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% (21of 200). The analysis also showed that there was higher positivity of HRV A and HRV B in the 0-1year 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

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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^{0}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^{0}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^{0}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 mixeach 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 wascovered 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.HRVA accounted for 47.5% (19/40) (Figure 1)while HRVB had53.5% (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 HRVA, HEX for HRVB, 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 HRVA, 11 were male (9.9%) and 10 were female (11.2%) with no significant difference (P = 0.761). While in HRVB, 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 19positives 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 HRVA (P = 0.15) and HRVB (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'-3')	Target gene/Amplicon size (bd)	Reference
HRVA-VP2F	CTG TTG AGG CGT GTG GGT AT	VP2 gene/224bp	Selected using SnapGene viewer software
HRVA-VP2R	TCC AGT CGA AGC AGT TGT CC		
HRVA-VP2F_PROBE	**FAM-**AGGTTAATGCAGATCACCCGTGGG-BHQ1		
HRVB-VP2F	AAC CAG ACA CCT CAG TGT GC	VP2 gene/236bp	Selected using SnapGene viewer software
HRVB-VP2R	GGC AAG TTG ATG CTC TGG GA		
HRVB-VP2F_PROBE	**HEX-**TTCCATTCTCTTGGCAGAACGGGA-BHQ1		
GAPDHF	GAAGGTGAAGGTCGGAGT	GAPDH (human)/ 206bp	Ng EKO *et al*. (2002)
GAPDHR	GAAGATGGTGATGGGATTTC		
GAPDHP	Cy3.5-CAAGCTTCCCGTTCTCAGCC-BHQ2		

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

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

Components	**20 µl reaction**
Luna universal probes one-step 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)

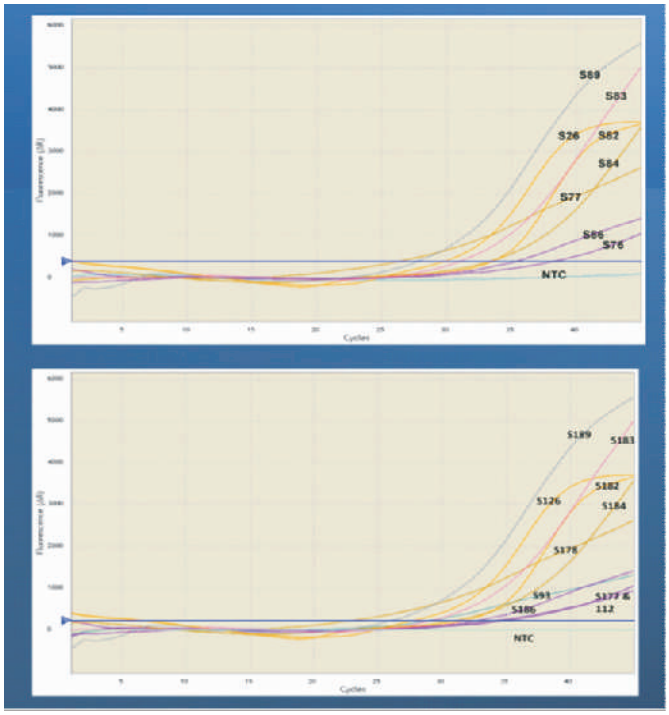

Figure 1: Optics graph shows RT-PCR of human Rhinovirus A results using FAM fluorescent dye. According to (threshold=417.525):- positive samples no. are 15, 26, 76, 77, 82, 83, 84, 86,89, 93, 111, 126, 177, 178, 182, 183, 184, 186, and 189 with CT values of 27.68-33.66. No amplification in no template control (NTC).

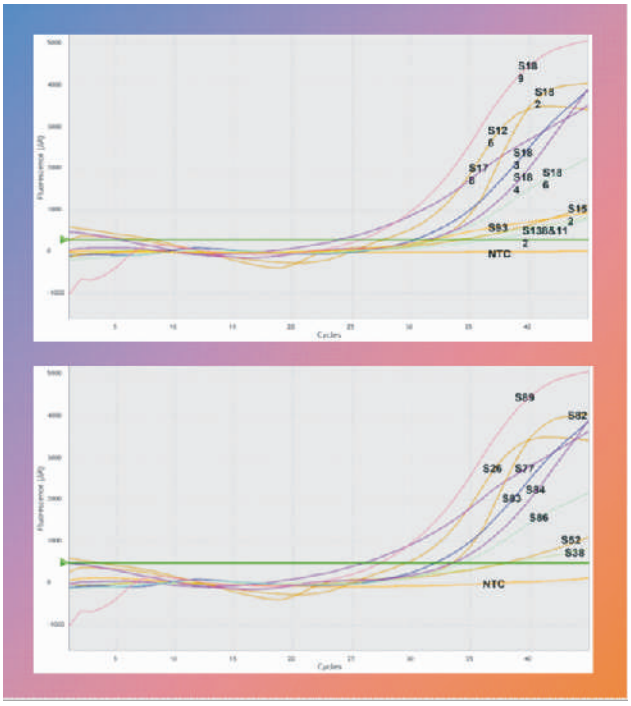

Figure 2: Optics graph shows RT-PCR of human Rhinovirus B results using FAM fluorescent dye. according to (threshold=417.525):- positive samples no. are 15, 26, 38, 52, 77, 82, 83, 84, 86,89, 93, 111, 126, 138, 152, 178, 182, 183, 184, 186, and 189 with CT values of 24.17-33.66. No amplification in no template control (NTC).

Table 3: Prevalence of Human Rhinovirus A and B in association with age, gender, ethnicity, and residential area.

Risk factors		Rhinovirus A		Rhinovirus B		
		Positive (%)	x^2(p-value)	Positive (%)	x^2(p value)	Total
Gender	male	9 (9.9%)	0.092 (0.761)	11 (8.1%)	0.562 (0.453)	20
	female	10 (11.2%)		10 (11.2%)		20
Age	0-1 years	9 (8.0%)	1.013 (0.152)	8 (7.1%)	6.875 (0.143)	17
	1-2 years	4 (10.3%)		4 (10.3%)		8
	2-3 years	2 (10.0%)		4 (20%)		6
	3-4 years	3 (18.8%)		4 (25%)		7
	4-5 years	1 (8.3%)		1 (8.3%)		2
Ethnicity	Yoruba	15 (9.1%)	7.794 (0.050)	16 (9.1%)	8.079 (*0.044)	31
	Hausa	1 (5.3%)		1 (5.3%)		2
	Igbo	2 (50.0%)		2 (50.0%)		4
	others	1 (16.7%)		2 (8.3%)		3
Residential area	urban	19 (10.3%)	0.234 (0.615)	20 (10.8%)	1.702 (0.192)	39
	rural	0		1 (6.7%)		1

X^2= wald Chi-square
P value ≤0.05= significance

rural areas tested positive for rhinovirus A and there was no positive case of rhinovirus B recorded for children from rural areas (Table 3). p-values of 0.615 and 0.192 showed that there was no significant difference between area of residence and rhinovirus A and B respectively.

In Table 3, p-value of 0.050 showed that there was no significant difference between the ethnicity of children and rhinovirus A while p-value of 0.044 showed that there was a significant difference between the ethnicity of children and rhinovirus B.

Discussion

In this study, we showed that RV infections were highly prevalent in group 0-1 year children from the study population. This report is in agreement with

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*., 2019which 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 Jiang*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 byRoyston 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 ina 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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