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GENETIC CHARACTERISTICS OF THE INFLUENZA A PANDEMIC (H1N1) 2009 VIRUS CIRCULATING IN ITALY

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ABSTRACT

Influenza viruses cause annual epidemics and occasional pandemics that have claimed the lives of millions. The emergence of new strains will continue to pose challenges to public health and the scientific communities. The recent flu pandemic, caused by a swine-origin A/H1N1 influenza virus, presents an opportunity to examine virulence factors, the spread of the infection and to prepare for major influenza outbreaks in the future.

As part of the intensified surveillance carried out during the influenza pandemic, the sequences of 133 pandemic A/H1N1 strains, from patients showing different clinical outcame (12 from fatal, 24 from severe and 97 from mild cases) have been examined at WHO National Influenza Centre (NIC) located at the National Institute of Health (Istituto Superiore di Sanità, ISS). Phylogenetic analysis of the new strain, showed that the virus circulating in Italy, combines genetic information related to different swine influenza viruses. Segments HA and NP are related to swine influenza viruses isolated in North America. Segments NA and M are related to swine influenza viruses isolated in Eurasia. Specific markers for virulence and pathogenicity have been evaluated in the viral genome. Sequence analysis of the isolates of the 2009 A/H1N1 viruses, to date, has not identified molecular features known to confer increased transmissibility or virulence in studies of other influenza A viruses, suggesting that previously unrecognized molecular determinants could be responsible for the transmission among humans.

Particular attention has been paid to the specific mutation in the viral haemagglutinin D222G, initially reported in association with fatal cases in several countries. On the basis of our observations, the majority of severe and fatal cases investigated in Italy did not carry the D222G substitution, and it was also observed in few mild cases, suggesting that this mutation is not required for a severe outcome.

We also describe a fatal case of myopericarditis presenting with cardiac tamponade in a previously healthy 11-year-old child, which has not shown the D222G mutation. Pandemic H1N1 2009 influenza A virus sequences were identified in throat and myocardial tissues and pericardial fluid, suggesting damage of myocardial cells directly caused by the virus.

The first case of oseltamivir-resistance among the influenza pandemic A/H1N1 strains circulating in Italy since the beginning of the pandemic is also reported.

ABSTRACT

I virus influenzali sono responsabili di epidemie annuali e di più rare pandemie occasionali, che in passato hanno causato la morte di milioni di persone. La comparsa di nuovi ceppi virali rappresenta una continua minaccia per la salute pubblica e una sfida per la comunità scientifica. La recente pandemia influenzale, causata da un virus di sottotipo A/H1N1 di origine suina, costituisce un ulteriore strumento per lo studio dei fattori di virulenza e della diffusione dell'infezione in preparazione a futuri eventi pandemici.

Nell'ambito dell'attività di sorveglianza virologica dell'influenza, intensificata durante la pandemia influenzale, le sequenze di 133 ceppi pandemici A/H1N1, isolati da pazienti che mostravano diversi esiti clinici (12 casi fatali, 24 gravi e 97 lievi) sono state esaminate presso il WHO-National Influenza Centre (NIC) dell'Istituto Superiore di Sanità (ISS).

L'analisi filogenetica dei ceppi in studio, ha mostrato che i virus pandemici circolanti in Italia, derivano dal riassortimento genetico di virus influenzali diffusi tra i suini. I segmenti genici HA e NP mostrano una stretta correlazione con i ceppi isolati nei suini del Nord America, mentre i segmenti M ed NA correlano maggiormente con i ceppi suini circolanti in Eurasia. La presenza di specifici marcatori molecolari per virulenza e patogenicità è stata valutata all'interno dell'intero genoma virale. L'analisi delle sequenze degli isolati analizzati, non ha evidenziato alcuna caratteristica molecolare coinvolta in una maggiore trasmissibilità o virulenza, suggerendo che preesistenti determinanti molecolari potrebbero essere responsabili della elevata trasmissibilità.

Particolare attenzione è stata dedicata alla mutazione nel gene della emagglutinina virale D222G, inizialmente riportata da molti paesi in associazione a casi clinici ad esito fatale. Sulla base delle nostre osservazioni, solo pochi tra i casi fatali e gravi, analizzati in Italia, presentano la sostituzione D222G, osservata anche in casi lievi, suggerendo che questa mutazione non è strettamente associata ad infezioni ad esito fatale.

In questo studio è inoltre descritto un caso di miocardite ad esito fatale in un bambina di 11 anni. Il virus pandemico, non presentante la mutazione D222G, è stato identificato nei tessuti della gola, del miocardio e nel fluido pericardico, il che suggerisce un danno alle cellule del miocardio causato direttamente dal virus.

Viene anche riportato il primo isolamento di un ceppo pandemico A/H1N1 resistente all'Oseltamivir in Italia a partire dall'inizio della pandemia.

KEYWORDS: influenza A virus, genetic reassortment, swine, pandemic, H1N1, molecular markers.

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1. INTRODUCTION

Influenza viruses are among the most common causes of human respiratory infections and they cause high morbidity and mortality.

Human epidemic influenza is caused by influenza type A and B viruses, which continually undergo antigenic change in their surface antigens. Seasonal influenza is an acute and recurring respiratory disease occuring, in particular, during winter months. The disease has high morbidity rates for people of all ages and particularly high mortality rates for children, adults over 60 years old, patients with chronic illnesses.

Influenza A viruses are also responsible for sporadic pandemics that usually cause higher mortality rates than seasonal influenza epidemics. Pandemic influenza occurs, at irregular and unpredictable intervals, when a new influenza A virus emerges for which there is little or no immunity in the human population; the virus causes serious illness and spreads easily from person-to-person worldwide, such as in 1918, when over twenty million deaths occurred. It is the result of a major antigenic change which occurs only in Influenza A viruses.

Based on history, influenza is and will continue to be a serious threat to public health. The rapid evolution of influenza viruses highlights the importance of surveillance in identifying novel circulating strains; to this aim, a global surveillance of influenza is actually maintained by a network of laboratories sponsored by the World Health Organization.

1.1. ORTOMYXOVIRIDAE

Influenza viruses are enveloped RNA viruses belonging to the Ortomyxoviridae family. According to the identity of two internal viral proteins, the nucleoprotein (NP) and matrix (M1) protein, influenza viruses are classified in three antigenically distinct groups, named A, B and C.

Influenza A and B viruses can cause serious diseases in humans, whereas only minor illness are due to type C viruses.

Type B and C are mainly found in humans, whereas influenza A viruses can infect a large spectrum of vertebrate hosts represented by both avian and mammalian species, human being included (Lamb et al., 1996).

Both the wide host range and the animal reservoir place influenza A viruses in a central position among the zoonotic emerging infectious desease (Morens et al., 2004).

1.1.1. STRUCTURE OF INFLUENZA A VIRUS

Influenza A virus has a lipid bilayer envelope, within which are eight RNA genomic segments, each of which is associated with the trimeric viral RNA polymerase (PB1, PB2, PA) and coated with multiple nucleoproteins (NPs) to form the viral nucleoproteins (vRNPs).

The outer layer of the lipid envelope is spiked with multiple copies of HA, NA and a small number of M2, whereas the M1 molecules keep vRNPs attached to the inner layer. According to the major differences of the main virus surface proteins, at the present time a total of 16 HAs (from H1 to H16) and 9 NAs (from N1 to N9) are known, and both HA and NA groups comprise different subtypes that, in general, are not cross-reactive serologically (Fouchier et al., 2005).

The influenza virus virions (Fig.1) are known to display a number of shapes, with the most abundant one being roughly spherical. The viral envelope is made up of a lipid bilayer that contains three of the viral transmembrane proteins: HA, NA, and M2. This lipid bilayer is derived from the host's plasma membrane and is known to contain both cholesterol-enriched lipid rafts and non-raft lipids (Scheiffele et al., 1992; Nayak et al., 2009). HA is the most abundant envelope protein at approximately 80 percent, followed by NA, which makes up around 17 percent of the viral envelope proteins. M2 is a very minor component of the envelope, with only 16 to 20 molecules per virion.

Sitting just underneath the viral lipid membrane is M1, which forms a matrix holding the viral ribonucleoproteins (vRNPs). These vRNPs are the core of the virus and are made up of the viral negative stranded RNAs, which are wrapped up around NP and very small amounts of NS2. At one end of the vRNPs are the three polymerase proteins (PB1, PB2 and PA) that make up the viral RNA polymerase complex (Nayak et al., 2004).

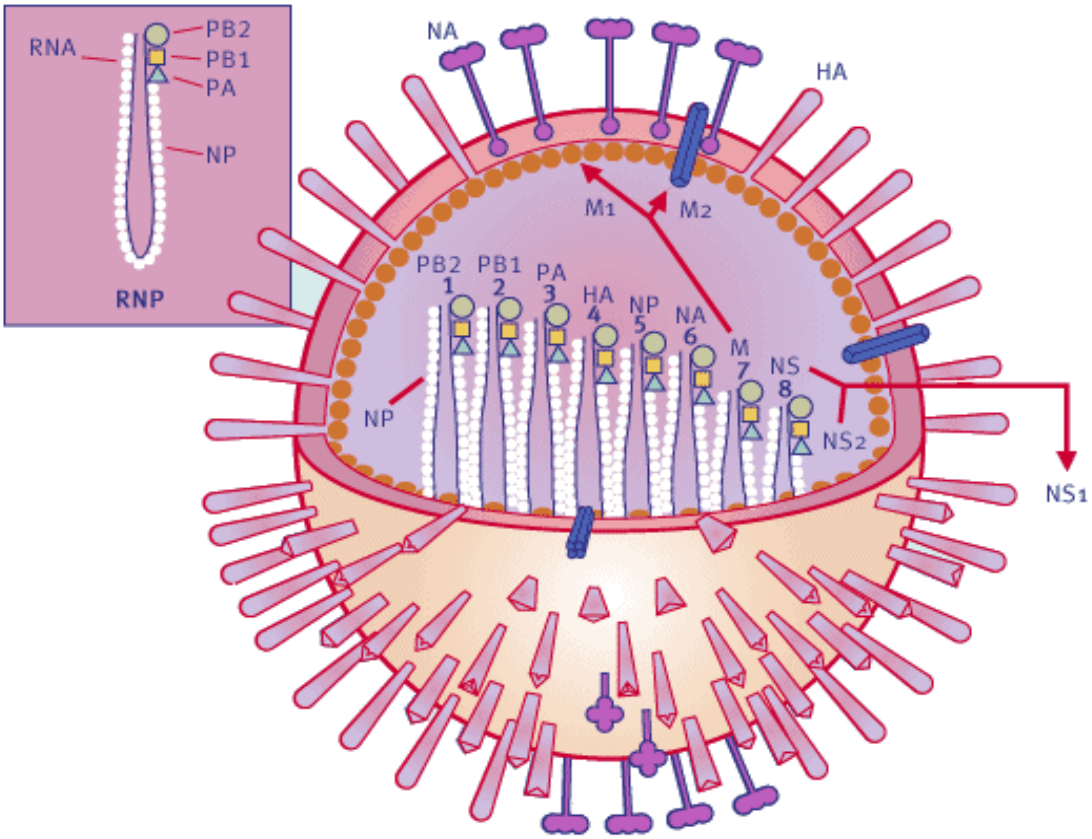


Fig. 1 Structure of the influenza virus virion.

Viral genome:

Influenza A is an enveloped virus with a genome of negative sense, single-stranded, segmented RNA. Influenza A contains eight segments that encode for 11 viral genes (Tab. 1). The three largest RNA segments encode the three viral RNA-dependent RNA polymerase (RdRP) proteins: polymerase acidic protein (PA), polymerase basic protein 1 (PB1) and PB2. The RNA segment for PB1 also encodes a small 87-residue nonstructural protein, PB1-F2, which has apoptotic functions (Chen et al., 2001).

The three intermediate-size RNA segments encode the hemagglutinin (HA), the neuraminidase (NA) and the nucleoprotein (NP). The larger of the remaining two segments encodes the M1 matrix protein and the M2 ion-channel protein, and the smaller one encodes two nonstructural proteins, NS1 and NS2 (also known as nuclear export protein, NEP) (Fields et al., 2007).

Gene segment	nucleotides	Protein product	molecular weight (dalton)	function
1	2341	**PB2**	86.000	recognizes and binds the 5'cap structures from host mRNAs for use as viral mRNA transcription primers
2	2341	**PB1**	83.000	responsible for elongation of the primed nascent viral mRNA and also as elongation protein for template RNA and vRNA synthesis
		PB1-F2	10.500	appears to enhance virus-induced cell death
3	2233	**PA**	85.000	induces proteolytic degradation of coexpressed proteins, suggesting a role in virion RNA synthesis
4	1778	**HA1**	50.000	responsible for attachment of virus to cells and fusion of virus and cell membranes; cleavage into HA1 and HA2 is required for infectivity
		HA2	30.000	
5	1565	**NP**	56.000	internal protein associated with RNA and polymerase proteins
6	1413	**NA**	50.000	responsible for release of virus from infected cells
7	1027	**M1**	27.000	involved in both virus assembly and budding
		M2	11.000	proton channel protein contributes to virus disassembly
8	890	**NS1**	27.000	non-structural protein, viral interferon antagonist
		NS2	13.000	contributes to the extra-nuclear exportation of newly synthesized RNPs

Tab. 1 Genes and gene products of Influenza A virus

Hemagglutinin:

The HA protein is an integral membrane protein and the major surface antigen of the influenza virus virion. It is responsible for binding of virions to host cell receptors and for fusion between the virion envelope and the host cell.

A newly synthesized ~70-kDa HA is cleaved into HA1 (~50-kDa) and HA2 (~27-kDa) subunits, which are disulfide linked. This cleavage is accomplished by host-produced trypsinlike proteases and is required for infectivity because virus-cell fusion is mediated by the free amino terminus of HA2.

HA molecules form homotrimers during maturation. The three-dimensional structure of a complete HA trimer has been determined. In essence, each HA molecule consists of a globular head on a stalk. The head is made up exclusively of HA1 and contains the receptor-binding cavity as well as most of the antigenic sites of the molecule. The stalk consist of all of HA2 and part of HA1. The carboxy-terminal region of HA2 contains the hydrophobic transmembrane sequence (Fig.2).

Owing to error prone viral RNA polymerase activity, influenza virus HA is subject to a very high rate of mutation. Selection for amino acid substitutions is driven at least in part by immune pressure, as the HA is the major target of the host immune response. Although the amino acids making up the receptor-binding site are highly conserved, the remainder of the HA molecule is highly mutable. In nature there are presently 16 recognized subtypes of HA (Murphy & Webster, 1996; Rohm et al., 1996, Fouchier et al., 2005).

HA is, also, the major determinant of host range restriction. The HAs of human influenza viruses preferentially bind sialyloligosaccharides terminated by N-acetylsialic acid linked to galactose by the α 2,6 linkages (NeuAcα 2,6Gal), whereas avian and equine influenza viruses preferentially recognize receptors terminating in an α 2,3 linkage (NeuAcα 2,3Gal). In the host

parenchyma of different species, presence, quantity and distribution of these cell receptors can vary influencing the viral tissue tropism; this is mailnly respiratory in mammals and both respiratory and intestinal in avian species (Horimoto et al., 2001; Zambon, 2001). In fact, NeuAcα 2,6Gal linkages are predominant in the human respiratory tract, whereas NeuAcα 2,3Gal are characteristic of horse trachea and duck intestine. The presence of both types of linkages in the epithelial cells of the pig trachea allows this species to be infected with human and avian viruses (Ito et al., 1998).

Neuraminidase:

The NA protein is also an integral membrane glycoprotein and a second major surface antigen of the virion. NA is a mushroom-shaped tetrameric protein, anchored to the viral membrane by a single hydrophobic sequence of some 29 amino acids near the N-terminus. Treatment of virions with pronase liberates a 200-kDa protein containing 4 identical and glycosylated polypeptides that have all of the antigenic and enzymatic properties of membrane-bound neuraminidase (Colman, 1994) (Fig. 2).

Like HA, NA is highly mutable with variant selection partly in response to host immune pressure. Nine subtypes of NA have been identified in nature (Murphy & Webster, 1996; Rohm et al., 1996). The host cell receptors for influenza A and B viruses are cell surface sialic acids. The predominant type of sialic acids is N-acetylneuraminic acid (Neu5Ac), which is the biosynthetic precursor for most of the other types. In nature, Neu5Ac is mostly linked to the penultimate galactose residues of carbohydrate side chains via α(2,3)- or α(2,6)-linkages.

Both Neu5Ac α(2,3)-Gal and Neu5Ac α(2,6)-Gal molecules can be recognized as a receptor by influenza viruses but human viruses prefer α(2,6)-linked sialic

acid, whereas avian and equine viruses predominantly recognize α(2,3)-linked sialic acid.

NA (belonging to the sialidase family) cleaves the α-ketosidic linkage between terminal Neu5Ac and the galactose residues on the host cell surface. Experimental evidence is available to support a role for the enzyme in facilitating release of progeny virions from the surface of infected cells, where they would otherwise aggregate as a result of interactions between the hemagglutinin and sialic acid on the surface of the infected cell and on the progeny virion envelope (Palese et al., 1974; Palese & Compans, 1976; Griffin & Compans, 1979; Griffin et al., 1983).

It also serves as an important antigenic site. The NA carries several important amino acid residues which, if they mutate, can lead to resistance against neuraminidase inhibitors.

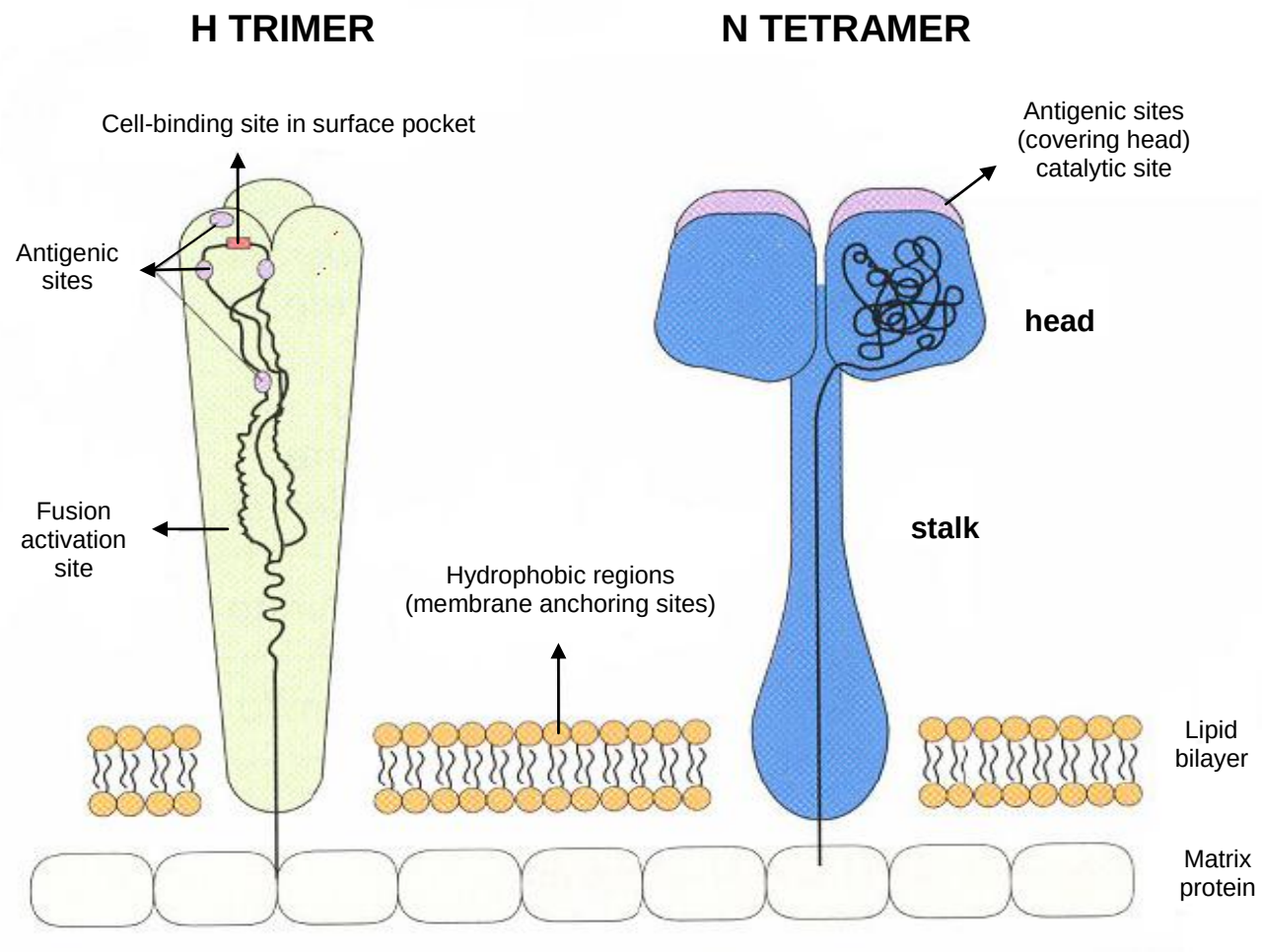


Fig. 2 Surface glycoprotein of the virion (HA and NA).

Other viral proteins:

The NP interacts with RNA as a structural component of the virion and also associates with the three polymerase proteins to form the transcriptase complex (Lamb, 1989). The NP has also been implicated in the switch from mRNA to vRNA synthesis in the infected cells (Schapiro & Krug, 1988; Portela & Digard, 2002).

The three polymerase proteins function as a complex in the nucleus of the infected cells, where transcription and replication of influenza virus RNA occur. The PA subunit of the influenza virus polymerase complex is a phosphoprotein that induces proteolytic degradation of coexpressed proteins, suggesting a role in virion RNA synthesis (Sanz-Ezquerro et al, 1998).

PB1 is responsible for elongation of the primed nascent viral mRNA and also as elongation protein for template RNA and vRNA synthesis, PB2 is known to function during initiation of viral mRNA transcription as the protein which recognizes and binds the 5'cap structures from host mRNAs for use as viral mRNA transcription primers (Nakagawa et al, 1996).

M1 protein is the most abundant protein in the influenza virus virion. Matrix protein forms a shell surrounding the virion nucleocapsids, underneath the virion envelope. It plays an important role in initiating progeny virus assembly. The mRNA for M2 is also transcribed from RNA segment 7. It is derived from the colinear (M1) transcript by splicing. M2 is an integral membrane protein, which acts as a proton channel to control the pH of the Golgi during HA synthesis and to allow acidification of the interior of the virion during virus uncoating (Yasuda et al., 1993).

The NS1 is a phosphoprotein and has two nuclear localization signals. In the nucleus, the NS1 interferes with the splicing as well as the nuclear export of cellular mRNAs. In the later time of the infection, the NS1 is present in the cytoplasm and associates with the polysomes. The NS1 binds to the 5'UTR of

some viral mRNAs and stimulates translation. In addition , NS1 is probably able to suppress the interferon response in the virus- infected cell leading to unimpaired virus production. The NS2 protein plays an important role in the nuclear export of the vRNPs interacting with the RNP-M1 complex and the nucleoprotein.

1.1.2. VIRAL LIFE CYCLE

A. Entry into the host cell

HA is a homotrimer that forms spikes on the viral lipid membrane. These spikes of HA bind to sialic acid found on the surface of the host cell's membrane (Webster et al., 1992; Skehel & Wiley, 2000) (Fig. 3).

The HA precursor, HA0, is made up of two subunits: HA1, which contains the receptor binding domain, and HA2, which contains the fusion peptide. These subunits are linked by disulphide bonds (Huang et al., 2003). Two major linkages are found between sialic acids and the carbohydrates they are bound to in glycoproteins: α(2,3) and α(2,6). These are extremely important for the specificity of the HA molecules in binding to cell surface sialic acid receptors found in different species. Viruses from humans recognize the α(2,6) linkage, whereas those from avians and equines recognize the α(2,3) linkages. Those from swine recognize both (Rogers & Paulson, 1983; Connor et al. 1994).. This explains the importance of swine being a good mixing vessel for avian and human influenza viruses, hence producing dangerous pathogenic viruses. Upon binding to the host cell's sialic acid residues, receptor-mediated endocytosis occurs and the virus enters the host cell in an endosome. The endosome has a low pH of around 5 to 6, which triggers the fusion of the viral and endosomal membranes. The low pH induces a conformational change in HA0, leading to maintenance of the HA1 receptor-binding domain but exposing the HA2 fusion peptide. This fusion peptide inserts itself into the endosomal membrane, bringing both the viral and endosomal membranes into contact with each other. The acidic environment of the endosome is not only important for inducing the conformation in HA0 and, thus, fusion of the viral and endosomal membranes but also opens up the M2 ion channel. M2 is a type III transmembrane protein that forms tetramers, whose transmembrane

domains form a channel that acts as a proton-selective ion channel (Holsinger et al., 1991; Pinto et al., 1992)

Opening theM2 ion channels acidifies the viral core. This acidic environment in the virion releases the vRNP from M1 such that vRNP is free to enter the host cell's cytoplasm (Bullough et al., 1994; Pinto & Lamb, 2006).

B. Transcription and replication of the viral genome

Influenza viral transcription and replication occurs in the nucleus; therefore, after being released into the cytoplasm, the vRNP enter the nucleus. The viral proteins that make up the vRNP are NP, PA, PB1, and PB2. All of these proteins have known nuclear localization signals (NLSs) that can bind to the cellular nuclear import machinery and, thus, enter the nucleus. To date, it is unclear which NLS is the most important for vRNP nuclear entry.

The influenza viral genome is made up of negative sense strands of RNA. In order for the genome to be transcribed, it first must be converted into a positive sense RNA to serve as a template for the production of viral RNAs. Replication of the genome does not require a primer; instead, the viral RNA dependent RNA polymerase (RdRp) initiates RNA synthesis internally on viral RNA, this is possible, as the extreme 5' and 3' ends of the genome exhibit partial inverse complementarity. Mature cellular messenger RNAs (mRNAs) have a 5' methylated cap and a poly (A) tail. It is known that the vRNPs have poly(A) tails but no 5' caps. It was determined that the 5'methylated caps of the viral mRNAs actually belonged to the cellular mRNAs. That discovery lead to the formulation of the "cap-snatching" mechanism (Plotch et al., 1981).

The viral RdRp is made up of three viral proteins: PB1, PB2, and PA. PB2 has endonuclease activity. It binds to the 5' methylated caps of cellular mRNAs

and cleaves the cellular mRNAs' 10 to 15 nucleotides 3' to the cap structure. This cellular capped RNA fragment is used by the viral RdRp to prime viral transcription (Li et al., 2001).

Cellular RNA Polymerase II (Pol II) binds to DNA and starts transcription. Six but two of the viral segments encode for one protein. Segments 7 and 8 encode for two proteins each due to splicing. Segment 7 encodes for M1 and M2; whereas, segment 8 encodes for NS1 and NS2. M2 and NS2 are the spliced products and generally are found in much lower abundance than NS1 and M1 (Amorim & Digard, 2006). The virus uses the host cell's splicing machinery to express both of these proteins (Engelhardt & Fodor, 2006).

Despite influenza's need for the cellular splicing machinery, it prevents the host cell from using its own splicing machinery for processing the host cell mRNAs. NS1 binds to U6 small nuclear RNAs (snRNAs) (Lu et al., 1994) and other splicing components, causing them to re-localize to the nucleus of infected cells. In this way, influenza is able to inhibit splicing of cellular mRNAs. It also has been shown to bind to a novel protein called NS1 binding protein (NS1-BP), causing it to re-localize to the nucleus in infected cells (Wolff et al., 1998).

The mechanism of polyadenylation of viral mRNAs is very unusual. Cellular mRNAs are polyadenylated through cleavage at the polyadenylation signal (AAUAAA) by cleavage and polyadenylation specificity factor (CPSF) and subsequent addition of a poly(A) tail at the 3' end of the mRNA. Viral mRNAs do not contain this sequence; instead, the viral RdRp remains bound to the 5' end of the template viral RNA, leading to steric blockage at the end of viral RNA synthesis (Hagen et al., 1994).

Each viral segment has a stretch of five to seven U residues approximately 17 nucleotides from the 5' end, and this forms the basis of the viral polyadenylation signal. Interestingly, NS1 inhibits the nuclear export of

cellular mRNAs by preventing cellular mRNAs from being cleaved at the polyadenylation cleavage site.

It is known that only negative sense vRNPs are exported from the nucleus (Shapiro et al., 1987). vRNPs appear to be exported out of the nucleus via the CRM1 dependent pathway through the nuclear pores. NP has been shown to interact with CRM1 directly, although no GTP hydrolysis activity could be detected. This indicates an unusual method of export if the binding of NP to CRM1 is critical for export of the vRNPs. M1 is known to interact directly with the vRNPs through the C-terminal end of the protein. It has been shown that the N-terminal portion of M1 can bind to NS2, thus masking the NLS. NS2 also has been shown to bind to CRM1 with the accompanying GTP hydrolysis that normally occurs in a CRM1- dependent export pathway. It is hypothesized that M1 binds to the negative sense vRNPs, as well as binding to NS2. In turn, NS2 binds to CRM1, and through this "daisychain" complex, the vRNPs are exported out of the nucleus (Akarsu et al., 2003).

A. Assembly and budding at the host cell's plasma membrane

After the vRNPs have left the nucleus, all that is left for the virus to do is form viral particles and leave the cell. Since influenza is an enveloped virus, it uses the host cell's plasma membrane to form the viral particles that leave the cell and go on to infect neighbouring cells. Virus particles bud from the apical side of polarized cells. Because of this, HA, NA, and M2 are transported to the apical plasma membrane. It has been shown through deletion and mutational analysis that the tail of M2 is extremely important in the formation of viral particles. Viruses that had the M2 tail deleted or partially mutated produced elongated particles.

M1, which is present underneath the lipid bilayer, is important in the final step of closing and budding off of the viral particle (Martin & Helenius, 1991).

There are two models that have been hypothesized to explain the packaging of viral genomic segments into virions: the random packaging model (Bancroft & Parslow, 2002) and the specific packaging model (Smith & Hay, 1982).

The former predicts that viral genomic segments are randomly packaged into virions; whereas, the latter predicts that there are signals present in the viral segments dictating which segments are to be packaged into the virions.

Packaging signals have been identified in the 5' and 3' non-coding and coding regions of some of the viral segments (Fujii et al.,2003), thus leaning toward the specific packaging model. One of the most important steps that must occur before the newly made viral particle can leave the plasma membrane is the cleavage of sialic acid residue from glyco-proteins and glycolipids. NA removes these sialic acids. Without this process, the viral particle would not be released from the plasma membrane .

HA, the major surface antigen, allows virus infection when it is cleaved at a specific site by host trypsin-like enzymes in two subunits HA1and HA2.

Cleavage of the HA by cellular trypsin like proteases is necessary for viral infectivity and liberates a hydrophobic, highly conserved amino terminus of HA-2. Under conditions of low pH such as exist in endosomal vesicles, a conformational change occurs in the HA which results in the amino terminus of HA-2 being exposed on the outside of the molecule.

The ability of the HA to be cleaved by cellular proteases has been correlated with virulence of avian influenza viruses in bird (Webster and Rott, 1987).

Host trypsine-like enzymes cleave HA in two subunits , HA1 and HA2. This proteolytic activation is essential for infectivity and spread of virions in the host organism. HP avian strains, to date represented by H5 and H7 subtypes, are cleaved in a broad range of tissues by ubiquitous proteases and cause systemic infections. Avian influenza viruses of the H5 or H7 subtypes with

genome sequences coding for multiple basic amino acids at the cleavage site of the haemagglutinin molecule similar to that observed for other HPAI viruses, indicating that the haemagglutinin molecule can be cleaved by a host ubiquitous protease.

On the contrary, LP avian influenza viruses (AIVs) are cleaved in only a few cell type, usually restricted to epithelial cells of the respiratory or gastrointestinal tract, and cause localized infections.In the HPAIVs the presence of multiple basic amino acid motifs at the HA cleavage site is a relevant virulence factor, lacking in most LPAIVs that usually have a single basic amino acid in this position.

When LP H5 or H7 subtypes infect reared land-based birds these virus can mutate to high pathogenicity, increasing virulence by the insertions of polybasic residues at the HA cleavage site.

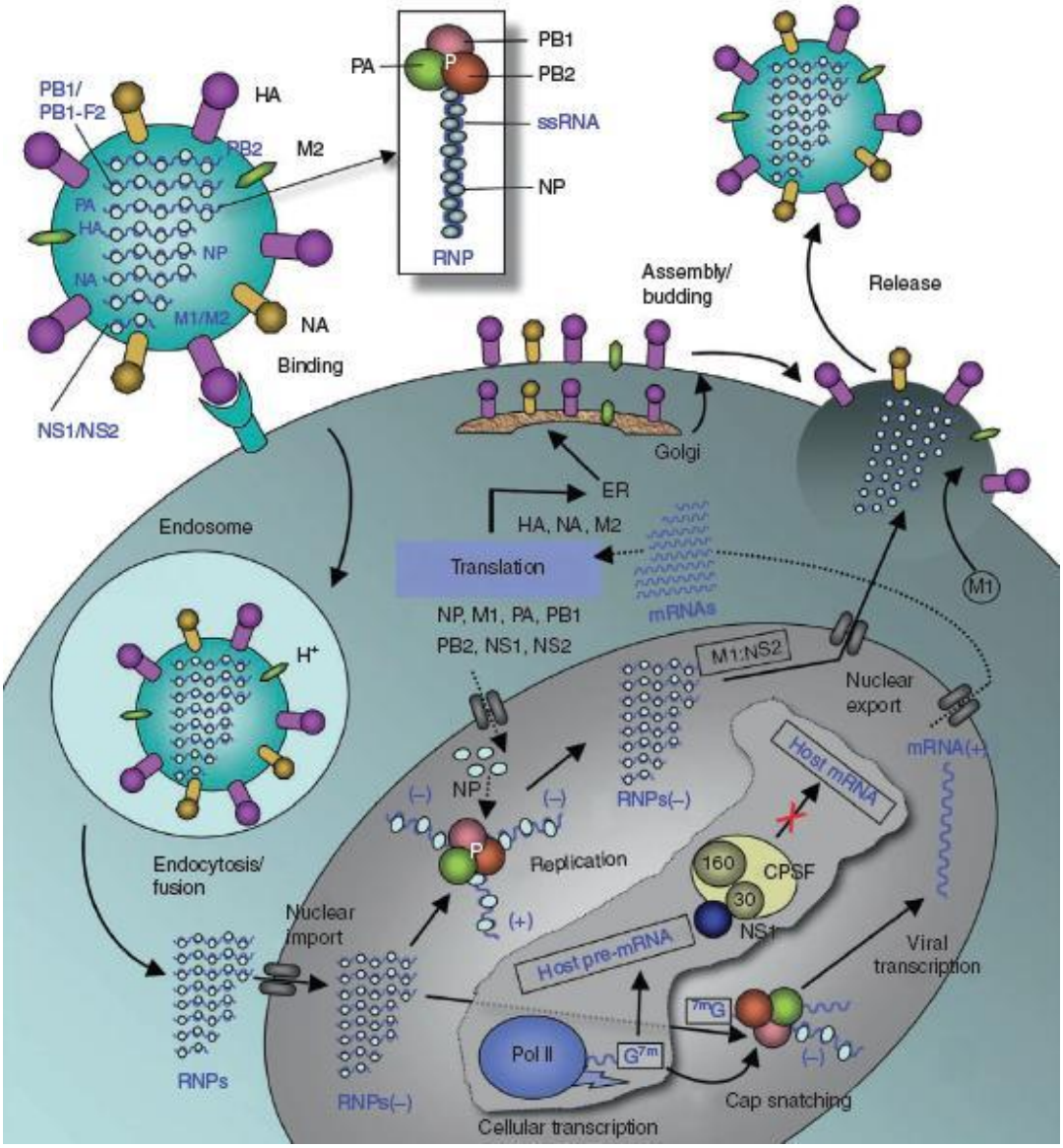


Fig. 3 Viral life cycle

1.2. ANTIGENIC VARIATION OF INFLUNZA A VIRUSES

The world of the RNA viruses includes biological organisms characterized by the highest mutation rate known in nature. RNA viruses have an intrinsically high RNA polymerase error rate, and during a replication cycle on average 1 error in 104 bases occurs. For this reason RNA virus populations can also be named quasispecies populations that include different genetic and phenotypic variants, arising during replication phases when competitive and selective mechanisms occur. Influenza viruses could represent quasispecies population in particular when they jump from their natural hosts (aquatic birds) to other non-reservoir hosts, moving from a well adapted host/parasite balance, where viruses show an apparent genetic stability, to an unstable evolutive relationship. During this interspecies transmission a rapid modification of the HA surface antigen usually occurs (Garcia et al., 1996) even if, during the outbreaks that occurred in Hong Kong in 1997, the initial rapid evolution of H5N1 viruses in chicken involved all gene products but the HA glycoprotein (Zhou et al., 1999). threet, and RNA recombination.

1.2.1. ANTIGENIC DRIFT

As previously described, genetic drift of influenza A viruses is due to point mutations in the RNA segments coding for all the virus gene products; however it is most relevant for the surface proteins HA and NA, causing the accumulation of small antigenic changes that represent the antigenic drift. This antigenic change mainly occurs in mammalian influenza viruses, and it is usually higher for human influenza A viruses compared to the swine and equine ones (Webster et al., 1992). In particular, in humans this process is responsible for annual epidemics due to the random emergence of variants

selected by the host immune response; it usually occurs during inter-pandemic periods, when viral variants belonging to the same HN subtype, such as H1N1 or H3N2, circulate (De Jong et al., 2000).

In avian hosts antigenic drift is very limited, in particular in the aquatic bird reservoir where a well adapted host/parasite balance exists at the population level, whereas it can occur in non-adapted avian hosts. This latter situation has been observed in Asia during the severe H5N1 chicken epidemics and in Mexico when H5N2 poultry outbreaks occurred between 1993 and 2002. Moreover, the widespread associated vaccination program likely facilitated the infection persistence in the field, allowing the emergence of virus lineages able to escape the population immune pressure (Hinshaw et al., 1980).

Antigenic drift results in minor antigenic changes (single or few aminoacids only) favoured by the high mutation rate of RNA viruses.

1.2.2. ANTIGENIC SHIFT

Antigenic shift is a major mutation due to mechanisms of genetic exchange and it is typical of segmented RNA viruses; it results from the simultaneous infection of a single cell with two or more antigenically distinct influenza A viruses which can exchange RNA segments and finally can originate novel strains and/or HN subtypes; reassortment of two influenza A viruses, each having eight RNA segments, can theoretically produce 256 different recombinations .

Human influenza pandemics can originate from genetic reassortment of viral RNA segments, causing antigenic shift of previously circulating HN serotypes.

For example, before 1968 an H2N2 influenza virus caused annual epidemics in the human population and when the new virus A/Hong Kong/68 (H3N2) appeared, it replaced the previously circulating H2N2 subtype and rapidly spread worldwide. Compared to the earlier H2N2 virus, the new pandemic strain had, as surface antigens, a new H3 haemagglutinin and an old N2 neuraminidase. Further genetic and biochemical characterization showed that six out of eight RNA segments of the Hong Kong pandemic virus derived from the previously circulating H2N2 virus and both the gene segments coding for the H3 haemagglutinin and PB1 protein were discovered to be recently introduced in the human population, likely from an avian donor (Horimoto & Kawaoka, 2001).

In this context, the presence in swine trachea cells of both α 2-3 and α 2-6 terminal sialic acid linkages make this species susceptible to both human and avian influenza A viruses and various evidence strongly indicates that pigs can represent, during viral co-infections, an ideal "mixing vessel", able to originate novel pandemic viruses (Lipatov et al., 2004).

The rarity of antigenic drift in waterfowl is a consequence of well adapted host/parasite interactions. However, ducks can increase the antigenic variability of avian influenza viruses (AIVs) by genetic reassortment. This event determines the genesis of recombinant viruses, as demonstrated in experimental and natural co-infections.

1.2.3. RNA RECOMBINATION

Finally, the third mechanism causing antigenic variability of influenza A viruses is RNA recombination; this is typical f other RNA viruses such as retroviruses and it was demonstrated for the first time for avian influenza during the outbreaks that occurred in Chile in 2002. On that occasion, an

H7N3 lowly pathogenic (LP) AIV mutated to high pathogenicity acquiring virulence by a 30 nucleotide insertion at the cleavage site. This event was likely due to recombination between the HA and nucleoprotein genes of the LPAIV (Suarez et al., 2004).

1.3. THE ECOLOGY OF INFLUENZA A VIRUSES

1.3.1 RESERVOIRS OF INFLUENZA A VIRUSES IN NATURE

The ecology of influenza A viruses is dynamic and complex involving multiple host species and viral genes.

Influenza A virus can infect a wide range of species, including both avian and mammalian hosts. At the present time, aquatic birds are generally indicated as the natural reservoir of the gene pool of influenza A viruses, which they perpetuate in nature, from one year to the next, by well-adapted host/parasite relationships. Influenza A viruses are preferentially endemic in water birds, such as ducks, geese, and shore birds (gulls), which usually do not fall ill from this infection. All 16 known hemagglutinin (HA) and 9 neuraminidase (NA) subtypes of influenza A viruses have been isolated from wild waterfowl and seabirds (Webster et al, 2006) (Fig. 4).

The presence of all subtypes influenza A viruses in wild aquatic birds poses serious health risks to a wide range of animal species. Few distinct subtypes have been isolated from pigs, horses, seals, whales and human beings (Fig. 5). Equines can be only infected with influenza viruses H3N8 and H7N7, although the latter has not been detected in horses in recent years and may have disappeared completely (Paillot et al., 2006). Dogs can also be infected with the H3N8 equine variant (Dubovi et al., 2008; Crawford et al., 2005). A variety of influenza viruses have been found in aquatic mammals (H1, H3, H4, H7, and H13 containing variants). The avian flu H5N1 outbreak in cats, leopards and tigers demonstrated that it was also possible to infect felids with this strain (Keawcharoen et al., 2004; Leschnik et al., 2007).

Asymptomatic infection in ducks are due to the circulation of lowly pathogenic avian influenza viruses (LPAIVs) mainly transmitted by the faecal-oral route (Webster et al., 1992). Through their movement and migrations,

wild birds carry the virus to various countries and environments where new hosts and/or parasite populations can be present. According to the main bird migratory routes, spatial and/or temporal segregation exists among wild bird populations of the world. This leads to the existence of numerous avian influenza strains that are basically grouped by several sublineages streaming in a few different geographical lineages, mainly represented by the Eurasian, Australian, North and South America clades (Suarez et al., 2004; Gorman et al., 1990). However, gene segment reassortment between different geographical clades have been reported, showing the existence of some contact areas among avian populations of different continents. The mixing, at a single location, of various populations of migratory birds could also explain the genetic variation recently described in AIVs isolated from natural reservoir species (Spackman et al., 2005; Chen et al., 2006): the selection pressure, naturally occurring at low level in these well adapted hosts, may increase when several bird groups having specific influenza immunity share different viruses.

Although some of these subtypes are non-pathogenic/nonvirulent within their natural hosts and have been present in these animal reservoirs for many centuries, various subtypes are highly virulent within their natural host species and to other species (Webby et al., 2007). For example, the changing role of the highly pathogenic avian influenza virus (HPAIV) H5N1 subtype in both wild and domestic ducks has recently been documented as a potential public health hazard because they are zoonotic agents with the theoretical ability – after genetic adaptation – of a human-to-human transmission (Hulse-Post et al., 2005).

Among domestic species utilized for meat and egg production, land-based birds (such as chickens and turkeys) are highly susceptible to avian influenza viruses. Moreover in these birds the well-adapted host/parasite interaction, typical of the aquatic avian reservoir, tends to change into an unstable balance

characterized by clinical disease ranging from mild respiratory symptoms to lethal illness forms able to kill 100% of the infected groups (Easterday et al., 1997). Fatal cases are usually due to the H5 and the H7 virus subtypes that, introduced in domestic avian species, can evolve into highly pathogenic (HP) strains responsible for systemic disease.

Commercial poultry farms, "wet markets", (where live birds and other animals are sold), backyard poultry farms, commercial and family poultry slaughtering facilities, swine farms, human dietary habits and the global trade in exotic animals have all been implicated in the spread of influenza A viruses (Greger, 2006). The "wet markets" of Southeast Asia, where people, pigs, ducks, geese and chickens (and occasionally other animals) are in close proximity pose a particular danger to public health (Webster, 2004; Bush, 2005; Greenfeld, 2006; Lau et al, 2007).

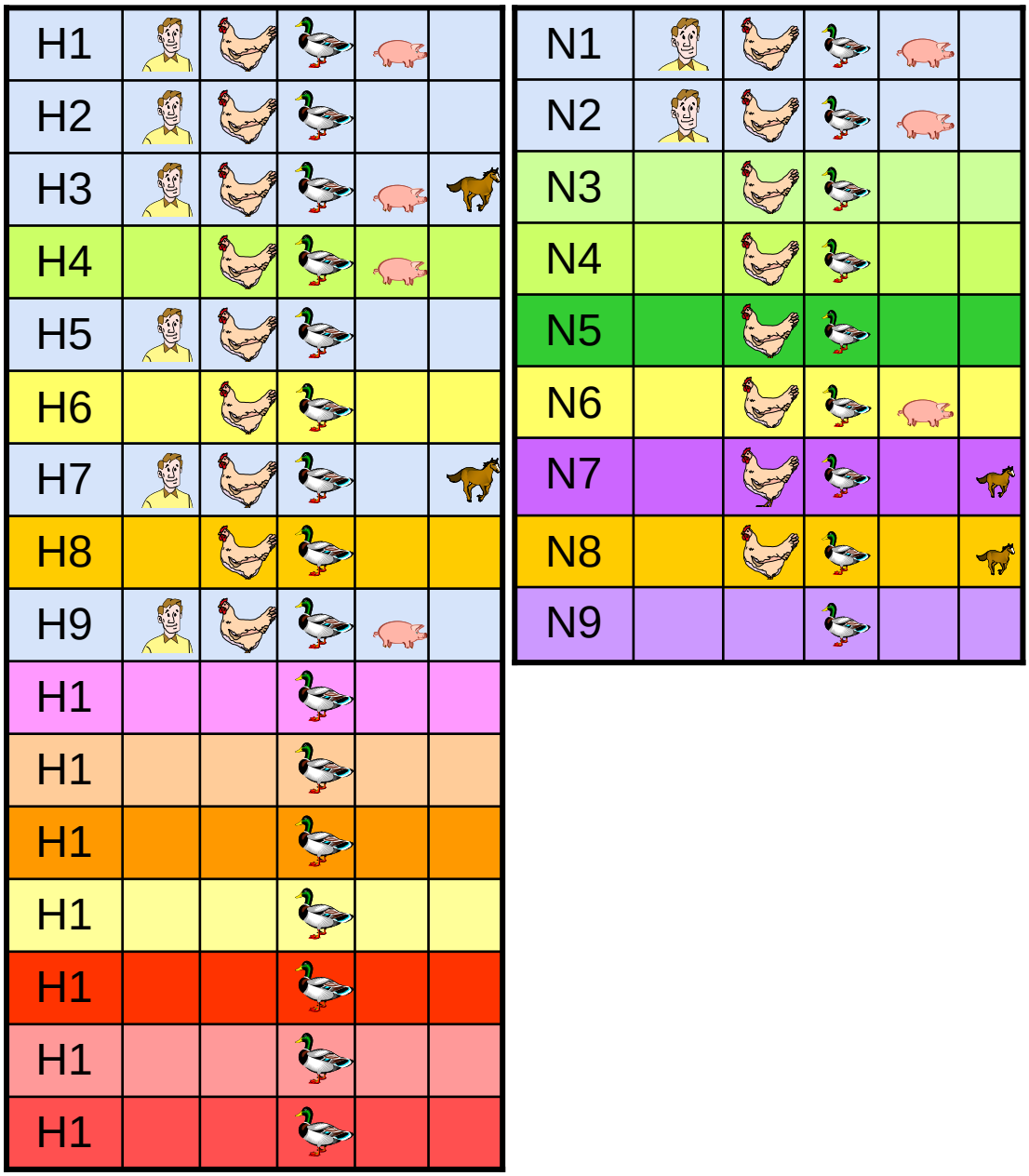


Fig. 4 Hemagglutinin (H) and neuraminidase (N) antigenic subtypes.

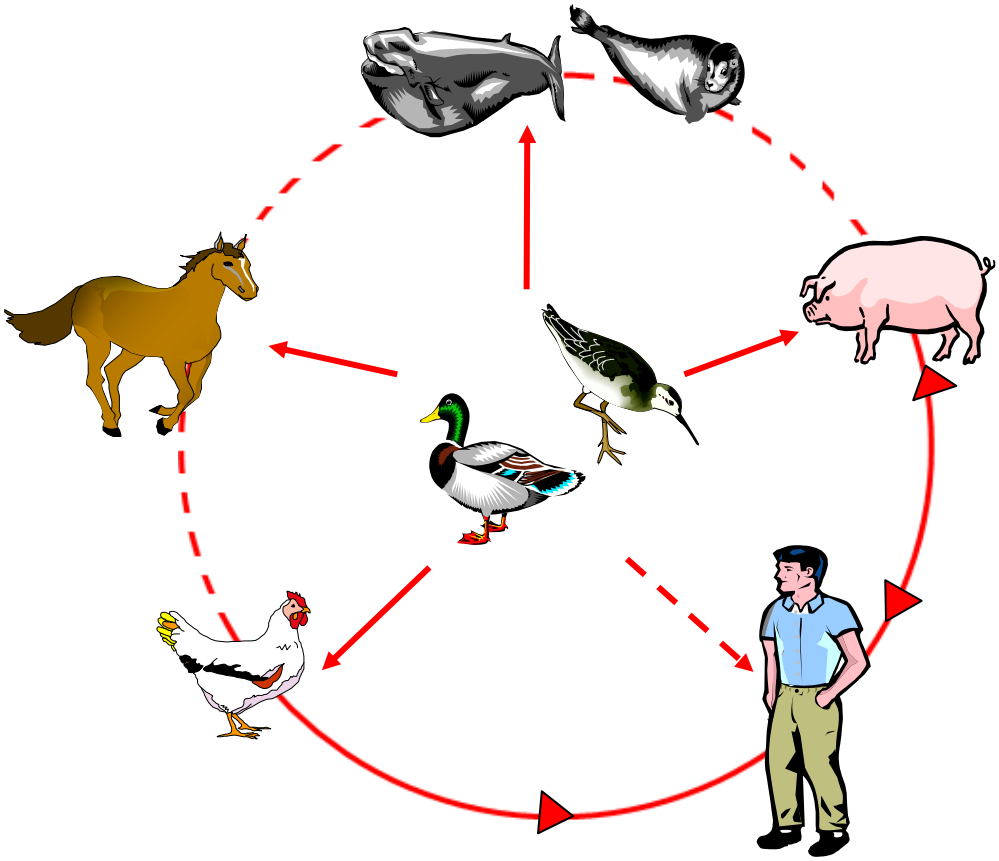


Fig. 5 Interspecies transmission of influenza A viruses.

Solid lines; frequent and/or confirmed transmission events.
Dotted lines; possible and/or occasional transmission events.

1.3.2. EMERGENCE OF PANDEMIC INFLUENZA VIRUSES

Pandemics are epidemics that rapidly spread on a worldwide scale, caused by pathogens against which humans have no immunity that infect a large part of the population and lead to associated serious illnesses.

Influenza pandemics occur when an influenza virus with a hemagglutinin, against which there is little or no existing immunity, emerges in the human population and efficiently transmits from human to human. Three human influenza pandemics occurred in the twentieth century, in 1918, 1957, and 1968. Human influenza pandemics are caused by emerging influenza viruses from non-human reservoirs. From the three influenza pandemics of the twentieth century, the 1918 pandemic was possibly caused by an influenza virus with an avian origin (Taubenberger et al., 2005; Rabadan et al., 2006) and the other two, in 1957 and 1968, were caused by new strains that were combinations of avian and human viruses through the process of reassortment (Lindstrom et al., 2004; Scholtissek et al., 1978)

There are two major reassortant hypotheses:

- the avian influenza virus transmits to humans first and then reassorts with human influenza viruses;
- both the avian and human influenza viruses infect and reassort in an unknown mammal, for example pigs; then the novel reassortant virus is transmitted to humans (Ito et al, 1998).

Introduction of an avian influenza virus in toto into the human population

Taubenberger and his colleagues (2005), after analyzing the complete genome of the 1918 Spanish flu virus, proposed that the 1918 virus was not a reassortant virus (like those of the 1957 and 1968 pandemics), but more likely an entirely avian-like virus which crossed the species barrier to humans without an intermediate mammal and infected and adapted to humans. However, it should be noted that recent studies have found that the receptors preferentially binding avian influenza viruses are located in the lower portions of the human respiratory system (Shinya et al., 2006; Gambotto et al, 2008), explaining how avian influenza viruses can directly infect humans and also why human-to-human transmission with non-adapted avian influenza viruses is rather limited. If changes were to occur in virus receptor binding affinity of avian influenza viruses that permitted replication of avian influenza viruses also in the upper human respiratory system, this would result in efficient human-to-human transmission and the possibility of a pandemic (van Riel, 2006).

The first documented instance of human infection with avian influenza A virus H5N1 occurred in Hong Kong in 1997. Infection was confirmed in 18 individuals, 6 of whom died. Infections were acquired by humans directly from chickens, without the involvement of an intermediate host. An influenza virus (H5N1) known to infect only birds previously was found to infect human causing disease and death. Prior to the human outbreak, the H5N1 virus was found to cause extensive death in chickens in three farms in Hong Kong (Shortridge et al., 1998) (Fig. 6).

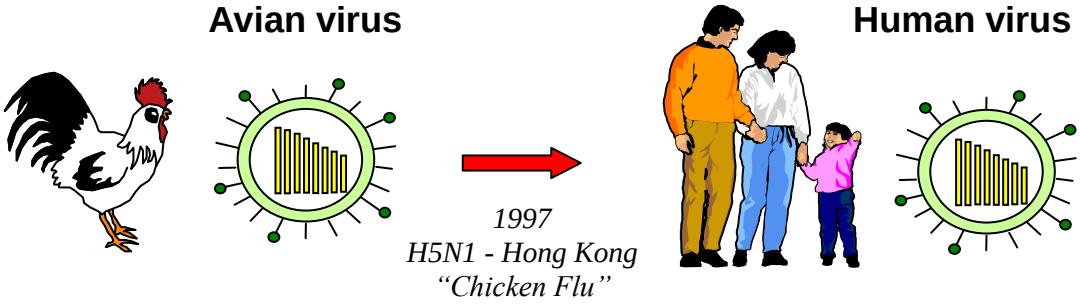


Fig. 6 Introduction of an avian influenza virus in toto into the human population.

- Then, in early 2003, a father and his nine-year-old son, who had been visiting relatives in Fujian Province in China, were hospitalized when they returned to Hong Kong with H5N1 infection. A second of the father's children – an eight-year-old girl – had died of an undiagnosed respiratory illness, while visiting in China. The father subsequently succumbed, the hospitalized boy recovered. The virus responsible was found to be a mutated strain of the H5N1 virus that had first surfaced in human beings in Hong Kong, in 1997.
- The largest outbreak of subtype H7 infections in humans to date occurred in the spring of 2003, when an HPAI (H7N7) virus was detected in commercial poultry farms in the Netherlands and necessitated the culling of >30 million birds (Fouchier et al., 2004). All internal genes of this virus were of avian origin and were found to be related to low pathogenicity viruses detected during surveillance of ducks in the region in 2000. Eighty-six persons involved in the culling operation and 3 of their family members who had not been in contact with infected poultry had virologically confirmed subtype H7 illness, which suggests that limited human-to-

human transmission of the avian virus also had occurred. Among these persons, 78 had conjunctivitis, 5 had conjunctivitis and respiratory symptoms, 2 had respiratory symptoms only, and 1 died, a veterinarian who had visited several infected farms and in whom an acute respiratory distress syndrome and pneumonia developed (Koopmans et al., 2003; Meijer et al., 2004).

From late 2003 H5N1 influenza viruses spread in an unprecedented manner across Asia, affecting poultry in Vietnam, Thailand, Indonesia, China, Japan, South Korea, Cambodia, and Laos. Hundreds of millions of chickens and ducks were culled in an effort to stop the spread. The outbreak appeared largely under control in March 2004. However, in July 2004 the virus reemerged in Thailand, Vietnam, and China and was isolated for the first time in Malaysia.

According to the World Health Organization (WHO) (http://www.who.int/ csr/disease/avian_influenza/country/cases_table_2009_05_15/en/ index.html)., as of May 2009, 424 human infections with H5N1 have been confirmed, resulting in 261 deaths. Although several family clusters of H5N1 virus infection have been described, sustained human-to-human infection has not occurred. Hence, these H5N1 viruses are characterized by a high mortality rate but inefficient spread among humans. It should be noted that 90% of H5N1 cases (348) and 91% of H5N1-related deaths (223) have been in only five countries: Indonesia, Vietnam, Egypt, Thailand and China. Nevertheless, since 2005, the WHO Pandemic Alert Level dealing with the H5N1 HPAIV epidemic has remained at Phase 3: “Human infection(s) with a new subtype, but no human-to-human spread, or at more rare instances of spread to a close contact” (WHO, 2005). Human-to-human transmissions of HPAIV H5N1 have been found within individual families in Thailand, Indonesia and most probably China (Ungchusak et al, 2005; Normile, 2007). The epidemic situation in these countries has come close to a Phase 4 Pandemic Alert:

“Small cluster(s) with limited human-to human transmission but spread is highly localized, suggesting that the virus is not well adapted to humans” (WHO, 2005). However, because the virus has remained localized within these families, the WHO has not raised the alert level.

The pig as a mixing vessel for influenza viruses

The obvious potential of creating novel reassortant influenza viruses in pigs has led to the "mixing vessel" theory (Fig. 7). The theory was first proposed by Scholtissek and his colleagues (1985) based on the understanding that human influenza A viruses do not spread easily to birds and vice versa, whereas the species barrier to pigs is rather low (Scholtissek, 1990; 1996). The antigenic and genetic similarities between certain subtypes of avian, swine and human influenza viruses and the susceptibility of swine to avian and human influenza viruses form the basis of this theory. Most avian and human influenza viruses preferentially bind to specific receptor types having SA α-2,3 Gal (avian receptor) - or SA α-2,6 Gal (mammalian receptor) - terminated saccharides, respectively (Rogers and Paulson, 1983; Rogers and D'Souza, 1989). Both receptors have been found in the tracheal epithelium of swine (Ito et al, 1998), providing solid molecular evidence for pigs as "mixing vessels" for human and avian influenza viruses, therefore, pigs are considered as an intermediate host for the adaptation of avian influenza viruses to humans or as mixing vessels for the generation of genetically reassortant viruses.

This phenomenon was responsible for the 1957 pandemic when the human H1N1 strain that had been circulating since 1918 reassorted to become a human H2N2 strain with new PB1, HA, and NA segments of avian origin. Also, in 1968, the reassortment of the PB1 and HA segments created a new human H3N2 strain which is currently co-circulating with the human H1N1 strain that reappeared in 1977 (Nakajima et al., 1978; Scholtissek et al., 1978). Analysis of the 1957 H2N2 pandemic strain found that the emergent virus resulted from the acquisition by previously circulating human H1N1 of three new gene segments of avian origin (the H2 gene, the N2 gene, and one other). Similarly, the 1968 pandemic H3N2 virus acquired two new genes from an avian virus closely related to viruses isolated from ducks in Asia in 1963.

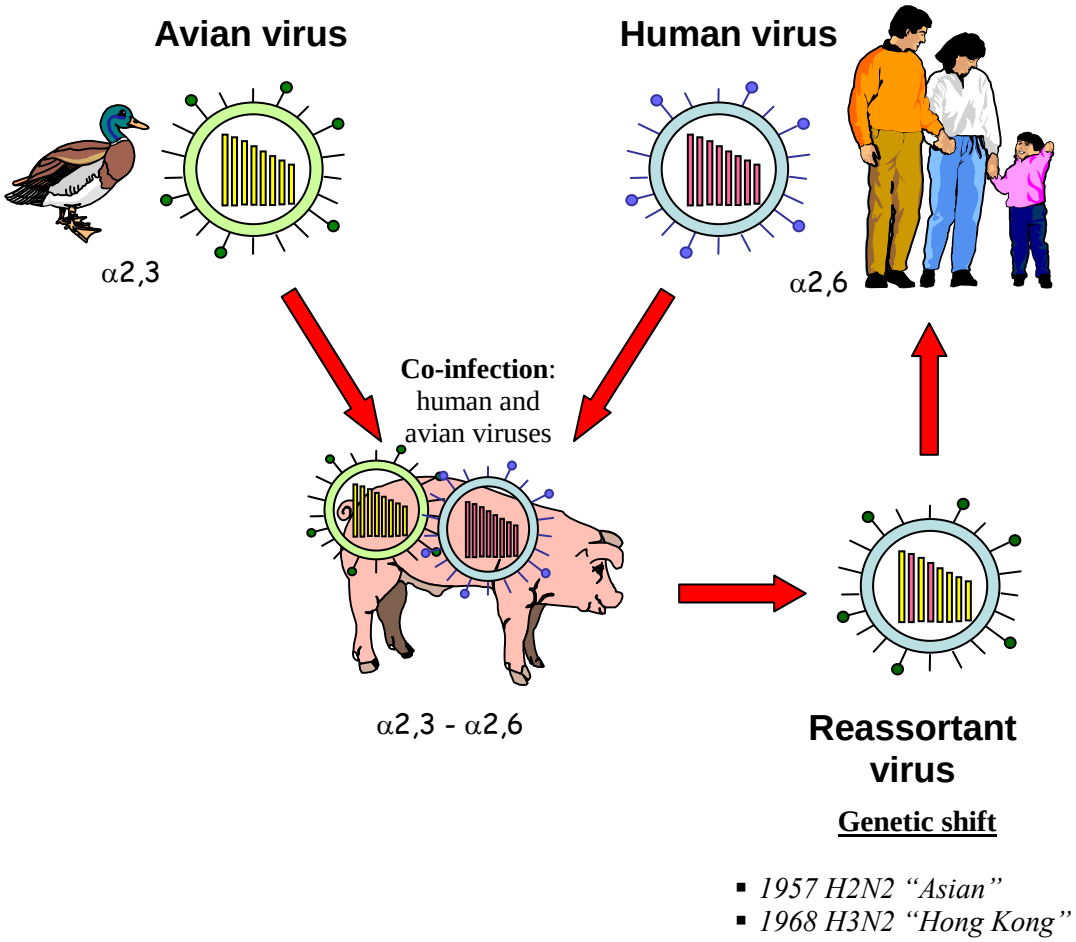


Fig. 7 Pig as a mixing vessel.

1.3.3. A NOVEL SWINE-ORIGIN INFLUENZA A VIRUS H1N1 IN HUMANS

During April 2009, a novel H1N1 virus was detected in epidemiologically unrelated cases of influenza-like illness in California and was subsequently recognized to be the cause of a major outbreak of respiratory disease in Mexico that had been ongoing for some weeks previously. The virus was found to be an H1N1 virus that was antigenically and genetically unrelated to human seasonal influenza viruses and genetically related to viruses known to circulate in swine.

As of 25th of April, the virus caused over 17919 deaths in 214 countries in the Americas, Europe, Asia and Australasia (WHO). On June 11, 2009, the World Health Organization raised its pandemic level to the highest level, Phase 6, indicating widespread community transmission on at least two continents. The virus contains a novel constellation of gene segments, the nearest known precursors being viruses found in swine and it likely arose through reassortment of two or more viruses of swine origin. H1N1, H1N2 and H3N2 subtype swine influenza viruses have occasionally infected humans before but such zoonotic transmission events did not lead to sustained human-to-human transmission in the manner this swine-origin influenza virus has done. Clinical disease generally appears mild but complications leading to hospitalization can occur, especially in those with underlying lung or cardiac disease, diabetes or those on immunosuppressive therapies. Children and young adults appear to be the most affected, perhaps reflecting protection in the elderly owing to exposure to H1N1 strains before 1957.

The current swine flu has emerged from reassortment of gene segments from North American and Eurasian swine strains that have been undetectably circulating in humans for a long period of time (Garten et al., 2009). The H1-subtype HA of swine flu differs substantially from recent H1 HAs of seasonal

influenza A viruses. Consequently, most of the human population lacks immunological protection against this virus, resulting in a pandemic. It is unusual for a pandemic virus to have the same HA subtype as currently circulating seasonal strains.

Genetic analysis of 2009 H1N1 viruses isolated in North America, Europe and Asia revealed quadruple reassortant swine influenza A viruses that have not been recognized previously in pigs or humans. The virus resulted from the reassortment of North American H3N2 and H1N2 swine viruses (triple reassortment viruses: avian/swine/human with Eurasian swine viruses) (Morens et al., 2009) . Sequence analysis also suggests that PB2 and PA genes originated from American H3N2 avian virus; a PB1 originated from H3N2: HA, NP, and NS genes originated from classical swine virus: and NA and M genes originated from Eurasian swine virus.

The NA and M gene segments are in the Eurasian swine genetic lineage. Viruses with NA and M gene segments in this lineage were originally derived from a wholly avian influenza virus and thought to have entered the Eurasian swine population in 1979 (Pensaert et al., 1981), continue to circulate throughout Eurasia, and have not been previously reported outside Eurasia. The HA, NP, and NS gene segments are in the classical swine lineage. Viruses that seeded this lineage are thought to have entered swine around 1918 and subsequently circulated in classical swine viruses and triple reassortant swine viruses (Olsen, 2002). The PB2 and PA gene segments are in the swine triple reassortant lineage. Viruses that seeded this lineage, originally of avian origin, entered swine in North America around 1998. Finally, the PB1 gene segment is in the swine triple reassortant lineage. This lineage of PB1 was seeded in swine from humans at the time of the North American swine triple reassortment events (Zhou et al., 1999) and was itself seeded from birds around 1968 (Kawaoka et al.,1989). However, the human-like PB1 gene and the avian-like PB2 and PA genes have been circulating in pigs since

1997/1998 (when triple reassortant swine viruses were first isolated), and have likely undergone adaptation to pigs. These viruses do not possess markers associated with high pathogenicity.

Figure 8 summarizes the host and lineage origins for the gene segments of the 2009 A/H1N1 virus.

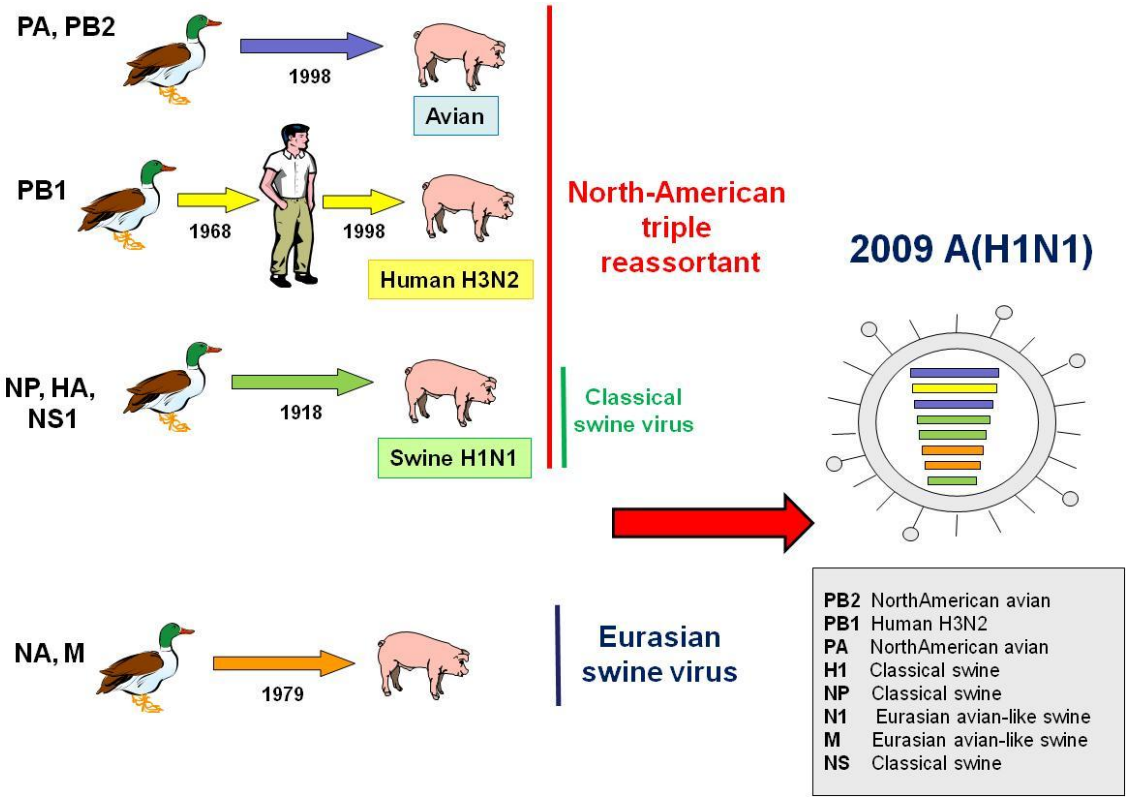


Fig. 8 Host and lineage origins for the gene segments of the 2009 A(H1N1) virus.

1.3.4. VIROLOGICAL INFLUENZA SURVEILLANCE IN ITALY AND THE 2009 PANDEMIC A/H1N1 VIRUS

In Italy, influenza surveillance is routinely based on integrated epidemiological and virological national networks. Seasonal virological surveillance is carried out by the WHO National Influenza Centre (NIC) located at the National Institute of Health (Istituto Superiore di Sanità, ISS), which coordinates the activities of 15 collaborating laboratories. In case of emergency, further 12 hospital laboratories are involved in the surveillance activities. The NIC performs quality control assessment and laboratory validation activities specifically aimed to strengthen the diagnostic capabilities of the Italian laboratory network. When a pandemic occurs, the major task of the NIC is to rapidly detect and/or confirm cases of influenza and perform virus characterisation. In response to the spread of the pandemic A/H1N1 virus in the United States and Mexico, virological surveillance activities throughout Italy were maintained effective beyond the usual deadline (week 17) of seasonal influenza surveillance. Since 28 April 2009, the Ministry of Health (MoH) undertook a number of actions, including the recommendations to enhance surveillance activities and laboratory confirmation of suspected and probable cases, which were published as a national guidance document [Ministry of Health of Italy. Influenza A (H1N1). Azioni del Governo [Actions of the government] [in Italian]. Available from: http://www.ministerosalute.it/dettaglio/approfondimentoFocusNuovo.jsp?id=13&sub=1&lang=it&area=influenzaA]. The main scope of the guidance was the early identification of individuals presenting with influenza-like illness and recent history of travel to the affected areas and the adoption of population distancing measures (early isolation of cases and precautionary school closure) and antiviral prophylaxis of close contacts of cases, in order to contain the spread of pandemic A/H1N1 virus cases in the country. In particular, a seven-

day period of isolation at home of travellers coming back from affected areas, although asymptomatic, was initially recommended. According to the above document, pharyngeal and/or nasal swabs should be collected by family and/or hospital doctors from each suspected case (i.e. a case fitting the clinical and epidemiological criteria (Commission Decision of 30 April 2009 amending Decision 2002/253/EC laying down case definitions for reporting communicable diseases to the Community network under Decision n° 21/19/98/EC. 2009/363/EC. Official Journal L 110/58. 01.05.2009. Available from:http://eurlex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2009:110:0058:0059:EN:PDF) and two separate aliquots of the samples should be sent – one to the regional reference laboratory and another one to the NIC. Since 20 May 2009, following the updated MoH recommendations, only specimens from probable cases (i.e. cases with positive test results for influenza A virus) should be sent for influenza pandemic A/H1N1 virus confirmation by NIC. The notification of confirmed cases of infection to the MoH is done by the NIC.

As of 10 June 2009, the number of cases of influenza pandemic A/H1N1 virus infection reached 27,737 in 74 different countries, with 141 deaths. On 11 June 2009 the WHO raised the level of pandemic alert to phase 6.

The pandemic influenza A/H1N1 virus, emerged in Italy in April 2009, has clearly been the predominant virus circulating among the population while seasonal influenza A/H1N1, A/H3N2 and B viruses circulated simultaneously to a lesser extent.

From week 18/2009 (April 2009) to week 18/2010 (April 2010), 13.309 influenza virus detections have been reported by the Italian laboratory network: 13.258 were influenza A (99,6%) and 51 (0,4%) were influenza B.

Of the influenza A viruses, 12.903 (97,3%) were subtyped, with 12.981 being pandemic A/H1N1 and 12 A/H3N2 (Fig. 9).

The pandemic virus has been characterized by mild and self –limiting disease in the over-whelming majority of cases. However, severe and fatal cases, many of them with primary viral pneumonia, have been occurring in age groups where such clinical outcomes are very rarely seen in seasonal influenza. It is important to better understand what viral and host-related factors determine this dichotomy.

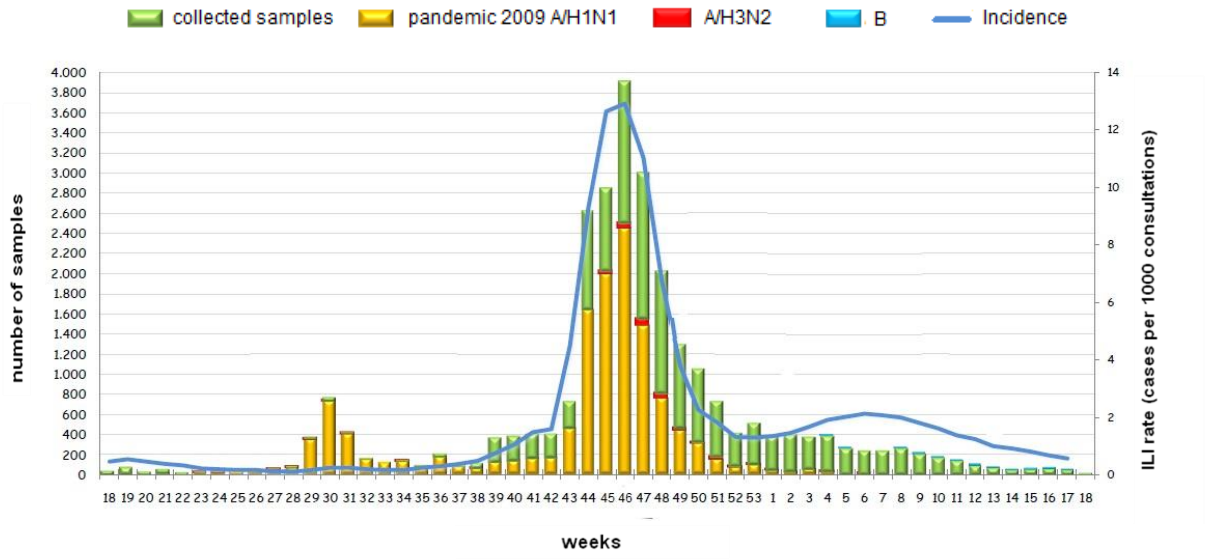


Fig. 9 Number of specimens collected and specimens positive for influenza, by type, subtype and by week of report, weeks 18/2009–18/2010.

2. AIM OF THE STUDY

The sequences of 133 pandemic A/H1N1 strains, from patients showing different clinical outcame (12 from fatal, 24 from severe and 97 from mild cases) have been examined at the Italian WHO National Influenza Centre (NIC) located at the National Institute of Health (Istituto Superiore di Sanità, ISS) in order to provide additional information on the genetic characteristics of pandemic A/H1N1 viruses circulating in Italy.

The aim of this study was to carry out a molecular characterization, by sequencing the entire genome of 133 pandemic A/H1N1 samples, collected between May 2009 and April 2010 in Italy, to determine the evolutionary relationships of their gene segments, compared to other recent pandemic A/H1N1 virus sequences obtained from GenBank and to some recent Italian swine and European human seasonal isolates.

Amino acid sequence analysis was also performed, with the aim to detect particular mutations potencially altering virus antigenicity or pathogenecity and to identify molecular markers of virulence and sensitivity to antivirals.

3. MATERIALS AND METHODS

Upper respiratory specimens such as nasopharyngeal aspirates or nasopharyngeal swabs, throat or nose swabs are suitable for the detection of influenza viruses.

Word Heath Organization recommends that suspected clinical cases of pandemic H1N1 influenza A infection are confirmed by:

(1) the isolation and identification of swine-origin influenza viruses (S-OIV),

(2) the detection of a fourfold rise of neutralization or haemagglutination inhibition antibodies to S-OIV,

(3) specific RT-PCR assays that differentiate S-OIV from seasonal influenza viruses.

(World Health Organization. WHO information for laboratory diagnosis of new influenza A(H1N1) virus in humans. http://www.who.int/csr/resources/publications/swineflu/WHO Diagnostic Recommendations H1N1 20090521.pdf)

3.1 MATERIALS

Respiratory specimens

Specimens for virus detection or isolation should be collected within 3 days after the onset of symptoms and rapidly transported to the laboratory.

A nasopharyngeal aspirate, nasal swab, nasal wash, nasopharyngeal swab, or throat swab are all suitable for diagnosis.

The timing of specimen collection is very important since the yield is the highest for respiratory specimens obtained within four days of onset of symptoms. Different types of respiratory specimens can be used. Nasal washes and nasopharyngeal aspirates tend to be more sensitive than pharyngeal swabs. Swabs should be transported in virus transport medium to prevent desiccation.

All specimens should arrive at the laboratory as soon as possible to avoid any degradation. Transportation in virus transport medium on ice or with

refrigeration at 2-8° C is recommended if any delay in transportation is expected.

Pharyngeal and/or nasal swabs collected by family and/or hospital doctors from each suspected case were sent to the NIC.

Blood specimens

Blood (whole blood, serum) specimens were also collected for the purpose of antibody serology (determining the presence of antibodies to influenza).

3.2 METHODS

3.2.1 ISOLATION METHODS

The S-OIV H1N1 can be isolated in MDCK cells in the presence of trypsin (as for other seasonal influenza viruses) or in embryonated hens egg. Virus culture is recommended to be carried out in BSL-3 or BSL-2 with BSL-3 practice.

A. Embryonated egg culture

Since this technique requires the supply of fertilized chicken eggs and special incubators it is no longer used for the routine diagnosis of influenza infection. However egg isolation provides high quantities of virus and is a very sensitive culture system. Reference laboratories therefore utilise this culture system to ensure high sensitivity and to enable the production of virus stocks for epidemiological monitoring.

Eggs are examined with an egg candler and place with blunt end up into egg trays. After discarding every egg that is infertile, has cracks, is underdeveloped, or appears to have a porous shell, punch a small hole in the shell over the air sac. Locating the embryo, 0,1 ml of specimens are inoculated into the allantoic cavity. After incubation at 37°C for 48 hours (the inoculated eggs must be held at 37°C and candled daily), eggs with dead or dying embryos are chilled to 4°C and the allantoic fluids harvested and tested for haemagglutination activity.

B. Cell culture

Most commonly Madin-Darby canine kidney (MDCK) cells are utilised to isolate influenza viruses.

Madin-Darby canine kidney (MDCK) cells were passed in minimal-essential medium (MEM) with 10% foetal bovine serum (FBS). Before infection, cells were washed with PBS and cultured in DMEM supplemented with 0,5 µg/mL TPCK (L-1-tosylamido-2-phenylethyl chloromethyl ketone)–treated trypsin (Sigma-Aldrich Corp., St. Louis, MO, USA), 4% bovine serum albumin, and antimicrobial drugs.

Cells were grown in 25 cm^2 flasks at 37°C in a humidified atmosphere containing 5% CO_2. The cultures were observed daily for morphologic changes and harvested 3 days after inoculation. The harvested cultures were clarified by centrifugation, and the supernatants were inoculated onto fresh MDCK cells as described for the initial inoculation; up to 3 additional passages were performed for samples that did not show evidence of influenza virus by hemagglutination or RT-PCR. Hemagglutination activity in the clarified supernatants was determined by using 0.5% turkey red blood cells and RT-PCR was performed as described below.

3.2.2. IDENTIFICATION OF VIRAL ISOLATE

Haemagglutination test (HA)

The traditional method for identifying influenza isolates takes advantage of the ability of the hemagglutinin (HA) to bind erythrocytes.

Serial twofold dilutions of antigen were made in 0,05 ml volumes in wells of plastic V-bottomed 96 wells microtitre plate to which an equal volume of 0,5% turkey red blood cells was added. The diluent used for the microtiter method was phosphate-buffered saline (pH 7,6).

The mixture was mixed and incubated at room temperature for 30 minutes. Hemagglutination occurs when the red blood cells are in suspension after the red blood cell control has settled completely.

HA titers were determined from the sedimentation pattern of the erythrocytes. HA titers were expressed as the reciprocal of the highest antigen dilution showing complete HA (one HA unit).

One HA unit in the haemagglutinin titration is the minimum amount of virus that will cause complete agglutination of the red blood cells. The last well that shows complete agglutination is the well that contains one HA unit.

3.2.3. ANTIGENIC CHARACTERIZATION

Subtype identification was done by hemagglutination inhibition (HI) and neuraminidase inhibition (NI) tests by using polyclonal chicken antisera against a panel of influenza A reference strains, which had been prepared in specific pathogen-free (SPF) chickens.

A. Haemagglutination inhibition test (HI)

HI assays are labour intensive and time consuming assays that require several controls for standardisation. However the assay reagents are cheap and widely available. 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 is the basis for the HI test. An 0.5% turkey red blood cell dilution is generally used. Serum is pre-treated to remove non-specific haemagglutinins and inhibitors. A viral haemagglutinin preparation that produces visible haemagglutination (usually 4 haemagglutination units) is then pre-incubated with two-fold dilutions of the serum specimen. The lowest dilution of serum that inhibits haemagglutination is the HI titre. HI has the added advantage that it is specific in differentiating between HA subtypes (Kendal et al.,1982).

The antigens used throughout this assay were harvests of virus-infected tissue culture or embryonated chicken eggs. All harvest materials were clarified by centrifugation at 1,200 X g for 10 min and at 4°C.

The sera were treated by adsorption with the erythrocytes to be used in the test and by the RDE of Vibrio cholerae.

For RDE treatment, one volume of undiluted serum was mixed with four volumes of RDE (100 units/ml) and incubated overnight in a water bath at 37° C. Three volumes of 2.5% trisodium citrate (dihydrate) was added. The mixture was heated at 56° C for 30 min, and the serum was raised to a starting

dilution of 1:10 by the addition of two volumes of PBS. These treatments were sufficient to remove or inactivate the nonspecific inhibitors and natural hemagglutinins from all of the sera.

A plastic microtitration plates containing 96 "V" bottom wells was employed. Each well received 0.025 ml of PBS; test serum in 0.025 ml amounts was serially diluted in twofold steps. All serum dilutions then received 0.025 ml of 4 virus hemagglutinin units. The antigens must have an HA titer of 4 HA units/25µl. This titer will hemagglutinate the first three wells of the back titration plate. If an antigen does not have an HA titer of 8, it must be adjusted accordingly by adding more antigen to increase units or by diluting to decrease units in the initial antigen standardization.

After incubation at room temperature for 1 h, 0.05 ml of 0.5% turkey erythrocyte suspension was added to each well.

Appropriate controls consisted of combinations of the different reagents with erythrocyte suspension.

The HI titer was the reciprocal of the highest test serum dilution that completely inhibited hemagglutination.

B. Neuraminidase inhibition test (NI)

The neuraminidase (NA) is the second most abundant glycoprotein on the surface of influenza viruses. There are 9 different antigenic subtypes of influenza A NA and antigenic drift occurs in the NA as it does in the HA. Immunity to NA plays a role in protection against influenza virus infection, and anti-NA antibodies prevent virus release from infected cells. NA is an enzyme (sialidase) that cleaves terminal sialic acid residues from cell surface receptors of the influenza virus. This activity enables release of virions from infected cells and removes sialic acid from newly synthesized HA and NA

molecules. The NI test was performed according to the protocol described in WHO Animal Influenza manual, 2002 (Neuraminidase assay and Neuraminidase inhibition assay).

The principal steps involved in the assay of neuraminidase are:

a) the release of free N-acetyl neuraminic acid (5-acetamido-3,5-dideoxy-N-acetyl-D-glycero-D-galacto-nonulosonic acid) from the fetuin substrate by the action of neuraminidase;

b) the conversion of N-acetyl neuraminic acid to fi-formyl pyruvic acid by periodate oxidation;

c) the formation of chromophore by thiobarbituric acid;

d) the extraction of chromophore into organic solvent for spectrophotometric analysis.

By this method the potency of the neuraminidase is determined and the standard dose (those giving an absorbance at 549 nm of 0.5 after 18 hours incubation with fetuin), for use in the NI test is selected.

The principal steps involved in the NI test are:

a) incubation of the standard neuraminidase dose with serial dilutions of test sera;

b) determination of the inhibitory effect of serum on neuraminidase activity

To identify the neuraminidase antigen of virus isolates, specific reference antisera should be used.

The NI titre of an antiserum is defined as the dilution of serum giving 50% inhibition of neuraminidase activity.

3.2.4. MOLECULAR CHARACTERIZATION

Polymerase chain reaction (PCR) is a rapid and sensitive method for the detection of Influenza A virus. The influenza virus genome is of RNA and this must be converted to complementary DNA (cDNA) in the first step following extraction of the viral RNA from samples. The RNA is then reverse transcribed (RT) using a viral reverse transcriptase enzyme to convert the target strand into cDNA. This DNA then is the target for PCR reactions using specific primers designed to bind to specific conserved regions in the genes of interest.

Methods for detecting swine origin influenza virus and differentiating it from seasonal H1N1 were performed. It is recommended that patient samples are tested by RT-PCR for an influenza A virus target (e.g. M gene) and for the swine origin influenza virus H1N1 HA in parallel. Specimens that are positive in both the M gene and swine origin influenza virus H1 and negative for seasonal influenza A H1 and H3 can be confirmed as swine origin influenza virus infection.

A. Detection of viral RNA

Isolation of viral RNA were performed using a Rneasy mini kit (QIAGEN), for infected MDCK cells and tissues, according to the manufacturer.

cDNA was synthesized from total RNA and an aliquot was used in PCR reaction. PCR products of amplifications were separated using 1.5% agarose gel and visualized by staining with ethidium bromide. Sample purification, RT-PCR reactions preparations and agarose gel analysis were performed in separated laboratories. Negative controls were processed and run with each assay.

B. Reverse transcription (RT)

Reverse transcription was carried out using SuperScript II, reverse transcriptase (RT) (Invitrogen) and the primer Uni12 (M) 5-AGCRAAAGCAGG-3, which is complementary to the conserved 3-end of all influenza A virus RNA segments. Briefly, 5 µl of the RNA preparation was mixed with 1 µl of 20 pmol Uni12 (M) and 1 µl of 2,5 mM dNTP. The mixture was incubated at 70°C for 5 min. Then, the following constituents including 4 µl of 5× cDNA buffer, 2 µl of 0.1M DTT, 1 µl of RNaseOUT ribonuclease inhibitor (40 units/µl), 0,75 µl of sterile, distilled water, and 0,25 µl of SuperScript II RT (200 unit/µl) were added into the tube. The reaction was carried at 42 ◦C for 60 min. and was terminated by heating at 100 ◦C for 5 min.

C. PCR amplification

A traditional RT-PCR assay, which was routinely used at NIC for seasonal surveillance and updated with specific primers (either suggested by CDC or designed by NIC) for pandemic A/H1N1 virus detection and sequencing, was employed.

The first step was based on the analysis of multiple sequence alignment of the influenza target genes in order the identify a conserved gene region. Primers utilized were selected with Primer Express software which uses a set of default parameters. The PCR reaction mixture contained 0,5 µl of Taq DNA Polymerase (5U/µl; Perkin Elmer) with 5 µl of 10× reaction buffer containing $MgCl_2$, 4 µl of dNTP (2.5 mM each), 1 µl of each of the primers (20 µM) and 10 µl of cDNA solution. Sterile, distilled water was added to reach a final volume of 50 µl. The amplification reaction was performed in a DNA thermal cycler.

The PCR conditions are summarized below:

1 step (hot start):

95°C	10 minutes

and 35 cycles:

94°C (denaturation)	45 seconds
52°C (annealing)	1 min. e 30 sec.
72°C (extension)	2 min. e 30 sec.

1 step:

72°C	5 minutes

3.2.5 SEQUENCE AND PHYLOGENETIC ANALYSIS

The resulting amplicons were analyzed by 1,5% agarose gel electrophoresis, stained with ethidium bromide and purified with Qiagen Qiaquick PCR Purification kit according to the manufacturer's instructions.

DNA sequencing was carried out using a Big Dye Terminator V.3.1 Cycle Sequencing Kit (Applied Biosystems) together with the corresponding inner primer, which was designed to ensure specificity for each sequence. Subsequently, any unincorporated labeled ddNTPs were removed by ethanol precipitation. The reactions were resolved on an ABI-Prism 310 Genetic Analyzer and sequences were edited using the SeqMan program (DNASTAR, Madison, WI) and the Bioedit Sequence Alignment Editor V.7.0.5.3 (Hall, 1999). Finally, sequences were aligned using ClustalX (Thompson et al., 1997). Phylogenetic trees were constructed based on continuous nucleotide sequence alignment, and genetic distances were calculated applying Kimura's two-parameter method using MEGA 3.1 (Kumar et al., 2004) and applied to construct neighbor-joining (NJ) trees. Confidence values for the tree topologies were evaluated by bootstrap analysis of 1,000 pseudo-replicate data sets.

3.2.6 REAL TIME (RT-PCR) FOR PANDEMIC H1N1 2009 INFLUENZA VIRUS

Real time PCR is a method that has been introduced relatively recently. The technology combines DNA amplification with detection of the products in a single tube while the reaction is in progress. In the case of influenza A viruses an extra reverse transcriptase step is required to convert the RNA into cDNA.

This one-step format is highly beneficial as it removes the significant contamination risk caused by opening tubes for post-PCR manipulation. It is also less time consuming than gel based analysis, highly specific and sensible. In order to assess whether the primer and probe sets, available at National Influenza Centre (NIC) for molecular influenza diagnosis, could be useful also to detect infection with the new influenza pandemic A/H1N1 virus, we performed sequence homology studies (by ClustalW program/ EMBL-EBI) of the matrix (M), hemagglutinin (HA), neuraminidase (NA) and nucleoprotein (NP) genes among influenza A(H1N1) strains of human and swine origin, downloaded from GenBank or available at the NIC database, together with the first complete viral genome sequence of the reference A/California/4/2009 (H1N1)v virus, made available in the publicly accessible GISAID sequence database (www.gisaid.org). Following the above studies, we decided to analyse the clinical samples collected from the Italian cases using a one-step in-house TaqMan (MGB)-real time RT-PCR (RRTPCR), already in use at NIC for the detection of the M gene of type A human influenza viruses. The viral gene sequence alignment analyses showed that the specific primers and probe set used by NIC in the RRT-PCR to detect the M gene of type A human influenza, was also able to detect the M gene of A(H1N1)v virus. The two primers corresponded to nucleotide positions 3-29 and 190-207, respectively, in the influenza A/California/6/09 sequence obtained from Gisaid

(EPI176497). Current TaqMan technology utilizes a minor groove binder (MGB) that forms a stable complex with the minor groove of single stranded DNA, increasing stability and specificity (Afonina et al., 1996; Uchiyama et al., 2004). Due to these qualities MGB probes are characterized by a shorter length and a higher melting temperature (Tm). The MBG-probe nucleotide positions were 152-167. The specific region recognised by the above primers was well conserved among human and swine strains, although a sequence discrimination between the two groups could be obtained on the basis of the sequence analysis of the final amplification M fragment (about 200 bp); along this region it was possible to highlight at least 12 nucleotide changes clearly distinguishing the pandemic A/H1N1 virus from the currently circulating human influenza isolates.

Primers and probe used for the above RRT-PCR were available at the website of the United Kingdom Health Protection Agency [Health Protection Agency, National Health Service, National Public Health Service for Wales. Real-time quadriplex PCR for the detection of influenza. National Standard Method. Available from: http://www.hpa-standardmethods.org.uk/documents/vsop/pdf/vsop25.pdf], although conditions used at NIC were adapted to a singleplex reaction.

Furthermore, each sample was also tested in a RRT-PCR assay specific for both seasonal A/H1 and A/H3 human subtypes.

This was the method initially employed to identify the novel A/H1N1 strain in the clinical material.

Since 12 May 2009, clinical samples have been tested by the specific RRT-PCR reagent kit from Centers for Disease Control and prevention (CDC) [World Health Organization (WHO). CDC protocol of realtime RTPCR for influenza A (H1N1). 30 April 2009. Available from: http://www.who.int/csr/resource/publications/swineflu/realtimeptpcr/en/index.html].

A CDC Realtime RT-PCR (rRTPCR) Protocol is available to rapidly detect the RNA of pandemic H1N1 2009 influenza virus from biological samples

(clinical specimens and/or cell surnatants/allantoic fluids) and provide an early warning for the presence of this virus in birds and mammals.

According to the "CDC Realtime RTPCR (rRTPCR) Protocol for Detection and Characterization of Swine Influenza" a panel of oligonucleotide primers and dual-labelled hydrolysis (Taqman®) probes were used in real-time RT-PCR assays for the in vitro qualitative detection and characterization of H1N1 pandemic viruses in respiratory specimens and viral cultures.

Each sample RNA extract was tested by three separate primer/probe sets:

- The InfA primer and probe set is designed for universal detection of type A influenza viruses and used to detect a conserved region of the influenza A virus Matrix gene.
- The swInfA primer and probe set is designed to specifically detect all swine influenza A viruses.
- The swH1 primer and probe set is designed to specifically detect H1N1 pandemic influenza A virus.

InfA primer and probe set are used to detect a conserved region of the influenza A virus Matrix gene; at the same time, in different wells, swInfA and swH1 primer and probe sets are utilized to specifically detect, by Nucleoprotein gene amplification, swine influenza A viruses and H1N1 pandemic viruses, respectively.

Primer and probe sets used are listed in Table 2.

Table 2 Primer and probe sets for influenza A/H1N1 pandemic virus.

Primers and Probes	Sequence (5'>3')	Working Concentration
InfA Forward	GAC CRA TCC TGT CAC CTC TGA C	40 µM
InfA Reverse	AGG GCA TTY TGG ACA AAK CGT CTA	40 µM
InfA Probe (1)	TGC AGT CCT CGC TCA CTG GGC ACG	10 µM
SW InfA Forward	GCA CGG TCA GCA CTT ATY CTR AG	40 µM
SW InfA Reverse	GTG RGC TGG GTT TTC ATT TGG TC	40 µM
SW InfA Probe 2 (2)	CYA CTG CAA GCC CA"T" ACA CAC AAG CAG GCA	10 µM
SW H1 Forward	GTG CTA TAA ACA CCA GCC TYC CA	40 µM
SW H1 Reverse	CGG GAT ATT CCT TAA TCC TGT RGC	40 µM
SW H1 Probe 2 (2)	CA GAA TAT ACA "T"CC RGT CAC AAT TGG ARA A	10 µM

(1) TaqMan® probes are labeled at the 5'-end with the reporter molecule 6-carboxyfluorescein (FAM) and with the quencher, Blackhole Quencher 1 (BHQ1) (Biosearch Technologies, Inc., Novato, CA) at the 3'-end.
(2) Taqman® probes are labeled at the 5'-end with the reporter molecule 6-carboxyfluorescein (FAM) and quenched internally at a modified "T" residue with BHQ1, with a modified 3'end to prevent probe extension by Taq polymerase.

To prevent carry over contamination, the preparation of the PCR mixture was performed in a separate area. All reactions were performed in triplicate.The reactions were carried out in a final volume of 25 µl, and the relative reaction mixture and the amplification procedure for the rRT-PCR are listed below. Three master mix were made up: one for Influenza A (Matrix gene), the second for swine influenza A (Nucleoprotein gene) and the third for H1N1 pandemic influenza.

Reagent	volume of reagent added per reaction
2X PCR Master Mix	12.5 µl
Forward Primer (40µM)	0.5 µl
Reverse Primer (40µM)	0.5 µl
Probe (10 µM)	0.5 µl
Superscript™ III RT/Platinum ® Taq Mix (Invitrogen)	0.5 µl
H2O	5.5 µl
Final volume	20 µl

Five µl of the sample RNA to the 20µl of master mix were added.

Thermocycling profile specifically designed for M and pandemic H1N1 primers and probes:

1 cycle: 50°C for 30 min, RT step
1 cycle 95°C for 2 min (Taq activation)
45 cycles: PCR amplification 95°C for 15 sec
55°C for 30 sec*

* Fluorescence data (FAM) should be collected during the 55°C incubation step.

4. RESULTS

As part of the intensified surveillance carried out during the current influenza pandemic, the HA sequences of 133 influenza pandemic A/H1N1 virus strains, identified from affected patients, were examined.
The sequences of neuraminidase and other genes of some of these viruses also have been analyzed.
All 133 strains had been obtained from clinical samples (nasal, pharyngeal, or nasopharyngeal swabs and/or tracheal aspirates) collected between May 2009 and April 2010 in the context of virologic surveillance, conducted by the Italian National Influenza Centre (NIC-ISS), in collaboration with the regional laboratory network. These samples were obtained and genetic characterization was performed, aimed to study the evolution of the pandemic strain and to detect particular mutations potencially altering virus antigenicity or pathogenecity.

4.1. EVOLUTIONARY CHARACTERIZATION

Preliminary studies showed that six genomic segments, including the HA, of the 2009 A/H1N1, virus circulating in Italy, were related to swine viruses from North America and the remaining two (coding for the NA and M proteins) were from swine viruses isolated in Europe and Asia.
Figure 10 shows the evolutionary relationships of the M1, HA, NA and NP gene segments of the first three pandemic A/H1N1 virus isolates, obtained in Italy from patients without epidemiological link, compared to other recent pandemic A/H1N1 virus sequences obtained from GenBank and to some recent Italian swine and European human seasonal isolates.

The phylogenetic trees confirmed that both the M and the NA gene segments of the 2009 A/H1N1 strains were closely related to the Italian swine strains.
In contrast, the HA and NP nucleotide sequences of these viruses appeared to be quite different from the Italian swine strains and more related to the swine strains belonging to the North-American lineage (A/Sw/Ohio/511445/07 in Figure 10), although forming a clade with human seasonal viruses.

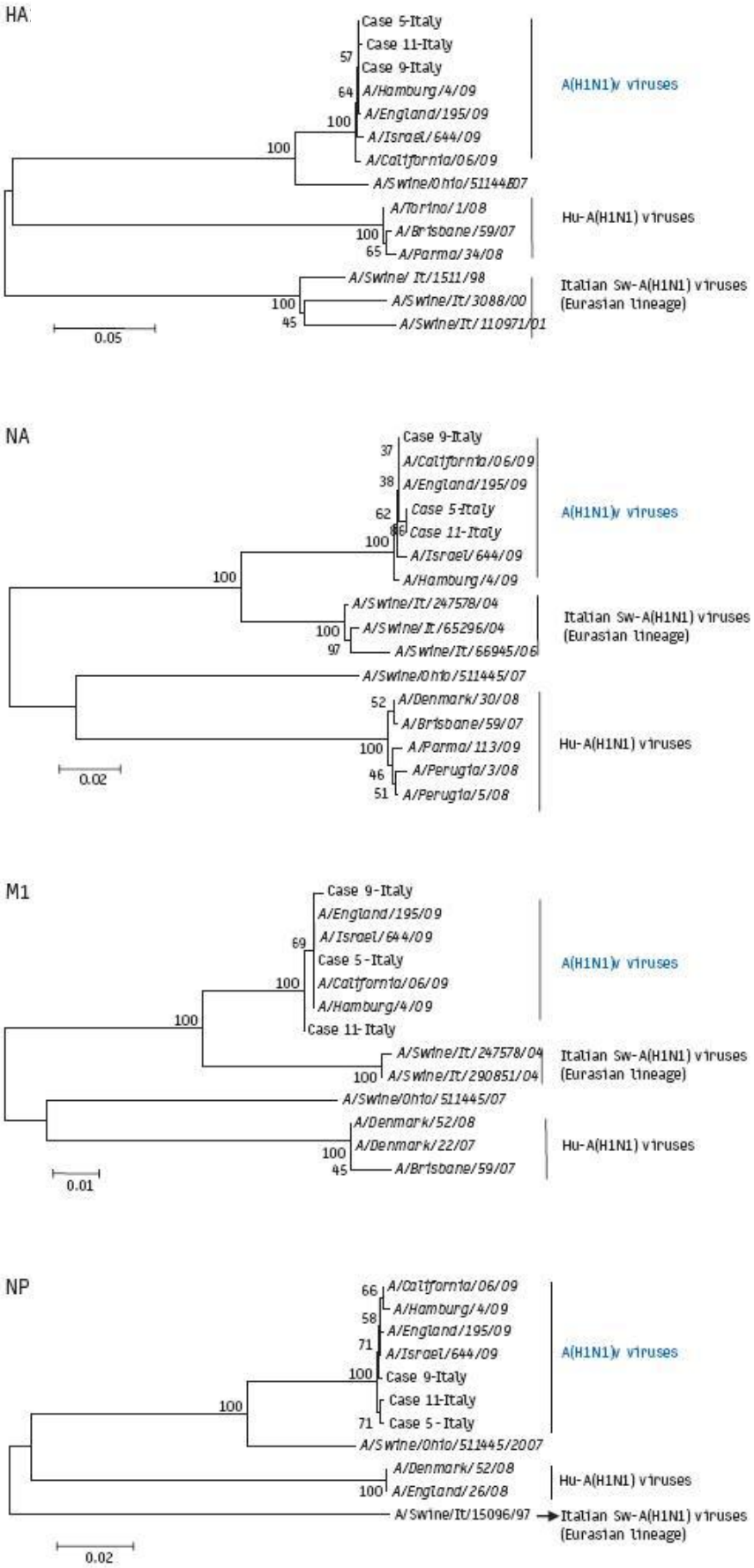


Fig. 10 Phylogenetic analysis of the M1, HA, NA and NP gene segments of the first three 2009 A/H1N1 italian virus isolates compared to recent Italian swine and European human seasonal influenza isolates.

MEGA software package (version 3.1) was used to estimate phylogenies from the nucleotide sequences and for the construction of phylogenetic trees, using Tamura-Nei method and the Neighbor-Joining algorithm. Bootstrap based on 1,000 replicates; Italian cases of A(H1N1)v virus are indicated in bold; GenBank sequences are in italics; Hu=human; Sw=swine

4.2. **ANALYSIS OF VIRAL PATHOGENICITY DETERMINANTS**

With the aim of assessing the correlation between the genetic changes and virus pathogenicity, the 133 sequences of pandemic H1N1 strains collected from patients showing different clinical course and outcome (12 from fatal, 24 from severe non fatal, and 97 from mild cases) were analyzed. Molecular characterization of all the 8 segments was carried out for known pathogenic markers predictive of adaptation to humans.The analysis of the genetic markers for some of severe and fatal cases are summarized in Table 3.

Table. 3 Common genetic markers for virulence and transmission in pandemic H1N1 and other influenza viruses.

Protein	aa	Pathogenicity		H1N1v 2009	SEVERE CASES		FATAL CASES			
		Low	High		Pat. N°1 8/2009	Pat. N°2 9/2009	Pat. N°3 10/2009	Pat. N°4 10/2009	Pat. N°5 1/2010	Pat. N°7 02/09
HA	Cleavage site	single Arg residue	multiple basic aa	single Arg residue	single Arg residue	single Arg residue	single Arg residue	single Arg residue	single Arg residue	single Arg residue
	222	D	G	D	G	G	D	D	D	D
PB1-F2	66	Asn	Ser	stop codon (11° aa)	stop codon (11° aa)	stop codon (11° aa)-	stop codon (11° aa)	stop codon (11° aa)	stop codon (11° aa)	stop codon (11° aa)
PB2	627	Glu	Lys	Glu	Glu	Glu	Glu	Glu	Glu	Glu
	701	Asp	Asn	Asp	Asp	Asp	Asp	Asp	Asp	Asp
NS1	92	Asp	Glu	Asp	Asp	Asp	Asp	Asp	Asp	Asp
	C-terminal aa	Arg-Ser-Glu-Val, deletion of 11 aa	Glu-Ser-Glu-Val	deletion of 11 aa	deletion of 11 aa	deletion of 11 aa	deletion of 11 aa	deletion of 11 aa	deletion of 11 aa	deletion of 11 aa

HA

Cleavage site

HA cleavage is essential for viral infectivity since exposure of the N-terminus of HA2 mediates fusion between the viral envelope and the endosomal membrane. HA cleavability is determined by the amino acid sequence at the cleavage site. Low pathogenic avian viruses and non-avian influenza viruses (with the exception of H7N7 equine influenza viruses) possess a single Arg residue at the cleavage site which is cleaved by proteases in the respiratory and/or intestinal organs and hence restricts viral replication locally. By contrast, highly pathogenic H5 and H7 viruses possess multiple basic amino acids at the HA cleavage site (Kawaoka & Webster, 1988). This motif is recognized by ubiquitous proteases, such as furin and PC6, and leads to systemic infections. For several outbreaks in poultry, increased pathogenicity of avian influenza viruses has been linked to the acquisition of multibasic HA cleavage sites, a finding that underscores the significance of the HA cleavage motif for virulence.

The presence of a multibasic cleavage site has been not observed in the pandemic A/H1N1 strains analyzed.

Receptor binding site

The differences in receptor-binding specificity are determined by the amino acid residues in the HA receptor-binding pocket.

The location of these amino acids in the structure of the HA glycoprotein is shown in Figure 11. It is notable that residues 203 and 222 are in the head of the HA and are closest to each other across the HA trimer interface.

Since its first appearance in April 2009, some mutations in the HA protein of pandemic A/H1N1 virus have appeared sporadically and have been reported in

several countries. In particular, the amino acid substitution aspartic acid to glycine in position 222 (D222G) in the HA1 subunit of the viral HA has been initially reported in Norway in association with fatal cases.

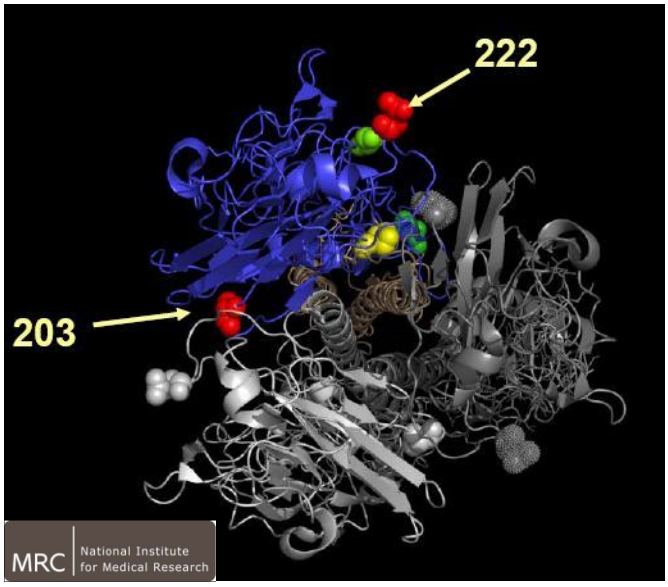
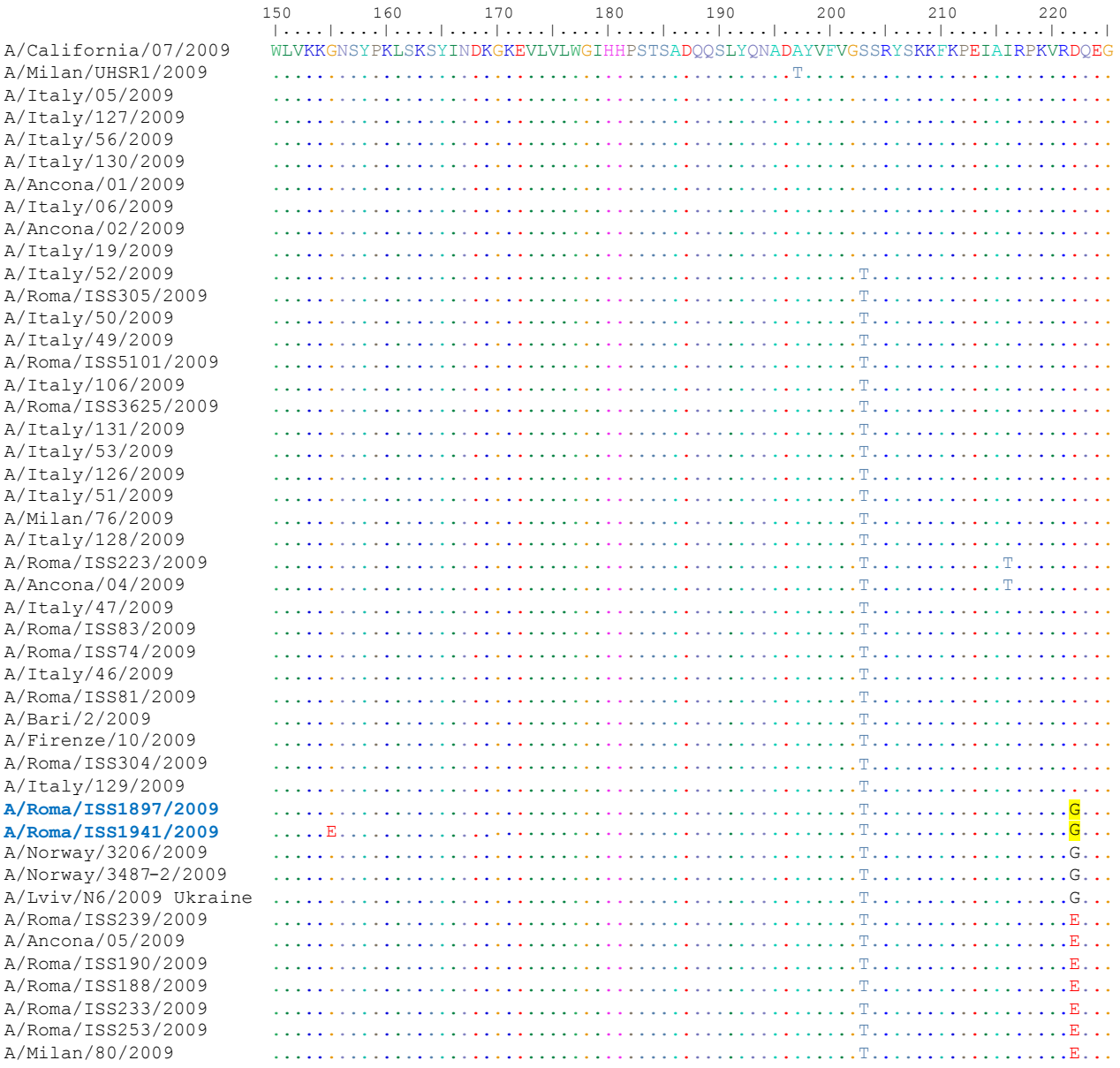


Fig. 11 Sites of H1N1 pandemic amino acid substitutions in HA head region.

In particular, for H1 HAs, amino acids at position 222 determine receptor binding specificity. HA-222Asp (found in human H1 HAs) confer binding to human-type receptors, whereas HA-222Gly (found in avian H1 HAs) confer binding to avian-type receptors (Stevens et al., 2004).

In Italy, heterogeneity was observed at residue 222 of the HA, with viruses displaying the substitutions either D222G or D222E (Fig. 12).

Fig.12 HA1 protein alignment for pandemic A/H1N1 viruses



Among 133 patients, 24 of whom had severe disease (i.e., requiring hospitalization), only 2 were infected with a virus showing the D222G change. The first patient, a man 25 years of age from northern Italy, had a febrile illness on August 17. One week later, he was admitted to an intensive care unit with severe pneumonia and acute respiratory distress syndrome, which resolved after treatment with extracorporeal membrane oxygenation.

Influenza pandemic A /H1N1 viral genome showing the D222G mutation was identified through direct sequencing of nasopharyngeal swab and tracheal aspirate collected on August 27.

No sequence could be retrieved from a nasal wash, which was obtained the same day and was positive in real-time RT–PCR for pandemic A/H1N1 2009 virus. No viral growth was detected in MDCK cells seeded with both clinical samples.

To identify possible transmission chains of the mutated virus, we analyzed the genome of the viral strain detected in the throat swab of the father of the index case-patient, 55 years of age, who was obese and had diabetes. He became moderately ill on August 25 but did not require hospitalization or antiviral treatment. The virus isolated in MDCK cells from the sample obtained on August 27 had the same HA mutation, D222G. Viral strains from index and contact cases were susceptible to oseltamivir, as determined by lack of the specific oseltamivir-resistance marker (His275Tyr) in neuraminidase sequences, which were identical in both viral strains. Additional samples analyzed from close contacts (i.e., 4 healthcare workers, 4 family members, and 2 friends) of the 2 patients all were negative for pandemic A/H1N1 virus.

Comparison of the 2 HA1 sequences from the index case-patient and his father showed an additional substitution in the latter (G155E), which is located close to the receptor-binding pocket. Furthermore, sequence analyses showed that all virus strains containing a change in HA1 position 222 (D222G or D222E)

also showed a second substitution in position 203 (S203T), when compared with the A/California/7/2009 vaccine strain (Fig.12).

However, the effect of this second mutation on the HA receptor-binding properties is still unclear. The HA1 genes from the index and contact case-patients shared 2 additional nucleotide changes, 1 synonymous substitution (T504C, N1 numbering) not found in any other sequence analyzed in the present study and another nucleotide change resulting in an amino acid substitution (P297S, N1 numbering), detected only in a small number of sequences from Italy.

Hemagglutination inhibition test of the isolated virus did not show substantial reduction in its reactivity with an antiserum against pandemic (H1N1) 2009 vaccine, as compared with the reactivity of an influenza virus A/California/7/2009 strain not carrying the G155E mutation (Table 4).

Table 4. Hemagglutination-inhibition test results of pandemic A/H1N1 2009 viruses

Viruses	*Postinfection ferret serum titer against*	
	A/Brisbane/59/2007 seasonal H1N1	A/California/7/2009 pandemic H1N1
A/Brisbane/59/2007 (H1N1)	1280	<10
A/California/7/2009 (H1N1v)	<10	1280
A/Roma/ISS1941/2009 Father's virus isolate (D222G+G155E)	<10	640
A/Rome/ISS11/2009	<10	320

Haemagglutination inhibition (HI) assays using post-infection ferret antisera have also shown that recent isolates continue to be antigenically similar to the prototype virus A/California/4/2009 and the vaccine virus A/California/7/2009

(data not shown). So far there has been little change in the amino acid sequences in the HAs of the 2009 A/H1N1 viruses isolated. The viruses analysed have shown a high level of genetic conservation, as illustrated in phylogenetic trees of the HA gene (Fig.13).

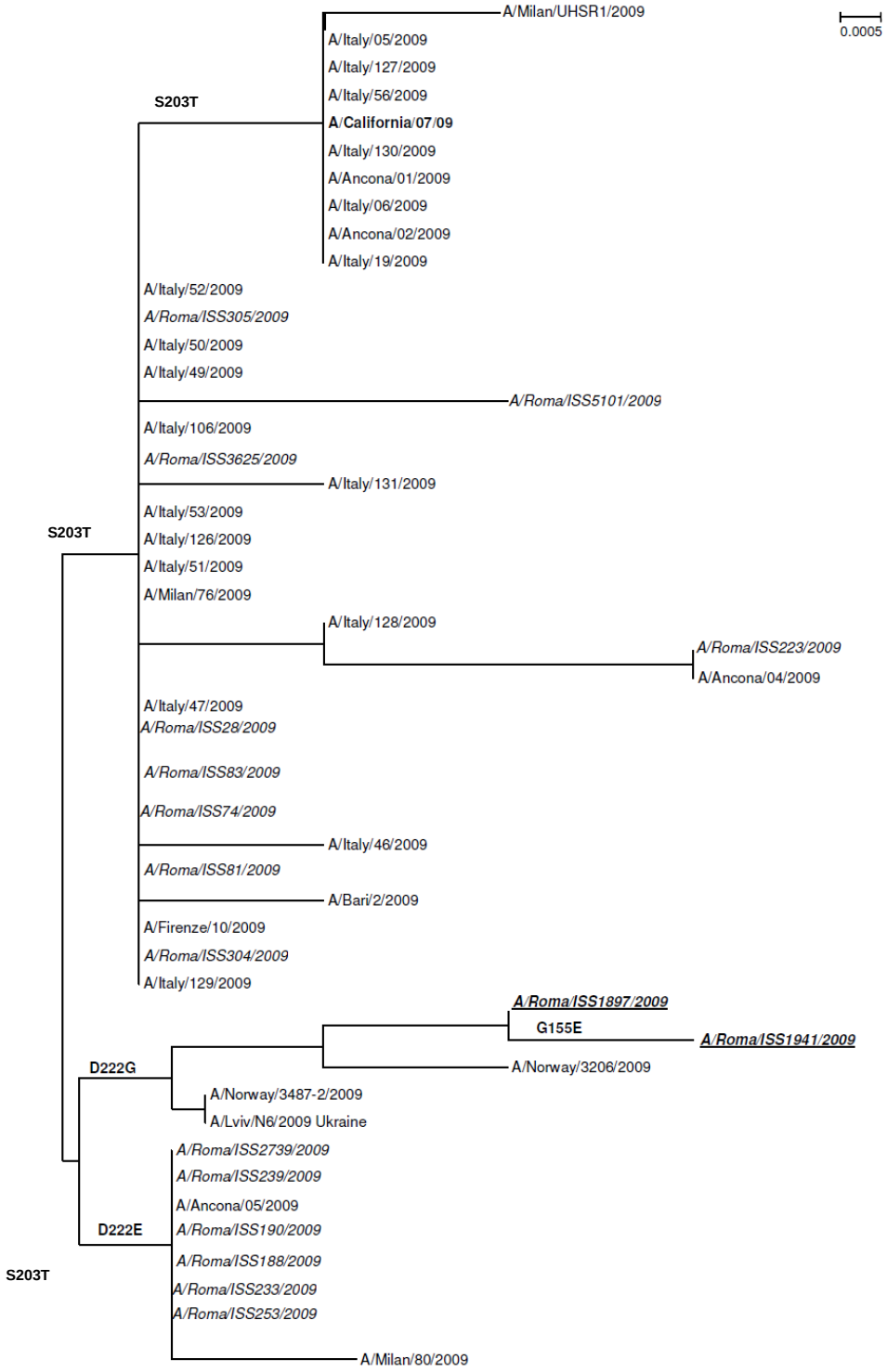


Fig. 13 Phylogenetic relationships of hemagglutinin (HA)1 sequences of pandemic (H1N1) 2009 viruses in Italy. The NIC-ISS sequences are in italics. The index case-patient and his father are indicated by underlined **boldface** italics. Amino acid mutations of interest in this study are reported on the nodes. All the NIC sequences were deposited in the GenBank database under the following accession numbers: (hemagglutinin sequences) GU451262–GU451280; GU576500–GU576502; GU576504; GU576506; GU576508; GU576510; GU576512; GU576514-GU57615; GU576517; GU576519; GU576521-GU576522; GU576524; GU576526-GU576527;GU576529; GU576531-GU576532; GU576534; GU576536-GU576540; GU585403–GU585443; (neuraminidase sequences) GU936490-GU936491. Scale bar indicates proportion of nucleotide substitutions per site.

PB1-F2

The PB1 gene of most avian and human influenza A viruses encodes a second protein, PB1- F2, that is expressed from the +1 reading frame 71. The length of PB1-F2 of swine influenza viruses differs depending on their origin; classical swine viruses possess truncated PB1-F2 proteins of 8-11 amino acids, while Eurasian avian-like swine viruses possess full length PB1- F2 proteins (87-89 amino acids).

The 2009 A/H1N1 virus encode a truncated PB1-F2 protein of 11 amino acids. PB1-F2 induces apoptosis, likely by interaction with two mitochondrial proteins enhances inflammation in mice, and increases the frequency and severity of secondary bacterial infections (McAuley et al., 2007). After comparing viruses from the Hong Kong 1997 H5N1 outbreak, one amino acid change (N66S) was found in the PB1-F2 sequence at position 66 that correlated with pathogenicity. This same amino acid change (N66S) was also found in the PB1-F2 protein of the 1918 pandemic virus.

A recent study demonstrated that the amino acid at position 66 of PB1-F2 affects the pathogenicity of an H5N1 virus in mice (Conenello et al., 2007). This finding is of great interest since the pandemic 1918 Spanish influenza virus possessed the “high pathogenic”-type amino acid, and its replacement attenuated this virus. In addition, both viruses with an S at position 66 (WH N66S and wt 1918) induced elevated levels of cytokines in the lungs of infected mice. These data show that a single amino acid substitution in PB1-F2 can result in increased viral pathogenicity and could be one of the factors contributing to the high lethality seen with the 1918 pandemic virus.

The current circulating pandemic A/H1N1 viruses do not contain the known molecular marker of pathogenicity PB1-F2, suggesting that it causes a mild disease compared to the three major pandemic viruses of the last century

PB2

Recently, the viral replication complex has been recognized as an important contributor to viral pathogenicity, likely by affecting viral growth. The amino acid at position 627 of the PB2 protein was first described by Subbarao et al. as a host range determinant, based on cell culture studies (Subbarao et al., 1993) Hatta et al.(2001) demonstrated that the respective amino acid change determined the pathogenicity of H5N1 influenza viruses in mice. Viruses with lysine at this position were pathogenic in mice, whereas those with glutamic acid were non pathogenic in these animals. Notably, almost all human influenza viruses possess lysine at this position, while most avian viruses possess glutamic acid at PB2-627. Lysine at position 627 of PB2 is now recognized as a determinant of viral pathogenicity in several mammalian species.

The amino acid at position 701 of PB2 has also emerged as a determinant of virulence (Gabriel et al., 2007)a role likely related to its facilitation of binding of PB2 to importin α (a cellular nuclear import factor) in mammalian cells 55. The recently emerged pandemic A/H1N1 viruses possess the 'low pathogenic'-type amino acids at positions 627 and 701 (i.e., Glu and Asp).

NS1

The four C-terminal amino acids of NS1 form a PDZ ligand domain motif that was identified by large-scale sequence analysis.

The PDZ domain is a common structural domain of 80-90 amino-acids found in the signaling proteins of bacteria, yeast, plants, viruses and animals.

Introduction of the PDZ ligand domains of highly pathogenic H5N1 viruses or the pandemic 1918 virus into an otherwise human virus conferred slightly increased virulence in mice (Jackson et al., 2008).

The pandemic A/H1N1viruses lack the 11 C-terminal amino acids of NS1 and hence, the PDZ domain motif. The biological significance of this finding is currently unknown.

4.3. DETECTION OF MOLECULAR MARKERS FOR ANTIVIRAL RESISTANCE

Two classes of antiviral drugs –ion channel inhibitors and neuraminidase inhibitors – are currently licensed for use against influenza A viruses.

Adamantanes (i.e., amantadine hydrochloride and rimantadine) block the ion channel formed by the M2 protein, which is critical in the release of vRNPs into the cytoplasm. Resistance to adamantanes arises quickly and frequently, and most circulating human H1N1 and H3N2 viruses, some H5N1 viruses, and most European swine H1N1, H1N2, and H3N2 viruses are resistant to adamantanes. The swine origin 2009 H1N1 viruses are also resistant to ion channel inhibitors (Novel Swine-Origin Influenza A (H1N1) Investigation Team, 2009).

Two neuraminidase inhibitors – oseltamivir and zanamivir – are currently licensed. Neuraminidase inhibitors interfere with the enzymatic activity of the NA protein, which is critical for the efficient release of newly synthesized viruses from infected cells.

In early 2008, drug susceptibility surveillance of influenza viruses in Europe revealed that some influenza A viruses (subtype H1N1) circulating during the winter season of 2007 and 2008 were resistant to the neuraminidase inhibitor, oseltamivir. This resistance arises due to a histidine to tyrosine substitution in the neuraminidase active site (H275Y in N1 nomenclature).

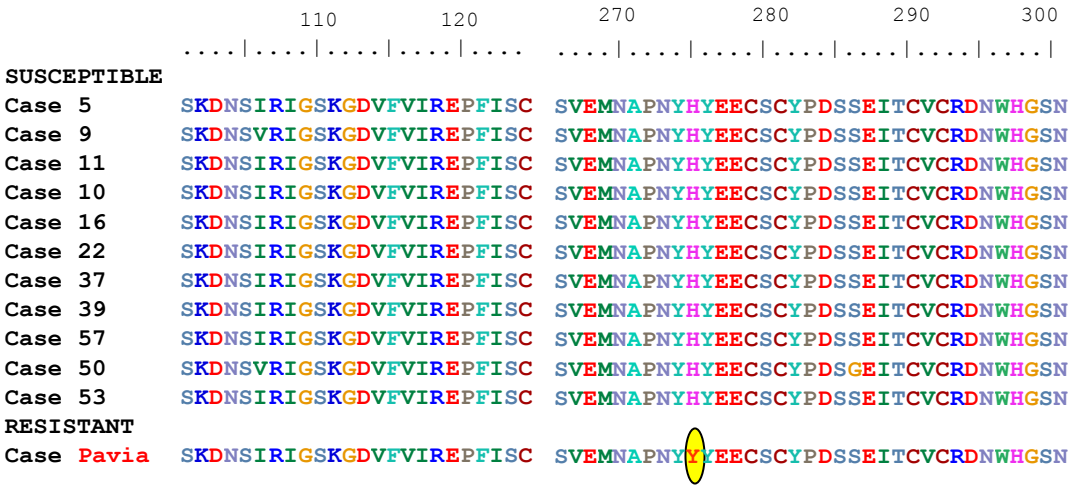
In contrast to recent seasonal A/H1N1 strains showing sustained NAI resistance (Meijer et al., 2009). strains of the pandemic A/H1N1 virus remain susceptible to NAIs and have only rarely undergone changes conferring resistance to oseltamivir. The target population mainly consists of hospitalized patients with severe respiratory syndrome and clustered cases of influenza in the community or schools. Among these cases, particular attention is focused on immunocompromised patients undergoing prolonged virus shedding,

requiring antiviral administration over long periods. The detection of changes at residues V116, I117, E119, H275, and N295 in the NA is known to alter NAI drug susceptibility.

We report on the detection of the first cases of the oseltamivir resistance-conferring mutation H275Y (Fig. 14).

Since the first case of the pandemic A/H1N1 virus was identified in Italy (May 2009) until the end of February 2010, A/H1N1 virus strains from clinical samples from NAI-treated or untreated patients of different ages and from different geographical areas have been screened for NAI resistance by the NIC. All viral strains, except the 2009 A/H1N1 virus - A/Pavia/21/2009, did not show mutations known to reduce susceptibility to oseltamivir or zanamivir. The pandemic influenza A/H1N1 strain with the neuraminidase H275Y mutation was detected in nasal secretions of a 2-year-old leukemic patient with influenza-like illness after 18 days of treatment with oseltamivir.

Fig.14 Italian influenza pandemic A/H1N1 isolate carrying the H275Y mutation associated with oseltamivir resistance



4.4. CASE REPORT: fatal myopericarditis associated with pandemic A/H1N1 influenza virus infection

This study also describe a fatal case of myopericarditis presenting with cardiac tamponade in a previously health 11-year-old child with no known risk factors, including obesity, for severe, complicated pandemic influenza. Pandemic H1N1 2009 influenza A virus sequences were identified in throat and myocardial tissues and pericardial fluid, suggesting damage of myocardial cells directly caused by the virus (Puzelli et al., 2010).

The patient developed a high fever (39.2°C) with coughing and vomiting on the evening of 28 October. The next morning, she was visited by a general practitioner, who prescribed antipyretic, antibiotic, and cortisone treatments. About 36 h after the onset of symptoms, the girl presented to the emergency unit of the district hospital with asthenia and dizziness. She was pale and afebrile at 35°C, with tachycardia of 140 beats/min and tachypnea of 32 breaths/min. Routine blood samples were analyzed. An urgent echocardiogram was performed, which demonstrated normal ventricle size, severely compromised pump function (ejection fraction, 20 to 30%), diffuse ipokinesia, and a small pericardial effusion, consistent with myopericarditis with a predominant myocarditic component. Because of the need for specialized care and treatment, the patient was transferred to a regional pediatric hospital, but during ambulance transport she had loss of consciousness and needed respiratory assistance. Clinical conditions worsened during transfer to the pediatric intensive care unit, the central pulse became absent, and the echocardiogram showed no contractile activity. The pathological exam showed changes such as increased ventricular size , the left/right ventricle size ratio was 6.28, versus a normal value of 3.1. Pulmonary congestion leading to pneumonic hepatization was also found.

Microscopic changes revealed mild inflammation: modest infiltration of histiocytes (CD68 positive) and myocellular necrosis were detected. PCR analysis of a nasopharyngeal swab collected at the intensive care unit was positive for the pandemic H1N1 2009 influenza virus. The sample was grown in MDCK-SIAT1 cells (hemagglutinating units [HAU], 64).

Molecular analyses of several samples of tissues and fluids collected during the autopsy were performed. The pandemic influenza virus was detected in bronchi, in the myocardial tissue, and in both the pericardium and pericardial fluid, whereas it was not found in the lungs or in other tissues or fluids, such as the pharynx, tonsils, and spleen (Table 5).

Table 5 Laboratory results

Clinical sample or tissue type	*Result[a] from:*	
	Real-time RT-PCR analysis[b] for pandemic A/H1N1	Isolation in MDCK Cells (HAU)
Rhinopharyngeal swab	**Positive**	**Positive (64)**
Pharynx	Negative	Negative
Tonsil	Negative	Negative
Bronchus	**Positive**	Negative
Lung	Negative	Negative
Spleen	Negative	Negative
Heart	**Positive**	**Positive (16)**
Pericardium	**Positive**	Negative
Pericardial fluid	**Positive**	Negative

[a] Positive results are listed in bold
[b] Real-time reverse transcriptase PCR (RT-PCR) analysis was performed using the CDC pandemic kit.

The virus was isolated from myocardial tissue but not from other tissues (the pharynx, tonsils, spleen, mesenteric lymphnodes, and liver). The patient was also examined for other common bacterial and viral respiratory agents (e.g.,

pneumococcus, mycoplasma, and respiratory syncytial virus [RSV], etc.), and the results were all negative.

Sequence analysis of the hemagglutinin (HA) showed a D222E mutation (H1 numbering). It is unclear whether and to what extent change at amino acid position 222, which is located in the receptor binding domain of the HA, influences receptor binding specificity and, ultimately, virus pathogenicity. With regard to neuraminidase (NA) genes, no significant mutations were detected, and the virus was found to be susceptible to NA inhibitors. The study findings suggest that cardiac tamponade and heart failure following myopericarditis in this young patient were due to direct tropism and damage caused by the 2009 H1N1 influenza virus. Cardiac involvement is not an uncommon complication of seasonal influenza. Elevated CK levels in 12% of patients affected by influenza but without cardiac symptoms and in up to 15% of patients with abnormal ECG patterns suggestive of myocarditis have been reported previously (Karjalainen et al., 1980). Myocardial damage or clinical myocarditis has also been reported in studies from Japan (Kaji et al., 2001) and Canada (Moore et al., 2006).

5. DISCUSSION

The pandemic A/H1N1 viruses, circulating in Italy between May 2009 and April 2010, are related to swine influenza A/H1N1 viruses recently circulating in pigs in North America and Europe/Asia.

Phylogenetic analysis confirmed that the HA and NP gene segments of these viruses appeared to be quite different from the Italian swine strains and more related to the swine strains belonging to the North-American lineage (A/Sw/Ohio/511445/07). By contrast, both the M and the NA gene segments were closely related to the Italian swine strains.

Antigenically the viruses are homogeneous and similar to North American swine A/H1N1 viruses but distinct from seasonal human A/H1N1.

The majority of reported illness has been in younger people aged less than 30 years presenting with mild symptoms. However, approximately 2% of patients have developed more severe illness and deaths have been associated with the infection, mainly among adults aged 30 to 50 years, many having underlying medical conditions. The cocrystal structure of the 1918 hemagglutinin with an antibody from a survivor of the 1918 Spanish flu, that neutralizes both 1918 and 2009 H1N1 viruses, reveals an epitope that is conserved in both pandemic viruses. Thus, antigenic similarity between the 2009 and 1918-like viruses provides an explanation for the age-related immunity to the current influenza pandemic (Xu et al., 2010). The 2009 A(H1N1) virus demonstrates that even subtypes already encountered in past human pandemics may constitute new pandemic threats.

The 2009 A/H1N1 viruses do not possess markers associated with high pathogenicity. Many of the molecular markers predicted to be associated with adaptation to a human host or to the generation of a pandemic virus, as seen in

1918 H1N1 or highly pathogenic H5N1, are not present in the 2009 A/H1N1 viruses characterized here.

All pandemic 2009 A/H1N1 viruses have a Glu at position 627 in the PB2 protein, which is unexpected because all known human influenza viruses have a Lys at this position, whereas Glu627 is typical for avian influenza viruses. PB2-627Lys infact seems to allow efficient replication not only in the lower, but also in the upper respiratory tract of mammals, a feature that may facilitate transmission.

The PB1-F2 protein has previously been associated with the increased pathogenicity of the 1918 virus and highly pathogenic H5N1 virus (Conenello et al., 2007). However, the PB1-F2 protein of the 2009 A/H1N1 viruses sequenced to date are truncated by the presence of a stop codon at position 11. The NS1 protein is also truncated, by a stop codon at position 220, which creates a deletion of the PDZ ligand domain, a protein-protein recognition domain involved in a variety of cell-signaling pathways that have been implicated in the pathogenicity of 1918 H1N1 and highly pathogenic H5N1 viruses (Jackson et al., 2008).

Together these data suggest that other previously unrecognized molecular determinants are responsible for the ability of the 2009 A/H1N1 virus to replicate and transmit in humans.

Identification of a pandemic A/H1N1 2009 virus strain carrying the D222G mutation and its association with the first fatal cases of influenza in Norway raised some concern about emergence of a viral strain with increased pathogenicity. On November 20, 2009, the Norwegian Institute of Public Health reported to the World Health Organization a mutation in the hemagglutinin (HA) of pandemic A/H1N1 2009 virus, consisting in a change of aspartic acid (D) with glycine (G) at aa 222. The same mutation has been also detected in Brazil, China, Japan, Mexico, Ukraine, the United States, France, and Spain (World Health Organization. Public health significance of

virus mutation detected in Norway. Pandemic (H1N1) 2009. Briefing note 17 [cited2009Nov20].http://www.who.int/csr/disease/swineflu/notes/briefing_20091120/en/).

The D-to-G mutation among the 1918 influenza virus variants (Stevens et al., 2006) correlated with a shift from α2-6–linked sialic acid preference to a dual α2-3/α2-6 specificity. However, whether such a mutation may alter receptor binding specificity in the pandemic A/H1N1 2009 virus is unknown. Although several pandemic A/H1N1 viral strains sharing this mutation were detected in fatal cases, the same mutation also was detected in some mild cases; conversely, viruses from numerous fatal cases have not shown the same mutation. The mutation appears to occur sporadically and spontaneously. On the basis of results from a retrospective HA1 sequence analysis performed on pandemic A/H1N1 2009 viral isolates in Italy, we reported on a transmission event of this virus carrying the D222G mutation.

A pandemic A/H1N1 virus strain carrying the D222G mutation was identified in a severely ill man and was transmitted to a household contact. Only mild illness developed in the contact, despite his obesity and diabetes. The isolated virus reacted fully with an antiserum against the pandemic vaccine strain.

Our findings suggest that the D222G mutated virus is to some extent transmissible. However, a number of close contacts were identified who did not acquire the infection. Whether the mutated virus may have a lower fitness for receptors in the high respiratory tract, which may affect transmission, remains undefined. Furthermore, the mutation, which was first found in a severely affected man, was transmitted to a family member (father), who had only a mild illness, despite risk for severe infection. The HA1 sequence from this latter case presented an additional amino acid substitution (G155E), which was not found in any other sequence analyzed in our study and rarely detected among all sequences available from GenBank. Recent data suggest that amino acid substitution at this position may be critical for the switch from dual α2-

3/α2-6 binding specificity to α2-6 linkage (Takahashi et al., 2009), which is preferentially recognized by human influenza viruses and expressed mainly in the upper respiratory tract. This change might be partially responsible for the milder influenza illness developed by the father of the index case-patient. In addition, the G155E mutation in laboratory-generated variants of pandemic A/H1N1 2009 virus has been associated with loss of antigenicity (Chen et al., 2010). In our study, the natural isolates of the virus carrying both the G155E and the D222G mutations had comparable antigenicity with the A/California/7/09-vaccine strain.

Amino acid substitution at residue 222 of the HA has been observed in a significant number of cases; substitutions D222E and D222G being the most common. The D222E substitution has been observed also in viruses from around the world and has very little affect on antigenicity. Viruses carrying the D222G substitution have been isolated from many individuals with severe infection but this substitution has not been observed in all severe cases and is not always associated with severe outcomes of infection.

The majority of severe and fatal cases investigated in Italy did not carry the D222G substitution and our data do not support the association of the D222G mutation with severe disease, suggesting that this mutation is not required for a severe outcome.

We also describe a fatal case of myopericarditis presenting with cardiac tamponade in a previously healthy 11-year-old child, which has not shown the D222G mutation. Pandemic H1N1 2009 influenza A virus sequences were identified in throat and myocardial tissues and pericardial fluid, suggesting damage of myocardial cells directly caused by the virus. In molecular studies conducted in the United States and in Italy, influenza virus accounted for 2 to 10% of all viral agents isolated from myocardial tissues (Calabrese et al., 2004). Influenza viruses A and B may play a role in myocarditis and/or

pericarditis, and sporadic cases of myocarditis due to the influenza virus have been reported over the past few decades (Mamas et al., 2008).

Overall, there is consistent evidence that influenza viruses may trigger cardiovascular death and that influenza vaccines reduce the risk of cardiac events in subjects with established cardiovascular disease. However, most of the evidence comes from observational and interventional studies of adults and the elderly in whom a cardiac event generally consists of a clinical sequela of primary respiratory infection. Cardiac involvement in influenza is usually reported to occur between 4 and 9 days after the onset of influenza symptoms and is characterized by worsening dyspnea, ECG abnormalities (i.e., ST elevation and Q waves), elevation of cardiac enzymes (e.g., the M [muscle type] and B [brain type] subunits of CK [CK-MB]), and impaired left ventricular function (Onitsuka et al., 2001). In some cases, pericardial effusions may result in cardiac tamponade. Myocyte damage may be due to a direct cytolytic effect of the virus or to the host immune response; the former mechanism usually plays the major role in cases with early myocardial involvement, whereas immune response mediated damage is most likely to occur in later phases of infection. In our case, cardiac involvement occurred early in the course of infection, on the third day after the onset of fever, and was associated with fever decrease by crisis. The timing of cardiac involvement, in combination with virus detection in myocardial and pericardial tissues and fluid, strongly suggests a direct effect of the 2009 pandemic influenza virus on the myocardium and pericardium.

A recent study has already reported a severe form of acute myocarditis in four children confirmed to be positive for the 2009 pandemic virus (Bratincsàk et al., 2010)

The first case of oseltamivir-resistance among the influenza A/H1N1v strains circulating in Italy since the beginning of the pandemic is also reported. The

emergence of drug resistance was associated with prolonged antiviral treatment in an immunocompromised patient with persistent virus replication, a recognized clinical setting favouring the emergence of resistance to single (Englund et al., 2009) or combined oseltamivir and zanamivir drugs (Baz et al., 2006). Nonetheless, the pandemic A/H1N1 - A/Pavia/21/2009 strain did not present mutations associated with zanamivir resistance. Importantly, no other resistance mutations were found among the pandemic A/H1N1 influenza strains from the 133 patients with ILI or pneumonia throughout different areas of Italy, suggesting that the great majority of the 2009 A/H1N1 influenza strains in Italy (either imported or locally transmitted) remain susceptible to oseltamivir and zanamivir. The low prevalence of drug-resistant strains reported in our and other European Countries indicates that drug resistant virus variants were not circulating in Europe during the period when the study was conducted.(Weekly update on oseltamivir resistance to pandemic influenza A (H1N1) 2009 viruses. World Health Organization; March2010,http://www.who.int/csr/disease/swineflu/oseltamivirresistant20100305.pdf.).

Efforts to control these outbreaks and real-time monitoring of the evolution of this virus should provide us with invaluable information to direct infectious disease control programs and to improve understanding of the factors that determine viral pathogenicity and/or transmissibility. Furthermore, the presence of mutation such as the D222G it may serve as a reminder that the generally very low virulence of the current pandemic virus is not a fixed characteristic, and that there is no reason for complacency in carrying out measures that limit infection with this virus at individual and population level. Further virological, clinical and epidemiological investigations are needed to ascertain the role of those mutations that may alter the virulence and transmissibility of the pandemic influenza A/H1N1 virus.

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