ORIGINAL ARTICLE

Early co-circulation of different clades of influenza A/H1N1v pandemic virus in Northern Italy

E. PARIANI, A. PIRALLA*, E. FRATI, G. ANSELMI, G. CAMPANINI*, F. ROVIDA*, A. RANGHIERO, E. PERCIVALLE*, A. AMENDOLA, F. BALDANTI*, A.R. ZANETTI
Department of Public Health-Microbiology-Virology, University of Milan, Italy; *Molecular Virology Unit, Virology and Microbiology Department, Fondazione IRCCS Policlinico San Matteo Pavia, Italy

Key words

Influenza A/H1N1v • Pandemic • Evolution

Summary

Introduction. *The spatial diffusion over time of pandemic influenza A/H1N1 virus (A/H1N1v) was surveyed in Northern Italy (nearly 10 million inhabitants) from April to December 2009, and the molecular characteristics of circulating viruses were analyzed to identify the appearance of drift variants. About 45% of analyzed samples were laboratory-confirmed cases of A/H1N1v. Sporadic cases occurred until the middle of June 2009, then, case numbers began to increase delineating distinct epidemiological phases of viral circulation.*
Methods. *RNA was extracted using RNeasy Mini kit (QIAGEN GmbH, Germany). Virological diagnosis of A/H1N1v infection was carried out by real-time RT-PCR assay. Sequence analysis of hemagglutinin (HA) gene was performed through a RT-PCR assay specific for a 995 bp fragment (nt. 64-1,058) in the HA1 domain. The nucleotide sequences were obtained by automated DNA sequencing. The HA1 sequences were aligned with other sequences collected from GenBank database by ClustalX software. The multiple sequence alignment was used to perform a basic phylogenetic analysis and a phylogenetic tree from HA sequences was constructed.*
Results. *The HA gene sequences of A/H1N1v analyzed segregated into three genetically distinct clades and were characterized by the appearance of amino acid variations that were progressively fixed in the field viral population under scrutiny.*
Conclusions. *These data suggest an early co-circulation of genetically distinct A/H1N1v variants and emphasize the importance of a close molecular surveillance to detect rapidly the spread of new viral variants and to define their epidemiological impact.*

Introduction

Influenza A (family *Orthomyxoviridae*, genus *Influenzavirus A*) is currently the greatest pandemic disease threat to humankind. Influenza A viruses are endowed with remarkable biological dynamism and well known for their propensity for rapid and unpredictable antigenic variation.
In late April 2009, a novel swine-origin influenza A/H1N1 virus - A/H1N1v - was identified in humans in Mexico and in the USA, initiating the first influenza pandemic of the 21st century. Genetic analysis of the whole genome of this new virus showed that A/H1N1v is a novel reassortant between North American and Eurasian swine influenza viruses. The novel virus possesses the polymerase basic-2 (PB2) and polymerase A (PA) genes of North American avian virus origin, the polymerase basic-1 (PB1) gene of human H3N2 virus origin, the hemagglutinin (HA), nuclear protein (NP) and non-structural (NS) genes of classical swine origin and the neuraminidase (NA) and matrix (M) genes of Eurasian swine virus origin [1]. The virus spread efficiently from person-to-person and on June 11, 2009, the World Health Organization (WHO) declared the phase 6 alert pandemic [2]. As the regional reference laboratory operating within the Italian Influenza Surveillance Network (INFLUNET, http://www.iss.it/iflu/), we were immediately engaged in laboratory surveillance and response to influenza pandemic. This gave a unique opportunity to examine the pandemic phenomenon using both the genetic and the epidemiological approach. The large-scale surveillance of A/H1N1v at a molecular level can be relevant to better define the evolutionary and spatial dynamics of this virus in human populations.
The aims of this study were to survey the spatial diffusion over time of A/H1N1v influenza virus in Lombardy (Northern Italy, nearly 10 million inhabitants over a total of about 60 millions at a national level) from April to December 2009 and to analyze phylogenetically the hemagglutinin gene of A/H1N1v viruses to identify the appearance of drift variants.

Methods

In Italy, influenza surveillance is routinely based on a nationwide sentinel surveillance network that consists of paediatricians and general practitioners, who survey approximately 2% of the general population, weekly reporting the number of new cases of influenza-like illness (ILI), and collecting respiratory samples for virological evaluation. The standard case definition of ILI is: abrupt onset of fever (> 38.0°C), one or more respiratory symptoms (non-productive cough, sore throat, rhinitis) and

one or more systemic symptoms (myalgia, headache, severe malaise).

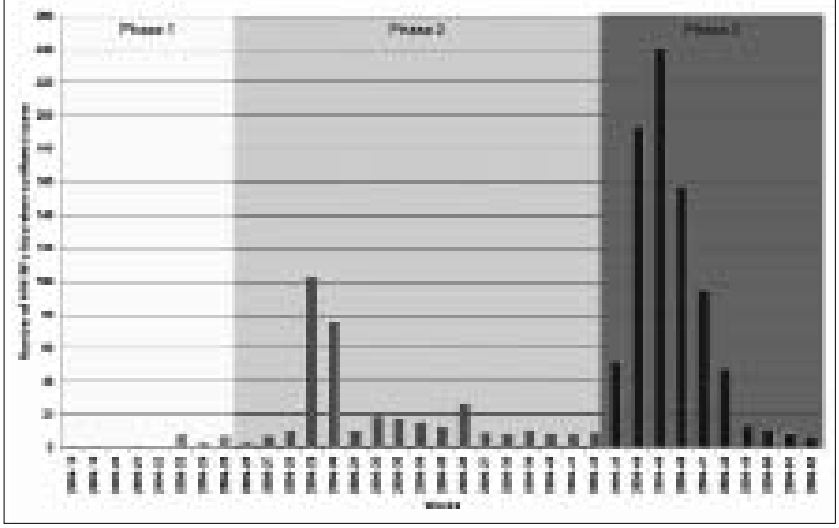
By April 26, 2009, surveillance activities were enhanced [3] and respiratory samples (nasal-pharyngeal swabs) were collected both from hospitalized patients suffering from severe ILI and from ILI outpatients. Specimens were sent to the regional reference laboratories for virological diagnosis of A/H1N1v infection and sequencing. On purpose, total RNA was extracted from each specimen using RNeasy Mini kit (QIAGEN GmbH, Germany). Virological diagnosis of A/H1N1v infection was carried out through a real-time RT-PCR assay [4]. Sequence analysis of HA gene was performed in 103 A/H1N1v viruses through a RT-PCR assay specific for a 995 bp fragment (nt. 64-1,058) in the HA1 domain [5]. Following the RT-PCR of the HA gene, amplicons were purified with NucleoSpin® Extract II (Macherey-Nagel GmbH, Germany). The nucleotide sequences were obtained by automated DNA sequencing based on fluorescent dye terminator on the genetic analyzer ABI PRISM 3100 (Applied Biosystem, USA). Then, the HA1 sequences were aligned with other sequences collected from GenBank database by ClustalX software, version 2.0.12. The multiple sequence alignment was used to perform a basic phylogenetic analysis. Phylogenetic tree from HA sequences was constructed by means of the Neighbor-Joining method and Poisson correction model, using MEGA package, version 4.1. A bootstrap analysis (n = 1,000) was performed. Sequences were deposited into GenBank, National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov), under accession numbers GQ14765, GQ246478, GQ259997-GQ260001,GQ330653-GQ330655,GQ374889,GQ374891, GQ374892,GQ422377-GQ422381,GU451262-GU451280, GU459082-GU459139,GQ387381-GQ387384,GQ258717, GQ166222, and GQ259996. The HA gene sequences used in the construction of phylogenetic tree were obtained from the NCBI Influenza Virus Sequence Database (http://www.ncbi.nlm.nih.gov/Genbank/index.html). Their accession numbers are GQ280797 and FJ969540.

Results

As of 31 December 2009, a total of 2,685 samples were analyzed and 1,184 (44.1%) laboratory-confirmed cases of pandemic A/H1N1v influenza were identified. Of the latter cases (median age: 17.1 years; range 15 days – 86 years), 52.5% were males. Figure 1 shows the temporal distribution of A/H1N1v confirmed cases. After the first case of A/H1N1v influenza occurred on May 3, 2009, sporadic cases occurred until the middle of June. Later on, case numbers began to increase delineating distinct epidemiological phases of viral circulation. During the first phase, spanning from week 18 to week 24-2009, 14 cases of A/H1N1v infection were laboratory-confirmed. Most (93%) cases (median age: 27.6 years; range 24-50 years) were reported among travellers returning from Mexico and the USA, while one case was secondary (contact with a travel-associated case within seven days of onset). During the second epidemiological phase (from week 25 to week 41-2009), 344 cases (46% males; median age: 16.7 years; range 4 months – 84 years) were laboratory-confirmed. Of them, 132 (38.4%) reported a history of travel to the UK in the seven days before the disease onset, and most of them were teenagers (median age: 14.7 years; range 3-47 years) attending English language summer-schools. Seventy-three (21%) cases were reported among travellers returning from a wide range of geographical areas (Americas, Oceania, and Asia). The first indigenously acquired infection was reported on July 16, 2009, and, since then, the number of autochthonously acquired cases steadily increased. On July 27, 2009, the Italian Ministry of Health decreed to stop laboratory confirmation of all probable cases [3]. The third epidemiological phase, spanning from week 42 to week 52-2009, corresponded to the pandemic wave that peaked in week 46-2009. During this period, 826 laboratory-confirmed cases (median age: 19.4 years; range 15 days – 86 years) were identified.

The HA sequences of the A/H1N1v viruses analyzed fell into three phylogenetically distinct clades (Fig. 2) marked by viral variants that appeared at the very beginning of pandemic and then co-circulated in the human population. All viruses belonging to clade I were A/California/07/2009-like and presented T197A amino acid variation in their HA sequences compared to A/California/04/2009. Afterwards, this mutation was maintained in the viral population under scrutiny. All viruses (n = 9) belonging to clade I were identified at the very beginning of the pandemic (May-June) in travel-

Fig.1. Temporal distribution of A/H1N1v laboratory-confirmed cases in Lombardy from 26 April to 31 December 2009.



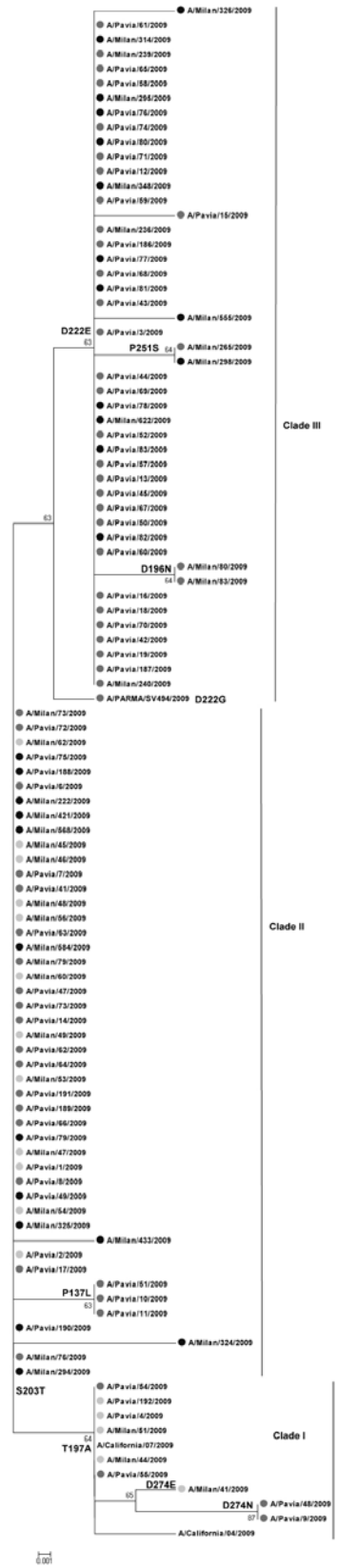


Fig. 2. HA1 phylogenetic tree. Sequences from the first epidemiological phase (May-June 2009) are labelled in light grey, the ones from the second epidemiological phase (July-August 2009) are labelled in dark grey, and the ones from the third phase (September-December 2009) are labelled in black. Major amino acid changes are reported in block letters.

lers returning from affected areas (Mexico, the USA and the UK). By the beginning of June, a new viral variant characterized by a further amino acid change (S203T) was identified and became representative of sequences (n = 46) included in clade II. The observed amino acid mutation was maintained in the viral population and this new variant co-circulated with the one previously recognized. Thirty-three (71.7%) sequences within clade II were identified in viral strains found in subjects returning from different geographic areas (Americas, Oceania, and Asia). The remaining thirteen (28.3%) sequences were identified in patients who did not travel abroad (autochthonous acquired disease). Clade II included sequences of viruses identified during the whole study period (from May to December 2009). From early July (week 27-2009), a novel viral variant, presenting the amino acid change D222E, was observed. This mutation, characterizing sequences (n = 48) included in clade III, fell in the HA receptor binding site and in the Ca2 epitope [6]. Twenty-eight (58.3%) viruses belonging to clade III were identified in autochthonous cases, while the remaining twenty (41.7%) in travellers returning from journeys in Europe. Clade III included sequences of viruses identified during the second and third epidemiological phase only (August-December).
Several mutations were observed at single-sequence level and occurred sporadically, suggesting that they were not fixed in the viral population under analysis. Altogether, all A/H1N1v viruses were antigenically characterized and resulted to be similar to A/California/07/2009, the WHO recommended vaccine strain.

Discussion

This study showed an early segregation of pandemic A/H1N1v influenza viruses in three distinct clades. Several viral variants were identified from the very beginning of the pandemic both in travellers returning from affected areas and in indigenous cases. This outline suggests that distinct viral variants appeared almost simultaneously in different geographical areas (Americas, Asia, Europe, and Oceania), and rapidly spread globally co-circulating over time and space.
The HA sequences of these viruses segregated into three distinct clades characterized by amino acid changes, which were fixed progressively in the viral population. A whole-genome phylogenetic analysis of A/H1N1v isolates collected globally [7] has revealed that at least seven major clades of the A/H1N1v influenza virus have co-circulated over time and space since April 2009. This study has demonstrated that, very early in its history, A/H1N1v virus has evolved, shifting from an initial mixed clade pattern to the predominance of one clade (clade 7) during the course of the pandemic. The viruses constituting this clade substituted all other clades in the later phases of the pandemic and become responsible for most of the pandemic burden worldwide [8]. Our findings, though relevant to a restricted area, showed a similar chronologi-

cal evolution of A/H1N1v virus. In fact, in agreement with these previous studies, the analysis carried out in our study indicated that the dominant A/H1N1v virus in Lombardy was clade 7. Indeed, the majority (91.3%) of analyzed sequences presented the amino acid change S203T in their HA, which is the hallmark of sequences constituting clade 7. Whether the predominance of this clade in the surveyed area is related to the adaptability of the virus or to a more efficient human transmissibility over other early clades has yet to be demonstrated. It is unclear whether differences in fitness or clinical severity exist among these co-circulating lineages, since no clear trend in the clinical aspects could be observed between the early and the late peaks of the epidemic [9]. Neither was a clear clinical impact demonstrated for HA variants. D222G/N substitutions [10] were observed sporadically with no evidence of their maintenance in the local viral population. In particular, the D222G mutation, whose significance in terms of pathogenicity needs to be verified, was detected in only one of the study sequences.

Overall, though several amino acid mutations were observed at or near the HA antigenic sites, all A/H1N1v viruses circulating in Northern Italy appeared to be antigenically related to A/California/07/2009 strain, thus potentially having no subsequent implications to vaccine efficacy.
Conversely, though a major limitation of our study is to provide data representing a restricted reflection of the evolution of the pandemic influenza A/H1N1v, our findings mostly replicate what has been described by others [7, 8], thus confirming the major evolutionary features of the pandemic influenza virus.

Conclusions

In conclusion, our data suggest an early co-circulation of genetically distinct A/H1N1v variants. Thus, a continuous molecular surveillance to detect rapidly the spread of new viral variants and to evaluate their epidemiological impact is critical.

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■ Correspondence: Alessandro R. Zanetti, Department of Public Health-Microbiology-Virology, University of Milan, via Pascal 36, 20133 Milan, Italy - Tel. +39 02 50315126 - Fax +39 02 50315120 - E-mail: alessandro.zanetti@unimi.it