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# Sequence and Phylogenetic Analysis of Influenza Virus (H1N1pdm2009) Circulating in Riyadh, Saudi Arabia

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## Abstract

**Influenza A virus (IAV) is the principal cause of seasonal flu and is often reported among pilgrims in Saudi Arabia (SA) due to their mass gatherings. The epidemiological, phylogenetic, and molecular details of A/H1N1pdm2009 in 200 clinical samples collected from hospitalized children in Riyadh during two epidemic seasons (2020/21 and 2021/22) are reported in this study. A total of 21 (10.50%) samples were positive for IAV, as determined using PCR. Fifteen isolates (71.42%) were identified as H1N1pdm2009: eight (53.33%) samples were from males, seven (46.67%) from females. The prevalence of H1N1pdm2009 isolates was significantly ($p < 0.05$) higher among the age group 15-64 years than the other age groups. A comparison of hemagglutinin (HA) and neuraminidase (NA) amino acid sequences between SA H1N1pdm and certain vaccine strains revealed 19 mutations relative to reference strain A/California/07/2009. Among them, eight (0.47%) were in HA, and eight (0.56%) were in NA sequences that differed from vaccine strains. All isolates of the 2020-2022 seasons exhibited N- and O-glycosylation sites comparable to vaccine strains. Phylogenetically their HA and NA genes are divided into different clades. Most of the studied isolates (five) belonged to clade 5a.1 of HA. These data identify the genetic makeup of circulating influenza virus subtypes.**

**Keywords:** Influenza A Virus, A/H1N1pdm2009, Phylogenetic Analysis, Recommended Vaccine Strains

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## INTRODUCTION

Acute respiratory tract infections (ARTIs) have long been recognized as a major source of death and morbidity worldwide. Every year, an estimated 3.9 million individuals die from ARTIs.[1] Annually, influenza, a serious respiratory virus infection, causes significant seasonal morbidity and mortality, usually during the winter. Annual influenza epidemics inflect one billion infected cases, three to five million episodes of serious illness, and estimated fatalities of 300,000-500,000.[2] Influenza viruses are members of the *Orthomyxoviridae* family with an RNA genome that is segmented single-stranded negative-sense[3]; categorized into four genera: influenza A (*Alphainfluenzavirus*), influenza B (*Betainfluenzavirus*), influenza C (*Gammainfluenzavirus*), and influenza D (*Deltainfluenzavirus*).[3] Only types A, B, and C affect people of all ages, causing mild to severe illness and being the cause of all previous influenza pandemics and seasonal epidemics.[2,4]

Influenza viruses possess two main glycoproteins with antagonistic activities: hemagglutinin (HA) and neuraminidase (NA). HA binds to the receptor for sialic acid on the cell surface, whereas NA protein releases it from host cells. Most influenza vaccinations target the HA and NA antigens on the viral surface. However, influenza vaccinations are now primarily HA-based on the market.[5] Due to the rapid antigenic shift/drift of the influenza HA and NA glycoproteins, any vaccination that aims to elicit the typical neutralizing responses to HA and/or NA must manage antigenic drift.[6,7] The influenza virus strains that spread across the population and mutate over time are subject to evolutionary change.[8]

The pandemic A/H1N1 (H1N1 pdm09) virus, which caused a worldwide outbreak in early 2009, was the product of a quadruple reassortment of IAV, which included two swine, one human, and one avian strain, presumably recombined *via* pigs as an intermediate mammalian host.[9] The relationship between the A/H1N1 pdm09 strain and human seasonal A/H1N1 is a significant cause of misunderstanding among the general public.[10,11] The A/H1N1 pdm09 HA was produced from the classic A/H1N1 swine lineage, which has diverged genetically and antigenically from the more quickly changing human seasonal A/H1N1 strain over the years.[12]

Saudi Arabia is among the nation's most susceptible to spreading and evolving respiratory viruses since it hosts two major yearly global religious gatherings.[13-16] The molecular and clinical epidemiological characteristics of the A/H1N1 subtype are currently poorly characterized in the Riyadh region.[17-19] Over time, the influenza virus strains circulating in the population shift and change.[8] The current study aims to identify the epidemiological features and genetic diversity of the A/H1N1pdm2009 in Riyadh during the winters of 2020/21 and 2021/22.

## MATERIALS AND METHODS

A total of 200 nasopharyngeal aspirates (NPAs) from patients with probable influenza infection and acute respiratory symptoms (rhinorrhea, cough, dyspnea, and fever) were collected during the epidemic years 2020/21 and 2021/22. Sampling was conducted during the fall-winter period between September and December of 2020/21 and 2021/22. Patients signed informed consent forms before they were permitted to participate in the study. The institutional review board (IRB) of the University Hospital provided the approval of the study protocol (Ethics Reference No. 20/5522/IRB). Data were collected and accessed for research in March 2021 and March 2022.

### Detection, sequencing, and typing of IAV

Clinical samples were processed using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) to extract viral RNA. IAV was identified and typed using the One-Step Ahead RT-PCR Kit

**Table 1.** Thermocycling conations in this study

| Step | Temp. | Time | Cycles |
|---|---|---|---|
| Reverse transcription | 50°C | 30 min. | 1 cycle |
| Initial PCR activation | 95°C | 15 min. | 1 cycle |
| Cycling: | | | |
| Denaturation | 94°C | 15 sec | 40 cycles |
| Primer Annealing | 52°C | 30 sec | |
| Extension | 72°C | 2 min | |
| Final extension | 72°C | 5 min | 1 cycle |
| Hold | 4°C | ∞ | |

(Qiagen, Hilden, Germany) using a GeneAmp 9700 thermal cycler (Applied Biosystems, USA). Thermocycling was carried out in this study, as mentioned in Table 1. For the identification of amplified PCR products, a 100 bp Plus DNA ladder (Qiagen in Hilden, Germany) was used for comparison by electrophoresis on a 1% agarose gel stained with ethidium bromide.

The HA and NA antigenic glycoprotein genes from A/H1N1pdm2009 were amplified using the same kit. Two overlapping primer sets were used to get complete HA and NA genes. The list of primers used in this study is shown in Table 2. The sequences of eight A/H1N1pdm2009 isolates were chosen to represent the whole positive sample from each of the two epidemic years (2020/21 and 2021/22). HA and NA gene sequencing was conducted commercially by Macrogen Inc., Seoul, South Korea.

**Sequence and phylogenetic data analysis**

The A/H1N1pdm2009 whole HA and NA genes were sequence edited, divergence analyzed, mutation sites identified, and amino acid changes predicted using the BioEdit 7.0 software (Ibis Biosciences, Carlsbad, CA). The sequence data of reference strains recommended by the World Health Organization (WHO) and reference strains from known clades were collected from the GISAID and GenBank® public databases (Supplementary Table 1). The potential N-glycosylation or O-glycosylation sites were predicted using NetNGlyc 1.0.[20,21] and NetOGlyc 3.1.[22] According to the neighbor-joining method, the phylogeny tree was created using MEGA 7.0 (Pennsylvania State University, University Park, PA, USA). The numbers at the internal nodes of the tree represent the bootstrap values of 1,000 replicates.[23]

**Statistical analysis**

Categorical variables were compared using Fisher's exact test. The Z-test with Bonferroni adjustment was used for the post-hoc comparison. Significance was considered at $P < 0.05$.

## RESULTS

**IAV detection and subtyping**

During the two study seasons (winters 2020/21 and 2021/22), the prevalence of IAV was 21 (10.50%), with A/H1N1pdm2009 isolates accounting for 15 (71.42%). Age categories were divided following the population at high risk for contracting ILI (influenza-like illness) or ARI (acute respiratory sickness) into four separate age categories: 0-4, 5-14, 15-64, and ≥65 years.[24,25] The frequency of A/H1N1pdm2009 isolates varied across age categories. The age group 15-64 years had a substantially greater prevalence of A/H1N1pdm2009 isolates than the other age groups ($p < 0.05$). Based on sex, there were more

**Table 2.** IAV testing, typing, and sequencing primers used in this study

| Primer description | Type/ subtype | Gene | Primer name | Sequence 5'-3' | Product Size (bp) | |
|---|---|---|---|---|---|---|
| Primers used for detection | IAV | M | M30F2/08 | ATGAGYCTTYTAACCGAGGTCGAAACG | 244 | 55°C |
| | | | M264R3/08 | TGGACAAANCGTCTACGCTGCAG | | |
| Primers used for typing | (H1N1) Pdm09 | | HKU-SWF | TGAGCTCAGTGTCATCATTTGA | 174 | 57°C |
| | | | HKU-SWR | TGCTGAGCTTTGGGTATGAA | | |
| Primers used for sequencing | H1N1 | HA | H1-F1 | AGCAAAAGCAGGGGAAAATAAAAGC | 1264 | 58°C |
| | | | H1-R1 | CCTACTGCTGTGAACTGTGTATTC | | |
| | | | H1-F2 | GGGAGAATGAACTATTACTGG | 979 | 50°C |
| | | | H1-R2 | AGTAGAAACAAGGGTGTTTTT | | |
| | NA | N1-F1 | | AGCAAAAGCAGGAGTTTAAAATG | 1099 | 56°C |
| | | | N1-R1 | CCTATCCAAACACCATTGCCGTAT | | |
| | | | N1-F2 | GGAATGCAGAACCTTCTTCTTGAC | 1073 | 58°C |
| | | | N1-R2 | ATATGGTCTCGTATTAGTAGAAACAA GGAGTTTTTT | | |

H1N1pdm2009 isolates in males, eight (53.33%), than in females, seven (46.67%) of all cases (Table 3).

### Sequence analysis of the *HA* gene of A/H1N1pdm2009 isolates

Using the Clustal W algorithm, eight A/H1N1pdm2009 study isolates' complete *HA* gene nucleotide sequences (1701 nucleotides) were sequenced and aligned with sequences from 45 local and international A/H1N1pdm2009 strains from different clades that were widely disseminated (Supplementary Table 1) in the Gene bank and GISAID databases. In the *HA* gene of A/H1N1pdm2009 isolates, 58 altered nucleotide sites (3.4%) were observed, although only 19

**Table 3.** Sample distribution across epidemic seasons, gender, and age groups

| | | No. of samples n (%) | Positive for IAV n (%) | Positive for H1N1 pdm2009 n (%) |
|---|---|---|---|---|
| Season | 2020/21 | 100 (50) | 9 (42.85) | 7 (33.33) |
| | 2021/22 | 100 (50) | 12 (57.14) | 8 (38.09) |
| | Total | 200 (100) | 21 (10.50) | 15 (71.42) |
| Gender | Male | 103 (51.5) | 12 (57.14)[a] | 8 (53.33)[a] |
| | Female | 97 (48.5) | 9 (42.85) | 7 (46.67) |
| Age in years | 0-4 | 35 (17.50) | 3 (14.29) | 2 (13.33) |
| | 5-14 | 41 (20.50) | 7 (33.33) | 4 (26.67) |
| | 15-64 | 73 (36.50) | 9 (42.86)[b] | 7 (46.67)[c] |
| | ≥65 | 51 (25.50) | 2 (9.52) | 2 (13.33) |

Data are displayed as percentages (%). [a]Significant difference ($p < 0.05$) from females. [b]Significantly different ($p < 0.05$) from age groups 0-4 and 65 years; [c]Significantly different (p 0.05) from age group 65 years

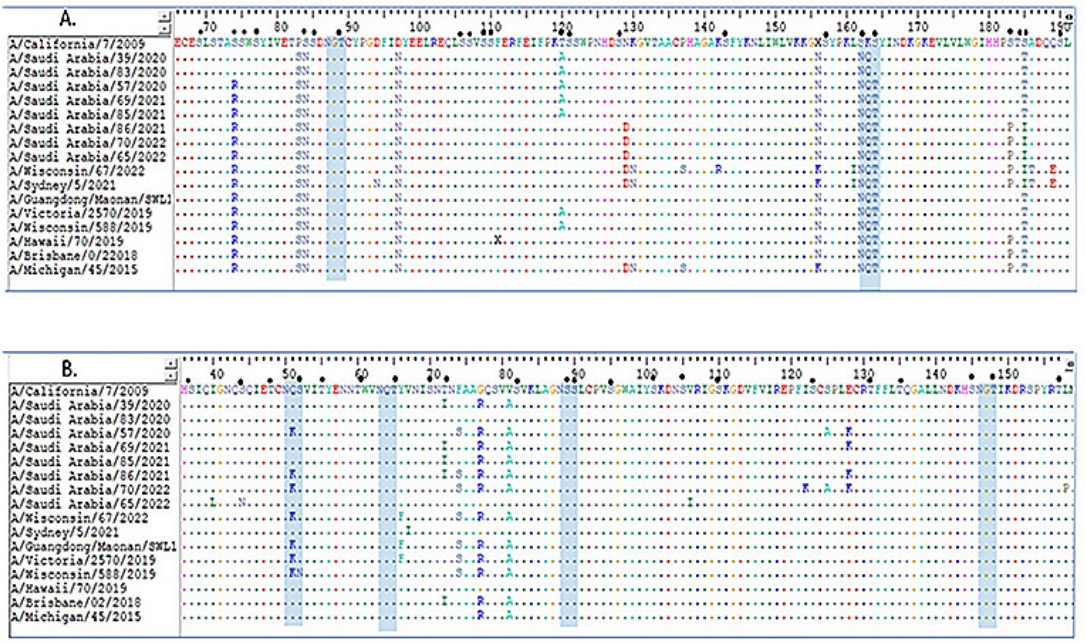


**Figure 1.** Alignment of amino acid sequences for two target proteins deduced from nucleotide sequences HIN1pdm2009. (A), HA amino acid sequences. (B), NA amino acid sequences. The A/California/7/2009 strain is used as a reference strain. Identical residues are shown by dots, whereas variations in amino acids are depicted by colored alphabets. Blue rectangles indicate the anticipated N-glycosylation sites. The expected O-glycosylation sites are represented by little filled circles

(32.75%) changed the amino acids. Multiple sequence alignment of amino acid sequences relative to the sequences in the prototype strain (A/California/07/2009) and current vaccine strain demonstrated that among 18 mutations, four (22.22%) had never been discovered before and were therefore thought to be unique mutations as opposed to the vaccine strains (T120A, D129N, P183S, S185T, and N2601D) (Table 4).

**Sequence analysis of the NA gene of H1N1pdm2009 isolates**

The study strain's NA gene sequence had 65 nucleotide mutation sites (4.61%) and 15 nucleotide substitutions that changed amino acids. The prepared NA gene sequences' nucleotide lengths were 1410 bp. However, eight (0.56%) had never been seen before and were regarded as distinct mutations, unlike the vaccine strains (Table 5).

**Analysis of amino acid sequence N- and O-glycosylation sites**

The number of sites for N-glycosylation on the HA protein of the A/H1N1pdm2009 isolates under investigation ranged between five and six. The A/H1N1pdm2009 isolates used in this study and the vaccine strains contained an N-glycosylation site. In contrast (Figure 1 A), the serine and threonine residues of the HA domain had substantial O-linked glycosylation. Two to four N-glycosylations were observed in the NA protein of A/H1N1pdm2009 sequences under investigation. Moreover, 31-36 O-glycosylation sites were observed (Figure 1 B).

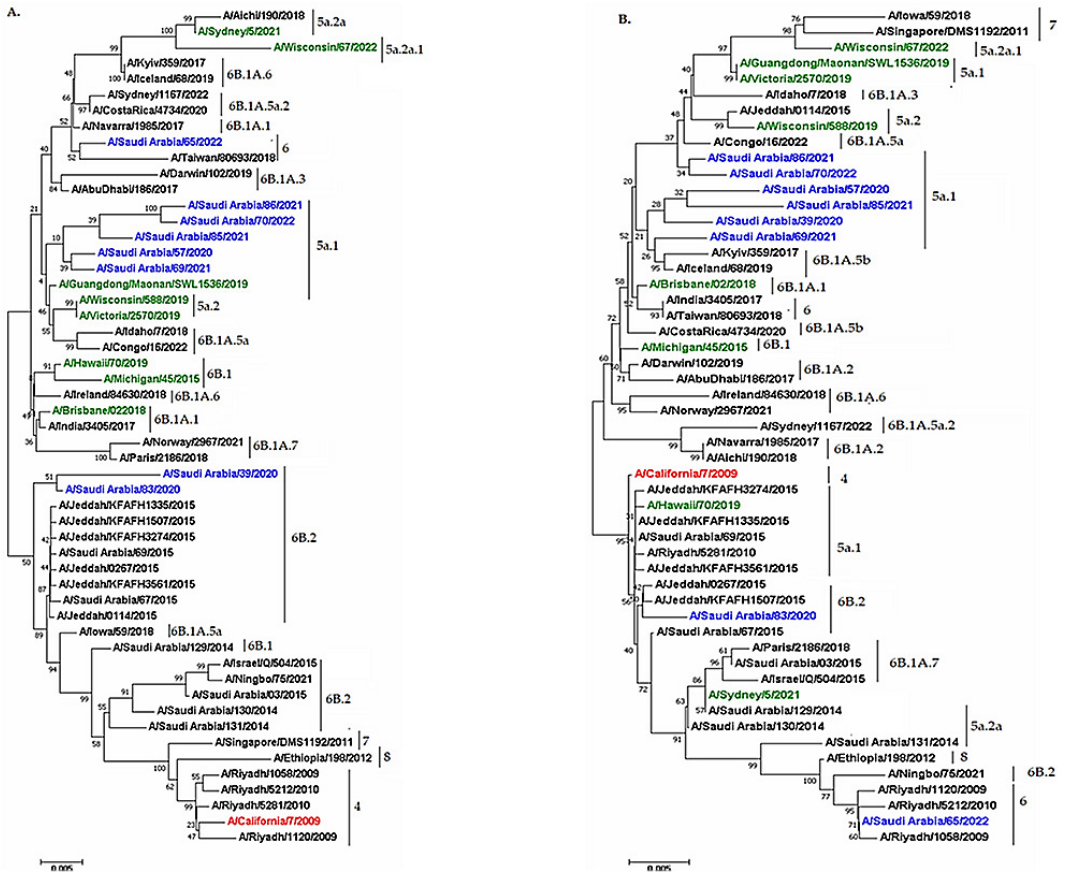


**Figure 2.** Phylograms of influenza H1N1pdm2009 viruses for two target genes. (A) phylogram based on based on *HA* gene. (B) phylogram based on *NA* gene. The neighbor-joining method was utilized by us of MEGA7 program. Study isolates are indicated with blue ink. Green represents the vaccine strain at the present time. The prototype strain is highlighted by red ink

**Table 4.** Changes in the amino acid in the HA1 domain of H1N1pdm2009 strains in comparison with the A/California/07/2009 strain

| Mutation sites | 74 | 83 | 84 | 97 | 120 | 129 | 156 | 162 | 163 | 164 | 183 | 185 | 203 | 216 | 222 | 223 | 256 | 260 | 289 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A/California/7/2009 | S | P | S | D | T | N | X | S | K | S | S | S | S | I | X | X | A | N | K |
| A/Saudi_Arabia/39/2020 | S | S | N | N | A | N | N | N | Q | S | S | T | T | T | D | Q | T | N | E |
| A/Saudi_Arabia/83/2020 | S | S | N | N | A | N | N | N | Q | S | S | T | T | T | D | Q | T | N | E |
| A/Saudi_Arabia/57/2020 | R | S | N | N | A | N | N | N | Q | T | S | T | T | T | D | Q | T | N | E |
| A/Saudi_Arabia/69/2021 | R | S | N | N | A | N | N | N | Q | T | S | T | T | T | D | Q | T | N | E |
| A/Saudi_Arabia/85/2021 | R | S | N | N | A | N | N | N | Q | T | S | T | T | T | D | Q | T | N | E |
| A/Saudi_Arabia/86/2021 | R | S | N | N | T | D | N | N | Q | T | P | I | T | T | D | Q | T | D | K |
| A/Saudi_Arabia/70/2022 | R | S | N | N | T | D | N | N | Q | T | P | I | T | T | D | Q | T | D | K |
| A/Saudi_Arabia/65/2022 | R | S | N | N | T | D | N | N | Q | T | P | I | T | T | D | Q | T | D | K |
| A/Wisconsin/67/2022 | R | S | N | N | T | D | K | N | Q | T | P | I | T | T | D | Q | T | E | E |
| A/Sydney/5/2021 | R | S | N | N | T | D | K | N | Q | T | P | I | T | A | D | Q | T | D | E |
| A/Guangdong/Maonan/SWL1536/2019 | R | S | N | N | T | N | N | N | Q | T | S | T | T | T | D | Q | T | N | E |
| A/Victoria/2570/2019 | R | S | N | N | A | N | N | N | Q | T | S | T | T | T | D | Q | T | N | E |
| A/Wisconsin/588/2019 | R | S | N | N | A | N | N | N | Q | T | S | T | T | T | D | Q | T | N | E |
| A/Hawaii/70/2019 | R | S | N | N | T | N | N | N | Q | T | P | T | T | T | D | Q | T | N | E |
| A/Brisbane/0/22018 | R | S | N | N | T | N | N | N | Q | T | P | T | T | T | D | Q | T | N | E |
| A/Michigan/45/2015 | R | S | N | N | T | D | K | N | Q | T | P | T | T | T | D | Q | T | N | E |

**Table 5.** Changes in the amino acid in the NA protein of Saudi Arabian H1N1 strains (seasons 2020-2022) in comparison with the vaccine strains

| Mutation sites | 51 | 72 | 74 | 77 | 81 | 128 | 253 | 257 |
|---|---|---|---|---|---|---|---|---|
| A/California/7/2009 | Q | T | F | G | V | E | Y | R |
| A/Saudi_Arabia/39/2020 | Q | I | F | R | A | E | N | K |
| A/Saudi_Arabia/83/2020 | Q | T | F | G | V | E | Y | R |
| A/Saudi_Arabia/57/2020 | K | T | S | R | A | K | N | K |
| A/Saudi_Arabia/69/2021 | Q | I | F | R | A | K | Y | R |
| A/Saudi_Arabia/85/2021 | Q | I | F | R | A | E | N | K |
| A/Saudi_Arabia/86/2021 | K | I | S | R | A | K | Y | R |
| A/Saudi_Arabia/70/2022 | K | T | S | R | A | K | Y | R |
| A/Saudi_Arabia/65/2022 | Q | T | F | G | V | E | Y | R |
| A/Wisconsin/67/2022 | K | T | S | R | A | E | Y | R |
| A/Sydney/5/2021 | Q | T | F | G | V | E | Y | R |
| A/Guangdong/Maonan/SWL1536/2019 | K | T | S | R | A | E | Y | R |
| A/Victoria/2570/2019 | K | T | S | R | A | E | Y | R |
| A/Wisconsin/588/2019 | K | T | S | R | A | E | Y | R |
| A/Hawaii/70/2019 | Q | T | F | G | V | E | Y | R |
| A/Brisbane/02/2018 | Q | I | F | R | A | E | Y | R |
| A/Michigan/45/2015 | Q | T | F | R | A | E | Y | R |

**Phylogenetic analysis**

To analyze the phylogeny of A/H1N1pdm2009 isolates, many reference sequences from GenBank and GISAID were used, including regional, global, and WHO-recommended influenza vaccine strains. The HA and NA gene sequences of the A/H1N1pdm2009 isolates were analyzed using phylogenetic methods, and the results revealed that the isolates were divided into three clades, with the majority belonging to the 5a.1 (Figures 2A and B).

## DISCUSSION

Regular international labor migration into and out of the country and the large annual religious assembly of pilgrims from around the globe have contributed significantly to the diversity and spread of respiratory viruses in the Kingdom of Saudi Arabia (KSA).[26] IAV-caused epidemics are to blame for all prior influenza pandemics and seasonal epidemics that have sparked public alarm around the world.[2,27] Even though earlier studies in KSA identified the subtypes that cause IAV infections, current knowledge of the regional and temporal distribution of the A/H1N1pdm2009 subtype in Riyadh is not fully documented. Here, we provide an analysis of the sequence and phylogeny of the A/H1N1pdm2009 subtype observed in clinical samples from Riyadh between 2020 and 2022.

In the current study, PCR-based detection demonstrated that IAV was observed in 21 (10.50%) with A/H1N1pdm2009 isolates accounting for 15 (71.42%) during the two study seasons (winters 2020/21 and 2021/22) in Riyadh. Furthermore, among the overall cases, we discovered that eight male cases (53.33%) had higher incidence rates of the A/H1N1pdm2009 subtype than seven female cases (46.67%). People aged 15 to 64 were observed to have a considerably greater incidence of H1N1 isolates ($p < 0.05$), consistent with a recent investigation that showed A/H1N1pdm2009 isolates in 43 (48.8%) of the 88 IAV-positive samples in Riyadh throughout five seasonal IAVs (2014-18 and 2019/20), and that 23 (53.5%) of these isolates were more common in males and those between the ages of 30 (46.2) and (15-64).[15] Similarly, between October 2015 and 2019, 526 (27.3%) cases of A(H1N1) were discovered in Jeddah. The patients' median age was 31, and 50% were female. The majority of instances (50.2%) involved adult patients aged 19 to 60.[14] During the Hajj gatherings from 2013 to 2015, seven of the 25 were subtyped as A/H1N1pdm2009.[28] During the 2019 Hajj season, 185 clinical samples were tested,

and 54 showed positive for the IAV. Of these, 27 samples were influenza A/H1N1 and 19/H3N2, four samples were influenza A (no type given), and four samples were influenza B.[16] Additionally, a study of 15 nations in the Eastern Mediterranean Region reported that the A/H1N1pdm09 subtype was the most widely distributed; 1666 (58.5%) were A/H1N1 subtypes, followed by A/H3N2 671 (23.6%).[29] The frequency of IAV fluctuated significantly throughout that time in many nations around the world, including Brazil 332 (23.62%).[30] China 312 (3.1%),[31] and Senegal 3993 (75%).[32]

In this study, considering multiple sequence alignment of amino acid sequences with respect to the sequences of the prototype strain (A/California/07/2009) and current vaccine strains, eight out of the 19 mutation sites in the *HA* gene have been observed (T120A, D129N, P183S, S185T, and N2601D) (Table 4). Early research indicated that P83S (100%) was the most frequent alteration in Cuban genomes. Further, S203T and D222E (Ca antigenic site), N156K and S162N (Sa antigenic site), and A186T and S203T were discovered in 85.7% of cases in comparison to the reference strain A/California/07/2009 (H1N1). Other significant alterations reported at antigenic sites included S203T and D222E (Ca antigenic site), N156K and S162N (Sa antigenic site), and A186T and S203T observed in 85.7% of patients as compared to the prototype strain A/California/07/2009 (H1N1).[33] According to a recent study, the amino acid substitutions of S101N, N146D, P100S, D114N, K180Q, S202T, and S220T represent the majority of Indian isolates relative to (A/California/07/2009).[34] According to previous reports.[35] The globular head domain of the HA protein in A/H1N1 viruses possesses five sites of antigenicity, namely Ca1, Ca2, Cb, Sa, and Sb. Even a single amino acid mutation can impact the virus's immunogenicity and the vaccine's capacity to protect these critical sites for establishing antigenicity.[36]

Vaccination remains the best method of protection against influenza virus infections and their consequences, particularly for at-risk populations such as small children, older adults, and persons with underlying medical conditions.[37-39] The HA and NA proteins were chosen as targets for the creation of a universal influenza virus vaccine. To improve the strategic control of influenza vaccination, a deeper comprehension of HA evolution and variation is required. Any vaccination strategy that aims to elicit the typical neutralizing responses to HA and/or NA must successfully manage antigenic drift. Therefore, IAV HA and NA antigenic drift should be considered as a possible target for a universal influenza vaccine. According to findings from HA sequence variation studies, HA changes in amino acids at Sa (residues 128 and 129, 156-160, 162-167) and Sb (residues 187-198) antigenic sites during infection are required for the selection of influenza vaccine strains.[6] The Saudi Ministry of Health offered the following strains of the trivalent inactivated influenza virus vaccine: A/Singapore/INFIMH-16-0019/2016(H3N2)-like virus, A/Michigan/45/2015(H1N1) pdm-09 virus, and influenza B/Colorado/06/2017-like virus (B/Victoria/2/87 lineage).[40] WHO has identified and recommended more than 28 vaccine strains.[41] Representative 2023-2024 northern hemisphere vaccine strain A/Wisconsin/67/2022 and the 25 (H1N1) pdm09 strains exhibited nucleotide and amino acid similarity of 98.41-99.22% and 98.41-99.36%, respectively.[11]

In the current study, phylogenetic analysis of HA and NA sequences of A/H1N1pdm2009 isolates divided into three clades, with the majority belonging to the 5a.1. An earlier study reported that all the A/H1N1pdm2009 strains belonged to clade 6 viruses, where 73 (92%) grouped into sub-clade 6b.1 and 7 (8%) grouped into clade 6b.2 in Riyadh, Saudi Arabia, between 2014 and 2015.[13] The A/H1N1 isolates have no significant connection to any strains used in influenza vaccines. Phylogenetic research, however, indicated that IAV strains did not typically cluster within regional isolates, which implies that travel may have contributed to the virus's global spread.[42] The WHO recommendations related to the influenza vaccines for utilization in both the northern and southern hemispheres were made with knowledge of the currently circulating viruses globally based on the year-round work of the WHO Global.[43]

The N-glycosylation sites A/H1N1pdm2009 isolates used in this study were from strains 5 and 6. Moreover, these N-glycosylation sites were

observed in the vaccine strains. Variations in the N- and O-linked glycosylation of the HA and NA proteins can have an impact on the host specificity, pathogenicity, and infectivity of an influenza strain by altering the biological characteristics of HA and NA or indirectly by reducing binding to the receptor and blocking antigenic regions of the protein. In previous studies, it was reported that seasonal viruses had more glycosylation sites on the head of the HA and NA than A/H1N1 pdm09, and that two glycosylation sites (glycosylation sites 50 and 68) on the stalk of the NA in A/H1N1 pdm09 were probably substituted by two more glycosylation sites (glycosylation sites 44 and 70) in the seasonal strains.[44,45] Furthermore, recent research has indicated that the HA globular head domain's glycosylation serves to physically cloak the antigenic sites, inhibiting antibody identification and enabling viral evasion of antibody-mediated neutralization.[6,46]

The small cross-sectional sample size of this study was the major limitation. Additionally, the study is unable to identify a cause for recurrent infections. For a deeper understanding of the A/H1N1pdm2009 circulation pattern, additional rigorous studies focusing on larger sample sizes scattered over KSA in successive epidemic seasons are required.

## CONCLUSION

PCR-based detection demonstrated that IAV was observed in 21 (10.50%), with A/H1N1pdm2009 isolates accounting for 15 (71.42%), during the two study seasons (winters 2020/21 and 2021/22) in Riyadh. In the overall number of cases, males (eight [53.33%]) were more likely to have the A/H1N1pdm2009 subtype than females (seven [46.67%]) ($p < 0.05$). Multiple sequence alignment of amino acid sequences relative to the sequences of the prototype strain (A/California/07/2009) and current vaccine strains showed that there were 19 mutations-eight (0.47%) in *HA* gene sequences and eight (0.56%) in NA gene sequences that differed from vaccine strains. These isolates shared N-glycosylation sites with the strains used in vaccines. The A/H1N1pdm2009 isolates were divided into three clades. These isolates primarily belonged to 5a.1. Additionally, a comparable grouping was observed in the NA phylogenetic tree and the HA tree. The A/H1N1pdm2009 isolates have no strong ties to any strains used in influenza vaccines. The current findings highlight the necessity to confirm the importance of these alterations and regularly check the effectiveness of vaccines considering new variants.

## SUPPLEMENTARY INFORMATION

Supplementary information accompanies this article at https://doi.org/10.22207/JPAM.18.4.11

**Additional file**: Additional Table S1.

## ACKNOWLEDGMENTS

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## FUNDING

None.

## DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript and/or in the supplementary files.

## ETHICS STATEMENT

The study was approved by the Institutional Review Board (IRB), University Hospital, with reference No. 20/5522/IRB.

## INFORMED CONSENT

Written informed consent was obtained from the participants before enrolling in the study.

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