

# Molecular surveillance of pandemic influenza A(H1N1) viruses circulating in Italy from May 2009 to February 2010: association between haemagglutinin mutations and clinical outcome

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**Haemagglutinin sequences of pandemic influenza A(H1N1) viruses circulating in Italy were examined, focusing on amino acid changes at position 222 because of its suggested pathogenic relevance. Among 169 patients, the D222G substitution was detected in three of 52 (5.8%) severe cases and in one of 117 (0.9%) mild cases, whereas the D222E mutation was more frequent and evenly distributed in mild (31.6%) and severe cases (38.4%). A cluster of D222E viruses among school children confirms reported human-to-human transmission of viruses mutated at amino acid position 222.**

## Introduction

Influenza viruses are known for their high evolutionary rate. Some mutations result in amino acid substitutions at key locations such as antigenic sites or the receptor-binding site of the haemagglutinin (HA) protein, thus potentially altering virus' antigenicity and/or pathogenicity. In most cases, infection with the 2009 pandemic influenza A(H1N1) virus has caused mild disease, but there have been sporadic cases with severe and fatal outcome. Since the first appearance of this virus in April 2009, certain mutations in the HA protein have been detected in several countries. In particular the substitution of aspartic acid with glycine in position 222 (D222G) in the HA1 subunit has been reported, initially in Norway, in association with fatal cases [1].

The Italian National Influenza Centre (NIC-ISS), in collaboration with the regional laboratory network and under the supervision of the Ministry of Health, conducted a study with the aim of assessing the distribution of the genetic changes in HA and their association

with disease severity, in a convenience sample of Italian subjects with confirmed diagnosis of pandemic influenza A(H1N1).

## Methods

We studied respiratory specimens, i.e. throat swabs and bronchoalveolar lavages (BAL), collected between May 2009 and February 2010 from patients with laboratory-confirmed pandemic influenza. The HA1 gene was amplified by RT-PCR using specific primers, as previously reported [2], and sequenced with the ABI Prism Big Dye Terminator Cycle Ready Reaction kit, version 3.1 (Applied Biosystems). The ClustalW algorithm included in the BioEdit software version 4.0 ([www.mbio.ncsu.edu/BioEdit/bioedit.html](http://www.mbio.ncsu.edu/BioEdit/bioedit.html)) was used to align and analyse the HA1 sequences. Fisher's exact test was used to assess the statistical significance of the associations.

## Results

Overall, 169 samples from individuals affected by pandemic influenza A(H1N1) were analysed. Of those, 98 were male and 71 female; the median age was 25 years (range: 1 to 77 years). In particular, 39% of samples were from subjects under 18 years of age, 58% from people aged 18-64 years and 3% from elderly people (over 65 years), as shown in Table 1. Of the 169 patients, 117 (69%) had mild disease, while 52 (31%) were classified as having severe disease according to guidance criteria of the World Health Organization (WHO) [3]. Among the severe or fatal cases, 58% were adults aged 18-64 years, whereas 35% were children younger than 18 years (Table 1).

The area of the HA gene coding for amino acid position 222 (in the H1 numbering system) was successfully amplified and sequenced in all 169 samples included in this study.

The overall results are reported in Table 2. Notably, the D222G change was detected in four of 169 individuals (2.4%). All these four mutant viruses were found in patients aged 18-64 years. In particular, this amino acid substitution was found in three of 52 severe/fatal cases but only in one of 117 mild cases (5.8% versus 0.9%). Although the odds ratio (OR) tended to be high (OR: 7.3; 95% confidence interval (CI): 0.7–72.1), the difference did not reach statistical significance (Fisher's exact test:  $p=0.08$ ). It must be highlighted that the one mutant pandemic influenza A(H1N1) virus isolated from a mild case, a household contact of one of the three severe cases carrying the D222G mutation [4], showed an additional change in the HA gene (G155E).

Another amino acid substitution in the same position of the HA, D222E, was detected in 57 of the 169 patients (33.7%). This common genetic change was similarly distributed between mild cases (37 of 117, 31.6%) and severe/fatal cases (20 of 52, 38.4%). Interestingly, five of the 37 mutant viruses in mild cases were isolated

from a cluster of seven children with influenza-like symptoms, returning to Italy from a school trip to England.

## Conclusions

We have previously described the only documented transmission event of a D222G mutant pandemic influenza A(H1N1) virus [4]; to the best of our knowledge, this mutation appears to be hardly ever transmitted. Less is known about the human-to-human transmissibility of D222E virus mutants. In the present study, we found that this mutation is much more frequent than the 222G mutation and equally distributed between severe and mild cases. Particularly we report here on a cluster of close contacts carrying the D222E substitution in a group of high school students with mild disease returning from England, suggesting inter-human transmission of D222E pandemic influenza A(H1N1) mutant viruses. However, the clinical significance of the D222E substitution remains uncertain [5].

It is of note that the D222G mutation was detected more commonly among viruses isolated from severe cases, which were about seven times more likely to have this genetic change than those isolated from mild cases; however, the difference did not reach statistical significance, probably due to limited study power. Further analyses are in progress in order to enlarge our data set.

The D222G variants were detected among adults (18-64 age group). Whether this was due to the fact that this age group had the highest number of cases (including severe ones), or to unidentified biological factors, remains undefined. In particular, due to the relatively limited number of cases with the 222G variant, definitive conclusions about possible age differences cannot be drawn.

Studies conducted in other countries, e.g. Norway and Scotland, also found D222G to be more common among severe than mild cases [1,6]. Although these

**TABLE 1**

Age and sex of pandemic influenza A(H1N1)-positive subjects, by clinical outcome, Italy, May 2009–February 2010 (N=169)

	Mild (n=117)	Severe (n=43)	Fatal (n=9)	All cases (N=169)
Age group (years)				
0-17	48 (73%)	15 (23%)	3 (4.6%)	66
18-64	68 (69%)	25 (26%)	5 (5.1%)	98
65+	1 (20%)	3 (60%)	1 (20%)	5
Sex				
Female	50 (70%)	16 (23%)	5 (7.0%)	71
Male	67 (68%)	27 (28%)	4 (4.1%)	98

Percentages refer to all cases in each age and sex group.

**TABLE 2**

Amino acid at position 222 of the HA1 gene of pandemic influenza A(H1N1) viruses, by clinical outcome, Italy, May 2009–February 2010 (N=169)

Amino acid at HA1 position 222	Clinical outcome <sup>a</sup>				
	Mild (n=117)	Severe (n=43)	Fatal (n=9)	Severe plus fatal (n=52)	All cases (N=169)
D222	79 (68%)	23 (53)	6 (67%)	29 (56%)	108 (64%)
E222	37 <sup>b</sup> (32%)	18 (42%)	2 (22%)	20 (38%)	57 (34%)
G222	1 <sup>c</sup> (0.9%)	2 <sup>c</sup> (4.7%)	1 (11%)	3 (5.8%)	4 (2.4%)

<sup>a</sup> Number of cases and the corresponding percentages (in parentheses) are reported within each specific clinical category.

<sup>b</sup> This group includes a cluster of five high school students, returning from England and likely sharing the source of infection.

<sup>c</sup> Two cases in these groups are epidemiologically linked: an index case with severe disease and a household contact with mild symptoms [4].

results indicate that the 222G variant may be more virulent, this association must be interpreted with caution as the same mutation was detected in mild cases, and mixed 222D and 222G virus populations were found in original samples and isolates from patients with severe disease [7]. Experiments in ferrets do not appear to support an association of the 222G substitution with virulence [5]. Moreover, the data could be biased by more frequent diagnostic sampling from the lower respiratory tract of severe cases.

*In vitro* studies show conflicting results. Studies conducted in the United States found the 222G mutation only in isolated viruses but not in the original clinical samples [5]. On the other hand, preliminary results from *in vitro* studies suggest that D222G substitution might enhance binding of HA to alpha2-3 sialic acid (avian-like) cell receptors, thus increasing the virus ability to infect human lung cells [5,8]. Moreover, studies from Liu *et al.* [9] and Chutinimitkul *et al.* [10] suggest an increased receptor affinity of the 222G variant for ciliated bronchial epithelial cells, which may explain enhanced disease in humans. Increased binding to macrophages and pneumocytes of the respiratory tract may indeed have an impact on disease severity since those cells are major producers of inflammatory cytokines upon viral antigen stimulation [11].

Another amino acid substitution (D222N) has been observed in a number of recent studies [1,5,7], however, no cases with this mutation have been identified in the samples analysed here. Finally, our data suggest that the D222G substitution is overall rather infrequent, even among severe cases. However, we confirm that it occurs with a higher frequency in severe cases. Whether this association is indicative of higher virulence or is the consequence of receptor-specific adaptive mutation needs to be further investigated.

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## References

1. Kilander A, Rykkvin R, Dudman SG, Hungnes O. Observed association between the HA1 mutation D222G in the 2009 pandemic influenza A(H1N1) virus and severe clinical outcome, Norway 2009-2010. *Euro Surveill.* 2010;15(9):pii=19498. Available from: <http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19498>

2. Surveillance Group for New Influenza A(H1N1) Virus Investigation in Italy. Virological surveillance of human cases of influenza A(H1N1)v virus in Italy: preliminary results. *Euro Surveill.* 2009;14(24):pii=19247. Available from: <http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19247>
3. Clinical management of human infection with pandemic (H1N1) 2009: revised guidance. Geneva: World Health Organization; November 2009. Available from: [http://www.who.int/csr/resources/publications/swineflu/clinical\\_management\\_h1n1.pdf](http://www.who.int/csr/resources/publications/swineflu/clinical_management_h1n1.pdf)
4. Puzelli S, Facchini M, Spagnolo D, De Marco MA, Calzoletti L, Zanetti A, et al. Transmission of the pandemic H1N1 2009 influenza virus presenting HA1 gene mutation at amino acid position 222 (D222G). *Emerg Infect Dis.* 2010;16(5):863-5.
5. Preliminary review of D222G amino acid substitution in the haemagglutinin of pandemic influenza A (H1N1) 2009 viruses. *Wkly Epidemiol Rec.* 2010;85(4):21-2.
6. Miller RS, MacLean AR, Gunson RN, Carman WF. Occurrence of haemagglutinin mutation D222G in pandemic influenza A(H1N1) infected patients in the West of Scotland, United Kingdom, 2009-10. *Euro Surveill.* 2010;15(16):pii=19546. Available from: <http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19546>
7. Mak GC, Au KW, Tai LS, Chuang KC, Cheng KC, Shiu TC, et al. Association of D222G substitution in haemagglutinin of 2009 pandemic influenza A (H1N1) with severe disease. *Euro Surveill.* 2010;15(14):pii=19534. Available from: <http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19534>
8. Stevens J, Blixt O, Glaser L, Taubenberger JK, Palese P, Paulson JC, et al. Glycan microarray analysis of the hemagglutinins from modern and pandemic influenza viruses reveals different receptor specificities. *J Mol Biol.* 2006;355(5):1143-55.
9. Liu Y, Childs RA, Matrosovich T, Wharton S, Palma AS, Chai W, et al. Altered receptor specificity and cell tropism of D222G haemagglutinin mutants from fatal cases of pandemic A(H1N1) 2009 influenza. *J Virol.* 2010 Sep 8. [Epub ahead of print].
10. Chutinimitkul S, Herfst S, Steel J, Lowen AC, Ye J, van Riel D, et al. Virulence-associated substitution D222G in hemagglutinin of 2009 pandemic influenza A(H1N1) virus affects receptor binding. *J Virol.* 2010 Sep 15. [Epub ahead of print].
11. Cheung CY, Poon LL, Lau AS, Luk W, Lau YL, Shortridge KF, et al. Induction of proinflammatory cytokines in human macrophages by influenza A (H5N1) viruses: a mechanism for the unusual severity of human disease? *Lancet.* 2002;360(9348):183.