Med Microbiol Immunol (2013) 202:277–284
DOI 10.1007/s00430-013-0291-4

ORIGINAL INVESTIGATION

Molecular and serological investigations of the Influenza A(H1N1) 2009 pandemic virus in Turkey

Meral Akcay Ciblak · Mustafa Hasoksuz · Melis Kanturvardar · Serkan Asar · Selim Badur

Received: 26 November 2012 / Accepted: 25 February 2013 / Published online: 13 March 2013

Abstract Intense research has been conducted on influenza A(H1N1)pdm09 virus to determine the virulence markers. Limited information on characteristics of pandemic virus has become available in Turkey since the pandemic. In this first report from Turkey, we investigated the molecular markers that have been associated with increased virulence and oseltamivir resistance. We also conducted serological studies in people after infection, vaccination, exposure, and no-exposure controls to determine the level of protection against the pandemic H1N1 influenza virus. Thirteen rRT-PCR positive samples were analyzed for presence of mutations that have been associated with host range, virulence, and antiviral resistance: substitution D222G in the HA, E627K in the PB2, and H275Y in the neuraminidase (NA). In addition, 135 serum samples from vaccinated, recovered, asymptomatic contacts, and control individuals were tested using hemagglutination inhibition (HI) assay. D222G was detected in nasal samples from two severe cases. No specified mutations in the PB2 and NA were identified. Additional substitutions, I216V, V321I, E374K, S203T in HA, V655I in PB2, and I163V in NA, were detected. HI testing from vaccinated individuals, recovered patients, asymptomatic contacts, and control individuals showed that 97.9, 99.7, 88.2, and 44.2 % had HI titers $\geq$40, respectively. Molecular markers promoting influenza A(H1N1)pdm09 to become a pandemic virus are still under investigation. Serological results confirm that younger, un-exposed individuals are at increased risk of pandemic virus infections. Influenza A(H1N1)pdm09 viruses are still in circulation around the globe. Therefore, these viruses need to be monitored closely for development of new markers including antiviral resistance mutations.

Keywords Pandemic · Vaccine · H1N1 · Serology · D222G · Influenza

M. Akcay Ciblak (✉) · M. Kanturvardar · S. Asar · S. Badur
Department of Microbiology and Clinical Microbiology, National Influenza Reference Laboratory, Istanbul Faculty of Medicine, Istanbul University, 34390 Capa, Istanbul, Turkey
e-mail: ciblakm@yahoo.com

M. Hasoksuz
Department of Virology, Faculty of Veterinary Medicine, Istanbul University, Istanbul, Turkey

Introduction

Influenza A viruses are the major pathogens which cause seasonal influenza epidemics annually and occasional pandemics arising from novel subtypes. Soon after its emergence in April 2009, the novel influenza A(H1N1) virus of swine origin spreads globally in a matter of weeks settling itself as the first pandemic virus of the twenty-first century [1]. The majority of human cases reported during the early phase of the pandemic ranged from mild to moderate in severity [2, 3]. However, severe and fatal cases were reported, predominantly in relatively young individuals and those with underlying conditions [4–6]. Besides underlying medical conditions, molecular virulence markers of the pandemic virus may have determined the outcome of the infection.

The first potential virulence marker associated with pandemic H1N1 2009 virus was reported from Norway in November 2009 where in 18 % of samples from patients with severe disease outcome, the D222G mutation in HA

gene was identified [7, 8]. This mutation apparently increases the binding affinity of HA to SAα 2,3 Gal receptors located abundantly in the upper respiratory tract of birds and the lower respiratory tract of mammals, leading to more severe disease [9–11]. Subsequent reports have strengthened the association of D222G with severe outcome [7, 12]. However, mild cases with this substitution have also been reported [13–15]. In addition to the HA gene segment, the PB2 gene segment of influenza A viruses has been implicated that in host-range restriction, a single amino acid at position 627 has been reported to regulate the activity of PB2 gene in a species-specific manner [16, 17]. An E627K substitution is required for an avian influenza virus to cross species barrier to mammals, whereas E627 is associated with reduced replication efficiency and pathogenicity in mammals [18, 19]. However, it is of importance to note that a significant proportion of H5N1 viruses isolated from humans and all of the 2009 pandemic viruses analyzed contained E627 [19]. Recently, two substitutions S590 and R591, namely SR polymorphism, have been reported in PB2 that are indicated in escape from host-range restriction caused by E627 [20].

Lack of pre-existing immunity in human population is a major factor determining the impact and severity of a pandemic. Some reports soon after the start of the pandemic revealed a degree of protection against the novel virus in people over 18 years of age, while seasonal influenza vaccination did not offer protection against the novel virus which was an indication that younger people were at elevated risk of the pandemic influenza and vaccines were needed for the novel virus [21].

In Turkey, the index pandemic case was detected in May 2009 [3]. Influenza A(H1N1)pdm09 viruses continued to be detected until the first week of January, 2010. During the pandemic, a total of 655 influenza deaths were reported nationwide. Testing a total number of 4,663 nasal samples by one of the two National Influenza Reference Laboratories by rRT–PCR resulted in 1,545 identifications of the pandemic influenza A(H1N1)pdm09 virus. A total of 13 virus isolates from mild, severely ill, and fatal cases were subsequently further analyzed. In addition, sera from vaccinated individuals, recovered patients, asymptomatic contacts, and control individuals in Turkey were tested in a hemagglutination inhibition (HI) assay, to quantify specific antibodies to pandemic influenza A virus (H1N1) 2009.

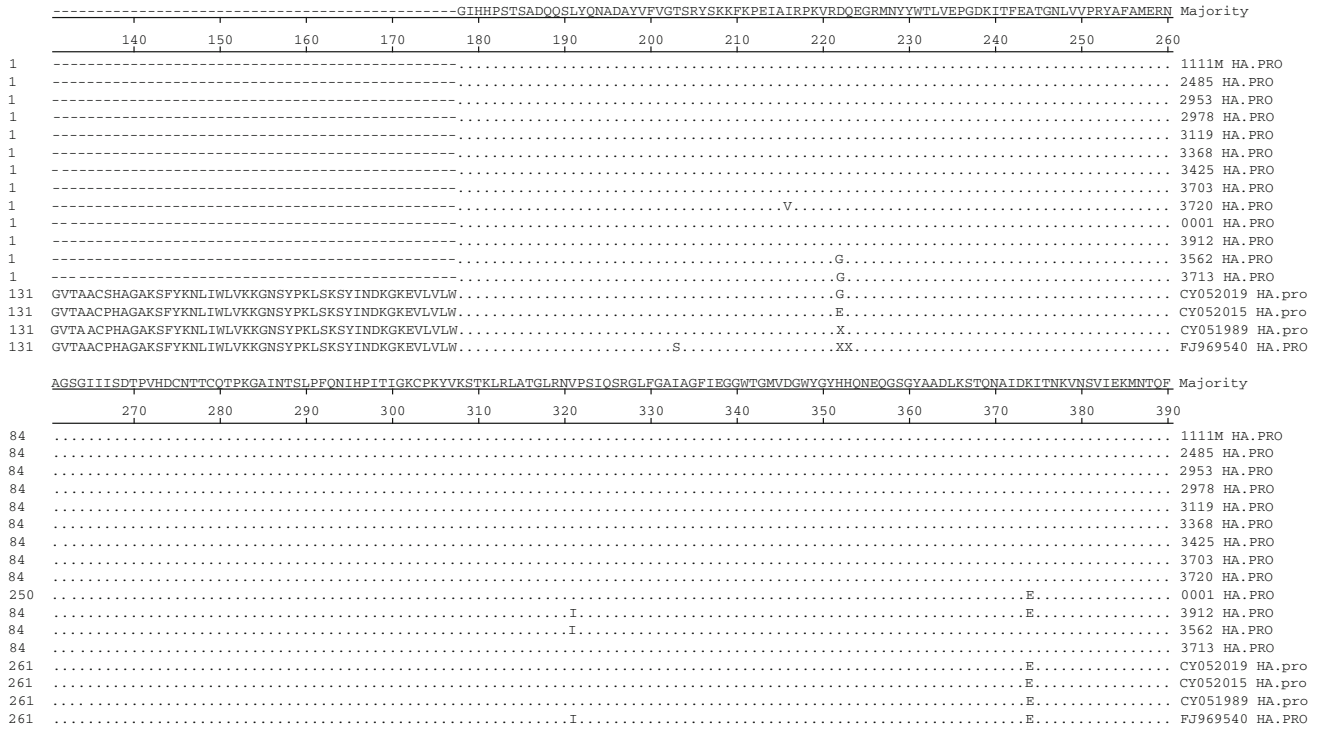

Fig. 1 Sequence alignment of HA gene by ClustalW program. HA gene sequences were aligned with HA sequences from NCBI GenBank with accession numbers CY05219, CY052015, CY051989, and FJ969540. Out of 13 samples, D222G substitution was detected in two samples from fatal cases (sample numbers 3562 and 3713). In addition, S203T substitution was detected in all 13 cases, E374K in 11 cases (shown as majority), I216V in a sample from a mild case (sample number 3720), V321I in a fatal and a mild case (sample numbers 3912 and 3562)

Materials and methods

Sample selection for mutational analysis

A total of 25 samples from mild, severe, and fatal cases that tested positive for pandemic H1N1 virus by the rRT-PCR provided by CDC, Atlanta, USA, were co-cultured in MDCK cells [22]. Virus was detected only in 6 samples from fatal, 3 samples from severely ill, and in 4 samples from mild cases. Therefore, a total of 13 samples were included in the study. Disease severity was defined based on the standard patient information form sent to the laboratory along with the samples and telecommunication with local health authorities who were the co-ordinators of sample flow from hospitals to the laboratory during the pandemic. Mild cases were those patients who developed influenza-like illness symptoms but neither developed pneumonia nor admitted to the hospital. Severe cases were patients admitted to hospital due to need for mechanical ventilation and severe disease-like pneumonia. All severely ill patients were administered oseltamivir up on admission to the hospital. Average time elapsed between the onset of symptoms and antiviral administration was 5 days, the earliest being 3 days.

Informed consent from all patients providing serum samples was obtained. Local ethical committee approved the study. Nasal swabs were sent to the laboratory within the context of National Surveillance.

Partial sequencing for mutational analysis

RNA extraction was performed as instructed by the manufacturer (Invitrogen, total RNA extraction kit, USA). Two pairs of primers were used for partial amplification of HA, neuraminidase (NA) segments, and one pair was used for partial amplification of the PB2 segment. For HA, HA1 forward: 5′ GGG CAT TCA CCA TCC ATC TAC T 3′ and HA1 reverse: 5′ TCG GCT GCA TAT CCT GAC C 3′ and HA2 forward: 5′ AGC CGA CCT GAA GAG CA 3′ and HA2 reverse: 5′ CCC CAG GGA GAC TAC CAG 3′ primer pairs were used to amplify 419–1626 region that produced a 1,226-bp product; for NA, NA1 forward: 5′ GGA CAG TCA GTG GTT TCC GT 3′ and NA1 reverse: 5′ CAT TAG GGC GTG GAT TGT CT 3′ and NA2 forward: 5′ TCC ACG CCC TAA TGA TAA GAC A 3′ and NA2 reverse: 5′ GGC CAA GAC CAA CCC ACA GT 3′ primer pairs were use to amplify 229–1373 region that produced a 1145-bp product,

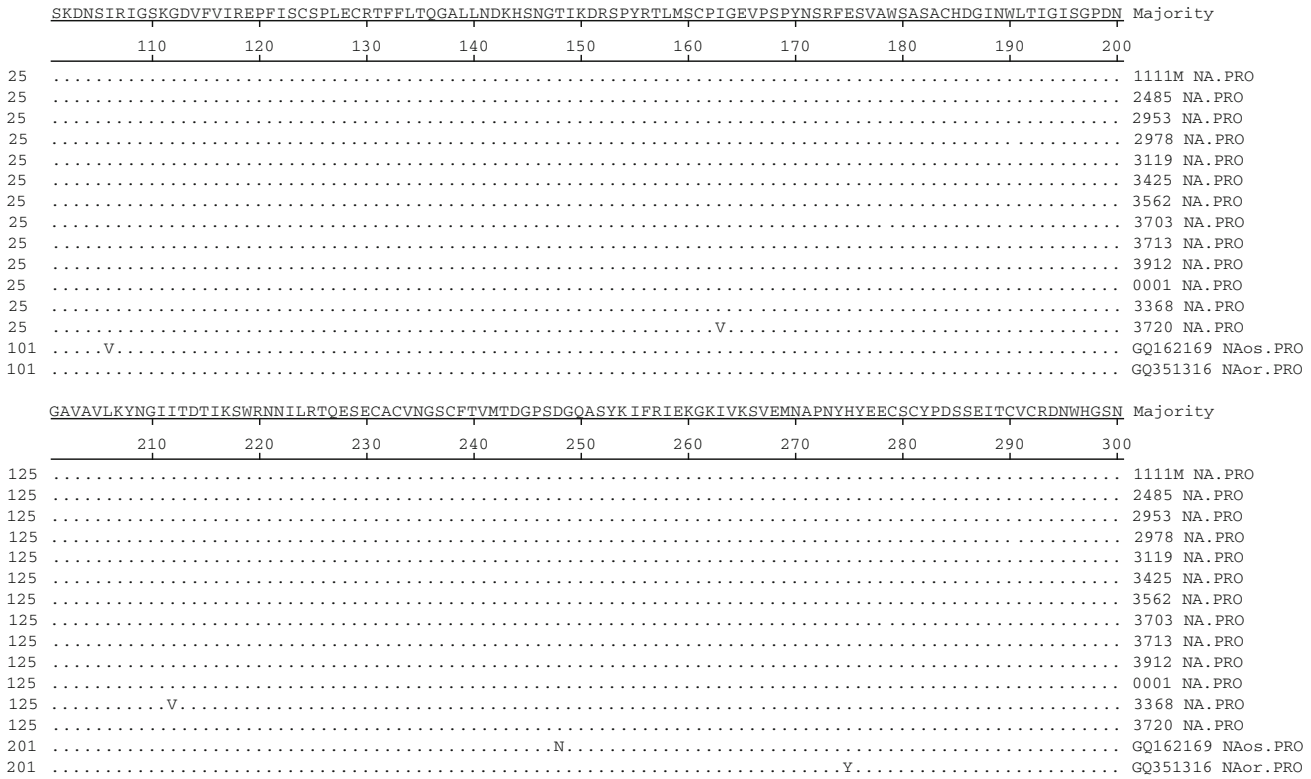

Fig. 2 Sequence alignment of NA gene by ClustalW program. NA gene sequences were aligned with GQ16216 and GQ351316 (with H275Y substitution) sequences obtained from GenBank. H275Y substitution was not detected in any of the 13 samples studied. Only sample (number 3720) from a mild case had I163V substitution in its NA gene

and for PB2, PB2 forward: 5′ GTG AAA CGC AAG GAA CTG AGA AG 3′ and PB2 reverse: 5′ TTG CCC AAA ATG AGA AAT CCT 3′ primer pair was used to amplify 1,571–2,111 region that produced a 541-bp product. PCR products were sequenced by REFGEN Gene Research and Biotechnology, Ankara, Turkey. Resulting gene sequences were aligned with HA, NA, and PB2 sequences from NCBI GenBank with accession numbers CY05219, CY052015, CY051989, FJ969540 for HA, GQ162169, GQ351316 for NA, and AY651719, HM014331 for PB2 gene by ClustalW program (DNA Star, Lasergene 9.1, USA).

Serological analysis

Sera from a total of 134 patients were tested by HI test using HI kit for serological testing provided by CDC, Atlanta, USA. Thirteen samples belonged to the patients who recovered from the laboratory-confirmed H1N1 infection, 51 were from patients who had received the pandemic vaccine, 43 were from close contacts of the patients with laboratory-confirmed H1N1, and 27 were from patients who visited the Istanbul University hospital for other causes before the emergence of the pandemic virus. These 27 samples were used as controls. Sera from the vaccinated persons were collected about 30 days postvaccination. None of the vaccinated subjects had developed influenza-like illness symptoms. Close contacts consisted of either parents taking care of their children, age range 30–39, siblings, age range 7–12, and intensive care unit care givers, age range 20–40. Only one of the close contacts had taken antiviral for prophylaxis. None of the close contacts had developed any influenza-like illness symptoms. Age range for controls was 21–68. All samples were studied in triplicate, and the HI testing was conducted as it was described in the protocol included within the kit.

Results

Sequencing

In the 13 samples studied by sequence analysis, the D222G substitution in HA was found in two of six samples from fatal cases (Fig. 1). Additional amino acid substitutions were found in the HA segment of the viruses: S203T in all 13 cases, E374K in 11 cases, I216V in a sample from a mild case, V321I in a fatal and a mild case. E627K in PB2 segment and H275Y in NA segment were not detected in any of the 13 samples studied (Figs. 2, 3). One sample had I163V substitution in its NA gene. All viruses had S590 and R591 polymorphism in their PB2 gene. All substitutions detected are summarized in Table 1.

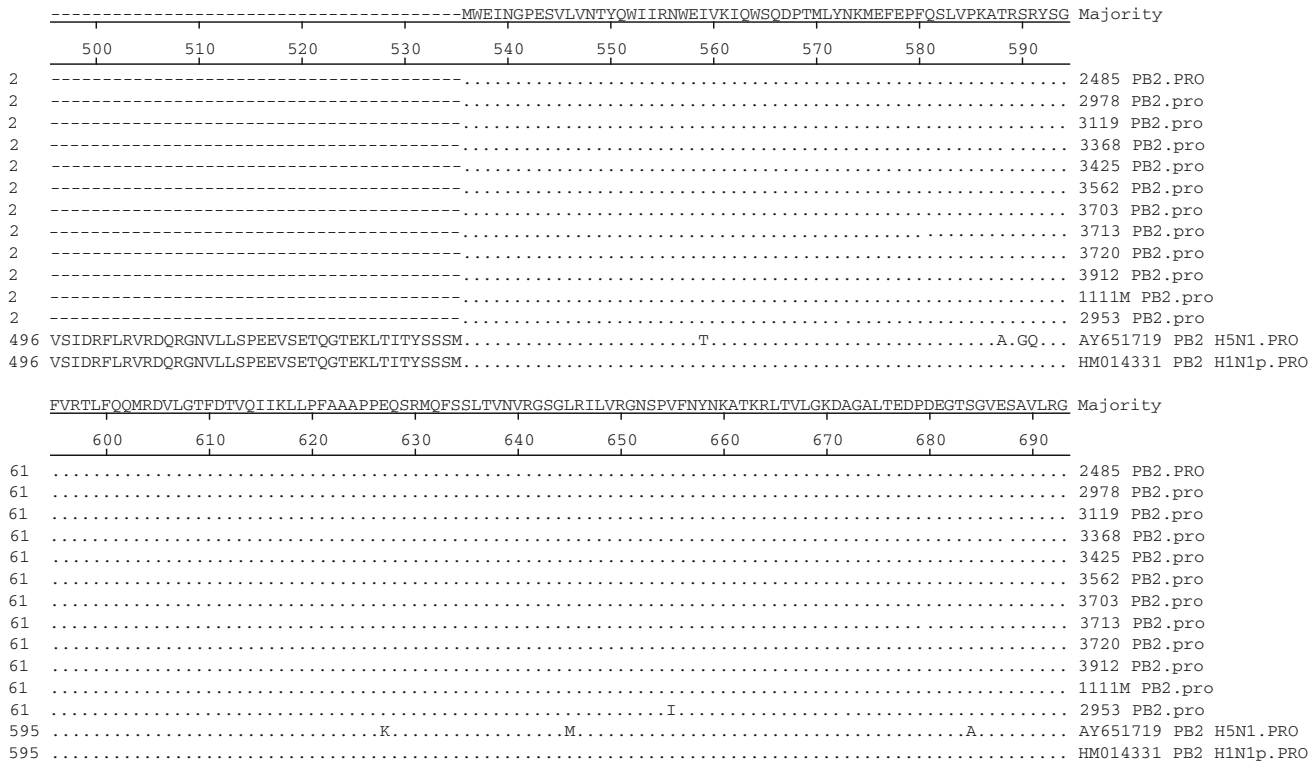

Fig. 3 Sequence alignment of PB2 gene by ClustalW program. PB2 gene sequences were aligned with AY651719 and HM014331 sequences obtained from GenBank. E627K was not detected in any samples studied. PB2 gene from all samples bared S590 and R591 (SR polymorphism)

Table 1 Partial sequencing results of selected samples and characteristics of the subjects

Isolates	Amino acid substitutions detected									Patients		
	HA D222G	HA S203T	HA E374K	HA V321I	HA I216V	NA H275Y	NA I163V	PB2 E627K	PB2 V655I	Age (year)	Sex	Clinical outcome
A/Istanbul/2953/2009/(H1N1)[a]	−	+	+	−	−	−	−	−	+	53	F	Fatal cases
A/Istanbul/3119/2009/(H1N1)	−	+	+	−	−	−	−	−	−	7	M	
A/Istanbul/3368/2009/(H1N1)	−	+	+	−	−	−	−	−	−	11 days	M	
A/Istanbul/3562/2009/(H1N1)[b]	+	+	+	+	−	−	−	−	−	26	F	
A/Istanbul/3703/2009/(H1N1)	−	+	+	−	−	−	−	−	−	28	F	
A/Istanbul/3713/2009/(H1N1)[c]	+	+	+	−	−	−	−	−	−	24	F	
A/Istanbul/1111 M/2009/(H1N1)	−	+	+	−	−	−	−	−	−	39	F	Severe cases
A/Istanbul/3425/2009/(H1N1)	−	+	+	−	−	−	−	−	−	90 days	M	
A/Istanbul/0001/2009/(H1N1)[d]	−	+	−	−	−	−	−	−	−	54	M	
A/Istanbul/2978/2009/(H1N1)	−	+	+	−	−	−	−	−	−	42	M	Mild cases
A/Antalya/3912/2009/(H1N1)[e]	−	+	−	+	−	−	−	−	−	10	F	
A/Manisa/2485/2009/(H1N1)	−	+	+	−	−	−	−	−	−	28	M	
A/Istanbul/3720/2009/(H1N1)[f,g]	−	+	+	−	+	−	+	−	−	36	M	
Total (13)	2	13	11	2	1	0	1	0	1			

Accession numbers for isolates submitted to GenBank: [a]KC514523, [b]KC514518, [c]KC514519, [d]KC514517, [e]KC514521, [f]KC514520, [g]KC514522

Serology

Out of 51 vaccinated subjects, 50 (97.9 %) had developed serum HI titers ≥40, with the majority showing HI titers >80 (Table 2). Almost all 13 subjects (99.7 %) who recovered from infection had developed serum HI titers ≥40. Of the 43 subjects, who were not vaccinated and had been in close contact with infected persons, 38 (88.2 %) had HI titers ≥40. Finally, 13 (44.4 %) out of 27 control subjects had antibody titers ≥40 (Table 2).

Discussion

This is the first report of data collected at the National Influenza Reference Laboratory in Istanbul, Turkey, on both molecular and serological properties of the 2009 pandemic influenza A(H1N1) virus that circulated in the country between 2009 and 2010. In Turkey, sentinel influenza surveillance was established in 2005 as part of a pandemic preparedness plan in which antiviral stockpiling and vaccine procurement strategy were well described.

Molecular characterization of the viruses isolated from 6 fatal influenza cases revealed the HA D222G substitution in 2 out of 6 fatal cases. This mutation was also found by others in viruses from critically ill patients [7, 8, 12]. The 2 deceased patients in the current study were epidemiologically unrelated and were otherwise healthy young females, aged 26 and 24, with no recorded co-morbidities. Both patients received oseltamivir treatment in the hospital. However, the oseltamivir resistance mutation H275Y in NA was not detected any of the 13 viruses characterized.

In addition to D222G, the current study detected S203T, E374K, V321I, and I216V in HA of analyzed viruses. In order to compare the prevalence of the additional mutations found in current study with the prevalence reported from other countries in the European region, 500 HA sequences of A(H1N1) viruses reported to NCBI GenBank database between 2009 and 2010 were analyzed.

Table 2 HI results of four different subject categories

Subject groups	HI Titers n/total (%)						HI Titer ≥40 n/total (%)
	40	40	80	160	320	>320	
Vaccinated (n:51)	1/51 (1.96)	21/51 (41.17)	12/51 (23.52)	6/51 (11.76)	9/51 (17.64)	2/51 (3.92)	50/51 (98.03)
H1N1 infected (n:13)	0/13 (0)	3/13 (23.07)	3/13 (23.07)	3/13 (23.07)	4/13 (30.7)	0/13 (0)	13/13 (100)
Close contacts of H1N1 infected (n:43)	5/43 (11.62)	8/43 (18.60)	7/43 (16.27)	12/43 (27.90)	7/43 (16.27)	4/43 (9.30)	38/43 (88.37)
Controls (n:27)	15/27 (55.5)	9/27 (33.3)	2/27 (7.40)	–	1/27 (3.70)	–	12/27 (44.44)

S203T is located in the antigenic epitope Ca1 and is positioned close to the receptor-binding pocket [23]. Studies with postvaccination sera showed reduced recognition of viruses that bared point mutations in antigenic sites Sa (N125D) and Ca1 (S203T) [23]. S203T substitution was detected in all viruses in the current study and 446 (89 %) out of 500 selected GenBank sequences. Another known antigenic site in HA gene is position 374, and E374K substitution has been reported in vaccine breakthrough infections [23, 24]. Eleven out of 13 (84.6 %) samples in current study and 415 out of 500 (83 %) HA sequences reported to NCBI GenBank from European region bared K374. Therefore, it would be interesting to know the extent to which S203T and E374K contributed to decreased efficacy, if any, of pandemic vaccine in Turkey and Europe.

Recombinant viruses with an HA I216K mutation increased human receptor affinity and related efficient airborne viral transmission in ferrets [25]. Whether mutation I216V has similar effect needs to be investigated. I216V was present in 1 (7.6 %) out of 13 samples in current study and in 3 (0.6 %) out of 500 sequences reported to GenBank from European region. Finally, V321I was present in 2 out of 13 (15 %) samples in current study and in 93 (18.6 %) out of 500 sequences in the selected GenBank sequences. The significance of this mutation is not known.

In PB2, all viruses had an E627 and one virus had a V655I mutation in current study. E627 was found to be a signature of avian-adapted influenza viruses, as most human influenza viruses carry K627 in their PB2 gene [17, 19]. The E627K substitution was shown to increase replication efficiency and pathogenicity in mammalian systems [17, 18]. Interestingly, a significant proportion of H5N1 viruses isolated from humans and all of the 2009 pandemic viruses analyzed contained E627 [19, 20]. It is therefore tempting to speculate why the pandemic H1N1 virus, like H5N1, carries the E627. Mehle et al. reported that substitutions at positions, G590S and Q591R in PB2 of H1N1 viruses could compensate for host-range restriction due to E627 [20]. In the current study, all 13 viruses bared the S590 and R591 along with E627 in their PB2 gene. These amino acids may be necessary for efficient virus replication in humans. Alternatively, E627K mutation might evolve in the next few years in the new H1N1 as a human adaptation. V655I substitution was not found in any of the 591 sequences reported to GenBank from European region indicating its sporadic nature.

Reports published soon after the emergence of novel A(H1N1)pdm09 virus suggested that this virus was able to spread due to lack of pre-existing immunity in the human population despite being of an H1 subtype [26]. Subsequent reports revealed some degree of protection against the novel virus in people over 18 years of age [21]. However, no protection was reported in younger children who received seasonal influenza vaccination starting in 2005. This suggested that seasonal influenza vaccination was not protective against the pandemic virus [21]. We investigated the seroprotection state of people in different categories of influenza virus exposure. In our study, 98.03 % vaccinated subjects, 100 % of the subjects recovered from A(H1N1)pdm09 infection, 88.37 % of the subjects who were in close contact with infected persons but did not develop influenza-like illness (ILI), and 44.4 % of the controls had HI titers of ≥40. The cutoff level for seroprotection is usually set at an HI titer of 40 for influenza viruses [27]. Considering the age range, 7–40, and the level of seroprotection of the contact group, the protection could come from an asymptomatic infection rather than from a past infection. This group of asymptomatic people might be important in spreading the virus. These data may also indicate that A(H1N1)pdm09 was more widespread in Turkey than suggested by laboratory confirmation of symptomatic cases [3, current study]. Sera used as controls were collected from patients reported to the hospital for other health problems long before the emergence of novel virus in Mexico. Majority of control patients with HI titers of ≥40 were over 50 years of age suggesting possible past infection with a virus reminiscent of 1918 H1N1 virus. The protective antibody levels of vaccinated and recovered

patients were similar indicating efficacy of the monovalent vaccine.

Influenza A(H1N1)pdm09 viruses currently circulate around the globe and molecular markers promoting this virus to become a pandemic virus are still under investigation. Therefore, any report on characteristics of the novel virus might be of importance. These viruses need to be monitored closely for development of new markers including antiviral resistance mutations. Investigation on influenza A(H1N1)pdm09 viruses isolated in 2011 epidemic season is still ongoing in our laboratory.

Established influenza virus surveillance plays an important role in pandemic preparedness. In Turkey, sentinel influenza surveillance was established in 2005, and there are two National Influenza Reference Laboratories with well-constructed infrastructure conducting the surveillance. Turkey also had developed pandemic preparedness plan in 2005 where antiviral stockpiling and vaccine procurement strategy were well described. Having a well-established surveillance and pandemic preparedness plan helped in early detection of the pandemic virus and aided the healthcare officials in taking proper measures for mitigating the pandemic.

Acknowledgments Authors thank Centers for Disease Control and Prevention, Atlanta, GA, USA, for providing HI kit for serological studies.

References

1. World Health Organization (2009) World now at the start of 2009 influenza pandemic http://who.int/mediacentre/news/statements/2009/h1n1_pandemic_phase6_20090611/en/index.html. Accessed 15 October 2012
2. World Health Organization (2009) Clinical features severe cases of pandemic influenza http://www.who.int/csr/disease/swineflu/notes/h1n1_clinical_features_20091016/en/index.html. Accessed 15 October 2012
3. Ciblak MA, Albayrak N, Odabas Y, BasakAltas A, Kanturvardar M, Hasoksuz M et al (2009) Cases of influenza A(H1N1)v reported in Turkey, May–July 2009. Eur Surveill 14(32):19304
4. Chowell G, Bertozzi SM, Colchero MA, Lopez-Gatell H, Alpuche-Aranda C, Hernandez M et al (2009) Severe respiratory disease concurrent with the circulation of H1N1 influenza. N Engl J Med 361:674–679
5. Echevarría-Zuno S, Mejía-Aranguré JM, Mar-Obeso AJ, Grajales-Muñiz C, Robles-Pérez E, González-León M et al (2009) Infection and death from influenza A H1N1 virus in Mexico: a retrospective analysis. Lancet 374:2072–2079
6. Pebody RG, McLean E, Zhao H, Cleary P, Bracebridge S, Foster K et al (2010) Pandemic Influenza A (H1N1) 2009 and mortality in the United Kingdom: risk factors for death, April 2009 to March 2010. Eur Surveill 15(20):19571
7. World Health Organization. Public health significance of virus mutation detected in Norway (November 2009) http://www.who.int/csr/disease/swineflu/notes/briefing_20091120/en/ Accessed 18 October 2012
8. Kilander A, Rykkvin R, Dudman SG, Hungnes O (2009) Observed association between the HA1 mutation D222G in the 2009 pandemic influenza A(H1N1) virus and severe clinical outcome, Norway 2009–2010. Eur Surveill 15(9):19498
9. Chutinimitkul S, Herfst S, Steel J, Lowen AC, Ye J, van Riel D et al (2010) Virulence-associated substitution D222G in the hemagglutinin of 2009 pandemic influenza A (H1N1) virus affects receptor binding. J Virol 84:11802–11813
10. Liu Y, Childs RA, Matrosovich T, Wharton S, Palma AS, Chai W et al (2010) Altered receptor specificity and cell tropism of D222G hemagglutinin mutants isolated from fatal cases of pandemic A(H1N1) 2009 influenza virus. J Virol 84:12069–12074
11. Belser JA, Jayaraman A, Raman R, Pappas C, Zeng H, Cox NJ et al (2011) Effect of D222G mutation in the hemagglutinin protein on receptor binding, pathogenesis and transmissibility of the 2009 pandemic H1N1 influenza virus. PLoS One 6(9):e25091
12. Miller RR, MacLean AR, Gunson RN, Carman WF (2010) Occurrence of haemagglutinin mutation D222G in pandemic influenza A (H1N1) infected patients in the West of Scotland, United Kingdom, 2009–10. Eur Surveill 5(16):19546
13. Melidou A, Gioula G, Exindari M, Chatzidimitriou D, Diza E, Malisiovas N (2010) Molecular and phylogenetic analysis of the haemagglutinin gene of pandemic influenza H1N1 2009 viruses associated with severe and fatal infections. Virus Res 151:192–199
14. Puzelli S, Facchini M, De Marco MA, Palmieri A, Spagnolo D, Boros S et al (2010) Molecular surveillance of pandemic influenza A(H1N1) viruses circulating in Italy from May 2009 to February 2010: association between haemagglutinin mutations and clinical outcome. Eur Surveill 15(43):19696
15. Wang B, Dwyer DE, Soedjono M, Shi H, Matlho K, Ratnamohan M et al (2011) Evidence of the circulation of pandemic influenza (H1N1) 2009 with D222D/G/N/S hemagglutinin polymorphisms during the first wave of the 2009 influenza pandemic. J Clin Virol 52:304–306
16. Almond JW (1977) A single gene determines the host range of influenza virus. Nature 270:617–618
17. Subbarao EK, London W, Murphy BR (1993) A single amino acid in the PB2 gene of influenza A virus is a determinant of host range. J Virol 67:1761–1764
18. Shinya K, Hamm S, Hatta M, Ito H, Ito T, Kawaoka Y (2004) PB2 amino acid at position 627 affects replicative efficiency, but not cell tropism, of Hong Kong H5N1 influenza A viruses in mice. Virology 320:258–266
19. Chen GW, Chang SC, Mok CK, Lo YL, Kung YN, Huang JH et al (2006) Genomic signatures of human versus avian influenza A viruses. Emerg Infect Dis 12:1353–1360
20. Mehle A, Doudna JA (2009) Adaptive strategies of the influenza virus polymerase for replication in humans. Proc Natl Acad Sci U S A 106:21312–21316
21. Centers for Disease Control and Prevention (2009) Serum cross-reactive antibody response to a novel influenza A (H1N1) virus after vaccination with seasonal influenza vaccine. Morb Mortal Wkly Rep 58:521–524
22. World Health Organization (2009) CDC protocol of realtime RTPCR for swine influenza A(H1N1). http://www.who.int/csr/resources/publications/swineflu/CDCrealtimeRTPCRprotocol_20090428.pdf. Accessed September 2012
23. Strengell M, Ikonen N, Ziegler T, Julkunen I (2011) Minor changes in the hemagglutinin of influenza A(H1N1)2009 virus alter its antigenic properties. PLoS One 6(10):e25848
24. Barr IG, Cui L, Komadina N, Lee RT, Lin RT, Deng Y et al (2010) A new pandemic influenza A (H1N1) genetic variant predominated in the winter 2010 influenza season in Australia New Zealand and Singapore. Eur Surveill 15(42):19692

25. Jayaraman A, Pappas C, Raman R, Belser JA, Viswanathan K, Shriver Z et al (2011) A single base-pair change in 2009 H1N1 hemagglutinin increases human receptor affinity and leads to efficient airborne viral transmission in ferrets. PLoS One 6(3):e17616
26. Capua I, Kajaste-Rudnitski A, Bertoli E, Vicenzi E (2009) Pandemic vaccine preparedness–have we left something behind? PLoS Pathog 5(6):e1000482
27. Wood JM (1998) Standardization of inactivated influenza vaccines. In: Nicholson KG, Webster RG, Hay AJ (eds) Textbook of influenza. Blackwell, Oxford, p 333