Original Article

Genetic diversity of the pandemic influenza A (H1N1) virus in Saudi Arabia

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Abstract

Introduction: Pandemic influenza A (H1N1) virus emerged and spread globally in the spring of 2009. Saudi Arabia also witnessed a severe H1N1 pandemic virus epidemic with considerable morbidity and mortality in different parts of the kingdom beginning in June 2009. The influenza A(H1N1)pdm09 virus was detected in samples collected between May 2009 and November 2010 from Makkah region. This study provides data on the viral diagnosis and genetic diversity of hemagglutinin (HA) and neuraminidase (NA) genes of influenza A (H1N1)pdm09 virus from Saudi Arabia.

Methodology: Nasopharyngeal swabs from 100 clinically infected patients in the peak of the outbreak were collected from Makkah region and processed for viral diagnosis by viral culture and real-time polymerase chain reaction (PCR). HA and NA genes of 10 selected samples were sequenced and analyzed.

Results: A total of 100 samples were collected; only 10 samples were found to be positive for influenza A virus infection by real-time PCR. Nucleotide sequence analysis of the HA and NA genes of influenza A (H1N1) from Saudi Arabia showed significant similarities with selected isolates. The phylogenetic tree constructed for both HA and NA genes formed close clusters with selected reference isolates.

Conclusions: Nucleotide sequence analysis and phylogenetic relationships of the HA and NA genes of influenza A (H1N1) virus from Saudi Arabia with selected reference isolates indicates that they were genetically close and most probably originated from influenza A(H1N1)pdm09.

Key words: Influenza A (H1N1) virus; HA and NA genes; genetic diversity; phylogenetic analysis.

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Introduction

Influenza is an acute respiratory disease caused by influenza A virus (H1N1). Influenza A H1N1 virus appeared simultaneously in humans and pigs in 1918 and has been circulating since then with an intermittent period in humans. The influenza virus was first isolated from pigs in 1930 and then from humans in 1933; separate swine and human lineages were readily distinguishable by antigenic analysis. Experimental infection of pigs with human H1N1 influenza viruses was found to produce very mild or no signs of disease [1]. H1N1 is a novel subtype of the influenza A virus that belongs to the family *Orthomyxoviridae*. It contains eight negative-sense, single-stranded RNA segments [2], which have been shown to be antigenically highly similar to a recently reconstructed human 1918 A (H1N1) virus [3] and likely share a common ancestor [4-5]. From 1930 to the late 1990s, these "classical swine influenza" viruses circulated in swine and remained relatively antigenically stable [6-7]. Influenza A (H1N1) viruses circulated in humans from 1918 until the A (H2N2) influenza pandemic of 1957. During this period, there was substantial antigenic drift of A (H1N1) viruses in humans away from the 1918 virus [3,8]. Influenza A (H1N1) viruses from the early 1950s reemerged in humans in 1977 [9]. From 1977 to 2009, there was substantial further antigenic evolution of the human A

(H1N1) viruses that was sufficient to warrant eight updates of the H1 component of the influenza virus vaccine [10]. The relative antigenic stasis of classical H1N1 influenza viruses in swine until 1998, during the time when substantial antigenic drift of H1 in humans was observed, created a substantial antigenic gap between classical swine H1 and human seasonal H1 viruses. Thus, swine have become a reservoir of H1 viruses with the potential to cause major respiratory outbreaks or even a possible pandemic in humans. The genomes of the last three pandemic influenza viruses (1918 H1N1, 1957 H2N2, and 1968 H3N2) all originated in whole or in part from nonhuman reservoirs, and the HA genes of all of the pandemic viruses ultimately originated from avian influenza viruses [11]. Since its reemergence in 2008, H1N1 has been reported to cause a variety of illnesses ranging from mild flu-like symptoms to severe multi-organ failure.

In 2009, cases of novel influenza A(H1N1)pdm09 virus were first identified in Mexico and the United States; the virus rapidly spread and caused a worldwide pandemic within a few months. Influenza pandemics occur when a novel influenza virus with surface antigens hemagglutinin (HA) and/or neuraminidase (NA), to which the majority of the human population has little or no preexisting immunity, emerges in humans and is able to be transmitted efficiently from person to person. Novel HA or NA can be introduced into the human population either through the direct transmission of an animal influenza virus to humans, through reassortment between human and animal viruses, or between different lineages of swine influenza viruses [11]. The World Health Organization reported that the virus had spread to 66 countries with 19,273 confirmed cases including 117 deaths. In the United States, the Centers for Disease Control reported 11,054 cases, including 17 deaths [12]. The outbreak strain was identified as a swine-origin influenza virus that resulted from a reassortment of two previously circulating strains: a triple-reassortant swine influenza that had been circulating in North America since 1998 and an H1N1 strain that had been circulating for decades in swine populations in Asia and Europe. The new strain comprised six segments from the North American lineage and two segments from the Eurasian lineage (novel swine-origin influenza A [H1N1] [S-OIV]). Based on genetic variations in HA and NA genes, they are classified into 16 HA subtypes and 9 NA subtypes [9,13]. HA and NA proteins are encoded by segments 4 and 6 of the viral genome, located on the virion surface, and are the primary target for the host immune response [14]. The HA protein is the most important determinant of virulence and host specificity because its binding site and binding pocket recognize sialic acid-containing cell surface receptors on host epithelial cells [15-18]. Swine play an important role in the ecology of influenza A viruses because they are susceptible to viruses of the avian and mammalian lineages as well. Sporadic human cases of S-OIV infections have been reported around the world with limited human-to-human transmission [19-24]. In April 2009, a SOIV A (H1N1) containing a unique combination of gene segments from both North American and Eurasian swine lineages was identified from human infections in Mexico and the United States. The virus rapidly spread worldwide, causing the first influenza pandemic of the 21st century [11,25]. The emergence of the 2009 A (H1N1) pandemic influenza [A(H1N1)pdm09] provided strong evidence that swine influenza viruses have public health importance and pandemic potential [26].

Influenza A (H1N1) virus cases were also reported in Saudi Arabia with the clinical features of the patients who were hospitalized between July 2009 and June 2010 in a tertiary care hospital in Khamis Mushyt, Saudi Arabia; the virus infection was confirmed by an RT-PCR test [27-28]. In another case, a young immunocompetent man presented with progressive shortness of breath and rapidly developed multi-organ dysfunction, including pancytopenia from H1N1 infection during the 2010-2011. Hemophagocytosis secondary to the H1N1 infection was observed in a bone marrow biopsy test [29].

Recently, on November 5, 2012, Saudi health authorities reported eight cases of H1N1 infections at the Al-Amal mental hospital in Riyadh. The infected patients were given Tamiflu vaccine while 27 other patients, including four nurses, suspected of having the influenza virus were quarantined. The latest H1N1 outbreak in the kingdom was discovered on November 5, 2012, but was not disclosed to avoid panic among Hajj pilgrims, according to spokesman of Health Affairs Directorate in Riyadh Saad Al Qahtani [27,30]. A Saudi boy vacationing with his family in Hong Kong was diagnosed with the virus but recovered [30]. This study was conducted based on this information. In this report, we present the diagnosis and genetic diversity of HA and NA genes of influenza A (H1N1) virus isolated from the Saudi population in Makkah region.

Methodology

Patients and samples

A total of 100 samples were collected from patients suspected of having influenza A (H1N1) virus infection between May 2009 and November 2010 from Makkah region (Saudi Arabia) during the 2009 pandemic. Acute respiratory symptoms were observed in patients as described by the World Health Organization (WHO) [31]. The patients ranged in age from 8 months to 70 years, both males and females. Nasopharyngeal swabs were taken and put into 3 mL of viral transport medium (VTM) (Becton Dickinson, Franklin Lakes, USA); the samples were immediately transferred to the laboratory in an ice box and were stored at the Special Infectious Agents Unit, King Fahd Medical Research Center, Jeddah, Saudi Arabia. The study was approved by the ethical committee of King Fahd Medical Research Center, approval number 010-CEGMR-01-ETH-restricted.

Viral RNA extraction and detection of influenza A (H1N1) virus

Viral RNA was extracted from cell culture by using a QIAmp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Primer selections for detection of influenza A virus are shown in Table 1. Complimentary DNA (cDNA) synthesis of viral RNA was done to screen influenza A virus from collected samples. One-step real-time RT-PCR assays were performed by using the extracted RNA with RealTime Ready Inf A/H1N1 Detection Set (Cat #05640393001, Roche, Mannheim, Germany) and RealTime Ready RNA Virus (Master mix Cat # 05619416001, Roche, USA). The kit contains two highly specific primer/probe mixes: one targeting the influenza A matrix protein 2 (M2) for the detection of all influenza A strains. The second primer/probe mix targets and specifically detects the hemagglutinin (HA) gene of the pandemic influenza A/H1N1-2009 strain. The RT-PCR reaction contained 10 µL of ~2 µg extracted viral RNA, 10 µL of 5X buffer, 2 µL of 10 mM dNTPs, and 2 µL of enzyme (5U/µL), in addition to 3 µL of 0.6 µM each primers and 2 µL of BSA in final volume of 50 µL. The RT-PCR was performed at initial holds at 60°C for 30 minutes, 95°C for 15 minutes in the initial denaturation step, followed by 45 cycles of amplification, which included denaturation for 30 seconds at 94°C, annealing for 60 seconds at 53°C, and extension for 120 seconds at 72°C. The final extension was completed at 72°C for 10 minutes. PCR products were purified by column purification using a QIAquick PCR Puirfication Kit (Qiagen, GmbH, Hilden, Germany).

Full-length PCR amplification of HA and NA genes

The positive samples were used to amplify the segments of NA and HA genes by RT-PCR using a Qiagen One-Step RT-PCR Kit (Qiagen, Chatsworth, USA) following the manufacturer's instructions. The PCR mixture contained 10 µL of ~2 µg extracted viral RNA, 10 µL 5X buffer, 2 µL of 10mM dNTPs, 2 µL enzyme (5U/µL), 3 µL of 0.6 µM each primers, and 2 µL of BSA (100 µM). The amplification reaction was performed in a thermo cycler (Eppendorf, Hamburg, Germany) under the following conditions: denaturation at 94°C for 15 minutes, followed by 40 cycles of denaturation at 94°C for 30 seconds, primer annealing at 53°C for 60 seconds, extension at 72°C for 2 minutes, and final extension at 72°C for 10 minutes.

Table 1. Primers used for detection of H1N1 virus

No.	Name	Sequences
Primers for HA segment		
1	H1-F1	5'-CCGCAAATGCAGACACATTA-3'
2	H1SWS1	5'-GTGTCATCATTTGAAAGGTTTGAGA-3'
3	Ca4/2009_B	5'-CTCAATCCTGTGGCCAGTC-3'
4	HA	5'-TAAACACCAGCCTCCCATTT-3'
5	H1-R1	5'-CCCATTAGAGCACATCCAGAA-3'
Primers for NA segment		
1	H1F1	5'-CCATTGGTTCGGTCTGTATG-3'
2	H1R1	5'-TGACCAAGCGACTGACTCAA -3'
3	H1F2	5'-TCCAATGGAACCATTAAAGACA-3'
4	H1R2	5'-TGACCAAGCGACTGACTCAA -3'
5	H1F3	5'-TGAGGAATGCTCCTGTTATCC -3'
6	H1R3	5'-CAGATTCTGGTTGAAAGACACC-3'

Sequencing and analysis of HA and NA genes of influenza A (H1N1) virus

Ten isolates of influenza A (H1N1) were selected for sequencing of HA and NA genes. The PCR products (1,619 and 1,550 bp) were purified using a PCR purification kit (GFX kit, Biocompare, Piscataway, USA). Cycle sequencing was performed, using forward and reverse primers to cover the full length of the PCR product, following the manufacturer's instructions (BigDye Terminator version 3.1 Cycle Sequencing Kit, Applied Biosystems, Austin**,** USA). The sequencing reaction mixture contained 4 μL of premix, 2 μL of ABI 5x sequencing buffer, 3.2 μM of sequencing primer, and 1 μL PCR product in a final volume of 20 μL. The amplification primers were used for sequencing by an initial denaturation at 96°C for 1 minute, followed by 30 cycles (10 seconds each) of denaturation at 96°C, annealing (5 seconds at 50°C), and extension (4 minutes at 60°C). The sequencing products were purified using the ethanol/EDTA precipitation method and analyzed with the ABI Prism 3100 (Avant Genetic Analyzer, Applied Biosystems) according to the manufacturer's instructions, at the Special Infectious Agents Unit, King Abdulaziz University.

The HA and NA gene nucleotide sequences were assembled, aligned together, and initially searched for similarity using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) [32]. Multiple sequence alignments were performed by the ClustalW program (http://www.ebi.ac.uk/clustalw). Sequence alignment results were further analyzed using the BioEdit program (http://www.mbio.ncsu.edu/BioEdit/) using nucleotide sequences of influenza A (H1N1) from Saudi Arabia with selected influenza A (H1N1) isolates from GenBank (Table 2).

Phylogenetic analysis of HA and NA genes of influenza A (H1N1) virus

A phylogenetic tree was constructed using the MEGA5 program from aligned nucleotide sequences with neighbor-joining and maximum parsimony methods using maximum composite likelihood for the DNA substitution test [33]. The complete HA and NA sequences of influenza A (H1N1) 2009 viruses isolated from humans in different countries were obtained from the GenBank (http://www.ncbi.nlm.nih.gov/genomes/FLU/Database/nph-select.cgi) for comparison and construction of phylogenetic trees. The phylogenetic trees were constructed using MEGA5 software with the 100 replicates bootstrap.

Results

Detection of influenza A(H1N1)pdm09 virus

A total of 100 samples were collected and tested for influenza A(H1N1)pdm09 virus from patients with suspected influenza A(H1N1)pdm09 virus infection. Among these, only 10 samples were found to be positive, detected by RT-PCR.

Sequence analysis

To understand the genetic diversity of influenza A (H1N1) virus collected from Saudi Arabia, the HA and NA genes from ten isolates were sequenced. The full length of HA gene was found to have 1,701 nucleotides, and NA had 1,423 nucleotides. The complete HA and NA gene of Saudi Arabian isolates were sequenced and submitted to GenBank (Table 2). Analysis of HA and NA genes of influenza A (H1N1) isolates were carried out on the basis of deduced nucleotide sequences with selected isolates from various countries submitted in GenBank (Tables 3 and 4).

Table 2. Accession numbers of HA and NA gene submitted to GenBank from Saudi Arabia

No.	Accession No. (HA)	Accession No. (NA)
1	KC297139	KC297149
2	KC297140	KC297150
3	KC297141	KC297151
4	KC297142	KC297152
5	KC297143	KC297153
6	KC297144	KC297154
7	KC297145	KC297155
8	KC297146	KC297156
9	KC297147	KC297157
10	KC297148	KC297158

Table 3. Virus isolates used for analysis of HA gene

No.	Virus	Place	Accession numbers
1	A/Saudi Arabia-Jeddah/3657/2010(H1N1)	Saudi Arabia	KC297139
2	A/Saudi Arabia-Jeddah/3659/2010(H1N1)	Saudi Arabia	KC297140
3	A/Saudi Arabia-Jeddah/1296/2009(H1N1)	Saudi Arabia	KC297141
4	A/Saudi Arabia-Jeddah/3647/2010(H1N1)	Saudi Arabia	KC297142
5	A/Saudi Arabia-Jeddah/3663/2010(H1N1)	Saudi Arabia	KC297143
6	A/Saudi Arabia-Jeddah/3666/2010(H1N1)	Saudi Arabia	KC297144
7	A/Saudi Arabia-Jeddah/3669/2010(H1N1)	Saudi Arabia	KC297145
8	A/Saudi Arabia-Jeddah/1365/2009(H1N1)	Saudi Arabia	KC297146
9	A/Saudi Arabia-Jeddah/3670/2009(H1N1)	Saudi Arabia	KC297147
10	A/Saudi Arabia-Jeddah/3673/2010(H1N1)	Saudi Arabia	KC297148
11	A/Singapore/ON802/2009(H1N1)	Singapore	CY124059
12	A/Ontario/29801/2009(H1N1)	Canada	CY060574
13	A/Malaysia/2076212/2009(H1N1)	Malaysia	CY118199
14	A/Jakarta/002/2009(H1N1)	Indonesia	JN412818
15	A/Helsinki/P15/2009(H1N1)	Finland	JQ173156
16	A/England/435/2009(H1N1)	England	HM567904
17	A/Scotland/8/2009(H1N1)	Scotland	HM567712
18	A/New York/3242/2009(H1N1)	New York	CY040742
19	A/Finland/565/2009(H1N1)	Finland	HQ228025
20	A/Chile/1586/2009(H1N1)	Chile	CY075171
21	A/Mum/NIV261/2009(H1N1)	India	HM204567
22	A/California/VRDL18/2009(H1N1)	California	CY055471
23	A/Argentina/HNRG13/2009(H1N1)	Argentina	CY053896
24	A/Nagasaki/HA-15/2009(H1N1)	Japan	AB530467
25	A/Bayern/66/2009(H1N1)	Bayern	CY045503
26	A/Moscow/IIV01/2009(H1N1)	Russia	GQ219586
27	A/Canada-ON/RV1527/2009(H1N1)	Canada	FJ998209
28	A/Stockholm/33/2009(H1N1)	Sweden	GQ360060
29	A/Firenze/10/2009(H1N1)	Firenze	GQ351319
30	A/Italy/85/2009(H1N1)	Italy	GQ351290
31	A/Hong Kong/419239/2009(H1N1)	Hong Kong	CY087292
32	A/British Columbia/GFA0401/2009(H1N1)	British Columbia	CY065762
33	A/Texas/42291877/2009(H1N1)	America	CY052399
34	A/swine/Brazil/4/2009(H1N1)	Brazil	JQ666848
35	A/Quebec/QC002461/2009(H1N1)	Quebec	JN171904
36	A/Athens/WRAIR2962N/2009(H1N1)	Greece	CY073254
37	A/Guam/NHRC0026/2009(H1N1)	Guam	CY069090
38	A/Mexico City/WR1312N/2009(H1N1)	Mexico	CY050059
39	A/Pernambuco/82/2009(H1N1)	Pernambuco	CY103941
40	A/Qingdao/333/2009(H1N1)	China	CY050267

Table 4. Virus isolates used for analysis of NA gene

No.	Virus	Place	Accession numbers
1	A/Saudi Arabia-Jeddah/3657/2010(H1N1)	Saudi Arabia	KC297149
2	A/Saudi Arabia-Jeddah/3659/2010(H1N1)	Saudi Arabia	KC297150
3	A/Saudi Arabia-Jeddah/1296/2009(H1N1)	Saudi Arabia	KC297151
4	A/Saudi Arabia-Jeddah/3647/2010(H1N1)	Saudi Arabia	KC297152
5	A/Saudi Arabia-Jeddah/3663/2010(H1N1)	Saudi Arabia	KC297153
6	A/Saudi Arabia-Jeddah/3666/2010(H1N1)	Saudi Arabia	KC297154
7	A/Saudi Arabia-Jeddah/3669/2010(H1N1)	Saudi Arabia	KC297155
8	A/Saudi Arabia-Jeddah/1365/2009(H1N1)	Saudi Arabia	KC297156
9	A/Saudi Arabia-Jeddah/3673/2010(H1N1)	Saudi Arabia	KC297157
10	A/Saudi Arabia-Jeddah/3670/2009(H1N1)	Saudi Arabia	KC297158
11	A/Singapore/TT145/2010(H1N1)	Singapore	JX309292
12	A/Ontario/35273/2009(H1N1))	Canadian province	CY060736
13	A/Blagovechensk/01/2009(H1N1)	Russia	HM173601
14	A/swine/Argentina/31215/2009(H1N1)	Argentina	CY044258
15	A/Stockholm/49/2009(H1N1)	Sweden	GQ906584
16	A/Toronto/T0106/2009(H1N1)	Canada	CY045956
17	A/Bayern/66/2009(H1N1)	Bayern	CY045505
18	A/Taiwan/126/2009(H1N1)	Taiwan	CY045236
19	A/Hong Kong/H090-781-V10/2009(mixed)	China	CY115458
20	A/Guangdong/45/2009(H1N1)	China	HQ011420
21	A/San Diego/INS10/2009(H1N1)	San Diego	CY056006
22	A/Lebanon/09L-23/2009(H1N1)	Lebanon	AB603635
23	A/Canada-BC/RV2949/2009(H1N1)	Canada	HQ240020
24	A/Saint-Petersburg/RII5/2009(H1N1)	Saint Petersburg	CY075481
25	A/Italy/142/2009(H1N1)	Italy	GU123909
26	A/California/VRDL5/2009(H1N1)	California	CY054717
27	A/Texas/45034157/2009(H1N1)	Texas	CY052609
28	A/Norway/3206-3/2009(H1N1)	Norway	CY052021
29	A/England/94200009/2009(H1N1)	England	JX625507
30	A/Reunion/2154-4-M4E/2009(H1N1)	French Island	JQ431279
31	A/Malaysia/2098038/2009(H1N1)	Malaysia	CY118221
32	A/Scotland/Edinburgh_20308/2009(H1N1)	Scotland	CY107510
33	A/Helsinki/Vi3/2009(H1N1)	Helsinki-Finland	JQ409144
34	A/Astana/S2/2009(H1N1)	Astana-Kazakhstan	JF789631
35	A/Kenya/0026/2009(H1N1)	Kenya	HQ214314
36	A/Korea/CJ04/2009(H1N1)	Korea	HM189491
37	A/Managua/5295.02/2009(H1N1)	Nicaragua	CY073595
38	A/Bonn/INS127/2009(H1N1)	Germany	CY062741
39	A/Belgrade/WRAIR2368N/2009(H1N1)	Belgrade	CY073192
40	A/Iran/16245/2009(H1N1)	Iran	HM581916

The HA and NA gene sequences showed high similarities; the nucleotide identity percentages ranged from 99.48% to 99.77% and 99.01% to 99.77% for NA genes with selected reference isolates. Saudi Arabian isolates also showed above 99% identity with the prototypic A/California/07/2009 H1N1 strain.

Phylogenetic analysis

Saudi Arabian isolates were compared with the selected H1N1 isolates from various locations. In the phylogenetic analysis of HA and NA genes, Saudi Arabian isolates showed that circulating influenza A(H1N1) viruses during the 2009–2010 season could be differentiated into different and independent genetic groups and formed close clusters with selected isolates of influenza A virus from various locations.

Analysis of hemagglutinin (HA gene)

The phylogenetic analysis of 10 isolates from Saudi Arabia was carried out on the basis of nucleotide sequences of HA segments and showed greater similarities with selected reference isolates. As shown in Figure 1, the viruses clustered into five main groups. In group 1, four Saudi Arabian isolates (KC297139, KC297140, KC297144, and KC297145) clustered with one isolate from England (HM567904). In group 2, only two isolates from Saudi Arabia (KC297143 and KC297147) formed close clusters with various areas (Moscow GQ219586, Finland HQ228025, Texas CY052399, Pernambuco CY103941, British Columbia CY065762, Helsinki JQ173156, Scotland HM567712, Argentina CY053896, and Firenze GQ351319). In group 3, only one Saudi Arabian isolate (KC297148) clustered with isolates from Singapore (CY124059) and India (HM204567). In the fourth group, two Saudi Arabian isolates (KC297141 and KC297146) formed close clusters with isolates from Quebec (JN171904). In group 5, only one Saudi Arabian isolate (KC297142) clustered with isolates from New York (CY040742), Canada (FJ998209), and Italy (GQ351290) (Figure 1).

Figure 1. The phylogenetic tree of the HA genes of 10 influenza A(H1N1)pdm09 viruses in Saudi Arabia and selected reference sequences. The length scale measured the number of substitutions per site.

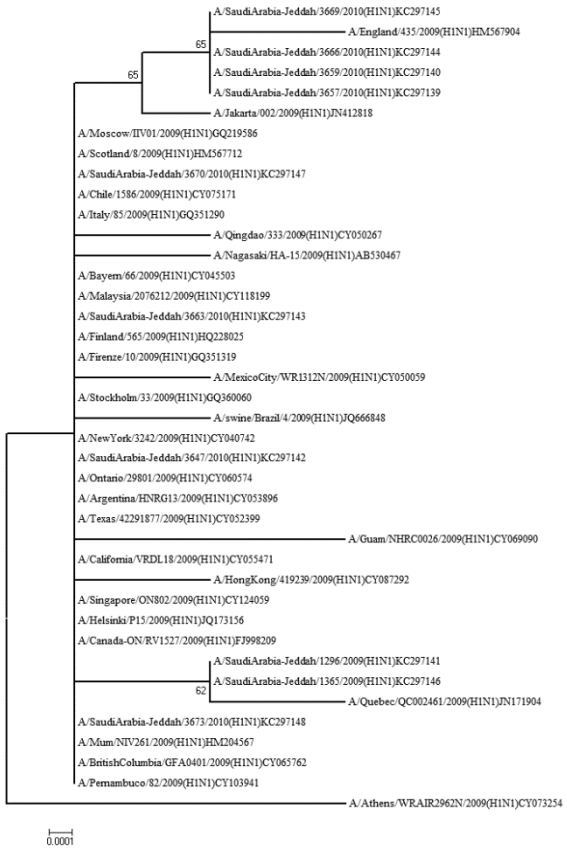
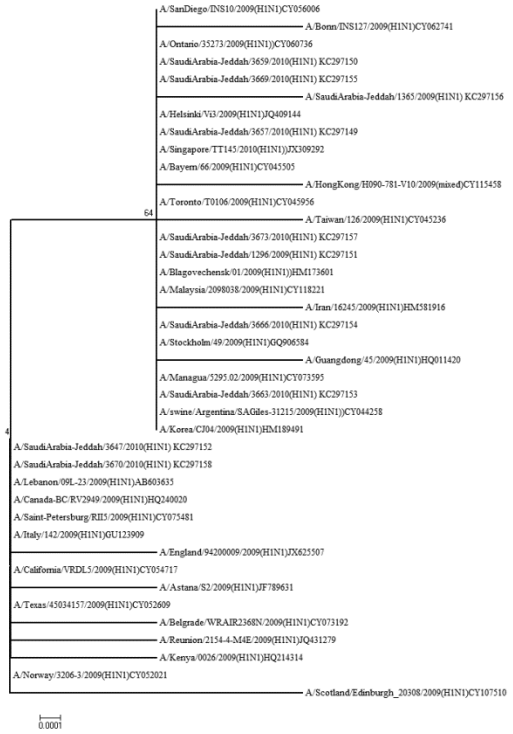


Figure 2. The phylogenetic tree of the NA genes of 10 influenza A(H1N1)pdm09 viruses in Saudi Arabia and selected reference sequences. The length scale measured the number of substitutions per site.



Analysis of neuraminidase (NA) gene

The phylogenetic analysis of Saudi Arabian isolates was carried out on the basis of nucleotide sequences of NA segments and showed greater similarities with selected reference isolates. As shown in Figure 2, the viruses clustered into six main groups. In group 1, Saudi Arabian isolate (KC297149) clustered with isolates from Korea (HM189491), Ontario (CY060736), and Singapore (JX309292). In group 2, only one isolate from Saudi Arabia (KC297155) formed close clusters with isolates from Helsinki (JQ409144) and Byem (CYO45505). In group 3, only two Saudi Arabian isolates (KC297151 and KC297154) clustered with isolates from Argentina (CY044258), San Diego (CY056006), and Toronto (CY045956). In the fourth group, two Saudi Arabian isolates (KC297153 and KC297157) formed clusters with isolates from Stockholm (GQ906584). In the fifth group, only one Saudi Arabian isolate (KC 297150) clustered with isolates from Malaysia (CY118221), Blagovechensk (HM173601), and Managua (CY073595). In the sixth and largest group, only two isolates from Saudi Arabia clustered with isolates from Lebanon (AB603635), Saint Petersburg (CY075481), Italy (GU123909), California (CY054717), Canada (HQ240020), and Norway (CY052021) (Figure 2).

Differences in amino acid

The comparison of amino acid sequences predicted from the HA and NA genes of Saudi Arabian isolates with prototypic A/California/07/2009 H1N1 strains showed a high degree of similarity. However, only six amino acid mutations were detected in HA and three in NA sequences of ten Saudi Arabian isolates.

Discussion

This study reports about the genetic diversity, phylogenetic relationships, and amino acid variations of HA and NA genes for the pandemic influenza A (H1N1)pdm09 virus isolated from a Saudi Arabian population between May 2009 and November 2010 from Makkah region, Saudi Arabia, with selected reference isolates. For this study, surveillance for A/H1N1 was initiated in Saudi Arabia. We provide evidence that, overall, the nucleotide sequences were fairly homogeneous on the basis of sequence similarity and phylogenetic relationship. We also further compared the amino acid sequences of A/California/07/2009 with Saudi Arabian isolates. Almost all residues were highly conserved in the Saudi Arabian isolates in both the HA and NA genes except a few mutations identified at different positions. Pandemic influenza A (H1N1) virus emerged and spread globally in the spring of 2009. In Saudi Arabia, influenza A (H1N1) virus infection was confirmed for the first time in June 2009 [27]. Pandemics are believed to arise when a novel avian or swine influenza HA and/or NA is acquired through reassortment between human, swine, and avian influenza viruses or by a non-human virus adapting to efficient human transmission [34]. The current pandemic H1N1pdm09 is the result of genetic reassortment of multiple gene segments from different lineages, and has a known evolutionary history of about a century [35]. However, the WHO reported that, so far, all of the circulating pandemic (H1N1) 2009 viruses are antigenically related to A/California/7/2009 [36]. Recently, outbreaks varying in severity throughout the UK were caused by a 2009 A(H1N1)pdm09 variant. Genomic analysis of the 2009 influenza A (H1N1) virus in humans indicated that it is closely related to common reassortant swine influenza A viruses isolated in North America, Europe, and Asia [37].

Our analyses were conducted on only ten isolates collected from Makkah region, which limited the possibility of greater genetic variability among them; our results also support less genetic variability. Nucleotide and amino acid sequence analysis and phylogenetic relationships of the HA and NA genes of Saudi Arabian isolates with selected reference isolates indicates that they are genetically very close and most probably originated from the same clade of A(H1N1)pdm09. The Saudi Arabian isolates clustered together both for the HA and NA genes and showed higher similarities with the selected reference isolate. Several reports have shown that influenza A(H1N1)pdm09 virus circulating in a geographic region may result from different original lineages that are more closely related in evolution [38-39]. Like all previous reassortment events, introduction of truncated HA and NA in circulating seasonal H1N1 can be a latent gateway for reassortment between the co-circulating pandemic and seasonal H1N1 strains. According to the last European Centre for Disease Prevention and Control report, eight genetic groups were presented; the new genetic group reported recently in a study was identified as A/Madrid/SO8189/2010 [40-41]. The severity of disease is very difficult to assess, as it can change with different geographic contexts and under different seasonal conditions, which favors the virus to adapt to a new host. Recently, this observation was demonstrated by the high spreading capacity of the

new influenza virus and its ability to compete with the previously dominant viral population, leading to an almost complete disappearance of the seasonal H1N1 virus [42-43]. Apart from an earlier report and confirmed case of infection with the new influenza A (H1N1) strain in Germany, in the peak period of the influenza epidemic, a different respiratory virus was detected in children [44-46].

Our findings confirm the genetic instability of influenza type A (H1N1) viruses and highlight the importance of continuous molecular surveillance for the effective management of influenza epidemics. Important disease management strategies can be employed by monitoring the disease spread (which mostly occurs during fall and winter seasons) and the disease severity.

Conclusions

Our study showed that viral isolation and RT-PCR are highly useful and sensitive for the detection of the novel influenza A(H1N1)pdm09 virus in Saudi Arabia. The nucleotide and amino acid sequence analysis and the phylogenetic relationship of HA and NA genes showed that the Saudi Arabian isolates were highly similar to selected reference isolates and most probably originated from the same clade of A(H1N1)pdm09. Thus, in addition to the HA and NA genes, molecular characterization of other gene segments of circulating influenza virus in the region is highly recommended for understanding genetic variation and reassortment events. Further antigenic analysis is also needed to assess the characteristics of the rest of the circulating viruses in Saudi Arabia, especially those that have variations in the antigenic and glycosylation sites of the HA and NA genes.

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