

High Genetic Diversity of Human Rhinovirus among Pilgrims with Acute Respiratory Tract Infections during 2019 Hajj Pilgrimage Season

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**Title:****High Genetic Diversity of Human Rhinovirus among Pilgrims with Acute Respiratory Tract Infections during 2019 Hajj Pilgrimage Season****Authors:**

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**Abstract**

**Objectives:** Acute Respiratory tract infections (ARI) due to Human Rhinoviruses (HRV) are common in pilgrims during the annual Hajj pilgrimage. The objective of this study was to investigate the genetic diversity of HRV among pilgrims with respiratory symptoms during Hajj 2019.

**Methods:** HRV infection was detected using multiplex real time RT-PCR. Cycle sequencing was performed on positive samples and the sequences were subjected to phylogenetic analysis.

**Results:** 19 HRV-positive respiratory samples were sequenced. All three serotypes of HRV were identified: HRV-A (13; [68.42%]) was more common than HRV-B (2; [10.53%]), and HRV-C (4; [21.05%]). HRV-A species were found to be of genotypes A101, A21, A30, A57, A23, A60 and A11. HRV-B species belonged to genotypes B4 and B84, and HRV-C species were of genotypes C15, C3 and C56.

**Conclusions:** Sequencing studies of respiratory tract viruses in pilgrims are important. We provide preliminary evidence of high diversity of HRV genotypes circulating in pilgrims in a restricted area during Hajj. This requires further clinical and sequencing studies of viral pathogens in larger consorts of overseas and local pilgrims.

**Key words:** Human rhinovirus; Hajj; respiratory viruses; Saudi Arabia; genetic diversity.

**Authors declaration:** All authors declare no conflicts of interest

## 1. Background

Acute Respiratory tract infections (ARTIs) are very common health problems in pilgrims who attend the annual Hajj pilgrimage (Aldossari et al., 2019, AlMazroa et al., 2010, Alzeer, 2009, Hashem et al., 2019, Wilder-Smith et al., 2005). They serve as potential epicenters to disseminate known and new virus strains among the susceptible populations, locally and overseas when they return to their home countries (Mani, 2017). Viral infections are known to cause epidemic or pandemic outbreaks such as Swine Flu or H1N1/09 Pandemic, SARS, Ebola, and more recently in late 2019, the coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) (Benkouiten et al., 2014, Boncristiani et al., 2009, Farrag et al., 2019, WHO-Emergencies preparedness, 2020).

Human rhinovirus (HRV) is responsible for over 50% of upper RTIs in children worldwide (Greenberg, 2016). It has long been known as the primary cause of common colds (Ljubin-Sternak et al., 2019, Vandini et al., 2019) and often cause upper RTIs (Jacobs et al., 2013, Vandini et al., 2019). HRV is also associated with exacerbations of chronic obstructive pulmonary disease (COPD), recurrent wheezing, and asthma development in childhood (Drysdale et al., 2017, Jacobs et al., 2013, Vandini et al., 2019). More recently, it has been associated with serious bronchiolitis in infants and children, and fatal pneumonia in immunocompromised adults and elders (Jacobs et al., 2013). Recurrent infections occur because of its enormous genetic diversity (Farrag et al., 2019, Greenberg, 2016, Vandini et al., 2019). Currently, three species of rhinovirus (A, B, and C) and more than 150 serotypes have been identified; with the most common serotypes are HRV-A and HRV-B (Farrag et al., 2019, Greenberg, 2016, Vandini et al., 2019).

There have been several reports of HRV infections in pilgrims with acute respiratory tract symptoms during the Hajj pilgrimage or upon their return to their home countries (Aberle et al., 2015, Al-Tawfiq et al., 2018, Alborzi et al., 2009, Barasheed O. et al., 2014, Barasheed Osamah et al., 2014, Benkouiten et al., 2019, Memish et al., 2014, Rashid et al., 2008). The prevalence of HRV ranged from 5.9–48.8% (Al-Tawfiq et al., 2018). Several reports highlight the significance of identifying the genetic diversity and typing of HRV and their global distribution (Briese et al., 2008, Huang et al., 2009, Kiang et al., 2008). However, little information is available about the genetic diversity of HRV circulating in Saudi Arabia during Hajj seasons. HRV is the most common viral infection among Hajj pilgrims who sought medical care for acute respiratory tract symptoms (Alsayed et al., 2021). In this study we performed molecular analyses of the genetic diversity and characterization of HRVs among pilgrims with ARTIs during the 2019 Hajj Season.

## **2. Methods:**

### **2.1. Ethics approval, Study design and Patient samples**

This cross-sectional analytical study was approved by the Research Ethics Committee (REC) of the Unit of Biomedical Ethics in the Faculty of Medicine King Abdulaziz University (KAU) (Reference No 569-20). Written consent forms were obtained from participants. Nasopharyngeal swabs (NS) were collected in viral transport media (VTM) from pilgrims with respiratory tract diseases who presented to the healthcare facilities in the holy places of Makkah, Saudi Arabia during the 2019 Hajj season. Demographic data including nationality, age, and gender from all participants were obtained. NS samples were stored at a temperature of  $-80^{\circ}\text{C}$  in the Special Infectious Agents Unit (SIAU), King Fahd Medical

Research Center (KFMRC) until they were tested. The molecular detection of HRV in addition to other respiratory pathogens was performed as described previously using FastTrack Respiratory 21 kit (FastTrack Diagnostics, UK) (Alsayed et al., 2021).

## 2.2. Reverse Transcription and PCR amplification for human Rhinovirus (HRV)

Complementary DNAs (cDNA) synthesis and PCR amplification was performed simultaneously in a single tube using One-Step RT-PCR System with Platinum<sup>TM</sup> Taq DNA Polymerase (Invitrogen, Thermo Fisher Scientific, USA). RT-PCR reaction contained 1 µl of extracted RNA, 12.5 µl of 2X Reaction mix (buffer, dNTP, and MgSO<sub>4</sub>), 8.5 µl of H<sub>2</sub>O, 1 µl of Taq polymerase enzyme, 0.5 µl of outer sense primer (OS-458) (CCGGCCCCCTGAATGYGGCTAA). and 0.5 µl of outer antisense primer (OAS-1125) (ACATRTTYTSNCCAAANAYDCCCAT) to amplify of the VP4-VP2 region of the genome, the final volume was 25 µl according to *Wisdom, A., et al* (Wisdom et al., 2009). The solution was incubated in the thermocycler (ABI Applied Biosystems, USA) at 55°C for 30 min followed by 72°C for 1 min, 1 cycle for cDNA synthesis and pre-denaturation step. PCR was performed for 40 cycles of amplification (denaturation at 95°C for 15 sec, annealing at 50°C for 30 sec, and extension at 72°C for 1 min) followed by a final extension 1 cycle at 72°C for 10mins.

## 2.3. Nested PCR Reaction

The Nested PCR Reaction was performed by using nested primers for the amplification of VP4-VP2 region of HRV. The PCR reaction was performed on 5 µl of 1<sup>st</sup> PCR product as template, the reaction mixture consisted of 12.5 µl of GoTaq Hot Start Green Master Mix (Promega, USA), 5.5 µl of nuclease free H<sub>2</sub>O, 1 µl of each primers (inner sense - 547 (IS) (ACCRACTACTTTGGGTGTCCGG) and inner antisense -1087 (IAS) (ACCRACTACTTTGGGTGTCCGG)) and 5 µl of the first PCR product, in a final volume of 25 µl. PCR reaction was performed in the thermocycler (ABI Applied Biosystems, USA)

with an initial denaturation step at 94°C for 30 sec 1 cycle, followed by 40 cycles of amplification (denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 2 min). A final extension 1 cycle at 72°C for 10 min. PCR products were subjected to gel electrophoresis using 1% agarose gel stained with ethidium bromide and visualized under UV light. The band of interest was cut out and PCR products (between 500 and 600 bp) were purified by column purification using a Norgen Biotek DNA gel extraction Kit (Norgen, Canada) according to manufacturer instructions.

#### **2.4. Cycle sequencing reaction and Phylogenetic analysis**

Samples were subjected to cycle sequencing for HRV VP4-VP2 genomic fragment in both forward and reverse directions using nested primers (inner sense -547 (IS) inner antisense -1087 (IAS). The BigDye Terminator version 3.1 Reaction Cycle Sequencing Kit (Applied Biosystems, USA) was used according to manufacturer's instructions. The sequencing reactions were carried out on a thermocycler (ABI Applied Biosystems, USA), under the following conditions: initial denaturation for 2 min at 96°C, followed by 30 cycles of 96°C for 30 sec, annealing for 15 sec at 55°C and extension for 4 min at 60°C. The sequencing products were purified using dry Sephadex® with 0.45mm MultiScreen-HV. Assembly of the generated forward and reverse sequences was performed using Geneious R09 software (Kearse et al., 2012). Sequences were blasted in GenBank of NCBI and related sequences were retrieved. Phylogenetic analysis was performed using MEGA x (v.10, Pennsylvania State University, University Park, PA, USA).

#### **2.5. Data curation**

GraphPad Prism software version 9 was used to create the graphs (GraphPad Software, La Jolla, CA, USA).



### 3. Results and Discussion

HRV is the most prevalent cause of RTI infection reported among Hajj pilgrims (Benkouiten et al., 2014, Benkouiten et al., 2013, Benkouiten et al., 2015, Gautret et al., 2016, Gautret et al., 2013, Gautret et al., 2014, Memish et al., 2012., Alsayed et al., 2021, HRVs are members of *Picornaviridae* family, with positive-sense, a single-stranded RNA of approximately 7,200 bp enclosed within the capsid. Viral genome composed of a 5' untranslated region (5' UTR), followed by a single, long open reading frame (ORF), and terminated by a short 3'UTR and poly A tail. The ORF encodes a polyprotein that is co-translationally cleaved into four structural viral particle proteins (VP4, VP2, VP3 and VP1) and seven non-structural proteins (2A, 2B, 2C, 3A, 3B, 3C and 3D) (Huang et al., 2009, Le Gall et al., 2008, Schibler et al., 2012).

We sequenced the positive samples in the VP4-VP2 region of the viral genome. All HRV-positive samples (n = 53) were subjected to DNA sequencing in the VP4-VP2 gene region. We were able to generate sequences for 19 samples, the remaining samples were not sequenced either because insufficient sample volume or because they had low viral load (as indicated by their high Ct values in real time PCR) (**Figure 1**). Phylogenetic analysis showed that out of these 19 sequences, 13 belonged to species A (68.42%), 2 to species B (10.53%), and 4 belonged to species C (21.05%). Samples that belonged to HRV-A were found to be for pilgrims from several countries (3 from Indonesia, 2 from India, 2 from Somalia, one from each of Bangladesh, Dominican Republic, Fiji, Sudan, Saudi Arabia and Turkey); while the two HRV-B samples were found to be for two pilgrims from Sudan. Genotype C samples were found to be for pilgrims from Indonesia, Morocco, Oman and Tunisia. Samples of HRV-A species were found to be of genotypes A7 (2 samples), A101 (2 samples), A21 (2 samples), A57 (2 samples), and A30, A23, A60, A51 and A11 (1 sample each). While HRV-B species' samples belonged to genotypes B4 and B84, the four samples of the HRV-C

species belonged to genotypes C15 (2 samples), C3 and C56. Sequences from this study were submitted to Genbank and were given the accession numbers OM103602-OM103620. The proportions of the three HRV species are consistent with previous studies that showed HRV-A and HRV-C strains to be generally more prevalent than HRV-B [38-44].

Nucleotide sequences generated from the positive samples of infected pilgrims were aligned with the HRV-A, HRV-B, and HRV-C reference sequences and other sequences from different countries retrieved from GenBank and subjected to phylogenetic analysis. Phylogenetic analysis showed that circulating HRV-A during the 2019 Hajj season could be differentiated into 9 HRV-A genotypes (namely A7, A11, A21, A23, A30, A51, A57, A60, A101) and formed close clusters with isolates of HRV from various locations. As presented in **Figure 2a**, in HRV genotype A101, two sequences from Somalia clustered with genotype 101 isolates from USA. Two sequences from Indonesia and Bangladesh clustered with HRV genotype A7 from USA, Spain, Germany and Singapore. Two sequences from Indonesia and Dominican clustered with HRV genotype A57 isolate from China. One isolate from Sudan clustered with genotype A30 isolates from USA. Two sequences from Indonesia and the Dominican Republic clustered with genotype A57 isolates from USA and China and two sequences from India and Turkey clustered with genotype A21 from Colombia, USA and China. One sequence from India clustered with genotype A60 isolates from Singapore, USA and Peru, one sample from Indonesia clustered with A23 isolates from USA and one sequence from Saudi Arabia clustered with isolates genotype A11 isolates from Singapore, Venezuela, Poland, Australia and USA. The 2 HRV-B sequences were from Sudan, one belonged to genotype B84 and clustered with isolates of the same genotype from Argentina, India and China, while the other belonged to genotype B4 sequence and clustered with isolates of the same genotype from USA and Australia (**Figure 2b**). As presented in **Figure 2c**, the clustering of HRV-C genotype sequences from this study: two sequences (one from

Indonesia and one from Oman) clustered with HRV genotype C15 isolates from Kenya, USA, Malaysia and Australia, and one Sequence from Morocco clustered with genotype C3 isolate from India and one sequence from Tunisia clustered with genotype C56 isolates from Italy. HRV-A sequences showed more diverse genotyping as they were distributed between 7 HRV-A genotypes. Studies showed that up to 20 HRV different serotypes might circulate simultaneously in a community (Lee et al., 2012, Monto, 2002, Monto et al., 1987) with the spectrum of serotypes changing from season to season (Lee et al., 2012). However, due to the small number of sequences available and the lack of continuing molecular surveillance of circulating HRV strains during Hajj season, it is difficult to rule out the possibility of other genotypes circulating among pilgrims. An association between HRV genotypes and the severity of the disease has been reported (Fawkner-Corbett et al., 2016, Marcone et al., 2014, Zhao et al., 2018). Our results showed no significant association of the HRV genotype with the viral genome copy number (as indicated by the Ct values in real time RT-PCR) of the recruited samples as shown in **figure 1**.

HRV infection may cause exacerbations of chronic respiratory disease and is associated with pneumonia, especially in children, the elderly, and immunocompromised patients (da Costa Souza et al., 2021). In our study, the majority (39/53; 73.58%) of HRV-infected individuals was over 60 years old. Thus particular attention must be given by healthcare workers at the Hajj to preventive healthcare and medical needs of elderly pilgrims. The disseminating of human-to-human HRV among pilgrims, in particular, may have been increased by the crowded conditions in the Hajj season (da Costa Souza et al., 2021). This has implications for transmission of other respiratory tract viruses including SARS-CoV-2 and influenza. Moreover, the pilgrims with HRV came from 21 countries, mainly from Indonesia (15 cases), India (9 cases) and Morocco (4 cases), followed by Somalia and Sudan (3 cases each). Then pilgrims coming from Saudi Arabia, Bangladesh, and Nigeria with 2

positive cases each. Finally, pilgrims coming from Turkey, Egypt, Syria, USA, France, Oman, Tunisia, Dominica, Ethiopia, Togo, Fiji, Chad, and Afghanistan with 1 positive case each.

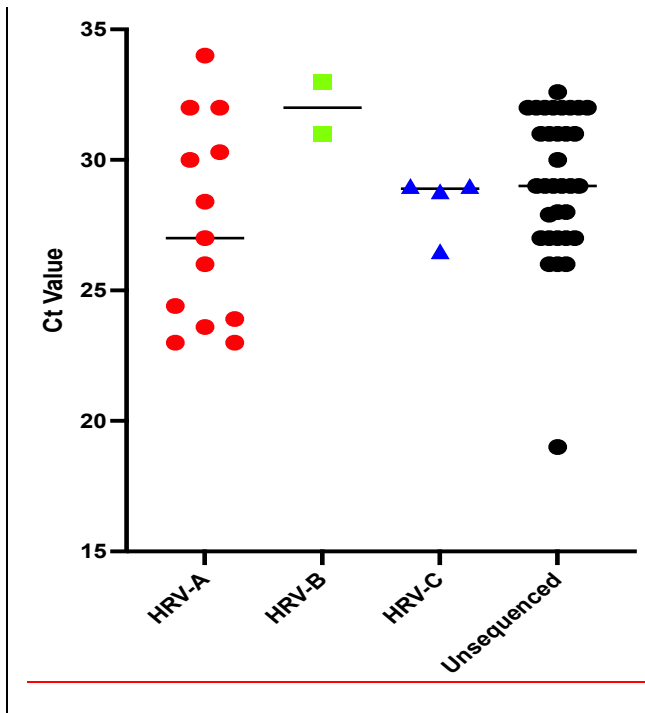
Our study had the limitation of the small number of HRV positive samples for molecular typing and our study only covered one Hajj season. The camps where the domestic pilgrims live during Hajj is entirely separated those of where international pilgrims are housed. Both groups of pilgrims mingle with each other only during their Hajj ritual activities and determining the transmission dynamics was not possible. Larger studies for HRV surveillance among pilgrims in different Hajj seasons and throughout the year among Umrah pilgrims are needed. Since over 2 million pilgrims from across the world visit Saudi Arabia each year large cohort studies are required to define the genotypic diversity and spread of respiratory tract viruses, and also define the association between rhinovirus infections and SARS-COV-2 (Dee et al., 2021). They should also be designed to define whether new strains of rhinovirus are being imported to KSA by pilgrims from overseas.

#### **4. Conclusions:**

In summary, we show that sequencing studies of respiratory tract viruses in pilgrims is important. We provide preliminary evidence of high diversity of HRV genotypes circulated in pilgrims a restricted area during Hajj. This high diversity in a small number of individuals and in the short time of Hajj season requires further study in parallel with genetic sequencing of other respiratory viruses such as SARS-CoV-2 and influenza so that preventive measures can be delineated more accurately.

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Figure legends:



**Figure 1.** Association of the HRV genotype with the genome copy numbers as indicated by the Ct values in real time RT-PCR.



comparison of those sequences with those from reference strains of species A, B and C. (A) Sequences of pilgrims patients with HRV type A. (B) Sequences of patients with HRV type B. (C) Sequences of patients with HRV type C. Those sequences without denotation are the reference sequences. Bootstrap values (1000 replicates).

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**Author Contributions:** Conceptualization, S.A.E.-K., T.A.A. A.Z. and E.I.A.; methodology, S.A.E.-K., S.M.A., H.A., A.M.H. and K.M.A.; validation, S.A.E.-K., S.M.A., T.A.A., L.H.B., A.A.F., and E.I.A.; formal analysis, S.A.E.-K., S.M.A., T.A.A., H.A., A.A.F., and E.I.A.; investigation, S.A.E.-K., S.M.A., T.A.A., A.M.H., H.A., A.A.F., A.Z. and E.I.A.; resources, S.A.E.-K., T.A.A, A.A.F., K.M.A., and E.I.A.; data curation, S.A.E.-K., S.M.A., T.A.A., A.Z. and E.I.A.; writing—original draft preparation, S.A.E.-K., S.M.A., and T.A.A.; writing—review and editing, All authors.; supervision, S.A.E.-K., T.A.A. and E.I.A.; project administration, S.A.E.-K., T.A.A. and E.I.A. All authors have read and agreed to the published version of the manuscript.

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**Informed Consent Statement:** Written consent forms were obtained from participants.

**Data Availability Statement:** All data related to the study are available within this manuscript.

**Conflicts of Interest:** The authors declare no conflict of interest.

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#### **Declaration of interests**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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