# "ANTIGENIC AND GENETIC CHARACTERIZATION OF INFLUENZA VIRUSES CIRCULATING IN CHENNAI DURING 2002-2007. SCREENING OF INDEGENOUS HERBS FOR ANTI-INFLUENZA VIRAL ACTIVITY AND SENSITIVITY OF THE ISOLATES TO AMANTADINE"

*A thesis submitted to*

*The Tamil Nadu Dr. M.G.R. Medical University*
*for the award of the degree of*

**DOCTOR OF PHILOSOPHY**

*By*

**Mr. A.KHALEEFATHULLAH SHERIFF,** M.Sc.,

**DEPARTMENT OF VIROLOGY**
**KING INSTITUTE OF PREVENTIVE MEDICINE**
**GUINDY, CHENNAI – 600 032**
**INDIA**

**JANUARY 2009**

# ACKNOWLEDGEMENT

The most pleasant part of a completed work is to put on record the debt of gratitude owed to people who made this task possible.

It gives me great pleasure to place on record my deep sense of gratitude and profound respect to my guide and supervisor **Dr. Nalini Ramamurty,** MD, Virologist, IVD, WHO-SEARO, New Delhi, for accepting me as her student and molding me to the standards required for ethical research and rational thinking. I sincerely acknowledge my indebtedness for her meticulous guidance, constant inspiration, constructive criticism, affectionate treatment and ever willingness to help me out of difficulties that has enabled me towards the completion of this study.

I wish to express my sincere gratitude to **Dr. J. Revathi**, MD, Director of King Institute of Preventive Medicine for allowing me to pursue my research in this esteemed institution.

I am highly indebted to **Dr.P. Gunasekaran,** MD, Deputy Director & Head, Department of Virology, an amicable personality, who had been always gracious to me with ideas, clues and constructive criticism which has helped me to finish my work with success.

It is my pleasant duty to thank **Dr.Shantha**, Professor of Microbiology, Stanley Medical College and **Dr.Saradha Suresh,** Director ICH, for being the Doctoral Committee Members and helping me in my preliminary works and add valuable comments.

I sincerely thank **Dr.A.C.Mishra,** Director – NIV, **Dr.D.A.Gadkari,** Consultant – NIV, **Dr.M.S.Chadha**, Deputy Director – NIV, **Dr.Varsha, Dr.Shailesh Pawar**, NIV for providing me prototype viral strains and helping me in sequencing of viral isolates, providing working literature and being kind enough to clear my doubts on practical applications.

I sincerely thank **Dr.Stephen Lindstorm,** Team Leader, Molecular Biology Division, Influenza Branch CDC Atlanta for providing me primer sequences and helping me during the course of molecular biology experiments.

It is also my profound duty to thank **Dr.Alexander Klimov**, for providing me the control antigens and reference antisera and helping me antigenic characterization.

I thank **Dr.Kaveri**, Deputy Director, Arbo Viral Annex, King Institute of Preventive Medicine for helping me concentrate on my thesis work undisturbed by the routine chores.

With immense gratitude, I sincerely thank **Mrs.S.Mohana,** Asst. Professor, King Institute of Preventive Medicine for helping me in herbal studies and for her constructive criticism.

I thank **Dr.Kavita Arunagiri,** Medical Officer, King Institute of Preventive Medicine for helping me during my course of study.

I record my sincere and whole hearted thanks to **Dr.Varalakshmi Elango,** MD, DMV, Clinical Trial Monitor, WHO providing helpful suggestions in presenting the data. Her constructive criticism, encouragement, valuable suggestion and technical questions have made me to learn and correct myself to metamorphose as successful research aspirant.

With deep sense of gratitude, I wish to express my heartfelt and sincere thanks to **Dr. Banumathi Selvam,** MD, Deputy Director, Blood Bank for her unstinted support, warmth and help throughout my course.

My sincere thanks to **Dr.Balathirupurasundari** for her help rendered in moulding my research career.

My sincere thanks to **Mr.D.Raja,** for his encouragement in difficult times of my life and his valuable guidance during the course of study.

I am thankful to **Dr.C.C.Harish,** for training me in Herbal Analysis.

I owe my sincere thanks and faithful regards to **Dr. Jayashri Mahalingam,** Scientist and their long lasting co-operation and help rendered during my course.

My sincere thanks **Mr.L.Mohammad Illiyas**, for his encouragement throughout my study.

I am immensely grateful to **Mrs.P.Padmapriya,** for being with me during the different phases of my work and standing with me at all times.

Acknowledgement will not be complete without me thanking **Mr.S.Gopalsamy,** Tutor, SRMC, who was there with me. Whenever I needed a helping hand and or any suggestions.

My sincere thanks are due to Late **Mr.P.K.Venkatesan, Mr.C.S. Damodharan, Mr.Sigamani, Mrs. V. Chandra, Mrs. N. Mythili, Mr.N.Karnan, Mr.D.Dhanakaran, Mr.V.P.Muthu, Mr. S.Raja** and **Mr.G.Pandurangan** and all the staff members of Department of Virology for extending their support at all times.

My sincere thanks to **Mr.V.Senthil Kumar, Mr.Raunak Kumar Das, Mr.K.Saravana Murali, Mr.B.V.Suresh Babu, Mrs. R.Kiruba** for their help during my study. I thank **Mrs.Deepa Murali** and **Ms.Mogana** helping me in formatting the data.

Thanks are due to **Ms.S.Gracy Fathima** for helping me through the sequence alignment and analysis.

I thank **Mrs. Sasikala** for sculpting me into a disciplined microbiologist and also for her efforts in evoking research interest in me.

Thanks are due to the **Mr. A. Venkatasan**, Librarian and **Mr. K. Selvaraj,** Asst. Librarian KIPM Central Library for permitting unrestricted access to utilize the facility.

The financial support by “Lady Tata Memorial Trust” Mumbai, ICMR, Delhi, CDC, Atlanta in the form of Research Fellowships is greatly acknowledged.

My faithful regards and gratitude to Almighty for granting me this beautiful life as human being and an opportunity to be born in the family I am a part of, whose ardent enthusiasm and moral support has been the pillars for building this thesis and my research career.

# ABBREVIATIONS

| | | |
|---|---|---|
| AMV | - | Avian Myeloblastosis Virus |
| MDCK | - | Madin Darby Canine Kidney |
| bp | - | Base Pair |
| *BSA* | - | *Bovine Serum Albumin* |
| CDC | - | Center for Disease Control |
| cDNA | - | Complimentary De-oxyribo Nucleic acid |
| CPE | - | Cytopathic effect |
| CTE | - | Cytotoxic effect |
| DMSO | - | Dimethyl sulphoxide |
| DNA | - | Deoxyribonucleic Acid |
| DPA | - | Direct Pre-infection Assay |
| EDTA | - | Ethylene Diamine Tetra Aceticacid |
| ELISA | - | Enzyme Linked Immunosorbent assay |
| EtBr | - | Ethidium Bromide |
| FCS | - | Fetal Calf Serum |
| HBSS | - | Hank's Balanced Salt Solution |
| MEM | - | Minimum Essential Medium |
| MMWR | - | Mortality Morbidity Weekly Report |
| MTT | - | Tertrazolium Blue Dye |
| PBS | - | Phosphate buffered saline |
| PCR | - | Polymerase Chain Reaction |
| RNA | - | Ribonucleic Acid |
| RPM | - | Revolution Per Minute |
| RT | - | Reverse Tanscriptase |
| SD | - | Standard Deviation |

| | | |
|---|---|---|
| Taq DNA polymerase | - | Thermus aquaticus DNA polymerase |
| TBE | - | Tris Borate EDTA |
| TCID | - | Tissue Culture Infectious Dose |
| TMB | - | Tri Methyl Benzidine |
| TPVG | - | Trypsin Phosphate Versene Glucose |
| Tris | - | Tri-hydroxymethyl amino methane |
| VIA | - | Virus Inactivation Assay |
| WHO | - | World Health Organization |

# CONTENTS

# 1. INTRODUCTION

Acute Respiratory Diseases (ARD) cause enormous morbidity and mortality, particularly in infants and children in developing countries[1]. ARD is responsible for about 4 million deaths and 500- 900 million episodes per annum in children globally[2-3]. In India, studies indicate that 600,000 children under the age of 5 die every year from ARD[4].

On an average an individual can contract several upper respiratory tract infections each year, most of which are caused by viruses. Many of these infections are uncomplicated and do not require medication, but for people with an immature or compromised immunity, an otherwise benign respiratory infection can develop into serious, life threatening conditions. *Influenza* virus infections are one of the most important causes of acute respiratory illness.

Among Acute respiratory illnesses, *Influenza* is one of the unconquered scourges of mankind as it causes frequent epidemics and periodic pandemics, and hence is a major public health problem. It is estimated that annually around 0.5-1 million people die and 600 – 1200 million become sick due to *Influenza* epidemics worldwide[5].

The history of *Influenza* epidemics and pandemics, can be traced back with some accuracy for the past three hundred years, and with less certainty before this time, it is apparent that outbreaks occur somewhere in the world in most years[6].

*Influenza* is an acute, highly contagious respiratory infection that has the potential to cause very serious illness. Typical clinical symptoms are characterized by a short incubation period, high attack rates, and the progression of the disease through the population[7]. Due to the major antigenic changes occurring with the *Influenza* A virus, epidemics occur every year on a seasonal basis. *Influenza* B is responsible for less frequent outbreaks[8].

Annual epidemics are due to antigenic drift; and pandemics, occurring at 10 to 50 years intervals, are due to new virus subtypes resulting from virus reassortment. Nothing has been introduced during the past 100 years to affect the recurrent pattern of epidemics and pandemics; and our future in the new century is clearly indicated by our past[6].

**Structure and Biology of *Influenza* viruses**

The *Influenza* viruses are classified under the family Orthomyxovridae. *Influenza* A virion is pleomorphic, with laboratory adapted strains assuming a spherical shape with a diameter of 80 -100 nm containing a single stranded segmented negative sense RNA genome with helical symmetry. Freshly isolated viruses are often filamentous. *Influenza* viruses are classified as *Influenza* A, B or C viruses based on the antigenic specificity of the nucleoprotein and matrix or M1 protein[6]. Of the three Immunologic types, Type A is highly variable and shows continuous antigenic variation and is a major cause of pandemics, Type B virus shows antigenic variation to a lesser degree which results in epidemics, whereas type C virus appears to be antigenically stable and causes sporadic upper respiratory tract infections[8].

The surface of the virus contains spikes about 10nm long projecting radially outward over the surface of the viral particles. The two surface proteins are haemagglutinins (HA) and neuraminidase (NA) which determine the antigenic variation of the virus as well as the host immunity. The HA, an epidemiologically important glycoprotein is a trimeric rod- shaped spike in the viral envelope[9]**.**The exposed portion contains the antigenic sites and binding sites for sialic acid residues in receptors of host cells or erythrocytes. The HA facilitates both attachment of the virus to the host cell receptors and penetration of the virus. Each HA monomer is cleaved proteolytically into 2 poplypeptide chains HA1 and HA2 held together by disulfide bonds. Cleavage is essential for infectivity and consequently important to spread of infection and pathogenicity[10-11]. The HA protein stimulates the production of neutralizing antibodies. Neuraminidase is a mushroom shaped tetramer that is anchored in the lipid envelope[12]. It cleaves terminal sialic acid residues from various glycoconjugates and plays an essential role in the release of virus from infected cells[13-14]. This reaction aids in increasing the infectivity and virulence of the virus.

Because of the segmented genome a phenomenon called genetic reassortment may occur when a cell is co infected with two different type A viruses. This leads to emergence of progeny virus with major changes in the surface glycoproteins[15-16].

**Antigenic Variation**

The surface glycoprotein antigens HA & NA show two types of antigenic variations: drift (minor) and shift (major). Studies on the subtyping of *Influenza* virus by immuno diffusion have demonstrated 15 HA and 9 NA subtypes. They occur in different combinations in variety of strains that infect humans, animals (equine, swine seals) and birds (avian) [16-22].

**Antigenic Drift (Minor antigenic variation)**

Gradual changes in the HA and NA antigens within a subgroup (antigenic drift) is the result of a gradual accumulation of point mutations leading to amino acid sequence changes that alter the antigenic sites of the molecule. Sequence alterations were found to be clustered into 4 or 5 regions of the HA molecule, thus providing evidence that amino acid residue changes in discrete regions resulted in antigenic alteration in the HA[23-25].

**Antigenic Shift (Major antigenic variation)**

Complete change in antigenic subtype of one or both surface glycoproteins, (antigenic shift), involves replacement of the gene coding for that subtype with that of another. Such events occur as a result of genetic reassortment between 2 viruses infecting the same host. When the HA gene is replaced and antigenic shift occurs, it is usually associated with worldwide pandemics [26].

**Mode of Transmission**

Transmission of *Influenza* in humans probably involves both respiratory infection by aerosols and droplets together with some contact transmission from contaminated surfaces. Unfortunately, there is insufficient solid data to determine which of these is the more important and to provide guidance in protection of front-line medical staff, particularly regarding the required porosity of facemasks, during a

pandemic. Nevertheless, *Influenza* virus is relatively short-lived on most surfaces[27] and clear examples of aerosol transmission have been described in the literature[28]. In addition, early experiments indicated greater infectivity by small particle aerosol than by nasal instillation of virus [29].

The virus is spread from person to person via small particle aerosols (less than 10μm diameter) through the respiratory tract. It can also survive for a short time on surfaces and can be spread by this route if the virus is introduced into the nasal mucosa before it loses infectivity. The incubation period is short, about 18 to 72 hours.

Virus concentration in nasal and tracheal secretions remains high for 24 to 48 hours after symptoms start and may last longer in children. Titers are usually high and so there are enough infectious virions in a small droplet to start a new infection[30].

**Pathogenesis**

*Influenza* is transmitted through inhalation of virus-containing droplets from infected individuals. The incubation period is dose dependent usually being 2 days. The viruses multiply in the ciliated columnar epithelium of the upper and lower respiratory tract, causing cell necrosis and sloughing. Peak virus shedding occurs from one day before to 3days after onset of illness.

**Clinical findings**

There is considerable variation in the severity of illness in different individuals. This is partly due to the age of those affected, general health, and immunization status relative to previous *Influenza* infections. Infections may be subclinical or may produce symptoms ranging from minor respiratory illness to fatal viral pneumonia. The "classic" symptoms of *Influenza* are rapid onset of malaise, fever abruptly rising to 100-106ºF and myalgia, usually with a non productive cough or sore throat. In children, nausea, vomiting and diarrhea are often observed. A long convalescence with hacking cough, lassitude and malaise is common. Mild and subclinical infections are also common[31].

## Complications

Complications of *Influenza* include primary viral pneumonia caused by the spread of virus to alveolar epithelium, secondary bacterial pneumonia, exacerbation of underlying chronic conditions. In young infants, severe *Influenza* resembles other severe respiratory infections with bronchitis, febrile convulsions and occasionally encephalitis. Other complications include otitis media, myositis, myocarditis, toxic shock syndrome and Reye's syndrome[32].

## Epidemiology

### Disease Impact

*Influenza* epidemics are regularly associated with excess morbidity and mortality. Usually expressed in the form of excess rates of pneumonia and *Influenza* associated hospitalization and death, during epidemics[33-34]. Pneumonia and *Influenza* deaths fluctuate annually in a predictable fashion, with peaks in winter and troughs in summer.

*Influenza* is usually associated with a U shaped epidemic curve. Attack rates are generally highest in the young whereas, mortality is highest in the elderly.

### Epidemic *Influenza*

An epidemic is an outbreak of *Influenza* confined to one location, such as a city, town or country. In a given community, epidemics of *Influenza* virus infection have a characteristic pattern. Epidemics begin abruptly; reach a sharp peak in 2-3 weeks and last 5-6 weeks[35].

### Pandemic *Influenza*

In contrast to the familiar pattern of epidemic *Influenza*, pandemics are severe outbreaks that rapidly progress and is associated with the emergence of a new virus to which the overall population possesses no immunity. Characteristics of pandemics include extremely rapid transmission with concurrent outbreaks worldwide; occurrence of diseases outside the usual seasonality, including the summer months; high attack rates in all age groups, with high levels of mortality particularly in healthy

young adults[36], and multiple waves of disease, immediately before and after the main outbreaks.

Serologic studies suggest that the pandemic of 1889 was associated with viruses of an H2N2 subtype, followed by a pandemic in 1901 due to an H3N8 subtype[37]**.** Spanish pandemic of 1918 was due to an H1N1 virus, which in turn was supplanted in the Asian Pandemic of 1957 with H2N2 viruses. In 1968, viruses of H3N2 subtype caused the Hong Kong pandemic. In 1977 viruses of H1N1 subtype were reintroduced through an unknown mechanism. These viruses are genetically identical to H1N1 viruses that were circulating in 1950. Since 1977, *Influenza* A viruses of both H1N1 and H3N2 are co-circulating.

Global epidemiology of *Influenza*: past & present (2000) was reported by Cox & Subbarao[38]. The tools of molecular virology have been applied to determine the origin of the pandemic viruses. Molecular methods have been used to investigate the avian H5N1 & H9N2 *Influenza* viruses that infected humans in Hong Kong. Starting from 1997 till date avian *Influenza* infections have spread through many countries.

**Human *Influenza***

*Influenza* viruses have shown a characteristic pattern of circulation throughout the world. In the 2002 - 03 season *Influenza* A accounted for the 56.4% of the isolates and *Influenza* B viruses accounted for the 43.6% of the isolates. Among *Influenza* A viruses A/H1 was predominant and isolated at a percentage of 70.3 and *Influenza* A/H3N2 accounted for 29.7 % of the total A viruses. *Influenza* A viruses were reported more frequently (range 57.8%-85.9%) than *Influenza* B viruses in the New England, East North Central, Pacific, Mountain, and Mid-Atlantic regions, and *Influenza* B viruses were reported more frequently (range: 51.1%-78.1%) than *Influenza* A viruses in the West North Central, West South Central, South Atlantic, and East South Central regions. Outbreaks of A/H3N2 were reported from Nepal in 2004[39] and in Japan in 2005 – 06.There have been reports of isolation of H1N1, H1N2 and H3N2 from Korea, H3 & H1 in China. In the United States, *Influenza* A (H1) viruses predominated overall, but *Influenza* A (H3) viruses were isolated more frequently than *Influenza* A (H1) viruses late in the season. Although *Influenza* A

(H1), A (H3), and B viruses co circulated worldwide, *Influenza* A (H3) viruses were the most commonly reported type in Europe and Asia[40]. There has been an outbreak of *Influenza* C virus infection in Japan in 2004 [41] .

During the 2006-07 season, *Influenza* activity peaked in mid-February in the United States and was associated with less mortality and lower rates of pediatric hospitalizations than during the previous three seasons. This report summarizes *Influenza* activity in the United States and worldwide during the 2006-07 *Influenza* season (October 1, 2006-May 19, 2007) and describes the composition of the 2007-08 *Influenza* vaccine[42]**.**

**Avian *Influenza***

Fowl plague was described in 1878 as a serious disease of chickens in Italy. It was determined in 1955 that fowl plague (FP) virus is actually one of the *Influenza* viruses and all known subtypes of *Influenza* A viruses infect birds. Avian *Influenza* A H5 and H7 viruses can be distinguished as "low pathogenic" and "high pathogenic" forms on the basis of genetic features of the virus and the severity of the illness they cause in poultry whereas *Influenza* H9 virus has been identified only in a "low pathogenicity" form.

Recent outbreaks of avian flu in have evoked considerable attention as there have been reports of transmission of these viruses to humans. From 1996 till date there have been numerous outbreaks of avian *Influenza* in birds (in more than 50 countries) and few instances of human infections (in13 countries).

The occurrence of human *Influenza* A (H5N1) has paralleled large outbreaks of avian *Influenza* A (H5N1)[43]. Bird flu virus H5N1 was isolated for the first time from a human patient in Hong Kong. The virus infected 18 patients after close contact with infected poultry, with six deaths. Fortunately the virus did not not spread from person to person. Within three days, Hong Kong's entire chicken population was slaughtered to prevent further outbreaks[44]. Human infections with avian *Influenza* A(H5N1) and A(H7N7) viruses were reported in Hong Kong and the Netherlands, respectively. Till date there have been reports of 391 human cases of avian *Influenza* of which 247 have been fatal.

***Influenza* viral activity In India**:

*Influenza* surveillance activity in India is extremely limited. National Institute of Virology, Pune which is a WHO collaborating centre has reported the isolation of 376 *Influenza* virus isolates comprising of 40 different antigenic variants of A/H3N2, A/H1N1 and B type viruses during the investigations of several outbreaks during 1976-2000. These strains circulated singly or in different combinations and the outbreaks occurred predominantly in the rainy seasons[45-54]. A study on the prevalence of *Influenza* viruses among the pediatric age group reported the circulation of *Influenza* A/H1N1 and B/Sichuan viruses and also that rainfall aids the transmission of flu[55]. *Influenza* laboratory network established by ICMR in 2004 has established that A/H1N1 and A/H3N2 are found to co circulate in the country with occasional isolation of *Influenza* B type viruses.

**Prevention**

**Vaccines**

A new vaccine is formulated annually with the types and strains of *Influenza* predicted to be the major problems for that year (predictions are based on worldwide monitoring of *Influenza*). The vaccine is multivalent consisting of the prevalent circulating strains of *Influenza* A (H1N1) virus, *Influenza* A (H3N2) virus and *Influenza* B virus. The vaccine is administered as a two dose schedule of 0.25 ml for children between 6mths to 3 years at an interval of atleast one month followed by one 0.25ml dose every year thereafter. For children aged 6 months–8 years who are receiving vaccine for the first time and those who only received 1 dose in their first year of vaccination should receive 2 doses in the following year. For children more than 3 years and adults one dose of 0.5ml of vaccine once a year is recommended.

The vaccine is an inactivated preparation of egg-grown virus. It is contraindicated for those with allergies to eggs. It has a short lived protective effect and so is usually given in the beginning of the flu season or winter so that protection is high during the peak months for flu. It needs to be given every year, because of the short lived nature of the protection and the most effective strains for the vaccine will change due to drift or shift[56].

## Chemotherapy

Although annual vaccination is the primary strategy for preventing complications of *Influenza* virus infections, antiviral medications with activity against *Influenza* viruses can be effective for the chemoprophylaxis and treatment of *Influenza*. Four licensed *Influenza* antiviral agents are available namely amantadine, rimantadine, zanamivir, and oseltamivir.

*Influenza* A virus resistance to amantadine and rimantadine can emerge rapidly during treatment[56]. On the basis of antiviral testing results conducted at CDC and in Canada indicating high levels of resistance, CDC and ACIP recommend that neither amantadine nor rimantadine be used for the treatment or chemoprophylaxis of *Influenza* A in the United States until susceptibility to these antiviral medications has been re-established among circulating *Influenza* A viruses[56]. Zanamivir [Relenza] and Oseltamivir [Tamiflu] can be prescribed if antiviral treatment of *Influenza* is indicated. Oseltamivir is approved for treatment of persons aged $\geq$1 year, and zanamivir is approved for treatment of persons aged $\geq$7 years.

There have been reports of self injury and delirium with the use of Tamiflu in pediatric patients (mostly from Japan). Though the relative contribution of the drug to these events is not known people with the flu, particularly children, may be at an increased risk of self-injury and confusion shortly after taking Tamiflu and should be closely monitored for signs of unusual behavior[56].

## Laboratory Diagnosis

Appropriate treatment of patients with respiratory illness depends on accurate and timely diagnosis. Early diagnosis of *Influenza* can reduce the inappropriate use of antibiotics and provide the option of using antiviral therapy. However, because certain bacterial infections can produce symptoms similar to *Influenza*, bacterial infections should be considered and appropriately treated, if suspected. In addition, bacterial infections can occur as a complication of *Influenza*[56]. Owing to its non-specific, clinical presentation, lack of definitive rapid diagnosis, *Influenza* virus infections are most often not detected early and mistaken for bacterial etiology.

Diagnostic tests available for detection of *Influenza* include viral culture, antigen detection and serological confirmation. The commonly employed diagnostic procedures are virus isolation and identification by cell culture, antigen detection by rapid strip tests, serology by Haemagglutination Inhibition and microneutralization tests and molecular diagnosis by RT PCR and Realtime RT PCR. Sensitivity and specificity of any test for *Influenza* might vary by the timing of specimen collection and the type of specimen tested. Among respiratory specimens for viral isolation or rapid detection, nasopharyngeal specimens are typically more effective than throat swab specimens. As with any diagnostic test, results should be evaluated in the context of other clinical and epidemiologic information available to health-care providers[57].

As it is well known that *Influenza* epidemics are caused by rapid evolution of the viral genome by antigenic shift or drift, monitoring antigenic and genetic variations of circulating *Influenza* viruses is critical for the selection of annual vaccine strains.

The major drawback in diagnosis of *Influenza* virus infections is non-availability of an universal cost effective rapid diagnostic test. The conventional virus isolation method though gold standard is tedious, requires tissue culture facility and takes a long time till definitive diagnosis is made. The currently available rapid antigen detection tests are not very sensitive[58]. Molecular techniques like PCR are being done but commercial PCR kits for specific identification of human *Influenza* virus are not available.

Of late *Influenza* viruses are showing resistance to amantadine a commonly used antiviral drug to combat these infections. Further the drug is expensive, and is in short supply. Therefore identification of a cost effective antiviral drug of synthetic or herbal origin is the need of the hour.

India has a rich heritage of using medicinal plants in Ayurveda, Siddha and other forms of traditional systems of medicine **(Gunapadam, 1998)**. The earliest mentions of the medicinal uses of plants are found in RIG VEDA, which is one of the oldest repositories of the human knowledge **(Chopra *et al*., 1958)**. Charaka Samhita

and Shusrutha Samhita are some of the important Indian classical works on the use of herbs in treating diseases. Literature evidence is available for Thulasi *(Ocimum sanctum)* to have anti- *Influenza* activity[59]**.**

With all the above lacunae in prevalence of *Influenza* activity in this part of the country, lack of rapid diagnostic tests and lack of effective, antiviral therapy as the background, the following were undertaken in the current study.

- ✓ Elucidation of seroprevalence of *Influenza* antibodies in all age groups of Chennai and emerging pattern of *Influenza* activity in Chennai.
- ✓ Antigenic Characterization of *Influenza* virus Isolates and the study of evolutionary pattern studied over the years 2002 to 2007.
- ✓ Characterization of viral isolates by HA gene sequencing.
- ✓ Standardization of Multiplex RT-PCR for rapid detection and identification of *Influenza* serotypes.
- ✓ Study of drug sensitivity pattern of the viruses for selection of appropriate antiviral agent.
- ✓ Study on the Antiviral activity of selected herbs throwing light on development of an alternate agent for treatment.

# 2. REVIEW OF LITERATURE

*Influenza* viruses (family *orthomyxoviridae*) cause highly contagious respiratory disease with potentially fatal outcomes. Symptoms include fever, headache, cough, sore throat, nasal congestion, sneezing, and body aches. *Influenza* viruses also cause local epidemics or worldwide pandemics with significant infection rates. Although the economic burden of *Influenza* is most prominent during pandemics, the combined annual costs of seasonal epidemics due to sick days, emergency room visits, and medications are significant. With the realization that avian *Influenza* viruses can be directly transmitted to humans, *Influenza* viruses are now considered a major, global health threat.

## NOMENCLATURE

*Influenza* viruses belong to the *orthomyxoviridae* family. Three types of *Influenza* viruses exist namely *Influenzavirus A*; *Influenzavirus B and Influenzavirus* C. *Influenza* A viruses are further classified into subtypes based on the antigenicity of their HA and NA molecules; currently, 16 HA subtypes (H1-H16) and 9 NA subtypes (N1-N9) are known. The present nomenclature system includes the type of virus, the host of origin (except for humans), the geographic site of isolation, the strain number, and the year of isolation, followed by the antigenic description of the HA and NA subtypes in parenthesis. For example, A/swine/lowa/15/30 (H 1 N 1) describes an *Influenza* A virus isolated from a pig in Iowa in 1930 with a strain number of 15 and an H1N1 subtype. Antigenic subtypes have not been identified for *Influenza* B and C viruses. Thogoto virus, Dhori virus, and ISAV do not cross-react antigenically.

## SEROARCHEOLOGY

Early studies suggested that the pandemic of 1889 to 1891 was caused by a virus of the H2N2 subtype, whereas that of 1900 had been attributed to an H3N8 strain[60]. More recent re-evaluation of the data indicates that the 1889 to 1891 pandemic was caused by an H3-like virus, and there is no compelling evidence that links the H2 subtype to a pandemic other than that of 1957[61]. The latter conclusion is

substantiated by the lack of protection among those who were at least 80 years old during the 1957 H2 pandemic. Seroarcheology has also linked the 1918/1919 pandemic to an H1 virus, a finding that has been confirmed by sequence determination of *Influenza* virus RNA from the lung tissues of victims[62-68]. Studies using antibodies to the NA protein suggest that in the late 1800s, viruses of the N8 subtype were circulating and were later replaced by the N1 and N2 subtypes. Thus, during the 1900s, only a limited number of virus subtypes (H1N1, H2N2, H3N2, H3N8, and recently also reassortant H1N2 viruses) have become established in humans.

## VIRUS ISOLATION

In 1930, Shope isolated the *Influenza* virus, A/swine/ Iowa/30, but the first human *Influenza* virus was isolated in 1933 by Wilson Smith, Sir Christopher Andrewes, and Sir Patrick Laidlaw of the National Institute for Medical Research in London, England. These investigators inoculated ferrets intranasally with human nasopharyngeal washes from an *Influenza* patient. The animals exhibited *Influenza* like disease, and the virus was transmitted to cage mates. One of their junior colleagues (later Sir Charles Stuart-Harris) became infected by these experimentally infected animals, and the virus was subsequently isolated from him. Because it was the first human *Influenza* virus, it was termed "*Influenza* A" virus. In 1940, an antigenically distinct virus was isolated and termed type B virus (B/Lee/40). The first *Influenza* C virus was isolated in 1947. "Fowl plague" was first described in 1878 as a disease that affected chickens in Italy. The causative agent was isolated in 1902 (A/chicken/Brescia/1902 [H7N7]); however, it was not until 1955 that Schafer recognized fowl plague virus as an *Influenza* virus.

## VIRUS PROPAGATION

*Influenza* viruses were first propagated in embryonated hens' eggs, which continue to be the system of choice for growth of large quantities of virus for vaccine production. Avian and equine strains of *Influenza* A viruses can be isolated from the allantoic cavity of 10-to-11-day-old embryonated eggs after 2 to 3 days of incubation at 33°C to 37°C. Human *Influenza* viruses have also been isolated from clinical

samples on inoculation into the allantoic or amniotic cavity of eggs and incubation at 33°C to 34°C. However, recent human viruses have proven difficult to be isolated with embryonated eggs. *Influenza* virus growth in embryonated eggs leads to the selection of antigenic variants that are characterized by mutations in the HA protein[69]. *Influenza* C viruses amplify in the amniotic, but not allantoic, cavity of eggs and are usually grown for 5 days in 7 to 8 day old embryonated eggs.

*Influenza* viruses can also be propagated in cell culture. Madin-Darby Canine Kidney (MDCK) cells support the efficient replication of many *Influenza* A and B viruses and can be used to isolate viruses from humans. Although many *Influenza* viruses can grow in African green monkey kidney (Vero) cells, they do so less efficiently than in MDCK cells. Cell culture systems based on MDCK [70] and Vero [71] cells are now being developed for *Influenza* virus vaccine production. *Influenza* viruses also replicate in a number of primary cell cultures, including monkey, calf, hamster, and chicken kidney cells, as well as in chicken embryo fibroblasts and primary human epithelial cells. With the exception of primary kidney cells, most other cell culture systems require the addition of trypsin to cleave the HA protein of human viruses (except highly pathogenic H5N1 viruses), a prerequisite for efficient replication.

Replication of *Influenza* viruses in eggs or cell culture is measured by testing the viruses ability to agglutinate erythrocytes haemagglutination (HA) or by use of molecular biology techniques, such as reverse transcriptase (RT)-polymerase chain reaction (PCR).

## EVOLUTION OF INFLUENZA VIRUSES

Phylogenetic analyses, together with the finding that viruses of all known HA and NA subtypes are maintained in avian species, led to the hypothesis that all mammalian *Influenza* A viruses are derived from the avian *Influenza* virus pool [72]. At both the nucleotide and amino acid levels, the evolutionary rates are significantly lower for avian *Influenza* viruses. In fact, in wild aquatic birds, *Influenza* viruses seem to be in an evolutionary stasis, suggesting optimal adaptation of these viruses to their hosts. In this situation, amino acid substitutions may not provide selective

advantages. Thus, although mutations occur with similar frequencies, they do not result in amino acid changes. In contrast, all gene segments of mammalian and land-based poultry viruses continue to accumulate amino acid substitutions.

For human *Influenza* A viruses, the evolutionary rates differ among the segments, probably reflecting differences in the selected pressure of the host. The human H3 HA genes are evolving much faster than the PB2, PB1, PA, NP, and M1 genes. In addition, the ratio of silent:total changes differs significantly among the different genes. For the human H3 HA gene, only 57% of all changes are silent, whereas for the human PB2 gene more than 90% are silent. Variants with mutations in HA may be selected because they confer an advantage by allowing the virus to escape the host immune response. Conversely, for the PB2 protein, only a limited level of immune pressure is imposed on this protein and, in addition, the structural constraints may be higher in this protein than in HA. The human M1 and M2 genes, encoded by an overlapping reading frame of the same segment, are under different selective pressures: For the M1 protein, 96% of changes are silent, in contrast to only 66% for the M2 protein[72]. The M1 protein is therefore well adapted to its mammalian hosts, whereas the M2 protein is under stronger selective pressure. The biological reason for the selective pressure on the M2 protein is unknown. The two proteins encoded by the NS gene also differ in their evolutionary rates, with NS1 showing more variation between alleles than NS2.

Extensive phylogenetic analyses have revealed host specific virus lineages for several viral proteins. The phylogenetic studies of the NP genes identified seven hostspecific lineages (a) human viruses, (b) classic swine viruses, (c) old H7N7 equine viruses (A/equine/ Prague/56), (d) recent equine viruses, (e) H13 gull viruses, (f) North American avian viruses, and (g) Eurasian avian viruses. Similar host specific virus lineages have been identified for other genes, but not for the HA and NA genes[72]. The phylogenetic tree of the PB1 gene differs from those of other *Influenza* virus genes. The PB1 genes of human H1N1 viruses cluster with classic swine viruses, whereas the PB1 genes of human H2N2 and H3N2 viruses form a different sublineage that reflects the introduction in 1957 and 1968 of avian PB1 genes[73]. The M gene[74]. differs significantly from the NP and PA genes in that the recent equine genes appear to have been derived very recently from North American

avian viruses, in contrast to the much older origin of recent equine NP and PA genes. The phylogenetic tree of the NS gene is divided into two alleles, A and B[75]. The "B allele" contains avian NS genes, and all mammalian NS genes belong to the "A allele."

The H3 HA gene has evolved in a single lineage since its introduction into the human population in 1968, with a mutation rate of 4 x $10^{-3}$ substitution per nucleotide per year and 5 x $10^{-3}$ amino acid changes per residue per year in HA1 [72.] Because most of these changes localize to the antigenic sites on the surface of the protein, the H3N2 virus component of *Influenza* virus vaccines needs to be updated frequently. For human and classic swine viruses, phylogenetic analyses indicate common ancestry. For avian viruses, two sublineages-an American and a Eurasian-can be defined for all eight segments[76], suggesting an important role for host species in the evolution of *Influenza* virus gene pools.

Significant differences in evolutionary rates exist for *Influenza* A, B, and C viruses. Type B viruses, but especially type C viruses, evolve more slowly than *Influenza* A viruses. Type B and C viruses seem to be near or at an evolutionary equilibrium in humans; genes of type A viruses were introduced into the human population less than 150 years ago, most likely from birds[72], and have not reached equilibrium. *Influenza* A viruses in humans evolve along single-branch a lineage, which suggests evolution by clonal selection[77] and limited cocirculation of sublineages. Cocirculation of sublineages has, however, been shown over limited periods. In contrast, the evolution of *Influenza* B and C viruses is characterized by the cocirculation of antigenically and genetically distinct lineage for extended periods of time. For *Influenza* B viruses, two lineages, the "B/Victoria lineage" (represented by B/Victoria/2/87) and the "B/Yamagata lineage: (represented by B/Yamagata/16/88) have been cocirculating for about 25 years with changing patterns of prevalence and geographic distribution.

## INFLUENZA VIRUS GENETICS

### Reassortment

Reassortment is the rearrangement of viral gene segments in cells infected with two different *Influenza* viruses. Reassortment can theoretically result in 256 different gene variations (i.e., the two parental genotypes and 254 new gene combinations). Reassortment occurs for *Influenza* A, B, and C viruses, but has not been observed among the different types of *Influenza* viruses.

The importance of reassortment to the generation of new *Influenza* virus strains is highlighted by the pandemics of 1957 and 1968, which were caused by reassortant viruses that contained HA, PB1, and NA or HA and PB 1 segments of avian virus origin in a human genetic background[73,78.] Moreover, reassortant H1N2 viruses emerged in humans in 2001 and have since become established in human populations. Further proof of the significance of reassortment comes from the emergence of a triple human/avian/swine reassortant that has spread throughout the North American pig population[79-81.] In addition, the H5NI viruses currently circulating in Asia, causing fatal infection in humans and having a profound impact on the poultry industry, arose from multiple reassortment events among avian *Influenza* viruses[82.]

### Recombination

Recombination has been detected in *Influenza* virus segments that contain genetic material from more than one origin. Two avian *Influenza* viruses of low pathogenicity have converted to high pathogenicity following the insertion of 21 nucleotides of the M segment[83-85] or 30 nucleotides of the NP segment[86] into the HA segment. The increased identification of recombinant viruses in recent years may indicate that recombination occurs more often than previously believed. In most cases, the event may be masked by the low biological fitness of the resulting viruses, whereas under selective pressure, recombination may provide a select advantage.

### Dominance

The cold adapted *(ca),* live attenuated A/Ann Arbor/6/60 strain 'interferes with the growth of other strains in co infection experiments, both *in vitro* and *in vivo.* This is

referred to as dominance[87], and this dominant phenotype seem to be linked to the M segment. Further coinfection experiments with this virus showed it to inhibit vRNA replication of the wild-type viruses. The molecular basis of this effect is not yet understood.

## INFLUENZA IN HUMANS-PAST PANDEMICS AND THE H5N1 EPIDEMIC

Pandemics are outbreaks that impact large geographic areas-typically on more than one continent-and large portions of the population in a short period of time. Pandemic outbreaks are the most dramatic manifestation of *Influenza*, attacking 20% to 40% of the world population and causing significant mortality. *Influenza* pandemics have occurred with 10- to 40-year intervals, although reliable records only date back to the 1918/1919 pandemic. The cumulative effects of epidemics in interpandemic periods, although is less dramatic, parallel those of pandemics.

### The Pandemic of 1918/1919-"Spanish *Influenza*" (H1N1)

The pandemic of 1918/1919 remains unprecedented in its severity. It killed more people than the World War I and reduced life expectancy in the United States by 10 years. AIDS has killed 25 million people in its first 25 years-the "Spanish *Influenza*" killed an equal number in 25 weeks (from September 1918 to March 1919). This pandemic occurred in three waves. In the spring of 1918, a mild respiratory disease started from Fort Funston, Kansas (now Fort Riley), and was attributed to a soldier that had been cleaning pig pens[76]. There is no mention of the presence of poultry in the camp at that time. The disease spread among soldiers from Fort Funston along the rail lines to other military bases and cities in the United States and by troopships to Europe. This first wave was highly contagious but caused few deaths and received limited attention in most parts of the world. In Spain, a neutral country without news censorship, the outbreak was covered extensively by news media and was soon referred to as the "Spanish *Influenza*." In late August, a virulent form emerged, probably in Western France that spread around the world, peaking between September and November. During that time, death tolls reached more than 10,000 people per week in some U.S. cities. About one-third of the U.S. population became sick, and the mortality rate was estimated to be over 2.5%, compared to less

than 0.1 % in typical *Influenza* outbreaks. In North America and Europe, mortality rates were in the order of 3 to 10 deaths per 1,000. Although these figures reflect the impact of the pandemic on the developed world, death rates are believed to have been significantly higher in African and Asian countries with more than 50 deaths per 1,000. In some isolated populations, the mortality rate reached 70%, likely because of the lack of previous exposure to *Influenza* virus. A third wave of similar impact to the second wave struck in the spring of 1919.

Typically, the onset of symptoms was sudden, with high fever, severe headache and myalgia, cough, pharyngitis, and coryza. Pathological findings were mostly restricted to the respiratory tract, and death was due to pneumonia and respiratory failure. There was no evidence of systemic viral infection. Most patients died of secondary bacterial pneumonia but some showed massive acute pulmonary hemorrhage or pulmonary edema, indicating the extreme virulence of the virus. The high rate of bacterial infections reflects the unavailability of antibiotics in 1918 and 1919.

The age-specific morbidity was similar to other pandemics, with children younger than 15 experiencing the highest infection rates. The mortality pattern, however, differed significantly from that of other *Influenza* virus outbreaks. In typical *Influenza* outbreaks, the highest death rates are observed in very young children and in the elderly. In 1918 and 1919, many deaths occurred among young adults. The death rate for the 15 to 35-year-old age group was 20 times higher in 1918 than in previous years and persons younger than 65 years accounted for more than 99% of excess deaths.

**The Pandemic of 1957- “Asian Influenza (H2N2)**

This pandemic originated in the Southern Chinese province of Guizhou in February 1957 and spread to Human Province and to Singapore and Hong Kong in March and April respectively. In May 1957, the causative agent of the outbreak, *Influenza* A virus of the H2N2 subtype, was isolated in Japan. A first wave struck the United States and the United Kingdom in October 1957, and was followed by a second wave in January 1958. The infection rate was highest in 5 to 19 year olds,

where it exceeded 50%. Both waves were characterized by heightened mortality with about 70,000 deaths in the United States and more than 1 million deaths worldwide.

Genetic and biochemical analysis indicated that the 1957 pandemic virus originated from reassortment between human and avian viruses. It contained H2 HA and N2 NA genes of avian virus origin[78]. Because the pandemic virus did not appear to be extraordinarily pathogenic, the increased mortality is attributed to the lack of pre-existing immunity among humans to the new surface glycoproteins of this virus. In addition to avian virus HA and NA genes the 1957 pandemic virus also possessed a PB 1 gene of avian virus origin[73]. The contribution of this gene segment to viral pathogenicity is unknown.

**The Pandemic of 1968-"Hong Kong *Influenza*" (H3N2)**

Eleven years after their emergence, viruses of the H2N2 subtype were completely replaced by those of the H3N2 subtype. The first signs of a new pandemic were noticed in Southern Asia in the summer of 1968, and a virus of the H3N2 subtype was isolated in Hong Kong in July 1968. This virus spread around the world during the following winter and the winter of 1969 to 1970. The attack rates reached 40% and were highest in the 10 to 14 year old age group. The excess mortality was estimated to be 33,800 in the United States.

The 1968 pandemic virus contained an avian virus HA protein of the H3 subtype that shared less than 30% sequence homology with its predecessor. However, preexisting antibodies to the N2 protein in human populations likely accounted for the moderate severity of the outbreak.

In addition to an avian H3 gene[78] the 1968 pandemic strain also acquired an avian virus PB1 gene[73] as did the 1957 pandemic strain. It is not clear whether the introduction of an avian. PB 1 gene into the human population contributed to the pathogenicity of the 1968 pandemic virus. The HA and PB 1 genes originated from viruses of the Eurasian avian lineage, in agreement with epidemiologic findings that Southern China was the likely origin of the pandemic.

## The Re-emergence of H1 N1 Viruses in 1977-"Russian *Influenza*"

The first signs of a new *Influenza* virus outbreak were noted in Tianjin, China, in May 1977. From November 1977 through mid-January 1978, young adults suffered from an *Influenza* virus outbreak in the Union of Soviet Socialist Republics and in China. The United States experienced a similar outbreak starting in mid-January 1978, and outbreaks in other countries occurred during the following winter. Among school-age children, the attack rates were more than 50%. Morbidity was almost exclusively limited to persons younger- than 25 years, suggesting that older individuals were protected by pre-existing immunity. This assumption was proven when the causative agent was identified as *Influenza* H1N1 virus (A/USSR/77) closely related to strains that had circulated in the early 1950s. This close relationship and the lack of mutations that are typically acquired during replication argue against maintenance of the virus in a nonhuman species. It is now believed that accidental release of this virus started the pandemic. In contrast 1968 when the newly emerging H3N2 viruses replaced the circulating H2N2 viruses, replacement ofH3N2 viruses did not occur in 1977 with the re-emergence of HI1 viruses; instead, both H1N1 and H3N2 viruses continue to circulate to this day. The long-lived protective immune response to *Influenza* in the elderly due to previous infection forms the basis for the use of live attenuated *Influenza* vaccines.

## THE H5N1 OUTBREAK ACROSS ASIA

Although not yet a pandemic, the outbreak ofH5N1 viruses across the world deserves discussion here because of its socioeconomic implications. The outbreak started in May 1997 in Hong Kong with the infection of a 3-year-old boy, who succumbed to the infection[88-89]. The causative agent was an H5N1 virus of entirely avian origin. This incident marked the first reported transmission of a wholly avian *Influenza* virus to a human with fatal outcome. In November and December 1997, 17 additional cases were reported, 5 of which had fatal outcomes. No conclusive evidence of human-to-human transmissions exists. These human cases accompanied an outbreak of *Influenza* in live bird markets in Hong Kong. In February 2003, two Hong Kong residents were infected with H5N1 virus. A 9-year-old boy became sick when his family traveled to Fujian Province in mainland China and was admitted to a

hospital in Hong Kong. He recovered from an H5N1 virus infection, but his 33-year-old father succumbed to the disease. The 8-year-old daughter died in a hospital in China during the families travel the cause of her death was not determined.

A new outbreak of H5N1 virus started in July 2003 in poultry in Vietnam, Indonesia, and Thailand, It has since spread to Cambodia, Laos, South Korea Japan, China, Malaysia, Mongolia, and Myanmar, and has led to the depopulation or death of more than 100 million poultry. During the past year, the virus has spread to Azerbaijan, Russia, Ukraine, Southeast Europe (including Turkey, Greece, and Romania, among others), Central Europe (including Italy, Austria, Switzerland, and Germany, among others), the Middle East (including Egypt, Israel Iran, Iraq, and Jordan), and Africa (including Nigeria, Niger, and Cameroon). In addition to far reaching economic consequences, direct avian-to-human transmission of highly pathogenic avian viruses has caused widespread public apprehension. Until the end of 2004 the mortality rate was as high as 70%, but it has since fall to about 50%. By April 3, 2006, the following case numbers had been reported: 7 cases with 5 fatalities in Azerbaijan, 5 fatal cases in Cambodia, 16 cases with11 fatalities in China, 4 cases with 2 fatalities in Egypt, 29 cases with 22 fatalities in Indonesia, 2 fatal cases in Iraq, 22 cases with 14 fatalities in Thailand, 12 cases with 4 fatalities in Turkey, and 93 cases with 42 fatalities in Vietnam. Although the viruses have not yet acquired the ability to spread efficiently among humans, isolated cases of human-to-human transmission may have occurred[90-91]. In addition to epidemiologic changes, H5Nl viruses are becoming more diverse genetically and antigenically, falling into two clades[92]. Avian H5N1 viruses isolated from the Indochina peninsula (Cambodia, Laos, Malaysia, Thailand, and Vietnam) tightly cluster within clade 1, whereas viruses isolated in china, Indonesia, Japan, and South Korea fall into clade 2, which is characterized by greater genetic divergence. All H5N1 viruses isolated in Russia, Southeast and Central Europe, the Middle East, and Africa belong to clade 2 and closely resemble the isolates that caused mortality among wild birds at Qinghai Lake in China.

During the 1997 H5N1 outbreak in Hong Kong, there was no evidence of systemic viral infection in infected individuals. More recent H5N1 viruses, however,

appear to cause systemic infection in humans, with virus being recovered from not only respiratory organs, but also from stool and cerebrospinal fluid[93]. Some of the currently circulation H5N1 viruses are resistant to the NA inhibitors oseltamivir and zanamivir. However, some patients treated with oseltamivir were infected with H5N1 viruses that were resistant to this drug[94-95]**.** These viruses contain an amino acid substitution at position 274 of the NA protein, which is known to confer resistance to oseltamivir[96]. It is of major concern that two patients treated with oseltamivir subsequently shed drug-resistant viruses and eventually died.

## EPIDEMIOLOGY

### INFLUENZA IN HUMANS

Since 1977, two *Influenza* A virus subtypes have been circulating together with *Influenza* B viruses. The prevalence of these three-groups of viruses varies geographically and temporally, making *Influenza* virus epidemiology more complex. The epidemiology of *Influenza* viruses is defined by the constant antigenic variation to escape the host immune response. In contrast to most other respiratory viruses, *Influenza* viruses posses two different mechanisms that allow them to reinfect humans and cause disease antigenic drift and antigenic shift.

### Antigenic Drift

Antigenic drift occurs as a result of point mutations in *Influenza* A and B viruses and refers to minor, gradual antigenic changes in the HA or NA proteins. In mammals, *Influenza* A virus drift variants result from the positive selection of spontaneous mutants by neutralizing antibodies. Antibodies to the "parental" strains can then no longer neutralize these variants. Antigenic drift has also been observed among *Influenza* viruses in land-based poultry, although to a lesser extent than in humans. Mutations in the human virus HA or NA amino acid sequence occur at a frequency of less than 1% per year. Nevertheless, antigenic drift variants can cause epidemics and typically prevail for 2 to 5 years before being replaced by a different variant.

**Antigenic Shift**

Antigenic shift involves major antigenic changes in which a new HA or NA subtype is introduced into the human population. The newly introduced proteins are immunologically distinct from the previously circulating strains and result in high infection rates in the immunologically naive population, leading to pandemics.

During the last century, four antigenic shifts occurred: in 1918, with the appearance of H1N1 viruses that caused the "Spanish *Influenza*" in 1957, when the H1N1 subtype was replaced with H2N2 viruses, causing the "Asian *Influenza*"; in 1968, when H3N2 viruses replaced the H2N2 subtype, leading to the "Hong Kong. *Influenza*"; and in 1977, when the H1N1 subtype reappeared ("Russian *Influenza*"). These new subtypes occurred suddenly and at irregular and unpredictable intervals.

An antigenic shift is caused by reassortment, typically between human and avian viruses. There is ample evidence for in vivo reassortment among human or avian strains, as well as between human and avian strains[72]. The 1957 and 1968 pandemics both resulted from reassortant between human and avian viruses. A second mechanism for antigenic shift is the direct transmission of avian or swine *Influenza* viruses to humans and their establishment in the human population. Although not conclusive, phylogenetic evidence suggests that the "Spanish *Influenza*" was caused by the introduction of an avian virus into the human population[62-68].

**MOLECULAR EPIDEMIOLOGY**

The study of the *Influenza* epidemiology has been augmented by the application of several molecular methods including Polyacrylamide Gel Electrophoresis of virion RNAs[97], protein comparison by electrophoretic and chromatographic procedures[98-99],nucleic acid hybridization[100-103], oligonucleotide mapping , nucleic acid and protein sequencing[104-108] and antigenic analysis using monoclonal antibodies[109].

The antigenic determinants of HA are located in the 39 portion (HA1) of the genomic RNA, which is approximately 1 kb in length. Vaccines derived from inactivated *Influenza* viruses have been used for prevention and control of *Influenza*

infection. However, the envelope proteins are continuously changing through the processes of shift and drift, giving rise to antigenic variants[110]. Consequently, vaccines effective in previous seasons eventually may not be protective for future epidemics, depending on the degree of divergence of the envelope proteins, especially the HA antigen. Faced with this challenge, a global surveillance effort was implemented[111-112] that monitors the antigenic changes of *Influenza* virus isolates worldwide through comparison with predominant strains from previous seasons by using the hemagglutination inhibition (HI) assay[113]. Isolates showing high degrees of hemagglutinin variation in terms of HI titers and exhibiting a tendency to spread are selected as candidates for vaccine components. Although it remains the standard assay, the HI test for HA variants is time-consuming, especially if many reference antisera are included in the test, and the assay for NA is even more tedious. Recently, *Influenza* laboratories across the world began to use PCR to amplify the HA and NA genes and to identify changes at the genetic level, usually by a sequencing of the amplicons[114- 122]. The WHO Collaborating centers for reference and research on *Influenza* have been active in the application of molecular techniques to WHO *Influenza* surveillance programme.

## TRANSMISSION OF AVIAN INFLUENZA VIRUSES TO HUMANS

Prior to 1997, the transmission of avian *Influenza* viruses to humans was not considered to be a serious human health threat. This assumption was based on the findings at avian viruses do not replicate efficiently in experimentally infected humans[123] and that no fatal cases of human infections had been reported during any outbreaks of highly pathogenic avian *Influenza* (HPAI). The differences in receptor-binding specificities between human and avian viruses were believed to provide a host range barrier that made the transmission of avian viruses to humans highly unlikely. The threat was not fully realized until 1997, when avian H5N1 viruses were transmitted to humans in Hong Kong and killed 6 of 18 patients[124,96]. The observed mortality rate of 33% is atypical for *Influenza* virus infections and has caused worldwide concern of a looming pandemic.

Most cases were mild and self-limiting, although a veterinarian developed fatal pneumonia and acute respiratory distress. In three cases, human-to-human transmission was documented and antibodies were detected in 59% of those in contact with infected poultry workers. In 2004, two people developed conjunctivitis and mild respiratory symptoms after an outbreak of an HPAI H7N3 virus in poultry in Canada[125]. These findings underscore the potential of H7 and H9 virus infection to humans, although human-to-human transmission has thus far been limited.

The recent surge in the number of human infections likely represents increased contacts of humans with infected poultry. The increase in poultry infected with highly pathogenic H5N1 avian *Influenza* viruses may result from both virus distribution by infected migratory birds and the movement of infected poultry.

## TRANSMISSION AMONG HUMANS

*Influenza* viruses do not cause persistent or latent infections; they are maintained in human populations by direct person-to-person spread during acute infections. In the northern hemisphere, epidemics generally peak between January and April, but may flare up as early as December or as late as May. In the southern hemisphere, outbreaks occur between May and September. The low relative indoor humidity during the winter months is believed to prolong the survival of *Influenza* in aerosols and is believed to be responsible for the seasonal pattern in the northern hemisphere. Seasonality in tropical and subtropical climates is believed to coincide with the onset of the rainy season. On a global scale, *Influenza* virus activity is detectable throughout the year, and viruses can be isolated in large cities year round.

The incubation period is about 3 days for *Influenza* A viruses and 4 days for *Influenza* B viruses. The most effective means of spread among humans are aerosols. Most aerosol droplets formed during sneezing or coughing are less than 2 μm in diameter and are preferentially deposited in the lower airways of the lung. Volunteers are readily infected by aerosol transmission[126] the human infectious dose of *Influenza*-A virus infection is 0.6 to 3 $TCID_{50}$ (dose required to infect 50% of

tissue culture) when delivered by aerosol[126], but 127 to 320 $TCID_{50}$ when delivered intranasally[127]. The often-sudden onset of epidemics suggests that an infected individual can transmit the virus to a relatively large number of people.

## INFLUENZA-RELATED MORBIDITY AND MORTALITY

### Morbidity

*Influenza* virus is estimated to cause about 50 million illnesses annually in the United States. Excess hospitalization of between 50,000 and 100,000 Americans per season were reported between the early *1970s* and the mid*1990s.* Direct costs include hospitalizations, medical fees, drugs, and testing, and were estimated in 1986 to be about $1 billion annually, while indirect costs such as loss of productivity reach $2 to $4 billion annually. The temporal curves of individual pandemics are similar in that virus introduction into a community is followed by a relatively sharp, single peak that represents school and work absenteeism, which is followed, slightly later, by excess mortality. Notable exceptions are the pandemics in 1918 and 1957, both of which were characterized by a second, more severe wave. This pattern may reflect virus adaptation to humans that resulted in increased replication and/or transmissibility.

A number of studies have documented how *Influenza* virus epidemics reduce school attendance and work productivity, increase doctor's visits and hospitalizations, and can cause increased mortality, particularly among high-risk groups. Children younger than age 2 years and the elderly have the highest hospitalization rates[128], reaching 1 per 270 for those older than 65 years, compared to 1 per 2,900 for the 1- to 44-year-old age group. The impact of *Influenza* on the elderly extends beyond *Influenza* and pneumonia illnesses to all respiratory conditions and to congestive heart failure. It is an important cause of respiratory infections in nursing homes. For children, 14% to 16% of those seeking medical care are infected with *Influenza* virus[129].

During interpandemic seasons, the overall infection rates are estimated to be 10% to 20% but can reach 50% in selected age groups or populations. The rate and severity of infection depend on the level of pre-existing immunity, the age of an individual, and the virulence of the virus, all of which vary greatly among

outbreaks[130]. Age-specific attack rates are highest in school-age children, and symptoms in this age group are usually more severe than in young adults[130]. Often, increases in school absenteeism mark the beginning of a new epidemic, suggesting that school age children play a critical role in disseminating *Influenza* viruses. Increased school absenteeism is typically followed by increases in work absenteeism. *Influenza* A viruses of the H1N1 and H3N2 subtypes, as well as *Influenza* B viruses, all cause similar symptoms; however, the frequency of severe infections is higher with H3N2 viruses. Reinfection with a closely related drift variant can occur[132], although the symptoms are usually less severe than after the first encounter with a particular virus strain[133].

**Mortality**

The increase in mortality during pandemics and epidemics is one of the hallmarks of *Influenza* virus infection. The term "excess mortality" was introduced by William Farr and describes the number of deaths observed during an outbreak in excess of the number of expected mortalities. The number of *Influenza* virus-related deaths, however, is difficult to determine because the death certificate may not indicate *Influenza* as a primary cause of death or because a laboratory diagnosis may not have been performed.

For three pandemics of the twentieth century, excess mortality was estimated to be between 20 and 50 million deaths for "Spanish *Influenza*" worldwide and 70,000 and 33,800 deaths in the United States for the "Asian *Influenza*" and "Hong Kong *Influenza*," respectively, Although excess mortality is highest during a pandemic, the cumulative deaths of interpandemic seasons usually exceed those of pandemic years. During epidemics, excess mortality is estimated to be more than 20,000 in the United States alone but can exceed 40,000 deaths. Excess mortality has been documented for both *Influenza* A and B virus outbreaks, and is usually higher with the former. Occasionally, *Influenza* B virus outbreaks will cause problems, as occurred in 1990. Since 1986, excess mortality typically results from H3N2 viruses, whereas H1N1 and type B viruses contribute to smaller extents.

Excess mortality affects all age groups but is highest in those older than age 65, who account for approximately 90% of excess mortalities during interpandemic seasons[134-135]. This age group thus has a 100-fold higher mortality rate than those younger than age 65[136]. During the 1968 pandemic, however, people younger than age 65 accounted for only 50% of *Influenza* virus-related deaths[146], and during the 1918 pandemic, excess mortality rates were extremely high not only in the elderly, but also in Young adults[138]. The reasons for these unusual patterns are unknown. The highest risk for pneumonia is seen in elderly who have cardiovascular and pulmonary conditions; other risk factors include metabolic or neoplastic diseases and pregnancy.

**Virulence related to strain difference**

Overall, the clinical manifestations of different *Influenza* A virus subtypes are remarkably similar, although some differences do exist. The most notable example is the 1918 H1N1 virus, which is epidemiologically associated with encephalitis lethargica, a feature that has not been associated with any other *Influenza* virus. Another example is the higher 'correlation of croup with H3N2 viruses compared to H2N2 viruses. In addition, the H1N1 viruses reintroduced into the human population in 1977 generally cause less severe illness than do H3N2 viruses.

**Interspecies Transmission**

The first report of interspecies transmission involving pigs dates back to 1938, when **Shope** presented serologic evidence for the transmission of a human virus to pigs. Further evidence for virus transmission between these two species came in 1976, when an H1N1 swine virus was isolated from a soldier who had died of *Influenza* at Fort Dix, New Jersey[139]. This virus was subsequently isolated from five other soldiers, and serologic studies suggest that more than 500 personnel were infected. Numerous other reports have described the transmission of swine viruses to humans, some cases of which resulted in fatal outcomes[140], and of the transmission of human viruses to pigs[141]. Of interest, most of the latter viruses were of the H3 subtype. Recently, avian H9N2 viruses have been introduced into pigs in Southeast Asia[142], and H5N1 viruses have been isolated from pigs in China[143] and Vietnam[144].

**Shu *et al*[145].**, surveyed 20 Chinese farm families who raised pigs and ducks to examine the possibility of interspecies transmission & genetic reassortment of *Influenza* viruses on farms in S. China. The study was conducted from Sep. 1992 to Sept. 1993 and 11 *Influenza* viruses (6in humans & 5 in ducks) were isolated. The human isolates were A (H3N2) type viruses. Serological studies on the farm pigs showed infection with the same subtype as humans & not with swine or avian viruses. Genotype analysis of duck & human isolates provide no evidence of reassortment but the transmission of avian viruses to humans was observed. All these results made them conclude that their findings support the concept that intermingling of humans, pigs & ducks on Chinese farms is favourable to the generation of new potentially hazardous strains of *Influenza* viruses.

## CLINICAL FEATURES AND PATHOGENESIS IN HUMANS

### Pattern of Virus Shedding

Human *Influenza* viruses replicate almost exclusively in superficial cells of the respiratory tract. *Influenza* virus is released from the apical surface of the cell, which may limit more systemic spread but facilitate accumulation of virus in the lumen of the respiratory tract for transmission to the next susceptible host[146]. Alveolar macrophages and dendritic cells can also be infected and play a role in the response to *Influenza* by processing antigens and presenting them for immune recognition. *Influenza* virus replicates throughout the respiratory tract, with virus being recoverable from the upper and lower tracts of people naturally or experimentally infected with virus. The site of optimal growth in the respiratory tract for some *Influenza* strains may be regulated by the prevalence of the α-2,3 or α-2,6 sialic linkage to galactose[147].

The pattern of virus replication in six adult volunteers administered an *Influenza* A/HK/68-like H3N2 virus, in relation to the onset of clinical symptoms, IFN response, and spout serum and nasal wash antibody responses, was studied[148]. Virus replication peaks about 48 hours after inoculation and declines slowly thereafter, with little shedding after days 6 to. 8. Peak virus titers in symptomatic adult volunteers range from $10^{3.0}$ to. $10^{7.0}$ $TClD_{50}$/mL of nasopharyngeal wash. There was a

positive correlation between the amount of virus shed and the severity of the clinical illness. Individuals who shed less than $10^{3.0}$ $TCID_{50}$/mL were either asymptomatic or had only minor upper respiratory tract symptoms. Even after infectious virus can no longer be recovered, viral antigen can be detected *for* several days in cells and secretions o*f* infected individuals. Viral antigen is detectable in conjunctival cells and secretions. In children, virus can be found for up to 13 days virus after the onset o*f* symptoms. The higher titred and more prolonged shedding in children contributes to their important role in spread o*f Influenza*.

**Pathology**

*Influenza* A virus induces changes throughout the respiratory tract, but the most significant pathology is present in the lower respiratory tract[149-150]. During bronchoscopy in uncomplicated *Influenza* infections, acute diffuse inflammation o*f* the larynx, trachea, and bronchi was observed with mucosal inflammation and edema. Light microscopic studies of infected cells show that columnar ciliated cells became vacuolated, edematous, and lose cilia before desquamating down to a one-cell-thick basal layer. Submucosal edema and hyperemia occur with an infiltration by neutrophils and mono nuclear cells. In more severe primary viral pneumonia, there is an interstitial pneumonitis with marked hyperemia and broadening *of* the alveolar walls, with a predominantly mononuclear leukocyte infiltration and capillary dilation and thrombosis. *Influenza* virus specific antigen is present in types 1 and 2 alveolar epithelial cells, as well as in intra-alveolar macrophages[151]. The pathological changes associated with the H5N1 virus include a hemophagocytic syndrome, renal tubular necrosis, lymphoid depletion, and diffuse alveolar damage with interstitial fibrosis[152].

Necrotizing changes may occur with rupture *of* walls of alveoli and bronchioles. *Influenza* virus has been obtained from lungs at autopsy in titers *of* $10^{2.0}$ to. $10^{5.7}$ 50% egg infectious doses per gram of tissue[153].

At a cellular level, *Influenza* virus shuts off cell protein synthesis and induces apoptosis as an additional mechanism of cell destruction[154]. From the third to fifth day after onset of illness, mitoses appear in the basal cell layer, and regeneration of the epithelium begins. During this time, reparative and destructive processes may be present simultaneously. Complete resolution of the epithelial necrosis probably takes up to. 1 month.

**Clinical Features**

*Adults*

Infection with *Influenza* A viruses results in clinical responses ranging from asymptomatic infection to primary viral pneumonia that rapidly progresses to a fatal outcome. The typical uncomplicated *Influenza* syndrome is a tra- cheobronchitis with some involvement of small airways[155]. The incubation period can be as short as 24 hours to 4 or 5 days, depending in part on the dose of virus and the immune status of the host[156]. The onset of illness is usually abrupt, with the occurrence of headache, chills, and dry cough, which are rapidly followed by high fever, significant myalgias, malaise, and anorexia. Substernal tightness and soreness can accompany the cough. The most-prominent sign of infection is fever that often peaks within 24 hours at 38°C to 40°C. The fever begins to decline on the second or third day of illness and is usually gone by the sixth day [155]. The elderly can have high fever, lassitude, and confusion without respiratory signs.

Physical findings in *Influenza* are confined to the respiratory tract. Nasal obstruction, rhinorrhea, and sneezing often occur, and pharyngeal inflammation without exudate is common. Conjunctival inflammation and excessive tearing may occur. Conjunctivitis was the hallmark of an H7N7 outbreak in the Netherlands[157]. Small cervical nodes can be felt in a minority of cases. Chest radiograph and auscultatory findings are usually normal although occasionally patchy rales and rhonchi are heard. As the fever declines, the respiratory signs and symptoms may become more intense. The cough frequently changes from dry and hacking to one that is productive of small amounts of mucoid or purulent sputum. After the fever and upper respiratory tract symptoms resolve, cough and weakness can persist for 1 to 2 additional weeks. Illness is more frequent and more severe in cigarette smokers. The loss of the mucociliary blanket is a factor in the predisposition to second sinusitis and bacterial pneumonia seen after *Influenza* infection. Although airflow in large airways is usually unaltered in uncomplicated *Influenza*, there is an increase in bronchial reactivity to chemical and particulate stimuli during natural infection. Small peripheral airways are often affected in otherwise uncomplicated *Influenza* A virus infections[158]. This small airway abnormality can persist after symptomatic illness has subsided. Alterations in pulmonary gas exchange, with a depression of the diffusing

capacity of carbon monoxide and an increase in the alveolar-arterial oxygen tension gradient are seen[159]. Tracheobronchial clearance of radiolabeled particles is depressed during acute *Influenza* virus infections but returns to normal levels about 1 month later. Although significant abnormalities in large and small airways can be demonstrated during acute infection and early convalescence, uncomplicated *Influenza* appears to cause little permanent damage in the lung, even in patients with chronic obstructive lung disease.

*Children*

The clinical manifestations of *Influenza* in children are generally similar to those in adults, but there are some distinct differences. Children have higher fever that may be accompanied by febrile convulsions[160]. At least 14% of fevers with respiratory tract symptoms that result in a pediatrician visit are caused by *Influenza* viruses[154]. Otitis media, croup, pneumonia, and myositis are more frequent in children than in adults. In approximately 12% of *Influenza*-infected seronegative children, otitis media develops. From 24% to 33% of children hospitalized with *Influenza* A virus infection have otitis media[161]. Although less well documented in children than in adults, bacterial sinusitis may follow acute *Influenza*.

Studies in children have shown that *Influenza* A viruses are important causes of laryngeotracheobronchitis (croup). Croup occurred predominantly in children younger than 1 year. *Influenza*-associated croup can be especially severe and occasionally requires intubation. In a prospective study of 121 susceptible (seronegative) young children seen during an H3N2 virus outbreak, 5 of 60 infected infants had clinical and radiographic evidence of pneumonia[162]. *Influenza* A virus infection has been shown to exacerbate asthma. Children (especially those younger than 3 years) have a higher incidence of gastrointestinal manifestations such as vomiting and abdominal pain. Infection of neonates can present as unexplained fever and can be life threatening[163]. With the avian H5N1 *Influenza*, there is a report of a fatal presentation with diarrhea and coma being the primary symptoms[164].

It is important to emphasize that *Influenza* A and B viruses are significant causes of serious lower respiratory tract disease that often lead to hospitalization in children and more rarely to death.

## LOWER RESPIRATORY TRACT COMPLICATIONS

Three distinct syndromes of severe pneumonia can follow *Influenza* infection in children or adults. Complications are most common in the elderly.

### Primary Viral Pneumonia

Primary *Influenza* virus pneumonia was first described in fatal cases caused by the 1957 Asian *Influenza* A virus (H2N2)[165 – 166], although retrospectively, it had been observed during previous *Influenza* A epidemics[167]. Primary viral pneumonia occurs predominantly in individuals at high risk for the complications of *Influenza* virus infection (i.e., the elderly or patients with cardiopulmonary disease), but 25% of the cases are in individuals without risk factors and an additional 13% in pregnant women. The typical case of primary viral pneumonia develops abruptly after the onset of *Influenza* illness. It progresses within 6 to 24 hours to a severe pneumonia with rapid respiration rate (30 to 60 respirations per minute), tachycardia (greater than 120 beats/min), cyanosis (in 80% of patients), high fever (average of 39°C), and hypotension. The illness may rapidly progress to hypoxemia and death in 1 to 4 days; the presence of frothy hemoptysis, tachypnea, and cyanosis portends a fatal outcome. Auscultatory findings include bilateral crepitant inspiratory rales. Examination by radiograph reveals mottled densities in two or more lobes. Diffuse symmetric interstitial infiltrates or areas of consolidation, cavitation, or pleural effusion suggest bacterial superinfection. Pathological findings in the trachea and bronchi are similar to those previously described during acute uncomplicated disease, but bronchiolitis and alveolitis are also is present[166]. The laboratory findings in primary viral pneumonia are not specific. The erythrocyte sedimentation rate is usually normal; a leukocytosis with band forms is seen in some cases, although lymphopenia may also be seen; and the partial pressure of arterial oxygen is depressed, often markedly.

In nonfatal cases, initial improvement occurs from 5 to 16 days after onset of pneumonia. Resolution of radiographic changes can take up to 4 months. Survivors can develop diffuse interstitial fibrosis with a decrease in diffusion capacity of carbon dioxide and a decrease in arterial oxygen tension. *Influenza* B virus can cause severe disease but has not been reported to be associated with fatal primary viral pneumonia

in normal individuals. The avian H5N 1 *Influenza* now occurring in Asia appears to follow a clinical pathway of primary viral pneumonia, although with a striking mortality of 51% in the first 112 documented cases[164, 168].

**Combined Viral-Bacterial Pneumonia**

Combined viral-bacterial pneumonia is quite common. The bacteria most often involved are *Streptococcus pneumoniae, Staphylococcus aureus,* and *Hemophilus Influenzae,* although other micro-organisms can play a role[169]**.** Clinically, this syndrome may be indistinguishable from primary viral pneumonia, except that the symptoms of pneumonia appear somewhat after the *Influenza* symptoms and chest radiographs are more apt to show pleural effusions. Virus has been recovered from the lungs and pleural fluid. The erythrocyte sedimentation rate is more frequently elevated than in primary viral pneumonia. Because there is no effective distinction between the two syndromes, the diagnosis depends solely on the demonstration of bacteria in the sputum, in fluid obtained at bronchoscopy, or in the pleural fluid. The case fatality rate for combined viral-bacterial pneumonia is 10% to 12%. Coinfection with *Influenza* and S. *aureus* can have a fatality rate of up to 42% (306).

There are strains of S. *aureus* that secrete proteases capable of activating the infectivity of *Influenza* virus by proteolytic cleavage of the HA. These strains play a synergistic role in experimental pneumonia in mice. Such protease-secreting bacterial strains could well be an added pathogenic factor in combined viral-staphylococcal pneumonias in humans. An apparent increase in combined viral-bacterial pneumonias has been noted with the 2003-2004 *Influenza* A H3N2 epidemic, perhaps coincident with the rapidly increasing impact of staphylococcal disease and emergence of methicillin-resistant S. *aureus*[170].

**Secondary Bacterial Pneumonia**

In this syndrome, an individual recovering from a typical *Influenza* illness develops shaking chills, pleuritic chest pain, and an increase in cough productive of bloody or purulent sputum. Cyanosis and a marked increase in respiratory rate are less common than with a primary viral pneumonia. Radiographic examination reveals local areas of lung consolidation but not diffuse pneumonia. Often, *Influenza* virus is

no longer recoverable, leukocytosis is common, and the erythrocyte sedimentation rate is elevated. This is generally amenable to antibiotic therapy, although a case fatality rate of about 7% has been described.

## EXTRAPULMONARY MANIFESTATIONS

### Viremia

Viremia is unusual in *Influenza* virus infections. Virus has been isolated from the blood in low quantity (present only in undiluted blood specimens) from a patient in the fourth day of *Influenza* illness[171]. In experimental or natural infection, virus has been recovered from the blood during the 1- to 3-day incubation period, but not thereafter. Virus was present in barely detectable amounts in the blood of two patients with fatal primary viral pneumonia on the sixth day after the onset of illness and was recovered at necropsy from the liver and spleen of one patient [172]. In addition, the presence of viremia has been inferred because virus was present in low titer in extrapulmonary tissues such as heart, liver, spleen, kidney, adrenal glands, and meninges obtained from patients dying of pneumonia. Viremia and systemic spread may be more common with the H5N1 *Influenza*, with PCR detection of virus from the blood in each of 6 patients tested[173].

### Myositis

*Cardiac Involvement*

*Influenza* recovery from the heart of patients with myocarditis associated with fatal pulmonary infection is rare. Clinical findings and cardiac function studies in patients with severe pulmonary infection suggest that myocardial dysfunction is not a direct result of *Influenza* A virus infection[28]. Both *Influenza* A and B virus-associated myopericarditis has been described.

### Reye Syndrome

Reye syndrome is a rapidly progressiye noninflammatory encephalopathy and fatty infiltration-of the viscera, especially the liver, which results in severe hepatic dysfunction on with elevated serum transaminase and ammonia levels. The onset of

the central nervous system (CNS) and hepatic symptoms usually begin as respiratory tract symptoms wane. The case fatality rate varied between 22% and 42%[173]. The etiology and pathogenesis of this syndrome is unknown. Histologically there is electron microscopic evidence of mitochondrial swelling[174]. This is seen following respiratory, varicella, and gastrointestinal viral infections. However, *Influenza* B virus infection is the most common antecedent infection. The disease associated with *Influenza* B virus infection occurs children ages 0 to 18 years (median age, 11 years) and is more frequently seen in a rural than in an urban environment. The administration of salicylates is a critical cofactor the development of Reye syndrome. The incidence of Reye syndrome after *Influenza* virus infection in the salicylate era was estimated to be between 0.37 and 0.88 cases 100,000 children younger than 18 years. This may have been underestimated because milder forms of Reye syndrome have been described. There has been a dramatic decrease in Reye syndrome cases in the United States associated with reduced use of salicylates, which are now widely recognized to be contraindicated for use in *Influenza* virus infection.

**Central Nervous System Involvement**

A wide spectrum of CNS disease has been observed during *Influenza* A and B virus infections in humans[175]**,** ranging from irritability, drowsiness, boisterousness, and fusion to the more serious manifestations of psychosis, delirium, and coma. Febrile convulsions leading to hospitalization occur in children with and without underlying CNS abnormalities. The pathogenesis of these CNS symptoms is unclear. Nonspecific metabolic effects such as hypoxia resulting from severe pulmonary infection may contribute to the CNS signs and symptoms. 'Two specific CNS syndromes have been suggested to accompany *Influenza* infection: *Influenzal* encephalopathy and post *Influenzal* encephalitis[176]. Encephalopathy occurs at the height of *Influenza* illness and may progress to[177] the cerebrospinal fluid (CSF) is usually normal the brain at autopsy shows severe congestion, and histologic changes are minimal. Lungs of such patients changes typical of *Influenza* and yield virus in high titre. A subset of *Influenza* encephalopathy has been described extensively in Japan and seen in other countries as acute necrotizing encephalopathy with bilateral thalamic and cerebellar involvement[178].

The post *Influenza* encephalitic syndrome is extremely rare and occurs from 2 to 3 weeks after recovery from *Influenza*. Recovery occurs in the majority of cases. The association of the post encephalitic syndrome with *Influenza* virus infection is less certain because virus is no longer recoverable and initial serum specimens may already reflect a rising antibody titer. *Influenza* A virus has only rarely been recovered from the brain or CSF, and attempts to isolate it from the CNS of individuals dying of primary viral pneumonia have been unsuccessful.

The syndrome of encephalitis lethargica followed by post *Influenza* encephalitic Parkinson disease was associated with the *Influenza* epidemics of 1918 and the epidemics following shortly thereafter. The epidemics of encephalitis lethargica followed by a year the epidemics of *Influenza*. These were followed in turn by post encephalitic Parkinson disease about one decade later[179].

Despite a limited association with *Influenza* vaccination, an increase in the incidence of the Guillain- Barre syndrome (GBS) has not been seen after *Influenza* A or B virus epidemics.

**Toxic Shock Syndrome**

Toxic shock syndrome has been seen in association with *Influenza* virus infection in humans and is believed to be the consequence of the bacterial exotoxin (TSST-l) secreted by colonizing S. *aureus* strains[180].

**Infection During Pregnancy**

Pregnant women in the second or third trimester have an increased risk of developing fatal *Influenza* disease. The increased mortality is generally seen during the years after introduction of a new pandemic strain, as in 1918 and 1957, although using a large data set; the impact can be appreciated in the interpandemic period[181]. Virus has not been recovered from the fetus, and the virus itself has not been implicated as a cause of congenital defects[182]**.** Although an increase in congenital anomalies and hematologic malignancies has been reported after *Influenza* virus infection in pregnancy, no consistent association between specific defects or malignancies and *Influenza* epidemics has emerged.

**Infection in Immunosuppressed Patients**

*Influenza* viruses can cause severe disease in immunocompromised individuals, but more often the illness resembles that seen in immunocompetent persons. Somewhat prolonged shedding of virus occurs in immunosuppressed persons.

*Influenza* B and C Virus Infections

*Influenza* B virus causes the same spectrum of disease as *Influenza* A. Severe illness can occur; however, the frequency of serious *Influenza* B virus infections requiring hospitalization is about fourfold less than that of *Influenza* A virus. *Influenza* B virus illness predominantly involves adolescents and school-age children, but adults and the elderly can also be involved[183-184]. Myositis, Reye syndrome, and gastrointestinal symptoms are more common with *Influenza* B than A virus infection.

*Influenza* C virus causes sporadic upper respiratory tract illness and is rarely associated with severe lower respiratory tract disease[185]. By early adulthood, most individuals (96%) have antibody to *Influenza* C virus[186]. Administration of *Influenza* C virus to volunteers induced mild coryza with some systemic symptoms.

**SURVEILLANCE**

A lot of emphasis is now placed on surveillance for *Influenza* in view of the threat of pandemic *Influenza*. Weekly summaries of *Influenza* activity are published in the, Mor*bidity and Mortality Weekly Report* from the Centers for Disease Control and Prevention (CDC) during the *Influenza* season and in the *Weekly Epidemiologic Record* published by the World Health Organization (WHO). Surveillance in the face of highly pathogenic H5N1 viruses has focused not only on human illness, but also on causes of mortality in domestic and wild birds. There continues to be a strengthening of routine surveillance particularly in China and Southeast Asia to identify the emerging strains that inform the choice of vaccine components for the coming *Influenza* season. The threat has propelled the countries around the world to prepare for an *Influenza* pandemic. The U.S. plan can be found at www.hhs.gov/nvpo/pandemicplan.

Additional components of surveillance include estimates of the burden and intensity of epidemics. Traditionally in the United States, this has been done by the CDC using weekly tracking of the proportion of deaths due to *Influenza* and pneumonia. Other estimates of the burden of *Influenza* have recently been published (187).

The seroepidemiology of H1N1 *Influenza* was studied by **Pyhala** and **Aho *et al*[188]., 1978-79**. They observed the rate of infection and reinfection in winter of 1978-79.The results of this study, according to the article had supported their earlier findings from the epidemic season of 1977-78.They observed that small children and pregnant women were affected only to a small extent. Elderly subjects possessed a low frequency of antibodies to the H1N1 *Influenza* in both pre epidemic and post epidemic sampling which contrasted the attack rate on young military servicemen, in whom the rate of reinfection was high. This indicated that the infection with the winter 1977-78 virus had conferred only modest protection against the closely related virus which caused the winter 1978-79 outbreak.

In one of the seroepdemiological studies on H1N1 *Influenza* virus in **1982 Pyhala and Aho *et al*[189]**, observed the widening of the affected population. It was found out that the H1N1 *Influenza* epidemic during the winter of 1980-81 affected a wider range of population than the previous epidemics of 1977-78 and 1978-79. The increase in the frequency of infection was due to antigenic drift in the surface protein of the virus. Small children and pregnant women were affected more in 1980-81 in contrast to the effect of the prior epidemics on them. This altered epidemiology was due to the development of adaptation of the virus to the human host.

A continuous surveillance of *Influenza* was carried out by **Rao, *et al*[48]., in 1980** in Pune, a city with a tropical climate. Three outbreaks of Acute Respiratory tract Infections (ARI) were investigated in March (hot season), in July- September (rainy season) and in Nov. (cold season), the first one was found to be associated with *Influenza A* H3N2 virus and the second with H3N2 and H1N1 and only a few *Influenza* viruses were isolated in the third outbreak. The illnesses had affected all the age groups, the paediatric age group being most affected. The study revealed that the combination of virus strains together with the wet weather contributed to the extensive outbreak during July-September.

Five outbreaks of acute respiratory diseases were observed by **Rao, *et al*[190].,** during the course of *Influenza* surveillance in Pune during 1983-85.Three of them occurred in rainy seasons, one in winter and the fifth in hot season. Specimens were processed in chick embryos, MDCK cell lines and LLC-MK2 cell lines and isolated *Influenza A* H3N2 in 1983,A H1N1 and A H3N2 in 1984 and A H3N2 in 1985.

Investigation on the outbreak caused by a new variant A H1N1 strain; A/Singapore/6/86 in the month of June 1986 was conducted by **Rao and Thite *et al*[50]** during the course of surveillance of *Influenza* in Pune, India. The study showed the high prevelance of anti bodies to the predecessor A H1N1 strains; A/USSR/90/77 and A/Chile/1/83(77% and 67% respectively) while it was 26% to the new A/Singapore/6/86 strain.

**Ghendon[191]** in his update on *Influenza* surveillance, **1991,** has given the main objectives of *Influenza* surveillance as the collection of *Influenza* virus isolates and analysis of their antigenic characteristics so that the most appropriate virus variants can be recommended as constituents of *Influenza* vaccines for the use in the future epidemiological season, collection and analysis of information on *Influenza* morbidity and mortality and earliest possible detection of *Influenza* epidemics. It was found that the objectives are being fulfilled only in certain countries and that the surveillance should be carried out worldwide and that the methods for surveillance need be improved and standardized.

During continuous surveillance of *Influenza* carried out from **1978-90** in Pune, India, 16 outbreaks of *Influenza* were investigated. Investigations showed that 10 of the epidemics occurred during the rainy season & the majority of the affected were children. The *Influenza* virus variants responsible for the outbreaks were identified as A H3N2 & A H1N1 by haemagglutination inhibition test. Statistical analysis given by **Rao *et al* [52];** showed that maximum were obtained during the rainy months.

In a surveillance carried out in the United States during the *Influenza* season from Oct 1988 - May 1989, *Influenza A* (H1N1) was the predominant strain while during the next season, *Influenza A* (H3N2) was the predominant[192].

In **1993-1994 Singh *et al* [193]., ** studied an outbreak of *Influenza* A subtype H3N2 occurred in Delhi during the month of July- Aug. Urban and Rural areas were affected & the attack rates were similar in children and adults. The mean age of the patients from whom the virus was isolated was found to be 2%. Of the 15 throat swabs collected 12 isolates were identified to be *Influenza A* (H3N2) virus, thus the predominance of H3N2 was recorded.

The importance of global surveillance for *Influenza* was put forth by **Cox *et al*[194] 1994**. They emphasized on the identification of the circulating strain of the virus, which in turn would help in updating the vaccine. Identification of the variants will also help in understanding the antigenic and genetic evolution of the virus. *Influenza* activity was found to occur in low-moderate levels, during Oct 1994-May 1995, in most parts of the world. The predominant strain was identified to be A (H3N2) followed by A (H1N1) during this period & in Australia they occurred most during March-August.

**Anchlan *et al*[195]., (1996)** studied the H1N1 *Influenza A* viruses circulating in the Mongolian population. Of the 4 *Influenza A* isolates of subtype A (H1N1) isolated from 1985 & analyzed by sequencing of RNA segments. The isolate from 1988 was found to be a derivative of reassortment between PR8 & USSR/77. One of the Mongolian isolates from 1991 (111/91) was similar to PR8 while another from 1991 (162/91) was related to H1N1 strain isolated around 1986 in the other parts of the world. From their reports it was evident that the mutational & evolutionary rates of the Mongolian strains are low & this could be a threat to human population as they retain their potential to reappear after several years to cause a pandemic as was in 1977.

The strains responsible for 1957 & 1968 pandemics were reassortants incorporating both avian & human *Influenza* viruses were suspected to have arosen in pigs. These pandemics were believed to have originated in China. A study was undertaken by **Zhou *et al*[196]., (1996)** in Nanchang region of central China to establish the prevalence types, seasonal pattern of humans & animals for evidence of interspecies transmission of *Influenza* viruses. The antibody profile was similar to those in the rest of the world. Evidence on transmission of avian H7 *Influenza* from

ducks was obtained. The serological survey on animals (pigs) showed that pigs were infected by human A (H1N1) & A (H3N2) *Influenza* viruses and not with swine viruses. There was no evidence for H2 *Influenza* viruses in humans after 1968.

In the world wide surveillance for *Influenza* activity from Oct 1995 through August 1996, the *Influenza* activity was found to have occurred at moderate to severe levels. A (H1N1) caused epidemics and A (H3N2) in Europe, Asia and N.A.A (H1N1) that caused an epidemic in Japan, was dominant in Canada, most region of the US & a few countries in Europe. *Influenza A* (H3N2) viruses predominated in China, most of Europe & a few regions in the US. The report summarizes that *Influenza* activity was at peak levels in southern hemisphere during March-August 1991.

An epidemiological study on *Influenza* pandemic carried out by **Gu, Yang & Jin**[197] in the rural areas of Shangdong & Henan. The cross sectional epidemiological study on the "relationship between 5 variables (fish, pig, duck, chicken & integrated fish farming) & *Influenza* pandemic provided results that suggested ducks played important role in ecology of *Influenza*.

**Mizuta *et al*[198]., ** studied the epidemiology of *Influenza* virus infections in children with ARI in Zambia between June 1993 & Sept 1995. A total of 3760 throat swabs were collected & processed for virus isolation. The results showed the isolation rate of *Influenza* virus type A (H3N2) was highest at 14.3% (20/139) in August 1993, at 15% (18/119) in June 1994 and at 25.4% (43/169) in July 1995. The results also revealed that *Influenza* viruses were one of the most important pathogens of ARI in children in the cool dry season (June-August) in Zambia.

The throat swabs/ nasal swabs from 456 patients with *Influenza* like illness were collected in Pune in 1998 & results were published by **Rao *et al*[54]., in 2000.** The isolates were identified to be a variant strain of *Influenza A* (H3N2) similar to the globally prevalent strain A/Sydney/05/97 (H3N2).

**Cameron *et al*[199]., (2000)** have reported on the close relation between the H9N2 subtype *Influenza* A viruses in poultry in Pakistan and the H9N2 viruses responsible for human infection in Hongkong. H9N2 viruses have been responsible

for disease in poultry in various parts of the world, recently. The analysis of HA genes of H9 isolates from poultry in various countries have shown a close relationship to the HA genes of viruses that infected 2 children in Hongkong. Analysis of the complete genome of a Pakistan isolate A/chicken/ Pakistan/2/99, showed that it was closely related to the human H9N2 isolates. The 6 genes coding for the internal component of the virus were similar to the corresponding genes of H5N1 viruses that caused 6 (out of 18) fatal cases of human infection. These findings make it important to check the ability of the avian-human transmission of the virus, which poses a possible pandemic threat.

United States, *Influenza* activity 2000. The report summarises the *Influenza* activity in the US from Oct1-Nov25, 2000. It was low during the period & viruses were isolated from 11 states. The isolates were identified to be *Influenza A* (H1N1), which matched the 2000-02 *Influenza* vaccine strain.

Epidemiology & evolution of *Influenza* viruses in pigs, **Brown[200], 2000.** Pigs serve as major reservoirs of H1N1 & H3N2 *Influenza* viruses. Swine husbandry practices provide continuous supply of susceptible pigs & regular contact with the other species, particularly humans. Pigs play important role as intermediate host for reassortment of *Influenza A* viruses of avian & human origin. This could always be a threat to outbreak of *Influenza.*

Global epidemiology of *Influenza*: past & present (**2000**) was reported by **Cox & Subbarao[38]** . Three pandemics have occurred in the 20$^{th}$ century: 1918 (H1N1) pandemic, 1957 (H2N2) pandemic, 1968 (H3N2) pandemic. The tools of molecular virology have been applied to determine the origin of the pandemic viruses. Molecular methods have been used to investigate the avian H5N1 & H9N2 *Influenza* viruses that recently infected humans in Hongkong.

## LABORATORY DIAGNOSIS

### Isolation and Propagation

*Influenza* viruses were first grown in embryonated eggs[29] but can be grown in a number of primary tissue culture systems. Cultivation of *Influenza* viruses in eggs is still the primary system for vaccine production and for the generation of large

quantities of virus used in laboratory studies. Currently, tissue culture systems, primary monkey kidney and MDCK cells, are generally used for the isolation of *Influenza* viruses from humans. Many, but not all strains of human *Influenza* A viruses, as well as most avian *Influenza* viruses, can be isolated directly in the allantoic cavity of embryonated eggs, although some human *Influenza* A and B viruses must first be isolated in the chicken embryo amniotic cavity and subsequently adapted to growth in the allantoic cavity. Highly pathogenic avian *Influenza* viruses are so virulent that virus kills the embryo before high titred virus can be recovered. Virus replication in eggs is detected by the ability of an aliquot of culture fluid to agglutinate erythrocytes, suggesting the presence of progeny. On initial passage in *eggs,* some *Influenza* viruses agglutinate guinea pig erythrocytes to higher titers than chicken erythrocytes, but on continued passage, *the* viruses usually agglutinate the latter to higher titers. Differentiation of *Influenza* from other hemagglutinnating viruses and strain specificity are determined by inhibition of HA by strain-specific antibody. *Influenza* viruses will replicate, cause cytopathology, and produce plaques in a number of primary cell cultures, including monkey kidney, calf kidney, hamster kidney, chicken kidney, and primary human respiratory epithelium. However, few cell *systems* other than primary kidney cells are suitable for plaque formation by *Influenza* viruses, unless trypsin is present to activate cleavage of the HA molecule. An interesting exception is that primary human respiratory epithelial cells will support the growth of *Influenza* virus and effectively cleave the *Influenza* HA[201].

*Influenza* viruses can be readily isolated from a variety of respiratory specimens. Embryonated hen's eggs are a practical isolation system but are less sensitive than cell culture. Primary Rhesus Monkey Kidney (PRMK) or cyanomolgous monkey kidney cell cultures are sensitive for most strains. Several cell lines, Madin- Darby Canine Kidney (MDCK) and LLC- MK2 are useful in primary isolation.

Virus replication is usually detected in cell culture by haemadsorption with guinea pig or chicken erythrocytes performed at fixed time after inoculation or when cytopathic effect (CPE) is detected. Identification of isolates can be done by HAI testing using antisera to current strains or by immunoflourescence (IF) or enzyme immuno assays (EIA) using type or subtype specific antisera **[6].**

**Rapid Viral Diagnosis**

A wide variety of rapid viral diagnostic tests are now available that greatly facilitate *the* diagnosis of *Influenza*. *They* have the greatest sensitivity in detecting *Influenza* virus in children because they shed *the* highest quantity of virus and in all ages are most sensitive early in the illness. In addition, all depend on the source and quality of the specimen. Characteristically, there is more virus present in the nasopharynx than the throat. They vary in ability to test for and distinguish between *Influenza* A and B viruses and in their adaptability to point of care use in physician's offices.

Direct detection of *Influenza* viral antigens in respiratory secretions can be performed within hours and can be accomplished with IF, EIA, radio immuno assay (RIA) and time resolved fluoroimmunoassay**.**

**Serology**

A serum antibody response can be documented in 80% of human *Influenza* infections[202]. After primary infection, HA-specific IgG, IgA, and IgM antibodies are *detected,* whereas in the secondary response, IgG and IgA antibodies predominate[203-204]. B cells producing *Influenza* virus-specific IgG, IgA, and IgM antibodies are present in the peripheral blood of normal individuals and of individuals undergoing infection with *Influenza* A viruses[205]. Traditionally, antibodies have been measured by hemagglution-inhibiting antibody (HAl) assays. There is a renewed interest in whether the direct measurement of virus neutralization may be a better correlate of immunity**.**

Serologic studies are not useful for rapid diagnosis of *Influenza* since paired acute and convalescent sera are usually needed. Commonly used systems include complement fixation (CF), HAI & enzyme linked immuno sorbant assay (ELISA), less commonly employed tests include neutralization (NT) or microneutralization, single radial hemolysis, radial immunodiffusion, passive haemagglutination and neuraminidase inhibition. HAI titers reflect subtype and strain specific antibodies directed to HA. HAI testing requires inactivation of non specific inhibitors in the sample and its sensitivity depends on the antigen variant employed. *Influenza* –

specific IgM antibodies are detectable in over 80% of infected persons but in only 36% of hospitalized adults at the time of admission.

**Yamane *et al*[206]**. in the year 1981 used the reversed single radial immunodiffusion (r-SRID) test for the assay of antibodies to *Influenza A* nucleoprotein. The test was employed during an epidemic of H3N2 and H1N1 viruses to estimate the infection ratio in junior high school students. The test was very convenient and highly sensitive in detecting the rise in antibody titer to *Influenza A* virus nucleoprotein. It was thus shown that the r-SRID technique using the avian origin *Influenza* A virus provided a simple and reproducible method for the assay of antibody level to *Influenza A* nucleoprotein in seroepidemiological study.

**Pyhala *et al*[207]., ** compared the sensitivity of the variants of *Influenza A*(H3N2)virus grown in embryonated egg and in Madin-Darby Canine Kidney(MDCK) cell lines for their use as antigens in seroepidemiology. In the test, the egg grown(A/Finland/13/85E) and MDCK(A/Finland/13/85/M).The clinical specimen obtained during the 1984-85 outbreak was grown in egg and MDCK cell lines. In an antigenic analysis it was found that the M-virus appeared to be more sensitive to Haemagglutination Inhibition antibodies against heterologous viruses than did the E-virus. The results of their study suggested that anti body to the MDCK grown virus is a more accurate indicator for the immune status of the immune status of a community than antibodies to egg grown virus variants.

New variants of *Influenza* A (H3N2) strains were isolated during an outbreak in Pune, India in the month of Feb.**1996**. In the work carried out by **Rao *et al*[53].**, throat & nasal swabs from patients with *Influenza* like illness were processed in MDCK & LLC-MK2 cell cultures. The isolates were similar to the recent circulating global strains A/Johanesburg/33/94 & A/Wuhan/359/95.

**Nelson *et al*[208]., (1999)** used multiantigen fluroscence immunoassay for the investigation of seroprevalence of respiratory virus infections in infant population using heel-prick blood samples collected on filter paper. The study aimed at measuring the IgG antibody specific to viruses which included RSV, *Influenza A* virus, Adeno virus & Para*Influenza* virus type 1,2,3 by indirect immunofluroscence

using multiviral assay developed for this purpose. The results showed that 9.4% of the infection was due to *Influenza A* virus. The method could be used for rapid diagnosis & seroepidemiological survey of viral respiratory infections in infants.

## REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION (RT-PCR)

As with many viruses, the *use* of PCR is increasing our ability to detect viruses. Particularly instructive is the ability to detect in a single multiplex assay the leading causes of viral respiratory disease[209]. As with viral culture, the results are not immediately available, and the assays must be carefully controlled to avoid cross contamination. Detection of viral nucleotide sequences by reverse transcriptase-polymerase chain reaction (RT- PCR) has been used in detecting *Influenza A* & B viruses in clinical samples [210].

In a study by **Matsuzaki Y**[211], a multiplex reverse transcription-polymerase chain reaction (RT-PCR) assay was developed to detect and identify subtypes of hemagglutinin H1 and H3 swine *Influenza* virus (SIV). Two oligonucleotide primer sets were prepared using published sequence data for H1N1 and H3N2. The PCR products with unique size characteristics of each subtype were sequenced, and the sequences were confirmed to be subtyping specific for H gene 1 or 3. These primer sets did not amplify when RT-PCR assay was performed for genomic DNA or RNA from other common swine pathogens. The RT-PCR assay was able to detect viral RNA up to 1 tissue culture infective dose of reference SIV H1N1 or H3N2. Results of this multiplex RT-PCR were comparable with those of the HAI test. These results suggest that multiplex RT-PCR can be a useful test for detection and sub typing of SIV in clinical samples.

Clinical presentation of infections caused by the heterogeneous group of the respiratory viruses can be very similar. Hence, there is a need for assays that can rapidly identify the most important viruses. A new multiplex reverse transcription nested-polymerase chain reaction (RT-PCR) assay that is able to detect and type different respiratory viruses simultaneously is described by Matsuzaki Y et al [212]. Primer sets were targeted to conserved regions of nucleoprotein genes of the *Influenza* viruses, fusion protein genes of respiratory syncytial viruses (RSV) and hexon protein

genes of adenoviruses. Individual *Influenza* A, B, and C viruses, RSV (A and B), and a generic detection of the 48 serotypes of adenoviruses were identified and differentiated by the size of the PCR products. Accurately amplifying RNA from *Influenza* and RSV prototype strains and DNA from all adenovirus serotypes demonstrates the use of this method for both laboratory routine diagnosis and surveillance of all these viruses.

A multiplex reverse transcription (RT)-PCR method has been developed. It is capable of detecting and sub typing *Influenza* A (H1N1 and H3N2) and B viruses as well as respiratory syncytial virus (RSV) types A and B in respiratory clinical samples taken as part of a national community-based surveillance program of *Influenza*-like illness in England and Wales **(Stockton J, Ellis JS, Saville M, Clewley JP, Zambon MC)**. The detection of each different pathogen depended on distinguishing five amplification products of different sizes on agarose gels following RT-PCR with multiple primer sets[213].

A one-step multiplex reverse transcription RT - PCR assay targeting the HA1 segment of the human hemagglutinin gene was developed as a rapid surveillance method by Daum LT etal. A researcher-blind study was performed using 112 randomly selected, culture-positive clinical samples collected through the Department of Defense (Global Emerging Infectious Surveillance (DOD-GEIS) *Influenza* network during the 2000-2001 *Influenza* season. The results confirm that this assay is a highly sensitive and timely surveillance tool for rapid detection and simultaneous sub typing of clinical *Influenza* specimens isolated worldwide [214].

## ANTIVIRALS

### Amantadine and Rimantadine

Amantadine hydrochloride and rimantadine, an analog of amantadine, are currently licensed for prophylactic and therapeutic use against *Influenza* A virus in humans in the United States. Recommendations for use are published yearly by the CDC.

Both have a tricyclic structure with an amine side group and have antiviral properties against all subtypes of *Influenza* A virus, but not against *Influenza* B or C viruses[215]. The concentration of amantadine and rimantadine that inhibits virus plaque

formation ranges from 0.2 to 0.4 μg per ml for sensitive *Influenza* A viruses and is greater than 100 μg per ml for *Influenza* B viruses. *The* former level of drug is found in the blood and nasal secretions after oral administration.

Resistant strains of *Influenza* A virus can be isolated *in vitro* and *in vivo.* In experimental studies, amantadine resistant mutants can spread to susceptible contacts and cause disease, including fatalities, indicating that acquisition of amantadine resistance is not associated with attenuation.

Amantadine and rimantadine are useful for prophylaxis against H1N1, H2N2, and H3N2 *Influenza* A virus infections in adults and children. During an epidemic involving both *Influenza* A H1N1 and H3N2 viruses, amantadine and rimantadine protected against *Influenza* like illness (78% and 65%, respectively), documented *Influenza* illness (91% and 85%), and *Influenza* A virus infection (74% and 66%) (135). Lower efficacy rates against documented illness (70%) and infection (39%) were observed for amantadine prophylaxis during an H1N1 virus epidemic.

Recommendations for prophylactic and therapeutic uses of amantadine include prophylactic use in individuals at high risk who have not been vaccinated, as well as hospital personnel who could spread infection in the hospital. Therapeutic use includes individuals with uncomplicated *Influenza* who have a high temperature of less than 48 hours duration it would be helpful to know that amantadine is useful in treating primary viral pneumonia, but efficacy in this clinical setting remains to be established.

After finding that a high percentage, 91%, of the circulating *Influenza* strains were resistant to amantadine and rimantadine in the winter of 2005 to 2006, the CDC published a health alert recommending against the' use of amantadine and rimantadine for the treatment or prophylaxis of *Influenza* in the United States during the 2005/2006 *Influenza* season[216]. The highly pathogenic H5N1 viruses are also resistant to amantadine and rimantadine.

**Detection of Amantadine resistance:**

The antiviral agent amantadine has been shown to be effective for treatment and prevention of human *Influenza* A virus infections, although treated individuals may excrete resistant viruses[217-219]. Single-amino-acid changes at four positions, 26,27, 30, and 31, within the transmembrane domain of the M2 protein can confer resistance[220- 222]. Resistant strains have been reported to account for one-third of viruses recovered from nursing home and household settings after persons were treated with amantadine, and they apparently can be transmitted [223-226]. Amantadine was approved for *Influenza* virus treatment in Japan in 1998, and sales then increased suddenly. Studies, have found a high frequency of resistant strains in nursing homes using the 50% tissue culture infective dose ($TCID_{50}$)/ 0.2-ml titration method with isolated viruses and showed predominant amino acid substitutions at position 31 (serine to asparagine [Ser-31-Asn]) in the M2 protein of resistant viruses[227]. However, the number of viruses examined is limited, and the correlation between indications of use and extent of resistant virus appearance was not clear. The detection of amantadine resistant strains with substitutions at three positions (amino acids 27, 30, and 31), using PCR-restriction fragment length polymorphism (PCR-RFLP) analysis, also enables direct analysis of nasopharyngeal swabs from patients.

**Neuraminidase Inhibitors**

The first NA inhibitors included DANA (2-deoxy-2,3dihydro-N-acetyneuraminic acid; Neu5Ac2en) and its N-trifluoroacetyl analog FANA, which were effective in *vitro* but did not inhibit replication of *Influenza* viruses in animals. After resolution of the structure of the NA, DANA served as the lead compound in the rational design of drugs targeting the NA. Replacement of the 4-hydroxyl group on DANA with a guanidino group filled the unoccupied pocket in the NA active site with an inhibition constant (Ki) of 2 x $10^{-10}$ mol/L[228]. Zanamivir is a specific inhibitor of the NA of all *Influenza* A and B viruses. Zanamivir must be administered intranasally or inhaled for optimal effect.

An additional NA inhibitor, oseltamivir, can be taken orally. This was achieved through the discovery and use of a hydrophobic pocket in the enzyme-active

center that could accommodate lipophilic groups necessary to improve the inhibitor's oral bioavailability. Oseltamivir is converted to oseltamivir carboxylate or GS 4071 in the body. The resulting.molecule not only retains the carboxylate bond of. sialic acid, but also makes use of a hydrophobic group . This group induces the binding cleft to form a hydrophobic; pocket, which holds the drug in place through hydrophobic interactions.

Recommendations for the use of each compound are published yearly by the CDC[229]. Oseltamivir is currently recommended for those as young as 1 year. Resistant variants to the NA inhibitors have been selected under drug pressure *in vitro,* but only with difficulty and at a much lower frequency than amantadine[230]. The emergence of resistance is first detected by appearance of mutations around the receptor-binding site of the HA, which compensates for reduced NA activity by lowering the affinity of binding to receptors. The second step in emergence of resistant mutants is selection of variants with mutations in the inhibitor-binding site of the NA. The most common substitution with zanamivir occurs at residue 119, where Glu was typically replaced by Gly and less frequently by Asp or Ala less frequently, substitutions occur at residues 292. Glu 119 is conserved among all *Influenza* viruses and is one of the "framework" residues, which do not directly participate in substrate catalysis but stabilize the enzyme structure. The interaction of Glu at residues 119 and 227 with the 4-guanidinium moiety of zanamivir provides the high potency and selectivity of the inbibitor, and changes at residue 119 weaken the interaction between the mutated NA and the drug. Characterization of these mutants reveals that the, functional integrity of their NA activity is impaired. They have reduced NA activity, are heat labile, and have a narrower range of pH optima. Thus, zanamivir-resistant mutants that contain structural changes in their NA and HA may not succeed in, nature.

**INDIGENOUS ANTIVIRAL HERBS**

All major civilization of the old world has given rich reference of herbs and other forms of naturally occurring substances as " anti-infectious" regardless of the agents associated with it. The significant development of non-formal drug therapies has initiated global scientists to work on authentication of existing herbs for antiviral property. References are available in the modern literature indicating the successful

usage of indigenous drug against diseases like flu, meningitis, encephalitis and myocarditis[231].

In recent years few herbs, either as isolated active principles like allicin, lycorine **(Norbert *et al*[232]., 1992, Margaretha Leven *et al*[233]., 1983)** or modified plant products like keelanalli[234], vallarai[235], karisilankani[236], turmeric[237], neem[238] are commercially available as antiviral agents .

From available Indian literature, very few plants have been scientifically documented to have antiviral properties. Tulsi (*Ocimum sanctum*) has been reported to have antiviral activity against Polio type 3 virus **(Parida *et al*[239]., 1997)**. Garlic extracts (*Allium sativum*) has been proven to have antiviral property against *Influenza* B and Herpes simplex viruses but not over Coxsackie B virus **(Yen Tsai *et al*[240]., 1985)**. The antiviral activity and virucidal effects of neem (*Azadirachata indica*) on all six serotypes of Coxsackie group B viruses and New Castle disease virus is one of the important screening works on *in vitro* antiviral drug evaluation **(Lalita Badam *et al*[241]., 1999)**. Extracts of kellanalli (*Phyllanthus amarus*) has been successfully patented for down regulating Hepatitis B viral transcription and replication **(Lee *et al*[242]., 1996)**.

Many marine plants and algae used in traditional system of medicine have also been screened for antiviral properties. Premanathan *et al.,* in 1992 has successfully documented 38 marine plants including seaweeds, sea grass and mangroves against Encephalomyocarditis virus (EMCV), New Castle disease virus, Vaccinia virus, Hepatitis B virus and Semiliki forest virus. Secondary metabolite from plant products like Taxol, Vincristine (Oncovin) has been successfully used in pharmaceutical formulations. Mangrove plants like Bruguiera *cylindrica, Cheilosporium spectabile* and *Rhizophora mucronata* were shown to have antiviral activity against Encephalomyocarditis virus (EMCV) and Semiliki forest virus **(Premanathan *et al*[243-244]., 1993 and 1994)**.

French native plants like *Bryonia dioica, Anthyllis vulneraria, Matricaria chamomilla* and *Matricaria inodara* are few herbs documented to exhibit antiviral activity against Polio and Herpes simplex virus **(Sugandha *et al*[245]., 1983)**. Extracts

of *Haemanthus albiflos* has been reported to inhibit Polio 1, Coxsackie B2, Echo 2, and Rota virus SAII propagation *in vitro* (Husson *et al*[246]., 1991).

From all the above literature, it is evident that many herbs used in traditional medicines are promising antivirals and are economic and reliable drug of choice in many developing countries. In present scenario major developed western countries have also turned towards the herbal resources of east as source of modern antiviral drugs.

## VACCINES

### Inactivated Vaccines

Inactivated *Influenza* A and B virus vaccines are licensed for parenteral administration in humans. The strains to be included each year for the annual vaccination program and recommendations for use are made by the WHO and the. US Public Health Service. Seed viruses for the A and B components of the vaccine are naturally occurring virus strains that replicate to high titers in the allantoic cavity of embryonated chicken eggs; the substrate used for vaccine production. Most commonly, the *Influenza* A components reassortant viruses that contain internal genes from the A/Puerto Rico/8/34(A/*PR/8/34;* H1N1)virus, which confers a high level of growth in eggs plus genes coding for surface antigens of the current *Influenza* A virus[247]. A number of techniques have now been described for deriving the high-yield recombinant vaccine by reverse genetics, which offers a more rapid and predict. Way of deriving vaccine virus and introducing attenuating mutations (e.g., removing the multibasic H5 cleavage site that is responsible for some of the virulence of the highly pathogenic avian *Influenza* viruses) [248-250].

In the manufacture of inactivated vaccine, virus present in allantoic fluid is purified and concentrated by zonal centrifugation or column chromatography and inactivated with formalin or beta-propiolactone. These purification procedures have greatly reduced the incidence of local and systemic reactions. The quantity of immunoreactive HA in each dose is standardized to contain the amount recommended by the Advisory Committee on Immunization Practices, which is usually 15 *µg* per component for adults and older children or 7.5 *µg* for children younger than 3 years.

The quantity of NA is not standardized because this glycoprotein is quite labile during the process of purification and storage. Each 0.5-mL dose of vaccine contains approximately 10 billion virus particles, and one egg yields one to three doses of vaccine. Vaccine also contains variable but small quantities of endotoxin, egg-derived protein, free formaldehyde, and most have thiomersal preservative, all of which do not appear to contribute to the reactogenicity or toxicity of the vaccines for humans. A vaccine has been licensed in Europe using the adjuvant MF-59 with evidence of slightly greater immunogenicity in the elderly. The route of immunization for all inactivated vaccines is intramuscular.

The preparation of an inactivated vaccine for the highly pathogenic H5N1 vaccines posed particular problems because it rapidly killed the chicken embryos before the yield of virus was sufficient for vaccine production. This was overcome by deleting the polybasic HA cleavage site and recombination with A/PR/8/34 genes. Experimental lots are now being tested in humans.

The vaccines currently in use are designated whole virus (WV) vaccine or subvirion (SV) (split or purified surface antigen are alternative terms) virus vaccine. The WV vaccine contains intact, inactivated virus, whereas the SV vaccine contains purified virus disrupted with detergents that solubilize the lipid-containing viral envelope, followed by chemical inactivation of residual virus. Experimental vaccines have been prepared and evaluated in humans that contain either baculovirus-expressed HA alone[251] or purified NA alone[252]. An experimental inactivated WV vaccine containing an NA antigen immunologically related to the epidemic virus and an unrelated HA appears to be less effective than conventional vaccine[253]. A veterinary approach of using an irrelevant NA (DIVA) to help distinguish vaccine-induced immunity from natural infection is being explored[254]. Only inactivated vaccines containing both relevant surface antigens are considered further.

It has been observed that human *Influenza* A and B viruses isolated in the allantoic cavity of embryonated chicken eggs can have altered antigenicity and possess mutations in the HA that promote growth in eggs[255]. Studies that have compared the immunogenicity of egg-passaged or cell culture grown viruses indicate that the egg-passaged viruses are less protective against the predominant virus

isolated from humans[256]. In humans, immunization with vaccines containing an HA made from egg-grown virus induces less neutralizing antibody to virus possessing the egg-adapted HA than to the homologous virus[257]. At present, egg-grown viruses are used in the inactivated virus vaccine, but there is an active program to develop cell culture grown alternatives.

**Vaccine Reactogenicity**

Reactogenicity of vaccines has been evaluated in four large scale, placebo-controlled trials in adults and children. The vaccines contained a monovalent *Influenza* A H1N1 virus or a mixture of H1N1, H3N2, and B viruses. In each instance, vaccine was standardized with respect to quantity of HA. Satisfactory serologic responses were achieved in children and adults with doses of vaccine that caused minimal clinical reactions. Reactions generally occurred within the first 24 hours and lasted for 1 to 2 days; these included systemic manifestations such as fever, malaise, myalgia, and headache, as well as local manifestations such as pain, erythema, induration, and tenderness at the site of inoculation.

Local or systemic allergic reactions to vaccine components occur rarely. It is believed that the majority of these reactions are due to residual egg protein. High-risk patients who have egg allergies may be immunized by using a specific protocol[258]. an increase in the number of reported cases of GBS occurred after the National *Influenza* Immunization Program from 1976 to 1977[259]. About 1 in 100,000 individuals who received the swine *Influenza* virus vaccine developed GBS, with a relative risk in vaccinees about four- to eightfold greater than that in unvaccinated subjects. *Influenza* virus vaccine produced after 1977 had not been associated with an increased risk of GBS, until a recent report implicated a low but definable excess risk (1 to 2 cases per 1 million vaccines) with the 1992 to 1993 and 1993 to 1994 vaccines[260]. The reason for the difference between 1977 and subsequent years is not known.

**Vaccine Immunogenicity**

Prior antigenic experience with *Influenza* A virus within the same subtype is an important determinant of the serum antibody response to SV and WV vaccines. An example occurred in 1977 when those younger than 24 years in 1977 represented an

unprimed population to H1N1 viruses, which had last circulated in 1957. In contrast, most persons 25 years or older probably had one or more infections with H1N1 viruses and thus represented a primed population. A dose of either SV or WV vaccine containing 20 to 30 *μg* of HA induced an acceptable level of antibody in primed adults. In contrast, two to three times as much vaccine was required to induce a similar antibody response in unprimed individuals (i.e., 60 *μg* or more of HA). In general increasing the dose of vaccine increases the level of antibody achieved, but there is a rather shallow dose-response curve with WV and SV vaccines in primed and unprimed individuals[261–262]. Responses to individual antigens in trivalent vaccines are as great as responses to the same components administered separately. The neuraminidase-inhibiting antibody response to WV and subunit vaccines in primed and unprimed individuals followed the same patterns as the HAI antibody response[263]. The serum antibody response of elderly subjects to inactivated virus vaccines is variable but is generally lower than that of younger subjects [264].

Parenteral administration of vaccine leads to an increase in nasal wash antibody in 25% to 50% of primed vaccines[265]. A correlation between an increase in serum and an. increase in nasal wash antibody has been observed, and the nasal wash response is dose dependent[266].

A systematic comparison of intranasal versus parenteral administration of vaccine in primed and-unprimed individuals using a vaccine of known HA content has not been performed, but intranasal administration of inactivated vaccine induces little systemic response[267].

**Vaccine Efficacy**

Parenteral vaccination with *Influenza* A and B viruses consistently induces resistance to illness and, to a lesser extent, infection with *Influenza* A and B viruses[268-271]. The resistance induced is manifested by a reduction in the frequency and severity of the illness, including otitis media in children[272]. Efficacy ranges from 60% to 80%, with the higher value seen after challenge with homologous virus and the lower value after challenge with virus that has undergone antigenic drift.

Although *Influenza* vaccine is currently recommended for annual vaccination of high-risk groups, most of the studies that have demonstrated efficacy have involved populations that received one or two doses of vaccine shortly before experimental or natural challenge. In one study in a boys' boarding school, the value of annual administration of vaccine was assessed over a 7-year period from 1970 to 1976, during which time three outbreaks occurred that involved different H3N2 drift viruses[273]. The protective effect of inactivated *Influenza* A vaccines was limited to boys after they received their first vaccination. As a consequence of the ineffectiveness of revaccination, the cumulative attack rate in the three epidemics was similar in vaccinated and unvaccinated boys. This raised a question concerning the current recommendation for annual vaccination with inactivated vaccine. However, subsequent studies have confirmed that annual vaccination with inactivated virus vaccines is indeed beneficial[274].

## Vaccine Recommendations[275]

Recommendations for vaccination have continued to expand. Annual vaccination is recommended for

- ✓ Persons at or older than 65 years of age
- ✓ Residents of nursing homes and other chronic care facilities that house persons of any age who have chronic medical conditions
- ✓ Adults and children who have chronic disorders of the pulmonary or cardiovascular systems, including asthma (hypertension is not considered a high-risk condition)
- ✓ Adults and children who have required regular medical follow-up or hospitalization during the preceding year because of chronic metabolic diseases (including diabetes-mellitus), renal dysfunction, hemoglobinopathies.. or immunosuppression (including immunosuppression caused by medications or by HIV)
- ✓ Adults and children who have any condition (e.g., cognitive dysfunction, spinal cord injuries, seizure disorders, or other neuromuscular disorders) that can compromise respiratory function or the handling of respiratory secretions or that can increase the risk for aspiration

- ✓ Children and adolescents (ages 6 months to 18 years) who are receiving long-term aspirin therapy and, therefore, might be at risk for experiencing Reye syndrome after *Influenza* infection
- ✓ Women who will be pregnant during the *Influenza* season
- ✓ Children ages 6 to 23 months
- ✓ Persons ages 50 to 64
- ✓ All health care workers
- ✓ Persons who can transmit virus to those at high risk.

**Live Viral Vaccines**

A live attenuated intranasally administered *Influenza* virus vaccine is licensed for use between the ages of 5 and 49 years. Licensure occurred after trials indicated high efficacy in the pediatric population [(276)] and effectiveness in adults [(277).] There are several major reasons for continued interest in expanding the use of live vaccines in humans. The live vaccines appear to have the potential to broaden the indications for *Influenza* vaccine and hence to have an impact on the overall morbidity and perhaps spread of the disease[278]. They appear to be safe in all ages, although some studies have suggested an increase in reactive airway disease after vaccination[279]. Second, local immunity is increasingly recognized to play a major role in resistance to most respiratory pathogens[280]. Respiratory tract infection with a live attenuated vaccine has been found to be the most efficient method of stimulating such immunity. Because infection of the respiratory tract stimulates both systemic and local immunity, and theoretically is a good way of stimulating cell-mediated immunity, all components of the host's immune response are brought into play. Direct comparisons of the efficacy of the two vaccines in children are now being done.

As previously indicated, *Influenza* A viruses (and, to a lesser extent, *Influenza* B viruses) are unique among viruses that infect the respiratory tract in that they undergo significant antigenic variation on virtually a yearly basis. The strategy for developing a live virus vaccine must take this property into account. It is not feasible to attenuate each new variant of *Influenza* virus that appears in nature by multiple passages in a heterologous host(s), a method that has been successfully used to

attenuate viruses that do not show antigenic variation. Therefore, a strategy is needed in which attenuation can be achieved in a single step by transfer of genes from an attenuated donor virus to each new epidemic or pandemic virulent virus[281].

Because resistance to *Influenza* A virus is mediated by the development of an immune response to the HA and NA glycoproteins, the genes coding for these surface antigens must come from the epidemic virus, whereas the attenuating genes are derived from the attenuated parent. This scheme is possible because of the segmented nature of the *Influenza* genome in which the HA and NA genes are derived from the virulent wild-type virus and the other genes are derived from the attenuated donor virus. In this approach, it is necessary that the genes that confer attenuation not code for the HA and NA glycoproteins, as these genes must be derived from the new epidemic or pandemic virus. The attenuated reassortant vaccine virus was intentionally shown to receive each of the six other internal genes from the attenuated donor virus because such reassortants would be expected to exhibit the same level of attenuation each time a new vaccine virus is produced. Many donor viruses have been evaluated for their ability to attenuate new epidemic *Influenza* A viruses reproducibly, including significant work in Russia with the A/Leningrad/ 134/ 17/57 strains[282]. However, only the A/ Ann Arbor/6/60 (A/AA/6/60; H2N2) cold-adapted *(ca)* donor virus shows considerable promise as a vaccine[283].

The ca reassortant vaccines induce resistance to experimental or natural challenge with wild-type *Influenza* A in adult and pediatric subjects who have had some experience with *Influenza* A viruses. In addition, they are highly immunogenic in seronegative subjects, and that the vaccine would be efficacious in this population. Recent very impressive efficacy, 93% overall, against culture-confirmed *Influenza* A/H3N2 and B was shown in a pediatric population ages 15 to 71 months after either 'one' or two doses of trivalent ca reassortant vaccine were given as a nasal spray[276] in the elderly, coadministration of live ca virus vaccine and inactivated virus vaccine may be more- efficacious than inactivated virus vaccine alone against -natural infection with *Influenza* A virus [284].

Because the populations to be immunized have such disparate experience with *Influenza* A viruses, it is not surprising that different vaccines (i.e., live vs.

inactivated virus vaccine) or different vaccine schedules would be needed for each population. The suggested use and' schedule of annual immunization with ca reassortant and inactivated virus vaccines and the reasons for the choice of vaccine in the pediatric, young adult, and elderly adult populations are (a) seronegative pediatric population older than 6 months may preferentially get live vaccine because this type of vaccine has been shown to be more immunogenic in this population; (b) previously primed children and adults younger than 65 years may get either vaccine because efficacies of live and inactivated vaccines are comparable for this population, although a live virus vaccine may be better accepted because of an easier route of delivery; and (c) elderly (older than 65 years) may get live plus inactivated virus vaccine because this combination is more efficacious in this population.

## Alternative Vaccine Approaches

A number of vaccine approaches are being considered. Including introducing attenuation by altering the interferon response genes[285]. A universal inactivated *Influenza* A vaccine has been pro-posed based on the extra cellular domain of the M2 protein and preclinical work on such a vaccine is promising[286].

# 3. SCOPE AND PLAN

*Influenza* viruses belong to the family *Orthomyxoviridae* and are the major etiological agents of upper respiratory tract infections. They are the major determinant of morbidity and mortality caused by respiratory disease and outbreak of infection sometimes occurs in world wide pandemics. *Influenza* A viruses are of greatest concern because of their property of antigenic change resulting in epidemics or pandemics. Type B virus shows antigenic variation to a lesser degree which results in epidemics, whereas type C virus appears to be antigenically stable and causes sporadic upper respiratory tract infections.

There is considerable variation in the severity of illness caused by *Influenza* in different individuals. This is partly due to the age of those affected, general health, and immunistation status relative to previous *Influenza* infections. Infections may be subclinical or may produce symptoms ranging from minor respiratory illness to fatal viral pneumonia. The "classic" symptoms of *Influenza* are rapid onset of malaise, feverishness and myalgia, usually with a non productive cough or sore throat. In children, nausea, vomiting and diarrhea are often observed.

*Influenza* surveillance activity in India is extremely limited. In a vast country like India with varied geographical diversity and huge population, this infection has not received specific attention. For the detection of newly emerging strains with pandemic potential, *Influenza* surveillance throughout the year is extremely important. This would enable health authorities to implement preventive measures to control the spread of the disease. During, the last several years of perennial episodes of *Influenza* like syndromes, no detailed investigation had been undertaken to implicate the presence and association of *Influenza* virus in particular. India has experienced *Influenza* pandemics in 1781, 1889 and 1918[287] Reports of isolation of virus strains similar to A/England/1/51(H1N1) between 1950-57 and pandemic strains of A/H2N2 and A/H3N2 from several areas have been recorded by the Pasteur Institute, Coonoor[288]. National Institute of Virology, Pune has been undertaking *Influenza* surveillance from 1976 and they have reported isolation of over 40 antigenic variants

of A/H1N1, A/H3N2 and B viruses. Though studies on *Influenza* had been done in different parts of the country there has been very little work done in Tamilnadu, particularly Chennai.

In this era where there is a looming threat of a flu pandemic it is always imperative to check the circulation of *Influenza* viruses in the community. This monitoring ensures prevention and effective check on the emergence of new antigenic variants and also gives an early warning of an epidemic or pandemic.

## PART-I SURVEILLANCE ANTIGENIC AND GENETIC CHARACTERIZATION OF INFLUENZA VIRUSES

### 3.1.1 Serosurveillance for Antibodies against *Influenza* Viruses In The Normal Population Of Chennai City.

Serosurveillance study is essential to know the status of exposure of the population exposed to the virus. Based on the immunity of the study population, surveillance study will help us to predict when an epidemic will arise, what the antigenic characteristics of the virus will be, the impact it will have and what age groups it will predominantly affect. It was therefore proposed to undertake a serosurveillance study to detect the exposure of the population to the commonly circulating *Influenza* viruses. . In the present study, the exposure of the population to *Influenza* viruses was determined by detecting the haemagglutinating IgG antibodies to the *Influenza* viruses in four age groups 0-5, 6-15, 16-45, and 46-60 by Haemagglutination Inhibition test. 50 samples from apparently healthy individuals of each age group were tested each year, from 2001 to 2006. HAI test was performed as per standard WHO protocols and using WHO reagent kit supplied by *Influenza* branch Centres for Disease Control and Prevention, Atlanta.

### 3.1.2 Antigenic Characterization

*Influenza* A/H1, A/H3 and B types are known to co circulate with one type predominating in a particular year or years. Virus isolation is the gold standard for diagnosis for *Influenza* virus infections.

Virus concentration in nasal and tracheal secretions remains high for 24 to 48 hours after symptoms start and may last longer in children. Titers are usually high and so there are enough infectious virions in a small droplet to start a new infection. Throat swab and nasopharyngeal swabs are likely to be positive in early infections.

In this study, virus isolation and antigenic characterization was attempted from throat and nasal swab samples from patients suffering from acute respiratory disease, attending the outpatient departments of major tertiary care hospitals.

Samples were collected with the consent of the patient or the guardian in case of young children. A total of 2536 samples were collected during the period 2002-2007, processed as per standard WHO protocols. The isolates were antigenically characterized by haemagglutination inhibition test using WHO reagent kit supplied by *Influenza* branch Centres for Disease Control and Prevention, Atlanta.

### 3.1.3 Neuraminidase Typing Of Influenza Isolates

*Influenza* A subtypes A(H1N1) and A(H3N2) have cocirculated in humans worldwide since the reappearance of A(H1N1) viruses in 1977[289-290] . Antigenic drift has also been reported for NA[291] and correlated with amino acid differences in the molecule[292]. Recently, reassortant *Influenza* A viruses with an H1N2 antigenic composition subtype[293] have been detected by surveillance programs in a number of countries. There have been few reported instances of viruses in which there had been genetic reassortment of internal genes[294-296] or of the surface antigen genes[297-299] between the A(H1N1) and A(H3N2) viruses infecting humans.

Surface antigen gene reassortment of these subtypes was detected in China during 1988/1989 where a small number of A(H1N2) viruses were isolated from the human population[297-298]. Outbreaks of A(H1N2) from Asian and other countries

during the 2001/02 – *Influenza* season[300-302], a report of A/H1N2 isolation in Yokahama[303] indicate that the A(H1N2) reassortant viruses are more numerous and more widespread than the strains reported from China in 1988/1989. Recently, viruses of this type have been identified in Europe, USA and south-east Asia, and in the UK they were more numerous than A(H1N1) strains during the 2001/2002 season suggesting that A(H1N2) viruses may become established on a continuing basis in the human population[304] .

Prior to the recent identification of A (H1N2) strains, routine laboratory studies of *Influenza* viruses generally classified the known circulating human *Influenza* A subtypes on the basis of HA type with the NA type usually being assumed. However the recent emergence and spread of *Influenza* A (H1N2) viruses which appear to have evolved by reassortment of circulating A(H1N1) and A(H3N2) strains, suggests the need for epidemiological studies to determine the neuraminidase type in order to differentiate between *Influenza* A (H1N2) and A(H1N1) strains..

The haemagglutination-inhibition assay that is routinely used for subtyping and antigenic characterization of *Influenza* strains does not distinguish between A(H1N1) and A (H1N2) strains; therefore, alternative tests are required for typing the NA. The standard test used to determine neuraminidase type is a rather cumbersome and time-consuming procedure (approximately 18 h) which relies on inhibition, by antibody, of *n*-acetyl neuraminic acid release from a substrate such as fetuin[305]. Alternative methods of neuraminidase assay have been described, one for example relying on the agglutination of neuraminidase-treated erythrocytes by lectins[306]**:** however, these have found little application in the laboratory. In addition, all of the methods that are based on antibody. Inhibition of neuraminidase activity may be subject to stearic hindrance from anti-haemagglutinin antibodies, if whole *Influenza* virus and antibody to whole virus is used. Recently, the reverse transcription polymerase chain reaction (RT-PCR) has been found to be useful as a definitive test for identifying both the haemagglutinin and neuraminidase subtype of *Influenza* viruses[307].

In the present study all *Influenza* A virus isolates were subjected to NA typing (N1&N2) by RT-PCR and a complete antigenic characterization was done.

### 3.1.4 Detection Of Antigenic Variants by RT-PCR RFLP

*Influenza* viruses undergo continuous antigenic variation and it is imperative to look for antigenic variants and also for the variation among strains isolated during a period of time. The restriction analysis of the RT-PCR products amplified from the isolates will help to detect any mutations that may accumulate and lead to the emergence of antigenic variants. Restriction analysis showed variation in *Influenza* A & B viruses thus emergence of epidemic variants.

### 3.1.5 Genetic Characterization of Influenza Virus Isolates

The use of molecular epidemiology has detected the existence of numerous genotypes endemic to different regions of the world. The molecular characterization further gives the information on the sequence diversity within a genotype, which is restricted to a single predominant lineage by epidemics.. Further, the type prevalent to one locality varies every year or in few years, probably due to the antigenic variation and development of immunity in the population.

In recent years, the potential resolving power of molecular diagnostic tools especially genomic sequencing has enhanced the precision and reliability of the *Influenza* virus surveillance worldwide. The envelope proteins induce protective antibodies following infection of an individual by the virus[308]. The antigenic determinant of HA is located in the 3' portion of the HA1 RNA. Vaccines are made from inactivated viruses, but as these envelope proteins change continuously changing giving rise to antigenic variants[309]. This property of the virus renders the vaccine of the previous season ineffective for the next season. Hence in this present study the *Influenza* A & B virus isolates were subjected to sequencing of HA1 genome. The sequences were compared with vaccine strains HA1 region sequences available.

## PART-2 MOLECULAR DIAGNOSIS

Since the unveiling of Polymerase Chain Reaction (PCR) method in October 1985 at American society of Human Genetics, PCR has been recognized as potential tool for sensitive, specific and rapid detection of minute quantity of genetic material in clinical specimens.

### 3.2.1 Evaluation of RT-PCR Directly From Specimens by Comparison With Virus Isolation

Routine virus isolation is time consuming and is very sensitive if samples are collected at appropriate time and transported in cold chain. PCR and Real-time PCR techniques have been accepted as better alternative and successfully employed for the rapid confirmation of other etiological agents including *Influenza* virus diagnosis. *Influenza* virus isolation from swabs is very sensitive and rapid ; RT-PCR is employed to detect the presence of *Influenza* genetic material in swab specimen that confirms the etiological agent associated with the infection. Hence, in the current study RT-PCR for rapid diagnosis of *Influenza* virus infection from throat and nasal swabs was standardized.

### 3.2.2. Standardization of Multiplex RT-PCR

The diagnostic tools are very expensive, standardization of multiplex RT-PCR for the detection and typing of *Influenza* viruses will be advantageous when compared with the RT-PCR available today as typing and subtyping of the virus can be performed in a single reaction tube thus saving on reagents. The multiplex RT-PCR was standardized by the use of KCl & Ammonium sulphate.

## PART 3: SENSITIVITY TO ANTIVIRALS:

### 3.3.1.Screening of Sensitivity to Antivirals by MIC:

Antiviral medications with activity against *Influenza* viruses can be effective for the chemoprophylaxis and treatment of *Influenza*. Four licensed *Influenza* antiviral agents are available namely amantadine, rimantadine, zanamivir, and oseltamivir. *Influenza* A virus resistance to amantadine and rimantadine can emerge rapidly during

treatment. As no published data is available in India on this aspect and to have an idea about the sensitivity pattern of the isolates, the isolates were tested for their sensitivity to the currently used M2 inhibitor (Amantadine) by minimum inhibitory assay (MIC) using MDCK cellline.

### 3.3.2 Detection of M2 Mutants by RT-PCR RFLP:

The resistance of the *Influenza* A isolates to Amantadine is due to the mutations occurring in the M2 protein which is the target for the drug. The genes coding for the M2 protein were amplified by RT-PCR and subjected to RFLP to detect any mutations in them. The results of sensitivity to antivirals by MIC were also confirmed.

### 3.3.3 Antiviral Screening of Common Herbs

Third world and developing countries are the worst affected by communicable diseases and require either mass preventive measures like vaccines or affordable antiviral drugs to combat the infection.

During the last decade, there has been a renaissance of herbal medicines globally. Numerous species of plants around the world are used for treating conditions ranging from minor abrasions to fatal diseases. The oldest reference for the use of herbs in India is found in Rig Veda dating back to $5^{th}$ century BC and Charaka Samhita a well-known Indian treasure on herbs and surgery. China is one of the contemporaries in herbal medicines to India and tremendous effort has been put forth in that country to validate its herbal resources on successful use as antimicrobials. Scanty reference is found in India on antiviral screening of herbs and natural products. Therefore in the present study screening of herbs listed in Ayurveda and Siddha literature to treat diseases of respiratory infections were chosen. Since most of the herbal medicinal products are prepared with water and ethanol, aqueous and ethanolic extracts of these herbs were tested *in vitro* for antiviral activity against *Influenza* A & B viruses.

# 4. MATERIALS AND METHODS

## 4.1 MATERIALS FOR SEROSURVEILLANCE:

### 4.1.a Specimens

Blood samples for serosurveillance were collected from normal healthy children attending ICDS units in Chennai and from adults for attending hospitals for reasons other than acute respiratory tract infections. 200 samples were collected during the year; 50 samples from each age group in four age groups (0-5, 6-15, 16-45, 46-60).

### 4.1.b Bleeding of guinea pigs for erythrocytes

- Healthy guinea pig
- 5 ml disposable syringe
- 18 guage needle
- surgical spirit, cotton
- 100 ml conical flasks with a small amount of Alsever's solution.

### 4.1.b Haemagglutination test:

- "U" bottom plates (96 wells),
- Variable volume pipetting device,
- Sterile tips,
- 25 µl dropper,
- Washed 0.75% guinea pig erythrocytes,
- PBS-pH: 7.2.
- Standard reference antigens-
  A/H1N1
  A/H3N2
  B/Victoria like
  B/Yamagata like

*Influenza* A(H3N2), A(H1N1) antigens consisted of infected allantoic fluid inactivated by Betapropionolactone(BPL). A final concentration of 0.1% sodium azide was added as a preservative and stored the liquid antigen at 4ºC

-Obtained from *Influenza* branch, CDC (Centres for Disease Control & Prevention), Alanta, Georgia, USA (The WHO *Influenza* Reagent Kit for the 2000-2008 seasons was used).

### 4.1.c Haemagglutination-Inhibition Test:

- Microtitre "U" bottom plates (96 wells).
- Reference sera- H1N1, H3N2, B/Victoria like B/Yamagata like
- Negative control sera obtained from CDC, Atlanta, Georgia,USA.
- Reference antigens obtained from CDC, Alanta, Georgia, and USA.
- Washed 0.75% guinea pig erythrocytes.
- 25-µl dropper, variable volume pipetting device.
- Sterile tips.
- PBS pH 7.2, 0.85 NaCl,
- Receptor Destroying Enzyme.

## 4.2 METHODOLOGY FOR SEROSURVEILLANCE STUDY

**Preparation of reagents**

### 4.2.a Alsever's solution for collection of erythrocytes:

| **Ingredients** | **g/l** |
|---|---|
| Dextrose | 20.5 |
| Sodium citrate dihydrate | 8.0 |
| Sodium chloride | 4.2 |
| Distilled water | 1 litre. |
| pH | 6.1+/- 0.1 |

pH was adjusted with 0.1 N NaoH or 1N HCL. The solution was sterilized by filtration through a membrane filter with 0.22 µm pore size.

### 4.2.b Phosphate Buffered Saline, pH 7.2 (PBS):

Stock 25 times concentrated (25X) phosphate buffer containing in 100ml was prepared:

2.74g dibasic sodium phosphate ($Na_2 HPO_4$)
0.79g monobasic sodium phosphate monohydrate ($NaH_2PO_4.H_2O$)
mixed with distilled water and made upto 100 ml

PBS was prepared by mixing & dissolving 40 ml of 25X phosphate buffer and 0.85% sodium chloride in distilled water & made upto 1 litre: pH 7.2+/-0.1. pH was adjusted with 1N NaoH or 1N HCl.

The PBS was sterilized by autoclaving.

The opened PBS, pH 7.2 was stored at 4°C and used within 3 weeks.

### 4.2.c Physiological Saline, 0.85% NaCl:

A 20 X stock solution was prepared by dissolving 170 g of NaCl in distilled water & made upto 1000 ml.

It was sterilized by autoclaving at 121°C.

Physiological saline 0.85% NaCl was prepared by adding 50 ml 20X stock solution to 950 ml distilled water.

This was again sterilized by autoclaving at 121°C.

Opened physiological saline was stored at 4°C and used within 3 weeks.

### 4.2.d Collection of serum samples:

Serum specimen was collected (3-5 ml of whole blood) from individuals of different age groups 0-5 years, 5-15 years, 15-45 years, and 45-60 years.

Specimens were centrifuged at 2500 rpm/15 minutes & the RBC's & serum were separated.

The serum was pipetted off & transferred to sterile 2 ml vials & the RBCs were discarded.

The sera were then stored at –20°C.

### 4.2.e Bleeding of guinea pigs for erythrocytes:

A healthy guinea pig was chosen for the collection of blood.

The animal was bled by cardiac puncture using sterile 5 ml disposable syringe & the blood was transferred to a container with a small amount of Alsever's solution to prevent clotting.

### 4.2.f Standardization of RBCs:

The final concentration of RBC's for HA/HAI is 0.75% for guinea pig erythrocytes. The following procedure was followed (as used by WHO collaborative centers for *Influenza*).

The RBCs in Alsever's solution were filtered through gauze before washing & centrifuged at 1200 rpm for 5 minutes.

Alsever's was aspirated & 50 ml PBS (pH 7.2) was added for wash. It was stirred gently to mix and centrifuged at 1200 rpm for 5 minutes to obtain packed cells. Supernatant was aspirated.

The wash was repeated with PBS three times.

At final wash the contents were centrifuged at 1200rpm for 10 minutes.

The packed cells were diluted to appropriate concentration based on packed cell volume. The concentration was checked and adjusted using a haemocytometer.

### 4.2.g Treatment of sera

**Removal of serum nonspecific inhibitors**

#### 4.2.g.1 RDE treatment procedure (WHO Manual for *Influenza* virus isolation and identification)

- The lyophilized RDE was reconstituted with 25 ml physiological saline.
- Three volumes of RDE was added to one volume of serum (eg. 0.3 ml RDE to 0.9 ml serum) & incubated at 37°C, overnight.
- The serum was then heated at 56°C for 30 minutes. Six volumes of physiological saline was added

#### 4.2.g.2 Removal of non specific agglutinins: (WHO Manual for *Influenza* virus isolation and identification)

- To one vol. of packed RBCs 20 volumes of RDE treated serum was added.
- The contents were thoroughly mixed & incubated at 4°C, mixed at intervals to resuspend cells.
- After 1 hour cells were centrifuged at 900xg for 5 minutes.
- The adsorbed serum was carefully removed without disturbing the packed cells.
- The serum was adsorbed with RBCs until the serum controls were negative.

### 4.2.h Haemagglutination (HA) Test: (WHO Manual for *Influenza* virus isolation and identification)

- The HA antigens were titrated before every HAI test.
- 50µl PBS (pH 7.2) was added to wells # 2 through # 10 of each lettered row on a U bottom microtitre plate.
- 100µl of each antigen was then added to the first well of the lettered row, which was subsequently diluted.

- Two or more RBCs control wells were prepared in wells at rows 11 &12 by adding 50µl of PBS. These served as indicators of a complete setting pattern without antigen.
- Serial two fold dilutions of each antigen were made by transferred 50µl from well to well & discarded the final 50µl after the $10^{th}$ row.
- The two fold dilution were 1:1 through 1:256.
- 50µl of RBC suspension was added to each well on plate. The contents of the well were mixed using a mechanical vibrator or by manually agitating the plates.
- The plates were incubated for 1 hour at room temperature (22°C-25° C) for guinea pig RBCs to allow complete settling of cells.
- The results were recorded & interpreted. (Figure 4.1)

**4.2.h.1 Preparation of standardized Ag for HAI test & "Back Titration" Protocol:**

The standard concentration of antigen for the HAI test is 4 HA units. The antigen dilution was calculated by dividing HA titer by 4. The dilution was calculated & prepared. The dilution prepared was recorded.

**4.2.i Haemagglutination Inhibition (HAI) Test: (WHO Manual for *Influenza* virus isolation and identification)**

The microtiter plate were labelled appropriately.

- 25 µl of PBS was added to wells B through H (B to H) of each numbered column.
- 50µl of each treated serum was added to the appropriate first well ( A1-A12) of the numbered column.

- Serial two fold dilution of the treated sera was prepared by transferring 25µl from the first well of the numbered columns 1-12 to successive wells. Finally 25µl was discarded after row H.

- 25µl of standardized Ag was added to the respective wells (A1-H12).

- 25µl of PBS was added instead of Ag to the set of treated sera for serum controls (A1-H12).

- The contents of the plates were mixed for 10 sec or by agitating the plates manually.

- The plates were covered and incubated at room temperature ($22^o$ to $25^oC$.) for 15 min.

- 50 µl of standardized RBCs were added to all wells and mixed as before.

- Cover the plates were covered and incubated at room temperature ($22^o$ to $25^oC$.) for 1 hour.

- The results were recorded. (Figure 4.2,3&4)

**4.2.i.1 Back Titration :**

Back titration was performed to verify HA units by performing the second HA test, using the standardized antigen dilution prepared .The diluted antigen was stored at 4°C and used on the same day.

100 µl of the standardised antigen was subjected to 2 fold dilutions.

The contents of the plate were mixed by shaking on a mechanical vibrator for 10 seconds or by agitating the plates manually.

50µl of standardized RBCs were added to wells & mixed as before.

The plates were covered and the RBCs were allowed to settle at room temperature (22-25°C) for 60 minutes. (Figure 4.2)

The results were recorded. The standardised antigen was used if it had a concentration of 8HAU/50 µl.

## 4.3 MATERIALS REQUIRED FOR MEDIA PREPARATION AND TISSUE CULTURE WORK

- Tissue Culture flasks 25 $cm^2$
- 5 ml, 10 ml, pipettes
- Minimum Essential Medium (MEM)
- Trypsin Phosphate buffered saline Versene Glucose (TPVG)
- Beaker with 10% hypo solution
- Gloves
- Spirit
- Cotton
- Label pad, Marker

## 4.4 METHODOLOGY FOR MEDIA PREPARATION AND TISSUE CULTURE WORK

**Preparation of Ingredients from Media**

**4.4.1 Penicillin and Streptomycin:** (Conc. 100 IU of penicillin and 100µg of streptomycin)

1 X $10^6$ units of crystalline penicillin and 1 gram 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. Stored at –20$^o$C.

**4.4.2 Kanamycin Acid Sulphate** (Conc: 20 mcg/ml)

1 gm of kanamycin was dissolved in 50 ml millipore double distilled water and mixed well to a final concentration of 20 mcg/ml and stored at –20$^o$C

### 4.4.3 Fungizone : Amphotericin B – 50 mgm (Conc.: 20mcg/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 mcg/ml and stored at -20°C.

### 4.4.4 L-Glutamine: 3%

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

### 4.4.5 7.5% Sodium-bi-carbonate solution

22 gms of sodium bicarbonate was dissolved in 200 ml of sterile millipore distilled water and filtered through whatman filter paper No.4, autoclaved at 10 lbs and stored at +4°C .

### 4.4.6 Fetal Bovine Serum

Fetal bovine serum was thawed at room temperature and inactivated at 56°C in water bath for ½ hour and cooled at room temperature. If floating particles were observed it was filter through Seitz filtered and stored at –20°C.

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

### 4.4.7.a Phosphate Buffered Saline

| | | |
|---|---|---|
| NaCl A.R | - | 8 gm |
| KCl A.R | - | 0.2 gm |
| $Na_2HPO_4$ A.R | - | 2.88 gm |
| $KH_2PO_4$ A.R | - | 0.2 gm |
| 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 autoclaved at 15 lbs for 15 mts.

**4.4.7.b 2% Trypsin**

2 gms of trypsin was dissolved in 100 ml sterile millipore distilled water stirred for 1/2 hrs with help of magnetic stirrer. The solution was filtered through membrane filter and stored at -20°C.

**4.4.7.c 0.2% EDTA (Versene)**

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

**4.4.7.d 10% Glucose A.R.- 10 ml**

1 gm of glucose was dissolved in 10 ml of sterile millipore distilled water and filtered 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.

**4.4.8 Hank's Balanced Salt Solution : For processing of samples**

| | | |
|---|---|---|
| HBSS 10X | - | 100 ml |
| Sterile Millipore distilled water | - | 887 ml |
| P&S | - | 1 ml |
| Kanamycin | - | 1 ml |
| Fungizone | - | 1 ml |
| 7.5% $NaHCO_3$ | - | 5 ml |
| Hepes buffer | - | 5 ml |

100 ml of HBSS was dissolved in 887 ml of millipore distilled water. 0.2 gm of Phenol Red was added and mixed well. All other ingredients were added, mixed and pH 7.2. Distributed into bottles and stored at +4°C.

### 4.4.9 0.4% Phenol Red

0.04 gms of Phenol red was dissolved in 10 ml of double distilled water. Sterilized by autoclaving at 10 lbs, 121°C, 15 minutes.

### 4.4.10 Trypan Blue for cell counting ( 0.1%)

0.1 gm of trypan blue was dissolved in 100 ml of phosphate buffered saline, filtered through whatman filter paper No.4 and stored at +4$^{o}$C.

### 4.4.11 TPCK Trypsin

20 mg of TPCK-trypsin was dissolved in 10 ml of sterile distilled water, filtered through 0.2 µm membrane and, stored in aliquots at -20$^{o}$C.

### 4.4.12 7.5% BSA fraction V

7.5 g of bovine serum albumin was dissolved in 100 ml of sterile distilled water, filtered through 0.2 µm membrane and stored in aliquots at -20$^{o}$C.

### 4.4.13 Media preparation for Tissue Culture

Media was prepared as in Table 4.1. All the ingredients were mixed well by shaking gently and pH was checked and adjusted to 7.2 to 7.4.

### 4.4.14 Preparation of MDCK Tissue Culture Flasks

**Subculture of MDCK Cells**

**Procedure:**

- One T-25 flask with a confluent monolayer of MDCK cells contains approximately $10^{7}$ cells.
- Medium was removed from the bottle using 10-ml pipette and discarded.

- 5 ml of 1x PBS without $Ca^{++}$ or $Mg^{++}$ was added to wash cell layer. The PBS pipetted out and discarded.

- 5ml of TPVG solution was added (for 25$cm^2$ flask) on the side opposite to the cell sheet, dispersed evenly on to the monolayer. The bottle was laid flat with the cell surface side down on the table for 2- 3 minutes.

- The TPVG was removed using pipette and the flask was placed in incubator until the cells detach from the surface (checked under microscope)

- The cells were resuspended in 5ml of growth medium. The suspension was aspirated using a pasteur pipette few times to break cell clumps.

- The cell concentration per ml was determined by counting the cells in haemocytometer.

**For preparation of 25$cm^2$ flask-**

Growth medium was dispensed to fresh sterile cell culture flasks to be seeded with cells (9ml/25$cm^2$ flask). The cell suspension was seeded to the TC flasks at 1 x $10^6$ cells/flask. Each flask was labeled with cell name, passage number and date of passage and incubated in 5% $CO_2$ environment for 48 hours.

## 4.5 MATERIALS FOR SAMPLE COLLECTION, VIRUS ISOLATION AND IDENTIFICATION:

### 4.5.1 Specimen for Virus isolation:

**3036** Throat and nasal swabs were collected from patients suffering from acute respiratory disease, attending the outpatient departments of major tertiary care hospitals.

Samples were collected from the following hospitals

- Institute of Child Health and Hospital for Children, Egmore, Chennai.
- Government General Hospital, Chennai.
- Government Stanley Hospital, Chennai.

- Government Saidapet Hospital, Chennai.
- Tambaram Sanatorium
- Periyar nagar Peripheral Hospital, Chennai.

**Inclusion Criteria**: The samples were collected from the patients who showed symptoms of fever, dry cough and myalgia for a duration of less than four days (acute phase). A copy of the proforma used for sample collection is enclosed.

**Exclusion criteria:** Patients with date of onset of illness more than one week and those who showed lower respiratory tract symptoms were excluded from the study.

### 4.5.2 Materials for Sample Collection:

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

### 4.5.3 Materials for Sample Processing

- Centrifuge
- Water bath
- Gentamycin
- Vortex
- 2 ml poly propylene vials.

### 4.5.4 Materials for Virus inoculation

- 48 hour old healthy, confluent, MDCK 25 $cm^2$Tissue Culture flasks
- 1ml, 10 ml, pipettes
- Virus growth medium
- Beaker with 4% hypochlorite solution
- Gloves
- Spirit

- Cotton
- Label pad, Marker
- Processed *Influenza* samples

### 4.5.5 Materials for HA test

As in section 4.1.b in addition to the control antigens, cell culture fluid from inoculated MDCK bottles was also titrated.

### 4.5.3 Materials for HAI test

As in section 4.1.c reference antisera was used instead of patients sera and HA positive cell culture fluids showing a titer of more than 8 HAU/50 µl was used as virus.

## 4.6 METHODOLOGY FOR VIRUS ISOLATION AND IDENTIFICATION

### 4.6.1 Methodology for Sample Collection: (WHO Manual for *Influenza* virus isolation and identification)

**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 - 3 ml of transport medium and the applicator stick was broken off.

**Throat swab**

Both tonsils and the posterior pharynx were swabbed vigorously, and the swab was collected into transport medium as described above.

Nasal- and throat swabs taken as described above were collected into the same vial of transport medium.

### 4.6.1 Methodology for Sample Processing: (WHO Manual for *Influenza* virus isolation and identification)

**Nasal, throat, and combined nasal/throat swabs.**

- 2ml 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 0.2ml gentamicin (10mg/ml) was added.
- Incubated at room temperature for 15 minutes.

Centrifuged at 1,000 rpm for 5 min, supernatant removed and inoculated an aliquot was stored at –70$^{o}$C.

### 4.6.2 Methodology for Virus isolation: (WHO Manual for *Influenza* virus isolation and identification)

#### 4.6.2.a Preparation of flasks

- The cells were checked for confluency and absence of contamination with microscope under 4X magnification.
- The growth medium was replaced with media for virus growth.
- Growth media was decanted into a beaker and washed three times with 6 ml of MEM containing 2 µg/ml of TPCK-trypsin.

#### 4.6.2.b Inoculation of flasks

- Media was removed from flask with sterile pipette.
- 500 ul of each specimen was inoculated into a T-25 flask using sterile pipettes. Inoculum was allowed to adsorb for 30 minutes at 37$^{0}$C.
- 9ml of complete media containing 2 µg/ml of TPCK-trypsin without calf serum was added to T-25 flasks.
- Flasks were observed daily for cytopathic effect (CPE).

#### 4.6.2.c Harvesting of flasks

- The cell culture flask was harvested if 3+ or 4+ CPE is observed by collecting supernatant fluid and adding 0.5% stabilizer bovine serum albumin. Even if no CPE is observed by day 6 or 7 the cells were harvested.

- Hemagglutination test was performed and if HA was positive HAI was put up and the virus typed. If no HA is present the sample was passaged one more time before reporting inability to recover virus from the specimen.

### 4.6.3 Methodology for Virus identification:

#### 4.6.3.a Haemagglutination (HA) Titration of Control Antigens and Field Isolates (WHO Manual for *Influenza* virus isolation and identification)

- U-shaped microtiter plates were used for titrating the viruses with guinea pig RBCs.

- 50 µl of PBS (pH 7.2) was added to #2 through #12 (A2 - H12) wells of each lettered row.

- 100 µl of each control antigen or field isolate was added to the first well of the lettered rows.

- RBC control well was prepared in column 11 and 12 by adding 50µl of PBS.

- Serial two fold dilutions were made by transferring 50 µl from the first well of lettered rows to successive rows. The final 50 µl was discarded.

- 50 µl of RBC suspension was added to each well of the plate.

- The plates were agitated thoroughly.

- Plates were incubated for 1 hour at room temperature ($22^{o}$to $25^{o}$C). Cell control was checked for complete settling of RBCs.

- The results were recorded. (Figure 4.1)

### 4.6.3.b Haemagglutnation Inhibition (HAI Test): Identification of Field Isolates: (WHO Manual for *Influenza* virus isolation and identification)

- Microtiter plates were labelled .
- 25 µl of PBS was added to wells B through H (B1 - H12) of each numbered column.
- 50 µl of each reference antisera was added to the first well of the appropriate numbered column.
- 50 µl of PBS was added to the first well of columns 6 and 7 (A6 - A7) for cell control.
- Serial twofold dilutions of the treated sera was prepared by transferring 25 µl from the first well of numbered columns 1-12 to successive wells. The final 25 µl was discarded after row H.
- 25 µl of standardized control antigen #1 (A/H1N1) was added to all wells of a complete set of diluted treated sera (Ex: A1 - H5). Continued with remaining standardized control antigens and field test antigens.
- 25 µl of PBS instead of antigen was added to serum control plate.
- The contents of the plates were mixed for 10 sec or by agitating the plates manually.
- The plates were covered and incubated at room temperature ($22^{o}$ to $25^{o}$C) for 15 min.
- 50 µl of standardized RBCs were added to all wells and mixed as before.
- Cover the plates were covered and incubated at room temperature ($22^{o}$ to $25^{o}$C) for 1 hour.
- The results were recorded. (Figure 4.2&3)

## 4.7 MATERIALS FOR NEURAMINIDASE TYPING OF INFLUENZA ISOLATES

**4.7.1 Viruses:** Viruses: 162 *Influenza* A type viruses isolated during the study period.

### 4.7.2 RNA Extraction:

- QIAamp Viral RNA Kit for isolation of viral RNA from cell culture supernatants, cell free fluids, serum and plasma.
- 100% ethanol
- Sterile 1.5ml microfuge tubes.
- Variable volume micropipettes.
- Vortex
- Centrifuge

### 4.7.3 Materials for RT-PCR

- Primers sequence given in Table 4.2
- Invitrogen SuperScript III Platinum One step RT-PCR kit.
- 0.2ml PCR tubes
- Pipettors (l-10,20,200,1000)
- gloves (powder free)
- 1.5ml microtubes (nuclease free)
- 0.6ml microtubes (nuclease free)
- Filter barrier tips (p10 to p1000)
- Thermal Cycler.
- Extracted RNA
- Gel casting tray
- Electrophoresis chamber with power supply
- UV Transilluminator
- 2% Agarose gel in 0.5% TBE
- Ethidium Bromide

- Gel loading buffer (50% glycerol, 25% Bromophenol blue and >25% Xylene Xyanole)
- Molecular weight marker (100-1000 DNA ladder).

## 4.8 METHODOLOGY FOR NEURAMINIDASE TYPING OF INFLUENZA ISOLATES

### 4.8.1 Methodology for RNA Extraction

#### 4.8.1.a 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°C. 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°C until the precipitate is 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 using 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
Gently mix by inverting the tube 10 times.

**Buffer AW1**

Buffer AW1 is supplied as a concentrate. When using for the first time, 19 ml of 100 % ethanol was added as indicated on the bottle. Buffer AW1 is stable for 1 year when stored closed at room temperature, but was used only until the kit expiration date.

**Buffer AW2**

Buffer AW2 is supplied as a concentrate. When using for the first time, 30 ml of 100 % ethanol was added to Buffer AW2 concentrate as indicated on the bottle.

Buffer AW2 is stable for 1 year when stored closed at room temperature, but was used only until the kit expiration date.

**4.8.1.b METHOD**

- 560 µl of prepared Buffer AVL containing carrier RNA was pipetted into a 1.5 ml microcentrifuge tube.
- 140 µl cell-culture supernatant, or throat swab specimen was added to the buffer AVL–carrier RNA in the microcentrifuge tube and mixed by pulse-vortexing for 15 sec and incubated at room temperature (25°C) for 10 min.
- The tubes were briefly centrifuged to remove drops from the inside of the lid.
- 560 µl of ethanol (100%) was added to the sample, and mixed by pulse-vortexing for 15 sec. After mixing, the tubes were briefly centrifuged to remove drops from inside the lid.
- 630 µl of the solution from the above step was carefully added to the QIAamp Mini column (in a 2 ml collection tube) without wetting the rim. The cap closed, and centrifuged at 6000 x g (8000 rpm) for 1 min.
- The QIAamp Mini column was placed into a clean 2 ml collection tube, and the tube containing the filtrate discarded.
- Step 6 was repeated.
- To the QIAamp Mini column 500µl of Buffer AW1 was added and centrifuged at 6000 x g (8000 rpm) for 1 min. The QIAamp Mini column was placed in a clean 2 ml collection tube and the tube containing the filtrate discarded.

- 500 µl of Buffer AW2 was added to the QIAamp Mini column and centrifuged at full speed (20,000 x g; 14,000 rpm) for 3 min.

- The QIAamp Mini column was kept in a new 2 ml collection tube and the old collection tube with the filtrate discarded centrifuged at full speed for 1 min.

- The QIAamp Mini column was placed in a clean 1.5 ml microcentrifuge tube, the old collection tube containing the filtrate discarded.

- To the QIAamp Mini column 60 µl of Buffer AVE was added and equilibrated to room temperature and incubated at room temperature for 1 min. centrifuged at 6000 x g (8000 rpm) for 1 min.

- Viral RNA is stable for up to one year when stored at –20°C until used for cDNA synthesis.

### 4.8.2 Methodology for RT-PCR

#### 4.8.2.1 Methodology for RT-PCR

**One StepRT-PCR Reaction Mix**

25 µl reaction volume containing 12.5 µl of 2X RT PCR master mix, 0.5 µl of RT mix 1.0 µl of forward and reverse primers 5µl of Nuclease free water and 5 µl of RNA was taken into the reaction mix.

**Reaction Cycle**

The reaction mix was kept at 50°C for 30 minutes for reverse transcription, then at 94°C for 5 minutes for reverse transcriptase inactivation and was subjected to the following thermocycling profile in a thermocycler (Perkin elmer cetus, USA).

DENATURATION- 94°C for 10 seconds
ANNEALING - 55°C for 10 seconds
TEMPLATE EXTENSION- 72°C for 1 minute
The cycle was repeated 35 cycles.

FINAL EXTENSION- 72°C for 5 minutes (Cycling conditions illustrated in figure 4.5)

#### 4.8.2.2 Product analysis by Agarose Gel Electrophoresis

The PCR amplified products were analyzed on a 1.5% agarose gel with intercalating ethidium bromide dye. 10 µl of the amplified product was mixed with 1 µl of 10X loading dye and loaded into the wells along with 1 µl of molecular weight marker (20-1000 bp ladder). The electrophoresis was run at 100 volts in 0.5X TBE buffer. The gel was visualized under UV transilluminator and the product was compared with molecular weight marker.

## 4.9 MATERIALS FOR RT-PCR RFLP ANALYSIS

### 4.9.1 Viruses

- Prototype virus strains of *Influenza* A & B viruses.
- 24 *Influenza* A/H3N2 isolates obtained in the year 2002,
- 20 representative isolates of *Influenza* A (H1N1) obtained during the year 2005
- 12 B Victoria lineage isolates
- 62 B Yamagata Lineage viruses obtained from 2002-2007

### 4.9.2 Materials for RT-PCR

RNA extraction as in section 4.7.2

RT-PCR as in section 4.7.3

Primers listed in table 4.3

#### 4.9.2.a Materials for RFLP analysis

Enzymes refer table 4.3a

#### 4.9.2.b Materials for gel electrophoresis

- Gel casting tray
- Electrophoresis chamber with power supply
- UV Transilluminator
- 2% Agarose gel in 0.5% TBE

- Ethidium Bromide
- Gel loading buffer (50% glycerol,.25% Bromophenol blue and >25% Xylene Xyanole).

## 4.10 METHODOLOGY FOR RT-PCR AND RFLP

### 4.10.1 Methodology for RT-PCR

**RNA Extraction refer section 4.8.1.a., 4.8.1.b.,**

**One Step RT-PCR Reaction Mix**

25 µl reaction volume containing 12.5 µl of 2X RT PCR master mix, 0.5 µl of RT mix 1 µl of forward and reverse primers 5µl of Nuclease free water and 5 µl of RNA was taken into the reaction mix.

**Reaction Cycle**

The reaction mix was kept at 50°C for 30 minutes for reverse transcription, then at 94°C for 5 minutes for reverse transcriptase inactivation and was subjected to the following thermocycling profile in a thermocycler (Perkin elmer cetus, USA).

DENATURATION- 94°C for 5 mins
ANNEALING - 42°C for 2 mins
TEMPLATE EXTENSION- 72°C for 3 mins.
The above steps comprised the first cycle
DENATURATION- 94°C for 1 mins
ANNEALING - 42°C for 1 mins
TEMPLATE EXTENSION- 72°C for 2 mins
The cycle was repeated 24 times.
FINAL EXTENSION- 72°C for 10 mins.

### 4.10.2 Methodology for RFLP

In a 0.5 µl eppendorf tube 1 µl of Reaction buffer, 1 µl of enzyme and 15 µl of PCR product (1µl of 10X BSA was added to tubes containing Sac I) was added and

incubated at 37$^{o}$ C for 1 hour. 8 µl of gel loading buffer was added to each tube at the end of the incubation time.

10 µl of the sample + 1 µl GLB mixture was loaded on to the well of 2% agarose gel with ethidium bromide. 100-1000 bp molecular weight marker was used. The gel was run for 90 minutes at 50 V and the results were observed.

## 4.11 MATERIALS FOR GENETIC CHARACTERIZATION OF INFLUENZA VIRAL ISOLATES

**4.11.1 Viruses:** 86 isolates; 34 Influenza A/H1, 32 Influenza A/H3 & 20 Influenza B virus isolates obtained during the study period

**4.11.2 RNA Extraction: as in section 4.7.2**

**4.11.3 Materials for RT-PCR and sequencing**

- Primers listed in table 4.4
- Invitrogen SuperScript III Platinum One step RT-PCR kit.
- 0.2ml PCR tubes
- Pipettors (l-10,20,200,1000)
- Gloves (powder free)
- 1.5ml microtubes (nuclease free)
- 0.6ml microtubes (nuclease free)
- Filter barrier tips (p10 to p1000)
- Thermal Cycler.
- Extracted RNA
- Gel casting tray
- Electrophoresis chamber with power supply
- UV Transilluminator
- 2% Agarose gel in 0.5% TBE
- Ethidium Bromide
- Gel loading buffer (50% glycerol,.25% Bromophenol blue and >25% Xylene Xyanole)
- Molecular weight marker (100-1000 bp DNA ladder).

- QIA quick PCR purification kit (QIAGEN)
- ABI PRISM Big Dye Terminator V3.1 cycle sequencing kit
- Dye ex 2.0 spin kit (Qiagen)
- Hi-diformamide (ABI)
- ABI PRISM 310

## 4.12 METHODOLOGY FOR GENETIC CHARACTERIZATION INFLUENZA VIRAL ISOLATES

### 4.12.1 Methodology for RNA Extraction as in section 4.8.1.a and 4.8.1.b.

### 4.12.2 Methodology for RT-PCR and sequencing

#### 4.12.2.a Methodology for RT-PCR

**One Step RT-PCR Reaction Mix**

100 µl reaction volume containing 50 µl of 2X RT PCR master mix, 2 µl of RT mix 20pm of forward and reverse primers and 10 µl of RNA and 36 µl of nuclease free water were taken into the reaction mix.

**Cycling Conditions**

The reaction mix was kept at 50°C for 30 minutes for reverse transcription, then at 94°C for 5 minutes for reverse transcriptase inactivation and was subjected to the following thermocycling profile in a thermocycler (Perkin elmer cetus, USA).

1. DENATURATION- 94°C for 1 min
2. ANNEALING - 50°C for 1 min
3. TEMPLATE EXTENSION- 72°C for 1 min
   The steps 1-3 were repeated 35 cycles.
4. FINAL EXTENSION- 72°C for 5 mins. (Cycling conditions illustrated in figure 4.6)

#### 4.12.2.b Methodology for sequencing

PCR products were subjected to electrophoresis on 2% agarose gel in TBE buffer. 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 ex 2.0 spin kit (Qiagen) and eluted DNA was dried in a vacuum centrifuge, resuspended in 20µl of hi-diformamide (ABI) and denatured at 94°C for 2 mins and quenched on ice. Sequencing was performed on ABI PRISM 310 sequence alignment and phylogenetic analysis were done using mega version 3.1.

## 4.13 MATERIALS FOR IDENTIFICATION OF INFLUENZA VIRUSES BY POLYMERASE CHAIN REACTION

### 4.13.1 Viruses and isolates

*Influenza* A/H1 and A/H3, B prototype Viruses

60 suspected samples of *Influenza* collected within 7 days of onset of symptoms

60 suspected samples of *Influenza* collected after 7 days of onset of symptoms

### 4.13.2 RNA Extraction as in section 4.7.2

### 4.13.3 Materials required RT-PCR

- Primers listed in Table 4.5
- 0.2ml PCR tubes
- Viral RNA
- 2.5mM dNTP's
- AMV Reverse Transcriptase.
- Taq DNA polymerase.
- Double Distilled DEPC treated water
- 5X RT buffer
- 10X PCR buffer
- RNase Inhibitor.

### 4.13.4 Materials required for gel electrophoresis

- Gel casting tray
- Electrophoresis chamber with power supply

- UV Transilluminator
- 1% Agarose gel in 0.5% TBE
- Ethidium Bromide
- Gel loading buffer (50% glycerol,.25% Bromophenol blue and >25% Xylene Xyanole)
- Molecular weight marker (100-1000 bp DNA ladder).

## 4.14 METHODOLOGY FOR IDENTIFICATION OF *INFLUENZA* VIRUSES BY POLYMERASE CHAIN REACTION.

**4.1.4.1 Methodology for RNA extraction as in section 4.8.1.**

**4.14.1.a cDNA synthesis**

25 µl reaction volume containing 4 µl 2.5 mM dNTP's, 2.5 µl 10X RT buffer, 1.0 µl(1.0µm) of forward primer 0.5 µl (25 U) AMV Reverse Transcriptase and 0.5 µl of RNAse inhibitor, 11.5 µl of Nuclease free water and 5 µl of RNA was made.

**Reaction Cycle**

The reaction mix was kept at 42°C for 45 minutes for reverse transcription in a thermocycler (Perkin elmer cetus, USA).

**4.14.1.b ds DNA synthesis and amplification**

- To the 25 µl cDNA synthesized in the previous step10µl 10X PCR buffer 4 µl 2.5mM of dNTPs 1µl (1.0µm) of reverse primer and 1µl (1U) of Taq DNA polymerase and 59 µl of Nuclease free water was added and a 100 µl reaction was set up and tubes were placed in Thermocycler, programmed for amplification.

**Reaction Cycle**

The reaction mix was subjected to the following thermocycling profile in a thermocycler (Perkin elmer cetus, USA).

DENATURATION- 94°C for 5 mins

ANNEALING - 42°C for 2 mins

TEMPLATE EXTENSION- 72°C for 3 mins

The above steps comprised the first cycle

DENATURATION- 94°C for 1 min.

ANNEALING - 42°C for 1 min.

TEMPLATE EXTENSION- 72°C for 2 mins.

The cycle was repeated 24 times.

FINAL EXTENSION- 72°C for 10 mins

#### 4.14.1.c Product analysis by Agarose Gel Electrophoresis

The PCR amplified products were analyzed on a 1% agarose gel with intercalating ethidium bromide dye. 10 µl of the amplified product was mixed with 1 µl of 10X loading dye and loaded into the wells along with 1 µl of molecular weight marker . The electrophoresis was run at 100 volts in 0.5X TBE buffer for 60 mins. The gel was visualized under UV transilluminator and the product was compared with molecular weight marker.

### 4.15 STANDARDIZATION OF RT-PCR FOR DETECTION OF INFLUENZA VIRUS FROM THROAT SWABS

120 randomly selected specimen collected during the study period were used for the study. The RNA isolation protocol as described in section 4.8.1.was followed. For RT-PCR the template used was 10 µl (instead of 5µl in for detection of isolates from the previous experiment), the RT-PCR was done in a single step as the sequencing PCR.

#### One Step RT-PCR Reaction Mix

25 µl reaction volume containing 12.5 µl of 2X RT PCR master mix, 0.5 µl of RT mix 1.0 µl of forward and reverse primers and 10 µl of RNA was taken into the reaction mix. RT-PCR cycling conditions were set up as in fig. 4.7. Product analysis by Agarose Gel Electrophoresis was done as in 4.14.1.c.

## 4.16 STANDARDIZATION OF MULTIPLEX RT-PCR FOR DETECTION OF INFLUENZA VIRUS FROM THROAT SWABS

The RNA isolation protocol as described in section 4.4

### 4.16.a cDNA synthesis

25 µl reaction volume containing 4 µl 2.5 mM dNTP's, 2.5 µl 10XRT PCR buffer, 1.0 µl of forward primers of *Influenza* A H1,H3 and B viruses and B 0.5 µl (25 U) AMV Reverse Transcriptase and 0.5 µl of RNAse inhibitor, 9.5 µl of Nuclease free water and 5 µl of RNA was made.

**Reaction Cycle**

The reaction mix was kept at 42°C for 45 minutes for reverse transcription in a thermocycler (Perkin elmer cetus, USA).

### 4.16.b ds DNA synthesis and amplification

- To the 12.5 µl cDNA synthesized in the previous step 5 µl 10XPCR buffer 2 µl 2.5mM of dNTPs 1µl of each reverse primer, 1µl (1U) of Taq DNA polymerase, 1 µl of KCl, 1 µl of ammonium sulphate 24.5 µl nuclease free water was added a 50 µl reaction was set up and tubes were placed in Thermocycler, programmed for amplification.

**Reaction Cycle**

The reaction mix was subjected to the following thermocycling profile in a thermocycler (Perkin elmer cetus, USA).

DENATURATION- 94°C for 5 mins
ANNEALING - 42°C for 2 mins
TEMPLATE EXTENSION- 72°C for 3 mins.
The above steps comprised the first cycle
DENATURATION- 94°C for 1 min
ANNEALING - 42°C for 1 mins

TEMPLATE EXTENSION- 72°C for 2 mins.

The cycle was repeated 24 times.

FINAL EXTENSION- 72°C for 10 mins

Product analysis by agarose gel electrophoresis was done as in 4.14.1.c.

## 4.17 MATERIALS FOR SCREENING THE SENSITIVITY OF INFLUENZA VIRUS ISOLATES TO ANTIVIRALS BY MIC:

**Viruses** As reference viruses, amantadine-resistant viruses which were produced through serial (five) passages of sensitive strains in media containing 2μg/ml amantadine.

**Isolates:** 162 *Influenza* A isolates obtained during the study period were used.

**Cell culture** requirements as in section 4.3.

## 4.18 METHODOLOGY FOR SCREENING THE SENSITIVITY OF INFLUENZA VIRUS ISOLATES TO ANTIVIRALS BY MIC

Drug susceptibility was determined by growing *Influenza* virus isolates in MDCK cell cultures in the presence of amantadine at a concentration of 1 μg/ ml ]. Ninety-six-well cell culture plates were seeded with MDCK cells in a concentration of 1 lakh cells per ml in medium supplemented with 10% fetal bovine serum, 0.2% bovine serum albumin, 25 m*M* HEPES, penicillin, and streptomycin were added to each well, and the plates were incubated at 37$^{o}$C in a humidified 5% $CO_2$ atmosphere for 48 hours. Then growth medium, was removed the monolayer washed thrice with virus growth media (described in virus isolation part). 100 μl of 1,10 and 100 $TCID_{50}$ dilutions of viruses in virus growth media were added to the confluent MDCK cell monolayer and the plates were incubated at 37$^{o}$C in a humidified 5% $CO_2$ atmosphere. Cell control, drug sensitive virus controls (Virus without the drug) and drug resistant virus control (amantadine Resistant Virus with drug) were also included. Amantadine-resistant viruses were produced through serial (five) passages of sensitive strains in the presence of amantadine (2μg/ml) in MDCK cell lines, the prototype *Influenza* A

viruses procured from NIV were used for drug sensitive controls. The plates were observed for 7 days and results were recorded.

## 4.19 MATERIALS FOR DETECTION M2 MUTANTS BY RT-PCR RFLP

**Viruses.** As reference viruses, we used amantadine-resistant viruses which were produced through serial (five) passages of sensitive strains in the presence of amantadine (2μg /ml) in vitro.

**Isolates**: 162 *Influenza* A isolates obtained during the study period were used.
Materials for RNA extraction as in section 4.7.2
Materials for RT-PCR and Gel electrophoresis as in section 4.7.3
Primers and enzymes used listed in table 4.6.

## 4.20 METHODOLOGY FOR DETECTION M2 MUTANTS BY RT-PCR RFLP

### One Step RT-PCR Reaction Mix

A 339 bp segment of M2 gene was first amplified by using M2F and M2R primers. 25 μl reaction volume containing 12.5 μl of 2X RT PCR master mix, 0.5 μl of RT mix 1.0 μm of forward and reverse primers 6μl of Nuclease free water and 5 μl of RNA was taken into the reaction mix.

### Cycling Conditions

The reaction mix was kept at 50°C for 30 minutes for reverse transcription, then at 94°C for 5 minutes for reverse transcriptase inactivation and was subjected to the following thermocycling profile in a thermocycler (Perkin elmer cetus, USA).

DENATURATION- 94°C for 30 sec.
ANNEALING - 55°C for 30 sec.
TEMPLATE EXTENSION- 72°C for 1 min.
The cycle was repeated 35 times.
FINAL EXTENSION- 72°C for 7 mins. (Cycling conditions illustrated in fig. 4.8)

**Nested PCR**

25µl reaction volume containing 12.5µl of 2 x PCR master mix, 1 µl of forward and reverse primers each, 1 µl of PCR product as template and 9.5µl of nuclease free water.

DENATURATION- 94°C for 30 sec.

ANNEALING - 50°C for 30 sec.

TEMPLATE EXTENSION- 72°C for 30 sec.

The cycle was repeated 30 times

FINAL EXTENSION- 72°C for 7 mins. (Cycling conditions illustrated in fig. 4.9)

**PCR-RFLP analysis**

Each 5µl aliquot of nested PCR product was treated with specific endonucleases. Those amplified with M2-27F and M2-27R were digested with 5 U of *Bsp*Lu11I for 2 h at 48°C in 1.5 µl of buffer recommended by the manufacturer and 8.0 µl of sterile distilled water. Those amplified by M2-30F and M2-30R were digested with 5 U of *Hha*I and M2-31F and M2-31R, were digested with *Sca*I, for 2 h at 37°C, respectively, with the same mixture ratio of buffer to distilled water. The digested samples were analyzed by electrophoresis using 4% agarose gels containing ethidium bromide. The restriction fragments were separated in 0.5 X Tris-borate-EDTA buffer at 100 V for 30 min and examined by transillumination before being photographed.

## 4.21 MATERIALS TO SCREEN HERBS FOR ANTIVIRAL ACTIVITY OF HERBS

**HERBS**

***Ocimum sanctum*** Linn: Labiatae

Commonly called Sacred Basil, Holy Basil.

**Tamil**-Krishna Tulsi,

**Sanskrit** –Ajaka, Brinda,

**Hindi**-Kala Tulsi,

These aromatic purple leaf herbs is seen in tropical and warm temperature of the world. Fresh leaves were collected form the outskirts of Chennai City.

**Glycyrrhiza glabra**

**Tamil** - Adhimadhuram
**Parts used** - Peeled root, leaves .
**Sanskrit** - Yashti- Madhu, Yashti-madhuka.
**Hindi** -Mulhathi, Jethi-madh

This tree is seen in tropical and warm temperature of the world. Dried powder was procured from Anna Hospital Chennai.

Cell culture requirements as in section 4.3.

## 4.22 METHODOLOGY TO SCREEN HERBS FOR ANTIVIRAL ACTIVITY OF HERBS

### 4.22.a Preparation of MDCK cell monolayer - 24 well microtitre plates

MDCK Cells of concentration 1 lakh cells/well was seeded in 24 well micro titer plates and incubated for 48 hrs at 36$^{o}$C in 5% $CO_2$ environment.

### 4.22.b Preparation of extracts

**Preparation of leaf powder**

Freshly collected leaves of ***Ocimum sanctum***, were cleaned, shade dried. The dried leaves were pounded to coarse powder in a low speed blender and stored in air tight opaque container till further extracted.

**Glycyrrhiza glabra** powder was commercially procured.

**Preparation of Aqueous and Ethanolic extract**

1 gm of the powdered leaves of Ocimum sanctum and powder of Glycyrrhiza glabra were soaked in 100ml of distilled water and 100ml of absolute ethanol separately. The mixture was kept in the rotary shaker for 48 hours. The contents were filtered through muslin cloth and the filtrate was dried at 55$^{o}$C. The sediments were

re-extracted as mentioned above. The dried extract was scraped and stored at 4$^{o}$C in air tight vials.

100 mg of each of the aqueous and ethanolic extracts of four herbs were dissolved in 10 ml of MEM. Working concentrations of extracts ranging from 10 micrograms to 1 mg were prepared freshly and filtered through 0 .45 microns filter before each assay.

### 4.22.c Preparation of stock virus (*Influenza* A & B pool)

- Confluent healthy monolayer MDCK Cell line 75cm$^2$ bottle was selected.
- The growth medium was decanted and the cell layer was rinsed gently in PBS. 600 µl (100 µl each) of known $TCID_{50}$ of *Influenza* A/H1N1, A/H3N2, B/Victoria and B/Yamagata viruses were mixed together.
- The viruses were allowed to adsorb on the cell monolayer for half an hour at 37$^{o}$C.
- Sufficient amount of medium was replenished and bottles were incubated at 37$^{o}$C, and observed daily for CPE
- The bottles were frozen on the day 4+ CPE observed.
- The bottles were frozen and thawn three consecutive times and centrifuges at 6000 rpm for 30 minutes and the supernatant was collected.
- The $TCID_{50}$ of the virus pool were estimated, aliquoted in 2 ml vials and stored at –80$^{o}$C

### 4.22.d Estimation of $TCID_{50}$ of *Influenza* A&B virus pool

- 1.8 ml of MEM without FCS was taken in 9 sterile tubes labeled from $10^{-1}$ to $10^{-9}$
- 200 µl of the *Influenza* A&B virus pool was added to the first tube ($10^{-1}$ dilution) using sterile pipette and mixed thoroughly.

- 200 µl from $10^{-1}$ dilution was added to the next tube and mixed. The dilution continued until 200 µl from $10^{-8}$ was added to $10^{-9}$.

- 100 µl from each dilution were added in a row 10 wells of A to H labeled from –1 to –8.

- Five wells each of $11^{th}$ and $12^{th}$ rows were used as cell control, to which 100 µl of MEM without FCS was added.

- 100 µl of MDCK cell suspension ($10^5$ cells /ml) was added to all the wells

- The plates were incubated at $37^oC$ in an incubator maintaining 5% $CO_2$ environment. The plates were observed on day 3, 5 and 7, CPE was recorded (figure4.12) and $TCID_{50}$ was estimated by Karbers formula.

### 4.22e Cytotoxicity Assay (Derrick J Dargan Vol.10 and Kailash Chandra *et al.*, 1998)

In order to study the antiviral activity of a new drug, it is important to determine whether antiviral activity can be uncoupled from the confounding effect to cellular toxicity. Cytotoxicity tests define the upper limit extract concentration, which can be used in subsequent antiviral studies.

- MDCK cells of concentration one lakh cells /well was seeded in 24 well micro titer plates and incubated for 48 hrs at $36^0$ C in 5% $CO_2$ environment.

- The growth medium is decanted and medium (W/O FCS) containing increasing concentration of the extracts (10 to 1000 µg) was added in tetrads.

- Cell Control containing extract free medium and neat extract controls were included .

- The plates were incubated at $37^oC$ in 5% CO2 environment, $3^{rd}$, $5^{th}$ and $7^{th}$ day reading was noted microscopically for cytotoxicity (Figure 4.13).

- Microscopic reading was confirmed by MTT assay.

## 4.23 ANTIVIRAL ASSAY

The test was performed in tetrads. Cell control, Virus control and back titration of the Virus used were performed simultaneously.

Concentration of extracts used: From the stock (100 mg/10 ml) varying concentration of non-toxic dose of the drug were prepared in minimum essential medium without FCS and filtered.

Virus concentration used: 1, 10, 100 $TCID_{50}$ of A&B virus pool was prepared in minimum essential medium (MEM) without FCS based on the initial $TCID_{50}$ of the virus pool.

The anti viral assay was performed with non-toxic doses of the extract by

- Direct Pre-infection Incubation (DPI) assay or viral inactivation assay **(Sidewell RA and Huffman T.H. 1971)**
- Viral Adsorption assay **(Derrick J. Dargan, Vol.10)**
- Pre- treatment assay **(Premanathan *et al.*, 1994)**

### 4.23.a Direct Pre- infection incubation (DPI) assay or viral inactivation assay

**Procedure**

- To the 24 well TC plates with confluent MDCK monolayer, varying concentration of the filtered extracts (50, 100, 200, 300 µg / 0.5 ml) to which 500 µl of 1, 10, 100 $TCID_{50}$ of the viruses were added individually and incubated for 0 hrs, ½, 1, 1½ & 2 hrs in 37°C. The extract virus mixture was then added to 24 well plates and incubated for seven days at 37°C in 5% $CO_2$ environment. 3rd and 5th day reading was noted microscopically for cytopathic effect (Figure 4.14).

- Cell control, Solvent control, Virus control was performed along the assay. The effect of room temperature and time exposure on the different $TCID_{50}$ of the virus used for the assay was determined simultaneously.

- Tertrazolium blue reduction (MTT) assay was performed.

**Inference**

- The concentration of extracts showing no cytopathic effect was recorded. The wells showing 25%,50% and 75% CPE was further subjected to Virus titration to determine the $TCID_{50}$ after the action of extracts on virus.

**4.23.b Viral Adsorption assay**

- 1,10,100 $TCID_{50}$ of the Viruses were separately adsorbed on MDCK cell monolayer of 24 well TC plate for one hour and incubated at 37$^{o}$C. The unbound viruses was washed with PBS (pH 7.2).

- The nontoxic concentration of the extracts were added to each of the wells. The plates were incubated for 7 days at 36$^{o}$C in 5% $CO_2$ environment (figure 4.15).

- Plates were microscopically examined for cytopathic effect.

- MTT assay was performed.

**Inference**

- The concentration of extracts showing no cytopathic effect was recorded. The wells showing 25%,50% and 75% CPE was further subjected to Virus titration to determine the $TCID_{50}$ after the action of extracts on virus.

**4.23.c Pre-treatment Assay**

- The varying nontoxic concentration of the extracts of were added to each of the wells and incubated for 2 hrs, 4 hrs, 6 hrs and 8 hrs 1,10,100 $TCID_{50}$ virus were separately added to each concentration after incubation time. The plates were incubated for 7 days at 37$^{o}$C in 5 % $CO_2$ environment (figure 4.16 ).

- The plates were microscopically observed on 3$^{rd}$ 5$^{th}$ and 7$^{th}$ day for Cytopathic effect.

- MTT assay was performed.

**Inference**

- The concentration of extracts showing no cytopathic effect was recorded. The wells showing 25%,50% and 75% CPE was further subjected to virus titration to determine the $TCID_{50}$ after the action of extracts on virus.

**4.23.d MTT assay (Alley *et al.*, 1989)**

**Principle**

MTT is cleaved by mitochondrial dehydrogenase in viable cells, yielding a measurable purple product formazan. This formazan production is proportional to the viable cell number and inversely proportional to the degree of cytotoxicity.

**Reagents**

MTT (3-(4,5-Dimethyl Thiazol-2yl)-2,5-diphenyl tetrazolium bromide)
Stock Solution (5 mg/ml)
MTT - 50 mg
PBS - 10 ml

Vortex for 20 minutes, filter sterilized with 0.45 microns filter. Wrapped the bottle in aluminum foil or paper to block light as MTT is light sensitive. Store at $4^oC$. Prepare fresh stock was prepared every month.

**Procedure**

To 24 well TC plate vero cells of 1 lakh cells/ml /well is seeded and incubated for 48 hrs at $36^oC$ in 5% $CO_2$ environment.

The cytotoxicity assay and antiviral assay was carried out as described in this chapter.

The supernatant was discarded and washed once with cold PBS.

1 ml of 5 mg/ml of MTT (F/S) was added to each well and Controls were treated in same way as tests.

The plate was incubated at $37^oC$ for 4 hrs.

1 ml of DMSO was added to each well after the incubation and aspirated to break the cells to dissolve the formazan crystals.

The color developed was read spectrophotometrically at 590 and 690 nm respectively.

The Correction value was calculated by

Absorbance at 690 nm – Absorbance at 590 nm.

Graph was plotted using the corrected OD at Y-axis and concentration of the extract in X-axis.

Cell control and extract control was included in each assay to compare the fall of cell viability in antiviral assessments.

# 5. RESULTS

## PART – I

## SURVEILLANCE, ANTIGENIC AND GENETIC CHARACTERIZATION OF INFLUENZA VIRUSES

### 5.1.1 Serosurveillance of Influenza in Chennai

A serosurvey for the presence of antibodies to the circulating *Influenza* A & B among the apparently healthy population of Chennai was done during 2001-2006.

In the present study, the exposure of the population to *Influenza* viruses was determined by detecting the haemagglutinating IgG antibodies to the *Influenza* viruses in four age groups 0-5, 6-15, 16-45, and 46-60 by haemagglutination inhibition test. 50 samples from apparently healthy individuals of each age group were tested each year, 2001 to 2006.HAI test was performed as per standard WHO protocols and using WHO reagent kit supplied by *Influenza* branch Centres for Disease Control and Prevention, Atlanta.

In the year 2001 the overall seropositivity for *Influenza* viruses was 36.45 % and it reduced in the year 2003 to 28.12% and increased in the following years to a high of 38.86% in 2006 (Table 5.1).

The seropositivity for *Influenza* A viruses was more than that of *Influenza* B viruses in the year 2001 in rest of the years seropositivity was more for *Influenza* B viruses.

Geometric mean titre was calculated and is shown in table 5.2. Standard Deviation values were analysed for *Influenza* A&B antibody titres. Applying the Mean +2SD, the baseline titre was calculated. The baseline titre was 1:40 for the *Influenza* A & B viruses (Table 5.2 & Fig 5.1).

The study puts into light the variation in the exposure of the study population to circulating *Influenza* viruses in different years thus indicating the circulation of

different strains during different periods. 2001 was dominated by A/H3N2 among the A type virus and B/Victoria lineage among the B type viruses. From then on there has been a steady increase in the exposure of the population to A/ H1N1 and B/Yamagata lineage viruses. A/H1N1 predominated from 2005 and B/Hong Kong predominated in 2006 (Table 5.1).

### 5.1.1.a Age and Gender wise distribution of Influenza antibodies

The seropositivity of the different age groups of the study population to *Influenza* is depicted in Table 5.3 & Fig 5.2. The results showed that the 6-15 years age group showed high exposure to *Influenza* in all the years. Statistical analysis of the results showed the exposure of 6-15 years age group was statistically significant(Chi square test $p<0.0001$) when compared with the other age groups whereas there was no significant difference among the different age groups studied.

Genderwise distribution of *Influenza* antibody titre of the study population has been depicted in table (5.4 & Fig 5.3). There was no significant difference in the antibody titres.

### 5.1.2 Virus isolation and antigenic characterization of Influenza virus isolates

A total of 3036 samples were collected during the period 2002-2007. Of these 236 were positive for *Influenza* viruses indicating 7.77% positivity. *Influenza* A Viruses predominated during 2002-2006; but in the year 2007 there was a change in the *Influenza* virus activity with predominance of *Influenza* B viruses (Table 5.5 & Fig 5.4).

*Influenza* A /H3N2 predominated the years 2002-2004 and A/H1N1 was first isolated in the year 2005 and predominated during 2005 and 2006 and in 2007 it was the predominant among the A type viruses isolated.

*Influenza* B Victoria lineage virus was isolated in the years 2002, 05, 06 & 07 it was the only B type virus isolated in the year 2002 whereas the other years were predominated by *Influenza* B Yamagata lineage virus.

Seasonal variation was observed in the isolation rates of the viruses (Table 5. 7 & Fig 5.6) but there was no significant difference in the gender wise distribution (Table 5. 8 & Fig. 5.7).

### 5.1.2.a A/H1N1 Viruses

A/H1N1 viruses are those, which were the etiological agents of the first *Influenza* pandemic (Spanish Flu) caused in 1918 that resulted in approximately 40 million deaths worldwide.

A/H1N1 viruses were not isolated in the first three years of our study but appeared for the first time in 2005. From then on 82 viruses were isolated during the period 2005-2007. The predominance was high in the year 2005 during which they constituted for 71.1% of the total isolates in the year 2006 the prevalence was slightly reduced to 60.86% and in 2007 their prevalence was just 18.75% (Table 5.5b).

A/H1N1 virus was the predominant type among the *Influenza* A type viruses isolated during the period 2005-2007. It accounted for 82 of the 100 *Influenza* A viruses isolated. Though it predominated there was co circulation of A/H3N2 viruses also during the same season (Table 5.5a1).

Virus isolation was high during the winter season and significant in the late winter and monsoon season but there was no isolation reported during the summer months (Tables 5.15-5.20). The isolates were obtained from all the age groups but the isolation rate was high in the Pediatric and geriatric age group (Table 5. 6 & Fig 5.5).

### 5.1.2.b A/H3N2 Viruses

A/H3N2 viruses were the etiological agents of the third *Influenza* pandemic (Hong Kong Flu) caused in 1968 that resulted in approximately 700,000 deaths worldwide.

A/H3N2 viruses are the only type of virus that was isolated in all the years of the study period. The virus predominated for a period of 3 years (2002-2004) and declined rapidly after that. It constituted for 86.67% of the isolates in the year 2002,

93.4% in 2003, and 100% in 2004. Its prevalence started to dip from the year 2005 during which 18.64%, 10.86% in 2006 and 3.12% were isolated in 2007 (Table 5.5c).

A/H3N2 virus was the only *Influenza* A type isolated during the period 2002-2004, though it was predominated by A/H1N1 virus from the year 2005 it continued to cocirculate along with A/H1N1 viruses in the years 2005-2007 also.

Viruses were isolated throughout the year including the summer months, though the isolation rate was high during the winter months (Tables 5.9-5.20). Majority of the isolates were obtained from the Pediatric and to a certain extent from the geriatric age group (Table5. 6 & Fig 5.5).

### 5.1.2.c Influenza B type viruses

Recent isolates of *Influenza* B viruses, for example, fall into two major phylogenetic lineages: the B/Victoria/2/87 lineage or the B/Yamagata/16/88 lineage. The former group of viruses was found primarily in East Asia during the last decade. In contrast, the B/Yamagata/16/88-like viruses have been found worldwide during the same period. The B/Victoria/2/87 lineage started to spread to other regions of the world from 2002 onwards.

In our study *Influenza* B types of both the lineages were isolated. Total of 74 *Influenza* B type viruses were isolated during the study period of which 62 (83.78%) were of the B/Yamagata/16/88 lineage and 12 (16.21%) were of the B/Victoria/2/87 lineage (Table 5.5 & Fig 5.4).

*Influenza* B/Victoria/2/87 lineage viruses were isolated during the years 2002 and 2005 – 2007. The isolation rate was high in the year 2002 where they accounted for 20 (13.34%) of the total isolates (Table 5.5 & Fig 5.4).

*Influenza* B/Yamagata/16/88 lineage viruses were isolated during the years 2003 and 2005 – 2007. The isolation rate was high in the year 2007 where they accounted for 71.8% of the total isolates and 92% of the *Influenza* B isolates (Table 5.5, 5.5a2 & Fig 5.4).

*Influenza* B type viruses were predominantly isolated during the winter months and rarely during the late winter and summer months (Tables 5. 9-5.20).

*Influenza* B/Victoria/2/87 lineage viruses were isolated predominantly from the pediatric population and from the geriatric population whereas the *Influenza* B/ Yamagata/16/88 lineage viruses were isolated from all the age groups predominantly from the paediatric middle age group (Table 5.6 & Fig 5.5).

### 5.1.2.d Clinical signs and symptoms of Influenza cases

Table depicts the clinical signs and symptoms of *Influenza* cases. 100% of suspected *Influenza* viral infections had fever, 93% had cough, 63 % of cases had headache, 92.1% of them showed nasal discharge and 72% of them presented with pharyngitis. 73.6% cases showed myalgia and 78% reported productive cough and 64% had symptoms of vomiting and diarrhea but only 8% of cases reported fever with sudden onset (Table5.21 & Fig 5.8).

Among the *Influenza* positive cases all had sudden onset of symptoms. Fever, cough was present in all the cases followed by headache (92.4%). The other symptoms that were predominantly observed were Myalgia (83.6%), nasal discharge (73%), pharyngitis (67%), Productive cough and vomiting and diarrhea were reported in (23%) and (39%) of the cases respectively. The presence of headache and myalgia along with sudden onset of symptoms, fever and cough were indications of *Influenza* infection among the acute respiratory Infections (Table 5.22 & Fig 5.9).

### Predisposing factors and association of chronic conditions in the elderly with Influenza infections

The presence of both cardiovascular and pulmonary disease was seen in 53% of the positive cases in the elderly. Cardiovascular and diabetes was associated with 30% of the cases pulmonary disease alone was found in the 12% of the cases and the rest 4.6% presented with cardiovascular disease alone (Table 5.23 & Fig 5.10).

### 5.1.3 NEURAMINIDASE TYPING OF INFLUENZA A ISOLATES:

Of the 162 *Influenza* A virus isolates typed for NA, the 82 *Influenza* A/H1 types generated the N1 neuraminidase type (708 bp product) and the 80 *Influenza* A/H3 types generated the N2 Neuraminidase type (615 bp product). There was no H1N2 or H3 N1 type indicating that there has not been any reassortment between the circulating *Influenza* serotypes (Figure 5.16 & 5.17).

### 5.1.4 DETECTION OF ANTIGENIC VARIANTS RT-PCR RFLP

Antigenic variation among the *Influenza* isolates was studied by RT-PCR and RFLP analysis.

- RT-PCR products obtained from prototype virus strains were subjected to RFLP.
- 24 *Influenza* A/H3N2 isolates obtained in the year 2002, 20 representative isolates of *Influenza* A (H1N1) obtained during the year 2005 and 12 B Victoria lineage isolates and 62 B Yamagata Lineage viruses obtained from 2002-2007 were subjected to RT-PCR RFLP analysis.
- *Influenza* A prototype viruses were digested with their respective enzymes but the H1N1 and H3N2 isolates obtained during the study were not digested.
- As the A isolates did not digest RFLP was not performed on isolates obtained after 2005.
- *Influenza* B prototype viruses and currently circulating strains were digested by their respective enzymes.
- This indicates that the *Influenza* A viruses have undergone drifting and mutated at the restriction site (Figure 5.18-5.21).

### 5.1.5 MOLECULAR CHARACTERIZATION OF INFLUENZA A&B ISOLATES BY HA REGION SEQUENCING

Representative samples of *Influenza* A&B viruses isolated from cases of acute respiratory infection were sequenced. The HA sequences of *Influenza* A&B viruses were edited by BioEdit analyzed by pairwise sequence alignment method using BLAST and Multiple sequence alignment by Clustal W. The similarity and divergence table was by Dismat module of EMBOSS. Aminoacid translation was done with the software DNA TRANS and all the phylogenetic analysis was carried out with MEGA 3.1.

Of the 236 *Influenza* viruses isolated during this study period 84 representative isolates were sequenced. Of this 86 isolates 34 were A/H1N1 (2005-2007) 32 (2002-2007) were A/H3N2, 7 were of B/Victoria lineage(2002, 2005-2007) and 13 were B/ Yamagata lineage viruses (2005-2007) (Table5.21).

The Gene bank database was used for comparison of Chennai strains with *Influenza* vaccine sequences. The use of CLUSTAL W for nucleotide and amino acid multiple sequence alignment using default setting was reported by Michael Lindberg *et al.* (2003), the same was adopted for this study. The use of neighbor joining method for the construction of specific tree topologies were similar to the methods adopted by **Oberste M.Steven *et al.* (1999c) and Hideyuki Kubo *et al.* (2002).**

#### *5.1.5.a* HA sequence analysis of the epidemic strains

**Influenza A/H1N1**

Figure 5.22 shows the phylogenetic relationships of HA for H1N1 strains, including 34 selected Chennai strains from 2005 to 2007 and three reference strains.

The isolates clustered into two distinct groups I and II with the group II being subdivided into group IIa and IIb. Eighteen isolates clustered with very high degree of similarity with A/H1/New Caledonia/20/99, the 2000 – 2007 vaccine strain into group I . Group II contained the remaining 16 isolates with eight of them in group IIa clustered with A/H1/New Caledonia/20/99 with a slightly less similarity than the group I isolates and the other eight isolates clustered with A/Solomon Islands/3/06 as

group IIb. The amino acid translation of the HA region sequence showed an average amino acid homology of 98.2% between the Chennai A/H1N1 serotypes. 7 site mutations were seen that are discussed in the next chapter.

### 5.1.5.b Influenza A/H3N2

Figure 5.23 shows the phylogenetic relationships of HA for H3N2 strains, including 32 selected Chennai strains from 2002 to 2007 and five reference strains.

The nucleotide sequence identity in terms of HA genes among Chennai 2002-2007 H3N2 isolates was 96.9 to 100%, based on a 789-nt HA segment .The isolates clustered into five distinct groups I to V. Five 2002 isolates were clustered to group I and were identical to the 00 –02 vaccine strain A/Panama/2007/99 (7). six isolates gathered from 2002 to 2004 clustered to group II, which was identical to the 2002-2003 vaccine strain A/Mos/5/97 (9–11). eleven isolates (2003–2004) were clustered in group III, together with the 2003–2005 vaccine strain A/fujian/10/99 (13–15) , four 2004–2005 isolates were clustered in group IV and were identical to the 2005–2006 vaccine strain A/California/411/02 and the remaining 6 isolates of the years 2005-2007 were clustered into group V and were identical to the 2006–2007 vaccine strain A/Wisconsin/411/02. The amino acid translation of the HA region sequence showed an average amino acid homology of 98.6% between the Chennai A/H3N2 serotypes. 14 site mutations were seen that are discussed in the next chapter.

### 5.1.5.c Influenza B type viruses:

Figure 5.24 shows the phylogenetic relationships of HA for *Influenza* B strains, including 20 selected Chennai strains from 2002 to 2007 and five reference strains. Two reference strains B/Victoria/2/87 and B/Yamagata/16/88 were included.

Identities among these 18 Chennai strains, based on a 396-nt HA segment, ranged from 87.3 to 100%. Notably, the Chennai *Influenza* B isolates accumulated significant variation in terms of HA nucleotide sequences (12.7% from 2002 to 2007), compared to the Chennai *Influenza* A isolates (3.7% for H1N1 from 2005–2006, and 3.9% for H3N2 from 2002 to 2007). Seven Chennai isolates 2002–2007 isolates were in line with the B/Victoria/2/87 lineage and formed group I, were clustered together

with the 2002–2004 vaccine strain B/Hong Kong/330/01 (14, 15). Thirteen other Chennai *Influenza* B isolates, together with the 2004–2005 vaccine strain B/Shanghai/361/02 (15), clustered into group II. They (including three vaccine strains) all experienced a characteristic deletion of N at position 189 of HA protein, as did B/Yamagata/16/88. On the other hand, all strains in cluster I retained this amino acid, as B/Victoria/2/87 did. The amino acid translation of the HA region sequence showed an average amino acid homology of 88.3% between the Chennai *Influenza* B serotypes. The mutations are discussed in the next chapter.

# PART - II

## 5.2 STANDARDIZATION AND EVALUATION OF RAPID MOLECULAR DIAGNOSTIC TOOL - RT-PCR

Molecular diagnosis for the rapid detection of *Influenza* viruses from tissue culture fluids as well as the specimen extracts by multiplex RT-PCR was standardized with minor modification of the original CDC, protocol. . The primers were selected in such a manner that they amplified a 517 bp product for *Influenza* A/H1N1, 785 bp for H3N2 and 618 bp product for B type viruses (Figure 5.25).

- The detection level of the RT-PCR was evaluated by subjecting 2 fold dilutions of standard *Influenza* A & B virus stocks ranging form 1000 to 0.001 $TCID_{50}$.

### 5.2.a Evaluation of RT-PCR directly from clinical samples in comparison with Virus Isolation

60 samples that were collected within 7 days of onset of illness and 60 samples that were collected after 7 days of onset of illness were selected and RT-PCR was performed. An uninoculated cell control and 10 samples collected from healthy individuals were also screened as negative controls .

- The results of the RT-PCR were compared with results of virus isolation (Table 25).
- Of the 60 samples collected within 7 days of onset 10 (16.66%) were positive. Of these only 6 samples were positive by virus isolation.
- Among the 60 samples collected after 7 days (up to 15 days) of onset 6 (10%) were positive and none of them was positive by virus isolation (Figure 5.26).
- Uninoculated cell control and the 10 samples that served as controls were negative by RT-PCR and virus isolation.

- RT-PCR when compared with virus isolation by tissue culture was sensitive in detecting *Influenza* viruses from samples collected within 7 days and detected *Influenza* in samples collected even after 7 days.

- The specificity, Sensitivity, Positive predictive value and negative predictive value were 60%, 100%, 92.5% and 100% (Table 25A). These values were found to be statistically significant (Chi square = 268.32; $p<0.001$).

### 5.2.b Standardization and Evaluation of Multiplex RT-PCR directly from clinical samples in comparison with RT-PCR.

60 samples that were collected within 7 days of onset of illness and 60 samples that were collected after 7 days of onset of illness were selected and Multiplex RT-PCR was performed. An uninoculated cell control and 10 samples collected from healthy individuals were also screened as negative controls.

- The results of the Multiplex RT-PCR were compared with results of strain specific RT-PCR.

- Of the 60 samples collected within 7 days of onset 10 (16.66%) were positive by both the methods.

- Among the 60 samples collected after 7 days (up to 15 days) of onset 6 (10%) were positive by both the methods.

- Uninoculated cell control and the 10 samples that served as controls were negative by both the methods.

- Multiplex RT-PCR when compared with RT-PCR was equally sensitive in detecting *Influenza* viruses from samples collected within 7 days and detected *Influenza* in samples collected even after 7 days (Figure 5.27).

- KCl when used in the concentration of 1 µM and 20 mM ammonium sulphate yielded accurate, sensitive and reproducible results.

# PART - III

## 5.3 SENSITIVITY TO ANTIVIRALS

### 5.3.1.a Screening of sensitivity to antivirals by MIC

- 162 *Influenza* A viruses were screened for their sensitivity to the M2 Inhibitor amantadine.
- Drug susceptibility was determined by growing *Influenza* virus isolates in MDCK cell cultures in the presence of amantadine at a concentration of 1 μg/ ml.
- Amantadine-resistant viruses were produced through serial (five) passages of sensitive strains in the presence of amantadine (2μg/ml) in MDCK cell lines.
- The prototype *Influenza* A viruses procured from NIV were used for drug sensitive controls. The plates were observed for 7 days and results were recorded.
- The drug resistant strains grew well even in the presence of the drug.
- The drug sensitive controls did not grow in the presence of drug.
- All the H1N1 isolates were sensitive and did not show any resistance.
- Of the 80 H3N2 Isolates 4 were resistant to amantadine. 60 viruses that were obtained during the years 2002-2004 did not show any resistance. Among the 11 isolates in 2005, 2 were resistant, of the 5 isolates in 2006 one was resistant and among the 2 isolates in 2007 one was resistant .
- Though there have been reports of high degree of resistance to amantadine in the US and Southeast Asian countries, this study did not showed only an emerging resistance pattern to amantadine.
- Amantadine not being used widely for the treatment of *Influenza* in India and this may be the reason behind the lack of resistance among the isolates for amantadine and the emerging resistance may be due to the importation of strains from the regions that show resistance.

### 5.3.1.b Detection M2 mutants RT-PCR RFLP

- Single-amino-acid changes at four positions, 26,27, 30, and 31, within the transmembrane domain of the M2 protein have been reported to confer resistance to amantadine among *Influenza* viruses.
- RT-PCR RFLP is the widely used and sensitive method for detecting any mutations in the genes. The same has been used to detect the mutations in the genes coding for M2 protein.
- The results of the MIC method were reconfirmed by performing a RT-PCR RFLP for detection of amantadine resistance.
- 162 *Influenza* A virus isolates that were screened for amantadine resistance by MIC method were subjected to RT-PCR RFLP.
- The drug resistant viruses did not show restriction pattern thus indicating mutation in the M2 protein.
- The drug sensitive controls showed restriction pattern.
- All the 82 H1N1 isolates showed restriction pattern similar to the drug sensitive controls thereby indicating that all H1N1 the isolates have not undergone any mutations and are sensitive to amantadine(Figure 5.28).
- Of the 80 H3N2 isolates 74 showed restriction pattern similar to the drug sensitive controls whereas 4 isolates did not show restriction digestion in the 31 amino acid position indicating resistance to amantadine(Figure 5.29).
- Thus the results of MIC are confirmed by RT-PCR RFLP analysis.

### 5.3.2 ANTIVIRAL ACTIVITY OF HERBS

**Herbs as antiviral agents**

Aqueous and ethanolic extracts of Ocimum sanctum (Fig 5.30) and Glycyrrhiza glabra(Fig 5.31) were screened for *Influenza* A & B virucidal activity. Toxicity of the extracts was assessed on MDCK (Table 5.26 & 27) cell line and nontoxic concentration was used for antiviral assay.

**Results of toxicity assay**

The Table 5.26 shows the evaluation of the toxicity of ethanol and solublizing agent used in the present study. The results revealed distilled water, 70% ethanol and 0.25% DMSO were non-toxic to MDCK cell line after seven days of observation. The test was repeated thrice at different periods for reproducibility, before use of the extracts in the drug assay (Table 5.26).

### 5.3.2.a Antiviral activity of *Ocimum sanctum*

The result of the toxicity assay of aqueous and ethanolic extracts of Ocimum is depicted in Table 5.27. The concentration above 500 µg of both extracts were toxic to MDCK cells (Fig 5.32). The results of viability of the cells assessed by MTT assay is illustrated in .

Table 5.28. shows the results of the antiviral assay by pretreatment assay. 100 µg to 300 µg of both aqueous and ethanolic extracts of Ocimum showed antiviral activity against 1,10,100 $TCID_{50}$ of *Influenza* A & B virus.

Table 5.29 shows the results of the antiviral assay by virus inhibition assay. 100 µg to 300 µg of both aqueous and ethanolic extracts of Ocimum showed complete inhibition of 1,10,100 of *Influenza* A & B virus. Table 5.29 & 5.30 show the results of varying the time exposure of virus to the extracts. 100 µg to 300 µg of both aqueous and ethanolic extracts of Ocimum showed no enhancement of virucidal activity.

Table 5.31 shows the results of the antiviral assay by virus adsorption assay. 100 µg to 300 µg of both aqueous and ethanolic extracts of Ocimum showed complete inhibition of 1,10,100 $TCID_{50}$ of *Influenza* A & B virus.

### 5.3.2.b Antiviral activity of *Glycyrrhiza glabra*

The results of the toxicity assay of aqueous and ethanolic extracts of *Glycyrrhiza glabra* are depicted in Table 5. 32 . The aqueous and ethanolic extracts of concentration above 500 µg were toxic to MDCK cells. The results of viability of the cells assessed by MTT assay are illustrated in Figure.

Table 5.33 shows the results of the antiviral assay by pre treatment assay. 100 µg of aqueous and ethanolic extract of Glyzzhrhizzia glabra showed antiviral activity against 1,10,100 $TCID_{50}$ of *Influenza* A & B virus.

Table 5.34 shows the results of the antiviral assay by virus inhibition assay. 100 µg to 300 µg of aqueous extract of ***Glycyrrhiza glabra*** showed complete inhibition of 1,10,100 $TCID_{50}$ of *Influenza* A&B viruses. Table 5.35 shows the results of varying time exposure of virus to the extracts. 100 µg to 300 µg of aqueous extract of ***Glycyrrhiza glabra*** showed no enhancement of virucidal activity.

Table 5.36 shows the results of the antiviral assay by virus adsorption assay. 100 µg to 300 µg of aqueous extract of ***Glycyrrhiza glabra*** showed complete inhibition of 1,10,100 $TCID_{50}$ of *Influenza* A&B viruses.

# 6. DISCUSSION

Epidemics of *Influenza* are one of the most feared outbreaks because of their potential threat to become a pandemic. During the last century three *Influenza* pandemics caused millions of deaths worldwide, social disruption and profound economic loss. *Influenza* experts agree that another pandemic is likely to happen. With the increase in global transport and communications as well as urbanizations and over-crowded conditions epidemics due to *Influenza* virus are likely to quickly spread around the world. The impact of the next pandemic is likely to be greatest in developing countries where health resources are strained and the general population is weakened by poor health and nutrition. Taking cognizance of this threat it is important to strengthen disease and virological surveillance as a corner stone of pandemic preparedness.

The present study was aimed at initiating *Influenza* surveillance in Chennai, generate data on the exposure of the population to the virus, study the molecular epidemiology, standardize molecular diagnosis, look in for antiviral resistance among the isolates and screen for antiviral activity of herbs.

This study is divided into three parts. First part deals with the seroepidemiology, virus isolation and genetic characterization of the isolates, the second part deals with standardization of molecular diagnosis and detection of antigenic variants, third part deals with the screening of isolates for antiviral resistance and screening of herbs for antiviral activity.

The data generated helped in identifying and characterizing the *Influenza* strains that are circulating in different regions of Chennai, the population at risk and the seasonality of *Influenza* activity. Continuation of this will help in rapid identification of any newly emerging *Influenza* strain with epidemic/pandemic potential.

# PART – I

## 6.1.1 SEROEPIDEMIOLOGY OF INFLUENZA IN CHENNAI, TAMILNADU

There have not been many reports of *Influenza* surveillance in South India, thus the epidemiology is not well understood. To understand the etiological significance of presence of antibody in the serum, determination of baseline titre for *Influenza* A&B Haemagglutinating antibodies in different age groups of normal healthy population in Chennai was undertaken for a period from 2001 – 2006.

In the study, it is seen that the population has been exposed to all the circulating four serotypes of *Influenza* A&B viruses. The overall antibody prevalence was 34.02% and the seropositivity to *Influenza* A (34.2%) and for B (33.19%).

The seropositivity varied every year based on the circulation of the viruses in the population. Among *Influenza* A viruses seropositivity to *Influenza* A/H3N2 was high during the years 2000-2004 and to *Influenza* A/H1N1 during the years 2005 and 2006 indicating the circulation of respective viruses during that period, which was later confirmed by the virus isolation studies initiated from the year 2002.

Among *Influenza* B viruses seropositivity to *Influenza* B/Victoria lineage was high during the years 2001-2005, and to *Influenza* B/Yamagata lineage was consistently increasing during the study period and it peaked in 2006.

Geometric mean titre and standard deviation were calculated for *Influenza* A& B antibodies. Applying the formula Mean + 2SD, the baseline titre for each serotype was calculated. Baseline titre of 1:40 for *Influenza* A & B was established.

The results of the serosurveillance study were validated by virus isolation data. It was observed that in the years 2002-2004 *Influenza* A H3N2 virus predominated and A/H1N1 virus from 2005 onwards, thus the seropositivity to the particular viruses was high during that period. Among the B viruses seropositivity to *Influenza* B/Victoria lineage viruses was consistent without much deviation and similarly isolation was also consistent, whereas as the seropositivity for *Influenza* B/ Yamagata

increased gradually during the study period and peaked in 2006. This was collaborated with virus isolation also.

The results of present study indicated that the school going age group of 6-15 was highly exposed. This can be attributed to the crowded environment in the schools that favor the increased transmissibility of the virus.

**B.L.Rao** did a study on the prevalence of antibodies in different age groups to pandemic and epidemic strains to *Influenza* in the year 1981-82. It was observed that the population in the older age group showed the presence of antibodies to more strains than the younger age group. But the striking factor of this study was that the GMT was higher for the currently circulating epidemic strains in the younger age group of 1-14 years, whereas the older age group showed the presence of antibodies to both epidemic and pandemic strains. In our study we had screened for the presence of antibodies to the currently circulating strains only and our result correlated with this study [310].

In the seroepidemiological study of *Influenza* performed by **L.R.Yeolekar** during the years 1997 - 1999 an increasing spectrum of antibodies in the older age group was observed. Seropositivity to the H1N1 viruses was observed in the 5-15 age group which were isolated later in the year 2000, thus indicating the susceptibility of the school going age groups to the virus and it also correlates with our study [311]. The study also demonstrated the absence of antibody to A/Singapore/1/57 (H2N2) strain in the age group < 25 years indicating the decreasing immunity to that serotype and raised concerns of its reintroduction as the serotype is circulating in wild birds.

A similar study carried out in Vellore by **Sridharan *et al*. (1999)** showed a seropositivity for *Influenza* A/H1N1(94.04%) and H3N2 (99.5%), and they further observed the high incidence of antibodies in all the age groups in the general population and said that this may be either be due to a recent epidemic or due to the high prevalence of *Influenza* A/H3N2 infection in Vellore population[311].

The current serosurveillance study in Chennai indicated the exposure of all the age groups to *Influenza* and also to the different serotypes. This indicates an active circulation of different types of *Influenza* viruses in the community. The school going

age group of 6-15 was the highly exposed followed by the geriatric age group of 55 and above this may be attributed to the fact that the paediatric and the geriatric age groups are susceptible to *Influenza* viruses because of the naïve and waning immunity respectively. The presence of antibodies even in the adult age group of 16-45 indicates that the *Influenza* strains circulating are continuously evolving and are capable of causing infection in all the age groups. The previous studies have shown that the antibodies to all the types are found in the older age group and to the currently circulating types in the younger age groups, as the exposure to the currently circulating strains was studied in our study it was observed that the school going age group was highly exposed to the circulating virus types.

### 6.1.2 VIROLOGICAL SURVEILLANCE OF INFLUENZA VIRUS

**Ghendon**[191] in his update on *Influenza* surveillance **1991**, has given the main objectives of *Influenza* surveillance as the collection of *Influenza* virus isolates and analysis of their antigenic characteristics so that the most appropriate virus variants can be recommended as constituents of *Influenza* vaccines for the use in the future epidemiological season, collection and analysis of information on *Influenza* morbidity and mortality and earliest possible detection of *Influenza* epidemics. It was found that the objective are being fulfiled only in certain countries and that the surveillance should be carried out worldwide and that the methods for surveillance need be improved and standardized.

The importance of global surveillance for *Influenza* was put forth by **Cox *et al***[38]., emphasized the need for identification of the circulating strain of the virus for updating the vaccine.

A six year study (2002-2007) was undertaken to screen the suspected acute respiratory disease (ARD) cases visiting the out patient departments (OPD) of different Hospitals in Chennai for *Influenza* viral etiology. The results of present study are based on the isolation of virus from cases of clinically confirmed *Influenza* like Illness (ILI). Virus isolation was done from samples collected within 4 days of onset. The processed samples were inoculated on MDCK cell line and the isolated virus was typed by Haemagglutinin Inhibition test (HAI) using antiserum provided by

CDC. Similar studies have been done by **Annick Dosseh *et al*[313], Shin-Ru Shih *et al*[314].** Both reported the use of MDCK cell line and WHO reagent kit for *Influenza* virus isolation and identification. The standard WHO protocol for *Influenza* virus surveillance was followed.

3036 suspected cases of acute respiratory disease (ARD) were screened during the period 2002 – 2007. 236 (7.77%) cases were confirmed as *Influenza* virus infection by Virus isolation. Year wise incidence was 12.5 % in 2002, 7.14% in 2003, 9.16% in 2004, 9.78% in 2005, 7.83% in 2006 and 5.8% in 2007 (Table 5.5a).

In the present study *Influenza* A/H1, A/H3, B/Victoria and B/Yamagata have been implicated as etiological agents associated with acute respiratory infection cases. *Influenza* A/H3 virus was isolated throughout the study period, A/H1 Viruses were isolated only in 3 years 2005 – 2007 and *Influenza* B viruses were isolated in the years 2002-2003 and 2005-2007. There were two localized outbreaks during the study period one in the year 2005 caused by *Influenza* A/H1 virus and one in the year 2007 due to *Influenza* B/Yamagata type. The overall isolation rate of *Influenza* A/H1 during the study period was 35.96%, followed by A/H3 with 35.08%, B/Yamagata 26.27% and B/Victoria 5.26% (Table 5.5b-f).

During continuous surveillance of *Influenza* for twelve years carried out by **Rao, *et al*[51].,** from **1978-90** in Pune, India, 16 outbreaks of *Influenza* were identified. The *Influenza* virus variants responsible for the outbreaks were recognized as A H3N2 & A H1N1.

A continuous surveillance of *Influenza* was carried out by **Rao, *et al.*, in 1980** in Pune. Three outbreaks of Acute Respiratory tract Infections (ARI) that were investigated the first one was found to be associated with *Influenza A* H3N2 virus and the second with H3N2 and H1N1 and only a few *Influenza* viruses were isolated in the third outbreak.

Five outbreaks of acute respiratory diseases were observed by **Rao,*et al.*,** during the course of *Influenza* surveillance in Pune during 1983-85. Specimens were processed in chick embryos, MDCK cell lines and LLC-MK2 cell lines and isolated *Influenza A* H3N2 in 1983,A H1N1 and A H3N2 in 1984 and A H3N2 in 1985.

A new variant A H1N1 strain; A/Singapore/6/86 was identified by Rao *et al* in June 1986 in Pune. The study showed the high prevalence of anti bodies to the predecessor A H1N1 strains; A/USSR/90/77 and A/Chile/1/83 (77% and 67% respectively) while it was 26% to the new A/Singapore/6/86 strain.

In a surveillance carried out in the United States during the *Influenza* season from Oct. 1988 - May 1989, *Influenza A* (H1N1) was the predominant strain while during the next season, *Influenza A* (H3N2) was the predominant.

In **1993-1994 Singh *et al.,*** reported an outbreak of *Influenza* A subtype H3N2 in Delhi during the month of July- Aug. Urban and Rural areas were affected & the attack rates were similar in children and adults. The mean age of the patients from whom the virus was isolated was found to be 19.6 years. 12 viruses that were isolated were identified to be *Influenza A* (H3N2) virus, thus the predominance of H3N2 was recorded.

*Influenza* activity was found to occur in low- moderate levels, during Oct. 1994- May 1995, in most parts of the world. The predominant strain was identified to be A (H3N2) followed by A (H1N1) during this period & in Australia they occurred most during March- August.

**Anchlan *et al.,* (1996)** studied the previous H1N1 *Influenza A* viruses circulating in the Mongolian population. The 4 *Influenza A* isolates of subtype A (H1N1) isolated from 1985 were analyzed by sequencing of RNA segments. The isolate from 1988 was found to be a derivative of reassortment between PR8 & USSR/77. One of the Mongolian isolates from 1991 (111/91) was similar to PR8 while another from 1991 (162/91) was related to H1N1 strain isolated around 1986 in the other parts of the world. From their reports it was evident that the mutational & evolutionary rates of the Mongolian strains are low & this could be a threat to human population as they retain their potential to reappear after several years to cause a pandemic as was in 1997.

### 6.1.2.a AGE WISE DISTRIBUTION OF INFLUENZA VIRUS INFECTED CASES

In this study samples were collected from the population of different age groups ranging from 1 year to 98 years and the mean age group of the population was 19.3 years.

Agewise distribution of *Influenza* virus infected cases showed significant statistical difference with 63.5% in 0-12 years, 11.8% in 13-55 years, 24.56% in >55 years. It indicates that the lower age group is highly susceptible to *Influenza* because of the lack of immunity and the older age group because of the waning immunity, the attack rate is high in the lower age group than the older age group can be attributed to the exposure of the geriatric age group to the viruses during their lifetime which has reduced the impact of the viruses.

Surveillance of *Influenza* carried out by **Rao*, et al.*, in 1980** in Pune showed that the acute respiratory illnesses had affected all the age groups, the pediatric age group being most affected. The study revealed that the combination of virus strains together with the wet weather contributed to the extensive outbreak during July-September.

The results matched with the observation of the study in **1993-1994 by Singh *et al.*,** who reported an outbreak of *Influenza* A subtype H3N2 that occurred in Delhi the attack rates were similar in children and adults. The mean age of the patients from whom the virus was isolated was found to be 19.6 years.

**Annick *et al.*,** suggested that *Influenza* is an all-age-group acute respiratory infection. In temperate regions, the elderly and the children are particularly at risk **Magdzik *et al*[315]** observed that no specific serotype was found to be associated with age and no clear evidence from previous studies on this issue is available.

**Lvov *et al.*, 1979** had reported the isolation of *Influenza* viruses from all age groups and reported that that the pediatric age group was more susceptible than the other age groups.

### 6.1.2.b GENDER WISE DISTRIBUTION OF INFLUENZA VIRUS INFECTED CASES

The analysis of male: female infection ratio was 1.1:1 and yielded no observable difference between the male and female population affected by *Influenza* virus. Both the sexes were equally infected. There have not been any reports on the gender based distribution of *Influenza* viruses but the previous Indian observation done by **Pal *et al*. (1973)** is same as the results of present study

### 6.1.2.c MONTH AND YEARWISE DISTRIBUTION OF INFLUENZA VIRUS INFECTED CASES

*Influenza* viruses do not cause persistent or latent infections; they are maintained in human populations by direct person-to-person spread during acute infections. In the northern hemisphere, epidemics generally peak between January and April, but may flare up as early as December or as late as May. In the southern hemisphere, outbreaks occur between May and September. The low relative indoor humidity during the winter months is believed to prolong the survival of *Influenza* in aerosols and is believed to be responsible for the seasonal pattern in the northern hemisphere. Seasonality in tropical and subtropical climates is believed to coincide with the onset of the rainy season. On a global scale, *Influenza* virus activity is detectable throughout the year, and viruses can be isolated in large cities year round.

From the six-year (2002 – 2007) observation, though *Influenza* viruses were isolated in all the months appreciable month wise variation in distribution of cases was noted. The notable feature that was observed in the study was the increased virus isolation during the early months of monsoon and winter. 30 (12.71%) cases were positive in the months of June and July which are the first two months of south west monsoon and 120 (50.8%) cases were positive in the months of October and November which are the first two months of winter which indicates the reduction in temperature favors the transmission of virus. The isolation of virus during the summer months indicates that the increase of relative humidity during the hot and rainy season is responsible for better survival of the virus . This is in concurrence with the observation by Annick et al that if cold weather is responsible

for the high sensitivity of the nasal mucosa and respiratory tract in favoring *Influenza* virus multiplication, perhaps the increase of relative humidity during the hot and rainy season is responsible for better survival of the virus.

A continuous surveillance of *Influenza* was carried out by **Rao, *et al*., in 1980** in Pune, a city with a tropical climate. Three outbreaks of Acute Respiratory tract Infections (ARI) that were investigated in March (hot season),in July-September(rainy season) and in Nov.(cold season). The study revealed that the combination of virus strains together with the wet weather contributed to the extensive outbreak during July-September.

Five outbreaks of acute respiratory diseases were observed by **Rao, *et al*.,** during the course of *Influenza* surveillance in Pune during 1983-85. Three of them occurred in rainy seasons, one in winter and the fifth in hot season.

The outbreak caused by a new variant A H1N1 strain; A/Singapore/6/86 in Pune, India in 1986, occurred during the month of June was conducted by **Rao and Thite**.

A similar reports by L.Rao *et al*. (1993), (2000) (2001) showed a predominance in the rainy season as 10 of the 16 outbreaks investigated were during the rainy season.

Thus the results of our study that *Influenza* virus activity predominates in the rainy season is collaborated by other workers.

**Fitzner *et al*[316]., (1999)** observed the change in seasonality of *Influenza* over the years of his study period. Though the viruses were isolated throughout the year peaks were seen during the later winter and spring months in the years 1993 and 1994 whereas peaks were seen during the summer months in the previous years. This study is in concurrence with our study which has shown the year round presence of the virus and peaks during different months of the year during the study period.

In a study conducted by **Anice *et al*[317]. (2008)** it had been proved that the *Influenza* virus transmission in guinea pigs is most efficient under cold and dry

conditions. They also reported the lack of aerosol transmission at 30°C and at all humidity tested. Conversely, transmission via the contact route was equally efficient at 30°C and 20°C. The results that contact or short-range spread predominates in the tropics and offer an explanation for the lack of a well-defined, recurrent *Influenza* season affecting tropical and subtropical regions of the world.

### 6.1.2.d1 CLINICAL SIGNS AND SYMPTOMS OF INFLUENZA CASES

All patients with suspected *Influenza* viral infections had fever. 63 % of cases had headache, 92.1% of them showed nasal discharge and 72% of them presented with pharyngitis. 73.6% cases showed myalgia and 78% reported productive cough and 64% had symptoms of vomiting and diarrhea but only 8% of cases reported fever with sudden onset. As the influenza virus infection mimics many virus infections it is difficult to diagnose influenza clinically.

But among the *Influenza* positive cases it was observed that all had sudden onset of symptoms. Fever, cough was present in all the cases followed by headache (92.4%). The other symptoms that were predominantly observed were Myalgia (83.6%), nasal discharge (73%), pharyngitis (67%), Productive cough and vomiting and diarrhea were reported in (23%) and (39%) of the cases respectively. The presence of headache and myalgia along with sudden onset of symptoms, fever and cough were indications of *Influenza* infection among the acute respiratory infections. These symptoms can be used as markers for accurate presumptive clinical diagnosis of influenza.

### 6.1.2.d2 PREDISPOSING FACTORS AND ASSOCIATION OF CHRONIC CONDITIONS IN THE ELDERLY WITH INFLUENZA INFECTIONS

*Influenza* viruses lead to severe complications and excessive hospitalization of the elderly. In our study the presence of both Cardiovascular and pulmonary disease was seen in 53% of the positive cases in the elderly. Cardiovascular and diabetes was associated with 30% of the cases pulmonary disease alone was found in the 12% of the cases and the rest 4.6% presented with cardiovascular disease alone.

The elderly and people suffering from medical conditions such as lung diseases, diabetes, cancer, kidney or heart problems, *Influenza* poses a serious risk. In these people, the infection may lead to severe complications of underlying diseases, pneumonia and death[327].

Elderly people are more likely to develop complications after Flu and incur excess hospitalization and mortality, due both to their age and other predisposing risk factors, including chronic respiratory and cardiovascular conditions[328].

Elderly those aged >75 years have the highest hospitalization rates and also the highest rate of *Influenza*-associated hospitalizations in all disease categories studied. The association between *Influenza* virus activity and hospitalization for acute respiratory diseases was least in those aged 15–39 yrs, a pattern also observed in the United States. Our results also show that *Influenza* is associated with excess hospital admissions for other chronic diseases such as cerebrovascular disease, ischemic heart disease, and diabetes[329].

Acute respiratory tract infection is reportedly associated with an increased risk of myocardial infarction and stroke.

### 6.1.3 NEURAMINIDASE TYPING OF INFLUENZA A ISOLATES

The sialidase activity of the NA protein removes sialic acid from sialyloligosaccharides, thereby serving two functions: (a) the removal of sialic acid from HA, NA, and the cell surface, facilitating virus release; and (b) the removal of sialic acid from the mucin layer, which likely allows the virus to reach the surface of the epithelial cells. The NA protein may also have a role in host range restriction and pathogenicity[330].

In addition to HA, antigenic drift has also been reported for NA[331] and correlated with amino acid differences in the molecule. The mechanism of NA antigenic drift is believed to be the same as for HA and can be mimicked in *vitro* with frequencies of variant selection similar to those found for HA. Studies with monoclonal antibodies and amino acid sequence analyses have revealed two to three antigenic sites.

*Influenza* A subtypes A(H3N2) and A(H1N1) have co circulated in humans worldwide since the reappearance of A(H1N1) viruses in 1977 **(Kendal 1979 & Stuart Harris 1985)**. Recently, reassortant *Influenza* A viruses with an H1N2 antigenic composition subtype **(PHLS, 2002)** have been detected by surveillance programs in a number of countries. There have been few reported instances of viruses in which there had been genetic reassortment of internal genes **(Xu *et al.*, 1993; Couch *et al.*, 1986; Young and Palese, 1979)** or of the surface antigen genes **(Li *et al.*, 1992; Guo *et al.*, 1992; Nishikawa and Sugiyama, 1983)** between the A (H1N1) and A (H3N2) viruses infecting humans.

The haemagglutination-inhibition assay that is routinely used for subtyping and antigenic characterization of *Influenza* strains does not distinguish between A (H1N1) and A (H1N2) strains; therefore, alternative tests are required for typing the NA. The standard test used to determine neuraminidase type is a rather cumbersome and time-consuming procedure (approximately 18 h) which relies on inhibition, by antibody, of *n*-acetyl neuraminic acid release from a substrate such as fetuin **(Aymard-Henry *et al.*, 1973)**. Alternative methods of neuraminidase assay have been described, one for example relying on the agglutination of neuraminidase-treated erythrocytes by lectins **(Fiszon and Hannoun, 1990)**, however, these have found little application in the laboratory. In addition, all of the methods that are based on antibody inhibition of neuraminidase activity may be subject to stearic hindrance from anti-haemagglutinin antibodies, if whole *Influenza* virus and antibody to whole virus is used. Recently, the reverse transcription polymerase chain reaction (RT-PCR) has been found to be useful as a definitive test for identifying both the haemagglutinin and neuraminidase subtype of *Influenza* viruses **(Poddar, 2002)**.

In the present study 162 *Influenza* A virus isolates were typed for NA, the 82 *Influenza* A H1 types had the N1 neuraminidase type and the 80 H3 types had the N2 Neuraminidase type. There was no H1N2 or H3N1 type indicating that there has not been any Reassortment between the circulating *Influenza* serotypes. The application of RT-PCR assay will also contribute to the rapid detection and diagnosis of recent reassortment events among the viruses. Our data indicates that, compared to neuraminidase inhibition testing one step RT-PCR assay offers a faster, accurate and specific tool for the subtyping all NA subtypes of *Influenza* A viruses. The product

can be sequenced and sequence information obtained can be helpful in determining the origin of the *Influenza* virus and can be interrogated for the presence of mutations conferring resistance to antiviral drugs. The prompt availability of this information is important for initiating an appropriate treatment and for the tracing and management of outbreaks.

Recently, *Influenza* laboratories across the world began to use PCR to amplify the HA and NA genes and to identify changes at the genetic level, usually by a sequencing of the amplicons.

**Chang *et al*[332]., in 2008** had devised a simple and rapid multiplex RT-PCR method to identify 12 HA subtypes (H1-H12) and 9 NA subtypes (N1-N9), which were commonly isolated in South Korea. Comparison of the visual images of amplification utilizing individual primer sets and multiplex primer sets revealed that multiplex PCR methods were as specific and efficient as individual PCR methods. In addition, the method had the advantages of being able to conserve time and reagents, considering that 3 to 4 subtypes could be distinguishably identified in a single amplification run for each of the NA and the HA genes, respectively. The multiplex RT-PCR assays developed in the study was able to differentiate 12 HA and all 9 NA subtypes of *Influenza* A viruses from cultured virus isolates and, more importantly, field specimens. They also concluded saying that these assays may facilitate *Influenza* virus diagnosis with easier identification and more rapid subtyping compared with other methods.

### 6.1.4 DETECTION OF ANTIGENIC VARIANTS RT-PCR RFLP:

Antigenic variation among the *Influenza* isolates was studied by RT-PCR and RFLP analysis.

RT-PCR products obtained from prototype virus strains were subjected to RFLP the, prototype strains did show a clear restriction pattern as they were isolated during the year 1999.

The *Influenza* A virus isolates did not show any restriction pattern indicating the mutations occurred in the restriction sites. *Influenza* B viruses showed restriction

pattern as they had not mutated at the restriction sites. Further sequencing has to be done to study the mutation occurred at the restriction site and it significance in the antigenicity of the viruses.

As the test was very cumbersome and very difficult to reproduce and there is a need for constant monitoring of the sequence and change enzymes accordingly the test is not being used presently for detection of antigenic variants.

### 6.1.5 MOLECULAR CHARACTERIZATION OF INFLUENZA A&B ISOLATES BY SEQUENCING HA REGION

Surveillance and monitoring antigenic changes in circulating *Influenza* viruses provide important information for annual selection of vaccine strains. *Influenza* A viruses have segmented genomes consisting of eight RNA segments which encode viral proteins. Because of immunological pressure, genetic variability is mostly confined to regions of the genome responsible for viral surface proteins[333]. In addition, identifying changes in the codons of the HA1 domain of the hemagglutinin gene, especially those under positive selection, can facilitate the prediction of future lineages[334]. However, other factors, including interactions of internal and surface proteins, are likely to affect viral fitness in a polygenic manner[335]. Molecular methods such as realtime PCR and sequencing are rapid and sensitive methods for characterizing the genetic composition and classification of the viruses. In this study, we analyzed 86 *Influenza* viruses isolated from 2002 to 2007 in Chennai. The first isolate of the season, one isolate in every month, and first middle and last isolate during the peak season were chosen for phylogenetic analysis.

Phylogenetic relationships of HA for 34 selected A/H1 Chennai strains from 2005 to 2007 and three reference strains were analyzed. The sequence identity among the Chennai isolates was 96.3 to 100%, based on a 495-nt HA segment. The most dissimilar pairs occurred between one 2007 isolate, A/Chennai/1/07, and two isolates isolated in 2005 and 2006, A/Chennai/5/05 and A/Chennai/1/06.

Of the 34 viruses isolated from 2005 to 2007, eighteen isolates shown in Fig.5.22 formed a group (group I) and clustered with A/H1/New Caledonia/20/99, the

2000 – 2007 vaccine strain[336-342], with a nucleotide sequence identity from 98.7 to 99.2%. The other eight 2006–2007 Chennai strains formed group IIa, with a slightly lesser identity of 96.3 to 100% with A/H1/New Caledonia/20/99, the 2000 – 2007 vaccine strain. Eight 2006 – 2007 isolates, shown in Fig.5.22 formed a subgroup IIb and clustered with A/Solomon Islands/3/06, the 2007-2008 vaccine strain[343] with a nucleotide sequence identity from 98.8 to 99.9%. One 2007 isolate, A/Chennai/3/07 in subgroup IIa, was 98.9 to 99.1% identical to the 2006 A/Chennai/6/06 isolate in the same subgroup. However, this isolate was less similar (96.2 to 96.9%) to the four 2006 isolates found in subgroup IIb.

The 2005 isolates were taken as baseline and mutation analysis was done as described by **Shin-Ru Shih *et al.*** All the 34 isolates showed a characteristic deletion mutation Lys (K) at amino acid 134 of the HA gene, as previously reported[344]. Only seven notable site mutations occurred in our isolates as compared to 11 by **Shin -Ru Shih *et al.*,** I 56 was converted to F, L to P between $E_{77}$ and $L_{78}$, $I_{79}$ to T, $V_{88}$ to A, $S_{137}$ to I, $R_{149}$ to M and $P_{186}$ to L. The sites L between $C_{52}$ and $L_{53}$, KGS between P125 and $S_{126}$, $G_{156}$ and $M_{166}$ were conserved. Among these changes, variation at position 156 and 78 have been reported to affect antigenicity[345-346].

Figure 5.23 shows the phylogenetic relationships of H3N2 isolates including the 32 chennai isolates and 5 vaccine strains. Among the A/H3N2 isolates nucleotide sequence identity in terms of HA genes among Chennai 2002-2007 H3N2 isolates were 96.9 to 100%, based on a 789-nt HA segment which slightly exceeded that among the 38 Chennai H1N1 isolates (96.2 to 100%) being investigated.

Four 2002 isolates were clustered to group I and were 98.4 to 98.6% identical to the 00 –02 vaccine strain A/Panama/2007/99[336-337]. Six isolates obtained during 2002 to 2004 clustered to group II, which was 99.1 to 99.4% identical to the 2002-2003 vaccine strain A/Moscow/10/99[338]. Twelve isolates (2002–2004) were clustered in group III, together with the 2003–2005 vaccine strain A/Fujian/411/32[339-340] with 97.7 to 99.9% identity. Four 2004–2005 isolates were clustered in group IV and were 99.2 to 99.7% identical to the 2005–2006 vaccine strain A/California/7/2004[341] and

the remaining 6 isolates of the years 2005-2007 were clustered into group V and were 99.2 to 99.7% identical to the 2006–2008 vaccine strain A/Wisconsin/67/2005[342-343].

The 2002 isolates were taken as baseline and mutation analysis was done. Fourteen mutations were observed among H3N2 viruses during the study period and among those 5 were found to affect antigenicity. $E_{62}$ to Q, $Q_{156}$ to H, $I_{144}$ to V, $A_{196}$ to V, $K_{276}$ to N, $N_{144}$ to R, $D_{225}$ to G, $Q_{75}$ to R, $K_{83}$ to N, $T_{131}$ to A, $P_{103}$ to Q, $K_{158}$ toH, $N_{137}$ to G, $N_{172}$ to N. Among these changes, variations at positions 62, 144, 156, 158, 172, 196, and 276 have been reported to affect viral antigenicity[347-349].

All the mutations that affected the viral antigenicity had occurred between late 2005-2007 as a result there was a spurt of A/H3N2 cases in the year 2008 (data not shown). The other mutations have been observed throughout the study period but did not affect the antigenicity. This is very evident as we look into the picture of A/H3N2 activity which was constant during the years 2002-2004 and decreased in 2005 as the population had garnered sufficient immunity and again mutations in the antigenic sites in 2005-2007 resulted in the increased cases of A/H3N2.

The phylogenetic analysis for *Influenza* B isolates, including 20 Chennai strains from 2002 to 2007 and five reference strains was done. Two reference strains B/Victoria/ 2/87 and B/Yamagata/16/88 were included, since they have been known as two major antigenic-genetic lineages of *Influenza* B viruses circulating worldwide since the mid-1980s[350]. Pairwise identities among these 18 Chennai strains, based on a 396-nt HA segment, ranged from 87.3 to 100%. The most dissimilar pairs occurred between one 2007 isolate, B/Chennai/4/07, and two 2002 isolates, B/Chennai/2/02 and B/Chennai/3/02. Notably, the Chennai *Influenza* B isolates accumulated significant variation in terms of HA nucleotide sequences (10.9% from 2002 to 2005).

Seven 2002–2007 Chennai isolates were in line with the B/Victoria/2/87 lineage and formed group I, were clustered together with the 2002–2003 vaccine strain B/Hong Kong/330/01[338]. The percent identities between these 7 isolates and B/Hong Kong/330/01 were between 98.4 and 99.4%.

Thirteen other Chennai *Influenza* B isolates, together with the 2003–2006 vaccine strain B/Shanghai/361/02[341-343], clustered into group II. The percent identities for these 13 isolates in comparison to B/Shanghai/361/02 ranged from 97.7 to 98.7%. These groups II isolates were in line with the lineage of B/Yamagata/16/88. Notably, they (including vaccine strains) all experienced a characteristic deletion of N at position 189 of HA protein, as did B/Yamagata/16/88. On the other hand, all strains in cluster I retained this amino acid, as B/Victoria/2/87 did.

2002 B/V/vss and 2005 B/Y/vss Mutations were also seen in other sites $H_{148}$ to T, $K_{162}$ to N, $I_{163}$ to V, $V_{172}$ to A, $R_{175}$ or $W_{175}$, $N_{176}$ to G, $K_{188}$ to R, $I_{201}$ to N, $Y_{205}$ to H, $E_{209}$ to Q, $G_{210}$ to S, $E_{223}$ to Q, $K_{228}$ to I and $K_{234}$ to N. Among these amino acid changes, frequent variations have been reported to occur at positions 162 and 175[351-352].

The mutations on the antigenic sites had occurred in the isolates which grouped with B/Yamagata lineage, thereby altering its antigenicity and resulting in an outbreak in 2006.

Over the seven years studied, strains isolated in Chennai and the vaccine strains matched exactly. For example, A/California/7/2004(H3N2) -like and A/New Caledonia/20/99(H1N1)- like strains were isolated in Chennai during the 2005–2006 season, which were the similar vaccine components of that period. B/Hong Kong/330/2001-like strains were also detected in the 2002–2003 season and were the vaccine strains of that year. Similarly, A/Fujian/411/2002(H3N2)-like strains were isolated in the 2003–2005 season and were the 2003–2005 vaccine strains. During the study period the vaccine strains were found in circulation a year before the vaccine is introduced, as vaccine manufacturing process generally takes 6 to 9 months after the vaccine strain is selected based on the epidemic strain circulated in the previous season. As a result, it is normal to see the strains circulating before a vaccine strain become available. This phenomenon has also been reported in United States[353 – 361] and European countries [362].

HA sequence comparison between the vaccine strains and Chennai strains provides an alternate way of evaluating how the endemic strains conform to the

vaccine strains. This approach provides a quantitative description of *Influenza* epidemiology over the often-used representation of phylogenetic relationships. It has been shown that the HA sequences correlate well with the *Influenza* antigenic properties and can be used to link the endemic strains and vaccine strains[363]. As shown in Fig the A/New Caledonia/20/99(H1N1)-like strain (a 2000–2007 vaccine isolates) prevailed from 2005 to 2007 and A/ Solomon Islands/3/2006 (H1N1)-like isolates (a 2006–2007 vaccine strain) prevailed from 2007. Figure  reveals that A/ Panama/2007/99 (H3N2)-like isolates (2000–2002 vaccine strain) isolated in Chennai in 2002, as well as A/Moscow/10/99(H3N2)-like isolates (2002–2003 vaccine strain) and A/Fujian/411/02(H3N2)-like isolates (2003–2005 vaccine strain) were circulating in 2002. As mentioned, it is normal to see a 1-year lag before a vaccine strain becomes available.

Phylogenetic study of recent human *Influenza* isolates from many other countries also revealed the 1-year lag phenomenon. However, sequence analysis of Chennai strains in the past 7 years have shown that epidemic strains often become the vaccine strains as many as 2 years later, for example A/Fujian/411/02(H3N2)-like strain (a 2003–2005 vaccine strain) circulating in 2002 itself. It would be interesting to study how many vaccine-like strains had circulated in 2 years ahead before the vaccine strain was introduced.

As observed during the study all B/Victoria/2/87 lineage Chennai strains exhibited high homology (98.4 and 99.4%) to the vaccine strain B/Hong Kong/330/01. Notably, during 2003-2007, some isolates closely matched B/Shanghai/361/02 (2004–2005 vaccine strain), some matched B/Hong Kong/330/01 (2002–2004 vaccine strain). The 2005-2007 *Influenza* B isolates were found to mix with the two well-known genetic lineages (clusters I and II, with B/Victoria/2/87 and B/Yamagata/16/88, respectively). It was reported as early as in 1999 that B/Victoria/2/87-like strains cocirculated with B/Beijing/184/93-like strains in some Asian countries but not in the United States, our data illustrated this cocirculation.

## PART – II

### 6.2.1 STANDARDIZATION AND EVALUATION OF RAPID MOLECULAR DIAGNOSTIC TOOL - RT-PCR

The two *Influenza* A subtypes which have been co-circulating in human populations since 1977 are *Influenza* A (H3N2) and A (H1N1). However, avian *Influenza* A (H5N1) virus infections in humans were reported in Hong Kong in 1997, where eighteen cases were confirmed. Two antigenically and genetically distinct lineages of *Influenza* B viruses, represented by B/Beijing/184/93 (or B/Panama/45/90) and B/Beijing/243/97 (or B/Victoria/2/87) viruses are currently circulating among humans. Co-circulation of multiple types and subtypes of *Influenza* viruses increases the difficulty of diagnosis and virus identification. Routine diagnostic methods for *Influenza* infections, including virus culture and antigen detection, are both highly sensitive and specific. However, the use of molecular techniques to directly detect virus in respiratory samples facilitates the investigation of respiratory outbreaks and provide rapid identification of viruses.

Viral culture has been the traditional method of identifying *Influenza* viruses. Virus growth in tissue culture is generally slow and requires a minimum of 3 weeks. In addition, the isolation of an *Influenza* virus from a specimen depends on a lot of factors like timing of collection, transport of sample in proper cold chain, sensitivity of the cell line used etc.

Clinical presentation of the *Influenza* like illness is difficult to distinguish from other respiratory infections because of the overlapping of symptoms with other viruses and bacteria thus resulting in unnecessary and inappropriate treatment. Therefore specific and rapid diagnosis of *Influenza* appears to be of paramount importance for a successful surveillance system.

Polymerase chain reaction offers an excellent insight and potential for sensitive, specific and rapid detection of minute quantities of genetic material in clinical specimens, thus providing an attractive alternate for the rapid diagnosis of *Influenza* viruses.

The objective of several studies was to detect the *Influenza* viruses and determine the serotype of the isolated strain both from the clinical specimen and isolates.

RT/PCR can be used for the detection of *Influenza* viruses in respiratory samples taken from patients with *Influenza*-like illness, or for the characterization of viruses grown in embryonated eggs or tissue culture.

In the present study, RT-PCR for the detection of *Influenza* virus was standardized with minor modification of CDC cycling conditions. Throat swab extracts and tissue culture isolates samples from suspected ARI cases were tested for the presence of *Influenza* virus RNA.

In this study, primer pairs specific for the hemagglutinin (HA) gene of currently circulating *Influenza* viruses are used. Primers BHAF and BHAR are *Influenza* B specific primers that amplify the HA gene of circulating *Influenza* B viruses (both B/Panama/45/90 and B/Victoria/2/87 lineages). Primers H1F-1 and H1R-1 are H1N1 specific and primers H3F and H3R are H3N2 specific. PCR product was examined by agarose gel electrophoresis and the type or subtype of the virus was determined.

**PCR was evaluated based on**

1. Detection limit of primers using tenfold dilutions of viral stock.
2. Comparison for concordance of the results of RT-PCR with the results of serology and virus isolation.
3. Evaluation of the detection limit of RT-PCR based on the day of onset of illness and sample collection.

**Selection of Primers**

The primers used in the study were designed by *Influenza* branch CDC Atlanta and were obtained from Gibco BRL. RNA was extracted from throat swab extracts and tissue culture isolates by using QiAmp viral RNA mini kit. The primers were for Haemagglutinin genes H1 and H3 and B genes. The primers for H1 gene yielded an

517 bp product, primers for H3 gene yielded 785 bp product and primers for BH gene yielded a 618 bp product. This region is known to contain conserved sequences characteristic of particular *Influenza* virus serotypes. The advantage of using these primer sets is that they confirm *Influenza* virus identity and the serotype also.

### 6.2.1.a Determination of the detection level of primers and specificity testing

The detection level of the primers was evaluated by subjecting 10 fold dilutions of standard *Influenza* A/H1, A/H3 and B type virus stocks ranging from 1000 to 0.001 $TCID_{50}$. The primers were able to detect 0.1 TCID50 of *Influenza* A and *Influenza* B type viruses. The performance of detection limit was to ensure the sensitivity of the standardized RT-PCR. The results of our study were similar to the study done by **Schweiger *et al*[364]., (2000)**.

**Holger F. Rabenau *et al*[365] (2002)** had reported the importance of determination of detection limit of an in-house PCR prior to its application in routine clinical diagnosis.

Specificity of present PCR system was assessed by inclusion of 10 throat swabs collected from normal persons and one tissue culture control. None of the negative samples, tested positive for *Influenza* PCR.

**Merisa Chesky *et al*[366]., (2000)**, reported the use of CSF, water and diluted viral culture sent by public health laboratory Oxford, UK to determine the sensitivity and specificity of the PCR assay.

### 6.2.1.b Evaluation of RT-PCR performed directly from clinical samples by comparison with virus isolation

The results of the RT-PCR of 60 samples that were collected within 7 days of onset of illness and 60 samples that were collected after 7 days of onset of illness were compared with results of virus isolation from throat swab extracts. The results showed RT PCR was more sensitive than the conventional virus isolation. RT-PCR detected 10 of the 60 samples (16.66% positivity) where as virus isolation detected only 6 cases (10% positivity) in the samples collected within 7 days of onset. Where

as the RT-PCR was able to detect 6 of the 60 (10%) samples collected even after 7 days in which the conventional virus isolation was not able to detect even single positive[367].

In order to control for false-positive and false-negative results, a second RT-PCR was performed with a fresh RNA extract for all RT-PCR-positive, culture-negative samples, RT-PCR-negative and culture-positive samples. The result of the second RT-PCR was always identical to the result of the initial RT-PCR.

### 6.2.2 Standardization and evaluation of multiplex RT-PCR performed directly from clinical samples in comparison with RT-PCR.

Primer binding is the most important step in PCR. Multiplexing involves the use of multiple pairs of primers to detect different serotypes or viruses in the same reaction. Standardization of multiplex PCR reaction requires optimization of cycling condition and reaction mix concentration.

It is known, that ammonium ions ameliorate primers annealing during the PCR, what helps to obtain higher amounts of specific product. Ammonium sulfate increases the ionic strength of reaction mixture, which alters denaturing and annealing temperatures of DNA and enzyme activity.

NH4+ ions in the buffer compete with the weak hydrogen bonds that form when nonspecific primer annealing occurs. These ions therefore destabilizes non specifically bound primers and enable highly specific annealing.

Incorporation of potassium chloride and ammonium sulphate as a components in PCR reaction mix has been reported to enhance primer binding in the multiplex PCR reaction. In our study various concentrations of potassium chloride with 20mM concentration of ammonium sulphate was used for optimization of multiplex RT-PCR.

The concentration of 0.5µM potassium chloride in the multiplex reaction resulted in good amplification of the individual types but did not amplify combination of viruses. Concentration of 1.0 µM potassium chloride in reaction mix yielded

good amplicons in both the sets. The concentration of 1.5 µM potassium chloride gave weak amplicons and there was no amplification in the 2.0µM potassium chloride. The results thus indicate that potassium chloride along with ammonium sulphate is required in optimum concentration for multiplex PCR reaction and seems inhibitory in higher concentrations. Therefore it is necessary optimize the concentration of potassium chloride along with ammonium sulphate for multiplex PCR reaction.

Interactions between K+ and NH4+ allow primer hybridization over a broader range of temperatures. K+ binds to the phosphate groups on the DNA backbone and therefore stabilizes the annealing of the primers to the template. NH4+, which exists as both ammonium ion and ammonia under thermal cycling conditions, can interact with the hydrogen bonds between the bases to principally destabilize the weak hydrogen bonds of mismatched bases. The combined effect of the two cations maintains the high ratio of specific to nonspecific primer: template binding over a wide range of temperatures.

The multiplex RT-PCR had to be done in two steps. The first step involving the synthesis of cDNA and followed by the nextstep amplification by PCR. One step PCR was not sensitive and resulted in nonspecific amplification also.Two step Multiplex RT-PCR was equally sensitive and specific as RT-PCR for detection of *Influenza* viruses. This reduces the reagents used thus making the test economical.

A similar kind of study on simultaneous detection and typing of *Influenza* viruses A and B by a nested reverse transcription-PCR: comparison to virus isolation and antigen detection by immunofluorescence and optical immunoassay (flu OIA) was done by **Bjorn Herrmann *et al.*, in 2000**[368]**.** They observed that RT-PCR detected 83 cases of *Influenza* A, compared to 66 cases detected by virus isolation and 68 cases detected by IF assay. The corresponding figures for the detection of *Influenza* B were 15, 12, and 11 cases, respectively. They concluded saying that RT-PCR provides a sensitive and specific method for detecting and typing *Influenza* viruses A and B. The rapid OIA is useful as a complementary test, but it cannot replace established methods without further evaluation.

**B. Schweiger *et al*[364]., in 2000** developed a fluorogenic PCR-based method (TaqMan-PCR) was developed for typing and subtyping of *Influenza* virus genomes in clinical specimens. The prospective studies performed in 1997–1998 and 1998–1999 revealed an overall increase in detection of *Influenza* viruses of approximately 12%. During the surveillance work, they found an overall detection rate of 15 to 18% by virus culture and 28 to 29% by the TaqMan-PCR for swabs that we received by community-based sampling during that period. They observed that the Taq- Man-PCR was much more sensitive than culture and revealed an excellent correlation for typing and subtyping of *Influenza* viruses when samples were positive by both methods. The processing of such a large number of specimens showed the reliability and practicability of the TaqMan-PCR, especially as results can be obtained within a few hours.

The results of this study compared well with the study of **Annie Ruest[369] *et al.* (2003)** who had evaluated Directigen Flu A_B Test, the quickvue *Influenza* Test, and Clinical Case Definition to Viral Culture and Reverse Transcription-PCR for Rapid Diagnosis of *Influenza* Virus Infection. They reported that RT-PCR detects more cases of *Influenza* than viral culture, and this greater accuracy makes it a more useful reference standard.

**Kate et al[370]., 2004** developed a rapid real-time multiplex PCR assay was developed for the detection of influenza A and influenza B viruses, human respiratory syncytial virus (RSV), parainfluenza virus 1 (PIV1), PIV2, PIV3, and PIV4 in a two-tube multiplex reaction which used molecular beacons to discriminate the pathogens.They observed that all of the culture-positive samples and an additional 5 influenza A virus, 6 RSV, 2 PIV1, 1 PIV2, 1 PIV3, and 3 PIV4 infections were detected by the multiplex real-time PCR.An observation similar to our study.

A multiplex reverse transcription (RT)-PCR method capable of detecting and subtyping influenza A (H1N1 and H3N2) and B viruses as well as respiratory syncytial virus (RSV) types A and B in respiratory clinical samples was developed by **J. Stockton *et al*[371] 1998**. They observed that for the typing and subtyping of influenza A and B viruses and RSV types A and B, the multiplex RT-PCR gave an excellent (100%) correlation with the results of conventional typing and subtyping with specific

antisera. It was also seen that the Multiplex RT-PCR accurately detected more than one viral template in the same reaction mixture, allowing viral coinfections to be identified with the same respiratory specimen. The Multiplex RT-PCR standardized by us also was able to detect more than one viral template in the same specimen.

In England in the 1995–1996 winter season a Multiple-target (multiplex) reverse transcription-PCR (RT-PCR) for detection, typing, and subtyping of the hemagglutinin gene of influenza type A (H3N2 and H1N1) and type B viruses was developed and applied prospectively to virological surveillance of influenza by **J. S. Ellis *et al*[372].** It was observed that there was 100% correlation between multiplex RT-PCR typing and subtyping and the influenza types and subtypes obtained from culture. There was also excellent correlation between the temporal detection of influenza A H3N2 and H1N1 viruses by multiplex RT-PCR and by culture. They reported an increased detection of 20% by RT-PCR when compared with virus isolation. Multiplex RT-PCR was successfully used in surveillance of influenza to provide accurate, sensitive diagnosis directly on clinical specimens sent through the post. Our study also showed an increase in detection by the use of RT-PCR and the same can also be implemented for routine surveillance.

The application of real-time PCR to clinical samples increases the sensitivity for respiratory viral diagnosis. In addition, results can be obtained within 6 h, which increases clinical relevance. Therefore, use of this real-time PCR assay would improve patient management and infection control.

# PART – III

## 6.3 SENSITIVITY TO ANTIVIRALS

Type A *Influenza* viruses represent important pathogens for humans, birds, horses, pigs, and other species. It is estimated that *Influenza* A viruses cause the death of approximately half a million individuals worldwide every year[373]. In the United States alone, 5%–20% of the population is infected with *Influenza* virus annually[374-376], and 36,000 people die from *Influenza*-associated illnesses a majority of the victims are elderly persons. Vaccination is the primary measure to control *Influenza* infections in humans. However, for individuals who cannot be or have not been vaccinated or when vaccine is not available, antiviral agent can provide an important alternative. Until recently, only amantadine and rimantadine derivative of Adamantane have been available for the prophylaxis and treatment of *Influenza* A infections[377-379] and have been shown to inhibit viral replication by blocking the proton channel formed by the M2 protein of *Influenza* A viruses. The channel activity is essential for uncoating the viral particle during early stages of viral replication[380]. A single substitution at 1 of 5 residues of the transmembrane domain of the M2 protein (positions 26, 27, 30, 31, or 34 has been shown to confer resistance to adamantane[381-382]. Amantadine- or rimantadine-resistant mutants are cross-resistant and show no evidence of fitness impairment[377]. Moreover, resistance emerges readily in the drug-treated patients, and resistant variants are transmissible[383-386].

Adamantanes have been used for many years because of their wide availability and low cost, yet the frequency of adamantine resistance among field isolates has been low until recently[387]. During the period from 1991 to 1995, surveillance for adamantane resistance among A(H3N2) viruses[388] revealed the global frequency of resistance to be as low as 0.8%. However, a recent study that included A(H3N2) viruses collected worldwide over the course of a decade showed that the incidence had increased to 12.3% by 2004[389]; by next year, it had reached 96%, 72%, and 14.5% in China, South Korea, and the United States, respectively. Resistance to adamantine among A(H3N2) viruses circulating in the United States had reached 92%[390], according to the results of testing of a limited number of viruses collected between October and December of 2005. With the alarming incidence of drug resistance, the

US Centers for Disease Control and Prevention (CDC) issued a public health alert recommending that clinicians avoid the use of adamantanes for the remainder of the 2005–2006 *Influenza* season[391].

As no published data is available in India on this aspect and to have an idea about the sensitivity pattern of the isolates, in the present study, we performed drug-susceptibility testing for A(H3N2) and A(H1N1) viruses collected in Chennai between 2002 to 2007. The isolates were tested for their sensitivity to the currently used M2 inhibitor (Amantadine) by minimum inhibitory assay (MIC) using MDCK cell line. Further to confirm the results of sensitivity to antivirals by MIC, the genes coding for the M2 protein were amplified by RT-PCR and subjected to RFLP to detect any mutations in them.

### 6.3.1 Screening of sensitivity to antivirals by MIC:

162 *Influenza* A viruses were screened for their sensitivity to the M2 inhibitor amantadine. Drug susceptibility was determined by growing *Influenza* virus isolates in MDCK cell cultures in the presence of amantadine at a concentration of 1 μg/ml. The drug resistant control strains grew well even in the presence of the drug. The drug sensitive controls did not grow in the presence of drug. All the H1N1 isolates were sensitive and did not show any resistance. Of the 80 H3N2 Isolates 4 were resistant to amantadine. 60 viruses that were obtained during the years 2002-2004 did not show any resistance. Among the 11 isolates in 2005, 2 were resistant (18.18%), of the 5 isolates in 2006 (20%) one was resistant and among the 2 isolates in 2007 one was resistant (50%). Thus indicating a growing resistnce of A H3N2 to amantadine..

Lack of amantadine resistance was reported by **Prud Homme *et al*[392]., *1997*** reported the lack of resistance of *Influenza* A viruses to adamantanes in Canada. Though Amantadine resistance of about 30% was reported in most of the countries during that time their study in Cananda did not find any resistance to Amantadine.

In a study on drug resistance conducted by **Thedi Ziegler *et al*[393].,(1999)** observed that only 0.8% of the viruses were resistant to antivirals. They also observed that only 2 of these resistant viruses were isolated from individuals who received amantadine or rimantadine treatment at the time the specimens were collected. These

results indicate that the circulation of drug-resistant *Influenza* viruses is a rare event, but surveillance for drug resistance should be continued. Our study is in concurrence with **Dyede *etal*[394]., 2007** who also reported an increase in the emergence of amantadine resistance.

Though there have been reports of high degree of resistance to amantadine in the US and Southeast Asian countries, our study showed only an emerging resistance pattern to amantadine. The 50% resistance shown in the year 2007 is only among the two isolates obtained but it cannot be taken as the exact picture of resistance as the number is few. Amantadine is not being used widely for the treatment of *Influenza* in India and this may be the reason behind the lack of resistance among the isolates for amantadine and the emerging resistance may be due to the importation of strains from the regions that show resistance.

### 6.3.2 Detection of M2 mutants by RT-PCR RFLP:

Single-amino-acid changes at four positions, 26, 27, 30, and 31, within the transmembrane domain of the M2 protein have been reported to confer resistance to amantadine among *Influenza* viruses[395-397]. RT-PCR RFLP is the widely used and sensitive method for detecting any mutations in the genes**.** The same has been used to detect the mutations in the genes coding for M2 protein. The results of the MIC method were reconfirmed by performing a RT-PCR RFLP for detection of amantadine resistance. The 162 *Influenza* A virus isolates that were screened for amantadine resistance by MIC method were subjected to RTPCR RFLP.

The drug resistant viruses did not show restriction pattern thus indicating mutation in the M2 protein. The drug sensitive controls showed restriction pattern. All the 82 H1N1 isolates showed restriction pattern similar to the drug sensitive controls thereby indicating that all H1N1 the isolates have not undergone any mutations and are sensitive to amantadine.

Of the 80 H3N2 isolates 76 showed restriction pattern similar to the drug sensitive controls whereas 4 isolates did not show restriction digestion in the 31 amino acid position indicating resistance to amantadine.Thus the results of MIC are confirmed by RT-PCR RFLP analysis.

**Prud Homme *et al.*, 1997** had reported the use of RT-PCR and sequencing for the detection of amantadine resistant isolates. They inferred that this method is very rapid, sensitive and requires only a minute quantity of RNA and can be used with RNA isolated from clinical specimen also.

In the year 2004–2005 *Influenza* season 15% isolates were found to be resistant and revealed that the highest frequency of resistance was detected among isolates received from Asia (93.4%). In the United States, it had reached 10.6%.97.5% had an amino acid change from Ser to Asn at residue 31 (Ser31Asn). This is the most common mutation known to confer resistance to adamantanes[389-390,398-399]

**Dyede *et al.*, 2007** in their Surveillance of Resistance to Adamantanes among *Influenza* A (H3N2) and A (H1N1) Viruses Isolated Worldwide had reported an increase in resistance to the drug as the years progressed. Their findings indicated that adamantane-resistant viruses carrying the Ser31Asn change in the M2 protein can efficiently compete with viruses lacking the substitution. Additionally, Ser31Asn was also the predominant amino acid change conferring resistance in the recent A (H1N1) viruses, whereas the report by Saito et al. [28] indicated that Val27Ala was the most predominant amino acid change in a set of 45 A(H1N1) viruses isolated between 1999and 2001.

In our study all the 4 amantadine resistant isolates had the Ser31Asn change in the M2 protein thus in complete concurrence with the study by **Dyede *et al***.

Though amantadine resistance in *Influenza* A viruses is almost 100% among the South East Asian countries, the report of low incidence of drug resistant viruses in our study may be because amantadine is very rarely being used for treatment in India..

The emergence and subsequent spread of adamantane resistance among A(H3N2) viruses may be attributed in part to more widespread use of those antivirals, given that resistant viruses typically emerge readily in treated patients and are transmissible[400]. In other countries, such as the United States, adamantanes are used less because a physician's prescription is required.

The adamantane-resistant A(H3N2) viruses were found in both major clades of viruses circulating from 2003 to 2006. The HA phylogenetic analysis indicates at least 2 independent introductions of resistant viruses into human populations. The data also showed no apparent association between resistance and a particular change(s) in the HA.

Adamantane resistance was first detected in Asia at a time when severe acute respiratory syndrome and avian flu outbreaks might have resulted in an increased use of antivirals in that densely populated area. These factors cannot, however, explain the continuous spread of resistant viruses worldwide and their dominance in a low or absent selective pressure due to drug use. It is possible that the spread of resistant mutants was a result of association with unknown advantageous mutations and a lack of fitness impairment of these mutant viruses. Further studies are needed to investigate possible mechanisms underlying the spread of the adamantane-resistant variants in human populations in regions where amantadine is not used.

### 6.3.3 DETERMINATION OF ANTIVIRAL ACTIVITY OF MEDICINAL HERBS

A large number of medicinal plants reported in traditional Indian System of medicine; Siddha and Ayurveda have been employed for centuries to treat infectious diseases. The herbs *Ocimum sanctum, Glycyrrhiza glabra* are few of them which have been used as herbal formulations to treat Respiratory disorders[401-402]. Although they are claimed to be useful in treating infectious diseases, little scientific evidence is available to substantiate the claim.

In the present study, aqueous and ethanolic extracts of these herbs were evaluated *in vitro* for antiviral activity against *Influenza* A&B viruses. In Siddha and Ayurvedic preparation, these herbs are used either as water extract or ethanolic extracts. The present study is intended to record and scientifically authenticate, if the crude extracts of these herbs have anti- *Influenza* A&B activity.

In this part of the study, the following were carried out

- Determination of non-toxic doses of extracts on MDCK cell line.
- Evaluation of anti- *Influenza* A&B activity of aqueous and ethanolic extracts of two herbs by pretreatment assay, virus inactivation assay or preincubation assay and viral adsorption or post-infection assay.

**Toxicity assessment of solvents and extracts on MDCK cell line**

Varying concentrations of ethanol used in extraction was first assessed for toxicity on Vero cell line and the non-toxic concentration was used for the study. In the present study distilled water, 70% ethanol were used for extraction and 0.025% DMSO was used as solublizing agent for ethanolic extracts in minimum essential medium (MEM) W/O FCS. The assay was repeated in tetrads, three different times. It was found to be reproducible.

Varying concentrations of the aqueous and ethanolic extracts of the herbs were screened for toxicity on Vero cells. The viability of the cells treated with drugs was assessed microscopically for extract induced morphological changes and were further evaluated by MTT assay. The aqueous and ethanolic extracts of the herbs exhibited toxicity at varying concentrations. The concentration $\leq$ 500 µg of both aqueous and ethanolic extracts of *Ocimum* and The concentration $\leq$ 600 µg of both aqueous and ethanolic extracts of *Glycyrrhiza* were non-toxic. to MDCK cell line. The non-toxic concentrations of the extracts were used for the antiviral screening.

General issues such as storage of extracts, extraction protocols were standardized in the laboratory. Long time storage of the extracts upto 1 month in $-20^{o}C$ showed no difference in the toxicity or antiviral pattern as opposed to the literature which showed that only fresh extracts exhibited high antiviral activity. **Norbert D. Webber *et al.* (1992)** reported that fresh garlic extracts were 100% toxic to HeLa cells and Vero cells and further reported that fresh extracts exhibited greater antiviral activity than the stored.

**Antiviral screening of herbs for anti- Influenza A&B virus activity**

In the two herbs screened for the antiviral activity against *Influenza* A&B viruses, Minimum of 100 μg/ml of aqueous and ethanolic extracts of Ocimum and *Glycyrrhiza* had antiviral activity against *Influenza* A&B viruses. There are very few *in vitro* and *in vivo* studies done with herbs against *Influenza* A&B viruses.

### 6.3.3.1 *Ocimum sanctum*

The minimum concentration of 100 μg/ml of aqueous and ethanolic extracts of Ocimum showed complete inhibition of 100 $TCID_{50}$ of *Influenza* A & B virus propagation by virus inactivation assay and virus adsorption assay. There was no observable enhancement of virus neutralization by varying the time of exposure of virus to the extracts. The extracts of Ocimum did not inhibit *Influenza* A & B virus by pre incubation or cell pretreatment assay, inferring that *Ocimum sanctum* inhibits virus propagation but has no inhibition on the penetration of the virus.

The complete inhibition of 100 $TCID_{50}$ of the virus by minimum concentration of 100 μg/ml shows that the aqueous and ethanolic extracts of Ocimum as potent virucidal drug. The results of the present investigation confirmed the anti *Influenza* activity of Ocimum, and previous studies have indicated the anti viral activity against other viruses too. **Parida *et al.* (1997)** showed 175 μg/ml ethanolic extract of Ocimum inhibited type 3 Polio virus replication by 99.9% and a higher concentration of 1.75 mg/ml of aqueous extract inhibited the same by 99.68%. Similar results on anti-Coxsackie B activity was observed in the study of **Lalita Badam *et al.* (1999)** with Neem (*Azadirachta indica A.Juss*) where 1000 μg/ml of methanolic extract of Neem was observed to inhibit all the six antigenic types of Coxsackie B virus by virus adsorption assay and virus inactivation assay, however the pretreatment assay showed no inhibition on the penetration virus into the cells.

The antiviral activity seen in Ocimum against *Influenza* A & B viruses may be due to the macromolecules like proteins, carbohydrates and other substance such as sterols, alkaloids, glycosides, saponins & tannins of ethanolic extract. Further, it is seen from the literature that the antiviral activity exhibited by most of the herbs have

synergistic effect of two or more metabolites along with glycoproteins rather than one active principle. The qualitative study showed the presence of naturally occurring flavanoids besides triterpenes and other secondary metabolites as the major phytochemicals involved in anti- *Influenza* A & B.

The herb *Ocimum sanctum* has been one of the well studied herb, for pharmacologically characteristics. The phytochemical documentation and bioactivity is well established **Surender Singh *et al*[406].,1996 and Gupta *et al.* (2002)** in their review, document vast number of therapeutic applications such as in cardiopathy, haemopathy, leucoderma, asthma, bronchitis, catarrhal fever, hiccups, genitourinary disorders, ringworm, verminosis and skin diseases.

### 6.3.3.2 *Glycyrrhiza glabra*

100 µg/ml of the aqueous and ethanolic extract of *Glycyrrhiza* completely inhibited 100 $TCID_{50}$ of *Influenza* A&B viruses by both virus inactivation assay and virus adsorption assay virucidal activity. . There was no observable enhancement of virus neutralization by varying the time of exposure of virus to the extracts.

**Utsunomiya T *et al* [407]., (1997)** had reported that Glycyrrhizin may protect mice exposed to a lethal amount of influenza virus through the stimulation of IFN – γ production by T cells, because T cells have been shown to be producer cells of IFN–γ stimulated with the compound.

**Harada S[408]., (2005)** had reported that Glycyrrhizin (GL) lowers the membrane fluidity, thus suppressing infection by HIV, Influenza A virus and vesicular stomatitis virus.

**Fiore C., Eisenhut *et al*[409]., (2007)** confirmed that the *Glycyrrhiza glabra* derived compound glycyrrhizin and its derivatives reduced viral activity in herpes simplex virus encephalitis and influenza A virus pneumonia.

The herbs showing anti- *Influenza* A&B activity should be qualitatively and quantitatively assessed for proteins, glycoprotein or conjugate proteins, carbohydrates, and other secondary metabolites and individually tested for antiviral

activity. The antiviral activity observed in these herbs may be due to the synergistic effect of one or more metabolite. It has been reported by studies of **Lalita Badam *et al.* (1999)** on Coxsackie B viruses and **Thyagarajan and Jayam[410] (1992)** on Hepatitis B virus suggesting no single compound but a battery of phytochemicals of secondary metabolite origin are responsible for antiviral activity. The antiviral activity found to occur at concentrations non-toxic to cell line may be used to screen large groups of herbs and natural products, yet, the therapeutic dose for commercial exploitation must be standardized by *in vivo* studies in animals. Nevertheless, these studies lend credence to the scientific authentication of the herbal plants as novel antiviral agents.

# 7. SUMMARY

*Influenza* A &B viruses have been implicated to be the major cause of acute respiratory infections in developed as well as developing countries causing significant morbidity and mortality and always hold a threat of pandemic. In a vast country like India with mosaic clinical problems around its teaming millions, this virus has not received specific attention and very few Indian literature is only available. Many Asian countries like China, Japan, Malaysia, Indonesia and Korea have documented *Influenza* activity and are doing continuous surveillance also. The present study records the *Influenza* viral etiology of ARI in Chennai for the year 2002-2007. The non existence of a continuous surveillance is the major reason for the lacunae in the knowledge on *Influenza* infections, therefore establishing a proper surveillance system, standardization of RT-PCR for rapid detection was done. Protocols for screening for antiviral resistance and studying the mutations were also standardized. Since antiviral vaccines and drugs available for *Influenza* are expensive, and limited, the study extends to an evaluation of two herbs for anti *Influenza* activity and detection of resistance of the isolates to known anti *Influenza* drug.

Based on the above aims and objectives, the present work was carried out between year 2002 and 2007.

# PART – I

## 7.1.1 SEROEPIDEMIOLOGY OF INFLUENZA IN CHENNAI, TAMILNADU

- 50 serum samples from apparently healthy individuals of each age group (0-5, 6-15, 16-45, and 46-60) were tested for the presence of haemagglutinating antibodies by Haemagglutination Inhibition test during the period 2001 to 2006. Overall *Influenza* antibody prevalence was 34.02%. Among *Influenza* A viruses, though H3N2 through 2002-2007 it was predominant in the years 2001 to 2004, while *Influenza* A/H1N1 was first detected in 2005 and predominant in the years 2005 and 2006. Among *Influenza* B viruses, *Influenza* B Victoria lineage was predominant in the years 2001 to 2005 and *Influenza* B/Yamagata Lineage was predominant in the years 2007.

- Baseline HA Ab titre of the population was calculated. The baseline titre was 1:40 for the *Influenza* A & B viruses.

- 6-15 years age group showed high exposure to *Influenza* in all the years. Statistical analysis of the results showed the exposure of 6-15 years age group was statistically significant(Chi square test $p<0.0001$) when compared with the other age groups whereas there was no significant difference among the different age groups studied.

- The genderwise distribution of the haemagglutinating antibodies to *Influenza* viruses showed no significant variation.

## 7.1.2 VIRUS ISOLATION AND ANTIGENIC CHARACTERIZATION OF INFLUENZA VIRUS ISOLATES

- In the present study *Influenza* A/H1, A/H3, B/Victoria and B/Yamagata have been implicated as etiological agent associated with Acute Respiratory infection cases.

- 236 *Influenza* virus isolates were obtained during the study period 2002-2008. Of the 236 isolates 82 were *Influenza* A H1N1 viruses, 80 were *Influenza* A/H3N2

viruses, 62 were of *Influenza* B/Yamagata lineage and 12 were of *Influenza* B/Victoria lineage.

- A/H1N1 viruses were not isolated in the first three years of our study but appeared for the first time in 2005. From then on 82 viruses were isolated during the period 2005-2007. The predominance was high in the year 2005 during which they constituted for 71.18% of the total isolates in the year 2006 the prevalence was slightly reduced to 60.86% and in 2007 their prevalence was just 18.75%.

- A/H3N2 viruses are the only type of virus that was isolated in all the years of the study period. The virus predominated for a period of 3 years (2002-2004). It constituted for 86.69% of the isolates in the year 2002, 93.4% in 2003, and 100% in 2004. Its prevalence started to dip from the year 2005 during which 18.64%, 10.86% in 2006 and 3.12% were isolated in 2007.

- In our study *Influenza* B type of both the lineages were isolated. Total of 66 *Influenza* B type viruses were isolated during the study period of which 54 (81.8%) were of the B/Yamagata/16/88 lineage and 12 (18.18) were B/Victoria/2/87 lineage.

- *Influenza* B/Victoria/2/87 lineage viruses were isolated during the years 2002 and 2005 – 2007. *Influenza* B/Yamagata/16/88 lineage viruses were isolated during the years 2003 and 2005 – 2007.

### 7.1.2a Agewise Distribution of Influenza Virus Infected Cases

- Age wise distribution of *Influenza* virus infected cases showed significant statistical difference with 63.13% in 0-12 years, 11.49% in 13-55 years, 25.42% in >55 years.

### 7.1.2b Genderwise Distribution of Influenza Virus Infected Cases

- The analysis of male: female infection ratio was 1.1:1 and yielded no observable difference between the male and female population affected by *Influenza* virus. Both the sexes were equally infected.

### 7.1.2c Month And Yearwise Distribution of Influenza Virus Infected Cases

- From the six-year observation, though *Influenza* viruses were isolated in all the months appreciable month wise variation in distribution of cases was noted. The notable feature that was observed in the study was the increased virus isolation during the early months of monsoon and winter.

- Highest positivity (19.10%) was observed in the winter season ( 1st October to 7 December) followed by South west monsoon season (1st June to 30 September.) (5%) and late winter (8th Dec to 28/29th Feb) and (4.32%).Very low isolation (1.77%) was observed in the summer months. (1st March to 31 May)

### 7.1.2d Clinical Signs and Symptoms of Influenza Cases

- 100% of suspected *Influenza* viral infections had fever. 63 % of cases had headache, 92.1% of them showed nasal discharge and 72% of them presented with pharyngitis. 73.6% cases showed myalgia and 78% reported productive cough and 64% had symptoms of vomiting and diarrhea but only 8% of cases reported fever with sudden onset.

- Among the *Influenza* positive cases all had sudden onset of symptoms. Fever, cough was present in all the cases followed by headache (92.4%). The other symptoms that were predominantly observed were Myalgia (83.6%), nasal discharge (73%), pharyngitis (67%), Productive cough and vomiting and diarrhea were reported in (23%) and (39%) of the cases respectively. The presence of headache and myalgia along with sudden onset of symptoms, fever and cough were indications of *Influenza* infection among the acute respiratory Infections

### 7.1.2e Predisposing factors and Association of chronic conditions in the elderly with Influenza infections

- The major predisposing factors and chronic factors that were associated with the elderly were Cardiovascular disease, pulmonary disease and diabetes. They were either associated individually or in combinations.

- The presence of a combination of Cardiovascular and pulmonary disease was seen in 53% of the positive cases in the elderly followed by the combination of Cardiovascular and diabetes which was associated with 30% of the cases. Pulmonary disease was found in the 12% of the cases and the rest 4.6% presented with cardiovascular disease.

### 7.1.3 Neuraminidase Typing of Influenza A isolates

- Of the 166 *Influenza* A Isolates typed for neuraminidase 84 were N1 type and 82 were N2. All the N1 types were H1 viruses and the N2 types were H3 isolates.The results indicate that there was no reassortment during the study period.

### 7.1.4 RT-PCR RFLP for detection of antigenic variants

- 24 *Influenza* A/H3N2 isolates obtained in the year 2002, 20 representative isolates of *Influenza* A (H1N1) obtained during the year 2005 and 14 B Victoria lineage isolates and 60 B Yamagata Lineage viruses obtained from 2002-2007 were subjected to RT-PCR RFLP analysis.

- *Influenza* A prototype viruses were digested with their respective enzymes but the H1N1 and H3N2 isolates obtained during the study were not digested.

- *Influenza* B type prototype viruses and isolates were digested.

- The study indicated that the A type viruses have undergone mutation and the B type viruses have not mutated.

### 7.1.5 MOLECULAR CHARACTERIZATION of INFLUENZA A&B ISOLATES BY HA GENE SEQUENCING

- Representative viruses of *Influenza* A/H1N1, A/H3N2, B/Victoria and B/Yamagata lineage from acute respiratory infection cases isolated during the study period were characterized by HA gene sequencing.

- Of the 236 *Influenza* viruses isolated during this study period 86 representative isolates were sequenced. Of this 86 isolates 34 were A/H1N1 (2005-2007) 32 (2002-2007) were A/H3N2, 7 were of B/Victoria lineage(2002, 2005-2007) and 13 were B/ Yamagata lineage viruses (2005-2007).

### 7.1.5a HA sequence analysis of Influenza A&B virus isolates

- The nucleotide sequence identity in terms of HA genes among Chennai 2005-2007 H1N1 isolates was 96.1 to 100%.
- The isolates clustered into two distinct groups I and II with the group II being subdivided into group IIa and IIb
- Eighteen isolates clustered with very high degree of similarity with A/H1/New Caledonia/20/99, the 2000 – 2007 vaccine strain into group I . Group II contained the remaining 16 isolates with eight of them in group IIa clustered with A/H1/New Caledonia/20/99 with a slightly less similarity than the group I isolates and the other eight isolates clustered with A/Solomon Islands/3/06 as group IIb.
- The nucleotide sequence identity in terms of HA genes among Chennai 2002-2007 H3N2 isolates was 96.9 to 100%.
- The isolates clustered into five distinct groups I to V.
- Five 2002 isolates were clustered to group I and were identical to the 00 –02 vaccine strain A/Panama/2007/99.
- Six isolates gathered from 2002 to 2004 clustered to group II, which was identical to the 2002-2003 vaccine strain A/mos/5/97.
- Eleven isolates (2003–2004) were clustered in group III, together with the 2003–2005 vaccine strain A/fujian/10/99.
- Four 2004–2005 isolates were clustered in group IV and were identical to the 2005–2006 vaccine strain A/California/411/02 and the remaining 6 isolates of the years 2005-2007 were clustered into group V and were identical to the 2006–2007 vaccine strain A/Wisconsin/411/02.
- The nucleotide sequence identity among in terms of HA genes among Chennai 2002-2007 *Influenza* B isolates ranged from 87.3 to 100%.

- Chennai *Influenza* B isolates accumulated significant variation in terms of HA nucleotide sequences (12.7%).

- The isolates clustered into two distinct groups I &II.

- Seven Chennai isolates 2002–2007 isolates were in line with the B/Victoria/2/87 lineage and formed group I, were clustered together with the 2002–2004 vaccine strain B/Hong Kong/330/01.

- Thirteen other Chennai *Influenza* B isolates, together with the 2004–2005 vaccine strain B/Shanghai/361/02 (15), clustered into group II.

## PART – II

## 7.2 STANDARDIZATION AND EVALUATION OF RAPID MOLECULAR DIAGNOSTIC TOOL - RT-PCR

- Molecular diagnosis for the rapid detection of *Influenza* viruses from tissue culture fluids as well as the specimen extracts by multiplex RT-PCR was standardized with minor modification of the original CDC, protocol. Throat swab and *Influenza* viral isolates were tested.

- RT-PCR was evaluated based on-detection limit of primers using tenfold dilutions of viral stock and comparison for concordance of the results of nested RT-PCR with the results of virus isolation.

### 7.2.1a Determination of the detection level of primers

- To ensure the sensitivity of the standardized RT-PCR for detection of viruses directly from specimen the detection level of the primers used was assayed.

- The detection level of the RT-PCR primers was evaluated by subjecting 10 fold dilutions of standard *Influenza* A and B virus stocks ranging form 1000 to 0.001 $TCID_{50}$.

- The primers were able to detect $0.1TCID_{50}$ of *Influenza* A & B type viruses.

### 7.2.1b Evaluation of RT-PCR in detection of viruses from specimen by comparison with virus isolation in confirmation of the etiology

- The results showed PCR was more sensitive than the conventional virus isolation.

- 16.66 % of the samples collected within 7 days of onset were positive by RT-PCR whereas only 10 % of those samples were positive by virus isolation.

- RT- PCR was able to detect *Influenza* RNA in samples that have been collected from day 1 to 15 days from the onset.

- RT-PCR can be regarded as confirmatory diagnosis for *Influenza* virus infection, as genetic material from antigen is detected which is more or less like demonstration of antigen itself, less labor-intensive and rapid.

### 7.2.2 Standardization of multiplex RT-PCR

- Multiplex RT-PCR was standardized with 1 μM concentration of KCl. 20 mM of ammonium sulphate.

- It was as sensitive and specific as RT-PCR.

- Multiplex RT-PCR can be regarded as confirmatory diagnosis for *Influenza* virus infection, as genetic material from antigen is detected which is more or less like demonstration of antigen itself, less labor-intensive, rapid and economical.

# PART – III

## 7.3 SCREENING OF ISOLATES FOR ANTIVIRAL RESISTANCE

In the present study, A(H3N2) and A(H1N1) isolates were tested for their sensitivity to the currently used M2 inhibitor (Amantadine) by minimum inhibitory assay (MIC) using MDCK cell line. The results obtained by MIC were confirmed by RT-PCR - RFLP.

### 7.3.1 Screening of isolates for Antiviral resistance by MIC

- 162 *Influenza* A viruses were screened for their sensitivity to the M2 Inhibitor Amantadine.
- Drug susceptibility was determined by growing *Influenza* virus isolates in MDCK cell cultures in the presence of amantadine at a concentration of 1 μg/ ml.
- All H1N1 isolates were sensitive and did not show any resistance.
- 4 H3N2 Isolates were resistant to Amantadine.
- H3N2 Isolates (60nos) isolated during the years 2002-2004 did not show any resistance.
- 2 of 11 in 2005, 1 of 5 in 2006 and 1 of the 2 H3N2 isolates in 2007 were resistant.

### 7.3.2 Detection M2 mutants RT-PCR RFLP:

- The drug resistant viruses did not show restriction pattern thus indicating mutation in the M2 protein.
- The drug sensitive controls showed restriction pattern.
- All the 82 H1N1 isolates showed restriction pattern similar to the drug sensitive controls thereby indicating that all H1N1 isolates have not undergone any mutations and are sensitive to amantadine.

- Of the 80 H3N2 isolates 76 showed restriction pattern similar to the drug sensitive controls whereas 4 isolates did not show restriction digestion in the 31 amino acid position indicating resistance to amantadine.

- Thus the results of MIC are confirmed by RT-PCR RFLP analysis.

### 7.3.3 Determination of Antiviral Activity of Medicinal Herbs

- In the present study, aqueous and ethanolic extracts of the herbs *Ocimum sanctum and Glycyrrhiza glabra* were evaluated *in vitro* for antiviral activity against *Influenza* A&B viruses.

#### 7.3.3a Toxicity assessment of solvents and extracts on MDCK cell line

- The toxicity of solvents, aqueous and ethanolic extract of the two herbs used were determined and nontoxic concentration was used in antiviral studies.

- Concentration $\leq$ 500 μg of aqueous and ethanolic extracts of *Ocimum sanctum* was found to be non-toxic.

- Long time storage of the extracts up to 6 months in $-20^{o}C$ showed no difference in the toxicity or antiviral pattern.

#### 7.3.3b Antiviral screening of *Ocimum sanctum* for anti-Influenza A & B virus activity

- Antiviral activity was evaluated by virus inactivation assay, virus adsorption assay and pretreatment assay.

- The minimum concentration of 100 μg/ml of aqueous and ethanolic extracts of Ocimum showed complete inhibition of 1,10 and100 $TCID_{50}$ of *Influenza* A & B virus propagation by both virus inactivation and virus adsorption assay. There was no observable enhancement of virus neutralization by varying the time of exposure of virus to the extracts.

### 7.3.3.3c Antiviral screening of *Glycyrrhiza glabra* for anti-Influenza A & B virus activity

- Antiviral activity was evaluated by virus inactivation assay, virus adsorption assay and pretreatment assay.

- The minimum concentration of 100 μg/ml of aqueous and ethanolic extracts of *Glycyrrhiza glabra* showed complete inhibition of 1,10 and100 $TCID_{50}$ of *Influenza* A & B virus propagation by both virus inactivation and virus adsorption assay. There was no observable enhancement of virus neutralization by varying the time of exposure of virus to the extracts.

# 8. CONCLUSION

- **Serosurvey for determination of Influenza Haemagglutinating antibody base-line titre**

The results of the serosurvey established the baseline neutralizing antibody titre of 1:40 for *Influenza* in normal healthy population.

Antibodies to *Influenza* A& B group were found to be elevated in the 6-15 years age group indicating high exposure to *Influenza* in indicating high circulation of *Influenza* A&B viruses in this age group. It is necessary to evaluate baseline titer periodically to assign the cut-off values for diagnostic purposes. It also indicates the outbreaks that might have occurred silently.

- **Influenza virus etiology of Acute Respiratory Infections (2002-2007)**

*Influenza* accounted for 7.77% of acute respiratory infections cases from 2002-2007 and *Influenza* A/H1N1 among *Influenza* A type and B/Yamagata type among the *Influenza* B type were found to be the predominant serotype.

Male and female children were equally affected

The notable feature that was observed in the study was the increased virus isolation during the early months of monsoon and winter. Simultaneous co-existence of *Influenza* A and B serotypes throughout the study period confirmed their endemic circulation.

There has been a continuous variation in the *Influenza* strains isolated in the study period. A/H3N2 predominated in the years 2002-2004 then H1N1 that was not circulating till then came into existence and predominated in the years 2005-2006. The year 2007 saw the predominance of the *Influenza* B type viruses for the first time. As the virus activity is constantly changing, there is a need for continuous surveillance.

**Clinical Manifestation**

The symptoms in a combination of cough, headache, and pharyngitis were found to predict an increased likelihood of *Influenza*.

**In Elderly**

Among the predisposing factors in the elderly, a combination of cardiovascular and pulmonary disease was predominantly associated with *Influenza* virus isolation followed by the combination of Cardiovascular and diabetics. Pulmonary disease was the major single predisposing factor to *Influenza* Virus infection followed by cardiovascular disease.

There was no specific clinical sign or symptom to be ascribed to either type or subtype in particular. The clinical manifestation observed in *Influenza* B type viruses was found to be less severe.

**Neuraminidase typing of Influenza A isolates**

No unusual changes in the neuraminidase types were detected among the *Influenza* A type in the study period. All the *Influenza* A types isolated were either A/H1N1 or A/H3N2. This indicates that there was no reassortment between the *Influenza* subtypes during the study period.

**RT-PCR RFLP**

The *Influenza* A virus isolates indicated drift in the sequence did not show any restriction pattern. Further sequencing has to be done to study the mutation occurred at the restriction site and it significance in the antigenicity of the viruses.

As the test is very cumbersome and very difficult to reproduce and there is a need for constant monitoring of the sequence and change enzyme accordingly the test cannot be recommended for detection of antigenic variants.

**Evaluation of Molecular Diagnostic assay for Influenza virus detection RT-PCR**

RT-PCR was found to be more sensitive with low virus requirement and showed no cross reactivity. RT-PCR was sensitive even when samples were collected within 0-15 days from onset. The detection limit of RT-PCR was found to be 0.1 $TCID_{50}$ showing very high sensitivity.

The role of virus isolation is of utmost importance as strains for vaccines need to be propagated. But RT-PCR can be used as first line of diagnosis and the positive samples can be inoculated and virus isolated for vaccine stock. Thus a combination of RT-PCR and virus isolation serves as the next best alternate. Multiplex RT-PCR provides us with an option of an economical rapid diagnostic tool.

**Sensitivity to Amantadine**

Resistance has started to emerge among the *Influenza* A/H3N2 type viruses. 4 isolates in the study period showed resistance but the resistance percentage is increasing every year. The results of MIC were in total concurrence with RT-PCR RFLP, therefore MIC can be used to screen large number of samples and the positives can be subjected to RT-PCR RFLP to study where the mutations have occurred and about the amino acid change. There is a need for continued surveillance for Amantadine resistance.

**Antiviral screening of two herbs**

The results of antiviral evaluation of two herbs showed that the aqueous and ethanolic extracts of herbs *Ocimum sanctum* and *Glycyrrhiza glabra* showed anti *Influenza* B activity. The extracts of the were both virucidal and inhibitory to viral replication.The virucidal effect of the all the three extracts were concentration dependent.

# 9. FUTUROLOGY

In a scenario where the threat of Avian *Influenza* is looming large the surveillance of *Influenza* is gaining significance.

- Laboratory based surveillance needs to be established throughout the state to understand the burden of the disease.
- Neuraminidase typing by RT-PCR may be made mandatory for early detection of reassortants.
- RT-PCR should be used rapid detection of the virus.
- Multiplex RT-PCR provides us with an option of an economical rapid diagnostic tool further standardization to make it an one step test will reduce the chance of cross contamination and also the time taken for the test.
- Screening for Amantadine resistance must be done for all the *Influenza* A isolates as there has been an increase in the Amantadine ressistance among the isolates.
- Though effective vaccine and antiviral drugs are available against *Influenza* viral infection, the cost, availability and resistance are the major drawback of them. Promising results have been shown by Ocimum and *Glycyrrhiza glabra when* were evaluated *in vitro* for antiviral activity. This has to be further characterized by phytochemical analysis. Evaluation of combined efficiency of herbs and synthetic drugs may throw light on new dimension in treating *Influenza* viral infection.

**Figure 1.1: Influenza Virus.**

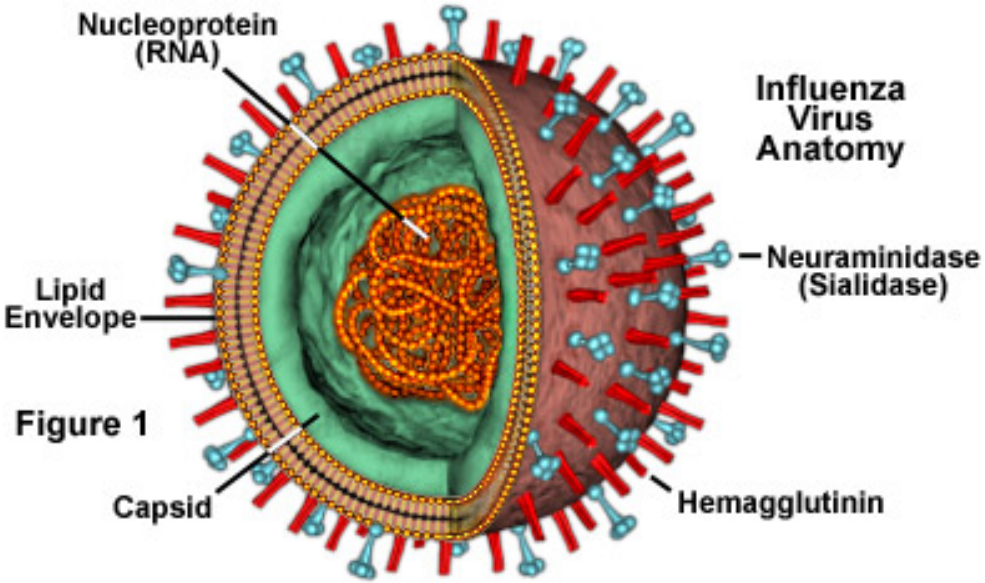
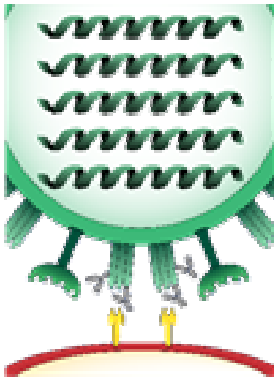


**Figure 1.2: Antigenic Drift in Influenza viruses.**



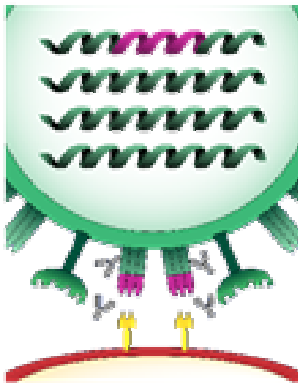


**Figure 1.3: Antigenic Shift in Influenza viruses.**

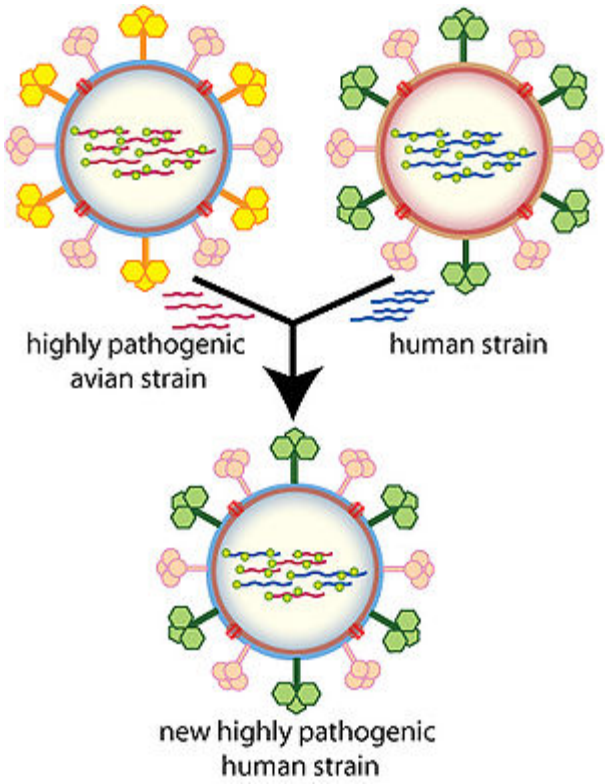
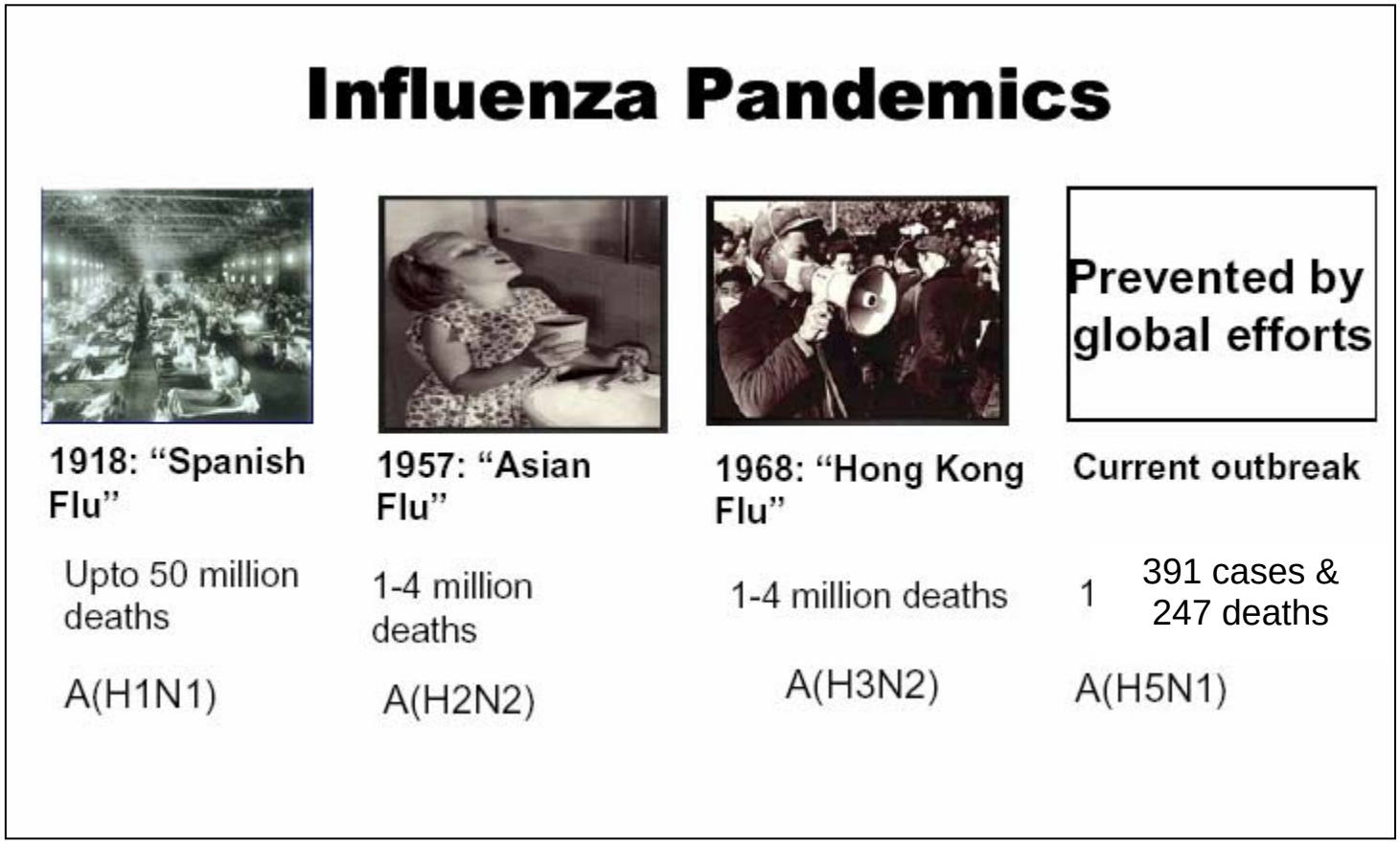

highly pathogenic
avian strain
human strain
new highly pathogenic
human strain

**Figure 1.4 Impact of Influenza Pandemics.**



## Figure 1.5: Countries affected by Avian influenza in poultry

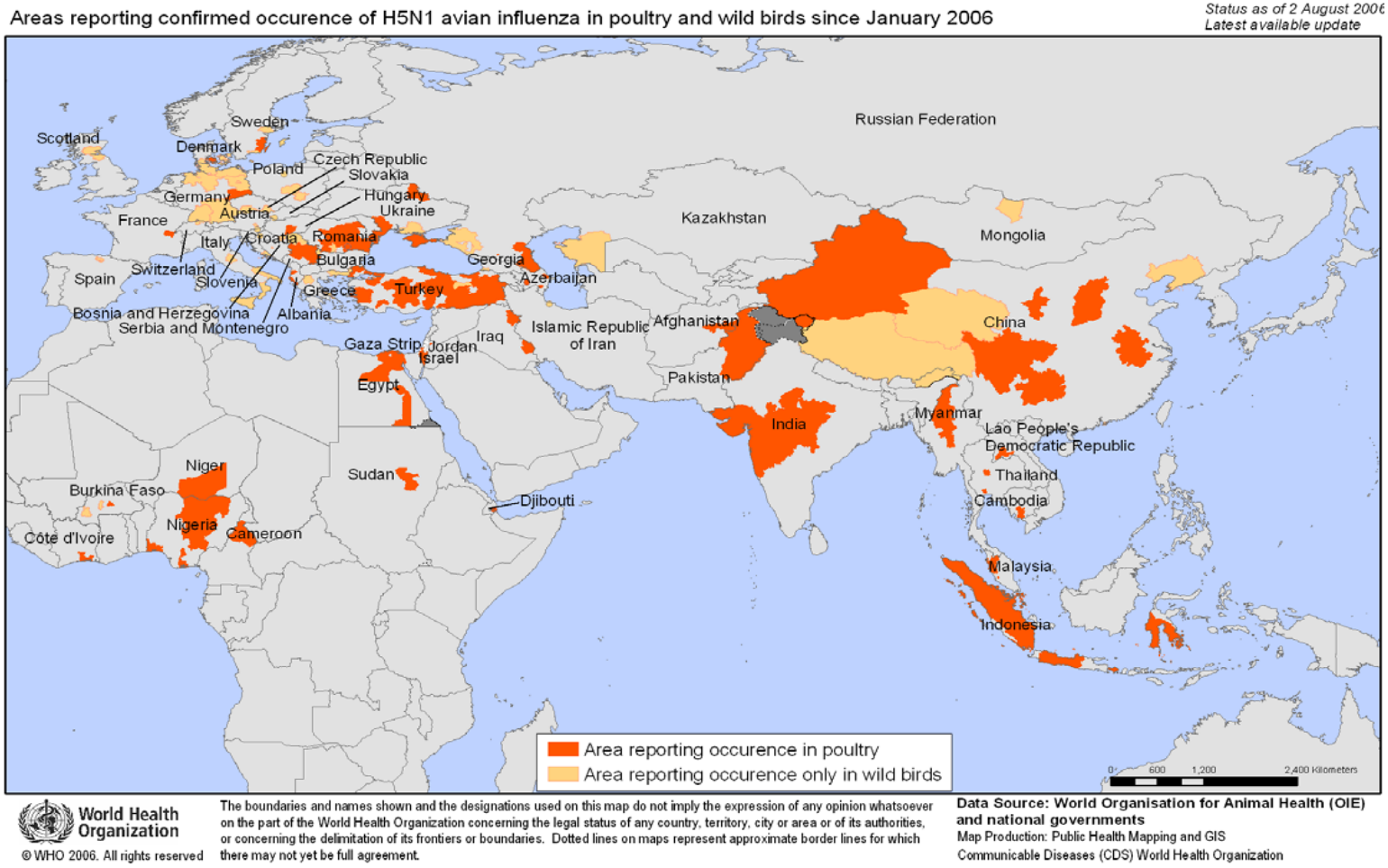


**Figure 1.6: Cumulative Number of Confirmed Human Cases of Avian Influenza A/(H5N1) Reported to WHO 16 December 2008**

| Country | 2003 | | 2004 | | 2005 | | 2006 | | 2007 | | 2008 | | Total | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | cases | deaths | cases | deaths | cases | deaths | cases | deaths | cases | deaths | cases | deaths | cases | deaths |
| Azerbaijan | 0 | 0 | 0 | 0 | 0 | 0 | 8 | 5 | 0 | 0 | 0 | 0 | 8 | 5 |
| Bangladesh | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 |
| Cambodia | 0 | 0 | 0 | 0 | 4 | 4 | 2 | 2 | 1 | 1 | 1 | 0 | 8 | 7 |
| China | 1 | 1 | 0 | 0 | 8 | 5 | 13 | 8 | 5 | 3 | 3 | 3 | 30 | 20 |
| Djibouti | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| Egypt | 0 | 0 | 0 | 0 | 0 | 0 | 18 | 10 | 25 | 9 | 8 | 4 | 51 | 23 |
| Indonesia | 0 | 0 | 0 | 0 | 20 | 13 | 55 | 45 | 42 | 37 | 22 | 18 | 139 | 113 |
| Iraq | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 2 | 0 | 0 | 0 | 0 | 3 | 2 |
| Lao People's Democratic Republic | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 2 | 0 | 0 | 2 | 2 |
| Myanmar | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 |
| Nigeria | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 |
| Pakistan | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 1 | 0 | 0 | 3 | 1 |
| Thailand | 0 | 0 | 17 | 12 | 5 | 2 | 3 | 3 | 0 | 0 | 0 | 0 | 25 | 17 |
| Turkey | 0 | 0 | 0 | 0 | 0 | 0 | 12 | 4 | 0 | 0 | 0 | 0 | 12 | 4 |
| Viet Nam | 3 | 3 | 29 | 20 | 61 | 19 | 0 | 0 | 8 | 5 | 5 | 5 | 106 | 52 |
| Total | 4 | 4 | 46 | 32 | 98 | 43 | 115 | 79 | 88 | 59 | 40 | 30 | 391 | 247 |

**Figure 2.1. Generation of a pandemic Influenza strain**

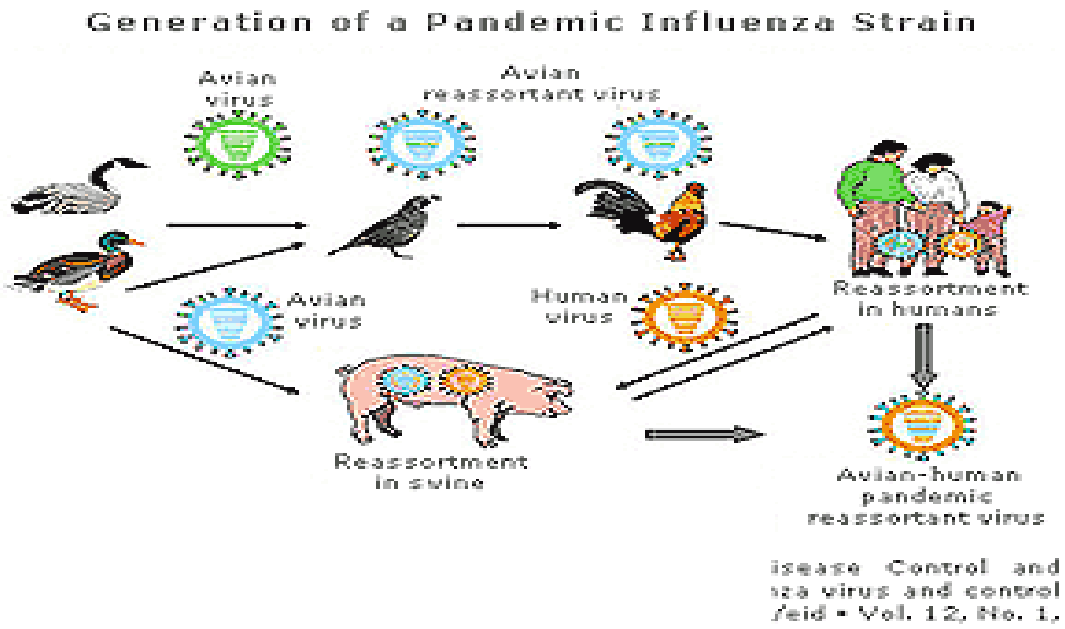


**Fig 4.1 Haemagglutination Test**

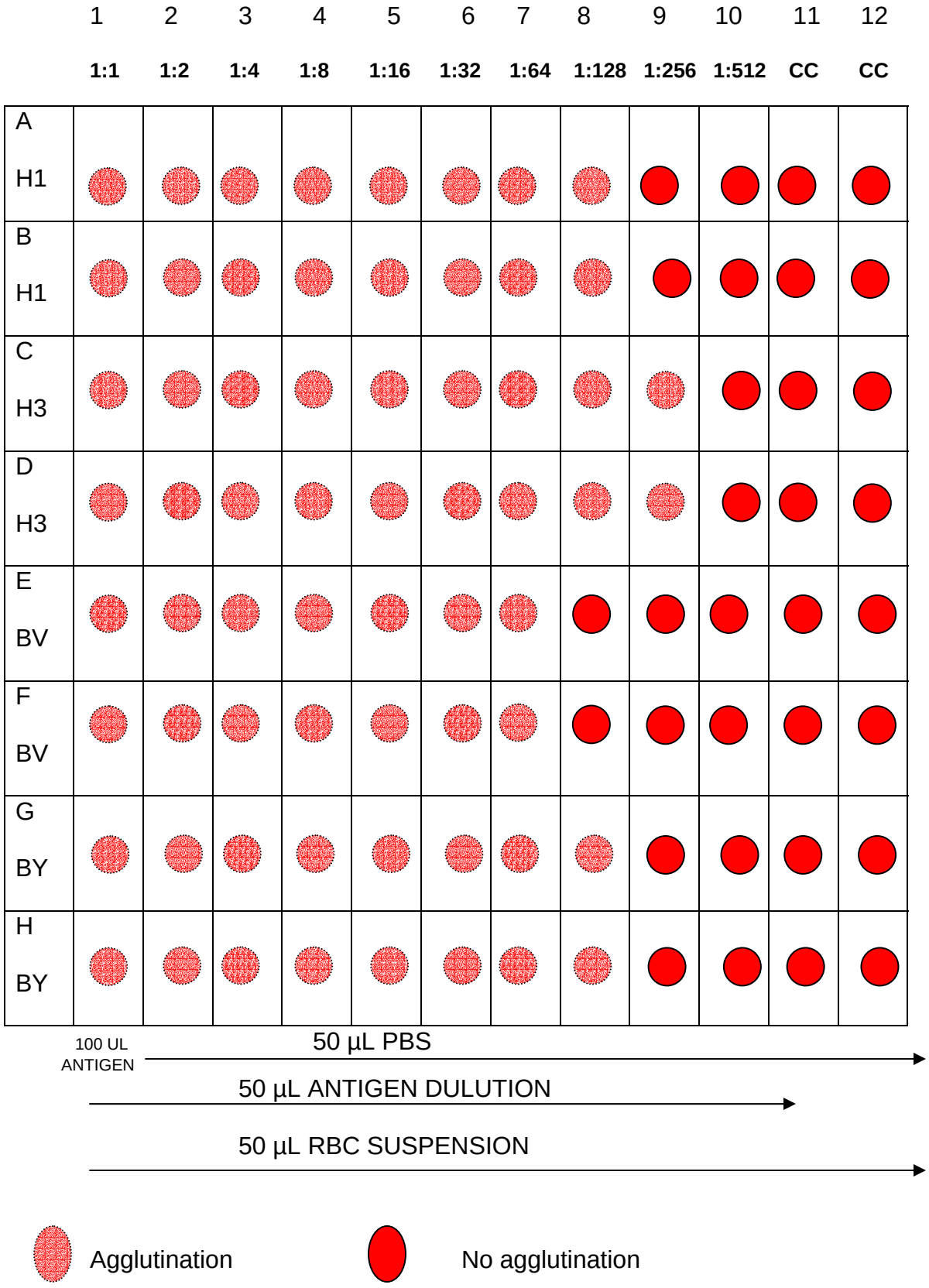


**Fig 4.2 Back Titration**

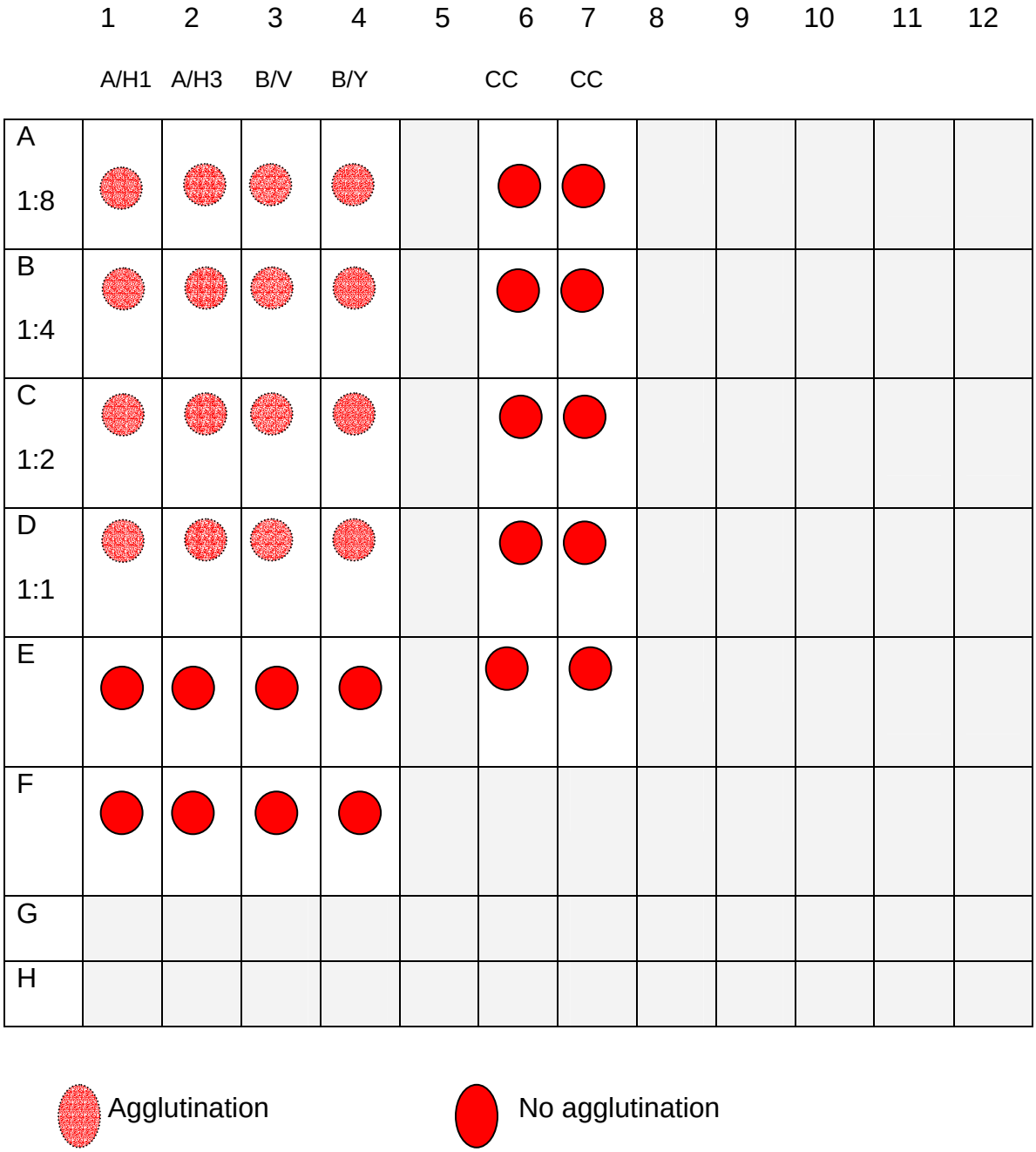


**Fig 4.3 Haemagglutination Inhibition Test**

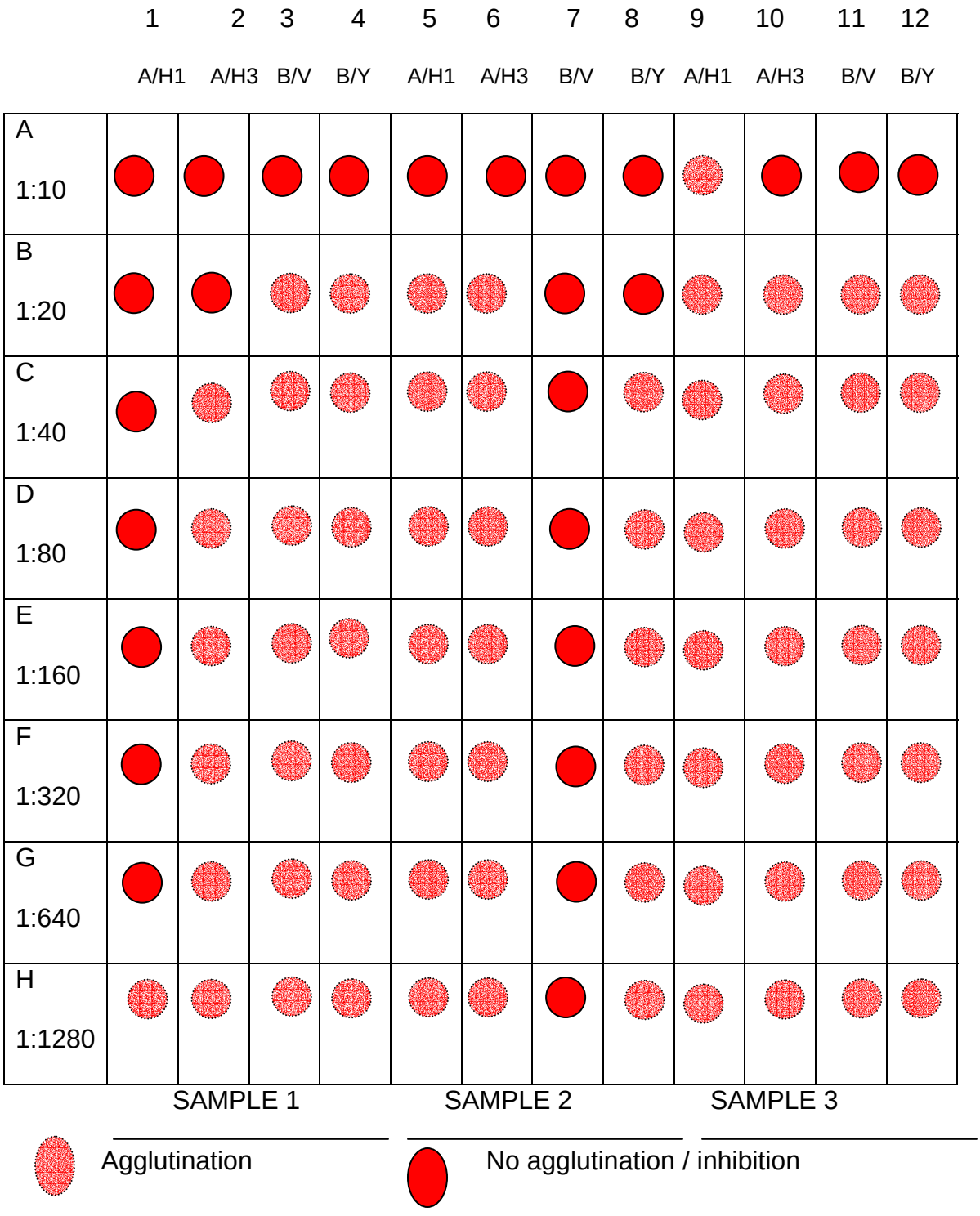


**Fig 4.4 Control Plate**

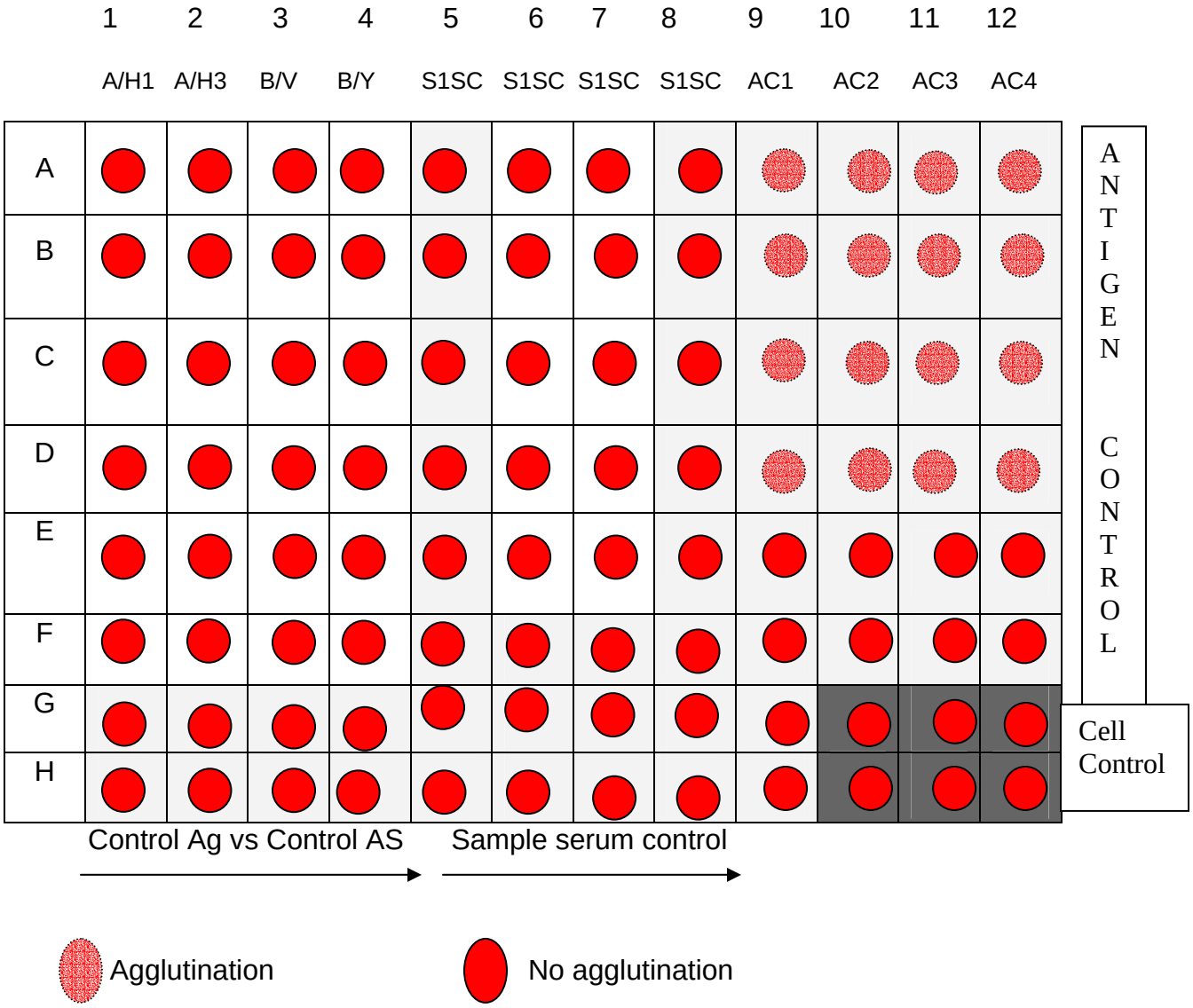


**Table 4.1 MEM Preparation Procedure**

| Ingredients | 10% Growth Media | Without FCS Maintenance Media | Without FCS Virus growth Media |
|---|---|---|---|
| MEM | 836 ml | 931 ml | 930 ml |
| P&S (Penicillin & Streptomycin) | 1 ml | 1 ml | 1 ml |
| Phenol red (0.4%) | 1 ml | 1 ml | 1 ml |
| Kanamycin | 1 ml | 1 ml | 1 ml |
| Fungizone | 1 ml | 1 ml | 1 ml |
| 3% L-Glutamine | 10 ml | 10 ml | 10 ml |
| Foetal Bovine Serum | 100 ml | NIL | NIL |
| 2.2g Sodium bicarbonate | 20 ml | 20 ml | 20 ml |
| Hepes buffer (IM) | 5 ml | 5 ml | 5 ml |
| 7.5% BSA Fraction V | 25 | 25 | 25 |
| TPCK Trypsin (2mg/ml) | NIL | NIL | 1 ml |
| Total volume | 1000 ml | 1000 ml | 1000 ml |

**Table 4.2: Primer sequence for amplification of NA gene**

| Primer | Target gene | Sequence | Base location | Size bp |
|---|---|---|---|---|
| A/N1 Fp | A/N1 | 5'-TTGCTTGGTCAGCAAGTGCA-3' | 505-524 | 708 |
| A/N1 Rp | | 5'-TTAGCTCAGGATGTTGAACG-3' | 1193-1212 | |
| A/N2 Fp | A/N2 | 5'-GGTGACGAGAGAACCTTATG-3' | 345-364 | 615 |
| A/N2 Rp | | 5'-CCTGAGCACACATAACTGGA-3' | 940-959 | |

**Fig. 4.5: RT-PCR Cycling conditions for amplification of NA gene.**

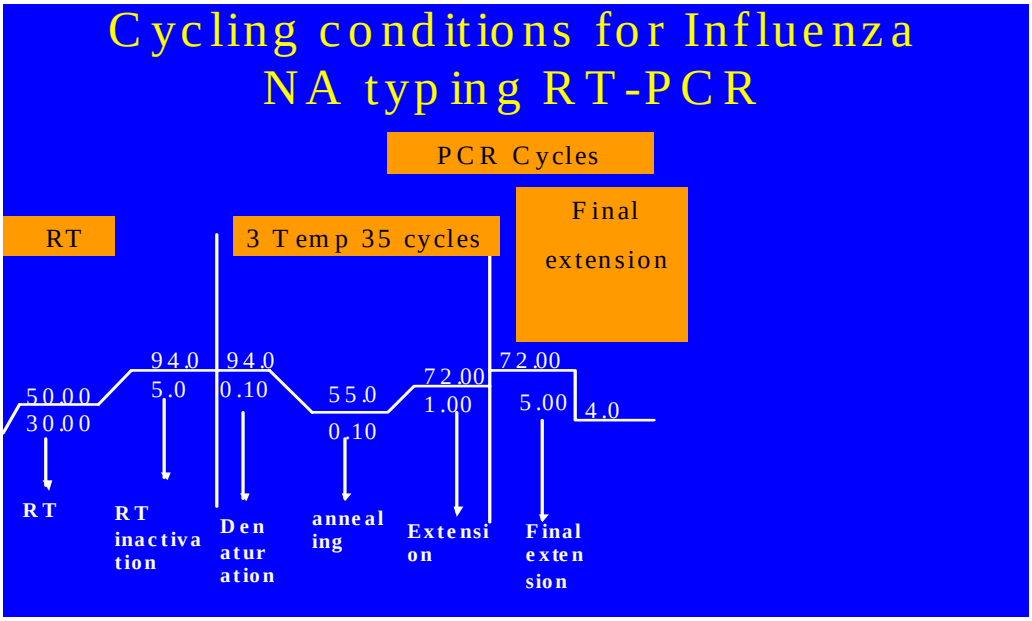


**Table 4.3. Primer sequence for amplification of HA gene targets for RFLP analysis**

| Virus | Primer | Sequence |
|---|---|---|
| A/H1N1 | H1HAF<br>H1HAR | 5'- AAG CAG GGG AAA ATA AAA -3'<br>5-'TGC CAT TAA CGG GAT TAC-3' |
| A/H3N2 | H3HAF<br>H3HAR | 5'- ACT ATC ATT GCT TTG AGC -3'<br>5'- ATG GCT GCT TGA GTG CTT- 3' |
| B Virus | BHAF<br>BHAR | 5'- GAA GGC AAT AAT TGT A -3'<br>5'- ACC AGC AAT AGC TCC GAA-3' |

**Table 4.3.a. Enzymes for RFLP analysis and their digested fragment lengths**

| Virus | Enzyme | Product in bases |
|---|---|---|
| A/H1N1 | Aci I | 651<br>442 |
| A/H3N2 | Ban I | 556<br>504 |
| B/Sichuan | Mae II | No cut<br>1104 |
| B/Hong Kong | Mae II | 671<br>433 |

**Table 4.4: Primer sequences for Influenza RT-PCR for sequencing**

| Virus | Primer | Sequence |
|---|---|---|
| A/H1N1 | H1HAF<br>H1HAR | 5'-AAG CAG GGG AAA ATA AAA-3'<br>5'-TAG ATT TCC ATT TGC CTC-3' |
| A/H3N2 | H3HAF<br>H3HAR | 5'-ACT ATC ATT GCT TTG AGC-3'<br>5'-CAG TAT GTC TCC CGG TTT-3' |
| B Virus | BHAF<br>BHAR | 5'-GAA GGC AAT AAT TGT A-3'<br>5'-GTT GAT TAC ATG GTG-3' |

**Fig. 4.6: Cycling conditions for Influenza RT-PCR for sequencing**

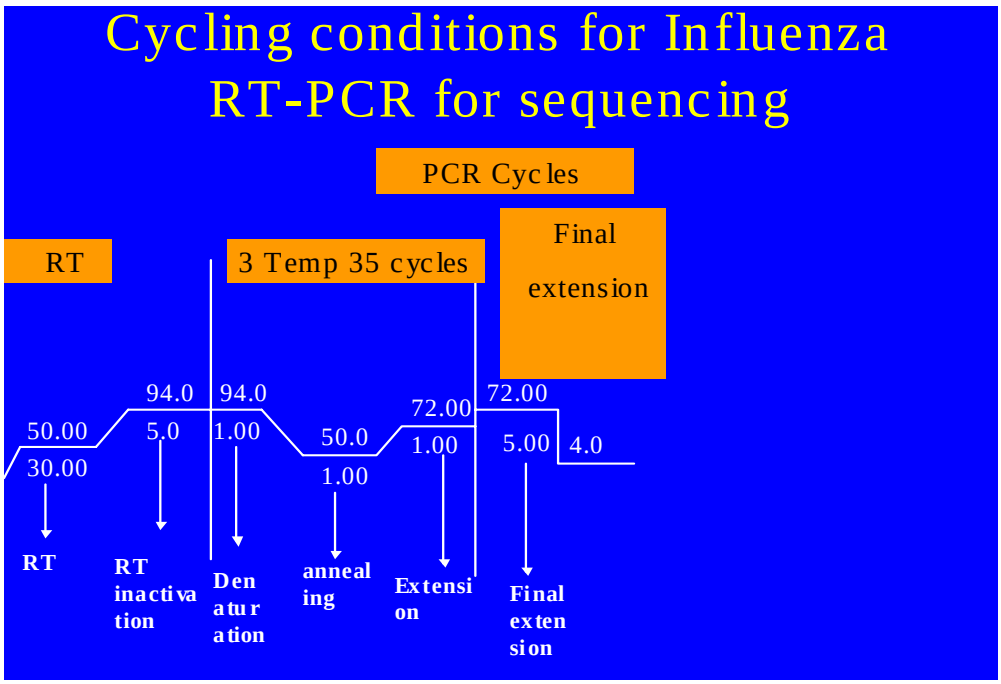


**Table 4.5: Primer sequences for Influenza Diagnostic RT-PCR**

| Virus | Primer | Region | Sequence |
|---|---|---|---|
| A/H1N1 | H1HAF<br>H1HAR | 6-523 | 5'- AAG CAG GGG AAA ATA AAA -3'<br>5'- CCG TCA GCC ATA GCA AAT -3' |
| A/H3N2 | H3HAF<br>H3HAR | 7-792 | 5'- ACT ATC ATT GCT TTG AGC -3'<br>5'- CAG TAT GTC TCC CGG TTT -3' |
| B Virus | BHAF<br>BHAR | 36-654 | 5'- GAA GGC AAT AAT TGT A -3'<br>5'- GTT GAT TAC ATG GTG – 3' |

**Fig. 4.7: Cycling conditions for Influenza Diagnostic RT-PCR**

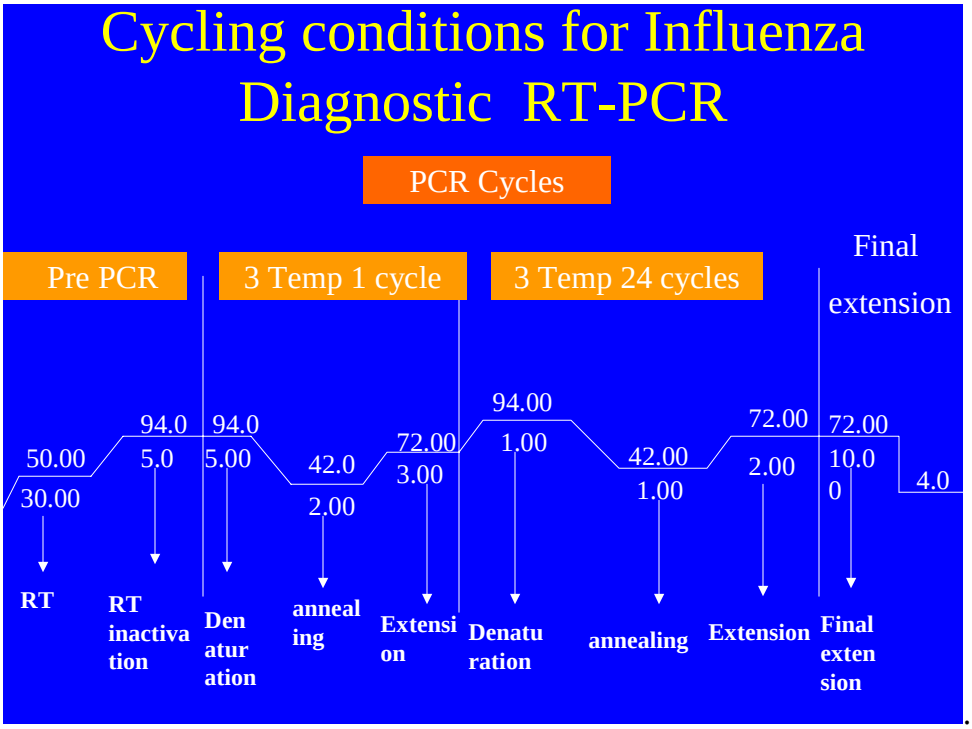


**Table 4.6:Primer sequences and Restriction enzymes for detection M2 mutants**

| Primer | Sequence | Base location | Size bp | Endo-nuclease | Fragment length |
|---|---|---|---|---|---|
| M2 F | 5’- CTAGTCAGGCCAGGCAAATG-3’ | 651 | 339 | NA | NA |
| M2 R | 5’- ACTGTCGTCAGCATCCACAG-3’ | 990 | | | |
| M2-27 F | 5’-GGGGGTGCAGATGCAACGATTCAAGTGACCCACAT-3’ | 732 | 154 | BspLU11 I | 32,122 |
| M2-27 R | 5’-TCCGTAGAAGGCCCTCTTTT -3’ | 885 | | | |
| M2-30 F | 5’-CTATCAGAAACGAATGGGGG -3’ | 717 | 95 | Hha 1 | 33,62 |
| M2-30 R | 5’-CCACAATATCAAGTGCAAGATCCCAATGATACGC -3’ | 811 | | | |
| M2-31 F | 5’-TCCTAGCTCCAGTGCTGGTC -3’ | 666 | 153 | Sca I | 37,116 |
| M2-31 R | 5’-GAAGAACCACAATATCAAGTGCAAGATCCCAATAGT -3’ | 818 | | | |

**Fig. 4.8: RT-PCR Cycling conditions for detection M2 mutants**

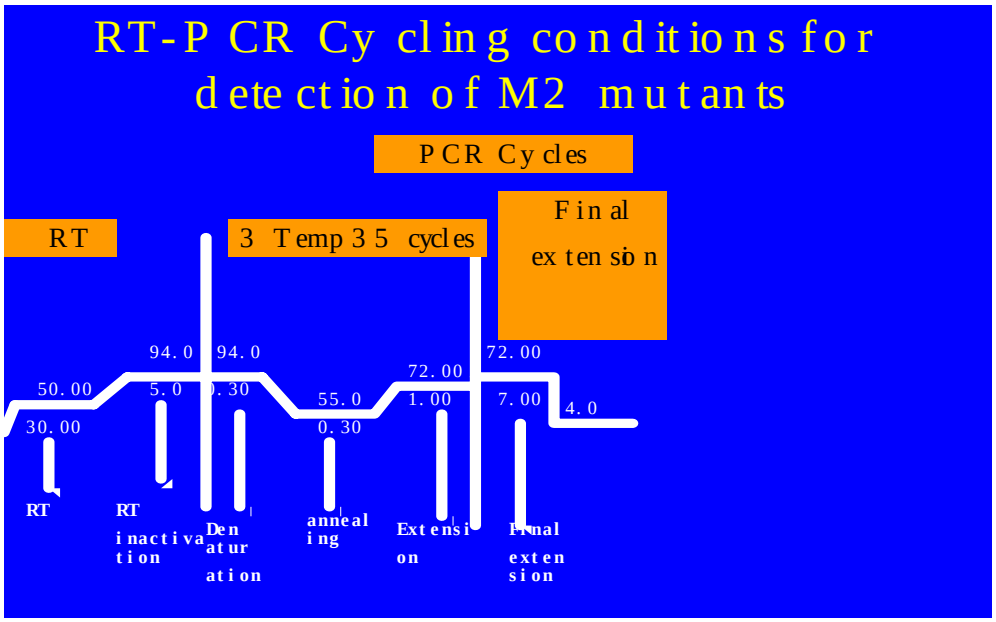



**Fig. 4.9: Nested PCR Cycling conditions for detection of M2 mutants**

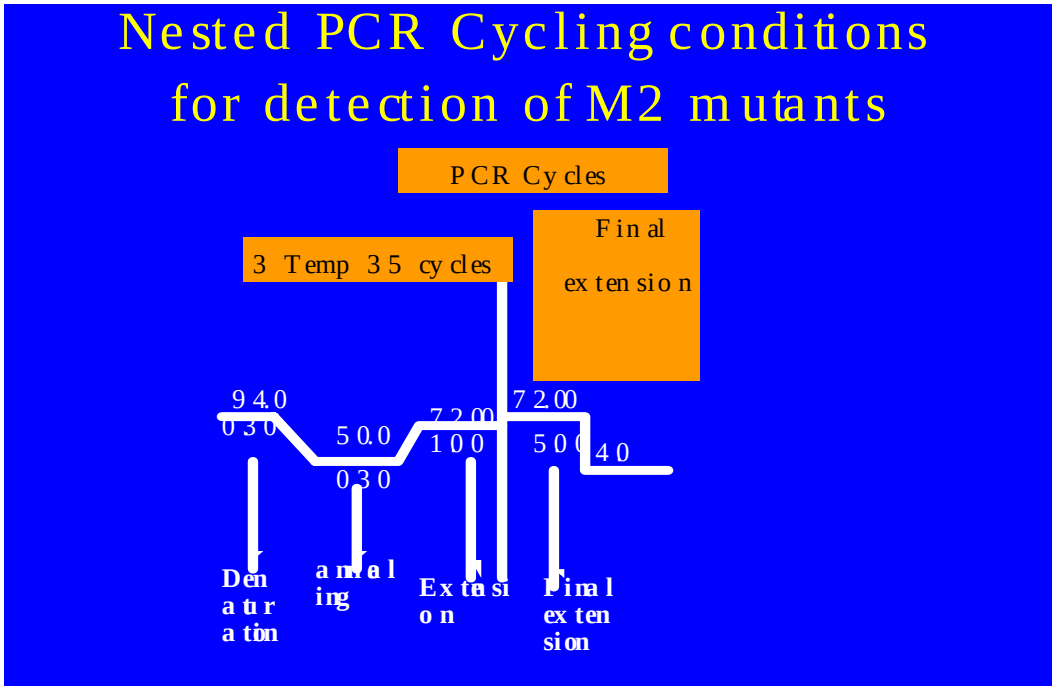


**Figure: 4.10 *Ocimum sanctum***

**Figure: 4.11 Glycyrrhiza glabra**

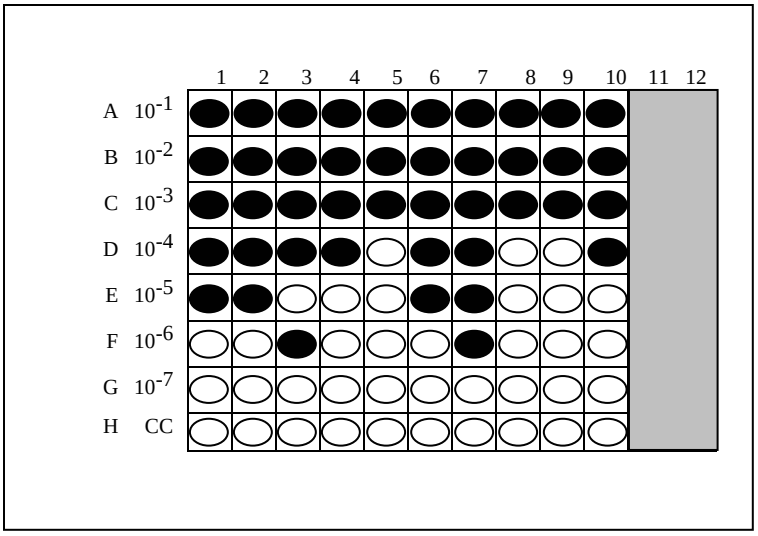
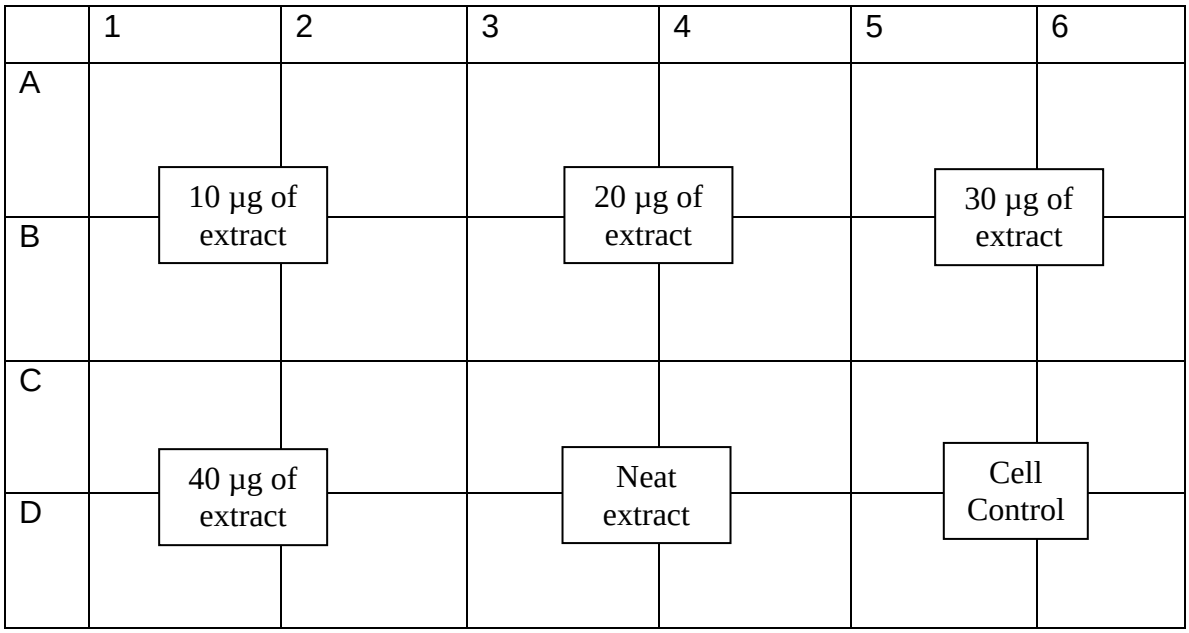
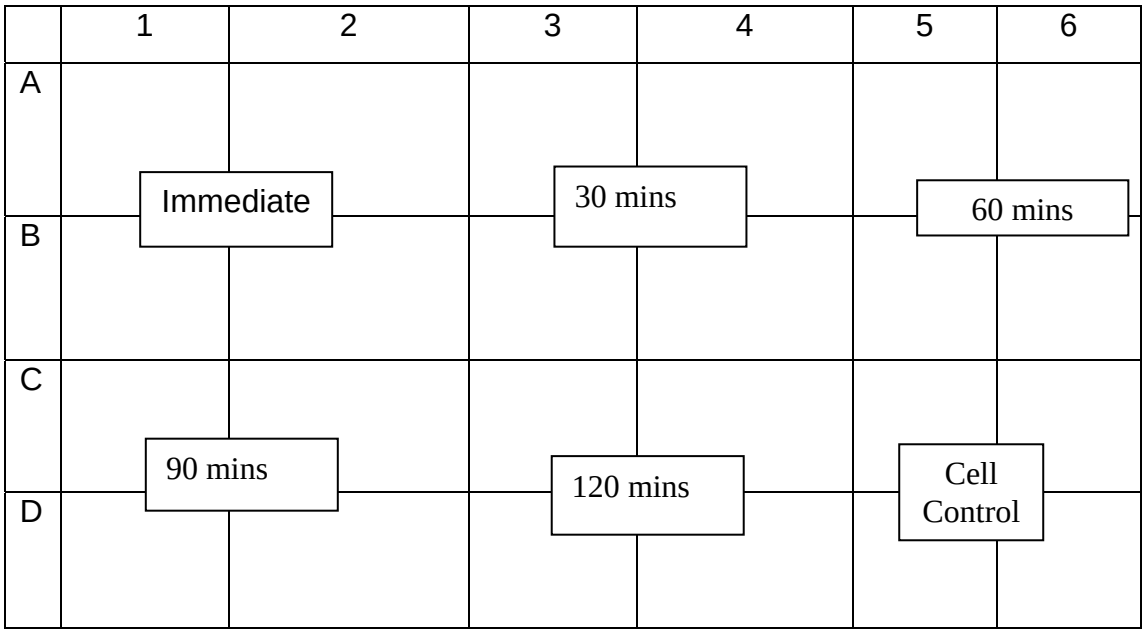



**Fig. 4.12: Estimation of TCID50 of Influenza A & B pool**




**Cell line:** **Extract used:** **Plate No:** **Date:**

**Figure 4.13 : Cytotoxicity assay protocol in 24 well micro tissue culture plate.**
**Above shown is an example of cytoxicity plate 5 such plates were put up with different concentrations of the extracts.**

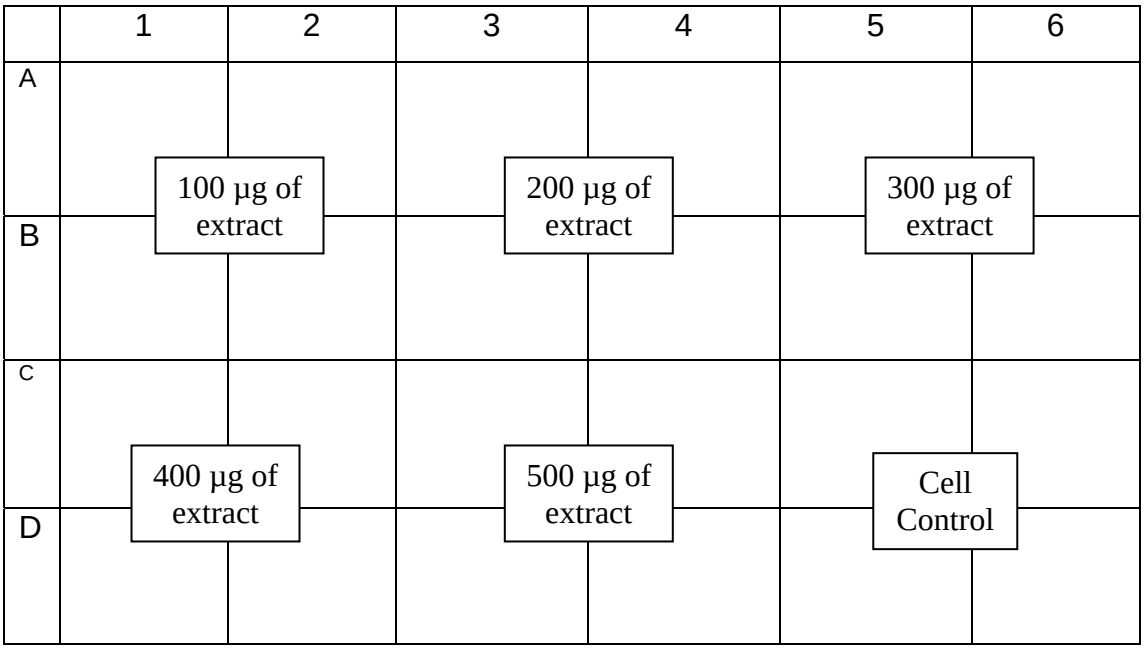



Cell line: Extract used: Virus used: Plate No: Date:

**Figure 4.14 : Protocol in 24 well TC Plates – Virus Inactivation Assay**

In each well 100 µg of extract (+) 100 TCID 50 of Influenza A&B virus pool was used

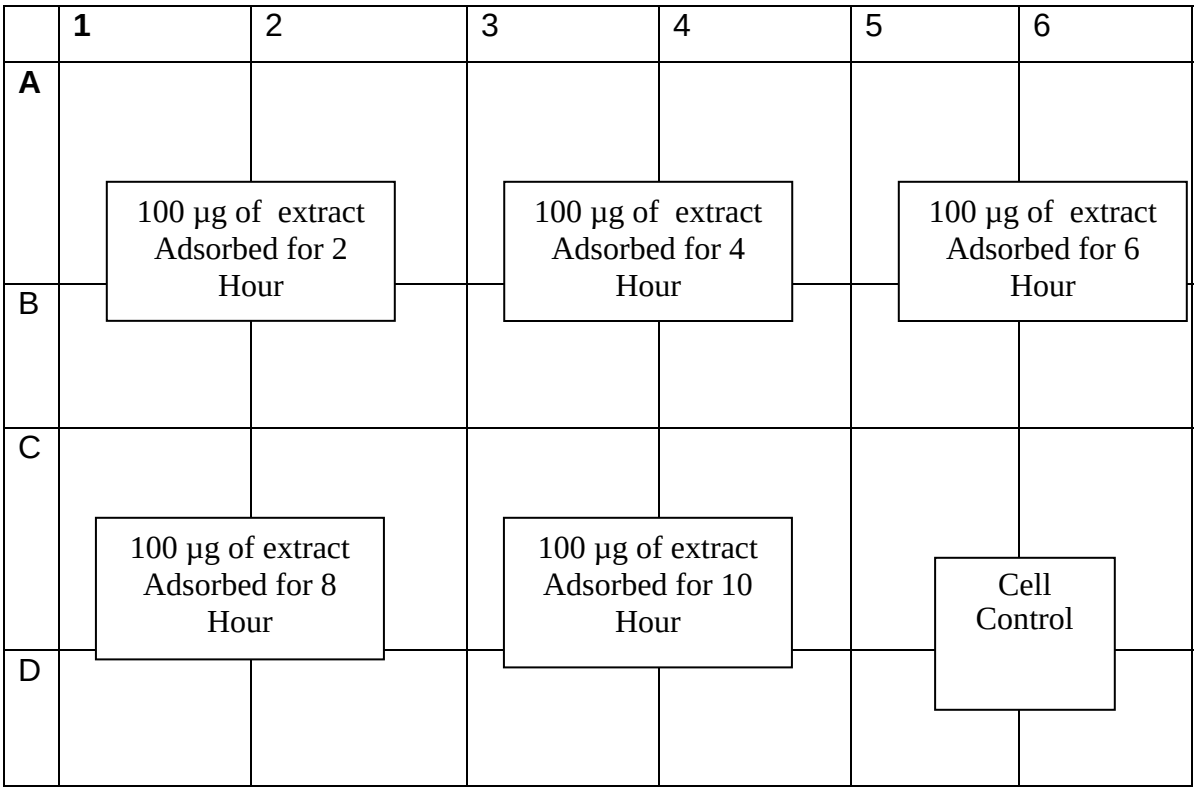
Above shown is an example of Direct Pre- infection incubation (DPI) assay plate 5 such plates were put up with different concentrations of the extracts.




Cell line: Extract used: Plate No: Date:

**Figure 4.15: Protocol in 24 Well TC Plates – Virus Adsorption Assay**

Each well contained 100 $TCID_{50}$ of Influenza A&B virus pool adsorbed for1 hour to which varying concentrations of the extract was added. Two more plates with the concentration of 1 and 10 $TCID_{50}$ of the viruses were put up.




Cell line: Extract used: Plate No: Date:

**Figure 4.16 : Protocol in 24 Well TC Plates – Pre-Treatment Assay**

The wells contained 100 $TCID_{50}$ of *INFLUENZA A & B* virus pool adsorbed for different time intervals HOUR to which 100 µg of the extract was added. Fourteen such plates with the concentration of 1 and 10 $TCID_{50}$ of the viruses and different concentrations of the drug were put up.

**Table 5.1: 2001 –2006 (% population exposed)**

| Antigen | 2001 | 2002 | 2003 | 2004 | 2005 | 2006 | Total |
|---|---|---|---|---|---|---|---|
| A/H1N1 | 49.1 | 32.6 | 20.83 | 18.14 | 32.43 | 44.06 | 32.86 |
| A/H3N2 | 56.6 | 36.6 | 25.83 | 32.46 | 32.32 | 29.66 | 35.05 |
| Total A | 52.85 | 34.6 | 23.33 | 25.3 | 32.37 | 36.86 | 34.2 |
| B/Vic | 28 | 48.5 | 40.83 | 42.44 | 40.58 | 38.33 | 39.78 |
| B/Yamagata | 12 | 25 | 25 | 29.6 | 32.66 | 43.45 | 27.95 |
| Total B | 20 | 36.75 | 32.9 | 36.02 | 32.62 | 40.89 | 33.19 |
| Total A&B | 36.45 | 35.6 | 28.12 | 30.66 | 34.47 | 38.86 | 34.02 |

Table 5.2: Geometric mean titre of the study population to influenza viruses during 2001 –2006

| Antigen | 2001 | 2002 | 2003 | 2004 | 2005 | 2006 |
|---|---|---|---|---|---|---|
| **A/H1N1** | 32.2 | 29.4 | 16.54 | 14.2 | 28.9 | 40 |
| **A/H3N2** | 43.5 | 30.6 | 20.12 | 28.52 | 29.6 | 26.39 |
| **B/Vic** | 22.3 | 41.3 | 33.41 | 33.1 | 34.7 | 33.4 |
| **B/Yamagata** | 10 | 22 | 21.36 | 23 | 30.5 | 37.34 |

Figure 5.1: Geometric mean titre of the study population for Influenza viruses during 2001-2006

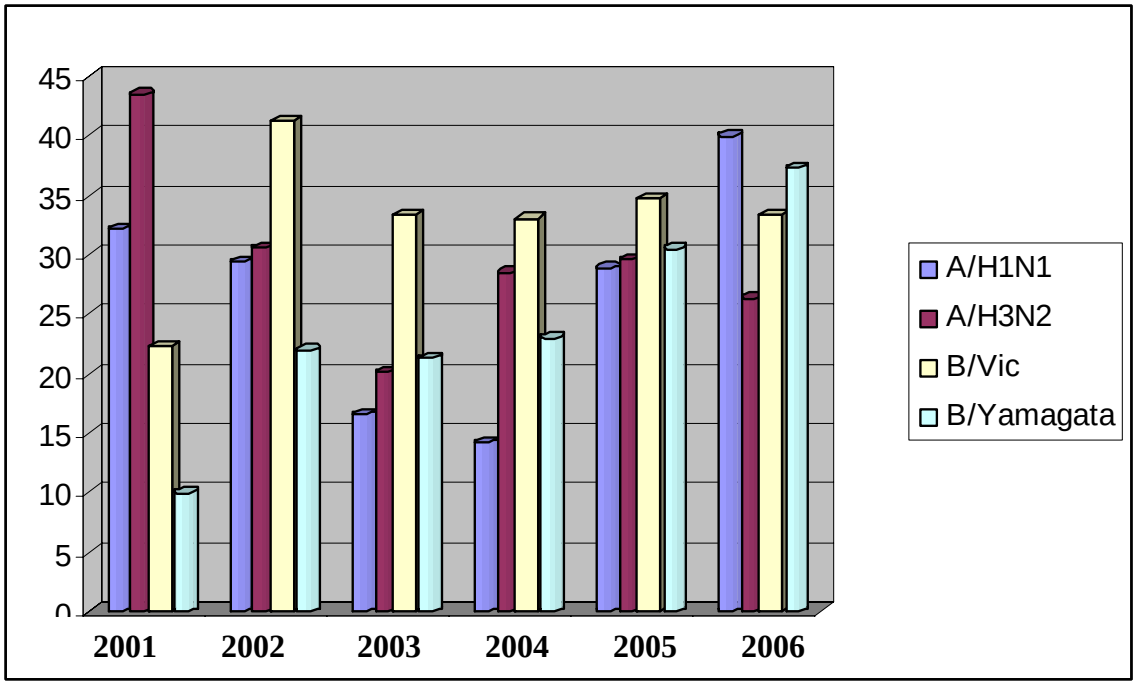


**Table 5.3: Yearwise / Agewise / Sero prevalence of Influenza A & B antibodies in the study population**

| Age | 2001 | | 2002 | | 2003 | | 2004 | | 2005 | | 2006 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Inf A | Inf B | Inf A | Inf B | Inf A | Inf B | Inf A | Inf B | Inf A | Inf B | Inf A | Inf B |
| **0-5** | 31.5 | 32.5 | 35 | 25.75 | 23 | 15 | 28.27 | 18.46 | 30.27 | 21.22 | 28 | 18.88 |
| **6-15** | **60.5** | **83.6** | **52.5** | **54.75** | **31.50** | **26.50** | **39** | **32.11** | **46.44** | **36.11** | **35.08** | **32.31** |
| **16-45** | 17.75 | 79.25 | 28 | 39.25 | 13 | 20 | 15.45 | 26.44 | 18.44 | 30.55 | 17.46 | 28.96 |
| **46-60** | 24.5 | 55.75 | 22.75 | 31.5 | 22.5 | 24 | 25.12 | 29.36 | 33 | 35.36 | 30.96 | 31.44 |

**Figure 5.2: Agewise distribution of Seropositivity of the study population for Influenza viruses from 2001-2006.**

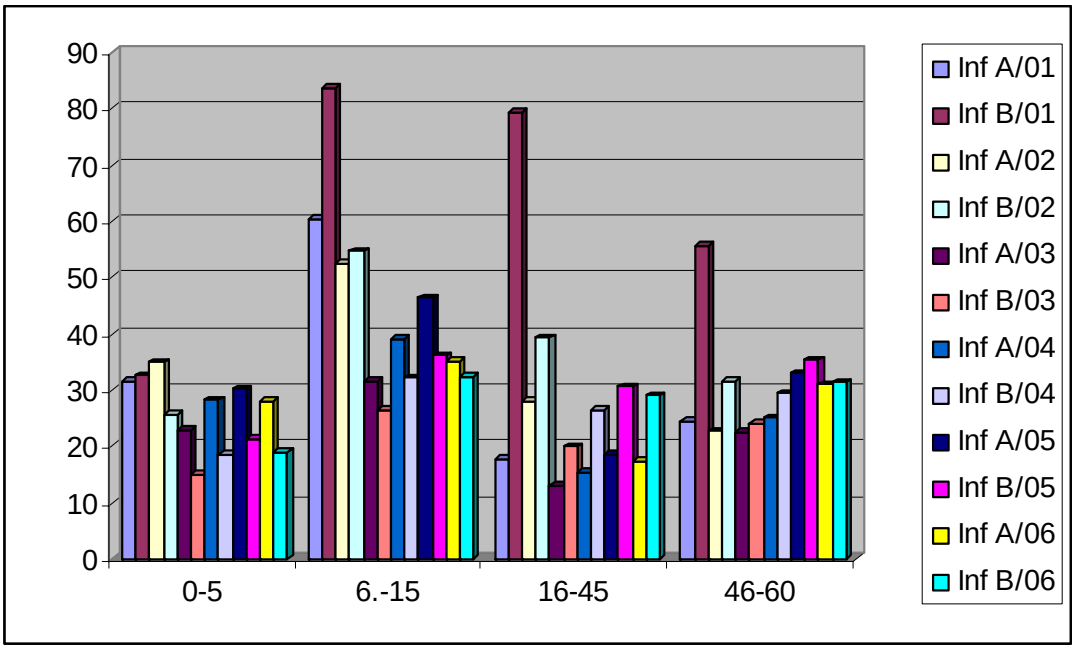


**Table 5.4: Year / Genderwise / Sero prevalence / Influenza A & B**

| Gender | 2001 | | 2002 | | 2003 | | 2004 | | 2005 | | 2006 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Inf A | Inf B | Inf A | Inf B | Inf A | Inf B | Inf A | Inf B | Inf A | Inf B | Inf A | Inf B |
| *Male* | 49.6 | 18.4 | 32.2 | 36 | 25.2 | 37.1 | 25.8 | 38.2 | 33.4 | 32 | 36.1 | 38 |
| Female | 52 | 21.2 | 33.5 | 38.3 | 21.9 | 32 | 24.3 | 34.1 | 35 | 33.7 | 37.4 | 43 |

**Figure 5.3: Genderwise distribution of Seropositivity of the study population for Influenza viruses from 2001-2006.**

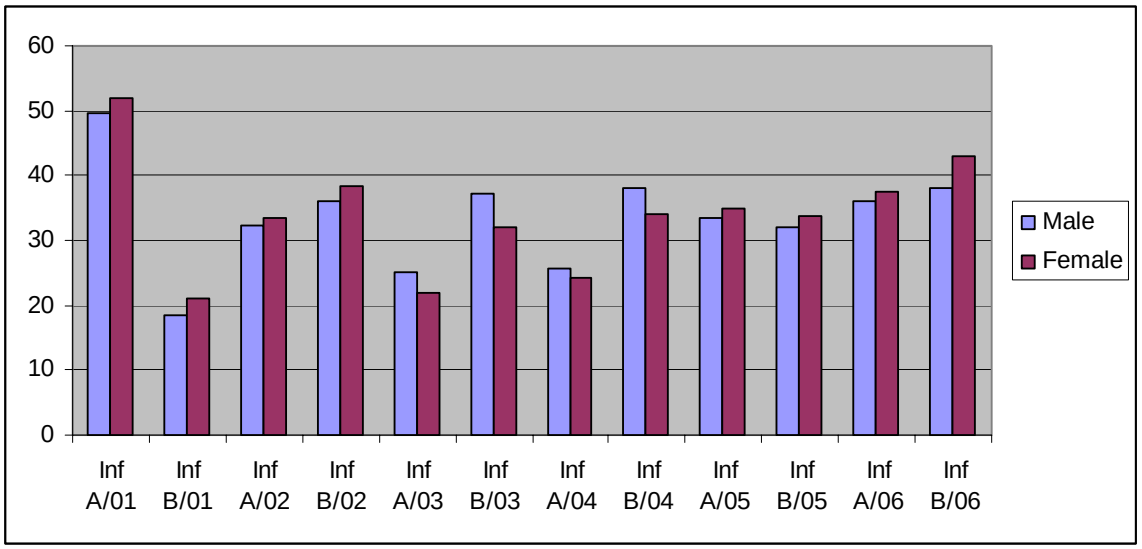


**Table 5.5 : Influenza Virus isolation data for the period from 2002-2007**

| Year | No. of Samples | Positives | % Positivity | No. of Isolates | | | |
|---|---|---|---|---|---|---|---|
| | | | | A/H1 | A/H3 | BV | BY |
| 2002 | 240 | 30 | 12.5 | Nil | 26 | 4 | Nil |
| 2003 | 210 | 15 | 7.14 | Nil | 14 | Nil | 1 |
| 2004 | 296 | 22 | 7.43 | Nil | 22 | Nil | Nil |
| 2005 | 603 | 59 | 9.78 | 42 | 11 | 2 | 4 |
| 2006 | 587 | 46 | 7.83 | 28 | 5 | 2 | 11 |
| 2007 | 1110 | 64 | 5.81 | 12 | 2 | 4 | 46 |
| **Total** | **3036** | **236** | **7.77** | **82** | **80** | **12** | **62** |

**Figure 5.4: Influenza Virus isolation data for the period from 2002-2007**

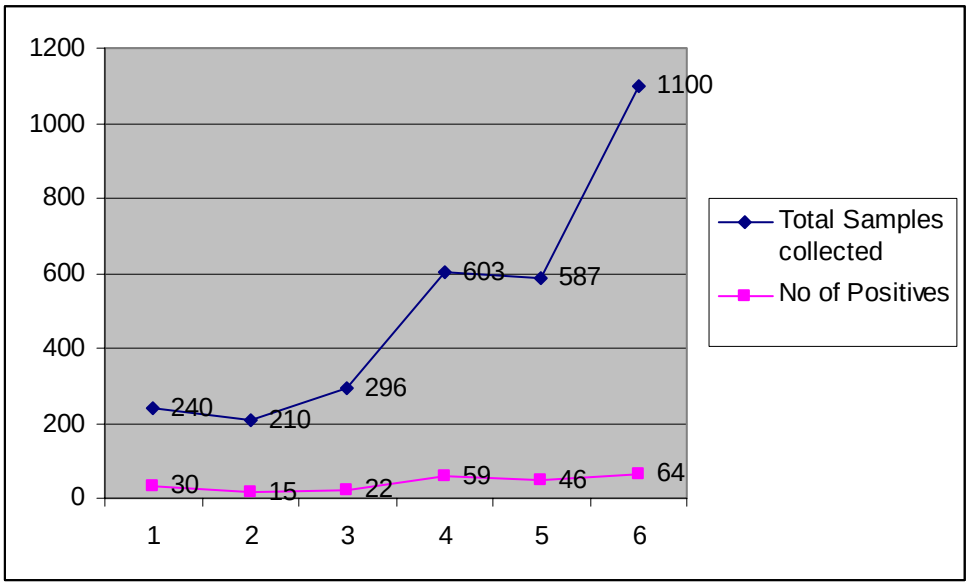


**Table 5.5a1: Yearwise isolation of influenza A viruses**

| Year | Total A Isolates | A/H1 positives | Positive % | A/H3 Positives | Positive % |
|---|---|---|---|---|---|
| 2002 | 26 | Nil | 0 | 26 | 100 |
| 2003 | 14 | Nil | 0 | 14 | 100 |
| 2004 | 22 | Nil | 0 | 22 | 100 |
| 2005 | 53 | 42 | 79.24 | 11 | 20.75 |
| 2006 | 33 | 28 | 84.84 | 5 | 15.15 |
| 2007 | 14 | 12 | 85.17 | 2 | 14.28 |
| **Total** | **162** | **82** | **50.6** | **80** | **49.4** |

**Table 5.5a2: Yearwise isolation of influenza B viruses**

| Year | Total B Isolates | B/VIC positives | Positive % | B/YAM Positives | Positive % |
|---|---|---|---|---|---|
| 2002 | 4 | 4 | 100 | 0 | 0 |
| 2003 | 1 | 0 | 0 | 1 | 100 |
| 2004 | 0 | 0 | 0 | 0 | 0 |
| 2005 | 6 | 2 | 33.4 | 4 | 66.6 |
| 2006 | 13 | 2 | 15.38 | 11 | 84.6 |
| 2007 | 50 | 4 | 8 | 46 | 88.4 |
| **Total** | **74** | **12** | **16.2** | **62** | **83.78** |

**Table 5.5b: Yearwise isolation of influenza A/H1N1 viruses**

| Year | Total Isolates | A/H1 positives | Positive % |
|---|---|---|---|
| 2005 | 59 | 42 | 71.18 |
| 2006 | 46 | 28 | 60.86 |
| 2007 | 64 | 12 | 18.75 |
| **Total** | **236** | **82** | **34.74** |

**Table 5.5c: Yearwise isolation of influenza A/H3N2 viruses**

| Year | Total Isolates | A/H1 positives | Positive % |
|---|---|---|---|
| 2002 | 30 | 26 | 86.67 |
| 2003 | 15 | 14 | 93.4 |
| 2004 | 22 | 22 | 100 |
| 2005 | 59 | 11 | 18.64 |
| 2006 | 46 | 5 | 10.86 |
| 2007 | 64 | 2 | 3.12 |
| **Total** | **236** | **80** | **33.89** |

**Table 5.5d: Yearwise isolation of influenza B viruses**

| Year | Total Isolates | B positives | Positive % |
|---|---|---|---|
| 2002 | 30 | 4 | 13.34 |
| 2003 | 15 | 1 | 6.67 |
| 2004 | 22 | 0 | 0 |
| 2005 | 59 | 6 | 10.16 |
| 2006 | 46 | 13 | 28.26 |
| 2007 | 64 | 50 | 78.12 |
| **Total** | **236** | **74** | **31.35** |

**Table 5.5e: Yearwise isolation of influenza B/VIC viruses**

| Year | Total Isolates | B/VIC positives | Positive % |
|---|---|---|---|
| 2002 | 30 | 4 | 13.34 |
| 2003 | 15 | 0 | 0 |
| 2004 | 22 | 0 | 0 |
| 2005 | 59 | 2 | 3.3 |
| 2006 | 46 | 2 | 4.34 |
| 2007 | 64 | 4 | 6.25 |
| **Total** | **236** | **12** | **5.08** |

**Table 5.5f: Yearwise isolation of influenza B/YAM viruses**

| Year | Total Isolates | YAM positives | Positive % |
|---|---|---|---|
| 2002 | 30 | 0 | 0 |
| 2003 | 15 | 1 | 6.67 |
| 2004 | 22 | 0 | 0 |
| 2005 | 59 | 4 | 6.77 |
| 2006 | 46 | 11 | 23.9 |
| 2007 | 64 | 46 | 71.8 |
| **Total** | **236** | **62** | **26.27** |

**Table 5. 6: Prevalence of Influenza virus strains in different age groups.**

| Age group | Total positives | A/H1 | Pos% | A/H3 | Pos% | B/Vic | Pos% | B/Yam | Pos% |
|---|---|---|---|---|---|---|---|---|---|
| **0-12** | 149 | 44 | **29.53** | 70 | **46.97** | 8 | 5.36 | 27 | 18.12 |
| **13-55** | 27 | 8 | 0 | 0 | 0 | 0 | 0 | 19 | **70.37** |
| **55&>** | 60 | 30 | 50 | 10 | 16.66 | 4 | 6.67 | 16 | 26.66 |
| **Total** | **236** | **82** | **34.75** | **80** | **33.8** | **12** | **5.08** | **62** | **26.2** |

**Figure 5.5: Prevalence of Influenza virus strains in different age groups**

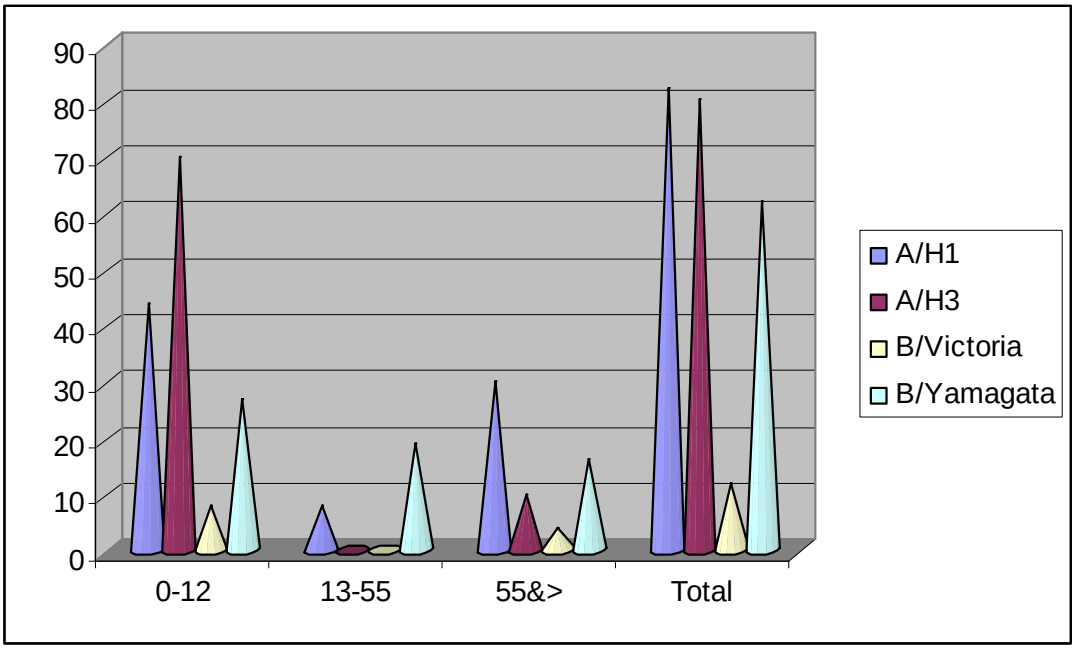


**Table 5.7: Seasonality of Influenza viruses**

| Season | No. of samples | No. positives | Positive % |
|---|---|---|---|
| Late winter & spring 8th Dec to 28/29th Feb | 532 | 23 | 4.32 |
| Pre monsoon / summer 1st March to 31 May. | 620 | 11 | 1.77 |
| South west monsoon 1st June to 30 September. | 1120 | 56 | 5 |
| Winter 1st October to 7 December. | 764 | 146 | 19.10 |
| **Total** | **3036** | **236** | **7.77** |

**Figure 5.6: Seasonality of Influenza viruses**

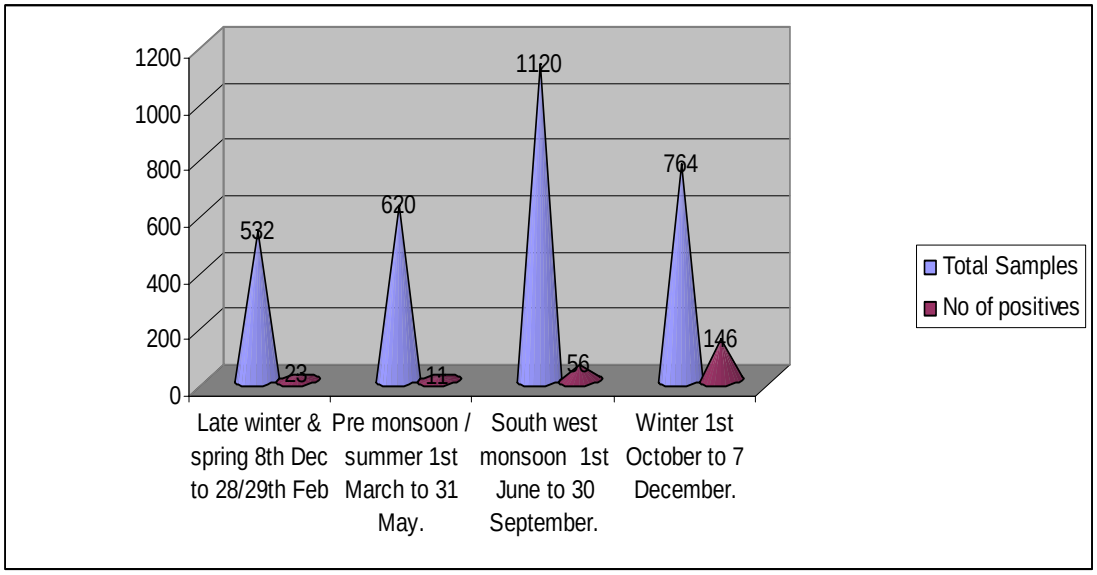


**Table 5.8: Genderwise distribution of Influenza viruses**

| Gender | Total Samples | Total Positives | % Positive |
|---|---|---|---|
| Male | 1596 | 130 | 8.14 |
| Female | 1440 | 106 | 7.36 |
| **Total** | **3036** | **236** | **7.77** |

**Figure 5.7: Genderwise distribution of Influenza viruses**

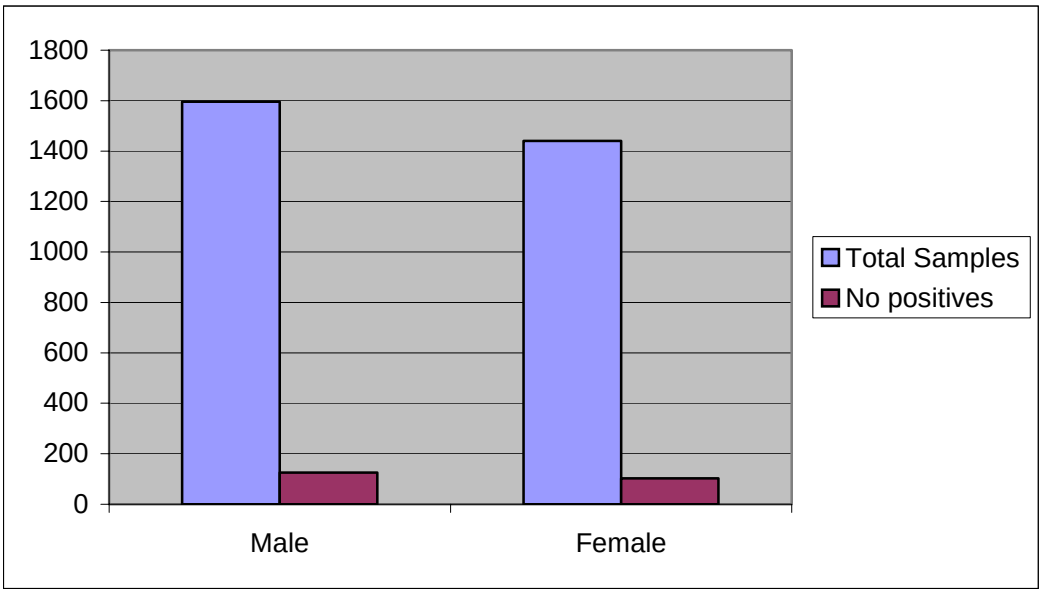


**Table 5.9: Monthwise isolation of Influenza 2002**

| Month | Specimens collected | Specimens Processed | Number isolated | Type |
|---|---|---|---|---|
| January | 20 | 20 | Nil | Nil |
| February | 20 | 20 | 2 | Type B (2) |
| March | 20 | 20 | Nil | Nil |
| April | 20 | 20 | Nil | Nil |
| May | 20 | 20 | Nil | Nil |
| June | 20 | 20 | 6 | H3N2 (6 ) |
| July | 20 | 20 | 6 | H3N2 (4 ), Type B (2) |
| August | 25 | 25 | 3 | H3N2 (3 ) |
| September | 12 | 12 | 5 | H3N2 (5 ) |
| October | 18 | 18 | 4 | H3N2 (4 ) |
| November | 20 | 20 | 4 | H3N2 (4 ) |
| December | 25 | 25 | Nil | Nil |
| **TOTAL** | **240** | **240** | **30** | **H3N2 (24 ), Type B (4 ), H3+BS (2)** |

**Table 5.10: Seasonwise isolation of Influenza Viruses in 2002**

| Season | No. of samples | No. of positives | Positive % |
|---|---|---|---|
| Late winter & spring 8th Dec to 28/29th Feb | **40** | **2** | **5** |
| Pre monsoon<br>1st March to 31 May. | **60** | **Nil** | **Nil** |
| South west monsoon<br>1st June to 30 September. | **77** | **20** | **25.9** |
| Winter<br>1st October to 7 December. | **45** | **8** | **17.8** |

**Table 5.11: Monthwise isolation of Influenza Viruses in 2003**

| ***Month*** | **Specimens collected** | **Specimens Processed** | **Number isolated** | **Type** |
|---|---|---|---|---|
| January | 20 | 20 | **Nil** | **Nil** |
| February | 20 | 20 | 2 | **H3N2 (2)** |
| March | 20 | 20 | **1** | **Type B (1)** |
| April | 21 | 21 | **Nil** | **Nil** |
| May | 14 | 14 | **Nil** | **Nil** |
| June | 20 | 20 | 1 | **H3N2 (1 )** |
| July | 20 | 20 | 3 | **H3N2 (3)** |
| August | 25 | 25 | 3 | **H3N2 (3 )** |
| September | 10 | 10 | **1** | **H3N2 (1 )** |
| October | 11 | 11 | **1** | **H3N2 (1 )** |
| November | 17 | 17 | 1 | **H3N2 (1 )** |
| December | 12 | 12 | **2** | **H3N2 (2 )** |
| **TOTAL** | **210** | **210** | **15** | **H3N2 (14), Type B (1)** |

**Table 5.12: Seasonwise isolation of Influenza Viruses in 2003**

| **Season** | **No. of samples** | **No. of positives** | **Positive %** |
|---|---|---|---|
| Late winter & spring 8$^{th}$ Dec to 28/29$^{th}$ Feb | 58 | 3 | 5.08 |
| Pre monsoon<br>1$^{st}$ March to 31 May. | 55 | 1 | 1.8 |
| South west monsoon<br>1$^{st}$ June to 30 September. | 75 | 8 | 10.7 |
| Early Winter<br>1$^{st}$ October to 7 December. | 28 | 3 | 10.7 |

**Table 5.13: Monthwise isolation of Influenza 2004**

| Month | Specimens collected | Specimens Processed | Number isolated | Type |
|---|---|---|---|---|
| January | 20 | 20 | Nil | Nil |
| February | 24 | 24 | Nil | Nil |
| March | 20 | 20 | Nil | Nil |
| April | 30 | 30 | 4 | H3N2 (4 ) |
| May | 28 | 28 | 4 | H3N2 (4) |
| June | 24 | 24 | 4 | H3N2 (4 ) |
| July | 20 | 20 | 1 | H3N2 (1 ) |
| August | 25 | 25 | 2 | H3N2 (2) |
| September | 35 | 35 | 2 | H3N2 (2 ) |
| October | 30 | 30 | 1 | H3N2 (1 ) |
| November | 20 | 20 | 2 | H3N2 (2) |
| December | 20 | 20 | 2 | H3N2 (2) |
| **TOTAL** | **296** | **22** | **22** | **H3N2 (22),** |

**Table 5.14 : Seasonwise isolation of Influenza Viruses in 2004**

| Season | No. of samples | No. of positives | Positive % |
|---|---|---|---|
| Late winter & spring 8th Dec to 28/29th Feb | 56 | 2 | 3.57 |
| Pre monsoon<br>1st March to 31 May. | 78 | 8 | 10.25 |
| South west monsoon<br>1st June to 30 September. | 104 | 9 | 8.6 |
| Early Winter<br>1st October to 7 December. | 55 | 5 | 5.45 |

**Table 5.15: Monthwise isolation of Influenza 2005**

| Month | Specimens collected | Specimens Processed | Number isolated | Type |
|---|---|---|---|---|
| January | 40 | 40 | Nil | Nil |
| February | 40 | 40 | Nil | Nil |
| March | 40 | 40 | Nil | Nil |
| April | 52 | 52 | 2 | H3N2(2) |
| May | 36 | 36 | Nil | Nil |
| June | 63 | 63 | Nil | Nil |
| July | 47 | 47 | 2 | H3N2 (2) |
| August | 45 | 45 | Nil | Nil |
| September | 45 | 45 | 5 | H3N2 (5) |
| October | 60 | 55 | 23 | H1N1 (19) B Type (4) |
| November | 57 | 52 | 21 | H1N1(18), H3N2 (2), B Type (1) |
| December | 78 | 78 | 6 | H1N1 (5), B Type (1) |
| **TOTAL** | **603** | **603** | **59** | H1N1 (42 ), H3N2 (11 ), Type B (6 ) |

**Table 5.16: Seasonwise isolation of Influenza 2005**

| Season | No. of samples | No. of positives | Positive % |
|---|---|---|---|
| Late winter & spring 8th Dec to 28/29th Feb | 95 | 1 | 1.05 |
| Pre monsoon<br>1st March to 31 May. | 128 | 2 | 1.56 |
| South west monsoon<br>1st June to 30 September. | 200 | 7 | 3.5 |
| Winter<br>1st October to 7 December. | 135 | 49 | 36.29 |

**Table 5.17: Monthwise isolation of Influenza 2006**

| Month | Specimens collected | Specimens Processed | Number isolated | Type |
|---|---|---|---|---|
| January | 66 | 66 | 4 | **H1N1 (3 ), Type B (1 )** |
| February | 62 | 62 | 3 | **H1N1 (1 ), Type B ( 2)** |
| March | 45 | 45 | **Nil** | **Nil** |
| April | 44 | 44 | **Nil** | **Nil** |
| May | 38 | 38 | **Nil** | **Nil** |
| June | 33 | 33 | 2 | **H1N1 (1 ), Type B ( 1)** |
| July | 74 | 74 | 3 | **H1N1 ( 1), H3N2 ( 1), Type B (1)** |
| August | 55 | 55 | **Nil** | **Nil** |
| September | 38 | 38 | 3 | **H1N1 (2 ), H3N2 (1 )** |
| October | 40 | 40 | 10 | **H1N1 (7 ), H3N2 ( 1), Type B (2 )** |
| November | 47 | 47 | 11 | **H1N1 (7), Type B (4)** |
| December | 45 | 45 | 10 | **H1N1 (6 ), H3N2 (2 ) Type B ( 2)** |
| **TOTAL** | **587** | **587** | **46** | **H1N1 (28 ), H3N2 (5 ), Type B (13)** |

**Table 5.18: Seasonwise isolation of Influenza 2006**

| Season | No. of samples | No. of positives | Positive % |
|---|---|---|---|
| Late winter & spring 8$^{th}$ Dec to 28/29$^{th}$ Feb | 188 | 12 | 6.38 |
| Pre monsoon<br>1$^{st}$ March to 31 May. | 127 | Nil | Nil |
| South west monsoon<br>1$^{st}$ June to 30 September. | 200 | 8 | 4 |
| Winter<br>1$^{st}$ October to 7 December. | 102 | 26 | 25.49 |

**Table 5.19: Monthwise isolation of Influenza 2007**

| Month | Specimens collected | Specimens Processed | Number isolated | Type |
|---|---|---|---|---|
| January | 41 | 41 | 2 | **H1N1 (2 ),** |
| February | 24 | 24 | 2 | **H1N1 (2 ),** |
| March | 50 | 50 | **Nil** | **Nil** |
| April | 60 | 60 | **Nil** | **Nil** |
| May | 62 | 62 | **Nil** | **Nil** |
| June | 111 | 111 | **Nil** | **Nil** |
| July | 116 | 116 | **Nil** | **Nil** |
| August | 94 | 94 | **Nil** | **Nil** |
| September | 103 | 103 | 4 | **H1N1 (2 ), Type B (2 )** |
| October | 90 | 90 | 13 | **H1N1 (1 ), Type B (12 )** |
| November | 173 | 173 | 29 | **H1N1 (3), Type B ( 26)** |
| December | 176 | 176 | 14 | **H1N1 (2 ), H3N2 (2 ) Type B ( 10)** |
| **TOTAL** | **1100** | **1100** | **64** | **H1N1 (12 ), H3N2 (2 ) Type B ( 50)** |

**Table 5.20 : Season wise isolation of Influenza in 2007**

| Season | No. of samples | No. of Positives | Positive % |
|---|---|---|---|
| Late winter & spring 8$^{th}$ Dec to 28/29$^{th}$ Feb | 95 | 7 | 7.36 |
| Pre monsoon<br>1$^{st}$ March to 31 May. | 172 | Nil | Nil |
| South west monsoon<br>1$^{st}$ June to 30 September. | 424 | 4 | 0.94 |
| Early Winter<br>1$^{st}$ October to 7 December. | 313 | 53 | 16.9 |

**Table 5. 21 Clinical signs and symptoms of suspected Influenza cases**

| ***Symptoms*** | **Reported Percentage** |
|---|---|
| Fever | 100 |
| Cough | 93 |
| Headache | 63 |
| Nasal Discharge | 92.1 |
| Pharyngitis | 72 |
| Myalgia | 73.6 |
| Productive cough | 78 |
| Vomitting | 64 |
| Diarrhea | 64 |
| Sudden onset of symptoms | 8 |

**Figure 5.8: Clinical signs and symptoms of suspected Influenza cases**

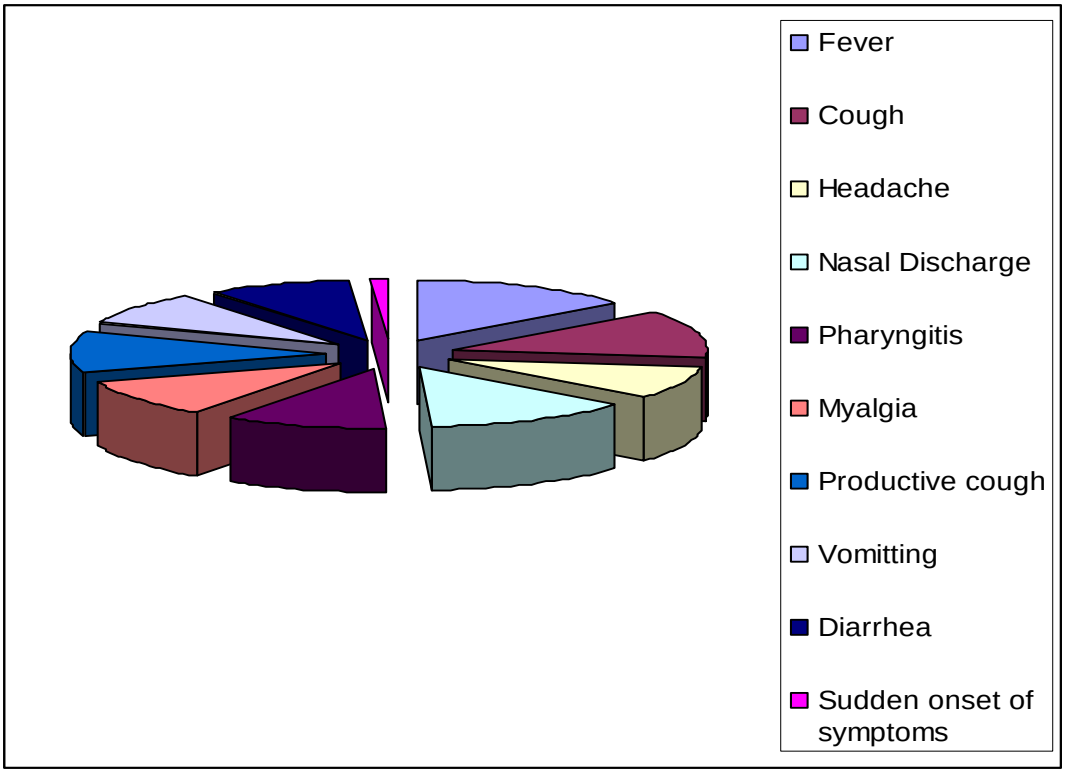


**Table 5.22: Clinical signs and symptoms of laboratory confirmed Influenza cases**

| Symptoms | Reported Percentage |
|---|---|
| Fever | 100 |
| Cough | 100 |
| Headache | 92.4 |
| Nasal Discharge | 73 |
| Pharyngitis | 67 |
| Myalgia | 83.6 |
| Productive cough | 78 |
| Vomitting | 23 |
| Diarrhea | 39 |
| Sudden onset of symptoms | 100 |

**Figure 5.9: Clinical signs and symptoms of laboratory confirmed Influenza cases**

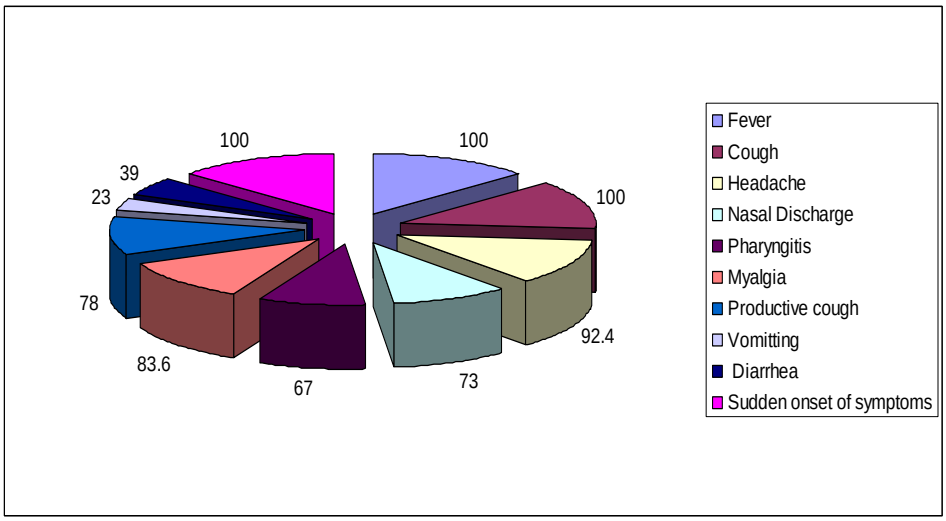


**Table 5. 23: Predisposing factors and Association of chronic conditions in the elderly with influenza infections**

| Predisposing factor | Reported Percentage |
|---|---|
| Cardio vascular and Pulmonary disease | 53 |
| Cardiovascular and diabetes | 30 |
| Pulmonary disease | 12 |
| Cardiovascular disease | 4.6 |

Figure 5. 10: Predisposing factors and Association of chronic conditions in the elderly with influenza infections

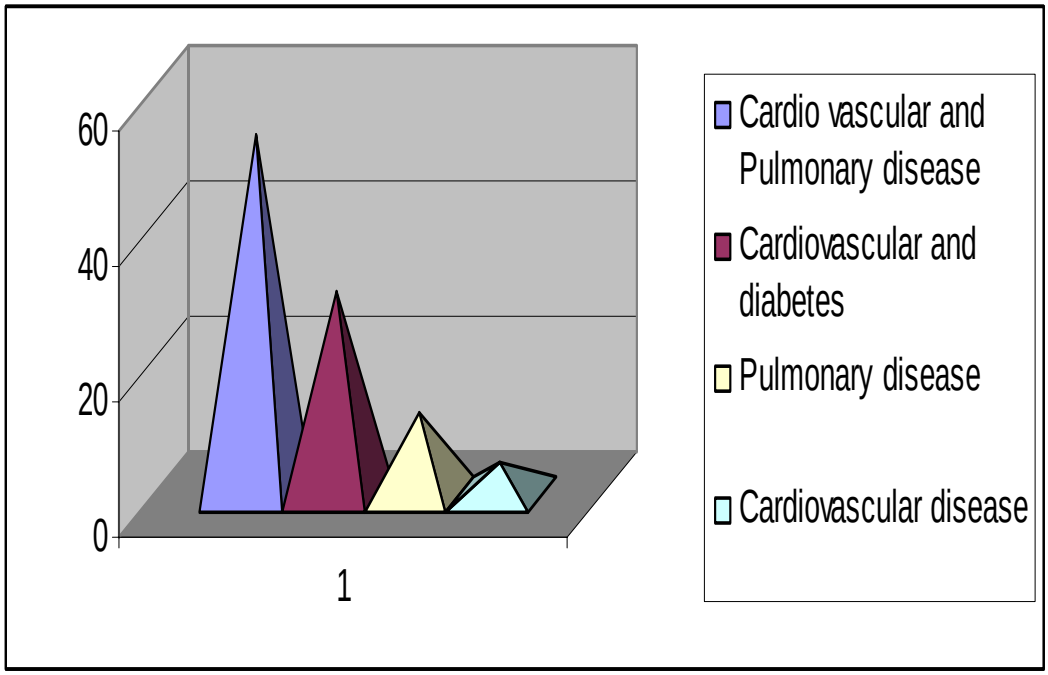


**Figure 5.11: Normal MDCK cell line**

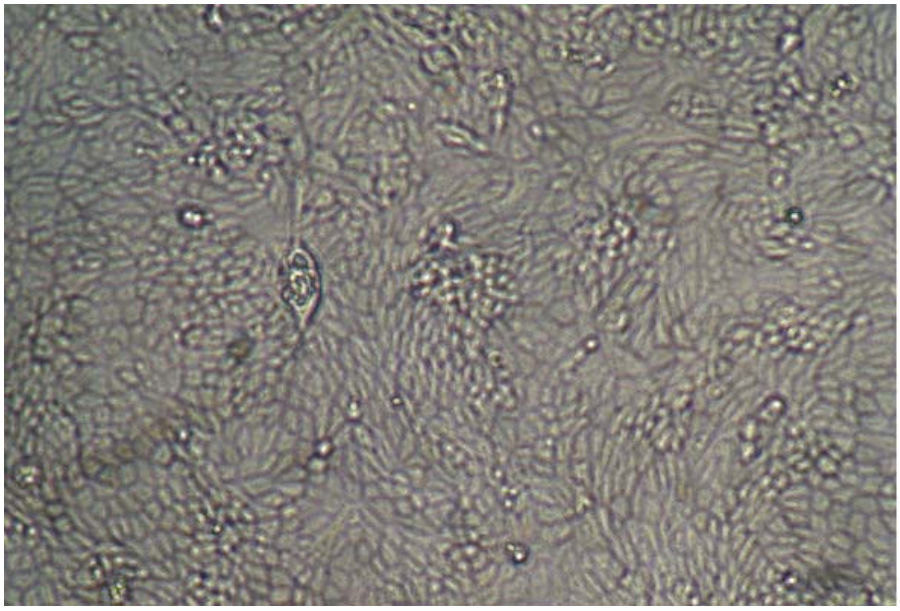


**Figure 5.12: MDCK cell line showing 1 + Cytopathic effect**

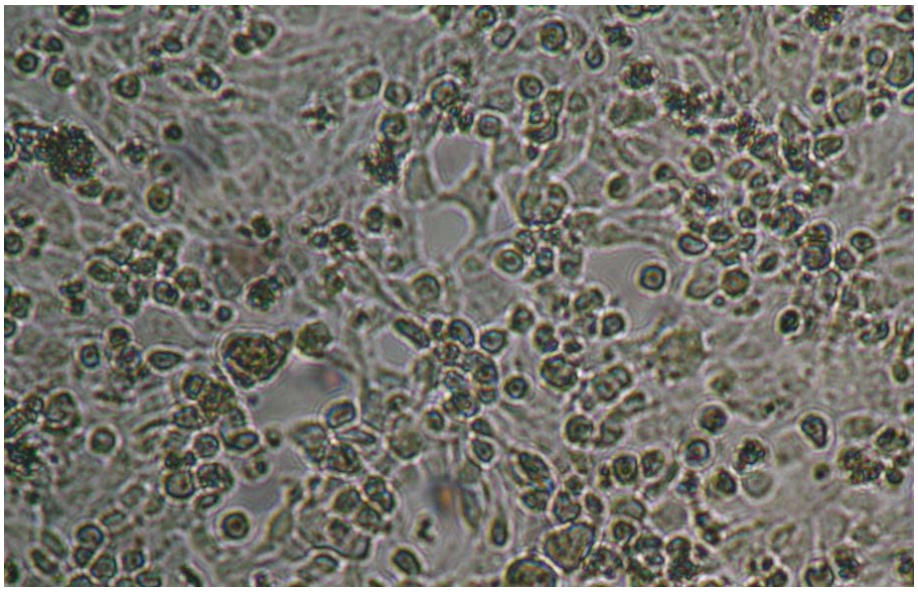


**Figure 5.13: MDCK cell line showing 2+ Cytopathic effect**

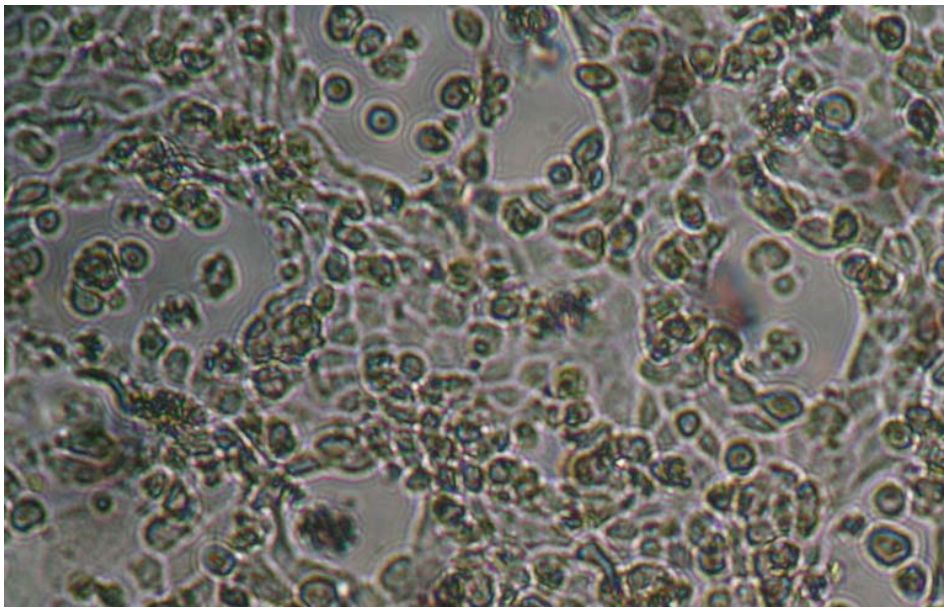


**Figure 5.14: MDCK cell line showing 3+ Cytopathic effect**

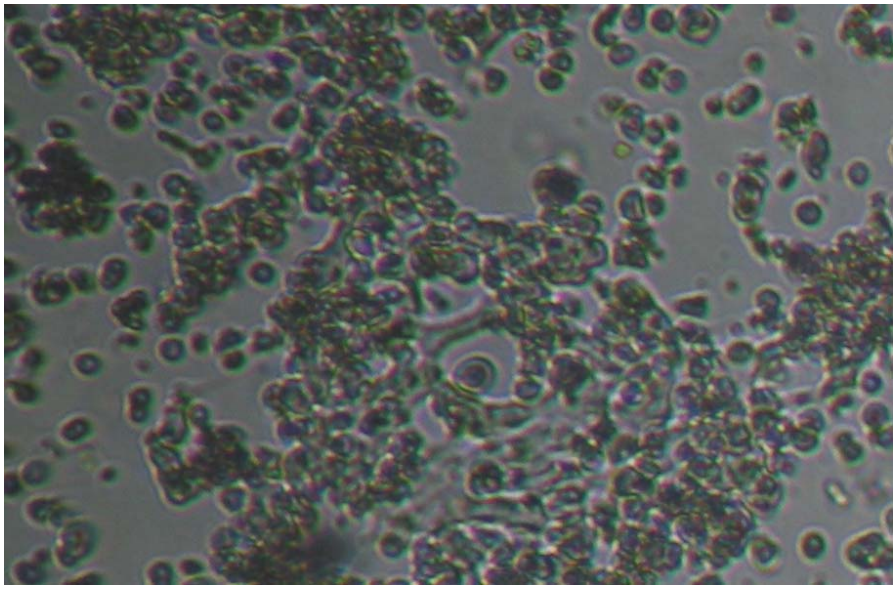


**Figure 5.15: MDCK cell line showing 4+ Cytopathic effect**

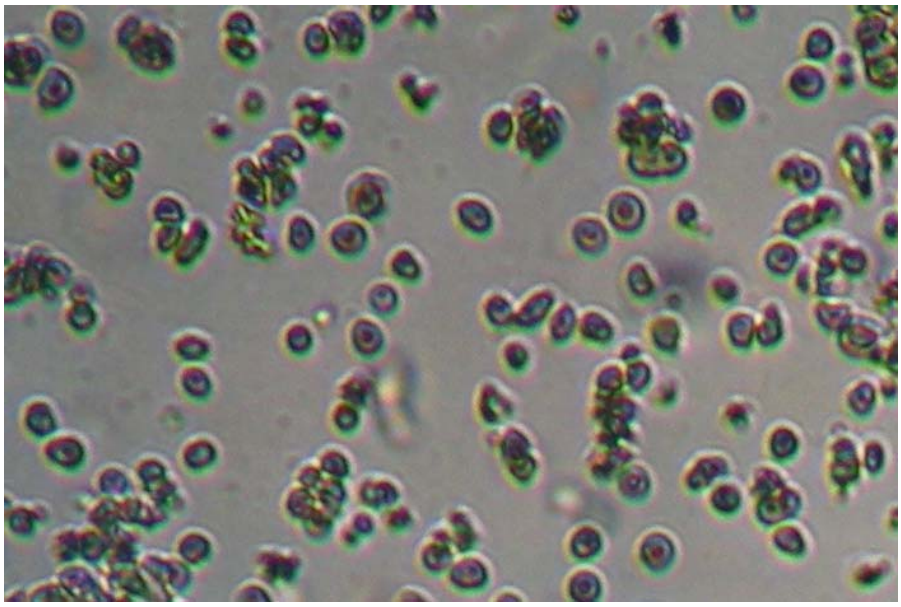


**Figure 5.16: Agarose gel analysis showing amplified N1 gene of A/H1N2 isolates**

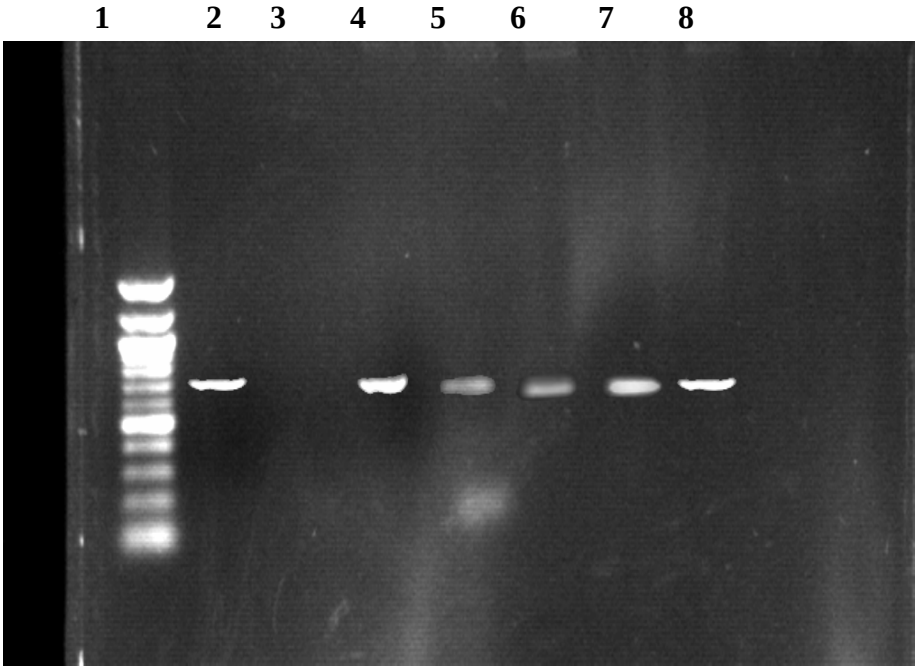



Figure 5.17: Gel Picture of Neuraminidase typing of A/H3N2 isolates.

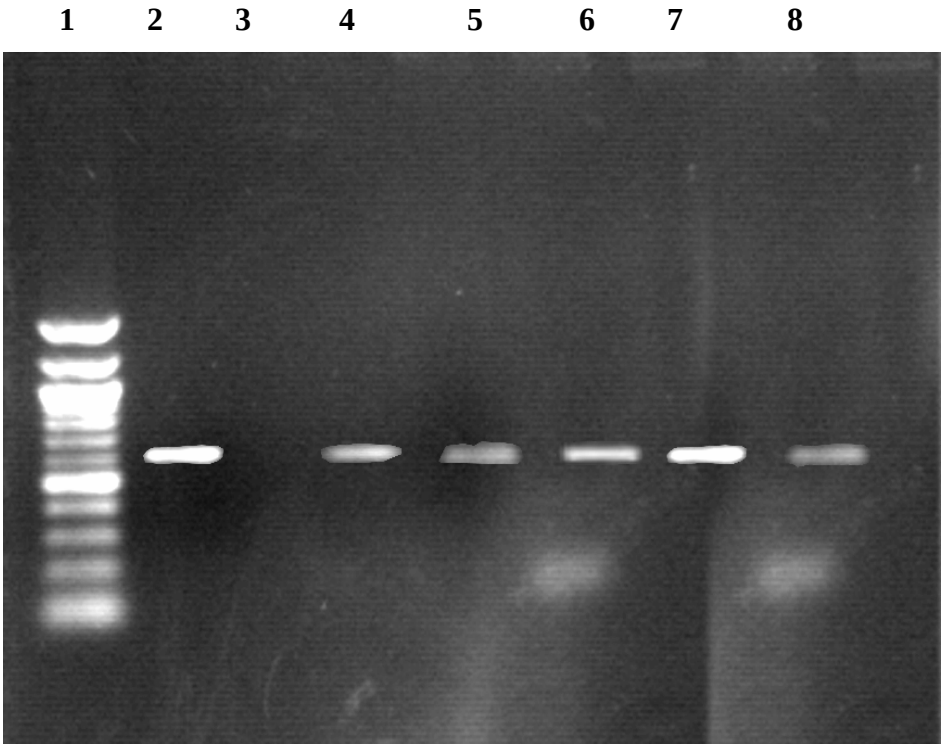


**Figure 5.16: Agarose gel analysis showing amplified N1 gene of A/H1N1 isolates.**

Lane 1. Molecular Weight Marker (100-1000 bp)
Lane 2. Influenza A/H1N1/ positive control (708 bp)
Lane 3. Negative control
Lane 4 to lane 8 Influenza A/H1N1 isolates (708 bp)

**Figure 5.17: Agarose gel analysis showing amplified N2 gene of A/H3N2 isolates.**

Lane 1. Molecular Weight Marker (100-1000bp)
Lane 2. Influenza A/H3N2 positive control (615 bp)
Lane 3. Negative control
Lane 4 to lane 8 Influenza A/H3N2 isolates (615 bp)

**Figure 5.18: RT-PCR RFLP analysis of Influenza A/H1N1 virus & isolates**

**1 2 3 4 5 6 7 8**

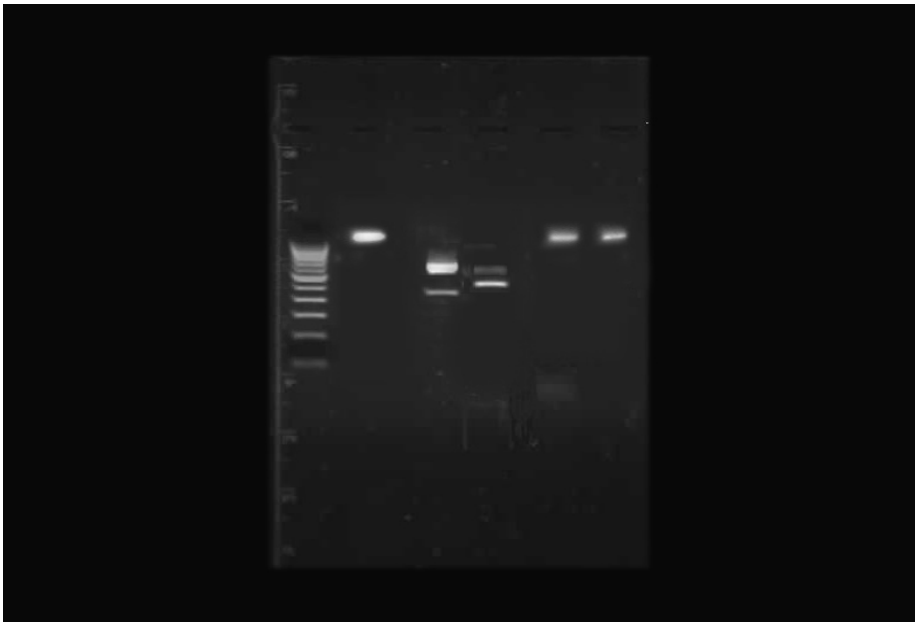


**Figure 5.19: RT-PCR RFLP analysis of Influenza A/H3N2 virus & isolates**

**1 2 3 4 5 6 7 8**

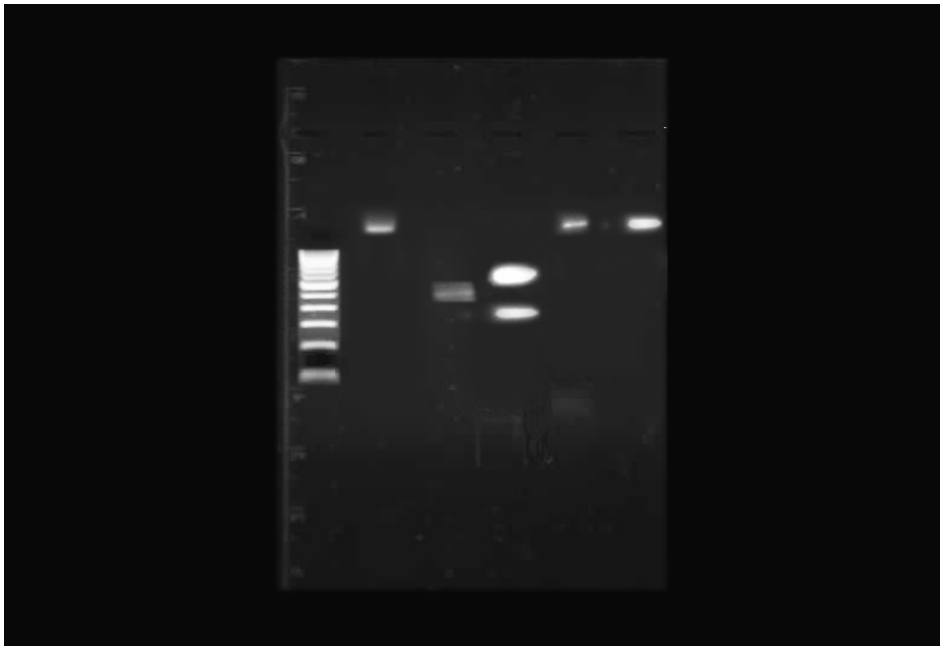


**Figure 5.18: RT-PCR RFLP analysis of Influenza A/H1N1 virus & isolates**

Lane 1- Molecular weight ladder 100-1000 bp

Lane 2- Influenza A H1N1 Prototype strain amplified product 1187

Lane 3- Negative control

Lane 4 - Influenza A H1N1 Prototype strain amplified product digested with Aci1 – 651 and 536 bp products

Lane 5- Influenza A H1N1 Prototype strain amplified product digested with Sac1 – 542 and 645 bp product.

Lane 6 - Blank

Lane 7- Influenza A H1N1 isolate amplified product treated with Aci-1. The product was not digested.

Lane 8- Influenza A H1N1 isolate amplified product treated with Sac-1. The product was not digested.

**Figure 5.19: RT-PCR RFLP analysis of Influenza A/H3N2 virus & isolates**

Lane 1- Molecular weight ladder 100-1000bp

Lane 2- Influenza A H3N2 Prototype strain amplified product 1177

Lane 3: Negative Control

Lane 4- Influenza A H3N2 Prototype strain amplified product digested with Ban-1 – 556 and 521 bp products

Lane 5- Influenza A H3N2 Prototype strain amplified product digested with Eco RV – 623 and 554 bp product.

Lane 6- Blank

Lane 7- Influenza A H3N2 isolate amplified product digested with Ban-1. The product was not digested.

Lane 8- Influenza A H3N2 isolate amplified product digested with Eco RV The product was not digested.

Figure 5.20: RT-PCR RFLP analysis of Influenza B/Victoria lineage virus & isolates.

**1 2 3 4 5 6 7 8**

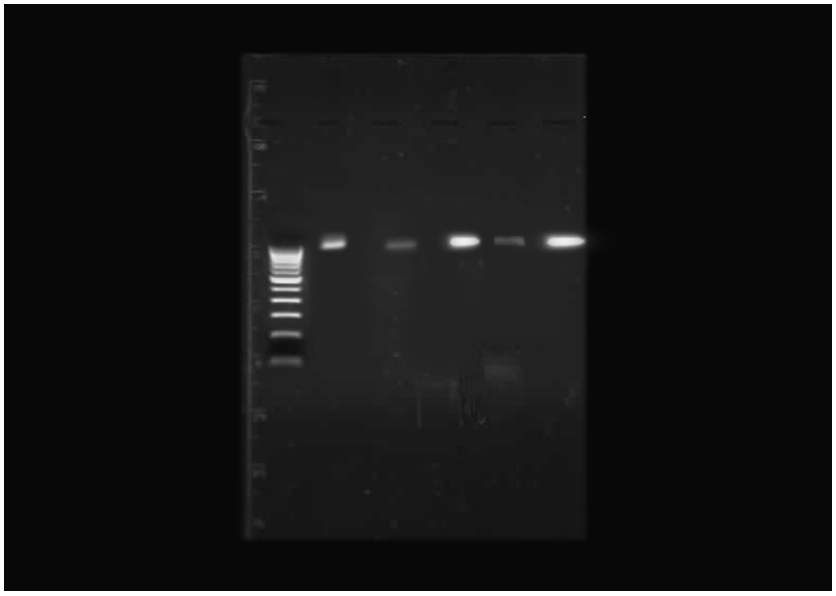


Figure 5.21: RT-PCR RFLP analysis of Influenza B/Yamagata lineage virus & isolates.

**1 2 3 4 5 6 7 8**

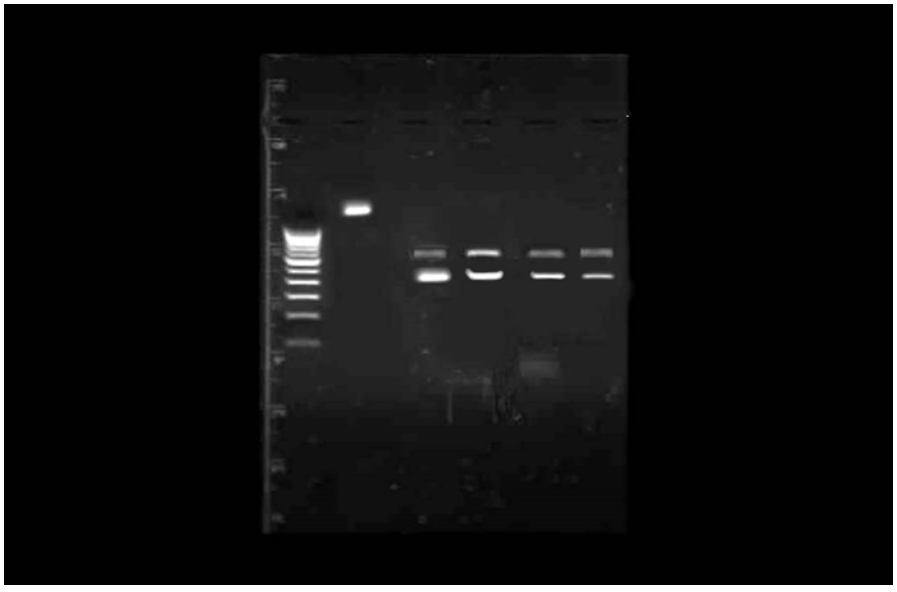


**Figure 5.20: RT-PCR RFLP analysis of Influenza B/ Sichuan virus & isolates.**

Lane 1- Molecular weight ladder 100-1000bp

Lane 2- Influenza B Sichuan Prototype strain amplified product 1104

Lane 3- Negative Control

Lane 4- Influenza B Sichuan Prototype strain amplified product treated with Mae-II. The product was not digested.

Lane 5-7 -Influenza B Sichuan isolate amplified product treated with Mae-II. The product was not digested.

**Figure 5.21: RT-PCR RFLP analysis of Influenza B/Hong Kong virus & isolates.**

Lane 1- Molecular weight ladder 100-1000bp

Lane 2- Influenza B Hong Kong Prototype strain amplified product 1104

Lane 3- Negative control

Lane 4- Influenza B Hong Kong Prototype strain amplified product digested with Mae-II yielding 671 and 433 bp product.

Lane 5- 7- Influenza B Hong Kong isolates strain amplified product digested with Mae-II yielding 671 and 433 bp product.

Table 5.24 Representative isolates selected from each year for genetic characterization

| Year | H1 | | H3 | | BV | | BY | |
|---|---|---|---|---|---|---|---|---|
| | **Total Positives** | **Rep isolates** | **Total Positives** | **Rep isolates** | **Total Positives** | **Rep isolates** | **Total Positives** | **Rep isolates** |
| 2002 | Nil | NIL | 26 | 6 | 4 | 3 | Nil | NIL |
| 2003 | Nil | NIL | 14 | 8 | Nil | NIL | 1 | 1 |
| 2004 | Nil | NIL | 22 | 9 | Nil | NIL | Nil | NIL |
| 2005 | 42 | 7 | 11 | 4 | 2 | 1 | 4 | 2 |
| 2006 | 28 | 15 | 5 | 4 | 2 | 1 | 11 | 6 |
| 2007 | 12 | 12 | 2 | 1 | 4 | 2 | 38 | 4 |
| **TOTAL** | **82** | **34** | **80** | **32** | **12** | **7** | **54** | **13** |

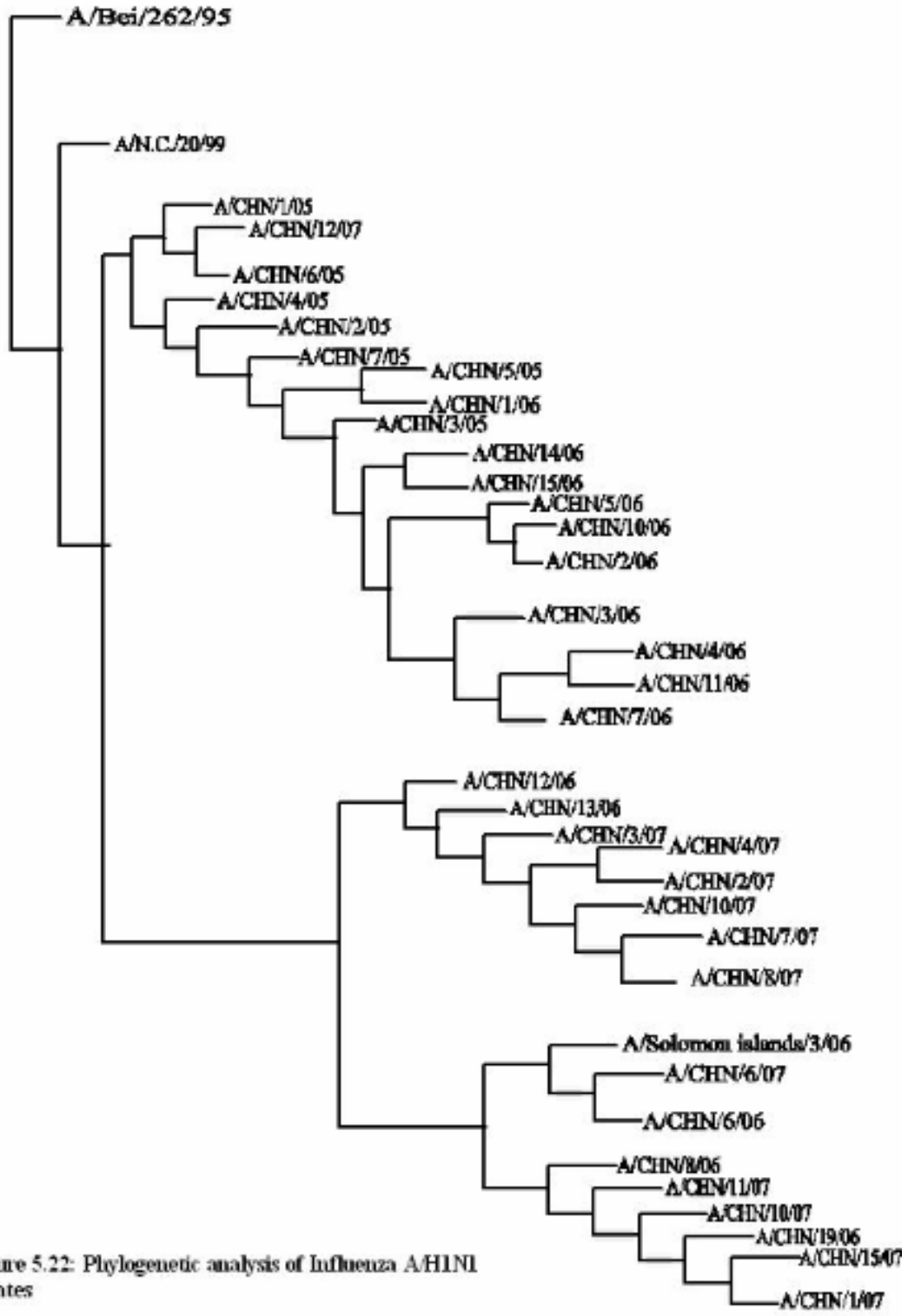



Figure 5.22: Phylogenetic analysis of Influenza A/H1N1 isolates

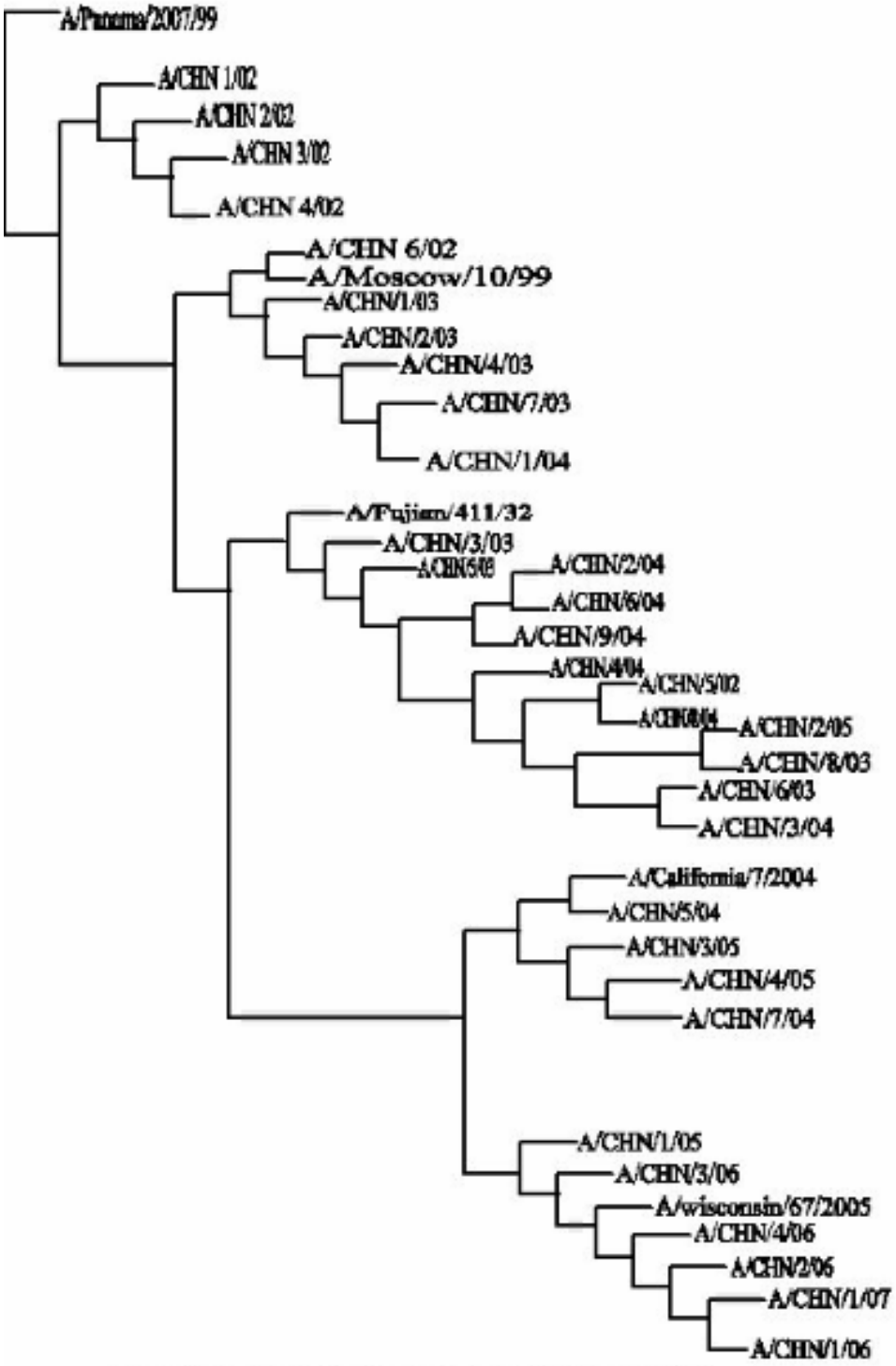



Figure 5.23: Phylogenetic analysis of Influenza A/H3N2 isolates

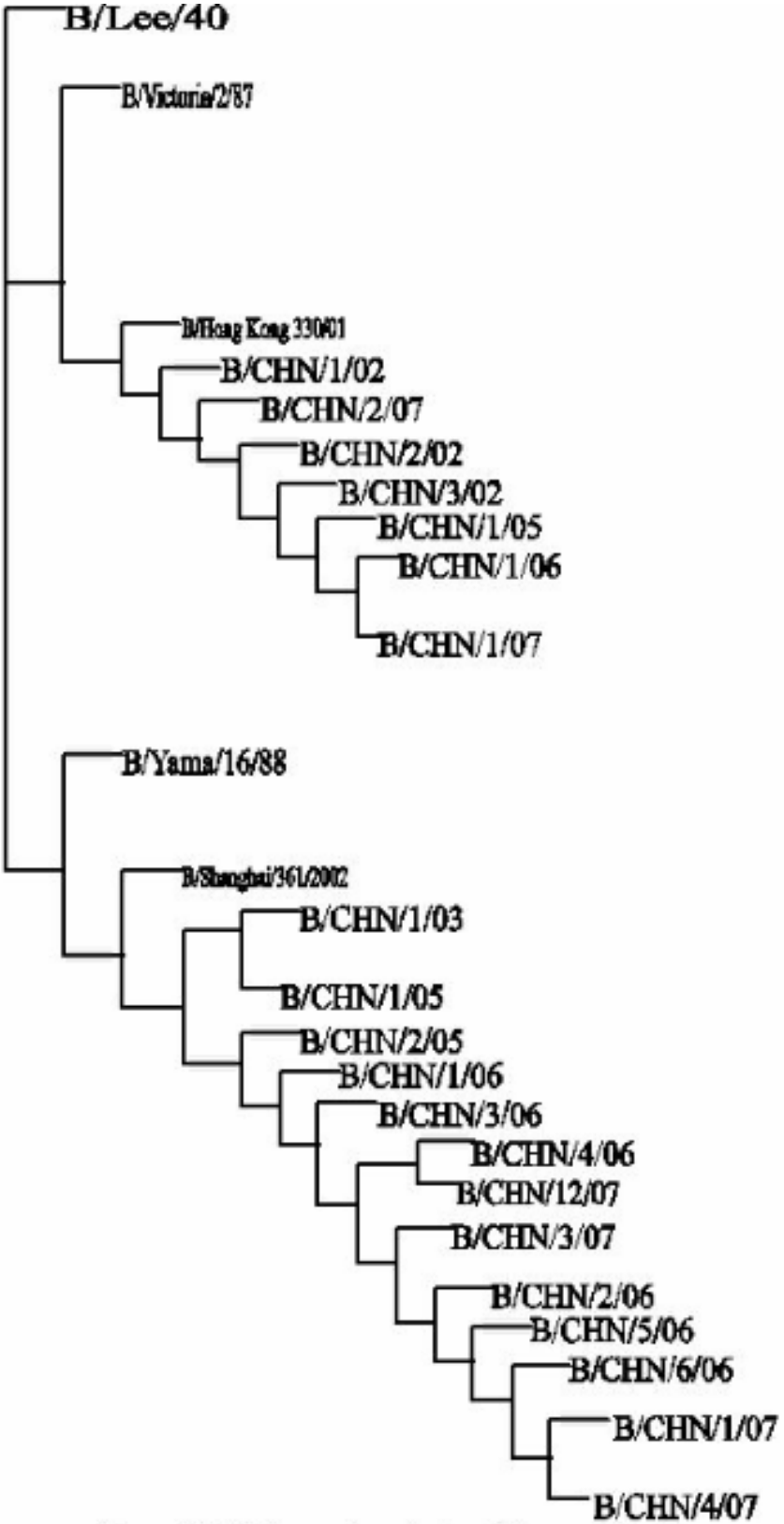



Figure 5.24: Phylogenetic analysis of Influenza B isolates

**Table 5.25: Positive percentage of RT-PCR compared with virus isolation.**

| *Date of Collection of samples* | *No. of Samples* | *No. Positive by viral culture* | *Positive %* | *No. Positive by RT-PCR* | *Positive %* |
|---|---|---|---|---|---|
| *< 7 Days* | *60* | *6* | *10* | *10* | *16.66* |
| *>7 Days* | *60* | *-* | *-* | *6* | *10* |
| *Healthy Individuals* | *10* | *-* | *-* | *-* | *-* |

**Table 5.25a: Evaluation of RT-PCR compared with virus isolation**

| **RT-PCR** | **Tissue culture Positive** | **Tissue culture Negative** | **Total** |
|---|---|---|---|
| Positive | 6 | 4 | 10 |
| Negative | 0 | 50 | 50 |
| Total | 6 | 54 | 60 |

**Figure 5.25:Diagnostic RT-PCR of Prototype Influenza viruses**

**1 2 3 4 5**

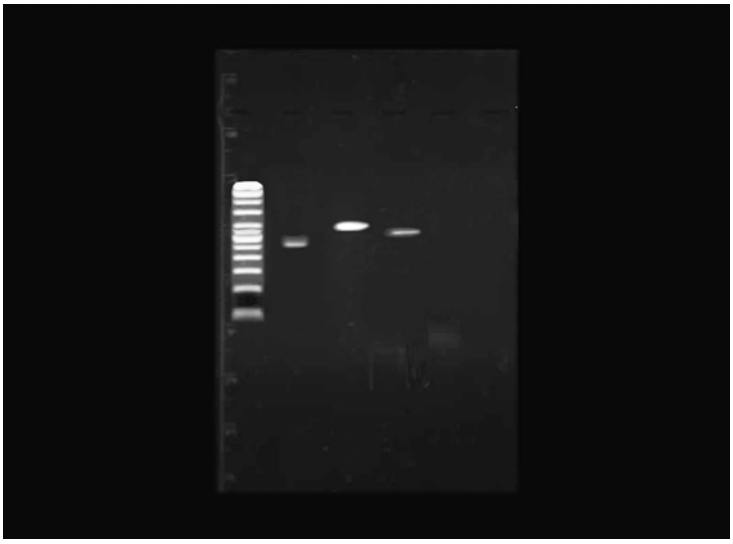


Figure 5.26: Diagnostic RT-PCR for detection of Influenza viruses from clinical samples.

**1 2 3 4 5 6 7 8 9**

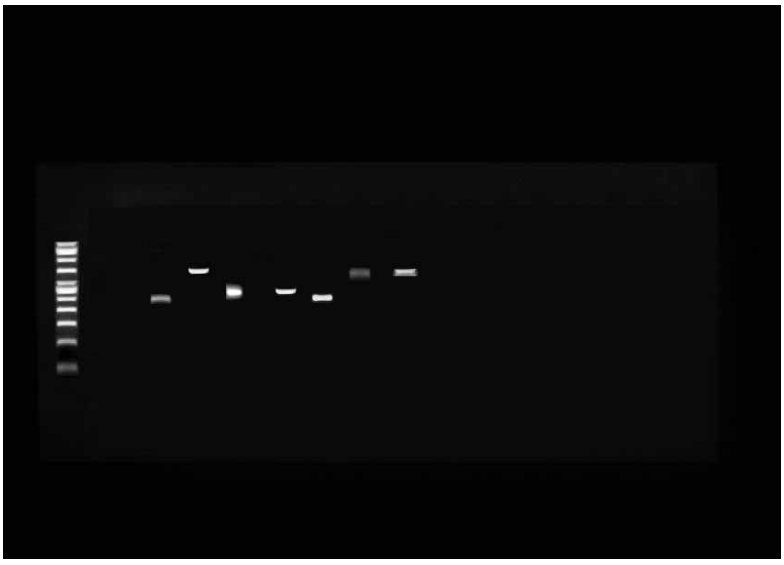


**Figure 5.25:** Diagnostic RT-PCR of Prototype Influenza viruses

Lane 1- Molecular weight ladder 100-1000 bp

Lane 2- Influenza A H1N1 Prototype strain specific amplified product 517 bp.

Lane 3- Influenza A H3N2 Prototype strain specific amplified product 785 bp.

Lane 4 - Influenza B Prototype strain specific amplified product 618 bp.

Lane 5- Negative control.

Figure 5.26:Diagnostic RT-PCR for detection of Influenza viruses from clinical samples.

Lane 1- Molecular weight ladder 100-1000 bp

Lane 2-Negative control

Lane 3- Influenza A H1N1 Prototype strain specific amplified product 517 bp.

Lane 4 -Influenza A H3N2 Prototype strain specific amplified product 785 bp.

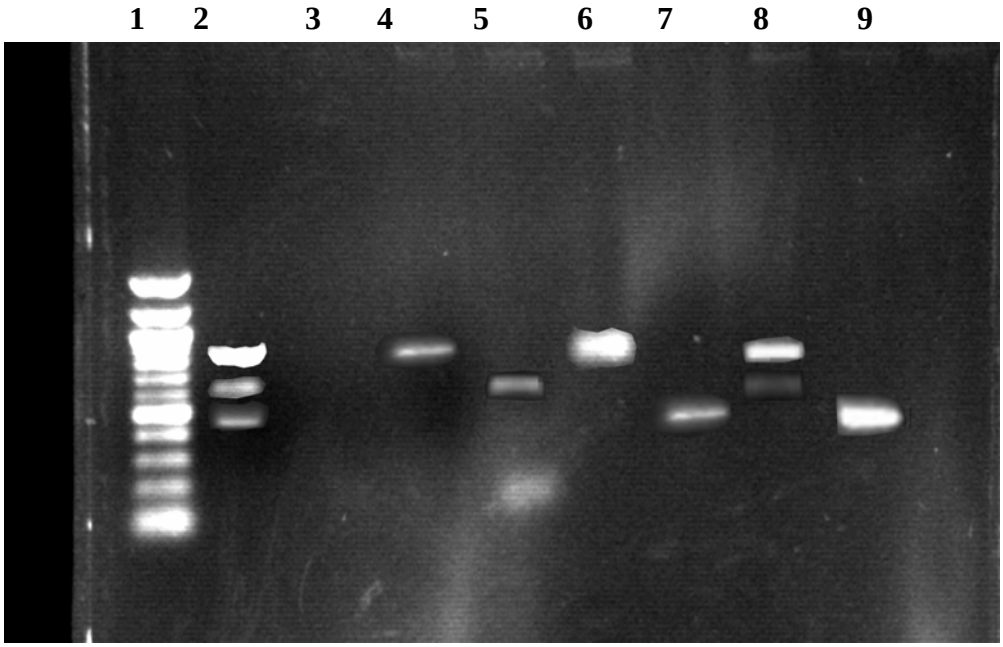
Lane 5 -Influenza B Prototype strain specific amplified product 618 bp.

Lane 6- Influenza A H1N1 virus detected from clinical sample

Lane 7- Influenza B virus detected from clinical sample

Lanes 8 & 9- Influenza A H3N2 virus detected from clinical sample

**Figure 5.27: Multiplex RT-PCR for detection of Influenza viruses from clinical samples**




Lane 1- Molecular weight ladder 100-1000 bp

Lane 2- Influenza A H1N1, H3N2 & B Prototype strain specific amplified products

Lane 3- Negative control

Lane 4 -Influenza A H3N2 virus detected from clinical sample

Lane 5 -Influenza B virus detected from clinical sample

Lane 6- Influenza A H3N2 virus detected from clinical sample

Lane 7- Influenza A H1N1 virus detected from clinical sample

Lane 8 – Influenza H3N2 & B virus detected from clinical sample

Lane 9 - Influenza A H1N1 virus detected from clinical sample

Figure 5.28: Detection of M2 mutants in A/H1 viruses by RT-PCR RFLP.

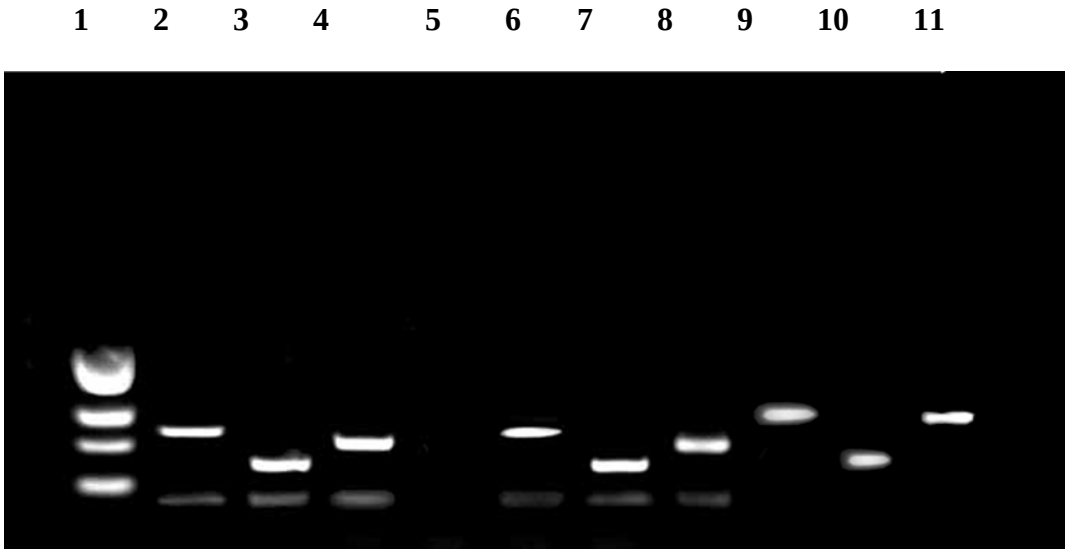



Figure 5.29: Detection of M2 mutants in A/H3 viruses by RT-PCR RFLP.

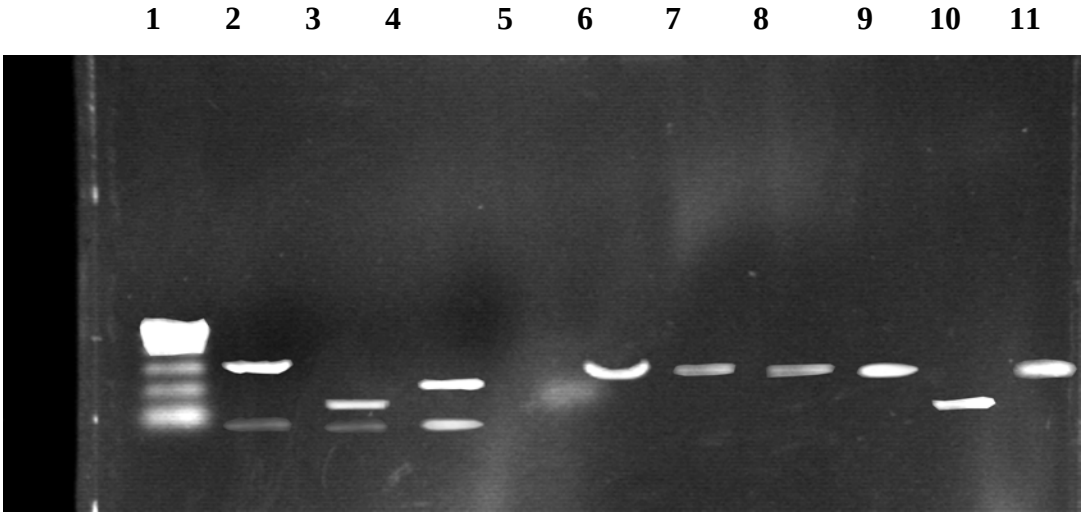



**Figure 5.28: Detection of M2 mutants in A/H1 viruses by RT-PCR RFLP.**

Lane 1- Molecular Weight Marker

Lane 2 - Influenza A H1N1 Amantadine sensitive prototype strain digested with BspLU11I (122 & 32).

Lane 3 - Influenza A H1N1 Amantadine sensitive prototype strain digested with Hha 1 (62 & 33)

Lane 4 - Influenza A H1N1 Amantadine sensitive prototype strain digested with Sca I (116 & 37)

Lane 5 – Negative control

Lane 6- Influenza A H1N1Amantadine sensitive virus isolate digested with BspLU11I (122 & 32)

Lane 7 - Influenza A H1N1 Amantadine sensitive virus isolate digested with Hha 1 (62 & 33)

Lane 8 - Influenza A H1N1 Amantadine sensitive prototype virus isolate with Sca I (116 & 37)

Lane 9 - Influenza A H1N1 Amantadine Resistant strain undigested product (154)

Lane 10 - Influenza A H1N1 Amantadine Resistant strain undigested product. (95)

Lane 11 - Influenza A H1N1 Amantadine Resistant strain undigested product. (153)

**Figure 5.29: Detection of M2 mutants in A/H3 viruses by RT-PCR RFLP.**

Lane 1- Molecular Weight Marker

Lane 2 - Influenza A H3N2 Amantadine sensitive prototype strain digested with BspLU11I (122 & 32).

Lane 3 - Influenza A H3N2 Amantadine sensitive prototype strain digested with Hha 1 (62 & 33)

Lane 4 - Influenza A H3N2 Amantadine sensitive prototype strain digested with Sca I (116 & 37)

Lane 5 – Negative control

Lane 6- 9 Influenza A H3N2 Amantadine Resistant virus isolates not digested with ScaI (153)

Lane 10 - Influenza A H3N2 Amantadine Resistant virus not digested with Hha I (95)

Lane 11 - Influenza A H3N2 Amantadine Resistant virus not digested with Sca I (153)

## Table 5.26: Toxicity Assay of Solvents on MDCK Cells

| S.No. | Solvents | Observation | | | |
|---|---|---|---|---|---|
| | | I Day | III Day | V Day | VII Day |
| 1. | Distilled Water | NT | NT | NT | NT |
| 2. | 70% Ethanol | NT | NT | NT | NT |
| 3. | 0.25% DMSO | NT | NT | NT | NT |

**Key : NT – Non-toxic**

**Table 5. 27: Results of toxicity assay of *Ocimum sanctum* extracts on MDCK cells 7th day observation**

| S. No | Name of the Herbal Extracts | 5 µg to 100 µg | 200 µg | 300 µg | 400 µg | 500 µg | 600 µg | 700 µg | 800 µg | 900 µg | 1 mg | Neat |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1. | *Ocimum sanctum* aqueous extract | NT | NT | NT | NT | NT | T | T | T | T | T | T |
| 2. | *Ocimum sanctum* ethtanolic extract | NT | NT | NT | NT | NT | T | T | T | T | T | T |

**NT : Non Toxic ,T. Toxic , +/-.Equivocal**

**Table 5.28: Results of Pretreatment assay, 100 µg to 300 µg Aqueous and Ethanolic extracts of *Ocimum sanctum* against 1,10,100 $TCID_{50}$ of Influenza A&B virus**

| Herb | Nature of the extract | $TCID_{50}$ of Influenza A&B virus | Concentration of Extracts in µg | *Varying time Exposure of Extract and cells** | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | 0 hrs | 2.0 hrs | 4.0 hrs | 6.0 hrs | 8.0 hrs |
| *Ocimum sanctum* | Aqueous | 1 $TCID_{50}$ | 100 to 300# | Complete Inhibition | | | | |
| | | 10 $TCID_{50}$ | | | | | | |
| | | 100 $TCID_{50}$ | | | | | | |
| | Ethanolic | 1 $TCID_{50}$ | 100 to 300# | Complete inhibition | | | | |
| | | 10 $TCID_{50}$ | | | | | | |
| | | 100 $TCID_{50}$ | | | | | | |

# Concentrations less than 100 µg did not show any protective activity

* Reading at end of 72 hrs

**Table 5.29: Results of Virus inhibition assay, 100 µg to 300 µg Aqueous and Ethanolic extracts of *Ocimum sanctum* against 1, 10, 100 $TCID_{50}$ of Influenza A & B viruses.**

| Name of herb | Nature of the Extract | $TCID_{50}$ of Influenza A&B virus | Concentration of the Extracts in µg* | | |
|---|---|---|---|---|---|
| | | | 100 # µg | 200 # µg | 300 # µg |
| ***Ocimum sanctum*** | Aqueous | 1 $TCID_{50}$ | Complete inhibition of virus | | |
| | | 10 $TCID_{50}$ | Complete inhibition of virus | | |
| | | 100 $TCID_{50}$ | Complete inhibition of virus | | |
| ***Ocimum sanctum*** | Ethanolic | 1$TCID_{50}$ | Complete inhibition of virus | | |
| | | 10 $TCID_{50}$ | Complete inhibition of virus | | |
| | | 100 $TCID_{50}$ | Complete inhibition of virus | | |

# Concentrations less than 100 µg did not show any protective activity

* Reading at end of 72 hrs

**Table 5. 30: Results of Varying time exposure of aqueous and ethanolic extracts of *Ocimum sanctum* vs 1,10,100 $TCID_{50}$ of viruses**

| Nature of the Extract | Concentration of the extracts in μg | Varying time exposure of extracts with 1,10,100 $TCID_{50}$ of Influenza A&B Viruses* | | | | |
|---|---|---|---|---|---|---|
| | | 0 hrs | 0.30 hrs | 1.00 hrs | 1.30 hrs | 2.00 hrs |
| **Aqueous *Ocimum sanctum*** | 100 # μg | CI | CI | CI | CI | CI |
| | 200 μg | | | | | |
| | 300 μg | | | | | |
| **Ethanolic *Ocimum sanctum*** | 100# μg | CI | CI | CI | CI | CI |
| | 200 μg | | | | | |
| | 300 μg | | | | | |

# Concentrations less than 100 μg did not show any protective activity

- Reading at end of 72 hrs ; CI – Complete inhibition of virus

**Table 5.31: Results of Virus adsorption assay of 100 µg to 300 µg of aqueous and ethanolic extracts of *Ocimum sanctum* against 1,10,100 $TCID_{50}$ of Influenza A&B Viruses.**

| Herb | $TCID_{50}$ of Influenza A&B Viruses | Log dilution of stock virus | Concentration of extracts in µg | Mean and SD of the residual viral titer on the day of CPE observed in virus controls* |
|---|---|---|---|---|
| **Aqueous Extract *Ocimum sanctum*** | 1 $TCID_{50}$ | $10^{-4.2}$ | 100 | Complete Inhibition |
| | | | 200 | |
| | | | 300 | |
| | 10 $TCID_{50}$ | $10^{-3.2}$ | 100 | Complete Inhibition |
| | | | 200 | |
| | | | 300 | |
| | 100 $TCID_{50}$ | $10^{-2.2}$ | 100 | Complete Inhibition |
| | | | 200 | |
| | | | 300 | |
| **Ethanolic Extract *Ocimum sanctum*** | 1 $TCID_{50}$ | $10^{-4.2}$ | 100 | Complete Inhibition |
| | | | 200 | |
| | | | 300 | |
| | 10 $TCID_{50}$ | $10^{-3.2}$ | 100 | Complete Inhibition |
| | | | 200 | |
| | | | 300 | |
| | 100 $TCID_{50}$ | $10^{-2.2}$ | 100 | Complete Inhibition |
| | | | 200 | |
| | | | 300 | |

CPE - Cytopathic Effect ; * Reading at end of 72 hrs

**Table 5. 32: Results of toxicity assay of extracts on MDCK cells 7th day observation**

| S.No | Name of the Herbal Extracts | 5 µg to 100 µg | 200 µg | 300 µg | 400 µg | 500 µg | 600 µg | 700 µg | 800 µg | 900 µg | 1 mg | Neat |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1. | *Glycyrrhiza glabra* aqueous extract | NT | NT | NT | NT | NT | NT | T | T | T | T | T |
| 2. | *Glycyrrhiza glabra* ethanolic extract | NT | NT | NT | NT | NT | NT | T | T | T | T | T |

NT : Non Toxic ,T. Toxic , +/-.Equivocal

**Figure: 5.30 MDCK Cell line showing toxicity**

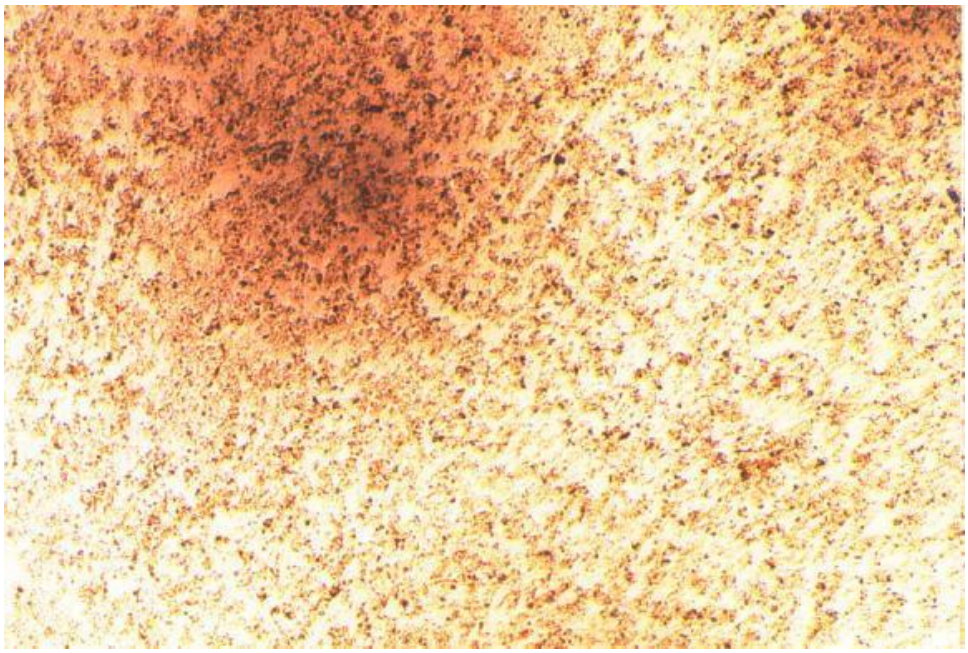


**Table 5.33: Results of Pretreatment assay, aqueous and ethanolic extracts of *Glycyrrhiza glabra* against 1,10,100 $TCID_{50}$ of Influenza A&B virus**

| Herb | Nature of the extract | $TCID_{50}$ of Influenza A&B virus | Concentration of Extracts in µg | *Varying time Exposure of Extract and cells** | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | 0 hrs | 2.0 hrs | 4.0 hrs | 6.0 hrs | 8.0 hrs |
| | Aqueous | 1 $TCID_{50}$ | 100 to 300[#] | Complete Inhibition | | | | |
| | | 10 $TCID_{50}$ | | | | | | |
| | | 100 $TCID_{50}$ | | | | | | |
| | Ethanolic | 1 $TCID_{50}$ | 100 to 300[#] | Complete inhibition | | | | |
| | | 10 $TCID_{50}$ | | | | | | |
| | | 100 $TCID_{50}$ | | | | | | |

[#] Concentrations less than 100 µg did not show any protective activity

* Reading at end of 72 hrs

**5.34: Results of Virus inhibition assay, 100 µg to 300 µg Aqueous and Ethanolic extracts of *Glycyrrhiza glabra* against 1, 10, 100 $TCID_{50}$ of Influenza A & B viruses.**

| Name of herb | Nature of the Extract | $TCID_{50}$ of Influenza A&B virus | Concentration of the Extracts in µg* | | |
|---|---|---|---|---|---|
| | | | 100 # µg | 200 # µg | 300 # µg |
| ***Glycyrrhiza glabra*** | Aqueous | 1 $TCID_{50}$ | Complete inhibition of virus | | |
| | | 10 $TCID_{50}$ | Complete inhibition of virus | | |
| | | 100 $TCID_{50}$ | Complete inhibition of virus | | |
| ***Glycyrrhiza glabra*** | Ethanolic | 1$TCID_{50}$ | Complete inhibition of virus | | |
| | | 10 $TCID_{50}$ | Complete inhibition of virus | | |
| | | 100 $TCID_{50}$ | Complete inhibition of virus | | |

# Concentrations less than 100 µg did not show any protective activity

* Reading at end of 72 hrs

**Table 5. 35: Results of Varying time exposure of aqueous and ethanolic extracts of *Glycyrrhiza glabra* vs 1,10,100 $TCID_{50}$ of viruses**

| Nature of the Extract | Concentration of the extracts in μg | Varying time exposure of extracts with 1,10,100 $TCID_{50}$ of Influenza A&B Viruses* | | | | |
|---|---|---|---|---|---|---|
| | | 0 hrs | 0.30 hrs | 1.00 hrs | 1.30 hrs | 2.00 hrs |
| **Aqueous *Glycyrrhiza glabra*** | 100 # μg | CI | CI | CI | CI | CI |
| | 200 μg | | | | | |
| | 300 μg | | | | | |
| **Ethanolic *Glycyrrhiza glabra*** | 100# μg | CI | CI | CI | CI | CI |
| | 200 μg | | | | | |
| | 300 μg | | | | | |

# Concentrations less than 100 μg did not show any protective activity

- Reading at end of 72 hrs ; CI – Complete inhibition of virus

**Table 5. 36: Results of Virus adsorption assay of 100 µg to 300 µg of aqueous and ethanolic extracts of *Glycyrrhiza glabra* against 1,10,100 $TCID_{50}$ of Influenza A&B Viruses.**

| Herb | $TCID_{50}$ of Influenza A&B Viruses | Log dilution of stock virus | Concentration of extracts in µg | Mean and SD of the residual viral titer on the day of CPE observed in virus controls* |
|---|---|---|---|---|
| **Aqueous Extract *Glycyrrhiza glabra*** | 1 $TCID_{50}$ | $10^{-4.2}$ | 100 | Complete Inhibition |
| | | | 200 | |
| | | | 300 | |
| | 10 $TCID_{50}$ | $10^{-3.2}$ | 100 | Complete Inhibition |
| | | | 200 | |
| | | | 300 | |
| | 100 $TCID_{50}$ | $10^{-2.2}$ | 100 | Complete Inhibition |
| | | | 200 | |
| | | | 300 | |
| **Ethanolic Extract *Glycyrrhiza glabra*** | 1 $TCID_{50}$ | $10^{-4.2}$ | 100 | Complete Inhibition |
| | | | 200 | |
| | | | 300 | |
| | 10 $TCID_{50}$ | $10^{-3.2}$ | 100 | Complete Inhibition |
| | | | 200 | |
| | | | 300 | |
| | 100 $TCID_{50}$ | $10^{-2.2}$ | 100 | Complete Inhibition |
| | | | 200 | |
| | | | 300 | |

CPE - Cytopathic Effect ; * Reading at end of 72 hrs

# REFERENCES


1. A Programme for controlling acute respiratory infections in children: Memorandum form a WHO meeting. Bull World Health Organ 1984; 62:47-58.

2. Grant J.P., The state of world children, Oxford, Oxford University Press, 1990;

3. Pio A., WHO programme on acute respitatory infections. Indian Journal of Paediatrics, 1998; 55: 197-205.

4. Kumar V, Need for national control programme for acute repiratory infections. Indian Journal of Paediatrics, 1987; 54: 145-148.

5. Lui KJ., Kendal AP. Impact of influenza epidemics on mortality in the United States from October 1972 to May 1985. Am J Pub Health, 1987; 77:712-6.

6. Walls HH., Harmon HW and Kendal AP. Characterisation and evaluation of monolonal antibodies developed for typing influenza A and influenza B Virus. J Clin Microbiol,1986; 23:240-5.

7. Layne SP, Beugelsdijk TJ, Patel CKN, Taubenberger JK, Cox NJ, Gust ID, et al. /a global lab against influenza. Science, 2001; 293:1729.

8. Rao BL. Epidemiology and control of influenza, National Medical Journal of India, 2003;16:3,143-149.

9. Wilson IA., Skehel JJ and Wiley DC . Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 A resolution. Nature, 1981; 289:365-67.

10. Lazarowitz SG., Choppin PW. Enchancement of influenza A and B viruses by rpoleotic cleavage of the haemagglutinin polypeptide. Virology, 1975; 68:440-54.

11. Klenk HD., Rott R and Orlich M. Activation of influenza A viruses by trypsin treatment. Virology, 1975; 68:426-39.

12. Colman PM., Varghese JN and Laver WG . Structure of the catalytic and antigenic sites in influenza virus neuraminidase. Nature, 1983; 303:41.

13. Colman,(). Nemaminidase: enzyme and antigen. P17. In Krug Rm (ed): The influenza viruses, 1989; Plenum new York.

14. Palese P., Tobita K and Veda M. Characterization of temperature sensitive influenza virus mutants defective in neuraminidase, 1974; Virology, 61:397-400.

15. Brooks GF , Butel Js Morse SA. Jawetz M(eds) Adelberg's medical microbiology .New York : Lange medical books / McGraw-Hill Medical Publishing Division, 2001; 459-69.

16. Wright PF, Webster RG Orthomyxoviruses. In:Knipe DM, Howley PM9eds) Field's Virology, Vol2. Philadelphia: Lippincott, Williams and Wilkins, 2001;1533-79.

17. Kendal AP, Pereira MS, Skehel JJ. Concepts and procedures for laboratory based Influenza Surveillance. Atlanta GA, USA: Centres for Disease Control , 1982; A7-A13.

18. Stuart-Harris CH, Schild GC , Oxford JS . influenza – the viruses and the disease. London : Edward Arnold , 1985; 41-138.

19. Hinshaw VS, Bean WJ, Geraci J, Fiorelli P, Early G, Webster RG. Characterization of two influenza A viruses from a pilot whale.J Virol 1986;58:655-6.

20. Rohm C, Zhou N Suss J, Mackenzie J, Webster RG. Characterization of novel influenza haemagglutinin, H15: criteria for determination of influenza A subtypes. Virology 1996;217:508-16.

21. Lvov DK. Zdanov VM, Sazonov AA, Braude NA, Vladimirtceva EA, Agafonova LV, etal. Comparison of influenza viruses isolated from man and from whales. Bull World health Organ 1978;56:923-30.

22. Kawaoka Y, Yamnikova S, Chambers TM, Lvov DK, Webster RG. Molecular characterization of a new hemagglutinin, subtype H14 of influenza A virus. Virology 1990;179:759-67.

23. Both GW., Seligh MJ and Cox NJ. Antigenic drift in influenza virus H3 haemagglutinin from 1968-1980; multiple evolutionary pathways and sequential amino acid changes at key antigenic sites. J Virol, 1983; 48:52-60.

24. Raymond FL., Laton AJ and Cox NJ. The antigenicity and evalution of influenza H1 haemaaglutinin from 1950-57 and 1977-83; two path ways from one gene. Virology, 1986; 148:275-87.

25. Wiley DC., Wilson IA and Skehel JJ. Structural identification of the antibody binding sites of Hong Kong influenza hemagglutinin and their involvement in antigenic variation . Nature, 1981; 289: 365-8.

26. Davenport FM., Hennessy AV and Francis T Jr. Epidemiologic and immunologic significance of age distribution of antibod to antigenic variants of influenza virus. J Exp Med, 1989; 98:641-56.

27. Buonagurio DA, Nakada S, Desselberger U, Krystal M, Palese P.Noncumulative sequence changes in the hemagglutinin genes of influenza C virus isolates. Virology 1985; 146:221-232.

28. Burch GE, Walsh JJ, Mogabgab W. A study of the response of the cardiovascular system to Asain influenza. *Am Rev Respir Dis* 1961; 83:68-78.

29. Burnett FM. Influenza virus on the developing egg. I changes associated with the development of and egg passage strain of virus. Br J exp Pathol. 1936; 17: 282-293.

30. Murray et al., Microbiology, 2006; 3rd Ed., Chapter 57.

31. Kilbourne ED. The influenza viruses and influenza. New york: Academic Press, 1975;315-481.

32. Brooks GF, Butel JS, Morse SA, Jawetz M (eds).Adelbergs Medical Microbiology.New York: Lange Medical Books/McGraw Hill Medical Publishing Division,2001;459-469

33. Glezen al. W P. Serious morbidity and mortality associated with influenza epidemics. Epidemiol.Rev. 1982;4:24-44.

34. Perotta DM, Decker M, Glezen W.P. Acute respiratory disease hospitalization as a measure of impact of epidemic influenza. Am. J. Epidemiol. 1985; 122:468-476.

35. Glezen WP, Couch RB. Intra pandemic influenza in Houston area, 1974-1976. N Engl. J. Med. 1978; 298:587-593.

36. Sinomen L, Clarke MJ, Schonberger LB etal. Pandemic versus epidemic influenza mortality. A pattern of changing age distribution. J. Inf Dis., 1998; 178: 53-60.

37. Masurel N, Marine WM. Recycling of Asian and Hong Kong Influenza A virus Haemagglutinin in man. Am J. Epidemiol, 1973;97:44-49

38. Cox NJ., Subbarao K (). Global Epidemiology of Influenza : Past and Present. Annu-Rev-Med, 2000; 51: 407-21.

39. Luke T. Daum, Michael W. Shaw, Alexander I. Klimov, Linda C. Canas, Elizabeth A. Macias, Debra Niemeyer, James P. Chambers, Robert Renthal, Sanjaya K. Shrestha, Ramesh P. Acharya, Shankar P. Huzdar, Nirmal Rimal, Khin S. Myint, and Philip Gould. Influenza A (H3N2) Outbreak, Nepal. Emerging Infectious Diseases, 2005; Vol. 11: No. 8:1186-91.

40. No author cited 2002 - 03 Influenza Season Summary Centers for Disease Control and Prevention, 1600 Clifton Rd, Atlanta, GA 30333, USA.

41. Matsuzaki Y, Abiko C, Mizuta K, Sugawara K, Takashita E, Muraki Y, Suzuki H, Mikawa M, Shimada S, Sato K, Kuzuya M, Takao S, Wakatsuki K,

Itagaki T, Hongo S, Nishimura H.A nationwide epidemic of influenza C virus infection in Japan in 2004. J.Clin Microbiol, 2007; Mar 45 (3): 783-8.

42. Centers for Disease Control and Prevention. Update: influenza activity—United States and worldwide, 2006–2007 season, and composition of the 2007–08 influenza vaccine. Morb. Mortal. Wkly. Rep. 2007; 56:789–794.

43. Avian flu http://www.cdc.gov/flu/avian/gen-info/avian-influenza.htm World Health Organization. WHO interim guidelines on clinical management of humans infected by influenza A(H5N1). February 20, 2004.

44. Avianflu http://www.cdc.gov/flu/avian/gen-info/avian-flu-humans.htm

45. Rao BL Gupta NP, Rao CVR, gogate SS, shah U. investigation on the outbreak of influenza by A/USSR/77 virus strain in Pune, maharashtra in 1978. Indian j Med Res, 1979;70:681-6.

46. Mohan rao CVR . Gupta NP Rao BL , Joshi BN. Clinical and laboratory investigation on theoutbreak of influenza by A/USSR/77 virus strain in Pune, maharashtra in 1978 . Indian j Med Res, 1980; 26:503-6.

47. Rao BL . Investigation of 1979 influenza outbreak caused by types A and B influenza viruses in Pune, India. Indian J Chest Dis Allied Sci, 198022:143-6.

48. Rao BL, Kadam SS, Pavri KM, Kothavale VS. Epidemiological, clinical and virological features of influenza outbreaks in Pune, India 1980. Bull World Health Organ, 1982; 60:639-42.

49. Rao BL. Investigation on the monsoon outbreak of influenza A (H3N2)virus strain in Pune, India , 1981. Indian J Med Res, 1983; 77:417-19.

50. Rao BL. Thite Ny . Virological and serological study on the new influenza variant A (H1N1) strain : A/ Singapore/6/86. Indian j Med Res, 1987; 86: 813-15.

51. Rao Bl. Influenza surveillance in Pune (India) during 1983-85. Indian J Med Res, 1987; 85:492-5.

52. Rao BL. Banerjee K. Influenza surveillance in Pune, India 1978-90. Bull World Health Organ, 1993; 71:177-81.

53. Rao BL, Wairagkar NS, Ranadive SN. Isolation of the new vriant influenza A (H3N2) strains during an outbreak in Pune, February 1996. Indian J Med Res, 1997; 106:223-4.

54. Rao BL, kadam SS. Isolation of recent variant influenza types A (H3N2) and B Strains in Pune, India during 1998. Indian J Med Res, 2000; 111:3-5.

55. Nalini Ramamurty, Lalitha C.Pillai, P.Gunasekaran, Varalakshmi Elango, Mohana, Padma Priya & A.Khaleefathullah Sheriff. Influenza activity among the paediatric age group in Chennai. Indian J Med Res, 2005;121:776-779.

56. Prevention and Control of Influenza: Recommendations of the Advisory Committee on Immunization Practices (ACIP) MMWR: 2006 Jul 28:55:1-42.

57. http://www.cdc.gov/flu/professionals/diagnosis/labrole.htm.27 August, 2007.

58. http://www .cdc.gov/flu/professionals/diagnosis/labprocedures.htm

59. Premnathan M., Chandra K., Bajpai S.K and Kathiresan K. A survey of some Indian Marine plants for antiviral activity. Botanica Marina, 1992; 35: 321-324.

60. Noble GR. Epidemiological and clinical aspects of influenza : Beare AS, ed. In: *Basic and Applied Inf/lIerlUl Research.* Boca Raton Fla: CRC Press: 1982;11-50.

61. D*owdle* WR. Influenza kvirus reqding revisited. *'Bull World Health . Org., 1999; 7:820-:.828.,*

62. Basler CF, Reid AH, Dybing JK, et al. From the cover : Sequence of the 1918 pandemic influenza virus nonstructural gene (NS) segment and characterization of recombinant viruses bearing the 1918 NS genes. *Proc Natl Acad Sci, USA* 2001; 98:2746-2751.

*63.* Reid AH, Fanning TG, Janczewski TA, Lourens RM, Taubenberger JK. Novel Origin of the 1918 pandemic influenza virus nucleoprotein gene. *J Virol*, 2004; 78:12462-12470.

*64.* Reid AH, Fanning TG, Janczewski TA, Lourens RM, Taubenberger JK. Novel Origin of the 1918 pandemic influenza virus nucleoprotein gene. *J Virol*, 2004; 78:12462-12470.

*65.* Reid AH, Fanning TG, Janczewski TA,McGall S, Taubenberger JK. Characterization of the 1918 "Spanish" influenza virus matrix gene segment. *J Virol*, 2002; 76:10717-10723.

*66.* Reid AH, Fanning TG, Janczewski TA, Taubenberger JK. Characterization of the 1918 "Spanish" influenza virus neuraminidase gene. *Proc Natl Acad Sci* USA, 2000; 97:6785-6790.

*67.* Taubenberger JK, Reid AH, Krafft AE, Bijwaard KE, Fanning TG.Initial genetic characterization of the 1918 "Spanish" influenza virus. Science, 1997; 275:1793-1796.

*68.* Taubenberger JK, Reid AH, Lourens RM, Wang Jin G, Fanning TG. Characterization of the 1918 influenza virus polymerase genes. Nature, 2005; 437:889-893.

69. Burnett FM. Influenza virus on the developing egg. I changes associated with the development of and egg passage strain of virus. Br J exp Pathol, 1936; 17: 282-293.

70. Brands R, Visser J, Medema J, Palache AM, Van Scharrenburg Gj. Influvac : A safe Madin Darby Canine Kidney (MDCK) cell culture based influenza vaccine. *Dev Biol Stand*, 1999; 98:93-100.

71. Kistner O, Barrett PN, Mundt W, Reiter M, Schober-Bendixen S, Dorner F. Development of a mammalian Cell (Vero) derived candidate influenza virus vaccine. *Vaccine*, 1998; 16:960-968.

72. Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y.Evolution and ecology of influenza A viruses. *Microbiol Rev,* 1992; 56:152-179.

73. Kawaoka Y, Krauss S, Webster RG. Avian-to-human transmission of the PB1 gene of influenza A viruses in the 1957 and 1968 pandemics. *J Virol,* 1989; 63:4603-4608.

74. Ito T.Gorman OT, Kawaoka Y, Bean WJ, Webster RG. Evolutionary analysis of the influenza A virus M gene with comparison of the M1 and M2 proteins. *J Virol,* 1991; 65:5491-5498.

75. Treanor JJ, Snyder MH, London WT, Murphy BR. The B allele of the NS gene of avian influenza viruses, but not the A allete, attenuates a human influenza. A virus for squirrel monkeys. *Virology*, 1989; 171:1-9

76. Donis RO, Bean WJ, Kawaoka Y, Webster RG. Distinct lineages of influenza virus H4 hemagglutinin genes in different regions of the world. *Virology,* 1989; 169:408-417.

77. Buonagurio DA, Nsselberger U, Krystal M, Palese P.Noncumulative sequence changes in the hemagglutinin genes of influenza C virus isolates. Virology, 1985; 146:221-232.

78. Scholtissek C, Rohde W, Von Hoyningen V, Rott R. On the origin of the human influenza virus subtypes H2N2 and H3N2. *Virology,* 1978; 87:13-20

79. Karasin AI, Schutten MM, Cooper LA, et al. Genetic charcterization of H3N2 influenza viruses isolated from pigs in North America, 1977-1999. evidence for wholly human and reassortant virus genotypes. *Virus Res,* 2000; 68:71-85.

80. Webby RJ, Swenson SL, Krauss SL., Gerrish PJ, Goyal SM, Webster RG. Evolution of Swine H3N2 influenza viruses in the United States. *J Virol,* 2000; 74: 8243-8251.

81. Zhou NN, Senne DA, Landgraf JS, et al. Genetic reassortment of avian, swine, and human influenza A viruses in American pigs. *J Virol,* 1999; 73:8851-8856.

82. Li KS Guan Y, Wang J, et al, Genesis of highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia . Nature, 2004; 430: 209-213.

83. Bowes VA, Ritchie Sj, Byrne S, Sojonky K, Bidulka JJ, Robinson JH. Virus characterization, clinical presentation, and pathology associated with H7N3 avian influenza in British Columbia broiler breeder chickens in 2004. *Avain Dis,* 2004; 48:928-934

84. Hirst M, Astell CR, Griffith M, et al. Novel avian influenza H7N3 strain outbreak, British Columbia. *Emerg Infect Dis,* 2004; 10:2192-2195.

85. Pasick J, Handel K, Robinson J, et al. Intersegmental recombination between the haemagglutinin and matrix genes was responsible for the emergence of a highly pathogenic H7N3 avian influenza virus in British Columbia. *J Gen Virol,* 2005; 86:727-731.

86. Suarez DL, Senna DA, Banks J, et al. Recombination resulting in virulence shift in avian influenza outbreak, Chile. *Emerg Infect Dis,* 2004; 10:693-699.

87. Whitaker-Dowling P, Lucas W, Youngner JS. Cold-adapted vaccine strains of influenza A virus act as dominant negative mutants in mixed infections with wild-type influenza A virus. *Virology,* 1990; 175-358-364.

88. Class EC, Osterhaus AD, Van Beek R, et al. Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. *Lancet,* 1998; 351:47-467.

89. Subbarao K, Klimov A, Katz J, et al. Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness. *Science,* 1998; 279:393-396.

90. Tran TH, Nguyen TL, Nguyen TD, et al. Avian influenza A (H5N1) in 10 patients in Vietnam. *N Engl J Med,* 2004; 350:1179-1188.

91. Ungchusak K, Auewarakul , Dowell SF, et al. Probable Person-to-person transmission of avian influenza A (H5N1). *N Engl J Med,* 2005; 352:333-340.

92. Chen H, Smith GJ, Zhang SY, et al. Avian Flu : H5N1 virus outbreak in migratory waterfowl. *Nature,* 2005; 436:191-192

93. de Jong MD, Bach VC, Phan TQ, et al. Fatal avian influenza A (H5N1) in a child presenting with diarrhea following by coma N Engl J Med, 2005; 352:686-691

94. de Jong MD, Tran TT, Truong HK, et al. Oseltamivir resistance during treatment of influenza A (H5N1) infection. *N Engl J Med,* 2005; 353:2667-2672.

95. Le QM, Kiso M, Someya K, et al. Avian flu : isolation of drug resistant H5N1 virus. *Nature,* 2005; 437:1108.

96. Gubareva LV, Bethell R, Hart GJ, Murti KG, Penn CR, Webster RG. Characterizatrion of mutants of influenza A virus selected with the neuraminidase inhibitor 4-guanidino-Neu5Ac2en. *J.Virol,* 1996; 70:1818-1827.

97. Palese ,P. The genes of influenza virus. Cell, 1977; 10: 1-10.

98. Lamemli, U.K. Cleavage of structural proteinsduring the assembly of the head of bacteriophage T4. Nature (London), 1970; 227: 680-685.

99. Laver ,W.G. et.al. Studies on the origin of pandemic influenza II. Peptide maps of the light and heavy polypeptide chains from the hemagglutinin subunits of A2 influenza viruses isolated before and after the appearance of Hong Kong influenza. Virology, 1972; 48: 445-455.

100. Hay, A.J. et.al., Transcription of the influenza virus genome. virology, 1977; 83: 337-355.

101. Ghendon, Y et.al., Genome analysis of influenza virus strains isolated during an epidemic of 1979-1980. Journal of general virology, 1981; 56:303-313.

102. Scholtissek, C et.al. Correlation between RNA fragments of fowl plague virus and their corresponding gene functions. Virology, 1976; 74: 332-344.

103. Bean, W.J. JR et.al Recombination of human influenza A viruses in nature. Nature (London), 1980; 284: 638-640.

104. deWatcher. R. & Fiers, W. Preparative two-dimensional polyacrylamid gel electrophoresis of $^{32}$P-labelled RNA. Analytical biochemistry, 1972; 49: 184-97.

105. Young, J.F, et.al., Advantages and limitations of the oligonucleotide mapping technique for the analysis of viral RNAs, In: D.H.L Bishop & R.W.Compansed., Replication of negative strain viruses. New York, Academic Press, 1981; 209-215.

106. Ortin, J. et.al., Genetic variability of Hongkong (H3N2) influenza viruses: spontaneous mutations and their location in the viral genome Gene,1980; 11. 319-331.

107. Nakagima, K. et.al. Recent human influenza A (H1N1) viruses are closely related genetically to strains isolated in 1950. Nature (London), 1978; 274: 334-339.

108. Young, J.F. et.al., Evolution of human influenza A viruses in nature: Sequential mutations in genomes of new H1N1, Cell, 1979;18: 73-83.

109. Webster , R.G. et.al. Analysis of antigenic drift in recently isolated influenza A (H1N1) viruses using monoclonal antibody preparations. Virology, 1979; 96: 258-264.

110. Cox, N. J., and C. A. Bender.. The molecular epidemiology of influenza viruses. Semin. Virol, 1995; 6:359–370.

111. Cox, N. J., T. L. Brammer, and H. L. Regnery. Influenza: global surveillance for epidemic and pandemic variants. Eur. J. Epidemiol, 1994; 10:467–470.

112. Hay, A. J., A. R. Douglas, D. B. Sparrow, K. R. Cameron, and J. J. Skehel. Antigenic and genetic characterization of current influenza strains. Eur. J. Epidemiol, 1994; 10:465–466.

113. Noble, G. R., H. S. Kaye, W. B. Yarbrough, B. K. Fiedler, C. J. Reed, M. B. Felker, A. P. Kendal, and W. R. Dowdle. Measurement of hemagglutination-inhibiting antibody to influenza virus in the 1976 influenza vaccine program: methods and test reproductivity. J. Infect. Dis., 1977; 136:S429–S434.

114. Bikour, M. H., E. H. Frost, S. Deslandes, B. Talbot, J. M. Weber, and Y. Elazhary. Recent H3N2 swine influenza virus with haemagglutinin and nucleoprotein genes similar to 1975 human strains. J. Gen. Virol, 1995; 76:697– 703.

115. Claas, E. C. J., M. J. W. Sprenger, G. E. M. Kleter, R. van Beek, W. G. V. Quint, and N. Masurel. Type-specific identification of influenza viruses A, B and C by the polymerase chain reaction. J. Virol. Methods,1992; 39:1–13.

116. Ellis, J. S., P. Chakraverty, and J. P. Clewley. Genetic and antigenic variation in the haemagglutinin of recently circulating human influenza A (H3N2) viruses in the United Kingdom. Arch. Virol, 1995; 140:1889–1904.

117. Ikonen, N., L. Kinnunen, T. Forsten, and R. Pyhala. Recent influenza B viruses in Europe: a phylogenetic analysis. Clin. Diagn. Virol, 1996;6:63–71.

118. Neumeier, E., H. Meier-Ewert, and N. J. Cox.. Genetic relatedness between influenza A (H1N1) viruses isolated from humans and pigs. J. Gen. Virol, 1994; 75:2103–2107.

119. Wright, K. E., G. A. R. Wilson, D. Novosad, C. Dimock, D. Tan, and J. M. Weber. 1995. Typing and subtyping of influenza viruses in clinical samples by PCR. J. Clin. Microbiol, 1995; 33:1180–1184.

120. Xu, X., E. P. Rocha, H. L. Regnery, A. P. Kendal, and N. J. Cox.. Genetic and antigenic analyses of influenza A (H1N1) viruses, 1986–1991. Virus Res., 1993; 28:37–55.

121. Xu, X., N. J. Cox, C. A. Bender, H. L. Regnery, and M. W. Shaw. Genetic variation in neuraminidase genes of influenza A (H3N2) viruses. Virology, 1996; 224:175–183.

122. Zhang, W., and D. H. Evans. Detection and identification of human influenza viruses by the polymerase chain reaction. J. Virol. Methods, 1991; 33: 165–189.

123. Beare AS, Webster RG. Replication of avian influenza viruses in humans. *Arch Virol* 1991; 119:37-42

124. Class EC, Osterhaus AD, Van Beek R, et al. Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. *Lancet,* 1998; 351:47-467.

125. Tweed SA, Skowroski DM, David ST, et al. Human illness from avian influenza H7N3, British Columbia. Emerg Infet Dis, 2004; 10:2196-2199.

126. Alford RH, Kasel JA, Gerone Pj, Knight V.Human influenza resulting from aerosol inhalation. *Proc Soc Exp Biol Med,* 1966; 122:800-804

127. Couch RB, Douglas RG Jr, Fedson DS, Kasel JA. Correlated Studies of a recombinant influenza-virus vaccine. 3. Protection against experimental influenza in man. *J Infect Dis,* 1971;124:473-480.

128. Glezen WP. Serious morbidity and mortality associated with influenza epidemics. *Epidemiol Rev,* 1982; 4:25-44.

129. Wright PF, Thompson J, McKee KT Jr, Vaughn WK, Sell SH, Karzon DT. Patterns of illness is in the highly febrile young child : epidemiologic, clinical, and laboratory correlates. *Pediatrics,* 1981; 67:694-700.

130. Jordon WS Jr.Badger GF, Dingle JH. A study of illness in a group of Cleveland families. XVI. The epidemiology of influenza, 1948-1953. *Am J hyg*, 1958; 68:169-189.

131. Dingle JH, Badger DF, Jordon WS Jr. *Illness in the Home : A Study of 25,000 Illness in a Group of Cleveland Families.* Cleveland, Oh : Press of Western Reserve University, 1964;162-187.

132. Frank AL, Taber LH, Glezen WP, Paredes A, Couch RB.Reinfection with influenza A (H3N2) virus in young children and their families. J *Infect Dis*, 1979; 140:829-836.

133. Glezen WP. Considerations of the risk of influenza in children and indications for prophylaxis. *Rev Infect Dis 1980;2:408-420.* Glezen WP Serious morbidity and mortality associated with influenza epidemics *Epidemiol Rev, 1982;4: 25-44.*

134. Barker WH, Mullooly Jp, Impact of epidemic type A influenza in a defined adult population. *Am J Epidemiol*, 1980; 112:798-811.

135. Sprenger MJ, Mulder PG, Beyer WE, Van SR, Masurel N.Impact of influenza on mortality in relation to age and underlying disease, 1967-1989. *Int J Epidemiol,* 1993 22:334-340.

136. Simonsen L. The global impact of influenza on morbidity and mortality. *Vaccine,* 1999; 17 (suppl 1) : S3-S10.

137. Cox NJ, Subbarao K.Global epidemiology of influenza: past and present. *Annu Rev Med,* 2000;51:407-421.

138. Noble GR. Epidemiological and clinical aspects of influenza. In:Beare AS, ed. In:Basic and Applied Influenza Research. Boca Raton, Fla : CRC Press: 1982;11-50

139. Kendal AP, Goldfield M, Noble GR, Dowdle WR. Identification and preliminary antigenic analysis of swine influenza-like viruses isolated during an influenza outbreak at Fort Dix, New Jersey. *J Infect Dis* 1977; 136 (Suppl): S381-S385.

140. Rota PA, Rocha EP, Harmon MW, et al. Laboratory characterization of a swine influenza virus isolated from a afatal case of human influenza. *J Clin Microbiol,* 1989; 27:1413-1416.

141. Castrucci MR, Campitelli L, Ruggieri A, et al. Antigenti and sequence analysis of H3 influenza virus haemagglutinins from pigs in Italy. *J Gen Virol*, 1994; 75:371-379.

142. Peiris JS, Guan Y, Makwell D, Ghose P, Webster RG. Shortridge KF. Cocirculation of avian H9N2 and contemporary "human" H3N2 influenza A viruses in pigs in southeastern china : potential for genetic reassortment" *J Virol*, 2001; 75:9679-9686.

143. Li H, Yu K, Yang H, et al. Isolation and characterization of H5N1 and H9N2 influenza viruses from pigs in China. *Chin J Prev Vet Med*, 2005;26.

144. Choi YK, Nguyen TD, Ozaki H, et al. Studies of H5N1 influenza virus infection of pigs by using viruses isolated in Vietnam and Thailand in 2004. J *Virol*, 2005; 79:10821-10825.

145. Shu LL, Zhou NN, Sharp GB, He SQ, Zhang TJ, Zou WW, Webster RG. An epidemiological study of influenza viruses among Chinese farm families with household ducks and pigs. Epidemiol Infect., 1996 Aug; 117(1):179-88.

146. Balu DM, Compans RW. Polarization of viral entry and release in epithelial cells. *Semin Virol*, 1996; 245-253.

147. Shinya K.Ebina M, Yamade S, Ono M, Kasai N, Kawaoka Y. Avian Flu : influenza virus receptors in the human airway. *Nature*, 2006; 440:435-436.

148. Richman DD, Murphy BR, Baron S, Uhlendorf C.Three strains of influenza A virus (H3N2) : interferon sensitivity in vitro and interferon production in volunteers. *J Clin Microbiol*, 1976; 3:223-226.

149. Hers JF, Mulder J. Broad aspects of the pathology and pathogenesis of human influenza. *Am Rev Respir Dis*, 1961; 83:84-97.

150. Walsh JJ, Dietlein LF, Low FN: Burch GE, Mogabgab WJ, Bronchotracheal response in human influenza. Type A. Asian strain, as studied by light and electron microscopic examination of bronchoscopic biopsies. *Arch Intern Med*, 1961; 108:376-388.

151. I Guarner J, Shieh WJ, Dawson J, et al. Immunohistochemical and in situ hybridization studies of influenza. A virus infection in human lungs. *Am J Clin Pathol,* 1999; 96:9363-9367.

152. To KF, Chan PK, Chan KF, et al. Pathology of fatal human infection associated with avian influenza A H57N1 virus. *J Med Virol,* 2001; 63:242-246.

153. Louria DB, Blumenfeld HL, Ellis JT, Kilbourne EB, Rogers DE. Studies on influenza in the pandemic of 1957-1958. II. Pulmonary complications of influenza. *J Clin Invest,* 1959; 38:213-265.

154. Hinshaw VS, Olsen CW, Dybdahl-Sissoko N, Evans D.Apoptosis : a mechanism of cell killing by influenza A and B viruses : *J Virol,* 1994;68:3667-3673.

155. Douglas R.G. Influenza in man. In : Kilbourne ED, ed. *Influenza Viruses and Influenza.* New York, NY : Academic Press: 1975;397-446.

156. Eccles R. Understanding the Symptoms of the common cold and influenza. *Lancet Infect Dis,* 2005; 5:718-725.

157. Fouchier RA, Schneeberger PM, Rozendaal FW, et al. Avian influenza A virus (H7N7) associated with human conjunctivitis and a fatal case of acute respiratory distress syndrome. *Proc Natl Acad Sci USA,* 2004; 101:1356-1361.

158. Hall Wj, Douglas RG Jr. Hyde RW, Roth FK, Cross AS, Speers DM.Pulmonary mechanics after uncomplicated influenza A infection. *Am Rev Respir Dis,* 1976; 113:141-148.

159. Horner GJ, Gray FD. Effect of uncomplicated, presumptive influenza on the diffusing capacity of the lung. *Am Rev Respir Dis,* 1973; 108:866-869.

160. Price DA, Postlethwaite RL Longson M. Influenzavirus A2 infections presenting with febrile convulsions and gastrointestinal symptoms in young children. *Clin* Pedialr *(Phila), 1976;15:361*367.

161. Paisley JW, Bruhn FW, Lauer BA, Mclntosh K. Type A2 influenza viral infections in children. *Am J Dis Child,* 1978; 132:34-36

162. Wright PF, Murphy BR, Kervina M, Lawrence EM, Phelan MA, Karzon DT. Secretary immunological response after intranasal inactivated influenza A virus vaccinations: evidence for immunoglobulin A memory. *Infect Immun,* 1983; 40-1092-1095.

163. Meibalane R, Sedmark GV, Sasidharan P, Garg P, Grausz JP. Outbreak of influenza in a neonatal intensive care unit. *J Pediatr*, 1977; 9:974-976.

164. Beigel IH, Farrar J, Han AM, et al. Avian influenza A (H5N.1-}infection in humans. *N Engl j Med,* 2005;353: 1374-1385.

165. Louria DB, Blumenfeld HL, Ellis JT, Kilbourne EB, Rogers DE. Studies on influenza in the pandemic of 1957-1958. II. Pulmonary complications of influenza. *J Clin Invest,* 1959; 38:213-265.

166. Martin CM, Kunin CM, Gottlieb LS, Barnes MW, Liu C, Finland M.Asian influenza-associated fatal cases. *AMA Arch Intern Med* ,1959; 103:515-531.

167. Goodpasture EW. The significance of certain pulmonary lesions in relation to the etiology of influenza. *Am J Med Sci,* 1919; 158:863-870.

168. Hien TT, de Jong M, Farrar J.Avian influenza – a challenge no global health care structures. *N Engl J Med,* 2004; 351:2363-2365.

169. Schwarzmann SW, Adler JL, Sullivan RJ Jr, Marine WM, Bacterial pneumonia during the Hong Kong influenza epidemic of 1968-1969. *Arch Intern Med,* 1971; 127: 1037-1041.

170. Podewillis LJ, Liedtke LA, McDonald LC, et. al., A national survey of severe influenza –associated complications among children and adults , 2003-2004. Clin Infect Dis, 2005; 40: 1693-1696.

171. Naficy K.Human influenza infection with proved viremia. Re-port of a case. *N Engl J Med,* 1963; 269:964-966.

172. Lehmann NI, Gust ID. Viraemia in influenza. A report of two cases, *Med J Aust* , 1971; 2:1166-1169.

173. Hurwitz ES, Nelson DB, Davis C, Morens D, Schonberger L.B. National surveillance for Reye syndrome : a five-year review : P*ediatrics*, 1982; 70-895-90.

174. Partin JC, Schubert WK, Partin JS. Mitochondrial ultrastructure in Reye's syndrome (encephalopathy and fatty degeneration of the viscera). *N Engl J Med*, 1971; 285: 1339-1343.

175. Hayase Y, Tobita K.Influenza virus and neurological diseases. *Psychiatry Clin Neurosci*, 1997;51:181-184.

176. Flewett TH, Hoult JG. Influenza encephalopathy and postinfluenza encephalitis. *Lancet*, 1958 ; 2:11-15.

177. Delorme L, Middleton Pj.Influenza A Virus associated with acute encephalopathy. *Am J Dis Child*, 1979; 133:822-284.

178. Weitkamp JH, Spring MD, Brogan T, Moses H, Block KC, Wright PF. Influenza A virus – associated acute necrotizing encephalopathy in the United States. *Pediatr Infect Dis J*, 2004;23:L259-263.

179. Ravenholt RT, Foege WH. 1918 influenza, encephalitis lethargica, Parkinsonism. *Lancet* 1982; 2:860-864.

180. MacDonald KL, Oserholm MT, Hedberg CW, et al. Toxic shock syndrome. A newly recognized complication of influenza and influenza like illness. *JAMA*, 1987; 257:1053-1058.

181. Neuzil KM, Reed GW, Mitchel EF, Simonsen L, Griffin MR. Impact of influenza on acute cardiopulmonary hospitalizations in pregnant women. *Am J Epidemiol*, 1998; 148: 1094-1102.

182. Karkinen-Jaaskelainen M, Saxen L. Maternal influenza, drug consumption, and congenital defects of the central nervous system. *Am J Obstet Gynecol*, 1974; 118:815-818.

183. Clark PS, Feltz ET, List-Young B, Ritter DG, Noble GR.An influenza B epidemic within a remote Alaska community. Serologic, epidemiologic, and clinical observations. *JAMA*, 1970;214:507-512

184. Glezen WP, Couch RB, Taber LH, et al. Epidemiologic observations of influenza B virus infections in Houston, Texas, 1976-1977. *Am J Epidemiol*, 1980; 111:13-22.

185. Moriuchi H, Katsushima N, Nishimura H, Nakamura K, Nu-mazaki Y.Community-acquired influenza C Virus infection in children. *J Pediatr*, 1991; 118:235-238.

186. O' Callaghan RJ, Gohd RS, Labat DD. Human antibody to influenza C virus : its age-related distribution and distinction from receptor analogs. *Infect Immun,* 1980; 30:500-505.

187. Dushoff J, Plotkin JB, Viboud C, Earn DJ, Simonsen L. Mortality due to influenza in the United States – an annualized regression approach using multiple-cause mortality data. *Am J Epidemiol,* 2006; 163:181-187

188. Pyhala R., Aho K seroepidemiology of H1N1 influenza: the infection and reinfection rate in winter 1978-79. J –Hyg (London), 1981, 86(1):27-33.

189. Pyhala R., Aho K and Visakorpi Sero epidemiology of H1N1 influenza striking differences in the attack rate among young people. Acta Pathol Microbiol Scand (B), 1979; 87 B (3): 161-4

190. B. Lalitha Rao. Influenza surveillance in Pune (India) during 1983-85. Indian J Med Res, 1987; 85, 492-495

191. Ghendon Y. Update / Le point: Influenza surveillance. Bull world Health Org, 1991;69 : 509-515.

192. Cox NJ, Subbarao K . Global Epidemiology of Influenza Past and Present . Annu-Rev-Med, 2000; 51: 407-21.

193. Singh J, Khare S, Prasad AK, Garg A, Singh NP, Sharma RS. An outbreak of Influenza (AH3N2) in Delhi, 1993. J Commun Dis, 1994; 26:14-18.

194. Cox NJ, Bramer TL, Regnery HL, Influenza: Global surveillance for Epidemic and Pandemic variants. Eur J Epidemiol, 1994;10 (4): 467-70.

195. Anchlan D, Ludwig S and Nya Madawa P. Previous H1N1 Influenza A viruses ciruculating in the Mangolian Population. Arch Virol. 1996; 141 (8): 1553-69.

196. Zhou N., He S and Zhang T Influenza infection in humans and Pigs in southeastern China. Arch Virol. 1996; 141 (3-4): 649-61.

197. Gu _ HX , Yang Zh, Jin-Xy. 1996. An Epidemiological study on influenza pandemic. Chung-Hu-Liu-Hsing-Ping-Hsueh-Tsa-Chih, 1996; 17 (2) 77-9

198. Mizuta K, Oshitani. Epidemiology of Influenza Virus infection in Children with acute respiratory infections in zambia. An-Trop-Paediatr, 1997; 17(2) 155-9.

199. Cameron KR, Brown IH, and Gregory V, (2007). H9N2 subtype Influeza A viruses in Poultry in Pakistan are closely related to the H9N2 viruses responsible for human infection in Hong Kong. Virology, 2000;278 (1): 36-41.

200. Brown IH (2000) Epidemiology and Evoultion of Influenza viruses in pigs. Vet Microbiol, 2000; 74 (1-2): 29-46.

201. Zhirnov, Kizler MR, Wright PI'. Cleavage of influenza A virus hemagglutinin in human respiratory epithelium is cell associated and sensitive to exogenous antiproteases. *J Virol*, 2002;76:8682-8689

202. Fox JP, Cooney MK, Hall CE, Foy HM. Influenza virus infections in Seattle *families*, 1975-1979.11. Pattern of infection in invaded households and relation of age and prior antibody to occurrence of infection and related illness. Am J *Epidemiol* 1982; 116: 228-242.

203. Brown TA, Murphy BR, Radl I. Haaijman JI. Mestecky I. Subclass distribution and molecular form of immunoglobulin A hemagglutinin

antibodies in *sera* and nasal secretions after experimental secondary infection with influenza A virus in humans. *Microbial, 1985;22:259-264.*

204. Murphy BR, Clements ML. The systemic and mucosal immune response of humans to influenza A virus. *Trop Microbiol lmmunoI, 1989;146:107-116.*

205. Callard RE. Specific in vit*ro* antibody response to influenza virus by human blood lymphocytes. *Nature], 979;282:734-736.*

206. Yamane N, Odagiri T and Arikava J. Reverse single radial immunodiffusion test: The method for the assay of the antibodies to Influenza A nucleoprotein. Tohoku J Exp Med, 1981; 133 (3): 245-55.

207. Pyhala R. Aho K, Egg grown and tissue culture grown Influenza A/H3N2 virus with special attention to their use as antigen in sero epidemiology. Epidemiol Infect, 1987, 99 (3): 745-53.

208. Nelson JK, Shields MD., and Stewart MC. Investigation of sero prevalence of respiratory virus infection in an infant population with multi antigen fluorescence immuno assay using heal prick blood sample collected on filter paper. Paediatr Res,1999; 45 (6): 799-802.

209. Iwane MK, Edwards KM, Szilagyi PG, et a!. Population-based surveillance for hospitalizations associated with respiratory syncytial virus, influenza virus, and parainfluenza viruses among young childr:en. *Pediatrics, 2004;113:1758-1764.*

210. Matsuzaki Y . Detection of influenza virus by RT-PCR Nippon Rinsho, 2000; Nov:58(11):2224-8.

211. Matsuzaki Y . Detection of influenza virus RT-PCR assay and others Nippon Rinsho., 2003; Nov:61(11):1909-13.

212. Matsuzaki Y .Detection of Respiratory pathogens by Multiplex RT-PCR. Nippon Rinsho, 2004; Jun:63(11):1206-11.

213. J. Stockton, J. S. Ellis, M. Saville, J. P. Clewley, and M. C. Zambon Multiplex PCR for Typing and Subtyping Influenza and Respiratory Syncytial Viruses Journal of Clinical Microbiology, Oct. 1998; . 2990–2995.

214. Daum, L. T., L. C. Canas, C. B. Smith, A. Klimov, W. Huff, W. Barnes, and K. L. Lohman. Genetic and antigenic analysis of the first A/New Caledonia/20/99-like H1N1 influenza isolates reported in the Americas. Emerg. Infect. Dis, 2002; 8:408–412.

215. Varghese IN, Laver WG, Co·lman PM. Structure of the influenza virus glycoprotein antigen neuraminidase at 2.9 A resolution. *Nature, 1983;303: 35-40.*

216. Anonymous. High levels of adamantane resistance among influenza A (H3N2) viruses and interim guidelines for *use* of antiviral agents-United States, 2005-2006 influenza season. *MMWR Morb Mortal Wkl' Rep, 2006;55:44-46.*

217. Hall, C. B., R. Dolin, C. L. Gala, D. M. Markovitz, Y. Q. Zhang, P. H. Madore, et al. 1987. Children with influenza A infection: treatment with rimantadine, Pediatrics 80:275–282.

218. Hayden, F. G., R. B. Belshe, R. D. Clover, A. J. Hay, M. G. Oakes, and W. Soo. Emergence and apparent transmission of rimantadine-resistant influenza A virus in families, N. Engl. J. Med, 1989; 321:1696–1702.

219. Hayden, F. G., S. J. Sperber, R. B. Belshe, R. D. Clover, A. J. Hay, and S. Pyke. Recovery of drug-resistant influenza A virus during therapeutic use of rimantadine. Antimicrob. Agents Chemother, 1991; 35:1741–1747.

220. Holsinger, L. I., D. Nichani, L. H. Pinto, and R. A. Lamb. Influenza A virus M2 ion channel protein: a structure-function analysis. J. Virol, 1994; 68:1551–1563.

221. Pinto, L. H., L. J. Holsinger, and R. A. Lamb.. Influenza virus M2 protein has ion channel activity. Cell, 1992; 69:517–528.

222. Wang, C., K. Takeuchi, L. H. Pinto, and R. A. Lamb. Ion channel activity of influenza A virus M2 protein: characterization of the amantadine block. J. Virol, 1993; 67:5585–5594.

223. Degelau, J., S. K. Somani, S. L. Cooper, D. R. P. Guay, and K. B. Crossley. Amantadine-resistant influenza A in a nursing facility. Arch. Intern. Med, 1992; 152:390–392.

224. Houck, P., M. Hemphill, S. LaCroix, D. Hirsh, and N. Cox. Amantadine resistant influenza in nursing homes. Arch. Intern. Med, 1995; 155:533–537.

225. Klimov, A. J., E. Rocha, F. G. Hayden, P. S. Shult, L. F. Roumillat, and N. J. Cox. Prolonged shedding of amantadine-resistant influenza A viruses by immunodeficient patients: detection by polymerase chain reaction-restriction analysis. J. Infect. Dis, 1995; 172:1352–1355.

226. Mast, E. E., M. W. Harmon, S. Gravenstein, S. P. Wu, N. H. Arden, R. Circo, G. Tyszka, A. P. Kendal, and J. P. Davis. Emergence and possible transmission of amantadine-resistant viruses during nursing home outbreaks of influenza A (H3N2). Am. J. Epidemiol, 1991; 134:988–997.

227. Masuda, H., H. Suzuki, H. Oshitani, R. Saito, S. Kawasaki, M. Nishikawa, and H. Satoh. Incidence of amantadine-resistant influenza A viruses in sentinel surveillance sites and nursing homes in Niigata, Japan. Microbiol. Immunol, 2000; 44:833–839.

228. Von Zstein M, Wu WY, Kok GB, et al. Rational design of potent sialidase-based inhibitors of influenza virus replication. *Nature,* 1993;363:418-423.

229. Harper SA, Fukuda K Uyeki TM, Cox NI. Bridges CB. Prevention and control of influenza. Recommendations of the Advisory Committee on Immunization Practices (ACIP). *MMWR Recolllm Rep* -2005;54: 1-40.

230. Gubareva LV, Kaiser L, Hayden FG. Influenza virus neuraminidase inhibitors. *Lancet, 2000 ;355:827-835.*

231. Subhuti Dharmanada. Viral myocarditis, Coxsackie virus infections. Director, Institute for Traditional Medicine, Portland, Oregon. Viral Myocarditis Coxsackie Virus Infec.html.

232. Norbert D. Weber, Douglass O. Andersen, James A .North, Byron K. Murray, Larry D. Lawson and Bronwyn G. Hughes. *In vitro* virucidal effects of *Allium stivum* (garlic) extracts and compounds. Planta Med., 1992; 58: 417-423.

233. Margaretha Leven, Dirk A., Van den Berghe and Arnold J.Vlietinck. Influence of Lycorine on growth pattern of three animal viruses. Planta Medica, 1983; 49: 109-114.

234. www.chennaiayurveda.com/herbs/tag/keelanelli

235. www.jnanadhara.cmredu.com/Herbalmint.doc

236. www.indiastudychannel.com/resources/42566-DISEASES-THROUGH-DRINKING-WATER.aspx

237. www.dhanvantri.com/Turmeric_Curcumin.htm

238. www.usingneem.com/ANTIVIRAL.pdf

239. Parida M.M., Pandya G., Bhargava R and Jana A.M. Assessment of *in vitro* antiviral activity of certain indigenous plants against Polio type–3 virus. Indian Journal of Virology, 1997; 13(2): 101-105.

240. Yen Tsai., Linda L. Cole., Larry E. Davis., Steven J. Lockwood. Antiviral Properties of Garlic : Invitro effects on Influenza B, Herpes Simplex and Coxsackie B viruses. Planta Medica., 1985; 55: 460-461.

241. Lalita Badam., Joshi S.P and Bedekar S.S. *In vitro* antiviral activity of Neem (*Azadirachta indica A.Juss*) leaf extracts against group B Coxsackie viruses. J. Communicative Disease, 1999; 31(2): 79-90.

242. Lee C.D., Ott M., Thyagarajan S.P., Shafritz D.A., Burk R.D and Gupta S. *Phyllanthus amarus* downregulates Hepatitis B virus mRNA transcription and replication. Eur. J. Clin. Invest, 1996; 26: 1069-1076.

243. Premnathan M., Kathiresan K., Chandra K and Bajpai S.K. Antiviral activity of marine plants against New Castle disease virus. Tropical Biomedicina, 1993; 10: 31-33.

244. Premnathan M., Kathiresan K and Chandra K. Antiviral activity of marine plants from India. Ind. J. Pharmacog, 1994; 32: 1-7.

245. Suganda A.G., Amoros M., Girre L. and Fauconnier B. Inhibitor effects of crude and semipurified extracts of native French plants on the multiplication of Herpes simplex 1 and Polio virus 2 in cell culture. Journal of Natural Products, 1983; 46(5): 626-632.

246. Husson G.P., Vilagines P., Sarrette B. and Vilagines R. Antiviral activity of Amaryllidceae extract over three enteroviruses. Plantes Medicinales et Phytotherapie, 1991; 25: 89-99.

247. Robertson IS, Nicolson C, Newman R, Major D, Dunleavy U, Wood JM. High growth reassortant influenza vaccine viruses: new approaches to their control. *Biologicals, 1992;20:213-220.*

248. Neumann G, Fujii K Kino Y, Kawaoka Y. An improved reverse genetics system for influenza A virus generation and its implications for vaccine production. *Proc Nat/ Acad Sci, USA,* 2005;102: 16825- 16829.

249. Neumann G, Watanabe T, Ito H, et aI. Generation of influenza A viruses entirely from cloned cDNAs, *Proc Natl Acad Sci USA,* ,1999; 96:9345--9350.

250. Nicolson C, Major , Wood JM, Robertson IS. Generation of influenza vaccine viruses on Vero cells by reverse genetics: an H5N 1 candidate vaccine strain produced under a quality system. *Vaccine, 2005;23:2943-2952.*

251. Lakey DL, Treanor IJ, Betts RF, et al. Recombinant baculovirus influenza A hemagglutinin vaccines are well tolerated and immonogenic in healthy adults. *J Infect Dis,* 1996; 174:838-841.

252. Johansson PRo Feldman S, Thompson JM, Mahoney *JD,* Wright PF. Immunity to influenza A virus infection in young children: a comparison of

natural infection, live cold-adapted vaccine, and inactivated vaccine. J *Infeet Dis, 1986;154:121-127.*

253. Ogra PL, Chow T. Beutner KR, et al. Clinical and immunologic evaluation of neuraminidase-specific inOuenza A virus vaccine in humans. *I Infect Dis,* 1977; 135:499-506.

254. Suarez DL, Lee CW, Swayne DE. Avian influenza vaccination in North America: strategies and difficulties. *Dev Biol Basel,* 2006;124:117-124.

255. Williams SP, Robertson IS. Analysis of the restriction to the growth of nonegg-adapted human influenza virus in eggs. *Virology,* 1993; 196:660-665.

256. Katz JM, Naeve CW, Webster RG, Host cell mediated variation in H3N2 influenza viruses. *Virology,* 1987; 156:386-395

257. Newman RW, Jennings R, Major DL, et aI. Immune response of human volunteers and animals to vaccination with egg-grown influenza A (HIN1) virus is influenced by three amino acid substitutions in the haemagglutinin molecule. *Vaccine, 1993;11:400*406.

258. Murphy KR, Stntnk Re. Safe administration of influenza vaccine In asthmatic children hypersensitive to egg proteins. J *Pediatr,* 1985; 106:931-933.

259. Safranek TL Lawrence DN, Kurland LT, et al. Reassessment of the association between Guillain·Barre syndrome and receipt of swine influenza vaccine in 1976-1977: results of a two-state study. Expert Neurology Group. Am *I Epidemiol, 1991;133:940*-951.

260. Lasky T, Terracciano G). Magder L, et al. The Guillain-Barre syndrome and the 1992-1993 and 1993-1994 influenza vaccines. *N Engl Med, 1998;339:1797-1802*

261. Mostow SR, Schoenbaum SC, Dowdle WR, Coleman MT, Kaye HS, Hierholzer Ie. Studies on inactivated influenza vaccines. II Effea'or increasing dosage'" on ~.antibody rp<f'on~pand ildvprsp reactions in man. *Am I EpidemioI, 1970;92:248-256.*

262. Palache AM, Beyer WE, Luchters G, Volker R, Sprenger ML Masurel N. In.fluenza vaccines: the effect of vaccine dose on antibody response in primed populations during the ongoing interpandemic period. A review of the literature. *Vaccine,* 1993; 11 :892-908 .

263. Kendal AP, Noble GR. Dowdle WR. Neuraminidase content of influenza vaccines and neuraminidase antibody responses after vaccination of immunologically primed and unprimed populations. *J Infect Dis,* 1977;136(suppl):S415-S424.

264. Palache AM, Beyer WE, Sprenger ML et al. Antibody response after influenza immunization with various vaccine doses: a double-blind, placebo-controlled, multi-centre, dose-response study in elderly nursing-home residents and young volunteers. *Vaccine,* 1993; II :3-9.

265. Mostow SR, Schoenbaum SC, Dowdle WR, Coleman MT, Kaye HS 1naaivated vaccines. Volunteer studies with very high doses of influenza vaccine purified by zonal ultracentrifugation. *Postgrad Med J, 1973;49:152-158.*

266. Wenzel RP, Hendley, Sande MA, Gwaltney JM, Jr. Revised (1972-1973) bivalent influenza vaccine. Serum and nasal antibody responses to parenteral vaccination. *JAMA, 1973;226:435*438.

267. Boyce TG, Hsu HH, Sannella EC, et al. Safety and immunogenicity of adjuvanted and unadjuvanted subunit Influenza vaccines administered intranasally to healthy adults. *Vaccine* 2000; 19:217-226.

268. Edwards KM, Dupont WD, WestrichMK, PlummerWD Jr, Palmer PS Wright PF. A randomized controlled trial of cold-adapted and inactivated vaccines for the prevention of influenza A disease. *J Infect Dis, 1994;169:68-76.*

269. Feery BJ. Evered MG, Morrison EI. Different protection rates in various groups of volunteers given subunit influenza virus vaccine in 1976. *J Infect Dis, 1979;139:237-241.*

270. Jennings LC, MacDiarmid RD, Miles JA A study of acute respiratory disease in the community of Port Chalmers. III. Efficacy of influenza virus subunit vaccines in 1973, 1974 and 1975. *N* Z *Med* J, 1980;92:230-233.

271. Meiklejoh NG, Eickhoff TC, Graves PJ. Antigenic drift and efficacy of influenza virus vaccines, 1976-1977. *I Infect Dis*, 1978; 138:618-624.

272. Heikkinen T, Ruuskanen, Waris M, ZieglerT Arola M, Halonen . P. Influenza vaccination in the prevention of acute otitis media in children . Am J *Dis Child, 1991;145:445-:448*

273. Hoskins TW, Davies JR, Smith AI. Miller CL, Allchin A. Assessment of mactivated influenza-A vaccine after three outbreaks of influenza A at Christ's Hospital. *Lancet, 1979;1:33-35.*

274. Edwards KM, DupontWD, WestrichMK, PlummerWD Jr, Palmer PS Wright PF. A randomized controlled trial of cold-adapted and inactivated vaccines for the prevention of influenza A disease. *J Infect Dis, 1994;169:68-76.*

275. Harper SA, Fukuda K Uyeki TM, Cox NI. Bridges CB. Prevention and control of influenza. Recommendations of the Advisory Committee on Immunization Practices (ACIP). *MMWR* - 2005;54: 1-40.

276. Belshe RB, Mendelman PM, Treanor I. et al. *The* efficacy of live attenuated, cold-adapted, trivalent, intranasal influenza virus vaccine in children. *N Engl J Med, 1998;338:405-]412.*

277. Nichol KL, Mendelman PM, Mallon KP, et.al., Effectiveness of live attenuated intranasal influenza virus vaccine in healthy , working adults: a randomed controlled trial . JAMA, 1999; 282: 137-144.

278. Piedra PA, Gaglani ML, Kozinetz CA., Herd immunity in adults against influenza –related illnesses with use of trivalent –live attenuated influenza vaccine (CAIV-T) in children. Vaccine, 2005; 23: 1540-1548.

279. Piedra PA, Gaglani ML, Riggs M, et. Al., Live attenuated influenza vaccine, trivalent, is safe in healthy children 18 months to 4 years, 5 to 9 years, and 10

to 18 years of age in a community based, non-randomized, open-label trial. Paediatrics, 2005; 116:397-407.

280. Boyce TG, Gruber WC, Coleman-Dockery SO, *et* al. Mucosal immune response to trivalent live attenuated intranasal influenza vaccine in children. *Vaccine, 1999;18:82-88.*

281. Murphy BR. Use of live attenuated cold-adapted infuenza A reassortant virus vaccines in infants, children, young adults, and elderly adults. *Infect Dis Clin Pract, 1993;2:174-181.*

282. Kendal AP. Cold-adapted live attenuated influenza vaccines developed in Russia: can they contribute to meeting the needs for influenza control in other countries? *Eur J Epidemiol*, 1997;13:591 609.

283. Belshe RB, Mendelman PM, Treanor I. et al. *The* efficacy of live attenuated, cold-adapted, trivalent, intranasal influenza virus vaccine in children. *N Engl JMed, 1998;338:405-]412.*

284. Treanor JJ, Mattison HR, Dumyati G, et. al., Protective efficacy of combined live intranasal and inactivated influenza A virus vaccines in the elderly. Ann Intern Med, 1992; 117: 625-633.

285. Falcon AM, Fernandez-Sesma A, Nakaya Y, Moran TM, Ortin J Garcia-Sastre A. Attenuation and immunogenicity in mice of temperature-sensitive influenza viruses expressing truncated NS 1 proteins. *J Gen Virol, 2005;86:2817-2821.*

286. Neirynck S, Deroo T, Saelens X, Vanlandschoot P, Jou WM, Fiers W. A universal influenza A vaccine based on the extracellular domain of the 'M' protein. *Nat Med J,* 1999;5: 1157-1163.

287. Rao BL. Epidemiology and control of Influenza . National Medical journal of India, 2003; 16: 3: 143-149.

288. Veerarhagavan N. Influenza virus isolation at the Government of India influenza center, Coonoor during 1950-1960. Bull. World Health organ, 1961; 24: 679-686.

289. Kendal, A.P., Joseph, J.M., Kobayashi, G., Nelson, D., Reyes, C.R., Ross et al., Laboratory-based surveillance of influenza virus in the United States during the winter of 1977–1978. Periods of prevalence of H1N1 and H3N2 influenza A strains, their relative rates of isolation. in different age groups, and detection of antigenic variants. Am. J. Epidemiol, 1980; 110, 449–461.

290. Stuart-Harris, C.H., Schild, G.C., Oxford, J.S., The epidemiology of influenza. In: Influenza: The Viruses and the Disease, 1985; 2nd ed. Edward.

291. Paniker CK. Serological relationships between the neuraminidases in influenza viruses. *] Gen Virol:* 1968;2:385-394.

292. Colman PM, Ward CWo Structure and diversity of influenza virus neuraminidase. *Curr Top Microbio Immunol*, 1985; 114: 177255.

293. PHLS, 2002. WHO announces the isolation of a new strain of influenza A(H1N2), and the vaccine composition for next winter Press release, 6 February 2002. http://www.phls.co.uk/news/bulletins/ 2002/020206id.htm.

294. Xu, X., Rocha, E.P., Regenery, H.L., Kendal, A.P., Cox, N.J.,.Genetic and antigenic analyses of influenza A (H1N1) viruses,1986–1991. Virus Res, 1993; 28, 37–55.

295. Couch, R.B., Kasel, J.A., Glezen, W.P., Cate, T.R., Six, H.R., Taber, L.H.,Frank, A.L., Greenberg, S.B., Zahradnik, J.M., Keitel, W.A., Influenza: its control in persons and populations. J. Infect. Dis, 1986; 153, 431–440.

296. Young, J.F., Palese, P., Evolution of human influenza A viruses in nature: recombination contributes to genetic variation of H1N1 strains. Proc. Natl. Acad. Sci. U.S.A., 1979; 76, 6547–6551.

297. Li, X.S., Zhao, C.Y., Gao, H.M., Zhang, Y.Q., Ishida, M., Kanegae, Y., Endo, A., Nerome, R., Omoe, K., Nerome, K., Origin and evolutionary

characteristics of antigenic reassortant influenza A (H1N2) viruses isolated from man in China. J. Gen. Virol, 1992; 73, 1329–1337.

298. Guo, Y., Xu, X., Cox, N.J., Human influenza A (H1N2) viruses isolated from China. J. Gen. Virol, 1992; 73, 383–388.

299. Nishikawa, F., Sugiyama, T., Direct isolation of H1N2 recombinant virus from a throat swab of a patient simultaneously infected with H1N1 and H3N2 influenza A viruses. J. Clin. Microbiol, 1983; 18, 425–427.

300. World Health organization. Recommended composition of influenza virus vaccines for 2002-2003 seasons. Wkly. Epidemiol. Rec, 2002; 77.

301. Xian Xu., Catherine, B.S., Mungall, B.A., Lindstorm, S.E., Subbarao, K., Cox, N.J., and Klimov 2002. (2002). International circulation of Human influenza A (H1N2) during the 2001-2002 influenza season. J.Infect.Dis., 186, 1490-1493.

302. Gregary.V Bennet.M., Orkan M.H., Al Hajjar, s., Varsano, N., Mendelson, E., Zambon, M., Ellis, J., Hay, A. and Lin, Y.P., Emergence of human influenza AH1N2 reassortant viruses in the human population during 2001. Virology, 2002;300, 1-7.

303. Kawakami, C., Munemura T., Saikusa, M., Noguchi Y., Fuji K., Takaoka M., Saito, T., Nakaya Y., Ito, R., Odagiri, T., Tashiro, M., The first identication of Influenza Viurs A/H1N2 in Japan Yokahama city. Infect. Agents surveillance Rep, 2002; 23, 198 (in Japanese).

304. Aeron C. Hurt, Ian G. Barr, Naomi Komadina, Alan W. Hampson. Novel means of identifying the neuraminidase type of currently circulating human A (H1) influenza viruses. Virus Research, 2004; 103: 79-84.

305. Aymard-Henry, M., Coleman, M.T., Dowdle, W.R., Laver, W.G., Schild,G.C., Webster, R.G., Influenza virus neuraminidase and neuraminidase-inhibition test procedures. Bull. World Health Organ, 1973; 48, 199–202.

306. Fiszon, B., Hannoun, C., Comparison of neuraminidases of the same subtype but from different species using a new method of titration. J. Virol. Methods, 1990; 27, 79–90.

307. Poddar, S.K., Influenza virus types and subtypes detection by single step single tube multiplex reverse transcription-polymerase chain reaction (RT-PCR) and agarose gel electrophoresis. J. Virol. Methods, 2002;99, 63–70.

308. Murphy, B. R., and R. G. Webster. 1996. Orthomyxoviruses, p. 1397–1445. *In* B. N. Fields, D. M. Knipe, P. M. Howley, R. M. Chanock, J. L. Melnick, T. P. Monath, and B. Roizman (ed.), Fields virology, 3rd ed. Lippincott-Raven Publishers, Philadelphia, Pa.

309. Cox, N. J., and C. A. Bender. The molecular epidemiology of influenza viruses. Semin. Virol, 1995; 6:359–370.

310. B Lalitha Rao. Prevalence of antibodies in different age groups to pandemic & epidemic strains of influenza virus. Indian J Med Res, 1984; Oct :80 :390-5.

311. Yeolekar LR, Kulkarni PB, Chadha MS, Rao BL. Seroepidemiology of influenza in Pune, India. Pune, India. Indian J Med Res., 2001;114:121-6.

312. Michael JS, Sridharan G. A pilot study of seroprevalence of influenza virus type A in Vellore, south India. *Indian J Med Res*, 2002; *115* : 173-5.

313. Annick Dosseh, Kader Ndiaye, Andre Spiegel, Monique Sagna, and Christian Mathiot. Epidemiological and Virological Influenza Survey in Dakar, Senegal: 1996–1998. *Am. J. Trop. Med. Hyg.*, 62(5), 2000; pp. 639–643.

314. Shin-Ru Shih *et al*., Laboratory-Based Surveillance and Molecular Epidemiology of Influenza Virus in Taiwan. Journal of Clinical Microbiology, Apr. 2005; p. 1651–1661.

315. Magdzik W, Brydak LB, 1996. Influenza: problem in Poland as elsewhere. *Archivum Immunologiae et Therapiae Experimentalis, 44:* 385–388.

316. KA Fitzner, SM McGhee, AJ Hedley, KF Shortridge Influenza surveillance in Hong Kong: results of a trial Physician Sentinel Programme, *HKMJ 1999;5:87-94.*

317. Anice *et al*. (2008) Anice C. Lowen,[1] John Steel,[1] Samira Mubareka,[1] and Peter Palese[1,2*–] High Temperature (30°C) Blocks Aerosol but Not Contact Transmission of Influenza Virus. Journal of Virology, June 2008, p. 5650-5652, Vol. 82, No. 11.

320. Price DA, Postlethwaite RL Longson M. Influenzavirus A2 infections presenting with febril convulsions and gastrointestinal symptoms in young children. *Clin* Pedialr *(Phila), 1976;15:361*-367.

321. Glezen WP. Considerations of the risk of influenza in children and indications for prophylaxis. *Rev Infect Dis 1980;2:408-420.* Glezen WP Serious morbidity and mortality associated with influenza epidemics *Epidemiol Rev, 1982;4:25-44.*

322. Meibalane R, Sedmak GV, Sasidharan P, Garg P, Grausz JP. Outbreak of influenza in a neonatal Intensive care unit. *J Pediatr,* 1977;91:974-976.

323. Beigel IH, Farrar J, Han AM, et al. Avian influenza A (H5N.1-}infection in humans. *N Engl* J *Med,* 2005;353: 1374-1385.

324. Paisley JW, Bruhn FW, Lauer BA, McIntosh K. Type A2 influenza viral infections in children. Am *J Dis Child, 1978;132:34-36.*

325. William T. Basco, Jr, MD, FAAP. The Underrecognized Burden of Influenza in Young Children. Medscape Pediatrics, 2006;8(2)

326. Arnold S. Monto, MD: Stefan Gravenstein, MD: Michael Elliott, MD:Michael Colopy, PhD: Jo Schweinle, MD. Clinical Signs and Symptoms predicting Influenza Infection. Arch Intern Med, 2000;160, NOV 27.

327. Gale Jurasek. What clinical symptoms predict the flu in children? Respiratory reviews.com, 2004,.9:(6).

328. Marla J. Friedman, DO: Magdy W. Attia, MD. Clinical Predictors of Influenza in Children. Arch Pediatr Adolesc Med, 2004;158:391-394

329. No author cited. World Health Organisation Fact sheet No. 211. Revised march 2003.

330. Chit Ming Wong et al.,. Influenza-Associated Hospitalization in a Subtropical City. PLoS Med, 2006; 3(4): e121.

331. Smeeth L, Thomas SL, Hall AJ, Hubbard R, Farrington P, et al. Risk of myocardial infarction and stroke after acute infection or vaccination. N Engl J Med, 2004; 351: 2611–2618.

332. Palese P, Tobita k, Ueda M, Compans RW, Characterization of temperature sensitive influenza virus mutants defective in neuraminidase. Virology, 1974; 61: 397-410.

333. Reid, A. H., T. G. Fanning, T. A. Janczewski, and J. K. Taubenberger. Characterization of the 1918 Spanish influenza virus neuraminidase gene.Proc. Natl. Acad. Sci, 2000; USA 97:6785–6790.

334. Bush, R. M., C. A. Bender, K. Subbarao, N. J. Cox, and W. M. Fitch. Predicting the evolution of human influenza A. Science, 1999; 286:1921–1925.

335. Holmes, E. C. et al., Whole-genome analysis of human influenza A virus revealsmultiple persistent lineages and reassortment among recent H3N2 viruses. PLoS Biol, 2005 ; 3:1579–1588.

336. Centers for Disease Control and Prevention. Update: influenza activity—United States and worldwide, 1999–2000 season, and composition of the 2000–01 influenza vaccine. Morb. Mortal. Wkly. Rep, 2000;49:375–381.

337. Centers for Disease Control and Prevention. Update: influenza activity—United States and worldwide, 2000–2001 season, and composition of the 2001–02 influenza vaccine. Morb. Mortal. Wkly. Rep, 2001; 50:466–470.

338. Centers for Disease Control and Prevention. Update: influenza activity—United States and worldwide, 2001–2002 season, and composition of the 2002–03 influenza vaccine. Morb. Mortal. Wkly. Rep, 2002; 51:503–506.

339. Centers for Disease Control and Prevention. Update: influenza activity—United States and worldwide, 2002–2003 season, and composition of the 2003–04 influenza vaccine. Morb. Mortal. Wkly. Rep, 2003; 52:516–521.

340. Centers for Disease Control and Prevention. Update: influenza activity—United States and worldwide, 2003–2004 season, and composition of the 2004–05 influenza vaccine. Morb. Mortal. Wkly. Rep, 2004; 53:547–552.

341. Centers for Disease Control and Prevention. Update: influenza activity—United States and worldwide, 2004–2005 season, and composition of the 2005–06 influenza vaccine. Morb. Mortal. Wkly. Rep, 2005; 54:631–634.

342. Centers for Disease Control and Prevention. Update: influenza activity—United States and worldwide, 2005–2006 season, and composition of the 2006–07 influenza vaccine. Morb. Mortal. Wkly. Rep, 2006;55:648–653.

343. Centers for Disease Control and Prevention. Update: influenza activity—United States and worldwide, 2006–2007 season, and composition of the 2007–08 influenza vaccine. Morb. Mortal. Wkly. Rep, 2007; 56:789–794.

344. Daum, L. T., L. C. Canas, C. B. Smith, A. Klimov, W. Huff, W. Barnes, and K. L. Lohman. Genetic and antigenic analysis of the first A/New Caledonia/20/99-like H1N1 influenza isolates reported in the Americas. Emerg. Infect. Dis, 2002; 8:408–412.

345. Caton, A. J., G. G. Brownlee, J. W. Yewdell, and W. Gerhard. The antigenic structure of the influenza virus A/PR/8/34 hemagglutinin (H1 subtype). Cell, 1982;31:417–427.

346. Kilbourne, E. D., C. Smith, I. Brett, B. A. Pokorny, B. Johansson, and N.Cox. The total influenza vaccine failure of 1947 revisited: major intrasubtypic antigenic change can explain failure of vaccine in a post-World War II epidemic. Proc. Natl. Acad. Sci USA, 2002;99:10748–10752.

347. Abe, Y., E. Takashita, K. Sugawara, Y. Matsuzaki, Y. Muraki, and S. Hongo. Effect of the addition of oligosaccharides on the biological activities and antigenicity of influenza A/H3N2 virus hemagglutinin. J. Virol, 2004; 78:9605– 9611.

348. Lee, M. S., and J. S. Chen. Predicting antigenic variants of influenza A/H3N2 viruses. Emerg. Infect. Dis, 2004; 10:1385–1390.

349. Lin, Y. P., V. Gregory, M. Bennett, and A. Hay. Recent changes among human influenza viruses. Virus Res. 2004; 103:47–52.

350. Centers for Disease Control and Prevention. Update: influenza activity—United States and worldwide, 2000–01 season, and composition of the 2001–02 influenza vaccine. Morb. Mortal. Wkly. Rep, 2001; 50:466–479.

351. Besselaar, T. G., L. Botha, J. M. McAnerney, and B. D. Schoub. Phylogenetic studies of influenza B viruses isolated in southern Africa: 1998– 2001. Virus Res. 2004; 103:61–66.

352. Pontoriero, A. V., E. G. Baumeister, A. M. Campos, V. L. Savy, Y. P. Lin, and A. Hay. Antigenic and genomic relation between human influenza viruses that circulated in Argentina in the period 1995–1999 and the corresponding vaccine components. J. Clin. Virol, 2003; 28:130–140.

353. Centers for Disease Control and Prevention. Update: influenza activity—United States and worldwide, 1994–95 season, and composition of the1995–96 influenza vaccine. Morb. Mortal. Wkly. Rep, 1995; 44:292–295.8.

354. Centers for Disease Control and Prevention. Update: influenza activity—United States and worldwide, 1995–96 season, and composition of the1996–97 influenza vaccine. Morb. Mortal. Wkly. Rep, 1996; 45:326–329.

355. Centers for Disease Control and Prevention. Update: influenza activity—United States and worldwide, 1996–97 season, and composition of the 1997–98 influenza vaccine. Morb. Mortal. Wkly. Rep, 1997; 46:325–330.

356. Centers for Disease Control and Prevention. Update: influenza activity—United States and worldwide, 1997–98 season, and composition of the 1998–99 influenza vaccine. Morb. Mortal. Wkly. Rep. Morb Mortal Wkly. Rep, 1998; 47:280–284.

357. Centers for Disease Control and Prevention. Update: influenza activity—United States and worldwide, 1998–99 season, and composition of the 1999–2000 influenza vaccine. Morb. Mortal. Wkly. Rep, 1999; 48:374–378.

358. Centers for Disease Control and Prevention. Update: influenza activity—United States and worldwide, 1999–2000 season, and composition of the 2000–01 influenza vaccine. Morb. Mortal. Wkly. Rep, 2000; 49:375–381.

359. Harmon, M. W. Influenza viruses,. *In* E. H. Lennette (ed.), Laboratory diagnosis of viral infections, 2nd ed. Marcel Dekker, New York,N.Y, 1992; 521–522.

360. Hierholzer, J., and R. A. Killington. Virus isolation and quantitation *In* B. W. Mahy and H. O. Kangro (ed.), Virology methods manual. Academic Press, San Diego, Calif, 1996;25–46.

361. Leland, D. S. Concepts of clinical diagnostic virology, *In* E. H. Lennette (ed.), Laboratory diagnosis of viral infections, 2nd ed. Marcel Dekker, New York, N.Y., 1992; p. 25.

362. Lin, Y. P., V. Gregory, M. Bennett, and A. Hay. Recent changes among human influenza viruses. Virus Res, 2004; 03:47–52.

363. Plotkin, J. B., J. Dushoff, and S. A. Levin. Hemagglutinin sequence clusters and the antigenic evolution of influenza A virus. Proc. Natl. Acad. Sci. USA, 2002; 99:6263–6268.

364. B. Schweiger. I. Zadow R. Heckler H. Timm, and G. Pauli. Application of a Fluorogenic PCR Assay for Typing and Subtyping of Influenza Viruses in Respiratory Samples. Journal Of Clinical Microbiology, Apr. 2000, p. 1552–1558.

365. Holger F.Rabenau et al., Rapid detection of enterovirus infection by automated RNA extraction and real-time fluorescence PCR. Journal of Clinical Virology, 2000; 25: 155-164.

366. Merisa Chesky, Rosana Scalco, Luciane Failace, Steven Read and Luiz Fernando Jobim. Polymerase chain reaction for the laboratory diagnosis of aseptic meningitis and encephalitis. Arq. Neurosiquiar, 2000; 58(3-B): 836-842.

367. http://www.protocol-online.org/biology-forums/posts/13593.html

368. Bjorn Herrmann, Christine Larsson, and Benita Wirgart Zweygberg. Simultaneous Detection and Typing of Influenza Viruses A and B by a Nested Reverse Transcription-PCR: Comparison to Virus Isolation and Antigen Detection by Immunofluorescence and Optical Immunoassay (FLU OIA). Journal Of Clinical Microbiology, Jan. 2001, Vol. 39, No. 1p. 134–138.

369. Annie Ruest, Sophie Michaud, Sylvie Deslandes, and Eric H. Frost. Comparison of the Directigen Flu A_B Test, the QuickVue InfluenzaTest, and Clinical Case Definition to Viral Culture and Reverse Transcription-PCR for Rapid Diagnosis of Influenza Virus Infection. Journal Of Clinical Microbiology, Aug. 2003, p. 3487–3493 Vol. 41, No. 8.

370. Kate E. Templeton, Sitha A. Scheltinga, Matthias F. C. Beersma, Aloys C. M. Kroes, and Eric C. J. Claas*. Rapid and Sensitive Method Using Multiplex Real-Time PCR for Diagnosis of Infections by Influenza A and Influenza B Viruses, Respiratory Syncytial Virus, and Parainfluenza Viruses 1, 2, 3, and 4. Journal of Clinical Microbiology, Apr. 2004, Vol. 42, No. 4 p. 1564–1569.

371. J. Stockton, J. S. Ellis, M. Saville, J. P. Clewley, and M. C. Zambon. Multiplex PCR for Typing and Subtyping Influenza and Respiratory Syncytial Viruses. Journal of Clinical Microbiology, Oct. 1998, Vol. 36, No. 10 p. 2990–2995.

372. J. S. Ellis, d. M. Fleming, and M. C. Zambon. Multiplex Reverse Transcription-PCR for Surveillance of Influenza A and B Viruses in England

and Wales in 1995 and 1996. Journal of Clinical Microbiology, Aug. 1997, Vol. 35, No. 8p. 2076–2082.

373. Englund JA. Antiviral therapy of influenza. Semin Pediatr Infect Dis, 2002; 13:120–8.

374. Fox JP, Cooney MK, Hall CE, Foy HM. Influenzavirus infections in Seattle families, 1975–1979. II. Pattern of infection in invaded households and relation of age and prior antibody to occurrence of infection and related illness. Am J Epidemiol, 1982; 116:228–42.

375. Glezen WP, Couch RB. Interpandemic influenza in the Houston area, 1974–76. N Engl J Med, 1978; 298:587–92.

376. Bridges CB, Winquist AG, Fukuda K, Cox NJ, Singleton JA, Strikas RA. Prevention and control of influenza: recommendations of the Advisory Committee on Immunization Practices (ACIP). MMWR Recomm Rep, 2000; 49:1–38.

377. Belshe RB, Smith MH, Hall CB, Betts R, Hay AJ. Genetic basis of resistance to rimantadine emerging during treatment of influenza virus infection. J Virol, 1988; 62:1508–12.

378. Dolin R, Reichman RC, Madore HP, Maynard R, Linton PN, Webber-Jones J. A controlled trial of amantadine and rimantadine in the prophylaxis of influenza A infection. N Engl J Med, 1982; 307:580–4.

379. Tominack RL, Hayden FG. Rimantadine hydrochloride and amantadine hydrochloride use in influenza A virus infections. Infect Dis Clin North Am, 1987; 1:459–78.

380. Mould JA, Drury JE, Frings SM, et al. Permeation and activation of the M2 ion channel of influenza A virus. J Biol Chem 2000; 275:31038–50.

381. Boivin G, Goyette N, Bernatchez H. Prolonged excretion of amantadine-resistant influenza a virus quasi species after cessation of antiviral therapy in an immunocompromised patient. Clin Infect Dis, 2002; 34: E23–5.

382. Hay AJ, Zambon MC, Wolstenholme AJ, Skehel JJ, Smith MH. Molecular basis of resistance of influenza A viruses to amantadine. J Antimicrob Chemother, 1986; 18:19–29.

383. Hayden FG, Belshe RB, Clover RD, Hay AJ, Oakes MG, Soo W. Emergence and apparent transmission of rimantadine-resistant influenza A virus in families. N Engl J Med, 1989; 321:1696–702.

384. Klimov AI, Rocha E, Hayden FG, Shult PA, Roumillat LF, Cox NJ. Prolonged shedding of amantadine-resistant influenza A viruses by immunodeficient patients: detection by polymerase chain reaction restriction analysis. J Infect Dis, 1995; 172:1352–5.

385. Shiraishi K, Mitamura K, Sakai-Tagawa Y, Goto H, Sugaya N, Kawaoka Y. High frequency of resistant viruses harboring different mutations in amantadine-treated children with influenza. J Infect Dis, 2003; 188: 57–61.

386. Hayden FG. Perspectives on antiviral use during pandemic influenza Philos Trans R Soc Lond B Biol Sci, 2001; 356:1877–84.

387. Belshe RB, Burk B, Newman F, Cerruti RL, Sim IS. Resistance of influenza A virus to amantadine and rimantadine: results of one decade of surveillance. J Infect Dis, 1989; 159:430–5.

388. Ziegler T, Hemphill ML, Ziegler ML, et al. Low incidence of rimantadine resistance in field isolates of influenza A viruses. J Infect Dis, 1999; 180: 935–9.

389. Bright RA, Medina MJ, Xu X, et al. Incidence of adamantane resistance among influenza A (H3N2) viruses isolated worldwide from 1994 to 2005; a cause for concern. Lancet, 2005; 366:1175–81.

390. Bright RA, Shay DK, Shu B, Cox NJ, Klimov AI. Adamantane resistance among influenza A viruses isolated early during the 2005–2006 influenza season in the United States. JAMA, 2006; 295:891–4.

391. Bright RA, Shay D, Breese J, Klimov A, Cox N, Ortiz JR. High Levels of adamantane resistance among influenza A (H3N2) viruses and interim guidelines for use antiviral agents: United States, 2005–2006 influenza season. MMWR Morb Mortal Wkly Rep, 2006; 55:44–6.

392. I.T. Prud'homme, O.Zoueva, J.M.Weber. Amantadine susceptibility in influenza A virus isolates: determination methods and lack of resistance in a Canadian sample, 1991-1994.Clinical and diagnostic virology, 1997; 8:41-51.

393. Thedi Ziegler,1,a Mark L. Hemphill, Marja-Liisa Ziegler, Gilda Perez-Oronoz, Alexander I. Klimov, Alan W. Hampson, Helen L. Regnery, and Nancy J. Cox. Low Incidence of Rimantadine Resistance in Field Isolates of Influenza A Viruses. The Journal of Infectious Diseases, 1999;180:935–9.

394. Varough M. Deyde, Xiyan Xu, Rick A Bright, Michael Shaw, Catherine B. Smith, Ye Zhang, Yuelong Shu, Larisa V. Gubareva, Nancy J. Cox, and Alexander I. Klimov. Surveillance of Resistance to Adamantanes among Influenza A(H3N2) and A(H1N1) Viruses Isolated Worldwide. The Journal of Infectious Diseases, 2007; 196:249–57.

395. Holsinger, L. I., D. Nichani, L. H. Pinto, and R. A. Lamb. Influenza A virus M2 ion channel protein: a structure-function analysis. J. Virol, 1994; 68:1551–1563.

396. Pinto, L. H., L. J. Holsinger, and R. A. Lamb. Influenza virus M2 protein has ion channel activity. Cell, 1992; 69:517–528.

397. Wang, C., K. Takeuchi, L. H. Pinto, and R. A. Lamb. Ion channel activity of influenza A virus M2 protein: characterization of the amantadine block. J. Virol, 1993; 67:5585–5594.

398. Barr IG, Hurt AC, Iannello P, Tomasov C, Deed N, Komadina N. Increased adamantane resistance in influenza A(H3) viruses in Australia and neighbouring countries in 2005. Antivir Res, 2007; 73:112–7.

399. Saito R, Sakai T, Sato I, et al. Frequency of amantadine-resistant influenza A viruses during two seasons featuring cocirculation of H1N1 and H3N2. J Clin Microbiol, 2003; 41:2164–5.

400. Aoki FY. Amantadine and rimantadine. In: Nicholson KG, Webster RG, Hay AJ, eds. In: Textbook of influenza. Oxford, UK: Blackwell Science, 1998;457–76.

401. Vohora S.B and Mishra G.V. Rational basis of the use of medicinal plants in skin diseases. Indian Drugs, 1998; 35(1): 1-17.

402. Gupta S.K., Jai Prakash and Sushma Srivastava. Validation of traditional claim of Tulsi, *Ocimum sanctum* Linn. as a medicinal plant. Indian Journal of Experimental Biology, 2002; July: 40: 765-773.

403. Denizoit F. and Lang R. Rapid colorimetric assay for cell growth and survival: modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J. Immunol. Methods, 1986; 89: 271-277.

404. Chiba K., Kawakami K. and Tohyama K. Simultaneous evaluation of cell viability by neutral red, MTT and crystal violet staining assays of the same cells. *Toxic. In Vitro*, 1998; 12: 251-258.

405. Yan-Yi Wang and Xiao-Xiang Zheng. A flowcytometry-based assay for quantitative analysis of cellular proliferation and cytotoxicity *in vitro*. Journal of Immunological Methods, 2002; 268: 179-188.

406. Surender Singh., Majumdar D. K and Yadav R.M. Chemical and pharmacological studies on fixed oil of *Ocimum sanctum.* Indian Journal of Experimental Biology, 1996, Dec: 34: 1212-1215.

407. Fiore C, Eisenhut M, Krausse R, Ragazzi E., Pellati D., Armanini D., Bielenberg J., Antiviral effects of *Glycyrrhiza* species. *Phytotherapy Research*, Sep 2007.

408. Harada S., The broad antiviral agent Glycyrrhizin directly modulates the fluidity of plasma membrane and HIV -1 envelope . *Biochem. J*, 2005; 392: 191 -199.

409. Utsunomiya T., Kobagashi M., Pollard B.R., Suzuki F., Glycyrrhizin, an active component of Licorice roots, reduced morbidity and mortaility of mice infected with lethal doses of Influenza virus. *Antimicrobial agents and chemotherapy*, 1997; 551 - 556.

410. Thyagarajan S.P. and Jayam S. Natural history of *Phyllanthus amarus* in the treatment of Hepatitis B. Indian Journal of Medical Microbiology, 1992; 10: 64-80.