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Original Article

A(H1N1)pdm09 hemagglutinin D222G and D222N variants are frequently harbored by patients requiring extracorporeal membrane oxygenation and advanced respiratory assistance for severe A(H1N1)pdm09 infection

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Background In patients with A(H1N1)pdm09 infection, severe lung involvement requiring admission to intensive care units (ICU) has been reported. Mutations at the hemagglutinin (HA) receptor binding site (RBS) have been associated with increased virulence and disease severity, representing a potential marker of critical illness.

Objectives To assess the contribution of HA-RBS variability in critically ill patients, A(H1N1)pdm09 virus from adult patients with severe infection admitted to ICU for extracorporeal membrane oxygenation support (ECMO) during influenza season 2009–2011 in Piemonte (4·2 million inhabitants), northwestern Italy, was studied.

Patients and methods We retrospectively analyzed HA-RBS polymorphisms in ICU patients and compared with those from randomly selected inpatients with mild A(H1N1)pdm09 disease and outpatients with influenza from the local surveillance program.

Results By HA-RBS direct sequencing of respiratory specimens, D222G and D222N viral variants were identified in a higher proportion in ICU patients (n = 8/24, 33·3%) than in patients with mild disease (n = 2/34, 6%) or in outpatients (n = 0/44) (Fisher's exact test $P < 0{\cdot}0001$; OR 38·5; CI 95% 4·494–329·9). Eleven ICU patients died (42%), three of them carrying the D222G variant, which was associated with RBS mutation S183P in two. D222G and D222N mutants were identified in upper and lower respiratory samples.

Conclusions A(H1N1)pdm09 HA substitutions D222G and D22 2N were harbored in a significantly higher proportion by patients with acute respiratory distress for A(H1N1)pdm09 severe infection requiring ICU admission and ECMO. These data emphasize the importance of monitoring viral evolution for understanding virus–host adaptation aimed at the surveillance of strain circulation and the study of viral correlates of disease severity.

Keywords A(H1N1)pdm09 virus, extracorporeal membrane oxygenation and influenza, hemagglutinin D222G and D222N variants.

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Background

Although the majority of A(H1N1)pdm09 infections present as mild or self-limited disease, severe lung involvement in patients requiring admission to intensive care units (ICU) has been responsible for high morbidity and mortality, and efforts have been made to identify A(H1N1)pdm09 correlates of severe disease.[1–3]

Changes in viral receptor binding affinity at the binding site (RBS) play a critical role in influenza virus tropism and virulence. Studies on viral correlates of disease severity identified that amino acid mutations in the viral hemagglutinin (HA) receptor binding domain could be potentially responsible for influenza outbreaks due to a switch in the receptor binding tropism with a higher affinity for respiratory cells in the lower than in the upper respiratory tract,

leading to a more aggressive disease.[4,5] Specific mutations in the A(H1N1)pdm09 HA occurred during virus–host adaptation have been shown to enhance viral binding to α-2,3 sialic acid receptors located in the lower respiratory tract.

Hemagglutinin receptor binding site sequence corresponding to codons 170 to 230 including positions 190 (helix), 220 (loop), and 130 (loop) is considered a major factor in determining HA binding in all A(H1N1)pdm09 strains.[6,7] This sequence is also involved in antigen recognition by the host, and mutations at this point could alter the antibody reactivity as well as the ability of the virus to infect cells. A(H1N1)pdm09 HA-RBS displays a considerably higher hydrophilic characteristic than the HA-RBS from other strains, probably due to some key amino acid substitutions mainly located between position 223 and 225 involved in binding strength and affecting surrounding residues. Most represented amino acid substitutions in the HA-RBS within A(H1N1)pdm09 clades have been recently described with a frequency of 3·5% at position 172, 5·3% at 183, 17·5% at 185, 31·6% at 205, 32% at 216, and 14·5% at 222.[8] Important polymorphisms at the HA-RBS, such as G155E, S183P and D222G, have been identified in animal models and then confirmed in humans to increase viral affinity for the α-2,3 receptors and are thus possibly associated with involvement and infection of the lower respiratory tract.[6,9]

During the 2009 A(H1N1)pdm09 outbreak, substitution of aspartic acid with glycine at position 222 (D222G) of HA 1 subunit of A(H1N1)pdm09 has been associated with increased virulence, disease severity, and fatal outcomes. This substitution was first observed in severe clinical cases of A(H1N1)pdm09 infection in Norway and then confirmed in fatal and severe outcomes of A(H1N1)pdm09 infection in Scotland, Spain, and Italy.[10–14] Important data on the evolutionary dynamics and temporal diversification of D222G strains have been reported also for the recent 2010–2011 season.[7]

To assess the contribution of HA-RBS variability in A(H1N1)pdm09 disease severity, we retrospectively investigated HA-RBS polymorphisms in critically ill adult patients requiring ICU admission for A(H1N1)pdm09 infection during the two influenza seasons 2009–2010 and 2010–2011. Virological data from patients requiring ECMO were compared with those from randomly selected inpatients with mild A(H1N1)pdm09 disease and outpatients reported by sentinel practitioners from the local influenza surveillance program within the National Surveillance Plan. Primary objective was to identify specific A(H1N1)pdm09 HA genetic correlates of disease severity and fatal outcome in order to improve the understanding of the clinical course of A(H1N1)pdm09 infection in critically ill patients.

Patients and methods

Study groups

During the two influenza seasons 2009–2010 and 2010–2011, respiratory samples for the detection of influenza viruses were processed at the Laboratory of Microbiology and Virology of the Department of Infectious Diseases, Amedeo di Savoia Hospital in Turin, which received 3986 respiratory samples covering a geographic area of 4·2 million inhabitants. Pandemic A(H1N1)pdm09 virus was identified in 1229 specimens (30%). Sixty-seven adult patients (5·5%) with A(H1N1)pdm09 severe complications required ICU admission in the two seasons; 26 of them (38·8%) were referred to the regional reference center for ECMO (ICU-ECMO group). Randomly selected A(H1N1)pdm09-infected inpatients ($n = 34$) hospitalized with mild disease and outpatients reported by sentinel practitioners from the local influenza surveillance program ($n = 44$) served as control groups.

A(H1N1)pdm09 molecular detection and HA polymorphism analysis

Respiratory specimens were processed for A(H1N1)pdm09 detection in a two-step procedure: a nucleic acid-based screening tests for type A and B influenza viruses by a commercial real-time PCR (Seegene Inc., Seoul, South Korea) followed by A(H1N1)pdm09 subtyping with a one-step real-time reverse transcription (RT) PCR. A modified WHO-CDC protocol (April 28, 2009, www.cdc.gov)[15] was applied for A(H1N1)pdm09 detection with specific MGB probes (Applied Biosystems, Foster city, CA, USA) and the combined identification of three viral genes: matrix for generic flu type A, swine flu type A nucleoprotein, and A(H1N1)pdm09 HA. Amino acid polymorphisms in A(H1N1)pdm09 HA-RBS were determined by direct HA sequencing from clinical specimens.[6,13] An internal HA fragment of 475 bp (from 446nt to 921nt according to A/California/07/2009 strain) was sequenced with the BigDye Terminator Cycle-Sequencing Reaction on the ABI Prism 3130 XL DNA sequencer (Applied Biosystems). We analyzed HA-RBS sequence corresponding to codons 170 to 230 including positions 190 (helix) and 220 (loop elements), based on reference data showing that these elements are major factors in determining HA binding in all H1N1/HA strains.[6,13] Sequences were assembled using SeqScape 2.0 (Applied Biosystems) and aligned with ClustalW using the A/California/07/2009, Acc. N. FJ966974 as reference strain. For phylogenetic analysis, the data set of our sequences was compared to sequences from England, France, Germany, and Norway, for a total of 455 sequences. The phylogenetic tree was constructed using the BioNJ method implemented in the PhyML software, with a bootstrap equal to 1000.[16–18] Amino acid substitutions were given for internal nodes. To study the frequency of viral polymorphisms, a data set of 2704 A(H1N1)pdm09 sequences

from Europe covering 2 years (May 2009–May 2011) obtained from the NCBI Influenza Virus Resources (http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html) was constructed. A (H1N1)pdm09 partial hemagglutinin sequences (373 bp, from 441nt to 863nt according to A/California/07/2009 strain) were aligned, and specific A(H1N1)pdm09 polymorphisms were analyzed with R statistical software and the package SeqinR, then referred to the entire data set.[19,20]

Statistical analysis

Analysis of mutation frequencies within different groups of patients and control groups was performed using the chi-square test (with Fisher's exact test where appropriate) and considered to be significant at a level of 0·05. Statistical analysis was performed using PASW Statistics 18.0.2 (SPSS Inc, Chicago, IL, USA).

Results

Detailed characteristics of the 26 ICU-ECMO patients admitted during the two influenza seasons from November 2, 2009, to March 31, 2010 (16 patients) and from January 14 to March 31, 2011 (10 patients), are given in Table 1. Median time from hospitalization to ICU admission was 2 days (average: 4·8, 95% CI: 2·5–7·1). Median APACHE score for all patients was 18·5 and comorbidities were present in the majority of them (22 patients, 84·6%), including obesity with a body mass index >30 (Kg/m^2) in 10 patients. All but two patients had fever, and leukocyte count was normal in 17 patients and $>10^3$ WBC/μl in 9 of them. At ICU admission, X-ray or CT scan revealed lung involvement in 18 patients (69%) and acute respiratory distress syndrome (ARDS) in 3 (11·5%). All patients were sampled for A(H1N1)pdm09 24–72 hours after hospital admission prior to ICU transfer for ECMO support. After A(H1N1)pdm09 detection, they received antiviral treatment within 24–48 hours (oseltamivir, standard dosage 75 mg BID increased to 150 mg BID in two patients), and average duration of oseltamivir treatment was 8 days (±5).

Clinical and microbiological evidence of bacterial or fungal co-infection was found in 14 patients (53%) (Table 2), and six of them died due to septic shock and multiorgan failure. Four patients suffered from extensive pneumonia due to *Aspergillus fumigatus* and two died.

Eleven of 24 patients died (42%): seven within the first 15 days and four within 45 days from ICU admission. Median length of ICU staying was 10 days for non-survivors and 19 days for survivors. Primary reported causes of death were multiorgan failure (nine patients), septic shock (eight patients, six with evident bacterial co-infection) and cardiac arrest (two patients). Comorbidity was the only significant variable that correlated with survival rates (Fisher's exact test $P = 15$, OR: 0·0667, 95% CI: 0·006667–0·6657).

One-hundred and ten respiratory samples from 60 inpatients and 44 outpatients were included in the study. Nucleic acid sequencing of A(H1N1)pdm09 HA was performed directly from respiratory samples [42 pharyngeal and 57 nasal swabs, 11 bronchoalveolar lavages (BAL)]. HA nucleotide sequence identity with the reference A/California/07/2009 ranged from 97·5% to 99·1%, with a maximum of 14 amino acid changes. Analysis of HA mutation revealed position 222 as the most variable site. Polymorphisms at this position were observed in 30 patients (28·8%); substitution D222G was found in 8 (7·7%), D222N in 2 (1·9%), and D222E in 20 (19·2%). D222G and D222N polymorphisms were detected in 8 ICU-ECMO patients, in only two inpatients with mild disease, and in none of the outpatients from the local influenza surveillance system. Statistical analysis showed that mutations D222G and D222N were harbored in a significantly higher proportion (33·3%) by patients with A(H1N1)pdm09 severe disease requiring ICU admission for advanced respiratory supply and ECMO (Fisher's exact test $P < 0·0001$; OR 38·5; 95%CI: 4·494–329·9) than by inpatients with mild disease (5·9%) or outpatients (0%) (Table 3). Moreover, the presence of D222G or D222N correlated with an older age (mean age ± SD for D222G or N carriers: 46·7 ± 7 versus 31·2 + 24 in non-carriers, $P = 0·002$). In ICU-ECMO patients, D222G and D222N mutants were identified in 4 BALs (36%) and in 4 (20%) upper respiratory specimens. In five ICU-ECMO patients, paired upper and lower respiratory samples were available for matched analysis, showing a perfect match of viral strains at the upper and lower respiratory level (Table 1, patient ID nos 2, 3, 5, 8, and 18). Viral shedding from the lower respiratory tract as evaluated on consecutive BALs ranged from 14 to 26 days (average 19·4 days). In ICU-ECMO patients, dichotomous variables (age > 50 years, BMI > 25, invasive ventilation at admission, bacterial/fungal co-infection, and survival) were tested for association with polymorphisms at position 222, but no significant association was found at univariate analysis (chi-square and Fisher's exact test).

Hemagglutinin polymorphisms at position different from D222 were analyzed across the study groups. The following polymorphisms were found: S185T ($n = 14$ patients, 2 ICU-ECMO, 10 inpatients, and two outpatients), A186T ($n = 3$ patients, 1 ICU-ECMO, and 2 outpatients), Q193H ($n = 1$ outpatient), A197T ($n = 3$, 1 ICU-ECMO and 2 inpatients), R205K in combination with I216V ($n = 23$, 3 ICU-ECMO, 14 inpatient, and 6 outpatients), I216E ($n = 1$ inpatient), K209T ($n = 1$ outpatient), M227K ($n = 1$ inpatient), and P236Q ($n = 1$ inpatient). None of these polymorphisms was significantly associated with ICU admission, disease severity and fatal outcome. Substitution S183P was identified in 10 patients (two ICU-ECMO and eight inpatients with mild disease), associated with D222G in 2 ICU-ECMO cirrhotic

Table 1. Characteristics of ICU patients hospitalized for severe complications of A(H1N1)pdm09 infection undergoing advanced respiratory support

Patient ID	Age	Sex	BMI	Comorbidity	X-ray/CT at ICU admission	Pattern of pneumonia	Invasive Ventilation	Inotropes/ Vasopressors	ECMO/DECAP	Specimen	Amino acid at HA-222	Other HA polymorphisms	Survival	Primary cause of death
1	44	M	40	None	ARDS	Interstitial	Yes	Yes	ECMO	BAL	D222G	No	Yes	
2	24	F	33	None	Bilateral pneumonia	Patchy	No	Yes	ECMO**	PS + BAL	D222E***	No	Yes	
3	34	M	31	Asthma	Bilateral pneumonia	Patchy	Yes	Yes	ECMO	NS + BAL	D222***	No	No	Septic shock MOF
4	36	M	48	None	ARDS	Interstitial	No	No	ECMO**	BAL	D222G	No	Yes	
5	32	F	32	None	Bilateral pneumonia, ARDS	Patchy	Yes	Yes	ECMO	NS + BAL	D222***	No	No	Septic shock MOF
6	55	M	24	HL	Bilateral pneumonia	Diffuse	Yes	Yes	ECMO	NS	D222	No	Yes	
7	24	M	25	None	Single-lung pneumonia	Patchy	No	Yes	ECMO**	NS + BAL	not done	No	Yes	
8	45	F	22	None	Bilateral pneumonia	Patchy	Yes	No	DECAP	NS + BAL	D222E***	No	Yes	
9	85	M	NA	NHL	ARDS	Interstitial	Yes	No	None	NS	D222E	No	No	Cardiac arrest
10	55	F	35	None	Bilateral pneumonia	Diffuse	Yes	Yes	DECAP	BAL	D222G	No	Yes	
11	49	F	28	HIV+	Bilateral pneumonia	Patchy	Yes	Yes	None	NS	D222	No	No	Septic shock MOF
12	67	M	NA	Asthma CKD	Single-lung pneumonia	Patchy	No	Yes	None	PS	D222E	No	Yes	
13	38	F	24	Asthma*	Bilateral pneumonia	Diffuse	Yes	No	None	NS	not done	No	Yes	
14	47	F	37	Diabetes	Bilateral pneumonia	Patchy	Yes	No	DECAP	PS	D222	No	No	Septic shock MOF
15	59	F	34	MM	Bilateral pneumonia	Diffuse	No	No	None	PS	D222E	No	Yes	
16	77	M	NA	MM	Bilateral pneumonia	Diffuse	Yes	Yes	DECAP	PS	D222E	No	No	Septic shock MOF
17	62	M	29	COPD		Patchy	Yes	No	None	NS	D222	S185T, A197T	Yes	

Table 1. (*Continued*)

Patient ID	Age	Sex	BMI	Comorbidity	X-ray/CT at ICU admission	Pattern of pneumonia	Invasive Ventilation	Inotropes/ Vasopressors	ECMO/DECAP	Specimen	Amino acid at HA-222	Other HA polymorphisms	Survival	Primary cause of death
18	35	F	NA	None	Single-lung pneumonia Bilateral pneumonia	Diffuse	Yes	No	None	NS + BAL	D222***	S205K, I216V	Yes	
19	50	F	19	Diabetes	Single-lung pneumonia	Diffuse	Yes	Yes	None	PS	D222G	No	No	Septic shock MOF
20	65	F	36	Diabetes, HBV cirrhosis	Bilateral pneumonia	Patchy	Yes	Yes	ECMO	BAL	D222	No	No	Septic shock MOF
21	39	M	35	None	Bilateral pneumonia	Interstitial	Yes	No	None	NS	D222	S205K, I216V	Yes	
22	48	F	NA	Cirrhosis, diabetes, CKD	Bilateral pneumonia	Patchy	Yes	Yes	None	PS	D222G	S183P	No	Septic shock MOF
23	64	M	25	Diabetes, COPD	Bilateral pneumonia	Interstitial	Yes	Yes	None	PS	D222	No	No	Cardiac arrest
24	37	M	NA	None	Bilateral pneumonia	Patchy	Yes	No	ECMO	BAL	D222G	S185T, A186T	Yes	
25	52	F	NA	MM	Single-lung Pneumonia	Patchy	Yes	Yes	ECMO	PS	D222N	S205K, I216V	Yes	
26	52	F	NA	HCV cirrhosis	Bilateral lung consolidation	Diffuse	Yes	No	ECMO	PS	D222G	S183P	No	Septic shock MOF

NA, not available; HL, Hodgkin lymphoma; NHL, non-Hodgkin lymphoma; CKD, chronic kidney disease; MM, multiple myeloma; COPD, chronic obstructive pulmonary disease; ARDS, acute respiratory distress syndrome; DECAP, extracorporeal CO_2 removal; BAL, bronchoalveolar lavage; PS, pharyngeal swab; NS, nasal swab; MOF, multi-organ failure.
*Patient also affected by colorectal polyposis.
**ECMO at admission in ICU.
***Confirmed on both samples.

Table 2. Co-infections in patients with A(H1N1)pdm09 infection who required advanced respiratory assistance and ECMO. See Table 1 for patient reference

Patient ID	Site of bacteria /fungi isolation	Microbial agent
1	BAS	*P. aeruginosa* *
3	BAL	*A. fumigatus*
5	BAS + blood	*A. baumannii**, *E. coli*
6	BAS + blood	*A. baumannii**, *S. marcescens*
7	BAS	*S. maltophilia*, *A. baumanni*, *Klebsiella spp***
8	BAS	*A. baumannii*
10	Blood	*E. faecalis*
17	BAL	*A. fumigatus*, *Pseudomonas* spp
18	BAL	*A. fumigates*
19	Blood	*K. pneumoniae***
20	BAL	*S. maltophilia**
22	Sputum + skin lesion	*K. pneumoniae***, *A. baumanii*, *E. cloacae**
25	BAS + blood	*E. cloacae, E. cloacae*
26	BAL + lung at autopsy	*A. fumigatus*

BAS, bronchoaspirate; BAL, bronchoalveolar lavage.
*Multidrug resistant strain.
**Multidrug resistant carbapenemase production strain (KPC).

Table 3. Amino acid at HA position 222 of A(H1N1)pdm09 virus in ICU-ECMO and other study groups. Percentage (in parenthesis) calculated for each group with respect to the total number of patients in that group

Amino acid at HA position 222	Total (*N* = 102)	ICU-ECMO Patients (*N* = 24)	Inpatients with mild disease (*N* = 34)	Surveillance group ** (*N* = 44)
D222 (wild type)	72	10 (41·6%)	32 (94%)	30 (68·1%)
D222E	20	6 (25%)	0 (0%)	14 (31·8%)
D222N	2	1* (4%)	1* (3%)	0* (0%)
D222G	8	7* (29%)	1* (3%)	0* (0%)

ICU-ECMO, intensive care unit-extracorporeal membrane oxygenation.
*Rate of ICU-ECMO patients carrying the D222G and D222N substitution significantly higher than the other groups (Fisher's exact test $P < 0{\cdot}0001$; OR 38·5; 95% CI: 4·494–329·9).
**Forty-four not-hospitalized individuals with mild A(H1N1)pdm09 influenza from the local surveillance program.

patients who died of sepsis and multiorgan failure following A(H1N1)pdm09 infection (Table 1, patients ID nos 22 and 26). Genetic analysis showed that A(H1N1)pdm09 strains from these patients belonged to a unique nosocomial cluster (Figure 1).[21] Substitution D222E was present in 20 subjects from all the study groups, but only in the season 2009–2010, while it disappeared in the following year. Considering all HA-RBS polymorphisms, ICU-ECMO patients and inpatients hospitalized with mild disease showed a significant higher number of mutations than outpatients (83·3% and 85·3% versus 59·0%, χ^2 8·29, $P = 0{\cdot}004$).

The phylogenetic analysis (Figure 1) of A(H1N1)pdm09 sequences from our series in comparison with other 353 European strains circulating from the year 2009–2011 showed the absence of distinct genetic D222G and D222N clusters in the two seasons apart from two very small clusters consisting, the first, of five strains (2 of them from the ICU-ECMO series, Table 1, patients ID nos 1 and 4) and another one (ICU-ECMO patients ID nos 22 and 26). According to major RBS amino acid substitutions, the following A(H1N1)pdm09 clades were identified: S185T (including 38 sequences: 8·3%), R205K + I216V (63 sequences: 13·8%), D222E (67 sequences: 14·7%), and S183P (36 sequences: 7·9%).

The study of the circulation of A(H1N1)pdm09 major polymorphisms on 2704 European sequences from European strains circulating from the year 2009–2011 showed for specific hot spots the following frequency: D222G, 2·8%; D222N, 0·8%; D222E, 16%; S183P, 2·2%, S185T, 10·2%, R205K, 5·5%; I216V, 4·8% (R205K + I216V: 5·5%) and A197T, 1·7%.

A(H1N1)pdm09 HA nucleotide sequences from ICU-ECMO patients were submitted to the NCBI GenBank with accession numbers CY098210–CY098218 and CY09 8275–CY098290.

Discussion

Worldwide surveillance studies on pandemic influenza have reported that A(H1N1)pdm09 infection is associated with a higher risk of severe disease, hospitalization and ICU admission than seasonal influenza, especially if there is a delay in antiviral treatment.[22–25] Herein, we report the clinical outcome and virological findings of patients admitted to ICU for advanced respiratory assistance and ECMO during the influenza seasons 2009–2010 and 2010–2011, in Piemonte, northwestern Italy (4·2 million inhabitants). In that period, 5·5% of patients with A(H1N1)pdm09 infection required ICU admission for severe complication and 38·8% of them were referred to the regional reference center for

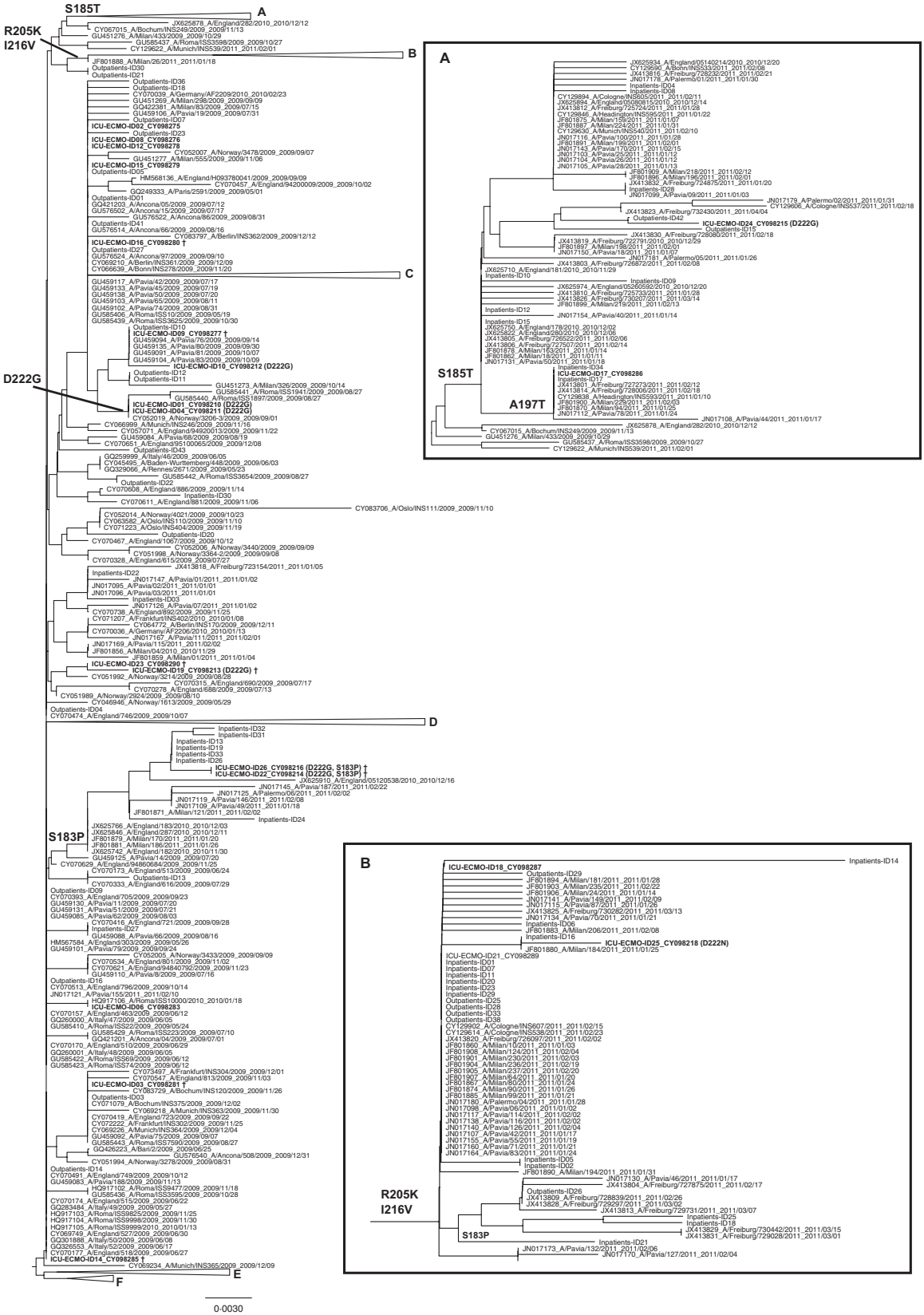

Figure 1. Phylogenetic tree of partial A(H1N1)pdm09 HA. Phylogenetic analysis of influenza A(H1N1)pdm09 partial hemagglutinin gene (373 bp, from 441nt to 863nt according to A/California/07/2009 strain) of 455 sequences. The tree was constructed by the BioNJ method implemented in the PhyML software, with bootstrap equal to 1000. Amino acid substitutions are given for internal nodes. Bootstrap values were omitted for tree readability. Sequence from outpatient ID24 was excluded from the tree reconstruction, because the sequence was too short. Main substitutions were reported on the branch of the respective clade. Details of collapsed sequences labeled with A and B are given magnified, because they form two important clades corresponding to polymorphism S185T and A197T (clade A), R205K + I216V (clade B). Collapsed clades are composed as follows: clade C (including D222E polymorphism): four strains from Italy, two from France, two from Germany, two from Norway, and 5 from England; clade D (corresponding to D222 wild type), from our series: 11 outpatients, 3 ICU-ECMO (ID5, ID11, ID20, who died), then 31 strains from Italy, two from France, 10 from Germany, nine from Norway, and 18 from England; clade E (corresponding to D222 wild type including reference strain A/California/07/2009), from our series: two outpatients, then 12 strains from Italy, four from France, five from Germany, two from Norway, and five from England; clade F (corresponding to D222 wild type): 1 from France, 3 from Norway, and 1 from England. †dead patients.

critical illness requiring ECMO support. These patients presented a rapid worsening of A(H1N1)pdm09 infection shortly after hospital admission with bilateral or single-lung pneumonia and ARDS that required advanced respiratory support including ECMO. A significant proportion of them (42%) died, and the majority of death occurred within the first 7 days after ICU admission.

As the natural tendency of influenza viruses to acquire point mutations at different genomic positions is very well known and changes at the RBS can alter antigenic properties and viral pathogenicity, we evaluated HA genetic correlates of disease severity in these critically ill patients and compared virological data with those from the control group.

Recent data have pointed out that HA-RBS sequence corresponding to codons 170 to 230 is a major factor in determining HA binding and antigen recognition.[6,13] Most represented hot spots for amino acid substitutions within A (H1N1)pdm09 clades are positions 183, 185, 205, 216, and 222.[8] Key amino acid substitutions at the RBS can possibly affect surrounding residues and alter antibody reactivity as well as the ability of the virus to infect cells. Of all mutations, substitutions at position 222 (either as G or N) have been detected only in patients with severe disease. Data from the A (H1N1)pdm09 worldwide surveillance have reported that substitutions D222G and D222N at HA-RBS have been detected in a significant proportion of severe cases: 6·3 % in a study from Spain, 14·7% from Norway, 8·7% from Finland, 8·3% from France, 6·9% from West Scotland, 4·1 % and 6·3% from two studies in Hong Kong, 30% and 5·8% from Italy, 15·4% from India and 21·7% from Greece.[5,10,13,14,26–30]

Our study highlighted that the highest number of HA polymorphisms were found at position 222 in ICU-ECMO patients compared with control groups. D222G and D222N RBS variants were detected in 33·3% of patients with A (H1N1)pdm09 severe infection requiring advanced respiratory support and ECMO, while these mutations were present at a significantly lower proportion (5·9%) in inpatients who did not require ECMO and were absent in viral isolates from outpatients of the local surveillance program.[31,32] D222N substitution was less frequent than D222G, as suggested by the very low circulation of this variant (0·8%) among the European A(H1N1)pdm09 sequences of the same period that we analyzed. On the contrary, the highly circulating variant D222E (16%) was detected in both severe and mild cases, confirming that it was not associated with A(H1N1)pdm09 disease severity.[33] Moreover, we observed D222E circulating strains only in the season 2009–2010 and not in the following one.

The increased virulence of D222G and D222N strains is postulated due to an enhanced binding affinity for the α-2,3 sialic acid receptor present at higher density on cell surface in the lower respiratory tract rather than for the α-2,6 sialic acid receptor located at higher density on upper respiratory tract cells.[34,35] Substitution D222G, which is located in the receptor binding cavity at the Ca antigenic site of HA, changes the properties of the RBS from acid and polar to neutral and non-polar, and experimental data have shown that this mutation causes a shift to a dual α-2,3/α-2,6-linkage specificity, enabling the mutated HA to bind to both upper and lower respiratory receptors, supporting the hypothesis that viruses with dual receptor binding specificity can replicate at high level in lung tissue producing a more aggressive disease.[36] Therefore, the complexity of A(H1N1) pdm09 virus–host interaction and adaptation is modulated by changes at the HA binding site.

In agreement with published data, we confirmed the higher proportion of D222G and D222N viral strains from patients with A(H1N1)pdm09 severe disease, but we additionally showed that the requirement of ECMO as advanced respiratory support was significantly associated with infection due to viruses carrying these HA substitutions at the RBS. Furthermore, we demonstrated the presence of D222G and D222N mutants in both upper and lower respiratory samples from critically ill patients.

In agreement with the rare circulation of these mutants during periods of high spread of influenza virus as shown by ours and other studies,[8] phylogenetic analysis identified only two very small clusters of D222G viruses, both including ICU-ECMO patients from our series, one of the two clusters due to the nosocomial spread of D222G variant.[21] Therefore, the high frequency of D222G strains in critically ill patients could be explained by the selection of these mutants in the

context of disease severity at the individual level instead of viral spreading.[5,37]

Among other HA-RBS substitutions associated with severe disease, the most significant was the mutation S183P, typical of all 1918 H1N1 viruses.[6–39] We identified S183P mutants only in 2011 circulating strains from 10 inpatients, two of them belonging to the ICU-ECMO series. In the two ICU-ECMO patients who died of A(H1N1)pdm09 complications, S183P was associated with D222G, whereas in inpatients with mild disease, S183P was alone and no outpatients from the local influenza surveillance system carried this mutation. Analysis of European A(H1N1)pdm09 strains identified only another virus from the year 2010 carrying the double mutations S183P and D222G from a severely infected patient who died in England (Figure 1, Acc. JX625910 GenBank notes). Therefore, the rare combination of the double mutant S183P and D222G (frequency: 0·1%) probably characterizes a more virulent strain associated with disease severity and case fatality.

We recognize that important limitations of our study were the lack of lower respiratory samples from all patients (BALs were available for only 10·8% of patients) as well as the lack of matched upper and lower respiratory samples (available for only five patients, none of them carrying mutation D222G). Therefore, in our series the presence of D222G and D222N mutations could be underestimated due to the potential higher tropism of these variants for the lower respiratory tract, even if there are recent data showing that D222G and D222N variants can be found in samples from the upper respiratory tract.[40,41] Our underestimation could also be due to the sensitivity of the technique applied for viral typing, that is, population-based DNA sequencing that did not allow to appreciate the composition of viral population at a quasi-species level. A recent work outlined a much higher detection rate of variants at position 222[40] with a highly sensitive ultra-deep pyrosequencing technology that found the presence of G and N variants in >40% of patients compared with <19% with the conventional population-based DNA sequencing. Other studies have confirmed that when ultra-deep pyrosequencing or clonal analysis are used for strain identification, the proportion of upper respiratory samples carrying D222 variants increases to levels similar to those of the lower tract.[5,42] Therefore, discrepancy between the detection of D222G and D222N variants in upper and lower respiratory tract must take into consideration the different sensitivity of analytical methods for viral detection and typing[40,42] and that population-based DNA sequencing is known to detect minor variants only if these represent a proportion higher than 15–20% of the whole population. All the above-mentioned studies showed a general agreement that D222G and D222N variants can be detected only in patients with severe disease.

Other limitations of our study are the lack of data on A (H1N1)pdm09 viral load quantification in the upper and lower respiratory tracts that did not allow information on potential viral transmission and the paucity of information on the time from the start of illness to sampling that is supposed to play an important role as D222G and D222N variants seem to be selected in the individual patient during the time course of the infection.[5,37] Comorbidities including obesity and secondary infection were highly represented in our ICU patients and had a major impact on patient survival, thus complicating the role of D222G and D222N mutants in determining disease severity.

In conclusion, the study of A(H1N1)pdm09 viral correlates of disease severity, including the detection and monitoring of HA variants at the RBS, emphasizes the importance of continuous monitoring of A(H1N1)pdm09 evolution. These data strongly support the epidemiological surveillance of strain circulation for understanding virus–host interaction and adaptation and viral correlates of disease severity.

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Addendum

VG and FDR performed the study design, VG wrote the manuscript. TR conducted HA sequencing analysis from biological samples. FC performed A(H1N1)pdm09 phylogenetic and polymorphisms analyses. FRD, RU, and MVR were in charge of patient ICU assistance and performed clinical analysis of the study. NP and MLS reviewed clinical charts and records. TA, MGM, EB, and GG carried out laboratory assays for A(H1N1)pdm09 identification. AC performed statistical analysis. MVR and GDP supervised the manuscript. All the authors read and approved the final manuscript.

Competing interest

None to declare.

Ethical approval

The study was approved by the institutional Review Board, Comitato Interaziendale A.O.U. San Giovanni Battista di Torino and A.O. C.T.O. Maria Adelaide di Torino (Protocol

Number. 0028847 – 20 April 2011) in agreement with the ethical standards of the Declaration of Helsinki. A(H1N1) pdm09 testing was performed for clinical, diagnostic and therapeutic purposes.

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