Journal of Infection and Public Health 12 (2019) 557–567

Contents lists available at ScienceDirect

Journal of Infection and Public Health

journal homepage: http://www.elsevier.com/locate/jiph

# Atypical influenza A(H1N1)pdm09 strains caused an influenza virus outbreak in Saudi Arabia during the 2009–2011 pandemic season

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ARTICLE INFO

*Article history:*
Received 4 December 2018
Received in revised form 22 January 2019
Accepted 30 January 2019

*Keywords:*
Influenza A(H1N1) virus
Phylogenetic
Saudi Arabia

ABSTRACT

*Background:* The triple assortment influenza A(H1N1) virus emerged in spring 2009 and disseminated worldwide, including Saudi Arabia. This study was carried out to characterize Saudi influenza isolates in relation to the global strains and to evaluate the potential role of mutated residues in transmission, adaptation, and the pathogenicity of the virus.
*Methods:* Nasopharyngeal samples (n = 6492) collected between September 2009 to March 2011 from patients with influenza-like illness were screened by PCR for influenza A(H1N1). Phylogenetic and Molecular evolutionary analysis were carried out to place the Saudi strains in relation to the global strains followed by Mutation analysis of surface and internal proteins.
*Results:* Concatenated whole-genome phylogenetic analysis along with hemagglutinin (HA) signature changes, that is, Aspartic Acid (D) at position 187, P83S, S203T, and R223Q confirmed that the Saudi strains belong to the antigenic category of A/California/07/2009. However, phylogenetic analysis revealed unusual strains of A(H1N1) circulating in Saudi Arabia, not belonging to any of known clades, appearing in five distinct groups well supported by group-specific mutations and novel mutation complexes. These cases had characteristic inter- and intragroup substitution patterns while few of their closest matches showed up as sporadic cases the world over. Specific mutation patterns were detected within the functional domains of internal proteins PB2, PB1, PA, NP, NS1, and M2 having a putative role in viral fitness and virulence. Bayesian coalescent MCMC analysis revealed that Saudi strains belonged to cluster 2 of A(H1N1)pdm09 and spread a month later as compared to other strains of this cluster.
*Conclusion:* Influenza outbreak in Saudi Arabia during 2009–2011 was caused by atypical strains of influenza A(H1N1)pdm09, probably introduced in this community on multiple occasions. To understand the antigenic significance of these novel point mutations and mutation complexes require functional studies, which will be crucial for risk assessment of emergent strains and defining infection control measures.



## Introduction

The influenza pandemic in 2009 was caused by the newly emerged triple assortment influenza A(H1N1) viruses, reported in patients with respiratory tract infections in Mexico and the United States [1,2]. Rapid human to human spread of these novel viruses affected more than 214 countries with a death toll of 18,449 by August 2010 [3]. Since August 2010, when WHO declared the end

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https://doi.org/10.1016/j.jiph.2019.01.067

of the (H1N1) 2009 pandemic (http://www.who.int/csr/don/2010_09_10/en/), this virus is treated as a seasonal virus, continuing to circulate with other seasonal viruses. Due to its devastating public health implications, keeping vigilance on the evolution of the virus has become increasingly important as this may influence selection of viruses to include in influenza vaccines.

Phylogenetic analysis of A(H1N1)pdm09 isolates collected globally have shown eight major clades co-circulating since April 2009 [4]. A(H1N1)pdm09 being a swine-originated influenza virus can undergo drastic genetic and phenotypic changes as a result of reassortment with other viruses and lead to the emergence of highly virulent and pathogenic strain. It has been suggested that A(H1N1)pdm09 is passing through continuous evolutionary phases [5], variants with higher fitness prevail and rapidly spread to the human population. Evolutionary analysis of contemporary gene sequences using phylogenetic and coalescent-based methods has been employed for different viruses which help in estimating viral divergence through time. Influenza A(H1N1)pdm09 strains were previously analyzed concatenated sequences which provided useful epidemiological information about the clusters circulated during the pandemic period [6]. This study paved the way for a subsequent study which effectively provided the estimates of the time of their transmission [7].

The HA and neuraminidase (NA) protein of influenza virus are the two most important envelope proteins of influenza viruses that continue to evolve under host immune pressure [5]. The HA gene is of special interest due to its role in viral entry into host cells and immune recognition. It plays a significant role in interspecies transmission, viral pathogenesis, and evasion of host immune response. If the virus acquires a segment in the HA gene from a different subtype as a result of reassortment, the resultant strain may possess pandemic potential [8,9]. Influenza virus uses its NA for two equally important purposes: first, to cleave sialic acid associated with mucins for mucosal penetration so as to reach functional receptors on target cells of the respiratory tract; second, the release of progeny viruses by breaking down sialic acid residues on the surface of infected cells [8–10]. The NA gene also exhibits genetic changes in its catalytic or framework sites of the protein, but not as frequent as HA [10]. The impact of amino- acid changes of HA and NA, therefore, have critical impacts on the epidemiology and clinical severity of the disease.

Rapid and accurate diagnosis as well as a timely delivery of appropriate vaccine are essential for the control of influenza spread. Least effective vaccines can still protect many people, but the influenza vaccine coverage in the general population and the compliance rate matters [11]. Data about influenza vaccine coverage in the Middle East, including Saudi Arabia is not available and presumed low as a survey of six major hospitals in Saudi Arabia showed lower compliance of ~38% even among health care practitioners [12].

Here we present evolutionary relationship of Saudi isolates with the global strains. We also investigated the genetic characteristics of the surface and internal genes in disease prognosis and circulation in Saudi Arabia during the 2009–2010 pandemic season.

## Materials and methods

### *Samples and clinical data collection*

Nasopharyngeal samples (n = 6492) were collected from patients with influenza-like illness who presented to King Abdulaziz Medical City in Riyadh City, Saudi Arabia during two consecutive seasons between September 2009 to March 2011. A total of 5215 (80.3%) samples were collected in 2009, 1137 (17.5%) samples in 2010, and 140 (2.16%) samples in 2011. Clinically a patient who had a combination of respiratory (cough, sore throat) and constitutional (fever, headache, muscle aches) symptoms was declared a possible case of influenza. In severe conditions patients required mechanical ventilation or admission to ICU for clinical management. Nasopharyngeal swabs were collected in 2 mL of viral transport medium and stored at −80 °C until processing. Detailed demographic and clinical data (symptoms, time of onset of symptoms, influenza vaccination status, and physical examination) were obtained from patients during their initial medical evaluation and according to the Saudi Ministry of Health (MOH) guidelines. Protocols for sample collection, storage and genomic analysis needed for the present study were approved by the Institutional Review Board of the institute and the clinical research was conducted according to the Declaration of Helsinki Principles.

### *Influenza virus detection*

Viral RNA was extracted directly from 400 μL of clinical sample (swab supernatant) using a QIAmp Viral RNA kit (Qiagen, Valencia CA, USA). Viral RNA was reverse transcribed into complementary DNA (cDNA) using the Superscript III Reverse Transcriptase (Invitrogen, USA) and stored at −80 °C. Influenza was confirmed in the sample by real-time ready influenza A(H1N1) Detection Kit (Roche Diagnostics, USA), containing specific primers and probes mix for detection of influenza A(H1) targeting the M2 gene using Roche Applied LightCycler.

### *Genomic amplification and sequencing*

Genomic amplification was performed on directly extracted RNAs from nasopharyngeal swabs without resorting to cell-culture virus isolation. All PCR amplifications of open reading frames (ORFs) was carried out in segments with the Hot start Taq DNA polymerase (Invitrogen, USA) with primers described previously [13] with the exception of HA and NA gene which were amplified using locally designed primers given in Table 2.

The PCR reaction started by denaturing at 95 °C for 2 min, and amplified in 45 cycles (ABI 9700, Applied Biosystems, CA, USA) under the following conditions, 95 °C for 30 s, 55 °C for 45 s, 72 °C for 1 min and a final extension at 72 °C for 15 min. The PCR products were visualized in 2% agarose gels and visualized by staining the gels with ethidium bromide. Gel purified DNA was sequenced in both directions by Sanger sequencing using a BigDye Terminator cycle sequencing kit (Applied Biosystem, California) and analyzed by 3100 automatic sequencer (Applied Biosystems). Sequences were assembled and manually corrected using Seqman implemented in DNASTAR Lasergene software.

### *Sequence alignment*

Phylogenetic analysis of the hemagglutin (HA) gene was carried out to place the Saudi isolates in the context of viruses responsible for epidemics in neighbouring countries and worldwide. Homologous BLAST search was carried out for each strain to determine the most closely related sequences. To have a composite phylogenetic tree we randomly removed sequences to create a dataset proportional to these newly isolated sequences, keeping in view that the homologous sequences stay in the final alignment.

Alignments of individual gene segments produced the following data sets for A(H1N1) Saudi viruses: PB2, 2280 bp; PB1, 2274 bp; PA, 2147 bp; HA, 1625 bp; NP, 1497 bp; NA, 1361 bp; M, 982 bp; NS, 863 bp. Phylogenetic analyses of each individual gene segment were performed to determine whether any of these sequences had a history of reassortment, placing the nucleotide sequences of vaccine and Hajj strains. Phylogenetic trees of these data were estimated using the maximum likelihood (ML) procedure in the RaxML version 8.2.10 [14] package, employing the GTR+G nucleotide substitution model and 100 bootstrap replicates.

*Bayesian evolutionary analysis*

To reconstruct the evolutionary history of Influenza AH1N1) in Saudi Arabia we built a concatenated alignment of Saudi H1N1 isolates and selected H1N1 reference sequences of 2009 influenza epidemic strains downloaded from sequence databases, that is, GenBank and GISAID. Due to narrow sampling time and low number of complete genome sequences, we were unable to precisely estimate the evolutionary rate. As reported earlier by Shiino et al., [7] normal prior distribution was used with a similar set of reference sequences because the substitution rate matched the analyzed sequence estimates. Evolutionary rates were then estimated using Bayesian Markov Chain Monte Carlo (MCMC) inference framework implemented in BEAST v1.8.0 [15]. We employed ModelGenerator (modelgenerator_v_85), an amino acid and nucleotide substitution model selection tool to compare different evolutionary models. Our analyses employed an HKY nucleotide substitution model and an uncorrelated lognormal relaxed molecular clock model. Bayes factors were compared, and model comparison test was run in Tracer v1.6 [16]. To measure the evolutionary change over time in our samples, we used both strict and relaxed (lognormal) molecular clock models and all included gamma-distributed rate. We used uniform prior distribution between 0 to 100 in our analysis. Each MCMC run contained 50 million states, sampled every 10,000 states. At least three MCMC runs were performed to ensure convergence and combined using log-combiner for better accuracy of parametric estimates. The obtained trees were summarized using TreeAnnotator v1.8.0 [15] and the Maximum clade credibility tree was visualized and annotated in Fig. 3 Tree v1.4.2.

*Mutational analysis*

The identification of mutations that were specific for the sequences (deduced amino acid sequences) from Saudi Arabia as compared to the reference sequence A/California/07/2009 was facilitated using the program BioEdit (available at www.mbio.ncsu.edu/bioedit/bioedit/html). The nucleotide sequences obtained in this study are available from GenBank under the accession numbers (LC092960 to LC093045).

## Results

Of the 6492 samples taken over the study period (September 2009 to March 2011), 1041 (16.03%) were positive for influenza A(H1N1) by PCR. The percent positivity in 2009 was 19.8% (1034/5215), which fell sharply in 2010 to 0.6% (7/1137) and no positive case detected in 2011. The age ranges of the patients, positive by RT-PCR were between 1 to 88 years. The distribution of the incident cases differed according to age: being highest in the age group 31–45 years, moderate in the age group 46–60 years and lowest in people aged >60 years (Fig. 1). Except for one patient who had a travel history within the Middle East, none of the other cases traveled outside of Saudi Arabia.

*Phylogenetic analysis*

The phylogenetic analysis shown in Fig. 2A was obtained by comparing Saudi sequences ($n = 43$) in the HA gene with reference sequences downloaded from GenBank/GISAID databases. Sample names are composed of the city of the case, internal laboratory code and the year of isolation. Analysis of the phylogenetic relationship with clade-specific global strains showed that Saudi strains did not fall into any recognized A(H1N1) clades (Fig. 2A). The viruses containing critical mutations were distributed throughout the phylogenetic tree in separate groups. Phylogenetic analysis of each gene segment did not show any evidence of reassortment and seemingly have the ancestry in the viruses of clade 1, quite different from the Hajj strains (Fig. 2B and Supplementary Fig. S4).

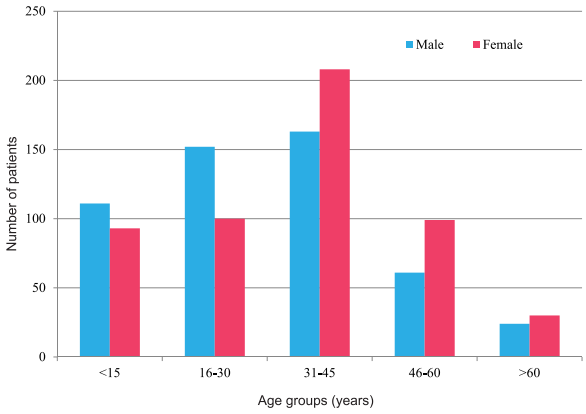


**Fig. 1.** Influenza A(H1N1)pdm09 positivity among different age groups in Riyadh, Saudi Arabia.

**Table 1**
Demographic and clinical characteristics of the sequenced samples (n = 49).

| Features | *n* (%) |
|---|---|
| Age (mean ± SD) | 19.5 ± 20.5 |
| Age range | 1–74 |
| <18 | 27 (55.1) |
| 18–59 | 21 (42.8) |
| ≥60 | 1 (2) |
| Gender | |
| Male | 23 (46.9) |
| Female | 26 (53) |
| Seasonal flu vaccination | |
| No | 46 (93.8) |
| Yes | 3 (6.1) |
| Exposure | |
| Sick household | 6 (12.2) |
| Other cases | 3 (6.1) |
| Flu symptoms | |
| Coughing | 24 (49) |
| Fever | 19 (38.7) |
| Shortness of breath | 13 (26.5) |
| Vomiting | 13 (26.5) |
| Rhinorrhea | 7 (14.3) |
| Diarrhea | 5 (10.2) |
| Headache | 5 (10.2) |
| Muscle aches | 5 (10.2) |
| Sore throat | 4 (8.2) |
| Comorbidity | |
| Cardiopulmonary | 11 (22.4) |
| Kidney disease | 3 (6.1) |
| Immunosuppressive disease | 1 (2) |
| Neurologic disease | 1 (2) |
| Admission | |
| Non-ICU | 35 (71.4) |
| ICU | 6 (12.2) |
| Death | 1 (2) |
| Hospital stay days | 6.3 ± 6.7 |

*Maximum clade credibility phylogeny*

Fig. 3 shows the MCC phylogeny obtained from the Bayesian molecular clock analysis of the concatenated full genome sequences of influenza A (H1N1) strains. The mean nucleotide substitution rate was $5.046 \times 10^{-3}$ substitution per site per year (95% credibility ranged $4.4 \times 10^{-3}$ to $5.21 \times 10^{-3}$). MCMC phylogeny grouped Saudi sequences in a distinct phylogenetic cluster within previously described cluster 2 of influenza A(H1N1)pdm09 [6,7]. The Bayesian chronological phylogeny also showed that cluster 1 had an older ancestor than cluster 2. Cluster 1 has previously been reported containing two sub-clusters, that is, 1.1, 1.2, and 1.3 [6].

Sub-cluster 1.1 was paraphyletic having four distinct microclusters (MC1-MC4). Microclusters were defined being monophyletic and possessing high posterior values >0.9. MC1 contained the vaccine strain from California and the estimated MRCA this cluster

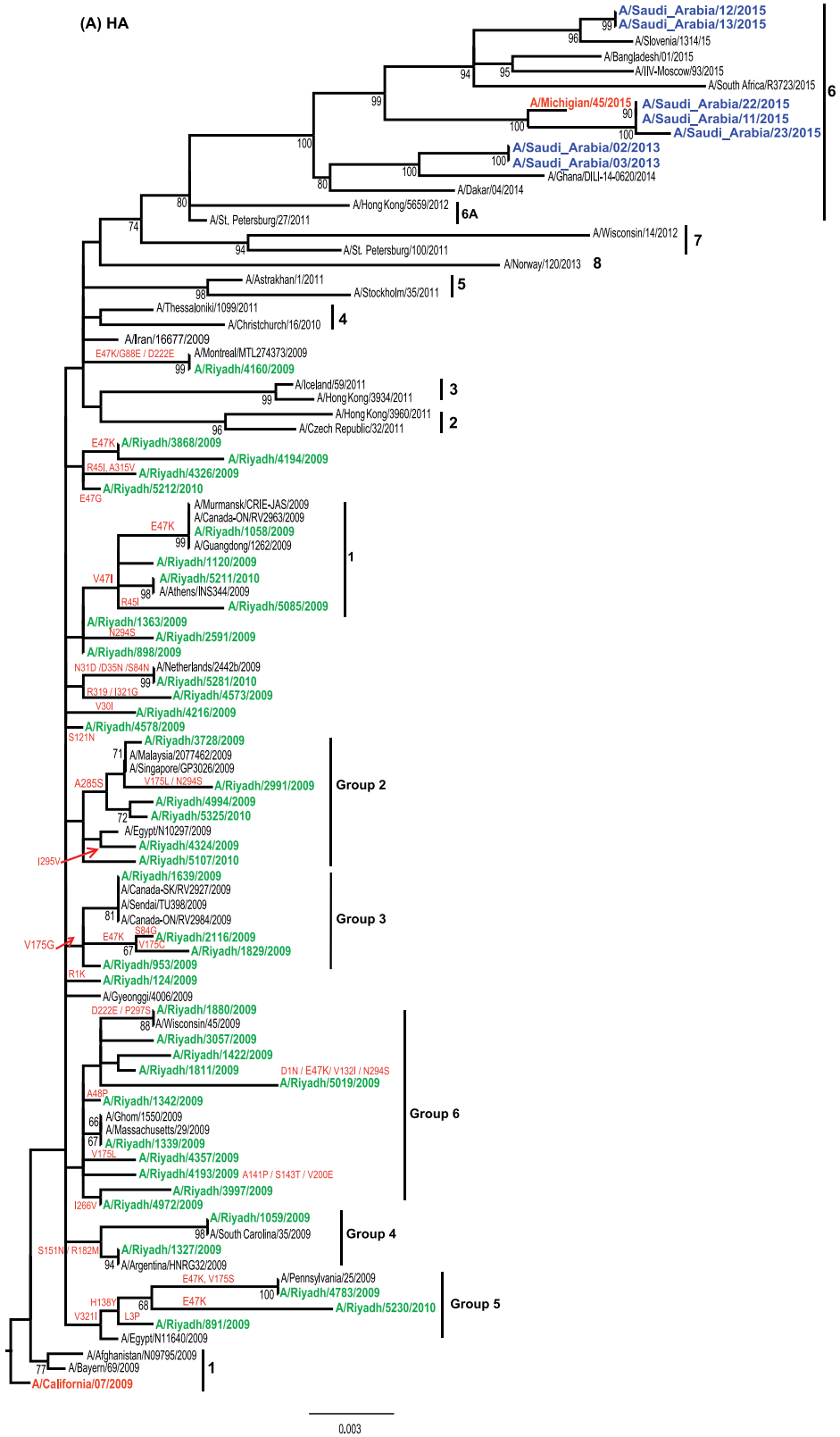


**Fig. 2.** Phylogenetic relationships of the HA and NA gene segments of A(H1N1) viruses, as indicated. Reference sequences in 2A are labelled by clades names and amino acid substitutions found are given for individual isolate or in group. Saudi strains isolated in this study are given in green colour. Sample names are composed of the city of the case, internal laboratory code and the year of isolation. Publicly available vaccine strains were included as out groups and shown in red. Phylogenies were rooted using the A/California/07/2009 sequence. Statistical support for individual nodes was estimated from 1000 bootstrap replicates, and values are represented as percentages on the nodes (values of ≥70% are shown). Scale bars are proportional to the number of nucleotide substitutions per site.

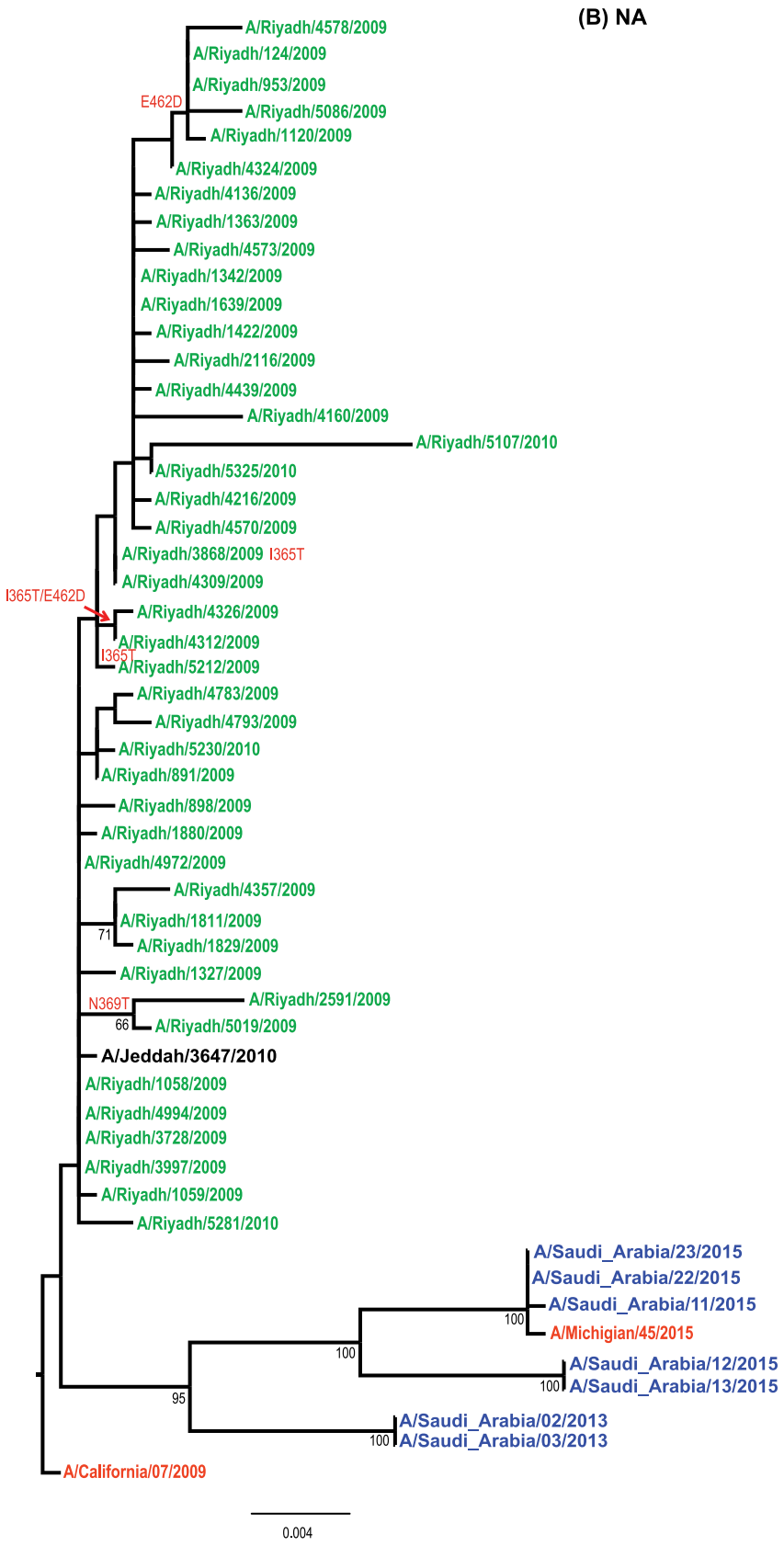


**Fig. 2.** (*Continued*)

was March 27, 2009 (95% HPD from March 17-April 19). MC2 contained sequences from Texas and the estimated MRCA of this cluster was April 8, 2009 (95% HPD from March 25-May 02). MC3 contained sequences from the diverse geographical background and the estimated MRCA of this cluster was February 23, 2009 (95% HPD from February 02-March 21). MC4 contained sequences from the

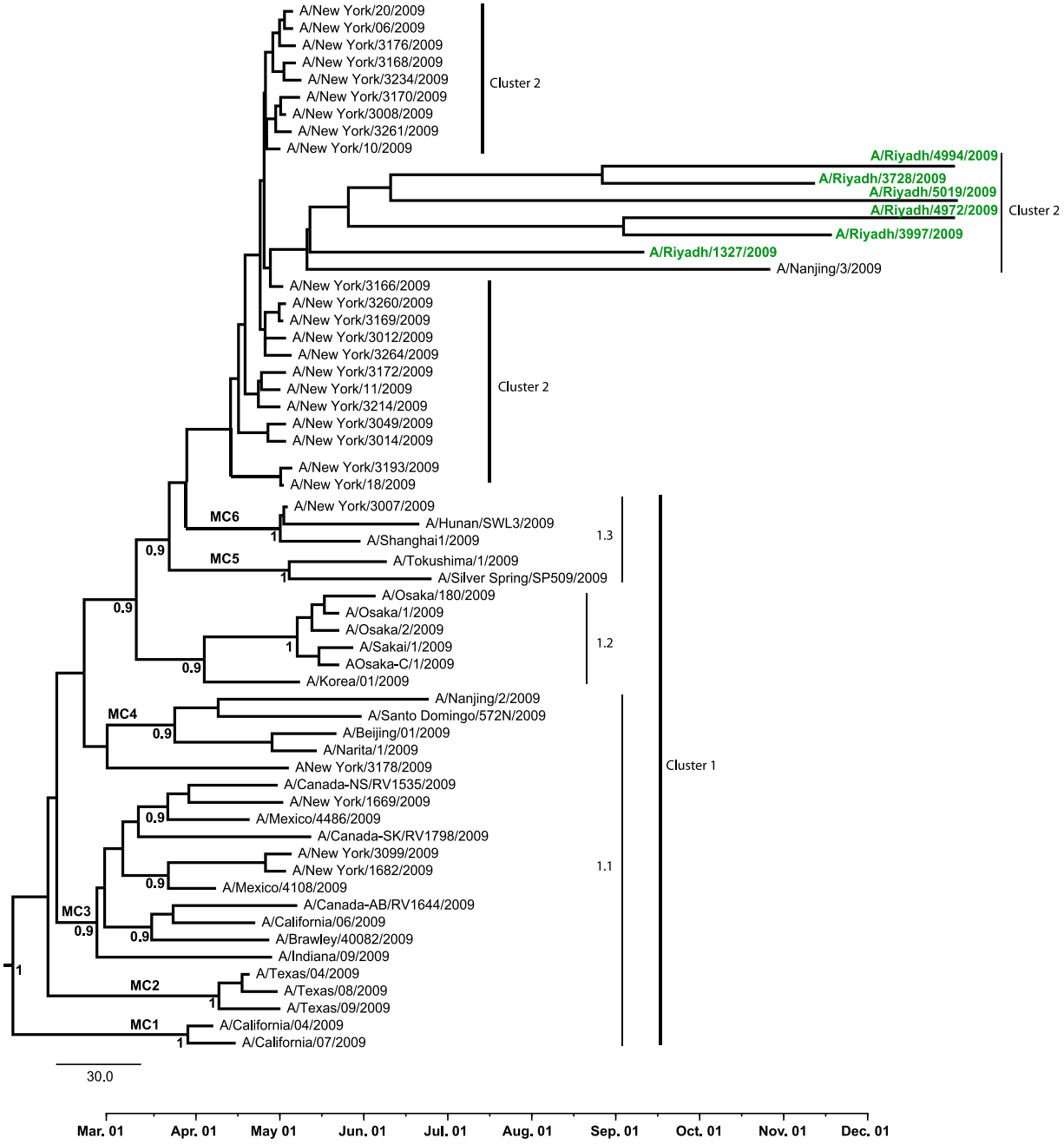


**Fig. 3.** Maximum clade credibility molecular clock phylogeny estimated from concatenated alignment. Nodes with a posterior probability >0.9 are labelled. Branch lengths represent time (see scale bar at the bottom of the figure). The scale bar indicating 30 days of the branch length. The clusters described are labeled on the right of the tree. The sequences from Saudi Arabia are colored in green. MC represents micro-cluster.

Asian region and the estimated MRCA of this cluster was February 27, 2009 (95% HPD from January 28-April 01). Subcluster 1.2 contained sequences mainly from Japan with an estimated MRCA of this cluster was April 03, 2009 (95% HPD from March 21-April 28) at which the Korean strain separates out while the MRCA of Japanese sequences was May 16, 2009 (95% HPD from May 10-May 29). Sub-cluster 1.3 contained two microclusters MC5 and MC6. The estimated MRCA of cluster MC5 was May 05, 2009 (95% HPD from April 23-June 05) and of MC6 was May 02, 2009 (95% HPD from April 26-May 15). The estimated MRCA of cluster 2 was April 15, 2009 (95% HPD from April 05-May 06). As previously reported [7], Viruses belonging to cluster 2 showed more continuous local chains of transmission. This MCMC phylogeny has shown that atypical Saudi isolates transmitted later in the season with an estimated MRCA of May 13, 2009 (95% HPD from May 01-June 25).

### *Sequence analysis of the surface genes*

Out of 1041 influenza A(H1N1) positive cases, genetic analysis was conducted in 49 HA and NA gene sequences with compatible sequence lengths (37 paired and 12 solitary sequences). Detail demographic and clinical data of these 49 cases is given in Table 1. The Saudi sequences had Aspartic Acid (D) at position 187 along with the signature changes P83S, S203T, R223Q in HA resembling the antigenic category of A/California/07/2009. Mutations observed in the HA gene of Saudi isolates with respect to the prototype A/California/07/2009 are presented in (Table 3) and

**Table 2**
Influenza primers in the H1 and N1 region used to amplify Saudi-H1N1 isolates.

| Name of Primer | Primer Sequence | Gene | Localization | PCR Product |
|---|---|---|---|---|
| H1-F1 | 5′-CCGCAAATGCAGACACATTA-3′ | HA | 61–80 | 543 |
| H1-R1 | 5′-CCCCATAGCACRAGGACTTC-3′ | HA | 604–585 | |
| H1-F2 | 5′-GAATGTRACAGTRACACACTCTG-3′ | HA | 137–159 | 947 |
| H1-R2 | 5′-CCTTCAATGAAACCAGCAATG-3′ | HA | 1084–1066 | |
| H1-F3 | 5′-CTAGAGGCCTATTTGGGGCC-3′ | HA | 1048–1067 | 645 |
| H1-R3 | 5′-CCCATTAGAGCACATCCAGAA-3′ | HA | 1693–1674 | |
| N1-F1 | 5′-CCAAAAGATAATAACCATTGGTTCG-3′ | NA | 12–36 | 735 |
| N1-R1 | 5′-TCCATTACTTGGTCCATCGGTC-3′ | NA | 747–726 | |
| N1-F2 | 5′-GCTGTGTTAAAGTACAACGGCATA-3′ | NA | 609–633 | 786 |
| N1-R2 | 5′-TGTCAATGGTAAATGGCAACTCAG-3′ | NA | 1395–1382 | |

HA = Hemagglutinin, NA = Neuraminidase.

**Table 3**
Amino acid change summary in the HA protein of Saudi Arabia A(H1N1)pdm09 isolates from 2009 to 2010 influenza season.

| Amino acid position | 35 | 45 | 47 | 83 | 84 | 138 | 175 | 187 | 203 | 222 | 223 | 266 | 285 | 294 | 321 | 374 | 478 | 509 | 538 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A/California/07/2009 | D | R | V | P | S | H | V | D | S | D | R | I | A | N | I | E | S | R | C |
| A/Riyadh/124/2009 | – | – | – | S | – | – | – | – | T | – | Q | – | – | – | V | – | – | – | – |
| A/Riyadh/891/2009 | – | – | – | S | – | Y | – | – | T | – | Q | – | – | – | – | – | – | – | – |
| A/Riyadh/898/2009 | – | – | – | S | – | – | – | – | T | – | Q | – | – | – | V | – | – | – | – |
| A/Riyadh/953/2009 | – | – | – | S | – | – | G | – | T | – | Q | – | – | – | V | – | – | – | – |
| A/Riyadh/1058/2009 | – | – | I | S | – | – | – | – | T | – | Q | – | – | – | V | K | – | – | – |
| A/Riyadh/1059/2009 | – | – | – | S | – | – | – | – | T | – | Q | – | – | – | V | – | N | M | S |
| A/Riyadh/1120/2009 | – | – | I | S | – | – | – | – | T | – | Q | – | – | – | V | – | – | – | – |
| A/Riyadh/1327/2009 | – | – | – | S | – | – | – | – | T | – | Q | – | – | – | V | – | N | M | – |
| A/Riyadh/1339/2009 | – | – | – | S | – | – | – | – | T | – | Q | – | – | – | V | – | – | – | – |
| A/Riyadh/1342/2009 | – | – | – | S | – | – | – | – | T | – | Q | – | – | – | V | – | – | – | – |
| A/Riyadh/1363/2009 | – | – | – | S | – | – | – | – | T | – | Q | – | – | – | V | – | – | – | – |
| A/Riyadh/1422/2009 | – | – | – | S | – | – | – | – | T | – | Q | – | – | – | V | – | – | – | – |
| A/Riyadh/1639/2009 | – | – | – | S | – | – | G | – | T | – | Q | – | – | – | V | – | – | – | – |
| A/Riyadh/1811/2009 | – | – | – | S | – | – | – | – | T | – | Q | – | – | – | V | – | – | – | – |
| A/Riyadh/1829/2009 | – | – | – | S | – | – | C | – | T | – | Q | – | – | – | V | K | – | – | – |
| A/Riyadh/1880/2009 | – | – | – | S | – | – | – | – | T | E | Q | – | – | – | V | – | – | – | – |
| A/Riyadh/2116/2009 | – | – | – | S | G | – | G | – | T | – | Q | – | – | – | V | K | – | – | – |
| A/Riyadh/2591/2009 | Y | – | – | S | – | – | – | – | T | – | Q | – | – | S | V | – | – | – | – |
| A/Riyadh/2991/2009 | – | – | – | S | – | – | L | – | T | – | Q | – | S | S | V | – | – | – | – |
| A/Riyadh/3057/2009 | – | – | – | S | – | – | – | – | T | – | Q | – | – | – | V | – | – | – | S |
| A/Riyadh/3728/2009 | – | – | – | S | – | – | – | – | T | – | Q | – | S | – | V | – | – | – | – |
| A/Riyadh/3868/2009 | – | – | – | S | – | – | – | – | T | – | Q | – | – | – | V | K | – | – | – |
| A/Riyadh/3997/2009 | – | – | – | S | – | – | – | – | T | – | Q | V | – | – | – | – | – | – | S |
| A/Riyadh/4160/2009 | – | – | – | S | – | – | – | – | T | E | Q | – | – | – | V | K | – | – | – |
| A/Riyadh/4193/2009 | – | – | – | S | – | – | – | – | T | – | Q | – | – | – | V | – | – | – | – |
| A/Riyadh/4194/2009 | – | – | – | S | – | – | – | – | T | – | Q | – | – | – | V | K | – | – | – |
| A/Riyadh/4216/2009 | – | – | – | S | – | – | – | – | T | – | Q | – | – | – | V | – | – | – | – |
| A/Riyadh/4324/2009 | – | – | – | S | – | – | – | – | T | – | Q | – | – | – | V | – | – | – | – |
| A/Riyadh/4326/2009 | – | I | I | S | – | – | – | – | T | – | Q | – | – | – | V | – | – | – | – |
| A/Riyadh/4357/2009 | – | – | – | S | – | – | – | – | T | – | Q | – | – | – | V | – | – | – | – |
| A/Riyadh/4573/2009 | – | – | – | S | – | – | L | – | T | – | Q | – | – | – | G | – | – | – | F |
| A/Riyadh/4578/2009 | – | – | – | S | – | – | – | – | T | – | Q | – | – | – | V | – | – | – | – |
| A/Riyadh/4783/2009 | – | – | – | S | – | Y | S | – | T | – | Q | – | – | – | – | K | – | – | – |
| A/Riyadh/4972/2009 | – | – | – | S | – | – | – | – | T | – | Q | V | – | – | V | – | – | – | – |
| A/Riyadh/4994/2009 | – | – | – | S | – | – | – | – | T | – | Q | – | S | – | V | – | – | – | – |
| A/Riyadh/5019/2009 | – | – | – | S | – | – | – | – | T | – | Q | – | – | S | V | K | – | – | – |
| A/Riyadh/5085/2009 | – | I | I | S | – | – | – | – | T | – | Q | – | – | – | V | – | – | – | R |
| A/Riyadh/5107/2010 | – | – | – | S | – | – | – | – | T | – | Q | – | – | – | V | – | – | – | – |
| A/Riyadh/5211/2010 | – | – | I | S | – | – | – | – | T | – | Q | – | – | – | V | – | – | – | – |
| A/Riyadh/5212/2010 | – | – | – | S | – | – | – | – | T | – | Q | – | – | – | V | – | – | – | – |
| A/Riyadh/5230/2010 | – | – | – | S | – | Y | A | – | T | – | Q | – | – | – | – | – | – | – | – |
| A/Riyadh/5281/2010 | N | – | – | S | N | – | – | – | T | – | Q | – | – | – | V | – | – | – | F |
| A/Riyadh/5325/2010 | – | – | – | S | – | – | – | – | T | – | Q | – | S | – | V | – | – | – | . |

have been annotated in (Fig. 2A). According to the amino acid substitution pattern, sequences were classified into five groups. Group 1 had a signature amino acid change in HA2 subunit of unknown function 'V47I' detected in 4/43 (9.3%) cases. Group 2 included sequences with variations in major glycosylation site of HA important in disease severity, that is, A285S present in 4/43 (9.3%) and I295V in one isolate (2.3%). A fickle substitution of unknown function was detected at residue 175 (V175S/C/G/L) in 8/43 (18.6%) isolate; out of which four sequences with V175G clustered in rather smaller group 3. Group 4 included 2/43 (4.6%) study sequences, two with double mutation S478N/R509M (S151N and R182M in HA2 subunit). One of the cases with double mutation S151N/R182M had underlying asthma and heart disease while the rest did not have comorbidity causative of influenza-like illness. Group 5 included five sequences, 3/43 (6.9%) from Saudi Arabia and one strain each from Egypt and USA. This group represented by isoleucine at position 321 (I321) which didn't change to V321, commonly found in the isolates from the post-pandemic period. A mutation in the Ca2 antigenic site H138Y was detected in combination as double mutation H138Y/L329P (L2P of fusion peptide in HA2 subunit) and H138Y/E47K in 2/43 (4.6%) and as triple mutant H138Y/E47K/H175S in one Saudi iso-

late along with the USA strain. One of the notable amino acid substitution E374K (E47K in HA2 subunit) was found in 9/43 (20.9%) isolates randomly distributed in the phylogenetic tree. Two out of nine cases with E47K mutation appeared to have underlying asthma along with the cardiac and renal disease. A diversity of other substitutions detected spatially or in combination with others were C538A/F/R (residue 311 in HA2 subunit) in 6/43 (13.9%), N294S in 3/43 (6.9%), D222E, I266V, D35Y/N, and S84G/N in 2/43 (4.6%) isolates. There was a case with double mutation R319K/I321G, and another one with triple mutation N31D/D35N/S84N (Fig. 2A).

Mutations observed in the NA gene of Saudi isolates with respect to the prototype A/California/07/2009 are presented in Table 4 and annotated in Fig. 2B. All of the Saudi isolates possessed V106I, N248D, and residue H275 (Table 4). The novel NA mutations detected in Saudi strains were E462D in 7/43 (16.2%), I365T in 5/43 (11.6%), and N369T in 2/43 (4.6%) isolates. We did not detect amino acid substitutions A247N, I223V or R [17], known to reduce the susceptibility to NAI in Saudi isolates.

*Sequence analysis of the internal genes*

The internal proteins of Saudi A(H1N1)pdm09 isolates analyzed revealed substitutions P224S in PA, V100I, and L122Q in NP, I123V in NS1 and N31 in M2, which were common to all Saudi isolates with available sequences in the specific regions. The other noteworthy substitutions included amino acid change in M2 protein E14G in 12/18 (66.6%) and an insertion of 8 amino acids in 15/18 (83.3%) cases between amino acid positions 151–152 in the NS1 protein (Table 4). Minor variations detected in internal genes included E188G (2/9; 22.2%), V400A (2/16; 12.5%), T588I (3/16; 18.7%) in PB2, V113A (2/16; 12.5%) in PB1, M579I (3/14; 21.4%), D478N (2/14; 14.3%), E610D (2/14; 14.3%) in PA, R38Q (2/5; 40%), V362A (2/17; 11.7%), and K400R (2/17; 11.7%) in NP (Table 5).

## Discussion

Surveillance studies from different parts of the world have shown that A(H1N1)pdm09 infection has different clinical pattern than seasonal influenza and is often associated with a higher risk of severe disease, hospitalization, and ICU admission. During 2009–2010, we confirmed a total of 1041 cases positive for A(H1N1), a higher proportion belonged to young age patients, while the incidence was quite low in the age group over 60 years. This is

**Table 4**
Amino acid change summary in the NA protein of Saudi Arabia A(H1N1pdm09 isolates from 2009 to 2010 influenza season.

| Amino acid Changes | 106 | 248 | 275 | 365 | 369 | 462 |
|---|---|---|---|---|---|---|
| A/California/07/2009 | V | N | H | I | N | E |
| A/Riyadh/124/2009 | I | D | H | – | – | D |
| A/Riyadh/891/2009 | I | D | H | – | – | – |
| A/Riyadh/898/2009 | I | D | H | – | – | – |
| A/Riyadh/953/2009 | I | D | H | – | – | D |
| A/Riyadh/1058/2009 | I | D | H | – | – | – |
| A/Riyadh/1059/2009 | I | D | H | – | – | – |
| A/Riyadh/1120/2009 | I | D | H | – | – | D |
| A/Riyadh/1327/2009 | I | D | H | – | – | – |
| A/Riyadh/1342/2009 | I | D | H | – | – | – |
| A/Riyadh/1363/2009 | I | D | H | – | – | – |
| A/Riyadh/1422/2009 | I | D | H | – | – | – |
| A/Riyadh/1639/2009 | I | D | H | – | – | – |
| A/Riyadh/1811/2009 | I | D | H | – | – | – |
| A/Riyadh/1829/2009 | I | D | H | – | – | – |
| A/Riyadh/1880/2009 | I | D | H | – | – | – |
| A/Riyadh/2116/2009 | I | D | H | – | – | – |
| A/Riyadh/2591/2009 | I | D | H | – | T | – |
| A/Riyadh/3728/2009 | I | D | H | – | – | – |
| A/Riyadh/3868/2009 | I | D | H | T | – | – |
| A/Riyadh/3997/2009 | I | D | H | – | – | – |
| A/Riyadh/4136/2009 | I | D | H | – | – | – |
| A/Riyadh/4160/2009 | I | D | H | – | – | – |
| A/Riyadh/4216/2009 | I | D | H | – | – | – |
| A/Riyadh/4309/2009 | I | D | H | T | – | |
| A/Riyadh/4312/2009 | I | D | H | T | – | D |
| A/Riyadh/4324/2009 | I | D | H | – | – | – |
| A/Riyadh/4326/2009 | I | D | H | T | – | D |
| A/Riyadh/4357/2009 | I | D | H | – | – | – |
| A/Riyadh/4439/2009 | I | D | H | – | – | – |
| A/Riyadh/4570/2009 | I | D | H | – | – | – |
| A/Riyadh/4573/2009 | I | D | H | – | – | – |
| A/Riyadh/4578/2009 | I | D | H | – | – | D |
| A/Riyadh/4783/2009 | I | D | H | – | – | – |
| A/Riyadh/4793/2009 | I | D | H | – | – | – |
| A/Riyadh/4972/2009 | I | D | H | – | – | – |
| A/Riyadh/4994/2009 | I | D | H | – | – | – |
| A/Riyadh/5019/2009 | I | D | H | – | T | – |
| A/Riyadh/5086/2009 | I | D | H | – | – | D |
| A/Riyadh/5107/2010 | I | D | H | – | – | – |
| A/Riyadh/5212/2010 | I | D | H | T | – | – |
| A/Riyadh/5230/2010 | I | D | H | – | – | – |
| A/Riyadh/5281/2010 | I | D | H | – | – | – |
| A/Riyadh/5325/2010 | I | D | H | – | – | – |

**Table 5**
Non-Synonymous mutations detected in Internal genes of Saudi Arabia A(H1N1)pdm09 isolates from 2009 to 2010 influenza season.

| Strain | PB2 | PB1 | PA | NP | NS1 | M2 |
|---|---|---|---|---|---|---|
| A/Riyadh/4994/2009 | – | V113I | P224S | V100I, L122Q | I123V | E14G |
| A/Riyadh/1327/2009 | E188G | – | P224S | V100I, L122Q | I123V | – |
| A/Riyadh/1339/2009 | E188G | – | M579I | V100I, L122Q | R38Q, I123V | E14G |
| A/Riyadh/3997/2009[a] | V400A, T588I | – | P224S, D478N, E610D | V100I, L122Q, K400R | I123V | – |
| A/Riyadh/1120/2009 | – | – | P224S | V100I, L122Q | I123V | E14G |
| A/Riyadh/4972/2009 | V400A, T588I | – | P224S, D478N, E610D | V100I, L122Q, K400R | I123V | E14G |
| A/Riyadh/1363/2009 | – | – | P224S, M579I | V100I, L122Q | I123V | E14G |
| A/Riyadh/4573/2009 | T588I | NA | P224S | V100I, L122Q, V363A | I123V | E14G |
| A/Riyadh/953/2009 | – | – | NA | NA | I123V | NA |
| A/Riyadh/1422/2009 | – | NA | P224S | – | I123V | E14G |
| A/Riyadh/1059/2009 | – | – | P224S | V100I, L122Q | I123V | E14G |
| A/Riyadh/1723/2009 | – | NA | P224S | V100I, L122Q, V363A | I123V | E14G |
| A/Riyadh/1433/2009 | – | – | P224S, M579I | H334P | I123V | E14G |
| A/Riyadh/3728/2009[a] | – | V113I | P224S | V100I, L122Q | I123V | E14G |
| A/Riyadh/5019/2009 | – | – | P224S | V100I, L122Q | I123V | – |
| A/Riyadh/5107/2009 | – | – | NA | V100I, L122Q | I123V | – |
| A/Riyadh/4326/2009 | – | NA | P224S | V100I, L122Q, H334P | I123V | E14G |
| A/Riyadh/5212/2009 | NA | NA | NA | V100I, L122Q | I123V | – |
| A/Riyadh/1880/2009[a] | NA | – | NA | V100I, L122Q | R38Q, I123V | – |

[a] All strains had 8 amino acid insertion in the NS1 protein between amino acid numbers 151 and 152 except marked strains; NA = Sequence not available.

consistent with previous reports which divulge that younger age people more likely are prone to catch influenza A(H1N1) infection, contrariwise to seasonal flu, which is heavily implicated in the older people [18,19]. The possible explanation is the availability of cross-reacting antibodies in the people aged 60 years and above, providing them some protection against the pandemic H1N1 virus [18–21].

Phylogenetic analysis revealed unusual strains of A(H1N1) circulating in Saudi Arabia, not belonging to any of known clades. Our phylogenetic analysis could not show any relationship with Hajj strains indicating that these viruses were evolved during the pandemic season and had nothing to do with mass gathering (Hajj) in Saudi Arabia. Consistent with previous findings our results provide additional evidence that the A(H1N1)pdm09 viruses diverged into two broadly distinct clusters (Fig. 3). MCMC analysis showed that tMRCA of all the strains was January 30, 2009, the time it was generated in swine and first swine to human transmission gave rise to the cluster 1. Our results stayed in agreement with previous findings and showed that tMRCA of cluster 1 preceded that of cluster 2 by two months [7]. iMCMC analysis revealed that Saudi strains belonged to cluster 2 of A(H1N1)pdm09 virus. tMRCA of Saudi strains succeeded to the other strains in this cluster by one month (Fig. 3). In general, the viruses belonging to cluster 2 appeared with rather less genetic difference between them as shown by short branch lengths but more successful continuous transmission. Long branching of Saudi strains suggests genome-wide rate increase of molecular evolution (compared to sister group) as seen in the form of novel mutation patterns in different genes (Tables 2–4) representing specific adaptive traits. Gene-specific rate variation having impact on the overall phylogeny is highly unlikely as individual gene-specific trees did not vary much as compared to reference strains (Supplementary Fig. S4).

The present molecular study indicates the changes in the HA gene sequences attributable to genetic evolution of the influenza A(H1N1)pdm09 viruses which circulated in Saudi Arabia during the season 2009–2011. HA phylogeny of 43 sequences identified 5 distinct groups of viruses, defined by specific mutations, co-circulating since the beginning of the 2009 season (Fig. 2A). Characteristic inter- and intragroup substitution complexes and their epidemiological information as given by HA phylogeny (Fig. 2A) illustrated that these strains introduced in this population on multiple occasions. Group 1 included strains with signature substitutions V47I in HA2 subunit. The role of V47I in the clinical severity of the disease is unknown; however, unlike Spanish study [22] where V47I was found in conjunction with E172K and K308E, this mutation was acting alone or in combination with an adjacent mutation R45I in our strains. None of these cases were vaccinated and found no comorbidities associated along with influenza-like illness. Group 2 contained sequences with substitutions between positions 284–299 (a major glycosylation site), potentially associated with clinical severity of disease [23]. None of the cases in this group had any kind of comorbidity ruling out other causes of their hospitalization except the influenza illness. Changes in the receptor binding site (RBS) residues between 170–230 are considered critical in antigen recognition and viral pathogenicity [23–27]. HA of the Saudi isolates, as in other H1N1pdm09 viruses possessed D187 in RBS, which is known to facilitate H1 viruses to bind human receptors and efficient virus transmission in humans [28]. Additionally, we detected a fickle substitution at residue 175 in eight isolates, four grouped with Japanese and Canadian isolates in group 3, while the rest were dispersed in the phylogenetic tree. None of these cases had any underlying comorbidity and there seems more likely influenza viral impact in their clinical state. Strains in group 4 with double S151N/R182M and triple mutation N31D/D35N/S84N have previously been reported from the United States (unpublished), Argentina [29]. Group 5 signature mutation I321 resides near the cleavage site and is well recognized for its role in the worst outcome of the disease [24,27,30]. This group had closely similar cases identified previously from Egypt and the United States (USA). The interactive role of additional mutations H138Y, a mutation in the antigenic site Ca2 [31], E47K, L3P, and V175S need to be elucidated. None of the cases in this group had any underlying comorbidity. One of the random mutations E47K found in nine of our isolates either alone or in combination is a well-diversified substitution across A(H1N1) clades except for clade 6B.2 and 3. E47K reportedly alter the salt bridge pattern stabilizing a region of HA oligomerization interface which is a known antigenic site and crucial for membrane fusion [32]. This mutation might have contributed to influenza virus stability and rapid transmission in Saudi Arabia. Accumulated evidence demonstrate that this change provides the influenza virus high adaptive capacity in tropical climates like Saudi Arabia [13,24,32–34] and transmissibility is quite common in the post-peak period [24]. The substitution at the 220-loop region (D222/G) is considered a potential marker of severity of infection with an increased likelihood of fatal outcomes [26]. Two of study isolates presented with apparently non-fatal change D222/E, however its role in viral fitness may not be ignored. Recently, an Italian study showed that viruses with D222E favored a successful transmission among children forming a distinct phylogenetic cluster [26]. Group 6 contained sequences with random single-, double-, triple and tetra-amino acid substitutions. To know the clinical significance of these combo-substitutions calls for functional studies.

Similar genetic analysis of the NA gene revealed amino acid changes V106I and N248D in all Saudi isolates (Table 3). The acquisition of double mutation results in low-pH-stable NA allows efficient adaptation of the pandemic A(H1N1)2009 virus and enhances virus replication in humans [35]. H275Y resistance mutation causes a substantial decrease in surface-expressed neuraminidase and its activity [36]. It has been reported that secondary mutations counteracted the diminishing effect of H275Y on neuraminidase activity in seasonal H1N1 [37]. In pandemic H1N1, H275Y found the rare but potential role of other mutations found in the internal genes leading to oseltamivir resistance cannot be excluded. We identified strains with novel mutations E462D, I365T, and N369T in the NA gene of quite a few Saudi strains. Whether these mutations have any role in enhancing surface expression of an oseltamivir-resistant influenza virus NA protein and/or in the replicative capacity and virulence, needs to be explored.

A(H1N1)pdm09 genetic variant strains in Saudi Arabia had characteristic genetic variations in the internal genes (Table 5), that is, V100I and L122Q in NP, P224S in PA, and I123V in NS. Substitution in the NP gene reflect the changes in the body domain of the protein considered to be involved in PB2 and PB1–NP interaction crucial for RNA replication [38,39]. The amino acid substitution V100I stimulated the raising of the pandemic alert from phase 4 to 6 and is a human to avian signature change causing enhanced viral fitness probably through increasing viral transmissibility or infectivity [38]. The phenotypic outcome of L122Q has not been established in A (H1N1), however, this mutation has been previously associated with increased replication capacity of H5N1 viruses. PA gene mutation 'P224S' is in the N-terminal domain of the protein. This region is required to perform endonuclease activity to initiate transcription; therefore, genetic changes in this region might affect virus replication potential [40]. I123V is located in the effector domain of NS1 considered to have a role in adaptation to the human host and to increase virulence [38]. The finding of 8 amino acids insertion in NS1 in Saudi isolates is novel and its importance in adaptation to the human host or virulence need to be established. E14G in M2 gene is a differential mutation less frequently found in swine- and avian-origin A(H1N1) influenza virus. In fact, the combination of E14-F55 is considered a molecular determinant associated with its human-to-human transmission. M2 stability and functionality

is based on its extracellular (M2e) and cytoplasmic (CT) domains where E14 lies in the extracellular domain and F55 in the cytoplasmic domain. It is suggested that the combined mutation of residues in M2e (E14), and CT (F55) may synergistically alter the structure and function of the M2 protein refining the virus to human specificity and thereby increase its human to human transmission and virulence [41,42]. Mutations in the polymerase basic protein 2 (PB2) of influenza viruses have been associated with virulence. Mutation T588I detected in 3 of Saudi isolates is of critical importance. It is known that A(H1N1)pdm/09 influenza virus develops high pathogenicity upon the acquisition of the PB2-T588I mutation. The variant is known to induce lower levels of host IFN-$\beta$ expression. In mammalian cells, this mutation significantly enhanced polymerase activity, and *in vivo*, the variant exhibited high viral replication and significant weight loss in a mouse model compared to the wild-type [43].

Some limitations of the current study were: samples were not tested other than influenza A(H1N1)pdm09 virus, therefore co-circulation of other influenza types could not be ruled out; sequence analysis was performed on a small sample size and a small group of isolates were amplifiable covering all gene segments; sensitivity testing for Oseltamivir including a large sample size would have provided subtle information about these atypical strains.

## Conclusion

Influenza outbreak in Saudi Arabia during 2009–2011 was caused by atypical strains of influenza A(H1N1)pdm09, probably introduced in this community on multiple occasions. These lineages with novel point mutations and mutation complexes are currently rare and geographically restricted, however, can potentially spread epidemically and internationally. To reveal the antigenic significance of these variations requires functional studies which will be decisive to estimate the risk of emergent strains and defining infection control measures.

## Author contributions

AK and MB have contributed equally and were involved in the analysis and study design, organizing the study plan and preparing the manuscript. FA, AS and HB were consulted in the study design, review and analysis of the results. AM, AA and WA were involved in the wet laboratory work. IA has been involved in the analysis, organizing and supervising the overall work.

## Funding

This study is funded by King Abdullah International Medical Research Center (KAIMRC)[RC10/003] and approved by the Ministry of National Guard Health Affairs, Saudi Arabia (MNG-HA, IRB) Committee with protocol # RC10/003.

## Ethical approval

Not required.

## Competing interest

The authors declare no competing interests.

## Acknowledgements

The authors would like to appreciate Ms. Zoe P. Camarig for her assistance in the data gathering, editing and proofreading of the manuscript.

## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jiph.2019.01.067.

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