Phylogenesis and Clinical Aspects of Pandemic 2009 Influenza A (H1N1) Virus Infection

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Abstract: During the spring of 2009, a new influenza A (H1N1) virus of swine origin emerged and spread worldwide causing a pandemic influenza. Here, 329 naso-pharyngeal swabs collected from patients with flu-like symptoms were analyzed by real-time PCR for the presence of H1N1 2009 pandemic virus. Twenty-five samples collected from immunocompetent and immunodepressed patients contained the H1N1 pandemic virus. Phylogenetic analysis of the hemagglutinin and neuraminidase genes showed no obvious differences in terms of similarity and/or homology between the sequences identified in immunocompetent individuals and those obtained from immunocompromised patients. Pre-existing clinical conditions may influence the outcome of H1N1 disease.

Keywords: H1N1 pandemic virus, swine flu, respiratory infections, phylogenetic analysis, influenza surface antigens.

In the early spring of 2009, Mexico and the United States reported clusters of human pneumonia cases caused by a novel H1N1 influenza A virus. This virus spread subsequently across the globe at an unprecedented rate, prompting the WHO to declare a pandemic in June 2009. Phylogenetic analysis has inferred that the virus is likely a reassortant between a North American triple-reassortant swine H1N1 or H1N2 virus and a Eurasian lineage H1N1 swine influenza virus [1, 2]. The analysis showed that the H1 of this reassortant originated from American pigs, while NA and MP were more likely from European pigs. All of the 2009 isolates appear homogeneous and cluster together, although they are distinct from classical human A (H1N1) viruses [3]. Bayesian molecular-clock analysis of each gene of this novel H1N1 virus [4] concluded that the mean evolutionary rate is typical of that of swine influenza viruses, but that the duration of unsampled diversity for each gene segment had means that ranged from 9.24 to 17.15 years, suggesting that the proposed ancestors of this virus may have been circulating undetected for nearly a decade. Inadequate surveillance and characterization of influenza A viruses that circulate in swine have been blamed for this evolutionary gap. Also a recent study showed that the domestic birds could act as intermediate hosts of H1N1 reassortants [5]. Multiple genetic groups have been recognized, including one recently predominant lineage [6], but any possible clinical importance of different lineages remains uncertain. To date, reassortment has not occurred with human influenza viruses. The level of pulmonary replication of the 2009 H1N1 virus has been higher than that of seasonal influenza A (H1N1) viruses in animals infected experimentally [7-9], but the 2009 pandemic strain generally lacks mutations that are associated with increased pathogenicity in other influenza viruses [10].

Here, a phylogenetic study was carried out on the H1N1 2009 pandemic viruses isolated from immunocompetent and immunocompromised patients admitted at Tor Vergata hospital for flu-like symptoms. Influenza-like illness was defined as the presence of fever >38º C and at least another constitutional symptom (asthenia, headache, malaise, sweating, and chills) and one respiratory symptom (cough, sore throat, nasal congestion).

From September 2009 through March 2010, 329 naso-pharyngeal swabs were collected from patients (127 females and 202 males) admitted at the Polyclinic Tor Vergata for flu-like symptoms. Patient's age ranged from 1 to 93 years old. The specimens were analysed at the Laboratory of Molecular Virology, Foundation Polyclinic Tor Vergata, Rome, Italy, for the presence of influenza viruses type A and B.

Viral RNA was extracted with the QIAamp RNA mini kit according to the manufacturer's instruction (QIAGEN S.P.A., Milan, Italy). The RNA was eluted in a volume of 60 µl and stored at 80°- C until analysis. The influenza virus was detected by qualitative real-time PCR using the Influenza virus A/B assay (Cepheid, Sunnyvalle, CA, USA) run on the SmartCycler platform (Cepheid, Sunnyvalle, CA, USA). Influenza A positive cases were re-amplified with primers targeting the hemaglutinin (HA) and neuraminidase (NA) genes of influenza A (H1N1) 2009 pandemic virus. The primer sequences were as follows: F-HA 5'- TGGGGCCATTGCCGGTTTCA-3' (nucleotide position 1041-1060) and R-HA 5'TGCCCCCAGGGAGACTACCA -3' (nucleotide position 1647–1628); F-NA 5'-GCCCAGACAAT GGGGCAGTGG-3' (nucleotide position 590-610) and R-NA 5'-CCGTCTGGCCAAGACCAACCC-3' (nucleotide position 1379-1359). Reverse-transcription and amplification was carried out using the OneStep RT-PCR Kit according to the manufacturer's instructions (QIAGEN S S.P.A., Milan, Italy).

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The PCR products were analyzed on a 2% agarose gel stained with ethidium bromide and visualized under ultraviolet light. All the necessary precautions were taken to avoid contamination.

The HA and NA amplified fragments were sequenced using the Genome Lab DTCS Quick Start Kit (Beckman Coulter, Fullerton, CA, USA) and run on a Beckman Coulter CEQ 8000 Genetic Analysis System (Fullerton, CA, USA) after column purification. The obtained sequences were submitted to the Genbank and matched against all deposited sequences (http://www.ncbi.nlm.nih.gov/BLAST).

An alignment with a set of reference sequences was obtained using CLUSTAL X [11] and manually edited with the Bioedit software [12]. The evolutionary model was chosen as the best-fitting nucleotide substitution model, according to the Hierarchical Likelihood Ratio Test (HLRT) implemented in the Model Test V3.0 software [13]. The statistical robustness and reliability of the branching order within each phylogenetic tree were confirmed by bootstrap analysis using 1000 replicates for the NJ tree and with the Zero Branch Length Test for the Maximum Likelihood (ML) tree. All calculations were performed with PAUP*4.0 software [14]. Trees were rooted as mid point root. The accession numbers of the sequences utilized for phylogenetic analysis including those referred to influenza A virus (H1N1) strains identified in our laboratory and labelled A/ROME/PTV1 to 25 are reported in Appendix Table **1**.

Twenty-five H1N1 2009 pandemic viruses were identified. Of these, six were from immunodepressed patients (five with hematological malignancies and one HIV-1 infected) and nineteen from immunocompetent individuals (Table **1**). A Maximum Likelihood phylogenetic tree was generated for the HA and NA genes (Figs. **1**, **2**). In the HA tree, a main clade was generated where the strains collected from Italian immunodepressed patients and Indian fatal cases [15] (in bold) intermixed with those obtained from immunocompetent Italian patients or Indian recovered cases (regular font). This is clearly shown by the strains A/Pune/NIV10278/2009 and A/Pune/NIV9355/2009 obtained from two fatal cases which cluster with the strain A/Blore/NIV236/2009 isolated from a recovered patient; and outside the main clade, by the strain A/Rome/PTV24/2010 isolated from an immunocompromised patient which clusters with the strain A/Rome/PTV12/2010 and A/Rome/PTV23/2010

Table 1. Clinical and Demographic Characteristics of the 25 Italian Patients Infected with the Pandemic H1N1 Influenza Virus

Patient	Isolate	Age Years/Sex	Isolation Date	Immuno Depression	H1N1 Influenza Outcome
1	A/Rome/PTV1/2010	28/F	01/11/2009	No	Recovered
2	**A/Rome/PTV2/2010**	49/F	01/11/2009	Yes	Recovered
3	A/Rome/PTV3/2010	8/M	02/11/2009	No	Recovered
4	A/Rome/PTV4/2010	41/M	04/11/2009	No	Recovered
5	A/Rome/PTV5/2010	19/M	04/11/2009	No	Recovered
6	A/Rome/PTV6/2010	57/M	04/11/2009	No	Recovered
7	A/Rome/PTV7/2010	50/M	06/11/2009	No	Recovered
8	**A/Rome/PTV8/2010**	51/M	07/11/2009	Yes	Recovered
9	**A/Rome/PTV9/2010**	62/M	12/11/2009	Yes	Recovered
10	A/Rome/PTV10/2010	45/F	12/11/2009	No	Recovered
11	A/Rome/PTV11/2010	40/F	13/11/2009	No	Recovered
12	A/Rome/PTV12/2010	55/F	13/11/2009	No	Recovered
13	A/Rome/PTV13/2010	53/M	13/11/2009	No	Recovered
14	A/Rome/PTV14/2010	17/M	15/11/2009	No	Recovered
15	A/Rome/PTV15/2010	41/M	18/11/2009	No	Recovered
16	A/Rome/PTV16/2010	75/F	18/11/2009	No	Recovered
17	A/Rome/PTV17/2010	27/F	20/11/2009	No	Recovered
18	**A/Rome/PTV18/2010**	12/M	23/11/2009	Yes	Recovered
19	A/Rome/PTV19/2010	23/M	23/11/2009	No	Recovered
20	**A/Rome/PTV20/2010**	27/F	24/11/2009	Yes	Recovered
21	A/Rome/PTV21/2010	37/M	24/11/2009	No	Recovered
22	A/Rome/PTV22/2010	19/M	24/11/2009	No	Recovered
23	A/Rome/PTV23/2010	51/M	25/11/2009	No	Recovered
24	**A/Rome/PTV24/2010**	52/F	25/11/2009	Yes	Recovered
25	A/Rome/PTV25/2010	30.6/F	31/12/2009	No	Recovered

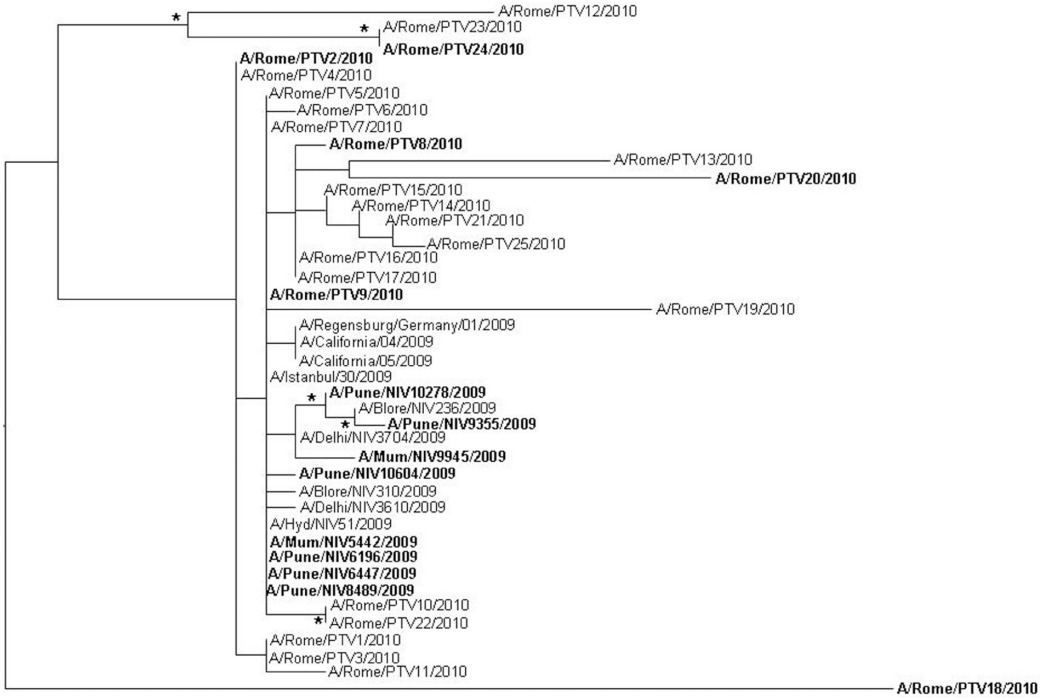

Fig. (1). Maximun likelihood phylogenetic analysis of H1N1 HA sequences. The data set included sequences isolated from patients admitted at Tor Vergata hospital. The tree was rooted by using the midpoint rooting method. Branch lengths were estimated with the best fitting nucleotide substitution model (HKY+I+G) according to a hierarchical likelihood ratio test, and were drawn to scale with the bar at the bottom indicating 0.0090 nucleotide substitutions per site. One asterisk (*) along a branch represents significant statistical support for the clade subtending that branch ($p<0.001$ in the zero-branch-length test and bootstrap support >75%). The strains isolated from severe cases are in bold.

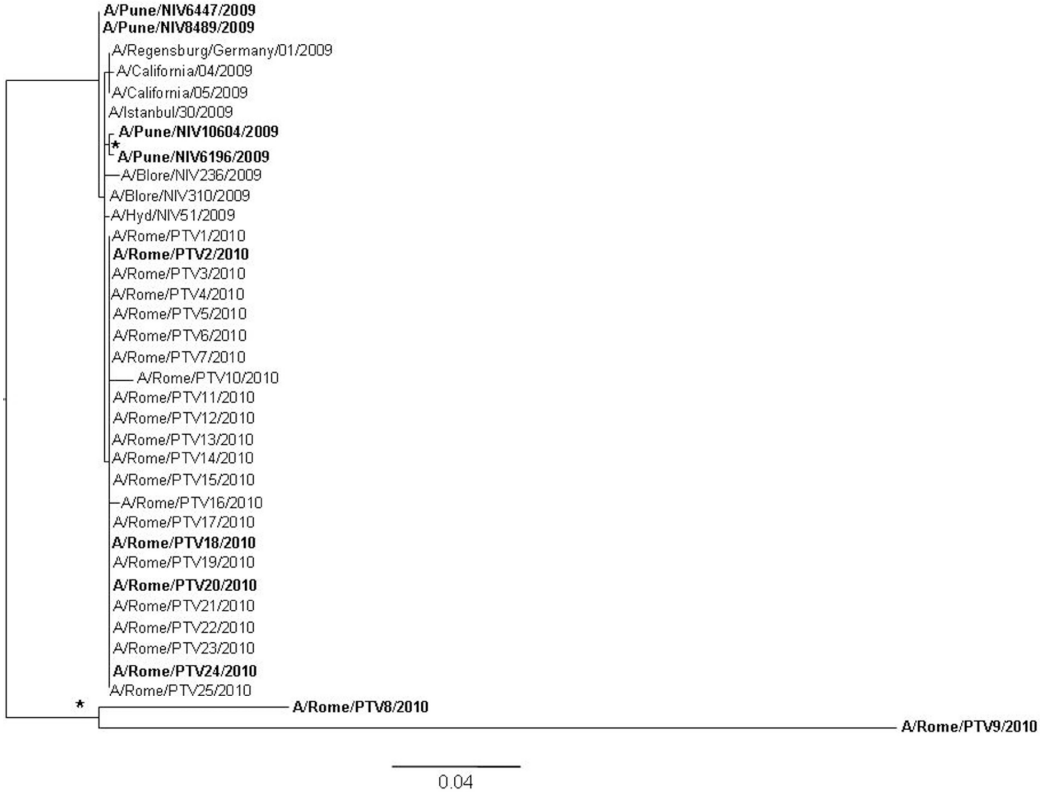

Fig. (2). Maximun likelihood phylogenetic analysis of H1N1 NA sequences. The data set included sequences isolated from patients admitted at Tor Vergata hospital. The tree was rooted by using the midpoint rooting method. Branch lengths were estimated with the best fitting nucleotide substitution model (HKY+I+G) according to a hierarchical likelihood ratio test, and were drawn to scale with the bar at the bottom indicating 0.04 nucleotide substitutions per site. One asterisk (*) along a branch represents significant statistical support for the clade subtending that branch ($p<0.001$ in the zero-branch-length test and bootstrap support .>75%). The strains isolated from severe cases are in bold.

obtained from immunocompetent individuals. Both clusters are statistically supported. A similar picture was observed with the NA tree (Fig. **2**). Even in this case the viral strains from immunocompromised or death patients intermixed with those isolated from immucompetent patients with the exception of two small clusters (A/Pune/NIV10604/2009, A/Pune/NIV6196/2009 and A/Rome/PTV8/2010, A/Rome/PTV9/2010) which were collected from two fatal cases and immunocompromised patients, respectively.

The results of this study indicate no obvious differences in viral sequences between the H1N1 pandemic viruses isolated from immunocompetent individuals and those identified in recovered immunocompromised patients and/or fatal cases. We can exclude that the lack of sequence variations observed in Italian immunocompetent and immunocompromised patients was due to contamination of virus sequences since the samples and sequencing were handled appropriately using separate rooms and including controls in each run as also shown by the phylogenetic trees.

Indeed, mutations that have been linked to fatal cases have also been detected in recovered cases [15, 16]; therefore their role in viral pathogenesis remains unclear. Preexisting specific severe clinical conditions may play an important role in determining the outcome of H1N1disease [17].

APPENDIX

Table A1. H1N1 2009 Pandemic Virus Sequences Used in this Study

Virus Strain	Accession no HA	Accession no NA	Age/Sex	Influenza Outcome	Country	Collection Date
A/California/04/2009	GQ280797	FJ969517	10/M	recovered	California	01/04/2009
A/California/05/2009	FJ966952	FJ966956	9/F	recovered	California	30/03/2009
A/Regensburg/Germany/01/2009	FJ974021	FJ984953	37/M	recovered	Germany	27/04/2009
A/Istanbul/30/2009	GQ200598	GQ200599	26/M	recovered	Turkey	14/05/2009
A/Pune/NIV10278/2009	GU292344	N	6/M	Death	India	Sep-2009
A/Pune/NIV10604/2009	GU292345	HM241726	3/F	Death	India	Sep-2009
A/Blore/NIV236/2009	GU292346	GU292381	2.5/ M	Recovered	India	26 /06/2009
A/Blore/NIV310/2009	GU292347	GU292382	9/F	Recovered	India	01/07/2009
A/Delhi/NIV3610/2009	GU292348	N*	12/M	Recovered	India	13 /08/2009
A/Delhi/NIV3704/2009	GU292349	N	13/M	Recovered	India	08/09/2009
A/Hyd/NIV51/2009	GU292350	GU292383	23/M	Recovered	India	13/05/2009
A/Mum/NIV5442/2009	GU292351	N	2 months/ F	Death	India	16/08/2009
A/Pune/NIV6196/2009	GU292352	GU292384	17/M	Death	India	16 /08/2009
A/Pune/NIV6447/2009	GU292353	GU292385	22/F	Death	India	17 /08/2009
A/Pune/NIV8489/2009	GU292354	GU292386	42/F	Death	India	22 /08/2009
A/Pune/NIV9355/2009	GU292355	N	20/M	Death	India	29 /08/ 2009
A/Mum/NIV9945/2009	GU292356	N	N/M	Death	India	3 /09/2009
A/Rome/PTV1/2009	HM625622	HM625647	28/F	recovered	Italy	01/11/2009
A/Rome/PTV2/2009	HM625623	HM625648	49/F	recovered	Italy	01/11/2009
A/Rome/PTV3/2009	HM625624	HM625649	8/M	recovered	Italy	02/11/2009
A/Rome/PTV4/2009	HM625625	HM625650	41/M	recovered	Italy	04/11/2009
A/Rome/PTV5/2009	HM625626	HM625651	19/M	recovered	Italy	04/11/2009
A/Rome/PTV6/2009	HM625627	HM625652	57/M	recovered	Italy	04/11/2009
A/Rome/PTV7/2009	HM625628	HM625653	50/M	recovered	Italy	06/11/2009
A/Rome/PTV8/2009	HM625629	HM625654	51/M	recovered	Italy	07/11/2009
A/Rome/PTV9/2009	HM625630	HM625656	62/M	recovered	Italy	12/11/2009
A/Rome/PTV10/2009	HM625631	HM625655	45/F	recovered	Italy	12/11/2009
A/Rome/PTV11/2009	HM625632	HM625657	40/F	recovered	Italy	13/11/2009
A/Rome/PTV12/2009	HM625633	HM625658	55/F	recovered	Italy	13/11/2009
A/Rome/PTV13/2009	HM625634	HM625659	53/M	recovered	Italy	13/11/2009
A/Rome/PTV14/2009	HM625635	HM625660	17/M	recovered	Italy	15/11/2009
A/Rome/PTV15/2009	HM625636	HM625661	41/M	recovered	Italy	18/11/2009
A/Rome/PTV16/2009	HM625637	HM625662	75/F	recovered	Italy	18/11/2009
A/Rome/PTV17/2009	HM625638	HM625663	27/F	recovered	Italy	20/11/2009
A/Rome/PTV18/2009	HM625639	HM625664	12/M	recovered	Italy	23/11/2009
A/Rome/PTV19/2009	HM625640	HM625665	23/M	recovered	Italy	23/11/2009
A/Rome/PTV20/2009	HM625641	HM625666	27/F	recovered	Italy	24/11/2009
A/Rome/PTV21/2009	HM625642	HM625667	37/M	recovered	Italy	24/11/2009
A/Rome/PTV22/2009	HM625643	HM625668	19/M	recovered	Italy	24/11/2009
A/Rome/PTV23/2009	HM625644	HM625669	51/M	recovered	Italy	25/11/2009
A/Rome/PTV24/2009	HM625645	HM625670	52/F	recovered	Italy	25/11/2009
A/Rome/PTV25/2009	HM625646	HM625671	30.6/F	recovered	Italy	19/01/2010

*N = data not available.

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