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Short Communication

Molecular characterization of influenza a viruses circulating in pediatric patients: A cross-sectional study

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Abstract

Influenza continues to be a major public health issue with the contribution of annual epidemics and sporadic outbreaks. The objective of the study is to investigate and characterize the circulating influenza A virus among pediatric patients over a period of 36 months, from March 2021 to March 2024 at a tertiary care hospital in Karad, India. A total of 160 pediatric patients aged from 1 year and 6 months to 5 years, presented with influenza-like illness, including fever, cough, breathlessness, and runny nose, were included in the study. Nasopharyngeal swab samples from both outpatient and inpatient respiratory illness cases were evaluated and processed for RNA extraction and real-time polymerase chain reaction (RT-PCR) detection of influenza A/B, including pandemic H1N1 and H3N2. Four of the samples tested positive for Influenza A and further nucleotide DNA sequencing and phylogenetic analysis were done at the ICMR-National Institute of Virology, Pune, using Miniseq NGS Platform and MEGA11 software. Sequence analysis revealed mutations in HA with especially three mutations at the receptor-binding domain (RBD); S137P, A141T, and R142K, which improve viral fitness and replication in respiratory epithelial cells. The R223Q mutation correlated with improved viral fitness and enhanced viral replication in human respiratory tract epithelial cells without altering the antigenic properties. Glycosylation site analysis revealed changes that alter virus-host interaction and display possible confirmation of oseltamivir's continued effectiveness. Phylogenetic analysis showed a unique evolving lineage revealing the necessity of regular vaccine updates. Robust surveillance is critical for early detection of emerging strains and guidance.

Keywords: Influenza A virus, Genomic characterization, Viral mutation, Hemagglutinin, Neuraminidase, Pediatric.

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1. Introduction

Influenza is a highly communicable viral infection that affects the respiratory tracts. The infections occur as seasonal epidemics and unpredictable pandemic on the global scale.[1] The seasonal influenza outbreak impacts about a billion people worldwide and approximately 3–5 million people experience severe illness due to this infection. According to the World Health Organization (WHO), "99% of deaths in children under 5 years of age with influenza-related lower respiratory tract infections are in developing countries".[2] These numbers are alarming for a developing nation like India. Among all the variants of the influenza viruses, influenza A viruses are the only known to be associated with global pandemics such as swine flu and bird flu.[3] The influenza A virus replicates predominantly in the respiratory epithelium, where the infectious particles are developed due to the cleavage of *hemagglutinin (HA)* protein.[4] The infection is transmitted through respiratory droplets and contact with contaminated surfaces. The infection spreads in the body even before the person is symptomatic for about up to 5 to 7 days after being infected.[5]

The virus rapidly mutates, eluding immunity and develops resistance to treatments, resulting in a lower vaccine efficacy. Mutations of the virus compel the urgency of broadly protective vaccines and new therapies.[6] The continuous evolution highlights the importance of

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surveillance and molecular characterization of circulating strains of influenza to guide development of vaccines and public health strategies.[7] Ongoing genomic analysis is important for identifying genetic (drift/shift) changes that impact viral virulence, host range, and susceptibility to antiviral drugs.[8] The objective of the present study was to identify and characterize the circulating strains of influenza A virus in pediatric patients in a tertiary care hospital setting.

2. Materials and Methods

We conducted a cross-sectional study to identify and conduct molecular characterization of circulating influenza A virus strains in pediatric patients in a tertiary care hospital in Karad, India between March 2021 and March 2024.

Patients who presented at the outpatient and inpatient department of the tertiary care hospital were screened. Pediatric patients aged from 18 months to 5 years presenting with influenza-like illness, along with symptoms including fever > 38□C, cough, breathlessness, and runny nose were included in the study. Patients who did not have the above symptoms and whose parents refused to give consent for this study were excluded from this study.

Nasopharyngeal swab samples from both outpatient and inpatient respiratory illness cases were evaluated for the presence of influenza viruses through real-time polymerase chain reaction (RT-PCR). Detection of influenza A/B (subtypes H1N1 pdm09 and H3N2) was done according to WHO protocols.[9,10]

The collected samples were processed for RNA extraction and real-time RT-PCR for the detection of influenza A and B viruses, including subtypes H1N1 pdm09 and H3N2. All samples were stored at -80^{o}C for further use. Four positive samples of Influenza A H1N1pdm09 virus were sent to Indian Council of Medical Research - National Institute of Virology (NIV), Pune for further confirmation and nucleotide sequencing as per the guideline published by NIV Pune. Whole genome nucleotide sequencing was performed using Miseq NGS Platform (Illumina, https://www.illumina.com). Neuraminidase (NA) nucleotide DNA sequence was analyzed for oseltamivir drug resistance using the method described by Potdar et al.[7] Phylogenetic and mutational analysis were done by using MEGA11 (https://megasoftware.net) with a Tamura-Nei nucleotide substitution model including 1,000 replicates bootstrap support for analysis of *HA* genes.[9,11] The whole genome was submitted to GISAID under accession numbers EPI_ISL_19201443–EPI_ISL_19201446. Glycosylation sites of the *HA* gene protein were analyzed by using Bioedit Count_GS software.

The study was conducted in accordance with the Declaration of Helsinki. As it was a pediatric study, written informed consent was obtained from parents or guardians or legal representatives of the patients before being enrolled in this study. The research proposal was approved by the Institutional Ethics Committee before initiating the study (KIMSDU/IEC/05/2021).

3. Results

Acute respiratory infection (ARI) samples had a higher total positivity i.e., 3.12% (5 positive samples out of 160) as compared with 1.25 % (2 positive samples out of 160) in severe acute respiratory infection (SARI) samples. In both the ARI and SARI cases, the highest positivity was especially during the months of June to October. Overall, a slightly higher male to female ratio was observed (male-to-female ratio 1:0.9). The mean age of the patients was 3.4 years.

Among the 160 samples analyzed, four samples were found to be positive for the influenza A virus. To assess antiviral susceptibility of the influenza A(H1N1) pdm09 particularly to drug oseltamivir, we examined the sequence of *NA* gene for the *H275Y* mutation associated with resistance. All *NA* gene sequences from the samples showed sensitivity to oseltamivir. Detailed sequence analysis of the *HA* gene revealed multiple mutations including *S83P*, *D94N*, *S137P*, *A141T*, *R142K*, *V173I*, *R223Q*, *E260D*, *A277T*, *D356E*, *V418I*, *and H451N***.** Mutations at positions 137, 141, and 142 were within the receptor-binding domain, which is critical for viral entry and immune system recognition. The *R223Q* mutation, present in all four samples, showed enhanced viral fitness for rapid replication in human epithelial cells of respiratory track without affecting antigenic properties (**Table 1**).

The phylogenetic tree of *HA* gene (**Figure 1**) shows that influenza A viral strain grouped with 6B.1A.5a.2a but showing separate clade. Phylogenetic analysis indicated that strains clustered with 6B.1A.5a.2a clade with signs of a unique evolving lineage, supporting frequent updates on vaccines (**igure 1**). The HA protein showed a total of nine glycosylation sites matching those found in vaccine strain at stalk (27, 28), side of head (40, 104), top of the head (179, 293), and HA-2 stalk (304, 498, 557). For the neuraminidase (N1) protein, eight glycosylation sites were identified as compared to vaccine strain which has seven sites. These include five sites on the stalk (42, 50, 58, 63, and 68) and three on head (88, 146, 235). The 50th glycosylation site showed a new addition in our viral strain, representing an effective change in glycosylation sequence which may influence virus-host interactions (**Table 1**).

This study highlighted the genomic characterization of influenza A virus in pediatric patients. These study findings provide key insights into genetic evolution of the virus as well as its public health implications. Mutations including *S137P*, *A141T*, and *R142K* in the receptor-binding domain of *HA* highlight that the virus's ability to adapt to the immune pressures within a host to facilitate infection and transmission.

Table 1: Mutation analysis of *haemagglutinin* gene mutation

S.No.	Sample ID	Mutation (Compared with A/Victoria/4897/2022) (Recommended vaccine strain) (20)
1	A/India/Karad-RSV-18/2023	*S83P, D94N, S137P, A141T, R142K, V173I, R223Q, E260D, A277T, D356E, V418I, H451N.*
2	A/India/Karad-RSV-20/2023	*S83P, D94N, S137P, A141T, R142K, R223Q, E260D, A277T, D356E, V418I, H451N.*
3	A/India/Karad-RSV-36/2023	*E21K, R45K, S137P, R142K, R223Q, E260D, A277T, D356E, K374E, V418I, N444K, H451N*
4	A/India/Karad-RSV-38/2023	*S83P, S85P, D94N, S137P, R142K, R223Q, E260D, H273Q, A277T, V321I, D356E, 418I, H451N*

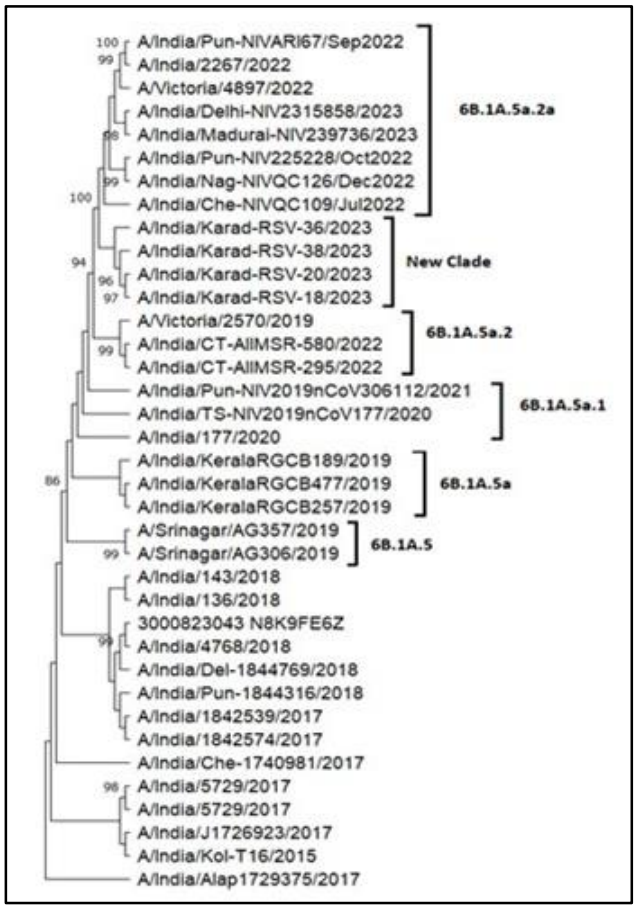

Figure 1: Phylogenetic analysis of influenza *haemagglutinin* gene

A/Victoria/4897/2022 is a current vaccine strain.

Mutation and their Impact on Virus Adaptation-*S83P, D94N, S137P, A141T, R142K, V173I, R223Q, E260D, A277T, D356E, V418I, H451N:* These mutations span various regions of the *HA* protein. Mutations in positions 137, 141, and 142 are located in the receptor-binding domain (RBD) of *HA*, which is crucial for binding to host cells. Changes in these positions could alter the virus's ability to infect cells or its recognition by antibodies.

E21K, R45K, S137P, R142K, R223Q, E260D, A277T, D356E, K374E, V418I, N444K, H451N: These mutations affect different regions of the *HA* protein. Notably, mutations in positions 21, 45, and 444 are outside the RBD but could still impact the overall structure or function of the protein, potentially influencing viral fitness or immune evasion.

S85P, H273Q, V321I: These mutations are also noteworthy. While *S85P* is close to position 83, which is in the RBD, *H273Q* and *V321I* are in other regions of the protein. *H273Q* could affect the *HA* protein's stability or other functions.

The *R223Q* mutation was seen in all the samples; however, there was no significant reduction of immune recognition indicating no major implications on the susceptibility to vaccine. Nine sites of glycosylation were involved in *HA*, whereas eight sites (and one more at position 50) were involved in *NA*, these mutations appear crucial for the adaptations by viruses towards immune evasion. However, none of the *NA* genes yielded any oseltamivir-resistant mutations indicating this antiviral treatment continues to be effective.

4. Discussion

In a sample of 593, 46% children ≤ 5 years of age tested positive for influenza. Molecular characterization of *HA* gene was performed, particularly the HA1 subunit of A(H1N1) pdm09, A(H3N2), and B influenza strains. During the 2012-2013 season 54.8% of influenza A virus circulating in children aged ≤5 years, the A(H1N1) pdm09 strain revealed its rapid replication and dynamic nature of influenza A virus.[12] In a study by Ram et al., among 16 million influenza-associated acute respiratory infection cases in children less than five years of age, 11.2% of outpatient cases and 7.1% of inpatient cases tested positive for influenza.[13] In another study carried out by Chao et al. in 519 patients, 320 tested positive for influenza A. Only about 40 percent were found positive according to the Influenza rapid diagnostic test (IRDT). Most of the influenza A virus infections were observed in children under the age of 6 years.[14] A study reported that out of 1495 cases, influenza viruses were identified in 28.2% samples. In the positive cases 71.5% were influenza A virus.[15] In our study, 4.37% of cases were positive for influenza A virus in children ≤5 years of age. Though positive rates are low compared with previously reported regional studies, it might be due to the COVID19 outbreak. During this outbreak influenza activity was relatively low due to public health measures like use of mask, social distancing, and increased vaccination efforts.

However, despite limitations, our study provides key insights into the genetic evolution of influenza A viruses highlighting the mutations occurring in the *HA* and *NA* genes. Phylogenetic analysis interprets the evidence of viral evolution and significance of vaccine updates, while further changes in glycosylation would also suggest immune evasion strategies. The variants identified in this study were still susceptible to oseltamivir. Continuous surveillance is important for early recognition of new strains and pointing the way toward relevant public health interventions-applied vaccination and antiviral strategies.

5. Ethical Statement

The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

6. Author Contributions

Dilip Hinge contributed to the concept and design of the study, participated in the acquisition, analysis, and interpretation of data, and drafted the manuscript. Satish Patil supervised the study and reviewed the manuscript for important intellectual content. Both authors developed and critically reviewed all drafts of the manuscript and take complete accountability of data and content published in this manuscript.

7. Data Availability Statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

8. Source of Funding

None.

9. Conflict of Interest

All authors have completed the ICMJE uniform disclosure form. The authors have no conflicts of interest to declare.

10. Acknowledgments

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