PREVALENCE OF HUMAN METAPNEUMOVIRUS AND NASAL CYTOKINE PROFILES AMONG CHILDREN UNDER TWO YEARS OF AGE SEEN AT MTRH AND HURUMA SUB-COUNTY HOSPITAL.

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DECLARATION.

This thesis is my original work and to the best of my knowledge has not been presented for a higher Degree in any other university.

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DEDICATION.

This thesis is dedicated to my parents for their continual support and also to the children who participated in the study and without whom; the study would not have been possible.

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Prevalence of Human Metapneumovirus and Nasal Cytokine Profiles among Children under two years of Age seen at MTRH and Huruma Sub-County Hospital.

ABSTRACT

Introduction: Human metapneumovirus (hMPV) with tropism for the respiratory tract epithelium, causes an acute respiratory tract infection (ARI) accounting for 5-10% of all ARIs. Patients may be asymptomatic, or exhibit symptoms ranging from mild upper respiratory tract symptoms to severe bronchiolitis and pneumonia in the lower respiratory tract. Diagnostic markers of the various stages of infection are not well elucidated. It is important to study the nasal immune responses to understand its implication in disease resolution and pathology.

Objective: To determine prevalence and the nasal cytokine levels in children below two years of age infected by hMPV.

Study Methodology.

A cross-sectional design was adopted and the study was conducted at Moi Teaching and Referral (MTRH) and Huruma Sub-County Hospitals, where children meeting the inclusion criteria were enrolled into the study. Formula for prevalence survey used gave 196 children. A pre- tested questionnaire was used to obtain bio-data. First nasal swab was collected for viral screening by RT-PCR and viral load quantification by Cycle Threshold (C_T) and the second for quantification of the cytokines by ELISA. Research approval was sought from IREC and only children whose parents consented were enrolled. Data was collected by questionnaire and laboratory print-outs. Analysis was done using Stata version 13 special editions. The test for association between categorical variables was done using Pearson's Chi Square test while the test for association between categorical and continuous variables was done using ANOVA test.

Results: There were more males than females at 53% with children under six months of age at 36% as the largest group. There were eight participants with hMPV C_T determined, placing prevalence at 4% with mean hMPV C_T of 28.34 and RNP C_T of 24.03±2.68. The entire sample of participants positive for hMPV had median cytokine levels being; IL-2, 57.11(IQR: 34.49-70-49), IL-4, 30.49(IQR: 8.07-60.84), IL-8, 396.61(IQR: 81.91-530.71), IL-10, 26.98(IQR: 7.55-42.65), and IL-12-p70, 44.93 (IQR: 23.59-70.38). There was a positive association between cytokines; IL-10 and IL-12p70 P=0.0005, IL-2 and IL-10 p= 0.007, IL-2 and IL-12p70 P=0.015

Conclusions: hMPV is not a common Acute Respiratory Infection etiological agent as it is demonstrated in one in 25 patients. Its infection is associated with IL-4 hyper responsiveness.

Recommendations: C_T values of hMPV could play an important role in diagnosis of upper respiratory tract infection due to hMPV.

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LIST OF ABBREVIATIONS.

hMPV	Human metapneumo Virus
ARI	Acute Respiratory tract Infection.
SARI	Severe Acute Respiratory tract Infection.
ILI	Influenza-like Infection.
MTRH	Moi Teaching and Referral Hospital.
IREC-	Institutional Review and Ethic Committee.
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction.
RNA	Ribonucleic acid.
RNP	RNase P
CTLs	Cytotoxic T cells.
APC	Antigen presenting cells.
DCs	Dendritic Cells.
NK	Natural killer.
moDCs	Monocyte derived Dendritic Cells.
TLR	Toll Like Receptors.
ELISA	Enzyme Linked Immunosorbent Assay.
URTI	Upper respiratory tract infection.
LRTI	Lower respiratory tract infection.
KEMRI	Kenya Medical Research Institute.
CDC	Center for Disease Control and Prevention.
hMPV C_T	Human metapneumo Virus Cycle Threshold.
RNP C_T	RNase P Cycle Threshold.

CHAPTER 1

INTRODUCTION

Acute respiratory tract infections (ARI) are important causes of illness and mortality worldwide. The World Health Organization (WHO) estimates that acute respiratory infections (ARI) cause nearly one million deaths per year, making a fifth of all deaths. Rates are even higher in developing countries, where pneumonia is responsible for an estimated 11% of all deaths among children under 5 years of age (WHO Report 2008; WHO 2012). Viral causes are also associated with a substantial proportion of ARI. Viral infections can exacerbate chronic or recurring respiratory conditions, including asthma, thus representing an additional burden of respiratory viruses (Weiss et al., 2010; Kusel et al., 2007). The role of respiratory viruses such as human metapneumo virus is well established in causing self-limiting upper respiratory tract infection as well as mild pneumonia and severe pneumonia (Ruuskanen, O et al 2011; Asad et al., 2013).

Human metapneumo virus is an etiological agent of ARI and was discovered a decade ago by Van den Hoogen in 2001.Human metapneumo virus (hMPV) is an enveloped, negative – stranded RNA virus of approximately 13KB and a member of the *pneumovirinae subfamily* of the paramyxoviruses (Biacchesi, S. 2004).Various studies have suggested that hMPV is responsible for 5–10% of ARIs in neonates and children (Agarwal et al., 2011; van Den Hoogen et al., 2004; Esper et al., 2004). Based on recent studies the prevalence of hMPV is placed at 5.7% in refugee populations in Kenya (Ahmed et al., 2012). Omballa V et al., 2009 Supplementary paper reports a prevalence of 5-15% in different urban and rural populations.

In general, hMPV infections have been observed mainly in children, the elderly and immunocompromised adults (Boivin et al., 2002; Falsey et al., 2003; Sumino et al.,

2005; Shahda et al., 2011). HMPV has a tropism for the respiratory tract epithelium. Patients may be asymptomatic, or symptoms may range from mild upper respiratory tract symptoms to severe bronchiolitis and pneumonia in the lower respiratory tract (Boivin et al., 2003; Cane et al., 2003).In the course of hMPV infection; mild upper respiratory illness occur as Influenza -like Illness (ILI) defined as fever ≥ 38℃ and cough or sore throat. While severe disease described as Severe Acute respiratory illness (SARI) defined as; admission to the pediatric ward with any of the following: respiratory rate > 60 per minute, severe chest in drawing, nasal flaring, grunting, fever ≥ 38°C, hypothermia < 35.5°C, or pulse oxygenation < 90%. For children >1 week and < 2months. For those aged >2 months and < 2 years defined as cough or difficulty breathing and any one of the following: respiratory rate > 50/min for infants 2 months to < 1 year old or > 40/min for children 1 to < 2 years old, chest in drawing or stridor in a calm child, unable to drink or breast feed, vomiting, convulsions, lethargic or unconscious, or pulse oxygen saturation < 90% (WHO 2010; Ahmed et al.2012).

Studies have shown that inflammatory cells such as neutrophils and natural killer cells have been implicated in infection resolution as well as immunopathology (Huck et al., 2007). A recent study suggested that a low serum antibody level is a risk factor for hMPV infection but not for disease severity in adults (Falsey et al., 2010). It would be important to report if the same applies for infants.

T cell profiles, CD4+ T cells, and CD8+ T cells, play an antiviral role, and the two subsets act together synergistically in hMPV clearance from the lung (Alvarez & Tripp, 2005; Kolli et al., 2008). Whilst CD4+ and CD8+ Cytotoxic T Lymphocyte responses serve to clear current infection and protect against future infection, they also contribute to clinical disease and lung pathology, although the pathophysiology is

not well-understood (Kolli et al., 2008). A potential role of CD4 + (IL-4 producing) and not CD8 + T cells in disease aggravation has been suggested by Darnoit et al., 2009.

Pulmonary inflammation is critical in viral control in the airways. Phagocytic cells such as neutrophils are recruited into the lungs; however, an exacerbated inflammatory response may result in severe lung disease (Kolli et al; 2010).This may aid in viral control or cause extensive immunopathology. A Finish study demonstrated increased levels of IL-8(Jartti et al; 2002) in mucus secretions of children infected by the virus. IL-8 is a known neutrophil chemo-attractant. It is important therefore to investigate if this phenomenon will be replicated in human nasal mucosa, by correlating the disease severity and levels of IL-8.

Studies have not fully explored the role innate and adaptive immunity in hMPV infection. It is therefore important to determine the nasal immune response and inflammatory makers that may correlate with disease severity, with the aim of distinguishing the various stages of infection. Immune profiles correlated with disease severity will identify the role of immune cells in viral clearance as well as in the infection induced immunopathology.

1.1 Problem Statement.

Acute respiratory tract infections are important causes of morbidity and mortality in the world. hMPV is a well-established viral causative agent of ARI. Patients may be asymptomatic, or exhibit symptoms ranging from mild upper respiratory tract symptoms to severe bronchiolitis, mild and severe pneumonia in the lower respiratory tract. The very young, elderly and immunocompromised adults suffer the greatest disease burden.

Research has been done to describe the immune responses elicited against the virus. Some of these studies have indicated infection induced pathology. Characterization of the innate and adaptive immune responses that determine viral clearance or immune pathology remain unclear, also, the nasal immune responses have not been extensively studied. It is therefore important to examine the nasal cytokine profiles as this will indicate implication of nasal-mucosal immune response against the viral infection. Diagnostic markers of the various stages of infection are not well elucidated.

1.2 Justification.

The study is intended to determine nasal inflammatory profiles with the expectation that information obtained for the study will help elucidate nasal immune responses in the course of hMPV infection. The intent is to characterize the immune response to hMPV, specifically to understand whether immune responses lead to a quicker resolution or immune induced pathogenesis in vulnerable children. Examination of the nasal cytokine profiles will indicate the implication of nasal immune response against the viral infection.

Data from study will help provide an insight on whether nasal-mucosal immune Reponses lead to mild illness and resolution of the infection as opposed to severe cases of pneumonia. Proper diagnosis will ensure proper treatment of children hence avoidance of unnecessary use of antibiotics.

For study site; Moi Teaching and Referral Hospital (MTRH) Eldoret would be the ideal study site as the patients that visit the hospital come from different socioeconomic backgrounds in Eldoret town thus with proper sampling will provide a

representative sample. Huruma Sub-County Hospital will provide the additional number of participants needed to obtain the expected study sample.

Children aged below two years are the target sample as previous studies have used this age group as based on high infection rates and also because adults infected by hMPV are likely to asymptomatic.

1.3 Research Questions.

1. What is the prevalence human metapneumovirus Eldoret?
2. What quantities of cytokines are induced after infection with hMPV?

1.4. Null Hypothesis.

The nasal inflammatory markers in acute respiratory tract infections of hMPV do not reflect symptomatic infection.

1.5. Broad objective

To determine the prevalence and nasal cytokine levels in children less than two years of age infected by human Metapneumovirus.

1.5.1 Specific objectives.

1. To determine the prevalence of hMPV infection in children below two years of age.
2. To determine hMPV and RNase P (RNP) cycle threshold (C_T) in nasal samples.
3. To quantify nasal cytokine levels by ELISA.
4. To correlate inflammatory markers in symptomatic hMPV infection.

CHAPTER 2

2.1 LITERATURE REVIEW.

Acute respiratory tract infections account approximately for one million deaths worldwide accounting for a fifth of deaths. These death rates are even higher in low income countries accounting for 11% of all deaths (WHO report 2012; Williams et al., 2002). Viral infections can exacerbate chronic or recurring respiratory conditions, including asthma, thus representing an additional burden of respiratory viruses (Weiss et al., 2010; Kusel et al., 2007). The role of respiratory viruses such as Human Metapneumovirus is well established in causing self-limiting upper respiratory tract infection as well as mild pneumonia and severe pneumonia (Ruuskanen, O et al 2011; Asad et al., 2013).Various studies have suggested that Human Metapneumovirus (hMPV) is responsible for 5–10% of acute respiratory tract infections in neonates and children (Agarwal et al., 2011; Van Den Hoogen et al., 2004). Molecular epidemiology studies conducted in Kenya place the prevalence at 5-15% in various populations.

2.1.1 Classification

hMPV was first described and classified in 2001 in children infected with acute respiratory tract infection(Van den Hoogen et al:,2001). hMPV is a member of the *Metapneumovirus* genus of the subfamily *Pneumovirinae*, family *Paramyxoviridae*, order *Mononegavirales* (Biacchesi et al:, 2004).

2.1.2 Morphology

hMPV is a pleiomorphic particle in the range of 150–600 nm, with short envelope projections in the range of 13–17 nm (Fig. 1 Fig.2). Similar to some other paramyxoviruses such as hRSV and parainfluenza virus isolates, nucleocapsids are rarely observed (Van Den Hoogen et al: 2001 Peret et al: 2002).

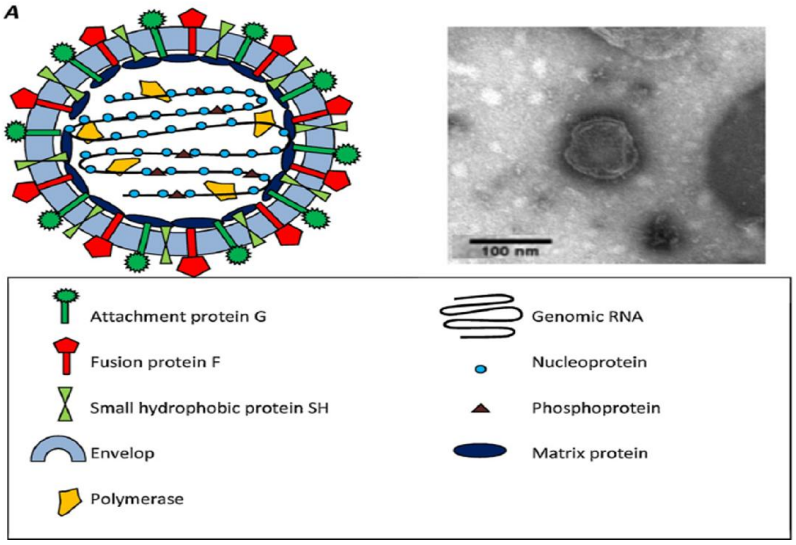

Figure.1 Schematic Representation of hMPV and an Electron Microscopic Image (F. Feuillet et al, 2012)

2.1.3 Genome

hMPV is a single negative sense RNA virus with a genome of 13Kb Fig.3 (Van den Hoogen:,2001, Biachessi et al:, 2003).The hMPV genome, which ranges in length from 13,280 to 13,335 nucleotides (nt) (Biacchesi et al :, 2003), contains eight genes in the order 3'-N-P-M-F-M2-SH-GL-5' and encodes nine proteins: M, matrix protein; F, fusion protein; M2-1, transcription elongation factor; M2-2, RNA synthesis regulatory factor; SH, small hydrophobic protein of unknown function; G, attachment glycoprotein; and L, viral polymerase.

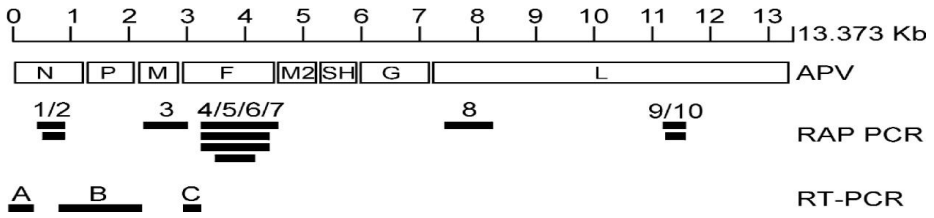

Figure.2 Schematic Representation of Genomic Fragments Obtained From hMPV (Van Den Hoogen 2002)

2.1.4 Phylogenetic diversity

hMPV isolates have been classified by phylogenetic analysis into two major genetic lineages termed subtypes A and B and are further subdivided into four subgroups (A1, A2, B1, and B2) (Agarwal et al., 2011; Wang et al., 2008; Arnott et al., 2011; Aberle JH et al., 2011; Gaunt et a., 2011; Biacchessi et al., 2003). Subtype A is the most dominant (Agarwal et al., 2011; Ablerle et al., 2011; Gaunt et al 2011).

2.1.5 Epidemiology

hMPV infections have been reported worldwide. Based on recent studies, the prevalence of hMPV is placed at 5.7% in refugee populations in Kenya where by the peak periods of incidence are November and December (Ahmed et al., 2012). Another study had similar reports of 5-15% in different urban and rural populations (Omballa V et al., supplementary paper). This rate is supported by findings in South Africa placing the prevalence at 5.8 %(Frank F. A. et al., 2003). hMPV was isolated in children and subsequently in the elderly (Boivin et al., 2002, Falsey et al., 2003; Van den Hoogen et al., 2003), and immunocompromised patients (Boivin et al., 2002; Pelletier et al., 2002; Cane et al., 2003). Young children, less than two years of age, are at risk for severe hMPV infection (Peiris et al., 2003; Van den Hoogen et al., 2003). Generally, all children are potentially exposed to the virus by age of five (Van den Hoogen et al., 2001).

2.1.6 Transmission

The shedding period and the mode of transmission are not known, but are suggested to be similar to other Paramyxoviruses, that is, transmission by aerosols (Hamelin et al., 2005; Semple et al., 2007). The incubation period has been estimated to be 4-6 days (Ebihara et al., 2004). Several studies have shown that cellular fusion during

infection by strains of hMPV from group A is stimulated at acidic pH, (Herfst S, et al., 2008; Easton AJ, et al., 2004).

2.1.7 Clinical features of hMPV infection

hMPV is an important respiratory tract pathogen causing both upper and lower respiratory tract infections in children (Ahmed et al., 2012; Bosis S et al., 2004; Jartti T et al., 2004; Peiris J S et al., 2003; Principi et al., 2004; Schildgen O., 2004; Semple M G et al., 2005; Van Den Hoogen., 2004; William J et al., 2004), who unlike adults, are rarely asymptomatic. Symptoms include; fever, cough, flu-like symptoms, sore throat, tachypnea, wheezing, and gastrointestinal symptoms such as nausea and vomiting. Less frequent symptoms include headache, muscle ache, rash, and conjunctivitis (Bastien et al., 2003; Wolf et al., 2006; IJpma et al., 2003). Severe lower respiratory tract disease is most frequently reported in very young, very old and in immunosuppressed patients (Fenwick et al., 2007).

2.1.8 Clinical and Radiological Diagnosis

Symptoms of cough, pharyngitis, tachypnea, wheezing, and prolonged expirium among patients with hMPV are similar to those found in RSV patients (Wolf et al., 2006). Radiological studies: More than 50% of hospitalized patients with an hMPV infection show pathological X-rays such as hyper expansion, peribronchitis, and atelectasis. Several respiratory viruses produce lobar or segmental pneumonias that are difficult to distinguish from bacterial lower respiratory tract infection (Wolf et al., 2006; Wilkesmann et al., 2007). In fact; neither clinical features nor radiological images permit a tentative differentiation between viral and bacterial pneumonias in infants and young children.

2.1.9 Laboratory diagnosis:

Currently, diagnosis of hMPV is based on molecular assays including conventional reverse transcription RT-PCR (van den Hoogen et al., 2004).The N gene is generally targeted in order to detect the 4 sub-groups of hMPV, since it is highly conserved among the different strains of hMPV (Maertzdorf J, et al.,2004). The superior sensitivity and specificity of RT-PCR has been shown. RT-PCR may detect as few as 5-10 viral genomes and is considered the gold standard method of diagnosis (Reina et al., 2007). Yet, in resource poor setting, RT-PCR may not be available in routine diagnostic laboratories (Fenwick et al., 2007).

Serological tests allow a retrospective diagnosis to confirm a recent infection, sero-conversion or, in case of pre-existing antibodies, a $\geq$ 4-fold increase in antibody titers must be demonstrated (Hamelin et al., 2004, 2005; van den Hoogen et al., 2004). In detection of hMPV, virus isolation in cell culture is used rarely because hMPV is difficult to detect due to its selectivity, slow growth, and mild cytopathicity (Boivin et al.,2002; Williams et al., 2004; Deffrasnes et al., 2005). Alternatively, detection can be performed by using an immunofluorescent bases assay: monoclonal antibodies targeted against viral antigens have been described allowing the detection of hMPV in nasopharyngeal samples (Gerna et al., 2006).

2.1.10. Viral load and Cycle Threshold C_T.

Viral load is an important measure in monitoring of infection. High viral copy numbers determine infectivity and the severity of infection. Data from previous studies revealed that there is a significant correlation between nasopharyngeal hMPV viral load and disease severity, that reflects the extent of an hMPV immune response (Huck et al., 2007), and that, a high viral load plays a role in clinical presentation (Bosis et al., 2008; Martin E.T. et al., 2012; Sarasini et al., 2006). Semi-quantitative RT-PCR (qRT-PCR) has the potential of estimating infectious virus levels that

correlate well with the standard curves using limiting dilutions of the purified PCR amplicons (Njenga et al., 2009; Martin et al., 2012; Fuller et al., 2013). For hMPV the association between the C_T values and severity is less clear (Fuller et al., 2013; Peng et al., 2010).

2.1.11 Infection and Specific Immune Response to hMPV

A viral infection results in immune responses, both innate and adaptive. During infection, the virus penetrates the epithelial cells and once the viral nucleocapsid is in the interior of the cytoplasm, proteins P, N and L dissociate from the viral RNA and in turn bind to each other forming the polymerase complex. The genomic RNA can thus serve as a matrix for viral transcription and replication, which occurs in the cytoplasm of infected cells. (Easton AJ, et al., 2004).The newly produced proteins P, N, L and M2 associate with neo synthesized viral genomes to form new nucleocapsids which will be incorporated into the virions during the budding at the surface of the cell membrane.

The envelope glycoproteins (F, G, SH) make their way via the Golgi apparatus to these zones of membranous accumulation and associate with them to be exposed at the surface of the infected cells (Mackay IM, et al., 2003).The progression of infection has also been observed in cell culture, via the viral-induced formation of syncytia.(Vargas, et al.,2004). By this mechanism, the infected cell merges with adjacent cells via the action of viral fusion proteins exposed at its surface, thereby allowing the spread of viral genomes (Herfst S, et al., 2008).

Infection is associated with airway epithelial cell changes and increased infiltration by inflammatory cells, the infiltrates being predominantly mononuclear cells in the lung interstitium. (Alvarez et al., 2004; Skiadopoulos et al., 2004; Wyde et al., 2005). Infection of the airway epithelial cells results in cell degeneration and or necrosis,

with ciliacytophthoria and round, red cytoplasmic inclusions on a background of haemosiderin laden macrophages, abundant neutrophils and prominent mucus (Vargas et al., 2004; Principi et al., 2006).

Re-infections with alternative subtypes may occur frequently in humans; children more than twelve years of age have higher virus neutralizing antibody titers than those aged five years (van den Hoogen et al., 2001). Furthermore, serological studies revealed that antibody titers measured by IF assays showed a greater range in individuals aged more than 2 years than in patients aged 6-24 months, suggesting a booster effect as a consequence of re-infection with the same or a closely related virus (Principi et al., 2006).

2.1.12. Adaptive Immune Response.

2.1.12.1. T cells (CD 8 and CD4)

Cytotoxic T cells play a role in control of hMPV infection. Studies by (Alvarez & Tripp, 2005; Kolli et al., 2008), show that CD4+ T cells, as well as CD8+ T cells, play an antiviral role, and the two subsets act together synergistically to effect hMPV clearance from the lung. Whilst CD4+ and CD8+ CTL responses serve to clear current infection and protect against future infection, they may also contribute to infection induced pathology, although the pathophysiology is not well-understood (Kolli et al., 2008).In humans, it has been shown that an hMPV-directed virus-specific memory response appears to persist for at least several years following clinical hMPV infection and, natural infection generates an IFN-γsecreting CTL response (Herd et al., 2008). Whilst recall of this response does not seem to prevent re infection, it may serve to halt serious lower respiratory-tract disease in healthy adults. This may not be the case in the very young, the elderly or immunocompromised individuals (Herd et al., 2010).

Moderate or no up regulation of Th2-type cytokine IL-10 was recorded following infection, this being consistent with the interpretation of a Th1-biased response (Guerrero-Plata et al., 2005; Huck et al., 2007). The matrix protein M-hMPV has been shown to induce Antigen Presenting Cell (APC) maturation, promoting their capacity to stimulate T lymphocytes and finally eliciting a predominant Th1 pattern of response (Bagnaud- Baule et al., 2011). It would be important; therefore, to assess the T cell associated cytokine profiles and correlate this with severity of the infection. This could be indicative of T cell subsets involved in immune response and predict the outcome of infection whether resolution or pathology.

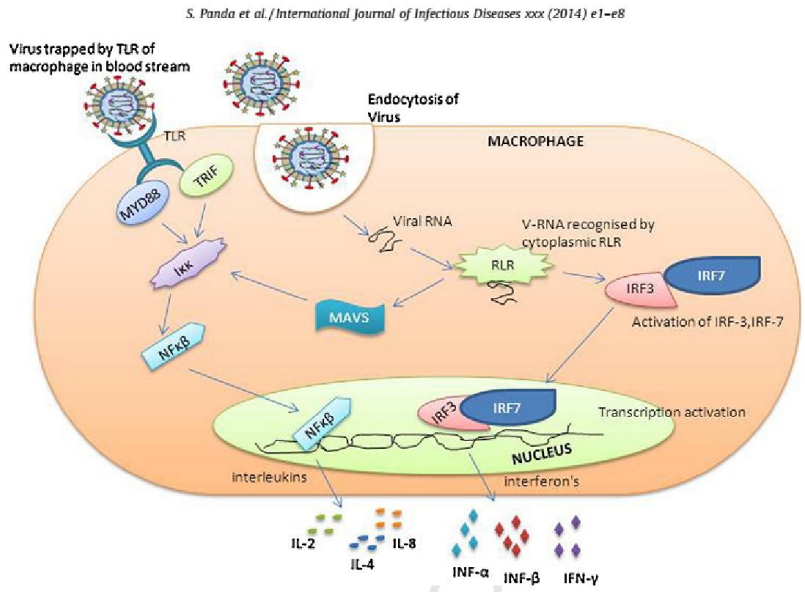

Figure.3: Pathogenesis of hMPV With Eventual Production of Cytokines

2.1.12.2 Cytokines.

Cytokines are immune modulators that play a role in activation and expansion of B cell and T Cells. Cytokines and chemokines such as; IL-1 ,IL-2, IL-12, IL-10, IFN-γ RANTES and MIP-β are key players in the migration of neutrophils, macrophages and lymphocyte (Moser B et al., 2001; Baggiollini M et al., 1994). hMPV infection is associated with enhanced expression of Th1-type (IL-12, IFN-γ) and antiviral (IFN-

β) cytokines (Herd et al.,2010). Most studies found up regulation of IFN-γ after hMPV infection (Guerrero-Plata et al., 2005; Hamelin et al., 2005; Huck et al., 2007). Severity of disease may not only be a consequence of inflammation but may be due to other mechanisms such as direct viral damage to the airways (Laham RF et al., 2004) and Th2 polarization leading to a state of pulmonary hyperactivity (Openshow PJ et al., 2001). Altered cytokine production with lower IFN-γ and higher IL-4 and IL-10 responses occurs in the elderly (Rink L et al., 1998). This age related shift: Th1-like (IFN-γ) to a Th2-like (IL-4 and IL-6) cytokine response is also supported by results from (Darnoit et al., 2009).

2.1.12.3 Dendritic Cells (DCs)

Cell type specific recognition of hMPV is through RIG-1 and TLR 7 for placmacytoid dendritic cells (pDCs). The F protein is thought to interfere with type 1 interferon production (Goutany et al., 2010). In culture, infection of dendritic cells (DCs) by hMPV resulted in maturation of the dendritic cells, with up regulation of MHC molecules and co-stimulatory molecules: CD83, MHC-I, MHC-II, CD38, CD80, CD86, HLA-DR (Vermaelen KY et al., 2001). This resulted in induction of cytokines such as high levels of IFN-γand low levels of IL-6 and TNF-α (Guerrero-Plata et al., 2005; Kolli et al., 2011), but minimal CD4 T cell activation (Guerrero-Plata, Casola, Suarez, et al.2006; Bagnaud- Baule et al.,2011). Some studies have described incomplete maturation of dendritic cells in response to hMPV (Tan et al., 2007).

TLR 4 plays an important role in hMPV-induced secretion of pro inflammatory cytokines and chemokines, as well as type I IFN in monocyte derived dendritic cells(moDCs), and hMPV G protein modulates cytokine and chemokine secretion in moDCs by targeting TLR4-dependent signaling (Kolli et al., 2011).

2.1.12.4 Antibody Response to hMPV.

Luseblink et al, 2010 found neutralizing antibodies against hMPV in all age groups with virus neutralization capacity. Neutralizing capacity increased in patients 10 to 29 years old before it reached a plateau. On average, the neutralizing effect was 10% higher for patients 29 to 69 years old than for patients less than 2 years old and 20% higher than for patients 2 to 9 years old. Starting with patients 70 to 79 years old, there was trend of decreasing neutralizing capacity and this was the same for children between 1 and 2 years of age. Sero prevalence of 100% has been reported in adults (Van den Hoogen et al., 2001). This may suggest that the hMPV-specific antibody response may contribute to protection against or reduction of the severity of the illness (Dranoit et al., 2007).

Neutralizing antibody titers are lower for younger than for older children, with proportions of 25% for 6- to 12-month-olds, 31% for 1- to 2-yearolds, 38% for 2- to 5-year-olds, and 75 to 100% for those over 5 years of age (Van Den Hoogen et al, 2001; Schildgen et al, 2011).

While most individuals have been exposed to hMPV by the age of 5, infection results in incomplete immunity and re infections occur in all age groups (van den Hoogen et al., 2001; Leung et al., 2005; Matsuzaki et al., 2008; Okamoto et al., 2010).

CHAPTER 3

3.1 Study Methodology.

3.1.1 Study Site

The site was Moi Teaching and Referral Hospital (MTRH) pediatric outpatient and inpatient units. MTRH was the ideal study site as it was a referral hospital that provided services to substantial number of Eldoret Town inhabitants. Patients who attended the hospital comprised of people from different socioeconomic backgrounds thus; a high probability that a representative sample was obtained. The second site was Huruma Sub-County Hospital. The two hospitals attended to 5-10 patients in a day who to fit the criteria. The laboratory assays were carried out in CDC/ KEMRI and AMPATH labs.

3.1.2 Study Design

The study design adopted was cross-sectional study. Samples were only collected once at the time of the study. This study design is useful in assessing prevalence.

3.1.3 Study Sample.

Study sample was calculated by

$$n = \frac{Z^2 P(1-P)}{d^2}$$

Where

n- The sample size

Z^2_{-Z} is the statistic confidence level; 95% therefore Z=1.96

d^2 -the precision; d=5%

p=prevalence of 0.15 was gotten from a previous study (Omballa et al., 2009) which placed prevalence between 5-15% in different urban and rural populations in Kenya. The highest prevalence was chosen.

$n= 1.96^2 *0.15(0.85)/0.05^2=196$

3.1.4 Sampling Methods.

Sampling methods used was convenience sampling; whereby a patient who visited the hospitals at the time of the study between 8:00AM and 5:00PM and were attended to by a clinician. Patients who presented with URTI or LRTI were recruited into the study.

The principal investigator or a trained research assistant sat in the same room with the clinician and upon diagnosis, they approached the parent or guardian and obtained consent and a child was enrolled into the study. A questionnaire was administered by the primary investigator and trained research assistant to obtain variables such as age, gender and number of siblings the participant had. This tool was adapted from an already pre-tested questionnaire used by KEMRI/CDC in influenza surveillance. Patients with LRTI were scored as per the WHO criteria. Children who met the above criteria and whose parents signed consent form were included into the study.

3.1.5 Inclusion Criteria:

Children aged two years old and under who presented clinically with URTI and LRTI case definitions after the clinicians diagnosis. The symptoms should have been experienced for less than seven days. It was also a prerequisite that the parents and guardians consented enrollment of their children in the study and they should have signed the consent form.

3.1.6 Exclusion Criteria:

Symptoms longer duration than 7 days as this was no longer considered an acute respiratory tract infection (ARI), or atopic children with asthma were excluded. Children whose parents and guardians did not sign the consent form were also excluded from the study.

3.1.7 Nasal Swabs.

Each study participant was sampled for 2 NP swabs, by use of nasal Nylon tipped flocked swab manufactured by COPAN Italia. The swab was inserted into the middle meatus of the nostril and turned carefully three times, then withdrawn.

The first NP swab was inserted immediately in viral transport medium (2mL). Storage done at -80^0C prior to virus detection by RT-PCR.

The second NP swab was inserted in 1ml tube containing physiological saline solution, in order to harvest nasal cells. This was also frozen at -80^0C and used for quantification of cytokines.

3.2 PCR; Viral diagnosis and viral load quantification.

RT-PCR was done to determine the viral load by determination of the Cycle threshold (C_T) levels. Data obtained was used to determine hMPV prevalence. RNase P (RNP) Cycle threshold was determined by RT-PCR and information data obtained was used to confirm the specimen integrity.

3.2.1 Procedure.

Viral nucleic acid was extracted from the nasal swab specimen. QIA amp RNA extraction kit will be used following the manufacturer's instruction manual. The extracted RNA was processed for PCR to detect presence of the virus. hMPV was confirmed by RT-PCR using primers targeted to the conserved region of hMPV nucleocapsid protein. The primers and probes were provided by CDC Atlanta and KEMRI CDC Nairobi. Ag-Path _ID One Step RT-PCR KIT manufactured by Life Technologies was used for PCR. The Real Time PCR cycling conditions for hMPV included Reverse Transcription at 45^0C for 10 min, followed by denaturation at 95˚C for 10 min, Enzyme activation at 95^0C for 0.15 minutes (Cycles), Annealing at 55˚C for 1 min, extension at 72˚C for 1 min and finally, data collection. Cycle threshold (C_T) represented the number of cycles need for amplification to occur.

Viral nucleic acid extracted from the swab was processed by RT- PCR to amplify RNP under the same cycling conditions as hMPV.

3.3 Cytokine Quantification

Cytokines were quantified using Enzyme linked Immunosorbent Assay (ELISA). IL-2, IL-4, IL-8 and IL-12p70 were quantified using Bio Legend ELISA Kits thus adopting a sandwich ELISA with Pre-coated with a capture antibody. The manufacture's' manual was followed. The absorbance was measured at 450 nm and standard curves prepared. IL-10 was also quantified by sandwich ELISA whereby the

plates were coated with capture antibody and incubated overnight then cytokine was quantified following the Manufacturers' manual. Absorbance was measured at 450nm and a standard curve was prepared.

3.4 DATA MANAGEMENT

3.4.1 Data Collection Tools

1. Data Collection tool included a questionnaire for obtaining the bio data. The questionnaire adapted was already pre-tested and Used by Centers for Disease Control (CDC) Nairobi in epidemiological studies for the virus and for routine Flu-surveillance. This tool captured the demographic data and the symptoms presented by the patient.

2. PCR result was recorded in Excel spread sheet showing the C_T values for the positive and negative samples.

3. The absorbance values from ELISA were recorded in Excel spread sheets.

3.4.2 Data Collection Methods.

For the laboratory assays, all the samples were assayed for PCR. Those who were PCR positive were assayed for cytokine profiles and the cytokine levels were described.

All questionnaires were verified for completeness and for purposes of data entry into spreadsheets, coding was done for the parameters that required yes and no answers.

3.4.3 Statistical Analysis.

Data analysis was done using Stata version 13 special edition. Categorical variables were summarized as frequencies and the corresponding percentages. Continuous

variables were summarized as mean and the corresponding standard deviation (SD) if it assumed the Gaussian distribution. If the continuous variable violated the Gaussian assumptions then it was summarized as median and the corresponding inter-quartile range (IQR). The assumptions for normality were assessed using Shapiro-Wilks test. Age was categorized as < 6 months, 6-12, months, 12-18 months, and >18 months. The test for association between categorical variables was done using Pearson's Chi Square test while the test for association between categorical and normally distributed continuous variables was done using one way analysis of variance (ANOVA). The relationship between the continuous variables was assessed using Spearman rank correlation coefficient since both or one of the variables violated the Gaussian assumptions. We reported the results and the associated p-values, and 95% confidence limits. Results were presented using graphs and tables.

3.5 ETHICAL CONSIDERATIONS.

Guardians and parents were requested to allow participation of their children in the study. They were informed in either English or Swahili of the procedures, their child's role in the study and that utmost confidentiality would be maintained and that, it was voluntary to participate or decline participation in the study. They were also informed that participation did not pose major risk to the child but rather, a slight discomfort would be experienced by the babies. Guardians who consented to their child's enrollment signed a consent form that would allow use of their child specimens. Two different specimens were collected from each child meeting the inclusion criteria. The samples were the 2 nasal swabs. Those guardians who neither consented nor signed the consent form were excluded from the study.

Approval of the study was obtained from the Institutional Research and Ethics Committee (IREC) and also from the collaborating institution (KEMRI/CDC) Nairobi.

Permission was sought from the hospital director at Moi Teaching and Referral for use of the institution as a study site.

Permission was also sought form Uasin Gishu county director for use of Huruma Sub-County Hospital as study site.

3.6 DISSEMINATION OF RESULTS

The results will be presented in scientific seminars and conferences such as the Annual Immunology conference. The findings will also be disseminated by publishing in scientific journals.

CHAPTER 4

4.1 RESULTS.

Data was collected between October of 2013 to January of 2014 and the findings are presented in figures and tables.

4.1.1 Demographic Data

There were a total of 196 participants whose data were included for analysis. The median age was 11(IQR: 6-17) months with a minimum of 1 month and a maximum of 24 months. The participants were distributed by age as shown in Figure 4.

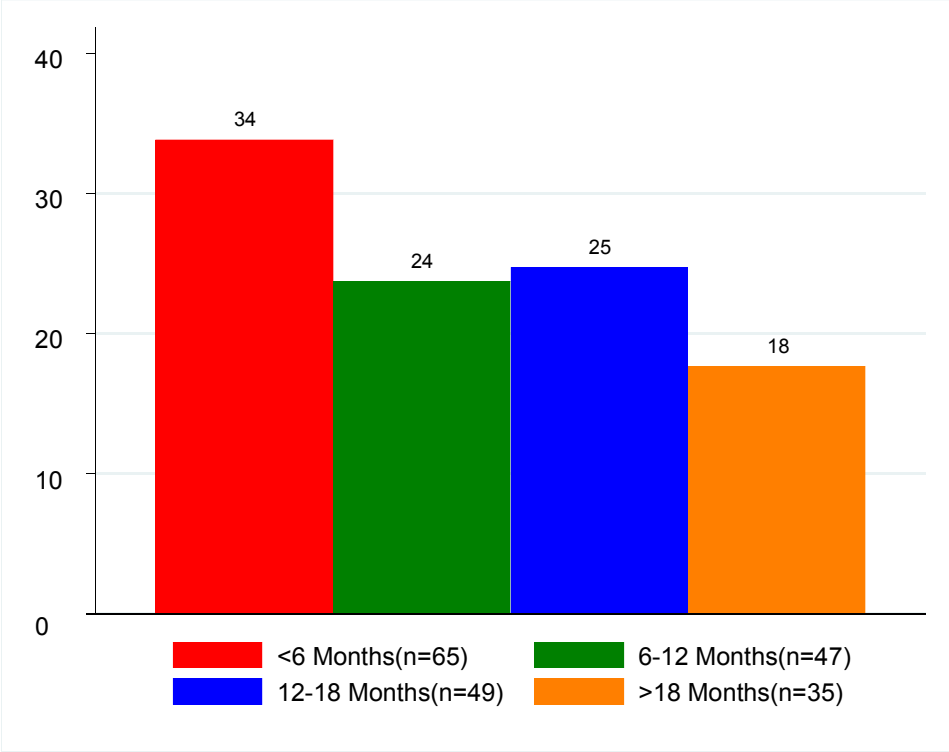

Figure 4: Distribution of Participants by Age

Figure 4 shows that majority of the children in the study were aged less than six months. This represented 67(34%). Relatively, the proportions in all the groups were similar. There were more males than females. Male represented 104 (53%).

The proportions of the male and female within each age group as seen in Table 1 were similar (P=0.785).

Table 1: Sex Distribution within Each Age Group

	Sex		
Age	**Female(n=92)**	**Male(n=104)**	**Total(n=196)**
<6 Months	28(31%)	37(36%)	65(34%)
6-12 Months	21(23%)	26(25%)	47(24%)
12-18 Months	25(27%)	24(23%)	49(25%)
>18 Months	18(19%)	17(16%)	35(18%)
Total	92(100%)	104(100%)	196(100%)
Pearson Chi Square value=1.07, Degrees of freedom=3, P=0.785			

4.1.2 PCR Findings

There were a total of eight participants positive for hMPV and whose C_T levels were determined as seen in table 2 below. The average hMPV C_T was 28.37.The average age for these patients was 13.38 months. Gender was equally distributed among these participants. The prevalence of hMPV infection was thus placed at 4%.

Table 2: The hMPV C_T Values from RT-PCR

Sex	**Age (months)**	**HMPV C_T**
F	17	20.24
M	2	22.29
F	17	24.21
M	24	25.66
F	14	27.42
M	4	27.85
M	17	39.27
F	12	40
Average	13.38	28.37

Table 3: Distribution of Symptoms in hMPV Infected Patients.

Symptom/ Patient Characteristic	**Present/Levels/ Number**	**n(%) / Mean(SD)**
Cough	Present	8(100%)
Sore throat	Present	1(12.5%)
Weight loss	Present	2(25%)
Lethargy	present	3(37.5%)
Difficulty in breathing	Present	1(12.5%)
Vomiting	Present	1(12.5%)
Diarrhea	Present	4(50%)
Temperature	Level	38.04(0.12)
Days with symptoms	Number	2.9(1.6)
Siblings present	Number	2(1)

All the participants who had hMPV C_T determined had a cough symptom while 25% weight loss and 37.5% were lethargic. Only one patient had a sore throat while another presented with vomiting. 50% of the participants presented with diarrheal symptoms. The average duration of the symptoms was 2.9±1.6 days and the average temperature was 38.04±0.12^{0}C. The average number of siblings the participants had was 2±1.

Samples were assayed for RNase P and any sample with RNase P-Cycle Threshold (RNP) $C_T < 40$ was rejected. The average RNP C_T was 24.03±2.68 with a minimum of 18.25 and a maximum of 34.08. Stratified by age, the median RNP C_T were as shown in Table 4.

Table 4: Distribution of RNP C_T within the Age Groups

Age group	Sample size	Mean ± SD	P
<6 Months	66	23.64 ± 2.34	0.166
6-12 Months	47	24.76 ± 2.85	
12-18 Months	49	23.99 ± 2.81	
>18 Months	34	23.88 ± 2.74	

The participants aged < 6 months had the lowest average RNP C_T of 23.64 ± 2.34. Those aged 6-12 months had the highest average RNP C_T values. The test for differences in RNP C_T among the four age groups was conducted. The results showed that the RNP C_T values in all the age groups were similar (P=0.166).

The Spearman's rank correlation was used to assess the correlation between age and RNP C_T. The results showed that the correlation is $r_{sp} = 0.023$, P=0.744, where r_{sp} – is the spearman rank correlation coefficient. There is very weak positive correlation, a demonstration that there is no linear relationship between the two.

4.1.3 ELISA Findings.

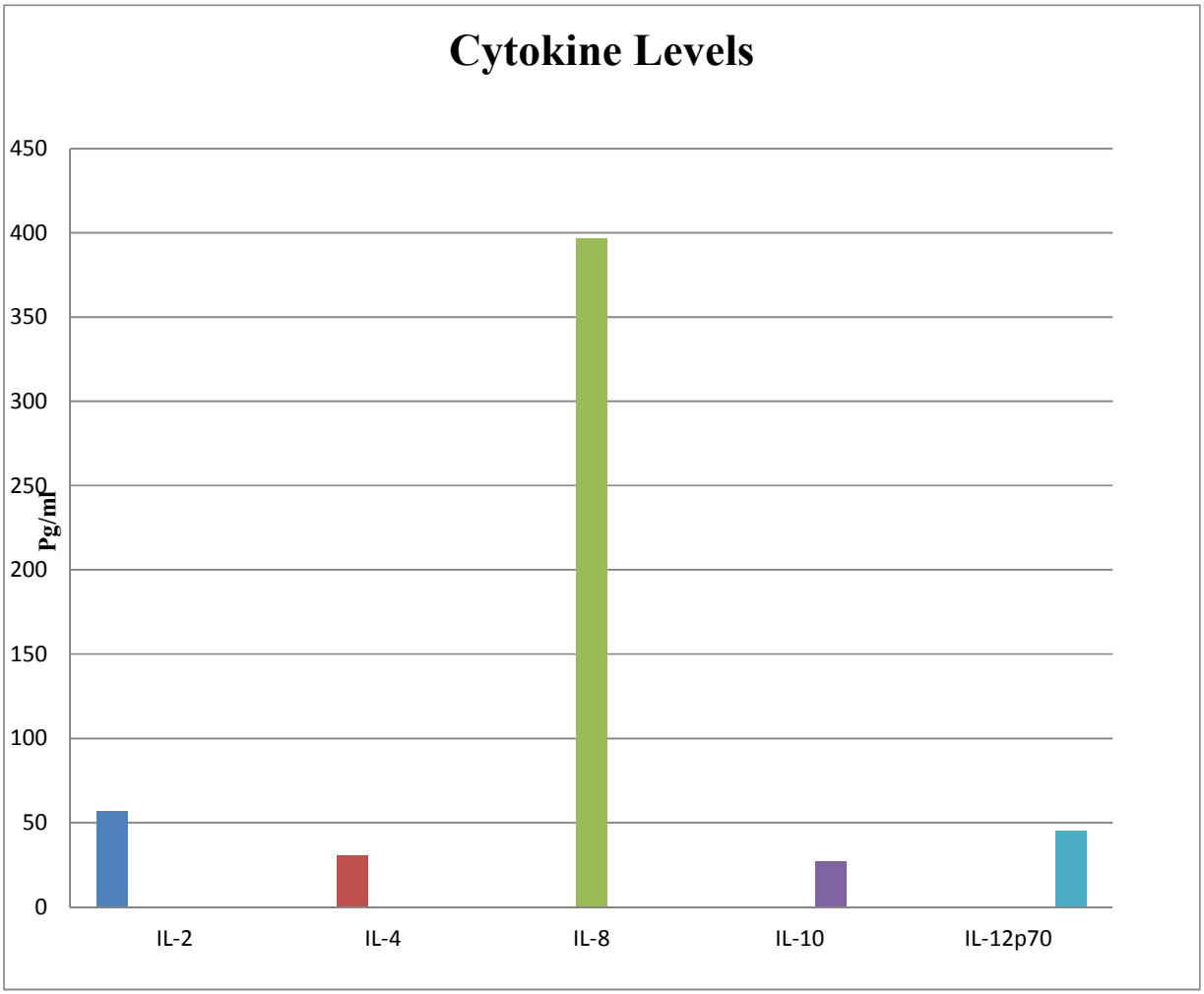

Figure 5: Median Cytokine Levels.

Distribution of the cytokine quantities induced in the course of hMPV infection in Figure 5. On comparison of all cytokines IL-8 appears to be induced in a greater magnitude.

Table 5. Distribution of Cytokine Quantities

Cytokine	Median (IQR) Levels
IL-2	57.11(34.49-70-49)
IL-4	30.49(8.07-60.84)
IL-8	396.61(81.91-530.71)
IL-10	26.98(7.55-42.65)
IL-12-p70	44.93 (23.59-70.38)

The sample of patients positive for hMPV was relatively small prompting the usage of median. The entire sample of participants positive for hMPV had their median levels of the cytokines shown in table 5.

Cytokine quantities were correlated with C_T levels.

Table 6: Association of hMPV C_T and Cytokine Quantities

CYTOKINE	Correlation Coefficient r	P value
IL-2	-0.167	0.693
IL-4	0.119	0.779
IL-8	-0.238	0.570
IL-10	0.048	0.911
IL-12p70	-0.238	0.570

IL-2, IL-8 and IL-12p70 correlated negatively with the C_T implying that high viral load was not associated with cytokines as shown in Table 6 above.

IL-4 and IL-10 had a positive association, implying that high viral load positively associated with cytokines, though no statistical significance was reported.

hMPV C_T was also correlated with the temperature the correlation coefficient r = 0.3696 and P= 0.184.

hMPV C_T was correlated with days patients presented with clinical symptoms. The correlation coefficient r= 0.570 and P= 0.069.

Results imply that the viral load associated with a positively with temperature and days the patients presented with symptoms, without statistical significance.

Table 7: Association of Cytokine Levels and Clinical Symptoms

Symptom	**IL-2**	**IL-4**	**IL-8**	**IL-10**	**IL-12p70**
	Median (IQR)	**Median (IQR)**	**Median ((IQR)**	**Median (IQR)**	**Median (IQR)**
Cough (8)	57.11(34.49-70-49)	30.49(8.07-60.84)	396.61(81.91-530.71)	26.98(7.55-42.65)	44.93(23.59-70.38)
Weight Loss (2)	38.82(34.49-43.16)	33.36(14.49-52.23)	403.0(39.99-766.0)	35.51(28.37-42.65)	57.66(44.93-70.38)
Lethargy (3)	57.11(34.49-58.83)	52.23(8.07-151.74)	239.42(81.91-766.0)	26.98(5.26-42.65)	40.41(23.59-70.38)
Vomiting (1)	57.11	151.74	81.91	5.26	23.59
Diarrhea (4)	38.82(32.81-172.59)	22.49(10.56-41.36)	404.69(218.30-589.39)	35.51(17.96-49.45)	57.66(26.31-103.52)

Association between the cytokine levels was calculated by use of Pearson's' Chi square and the cytokine levels were as presented in table 7 above. The statistical significance was not calculated due to the limited sample size. An interquartile range for vomiting was not worked out since only one participant presented with this symptom hence the representation of the participants' actual cytokine levels.

Table 7 in the previous page shows how specific clinical symptoms in patients who had their hMPV C_T determined associated with cytokines. Cough, lethargy and diarrhea associated with high levels of IL-8 whereas vomiting associated with high levels of IL-4 but with very low levels of IL-10.

Table 8. Association between Cytokine Levels and Patients characteristics.

Cytokine	**Correlation coefficient.**	**Correlation Coefficient**
	(Fever)	**(Days with symptoms)**
IL-2	-0.37	-0.05
IL-4	0.28	0.04
IL-8	-0.09	-0.23
IL-10	-0.1	0.02
IL-12-p70	-0.17	0.03

There was negative correlation between fever, and IL-2, IL-8, IL-10, and IL12-P70 as seen in table 8. There was a positive correlation between fever and IL-4 implying high levels of IL-4 is positively associated with fever. Symptoms duration had negative correlation with IL-2, and IL-8 but a positive correlation with IL-4, IL-10, and IL12-P70. The implication is that high levels of IL-2 and IL-8 had no association with the duration of illness while for the other cytokines; IL-4, IL-10 and IL-12p70 were positively associated with the duration of the illness.

Association between cytokines was calculated and correlation between IL-4 and IL12-P70 was -0.28 but not statistically significant, P=0.502. This implies that an increase in IL-4 will not lead to an increase in IL-12P70.

There was a statistically significant association between IL-10 and IL-12P70 r= 0.94, **P=0.0005.** This strong positive correlation implying that an increase in IL-10 was positively associated with an increase in IL12-P70. IL-2 associated with IL-10, and IL-12P70, **P=0.007**, and **0.015** respectively.

CHAPTER 5

DISCUSSION

This prospective study aimed to determine prevalence of hMPV and assess the nasal cytokine profiles. hMPV was found not to be an important etiological factor of acute respiratory illness with patients exhibiting mild clinical illness.

Our study demonstrates hMPV circulation amongst the Eldoret populations. hMPV was detected and the prevalence was placed at one in twenty five patients thus was slightly lower than reports from other study which placed the prevalence between 5-10% in refugee populations in Kenya where the peak incidence periods were November and December (Ahmed et al., 2012). Another study in Kenya also placed the prevalence in different urban and rural populations at 5-15 %(Omballa V., Supplementary paper 2009). Our data supports the assumption that clinical manifestation associated most commonly with hMPV for the out patients included; cough and fever which were seen in all patients who presented clinically with mild upper respiratory illness. In our study, this was reflected by all (100%) of the hMPV-positive samples; similar results are reported by other studies (Mona S. Embarek Mohamed et al.2014; Reiche J. et al., 2014). Never the less, lower respiratory infection such as pneumonia can be presented clinically.

Quantitative Real-Time PCR (qRT-PCR) was used to measure the amounts of virus in a clinical specimen and the cycle threshold (C_T) for viral load determination. Semi-quantitative RT-PCR (qRT-PCR) has the potential of estimating infectious virus levels that correlate well with the standard curves using limiting dilutions of the purified PCR amplicons (Njenga et al., 2009; Martin ET et al., 2012; Fuller et al., 2013; Life Technologies 2011). In order to confirm the specimen integrity, each

sample was evaluated for the human housekeeping gene *RNase P (*RNP). Younger children had lower C_T values than older children but this was not statistically significant. Generally the average hMPV C_T value was low, this, being indicative of higher viral loads. The lower C_T values observed was inconsistent with that found in another study (Fuller et al., 2013), this could be due to the varied swabs used; Nylon vs polyester tipped swabs. It is also possible that the lower C_T values reported in our study were due to the point in time in the course of infection. On correlating the viral load and fever, we report no association this being consistent with findings from a previous study. (Houben et al,.2010). We report a positive association between C_T values and days since onset though the association was not statistically significant.

Our study characterized inflammatory markers and how they relate with specific clinical symptoms. The inflammatory markers we characterized were elevated with the exception of IL-12p70 whereby low levels were observed when compared to the serum levels in healthy children (Giulio et al., 2013). It is possible that inflammation leads to resolution of infection by direct antiviral activity or by inducing innate and adaptive immunity. IL-4 hyper responsiveness was observed and this could be indicative of TH2 bias. It is therefore possible that IL-4 could play an important role in hMPV infection pathophysiology as opposed to viral clearing. This finding is consistent with reports from other studied that reported Th-2 biased cytokine response (Hamelin et al., 2005; Alvarez &Tripp., 2005). High levels of IL-8 in mild disease, this could indicate that IL-8 is important in infection resolution since, IL-8 is a portent neutrophil chemotactic and activating factor. Moreover, regulated recruitment and clearance of neutrophils is a hallmark of competent host defense. In study of RSV, IL-8 is thought to play an essential role in pathogenesis of severe RSV infections (Hall, 2001). This may not be the case in hMPV, since all the participants had high levels of

IL-8 despite the mild disease. Low IL-12p70 levels were observed in our study. IL-12 plays a vital role in inducing antiviral interferon; it is likely that this cytokine plays a critical role in viral clearance. Similar findings have been reported whereby, hMPV infection is associated with enhanced expression of Th-1 type (IL-12 and IFN-γ) and antiviral (IFN-γ) (Herd et al., 2010). The low levels of IL-12 observed could be explained by inhibition of T cell proliferation by hMPV infected dendritic cells (PF Cepedes et al., 2013). We also sought to understand the role of IL-10 a regulatory cytokine. We observed slightly elevated levels of IL-10. This cytokine was associated with a significant positive correlation with IL-12p70 in that, an increase in IL-12p70 was associated with increased IL-10. This may imply the regulatory role of IL-10 to IL-12. Consistent findings have been reported on IL-10 levels (Laham et al., 2004). IL-2 is vital in promoting clonal expansion of T cells we measured IL-2 quantities to understand how hMPV induces this cytokine. We report moderate elevation of IL-2. A recent study reported similar findings with low IL-2 production in adults infected with the virus. The low levels were observed from day 1 of infection to day 12 (Talaat et al., 2013). Lower levels of IL-2 reported could be because hMPV is thought to infect dendritic cells and interfere with their capacity to activate T cells (Le Nouen et al., 2009); also, hMPV interferes with DCs function in priming antigen-inexperienced T cells (P F Cepedes et al., 2013). On relating IL-2 with IL-10 and IL-12p70 there was a statically significant correlation implying that an increase in IL-2 associated with subsequent increase in IL-10 and IL-12P70, showing that IL-2 functions in expansion of the T cell lineage.

We associated the cytokine levels with some clinical symptoms. Fever associated with a negative correlation with IL-2, IL-8, IL-10 and IL-12P70 but a positive correlation with IL-4. This implies that a high level of IL-4 is associated with fever. It

is possible that these symptoms occurred as result of the viral infection rather than higher cytokine levels as a recent study reported fever to be associated with virus-positive exacerbation in infants (Kapur N,Mackay IM, Sloots TP,et al.2013). IL-8 on the other hand did not associate with fever inasmuch as IL-8 is known to be pyrogenic (Zamporino et al. 1994). We sought to understand if duration of disease was in any way associated with the quantities of cytokines secreted by the infected children. We related the number of days with symptoms to quantities of cytokines. IL-2 and IL-8 had a negative correlation while IL-4, IL-10 and IL-12p70 had a positive correlation. The implication is that IL-2 and IL-8 are not associated with duration of illness. High levels IL-4, IL-10 and IL-12P70 are associated with duration of illness. Data from previous study indicate that IL-4 peak on day 5 of infection (Talaat et al., 2013); it is therefore possible that we would have observed higher IL-4 quantities had most of our patients been ill for a longer time. The average duration of disease in our study was 3 days. IL-10 and IL-12P70 may associate positively with the duration of illness. We speculate that these two cytokines are likely to peak at the same time. Data from our study indicate a statistical significant correlation between IL-10 and IL-12P70. A recent study reported that peak secretion of IL-10 may coincide with viral shedding which peaks between day 5 and 10 (Talaat et al., 2013). Upon comparing the median cytokine levels in patients who had lost weight and those who did not, we report that patients who lost weight had higher cytokine levels compared to those who did not for all cytokines except IL-2. We cannot however report that weight loss is associated with higher cytokine levels since weight loss could be as a result of the clinical manifestation of hMPV infection.

Our data indicate no association between the viral load and cytokine quantities. We correlated cytokine quantities with the C_T and report no association.

5.2. LIMITATIONS

1. Children with unknown asthma may not have been excluded from the study.

2. There were no patients who tested positive for hMPV and presented with severe illness this rendered it impossible to compare immune responses and PCR C_T in upper respiratory tract and severe, lower respiratory tract ill patients.

3. The study participants were not screened for co-infections.

4. The study was done between October, 2013 and January, 2014 and may not be representative of the annual rates of infection.

CHAPTER 6

6.1 CONCLUSIONS

1. hMPV is not a common etiology for Acute respiratory illness as it is only demonstrated in one in 25 of patients.
2. Cycle threshold from qRT-PCR is an important diagnostic assay for acute respiratory illness.
3. There is cytokine elevation in nasal secretion of children infected with hMPV.
4. hMPV infection is associated with IL-4 hyper responsiveness.

6.2 RECOMMENDATIONS

1. C_T values of hMPV could play an important role in diagnosis of Upper respiratory tract infections in a clinical setting.

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APPENDICES

TIME PLAN

YEAR	2013	2014/2015
MONTH	S O N D	J F M A M J J A S O N
Proposal writing	X XX	
Proposal presentation	X	X XX
Ethical approval		X
Sourcing for study participants		X
Sample collection		X X
Lab; PCR and serology.		X XX
Data processing and analysis.		X X
Thesis writing		X X
Thesis Presentation		X X
Defense of Thesis		X

BUDGET

	PRICE (Ksh.)	TOTAL (Ksh.)
RT-PCR KIT for 500 rxn	@ 50,000	50,000
PCR Extraction Kit	@ 25,000	25,000
Laboratory consumables	40,000	40,000
ELISA KIT	@25,000	50,000
Stationary and computer services	10,000	10,000
TOTAL		175,000

CONSENT FORM

My Name is Imelda Leitich and I am a Masters student in the department of immunology. I am undertaking a research titled: **Human Metapneumo Virus-Induced Nasal Immune Responses in Children Under 2 Years Attending Moi Teaching and Referral Hospital, Eldoret.** This virus causes acute respiratory infection that may be mild or severe. I am requesting to enroll you child to aid in this research. Two nasal swabs will be obtained for viral detection and enumeration of nasal immune cells in the lab.

The process of obtaining the samples will not pose any risk to the child. Sterile nasal swabs will be used thus minimizing any risk to infection. There will be a mild discomfort. The specimen taken will be used solely for research purposes. You are assured of confidentiality, while in the lab, samples will have codes and no names of the child will appear on the samples.

The blood samples and glass slide smears will be stored for another three years incase reference from this information will be needed for publications. Nasal swabs for Identification of the virus will also be stored for three years at -80^0C. Nasal swabs in normal saline cannot be stored as refrigeration will result in tissue destruction and will therefore be destroyed by incineration after the cells have been extracted. Glass slides prepared by cytospin smears for staining will be stored in a lockable safety cabinet at room temperature. After three years, all samples: Nasal swabs and Glass slides will be incinerated.

You are assured that enrollment to the study is voluntary and there are no adverse effects for refusal. All questions you have about the research will be answered to the best of my ability and I can also be contacted through Cell number: 0725- 893467. If you accept to enroll your child to the study, you will be required to sign a written informed consent.

CONSENT FORM

I have been informed that all personal information conveyed to the researcher will be treated with utmost confidentiality.

I have been informed by the researcher of the procedures, risks and benefits that the study is expected to yield to humanity. 2 sterile nasal swabs will be used to obtain nasal secretion. This will cause minor discomfort but no pain. I have been informed that no harm will be caused by this procedure to my child.

I have been informed that the purpose of the specimen obtained will be solely for research and I will be given information arising out of this study. I have also been informed that sample taken from my child will be stored for three years incase reference information is need for publication and after these three years, all samples and slides will be incinerated.

I have been informed that refusal to participate in the study will in no way hinder receipt of services I am seeking from the health institution. I have also been informed that I am at will to withdraw from participation without any penalty.

I am therefore making an informed consent on behalf of my child in allowing specimens to be obtained and used for purposes of research.

Signature of Parent or
Guardian……..Date…….

Signature of the
witness…….....Date……

CONSENT SWAHILI (FOMU IDHINI)

Jina langu ni Imelda Leitich na nina Masters mwanafunzi katika idara ya immunology Chuo Kikuu cha Moi. Mimi ningependa kufanya utafiti yenye jina: **Human Metapneumo Virus-Induced Nasal Immune Responses in Children Under 2 Years Attending Moi Teaching and Referral Hospital, Eldoret.**Virusi vinavyosababisha homa inayoweza kuwa kali. Mimi nakuomba kuandikisha mtoto wako katika utafiti huu. Swabs mbili ya pua itachukuliwa itumiwe kwa kutambua virusi na kuhesabu seli katika maabara.

Mchakato wa kupata sampuli sio hatari kwa mtoto. Kutakuwa na usumbufu mdodgo. Specimen zinachukuliwa zitatumika kwa madhumuni ya utafiti.Wewe ni uhakika ya usiri,katika maabara, sampuli itakuwa na codes na hakuna majina ya mtoto itaonekana kwenye sampuli.

Sampuli zitahifadhiwa kwa miaka mingine mitatu kama zitahitajika kwa machapisho. Swabs ya pua pia zitahifadhiwa kwa muda wa miaka mitatu katika -80C. Baada ya miaka mitatu, kila sampuli: swabs pua na slides itakuwa incinerated.

Wewe ni unahakika kwamba uandikishaji katika utafiti ni kwa hiari na hakuna athari mbaya kwa kukataa. Maswali yote una kuhusu utafiti yatajibiwa kwa kadri ya uwezo wangu na pia, tunaweza kuwasiliana kupitia namabri ya simu: 0725- 893467. Kama wewe umekubali kuandikisha mtoto wako ili kusaiadia kwa utafiti, wanapaswa kutia saini kwakuandika kwenye taarifa ridhaa.

FOMU IDHINI

Nimefahamishwa kwamba taarifa za kibinafsi atakayo ambiwa mtafiti itahifadhiwa kwa usiri mkubwa.
Nimefahamishwa utaratibu wa utafiti, hatari na faida na kwamba utafiti inatarajiwa kuwa ya manufaa kwa binadamu. Swabs za pua zitatumika ili kupata kamasi. Hii itasababisha usumbufu madogo lakini hakuna maumivu. Nimefahamishwa kwamba hakuna madhara yanaweza kusababishwa na taratibu hii kwa mtoto wangu.

Nimefahamishwa kuwa lengo la kuchukuwa specimen ni kwa ajili ya utafiti na mimi nitapewa taarifa itakayojitokeza kutokana na utafiti huu. Mimi pia nimejulishwa kuwa sampuli zilizochukuliwa kutoka mtoto wangu itawekwa kwa miaka mitatu iwapo itaitajika kwa uchapishaji. Baabaye sampuli zote zitakuwa incinerated.

Nimefahamishwa kwamba kukataa kushiriki katika utafiti ni kwa mapenzi yangu na hakutakuwa na madhara ya kukatazwa matibabu ninayotafuta kutoka taasisi hii ya afya. Mimi pia nimambiwa kwamba mimi niko huru kuondoka kutoka ushiriki wa utafiti kwa hiari yangu.
Mimi basi napeana ruhusa kwa niaba ya mtoto wangu, specimen ichukuliwe ili kutumika kwa madhumuni ya utafiti.

Saini ya Mzazi au
Guardian……..Tarehe…….....

Saini ya mshahidi.....Tarehe.....