Università degli Studi di Trieste

PhD program in MOLECULAR MEDICINE

PhD Thesis

MOLECULAR EPIDEMIOLOGY OF INFLUENZA VIRUSES IN THREE CONSECUTIVE EPIDEMIC SEASONS, 2005-2007

Tatiana Rossi

Anno Accademico 2005-2007 (XX ciclo)

I thank prof. Campello and prof. D’Agaro for kindly
proof reading the manuscript.
I thank dr. Ansaldi for the help to writing the results.

Table of contents:

ACKNOWLEDGMENTS p. 1

TABLE OF CONTENTS p. 2

ABBREVIATIONS p. 4

1. INTRODUCTION p. 6

1.1 HISTORY OF INFLUENZA p. 6
1.2 THE CLINICAL MANIFESTATIONS OF INFLUENZA p. 8
1.3 THE INFLUENZA VIRUSES p. 10
1.4 INFLUENZA EPIDEMIOLOGY AND ECOLOGY p. 15
1.5 THE AVIAN FLU p. 16
1.6 THE IMMUNE SYSTEM AND IMMUNITY OF INFLUENZA p. 20
1.7 THE GENETIC VARIABILITY OF THE VIRUS p. 23
1.8 THE PREVENTION AND THE SURVEILLANCE p. 27
1.9 OTHER RESPIROVIRUSES p. 30

2. AIMS OF THE STUDY p. 32

3. MATERIALS AND METHODS p. 33

3.1 SURVEILLANCE NETWORK AND SAMPLES COLLECTIONS p. 33
3.2 PROCESSING SAMPLES p. 34
3.3 VIRAL ISOLATION
AND IMMUNOFLUORESCENCE ASSAY (IFA) p. 36
3.4 HEMAGGLUTINATION ASSAY p. 37
3.5 HEMAGGLUTINATION INHIBITION TEST p. 39
3.6 RNA EXTRACTION AND RT-PCR p. 42
3.7 REAL-TIME PCR p. 44
3.8 SEQUENCING p. 46
3.9 PHYLOGENETIC ANALYSIS p. 48
3.10 PREDICTION OF GLYCOSYLATION SITES p. 49
3.11 DETECTION OF ADENOVIRUS AND RSV p. 50

4. RESULTS p. 52

4.1 VIROLOGICAL AND EPIDEMIOLOGICAL DATA p. 52

4.2 ANTIGENIC AND GENETIC CHARACTERIZATION OF THE STRAINS p. 60

4.3 PRELIMINARY RESULTS OF 2007-08 SEASON p. 73

5. DISCUSSION p. 75

6. BIBLIOGRAPHY p. 78

Abbreviations

ADCC Antibody dependent cell-mediated cytotoxicity

ASC Antibody secreting cell

BM2 Influenza B matrix 2 protein

HA Hemagglutinin

HI Hemagglutination inhibition

IFN Interferon

Ig Immunoglobulin

IL Interleukin

ILI Influenza like illness

M1 Matrix protein 1

M2 Matrix protein 2 (Membrane ion channel protein)

MHC Major histocompability complex

NA Neuraminidase

FLU Influenza

ARI acute respiratory illness

MDCK-SIAT1 Madin Darby Canine Kidney, Sialyltransferase 1

IFA immunofluorescence assay

HI hemagglutination inhibition

EISS European Influenza Surveillance Scheme

CIRI Centro interuniversitario di ricerca sull'influenza

NK Natural killer cell

NP Nucleoprotein

NS1 Non structural protein 1

NS2 Non structural protein 2 (renamed NEP)

nt Nucleotide

PBS Phosphate Buffer Saline

PB1 Protein basic 1

PB1-F2 Protein basic 1 – frame 2

PB2 polymerase B2

RNP Ribonucleoprotein

SAá-(2,3)Gal Sialic acid α2,3 Galactose

SAá-(2,6)Gal Sialic acid α2,6 Galactose

Tc Cytotoxic T-cell

TcR T-cell receptor

Th T helper cell

TNF Tumor necrosis factor

WHO World health organization

HPAI Highly Pathogenic Avian Influenza

IAV Influenza A virus

LPAI Low Pathogenic Avian Influenza

PA polymerase A

RT-PCR Reverse Transcriptase Polymerase Chain Reaction

vRNP Viral ribonucleoprotein

CTL Cytotoxic T Lympocites

1.1 HISTORY OF INFLUENZA

Epidemics of influenza occur in most countries in some years and in some countries in most years.

Although historical data on influenza are difficult to interpret because its symptoms are not peculiar, evidence of pandemics is present in the historical record which include 10 probable and three possible pandemics since 1590 AD and allusion to earlier possible pandemics is suggested throughout history (Potter. 2001; Potter. 1998).

The earliest written reports of a flu disease likely were made by Hippocrates roughly in 412 BC but the first influenza pandemic agreed by all authors occurred in 1580 AD which began in Asia and spread to Europe via Africa.

But the most famous pandemic was the so called Spanish flu pandemic (type A influenza, H1N1 subtype), which lasted from 1918 to 1919. This pandemic has been described as "the greatest medical holocaust in history" and current estimes say 50-100 million people worldwide were killed (Waring 1971). The origin of pandemic is not known; reviewers have commentated on a possible origin in China but the origin in USA could be more likely (Crosby 1989).

Anyway the entire world from America to Europe, Africa and Asia was involved by this pandemic.

This death toll caused by extremely high infection rate of up 50 % and the extreme severity of the symptoms, suspected to be caused by cytokine storms (Patterson et al. 1991).

Bacterial pneumonia, a secondary infection caused by influenza and also the virus directly, causing massive haemorrhages and oedema in the lung, had killed many people.

An unusual feature of this pandemic was that it mostly killed young adults (aged 20 to 40 years), an age group that is generally the last susceptible to serious complications from influenza infection; in more than half of the deaths occurred in this age group (Taubengerger et al 2001; Kobasa et al 2005).

The research on this storic virus and the comparison of several of the 1918 virus genes with contemporary avian and human ones indicates an avian origin of the virus but not a direct transmission from avian to human.

The Spanish flu spread from continent to continent and returned in three major waves during next years with increasing virulence.

The world experienced two more pandemics during 20 th century but less devastating than the 1918-1919 one.
During 1957-58 exploded a pandemic called “Asian flu” caused by H2N2 subtype virus while during 1968 was a Hong Kong pandemic caused by H3N2 subtype.
The Asian flu originated in China was caused by a reassortment between the H1N1 circulating prior to 1957 and an avian (wild ducks) virus. (Kobasa et al 2005)
It caused about 1-1,5 million of deaths.
In 1968 the H2N2 viruses reassorted with avian viruses and caused pandemic called Hong Kong, causing about 1 million of deaths (Scholtissek et al 1978; Kawaoka et al 1989). This pandemic was caused by the H3N2 virus generated.
However, history indicates certain features which make influenza pandemics more likely: firstly epidemics tend to occur in winter mouths which a cold and damp climate, secondly the epidemic occurs likely when a variant virus appears with antigenic changes from previous strain and when the cross-reacting antibody, acquired by previous infection is low.

1.2 THE CLINICAL MANIFESTATIONS OF INFLUENZA

Influenza, commonly known as FLU, in humans caused symptoms as: fever, sore throat, muscle pain, severe headache, coughing, weakness, general discomfort, nasal congestion and sometimes nausea and vomiting.

In more serious cases influenza causes pneumonia, which can be fatal particularly in young children and in old people.

An individual can fall sick more than once during his life. The reason of these repeated infections is that influenza virus mutates frequently and leaves an individual at best only partially protected against recurrent infection with new influenza strains. Influenza is transmitted via droplets expelled upon sneezing and coughing (Nicholson et al 1998).

Influenza virus can be infectious for about 1 week at human body temperature, over 30 days at 0° C at indefinitely at very low temperature.

The incubation period is usually 2-3 days before onset of illness, but it can be as long as 7 days. The patient is generally contagious during the febrile phase, but cases of viral spread have been observed seen prior to symptoms.

Healthy people usually recover within one week of bed rest without any medical intervention. In the very young, the elderly and people with underlying medical problems (diabetes, cancer, neurological diseases, kidney, cardio or respiratory diseases) influenza poses a serious risk, and infection may lead to hospitalisation and in some cases death (Nguyen Van Tan 1998).

The cause of death can be the virus itself (viral pneumonia) or secondary infection (often bacterial pneumonia) as the cells of the epithelia are damaged by virus replication. Chronic cardiac and pulmonary disease predispose to secondary bacterial pneumonia, as does older age. Institution of an appropriate antibiotic regimen is usually sufficient for a prompt treatment response.

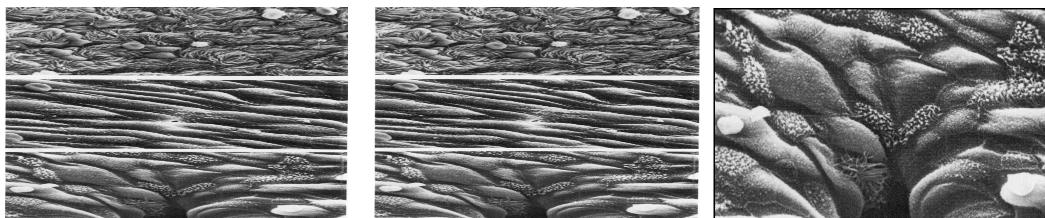


Fig.1 Tracheal mucosa before and after influenza infection (Lycke and Norrby Textbook of Medical Virology 1983)

Influenza may exacerbate heart or lung diseases or other chronic conditions. Influenza infection has also been associated with encephalopathy (McCullers JA et al. 1999, Morishima. et al. 2002), transverse myelitis, myositis, myocarditis, pericarditis, and Reye's syndrome. Common symptoms of the flu such as fever and headache come from amount of proinflammatory cytokines and chemokines (IF, TNF) produced by influenza-infected cells. In patients with chronic bronchitis, clinical influenza infection may lead to a permanent loss of pulmonary function. In children, influenza-induced asthma may continuously deteriorate during the first two days of illness and reconvalescence is typically longer (at least seven days) (Kondo et al 1991). Influenza virus is also implicated in the pathogenesis of asthma attacks in adults (Techtahl et al 1997). Myositis is a rare complication of influenza B virus infection, and to a lesser extent influenza A. It has mainly been reported in children, with boys being more commonly affected than girls. The median interval between the onset of influenza and the onset of benign acute childhood myositis is 3 days (Agyeman et al. 2004). An other complication of Influenza infection could be the Reye's syndrome characterised by the combination of liver disease and non-inflammatory encephalopathy. It is a non-specific clinical pathological entity and a descriptive term which covers a group of heterogeneous disorders. It is almost always associated with previous viral infections, such as influenza, cold, or chickenpox. Reye's syndrome is a serious complication that may occur in children, in particular with influenza B virus. There is a strong link between the administration of aspirin and Reye's syndrome (Starko et al. 1980) Influenza virus normally infects the epithelial cells of the upper respiratory tract, but the virus that caused the H5N1 outbreaks in 1997 (To et al 2001) and 2003 (Peiris et al 2004) has been shown to have an unusually broad cellular tropism indeed virus was detected in lungs, spleen, heart, brain and colon of diseased individuals.

A special composition of the cleavage site of the hemagglutinin (HA), which needs to be cleaved to produce an infectious virus, may have contributed to the systemic spread of H5N1. Similar to the virus that caused the 1918 pandemic (Kobasa et al 2001), H5N1 has also resulted in high systemic cytokine levels, which may have contributed to the pathogenicity observed .the symptoms of avian flu infection are non-specific and may also be associated with the currently circulating human influenza virus subtypes, H1N1 and H3N2 but severe diarrhoea was reported in two reports such as peculiar symptoms.

1.3 THE INFLUENZA VIRUSES

The first influenza virus A was isolated in 1931 in pigs by Richard Schope and within only two years was then isolated in humans in UK.

Influenza virus is a RNA virus belongs to the family of *Orthomyxovirida.*

There are three influenza genera: *Influenzavirus A*, *Influenzavirus B* and *Influenzavirus C*, which are divided on the basis of antigenic differences in the internal proteins, matrix (M1) and nucleoprotein (NP). The three genera differ in epidemiology, host range and pathogenicity. Influenza A and B viruses are important human pathogens, whereas influenza C infection results only in a mild respiratory infection in man. Influenza C viruses are only rarely isolated, but by early adulthood 96% of the human population have antibodies directed against influenza C, indicating that infection with influenza C is common (O' Callagan et al. 1980). Influenza B viruses are mainly found in man, whereas influenza A viruses are found in a range of vertebrates withwaterfowl being the most important host (Nicholson KG. et al 2003). Only influenza A may cause pandemics.

The influenza A can be subdivided into different types based on the antigenic properties of its surface glycoproteins: the hemagglutinin (HA) and the neuraminidase (NA) and an individual strain is designed by letters H and N, each followed by the number of the subtype. Currently, there are 16 HA and 9 NA subtypes recognised by the WHO.

The 16th HA subtype was detected in gulls in Sweden in 2005 (Fouchier et al. 2005).

Influenza A viruses, infecting a wide variety of animal species, represents a threat of transmission to humans from other species and a emerge of new virus in humans, hence the importance of studying especially this type of virus.

Influenza A virus consists in a lipid-enveloped particle (diameter 18-20 nm) containing eight segments negative-sense single strand RNA (Fig. 2).

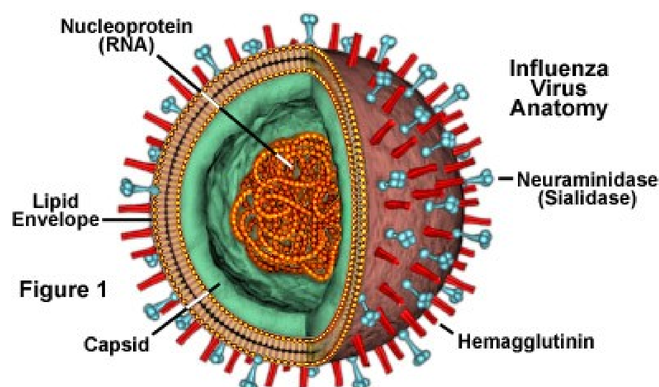


Fig. 2 Influenza virus structure

The segmented genome is an important feature of influenza A in fact it allows that different segments from different strains to mix, generating a novel virus, completely unknown by immunity of population (Webster 1970). This process is called reassortment and could cause a pandemic if it involved avian and human genes.

The largest segment is assigned segment number 1 and the remaining segments are numbered according to decreasing segment size. Each segment is encapsulated by the NP to form a ribonucleoprotein complex (RNP).

In the viral envelope of influenza A viruses there are three integral viral proteins: the HA, NA and an ion channel protein named M2. The peripheral matrix protein (M1) lines the viral envelope in close proximity to the viral genome and is hypothesized to interact with cytoplasmic tail of the surface glycoproteins (Zhang et al 1996).

The hemagglutinin is encoded by segment 4, the neuraminidase by segment 6, the remaining six segment encode for one or more viral protein (Tab.1).

Segment number	vRNA segment length, nt[a]	Gene product	Polypeptide length, aa[b]	Function
1	2341	PB2	759	Polymerase activity, RNA cap binding
2	2341	PB1	757	Transcriptase activity
		PB1-F2	87	Modulates immune response
3	2233	PA	716	Kinase and trancriptase activity, chain elongation
4	1778	HA	566	Receptor binding, entry, fusion with endosome, trimeric structure in the virion
5	1565	NP	498	Encapsidates RNA in RNP
6	1413	NA	454	Virion release, receptor cleavage, tetrameric structure in the virion
7	1027	M1	252	Viral matrix protein
		M2	97	H^+ ion channel, tetrameric structure in virion
8	890	NS1	230	Controls mRNA splicing and transport
		NS2	121	RNP nuclear export

a) nt, nucleotide; b) aa, amino acid

Tab. 1Influenza genome segments

The best characterized viral proteins are the HA and NA, two glycoproteins presented on the virus surface.

The HA is a lectin involves in the binding of the virus to target cell and entry of the viral genome in the host cell. The hemagglutinin is the most abundant protein in the viral envelope and it is named derived from its ability to agglutinate erythrocytes.

It is sintetized as a precursor, named HA0, that is proteolitically cleaved by the host cell proteases into two subunits disulfide-linked: HA1 (328 residues) and HA2 (221 residues). This process is fundamental for the HA fusion activity (Skehel et al 2000).

The mature HA is a homotrimer, consisting in HA1-HA2 complex, it causes the attachment of the virus to the host cell, interacting with the terminal sialic acid residues on the host glycoproteins (Fig. 3).

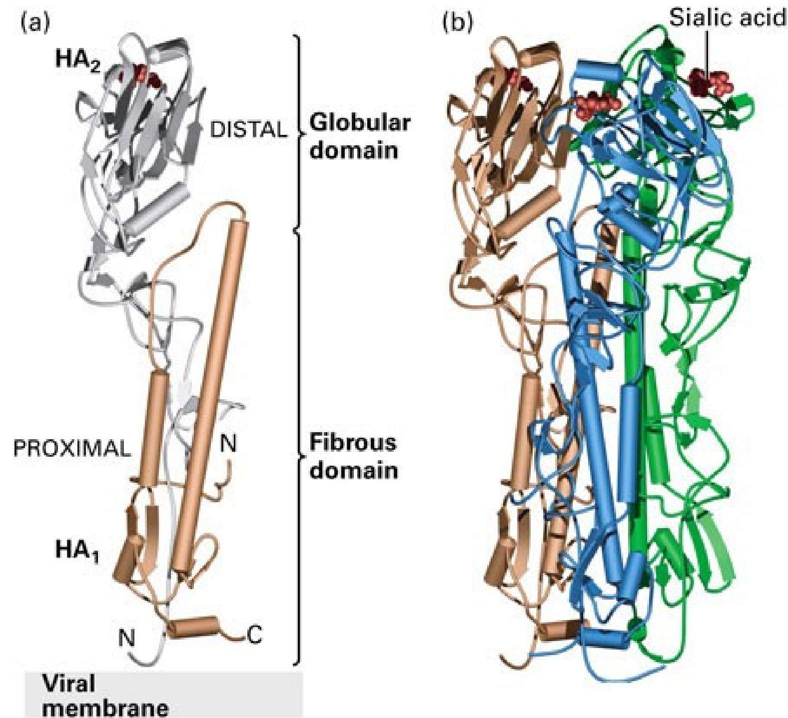


Fig. 3 HA structure

Once attached the virus is endocited and the acid ph of endosomes causes a conformational modification of HA that exposing the N-terminus of HA2, mediates the fusion between the viral and the endosomial membrane (Fig. 4).

The affinity of the HA for different types of sialic acid is a mutter of investigation by the scientific researchers, infact the HA of human viruses preferentially recognizes the terminal N-acetylneuraminic acid that is bound to galactose by an α2,6 linkage (NeuAcα2,6Gal), the major type of sialic acid found on the epithelial cells of the human trachea hemagglutinin. On the other hand, avian viruses preferentially recognize NeuAcα2,3Gal, which differs from the human receptor by only the α2,3 linkage between the sialic acid and galactose (Ito et al. 1998).

Pig's trachea express both the avian and human virus receptors so pigs are susceptible to both infections and for this reason they could play a fondamental role in a generation of a novel pandemic virus strain, as a "mixing vessel" (Scholtissek et al 1988).

The HA presents five antibody-binding regions or epitopes, named A to E, involving in the antibody recognition. Epitopic residues shows greater variability, higher ratios of replacement to silent mutations, and grater correlation with future philogenetic evolution (Plotkin et al., 2003).

That's why the HA sequence is considered antigenically important and it was object of many molecolar studies.

While the HA plays a role in the early stage of the viral infection, the NA has a tardive role, and it's a protagonist of the continuation of infection and the releasing of the virions.

NA is a tetramer (453 residues) with enzymatic sites that cleaves terminal sialic residues from newly syntetized viral particles, facilitating their releasing from the host cells in order to prevent the aggregation of virus particles (Kaverin et al. 1998) and to promote virus release and spread (Stray et al. 2000).

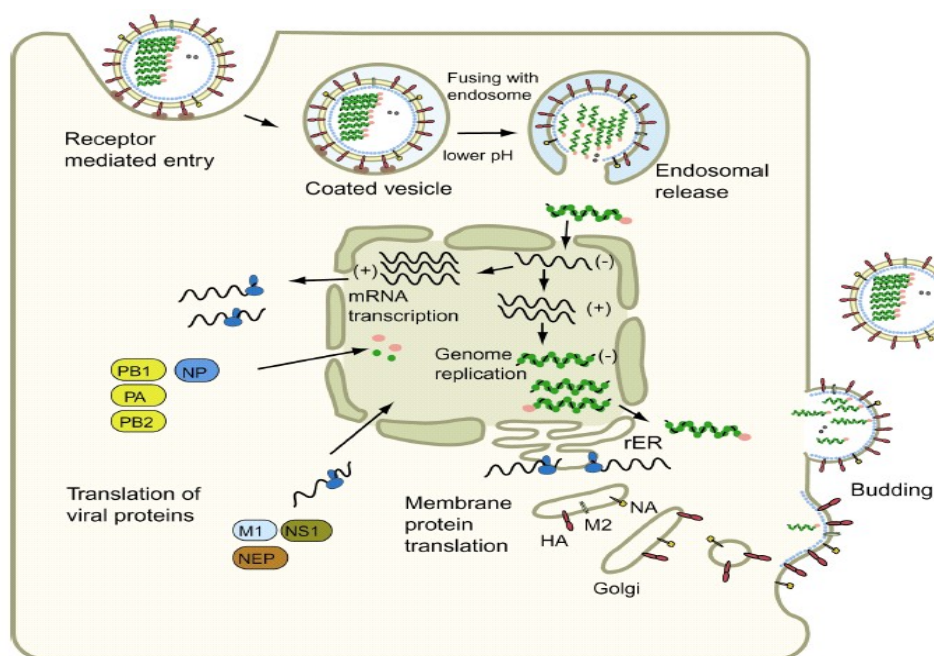


Fig. 4 Life cycle of Influenza viruse

Both HA and NA are recognized by antibodies and are targets for antiviral drugs.

The guidelines for influenza virus nomenclature establish by the WHO (WHO 1980) names the virus isolate in the following order: the influenza genus (e.g. A, B or C), then the host (omitted if human), followed by the place of isolation, strain number and year of isolation (for example: A/Wisconsin/67/05).

HUMAN INFLUENZA VIRUSES:

The most important human influenza viruses are H1N1 and H3N2 and B subtypes that caused and continue to cause epidemic every season.

- H1N1: its variant was responsible for the Spanish flu pandemic that in 1918-19 killed some 50 to 100 million people. It is currently endemic in both human and pig population.
- H3N2: it evolved from H2N2 (that caused Asian flu pandemic in 1957) by antigenic shift and caused the Hong Kong flu pandemic. It is currently endemic in both human and pig population. During 2004-2005 a strain called A/California/7/2004 caused an epidemic in Italy and in FVG with peak of incidence of 16‰ (D'Agaro et al. 2007)
- B: it has particular evolutionary characteristics such as co-circulation of multiple lineages at one time. It has slower HA1 evolutionary rates than influenza A viruses, and its evolution seems to be related with insertion-deletion mechanisms and genetic reassortment (Lindstrom et al. 1999). The analysis of the influenza B HA1 genes revealed the existence and co-circulation of two distinct lineages since the mid 1980s until 1990: B/Victoria/2/87 (Vic87) and B/Yamagata/16/88 (Yam88) lineages (Rota et al. 1992) so the B viruses was distinct in Victoria-liked and Jamagata-liked.

1.4 INFLUENZA EPIDEMIOLOGY

Influenza virus is one of the major causes of disease worldwide. During the annual influenza outbreaks, it is estimated that 5-15% of the world's population are infected resulting in one million deaths every year (WHO 2002; Yewdell et al 2002).

In the northern hemisphere the annual influenza outbreaks usually starts during the winter months. In the tropic and subtropics on the other hand, influenza virus is isolated all year around. Is not completely clear why outbreak of the flu occurs seasonally than during all the year. The possible explanation could be that in the winter people are indoors more often and they are in close contact promoting the transmission person to person, or could be the cold temperature and the humidity.

An alternative explanation to motive the seasonality of influenza could be a decrease of vitamin D levels, especially on the skin (Cannell et al. 2006).

The number of suspected influenza cases in periods of known influenza spread, designated Influenza like illness (ILI) is a frequently used measure of epidemiological activity by international and national authorities (Fleming et al. 2000). The numbers of ILI cases are reported by general practitioners (GP) with patients suffering from typical influenza symptoms and the number of ILI is a good estimate of the magnitude of circulating influenza (Stephenson et al. 2002). The definition of ILI that corresponds best with laboratory confirmed influenza is a sudden on set of fever, cough and fatigue (Thursky et al. 2003). Although the influenza incidence can vary strongly between seasons the estimate for influenza related deaths worldwide is about 1 million people each year and in USA about 60-70% of deaths occur in people above 65years old. However, the total number of influenza related deaths worldwide is difficult to estimate, due to a lack of knowledge about influenza epidemics in developing countries. During an influenza outbreak there is substantial morbidity.

1.5 AVIAN FLU

The virus was not classified as influenza virus until 1955, yet, outbreaks before the recognition were already described.

By middle of the twentieth century, highly pathogenic avian influenza was diagnosed in Europe, Middle East, Russia, Asia, North Africa, North and South America.

During this time also, a milder form of the disease was recognized in chickens, domestic ducks and turkeys causing respiratory distress (Thang et al 2003).

In 1972, surveillance of Newcastle disease in migratory birds led to coincidental isolation of AIV (Avian Influenza Virus) but there were reports of AIV outbreaks in wild aquatic birds in 1961 in South Africa (Slemons et al. 1974).

Surveys revealed that many wild birds were positive for AIV infection but did not show any clinical sings of the disease. It was established, since then, that healthy wild birds are primordial reservoirs of AIV. They harbour the virus without causing disease to them. These birds act as silent reservoirs that can shed viruses in huge amounts which can further infect other poultry and mammals as well .

This was a very important finding because it led to an active surveillance of AIV in wild birds.

Avian influenza virus has been found in many wild bird species, most commonly in wild waterfowls with the highest frequency of isolation from mallard ducks.

Other families of wild birds that shed avian influenza virus are geese, swans, gulls, terns, waders, cormorants, quails. All 16 HA and nine NA subtypes are found in these birds. The mode of transmission is through feco-oral route (Fouchier et al. 2005). During migration of these birds, they can infect other waterfowls.

For instance, in 1998, five hemagglutinin subtypes (H2, H3, H6, H9, and H12), six neuraminidase subtypes (N1, N2, N4, N5, N6, and N8), resulting to nine HA-NA combinations were isolated from the resident ducks in the eastern shore of Maryland. The role of migratory birds in the biological evolution of a low pathogenic to a high pathogenic form of the virus is evident in the current situation of the circulation of the subtype H5N1 in Asia, Europe, Middle East and some parts of Africa.

The H5N1 can be traced from 1996 when a high pathogenic (HPAI) form of this subtype was isolated from a farmed goose in Guangdong Province, China. In 1997, analysis of sequences of all eight segments of A/Goose/Guangdong/1/06 revealed that the HA gene of the virus was genetically similar to those H5N1 viruses isolated in wet market in Hong Kong during 1997 (Lu et al 2006; Xu et al 1999).

At the same time in Hong Kong eighteen persons were seriously infected, 6 fatally; this was the first evidence of H5N1 causing fatal disease in man (lethality rate of 33%).

The rapid killing of Hong Kong's entire poultry population (1,5 million of birds) had diverted a possible pandemic.

The virus was quiescent until 2003 when for first time, in a Thailand zoo, was isolated from tissue of two tigers and two leopards that eated chicken infected carcasses (Fig. 5).

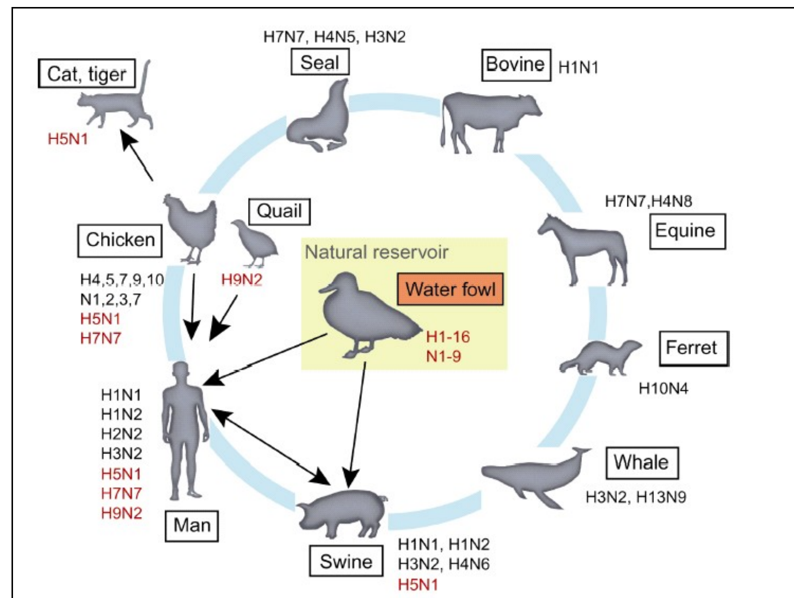


Fig 5. Ecological network of influenza viruses

From 2003 to 2005 were reported 112 cases of H5N1 infections with 57 deaths in humans in the NE Asia (Indonesia, Vietnam, Thailand, Cambodia-WHO oct 11 2005).

Fortunately H5N1 has not demonstrated easy transmissibility between animals and people and between persons (WHO Committee, 2008).

But the H5N1 had continued to expand its geographic range, infecting migratory birds in Russia and Kazakhistan in 2005 and during 2006 Bulgaria, Greece, Slovenia Iran, Austria, UK, Germany and Italy had reported isolations from swans.

Since May 2005, the numbers of both affected countries and confirmed cases of influenza A (H5N1) virus infection (340 cases as of December 14, 2007) have increased, in part because of the spread of clade 2.2 viruses across Eurasia and to Africa (Committee of Second WHO Consultation on Clinical Aspects of Human Infection with Avian influenza (H5N1), Jan 2008; Webster et al 2006) (Tab 2).

Recently the scientists focus your attention on a domestic ducks, which excreted H5N1 in its pathogenic form, therefore showed no signs of illness. This results showed that H5N1

can revert to a non pathogenic form in order to maintain itself in its natural host and that the domestic ducks in southern China had a central role in the maintenance of this virus, increasing the dissemination of the virus in Asia (Lu et al 2006; Isoda et al 2006).

Country	2003		2004		2005		2006		2007		2008		Total	
	C	D	C	D	C	D	C	D	C	D	C	D	C	D
Azerbaijan	0	0	0	0	0	0	8	5	0	0	0	0	8	5
Cambodia	0	0	0	0	4	4	2	2	1	1	0	0	7	7
China	1	1	0	0	8	5	13	8	5	3	0	0	27	17
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	0	0	43	19
Indonesia	0	0	0	0	20	13	55	45	42	37	10	8	127	103
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	1	1	0	0	1	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	1	1	102	48
Total	4	4	46	32	98	43	115	79	86	59	11	9	360	226

Tab 2 Cumulative Number of Confirmed Human Cases of Avian Influenza A/(H5N1) Reported to WHO (updated 12 Feb 2008).

Only viruses of the Influenza A genus have been isolated from birds and termed avian influenza (AI); but avian influenza can be divided into two groups: the virulent viruses causing highly pathogenic avian influenza (HPAI) with flock mortality as high as 100%, and the second group that causes milder respiratory disease (low pathogenic avian influenza LPAI). The HPAI was restricted to subtypes H5 and H7, although not all H5 and H7 viruses cause HPAI (Alexander et al 2007).

LPAI viruses replicate in limited tissue where host proteases, such as trypsin-like enzymes, are found. HPAI viruses possess inserted multiple basic amino acid residues in their HAO (HA uncleaved) that permits a cleavage of HAO in HA1 and HA2 by ubiquitous proteases such as furin. For this reason HPAI viruses can replicate in a broad range of tissues, showing an high aggressiveness (Isoda et al 2006).

In particular LPAI has a single Arg at cliveage site and an another basic aminoacid at position -3 or -4, this molecular feature permits the attack of only trypsine-like enzymes and thus restricts to replication at sites in the host where those enzymes are found (respiratory and intestinal tract) (Stieneke-Grober et al 1992). The HPAI viruses possess

inserted multiple basic amino acids (Arg and Lys) attackable by ubiquitous proteases (Alexander et al 2007).

Further investigation revealed that pathogenicity is also determined by aminoacids 97,108,126,138,212 and 217 of HA and by an additional glycosilation site within the NA protein globular head (Hulse DJ. et al 2004).

The NS (Non Structural) gene also contributes to pathogenicity by disarming the interferon-based defence system of the host (Russell et al. 2005).

In particular in carboxy terminus of NS1 of the HPAI viruses there is a sequence Glu-Ser-Glu-Val (ESEV), by contrast in low virulent viruses there is a different sequence (Karen et al. 2006).

Learning the pathogenetic features and the precise molecular changes of AI viruses that allow the crossing the species barrier is essential to prevent a possible pandemic in people.

A deletion in the stalk of the NA molecule and increased glycosilation of the HA globular head are thought to be associated with adaptation to chickens.

The HA proteins is determinant to the host contact and in the attachment to it.

The HA of avian influenza contain Gln226 and Gly228 which form a narrow receptor binding the α2,3 sialic acid. The human HA contain Leu226 and Ser228 which prefers binding the α2,6 sialic acid. The avian virus strains gained human transmissibility altering the binding site sequence preferring the human receptors (Russell et al. 2005).

The virus responsible for the 1918 pandemic was a H1N1 virus that resembled swine viruses (Reid et al. 1998; Tumpey et al. 2004), although a more methodical sequencing revealed that the virus most likely was transmitted to man from an avian source *in toto* and was not a reassortant (Taubenberger, et al 2005; Reid, et al 2004).

Cell surface receptors for both human and avian influenza viruses were identified in pig trachea, this could induce to consider the pig as a vessel of reassortment for human and avian viruses.

1.6 THE IMMUNE SYSTEM AND IMMUNITY OF INFLUENZA

The immune system can be divided into two interconnected parts, the innate and the adaptive system. The innate system responds to every antigen similarly and does not generate any immunological memory. The main function of the innate immune system is contain the pathogen until the adaptive immune system is activated and fully functional. The adaptive arm of the immune system has the capacity to selectively identify a particular antigen and a memory response is induced to allow a more rapid response upon re-encounter of the same antigen.

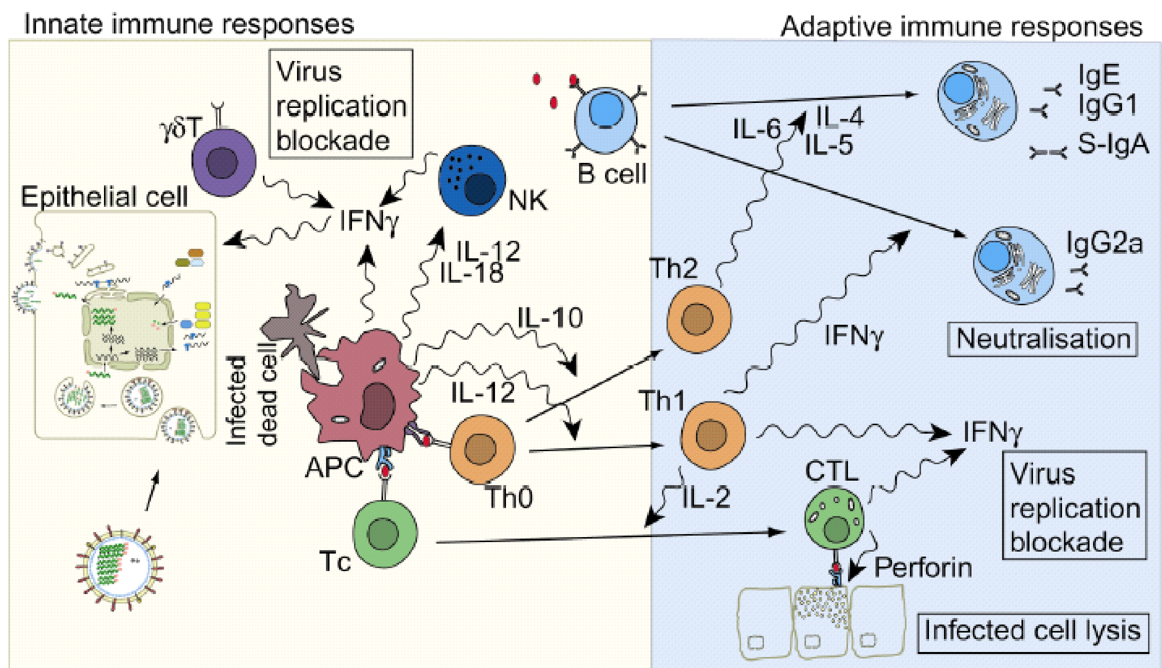


Fig. 6 Human immunity system

The protagonists of innate immunity are NK (Natural killer) cells, the neutrophiles, the basophiles, the macrophages, the cytokines and the complement.

The adaptative immunity consists of humoral component and cell-mediated one.

The cell-mediated component is composed by T-cell, CD4 (T-helper) and CD8 positive (T-cytotoxic) while the humoral response consists of B-cells that produce and secrete antibodies.

The first line of defence against all respiratory pathogens is an intact mucosal membrane with the cilia and mucosal secretions that remove foreign particles. Secreted interferon (IFN) from infected cells can turn on a range of antiviral genes in nearby cells (Garcia-Sastre 2001).

If the virus succeeds to enter the cell and starts to replicate, a range of anti viral genes are turned on. One important protein that is activated is the protein kinase R (PKR) (Ito et al 1999).

This protein recognises dsRNA, which does not normally exist in a cell, and is therefore utilised as a hallmark of a viral infection.

Recently, a toll like receptor (TLR)–7 in the endosomes was found to bind influenza (ss)RNA and induce IFN-α production (Diebold et al 2004)

Natural killer (NK) cells are involved early in fighting infection and an increase in NK activity is detected after influenza infection (Lewis et al 1986). NK cells have several functions; producing cytokines (e.g. IFN-γ) and mediating cellular cytotoxicity.

The Nkp46 and Nkp44 receptors found on NK cells are reported to bind HA (Mandelboim et al 2001).

Also macrophages, neutrophiles, basophiles and mast cells are involved in early response to infection. A complex mechanism is activated, including opsonisation of antigen, cytokines secretion, phagocytation and a important inflammatory response.

Another system linking the innate and the adaptive immune system is the complement system. Complement is important in resolving and solubilizing antigen-antibody complexes facilitating their elimination. The complement system also recruits phagocytic cells by acting as an opsonising and inflammatory initiator. Additionally, complement together with antibody, permeabilizes membranes and contributes to the destruction of target cells.

The cell-mediated immune response consists of two main cell lineages, CD4 and CD8 positive T-cells. The cells have a T-cell receptor, which can recognise antigen that is bound to the major histocompability complex (MHC) II on professional antigen presenting cells. After recognising an antigen, the cells begin to divide and give rise to effectors cells, whose main task is to secrete cytokines :Th2 that produce IL-4, IL-5, IL-6 and IL-10, which stimulate B-cells to produce antibody and induce a humoral immune response and Th1 cells that produce cytokines as IL-2 and IFN-γ.

CD8+ T-cells (also known as cytotoxic T-cells, Tc) and macrophages are activated and supported by Th1 cells.

After an antigen is presented by MHC I and recognised by the TcR, the activated CD8+ cell differentiates into a CTL (Cytotoxic T Lympocites).

The mechanism of cell lysis consists in firstly, the perforin/granzyme mediated pathway.

Perforin forms a pore in the target membrane allowing the granzyme pass into the target cell, leading to cell death.
Perforin activity has been shown to be important in protection from influenza infection as knock-out mice for perforin (-/-) show an increased susceptibility to influenza infection and prolonged viral shedding (Liu et al 2003).
The humoral immune system consists of B-cells that produce and secrete antibodies. After encountering an antigen, B-cells differentiate into plasma cells and memory cells. This process that is aided by cytokines produced by Th-cells (IgA is the major class of immunglobulin in the mucosa.
Antibody secreting cells, located in the mucosal tissue synthesise a peptide called J chain, which allows the IgA to dimerize and be actively transported across the mucosa, utilizing the pIgR (a poly immunoglobulin receptor) on epithelial cells.
The IgG2a subclass is important in resolving viral infection and is indeed detected at high serum antibody concentrations after a range of viral infections including influenza.
Mucosal immunity is not an independent part of the immune system, rather a function of the innate, humoral and cell-mediated acting in concert.
In the case of influenza, the cells of the mucosa are the site of both infection and the immune response to the virus IgA is constantly secreted in its dimeric form, dIgA, across the epithelial surfaces of the mucosa. Epithelial cells have (pIgR) that binds antibody containing a J chain and the antibody is secreted after a protease has cleaved the pIgR, leaving secretory component (SC).
This dIgA with bound SC is termed S-IgA. S-IgA and the passively derived IgG from serum neutralise virus by forming antigen-antibody complexes, which block the receptor-binding site on HA. S-IgA can also bind viral proteins when it is transported through the epithelial cell (intracellularly) and thereby reduce the efficiency of the viral assembly.
The consensus now emerging is that IgA is important in immunity of the upper respiratory tract whereas IgG prevents viral pneumonia, clinical illness and viral shedding (Renegar, et al 1991; Tamura, et al 2004).
In the lower respiratory tract, IgG more readily diffuses across the alveolar wall than across the mucosa and the pIgR, vital for S-IgA secretion, is only sparsely expressed in the lungs.

1.7 THE GENETIC VARIABILITY OF THE VIRUS

The influenza virus is able to evade the host immune system as it continuously undergoes antigenic evolution through the genetic processes called ANTIGENIC DRIFT and ANTIGENIC SHIFT.

Antigenic drift

The three influenza genera differ in the magnitude of genetic variability but all genera undergo mutations leading to changes in the amino acid sequence, a process called antigenic drift.

New drifted influenza A variants replace previously circulating strains each year causing the seasonal epidemic, whereas influenza B and C accumulate fewer mutations and they are more antigenically stable.

The slower evolution of influenza B and C viruses may be attributed to having a single host to replicate in. A hypothesis explaining this has been proposed; both influenza B and C viruses have been coevolving with humans longer and have undergone host specific adaptations.

Antigenic drift is the gradual evolution of viral strains due to frequent mutations and it occurs on average 2-8 years in response to selection pressure to evade human immunity (Carrat et al 2007, Smith et al 2004, Koelle et al 2006).

The process of antigenic drift involves point mutations within antibody-binding sites in the HA protein, the NA protein, or both, which potentially occur each time the virus replicates (Boni et al 2004).

Influenza virus such as all RNA viruses in general usually have very high frequencies of copy errors during replication as the RNA dependent RNA polymerase does not have the ability to proof read. The average number of mutations in influenza virus per genome per replication cycle is 1.0, compared to 0.0027 for yeast (*Saccharomyces cerevisiae*). More than two mutations per genome per replication often lead to extinction of a species (Drake et al 1998).

Influenza viruses are unique among the respiratory viruses as they have a substantial antigenic variation. The general mutation rate for influenza A HA and NA is 0.4% and 0.7%, respectively, per year (Smith et al 1987).

Mutations, especially in the HA which contains the main antigenic determinants; allow the virus to escape the host’s immunological memory.

Most of these mutations are 'neutral' as they do not affect the conformation of the proteins; however, some mutations cause changes to the viral proteins such that the binding of host antibodies is affected. Consequently, infecting viruses can no longer be inhibited effectively by host antibodies raised to previously circulating strains, allowing the virus to spread more rapidly among the population.

Frequency of nucleotide and amino acid substitutions for the haemaglutinnin gene and protein, respectively, for A (H3), A (H1) and B influenza virus strains [20,22–24]

Influenza virus type	Nucleotide substitutions (per site per year)	Amino acid substitutions (per site per year)
A (H3) [22]	0.0057	0.0097
A (H1) [23]	0.0038	0.0058
B[a] [24]	0.0014/0.0024	0.0022/0.0034

[a] The two different values correspond to results from co-circulating lineage III and lineage II influenza B viruses, respectively.

Tab.3 Frequency of nucleotide and amino acid changes for the HAs

Antigenic drift occurs in all strains of A and B viruses, although the observed evolutionary patterns vary dependent on the strain. For influenza A (H1) and B viruses, drift variants often co-circulate with multiple co-existing lineages, allowing the re-emergence of old strains. In contrast, influenza A (H3) subtype viruses undergo antigenic drift much more often and the new variants tend to replace the old ones.

In line with this, fixation rates have been calculated that indicate how often nucleotide and amino acid substitutions occur in the different virus types, with the rates being highest for A (H3) (Tab. 3). Nucleotide substitution rates alone do not tell the whole story, however, as some regions of the surface proteins are more susceptible to change than others. Indeed, as many as 35% of the substitutions have been reported to occur at only 18 of the 329 codons of the A (H3) virus. The fixation rate at these 18 sites is 0.053 substitutions per site per year, revealing the importance of a small group of codons to the evolution of the influenza virus.

Five antibody-binding sites have been located in this region thus antigenic drift involving non neutral point mutations of these epitopes can be expected (Treanor 2004). In particular amino acids changes could comport a variation of the glycosilation sites and create escape mutants.

The glycosylation has a variety of important functions including receptor binding, infectivity, virus release and neurovirulence HA undergoes post-translational, host-cell

dependent glycosylation that is crucial to the proper folding and trafficking of the molecule during infection. Carbohydrate positioned around the globular head can mask antigenic sites from immune recognition (Wagner et al. 2000).

Antigenic shift:

The antigenic shift consists in a emerge of a new virus that have never been present in human circulation, results from the replacement of HA (and less frequently NA) subtype with novel ones (Glezen et al 1996). This phenomenon causes the pandemics.

It occurred only in Influenza A viruses and it is estimated to occur approximately three times every 100 years (Potter et al 2001) in fact three pandemics occurred during the 20th century (1918, 1957 and 1968).

There are 3 theories as to how antigenic shift arise: the reassortment, the recirculation of existing subtypes and a gradual adaptation of animal viruses to human transmission (Fig. 7).

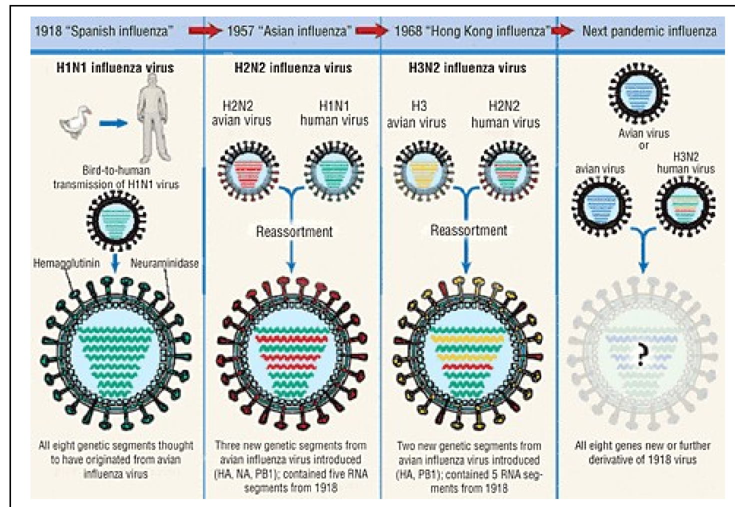


Fig. 7 Antignic shift

The reassortment is the most important process that contributes to major shift in influenza antigenicity and consists in a mixing of genetic material between different viral strains.

It occurs due to co-circulation of different influenza A subtypes and influenza A and B viruses (Carrat et al 2007) and it is of particular importance in evolution of H3N2 viruses (Schweiger et al 2006). Genetic reassortment is also possible between co-infecting influenza A subtypes from different species; it is feasible that the reassortmen between human and avian virus strains will produce a virulent one.

Once a virus undergone antigenic shift it however remains susceptible to antigenic drift as occurs with any influenza virus. Currently the scientists though that the highly pathogenic A/H5N1 avian influenza strain submitted to antigenic drift, could acquire the human to human transmissibility, causing a human pandemic (Smith. et al 2006).
At today, fortunately, although A/H5N1 is undergone considerable drift since 1996, it not yet able to diffuse between persons.

1.8 THE SURVEILLANCE of INFLUENZA VIRUSES

The burden of annual influenza infection is substantial, both in terms of illness, lives lost and economic impact on society.

Continued and focused research efforts are needed in order to understand the immunology, epidemiology, ecology and the aetiology of influenza viruses. Despite many years of studies, we still lack some knowledge about influenza and the infection it causes, and the subsequent immune response.

The most important tools to control and to try to limit the influenza incidence are the surveillance, the antiviral drugs and the vaccines.

All those measures are connected each other and they are continuously evolving.

What we know is that the vaccine that is produced today provides a satisfactory protection with a protective effectiveness of 70-90 percent against laboratory confirmed influenza in healthy adults.

However, there is still room for improvement; especially in the elderly the efficacy is not optimal. This is also true for the antiviral drugs as they too must be refined and new drugs need to be developed to fight resistant viruses.

Integrated clinical and laboratory based surveillance is the best tool to control the virus behaviour, to prevent a possible pandemic, and to update the vaccine composition and the antiviral drugs to increase their effectiveness.

In 1944 Thomas Francis jr discovered the first life vaccine for influenza, posing the first great step towards prevention.

The **WHO Global Influenza Surveillance Network** was established in 1952 and the first international Influenza centre was based in London. In 1957, virus isolates and serology reports from throughout the nation began to be collated centrally, though these were generally few in number and not always delivered promptly. In 1967, information began to be gathered from patients consulting in sentinel practices (Flemming DJ et al. 2003).

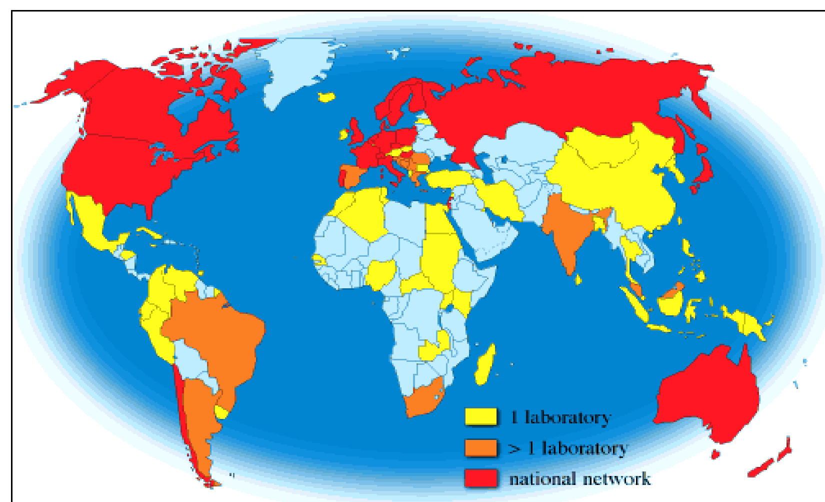
Indeed there was need for obtaining timely information and opened up opportunities for estimating the impact of influenza in primary care. When sentinel practice-based surveillance was first introduced in the UK, an attempt was made to distinguish clinically between influenza-like illness (ILI) and what was referred to as epidemic influenza. Data on both were reported separately from other respiratory infections such as acute bronchitis.

From the surveillance perspective, however, reports of an increased incidence of ILI provided an earlier indication of influenza epidemics.

During the 1970-1980 years also in Netherlands, Scotland, Portugal, France, Spain and other European states were established surveillance scheme similar to UK.

Influenza virus infection may cause a spectrum of clinical illness well beyond the usual ‘case definition’ wherefore it’s important to distinguish the ILI cases from the ARI (acute respiratory infections) ones. Ideally, both should be separately recorded.

At present the WHO network comprises 4 WHO Collaborating Centres (WHO CCs: in Japan, Australia, USA and in England) and 122 institutions in 94 countries, which are recognized by WHO as WHO National Influenza Centres (NICs). These NICs collect specimens in their country; perform primary virus isolation and preliminary antigenic characterization. They ship newly isolated strains to WHO CCs for high level antigenic and genetic analysis, the result of which forms the basis for WHO recommendations on the composition of influenza vaccine for the Northern and Southern Hemisphere each year. The WHO Influenza Surveillance Network serves also as a global alert mechanism for the emergence of influenza viruses with pandemic potential.



SURVEILLANCE IN EUROPE

In order to monitoring the influenza strains in UE in 1996 the European Influenza Surveillance Scheme (EISS) was created.

The main objective of EISS is to help reduce the burden of disease associated with influenza in Europe by collecting and exchanging timely information on influenza activity.

In 2003 as part of the evolution of EISS, the Community Network of Reference Laboratories for Human Influenza in Europe (CNRL) was established.

As of October 2005, the CNRL comprises 38 laboratories of which at least one is located in each of the 28 countries in EISS. All WHO National Influenza Centres in the 28 countries are included in the CNRL.

The laboratories in the CNRL should be able to perform direct detection, culture, typing, subtyping and strain characterisation of influenza viruses, diagnostic serology and the creation of archives for clinical specimens and virus isolates. Influenza surveillance data is published by EISS on its website (www.eiss.org) and in the EISS weekly electronic bulletin and integrates virological information produced by the laboratories with information on the incidence of influenza-like-illness and/or acute respiratory infection.

SURVEILLANCE IN ITALY

The Italian surveillance Network was called Influnet (www.influnet.it), coordinated by Health Ministry.

The Regional Sanity institutions, with the Inter-University Centre for Research on Influenza and Viral Infections (CIRI-IV) and the Istituto Superiore di Sanità (ISS), collaborate to performe the virological and thepidemiological surveillance in Italy (D’Agaro et al 2007).

1.9 OTHER RESPIROVIRUSES

Adenovirus:

Human adenoviruses (HAdVs) belong to a group of non-enveloped icosahedral DNA viruses that infect a broad range of vertebrate species (Davison et al 2003) and replicate in the nucleus of the cell .

These viruses are included in the genus Mastadenovirus of the family Adenoviridae and comprise more than 50 serotypes clustered in 6 species designated A through F. Primary targets of HAdV infection are the respiratory, gastrointestinal and urinary tracts as well as the eye (Goncsalves et al 2006; Kojaoghlanian et al 2003).

Infections of immunocompetent individuals usually stay localised and take a subclinical course. Occasionally, more severe disease affects the brain, heart, kidney or liver. Species of HadVs display a certain degree of tissue specificity. Accordingly, several clinical syndromes are associated with particular groups of HAdVs. For instance, HAdV serotypes 2 (HAdV-2) and 5 (HAdV-5) from species C are responsible for 5% to 10% of upper respiratory tract illness observed in children. In contrast, species F HAdV-40 and HAdV-41 are typically associated with gastrointestinal infections and comprise the major cause of HAdV-induced diarrhoea. Furthermore, several species D HAdVs including serotypes 8, 19 and 37 are linked to a relatively severe and highly contagious form of epidemic keratoconjunctivitis. Normally, HAdV infections are self-contained.

However, during the past two decades the increase in the number of immunocompromised individuals due to a rise in organ and bone marrow transplantations and the HIV/AIDS pandemic has resulted in a higher frequency of HAdV-related mortality. In this regard it is of note that HAdVs, such as the species B serotype 35, rarely detected in the healthy population, can be readily isolated from individuals with impaired immunity (Kojaoghlanian et al, 2003).

In parallel, HAdVs are becoming more prominent through continuous development as gene transfer systems or vectors. In fact, several of the most versatile and efficient gene transfer systems are based on recombinant HAdVs.

Long inverted terminal repeat (ITR) sequence in which the viral origin of replication is embedded. On the basis of the kinetics of viral gene expression, the HAdV DNA can be divided into early properties.

The prevalence of AdV infections is high, as revealed by serological studies (Shenk T. et al 2001). The most common sites of infection in immunocompetent individuals include the gastrointestinal tract, the upper respiratory tract, and the eyes (Mitchell et al. 2001). In the

presence of a functional immune system, AdV infections are not associated with life-threatening disease, but latent infections with persistence of the viral genome, involving particularly species C, have been found in about 80% of the individuals investigated (Garnett. et al. 2001; Ebner et al. 2005).

The virus capsid is composed of three different proteins: 12 fiber attachment proteins associated with 12 penton base proteins, which are involved in the recognition and the interaction with cellular receptors, and 240 hexon proteins comprising 919 to 968 amino acids (aa), which form the main capsid component the hexon protein are projected away from the virus surface and contain serotype-specific epitopes. Owing to its hypervariable regions, the hexon protein is the most important part of the adenovirus proteome for the classification and recognition of individual serotypes.

Respiratory syncytial virus (RSV)

RSV is a negative-sense, enveloped RNA virus. The virion is variable in shape and size (average diameter of between 120 and 300 nm), is unstable in the environment (surviving only a few hours on environmental surfaces), and is readily inactivated with soap, water and disinfectants. It is the most common cause of bronchiolitis and pneumonia among infants and children under 1 year of age. Illness begins most frequently with fever, runny nose, and cough. RSV also causes repeated infections throughout life and severe lower respiratory tract disease especially among the elderly or among those with compromised cardiac, pulmonary, or immune systems.

Human RSV exists as two antigenic subgroups, A and B. The F (fusion) and G (attachment) proteins are two transmembrane envelope glycoproteins and are the most important antigens that induce RSV-neutralizing antibodies.

Two antigenic soubgroup were distinguished on the basis of antigenic dimorphism which is observed in whole genome but in particular in the ectodomain of G protein.

2. AIMS OF THE STUDY

The virological and epidemiological surveillance of influenza viruses is essential to understand the ecology and epidemiology of influenza viruses, to determine the multifaceted nature of its virulence and to assess the evolutionary relationship between the existing and emerging strains.

The objectives of this study were:

- To carrying out a point of epidemiological surveillance of human influenza in Friuli Venezia Giulia comparing three consecutive seasons.
- The second objective of the study was to analyse the sequences of HA1 region of the influenza strains to understand the virus evolution. We also performed a phylogenetic evaluation on the Friuli-Venezia-Giulia strains to predict the viral evolution.
- To verify the amino acidic changes in this region, with particular reference to the most variable sites of the molecule.
- Comparing the genetic results with the serological ones to identify similarities and discrepancies.
- The third point was to verify the role of other respiratory viruses in the ILI aetiology.

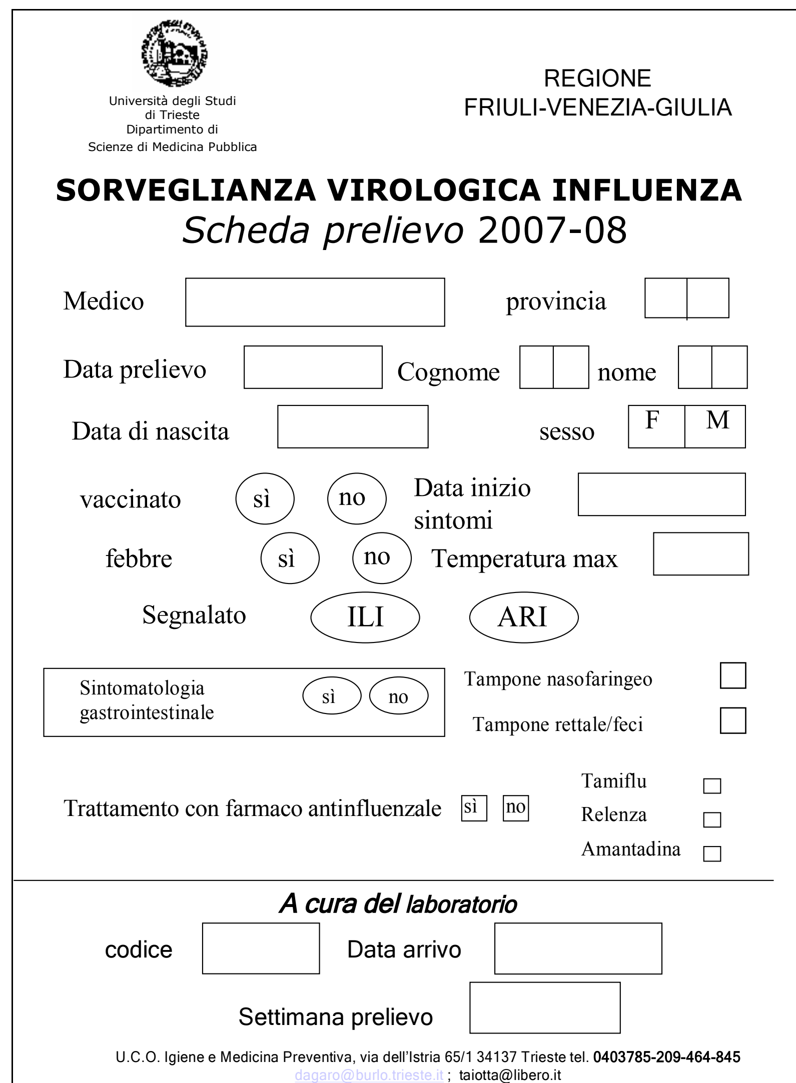
3.1 SURVEILLANCE NETWORK AND SAMPLE COLLECTION

The virological surveillance in Friuli Venezia Giulia region is made up by a network of general practitioners and paediatricians (about 25) located in all regional territory. They survey about 2 % of total population reporting the cases of ILI and taking nasopharyngeal swabs from the patients for the viral evaluation.

The swabs called Virocult® (Medical Wire and Equipment, Corsham, U.K.) are specific for virus's collection and they are provided to the physicians by our laboratory .

Than they compile a surveillance schedule (provided at first of every season) with the patient dates, symptoms and other informations and mail the samples to our lab (Fig. 1).

The samples collected in Trieste are taken directly.



Università degli Studi di Trieste
Dipartimento di Scienze di Medicina Pubblica

REGIONE FRIULI-VENEZIA-GIULIA

SORVEGLIANZA VIROLOGICA INFLUENZA
Scheda prelievo 2007-08

Medico ___ provincia ___

Data prelievo ___ Cognome ___ nome ___

Data di nascita ___ sesso F M

vaccinato sì no Data inizio sintomi ___

febbre sì no Temperatura max ___

Segnalato ILI ARI

Sintomatologia gastrointestinale sì no

Tampone nasofaringeo ☐
Tampone rettale/feci ☐

Trattamento con farmaco antinfluenzale sì no
Tamiflu ☐
Relenza ☐
Amantadina ☐

A cura del laboratorio

codice ___ Data arrivo ___

Settimana prelievo ___

U.C.O. Igiene e Medicina Preventiva, via dell'Istria 65/1 34137 Trieste tel. 0403785-209-464-845
dagaro@burlo.trieste.it ; taiotta@libero.it

Fig. 1 Influenza surveillance schedule

3.2 PROCESSING OF SAMPLES

The swabs were re-suspended in Hank's Balanced Solution and vortexed for few seconds. Than the solution transferred into 15 ml polypropylene tubes and centrifuged for 10 minutes at 1800 rpm.

200 µl of this seupernatant is disposed into 1,5 ml tube for the acid nucleics extraction, the rest is supplemented with a mixture of penicillin, streptomycin and amphotericin for almost 30 minutes at 4°C temperature. Than 200 µl is sowed on MDCK-SIAT1 (Canine Cocker Spaniel Kidney Sialic Acid Over Expression) layer into cell cultures tubes.

Sowing on specific cell lines is a good way for isolation and to study the influenza strains and MDCK-SIAT1 represent a suitable system for viral isolation and also to testing the sensitivity of human influenza virus to neuraminidase inhibitors (NAI) due to their over expression of sialyl-alpha2,6-galactose moieties. This cell line has been derived by the stable transfection of MDCK cells with the cDNA of human 2,6-sialtransferase (SIAT1). The cells express two fold-higher amounts of 6-linked sialic acids and two fold-lower amounts of 3-linked sialic acids than parent MDCK cells.

The medium used to cell growth cointains: (vol 100 ml)

- 2 ml "Geneticin Liquid (G418 Sulfate)";50mg/ml **(**GIBCO)
- 10 ml Fetal Bovine Serum (FBS) (SIGMA)
- 1 ml L- Glutamine (conc. 200mM) (SIGMA)
- 0.1 ml antibiotics (streptomycin, 100 mg/ml and penicillin, 100 U/ml). (conc.:100000 U/ml) (SIGMA)
- 86.9 ml SIGMA "Dulbecco's Modified Eagle's Medium" (SIGMA)
- 1 ml Na Pyruvate (SIGMA)

The medium used for infection is composed by: (vol 100 ml)

- 1,3 ml bovine seric albumin (BSA) (SIGMA; conc. 7,5%)
- 1 ml L- Glutamine (conc. 200mM) (SIGMA)
- 0.1 ml antibiotics (streptomycin, 100 mg/ml and penicillin, 100 U/ml). (conc.:100000 U/ml) (SIGMA)
- 97,55 ml "Dulbecco's Modified Eagle's Medium" (SIGMA)
- 1 ml Na Pyruvate (SIGMA)

- 50 μl TPCK-trypsin (conc. 1 mg/ml) (0,5 μg/ml) (SIGMA)

The trypsin presence is to promote the viral spread.
At the same time they sowed also to other cell lines (LLC-MK2 and Hep-2) to isolate other respiroviruses like Adenovirus and RSV (Respiratory Syncytial Virus).
The cell coltures were checked daily by microscope to see an eventual cytopathic effect of the virus.

3.3 Viral isolation and immunofluorescence assay (IFA):

Specimens treated with antibiotics were inoculated into tubes containing monolayers of HEp-2 (for Adenovirus and RSV isolation) and MDCK-SIAT1 for Influenza viruses). The cultures were examined every day for Influenza Adenovirus and RSV cytopathic effects.

For Adenovirus and RSV we had performed the direct IFA using monoclonal FITC-conjugated antibodies (Argene Biosoft).

We had transfer the cell colture into 15 ml polypropylene tubes and centrifuged it for 10 minutes at 1800 rpm.

Than the supernatant was conserved in cryovials on -80°C while the pellet was re-suspended with 200-500 µl of physiological solution. Than, we had spotted some cellular suspension on the glass support until the drop was dried.

Than the cellules were fixed in acetone/ methanol (+4°C) solution for 10 minutes, than we added 100 µl of antibody solution (for Adenovirus and RSV) on every sample and after an incubation of 30 minutes at 37°C we had make three washings of 5 minutes with PBS.

In the last washing we added in the PBS 100 µl of Evan's Blue.

For Influenza viruses indirect IFA was used, therefore, after the antibodies incubation (WHO) had incubated the samples with 100 µl FITC for 30 minutes at 37°C. Than the samples were washing three times in PBS. At last, when the glass was dried we had cover it with a cover glasses and a drop of glycerol. The specimens were examined under a fluorescence microscope with a halogen lamp (Fig 2a, 2b).

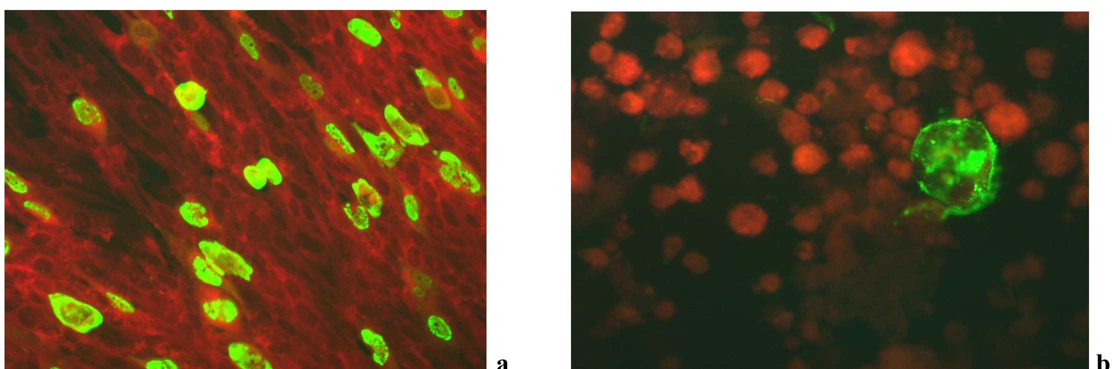


Fig. 2a) IFA positve for Adenovirus 2b) IFA positve for RSV

3.4 HEMAGGLUTINATION ASSAY

To confirm a viral isolation of Influenza strains we had performed the Hemagglutination Assay.

We had used a 96 wells plate, testing every samples in double. Using a multichannel pipettor we placed 25 µl of PBS (Phosphate-buffered saline) starting by the second well.

Than we placed 25 µl of supernatant of MDCK-SIAT1 positive culture on the first four wells, than, starting by the second well, remove 25 µl of fluid and placed into the third well, gently mixed by pipetting, This was repeated from the second row of wells until the last, thus producing two-fold serial dilutions. The last 25 µl of the mixture were discarted.

Than we added 25 µl of PBS on all the wells and 50 µl of red blood cells (RBCs)Rh negative solution (0,5% in PBS). The plate, briefly agitated, was incubated at room temperature for 45 minutes to 1 hour. The presence of virus produced agglutination of the red blood cells and the mixture in the well appeared diffusely pink in color. Negative results were indicated by the presence of red "button" on the bottom of the well due to settling of the red blood cells (Fig. 3).

All the virus strains collected were named according with the guidelines for influenza virus nomenclature establish by the WHO (for example A\trieste\01\08) and conserved in vials at -80°C of temperature to perform any other test.

Red blood cells were maintained in Alsever's solution at +4°C.

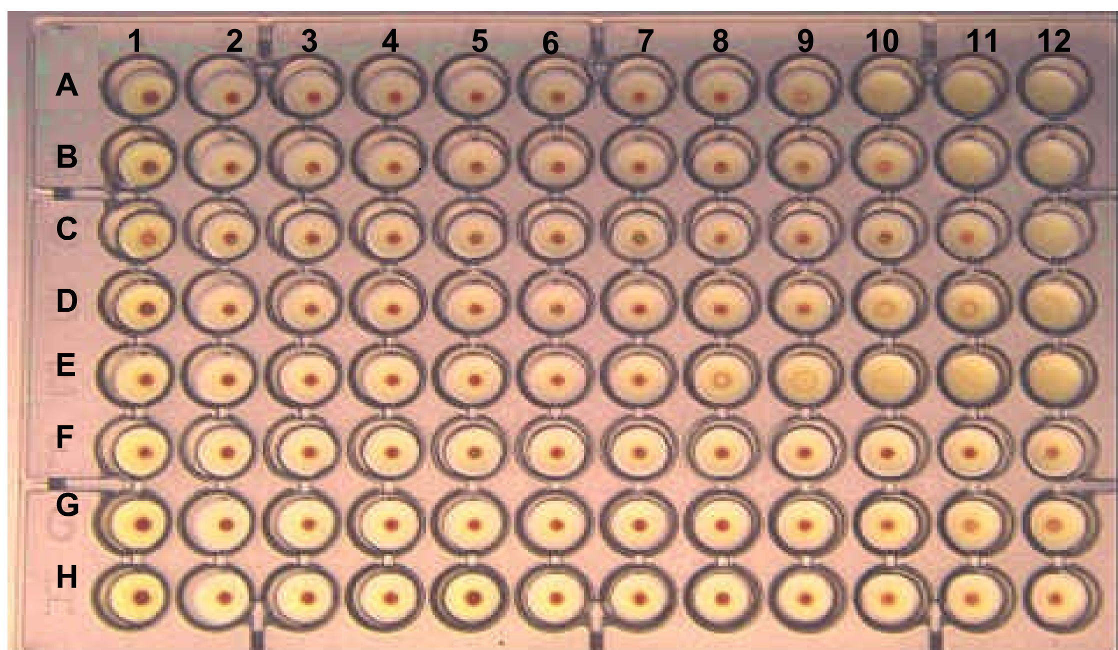


Fig. 3 HA assay plate

Preparation of reagents and solutions

PBS

Firstly the stock 25 times concentrated (25X) phosphate buffer was prepared; for 100 ml it contains: 2.74 g dibasic sodium phosphate (Na_2HPO_4) and 0.79 g monobasic sodium phosphate monohydrate ($NaH_2PO_4 \times H_2O$).

To prepare PBS, mix and dissolve in deionised, distilled water, and q.s. to 1 litre: 40 ml of 25X phosphate buffer and 8.5 g of sodium chloride (NaCl).

After thorough mixing, check pH = 7.2 plus or minus 0.1. Adjust pH with 1 N NaOH or 1 N HCl, if necessary.

Than the solution was filtered to sterilize and stored at 4°C.

ALSEVER'S

Was weigh out, dissolved in distilled water, and q.s. to 1 litre: 20.5 g dextrose, 8.0 g sodium citrate dehydrate ($Na_3C_6H_5O_7 \times 2H_2O$), 4.2 g sodium chloride (NaCl), 0.55 g citric acid ($C_6H_8O_7$)

After thorough mixing, check pH = 6.1 plus or minus 0.1. Adjust pH with 1 N NaOH or 1 N HCl, if necessary. Than the solution was filtered to sterilize and stored at 4°C.

3.5 Hemagglutination inhibition test

The hemagglutinin (HA) protein agglutinates erythrocytes; hence, the derivation of its name. The traditional method for identifying influenza field isolates takes advantage of this property. Specific attachment of antibody to the antigenic sites on the HA molecule interferes with the binding between the viral HA and receptors on the erythrocytes.

This effect inhibits hemagglutination and s the basis for the hemagglutination inhibition (HI) test.

The virus isolates obtained by cell culture were characterized to HA-subtype with a hemagglutination inhibition assay using human erythrocytes and subtype-specific hyperimmune rabbit antisera raised against all known HA subtypes.

In general, a standardized quantity of HA antigen is mixed with serially diluted antisera, and red blood cells are added to determine specific binding of antibody to the HA molecule.

Treatment of Reference Antisera for Inactivation of Non specific Inhibitors

Reference antisera to influenza viruses must be treated with receptor destroying enzyme (RDE) to remove nonspecific inhibitors.

Reconstitute lyophilized reference antisera with sterile distilled H2O to volume indicated on label. Store reconstituted antisera at -20°C to - 70°C.

Reconstitute and store RDE (receptor destroying enzyme) with 25 ml physiological saline, 0.85% NaCl. Aliquot and store it at -20°C to -70°C.

Add 3 vol of RDE to 1 vol serum (0.9 ml RDE + 0.3 ml serum).This volume is sufficient for testing 50-55 field isolates.

Incubate overnight in a 37°C waterbath.

Than heat in a 56°C waterbath for 30 min to inactivate remaining RDE.

Allow antisera to cool to room temperature. Add 6 vol (1-8 ml) of physiological saline, 0.85% NaCl. The final dilution of antisera is 1:10.

HA Titration of Control Antigens and Field Isolates

On V-shaped 96-well microtiter plates, add 50 µl of PBS (pH 7.2) from 2 to 12 row.

Add 100 µl of each control antigen or field isolate to the first well (A1-F1) of the lettered rows except rows G & H. Prepare an RBCs control well in row H (H1) by adding 100 µl of PBS.

Make serial twofold dilutions by transferring 50 µl from the first well of lettered rows to successive rows. Discard the final 50 µl. Add 50 µl of RBCs suspension to each well on the plate. Mix the plate by a mechanical vibrator.

Incubate the plates at room temperature (22°C to 25°C). Check cell control for complete settling of RBCs.

Preparation of Standardized Antigen for the HAI Test and "Back Titration"

A "unit" of hemagglutination is not a measure of an absolute amount of virus, but is an operational unit dependent on the method used for HA titration. An HA unit is defined as the amount of virus needed to agglutinate an equal volume of a standardized red blood cell suspension.

Determine the volume of standardized antigen needed for the HAI test.

For example, 1 ml of antigen will test 5 sera, each of which is diluted in 8 wells, with 25 µl of antigen added to each well (5 sera X 8 wells X 25 µl = 1 ml of standardized antigen). Prepare an additional 1.0 ml additional volume for "back titration" and wastage.

The standard for the HAI test is 4 HA units of virus/antigen added to twofold dilutions of antisera. Since we are adding 25 µl of antigen in the test, we need a virus dilution that contains 4 HA units/ 25 µl or 8 HA units/50 µl. Calculate the antigen dilution by dividing the HA titer (which is based on 50 µl) by 8 because you wish to have 8 HA units/50 µl. For example, an HA titer of 160 divided by 8 is 20. Mix 1 part of virus with 19 parts PBS to obtain the desired volume of standardized antigen Add 0.1 ml antigen to 1.9 ml of PBS). Calculate and prepare dilution.

Keep a record of the dilution prepared. Perform a "back titration" to verify units by performing a second HA test using the standardized antigen dilution preparation. Store the diluted antigen at 4°C and use within the same day. Record the results.

Standardized antigens must have an HA titer of 4 HA units/25µl. This titer will hemagglutinate the first four wells of the back titration plate. If an antigen doesnot have an HA titer of 8, it must be adjusted accordingly by adding more antigen to increase units or by diluting to decrease units. For example, if complete hemagglutination is present in the fifth well, the virus now has a titer of 16 and the test antigen should be diluted twofold. Conversely, if hemagglutination is only present to the third well, the antigen has a titer of 4 and an equal volume of virus must be added to the test antigen as was used when the antigen was initially diluted. This will double the concentration of virus in the test antigen to give a titer of 8. Continue adjusting the concentration of antigen until 4 HA units/25µl (8

units/50µl) is obtained. It is acceptable for the final dilution to have complete hemagglutination in 3 or 4 wells.

HA-HAI Test: Identification of Field Isolates

Label appropriate microtiter plates with virus and sera names._Add 25 µl of PBS to wells B through H (B1 - H12) of each numbered_column.

A 50 µl of each serum diluted 1:10 or 1:40 to the first well of the appropriate numbered column. For example, serum #1 should be added to well A1 and well A8; serum #2 to A2 and A9, etc.

Add 50 µl of PBS to the first well of columns 6 and 7 (A6-A7) for cell control.

Prepare serial twofold dilutions of the treated sera by transferring 25 µl from the first well of numbered columns 1-12 to successive wells.

Discard the final 25 µl after row H. Add 25 µl of standardized control antigen #1 to all wells of a complete

set of diluted treated sera (Ex: A1 - H5). Continue with remaining standardized control antigens and test antigens. Add 25 µl of PBS instead of antigen to serum control plate.

Mix the contents of the plates by shaking on a mechanical vibrator for10 seconds.

Cover the plates and incubate at room temperature (22°C to 25°C) for15 min.

Add 50 µl of standardized RBCs to all wells. Mix as before.Cover the plates and allow the RBCs to settle at room temperature (22°C to 25°C) for the appropriate time according to the RBCs being used. Record the HAI titers. The HAI titer is the reciprocal of the last dilution of antiserum that completely inhibits hemagglutination. To identify a field isolate, compare the results of the unknown field isolates to those of the antigen control. An isolate is identified as a particular type or subtype if the field isolate reacts with one reference antiserum to a fourfold or greater HAI titer than to other antisera. The higher titer is assumed to be homologous.

In our lab the HAI test were performed in order to effect a general antigenic characterisation and to confirm the molecular one done by sequencing.

The specific antigenic characterization, to identify the antigenic identity of the strains compaining with the vaccine and reference strains is perormed in WHO Collaborating Centre for Influenza in London, because of Dr. Alan Hay's kindness.

3.6 RNA\DNA Extraction and RT-PCR

The nucleic acids extraction was performed using a kit called QIAmp Viral RNA Mini kit (Qiagen, Valencia, CA) and the following protocol.

In 1.5ml tube, 560 µl of the AVL solution (with RNA carrier) was added to 200 µl of sample, vortexed for 15 seconds and incubated for 10 minutes.

Than we had added 560 µl of Ethanol 100% and we vortexed the solution.

630 µl of mix was transferred to spin columns and centrifuged at 6000x g for 1 minute.

Than the eluated solution is discarded. The action was repeated with the remaining solution.

In the column was added to 500 µl of AW1 buffer and it was centrifuged at 6000x g for 1 minute, descanting the eluted solution.

Than in the column was added to 500 µl of AW2 buffer and it was centrifuged at 20000 x g for 3 minute, descanting the eluted solution.

At last the nucleic acids was eluted using 60 ul of buffer AVE, centrifugating the column for 1 minute at 6000 x g and were conserved on ice or at –80°C temperature.

RT-PCR and PCR :

To verify the presence of influenza viruses genome we performed RT-PCR and PCR (two steps), using a kit called INFLUCHECK (Euroclone).

The extracted RNA was submitted to simultaneous reverse transcription, and the first amplification of 4 different genome regions was carried out using the outer primers deduced from the Matrix Protein (MP) region of type A virus, the MP region of type B virus, type-1 HA region and type-3 HA region.(Zhang WD et al 1991).

2 µl RNA was mixed with 1 µl of each of A and B primers (or H1 and H3) and 46 µl of sterile water using RT-PCR Beads provided by the kit.

The tube was placed in the thermal cycler (GeneAmp PCR System Perkin Elmer 9700) and incubated on at 42°C for 45 minutes and at 95°C for 5 minutes then subjected to 30 cycles of 93°C for 1 minute for denaturation, 48°C for 1 minute for primer annealing and 72°C for 1 minute for polymerization. After 30 cycles, the sample was then further subjected to 72°C for 7 minutes before putting it to 4°C.

Then 2 µl of first amplification products underwent a second round of amplification with 1 µl of inner primers and 22 µl of sterile water using PCR Beads.

The tube was placed in the thermal cycler and incubated at 95°C for 5 minutes then subjected to 30 cycles of 93°C for 1 minute, 48°C for 1 minute and 72°C for 1 minute Then it was subjected to 72°C for 7 minutes before putting it to 4°C.

The PCR product was visualized by 8% polyacrilamide gel electrophoresis after ethidium bromide staining.

The fragment lenghts are: influenza A 401 bp, influenza B 302 bp, influenza HA3 591 bp and influenza HA3 944 bp.

3.7 Real Time PCR

We used two Real Time PCR protocols: one for the detection of Influenza A and B and ona to subtyping the influenza A into the different subtypes.

The reaction mix was prepare on ice and was the following:

25 µl Master Mix 2x,

1,25 µl 40x MS RNAse mix,

3 µl Primer Forward (600 nM)

3 µl Primer Reverse (600 nM)

0.2 µl Probe (200 nM)

15,25 µl RNAse free water

5 µl RNA

The Real Time PCRs were performed using TaqMan® One-Step RT-PCR Master Mix Reagents Kit (ABI, Applied Biosystem) in a total volume of 50 µl and at the following temperature conditions: at 42 °C for 40 minutes, 95°C for 10 minutes, then subjected to 45 cycles of 95°C for 15 seconds, 55°C for 30 seconds, 72 °C for 30 seconds.

The primers used were:

1) ref. http://www.cdc.gov/flu/ (Real Time RT-PCR Protocol for Influenza)

Flu A Forward GAC CRA TCC TGT CAC CTC TGAC

Flu A Reverse GGG CAT TYT GGA CAA AKC GTC TAC G
Flu A Probe1 TGC AGT CCT CGC TCA CTG GGC ACG
H1 HA Forward AAC TAC TAC TGG ACT CTG CTG GAA
H1 HA Reverse CCA TTG GTG CAT TTG AGG TGA TG
H1 HA Probe2 TGA YCC AAA GCC TCT ACT CAG TGC GAA AGC
H3 HA Forward AAG CAT TCC YAA TGA CAA ACC
H3 HA Reverse ATT GCR CCR AAT ATG CCT CTA GT
H3 HA Probe1 CAG GAT CAC ATA TGG GSC CTG TCC CAG
H5 HA Forward TGG AAA GTG TAA RAA ACG GAA CGT
H5 HA Reverse TGA TTG CCA GYG CTA GGG AAC T
H5 HA Probe1 2* TGA CTA CCC GCA GTA TTC AGA AGA AGC AAG ACT AA
H5HA Probe2 2* CAA CTA TCC GCA GTA TTC AGA AGA AGC AAG ATT AA
EuH7 HA Forward GCT TCA GGC ATC AAA ATG CAC AAG G
EuH7 HA reverse CAT TGC TAC YAA GAG TTC AGC RT
EuH7 HA Probe2 ACC ACA CTT CTG TCA TGG AAT CTC TGG TCC A
H9 HA Forward CAA GCT GGA ATC TGA RGG AAC TTA CA
H9 HA Reverse GCA TCT GCA AGA TCC ATT GGA CAT
H9 HA Probe1 CCC AGA ACA RGA AGG CAG CAA ACC CCA TTG
Flu B Forward TCC TCA ACT CAC TCT TCG AGC G
Flu B Reverse CGG TGC TCT TGA CCA AAT TGG

Flu B Probe1 CCA ATT CGA GCA GCT GAA ACT GCG GTG
RNP Forward AGA TTT GGA CCT GCG AGC G
RNP Reverse GAG CGG CTG TCT CCA CAA GT
RNP Probe1 TTC TGA CCT GAA GGC TCT GCG CG
All those probes were 5’ FAM and 3’ BHQ1.

2) Ref Valle L. et al. 2006
FLUAV_FOR 5’- ACA AgA CCA ATC CTg TCA CCT CT-3’
FLUAV_REV 5’- ggC ATT TTg gAC AAA gCg TCT AC-3’
FLUAV_TM 5’-FAM- CAg TCC TCg CTC ACT ggg CAC ggT(p)-BHQ1-3’
FLUBV_FOR 5’- CCA gTg ggA CAA CCA Ga-3’
FLUBV_REV 5’- TgC TCT TTC Cgg ggA Tg-3’
FLUB_TM 5’-JOE- ATC ATC AgA CCA gCA ACC CTT gCC (p)- BHQ1-3’

3.8 SEQUENCING

To characterize the influenza strains and in order to evaluate eventual modifications we had performed the sequence analysis of the globular head region of the HA protein (HA1 sub-unit) using a primer set kindly provided by Dr. Alan Hay, WHO Collaborating Centre for Influenza, London, UK.

After the RNA extraction (see section 3.5) from 200 µl of supernatant of positive cultures we had prepared cDNA incubating 5 µl of RNA, 1 µl of Primer (forward or reverse) of 20 pmol/µl concentration and 44 µl of sterile water, using Ready-To-Go™ RT-PCR Beads (Amersham Biosciences).

The thermal profile was: 42°C for 60°C and 95°C for 5 minutes.

Then the sequencing PCR was performed: 5 µl of cDNA was mixed with 1 µl each of forward and reverse primers (Sigma) of 20 pmol/µl concentration, 8 µl of dNTP's (200 µM; Promega), 5.0 µl of 10x buffer (ABI), 3.5 µl of MgCl2, 0.5 µl Gold Taq Polymerase (ABI) and 26 µl of sterile water.

The sample was incubated at 95°C for 10 minutes and then subjected to 45 cycles of 95°C for 30 seconds, 50°C for 30 seconds for primer annealing and 72°C for 2 minutes. After 30 cycles, the sample was then further subjected to 72°C for 10 minutes before putting it to 4°C. The amplicons (950 bp) were detected by agarose 1% gel running.

The primers used in the sequencing PCR were summarized below:

H3A1R1 GTCTAT VCAT TCC CTC CCA ACC ATT
H3A1F9 CAG GGG ATA ATT CTA TTA ACC ATG
H3HAF567 CTG AAC GTG ACT ATG CCA AAC AAT
H3HAR650 TTG GTC ACT GTC CGT ACT CGC GTG
BHA1F1 AAT ATC CAC AAA ATG AAG GCA ATA
BHAR1 ATC ATT CCT TCC CAT CCT CCT TCC
BHAF458 AGA AAA GGA ACC AGG AGG ACC CTA
BHAR652 GGA ACC CCC AAA CAG TAA TTT GGT
H1HA1F6 AAG CAG GGG AAA ATA AAA
H1HA1R1193 GTA ATC CCG TTA ATG GCA
H1R365 TTC CTC ATA CTC GGC GAA
H1R1110 CCA TCC ATC TAT CAT CAT TCC

Than the amplicons were purified using Microcon microconcentrator 100 (Amicon, Beverly, MA) prior sequencing.

The purified template was mixed with 1 µl of each of primers, (3,2 pmol/ul concentration) and 8 µl BigDye-Terminator v 3.1 (Cycle Sequencing Ready Reaction Kits, Applied Biosystems, Inc. Foster City, CA) and sterile water for a total volume of 20 µl.

The tube was placed in the thermal cycler and incubated on at 96°C for 10 minutes, 50°C for 5 seconds, 60°C for 4 minutes (25 cycles), before putting it to 4°C.
Than, after singly purification by Centrisep-spincolumn (Princeton) or by a multiple method (Millipore Plate purification- Montage Seq 96 sequencing reaction Cleanup Kit Millipore), were added 20 μl of HiDi formammide (ABI) the samples were denaturated in the thermal cycler at 95°C for 5 minutes.
Finally the sequences were maintained on ice (or at -20°C) for at least 10 minutes and analysed by ABI 310 (or 3130) genetic analyzer (ABI) with 80 cm capillares.

3.9 PHYLOGENETIC ANALYSIS

The assemblage of sequences obtained was carried out by the Sequencer package 4.5 of Gene Codes Corporation (Ann Arbor, MI).

The multiple alignments were performed by ClustalW (MEGA package).

The phylogenetic trees are created using MEGA package, version 3.1 (Pennsylvania State University, PA) and are based the Neighbor-Joining (NJ) method.

The NJ method is the most used distance method basing on algorithmic to construct the tree from the data. This distance method convert aligned sequences into a distance matrix of pairwise differences (distances) between the sequences.

The matrix is much like the tables of "percentage homology".

This distance method uses that matrix as the data from which branching order and branch lengths are computed.

Once the tree obtained, its reliability was estimated using the "bootstrap" method.

The bootstrap value gives the probability that the members of a given clade are always members of the clade.

This method are based on repeated multiple alignment (Barry 2008).

Some sequences generated in this study are deposited in GenBank under accession numbers from EF692535 to EF692581 (D'Agaro et al. 2007).

In addition to the sequence data determined in this study, the sequences of HA genes, used in the construction of the phylogenetic tree, were obtained from the Influenza Sequence Database (http://www.ncbi.nlm.nih.gov/genomes/FLU/Database/multiple.cgi).

3.10 Prediction of glycosylation sites:

Potential N-linked glycosylation sites were predicted using nine artificial neural networks with the chracterizeNetNGlyc 1.0 Server.

A threshold value of >0.5 average potential score was set to predict glycosylated sites.

The N-Glycosite prediction tool was used to visualise the fraction of isolates possessing certain glycosylated sites along the sequence alignment.

An N-linked glycosylation site pattern, NX[ST] (where X can be any amino acid), is called a sequon. This pattern forms the basis for most of the analyses on this web site. The extent of N-linked glycosylation of a particular N-linked glycosylation site, however, can be influenced by the content in which it is embedded, and could be expanded to a 4-amino acid NX[ST]Y pattern, where the amino acid in the X or Y position of NX[ST]Y pattern can be important determinants of N-linked glycosylation efficiency. For example, a proline in position X or Y strongly disfavors N-linked glycosylation. Thus we provide NX[ST] or NX[ST]Y summaries (http://hcv.lanl.gov/content/sequence/GLYCOSITE/glycosite.html).

3.11 Detection of adenovirus and RSV

On the same samples collected by the practitioner's network, we had performed a molecular study on other important respiroviruses responsible of ILI and ARI such as Adenovirus and RSV.

Adenovirus detection:

After the nucleic acids extraction (see 3.5 section), we had performed PCR amplifying a region of the hexon gene to detect the HAdV infection (Allard et al 1990) as the following:10 µl of DNA was mixed with 1 µl each of forward and reverse primers (Sigma) of 20 pmol/µl concentration, 8 µl 50X dNTP's (Promega), 5.0 µl 10x Buffer (ABI),4 µl of MgCl2 (ABI), 0.3 µl Taq Gold Polymerase (ABI) and 20.7 µl millipore water in a 200 µl tube. The tube was then placed in the thermal cycler at 95°C for 10 minutes and then subjected to 45 cycles of 95°C for 30 seconds for denaturation, 66°C for 30 seconds for primer annealing and 72°C for 45 seconds. Than the sample was subjected to 72°C for 7 minutes before putting it to 4°C.

The PCR product was then ran on a 8% polyacrilamide gel to determine if the desired gene was amplified. The amplification product is of 308 bp.

RSV detection:

The RSV detection based on Real Time PCR tecnique, using the following primers and probes directed against F protein that differs the RSV A group and B (Valle L. et al. 2006).

RSVA_FOR 5’- CCA TAT ATT gAA CAA CCC AAA AgC ATC 3’
RSVA_REV 5’- TgT ACC TCT gTA CTC TCC CAT TAT 3’
RSVA_TM 5’-FAM- Agg CCA gCA gCA TTg CCT AAT ACT ACA(p)- BHQ1-3’
RSVB_FOR 5’- ACC ATA TAT TgA ACA ATC CAA AAg CAT C 3’
RSVB_REV 5’- TgT ACC TCT ATA CTC TCC CAT TAT gC 3’
RSVB_TM 5’-FAM – ACC TgC TgC ATT gCC TAg gAC CAC(p)- BHQ1-3’

The reaction mix was prepare on ice and was the following:

25 µl Master Mix 2x,
1,25 µl 40x MS RNAse mix,
3 µl Primer Forward (600 nM)
3 µl Primer Reverse (600 nM)
0.2 µl Probe (200 nM)
15,25 µl RNAse free water
5 µl RNA

The Real Time PCRs were performed using TaqMan® One-Step RT-PCR Master Mix Reagents Kit (ABI, Applied Biosystem) in a total volume of 50 µl and at the following temperature conditions: at 42 °C for 40 minutes, 95°C for 10 minutes, then subjected to 45 cycles of 95°C for 15 seconds, 55°C for 30 seconds, 72 °C for 30 seconds.

4. RESULTS

2004-2005 SEASON:

The 2004-2005 influenza epidemic started in the second week of December 2004 and the last cases were reported in end of March 2005. After a first low peak in December 2004 the epidemic curve was characterized by the maximum peak in weeks 7/8 (16.4 ‰).

It was the highest recorded in the Friuli-Venezia Giulia region over the last eight years, since the epidemiological surveillance has been started (Fig. 1).

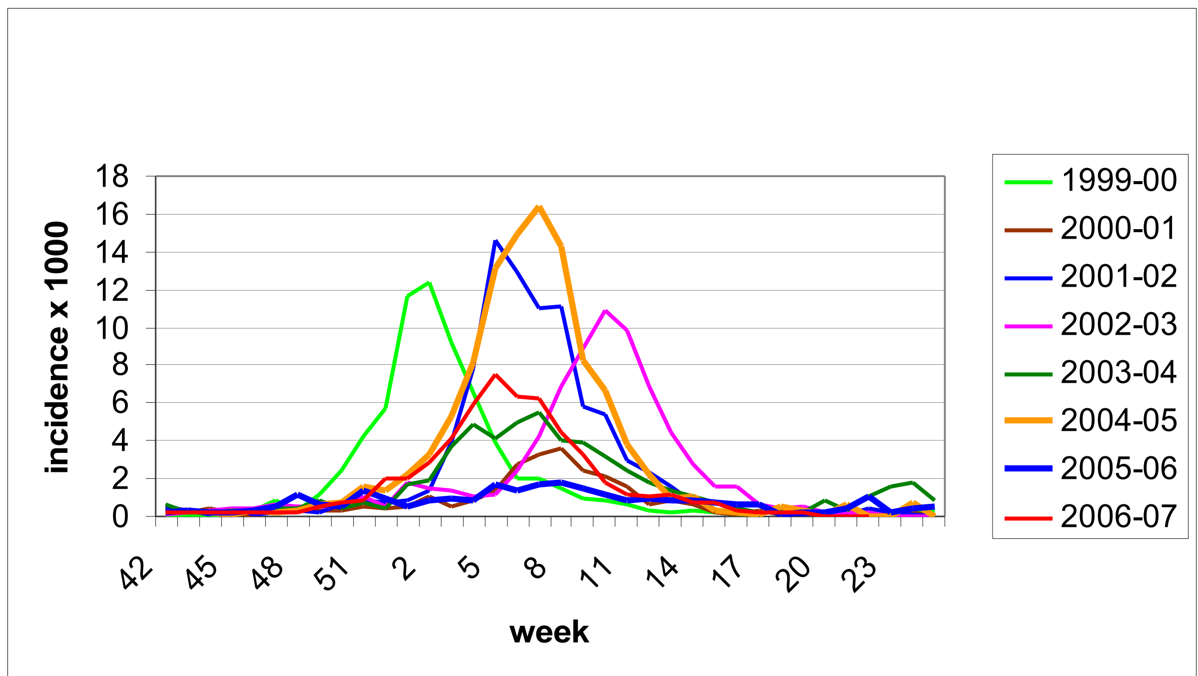


Fig. 1 ILI incidence in the Friuli Venezia Giulia region in the years 1999-2007

During the epidemic season 319 nasopharyngeal samples had been collected from the sentinel physicians and paediatricians distributed all over the Friuli Venezia Giulia region and analyzed by the Influenza Regional Reference Laboratory, namely the Virology unit of the UCO Igiene e Medicina Preventiva.

The largest number of positive specimens was detected between week 4/2005 and 12/2005 (Fig. 2).

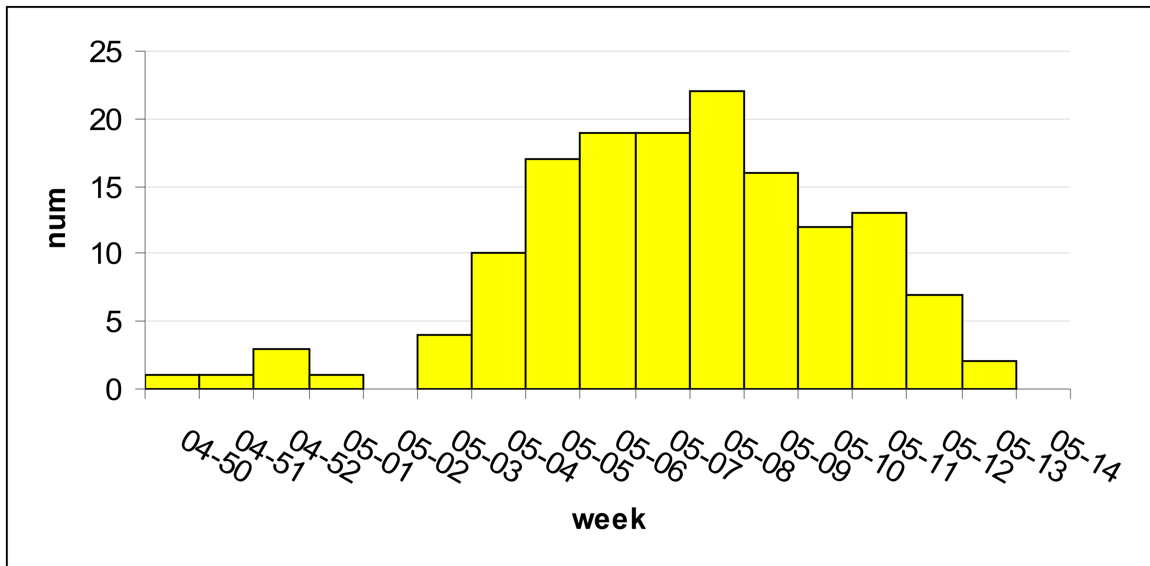


Fig. 2. Histogram of influenza positive samples during 2004-2005 season

As many as 147 samples resulted positive for Influenza footprints by RT-PCR analysis with a positivity rate of 46%: 88 (60 %) were type A influenza and 59 (40 %) were type B. All the influenza A viruses were haemagglutinin-subtyped; 85% belonged to the H3 and 15% to the H1 subtype. The predominant circulating viruses in the region were mostly influenza A(H3) (Fig. 3) but influenza B viruses presented high level circulation, too.

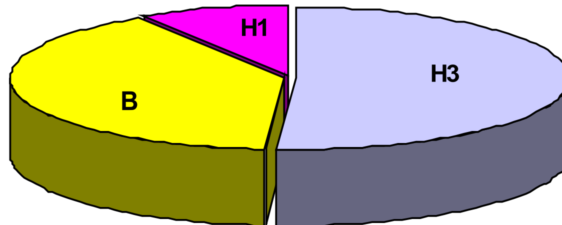


Fig. 3 Influenza viruses subtypes

The temporal distribution of influenza positive samples showed a complex pattern sustained by different types/subtypes of viruses. During the entire epidemic, sporadic H1N1 infections were detected. However, according to the influenza type and subtype characterization, the epidemic wave was composed of a biphasic curve with a first mode in the first half of January to the first half of February (weeks 05-04 to 05-06), sustained mainly by the H3N2 subtype, and a second one in the middle of March (weeks 05-08 to 05-12), due almost exclusively to influenza B virus.

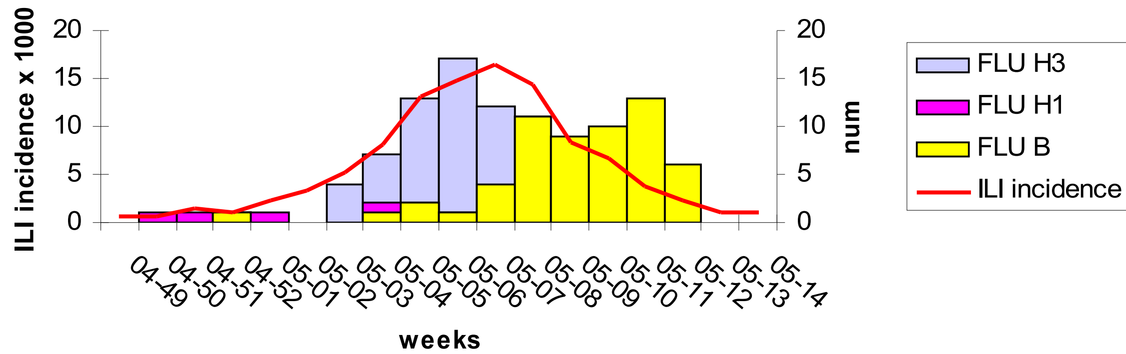


Fig. 4 Temporal distribution of influenza subtypes

The figure 5 shows incidence rates and viruses distribution according to age groups. High morbidity rates were recorded in the two children groups (about 40‰), and a substantial rate of infection was recorded in the adult group (peak rate 14‰). The incidence of ILI in subjects aged over 65 was lower, about 6‰, but with an important relevance considering the high level of vaccination in this age group. The virological pattern differed substantially according to age.

The influenza B occurred especially in children and in adults people (respectively 37% and 44% of positivity) but was demonstrated also in the oldest patients accounting for 4 of the 15 flu viruses demonstrated in this group. The H3 subtype was detected for more of 50% in adults, moreover, the 75% of the viruses isolated in subjects with more than 65 years belonged to this subtype. During the entire season, scattered isolation of H1N1 strains occurred from children and adults, but never from the oldest subjects. In very young children influenza positive rate was almost low, less then 10%.

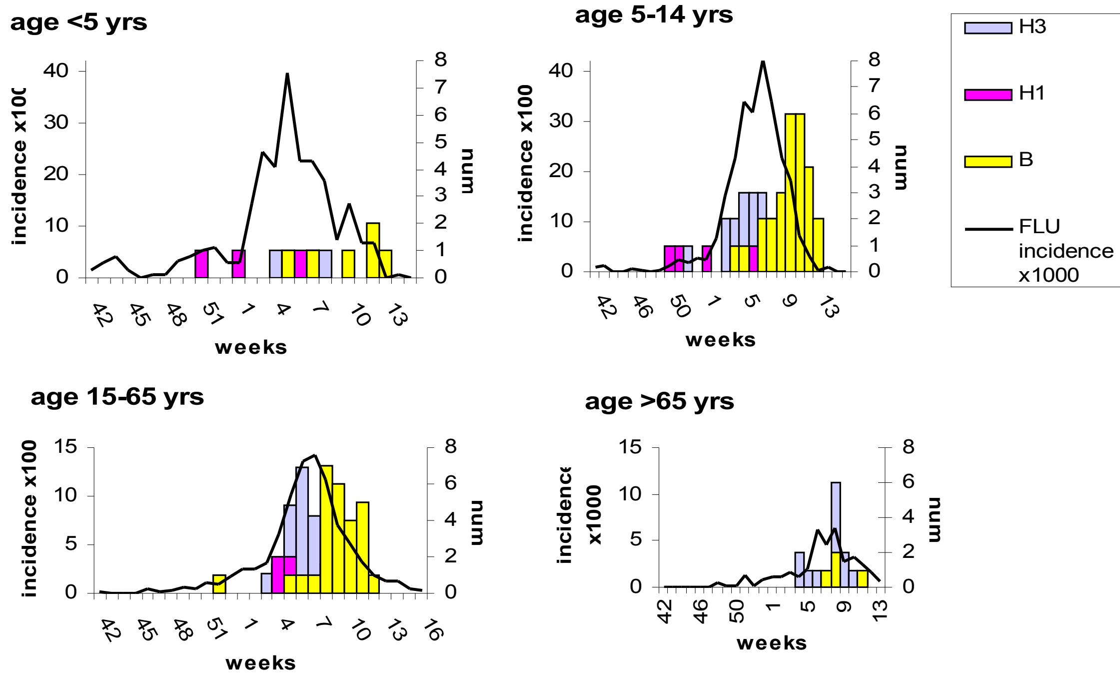


Fig. 5 Influenza subtypes incidence according to age groups.

We isolated 106 influenza strains in MDCK cell cultures, 61 were identified as influenza A subtype H3, 35 as influenza B and 10 as influenza A H1 subtype; the efficiency of viral isolation as compared to RT-PCR was very high, about 72%. The RSV and the adenoviruses were sporadically detected during the entire season with a positivity rate of about 5%. The distribution by age of the proportion of RSV and adenovirus positive samples is reported in Fig. 6. Adenovirus was found in all the age groups with a similar pattern while RSV infections were demonstrated mainly in children with less than 5 yrs and in this group 20% of the analyzed samples resulted RSV positive.

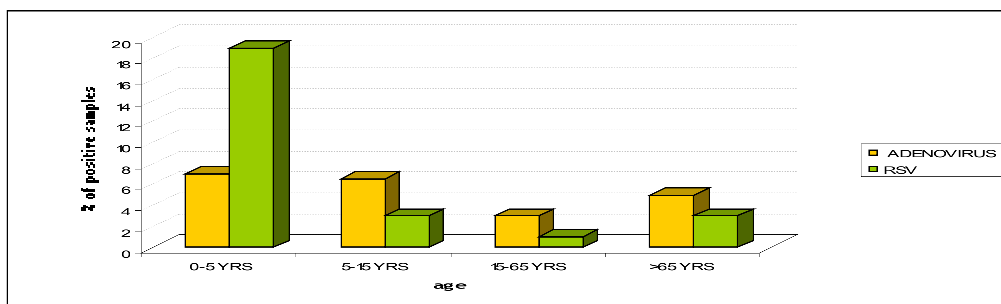


Fig. 6. Adenovirus and RSV positive samples according to age groups

2005-2006 SEASON

The 2005-2006 influenza surveillance in FVG was conducted from October 2005 to May 2006 but this season was characterised by the substantial absence of the influenza epidemic.

During this season 303 nasopharyngeal samples had been collected but only 8 samples were positive for influenza viruses: 3 were identified as H3, 1 as H1 and 4 were B.

The peak of ILI incidence was very low (1,77 ‰) and only 5 strains were isolated in cell culture. The ARI did not present a clear peak of incidence, too.

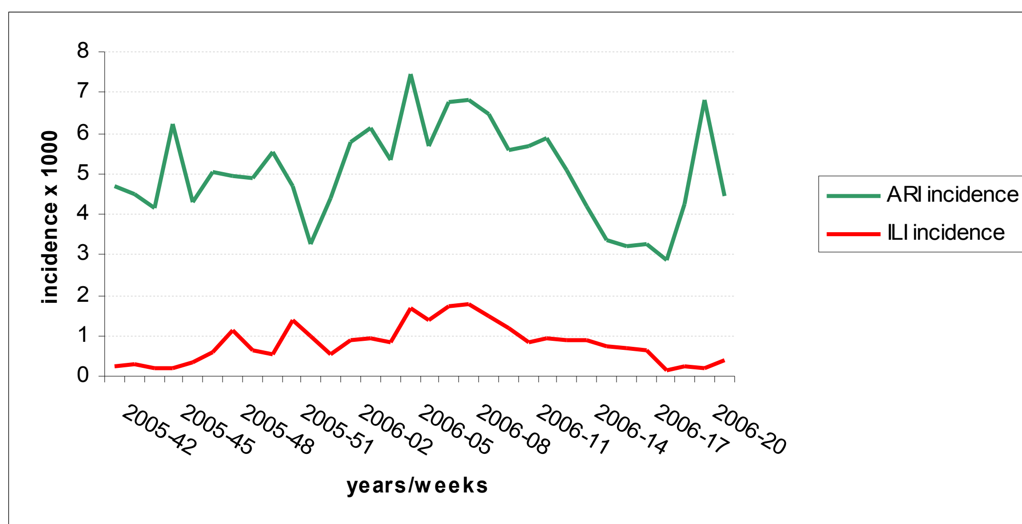


Fig. 7. ILI and ARI incidence in FVG during 2005-06 season

While the influenza viruses circulation was very restricted, the RSV and adenovirus had been detected during the entire season. In fact, during the virologic surveillance, 36 samples were PCR positive for adenovirus (12%) and 22 samples for RSV (7%). Moreover, 16 adenovirus strains were isolated in cell culture with an isolation rate of 44% respect to PCR amplification.

In the figure 8 the age specific prevalence of adenovirus and RSV are compared.

In this epidemic season adenovirus infections were demonstrated mainly in the youngest age groups; in particular in the children aged <5 yrs the adenovirus positive samples accounted for about a quarter of the total of the samples examined in this group. The pattern of age specific RSV positivity presented a bimodal distribution comprising the youngest and the oldest age groups with rates of 12% and 6% respectively .

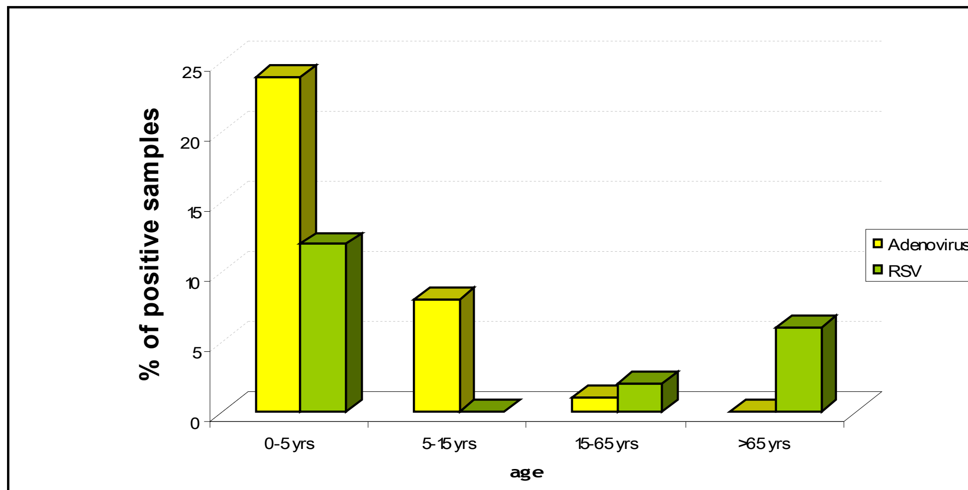


Fig. 8 Adenovirus and RSV positive samples according to age groups.

2006-2007 SEASON

The 2006-2007 first influenza virus was isolated in the mid-November 2006 but the influenza epidemic started later, in the first week of 2007.

During this season 302 nasopharyngeal samples from all the FVG region were analyzed.

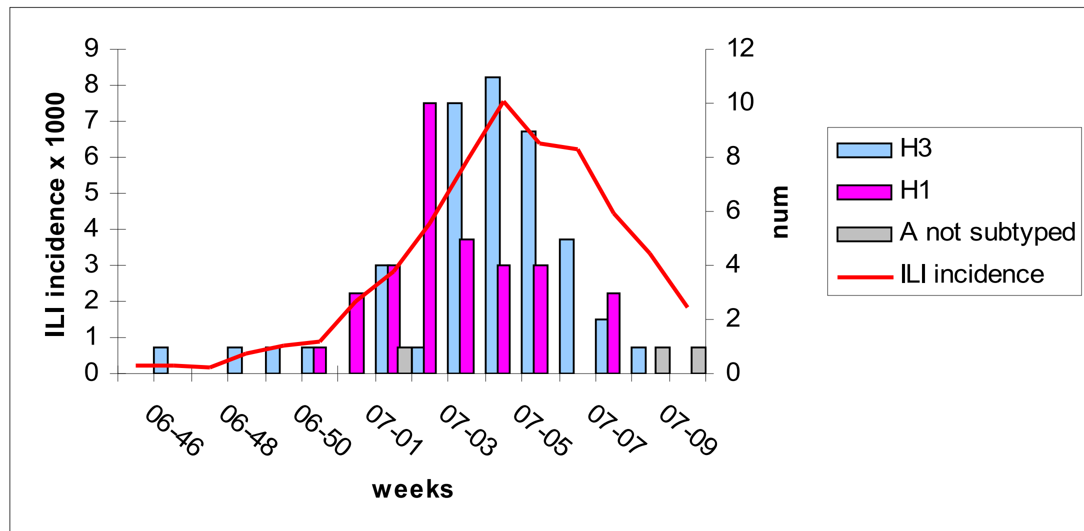


Fig. 9. 2006-07 Influenza epidemic

A total of 84 (28%) specimens resulted positive for influenza virus by RT-PCR: 47 (56 %) were typed as H3 and 34 (40 %) as H1, three influenza A samples could not be subtyped.

The epidemic of influenza resulted of middle intensity with a peak of ILI incidence of 7.5% (Fig. 9).

The strains isolated in cell culture were 43 (52% rate of isolation of the RT-PCR positive samples), 18 were H1 and 25 H3. No B virus could be detected.

The age distribution of the two influenza A subtypes (Tab. 1) does not shows significant differences even if a higher proportion of H3 viruses has been found in young adults and in the oldest group. As in the previous epidemics, a lower proportion of influenza positive samples was found in children under 5 years in this season, too

	<5 yrs	5-14 yrs	15-65 yrs	>65 yrs
total	128	51	106	14
Positive FLU	21 (16%)	21 (41%)	38(36%)	4 (29%)
FLU H3	11	11	22	3
FLU H1	9	10	14	1

Tab. 1 Influenza viruses distribution according with age groups.

In fig 10 the age distribution of the positivity for adenovirus and of RSV is reported; the pattern of the prevalence of RSV positive samples reproduces the bimodal pattern already observed in the former season but with higher rates. Adenovirus infections were

demonstrated in all the age groups with detection rates ranging from 6 to 18% in the 15-65 and 0-5 cohorts, respectively.

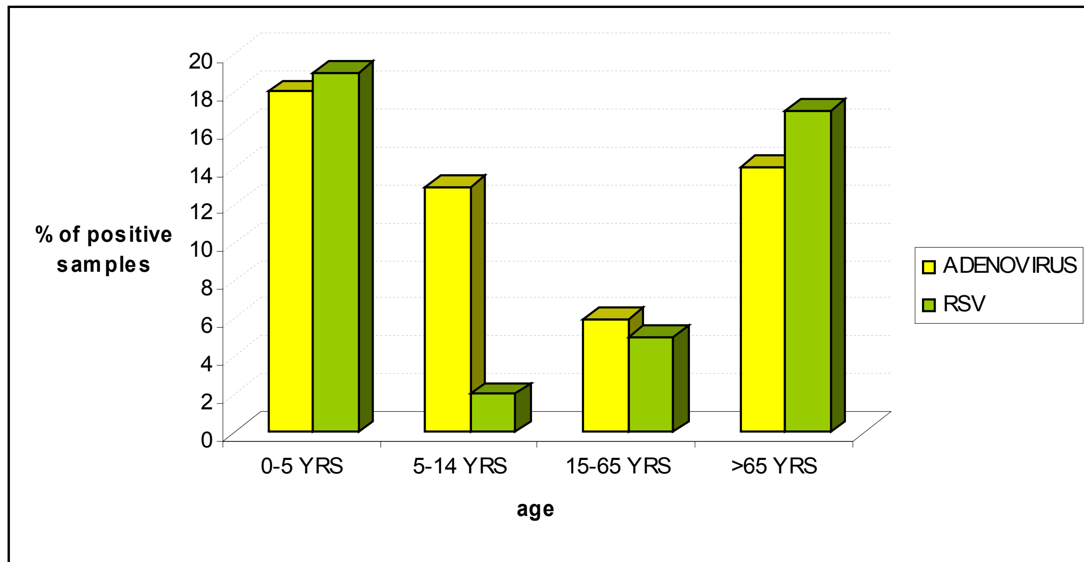


Fig. 10 Adenovirus and RSV positive samples according to age groups

4.2 ANTIGENIC AND GENETIC CHARACTERIZATION OF THE STRAINS

AH3N2 VIRUSES:

The antigenic and genetic characterization was performed on most of the 61 H3N2 strains isolated in the 2004-05 season. The antigenic characterization by the Hemagglutination Inibition test on the H3 viruses isolated during the 2004-05 season showed a relatively low inhibition of the Trieste isolates with the antiserum directed to the vaccine strain A/Wyoming/3/2003 (A/Fujian/411/2002-like virus) demonstrating a greater antigenic homology with the recent strains such as A/Oslo/807/04, and A/California/7/04 (Tab.2).

Viruses	Isolation Date	A/Wy 3/03 F11/03	A/Chch 28/03 F3/04	A/Well 1/04 F13/04	A/Shan 1219/04 F14/04	A/Oslo 807/04 F20/04	A/Fin 486/04 F22/04	A/Cal 7/04 F2/05	A/Sing 37/04 F3/05
A/Wyoming/3/2003		**2560**	2560	320	640	640	640	640	160
A/Christchurch/28/2003		1280	**2560**	160	320	320	320	320	80
A/Wellington/1/2004		1280	640	**640**	1280	640	640	640	640
A/Shantou/1219/2004		320	640	320	**1280**	640	320	320	640
A/Oslo/807/2004		320	160	160	320	**640**	320	320	320
A/Finland/486/2004		80	160	80	640	1280	**320**	320	320
A/California/7/2004		640	1280	320	1280	2560	640	**1280**	640
A/Singapore/37/2004		320	320	160	1280	2560	1280	1280	**1280**
A/Trieste/4/ 05	14.1.05	640	640	160	1280	2560	640	640	640
A/Trieste/6/ 05	17.1.05	640	640	160	1280	2560	1280	1280	640
A/Trieste/8/ 05	17.1.05	640	1280	320	1280	2560	1280	1280	1280
A/Trieste/23/2005	28.1.05	160	160	160	320	640	320	640	320
A/Trieste/24/2005	28.1.05	320	320	160	320	1280	320	1280	320
A/Trieste/27/2005	27.1.05	160	160	80	160	160	160	640	160
A/Trieste/29/2005	31.1.05	160	320	160	320	640	320	640	320
A/Trieste/31/2005	1.2.05	640	160	160	640	640	640	1280	320
A/Trieste/35/2005	1.2.05	320	320	160	640	1280	640	1280	320
A/Trieste/39/2005	3.2.05	320	320	160	640	640	320	640	320
A/Trieste/48/2005	8.2.05	160	320	160	640	1280	640	1280	320
A/Trieste/55/2005	15.2.05	160	80	40	160	160	80	320	80
A/Trieste/57/2005	22.2.05	640	160	160	320	640	160	640	320
A/Trieste/59/2005	22.2.05	320	160	160	320	640	160	640	320
A/Trieste/61/2005	21.2.05	160	80	80	160	320	160	640	160
A/Trieste/63/2005	19.2.05	160	80	80	160	320	160	640	160
A/Trieste/65/2005	28.2.05	320	160	80	160	640	320	640	160

Tab. 2 Hemaggutination inhibition titre of some representative 2004-05 strains.

In order to relate the antigenic and genetic characteristics of the H3N2 viruses sequence and phylogenetic analysis of the HA1 region of the isolated viruses was performed. The

analysis of the phylogenetic relationships with some reference strains confirmed the serological results; indeed the viruses isolated in our region resulted similar to the A/California/7/04 strain characterized by the aminoacidic changes: N145K, D188N, D190V, S193N, S227P when compared with the formerly circulating Wyoming/03/03 sequence (Tab 3).

In particular, this new variant presented an aminoacidic change (K145N) which could add a glycosilation site in the antigenic site A, resulting in a minor reactivity to vaccine induced antibodies.

CODON	*Nr of strains*	138	145	188	190	192	193	201	214	219	225	226	227
ANTIGENIC SITE		**A**	**A**	**B**	**B**	**B**	**B**	**D**	**D**	**D**		**D**	**D**
PANAMA200799		A	K	D	D	I	S	R	I	S	G	V	S
CHRISTCH.2803		S	K	D	V	I	S	R	I	Y	D	I	S
FUJIAN41102		A	K	D	D	I	S	R	I	S	D	V	S
WELLINGT.0104		S	K	D	V	I	N	R	I	Y	D	V	P
WYOMING0303		A	K	D	D	I	S	R	I	Y	D	I	S
CALIFORNIA704		A	N	N	V	I	N	R	I	Y	D	I	P
TRIESTE0205	*46*	A	N	N	V	I	N	R	I	Y	D	I	P
TRIESTE1405	*1*	A	N	N	V	I	N	R	T	Y	D	I	P
TRIESTE3105	*1*	A	N	N	V	I	N	K	T	Y	D	I	P
TRIESTE3205	*1*	A	N	N	V	V	N	K	T	Y	D	I	P
TRIESTE3405	*1*	A	N	N	V	V	N	K	T	Y	D	I	P
TRIESTE4605	*2*	A	N	N	V	V	N	K	T	Y	D	I	P
TRIESTE4805	*1*	A	N	N	V	V	N	K	T	Y	D	I	P
TRIESTE6105	*1*	A	N	N	V	V	N	K	T	Y	D	I	P
TRIESTE6405	*1*	A	N	N	V	V	N	K	T	Y	D	I	P

Tab. 3 Amino acids changes of the 2004-2005 H3N2 viruses

In the phylogenetic tree (Fig. 11) the sequences of H3N2 viruses isolated in the three epidemic seasons in Friuli Venezia Giulia region are compared with some reference strains. The California7/04 genetically related viruses, associated with the 2004-05 epidemic had continued to circulate during the 2005-06 season but with a very low incidence.

The H3N2 viruses evolution was characterised by an appearance of a viruses belonging to the A/Wisconsin/67/2005 clade, the strain introduced in the vaccinal composition of 2006-07 season.

Despite the viruses isolated in Trieste during 2006-07 season (n=25) are quite genetically related to the vaccine strains, they represented a complex cluster of the tree, due to some aminoacidic changes in the HA1 region.

Strains/codons	6	9	10	25	45	50	122	140	142	145	157	171	173	193	221
ACalifornia704	N	S	T	I	S	G	N	K	R	N	L	N	K	S	P
Awellington104	N	S	T	I	S	G	N	K	R	K	L	N	K	S	P
AFujian41102	N	S	T	I	S	G	N	K	R	K	L	N	K	S	P
A/Wisconsin/67/2005	N	S	T	I	S	E	D	K	R	N	L	N	K	F	P
A/Winsconsin/01/06	N	S	T	I	S	E	N	K	R	N	S	N	K	F	P
A/Nepal/21/06	I	S	T	I	S	E	N	K	G	N	L	N	E	F	P
A/Brisbane/10/2007	N	S	T	I	S	E	N	I	R	N	L	N	K	F	P
Atrieste0307	N	S	T	I	S	E	N	K	R	N	L	D	K	F	P
Atrieste1907	N	S	T	I	S	G	N	K	R	N	L	N	K	F	P
Atrieste3107	N	S	T	I	S	E	N	K	R	N	L	N	K	F	P
Atrieste4007	N	N	T	I	S	G	N	K	R	N	L	N	K	F	P
Atrieste4207	N	S	A	I	S	E	N	K	R	N	L	N	K	S	P
Atrieste1707	N	S	T	I	S	G	N	K	R	N	L	N	K	F	P
Atrieste2807	N	S	T	I	S	G	N	K	R	N	L	N	K	F	H
Atrieste2907	N	S	T	I	S	G	N	K	R	N	L	N	K	F	H
Atrieste2307	N	S	T	I	S	E	N	I	R	N	L	N	K	F	P
Atrieste3007	N	S	T	I	S	G	N	K	R	N	L	N	K	F	P
Atrieste3207	I	S	T	V	S	G	N	K	G	N	S	N	E	F	P

Tab. 4 Amino acids changes of the 2006-2007 H3N2 viruses

All of the strains isolated in the 2006/07 epidemic season maintained the K145N aminoacidic change, but some of them acquired other changes related with new reference strain. In particular A/Trieste32/07,-33/07 acquired N6I, G142R, and E173K, like the A/Nepal/21/06 strain. The A/Trieste/25/07, -23/07 and 27/07 strains presented the same amino-acidic changes as A/Brisbane/10/2007, namely: G50E and K140I (Tab 4).

Indeed, some of the viruses such us A/Trieste/23-28-32/2007, showed a HI pattern of reactivity very peculiar, showing very low titres inhibition with the antisera directed to the prototype strains suggesting antigenic changes that are not explained by a specific pattern of HA1 sequence (Tab. 5).

Other strains as A/Trieste/27-29-30-31/2007 are inhibited by ferret antisera to A/Wisconsin/67/05 at higher dilutions although presenting HA1 sequences quite similar to the poorly inhibited strains.

Viruses		Post infecton ferret sera A/Cal 7/04 F31/05	A/HK 4443/05 F24/05	A/Hiro 52/05 F2/06	A/Wis 67/05 F6/06	A/Slov 134/06 F9/06	A/Bay 4/06 F11/06	A/Berlin 2/06 F8/06	A/Lyon 636/06 F12/06
A/California/7/2004		**2560**	640	320	640	160	1280	320	640
A/HongKong/4443/2005		640	**640**	640	640	160	640	160	320
A/Hiroshima/52/2005		1280	1280	**2560**	2560	320	640	80	640
A/Wisconsin/67/2005		1280	2560	2560	**5120**	320	1280	80	320
A/Slovakia/134/2006		1280	1280	640	1280	**320**	1280	160	320
A/Bayern/4/2006		640	320	640	320	160	**1280**	320	320
A/Berlin/2/2006		320	320	320	160	80	320	**80**	160
A/Lyon/636/2006		1280	640	640	640	320	1280	320	**640**
	N Strain								
A/Trieste/3c/2007	3	80	80	160	80	40	80	40	40
A/Trieste/19c/2007	3	80	160	160	160	40	20	10	<
A/Trieste/23c/2007	3	<	80	80	80	10	<	10	<
A/Trieste/27c/2007		640	320	320	640	160	320	40	320
A/Trieste/28c/2007		<	80	80	80	20	<	10	<
A/Trieste/29c/2007		320	640	640	640	320	320	80	160
A/Trieste/30c/2007		320	320	320	320	160	160	20	<
A/Trieste/31c/2007		160	320	160	320	20	80	10	<
A/Trieste/32c/2007		80	80	160	80	80	80	40	40

Tab 5 Hemaggutination inhibition titre of 2006-07 strains

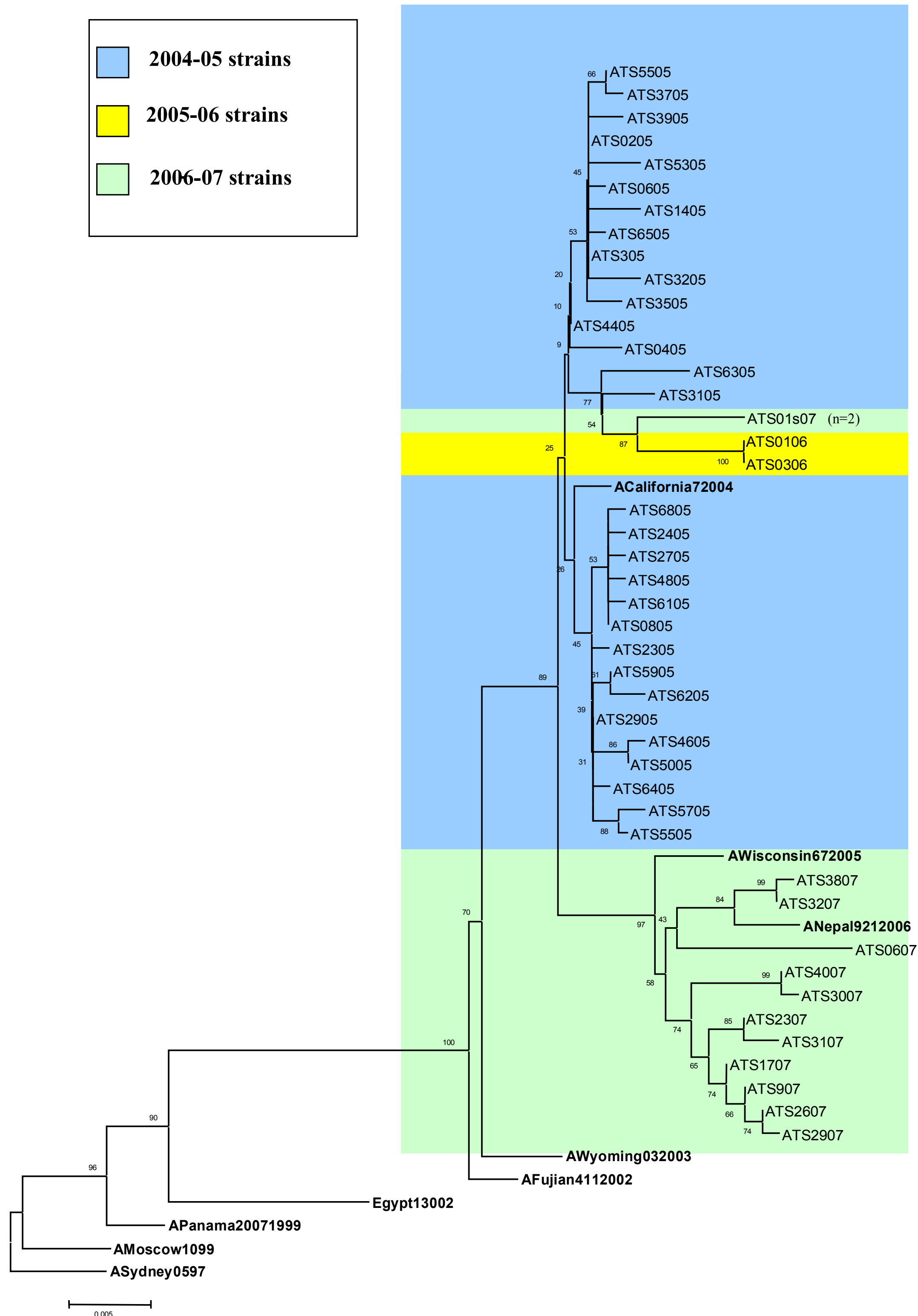


Fig. 11 Phylogenetic tree of H3 HA1s

AH1N1 VIRUSES:

During the three epidemic seasons we isolated 28 H1N1 viruses and we could verify and analyze their antigenic and genetic features in order to evaluate the viruses evolution.

The H1N1 viruses isolated in 2004-2005 season analyzed by the HI tests, with post-infection ferret antisera, resulted antigenically closely related to the current vaccine strain, A/New Caledonia/20/99 and to recent reference strains, such as A/Hong Kong/2637/04 and A/Netherlands/128/04 (Tab. 6).

This serological result was confirmed by the sequence analysis that not showed genetic significant modifications from the vaccine reference strain in terms of aminoacidic changes (Tab. 8).

	Post infection ferret sera					
Viruses	**A/Beij 262/96**	**A/NC 20/99**	**A/Eg 96/02**	**A/Chile 8885/02**	**A/HK 2367/04**	**A/Neth 128/04**
A/Beijing/262/96	**320**	640	160	160	640	640
A/New Caledonia/20/99	160	**1280**	640	640	1280	1280
A/Egypt/96/02	80	640	**640**	320	640	640
A/Chile/8885/02	40	640	320	**640**	640	640
A/Hong Kong/2637/04	40	320	320	320	**640**	640
A/Netherlands/128/04	80	640	320	320	1280	**2560**
A/Trieste/32/ 04	160	640	640	320	1280	2560
A/Trieste/33/ 04	80	640	640	320	1280	1280
A/Trieste/1/ 05	80	640	320	320	1280	1280
A/Beijing/262/96	**1280**	640	320	320	1280	640
A/New Caledonia/20/99	320	**1280**	640	1280	1280	1280
A/Egypt/96/02	320	1280	**1280**	640	640	640
A/Chile/8885/02	160	640	320	**640**	640	640
A/Hong Kong/2637/04	80	640	640	320	**640**	640
A/Netherlands/128/04	160	640	640	320	1280	**2560**
A/Trieste/36/2005	160	640	640	320	640	2560
A/Trieste/47/2005	320	1280	640	640	1280	2560
A/Trieste/60/2005	320	1280	640	640	1280	2560

Tab. 6 Hemaggutination inhibition titre of 2004-05 strains

The 2004-05 was characterized by a reduced and sporadic circulation of H1N1 while during the 2006-07 season, we could demonstrate H1N1 amplification products in 34 samples and we isolated 18 H1N1 strains in MDCK cultures.

The antigenic characterization put in evidence that all the strains isolated in this season were antigenically not related with the vaccine strain A/New Caledonia/20/99 while most of them presented high level inhibition with sera directed to the new emerging strain A/Solomon Islands/3/2006 (Tab. 7).

The strain A/Trieste/02/2007 was closely related with a recent circulating strain called A/Fukushima/141/2006 (A/Solomon Islands/3/2006-like), while other strains, such as A/Trieste/18c/2007, A/Trieste/16c/2007 and A/Trieste/10c/2007 were antigenically related with all of the recent strains (A/Fukushima/141/2006, A/Fukushima/97/2006, A/Hong Kong/2652/2006).

Viruses	**A/NC 20/99**	**A/Thess 24/05**	**A/Egypt 39/05**	**A/Fuk 97/06**	**A/HK 2652/06**	**A/SI 3/06**	**A/Fuk 141/06**
A/New Caledonia/20/99	**320**	640	1280	320	80	80	160
A/Thessaloniki/24/05	640	**2560**	2560	320	160	160	160
A/Egypt/39/2005	160	640	**1280**	320	40	160	160
A/Fukushima/97/2006	<	<	160	**1280**	640	1280	640
A/Hong Kong/2652/2006	<	<	160	640	**320**	640	640
A/Solomon Islands/3/2006	80	160	160	640	320	**640**	320
A/Fukushima/141/2006	40	80	320	640	320	1280	**640**
A/Trieste/2c/2007	<	40	80	160	160	160	320
A/Trieste/4c/2007	<	40	80	160	160	320	320
A/Trieste/5c/2007	<	80	40	160	160	160	160
A/Trieste/7c/2007	40	40	80	320	160	320	320
A/Trieste/8c/2007	40	80	160	320	320	640	320
A/Trieste/9c/2007	40	40	160	320	160	640	320
A/Trieste/10c/2007	40	80	160	640	320	640	640
A/Trieste/11c/2007	40	160	80	320	160	320	160
A/Trieste/12c/2007	80	80	160	320	320	640	320
A/Trieste/13c/2007	<	40	80	320	160	320	320
A/Trieste/14c/2007	40	80	160	320	320	320	320
A/Trieste/15c/2007	40	40	80	320	160	320	320
A/Trieste/16c/2007	40	40	80	320	160	320	320
A/Trieste/18c/2007	40	40	80	640	640	640	320
A/Trieste/34c/2007	40	80	160	320	160	320	320

The serological results were confirmed by the aminoacidic changes deduced by the sequence analysis and reported in tab 7.
In particular the amino acidic changes revealed a close correlation between the antigenicity of A/Solomon Island/3/2006 and the presence of K140E change in comparison with the A/ New Caledonia/20/99 (Fig 12).
All of Trieste strains presented this specific change accounting for the low antigenic correlation with the A/New Caledonia/20/99 strain. But also other changes were observed namely: V165A, R208K, W251R.
But most of the viruses isolated in 2006/07 presented amino acidic differences also with respect to the A/Solomon Island/3/2006 confirming the antigenic data above reported. Indeed most of the amino acidic sequences of the isolates are characterized by four common changes: D35N, T82K, H94Q, K145R, R188K differing by the A/Solomon Island/3/2006 sequence (Tab. 8).
Some of them (K145R and R188K) were present in A/Egypt/96/02 strain sequence.

Strains\codons	N strains	35	82	94	140	145	165	188	208	251	252	266	273
A/NewCaledonia/20/99		D	T	Y	**K**	R	V	R	R	W	Y	T	E
A/Solomon/Island/3/06		D	T	H	**E**	K	A	R	K	R	Y	N	E
A/Egypt/96/02		D	T	Y	**K**	R	A	R	R	W	Y	T	E
ATrieste4705		D	K	Y	**K**	R	A	R	R	R	F	T	E
ATrieste0105	4	D	T	Y	**K**	R	A	R	R	R	F	T	E
ATrieste3605		D	T	Y	**K**	R	A	R	R	R	F	T	E
ATrieste0705	3	D	T	Y	**K**	R	A	R	R	R	F	T	E
ATrieste3404		D	T	Y	**K**	R	A	R	R	R	F	T	E
ATrieste1107	12	N	K	Q	**E**	R	A	K	K	R	Y	N	K
ATrieste4107		N	K	Q	**E**	R	A	K	K	R	Y	N	K
ATrieste0207		N	K	Q	**E**	R	A	K	K	R	Y	N	K
ATrieste1007		N	K	Q	**E**	R	A	K	K	R	Y	N	K
ATrieste1307		N	K	Q	**E**	R	A	K	K	R	Y	N	K

Tab. 8 Amino acids changes of the 2004-05 and 2006-07 H1N1 viruses

The H1N1 phylogenetic tree (Fig.12) indicate that the 2004-05 strains (in light blue) are genetically related with A/New Caledonia/20/99 strain, while the 2006-06 strains are related with the new variant of the virus, namely A/Solomon Island/3/2006. The changes between our isolates and the prototype led the clustering of the 2007 Trieste isolates in a distinct branch derived from the A/Solomon Island/3/2006. This cluster is supported by a high bootstrap value (99).

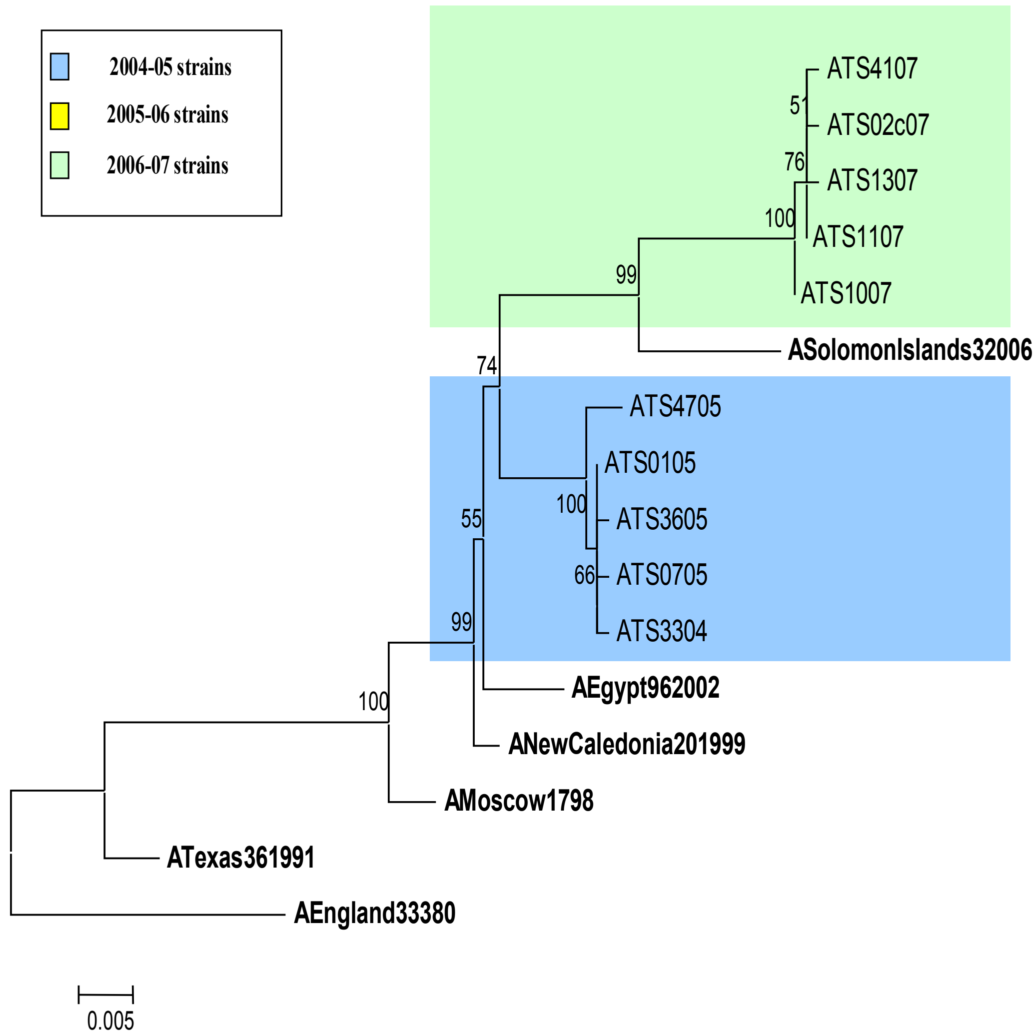


Fig. 12 Phylogenetic tree of H1 HA1s

B VIRUSES:

In the second part of the 2004-05 epidemic was sustained by B influenza wave that had brought about 35 B strains isolated in FVG.

The main of B influenza strains (n=23) were antigenically related to the B/Shandong7/97 strain in the Victoria-lineage, presenting high HI titre against sera directed to this variant, while only 5 strains were inhibited by sera directed to Yamagata-like viruses (in red; Tab. 9).

Viruses	Isolation date	B/Shan[2] 7/97	B/Shan 7/97	B/Tehr 80/02	B/Bris 32/02	B/Sich 379/99	B/Shai 361/02	B/Jiang 10/03
B/Shandong/7/97		**640**	**320**	160	160	<	<	<
B/Tehran/80/02		320	160	**320**	160	<	<	<
B/Brisbane/32/02		320	320	160	**160**	<	<	<
B/Sichuan/379/99		<	<	<	<	**320**	320	40
B/Shanghai/361/02		<	<	<	<	320	**640**	80
B/Jiangsu/10/03		<	<	<	<	80	80	**320**
B/Egypt/144/2005		<	<	<	<	160	320	40
B/Trieste/1/ 05	18.1.05	2560	<	40	<	<	<	<
B/Trieste/3/2005	25.1.05	<	<	<	<	80	80	80
B/Trieste/6/2005	8.2.05	640	80	40	<	<	<	<
B/Trieste/9/2005	14.2.05	640	80	40	<	<	<	<
B/Trieste/10/2005	14.2.05	640	160	40	<	<	<	<
B/Trieste/11/2005	15.2.05	640	160	40	<	<	<	<
B/Trieste/12/2005	15.2.05	640	320	<	<	<	<	<
B/Trieste/13/2005	16.2.05	<	<	<	<	<	<	<
B/Trieste/14/2005	21.2.05	320	320	<	<	<	<	<
B/Trieste/20/2005	1.3.05	640	160	80	<	<	<	<
B/Trieste/22/2005	1.3.05	640	80	40	<	<	<	<
B/Trieste/23/2005	4.3.05	<	<	<	<	40	80	80
B/Trieste/25/2005	7.3.05	640	80	40	<	<	<	<
B/Trieste/27/2005	9.3.05	<	<	<	<	40	80	80

Tab. 9 Hemaggutination inhibition titre of 2004-05 strains

The sequencing analysis and the phylogenetic tree confirmed the serological results except for the B/Trieste 13/05 (Victoria-like) that seems no antigenically related to the B/Shandong7/97 variant, while its sequence clustered with the other strains, B/Shandong7/97-correlated.

The other Victoria-like strains represent a compact clade of the tree, with identical (n=21) or very similar sequences (Fig. 13).

The Yamagata-like strains resulted similar to B/HongKong/1434/2002 virus.

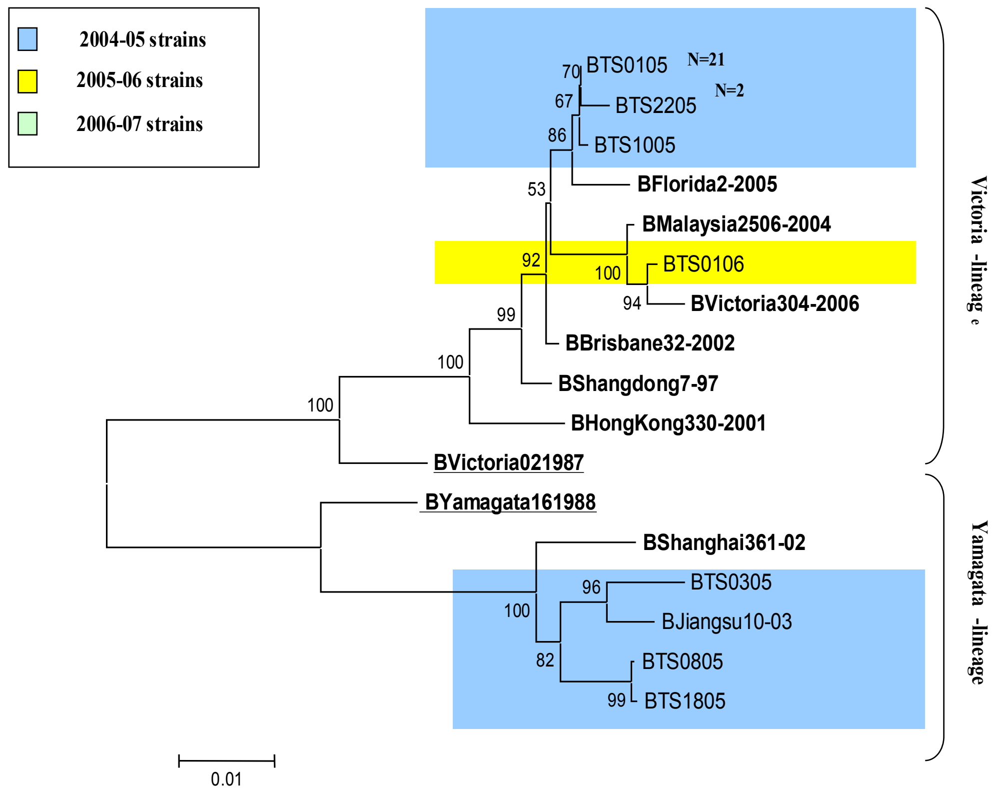


Fig. 13 Phylogenetic tree of B isolates

Glycosylation patterns

Seven potential N-glycosylation sites in H3 HA1 have been constant since 1999, namely: 8, 22, 63, 133, 165, 246, and 285. These glycosylation sites have been conserved in our dataset from 1999 to 2006.

Strains/ Glyc.site	8	22	38	46	63	122	126	133	144	165	246	285
Moscow1099	V	V	V	-	V	V	V	V	-	V	-	V
Fujan41102	V	V	V	-	V	V	V	V	V	V	V	V
California72/04	V	V	V	-	V	V	V	V	V	V	V	V
Wisconsin67/05	V	V	V	-	V	-	V	V	V	V	V	V
Nepal92/06	V	V	V	-	V	V	-	V	V	V	V	V
04-05Trieste str	V	V	V	-	V	V	V	V	V	V	V	V
05-06 Trieste str	V	V	V	-	V	V	V	V	V	V	V	V
A/TS/01, 37/07	V	V	V	V	V	V	V	V	V	V	V	V
06-07 Ts str.	V	V	V	-	V	V	V	V	V	V	V	V
A/TS/03,06,36/07	V	V	V	-	V	V	-	V	V	V	V	V

Tab. 20 Glycosylation sites

The A/Moscow/10/99(H3N2)-like viruses from the 1999-2000 season possessed three additionally predicted sites 38, 122 and 126 but did not posses the 246.

The A/Fujian/411/02(H3N2)-like viruses from the 2002-2003 season possessed eleven potential glycosylation sites, they kept the newly introduced sequon at position 144, maintaining the 246 and the 126 site.

Both position 126 and 144 are located at HA antigenic site A.

The A/California/7/04(H3N2) and A/Wisconsin/67/05(H3N2)-like viruses from 2004 to 2006 have the same ten glycosylation sites as the A/Wellington/1/04(H3N2)-like viruses.

Most of 2004-05 strains isolated in Trieste presented a glycosylation pattern related with the California/7/04 one maintaining eleven sites. The same features are detected in the two 05-06 strains.

Most of 2006-07 strains presented the same glycosylation site of the strains of the previous season (eleven). Three strains such as the A/Trieste/03/07 had the same pattern of the reference strain called A/Nepal92/06, without the 126 site.

Two strains, namely A/Trieste/01/07 and A/Trieste/37/07, had twelve sites, with an additional site: 46.

The H1 HA strains from 1999 to 2006 eight predicted potential N-linked glycosylation sites in HA1 (11, 12, 23, 54, 87, 125, 159, 286).

All the strains isolated in Trieste during the study period maintained those sites, and they did not present any additional sites.

PRELIMINARY RESULTS OF 2007-08 SEASON:

The 2007-08 surveillance season started at the middle of October until end of April 2008. At the moment (middle of March) we revealed a epidemic of influenza substained by two subtypes of virus, namely H1N1 and B.

We had so far detected only sporadic cases of H3N2 We had collected 240 samples and 83 samples resulted positive for influenza with a positivity rate of 35 %.

The temporal distribution of influenza positive samples showed a complex pattern sustained by H1N1 and B viruses (Fig. 14).

During the entire epidemic, sporadic H3N2 infections were detected.

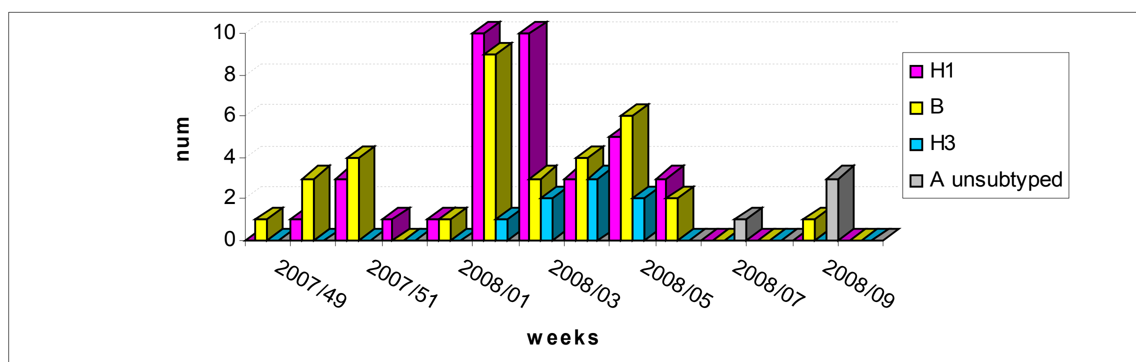


Fig. 14 Temporal distribution of influenza positive samples during the season 2007-08

We isolated 36 influenza strains in MDCK cell cultures, 19 were identified as influenza A subtype H1, 5 as subtype H3 and 12 as influenza B.

We had performed a partial genetic characterization on H1N1 and B viruses, with the respective phylogenetic trees.

As we can see in the tree of the H1N1 viruses, the Trieste isolates belonged to Yamagata-lineage, and are genetically related to B/Jiangsu/10/03 reference strain (Fig. 15).

The 2007-08 vaccine contained the B/Malaysia/2506/04 strain (Victoria–like virus).

The H1N1 tree presented a compact cluster formed by Trieste strain, derived from A/SolomonIsland/32006 but genetically almost distant from it (Fig. 16)

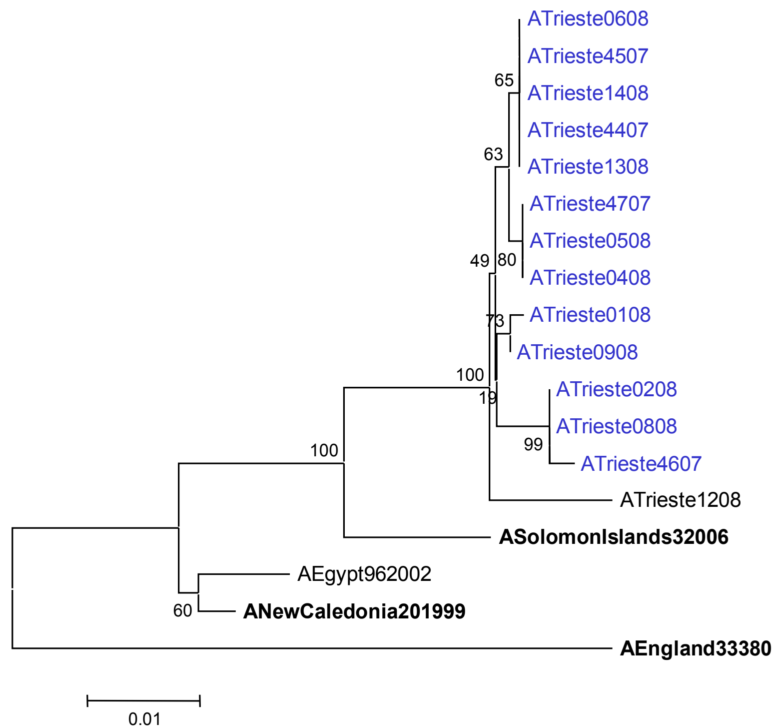


Fig. 15 Phylogenetic tree of H1N1 strains of 2007-08 season

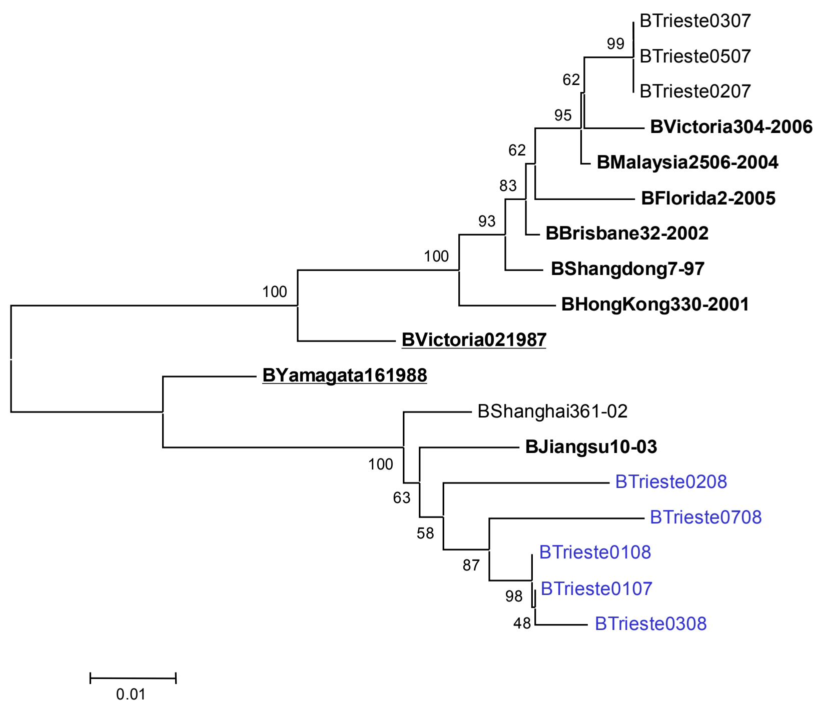


Fig.16 Phylogenetic tree of B strains of 2007-08 season

5. Discussion

The introduction of the molecular techniques in the virologic surveillance led to an enormous improvement of the analytic power in detection and characterization of Influenza. The application of highly sensitive PCR methods and of the direct sequencing of amplicons revealed a more detailed and reliable picture of the real circulation of Influenza viruses in the population. The results of the virologic surveillance of Influenza in the Friuli Venezia Giulia reflect the circulation of the virus in the Northern Hemisphere and, in particular, in Europe although, sometimes, specific patterns may be observed.

The epidemiology of Influenza in the three considered epidemic seasons has been characterized by a first high incidence epidemic in 2004/05 sustained by both A and B viruses, a substantial absence of the influenza epidemic in 2005/06 and by a medium intensity epidemic in 2006/07 with an interesting pattern of circulation of Influenza viruses. The H3N2 was the prevalent subtype in Friuli Venezia Giulia during two seasons in the study period, and the high incidence reported was justified by the antigenic and genetic features of the circulating strains.

On the whole hemagglutinin of H3N2 viruses formed seasonal phylogenetic clusters but different lineages, co-circulating within the same season, were also observed. The evolution has been stochastic, influenced by small "jumps" in genetic distance rather than constant drift, especially with the introduction of the California-like viruses in 2004-2005 season.

An evolutionary stasis-periods were observed in 2005-06 which might indicate well fit viruses. During 2006-07 season most of H3N2 strains were related with an emerging strains, similar to the A/Wisconsin/67/2005 prototype, nevertheless showing genetic variations in comparison with it. The complex genetic results are confirmed by the antigenic data suggesting that the H3N2 viruses are evolving continuously as demonstrated also by the emergence of a new variant in the recent 2007-08 epidemic, namely the A/Brisbane/10/07.

The antigenic site B (such 188, 190, 192, 193 codons) in H3N2 HA was the preferred site for genetic change during the study period, in paricular during 2004-05 season, probably because the site A has been masked by glycosylations.

The glycosylation sites are an important focal point because the virion surface glycoprotein attaches the virus to its receptors on host cells and fuses the viral envelope with the membranes of endocytic vesicles to initiate the infectious process. It is also the virion component that stimulates the formation of protective antibodies. The nature and extent of

glycosylation of the HA are thus implicated in altering the receptor binding properties and in the emergence of viral variants with enhanced cytopathogenicity and virulence and in masking its antigenic sites.

The N-linked glycosylation pattern varied during the study period and the H3N2 isolates from 2004 to 2007 were highly glycosylated with eleven predicted sequons in HA1, the highest amount of glycosylations observed in the last years.

H1N1 viruses, after sporadic cases during 2004-05 season, restarted to circulate in the 2006- 2007 season.

After years of little genetic change in the H1N1, the 2006-2007 viruses resulted antigenically and genetically distinct by the vaccine strain A/New Caledonia/20/99; the total effect of the genetic modifications led to the spread of the new variants in the population as a drifted variant. Among the different H1N1 viruses circulating in the 2006/07 season, the WHO Recommendations for Influenza Vaccine Composition introduced the A/SolomonIslan/03/06 as the new H1N1 vaccine strain. The analysis of the H1N1 viruses isolated in 2007/08 revealed that the directions of the evolution were rather different; we characterized the new viruses resembling to the H1N1 in FVG in the last epidemic season. As a consequence of this, the H1N1 strain recommended for the vaccine composition for the 2008/09 season was replaced by the A/Brisbane/59/2007 virus.

During the study period, with the exception is the 2006-2007 season, the H3N2 strains have undergone changes more frequently in the antigenic sites in HA than H1N1 strains did, thereby evading the host immune system more efficiently than H1N1.

Key positions in H3 HA for antibody antigenic change are 144 and 145 located in antigenic site A.

Changes in the 140-146 region, corresponding to the antigenic site A, are relevant to determine antigenically distinct viruses of epidemic significance. The residue (N144) has remained unchanged since the A/Fujian/411/02’s introduction. Several amino acid substitutions introduced with the A/California/7/04-like viruses were retained in the following seasons suggesting they may be important for viral escape from the host immune system and the overall fit of the virus. With the A/California/7/04(H3N2)-like viruses in 2004-2005, site 145 has changed from K and to N.

It was therefore suggested that substitutions at this site alone could have a large antigenic effect. Antigenic site A, located in a loop, makes few contacts with the rest of the structure, therefore 144 and 145 may change drastically without influencing the overall shape of the HA molecule.

Another region in HA, corresponding to position 225 to 227, that influence the antigenic site D, has drastically changed during the study period and in particular in the antigenic change from Fujian- like viruses to California-like viruses. The vaccine composition was subsequently changed for the 2005-2006 season to include the new variant.
The 2006-2007 vaccine composition was again changed to include A/Wisconsin/67/05.
The observed difference from circulating California- like to Wisconsin like viruses involved only three substitutions at three antigenic sites in HA.

Influenza B viruses are more genetically stable than influenza A with a minor impact to the population. Only during the 2004-05 season we detected a high circulation of B viruses. The majority of them were B/Hong Kong/330/2001-like (B/Victoria/2/87 lineage) viruses distinct from the B/Shanghai/361/02 vaccine virus and only five viruses were related to the Yamagata lineage.
The simultaneous circulation of viruses belonging to the both lineages was generally recorded, but alternating dominance of one lineage. Accordingly, the vaccine component for B virus influenza was adapted to these fluctuating events, namely, Victoria-like in 2002/03 and 2003/04 seasons, Yamagata-like in the season 2004/05. The reasons of such curious pattern are yet not clear but a possible explanation could rely on a partial immunity saturation of the population, due to the intensity of circulation of one variant during the preceding seasons.
This particular epidemiological feature, in which a lineage of B virus is unable to take a solid and stable role in the epidemiological circulation as happened all over the 1990s, suggests that the introduction of strains belonging to both B lineages in the composition of the influenza vaccine may be advisable.
Finally, Adenovirus and RSV infections seem to have a relevant role in the aetiology of ILI only in childhood, in particular in the age group of less than 5 yrs when the positivity rate for Adenovirus and RSV in the three considered seasons ranged 6-23% and 12-18% respectively; this observation may explain the low efficiency of Influenza virus detection in this age group.

Bibliography

Potter CW. A history of influenza. J Appl Microbiol. 2001;91(4):572-9.

Potter CW. Chronicle of influenza pandemics. Textbook of Influenza. ed. Nicholson, K.G., Webster, R.F. and Hay. 1998 A.J. pp 3-18. Oxford: Blackwell Science Ltd.

Waring J.I. A history of Medicine in South Carolina 1900-70. 1971 p33. South Carolina Medical association.

Crosby, A.W. 1989 America's forgotten pandemic. The Influenza of 1918. Cambridge University Press.

Patterson KD, Pyle GF The geography and mortality of the 1918 influenza pandemic. Bull Hist Med. 1991 Spring;65(1):4-21.

Taubenberger JK, Reid AH, Janczewski TA, Fanning TG. Integrating historical, clinical and molecular genetic data in order to explain the origin and virulence of the 1918 Spanish influenza virus. Philos Trans R Soc Lond B Biol Sci. 2001 Dec 29;356(1416):1829-39.

Kobasa D, Kawaoka Y. Emerging influenza viruses: past and present. Curr Mol Med. 2005 Dec;5(8):791-803.

Scholtissek C, Rohde W, Von Hoyningen V, Rott R. On the origin of the human influenza virus subtypes H2N2 and H3N2. Virology. 1978 Jun 1;87(1):13-20.

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 Nov;63(11):4603-8.

Nicholson KG, Nguyen-Van-Tam JS, Ahmed AH, Wiselka MJ, Leese J, Ayres J, Campbell JH, Ebden P, Eiser NM, Hutchcroft BJ, Pearson JC, Willey RF, Wolstenholme RJ, Woodhead MA.
Randomised placebo-controlled crossover trial on effect of inactivated influenza vaccine on pulmonary function in asthma. Lancet. 1998 Jan 31;351(9099):326-31.

Nguyen-Van-Tam JS. Epidemiology of Influenza. In: Nicholson KG, Webster RG, Hay AJ. , editors. Textbook of Influenza. Malden: Blackwell Science; 1998. pp. 181–206.

McCullers JA, Facchini S, Chersney PJ, Webster RG. Influenza B virus encephalitis. Clin Inf. Dis. 1999 Apr;28(4)898-900.

Morishima T, Togashi T, Yokota S, Okuno Y, Miyazaki C, Tashiro M, Okabe N. Encephalitis and encephalopathy associated with an influenza epidemic in Japan. Clin Infect Dis. 2002 Sep 1;35(5):512-7.

Kondo S, Abe K. The effects of influenza virus infection on FEV1 in asthmatic children. The time-course study. Chest. 1991 Nov;100(5):1235-8.

Teichtahl H, Buckmaster N, Pertnikovs E. The incidence of respiratory tract infection in adults requiring hospitalization for asthma. Chest. 1997 Sep;112(3):591-6.

Agyeman P, Duppenthaler A., Heininger U., Aebi C. Influenza-associated myositis in children. Infection. 2004. Aug; 32(4): 199-203

Starko KM, Ray CG, Dominguez LB, Stromberg WL, Woodall DF. Reye's syndrome and salicylate use. Pediatrics. 1980 Dec;66(6):859-64.

To KF, Chan PK, Chan KF, Lee WK, Lam WY, Wong KF, Tang NL, Tsang DN, Sung RY, Buckley TA, Tam JS, Cheng AF. Pathology of fatal human infection associated with avian influenza A H5N1 virus. J Med Virol. 2001 Mar;63(3):242-6.

Peiris JS, Yu WC, Leung CW, Cheung CY, Ng WF, Nicholls JM, Ng TK, Chan KH, Lai ST, Lim WL, Yuen KY, Guan Y. Re-emergence of fatal human influenza A subtype H5N1 disease. Lancet. 2004 Feb 21;363(9409):617-9.

Kobasa D, Wells K, Kawaoka Y. Amino acids responsible for the absolute sialidase activity of the influenza A virus neuraminidase: relationship to growth in the duck intestine. J Virol. 2001 Dec;75(23):11773-80.

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 Nov;30(2):500-5.

Nicholson KG, Wood JM, Zambon M. Influenza. Lancet. 2003 Nov 22;362(9397):1733-45.

Fouchier RA, Munster V, Wallensten A, Bestebroer TM, Herfst S, Smith D, Rimmelzwaan GF, Olsen B, Osterhaus AD. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. J Virol. 2005 Mar;79(5):2814-22.

Webster RG. Estimation of the molecular weights of the polypeptide chains from the isolated hemagglutinin and neuraminidase subunits of influenza viruses. Virology. 1970 Mar;40(3):643-54.

Zhang J, Lamb RA. Characterization of the membrane association of the influenza virus matrix protein in living cells. Virology. 1996 Nov 15;225(2):255-66

Skehel JJ, Wiley DC. Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. Annu Rev Biochem. 2000;69:531-69.

Ito T, Couceiro JN, Kelm S, Baum LG, Krauss S, Castrucci MR, Donatelli I, Kida H, Paulson JC, Webster RG, Kawaoka Y. Molecular basis for the generation in pigs of influenza A viruses with pandemic potential. J Virol. 1998 Sep;72(9):7367-73.

Scholtissek C, Müller K. Interference between influenza A viruses with a cleavable and a noncleavable hemagglutinin; pH-stability after mixed infection.
Arch Virol. 1988;101(1-2):119-23.

Plotkin J, Dushof J.Codon bias and frequency-dependent selection on the hemagglutinin epitopes of influenza A virus. PNAS 2003; 100 (12):7152-57

Kaverin NV, Gambaryan AS, Bovin NV, Rudneva IA, Shilov AA, Khodova OM, Varich NL, Sinitsin BV, Makarova NV, Kropotkina EA. Postreassortment changes in influenza A virus hemagglutinin restoring HA-NA functional match. Virology. 1998 May 10;244(2):315-21.

Stray SJ, Cummings RD, Air GM. Influenza virus infection of desialylated cells. Glycobiology. 2000 Jul;10(7):649-58.

WHO 1980. A revision of the system of nomenclature for influenza viruses: a WHO memorandum. Bull WHO 1980;58(4):585-91

D'Agaro P, Rossi T, Burgnich P, Molin GD, Coppola N, Rocco G, Campello C. The molecular epidemiology of influenza viruses: a lesson from a highly epidemic season. J Clin Pathol. 2008 Mar;61(3):355-60.

Lindstrom SE, Hiromoto Y, Nishimura H, Saito T, Nerome R, Nerome K.
Comparative analysis of evolutionary mechanisms of the hemagglutinin and three internal protein genes of influenza B virus: multiple cocirculating lineages and frequent reassortment of the NP, M, and NS genes. J Virol. 1999 May;73(5):4413-26.

Rota PA, Hemphill ML, Whistler T, Regnery HL, Kendal AP. Antigenic and genetic characterization of the haemagglutinins of recent cocirculating strains of influenza B virus.J Gen Virol. 1992 Oct;73 (10):2737-42.

WHO 2002, Draft WHO guidelines on the use of vaccines and antivirals during influenza pandemics. Wkly Epidemiol Rec, 2002. 77(47): p. 394-404

Yewdell J, García-Sastre A. Influenza virus still surprises. Curr Opin Microbiol. 2002 Aug;5(4):414-8.

Cannell JJ, Vieth R, Umhau JC, Holick MF, Grant WB, Madronich S, Garland CF, Giovannucci E. Epidemic influenza and vitamin D. Epidemiol Infect. 2006 Dec;134(6):1129-40.

Fleming DM, Zambon M, Bartelds AI. Population estimates of persons presenting to general practitioners with influenza-like illness, 1987-96: a study of the demography of influenza-like illness in sentinel practice networks in England and Wales, and in The Netherlands. Epidemiol Infect. 2000 Apr;124(2):245-53.

Stephenson I, Zambon M. The epidemiology of influenza. Occup Med (Lond). 2002 Aug;52(5):241-7.

Thursky K, Cordova SP, Smith D, Kelly H. Working towards a simple case definition for influenza surveillance. J Clin Virol. 2003 Jul;27(2):170-9.

Tang Y, Lee CW, Zhang Y, Senne DA, Dearth R, Byrum B, Perez DR, Suarez DL, Saif YM. Isolation and characterization of H3N2 influenza A virus from turkeys. Avian Dis. 2005 Jun;49(2):207-13.

Slemons RD, Johnson DC, Osborn JS, Hayes F. Type-A influenza viruses isolated from wild free-flying ducks in California. Avian Dis. 1974 Jan-Mar;18(1):119-24.

Fouchier RA, Munster V, Wallensten A, Bestebroer TM, Herfst S, Smith D, Rimmelzwaan GF, Olsen B, Osterhaus AD. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. J Virol. 2005 Mar;79(5):2814-22.

Lu JH, Zhang DM, Wang GL. Highlight the significance of genetic evolution of H5N1 avian flu. Chin Med J. 2006 Sep 5;119(17):1458-64.

Xu X, Subbarao, Cox NJ, Guo Y. Genetic characterization of the pathogenic influenza A/Goose/Guangdong/1/96 (H5N1) virus: similarity of its hemagglutinin gene to those of H5N1 viruses from the 1997 outbreaks in Hong Kong. Virology. 1999 Aug 15;261(1):15-9.

WHO 2008; Committee of Second WHO Consultion on Clinical Aspects of Human Infection with Avian influenza (H5N1), Jan 2008

Webster RG, Peiris M, Chen H, Guan Y. H5N1 outbreaks and enzootic influenza. Emerg Infect Dis. 2006 Jan;12(1):3-8.

Isoda N, Sakoda Y, Kishida N, Bai GR, Matsuda K, Umemura T, Kida H. Pathogenicity of a highly pathogenic avian influenza virus, A/chicken/Yamaguchi/7/04 (H5N1) in different species of birds and mammals. Arch Virol. 2006 Jul;151(7):1267-79.

Alexander ME, Bowman CS, Feng Z, Gardam M, Moghadas SM, Röst G, Wu J, Yan P. Emergence of drug resistance: implications for antiviral control of pandemic influenza.Proc Biol Sci. 2007 Jul 22;274(1619):1675-84.

Stieneke-Gröber A, Vey M, Angliker H, Shaw E, Thomas G, Roberts C, Klenk HD, Garten W. Influenza virus hemagglutinin with multibasic cleavage site is activated by furin, a subtilisin-like endoprotease. EMBO J. 1992 Jul;11(7):2407-14.

Hulse DJ, Webster RG, Russell RJ, Perez DR. Molecular determinants within the surface proteins involved in the pathogenicity of H5N1 influenza viruses in chickens. J Virol. 2004 Sep;78(18):9954-64.

Russell CJ, Webster RG. The genesis of a pandemic influenza virus. Cell. 2005 Nov 4;123(3):368-71.

Karen Y, Noah DL, Rao P, Kuo RL, Krug RM. The CPSF30 binding site on the NS1A protein of influenza A virus is a potential antiviral target. J Virol. 2006 Apr;80(8):3957-65.

Reid AH, Fanning TG, Hultin JV, Taubenberger JK. Origin and evolution of the 1918 "Spanish" influenza virus hemagglutinin gene. Proc Natl Acad Sci U S A. 1999 Feb 16;96(4):1651-6.

Tumpey TM, García-Sastre A, Taubenberger JK, Palese P, Swayne DE, Basler CF. Pathogenicity and immunogenicity of influenza viruses with genes from the 1918 pandemic virus. PNAS U S A. 2004 Mar 2;101(9):3166-71.

Taubenberger JK, Reid AH, Lourens RM, Wang R, Jin G, Fanning TG. Characterization of the 1918 influenza virus polymerase genes. Nature. 2005 Oct 6;437(7060):889-93.

Reid AH, Taubenberger JK, Fanning TG. Evidence of an absence: the genetic origins of the 1918 pandemic influenza virus. Nat Rev Microbiol. 2004 Nov;2(11):909-14.

García-Sastre A. Inhibition of interferon-mediated antiviral responses by influenza A viruses and other negative-strand RNA viruses. Virology. 2001 Jan 20;279(2):375-84.

Diebold SS, Kaisho T, Hemmi H, Akira S, Reis e Sousa C. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. Science. 2004 Mar 5;303(5663):1529-31.

Lewis DE, Gilbert BE, Knight V. Influenza virus infection induces functional alterations in peripheral blood lymphocytes. J Immunol. 1986 Dec 15;137(12):3777-81.

Mandelboim O, Lieberman N, Lev M, Paul L, Arnon TI, Bushkin Y, Davis DM, Strominger JL, Yewdell JW, Porgador A.Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells. Nature. 2001 Feb 22;409(6823):1055-60.

Liu H, Andreansky S, Diaz G, Turner SJ, Wodarz D, Doherty PC. Quantitative analysis of long-term virus-specific CD8+-T-cell memory in mice challenged with unrelated pathogens. J Virol. 2003 Jul;77(14):7756-63.

Renegar KB, Small PA Jr. Immunoglobulin A mediation of murine nasal anti-influenza virus immunity. J Virol. 1991 Apr;65(4):2146-8.

Tamura S, Kurata T. Defense mechanisms against influenza virus infection in the respiratory tract mucosa. Jpn J Infect Dis. 2004 Dec;57(6):236-47.

Carrat F, Flahault A. Influenza vaccine: the challenge of antigenic drift. Vaccine. 2007 Sep 28;25(39-40):6852-62.

Smith DJ, Lapedes AS, de Jong JC, Bestebroer TM, Rimmelzwaan GF, Osterhaus AD, Fouchier RA. Mapping the antigenic and genetic evolution of influenza virus. Science. 2004 Jul 16;305(5682):371-6.

Koelle K, Cobey S, Grenfell B, Pascual M. Epochal evolution shapes the phylodynamics of interpandemic influenza A (H3N2) in humans. Science. 2006 Dec 22;314(5807):1898-903.

Boni MF, Gog JR, Andreasen V, Christiansen FB. Influenza drift and epidemic size: the race between generating and escaping immunity. Theor Popul Biol. 2004 Mar;65(2):179-91.

Drake JW. Rates of spontaneous mutation among RNA viruses. Proc Natl Acad Sci U S A. 1993 May 1;90(9):4171-5.

Smith GL, Levin JZ, Palese P, Moss B. Synthesis and cellular location of the ten influenza polypeptides individually expressed by recombinant vaccinia viruses. Virology. 1987 Oct;160(2):336-45. Erratum in: Virology 1988 Mar;163(1):259.

Treanor J. Influenza vaccine outmaneuvering antigenic shift and drift. N Engl J Med. 2004 Jan 15;350(3):218-20.

Glezen WP. Emerging infections: pandemic influenza. Epidemiol Rev. 1996;18(1):64-76.

Wagner, R. et al. (2000) Interdependence of hemagglutinin glycosylation and neuraminidase as regulators of influenza virus growth: a study by reverse genetics. J. Virol. 74, 6316–6323

Potter CW. A history of influenza. J. Appl Microbiol. 2001 Oct;91(4):572-9.

Schweiger B, Bruns L, Meixenberger K. Reassortment between human A(H3N2) viruses is an important evolutionary mechanism. Vaccine. 2006 Nov 10;24(44-46):6683-90.

Smith GJ, Naipospos TS, Nguyen TD, de Jong MD, Vijaykrishna D, Usman TB, Hassan SS, Nguyen TV, Dao TV, Bui NA, Leung YH, Cheung CL, Rayner JM, Zhang JX, Zhang LJ, Poon LL, Li KS, Nguyen VC, Hien TT, Farrar J, Webster RG, Chen H, Peiris JS, Guan Y. Evolution and adaptation of H5N1 influenza virus in avian and human hosts in Indonesia and Vietnam. Virology. 2006 Jul 5;350(2):258-68.

Davison AJ, Benko M, Harrach B. Genetic content and evolution of adenoviruses. J Gen Virol. 2003 Nov;84 (11):2895-908.

Goncalves MA, de Vries AA. Adenovirus: from foe to friend. Rev Med Virol. 2006 May-Jun;16(3):167-86.

Kojaoghlanian T, Flomenberg P, Horwitz MS. The impact of adenovirus infection on the immunocompromised host. Rev Med Virol. 2003 May-Jun;13(3):155-71.

Mitchell LS, Taylor B, Reimels W, Barrett FF, Devincenzo JP. Adenovirus 7a: a community-acquired outbreak in a children's hospital. Pediatr Infect Dis J. 2000 Oct;19(10):996-1000.

Garnett CT, Erdman D, Xu W, Gooding LR. Prevalence and quantitation of species C adenovirus DNA in human mucosal lymphocytes. J Virol. 2002 Nov;76(21):10608-16.

Ebner K, Pinsker W, Lion T. Comparative sequence analysis of the hexon gene in the entire spectrum of human adenovirus serotypes: phylogenetic, taxonomic, and clinical implications. J Virol. 2005 Oct; 79(20):12635-42. Erratum in: J Virol. 2006 Mar;80(5):2586.

L. Valle, D. Amicizia, S. Bacilieri, F. Banfi, R. Riente, P. Durando, L. Sticchi, R. Gasparini C. Esposito G, Icardi, F. Asaldi Performance testing of two new one-step real time PCR assays for detection of human influenza and avian influenza viruses isolated in humans and Respiratory Syncytial Virus. J. of Preventive Medicine and Hygiene 2006; 47: 127-133.

Allard. A., Girones R., Per Juto, Wandell G.1990 Polymerase Chain Reaction for Detection of Adenoviruses in Stool Samples. J. of Clin. Microb., Dec. 1990;28 (12):2659-2667

Barry G. Hall 2008. Phylogenetic trees made easy: how-to manual. Third ed. Sinauer ed.

Links

http://www.who.int/csr/disease/influenza/en/

http://www.influnet.it/

http://www.cirinet.it

http://www.eiss.org/public/present.htm

http://www.ncbi.nlm.nih.gov/genomes/FLU/Database/multiple.cgi

http://hcv.lanl.gov/content/sequence/GLYCOSITE/glycosite.html