Original Article

Epidemiological, Demographic, and Molecular Characteristics of Laboratory-Confirmed Pandemic Influenza A (H1N1) Virus Infection in Turkey, May 15–November 30, 2009

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SUMMARY: A total of 19,973 clinical specimens obtained from suspected cases of pandemic influenza A virus infection were analyzed by real-time reverse transcription-polymerase chain reaction. Mutations in hemagglutinin (HA) gene and alteration at position 275 in neuraminidase (NA) gene of the randomly selected 29 isolates were detected by sequencing analysis. The virus RNA was detected in 47.3% of the clinical specimens. The pandemic flu cases increased from the 42nd week and peaked in the 46th week of 2009. This intensity continued to the end of the study period. Pandemic flu mainly affected children in the 5–14 year age group, without any gender predominance. The analyzed strains had ≥98.9% homology with vaccine strains and with each other. More than 37% of the isolates had mutation at position D222E/N on HA gene. There was no isolate harbored mutation at the position H275Y of the NA gene, indicating that the virus isolates currently circulating in Turkey are sensitive to oseltamivir.

INTRODUCTION

Pandemic influenza A (H1N1) virus is a member of the orthomyxoviruses, and has an envelope and a negative-sense segmental RNA genome. Because of the segmental structure of RNA, mutation and reassortment of the gene segment have frequently occurred among different animal and human strains of the influenza A virus. Therefore, several pandemic strains of the influenza A virus such as Spanish influenza H1N1 in 1918–1920, Asian influenza H2N2 in 1957–1958, and Hong Kong influenza H3N2 in 1968–1969, have occurred at intervals of 10 to 50 years since the beginning of the 19th century (1). A new pandemic influenza A (H1N1) virus was described on April 15 and April 17, 2009 in specimens obtained from two epidemiologically unrelated patients in the United States (2). This virus contains gene segments from human, swine, and avian strains (2,3). Two of the polymerase components (PB2 and PA genes) originate from an avian virus, one (PB1) from the human H3N2, hemagglutinin (HA), nucleoprotein (NP), and nonstructural (NS) genes originate from the classical swine virus, and neuraminidase (NA) and matrix/membrane proteins (M) genes originate from Eurasian swine virus (4).

Pandemic influenza A (H1N1) virus infection has continuously increased worldwide. Up to the 48th week of 2009, more than 207 countries around the world reported laboratory-confirmed cases, including more than 8,768 deaths. The highest mortality was reported in the World Health Organization (WHO) Regional Office for the Americas (5,878 deaths) followed by Europe (≥918 deaths), South-East Asia (766 deaths), Western Pacific (706 deaths), the Eastern Mediterranean (392 deaths), and Africa (108 deaths) (5). In Turkey, the first laboratory-confirmed human case was reported on May 15, 2009. This and the first few cases that followed were imported from outside of the country. Until the 42nd week of 2009, the number of cases originating from various provinces of Turkey was at a limited level. After the beginning of 2009–2010 education period epidemics were reported in various elementary schools, and by the end of the 48th week of 2009 the number of laboratory-confirmed cases reached more than 9,500.

In Turkey, all pandemic influenza A (H1N1) virus infections were reported to the Ministry of Health (MoH). Nasopharyngeal and/or nasal swabs of the suspected cases were sent to one of the following laboratories: Reflk Saydam National Public Health Agency (RSNPH), National Influenza Center in Ankara, National Influenza Reference Laboratory at Istanbul University, Faculty of Medicine in Istanbul; and regional public health laboratories. At first, clinical specimens were obtained from all suspected cases. However, since the number of cases increased tremendously after the 47th week of 2009, clinical specimens were collected only from patients requiring hospitalization and from outbreak cases. The clinical samples have been tested by real-time reverse transcription-polymerase chain reaction (RT-PCR). Data regarding demographic, epidemiological, and clinical information of the suspected cases are recorded by local healthcare institutes and the patients’ information forms are sent to laboratories together with the clinical specimens.

Since the first case was described in California, several investigations have been performed in many different countries to analyze clinical, epidemiological, and
molecular characteristics of this pandemic influenza A (H1N1) virus (6–10). Molecular studies are critical for distinguishing 2009 pandemic influenza A (H1N1) virus from other circulating influenza viruses. Furthermore, molecular analysis of this new virus is critical for the monitoring of modifications in virus genome related to pathogenesis, and susceptibility to antiviral drugs. All such efforts provide significant improvements to the effective management of patients and development of more protective vaccines to control the infection.

In Turkey, up to now there has been only one published study reflecting the characteristics of pandemic influenza A (H1N1) virus infections; a study of the first 128 cases diagnosed from April 29 to July 17, 2009 (11). The aim of the current study was to gain an insight to the epidemiological and demographic characteristics of the laboratory-confirmed cases diagnosed from May 15, 2009, the date of the first index patient, to the end of the 48th week of 2009, and to search mutations in the receptor-specific region of the HA1 domain and resistance to NA inhibitor of the 29 viral strains randomly selected among the isolates of the patients admitted to RSNPHA, Ankara, Turkey.

MATERIALS AND METHODS

Patients: In this study, a total of 19,973 clinical specimens obtained from the cases suspected with pandemic influenza A virus infection were analyzed from the 18th week to the end of the 48th week of 2009. Nasal or nasopharyngeal samples were taken from suspected cases. All clinical samples were inserted into viral transport medium (Virotact; Medical Wire & Equipment, Wiltshire, UK) and transported to the laboratories in a triple biological safety system by courier. Patients’ information including age, gender, work place, school, city of residence, and clinical findings were recorded in a form prepared by the MoH and sent to the laboratory with the clinical specimens.

Laboratory diagnosis: (i) RNA extraction: The clinical specimens arriving in transport medium were gently mixed by vortex and 200 or 400 μl of the samples were transferred into a 1.5-ml microcentrifuge tube in a biological safety cabinet. Following the kits instructions, RNA extraction was performed using either a Total Nucleic Acid Isolation Kit (Roche, Mannheim, Germany) or an EZ1 Virus Mini Kit (Qiagen GmbH, Hilden, Germany).

(ii) PCR amplification: Search for viral RNA was conducted using either an in-house real-time RT-PCR protocol provided by the Centers for Disease Control and Prevention (CDC) or a commercial real-time RT-PCR kit (Qiagen). The commercial kit provided the opportunity to search an 80-base pair region of pandemic influenza A virus (H1N1) using the Rotor-Gene 6000 instruments. Using in-house real-time RT-PCR method, the clinical samples were first analyzed for influenza A M gene, pandemic influenza A (H1), and for RNaseP as an external internal control in the same run. If any sample yielded a positive result for influenza A virus but was negative for pandemic influenza A (H1), those samples were tested for seasonal influenza A (H1) and influenza A (H3), using subtype-specific primers for the HA gene segments. In-house real-time RT-PCR was performed using the ABI 7500. The 25-μl PCR mixture contained 5 μl of extracted RNA, 1 μl each of forward and reverse primers, 0.5 μl SuperScript III RT/Platinum Taq DNA polymerase mix, 12.5 μl of 2× Master mix, and 4 μl nuclease-free water. Amplification conditions were as follows: reverse transcription at 50°C for 30 min, Taq inhibitor activation at 95°C for 2 min and 45 cycles at 95°C for 15 s, 55°C for 30 s.

(iii) Nucleotide sequence of HA gene: Sequences of HA gene of 29 strains randomly selected among the laboratory-confirmed cases were determined using the forward and/or reverse primers designed by the WHO for sequencing of this virus. Information of the primers is available at http://www.who.int/csr/resources/publications/swineflu/GenomePrimer_20090512.pdf. The extracted RNA was amplified by in-house RT-PCR using the one-step RT-PCR system (Invitrogen, Carlsbad, Calif., USA). Each segment of HA gene was first amplified using fragment-specific reverse and forward primers of HA gene. Amplification conditions were as follows: cDNA synthesis at 48°C for 45 min and initial denaturation at 94°C for 2 min and 30 cycles at 94°C for 20 s, 50°C for 30 s, 72°C for 1 min with an additional extension step at 72°C for 7 min. After purification of amplicons with Agencourt Ampure (Beckman Coulter, Beverly, Mass., USA), sequence reactions were setup with M13 primers. Each sequence reaction mixture consisted of 3.5–5 μl of purified amplion, 5 pmol primer, and 4 μl of Dye terminator cycle sequencing quick start kit (Beckman Coulter). The sequence reaction was done as follows: initial denaturation at 94°C for 3 min followed by 30 cycles consisting of denaturation at 96°C for 20 s, annealing at 55°C for 20 s, and elongation at 60°C for 4 min. The PCR products were purified with Dye-Terminator removal kit (Agencourt CleanSeq; Beckman Coulter) and 20 μl of purified product was sequenced in the Beckman Coulter 8000 instrument. The sequence results were subjected to BLAST analysis using the National Center for Biotechnology Information (NCBI) GenBank. The base numbering of HA gene in the present study is for H1 gene. Therefore, position 222 in H1 HA corresponds to position 225 in H3 HA.

(iv) Nucleotide sequence for NA mutation: Since a mutation which results in a histidine-to-tyrosine substitution at position 275 (H275Y) in the NA gene, is known to be associated with oseltamivir resistance (12,13), a set of primers was used to determine the alterations at this position of the NA gene (14). The viral RNA was extracted from the original clinical specimens or Madin-Darby canine kidney (MDCK) cell isolates. Amplification and sequencing conditions of NA gene were similar to those used for HA gene, except the used primers. Sequence data was analyzed to find out whether there was a mutation at codon 275 or not. The “CAC” base sequence at position 275 was evaluated as susceptible to oseltamivir. If the strain was resistant to oseltamivir, this base sequence would be “TAC” or “TAT” (12).

Statistical analysis: The distribution of pandemic influenza A (H1N1) virus positivity among different age groups and genders were compared by χ² test. Homology between the sequence results of the studied isolates and A/California/07/2009 (H1N1) strain and homology of each isolate with the other studied isolates were
evaluated using MEGA 4.1 software (Beta 3) Alignment Explorer. The available HA gene sequence results of the studied isolates were compared with the same length of the corresponding HA gene sequences of the A/California/07/2009 (H1N1) virus strain and we also compared the sequence results of the studied isolates with each other. Homology was calculated as a percentage in accordance with the number of identical nucleotides between the compared sequences.

RESULTS

A total of 9,459 laboratory-confirmed pandemic influenza A (H1N1) virus infections were defined among the 19,973 clinical specimens analyzed in Turkey up until the end of the 48th week of 2009. The overall rate of laboratory-confirmed cases was 47.36%. Up to the 42nd week of 2009, pandemic influenza cases were reported rarely and from a limited number of provinces; mainly in the two biggest cities, Ankara and Istanbul. The first reports of local outbreaks from primary schools were in the 42nd week of 2009. In the 43rd week, the number of cases increased tremendously and reached the highest level during the 46th week of the year (Fig. 1). By the 48th week of 2009, all 81 provinces in Turkey had confirmed pandemic influenza cases, with 33.3% of cases collected from Ankara and Istanbul.

Demographic data was available for the 9,588 suspected cases analyzed during the study period. More than half of these cases (51.9%) belonged to two age groups, 5–14 years old (n = 2,380, 24.8%) and 25–44 years old (n = 2,600, 27.1%). The 15–24 year age group included 19.4% (n = 1,861) of the suspected cases. The sampling rates for the other age groups were as follows: 10.9% (n = 1,041) in 1–4 years old, 10.1% (n = 966) in 45–64 years old, 4.1% (n = 397) in ≥1 year old; and 3.6% (n = 343) in >64 years old. Of the 9,588 suspected cases, 4,121 were real-time RT-PCR positive. The mean age of the 4,121 PCR-positive cases was 20 years and 48.5% of them were male. The highest PCR positivity rate belonged to the 5–14 year age group (30.5%), followed by the 15–24 year age group (21.9%), the 25–44 year age group (20.7%), the 1–4 year age group (10.6%), the 45–64 year age group (7.5%), the 0–11 month age group (3.4%), and the >65 year age group (2.0%) (Fig. 2). The positivity rates of the 5–14, 15–24, and 25–44 year age groups were significantly higher than the rates of the other age groups (P = 0.001).

HA genes of the 29 pandemic influenza A (H1N1) viruses were sequenced. Of these 29 strains, 14 belonged to patients diagnosed in summer, 2009; the remaining 15 were selected from among the positive cases in autumn, 2009. These strains were randomly selected from different parts of Turkey. Twenty of the isolates were obtained from males and nine were from females. Eight of these pandemic virus isolates belonged to the deceased patients. Based on the number of identical nucleotides between the compared sequences, there was 98.96–100% homology between California strains and the tested isolates (Table 1). Moreover, genetic homology between each of the tested isolates ranged from 99.01 to 100%.

Mutation analysis of the receptor binding domain of HA1 gene was available for 25 strains. Nine of the isolates (37.5%) had mutations at position D222E/N and P83S. Eight of the nine mutant isolates had D222E alteration, and one had D222N alteration. In addition to these mutations, these isolates also had mutations at position P83S, S203T, and I302V (Fig. 3). Two of these mutant isolates belonged to the deceased patients, two isolates were obtained from females, and six were from males (Table 1).

Among the 29 pandemic influenza A (H1N1) virus isolates, there were no isolates with amino acid substitution at position 275 in the NA gene. All isolates had a base sequence of “CAC” at the 275th codon. The analyzed virus strains belonged to patients who had not been treated previously with oseltamivir. Demographic and epidemiological characteristics of the patients are shown in Table 1.

DISCUSSION

This study was conducted to analyze the epidemiologic and demographic characteristics of pandemic A influenza (H1N1) virus infections in Turkey between May 15 and November 30, 2009, and to determine the mutations in the HA gene and resistance to oseltamivir among the selected isolates. During the study period, more than 47% of the study population had confirmed pandemic influenza A (H1N1) virus infection which
spread throughout all of the provinces of Turkey. The number of cases began to increase in the 42nd week of 2009, reaching its peak in the 46th week, with this increase continuing for the duration of the study period. Children aged 5–14 seemed to have an increased risk for contracting pandemic flu. Gender was not considered to be a significant risk factor for pandemic influenza A (H1N1) virus infection. Sequencing of the HA gene showed that Turkey had a homogenous pandemic influenza A (H1N1) virus population with more than 98.9% similarity between the vaccine strain and the tested strains, with all tested isolates susceptible to oseltamivir. Interestingly, more than 37% of the pandemic influenza A virus isolates in Turkey had a mutation at position D222E/N in the HAI domain.

Since pandemic influenza A virus is easily transmitted from infected people to healthy people, its positivity rate rapidly increases all over the world (5–7,11). In Turkey, only a few cases were reported in only a limited number of provinces during the summer. By the start of the education period, outbreaks were occurring in elementary schools and the rate of the laboratory-confirmed cases increased tremendously to 47%, with confirmed cases observed all over the country. In agreement with previous reports (7,15), there were no statistically significant differences between the PCR positivity rates obtained in males and females (46.5% versus 48.5%, respectively, $P = 0.096$). However, it has been reported that this virus mostly affects young adults and persons aged $<24$ years (3,7), with the mean age of patients ranging from 20 to 25 years old (6,8). Similar to this data, the mean age of our laboratory-confirmed cases was 20 years old and 66.4% of the positive cases were $<24$ years old. Additionally, a significant portion (one-fifth) of our cases was in the young adult group, whereas people aged $\geq$ 65 years had the lowest rate. The lower attack rate of pandemic influenza A (H1N1) virus infections in older people can be explained by the presence of a cross-reactive antibody (3,7). Although we do not have any laboratory-test results regarding the cross-reactive antibody in our study population, the lower positive rate in the older age group supports the view of previous cross immunity within this age group in Turkey.

Several studies have been performed to evaluate the mutations which may result in resistant and/or more virulent strains during the spread of this new virus among human populations (16–18). Researchers have mainly focused on monitoring the mutations in HA gene which is very important for viral attachment and immunization. Sequencing studies showed that mutations have been predominately observed in HAI domain.
of the HA gene and mostly clustered in five main antigenic regions (16,17). Amino acid changes at codons 138, 163, 187, 189, 190, 194, 225, 226, or 228 (H3 numbering) in the HA receptor-linked domain are responsible for receptor binding capacity to either α2-6-sialyl (N-acetyllactosamine) residues (α2-6SA; human-linked receptor) or α2-3-sialyl galactose moiety (α2-3SA; avian like receptor) (17). Recently, some of these mutations especially D222G/N/E in HA1 domains (corresponding to position 225 in H3 HA) have been reported among the pandemic influenza A (H1N1) virus strains from different countries, such as Norway, China, Japan, Ukraine, the United States, and the Netherlands (18). Although currently available data showed that the D222G substitution does not appear to pose a major public health issue, this substitution has been frequently observed in the HA gene of pandemic influenza A (H1N1) viruses isolated from severe and fatal cases in several countries (19). While the prevalence of D222G substitution is <1.8%, it increased to 7.1% among the isolates of fatal cases (19). On the other hand, the frequency of D222E substitution did not differ significantly between mild and severe cases, while D222N was observed in very few cases (20). In our study, we were able to evaluate the mutation in HA1 domain in 24 of the 29 isolates sequenced. We found a mutation at position 222 in the nine isolates (37.5%) with eight of these isolates having an alteration from aspartic acid (D) to glutamic acid (E), and one having an aspartic acid-asparagines (N) alteration. In our study there was no isolate with a D222G substitution, however D222E substitution seems to be predominant in Turkish isolates. Although it was indicated that the clinical significance of the D222E and D222N substitution remains uncertain (19), two of the mutant viruses (D222N, D222E) in our study were isolated from deceased patients. Interestingly, we found mutation of D222N in only one fatal case. Since we only had one case, it is very difficult to speculate about the importance of this mutation on the fatality; however this result supports previous data. Kilander et al. (20) found D222N alteration in only a few cases in Norway (4/266 cases), however three of the isolates having this
mutation were isolated from severe and fatal cases. In Ukraine, two of the five sequenced viruses had D222N substitution and both of these viruses were isolated from fatal cases (http://www.recombionomics.com/News/12090091/D225N_Ukraine.html). In Brazil, two isolates of fatal cases also had D222N substitution (http://www.recombionomics.com/News/11090902/Ukraine_1918.html). Although the number of strains analyzed in our study was limited, since our sequenced isolates were collected from patients of different ages, gender, geographic origin, and survival, we can conclude that these results may represent the current situation of Turkish isolates. However, further analyses on additional strains from different patients including fatal cases must be continued to highlight the real frequency of the mutation at 222 and the relationship between mutations and virulence.

Comparison of the sequence data of the patients’ isolates to the vaccine virus A/California/07/2009 (H1N1) provides valuable information for understanding the efficacy of the vaccines. In the WHO report Pandemic (H1N1) 2009-update 78, it was reported that all pandemic influenza viruses analyzed worldwide were antigenically and genetically closely related to the vaccine strain (18). In concurrence with this data, the sequencing results of the 29 pandemic A (H1N1) virus isolates in Turkey were closely related to the sequence of the vaccine strain. The homology rate was similar among the isolates collected from different provinces, gender, ages, and patient outcome. This data showed that the current pandemic A virus vaccines cover all pandemic virus strains that were circulating in Turkey.

Based on present data, it has been accepted that pandemic influenza A (H1N1) strains are mainly susceptible to NA inhibitor but resistant to adamantane (21). However, once oseltamivir resistance develops, this resistant virus becomes predominant (22). For this reason, tracking oseltamivir resistance among pandemic influenza A virus isolates is of great importance. Up to December 11, 2009, a total of 102 oseltamivir-resistant isolates were reported from 31 countries, and worldwide more than 10,000 isolates tested for oseltamivir resistance were found to be sensitive (18). A study which conducted analysis of more than 1,000 pandemic influenza A isolates found that only 3 strains from Denmark, China, and Japan were resistant to oseltamivir but susceptible to zanamivir (23). It was reported that the patients infected with these resistant variants did not have severe illness and all recovered. A case report study described a 16-year-old girl who was infected with an oseltamivir-resistant pandemic influenza virus in Hong Kong (21). In the Netherlands, oseltamivir resistance was found in 11 strains, 5 of which were isolated from fatal cases (http://www.recombionomics.com/News/12110901/H274Y_Fatal_Netherlands_Spike.html). On the other hand, similar to our study, in New Zealand all pandemic influenza A (H1N1) virus strains tested were found to be as sensitive to oseltamivir (24). Additionally, the 13 isolates of the patients diagnosed in California were tested for resistance to antiviral drugs and all viruses were susceptible to oseltamivir and zanamivir (13). Similar to the previous studies, our data confirmed that the NA inhibitors are important antiviral drugs for controlling influenza A (H1N1) virus epidemics and/or pandemics in Turkey (25). However, since some countries reported mutations at position H275Y and there is a possibility of transmission of the resistant isolates to other patients, oseltamivir resistance should be closely followed, especially in patients at risk of developing resistance. Patients having prolonged postexposure prophylaxis or treatment with subtherapeutic dosages are at risk of developing resistance to oseltamivir (26, 27).

In conclusion, pandemic influenza A (H1N1) virus mainly affected children aged 5–14 years old in Turkey, without any gender predominance. Molecular studies indicated that the current vaccine prepared from A/California/07/2009 (H1N1)-like virus covered all pandemic A (H1N1) virus strains in Turkey, and all pandemic influenza A (H1N1) viruses currently circulating in Turkey were susceptible to oseltamivir. Observation of mutations in the receptor binding domain enables us to closely track alterations in new isolates in Turkey.

REFERENCES


