STUDY ON THE EPIDEMIOLOGY AND CLINICAL PICTURE OF HUMAN PARAINFLUENZA VIRUS IN CHENNAI. STANDARDIZATION OF RAPID DIAGNOSTIC TOOL AND TO STUDY THE EFFECT OF HEMAGGLUTININ NEURAMINIDASE INHIBITORS ON THE ISOLATES

A thesis submitted to

The Tamil Nadu Dr.M.G.R. Medical University

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CERTIFICATE

This is to certify that the thesis entitled **"STUDY ON THE EPIDEMIOLOGY AND CLINICAL PICTURE OF HUMAN PARAINFLUENZA VIRUS IN CHENNAI STANDARDIZATION OF RAPID DIAGNOSTIC TOOL AND TO STUDY THE EFFECT OF HEMAGGLUTININ NEURAMINIDASE INHIBITORS ON THE ISOLATES"** is based on the results of the work carried out by C.P.Indumathi for Ph.D degree under my supervision and guidance. This work has not been submitted to any degree or diploma of any other university.

Date: 30.03.2016

Place: Chennai.

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DECLARATION

I hereby declare that the thesis entitled **"STUDY ON THE EPIDEMIOLOGY AND CLINICAL PICTURE OF HUMAN PARAINFLUENZA VIRUS IN CHENNAI. STANDARDIZATION OF RAPID DIAGNOSTIC TOOL AND TO STUDY THE EFFECT OF HEMAGGLUTININ NEURAMINIDASE INHIBITORS ON THE ISOLATES"** submitted to the Tamil Nadu Dr. M.G.R. Medical University for the award of the degree of Doctor of Philosophy is the original and independent work carried out by me in the Department of Virology, King Institute of Preventive Medicine & Research, Guindy, Chennai-32.

C.P. Indumathi

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Date: 30.3.16

Place: CHENNAI

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C.P. INDUMATHI

Dedicated to

my beloved

Parents, Husband

Research Guide

&

Friends…..

CONTENTS

ABBREVIATIONS

µg	-	Microgram
µl	-	Microlitre
µM	-	Micromolar
Ab	-	Antibody
Ag	-	Antigen
ARTI	-	Acute respiratory tract infection
BP	-	Base pair
BSA	-	Bovine serum albumin
CDC	-	Centre for Disease Control
CPE	-	Cytopathic effect
DNA	-	Deoxyribonucleic acid
EDTA	-	Ethylene Diamine Tetra Acetic acid
F gene	-	Fusion gene
FCS	-	Fetal calf serum
FITC	-	Fluorescein Isothiocyanate
HAD	-	Hemadsorption
HAI	-	Hemadsorption inhibition
HN gene	-	Hemagglutinin Neuraminidase gene
HPIV-1	-	Human parainfluenza virus 1
HPIV-2	-	Human parainfluenza virus 2
HPIV-3	-	Human parainfluenza virus 3
HPIV-4	-	Human parainfluenza virus 4
HTS	-	High throughput screening
IFA	-	Immunofluorescence assay
IL - 1	-	Interleukin - 1
ILI	-	Influenza like illness

IP	-	Inpatient
IU	-	International Unit
KDa	-	Kilo dalton
LRI	-	Lower respiratory infection
M	-	Membrane protein
m.o.i.	-	Multiplicity of infection
Mab	-	Monoclonal antibody
MEGA	-	Molecular Evolutionary Genetics Analysis
MEM	-	Minimum essential medium
mL	-	Millilitre
mM	-	Millimolar
mRT-PCR	-	Multiplex reverse transcription polymerase chain
MTT	-	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NCBI	-	National Centre for Biotechnology Information
NIV	-	National Institute of Virology
nm	-	Nanometer
NP	-	Nucleocapsid
NR	-	Neutral red
nt	-	Nucleotide
OD	-	Optical Density
OPD	-	Out patient department
PBS	-	Phosphate buffered saline
PDB	-	Protein data bank
PFU	-	Plaque forming units
PIV	-	Parainfluenza virus
RBC	-	Red blood cells
RBV	-	Ribavirin

RCSB	-	Research collaborate structural biology
RFU	-	Relative fluorescence units
RNA	-	Ribonucleic acid
RPM	-	Revolution per minute
RT-PCR	-	Reverse transcription - polymerase chain reaction
SARI	-	Severe acute respiratory tract infection
SD	-	Standard Deviation
SeV	-	Sendai virus
TAE	-	Tris Acetate EDTA
TBE	-	Tris Borate EDTA
TCID	-	Tissue culture infective dose
TPVG	-	Trypsin Phosphate Versine Glucose
URI	-	Upper respiratory infection
WHO	-	World Health Organization

Introduction

INTRODUCTION

1.1 HUMAN PARAINFLUENZA VIRUS

Human parainfluenza viruses are the viruses that cause human parainfluenza. They are group of four distinct serotypes of enveloped single–stranded RNA viruses belonging to the paramyxovirus family[1,2]. Human parainfluenza viruses are important pathogens associated with mild upper respiratory tract illness in older children and adults, but in infants and young children they are major cause of morbidity, producing lower respiratory tract illness such as croup, bronchiolitis and pneumonia[3,4,5]. The Paramyxovirus family includes numerous viruses that are of importance to animal and human health[6]. It has been estimated to account for 40% of acute lower respiratory tract illnesses in childhood from which a virus is recoverable[7] and only pediatric respiratory illnesses were 20% hospitalized[8]. HPIVs are ubiquitous, as shown by their isolation in many parts of the world with different climates[9,10].

Human parainfluenza viruses (HPIVs) types 1, 2, and 3, cause the majority of childhood cases of croup, bronchiolitis and pneumonia worldwide[11]. HPIV-3 alone is responsible for approximately 11% of pediatric respiratory hospitalizations in US[12,13] and is the predominant cause of croup in young infants, while HPIV-1 and 2 tend to infect older children and adolescents. An estimated five million lower respiratory tract infection (LRI) occur each year in the United States in children under 5 years old, and HPIVs have been isolated in up to one third of these infections[14] and community acquired pneumonia was 0.026 episodes/child-year, resulting in 2 million new cases in children less than 5 years of age[15]. Acute respiratory infections (ARI) are the main cause of childhood hospitalization and death worldwide, particularly in industrializing countries. In 2000, global estimates of child death showed that 1.9 million children died from ARI and 70% of those occurred in industrializing countries[16].

HPIVs are most classically associated with croup but can also cause a wide spectrum of respiratory illness.There is less information about their role in upper

respiratory tract illness and associated otitis media[17]. Primary infection with HPIV often causes serious illness in children, while subsequent infections, although frequent, are usually less severe[18]. HPIV-3 has been implicated as a cause of severe disease in the immunocompromised patient[19,20] and rare cases in patients with meningitis[21].

Infection due to HPIV-1, 2 and 3 are second only to Respiratory syncytial virus (RSV) as a cause of hospitalization (2-17%) for acute respiratory infection among children aged younger than 5 years in the United States[1,22-24]. One estimate suggested that pneumonia accounted for 19% of the 10.6 million yearly deaths in children younger than 5 years in 2000 – 2003, and was the leading cause of childhood mortality among this age group globally[25]. Human parainfluenza viruses (HPIVs) are not only a common causative agent of ARI among infants and young children, but these viruses are also associated with nosocomial acute respiratory illness in the immunocompromised, hematopoietic stem cell transplant patients[26-28].

History

Human parainfluenza viruses (HPIV) were first discovered in the late 1950s. Over the last decade, considerable knowledge about their molecular structure and function has been accumulated. This has led to significant changes in both the nomenclature and taxonomic relationships of these viruses[29].

When three different viruses recovered from children with lower respiratory disease proved to be unique and easily separated from the myxoviruses (Influenza virus), they closely resembled. 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”[29].

Isolated and identified over 25 years ago, the Human parainfluenza viruses are subdivided into four major serotypes like serotype l, 2, 3 and 4[30-32]. Homotypic re-infection occurs, most commonly with type 3, slightly less often with type 1, and rarely with type 2[33]. All serotypes are ubiquitous, and infections are reported at all

times of the year. In addition, there are epidemics such that in the U.K. epidemics of types 1 and 2 occur predominantly in the winter, while epidemics of type 3 have a distinct summer peak.

The first parainfluenza virus type 1 was discovered in 1952, in Japan Sendai virus. PIV type was isolated from children with acute laryngotracheobronchitis (croup) in 1955. HPIV type 3 was isolated from children with respiratory tract infection in 1960. Type 4 was isolated from children with mild respiratory tract infection in 1965[34].

Global Scenerio

HPIV are common community-acquired respiratory pathogens without ethnic, socioeconomic, gender, age, or geographic boundaries. Many factors have been found that predispose to these infections, including malnutrition, overcrowding, vitamin A deficiency, lack of breast feeding and environmental smoke or toxins[35-39].

HPIV-1 to HPIV-3 has been found in many as one-third of LRI infections[40]. In addition, HPIV cause upper respiratory infection (URI) in infants, children and adults to a lesser extent, LRI in the immunocompromised, those with chronic diseases (e.g., heart and lung disease and asthma) and the elderly[40-46]. HPIV-2 has been reported to cause infections biennially with HPIV-1 or alternate years with HPIV-1 or to cause yearly outbreaks[47,48].

HPIV-2 activity occurred 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 nonimmunocompromised 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 3. This may be due to difficulties in isolation and detection[29].

As many as 6,000 children younger than 18 years may be hospitalized each year in the United States because of HPIV-2. 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 over shadowed 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[29].

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[42,43,49,50].

The epidemiologic features and clinical manifestations of HPIVs and other common respiratory pathogens in children and adults with acute respiratory tract illness (ARTI) in Guangzhou, Southern China, which might help to establish clinical distinctions between different HPIV types. HPIV were identified 3.7% positives[51].

In England and Wales, HPIV-3 causes yearly epidemics in the summer[52]. In the Northeast of England, a hospital-based study of the epidemiology of respiratory infections among pediatric inpatients over a six-year period showed that HPIV- 1 and HPIV-2 epidemics occurred together, but only every 2 years[53]. In tropical countries, there have been fewer studies of HPIV and most of them do not demonstrate a seasonal pattern of HPIV infections[54,55].

HPIV-3 infection occurs world-wide in the United Kingdom, follows an epidemic pattern with peak incidence occurring in the summer months in 21-32 weeks[52]. Many sporadic cases of infection are observed between the peak periods and these may account for the earlier long-held view that HPIV-3 was endemic[52].

Indian scenario

Prevalence of Human parainfluenza virus was observed in Lucknow (India) during the period of September 1986 to June 1988. Sporadic cases of parainfluenza virus type 1 were identified 2.5% and parainfluenza virus type 3 were identified 3.2%[56]. Respiratory viral infections detected by multiplex PCR among pediatric

patients with lower respiratory tract infections seen at an urban hospital in Delhi from 2005 to 2007. Multiplex PCR detected respiratory viruses which showed 22 cases for HPIV-3 and 17 for HPIV-2, 10 for HPIV-1[57].

The spectrum of respiratory viruses circulating in Eastern India prospective surveillance among pediatric patients with Influenza-like illness during 2010-2011. Nasal swab, throat swab were tested for HPIV1-4 by conventional RT-PCR. The study which showed 0.3% for HPIV-1, 6% for HPIV-2 and 1% for HPIV-3[58].

Throat swabs were collected from one child aged under 5 years in each of 164 families in 4 villages in Bengal over two years. Of the 4171 clinical swabs, 11% tested positive for parainfluenza virus, 32% for Adenovirus and 2.5% for RSV. Parainfluenza viruses were twice as common and adenovirus were four times as common in India[59].

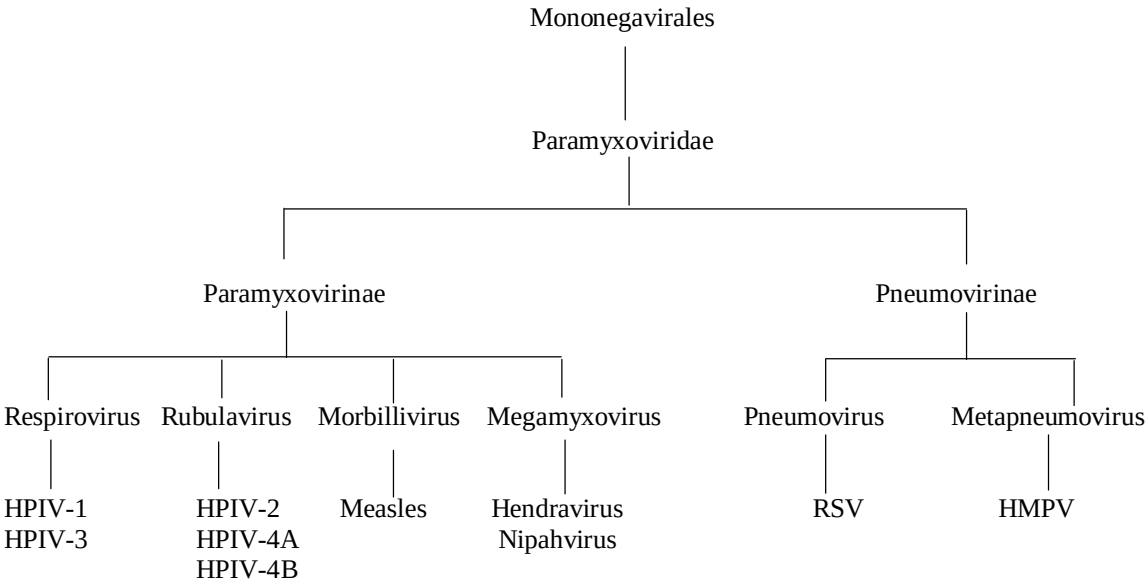
Interactions with the environment

Environmental conditions such as temperature, humidity, pH, and the composition of the storage fluid easily affect HPIV. Viral survival markedly decreases at temperature above 37 ºC, until at 50 ºC almost all virus is inactivated within 15 minutes[60]. Room temperature storage of myxoviruses has demonstrated considerable survival variability by decreasing titers by >50% in as little as 2 hours or as long as 1 week, but this rate averages closer to hours than days[61-64].

HPIV have their greatest stability at 4 ºC or if frozen (e.g., -70 ºC). Even though freezing causes loss of infectivity and virus destruction (>90% at times), the small amount of infectivity that is left must be sufficient for virus recovery[60,65]. HPIV-1 can be recovered even after 26 years of being frozen (-70 ºC), with a recovery rate greater then 90%[29].

The addition of several reagents (e.g., 0.5% bovine serum albumin, skim milk, 5% dimethyl sulfoxide, or 2% chicken serum) to HPIV prior to freezing has been shown to prolong survival [60, 66,67]. In addition, optimal stability of HPIV occurs at physiologic pH (7.4 to 8.0), while infectivity is rapidly lost at pH 3.0 to 3.4[63,68], under low humidity[69], and with virus desiccation[70,71]. HPIV and all myxoviruses are inactivated by ether[72].

Classification of Human parainfluenza virus



All HPIV are members of *Paramyxovirinae* subfamily in the large *Paramyxoviridae* family and classified into different genera. Thus animal and human PIV1and PIV3 belong to respirovirus (former Paramyxovirus) genus whereas PIV2 and PIV 4 are members of the *Rubelavirus* genus. The human pathogen mumps virus is also a members of the *Rubela virus* genus. These two genera together with the *Megamyxovirus* genus (to which the recently identified Hendra and Nipahviruses belong) and *Morbillivirus* genus (which includes measles virus) form part of *Paramyxovirinae* subfamily. The *Pneumovirinae* subfamily contains *Pneumovirus* genus the most important member being respiratory syncytial virus (RSV) as well as *Metapneumovirus* genus, to which metapneumovirus (hmpv) belong[73].

Paramyxoviruses, particularly respiratory syncytial virus (RSV), human metapneumovirus, and the human parainfluenza viruses (HPIVs), cause the majority of childhood croup, bronchiolitis, and pneumonia[74]. In adults, these viruses cause about two-thirds of respiratory illnesses, with high mortality in immunocompromised persons[75]. HPIV-3 accounts for 90% of the respiratory illnesses in hematopoietic stem cell transplant patients[76]and carries high mortality[76-81].

The HPIV groups are traditionally given a high place in the pantheon of respiratory viruses as a cause of lower respiratory tract illness and hospitalization. All three types cause croup, a life-threatening infection due to respiratory embarrassment[82]. HPIV-3 is an important pathogen in very young children. It is second only to RSV as a cause of serious lower respiratory need to time paired sera around each respiratory tract disease in infants[7]. The use of corticosteroids and nebulized epinephrine to treat croup requiring urgent medical care has decreased croup related hospitalization significantly and also explains a reported decrease in the contribution of HPIV-1 to overall HPIV-attributable hospitalization[83-85].

There are no effective vaccines or treatments for the HPIVs. Remarkably, while strategies of passive immunoprophylaxis for RSV protect infants at greatest risk[86] and effective antiviral drugs and vaccines are available for influenza[87,88] there are no vaccines or drugs for the HPIVs[74,89].

The study provides useful information on the prevalence, clinical profiles, and epidemiology of specific viral aetiology in patients with ARTI attending Government hospitals in Chennai, and will contribute to the diagnosis, treatment, and prevention of ARI in patients. Our findings address the impact of routine infections with these viruses and how one might assess the impact of prevention of HPIV illness. The present study, aim to screen and detect the prevalence of human parainfluenza virus in different age groups by PCR, epidemiology and its clinical characteristics. Till date, there is no report on detection of HPIV in Tamilnadu (Chennai).

In addition, HPIV surveillance to the entire range of acute respiratory tract infections so that indication based public health actions can be planned and carried out. This review is based on a thorough search of the literature. Earlier investigators occasionally used a cordless of tests that would comprise viruses causing acute respiratory tract infections. Not only did the studies lack stability, they also differed from one another in respect to the viral diagnostic methods employed, and the range of etiologies for which diagnostic tests were comprised. For instance, investigators exploring lower and upper respiratory tract infections caused by Human parainfluenza virus were also reported from the same region in the past.

Aim and Objective

AIM AND OBJECTIVE

AIM

1. Surveillance of Human parainfluenza viruses in Chennai.
2. Evaluate acute respiratory tract infections, URI, LRI patients for Human Parainfluenza virus etiology by Multiplex RT-PCR.
3. Genetic characterization of HPIV detected in Chennai.
4. Sequencing and phylogenetic analysis of circulating strains.
5. To isolate HPIV using LLC-MK2, A549 and MDCK cell lines.
6. Antiviral activity of Hemagglutinin Neuraminidase inhibitor (4-GU-DANA) and Nucleoside inhibitor (Ribavirin) against Human parainfluenza virus determined in LLC-MK2 and A549 cells.
7. Standardization of hemadsorption inhibition assay, plaque inhibition assay for HPIV and neuraminidase inhibition assay to inhibit the growth of HPIV.
8. Screened and antiviral activity of Licorice against HPIV.
9. Docking study of Zanamivir with receptors.

OBJECTIVE

Human parainfluenza viruses (HPIVs) are medically important respiratory pathogens and are second only to respiratory syncytial virus (RSV) as a major cause of lower respiratory tract (LRT) illness in infants and young children.Prevalence study would be the first of its kind to report HPIV occurrence in Chennai as not many studies have been done. Prevalence study will help to identify the circulating HPIV serotypes in Chennai population.

Mortality induced by HPIV is unusual in developed countries and is seen almost entirely in young infants, the immunocompromised and the elderly. The preschool population in developing countries has considerable risk of HPIV induced death. To address these problems Multiplex reverse transcription (RT-PCR) assay

can be a sensitive, specific tool for the rapid and early diagnosis of HPIV infections and for simultaneous detection of HPIV serotype.

Genetic characterization of HPIV serotypes is important to know the mechanism resulting in genetic diversity of HPIV and for controlling the pathogen. Phylogenetic analysis would confirm the similarity of HPIV strains and the sequence data may help in understanding virus diversity and evolution. This is to elucidate the genetic characteristics and phylogeny of Chennai strains, the hemagglutinin neuraminidase gene and nucleocapsid gene sequence of selected strains to analyse and to compare the other strains from different countries worldwide

Viral isolation was tried in the three cell lines, LLC-MK2, A549 and MDCK cell cultures, to identify the most effective cell line for the recovery of these viruses from clinical specimen.Virus infection of cultured cells (LLC-MK2 and A549) can be monitored by hemadsorption assay and quantitated by plaque assay.

Three methods were used to detect the cell viability in vitro by (i) cell proliferation assay (MTT assay) (ii) increase in neutral red (NR) dye uptake into cells (iii) trypan blue exclusion method

The antiviral activity of 4-GU-DANA (HN Inhibitor) and Ribavirin (Nucleoside inhibitor) against Human Parainfluenza virus detected and to elicit IC_{50} by hemadsorption inhibition assay, plaque inhibition assay and neuraminidase inhibition assay.

Review of Literature

REVIEW OF LITERATURE

Viruses are obligate parasites that can infect all living organisms. Composed of either DNA or RNA, these subcellular organisms have no metabolic activity outside the host cell and so are totally dependent on these cells for life[90].

3.1 INFLUENZA-LIKE ILLNESS

Influenza-like illness (ILI), a nonspecific respiratory illness defined as fever >38 ºC with cough and/or pharyngitis. The diagnosis of person with a 12-36 hours onset of painful retro-sternal cough, fever, and fatigue or malaise during a time when influenza viruses are known to be circulating, the same symptoms may be due to other viruses at other times. In addition to influenza, viruses known to cause ILI include respiratory syncytial virus, rhinovirus, adenovirus, parainfluenza viruses, human coronaviruses (including the virus that causes severe acute respiratory syndrome) and the human metapneumovirus. Many viruses are known to cause clinical illness that is difficult to distinguish from influenza. Among the most common of these are respiratory syncytial virus (RSV) and picornaviruses (small RNA viruses that include enteroviruses and rhinoviruses). Rhinoviruses are the most common of the picornaviruses to cause an ILI.

3.1.1 Structural Organisation

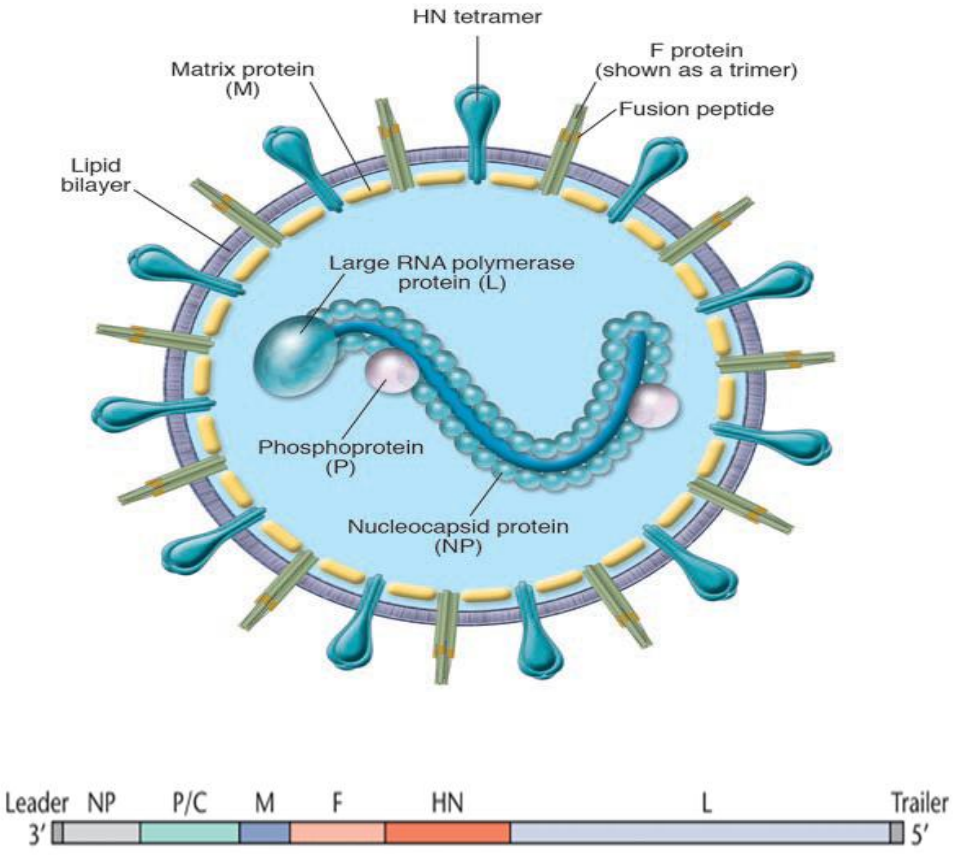
The Human Parainfluenza virus genome are enveloped, single stranded, nonsegmented negative sense RNA must be transcribed into message-sense RNA before it can be translated into protein. Like all negative-stranded RNA viruses the HPIV encode and package an RNA-dependent RNA polymerase in the virion particles[74].

Parainfluenza viruses are pleomorphic, spherical virions that range in average from 120-300 nm diameter with helical nucleocapsid 12-17 nm in diameter with a genome encompassing ~15,000 nucleotides. These are organized to encode at least six common structural proteins (3'N-P-C-MF-HN-L-5'). 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[91-93].

The virion consists of a nucleocapsid (also called the ribonucleoprotein or RNP) packaged in a lipid envelope that is derived from the host cell plasma membrane during budding. Nucleocapsid core is filamentous or herringbone-like, has helical RNA tightly associated with Nucleoprotein (NP) and also Phosphoprotein (P) and Large protein (L). The envelope bears spike like surface projections composed of the tetrameric HN or trimeric F transmembrane glycoprotein. The inner surface of the envelope is coated with the nonglycosylated matrix (M) protein[94] shown in the Figure 3.1.

Figure 3.1 Structure of Human parainfluenza virus



Hemagglutinin Neuraminidase gene

The hemagglutination activity of the HN protein mediates adsorption of virus to the host cell to initiate infection. The hemagglutinin neuraminidase (HN protein) (Molecular Weight 69,000 to 82,000 KDa). The HN proteins of HPIV are different from the receptor binding glycoproteins of other members of the paramyxovirus family in that they possess both hemagglutinating (sialic acid containing receptor-binding) and neuraminidase (sialic acid containing receptor cleaving) activities. The parainfluenza HN proteins are oriented such that their amino termini extend into cytoplasm, while C termini are extracellular. The HN protein is found on the lipid envelope of HPIV and infected cells there it most probably exists as a tetramer composed of disulfide-linked dimmers and functions in virus-host cell attachment via sialic acid receptors, suggesting that it has neuraminidase activity (important for virus release from cells)[74]. There are significant differences in the number of HN glycosylation sites between HPIV types and among strains within one type. 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 and 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. It involves both surface glycoproteins and varies between HPIV types. Fusion protein-mediated cell fusion is affected by the affinity of the HPIV-3 HN to its receptors[29].

Fusion gene

The viral envelope thought to fuse directly with the plasma membrane of the cell, mediated by the viral fusion protein (F protein) during viral infection releasing the nucleocapsid into the cytoplasm[74]. The molecule contains a cytoplasmic domain, a membrane spanning region, a stalk region, and a globular head contains the primary sialic acid-binding site and the neuraminidase active site. The molecular weight of fusion protein 60,000 to 66,000 KDa)[29]. F protein-mediated cell fusion is affected by the affinity of the HPIV-3 HN to its receptor(s) [95]. 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[96].

The 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. The enzymes responsible for this proteolytic cleavage in humans but trypsin are most frequently used in vitro[97].

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-1,2,3 in vitro require both HN and F for fusion and hemolysis[97-100]. 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[101-104].

Matrix gene

The membrane/matrix protein is located on the inner surface of HPIV envelope and is thought to play an important role in virion assembly. The membrane protein (M) (MW 28,000 to 40,000 KDa) 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., glcosylation or phosphorylation) and the MW as calculated from cloning and sequencing data can differ considerably from that seen on gel electrophoresis[29]. 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[105-108]. A mature Sendai virion contains approximately 3000 copies of the M protein[109].

Nucleocapsid gene

The virion consists of a filamentous, herringbone-like nucleocapsid core surrounded by a lipid envelope with virus specific glycoprotein spikes. The nucleocapsid is composed of the genome, a single-stranded RNA molecule of negative sense polarity, which is tightly coated with the nucleocapsid protein (NP). The nucleocapsid structure also contains two other proteins, the phosphoprotein (P protein) and the large protein (L protein), which occur discontinuously as clusters. The NP is the most abundant protein in the virion, and it is believed to be responsible, together with the P and L proteins, for RNA-dependent RNA polymerase activity[110]. Nucleocapsid proteins are important for transcription and replication of viral genome. The first gene at the 3' end of the genome is the NP gene, which is highly conserved among PIV[111-113].

The cellular receptors for the PIV are N-acetylneuraminic acid (sialic acid) in a terminal linkage to cellular glycoproteins and glycolipids. In the nucleocapsid, the viral genome is tightly bound along its entire length with the nucleoprotein N at a ratio of 1 protein molecule per six nucleotides (~2,500 to 2,700 protein molecules) and molecular weight 66,000 to 70,000 are closely associated with the viral RNA (vRNA)[29].

Large gene

The largest HPIV protein is the “large” (L) nucleocapsid protein (polymerase, MW 175,000 to 251,000 KDa). It also contains approximately 300 copies of the phosphoprotein P and approximately 40 copies of the major polymerase large (L) protein. Within the virion, the nucleocapsid is wound about itself however, when it is released by disruption of virus, it assumes a linear, flexible conformation that is approximately 1,000 nm in length. The nucleocapsid has an

RNA-dependent RNA polymerase that consists of the P and L proteins in association with N-bound genome[29].

Phosphoprotein gene

The phosphoprotein (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)[114-116]. HPIV-2 (and maybe HPIV-3) has an additional nonstructural protein (V), which HPIV-1 does not contain[114, 117,118]. This protein appears to have several functions like binds N and may play a role in regulating viral replication[119]. 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[120].

3.2 LIFE CYCLE OF HUMAN PARAINFLUENZA VIRUS

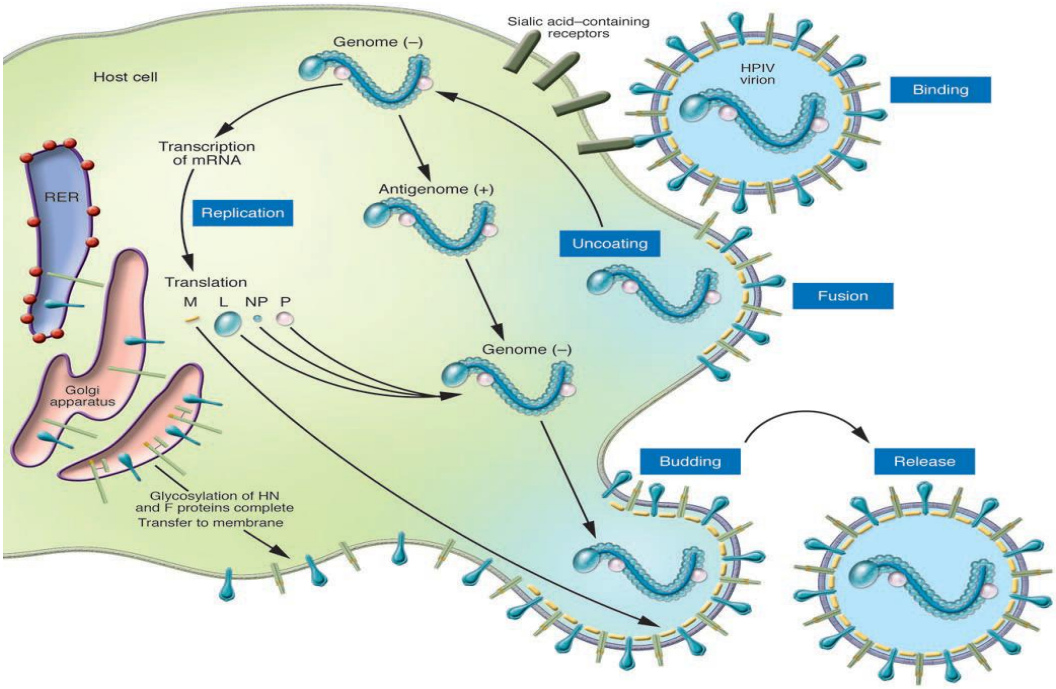
The first step in infection of a cell by all HPIVs is binding to the target cell, via interaction of the viral receptor-binding molecule hemagglutinin-neuraminidase (HN) with sialic acid–containing receptor molecules on the cell surface (Figure 3.2). The viral envelope then is thought to fuse directly with the plasma membrane of the cell, mediated by the viral fusion protein (F protein), releasing the nucleocapsid into the cytoplasm[121,122].

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. 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 the plasma membrane containing viral glycoproteins in order to set the scenario for budding shown in the Figure 3.2[123].

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[124,125].

Figure 3.2 Life cycle of Human parainfluenza virus



3.3 MODE OF TRANSMISSION

HPIV transmission has been specifically investigated in only a few studies. Air-sampling experiments have shown that HPIV-1 could be recovered from only 2 of 40 infected children at a distance of 60 cm[126]. 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[127]. It is thought that HPIV is transmitted by similar modalities. HPIV-1, 2 and 3 have all been shown to survive for up to 10 h on nonporous surfaces and 4 h on porous surfaces[71].

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[70]. Spread from an infected person to other people through the air by coughing and sneezing, close personal contact, such as touching or shaking hands, and touching objects or surfaces with the viruses on them touching the mouth, nose, or eyes. However, the amount of virus excreted from an acutely infected child may be more than 10 times greater then that tested[62]. HPIV can be efficiently removed from surfaces with most common detergents, disinfectants, or antiseptic agents.

3.3.1 Nosocomial infections

HPIV were spread to doctors, offices, hospitals, and chronic care facilities are institutions where respiratory viruses are frequently transmitted between patients. The populations at greatest risk for HPIV infection are young preschool children, the immunocompromised, and the elderly[128]. Children infected with HPIV-3 will transmit this virus to a minimum of 20% of uninfected control children residing on the same ward[129]. About one-third of these will develop mild respiratory symptoms, but some will experience serious LRI and even death[48,129]. Serious sequelae are most common in patients with underlying medical problems. The mean length of hospitalization was increased by many days, even for those with only mild symptoms, because of unnecessary tests and therapies due to their new signs and symptoms.

3.4 HPIV INFECT WITH HOST RANGE

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[66,130-132]. However, fatal disease is caused by infection with HPIV-1,2,3 in newborn ferrets[132]. HPIV-3 causes clinically asymptomatic infection in cotton rats and hamsters, but on autopsy significant respiratory pathology and virus replication can be demonstrated[133,134]. HPIV-3 induces hyper reactivity in the tracheas of guinea pigs and neonatal hydrocephalus in hamsters whose mothers were intravenously inoculated[135,136]. Primates are easily infected with HPIV, but almost all infections are asymptomatic. Chimpanzees, macaques, and squirrel, owl, and rhesus monkeys have been asymptomatically infected with HPIV-3 or HPIV-4[137-139], and only marmosets have developed symptomatic upper respiratory infections (URI) with HPIV-3 and Sendai virus [140].

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[141-143]. Simian viruses 5 and 41 are related to HPIV-2 and infect primates[144-146]. 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[147,148]. Bovine PIV-3 has been associated with “shipping fever” in cattle, and is antigenically related to HPIV-3[149]. This virus or similar viruses may also infect horses, sheep, goats, water buffaloes, deer, dogs, cats, monkeys, guinea pigs, rats, and pigs[134,150,151].

Some PIV can infect nonmammalian species. A Rubella virus, Newcastle disease virus, infects poultry, penguins, and other birds and has been responsible for conjunctivitis in bird handlers and laboratory workers[152-154]. There have been reports of human infections by some of the other nonhuman PIV, but these have not been well established[146,149,155,156].

3.5 CLINICAL FEATURE

HPIV have been associated with upper and lower respiratory tract illness. However, there is a strong relationship between HPIV-1,2 and 3 and specific clinical syndromes, age of child, and time of year. 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. The incubation period, the time from exposure to HPIV to onset of symptoms, is generally 2 to 7 days[29].

3.5.1 Croup

Croup is a generic term that encompasses a heterogeneous group of illnesses affecting the larynx, trachea, and bronchi. 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. It manifests variously as a barking cough, hoarseness, stridor and respiratory distress, with or without concomitant symptoms of viral upper respiratory infection. 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[157].

The incubation period, the time from exposure to HPIV to onset of symptoms, is generally 2 to 7 days.

- HPIV-1 and 2 are most often associated with croup. HPIV-1 often causes croup in children, whereas HPIV-2 is less frequently detected. Both types can cause upper and lower respiratory tract illnesses. People with upper respiratory tract illness may have cold-like symptoms.

- HPIV-4 is not recognized as often, but may cause mild to severe respiratory tract illnesses[158].

3.5.2 Bronchiolitis

Bronchiolitis is inflammation of the bronchioles. The smallest air passages of the lungs. It usually occurs in children less than two years of age with the majority being aged between three and six months[159].

The predominant symptoms include fever, expiratory wheezing, tachypnea, retractions, air trapping, rales and shortness of breath which can cause some children difficulty in feeding. The peak incidence of bronchiolitis (81%) is in the first year of life and then dramatically declines until it virtually disappears by school age. This syndrome is diagnosed in approximately 25 to 30% of lower respiratory infection (LRI) in childhood but makes up a larger percentage in the first year or two of life. At least 90% of cases of bronchiolitis are thought to be viral in origin and a viral identification rate as high as 83% has been reported[160]. All four types of HPIV can cause bronchiolitis, but HPIV type 1 and 3 have been reported most commonly. Each of these two groups appears to cause 10 to 15% of cases of bronchiolitis in nonhospitalized children. However, in hospitalized children, HPIV-3 causes many more cases than HPIV-1 (three or four times as many)[29]. HPIV-3 is more often associated with bronchiolitis, bronchitis, and pneumonia[158].

3.5.3 Pneumonia

Pneumonia is an infection in one or both lung. It can be caused by fungi, bacteria or viruses. Pneumonia causes inflammation in lung's air sacs or alveoli. The alveoli fill with fluid or pus, making it difficult to breath. Pneumonia symptoms can vary from mild to severe, depending on the type of pneumonia, age and health. The most common symptoms are (i) cough (with some pneumonias may have cough up greenish or yellow mucus, or even bloody mucus), (ii) fever (which may be mild or high), (iii) shaking chills, (iv) shortness of breathe, (which may only occur when the climb stairs) (v) headache, (vi) muscle pain, (vii) weakness[161].

Pneumonia is classically diagnosed by the presence of fever, rales and evidence of pulmonary consolidation on physical examination or x ray. Pneumonia diagnosed 29-38% of children hospitalized with LRI and in 23% treated as

outpatients[47,162-164]. The peak incidence for pneumonia is in the second and third years of life. Viruses shown to cause upto 90% of LRI, especially in the first year and this percentage decreases to 50% by school age[160,162,165,166]. After 9-10 years of age, viruses cause a decreasing but still significant amount of pneumonia in immunocompetent individuals. In adulthood, pneumonia cause 12% have been documented[50,167-169]. HPIV-1 and 3 cause 10% outpatient pneumonias, but similar to bronchiolitis, HPIV-3 causes a larger percentage of cases in hospitalized patients. Pneumonia can be caused by both HPIV-2 and 4, but the incidence of disease is not well described. HPIV-1 infection has been associated with secondary bacterial pneumonias in the elderly[170].

3.5.4 Tracheobronchitis

Tracheobronchitis is defined as inflammation of the airways between the larynx and the bronchioles. Clinically, this syndrome is recognized by an increase in the volume and purulence of the lower respiratory tract secretions and is frequently associated with signs of variable airflow obstruction. The most common symptoms are cough and large airway noise on auscultation, but patients may have fever and URI. Children are infected with LRI receive tracheobronchitis of about 20-30%. This infection occurred in the first 5 years of life, but tracheobronchitis are fairly evenly diagnosed throughout school age and adolescence[160]. More than 25% of the agents identified to cause tracheobronchitis have been HPIVs. HPIV-3 is more common highly infected than HPIV-1 or 2.

In other studies have recorded tracheobronchitis as the most common diagnosis in patients with HPIV-4 infections. Tracheobronchitis is used more commonly in patients with chronic diseases. Any single HPIV can cause more than LRI syndromes to occur simultaneously or progressively in the same child. In 5 to 20% of LRI cases, two viruses can be detected and may be associated with more severe disease[168].

3.5.5 Otitis media

Otitis media is a group of inflammatory diseases of the middle ear[171]. Otitis media shown to be associated with viral respiratory tract infections in 30-60% cases. Viruses may work synergistically with bacteria to initiate otitis media or prolong symptoms, and occasionally they are found to be the only cause of disease[172]. HPIV-3 is the most frequently reported HPIV associated with otitis media[173]. HPIV have been found in 1% of middle ear effusions and in 2% of nasopharyngeal secretions in children with acute otitis media[174].

3.6 PATHOGENESIS

Parainfluenza viruses enter new susceptible hosts by inhalation of virus-laden droplet nuclei which are expelled into the air from the mouths or noses of infected patients. Virus multiplication occurs throughout the tracheobronchial tree, inducing catarrhal inflammation with excessive production of mucus. The aryepiglottic folds (vocal cords) of the larynx become grossly swollen, causing obstruction to the inflow of air, which is manifested by inspiratory stridor and indrawing of the soft tissues around the rib cage. Infection does not regularly spread beyond the respiratory tract[175].

The virus adsorbs to the respiratory epithelial cells by specifically combining with neuraminic acid receptors in the cell through its hemagglutinin. Subsequently, the virus enters the cells following fusion with the cell membrane, mediated by F1 and F2 receptors. The virus replicates more rapidly than mumps and measles viruses in the cell cytoplasm and causes formation of multinucleated giant cells. These giant cells, each of which contains 2-7 nuclei usually develop late in the infection. The virus also causes the formation of single and multilocular cytoplasmic vacuoles and basophilic or eosinophilic inclusions. The virus causes inflammation of the respiratory tract leading to secretions of high level inflammatory cytokines, usually 7-10 days after initial exposure. Paramyxoviruses are known to induce apoptosis in tissue culture cells[176].

The focal tissue destruction caused by HPIV is usually mild and rapidly repaired and, in many infections, may not even be detectable. Infection in immunocompromised hosts is an exception where giant-cell pneumonia can lead to death. Disease severity has been correlated with HPIV shedding in children[62], but not in adults[16]. Airways inflammation, necrosis and sloughing of respiratory epithelium, edema and excessive mucus production are the noted pathological features associated with human parainfluenza virus infection[177]. HPIV-1 and other respiratory viruses have been shown to have procoagulant activity that may play a role in cardiovascular disease[178]. Also, PIVs have been shown to enhance the appearance of pulmonary edema in hypoxic rats, suggesting that they may be a cause of high-altitude pulmonary edema[179].

HPIV infection associated with the accumulation of ions and fluid in the respiratory tract. This may at least partly be due to a direct effect on ion transport by the respiratory epithelium (activation of Cl^- secretion and inhibition of Na^+ absorption), as shown in a model of SeV infection of tracheal epithelium[180]. The role of HN receptor interaction in the process has been demonstrated in experiments using variants of HPIV-3 that contain single amino acid changes in HN. These altered HN molecules shows increased avidity for sialic acid receptors, and as a result the respective variants are highly fusogenic and destroy a cell monolayer more rapidly than wild type (wt) HPIV. More importantly they cause alveolitis and more severe interstitial pneumonitis than wt HPIV-3 in a reliable cotton rat model for PIV infection[181].

3.7 IMMUNE RESPONSE

Host defense against HPIV is mediated largely by humoral immunity to the two surface glycoproteins HN and F[182,183]. Most children are born with neutralizing antibody to all four types of HPIV, but these titers quickly fall during the first 6 months of life. HPIV-3 infects approximately two-thirds of children during their first year of life, causing symptomatic disease in about one-third. Virtually all children by the age of 3-years will demonstrate serologic evidence of infection. Monoclonal antibodies ae preferentially formed to epitopes on the HN virus. In experimental animals, antibodies raised to an HN vaccinia construct were considerably more

protective than antibodies to protein F. After several infections, antibodies may develop that cross neutralize different parainfluenza strains[183].

These immunologic targets, children and adults are repeatedly infected with parainfluenza viruses over the course of a life time. Reinfection is more likely to solely involve the upper respiratory tract, with sparing of the lower respiratory tract after the first or second exposure in individuals with immunocompetence[1]. Prior infection in animal models blocks virus recovery on subsequent challenge. Experimental infection of adults with wild-type viruses is modulated by the level of immunity[184], and infection of seropositive children with live-attenuated vaccines is more difficult than ready infection of immunologically naïve children. In children and adults, recovery of virus is dramatically lowered by recent past exposure to the virus[185].

The most important component of resistance appears to be mucosal immunity. In animal models, greater protection is afforded with intra nasal than with systemic administration of parainfluenza type 3 glycoproteins. In addition, passive IgA antibody delivered into the respiratory tract of mice provides greater protection than IgG. In adult, after an experimental parainfluenza type 1 challenge, reisolation of virus was inversely correlated with the detection of local neutralizing antibody in secretions and not with serum antibody[186]. Immunity to HPIV-1 and 2 develops later than immunity to HPIV-3, increasing more rapidly during the second and third years of life. It is not until school age that a significant percentage of the general population develops antibody levels to HPIV-4[29].

Secretory IgA plays an important but not fully defined role in protection against natural infections with HPIV. After natural infection with HPIV, most children and adults develop measurable levels of this antibody[33,186-188]. This has been shown to be correlated with disease prevention and amelioration in adults[186,189] . However, in infants, secretory IgA levels did not correlate with the ability of nasal secretions to neutralize infection or ameliorate disease[188]. Cytotoxic T-lymphocyte responses appear to be important in the clearance of virus from the lower respiratory tract during infections with HPIV-3[133,190,191] and mouse PIV-1[192,193].

In children, prior natural infection blocks the replication of live-attenuated, intranasal administered virus vaccines, which replicate freely in naïve children, including children in the first 6 months of life with passively acquired maternal serum antibody. IgA antibody has the property of being transcytosed across epithelial cells from the basolateral surface to release at the apical surface into the respiratory tract. Antibody and virus have been proposed to potentially localize within cells and result in intracellular inhibition of virus assembly and release[194].

In addition to prevention of reinfection, immunity is involved in termination of primary infection. In animal models, the role of $CD8^+$ T-cells is critical in virus clearance. Lymphoid cells, some of which are virus specific accumulate in the regional peribronchial lymph nodes during acute infection. The lymphoid cells presumably contribute to the establishment of immunologic memory. The cells active in cytotoxic destruction of virally infected cells appear to accumulate in the airways and can be found in bronchoalveolar lavage fluid[195]. The severity of disease in individuals with T-cell deficits suggests the importance of T-cell immunity in clearance of infection. Parainfluenza virus type 3 has been to downregulate granzymeB, one of the perforins that mediates cytotoxicity, thus suggesting a mechanism of immune modulation by parainfluenza viruses[196].

3.8 EPIDEMIOLOGY

A number of studies have tested the effect of respiratory viral infections in pediatric practice[1]. Parainfluenza type 3 is the most frequently recovered of the parainfluenza virus types in longitudinal studies of respiratory illness in children. Roughly half as many parainfluenza type 1 isolates of parainfluenza type 2 are found as type 3[1]. Parainfluenza viruses vary in their seasonal epidemiology by type. Parainfluenza type 3 is endemic, with isolation throughout the year however, a distinct peak is seen in the spring months of April and May[7].Parainfluenza virus types 1 and 2 cause annual fall epidemics of disease and often alternate in years, so an individual type may be seen only every 2 years. Parainfluenza virus types 4A and 4B are isolated so seldom that their seasonality is not well described[197].

In the unique environment of over wintering on the South pole where 20 people were isolated for 6 months, parainfluenza virus types 1 and 3 were repeatedly isolated through the quarantined period, which suggests that persistent or repeated infection was spread. In tissue culture cells, persistent parainfluenza virus type 3 infection can also be established[198]. The first reported outbreak of HPIV-4 infection occurred in Hong Kong in the autumn of 2004, involving 38 institutionalized children and 3 staff members during a 3-week period in a developmental disabilities unit[199].

In India, Chennai, 232 clinical samples were detected by mRT-PCR. Among these 26 (11.2%) were positive by mRT-PCR and nine (34.6%) showed cytopathic effect with syncytium formation for HPIV and all were HPIV-3 serotype, other serotypes like 1,2,4 were negative[200].

In China, 178 HPIV cases were identified. Ninety nine samples were positive for HPIV-3, 58 for HPIV-1, 19 for HPIV-2 and 8 for HPIV-4. Some samples were positive for multiple HPIV types, therefore the sum of these segments are more than 178 and positive for HPIV ranged from one month to 78 years[51]. In Spain 201 nasopharyngeal aspirate specimens from pediatric patients hospitalized for lower respiratory illness were tested by mRT-PCR assay detected 64 HPIVs, while only 42 of them HPIV grew in cell culture. Among four serotypes HPIV-3 is highly predominantly identified[231].

Internationally, HPIV-1,2,3, and 4 have worldwide distribution, and epidemics are known to occur, particularly with HPIV-1. Parainfluenza viruses are responsible for disease throughout the year, but winter outbreaks of respiratory tract infections, especially croup, in children throughout the temperate zones of the Northern and Southern hemispheres represent peak periods of prevalence. Most infections are endemic, but sharp small epidemics involving HPIV-1 and 2 occasionally occur[199]. For the influenza like illnesses reported, the main etiologic agents in the early epidemic period were noninfluenza viruses, and among these noninfluenza viruses, HPIV accounted for about 24% of the infections[202]. In a study from Southern China, seasonal peaks due to HPIV-1 and 3 were observed during autumn, while the HPIV-2 and 4 were detected less frequently, with their incidence

increasing with the decline in the frequency of HPIV-1 and 3[51]. Epidemiology of Human parainfluenza virus was shown in the Table 3.1.

The morbidity, costs, and epidemiological features of lower respiratory tract infections in African-American children (LRIs) due to Human parainfluenza virus types 1 and 2 (HPIV-1 and 2), evaluated 1,213 children < 6 years of age were admitted to the hospital for LRIs during the fall quarter of 1991.

Table 3.1: Epidemiology of HPIV in different countries

Country	HPIV %	Years	Age
Israel[203]	1.1%	2004-2006	<17 years
Italy[204]	6.0%	2004-2007	<5 years
Netherland[205]	2.6%	2005-2008	<5year old
Portugal[206]	21%		<24 months
South Korea[207]	7.8%	2004	5 year old
Hongkong[208]	1.2%	2009	2 years old 1monthto 94 years
America[209]	13%	1991	<6years
Latin America[210]	3.2%	2006-2010	5years
Brazil[211]	7.6%	2005-2007	7.9 months and median hospital stay of six days.
Western Australia[212]	5.7%	1997-2005	5 years
Dhaka[213]	15%	2001	<13 years
Senegal[214]	2%	2009	<5 years
China[215]	3%	2011-2013	5-60 years
Guangzhou[216]	4.9%	2006-2009	<5 years
Beijing[217]	12%	2007-2010	< 1yers
Shanghai[218]	42.6%	2009-2010	<3 years
Six Provinces[219]	7.1%	2009-2012	<5 years
Ganzu Province[220]	22.1%	2011	1 month-12years
Changsha[221]	13.7%	2007-2008	<5 years

3.9 SIGNS AND SYMPTOMS

The following are the most common symptoms of HPIV infections. However, each child may experience symptoms differently or from one type of infection to another.

- Runny nose
- Redness or swelling of the eyes
- Barky (seal-like) cough
- Noisy, harsh breathing
- Ear pain
- Hoarse voice when speaking or crying
- Rattling felt over the chest or back when breathing
- Wheezing
- Fever
- Irritability
- Decreased appetite
- Vomiting
- Diarrhea

Human parainfluenza viruses (HPIVs) commonly cause upper and lower respiratory illnesses in infants and young children, but anyone can get infected. After get infected, it takes about 2 to 7 days before to develop the symptoms.

Symptoms of upper respiratory illness may include

- fever
- runny nose
- cough
- Sneezing
- Nasal discharge
- Nasal congestion
- Sore throat
- Nasal breathing
- Stuffy nose

Symptoms of severe lower respiratory illness may include

- Croup [an infection of the vocal cords (larynx), windpipe (trachea) and bronchial tubes (bronchi)],
- Bronchitis (swelling of the main air passages that connect the windpipe to the lungs),
- Bronchiolitis (swelling in the smallest air passages in the lungs), or
- Pneumonia (an infection of the lungs).

Most often, the symptoms of parainfluenza viruses aren't severe enough to cause concern in healthy adults. However, they can be life-threatening in an infant, older person, or anyone else with a compromised or weakened immune system[222].

3.10 LAB DIAGNOSIS

3.10.1 Collection and preparation of clinical samples

Diagnosis of HPIV infection can be performed utilizing a wide variety of methodologies. A critical step in the diagnosis of HPIV infection, regardless of the methodology being utilized is sample collection. Initial infection and replication of HPIV takes place in the nasopharynx and oropharynx and thus these are the most appropriate collection sites for detection of HPIV. In addition to the sample site, the time of test collection is important because HPIV shed at much higher titers early in infection and then declines quickly. Children usually have higher viral loads, so isolation of HPIV is generally easier from children than adults[223].

The infections in adults were 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 4[97,224]. 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, 2 and 3 for many months[225].

Throat swabs, nasopharyngeal swabs, nasal washes, and nasal aspiration have all been used successfully to recover HPIV[18,62,226-228]. The few studies to yield high rates of viral recovery (HPIV-1 and 3) used nasal washes or nasal

aspirates[62,229], 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 8.0 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 h is anticipated, the specimen should be frozen. Centrifugation at 1,000X *g* prior to inoculation is also helpful in removing debris[29].

Tissue culture

Cell cultures were suitable for virus isolation, with facilitated control of contamination with antibiotics and clean-air equipment and decreased use of experimental animals. Susceptible cell lines for culturing HPIV are Lewis lung carcinoma monkey kidney (LLC-MK2), Human lung adenocarcinoma (A549), African green monkey (CV1) and Madin-Darby Canine Kidney (MDCK). More than one cell culture passages were required to observe cytopathic effect (CPE). HPIV has the best growth in primary monkey kidney cells. The clinical virology laboratories, the most popular cell line for growing HPIV were rhesus MK cells. However, this cell line is not always the most sensitive for HPIV, especially for HPIV-2[230]. Human laryngeal epidermoid carcinoma (HEp-2) cells, human lung mucoepidermoid carcinoma (NCI-H292) cells and human embryonic lung fibroblast (Fp) cell cultures were used for primary viral isolation[231].

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. 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 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 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[29].

Virus Isolation

HPIVs were isolated more easily in epithelial cell lines than in fibroblast cell lines. The addition of an exogenous protease (trypsin) to the cell culture medium facilitates virus recovery for some serotypes and strains of HPIV. Virus isolation in cell cultures has long served as the "gold standard" for virus detection and it is the best method compared to all other methods[232].

Hemadsorption assay

The hemadsorption (HAD) technique is used primarily to detect viruses that produce little or no cytopathic effect (CPE) in tube culture monolayers. Using guinea pig RBC, it is used to screen inoculated cell cultures for the presence of influenza, parainfluenza, mumps and Newcastle disease viruses[233]. Hemadsorption is the ability of cells infected with an enveloped virus containing a hemagglutinin in its envelope to adsorb red blood cells. As the virus reproduces, these hemagglutinins (glycoproteins) are inserted into the plasma membrane of the infected cell. These modified areas of the cell surface are the sites at which progeny virus particles will mature. If agglutinable red blood cells are brought into contact with hemagglutinin-containing surfaces of cultured cells, the red blood cells will specifically bind to the infected cells a phenomenon is known as hemadsorption[234].

If hemadsorbing virus is present, by microscopy we can observe the erythrocytes adhering in clumps to the infected areas of the cell monolayer. Erythrocytes will not adhere to uninfected cells or to cells infected by nonhemadsorbing viruses. The CPE might not be very characteristic but can be detected by the affinity to erythrocytes to adhere to the infected cells[29].

Multiplex Reverese transcription Polymerase chain reaction (Multiplex RT-PCR)

Multiplex PCR refers to the use of polymerase chain reaction to amplify several different DNA targets (genes) simultaneously (as if performing many separate PCR reactions all together in one reaction). This process amplifies genomic DNA samples using multiple primers and a temperature mediated DNA polymerase in a thermal cycler. Annealing temperature for each of the primers sets must be optimized to work correctly within a single reaction, amplicon sizes, that is their base pair length, should be different enough to form distinct bands when visualized by gel electrophoresis[231].

Plaque assay

One of the most important procedures in virology is measuring the virus titer – the concentration of viruses in a sample. A widely used approach for determining the quantity of infectious virus is the plaque assay. This technique was first developed to calculate the titers of bacteriophage stocks. Renato Dulbecco modified this procedure in 1952 for use in animal virology, and it has since been used for reliable determination of the titers of many different viruses.

To perform a plaque assay, 10-fold dilutions of a virus stock are prepared, and 0.1 mL aliquots are inoculated onto susceptible cell monolayers. After an incubation period, to allow virus to attach to cells, the monolayers are covered with a nutrient medium containing a substance, usually agar that causes the formation of a gel. When the plates are incubated, the original infected cells release viral progeny. The spread of the new viruses is restricted to neighboring cells by the gel. Consequently, each infectious particle produces a circular zone of infected cells called a plaque. Eventually the plaque becomes large enough to be visible to the naked eye. Dyes that stain living cells are often used to enhance the contrast between the living cells and the plaques. Only viruses that cause visible damage of cells can be assayed in this way. Plaques are generally counted manually and the results, in combination with the dilution factor used to prepare the plate, are used to calculate the number of plaque forming units per sample unit volume (pfu/mL). The pfu/mL

result represents the number of infective particles within the sample and is based on the assumption that each plaque formed is representative of one infective virus particle[235].

Immunofluorescence assay

Immunofluorescence is a technique using fluorescent dyes, fluorescence microscope to detect targets. This technique uses the specificity of antibodies to their antigen target using fluorescent dyes to detect specific biomolecule targets within a cell and therefore allows visualization of the distribution of the target molecule through the sample. Immunofluorescence is a widely used example of immunostaining and is a specific example immunohistochemistry that makes use of fluorophores to visualize the location of the antibodies/antigens[236].

There are two types of techniques including direct and indirect immunofluorescence which are performed depending in clinical scenario. DIF techniques can also be used to detect nonantibody targets in the skin, such as infectious organisms. In this case, a fluorophore-labeled primary antibody directed against the suspected antigen, this is used to detect the presence or absence of the organism. This technique is rapid and quite specific, but, owing to the limited number of antibodies that can bind to the specific target, it may be less sensitive than other microbiologic techniques. Indirect immunofluorescence utilizes a two-step technique, in which a primary, unlabeled antibody binds to the target, after which a fluorophore-labeled second antibody (directed against the Fc portion of the primary antibody) is used to detect the first antibody. This technique is more complicated and time consuming than direct immunofluorescence (because it requires a second incubation period) however, it is more sensitive because more than one secondary antibody can bind to each primary antibody, which amplifies the fluorescence signal[237].

Morbidity and mortality

Mortality caused by HPIV in developed regions of the world remains rare. Where mortality has occurred, it is principally in the three core risk groups (very

young, elderly and immunocompromised. Long term changes can however be associated with airway remodeling and are believed to be a significant cause of morbidity[238]. 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 total deaths in preschool children age group and HPIV causes at least 10% of the LRI[239].

3.11 PREVENTION

Currently, there is no vaccine to protect against Human parainfluenza virus infection. To reduce the risk of HPIV and other respiratory viral infections by washing hands often with soap and water avoiding touching our eyes, nose or mouth, avoiding close contact with people who are sick and keeping objects, surfaces clean and disinfected. Breast feeding may protect babies from HPIVs during their first few months of life, because mothers may have protective antibodies to HPIVs in their breast milk. Formalin-killed whole HPIV-1, 2 and 3 vaccines used in field trials were failed to protect children against natural infection in the late 1960[240,241]. The majority of children developed antibodies to all three HPIV serotypes, but the levels were considerably lower than those seen following natural infection[241]. Additional studies revealed that some of the antibody produced against the inactivated viruses may not have neutralized native virus and that no significant mucosal immunity developed[242].

3.12 TREATMENT

There was no specific treatment that can eliminate HPIV from the body. When infected with HPIV just to have let it run its course. Most people with HPIV illness will recover on their own. To relieve symptoms for the following medications can be used–taking acetaminophen, ibuprofen and other over the counter medications for pain and fever, using a rook humidifier or taking a hot shower to help ease a sore throat and cough. People who are sick should be encouraged to drink plenty of liquids and stay home and rest. If people were illness caused by

HPIV then antibiotics will not make better. Antibiotics are only effective against bacteria. Antiviral agents are of uncertain benefit for treatment of HPIV infection.

The first inactivated vaccines for PIV-1, 2 and 3 were developed in the late 1960, however the antibody responses were variable and no protection against disease was observed[243]. Another approach used selected variants of human viruses with reduced virulence such as cold-adapted, temperature sensitive mutants of PIV-3 had been used as cold-adapted mutants were produced by serial passages under suboptimal conditions in cell cultures. These viruses seem to have stable phenotype, are attenuated when evaluated in animal models, and induce resistance to wild type PIV-3 challenge in experimental animals[244].

Human parainfluenza virus, neither inactivated nor live vaccines are available. Even though no data are presented from studies with humans, formalin inactivated vaccines for PIV in animals intensify the symptoms of infection upon challenge with homologous virus[245] as formerly shown in humans and animal models specified inactivated vaccines for respiratory syncytial virus (RSV) (a close relative of PIV)[246,247]. This development of pathogenicity was not observed for a live attenuated candidate vaccine, which showed to be effective in animal studies[248]. Thus for the prevention for Influenza virus and human parainfluenza virus infections, live attenuated vaccines are prospective to be more effective than inactivated vaccines.

Recent studies involved mice used vectored vaccines against HPIV-1 and 3 suggest that brief cross protection between these viruses may occur via cell mediated immunity associated with internal proteins common to both viruses[249,250]. The significant cross protection does not appear to occur in humans[251]. Recombinant DNA technology also hastened the development of vaccines for HPIV-1 and 2. Recombinant, chimeric parainfluenza viruses containing the internal genes of HPIV-3 (either wild type or cp45) and the HN and F genes of HPIV-1 (rPIV3-1) or HPIV-2 (rPIV3-2)[252,253].

5.13 ANTIVIRALS

Zanamivir and Ribavirin

Zanamivir is a neuraminidase inhibitor used in the treatment and prophylaxis of influenza caused by influenza A and B viruses. It was established by Australian biotech and accredited to Glaxo in 1990 and approved at United States in 1999. Zanamivir only used as a treatment for influenza and was approved for prevention of influenza A and B[254]. Its molecular weight has 332.3 g/mol and molecular formula $C_{12}H_{20}N_4O_{7.}$

Ribavirin (RBV, also known as virazole), 1-ß-D-ribofuranosyl-1,2,4-triazole-3-carboxamide, is the first synthetic, broad-spectrum antiviral nucleoside analog which has been shown to exhibit antiviral activity against many RNA and DNA viruses both in vitro and in vivo[255-259]. The discovery of the broad spectrum antiviral activity of ribavirin in 1972 and suggested that the active form of ribavirin is the monophosphate (RMP)[256]. Its molecular weight 244.2 KDa and molecular formula as $C_8H_{12}N_4O_{5.}$

Neuraminidase Inihibitor

The establishment of human parainfluenza virus type 3 (HPIV-3) infections entail the interaction of viral hemagglutinin neuraminidase (HN) glycoprotein with sialic acid receptor. 4-Guanidino-2,4-dideoxy-2,3-dehydro- N- acetylneuraminic acid (4-GU-DANA; zanamivir) is a sialic acid transition state analog was evaluated for the antiviral (inhibiting) activity. The (4-GU-DANA) 4-guanidino-Neu5Ac2en assists as a clinically effective antiinfluenza agent[260], whereas Zanamivir inhibits Human parainfluenza virus neuraminidase activity, it does not prevent release of virus from the infected cell surface but it does in the case of Influenza viruses, instead it blocks interaction between the Parainfluenza virus HN protein and its receptor and thus surprisingly aids in the release of newly assembled virions from the infected cell[261].

Nucleoside inhibitor

Ribavirin is a carboxamide group can sort the native nucleoside drug resemble adenosine or guanosine, depending on its rotation. For this reason when ribavirin is incorporated into RNA, as a base analog of either adenine or guanine, it pairs equally well with either uracil or cytosine, including mutations in RNA-dependent replication in RNA viruses. Such hypermutation can be lethal to RNA viruses[262,263].

Ribavirin was first approved for use in humans to treat respiratory syncytial virus infection in children. Ribavirin used to treat other viruses[264,265]. Ribavirin inhibition of virus replication in the cells, mainly by inhibition of transcription, resulting in inhibition of viral protein synthesis, of cell-to-cell spread of virus and virus budding from cells[266].

Medicinal plants (*Glycyrrhiza glabra*)

Many traditional medicinal plants have been reported to have strong antiviral activity and some of them have already been used to treat animals and people who suffer from viral infection[267-270]. The antiviral agent development was started after the Second World War in Europe and in 1952 the Boots drug company at Nottingham, England, examined the action of 288 plants against influenza A virus in embryonated eggs. They found that 12 of them suppressed virus amplification[271]. During the last 25 years, there have been numerous broad-based screening programmes initiated in different parts of the globe to evaluate the antiviral activity of medicinal plants for in vitro and in vivo.

In China, licorice (Gan Cao) has been reported as one of the oldest and most commonly prescribed traditional medicine, used in the treatment of several diseases[272]. *G. glabra* natural habitat are Southwest and Central Asia as well as subtropical and temperate areas of the planet, including Europe. The root is termed licorice and has a sweet odor and smell. The genus *Glycyrrhiza* (*Leguminosae*) includes about 30 species such as *G. glabra*, *G. uralensis*, *G. inflata*, *G. aspera*, and Persian and Turkish licorices, which are determined as *G. glabra* var. *violace*.

The first report that indicated an antiviral property of licorice constituents dates back to 1979[273]. In that research, the scientists recognized glycyrrhizic acid and its antiviral activity in vitro, which suppressed the growth and cytopathic effects (CPE) of numerous DNA and RNA viruses[274]. Some reports indicated that a few minor constituents of *G. glabra* such as liquiritigenin and isoliquiritigenin might have some pharmacological functions[272].

Medicinal plants have been used all over the world for their therapeutic benefits, although their use remained restricted to China, India, Japan, Pakistan, Sri Lanka, Thailand and a number of African countries[275]. Similarly, the developed nations are also encouraging the use of natural medicinal products in their health care systems. Natural medicinal products in the forms of herbs have been commercially added in the dietary supplement industry as well as in holistic medicine in the United States. It has been estimated that one-third person in the United States has tried some form of natural medicine at least once[276].

The traditional sources for the use of *Glycyrrhiza* species as an herbal medicine are reported in ancient manuscripts from China, India, and Greece. Its use for symptoms of viral respiratory tract infections and hepatitis has been documented. Randomized controlled trials of the *Glycyrrhiza glabra* derived compound "glycyrrhizin," and its derivatives showed reduced hepatocellular damage in chronic hepatitis B- and C-infected patients. In hepatic cirrhosis induced by hepatitis C virus, the risk to develop hepatocellular carcinoma was reduced in those infected patients who administered with glycyrrhizin[277]. Glycyrrhizin (licorice root extract) has anti-inflammatory and antioxidant activities. Glycyrrhizin inhibits $CD4^+$ T-cell and tumor necrosis factor (TNF) - mediated cytotoxicity[278]. Glycyrrhizin has a membrane stabilizing effect[279] and also stimulates endogenous production of interferon[280]. β glycyrrhetinic acid, an active constituent of glycyrrhizic acid, shows anti-viral activity against a number of DNA and RNA viruses, possibly due to activation of nuclear factor (NF-κB and induction of IL-8 secretion)[281].

Licorice can treat respiratory tract infections, such as a sore throat, cold, cough and asthma. Its anti-inflammatory and antioxidant properties help reduce inflammation of the bronchial tubes and calm the airways. It even loosens and thins

mucus in airways, which eases congestion and coughing. In addition, its antimicrobial, antibacterial and antiviral properties fight microbes that cause respiratory illnesses and an overproduction of mucus. They are also effective in treatment of pain and cramps during menstrual cycle and cure the complaints of indigestion, acidity and stomach pain[282].

Molecular docking is used to predict the structure of the intermolecular complex formed between two molecules. Molecular docking as an important role in lead discovery and design. Molecular docking a key tool in structural molecular biology and computer assisted drug design. The goal of ligand-protein docking to predict the predominant binding modes of a ligand with a protein of known 3 dimensional structure. It has long been recognized that a simplistic rigid 'lock-and-key' model of ligand-receptor interaction an inadequate and incorporation of ligand and receptor flexibility is required for accurate docking. While ligand flexibility has been addressed by a variety of algorithms, receptor flexibility remains a formidable challenge[283]. The computational strategies to permeate all aspects of drug discovery[284,285] such as virtual screening (VS)6 techniques for hit identification and methods for lead optimization. Compared with traditional experimental high-throughtput screening (HTS), VS a more direct and rational drug discovery approach and has the advantage of low cost and effective screening[285,286]. It also predicts the strength of the binding, the energy of the complex; the types of signal produced and calculate the binding affinity between two molecules using scoring functions. Scoring functions as evaluated a particular pose by counting the number of favorable intermolecular interactions, such as hydrogen bonds, hydrophobic contacts[287]. The most interesting case is the type protein-ligand interaction, which has its applications in medicine. *Insilico* drug discovery serve a significant role in the development of novel drugs for pharmaceutical and clinical industry. It reduces the duration for finding novel lead solution tomany infectious diseases[288].

Scope and Plan

SCOPE AND PLAN

4.1 SURVEILLANCE OF HUMAN PARAINFLUENZA VIRUS BY MULTIPLEX RT-PCR

Acute lower respiratory tract illness are the foremost cause of morbidity and mortality in children worldwide, with the highest number of deaths ensued in developing countries[289]. Pneumonia are the leading cause of death in children under five years old >30 000 per annum[283 290] and account for 30 to 90% of ARI in young children[284,285 291,292]. RSV, PIV-1-3, Influenza viruses are the most common viral agent being detected in 63.1% of China[286 293].

Human parainfluenza viruses (HPIVs) are medically important respiratory pathogens and are second only to respiratory syncytial virus (RSV) as a major cause of lower respiratory tract (LRI) illness in infants and young children. There is no information available on the prevalence of HPIV in Chennai. Prevalence study would be the first of its kind to report HPIV occurrence in Chennai, Tamilnadu as not many studies have been done.

4.1.1 Standardization of multiplex Reverse Transcription PCR

Mortality induced by HPIV is unusual in developed countries and is seen almost entirely in young infants, the immunocompromised and the elderly. The preschool population in developing countries has considerable risk of ARI induced death. To address these problems Multiplex reverse transcription PCR (RT-PCR) assay can be a sensitive, specific tool for the rapid and early diagnosis of HPIV infections and for simultaneous detection of HPIV serotype.

In the present study the throat and nasal swabs were collected from symptomatic patients from January 2011 to December 214 based on the date of onset of illness. The samples were collected at different age groups 0-10yrs, 11-20yrs, 21-30yrs, 31-40yrs, 41-50yrs and above 50yrs. Prevalence study will help to identify the circulating HPIV serotypes in Chennai population.

The classic diagnostic methods like viral isolation and serology results obtained to delay several weeks before test results are available[48]. Rapid diagnostic to assist the clinician in making therapeutic decisions and to prevent nosocomial infections.

In the present study the samples were initially processed for the cDNA conversion and molecular technique Multiplex-PCR based in reverse transcription establish to rapid diagnosis with expected high sensitivity. Multiplex RT-PCR were used to detect respiratory viruses like HPIV, if positives were observed in multiplex PCR further confirmed by single RT-PCR. Multiplex PCR allowed the detection of several viruses simultaneously and consume less reagents, samples and time than single PCR assays, which can be an important consideration for high volume diagnostic laboratories.

4.1.2 Genetic characterization, sequencing and phylogenetic analysis

Human parainfluenza viruses are greatest characteristically associated with croup but can also cause a wide spectrum of respiratory illness.There is less information about their role in upper respiratory tract illness and associated otitis media[17].

In the present study, all the clinical samples were subjected to multiplex RT-PCR for HPIV-1,2,3 and 4 for the more sensitivity. The genetic characterization of the HPIV was performed to find out the genotypic characterization and mutational events were analyzed during the study period. Genetic characterization of HPIV serotypes is important to know the mechanism resulting in genetic diversity of HPIV and for controlling the pathogen. Phylogenetic analysis would confirm the similarity of HPIV strains and the sequence data may help in understanding virus diversity and evolution. This is to elucidate the genetic characteristics and phylogeny of Chennai strains, the Hemagglutinin Neuraminidase gene and Nucleocapsid gene sequence of selected strains to analyse and compare the other strains from different countries worldwide.

The Chennai strains were circulatory in different areas was identified. Sequencing was performed on ABI Prism 310 and sequence alignment and phylogenetic analyses will be done using software Mega version 6. Phylogenetic tree was constructed with the neighbor-joining method, with a bootstrap analysis of 1000 replicates, using software[294]. Multiple sequence alignment was performed by ClustalW tool was used to identify sequence alignment, mutations and amino acid alterations.

4.2 COMPARISON BETWEEN THREE CELL LINES BY HPIV ISOLATES

Upper and lower respiratory tract infection caused by Human parainfluenza virus. A primary and secondary cell lines support the growth of HPIV, the most common viral isolation was tried in the three cell lines, LLC-MK2, MDCK cell cultures and human adenocarcinoma (A549) cell line to identify the most effective cell line for the recovery of these viruses from clinical specimen. Identify the most appropriate cell line for isolation of HPIV and to compare three different cell lines. Virus isolation in cell cultures has long served as the “gold standard” for virus detection, and it is the best method compared to all other methods[9]. In the present study the comparative analysis of different cell lines in the isolation of Human parainfluenza virus and assess the factors affecting viral isolation and determination of the most desirable cell line that is likely to yield the best outcome.

Virus infection of cultured cells (LLC-MK2 and A549) can be monitored by hemadsorption assay, quantitated by plaque assay and evaluate neuraminidase activity.

4.3 SENSITIVITY OF ANTIVIRALS

4.3.1 Antiviral activity of Zanamivir and Ribavirin against HPIV

HPIVs are subsequent to Respiratory Syncytial Virus (RSV) as the viral cause of severe acute respiratory tract infections in young children. The establishment of human parainfluenza virus type 3 (HPIV-3) infection entails the interaction of viral hemagglutinin neuraminidase (HN) glycoprotein with sialic acid

receptor. 4-Guanidino-2,4-dideoxy-2,3-dehydro- N- acetylneuraminic acid (4-GU-DANA; zanamivir) is a sialic acid transition state analog was evaluated for the antiviral (inhibiting) activity, specificity and sensitivity of human parainfluenza virus in persistently infected LLC-MK2 and A549 cells.

Ribavirin (RBV, also known as virazole), 1-ß-D-ribofuranosyl-1,2,4-triazole-3-carboxamide, is the first synthetic, broad-spectrum antiviral nucleoside analog, Human parainfluenza virus type 2 is an important respiratory tract pathogen of infants and children. There are no vaccines or antiviral currently approved for prevention or treatment of HPIV-2 infection. Towards developing an antiviral activity to combat HPIV2 infection, our present study established nucleoside analog Ribavirin used to reduce viral growth in LLC-MK2 and A549 cells.

Cytotoxicity of Zanamivir and Ribavirin were performed by MTT assay, Neutral red uptake assay and trypan blue exclusion method.

4.3.2 Antiviral screening of HN inhibitor and nucleoside inhibitor against HPIV

In the present study, the efficacy of 4-GU-DANA on HPIV-3 was assessed through Hemadsorption inhibition assay, plaque reduction assay and neuraminidase inhibition assay. The nucleoside inhibitor Ribavirin was evaluated by Hemadsorption inhibition assay and plaque reduction assay.

4.3.3 Nucleoside inhibitor against HPIV-2 by RT-PCR

The effects of the drug on HPIV-2 infection in which there is almost total culture destruction and high levels of released infectious virus and also to investigate the effect of the drugs on viral genome synthesis, to prepared and analyzed viral RNA by reverse transcription polymerase chain reaction. Synthesized cDNA using random primers and performed PCR to elucidate the effect of RBV on mRNA synthesis.

4.4 ANTIVIRAL ACTIVITY OF LICORICE AGAINST HPIV

Several hundred plant and herb species that have potential as novel antiviral agents have been studied. A wide variety of active phytochemicals, including the flavonoids, terpenoids, lignans, sulphides, polyphenolocs, furyl compounds, alkaloids, polyines, thiophenes, proteins and peptides have been identified[288 295]. In China, Greece, and India confirm the historical background of the *Glycyrrhiza* species use[289,296]. In China licorice has been reported as one of the oldest and most commonly prescribed traditional medicine, which has been used in the treatment of several diseases[290 297]. The first report indicated an antiviral property of licorice constituents dates back to 1979[291 298]. Glycyrrhizic acid and its antiviral activity in vitro, which suppressed the growth and cytopathic effect of numerous DNA and RNA viruses[289 295].

There were no much data available for treating HPIV by natural herbs (Licorice root) in Tamilnadu particularly in Chennai. To address these problems, in the present study the effects of Glycyrrhizic acid from Licorice root on HPIV to determine the efficacy and the effect of the elapsed treating with LLC-MK2 and A549 cells. To study the efficacy of Glycyrrhizic acid (commercially available) against Human parainfluenza virus type 2 and 3 and to study the efficacy of medicinal plants had a variety of chemical constituents which have the ability to inhibit the replication cycle of various types of DNA or RNA viruses. To screened the efficacy of Licorice root on HPIV assessed through hemadsorption inhibition assay, plaque reduction assay and neuraminidase inhibition assay.

Materials and Methods

5. MATERIALS AND METHODS

5.1 SAMPLE COLLECTION AND PROCESSING

5.1.1 Materials for sample collection

- Ice packs and vaccine carriers
- Viral transport medium (HBSS)
- Sterile viscose /dacron swabs
- Tongue depressors
- Screw capped tubes

5.1.2 Methodology for sample collection

Samples were collected from Seven Government outpatient departments (OPD) of tertiary care Government hospitals in Chennai (Institute of Child Health and Hospital (ICH), Royapettah Government Hospital (RGH), Saidapet Government Hospital (SGH), Rajiv Gandhi Government Hospital (RGH), Government peripheral hospital (GPH), Kilpauk Medical College (KMC) and Stanley Medical College (SMC). The samples were collected after obtaining informed consent and standard laboratory request form was duly filled with necessary details.

Criteria for sample collection

Inclusion criteria

Nasal and throat swabs were collected from patients with acute respiratory symptoms who had visited outpatient clinics and hospitals at Chennai from January 2011 to December 2014. Patients of all age groups were selected according to a set of criteria that included respiratory symptoms such as chills and rigor, nasal discharge, cough, ear discharge, fatigue, breathlessness, expectoration, headache, body ache, vomiting, diarrhoea, seizure, wheezing and a body temperature above 38 ºC.

Symptoms, history of illness, results of laboratory investigations and demographic data were recorded for each patient, using a standardized form.

Clinical information of patients with virus information was reviewed retrospectively from records ofHuman parainfluenza virus samples.

Exclusion criteria

The samples from patients without fever and other symptoms were excluded.

Nasal swab

A dry swab was inserted into the nostril parallel to the palate and left in place for a few seconds. Then it was slowly withdrawn with a rotating motion. Specimens from both nostrils were obtained with the same swab. The tip of the swab was put into a vial containing 2 mL of viral transport medium (containing veal infusion broth, NaCl, protease peptone) and the applicator stick was broken off. Specimen was stored at 4 ºC.

Throat swab

Both tonsils and the posterior pharynx were swabed vigorously and the swab was collected into viral transport medium and the applicator stick was broken off. Specimen was stored at 4 ºC.

Collection of swabs

- ❖ Collection of nasal swabs from both nostrils in infants < 1 year old.
- ❖ Collect nasal swab from both nostrils in patients >1 year old and having predominant symptoms of nasal discharge (running nose).
- ❖ Collect throat swab (two swabs) from patients greater than 1 year old with no nasal discharge.

5.1.3 Material for Sample processing

- Centrifuge
- Amphotericin B
- Penicillin, Streptomycin
- Vortex mixer
- 2 mL poly propylene vials.

5.1.4 Methodology for sample processing

Nasal and throat swabs

- 2 mL collection vials with swab were agitated vigorously on vortex mixer. The fluid was expressed by squeezing the swab to the inner wall of the vial.
- The swab was removed from the collection vial and antibiotics (Amphotericin B, Streptomycin, Penicillin) were added.
- It was incubated at room temperature for 15 minutes.
- It was centrifuged at 2500 rpm for 15 minutes, supernatant was removed and aliquoted into vials and stored at -70 ºC.

5.1.5 Molecular characterization of Human parainfluenza virus

Materials for RNA extraction

The QIAampViral RNA kit (QIAGEN) was used for the extraction of RNA from clinical samples and cell culture supernatants following materials were provided in the kit.

- QIAamp mini spin columns
- Collection tubes (2 mL)
- Viral lysis Buffer (AVL)
- Wash Buffer AW1 (concentrate)
- Wash Buffer AW2 (concentrate)
- Elution Buffer (AVE)
- Carrier RNA (poly A)
- 99% Ethanol
- 1.5 mL micro centrifuge tubes
- Vortex mixer
- Micro centrifuge
- Miscellaneous – Pipette (1000 µL, 100 µL), disposable sterile tips, tissue papers

5.1.6 Preparation of reagents

Addition of carrier RNA to Buffer AVL

310 µL Buffer AVE was added to the tube containing 310 µg lyophilized carrier RNA to obtain a solution of 1 µg/µL, dissolved thoroughly, divided it into conveniently sized aliquots, and stored at -20 ºC. Freeze thawing the aliquots of carrier RNA was not done for more than 3 times.

Buffer AVL was checked for precipitate, and if necessary incubated at 80 ºC until the precipitate were dissolved. The volume of buffer AVL–carrier RNA mix needed per batch of samples was calculated by selecting the number of samples to be simultaneously processed. Volumes were calculated used in the following.

Sample calculation:

n x 0.56 mL = **y** mL
y mL x 10 µL/mL = **z** µL
Where: **n** = number of samples to be processed simultaneously
y = calculated volume of Buffer AVL
z = volume of carrier RNA–Buffer AVE to add to Buffer AVL
It was mixed gently by inverting the tube for 10 times.

AVL Buffer

The sample was lysed under highly denaturing conditions provided by AVL buffer to inactivate RNases and to ensure isolation of intact viral RNA. The AVL buffer contains guanidine thiocyanate and carrier RNA. Guanidine thiocyanate, a cationic chaotropic agent denatures macromolecules, inactivates enveloped and nonenveloped viruses and irreversibly inactivates degrading nucleases.

Carrier RNA enhances binding of viral nucleic acids to QIAamp mini membrane (silicon membrane which have greater affinity to nucleic acids).

Buffer AW1

Buffer AW1 were supplied as a concentrate. When the buffer used for the first time, 19 mL of 100 % ethanol was added to Buffer AW1 concentrate.

Buffer AW2

Buffer AW2 were supplied as a concentrate. When the buffer used for the first time, 30 mL of 100 % ethanol was added to Buffer AW2 concentrate.

5.1.7 Methodology for RNA extraction

560 µL of AVL buffer prepared containing carrier RNA was pipetted into a 1.5 mL micro centrifuge tube. (In case of larger sample volume exceeding 140 µL then the amount of AVL buffer or carrier RNA would be increased proportionally, e.g 280 µL sample will require 1120 µL of AVL buffer or carrier RNA).

- 140 µL of sample was taken and vortex - spin for 15 seconds.
- Incubated at room temperature for 10 minutes.
- Briefly centrifuge the 1.5 mL micro centrifuge tube to remove drops from the inside of the lid.
- 560 µL of ethanol was added (96-100%) to the sample and mix by pulse vortexfor 15 seconds briefly centrifuge to remove drops from inside the lid.
- Carefully apply 630 µL of solution from step 5 to column. Centrifuge at 8000 rpm for 1 minute. Place column into a clean 2 mL collection tube and discard the tube containing the filtrate.
- Repeat STEP 6.
- 500 µL of Buffer AW1 was added and centrifuge at 8000 rpm for 1 minute. Place column into a clean 2 mL collection tube and discard the tube containing the filtrate.

- ❖ 500 µL of Buffer AW2 was added and centrifuge at full speed (14000 rpm) for 4 minutes.

- ❖ Place the column in a clean 2 mL collection tube and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 minute.

- ❖ Placed the column in a clean 1.5 mL micro centrifuge tube. 60 µL of Buffer AVE was added and incubate at room temperature for 1 minute. Centrifuge at 8000 rpm for 1 minute.

- ❖ In case of storage, store RNA at -20 ºC or -70 °C till further use.

5.1.8 Materials for cDNA conversion

(i) Genomic DNA elimination reaction components

- Genomic DNA wipeout buffer 7X
- Template RNA 1µg
- RNase free water

(i) Reverse-transcription reaction components

- Quantiscript Reverse transcriptase
- Quantiscript RT buffer 5X
- RT primer mix
- Template RNA(entire genomic DNA elimination reaction)

5.1.9 Methodology for cDNA conversion

- ❖ Thaw template RNA on ice. Thaw genomic DNA Wipeout Buffer, Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, RT Primer Mix, and RNase-free water at room temperature (15–25 ºC).

- ❖ Prepare the genomic DNA elimination reaction on ice according to Table 5.1.

Table 5.1: Genomic DNA elimination reaction components

Component	Volume/reaction (IX)
Genomic DNA wipeout buffer, 7X	2 µL
Template RNA	Variable (upto 1 µg)
RNase-free water	variable
Total volume	14 µL

- Incubate for 2 minutes at 42 ºC. Then place immediately on ice.
- Prepare the reverse-transcription master mix on ice according to Table 5.2.

Table 5.2: Reverse-transcription reaction components

Component	Volume/reaction (IX)
Reverse-transcription master mix	
Quantiscript Reverse transcriptase	1 µL
Quantiscript RT buffer, 5X	4 µL
RT primer mix	1 µL
Template RNA	
Entire genomic DNA Elimination reaction (step 3)	14 µL (add at step 5)
Total volume	**20 µL**

- Add template RNA from step 3 (14 µl) to each tube containing reverse-transcription master mix.
- Incubate for 15 minutes at 42 °C.
- Incubate for 3 minutes at 95 °C to inactivate Quantiscript Reverse Transcriptase.

5.1.10 Materials for Multiplex RT-PCR

- Taq polymerase master mix (Phusion DNA PCR Kit, New England, BioLabs)
- Primers (designed to amplify hexon gene)
- Nuclease free water
- Positive control
- Microcentrifuge tube (1.5 mL)
- PCR tubes
- Sterile tips
- Micropipettes (0.5-10 µL, 20-200 µL)
- Microcentrifuge
- Template (cDNA)
- Thermal cycler (ABI Gene Amp PCR System 9700)

5.1.11 Methodology for multiplex PCR

❖ Each sample was tested by the multiplex primer sets. RNA were screened with four targets namely HPIV (HN gene) for serotype 1, 2 and 3. Phosphoprotein for HPIV serotype 4.

❖ Reaction assay mixtures were made in 1.5 mL micro centrifuge tube as a cocktail. Water, extracted RNA and positive template controls were then added to the appropriate test reactions and controls.

❖ Master Mix: The amount of each reagent was added for primer set which shown in the Table 5.3 and calculated accordingly. The following materials were depicts the calculation for 1 reaction.

HPIV – Target Hemagglutinin neuraminidase (HN) gene

2X PCR Master Mix	12.5 µL
HPIV1 (F)	0.2 µL
HPIV1 (R)	0.2 µL
HPIV2 (F)	0.2 µL

HPIV2 (R)	0.2 µL
HPIV3 (F)	0.2 µL
HPIV3 (R)	0.2 µL
HPIV4 (F)	0.2 µL
HPIV4 (R)	0.2 µL
Nuclease free water	4.9 µL
Template	1.0 µL
Total volume	20.0 µL

Cyclic condition

Initial Denaturation	-	94 ºC for 15 minutes	
Denaturation	-	94 ºC for 30 seconds	35 cycles
Annealing	-	55 ºC for 30 seconds	
Extension	-	72 ºC for 1 minute	
Final Extension	-	72 ºC for 10 minutes	
Cooling hold	-	4 ºC	

Table 5.3: Oligonucleotide primers of Human parainfluenza virus HN gene

Primers	Sequence(5´-3´)	Base pairs	Gene
HPIV-1 F^{299}	CCGGTAATTTCTCATACCTATG	317	HN
HPIV-1R^{299}	CCTTGGAGCGGAGTTGTTAAG		HN
HPIV-2 F^{299}	AACAATCTGCTGCAGCATTT	507	HN
HPIV-2 R^{299}	ATGTCAGACAATGGGCAAAT		HN
HPIV-3 F^{299}	CTCGAGGTTGTCAGGATATAG	189	HN
HPIV-3 R^{299}	CTTTGGGAGTTGAACACAGTT		HN
HPIV-4A F^{231}	ATGATGGTGGAACCAAGATT	451	P
HPIV-4A R^{231}	AACCAGGGAAACAGAGCTC		P
HPIV-4B F^{231}	CTGAACGGTTGCATTCAGGT	451	P
HPIV-4B R^{231}	AGGACTCATTCTTGATGCAA		P

HPIV – Target Nucleocapsid (N) gene

Oligonucleotide primers for (HPIV) Nucleocapsid gene primer set which shown in the Table 5.4

2X PCR Master mix	12.5 µL
HPIV 1 (F)	1 µL
HPIV 1(R)	1 µL
HPIV 2 (F)	1 µL
HPIV 2 (R)	1 µL
HPIV 3 (F)	1 µL
HPIV 3 (R)	1 µL
RNase free water	0.5 µL
Template	1 µL
Total	20 µL

Cyclic condition

Initial Denaturation	-	94 ºC for 3 minutes	
Denaturation	-	94 ºC for 1 minute	35 cycles
Annealing	-	53 ºC for 1 minute	
Extension	-	72 ºC for 1 minute	
Final Extension	-	72 ºC for 7 minutes	
Cooling hold	-	4 ºC	

Table 5.4: Oligonucleotide primers for Human parainfluenza virus Nucleocapsid gene (N)

Primers	Sequence (5´-3´)	Base pairs	Gene
HPIV-1 F[57]	TCTGGCGGAGGAGCAATTATACCTGG	84	N
HPIV-1R[57]	ATCTGCATCATCTGTCACACTCGGGC		
HPIV-2 F[57]	GATGACACTCCAGTACCTCTTG	197	N
HPIV-2 R[57]	GATTACTCATAGCTGCAGAAGG		
HPIV-3 F[57]	GATCCACTGTGTCACCGCTCAATACC	266	N
HPIV-3 R[57]	CTGAGTGGATATTTGGAAGTGACCTGG		

- ❖ After the addition of water the reaction mixture was spin down and placed in the bench top cooler.
- ❖ Appropriate PCR tubes were arranged in the cold block.
- ❖ 20 µL of master mix were dispensed respectively.
- ❖ Addition of template, positive control, reagent blank and negative controls was added.

5.1.12 Materials for gel electrophoresis

- Agarose gel casting tray
- Electrophoresis chamber
- Gel casting tray
- Electrode leads
- Gel doc system
- Agarose gel powder
- 5X TBE
- Ethidium bromide (10 mg/mL)
- Gel loading dye (6X)
- Molecular weight marker (1000bp)
- Weighing balance

5.1.13 Methodology of gel electrophoresis

- 1.5 g of agarose were weighed in gel preparing bottle, 100 mL of 0.5X TAE buffer was added, mix well and boil it in microwave for 90 seconds or till agarose melts properly.
- Let it cool down to 55 ºC then 3 µL of 1% ethidium bromide were added and mixed well.
- Gel was poured carefully in casting tray. Prevent formation of air bubble.
- Gel was solidified for 30 minutes.
- Comb was removed carefully and placed the gel such that the wells are towards cathode.
- 0.5X TAE buffer was poured in the gel apparatus and ensure that the gel was properly covered with buffer.
- For loading, 2 µL of 6X loading dye was mixed with 5 µL of PCR product and samples were loaded carefully in the well. 2 µL of molecular weight marker was added in one of the well.
- Lead was closed on chamber and attached electrodes. Gel apparatus was allowed at 80V for 40 minutes or till molecular weight marker resolves properly.
- The gel was imaged under gel documentation and the product was compared with molecular weight marker for appropriate band.

5.1.14 Sequence Analysis

PCR product was sequenced according to the standard protocols. Amplicons were purified using QIA quick PCR purification kit (Qiagen) and subjected to cycle sequencing using ABI Prism Big Dye terminator V3.1 cycle sequencing kit. Post-cycle sequencing purification was done using Dye Ex2.0 spin kit (Qiagen).

Sequencing was performed on ABI Prism 310 and sequence alignment and phylogenetic analyses will be done using Mega version 5[294].

5.2 CELL CULTURE

5.2.1 Materials for media preparation and tissue culture work

- Inverted Microscope
- Incubator
- Tissue culture flasks 25cm^2
- Pipettes
- Phosphate buffered saline (PBS)
- Minimum Essential Medium
- Trypsin Phosphate buffered saline Versene Glucose (TPVG)
- Fetal calf serum
- Penicillin and streptomycin (P&S)
- Glutamine vial (5 mL)

Preparation of ingredients for Media

Penicillin and Streptomycin (Concentration: 100 IU of penicillin and 100 µg of streptomycin)

1X10^6 units of crystalline penicillin and 1g of streptomycin were dissolved in 100 mL of PBS. 1 mL of this stock was added to 100 mL of medium to give a final concentration of 100 units penicillin and 100 µg of streptomycin and stored at -20 ºC.

Kanamycin Acid Sulphate (Concentration: 20 µg/mL)

1 g of kanamycin was dissolved in 50 mL millipore double distilled water and mixed well to a final concentration of 20 µg/mL and stored at -20 ºC.

Fungizone : Amphotericin B – 50 mg (Concentration: 20 µg/mL)

50 mg of amphotericin B was dissolved in 5 mL of sterile millipore distilled water and 1 mL of this stock was diluted to 100 mL of sterile millipore distilled water to a final concentration of 20 µg/mL and stored at -20 ºC.

3% L-Glutamine

6 g of L-Glutamine was dissolved 200 mL of sterile millipore distilled water and mix well. It was filtered through 0.22 micron membrane filter and stored at -20 °C.

7.5% Sodium-bi-carbonate solution

22 g of Sodium Bicarbonate was dissolved in 200 mL of sterile Millipore distilled water and filter through Whatman filter paper No.4, autoclavedat 10 lbs for 10 minutes and stored at +4 °C.

Fetal Bovine Serum

Fetal bovine serum was thawed at room temperature and inactivated at 56 °C in water bath for 30 minutes and cooled at room temperature. If floating particles were observed it was filter through Seitz filter and stored at -20 °C.

Trypsin, PBS, Versene, Glucose solution : (TPVG)

Phosphate Buffered Saline

Sodium chloride (NaCl)	-	8 g
Potassium chloride (KCl)	-	0.2 g
Dibasic sodium phosphate anhydrous (Na_2HPO_4)	-	2.88 g
KH_2PO_4 A.R	-	0.2 g
pH	-	7.4

All the above ingredients were dissolved in 1000 mL of sterile distilled water and filtered through Whatman filter paper No.4 and autoclave at 10 lbs for 10 minutes.

2% Trypsin

2 g of trypsin was dissolved in 100mL sterile millipore distilled water stirred for half an hour with help ofmagnetic stirrer. The solution was filteredthrough membrane filterand stored at -20 °C.

0.2% EDTA (Versene)

200 g of EDTA was dissolved in 100 mL of sterile millipore distilled water and autoclaved at 15 lbs / 15 minutes.

10% Glucose

1 g of glucose was dissolved in 10 mL of sterile millipore distilled water and filter through Whatman filter paper and autoclaved at 10 lbs / 15 minutes.

TPVG – 1000 mL

PBS	-	840 mL
2% Trypsin	-	50 mL
0.2% EDTA	-	100 mL
10% Glucose	-	5 mL
P&S	-	5 mL

All the ingredients were mixed and pH was adjusted to 7.4 and stored at -20 °C.

0.4% Phenol Red

0.04 g Phenol red was dissolved in 10ml of double distilled water. Sterilized by autoclaved at 10 lbs for 15 minutes at 121 ºC.

HEPES

115 g HEPES buffer were dissolved in 500 mL of Millipore distilled water and mixed well. Autoclave at 121 ºC, 15 lbs for 15 minutes and allowed cool. pH was adjusted to 7.2 for 250 mL and pH 7.4 for the remaining 250mL. HEPES buffer were aliquoted in 125 mL bottles and mentioned the pH in the label, stored at +4 ºC.

Trypan Blue for cell counting (0.1%)

0.1 g of Trypan Blue was dissolved in 100 mL of phosphate buffered saline, filtered through Whatman filter paper No.4 and stored at +4 ºC.

Methods for the media preparation

The following Table 5.5 illustrates the composition of media and its preparation.

Table 5.5: MEM Preparation Procedure

Ingredients	10% Growth Media	2% Maintenance Media
MEM	861 mL	941 mL
P&S (Penicillin & Streptomycin)	1 mL	1 mL
Phenol red (0.4%)	1 mL	1 mL
Kanamycin	1 mL	1 mL
Fungizone	1 mL	1 mL
3% L-Glutamine	10 mL	10 mL
Fetal Bovine Serum	100 mL	20 mL
2.2g Sodium bicarbonate	20 mL	20 mL
HEPES buffer (IM)	5 mL	5 mL
Total volume	1000 mL	1000 mL

All the ingredients were mixed well by shaking gently and pH was checked and adjusted to 7.2 to 7.4.

Freezing Media

20% FCS with 10% DMSO

MEM	-	70 mL
FBS	-	20 mL
DMSO	-	10 mL

All ingredients were mixed well and aliquoted in 5 mL aliquots in vials and stored at -20 ºC.

5.2.2 Maintenance of Cell Lines

- Tissue culture bottles that showed confluent monolayer were selected by observing them under an inverted microscope.
- Growth medium was removed from the bottle, washed with PBS/MEM without FCS and 5 mL of TPVG (for 25 cm^2) was added dispersing evenly on the monolayer and left in contact with the cells for 2-3 minutes.
- TPVG was removed and the bottle was incubated at 37 °C, until all the cells were detached from the surface.
- The cells were re-suspended in 5 mL of growth media. The suspension was aspirated few times to break cell clumps.
- The cell concentration was determined by counting the cells in haemocytometer.

5.2.3 Cell Counting

- A 0.2 mL of the cell suspension was diluted in 0.2 mL of Trypan blue (0.1% trypan blue).
- It was mixed well with pipette and sufficient volume was aspirated to fill haemocytometer immediately.

- The viable cells (nonviable cells are stained blue and viable cells remain unstained) were counted in each of the four corners of both chambers, avoiding cells lying on the top and to the left.

- If cell clumping was observed, it was discarded and original cell suspension was re-suspended.

- The total number of cells in the suspension was calculated using the following formula.

  Average cell count x dilution factor x 10^4 = - lakhs/mL

  Or C_1 = t x tb x ¼ x 10^4

Where C_1 = initial cell concentration per ml
t = total viable cell count of 4 squares
tb = correction to the Trypan blue dilution
¼ = correction to give mean cells per corner square.
10^4 = conversion factor for counting chamber.

Based on the cell count, sterile flasks/tubes were seeded and incubated at 37 ºC. Cell lines were not passaged beyond 15 times.

5.2.4 Cell seeded in tissue culture tube

The virus isolation was attempt for all processed samples in LLC-MK2 (Rhesus monkey kidney cell line), A549 (Human adenocarcinoma cell line) and MDCK (Madin Darby Canine Kidney epithelial cell line). The cell line was obtained from National Institute of Virology (NIV), Pune and maintained in tissue culture lab facility available in the department of virology, KIPM & R.

Growth medium was dispensed into fresh sterile cell culture tube to be seeded with cells (1 mL). LLC-MK2, A549, MDCK cell lines were propagated independently at a final concentration of 100,000 cells/mL. Each tube was labeled with name of cell line, passage number and date of passage and incubated in horizontal position of stationary racks at 37 ºC in 5% CO_2 environment for 48 hours.

5.2.5 Virus Isolation

- ❖ Growth medium were discarded from tissue culture tube with monolayer of LLC-MK2, A549 and MDCK cells.
- ❖ The tubes were marked with sample number, passage number and date of inoculation (used two control tubes per rack).
- ❖ Growth medium was pipetted out from the tubes and cells were washed twice with PBS
- ❖ 0.2 mL of processed samples was inoculated into tissue culture tubes.
- ❖ Inoculum was allowed adsorption for 1 hour in horizontal position of stationary racks at 37 ºC for 5% CO_2.
- ❖ 1 mL of fresh cell culture medium was added to each tube at the end of adsorption period.
- ❖ Incubated the inoculated tubes at 37 ºC.
- ❖ Cell monolayers were observed for cytopathic effect every 24 hours.
- ❖ HPIV positive clinical isolates demonstrated focal rounding and destruction, occasional syncytia on initial isolation.

5.2.6 Hemadsorption assay (HAD) in tissue culture tube

Materials

- Infected cell culture tube
- Human RBC in Alsever's solution
- Sterile Phosphate buffered saline
- Inverted microscope
- Centrifuge
- Centrifuge tube-15 mL
- Refrigerator

Preparation of 10% stock human RBC suspension

- 5 mL of blood were taken, transfer to 15 mL tube and PBS were added equally.
- Centrifuged at 3000 rpm (700g) for 5 minutes at room temperature, aspirate supernatant and buffy coat.
- RBCs were suspended in cold (2-8 ºC) PBS. Washed with cold PBS until supernatant was cleared (two-three times).
- PBS was aspirated after last wash. Measured remaining volume of cells using graduations on centrifuge tube. Sufficient amount of PBS were added to make a 10% cell solution.
- Hemadsorption were made used 0.4% RBCs solution prepared with cell maintenance medium.
- Stock suspension were stored at 37 ºC and used within seven days of preparation.

Preparation of 0.4% working human RBC suspension

- The working suspension was prepared from 10% suspension on the day of testing.
- 0.4 mL of the 10% suspension was added to 9.6 mL of PBS.

5.2.7 Methodology

- Maintenance medium were aspirated form the tubes to be tested so that the cell monolayer were exposed.
- Medium were transferred in the tube to another sterile, labelled capped tube and place medium at 4 ºC.
- 0.2 mL of 0.4% RBC suspension was added to each culture tube were tested.
- Tubes were incubated horizontally at 4 ºC for 30 minutes. RBC suspension was distributed over the monolayer.

- ❖ Incubation was followed to dislodge RBC lying on the cell sheet.
- ❖ Tubes were examined immediately with inverted microscope with 40X objective.
- ❖ All tubes were read as soon as possible after their removal from the fridge.
- ❖ RBCs were poured then cells were washed with PBS and maintenance medium were added on those that require further incubation.
- ❖ Positive HAD test showed RBCs firmly attached to the monolayer.
- ❖ Negative HAD test showed minimal RBCs attached to the monolayers, with almost all cells floating above the monolayers.

5.2.8 Virus sensitivity

Materials

- Inverted microscope
- TC flasks of LLC-MK2, A549 and MDCK cell line
- 96 well culture plates
- Ice pack
- Barrier tips
- Vortex
- Gloves
- -80 ºC freezer
- CO_2 incubator

Methodology

Preparation of stock virus

- ❖ Sensitivity test were to be performed in LLC-MK2, A549 and MDCK cells at revival. Standard HPIV were procured from NIV, Pune.

- Microscopically was examined 48-72 hours monolayer T25 flask culture of LLC-MK2, A549 and MDCK cells.
- Growth medium was pipette out from the cell culture flasks and cells were washed twice with PBS.
- 0.5 mL of standard virus was transferred to the 25 cm^2 flask.
- The virus was allowed to adsorb for one hour at 37 ºC.
- 5 mL of maintenance media were added and incubated the inoculated flask at 37 ºC.
- Bottles were examined daily microscopically for identified cytopathic effect (CPE).
- If CPE were observed 3+ to 4+ harvest the supernatant using a 5mL pipette, mixed the contents of the flask and transferred to a 15mL centrifuge tube.
- Centrifuged at 1,500 rpm for 10 minutes.
- Supernatant were collected and aliquoted viral stocks in 1.5 mL vials.

5.2.9 Titration of standard Human parainfluenza virus in three different cell lines

- LLC-MK2 cell suspension were prepared approximately 3-5 X 10^5 cells/mL, for A549 2-3X10^4 cells/ml, for MDCK 5-6 X 10^5 cells/ml in each cell line used separate 96 well tissue culture plate.
- 100 µL of cell suspension were added to each well of 96 well flat bottom tissue culture plates using multi-channel pipette.
- Incubated at 37 ºC incubator for 1-2 days until confluent monolayer were formed.

Virus dilutions

- 5 mL of glass dilution tubes were arranged as 8 tubes and label the dilution tubes $10^{-1 \text{ to}}\, 10^{-8}$.
- 1.8 mL of maintenance medium was added to tube1-tube 8.
- 0.2 mL of original virus suspension were added to the first tube used a sterile micropipette and vortex gently.
- Used another pipette tip, transferred 0.2 mL to the second tube and discard pipette tip. The dictum were mixed, dispensed and discard.
- Dilution steps were repeated, transferred 0.2 mL each time and always changed pipette tip between dilutions, up to 8 (dilution 10^{-8}).

96 well plate

- Cell growth medium were decanted and 100 µL of maintenance medium were added to all the wells.
- 100 µL of virus dilutions were added (10^{-1} to 10^{-8}) to wells 1 to 10 of rows A to G, 10 wells per dilutions, starting from the highest dilution to the lowest.
- Maintenance medium were added to well A12 to H12 in rows A to F, for the cell controls.
- Incubated at 37 ºC in CO_2 incubator. Development of CPE were examined, used an inverted microscope.
- For a valid test the cell control showed complete monolayer of healthy cells. Calculate the $TCID_{50}$ of the virus using Reed and Meunch method.

5.2.10 Plaque assay

Materials

- 24 well plate
- Cell growth media
- PBS, without calcium and magnesium
- Agarose
- Microfuge tubes
- Microwave oven
- Vortex
- Various tissue culture grade sterile bottles of appropriate size (100-250 mL)
- 0.2% crystal violet (in 20% ethanol) made up from stock 5% crystal violet in 100% methanol.
- Methanol : Acetic acid solution (3:1)

5.2.11 Methodology

- ❖ Cells were seeded at $4.1X10^5$ cells/mL for LLC-MK2 and $2.1X10^6$ cells/mL for A549 cells.
- ❖ 2.0 mL of growth media were added with cells to each well. Jiggle the plate to evenly distribute cell, 12 O' clock to 6 O' clock and 9 O' clock to 3 O' clock (left-right, top-bottom).
- ❖ Incubated plates at 37 ºC 5% CO_2 incubator for 48 hours.

Preparation of virus dilutions

- Prepared eight tubes, each tube will contain 1000 µL of MEM (no serum) except first tube.
- 990 µL of MEM (no serum) was added to the first tube.
- 10 µL of HPIV strains was added to the first tube and vortex gently.
- Repeated the dilution process through all eight tubes.
- The tubes had effective dilutions of virus 10^{-1}(1/10), 10^{-2}(1/100), 10^{-3} (1/1000), 10^{-4}(1/10,000), 10^{-5}(1/100,000), 10^{-6}(10,00,000), 10^{-7}(100,00,000), 10^{-8}(1/10,00,00,000).

Infection of the monolayer

- 1 mL of growth media were discarded from each well. One mL of media should now remain on each monolayer.
- 100 µL of each dilution were added in duplicate to each well, allowed the virus flow gently into the media.
- Infected monolayers were incubated at 37 ºC for one hour, mildly shaken the plates gently several times during the adsorption period.

5.2.12 Agarose overlay

- 1% agarose were prepared in distilled water by autoclaved at 121 ºC for 20 minutes.
- Agarose were stored on the shelf at room temperature or used immediately after equilibrating in a 65 ºC water bath.
- Alternatively, 100 mL aliquots of solidified agarose was melted in a microwave for about 1 minute and allowed cool at 65 ºC in a water bath.
- Plaquing media were warmed at 37 ºC water bath until equilibrated.
- Gently draw media out of each LLC-MK2 and A549 infected monolayer well and discard.
- Equal volume of media (i.e. 2 plates, 48 wells, 96 mL) in a 37 ºC pre-warmed container and equal volume agarose were added to the bottle with swirling (1:1) and vigorously shaken to mix with each other.
- With a new pipette, 2 mL of the agarose/growth media to each well were added, pipetting it down the side of the well. Plate was allowed to solidify for 15 minutes in the level hood at room temperature as the agarose overlay turns solid.
- Plates were incubated at 37 ºC and 5% CO_2 humidified atmosphere.

- ❖ Plaques were visualized at days 10 after infection and to desire can stain the monolayer to visualize and plaques were counted on the final day of plaque development.
- ❖ With the naked eye, white dots were visualized on the monolayer. These dots may be more easily visualized by viewing the plate with oblique light. It was critical to confirm that the dots are plaques by inspection under a microscope.
- ❖ Plaques were counted and verified by stained the cells to facilitate visualization.
- ❖ Plaques were stained to fix the cells through agarose overlay by addition of 2 mL Methanol: Acetic acid solution (3:1 Methanol: Acetic acid). Plates were allowed for 30 minutes at room temperature.
- ❖ Under gently flowed with warm tap water, carefully rinsed out agarose layer. Direct stream of water to side of well and should gently slid agarose layer off. Agarose layer were discarded.
- ❖ Minimal amount of 0.2% crystal violet were added to each well, just enough to cover bottom. Allowed to slow for roughly 30 minutes on orbital shaker.
- ❖ Gently rinsed each well with water. Tap off excess water, allowed monolayer to dry and plaques were counted.

5.2.13 Calculation of plaque forming units (PFU)

The viral titer is a quantitative measurement of the biological activity of a virus and is expressed as plaque forming units (pfu) per mL. To calculate the viral titer, count the number of well isolated plaques.

Formula

$$PFU/mL = \frac{\text{Average number of plaques}}{D \times V}$$

D = Dilution factor

V = Volume of diluted virus added to the well.

Multiplicity of infection

Multiplicity of infection (m.o.i.) exists the average number of virus particles which infect a cell. In order to calculate the MOI, first determine the number of cells are infecting, and the titer of the virus inoculated on them.

5.2.14 Neuraminidase treatment

Materials

- Infected cells
- 24 well plate
- Neuraminidase obtained from (*Clostridium perfringens)* (Type X, Sigma scientific, N-2133)
- CO_2 Incubator

Methodology

- ❖ Growth medium was dispensed into 24 well plates to be seeded with cells (1ml). LLC-MK2, A549 cell lines were propagated independently at a final concentration of 100,000 cells/mL.
- ❖ Monolayers were incubated at 37 ºC in 5% CO_2 environment for 48 hours.
- ❖ Monolayer cells were infected with HPIV at an MOI 5.
- ❖ Following aspiration of the inoculum 90 minutes later the medium was replaced with 1mL of serum free medium with containing 1U of *Clostridium perfringens* neuraminidase and cells were incubated at 37 ºC for 18 hours.
- ❖ After neuraminidase treatment further to ensure the hemadsorption assay

5.2.15 Hemadsorption assay

Materials

- Infected cells
- Neuraminidase*(Clostridium perfringens)*(type X, Sigma scientific, N-2133)

- Guinea pig RBC in Alsever's solution
- Sterile Phosphate buffered saline
- Inverted microscope
- Centrifuge
- Centrifuge tube-15mL
- Refrigerator

Methodology

- ❖ Growth medium was dispensed into 24 well plates to be seeded with cells (1 mL). LLC-MK2, A549 cell lines were propagated independently at a final concentration of 100,000 cells/mL.
- ❖ Monolayers were incubated at 37 ºC 5% CO_2 environment for 48 hours.
- ❖ Monolayer cells were infected with HPIV at an MOI 0.1.
- ❖ Incubated at 37 ºC 5% CO_2, for one hour.
- ❖ After one hour adsorption period the medium was replaced with 1 mL of serum free medium with containing 0.1U of *Clostridium perfringens* neuraminidase and cells were incubated at 37 ºC for 18 hours.
- ❖ 0.5% human RBC – 300 µL added every well.
- ❖ Incubated at 4 ºC for 2 hours.
- ❖ The cell monolayers were then washed four times with cold serum free medium and photographed.
- ❖ Unbound erythrocytes were removed by washing, and the percentage of hemadsorption positive cells were determined.
- ❖ Level of adsorbed RBC was estimated.
- ❖ Hemadsorption was determined after removing the enzyme.
- ❖ Quantification of the bound RBC was achieved by RBC lysis with 250 mL of 50 mM ammonium chloride (NH_4Cl).
- ❖ Transferred into 96 well plates and the optical density (OD_{540}) absorbance at 540 nm using multiwell plate reader.

5.2.16 Neuraminidase assay

Materials

- Black 96 well flat bottom plates
- Adhesive plate sealers
- Human parainfluenza virus (HPIV) isolates
- MES buffer (325 mM 2-Morpholinoethanesulfonic acid) (Sigma Life science M-2933)
- 100 mM Calcium chloride
- MUNANA (2'2'-(4-Methylumbelliferyl)-a-D-N-acetylneuraminic acid sodium salt hydrate as a substrate (Sigma Aldrich M8639)
- 4-methylumbelliferone sodium salt (Sigma Life science M 1508)
- 0.1% Bovine serum albumin
- Neuraminidase sourced from *Clostridium perfringens* (Type X, Sigma scientific, N-2133)
- 1 M glycine
- Absolute ethanol
- Sodium hydroxide
- Distilled water
- Spectrofluorometer (355 nm and 460 nm filters)
- Plate shaker
- Incubator
- Aluminum foil

Preparation of buffers and solution

All solutions and buffers ought to be stored at room temperature unless otherwise stated. Working solutions for use in the assay were prepared from master stock solutions where stated, for accuracy. The working solution of MUNANA (substrate) must be made freshly for each assay.

Preparation of bacterial neuraminidase

Neuraminidase	**:**	8.3 µg/mL 0.1% cold BSA

Master stock solution and buffers

MES buffer	**:**	12.6 g MES buffer in 200 mL of distilled, pH to 6.5 concentrate NaOH
100mM $Cacl_2$	**:**	1.11 g calcium chloride in100 mL distilled
1M glycine	**:**	7.5 g Glycine in 100 mL distilled water
100µM MUNANA	**:**	0.97882 mg in 2 mL of MES buffer stored at - 20 ºC

Working solutions and buffers

MES assay buffer	**:**	32.5 mM MES	:	50 mL of 325 mM MES stock solution
		4 mM $CaCl_2$	:	20 mL of 100 mM $CaCl_2$
		Distilled water	:	430 mL
100 µM MUNANA	:	100 µL of 100 µM stock solution with 2.9 mL assay buffer		
Stop solution (500 mL)	:	0.1 M Glycine	:	50 mL (1M stock solution)
		25% Ethanol	:	125 mL absolute ethanol
	:	Distilled water	:	325 mL pH to 10.7 to a concentrated NaOH

Methodology

Stability of bacterial neuraminidase

- Neuraminidase (16.7 µg/mL) *Clostridium perfringens* was prepared in 0.1% BSA.
- The stability of neuraminidase (NA) in BSA was monitored by determining the reaction activity of NA using optimized conditions.

4-Methylumbelliferone sodium salt standard curve

- 100 µL stop solution was added in all the wells.
- 100 µL 4-Methylumbelliferone sodium salt at 20 µM concentration were added in first well A1 to A12.
- Serial dilute in first well to take 100 µL from A1 to B1 and from B1 to C1 and so on up to G1, finally 100 µL were discarded given in the table below.
- The intensity of fluorescence was measured at an excitation wavelength of 355 nm and an emission wavelength of 460 nm.
- The volume of 100 µL essentially measured as this was equal to the final volume which measured in NA activity and IC_{50} assays.

5.2.17 Neuraminidase determination (MUNANA assay)

- ❖ 20 µL of MES assay buffer were added in each well.
- ❖ 20 µL of HPIV reference strains (10^{-3}) were added to first well A1 and B1, HPIV reference strains (10^{-4}) for first well C1 and D1 represented in the Table 5.6.
- ❖ 20 µL of clinical HPIV strains (clinical samples 10^{-3}) were added to first well E1 and F1, clinical samples (10^{-4}) for first well G1 and H1.
- ❖ Buffer and virus were mixed properly by up and down several times.
- ❖ Viruses were serial diluted by down the plate by carrying over 20 µL from row A1 to row A2 and so on, stopping at row A10. Discard 20 µL from A10. The final column of the plate contains buffer only as a blank control.
- ❖ 3 mL of MUNANA substrate were prepared as working stock (100 µM) per plate and 30 µL were added to each well including the blank column A11-H11.
- ❖ Plate were sealed and incubated at 37 ºC for 60 minutes with shaken in the dark.

- ❖ Reaction was terminated by addition of 150 µL stop solution to all wells.
- ❖ Plate reading was taken within 30 minutes with addition of stop solution.
- ❖ Relative fluorescence units were observed at a 365 nm excitation wavelength and a 450 nm emission wavelength.
- ❖ The data plotted as relative fluorescence units (RFU) against virus with the mean blank (buffer only) value subtracted.

Table 5.6: Determination of enzyme activity Neuraminidase assay

	1	2	3	4	5	6	7	8	9	10	11 Buffer only	12 4MUSS
A 10^{-3}	→									→	↓	↓
B 10^{-3}	→									→		
C 10^{-4}	→									→		
D 10^{-4}	→									→		
E 10^{-3}	→									→		
F 10^{-3}	→									→		
G10^{-4}	→									→		
H 10^{-4}	→									→	↓	↓

5.3 COMPOUNDS USED AGAINST HPIV

(i) Neuraminidase inhibitor (Zanamivir) (Sigma Life science SML 0492)

(ii) Nucleoside analogue (Ribavirin) (Sigma Life science R9644)

Zanamivir was performed used at 100 µM to 1000 µM concentration against Human parainfluenza virus for cytotoxic activity.

Ribavirin was performed at 100 µM to 1000 µM concentration against Human parainfluenza virus for cytotoxic activity.

5.3.1 Cytotoxicity assay

The effects of the drug on the growth of uninfected LLC-MK2 and A549 cells in 96 well plates was determined by using a live/dead viability/cytotoxicity. Cell viability and cytotoxicity assays were used for drug screening and cytotoxicity tests of chemicals. Three methods were used to proliferate the cell viability in vitro by

(i) Cell proliferation assay (MTT assay)
(ii) Neutral red dye uptake into cells
(iii) Trypan blue exclusion method

Different concentration of test drugs were evaluated in LLC-MK2 and A549 cell line in 96-well flat-bottomed microplates at 24h, 48h and 72hours.From these results, 50% cytotoxic end points (50% cell-inhibitory concentration [IC_{50}])were determined. Later, the compounds were assayed for toxicity in actively proliferating in both the cell lines.

Materials

- 96 well plate
- MTT
- Trypan blue
- Neutral red
- Zanamivir (Sigma Life science SML 0492)
- Ribavirin (Sigma Life science R9644)

(i) MTT assay (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole)

Materials

- MTT
- PBS
- 96 well plate
- Cell lines (LLC-MK2 and A549)

- Multiwell plate reader
- Micropipette and tips
- Vortex

Preparation of MTT

Procedure

- ❖ Cells were prepared at $4.1x10^5$ cells/mL for LLC-MK2 and $2.1x10^6$ cells/mL for A549 cells in 96 well plates. Cells were added 100 µL/well.
- ❖ 2.0 mL of growth media were added with cells to each well.
- ❖ Plates were incubated at 37 ºC in 5% CO_2 incubator for 48 hours.
- ❖ Growth medium were removed and 0.01 to 1000 µM concentration of compound were added on the growth of uninfected LLC-MK2 and A549 cells in 96 well plates.
- ❖ 100 µM (from 1mM concentration) concentration of compound were added in row A1 to A9 and B1 to B9 duplicate plates, 200 µM concentration in row C1 to C9, 300 µM concentration in row D1 to D10 duplicate plates and so on. Add 100 µL of MTT alone were added in row A10 to H10. A11 to H11 as drug control and A12 to H12 consider as cell control shown in the table below.
- ❖ Plates were incubated at 37 ºC in 5% CO_2 incubator for 72 hours.
- ❖ Photograph were taken at 0 hours, 48 h and 72 h.
- ❖ Medium was removed and 50 µL of MTT were added in all the wells including drug and cell control.
- ❖ Incubated at 37 ºC for 2 hours.
- ❖ Cells were viewed periodically under an inverted microscope for presence of intracellular punctuate purple precipitate.

- ❖ When the purple precipitate was clearly visible under the microscope 100 µL of Dimethylsulfoxide were added to all wells, including drug and cell controls. Gently swirled and not to be shaken.
- ❖ The plate was mixed on an orbital shaker until the reaction product was completely dissolved.
- ❖ Plates were revolved to crystal formation to be viewed under microscope.
- ❖ The coloured MTT formazan reaction product were measured at 570 nM with a Vmax muliwell plate reader.
- ❖ Average values were determined from duplicate readings and subtract the average value for the blank. Plot absorbance against number of cells/mL.
- ❖ Absorbance values that are lower than the control cells indicate a reduction in the rate of cell proliferation. Conversely a higher absorbance rate indicates an increase in cell proliferation. Rarely, an increase in proliferation may be offset by cell death, evidence of cell death may be inferred form morphological changes.

(ii) Neutral red uptake assay

Materials

- Neutral red
- 96 well plate
- Distilled water
- Cell cultures
- CO_2 incubator
- Compound
- PBS
- MEM media
- 1% Glacial acetic acid
- 50% Ethanol
- Multiwell plate reader

Preparation of Neutral red

0.33 g of Neutral red powder was added in 100 mL of distilled water, filter sterilized and stored at room temperature, protected from light. On the day of use, the reagent was diluted in media to obtain a final concentration of 33 µg/mL.

Preparation of Neutral red desorb

1% Glacial acetic acid : 0.5 mL of glacial acetic acid in 45.5 mL of water.
50% Ethanol : 50 mL of ethanol in 100 mL of water.
1% glacial acetic acid and 50% ethanol solution made in water.

Procedure

- Cells were prepared at $4.1X10^5$ cells/ml for LLC-MK2 and $2.1X10^6$ cells/mL for A549 cells in 96 well plates. Cells were added 100 µL/well.
- 2.0 mL of growth media were added with cells to each well.
- Plates were incubated at 37 ºC in 5% CO_2 incubator for 48 hours.
- Growth medium was removed and 0.01 to 1000 µM concentration of compound were added on the growth of uninfected LLC-MK2 and A549 cells in 96 well plates.
- 100 µM (from 1 mM concentration) concentration of compound were added in row A1 to A9 and B1 to B9 duplicate plates, 200 µM concentration in row C1 to C9, 300 µM concentration in row D1 to D9 duplicate plates and so on. Add A11 to H11 as drug control and A12 to H12 consider as cell control shown in the table below.
- Incubated at 37 ºC in 5% CO_2 incubator for 72 hours.
- At 24 hours later cells were washed with pre warmed PBS and add 125 µL of Neutral red reagent in each well.
- The plates were incubated at 37 ºC 5% CO_2 incubator for 5 hours.
- After incubation plates were taken out, gently washed three times with PBS.

- ❖ 100 µL of Neutral red were added to desorb in all wells.
- ❖ Plates were protected light and shaken on a shaker at 60 rpm of 30 minutes followed by keeping the plates still for 5 minutes.
- ❖ Plates were taken to 96 well plate reader and their absorbance measured in each well at 540 nm.

(iii) Trypan blue exclusion method

Materials

- Trypan blue dye
- PBS
- Glass slide
- Cover slip
- 1ml pipette
- Distilled water
- Haemocytometer

Preparation of trypan blue (0.4%)

0.4% trypan blue solution in phosphate buffered saline and store at room temperature.

Procedure

- ❖ Cells were prepared at $4.1x10^5$ cells/mL for LLC-MK2 and $2.1x10^6$ cells/mL for A549 cells in 96 well plates. Cells were added 100 µL/well.
- ❖ 2.0 mL of growth media were added with cells to each well.
- ❖ Plates were incubated at 37 ºC in 5% CO_2 incubator for 48 hours.
- ❖ Growth medium was removed and 0.01 to 1000 µM concentration were added on the growth of uninfected LLC-MK2 and A549 cells in 96 well plates.
- ❖ 100 µM (from 1 mM concentration) concentration of compound were added in row A1 to A9 and B1 to B9 duplicate plates, 200 µM concentration in row

C1 to C9, 300 µM concentration in row D1 to D9 duplicate plates and so on. Add A11 to H11 as drug control and A12 to H12 consider as cell control shown in the table below.

- Incubated at 37 ºC in 5% CO_2 incubator for 72 hours.
- Medium was removed and washed with PBS.
- Trypsin were added and allowed for 3 minutes and trypsin was discarded.
- Maintenance media were added scarped the cells and cell suspension with drug were transferred to the tubes.
- Cell density of suspension was determined by haemocytometer.
- 0.1 mL of trypan blue stock solution was added to 1 mL of cells.
- If cells were exposed to Trypan blue for extended periods of time, viable cells, as well as non-viable cells, may begin to yield up dye.
- With the cover – slip in place, used a Pasteur pipette or other suitable device to transfer a small amount trypan blue cell suspension mixture to both chambers of the haemocytometer.
- Carefully touch the edge of the cover slip with pipette tip and allowed each chamber to fill by capillary action.
- Starting with chamber 1 of the haemocytometer, count all the cells in the 1mm center square and four 1mm corner square.
- Non-viable cells resolve stain blue. Keep a separate count of viable and non-viable cells.
- Cells were counted on top and left touching middle line of the perimeter of each square, cells touching the middle line at bottom and right sides were not counted.
- Numbers of blue staining cells were counted and number of total cells. Cell viability ought to be at least 95% of healthy log-phase cultures.

Calculation

% of viable cells= (1.0-(Number of blue cells ÷ Number of total cells) X 100

Calculate the number of viable cells per mL of culture, use formula. Correct for the dilution factor.

Number of viable cells x 10^4x 1.1=cells/mL culture.

5.4 ANTIVIRAL ACTIVITY OF ZANAMIVIR AND RIBAVIRIN AGAINST HPIV

(i) Hemadsorption inhibition assay
(ii) Plaque reduction assay
(iii) Neuraminidase inhibition assay

5.4.1 Hemadsorption Inhibition assay

Materials

- Infected cells
- 24 well plate
- Cold serum free media
- Human RBC
- Neuraminidase sourced from (*Clostridium perfringens*Type X, Sigma scientific, N-2133)
- Various concentrations of drugs
- Incubator
- Phosphate buffered saline
- Ammonium chloride (NH_4Cl)
- 96 well plate
- Elisa reader

Procedure

❖ LLC-MK2 and A549 cells were seeded in 24 well plates ($4x10^5$ to $6x10^5$ cells/well).

- ❖ Cells were infected with HPIV at an MOI (0.1) in 24 well plates.
- ❖ Incubated at 37 ºC for 90 minutes.
- ❖ The medium was replaced with 1ml of serum free medium containing 0.1U of *Clostridium perfringens.*
- ❖ Incubated at 37 ºC for 18 hours.
- ❖ The medium was aspirated and replaced with 0.5% human RBC in serum free medium containing various concentration of drugs (100 µM - 1000 µM).
- ❖ Cell monolayers were leveled with bubble level and placed at 4 ºC for two hours.
- ❖ The wells were washed with cold serum free medium.
- ❖ Quantification of the bound RBC was achieved by RBC lysis with 250 mL of 50 mM NH_4Cl.
- ❖ Absorbance was read at 540nm on enzyme linked immunosorbent assay reader.

5.4.2 Plaque reduction assay

Materials

- 24 well plate
- Cell growth media
- Various concentrations of drugs
- PBS, without calcium and magnesium
- Agarose
- Microfuge tubes
- Microwave oven
- Vortex
- Various tissue culture grade sterile bottles of appropriate size (100-250 mL)
- 0.2% crystal violet (in 20% ethanol) made up from stock 5% crystal violet in 100% methanol.
- Methanol : Acetic acid solution (3:1)

Procedure

- LLC-MK2 and A549 cells were seeded in 24 well plate ($4x10^5$ to $6x10^5$ cells/well).
- 1 mL of growth media were discarded from each well. One ml of media ought now remain on each monolayer.
- Cells were inoculated with 100 PFU of HPIV in the presence of various concentrations of drugs (100µM-1000 µM).
- Plates were incubated at 37 ºC for one hour, mildly shake the plates gently several times during the adsorption period.
- 1% agarose were prepared in distilled water by autoclaving at 121 ºC for 20 minutes.
- 100 mL aliquots of solidified agarose can be melted in a microwave for about 1 minute and cooled at 65 ºC in a water bath.
- Warm the plaquing media at 37 ºC water bath until equilibrated.
- Gently draw media out of each LLC-MK2 and A549 infected monolayer well and discard.
- Equal volume of media (i.e. 2 plates, 48 wells, 96 ml) in a 37 ºC pre-warmed container and equal volume agarose were added to the bottle with swirling (1:1). Vigorously to mix.
- With a new pipette, 2 mL of the agarose/growth media were added to each well, pipetting it down the side of the well. Plates were allowed to solidify for 15 minutes in the level hood at room temperature as the agarose overlay turns solid.
- Incubated the plates to a humidified incubator at 37 ºC in 5% CO_2 incubator.
- Plaques were visualized by day 10 after infection and to desire can stain the monolayer to visualize and plaques were counted on the final day of plaque development.

- ❖ With the naked eye, white dots were visualized on the monolayer. These dots may be more easily visualized by viewed the plate with oblique light. It was critical to confirm that the dots are plaques by inspection under a microscope.
- ❖ Plaques were counted and verified by staining cells to facilitate visualization.
- ❖ Plaques were stained with fixed cells through agarose overlay by addition of 2 mL Methanol:Acetic acid solution (3:1 Methanol:Acetic acid). Allowed for 30 minutes at room temperature.
- ❖ Under gently flowing warm tap water, carefully rinse out agarose Layer. Direct stream of water to side of well and should gently slid agarose layer off. Agarose layer were discarded.
- ❖ Minimal amount of 0.2% crystal violet were added to each well, just enough to cover bottom. Slowed for roughly 30 minutes on orbital shaker.
- ❖ Gently rinsed each well with water. Tap off excess water, allowed monolayer to dry and plaques were counted.
- ❖ Plaques in the control (no drug) and experimental wells were counted under microscope.
- ❖ For experiments presenting plaque area measurements as a function of drug concentration were measured under microscope.

5.4.3 Neuraminidase inhibition assay

Materials

- Black 96 well flat bottom plates
- Adhesive plate sealers
- HPIV isolates
- 4-GU-DANA (Zanamivir)
- 325 mM 2-Morpholinoethanesulfonic acid (MES) (Sigma Life science M2933)
- 100 mM Calcium chloride

- MUNANA (2’2’-(4-Methylumbelliferyl)-a-D-N-acetylneuraminic acid sodium salt hydrate (Sigma Aldrich M8639)
- 4-methylumbelliferone sodium salt (Sigma Life science M1508)
- Neuraminidase sourced from *Clostridium perfringens* (Type X, Sigma scientific, N-2133)
- 1 M glycine
- Absolute ethanol
- Sodium hydroxide
- Distilled water
- Spectrofluorometer (355 nm and 460 nm filters)
- Plate shaker
- Incubator
- Aluminum foil

Methodology

4-Methylumbelliferone sodium salt standard curve

- ❖ 100 µL stop solution was added in A11 to G11wells.
- ❖ 100 µL 4-Methylumbelliferone sodium salt was added in first A11 only.
- ❖ Serial dilution was done in first well to take 100 µL from A11 to B11and from B11 to C11 and so on up to G11, finally 100 µL discard given in the table below.
- ❖ Fluorescence activity was measured the 4-MUSS titration series.
- ❖ The volume of 200 µL necessity is measured as this was equal to the final volume which measured in NA activity and IC_{50} assays.

Neuraminidase determination (MUNANA assay)

- ❖ HPIV strains were diluted appropriately in MES assay buffer.
- ❖ 100 µl of diluted virus were added to 2 columns from A-G of a black flat bottomed 96 well plate (column 1,2 wells A-G virus 1 for Reference strains, column 3,4 wells A-G virus for clinical sample shown in the Table12.3.

- ❖ 10 fold dilutions of drug were prepared in an eppendorf tubes.
- ❖ 10 µL of each drug dilution were added to a full row of a 96 well plate (Row A1-12) 100nm, row B1-12:100nm, row C1-12:10nm). Ensure that the virus and drug were mixed properly.
- ❖ Plate were sealed and incubated at 37 ºC for 30 minutes with shaking.
- ❖ 3 mL of MUNANA working stock (100 µM) per plate were prepared and 30 µL of substrate were added to each well including the blank row H, ensuring virus or drug and substrate mix.
- ❖ Plate were sealed and incubated for 60 minutes at 37 ºC with shaking, in the dark.
- ❖ The reaction was terminated by addition of 150 µL stop solution to all wells.
- ❖ The plates are plotted as Relative Fluorescence units (RFU) against neuraminidase inhibitor concentration, with the mean blank (buffer only) value subtracted.
- ❖ Neuraminidase inhibition assay was defined as the concentration of the compound required to reduce the NA activity of the treated virus to 50% of that of the control virus.
- ❖ IC_{50} was calculated by plotting the percentage of fluorescence inhibition (relative to that for the control) versus the log concentration of the compounds.
- ❖ The data provided represent the mean value ±SDs from at least three independent experiments.

5.4.4 Detection of messenger RNA (mRNA) of HPIV-2

Materials

- Infected cell culture
- RNA extraction kit
- cDNA conversion kit

- Taq polymerase master mix (Phusion DNA PCR Kit (New Englands BioLabs))
- Nuclease free water
- Positive control
- Microcentrifuge tube (1.5 mL)
- PCR tubes
- Sterile tips
- Micropipettes (0.5-10 µL, 20-200 µL)
- Microcentrifuge
- Ribavirin
- Nucleocapsid gene (primer set)
- Thermal cycler (Gene Amp PCR System 9700 (ABI))
- Agarose

Procedure

RNA extraction as in section 5.1.7

cDNA conversion as in section 5.1.8

- ❖ Cells were cultured at 37 ºC in humidified atmosphere with 5% CO_2 in 24 well plate.
- ❖ Monolayer of cells were infected with HPIV at an m.o.i.(1)
- ❖ 3.1 µM of Ribavirin were added.
- ❖ Incubated at 37 ºC with 5% CO_2 for 8 days.
- ❖ Observed if compound able to inhibit the HPIV 2 by microscope.
- ❖ RNA was extracted from cultured cells ($2x10^6$ cells) used QIAmp viral Qiagen RNA Extraction kit.
- ❖ cDNA was synthesized using random primer.
- ❖ PCR were performed with forward and reverse primers for nucleocapsid protein (NP).

Table 5.7: Oligonucleotide primers for Nucleocapsid gene – HPIV type 2

Primers		Sequences	Basepairs	Genes
HPIV-2	Forward[300]	5'CATGGCCAAGTACATGGCTC3'	400bp	Nucleocapsid protein
	Reverse[300]	5'CCTCCGAGTATCGATTGGATTGAA3'		

5.5 ANTIVIRAL ACTIVITY OF GLYCYRRHIZA GLABRA (GLYCYRRHIZIC ACID) AGAINST HPIV

A purified compound Glycyrrhizic acid from Licorice root were purchased from Sigma Aldrich (G2137)

5.5.1 Cytotoxic assay as in section 5.4.1

5.5.2 Antiviral activity of Glycyrrhizic acid

(i) Hemadsorption inhibition assay as in section 5.4.2

(ii) Plaque reduction assay as in section 5.4.2

(iii) Neuraminidase inhibition assay as in section 5.4.2

5.6 INSILICO ANALYSIS FOR VIAL PROTEIN AGAINST 4-GU-DANA

Ligand and receptor selection

The five different hemagglutinin neuraminidase proteinof HPIV type 3 was retrieved from the RCSB Protein Data Bank. The PDB was a key resource in areas of structural biology and a key repository for 3-dimensional structure data of HN Human parainfluenza virus type 3 strain complex with 4-GU-DANA. The PDB ID viz., 1V2I, 1V3b, 1V3d, 1V3e and 4MZA a resolution factor and the method of incorporation are X-ray diffraction method $2.2A^0$, $2.0A^0$, $2.28A^0$, $1.89A^0$ and $1.65A^0$ respectively. The interactions between HN glycoprotein receptors from Human parainfluenza virus type 3 and lead binding via computational docking methods. The binding interactions between 4-GU-DANA and HN glycoprotein were assessed by docking methods used Auto dock vina software.

Autodock

An adequate 3-dimensional model of the receptor pocket was planned to dock ligands to identify the compounds of 4-GU-DANA were docked with five different kinds of HPIV type 3 HN receptors proteins to find out the antihuman parainfluenza virus activity by molecular docking parameters. Each receptor were linked to a particular cellular biochemical pathway although several receptors are found in most cells, each receptor were bound only with ligands of a specific structure, abundant like how docks only accept specifically shaped keys. When a ligand binds to its corresponding receptor, it activates or inhibits the receptor associated biochemical pathway. Finally, the study analysed the types of interactions involved between Zanamivir with five different receptor sites of HPIV type 3 HN protein and to analysed hydrophobic, electronic and steric characteristics of Zanamivir, the role of water molecules in drug receptor interactions.

Equipments used in this study

Name		**Manufacturer**
1. Inverted microscope	-	Nikon
2. Centrifuge	-	Eppendorf and Remi
3. CO_2 Incubator	-	Binder
4. Vortex mixer	-	Remi
5. Micropippettes	-	Gilson / Finn pipette
6. Spectrofluorometer	-	Perkin Elmer
7. Thermocycler	-	Applied Biosystems
8. Gel documentation system	-	Alpha imager

Statistical analysis

The Data was statistically analyzed by the software IBM SPSS.23 version. Specific type distribution was assessed by ANOVA and posthoc Dunnet analysis were performed in various variables such as sex, age, monthandpositivity were performed. Degrees of freedom were set as 0.05 level. Comparison with different viral species was analyzed. Further Linear regression were performed for yearwise analysis ofsignificance in samples. Ms-Excel was used for the graphical plotting of variables in different year and seasons using standard error mean.

Results and Analysis

RESULTS AND ANALYSIS

6.1 SURVEILLANCE OF HUMAN PRAINFLUENZA VIRUS IN CHENNAI

6.1.1 Surveillance

Samples were collected from symptomatic patients referred from different Government hospitals in Chennai and were detected for Human parainfluenza virus by Multiplex reverse transcription PCR. The positivity of HPIV-2 and 3 were tabulated in the Table 6.1.

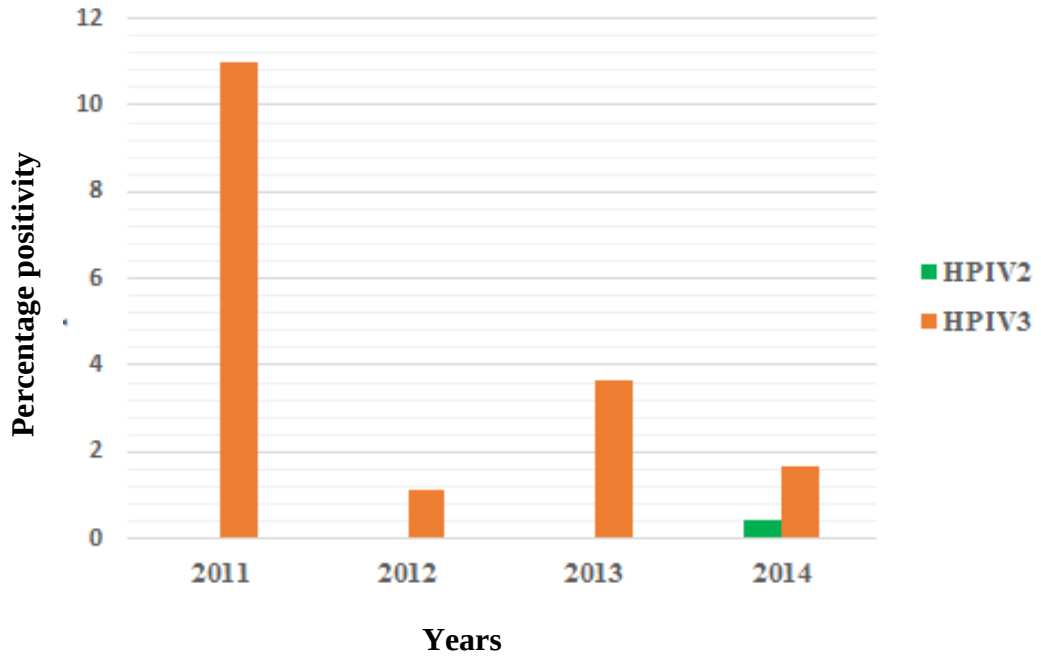
Table 6.1: HPIV positives by Multiplex RT-PCR

Year	Total No. of samples screened	HPIV-2 Positives	HPIV-3 Positives
2011	236	-	26
2012	264	-	3
2013	191	-	7
2014	240	1	4
Total	**931**	**1**	**40**

Among the four years 2011 to 2014, 2011 had increased positivity percentage of HPIV-3 but HPIV-2 positivity was seen only in 2014. HPIV serotypes 1 and 4 were not detected during this period, since they are rare when compared to the other two strains.

The study describes that the prevalence of circulating HPIV were detected in Chennai. HPIV type 3 was more predominant in all the years followed by HPIV type 2 was shown in the Figure 6.1.

Figure 6.1: Year wise Distribution of HPIV positive percentage by mRT-PCR from 2011-2014



The highest positive percentage of HPIV-3 was observed in the year 2011 and it decreased in the subsequent years. But, in 2013 positive percentage of HPIV-3 was higher than in 2012 and 2014. The positive rate of HPIV type 3 in 2011 (11.0%), 2012 (1.1%), 2013 was 3.6% and 2014 was 1.6%. Only one positive for HPIV type 2 were identified in 2014. In subsequent years, HPIV-2 was not identified.

6.1.2 Survival of Human parainfluenza virus causing acute respiratory tract infection in Chennai

Throat and nasal swabs were collected from patients with ILI/SARI belonging to different age groups during January 2011 to December 2014, attending outpatient departments (OPD) and IP respectively of tertiary care Government Hospitals in Chennai. Year wise distribution of HPIV in seven different Government Hospitals from clinical samples were screened shown in the Table 6.2.

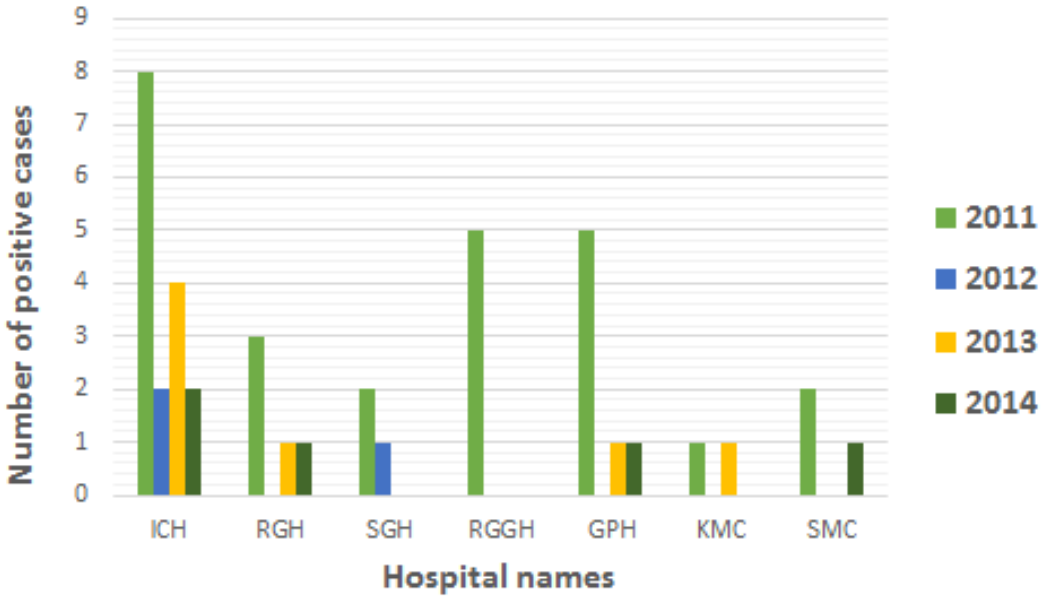
Table 6.2: HPIV screened in different Government Hospitals in Chennai from 2011 to 2014

Hospitals Name	Total number of samples screened			
	2011	**2012**	**2013**	**2014**
Institute of Child Health (ICH)	52	54	32	50
Royapettah Government Hospital (RGH)	22	30	25	37
Saidapet Government Hospital (SGH)	26	34	22	32
Rajiv Gandhi Government Hospital (RGH)	39	46	24	23
Government Peripheral Hospital (GPH)	42	35	30	38
Kilpauk Medical College (KMC)	31	20	27	33
Stanley Medical College (SMC)	24	45	31	27
Total	**236**	**264**	**191**	**240**

Among the seven Government Hospitals, Institute of Child Health (ICH) showed high positivity rate when compared to other Hospitals. In 2011, eight positivity samples seen in ICH and showed high positive cases were screened when compared to other years.

In 2012 there were no positive cases observed in Rajiv Gandhi Government Hospital, Government Peripheral Hospital, Kilpauk Medical College and Stanley Medical College and Hospital. In 2013 and 2014, positive cases of HPIV were not identified in Saidapet Government Hospital and Rajiv Gandhi Government Hospital was shown in the Figure 6.2.

Figure 6.2: Positive cases of HPIV were confirmed by laboratory diagnosis in Government Hospitals



6.1.3 Age wise distribution of HPIV

Clinical samples were received from patients belonging to all age group and were classified into 0-10, 11-20, 21-30, 31-40, 41-50 and above 50.

The prevalence in different age group was analyzed during the study period from 2011 to 2014 which represented in the Figure 6.3 to 6.6.

Figure 6.3: Epidemiology of HPIV in 2011

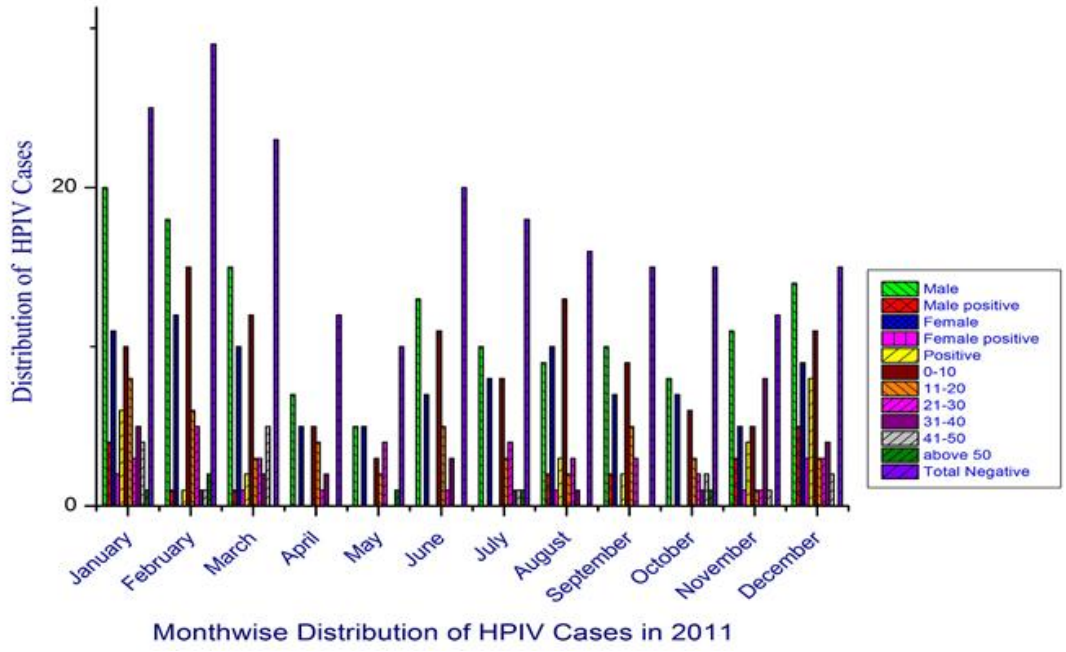


Figure 6.4: Epidemiology of HPIV in 2012

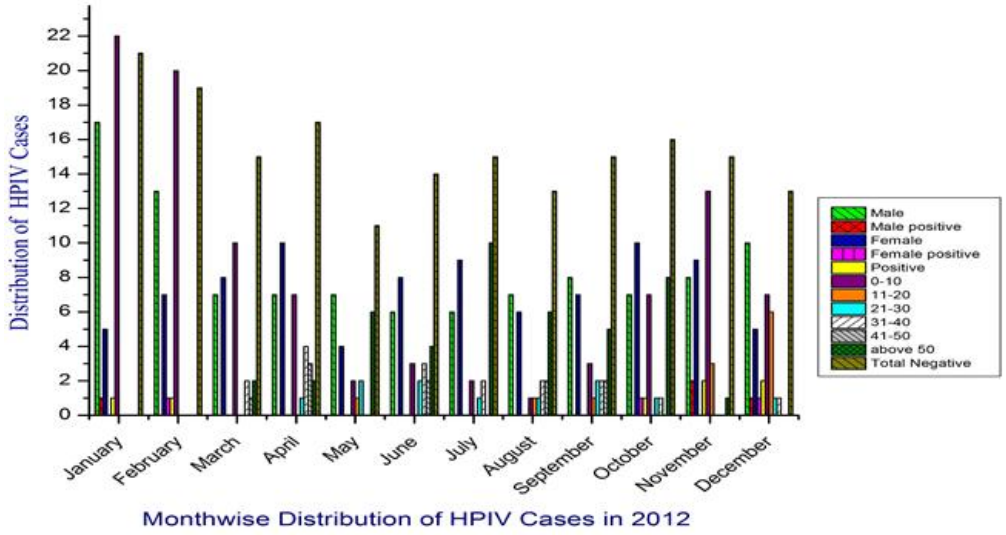


Figure 6.5 Epidemiology of HPIV in 2013

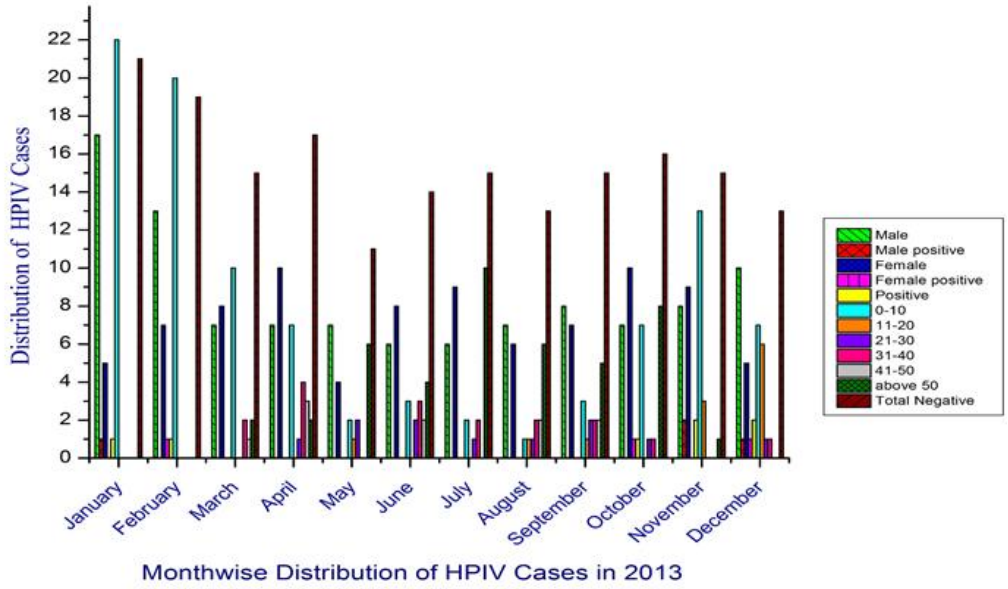
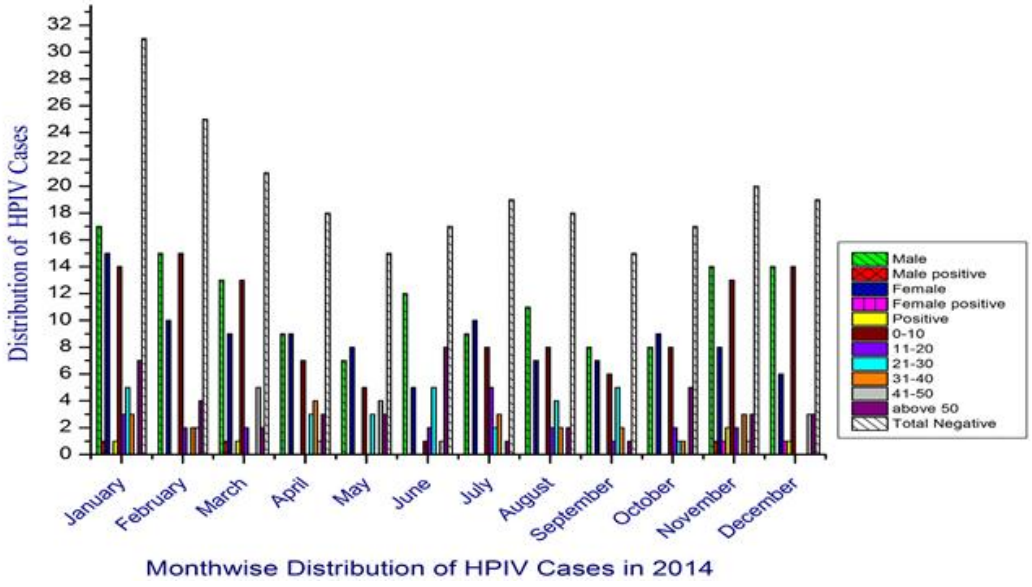


Figure 6.6: Epidemiology of HPIV in 2014



Among the various age groups subjected to testing, positivity was predominantly seen in the pediatric age group during the study period shown in the Table 6.3. The Oneway Anova of HPIV susceptible cases and positivity were analysed during 2011 to 2014 in different age groups. There was significant

difference between the groups were observed in the distribution of HPIV in the study period. The Post hoc Dunnet statistical analysis inferred that there was significant difference within the group and there was no significant difference between the groups shown in the Table 6.4.

Table 6.3: Statistical Analysis (ANOVA) of Human parainfluenza virus during 2011-2014

		Sum of Squares	Mean Square	F	Sig.
Male	Between Groups	121.667	40.556	2.716	0.056
	Within Groups	657.000	14.932		
	Total	778.667			
Female	Between Groups	159.896	53.299	8.880	0.000
	Within Groups	264.083	6.002		
	Total	423.979			
FemalePositives	Between Groups	2.417	.806	2.287	0.092
	Within Groups	15.500	.352		
	Total	17.917			
MalePositives	Between Groups	13.154	4.385	4.125	0.013
	Within Groups	39.333	1.063		
	Total	52.488			
Negatives	Between Groups	273.083	91.028	4.168	0.011
	Within Groups	960.833	21.837		
	Total	1233.917			
zeroto10yr	Between Groups	116.833	38.944	1.530	0.220
	Within Groups	1119.833	25.451		
	Total	1236.667			
Elevento20yrs	Between Groups	32.083	10.694	1.797	0.162
	Within Groups	261.833	5.951		
	Total	293.917			
Twentyoneto30yrs	Between Groups	25.229	8.410	3.116	0.036
	Within Groups	118.750	2.699		
	Total	143.979			
thirtyoneto40yrs	Between Groups	5.833	1.944	.706	0.554
	Within Groups	121.167	2.754		
	Total	127.000			
fouty0neto50	Between Groups	2.500	.833	.349	0.790
	Within Groups	105.167	2.390		
	Total	107.667			
Above50yrs	Between Groups	80.917	26.972	4.088	0.012
	Within Groups	290.333	6.598		
	Total	371.250			

* The mean difference is significant at the 0.05 level.

a Dunnett t-tests treat one group as a control, and compare all other groups against it.

Table 6.4: Posthoc Dunnet analysis of Human parainfluenza virus during 2011-2014

Dependent Variable		Std. Error	Sig.
Male	Dunnett t (2-sided)[a] 2011-12	1.57754	.662
	2012-13	1.57754	.993
	2013-14	1.57754	.190
Female	Dunnett t (2-sided)[a] 2011-12	1.00016	.005
	2012-13	1.00016	.240
	2013-14	1.00016	.461
Male positive	Dunnett t (2-sided)[a] 2011-12	.54882	.626
	2012-13	.54882	.269
	2013-14	.54882	.427
Female Positives	Dunnett t (2-sided)[a] 2011-12	.24231	.112
	2012-13	.24231	.973
	2013-14	.24231	.973
Negatives	Dunnett t (2-sided)[a]2011-12	1.90775	.565
	2012-13	1.90775	.536
	2013-14	1.90775	.079
Zero to10 yrs	Dunnett t (2-sided)[a]2011-12	2.05956	.997
	2012-13	2.05956	.362
	2013-14	2.05956	.876
Eleven to 20yrs	Dunnett t (2-sided)[a] 2011-12	.99589	.126
	2012-13	.99589	.568
	2013-14	.99589	1.000
Twenty one to30yrs	Dunnett t (2-sided)[a]2011-12	.67068	.869
	2012-13	.67068	.966
	2013-14	.67068	.101
Thirty one to 40yrs	Dunnett t (2-sided)[a]2011-12	.67747	.639
	2012-13	.67747	.999
	2013-14	.67747	.967
Fourthy one to 50yrs	Dunnett t (2-sided)[a]2011-12	.63116	.960
	2012-13	.63116	.768
	2013-14	.63116	.680
Above 50yrs	Dunnett t (2-sided)[a] 2011-12	1.04869	.018
	2012-13	1.04869	.274
	2013-14	1.04869	.997

* The mean difference is significant at the 0.05 level.

a Dunnett t-tests treat one group as a control, and compares all other groups against it.

6.1.4 Symptom wise distribution of HPIV

Human parainfluenza viruses are important pathogens associated with mild upper and lower respiratory tract illness in infants and young children such as fever, nasal discharge, cough, sore throat, breathlessness, wheezing, and pneumonia.

Symptom wise circulation of HPIV during the period 2011 to 2014 which are shown in the Figure 6.7 to 6.10.

Figure 6.7: Symptoms of HPIV cases in 2011

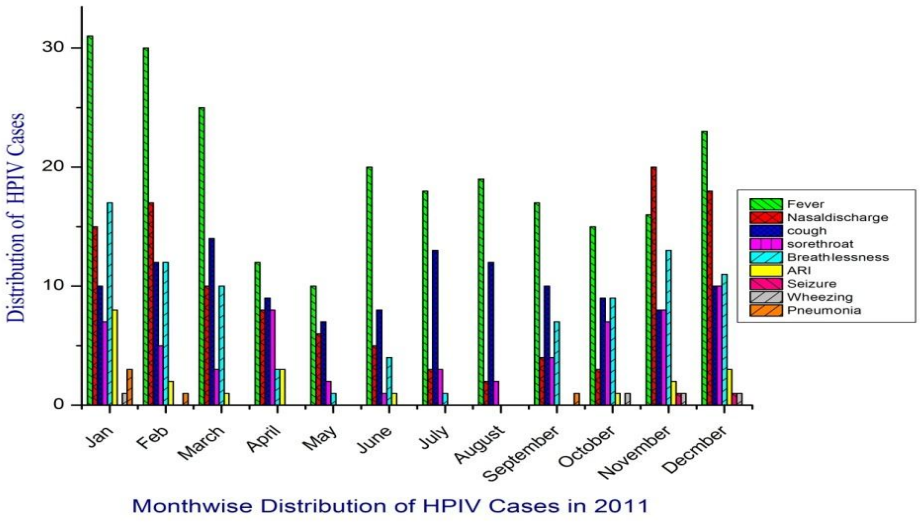


Figure 6.8 Symptoms of HPIV cases in 2012

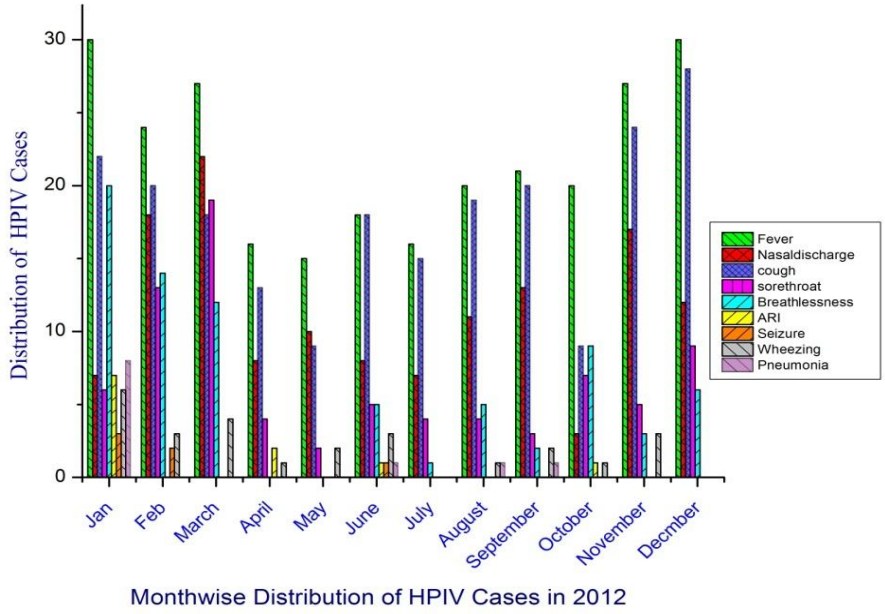


Figure 6.9: Symptoms of HPIV cases in 2013

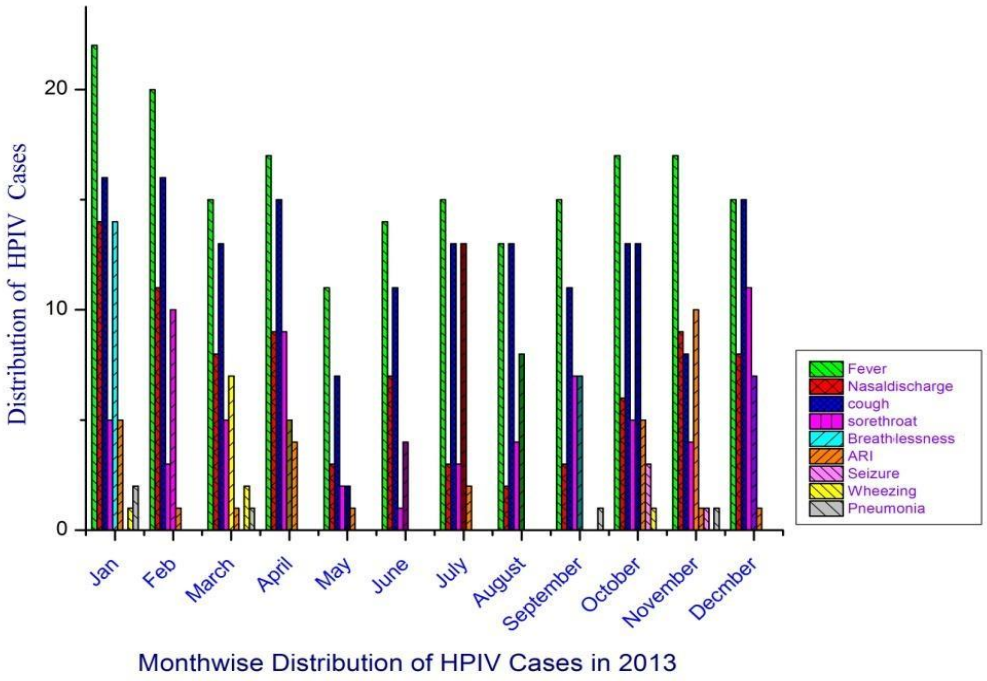
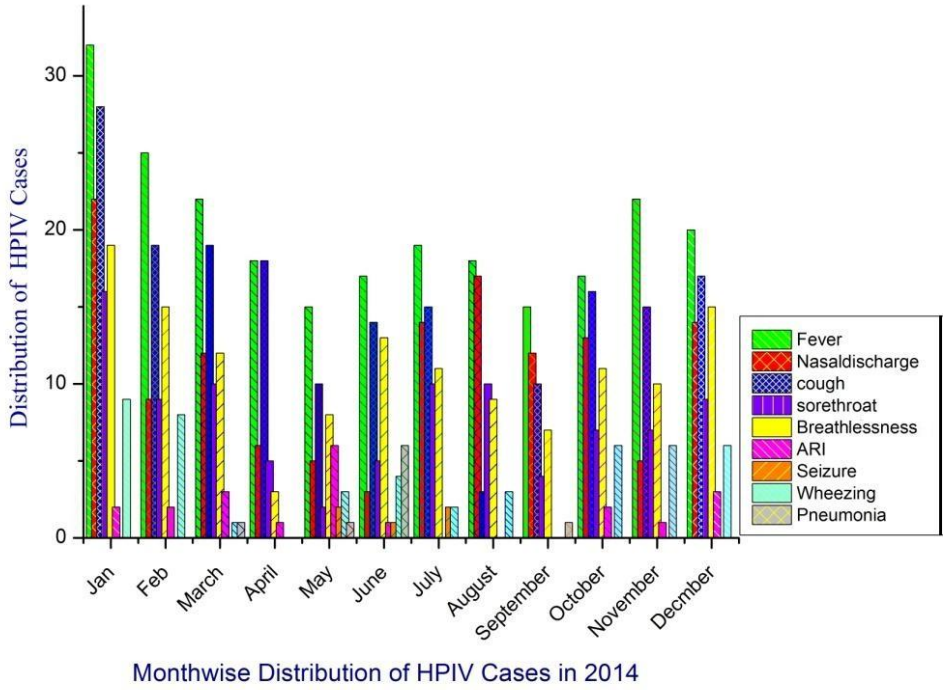


Figure 6.10 Symptoms of HPIV cases in 2014

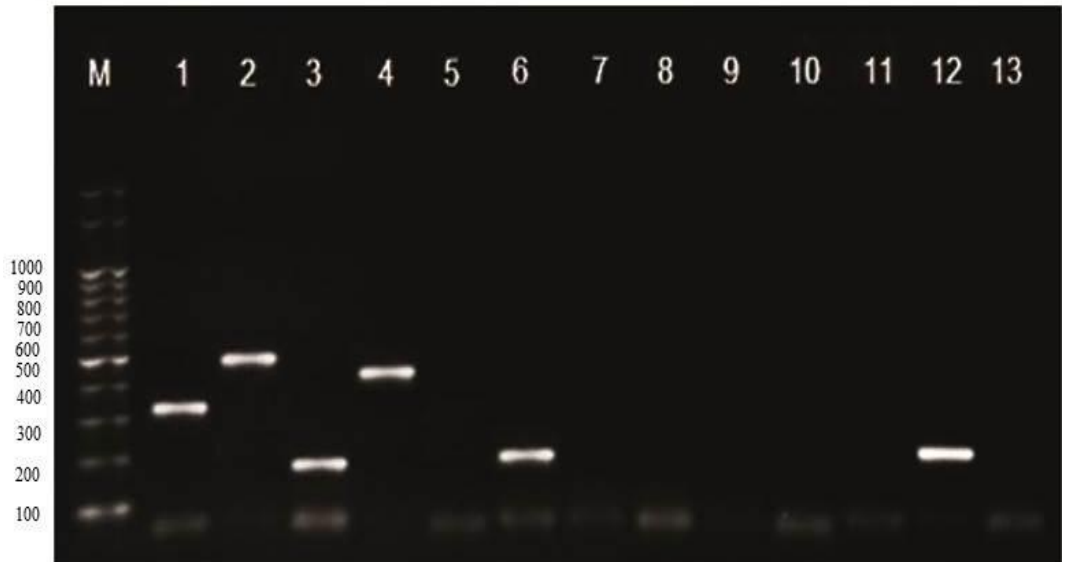


6.1.5 Molecular characterization of HPIV

All 931 clinical samples were subjected to diagnosis of Human parainfluenza virus by multiplex reverse transcription polymerase chain reaction (mRT-PCR).

Among 931 clinical samples, 40 samples were confirmed positive for HPIV-3 Haemagglutinin Neuraminadase (HN) was characterized by the amplified product of 189 bp which represent in the Figure 6.11.

Figure 6.11 : Agarose gel analysis of Human parainfluenza virus type 3 (HN) Lane M: Molecular weight marker, Lane 1: HPIV-1 (Positive control), Lane 2: HPIV-2 (PC), Lane 3: HPIV (PC), Lane 4: HPIV-4 (PC), Lane 5: Negative, Lane 6: HPIV-3 (clinical sample), Lane 7 to 11: Negative, Lane 12: HPIV-3 (Clinical sample), Lane 13: Negative control.

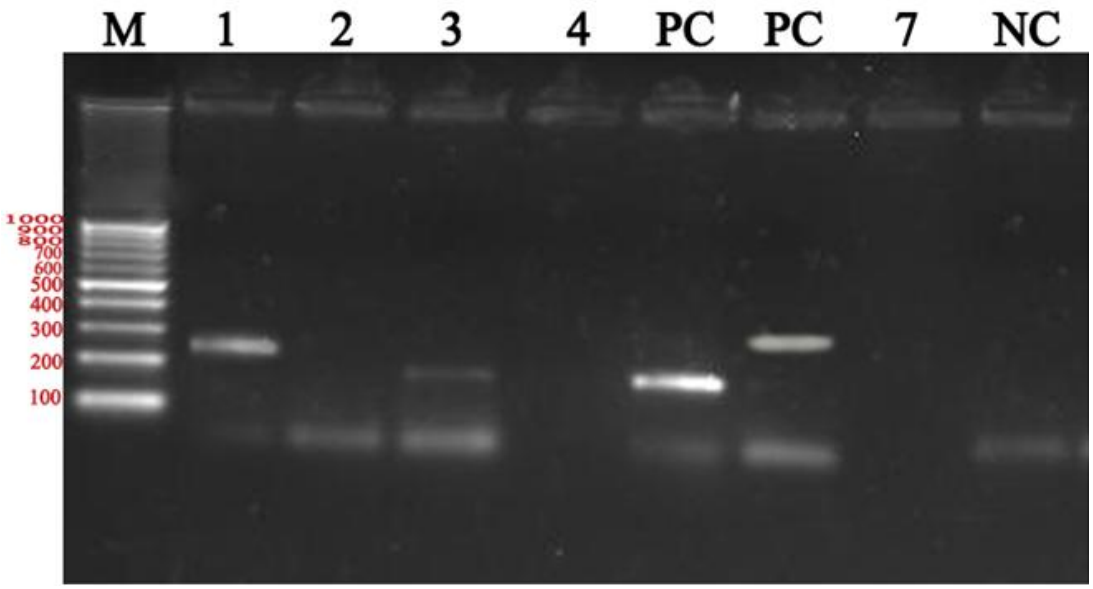


Among the 41 positives, five representative samples from different age groups and three geographical regions within Tamilnadu were chosen and subjected to sequencing. These sequences of HPIV-2 and 3 strains submitted to Genbank, National Center for Biotechnology Information (NCBI). The accession numbers for HPIV-3 Hemagglutinin neuraminidase gene (JQ901411-JQ901413).

All 931 clinical samples were imperiled to Human parainfluenza virus by multiplex reverse transcription polymerase chain reaction (mRT-PCR). Among 931

clinical samples, only one sample was confirmed positive for HPIV type 2 (Nucleocapsid gene) which was further characterized by the amplified product of 197 bp which represent in the Figure 6.12. The amplified product was subjected to sequencing, this sequence of HPIV-2 strains submitted to NCBI. The accession numbers for HPIV-2 Nucleocapsid gene (gi:672424560) and HPIV-3 Nucleocapsid gene (gi:692112437).

Figure 6.12: Agarose gel analysis of HPIV-2 and 3 (N gene) Lane M: Marker, Lane 1: HPIV-3 (clinical sample), Lane 2: Negative, Lane 3: HPIV-2 (clinical sample) Lane 4: Negative, Lane 5: HPIV-2 (PC), Lane 6: HPIV-3 (PC), Lane 7: Negative, Lane 8: Negative control.



6.1.6 Sequence analysis of HPIV (HN gene)

Different sequences were retrieved from National Centre for Biotechnology information (NCBI) and shown in the Table 6.5. Sequence from other countries was mentioned as strain name and year.

Table 6.5: Strains of Human parainfluenza virus type 3 reported from present study and other countries

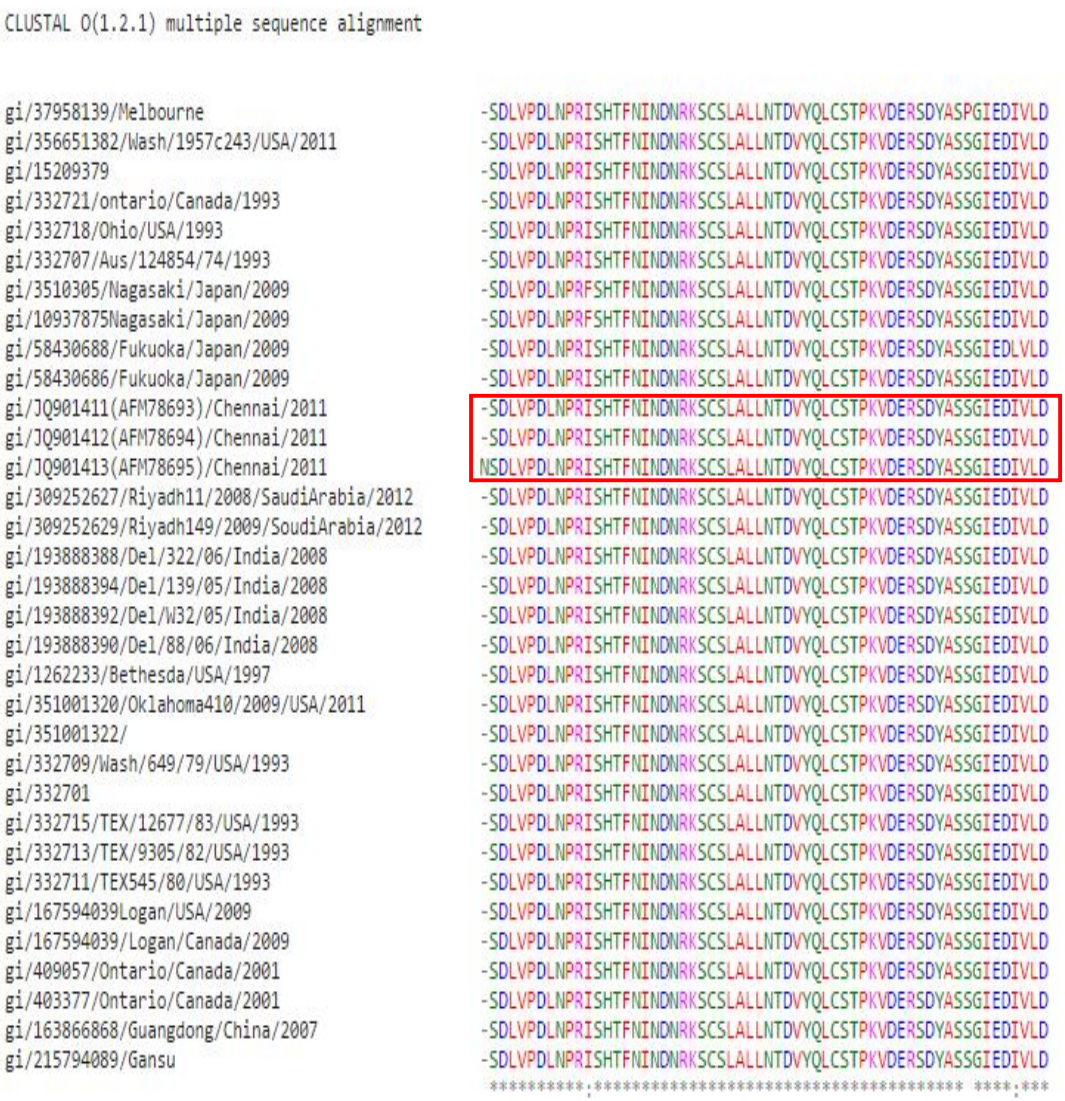
GI and Accession No	Strain/Country	Year
gi332709, AAA46848	USA	1993
gi332701, AAA46844	USA	1993
gi351001320, AEQ39012	Oklahoma 10/2009/USA	2011
gi1262233, AAB48689	JS/USA	1997
gi193888390, ACF28540	Del/88/06/India	2008
gi193888394,ACF28542	Del/139/05/India	2008
gi193888388,ACF28539	Del/322/06	2008
gi309252629, ADO60288	Riyadh149/2009/SaudiArabia	2012
gi309252627, ADO60287	Riyadh11/2008/SaudiArabia	2012
gi332715, AAA46851	USA	1993
gi332713,AAA46850	USA	1993
gi332711, AAA46849	USA	1993
gi167594039, ABZ85673	14702/Canada	2009
gi409057, AAA46855	Canada-Ontario	2001
gi163866868,ABY47606	ZHYMgz01China	2007
gi37958139,AAP35240	Australia-Melbourne	2004
gi215794089, ACJ70090	China- Gansu Provence	2011
gi58430688,BAD89145	Fukuoka-Japan	2009
JQ901411(AFM78693	Chennai, India	2011
JQ901412(AFM78694)	Chennai, India	2011
JQ901413(AFM78695)	Chennai, India	2011
gi3510305,BAA32574	GP/Japan-Nagasaki	2009
gi10937875,NP_067152	GP/Japan-Nagasaki	2009
gi356651382,AET35008	Wash/1957c243/USA	2011
gi15209379,CAC51081	MK9/UK	2001
gi332721,46856	Canada-Ontario	1993
gi332718, AAA46853	USA-Ohio	1993
gi 332707, AAA46847	Australia	1993
gi168481518, ACA24945 /swine	USA	2009

6.1.7 Identification of mutations for HPIV-3 (HN gene - 295 and 297)

Multiple sequence alignment of HPIV-3 by ClustalW tool was used to identify the mutations and amino acid alterations. The study sequence, USA, Oklahoma, China, Saudi, Riyadh strain were identified for mutation at position 295 residue and further the Histidine (H) replaced by tyrosine (Y), while other countries like Washington, Canada, USA existed as tyrosine (Y) was replaced by Histidine (H) amino acid as shown in the Figure 6.13. Tyrosine contains reactive hydroxyl group likely to be involved in interactions with non-carbon atoms. Amino-acid changes are functionally important if mutation in the position 295 forms the functional part of the protein, leading to functional variability.

At 297 residue the query sequence and Japanese sequence were similar by way of Glycine (G) being replaced by Serine (S) amino-acid which can also act as protein functional center, whereas from other countries were characterized by Serine (S) being replaced by Glycine (G) which signified in the Figure 6.13. Had the conserved Glycine forming the functional part had changed to any other amino acid, the change could had a drastic impact on function.

Figure 6.13: Multiple sequence alignment of HPIV-3 HN gene sequence with amino acid alterations



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gi/37958139/Melbourne                                IVNYDGSIST
gi/356651382/Wash/1957c243/USA/2011                  IVNHDGSIST
gi/15209379                                          IVNHDGSIST
gi/332721/ontario/Canada/1993                        IVNHDGSIST
gi/332718/Ohio/USA/1993                              IVNHDGSIST
gi/332707/Aus/124854/74/1993                         IVNHDGSIST
gi/3510305/Nagasaki/Japan/2009                       IVNYDGSIST
gi/10937875Nagasaki/Japan/2009                       IVNYDGSIST
gi/58430688/Fukuoka/Japan/2009                       IVNYDGSIST
gi/58430686/Fukuoka/Japan/2009                       IVNYDSSIST
gi/JQ901411(AFM78693)/Chennai/2011                   IVNYDSSIST
gi/JQ901412(AFM78694)/Chennai/2011                   IVNYDSSIST
gi/JQ901413(AFM78695)/Chennai/2011                   IVNYDSSIST
gi/309252627/Riyadh11/2008/SaudiArabia/2012          IVNYDGSIST
gi/309252629/Riyadh149/2009/SoudiArabia/2012         IVNYDGSIST
gi/193888388/Del/322/06/India/2008                   IVNYDGSIST
gi/193888394/Del/139/05/India/2008                   IVNYDGSIST
gi/193888392/Del/W32/05/India/2008                   IVNYDGSIST
gi/193888390/Del/88/06/India/2008                    IVNYDGSIST
gi/1262233/Bethesda/USA/1997                         IVNYDGSIST
gi/351001320/Oklahoma410/2009/USA/2011               IVNYDGSIST
gi/351001322/                                        IVNYDGSIST
gi/332709/Wash/649/79/USA/1993                       IVNYDGSIST
gi/332701                                            IVNYDGSIST
gi/332715/TEX/12677/83/USA/1993                      IVNYDGSIST
gi/332713/TEX/9305/82/USA/1993                       IVNYDGSIST
gi/332711/TEX545/80/USA/1993                         IVNYDGSIST
gi/167594039Logan/USA/2009                           IVNYDGSIST
gi/167594039/Logan/Canada/2009                       IVNYDGSIST
gi/409057/Ontario/Canada/2001                        IVNYDGSIST
gi/403377/Ontario/Canada/2001                        IVNYDGSIST
gi/163866868/Guangdong/China/2007                    IVNYDGSIST
gi/215794089/Gansu                                   IVNYDGSIST
                                                     ***:*.****
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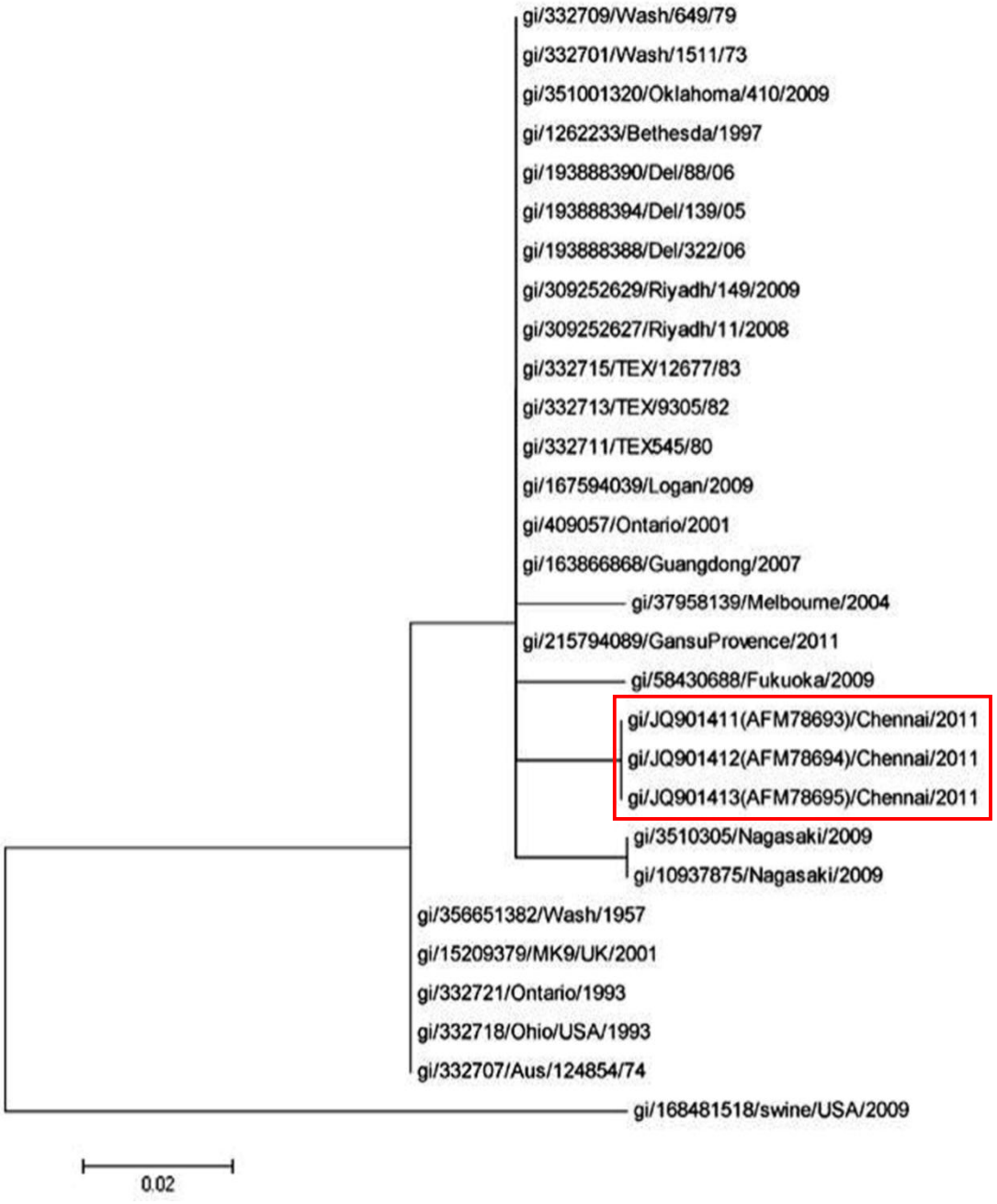
6.1.8 Comparison sequence of HPIV-3 (HN gene) with other strains

Phylogenetic analysis of the Chennai strains Figure 6.14 showed higher similarity to strains of Nagasaki, Fukuoka and Melbourne strains (97% similarity) and formed a same clade. The other strains were significantly related to Washington (1973 and 1979), Oklahoma (2009), Bethesda (1997), New Delhi (2005, 2006), Riyadh (2008 & 2009), Texas (1980, 1982 and 1983), Logan (2009), Ontario (2001) and Guangdong (2007). Phylogenetic analysis explains the similarity of Chennai (2011) strains to other strains from different countries. The similarity confirms the circulation of HPIV in Chennai and also its similarity to the strains in different countries. In this phylogenetic analysis swine (2009) strain was used as an out group.

Figure 6.14: Phylogenetic analysis of Human parainfluenza virus type 3 HN gene



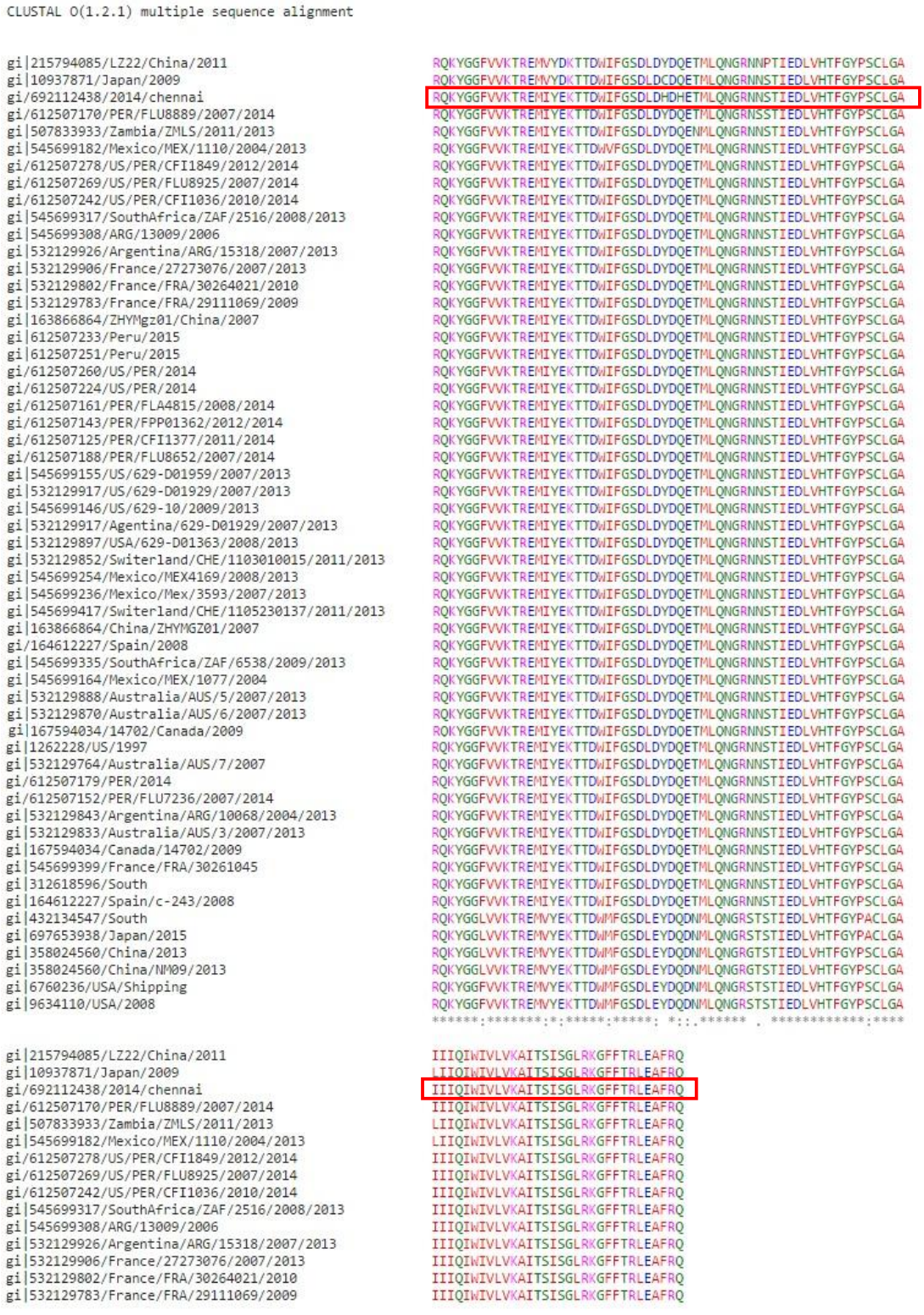
6.1.9 Sequence analysis of HPIV-3 (Nucleocapsid gene)

Based on sequence analysis various sequence of HPIV-3 (N) gene from different countries in different years were retrieved from NCBI data base and aligned.

6.1.10 Mutations identified at 138 and 140 residue in HPIV 3 Nucleocapsid gene

At 138 residue the study sequence were recognized by way of tyrosine (Y) replaced by Histidine (H) amino-acid which can act as protein functional centers, whereas other countries showed Histidine (H) and Tyrosine (Y) which signified in the Figure 6.15. The conserved Tyrosine changed to any other amino acid, the change had a drastic impact on function.

Figure 6.15: Multiple sequence alignment by ClustalW of HPIV–3 (N gene) sequence with amino acid alterations



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gi|163866864/ZHYMgz01/China/2007                          IIIQIWIVLVKAITSISGLRKGFFTRLEAFRQ
gi|612507233/Peru/2015                                    IIIQIWIVLVKAITSISGLRKGFFTRLEAFRQ
gi|612507251/Peru/2015                                    IIIQIWIVLVKAITSISGLRKGFFTRLEAFRQ
gi/612507260/US/PER/2014                                  IIIQIWIVLVKAITSISGLRKGFFTRLEAFRQ
gi/612507224/US/PER/2014                                  IIIQIWIVLVKAITSISGLRKGFFTRLEAFRQ
gi/612507161/PER/FLA4815/2008/2014                        IIIQIWIVLVKAITSISGLRKGFFTRLEAFRQ
gi/612507143/PER/FPP01362/2012/2014                       IIIQIWIVLVKAITSISGLRKGFFTRLEAFRQ
gi/612507125/PER/CFI1377/2011/2014                        IIIQIWIVLVKAITSISGLRKGFFTRLEAFRQ
gi/612507188/PER/FLU8652/2007/2014                        IIIQIWIVLVKAITSISGLRKGFFTRLEAFRQ
gi|545699155/US/629-D01959/2007/2013                      IIIQIWIVLVKAITSISGLRKGFFTRLEAFRQ
gi|532129917/US/629-D01929/2007/2013                      IIIQIWIVLVKAITSISGLRKGFFTRLEAFRQ
gi|545699146/US/629-10/2009/2013                          IIIQIWIVLVKAITSISGLRKGFFTRLEAFRQ
gi|532129917/Agentina/629-D01929/2007/2013                IIIQIWIVLVKAITSISGLRKGFFTRLEAFRQ
gi|532129897/USA/629-D01363/2008/2013                     IIIQIWIVLVKAITSISGLRKGFFTRLEAFRQ
gi|532129852/Switerland/CHE/1103010015/2011/2013          IIIQIWIVLVKAITSISGLRKGFFTRLEAFRQ
gi|545699254/Mexico/MEX4169/2008/2013                     IIIQIWIVLVKAITSISGLRKGFFTRLEAFRQ
gi|545699236/Mexico/Mex/3593/2007/2013                    IIIQIWIVLVKAITSISGLRKGFFTRLEAFRQ
gi|545699417/Switerland/CHE/1105230137/2011/2013          IIIQIWIVLVKAITSISGLRKGFFTRLEAFRQ
gi|163866864/China/ZHYMGZ01/2007                          IIIQIWIVLVKAITSISGLRKGFFTRLEAFRQ
gi/164612227/Spain/2008                                   LIIQIWIVLVKAITSISGLRKGFFTRLEAFRQ
gi|545699335/SouthAfrica/ZAF/6538/2009/2013               LIIQIWIVLVKAITSISGLRKGFFTRLEAFRQ
gi|545699164/Mexico/MEX/1077/2004                         LIIQIWIVLVKAITSISGLRKGFFTRLEAFRQ
gi|532129888/Australia/AUS/5/2007/2013                    LIIQIWIVLVKAITSISGLRKGFFTRLEAFRQ
gi|532129870/Australia/AUS/6/2007/2013                    LIIQIWIVLVKAITSISGLRKGFFTRLEAFRQ
gi|167594034/14702/Canada/2009                            LIIQIWIVLVKAITSISGLRKGFFTRLEAFRQ
gi|1262228/US/1997                                        LIIQIWIVLVKAITSISGLRKGFFTRLEAFRQ
gi|532129764/Australia/AUS/7/2007                         LIIQIWIVLVKAITSISGLRKGFFTRLEAFRQ
gi/612507179/PER/2014                                     LIIQIWIVLVKAITSISGLRKGFFTRLEAFRQ
gi/612507152/PER/FLU7236/2007/2014                        LIIQIWIVLVKAITSISGLRKGFFTRLEAFRQ
gi|532129843/Argentina/ARG/10068/2004/2013                LIIQIWIVLVKAITSISGLRKGFFTRLEAFRQ
gi|532129833/Australia/AUS/3/2007/2013                    LIIQIWIVLVKAITSISGLRKGFFTRLEAFRQ
gi|167594034/Canada/14702/2009                            LIIQIWIVLVKAITSISGLRKGFFTRLEAFRQ
gi|545699399/France/FRA/30261045                          LIIQIWIVLVKAITSISGLRKGFFTRLEAFRQ
gi|312618596/South                                        LIIQIWIVLVKAITSISGLRKGFFTRLEAFRQ
gi|164612227/Spain/c-243/2008                             LIIQIWIVLVKAITSISGLRKGFFTRLEAFRQ
gi|432134547/South                                        LIIQIWIILVKAITSISGLRKGFFTRLEAFRQ
gi|697653938/Japan/2015                                   LIIQIWIILVKAITSISGLRKGFFTRLEAFRQ
gi|358024560/China/2013                                   LIIQIWIILVKAITSISGLRKGFFTRLEAFRQ
gi|358024560/China/NM09/2013                              LIIQIWIILVKAITSISGLRKGFFTRLEAFRQ
gi|6760236/USA/Shipping                                   LIIQVWIILVKAITSISGLRKGFFTRLEAFRQ
gi|9634110/USA/2008                                       LIIQVWIILVKAITSISGLRKGFFTRLEAFRQ
                                                          :***:**:**********************
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6.1.11 Comparison sequence of HPIV-3 (N gene) with other strains

Phylogenetic analysis of the Chennai strains Figure 6.16 showed that it was highly similar to strains from Switzerland, USA, South Africa, Argentina, France, China, Peru, Mexico strains and formed a same clade. The other strains that were significantly related to Japan (2009) and China (2011). The current study the sequences were related to strains of Spain, South Africa, Mexico (2004), Canada, Australia and highly distance existed as Zambia strain. Phylogenetic analysis explains the similarity of Chennai (2011) strains to strains from different countries confirming that the circulation of HPIV in Chennai could have come from different countries. In this phylogenetic analysis Bovine parainfluenza virus (2013) strain was used as an out group.

Figure 6.16: Human parainfluenza virus type 3 Nucleocapsid gene

6.1.12 Sequence analysis of HPIV-2

Based on sequence analysis various sequence of HPIV-2 nucleocapsid gene from different countries in different years were retrieved from NCBI data base and aligned.

6.1.13 Mutations identified in HPIV-2 (Nucleocapsid gene)

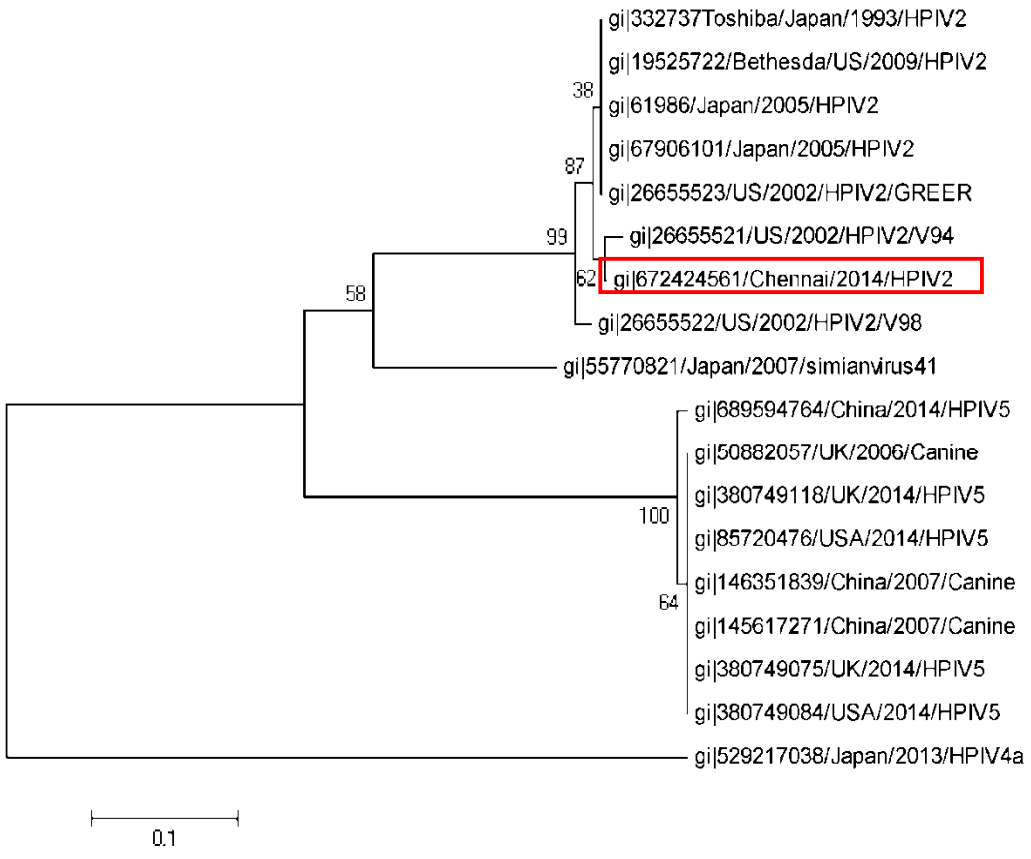
Amino acid aletrations were identifed shown in the Figure 6.17.

Figure 6.17: Multiple sequence alignment of HPIV-2 (N gene) by ClustalW

6.1.14 Comparison sequence of HPIV-2 (N gene) with other strains

Phylogenetic analysis of the HPIV-2 (NP) isolated from Chennai strains Figure 6.18 showed that Chennai strains were highly similar to strains from US (V94 strain). The study sequence were significantly related with US (Greer strain), Japan, Bethsida strain arranged as same clade. The study sequence were similar with US (V98 strain), Japan (Simian vius 41), United Kingdom, US, China strains. In this phylogenetic analysis Japan (Human parainfluenza virus 4A 2013) strain was used as an out group.

Figure 6.18: Phylogenetic analysis of Human parainfluenza virus type 2 nucleocapsid gene

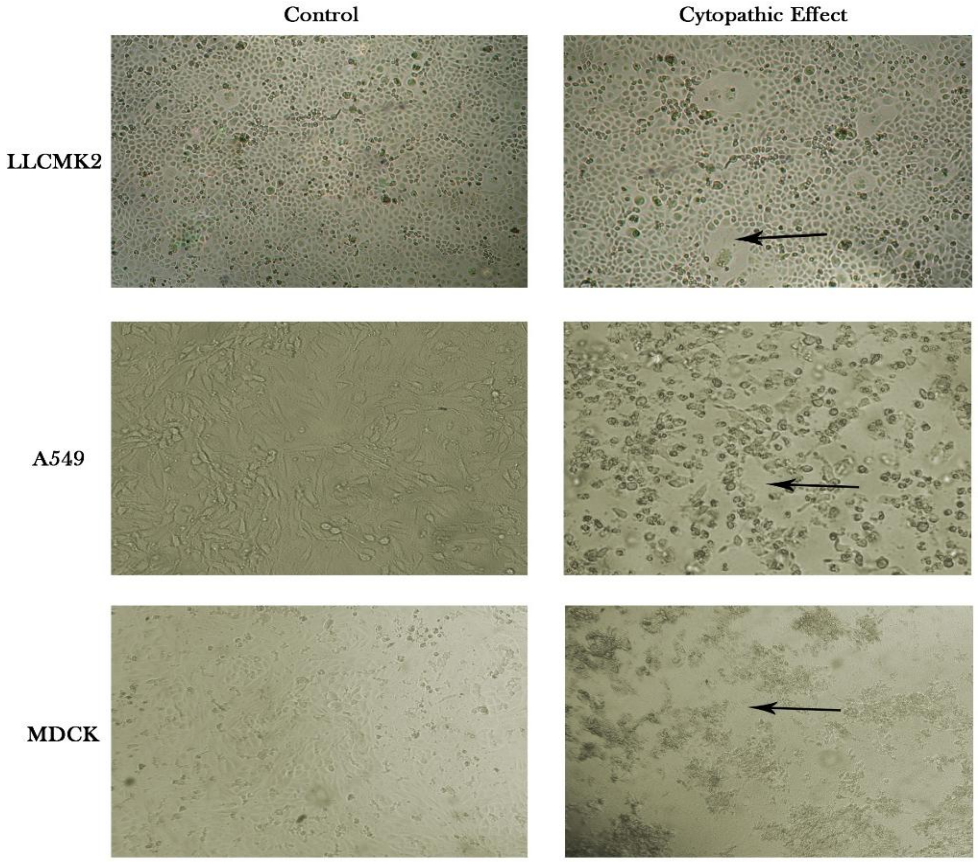


6.2 ISOLATION OF HUMAN PARAINFLUENZA VIRUS FROM THROAT AND NASAL SWAB

6.2.1 Viral isolation of HPIV in three cell lines

All clinical samples were subjected to viral isolation in respective cell lines namely LLC-MK2, A549 and MDCK cell lines in tissue culture tube. Out of 931 samples, thirty four (3.6%) were positive for HPIV and were confirmed by mRT-PCR. There were seven samples negative by viral isolation but positive by mRT-PCR. In LLC-MK2, cytopathic effect were observed as syncytium formation, in A549 which showed cell rounding formation and MDCK identified as degeneration of cells were represented in the Figure 6.19. Statistically significant difference was observed between LLC-MK2, with A549 and MDCK cell lines.

Figure 6.19: Cytopathic effect of HPIV in LLC-MK2, A549 and MDCK cell lines



Negatives were discarded after ninth passages, and the samples showed mild changes in the cell morphology were passaged further. If these samples produced clear CPE were confirmed by PCR, if CPE were not seen after two more passages, these were checked by PCR and if negative, discarded. Clear cytopathic effect was observed in the ninth passage in thirty two samples and two samples showed CPE in fifth passage. These samples were reconfirmed by RT-PCR as HPIV type 2 and 3.

6.2.2 Yearwise distribution of HPIV in cell lines

Among 931 clinical samples, 33 were positive for HPIV type 3 and only one sample was positive for HPIV type 2. The yearwise analysis showed all 34 samples were isolated in LLC-MK2, 15 were showed CPE in A549 and 5 were grown in MDCK cell lines. Only one sample of HPIV 2 was grown in all three cell lines (LLC-MK2, A549 and MDCK). In 2011, the viral isolation rate was comparatively higher when compared to 2012 and 2014. In 2013 the isolation rates were marginally better despite improving on viral isolation techniques.

6.2.3 Sensitivity of viral titer for HPIV

The tissue culture infective dose of HPIV was performed using standard HPIV type 2 and 3 (Greer HPIV-2 and D-10025 HPIV 3) in LLC-MK2, A549 and MDCK cells were calculated by Reed and Meunch method.

For HPIV type 2 and 3 sensitivity test was done in LLC-MK2, A549 and MDCK cell lines in 96 well plates. $TCID_{50}$ for HPIV in three cell lines were shown in the Table 6.16.

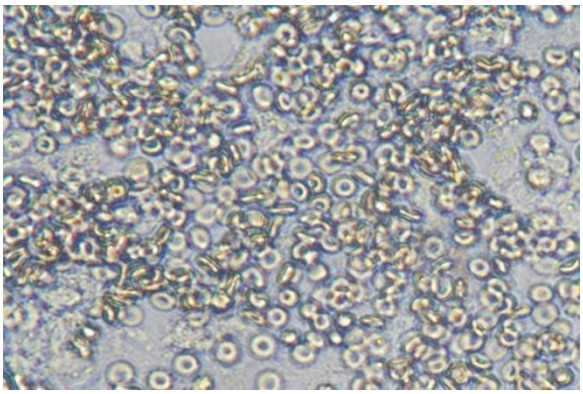
Table 6.16: Titration of Human parainfluenza virus type 2 and 3 in three different cell lines

Cell lines	CPE was observed in following days of inoculation	HPIV-2 ($TCID_{50}$)/0.1ml	HPIV-3 ($TCID_{50}$)/0.1ml
LLC-MK2 (passage 9)	Day 5	$1x10^{-4}$	$1x10^{-5}$
A549 (passage 5)	Day 7	$1x10^{-3}$	$1x10^{-6}$
MDCK (passage 8)	Day8	$1x10^{-2}$	$1x10^{-2}$

6.2.4 Detection of HPIV by Hemadsorption assay

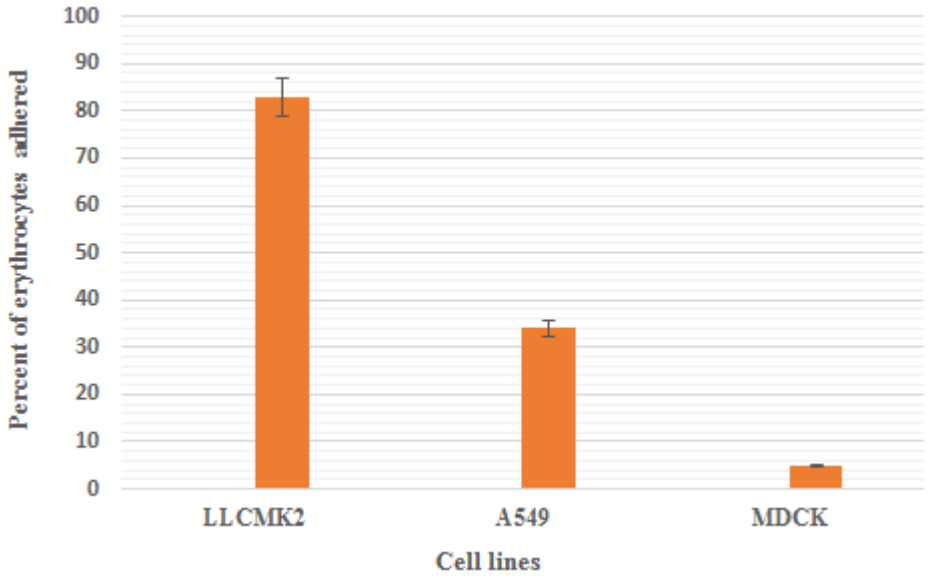
HPIV isolated samples from three different cell lines were confirmed by hemadsorption assay in tissue culture tube. All 34 samples which might be erythrocytes were adhered in cell monolayer of culture tube were embodied in the Figure 6.20.

Figure 6.20: Erythrocytes adhered to the monolayer



Of the total 34 isolates subjected to hemadsorption assay, 34 (82.9%) showed results by hemadsorption assay in LLC-MK2. In A549, 34% of the isolates showed positive results. Whereas in MDCK 4.8% showed erythrocyte adhered percentage error bars on to the cell line which revealed in the Figure 6.21.

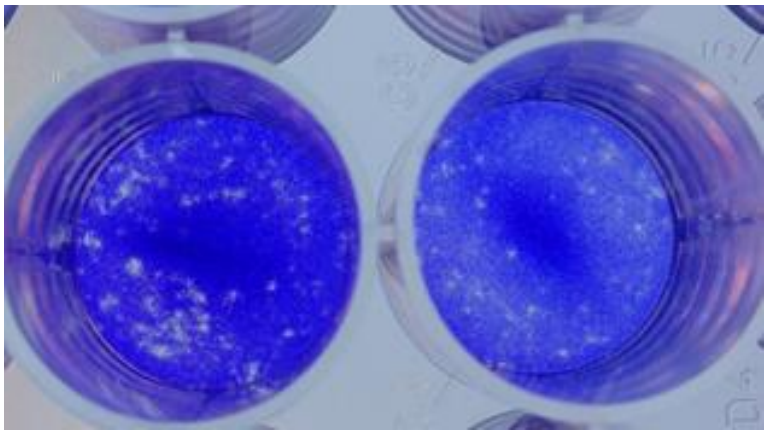
Figure 6.21 Percentage of HPIV positive by Hemadsorption assay



6.2.5 Confirmation of cytopathic effect (CPE) by plaque assay

Plaques were visualized by day five to ten after infection, and the monolayer stained to visualize and count the plaques on the final day which shown in the Figure 6.22. By naked eye examination there were white dots on the monolayer. These dots may be more easily visualized by viewing the plate with oblique light falling upon the plate. Plate count was verified by staining to assist visualization of plaques. Plaque forming units were calculated in LLC-MK2 and A549 cells which is shown in the Table 6.7.

Fig 6.22: Confirmation of CPE by plaque assay



Calculation:

$$\text{PFU/ml} = \frac{\text{Average number of plaques}}{\text{D x V}}$$

$$= \frac{38}{10^{-4}\text{x}0.1} = 3.8\text{x}10^{6}$$

Table 6.7: Plaque forming units calculated in LLC-MK2 and A549 cells

Virus	LLC-MK2 (PFU/ml)	A59 (PFU/ml)
HPIV-2	$3.8\text{x}10^{6}$	$4.3\text{x}10^{7}$
HPIV-3	$3.1\text{x}10^{7}$	$5.4\text{x}10^{8}$

Multiplicity of infection (m.o.i.)

Infected cells were determined and titer of the virus inoculated on them. $2.5x10^4$ LLC-MK2 cells are seeded of a 24 well plate, 250 µL of 10^4 PFU/mL were used to infected it, MOI are

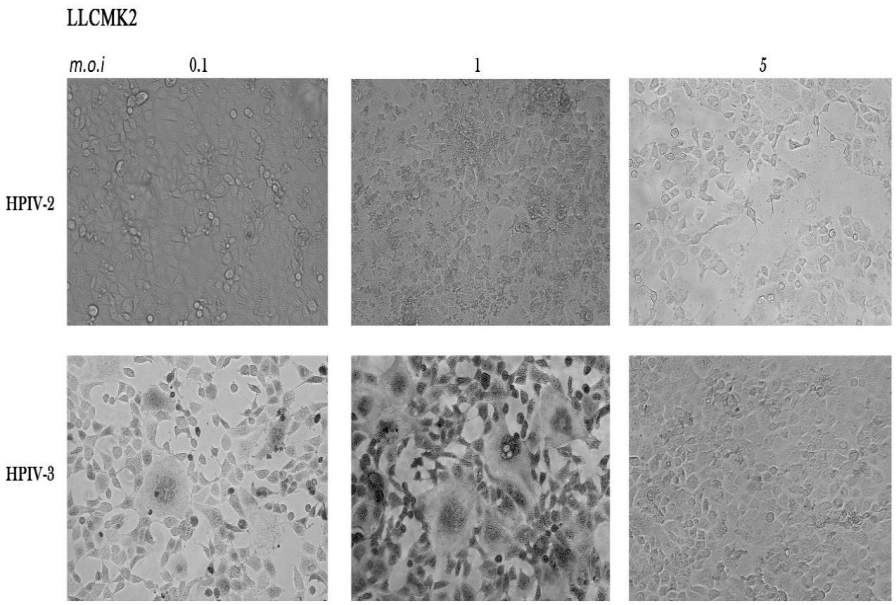
$$m.o.i = \frac{PFU}{\text{Number of cells}}$$

$$m.o.i = \frac{10^4 \times 0.25}{2.5x10^4} = 0.1 \text{ m.o.i.}$$

6.2.6 Receptors interaction-difference between HPIV-2 and 3 with high m.o.i. in LLC-MK2

HPIV-3 infection with high m.o.i. blocks fusion in LLC-MK2 cells. For HPIV-2 infection with high m.o.i. was not blocked fusion in LLC-MK2 cells represented in the Figure 6.23. The fusion mediated by HPIV-2 increases with increasing m.o.i. Cells were infected with HPIV-2 and 3 at the indicated m.o.i. (0.1, 1, 5). The degree of fusion at higher m.o.i. differs dramatically between HPIV-2 and 3. These results provided indication of foremost differences the receptor interactions between Human parainfluenza viruses.

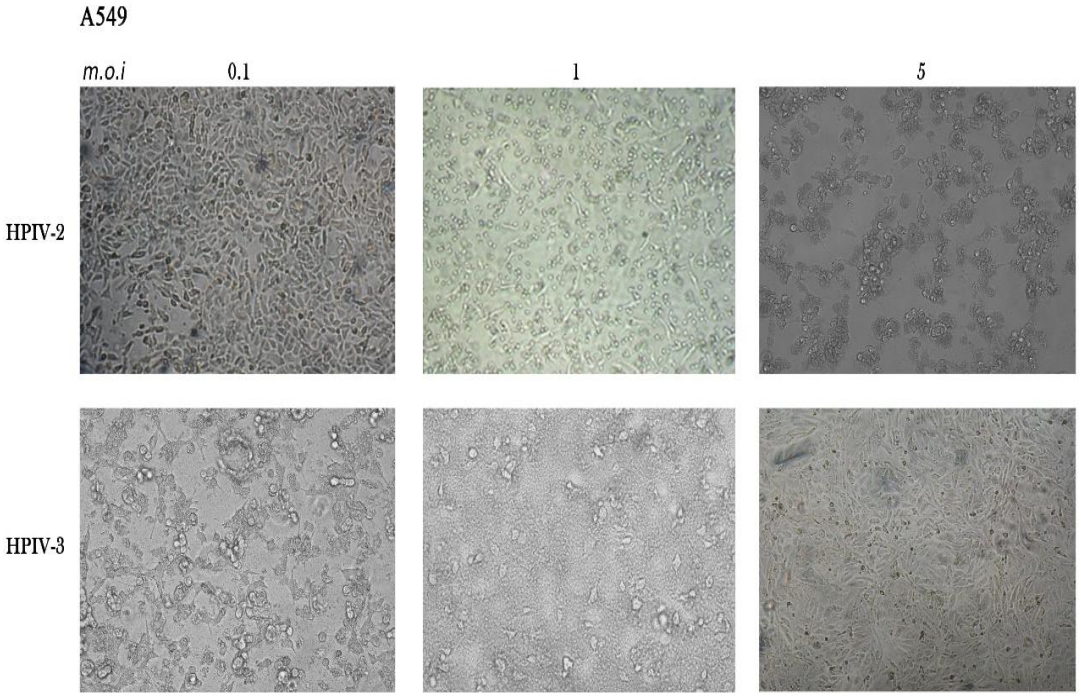
Figure 6.23: High m.o.i. of HPIV in LLC-MK2 cells



Receptors interaction - difference between HPIV-2 and 3 with high m.o.i. in A549

HPIV-3 infection with high m.o.i. blocks fusion in A549 cells displayed in the Figure 6.24. For HPIV-2 infection with high m.o.i. did not block fusion in A549 cells. HPIV-2 and 3 at the indicated m.o.i. (0.1, 1, 5). The Figure 6.24 showed that the gradation of fusion at higher m.o.i. differs intensely between HPIV-2 and 3. It was indicated that the receptor interactions between HPIV-2 and 3.

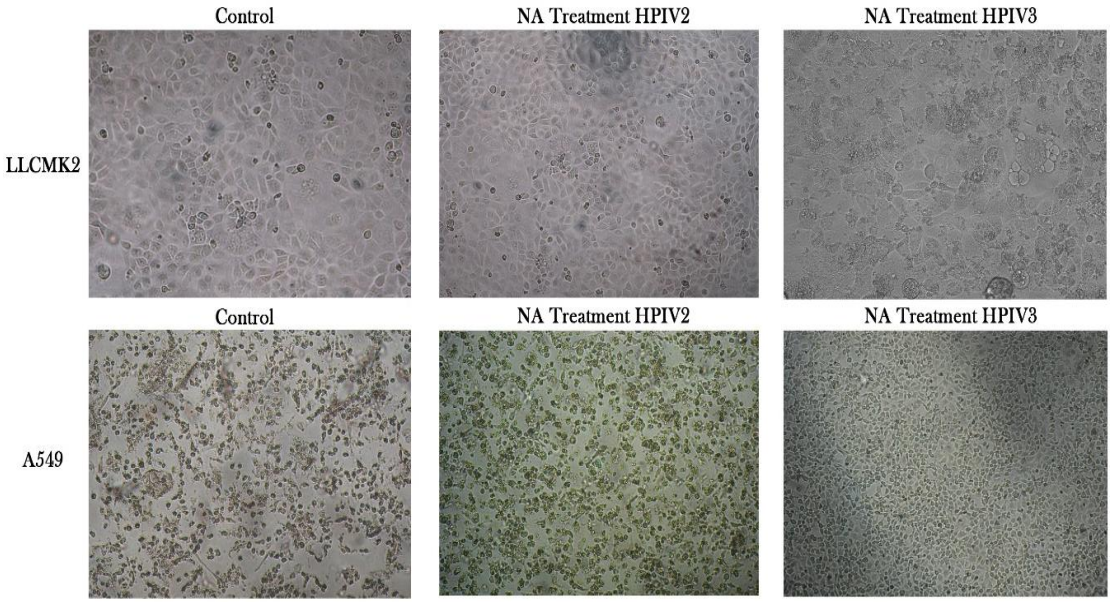
Figure 6.24: High m.o.i. of HPIV in A549 cells



6.2.7 Neuraminidase treatment - difference between HPIV-2 and 3 with low multiplicity of infection in LLC-MK2 and A549 cells

Effect of bacterial neuraminidase on the degree of cell fusion produced HPIV. Cells were infected with HPIV-2 and 3 in LLC-MK2 cells at low m.o.i. and treated with 0.1U *Clostridium perfringens* (which has a wide range of sialic acid linkage specificity). Figure 6.25 shows a clear difference between HPIV-2 and 3. Fusion mediated by HPIV-3 was inhibited by neuraminidase facilitated by HPIV-2 seems unpredicted. Neuraminidase was added after 90 minutes. Figure 6.25 shows that the extent of cell fusion in infected cells incubated with or without bacterial neuraminidase.

Figure 6.25: Treatment with bacterial neuraminidase with HPIV-2 and 3 in LLC-MK2 and A549 cells

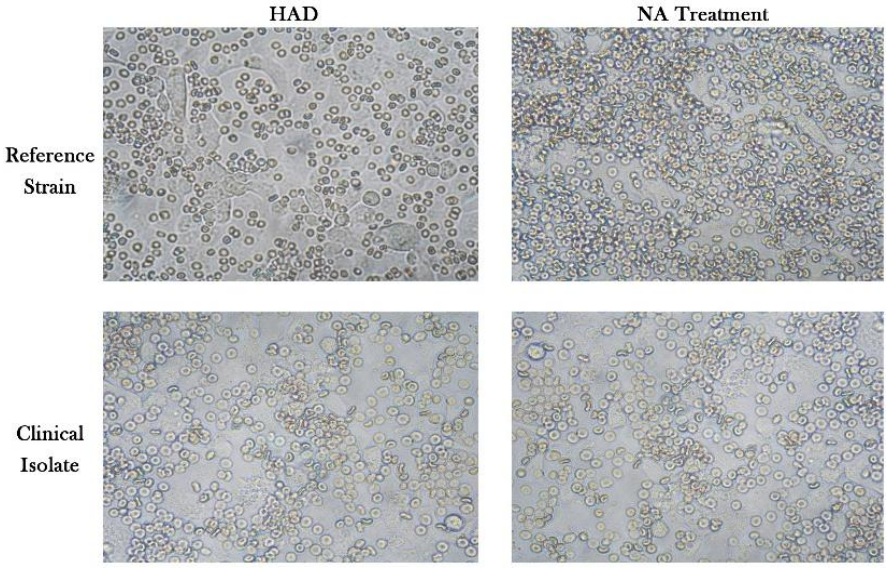


6.2.8 Quantification of hemadsorption assay (HAD)

The ability of HPIV to bind sialic acid receptors were assessed by a quantitative HAD assay in 24 well plates. Hemadsorption activity was done in infected cells if virus was present, erythrocytes were adhered on the monolayer and unbound erythrocyte are floated were seen in reference and clinical strain.

Post treatment of *C.perfringens* neuraminidase treatment, erythrocyte binding was greatly enhanced at 37 ºC on cells infected with HPIV were represented in the Figure 6.26.

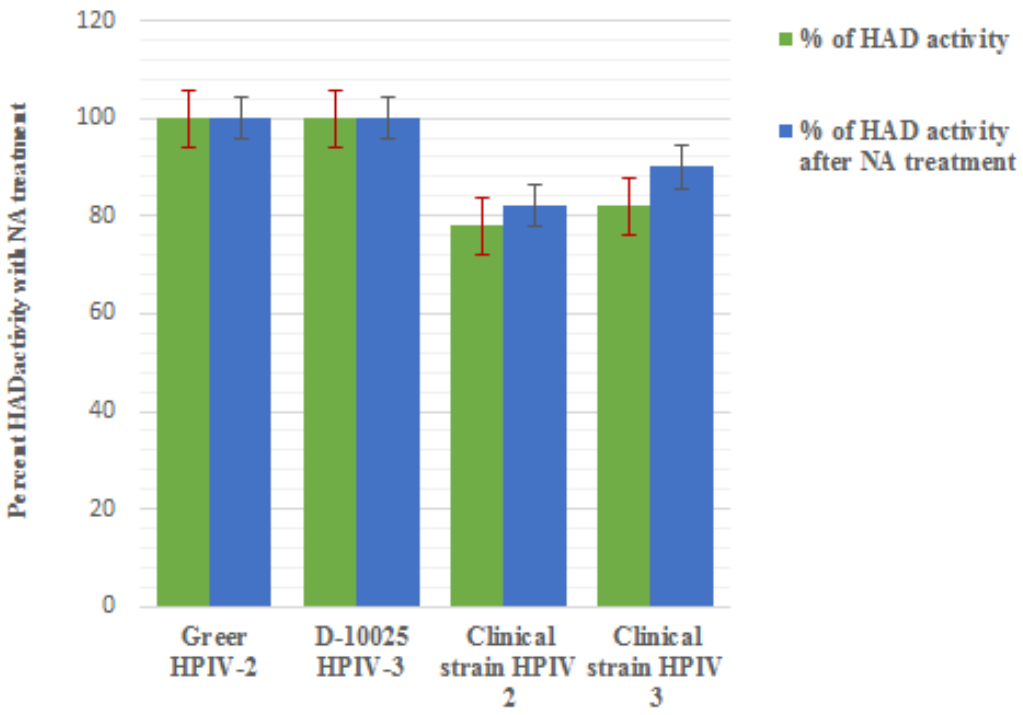
Figure 6.26: Interactions of receptor binding to HPIV with or without NA treatment by hemadsorption assay



Cells were infected with HPIV-2 and 3 at m.o.i (0.1) leading to 100% HAD activity present in reference strain but in clinical strain slightly decreased as 78% HAD activity for HPIV-2 and 82% of HPIV-3 at 37 ºC. Cells were infected with HPIV after adsorption period and treated with 0.1U of *C.Perfringens* neuraminidase which showed the percentage of HAD activity after NA treatment.

Erythrocytes were highly increased after NA treatment which shows 82% of HPIV-2 and 90% for HPIV-3, this percentage was not statistically significant standard error which represent in the Figure 6.27. The activity was determined after removing the enzyme by wide-ranging cell. Cells infected with HPIV with *C.Perfringens* treatment by HAD assay to enhance erythrocyte binding for HPIV-2 (82%) and HPIV-3 (90%).

Figure 6.27: Percentage of cells covered with erythrocytes and after NA treatment for HPIV-2 and 3



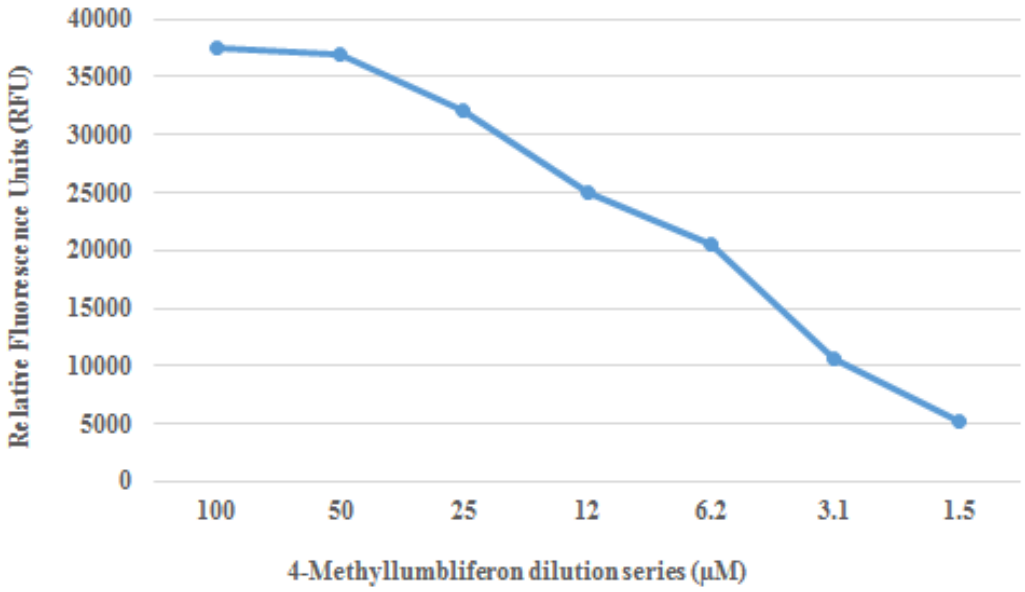
6.2.9 Determination of neuraminidase activity by Neuraminidase assay

4-Methylumbelliferone sodium salt 4-MU standard curve

Various concentration of 4-MU in stop solution which was stated by determination of fluorescence activity measured. It was specified that the 4-MU concentration increased the fluorescence intensity also increased progressively. Blank samples of buffer engendered signal around 10 RFU and an active series

to identify increased concentrations of 4-MU fluorescence. At 25 µM concentration of 4-MU exists the necessities for determination of relative fluorescence units (RFU) which was represented in the Figure 6.28. A standard curve was produced for 4-MU diluted in enzyme buffer at final concentrations of 0.05 µM to 50 µM. Background-corrected RFU was converted to 4-MU concentrations and used determined the percentage of substrate expended during the reaction.

Figure 6.28: 4-MU at different concentration

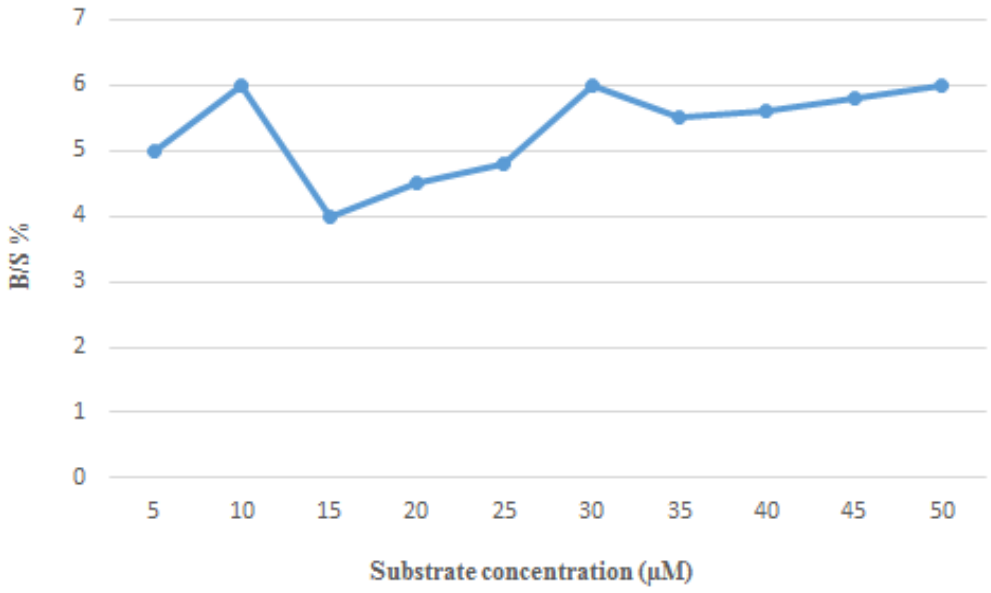


6.2.10 MUNNA based fluorescence activity

Various concentrations of MUNNA as a substrate were incubated with neuraminidase at 37 ºC for 3, 5, 10 and 15 minutes. If substrate concentrations increased, the fluorescence intensity also increased gradually which shown in the figure (linear regression). The enzymatic reaction rate was constant at 20 minutes when substrate concentrations are used as 5,10,15,20,25,30,35 and 40 µM. The various concentrations of substrate were used and the value of background ratio was found as the lesser.

The substrate fluorescence did not distinctly interference with the fortitudes which increased the sensitivity of detection which was referred in the Figure 6.29. The signal to background ratio were determined as the fluorescence intensities restrained after 20 minutes incubation period with deactivated and active enzyme. In order to select the substrate concentrations which are used at 25 µM.

Figure 6.29: Standardization of MUNNA with different concentrations



6.2.11 4-Mu with MUNNA based fluorescence intensity

The fluorescence activity of absorption and emission between the MUNNA substrate and 4-MU in the neuraminidase enzyme assay showed effects in a MUNNA concentration dependent nonlinear interference with 4-MU concentration fortitude in the assay. The Relative fluorescence units (RFU) and the correction factor were empirically determined, using control measurements of fluorescent with 4-MU alone, mixtures of 4-MU and MUNNA. The correction factor was intended by dividing the fluorescence signal formed by 4-MU in the presence of different concentration of MUNNA. The fluorescence signal produced by 4-MU alone (in RFU) were shown in the Table 6.8.

Neuraminidase enzymatic reactions were determined by spectrofluorometer. The correction factors for each MUNNA concentration were used to correct RFU

values at the equivalent MUNNA concentrations. Determination of NA activity comprises the use of multiple concentrations of MUNNA and subject to nonlinear interference of 4-MU quantification by the spectroscopic interference supplementary with micromolar concentrations of MUNNA. The 4-MU standard curves were generated for all enzyme activity measurements in which the MUNNA substrate was used.

The MUNANA substrate indicated that major fluorescence at the 4-MU excitation and emission wavelengths and at the MUNNA concentration series used in the neuraminidase enzyme activity assay, which produced generous nonlinear interference with MUNNA and 4-MU fluorescence measurements which represent in the Table 6.8.

Table 6.8: Calculation of correction factor

	Fluorescence (RFU)		**Fluorescence MUNNA+MU (RFU)**		
Concentrations of MUNNA	**MUNNA only[a]**	**MU only[b]**	**MUNNA+MU[c]**	**Blanked[d]**	**Correction factor[e]**
Column1	**Column2**	**Column3**	**Column4**	**Column5**	**Column6**
2000	13642	10179	20531	6889	0.67
1000	7821	10000	16532	8711	0.84
500	3561	10323	10337	6776	0.65
250	1554	10542	10526	8972	0.87
125	1278	10428	10342	9064	0.88
50	537	10254	10236	9699	0.94
25	425	10339	10339	9914	0.96
12.5	247	10078	10000	9753	0.94
6.25	142	10267	10112	9970	0.97
3.1	123	10371	9891	9768	0.95
1.5	82	10256	10254	10172	0.98

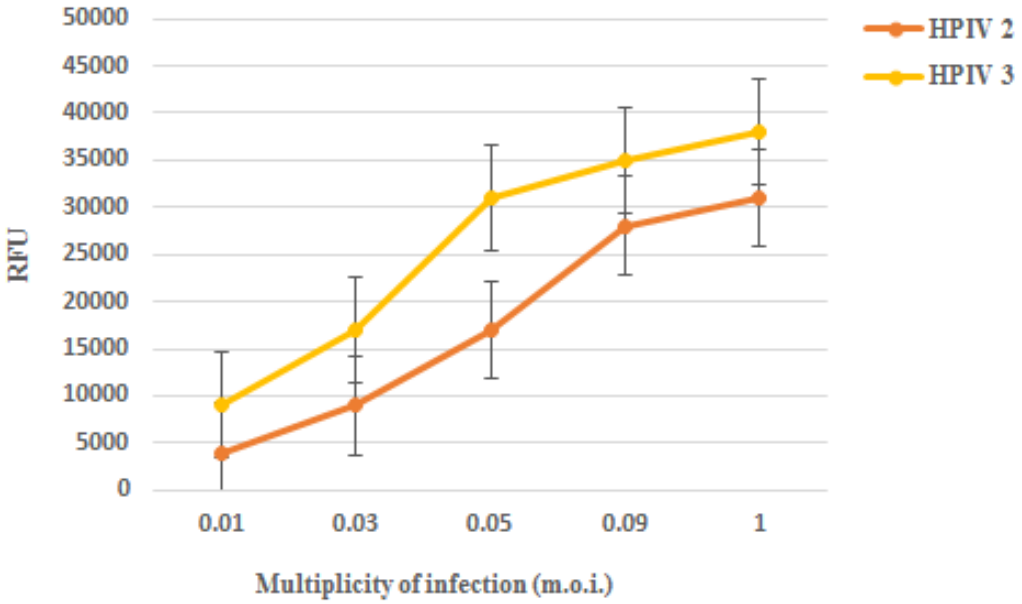
a. Fluorescence of MUNNA only in enzyme buffer at 1.5 to 2000µM (column1).
b. Fluorescence of 4-MU only in enzyme buffer at 12µM concentration. Mean (±SD) 4-MU fluorescence in enzyme buffer was 10277±09 RFU.
c. Fluorescence of 4-MU in enzyme buffer at 12µM concentration in the presence of 1.5-2000 µM MUNNA concentrations (column 1).
d. RFU values of MUNNA fluorescence from column 2 subtracted from the values in (RFU) obtained in the mixture of 4-MU and MUNNA column 4.

6.2.12 Neuraminidase activity

Human parainfluenza virus type-2 and 3 were diluted with MES buffer with MUNNA as a substrate for one hour to measure the fluorescence intensity after addition of stop solution. The readout relative fluorescence unit (RFU) was directly proportional to the amount of virus was auxiliary to each assay well. Various concentrations of HPIV type-2 and 3 (m.o.i.) were used to perform neuraminidase assay, for HPIV-2 RFU were 35000 and HPIV-3 (38000) and statistically significant ($p<0.0001$).

Substrate concentrations of MUNNA at 25 µM were used in the phenotypic assay for determination of susceptibility in Human parainfluenza virus type-2 and 3 to Neuraminidase inhibitors and the spectroscopic interference was similar across different 4-MU fluorescence tested shown in the Figure 6.30.

Figure 6.30 Neuraminidase activity of HPIV-2 and 3

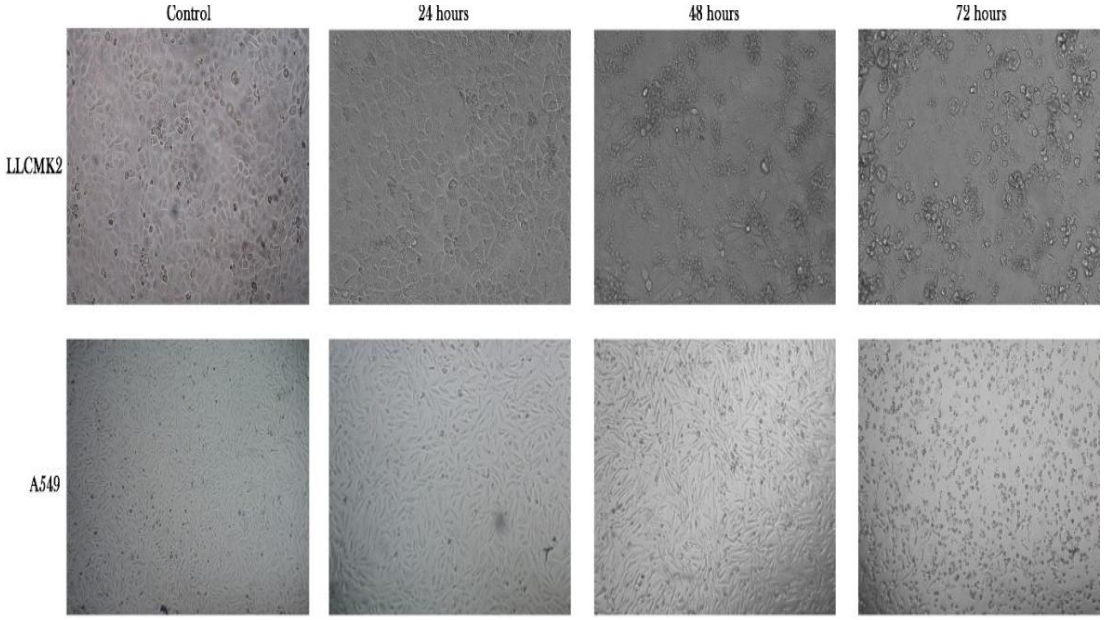


6.3 ANTIVIRAL ACTIVITY OF ZANAMIVIR AND RIBAVIRIN AGAINST HPIV-2 AND 3

6.3.1 Cytotoxicity of Zanamivir in LLC-MK2 and A549 by MTT and Neutral red uptake assay

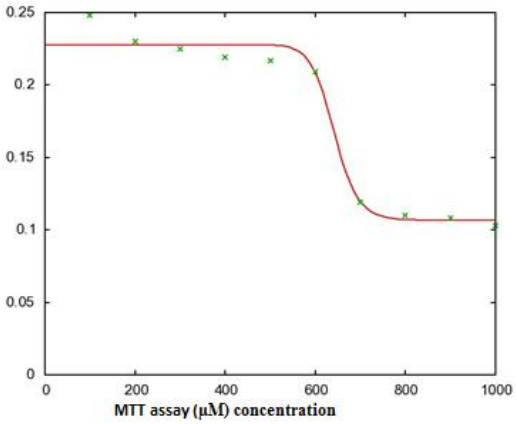
Various concentrations of drugs were evaluated in LLC-MK2 and A549 cell line in 96-well flat-bottomed microplates. The cell morphology were changed at 24 h, 48 h and 72 h which shown in the Figure 6.31.

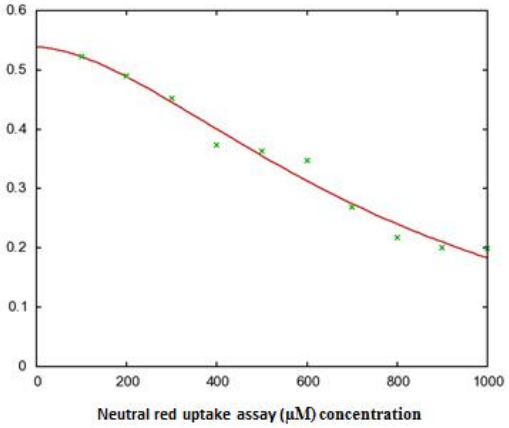
Figure 6.31: Cytotoxicity of Zanamivir in LLC-MK2 and A549 cells at 24, 48 and 72 hours



Cytotoxic end points (50% cell-inhibitory concentrations [IC_{50}]) in LLC-MK2 were determined at 641 µM in MTT assay and 807 µM in neutral red uptake assay which represent in the Figure 6.32. The Hill coefficient percentage engendered for MTT (24.5%) and NR (53.6%).

Figure 6.32: IC_{50} concentrations of Zanamivir in LLC-MK2 cells by MTT assay and Neutral red uptake assay





Results:

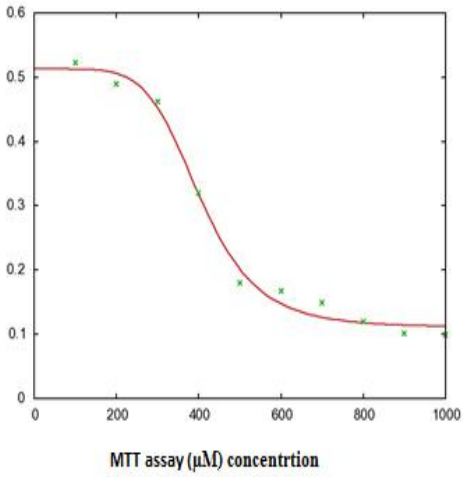
Minimum	0.106664 +/- 0.006103 (5.722%)
Maximum	0.227925 +/- 0.00462 (2.027%)
IC_{50}	641.89 +/- 15.8 (2.462%)
Hill coeff.	24.5885 +/- 7.806 (31.75%)

Results:

Minimum	-0.0623621 +/- 0.38 (609.3%)
Maximum	0.538393 +/- 0.04002 (7.433%)
IC_{50}	807.059 +/- 562.8 (69.73%)
Hill coeff.	1.72064 +/- 0.9222 (53.6%)

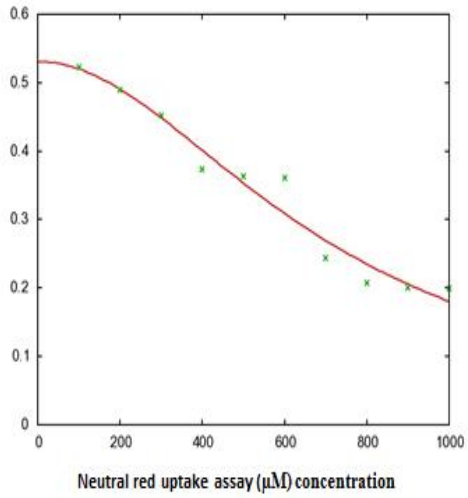
Cytotoxic end points (50% cell-inhibitory concentrations [IC_{50}]) in A549 were determined at 404 µM in MTT assay and 700 µM in neutral red uptake assay which represent in the Figure 6.33. The Hill coefficient percentage engendered for MTT (16.9%) and NR (61.6%).

Figure 6.33: IC_{50} concentrations for Zanamivir in A549 cells by MTT assay and Neutral red uptake assay



Results:

Minimum	0.110454 +/- 0.01202 (10.88%)
Maximum	0.512955 +/- 0.01458 (2.842%)
IC_{50}	404.392 +/- 13.06 (3.23%)
Hill coeff.	5.79741 +/- 0.9851 (16.99%)



Results:

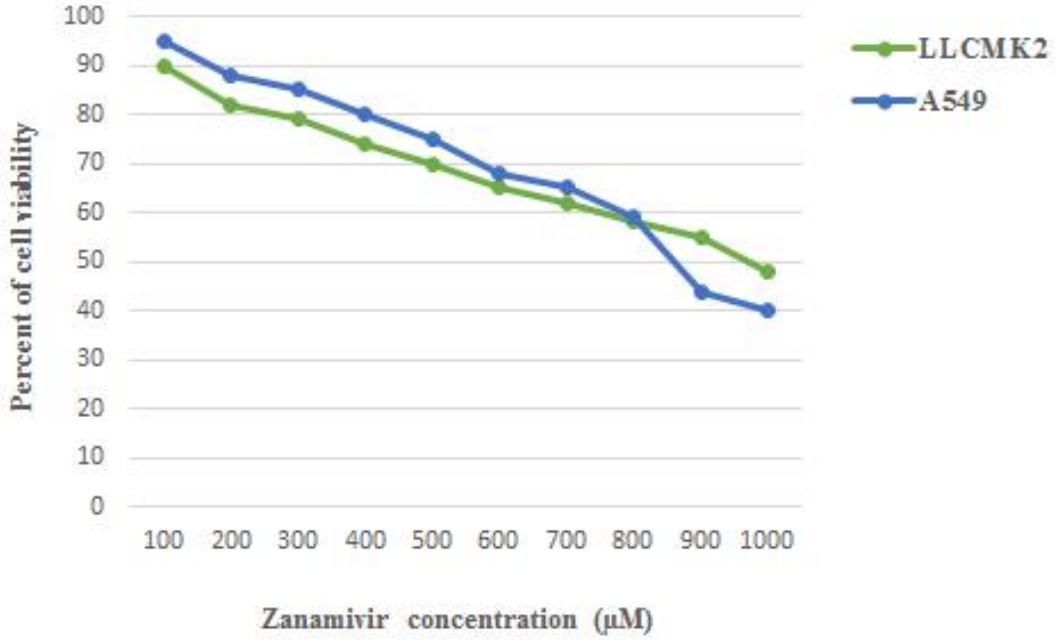
Minimum	0.00687879 +/- 0.3105 (4514%)
Maximum	0.530895 +/- 0.04453 (8.388%)
IC_{50}	700.9 +/- 412.2 (58.81%)
Hill coeff.	1.97924 +/- 1.219 (61.61%)

6.3.2 Cytotoxicity of Zanamivir in LLC-MK2 and A549 cells by trypan blue exclusion method

The counting chamber of a haemocytometer was allocated by grating lines that ascertain the chamber parts to be used in cell counting.

Four corners were counted with viable (unstained cells) and non-viable (stained cells) which represent in the Figure 6.34 and percentage of compound cytotoxicity for LLC-MK2 were progressed as 50% at 1000 µM and A549 were generated as 50% cytotoxicity at 900 µM which embodied in the Figure 6.34.

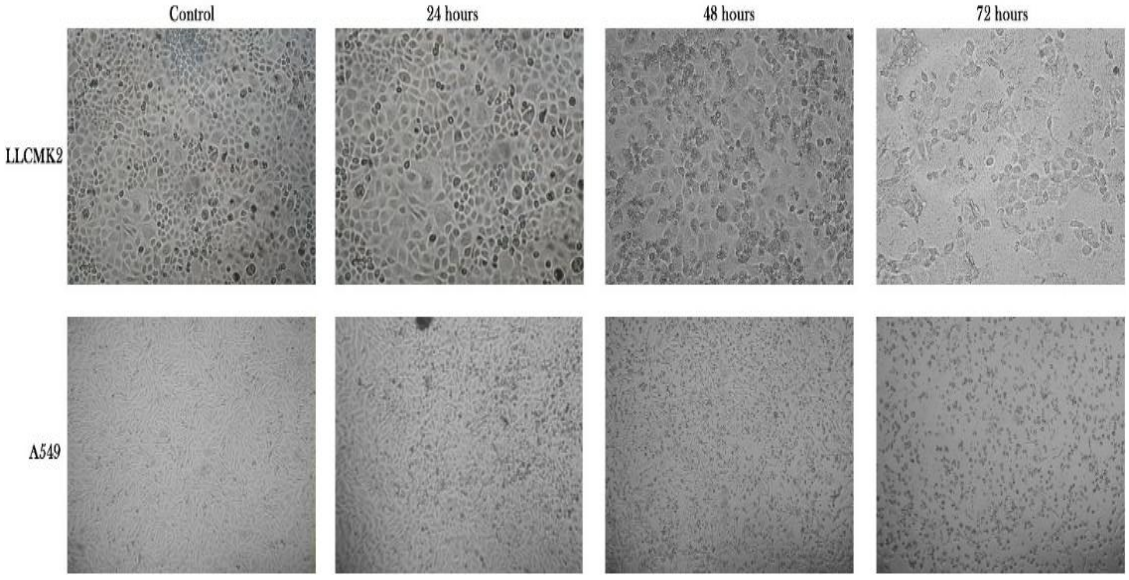
Figure 6.34: Percentage of cell viability of Zanamivir in LLC-MK2 and A549 cells by trypan blue exclusion method



6.3.3 Cytotoxicity of Ribavirin in LLC-MK2 and A549 against HPIV by MTT and Neutral red uptake assay

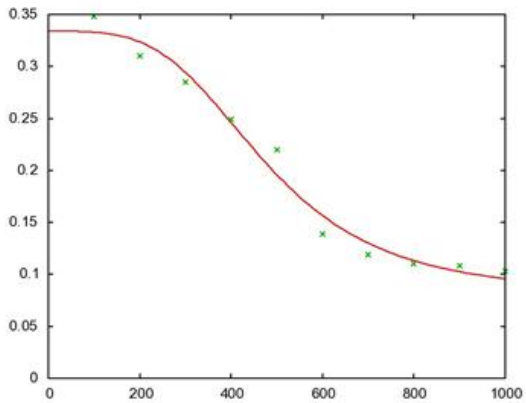
Several concentrations of drugs were assessed in LLC-MK2 and A549 cell line in 96-well flat-bottomed microplates. Cytotoxicity were identified after incubation period at 24 h, 48 h and 72 h which shown in the Figure 6.35.

Figure 6.35: Cytotoxicity of Ribavirin in LLC-MK2 and A549 cells at 24, 48 and 72 hours



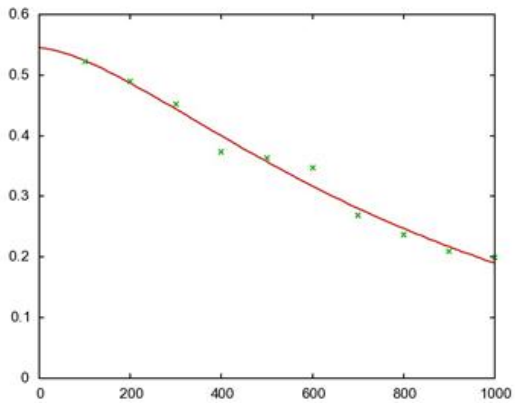
Cytotoxic end points (50% cell-inhibitory concentrations [IC_{50}]) for Ribavirin in LLC-MK2 were determined at 476 µM in MTT assay and 918 µM in neutral red uptake assay which represent in the Figure 6.36. Hill coefficient percentages were generated for MTT as 27.8% and Neutral Red as 53.1%.

Figure 6.36: IC_{50} concentrations of Ribavirin in LLC-MK2 by MTT assay and Neutral red uptake assay



Results:

Minimum	0.0796844 +/- 0.0247 (31%)
Maximum	0.333933 +/- 0.01443 (4.321%)
IC_{50}	476.793 +/- 37.36 (7.836%)
Hill coeff.	3.64046 +/- 1.015 (27.89%)

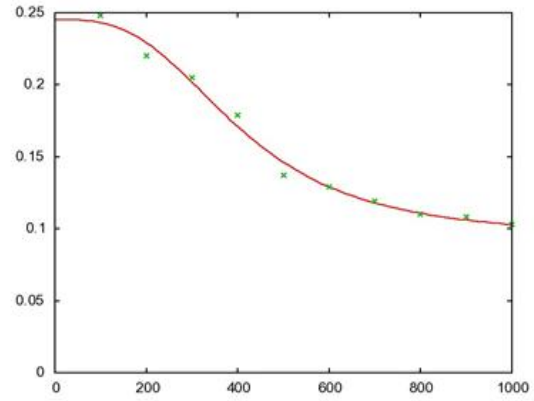


Results:

Minimum	-0.121854 +/- 0.4846 (397.7%)
Maximum	0.545297 +/- 0.04096 (7.512%)
IC_{50}	918.662 +/- 802.9 (87.39%)
Hill coeff.	1.53597 +/- 0.8171 (53.19%)

Cytotoxic end points (50% cell-inhibitory concentrations [IC_{50}]) for Ribavirin in A549 were determined at 405 µM in MTT assay and 753 µM in neutral red uptake assay which represent in the Figure 6.37. Hill coefficient percentage was generated for MTT as 20.68% and Neutral Red as 34.7%.

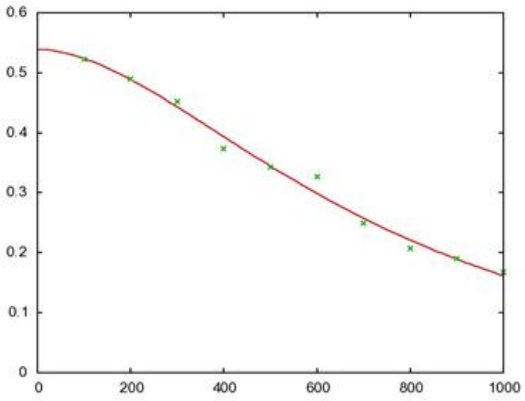
Figure 6.37: IC_{50} concentrations for Ribavirin in A549 MTT assay and Neutral red uptake assay



MTT assay (µM) concentration

Results:

Minimum	0.0935651 +/- 0.01003 (10.72%)
Maximum	0.245313 +/- 0.007644 (3.116%)
IC_{50}	405.299 +/- 26.06 (6.43%)
Hill coeff.	3.02225 +/- 0.6251 (20.68%)



Neutral red uptake assay (µM) concentration

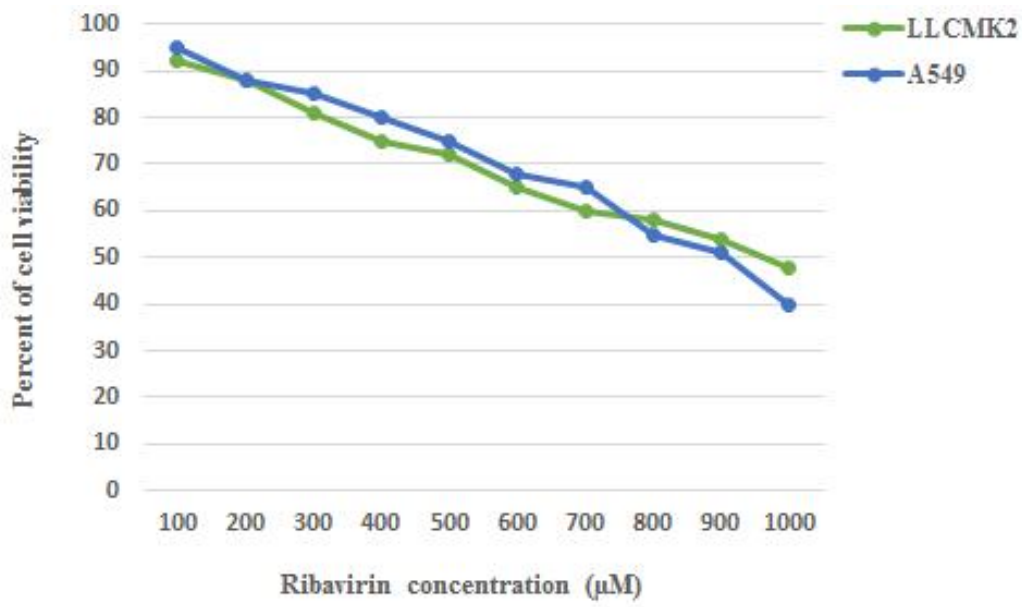
Results:

Minimum	-0.0642148 +/- 0.225 (350.4%)
Maximum	0.538876 +/- 0.02753 (5.108%)
IC_{50}	753.55 +/- 298.7 (39.64%)
Hill coeff.	1.80812 +/- 0.6275 (34.7%)

6.3.4 Cytotoxicity of Ribavirin in LLC-MK2 and A549 cells by trypan blue exclusion method

The counting chamber of a haemocytometer was allocated by grating lines that establish the chamber parts to be used in cell counting. Four corners were counted with viable (unstained cells) and non-viable (stained cells) which epitomize the percentage of compound cytotoxicity for LLC-MK2 were progressed as 50% at 900 µM and A549 were generated as 50% cytotoxicity at 800 µM which embodied in the Figure 6.38.

Figure 6.38: Cytotoxic percentage of ribavirin by trypan blue exclusion method

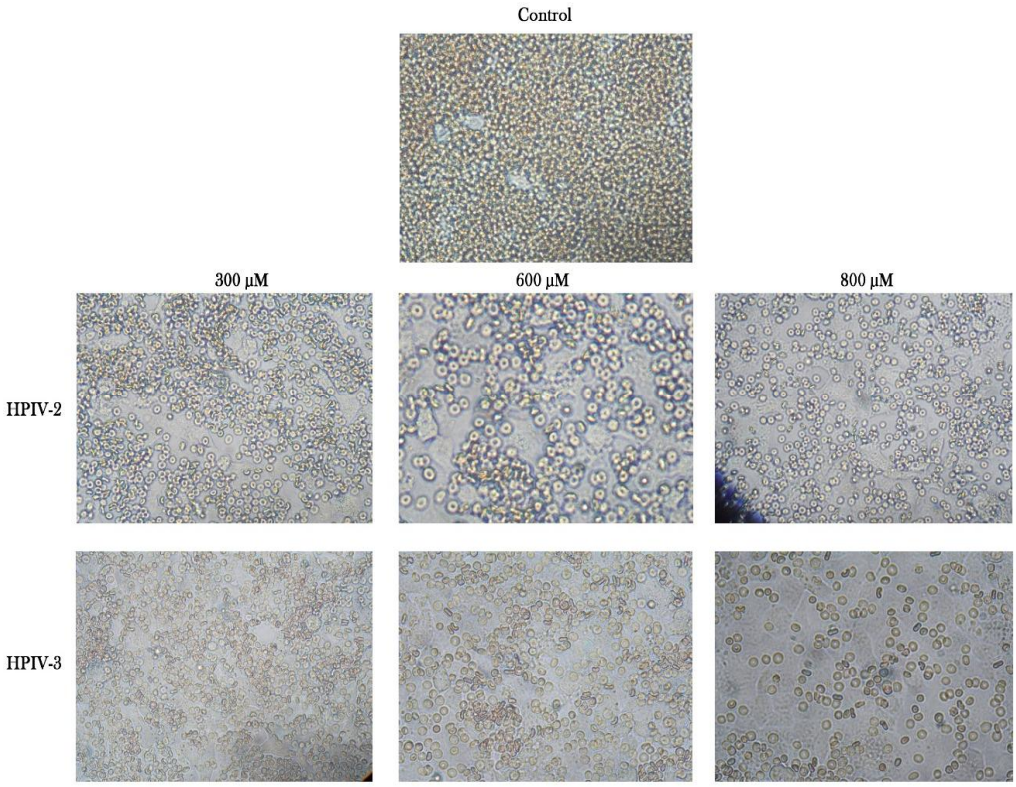


6.4 ANTIVIRAL ACTIVITY OF NEURAMINIDASE INHIBITOR AND NUCLEOSIDE INHIBITOR

6.4.1 Antiviral activity of 4-GU-DANA against HPIV by hemadsorption inhibition assay (HAD)

The inhibitory activity of 4-GU-DANA on sialic acid receptor was determined by hemadsorption assay. The establishment of HPIV-2 and 3 infection in the host erythrocytes with the adherence of sialic acid receptors in the presence of the 4-GU-DANA (at various concentrations) was evaluated for the inhibitory activity of virus in cell lines which shown in the Figure 6.39.

Figure 6.39: Inhibition of HPIV by hemadsorption inhibition assay at different concentrations



4-GU-DANA effectively blocks the HPIV-2 and 3 attachments over the adherence of erythrocytes to the monolayer and the inhibition of receptor binding at 600 μM shows the inhibition percentage was obtained as 77% for HPIV-2 and 78% for HPIV-3 but in reference strain 79% for HPIV-2 and 80% (HPIV-3). At various concentrations of 4-GU-DANA at 800 μM of hemadsorption inhibition percentage was obtained as 83% for HPIV-2 and 86% (HPIV-3) but in clinical isolates 80% for HPIV-2 and 82% (HPIV-3).

4-GU-DANA efficiently indicates the HPIV-3 (reference strain and HPIV-3 isolates) with the adherence of erythrocytes to the monolayer shown in the Table 6.9. The reference strain of HPIV-3 in 4-GU-DANA blocks the hemadsorption with complete inhibition of erythrocyte binding at 300 μM (LLC-MK2) and 200 μM concentrations in A549 cells, but in the clinical isolate 500 μM (LLC-MK2) and 400 μM concentration in A549 cells.

6.4.2 Antiviral activity of 4-GU-DANA against HPIV by plaque reduction assay

The efficacy of 4-GU-DANA on viral entry and replication was assessed by plaque reduction assay. The HPIV-3 inhibitory activities of 4-GU-DANA at various concentrations were evaluated in A549 ($3.1x10^5$) and LLC-MK2 ($4.1x10^5$) cell monolayers with approximately 200 Plaque Forming Units (PFU). The HPIV 3 was infected with established monolayer of A549 and LLC-MK2 cells which induced cytopathic effect were observed. Then the CPE confirmed strain was continued for plaque reduction assay. The formed plaque was kept for 90 minutes adsorption period, the monolayers were overlaid with agarose containing 4-GU-DANA which revealed in the Table 6.9. The 4-GU-DANA was screened at the various concentrations from 100 to 1000 μM. The 500 μM showed maximum reduction of plaque formation (72%) reference strain and clinical strain (60%) in LLC-MK2 cells Figure 6.40. Similarly the A459 cells illustrate (85%) reference strain and (79%) clinical strain were plaque formation Figure 6.41. The viral inhibition was higher in the A459 cells than LLC-MK2.

Table 6.9: Effects of Neuraminidase inhibitor by plaque reduction assay

Hemadsorption (HAD)	Viral entry and binding			
4-GU-DANA (µM)	Reference strain[a]		Clinical strain[b]	
Concentration	LLCMK2	A549	LLCMK2	A549
100	-	-	-	-
200	-	-	-	-
300	++	++	-	-
400	+++	+++	-	++
500	+++	+++	+++	+++
(600-1000)	+++	+++	+++	+++

Reference strain of Human parainfluenza virus type-3 (HPIV-3) with 4-GU-DANA (+) indicates the hemadsorption inhibition activity and (–) indicates partial Hemadsorptioninhibition activity.

[b]Clinical strain of Human parainfluenza virus type-3 (HPIV-3) with 4-GU-DANA (+) indicates the Hemadsorption inhibition activity and (–) indicates partial Hemadsorption inhibition activity

Figure 6.40: Plaque reduction (%) for HPIV-3 in LLC-MK2 cells

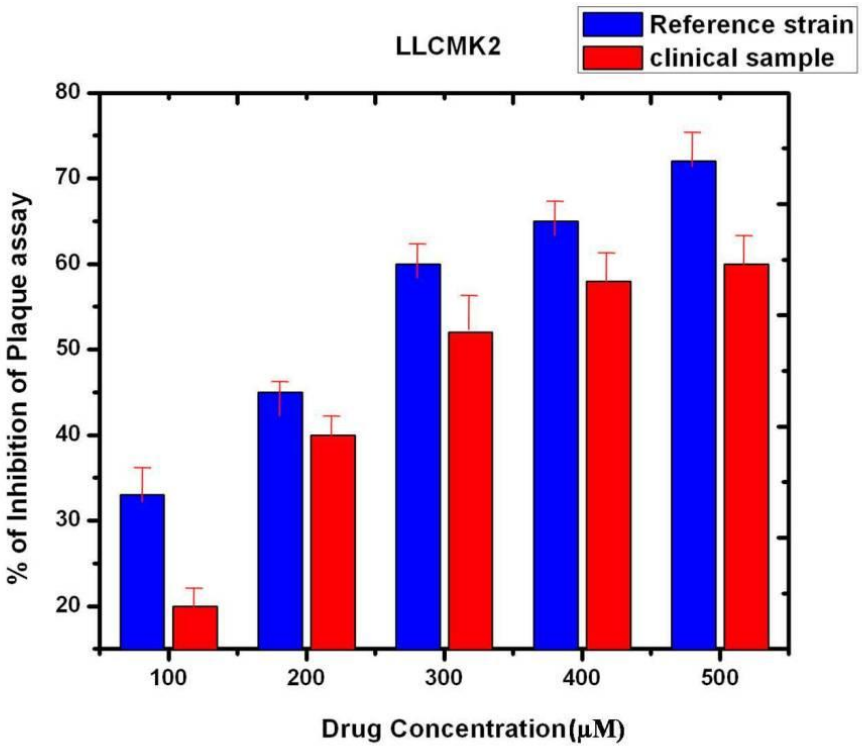
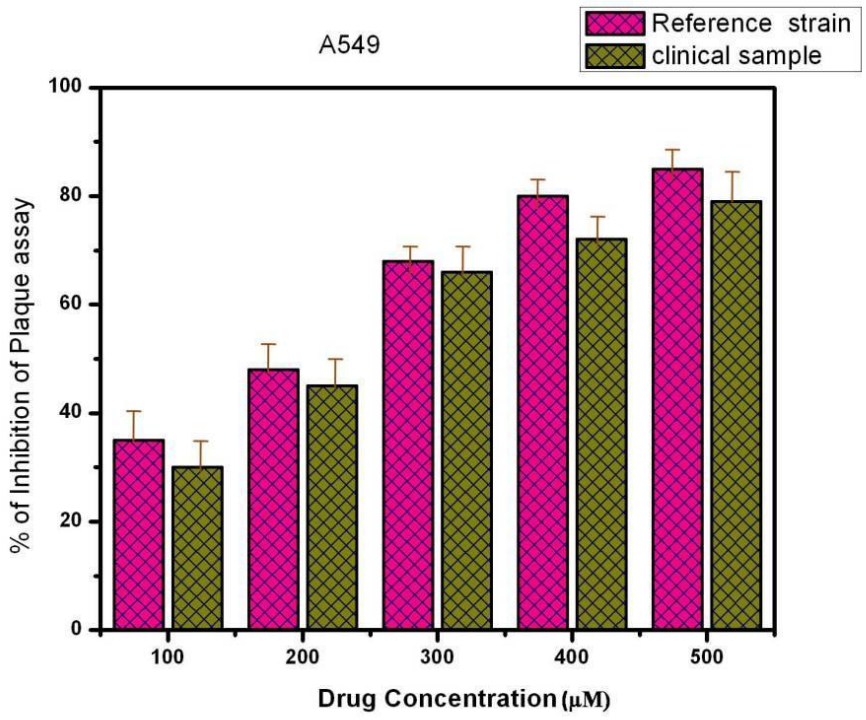


Figure 6.41: Plaque reduction (%) for HPIV-3 in A549 cells



Plaque reduction for HPIV-2 in LLC-MK2 cell line for 4GUDNA at 500 µM concentration showed maximum reduction of plaque formation (75%) in reference strain and (60%) in clinical strain. Similarly, the A459 cells illustrate (75%) reference strain and (67%) clinical strain with plaque formation. The viral inhibition was higher in the A459 cells than LLC-MK2. 50% Inhibitory concentration of Zanamivir was performed at 300 µM, 200 µM in LLC-MK2 and A549 cells respectively.

6.4.3 Antiviral activity 4-GU-DANA against HPIV-2 and 3 by Neuraminidase inhibition assay (NAI)

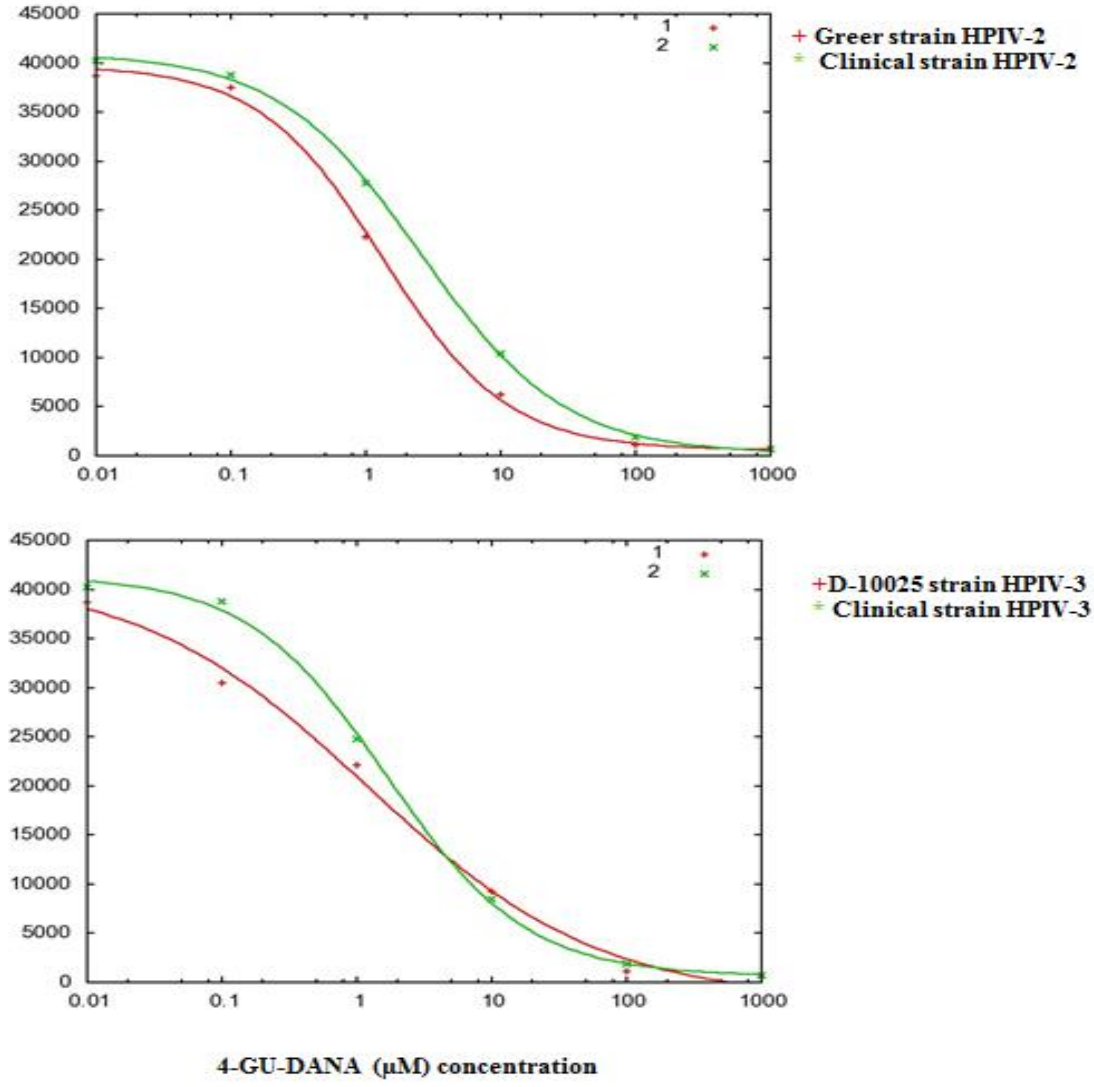
Cytotoxicity of 4-GU-DANA generated at 641 µM by MTT assay. But in neuraminidase inhibition assay, compound concentrations were used from lower to higher concentrations (0.1-1000 µM). Virus preparation of HPIV type 2 and 3 m.o.i. (1) was used to perform the NAI assay. Because HPIV were accomplished and standardized in neuraminidase assay. Neuraminidase activity as nanomoles per minute per milligram of protein used for ensured the NAI assay.

The inhibition of neuraminidase activity presented as a function of the 4-GU-DANA concentrations. Virions released were high in reference strain when comparable to clinical isolates it seems that exogenous neuraminidase treatment were occurred. After NA treatment to destroy the receptors to which HN binds thus enhancing the elution of progeny clinical isolates (HPIV-2 and 3) virions from the cell surface.

The neuraminidase inhibition assay for HPIV-2 and 3 was performed in triplicates and the results revealed substantial differences in absolute neuraminidase activity. In NAI assay, reference strains used smaller concentrations when compared to clinical strains which inhibit the HPIV-2 and 3.

The IC_{50} concentration for 4-GU-DANA against HPIV-2 at 2.5 µM and HPIV-3 at 1.6 µM. 4-GU-DANA concentrations were used lesser amount for HPIV-2 when compared to HPIV-3. At different micromolar concentrations of 4-GU-DANA against HPIV-2 and 3 were used to inhibit the neuraminidase activities which are represented in the Figure 6.42.

Figure 6.42: Inhibition by 4-GU-DANA of neuraminidase activity on HN of HPIV-2 and 3



The IC_{50} values are calculated used IC_{50} tool kit and multi IC_{50} plotting tool kit. Hill coefficient range and IC_{50} values are depicted in the Table 6.10.

Table 6.10: IC_{50} concentration of 4GU-DANA against HPIV-2 and 3

Virus	Strains	Minimum	Maximum	IC_{50}	Hill coefficient
HPIV 2	Greer strain	591.926±754.5	39724.1±956.8	1.3	0.944705±0.1101
	Clinical/Chennai isolates	209.858±475	40980.9±505	2.5	0.820009±0.04457
HPIV 3	D-10025 strain	1847.15±2728	41530.1±0.6154	1.2	0.506028±0.1405
	Clinical Chennai isolates	615.781±789.7	41405.2±957.7	1.6	0.836583±0.08466

Based on the studies, the inhibition of HPIV-2 (Greer strain HPIV-2) and clinical isolates were determined by neuraminidase inhibitor (4-GU-DANA) in which the 50% inhibitory concentrations in A549 was identified as bars indicated the percent inhibition of neuraminidase activity (nanomoles/minute/mg of protein) as a utility of micromolar concentrations of 4-GU-DANA. In LLC-MK2 percentage of neuraminidase inhibitory concentration were identified more or less similar.

Consistent with the analyses of 4-GU-DANA by Neuraminidase inhibition activity on reference strain (D-10025 strain HPIV-3) and clinical isolates in which known as 50% inhibitory concentrations in A549 was recognized as bars indicated the percent inhibition of neuraminidase activity by way of utility on micromolar concentrations of 4-GU-DANA. In LLC-MK2 percentage of neuraminidase inhibitory concentration were identified more or less similar.

6.4.4 Antiviral activity of Ribavirin against HPIV by hemadsorption inhibition assay (HAD)

The inhibitory activity of 1-β-D-ribofuronosyl-1,2,4 triazole-3-carboxamide (Ribavirin) on nucleocapsid was determined by hemadsorption assay. The founding of HPIV-2 and 3 infections in the host erythrocytes with the adherence of receptors in the presence of the Ribavirin (at various concentrations) was evaluated for the inhibitory activity of HPIV-2 and 3.

Ribavirin efficiently blocks the HPIV-2 and 3 attachments over the adherence of erythrocytes to the monolayer and the inhibition of adherence at 400 µM shows the inhibition percentage was 77% for HPIV-2 and 75% for HPIV-3 but in reference strain 80% for HPIV-2 and 82% (HPIV-3). At various concentrations of Ribavirin at 800 µM of hemadsorption inhibition percentage was 83% for HPIV-2 and 86% (HPIV-3) but in clinical strain 80% for HPIV-2 and 82% (HPIV-3) with means ± SD for measurement for three to six wells which revealed in the Figures 6.43 and 6.44.

Figure 6.43: Inhibition by Ribavirin on HAD cells expressing of HPIV

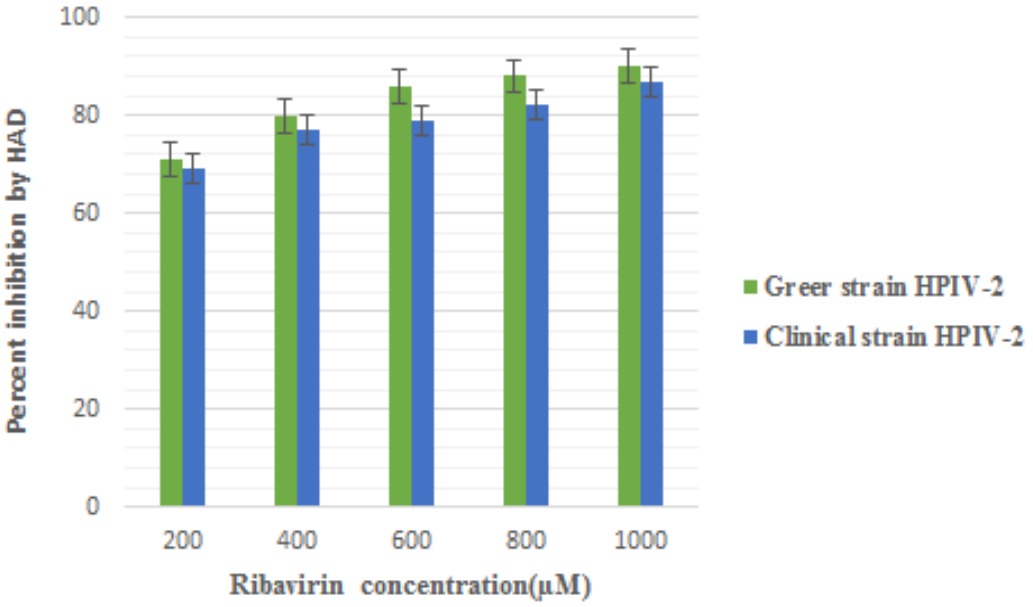
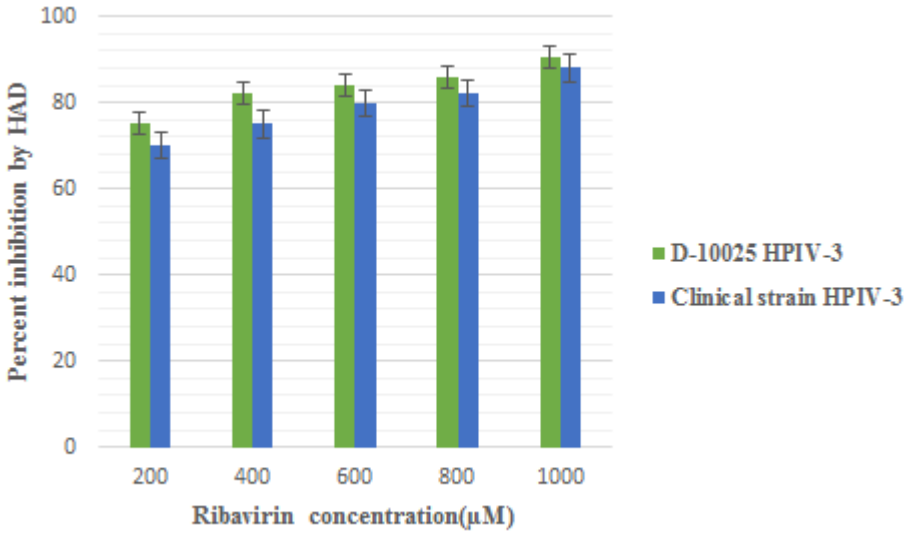


Figure 6.44: Inhibition activity of HPIV-3 by HAD



6.4.5 Effects of Nucleoside inhibitor by plaque reduction assay

The ribavirin was assessed for the HPIV- 2 and 3 plaque reduction during pre and post adsorption periods in two different cell lines. Table 6.11 shows that there was no significant reduction in plaque number due to the presence of ribavirin during the adsorption period of 90 minutes. The plaque area was strikingly reduced by addition after the adsorption period of ribavirin in LLC-MK2 and A549 cells for HPIV type 2 and 3. This study demonstrates that the incubation of nucleoside analogue inhibits the viral replication or entry in cells. Human parainfluenza virus type 2 was significantly resembled with HPIV type 3.

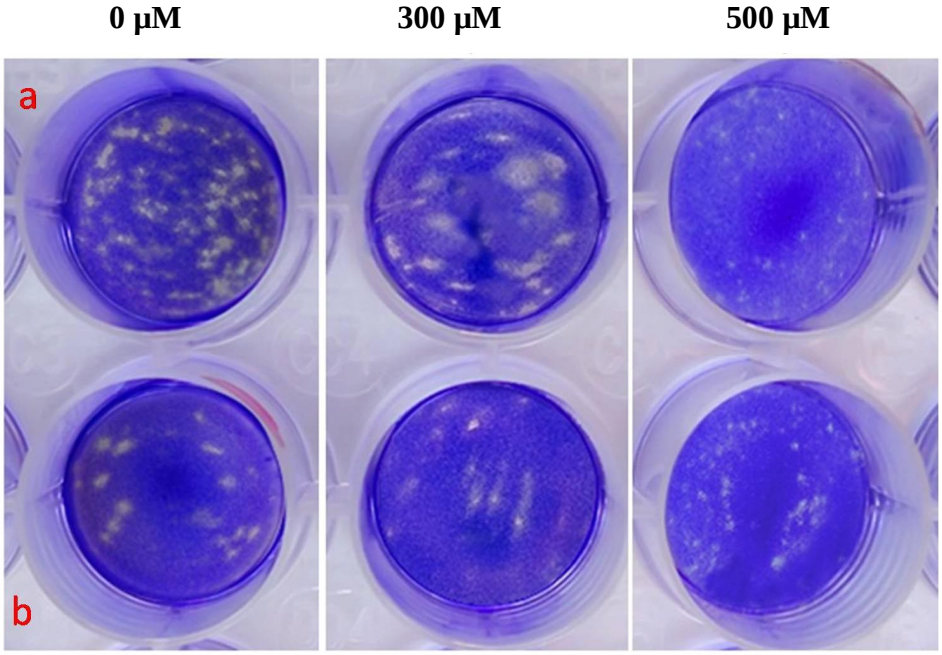
Table 6.11: Effect of 4-GU- DANA on inhibition of HPIV-3 viral entry and replication in different intervals

Plaque reduction assay % reduction of plaque number	Viral entry and binding			
4-GU-DANA (µM)	Reference strain[a]		Clinical strain[b]	
Time Intervals	LLC-MK2	A549	LLC-MK2	A549
0 min	16%	15%	14%	15%
90 min	20%	22%	19%	20%
180 min	72%	85%	60%	79%

[a]Reference strain of Human parainfluenza virus type-3 (HPIV-3) with 4-GU-DANA on plaque reduction assay;[b]Clinical strain of Human parainfluenza virus type-3 (HPIV-3) with 4-GU-DANA on plaque reduction assay.

The difference in the plaque enlargement and formation were observed in the absence of inhibitor and plaque reduction was shown in the presence of ribavirin shown in Figure 6.45.

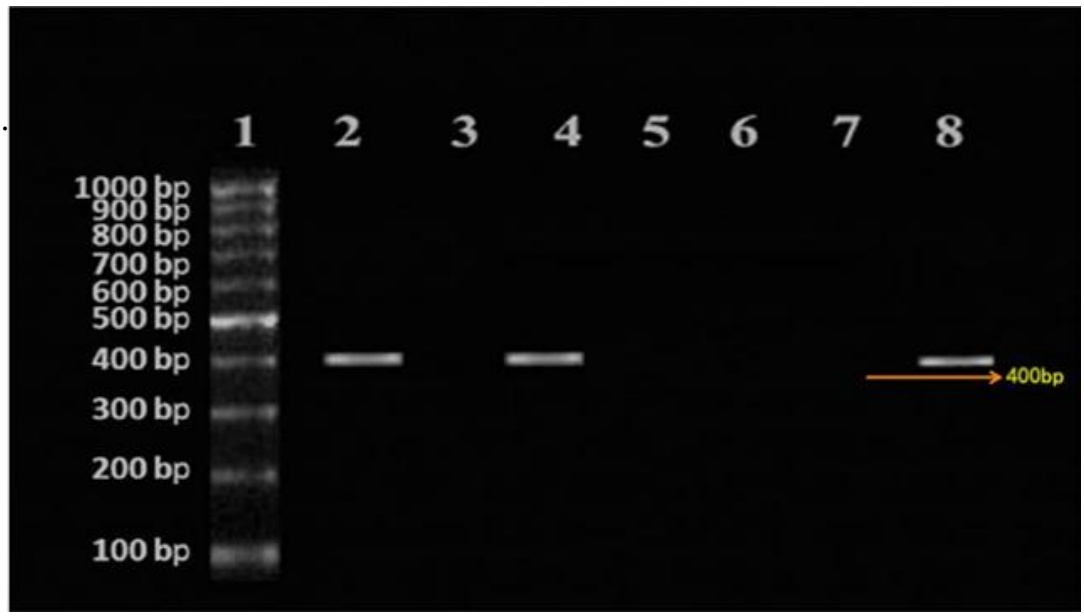
Figure 6.45: Inhibition of HPIV-3 plaque formation by reduction of Ribavirin at various concentrations



6.4.6 Ribavirin inhibit viral genome RNA synthesis

RNA was prepared from infected cells at 8 days post infection, mRNA synthesis were analyzed from cDNA was synthesized using Random primer and PCR performed using HPIV-2 specific primers for NP genes. The number of base pairs between forward and reverse primer of NP gene was 400 bp. Viral RNA synthesis of cell cultured with Ribavirin at 2.5 µM was analyzed and completely inhibit RNA synthesis with NP gene of HPIV-2 was detected by PCR which is represented in the Figure 6.46. But Ribavirin concentration at 1.5 µM did not inhibit virus RNA synthesis of nucleocapsid (NP) gene were detected just the same as in HPIV-2 infected positive controls.

Figure 6.46: Lane 1: Molecular marker, Lane 2: mRNA detected in the virus-infected cells, Lane 3: Ribavirin treated infected cells, Lane 4: mRNA detected in the virus infected cells, Lane 5, 6: Ribavirin treated infected cells, Lane 7 : Negative control, Lane 8: mRNA detected in the virus infected cells.

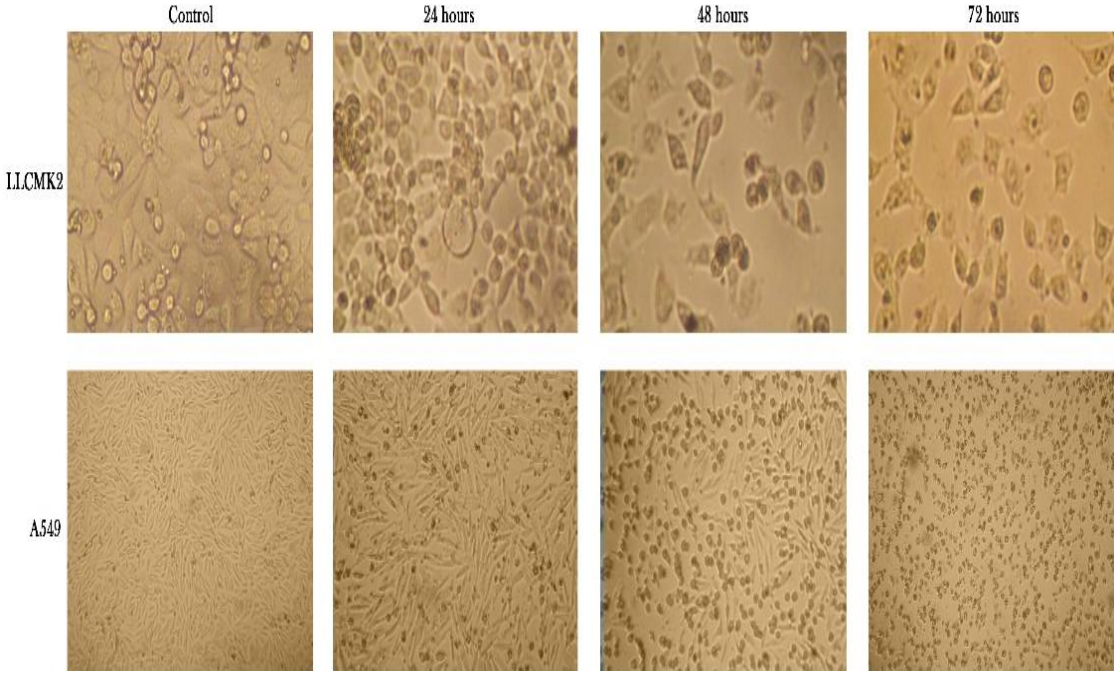


6.5 CYTOTOXIC ACTIVITY OF GLYCYRRIHIC ACID

6.5.1 Cytotoxicity of *Glycyrrihic acid* from Licorice in LLC-MK2 and A549 cells by MTT assay and Neutral red uptake assay

Various concentrations of compounds were evaluated in LLC-MK2 and A549 cell line in 96-well flat-bottomed microplates. The cell morphology were changed at 24 h, 48 h and 72 h which shown the Figure 6.47.

Figure 6.47: Cytotoxicity of Licorice cytotoxicity in LLC-MK2 at 24, 48 and 72 hours



Cytotoxic end points (50% cell-inhibitory concentrations [IC_{50}]) in LLC-MK2 were determined at 31 µM in MTT assay and 85.5 µM in neutral red uptake assay which represent in the Figure 6.48. The Hill coefficient percentage engendered for NR 68.83% which is represented in the Table 6.12.

Figure 6.48: IC_{50} concentrations of *Glycyrrihic acid* in LLC-MK2 cells by MTT assay and Neutral red uptake assay

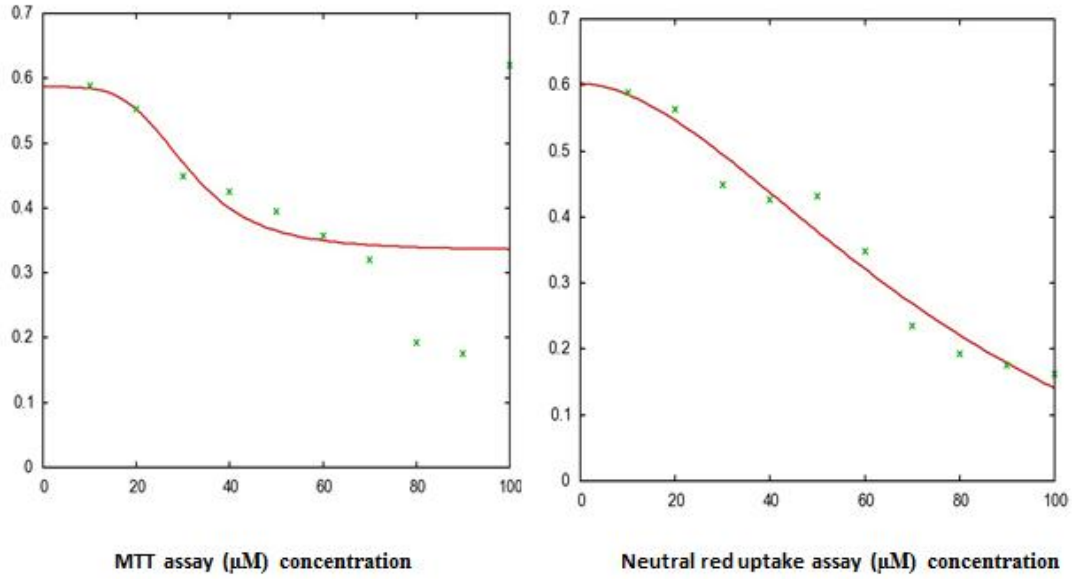


Table 6.12: Cytotoxicity of *Glycyrrihic acid* from Licorice in LLC-MK2 by MTT assay and Neutral red uptake assay

MTT assay		Neutral red assay	
Maximum	0.58656 +/- 0.156 (26.59%)	Maximum	0.602592 +/- 0.06267 (10.4%)
Minimum	0.334845 +/- 0.09321 (27.84%)	Minimum	0.58656 +/- 0.156 (26.59%)
IC_{50}	31.0454 +/- 18.44 (59.39%)	IC_{50}	85.5709 +/- 80.84 (94.47%)
Hill coefficient	4.18506 +/- 9.342 (223.2%)	Hill coefficient	1.78831 +/- 1.231 (68.83%)

Cytotoxic end points (50% cell-inhibitory concentrations [IC_{50}]) were determined at 45.1 µM in MTT assay and 92.1 µM in neutral red uptake assay which represent in the Figure 6.49. The Hill coefficient percentage has engendered for NR 68.8% which is represented in the Table 6.13.

Figure 6.49: IC_{50} concentrations of Glycyrrihic acid in A549 cells by MTT assay and Neutral red uptake assay

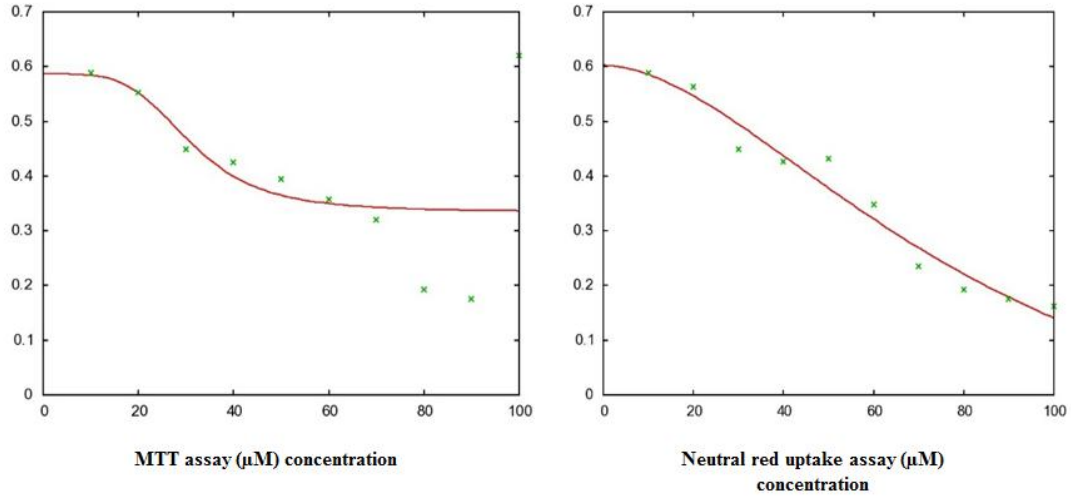


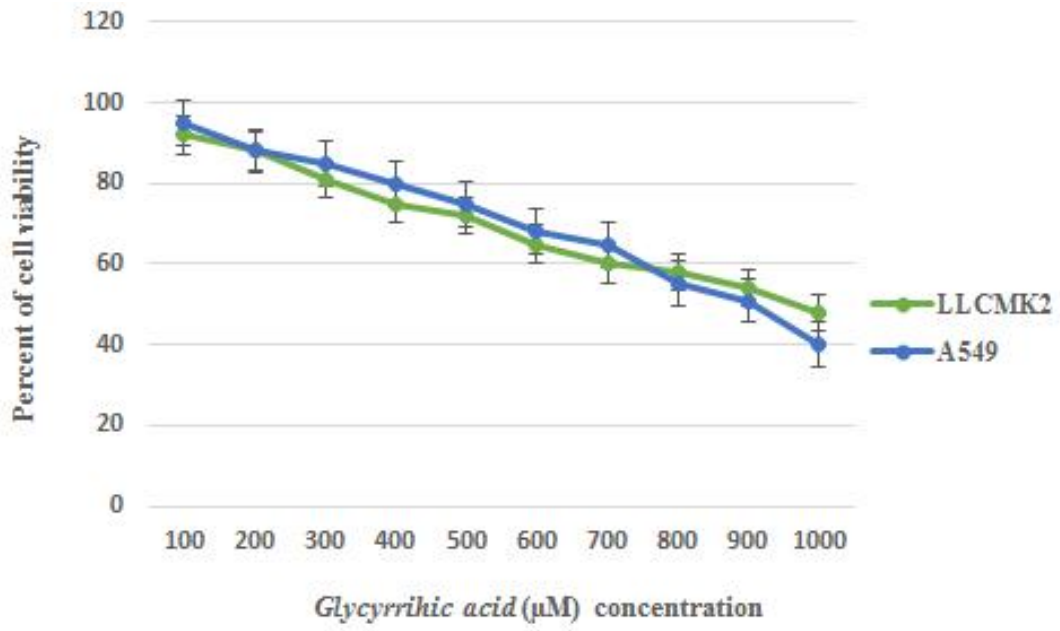
Table 6.13: Cytotoxicity of *Glycyrrihic acid* from Licorice in A549 cells by MTT assay and Neutral red uptake assay

MTT assay		**Neutral red assay**	
Maximum	1.31365 +/- 176.4 (1.343e+04%)	Maximum	0.600061 +/- 0.06191 (10.32%)
Minimum	-0.521885 +/- 204.8 (3.924e+04%)	Minimum	-0.290879 +/- 0.8879 (305.2%)
IC_{50}	45.1273 +/- 7209 (1.597e+04%)	IC_{50}	92.1164 +/- 95.59 (103.8%)
Hill coefficient	0.223242 +/- 46.6 (2.087e+04%)	Hill coefficient	1.80535 +/- 1.244 (68.89%)

Cytotoxicity for *Glycyrrihic acid* in LLC-MK2 and A549 cells by trypan blue exclusion method

The counting chamber of a hemocytometer was allocated by grating lines that ascertain the chamber parts to be used in cell counting. Four corners were counted with viable (unstained cells) and non-viable (stained cells) and percentage of compound cytotoxicity for LLC-MK2 were progressed as 50% at 90 µM and A549 were generated as 50% cytotoxicity at 80 µM which embodied in the Figure 6.50.

Figure 6.50: Cell viability by trypan blue exclusion method



***Glycyrrihic acid* from Licorice inhibit HPIV-2 and 3 by hemadsorption inhibition assay**

The inhibitory activity of *Glycyrrihic acid* on HN gene was determined by hemadsorption assay. The HPIV-2 and 3 infections in the host erythrocytes with the adherence of receptors in the presence of the *Glycyrrihic acid* (at various concentrations) was evaluated for the inhibitory activity of HPIV-2 and 3. Infected cells which exhibit HAD activity without neuraminidase pretreatment. This activity

as well as the similar activity found after neuraminidase pretreatment, was inhibited 50 and 80% at *Glycyrrihic acid* concentrations of 10 and 50 µM. This activity were performed as mean ± standard deviations of results from duplicate culture wells which shown in the Table 6.14.

Table 6.14: Inhibition of HPIV-2 and 3 infectivity by *Glycyrrihic acid*

HN	Neurami-nidase treatment	% inhibition of HAD activity					
		10 µM	**30 µM**	**50 µM**	**70 µM**	**90 µM**	**100 µM**
Greer HPIV-2	+	27.3±5	52.4±4	89.4±7	90.3±2	95.8±5	98.4±2
Greer HPIV-2	-	22.8±7	51.7±8	87.5±4	85.1±5	93.6±1	89.1±3
D-10025 HPIV-3	+	25.3±5	51.1±2	85.4±4	88.7±5	90.4±2	97.8±6
D-10025 HPIV-3	-	22.4±3	50.4±9	82.6±4	84.6±7	88.1±7	90.5±9
Clinical strain HPIV-2	+	32.4±4	54.4±6	80.4±2	87.2±1	89.6±5	90.8±5
Clinical strain HPIV-2	-	28.1±7	52.4±7	75.2±4	83.6±8	85.7±4	87.4±2
Clinical strain HPIV-3	+	35.8±2	57.5±6	85.9±7	85.1±2	86.7±5	95.7±1
Clinical strain HPIV-3	-	30.4±5	55.8±3	82.1±6	80.7±2	83.8±1	93.8±4

***Glycyrrihic acid* from Licorice inhibit HPIV-2 and 3 by plaque reduction assay**

The ability of Licorice on viral entry and replication was assessed by plaque reduction assay. Drug treatments starting at time 0 after infections not only inhibit virus growth, but also prevent CPE completely. Consequently, there was no difference found, at microscope examination, between drug treated infected cells, and uninfected controls. Drug treatments starting 4 hours after infection when synthesis of virus macromolecules is in progress, still inhibit virus growth completely, while only reducing the extent of CPE.

The HPIV-2 and 3 inhibitory activities of Licorice at various concentrations were evaluated in A549 ($3.1x10^5$) and LLC-MK2 ($4.1x10^5$) cell monolayers with

approximately 200 Plaque Forming Units (PFU). Effect of Licorice on viral entry and replication cells were infected with HPIV-2 & 3 with the addition of Licorice in concentrations ranging from 10 to 100 µM. Plaque formation preceded under an agarose overlay for 48 hours and plaques were visualized and counted. The Licorice was screened at the various concentrations from 10 µM to 100 µM. The concentration of Licorice 70 µM showed maximum reduction of plaque formation (88%) reference strain (D-10025 strain) and (86%) clinical strain in LLC-MK2 cells. Similarly the A549 cells illustrate (90%) reference strain and (87%) clinical strain were plaque formation. The viral inhibition was higher in the A549 cells than LLC-MK2.

6.4.3 Antiviral activity of *Glycyrrihic acid* against HPIV-2 and 3 by Neuraminidase inhibition assay

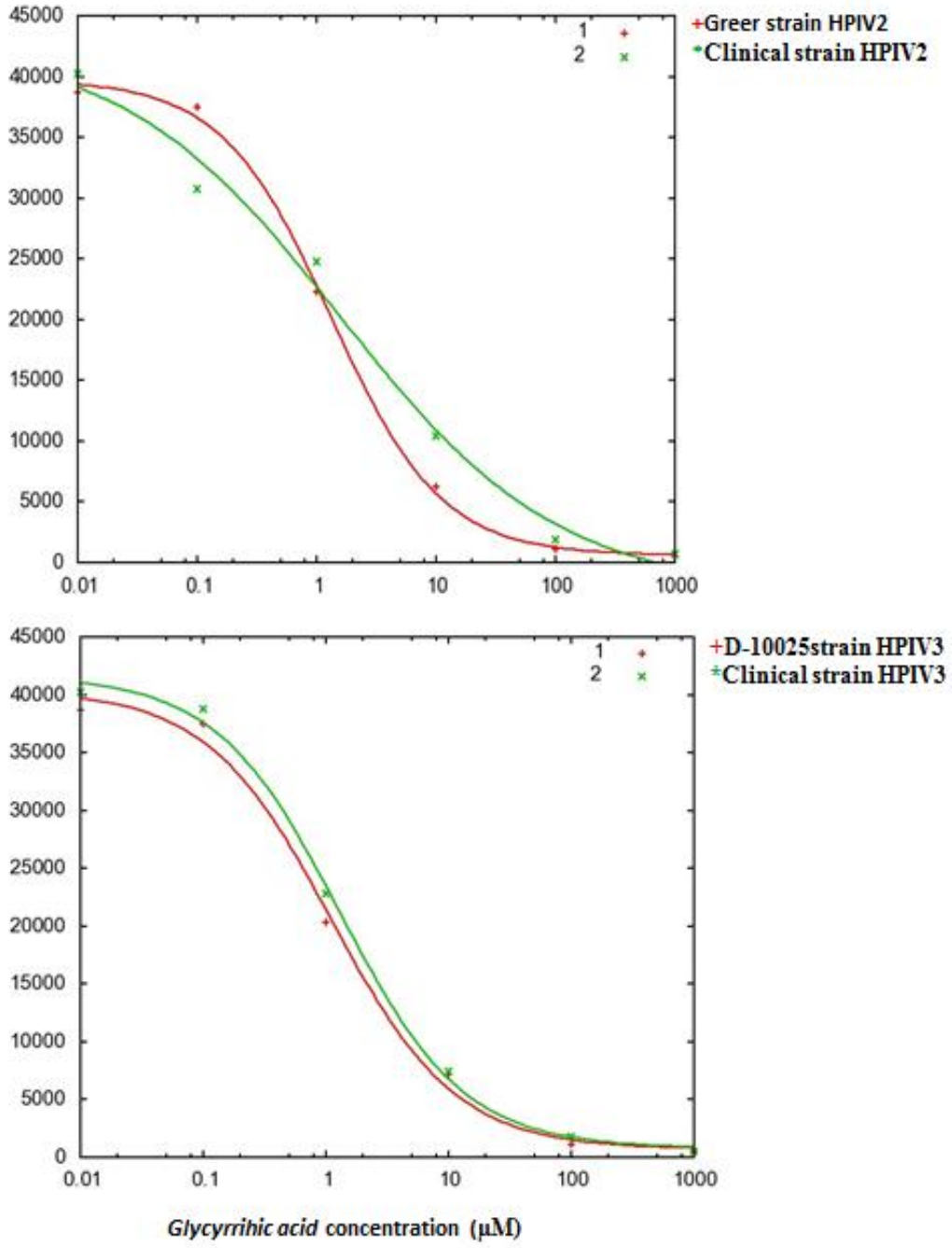
Cytotoxicity of *Glycyrrihic acid* from Licorice generated at 31 µM by MTT assay. But in neuraminidase inhibition assay, compound concentrations were used from lower to higher concentrations (0.1-1000 µM). Virus preparation of HPIV type 2 and 3 m.o.i. (1) were used to perform the NAI assay. Because HPIV were accomplished and standardized in neuraminidase assay. Neuraminidase activity as nanomoles per minute per milligram of protein used for ensured the NAI assay.

The inhibitory concentration of neuraminidase activity presented was considered as antiviral activity of *Glycyrrihic acid*. Virions released were high in reference strain when comparable to clinical isolates it seems that exogenous neuraminidase treatment were occurred. After NA treatment to destroy the receptors to which HN binds thus enhancing the elution of progeny clinical isolates (HPIV-2 & 3) virions from the cell surface.

The neuraminidase inhibition assay for HPIV-2 and 3 were performed in duplicate revealed substantial differences in absolute neuraminidase activity. In NAI assay, reference strains used smaller concentrations when compared to clinical isolates which inhibit the HPIV-2 and 3. The IC_{50} concentration for HPIV-2 at 1.5 µM and HPIV-3 at 1.2 µM. *Glycyrrihic acid* concentrations were used high concentration for HPIV-2 when compared to HPIV-3. At various micromolar

concentrations of *Glycyrrihic acid* showed minimum inhibitory concentration against HPIV-2 and 3 were performed, represented in the Figure 6.51.

Figure 6.51: Reduction of neuraminidase enzyme by *Glycyrrihic acid* against HPIV-2 and 3.



The IC_{50} values are calculated used IC_{50} tool kit and multi IC_{50} plotting tool kit. Hill coefficient range and IC_{50} values are depicted in the Table 6.15.

Table 6.15: IC_{50} concentration of HPIV-2 and 3

Virus	Strains	Minimum	Maximum	IC_{50}	Hill coefficient
HPIV 2	Greer strain	591.926±754.5	39724.1±956.8	1.3	0.944705±0.1101
	Clinical/Chennai isolates	-2358.66±4739	42872.9±6655	1.5	0.475177±0.1999
HPIV 3	D-10025 strain	691.922±1506	40407.8±2142	1.1	0.856412±0.1941
	Clinical/Chennai isolates	799.159±1040	41623.3±1376	1.2	0.86729±0.126

Glycyrrihic acid was based on the studies on inhibition of HPIV-2 and 3 (Greer strain HPIV-2 and D-10025 HPIV-3) and clinical isolates were determined by in which the 50% inhibitory concentrations at 1.3 µM for HPIV-2 reference strain and 1.5 µM for clinical isolate was identified as minimum concentration of compound when compared to HPIV-3.

The percent inhibition of neuraminidase activity (nanomoles/minute/mg of protein) against HPIV-2 and 3 in A549 cells. At 3.5 µM 88% for clinical strain and 97% for reference strain (D-10025) as a utility of micromolar concentrations of *Glycyrrihic acid* in A549 cells. In LLC-MK2 occurred at 3.5 µM 85% neuraminidase inhibition for HPIV-2 and 86% for HPIV-3 similar more or less and are the means (with standard error bars) of at least three experiments which is represented in the Figures 6.52 to 6.55.

Figure 6.52: Inhibition of HPIV-2 by *Glycyrrihic acid* in A549 cell line

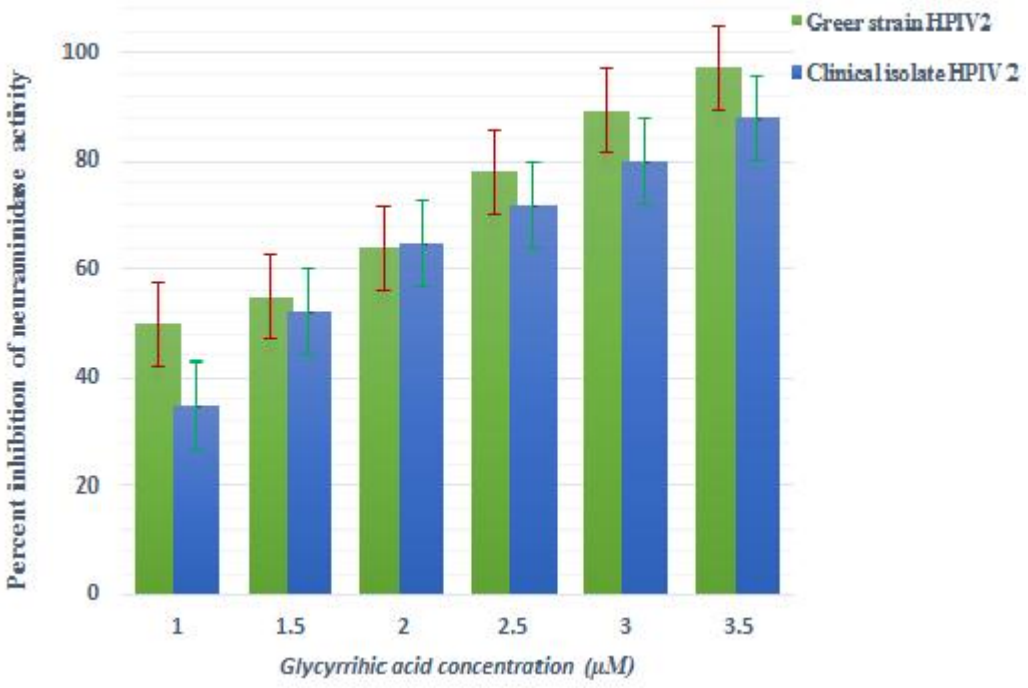


Figure 6.53: Inhibition of HPIV-3 by *Glycyrrihic acid* in A549 cell line

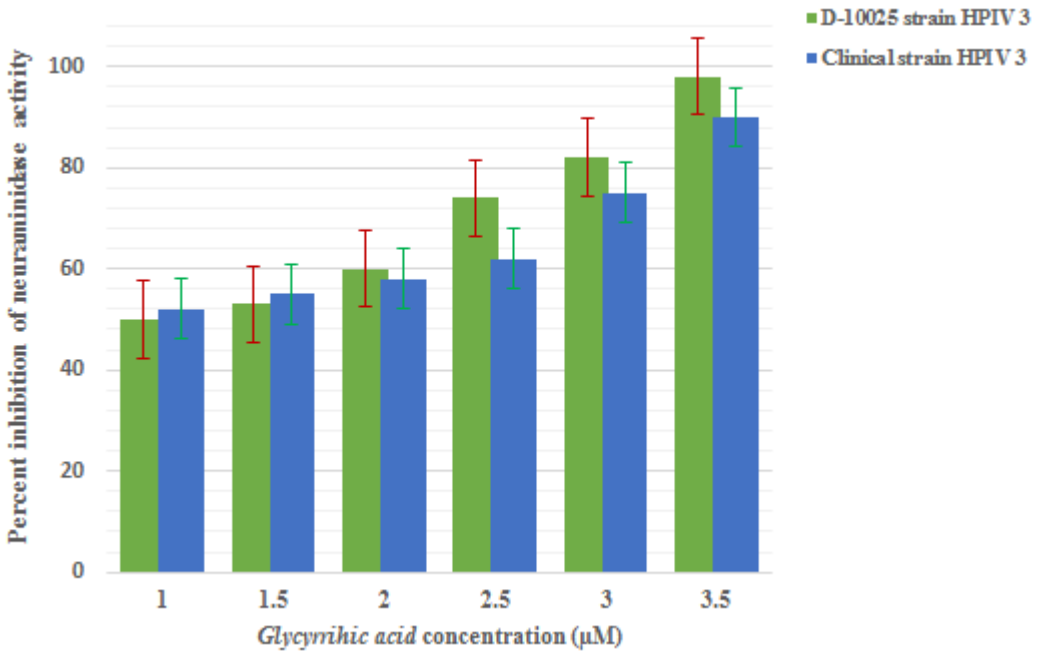


Figure 6.54: Inhibition of HPIV-2 by *Glycyrrihic acid* in LLC-MK2 cell line

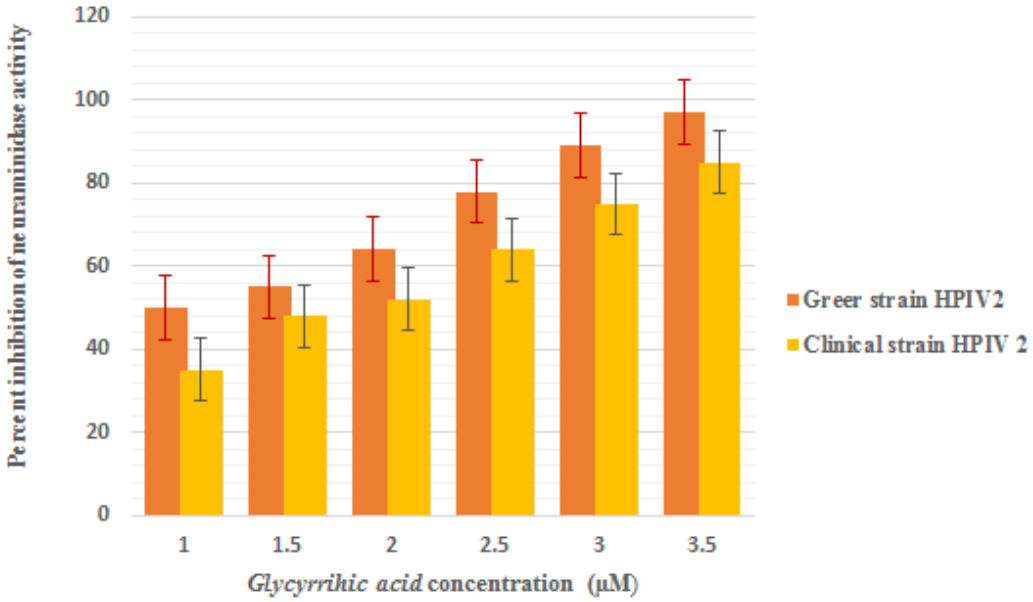
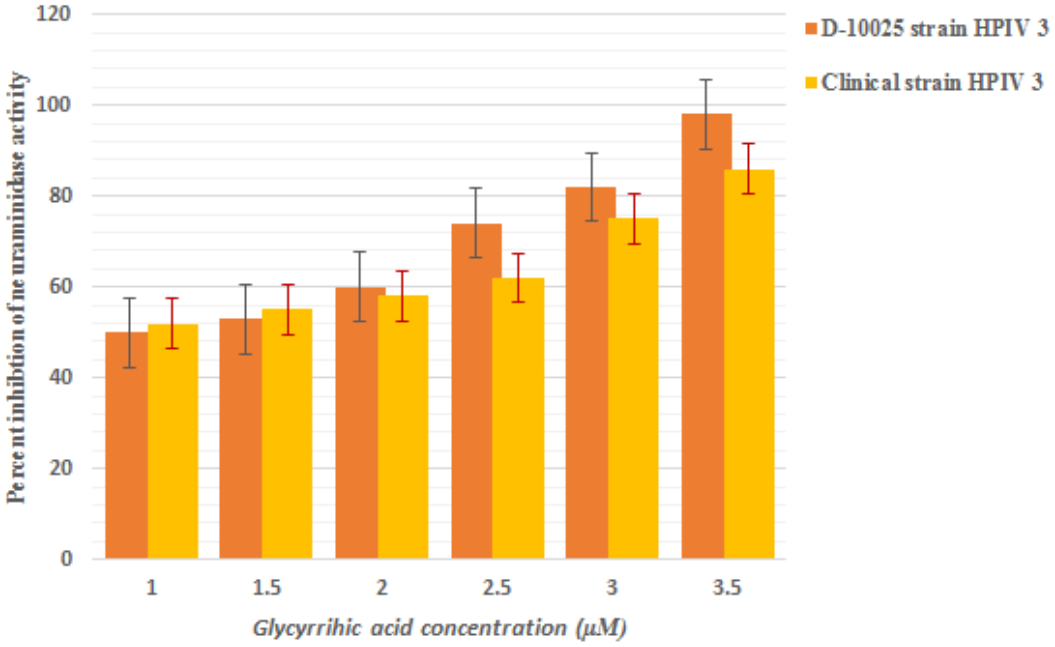


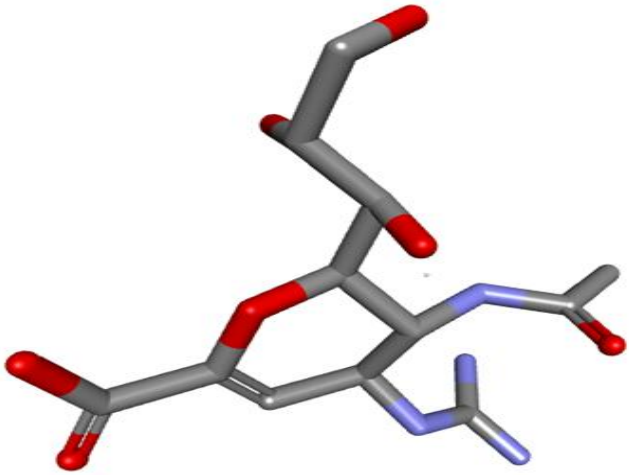
Figure 6.55: Inhibition of HPIV-3 by *Glycyrrihic acid* in LLC-MK2 cell line



6.6 MOLECULAR DOCKING

In the previous Chapter, the Zanamivir *in vitro* antiviral activity was evaluated against HPIV-3 through HAD inhibition assay, plaque assay and NAI assay. The mechanism behind the antiviral activity was still not understood clearly. In this present chapter, the molecular mechanism of Zanamivir antiviral activity on the HPIV-3 receptors was elucidated through *insilico* docking. The ligand structure of Zanamivir was first prepared used Ligprep packages presented in the Figure 6.56.

Figure 6.56: 3-dimensional structure of Zanamivir



Similarly the structures of HPIV-3 (HN) glycoprotein receptors were mined from RCSB and were optimised for In silico docking through Discovery Studio tools. The water molecules and the unpaired hydrogen bonds were removed and presented in the Figure 6.57. The HN receptors of glycoprotein, PDB ID viz., A:1V2I, B:1V3b, C:1V3d, D:1V3e and E:4MZA were displayed in the Figure 6.58 A:Structure of hemagglutinin-neuraminidase glycoprotein 1V2I, B: 1V3b, C: 1V3d, D:1V3e and E: 4MZA.

Figure 6.57: 3D structure of HPIV type 3 HN protein

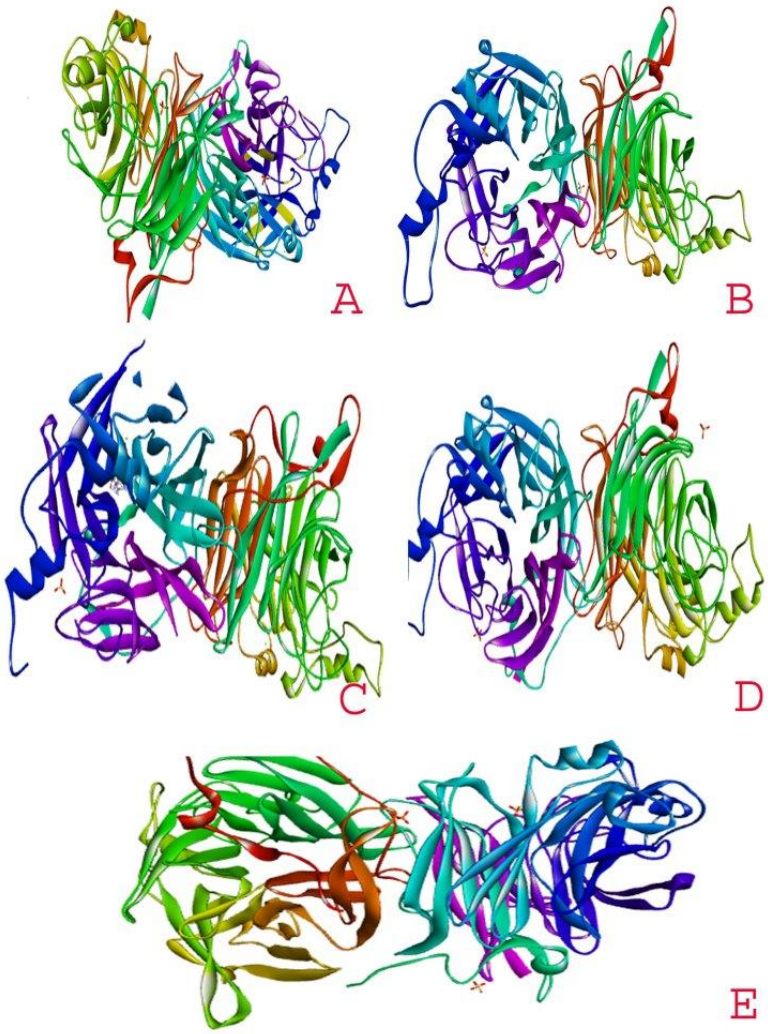
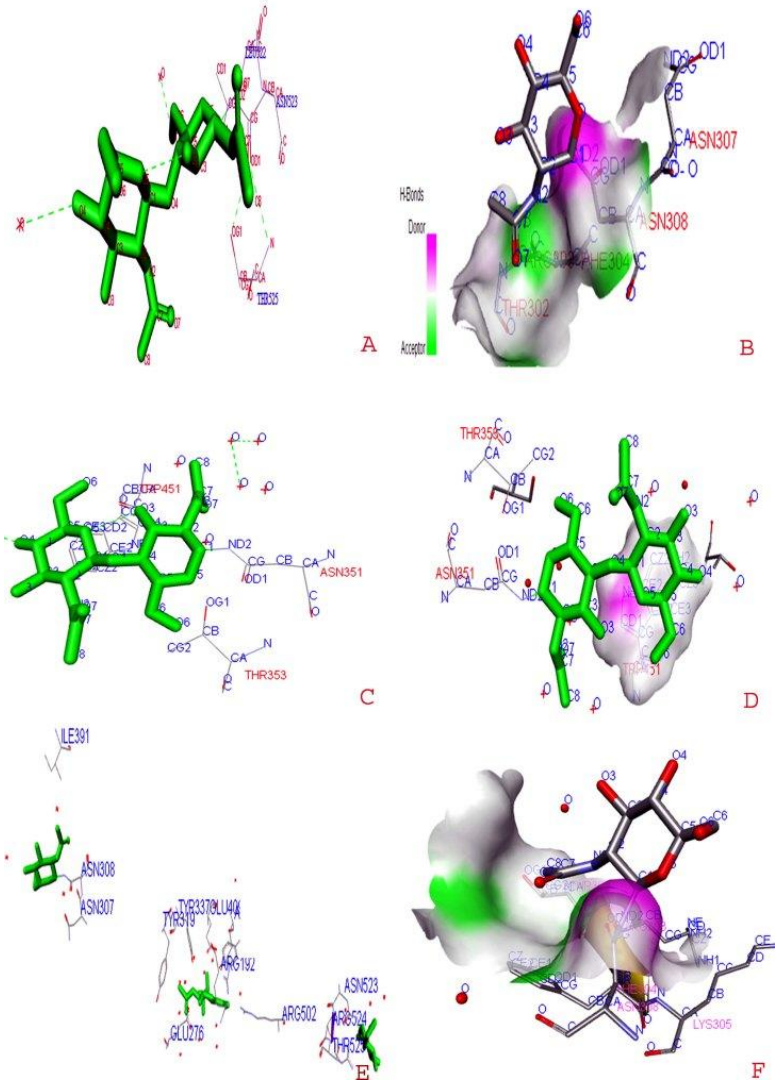
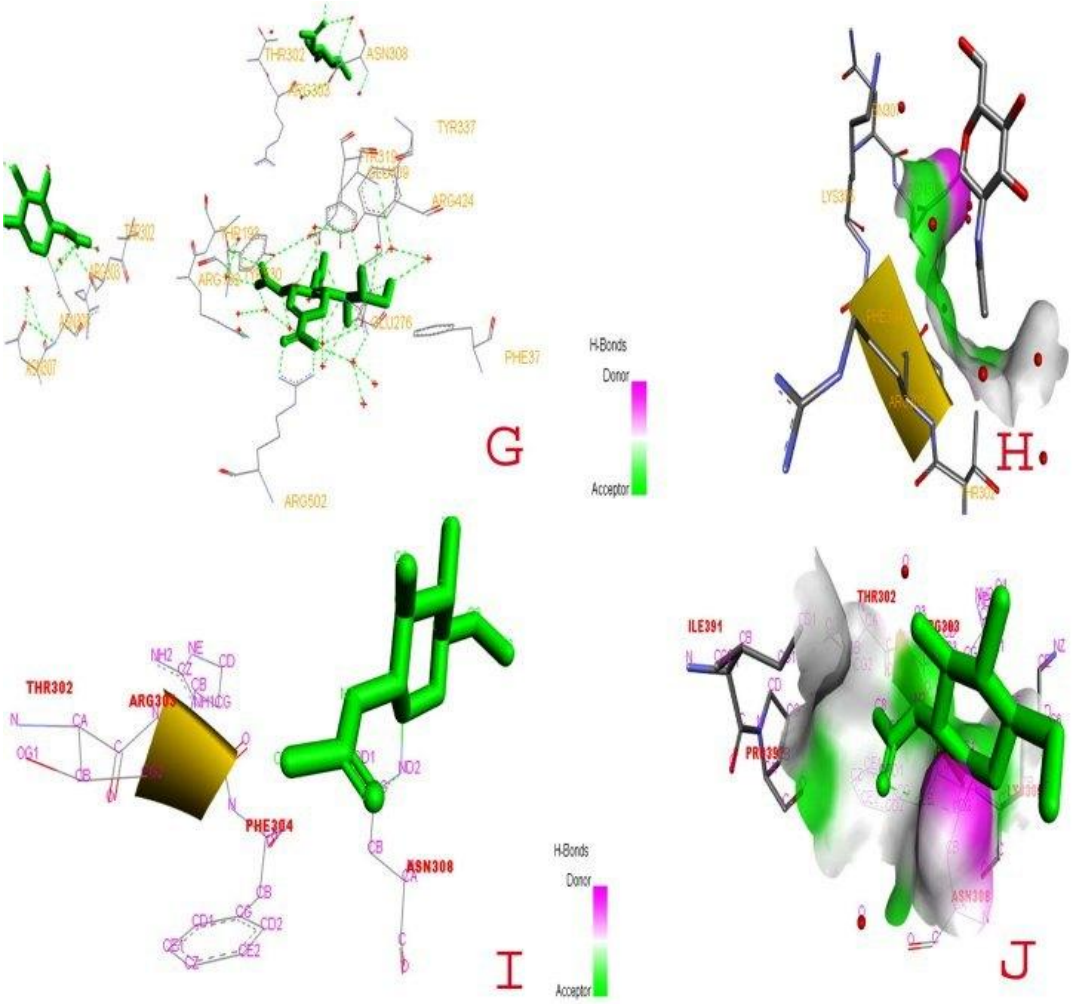


Figure 6.58: Ligand pocket prediction and Insilico docking of HPIV-3 targets with Zanamivir ligand



A: Zanamavir binding pockets with 1V2I, B:Insilico docking of Zanamavir and binding pockets with1V2I, C: Zanamavir binding pockets with 1V3b, D: Insilico docking of Zanamavir and binding pockets with 1V3b, E: Zanamavir binding pockets with 1V3d, F:Insilico docking of Zanamavir and binding pockets with1V3d,



G: Zanamivir binding pockets with 1V3e, H: In silico docking of Zanamivir and binding pockets with 1V3e,I: Zanamivir binding pockets with 4MZA, J:Insilico docking of Zanamivir and binding pockets with 4MZA

The active binding pockets were found as CA, CB, CG, CE2, CG2 and ND2. Similarly, the active sites of receptors of HPIV-3 glycoprotein 1V2i were found as ASN 307,308 and THR 302,304. The active site of 1V3b was predicted as ASN 351, THR 353 and 358 TRP 451. The active site of 1V3d was found as ASN 307, 308, ILE 391, GLU 276, ARG502,524, TYR19,337 and LYS305. The active site of 1V3e was found TYR302, ASN308, ARG303,307,308,424, GLU276, THR193 and GLU276. The active site 4MZA was found as THR302, ARG303, PHE304,

ASN308, PRO392 and LYS309. The In silico docking of HN glycoproteins were docked with Zanamivir presented in the Table 16.

Table 6.16: In silico docking of Zanamivir to HN glycoprotein

Rank	Est free energy of binding	Esti inhibition constant (Ki)	Vdw+H bond+ desolv energy	Electro-Static energy	Total intermo-lecular energy	Frequency	Interact surface ±
1v2I	11.81 kcal/mol	496.10µM	0.50 kcal/mol	-2.03 kcal/mol	1.41 kcal/mol	96.60%	-58.71
1v3e	138.78 kcal/mol	456.0 µM	-0.65 kacl/mol	-0.87 kcal/mol	-2.88 kcal/mol	84.95%	-219.74
1v3b	145.03 kcal/mol	458.90 µM	-0.68 kcal/mol	-0.80 kcal/mol	-2.94 kcal/mol	81.77%	-229.16
1v3d	103.43 kcal/mol	514.20 µM	-0.31 kcal/mol	-1.47 kcal/mol	-0.98 kcal/mol	77.13%	-30.67
4MZA	60.58 kcal/mol	496.20 µM	-0.43 kcal/mol	-0.92 kcal/mol	1.81 kcal/mol	58.89%	-134.63

Among the four receptors, 1V2i the docked well with 96.6% of frequency and 1.41 kcal/mol of total inter molecular energy and inhibition constant of 496.10µM. The best free energy binding was found as 11.81kcal/mol, the 1V2i showed highest fit and docking score of 11.81 kcal/mol than all the other receptors. Next to that the best docking of glycoprotein receptors 1V3e was found as 84.95 docking score, -2.88 total inter molecular energy, electrostatic energy (-0.87) and the free energy binding was found as 138.78 kcal/mol.

The 1V3b HN glycoprotein receptor demonstrates that good docking with Zanamivir scored 81.77% docking frequency and total inter molecular energy was found as -2.94 kcal/mol, electrostatic energy -0.8.kcal/mol and the free energy binding was found as 145.3 kcal/mol. The 1V3d receptor scores docking on 77.13% frequency and -0.98 total inter molecular energy, -1.47 kcal/mol as electrostatic energy and free energy binding was found as 103.43kcal/mol. Finally the fifth receptor in the study 4MZA scored as docking frequency 58.89% this is the lowest docking frequency with compared to other receptors. The total intermolecular energy was found as 1.81 kcal/mol, electrostatic energy -0.92 kcal/mol and free energy binding was found as 60.58 kcal/mol.

Discussion

DISCUSSION

The group human parainfluenza viruses were traditionally given a high place in the pantheon of respiratory viruses cause upper & lower respiratory tract illness and hospitalization. All four types cause croup, a life threatening infection due to respiratory embarrassment. HPIV-3 is an important pathogen especially in younger children. Our findings address the impact of routine infections with these viruses and how one might assess the impact of prevention of HPIV illness.

Respiratory viruses, a major cause of acute respiratory tract infection causing significant global human morbidity and mortality, especially in infants, at times can be serious when caused by HPIV. This is demonstrated by their activity during 2011-2014 as the occurrence had showed distinct seasonal peaks for both HPIV-2 and 3. The conclusions from the present data allowed us to confirm earlier studies and to use this data to examine discrepancies in HPIV trends that was previously reported.

HPIV type 3 positivity was seen form August-February, with peak positivity in November. In the study period, out of four years, 2011 had increased positivity rate of HPIV-3, less positive percentage occurred next three years. HPIV type 2 positivity was detected only in 2014 and during the remaining three years HPIV-2 could not be detected. Several factors may account for this disparity especially the climatic conditions. HPIV serotype 4 was not detected during this period by both multiplex PCR and viral isolation, since it is rare to isolate from cell line. The study indicated that the prevalence of Human parainfluenza virus were analyzed by their clinical profiles. Among 4 serotypes, HPIV-3 was more readily recognized in Chennai and followed by HPIV-2 whereas there were no outcomes from the other serotypes (HPIV-1, 4) during the four years study period.

The respiratory viruses namely Respiratory syncytial virus (RSV), Human parainfluenza virus and Influenza viruses A and B, RSV has been documented to be the most common cause of respiratory infection followed by human influenza virus

and HPIV[301]. Human parainfluenza viruses are the utmost common cause of lower respiratory disease in young children following respiratory syncytial virus. These viruses infect subjects from all age groups.HPIV constitutes common viral cause of community-acquired pneumonia in healthy adults. But the most common viral sources susceptible HPIV was from the pediatric age group[50,302]. The serotypes of HPIV-1, 2, 3 and 4 spread by direct close contact between persons, by hand shaking, and through large aerosol. Human parainfluenza virus principally attacks the respiratory epithelium cells. Infected cells are unique from normal cells due to their change in morphology, together with focal rounding and increase in size of the cytoplasm and nucleus. The previous reports referred multinucleated giant cell[129]. Though HPIV caused varied illness at short rates, patients frequently desired severe symptoms particularly those undergoing bone marrow transplant[128,303].

In USA, 12% of lower respiratory tract infections which were annually reported are caused by HPIV-3. Globally 10% of LRI in preschool children were caused by HPIV and 25 to 30% were leading in death from these infections[29,304]. In the previous studies, the epidemiologic and phylogenetic data about HPIV were circulated in Central and South America[305]. In Japan between 2001 to 2011, HPIV strains were detected among subjects[306]. Whereas an insufficient epidemiological studies and facts of HPIV were stated in an article from China covering a short period of time[307,308]. The prevalence of HPIV in China during that period were 3.7%in Malaysia (3.4%). In most of the studies, HPIV-1 and HPIV-3 were common in China, Japan, Malaysia and Brazil[309-312]whereas HPIV-2 and HPIV-4 were highly predominant in North America and Canada[29,313]. In United States, the most common HPIV types associated with respiratory illness are HPIV-1 and 3, while HPIV-2 and 4 were less frequently detected[2].

In the present study, Human parainfluenza virus positivity was higher in males when compared to females. The younger age group, of 0-10 years had higher positivity as when compared to other age groups. The percentage of HPIV-3 was 40% in the first year of life[29]. The present study indicates that HPIV-3 was first isolated from 3 months old boy baby in Child Trust Hospital (Chennai). It is well

known that Influenza infections are more common in the pediatric age group, likewise in existed study there was a predominance of HPIV among 1-5 age group and it was observed that the school going age group was less affected but some positivity occurred by the age of above 50 years.

The age wise circulation of HPIV-3 were detected from children less than 12 months age group causing mainly bronchiolitis and pneumonia[18,314,315]. The patients median age was 20 months for HPIV-4 infections and 7-11 months for HPIV-1,2 and 3 infections, but the clinical manifestations did not differ significantly between HPIV-1,2,3 and 4 infections[316]. It was known that influenza virus infections are more common in the paediatric age group, likewise in our study there was a predominance of HPIV among 1-5 age group. HPIV-3 was the prevalent serotype.

The molecular characterization of HPIV type 3 strains were analysed by sequencing the Hemagglutnin neuraminidase (HN) gene and Nucleocapsid (NP) gene. The study indicates that the HPIV type 3 was diagnosed by RT- PCR against HN region with product size of 189 bp and was developed as markers for diagnosis. Similarly for HPIV type 3 NP gene with target of 266 bp was marked for diagnosis. In the present study for HPIV type 2 NP gene region with product size of 197 bp was used for diagnosis

In the previous studies, the respiratory specimen were subjected to multiplex semi-nested PCR assay which revealed that 31 (4.8%) samples were positive for HPIV, of this 5 patients had infected with HPIV-1, 15 with HPIV-3, 7 with HPIV-4A and four samples with 4B. HPIV-2 were not identified in the samples[317]. But in our present study, only one sample was positive for HPIV-2 by multiplex RT-PCR in 2014. HPIV type 2 (Nucleocapsid gene) was used to detect virus by multiplex RT-PCR. In the existing study, among the four serotypes, HPIV-3 was revealed high as it was predominantly detected when compared to other serotypes. The similar studies was identified in Canada by multiplex RT-PCR[51,318]. Community acquired respiratory virus infections including HPIV-3 substantially cause morbidity and mortality after stem cell transplantation[319-322].

The prominent cause of severe lower respiratory tract infection in infants and young children caused by Respiratory syncytial virus (RSV)[323] followed by Human parainfluenza virus -3 (HPIV-3)[324]. Rapid tools for the detection of direct antigen test often lack sensitivity and thus entail confirmation by virus isolation or indirect antigen testing following specimen culture[325]. Specimen integrity and the number of intact cells present in the specimen are vital for a consistent direct immunofluorescence assay[326]. Direct antigen tests may fail to detect emerging variants having altered amino acid sequences on envelope or outer capsid proteins[327]are responsible for negative results. To reduce these problems in our study, a molecular diagnostic technique was developed to permit the rapid and sensitive detection of the respiratory viruses, HPIV involved in lower respiratory tract illness. In our study, Multiplex RT-PCR assay was capable to identify a greater number of positives when compared to the positivity by viral isolation[201,328,329].

In previous studies, it was identified that the point mutation at residue of 278 and 281, coding for a single amino acid substitution in the HN protein[328]. In this study, two novel mutations at amino acid residue of 295 and 297 were seen. By phylogenetic analysis of HPIV-3 HN gene, it was shown to be related to Fukuoka /2009, Nagasaki2009 and Washington 64979 strains.

The year wise distribution of acute respiratory infection (ARI) cases were from 2011 to 2014, which totally included 931 symptomatic subjects with ARI from whom samples were collected and tested for Human parainfluenza viruses. Among the symptomatic cases, 41 samples were confirmed positive by multiplex RT-PCR, thirty three positive for HPIV-3 and only one positivity were detected and HPIV-2 strains which could be isolated representing one third of the total. Detection of HPIV in the remaining four specimens by multiplex PCR and not by isolation was owed to the high sensitivity of mRT-PCR assay[25,330].

In this present study, the most common type of presentation was fever, followed by sore throat and cough like any other ILI. Meanwhile samples were not collected from patients with SARI (severe acute respiratory illness) and long duration follow up was not done, the proportion of hospitalization among the ILI

cases was not assessed in our study. Additional studies that embrace hospitalized controls are needed to elucidate the clinical status of HPIV infection in adults with community-acquired lower respiratory tract infection. The rate of infection were moderately the same in age groups from young infants to adults.

A high proportion of males were predominantly found to be infected with respiratory viruses as compared to females in our study which was similar to already reported study in Delhi, Chennai[57,200].

Parainfluenza virus occurred throughout the year, with seasonal predominance with respect to the different serotypes. In United States, HPIV-1 infections occur primarily in the fall of odd numbered years, while PIV-2 infections were most commonly detected in the fall of even numbered years showing that infection sets in with fall in immunity[2]. HPIV-3 infections occur year round, but peaks of illness occur each spring. Because PIV-4 infections are infrequently recognized, a clear seasonality has not been elsewhere established. Seasonal variation of PIV has not been reported in other developing and tropical countries. HPIV are the most common cause of PIV infection, with HPIV-1,2 and 4 infections occurring at decreasing frequency[331].

In temperate regions, every year a summer epidemic of parainfluenza virus type 3 infection has occurred, with the peak frequently in July. Type 1 appears to follow a biennial pattern, with epidemics usually centered on the autumn of alternate years. Type 2 coincides with the type 1 variations, with small outbreaks happening in the winter of the same years. Type 4A infections were gathered composed during the autumn and winter of 1971 or 1972[332].

HPIV-1 was reported to cause infections biennially fall and epidemics occurred during this period as per previous stidues[164,333,334]. HPIV-2 cause infections biennially with HPIV-1 to alternate years with HPIV-1 or to cause yearly outbreaks[332,335]. HPIV-3 reported to occurred annually during April to June in the United States[2]. In other study HPIVs were isolated throughout the year. Seasonal peaks of HPIVs, driven mostly by HPIV-1 and 3, occurred in the time when autumn

turned to winter and summer turned to autumn[51].In temperate climates, HPIV-3,4 were detected in spring, summer and late fall and seasonal incidence varied for HPIV-1,2 in Chinese children[321]. The first reported outbreak of HPIV-4 infection occurred in Hong Kong in the autumn of 2004 involving 38 institutionalized children and 3 staff members during a 3 week period in a developmental disabilities unit[336]. These studies are mostly from temperate regions but for Hong Kong which are different from our seasonal pattern. The different geographic locations might lead to the different seasonal distributions of HPIV.

In our study, HPIV-3 positivity was seen during the monsoon months of August-September and post monsoon months of November-January, with peak positivity in November. But in Northern hemisphere HPIV infections were more frequent from January to April[337]. Our study the prevalence of HPIV-2 during the four years observation was detected in the winter season.

Further the epidemiological studies indicate that there is an increase in the HPIV cases from 2011-14. But the number of positive cases was highly significant in the year 2011. In 2012, the number of cases were screened were more, but less number of positives were confirmed. Further, the viruses were characterized molecularly and genotypically for developing new markers for diagnosis. The positive samples were cultured in the LLC-MK2, A549 cell lines. Then the specificity and sensitivity of the cell passages were characterized.

Among the three cell lines, LLC-MK2 showed higher and better positivity rate when compared to the other two cell lines. But in the case of antiviral activity, A549 cell lines showed better results when compared with LLC-MK2. In previous study the highest rate of isolation of Human parainfluenza virus has been were in LLC-MK2 cell line[338.] Our findings, identified LLC-MK2 revealed cytopathic effect in earlier passages and obtained isolation rate was high when compared other two cell lines. In one of the study, of the 129 throat samples only 9 (7%) HPIV were isolated in Rheses monkey kidney cell line[339] and the percentage of isolation in the cell lines is much lesser compared to multiplex PCR. But certain studies have showed canine kidney cell line to be the most suitable cell line for the isolation of

respiratory viruses[340].In our study, LLC-MK2 showed better success rate in isolation when compared to A549 and MDCK cell lines. According to a study, MDCK had moderate success in isolating human parainfluenza viruses but was unsuitable because of inadequate production heamagglutinin and hence Hemadsorption assay results were not satisfactory when compared to LLC-MK2[338].

In the present study, the HPIV prevalence in Chennai was investigated during the study period of 2011 to 2014 was not statistically significant. The number of positives was high from subjects referred to from Institute of child health government hospital (ICH) followed by Government Peripheral Hospital and Rajiv Gandhi Government Hospital. The study also shed some light upon the spread of HPIV infection by direct close contact, fomites being responsible for the spread of infection as most positive cases from crowded areas in North Chennai, Central and South Chennai.

The molecular phylogeny of HPIV-3 (HNgene) JQ901413.1 was very closer to gi/3510305/GPJapan/2009/HN,gi/58430688/FukukuokaJapan/2009/HN and gi/37958139/Australia/Melborne/2004/HN glycoprotein. This indicted that closer clustering, even though the isolates were from geographically distant location or different countries. This established that resemblances among the isolates circulating in the years.

In our findings, phylogenetic analysis of HPIV-3 (N gene) gi:692112437 was very closer to gi/545699417/Switzerland/CHE/2011/NP, gi/612507269/US/2000/ NP, gi/545699317/South Africa/2000/NP. This studies indicated that Chennai sequence were similar to European strains and Africa stains as well. This was one of the first few studies characterizing HPIV strains from Chennai and comparing them with other strains from different continents.

Moreover based on the present study, the circulation of HPIV strains were much less when compared to other common respiratory viruses in Chennai, Tamilnadu. Phylogeny sequence of HPIV-2 (Nucleocapsid gene) strains were highly closer to clade of gi/26655521/US/V94 strain, gi/26655523/US/Greer strain,

gi/26655522/US/V98strain. The mentioned clades were similar to KJ939327.1 gi/672424506 HPIV-2 strains.

Hemadsorption assay (HAD) and Hemadsorption Inhibition assays (HAI) have been widely used for the detection of Paramyxoviruses family of Human parainfluenza virus[125,341,342]. These assays exploit the ability of the HN glycoprotein with Neuraminidase treatment (prevent cell to cell fusion) to bind and adhere human erythrocytes on the monolayer[29]. For quantitatively HPIV was detected by HAD assay, RBC were lysed in ammonium chloride and absorbance in multiwell plate reader[342]. In the Hemadsorption inhibition assay from infected individuals prevent the adherence of erythrocyte which subsequently floated on the monolayer.

Plaque assays were used in cell culture monolayers beneath overlay media are used for quantification of HPIV and antiviral substances[343-345]. Infected cells were stained with crystal violet and dots may be more easily envisioned by viewing the plate with oblique light in LLC-MK2 and A549. Plaque forming units were calculated in LLC-MK2 and A549 cell lines. MDCK cells were not used for further analysis because viral titer was very high when compared to other two cell lines. Infected cells were determined and m.o.i. calculated. High m.o.i. of HPIV-3 were able to block fusion but in HPIV-2 did not block fusion in LLC-MK2. Such similar effects were identified in A549 cell line too. Infected cells at low m.o.i. for HPIV-3 with subsequent bacterial treatment inhibited fusion aided by HPIV-2 and this effect was at low m.o.i. for HPIV-3 was identified fusion aided by HPIV-2 and this similar effects was seen in CV-1 cells. Related such results were observed in previous studies[346].

Formerly, Neuraminidase inhibition assay was accompanied, the activity of each viral NA was measured by a standard fluorometric assay with 2-(4-methylumbelliferyl-)-DN-acetylenuraminic acid as a substrate were used[347,348]. Numerous concentration of MUNNA as a substrate with neuraminidase and incubate for different minutes at 37 ºC, the substrate concentration increased the fluorescence intensity also increased progressively. The enzymatic reaction rate was constant 20 min when substrate concentrations are used from 5 µM to 40 µM. The signal to

background ratio were determined as the fluorescence intensities restrained after 20 minutes incubation period with deactivated and active enzyme. Substrate concentration were used as 25 µM. In the present study substrate concentration may vary with interference reduced were indicated at 20 µM for the substrate concentration and fluorescence intensities were measured after 15 minutes incubation with deactivated and active enzyme[349].

Substrate concentrations of MUNNA as 25 µM were used in the phenotypic assay for determination of susceptibility of Human parainfluenza virus type 2 and 3 to at 25 µM concentration of 4-MU which chances the rations for determination of relative fluorescence units (RFU). Background-corrected RFU was converted to 4-MU concentrations and used determined the percentage of substrate expended during the reaction.

The affinity of the HN receptor binding pocket for 4-GU-DANA plays a pivotal role for affinity and for HAD based assay for binding inhibition. A study done earlier had showed T193I mutation in HN resulted in decreased sensitivity to 4-GU-DANA. In their study a concentration 10 mM was used to inhibit HPIV-3, another article wherein H552Q mutation was identified also showed resistance with 10 mM concentration of4-GU-DANA[350].

Another study which revealed that variant ZM1 strain was found to be resistant to 4-GU-DANA. The various drug concentrations used 1 mM, 3 mM, 12.5 mM for which 9.2%, 19.7%, 43.5% respectively were resistant. When 4-GU-DANA concentration was raised to 15 mM there was inhibition of the drug by ZM1 variant. Another variant C0 showed sensitivity to be 4-GU-DANA with HPIV-3 inhibition at 1 mM concentration which inhibited the binding activity by 89.2%[351]. Additional variant C22 which showed the HPIV-3 inhibition binding activity percentage was revealed 50% and 80-90% at 2 mM and 5 mM respectively[261].

Our study findings, 4-GU-DANA effectively blocks the HPIV-2 and 3 mediated adherence of erythrocytes to the monolayer, which shown as inhibition

percentage. The inhibition percentage was higher when compared to the previous studies. The present analysis of our study showed lesser concentration of 4-GU-DANA showed high inhibition percentage inhibiting HPIV-2 and 3 HN gene.

Antiviral activity of 4-GU-DANA were determined based on plaque reduction assay to inhibit the replication of viral growth. In preceding studies, the variant C-0 which inhibit the plaque enlargement were showed at 1 mM 4-GU-DANA concentration of about 99%, another variant ZM1 which is resistant to various concentration other variant C22 which shows plaque reduction percentage 47.7% at 15 mM concentration[261]. Our verdicts where in at 500 µM of 4-GU-DANA maximum reduction of plaque formation by 72% in LLC-MK2 and 79% plaque reduction in A549 cell line was seen. Among two cell lines A549 was highly predominant to prevent the replication of HPIV-2 and 3 when compared to LLC-MK2. The outcome of our study limited concentration of 4-GU-DANA to be used which is of plaque reduction.

Neuraminidase inhibition activity depends upon 4-GU-DANA with ZM1 variant was highly resistant to neuraminidase inhibitory effect. Other variant C-0 in which neuraminidase activity was achieved at 0.5 mM for 50% inhibition. But 5 and 10 mM 4-GU-DANA indicating 10 to 20 fold increase in resistance of variant ZM1. 4-GU-DANA at 5 mM concentration which variant C-0 neuraminidase were nearly entirely inhibited at 95.7%[351]. In another study, ZM1 which showed 43.5% inhibition which was much lesser[351]. In afore mentioned studies the variant C-0 was existed completely inhibit the neuraminidase activity whereas C28a, P111S which represented as less than 1% and 30% was reduced neuraminidase activity respectively[261].

In our study, the antiviral activity of Ribavirin against HPIV-2 and 3 were assessed by plaque reduction assay, which denoted the 600 µM concentration extremely 70% reduction of plaque formation in LLC-MK2 and 75% plaque reduction in A549 cell line. Among the two cell lines, A549 did not support the replication of HPIV-2 and 3.

Ribavirin, a synthetic nucleoside analog with broad antiviral activities against DNA and RNA viruses. Antiviral activity of Ribavirin against HPIV-2 was conventionally detected by synthesis of cDNA used random primer. PCR was performed to elucidated the effect of RBV on mRNA synthesis. The existing outcome intend that the inhibitory effect of RBV at 500 µM on HPIV-2 growth was arbitrated by inhibition of virus replication in the cells, generally by inhibition of viral transcription. Viral mRNA was not noticed in Ribavirin treated cells, specified that the inhibition of viral protein synthesis was interceded by inhibition of transcription by Ribavirin. In previous studies similar outcomes was observed with 1 mg/mL[300].

The effect of Glycyrrhizic acid (Phytal compound) was evaluated in the present study for the broad spectrum of antiviral activities and pharmacological effects and multiples site of actions. Licorice compound (Glycyrrihic acid) was determined 50% tissue culture infectious dose ($TCID_{50}$) in LLC-MK2 and A549 cells. In the current study, Glycyrrihic acid compound was assessed for the antiviral activity efficacy by elapsed incubation time and treatment of the cells infected with HPIV type 2 and 3. The Glycyrrihic acid concentration was extended from 10-100 µg/mL for antiviral determination. The similar study was not conducted elsewere. The Licorice *(Glycyrrhiza glabra)* against HPIV-2 and 3 in LLC-MK2 and A549 cell lines were first reported in this study. Glycyrrihic acid from (Licorice root) concentration 70 µM showed maximum reduction of plaque formation was about 86% in LLC-MK2 and 87% in A549 cell lines. Among two cell lines, A549 was highly sensitive to identify the plaque reduction formation. This study was the first report that the licorice antiviral activity was evaluated *invitro* against HPIV by Hemadsorption inhibition assay, Plaque reduction assay and neuraminidase inhibition assay.

In previous studies, Glycyrrihic acid is active against Epstein-Barrvirus[352]. The replication of Epstein-Barr virus in superinfected Raji cells in a dose-dependent manner, IC_{50} values for viral inhibition at 0.04 mM concentration[353], for Varicella Zoster virus inhibit concentration at 0.71 mM[354] and for Human immunodeficiency

virus inhibit concentration at 0.15 mM[355]. Clinically, Glycyrrihic acid used to treat patients with chronic active hepatitis virus[356]. Glycyrrihic acid endowed inhibitory activity on growth of Herpers simplex virus, New castle disease virus, Vaccinia virus and Vesicular stomatitis virus at affective concentration at 8 mM, 4 mM and glycyrrhizic acid were ineffective inhibitory activity in Polio virus[357].

Neuraminidase inhibition activity upon with Glycyrrihic acid were stripped with HPIV- 2 and 3 was highly sensitive to neuraminidase inhibitory effect. The 50% (IC_{50}) inhibitory concentration of Glycyrrihic acid were identified as 95%, 97% to inhibit the growth of HPIV-2 and 3 respectively. In addition, based on the proposed studies it speculated that the suppression of HPIV-2 and 3 replication in LLC-MK2 and A549 cells. Licorice from vital compound glycyrrhizic acid were performed at minimum concentration to inhibit the growth of HPIV type 2 and 3 when compared to Ribavirin and Zanamivir.

High throughput screening (HTS) have been employed for many small molecules to identify the effective drugs which inhibit the activity of viral receptors (HPIV-3). Nearly 32000 compounds were identified as potent drugs against several viral receptors[358]. In the present study, focused on the molecular mechanism of antiviral activity in Zanamivir small molecule against the HN receptor glycoprotein was elucidated. The zanamivir is chemically 4-GU-DANA (4-Guanidino-2,4-dideoxy-2,3,-dehysro-Nacetylneuraminic acid) was evaluated for the molecular interactions at binding site. The study of molecular interaction helps to understand the mechanism of action on ligand and receptor docking. The involvement and significance of ligand receptor binding interpreted the biological activity of lead molecules involved in defense activities against viral pathogens[359]. The parainfluenza strains changing in their genome in recent years due to environmental surrounding and unique antiviral drugs in the control of this infection is very important to prevent mortality and morbidity[360]. Surveillance programme on the Human parainfluenza viruses circulating in different part of the world is very important to monitor and control the resistant viral strains. Similarly, the elucidation

on mechanism of inhibition with HN receptors and antiviral drugs is extremely important in the effective controlling of resistant viral strains[361].

Consequently in the present study, the active sites of glycoprotein receptors 1V2i indicates the amino acid residues such as ASN 307,308, THR 302 and 304 are present in the docking sites. Similarly the active site of 1V3b was predicted as ASN 351, THR 353,358 TRP 451. The active site of 1V3d indicated the residues such as ASN 307,308, ILE 391, GLU 276, arginine 502,524, Thyrosine 319, 337 and Lysine 305. The active site of 1V3e demonstrate that the presence of amino acid viz., tyrosine302, Aspargine308, Arginine303,307,308,424, Glutamic acid 276, Threonine 193, glutamic acid 276. The active site of 4MZA was revealed that amino acid such as thronine302, Arginine303, Phenylalanine304, Aspargine308, proline392, lysine309 was found. The In silico docking of Zanamivir with all the five receptors demonstrates significant In silico antiviral activity. The Zanamivir revealed effective docking was highly significant in 1V2i. 1V3b and 1V3e and this indicate the moderate docking with Zanamivir ligand.

Summary

SUMMARY

Human parainfluenza viruses are a group of viruses that cause different types of respiratory infections and are most common in children and infants.

Throat and nasal swabs were collected from symptomatic patients in Chennai within three days onset of illness were determined the prevalence of HPIV by Multiplex reverse transcription PCR. Epidemiology of specific viral etiology in patients was observed throughout the years. The age wise distribution of HPIV cases were analyzed and divided into 0-10, 11-20, 21-30, 31-40, 41-50 and above 50. The prevalence in different age groups was statistically analyzed by standard error mean. Their positivity was observed in all the years during monsoon months of August to September and post monsoon months of November to February. Among the four serotypes HPIV type 3 is highly predominant in all the years (2011-2014). HPIV-2 positivity were occurred rarely in 2014. This study validates the prevalence of HPIV infection in Chennai and indicates the circulating serotypes and HPIV strains.

The PCR products were sequenced and submitted to genbank and assigned the accession number. Different sequences were retrieved from NCBI and aligned as FASTA format. Mutations were identified by multiple sequence alignment of HPIV by ClustalW tool. Amino acid alterations were identified in HPIV-3 (HN gene) at residue 295 which Histidine replaced by Tyrosine and at 297 which Serine replaced by Glycine. Another mutations were identified in HPIV-2 (N gene) at residue 138 which Histidine replaced by Tyrosine and at 140^{th} residue identified amino acid alteration which Histidine replaced by Glutamine. The phylogenetic analysis were identified the homology of Chennai strains with other strains. HPIV type 3 (HN) strain was clustered with Fukuoka /2009, Nagasaki 2009 and Wash 64979. HPIV type 3 (NP) strain was grouped with Switzerland/2013, US/2000, and South Africa/2000. HPIV type 2 was compared with Greer strain and HPIV2/V94.

All clinical samples were cultured in the LLC-MK2, A549 and MDCK cell lines. Then specificity and sensitivity of the cell passages were characterized for clinical isolates. The number of positive cases were highly significant in the year

2011 followed by 2013. Out of 931 samples, 38 were isolated by LLC-MK2, 15 samples identified CPE in A549 and only 5 samples were grew in MDCK cells. Among the three cell lines LLC-MK2 was highly predominant for the isolation of HPIV. Positive percentage remained very small in MDCK cell line thus for further confirmation were not studied, whereas other two cell lines LLC-MK2 and A549 performed further confirmation. The isolated Human parainfluenza virus type 2 were more sensitive in the early passages of 8 and 9 at day five for LLC-MK2, highly compatible in the 5^{th} passage at day 7 for A549 and 8^{th} passage at day 8 for MDCK passage at day 8 for rest of the passages were less sensitive and specificity. HPIV-3 was more accustomed in the 9^{th} passage at day 5 for LLC-MK2.

The virus isolated samples were performed by hemadsorption assay (HAD) was aimed at confirmation of cytopathic effect were identified in LLC-MK2, A549 cell lines. Among the two cell lines LLC-MK2 was highly predominant to detect erythrocytes adhered on the monolayers and followed A549 cell lines. Cells infected with HPIV with *C.Perfringens* treatment by HAD assay to enhance erythrocyte binding for HPIV-2 (82%) and HPIV-3 (90%) in 24 well plate. Virus isolated samples were confirmed by plaque assay. Plaque was observed after 8-10 days of incubation. Interactions of receptors between HPIV-2 and 3 with m.o.i. in LLC-MK2 appeared as fusion were not blocked in HPIV-2 whereas HPIV-3 achieved fusion were blocked syncytium was not formed. Similar outcomes remained in A549 cells. Further the study evaluated the neuraminidase enzyme activity of HPIV. 4-MU concentrations and used determined the percentage of substrate expended during the reaction. The signal to background ratio were determined as the fluorescence intensities restrained after 20 minutes. The substrate concentrations were used at 25 µM. Various concentration of HPIV- 2 and 3 (m.o.i.) with bacterial neuraminidase were performed by neuraminidase activity which are statistically significant.

Cytotoxicity of 4-GU-DANA at the concentrations less than 400 µM in A549 and greater than 641 µM in LLC-MK2 by MTT assay. Cytotoxicity of Ribavirin at the concentrations less than 405 µM in A549 and greater than 476 µM in LLC-MK2 by MTT assay. Cytotoxic percentage of Ribavirin were appears as

high when compared with HN inhibitor. Cytotoxicity of glycyrrhizic acid form Licorice at concentration 31 µM in LLC-MK2 and 45 µM in A549 were identified. Indicates that MTT obtained better results compared with other dyes.

Antiviral activity of neuraminidase inhibitor (4-GU-DANA) against HPIV by Hemadsorption inhibition assay was performed and ability to interfere with receptor interaction of HPIV-2 and 3 blocks hemadsorption activity at 600 µM seemed as 77% and 78% respectively. 4-GU-DANA inhibits receptor binding for HPIV-2 and 3 at 500 µM (60%) inhibit plaque formation in LL-CMK2 and (67%) and (79%) inhibit plaque reduction in A549 cells. In neuraminidase inhibition assay, less concentrations which inhibit the HPIV-2 and 3. The IC_{50} concentration for HPIV-2 at 2.5 µM and HPIV-3 at 1.6 µM. 4-GU-DANA concentrations were obtained less deliberation for HPIV-3 when compared to HPIV-2.

Antiviral activity of nucleoside inhibitor (Ribavirin) against HPIV type 2 and 3 by hemadsorption inhibition assay was performed to inhibit the adherence of erythrocytes to the monolayer of HPIV type 2 and 3 at 400 µM (77%) and (75%) individually. Ribavirin inhibits replication for HPIV-2 and 3 at 400 µM during pre and post adsorption period in LLC-MK2 and A549 cells. There was no significant reduction in plaque number due to existence of ribavirin during the adsorption period of 90 minutes. The plaque area was reduced by addition after the adsorption period. Further confirmed inhibition of HPIV-2 by molecular characterized, observed infected cells without drug showed band by molecular characteristics. RBV treated infected cells, band cannot be seen and indicating that RBV inhibited transcription of viral genome.

Cytotoxicity of *Glycyrrihic acid* from Licorice generated at 31 µM for LLC-MK2 and 45 µM for A549 by MTT assay. Antiviral activity of *Glycyrrihic acid* against HPIV-2 and 3 by hemadsorption inhibition assay was achieved to inhibit the erythrocyte adherence to the monolayer of HPIV-2 and 3 at 100 µM inhibition percentage was occurred 90% and 95% respectively. *Glycyrrihic acid* compound inhibits plaque formation at 70 µM replication of viral growth percentage was 86% for LLC-MK2 and 87% for A549 cells. Further confirmed by

neuraminidase inhibition assay were performed at less concentration for HPIV-2 and 3 at 1.5 µM and 1.2 µM respectively.

Neuraminidase inhibitor (Zanamivir) and nucleoside inhibitor (Ribavirin) were performed for antiviral activity against HPIV-2 and 3. Among these inhibitors Ribavirin has highly preferable with less concentration when compared to HN inhibitor. Among these three compounds natural glycyrrhizic acid from Licorice root were performed and observed very minimum concentration to inhibit both serotype of Human parainfluenza virus-2 and 3. The following inhibitors namely Neuraminidase inhibitor (Zanamivir) and nucleoside inhibitors (Ribavirin) maximum concentration was used to inhibit the HPIV-2 and 3. Finally, the phytal compound glycyrrhizic acid from licorice showed comparatively high inhibition on the viral growth in *invitro* screening. The Zanamivir nucleoside analog was elucidated for the mechanism of antiviral activity. The HN receptor of HPIV was docked with ligand Zanamivir using Autodock programme. All the HN receptor was significantly docked by Zanamivir. The 1V2i receptor was prominently docked with (95%) high frequency and good dock score.

Conclusion

CONCLUSION

The HPIV group of viruses is conventionally specified a high place in the pantheon of respiratory viruses as a cause of upper and lower respiratory tract illness. All three types can cause croup, a serious infection due to respiratory mortification. A comprehensive analysis of the potential impact of respiratory viruses in Chennai among symptomatic patients with acute respiratory tract infections were analyzed. This shows that HPIV should also be screened as a routine when screening for other viral agents in the community.

The present study throws light on the presence of HPIV existence and its persistence. This study would be the first of its kind to report HPIV occurrence in Chennai as not many studies have been reported, owing to the difficulties in isolating HPIV and the lack of general awareness of its clinical significance and respiratory ailments. The role of human parainfluenza virus serotypes 1,2,3 and 4 as a causative agent of upper and lower respiratory tract infections during the period 2011-2014, their epidemiology and molecular characterization were analyzed in great detail. The commonly occurring strains among HPIV were identified in this study, further correlation with the signs and symptoms can be done in future to understand their association better. One of the main setback was follow up of the positive subjects were not done hence comments on the mortality and morbidity could not be made. This association could help the scientists comprehend the disease condition better.

Among the four serotypes HPIV screened, HPIV- 2 and 3 was found to be the most prevalent and other serotypes were not detected. The predominance of HPIV among 1 to 5 age group and the observation that the school going age group was less affected looks very significant as higher positivity is usually expected among school going children. We had some positivity in the age group of >50 years. There are studies from the west saying that HPIV is common among the >50 age group which was observed in our study. Infection among the extremes of age shows absence of immunity or lesser immunity being the reason for increase in infection among this age group. It is obvious that any infection which spreads through

aerosols and fomites is found to be high among school children because of close proximity this can be expected when there is very active spread of infection or better immunity among this group and adults may be due to improved immunity among them.

Out of seven Government hospitals in Chennai, Institute of Child Health had high prevalence of acute respiratory tract infection caused by Human parainfluenza viruses during the study period 2011-2014. Periodically followed by Government Peripheral Hospital and Rajiv Gandhi Government Hospital. ICH and RGGH being pediatric and adult tertiary care were premier referral centre indicates higher positivity on investigation is as expected.

Season wise circulation of the virus established that the HPIV positives were prevalent in monsoon months of August to September and post monsoon months of November to January, with elevated positivity in November. In our study HPIV positivity were seen during the monsoon months of August-September and post monsoon months of November-January, with peak positivity in November. This could be due to rainfall, lower temperature and increase in humidity. Since Chennai has high humidly all through the year, increase in the rainfall and fall in the temperature could be the major reason for increase in positivity among the monsoon and in post monsoon months. Hence preparedness by the public health authorities prior to these months will help in preventing HPIV along with other Influenza infections.

In the present study, the etiological role and prevalence of Human parainfluenza virus among the other infections in the community was evaluated. Clinicians should be aware of the possibility of the other causes of ILI that may be similar to Human Influenza infections. HPIV may also be an emerging infectious disease which could be included in testing of samples along with other common respiratory viruses. Multiplex Reverse Transcription Polymerase Chain Reaction assay could be used for the accurate diagnosis and detection of HPIV. Further, expanded surveillance throughout the country will help in better epidemiological analysis, for evolving and implementation of effective public health program in controlling HPIV induced respiratory infections. However, further study is needed to

refine the ability to make epidemiologic references based on sequence similarities. Since were able to detect HPIV-2 and 3 only, and HPIV- 1 and 4 were not detected, further surveillance studies can be taken up by authorities as this could be potential causative agent for respiratory infections with complications and not responding to antiviral agents against Influenza and RSV.

The molecular characterization of HPIV was performed by mRT-PCR assays targeting HPIV in symptomatic patients with acute respiratory tract infections. The unique aspect of this study was identification of HPIV and its associated clinical profiles in patients with ARI. The pattern differed from those in previous reports from different areas due to geographical and climatic pattern. Long term follow up studies will help in better understanding of correlation between clinical patterns and the agent. Since molecular detection of HPIV is technically easy especially in referral centres, screening can be done after ruling out other respiratory viral infections.

Sequence analysis of HPIV type 3 (HN gene), the strains were found to be similar to the isolates from different countries of Nagasaki, Fukuoka and Melbourne strains. By phylogenetic analysis Swine strain was used as an out-group, mutations were identified at 295 and 297 amino acid residues. Human parainfluenza virus type 3 (N gene) was very close to the clades of Switzerland, USA, South Africa, Argentina strains on sequence analysis. Bovine parainfluenza viral strains were placed as an outgroup. Phylogenetic analysis of HPIV type 2 (N gene) were predominantly homologous to US (V94 strain) and US (Greer strain). Human parainfluenza virus 4A considered as an out-group. This indicates that Chennai strains were comparable with strains from other countries and there were no evidence new or mutated strain circulation of HPIV in Tamilnadu, Chennai.

LLC-MK2 cell lines can be used for large scale production of HPIV, as in vaccine production. Human Lung carcinoma, A549 cannot be used for vaccine production because of its carcinogenic potential. Thus LLC-MK2 would be an acceptable single alternative to primary monkey kidney cells. To conclude the ability of a clinical laboratory to detect Human parainfluenza virus in clinical specimens efficiently and accurately has important implications mainly to detect

mutations, further strain evolution and large scale production for vaccine development. LLC-MK2 is more sensitive to the virus when compared to the other cell lines.

Antiviral activity of Hemagglutinin Neuraminidase inhibitor and Nucleoside inhibitor against HPIV type 2 and 3 were assessed. Among these inhibitors Nucleoside inhibitor (Ribavirin) efficiently inhibited the replication and transcription of HPIV. Commercially available natural herbal product of Licorice was used at less concentration to inhibit the replication of HPIV. But 4-GU-DANA and Ribavirin were used at higher concentration to inhibit the growth of Human parainfluenza virus. In this age of antibiotics wherein antiviral agents are used to treat viruses when required, these potential agents can be considered as therapeutic agents. HPIV is an emerging virus capable of causing respiratory tract infections with significant morbidity emphasis may be placed upon this agent when screening of respiratory viruses.

Finally, the mechanism of antiviral activity of Zanamivar was elucidated through insilico molecular docking. The Zanamivar was docked with 5 receptors of HPIV-3 envelope proteins. Among the receptors, the 1V2I receptor with Zanamivar revealed high docking score and 90% of docking frequency. From the insilico docking the 1V2I was the best molecular target for Zanamivar in the prevention of HPIV infection.

Recommendations

RECOMMENDATIONS

- In this study Human parainfluenza virus infections among Chennai population has been proven, hence routine screening for HPIV among acute respiratory tract infections will have to be routenly monitored.

- HPIV infection were occured during monsoon and post monsoon months hence health awareness activities by public health authorities are usually geared up prior to monsoon, will have an effective role in the control and prevention of HPIV infections.

- In the established virology laboratories in our country, screening for HPIV can be taken up for confirming the presence of HPIV, as the multiplex PCR proposed can be used to screen for the common strains of HPIV effectively.

- In this era of antibiotics the suggested antiviral agents can be considered as potential therapeutic agents, with docking studies proving the antiviral activity of Zanamavir, these agents can be subjected to further research and, clinical trials and can be used as active anti HPIV agents.

- Isolation of HPIV, strains isolated from our local areas can give a wealth of information with respect to the developments of mutations and other genetic changes and can aid in the development of vaccines.

- Glycyrrhizic acid from licorice, being effective at very low quantity can be considered as a low cost effective agent HPIV as shown in our study.

- Surveillance activities for HPIV along with other commonly occurring respiratory viruses can be taken up by public health authorities in coordination with established virology laboratory in the country. This would provide valuable information to the public health authorities and clinicians.

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Isolation & molecular characterization of human parainfluenza virus in Chennai, India

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***Background & objectives*: Human parainfluenza virus (HPIV) accounts for a significant proportion of lower respiratory tract infections in children as well as adults. This study was done to detect the presence of different subtypes of HPIV from patients having influenza like illness (ILI).**

***Methods*: Throat and nasal swabs from 232 patients with ILI who were negative for influenza viruses were tested by multiplex reverse transcription polymerase chain reaction(mRT-PCR) for the detection of human parainfluenza virus. All samples were inoculated in rhesus monkey kidney (LLC-MK2) cell line.**

***Results*: Of the 232 samples, 26(11.2%) were positive by mRT-PCR and nine (34.6%) showed cytopathic effect with syncytium formation for HPIV and all were HPIV-3 serotype, other serotypes like 1,2,4 were negative. The HPIV-3 strains (*HN* gene) were sequenced and analysed. Two novel mutations were identified at amino acid residues 295 and 297.**

***Interpretation & conclusions*: The mRT-PCR assay offers a rapid, sensitive and accurate diagnostic method for detection of HPIV which enables early detection and control. In our study there was a predominance of HPIV among 1-5 yr age group and the school going age group was less affected. Further studies need to be done to characterize HPIV isolated from different parts of the country.**

Key words Human parainfluenza virus - mRT-PCR - isolation - ILI - prevalence

Human parainfluenza virus (HPIV) is known to cause acute respiratory infections (ARI) including lower respiratory tract infection, which is a leading cause of morbidity and mortality in infants and young children world-wide[1-3]. HPIV belongs to the *Paramyxoviridae* family, subfamily Paramyxovirinae, and is classified into four serotypes (HPIV-1, HPIV-2, HPIV-3, and HPIV-4). Serotype 4 can be further subdivided into two antigenic subtypes, HPIV-4A and HPIV-4B[4]. Infection with HPIV in immuno-compromised children is known to be associated with a range of diseases, from mild upper-respiratory symptoms to severe disease requiring mechanical ventilation and leading to death[5]. Of the four recognized serotypes, HPIV-3 is most

commonly associated with serious lower respiratory tract illness, followed by HPIV-1 and HPIV-2; HPIV-4 is rarely associated with serious illness[6]. HPIV is second only to respiratory syncytial virus (RSV) as a cause of hospitalizations for acute respiratory infection among children aged <5 yr; 2-17 per cent of such hospitalizations are due to HPIV infection[6]. It is important to know the mechanism resulting in genetic and antigenic diversity of HPIV for controlling the pathogen.

The use of classic diagnostic methods like viral isolation and serology is time consuming and takes several weeks till the results are available, and hence these methods are less useful for making therapeutic decisions[7]. Cell culture, often considered to be the gold standard, is delicate and sometimes too slow for it to be useful for diagnosis. Direct antigen detection methods are widely used for rapid diagnosis of HPIV infections[8-10], but results can be variable[5,11]. Multiplex reverse transcription polymerase chain reaction (mRT-PCR) assay can be a sensitive and specific tool for rapid diagnosis of HPIV infections[5,12,13]. In this study, mRT-PCR was performed for the simultaneous detection of HPIV-1,2,3 and 4 in samples collected from patients with influenza like illness (ILI). The isolation positive samples were sequenced and analysed.

Material & Methods

Clinical samples: Throat and nasal swabs were collected during January 2011 to August 2012 from patients with ILI belonging to different age groups attending outpatient departments (OPD) of tertiary care government hospitals in Chennai, Tamil Nadu, India [Institute of Child Health and Hospital (52 samples), Royapettah Government Hospital (20 samples), Saidapet Government Hospital (26 samples), Rajiv Gandhi Government Hospital (39 samples), Government Peripheral Hospital (42 samples), Kilpauk Medical College (29 samples) and Stanley Medical College (24 samples)].

Sample collection and processing: The study was conducted in the department of Virology, King Institute of Preventive Medicine and Research, Chennai. A total of 232 throat and nasal swabs were collected from infants, children, adolescents and adults. These samples were collected from patients with symptoms like fever, chills/rigors, nasal discharge, cough, sore throat, breathelessness and headache. Clinical samples were collected in 3 ml of cold viral transport medium (Hank's balanced salt solution) containing 0.5 per cent gelatin and transported in cold chain to the laboratory.

Nucleic acid extraction: QIAmp viral Qiagen RNA extraction kit was used for RNA extraction from clinical samples and Invitrogen superscript III platinum one step RT-PCR system for the detection of RNA. Oligonucleotide primers against hemagglutinin neuraminidase (*HN*) gene were used to detect HPIV-1, 2, 3 [14] and for HPIV-4, primers were directed against phosphoprotein gene[15] (Table I).

Table I. Oligonucleotide primers for human parainfluenza virus

Primers	Sequence	Base pairs	Gene
HPIV-1 F[14]	CCGGTAATTTCTCATACCTATG	747-768	*HN*
HPIV-1R	CCTTGGAGCGGAGTTGTTAAG	694-714	*HN*
HPIV-2 F[14]	AACAATCTGCTGCAGCATTT	803-822	*HN*
HPIV-2 R	ATGTCAGACAATGGGCAAAT	620-639	*HN*
HPIV-3 F[14]	CTCGAGGTTGTCAGGATATAG	632-652	*HN*
HPIV-3 R	CTTTGGGAGTTGAACACAGTT	900-920	*HN*
HPIV-4A F[15]	ATGATGGTGGAACCAAGATT	240-259	*P*
HPIV-4 A R	AACCAGGGAAACAGAGCTC	1103-1084	*P*
HPIV-4B F[15]	CTGAACGGTTGCATTCAGGT	333-351	*P*
HPIV-4B R	AGGACTCATTCTTGATGCAA	1103-1084	*P*

Superscript numerals denote reference numbers

Optimized reagents and PCR cycling condition for HPIV: For primary PCR amplification, RNA was added to PCR mixture containing buffer, water, Taq polymerase, primers to a final volume of 50 µl. Amplification was performed on ABI Thermal Cycler, USA, with cyclic conditions of 50°C for 30 min, 94°C for 15 min, 35 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for one min and 72°C for 10 min for elongation[14]. PCR products were visualized in 1.5 per cent agarose gel electrophoresis and molecular weight marker of 100 bp used. Expected band sizes for HPIV-1,2,3 and 4 were 371, 507, 189 and 451 bp, respectively.

Virus and reagents: The human parainfluenza virus was propagated in rhesus monkey kidney cell line LLC-MK2 [National Institute of Virology (NIV), Pune] at 37°C with 5 per cent CO_2 in humidified conditions. The cells were maintained in Eagles minimal essential medium (Sigma, USA), supplemented with 10 per cent foetal bovine serum (FBS, Hi media, India) and 0.01 per cent antibiotic-antimycotic solution penicillin, streptomycin, kanamycin and fungizone, and trypsin-EDTA.

Viral isolation: All samples were inoculated into tissue culture T 25cm^2 flasks of LLC-MK2 cell line (NIV, Pune), allowed to adsorb for one hour and incubated at 37°C. Cell monolayers were observed for cytopathic effect (CPE) every 48 h. HPIV positive clinical isolates demonstrated focal rounding and destruction, occasional syncytia on initial isolation. The samples which showed CPE were confirmed by mRT- PCR.

Sequence analyzing: Three representative *HN* genes of HPIV were detected by mRT-PCR and isolation positive samples were sequenced and analysed. The HPIV-3 standard strain was downloaded along with strains from different countries from NCBI database (*www.ncbi.nlm.nih.gov*). Strain-gi/168481518/swine/USA/2009 was used as out group for construction of phylogenetic tree. All the sequences were analyzed by MEGA (version 5) program (*mega.software.informer.com/5.0/)* using maximum likelihood method with p distance[16].

Results

Evaluation of multiplex reverse transcription PCR with clinical specimens: Oligonucleotide primers were used to amplify *HN* gene of HPIV (Table I). A total of 232 samples were tested for HPIV by mRT-PCR, of which 26 (11.2%) were positive for HPIV-3, other serotypes were negative. Positive samples showed amplicon size 189 bp for HPIV-3 (Fig. 1). Twenty mRT-PCR positives were among the paediatric age group (less than 12 yr) and six positives were in the group more than 12 yr.

Symptoms analyses for HPIV cases: In this study, 151 males were tested and 16 (10.5%) were positive. Among the 81 females tested, 10 (12.3%) were positive for HPIV-3 by mRT-PCR. Among all the signs and symptoms presented by the HPIV positive cases sore throat and, breathlessness were significant when compared with the negative cases (P<0.05) (Table II).

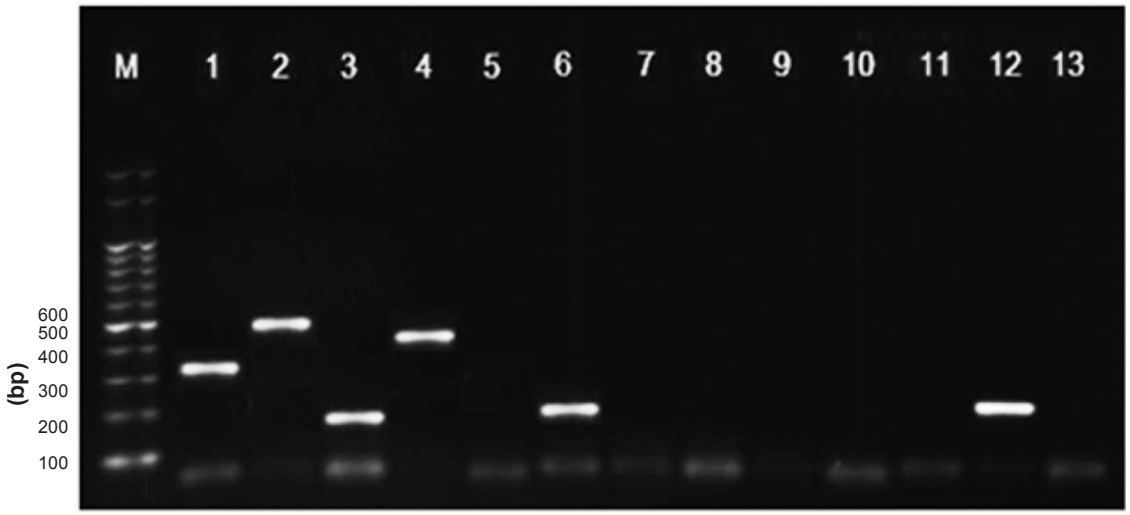

Fig. 1. Results of multiplex reverse-transcription (RT)-PCR with clinical samples and controls. Lane M, Marker; Lane 1, HPIV-1(Positive control); Lane 2, HPIV-2(PC); Lane 3, HPIV-3(PC); Lane 4, HPIV4(PC); Lane 5, Negative; Lane 6, HPIV3(clinical sample); Lanes 7 to 11, Negative; Lane 12, HPIV-3 (clinical sample); Lane 13, Negative control.

Table II. Clinical manifestations of human parainfluenza virus

Signs/symptoms	Samples positive for human parainfluenza virus (n=26)	Samples negative for human parainfluenza virus (n=206)
Fever	26	206
Chills/rigors	5 (19.2)	49 (23.7)
Nasal discharge	18 (69.2)	160 (77.6)
Cough	23 (88.4)	198 (96.1)
Sore throat	14 (53.8)	45 (21.8)
Breathelessness	12 (46.1)	63 (30.5)
Headache	6 (23)	70 (33.9)

Values in parentheses are percentages

Comparison of multiplex reverse transcription PCR with tissue culture techniques: Viral isolation was attempted using LLC-MK2 cell lines. Of the 232 samples subjected to viral isolation in LLC-MK2, nine (34%) were positive. Negatives were discarded after three passages, and the samples that showed mild changes in the cell morphology were passaged further. If these samples produced clear CPE these were confirmed by PCR, if CPE was not seen after two more passages, these were checked by PCR and if negative, discarded. Clear CPE was observed in the ninth passage in seven samples and two samples showed CPE in 5th passage. These samples were reconfirmed by RT-PCR as HPIV-3.

Virus and sequence analysis: Among the 26 positives, three representative samples from different age groups and three geographical regions within Tamil Nadu were chosen and subjected to sequencing. The sequences of HPIV-3 strains were submitted to NCBI. Their accession numbers are (JQ901411-JQ901413).

Comparison of sequence with other strains

Sequences of HPIV strains from different countries were retrieved and compared with our strains using BLAST (*blast.ncbi.nlm.nih.gov*). (Table III). Phylogenetic analysis of our strains (Fig. 2) showed that these were highly similar to strains from Nagasaki, Fukuoka and Melbourne strains (97% similarity) and formed a same clade, The other strains that were significantly related were Washington (1973 and 1979), Oklahoma (2009), Bethesda (1997), Delhi (2005 and 2006), Riyadh (2008 and 2009), Texas (1980, 1982 and 1983), Logan (2009), Ontario (2001) and Guangdong (2007). The similarity confirmed the circulation of HPIV in Chennai and also its similarity to the strains in different countries.

Amino acid analysis: In our strains two mutations were identified at 295 and 297 amino acid residue. At 295 residue, glycine was replaced by serine, which can act as protein functional centres and hydrophobic amino acid. At position 297, histidine was replaced by tyrosine. Histidine is an essential amino acid with a positively charged imidazole functional group.

Discussion

It is known that among respiratory viruses namely RSV, HPIV and influenza viruses A and B, RSV has been documented to be the most common pathogen[17] followed by human influenza virus and HPIV. Among the HPIVs, serotype 3 has been predominantly reported[2,18] as also found in the present study.

In our study HPIV positivity was seen during the monsoon months of August-September and post monsoon months of November-January, with peak positivity in November. But in northern hemisphere HPIV infections were more frequent from January to April[19]. In temperate climates, HPIV-3,4 were detected in spring, summer and late fall and seasonal incidence varied for HPIV-1,2 in Chinese children[20].

A high proportion of males were found to be infected with respiratory viruses as compared to females in our study which was similar to another study in Delhi[21]. The patients' median age was 20 months for HPIV-4 infections and 7-11 months for HPIV-1, 2 and 3 infections, but the clinical manifestations did not differ significantly between HPIV-1, -2, -3, and -4 infections[20]. It is known that influenza virus infections are more common in the paediatric age group, likewise in our study there was a predominance of HPIV among 1-5 age group. HPIV-3 was the prevalent serotype.

In this study the most common mode of presentation was fever, followed by sore throat and cough like any other ILI. Since samples were not collected from patients with SARI (severe acute respiratory illness) and long term follow up was not done, the rate of hospitalization among the ILI cases was not assessed in our study. Additional studies that include hospitalized controls are needed to clarify the clinical importance of HPIV infection in adults with community-acquired lower respiratory tract infection. One of the limitations of our study was that screening for RSV was not undertaken, as this could have been of

Table III. Strains of human parainfluenza virus reported from other countries and from the present study

GI and Accession No.	Strain/country	Year
gi332709, AAA46848	USA	1993
gi332701, AAA46844	USA	1993
gi351001320, AEQ39012	Oklahoma410/2009/USA	2011
gi1262233, AAB48689	JS/USA	1997
gi193888390, ACF28540	Del/88/06/India	2008
gi193888394,ACF28542	Del/139/05/India	2008
gi193888388,ACF28539	Del/322/06	2008
gi309252629, ADO60288	Riyadh149/2009/Soudi Arbia	2012
gi309252627, ADO60287	Riyadh11/2008/Soudi Arbia	2012
gi332715, AAA46851	USA	1993
gi332713,AAA46850	USA	1993
gi332711, AAA46849	USA	1993
gi167594039, ABZ85673	14702/Canada	2009
gi409057, AAA46855	Canada-Ontario	2001
gi163866868,ABY47606	ZHYMgz01China	2007
gi37958139,AAP35240	Australia-Melbourne	2004
gi215794089, ACJ70090	China- Gansu Provence	2011
gi58430688,BAD89145	Fukuoka-Japan	2009
JQ901411(AFM78693	Chennai, India (present study)	2011
JQ901412(AFM78694)	Chennai, India (present study)	2011
JQ901413(AFM78695)	Chennai, India (present study)	2011
gi3510305,BAA32574	GP/Japan-Nagasaki	2009
gi10937875,NP_067152	GP/Japan-Nagasaki	2009
gi356651382,AET35008	Wash/1957c243/USA	2011
gi15209379,CAC51081	MK9/UK	2001
gi332721,46856	Canada-Ontario	1993
gi332718, AAA46853	USA-Ohio	1993
gi 332707, AAA46847	Australia	1993
gi168481518, ACA24945/swine	USA	2009

Source: *BLAST: http://www.blast.ncbi.nlm.nih.gov*

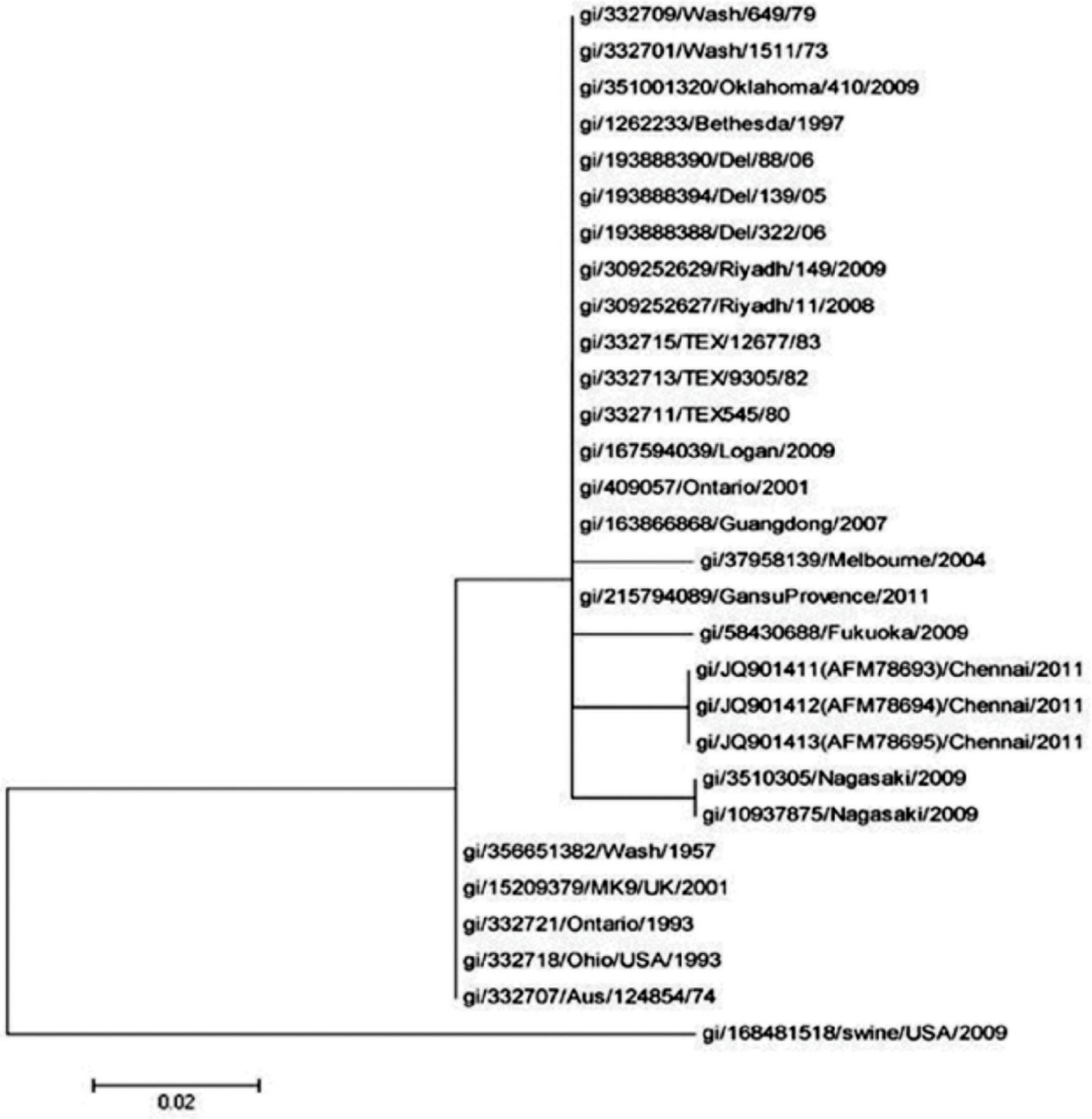

Fig. 2. Phylogenetic analysis of the deduced amino acid sequences of hemagglutinin neuraminidase gene of HPIV-3 to members of the family *Paramyxoviridae*. The tree was constructed by the maximum likelihood method with p distance.

great relevance in terms of the role of other respiratory pathogens in causing ILI.

In our study mRT-PCR assay was able to identify a greater number of positives in clinical specimens than cell culture as reported earlier[4,11,15]. Of the 26 specimens that were mRT-PCR positive, only nine HPIV-3, strains could be isolated which was about one third of the total. Detection of HPIV in the remaining 17 specimens by mRT-PCR and not by isolation was due to the high sensitivity of mRT-PCR assay[22]. Hence compared to other methods, mRT-PCR seemed to be a better method for detecting HPIV in suspected cases. There are similar studies indicating mRT-PCR to be a better diagnostic aid when compared with viral isolation and immunoflouresence tests[15]. The advantages are that mRT-PCR does not require cell line maintenance, as the isolation of HPIV requires multiple passages which is not only time consuming but also cost ineffective.

It was earlier identified that the point mutation at residues 278 and 281 coded for a single amino acid substitution in the HN protein[23]. Another study also conferred the mutations at the residues in threonine

193 isoleucine and isoleucine 567 valine[24]. The viral neuraminidase alters the host cells surface, modulating the number of available sialic acid receptors and thus determining the outcome of infection, fewer sialic acid receptors are available to interact with other viral HN molecules. Other studies have shown one point mutation in the *HN* gene corresponding to a single amino acid change in the HN glycoprotein, which converts aspartic acid 216 to an asparagine and proline 111 to a serine[25,26]. We found two novel mutations at amino acid residues of 295 and 297.

In conclusion, mRT-PCR assay could be used for the accurate diagnosis and detection of HPIV. Further, expanded surveillance throughout the country will help in better epidemiological analysis, for implementation of better public health programmes in controlling virus induced respiratory infections.

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