A SURVEY OF HUMAN PARAINFLUENZA VIRUS AMONG CHILDREN IN KADUNA METROPOLIS, NIGERIA

BY

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(M.Sc/SCIE/05755/2010-2011)

DEPARTMENT OF MICROBIOLOGY,

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A THESIS SUBMITTED TO THE POST-GRADUATE SCHOOL, AHMADU BELLO UNIVERSITY, ZARIA, NIGERIA.

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DEPARTMENT OF MICROBIOLOGY,
FACULTY OF SCIENCE,
AHMADU BELLO UNIVERSITY, ZARIA, NIGERIA.

MARCH, 2015.

DECLARATION

I declare that the work in this thesis, entitled "A Survey of Human Parainfluenza Virus among Children in Kaduna Metropolis, Nigeria" has been performed by me in the Department of Microbiology, Faculty of Sciences, Ahmadu Bello University, Zaria, under the supervision of Prof. (Mrs) V.J Umoh and Prof. S.A Ado. The information derived from the literature has been duly acknowledged in the list of references provided. No part of this thesis was previously presented another degree or diploma at any university.

Bonire, Folashade Sarah	Signature	Date

CERTIFICATION

This thesis entitled "A Survey of Human Parainfluenza Virus among children in Kaduna Metropolis, Nigeria" by Bonire, Folashade Sarah meets the regulations governing the award of the degree of Master of Science of the Ahmadu Bello University, Zaria and is approved for its contributions to knowledge and literary presentation.

Prof. (Mrs) V.J. Umoh Chairman, Supervisory Committee	Signature	Date
Prof. S.A. Ado Member, Supervisory Committee	Signature	Date
Prof. S.A. Ado Head, Department of Microbiology	Signature	Date
Prof. A.Z. Hassan Dean, School of Postgraduate Studies	Signature	Date

DEDICATION

I dedicate this work to God Almighty, My King and My Lord in whom I live, move and have my being and to my Beloved Parents of blessed memory, Prof. and Mrs J.J. Bonire. You were indeed the best parents any child could ever ask for. Your legacy will always remain.

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My utmost gratitude goes to God Almighty, my Father and Friend, the essence of my existence, giving my life new meanings and opening my eyes each day to the awesome reality of who you are and what you mean to me. For all I have ever been and ever will be in life, I owe it all to you,

To my Supervisors, Prof (Mrs) V.J Umoh and Prof S.A Ado for your profound assistance and in-depth contributions to this work. I appreciate you greatly.

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To my beloved husband and best friend, Oluwaseun Adebayo Ojeleye for your support to my life, for your timely and painstaking assistance in gathering materials, proof reading and thorough editing of this work despite your tight schedules. I cherish you dearly. To my children, Oluwatimilehin and Oluwatumininu Ojeleye, my bundles of joy. I love you my babies.

My indebtedness and sincere gratitude goes to my dearest parents, Late Prof and Mrs J.J Bonire, for every sacrifice, for your unrelenting prayers and unending support and words of advice through the course of every pursuit of my life. Your legacy still remains and you will always live in my heart.

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ABSTRACT.

Respiratory infections have a significant impact on health worldwide. The great majority of respiratory infections are of viral origin of which greatly includes Human Parainfluenza viruses. They are rapidly growing viruses that cause significant human and veterinary disease. This study was done to carry out a survey of Human Parainfluenza Virus in children aged 1-12years in Kaduna Metropolis, Nigeria using the Enzyme Linked Immunosorbent Assay Diagnostic kits. The seroprevalence was determined to be 76.6%. Throat swabs were obtained from thirty (30) children with respiratory symptoms such as cold, catarrh, cough and fever. The swabs were cultured aseptically in 10-day old embryonated eggs incubated at 37^{o}C for 24 – 48 hours. The allantoic fluid (which was the viral culture) was harvested and tested for the presence of parainfluenza virus using the haemagglutination assay procedure. Of the thirty samples, ten showed agglutination of various titers; 1:256, 1:128, 1:256, 1:16, 1:16, 1:128, 1:128, 1:32, 1:64, 1:64, thereby indicating the presence of the virus with a prevalence of 33.3%. The antibody level in the highly positive sera samples were tested for by using the Haemaglutination Inhibition Method using the viral antigen isolated with titre 1:256. Risk and demographic factors such as Age of the children (χ^2=17.408, p=0.001), parental occupation(χ^2=10.116, p=0.039),Parental educational status, Vitamin A deficiency, Frequency of eating, Household size, Duration of breastfeeding (χ^2=8.439, p=0.015), environmental smoke, respiratory symptoms (χ^2=5.116, p=0.024), Fever, sickle cell and underlying diseases were analysed as well. Age, Parental occupation, Duration of breastfeeding, Presence of respiratory symptoms were significantly associated with the infection. An antibody level of 1:64 ie 64HAIunits/ml of blood was found in only one sample. ELISA in this study was discovered to be a more sensitive diagnostic method than Haemagglutination Inhibition. Observation from the study showed the importance of Human Parainfluenza Virus as an agent of respiratory tract infection in children. As antiviral drugs are not readily available, preventive measures such as eating of balanced diet, proper hygienic practices and parental supervision should be adhered to in the control of the infection.

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CHAPTER ONE

1.0 INTRODUCTION

Human parainfluenza viruses (HPIVs) have been associated with every type of upper and lower respiratory tract illness, including common cold with fever, laryngotracheobronchitis (croup), bronchiolitis, and pneumonia. The rate of HPIV detections is variable depending on the pathology (upper or lower tract infections) and whether one simulates ambulatory or hospitalized children. A few articles have focused on this virus in the last years (Farichock *et al*., 2010; Lee *et al* 2010; El Feghaly *et al*., 2010). HPIVs are also a cause of community-acquired respiratory tract infections of variable severity in adults. The incubation period of HPIV infection generally lasts 1-7 days. Weinberg *et al* found that HPIV accounted for 6.8% of all hospitalizations for fever, acute respiratory illnesses, or both in children younger than 5 years (Weinberg *et al*., 2009). All HPIV types are strongly correlated with specific clinical syndromes, ages, and times of year, though the lack of epidemiologic data on HPIV-4a and HPIV-4b has so far prevented a clear understanding of the true clinical significance of these serotypes. HPIV-1 and HPIV-2 are the pathogens most commonly associated with croup, and HPIV-3 is the pathogen most commonly associated with bronchiolitis and pneumonia in infants and young children (Pickering *et al.,* 2009).

Respiratory infections have a significant impact on health worldwide. The great majority of respiratory infections are of viral origin. However, 10%–50% of patients will develop a secondary bacterial infection; children are mainly afflicted with acute otitis media, sinusitis, or pneumonia. In very young or older individuals or individuals with chronic medical conditions, viral respiratory infections may induce a severe illness (Hayden *et al*., 2006). Most acute respiratory infections are caused by Rhinovirus, Respiratory Syncytial Viruses,

Enteroviruses, Influenza A and B viruses, Parainfluenza viruses, or Adenovirus (Allander *et al*., 2007).

Respiratory disease accounts for an estimated 75 to 80% of all acute morbidity in the US population. Most of these illnesses (approximately 80%) are viral. If episodes not requiring medical attention are included, the overall average is three to four illnesses per year per person, although incidence varies inversely with age (the frequency is greater among young children). Seasonality is also a feature; incidence is lowest in the summer months and highest in the winter. The viruses that are major causes of acute respiratory disease (ARD) include influenza viruses, parainfluenza viruses, rhinoviruses, adenoviruses, respiratory syncytial virus (RSV), and respiratory coronaviruses (Ray, 2004).

In addition to the ability to cause a variety of ARD syndromes, this somewhat heterogeneous group of viruses shares a relatively short incubation period (1–4 days) and a person-to-person mode of spread. Transmission is direct, by infective droplet nuclei, or indirect, by hand transfer of contaminated secretions to nasal or conjunctival epithelium. All of these agents are associated with an increased risk of bacterial superinfection of the damaged tissue of the respiratory tract, and all have a worldwide distribution (Ray, 2004).

In the past few decades, infectious diseases have moved steadily back up the health agenda, prompting new emphasis on developing strategies for prevention and control. In most developing countries, a significant proportion of these disease burdens are respiratory infections. A hallmark of infection by respiratory viruses is productive infection of and the subsequent destruction of the airway epithelium (Gorski *et al.*, 2012). Human parainfluenza viruses (HPIVs) are a common cause of acute respiratory illness throughout life. Infants, children, and the immunocompromised are the most likely to develop severe disease (Schomacker *et al*., 2012).

HPIVs are spread from person to person by direct contact with infected secretions through respiratory droplets or contaminated surfaces or objects. Infection can occur when infectious material contacts mucous membranes of the eyes, mouth, or nose, and possibly through the inhalation of droplets generated by a sneeze or cough. HPIVs can remain infectious in airborne droplets for over an hour. HPIVs are ubiquitous and infect most people during childhood. Serologic surveys have shown that 90% to 100% of children aged 5 years and older have antibodies to HPIV-3, and about 75% have antibodies to HPIV-1 and -2 (Hayden, 2006).

Human parainfluenza viruses (HPIVs) are common community-acquired respiratory pathogens without ethnic, socio-economic, gender, age or geographical boundaries. Many factors have been found that predispose individuals to these infections, including malnutrition, overcrowding, vitamin A deficiency, concomitant diseases (e.g. diarrhoea, heart disease, asthma), day care attendance, lack of breast feeding and environmental smoke or toxins (Laurichesse *et al.*, 1999; Rudan *et al.*, 2008).

Acute respiratory infections cause 3 to 18% of all admissions to pediatric hospitals, and HPIV can be detected in 9 to 30% of these patients depending on the time of year. There are between 500,000 and 800,000 LRI hospitalizations (in persons younger than 18 years) in the United States each year, with approximately 12% being for HPIV-1, HPIV-2, and HPIV-3 infections. This is second only to respiratory syncytial virus (RSV) as a cause of hospitalization for viral lower respiratory tract infections. Immunity to HPIV is incomplete, and infections occur throughout life; however, less is known about infections in adults. Therefore, the majority of the clinical discussion will focus on children (Rudan *et al*., 2008).

1.1 STATEMENT OF THE PROBLEM

Although respiratory viral infections are much studied in developed countries and their impact on health care is well understood, there is a gap in information on the burden of respiratory viral infections in developing countries. From the public health point of view, it would be valuable to know which viruses are the most common causative agents, what their disease manifestations are, how often virus alone causes severe respiratory infection, and how severe lower respiratory infections could be prevented (Rudan and Conseus, 2005).

The great majority of respiratory infections are of viral origin. However, 10%–50% of patients will develop a secondary bacterial infection. Patients are often diagnosed and treated for the bacterial infection and the underlying viral cause may be left untreated (Hayden, 2006).

Acute Respiratory Infection symptoms (cough and difficult/fast breathing) frequently overlap with those of malaria which sometimes make diagnosis difficult. In Nigeria, children with these pneumonia symptoms which can be caused by HPIVs are frequently overlooked by the home management strategy that seeks to treat all childhood fevers as malaria (Ukwaja *et al.*, 2010). The majority of deaths occur in Africa and South-East Asia (Rudan *et al*., 2008).

In Nigeria, the incidence of clinical pneumonia, a respiratory tract infection among children ranges from 0.34 to 1.3 episodes per child-year (Akanbi *et al*., 2009). Over 6 million new cases are estimated annually with expected mortality of 204 000 children (Rudan *et al*, 2008). However, many children with pneumonia do not receive timely, appropriate treatment at health facilities, (UNICEF, 2007) especially children from poorer families – for economic and social reasons (Schellenberg *et al*., 2003). Hence, many pneumonia cases are treated at home or seek care too late. In Uganda, pneumonia contributes to the death of one-third of

children with acute respiratory infections, who die at home without prior contact with the formal health sector (Kallander *et al.*, 2005).

1.2 JUSTIFICATION

There is need for children coming down with fever like symptoms to be tested for respiratory tract infections and not just other diseases like malaria and typhoid fever.

Most clinical cases of respiratory infections are regarded as bacterial and there is a tendency of not focusing enough attention to viral causes of respiratory tract infections. Sometimes the viruses cause only a runny nose and other symptoms that may be diagnosed as a simple cold rather than Human Parainfluenza Infection.

Unlike influenza (which has the WHO Collaborating Centre for Reference and Research), there is limited centralised service for parainfluenza diagnosis and study within continents like Africa and Australia (Rudan and Conseus, 2005).

Many factors have been found that predispose individuals to these infections, including malnutrition, overcrowding, vitamin A deficiency, lack of breast feeding and environmental smoke or toxins (Laurichesse *et al*., 1999). These factors are very common in Nigeria and therefore there is a high possibility of many children coming down with the infection.

With increases in morbidity and persistence, similar clinical manifestations despite recommended therapy against parainfluenza infection, the non-availability of adequate literature and information coupled with increasing outbreaks of respiratory tract infections within this community underscores the need to investigate the seroprevalence of parainfluenza virus in children within the community. This work seeks to find out to survey the presence of Human Parainfluenza Virus in children aged 1-12years in Kaduna Metroplois as well as the risk and demographic factors associated with the infection.

1.3 AIM AND OBJECTIVES

The aim of the study was to conduct a survey of Human Parainfluenza Virus in children (1-12 years) in selected hospitals in Kaduna Metropolis, Nigeria.

The specific objectives were;

1. To determine the risks and demographic factors associated with the infection in the study area.
2. To determine the presence and titre values of the Human Parainfluenza Virus IgG among the children.
3. To examine clinical samples of children in the study area for Human Parainfluenza Viruses.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 History of the Human ParainfluenzaVirus

These viruses were first discovered in the late 1950s, when three different viruses recovered from children with lower respiratory disease proved to by unique and easily separated from the myxoviruses (influenza virus) and they closely resembled(Kingsbury.,1985). This new family of respiratory viruses grew poorly in embryonated eggs and shared few antigenic sites with influenza virus. In 1959, a fourth virus was found that also met these criteria, and a new taxonomic group was created called "parainfluenza viruses."(Chanock *et al*., 1985)

HPIV is genetically and antigenically divided into types 1 to 4. Further major subtypes of HPIV-4 (A and B and subgroups/genotypes of HPIV-1 and HPIV-3 have been described (Henrickson., 1999). HPIV-1 to HPIV-3 are major causes of lower respiratory infections in infants, young children, the immunocompromised, the chronically ill, and the elderly (Hoogen *et al*., 2001)). These medium-sized viruses are enveloped, and their genomes are organized on a single negative-sense strand of RNA. The majority of their structural and biological characteristics are similar, but they each have adapted to infect humans at different ages and cause different diseases. These viruses belong to the *Paramyxoviridae* family, which is a large rapidly growing group of viruses that cause significant human and veterinary disease. In fact, this virus family is one of the most costly in terms of disease burden and economic impact to our planet. Recently discovered members of the *Paramyxoviridae* (megamyxoviruses [Hendra and Nipah viruses] and metapneumovirus) emphasize this point. (Hoogen *et al*., 2001).

There are now two genera of HPIV, *Respirovirus* (HPIV-1 and HPIV-3) and *Rubulavirus* (HPIV-2 and HPIV-4). Both genera (paramyxoviruses) can be separated morphologically from influenza virus (myxoviruses) by their nonsegmented thick nucleocapsids (17 nm versus 9 nm). Other genera of the *Paramyxoviridae* can be physically distinguished from HPIV by the absence of a neuraminidase (morbilliviruses, e.g., measles virus and distemper virus) or a thiner nucleocapsid (pneumoviruses, e.g. respiratory syncytial virus [RSV], or metapneumoviruses). The megamyxoviruses are still being described but appear more closely related to morbilliviruses phylogenetically than to the HPIV (Henrickson, 2003).

Human parainfluenza viruses (HPIVs) have been associated with every type of upper and lower respiratory tract illness, including common cold with fever, laryngotracheobronchitis (croup), bronchiolitis, and pneumonia. HPIVs are also a cause of community-acquired respiratory tract infections of variable severity in adults. The incubation period of HPIV infection generally lasts 1-7 days. Weinberg *et al* found that HPIV accounted for 6.8% of all hospitalizations for fever, acute respiratory illnesses, or both in children younger than 5 years (Weinberg *et al.*, 2009).

All HPIV types are strongly correlated with specific clinical syndromes, ages, and times of year, though the lack of epidemiologic data on HPIV-4a and HPIV-4b has so far prevented a clear understanding of the true clinical significance of these serotypes. HPIV-1 and HPIV-2 are the pathogens most commonly associated with croup, and HPIV-3 is the pathogen most commonly associated with bronchiolitis and pneumonia in infants and young children (American Academy of Pediatrics, 2009).

2.2 Structural Organization of HPIV

Human parainfluenza viruses are typical members of the family Paramyxoviridae. They are a large rapidly growing group of viruses that cause significant human and veterinary diseases. These RNA viruses are pleomorphic enveloped particles (Figure1 and plate1) that are 150-300 nm in diameter, and their genome are organized on a single negative-sense strand of RNA which is complementary to mRNA (Raija and Timo, 1994; Henrickson, 2003). Their envelope is derived from the host cell that they last infected (Kingsbury,1985). The viral genome of Paramyxoviruses is linear, negative sense, single stranded, non segmented RNA, about 15 kilo bases (kb) in size (Brooks *et al*., 2007). Virions with a positive polarity have been reported, but these are thought to be noninfectious (Kingsbury, 1985).

2.3 Genomic Organization

The HPIV genome contains approximately 15,000 nucleotides (Wechsler *et al*., 1985). These are organized to encode at least six common structural proteins (3′-N-P-C-M-F-HN-L-5′) (Tashiro *et al*., 1985). During the last decade, reverse genetic systems were developed that demonstrated a "rule of six" for HPIV. Simply stated, this means that the most efficient replication and transcription of HPIV takes place when the genome is divisible by 6, although exceptions have been found (Henrickson., 2003).

2.4 The Human Parainfluenza Virus Life Cycle

HPIVs are members of the Respirovirus and Rubulavirus genera within the Paramyxoviridae family. The viruses are roughly spherical in shape, approximately 150–400 nm in diameter, and have an envelope composed of host cell lipids and viral glycoproteins derived from the plasma membrane of the host cell during viral budding.

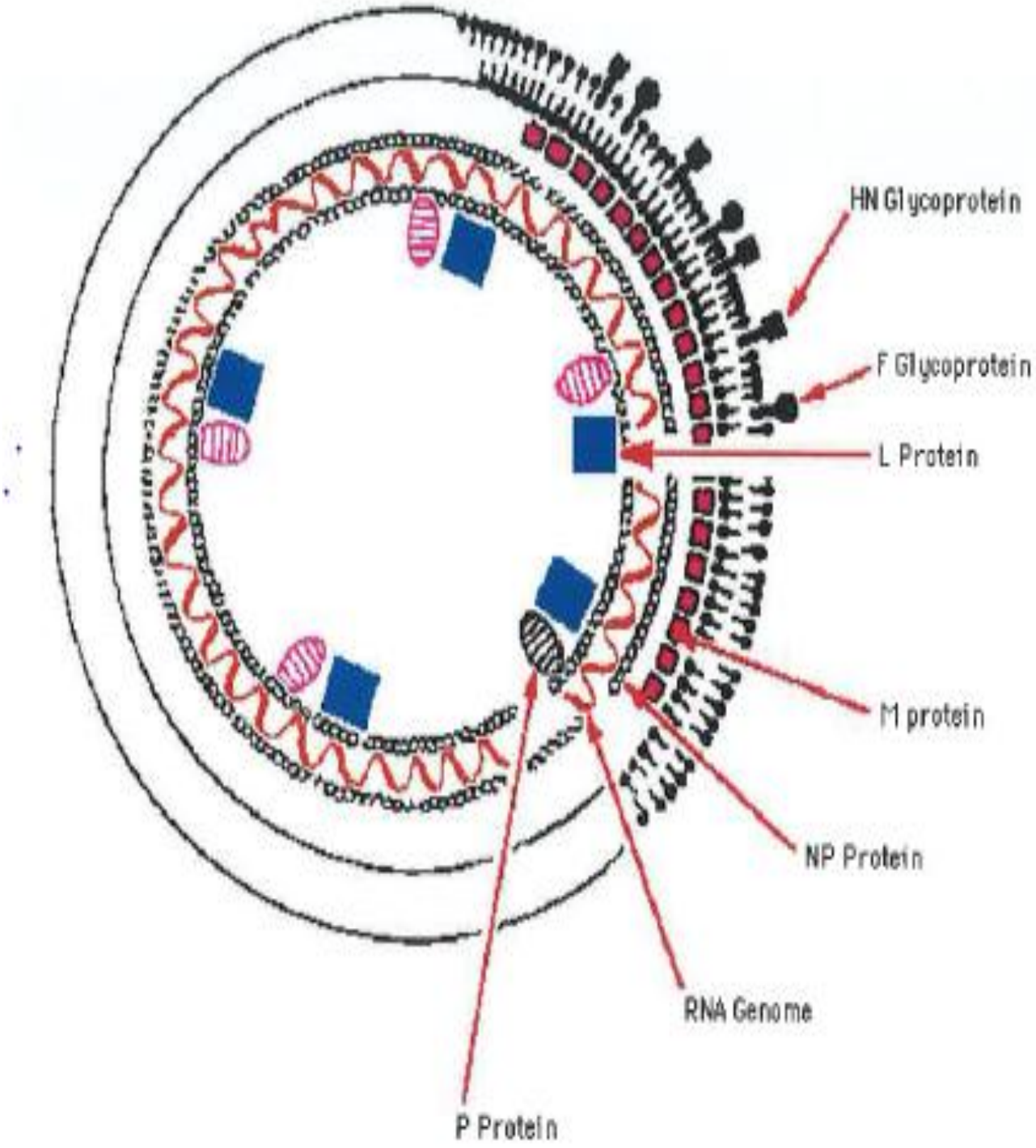

Fig 2.1- Structure of Human Parainfluenza Virus

(Taken from www.microbios.com.ar/galeria-u2.htm)

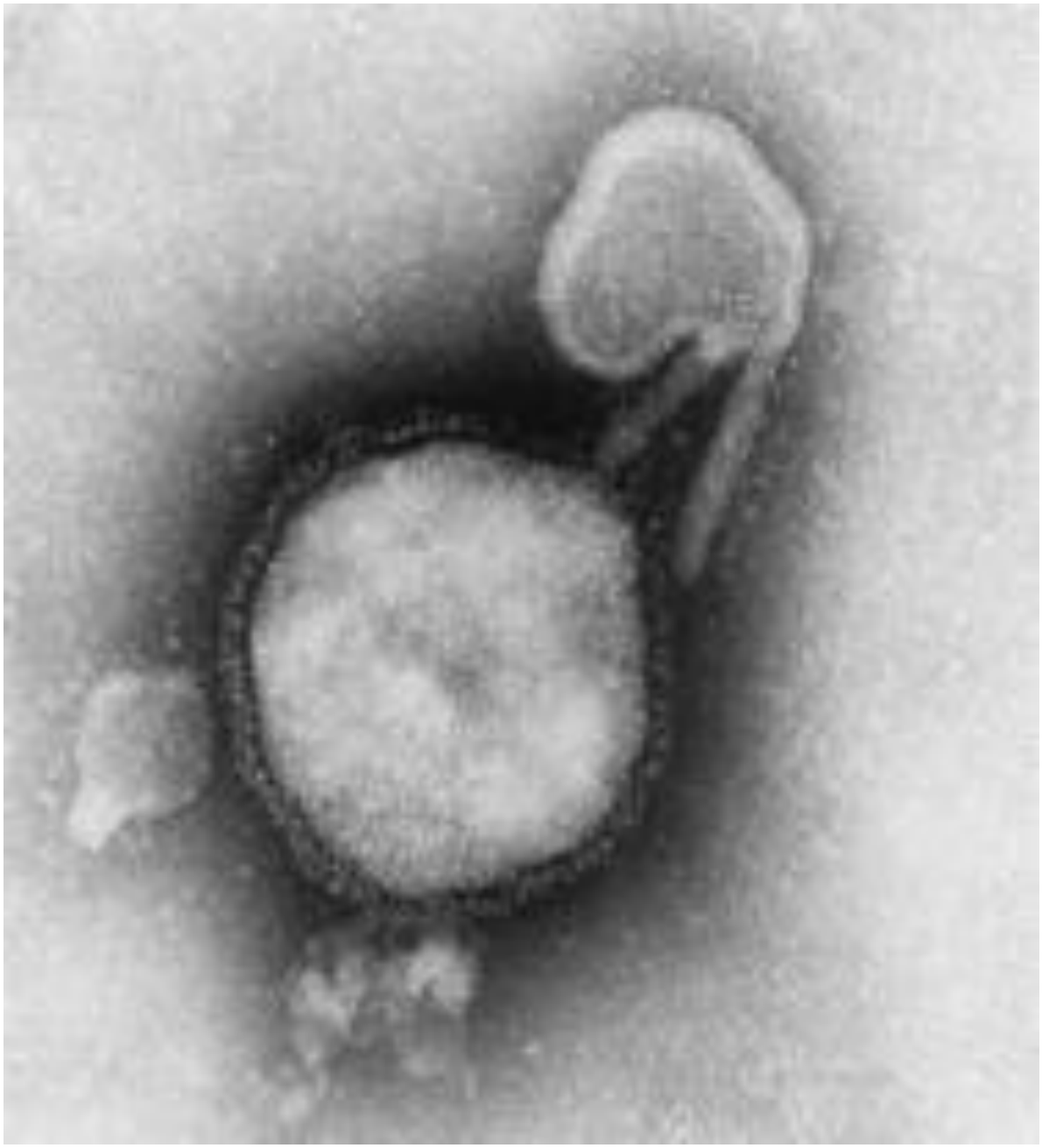

Plate 1. Electron micrograph of HPIV-3. Surface glycoproteins are easily visualized. Magnification, ×275,000. (Taken from Henrickson, 2003)

The HPIV genome is single-stranded, negative-sense RNA that must be transcribed into message-sense RNA before it can be translated into protein. Like all negative-stranded RNA viruses, the HPIVs encode and package an RNA-dependent RNA polymerase in the virion particles (Lamb *et al.*, 1976). The RNA genome is approximately 15,500 nucleotides in length and is encapsidated by the viral nucleocapsid protein, forming helical nucleocapsids (Choppin *et al.*, 1975). The first step in infection of a cell by all HPIVs is binding to the target cell, via interaction of the viral receptor-binding molecule (hemagglutinin-neuraminidase [HN]) with sialic acid–containing receptor molecules on the cell surface. The viral envelope then is thought to fuse directly with the plasma membrane of the cell, mediated by the viral fusion protein (F protein), releasing the nucleocapsid into the cytoplasm (Lamb *et al.*, 1993, Plemper *et al.*, 2003). The nucleocapsid released into the cytoplasm after fusion contains the genome RNA in tight association with the viral nucleocapsid protein, and this RNA/protein complex is the template both for transcription and for replication of the genome RNA that is packaged into progeny virions.(plate 2) The 6 viral genes encode the 2 surface glycoproteins HN and F; the matrix protein, which is involved in assembly and budding; the RNA polymerase proteins and a protein that encapsidates the RNA; and, through alternative reading frames and/or RNA editing, 1 or more proteins that are expressed only in the infected cell and whose roles include evasion of the host immune response. Virions are formed, according to the prevailing model for virion assembly, when newly assembled nucleocapsids containing the full-length viral RNA genome along with the polymerase proteins bud out through areas of the plasma membrane that contain the F and HN proteins and the matrix protein. In polarized epithelial cells, the viruses bud from the apical surface of the cell. The matrix protein binds to the nucleocapsid and also interacts with the cytoplasmic tails of the HN and F proteins, in this way mediating the alignment of the nucleocapsid with the areas of

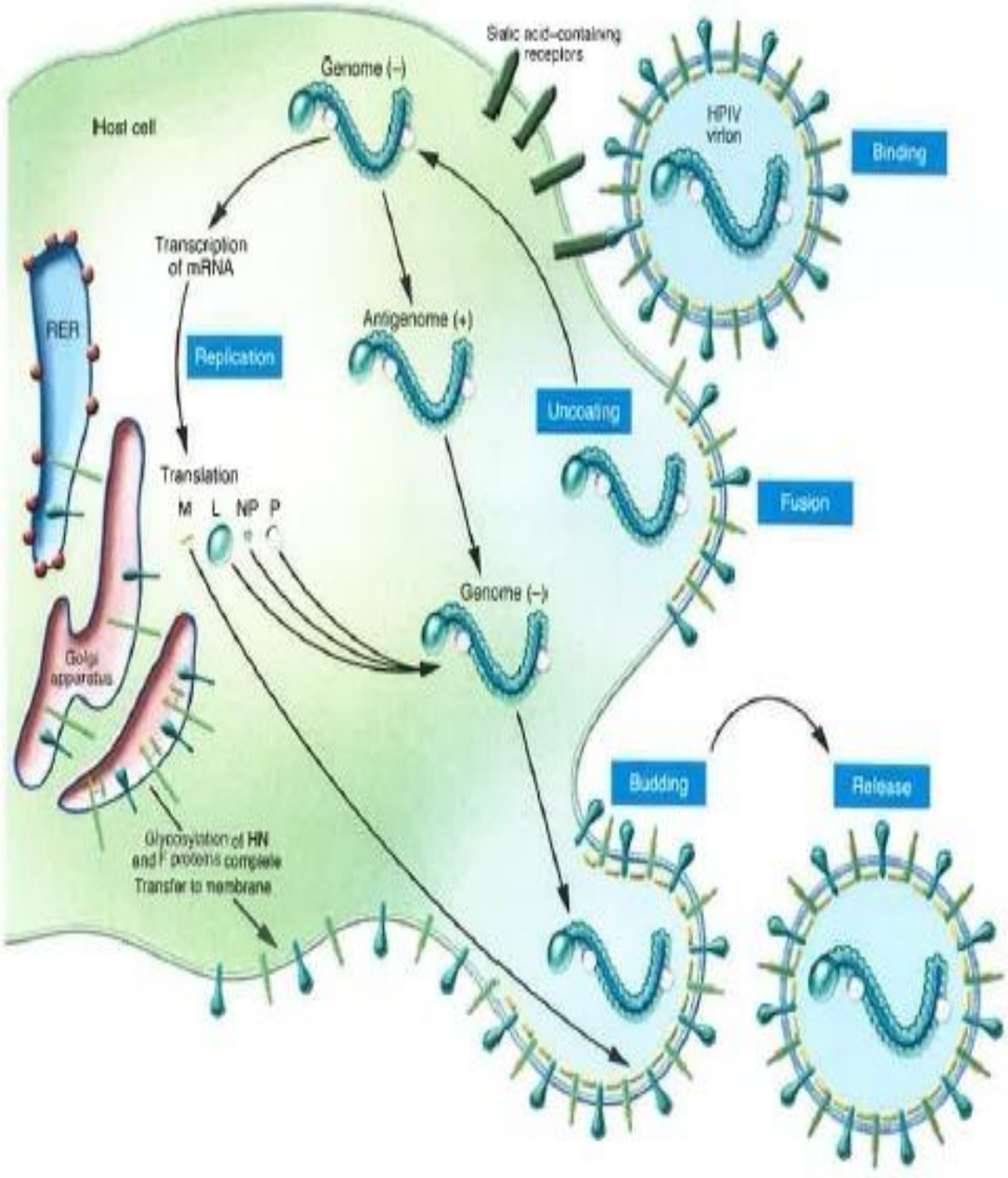

Plate2- Schematic representation of the lifecycle of the Parainfluenza virus.

(Taken from Moscona, 2005)

the plasma membrane containing viral glycoproteins in order to set the scenario for budding (Ali *et al*., 2000). The neuraminidase or receptor-cleaving activity of the HN molecule cleaves sialic acid–containing receptor moieties that would attach the viral HN protein to the cell surface and allows the release of newly budded particles from the cell to begin a new round of infection (Huberman *et al*., 1995, Porotto *et al*., 2001).

2.5 Viral Replication of HPIV.

The first event in viral reproduction is the fusion of the virus and host cell lipid membranes. This is followed by the expulsion of the HPIV nucleocapsid into the cytoplasm of the cell. Once in the cytoplasm, transcription takes place by using virus-specific RNA-dependent RNA polymerase (L protein). Cellular ribosomal machinery then translates the viral mRNAs into viral proteins. These direct the full-length replication of the virus genome, first into a positive-sense RNA strand and then into the appropriate negative strand. Once produced, these single negative-sense strands of RNA are then encapsidated with NP and may be used in further rounds of transcription and replication or may be packaged for export as a new virion (Henrickson, 2003).

2.6 HPIV Proteins

The viral envelope of HPIVs contains two major surface glycoproteins: the hemagglutinin-neuraminidase (HN) and the fusion (F) proteins (Vainionpaa *et al*., 1994). The HN glycoprotein regulating the interaction between virus and host cells, has dual biological functions of hemagglutinin and neuraminidase activities and also plays an essential role in promoting fusion by the F protein. In addition, the HN glycoprotein possesses the largest antigenic and genetic differences among HPIV types and strains within one type (Palermo *et al*., 2009). Therefore, the HN glycoprotein, which is the target for protective humoral

immunity, has been broadly used for typing HPIVs for molecular epidemiological investigations (Jalal *et al*., 2007; Lau *et al*., 2009).

Electrophoresis demonstrates great similarity in protein size between the four major HPIV types except for the phosphoprotein (P). HPIV-2 and HPIV-4 have a phosphoprotein with a molecular weight (MW) of 49,000 to 53,000 versus 83,000 to 90,000 for HPIV-1 and HPIV-3 (Komada *et al*., 1989; Spriggs *et al*., 1986; Yoshida *et al*., 1985). The largest HPIV protein is the "large" (L) nucleocapsid protein (polymerase, MW 175,000 to 251,000), followed by P (HPIV-1 and HPIV-3). These two viral proteins and the nucleocapsid protein (N or NP) [MW 66,000 to 70,000] are closely associated with the viral RNA (vRNA). Two surface glycoproteins are found in all HPIV: the hemagglutinin-neuraminidase (HN) (MW 69,000 to 82,000) and the fusion protein (Fo) (MW 60,000 to 66,000). Finally, the membrane protein (M) (MW 28,000 to 40,000) is strongly associated with and found just beneath the viral membrane. It is important to note that some of these proteins undergo extensive posttranslational modification (e.g., glycosylation or phosphorylation) and the MW as calculated from cloning and sequencing data can differ considerably from that seen on gel electrophoresis. The P gene of some paramyxoviruses produces many small nonstructural proteins from multiple overlapping reading frames. Additional editing of the mRNA may occur to produce these proteins. For example, HPIV-1, HPIV-2, and HPIV-3 encode a nonstructural protein (C) (Matsuoka *et al*., 1991). HPIV-2 (and maybe HPIV-3) has an additional nonstructural protein (V), which HPIV-1 does not contain (Durban *et al*., 1999). This protein appears to have several actions. It binds N and may play a role in regulating viral replication (Watanabe *et al*., 1996). However, the V protein distribution (nuclei and cytoplasm) does not appear to be similar to that of the N or P protein (cytoplasmic granules) within virus infected cells (Nishio *et al*., 1999). Another work suggests that slowing the cell cycle might be one way the V protein favors viral replication (Lin *et al*., 2000). In addition, it

may be involved in *Rubulavirus* inhibition of the interferon response by inducing the degradation of STAT1 and/or STAT2 (Gotoh *et al*., 2001). The C protein of PIV-1 (Sendai virus) may perform the same function (Nishio *et al*., 2001). This interferon interaction with V and C proteins may be important in determining the host range for some HPIV. HPIV-3 appears to have a unique nonstructural protein, D, which is a chimera between the upstream half of P and a second internal open reading frame (Landry *et al*., 2000). vRNA, together with the N, P, and L proteins, form the nucleocapsid core of HPIV. The N binds to the vRNA (one N protein to six nucleotides), making a template that allows the L (RNA-dependent RNA polymerase) and P proteins to transcribe and eventually replicate the HPIV genome (Chanock, 1985). The P protein of HPIV is probably a homotrimer (Curran *et al*., 1998). The surface glycoproteins (HN and F) interact with the M protein, which may direct their insertion and aggregation at specific cell membrane locations. The M protein also appears to play a role in attracting completed nucleocapsids to areas of infected cell membrane that will soon become viral envelope and may be involved in viral budding (Patterson *et al.*, 1988).

The HN protein is found on the lipid envelope of HPIV and infected cells (Thompson *et al*., 1983). There it most probably exists as a tetramer and functions in virus-host cell attachment via sialic acid receptors, suggesting that it has neuraminidase activity (important for virus release from cells). There are significant differences in the number of HN glycosylation sites between HPIV types and among strains within one type (Henrickson *et al*., 1999). This may be part of the strategy used by HPIV to escape immune detection. The terminal sialic acid sequences important for HN binding of HPIV are just beginning to be worked out (Suzuki *et al*., 2001). It appears that HPIV-1 HN is more limited in its binding than HPIV-3 HN, which may be important for host and tissue range. It is the binding of the HN protein to receptors on red blood cells that creates the well-recognized hemagglutination or hemadsorption of paramyxoviruses. Elegant work by Moscona and Paluso has demonstrated that the HN-cell

receptor interaction is specific and complex. It involves both surface glycoproteins and varies between HPIV types. F protein-mediated cell fusion is affected by the affinity of the HPIV-3 HN to its receptor(s) (Moscona *et al*., 1992). In addition, cell-to-cell fusion requires a minimum density of receptors which is greater than the density needed for virus membrane-cell membrane fusion (infection). The enzymatic removal of sialic acid receptors from HPIV can create persistently infected tissue cultures. This is one explanation for HPIV-3 persistence in vitro, but in vivo persistence may have additional mechanisms. Persistent infection with HPIV-2 appears to use a different mechanism (Ah-Tye *et al*., 1999).

As mentioned above, the HPIV F protein is integral in virus-host cell membrane fusion. It is this fusion of membranes which allows the viral nucleocapsid to enter and infect a host cell. Also, this protein is needed in membrane fusion between host cells (syncytial formation) and causes hemolysis. Initially an inactive precursor (F0) is made, which must be cleaved by an endopeptidase to yield the active F protein, which is composed of two disulfide-linked molecules (F1 and F2). The new N terminus on F1 is highly hydrophobic and is thought to make the first contact with the lipid membrane during virus-cell fusion. Furin and Kex2 have been proposed as the enzymes responsible for this proteolytic cleavage in humans (Ortmann *et al*., 1994), but trypsin is most frequently used in vitro. The host range and virulence of HPIV is strongly influenced by the enzymes that cleave the Fo precuror. The ability of the F protein to independently induce both fusion and hemolysis varies among the different HPIV types. HPIV-1, HPIV-2, and HPIV-3 in vitro require both HN and F for fusion and hemolysis (Ortmann *et al*., 1994). The structure and location of the physical interactions between the HN and F proteins responsible for their functional interactions, including fusion promotion, oligomer formation, and cell surface expression, are still being determined (Okamoto *et al*., 1997).

The HPIVs are enveloped, non-segmented, negative-strand RNA viruses of subfamily Paramyxovirinae , family Paramyxoviridae (Karron *et al*., 2007; Henrickson, 2003). HPIV1 and 3 belong to genus Respirovirus, while HPIV2 and 4 belong to genus Rubulavirus (Henrickson, 2003). The genomes of HPIV1, 2, and 3 are similar in size (15.5-15.7 kb), whereas that of HPIV4 is somewhat larger (17.4 kb) (Yea *et al*., 2009). They share the same order of 6 genes: 3'-N-P-M-F-HN-L, which are transcribed sequentially into separate mRNAs. There are two viral surface proteins: the hemagglutinin-neuraminidase (HN) protein, which mediates attachment to sialic acid residues on host cell membranes and cleavage of these residues during release, and the fusion (F) protein, which mediates the fusion of the viral envelope with the host cell membrane. These are the viral neutralization antigens and major protective antigens. The N protein coats the genomic RNA, forming a highly stable nucleocapsid. The phosphoprotein (P) and the large polymerase protein (L) are associated with the nucleocapsid, while the matrix protein (M) coats the inner surface of the envelope (Karron *et al*., 2007).

The P gene also encodes additional proteins that vary among viruses. These are called accessory proteins because they are not essential for virus replication in vitro. The most notable are the C and V proteins, which suppress host innate immune responses as described below. C proteins are expressed by a separate ORF that overlaps the P ORF and are encoded by HPIV1 and 3, but not by HPIV2 and 4, as is characteristic of their respective genera. The sequence of the C protein is not highly conserved and lacks evident motifs. Expression of the V protein depends on a mechanism called "RNA editing", whereby the polymerase stutters at a specific motif during transcription of the P gene to create mRNA species with frameshifts that link the upstream half of the P ORF to an internal V ORF encoding a protein domain with a characteristic zinc finger motif containing seven cysteine residues. Thus, V is a chimeric protein with an N-terminal domain encoded by the P ORF and a C-terminal domain

encoded by the V ORF. All members of Paramyxovirinae express a V protein with the curious exceptions of HPIV1 and 3, which contain a relic V ORF that is not expressed in the case of HPIV1 (Rochat *et al*., 1992) and is either not expressed or is expressed at a low level by HPIV3 (Durbin *et al.,* 1999; Galinski *et al.,*1992). The zinc finger motif of the V protein is conserved among all members of Paramyxovirinae and is necessary for suppression of both IFN induction and IFN signalling (Schomarker *et al*., 2012).

2.7 Antigenicity and Serotype of HPIV

HPIV has four predominant serotypes. Serologic and antigenic analysis of all of the species within the *Paramyxovirinae* subfamily demonstrates four basic genera: (i) HPIV-1, HPIV-3, Sendai virus, and bovine PIV-3; (ii) HPIV-2, HPIV-4, mumps virus, and simian viruses 5 and 41; (iii) measles and distemper viruses (Henrickson *et al.,* 1996); and (iv) the new megamyxoviruses. The HPIV all induce variable levels of heterotypic antibody during infection and have common antigens. This often makes it impossible to determine whether serologic positivity represents specific amnestic responses or cross-reactions to similar antigens on different HPIVs. However, specific hyperimmune animal serum (e.g., hamster or guinea pig) can usually differentiate between these viruses in standard hemagglutinin inhibition (HI), hemadsorption inhibition (HAdI), complement fixation, or neutralization tests or enzyme-linked immunosorbent assay (ELISA) (Canchola *et al*., 1965).

Two major subtypes of HPIV-4 (A and B) were discovered shortly after this virus was first identified 40 years ago. HAdI and neutralization assays could easily distinguish these subtypes, but complement fixation could not (Canchola *et al*., 1964). During the same decade, several HPIV-2 strains were isolated that could be differentiated serologically from the type stain, and then about 10 years ago studies demonstrated significant antigenic variation between different HPIV-2 clinical isolates (Ray *et al*., 1992). At about the same

time, strains of HPIV-1 were reported that could be separated from the type strain by ELISA, HI, and neutralization assays (Henrickson., 1999). Molecular analyses of all four types have demonstrated more antigenic and genetic heterogeneity than was initially appreciated (Komada *et al*., 1990). In fact, the data suggest that all four major HPIV serogroups (HPIV-1 to HPIV-4) have subgroups or populations that have unique antigenic and genetic characteristics. Even HPIV-4 subtypes A and B demonstrate this variability (Komada *et al*., 1990). The variability and changes seen in HPIV suggest an evolutionary pattern similar to that of influenza B virus. Polyclonal serologic testing can detect most HPIV strains using common "type" antigens, but subgroups of HPIV-1 (A, C, and D) and HPIV-3 have been reported with progressive antigenic change (Henrickson, 1999). In addition, HPIV-1 strains isolated over the last 12 years demonstrate persistent antigenic and genetic differences compared to the 1957 type strain, including differences between genotypes within the same epidemic and same geographic location (Henrickson, 1999). This progressive antigenic change (although slow) will cause standard reference sera raised to HPIV isolates from the 1950s, or antigen prepared from these same "type" strains, to not universally react in routine serologic assays in the future. Examples of this have already occurred, leading to failure of commercial diagnostic products. Investigators now often use more recent strains of HPIV as sources of antigen or genomic material.

Viral replication is initiated only after successful entry into a cell by attachment and fusion between the virus and the host cell lipid membrane. Viral RNA (vRNA) is initially associated with nucleoprotein (NP), phosphoprotein (P) and the large protein (L). The hemagglutinin-neuraminidase (HN) is involved with viral attachment and thus hemadsorption and hemagglutination. Furthermore, the fusion (F) protein is important in aiding the fusion of the host and viral cellular membranes, eventually forming syncytia (Moscona, 2005)

Initially the F protein is in an inactive form (F_0) but can be cleaved by proteolysis to form its active form, F_1 and F_2, linked by di-sulphide bonds. Once complete, this is followed by the hPIV nucleocapsid entering the cytoplasm of the cell. Subsequently, genomic transcription occurs using the viruses own 'viral RNA-dependant RNA polymerase' (L protein). The cells own ribosomes are then tasked with translation, forming the viral proteins from the viral mRNA (Moscona, 2005). Towards the end of the process, (after the formation of the viral proteins) the replication of the viral genome occurs. Initially this occurs with the formation of a positive-sense RNA (intermediate step necessary for producing progeny) and finally negative-sense RNA is formed which is then associated with the nucleoprotein. This may then be either packaged and released from the cell by budding or it can be used for subsequent rounds of transcription and replication. The pathogenicity of HPIV is mutually dependant on the virus having the correct accessory proteins which are able to elicit anti-interferon properties. This is a major factor in the clinical significance of disease. The observable and morphological changes which can be seen in infected cells includes the enlargement of the cytoplasm, decreased mitotic activity and 'focal rounding,' with the potential formation of multi-nucleate cells (Moscona, 2005)

2.8 Pathogenesis of the infection

HPIV infection usually initiates at the epithelium of the Upper respiratory tract (URT) following exposure by contact or inhalation. Infection frequently spreads to the paranasal sinuses, larynx and bronchi, and obstruction of the Eustachian tubes can lead to otitis media. Each of the HPIVs has been associated with a similar broad spectrum of respiratory tract disease including the common cold, croup, bronchitis, bronchiolitis, and pneumonia, but certain serotypes are more frequently associated with certain illnesses (Counihan *et al.*, 2001, Rihkanen *et al.*, 2008). For example, HPIV1 and 2 are more frequently associated with

laryngotracheobronchitis (croup), and HPIV3 is more likely than HPIV1 or 2 to spread to the smaller airways, causing bronchiolitis and pneumonia resembling that of Respiratory Syncytial Virus (Welliver *et al*., 1986; Karron *et al*., 2007., Parott *et al*., 1959). Apnea also is associated with HPIV LRI in the first few months of life (Weinberg *et al*., 2009). Treatment for HPIV disease is largely supportive except in the case of croup, where effective symptomatic relief can be achieved with corticosteroids and nebulized epinephrine (Russell *et al*., 2011, Bjornson *et al*., 2011). Infections are self-limiting. HPIV infection is rarely fatal in otherwise healthy individuals, but mortality can be high in severely immunosuppressed individuals such as hematopoietic stem cell transplant recipients (Campbell *et al*., 2010; Srinivasan *et al*., 2011). It has been hypothesized that severe HPIV infections in young infants may sometimes have long-term effects on lung function and immunity, but this remains unclear (Schomarker *et al*., 2012).

2.8.1 Contributions of the HPIV C and V accessory proteins to pathogenesis

For cytoplasmic RNA viruses such as the HPIVs, the most potent stimulus for innate immunity is viral RNA synthesis, which can strongly activate host cytoplasmic sensors such as MDA5, RIG-I, and PKR. Downstream effects include: signal transduction that activates transcription factors IRF3 and NFκ B and induces IFNβ and pro-inflammatory cytokines; suppression of cellular translation by PKR; and IFN-induced signaling to induce an antiviral state (Takaoka *et al*., 2006; Goodbourn *et al*., 2009). These responses can strongly restrict HPIV replication but are inhibited in multiple ways by the viral V and C proteins (Horvath *et al*., 2004). The C ORF of HPIV1 encodes four C proteins called C', C, Y1, and Y2 that originate from different translational start sites but share a common C-terminus (Curran *et al*., 1989; Boeck *et al*.,1992). HPIV1 C proteins have been shown to prevent IFN induction (Boonyaratanakornlit *et al*., 2011), IFN signaling (Van Cleve *et al*., 2006), and apoptosis (Bartlett *et al*., 2008).

The HPIV1 C proteins do not appear to interact with or directly inhibit the signal transduction pathways leading to activation of IRF3 and NFκB: instead, they modulate and reduce viral RNA synthesis to prevent the accumulation of dsRNA, thus avoiding activation of MDA5 and PKR (Boonyaratanakornkit *et al*., 2011). With regard to Interferon (IFN) signaling, the HPIV1 C proteins have been shown to bind to STAT1 (but not STAT2), but not to interfere significantly with phosphorylation or stability of either STAT1 or 2. Rather, they sequester STAT1 in complexes that accumulate in the cytoplasm associated with late endosomes, thus preventing STAT1 translocation to the nucleus. The mechanism by which the HPIV1 C proteins inhibit apoptosis remains unclear. Infection of respiratory epithelial cells with an HPIV1 mutant engineered to not express the C proteins resulted in changes in the expression of hundreds of cellular genes controlled by IRF3, NFκ B, and other factors, illustrating that C normally has a broad impact on the host response (Boonyaratanakornkit *et al*., 2009). HPIV3 encodes a single C protein that is important for efficient replication in experimental animal models (Durbin *et al*., 1999)), but is less well characterized than that of HPIV1. The HPIV3 C protein down-regulates viral RNA synthesis (Malur *et al*., 2004), which may reduce the innate response. HPIV3 C inhibits IFN signaling by binding to STAT1 and inhibiting its phosphorylation (Caignard *et al*., 2009; Malur *et al*., 2005). Interestingly, the HPIV3 C protein enhances activation of the MAPK/ERK pathway, which promotes the cellular response to growth factors and is necessary for efficient viral replication (Caignard *et al*., 2009). The HPIV2 V protein has been shown to inhibit IFN induction, IFN signaling (Poole *et al*., 2002), and apoptosis (Schaap *et al*., 2010). The HPIV2 V protein inhibits IFN induction in two ways: by binding to and inhibiting the activation of MDA5 (Childs *et al*., 2007), and by serving as an alternative, competing substrate for cellular kinases in the signal transduction for IRF3 activation (Lu *et al*., 2008). HPIV2 V protein also appears to reduce the production of viral RNA (Nishio *et al*., 2008), reminiscent of the C proteins noted above.

HPIV2 V protein inhibits IFN signaling by binding to and promoting the degradation of STAT2 (Schaap *et al*., 2010). Overall, inhibition of IFN induction appears to be more important for efficient HPIV2 replication in vivo than inhibition of IFN signaling: recombinant HPIV2 viruses carrying V protein point mutations allowing IFN induction are attenuated in non-human primates (Schaap *et al*., 2011), while HPIV2 viruses carrying V protein point mutations allowing IFN signaling are not attenuated (Schaap *et al.*, 2010). This difference may reflect the ability of secreted IFN to affect cells beyond those directly infected with virus; while virus mediated inhibition of IFN signaling operates only in infected cells. The ability of HPIV4 to interfere with host innate immunity is less well characterized (Schomacker *et al*., 2012).

Surprisingly, HPIV4 does not appear to interfere with IFN signaling (Nishio *et al*., 2005). It is possible that this contributes to its reduced virulence. It seems likely that HPIV4 has the ability to inhibit IFN induction, although investigation of this has not been reported (Schomarker *et al*., 2012).

2.8.2 Tissue tropism and cytopathology of HPIV

HPIVs replicate to high titre in the epithelial cells that line the respiratory tract (Schaap-Nutt *et al*., 2008, Bartlett *et al*., 2008). In an in vitro model of well-differentiated mucociliary human airway epithelium (HAE) (Pickles *et al*., 1998), HPIV1, 2, and 3 infected only the superficial ciliated cells on the apical (lumenal) face and did not spread to the underlying basal cells or goblet cells (Bartlett *et al.* 2008; Schaap-Nutt *et al*., 2010). The release of progeny HPIV1, 2, and 3 occurred solely at the apical surface. This selective tropism is consistent with the absence of viremia in otherwise healthy individuals. This tropism also may limit exposure of viral antigen to antigen presenting cells, possibly leading to reduced

immune responses that may allow re-infection. Low infectivity of HPIV3 for human dendritic cells may have a similar effect (Le Nouen *et al*., 2009).

HPIV infection of non-polarized cell lines in vitro is characterized by syncytium formation leading to cytopathology. In contrast, HPIV infection of HAE cultures causes minimal grosscytopathology, with no evidence of cell-to-cell fusion, in contrast to the rapid tissue destruction caused by influenza A virus (Bartlett *et al*. 2008; Schaap-Nutt *et al*., 2010; Palermo *et al*., 2009). In explanation for the lack of syncytium formation, the HPIV3 F protein was found to be localized solely on the apical surface of these polarized cultures and thus did not contact neighboring cells. In post-mortem studies of human lung tissue, syncytium formation has been observed in the lungs of HPIV-infected individuals with severe immune deficiency or immunosuppression but is uncommon in immunocompetent patients (Downham *et al*., 1975; Delage *et al*., 1979; Aherne *et al.*, 1970; Jarvis *et al.*, 1979; Weintrub *et al.*, 1987). HPIVs also strongly suppress apoptosis, which would reduce cytopathology. Viremia and infection beyond the superficial respiratory epithelium are rare with HPIV infection. One likely factor is the directional budding of virions noted above. Another important factor is host immunity: systemic spread of HPIV has been detected only in severely immunocompromised patients (Campbell *et al*., 2010; Srinivasan *et al*., 2011; Jarvis *et al*., 1979).

2.8.3 Factors contributing to HPIV pathogenesis

Young age and lack of prior exposure to the infecting HPIV serotype are two major factors associated with HPIV LRI. Young infants are at greater risk in part because their smaller airways are more susceptible to obstruction, and their immune responses are reduced due to immunological immaturity and the presence of HPIV-specific maternal antibodies that suppress antibody responses (Karron *et al*., 2007; Crowe *et al*., 2003). Host genetic factors, particularly those affecting innate and inflammatory responses, likely also play a role, as has

been investigated in greater detail for Respiratory Syncytial Virus (Karron *et al.*,2007; Miyairi *et al*., 2008). It is generally thought that the respiratory pathology observed in vivo can be attributed to

(i) high levels of virus replication and resulting direct virus-mediated effects, and

(ii) the host response to infection.

However, the relative contributions of these two factors are unclear and may be situational. While HPIV infection is not rapidly cytopathic in HAE cultures, infection appears to accelerate the normal mechanism of epithelial cell shedding and replacement (Zhang *et al*., 2005). The number of mucin-containing cells in HAE cultures increases during HPIV infection, consistent with the increased mucus production seen in HPIV-infected individuals. Also, HPIV infection inhibits ciliary motility in HAE cultures, an effect that in vivo would impede clearance of mucus and exfoliated cells from the airways. Airway inflammation and obstruction are certainly important components of HPIV pathogenesis. The histopathology of RSV bronchiolitis and pneumonia, which has been studied in more detail, also is characterized by obstruction of the small airways by inflammatory cell debris, edema, and external compression of the bronchioles from hyperplastic lymphoid follicles (do Carmo *et al*., 2010; Johnson *et al*., 2007).

2.9 Immune responses

HPIV infection induces innate immune responses, serum and mucosal antibody responses, and CD8+ and CD4+ T-cell responses that restrict replication and clear HPIV infection. Neutralizing antibodies that target the HN and F glycoproteins are the most important determinants of long-term protection from HPIV disease (Johnson *et al*., 2007). Serum IgG antibodies provide the most durable protection against re-infection, but transport to the respiratory lumen is mostly non-specific and inefficient, and protection depends on high serum titers. Maternal IgG is thought to provide protection to infants for the first weeks or

months of life. Studies in adults showed that detection of mucosal neutralizing antibody titers was a better correlate of protection than serum titers (Smith *et al*., 1966). Mucosal IgA is transported efficiently through the epithelium to the airway lumen and can neutralize virus within infected epithelial cells, though IgA protection may be relatively short-lived compared with serum neutralizing antibodies. More than one infection is likely needed to maintain protective titers of mucosal IgA. The difficulty in maintaining protective levels of IgA and IgG in the respiratory lumen is an important factor in the ability of the HPIVs to re-infect (especially in the URT) throughout life. However, immunity from prior exposure typically limits re-infections to mild disease (Glezen *et al*., 1984).

As discussed earlier, virus-encoded IFN antagonists permit HPIVs to replicate efficiently in vivo; however, Interferon(IFN) production and activity are usually not completely blocked and probably contribute to host defense. Many patients with primary HPIV infections develop a detectable IFN response during the acute stage of illness (Malur *et al*., 2004). The induction of T-cell responses also contributes to viral clearance (Caignard *et al*., 2009; Malur *et al*., 2005). Cellular immunity confers some resistance to reinfection but this effect wanes over a period of weeks to months (Poole *et al*., 2002). Mucosal antibodies that neutralize virus infectivity appear to be the best correlates of protection against HPIV disease in adults (Parisien *et al*., 2001) and may also correlate with protection in children, though measurement of mucosal immunity in children has been difficult. IgA has the advantage of being specifically transported through the epithelium to the lumenal surface and IgA is also able to neutralize the virus within infected epithelial cells. Although local IgA plays a key role in resistance against reinfection, this protective effect is also relatively short-lived and two or more infections might be needed in order for mucosal IgA to persist long term (Parisien *et al*., 2001; Schaap *et al*., 2010). Long-term resistance in the URT is less complete than resistance in the LRT and most individuals experience multiple HPIV and RSV

infections in the URT throughout life (Andrejeva *et al*., 2004). Serum neutralizing antibodies seem to provide long-term resistance to virus replication. By analogy with RSV, protection conferred by serum antibodies is likely to be more effective in the LRT than in the URT (Childs *et al*., 2007). In addition, young infants possess maternally-derived serum IgG antibodies that are transferred across the placenta during the last trimester of pregnancy and provide some protection during the first months of life. Immune responses in infants are reduced in magnitude, effectiveness and durability compared with older children due to immunologic immaturity and the immunosuppressive effects of maternal antibodies, as reponed below (Lu *et al*., 2002; Nishio *et al*., 2008; Andrejeva *et al*., 2002). Immunity induced by primary infection with HPIV often does not prevent symptomatic reinfection for more than a few months, especially in infants. However, illness upon reinfection is generally milder than with primary infection, with restricted virus replication and infrequent progression co ALRI (Karron *et al*., 2007).

2.10 Inflammatory responses

HPIV infection of the airway epithelium causes extensive changes in cellular gene expression and stimulates increased production of numerous cytokines and chemokines that either have antiviral functions themselves or attract and activate cells that mediate an immune response (Boonyaratanakornkit *et al*., 2009; Schaap-Nutt *et al*.,2010). Microarray analysis of epithelial cells infected with HPIV1 has indicated that the NF-κB, IRF3 and type 1 IFN pathways play a major role in regulating the cellular antiviral and inflammatory response to HPIV1 infection, and the HPIV1 C proteins normally suppress activation of these pathways (Boonyaratanakornkit *et al*., 2009). Correlating with the microarray data, elevated nasal wash concentrations of inflammatory cytokines, including IL-8/CXCL8, MIP1α+1β/CCL3+4, RANTES/CCL5, and CXCL9, have been described in case series of children with HPIV disease (El Feghaly *et al*., 2010; Gern *et al*., 2002). Patients with primary HPIV infections

also develop detectable IFN responses during acute infection (Hall *et al*., 1978). CXCR3 ligands such as IP-10 and I-TAC, which attract activated Th1 cells to the infected epithelium, are the dominant chemokines known to be produced during HPIV infection and are secreted by the airway epithelium as well as by monocytes and neutrophils (Bafadhel *et al*., 2011, Sumino *et al*., 2010, McNamara *et al*., 2005). High concentrations of IL-8 and IP-10, in particular, have been correlated with more severe HPIV disease (El Feghaly *et al*., 2010). It is unclear whether the association between the magnitude of inflammatory responses and disease severity reflects a specific pathogenic feature of these viruses, or the magnitude of virus replication resulting in increased immune responses, or both (Schomacker *et al*., 2012).

2.11 Pathology and Clinical Features of HPIV.

Human parainfluenza viruses (HPIVs) have been associated with every type of upper and lower respiratory tract illness, including common cold with fever, laryngotracheobronchitis (croup), bronchiolitis, and pneumonia. HPIVs are also a cause of community-acquired respiratory tract infections of variable severity in adults. The incubation period of HPIV infection generally lasts 1-7 days. Weinberg *et al* found that HPIV accounted for 6.8% of all hospitalizations for fever, acute respiratory illnesses, or both in children younger than 5 years. (Weinberg *et al*., 2010). All HPIV types are strongly correlated with specific clinical syndromes, ages, and times of year, though the lack of epidemiologic data on HPIV-4a and HPIV-4b has so far prevented a clear understanding of the true clinical significance of these serotypes. HPIV-1 and HPIV-2 are the pathogens most commonly associated with croup, and HPIV-3 is the pathogen most commonly associated with bronchiolitis and pneumonia in infants and young children (Pickerling *et al*., 2009). Patients with HPIV infection typically present with a history of coryza and low-grade fever; they then develop the classic barking cough associated with croup. Symptoms of croup include the following: Fever, Barking cough, Coryza, Stridor, Retractions, Tachypnea (when lower airways become involved),

Irritability. Children with croup are usually more symptomatic at night. Coughing often awakens them from sleep. The reasons why symptoms are worse at night are unknown. HPIV infections can also present as bronchiolitis or pneumonia. The typical presentation includes the following: Fever, Coryza, Tachypnea, Coughing, Wheezing (López Pérez *et al*., 2009). HPIV infection is associated with a broad range of findings, which may include fever, nasal congestion, pharyngeal erythema, nonproductive to minimally productive cough, inspiratory stridor, rhonchi, rales, and wheezing.

2.11.1 Croup

Croup is a generic term that encompasses a heterogeneous group of illnesses affecting the larynx, trachea, and bronchi. It affects about 3% of children in a given year, usually between ages 6 months and 3 years. HPIV-1 is the most common cause of croup; between them, HPIV-1, HPIV-2, and HPIV-3 account for almost 75% of all cases. Symptoms of croup include fever, hoarse barking cough, laryngeal obstruction, and inspiratory stridor. Croup scoring systems have been developed to aid in grading the severity of infection. Factors addressed in such systems include stridor, retractions, air entry, color, and level of consciousness. However, these croup scoring systems were developed before the advent of pulse oximetry. Pulse oximetry may be beneficial in grading severity of illness, response to management, and disposition.

2.11.2 Bronchiolitis

All 5 serotypes of HPIV can cause bronchiolitis, but the ones most commonly associated with this condition are HPIV-1 and HPIV-3, each of which appears to cause 10-15% of bronchiolitis cases in non hospitalized children. The incidence of bronchiolitis peaks during the first year of life (with 81% of cases occurring during this period) and then declines dramatically until it virtually disappears by school age. Predominant features include fever, expiratory wheezing, tachypnea, retractions, rales, and air trapping.

2.11.3 Pneumonias

HPIV-1 and HPIV-3 each cause about 10% of outpatient pneumonia cases, but as with bronchiolitis, HPIV-3 causes a larger percentage of cases in hospitalized patients. HPIV-2 and HPIV-4 can both cause pneumonia, but the incidence of disease attributable to these serotypes is not well described. HPIV-1 infection has been associated with secondary bacterial pneumonias in elderly persons. Features of pneumonia include fever, rales, and evidence of pulmonary consolidation.

2.11.4 Tracheobronchitis

More than 25% of the agents identified as causing tracheobronchitis have been HPIVs. (HPIV-3 is more commonly associated with tracheobronchitis than HPIV-1 or HPIV-2 is.) Tracheobronchitis is the most common feature seen in persons with HPIV-4 infections.

2.11.5 Other infections

HPIVs routinely cause otitis media, pharyngitis, and conjunctivitis coryza, and these can occur either singly or in combination with a lower respiratory tract infection (LRTI). HPIV-3 is the most frequently reported HPIV associated with otitis media.

2.11.6 Infections in immunocompromised patients

The growing number of patients who receive intense immunosuppression after undergoing transplantation of bone marrow and solid organs has highlighted the role of HPIVs as potential opportunistic pathogens. HPIV-2 causes giant cell pneumonia in persons with severe combined immunodeficiency diseases (SCIDs). HPIV-3 has been found in persons with SCIDs and acute myeloid leukemia (AML), as well as in patients who have undergone bone marrow transplantation (BMT). The natural history of HPIV in patients infected with HIV is generally less severe than that in transplant recipients.

2.11.7 Complications

Complications of HPIV infection may include the following:Acute respiratory distress syndrome (ARDS) and exacerbation of nephritic syndrome, Serious morbidity in immunocompromised hosts (eg, transplant recipients) – Posttransplant HPIV infection is a cause of serious lower respiratory tract involvement in both adults and children who undergo Bone Marrow Transplant. Rare complications include Guillain-Barré syndrome and Meningitis

2.12 Host range

The main host remains the human. Despite this, infections have been induced in other animals (both under natural and experimental situations) although these were always asymptomatic. HPIV can infect many different animals both naturally and under experimental conditions. Asymptomatic infection can be induced in hamsters, guinea pigs, and adult ferrets by all four HPIV types (Ray *et al.*, 1990). However, fatal disease is caused by infection with HPIV-1 to HPIV-3 in newborn ferrets (Mascoli *et al.*, 1976). Interestingly, HPIV-3 causes clinically asymptomatic infection in cotton rats and hamsters, but on autopsy

significant respiratory pathology and virus replication can be demonstrated (Porter *et al*., 1991). HPIV-3 induces hyperreactivity in the tracheas of guinea pigs and neonatal hydrocephalus in hamsters whose mothers were intervenously inoculated (Takano *et al*., 1991). Primates are easily infected with HPIV, but almost all infections are asymptomatic. Chimpanzees, macaques, and squirrel, owl, patas, and rhesus monkeys have been asymptomatically infected with HPIV-3 or HPIV-4 (VanWyke Coelingh *et al*., 1990), and only marmosets have developed symptomatic upper respiratory infections (URI) with HPIV-3 and Sendai virus (Hawthorne *et al*., 1982). There are numerous PIV closely related to HPIV that have adapted to other mammalian species. HPIV-1 has antigenic, genetic, and pathophysiologic homology to Sendai virus, which infects mice, hamsters, and pigs. Simian viruses 5 and 41 are related to HPIV-2 and infect primates (Randall *et al*., 1988). Another virus related to both HPIV-2 and simian virus 5 is canine parainfluenza virus (CP2), which causes croup and lower respiratory infection (LRI) in dogs (Baumqartner *et al*., 1989). Bovine PIV-3 has been associated with "shipping fever" in cattle, and is antigenically related to HPIV-3. This virus or similar viruses may also infect horses, sheep, goats, water buffaloes, deer, dogs, cats, monkeys, guinea pigs, rats, and pigs (Lyon *et al*., 1997). Some PIV can infect nonmammalian species. A rubulovirus, Newcastle disease virus, infects poultry, penguins, and other birds and has been responsible for conjunctivitis in bird handlers and laboratory workers.

2.13 Mode of Transmission

HPIVs can be transferred through direct person-to-person contact (with infected secretions) and via respiratory droplets (Chanock *et al*., 2001). Some sources, however, suggest that person-to-person transfer through contact is less likely since HPIVs do not survive well outside the host, and instead transfer through contaminated surfaces is more likely

(Henrickson, 2003). They are also transmitted by large-droplet spread. The exact period of communicability is not known; however, HPIV3 (the most infective HPIV) is known to shed from the oropharynx for about 3 to 10 days during initial infection. Shedding rates are lower for subsequent infections. In rare cases, HPIV3 has been observed to shed for periods as long as 3 to 4 weeks. Air-sampling experiments have shown that HPIV-1 could be recovered from only 2 of 40 infected children at a distance of 60cm (McLean et al., 1967). Therefore, transmission is unlikely to take place by small-particle aerosol spread. Close-contact transmission and surface contamination by RSV takes place by aerosolization of large droplets. Furthermore, contaminated surfaces may then lead to direct self-inoculation. It is thought that HPIV is transmitted by similar modalities. HPIV1, HPIV-2, and HPIV-3 have all been shown to survive for up to10h on non porous surfaces and 4h on porous surfaces. However, HPIV-3 experimentally placed on fingers has been shown to lose more than 90% of its infectivity in the first 10 minutes and could not be transferred to other fingers. Person to-person spread by direct hand contact appears to be an unlikely means of transmission. However, the amount of virus excreted from an acutely infected child may be more than 10 times greater than that tested. HPIV can be efficiently removed from surfaces with most common detergents, disinfectants, or antiseptic agents (Henrickson, 2003). Parainfluenza viruses are highly contagious, a leading cause of acute respiratory infection (ARI) in children, often reoccurring, and currently controlled by non-pharmaceutical interventions. In a study of infection and reinfection of a prototypic murine parainfluenza virus after contact or airborne transmission, it was revealed that the mode of transmission determines the dynamics of primary infection. Additionally, higher levels of protection from reinfection are induced in individual respiratory tissues by higher levels of primary infection in those same tissues. Natural infection after either contact or airborne transmission tends to initiate in the Upper respiratory tract (URT), but not the lungs. Complete protection from infection in the URT

was afforded by URT biased non-pathogenic infection after low-dose intranasal vaccination. Overall, the data suggested that parainfluenza virus transmission may be effectively controlled by handwashing, disinfection of surfaces, and environmental control of short-range transmission, in addition to the development of live attenuated vaccines that target the URT (Burke *et al*., 2013). One potential factor influencing the magnitude, tropism, and clinical impact of reinfection may be the mode of transmission and dynamics of primary infection, as described here for SeV transmission in mice. Thus, we hypothesize that the mode of primary infection of respiratory paramyxoviruses may also influence the severity of reinfection in other species including humans (Burke *et al*., 2013).

2.14 Interactions with the Environment

Parainfluenza viruses last only a few hours in the environment and are inactivated by soap and water. Furthermore, the virus can also be easily destroyed using common hygiene techniques and detergents, disinfectants and antiseptics. Environmental factors which are important for HPIV survival are pH, humidity, temperature and the medium the virus in found within. The optimal pH is around the physiologic pH values (7.4 to 8.0), whilst at high temperatures (above 37°C) and low humidity, infectivity reduces. (Hambling, 1964) The majority of transmission has been linked to close contact, especially in nosocomial infections. Chronic care facilities and doctors surgeries are also known to be transmission 'hotspots' with transmission occurring via aerosols, large droplets and also fomites (contaminated surfaces). The exact infectious dose remains unknown. The virus is ubiquitous; infections occur as epidemics as well as sporadically. Parainfluenza viruses are sensitive to detergents and heat but can remain viable on surfaces for up to 10 hours.

2.15 Epidemiology

Parainfluenza viruses are transmitted from person to person by direct contact and exposure to contaminated nasopharyngeal secretions through respiratory tract droplets and fomites.

Parainfluenza viral infections produce sporadic infections as well as epidemics of disease. Seasonal patterns of infection are distinctive, predictable, and cyclic. Different serotypes have distinct epidemiologic patterns (American Academy of Paediatrics, 2006).

Human PIVs are common community-acquired respiratory pathogens responsible for upper and lower respiratory infections throughout the world without any ethnic, socioeconomic, gender, age, or geographic boundaries; however, morbidity and mortality rates are higher in developing countries as compared to developed countries. The majority of infections and deaths are observed among young infants, immunocompromised, and elderly individuals. Malnutrition, Overcrowding, Vitamin A deficiency, lack of breast feeding, and environmental contaminants are factors that can predispose to these infections. The World Health Organisation estimates that acute respiratory infections (ARIs) are responsible every year worldwide for the deaths of 4 million children below 5 years. Paramyxoviruses are one of the major respiratory pathogens in this age group (Brooks *et al*., 2007). It has been estimated that 12% of the 500,000 to 800,000 lower respiratory infection (LRI) cases reported annually in USA are caused by HPIV1-3. It has also been estimated that, 10% of the total LRIs in preschool children are caused by HPIVs worldwide and 25 to 30% of these result in death. Nosocomial infections are also common, especially among young infants; with HPIV3 being the most frequently transmitted among the four HPIVs. (Mufson *et al*., 1973, Henrickson 2005). Nearly all children are infected with HPIV3 by age 2 and with HPIV1 and HPIV2 by age 5 (Parrott *et al*., 1962).

Although the four serogroups of HPIV1-4 have different seasonal peaks, infections caused by these viruses tend to be diagnosed throughout the year (Henrickson, 2005). HPIV1 causes biennial epidemics which peak during the fall season. During these epidemics the majority of infections (50%) occur in children aged 7 to 36 months and peaking during the second and third year of life. HPIV2 also causes biennial infections, either with HPIV1 or during

alternate years from HPIV1, or annual epidemics, which peak during fall to early winter. The majority of infections (60%) caused by HPIV2 occur in children younger than 5 years of age and peak between the first two years of life. Outbreaks caused by HPIV3 tend to occur yearly and peak during early spring to summer (for North America and Europe). The majority of these infections (40%) occur in children during the first year of life. Little is known about the epidemiology of hPIV4 due to the small number of studies conducted. Generally, it has been noted that the rate of infection is relatively the same in age groups from young infants to adults. An outbreak of hPIV4 within a developmental disabilities unit involving 38 institutionalized children and 3 staff members has also been described (Lau *et al*., 2005).

In North America, the annual incidence of pneumonia per 1000 children ranges from 30 to 45 among those less than 5 years old, 16 to 20 among those 5 to 9 years old and 6 to 12 among older children and adolescents. In developing countries including Nigeria, acute respiratory infections cause up to 5 million deaths annually especially among children less than 5 years old. (Grant., 1990). Biennial fall epidemics are the hallmark of HPIV-1 and occur in both hemispheres. Reports from the United States have suggested that a minimum of 50% of croup cases `are caused by this virus. During each HPIV-1 epidemic, an estimated 18,000 to 35,0000 U.S. children younger than 5 years are hospitalized. Some of these children have bronchiolitis, tracheobronchitis, pneumonia, and febrile and afebrile wheezing. The majority of infections occur in children aged 7 to 36 months, with a peak incidence in the second and third year of life. HPIV-1 can cause LRI in young infants but is rare in those younger than 1 month. The full burden of HPIV-1 in adults and the elderly has not been determined, but several studies have shown this virus to cause yearly hospitalizations in healthy adults and perhaps play a role in bacterial pneumonias and deaths in nursing home residents. HPIV-1 to HPIV-3 has all been found to occur at low levels in most months of the year, similar to RSV and influenza virus (Henrickson., 1998).

HPIV-2 has been reported to cause infections biennially with HPIV-1 or alternate years with HPIV-1 or to cause yearly outbreaks. We see some HPIV-2 activity every year in Milwaukee, Wis. The peak season for this virus is fall to early winter. HPIV-2 causes all of the typical LRI syndromes, but in non-immunocompromised or chronically ill children croup is the most frequent syndrome brought to medical attention. LRI caused by this virus has been reported much less frequently than with HPIV-1 and HPIV-3. This may be due to difficulties in isolation and detection. As many as 6,000 children younger than 18 years may be hospitalized each year in the United States because of HPIV-2 (Henrickson, 2003). About 60% of all HPIV-2 infections occur in children younger than 5 years, and although the peak incidence is between 1 and 2 years of age, significant numbers of infants younger than 1 year are hospitalized each year. HPIV-2 is often overshadowed by HPIV-1 or HPIV-3 infections, yet in any one year or location it can be the most common cause of parainfluenza LRI in young children. (Henrickson, 2003)

Young infants (younger than 6 months) are particularly vulnerable to infection with HPIV-3. Unlike the other HPIV, 40% of HPIV-3 infections are in the first year of life. Brochiolitis and pneumonia are the most common clinical presentations. Only RSV causes more LRI in neonates and young infants. HPIV-3 has caused outbreaks within neonatal intensive care units, and the natural history of this virus in the neonate is currently being studied. Approximately 18,000 infants and children are hospitalized each year in the United States because of LRI caused by HPIV-3. This virus causes yearly spring and summer epidemics in North America and Europe and is somewhat endemic, especially in the immunocompromised and chronically ill. Although the exact reasons for the different seasonality of the HPIV types is unknown, differences in ambient climate conditions have been proposed as one hypothesis.

Only a small number of studies have reported on the isolation or epidemiology of HPIV-4. These cases and data are distributed fairly equally between infants younger than 1 year, preschool children, and school age children and adults. Seroprevalence studies have demonstrated that 60 to 84% of infants have significant antibody levels after birth (presumably maternal in origin). These levels drop to 7 to 9% by 7 to 12 months of age and stay low for several years before increasing to about 50% by 3 to 5 years of age. Antibody levels to HPIV-4 continue to rise throughout childhood until approximately 95% of adults have antibody to HPIV-4 A and 75% have antibody to HPIV-4 B. Interestingly, the majority of HPIV-4 clinical isolates appear to be subtype B. All of the different respiratory tract syndromes can be caused by HPIV-4. Although hospitalization of infants and young children secondary to HPIV-4 LRI has been reported, serious disease is either rare or difficult to diagnose based on the seroprevalence (Lindquist *et al*., 1997)).

2.15.1 Morbidity and mortality.

Children who were tested years after having croup have demonstrated increased bronchial reactivity (Loughlin, 1979). Similarly, children with LRI caused by RSV have been shown to have chronic pulmonary abnormalities including decreased gas exchange (Hall, 1984), restrictive lung disease (Henry *et al*., 1984), obstructive lung disease, and hyperreactivity (Tuffaha *et al*., 2000). Even adults have been reported with long-term lung disease following HPIV LRI (Ortmann *et al*., 1994). These viruses clearly cause acute inflammatory changes directly to airways and also are capable of inducing responses in the immune system (e.g., humoral, cellular cytokine production and interaction with allergens) that lead to acute pulmonary changes(Tuffaha *et al*., 2000). The exact role of HPIV or other common respiratory viruses in chronic respiratory illness (e.g., asthma and chronic obstructive pulmonary disease is still being determined. This includes looking at the question of biased

selection of susceptible or at-risk individuals (phenotype expression) in long-term follow-up studies. However, persistent changes in lung mechanics and hyperresponsiveness have been demonstrated in animal models of PIV LRI (Quan *et al*.,1990, Sorkness *et al*., 1991), suggesting that HPIV LRI may lead to chronic pulmonary damage in some hosts.

As stated above, mortality induced by HPIV is unusual in developed countries and is seen almost entirely in young infants, the immunocompromised, and the elderly. However, the preschool population in developing countries has considerable risk of HPIV-induced death. Whether by primary viral disease or by facilitating secondary bacterial infections in malnourished children, LRI causes 25 to 30% of the deaths in this age group and HPIV causes at least 10% of the LRI (Berman, 1991).

2.15.2 Economic burden of the infection

The socioeconomic conditions within a country are the biggest determinants of the ultimate cost of HPIV infection. In undeveloped regions, the cost is in human lives. In the United States, mortality is unusual and the cost of these viruses is seen mostly in health care utilization, hospitalization, lost productivity for parents, and, perhaps, long-term Respiratory tract sequelae in children. Apparent is lost time both from work and home secondary to HPIV infections has not been quantitated but is considerable. Extrapolations based on hospitalizations a decade ago indicate that the yearly cost of HPIV-1 and HPIV-2 infection could exceed $186 million nationally (Henrickson *et al*., 1994). Another estimate based on a computer generated sampling of a national data base (0.5%) for croup caused by HPIV-1 resulted in an estimate of $30 million per croup epidemic for this subset of disease caused by this one virus (Marx *et al*., 1997). Separate and independent extrapolations using more recent data from Children's Hospital of Wisconsin (CHW) suggest that the yearly cost of hospitalizations for HPIV infections in the United States exceeds $200 million. In addition,

this estimate does not take into account URI caused by these viruses, the fact that HPIV-3 causes longer and more costly stays than HPIV-1 or HPIV-2, or the economic burden of infections in adults and immunocompromised patients (Henrickson, 2003). In the poorest regions of the world, HPIV infection can be measured in terms of mortality. In the developed world where mortality remains rare, the economic costs of the infection can be estimated. Estimates from the USA are suggestive of a cost in the region of $200 million per annum. (Henrickson, 2003).

2.16 LABORATORY DIAGNOSIS

2.16.1 Collection and Preparation of Clinical Specimens

The nasopharynx and oropharynx are primary locations of initial HPIV replication. Virus is shed at high titer early in an infection and then sharply declines. Experimental infections in adults have demonstrated that illness usually starts about 3 to 4 days after inoculation and lasts from 3 to 17 days, with an average of 4 days for HPIV-1 and 6 to 13 day for HPIV-2 and HPIV-4 (Tyrrell *et al.*, 1969). Virus recovery in adults is much more difficult than in children. This is partly due to the several-log unit increase in viral titer produced by children compared to adults. Children shed virus usually from 3 to 4 days prior to the onset of clinical symptoms until approximately 10 days past (Frank *et al*., 1989). However, HPIV-3 has been isolated in children from as early as 6 days before to 6 weeks past the first symptom. Immunocompromised patients and adults (mostly with chronic lung disease) have persistently shed HPIV-1, HPIV-2, and HPIV-3 for many months (Gross *et al*., 1973). Throat swabs, nasopharyngeal swabs, nasal washes, and nasal aspiration have all been used successfully to recover HPIV (Frayha *et al*., 1989). The optimal method of collecting clinical samples for HPIV has not been well studied but depends on the method of detection used (e.g., PCR or tissue culture), the age of the patient, and the general health of the patient (i.e.

immunocompromised or with chronic lung disease). The few studies to yield high rates of viral recovery (HPIV-1 and HPIV-3) used nasal washes or nasal aspirates (Wong *et al*., 1982). These methods are therefore recommended even in young infants for optimal virus isolation.

HPIV lose infectivity rapidly when the temperature rises above 4 to 8°C. Specimens (swabs or 2- to 4-ml nasal wash aspirates) should be collected and placed in viral transport medium (2 to 3 ml), either veal infusion broth or minimum essential medium supplemented with some protein source (not serum) such as 0.5% bovine serum albumin. The transport medium should contain antibiotics and antifungal agents to decrease contamination and be buffered to yield a pH of 7.5 to 80 after addition of the clinical sample. Ideally, the specimen in transport medium should be kept at 4°C until tissue culture inoculation. If a delay of more than 24 hours is anticipated, the specimen should be frozen. Centrifugation at 1,000g prior to inoculation is also helpful in removing debris.

It is rare to have to process non respiratory specimens for HPIV. CSF can be directly plated onto tissue culture cells. If it is going to be frozen, transport medium should be added 1:1 prior to freezing. Transport medium with extra antibiotics can be used to dilute stool specimens or for rectal swabs. Standard transport medium can be used to dilute tissue homogenized in a dounce prior to testing.

2.16.2 Serum Samples

The first serum sample (acute phase) should be collected as soon into the person's viral illness as possible. The second serum sample (convalescent phase) should be drawn when the peak antibody production occurs. For neutralizing antibody, this occurs between 3 and 5 weeks after infection. Both samples should have the serum removed as quickly as possible

and then be stored at either 7 or 20°C in a non-frost-free freezer. Both the acute- and convalescent-phase samples should be tested at the same time.

2.16.3 Virus Isolation

Tissue culture HPIV has the best growth in primary monkey kidney cells. In a survey of clinical virology laboratories, the most popular cell line for growing HPIV was rhesus MK cells. However, this cell line is not always the most sensitive for HPIV, especially for HPIV-2 (Frank *et al*., 1973). Cynomolgus and African green monkey cell lines are also used. An easy to-use secondary cell line (LLC-MK2) is excellent for continued passage and almost as good as PMK cells for primary isolation. LLC-MK2 cells need trypsin added to the maintenance medium to recover all HPIV serotypes. Trypsin may be helpful even for PMK cell lines. Both PMK and LLC-MK2 cells may both be required for optimal isolation of HPIV. Most strains of HPIV-4 and even some strains of HPIV-2 and HPIV-3 are detected 10 days or longer after inoculation.PMK cells may start to degenerate between 10 and 14 days, especially if trypsin is in the medium. One method to maximize HPIV recovery is to infect both LLC-MK2 and PMK cell lines and perform initial detection methods (e.g., F and HAd) on the PMK cells. After 10 days, if these tests are negative and further testing of the PMK cells is not possible, the LLC-MK2 cells are in reserve for testing at 2 and 3 weeks.

Isolation in animals. Eggs are a very poor growth medium for HPIV. It is only after many repeated passages and much presumed antigenic change that HPIV can be detected in embryonated eggs. Only three isolates of HPIV-2 have ever been reported to have undergone primary isolation in eggs (VonEuler *et al*., 1963, Henrickson, 2003).

Detection and typing. Cytopathic effect (CPE) is rarely demonstrated during primary isolation of HPIV in tissue culture, and so this is not a reliable or useful method to detect positive cultures. HAd of guinea pig red blood cells directly onto the tissue culture monolayer

is the most common method to detect the presence of HPIVs. The respiroviruses are quickly and easily detected using HAd. Most HPIV-1 isolates are detected by day 4 (Henrickson., 1994), and HPIV-3 takes only a little longer (Canchola *et al*., 1965). However, the rubulaviruses are much slower. Only 35% of HPIV-2 isolates are HAd positive by day 7 (Henrickson *et al*., 1994), and HPIV-4 may take 3 weeks or longer (Canchola *et al*., 1964). Interestingly, the neuramindase of HPIV-4 appears to be temperature sensitive because this virus hemadsorbs better at room temperature or 37°C while all of the other serotypes react well at 4°C (Gardner, 1969). Immunoflorescense (IF) is the most rapid and accurate method currently available to detect and type HPIV in tissue culture (Henrickson, 2003).

HPIV isolates are detected much faster using this technique than using to HAd, and IF detects positive cultures that never develop HAd. This is especially true for HPIV-2 (Henrickson *et al*, 1994). In addition, further typing of HPIV is unnecessary when IF detection is used. This can save time and money. Tissue culture isolation of HPIV is being done less and less. However, high risk populations may continue to have a need for this method. A typical testing plan (all year round) would be as follows. IF detection is performed on all inoculated PMK tissue culture cells on days 2 or 3, 4 or 5, and 10 and on MK2 cells on days 14 and 21. A MAb pool reagent could be used such that only a single test had to be done per culture (Stout *et al*., 1989). Also, the addition of centrifugation (e.g., shell vial) to the above IF schedule may speed HPIV detection by 1 day or more (Rabalais *et al*., 1992). If desired, HAd could be performed first and then IF could be performed only on those that were positive. It is important to remember that primary screening with HAd will miss some HPIV. Other useful methods of detecting and typing HPIV in tissue culture include HAdI, HI, complement fixation, and neutralization (Henrickson *et al*., 1996). These methods are currently more useful for research purposes since they are time-consuming and costly compared to IF (except HI). On primary isolation, most strains of HPIV do not release enough free viruses

into the medium to react in the HI assay, limiting the usefulness of this relatively easy assay in clinical virology. Until commercial MAb IF reagents became widely available, Haemadsorption inhibition (HAdI) was the method of choice for HPIV serotype identification. This meant that each identification took an additional 3 to 5 days after the culture was identified as positive. Although tissue culture is the current "gold standard," the new molecular assays like PCR are rapidly replacing this time-honored method in clinical virology (Henrickson, 2003).

2.16.4 Serologic Diagnosis

There are many methods to detect antibodies to HPIV, including ELISA, radioimmunoassays, HI, complement fixation, western blotting, and neutralization assays. The development of heterologous antibody to closely related HPIV serogroups during any HPIV infection is a persistent problem in trying to make a serological diagnosis of acute HPIV (Chanock *et al*., 1958). This problem is greatest when trying to separate HPIV-1 infection from HPIV-3 infection, but mumps virus antibody also cross-reacts with these two viruses (Frankova *et al*., 1988). A large number of children's sera that test positive by ELISA and HI toHPIV-3 will have similar titers to HPIV-1.Although the least sensitive of the serologic assays, complement fixation have the smallest number of problems with heterologous cross-reactivity. On the other hand, ELISA is by far the most sensitive assay and detects the largest number of titer rises in patients with respiratory illness but is the least specific and detects many dual HPIV antibody titers. HPIV-2 demonstrates the least amount of serologic cross-reactivity (Vuorinen *et al*., 1989). A fourfold rise or drop in titer is generally thought to signify acute infection if the testing is performed at the same time on paired acute- and convalescent-phase serum samples. A positive serologic rise in titer to any of the HPIV types (even if without heterologous antibody demonstrated) only indicates infection with HPIV (Chanock *et al*., 1979). It should not be used to determine the HPIV serotype. Only virus

isolation and typing with Monoclonal Antibodies (or molecular identification) can accurately establish the specific serotype.

2.16.5 Direct Examination for Viruses

Electron microscopy can easily demonstrate HPIV (Plate 1). However, many paramyxoviruses appear the same (e.g., mumps virus). No large study looking at the diagnostic utility of electron microscopy for HPIV infection has been published and less expensive methods of diagnosis have been developed. Methods to detect HPIV antigen using ELISA, radioimmunoassay, and fluoroimmunoassays have all been developed (Henrickson, 2003). Investigators using these methods have reported sensitivities between 75 and 95% (polyclonal sera), with the MAb-FIA demonstrating slightly higher detection of HPIV-1 and HPIV-2 (94–100%). Most clinical virology laboratories will find these methods difficult to perform until reagents are widely available. For more than two decades, HPIV has been detected by IF (Blanding *et al*., 1989). Originally these techniques involved the production of serotype specific antisera in various animal species by each individual laboratory and subsequent conjugation to fluorescein isothiocyanate. Now most laboratories use of commercially produced MAbs (single or pooled). The detection of HPIV by direct IF staining of clinical material has yielded highly variable and sometimes disappointing results. The best results have been demonstrated for HPIV-3, with most studies reporting sensitivities between 50 and 75% (range, 31 to 100%). In studies that used only commercially available MAbs the sensitivities range between 31 to 93% of culture-positive specimens. Studies detecting HPIV-1 showed sensitivities ranging between 0 and 92%, and those detecting HPIV-2 showed sensitivity ranging between 50 and 100%, with most sensitivities falling between 50 and 83%. Differences in specimen type, collection, processing, reagents (antibody), and testing procedures help explain the reported variability in sensitivities. However, specificity appears to be uniformly excellent. The use of Immunofluorescense to detect HPIV-3 has

demonstrated a rate for IF-positive/culture-negative specimens as high as 16%. It is not known whether these are all false positives or positive specimens without viable virus.

The shell vial assay is another method for the rapid identification of HPIV. In this assay, tissue culture is grown on slides and then centrifugation is used initially to speed viral absorption and cell infection. Detected is performed by standard IF of the tissue culture cells, typically at 48 hours and 5 days. Although variable results have been reported, the majority of data appear to demonstrate improved detection using this method. Influenza virus and RSV are the two viruses most widely studied by this method. Detection of HPIV has been compared using shell vials and standard tissue culture, with sensitivities averaging 84%, but most of the viruses tested were HPIV-3 (Henrickson, 2003).

Standard IF detection is done visually using a fluorescent microscope with appropriate mirrors and filters for fluorescein isothiocyanate. Fluorescence can also be detected using flow cytometry analysis. This automated process analyzes the fluorescent antibody binding to cell surfaces and reports this binding quantitatively. The minimum number of cells needed (2,000 cells), the lengths of time, and the cost make this technique more useful in a research setting.

2.16.6 Genomic Detection

HPIV RNA can be detected directly by performing a Northern hybridization or a dot blot analysis using virus specific DNA probes. This takes many hours to complete, yields less uniform results, and, even with sensitive detection methods, appears to be most useful as a research tool. The amount of HPIV RNA in many clinical samples is not great enough to allow detection without either biological (tissue culture) or molecular amplification. After conversion of RNA into DNA, PCR can be used easily and efficiently to detect small numbers of virus in different fluids. Several studies demonstrating PCR to be sensitive and

specific in detecting HPIV have been published (Echevarria *et al*., 2000, Eugene-Ruellan *et al*., 1998, Fan *et al*., 1996, Freymuth *et al*., 1997). A multiplex RT-PCR assay for detecting HPIV-1, HPIV-2, and HPIV-3 was developed in 1995. Currently, there is one commercial multiplex PCR assay available for detecting HPIV-1, HPIV-2, and HPIV-3, along with RSV A, RSV B, influenza A virus, and influenza B virus. This RT-PCR-EHA has a reported sensitivity of 95 to 100% and a specificity of 97 to 100% compared to tissue culture and yields results in about 7 hours (Barenfranger *et al*., 2000).

Real-time PCR methods have been developed for HPIV-1, HPIV-2, and HPIV-3 and reagents for multiplex real-time PCR are available commercially. Recently, protocols for the duplex detection of HPIV-1 andHPIV-3 have been developed for isothermic amplification using nucleic acid sequence-based amplification. However, to use these protocols, HPIV primers and probes have to be designed by each laboratory, and royalties may have to be paid to the patent holders (Henrickson, 2003)

2.17 Prevention of HPIV.

Despite decades of research, no vaccines currently exist (Sato *et al*., 2008). Recombinant technology has however been used to target the formation of vaccines for HPIV-1, 2 and 3 serotypes and has taken the form of several live-attenuated intranasal vaccines. Two vaccines in particular were found to be immunogenic and well tolerated against HPIV-3 in phase I trials. HPIV 1 and 2 vaccine candidates remain less advanced (Durbin *et al*., 2003)

Vaccine techniques which have been used against HPIV are not limited to intranasal forms, but also viruses attenuated by cold passage, host range attenuation, chimeric construct vaccines and also introducing mutations with the help of reverse genetics to achieve attenuation (Schaap-Nutt *et al*., 2011). Maternal antibodies may offer some degree of

protection against HPIV during the early stages of life via the colostrum in breast milk. (Nishio *et al*., 2008)

2.18 Approaches to vaccine development & obstacles along the way

The first attempts at developing a HPIV vaccine were undertaken in the early 1960s, not long after the discovery of these viruses. It was shown that protection against challenge with wild type (WT) HPIV1 correlated with the presence of neutralizing nasal mucosal antibodies (Schaap-Nutt *et al*., 2010). Serum antibodies also contributed to protection, but only when the serum antibody titer was high. A formalin-inactivated (FI) HPIV1 vaccine that induced neutralizing antibody titers in the serum, but not the nasal mucosa, did not confer protection (Parisien *et al*., 2001, Schaap-Nutt *et al*., 2010). FI vaccines against RSV and measles were also evaluated clinically in the 1960s and conferred only transient (measles virus) or insignificant (RSV) protection; instead, enhanced and atypical disease was observed following natural infection of vaccinees with WT virus. Of the 23 FI-RSV vaccinees who were infected with WT RSV during an outbreak following vaccination, 18 needed to be hospitalized and two infants died of pneumonia. As a result of the failure of these FI vaccines, new attempts at developing HPIV and RSV vaccines for use in children have focused, for many years, on live-attenuated approaches. This is because preclinical and clinical data demonstrate that disease enhancement is not associated with live vaccines (Schaap-Nutt *et al*., 2011). Only very recently has the interest in developing protein-based non live vaccines re-emerged and several vaccine developers have invested in the development of non live RSV vaccines.

At first consideration, HPIVs appear to be an easy target for vaccine development because these viruses cause acute, self-limiting disease and do not establish persistent infection. However, vaccine development has proved to be far from trivial and several obstacles need to be overcome;

First, immunity induced by a single infection with WT HPIV does not prevent symptomatic reinfection. Most children and adults experience multiple symptomatic infections. However, reinfections generally induce milder disease and significant LRI is infrequent.

Second, severe HPIV3 disease often occurs in young infants under 6 months of age and a robust antibody response to the viral surface glycoproteins is induced less frequently in this age group than in older infants (Nishio *et al*., 2005, Zhang *et al*., 2005). In addition, young infants have a less diverse B-cell repertoire and less efficient antibody affinity maturation (Schaap-Nutt *et al*., 2010, Bartlett *et al*., 2008). Although they can mount a protective immune response, as indicated by restriction of a second dose of live vaccine, measurable correlates of protection are not well defined (Lu *et al*., 2008).

Third, maternal antibodies can suppress the immunogenicity of both parenterally administered nonlive and mucosally-delivered live vaccines (Andrejeva *et al*., 2002, Pickles *et al*., 1998, Le Nouen *et al*. 2009).

In summary, one would want an ideal HPIV vaccine to be immunogenic in young infants in the presence of maternal IgG, to protect against LRI during the first infection with WT virus and to be well tolerated and safe. A live-attenuated intranasally-administered HPIV or RSV vaccine would probably need to be given in two or three doses, perhaps at 2, 4 and 6 months of age to fit the pediatric vaccination schedule.

2.19 Treatment

Specific antiviral therapy is not available. Most infections are self-limited and require no treatment. Monitoring for oxygenation and hypercapnia for more severely affected children with lower respiratory tract disease may be helpful. Epinephrine aerosol commonly is given to severely affected, hospitalized patients with laryngotracheobronchitis to decrease airway obstruction. Parenteral dexamethasone in high doses (>0.3 mg/kg), oral dexamethasone (0.15–0.6 mg/kg), and nebulized corticosteroids have been demonstrated to lessen the

severity and duration of symptoms and hospitalization in patients with moderate to severe laryngotracheobronchitis. Oral dexamethasone (0.15 mg/kg) also is effective for outpatients with less severe croup. Management otherwise is supportive. Antimicrobial agents should be reserved for documented secondary bacterial infections. (American Academy of Paediatrics., 2006). Ribavirin is one medication which has shown good potential for the treatment of HPIV-3 given recent in-vitro tests (in-vivo tests show mixed results). Ribavirin is a broadscale anti-viral and is currently being administered to those who are severely immuno-compromised, despite the lack of conclusive evidence for its use. Protein inhibitors and novel forms of medication have also been proposed to relieve the symptoms of infection.

Furthermore, antibiotics may be used if a secondary bacterial infection develops. Corticosteroid treatment and nebulizers and also a first line choice against croup if breathing difficulties ensue.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study area

This study was conducted in Kaduna Metropolis, Kaduna State which is located in the Northern Guinea Savanna ecological zone. It occupies almost the entire central portion of the Northern part of Nigeria and share common borders with Zamfara, Katsina, Niger, Kano, Bauchi, Nassarawa and Plateau States. To the Southwest, the state shares border with the Federal Capital Territory, Abuja. The global location of the state is between longitude $06^{o}00$ and $09^{o}00$ easts of the Greenwich Meridian and also between latitude $09^{o}00$ and $11^{o}30$, north of the equator. The state occupies an area of about 48,473.2 square kilometres. It has a population of 6,066,562 people (NBS, 2007); and a projected population of 6,527,620 in 2009. Kaduna State is the successor to the old Northern Region of Nigeria, which had its capital at Kaduna. In 1967 this was split up into six states, one of which was the North-Central State, whose name was changed to Kaduna state in 1976. This was further divided in 1987, losing the area now part of Katsina State.

The study was conducted in four major hospitals in Kaduna Metropolis which included; Barau Dikko Specialist Hospital, Yusuf Dantsoho Memorial Hospital, Kawo General Hospital, Gwamna-Awan General Hospital Kaduna situated in North and South regions of Kaduna Metropolis.

3.2 Study population

The study population included children (1-12years) attending the hospitals selected for the study whose parents gave their consent to be part of the study.

3.3 Sample Size

The sample size will be determined using the following equation as described by Naing *et al*., (2006)

$$n=\frac{Z^2pq}{d^2}$$

Where;

n=number of samples collected from the children

Z=standard normal distribution at 95% confidence limit=1.96

P=prevalence of seroprevalence 11.8% (Calvo et al., 2011)

q=1-P

d=absolute desired precision=0.05%

Therefore,

$$n=\frac{(1.96)^2\times 0.118\times (1-0.118)}{(0.05)^2}$$

=159.927

=160 samples

However, the sample size was increased to 376 to minimize errors.

Sample size therefore= 376 samples.

3.4 Ethical approval

Ethical approval was obtained from the ethical committee of the Kaduna State Ministry of Health.

3.5 Inclusion and Exclusion Criteria

3.5.1 Inclusion Criteria.

Children (1-12years) whose parents accepted and gave their consent to be part of the study.

3.5.2 Exclusion Criteria

The exclusion criteria included all children less than a year, above 12years and children within (1-12years) with whose parents did not give consent to be part of the study.

3.6 Study design

The study design was a descriptive cross sectional and experimental study which combines the use of administered structured questionnaire in the analysis of serum samples collected from consented patients. The essence of the research was explained to the target population and their verbal consent was obtained, after which structured questionnaire were administered and blood samples collected. A total of three hundred and eighty (376) blood samples were collected of which 120 samples was collected from Barau Dikko Specialist Hospital, One hundred (100)samples each from Yusuf Dantsoho Memorial Hospital and Gwamna Awan Hospitals and 56 samples from Kawo General Hospital. Analysis of the serum samples was carried out subsequently.

3.7 Questionnaire administration to determine the risk and demographic factors associated with the infection

A structured questionnaire was administered to the parents of the children who met the selection criteria who had consented to be part of the study. The questionnaire contained information on risk factors (malnutrition, inadequate breast feeding, vitamin A deficiency etc) and demographic factors (parental occupation, parental educational status, etc) associated with the prevalence of Human Parainfluenza Virus in their children. This was done just before the blood sample of the child was collected.

3.8 Sample collection

Blood samples were collected from the patients with the assistance of a Laboratory Scientist. Three hundred and seventy six blood samples were collected from children in the selected hospitals in Kaduna Metropolis.

All materials (e.g sterile plain containers, serum storage screw-capped container, syringes and needles, tourniquet, cotton wool etc)required for collection of venous blood were assembled and labelling of the sterile bottles with the patient's identification and date was done. About two millilitres (2 ml) of blood sample was collected from the antecubital vein by venupuncture and dispensed into sterile plain labelled containers and allowed to clot for 30 minutes and then centrifuged at 1000rpm for 10minutes to separate the serum. The serum was carefully removed with a transfer pipette in order to avoid extracting red cells and was transferred aseptically into the sterile labelled serum storage screw-capped container. The serum was stored at -20^0 C until required for analysis.

3.9 Laboratory analysis

Serum samples were analysed for the presence of human parainfluenza virus antibody using the ELISA Test kits (IMMUNOLAB GmbH, Otto-Hahn-Str. 16, D-34123, Kassel Parainfluenza 1/2/3 IgG antibody ELISA kit). The procedure used is as described below;

3.9.1 ELISA assay procedure

Principle of the Assay

The Immummunolab Parainfluenza 1/2/3 IgG antibody test kit(IMMUNOLAB GmbH, Otto-Hahn-Str. 16, D-34123 Kassel) is based on the principle of the Enzyme Immunoassay (EIA). Parainfluenza 1/2/3 antigen is bound on the surface of the microtitre strips. Diluted patient serum or ready to use standards were pippeted into the wells of the microtitre plate. A

binding between the binding of the IgG antibodies of the serum and the immobilized Parainfluenza 1/2/3 antigen took place. After one hour incubation at room temperature the plate was rinsed with diluted wash solution, in order to remove the unbound material. Then ready-to-use anti-human-IgG peroxidase conjugate was added and incubated for 30minutes. After further washing step, the substrate (TMB) solution was pippeted and incubated for 20minutes, inducing a development of a blue dye in the wells. The color development was terminated by the addition of a stop solution, which changed the color from blue to yellow. The resulting dye intensity was measured spectrophotochemically at the wavelength of 450nm. The concentration of IgG antibody is directly proportional to the intensity of the color.

3.9.1.1 Reagent preparation

Wash buffer

The wash concentrate was brought to room temperature and poured into a measuring cylinder; the volume was made up to 600mls with distilled water.

Assay procedure

All samples and reagent kits were brought to room temperature (20-25^{o}C) and mixed thoroughly by gentle swirling before use samples were numbered according to the microtitrewells. The coated wells were placed on a holder. Approximately, 5µl of the sample, negative control, positive control and cut-off control was added to 200µl of the sample diluents to make 1 in 40 dilution. About 100µl diluted sera and controls was dispensed into appropriate wells. For the reagent blank, 100µl sample diluents were dispensed in 1A well position. All the wells were incubated for 60mins at room temperature. The liquid from the wells was removed and wells washed three times with washing buffer. A quantity of 100µl of

the enzyme conjugate was dispensed into each and the plate incubated at room temperature for 30minutes. Excess enzyme conjugate was removed by washing each well with washing buffer three times. Approximately, 100µl of chromogenic substrate was dispensed into each well and incubated at room temperature for 20minutes. Then 100µl of 2M HCL was added to stop the reaction. The results were obtained using a microplate reader at an optical density of 450nm (Collins *et al,* 1996).

3.9.2 Viral Cultivation

Exactly 30 throat swabs were obtained from the children with apparent respiratory infection symptoms using sterile swab sticks. Swabs was placed in a solution containing normal saline and shaken to release the virus particles into the solution and then transported to the refrigerator and stored at -20^{o} C until when ready to use. Ten-day old embryonated eggs obtained and maintained at room temperature were disinfected with methylated spirit and then candled by placing the eggs in the candle box. The airspace and veins were noted, which was a portion about 1cm below the airspace. The solution was centrifuged at 10000rpm for 3-5minutes. The supernatant was discarded and then 0.01ml of the sediment was inoculated into the amniotic/allantoic cavity of the embryonated eggs by puncturing the space 1cm below the airspace and the tip of the airspace. A needle containing the inoculum was inserted directly about one inch into the eggs. The content of the egg was released carefully and gently to avoid oozing out. The punctured parts was sealed with candle wax and incubated at 34-37^{o}C for 3days. After incubation, the portion of the airspace was cut-off; the chorioallantoic membrane was also punctured and removed. The allantoic fluid (which is the virus suspension) was harvested into a sterile container and refrigerated at -20^{0}C until when ready to use (Chanock et al., 1961).

3.9.3 Parainfluenza Virus Detection Using the Haemagluttination Method

In order to test the antibody level in the positive samples, Thirty (30) throat swab samples were tested for haemagglutination using the following procedure;

An aliquot of 0.2ml of phosphate-buffered saline (pH 7.2-7.4) was added into each of twelve 12×75mm serological tubes. Doubling dilutions of the virus suspension was made using 0.2ml transfers. The last tube contained 0.2ml saline only. Aliquot of 0.2ml of a 0.25 percent sheep erythrocyte suspension was added to each of the tubes and mixed well. The tubes were incubated for 30-60 minutes. Without disturbing the content of the tubes, the evidence of haemagluttination patterns was carefully observed by holding the rack overhead with the bottoms of the tubes at eye levels. The control tube was observed for a negative reaction which was a smooth settling of the cells into either a solid round button or ring. The highest dilution of virus showing definite agglutination was recorded. This is one unit of virus. In the inhibition tests, four units are used. The dilution of virus that contained four units in 0.2ml was recorded (Canchola *et al*., 1965).

3.9.4 Haemaglutination Inhibition Test

Sixteen (16) positive sera samples with the highest concentration (based on ELISA) of antibody to human parainfluenza virus were selected and the antibody titre was quantified by haemagglutination inhibition method using the antigen isolated with the highest agglutination titre. The following procedure was used;

Doubling dilutions of an antiparainfluenza serum in 0.25ml quantities from 1:2 through 1:1024(ten tubes) were made. Aliquot of 0.25ml of saline was placed into each of two more tubes. Exactly 0.25ml of virus suspension diluted to contain 4units was added into each of the serum dilution tubes and into one of the control tubes. This was mixed well and incubated at room temperature for 30minutes. Aliquot of 0.5ml of 0.25percent sheep erythrocytes was

added to each tube. The tubes was agitated thoroughly and incubated at room temperature for 30-60minutes and observed for haemaglutination. The titre of the antiserum is the highest dilution of serum which completely inhibits haemaglutination by the four units of virus and this was recorded (Canchola *et al.*, 1965).

3.10 Data Analysis

Descriptive statistics and Chi-square test were used to analyse the data. Chi-square is one of the most useful of the non-parametric statistics. Chi-square is specified as;

$$\chi^2 = \sum \frac{(0-E)^2}{E}$$

where;

O=observed value

E=Expected value

If the p-value for the calculated χ^2 is P>0.05, null hypothesis is accepted. If calculated χ^2<0.05, null hypothesis is rejected.

CHAPTER FOUR

4.0 RESULTS

It was discovered that there was significant association (p = 0.039) between parents' occupation and the HPIV infection. Children whose parents were teachers had the highest infection rate of 84.1% followed by those whose mothers were fulltime housewives (81.1%), 76.9% of civil servants' children were infected while children of self-employed parents had the least infection (70.9%) (Table 4.1).

Similarly, test of association of parents' level of formal education with the infection was carried out. Though there was no significant association (χ^2 = 4.768, p = 0.092), children of parents with primary education had the highest rate of infection; those with tertiary level of education, their children had 78.9% infection rate and the least (70.9%) was observed amongst children of parents with secondary level of education (Table 4.1).

Table 4.2 presents the result of the assessment of the association of number of meals eaten daily; this shows no significant association (χ^2 = 2.132, p = 0.344) with human parainfluenza virus infection but the daily eating pattern revealed that children that ate once a day had the least rate of infection while those that ate twice daily had 80.0%. Household size was not significantly associated (χ^2 = 0.194, p = 0.907) but households with 7-12 persons had higher (77.0%) infection rate compared to those with 1-6 persons where the infection rate was 75.9% (Table 4.2).

Other risk factors assessed include but not limited to vitamin A deficiency, duration of breastfeeding, whether or not parents smoke. There was no significant association between vitamin A and human parainfluenza virus infection in children (Table 4.2). Table 4.2 further shows significant association (p = 0.015) between duration of breastfeeding and the infection. Children who were breastfed for 1-6 months had 41.7% infection rate followed by those

Table 4.1: Association of demographic data with human parainfluenza virus infection in children.

Parameter	No. of sample tested	No. Positive (%)	χ^2	P
Age group (years)				
1-3	151	99(65.6)	17.408	0.001**
4-6	76	65(85.5)		
7-9	84	69(82.1)		
10-12	65	55(84.6)		
TOTAL	**376**	**288(76.6)**		
Parents' Occupation				
Civil servant	52	40(76.9)	10.116	0.039*
Teacher	69	58(84.1)		
Housewife	90	73(81.1)		
Self-employed	165	117(70.9)		
TOTAL	**376**	**288(76.6)**		
Education				
Primary	81	67(82.7)	4.768	0.092
Secondary	148	105(70.9)		
Tertiary	147	116(78.9)		
TOTAL	**376**	**288(76.6%)**		

χ^2 = chi square
p = level of significance
* = significant association exists at $p < 0.05$
** = significant association exists at $p < 0.01$

Table 4.2: Effect of household number and nutrition on human parainfluenza virus infection.

Parameter	No. of samples tested	No. Positive (%)	χ^2	P
Daily eating				
Once	16	10(62.5)	2.132	0.344
Twice	55	44(80.0)		
Thrice	305	234(76.7)		
TOTAL	**376**	**288(76.6)**		
Household number				
1-6	137	104(75.9)	0.194	0.907
1-12	239	184(77.0)		
TOTAL	**376**	**288(76.6)**		
Vitamin A				
Yes	294	226(76.9)	0.057	0.812
No	82	62(75.6)		
TOTAL	**376**	**288(76.6)**		
Breastfeeding				
1-6 months	12	5(41.7)	8.439	0.015*
7-12 months	98	76(77.6)		
>12 months	266	207(77.8)		
TOTAL	**376**	**288(76.6)**		

χ^2 = chi square
p = level of significance
* = significant association exists at $p < 0.05$
** = significant association exists at $p < 0.01$

breastfed for 7-12 months (77.6%) and 77.8% for those that were breastfed for more than 1 year.

Children of parents that smoke had higher (80.0%) infection with human parainfluenza virus than those of non-smoking parents (76.4%) though smoking was not significantly associated with the infection. In like manner, environmental smoke was not significantly associated with the infection (Table 4.3).

Out of the 319 children with respiratory symptoms, 251 (78.7%) had human parainfluenza virus infection while 37(64.9%) of those without respiratory symptoms did not have the infection. Significant association ($p = 0.024$) was observed (Table 4.4). Fever, sickle cell and underlying diseases showed no significant association with the infection in children (Table 4.4).

The ELISA result showed that 288(76.6%) samples tested positive to Human Parainfluenza Virus IgG antibody while 88(23.4%) samples tested negative to HPIV IgG antibody giving a total seroprevalence of 76.6%.

Ten of the thirty samples tested for haemaggglutination showed agglutination with the titre profile shown in Table 4.5. In Table 4.6, the antibody level specific for parainfluenza virus antigen isolated and used (which had a titre of 256 ie 64HI unit) was 1:64 ie 64HAI units/ml of blood and was found in one sample only.

Table 4.3: Effect of smoking on human parainfluenza infection.

Parameter	No. of samples tested	No. Positive (%)	χ^2	P
Parental smoking				
Yes	25	20(80.0)	0.173	0.677
No	351	268(76.4)		
TOTAL	**376**	**288(76.6)**		
Environmental smoke				
Yes	195	143(73.3)	0.405	0.121
No	181	145(80.1)		
TOTAL	**376**	**288(76.6)**		

χ^2 = chi square
p = level of significance

Table 4.4: Relationship between symptoms, risk factors and human parainfluenza virus.

Parameter	No. of sample tested	No. Positive (%)	χ^2	P
Respiratory symptoms				
Yes	319	251(78.7)	5.116	0.024*
No	57	37(64.9)		
TOTAL	**376**	**288(76.6)**		
Fever				
Yes	275	209(76.0)	0.203	0.653
No	101	79(78.2)		
TOTAL	**376**	**288(76.6)**		
Sickle cell				
Yes	29	26(89.7)	2.990	0.084
No	347	262(75.5)		
TOTAL	**376**	**288(76.6)**		
Underlying diseases				
Diarrhoea	21	17(81.0)	4.450	0.217
Asthma	6	6(100.0)		
Blood diseases	7	258(75.4)		
None	342			
TOTAL	**376**	**288(76.6)**		

χ^2 = chi square
p = level of significance
* = significant association exists at $p < 0.05$

Table 4.5: Haemaglutination titres of the virus present in the throat swabs.

Throat swab number	**Haemagglutination titre**
1	1:256
2	-
3	1:128
4	-
5	1:256
6	1:16
7	1:16
8	1:128
9	-
10	-
11	-
12	1:128
13	-
14	-
15	1:32
16	-
17	-
18	-
19	-
20	-
21	-
22	1:64
23	1:64
24	-
25	-
26	-
27	-
28	-
29	-
30	-

Table 4.6: Quantified antibody titre level to human parainfluenza virus using haemagglutination inhibition.

Serum Sample	Inhibition titre
01	-
02	-
03	-
04	-
05	-
06	1:64
07	1:4
08	1:16
09	1:16
10	1:16
11	1:4
12	1:16
13	1:4
14	1:16
15	-
16	1:4

CHAPTER FIVE

5.0 DISCUSSION

The study was conducted to assess the prevalence of Human Parainfluenza virus in children between the ages of 1 and 12 in Kaduna Metropolis, Nigeria. The IgG antibody was measured to determine the proportion of sero-positivity and the level of the antibody with deference to such factors as age of children, parental occupation, highest educational level of the parent, environmental pollution (smoke), duration of breastfeeding, presence of respiratory symptoms, sickle cell disease, and any other underlying diseases.

The study revealed a rate of 76.6% of Human Parainfluenza Virus in the population of children in Kaduna Metropolis. There is an increase in prevalence rate as compared with previous results from Sale *et al*., (2010) which showed a prevalence of 46.5% among children aged 1-5years in Zaria, Kaduna and Calvo *et al*., (2011) in Spain with prevalence 11.8% can be due to the fact that the ELISA kit used in this study tested for IgG antibodies for Parainfluenza 1, 2 and 3. This means that the children may have had the IgG antibody however may not have come down with the infection. Also the study was carried out during the Harmattan (equivalent to the winter season) which usually is the peak period of the infection in children especially (Ray, 2004). The result is however similar to the report of Glezen and Denny (1997) which showed that 75% of children aged 5 years had antibodies to HPIV-1 and 2 as well as that of Akinloye *et al*., (2011) in Ibadan who discovered that 80% of children have antibodies to the virus by ten years of age. Also there is a possibility that some of the children were immunocompromised (were HIV positive) since there was no screening for HIV virus before their samples were taken and this may be responsible for the high seropositivity as no HIV test was done for the children before their blood sample was collected.

Parainfluenza virus infections are primarily childhood diseases, the highest age-specific attack rates for croup occur in children below the age of 3 years. Type 3 infections occur earliest and most frequently, so that about 50% of children are infected during the first year of life and almost all by 6 years, as determined by seroepidemiological studies. Antibodies against types 1 and 2 are acquired less rapidly but 80% of children have antibodies by 10 years of age.

The study also revealed that there was increase in seropositivity with age. This could be as a result of reinfection of older children that occurs in the presence of antibodies elicited by an earlier infection. Since there is no permanent immunity to the infection those antibodies modify the disease, as such reinfection usually present simply as nonfebrile upper respiratory infection. This agrees with the findings of Brooks *et al*., (2007) as well as that of Sale *et al*., (2010). Infection with the human paramyxoviruses such as the HPIVs can occur throughout life Hall (2001); however unlike primary infection in the very young, subsequent infections are often milder or subclinical Hall (2001). The mechanism behind the ability of these viruses to reinfect has been attributed to the incomplete and waning immunity that develops after primary infection with specific emphasis being placed on the serum neutralizing antibody and mucosal IgA levels (Okamoto *et al*., 2010; Kawasaki *et al*., 2004).

In terms of the occupation of parents of the children in relation to human parainfluenza virus infection, it was discovered that there was significant association ($p = 0.039$) between parents' occupation and the infection. Children whose parents were teachers had the highest infection rate of 84.1% followed by those whose mothers were fulltime housewives (81.1%), 76.9% of civil servants' children were infected while children of self-employed parents had the least infection (70.9%). This may possibly be explained by the fact that some of these teachers may have come in contact with other children in their schools who could have been

infected and so passed it on to their children at home since HPIV infection is transmitted via contact and air particles (Burke *et al.*, 2013).

The test of association of parents' level of formal education with the infection showed there was no significant association. Children of parents with primary education had the highest rate of infection; those with tertiary level of education, their children had 78.9% infection rate and the least (70.9%) was observed amongst children of parents with secondary level of education. The parents with the primary education as the highest educational status are more likely not to be informed and mindful of methods of controlling the transmission of the virus such as the use of disinfectant, proper hygienic practices and environmental control of short-range transmission. This agrees with the findings of Rudan *et al*., 2008 who stated that Mother's education is a possible risk factor of the pneumonia an infection caused by Parainflenza virus 3 specifically.

There was no significant association between the number of meals eaten daily with Human Parainfluenza Virus infection. The daily eating pattern revealed that children that ate once a day had the least rate of infection while those that ate twice daily had 80.0%. The sample size was limited for children that ate once a day than for the others. Also, the other risk factors such as contact with infected persons and fomites, (Burke., 2013) may have been more common among the older children that made them more susceptible to the infection.

There was no significant association between sources of vitamin A and human parainfluenza virus infection in children. Household size was not significantly associated. Households with 7-12 persons had higher (77.0%) infection rate compared to those with 1-6 persons where the infection rate was 75.9%. This finding suggests that overcrowding which results in indoor air pollution and eventually person to person contact is a risk factor for contacting the HPIV infection. This finding agrees with that of Rudan *et al*., (2008).

Duration of breastfeeding and the infection was statistically significant. Children who were breastfed for 1-6 months had 41.7% infection rate followed by those breastfed for 7-12 months (77.6%) and 77.8% for those that were breastfed for more than 1 year. This could be due to the fact that a larger number of the children involved in the study were breast fed for 1-2years than for 6-12 months and 1-6 months. Also some of the children were already above 1-2years so they may have been more exposed to other means of transmission of the virus. The action of maternal antibodies in stronger in children for the first six months of life than when they get older (Schomacker., 2012).

Children of parents that smoke had higher infection with human parainfluenza virus than those of non-smoking parents though smoking was not significantly associated with the infection. In like manner, environmental smoke was not significantly associated with the infection and this is consonance with the finding of Johnson *et al*., (2008). This is in contrast with the findings of Laurichesses *et al.,* (1999) who identified environmental smoke as a predisposing factor to the infection in England and Wales. This contrast may also be due to the fact that there is a larger number of smoking parents in such a place than in Nigeria where smoking is not so socially acceptable.

The results showed that of the 29 subjects tested to have sickle cell disease, seropositivity was observed in 26 (89.7%) but was not statistically significant. This could be due to the fact that there was a limited number of sickle cell individuals involved in the study and this finding agrees with that of Sale *et al*., (2010).

Fever and underlying diseases showed no significant association with the infection in children. This is in contrast with the findings of Laurichesses *et al*., (1999) and Rudan *et al*., (2008) who identified concomitant diseases(underlying diseases) as a predisposing factor to the infection in England and Wales. This could be due to the fact that there were a limited

number of children with concomitant diseases involved in the study. Also fever and concommitant diseses such as Diarrhoea, Heart disease etc could have been caused by other microbial agents than Human parainfluenza virus.

Out of the 319 children with respiratory symptoms, 251 (78.7%) had human parainfluenza virus infection while 37(64.9%) of those without respiratory symptoms did not have the infection. Significant association was observed. Parainfluenza infection is a respiratory tract infection and as such patients should show respiratory symptoms such as cold, cough and catarrh. Early infections however may not show such symptoms. Also a large proportion of the children studied had respiratory symptoms due to the season when the sampling was done i.e the Harmattan season which resulted to the high seropositivity. This result however, is in contrast to the findings of Sale *et al*., (2010) whose findings showed that respiratory symptom is not a statistically significant to HPIV infection. The result agrees with the findings of Brooks *et al*., (2007).

Ten of the samples indicated positive to HPIV virus implying that the other twenty must have had other microbial agents. This result is in contrast with previous literature (Chanock *et al*., 1985, Henrickson., 2003) that stated that Parainfluenza viruses grew poorly in embryonated eggs. The haemagglutination procedure is a phenomenon whereby erythrocytes of a variety of species (sheep red blood cells in this case) can be agglutinated through the agency of microorganisms or their products. The erythrocyte merely serves as an indicator of an antigen-antibody reaction.

The antibody level specific for parainfluenza virus antigen isolated and used was 1:64 i.e. 64HAI units/ml of blood and was found in one sample only. HPIVs have antigenic variation so it could be that the viral antigen used for HAI was not specific for the antibodies in the other fifteen (15) highly positive samples used. The serum may also have been more

immunogenic than the fifteen other sera samples and so was easily detected by HAI. The result also suggests that ELISA is a more sensitive method of diagnosis than Haemaggglutination Inhibition.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

From the study, the following conclusions were made:

1. Observation from the study showed the importance of Human Parainfluenza Virus 1, 2 and 3 as an agent of respiratory tract infections in children.
2. Age, Parental occupation, duration of breastfeeding and presence of respiratory symptoms are important demographic and risk factors of Human Parainfluenza Infection in children.
3. The Seroprevalence of Human Parainfluenza virus among children (1-12years) in Kaduna Metropolis is 76.6%.
4. HPIVs can be cultured successfully in embryonated eggs. ELISA in this study is a more sensitive method of diagnosis of HPIV than Haemagglutination Inhibition Method.

6.2 RECOMMENDATIONS.

1. Public health education and enlightenment programs which emphasize on the importance and need to promote key hygienic practices such as thorough hand washing and surface hygiene. To do this it is necessary to gain acceptance that the home and the school community settings will always contain potentially harmful microbes and that good hygiene is not about eradication but about targeting the correct measures at the times that matter. In order to reduce infection risks though at the same time, it is essential to ensure that improvements to domestic hygiene do not produce secondary problems such as chemical pollution and antimicrobial resistance.

2. Eating of balanced diet is recommended for children as it will boost their immunity to infections such as HPIV.
3. Parents especially the teachers are encouraged to keep a close watch on their children at home and around the home especially after playing with other children (as person-person transmission of HPIV is possible). In cases where the services of house helps are employed, such should be properly enlightened.
4. As this infection is at its peak in the Harmattan Season, control measures should be adhered to especially at this season. Children should be properly protected as well at such periods. They should also be properly attended to should they begin to show symptoms such as cold, cough and catarrh.
5. Parents are discouraged from smoking as it is not detrimental to their health only but to that of their children.
6. Mothers are encouraged to breastfeed their children for six months exclusively and even up to two years and should pay heed to other important factors such as eating of balanced diet and hygienic practices as breastfeeding alone cannot guarantee the total wellbeing of the child.
7. This study suggests the need for rapid, easy and less expensive methods of diagnosis for clinical management and availability of vaccines. A better understanding of HPIV pathogenesis will aid the design and evaluation of live attenuated HPIV vaccines and therapeutic drugs.
8. Fever is a common symptom of HPIV infection and as such should not just be regarded and treated as Malaria or Typhoid Fever which is a very common practice in our hospitals.
9. Viral cultivation of HPIV in cell culture (embryonated eggs) is possible and can be included in the methodology of the study of other paramyxoviruses when necessary.

10. Efforts should be aimed at decreasing nosocomial infection. Hand hygiene should be emphasized. Simple hygienic measures such as washing of hands, regular disinfection of fomites and avoidance of overcrowding as well as healthy eating habits are good preventive measures.

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APPENDICES

I: Structured Questionnaire

Section A: BIODATA

Serial number: .....

Age: .....

Occupation of Parent: .....

Highest Educational Level of Parent: Primary ( ) Secondary ( ) Tertiary ( )

Section B: Epidemiological Risk Factors

1. How many times does your child eat in a day? Once { } Twice { } Three times { }
2. How many people live in your household? 1-6{ } 6-12{ }
3. Is Vitamin A (e.g. carrots and palm oil) a regular part of your child's diet? Yes { } No { }
4. How long did you breastfeed your child? 0-6 months { } 6-12 months { } 1-2years{}
5. Do you smoke? Yes { } No { }
6. Do you have history of sickle cell in your family? Yes { } No { }
7. Do you frequently use firewood for cooking in your house or live in an industrialized or a very busy area? Yes { } No { }
8. Does your child have cough, cold or catarrh? Yes { } No { }
9. Does your child have fever? Yes ( ) No ( )
10. Does your child have history of Heart disease, Yes { } No { }
    Lung disease Yes { } No { }
    Blood disease Yes { } No { }
    Asthma Yes { } No { }
    Diarrhoea Yes { } No { }

II: Haemaglutination titres of the virus present in the throat swabs.

Sample No	Titres											
	1:2	**1:4**	**1:8**	**1:16**	**1:32**	**1:64**	**1:128**	**1:256**	**1:512**	**1:1024**	**1:2048**	**1:4096**
01	+	+	+	+	+	+	+	+*	-	-	-	-
02	-	-	-	-	-	-	-	-	-	-	-	-
03	+	+	+	+	+	+	+*	-	-	-	-	-
04	-	-	-	-	-	-	-	-	-	-	-	-
05	+	+	+	+	+	+	+	+*	-	-	-	-
06	+	+	+	+*	-	-	-	-	-	-	-	-
07	+	+	+	+*	-	-	-	-	-	-	-	-
08	+	+	+	+	+	+	+*	-	-	-	-	-
09	-	-	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-	-	-	-	-	-
12	+	+	+	+	+	+	+*	-	-	-	-	-
13	-	-	-	-	-	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-	-	-	-	-	-
15	+	+	+	+	+*	-	-	-	-	-	-	-
16	-	-	-	-	-	-	-	-	-	-	-	-
17	-	-	-	-	-	-	-	-	-	-	-	-
18	-	-	-	-	-	-	-	-	-	-	-	-
19	-	-	-	-	-	-	-	-	-	-	-	-
20	-	-	-	-	-	-	-	-	-	-	-	-
21	-	-	-	-	-	-	-	-	-	-	-	-
22	+	+	+	+	+	+*	-	-	-	-	-	-
23	+	+	+	+	+	+*	-	-	-	-	-	-
24	-	-	-	-	-	-	-	-	-	-	-	-
25	-	-	-	-	-	-	-	-	-	-	-	-
26	-	-	-	-	-	-	-	-	-	-	-	-
27	-	-	-	-	-	-	-	-	-	-	-	-
28	-	-	-	-	-	-	-	-	-	-	-	-
29	-	-	-	-	-	-	-	-	-	-	-	-
30	-	-	-	-	-	-	-	-	-	-	-	-

Key: +*: Haemaglutination titre +: agglutination -: no agglutination

III: Haemaglutination Inhibition values of the highly positive sera samples. Quantified antibody titre level to human parainfluenza virus using haemagglutination inhibition.

Serum number	**Dilution titres**											
	1:2	**1:4**	**1:8**	**1:16**	**1:32**	**1:64**	**1:128**	**1:256**	**1:512**	**1:1024**	**1:2048**	**1:4096**
01	-	-	-	-	-	-	-	-	-	-	-	-
02	-	-	-	-	-	-	-	-	-	-	-	-
03	-	-	-	-	-	-	-	-	-	-	-	-
04	-	-	-	-	-	-	-	-	-	-	-	-
05	-	-	-	-	-	-	-	-	-	-	-	-
06	+	+	+	+	+	+*	-	-	-	-	-	-
07	+	+	-	-	-	-	-	-	-	-	-	-
08	+	+	+	+	-	-	-	-	-	-	-	-
09	+	+	+	+	-	-	-	-	-	-	-	-
10	+	+	+	+	-	-	-	-	-	-	-	-
11	+	+	-	-	-	-	-	-	-	-	-	-
12	+	+	+	+	-	-	-	-	-	-	-	-
13	-	+	-	-	-	-	-	-	-	-	-	-
14	+	+	+	+	-	-	-	-	-	-	-	-
15	_	-	-	-	-	-	-	-	-	-	-	-
16	+	+	-	-	-	-	-	-	-	-	-	-

+: no agglutination -: agglutination +*:Haemagglutination Inhibition value

IV: Respondent's Consent Form

I understand that I have been asked by Kaduna State Ministry of Health, Kaduna State alongside the Department of Microbiology, Ahmadu Bello University, Zaria to participate in a research project designated to know the Sero-prevalence of Parainfluenza Virus in children with Respiratory Tract Infections in Kaduna Metropolis.

I understand that during this study, I will be asked a permission to take my child's blood sample and asked some health related questions about my family and child and that my responses will be in a questionnaire form. I also understand that my participation in the study will be kept confidential; my identity will be revealed only to those performing or supervising the research. I understand that I would never be identified by name in any publication resulting from this study. I realize that I will not benefit directly from this study. However, with my participation, I hope to help the investigators to understand sero-prevalence of Parainfluenza virus in children with Respiratory Tract infections.

I have read this consent form or it has been read/explained to me. I have had the opportunity to ask questions about the research and my questions have been answered to my satisfaction.

I hereby consent voluntarily to participate in this study. I understand that I am free to withdraw my consent or to discontinue participation in this research project at any time without it affecting me.

Respondent:

Name:_____

Date:_____

Signature/Thumb print:_____

Interviewer

Name:_____

Date:_____

V: Ethical Approval

MINISTRY OF HEALTH, KADUNA STATE

All Communications to be Addressed to:
THE HON. COMMISSIONER
Quoting Reference and Date
Tel: (062) 248084
(062) 248252

Independence Way,
P.M.B. 2014,
Kaduna.
Kaduna State, Nigeria

MOH/ADM/744/VOL.1/208 **14th December, 2012.**

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ETHICAL APPROVAL

I have been directed to convey the Ministry's approval to Folashade Sarah Bonire, an M.Sc. student of Microbiology Department, ABU – Zaria, Kaduna State.

Topic: **SERO - PREVALENCE OF PARA INFLUENZA IN CHILDREN IN SOME SELECTED FACILITIES IN KADUNA.**

It is expected that necessary assistance be accorded to her in the process of her research, please.

However, it is mandatory for the researcher to submit a copy of her research finding(s) to the office of the Honorable Commissioner, Health as soon as it is concluded as you have the assurances of my highest regards.

14/12/12.

JACOB KAYIT MUSA
SECRETARY, ETHICAL COMMITTEE