

**DETECTION AND CHARACTERIZATION OF HUMAN PARAINFLUENZA
VIRUSES IN GHANAIAN CHILDREN BELOW FIVE YEARS WITH ACUTE
LOWER RESPIRATORY TRACT INFECTIONS**

By

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**This thesis is submitted to the University of Ghana, Legon in partial fulfilment of the
requirement for the award of MPhil Microbiology degree**

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DECLARATION

I, Comfort Akorfa Fiave do hereby declare that with the exception of cited references, all the work described and the formation given in this thesis was done by me under the supervision of Dr. Theophilus K. Adiku of the Department of Microbiology, University of Ghana Medical School, College of Health Sciences and Prof. William K. Ampofo of the Department of Virology, Noguchi Memorial Institute for Medical Research, Legon.

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ABSTRACT

Worldwide, acute lower respiratory tract infections in young children are one of the leading causes of childhood mortality. The need to investigate respiratory viruses associated with acute lower respiratory tract infections, more especially all four types of human parainfluenza viruses in Ghana has become crucial due to the limited information available on these viruses. Thus the aim of this study was to detect and characterize human parainfluenza viruses in children that present with acute lower respiratory tract infections at Princess Marie Louise Children's Hospital in Accra, Ghana. From March to July 2013, nasopharyngeal swabs were collected from 71 children and the presence of human parainfluenza viruses was investigated. Ribonucleic acid extracts of the nasopharyngeal swabs were subjected to one-step real time reverse transcriptase polymerase chain reaction. Positive samples were further analysed molecularly. Also, the demographics of participants, risk factors for human parainfluenza viruses infection as well as clinical presentations were also evaluated. A 19.7% positive test rate was recorded for the human parainfluenza viruses. All four types of the virus were detected but with different frequencies (five were type 1, one was type 2, six were type 3 and two were type 4). No multiple serotype infection of human parainfluenza viruses was detected in the positive cases. Positivity was highest (57.1%) in children who were less than one year old. Males and females were equally infected. Phylogenetic analysis of the human parainfluenza viruses sequenced revealed that the strains within each type were closely related and clustered with reference strains. Age and having siblings with respiratory tract infections were risk factors significantly associated with the human parainfluenza viruses' infection. In conclusion, all four types of human parainfluenza viruses are in circulation in Ghana and were associated with acute lower respiratory tract infections.

DEDICATION

This work is dedicated to the glory of God Almighty for journeying with me throughout this research period. I also dedicate this thesis to my family for their love and priceless support. Lastly I dedicate this thesis to my supervisors for their enormous contributions towards the success of this work.



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LIST OF ABBREVIATIONS

A	Adenine
ABI	Applied Biosystems
ALRTIs	Acute Lower Respiratory Tract Infections
C	Cytosine
CDC	Centre for Disease Control and Prevention
cDNA	Complementary deoxyribonucleic acid
Ct	Threshold cycle
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
ddATP	dideoxyadenosine triphosphate
ddCTP	dideoxycytidine triphosphate
ddGTP	dideoxyguanosine triphosphate
ddTTP	dideoxythymidine triphosphate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
dsDNA	double stranded deoxyribonucleic acid
dTTP	deoxythymidine triphosphate
ELISA	Enzyme- linked immunosorbent assay
F	Fusion protein
g	Gram
G	Guanine

HPIVs	Human parainfluenza viruses
L	Large polymerase protein
LRT	Lower respiratory tract
LRTIs	Lower Respiratory Tract Infections
M	Matrix protein
ml	Millilitre
mRNA	Messenger ribonucleic acid
nm	Nanometre
n.d.	No date
NFW	Nuclease free water
NP	Nucleocapsid protein
OR	Odds ratio
P	Phosphoprotein
pmol	Picomole
RNP	Ribonucleoprotein
rpm	Revolutions per minute
rRT-PCR	real time Reverse Transcriptase Polymerase Chain Reaction
RTIs	Respiratory Tract Infections
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SH	Small hydrophobic protein
T	Thymine
TAE	Tris acetate EDTA (EDTA= ethylenediaminetetra-acetic acid)
UNAIDS	The Joint United Nations Programme on HIV/AIDS

URT	Upper respiratory tract
URTIs	Upper respiratory tract infections
UV	Ultra violet
WHO	World Health Organisation
μl	Microlitre
μM	Micro molar

CHAPTER ONE

1.0 INTRODUCTION

Respiratory tract infections (RTIs) over the decades have been a major public health concern, affecting both adults and children alike. These RTIs may progress to upper or lower respiratory tract diseases. Examples of some upper respiratory tract diseases include coryza and pharyngitis. Some lower respiratory tract diseases also include pneumonia, bronchopneumonia, bronchiolitis, bronchitis and tracheobronchitis.

Upper respiratory tract infections (URTIs) though the most frequent RTIs in children (Nokso-Koivisto *et al.*, 2006) is most often less severe and self-limiting compared to lower respiratory tract infections (LRTIs). LRTIs are life threatening (Henrickson *et al.*, 2004) demanding thorough medical intervention. Acute lower respiratory tract infections (ALRTIs) thus have become serious public health concern worldwide due to its severity.

ALRTIs are one of the leading causes of infectious diseases globally especially in children resulting in death (Nascimento-Carvalho, 2001; Jin *et al.*, 2012). The World Health Organization (WHO) statistics in 2002 showed LRTIs alone to be responsible for 26% of deaths caused by infectious diseases globally (Rutherford, 2009). Majority of these deaths due to LRTIs are in children under five years of age (Tregoning *et al.*, 2010). Also, one-third of children are estimated to develop lower respiratory tract infections within their first twelve months of existence (Kusel *et al.*, 2006).

Several aetiological agents have been associated with ALRTIs of which the majority are viruses (Juvén *et al.*, 2000; Erdman *et al.*, 2003). Some of the viruses associated with

ALRTIs are respiratory syncytial virus, human parainfluenza viruses, influenza virus, adenovirus and human metapneumovirus (Ordas *et al.*, 2006; Mahony, 2008; Nicholas *et al.*, 2008; Ou , 2009; Pavia , 2011; Guerrier, 2012). Of these viruses, respiratory syncytial virus has been found to be the most important in causing lower respiratory tract diseases (Berman, 1991; Karron, 2008). After respiratory syncytial virus, human parainfluenza viruses (HPIVs) have been reported as the second most common viral agent in ALRTIs in children (WHO, 2005; Vachon *et al.*, 2006; Schmidt *et al.*, 2011). HPIVs are single stranded, negative sense RNA viruses that cause severe disease in infants, the elderly and immune-suppressed patients (Hall, 2001). Four types of HPIVs: HPIV-1, HPIV-2, HPIV-3 and HPIV-4 are known to exist (Reed *et al.*, 1997).

In the developed countries, HPIVs have been well documented to be significant causes of ALRTIs often requiring hospitalization. An estimated rate of 0.32 to 1.6 per 1000 children has been reported as the hospitalization rate in children less than five years due to HPIV-1 alone in the United States (Beck *et al.*, 2012). Mahony (2008) also reported HPIVs to be responsible for about 15-30% of respiratory tract diseases in children that require hospitalization.

Unfortunately in Sub-Saharan Africa, limited studies have been done on HPIVs. The few studies conducted either did not include all four types or used comparatively less specific techniques. In Kenya, it was found that 7.5% of pneumonia patients tested positive for HPIVs (Berkley *et al.*, 2010). In West Africa, few studies were conducted to determine the contribution of HPIVs in respiratory tract infections among young children. HPIV positive test rates of 5.9% and 2.4% were found in Gambia and Senegal respectively (Mulholland *et al.*, 1999; Niang *et al.*, 2010). In Nigeria, 46.4% of children between 1 to 5 years were seropositive for HPIV-2 antibody (Sale *et al.*, 2010). In Ghana, work done by Kwofie *et al.*,

(2012) recorded HPIV prevalence of 3.1% in nasopharyngeal swabs taken from children hospitalized for ALRTIs. HPIV-4 was however not included in their study.

This research therefore seeks to provide detailed information on all four types of HPIVs as one of the viral aetiological agents that cause ALRTIs in Ghana, using real time reverse transcriptase polymerase chain reaction (rRT-PCR).

1.1 Problem statement

ALRTIs are one of the leading causes of infectious diseases resulting in millions of deaths in children less than five years old, especially in the developing countries. About 19% of all deaths in children less than five years are due to pneumonia (Bryce *et al.*, 2005; Rudan *et al.*, 2008). Seventy percent of such deaths are in Africa and South East Asia. Several studies have been carried into the bacteriological agents of ALRTIs. However, there is a gap in information on viruses, especially the four types of HPIV that cause ALRTIs in Ghana. Currently in Ghana, laboratory diagnosis of ALRTI cases in health facilities focuses on the detection of bacteria as the possible etiological agents. This is as a result of inadequate facilities for virological investigation in our health institutions; leading to the absence of detailed information on the possible roles that HPIVs play in ALRTIs. The situation will not improve unless virological tests are included in laboratory diagnoses in our health facilities. This study therefore will support the importance of regular laboratory diagnosis of HPIV in ALRTI patients.

1.2 Justification

In Ghana, limited work has been done on all four types of HPIV as well as their molecular characterization. This research will add to the body of knowledge on HPIVs in Ghana. It may

also provide the basis to include HPIVs in the diagnostic profiles for ALRTIs in Ghanaian health facilities since the contribution of HPIVs in ALRTI cases will be described. This could improve the management options available for ALRTIs cases seen in Ghana.

1.3 Main Objective

To detect and characterize HPIVs in ALRTIs cases seen in children below five years that visit Princess Marie Louise Children's Hospital in Accra.

1.3.1 Specific Objectives

- To detect HPIVs in ALRTIs cases
- To characterize the HPIVs using molecular tools
- To assess the risk factors associated with the HPIVs infection
- To determine the clinical characteristics of the HPIV positive patients

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 History of human parainfluenza virus discovery

In the late 1950s, four HPIVs were discovered in patients who suffered from respiratory tract infections. HPIV-1 to HPIV-3 were isolated from children that suffered from lower respiratory tract infections. HPIV-1 was however the first to be discovered in Japan (Henrickson, 2003; Tying, 2005). HPIV-4 was also first isolated from a male college student who had mild upper respiratory tract infection (Johnson *et al.*, 1960 as cited in Lau *et al.*, 2005). The newly discovered HPIVs possessed non segmented thick nucleocapsids (Chanock and McIntosh, 1985), did not grow well in embryonated egg but grew well in monkey kidney tissue (Henrickson, 2003).

2.2 Classification

HPIVs belong to the order Mononegavirales, family Paramyxoviridae and subfamily Paramyxovirinae. It consists of two genera (Respirovirus and Rubulavirus) (Lamb and Kolakofsky, 1996). HPIV-1 and HPIV-3 belong to the genus Respirovirus whiles HPIV-2 and HPIV-4 belong to the genus Rubulavirus (Collins *et al.*, 1996; Lamb and Kolakofsky, 1996). Parainfluenza viruses can infect humans and animals and cause diseases in both. HPIVs also have been shown to infect and cause severe disease in some animals (Henrickson, 2003; Sale *et al.*, 2010).

2.3 Structure

HPIVs are pleomorphic enveloped viruses that range from 150nm to 300nm in diameter. As seen in Figure 2.1 below, the virus is made of a thick non-segmented nucleocapsid core which is surrounded by lipid bilayer envelope derived from the plasma membrane from its host cell (Choppin and Compans, 1975). The nucleocapsid core consists of the genome which is surrounded by nucleocapsid proteins (NP), phosphoprotein (P) and the large RNA polymerase (L) (Masters and Banerjee, 1988 as cited in Moscona and Peluso, 1991). Beneath the lipid bilayer is the matrix protein (M). Two surface glycoproteins are found on the viral envelope: the hemagglutinin-neuraminidase (HN) and the fusion proteins (F). These glycoproteins extend from the membrane surface to a length of about 8nm to 12 nm and can be viewed by electron microscopy (Lamb and Kolakofsky, 1996).

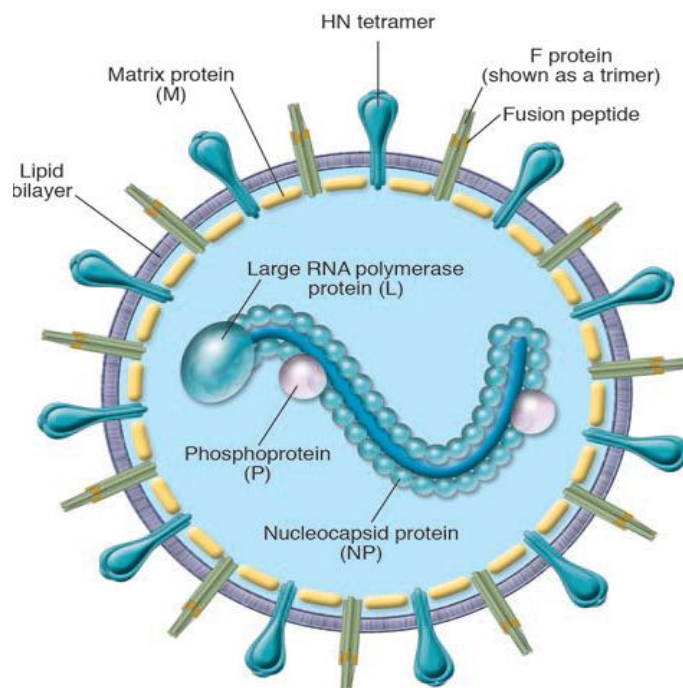


Figure 2. 1: Schematic diagram showing the structure of parainfluenza virus

Adapted from “Entry of parainfluenza virus into cells as a target for interrupting childhood respiratory disease” by A. Moscona, 2005, *Journal of Clinical Investigation*, 115(7), 1698.

2.4 Structural proteins

The genome of HPIVs is said to encode six structural proteins and at least one non-structural protein (Hall, 2001). These proteins play important functions in the viral life cycle. They include nucleocapsid protein, phosphoprotein, matrix protein, fusion protein, hemagglutinin-neuraminidase protein and large polymerase protein.

2.4.1 Nucleocapsid protein (NP)

Located in the nucleocapsid core is the nucleocapsid protein. It is the most abundant structural protein found in the HPIV (Hamaguchi *et al.*, 1983). It wraps tightly around the viral RNA, usually one nucleocapsid protein binds to six nucleotides to form a stable RNase resistant nucleocapsid (Moscona and Peluso, 1991; Henrickson, 2003). The nucleocapsid protein functions together with the L and P proteins for transcription and replication of the viral genome (Vainionpää and Hyypiä, 1994).

2.4.2 Phosphoprotein (P)

The phosphoprotein is the second largest structural protein found in the virus. This structural protein has size differences among all four types of the HPIVs. The P of HPIV-1 and HPIV-3 was found to have a molecular weight ranging from 83000 to 90000 Daltons while that of HPIV-2 and HPIV-4 ranged from 49000 to 53000 Daltons (Henrickson, 2003). The P helps in RNA polymerase activity (Hamaguchi *et al.*, 1983).

2.4.3 The matrix (M)

The M protein found beneath the lipid bilayer is very hydrophobic in nature (Vainionpää and Hyypiä, 1994). It attracts completed nucleocapsids to infected cell membrane sites that will become the viral envelope thus mediating the interaction between the nucleocapsids and glycoproteins which are found on the envelope. It has been suggested that the M protein may also play a role in viral budding (Henrickson, 2003).

2.4.4 Fusion protein (F)

This is one of the two glycoproteins found on the viral envelope. The F protein is initially synthesized in a biologically inactive form (F₀). Cellular proteases (Furin and Kex2 are responsible for proteolytic cleavage in man) cleave this inactive form post translationally to an active form (Ortmann *et al.*, 1994). The active form consists of two disulfide-linked molecules (F₁ and F₂). The F protein functions in the fusion of virus membrane with the host cell membrane. This allows the viral nucleocapsid to enter the host cell (Moscona, 2005). The F protein has some immunologic properties such as increased variation in specific regions that may help the virus to escape host immune system (Beck *et al.*, 2012).

2.4.5 Hemagglutinin-Neuraminidase protein (HN)

The HN protein is the second surface glycoprotein found on the viral envelope and on infected cells (Parks and Lamb, 1990). It is fixed into the viral envelop membrane by a hydrophobic transmembrane domain (Wang *et al.*, 2001). The HN protein functions in virus-host cell attachment. This protein identifies and binds to the sialic acid-containing receptors on the host cell surface. The HN protein cell receptor interaction is specific and complex. It involves the F protein as well and it differs between the HPIV types (Moscona and Peluso, 1992). The HN protein of HPIV 1 has been shown to permit several amino acid substitutions. Its ability to permit different amino acid substitution (mutation) outside its key functional domains is one of the ways to escape the host immune response (Beck *et al.*, 2012).

2.4.6 Large polymerase protein (L)

It is the largest structural protein in the virion and is loosely associated with the RNA genome. It functions in the replication of the viral genome (Moscona and Peluso, 1991).

2.5 Genome organization

HPIVs have a linear genome organization. The genome consists of non-segmented, single stranded RNA with a negative polarity (Karron and Collins, 2007). On the average, the RNA genome consists of 15,000 nucleotides (Galinski *et al.*, 1991). As depicted in Figure 2.2 the genome generally encodes at least six main structural proteins in the unvarying order 3'-N-P-M-F-HN-L-5' (Cote *et al.*, 1987; Vainionpää and Hyypiä, 1994; Schmidt *et al.*, 2011). Each gene encodes one major protein with the exception of the phosphoprotein gene which produces one or more accessory proteins (non-structural) from multiple overlapping reading frames (Galinski, 1992). These non-structural proteins result from additional editing of the mRNA (Henrickson, 2003).

The sequence analysis of HPIV-3 genome and some other viruses within the paramyxoviridae family showed that the 3' ends of the viral genome contains extracistronic noncoding region which is about 50 nucleotides long while the 5' end contained extracistronic region which is also about 40 nucleotides long (Vainionpää and Hyypiä, 1994). Among the paramyxoviruses, the first 20 nucleotide at the 3' end are extremely conserved, this indicates their importance in initiating transcription and replication. "The adjacent genes within the genome are separated by intercistronic regions which contain transcriptional control sequences found at the beginning and end of every gene" (Vainionpää and Hyypiä, 1994). These control sequences are conserved and transcribed into mRNAs. Work done by Beck *et al.*, (2012) confirms this fact. In their work, the genome of 40 HPIV-1 were sequenced and they found the intercistronic or intergenic sequences to be very much conserved with no changes in all 40 HPIV-1 sequenced as well as the HPIV 1 sequences obtained from the GenBank.

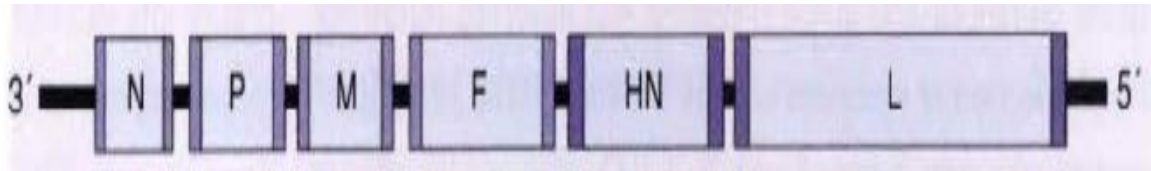


Figure 2. 2: Genomic organization of parainfluenza virus

Adapted from “Progress in the development of PIV vaccine” by Schmidt et al., 2011, *Expert Review of Respiratory medicine*, 5(4), 17.

2.6 Parainfluenza virus life cycle

As shown in Figure 2.3, the life cycle of HPIVs involves different stages (1 to 7) which are described below.

Attachment, fusion, penetration and uncoating

The HPIV first uses its hemagglutinin-neuraminidase glycoprotein to bind to the sialic acid containing receptor molecules on the host cell surface (Step 1, Figure 2.3). The fusion protein then facilitates fusion of the viral envelope with the host cell plasma membrane thus releasing the viral nucleocapsid into the host cell cytoplasm (Steps 2 and 3, Figure 2.3) (Moscona, 2005).

Transcription, translation and replication

HPIVs consist of negative sense RNA genome hence must be transcribed to a positive sense RNA (Step 4, Figure 2.3) by the viral RNA dependent polymerase. This positive sense intermediate RNA acts as mRNA which is then translated into viral proteins. A full length negative strand is also copied from this positive sense RNA intermediate template (Step 5, Figure 2.3) (Jawetz, 2007).

Maturation

In the cytoplasm, nucleocapsid proteins encapsidate this full length negative strand RNA (Step 6a, Figure 2.3) to form newly assembled nucleocapsids (Henrickson, 2003) that bud out through areas of the host plasma membrane that contain the fusion, hemagglutinin-neuraminidase and the matrix proteins (Step 6b, Figure 2.3) (Moscona, 2005). The new virus is then finally released to infect new cells (Step 7, Figure 2.3) (Moscona, 2005).

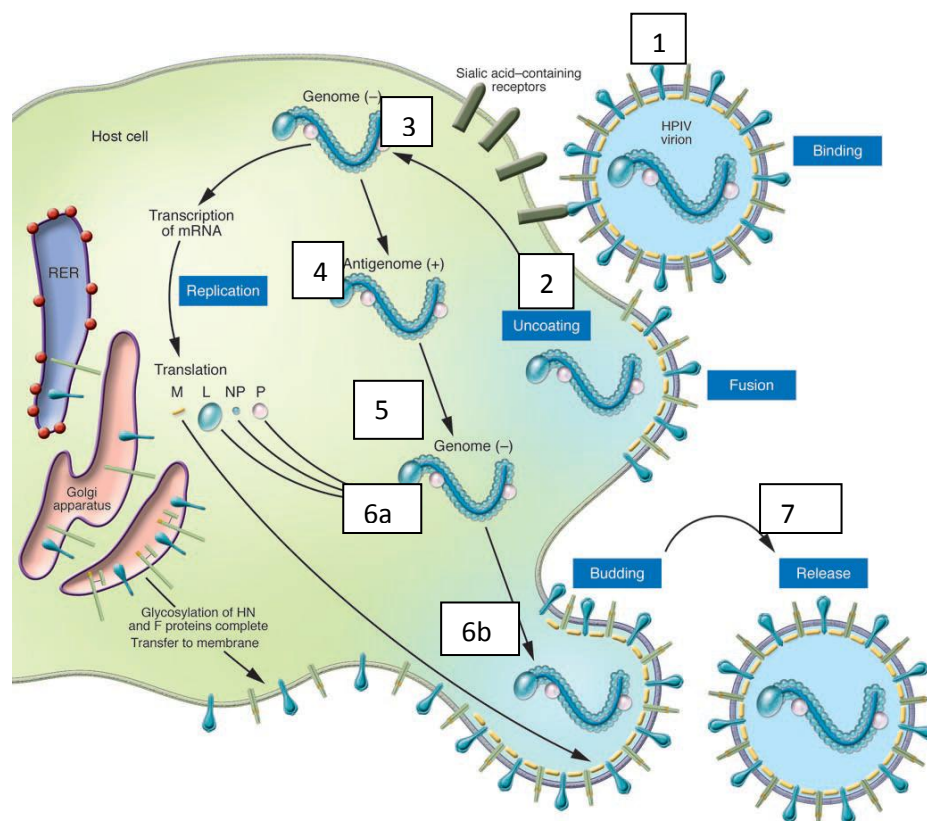


Figure 2. 3: Schematic diagram of the life cycle of parainfluenza virus

Adapted from “Entry of parainfluenza virus into cells as a target for interrupting childhood respiratory disease” by A. Moscona, 2005, *Journal of Clinical Investigation*, 115(7), 1690.

2.7 Human parainfluenza virus types

HPIVs have been antigenically and genetically grouped into four types which belong to the genus *Respirovirus* or *Rubulavirus* (Henrickson, 2003). The *Respirovirus* consists of HPIV-1 and HPIV-3. The *Rubulavirus* consists of HPIV-2 and HPIV-4. HPIV-4 has two subtypes; A and B (Henrickson, 2003).

All the four types of HPIV can cause lower respiratory tract diseases. Some of these diseases include pneumonia and bronchiolitis. These diseases are common in children however adults can also be equally infected with the HPIVs. In adults, virus infection usually results in mild disease usually manifesting as upper respiratory tract symptoms (Fry *et al.*, 2006). However the viral infection can also cause severe disease in the adult often requiring hospitalization.

In a study conducted by Marx and his colleagues (1999) in Franklin and Summit counties of Ohio on adults hospitalized for lower respiratory tract infections, HPIV-1 and HPIV-3 were the most common HPIVs found. Their study however did not emphasize the importance of HPIV-2 in causing community acquired lower respiratory tract infection in adults because the study period did not include the HPIV-2 outbreak season. If the studies had been conducted to include the HPIV-2 outbreak season, the percentage contribution of HPIV-2 would have been known.

In a work published by Sale *et al.*, (2010) HPIV-2 was shown to have a seroprevalence of 46.4% in 379 children aged 1-5 years. Their studies also did not show the percentage contributions of the other HPIV serotypes.

Most of the researches studies have been on HPIV-1 to HPIV-3. HPIV-3 and at times HPIV-1 are the most predominant detected in HPIVs research studies. Very little is known about

HPIV-4 (its epidemiology, seasonality and clinical manifestation). The scanty information on HPIV-4 could probably be due to the fact that HPIV-4 is usually associated with mild respiratory illness (Killgore and Dowdle, 1970; Collins *et al.*, 1996) and also most laboratories do not include it in their diagnosis. HPIV-4 was however shown by Lau *et al.*, (2005) to be associated with severe respiratory tract infections. Vachon *et al.*, (2006) reported HPIV-4 detection rate of 43% (9 positive patients) among all HPIVs detected between the period of October 2004 to March 2005. This was an increment in the HPIV-4 detection rate over the previous years.

2.8 Differences between the types

HPIVs differ antigenically and genetically hence their division into the four main types though they all have like morphologies (Henrickson, 2003).

The phosphoprotein size differentiates between the four types of HPIVs since the size of the other structural proteins in the virus are similar in size. Basically, HPIV-1 and HPIV-3 have a phosphoprotein molecular weight ranging from 83,000 to 90,000 Daltons while HPIV-2 and HPIV-4 have phosphoprotein of molecular weight between 49,000 to 53,000 Daltons (Henrickson, 2003).

On the genomic level, HPIV-3 lacks small hydrophobic (SH) gene which is located between the F and HN proteins. The SH gene encodes small integral membrane protein which is solely found in infected cells (Vainionpää and Hyypiä, 1994).

Six antigenic sites (A to F) exist on the HN glycoprotein of HPIV-3. Sites A, B and C together have 11 epitopes which function in hemagglutination and neutralization. Coelingh *et al.*, (1985) and Rydbeck *et al.*, (1987) reported the sites involved in hemagglutination and neutralization to be very much conserved in HPIV-3 whereas HN protein variation and evolution exist in HPIV1 (Henrickson, 1991; Henrickson and Savatski, 1992).

2.9 The Nasopharynx

Rajion *et al.*, (2012) described the nasopharynx as a fibromuscular tissue found at the back of the human nose (Figure 2.4). Brown and his colleagues in 2010 described the surgical anatomy of the nasopharynx to be a cuboidal midline chamber which stretches out from the skull's base to the position of the soft palate. It opens anteriorly to the nasal cavity and posteriorly to the oropharynx and functions in respiration, chewing and swallowing, smelling and in aids in speech.

The nasopharynx as well as the oropharynx are the main areas where HPIV initially replicates (Henrickson, 2003), making these locations good sites to obtain samples for viral testing. It is a challenge to take oropharyngeal samples from children less than five years as it is more difficult to keep the mouth of these little children open to obtain oropharyngeal swabs. It is however more convenient to obtain nasopharyngeal swabs. Epitheloid cells are found in the nasopharynx as well as in the oropharynx and these cell turn to support the growth of human parainfluenza viruses (Henrickson *et al.*, 2004).

2.10 Transmission

The virus can be transmitted by the inhalation of infected droplets which are generated through coughing or sneezing. The chance of transmission through large droplets of aerosol is higher as compared to that of small particle aerosol spread. HPIV-1, HPIV-2 and HPIV-3 can survive for about ten hours on non-porous surface and about four hours on porous surface (Brady *et al.*, 1990). There is the likelihood of self-inoculation when one comes into direct contact with these contaminated surfaces. HPIV-3 loses 90% of its infectivity when placed on the fingers within the first 10 minutes hence person to person spread by direct hand contact is less likely to occur (Henrickson, 2003).

2.11 Pathogenesis

The virus binds to the respiratory epithelium upon entry. The HN proteins found on the lipid envelop helps in attachment on reaching the epithelia cells (Henrickson, 2003). Both the HN and F proteins are involved in cell membrane fusion and are HPIV type specific (Hu *et al.*, 1992). Infected cells show change in morphology. HPIVs can infect different anatomical parts of the respiratory tract from the nose down to lungs resulting in diverse clinical presentations and different levels of disease severity depending on the HPIV type (Jawetz *et al.*, 2007). An immunocompromised patient cannot clear off the HPIVs and is at risk of persistent infection (McIntosh *et al.*, 1984).

2.12 Clinical presentations of HPIV infections

Each of the HPIV types can be associated with a particular clinical feature (Henrickson, 2003). Croup is a common clinical presentation with HPIVs. HPIV-1 causes mostly acute croup in infants and young children. HPIV-2 causes croup which is often less severe. HPIV-3 causes severe croup, pneumonia and bronchitis in children less than one year. HPIV-4 causes mild upper respiratory tract infections (Mahony, 2008). Vachon *et al.*, (2006) however reported HPIV-4 to be associated with bronchiolitis in 3 children.

In a study conducted to determine the seroprevalence of HPIV-2 in children, other clinical symptoms such as wheezing and rhonchi were seen (Sale *et al.*, 2010, Morgan *et al.*, 2012).

Fever, cough, sputum production and tachypnea, rales, chest pain and hemoptysis, difficulty in breathing and chest indrawing were the signs and symptoms presented by patients that tested positive for HPIVs according to work done by Morgan and colleagues (2012) and Shafik *et al.*, (2012).

2.13 Risk factors predisposing individuals to HPIVs infection

Overpopulation, lack of breast feeding, malnourishment, smoking, vitamin A deficiency, age and parental history of asthma are possible factors predisposing individuals to HPIV infection (Cushing *et al.*, 1998; Laurichesse *et al.*, 1999; Berlinger *et al.*, 2003; Harberg *et al.*, 2007; Quigley *et al.*, 2007; Ahmed *et al.*, 2012).

The mode of HPIV transmissions through the inhalation of large aerosol droplets from sneeze or coughs and also through self-inoculation by contact with contaminated surfaces explains over-crowding as a predisposing factor (Henrickson, 2003).

During breast feeding, a nursing mother transfers maternal antibodies to her baby; these antibodies may protect the baby from HPIV infection. The absence of breastfeeding increases the risk of getting infected with HPIV (Human parainfluenza viruses, n.d.).

Age is a predisposing factor to LRTIs and as such plays an important role in predisposing individuals to HPIV infection. Children at very tender ages have premature immune systems and are very likely to be at risk of infection (Holt, 2005). In a study conducted by Franz *et al.*, (2010) which looked at the relationship between the viral load of respiratory pathogens and the severity of disease in LRTI hospitalized children, it was observed that majority of children who had LRTI were less than two years.

Serologic surveys showed 90% to 100% of children at 5 years and above to have antibodies to HPIV-3. 75% of children 5 years and above also have antibodies to HPIV-1 and HPIV-2 (Human parainfluenza viruses, n.d.). The presence of the antibodies however does not prevent future infection with HPIV. Reinfection with HPIV results in mild upper respiratory tract infection (Mäkelä *et al.*, 1998). This could be as a result of the modification of the disease by the already existing antibodies (Sale *et al.*, 2010).

It was found in Milwaukee that white children had a much higher HPIV incidence than black children (Henrickson *et al.*, 1994). This could raise the concern of questioning races as a risk factor to HPIV infection.

2.14 Seasonality of HPIVs

All the different HPIV types have seasonal occurrences though this present study does not address HPIVs' seasonality. In the United States, HPIV-1 causes outbreaks in fall. Large outbreaks of HPIV-1 occur in odd numbered years and smaller out breaks in even numbered years. HPIV-2 outbreaks occur annually or biennially in fall. HPIV-3 outbreaks occur annually during spring and early months in summer (HPIV Seasons, 2012).

In the tropics however, very few studies have been carried out on HPIVs and these studies hardly document the seasonality of the HPIVs. In a study carried out in Brazil by Fe and colleagues from January 2001 to December 2006, the seasonal occurrence of HPIV-3 was in the dry months. Cases of HPIV-3 occurred from September to November. The other HPIV serotypes did not show any seasonal occurrence (Fe *et al.*, 2008).

In a study on HPIVs in rural Thailand between 2003 and 2007, HPIV-1 and HPIV-3 were found to be circulating throughout the study period. HPIV-3 was detected very frequently from January to May and at times, extended to July. HPIV-1 was detected in low numbers throughout, but its frequency was a bit higher between October 2003 to July 2005 and from October 2005 to July 2006. The peak period for the detection of HPIVs tended to be between January to April but was not restricted to a specific type (Morgan *et al.*, 2012).

2.15 Diagnosing HPIVs

Nasopharyngeal aspirates, nasopharyngeal swabs, nasal wash, throat swabs, bronchoalveolar lavage fluid and bronchial washing are some of the respiratory specimens that can be tested for the diagnosis of human parainfluenza viruses (Storch, 2000; Henrickson, 2003). HPIVs are shed in high titres during the early stage of infection (Henrickson, 2003) hence it is advisable to collect samples during this period to maximize viral detection. The type of specimen collected depends on factors such as the age of the child or the type of test to be performed. For example, in testing for HPIV in an infant, it is convenient to obtain a nasopharyngeal swab than a throat swab. Also in direct viral detection, it is appropriate to collect samples that contain the virus itself as against a blood sample which contains antibodies to the HPIVs.

Diagnoses of HPIVs can be divided into 3 broad groups. These include:

- Direct detection; this involves the direct detection of antigens, viral nucleic acid or the detection of the virus particle itself. Direct detection of the virus itself is possible by the use of electron microscope.
- Viral isolation; this is achieved by means of cell culture. The use of cell culture in diagnosing HPIVs takes a long duration (days) to produce result hence it is not useful as a prompt diagnostic tool. HPIVs grow best in primary monkey kidney cells however, other cell lines such as Vero, Hep-2 and HeLa also supports its growth (Henrickson, 2003). HPIVs do not usually show cytopathic effects, hence test such as hemadsorption, immunofluorescence and hemagglutinin inhibition are employed in detecting an HPIV positive culture.

- Measurement of immune response: this has to do with the measurement of humoral and cellular immune responses. Acute and convalescent antibody titres (antibodies to the HN and F proteins of HPIVs) are useful in the detection by humoral response.

(Storch, 2000; Henrickson, 2003).

These diagnostic methods do have their advantages and disadvantages. Tests such as ELISA, hemagglutinin inhibition, complement fixation, hemadsorption, immunofluorescence which fall under either direct antigen detection or serology have been very useful in diagnosing HPIVs infections. Though these methods produce rapid results when compared to viral culture (Storch, 2000), there are limitations. Their sensitivity and specificity is less when compared with molecular techniques which detect presence of HPIVs nucleic acids (Cupic *et al.*, 2007). All HPIVs have common antigens and they induce varying levels of heterotypic antibodies (Parrott *et al.*, 1962; Henrickson, 2003). This makes it difficult to determine whether a positive serologic test is as a result of cross-reactions to similar antigens on a different HPIV type especially in acute cases of HPIV infection. Heterologous antibodies have been produced against HPIV-1 and HPIV-3, making serologic diagnosis nonspecific. Antibodies from some other members of the paramyxovirinae family, for example antibodies from mumps virus can also cross-react with antigens from HPIV-1 and HPIV-3 (Henrickson, 2003).

The advent of nucleic acid detection methods (molecular techniques) overcomes the setbacks of less sensitivity and less specificity of the serologic HPIV diagnostic tools. These molecular diagnostic tools though expensive and not routinely used in hospital laboratories can be used as a research tool to provide information on HPIVs. Some HPIVs nucleic acid detection methods include polymerase chain reaction (PCR) and northern blot analysis.

The HIVs diagnostic tool used in this current research was a PCR (specifically real time reverse transcriptase PCR). HIVs contain RNA as their genetic material and have to be transcribed in the reverse mode to a cDNA. PCR was developed by Kary Mullis and colleagues in the mid-1980s (Kubista *et al.*, 2006). This method is highly sensitive and specific. It can amplify any small amount of HIV in a clinical sample after the HIV RNA has been reverse transcribed to a cDNA. The PCR amplifies HIV cDNA through a number of processes which is dependent on temperature cycling. First the HIV cDNA is denatured at a high temperature, then forward and reverse primers anneal to specific sequences on the denatured cDNA at a lowered temperature. The cDNA is then elongated through the incorporation of deoxynucleoside triphosphates by a heat stable DNA polymerase at about 72°C (Kubista *et al.*, 2006). Detection of amplified products is achieved at the end of the reaction through post-PCR analysis which includes gel electrophoresis and gel imaging (Invitrogen Corporation, 2008). Real time PCR was developed in 1992 by Higuchi and colleagues. Unlike the traditional PCR that detects amplification only at the endpoint; in real time PCR, amplification can be monitored as the reaction progresses in real time (Invitrogen Corporation, 2008). Real time PCR is advantageous over traditional PCR in that the initial amount of genetic material in the clinical sample can be quantified. It is faster as there is no need for a post-PCR analysis and rate of contamination is also lesser. Probe is used in real time PCR but absent in traditional PCR.

2.16 Principle of the real time PCR method

This method makes use of two oligonucleotide primers that anneal to specific sequences on the denatured cDNA or DNA that is to be amplified. A probe bearing reporter fluorophore and quencher fluorophore hybridizes to specific sequences found between the forward and reverse primers binding sites. Quencher fluorophore absorbs or suppresses any fluorescent

emission from the reporter fluorophore because of their close proximity. Strand extension is achieved through the incorporation of deoxynucleoside triphosphates by DNA polymerase. When the DNA polymerase reaches the annealed probe, the probe is cleaved as a result of the 5' exo-nuclease activity of the polymerase. The reporter fluorophore is thus released from the quencher fluorophore (Figure 2.5). Because the reporter fluorophore is no longer in close proximity to the quencher fluorophore, fluorescent emission from the reporter increases (Kubista *et al.*, 2006; Applied Biosystems, n.d.).

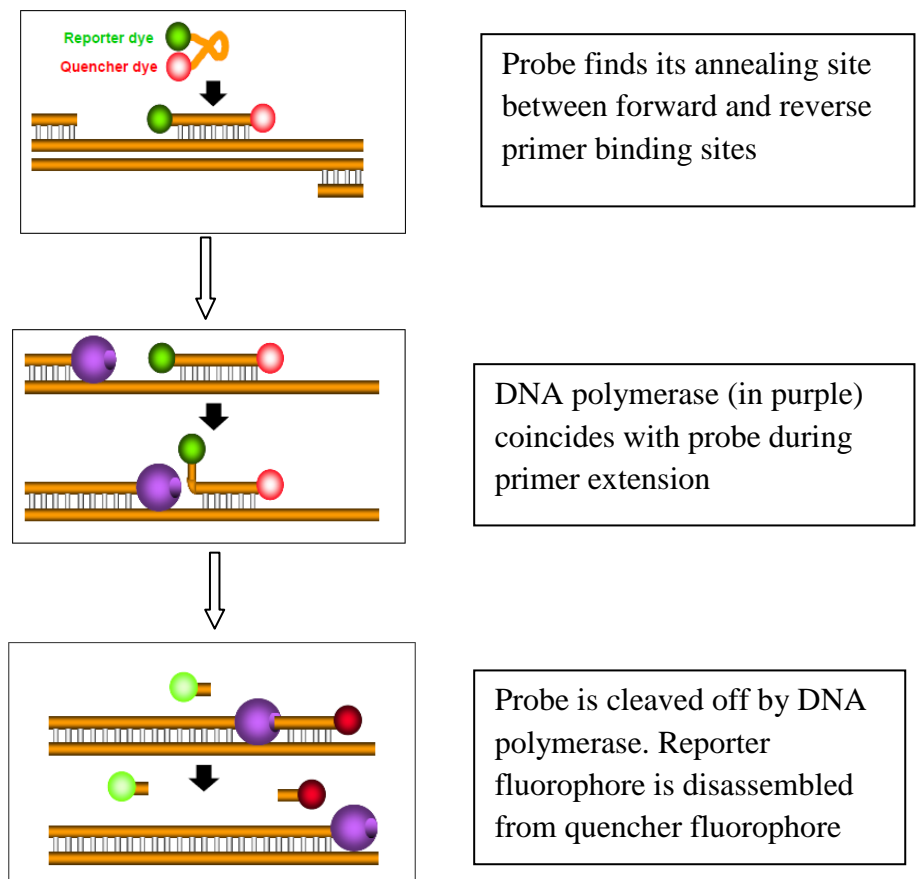


Figure 2. 4: Steps involved in the binding of probe to its cleavage during real time PCR.

Adapted from "Traditional PCR versus real time PCR" by Applied Biosystems, n.d.

Increase in fluorescence signal is an indication of increase in the amount of amplicon produced during the exponential phase of the PCR. A fluorometer measures the change in fluorescence and combines it with the thermal cycling. An amplification curve is then generated by plotting fluorescence against cycle number for each sample throughout the PCR reaction (Invitrogen Corporation, 2008).

During early amplification, fluorescence signal is indistinguishable from the background. The point at which the fluorescence signal passes from insignificant levels to well distinguishable levels is known as the threshold cycle (Ct). This Ct can be used to determine the initial quantity of DNA in a sample. The more DNA in a sample, the lower the Ct and vice versa (Invitrogen Corporation, 2008). There are three phases in a PCR. These phases are reflected in the amplification curve, these include;

- Exponential phase: in this phase, all reagents are present in excess amount, DNA polymerase efficiency is high. There is doubling of amplicon at every amplification cycle
- Linear phase: reaction begins to slow down at this stage as the reagents are being used up
- Plateau phase: at this stage, the reaction stops as very crucial components in the reaction for example the DNA polymerase are used up.

(Invitrogen Corporation, 2008; Applied Biosystems, n.d).

2.17 Viral Burden in ALRTIs

HPIVs cause respiratory tract diseases both in children and adults. Several studies have been carried out on these viruses in the developed countries where they have been found to be the

second most important in causing lower respiratory tract infections (WHO, 2005; Schmidt *et al.*, 2011).

In Ghana, Isomura *et al.*, (1986) tested for antibodies to HPIV-1, HPIV-2 and HPIV-3 in preschool children using hemagglutination inhibition test. They found that percentage seropositivity to HPIVs antibody increased with age. Forty-eight percent, 13% and 54% of the preschool children had antibodies to HPIV-1, HPIV-2 and HPIV-3 respectively. Among those in early infancy, seropositivity was greatest (26.7%) to HPIV-3. Children above three years were found to have antibodies to at least one type of the HPIV. Presence of antibodies to more than one type of the HPIVs increased with age. Presence of antibodies to the different HPIVs is an indication of HPIV infection. Kwofie *et al.*, (2012) also used real time PCR to detect HPIV positive test rate of 3.1 % in Ghanaian children who were hospitalized for ALRTIs.

Ahmed *et al.*, (2012) carried out studies on 6,264 refugees who presented with influenza-like illness and severe acute respiratory infections in two camps in Kenya to determine the epidemiology of respiratory viral infections. Various viral etiological agents were found using RT-PCR. HPIVs constituted 9.4% of the respiratory viral infections. The proportions of the individual HPIV types were however not specified. Also, the study was not limited to children under the age of five but ranged from 1 month to 84 years with the under one year group constituting 36.6%, the majority of the patients (45.4%) were between 1 year to 4 years, emphasizing the vulnerability of this age group to respiratory tract infections. Children under the age of 1 year amounted to 41.1% of the total HPIV infection while those of RSV and adenovirus were 47% and 38.1%. Shafik and colleagues (2012) also found that HPIV positivity was highest in children less than year.

In a study by Morgan and colleagues (2012), 10,097 hospitalized patients in two provinces of Thailand were enrolled over four years to investigate HPIVs associated lower respiratory tract infections. The presence of HPIVs was diagnosed in patients using different diagnostic methods (RT-PCR, real time RT-PCR, virus isolation and indirect enzyme immunoassay). Five percent of the patients were positive for the virus. This percentage was not only limited to children but covered a wide age range with the elderly (more than 65 years) inclusive. Work done by Franz *et al.*, (2010), also detected 20 HPIVs in 404 nasopharyngeal samples representing 5% positive test rate.

2.18 Phylogenetic Analysis

Phylogenetic analysis is the means of inferring the evolutionary relationship between organisms. This relationship between organisms is usually shown as a branching treelike diagram (phylogenetic tree) (Brinkman, 2001). Phylogenetic trees can be built using morphological features, behavioural features or molecular data of organisms. Molecular data includes protein and DNA sequences. The use of molecular data has surpassed morphological or behavioural characters in the construction of phylogenetic trees. The use of molecular data can help determine the functions of genes. In constructing a phylogenetic tree using molecular data, it is very important to ensure that the sequences used are homologous (Brinkman, 2001). Phylogenetic analysis has been used in determining the evolutionary relationship between circulating strains of HPIVs and reference strains. Several studies have determined the nucleotide sequences of specific genes within the HPIV genome and compared them to reference strains in Genbank.

CHAPTER 3

3.0 METHODOLOGY

3.1 Study design

This study was cross-sectional on children less than five years with acute lower respiratory tract infections.

3.2 Study site

The study was conducted at Princess Marie Louise Children's Hospital (PML). The PML is one of the government hospitals in the Accra metropolis in the Greater Accra region of Ghana. It is located in the Arena community within the Ashiedu Keteke sub metro of the Greater Accra region of Ghana.

The Princess Marie Louise Children's hospital primarily provides health care for children in the Greater Accra region and beyond. The hospital comprises the out patients department, recovery ward, dietetics centre, emergency ward, laboratory department, records and administrative unit, laundry and a recreational area for the children.

3.3 Target population of the study

The target population of the study were children with respiratory illness who were seen at the PML between March to July 2013.

3.4 Case definition

The case definition for ALRTI adopted was in accordance with Shafik *et al.*, (2012) and WHO and UNAIDS, (n.d.).

This included presence of cough or difficult breathing plus any of the following: chest indrawing, inability to drink or breastfeed, vomiting and fast breathing. Fast breathing was dependent on child's age as defined below:

- >60 respiration /minute for infant less than 2 months
- >50 respiration /minute for infant aged 2 months to less than 1 year
- >40 respiration/ minute for child aged 1 year to less than 5 years

3.5 Inclusion criteria

Children under five years who met the case definition or under-fives who were diagnosed of any lower respiratory tract disease (for example bronchopneumonia, pneumonia and bronchitis) were deemed eligible for the study.

Patient folders were used for recruitment. A child was recruited only after the parent or guardian had given their written consent.

3.6 Exclusion criteria

Children below five years who did not meet the inclusion criteria and children who were five years and above were not included in the study.

3.7 Sample size

The sample size was calculated based on HPIV prevalence of 3.8%. This HPIV prevalence is an average of the prevalence in Gambia, Senegal and Ghana (Mulholland *et al.*, 1999; Niang *et al.*, 2010; Kwofie *et al.*, 2012). This HPIV prevalence, a z score of 1.96 at a 95% confidence interval and an allowable error (e) of 5% were substituted into formula:

$$n = \frac{z^2 p(1-p)}{e^2}, \text{ (Godden, 2004).}$$

The minimum sample size was determined as 56. However a total of 71 nasopharyngeal swabs were collected from consented participants.

3.8 Sampling method

A simple random sampling method was used to recruit children into the study once their care givers consented for them to be enrolled into the study.

3.9 Procedures

3.9.1 Questionnaires

Structured questionnaires were administered to document the children's demographics, risk factors to HPIV infection and their clinical features.

3.9.2 Sample collection

Nasopharyngeal swabs were obtained from children whose guardians or parents gave written consent for their wards to participate in the study by an expert.

Samples were collected using Copan Flocked Swabs from Copan Diagnostics Incorporated, USA. The Copan Flocked Swab was made of a molded plastic applicator shaft with the tip of

the applicator coated with nylon fibers that were perpendicularly arranged from a flocking process. The perpendicularly arranged fibers allow correct collection of cells.

Using the swab still in the wrapper, half the distance from the base of the nose to the ear lobe was estimated. The child's head was tilted back a little after which the sterile swab was taken out of the wrapper and then gently inserted along the floor of the nose through the nostril to the nasopharynx with the estimated distance in mind. The swab was then rotated 3 times and held in position for ten seconds to allow picking of epithelial cells in the nasopharynx after which the swab was gently brought out. The cap of a sterile tube containing universal transport medium (UTM) (Copan Diagnostics Incorporated, USA) was opened and the swab was rotated few times in the UTM and was broken off at the prescored break off point. The sterile tube cap was replaced and screwed tightly. The sample was then transported on ice immediately to the Virology Laboratory of the Noguchi Memorial Institute for Medical Research (NMIMR), Legon. Sometimes, samples were kept in a refrigerator at 4°C at the PML and transported to the NMIMR within three days. At the laboratory, the samples were vortexed and transferred into cryovials and kept at -80°C for RNA extraction.

3.9.3 RNA extraction from samples

Ribonucleic acid was extracted using QIAmp Viral RNA mini kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. The kit consisted of lysis buffer, RNA carrier, spin column, collection tubes, wash buffer 1, wash buffer 2, elution buffer and a manual.

Extraction procedure

One hundred and forty microlitres of well mixed nasopharyngeal swab was added to 560µl of lysis buffer in an extraction tube, the cap was closed securely and then pulse vortexed for about 15 seconds. The mixture was then incubated at room temperature for 10 minutes.

Five hundred and sixty microlitres of absolute ethanol was added and then mixed by pipetting up and down. Six hundred and thirty microlitres of the mixture was transferred into a spin column, the cap was tightly closed and spun for 1 minute at 8 revolutions per minute (rpm) in an Eppendorf centrifuge. The filtrate was discarded and the spin column placed into a new collection tube. The remaining 630 μ l of the mixture was transferred onto the spin column and spun for 1 minute at 8 rpm. The filtrate plus the collection tube was discarded. The spin column was again placed into a new collection tube, 500 μ l of wash buffer 1 (W1) was added, spun for 1 minute at 8 rpm and filtrate plus the collection tube discarded. The spin column was again placed into another collection tube, 500 μ l of wash buffer 2 (W2) was added and spun for 3 minutes at 13.2 rpm (maximum speed). The filtrate was discarded and the collection tube reused for another spin for 1 minute at 8 rpm. The collection tube together with the filtrate was discarded. The spin column was finally placed into an elution tube. Sixty microlitres of elution buffer was added to the spin column and spun for 1 minute at 8 rpm. The spin column was discarded and the eluted RNA was frozen at -20°C for later use.

3.9.4 RNA extraction from HPIV positive control isolates

The NMIMR Virology Department obtained reference HPIV isolates from the CDC, USA. These isolates were subjected to the RNA extraction procedure described above. An extraction control (contained all reagents needed in the extraction with the exception of the isolates) was included to check for contamination of the RNA. The extracts were then kept in -20°C freezer for later use.

3.10 Primer and probe rehydration

Primers and probes used in this work were designed according to the CDC protocol “Real-Time RT-PCR Assays for Non-Influenza Respiratory Viruses, 2010”. Primers and probes

were received in lyophilised form and were rehydrated with nuclease free water (NFW) to get a concentration of 100pmol/μl.

3.11 Working concentrations for the primers and probes

The working concentrations for the various primers and probes were established according to the CDC protocol "Real-Time RT-PCR Assays for Non-Influenza Respiratory Viruses, 2010".

The mol ratio $C_1V_1 = C_2V_2$ was used in calculating the volumes of the rehydrated primers and probe to be added to nuclease free water to get the working concentration.

C_1 was the initial concentration of the rehydrated primers or probes. In all instances the initial concentration of the primers or probes was 100pmol/μl. C_2 was the working concentration as stated in the protocol. V_2 was the volume of the working concentration. V_1 was the unknown volume of rehydrated primers or probes to be added.

3.12 Testing positive RNA controls using real time RT-PCR

In line with the CDC protocol, the rRT-PCR protocol for HPIV-1 to HPIV-4 was established. A master mix was prepared for each of the four human parainfluenza viruses (HPIV 1-4) and RNP. Twenty microlitres each of the master mix was dispensed into their respective positions on a PCR plate. To each 20μl of the appropriate master mix, 5μl of the appropriate extracted RNA (positive control) was added. Cycling conditions in the Applied Biosystems Incorporated (ABI) 7300 Real time PCR were set and the samples were tested. The cycling conditions were; 10 minutes at 45°C, 10 minutes at 95°C and 45 cycles of [15 seconds at 95°C and 1 minute at 55°C]. The reagents used in the master mix for the RNA controls were the same as that used for the test samples.

3.13 Serial dilutions

The extracted RNAs (for the four parainfluenza viruses) were later diluted into concentrations of 1/10, 1/100, 1/1000 and 1/10000. Real time RT-PCR was run on the diluted RNAs and their Ct values recorded.

3.14 Testing clinical samples using real time RT-PCR

3.14.1 Master Mix Preparation

The protocol used was “Real-time RT-PCR Assays for Non-Influenza Respiratory Viruses, 2010” from CDC. The ABI AgPath-ID One Step RT-PCR kit was used in the master mix preparation.

Table 3. 1: Master mix constituents for one reaction

Reagents	Volume for 1 reaction
2X RT-PCR Buffer	12.5µl
25X RT-PCR Enzyme Mix	1.0µl
50X Forward primer	0.5µl
50X Reverse primer	0.5µl
50X probe	0.5µl
Nuclease-free water	5.0µl
Total	20.0µl

Real time RT-PCR was performed using the ABI 7300 and 7500 fast thermocycler. The hemagglutinin-neuraminidase gene of HPIV-1 to HPIV-3 and the nucleocapsid protein gene of HPIV-4 were amplified from the RNA extracts using rRT-PCR to detect the presence of HPIVs. Five microlitres of extracted RNA was added to 20µl of the master mix for each target. Negative and positive controls were included in each run. The negative control was

nuclease free water. Positive controls were HPIV-1 to HPIV-4 RNA that was extracted from the isolates obtained from CDC.

Amplification conditions for rRT-PCR were 10 minutes at 45°C for reverse transcription. Enzyme activation at 95°C for 10 minutes and 45 cycles of [15 seconds at 95°C for denaturation and 1 minute at 55°C for primer annealing]. All samples were tested for RNP and all four HPIVs.

3.15 Conventional PCR

The rRT-PCR forward and reverse primers were used to amplify the hemagglutinin-neuraminidase gene of HPIV-1 to HPIV-3 and the nucleocapsid gene of HPIV-4 from the extracted RNA of samples that tested positive by rRT-PCR. A 20µl master mix was prepared using Qiagen One-Step RT-PCR Kit. The master mix consisted of 5X RT-PCR Buffer, dNTP mix, forward and reverse primers, enzyme mix and nuclease free water. Five microlitres of RNA template was used in the amplification. DNA synthesis was achieved under the following conditions: 50°C for 30 minutes for reverse transcription, enzyme activation at 95°C for 15 minutes, forty cycles of [denaturation at 94°C for 30 seconds, primer annealing at 55°C for 30 seconds and extension at 72°C for 2 minutes] followed by a final extension at 72°C for 10 minutes and a holding temperature of 4°C.

3.15.1 Gel electrophoresis

The PCR amplicons were run on a 1.5% (W/V) agarose gel. The 1.5 % (W/V) agarose gel was prepared by dissolving 1.5 g of agarose powder in 100ml of 1X TAE buffer. Five microlitres of ethidium bromide was added to the solution and the gel was cast using a 16 well comb.

Five microlitres of amplified conventional PCR products were mixed with 2 μ l of loading dye and then loaded onto the 1.5% (W/V) agarose gel. Electrophoresis was run in 1X TAE buffer at 100V for about 20 minutes at which time the amplicons were expected to run up the gel.

Amplified genes were viewed under UV using Kodak Gel Logic 100 imaging system. Positivity was determined by the size of the amplicon. Amplicon size was originally determined using HPIV positive controls based on a 100 molecular weight marker. A BLAST of the sequences generated from the HPIV controls showed true HPIV gene.

3.16 Purification of PCR amplicons

HPIV positive PCR amplicons were purified using QIAquick PCR Purification kit (Qiagen, Hilden, Germany). Purification was done to get rid of excess primers and nucleotides that were not used in the amplification step.

Hundred microlitres of buffer PB was added to each 20 μ l of PCR amplicon (5 volume of buffer to one volume of PCR amplicon). QIAquick spin column was placed into a 2 ml collection tube. The solution of buffer PB and PCR amplicons was added to the spin column and centrifuged at 13.2 rpm (maximum speed) for 1 minute so as to bind the dsDNA to the membrane in the spin column. The flow through was discarded and the spin column placed back into the same collection tube. dsDNA bound to the membrane in the spin column was then washed with 0.75 ml buffer PE (which was diluted with 100% ethanol) and spun at 13.2 rpm for 1 minute. The flow through was discarded and the collection tube reused for an additional minute centrifugation to get rid of any residual ethanol. The QIAquick columns were placed into well labelled RNase and DNase free 1.5 ml microcentrifuge tube. Thirty microlitres of elution buffer EB was added to the centre of the QIAquick membrane, allowed

to stand for a minute and then centrifuged to elute purified dsDNA. The purified dsDNA was stored at -20°C for later use.

3.17 Cycle sequencing of purified amplicons

The ABI Big Dye Terminator v 3.1 sequencing kit was used in the cycle sequencing. The Sanger DNA sequencing method was used (Sanger *et al.*, 1977). An 8 µl master mix was prepared for each target. The master mix consisted of 2µl nuclease free water, 2µl sequencing buffer, 2µl of the appropriate primer, 2µl Big Dye Terminator (the Big Dye terminator consisted of a mixture of 2',3'- dideoxynucleoside triphosphates: ddATP, ddCTP, ddGTP and ddTTP, 3'- deoxynucleoside triphosphates: dATP, dCTP, dGTP and dTTP, DNA polymerase and MgCl₂). Eight microlitres each of the master mix was dispensed into righty labelled PCR tubes. Two microlitres of the appropriate purified PCR amplicon was added to the corresponding labelled PCR tube and mixed well. The PCR tubes were tightly closed and incubated in a 2700 Geneamp thermal cycler. Cycling conditions were enzyme activation at 94°C for 2 minutes. Twenty-five cycles of [94°C for 30 seconds for denaturation, 50°C for 15 seconds for primer annealing and 60°C for 4 minutes for extension] and a holding temperature of 4°C.

3.18 Purification of cycle sequencing products

Cycle sequencing PCR products were purified using Agencourt Cleanseq Dye Terminator Removal Procedure (Agencourt Bioscience).

Eight microlitres of magnetic beads was added to each of the cycle sequencing products. Forty-two microlitres of 85% ethanol was then added and left on a magnetic field for 3

minutes. DNA which is negatively charged binds to the magnetic particles and in turn gets attracted to the magnetic field forming a brown ring which is attached to the walls of the PCR tube. The rest of the liquid in the PCR tube was pipetted out with the PCR tube still in the magnetic field. Next, 100µl of 85% ethanol was added to each tube and kept on the magnetic field for another 3 minutes. The ethanol was then pipetted out ensuring that PCR tube was totally empty. The tube was allowed to air dry for 10 minutes still on the magnetic field. This was followed by the addition of 50µl of distilled water to each tube. The tubes were taken off the magnetic field and left on a plastic rack for 5 minute within which the magnetic beads were expected to fall off the walls and settle at the bottom of the PCR tube since there was no field holding the beads onto the walls. At this point DNA is expected to dissolve into the distilled water. The tubes were placed back onto the magnetic field once more for 2 minutes for the magnetic beads to get attracted to the field so that the soluble DNA can be pipetted out. Finally, 40µl of the clear solution was pipetted into a 96 well plate, covered with a plate septum and immediately loaded onto the ABI 3130 genetic analyzer to read the sequences generated.

3.19 Data handling

All data was handled anonymously and confidentially. Samples bore identification numbers and not participant's name. Questionnaires were filled and kept secured until data entry.

3.20 Statistical analysis

Appropriate measures of centrality were used to summarize quantitative variables. Tables were drawn to summarize qualitative data. Chi square test was used to check for test of association. Logistic regression was used to calculate the odds ratio for risk factors and HPIV

positivity. P values less than 0.05 were considered statistically significant. All data were analysed using Microsoft Office Excel 2007, SPSS version 20 and Stata 12.

Sequences generated were aligned with ClustalW 1.7 software (Thompson *et. al.*,, 1994). A BLAST was performed in Genbank to retrieve sequences similar to that generated and were also aligned with the ClustalW 1.7 software. Aligned sequences were imported to MEGA 5.2 for phylogenetic analysis.

3.21 Ethical issues

3.21.1 Ethical consideration

The research proposal was submitted to the Research and Ethical Review Committee of University of Ghana Medical School for review and approval. Permission was obtained from the Medical Superintendent of Princess Marie Louise Children's Hospital before sample collection.

3.21.2 Consent

Written voluntary consent forms were signed by guardians or parents of the participating children. In cases where guardians or parents could neither read nor write, the information in the consent forms were translated into local language and their thumb prints taken.

CHAPTER FOUR

4.0 RESULTS

4.1 Study participants

A total of 71 samples were collected from children between March and July 2013. Thirty-five of the participants were females (median age was 15 months; range was 1 month to 48 months) and 36 were males (median age was 14.5 months; range was 1 month to 39 months). Out of these 71 participants, 31 of them were less than 1 year old (Table 4.3)

4.2 Detection of HPIVs among participants

All 71 samples were tested for the presence of all four HPIVs by one step rRT-PCR. Samples that had Ct values less than 37 were considered positive. Fourteen of the samples tested positive for HPIVs (Appendix A: Table A1) representing HPIVs positive test rate of 19.7%. There were however different proportions for each of the HPIV types (Figure 4.1) in the 71 samples. No multiple serotype infections of HPIVs was detected in the positive cases

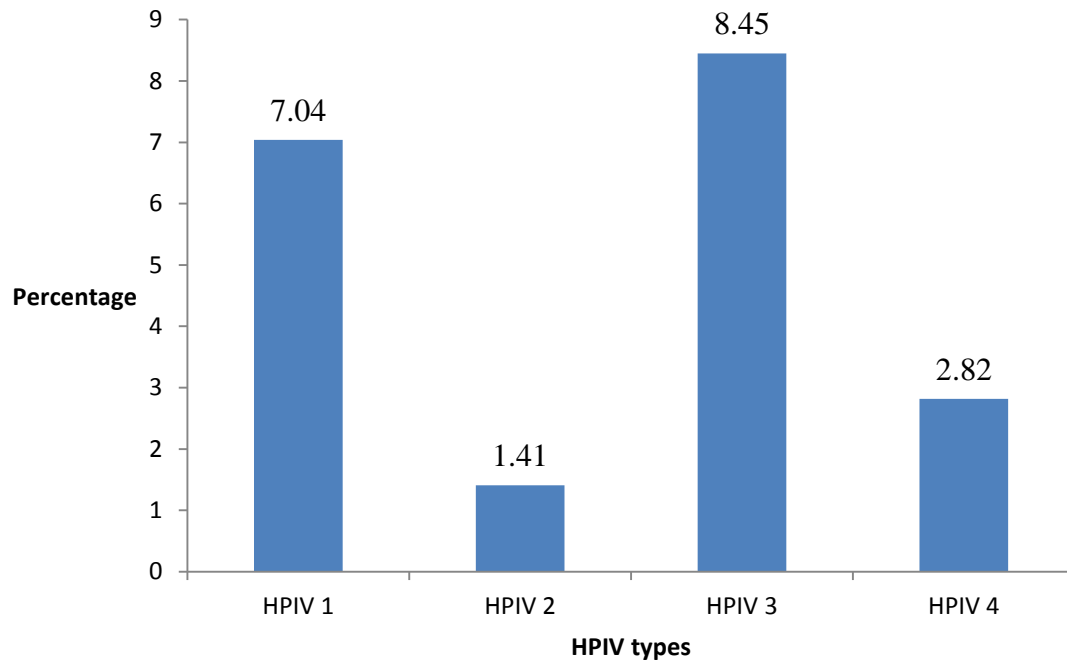
Figure 4.1: Proportions of each HPIV type in the 71 samples

Figure 4.1 shows that the HPIVs had different detection rates that were statistically insignificant ($P > 0.05$), with HPIV-3 being the most detected and HPIV-2 being the least detected.

Table 4.1: Duration from onset of symptoms to day of sample collection for patients who tested positive for the various HPIV types.

HPIV types	Range of days from the onset of symptoms to sample collection
1	3-7
2	8
3	3-5
4	3

Duration from onset of symptoms to sample collection for all positives was in the range of 3 to 8 days.

Table 4.2: Monthly distribution of cases that met the case definition and those associated with HPIVs during the study period.

Months	No. of samples	HPIV-1	HPIV-2	HPIV-3	HPIV-4	Total HPIVs detected
March	4	0	0	0	0	0
April	22	1	0	2	0	3
May	20	1	1	1	2	5
June	12	1	0	1	0	2
July	13	2	0	2	0	4
Total	71	5	1	6	2	14

Table 4.2 shows the monthly distribution of samples collected and associated with HPIVs. The highest number of enrolled participants was seen in April. Highest HPIV detection was in the month of May.

Table 4.3: Distribution of HPIVs positive cases against age and gender

Parameter	No. tested	HPIV positives				Total positives No. (%)	P-value
		HPIV-1 No. (%)	HPIV-2 No. (%)	HPIV-3 No. (%)	HPIV-4 No. (%)		
Age(Months)							
0-6	18	0(0)	0(0)	4(66.7)	2(33.3)	6(33.3)	0.67
7 – 11	13	1(50)	1(50)	0(0)	0(0)	2(15.4)	
12 – 23	19	2(50)	0(0)	2(50)	0(0)	4(21.1)	
24 – 48	21	2(100)	0(0)	0(0)	0(0)	2(9.5)	
Gender							
Male	36	4(57.1)	1(14.3)	2(28.6)	0(0)	7(19.4)	1
Female	35	1(14.3)	0(0)	4(57.1)	2(28.6)	7(20)	
Total	71	5	1	6	2	14	

8 out of the 14 HPIVs positives were in children less than 1 year old, 6 out of this 8 were detected in children up to 6 months. Almost equal percentages of males and females were infected with HPIV.

4.3 Molecular characterization

4.3.1 Gel electrophoresis

Figure 4.2: Gel photograph of amplified HN gene of HPIV-3

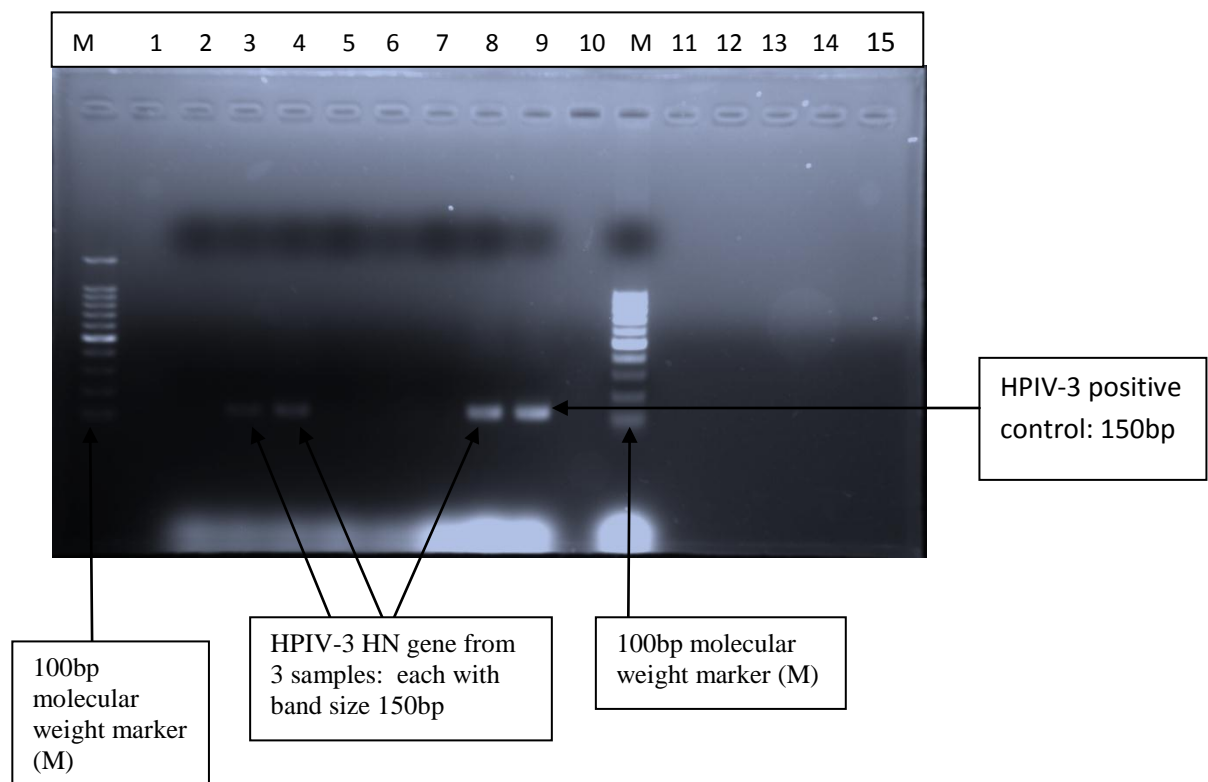


Figure 4.2 shows the HN gene of HPIV-3. Negative control was kept in lane 2 and it showed no band. Lane 9 shows a HPIV-3 positive control which showed a band size of 150 base pairs. Lanes 3, 4 and 8 contained samples that were positive for HPIV-3 HN gene with band sizes of 150 base pairs each. Lanes 4, 6 and 7 showed no band.

4.3.2 Phylogenetic analysis

Figure 4.3: Phylogenetic analysis of HPIV-1 to HPIV-4.

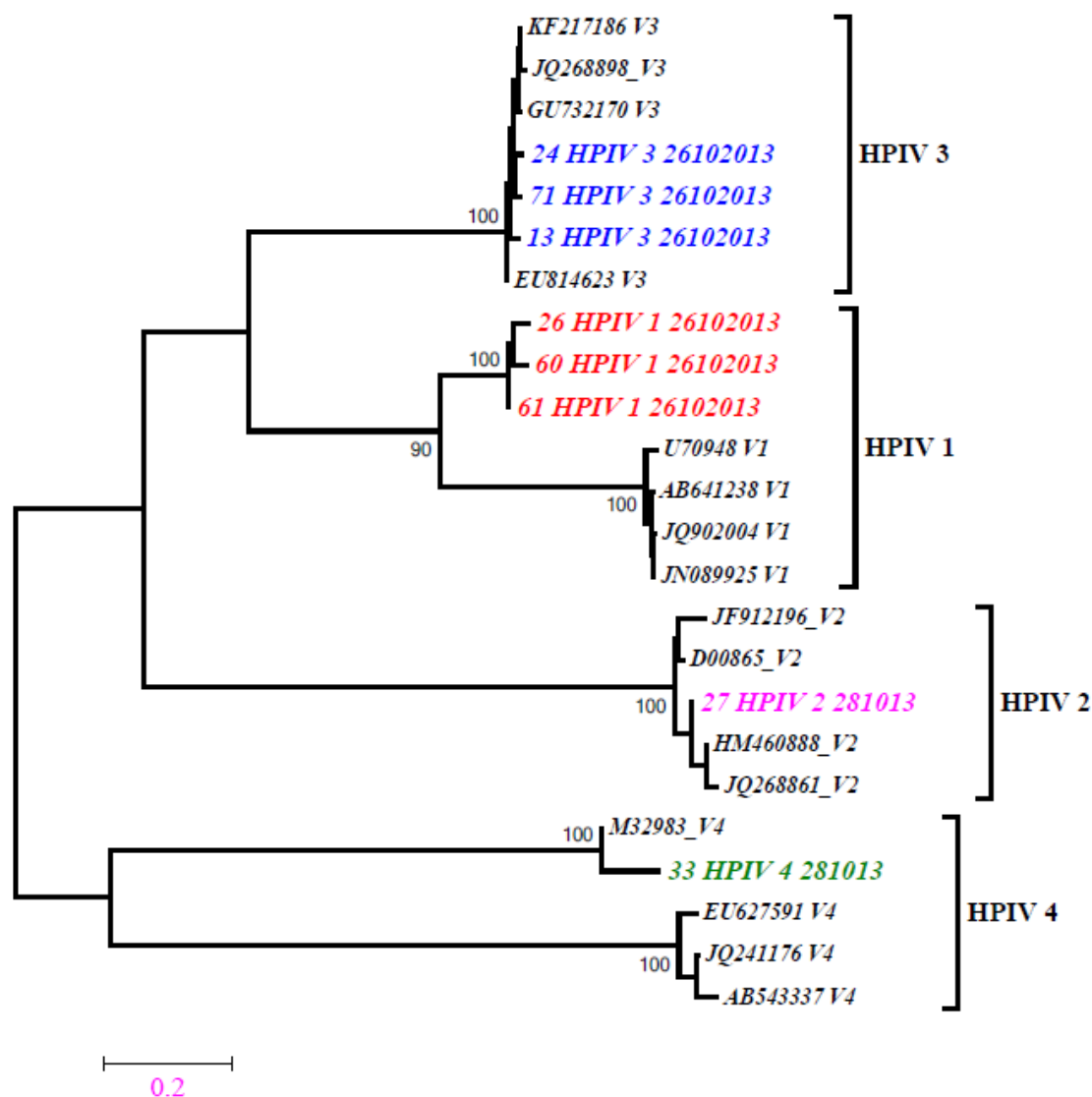


Figure 4.3 shows a phylogenetic tree of 8 HPIVs that were sequenced from clinical samples. These 8 were the only ones whose sequences were generated by the ABI genetic analyser. These are: three HPIV-1, one HPIV-2, three HPIV-3 and one HPIV-4. The tree was constructed from the HN genes of HPIV-1 to HPIV-3 and the NP gene of HPIV-4 using distance method and the neighbour-joining algorithm. Clinical HPIV strains are identifiable by the sample identification number, followed by HPIV 1, HPIV 2, HPIV 3 or HPIV 4 and next by the date of analysis. Reference strains are written in black while clinical strains are coloured.

4.3 Risk factors for HPIV infection and clinical features of participants

4.3.1 Risk factors

Table 4.4: Assessment of risk factors against the HPIVs

Risk factor	Factor present	Positive No. (%)	Negative No. (%)	Crude OR	P value	95% CI	Adjusted OR	P value	95% CI
Age category									
	0-6	6(33.3)	12(66.7)	1					
	7-11	2(15.4)	11(84.6)	0.364	0.039	(0.139, 0.950)	0.246	0.009	(0.087, 0.700)
	12-23	4(21.1)	15(78.9)	0.533	0.118	(0.242, 1.174)	0.569	0.196	(0.242, 1.336)
	≥24	2(9.5)	19(90.5)	0.211	0.001	(0.082, 0.538)	0.155	0.000	(0.056, 0.428)
Exclusive breastfeeding	Yes	9(17.3)	43(82.7)	0.586	0.117	(0.301, 1.142)	0.671	0.318	(0.307, 1.468)
	No	5(26.3)	14(73.7)	1					
Environmental smoke	Yes	6(15.4)	33(84.6)	0.545	0.06	(0.290, 1.026)	0.485	0.085	(0.213, 1.106)
	No	8(25.0)	24(75.0)	1					
Passive smoke	Yes	6(15.4)	33(84.6)	0.545	0.06	(0.290, 1.026)	0.909	0.812	(0.413, 2.000)
	No	8(25.0)	24(75.0)	1					
Sibling with RTI	Yes	10(25.0)	30(75.0)	2.25	0.019	(1.141, 4.438)	2.584	0.011	(1.241, 5.379)
	No	4(12.9)	27(87.1)	1					

P=0.0002 Pseudo R²=0.1155

The result in Table 4.4 showed that globally the logistic regression model used was statistically significant at the 5% threshold level. The model gave a pseudo R² of 0.12, an indication that the risk factors are able to explain about 12% of the probability of the variations of being positive or negative for HPIVs. Among all the risk factors, only age and having siblings with RTI were significantly associated with HPIV positivity.

4.3.2 Clinical features of participants

Table 4.5: Distribution of the various respiratory illnesses in the HPIV positive cases

HPIV types	Diagnosis				Total
	Bronchopneumonia	Pneumonia	Bronchiolitis	RTI	
HPIV-1	3	1	0	1	5
HPIV-2	1	0	0	0	1
HPIV-3	5	0	1	0	6
HPIV-4	2	0	0	0	2
Total	11	1	1	1	14

P value of diagnosis = 0.000

Table 4.5 shows the distribution of the various diagnoses in the HPIV positives. Bronchopneumonia was the most prevalent respiratory diagnosis. HPIV-1 to HPIV-4 were all associated with bronchopneumonia with HPIV-3 dominating.

Table 4.6: Percentage presentation of symptoms by HPIV positive patients

Symptoms	No. (%)
Cough	14(100)
Fast breathing	12(85.7)
Nasal discharge	11(78.6)
Difficulty feeding	6(42.9)
Difficulty in breathing	6(42.9)
Fever	6(42.9)
Diarrhoea	5(35.7)
Vomiting	3(21.4)

P=0.059 Total number of positives=14

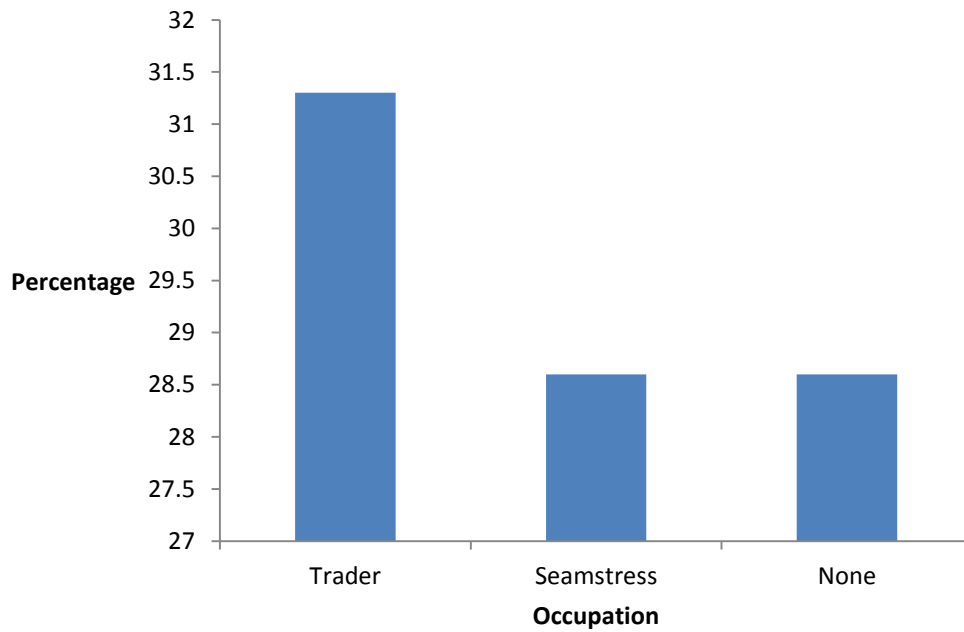
Table 4.6 shows the diverse symptoms that HPIV positive patients presented with. Vomiting was the least occurring symptom and cough the most predominant.

Table 4.7: Association between education and HPIV status

Parents' educational level	Number	HPIV status	
		Negative No. (%)	Positive No. (%)
None	19	15(78.1)	4(21.1)
Primary	16	12(75.0)	4(25.0)
JHS	22	18(81.8)	4(18.2)
SHS	8	6(75.0)	2(25.0)
Tertiary	6	6(100.0)	0(0)
Total	71	57	14

P-value=0.745

Table 4.7 shows that there was no association between education and HPIV status. The majority of parents were either not educated or had only basic education (primary and JHS: junior high school). No child was positive for parents who had tertiary education.

Figure 4.4: Occupation of parents whose children tested positive for the HIVs

P-value=0.956

Figure 4.4 shows the occupation of parents (main care provider) whose children tested positive. The majority of the parents of HIV positive children were traders (31.3%). Equal proportions (28.6%) were either seamstresses or unemployed.

CHAPTER FIVE

5.0 DISCUSSION

Human parainfluenza viruses are important viral aetiological agents in ALRTIs, especially in children under the age of five years. In the current study, HPIVs were investigated in 71 children that presented with ALRTI at the Princess Marie-Louise Children's Hospital in Accra from March to July 2013. The ages of the children in this study were from 1 month to 48 months (4 years) with the majority (44%) being less than one year old. This finding is similar to those of several other studies (Kusel *et al.*, 2006; Ahmed *et al.*, 2012; Lu *et al.*, 2013) which also showed the same trend, having the majority of affected children being the less than 1 year old. This could be due to the fact that the young children have a premature immune system and hence are more prone to infections (Holt, 2005).

The Ct values obtained as well as the characteristics of the amplification curves (Appendix A: Table A1) from the real time RT-PCR showed that the samples were true positives. Fourteen children tested positive; hence the HPIVs positive test rate was 19.7%. This rate was higher than what was detected in several other studies which used PCR in testing samples. For example the HPIVs positive test rate seen in the current study was much higher than what was found in Senegal and Ghana (Niang *et al.*, 2010; Kwofie *et al.*, (2012). Ahmed and colleagues (2012) in Kenya also had a HPIV positive test rate lower than what was found in the current study. This high rate necessitates prompt medical attention to viral respiratory pathogens especially HPIVs.

No significant difference ($p>0.05$) was observed among the various HPIV detection rates. HPIV-3 had the highest occurrence. This was followed by HPIV-1, HPIV-4 and HPIV-2

respectively (Figure 4.1). This is similar to the findings of Morgan *et al.*, (2012) in Thailand where HPIV-3 was the most highly detected followed by HPIV-1 and then HPIV-2. Their study however did not include HPIV-4. In Ghana, Kwofie *et al.*, (2012) also found HPIV-3 to be of the highest predominance followed by HPIV-1. A similar trend was observed by Weinberg *et al.*, (2009). The predominance of HPIV-3 among all the HPIVs buttresses it as the most common HPIV detected in children and it agrees with literature which states HPIV-3 to be the most common of the HPIVs (Henrickson, 2003). The reason for its predominance appears complex however an explanation to its occurrence could be one possible means to prevent or minimize its infection. HPIV-2 had the least predominance of 1.41%. This observation is similar to what was found in the work done by Shafik *et al.*, (2012) where they looked at the viral etiological agents of lower respiratory tract infections among the under five year old Egyptian children. Different viruses were detected in their study but with regard to HPIV, type 2 was the least dominant. Very little information is available on the prevalence of HPIV-4 mostly because most laboratories do not include it in their diagnosis (Lau *et al.*, 2005) and it is also reported to cause mild upper respiratory illness. Surprisingly, the current study presented HPIV-4 to have a positive test rate two times higher than that of HPIV-2. HPIV-4 could be associated with more ALRTIs, therefore underestimated. More research on this HPIV type would be very useful in the near future.

No HPIV multiple serotype infections were detected in the samples. This was not the case in the work done by Morgan *et al.*, (2012) where multiple serotype infections were detected in two patient samples; HPIV-1 and HPIV-2 were detected in one sample, then HPIV-2 and HPIV-3 in another.

The period from the onset of clinical symptoms to the day of sample collection for patients who tested positive for the HPIVs ranged from 3 to 8 days (Table 4.1). Three days was the

most frequent. Frank *et al.*, (1981) reported that children shed HPIVs from 3 to 4 days prior to the onset of clinical symptoms and continue shedding the virus for about 10 days after the onset of clinical symptoms. In this study, the duration from the onset of symptoms to sample collection (3 to 8 days) among the HPIV positive patients were all within the 10 days of viral shedding after the onset of clinical symptoms. This probably made the detection of HPIVs among the patients highly possible.

Majority of ALRTIs cases were seen in April and May with moderate ones in June and July (Table 4.2). One might have predicted that more HPIV positive cases be found in the month of April (the month with the highest ALRTI cases) but interestingly, the highest HPIV positive cases were detected in May. It was in May that all four types of the HPIVs were detected with HPIV-4 being the most dominant. Only HPIV-1 and HPIV-3 were detected in the other months. The next month of a higher HPIVs detection was in July and virus types found were HPIV-1 and HPIV-3. The duration of sample collection was not long enough to emphatically state that these months are the peaks of HPIV detection though some studies showed the peak of HPIV detection to be between January to April (Morgan *et al.*, 2012). The months of HPIV detection were all within the rainy season and agree with work done by de Arruda *et al.*, (1991). A clearer picture however may have been gotten if the sample collection had covered the dry season. Alternatively, the peak period for HPIVs can be determined by looking at the peak periods of ALRTIs cases from hospital records and then testing for the HPIVs during those times.

Males and females were almost equally infected with HPIVs. This is an indication of the fact that both males and females stand an equal chance of infection from the HPIVs. There were differences though not significant in the numbers of the HPIV types that infected each sex

(Table 4.3). Almost all the HPIV-1s were found in males while more than half of the HPIV-3 infected females. This observation is in line with work done by Kwofie *et al.*, (2012) and in contrast to the observation by Weinberg *et al.*, (2009). All the HPIV-2 and HPIV-4 were found in a male and in females respectively.

Amplified HN and NP genes from HPIV positive samples were determined by the size of amplicon (Figure 4.2 and Appendix C; Figure C1). A BLAST performed in genbank after the sequences were generated confirmed that the amplicon were truly that of HPIV HN and NP genes. Eight HPIV samples tested positive by conventional PCR.

Phylogenetic analysis of the HPIVs sequenced generated in this study showed that the individual strains within an HPIV type clustered closely together and with reference strains. This is an indication that the individual strains within a type were closely related (Figure 4.3). Contrary to the study by Terrier *et al.*, (2008), the nucleotide sequences of the F and HN genes of HPIV-2 isolates showed differences when compared with a HPIV-2 prototype strain and consequently fitted in a cluster different from that of the HPIV-2 prototype.

Several factors predispose young children to viral respiratory tract infections. The present study assessed risk factors such as age, exclusive breastfeeding, passive smoking, environmental smoking and siblings with RTIs. Univariate and multivariate analysis of the risk factors proved age and siblings with RTI to be the only significant risk factors associated with HPIV infection.

Age plays a crucial role in the development of immunity. It is known that young children have a premature immune system and thus more prone to diseases (Holt, 2005). In this study, more than half (57.1%) of the HPIV positive patients were within the first year of life (Table

4.3) with the median age being 8.5 months. This finding is in line with that of Morgan *et al.*, (2012) where the less than one year olds were the most infected with HPIV. Contrary to Weinberg *et al.*, (2009), children within the first five months of life and those within the second year had equal HPIV positive test rates. Out of the 57.1% HPIV positive patients who were less than one year, the greater part was in their first 6 months of life. The observation in this age group (first six months) could be due to the fact that maternal neutralizing antibodies tend to decrease significantly in children during the first 6 months of life making them more vulnerable to infection (Henrickson, 2003). Among the children within 6 months old, HPIV-3 was the most common HPIVs detected. HPIV-3 infection has been reported to occur earlier in life than the other HPIVs and infants younger than 6 months are the most vulnerable to HPIV-3 infection (Parrott *et al.*, 1962, Henrickson, 2003).

In Table 4.3, it was observed that a lesser number of children were positive for HPIVs as they advanced in age. This was confirmed in the assessment of age against HPIV positivity (Table 4.4). There was a reduced risk of getting infected with HPIVs as one grows older and this was statistically significant. The decreased risk of HPIV positivity as one advanced in age may be due to the presence of antibodies from previous HPIV infections and the development of the immune system. Both cellular immunity as well as humoral immunity to the HN and F glycoproteins protect against HPIV infection (Henrickson, 2003). Anti-HN antibodies alone do not protect against HPIVs but a concurrent increase in anti-F antibody (Henrickson, 2003). The highest HPIV positive test rate among the less than one year group may also be dependent on the fact that the greater parts of enrolled patients were less than one year old.

Breastfeeding is said to transfer maternal antibodies to infants to reduce infections. In this current study, a higher percentage of the children enrolled were exclusively breastfed for the first 6 months as against a lower number that was not. In the assessment of children

exclusively breastfed for the first 6 months of life against those not exclusively breastfed, it was observed that those exclusively breastfed were at a reduced risk of HPIV infection (odds ratio: 0.671) than those not exclusively breastfed. In other words there was a 23.9% reduced risk of having HPIV infection in children who were exclusively breastfed compared to those not exclusively breastfed. The p-value (0.318) however indicates that the probability of being at a lesser risk of HPIV infection if exclusively breastfed was due to chance (Table 4.4). Cushing *et al.*, (1998) found that exclusive breastfeeding for the first 6 months of life significantly reduced the risk of suffering from lower respiratory tract infection. In their study also, the annualized incidence rate for lower respiratory tract infection was higher for partial breastfeeding than exclusive breastfeeding during the first 6 months of life.

More than half of the children were exposed to environmental smoke and passive smoke. Assessment of environmental smoke and passive smoke in relation to HPIV positivity showed that those exposed to either passive smoke or environmental smoke were at a reduced risk of HPIV infection than those who were not exposed. The likelihood of being at a reduced risk of HPIV infection when exposed to either environmental or passive smoke was however statistically insignificant ($p > 0.05$). This is contrary to the report of Laurichesses *et al.*, (1999) that cited environmental smoke as a predisposing factor to HPIV infection.

Koehoorn *et al.*, (2008) found out that having older siblings and sharing room with them increases the likelihood of viral transmission. In the current study, it was observed that children who had siblings with a respiratory tract illness were at a higher risk (odds ratio = 2.584) of HPIV infection when compared with those who did not have siblings with a respiratory tract illness and the difference was statistically significant (Table 4.4). The mode of HPIV transmission (through direct contact with contaminated surfaces or inhalation of

infective particles in the air) predisposed these younger ones to infection when in close contact with siblings that shed the virus.

The majority of patients positive for HPIV in this current study were diagnosed have bronchopneumonia. Pneumonia, bronchiolitis and the unspecified RTI were the least diagnosed (Table 4.5). Contrary to the study by Weinberg *et al.*, (2009), croup was the most common diagnosis due to HPIV -1, HPIV-2 and HPIV-3. Ren *et al.*, (2011) also reported that majority of their HPIV positive patients were diagnosed of pneumonia followed by bronchopneumonia. Kwofie *et al.*, (2012) reported all HPIVs detected in their study to be associated with pneumonia. All the four types of HPIVs in this current study were associated with bronchopneumonia. HPIV-3 was associated with almost all the diagnoses. HPIV-4 was reported by Vachon *et al.*, (2006) to be associated with bronchiolitis but this case of bronchiolitis was associated with HPIV-3.

About 90% of the enrolled children presented with cough (Appendix B; Table B2). This observation was similar to that of Shafik *et al.*, (2012) where 98% of LRTI patients presented with cough. It was also observed that all the HPIV positives presented with cough (Table 4.6) emphasizing it as an important and common symptom associated with respiratory tract infections. Fast breathing and nasal discharge were also highly detected among the HPIV positive children. There was no significant difference ($p>0.05$) between the presence and absence of each symptom in relation to HPIV positivity with the exception of fast breathing, vomiting and nasal discharge. The percentage presentation of difficulty in breathing observed in the ALRTI cases in this study (Appendix B; Table B2) was lower than that reported by Shafik *et al.*, (2012).

A general observation with respect to education was that a small percentage of children with ALRTIs were from parents who had a higher education (tertiary). Also, none of the HPIV positive children were from parents who had tertiary education (Table 4.7). With a higher education, parents are able to understand the importance of implementing preventive measures that minimize the spread of HPIV. There was however no association between education and HPIV positivity ($P>0.05$).

Most of the parents of the HPIV positive children were traders who probably worked in overcrowded places thus predisposing their children to infection (Figure 4.4). Contact with contaminated surfaces and inhalation of aerosol droplets which most often is higher in overcrowded places are means of HPIV transmission.

5.1 CONCLUSION AND RECOMMENDATIONS

5.1.1 Conclusion

In conclusion:

- The study detected all four types of HPIVs in children less than five years of age that presented with ALRTIs.
- Children less than one year were the most infected with the HPIVs.
- Phylogenetic analysis showed the strains within each HPIV type to be closely related. HPIVs clustered with reference strains.
- Age and having siblings with RTI were risk factors significantly associated with HPIV infection.
- Bronchopneumonia was the most common diagnosis among patients that tested positive for HPIVs

5.1.2 Recommendations and further studies

- Large scale investigation of HPIVs in ALRTI cases at different sampling sites for longer periods is needed to establish the seasonal pattern of the virus in Ghana.
- The study was conducted using a sensitive, specific but expensive diagnostic assay (rRT-PCR) which is not cost effective for routine test in hospital laboratories. It is recommended that this study be repeated using other relatively less expensive diagnostic assays (example; ELISA) and the results evaluated with that of the rRT-PCR. Based on the outcome, a conclusion can be reached on the implementation of the most appropriate diagnostic assay for routine testing.

- Continuous HPIV surveillance as well as molecular characterization is essential to closely monitor antigenic and genetic differences in the HPIV viral genome. This will be useful in the candidate vaccines being tested.
- More studies on HPIV-4 are necessary to generate enough information on this HPIV type.

5.1.3 Limitations of the study

- A control group was not included in this study. The detection of HPIV in the respiratory samples does not necessarily prove their involvement in the ALRTI. However the detection of HPIVs in a significantly higher proportion of ALRTI cases or the inability to detect HPIV in non-ALRTI cases will strengthen the role of the HPIVs in these ALRTI cases.
- The study was limited to only one sampling site hence the study outcome may not reflect the real HPIVs situation in the entire country.

REFERENCES

- Ahmed, J. A., Katz1, M. A., Auko, E., Njenga, M. K., Weinberg, M., Kapella, B. K., Burke, H., Nyoka, R., Gichangi, A., Waiboci, L.W., Mahamud, A., Qassim, M., Swai, B., Wagacha, B., Mutonga, D., Nguhi, M., Breiman, R. F. and Eidex, R.B. (2012). Epidemiology of respiratory viral infections in two long-term refugee camps in Kenya, 2007-2010. *Biomed Central Infectious Diseases*, 12(7), 1 - 8.
- Applied Biosystems. (n.d.). Real-time PCR versus traditional PCR. Available from http://www6.appliedbiosystems.com/support/tutorials/pdf/rtpcr_vs_tradpcr.pdf. Retrieved on 12/03/2014.
- Beck, E. T., He, J., Nelson, M. I., Bose, M. E., Fan, J., Kumar, S. and Henrickson, K. J. (2012). Genome Sequencing and Phylogenetic Analysis of 39 Human Parainfluenza Virus Type 1 Strains Isolated from 1997–2010. *PLoS One*, 7(9), e46048.
- Belanger, K., Beckett, W., Triche, E., Bracken, M. B., Holford, T., Ren, P., McSharry, J. E., Gold, D. R., Platts-Mills, T. A. and Leaderer, B. P. (2003). Symptoms of wheeze and persistent cough in the first year of life: Associations with indoor allergens, air contaminants, and maternal history of asthma. *American Journal of Epidemiology*, 158(3), 195 - 202.
- Berkley, J. A., Munywoki, P., Ngama, M., Kazungu, S., Abwao, J., Bett, A., Lassauniere, R., Kresfelder, T., Cane, P. A., Venter, M., Scott, J. A. and Nokes, J. D. (2010). Viral etiology of severe pneumonia among Kenyan young infants and children. *The Journal of the American Medical Association*, 303(20), 2051 - 2057.
- Berman, S. (1991). Epidemiology of acute respiratory infections in children of developing countries. *Reviews of Infectious Diseases*, 13(Suppl 6), S454 - 462.
- Brady, M. T., Evans, J. and Cuartas, J. (1990). Survival and disinfection of parainfluenza viruses on environmental surfaces. *American Journal of Infection Control*, 18, 18-23.

- Brown, J. J., Willard, D. D. S. and Fee, E. J. (2010). Surgical resection of the nasopharynx. *Operative Techniques in Otolaryngology*, 21, 26 - 34.
- Brinkman, F. S. L. and Leipe, D.D. (2001). Phylogenetic analysis. In: A. D. Baxevanis, B. F. F. Quellerie. *Bioinformatics: A practical guide to the analysis of genes and proteins* (2nd ed). John Wiley and Sons, Incorporated. New York. Pp. 323 - 356.
- Bryce, J., Boschi-Pinto, C., Shibuya, K., Black, R. E., and the WHO Child Health Epidemiology Reference Group. (2005). WHO estimates of the causes of death in children. *Lancet*, 365, 1147 - 1152.
- Centre for Disease Control and Prevention. Real-time RT-PCR assays for non-influenza respiratory viruses. (2010).
- Chanock, R. M. and McIntosh, K. (1985). Parainfluenza viruses. In: B. N. Fields (Ed). *Virology*. Raven Press. New York. Pg. 1241.
- Choppin, P.W. and Compains, R. W. (1975). Reproduction of paramyxoviruses. In: H. Franenkel-Conrat, R. R. Wagner (Eds). *Comprehensive Virology*. Plenum Press. New York. Pp. 95 - 178.
- Coelingh, K. J., Winter, C. C. and Murphy, B. R. (1985). Antigenic variation in the hemagglutinin-neuraminidase protein of human parainfluenza type 3 virus. *Virology*, 143, 569 - 582.
- Collins, P. L., Chanock, R. M. and McIntosh, K. (1996). Parainfluenza viruses. In: B. N. Fields, D. M. Knipe and P. M. Howley (Eds). *Fields Virology* (3rd ed.). Lippincott-Raven Press. Philadelphia. Pp. 1205 - 1210.
- Cote, M., J., Storey, D. G., Kang, C. Y. and Dimock, K. (1987). Nucleotide sequence of the coding and flanking regions of the human parainfluenza virus type 3 fusion glycoprotein. *Journal of General Virology*, 68, 1003 - 1010.

- Cupic, M., Lazarevic I., Knezevic, A. and Stanojevic, M. (2007). Conventional and molecular methods in diagnosis and monitoring of viral infection. *Srp Arh Celok Lek.* 135(9-10), 589 - 593.
- Cushing, A. H., Samet, J. M., Lambert, W. E., Skipper, B. J., Hunt, W. C. Young, S. A. and McLaren, L. C. (1998). Breastfeeding reduces risk of respiratory illness in infants. *American Journal of Epidemiology*, 147(9), 863 - 870.
- de Arruda, E., Hayden, F. G., McAuliffe, J. F., de Souza, M. A., Mota, S. B., McAuliffe, M. I. Geist, F. C., Carvalho, E. P., Fernandes, M. C., Guerrant, R. L., *et al.* (1991). Acute respiratory viral infections in ambulatory children of urban northeast Brazil. *Journal of Infectious Disease*, 164, 252 - 258.
- Erdman, D. D., Weinberg, G. A., Edwards, K. M., Walker, F. J., Anderson, B. C., Winter, J., Gonzalez, M. and Anderson L.J. (2003). Gene scan reverse transcription-PCR assay for detection of six common respiratory viruses in young children hospitalized with acute respiratory illness. *Journal of Clinical Microbiology*, 41(9), 4298 - 4303.
- Fe, M. M. M., Monteiro, A. J., Moura, F. E. A. (2008). Parainfluenza virus infections in a tropical city: Clinical and epidemiological aspects. *Brazilian Journal of infectious Diseases*, 12, 192 - 197.
- Frank, A. L., Taber, L. H., Wells, C. R., Wells, J. M., Glezen, W. P. and Pareues, A. (1981). Patterns of shedding myxoviruses and paramyxoviruses in children. *Journal of Infectious Disease*, 144, 433 - 441.
- Franz, A., Adams ,O., Willems, R., Bonzel, L., Neuhausen, N., Schweizer-Krantz, S., Ruggeberg, J. U., Willers, R., Henrich, B., Schrotten, H., Tenenbaum, T., (2010). Correlation of viral load of respiratory pathogens and co-infections with disease severity in children hospitalized for lower respiratory tract infection. *Journal of Clinical virology*, 48, 239 - 245.

- Fry, A.M., Curns, A. T., Harbour, K., Hutwagner, L., Holman, R. C., Anderson, L. J. (2006). Seasonal trends of human parainfluenza viral infections: United States, 1990-2004. *Clinical Infectious Diseases*, 43(8), 1016 - 1022.
- Galinski, M. S. (1991). Annotated nucleotide and protein sequences for selected paramyxoviridae. In D. W. Kingsbury (Ed). *The paramyxoviruses*. Plenum Press. New York. Pp. 537 - 568.
- Galinski, M. S., Troy, R. M. and Banerjee, A. K. (1992). RNA editing in the phosphoprotein gene of the human parainfluenza virus type 3. *Virology*, 186, 543 - 550.
- Godden, B. (2004). Sample size formulas. Available from <http://williamgodden.com/samplesizeformula.pdf>.
- Guerrier, G., Goyet, S., Chheng, E. T., Rammaert, B., Borand, L., Te, V., Try, P. L., Sareth, R., Cavailler, P., Mayaud, C., Guillard, B., Vong, S., Buchy, P. and Tarantola, A. (2012). Acute viral lower respiratory tract infections in Cambodian children. Clinical and epidemiological characteristics. *Pediatric Infectious Diseases*, 32, e8 - 13.
- Hall, B. C. (2001). Respiratory Syncytial virus and Parainfluenza virus. *The New England Journal of Medicine*, 344(25), 1917 - 1928.
- Hamaguchi, M., Yoshida, T., Nishikawa, K., Naruse, H. and Nagai, Y. (1983). Transcriptive complex of Newcastle disease virus. Both L and P proteins are required to constitute an active complex. *Virology*, 128, 105 - 117.
- Harberg, S. E., Stigum, H., Nystad, W. and Nafstad, P. (2007). Effects of pre and postnatal exposure to parental smoking on early childhood respiratory health. *American Journal of Epidemiology*, 166 (6), 679 - 686.
- Henrickson, K. J. (1991). Monoclonal antibodies to human parainfluenza virus type 1 detect major antigenic changes in clinical isolates. *Journal of Infectious Disease*, 164, 1128 - 1134.

- Henrickson, K. J. (2003). Parainfluenza Viruses. *Clinical Microbiology Reviews*. 16(2), 242 - 264.
- Henrickson, K. J., Hoover, S., Kehl, K. S. and Hua, W. (2004). National disease burden of respiratory viruses detected in children by polymerase chain reaction. *Pediatric Infectious Disease Journal*, 23(1Suppl), S11 - 8.
- Henrickson, K. J., Kuhn, K. J. and Savastski, L. L. (1994). Epidemiology and cost of human parainfluenza virus type one and two infections in young children. *Clinical Infectious Diseases*, 18, 770 - 779.
- Henrickson, K. J. and Savastski, L. L. (1992). Genetic variation and evolution of human parainfluenza virus type 1 hemagglutinin neuraminidase: analysis of 12 clinical isolates. *Journal of Infectious Disease*, 166, 995 - 1005.
- Higuchi, R., Dollinger, G., Walsh, P.S. and Griffith, R., 1992. Simultaneous amplification and detection of specific DNA-sequences. *Bio-Technology*, 10 (4), 413 - 417.
- Holt, P. G. (2005). Developmental factors as determinants of risk for infections and atopy in childhood. *European Respiratory Review*, 14(95), 69 - 73.
- HPIV Seasons. (2012). Available from <http://www.cdc.gov/parainfluenza/seasons.html>. Retrieved on: 8/06/2012.
- Hu, X. L., Ray, R. and Compans, R.W. (1992). Functional interactions between the fusion protein and hemagglutinin-neuraminidase of human parainfluenza viruses. *Journal of Virology*, 66, 1528 - 1534.
- Human parainfluenza viruses. (n.d.). Available from <http://www.cdc.gov/ncidod/dvrd/revb/respiratory/hpivfeat.html>. Retrieved on 8/06/2012.

- Invitrogen Corporation. 2008. Real-time PCR: from theory to practice. Available from <http://corelabs.cgrb.oregonstate.edu/sites/default/files/RealPCR.FromTheorytoPractice.pdf>. Retrieved on 24/03/2014.
- Isomura, S., Biritwum, R. B. and Nerome, K. (1986). Seroepidemiological study of parainfluenza viruses in preschool children in Ghana. *Journal of Tropical Pediatrics*, 32, 181 - 182.
- Jawetz, E., Melnick, J. L. and Adelberg, E. A. (2007). *Medical Microbiology* (24th ed.). McGraw Hill Publishers. New York. Pp. 546 - 554.
- Jin, Y., Xie, Z. P., Yan, K. L., Gao, H. C., Song, J. R., Yuan, X. H., Cheng, W. X., Hou, Y. D. and Duan, Z. J. (2012). Newly identified respiratory viruses associated with acute lower respiratory tract infections in children in Lanzou, China, from 2006 to 2009. *Clinical Microbiology and Infection*, 18(1), 74 - 80.
- Johnson, K. M., Chanock, R. M., Cook, M. K. and Huebner, R. J. (1960). Studies of a new human hemadsorption virus. I. Isolation, properties and characterization. *American Journal of Hygiene*, 71, 81 - 92.
- Juvén, T., Mertsola, J., Waris, M., Leinonen, M., Meurman, O., Roivainen, M., Eskola, J., Saikku, P., Ruuskanen, O. (2000). Etiology of community-acquired pneumonia in 254 hospitalized children. *Pediatric Infectious Disease Journal*, 19, 293 - 298.
- Karron, R. A. (2008). Respiratory syncytial virus and parainfluenza virus vaccines. In: S. A. Plotkin, W. A. Orenstein, P. A. Offit (Eds). *Vaccines* (5th ed). Saunders- Elsevier. Philadelphia. Pp. 1283 - 1293.
- Karron, R. A. and Collins, P. L. (2007). Parainfluenza viruses. In B. N. Fields, D. M. Knipe and P. M. Howley. (Eds). *Virology*. (5th ed.). Lippincott-raven Press. New York. Pp. 1497 - 1526.

- Killgore, G. E. and Dowdle, W. R. (1970). Antigenic characterization of parainfluenza 4A and 4B by the hemagglutination-inhibition test and distribution of HI antibody in human sera. *American Journal of Epidemiology*, 91, 308 - 316.
- Koehoorn, M., Karr, C. J., Demers, P.A., Lencar, C., Tamburic, L. and Brauer, M. (2008). Descriptive epidemiological features of bronchiolitis in a population-based cohort. *Pediatrics*, 122(6), 1196 - 1203.
- Kubista, M., Andrade, J. M., Bengtsson, M., Forootan, A., Jonak, J., Lind, K., Sindelka, R., Sjoback, R., Sjogreen, B., Strombom, L., Stahlberg, A. and Zoric, N. (2006). The real-time polymerase chain reaction. *Molecular Aspects of Medicine*, 27, 95 - 125.
- Kusel, M. M, de Klerk, N. H., Holt, P. G., Keadze, T., Johnston, S. L. and Sly, P. D. (2006). Role of respiratory viruses in acute upper and lower respiratory tract illness in the first year of life: a birth cohort study. *Pediatric Infectious Disease*, 25(8), 680 - 686.
- Kwofie, T. B., Anane, Y., Nkrumah, B., Annan, A., Nguah, S. B. and Owusu, M. (2012). Respiratory viruses in children hospitalized for acute lower respiratory tract infection in Ghana. *Virology Journal*, 9, 78.
- Lamb, R. A. and Kolakofsky, D. (1996). Paramyxoviridae: The viruses and their replication. In B. N. Fields, D. M. Knipe and P. M. Howley (Eds). *Fundamental virology* (3rd ed). Lippincott-Ravens. New York. Pp. 577 - 604.
- Lau, S. K. P., To, W., Tse, P. W. K., Chan, A. K. H., Woo, P. C. Y., Tsoi, H., Leung, A. F. Y., Li, K. S. M., Chan, P. K. S., Lim, W. W. L., Yung, R.W. H., Chan, K. and Yuen, K. (2005). Human Parainfluenza virus 4 outbreak and the role of diagnostic tests. *Journal of Clinical Microbiology*, 43(9), 4515 - 4521.
- Laurichesse, H., D. Dedman, J. M., Watson, J. M. and Zambon, M. C. (1999). Epidemiological features of parainfluenza virus infections: Laboratory surveillance in

- England and Wales, 1975-1997. *European Journal of Epidemiology*, 15 (5), 475 - 484.
- Lu, MP., Ma LY., Zheng, Q., Dong, LL., Chen, ZM. (2013). Clinical characteristics of adenovirus associated lower respiratory tract infection in children. *World Journal of Pediatrics*, 9(4), 346 - 349.
- Mahony, J. B. (2008). Detection of respiratory viruses by molecular methods. *Clinical Microbiology Reviews*, 21(4), 716 - 747.
- Marx, A., Gary, H. E., Marston, B.J., Erdman, D. D., Breiman, R. F., Torok, T. J., Plouffe, J. F., File, T. M and Anderson, L. J. (1999). Parainfluenza virus infection among adults hospitalized for lower respiratory tract infection. *Clinical Infectious Diseases*, 29, 134 - 140.
- Mäkelä, M. J., Puhakka, T., Ruuskanen, O. Leinonen, M. Saikku, P., Kimpimäki, M., Blomqvist, S. and Hyypiä, T. (1998). Viruses and bacteria in the etiology of the common cold. *Journal of Clinical Microbiology*, 36, 539 - 542.
- Masters, P. S. and Banerjee, A. K. (1988). Replication of nonsegmented negative strand RNA viruses. In J. J. Holland and P. Ahlquist (Ed), *RNA genetics*. CRC Press. Boca Raton. Pp. 137 - 158.
- McIntosh, K., Kurachek, S. C., Cairns, L. M., Burns, J. C. and Goodspeed, B. (1984). Treatment of respiratory viral infection in an immunodeficient infant with ribavirin aerosol. *American Journal of Childhood Diseases*, 138, 305-308.
- Morgan, O. W., Chittaganpitch, M., Clague, B., Chantra, S., Sanasuttipun, W., Prapasiri, P., Naorat, S., Laosirithavorn, S., Peret, T. C. T., Erdman, D. D., Baggett, H. C., Olsen, S. J., Fry, A. M. (2012). Hospitalization due to human parainfluenza virus-associated lower respiratory tract illness in rural Thailand. *Influenza and Other Respiratory Viruses*, 7(3), 280 - 285.

- Moscona, A. (2005). Entry of parainfluenza virus into cells as a target for interrupting childhood respiratory disease. *Journal of Clinical Investigation*, 115(7), 1688 - 1698.
- Moscona, A. and Peluso, R. W. (1992). Fusion properties of cells infected with human parainfluenza virus type 3: Receptor requirements for viral spread and virus-mediated membrane fusion. *Journal of Virology*, 66, 6280 - 6287.
- Moscona, A. and Peluso, R.W. (1991). Properties of human parainfluenza virus type 3 RNA polymerase/replicase activity in vitro: Consensus with other negative-stranded RNA viruses. *Journal of Virology*, 65(8), 4470 - 4474.
- Mulholland, E. K., Ogunlesi, O. O., Adegbola, R. A. (1999). Etiology of serious infections in young Gambian infants. *Pediatric Infectious Disease Journal*, 18(10), S35 - 41.
- Nascimento-Carvalho, C. M. C. (2001). Etiology of childhood community acquired pneumonia and its Implications for vaccination. *The Brazilian Journal of Infectious Diseases*, 5(2), 87 - 97.
- Niang, M., Diop, O., Sarr, F. (2010). Viral etiology of respiratory infections in children under 5 years old living in tropical rural areas of Senegal: The EVIRA project. *Journal of Medical Virology*, 82, 866 - 72.
- Nicholas, W. G., Campbell, P. A. J., Boeckh, M. (2008). Respiratory viruses other than influenza virus: impact and therapeutic advances. *Clinical Microbiology Reviews*, 21, 274 - 290.
- Nokso-Koivisto, J., Hovi, T. and Pitkaranta A. (2006). Viral upper respiratory tract infections in young children with emphasis on acute otitis media. *International Journal of Pediatric Otorhinolaryngology*, 70, 1333 - 1342.
- Ordas, J., Boga, J.A, Alvarez-Arguelles, M., Villa, L., Rodriguez, C., Ona, M., Rodrigue, J. and Melon, S. (2006). Role of metapneumovirus in viral respiratory infections in young children. *Journal of Clinical Microbiology*, 44(8), 2739 - 2742.

- Ortmann, D., Ohuchi, M., Angliker, H., Shaw, E., Garten, W. and Klenk, K. S. (1994). Proteolytic cleavage of wild type and mutants of the F protein of human parainfluenza virus type 3 by two subtilisin-like endoproteases, furin and Kex2. *Journal of Virology*, 68, 2772 - 2776.
- Ou, S.Y., Lin, G. Y., Wu, Y., Lu, X. D., Lin, C. X and Zhou, R. B. (2009). Viral pathogens of acute lower respiratory tract infection in hospitalized children from East Guangdong of China. *Zhongguo Dang Dai Er Ke Za Zhi*, 11(3), 203 - 206.
- Parks, G. D., and Lamb, R. A. (1990). Defective assembly and intracellular transport of mutant paramyxovirus hemagglutinin-neuraminidase proteins containing altered cytoplasmic domains. *Journal of Virology*, 64, 3605 - 3616.
- Parrott, R. H., Vargosko, A. J., Kim, H. W., Bell, J. A. and Chanock, R. M. (1962). Myxoviruses: Parainfluenza. *American Journal of Public Health and the Nation's Health*, 52(6), 907 - 917.
- Pavia, A. T. (2011). Viral infections of the lower respiratory tract: old viruses, new viruses and the role of diagnosis. *Clinical Infectious Disease*, 52 (suppl 4), S284 - S289.
- Quigley, M. A., Kelly, Y. J. and Sacker, A. (2007). Breastfeeding and hospitalization for diarrheal and respiratory infection in the United Kingdom Millennium Cohort Study. *Pediatrics*, 119(4), e837 - 842.
- Rajion, Z. A., Al-Khatib A. R., Netherway, D.J., Townsend, G. C., Anderson, P. J., McLean, N. R. and Samsudin, A. R. (2012). The nasopharynx in infants with cleft lip and palate. *International Journal of Pediatric Otorhinolaryngology*, 76, 227 - 234.
- Reed, G., Jewett, P. H., Thompson J., Tollefson, S. and Wright, P.F. (1997). Epidemiology and clinical impact of parainfluenza virus infections in otherwise healthy infants and young children less than 5 years old. *Journal of Infectious Diseases*, 175, 807 - 813.

- Ren, L., Gonzalez, R., Xie, Z., Xiong, Z., Liu, C., Xiang, Z., Xiao, Y., Li, Y., Zhou, H., Li, J., Yang, Q., Zhang, J., Chen, L., Wang, W., Vernet, G., Paranhos-Baccala, G., Shen, K. and Wang, J. (2011). Human parainfluenza virus type 4 infection in Chinese children with lower respiratory tract infections: a comparison study. *Journal of Clinical Virology*, 51, 209 - 212.
- Rudan, I., Boschi-Pinto, C., Biloglav, Z., Mulholland, K. and Campbell, H. (2008). Epidemiology and etiology of childhood pneumonia. *Bulletin of the World Health Organization*, 86, 408 - 416.
- Rutherford, G. W. (2009). Emerging infectious diseases and global Health, University of California, San Francisco, Global Health Sciences. 1 - 12.
- Rydbeck, R., Love, A., Orvell, C. and Norrby, E. (1987). Antigenic analysis of human and bovine parainfluenza virus type 3 strains with monoclonal antibodies. *Journal of General Virology*, 68, 2153 - 2160.
- Sale, J., Ahmad, A. A., Idris, H. W., Aliyu, A. M. and Rogo, L. D. (2010). Seroprevalence of human parainfluenza virus type 2 infection among children (1-5 years) in Zaria, Kudu State, Nigeria. *Bayero Journal of Pure and Applied sciences*, 3(1), 6 - 9.
- Sanger, F., Nicklen, S and Coulson, A. R. (1977). DNA sequencing with chain terminating inhibitors. *The proceedings of the National Academy of Sciences*, 74(12), 5463 - 5467.
- Schmidt, A. C., Schaap-Nutt, A., Bartlett, E. J. Schomacker, H., Boonyaratanakornkit, J., Karron, R. A., and Collins, P. L. (2011). Progress in the development of PIV vaccine. *Expert Review of Respiratory Medicine*, 5(4), 515 - 526.
- Shafik, C. F., Mohareb, E. W., Yassin A. S., Amin, M. A., El-Kholy, A., El-Karaksy, H and Youssef, F. J. (2012). Viral etiologies of lower respiratory tract infections among Egyptian children under five years of age. *Biomed Central Infectious Diseases*, 12(350), 1471 - 2334.

- Storch, G. A. (2000). Diagnostic Virology. *Clinical Infectious Diseases*, 31, 739 - 751.
- Terrier, O., Cartet, G., Ferraris, O., Morfin, F., Thouvenot, D., Hong, S. S. and Lina, B. (2008). Characterization of naturally occurring parainfluenza virus type 2 (hPIV-2) variants. *Journal of Clinical Virology*, 43, 86 - 92.
- Thompson J. D., Higgins D. G. and Gibson, T. J. (1994). ClustalW: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Research*, 22, 4673 - 4680.
- Tregoning, J. S. and Schware, J. (2010). Respiratory viral infections in infants: Causes, clinical symptoms, virology and immunology. *Clinical Microbiology Reviews*, 23(1), 74 - 98.
- Tyring, S. K. (2005). *Antiviral agents, vaccines and immunotherapies*. Marcel Dekker/CRC Press. New York. Pg. 359.
- Vachon, M. L., Dionne, N., Leblanc, E., Moisan, D., Bergeron, M. G. and Boivin, G. (2006). Human parainfluenza type 4 infections, Canada. *Emerging Infectious Diseases*, 12(11), 1755 - 1758.
- Vainionpää, R., Hyypiä, T. (1994). Biology of parainfluenza viruses. *Clinical Microbiology Reviews*, 7(2), 265 - 275.
- Wang, M. W., Tong, L., Grant, D., Cihlar, T. (2001). Expression and characterization of soluble parainfluenza virus type 1 hemagglutinin-neuraminidase glycoprotein. *Journal of Virological Methods*, 98, 53 - 61.
- Weinberg, G. A., Hall, C. B., Iwane, M. K., Poehling, K. A., Edwards, K. M., Griffin, M. R., Staat, M. A., Curns, A. T., Erdman, D. D. and Szilagyi, P. G. (2009). Parainfluenza virus infection of young children: estimates of the population-based burden of hospitalization. *Journal of Paediatrics*, 154, 694 - 699.

World Health Organisation and The Joint United Nations Programme on HIV/AIDS. (n.d.)

World Health Organisation Recommended Surveillance Standards (2nd ed).

Available from

<http://www.who.int/csr/resources/publications/surveillance/whocdscsr992.pdf>.

Retrieved on 07/03/2013.

World Health Organization. (2005). Make any mother and child count. Geneva. Available

from <http://www.cdc.gov/ncidod/dvrd/revb/respiratory/hpivfeat.html>. Retrieved on

8/06/2012.

APPENDICES

APPENDIX A

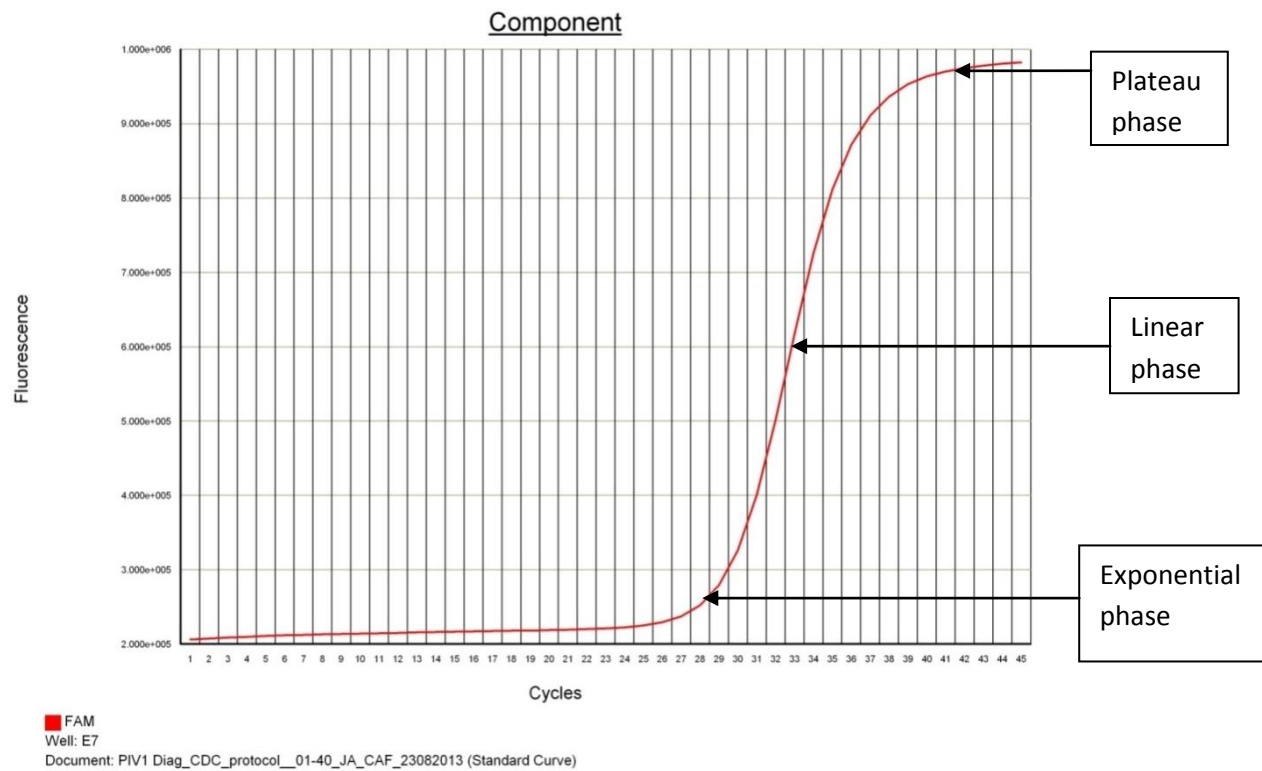
Figure A 1: A linear view amplification curve of a HPIV positive sample

Table A 1: Ct values of the 14 samples that tested positive for the human parainfluenza viruses.

Sample ID	Ct
PIV-13-13	15.42
PIV-13-24	31.48
PIV-13-26	26.88
PIV-13-27	25.28
PIV-13-30	35.11
PIV-13-32	22.53
PIV-13-33	25.37
PIV-13-39	36.68
PIV-13-50	31.21
PIV-13-56	36.26
PIV-13-60	29.94
PIV-13-61	28.89
PIV-13-69	34.33
PIV-13-71	15.57

Table A1 shows the Ct values of all samples that tested positive for the HPIVs using rRT-PCR. With the CDC protocol used (Real-Time RT-PCR Assays for Non-Influenza Respiratory Viruses, 2010), the Ct value for a weak positive sample is greater than or equal to 37. The Ct values for the samples that tested positive were all less than 37 and they showed sigmoid curves with exponential, linear and plateau phases (Appendix A, Figure A1). These features are characteristic of a true detection by rRT-PCR.

Table A 2: A plate map for one type of the human parainfluenza virus

	1	2	3	4	5	6	7	8	9	10	11	12
A	NC	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	PC
B	NC	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20	PC
C	NC	S21	S22	S23	S24	S25	S26	S27	S28	S29	S30	
D	NC	S31	S32	S33	S34	S35	S36	S37	S38	S39	S40	
E		S41	S42	S43	S44	S45	S46	S47	S48	S49	S50	
F		S51	S52	S53	S54	S55	S56	S57	S58	S59	S60	
G		S61	S62	S63	S64	S65	S66	S67	S68	S69	S70	
H		S71										

NC: Negative control PC: Positive control S: Sample

Appendix B

Table B 1: Risk factors for HPIV positive cases

ID	Age	Exclusive breastfeeding	Passive smoking	Environmental smoking	Siblings with ALRTI	No. in a room	No. of windows to a room
HPIV-13-13	6	No	No	No	Yes	4	3
HPIV-13-24	23	Yes	Yes	Yes	No	5	2
HPIV-13-26	24	Yes	No	No	Yes	3	1
HPIV-13-27	7	Yes	Yes	Yes	Yes	4	2
HPIV-13-30	10	No	No	No	Yes	3	1
HPIV-13-32	5	Yes	Yes	No	Yes	4	2
HPIV-13-33	5	No	No	No	No	3	2
HPIV-13-39	2	No	No	No	Yes	5	3
HPIV-13-50	18	Yes	Yes	Yes	No	3	1
HPIV-13-56	23	Yes	Yes	Yes	Yes	4	2
HPIV-13-60	12	No	No	No	Yes	3	3
HPIV-13-61	24	Yes	No	Yes	Yes	7	2
HPIV-13-69	2	Yes	Yes	Yes	Yes	5	2
HPIV-13-71	1	Yes	No	No	No	5	2

Table B 2: Symptoms presented by participants

Symptom	Number screened	Number positive	% positive per group	P value
Cough				
Absence	7	0		
Presence	64	14	21.9	
Difficulty in breathing				
Absence	41	8	19.5	0.593
Presence	30	6	20	
Fast breathing				
Absence	18	2	11.1	0.008
Presence	53	12	22.6	
Fever				
Absence	44	8	19.5	0.593
Presence	27	6	20	
Diarrhoea				
Absence	43	9	20.9	0.285
Presence	28	5	17.9	
Vomiting				
Absence	38	11	28.9	0.03
Presence	33	3	9.1	
Difficulty in feeding				
Absence	29	8	27.6	0.593
Presence	42	6	14.3	
Nasal discharge				
Absence	27	3	11.1	0.03
Presence	44	11	25	

Total number of patients=71

Total number of HPIV positives=14

Figure B 1: Percentage distributions of diagnoses of children that met the inclusion criteria

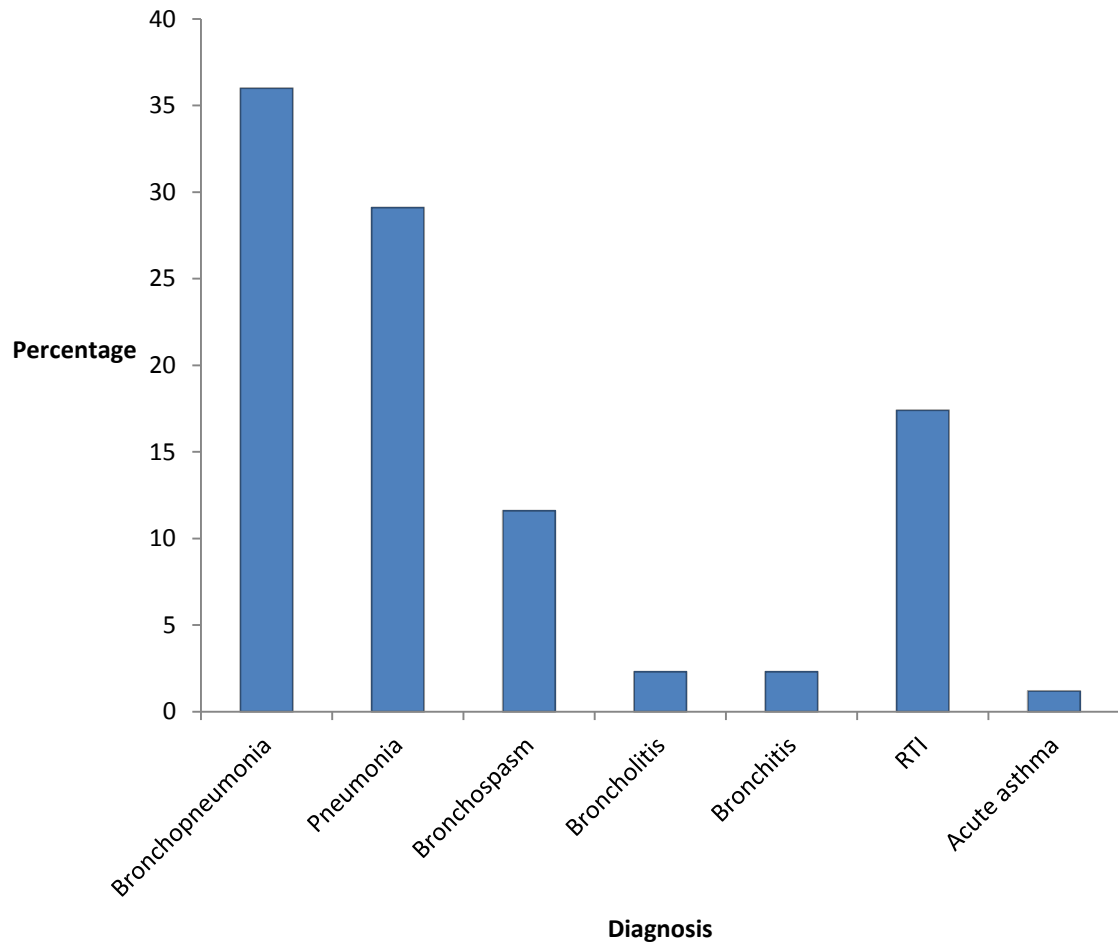
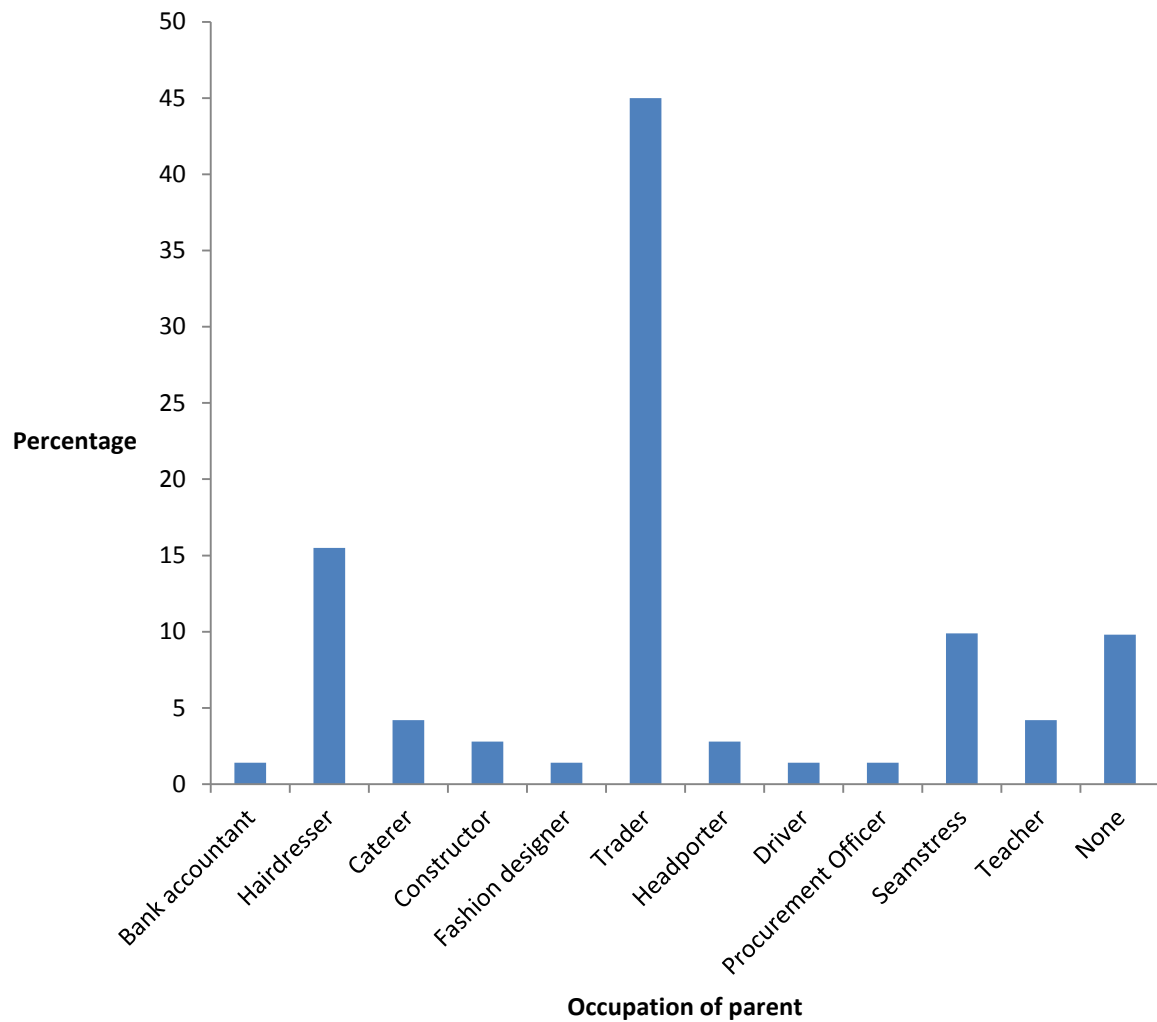


Figure B 2: Occupation of parents whose children participated in the study

Appendix C

Table C 1: Working concentrations of HPIVs primers and probes as well as the quantity of NFW that was used to rehydrate primers and probes to get a concentration of 100pmol/ μ l.

Primers and probes	Amount of NFW(μ l)	Working concentration(μ M)	Primer Sequence (5' > 3')
HPIV-1 Forward	190	25	AGT TGT CAA TGT CTT AAT TCG TAT CAA T
HPIV-1 Reverse	171	25	TCG GCA CCT AAG TAA TTT TGA GTT
HPIV-1 Probe	268	2.5	ATA GGC CAA AGA "T"TG TTG TCG AGA CTA TTC CAA
HPIV-2 Forward	186	37.5	GCA TTT CCA ATC TAC AGG ACT ATG A
HPIV-2 Reverse	123	37.5	ACC TCC TGG TAT AGC AGT GAC TGA AC
HPIV-2 Probe	292	2.5	CCA TTT ACC "T"AA GTG ATG GAA TCA ATC GCA AA
HPIV-3 Forward	172	37.5	TGG YTC AAT CTC AAC AAC AAG ATT TAA G
HPIV-3 Reverse	173	25	TAC CCG AGA AAT ATT ATT TTG CC
HPIV-3 Probe	152	10	CCC RTC TG"T" TGG ACC AGG GAT ATA CTA CAA A
HPIV-4 Forward	162	15	CTG CCA AAT CGG CAA TTA AAC
HPIV-4 Reverse	158	15	CTG GCA GCA ATC ATA AGR TGA TTC
HPIV-4 Probe	122	5	CA TTA TTA TCT CTG C"T"T TCC TTA CAG GCC ACA TCA

Probes have FAM (6-carboxy-fluorescein) at the 5' end and BHQ (Black hole quencher) at the 3' end.

Table C 2: Conventional PCR mastermix for the various targets (HPIV-1 to HPIV-4)

Kit used: Qiagen One-Step RT-PCR Kit

HPIV- 1

Reagents	X1
5X RT-PCR Buffer	5.0µl
dNTP mix	1.0µl
Primers F(25µM)	0.6µl
Primers R(25µM)	0.6µl
Enzyme mix	1.0µl
NFW	11.8µl
Total	20.0µl

HPIV- 2

Reagents	X1
5X RT-PCR Buffer	5.0µl
dNTP mix	1.0µl
Primers F(37.5µM)	0.4µl
Primers R(37.5µM)	0.4µl
Enzyme mix	1.0µl
NFW	12.2µl
Total	20.0µl

HPIV-3

Reagents	X1
5X RT-PCR Buffer	5.0 μ l
dNTP mix	1.0 μ l
Primers F(37.5μM)	0.4 μ l
Primers R(25μM)	0.6 μ l
Enzyme mix	1.0 μ l
NFW	12.0 μ l
Total	20.0 μ l

HPIV- 4

Reagents	X1
5X RT-PCR Buffer	5.0 μ l
dNTP mix	1.0 μ l
Primers F(15μM)	1.0 μ l
Primers R(15μM)	1.0 μ l
Enzyme mix	1.0 μ l
NFW	11.0 μ l
Total	20.0 μ l

Figure C 1: Gel photograph of the HN and NP genes of HPIV-1 and HPIV-4 positive samples

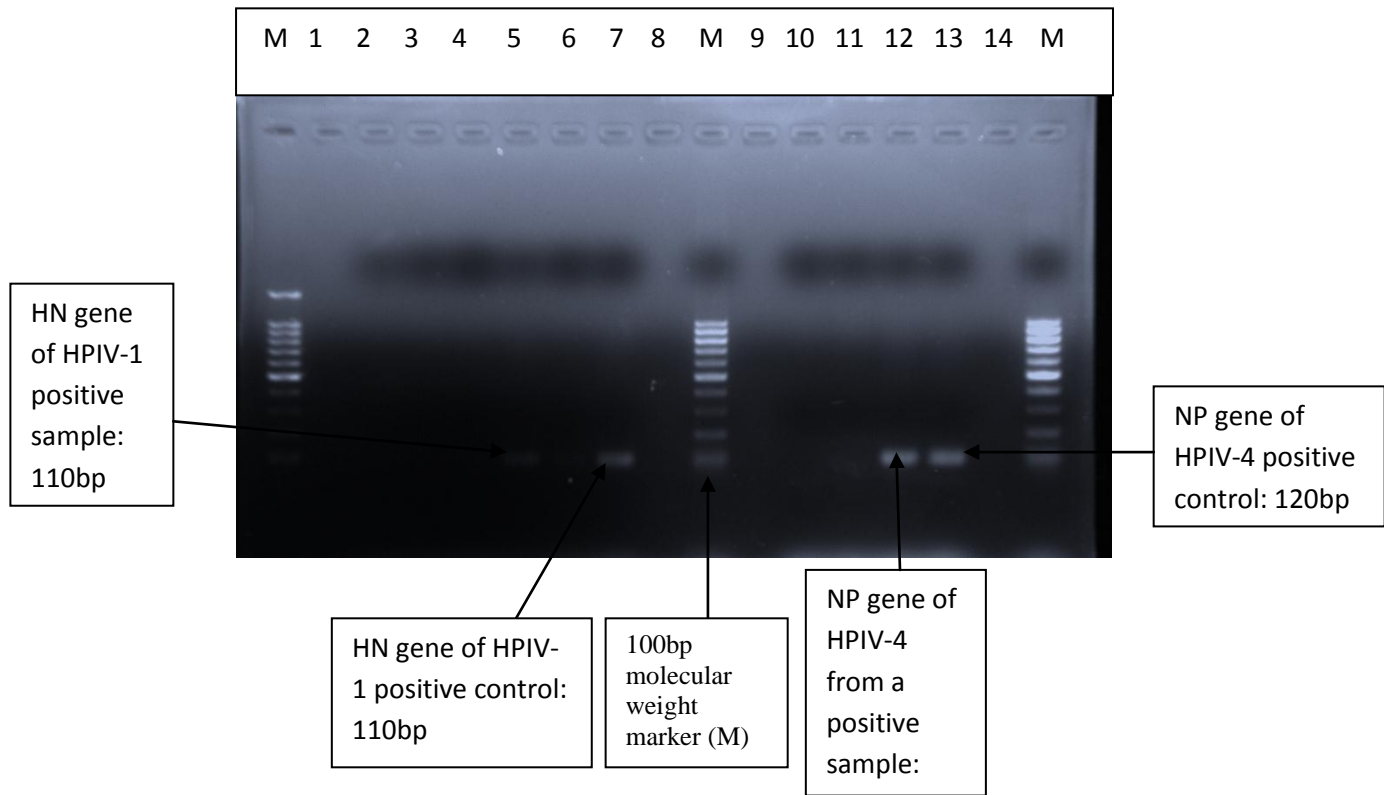


Figure C 2: Aligned human parainfluenza virus sequences



Appendix D

INFORMED CONSENT FORM FOR PARENTS OR GUARDIANS WHOSE CHILDREN ARE BELOW FIVE YEARS AND HAVE ACUTE LOWER RESPIRATORY TRACT INFECTIONS

Name of organisation: University of Ghana Medical School, Korle-Bu, Accra.

Project Title: Detection and Characterization of Human Parainfluenza Viruses in Ghanaian Children below Five years with Acute Lower Respiratory Tract infections

This is a research which is conducted at the Microbiology Department of the University of Ghana Medical School. The research aims at the detection and characterizing of human parainfluenza viruses (pathogens that cause respiratory diseases particularly in children).

Your child has a respiratory disease and I would like to take a sample of the discharge from the back of his or her nose using a sterile swab. You can decide whether or not your child should participate in the research.

Your child age falls below 5 years which makes him or her at risk of infection caused by this pathogen (human parainfluenza virus). This explains why I would like to involve him in the research.

Discomfort and Risk

There is mild discomfort in obtaining samples from your child. Maximum care will be taken by qualified personnel in collecting the samples. You may however feel the discomfort of being asked to answer some questions.

Benefit

The results of the research will help us find out the percentage of acute lower respiratory tract infections (ALRTIs) caused by human parainfluenza viruses and will help manage respiratory infections better which will benefit the society.

Confidentiality

The information about the research will be held highly confidential. Numbers will be assigned to any information collected from your child and the identity will not be revealed. Findings of the study will be reported but not with your child's name assigned to it.

Right to Refuse or Withdraw

Your decision to allow your child to partake in the research is entirely voluntary and you can withhold your consent without fear. This will in no way prevent your child from receiving treatment.

Contact

This proposal has been reviewed and approved by the University of Ghana Medical School Ethical and Protocol Review Committee which ensures that ensures that the participants of the study are protected from harm. If you have any concerns about your child's participation, you can contact the chairman of the Ethical and Protocol Review Committee of the College of Health Sciences, Korle-Bu, Accra.

My child has been invited to participate in this research. I have read the foregoing information or have been explained to me. I have had the opportunity to ask questions and have been answered to my satisfaction.

I consent voluntarily to my child's participation in the research.

Name of participant:

Name of parent or guardian:

Signature:

Thumb print (if illiterate):

Date:

Place:

Appendix E

HPIV SURVEY QUESTIONNAIRE

Form number:

1. Age:
2. Sex : M/F
3. Name:
4. Date of visit: _____ Date of sample collection: _____
5. Hospital Folder Number:
6. Place of residence
7. Educational background of child: a)None b)Crèche c)Nursery d) Primary
8. Educational background of parent/guardian: a)None b) Primary c) J.H.S d) Secondary e) Tertiary
9. Parent /guardian occupation:
10. Respiratory symptoms presented by child: a) Fever b) Fast breathing c) Sputum production d) Cough e)Nasal flare f) Chest pain
11. Other symptoms.....
12. Duration of symptom:
.....
13. Non respiratory symptoms presented by child: a) Headache b) Confusion
c)Myalgia d)Abdominal pain e) Nausea f)Vomiting g) Diarrhoea
14. Has child been on any medication for treatment of illness, please specify:
.....

15. Medical history, has your child suffered from any previous disease:

Please specify.....

16. Feeding history up to 6 months: a) Exclusive breast feeding b) others

.....

17. Does child sleep alone: a) Yes b) No

18. If no, how many in a room:

19. How ventilated are the rooms/ number of windows to a room:

20. Is there any sibling with RTI: a) Yes b) No

21. Is there any form of passive smoking: a) Yes b) No

22. Is there any form of environmental smoking: a) Yes b) No

23. Medical examination (to be documented from patient folder):

Respiratory rate:

Temperature:

24. Clinical diagnosis (to be documented from patient folder)