

Molecular detection of Human Rhinovirus Infection among Children with Respiratory infection attending University of Ilorin Teaching Hospital, Ilorin, Nigeria.

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Abstract

Human rhinoviruses (HRV) are the most common cause of the common cold and infections are asymptomatic, as a result, these viruses are mostly ignored. In addition, there is no diagnostic strategy for these viruses. However, reports have shown an association between HRV infections in early life and the consequent induction of asthma. It is therefore pertinent to conduct research to assess the baseline epidemiology knowledge of these viruses in the community. The present study aimed to use molecular methods to investigate the prevalence of Human Rhinoviruses A and B among infants and children under 5 years attending the outpatient pediatric clinic at the University of Ilorin Teaching Hospital between October and December 2021.

A total of 200 nasopharyngeal (NP) and oropharyngeal (OP) samples from participants with symptoms of respiratory infection were collected. The samples were tested for HRV A and B using real-time reverse transcription polymerase chain reaction using primers that were designed HRV- VP4/VP2 coding region.

A prevalence of 20% was recorded, 40 samples out of the 200 tested positive for both Human Rhinoviruses A and B. HRVA accounted for 9.5% (19 of 200) while HRVB was 10.5% (21 of 200). The analysis also showed that there was higher positivity of HRV A and HRV B in the 0-1 year age group. Of the 21 positive for HRV B, age 0-1 year accounted for 42.8% (9/21) while 47% (8/19) were positive for HRV A. The study showed that there is high prevalence of HRV infection among children between 0-5 years of age. It is therefore important to continue to monitor in order to prevent potential underlying respiratory conditions such as asthma among this susceptible age group.

Keywords: Human Rhinovirus, Respiratory infection, qPCR

Introduction

Human rhinovirus (HRV) is one of the leading causes of upper respiratory tract infections (URI) associated with common cold but may also lead to more severe respiratory illness in vulnerable populations.⁹ Human Rhinovirus is a non-enveloped, positive-sense, single-stranded RNA virus, classified under the genus *Enterovirus*, family *Picornaviridae*, with a genome ~7.2 kb long. It is characterized by high genetic and antigenic diversity with 169 HRV genotypes distributed across 3 species (A, B, and C): HRV-A (80), HRV-B (32), and HRV-C (57) genotypes.⁹

Most HRV cases are mild, non-specific, and self-limiting infections,⁶ they contribute to substantial economic losses through missed school and workdays.⁶ HRV is a common reason for prescribing antibiotics, which contributes greatly to antibiotic resistance.⁹ The virus can be transmitted via inhalation of contaminated aerosols with the virus, for example, during physical contact with infected persons, or self-inoculation via touching of infected surfaces or objects.⁶ Children are the most susceptible population that drives the transmission and persistence of HRV in populations.⁹ HRV has an incubation period ranging from 1 to 7 days and a symptom duration of 7 to 14 days.⁸

The laboratory diagnosis of HRV infections is important not only for epidemiological purposes but also for optimizing the medical management of patients. Detection of HRV by culture is slow and complex for HRVA and HRVB, HRVC has not been successively cultured *in vitro* to date.¹⁰ Serologic diagnosis is virtually impossible due to the number of serotypes, and rapid antigen test kits are not available.⁷ Molecular methods such as real-time RT-PCR appear to be the most suitable method, combining short analysis time, high sensitivity, semi-quantification of viral load, and the detection of most respiratory viruses with multiplex methods.³

Human rhinoviruses are distributed worldwide but, the peak HRV incidence in the tropics occurs during the rainy season, from June to October.^{12,16} The only known host of HRV is humans and it is the commonest viral infection that affects children with respiratory symptoms at an early age.¹

Efforts for the development of effective vaccines against human rhinovirus has been frustrated due to a large number of HRV serotypes. As a result, there is no effective vaccine for HRVs. Though, there

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are some preventive measures that can be taken in order to avoid Human Rhinovirus infection these include; washing hands, using respiratory hygiene/cough etiquette, avoiding touching your eyes, nose, and mouth with unwashed hands, and limiting contact with infected people. However, these preventive measures are hard to implement in the most susceptible population (children).

It has been shown that infection with Human Rhinoviruses at an early age can predispose to long-term respiratory conditions such as asthma.¹³ Therefore, the surveillance of these viruses among these susceptible age groups is important in order for proper patient management.

Ethical Approval

Ethical approval for the study was obtained from the University of Ilorin Teaching Hospital, Ilorin, Kwara State. Informed written consent was obtained from the parents/guardians of the of the participants after highlighting to them the background and importance of the study.

Sample size

Two hundred nasopharyngeal (NP) and oropharyngeal (OP) samples from participants with symptoms of respiratory infection were collected for the study of Human Rhinovirus at the University of Ilorin Teaching Hospital between October to December 2021.

Materials and methods

Sample collection

A random sampling method was adopted in the study. Patients aged 0-5 years attending Pediatrics unit of the University of Ilorin Teaching Hospital were enrolled. A total of 200 Nasopharyngeal and Oropharyngeal swabs were collected from patients with acute respiratory symptoms such as runny nose, mild fever, nasal congestion, cough, sore throat, and sneezing. The samples were obtained by inserting a commercially purchased sterile swab into the nostril and mouth to a depth of 2-4 CM and retracting it in a slow rotating motion to trap epithelial cells in the swab. The swabs were stored in 2ml of Viral Transport Medium (VTM) and transported to the laboratory with ice packs after collection. The samples were adequately stored at -80°C until analysis.

Extraction of nucleic acid from samples

Extraction was done using a commercial viral RNA kit (Zymo product with cat no: R1035, Lot no: 209610) following the manufacturer's instructions. Briefly, 400µl of Viral RNA Buffer was added to 200µl of sample in a 2ml Eppendorf tube. The mixture was then transferred into a Zymo-spin column in a collection tube and centrifuged at 16000rcf for 2

minutes. The spin column was transferred into a new collection tube. 500µl of the Viral Wash Buffer was added to the spin column and centrifuged at 16000rcf for 30 seconds and the flow-through was discarded. The step was repeated. 500µl of ethanol (100%) was added to the spin column and centrifuged at 16000rcf for 1 minute to ensure complete removal of the wash buffer and the flow-through was discarded. The column was carefully transferred into a nuclease-free tube. The RNA was eluted in 15µl of DNase/RNase-free water was added directly to the column matrix and centrifuged for 30 seconds and the elute was collected in a 1.5 ml Eppendorf tube. The eluted RNA was stored at -80°C.

Primers and probes design and synthesis

Primers for Human Rhinovirus A and B were designed from the VP2 region using sequences available in the NCBI GenBank. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control for the PCR reaction to check the presence of inhibitors of the RT-PCR assay.¹¹ Both fluorogenic probes for Human Rhinoviruses A and B consisted of oligonucleotides with the 3' reporter dye 6-carboxyfluorescein (FAM) and hexachlorofluorescein (HEX) with 5' quencher dye black hole quencher 1 (BHQ1) (Table 1). Primers and probes were synthesized by Inqaba biotech, South Africa.

Reconstitution of primers and probes

Primers and probes were reconstituted according to the manufacturer's instructions, in nuclease free water for all primers and probes. They were reconstituted to stock concentrations of 100µM for probes and 1000µM for primers. A 10µM working stock of each of the primers and probes was made and stored at -20°C.

Preparation of master mix

The Luna Universal Probe One-Step RT-qPCR Kit (New England, BIOlabS, E3006) was used to prepare the reaction for the PCR and it was prepared according to the manufacturer's instructions. The master mix was prepared using One-Step reaction mix, WarmStart RT enzyme mix, primers, and probes. The reaction was prepared for each sample as stated in Table 2.

Multiplex polymerase chain reaction-reverse transcription (Multiplex RT-qPCR)

14µl of the master mix each well of the 96-well qPCR plate. 6µl of RNA template from each sample was added into the respective well of the qPCR plate and the plate was covered with a sealant. The following thermal cycling protocol was used for the qPCR: 50°C for 15min (cDNA synthesis), 95°C for 2min (reverse transcription), and 40 cycles of 95°C for 10s and 50°C for 20s and 60°C for 30s (PCR amplification). Reaction

products were detected on AriaMx Real-time PCR System (Agilent). There was No template control (NTC) for each PCR run.

Statistical analysis

Data were analyzed using Statistical Package for Social Sciences (SPSS) version 26. Sociodemographic data were summarized using descriptive statistics such as mean, frequency, percentage, and cumulative percentage was generated. Categorical information was analyzed with parametric and non-parametric test as adequate. Binary Logistic analysis was used to analyze the association of sociodemographic factors with Human Rhinovirus infection in children below five years as sampled.

Results

A total of 200 Nasopharyngeal and Oropharyngeal swab samples were screened for HRV A and B. Of these, 40 samples (20%) were positive for both HRV A and B. HRV A accounted for 47.5% (19/40) (Figure 1) while HRV B had 53.5% (21/40) (Figure 2).

The threshold for the positive samples is

417.525 with the CT values ranging from 27.68-33.66 using FAM for HRV A, HEX for HRV B, and ROX for GAPDH fluorescence dye for 45 cycles at 60°C for 30 seconds. The channel probes show fluorescence if there is amplification.

The analysis revealed that out of the 21 that tested positive for HRV A, 11 were male (9.9%) and 10 were female (11.2%) with no significant difference ($P=0.761$). While in HRV B, 9 (8.1%) males tested positive, and 10 (11.2%) females tested positive with no significant difference as well ($P=0.453$) (Table 3).

In regards to the age distribution of the participants, out of the 21 total positive for human rhinovirus A, age group 0-1 year recorded the highest positivity of 42.8% (9/21) and in all 19 positives for human rhinovirus B, highest positivity of 47% (8/19) was also recorded in age group 0-1 year. However, there was no significant difference between the different age groups for HRV A ($P=0.15$) and HRV B ($P=0.14$) positivity.

Children residing in urban areas recorded 20 (10.8%) positive cases of rhinovirus A and 19 (10.3%) positive cases of rhinovirus B. only 1 (6.7%) child from

Table 1 : Oligonucleotide sequences used in the Multiplex RT- qPCR Process for Human Rhinovirus

Primers name	Oligonucleotide sequences (5')	Target gene/Amplicon size (bp)	Reference
HRV A-VP2F	CTG TTG AGG CGT GTG GGT AT	VP2 gene/224bp	Selected using SnapGene viewer software
HRV A-VP2R	TCC AGT CGA AGC AGT TGT CC		
HRV A-VP2F_PROBE	FAM-AGGTTAATGCAGATCACCCGTGGG BHQ1		
HRV B-VP2F	AAC CAG ACA CCT CAG TGT GC	VP2 gene/236bp	Selected using SnapGene viewer software
HRV B-VP2R	GGC AAG TTG ATG CTC TGG GA		
HRV B-VP2F_PROBE	HEX-TTCCATTCTCTTGGCAGAACGGGA BHQ1		
GAPDH F	GAAGGTGAAGGTCGGAGT	GAPDH (human)/ 206bp	Ng EKO <i>et al.</i> (2002)
GAPDH R	GAAGATGGTGATGGGATTTC		
GAPDH P	Cy3.5-CAAGCTTCCCGTTCTCAGCC BHQ2		

Key: HRV, Human Rhinovirus; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

Table 2: Master Mix Preparation for the Multiplex RT-PCR Reaction

Components	20 µl reaction
Luna universal probes onestep reaction mix	10
Luna warm start RT enzyme mix	1 µl
Forward primers	0.4 µl (0.2 µM)
Reverse primers	0.4 µl (0.2 µM)
Probes	0.2 µl (0.1 µM)

the previous research of Oluwasemowo *et al.* 2020 that showed that human rhinovirus is a common aetiological agent that causes respiratory infection in infants and children. This may be due to immature or underdeveloped adaptive Immune response in infants and young children.

A prevalence of 20% for Rhinovirus A and B infection was determined in this study. This is in line with Baillie *et al.*, 2019 which showed that a total of 2,120 hospitalized cases with severe/very-severe pneumonia were enrolled across the three sub-Saharan African sites. Of these children, 439 (21%) tested positive for HRV.¹⁶

This study also established that HRVA is more prevalent with a total of 21 (10.5%) than HRVB with a total positive of 19 (9.5%). Oluwasemowo *et al.*, 2021 reported the same pattern in their study conducted in two communities in southwest Nigeria by showing that HRVA is more (21.5%) than HRVB (7.1%).² A study by Jianget *et al.*, 2020 showed that human rhinovirus A is more associated with respiratory tract infection, accounting for 68.13% of the total than HRVB with 20% of the total positive, which is also in agreement with our findings.⁵ This may be attributed to rhinovirus B having a slower and lower replication than HRVA therefore, causing less infection as reported by Royston and Tapparelin 2016.¹⁴

As a result of genetic, hormonal, and physiological variations that exist between males and females, it is typically discovered that the HRV infection rate is higher among males than females. The reason could be adduced to the innate and adaptive immunity being higher in females than in males, and this could result in a speed-up clearance of viruses.¹⁵ In our current study, there was a slight male predominance, but with no significant difference.

Conclusion

The current study has demonstrated the importance of using molecular techniques to detect Rhinovirus infections, especially when identifying multiple strains. The method may be cumbersome but it is considered an excellent method with adequately high sensitivity. A further study is required to detect the HRV A and B subtypes to determine which serotype is prevalent in our community.

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References

1. Kolawole OM, Adeyemi BJ, Gobir AA. Prevalence of human rhinovirus infection in children with acute respiratory symptoms in Ilorin, Nigeria. *Nigerian Health Journal*. 2015;15(2):62-8.

2. Oluwasemowo OO, Nejo YT, Abokede JO, Lawson M, Motayo BO. Genotypes of rhinovirus detected among children in two communities of south-west Nigeria. *Virus genes*. 2021 Jun;57(3):276-9.
3. Caliendo AM. Multiplex PCR and emerging technologies for the detection of respiratory pathogens. *Clinical Infectious Diseases*. 2011 May 1;52(suppl_4):S326-30.
4. Faux CE, Arden KE, Lambert SB, Nissen MD, Nolan TM, Chang AB, Sloots TP, Mackay IM. Usefulness of published PCR primers in detecting human rhinovirus infection. *Emerging infectious diseases*. 2011 Feb;17(2):296.
5. Jiang H, Yang T, Yang C, Lu Y, Yi Z, Zhang Q, Wang W. Molecular epidemiology and clinical characterization of human rhinoviruses circulating in Shanghai, 2012-2020. *Archives of Virology*. 2022 Apr;167(4):1111-23.
6. Jacobs SE, Lamson DM, St. George K, Walsh TJ. Human rhinoviruses. *Clinical microbiology reviews*. 2013 Jan;26(1):135-62.
7. Kennedy JL, Turner RB, Braciale T, Heymann PW, Borish L. Pathogenesis of rhinovirus infection. *Current opinion in virology*. 2012 Jun 1;2(3):287-93.
8. Loeffelholz MJ, Trujillo R, Pyles RB, Miller AL, Alvarez-Fernandez P, Pong DL, Chonmaitree T. Duration of rhinovirus shedding in the upper respiratory tract in the first year of life. *Pediatrics*. 2014 Dec;134(6):1144-50.
9. Luka MM, Kamau E, Adema I, Munywoki PK, Otieno GP, Gicheru E, Gichuki A, Kibinge N, Agoti CN, Nokes DJ. Molecular epidemiology of human rhinovirus from 1-year surveillance within a school setting in rural coastal Kenya. In *Open forum infectious diseases 2020 Oct (Vol. 7, No. 10, p. ofaa385)*. US: Oxford University Press.
10. McCulloch DJ, Sears MH, Jacob JT, Lyon GM, Burd EM, Caliendo AM, Hill CE, Nix WA, Oberste MS, Kraft CS. Severity of rhinovirus infection in hospitalized adults is unrelated to genotype. *American journal of clinical pathology*. 2014 Aug 1;142(2):165-72.
11. Ng EK, Tsui NB, Lam NY, Chiu RW, Yu SC, Wong SC, Lo ES, Rainer TH, Johnson PJ, Lo YD. Presence of filterable and nonfilterable mRNA in the plasma of cancer patients and healthy individuals. *Clinical chemistry*. 2002 Aug 1;48(8):1212-7.
12. Da Costa Souza L, dos Santos EM, Goreth M, Nagata T. 4.1 Rhinovirus incidence in association with climate factors in Brasília, Brazil. *Rinovírus no Distrito Federal: caracterização epidemiológica e molecular por sequenciamento de alta performance*. 2021:87.
13. Jackson DJ, Gern JE. Rhinovirus infections and their roles in asthma: etiology and exacerbations.

The Journal of Allergy and Clinical Immunology: In Practice. 2022 Mar 1;10(3):673-81.

14. Royston L, Tapparel C. Rhinoviruses and respiratory enteroviruses: not as simple as ABC. *Viruses*. 2016 Jan 11;8(1):16.

15. Klein SL. Sex influences immune responses to viruses, and efficacy of prophylaxis and treatments for viral diseases. *Bioessays*. 2012 Dec;34(12):1050-9.

16. Baillie VL, Moore DP, Mathunjwa A,

Morailane P, Simões EA, Madhi SA. Molecular subtyping of human rhinovirus in children from three sub-Saharan African countries. *Journal of clinical microbiology*. 2019 Sep;57(9):e00723-19.

17. Morobe JM. *Investigating Origins and Spread of Human Rhinovirus in Kilifi, Rural Coastal Kenya by Analysis of Molecular Sequence Data* (Doctoral dissertation, JKUAT-IBR) 2020.