showing results (Ct values) of 7 samples that were HCoV positive. A cut-off Ct value ≤37 was used.

ID	Ct Value	HCoV Type
ILI-014	32.83	229E
ILI-042	28.10	NL63
ILI-060	34.38	HKU1
ILI-088	31.28	HKU1
ILI-090	32.07	OC43
SARI-019	29.32	229E
SARI-084	29.70	HKU1

Appendix F: Gel photograph of 7 positives samples amplified

Gel photograph of 7 positives amplified: M; 100 base pair marker, PC; Positive control, NC; Negative control, S; samples .S1 and S2 were amplified with HCoV 229E primers. S3 was amplified for OC43. S4 was amplified for NL63. S5, S6, S7 were amplified for HKU1.



Appendix G: Picture, extraction of RNA from clinical samples.

All clinical Samples were handled under Biosafety Level 2 (BSL2) practices and procedures.

Appendix H: Picture, preparation of Master Mix for rRT-PCR.

Master mixes were prepared in a segregated (clean) area to prevent contamination and ensure integrity of results.

Appendix I: Picture, loading samples into thermal cycler for conventional RT-PCR