ISOLATION AND MOLECULAR CHARACTERIZATION OF HUMAN METAPNEUMOVIRUS FROM CHILDREN WITH ACUTE RESPIRATORY TRACT INFECTIONS AT KENYATTA NATIONAL HOSPITAL, KENYA IN 2008

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Isolation and Molecular Characterization of Huma Metapneumovirus from Children with Acute Respiratory Tract Infections at Kenyatta National Hospital, Kenya in 2008

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

Signature…… Date……..

Sammy Nyongesa. Sichangi

This thesis has been submitted with my approval as university supervisor.

Signature…… Date……..

Prof Zipporah Ng'ang'a
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DEDICATION

I dedicate this work to my parents Patrick and Hellen Sichangi, my brothers Michael and Jimmy, for their love and support always.

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LIST OF ABBREVIATIONS / ACRONYMNS

aa	amino acid
APV	Avian Pneumovirus
ARTI	Acute Respiratory Tract Infections
BAL	Bronchoalveolarlavage
BLAST	Basic Local Alignment Search Tool
bp	base pairs
cDNA	complementary DNA
DFA	Direct Fluorescent Antigen
EIA	Enzyme Immuno Assay
Gp	glycoprotein
hMPV	human Metapneumovirus
hRSV	Human Respiratory Syncytial Virus
IV	Influenza Virus
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KEMRI	Kenya Medical Research Institute
KNH	Kenyatta National Hospital
LMICs	Low and medium income countries
LRTI	Lower Respiratory Tract Infection
MK	Monkey Kidney cells
NCBI	National Centre for Biotechnology Information
NL-N	Nucleoprotein gene of hMPV

NPA	Nasopharyngeal aspirates
nt	nucleotide
PIV	Parainfluenza Virus
RNA	Ribonucleic Acid
RNP	Ribonucleoparticle
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SARS	Severe Acute Respiratory Syndrome
under-5s	children under five years.
URTI	Upper Respiratory Tract Infection
UN	United Nations
VTM	Virus Transport Medium
WHO	World Health Organization

ABSTRACT

Human metapneumovirus (hMPV) is a respiratory viral pathogen that is associated with acute respiratory tract infections (ARTI) in very young children, elderly persons, and immunocompromised patients. Two subtypes of hMPV exist (A and B) which are further divided into subgroups (A1 and A2, and B1 and B2). South Africa is the only African country where hMPV has been isolated and studied Important questions remain on the contribution of hMPV to ARTIs in Kenya .This study aimed at to detect and study the molecular characterization of hMPV from children with ARTI at KNH, in 2008. One hundred and fifty archived nasopharyngeal aspirates from hospitalized children below 5 years with ARTI at Kenyatta National Hospital in 2008 were used. The samples were cultured on LLC-MK2 cells and viral RNA extracted from CPE positive cells. Conventional PCR and sequencing was done for F and G genes. Phylogenetic analysis of the F gene was done by neighbor-joining algorithm using MEGA version (5.02). Of the 150 samples, 7 (4.67%) were PCR positive for both F and G gene proteins. Pylogenetic analysis of the F gene showed that two subtypes; A2b and B2 were in circulation. Subtype A2 accounted for 83.3 % of the isolates. All the Kenyan isolates were closely related to the Chinese isolates suggesting that they had a common origin. The detection of hMPV suggests its involvement in pediatric pneumonia in Kenya The presence of two subtypes (A2 and B2) during the same epidemic period (2008) might suggest that the epidemic was shifting to favor either A2 or B2 strains in the subsequent seasons

CHAPTER ONE

1.0 INTRODUCTION

1.1 The global burden of acute respiratory infection

Acute Respiratory Tract Infection (ARTI) is a serious life-threatening and death-associated illness among pediatric patients worldwide and particularly in developing countries (Williams *et al.*, 2002). Pneumonia kills more children than any other illness, more than AIDS (Acquired Immune Deficiency Syndrome), Malaria and Measles combined (WHO, 2006). Acute respiratory tract infections are the most common illness regardless of age or gender (Monto, 2002). For children below five years old (under-5s), respiratory tract infections are ranked as the second leading cause of death regardless of the geographical region (Murray *et al.*, 2001). It is estimated that more than 150 million episodes of pneumonia occur every year among under-5s in developing countries, accounting for more than 90 percent of all new cases worldwide. In these countries, acute pneumonia is responsible for 19-23% of deaths among this age group (Black *et al.*, 2003), over 0.8 million (42%) of these ARI-associated deaths occur in Africa (WHO/UNICEF, 2006). Another 0.5 million children die in India, contributing 25% of the annual global ARTI deaths (Williams *et al.*, 2002).

1.2 The burden of pediatric respiratory illness in developing countries

The incidence of pneumonia in under-5s in industrialized countries is estimated at 0.05 episodes per child-year. In contrast, the incidence in low-and-middle-income countries (LMICs) is approximately 0.3 episodes per child-year, which translates into over 150 million new episodes annually (Rudan *et al.*, 2008). The regions with the highest

incidence are South-East Asia and sub-Saharan Africa (SSA). The incidence varies with the prevalence of several risk factors, these include; malnutrition, low birth weight, non-exclusive breastfeeding, indoor air pollution, and crowding (WHO/UNICEF, 2006). The incidence also varies with age and is higher in infants than in toddlers, i.e. young children less than 12 months old.

There are major differences between developed and developing countries that have impacted on the burden of pediatric ARTI. In the developing countries, children account for a relatively higher proportion of the population. In 2005, children under 15 years of age comprised approximately 31% of the total population in developing countries, compared to 17% in developed nations (UN, 2005)

The prevalence of some infectious agents may differ as a result of socioeconomic, medical or geographic factors. For example measles occurs almost exclusively in developing countries (Bryce *et al*., 2005). Similarly the incidence of severe pneumococcal disease including pneumonia has declined substantially in developed countries with routine immunization of young children after the introduction of the (PCV 7 and PCV 13) pneumococcal conjugate vaccines. (Whitney *et al*., 2003; Kyaw *et al*., 2006)

The pediatric HIV epidemic is now largely confined to developing countries especially those in sub-Saharan Africa, where almost 2 million HIV-infected children live (UNAIDS, 2006). The impact of the HIV epidemic on childhood respiratory illness has been compounded by poor access and unavailability of preventative strategies and limited availability of highly active antiretroviral therapy (HAART) in these countries.

As a result, HIV-associated lung disease is a major cause of childhood morbidity, hospitalization and mortality in sub-Saharan Africa with 90% of HIV- infected children developing respiratory illnesses during the course of their HIV disease (ZarHJ, 2004).

The contribution of tuberculosis (TB) to childhood pneumonia has also been demonstrated. Despite the fact that the burden of childhood tuberculosis is difficult to quantify, it is estimated to account for 15-20% of the TB caseload in developing countries, where the major global TB burden exists (Donald *et al.*, 2002).

Socio-economic factors including nutrition impact on child lung health, influencing the epidemiology and severity of the illness. Co-morbid malnutrition occurs in almost half of all children dying from pneumonia (Bryce *et al*., 2005).

Environmental determinants of child lung health for example overcrowded living conditions, exposure to biomass fuel or passive smoke exposure may be risk factors that are more prevalent in the developing country settings (Monto *et al.*, 2002).

1.3 Etiological agents associated with childhood pneumonia

Different studies have demonstrated the etiology of ALRI as complex (Ekalaksananan *et al.*, 2001; Noyala *et al*., 2005). The etiologic agents involved in the occurrence of this infection are bacteria, viruses and fungi.

Although the clinical manifestations of respiratory tract disease are easily recognized, the etiological agent responsible for disease is often not identified. Even with the

advancement of diagnostic tools, the causative agent of about 30 % of respiratory tract infections go undiagnosed (Davies *et al.*, 1996). In nearly half of upper respiratory illnesses (URI) in children, an infectious cause cannot be determined (Nokso *et al.*, 2002). The etiology of a majority of LRTI is thought to be viral (File *et al.*, 2003), yet in only 40% of cases can a viral agent be identified (Louie *et al.*, 2005).

The major bacterial agents for pediatric pneumonia are *Streptococcus pneumoniae* (SPN) and *Haemophilus influenzae* type b (UNICEF/WHO, 2006). Other bacterial pathogens include; *Mycoplasma pneumoniae, Staphylococcus aureus, Legionella pneumophila* and *Chlamydophila pneumoniae*.

Studies done in recent years have demonstrated the increased role of viral etiologic agents of pneumonia among pediatric patients (Costa *et al.*, 2006). Of all the common respiratory viruses causing a wide range of illnesses, from mild infections of the upper respiratory tract to pneumonia, respiratory syncytial virus (RSV) undoubtedly causes the most severe illness and is responsible for a large proportion of hospitalizations in infants and young children in industrialized countries (Kim *et al.*, 2000; Straliotto *et al.*, 2002; Iwane *et al.*, 2004). Human parainfluenza viruses (PIV), particularly types 1, 2 and 3, are second to RSV in causing severe viral lower respiratory infection in children (Girard *et al.*, 2005). PIV involve the lower airways less frequently than RSV (Iwane *et al.*, 2004). The difference between hospitalization rates for RSV and PIV is particularly striking for the first six months of life. Seasonal influenza also causes a significant number of acute respiratory infections, including pneumonia, among children (McIntosh *et al.*, 2002). Other pneumonia viral etiologic agents include:

Adenovirus (AdV), Human Rhino Virus (HRV), hMPV (Human Metapneumovirus), human Corona virus (hCoV) and human Boca virus (hBoV). These viruses can form dual or triple infection (Kim *et al*., 2000, Chkhaidze *et al*., 2006) or result in co-infections with other bacterial etiologic agents of pneumonia in children (Nacul *et al*., 2005). Respiratory viruses have been shown to play an important role in the pathogenesis of pneumonia by predisposing to secondary bacterial infections, a feature especially associated with influenza virus types A and B (McCullers *et al*., 2006).

In general, the true burden of the various organisms causing pneumonia is inadequately documented in LMICs due to lack of surveillance systems and diagnostic facilities (Madhi *et al*., 2008).

1.4 Human Metapneumovirus

Human metapneumovirus is a recently discovered etiological agent of ARTI. It was for the first time detected in the Netherlands from nasopharyngeal aspirates taken over a 20-year period from 28 hospitalized children and infants with respiratory tract infections who had signs and symptoms similar to those of RSV infection (Van den Hoogen *et al*., 2001). Earlier recognition of hMPV was delayed because it has been difficult to detect in cell culture due to its slow growth and mild cytopathic effect (CPE). This virus was distinct from common respiratory viruses, as immunological assays using virus-specific antibodies and PCR-based methods using virus genome-specific primers failed to identify this agent.

A lot of progress has been made in the molecular detection of hMPV (Cote *et al.*, 2003; Ebihara *et al*, 2004; Leung *et al.*, 2005). This has led to a better understanding of its genetic diversity (Boivin *et al*., 2002; Ludewick *et al.*, 2005) and in documenting its varied clinical presentation (Falsey *et al*., 2003; Principi *et al*., 2006). The genetic characterization of this agent remained a mystery until the tools of molecular biology were applied to identify portions of the genomic sequence. Using randomly primed PCR, Dutch researchers were able to obtain genomic sequence of this novel pathogen. Based on limited sequence data, this virus appeared to be closely related to the avian pneumovirus, a member of the *Metapneumovirus* genus, and it was called Human Metapneumovirus (van den Hoogen *et al*., 2001). Like RSV, hMPV has two types, A and B, and each type comprises two subtypes (A1, A2, B1 and B2). The hMPV types and subtypes have been differentiated phylogenetically (van den Hoogen *et al.*, 2001).

Human Metapneumovirus has continuously been detected from patients with respiratory diseases in different countries and continents, suggesting that hMPV may be circulating worldwide (Freymuth *et al*., 2003; Peret *et al*., 2002). hMPV is now recognized as an important causative agent of ARI in children, both in the community and in hospitalized cases. The rate of hospitalization for hMPV infections is lower than for RSV infections but higher than that observed for influenza and PIV viruses (Principi *et al*., 2006). Available data shows that hMPV is responsible for approximately 5-8% of ARI hospitalizations and 2-6% of community ARI cases in the under-5 in industrialized countries (Van den Hoogen *et al*., 2004). Hospital- based studies of the under-5 in LMICs have shown similar occurrence (Noyola *et al*., 2005), but few studies report on hMPV pneumonia in the community (Brooks *et al*., 2007).

1.5 Clinical features of Human Metapneumovirus infection

Most hMPV infections occur in the under-5, with children less than 2 years of age being the most at risk for serious infections. It causes a variety of clinical syndromes in children that are typical of the paramyxoviruses, including upper and lower respiratory tract illnesses. The clinical characteristics of hMPV infections are not distinctive; it is therefore not possible to differentiate it from other respiratory viruses on clinical grounds (Stockton *et al.,* 2002). In a study conducted at Vanderbilt University Medical Center on the association of the virus in a cohort of 2000 subjects of age 0–5 years, followed during a 25-year period revealed that hMPV was associated with common cold (complicated by otitis media in one third), bronchiolitis, pneumonia, croup and exacerbation of reactive airway disease (Williams *et al.,* 2004). Hoarseness has also been observed more common in hMPV than RSV infections (Falsey *et al.,* 2003). When compared with RSV, infection with hMPV tends to occur in slightly older children. Co-infection with both viruses is generally associated with worse disease (Semple *et al*., 2005). In some studies in children with asthma, hMPV was found more frequently than RSV (Peiris *et al.,* 2003). Acute wheezing and asthma exacerbations have been associated with hMPV infection (Jartti *et al.,* 2002). Arabpour *et al* (2008) studied the common signs, symptoms and clinical diagnosis in children hospitalized with hMPV in India. Common manifestations associated with *paramyxoviridae* infections such as RSV and measles were notably present as shown in Table 1.5

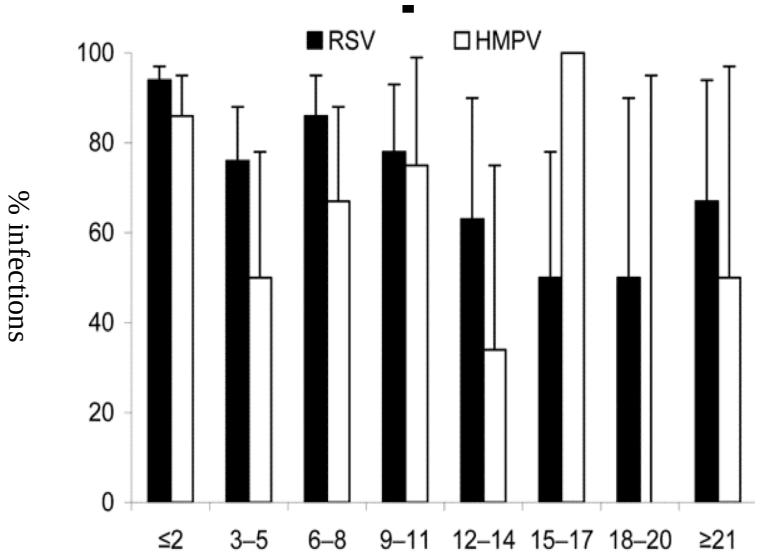
Table 1.5: Distribution of signs, symptoms and clinical diagnosis in children with Human Metapneumovirus.

Clinical diagnosis	**Symptoms**	**Signs**
Pneumonia 9 (13.2%) Bronchiolitis 19 (27.9%) Bronchitis 14 (20.6%) Laryngitis 8 (11.8%) Croup (Laryngio trachio bronchitis) 4 (5.9%)	Cough 96.5% Coryza 82.4% Fever 75% Irritability 69.1% Wheezing 64.7% Aneroxia 53% Vomiting 17.6% Diarrhoea 13.2%	Ronchi 73.5% Rhinitis 72.1% Pharyngitis 52.9% Abnormal tympanic pain 32.4% Rales 11.9%

(Adapted from Arabpour *et al.*, 2008)

1.6 Human Metapneumovirus and associated co-infections in children

The seasonal distributions of hMPV and RSV have been shown to overlap, therefore potential for dual infection exists. Several studies have found co-infection rates greater than 10% (Viazov *et al.*, 2003). There is however an increase in cases of hMPV and other respiratory tract viruses. This might be due to increased use of molecular techniques for diagnosis e.g real time PCR (Kehl *et al*., 2001). The rate of hMPV and RSV coinfections in children below 2 years of age is illustrated in figure 1.6



Source (www.cdc.gov)

Figure 1.6: Co-infections of RSV and hMPV in children in Yemen, 2006

This study showed co-infections to be common up to about two years of age. RSV accounted for most cases of RTI in the children, however hMPV was responsible for a higher percentage of infections in children between the ages of 15 to 17 months. Some patients with severe acute respiratory syndrome (SARS) have been found to be positive for hMPV, although it is not clear whether infection with hMPV aggravates illness in patients with SARS or whether its presence is mere coincidence (Ebihara *et al*., 2003).

hMPV infection can be severe since the virus has been isolated from the lungs of a previously healthy man who died from acute pneumonia (Rohde *et al*., 2005). There are reports that associate hMPV infection with encephalitis (Schildgen *et al*., 2005)

and in one example hMPV was detected in a patient who died of complications associated with encephalitis (Hata *et al*., 2007). In this case, viral RNA was detected in both the brain and lung tissues, suggesting that under certain circumstances, the virus can leave the confines of the respiratory airway and infect other tissues.

1.7 Pathogenesis Human Metapneumovirus Infection

Based on the model of infection of *paramyxoviridae* in the host cell, hMPV infection involves the interaction of viral G (attachment) protein with a molecule or molecules on the host cell surface (Levine *et al.*,1987). Cellular glycosaminoglycans or heparin-like molecules are involved in virus attachment and entry (Feldman *et al.*, 2000). Following G-protein attachment, the F (Fusion) glycoprotein promotes pH-independent fusion between the cell membrane and the virus envelope. The F protein is then cleaved by cellular proteases thus becoming functional. The hydrophobic amino terminal region of the F1 component triggers the fusion process, which introduces the internal components of the virion into the cytoplasm of the host cell, where the remainder of the infectious cycle takes place.

Animal models using primates (*Cynomolgus* and *Rhesus macaques*, African green monkeys) and rodents (hamsters, cotton rats, mice) have shown that hMPV replicates in the upper and lower respiratory tracts. Peak viral titers are found around day 4–5 after infection and decrease thereafter, (Alvarez *et al.*, 2004; Kuiken *et al.*, 2004, Skiadopoulos *et al.*, 2004). Hamelin *et al*, (2005), has demonstrated that hMPV may present an initial biphasic replication pattern in lungs of BALB/c mice, with hMPV

RNA still detectable more than 180 days after infection. Such persistence could be explained by an aberrant T helper cell type 2-like immune response, with impaired virus clearance after primary hMPV infection (Alvarez and Tripp, 2005). Increase in many cytokines and chemokines such as interleukin IL-2, IL-8, IL-4, INF-γ, macrophage inflammatory protein 1α and monocyte chemotactic protein has been observed in the lungs or bronchoalveolar lavage (BAL) of both mice and cotton rats in response to hMPV challenge (Alvarez *et al.,* 2004; Hamelin *et al.,* 2005).

In humans, hMPV infection has also been associated with an increase of IL-8 in upper respiratory tract secretions and with chronic inflammatory changes of the airways, with presence of, intra alveolar foamy and hemosiderin-laden macrophages (Vargas *et al.,* 2004). When compared with RSV, hMPV infection in humans seem to induce lower levels of inflammatory cytokines such as IL- 12, tumor necrosis factor α, IL-6 and IL-1β (Laham *et al.,* 2004).

1.8 Epidemiology of Human Metapneumovirus

Since its initial description in 2001 (Vargas *et al.,* 2004), hMPV has been isolated from individuals of all ages with ARI, and has been identified in every continent as shown in table 1.8. Most of these studies have been conducted in the temperate regions, this is largely due to the availability of sensitive equipment such as real time PCR. In some areas, the virus has been detected through out the year (Freymuth *et al.,* 2003). The virus is therefore not endemic in the temperate regions alone (Percivalle *et al.,* 2005). Most of these studies have been conducted on pediatric patients who are the

most vulnerable.

Table 1.8: showing studies conducted in different geographical regions

Geographical region	Studies
Europe	United Kingdom (Stockton *et al.*, 2002) Finland (Jartti *et al.*, 2002) Italy (Maggi *et al.*, 2003) France (Freymuth *et al.*, 2003) Germany (Viazov *et al.*, 2003) Spain (Vicente *et al.*, 2003) Norway (Dollner *et al.*, 2004).
North and South America	Canada (Boivin *et al.*, 2002) United States (Esper *et al.*, 2003, Falsey *et al.*, 2003) Brazil (Cuevas *et al.*, 2003)
Australia	(Nissen *et al.*, 2002)
Asia	HongKong (Peiris *et al.*, 2003) Japan (Ebihara *et al.*, 2004) Thailand (Samransamruajkit *et al.*, 2005) India (Rao *et al.*, 2004) Korea (Kim and Lee, 2005).
Africa	South Africa (Ludewick *et al.*, 2005).

In young hospitalized children hMPV has been detected in 5-10% of ARTI cases. (Peret *et al.*, 2002, Bastein *et al.*, 2003, Bovin *et al.*, 2003). However, the rates of hospitalizations due to hMPV infection have been shown to vary from 7-43% (Maggi

et al., 2003). The rates of detection of hMPV in adults are usually lower than children with rates of about 3% in the community (Stockton *et al.,* 2002). It is interesting to note that detection rates of hMPV have generally been higher in retrospective studies than in prospective studies, an observation consistent with a degree of selection bias (Hamelin *et al.,* 2004).

1.9 Seroprevalence of Human Metapneumovirus

Sera taken from patients in the late 1950s showed the presence of hMPV-specific antibodies. This retrospective study suggested that hMPV was not a new virus, but had been circulating in the human population for at least 50 years prior to its isolation in the Netherlands (Sugrue *et al*., 2006). The seroprevalence of hMPV-specific antibody in infants below 3 months of age is over 90%, indicating that maternally derived antibodies are present in young children (Kahn *et al***.,** 2006).

1.10 Seasonality of Human Metapneumovirus and other respiratory viruses

Infections with respiratory viruses exhibit distinct seasonal patterns in most temperate regions. Typically, RSV and influenza cause annual recurrent well-defined epidemics during the cold months (Stensballe *et al*., 2003). The activity of hMPV has been shown to be greatest in winter and spring in the northern hemisphere (Van den Hoogen *et al*., 2004) and autumn through spring in the southern hemisphere (Lambert *et al*., 2007), but data are still limited as year-round surveillance has not been extensively undertaken. There are reports suggesting a biennial epidemic pattern of early and late

hMPV occurrence in several European countries (Weigl *et al*., 2007). PIV type 3 infections occur year round with outbreaks usually occurring in spring, while type 1 and 2 demonstrate a biennial pattern with epidemics in the fall or early winter, sometimes in alternate years.

Different studies have shown that RSV infections peaked during the cold months in temperate regions in the southern hemisphere, seemingly independent of rainfall. In sub-tropical and tropical locations with seasonal rainfall, RSV tends to occur in relation to the rainy season, however, in locations closer to the equator with perennial rainfall, RSV activity is almost continuous and with varying peaks of infection (Weber *et al*., 1998). Influenza is also reported to be detectable throughout the year in tropical and sub-tropical regions with less predictable timing of outbreaks, although there are reports of a biannual pattern of outbreaks with considerable activity between epidemic periods (Simonsen *et al*., 1999).

In a three-year study in South Africa, hMPV was seen in yearly epidemics, peaking during autumn and winter (Ludewick *et al*., 2005). There are however no reports on seasonality of hMPV in other African countries.

1.11 Diagnosis of Human Metapneumovirus

1.11.1 Specimen types

The specimens used for the laboratory diagnosis of suspected etiologic agents are, nasal swabs (NS), nasopharyngeal aspirates, oropharyngeal swabs, bronchoalveolar lavage (BAL) or lung aspirates, and induced sputum (IS).

1.11.2 Virus isolation

Many hMPV strains show reliable cytopathic effect (CPE) in tertiary monkey kidney cell line/LLCMK-2 cells (Peret *et al.,* 2002). The CPE is quite variable, some strains show syncytia formation and other only produce rounding (Boivin *et al.,* 2002). CPE is usually observed after 10-21 days. Shell vial culture using commercial LLCMK-2 cells for rapid isolation of hMPV has also been described (Reina *et al.,* 2007). Some reports have shown that hMPV can replicate efficiently in Hep-2 cells but does not produce good CPE (van den Hoogen *et al.,* 2004). Confirmation of CPE is done by RT-PCR or indirect immunofluorescence staining using hMPV specific antibodies.

1.11.3 Direct immunofluorescence assay

Direct immunofluorescence assay (DFA) using virus specific antibodies is a rapid method to detect respiratory viruses. Commercially available hMPV specific antibodies have been developed for direct immunofluorescence assays. DFA is however not as sensitive as RT-PCR in the detection of hMPV (Ebihara *et al.,* 2005, Landry *et al.,* 2005; Percivalle *et al.,* 2005).

1.11.4 Molecular diagnostics

Real time PCR (RT-PCR) is a powerful method for detecting hMPV and other respiratory viruses, it allows amplification and quantification of the pathogens in clinical samples. The assay has been developed to detect genes of all known

hMPV lineages (Maertzdorf *et al.,* 2004). RT-PCR is a more sensitive, specific, and rapid method to detect this virus in clinical samples than conventional PCR (Mackay *et al.,* 2003). Nucleic acid sequence based amplification assay (NASBA) has also been developed for detection of hMPV infection in respiratory specimens (Dare *et al.,* 2007).

1.11.5 Serology

Enzyme-linked immunosorbent assay (ELISA) can also be used for hMPV serological testing (Falsey *et al.,* 2003; Hamelin *et al.,* 2005). Recently, ELISA methods using viral N or F protein expressed in prokaryotic (Hamelin and Boivin 2005) or recombinant vesicular stomatitis virus (VSV) (Leung *et al.,* 2005) and recombinant baculovirus system (Liu *et al.,* 2007) have been developed to detect antibodies against hMPV. Use of these assays in sero-epidemiologic studies might be helpful for detection of antibody response against hMPV infections thus increasing the understanding of the human immune responses to hMPV and permitting better understanding of the epidemiology of this virus. (Prins and Wolthers, 2004).

1.12 Vaccine candidates for Human Metapneumovirus

Several promising vaccine candidates have been developed and tested in animal models. A live recombinant human parainfluenza virus that contains the hMPV F gene has been shown to induce hMPV-specific antibodies and to protect experimental animals from hMPV challenge (Skiadopoulos *et al.,* 2004). A chimeric bovine/human

parainfluenza virus 3 expressing the hMPV F elicits neutralizing antibodies against both Parainfluenza virus and hMPV (Tang *et al.,* 2005).

1.13 Treatment of Human Metapneumovirus

The antiviral activity of ribavirin to inhibit the replication of hMPV is equivalent to that observed with RSV. Other compounds, such as Sulfated Sialyl lipid (NMSO3), a sulphated sialyl lipid that has been shown to have potent antiviral activity against RSV in tissue culture cells, have been shown to have anti-hMPV activity *in vitro* (Wyde *et al.,* 2004). It is likely that an hMPV-neutralizing monoclonal antibody for prophylaxis of high-risk infants (similar to the anti- RSV F humanized monoclonal antibody currently used for prevention of severe RSV disease) will be developed and tested. The progress towards an effective antiviral strategy for hMPV is currently limited by the scant data on pathogenesis of the virus in the natural host (Wyde *et al.,* 2004).

1.14 Classification of Human Metapneumovirus

Human metapneumovirus is an enveloped virus with a non-segmented negative-sense single stranded RNA genome. Based on the International Committee on taxonomy of viruses (ICTV) (Table 1.14), hMPV belongs to the family *Paramyxoviridae,* subfamily *Pneumovirinae*, and is the only known human pathogen of the genus *Metapneumovirus.* Genetically, it is closely related to the avian pneumovirus type C. Clinically, it resembles the respiratory syncytial virus (RSV), a common respiratory pathogen classified in the family *Paramyxoviridae,* and subfamily *Pneumovirinae*.

Table 1.14: Classification of Human Metapneumovirus

Order: *Mononegavirales*
Family: *Paramyxoviridae*
Sub family: *Pneumovirinae*
Genus: *Metapneumovirus*
Species: *Human metapneumovirus*

Adapted from ICTV, 2009

1.15 The pneumovirus structure

Paramyxoviridae (figure 1.15) are enveloped viruses composed of a nucleocapsid and a matrix protein. These viruses are similar in morphology to the influenza viruses. The virus capsid is enveloped and mature naturally by budding through the membrane of the host cell. The virus shapes can be spherical to pleomorphic, filamentous and other forms. The sizes of these viruses are 150-200 nm in diameter and 1000-10000 nm in length.

The envelope surface is evenly covered with hemagglutinin-neuraminidase (HN) and fusion protein (F), which are embedded in a lipid bilayer. The lipid bilayer is comprised of hemagglutinin-neuraminidase (HN), or hemagglutinin (H), or surface glycoproteins (GP), or fusion proteins.

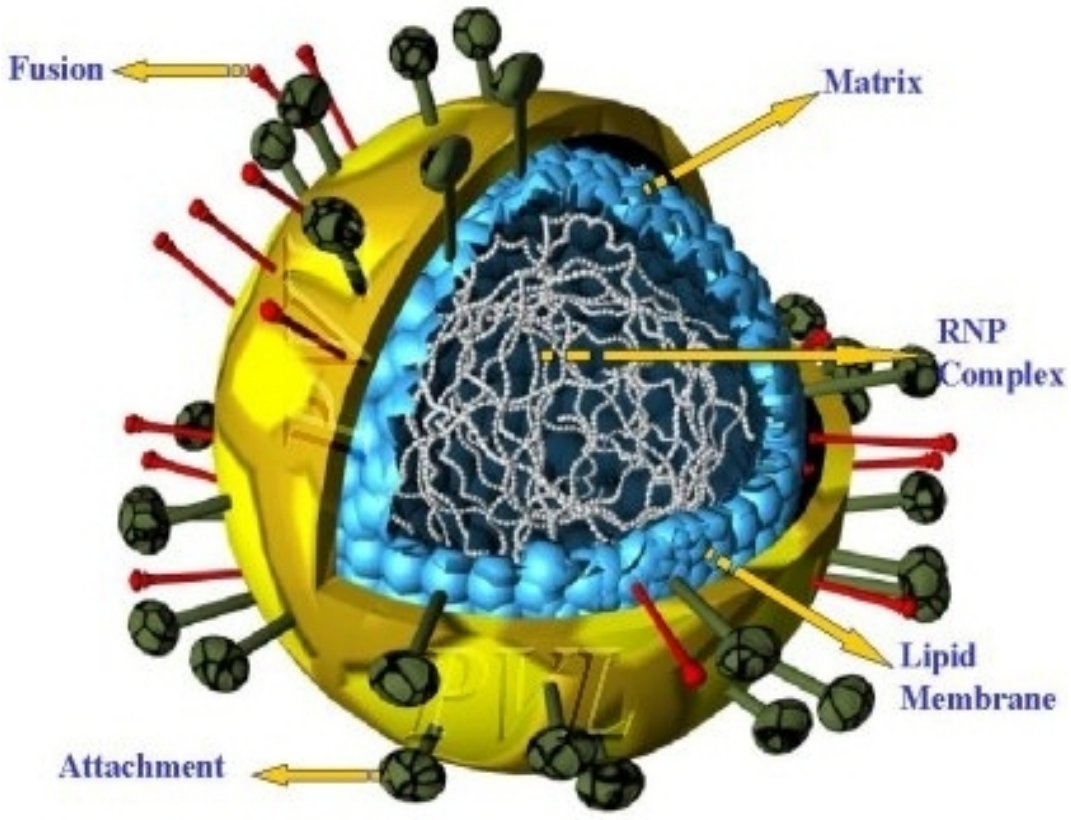


Figure 1.15: 3D image of Pneumovirus reconstruction originates from the University of Warwick, Pneumovirus Laboratory, UK.

The surface of viruses have spike-liked projections, 8-12 nm in length and 6-10 nm apart (depends on the genus). The capsid or nucleocapsid is elongated and exhibits helical symmetry. The nucleocapsid is filamentous, flexuous, and varies in length with the usual being 600-800 nm (depends on the genus), and width of 13-18 nm. The basic helix is obvious. Pitch of helix is 5.5-7 nm (depending on the subfamily).

1.16 The problem Statement

Acute Respiratory Tract Infections (ARTI) remain a leading cause of illness and death among children under five years of age (Williams, 2002). Childhood pneumonia is a serious public health problem in Kenya (WHO, 2003; CBS:MOH, 2004). Even with extensive use of highly sensitive assays such as real time PCR, a substantial proportion of ARTI cannot be attributed to any known pathogen. hMPV is a novel virus, having been detected for the first time in 2001(van den Hoogen *et al*., 2001) in Netherlands. Not much is known about its presence and molecular epidemiology outside temperate countries, especially in Sub-Saharan Africa (SSA). Its contribution in ARTI in these countries is not known since it is not tested during laboratory diagnosis of patients with ARTI. In Kenya most studies are centered on other viral pneumonia pathogens such as RSV and Influenza viruses. There is no known published report on the presence, molecular epidemiology of hMPV and its association with ARTI in Kenya.

1.17 Justification

Different studies have identified hMPV as an important cause of hospitalization for ARTI in young children (Boivin *et al*., 2003). For effective treatment and management of the pediatric patients with ARTI, it is important to detect the causative agent. It is therefore important to establish the presence of hMPV in these patients. Determining the types and studying the molecular epidemiology of hMPV isolates from Kenya will establish their relationship with other global isolates. The research findings can be

useful in future epidemiological studies and vaccine designs that are necessary in mitigating the burden of ARTI in pediatric patients.

1.18 Research Questions

i. What is the positivity rate of hMPV in archived respiratory samples collected from pediatric patients at Kenyatta National Hospital in 2008?

ii. Which types and subtypes of hMPV are responsible for infection among children with ARTI at Kenyatta National Hospital?

iii. What is the phylogenetic relationship between hMPV isolates in the study with other global isolates?

1.19 Study objectives

1.19.1 General Objective

- To detect and study the molecular characterization of hMPV from children with ARTI at KNH, in 2008.

1.19.2 Specific Objectives

2 To detect hMPV from nasopharyngeal samples of children with ARTI at KNH.

3 To sequence the F and G genes of the hMPV isolates.

4 To determine the types and subtypes of the hMPV isolates.

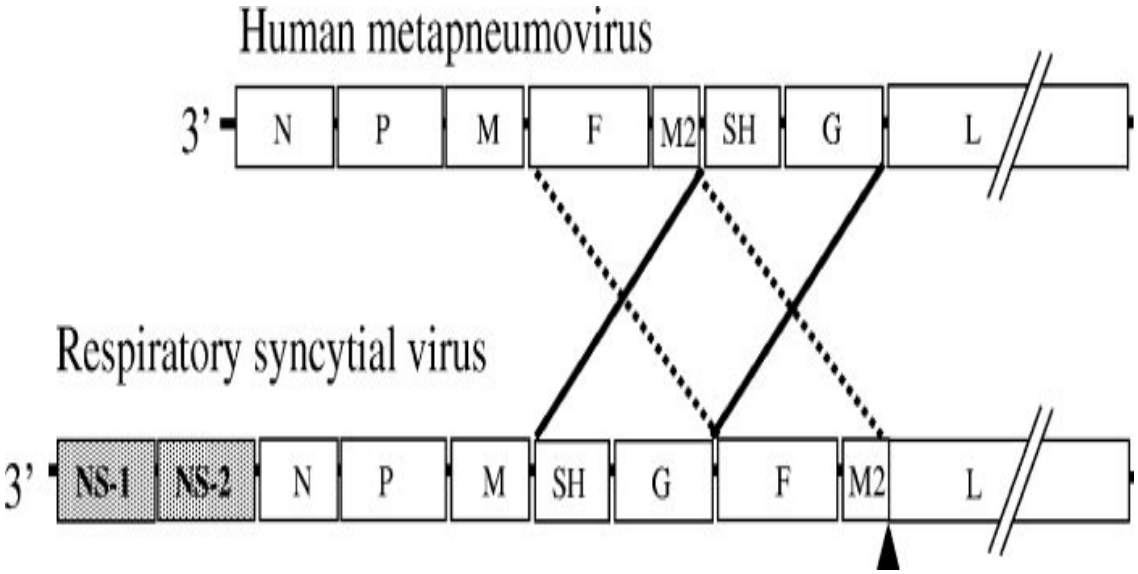
5 To compare the phylogenetic relationship of the Kenyan hMPV sequences with others from Europe, Asia, America and other parts of Africa.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 *Pneumovirinae* genomic organization

The full length sequences of at least 4 hMPV genomes have been reported (Van den Hoogan *et al.,* 2002; Biacchesi *et al.,* 2003; Herfst *et al.,* 2004). The genome of the virus is approximately 13 kb in length (Easton *et al.,* 2004). It is predicted to encode 9 proteins in the order 3-N-PM- F-M2-SH-G-L-5 (the M2 gene is predicted to encode 2 proteins, M2-1 and M2-2, using overlapping open reading frames, as in RSV) (Khan *et al.,* 2005) illustrated in figure 2.1.



(Adapted from Khan *et al*., 2005)

Figure 2.1: The genomic maps of *pneumovirinae*; human metapneumovirus and respiratory syncytial virus.

The genome also contains noncoding 3'leader which has the viral promoter, 5' trailer and intergenic regions, consistent with the organization of RSV (Herfst *et al.*, 2004). However there are some differences in the genome of APV and hMPV as compared to

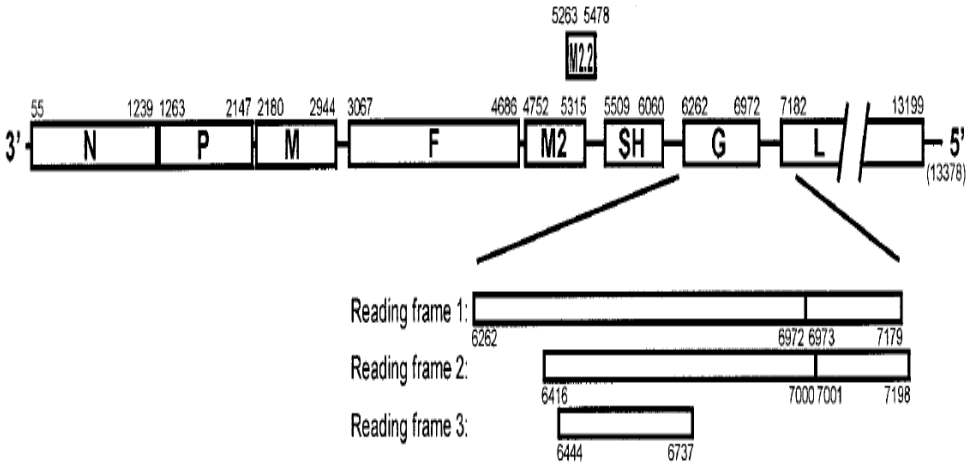
RSV, they lack the 2 nonstructural proteins NS1 and NS2 located at the 3' end of RSV genomes. These proteins counteract host interferons; therefore the lack of these genes in the Metapneumoviruses may have important implications for the relative pathogenicity of these viruses compared with RSV strains (Collins *et al.*, 2001). The key similarities between these two viruses are; their negative-strand RNA genomes contain open reading frames (ORFs) that encode three putative viral envelope glycoproteins, the F (fusion), G (glycosylated) and SH (short hydrophobic) proteins. These proteins are integral membrane proteins on the surface of infected cells and virion particles.

The F protein appears to be a classic viral fusion protein, with a predicted nonfurin F1/F2 cleavage site near a hydrophobic fusion peptide and 2 heptad repeats in the extra cellular domain that facilitate membrane fusion. The F gene is the most conserved of the three putative genes in both viruses (van den Hoogan *et al.*, 2002). It contains several features that are conserved among the F proteins of paramyxoviruses, which includes a characteristic distribution of cysteine residues, a putative cleavage site (the distinguishing feature of fusion proteins) and fusion domains. The predicted G protein of hMPV exhibits the basic features of a glycosylated type II mucin-like protein but interestingly lacks the cluster of conserved cysteines sometimes termed the "cysteine noose" that is found in the RSV and APV G proteins. G gene of hMPV tends to be highly variable like RSV G gene, which may be due to host immune selection pressure (Crowe, 2004). RSV G gene is heavily glycosylated with both O- and N-linked sugars (Collins *et al*., 1996). The predicted amino acid sequence suggests that the same is true for hMPV. The SH gene of hMPV encodes a protein of 180 amino acids (aa), that is

substantially longer than the corresponding protein of 64 aa in RSV (Khan *et al.*,2005). The function of the SH proteins of both the viruses remains unknown. The N (nucleoprotein), P (phosphoprotein) and L (large, polymerase) proteins are replication proteins in the nucleocapsid. The viral genome interacts with these proteins to form the virus ribonucleoparticle (RNP) a characteristic structure found in all *paramyxoviridae.* The M2-1 and M2-2 proteins are regulatory proteins and the M (matrix) protein may coordinate viral assembly of viral nucleocapsids with envelope proteins (Crowe, 2004).

2.2 Genomic Structure of Human Metapneumovirus

The hMPV genome is thought to express eight proteins as illustrated in Figure 2.2. By analogy with other *paramyxoviruses,* they can be classified as either integral membrane proteins, which are embedded in the virus envelope, or internal proteins, which associate with the virus polymerase beneath the virus envelope.



Adapted from (van den Hoogen., 2002)

Figure 2.2: Genomic map of hMPV isolate 00-1. The putative ORFs and the nt

positions of the start and stop codons are indicated. The double lines crossing the L ORF indicate the shortened representation of the L gene. The three reading frames below the map indicate the primary G ORF (nt 6262–6972) and overlapping potential secondary ORFs.

2.3.1 Nucleoprotein Open Reading Frame

As shown earlier, (van den Hoogen *et al*., 2001) the first gene (N protein) in the genomic map of hMPV codes for a 394-aa protein. The length of the N ORF is identical to the length of the N ORF of APV-C and is smaller than those of other paramyxoviruses (Barr *et al*., 1991). Analysis of the aa sequence revealed the highest percentage of sequence identity with APV-C at 88%. Very low sequence identity of 7–11% was shown with other paramyxoviruses (Barr *et al*., 1991). The region between aa residues 160 and 340 is highly conserved among metapneumoviruses and to a lesser extent in the Pneumovirinae (Miyahara *et al*., 1992; Li *et al*., 1996). This is in agreement with hMPV being a metapneumovirus, showing 99% similarity with APV-C.

2.3.2 Phosphoprotein Open Reading Frame

This ORF encodes for a 294-aa protein. The P gene of hMPV contains one substantial ORF and in that respect is similar to P from many other paramyxoviruses (Lamb and Kolakofsky, 1996; Sedlmeier and Neubert,1998). The hMPV P ORF lacks cysteine residues but is rich in glutamate residues as has been described for APVs. It has been suggested that a region of high similarity between all pneumoviruses (aa185–241) plays

a role either in the RNA synthesis process or in maintaining the structural integrity of the nucleocapsid complex (Ling *et al*., 1995). This conserved region may represent the domain that interacts with the polymerase protein. This region of high similarity is also found in hMPV showing 100% similarity with APV-C, 93% with APV-A and -B, and approximately 81% with RSV (van den Hoogen *et al.*, 2001).

2.3.3 Matrix protein Open Reading Frame

This ORF encodes for a 254-aa protein, the M ORF of hMPV is similar in size with the M ORFs of other metapneumoviruses and shows high aa sequence identity with the matrix proteins of APV (76–87%), lower identity with those of RSV (37–38%).

Two small secondary ORFs in the M ORF of hMPV exist, a small ORF of 54 aa residues is found within the major M ORF, starting at nt 2281, and also a small ORF of 33 aa residues is found overlapping with the major ORF of M, starting at nt 2893(van den Hoogen *et al.*, 2001). Similar to the secondary ORFs of RSV and APV there is no significant sequence identity between these secondary ORFs and secondary ORFs of the other pneumoviruses, and apparent start and stop signals are absent.

2.3.4 Fusion protein Open Reading Frame

The F gene of hMPV encodes a 539-aa protein. One of the conserved features among F proteins in hMPV and also seen in paramyxoviruses is the distribution of cysteine residues (Yu *et al*.,1991). The metapneumoviruses share 12 cysteine residues in F1 (7 are conserved among all paramyxoviruses) and 2 in F2 (1 is conserved among all paramyxoviruses). Of the three potential N-linked glycosylation sites present in the F

ORF of hMPV, none are shared with RSV and two (positions 66 and 389) are shared with APV. The third, unique, potential N-linked glycosylation site for hMPV is located at position 206. The F protein of hMPV has typical fusion protein characteristics consistent with those described for the F proteins of other *paramyxoviridae* family members (Morrison, 1988). F proteins of *paramyxoviridae* members are synthesized as inactive precursors (F0) that are cleaved by host cell proteases that generate N-terminal F2 subunits and large C-terminal F1 subunits. The cleavage site is conserved among all members of the *paramyxoviridae* family (Collins *et al.*, 1996). The hMPV F protein has only one cleavage site that contains the residues RQSR. The hydrophobic region at the N-terminus of F1 functions as the membrane fusion domain and displays a high degree of sequence similarity among paramyxoviruses, and to a lesser extent among the pneumoviruses (Morrison, 1988; Horvath and Lamb, 1992). This hydrophobic region (position 137–159,) is conserved between hMPV and APV-C, which is in agreement the region being highly conserved among the metapneumoviruses (Seal *et al*., 2000). Adjacent to the fusion peptide and transmembrane segment are two regions that contain heptad repeats (HRA and HRB) that are relatively poor in glycines. They lack helix-breaking prolines, and contain charged aa side chains in all heptad positions except a and d. These heptad regions have been shown to be necessary for viral fusion (Russell *et al*., 2001).

2.3.5 The 22K protein Open Reading Frame

This is the first major ORF representing the M2-1 protein that enhances the processing of the viral polymerase (Collins *et al.*, 1996). The M2-1 gene for, located adjacent to

the F gene, encodes for a 187-aa protein, and reveals the highest percentage of sequence identity of 84% with M2-1 of APV-C Comparison of all pneumovirus M2-1 proteins revealed the highest level of conservation in the N-terminal half of the protein (Ahmadian *et al.*, 1999). The hMPV M2-1 protein contains 3 cysteine residues that are located within the first 30 aa residues that are conserved among all pneumoviruses. Such a concentration of cysteines is frequently found in zinc-binding proteins (Cuesta *et al.*, 2000). The secondary ORFs (M2-2) which is 71 aa residues in length, overlap with the M2-1 ORFs of pneumoviruses are conserved in location but not in sequence and are thought to be involved in the control of the switch between virus RNA replication and transcription (Collins *et al*., 1990; Ahmadian *et al*., 2000).

2.3.6 Small hydrophobic protein Open Reading Frame

This gene is located adjacent to M2 of hMPV and encodes for a 183-aa SH protein. The SH ORF of hMPV contains 10 cysteine residues, and 2 N-linked glycosylation sites (aa 76 and 121) In all pneumovirus SH proteins, the hydrophobic domain is flanked by basic aa residues, which are also found in the SH ORF for hMPV aa 29 and aa 54 (van den Hoogen *et al.*, 2001).

2.3.7 Attachment glycoprotein Open Reading Frame

The G ORF of hMPV is located next to the SH gene and encodes a 236-aa protein (nt 6262–6972). A secondary small ORF is found immediately following this ORF, potentially coding for 68 aa residues (nt 6973–7179) but lacks a start codon. A third potential ORF in the second reading frame of 194 aa residues overlaps both of these

ORFs but also lacks a start codon (nt 6416–7000). This ORF is followed by a potential fourth ORF of 65 aa residues in the same reading frame (nt 7001–7198), also lacking a start codon. Finally in the third reading frame, there exists a potential ORF of 97 aa residues also lacking a start codon (nt 6444–6737). Unlike the first ORF, the other ORFs do not have apparent gene start or gene end sequences. Although the 236 aa G ORF probably represents at least a part of the hMPV attachment protein (van den Hoogen *et al*., 2001).

2.3.8 Polymerase protein Open Reading Frame

The L gene of hMPV encodes for a 2005-aa protein that shares 64% aa sequence identity with APV-A, 44% with RSV. It has six conserved domains from which domain III has four core polymerase motifs that are thought to be essential for polymerase function. These motifs (A, B, C, and D) are well conserved in the hMPV-L protein (van den Hoogen *et al*., 2001).

2.4 Genetic and antigenic diversity

The classification and naming of types and subtypes, strains, variants and isolates of hMPV is still evolving. Based on genomic sequences and phylogenetic analyses, there are two major types of hMPV, designated A and B (Biacchesi *et al.*, 2003; van den Hoogen *et al.*, 2003). These analyses are based on sequencing of the N, M, F, G, or L gene, and the genotype groupings are concordant regardless of which gene is studied (Bastein *et al.*, 2003; Biacchesi *et al.*, 2003; Boivin *et al.*, 2004; Peret *et al.*, 2004). Full length sequences of genomes from viruses representing the 2 major types show that the

diversity between hMPV subgroup A and B sequences is greatest for the SH and G proteins (59 and 37% identity, respectively), (Biacchesi *et al.*, 2003; Herfst *et al.*, 2004) and is more than RSV subgroup A and B. hMPV F protein, which is predicted to be the principal target of protective antibodies, is more conserved in hMPV strains than RSV. The overall level of genome nucleotide sequence identity and aggregate proteome amino acid sequence identity between the two hMPV subgroups is 80 and 90%, respectively, similar to the respective values for RSV A and B groups (Hamelin *et al.*, 2004).

Based on the sequence analysis of two surface glycoprotein encoding genes namely F and G genes each of the major lineages are further subdivided into two genetic subtypes known as A1, A2 and B1, B2, the significance of this diversity is unclear at this time (Boivin *et al.*, 2004). The previously reported A2 lineage has been shown to consist of two clusters namely, A2a and A2b. Among all the sub-lineages of hMPV the A2 sub-lineage shows the greatest diversity (Huck *et al.*, 2006). Vicente *et al*, (2006) suggested that hMPV type A might be more pathogenic than type B, causing greater clinical severity in children whereas no differences in disease severity associated with either genotype (Agapov *et al.*, 2006) was observed in another study.

The genetic diversity in hMPV strains also affects antigenic diversity, but to what extent has not been resolved. Studies with experimental infection of animals have suggested that the two lineages exhibited 48% antigenic relatedness based on reciprocal cross-neutralization assay with post-infection hamster sera (Skiadopoulos *et al* 2004). However cross protection studies in hamsters and nonhuman primates have shown that

each strain provided a high level of resistance to reinfection with the homologous or heterologous strain, supporting the conclusion that the two hMPV genetic lineages are highly related antigenically and are not distinct antigenic subtypes or subgroups (Herfst *et al.,* 2004). The hMPV F protein is a major antigenic determinant that mediates extensive cross-lineage neutralization and protection (MacPhail *et al.,* 2004; Skiadopoulos *et al.,* 2004). The extent of cross-protection in rodents cannot be extrapolated directly to the human situation because the animals are only semi permissive hosts for hMPV replication. Although difficult to assess, the extent of cross-protection is important to estimate because vaccine developers will choose to develop either a monovalent or a bivalent vaccine formulation based on this factor.

CHAPTER THREE

3.0 MATERIAL AND METHODS

3.1 Study site

The study samples were obtained from the pediatrics ward of Kenyatta National Hospital (KNH), analysis was done at the KEMRI Centre for Virus Research, ARI unit laboratory. KNH is situated in Kenya's capital, Nairobi. It is the oldest hospital in Kenya, having been founded in 1901 as the Native Civil Hospital. It is currently the largest National referral, teaching and research hospital in east and central Africa, having a bed capacity of 1,800, an annual outpatient attendance of 600,000 visits and an average annual inpatient attendance of 89,000 patients (MOH, 2007).

3.2 Study design

This was a retrospective laboratory based study, where archived nasal-wash specimens from hospitalized children below 5 years that had symptoms of ARTI at the pediatrics ward of KNH in 2008 were used. The samples were transferred to KEMRI, Centre for Virus Research, ARI unit laboratory in 2008. This study was done using archived samples that had tested negative for other respiratory viruses tested in 2008. The study was done in 2009 and 2010 at KEMRI, Centre for Virus Research, ARI unit laboratory.

3.3 Sample size determination

Fischer's *et al*. (1998) exact test formula was used to determine the sample size.

$$n_o = \frac{z^2 pq}{e^2}$$

where n_0 is the sample size, Z^2 is the abscissa of the normal curve that cuts off an area at the tails (equals the desired confidence level, which is 95%), e is the desired level of precision, p is the estimated prevalence, and q is (1-p)

The prevalence of hMPV in Kenya is not known, but using the estimate prevalence of 6-10 % outside the temperate regions given by (Van den Hoogen *et al*., 2004) p was taken to be 10%

$$n_o = \frac{1.96^2(0.1 \times 0.9)}{(0.05)^2}$$

= 139 respiratory samples

A minimum of 139 samples were required for the study, but all the available 150 samples were used.

3.4 Ethical considerations

Clearance for the study was gotten from Kenya Medical Research Institute Scientific Steering Committee (SSC number 1648) and the Ethical Review Committee (Appendix V and VI). Human experimentation guidelines for the conduct of clinical research were followed. Confidentiality was maintained by use of patients study identification numbers instead of names.

3.5 Demographic Characteristics of the Patients

The demographic information was obtained from the patients records used during recruitment in 2008. Of the one hundred and fifty (150) samples used for this study 65.3% (n = 98) were collected in April and 34.7% (n = 52) collected in May of 2008. In terms of sex distribution of patient samples, 81 representing 54% of were males while 69 representing 46% were females. The ages of the patients ranged from 1 to 61 months, with a mean of 10.61 months. Majority of the patients were in the 1-12 month age cluster (n=41, 27.33%), followed by 12-24 months age cluster (n = 37, 24.67%).

Table 3.5: The demographic characteristic of the patients whose samples were used in the study.

Age in months	**Sex**		**Total**
	Male	**Female**	
1-12 months	19	22	41
13-24 months	21	16	37
25-36 months	13	11	24
37-48 months	16	9	25
49-60 months	9	6	15
>61months	3	5	8
Total	81	69	150

3.6 The nasopharyngeal samples storage

The samples had been stored at -70^{0}C in clearly labeled 0.5ml cryovail tubes. The samples were thawed and immediately frozen after inoculation to the LLC-MK2 cell line.

3.7 Virus culture

Virus culture was done according to Abiko *et al*.(2004). In brief, 120 µl of sample were inoculated into 24 well plates in a semi-confluent monolayer of rhesus macaque kidney (LLC-MK2) cells in 1 ml of Opti-MEM I medium (Invitrogen GIBCO, Burlington, Ontario, LLC-MK2 cells Canada) containing 2 µg of trypsin (Sigma, Oakville, Ontario, Canada) per ml and 10 µg gentamycin. Mock-infected cells served as a negative control in parallel. The plates were incubated at 33°C with 5% CO_2 and examined for CPE using an inverted microscope every other day for 21 days. CPE was characterized by presence of round refringent cells with subsequent detachment from the monolayer and clusters of small syncytia. DNAse/RNAse free barrier tips were used to harvest the cell culture supernatant. The supernatant were transferred into cryovail tubes and frozen at -70^{0}C until use.

3.8 Human Metapneumovirus isolation and RNA extraction

The cryovails were thawed at room temperature. Viral RNA was extracted using a QIAamp viral RNA minikit (Qiagen, Hilden, Germany) according to the manufacturers instructions (Appendix II). In brief, 200 µl of infected cell culture supernatants were

mixed with 560 µl of Buffer AVL containing Carrier RNA and incubated at 25°C for 10 min. 560 µl of ethanol (100%) was added to each sample and vortexed for 15 sec. The resulting mixture was transferred into to the QIAamp spin column (in a 2-ml collection tube). The tubes were centrifuged at 8000 rpm for 1 min. Washing was done twice using 500 µl of wash buffer one (AW1) and wash buffer two (AW2) and centrifuged at full at 14,000 rpm for1 and 3 min respectively. 60 µl of elution buffer (AVE) added into each tube. The tubes centrifuged at 8000 rpm for 1 min. The extracted viral RNA was stored at–70°C until usage.

3.9 Complementary DNA synthesis of RNA

8 µl of RNA eluate, was used for complementary DNA **(**cDNA) synthesis using random hexamers and Superscript II Reverse Transcriptase (Invitrogen,Grand Island, NY); In brief, 6 µl of molecular grade water was mixed with 4 µl of 5x VILO reaction mix and 2 µl of 10x Superscript reverse transcription (RT) enzyme. 8 µl of the extracted RNA was added into the mixture and vortexed briefly for 15 sec. The tubes were incubated at 25°C for 10 min, then heated to 42°C for 60 min., and 70 °C for 5 min. The cDNA was stored at –20°C until use.

3.10 Detection of Human Metapneumovirus G gene

hMPV G gene detection was according to Ludewick *et al*.(2005) using 4 µl of cDNA to obtain a total of 50µl PCR reaction volume. In brief, the G gene open reading frame (ORF) was amplified using the following primers at 10µM concentration: HMPVGunivF: 5'-GAGAACATTCGRRCRATAGAYATG-3' (nucleotide position

6262-6285) and HMPVGunivR: 5'-AGATAGACATTRACAGTGGATTCA-3' (nucleotide position 7181-7204) under the following conditions: 95^0C for 3 min. 94^0C for 1 min. 59^0C for 1 min, and 38 cycles at 72^0C for 2 min. The final extention was at 72^0C for 7 min. To detect the band of interest of 942 bp, PCR products were analyzed by gel electrophoresis using 2% agarose gel in 0.5% TBE at 90 V for 40 min.

3.11 Detection of Human Metapneumovirus F gene

hMPV F gene detection was according to Ludewick *et al*. (2005) using 4 µl of cDNA to obtain a total of 50µl PCR reaction volume. In brief amplification with the following primers at 10µM concentration: 5'ATGTCTTGGAAAGTGGTG-3' (corresponding to nucleotide position 3052-3069) and 5'-CCATGTAAATTACGGAGCT-3' (nucleotide position 3844-3862) under the following conditions: 94^0C for 2 min. 94^0C for 30 sec. 45^0C for 45 sec. 35 cycles at 68^0C for 1 min. The final extention was at 68^0C for 7 min. To detect the band of interest of 810 bp PCR products were analyzed by gel electrophoresis using 2% agarose gel in 0.5% TBE at 90 V for 40 min.

3.12 Purification of PCR Products

PCR products were purified using QIAquick PCR Purification Kit (Qiagen Hilden, Germany) according to the manufacturers instructions (Appendix III). In brief; 100 µl of Buffer PB was mixed with 20 µl of the PCR sample. The mixture was transferred into QIAquick spin column and centrifuged for 14000 rpm for 60 sec. 750 µl of buffer PE was added to the QIAquick column and centrifuge for 60 sec. 30 µl elution buffer

(EB) (was added to the center of the QIAquick membrane and centrifuge the column for 60 sec. Purified PCR products were kept at -20^{0}C until use.

3.13 Quantification of purified PCR products

The purified PCR products were quantified using a NanoDrop. The 260:230 and 260:280 ratios were used to determine the purity of DNA. A minimum of 10 ng/µl with 260:280 and 260:230 ratios between between 1.7 and 2 was good for sequencing.

3.14 Sequencing of Human Metapneumovirus F and G genes

The sequencing of the F and G genes was according to Ludewick *et al*. (2005). In brief, purified PCR products were sequenced in both directions by using the respective sets of primers (5'ATGTCTTGGAAAGTGGTG-3' and 5'-CCATGTAAATTACGGAGCT-3). Purified PCR products were taken to International Livestock Research Institute (ILRI) in Nairobi where sequencing was done.

3.15 Gene bank sequences

Reference and other global sequences were obtained from National Center for Biotechnology Information (NCBI) through the Basic Local Alignment Search Tool (BLAST). The search was limited to full and partial F-gene sequences. 10 sequences had their subtypes information and were used for typing of the study's sequences. The accession number for the sequences is shown in table 3.15.

Table 3.15: showing the accession numbers, type and subytypes of F gene sequences from different continents that were used in the study.

NCBI hMPV accession number	**Origin/type**
AY145301.1| CAN00-16 (F)-gene AY145297.1| CAN00-12 (F)-gene AY145296.1| CAN97-83 (F)-gene AY145299.1| CAN00-14 (F) gene-**A1** AY145295| CAN97-82 (F) gene-**B1** AY145290.1|CAN98-76 (F) gene-**B2**	North America
AY694758.1| RSA/1/01 (F)-gene AY694735.1| RSA/20/01 (F)-gene AY694715.1| RSA/26/00 (F)-gene AY694713.1| RSA/44/00 (F)-gene AY694740.1| RSA/7/01 (F)-gene	Africa
AY304362.1| NL/1/94 (F) gene-**B2** AY304360.1|NL/17/00 (F) gene-**A2**	Europe
gi|Br06mpvgdn61 (F)-gene gi Br06mpvgdn72 (F)-gene gi Br06mpvgdn67 (F)-gene DQ362942.1| Arg/4/00 (F)-gene DQ362941.1| Arg/3/00 (F)-gene	South America
AY622381.1| JPY88-12 (F) gene GQ386856/TJ08-01(F)-gene-**B2** GQ386855/TJ06-09 (F)-gene-**B1** GQ386854/TJ06-03(F)-gene-**A2b** GQ386853/TJ06-02(F)-gene-**A2a** GQ386852/TJ09-03(F)-gene-**A2b**	Asia

3.16 Phylogenetic Analysis of Human Metapneumovirus isolates

Sequence assembly, nucleotide and amino acid visualization was also obtained using BioEdit version 7.0.9.0. Sequence alignment was done using ClastaW software. Phylograms were generated using Mega version 5.03 through the maximum likelihood with 500 bootsrap replications.

3.17 Study limitations

It was not possible to get hMPV RNA or cDNA from other laboratories in the country, therefore the study did not have a positive control.

The archived frozen specimens used had been collected in 2008 for a different study. These samples having been freeze thawed on different occasions, might have affected the integrity of the samples and the eventual viral RNA present.

Finally it was not possible to study the G-gene that is associated with genetic variability in hMPV, due to the failure of the sequencing assay.

CHAPTER FOUR

4.0 RESULTS

4.1 Human Metapneumovirus isolation by culture

Cytopathic effect (CPE) was evident in 21 (14%) of the 150 samples inoculated on the LLC-MK2 cell lines. The CPE were apparent after a mean incubation time of 16.67 days. CPE positive cells were evident as small, granular, round up cells (plate 4.1a and 4.1b).

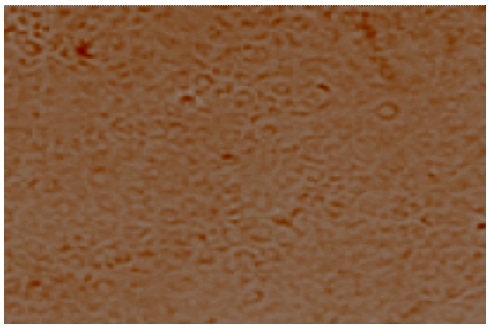


Plate 4.1a: Negative control showing uniform monolayer of LLC-MK2 cell line.

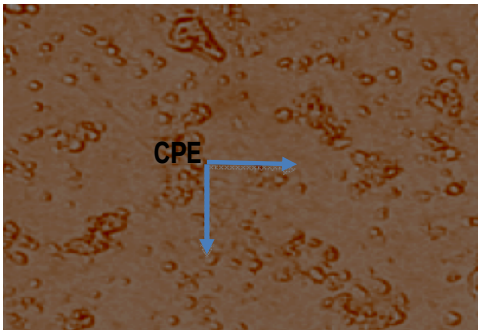


Plate 4.1b: Infected LLC-MK2 cell line showing granular round up cells (CPE) characteristic of hMPV

4.2 PCR Amplification of hMPV F and G genes

The amplified PCR products were subjected to gel electrophoresis. The bands of interest were 810 bp and 942 bp for the F and G genes respectively (Plate 4.2).

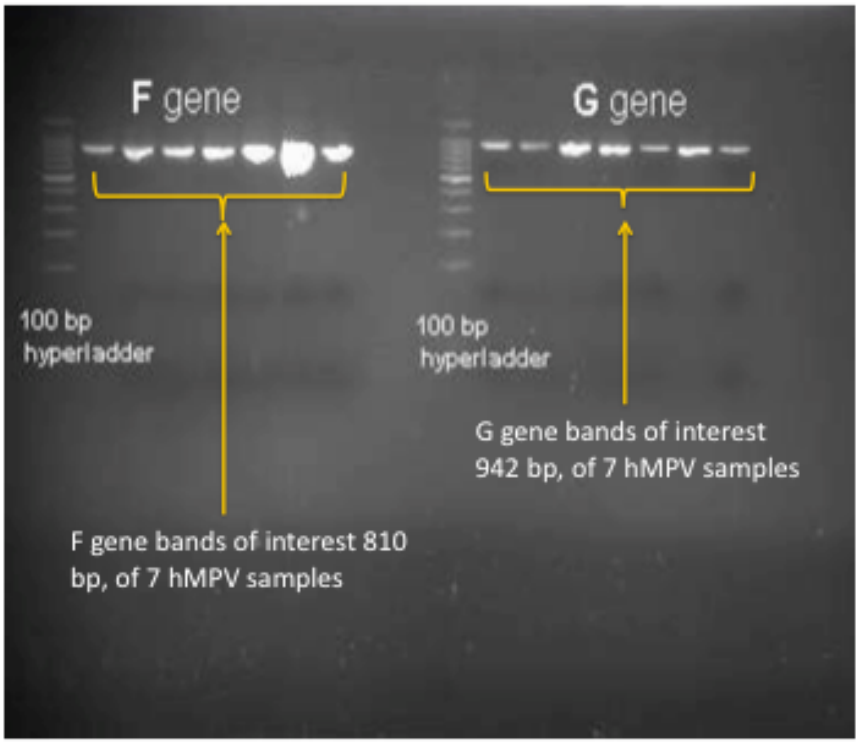


Plate 4.2: gel photo of PCR products positive for F and G protein genes. Product size was 810 bp and 942 bp for the F and G genes respectively.

Out of the 21 CPE positive samples, 7 (33.3%) were hMPV PCR positive for both G and F genes, this gave 4.67% PCR positive results for all the 150 samples used in the study.

4.3 Nanodrop Quantification

The DNA concentration had a mean of 56.2 ng/µl and 44 ng/µl for the F and G genes respectively. Good quality DNA was obtained, the mean for 260:280 and 260:230 ratios for F gene were 1.75 and 1.53 respectively and 1.78 and 1.52 respectively for G gene. The nanodrop quantification results are shown in tables 4.3a and 4.3b.

Table 4.3a: Nanodrop Quantification and Purity of F gene PCR Products.

Sample	260/280	260/230	Conc. ng/µl
S-1	1.74	1.64	70.75
S-2	1.81	1.56	66.41
S-3	1.72	1.69	52.04
S-4	1.77	1.71	56.52
S-5	1.82	1.61	49.35
S-6	1.92	1.63	59.19
S-7	1.65	1.58	39.14

Table 4.3b: Nanodrop Quantification and Purity of G gene PCR Products.

Sample	260/280	260/230	Conc. ng/µl
S-1	1.83	1.73	50.35
S-2	1.67	1.63	47.73
S-3	1.91	1.54	33.27
S-4	1.85	1.73	41.04
S-5	1.88	1.64	64.39
S-6	1.67	1.56	29.83
S-7	1.94	1.52	41.65

4.4 F and G genes sequencing

Despite detection of the G gene (plate 4.2), none of the G gene sequencing results gave good quality sequences for analysis. However, six out of the seven F gene samples gave

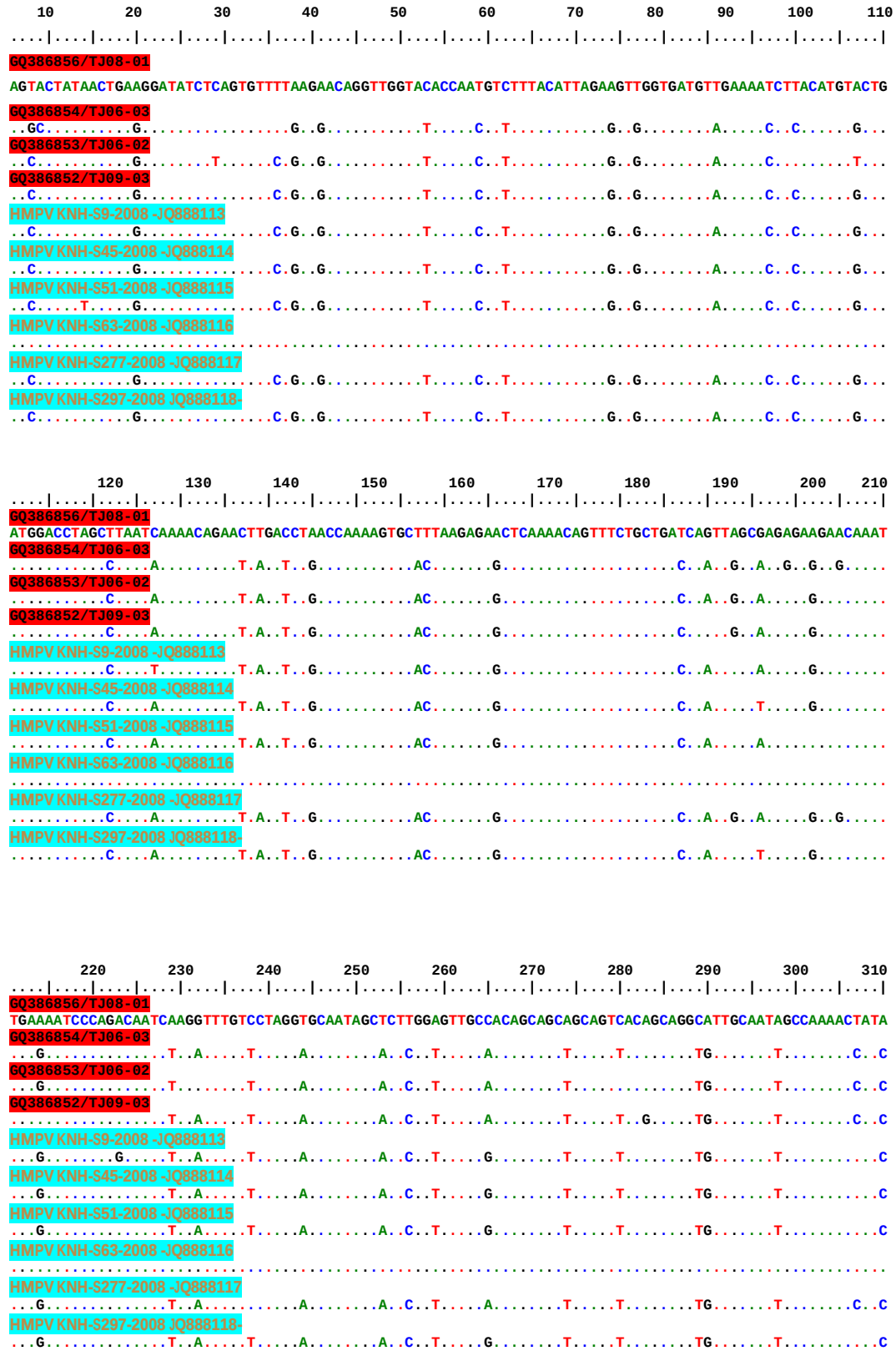
good sequences. The F gene sequences were uploaded to NCBI and were given the following accession numbers;

i. HMPV KNH-S9-2008 JQ888113
ii. MPV KNH-S45-2008 JQ888114
iii. HMPV KNH-S51-2008 JQ888115
iv. HMPV KNH-S63-2008 JQ888116
v. HMPV KNH-S277-2008 JQ888117
vi. HMPV KNH-S297-2008 JQ888118

The nucleotide sequences are shown in Appendix IV**.**

4.5 Nucleotide sequence alignment

The partial F gene nucleotide sequence alignment of the Kenyan strains with the reference sequences from Tianjin province in China generated by BioEdit version 7.0.9.0. showed the positions associated with similar and different nucleotide sequences. Reference sequences from isolate GQ386856/TJ08-01 for subtype B2 showed 99.8% homology with HMPV KNH-S63-2008 JQ888116. There was also an established nucleotide homology pattern between GQ386852/TJ09-03 and GQ386854/TJ06-03 for A2, with the Kenyan isolates (HMPV KNH-S9-2008 JQ888113, HMPV KNH-S45-2008 JQ888114, HMPV KNH-S51-2008 JQ888115, HMPV KNH-S277-2008 JQ888117, HMPV KNH-S297-2008 JQ888118). There was 100% nucleotide homology between isolates HMPV KNH-S45-2008 JQ888114 and HMPV KNH-S297-2008 JQ888118. Isolate HMPV KNH-S277-2008 JQ888117 had 98.7% nucleotide homology with isolate GQ386854/TJ06-03.


10 20 30 40 50 60 70 80 90 100 110
GQ386856/TJ08-01
AGTACTATAACTGAAGGATATCTCAGTGTTTTAAGAACAGGTTGGTACACCAATGTCTTTACATTAGAAGTTGGTGATGTTGAAAATCTTACATGTACTG
GQ386854/TJ06-03
GQ386853/TJ06-02
GQ386852/TJ09-03
HMPV KNH-S9-2008 -JQ888113
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HMPV KNH-S51-2008 -JQ888115
HMPV KNH-S63-2008 -JQ888116
HMPV KNH-S277-2008 -JQ888117
HMPV KNH-S297-2008 JQ888118-
120 130 140 150 160 170 180 190 200 210
GQ386856/TJ08-01
ATGGACCTAGCTTAATCAAAACAGAACTTGACCTAACCAAAAGTGCTTTAAGAGAACTCAAAACAGTTTCTGCTGATCAGTTAGCGAGAGAAGAACAAAT
GQ386854/TJ06-03
GQ386853/TJ06-02
GQ386852/TJ09-03
HMPV KNH-S9-2008 -JQ888113
HMPV KNH-S45-2008 -JQ888114
HMPV KNH-S51-2008 -JQ888115
HMPV KNH-S63-2008 -JQ888116
HMPV KNH-S277-2008 -JQ888117
HMPV KNH-S297-2008 JQ888118-
220 230 240 250 260 270 280 290 300 310
GQ386856/TJ08-01
TGAAAATCCCAGACAATCAAGGTTTGTCCTAGGTGCAATAGCTCTTGGAGTTGCCACAGCAGCAGCAGTCACAGCAGGCATTGCAATAGCCAAAACTATA
GQ386854/TJ06-03
GQ386853/TJ06-02
GQ386852/TJ09-03
HMPV KNH-S9-2008 -JQ888113
HMPV KNH-S45-2008 -JQ888114
HMPV KNH-S51-2008 -JQ888115
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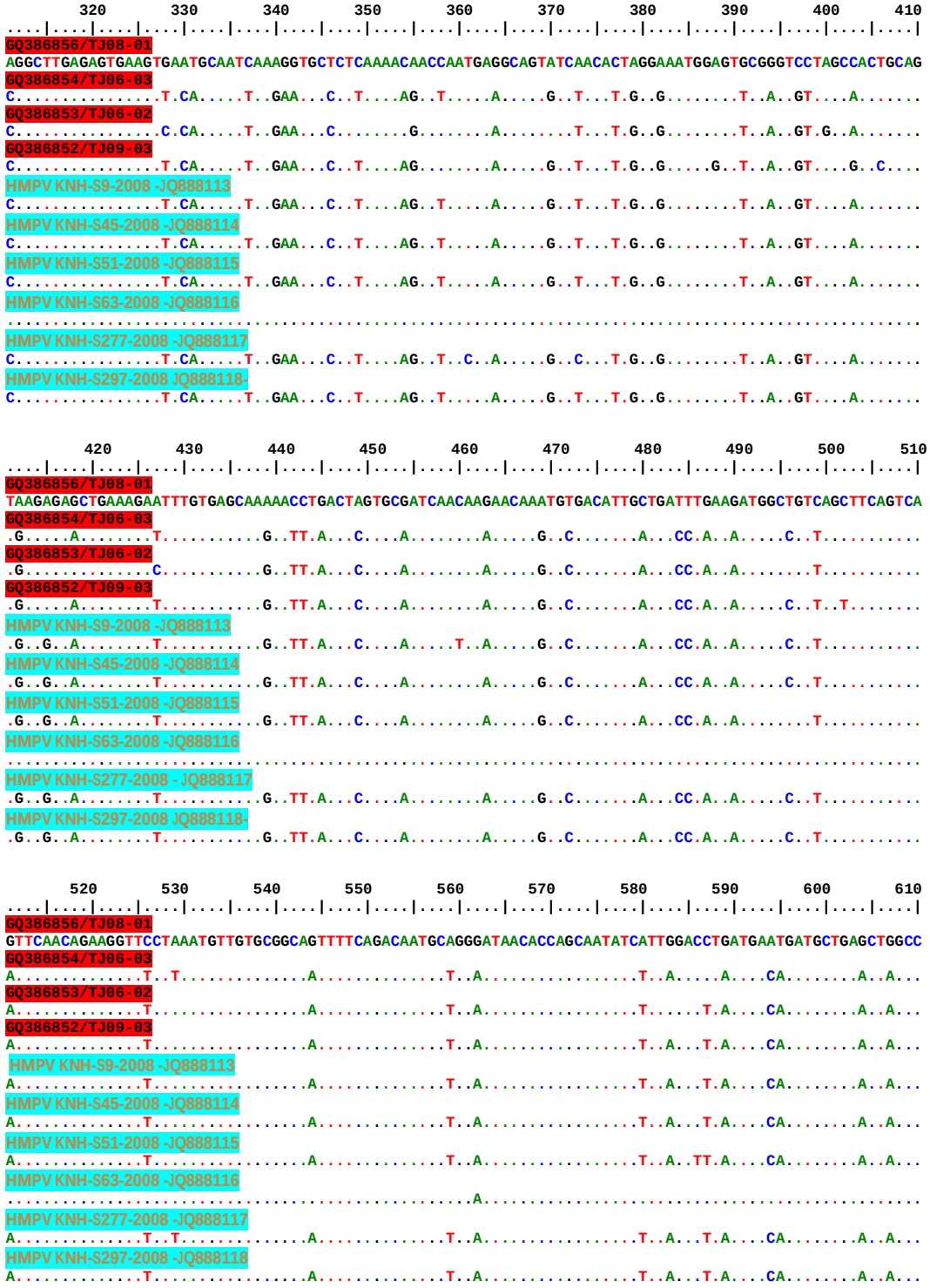
HMPV KNH-S277-2008 -JQ888117
HMPV KNH-S297-2008 JQ888118-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GQ386856/TJ08-01
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GQ386854/TJ06-03
GQ386853/TJ06-02
GQ386852/TJ09-03
HMPV KNH-S9-2008 -JQ888113
HMPV KNH-S45-2008 -JQ888114
HMPV KNH-S51-2008 -JQ888115
HMPV KNH-S63-2008 -JQ888116
HMPV KNH-S277-2008 -JQ888117
HMPV KNH-S297-2008 JQ888118-
420 430 440 450 460 470 480 490 500 510
GQ386856/TJ08-01
TAAGAGAGCTGAAAGAATTTGTGAGCAAAAACCTGACTAGTGCGATCAACAAGAACAAATGTGACATTGCTGATTTGAAGATGGCTGTCAGCTTCAGTCA
GQ386854/TJ06-03
GQ386853/TJ06-02
GQ386852/TJ09-03
HMPV KNH-S9-2008 -JQ888113
HMPV KNH-S45-2008 -JQ888114
HMPV KNH-S51-2008 -JQ888115
HMPV KNH-S63-2008 -JQ888116
HMPV KNH-S277-2008 - JQ888117
HMPV KNH-S297-2008 JQ888118-
520 530 540 550 560 570 580 590 600 610
GQ386856/TJ08-01
GTTCAACAGAAGGTTCCTAAATGTTGTGCGGCAGTTTTCAGACAATGCAGGGATAACACCAGCAATATCATTGGACCTGATGAATGATGCTGAGCTGGCC
GQ386854/TJ06-03
GQ386853/TJ06-02
GQ386852/TJ09-03
HMPV KNH-S9-2008 -JQ888113
HMPV KNH-S45-2008 -JQ888114
HMPV KNH-S51-2008 -JQ888115
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HMPV KNH-S277-2008 -JQ888117
HMPV KNH-S297-2008 -JQ888118

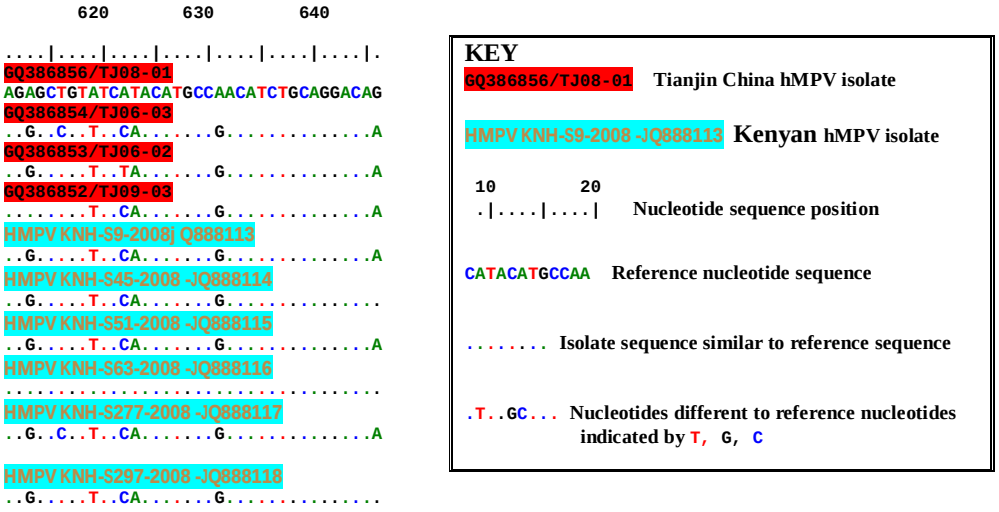


Figure 4.5: Alignment of the predicted nucleotide sequences of the partial hMPV F gene sequences of Kenyan and Tianjin, China isolates. A 646 nucleotide sequence of GQ386856/TJ08-01 for genotype B2 is the reference sequence. GQ386853/TJ06-02 for A2a, GQ386854/TJ06-03 and GQ386852/TJ06-03 for A2b. Only residues that differ from isolate GQ386856/TJ08-01 are shown; identical nucleotides are represented by periods.

The nucleotide sequence positions with noticeable similarities and differences based on the Kenyan isolates sequences are summarized in table 4.5.

Table 4.5: Nucleotide positions with differences between hMPV reference and Kenyan sequences.

Nucleotide Position	14	127	193	201	202	205	223	224	265	312	361	373	460	529	586	616	646
GQ386856/TJ08-01-B2	A	C	A	G	A	A	A	C	C	T	T	A	C	A	C	T	G
GQ386852/TJ06-03-A2	A	A	G	A	G	G	A	T	A	C	T	T	C	T	C	C	A
GQ386853/TJ06-02-A2	A	A	G	A	G	A	A	T	A	C	T	T	C	A	C	T	A
GQ386854/TJ06-03-A2	A	A	G	A	G	A	A	T	A	C	T	T	C	A	C	T	A
HMPV KNH-S9-2008 -JQ888113	A	T	A	A	G	A	G	T	G	T	T	T	T	A	C	T	A
HMPV KNH-S45-2008 -JQ888114	A	A	A	T	G	A	A	T	G	T	T	T	C	A	C	T	G
HMPV KNH-S51-2008 -JQ888115	T	A	A	A	A	A	A	T	G	T	T	T	C	A	T	T	A
HMPV KNH-S63-2008 -JQ888116	A	C	A	G	A	A	A	C	C	T	T	A	C	A	C	T	G
HMPV KNH-S277-2008 -JQ888117	A	A	G	A	G	G	A	C	A	C	C	C	C	T	C	C	A
HMPV KNH-S297-2008 -JQ888118	A	A	A	T	G	A	A	T	G	T	T	T	C	A	C	T	G

KEY

X Nucleotides characteristic to B2 reference isolate GQ386856/TJ08-01

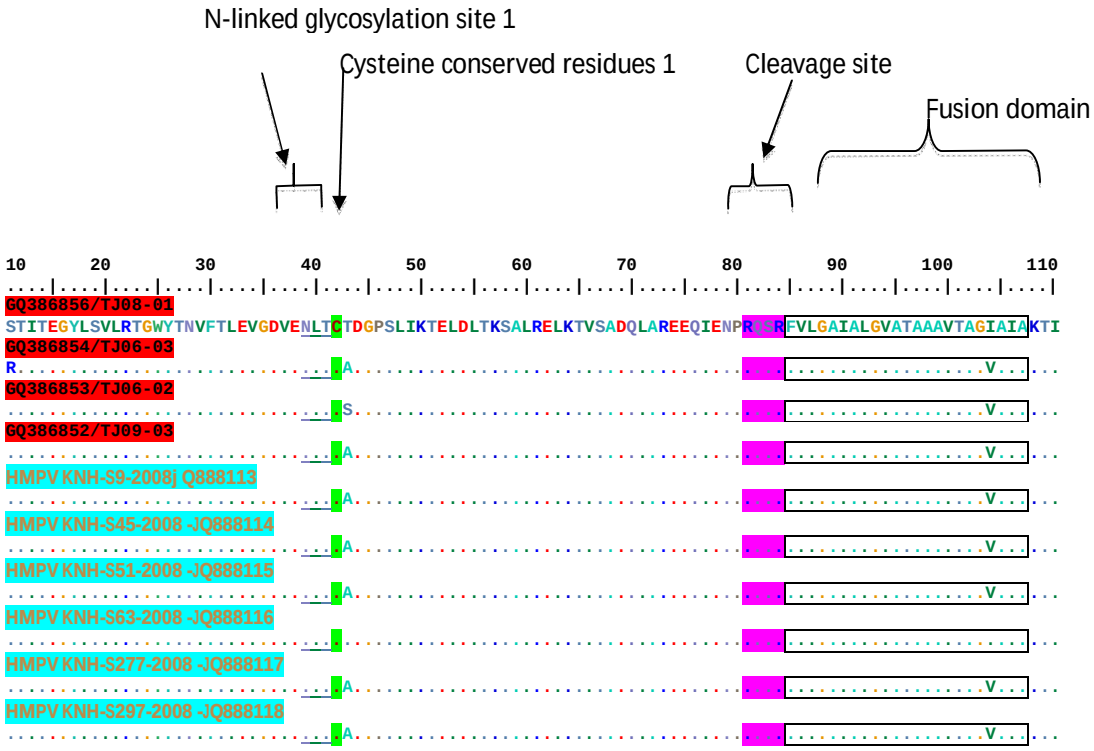
X Nucleotides different from A2 reference isolates GQ386853/TJ06-02 for A2a, GQ386852/TJ06-03 and GQ386854/TJ06-03

X Nucleotides not in A2 or B2 reference isolates

There was 100% homology shown at all the identified 17 nucleotide positions between isolate GQ386856/TJ08-01 and HMPV KNH-S63-2008 JQ888116. There was 100% homology between GQ386852/TJ06-03 and HMPV KNH-S277-2008 -JQ888117 with nucleotides G, T and C at positions 205, 259 and 616 respectively. All the other isolates had A, A and T at similar positions. Isolates HMPV KNH-S9-2008 JQ888113, HMPV KNH-S45-2008 JQ888114, HMPV KNH-S51-2008 JQ888115 and HMPV KNH-S297-2008 JQ888118) had A and T at positions 193 and 312 respectively, which were characteristic nucleotides for the B2 isolate GQ386856/TJ08-01.

4.6 Amino acid sequence alignment

The partial F gene amino acid sequence alignment showed more conserved sequence identities within the reference and Kenyan isolates. The different nucleotide sequences within the isolates figure 5 did not result in any subtype specific amino acid change as in figure 4 6



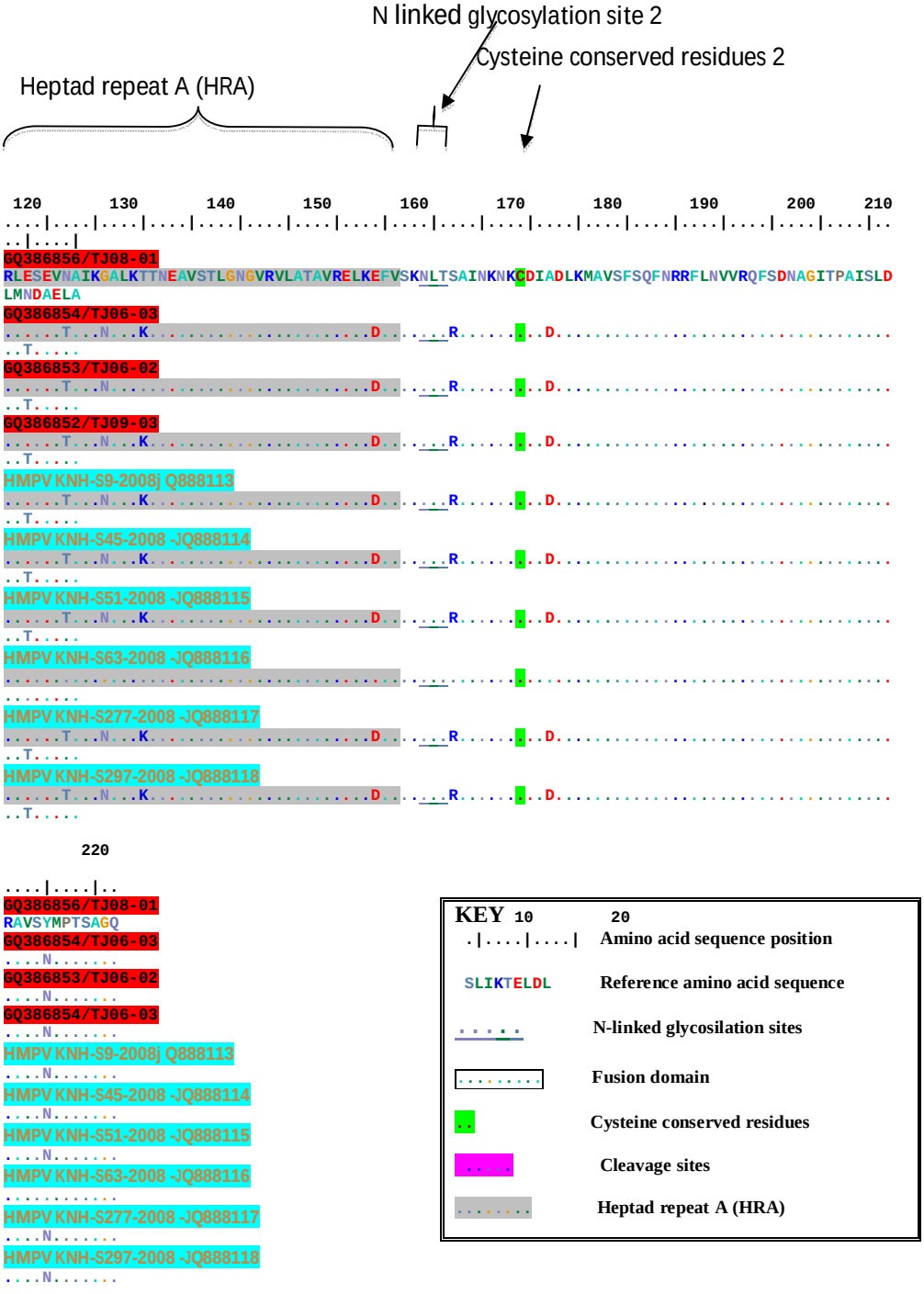


Figure 4 6: Alignment of the predicted amino acid sequences of the hMPV F genes of the Kenyan and Tianjin, China isolates. A 222 amino acid sequence of GQ386856/TJ08-01 for genotype B2 is the reference sequence. GQ386853/TJ06-02 for

A2a, GQ386854/TJ06-03 and GQ386854/TJ06-03 for A2b. There was 100% homology between subtype B2 isolate GQ386856/TJ08-01 and HMPV KNH-S63-2008 JQ888116 as shown in figure 4.2 There was also 100% amino acid sequence homology between Kenyan isolates (HMPV KNH-S9-2008 JQ888113, HMPV KNH-S45-2008 JQ888114, HMPV KNH-S51-2008 JQ888115, HMPV KNH-S277-2008 JQ888117, HMPV KNH-S297-2008 JQ888118), and the reference A2 isolates (GQ386853/TJ06-02, GQ386854/TJ06-03 and GQ386854/TJ06-03). There were however noticeable amino acid differences between subtypes A2 and B2 at 8 positions as shown in table 4.6.

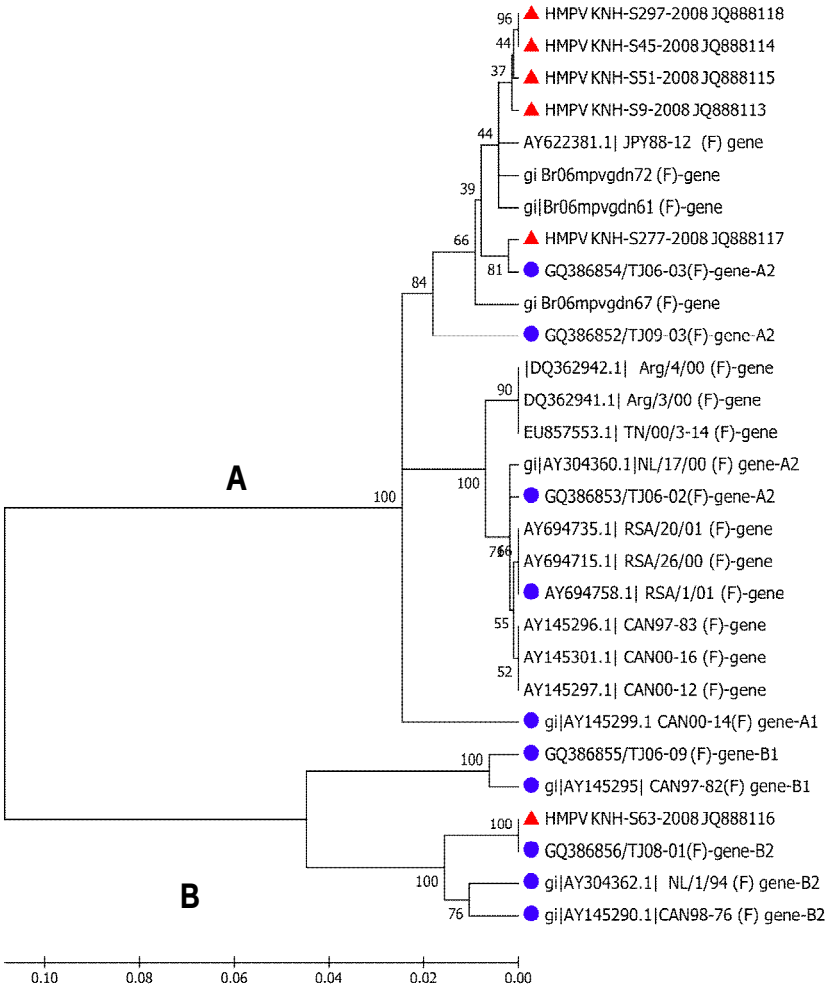
Table 4.6: List of subtype specific amino acids differences from the Kenyan isolates, the respective amino acids are shown in Appendix I**.**

Position	**43**	**104**	**117**	**121**	**149**	**157**	**167**	**205**
Subtype A2	A	V	T	N	D	R	D	T
Subtype B2	T	I	N	G	E	S	A	N

Conserved cysteine positions were identified at positions 42 and 169 in all the Kenyan and Tianjin China isolates as shown in figure 6. It was also possible to identify one of the two fusion domain regions (positions 85-112), which are also highly conserved in *paramyxoviridae*. The heptad repeat region A (position 111 to 151) and one of the two cleavage sites (position 81-84) was also highly conserved.

4.7 Phylogenetic analysis of Isolates

A Phylogram diagram was generated by MEGA 5(figure 4.7) was used for genotyping and studying the molecular epidemiology of the isolates



Key

Known hMPV subtypes from

Kenyan isolates

Figure 4.7: Phylogenetic tree for Kenyan and worldwide hMPV strains based on partial F gene sequences. The nucleotide sequences of the F gene were aligned by CLUSTAL W. Phylogenetic and molecular evolutionary analyses were conducted using MEGA Version 5.02, Tamura-Nei model and neighbor joining algorithm at (500 replications).

Five out of the six (83.3%) the Kenyan isolates; (HMPV KNH-S9-2008 JQ888113, HMPV KNH-S45-2008 JQ888114, HMPV KNH-S51-2008 JQ888115, HMPV KNH-S277-2008 JQ888117, HMPV KNH-S297-2008 JQ888118) clustered with the subtype A2 from China.Isolate (HMPV KNH-S63-2008 JQ888116) clustered with the B2 subtype from China. Further comparison of the Kenyan sequences with 25 isolates from South Africa, Canada, Singapore, Tianjin- China, Brazil and Netherlands showed that the Kenyan isolates clustered with isolates from China and Brazil.

CHAPTER FIVE

5.0 DISCUSSION

5.1 Human Metapneumovirus detection

In earlier studies on different respiratory viruses, the study samples had been freeze thawed several times, therefore the virus culture on LLC-MK2 cells was important for the multiplication of viral copies, for the final extraction of sufficient viral RNA for the molecular assays. The high rate (67%) of CPE positive virus culture cells being negative by PCR confirms the superiority of molecular techniques in diagnosis (Parcivelle *et al*., 2005). The false positive cultures might have been due to presence of other viruses in the samples. This can also be due to the difficulty in determining clear CPE for hMPV, given that it is a slow growing virus that does not form clear synsytia, cell death in some wells might have been confused to be CPE.

PCR products concentrations for both F and G gene were high with means of 56.2 and 44 ng/µl respectively as shown in tables 5a and 5b. The purity depicted by 260:280 ratios for both gene were good with means of 1.75 and 1.78. Therefore the purified PCR products taken for sequencing at ILRI were of good quality. The G gene sequences obtained were short (< 80 reads) and therefore they could not form contigs for sequence alignment. The primer used for the G gene PCR amplified the expected 942 bp region (plate 3), but secondary primer sets targeting a shorter region inside the 942 bp region could have increased the sensitivity of the sequencing PCR and the eventual sequencing. It was not possible to re-design primers for the nested PCR due to time. The F gene sequencing PCR was successful for 6 samples using the same

primer sets, therefore suggesting that the difference in the success rate between F and G gene sequencing was dependent on the primer design.

5.2 Human Metapneumovirus in Pediatric Patients

The etiology of viral childhood pneumonias have been studied extensively during the last 3- 4 decades and there is a large body of research contributing to this field both in industrialized countries as well as in developing countries. Studies of viral etiology of acute lower respiratory tract infections in tropical and developing countries published up to 1996 identified virus in 9% to 64% of hospital-based studies and 11% to 36% of community-based studies (Weber *et al*., 1998).

In Africa, studies of hMPV have only been done in South Africa (Ludewick *et al*., 2005). This shows that hMPV has not been studied extensively in childhood in Africa, where 42% of ARTI associated deaths in children below 5 years occur (Williams *et al*., 2002). For this reason it was not possible to obtain positive controls for the assays in other laboratories in Kenya, however other quality control measures were taken to ensure the integrity of the assays. The primers used were queried using BLAST to determine their ability to detect hMPV. Negative controls were also used during extraction, virus culture and PCR check for contamination. Even though less sensitive techniques were used for detection (culture and conventional PCR), and the fact that the samples had been freeze thawed before, the detection of hMPV in 7 (4.67%) of samples as shown in plate 3, was a breakthrough that confirms the involvement of the virus in pediatric pneumonia in Kenya. This however, does not rule out the involvement of other viral or bacterial pathogens in these pneumonia cases since co-infections are common (Viazov *et al*., 2003, Ebihara *et al*., 2003).

These results might also not exhaustive of all the hMPV cases that might have been present in the recruited pediatric patients in 2008 since the samples used had already tested negative for other respiratory viruses tested in previous studies. Therefore samples that might have had multiple respiratory viruses infections including hMPV were not analyzed.

Studies from different countries (Stockton *et al.,* 2002; Cuevas *et al.,* 2003; Ludewick *et al.,* 2005) have shown that most infections with hMPV occur in children younger than 1 year of age, this was also true this study where 3 (42.86%) were PCR positive. 5(71.43%) out of the 7 PCR positive samples were from males, females accounted for (28.57%) of the cases. Young age acts as a metafactor reflecting the interplay of factors causing disease following viral infection. Age has an effect on the size of the child, particularly airway size, transmission dynamics (due to multiple close contacts between small children), and immune experience, all of which contribute to an increased severity of infection. (Hall *et al*., 2009). These results provides further evidence of the role of hMPV in childhood pneumonia and the significant burden it may present to health care systems in developing countries.

5.3 Human Metapneumovirus types and subtypes detected

From the partial F-gene amino acid and nucleotide sequence alignment, it was evident that the identified nucleotide differences did not impact on the diversity of the isolates within the same subtype as shown in figures 4.5 and 4.6. The nucleotide differences when translated resulted in similar amino acid (100%) within the each subtype. Even though it was not possible to determine the ratio of synonymous to non-synonymous

changes, the similarity in amino acid sequences suggests that it was not significant.

However, 8 positions showing different amino acid sequences (table 4.6), could explain the difference between hMPV type A and B. These regions could be targeted when designing primers for typing of hMPV.

Phylogenetic analysis of the isolates supported by a boostrap value of 100 (figure 4.7) showed the existence of two main types (A and B). They are subdivided further into two subtypes A2 and B2. Subtype A2 was subdivided further to sub-subtypes A2a and A2b. These results confirm the presence of two distinct genetic clusters (Boivin *et al*., 2004, Ludewick *et al*., 2005). The Kenyan isolate sequence HMPV KNH-S63-2008 JQ888116 is a subtype B2 lineage. The other five representing 83.3% of Kenyan isolate sequences; HMPV KNH-S9-2008 JQ888113, HMPV KNH-S45-2008 JQ888114, HMPV KNH-S51-2008 JQ888115, HMPV KNH-S277-2008 JQ888117 and HMPV KNH-S297-2008 JQ888118 are sub-subtype A2b. Huck *et al*. (2006) described the existence of 2 lineages of subtype A2 (A2a and A2b) with A2b being detected at a higher rate, this was also the case in this study. The difference in the frequency of detection of circulation subtypes in this study might suggest that the epidemic was shifting. This phenomenon of genotype switching between epidemic seasons has been described before (Agapov *et al*., 2006). Switching of the predominantly circulating genotype in a population is widely discussed, but poorly understood in terms of what drives these events or the mechanisms by which they occur. It is hypothesized that switching of the predominant circulating subtype of hMPV is brought about by short-lived subtype-specific herd immunity in a population generated over one or two seasons, which favors dissemination of the

alternate subtype in a subsequent season (Agapov *et al.*, 2006). Circulation of multiple lineages may suggest an attempt at evasion of preexisting immunity (Padhi *et al.*, 2008). The findings in this study might suggest emergence of either subtype A2 or B2 viruses which may eventually have led to the displacement of subtype A2 or B2 as the dominant viral strain in subsequent years. It was not possible to predict the direction towards which the circulating subtypes were shifting.

The absence of subtypes A1 and B1 may have been due to pre-existing community immunity in the previous years. Proliferation occurs when the genotype is of minimal susceptibility to the adaptive immune responses of the host population, and a regression in circulating frequency occurs as the host population is increasingly exposed (Padhi *et al.*, 2008).

5.4 Molecular Epidemiology of Human Metapneumovirus Isolates from Kenya

The phylogram in figure 4.7 showed the clustering of the Kenyan A2b isolates with Brazilian and Chinese isolates. This might suggest that the A2b hMPV isolates from Kenya(HMPV KNH-S9-2008 JQ888113, HMPV KNH-S45-2008 JQ888114, HMPV KNH-S51-2008 JQ888115, HMPV KNH-S277-2008 JQ888117, HMPV KNH-S297-2008 JQ888118) have similar origin with those of Brazil and China. The Kenyan B2 Isolate HMPV KNH-S63-2008 JQ888116 also clustered with Chinese isolate GQ386856/TJ08-01, supported by boostrap value of 100. The clustering of the Kenyan isolates subtypes B2 and A2 on both sides of the phylogram shows how dynamic hMPV infections in the same population can be.

It is not possible to explain which country acquired the virus from the other. These findings can only emphasize the role of international travel in the spread of respiratory viruses.

5.5 Conclusions

- It is possible that the role of respiratory viruses in the global ARI burden has been underestimated because of the less sensitive viral diagnostic assays used particularly in the developing countries. The detection of 7(4.67%) hMPV cases suggests the involvement of the virus in pediatric pneumonia in Kenya.

- Phylogenetic analysis based on F gene sequences of six isolates confirmed co-circulation of hMPV subtypes A2a and B2 in the patients in 2008. Subtype A2a was however dominant, with 5 (83.3%) of the isolates.

  Sequencing data of the G gene would have given the same typing results, however being highly variable the data would have shown the effect of the host on virus evolution.

- Pylogenetic trees generated by MEGA 5.02, showed the close clustering of the Kenyan A2 isolates with the Brazilian and Chinese isolates. The B2 isolate on the other hand clustered with Tainjan, China isolate. These findings show the role of international travel in the spread of respiratory pathogens.

5.6 Recommendations

- The association of hMPV in with ARTI in pediatric patients in Kenya should be conducted in a prospective study. larger sample size and longer sampling periods should be considered in order to determine the prevalence and monthly trends of hMPV.
- Studies targeting other hMPV genes e.g G, L, M and N should be conducted. This will help in determining targets for possible interventions e.g vaccine and drug design.

- Further studies describing the molecular epidemiology of respiratory viruses including hMPV should be conducted in order to understand the global relatedness of the viruses.

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APPENDICES

Appendix I

Single and 3-letter codes for the 20 naturally occurring amino acids

Amino acid	Three letter code	Single letter code
Alanine	Ala	A
Cysteine	Cys	C
Aspartic Acid	Asp	D
Glutamic Acid	Glu	E
Phenylalanine	Phe	F
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Lysine	Lys	K
Leucine	Leu	L
Methionine	Met	M
Asparagine	Asn	N
Proline	Pro	P
Glutamine	Gln	Q
Arginine	Arg	R
Serine	Ser	S
Threonine	Thr	T
Valine	Val	V
Tryptophan	Trp	W
Tryrosine	Tyr	Y

Appendix II

QIAamp Viral RNA Mini Spin Protocol (QIAamp Viral RNA Mini Kit Handbook 01/99)

Important to note:

• Equilibrate samples to room temperature (15–25°C).

• Equilibrate Buffer AVE to room temperature for elution in step 10.

• Check that Buffer AW1, Buffer AW2, and Carrier RNA have been prepared according to the instructions.

• Re-dissolve precipitate in Buffer AVL/Carrier RNA by heating, if necessary, and cool to room temperature before use.

•All centrifugation steps are carried out at room temperature.

• Close each spin column in order to avoid cross-contamination during centrifugation.

Preparation of reagents

Addition of Carrier RNA to Buffer AVL

Check Buffer AVL for precipitate, and if necessary incubate at 80°C until the precipitate is dissolved.

Add 1 ml of Buffer AVL to one tube of lyophilized Carrier RNA. Dissolve Carrier RNA thoroughly. Transfer to the Buffer AVL bottle, and mix thoroughly before using Buffer AVL for the first time.

Note: If less Carrier RNA has been shown to be better for your particular amplification system, add 1 ml of Buffer AVL to one tube of lyophilized Carrier RNA, as described above, and transfer only the required aliquot to the Buffer AVL bottle. For example, if 1 µg Carrier RNA per ml of Buffer AVL has been shown to provide optimal RT-PCR efficiency, transfer 100 µl of reconstituted Carrier RNA to the Buffer AVL bottle. Discard the unused portion of the reconstituted Carrier RNA.

Buffer AW1

Buffer AW1 is supplied as a concentrate. Before using for the first time, add the appropriate amount of ethanol (96–100%) to Buffer AW1 concentrate as indicated on the bottle

Buffer AW2

Buffer AW2 is supplied as a concentrate. Before using for the first time, add the appropriate amount of ethanol (96–100%) to Buffer AW2 concentrate as indicated on the bottle.

Protocol

1. Pipette 560 µl of prepared Buffer AVL containing Carrier RNA into a 1.5-ml micro- centrifuge tube.

If the sample volume is larger than 140 µl, increase the amount of Buffer AVL/Carrier RNA proportionally (e.g., a 280-µl sample will require 1120 µl Buffer AVL/Carrier RNA).

2. Add 140 µl plasma, serum, urine, cell-culture supernatant, or cell-free body fluid to the Buffer AVL/Carrier RNA in the microcentrifuge tube. Mix by pulse-vortexing for 15 sec.

To ensure efficient lysis, it is essential that the sample is mixed thoroughly with Buffer AVL to yield a homogeneous solution. Frozen samples that have only been thawed once can also be used.

3.Incubate at room temperature (15–25°C) for 10 min.

Viral particle lysis is complete after lysis for 10 min at room temperature. Longer incubation times have no effect on the yield or quality of the purified RNA. Potentially infectious agents and RNases are inactivated in Buffer AVL.

4. Briefly centrifuge the 1.5-ml microcentrifuge tube to remove drops from the inside of the lid.

5. Add 560 µl of ethanol (96–100%) to the sample, and mix by pulse-vortexing for 15 sec. After mixing, briefly centrifuge the 1.5-ml microcentrifuge tube to remove drops from inside the lid.

Only ethanol should be used since other alcohols may result in reduced RNA yield and purity. If the sample volume is greater than 140 µl, increase the amount of ethanol proportionally (e.g., a 280-µl sample will require 1120 µl of ethanol). In order to ensure efficient binding, it is essential that the sample is mixed thoroughly with the ethanol to yield a homogeneous solution.

6.Carefully apply 630 µl of the solution from step 5 to the QIAamp spin column (in a 2-ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp spin column into a clean 2-ml collection tube, and discard the tube containing the filtrate.

Centrifugation is performed at 6000 x g (8000 rpm) in order to limit microcentrifuge noise. Centrifugation at full speed will not affect the yield or purity of the viral RNA. If the solution has not completely passed through the membrane, centrifuge again at a higher speed until all of the solution has passed through.

7.Carefully open the QIAamp spin column, and repeat step 6.

If the sample volume was greater than 140 µl, repeat this step until all of the lysate has been loaded onto the spin column.

8. Carefully open the QIAamp spin column, and add 500 µl of Buffer AW1. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp spin column in a clean 2-ml collection tube (provided), and discard the tube containing the filtrate.

It is not necessary to increase the volume of Buffer AW1 even if the original sample volume was larger than 140 µl.

9. Carefully open the QIAamp spin column, and add 500 µl of Buffer AW2. Close the cap and centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min. Continue directly with step 10, or to eliminate any chance of possible Buffer AW2 carryover, perform step 9a, and then continue with step 10.

Note: Residual Buffer AW2 in the eluate may cause problems in downstream applications. Some centrifuge rotors may vibrate upon deceleration, resulting in flow-through, containing Buffer AW2, contacting the QIAamp spin column. Removing the QIAamp spin column and collection tube from the rotor may also cause flow- through to come into contact with the QIAamp spin column. In these cases, the optional step 9a should be performed.

9a. **(Optional):** Place the QIAamp spin column in a new 2-ml collection tube (not provided), and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.

10. Place the QIAamp spin column in a clean 1.5-ml microcentrifuge tube (not provided). Discard the old collection tube containing the filtrate. Carefully open the QIAamp spin column and add 60 µl of Buffer AVE equilibrated to room temperature. Close the cap, and incubate at room temperature for 1 min. Centrifuge at 6000 x g (8000 rpm) for 1 min.

A single elution with 60 µl of Buffer AVE is sufficient to elute at least 90% of the viral RNA from the QIAamp spin column. Performing a double elution using 2 x 40 µl of Buffer AVE will increase yield by up to 10%. Elution with volumes of less than

30 µl will lead to reduced yields and will not increase the final concentration of RNA in the eluate.

Viral RNA is stable for up to one year when stored at –20°C or –70°C.

Appendix III

QIAquick PCR Purification Kit Protocol using a microcentrifuge (QIAquick Spin Handbook 03/2008)

This protocol is designed to purify single- or double-stranded DNA fragments from PCR and other enzymatic reactions (see page 8). For cleanup of other enzymatic reactions, follow the protocol as described for PCR samples or use the MinElute Reaction Cleanup Kit. Fragments ranging from 100 bp to 10 kb are purified from primers, nucleotides, poly- merases, and salts using QIAquick spin columns in a microcentrifuge.

Important points before starting

■Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).

■ All centrifugation steps are carried out at 17,900 x g (13,000 rpm) in a

conventional tabletop microcentrifuge at room temperature.

■Add 1:250 volume pH indicator I to Buffer PB (i.e., add 120 µl pH indicator I to 30 ml Buffer PB or add 600 µl pH indicator I to 150 ml Buffer PB). The yellow color of Buffer PB with pH indicator I indicates a pH of □7.5.

■Add pH indicator I to entire buffer contents. Do not add pH indicator I to buffer aliquots.

■If the purified PCR product is to be used in sensitive microarray applications, it may be beneficial to use Buffer PB without the addition of pH indicator I.

Procedure

1. Add 5 volumes of Buffer PB to 1 volume of the PCR sample and mix. It is not necessary to remove mineral oil or kerosene.

For example, add 500 µl of Buffer PB to 100 µl PCR sample (not including oil).

2.If pH indicator I has been added to Buffer PB, check that the color of the mixture is yellow.

If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow.

3.Place a QIAquick spin column in a provided 2 ml collection tube.

4.To bind DNA, apply the sample to the QIAquick column and centrifuge for 30–60s.

5. Discard flow-through. Place the QIAquick column back into the same tube.

Collection tubes are re-used to reduce plastic waste.

6.To wash, add 0.75 ml Buffer PE to the QIAquick column and centrifuge for 30–60s.

7.Discard flow-through and place the QIAquick column back in the same tube. Centrifuge the column for an additional 1 min.

IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.

8.Place QIAquick column in a clean 1.5 ml microcentrifuge tube.

9.To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.

IMPORTANT: Ensure that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA. The average eluate volume is 48 µl from 50 µl elution buffer volume, and 28 µl from 30 µl elution buffer.

Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water, make sure that the pH value is within this range, and store DNA at –20°C as DNA may degrade in the absence of a buffering agent. The purified DNA can also be eluted in TE buffer (10 mM Tris·Cl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.

10.If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.

Appendix IV

Study sequences

HMPV KNH-S9-2008 JQ888113

TATACCAACGTTTTTACATTAGAGGTGGGTGATGTAGAAAACCTCACATG
TGCTGATGGACCTAGCCTAATTAAAACAGAATTAGATCTGACCAAAAGT
GCACTAAGAGAGCTCAAAACAGTTTCTGCTGACCAATTAGCAAGAGAGG
AACAAATTGAGAATCCCAGGCAATCTAGATTTGTTCTAGGAGCAATAGC
ACTCGGTGTTGCGACAGCAGCTGCAGTTACAGCAGGTGTTGCAATTGCCA
AAACTATCCGGCTTGAGAGTGAAGTTACAGCAATTAAGAATGCCCTTAA
AAAGACTAATGAAGCAGTGTCTACATTGGGGAATGGAGTTCGAGTGTTA
GCAACTGCAGTGAGGGAACTGAAAGATTTTGTGAGCAAGAATTTAACTC
GTGCAATCAATAAAAACAAGTGCGACATTGATGACCTAAAAATGGCCGT
TAGCTTCAGTCAATTCAACAGAAGGTTTNTAAATGTTGTGCGGCAATTTT
C

HMPV KNH-S45-2008 JQ888114

TATACCAACGTTTTTACATTAGAGGTGGGTGATGTAGAAAACCTCACATG
TGCTGATGGACCTAGCCTAATAAAAACAGAATTAGATCTGACCAAAAGT
GCACTAAGAGAGCTCAAAACAGTTTCTGCTGACCAATTAGCTAGAGAGG
AACAAATTGAGAATCCCAGACAATCTAGATTTGTTCTAGGAGCAATAGC
ACTCGGTGTTGCGACAGCAGCTGCAGTTACAGCAGGTGTTGCAATTGCCA
AAACTATCCGGCTTGAGAGTGAAGTTACAGCAATTAAGAATGCCCTTAA
AAAGACTAATGAAGCAGTGTCTACATTGGGGAATGGAGTTCGAGTGTTA
GCAACTGCAGTGAGGGAACTGAAAGATTTTGTGAGCAAGAATTTAACTC
GTGCAATCAACAAAAACAAGTGCGACATTGATGACCTAAAAATGGCCGT
TAGCTTCAGTCAATTCAACAGAAGGTTTCTAAATGTTGTGCGGCAATTTT
C

HMPV KNH-S51-2008 JQ888115

TATACCAACGTTTTTACATTAGAGGTGGGTGATGTAGAAAACCTCACATG
TGCTGATGGACCTAGCCTAATAAAAACAGAATTAGATCTGACCAAAAGT
GCACTAAGAGAGCTCAAAACAGTTTCTGCTGACCAATTAGCAAGAGAAG
AACAAATTGAGAATCCCAGACAATCTAGATTTGTTCTAGGAGCAATAGC
ACTCGGTGTTGCGACAGCAGCTGCAGTTACAGCAGGTGTTGCAATTGCCA
AAACTATCCGGCTTGAGAGTGAAGTTACAGCAATTAAGAATGCCCTTAA
AAAGACTAATGAAGCAGTGTCTACATTGGGGAATGGAGTTCGAGTGTTA
GCAACTGCAGTGAGGGAACTGAAAGATTTTGTGAGCAAGAATTTAACTC
GTGCAATCAACAAAAACAAGTGCGACATTGATGACCTAAAAATGGCTGT
TAGCTTCAGTCAATTCAACAGAAGGTTTCTAAATGTTGTGCGGCAATTTT
C

HMPV KNH-S63-2008 JQ888116

TACACCAATGTCTTTACATTAGAAGTTGGTGATGTTGAAAATCTTACATG
TACTGATGGACCTAGCTTAATCAAAACAGAACTTGACCTAACCAAAAGT
GCTTTAAGAGAACTCAAAACAGTTTCTGCTGATCAGTTAGCGAGAGAAG
AACAAATTGAAAATCCCAGACAATCAAGGTTTGTCCTAGGTGCAATAGCT
CTTGGAGTTGCCACAGCAGCAGCAGTCACAGCAGGCATTGCAATAGCCA
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AACAACCAATGAGGCAGTATCAACACTAGGAAATGGAGTGCGGGTCCTA
GCCACTGCAGTAAGAGAGCTGAAAGAATTTGTGAGCAAAAACCTGACTA
GTGCGATCAACAAGAACAAATGTGACATTGCTGATTTGAAGATGGCTGTC
AGCTTCAGTCAGTTCAACAGAAGGTTCCTAAATGTTGTGCGGCAGTTTTC

HMPV KNH-S277-2008 JQ888117

TATACCAACGTTTTTACATTAGAGGTGGGTGATGTAGAAAACCTCACATG
TGCTGATGGACCTAGCCTAATAAAAACAGAATTAGATCTGACCAAAAGT
GCACTAAGAGAGCTCAAAACAGTTTCTGCTGACCAATTGGCAAGAGAGG
AGCAAATTGAGAATCCCAGACAATCTAGATTTGTCCTAGGAGCAATAGC
ACTCGGTGTTGCAACAGCAGCTGCAGTTACAGCAGGTGTTGCAATTGCCA
AAACCATCCGGCTTGAGAGTGAAGTTACAGCAATTAAGAATGCCCTTAA
AAAGACTAACGAAGCAGTGTCCACATTGGGGAATGGAGTTCGAGTGTTA
GCAACTGCAGTGAGGGAACTGAAAGATTTTGTGAGCAAGAATTTAACTC
GTGCAATCAACAAAAACAAGTGCGACATTGATGACCTAAAAATGGCCGT
TAGCTTCAGTCAATTCAACAGAAGGTTTCTTAATGTTGTGCGGCAATTTTC

HMPV KNH-S297-2008 JQ888118

TATACCAACGTTTTTACATTAGAGGTGGGTGATGTAGAAAACCTCACATG
TGCTGATGGACCTAGCCTAATAAAAACAGAATTAGATCTGACCAAAAGT
GCACTAAGAGAGCTCAAAACAGTTTCTGCTGACCAATTAGCTAGAGAGG
AACAAATTGAGAATCCCAGACAATCTAGATTTGTTCTAGGAGCAATAGC
ACTCGGTGTTGCGACAGCAGCTGCAGTTACAGCAGGTGTTGCAATTGCCA
AAACTATCCGGCTTGAGAGTGAAGTTACAGCAATTAAGAATGCCCTTAA
AAAGACTAATGAAGCAGTGTCTACATTGGGGAATGGAGTTCGAGTGTTA
GCAACTGCAGTGAGGGAACTGAAAGATTTTGTGAGCAAGAATTTAACTC
GTGCAATCAACAAAAACAAGTGCGACATTGATGACCTAAAAATGGCCGT
TAGCTTCAGTCAATTCAACAGAAGGTTTCTAAATGTTGTGCGGCAATTTT
C

Appendix V:

Scientific Steering Committee approval letter

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840 - 00200 NAIROBI, Kenya
Tel: (254) (020) 2722541, 2713349, 0722-205901, 0733-400003; Fax: (254) (020) 2720030
E-mail: director@kemri.org info@kemri.org Website:www.kemri.org

ESACIPAC/SSC/6138 **16th December, 2009**

Sammy N. Gichangi

Thro'
Director, CVR
NAIROBI

forwarded
FOR DIRECTOR
CENTRE FOR VIRUS RESEARCH
P. O. Box 54628
NAIROBI
17/12/2009

REF: SSC No. 1648 (Revised) – Isolation and characterization of human metapneumovirus from children with acute respiratory tract infections at Kenyatta National Hospital, Kenya

I am pleased to inform you that the above mentioned proposal in which you are the PI, has been given provisional approval by the KEMRI Scientific Steering Committee (SSC) and has been forwarded to the Ethical Review Committee (ERC) for consideration.

Kindly submit 4 copies of the revised protocol to SSC for onward transmission to ERC Office.

The SSC however, advises that work on this project can only start when ERC approval is received.

Sammy Njenga, PhD
SECRETARY, SSC

CENTRE FOR VIRUS RESEARCH
17 DEC 2009

Appendix VI:

Ethical Review Committee approval letter

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840 - 00200 NAIROBI, Kenya
Tel: (254) (020) 2722541, 2713349, 0722-205901, 0733-400003; Fax: (254) (020) 2720030
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KEMRI/RES/7/3/1 **February 11, 2010**

TO: **SAMMY SICHANGI (PRINCIPAL INVESTIGATOR)**
ITROMID STUDENT
EMAIL: sammysakini@yahoo.com

THRO': **DR. FREDERICK OKOTH,**
THE DIRECTOR, CVR,
NAIROBI

RE: **SSC PROTOCOL NO. 1648 (*RE-SUBMISSION*): ISOLATION AND CHARACTERIZATION OF HUMAN METAPNEUMOVIRUS FROM CHILDREN WITH ACUTE RESPIRATORY TRACT INFECTIONS AT KENYATTA NATIONAL HOSPITAL, KENYA.**

Make reference to your letter dated February 10, 2010. Thank you for your response to the issues raised by the Committee. This is to inform you that the issues raised during the 174th meeting of KEMRI/National Ethical Review Committee held on 19th January 2010, have been adequately addressed.

Due consideration has been given to ethical issues and the study is hereby granted approval for implementation effective this **11th day of February 2010,** for a period of twelve (12) months.

Please note that authorization to conduct this study will automatically expire on **10th February 2011.** If you plan to continue with data collection or analysis beyond this date, please submit an application for continuing approval to the ERC Secretariat by **28th December 2010**.

You are required to submit any amendments to this protocol and other information pertinent to human participation in this study to the ERC prior to initiation. You may embark on the study.

Yours sincerely,

R.C.Kithinji

R. C. KITHINJI,
FOR: SECRETARY,
KEMRI/NATIONAL ETHICS REVIEW COMMITTEE