Isolation, Identification and Characterization of Influenza Viruses from Upper Respiratory Tract Infections (URTI)

A Dissertation submitted to the

Department of Biological Sciences, Quaid-i-Azam University

Islamabad, Pakistan

In partial fulfillment of the requirements for the Degree of

Master of Philosophy (M. Phil) in Microbiology

By

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2001

IN THE NAME OF ALLAH, THE COMPASSIONATE,
THE MERCIFUL.

To my parents. . .for their endless efforts.

CERTIFICATE

The Department of Biological Sciences, Quaid-i-Azam University Islamabad, accepts this Dissertation by Anis Ahmad Khan in its present form as satisfying the Dissertation requirements for the Degree of Master of Philosophy in Biology (Microbiology).

Internal Examiner: _____

External Examiner: _____

Chairman: _____

Dated: 01 / 09 /2001

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ACKNOWLEDGEMENTS

I am extremely thankful to Almighty Allah for all His blessings and guidance in my life and all respects to His Holy Prophet Muhammad (PBUH) who taught us eternal truth.

My sincere attributes and heartiest obligations are for my supervisor Dr. A. Hameed whose guidance, patience and kind attitude has helped me during my whole M. Phil. Thanks also due to Dr. Safia Ahmed for her candid and cooperative attitude during my education era at Campus.

I would like to thank Dr. Maqbool Ahmad, Chairman of the Biology Deptt. for his consistent encouragement and in allowing me to take my research project at NIH.

I am also thankful to Dr. Athar Saeed Dil executive director NIH and Dr. Burgees Mazhar Qazi, chief PHD, for allowing to me work at NIH and providing me necessary lab. facilities.

I feel great pleasure in expressing my heartiest obligation to S. Sohail Z. Zaidi, Head of Virology (NIH), as he provided me every guidance and facilities, which I needed during my research work. Additional thanks to Dr. Fazl-e- Subhan for all assistance he has given me at NIH.

I am especially thankful to Dr. Humayun Asghar and Dr. Zarina Fazli for their moral guidance and valuable help in my research.

Acknowledgements are also for my friends and lab. fellows at NIH and at campus for their valuable suggestions and assistance during my M. Phil.

At last but not the least, I acknowledge my efforts to the patients whom participation in the study helped me to prepare this text.

My due regards and thanks are reserved for my brothers and sisters whose caring and loving attitude helped me a lot to get through the miseries and never let me track out at any brim of my life.

Anis Ahmad Khan

TABLES AND FIGURES

ABBREVIATIONS

AIIMS	All India Institute of Medical Sciences
ALRTI	Acute lower respiratory tract infection
AR	Allergic rhinitis
ARI	Acute respiratory infection
AURTI	Acute upper respiratory tract infection
BHK	Baby hamster kidney cells
CK	Chicken kidney cell
CNS	Central nervous system
CPE	Cytopathic effect
DIF	Direct immunofluorescence assay
DMSO	Dimethyl Sulfoxid
DNA	Deoxy ribo nucleic acid
EIA	Enzyme-linked immuno-assay
EIA-DB	Dot-blot EIA
ESK	Embryonic swine kindney cells
FGSH	Federal Government Services Hospital
H	Hemagglutinin
HA	Hemagglutination
HAD	Hemadsorption
Hep-2	Human epithelial carcinoma cells
HI/HAI	Hemagglutination inhibition test
HAU	Hemagglutination units

LLCMK-2	Secondary rhesus monkey kidney cells
MDCK	Madin-Darby canine kidney cells
MEM	Minimal essential medium
mRNA	Messenger RNA
N	Neuraminidase
PAP	Peroxidase-antiperoxidase staining method
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PIMS	Pakistan Institute of Medical Sciences
PIV	Para-influenza virus
RBCs	Red blood cells
RDE	Receptors destroying enzyme
RNA	Ribo Nucleic acid
RSV	Respiratory syncytial viruses
RT-PCR	Reverse transcription polymerase chain reaction
SVC	Shell vial culture
USA	United States of America
WHO	World Health Organization

ABSTRACT

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Acute respiratory infection (ARI) is the major cause of morbidity and mortality throughout the world, especially in young children of developing countries. Majority of the infections are due to viral pathogens of which most common are influenza-, adeno-, para-influenza- and respiratory syncytial viruses (RSV). To find out the viral etiology in ARI cases in Rawalpindi and Islamabad, 80 samples were collected from patients attending the outdoor clinic of Federal Government Services Hospital (FGSH) and indoor patients of Pakistan Institute of Medical Sciences (PIMS) with not more than three days of clinical history of ARI. Male to female ratio was 2:1. Age distribution in children ranged from five weeks to 12 years. Adults/elderly patients ranging in age from 12 to 70 years were also examined for the presence of respiratory viruses. The patients were presenting clinical symptoms like running nose, cough, strider, headache, fever including bronchitis/bronchiolitis and pneumonia. Few patients were also complaining underlying diseases like asthma, gastroenteritis, and cardiovascular and renal diseases. All the samples were inoculated in Madin-Darby Canine Kidney (MDCK), Secondary Rhesus Monkey Kidney (LLCMK-2) and Human Epithelial Carcinoma (Hep-2) cell lines. Of 80 throat swab specimens, 11 (13.75%) produced hemadsorption in MDCK and LLCMK-2 cell lines, which on subsequent sero-typing were identified as influenza virus A/H3N2 type. No adeno-, para-influenza- and RSV viruses were isolated. Five of 11 influenza virus isolates were detected from hospitalized patients (children), demonstrating that influenza might be the leading cause of pediatric hospitalization during winter. High titer [256^{+} Hemagglutination Unit (HAU)] of influenza virus was obtained in MDCK cells than LLCMK-2 cells [128^{+} HAU] in relatively shorter time (<24 hours) in the presence of trypsin. PCR offers an alternate to culture for influenza detection but it does not characterize influenza antigenic variants. For this constantly evolving and re-emerging pathogen, such characterization is important. Viral culture is efficient technique for influenza diagnosis and is the only technique that helps in fully characterization of new variants. The study demonstrates that influenza viruses are important pathogens along other respiratory viruses in ARI in rainy and cold seasons. Effective methods of prophylaxis are needed not only for high-risk patients but also for healthy young children.

INTRODUCTION

INTRODUCTION

The respiratory tract is subject to infection with numerous viruses containing RNA or DNA genomes (Avila, *et al*; 1990), which produce clinical syndromes ranging in severity from simply uncomfortable to life threatening. Most of the morbidity and mortality associated with these infections occur in early childhood throughout the world, especially in developing countries (Denny and Loda, 1986; Hijaji, *et al*; 1996). The incidence rate of acute respiratory tract infections (ARI) in children under five in developing countries, ranging from 3-10 episodes per year (Tupasi, *et al*; 1988; Wafula, *et al*; 1990). An attack rate of 3-7 episodes of ARI/child/year has been reported from India of which, 10-15 % are lower respiratory tract infections (Reddiah and Kapoor, 1988). In developing countries majority of lower respiratory tract infections are due to viral pathogens of which most common are influenza viruses, respiratory syncytial viruses (RSV), para-influenza viruses (PIV) and adenoviruses (Steinhoff, *et al*; 1985; Shann, 1986; Isabella, *et al*; 1990).

Influenza viruses of the family Orthomyxoviridae are enveloped viruses that contain single-stranded RNA of negative polarity (Lamb and Krug, 1996). There are three types of influenza –A, B and C – which are distinguished by serological responses to their internal proteins. The natural hosts for influenza A are aquatic birds, although various mammals, including humans, horses and pigs are also hosts. Influenza B is restricted to humans, although it has been isolated from pigs (Manuguerro, 1993). Influenza C causes a less severe illness than either influenza A and B, more akin to the common cold (Joosting, *et al*; 1968).

The virus membrane contains 400-500 spikes, which are anchored in and also project out of the influenza A or B virus particle. Each of these projections consists of a glycoprotein, either rod-shaped, haemagglutinin (H) or Mushroom-shaped, Neuraminidase (N). The H and N spikes occur in the ratio of about 8:1; the H glycoprotein constitutes about 40 % of the total mass of virus particle (Ruigrok, 1998). The eight-segmented virus genome is transcribed to complementary mRNA by a virus-

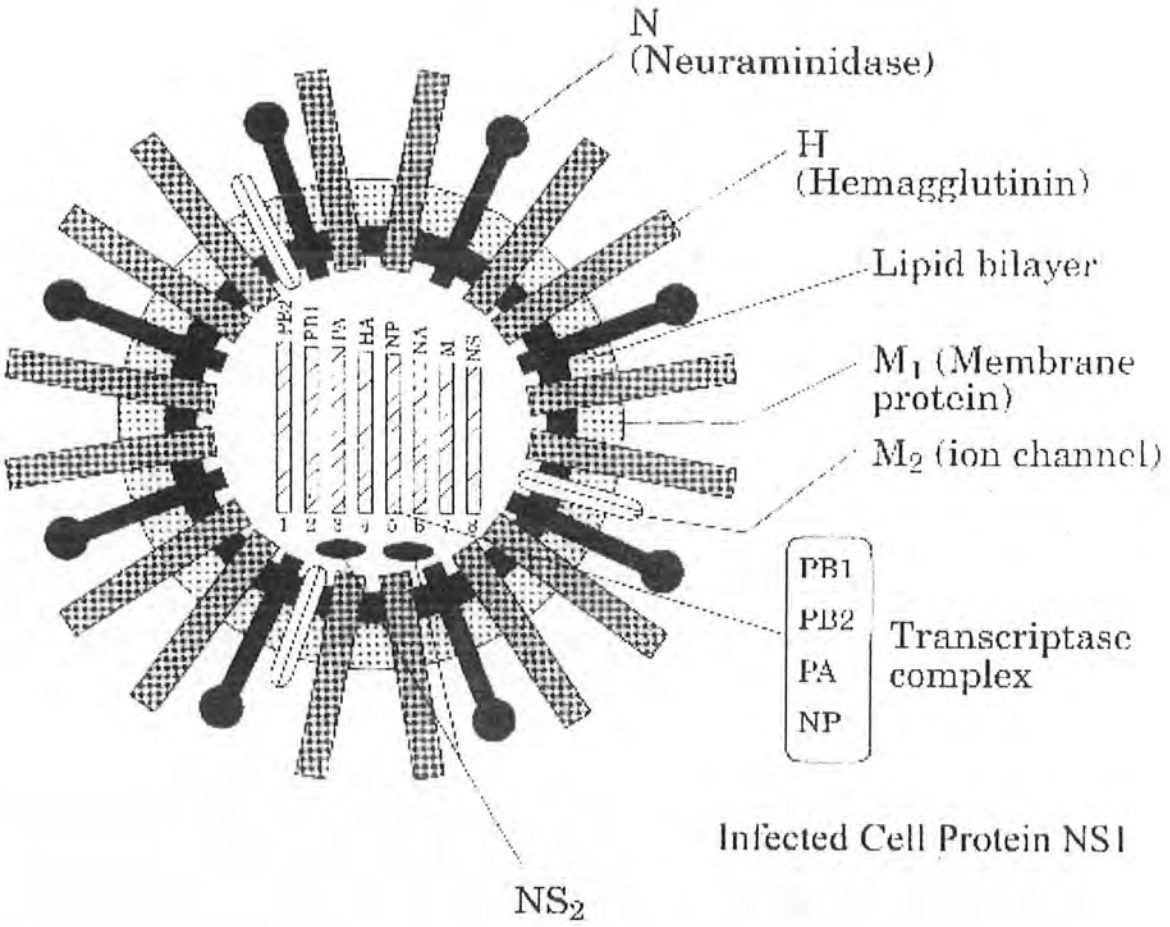


Figure 1. Structure of influenza A virus particle derived from Lamb and Krug, 1996.

associated transcriptase. The segmentation of the RNA is extremely important as it is responsible for genetic reassortment (recombination) resulting in mixed infections with different strains of influenza A viruses (Alexander, 1982). There are fifteen H and nine N subtypes recognized to date, and most of the possible combinations have been isolated from avian species.

The antigenic variability of influenza A takes two forms - antigenic drift and - antigenic shift. Antigenic drift occurs when the genes encoding the viral surface antigen, the N and the H, undergo stepwise mutation potentially each time the virus replicates. Eventually, these proteins on the virus particle become sufficiently different that host antibodies can't neutralize the virus resulting in a variant capable of causing severe illness. Antigenic shift occurs when two different viruses, possibly each from a different host species, co-infect a single host (Shull, 1994). By reassortment of genome segments a new virus is created with an unpredictable pathogenecity, which may lack the requisite virulence factors or may possess full virulence for humans. Such a virus has pandemic potential because it may be intrinsically pathogenic in humans and have surface antigens against which the human population lacks any significant immunity (Zambon, 1999).

Avian influenza strains more frequently establish long-term infection in swine (Pensaert, *et al*; 1981; Scholitissek, *et al*; 1983; Guan, *et al*; 1996). Pigs are sensitive to infection and colonization of human influenza viruses (Tumova, *et al*; 1980; Mancini, *et al*; 1985; Morin, *et al*; 1990), so it is not surprising that reassortments between human and swine have been isolated from pigs (Shu, *et al*; 1994). Until recently, therefore, the consensus was that the pig is the most probable 'genetic mixing vessel', giving birth to pandemic strains (Webster, *et al*; 1992; Scholitissek, *et al*; 1988; Scholitissek, *et al*; 1998). It has been demonstrated that man is also susceptible to infection with swine influenza viruses (de Jong, *et al*; 1986, Claas, *et al*; 1994). Hong Kong H5N1 episode has provided evidence that man himself can also act as a mixing vessel (de Jong, *et al*; 1997; Claas, *et al*; 1998; Yuen, *et al*; 1998).

Influenza pandemics have occurred three times in the present century: in 1918, when subtype H1N1 was introduced, starting in U.S.A ('Spanish Influenza'); in 1957, when

subtype H2N2 emerged in south-east Asia ('Asian Influenza'); and in 1968, when subtype H3N2 established itself in the human population, starting in the same area ('Hong Kong Influenza'), (Potter, 1998). As recently in 1997, the pandemic threat was almost realized in Hong Kong, when an avian subtype (H5N1) of influenza A was found to be associated with serious disease in humans (Claas, *et al*; 1998).

In the years between influenza pandemics, the process of antigenic drift continued to produce epidemics of influenza throughout the world. Influenza epidemics nearly always occur during the winter in temperate climate, although the significance of this is not fully understood. The cold, damp conditions in winter may favor virus survival out side the host airway and there may be behavioral influences, such as people spending longer times together indoors. School children and the children attending day care centers are also thought to play role in the spread of influenza; their activities, sometimes described as 'seeding' the local community. In equatorial climates influenza may not have such sharply defined seasonality (Van Tam, 1998).

Influenza viruses are shed into respiratory secretions, which are then coughed or sneezed into the air and transmitted to the next host. Infection occurs in the cells of bronchial epithelium, where the first cycle of replication takes 4-6 hours. Very high titers of virus are shed during the initial infection period. This combined with the short incubation period, produces the characteristically explosive nature of influenza outbreaks. Estimates of attack rates in outbreaks vary from 10 % to nearly 100 %, depending on type of community, age of individuals, vaccination rate and methods of diagnosis (Van Tam, 1998).

Acute influenza infection is characterized by abrupt onset of symptoms that include: fever (in the region of 38-40°C) or feverishness, chills, cough, headache, myalgia, sore throat, malaise, anorexia, and many other nonspecific symptoms. None of the acute features are pathognomonic but the continuation of respiratory and systemic symptoms, at a time when influenza activity has already been confirmed in the locality, is likely to lead to accurate diagnosis in 60-70 % of medical consultations (Nicholson, 1998).

In patients with preexisting respiratory or cardiovascular disease and in frail elderly influenza infection can be more serious and it may lead to excess or 'hidden' deaths. These deaths are not usually attributed to influenza infection, even though many of them are caused by either viral or bacterial pneumonia; secondary to influenza infection. It is estimated that between 5,000 to 29,000 deaths annually were caused by influenza in the U.K between 1975 and 1990- five times the number directly attributed to influenza on death certificates (Nicholson, 1996).

The exact details of the pathogenicity of influenza viruses infection in humans are still incompletely understood. It is believed that H plays a critical role in the pathogenesis of influenza. The nascent H cleavage at specific regions by host trypsin like enzymes, resulting in two subunits, H1 and H2, which are linked by a disulphide bridge (Chen, *et al*; 1998). The insertion of multiple amino acids near the cleavage site (Kawaoka, *et al*; 1988) make the unsplit H susceptible to cleavage not only by the trypsin like enzymes, but also by other proteolytic enzymes which are universally present, therefore, rendering the virus infectious for many organs, including heart and brain. No insertion was found, however, at the cleavage site of the H1N1 virus (Reid, *et al*; 1999; Webster, 1999).

The explanation of high pathogenecity of H1N1 was found in the N, which was shown to have acquired a lysine residue (Goto and Kawaoka, 1998). This mutation endowed N the ability to bind host plasminogen, which after activation to plasmin, is capable of cleaving H. The 1918 H1N1 virus, however, does not show this mutation (Taubenberger, 1999). Normally N functions as an enzyme that cleaves sialic acid from the oligosaccharide chains anchored in the cell membrane, thus enabling the newly formed influenza virus particles to detach from the host cell membrane.

Natural defense measures, which provide resistance to infection include: (1) the mucus blanket that protects epithelium and the continuous beating of cilia that clears invaders from a healthy respiratory tract, (2) soluble mannose-binding lectins, lung surfactants, and sialylglycoproteins present in the mucus and (3) alveolar macrophages. These natural defense mechanisms may be sub-optimal in aged, the premature, the pregnant, the pulmonary invalid, the immunocompromised or the smoker. Anti-H antibodies may intercept and neutralize infecting virions, if the individual has been infected within the

past few years by a closely related strain of the same influenza H subtype. Secretory IgA is generally believed to be the most relevant antibody in the upper respiratory tract at least, but serum IgG may provide protection in the lung. If pre-existing antibody proves inadequate to block the establishment of infection, recovery is dependant on cell-mediated immunity (Fenner and White, 1994).

Vaccines protect 30-90 % of the vaccinates against complications and deaths from influenza (Gross, *et al*; 1995; Nichol, 1998). The high variability of the virus, time required to prepare new vaccine and also high costs of vaccines limit possibilities to control influenza transmission and outbreaks through vaccination (Leese and Tamblyn, 1998). Research is continued in the development of live influenza vaccines (Keitel and Piedra, 1998) virus vectors (Berglund, *et al*; 1999) and influenza virus DNA vaccines (Webster, 1999).

Antiviral drugs like amantadine and rimantadine (Aoki, 1998) and the recently developed influenza virus neuraminidase inhibitor Zanamivir and oseltamivir (Penn, 1998; Calfee and Hayden, 1998; Patriarca, 1999; Hayden, *et al*; 1999) play a more important role in the control of the disease, because they are generally broadly active against all influenza A subtypes. However, the use of amantadine and rimantadine has been limited because of the considerable frequency of adverse neurological and gastrointestinal effects and the rapid emergence of resistant strains. Zanamivir is only active when treatment is started within 30 hours of the disease onset (Hayden, *et al*; 1997; Monto, *et al*; 1999). Naturally circulating influenza virus variants sensitive to zanamivir have already been found (Woods, *et al*; 1993). These restrictions imply that antivirals can only be valuable adjuncts to vaccines (Osterhaus, and de Jong, 2,000). Moreover, their efficacy is partly dependant on pre-existing antibodies (Aoki, 1998 and Glezen, 1996).

The purpose of the present study is to investigate the local community for acute respiratory tract infections with a major focus on influenza virus isolation and identification. The study will help to know the strains of influenza virus, which are prevalent in Rawalpindi/Islamabad region, so that effective methods of prophylaxis can be developed not only for young and elderly but also for healthy young children.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Infection with influenza viruses can produce a spectrum of clinical responses ranging from febrile upper respiratory illnesses to central nervous system (CNS) involvement. During the 20th century, influenza and pneumonia have accounted, on average, for 44 % of all deaths caused by all of infectious diseases in the USA, with most influenza-related deaths occurring in people with underlying medical conditions or in those over 65 years of age (Armstrong, *et al*; 1999). The 1918 influenza pandemic, one of the most devasting infections disease outbreaks in history, accounted 20-40 million deaths worldwide (Nguyen-Van-Tam, 1998) with disproportionate mortality among children, adolescents, young and middle aged adults, although, relatively, the elderly were spared (HMSO, 1920). After the first influenza virus was isolated in 1933, several examples of influenza-associated encephalopathy have also been reported (Balisteri, 1996).

Epidemiology

Chatterjee, *et al*; 1996, carried out studies on incidences of influenza in the city of Calcutta, India, by virus isolation and strain identification. Over 3,500 patients with acute respiratory infections were examined and 1950 throat swab specimens were inoculated in embryonated chicken eggs. Only 339 hemagglutinating agents were isolated, in which 233 strains were of influenza A virus; 62.66 % of these strains were identified as H3N2, 34.76 % as H1N1 and 0.86 % H2N2. This observation indicated that H3N2 is the major prevalent strain followed by H1N1 strain of influenza A virus in Calcutta.

Chappuis, *et al*; 1996, conducted influenza surveillance in Switzerland for eight consecutive years (1987-1995) and found that influenza A viruses predominated during five winter seasons and influenza B viruses during three winter seasons. New strains detected at the end of the season made it possible to forecast the type and subtype of virus, which would have to predominate the next season. Isolation rates for A/H3N2 viruses were significantly higher in the 10-19 years and 60-plus age groups, whereas there was no statistical difference between age groups for A/H1N1 viruses. A decreasing isolation rate, corresponding to increasing age, was found for influenza B viruses.

Increased mortality was observed in patients over 65 years during the period of high influenza A/H3N2 activity.

Sugaya, *et al*; 1996, conducted a study in the institution for mentally handicapped people in Japan where the mixed epidemic caused by influenza A (H3N2) and B spared none of residents or staff members who were not immunized before. During the influenza outbreak (A/Beiging/32/92-like strain) in January, 37.0 % of the residents and 31.4 % of the staff had an influenza-like illness. During the influenza B outbreak (B/Panama/45/90 and B/Beiging/184/93 like strain) in late February, 59.0 % of the residents and 24.3 % of the staff had an influenza-like illness. They concluded that mixed epidemics probably have a severe impact on institutionalized high-risk people adversely affecting them almost twice as much as influenza epidemics caused by single virus.

Mizuta, *et al*; 1997, carried out a study of acute respiratory tract infections (ARI) in Lusaka, Zambia between 1993-1995. A total of 86 Influenza viruses were isolated from 3,760 throat swab specimens collected from children under 5 years of age, out of which 53 isolates were of influenza A/H3N2 and 34 isolates were of influenza B. The isolation rate of influenza viruses was the highest (14.3 %) in August 1993 as compared to June 1994 (15.1 %) and July 1995 (25.4 %). It was concluded that influenza virus infections are one of the most important pathogens of ARI in children in the cool and dry seasons in Zambia.

Suzuki, *et al*; 1997, studied influenza viruses in Thailand by isolating and comparing their antigenic features with those of Japanese isolates. During 1991 to 1994, thirty-two strains were isolated from 186 throat swab specimens. Twenty-one of thirty-two were of type A, subtype H3N2, and eleven strains were type B. It was found that isolates of type A, subtype H3N2, drifted antigenically from A/Beijing/352/89- like to A/Kitakyusyu/159/93- like variants used as reference strains for comparison. The type B isolates in 1991 were suspected to be antigenically different from those of B/Bangkok/163/90, Thailand, in HI tests. These isolates were similar to B/Mic/1/93, isolated in the later half of the epidemics in Japan in winter 1992/1993. It was concluded that continuous survey of influenza viruses is necessary so that one can understand the factors, which lead to the appearance of new epidemic strains.

Flemming, *et al*; 2,000, provided estimates of the consulting population in England, Wales and in Netherlands, during influenza epidemics between 1987-1996. Most of the people, especially in the age groups between 0-4 and 65 years were associated with influenza A/H3N2 virus infection as compared to influenza B. In the intervening age groups, population estimates were more consistent regardless of the virus type. There was substantial increase in the number of persons reporting other respiratory illnesses during influenza epidemics. It was concluded that population estimates of the consulting population provide the only secure basis for which health services utilization during influenza epidemics can be estimated.

To demonstrate the impact of influenza epidemics on pediatric hospital admissions, a study was conducted at an urban general hospital in Japan during a 4-month period from December to March per year from 1991 to 1998. During the 1997-98 influenza type A (H3N2) epidemic, 26.3 % of the under 16 years old patients were diagnosed as suffering from influenza. During the peak of the epidemic, as many as 50-70 % of the admissions were attributable to influenza type A (H3N2). In the seven winters from 1991 to 1998, 14.0 % of all admissions were associated with influenza viruses' infection (mean age 4.4 years) and 17.5 % were due to RSV. Among the patients with influenza, 74.5 % of the cases were previously healthy children. It was concluded that influenza and RSV infection are leading causes of pediatric hospital admissions during winter. Effective methods of prophylaxis are needed not only for high-risk patients but also for healthy young children (Sugaya, *et al*; 2,000).

Infection and Pathogenecity

Acute asthma is considered a complication of respiratory viral infections. This investigation assessed the effects of influenza A virus infection on both patency and responsiveness of the lower airways. Subjects with allergic rhinitis (AR) and without AR were intranasally inoculated with influenza A virus and monitored for eight days for changes in symptoms, signs and airway physiology. All subjects were infected after inoculation. Significant increase in nasal symptoms and secretion weights were observed, with peak effects on day 3 and 4. Cough was a relatively minor symptom, and none of the subjects developed wheezing. Likewise, there were no effects on allergy

status and no significant changes in the measured functions of lower airways. Under the experimental conditions influenza A virus infection did not produce detectable alternation in lower airway function in health AR and non-AR subjects (Skoner, *et al*; 1996).

Chang, *et al*; 1996, isolated influenza B virus from a throat swab taken from a 2-month-old infant. Infant presented with acute symptoms suggestive of septic shock but recovered after vigorous resuscitation the next day. It was primarily considered as a case of bacterial infection but bacterial cultures of blood, urine and spinal fluid showed no growth. It was concluded that the clinical signs and symptoms of influenza in infants may be indistinguishable from bacterial sepsis.

Everts, *et al;* 1996, described two outbreaks of influenza A that occurred in wards of elderly patients at the princess Margret Hospital in Christchurch. Infected patients and staff were identified clinically by viral antigen detection, culture of throat and naso-pharyngeal swabs or serology. Overall the attack rate of clinical influenza in two wards affected was 48 and 58 %, respectively. Of all the influenza patients, 46 % developed lower respiratory tract infections and there were two deaths (7 %) at least in part attributed to influenza. Patients in single room were not protected from infection. Vaccination rate among patients was 18 %. Immunized patients had a lower attack rate (21%) than those who had not received influenza vaccine that season (40 %). The attack rate among staff in each ward was 69 and 32 %, respectively, and the overall staff vaccination rate was 12 %. It was concluded that these outbreaks have severe consequences of influenza in an institutional setting.

Examination of clinical specimens for diagnosis during a three-year period (August 1993-July 1996) at King Faisal Hospital and Research Center in Riyadh revealed a wide spectrum of diseases associated with the isolation of five respiratory viruses. Severity of disease ranged from mild upper respiratory illness to threatening lower respiratory illnesses including bronchitis and pneumonia. Of the 256 isolates, RSV accounted 28.5%, adenoviruses 27.3 %, influenza 23.8 %, entero viruses 15.5 % and Para-influenza for 2.3%. Viruses were found more frequently in children attending emergency or pediatric wards than in outpatients (Al-Hajjar, *et al*; 1998).

The transmission of avian H5N1 influenza viruses to 18 humans in Hong Kong in 1997 with six deaths established that avian influenza viruses can transmit to and cause lethal infections in humans. The perpetuation of H5N1 influenza viruses in the poultry markets in Hong Kong and the transmission of these viruses to other avian species and mammals including humans emphasize the importance of these markets in the epidemology of influenza (Shortridge, *et al*; 1998).

Yuen, *et al*; 1998, described the clinical presentations of 12 patients, infected with avian influenza A and options for rapid viral diagnosis. Clinical presentation was that of influenza like illness with evidence of pneumonia. Seven patients, older than 13 years, had severe disease (four deaths), whereas children under 5 years had mild symptoms with the exception of one who died with Reye's syndrome associated intake of aspirin. Gastrointestinal manifestations, raised liver enzymes and renal failure were unusually prominent. Factors associated with severe diseases included older age, delay in hospitalization, lower-respiratory tract involvement and a low total peripheral blood cell count or lymphopenia at admission. An H5 specific reverse-transcription PCR assay (RT-PCR) was useful for rapid detection of virus directly in respiratory specimens. A commercially available enzyme immunoassay was more sensitive than direct immuno-fluorescence.

Interspecies Transmission

In May 1997, a young boy died of viral pneumonia and multiorgan failure because of an outbreak of highly pathogenic H5N1 avian influenza on farms in Hon Kong New Territories. Shortridge, *et al*; 1997, working on the virus, highlighted the role of chicken in many live poultry markets as the source of the virus for humans. This perspective from Hong Kong SAR marks the coming-of-age of acceptance of the role of avian hosts as source of pandemic influenza viruses.

Preceding this incident, an avian influenza outbreak of high mortality was reported from three chicken farms in Hong Kong and the virus involved was also found to be H5 subtype. Claas, *et al*; 1998, carried out antigenic and molecular comparison of the influenza A H5N1 virus isolated from boy with the viruses isolated from outbreak.

Differences were observed in the antigenic reactivities of the viruses by hemagglutination inhibition assay. However, nucleotide sequence analysis of all genes showed that human virus A/Hong Kong/156/97 was genetically closely related to the avian A/Chicken/Hong Kong/258/97.Transmission of the virus from infected chickens to child without another intermediate mammalian host acting as "mixing vessel" illustrated the importance of intensive global influenza surveillance.

Avian influenza virus was not known to cause systemic infection in humans before. KU and Chan, 1999, reported a three-year-old boy with good past health who developed pneumonia caused by H5N1 avian influenza A virus (A/Hong Kong/156/97). The virus was isolated from a tracheal aspirate. Patient was also associated with other complications like Reye's syndrome, adult respiratory distress syndrome and multiple organ system failure.

de-Jong, *et al*; 2000, reported that the circulation of numerous influenza virus subtypes in water birds constitutes a continuous threat with respect to emergence of a future influenza pandemic. The interspecies barrier is high but has already been breached three times this century, notably in 1918, 1957, and 1968. Still, it is impossible to predict when another pandemic will occur. It was concluded that continuous and intensive surveillance of influenza in man, pigs, and birds may provide the opportunity to prepare vaccine timely in sufficient amounts.

Laboratory Diagnosis

Influenza virus strains for use as an inactivated vaccine have been successfully grown in different cell lines. Increasing titers were obtained with BHK-21/BRS, VERO and MDCK cells. MDCK cells grown in serum free medium before and during the virus isolation phase was found to yield high titers in the presence of trypsin (Merten, 1996).

Reina, *et al*; 1996, evaluated the efficacy of two commercial rapid methods for antigenic detection. A dot-blot enzyme immunoassay (EIA-DB) [Directigen Flu A, Becton-Dickinson, USA] and a direct immunoassay (DIF) [Monofwokit influenza A, Diagnostic Pasteur, France] were compared with the shell vial culture in the MDCK cell line, incubated 2 to 3 days and stained with monoclonal antibody clone TA-52 for the

diagnosis of lower respiratory tract infection caused by influenza A virus. A statistically significant difference was observed between the sensitivity of the EIA-DB and the DIF methods. In view of the results, the use of EIA-DB as a screening method was recommended when infection by the influenza A is suspected. But to obtain the maximum diagnostic yield, it was suggested that all samples would have to be inoculated in a shell vial culture with the MDCK cell line.

During January-February, 1996, an outbreak of influenza-like illness occurred in Pune. The throat and nasal swabs collected from patients during this outbreak were processed in MDCK and LLCMK2 cell cultures and influenza (H3N2) viruses were isolated. They were identified as being similar to the circulating global strains A/Johannesburg/33/94 and A/Wuhan/359/95 (Rao, *et al*; 1997).

A comparative study of the MDCK, VERO, and MRC-5 cell lines in the isolation of influenza A virus was conducted. Seven hundred and forty six samples were studied, out of which 63 influenza A (IA) viruses were isolated. The MDCK line displayed 100% sensitivity, the VERO and MRC-5 cell lines displayed 71.4 and 57.1% sensitivity, respectively. The quantitative sensitivity analysis showed the MDCK line to be superior to the other lines. It was concluded that the MDCK line is one of the most recommendable for the isolation of the Influenza A virus from respiratory samples (Reina, *et al*; 1997).

Ciappi, *et al*; 1997, reported the isolation of two influenza A (H3N2) virus strains, which were unable, in the first passage in MDCK cell culture, to agglutinate chicken erythrocytes, though reacting with guinea pig and turkey red blood cells. This observation demonstrates that the occurrence of this phenomenon is not exclusive to influenza A (H1N1) viruses. When molecular basis of this phenomenon was investigated by analyzing the nucleotide sequence of the HA-1 region, it was found that the substitution of amino acid 138 (Ala→Ser) in HA-1 region during isolation in MDCK cells is responsible for the change in hemagglutination characteristics.

Ueda, 1998, established a new rapid staining method [peroxidase-antiperoxidase (PAP) with two subtype-specific murine monoclonial antibodies] for identification of influenza A and B virus strains. A total of 160 strains were examined; 158 strains were identified to

be the same by hemagglutination-inhibition (HI) test and the PAP method. In contrast to the results of HI test, two strains revealed to be a mixture of two subtypes of H1 and H3 by the PAP method. Further analysis of clinical specimens was done by PAP method by directly inoculating them into MDCK cells in microplates. After hours of incubation, the types and sub-types of viruses in 52 out of 152 specimens were clearly identified. It was concluded that since the reactivity of the two monoclonal antibodies are not influenced by the antigenic drift of influenza virus, the newly developed method should be applicable not only for rapid diagnosis but also for the epidemiological study of influenza.

Magnard, *et al*; 1999, processed 154 samples collected during the peak of influenza epidemic (1997-1998) in south of France for influenza using antigen detection (ELISA-immunocapture assay), two different nested RT-PCR assays and cell culture. They found that the PCR assay offered an alternate to culture for influenza detection. Nevertheless, culture is efficient for influenza diagnosis and is only technique that allows the reference centers to collect viral strains and characterize fully new variants

Shih, 1999, studied the effectiveness of centrifuge-enhanced shell vial culture (SVC) containing MDCK cells, combined with IF staining in 24 hours. The results showed that this technique rapidly detects and identifies RT viruses than the conventional tube culture system with multiple cell lines would ordinarily detects RV within 3-30 days.

Maitreyi, *et al*; 2,000 studied the role of viruses in acute lower respiratory tract infections (ALRTI) in children during September 1995 to April 1997 attending pediatric clinic of All India Institute of Medical Sciences (AIIMS). Virus isolation was done by centrifugation-enhanced cultures using HEP-2, LLCMK-2 and MDCK cells. The viruses were identified at 24-48 hours post inoculation by IF staining using monoclonal antibodies to RSV, PIV, influenza virus and adenoviruses. Out of 200 specimens, 44.5 % were positive for one or more viral pathogens. RSV was detected in 17 % in all ALRTI cases followed by influenza viruses in 14.5 %, Para-influenza viruses in 11.5 % and adenoviruses 1.5 %. It was concluded that respiratory viruses accounted for 44.5 % of cases of ALRTI in India and the results of viral etiology could be given in 24-48 h using

centrifugation enhanced cultures. RSV was the most common agent associated with ALRTI in children less than 5 years of age with greater association with bronchiolitis.

The susceptibilities of culture cells to 12 avian influenza virus strains were determined with 10 established cell lines including MDCK and ESK cells and three primary culture cells. The established cell lines derived from embryonic swine kidney (ESK) and chicken kidney (CK) primary culture cells were more sensitive to the avian influenza viruses than the other 11 cells. The ESK cells had a particularly higher infective titer than the MDCK cells with and without trypsin supplement in culture medium, and dispersion of the infective titers was narrower than that of the MDCK cell. It was concluded that ESK cell is a suitable candidate for routine work on avian influenza viruses in laboratories (Sugimura, *et al*; 2000).

Control and Treatment

Govaert, *et al*; 1994, conducted a study to determine the immune response to influenza vaccination in elderly people, using a randomized, double blind, placebo-controlled trial. Venous blood was taken from 1838 from people aged 60 years and older, prior to injection with the influenza vaccine or a placebo. A second blood sample was taken three weeks later. The antibody reaction was measured with the percentage of participants who had a protective antibody titer before and after vaccination and for all sera of each strain. A protective antibody titer was found in 43-68 % of those who had received the vaccine, depending on the strain investigated. Patients potentially at risk showed a response similar to the other participants. They concluded that influenza vaccination in elderly people provides a reasonable good immune response.

Atmar, *et al*; 1995, compared a trivalent cold-adapted recombinant (CR) influenza virus vaccine containing types A and B viruses with monovalent vaccines of each virus in a double-blind, placebo-controlled trial. Adults with a wide range of pre-existing antibody titers received one dose intranasally of trivalent vaccine; monovalent A/H1N1, A/H3N2, or B vaccine or placebo. All vaccines were well tolerated. Serum antibody response frequencies and postvaccination geometric mean antibody titers were similar for recipients of trivalent or the corresponding monovalent vaccine for each of the vaccine

components. The antibody responses of trivalent vaccine recipients demonstrated that response to one vaccine virus did not adversely affect the likelihood of response to the other viruses. However, this study failed to find serologic evidence of interference between vaccine viruses, suggesting that trivalent CR influenza virus vaccine may be useful for preventing influenza in adult populations.

A standardized elderberry extract, Sambucol (SAM), reduced hemagglutination and inhibited replication of different human influenza viruses type A and B strains and animal strains in MDCK cells. A placebo-controlled, double blind study was carried out on a group of individuals living in an agricultural community (Kibbutz) during an outbreak of influenza B/Panama in 1993. Fever, feeling of improvement, and complete cure were recorded during 6 days. Sera obtained in the acute and convalescent phases were tested for the presence of antibodies to influenza A, B, respiratory syncytial, and adenoviruses. Higher hemagglutination inhibition (HI) titers to influenza B were observed in the group treated with SAM than in the control group. A significant improvement of the symptoms, including fever, was seen in 93.3 % of the cases in the SAM-treated group within 2 days, whereas in the control group 91.7 % of the patients showed an improvement within 6 days. A complete cure was achieved within 2 to 3 days in nearly 90 % of the SAM-treated group and within at least 6 days in the placebo group. Considering the efficacy of the extract in vitro on all strains of influenza virus tested, the clinical results, its low cost and absence of side-effects, it was concluded that this preparation could offer a possibility for safe treatment for influenza A and B (Zakay-Rones, *et al*; 1995).

Induction of local antibody responses to influenza A virus hemagglutinin by co-administration of two vaccines was investigated. Fifty elderly nursing home residents received inactivated trivalent influenza virus vaccine intra-muscularly and simultaneously were randomized to receive either bivalent live attenuated influenza vaccine or saline placebo intranasally in a blinded fashion. More significant increases in anti –H1 and -H3 IgA antibodies were detectable in nasal wash specimens of subjects who received live attenuated virus vaccine than those who received intranasal placebo. The increased anti-hemagglutinin IgA antibody response was of longer duration in recipients of live attenuated vaccine. The change in antibody titers after vaccination was positively correlated with total blood lymphocyte counts measured before vaccination in both

vaccine groups. A possible advantage of administering live attenuated with inactivated virus vaccines was found because of local antibody responses (Gorse, *et al*; 1996).

Hayden, *et al*; 1997, assessed the therapeutic activity of zanamivir in adults with acute influenza. They conducted separate, randomized, double blind studies in 38 centers in North America and 32 centers in Europe during influenza season of 1994-1995. Adults with acute infection were randomly assigned to one of the three treatments: zanamivir by inhalation or intranasal spray; zanamivir by inhalation plus placebo spray or placebo by both routes. Treatments were self-administered, twice daily, for five days. The results showed that the group, given inhaled intranasal zanamivir had significantly lower viral titer than those in placebo group. The topically administered zanamivir was well tolerated. It was concluded that in adults with influenza A or B virus infections, direct administration of a selective neuraminidase inhibitor, zanamivir, to the respiratory tract is safe and reduces symptoms, if begun early.

Monto, *et al*; 1999, evaluated zanamivir activity in seven, similarly designed, placebo-controlled studies for the treatment of influenza. Pooled analysis was conducted to evaluate efficacy more precisely in terms of the alleviation of symptoms in population subgroups and for secondary endpoints. Results showed significant reductions in antibiotic use, time to return to normal activities and use of relief medication. In addition, reduction in symptom scores was apparent shortly after commencing zanamivir treatment. By the evening of the second day of treatment, the median total symptom score had fallen by 44 % in zanamivir recipients compared with 33 % in placebo recipients. These results highlighted the groups likely to show greatest benefit from zanamivir treatment, and confirmed the clinical relevance of the treatment benefit.

de Jong, *et al*; 2000, reported that the occurrence of another pandemic in the near future cannot be excluded if sufficient vaccine may not be available to counteract its consequences. During interpandemic periods, important antigenic drift variants sometimes arise and the production of correspondingly adapted vaccine becomes impossible because of a shortage of time. It was suggested that these problems could be solved by increasing influenza surveillance and by adopting new ways of vaccine composition, production, formulation, presentation and delivery.

MATERIALS AND METHODS

MATERIALS AND METHODS

A- Cells and Cell Culture

Three types of cells (MDCK, LLCMK-2 and Hep-2) were used for isolation of influenza viruses. These cells were stored in culture medium containing 10 % dimethyl sulfoxid (DMSO) in liquid nitrogen.

i) Reconstitution of Frozen Cells

Ampoules taken out of liquid nitrogen were shifted in a water bath at 37°C and as soon as the contents were melted the ampoules were wiped off with 70 % ethanol. The cells were aspirated in a syringe already containing 1ml of the minimal essential medium (10% MEM) and added in 75 cm^2 tissue culture flasks containing 10 ml of 10 % MEM (appendix-I) under constant but gentle shaking. These flasks were incubated at 37°C for 24 hours.

After 24 hours the culture medium was replaced with fresh 10 % MEM to remove the dead cells and DMSO. The cells were observed daily for quality and contamination.

ii) Preparation of Culture Tubes

To prepare culture tubes medium was poured off from each flask and the monolayer was washed twice with PBS (appendix-II). Trypsin 3 ml (0.25 %) was added to each flask and kept in incubator for 5 minutes. Flasks were examined at regular intervals to observe the dispersion of cells. Trypsin was poured off and the flasks were tapped few times vigorously to disperse cells completely. MEM (10%) 10 ml was added in the flasks and aspirated few times. An equal volume (0.5 ml) of cell suspension was mixed with 0.5 ml of typan blue and the yield/ml was determined for each flask. To prepare culture tubes for virus isolation 55,000 cells/ml were added in each tube and these tubes were incubated at 37°C. The rest of cells were used for preparation of flasks of further use. Same procedure was adopted for preparation of culture tubes of Hep-2, LLCMK-2 and MDCK cells. Cell lines were examined daily for cells confluency and absence of contamination.

B- Collection of Specimens

Two cohorts of patients were studied during influenza outbreak of winter 2,000-2,001. In the first cohort (September to November 2,000) 40 patients between the age of 12-70 years presenting with acute upper respiratory tract infection attending the out patients department (OPD) of the Federal Government Services Hospital (FGSH) were enrolled. During the second cohort (December 2,000 to February 2,001), 40 patients children <12 years hospitalized due to acute upper/lower respiratory tract infection at Children Hospital, Pakistan Institute of Medical Sciences (PIMS) were studied.

A complete clinical history and consent was obtained from adult patients and parents of infants before taking the specimens. Clinical data collected includes gender, age, presence of underlying disease such as congenital heart disease, broncho-pulmonary dysphasia or immunodeficiency, the presence of wheezing, cough, strider, respiratory rate, pulse rate, temperature etc. The consent and History form is given in appendix III & IV.

Throat swabs were obtained with soft sterile Dacron tipped applicators in 2.5 ml transport media (appendix-V), using disposable sterile tongue depressors and immediately sent in cold conditions to the laboratory.

C- Virus Isolation

i) Processing of Specimens

Bottles containing throat swabs were shaken vigorously, throat swabs were taken out and homogenized suspensions were transferred to sterile tubes corresponding to the number of specimens and centrifuged for 10 minutes at 1000 rpm. Supernatant was decanted from tubes and kept on ice until used for inoculation.

ii) Inoculation of samples in MDCK, LLCMK-2 and Hep-2 Cells

The culture tubes were observed for growth of cells and marked in duplicate according to the number of specimens. Growth medium was replaced with 1 ml of maintenance medium (2 % MEM). For MDCK and LLCMK-2 cells 0.5 µg/ml trypsin was also added in maintenance medium. Supernatant (200 µl/tube) was added in culture tubes in

duplicate corresponding to the number of specimen and incubated at 37°C. The tubes were examined at 2,3,5 and 7 days for cytopathic effect (CPE). For Hep-2 cells media was changed at every third day.

D- Hemadsorption Test

Influenza viruses like some other viruses do not show cytopathic effect (CPE) in a cell culture and their presence in the cell culture is detected by applying red cells suspension to the monolayer. Such viruses modify the cell surfaces of cells in culture by the inclusion of their proteins, some of which will attach to red cells and make them to stick. Hence showing hemadsorption in cell culture (Shelokov, *et al*; 1958).

i) Preparation of RBCs Suspension

Chicken blood (5 ml) was collected in 15 ml of Alsever Solution (appendix-VI) and centrifuged at 1200 rpm for 5 minutes. Supernatant was aspirated and residue was washed three times with PBS (pH 7.2) to get packed RBCs. At final washing centrifugation was done at 1200 rpm for 10 minutes. Supernatant was aspirated and packed RBCs were diluted to 0.5 % v/v in PBS and stored at 4°C until used.

ii) Procedure of Hemadsorption Test

Culture fluid was aspirated from tubes and discarded into disinfectant solution. Six drops (100 µl) of RBCs were added to infected and control tubes with a Pasteur pipette. RBCs were spread over the cell sheet and the tubes were incubated at 4°C for 20 minutes. Tubes were washed twice with PBS to remove unadsorbed RBCs and examined under microscope.

E- Identification/Characterization of Influenza Viruses

Influenza viruses after detection in the cell culture by hemadsorption test were identified and characterized using hemagglutination inhibition test (HAI). When RBCs are mixed with influenza virus in appropriate ratio, the virus bridges the RBCs and changes their normal settling pattern. This is called hemagglutination (HA). The traditional method for identifying influenza field isolates takes advantage of this property. Specific attachment of antibody to antigenic sites on the H molecule interferes with the binding between the

viral H protein and the receptors on the RBC membrane. This effect inhibits hemagglutination and is basis for hemagglutination inhibition (HAI) test.

The HAI test was developed by Hirst (1941) and later modified by Salk (1944). In general this test is performed by mixing the standardized viral antigen with serial dilutions of serum, adding a red blood cell (RBC) suspension in microtiter plates to determine specific binding of antibody to the H molecule.

i) Reagents and Solutions

HAI kit reagents, supplied by WHO Collaborating Center for reference and research on influenza, were reconstituted according to the instructions provided and stored at -20°C (appendix-VII). Lyophilized sera and solutions needed for this titration procedure were stored at 4°C.

ii) Determination of Hemagglutination (HA) Titer of Reference Antigens and Field Isolates

Reference control antigens and field isolates were titrated before every HAI test (Figure: 2). Each control reference antigen and field isolates were diluted 1:10 in PBS and titrated in separate V-bottom plates. PBS (50 µl) was added to wells #2 through 10 of each letterd row in a microtiter plate. In the first well of the lettered row, 100 µl of (1:10 dilution) each field isolate was added. Two wells in row 11 and 12 were added with 50 µl of PBS and used as RBCs control. Two-fold serial dilutions of each field isolate/reference antigen were made transferring 50 µl from well to well and discarding the final 50µl after row 10. RBCs (50 µl) suspension (0.5 %) was added to each well and mixed by manually shaking the plates. The plates were incubated at room temperature (22 to 25°C) for 30 minutes and the results were recorded. The dilution of virus/reference antigen that caused complete hemagglutination was considered as the end point of HA titration.

iii) Preparation of Standardized Antigen for Hemagglutination Inhibition (HAI) Test

A "unit" of hemagglutination is an operational unit defined as the amount of virus needed to agglutinate an equal volume of a standardized RBCs suspension. The standard for the

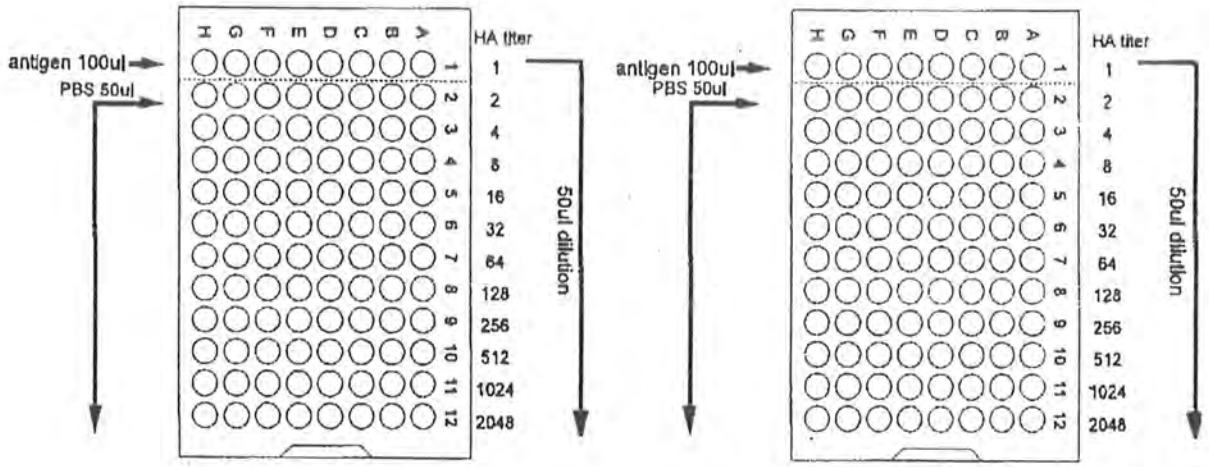


Plate # 1

Reference Antigens

A- Influenza A (H3) Control Antigen

B- Influenza A (H1) Control Antigen

C- Influenza B Control Antigen (B/Beijing/184/93-like)

D- Influenza B Control Antigen (B/Beijing/243/97-like)

G- Blank

H- Cell Control

Plate # 2

Field Isolates

A- Field Isolate # 1

B- Field Isolate # 2

C- Field Isolate # 3

D- Field Isolate # 4

G- Blank

H- Cell Control

Figure 2. Flow Diagram of Hemagglutination (HA) Assay.

HAI test is a 4 HA units of antigen added to two fold dilutions of antisera. This antigen dilution was calculated by dividing the HA titer by 8. Volume of standardized antigen (4 HA units/25 µl or 8 HA units/50 µl) needed for the HAI test and "back titration" was prepared and kept in record.

Back titration was performed to verify units by performing a second HA test using calculated antigenic dilution. The complete HA in first three wells and partial HA in the fourth well confirmed the 4 HA titer of antigenic dilution. The diluted antigen was stored at 4°C and used within the same day.

iv) Serotyping of Influenza Isolates

a) Treatment of Anti-Sera

Anti-sera from many animal species contain non-specific inhibitors to hemagglutination, which unless removed, can give false positive results. To remove these non-specific inhibitors anti-sera were treated with receptor destroying enzyme (RDE) provided in the kit. Three volumes of RDE was added to one volume of serum (0.3 ml RDE + 0.1 ml serum) and incubated overnight. In the following day anti-sera were heated in water bath at 56°C for 30 minutes. Physiological saline (appendix-VIII) 6 volume (0.6 ml) was added in each treated anti-sera tube and used for HAI.

b) Procedure of Hemagglutination Inhibition (HAI) Test

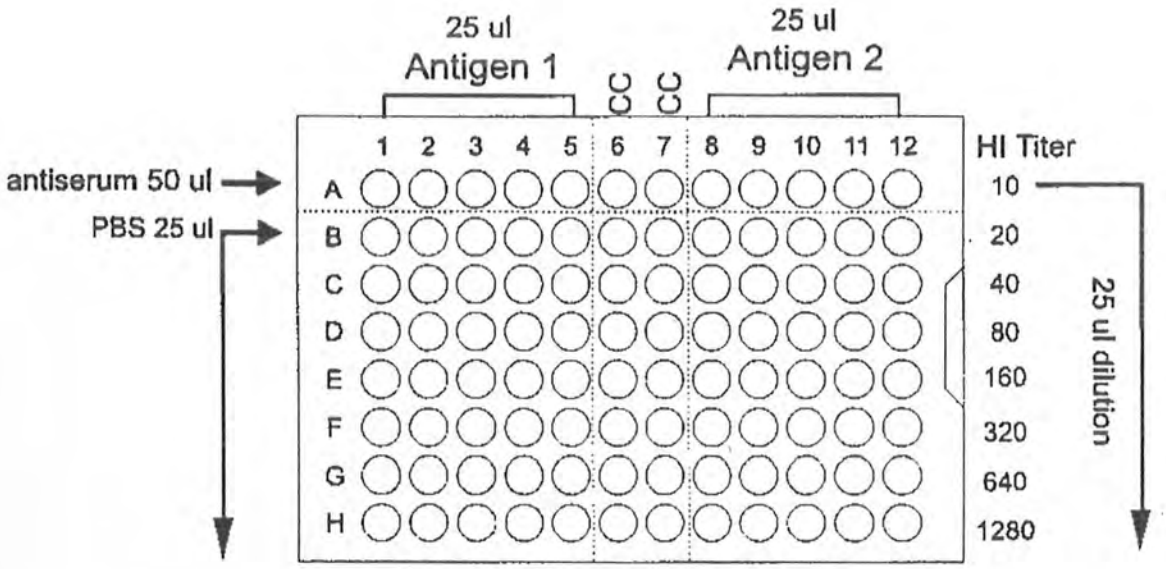
Figure 3 is a flow diagram for the HAI test. This scheme was used for two antigens per plate and a complete set of four reference antisera and a negative control serum. One extra plate was used for the serum control in which PBS was added instead of antigen.

Dilutions of RDE treated antisera were prepared by first adding 25 µl of PBS to wells B through H (B1-H12) of each numbered column. 50 µl of each treated anti-serum was added to the first well of the appropriate numbered column, i.e. serum #1 was added to well A1 and well A8; serum #2 to A2 and A9, etc. The starting dilution of the RDE treated serum was 1:10. PBS (50 µl) was added to wells A6 and A7 and used for RBCs control. Serial two-fold dilutions were prepared of the treated sera by transferring 25 µl from the first well of numbered columns to successive wells. The final 25 µl was discarded after row H. Field test antigen/standardized control antigen (25 µl) was added

from #1 to all wells of a complete set of diluted treated sera (A1-H5). PBS (25 µl) was added instead of antigen to serum control plate. The contents of the plates were mixed by manually shaking the plates. The plates were covered and incubated at room temperature (22° to 25°C) for 15 minutes. Standardized RBCs 0.5 % (50 µl) were added to all wells and mixed as before. The plates were covered again and the RBCs were allowed to settle at room temperature for one hour and the results were recorded. Procedure was repeated for remaining field test antigens and standardized control antigens. The HAI titer was the last dilution of antiserum that completely inhibited HA.

F- Comparison of Growth of Influenza Virus in MDCK and LLCMK-2 Cells

Two sets of 18 tubes, one of each cell type, were inoculated with the isolated influenza strain (as described previously) and incubated at 37°C growth of influenza virus was checked for nine consecutive days. Each day a pair of tubes of each cell type was taken out from the incubator and examined for cells confluency. The culture tubes were frozen for one hour at -20°C. After one hour tubes were taken out from the freezer and allowed to thaw at room temperature. The influenza virus particles thus liberated were titrated by standardized hemagglutination procedure and the virus titer thus determined was recorded.



Wells # 1, 8- Influenza A (H3) Reference Antiserum

Wells # 2, 9- Influenza A (H1) Reference Antiserum

Wells # 3, 10- Influenza B Reference Antiserum (B/Beijing/184/93-like)

Wells # 4, 11- Influenza B Reference Antiserum (B/Beijing/243/97-like)

Wells # 5, 12- Negative Control Antiserum

Wells # 6, 7- Cell Control

Figure 3. Flow Diagram of Hemagglutination Inhibition (HAI) Assay of field isolates and reference control antigens.

RESULTS

RESULTS

A total of 80 patients who fulfilled the inclusion criteria were enrolled in this study. Influenza viruses A/H3N2 Type were isolated in 11 (13.75 %) of throat swabs collected from the out patients (adults/elderly) and hospitalized patients (children) during the study period September 2,000 to February 2,001. Male to female ratio was 2:1 (figure: 4).

Age Distribution and Clinical Features

The age distribution of 40 children in which viral etiology was studied ranged from five weeks to 12 years; 28 (70 %) of these were < 2 years. Only five of the 40 patients were detected as influenza positive (figure: 5). However, all the cases were presenting with typical symptoms of bronchitis/bronchiolitis or pneumonia. Patients were identified for underlying diseases like asthma, gastroenteritis, hemophilia, tonsillitis and thalassaemia. There was one influenza-infected patient presenting with underlying disease of asthma and tonsillitis while rest of patients with influenza infection were free of any such disease (table: 1). No RSV or Para-viruses were detected in the study.

Forty throat swab specimens taken from adults/elderly patients were processed for influenza-, adeno viruses presence. Six out of 40 specimens were identified as having influenza viruses while no adeno-virus was detected (figure: 5). Few influenza-associated patients were also complaining for underlying diseases like asthma, diabetes, renal-syndrome gastroenteritis and blood pressure along respiratory tract infection (table: 1).

Seasonal Distribution of Influenza Viruses

Seasonal patterns were observed during the study period. Significant numbers of influenza virus cases were identified in September and October; there were two influenza cases detected in November while no influenza virus was isolated in December. However, two more influenza virus cases were identified in the following months, one each in January and February (Fig. 6).

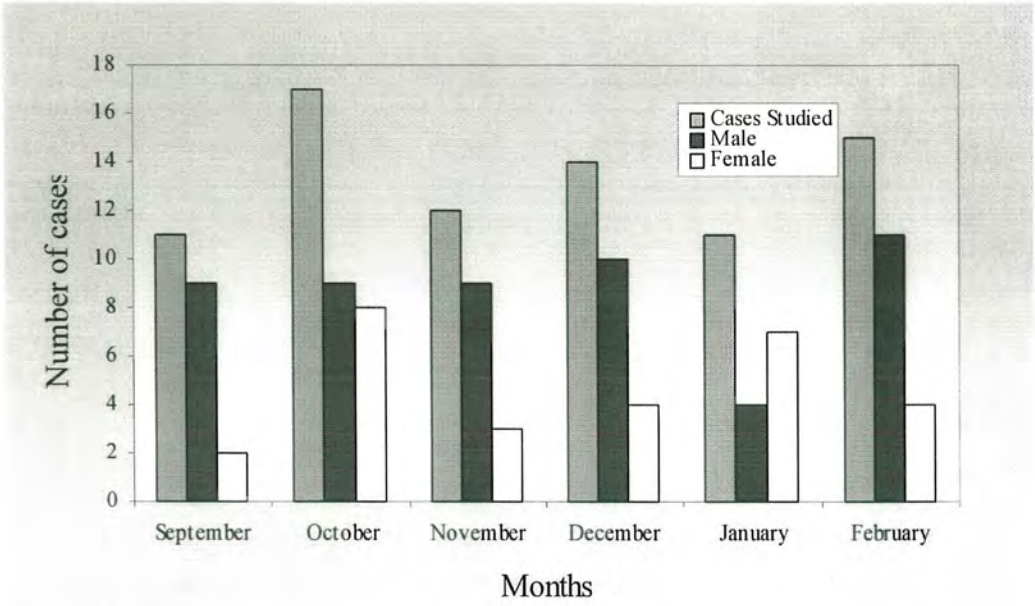


Figure 4. Seasonal Distribution of ARI Cases (AURTI/ALRTI) in Islamabad.

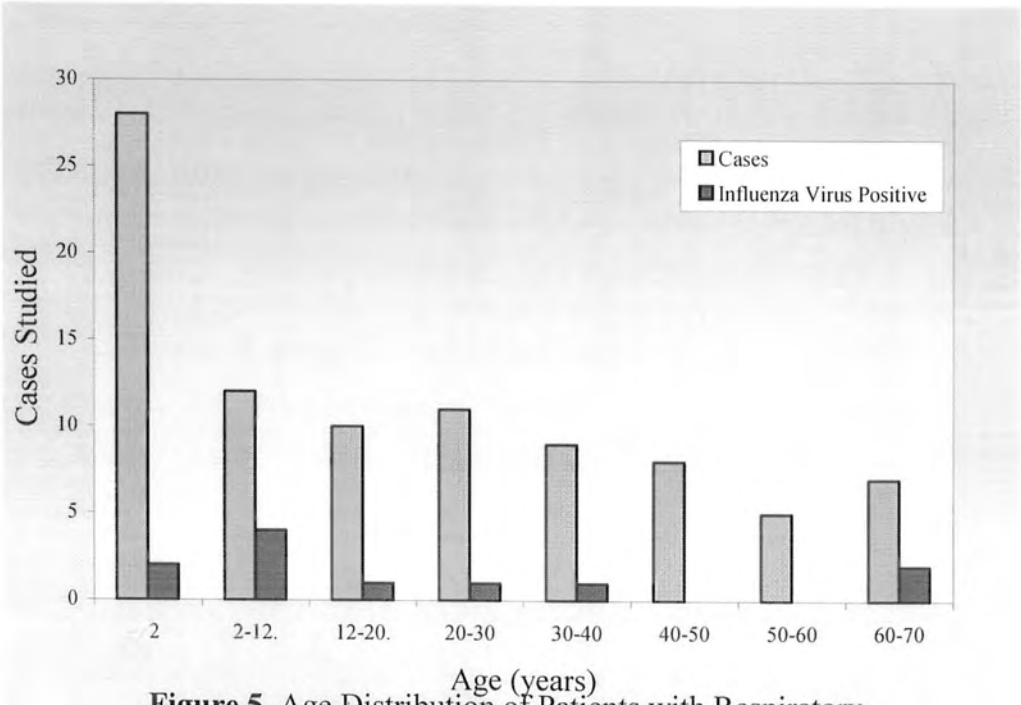


Figure 5. Age Distribution of Patients with Respiratory Illness.

Table 1. Clinical features observed in patients with influenza infection

S. No.	Age (Yrs.) /Sex	Clinical Syndrome	Underlying diseases	Mortality/ Morbidity
1	2/F	Pneumonia	Asthma	Recovery
2	2/F	Pneumonia	NA	Recovery
3	12/F	URTI	Asthma, Tonsillitis	Recovery
4	10/M	URTI	NA	Recovery
5	12/F	URTI	NA	Recovery
6	16/M	URTI	NA	Recovery
7	30/M	URTI	Asthma, Gastroenteritis	Recovery
8	34/M	URTI	Asthma, Gastroenteritis	Recovery
9	55/M	URTI	Asthma, Blood-Pressure	Recovery
10	57/F	URTI/LRTI (Pneumonia)	Diabetes, Renal-Syndrome	Recovery
11	66/M	URTI	Blood Pressure, Gastroenteritis	Recovery

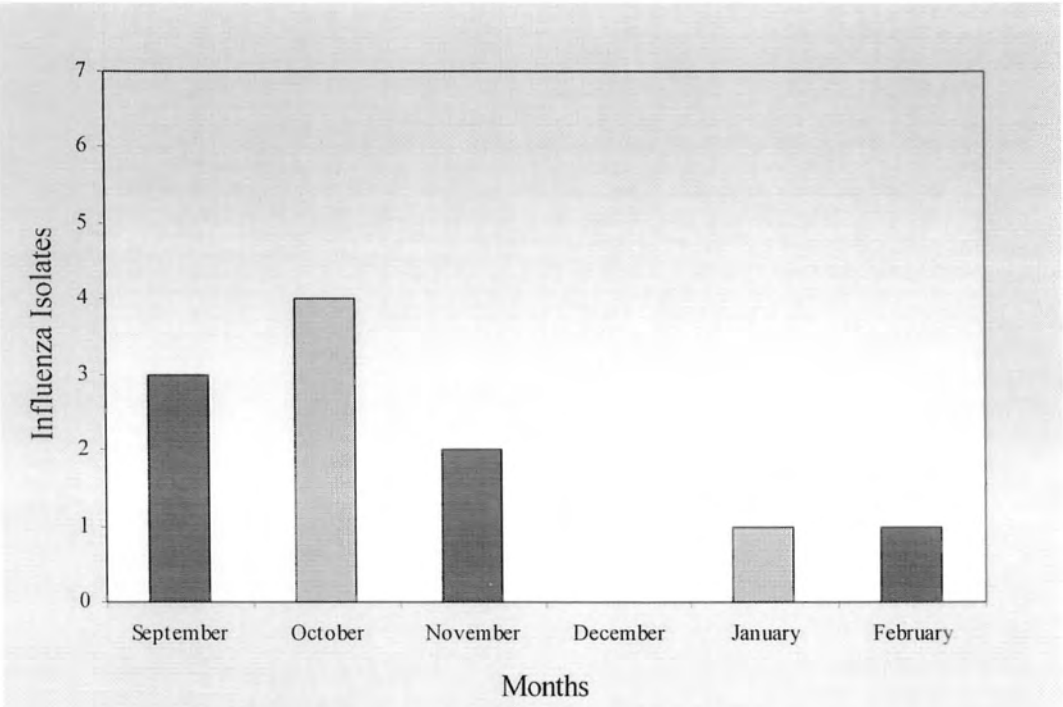


Figure 6. Seasonal Distribution of Influenza Virus.

Virus Isolation

MDCK, LLCMK-2 and Hep-2 cells were cultured with a cell count of 55,000 cells/ml in culture tubes. These cells were microscopically examined for their confluency each day. MDCK and LLCMK-2 cells gave more than 90 % confluency in culture tubes after 48 hours, while Hep-2 cells having faster growth rate showed islands after 24 hours. Hep-2 cells showed infection but no specific pattern of infection was observed for any respiratory virus to be identifiable (figures: 7a, 7b). However, all 11 isolates were successfully grown on both MDCK and LLCMK-2 cells in presence of trypsin (figure: 8a, 8b).

Hemadsorption (HAD) Test

Influenza viruses growing in the cell culture are capable of causing the adsorption of erythrocytes to the surface of infected cells. This phenomenon of hemadsorption has been proven to be particularly valuable in detecting the presence of influenza viruses in cell culture, which multiply within cells without producing a microscopically detectable cytopathic effect. All of the 11 isolates produced hemadsorption with chicken RBCs (figure: 8a, 8b). The specimens were then processed further for HAI for confirmation and typing of Influenza viruses.

Hemagglutination (HA) and Hemagglutination Inhibition (HAI) Test

Hemagglutination (HA) was observed when chicken RBCs were mixed in appropriate ratio to the virus particles (figure: 9a). HA titration was performed to calculate 4HA units for each field isolate and reference test antigen needed in serotyping of influenza virus isolate (table: 2).

Hemagglutination inhibition (figure: 9b) occurs when antibodies interfere in the binding of viral H protein to the receptors on the RBCs surface. The specificity of antibodies for each type of virus particle helps in identifying the influenza strains present in field isolates. HAI titration of all isolates with a set of five antisera provided the evidence of influenza virus A/H3N2 circulating in this region (table: 3).

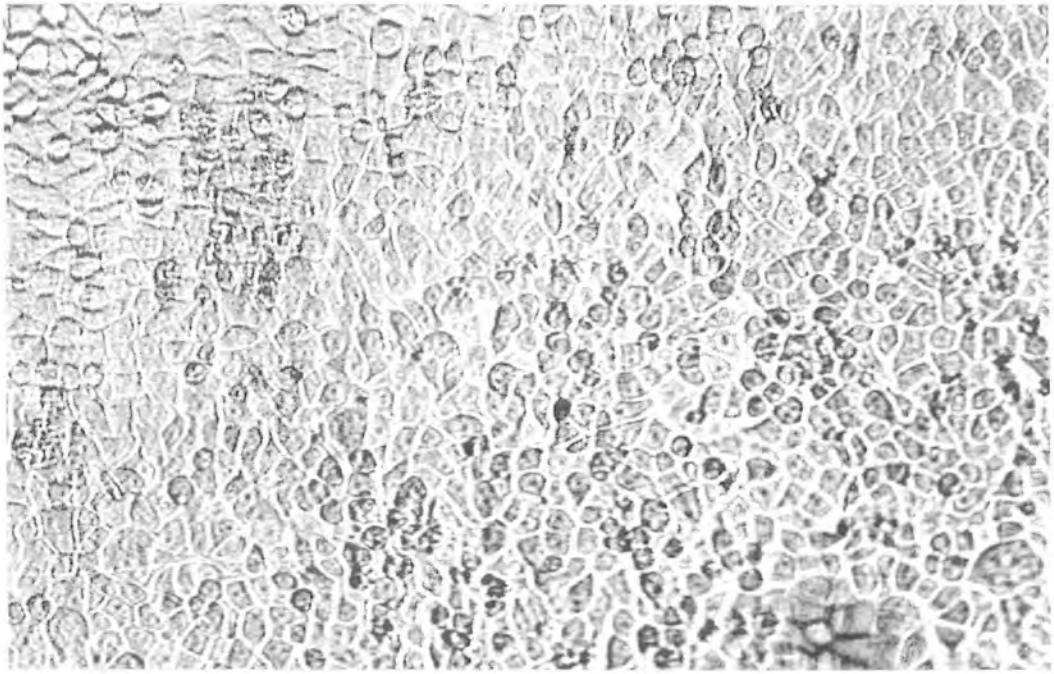


Fig 7a. Uninfected Hep-2 cells

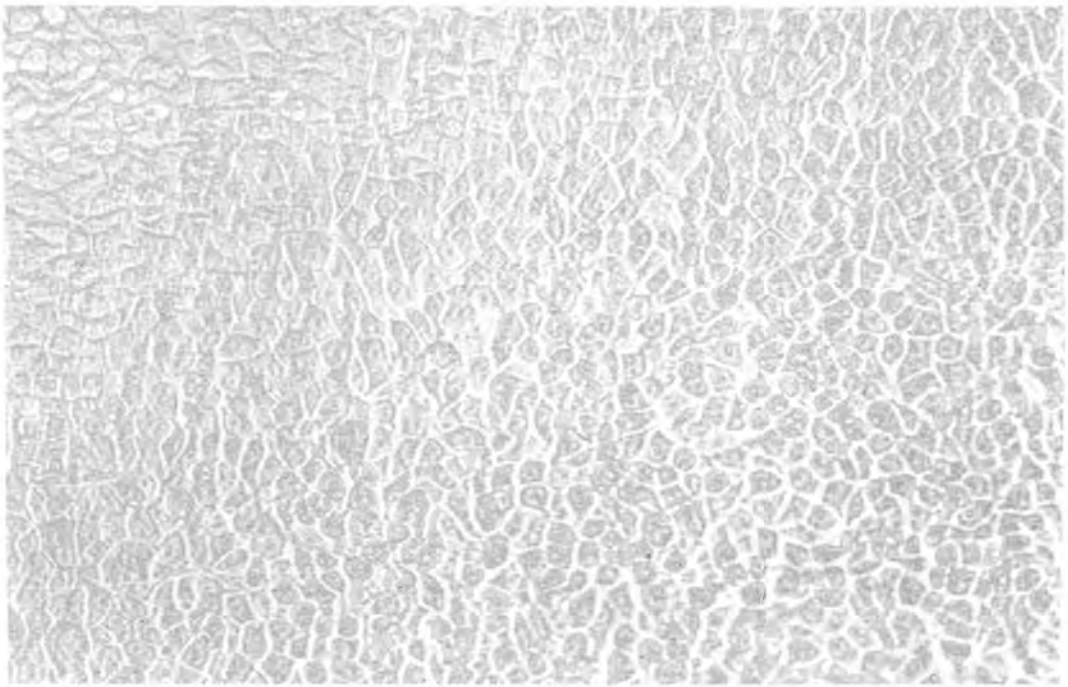


Figure 7b. Infected Hep-2 Cells

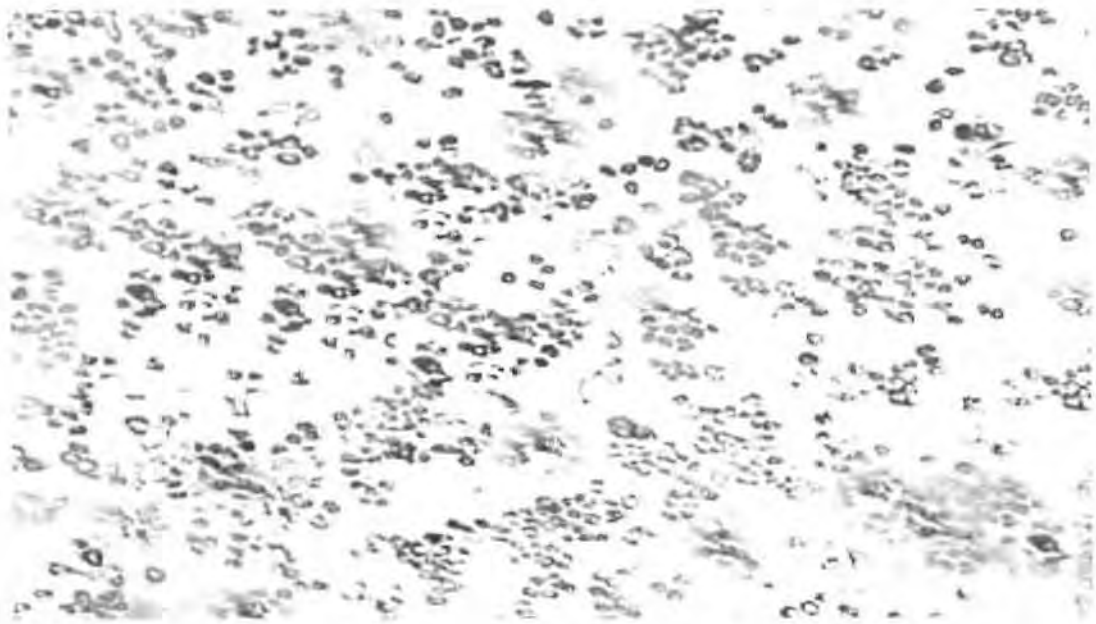


Fig 8a. Hemadsorption in MDCK Cells.

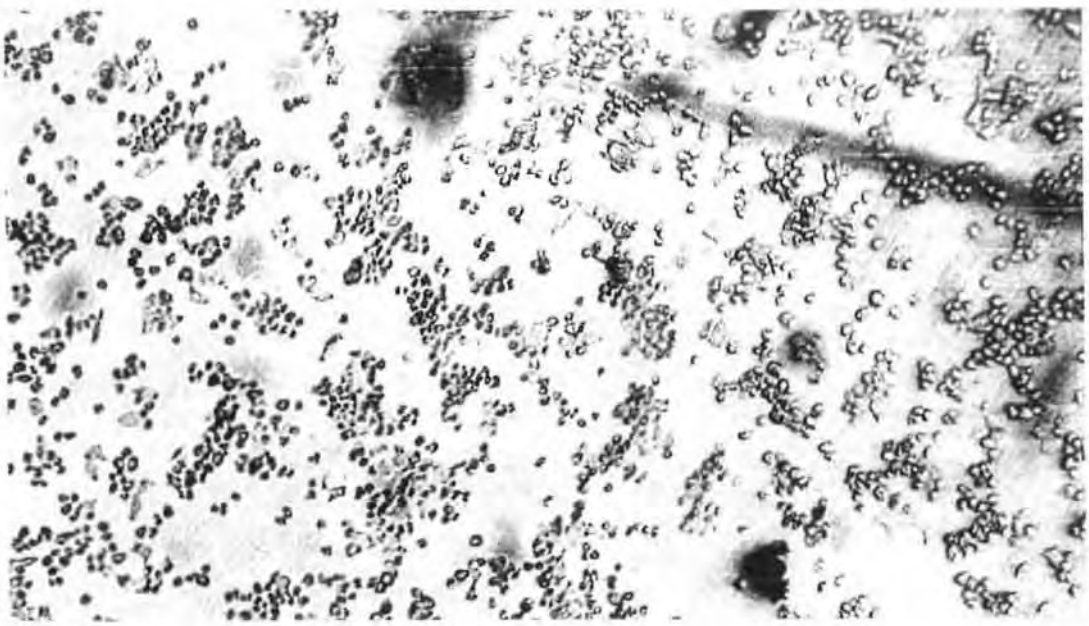


Figure 8b. Hemadsorption in LLCMK-2 Cells.

Table 2. Determination of Hemagglutination Units (HAU) of Influenza Virus Isolates.

Reference Antigens	Hemagglutination (HA) titer	4 HA Unit (HA Titer ÷ 8)
A(H1N1)	2560	1:320
A(H3N2)	2560	1:320
B/Beijing/184/93	1280	1:160
B/Beijing/243/97	1280	1:160
Influenza Virus Isolates		
Isolate #1	256	1:32
Isolate #2	256	1:32
Isolate #3	128	1:16
Isolate #4	256	1:32
Isolate #5	128	1:16
Isolate #6	256	1:32
Isolate #7	256	1:32
Isolate #8	128	1:16
Isolate #9	256	1:32
Isolate #10	256	1:32
Isolate #11	128	1:16

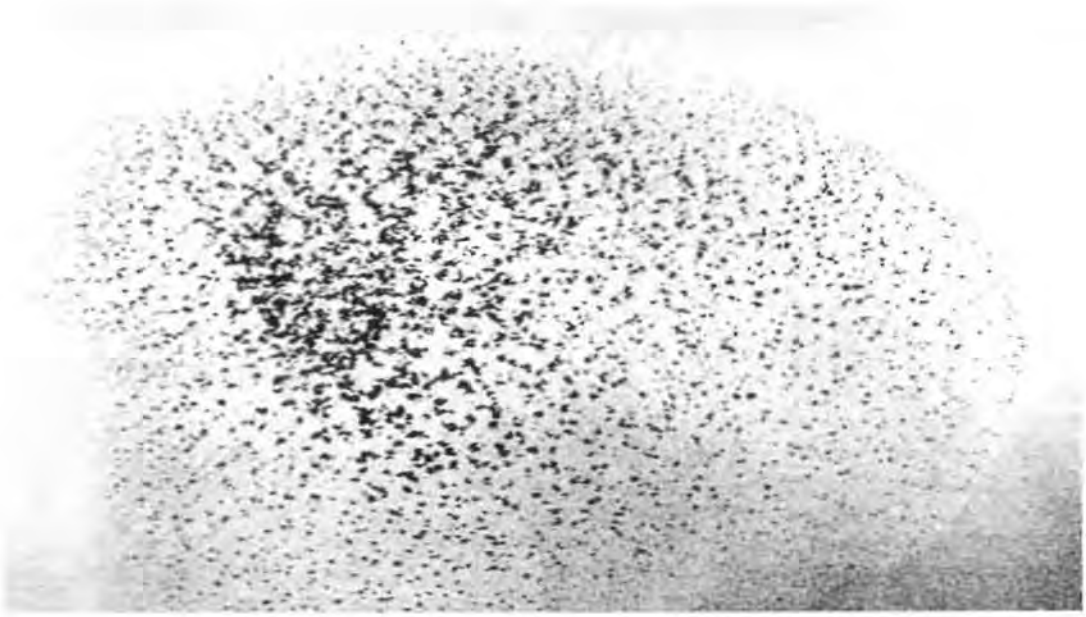


Figure 9a. Hemagglutination caused by Influenza Virus.

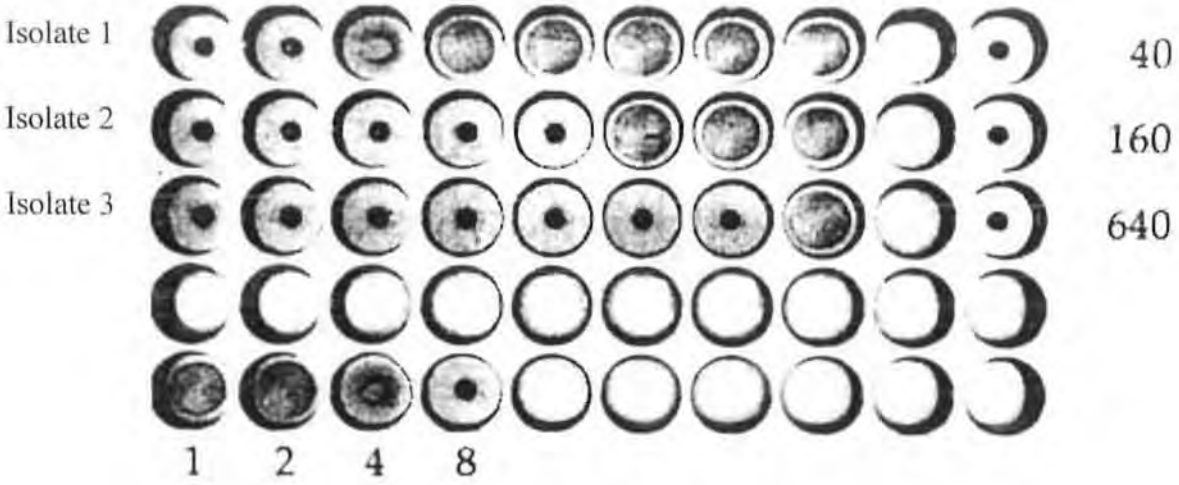


Figure 9b. Hemagglutination Inhibition caused by three isolates of influenza A/H3N2. Titers are expressed in reciprocal of dilutions. Bottom line indicates hemagglutination reaction in first two wells, a partial hemagglutination has occurred when influenza virus was diluted ¼. Wells showing buttons indicate absence of hemagglutination.

Table 3. Determination of S erotypes of Influenza Virus Isolates.

Hemagglutination Inhibition Assay Reference Antisera					
Reference Antigens	1. A (H1N1)	2. A (H3N2)	3. Bj/184	4. Bj/243	5. Negative
A(H1N1)	2560	80	10	<10	<10
A(H3N2)	<10	1280	20	<10	<10
B/Beijing/184/93	<10	<10	320	<10	<10
B/Beijing/243/97	<10	<10	<10	640	<10
Influenza Virus Isolates					
Isolate #1	<10	40	10	<10	<10
Isolate #2	<10	640	<10	<10	<10
Isolate #3	<10	160	<10	<10	<10
Isolate #4	<10	640	<10	<10	<10
Isolate #5	<10	320	<10	<10	<10
Isolate #6	<10	1280	<10	<10	<10
Isolate #7	<10	640	<10	<10	<10
Isolate #8	<10	1280	<10	<10	<10
Isolate #9	<10	160	<10	<10	<10
Isolate #10	<10	320	<10	<10	<10
Isolate #11	<10	640	<10	<10	<10

Comparison of Growth of influenza virus in MDCK and LLCMK-2 Cells

Forty-eight hours cultured cells of both cell types produced influenza virus titers periodically at 72 hours of their infection with the isolated influenza A/H3N2. Influenza virus titer (256^{+} HAU) was obtained in MDCK cells at the 6^{th} day of infection. Same titer was obtained in 7^{th} day. In the following 8^{th} and 9^{th} day virus titer fell in quick succession due to destruction of cells. LLCMK-2 cells showed influenza titer (128^{+} HAU) at the 7^{th} and 8^{th} day post-infection but at 9^{th} day virus titer also fell periodically for this cell type (figure: 10).

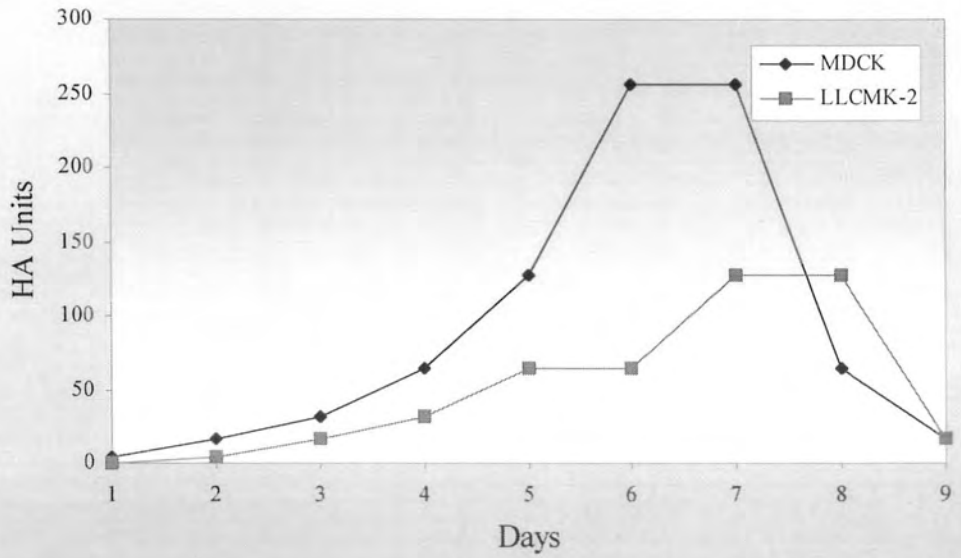


Figure 10. Comparison of Growth of Influenza Virus in MDCK and LLCMK-2 Cells.

DISCUSSION

DISCUSSION

During the last century influenza epidemics resulted in thousands of deaths worldwide. Most of these deaths were those, which were not directly attributed to influenza partly because of scarcity of diagnostic facilities or lack of rapid diagnostic procedures to people especially of developing countries.

In South Asia, the year is divided into 3 seasons, i.e., hot (March to May), Rainy (June to October) and cool (November to February). In general, the number of influenza patients increases in the rainy season (Takahashi, *et al*; 1981, Yamazi, *et al*; 1983) and during winter (Reddiah and Kapoor, 1988; Ghafoor, *et al*; 1990). Throat swabs were collected from patients during both rainy and cool seasons in the present study. Samples collected during September and October provided six influenza viruses but later in the cool season decline in influenza virus isolation was obtained. One possibility may be the haphazard climatic changes in the following cold months, however, influenza viruses have been isolated both during warm and cold months (Huq, *et al*; 1990).

Rapid diagnosis of respiratory viruses is essential for initiating antiviral drugs, if any, in time and also to reduce the use of unnecessary antibiotics. The most rapid and reliable way to diagnose influenza virus infection is virus isolation. Nevertheless, this method has its limitations. Firstly, specimens should be transported to and processed in a virological laboratory as soon as possible. Secondly, if the specimens are taken too late in the course of disease, virus isolation will be difficult (Rothbrath, *et al*; 1999). Specimens were collected from patients during acute phase of virus infection, as there usually is abundant shedding of virus and viral proteins (Mcintosh, 1996). In the present study, the influenza virus was isolated on tissue culture and later on identified and characterized by hemagglutination (HA) and hemagglutination inhibition (HAI) tests. MDCK, LLCMK-2 and Hep-2 cells were used for isolation of respiratory viruses. MDCK produced rather rapidly influenza virus after infection, with very high titers (256^{+}HA) as also demonstrated by Meguro, *et al*; 1979; Bartholoma and Torbes, 1989; Mills, *et al*; 1989; Merten, *et al*; 1996; Reina *et al*; 1997. LLCMK-2 also showed sensitivity to influenza

virus infection but less titer (128^{+}HA) was obtained in relatively (>24 hours) more time than MDCK cells.

The HAI test was confirmed to be a reliable diagnostic method during the 1997-98 epidemic and showed 80 % to 90 % sensitivity (Mitamura, *et al*; 1998). We remained unable to isolate any other respiratory viruses using Hep-2 cells. One possibility is the early commencement of antibiotics that may have suppressed the isolation of these viruses. Second possibility lies in the fact that the ARI infections in these patients may be bacterial as clinical signs and symptoms of viral infection especially in infants may be indistinguishable from bacterial sepsis (Ghafoor, *et al*; 1990; Chang, *et al*; 1996).

Few influenza patients were presenting with underlying diseases like asthma, gastroenteritis, renal syndrome, heart disease and tonsillitis. Nicholson, 1996, demonstrated that in patients with pre-existing respiratory and cardiovascular diseases and in frail elderly influenza infection can be more serious and it may lead to excess or 'hidden' deaths. Asthma predominated among the patients who had an underling disease. It is considered that asthmatic patients may be much more susceptible to influenza virus infection (Sugaya, *et al*; 2000).

With the development of antiviral therapy, clinical laboratories use rapid diagnostic methods like PCR. As influenza culture requires minimum of 4 days, it looks unsuitable for such a purpose. Although PCR is much more sensitive for influenza virus diagnosis, optimized culture method can also give an almost equivalent detection rate (Magnard, *et al*; 1999). This requires good quality MDCK cells, careful checking and detection of influenza in culture.

PCR detects but does not characterize influenza antigenic variants. For this constantly evolving and re-emerging pathogen, such characterization is important. Moreover, influenza strains that may undergo antigenic drifts or antigenic shift will require a new set of optimized primers in order to be detected by PCR. The potential problem was highlighted by the detection of the first H5N1 strain from the 3-year-old boy in May 1997 in Hong Kong. This influenza A/H5N1 strain was identified initially by an immuno-fluorescence assay and was not detected by H1 and H3 specific RT-PCR assays (Subbaro, *et al*; 1998).

The children who were included in the present study had typical symptoms of bronchiolitis/bronchitis or pneumonia. Five out of 11 influenza virus isolates were isolated from children. The results demonstrate that influenza epidemics have significant impact on pediatric hospitalization along RSV infection. During the 1997-98 season, the largest epidemic in Japan in the last 10 years, more than 50 % of the admissions for three consecutive weeks were for influenza Type-A (H3N2). A similar experience was reported during the 1989-90 epidemic (Sugaya, *et al*; 1992). At the peak of epidemics, influenza viruses appeared to interfere with the spread of other major respiratory viruses particularly RSV (Glezen, *et al*; 1973, 1980), in making it impossible for both viruses to reach the peaks of their epidemics during the same period (Anestad, 1982; Anestad, 1987).

Ghafoor, *et al*; 1990, reported the incidence of respiratory viruses identified in children who attended the out-clinic or in-patients of two major hospitals at Rawalpindi and Islamabad. Their data showed 25 times more RSV patients than influenza, suggesting that the impact of influenza in children was much less than in the present study. Because Ghafoor, *et al*; 1990, conducted a broad based study and their findings may also be attributable to the differences in the sensitivity of diagnostic methods used in respiratory viruses identification. So, findings presented here may not reflect the actual frequency of respiratory viruses at large.

Influenza epidemics and pandemics have a huge impact on both society and individual sufferers. Scarce resources and increasing medical costs highlight the need to quantify the burden of diseases such as influenza and subsequently to make accurate economic assessments of the available interventions (Szucs, 1999). Furthermore, the full pathogenic profile of this highly labile and species-mobile pathogen remains incompletely understood. Nevertheless, the ultimate arrival of a new pandemic of influenza is a certainty. There is no vaccination strategy developed against respiratory infections being applied in this part of world. It will be unjust to the lives especially of children of this region if effective and solid measures would not be taken to combat with this fatal infection. International health organizations and NGO's can play an important role in this regard.

It is, therefore, concluded that respiratory viruses are important pathogens and are responsible for a wide variety of diseases, with influenza being the most common agent along respiratory syncytial-, paramyxo- and adeno- viruses. The use of PCR is to have a sensitive test that assesses rapidly the diagnosis of influenza in patients presenting with acute respiratory illness, but it may not prevent attempts to cultivate the virus; viral culture remaining the reference technique (the gold standard) for influenza diagnosis. We believe that effective methods of prophylaxes should be developed not only for high-risk patients but also for healthy young children. Efforts should be made in the future to conduct large prospective study in ARI patients in different age groups and from different geographical areas of our country in order to achieve the better understanding of the epidemology of respiratory viruses in this region.

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APPENDIX

APPENDIX

I. Minimal Essential Medium, 10 %, (100 ml)

A 10X stock solution was prepared by dissolving 9.53 gm of MEM powder in1000 ml of de-ionized distilled water and sterilized by autoclaving. 10% MEM was prepared according to the following composition:

a.	F.B.S. (Fecal Bovine Serum)	=	10 ml
b.	Penicillin/Streptomycin	=	1 ml
c.	L-Glutamine	=	0.06 ml
d.	Fungizone	=	1 ml
e.	10X MEM	=	10 ml
f.	Non-Essential Amino Acids	=	1 ml
g.	Triple distilled water	=	up to 100 ml

To prepare 5 % and 2 % MEM only the amount of FBS is changed according to the percentage. Medium was sterilized by autoclaving and stored at 4°C until used.

II. Phosphate Buffered Saline (PBS), pH 7.2

Phosphate buffered saline was prepared according to the following composition:

NaCl	=	8.00 g
KCl	=	0.20 g
KH_2PO_4	=	0.12 g
Na_2HPO_4	=	0.91 g
Triple Distilled H_2O	=	Upto1000 ml

pH of the solution was adjusted and sterilized by autoclaving. PBS was stored at room temperature for not more than 3 days.

III. Consent Letter

I Mr./Ms./Mrs.----------S/O or D/O or W/O----------
declare that I have been informed about the research to be carried out on my sample. I am willing to give consent to collect throat swab.

Signature of Patient: ----------

Dated: ----------

IV. History Form for Patients with Respiratory Infection

A) Patients Identification

Name: ---------- Age: ---------- Sex: ----------

Occupation: ---------- Marital Status: ----------Income: ----------

Address: ----------

No. of family members:---------- Male:---------- Female:----------

Educational Status: Read/Write/Primary/Secondary/Degree

B) History of Illness

Date of onset of symptoms: ----------

Date of OPD visit: ----------

C) Symptoms

Fever:	N/Y	Nasal Discharge:	N/Y
Cough:	N/Y	Rash:	N/Y
Difficulty in Breathing:	N/Y	Diarrhea:	N/Y

D) Examination

Temperature: ---------- Pulse Rate: ----------

Nasal Discharge: ----------Respiratory Rate: ----------

Cough:	Heard	N/Y
	Wheezing	N/Y
	Whooping	N/Y
Rash:	N/Y	In drawing of Chest: N/Y

E) Treatment given if any

1)----------

2)----------

3)----------

4)----------

Name of Hospital: ----------

Examiner: ----------

V. Virus Transport Medium (VTM), 500 ml

Virus Transport medium was prepared according to the following composition:

a.	Albumin	=	2.5 ml
b.	Fungizone	=	5 ml
c.	Penicillin/Streptomycin	=	10 ml
d.	P.B.S. Phosphate Buffer Saline	=	up to 500 ml

Medium was sterilized by autoclaving and divided into sterilized scotch bottles (2.5ml/bottle) and stored at -20°C.

VI. Alsever Solution

Alsever Solution was prepared according to the following composition:

Dextrose	=	20.50 g
NaCl	=	4.20 g
Citric Acid	=	0.55 g
Sodium Citrate	=	8.00 g
Distilled water	=	1000.00 g

pH of the solution was adjusted at 7.2 and sterilized by autoclaving. Alsever solution was stored at 4°C.

VII. Influenza Reagent Kit Contents

(HAI reagents for identification of field Isolates)

Influenza A reagent list for HAI:

1 @ 10 ml - Influenza A (H1N1) control antigen, Lot 99-0012N

2 @ 1 ml - Influenza A (H1N1) reference antisera, Lot 980-0045L

1 @ 10 ml - Influenza A (H3N2) control antigen, Lot 99-0018N

2 @ 1 ml - Influenza A (H3N2) reference antisera, Lot 99-0038L

Influenza B reagent list for HAI:

1 @ 10 ml - Influenza B control antigen (B/Beijing/184/93-like), Lot 990-0028N

1 @ 1 ml - Influenza B reference antisera (B/Beijing/184/93-like), Lot 99-0043L

1 @ 10 ml - Influenza B control antigen (B/Beijing/243/97-like), Lot 99-0034N

1 @ 2 ml - Influenza B reference antisera (B/Beijing/243/97-like), Lot 98-0054L

Other reagents for HAI:

1 @ 1 ml - Influenza Negative Control Serum, Lot 98-0002L

1 @ 25 ml - Receptor Destroying Enzyme (Denka)

Antigens and lyophilized antibodies were stored at 4°C and reconstituted antibodies in aliquots at -20°C.

VIII. Physiological Saline, 0.85 %

a. A 20X stock solution was prepared by dissolving 170 g of NaCl in de-ionized water 1000 ml and sterilized by autoclaving.

b. To prepare physiological saline, 0.85 % NaCl, 50 ml of 20X stock solution was added to 950 ml de-ionized water and sterilized by autoclaving.

c. Physiological saline was stored at 4°C for no longer than 3 weeks.