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Genetic diversity and molecular analysis of human influenza virus among pilgrims during Hajj

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ABSTRACT

The risk of transmission of respiratory tract infections is considerably enhanced at mass gathering (MG) religious events. Hajj is an annual Islamic MG event with approximately 3 million Muslim pilgrims from over 180 countries concentrated in Makkah, Saudi Arabia. This study aimed to investigate the genetic diversity of influenza viruses circulating among pilgrims during the Hajj pilgrimage. We performed a cross-sectional analytical study where nasopharyngeal swabs (NPs) from pilgrims with respiratory tract illnesses presenting to healthcare facilities during the 2019 Hajj were screened for influenza viruses. Influenza A subtypes and influenza B lineages were determined by multiplex RT–PCR for positive influenza samples. The phylogenetic analysis was carried out for the hemagglutination (HA) gene. Out of 185 nasopharyngeal samples, 54 were positive for the human influenza virus. Of these, 27 were influenza A H1N1 and 19 H3N2, 4 were untypable influenza A, and 4 were influenza B. Phylogenetic analysis revealed that the H1N1 and H3N2 strains differentiated into different and independent genetic groups and formed close clusters with selected strains of influenza viruses from various locations. To conclude, this study demonstrates a high genetic diversity of circulating influenza A subtypes among pilgrims during the Hajj Season. There is a need for further larger studies to investigate in-depth the genetic characteristics of influenza viruses and other respiratory viruses during Hajj seasons.

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1. Introduction

Influenza viruses are among the most significant causes of public health concern particularly during mass gathering events. The possibility of both epidemic (seasonal flu) and pandemic influenza still exists [1–5]. Influenza infections impose a large burden to the healthcare and economic systems worldwide due to their associated morbidities and mortalities [6–8]. According to the world health organization (WHO), between 20 % and 30 % of children and between 5 % and 10 % of adults are infected every year by influenza viruses leading to 3 to 5 million serious illness [9]. Indeed, seasonal influenza epidemics are estimated to cause about 650,000 fatalities annually worldwide [4,10].

Historically, many influenza epidemics and pandemics have been reported. Influenza A virus (H1N1) "Spanish flu" was first reported in 1918 and was responsible for 500 million infections worldwide, with about 50–100 million deaths [11,12]. Since then, H1N1 subtype viruses have continued to cocirculate with influenza A (H3N2) viruses which first emerged in the 1968 pandemic [13]. Avian influenza virus A H5N1 was first reported in 1997 and is causing severe human illness since then [14]. Since the early 1980s, two antigenically and genetically distinct lineages of influenza B viruses, (B/Yamagata/16/88 viruses and B/Victoria/2/87) have co-circulated in humans and accounted for nearly one-fourth of all influenza cases occurring annually in the world [3,15–18]. Co-circulation of multiple subtypes/lineages of influenza viruses provides an opportunity for genetic reassortment offering an important evolutionary mechanism for human influenza viruses [13].

Saudi Arabia hosts about 3 million pilgrims and more than 20 million religious' tourists from more than 180 countries annually [19–22]. Overcrowding of individuals with close contact within a confined area increases the risk of spreading respiratory infections among pilgrims including influenza viruses [23–26]. The first report on the prevalence of influenza viruses in Hajj was among Tunisian pilgrims in the 1975 Hajj season which reported about 4 % (37 of 950) influenza cases [27,28]. This was followed by several studies investigating the prevalence of influenza viruses and other respiratory pathogens among Hajj pilgrims. A longitudinal analysis conducted over 15 years in all related publications between 2003 and 2018 showed a wide variation in the prevalence rates of influenza viruses ranging from 0.6 to 15.8 % for influenza A viruses and from 0 to 11.5 % for influenza B viruses [29]. Due to the concern of the 2009 pandemic swine A H1N1 strain (H1N1pdm09), the Saudi authorities recommended influenza A(H1N1) pdm09 and seasonal flu vaccinations for all Hajj pilgrims in 2009. Despite this, it was estimated that only about 30 % and 53 % of pilgrims received the vaccines, respectively [28]. Saudi health authorities reported 73 cases of H1N1 including 5 deaths (4.9 %) during the 2009 Hajj season. This low number of cases demonstrated the effectiveness of the control measures implemented during the 2009 Hajj season [28]. Currently, the Saudi government mandates flu vaccination as a pre-requirement for all domestic pilgrims whereas it is highly recommended for international pilgrims [30]. It is advised for international pilgrims to receive the southern hemisphere vaccines due to the potential mismatch between the strain of the northern hemisphere vaccine and the circulating strains during Hajj [31].

Although important, the molecular investigations of influenza viruses and respiratory infections among attendees of mass gathering events are largely understudied. More recently, some reports linked the Kumbh Mela "Hindu pilgrimage" to the spread of COVID-19 variant B.1.617.2 (Delta) which had a major impact on the healthcare system in India [32]. Several reports have investigated the prevalence and circulation respiratory infections during Hajj seasons [33]. In previous studies [23,34], we investigated the prevalence of respiratory viral infections in a cohort of pilgrims where we showed that Human rhinovirus (HRV) was the most common viral infection (42.06 %), followed by influenza A (H1N1) (21.43 %), and influenza A other than H1N1 (18.25 %). Other viruses were identified like CoV-229E (19.84 %), CoV-OC43 (3.97 %), CoV-HKU1 (3.17 %), and CoV-NL63 (0.79 %) [34]. We also investigated the genetic diversity of human rhinovirus in pilgrims [35] and showed that the virus has 3 genotypes (HRV-A, -B and –C) with high genetic diversity among all genotypes. In continuation of our previous investigations of respiratory infections in the Hajj season, this study aimed to identify the molecular characteristics and investigate the genetic diversity of influenza viruses circulating among pilgrims during the 2019 Hajj pilgrimage.

2. Methods

2.1. Study design and respiratory virus detection

This is a cross-sectional analytical study for the investigation of respiratory infections in pilgrims of the 2019 Hajj season. As detailed previously [34], nasopharyngeal swabs (NS) were collected in viral transport media (VTM) and stored at −80 °C for screening of respiratory viruses among pilgrims presenting to healthcare facilities in the holy places with respiratory symptoms during the 2019 Hajj. The molecular detection of respiratory viruses was performed using Fast-Track respiratory pathogens 21 kit (Fast-track Diagnostics, Luxembourg).

2.2. Detection of influenza a sub-types and influenza B lineages

All samples that tested positive for influenza A other than H1N1 were tested first for H3N2 influenza. Subsequently, all influenza A H3N2 negative samples were tested for H5NX influenza. The procedure was performed according to Althaqafi et al. [36] using primers and probes targeting the subtype-specific haemagglutinin gene (HA) according to the WHO protocol [37]. All Extracted RNA samples that tested positive for influenza B virus were further utilized for influenza B Victoria and Yamagata lineage characterization according to WHO standard protocol [37]. Target amplification was performed using QuantiFast Probe RT-PCR + ROX™ Vial Kit (Qiagen, Hilden, Germany). Amplification was performed on the QuantStudio 12K Flex Real-Time PCR System (Applied biosystem—Thermo Fisher Scientific, Waltham, USA).

2.3. Reverse Transcription and PCR amplification for influenza a (H1N1) and (H3N2) (H5Nx) and influenza B

RT-PCR was performed using One-Step RT-PCR System (Invitrogen, Thermo Fisher Scientific, USA). The RT-PCR reaction contained 1 μl of extracted RNA, 12.5 μl of 2X Reaction mix (buffer, dNTP, and MgSO4), 8.5 μl of H_2O, 1 μl of Taq polymerase enzyme, 0.5 μl of each of the forward and reverse primers according to the WHO protocol and our previously published report in a final volume of 25 μL [36,37]. The RT-PCR was performed in the thermocycler (ABI Applied Biosystems, USA). The initial RT step at 55 °C for 20 min, followed by denaturation at 95 °C for 3 min, then 45 cycles of amplification, denaturation step for 15 s at 95 °C, annealing at 57 °C for 15 s, and extension for 1 min at 72 °C. The final extension step was completed at 72 °C for 2 min. PCR products were subjected to gel electrophoresis using 1 % agarose gel and visualized under UV light using the ethidium bromide stain. The band of interest was cut out and PCR products (1619 and 1550 bp) were purified by column purification using a Norgen Biotek DNA gel extraction Kit (Norgen, Canada).

2.4. Cycle sequencing reaction and phylogenetic analysis

Influenza A (H1N1 and H3N2) samples were subjected to cycle sequencing of H1 and H3 genes, using forward and reverse primers to cover the full length of the PCR product, and Bigdye Terminator version 3.1 Reaction Cycle Sequencing Kit (Applied Biosystems, USA) according to manufacturer's instructions. The sequencing reaction mixture contained 0.32 μL of each of the forward and reverse primers, 16.43 μl of water, 1.75 μl of sequencing buffer, 0.5 μl of Sequencing reagent, and 1 μL PCR product in a final volume of 20 μL. The sequencing reactions were carried out in a thermocycler (ABI Applied Biosystems, USA), as follows: initial denaturation for 2 min at 96 °C, followed by 30 cycles of 96 °C for 30 s, annealing for 15 s at 55 °C and extension for 4 min at 60 °C. The cycle sequencing products were purified using the dry Sephadex® with 0.45 mm MultiScreen-HV. Purified sequencing products were detected on an ABI 3500 sequence analysis system in both forward and reverse directions. Assembly of the forward and reverse sequence and phylogenetic trees were generated using Geneious software [38]. The generated sequences were multiple aligned using Clustal omega and phylogenetic trees were produced using the Maximum-Likelihood method with 1000 bootstrap replicates.

2.5. Data availability Statement

The generated sequences were submitted to Genbank and were given the accession numbers OM817519-OM817529 for the H1N1 sequences and OM818352-OM818365 for the H3N2 sequences.

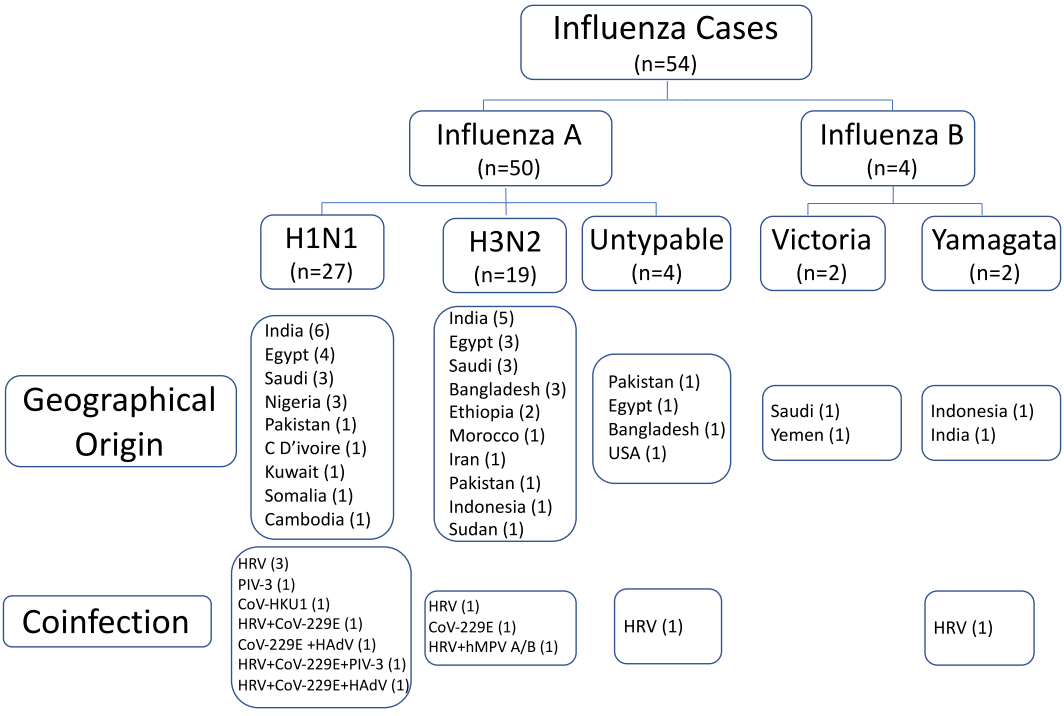

Fig. 1. Flow chart for the distribution of detected influenza subtypes and lineages in the recruited cases. The geographical origin of the pilgrims and the coinfection status are also shown.

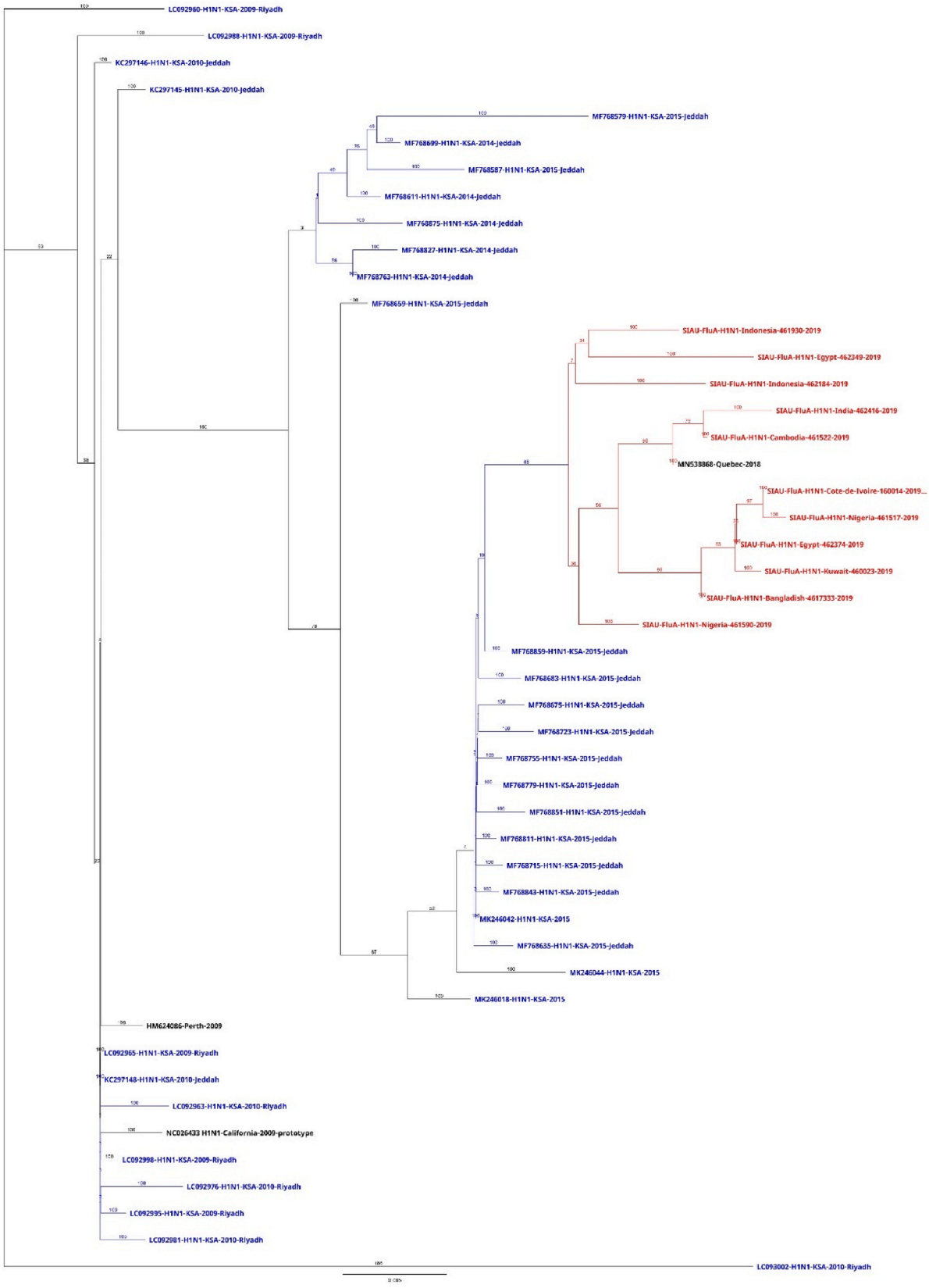

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Fig. 2. A Maximum Likelihood Phylogenetic relationship of the hemagglutination (HA1) of influenza A H1N1 sequences detected among pilgrims patients during Hajj 2019 pilgrimage and selected reference gene sequences. The phylogenetic tree was constructed using Geneious Prime software. Bootstrap value (1000 replicates) shown on branches. Scale bars represent relative evolution distance. Red taxa represent sequences from this study, while blue taxa represent Saudi influenza A virus (H1N1) sequences previously deposited in Genbank. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3. Results

3.1. Characteristics of the influenza positive patients among the pilgrims

In a previous study [34], we showed that Influenza viruses were the second most prevalent respiratory viral infection in the 2019 Hajj season (54/126, 42.85 %). Out of all influenza-positive cases (n = 54); influenza A viruses comprised 92.59 % (n = 50/54). Of these, 27 cases were H1N1 (50.00 %); 19 cases were H3N2 (35.19 %); and 4 cases were untypable influenza A cases (7.41 %). The mean age for influenza A patients was 57.90 years ± 12.97 (ranging from 22 to 82 years). The majority (31/50, 62.00 %) of positive cases were aged 60 years and older with males representing 60 % (n = 30/50) of them.

Influenza B viruses comprised about 7.41 % (n = 4/54) of the influenza cases. Influenza B(Victoria)–lineage viruses and influenza B (Yamagata)–lineage viruses were equally prevalent in Hajj 2019 (n = 2; 50 %). The mean age for influenza B patients was 42.5 years ± 14.97 (ranging from 22 to 58 years). All influenza B-infected cases were younger than 60 years old, with females representing 75 % (n = 3/4). Fig. 1 describes the distribution of Influenza infections among pilgrims, their country of origin and the detected viral coinfections.

Influenza A (H1N1) patients participating in this study descended from 11 countries: India (n = 6) Indonesia and Egypt (n = 4), Saudi Arabia and Nigeria (n = 3), Pakistan, Côte d'Ivoire, Kuwait, Somali, and Cambodia (n = 1) (Fig. 1). Influenza A (H3N2) patients participating in this study descended from 10 countries, mainly from Egypt (n = 5) followed by India (n = 3). Then two positive cases were detected from Saudi Arabia, Bangladesh and Ethiopia (n = 2 for each). 1 case was found from each of Morocco, Iran, Pakistan, Indonesia, and Sudan. For the untypable influenza samples, we were not able to confirm their status with another confirmatory assay due to insufficient sample volume. Thereby, we referred to these samples as untypable influenza A cases. These 4 untypable cases were equally distributed between Pakistan, Egypt, Bangladesh, and the United States (Fig. 1).

Influenza B cases were geographically distributed between Indonesia, India, Saudi Arabia, and Yamen with 1 case each. Influenza B (Victoria)–lineage viruses were found in cases from Saudi Arabia and Yamen, while influenza B(Yamagata)–lineage viruses were found in cases from Indonesia and India (Fig. 1).

3.2. Phylogenetic analysis

To understand the genetic diversity of influenza A and B viruses collected during the Hajj 2019 season, the HA gene of the recruited samples was sequenced. We were able to generate sequences for 11 and 14 samples of influenza A H1N1 and H3N2, respectively. The generated sequences were aligned with other HA sequences of influenza A H1N1 and H3N2 in GenBank and subjected to phylogenetic analysis. Several attempts were made to sequence all positive samples of influenza B; however, we were unable to produce any sequences due to insufficient sample volume or low viral load as indicated by high Ct values in real-time PCR.

Pilgrims' strains showed that the circulating influenza H1 and H3 viruses during the Hajj 2019 season could be differentiated into different and independent genetic groups and formed close clusters with selected influenza strains from various locations.

The generated influenza A virus (H1N1) sequences generated from this study clustered with the Quebec 2018 vaccine strain (MN538868). The generated sequences also clustered with previously circulating sequences reported from Jeddah in 2014 and 2015 but away from sequences previously reported form Jeddah and Riyadh in 2009 and 2010 and the prototype H1N1 California prototype (Fig. 2).

Mutation analysis of the generated H1N1 sequences compared to the prototype sequence Influenza A virus (A/California/07/2009 (H1N1)) (accession number NC026344) (Table S1) showed 54 nucleotide changes including 23 non-synonymous mutations resulting in amino acid changes and 31 synonymous mutations with no amino acid changes.

Phylogenetic analysis of the H3N2 sequences showed a more diverse distribution. Three from the H3N2 strains from this study clustered with a strain from China 2017, 2 strains with a strain from Iran 2019, 2 strains with a strain from Chile 2018, 1 strain with strains from Australia 2018 and 2 strains with a strain from the USA 2020. The generated sequences clustered separately from local isolates reported from Saudi Arabia in 2016–2020 but away from local sequences reported in 2013–2015. The sequences also clustered with reported sequences around the world from 2017 to 2020 (Fig. 3).

Mutation analysis of the generated H3N2 sequences compared to the prototype sequence prototype Influenza A virus (A/Texas/50/2012(H3N2)) GenBank accession number KJ942616 (Table S2) showed 52 nucleotide changes including 26 non-synonymous mutations resulting in amino acid changes and 26 synonymous mutations with no amino acid changes.

4. Discussion

Influenza virus infection is among the most significant causes of public and global health concerns. Pilgrims suffer from extreme weather conditions during their stay in Makkah and the holy places where the temperature might exceed 45 °C. They are also exposed

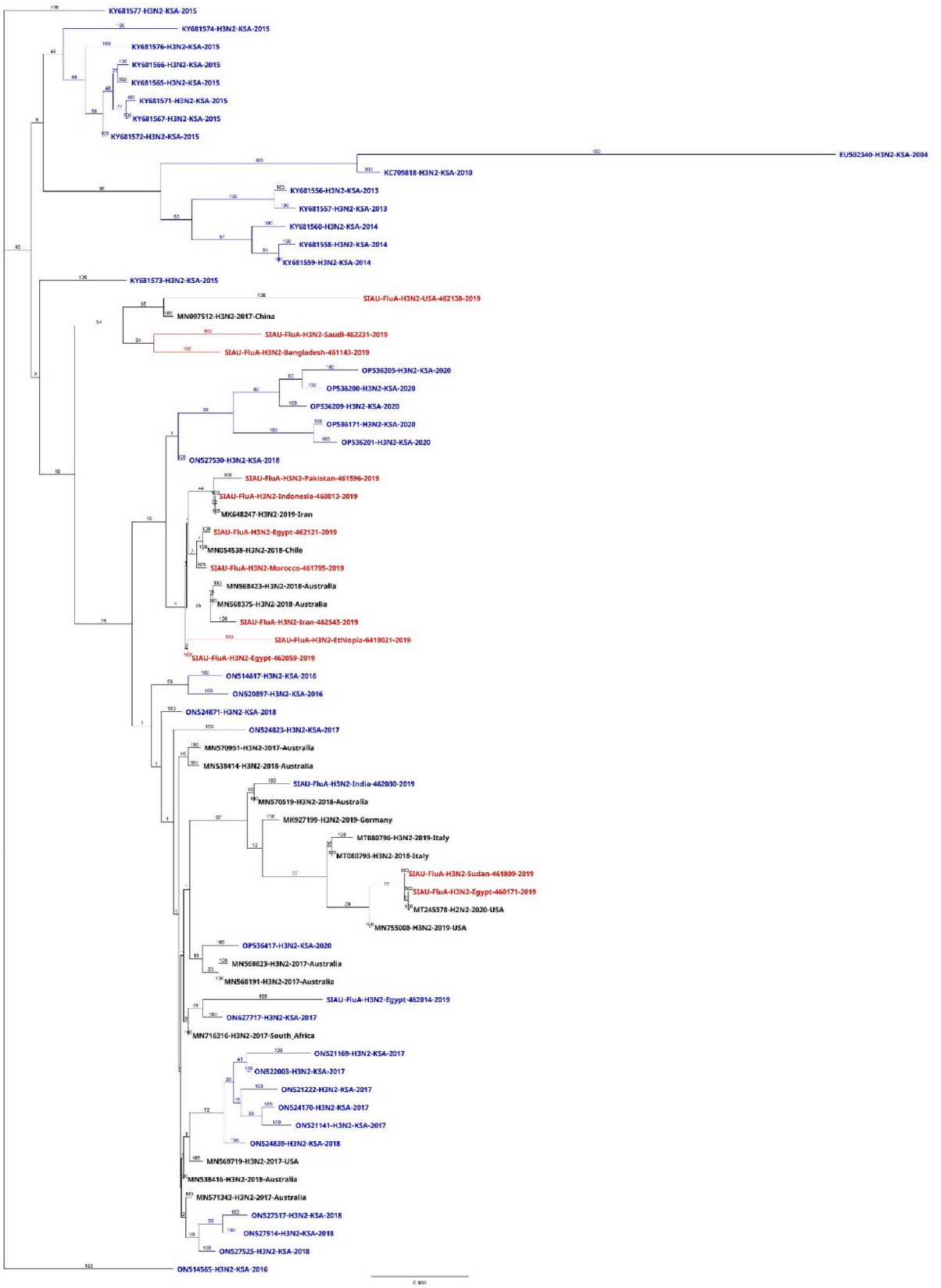

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Fig. 3. A Maximum Likelihood Phylogenetic relationship of the hemagglutination (HA1) of 16 influenza A (H3N2) virus sequences detected among pilgrims patients during Hajj 2019 pilgrimage and selected reference gene sequences. The phylogenetic tree was constructed using Geneious software. Bootstrap value (1000 replicates) shown on branches. Scale bars represent relative evolution distance. Red taxa represent sequences from this study, while blue taxa represent Saudi influenza A virus (H3N2) sequences previously deposited in Genbank. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

to hard physical stress due to the long walks during the Hajj rituals. These harsh conditions make them more susceptible to respiratory infections [39,40]. The potential of multiple infections and genetic reassortment substantially increases in high crowding index gatherings. The increased potential of reassortment in influenza viruses due to their segmented genome might result in progeny viruses with novel genetic and antigenic characteristics [13]. Molecular studies remain crucial for the genetic characterization of circulating influenza strains, particularly during mass gathering events.

In our previous study [41], the overall prevalence of influenza cases was 42.85 % (54/126) with influenza A H1N1 as the predominant strain of influenza (27 out of 126, 21.43 %) followed by influenza A H3N2 (19 out of 126, 15.08 %) while the untypable influenza A samples in this study had the lowest prevalence among influenza A strains (4 out of 126, 3.17 %). Influenza B virus was detected in 4 cases (3.17 %) with Yamagata lineage and Victoria lineage equally represented among pilgrims with 2 cases each.

Several studies have shown that seasonal influenza infection is associated with the age group of infected individuals [42–45]. Elderly aged more than 60 years which represents most pilgrims are at a higher risk for complications, hospitalizations, and death. This is particularly true for those with comorbidities such as diabetes mellitus or chronic heart disease. In our study, patients with influenza A viruses were in the age range of 22–82 years, with the majority being over 60. While patients with influenza B viruses were all under 60 years and ranged in age from 22 to 58 years.

In this study, the clustering of influenza A H1N1 with circulating strains from Jeddah in 2014–2015 but separate from those circulating in 2009 and 2010 indicates the change in circulating strains in Jeddah over time. The same trend was observed for influenza A H3N2, where we identified H3N2 lineages that did not cluster with previously identified strains from Saudi Arabia. This continuous change of the influeza strains with time warrants the need for continuous surveillance for the circulating influenza strains particularly in the times of mass gatherings like Hajj and mass sports and recreational events. The high genetic diversity of the influenza sequences in this study increases the potential of genetic changes that might take place leading to the possible emergence of new strains and spread locally and globally. A novel influenza virus might emerge periodically and spread rapidly among susceptible populations and lead to epidemics or pandemics. Mass gatherings provide a favorable environment for genetic reassortment events due to the enhanced possibility of multiple strain or type co-circulation. We detected the co-circulation of at least 3 types of influenza viruses, influenza A H1N1, H3N2, and influenza B in a short period of a few days and in a confined geographical area. These findings highlight the need for continuous monitoring and genetic surveillance of influenza viruses annually during the Hajj season. This is particularly important as many respiratory infections are of global concern. Examples of these viruses include COVID-19 and Influenza A H5. With regards to the untypable influenza A cases in this study, it was hard to draw comprehensive conclusions about them but they might include influenza strains with the potential of spread in mass gatherings like Hajj. In addition, this study was conducted on a relatively small sample size and samples were collected during a single Hajj season. Hence, large, controlled cohort studies (pre-Hajj, during Hajj, and post-Hajj) for multiple Hajj seasons are required to estimate the role of influenza viruses in respiratory tract infections (RTIs). Another limitation of the study was the technical difficulties faced to generate sequences for the influenza B-positive cases.

This study demonstrates a high genetic diversity of circulating influenza A subtypes among pilgrims during the 2019 Hajj Season. Our data highlight the importance of conducting larger and continuous annual surveillance of respiratory viruses during the Hajj season to early identify potential outbreaks. Indeed, the high genetic diversity of influenza viruses in this small number of samples can potentially increase the possibility of genetic changes in the viral genomes and the emergence of new strains. In addition to active surveillance of respiratory viruses, the implementation of infection control and preventive measures such as wearing masks and influenza vaccination should be implemented in this mass gathering event.

5. Institutional Review Board statement

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board of Research Ethics Committee (REC) at King Abdulaziz University (Reference No 569-20)

Informed consent statement

Informed consent was obtained from all subjects involved in the study.

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Data availability statement

The generated sequences were submitted to Genbank and were given the accession numbers OM817519-OM817529 for the H1N1 sequences and OM818352-OM818365 for the H3N2 sequences.

CRediT authorship contribution statement

Sherif A. El-Kafrawy: Writing - review & editing, Writing - original draft, Visualization, Validation, Supervision, Software, Resources, Methodology, Investigation, Formal analysis, Data curation. **Salma M. Alsayed:** Writing - review & editing, Writing - original draft, Visualization, Methodology, Formal analysis, Data curation. **Arwa A. Faizo:** Writing - review & editing, Funding acquisition, Formal analysis, Data curation. **Leena H. Bajrai:** Writing - review & editing, Visualization, Data curation. **Norah A. Uthman:** Writing - review & editing, Visualization, Methodology. **Moneerah S. Alsaeed:** Writing - review & editing, Visualization, Methodology. **Ahmed M. Hassan:** Writing - review & editing, Visualization, Methodology, Formal analysis. **Khalid M. Alquthami:** Writing - review & editing, Visualization, Conceptualization. **Thamir A. Alandijany:** Writing - review & editing, Writing - original draft, Project administration, Funding acquisition, Data curation. **Alimuddin Zumla:** Writing - review & editing, Conceptualization. **Esam I. Azhar:** Writing - review & editing, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e23027.

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