

Rapid communications

VIROLOGICAL SURVEILLANCE OF HUMAN CASES OF INFLUENZA A(H1N1)v VIRUS IN ITALY: PRELIMINARY RESULTS

Surveillance Group for New Influenza A(H1N1) Virus Investigation in Italy^{1,2,3,4}

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In this report we describe the findings of laboratory-based surveillance of human cases of influenza A(H1N1)v virus infection in Italy, following the recent worldwide detection of this new virus among human population and the decision of the World Health Organization (WHO) to raise the level of pandemic alert.

Background

In late April 2009, in California, the United States, the Centers for Disease Control and Prevention (CDC) identified two human cases of infection with a new swine-like influenza virus A(H1N1), recently named influenza A(H1N1)v virus [1]. The virus isolates showed a unique combination of gene segments, not identified previously among either human or swine influenza A viruses. Similar virus strains were identified in Mexico [2], where a large outbreak of influenza-like illness had been ongoing since mid-March. On 25 April 2009, the World Health Organization (WHO) declared the outbreak as a 'Public Health Event of International Concern' (PHEIC) under the International Health Regulations (2005) [3]. As of 10 June 2009, the number of cases of influenza A(H1N1)v 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.

Hereby we report the characteristics of the first 54 cases of influenza A(H1N1)v virus infection identified in Italy and describe the virological surveillance activities carried out by the National Influenza Centre and the Italian Surveillance Influenza Network (INFLUNET).

Enhanced influenza surveillance

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 A(H1N1)v 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 [4]. The case definitions used were based on those adopted by the European Commission [5]. 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 A(H1N1)v 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 [5]) 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 [4], only specimens from probable cases (i.e. cases with positive test results for influenza A virus) should be sent for influenza A(H1N1)v confirmation by NIC.

The notification of confirmed A(H1N1)v cases of infection to the MoH is done by the NIC.

Laboratory confirmation of cases of influenza A(H1N1)v virus infection

The well-established seasonal surveillance network made it possible to identify the first suspected cases of influenza A(H1N1)v virus infection in Italy as early as 27 April 2009. However, although

WHO had promptly provided the national influenza centres with updated molecular diagnostic protocols for influenza A(H1N1)v virus detection, at the time no specific diagnostic reagents were available at the Italian NIC. For this reason, a differential diagnostic test was urgently needed in order to confirm the cases reported by the collaborating laboratories.

In order to assess whether the primer and probe sets, available at NIC for molecular influenza diagnosis, could be useful also to detect infection with the new influenza A(H1N1)v 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 (RRT-PCR), already in use at NIC for the detection of the M gene of type A human influenza viruses. Primers and probe used for the above RRT-PCR were available at the website of the United Kingdom Health Protection Agency [6], although conditions used at NIC were adapted to a singleplex reaction. To confirm the results, the amplified product of the M gene (about 200bp) was sequenced and used for a differential diagnostic analysis to discriminate between

seasonal and A(H1N1)v viruses. Furthermore, each sample was also tested in a RRT-PCR assay specific for both seasonal A/H1 and A/H3 human subtypes. 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 A(H1N1)v virus detection and sequencing, was also employed.

Since 12 May 2009, clinical samples have been tested by the specific RRT-PCR reagent kit from CDC [7]. Virus isolation attempts of laboratory-confirmed cases were also performed, and genes coding for viral protein M, HA1, NA and NP of the first three virus isolates were sequenced and phylogenetically analysed.

Results

Clinical and epidemiological findings of virologically confirmed cases

Information on the epidemiological characteristics and the geographical distribution of the 54 cases of influenza A(H1N1)v virus infections, reported in Italy up to 10 June, is summarised in Figure 1.

Of the 54 confirmed cases, all of whom presented with a self-limiting influenza-like illness (ILI), six were reported among travellers returning from Mexico, 42 in travellers from the United States, two from Canada and one from the Bahamas. Only three cases were due to in-country transmission (specifically household transmission). About 30% of patients were isolated in hospital and 70% were advised to stay at home for the period of seven days. All 54 patients received antiviral treatment.

Figure 2a shows the distribution of all samples analysed and the laboratory-confirmed cases by day of sample collection, whereas Figure 2b shows the distribution of cases by day of symptoms onset and travel history. The median age of the patients was 27.5 years (Figure 1), ranging from 2 to 69 years, and 28 (52%) of the confirmed cases were females. Thirty-three cases were identified in central Italy, 19 in the north and only two in the south of the country. Interestingly, 12 of the cases identified in central Italy involved a group of high-school students from two schools in Rome, returning from a United Nations meeting held in New York and travelling back to Italy on 19 May on the same flight. The index case was a girl who showed typical ILI-symptoms as early as 15 May when still in New York, but whether she was the source of infection for the other students or whether they had acquired the infection during the meeting attended by about 10,000 students from all over the world remains unknown. One of the students was asymptomatic, 11 developed mild clinical symptoms consistent with those of seasonal influenza. Following these cases, the two schools in Rome were closed for one week.

Specificity analysis of the primer and probe sets and laboratory results

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). 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

FIGURE 1

Geographical distribution and epidemiological characteristics of cases of laboratory-confirmed A(H1N1)v virus infection in Italy, by 10 June 2009 (n=54)

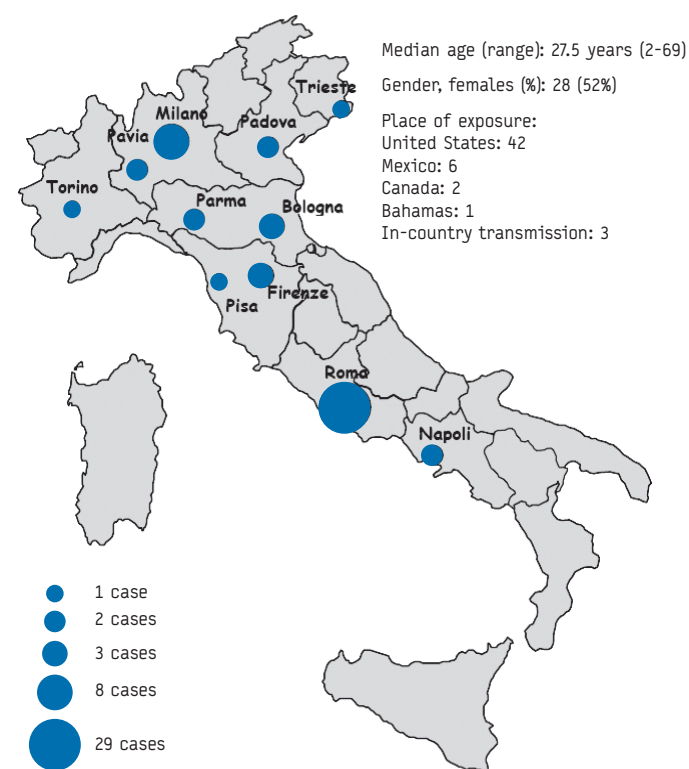
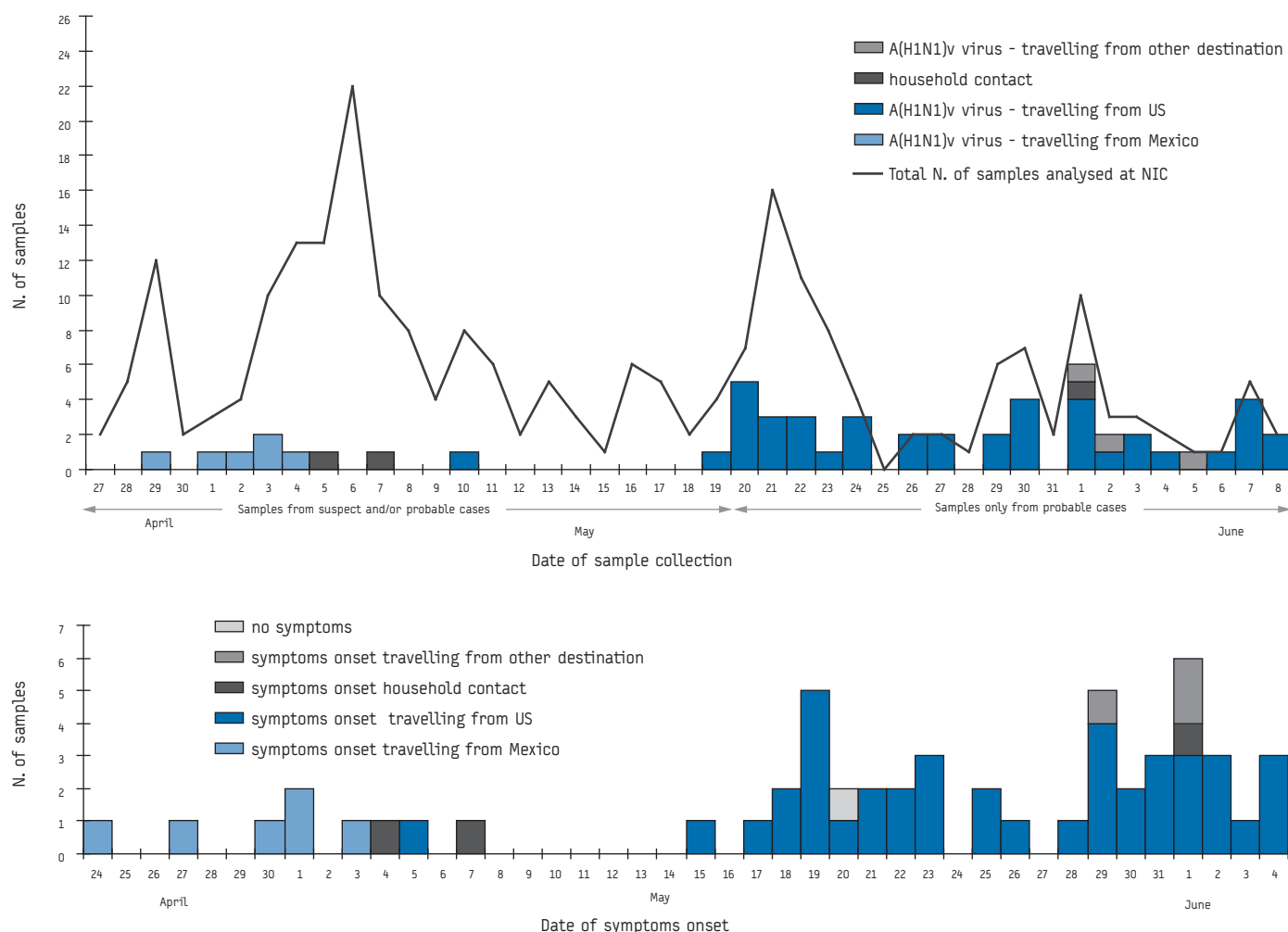


FIGURE 2

Number of clinical samples analysed at the National Influenza Centre and of laboratory-confirmed cases of influenza A(H1N1)v virus infection by (A) day of sample collection and (B) day of symptoms onset; Italy, as of 10 June 2009



(about 200 bp); along this region it was possible to highlight at least 12 nucleotide changes clearly distinguishing the A(H1N1)v virus from the currently circulating human influenza isolates. This was the method initially employed to identify the novel A(H1N1) strain in the clinical material. When the regional laboratories were able to provide viral sequences, a confirmatory BLAST analysis was performed by the NIC to confirm A(H1N1)v virus cases.

Sequence analyses

Preliminary studies showed that six genomic segments of the virus, including the HA, 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 [8,9]. Figure 3 shows the evolutionary relationships of the M1, HA1, NA and NP gene segments of the first three A(H1N1)v virus isolates, obtained in Italy from patients without epidemiological link, compared to other recent A(H1N1)v 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 new A(H1N1) strains were closely related to the Italian swine strains. In contrast, the HA1 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 3), although forming a clade with human seasonal viruses.

Antiviral susceptibility

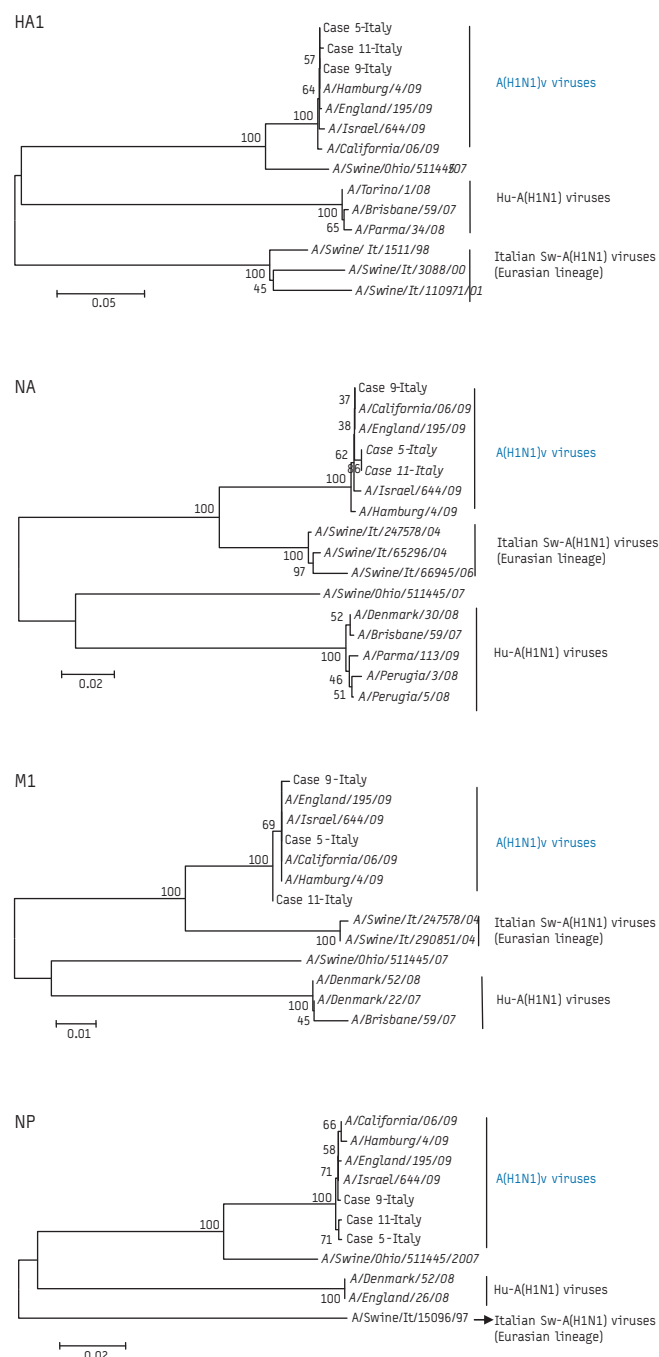
The sequence analyses of the NA and M genes, respectively, revealed that the above mentioned three A(H1N1)v virus isolates were resistant to adamantanes and sensitive to both neuraminidase inhibitors (oseltamivir and zanamivir).

Discussion

During a period of over one month between 27 April and 10 June, 54 laboratory-confirmed cases of influenza A(H1N1) v virus infection were identified in Italy. With the exception of

FIGURE 3

Phylogenetic analysis of the M1, HA1, NA and NP gene segments of the first three A(H1N1)v virus isolates obtained in Italy in May 2009 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

three secondary cases (in-country transmission) in Rome, all cases of influenza A(H1N1)v virus infection reported in Italy, in this first phase, were acquired abroad, during travel to affected areas (Mexico, the United States, Canada, the Bahamas) and involved mainly young adults.

Laboratory-based surveillance represented a useful tool for early detection of influenza A(H1N1)v cases among travellers and their close contacts. Most laboratories developed methodologies for a rapid diagnosis of this novel virus infection, in close collaboration with the NIC which provided support for definitive diagnosis and data collection. It is expected that the sustainability of this system will decrease as the epidemic spreads and syndromic surveillance will prevail.

The very limited in-country transmission suggests that early diagnosis, antiviral prophylaxis and social distancing, including precautionary school closure, may have contributed to contain the spread of infection in the first phase of the epidemic. However, containment strategies are not realistic in the long-term, and mitigation remains the only option as the epidemic progresses.

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