

# Molecular and Conventional Methods of Detecting Respiratory Viruses among Children with Lower Respiratory Tract Infection in Sokoto, Nigeria

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## ABSTRACT

**Background:** Most infections of respiratory tract are caused by viruses but bacteria with viruses contributing to a higher proportion of infection.

**Objectives:** The aim of the study was to assess the epidemiology of respiratory viral infections among children less than five years of age hospitalized with acute lower respiratory tract infections (ALRTIs) at Specialist Hospital, Sokoto using conventional and molecular detection methods.

**Methods:** The cross-sectional study was designed to investigate the occurrence of respiratory viruses including (RSV), human Metapneumovirus (HMPV), influenza virus A and B (IFV-A and B), parainfluenza virus 1, 2, 3 and 4 (PIV 1, 2, 3 and 4), human rhinoviruses (HRV), human enterovirus (EV), human coronaviruses (HCoV) 229E and OC43, human Boca virus (HBoV) and human adenovirus (HAdV) among hospitalized children with acute lower respiratory tract infections (ALRTIs), at Specialist Hospital, Sokoto, from June 16 to December 21, 2010. The present study was also designed in part to assess the performance of the conventional methods against molecular methods.

**Results:** Etiologic agents were detected in 158 (95.8%) of the patients. Single virus was detected in 114 (67.9%) patients; 46 (27.9%) were co-infected with different viruses including double-virus infections in 37 (22.4%) and triple-virus infections in 9 (5.5%) cases. RSV (50.3%), with predominance of group B, played a major role. Other etiological agents including HAdV, HMPV, IFV-A, PIV 1-3, HBoV, HCoV-OC43 and EV were detected in 14.5, 9.6, 9.1, 4.8, 3.6, 2.4 and 1.8 percent of the samples, respectively.

**Conclusion:** Our results demonstrated the potential usefulness of molecular detection methods compared with conventional methods for the diagnosis of ARTIs among hospitalized children. This is the first report of HMPV, HBoV and HRV infections among hospitalized children in Sokoto.

**Keywords:** Conventional Method; Molecular Detection; Respiratory Viruses;

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## Introduction

Most infections of respiratory tract are caused by viruses but bacteria with viruses contributing to a higher proportion of infection. Among the viruses, respiratory syncytial virus (RSV), parainfluenza virus (PIV), influenza virus (IFV), adenovirus (AdV), and human rhinoviruses (HRV) are the major causes of acute respiratory infection (ARI)



in children whereas human Metapneumovirus (HMPV), and human coronaviruses (HCoV),<sup>1</sup> however Boca virus (HBoV), parvovirus type 4 and 5, and Mimi virus have been implicated as etiological agents of acute lower respiratory tract infections (ALRTIs) even though at a lower frequency.<sup>2-4</sup> Laboratory confirmation is required for aetiological diagnosis of respiratory virus infections since clinical features alone are not specific enough to differentiate the viral agents on clinical background.<sup>3</sup> Sensitive and rapid diagnosis of respiratory infections among hospitalized children is a cost-effective procedure and it is pivotal to direct active treatment early in the course of the illness following detection, to reduce unnecessary antibiotic prescription, and to limit nosocomial transmission to high-risk patients.<sup>5-8</sup> On the other hand, assessment of ALRTIs morbidity by specific etiological agents using sensitive detection methods in hospitalized patients is important for the evaluation of agent-specific interventions such as vaccination against RSV.<sup>6</sup> Extensive detection of infectious agents will also expand our knowledge about aetiology of pneumonia and emphasize which etiological agents should be considered for vaccine.<sup>9</sup> Recent developments in molecular diagnosis of respiratory viruses and discovery of new viruses have renewed the interest in epidemiology of respiratory viruses. However, there is still a considerable deficiency in the etiologic diagnosis of ALRTI.<sup>10</sup> Looking into the Sokoto data, far too little attention has been paid on the epidemiology of respiratory viral infections.

Therefore, in this study, the goal is to develop a panel of tests to detect classical and newly discovered respiratory viruses including IFV, RSV, PIV, AdV, HMPV, HRV, EV, HCoV, and HBoV among children below five years old, for the diagnosis of ALRTI admitted to Specialist Hospital, Sokoto using both conventional methods including direct immunofluorescence assay, cell culture and shell vial culture and molecular diagnostic techniques including multiplex PCR and sequencing.

## Materials and Methods

### Study Design

The survey was conducted at paediatric wards, in Specialist Hospital, a government-funded multi-specialty hospital located in Sokoto South local government of Sokoto state, North western Nigeria. The participants were children more than one-month-old and less than 5 years of age who were admitted to the hospital from June 16, to December 21, 2010 with the diagnosis of ALRTI. Patients with congenital or acquired immunosuppressive conditions, with conditions that posed a potential hazard in obtaining the nasopharyngeal samples (e.g. bleeding diathesis, severe respiratory compromise) as determined by the clinicians and children with incomplete data or inadequate samples were excluded from the study. Ethical approval was obtained from Specialist Hospital Sokoto. Informed consent was also obtained from parents of the patients involved.

Nasopharyngeal aspirate was collected by the doctors involved in the study and sent to the Medical Microbiology laboratory of the Hospital where it was initially processed and the cell pellets were transported to University Putra Malaysia for further analyses.

### Specimen Processing

Nasopharyngeal aspirate (NPA) was taken through both nostrils by inserting a disposable catheter (no. 6 or no. 8) connected to a mucus extractor.

NPAs were transported by viral transport medium (VTM) to the laboratory and refrigerated at 4°C to 8°C until proceed.

In order to avoid repeated freezing and thawing, all NPAs were processed upon receipt.

The samples were vortexed vigorously for 15 seconds and centrifuged with free swinging bucket rotor at 600xg for 7 minutes.

The supernatant was collected and set aside for virus isolation and genome extraction.

The cell pellet was used for Direct Immunofluorescence Assay (DFA).

They were washed several times to remove mucus layer in order to avoid nonspecific fluorescence.



### Immunological Assays

The D3 Ultra 8 Direct Immunofluorescence Assay (DFA) Respiratory Virus Screening & Identification kit (Diagnostic Hybrids Inc. (DHI), USA) that contains a blend of murine fluorescein isothiocyanate (FITC)-conjugate monoclonal antibodies (MAbs) was used as a first step of detection which made it possible to detect eight common viruses including RSV, human Metapneumovirus (HMPV), influenza virus (IFV) type A and B, parainfluenza virus 1-3 (PIV1-3), human adenovirus (HAdV) using DFA.

When the DFA was negative, the samples were inoculated onto shell vial culture (SVC). R-Mix™ Ready cells (DHI, USA), ready-to-use mixed cell monolayers comprising mink lung cells (Mv1Lu) and human Adenocarcinoma cells (A549), were used as manufacturer's recommendations.

### Genome Extraction and Reverse Transcription

Viral RNA/DNA was extracted from filtered supernatant of NPA using MagMAX Viral RNA Isolation Kit (Applied Biosystems, Ambion, USA) according to the manufacturer's instructions. The concentration and the purity of the extracts (A260/A280 nm and A260/A230 nm) were measured by using NonoDrop (ThermoFisher scientific, USA). The first strand cDNA synthesis was carried out on RNA extracts in a final volume of 20 µl by random hexamer primer using RvertAid™ H Minus First Strand cDNA Synthesis kit (Fermentas, USA) following the manufacturer's instructions. The samples were incubated first for 5 min at 25°C and followed by 60 min at 42°C. The reaction was terminated by heating at 70°C for 5 min.

### Polymerase Chain Reaction

Three multiplex RT-PCR (MP/RT-PCR1-3) and subsequently two hemi-nested multiplex PCR assays (HNMP/PCR 1-2) were carried out for the molecular detection of RNA viruses. MP/RT-PCR1 targeted influenza viruses A and B, RSV (types A and B), HMPV (A and B). MP/RT-PCR2 detected parainfluenza virus types 1-4 (PIV 1-4). The MP/RT-PCR 3 contained primers for the detection of HRV, EV, HCoV OC43 and 229E. An

internal control consisting of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was included in MP/RT-PCR2. The presence of Human Boca virus (HBoV) and HAdV were individually investigated in the samples by singleplex PCR and nested PCR respectively.<sup>11, 12</sup> Primer set of HAdVhexF1/AdhexR1 and nested primer set of AdhexF2/AdhexR2 targeted HAdV hexon gene hyper-variable regions 1-6 (HVR1-6) were used for detection of HAdVs. The PCR products were fractionated by electrophoresis of 2.5% agarose gel and visualized using ethidium bromide under UV light. pCR®2.1-TOPO® plasmid vector [TOPO TA cloning® kit (Invitrogen, USA)] was used for the validation of the multiplex PCR.

### Virus Culture

HEp-2 (ATCC CCL-23, USA), MRC-5 (ATCC CCL-171, USA), Vero (ATCC (CCL-81, USA) and HeLa (ATCC, USA) were purchased from American Type Culture Collection (ATCC). The cell culture procedures were based on ATCC guidelines. The entire samples positive for RSV were cultured on Vero and HEp-2 cell lines. HAdV positive samples inoculated onto HeLa and HEp-2 cells. MRC-5 and HeLa cell line were used to inoculate samples positive for HRV. The cell cultures with characteristic CPE for RSV and HAdV were harvested and confirmatory testing was performed with DFA. The tube cultures showed CPE for HRV were harvested and confirmed by PCR. The second blind passage was performed after a week in a case with no characteristic CPE.

### Statistical Analysis

Data were analysed using the SPSS version 16.0. All *P*-values were two-tailed and *P*-values of < 0.05 were considered statistically significant. Comparisons between the results obtained by molecular methods and conventional methods were evaluated by McNemar's test and Paired-samples *t*-test.

### Results

**Conventional Methods:** A total of one hundred and sixty-five children less than five years of age



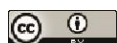
who fulfilled the inclusion criteria as outlined above and hospitalized with ALRTIs during a 20-week period between June 16, and December 21, 2010 were enrolled in the study. DFA was the initial step of conventional detection of common respiratory viruses which made it possible to diagnose eight viruses including IFV A & B, PIVs 1-3, RSV, HMPV and HAdV. Due to the unavailability of specific monoclonal antibodies for HRV, EV, HCoV, PIV4 and Hob, they were excluded from immunological detection. A positive reaction was one in which bright apple-green fluorescence was observed in the infected cells. For each virus the pattern of fluorescence staining was specific and used as confirmatory purpose. Eighty-eight (53.9%) of the 165 NPA samples were found DFA positive and distributed as follows: 67 (40.6 %) for RSV, 9 (5.5 %) for HMPV, 4 (2.4 %) for HAdVs, 3 (1.8 %) for IFV A and 6 (3.61%) for PIVs 1-3. Detection of RSV, HMPV, PIV1-3, IFV-A & B, HAdV in shell vial culture were attempted as the second stage of the conventional method on the remaining 76 NPA samples found DFA negative. Seven additional viruses were detected and a total of 95 (57.6%) samples were found to contain a virus by DFA followed by shell-vial culture. Therefore, this method improved the detection of some viruses which were missed by the DFA: 4 RSV and one case each for HAdV, HMPV and PIV2. For RSV, CPE was detected in 31 of the 83 specimens representing a 37% recovery. Twenty-four samples positive for HAdVs were inoculated into HeLa and HEp-2 cell lines. Five of twenty-four samples (21%) showed characteristic CPE. The HRV positive samples detected using RT-PCR were isolated by inoculation in semi-confluent monolayers of HeLa and MRC-5 cell lines. Of the 54 samples, 26 were recovered in cell culture.

### Molecular methods

The ability of the multiplex method to detect multiple viruses distinctively in the same reaction tube was evaluated by testing a mixture of cloned plasmids of targeted viruses. Analysis of the PCR products showed that each multiplex method simultaneously detected all three control viruses

included in each reaction tubes as well as the internal control with the expected band sizes. In the presence of all primer sets in the multiplex reaction, no mispriming was observed in the positive and negative control tubes. The specificities of PCR products of MP/RT-PCR were confirmed further by nucleotide sequence analysis. Multiplex RT-PCR (MP/RT-PCR 1-3) and subsequently two hemi-nested multiplex PCR (HNMP/PCR 1-2) were carried out on nucleic acids extracted from 165 clinical specimens. The specific products were clearly separated and identified on a 2.5% Seakom agarose gel, both for virus control and for clinical specimens. In total, 154 samples (93.3 %) from the panel of 165 were positive for the viruses and 11 (6.7%) of specimens were negative using this method. In the first stage of the assay using MP/RT-PCR1-3, almost all the viruses were detected 177/183 (97%). In the second stage six (6/183, 3%) extra viruses were detected by HNMP/PCR1-2. Using common and nested PCR, of the 165 samples tested, 6 were found to be positive for HBoV and 24 (14.5%) for adenovirus. Of these positive samples, single infection was documented in one HBoV and 3 cases of HAdV infection. In total, 158 samples (95.8%) were positive for respiratory viruses using the molecular method while 7 (4.2%) were negative. All the NPA samples tested by PCR, presented a GAPDH amplification band, indicating that there was no PCR inhibitors in the reactions. Therefore, false negative results were excluded using an internal control.

Further classification of 67 RSV strains showed that subgroups A and B comprised 11/67 (16.4%) and 56/67 (83.6%), respectively. The second hypervariable region at the carboxyl-terminal of the G gene was amplified and sequenced in order to do phylogenetic study. Phylogenetic analysis of the 32 sequenced samples showed that all nine RSV-A strains were clustered within NA1 genotype while the remaining twenty-three strains of the RSV-B subgroup could be grouped into newly discovered BA10 and BA9 genotypes. Phylogenetic study of the thirty-six HRV strains at VP4/VP2 region confirmed the broad genetic diversity of circulating HRV. HRV-A strains repr-



-esented the majority of the detections, 22/36 (61%). Recently discovered HRV-C group was substantially implicated as etiological agent among studied patients, 14/36 (39%).

### Multiple Viral Infections

One hundred and twelve patients (67.9%) were identified with a single virus; RSV (30%), HRV (22%), IFV-A (6.1%) and HMPV (4.8%) were the most frequently detected sole viruses in the samples respectively. No single infection due to HCoV was observed. Multiple respiratory viral infections were documented in 46 (28%) of samples; 37 (22.4%) were double infections and 9 (5.5%) as triple infections. Of the 46 patients with multiple viral infections, the most frequent was RSV (34, 73.9%), followed by HAdV (21, 45.6%) and HRV (18, 39.1%). Dual infections of RSV with HAdV and HRV were the most prevalent multiple viral infections found in the study (13/46, 28% and 11/46, 24%, respectively). Out of 165 patients

whose blood culture were taken, bacterial infections were documented in only 5 (3%) cases including one patient with single infection (0.6%) with  $\alpha$ -hemolytic *Streptococcus viridans*. Four other samples were co-infected with viruses and considered as nosocomial infections (blood cultures results were positive after 48 hours) were as follows: two RSV/*Burkholderia cepacia*, one HRV/*B. cepacia* and one HRV/ coagulase-negative staphylococcus. *M. pneumonia* infections were not identified in any of the total 165 patients.

### Conventional Methods versus Molecular Methods

In total, 158 specimens (95.8%) from the panel of 165 were positive for some viruses by combination of conventional and molecular methods, and 7 (4.2%) specimens were negative for any viruses. The comparison between conventional and molecular methods was depicted in Table 1.

**Table 1: Comparison of Conventional and Molecular Methods**

Variables	Conventional Assays <sup>a</sup>	Molecular Assay	<i>p</i> value
No. of detectable viruses	9	14	
No. of NPA tested	165	165	
No. (%) of positive NPA	114 (69.1)	158 (95.8)	<0.001 <sup>d</sup>
No. of virus detected	114	213	<0.001 <sup>e</sup>
No. of extra virus detected	0	92	
No. of samples with co-infections	0	46 (27.9)	
<b>No. of positive NPA by virus types</b>			
<b>Conventional viruses</b>	<b>94 (56.9)</b>	<b>146 (88.5)</b>	
RSV	71 (43.0)	83 (50.3)	<0.001 <sup>d</sup>
HMPV	11 (6.7)	16 (9.7)	0.062 <sup>d</sup>
IFV-A	3 (1.8)	15 (9.1)	<0.001 <sup>d</sup>
IFV-B	0	0	Nil
PIV1	1 (0.6)	1 (0.6)	Nil
PIV2	2 (1.2)	3 (1.8)	Nil
PIV3	1 (0.6)	4 (2.4)	Nil
HAdV	5 (3.0)	24 (14.5)	<0.001 <sup>d</sup>
<b>Unconventional viruses</b>	<b>27 (16.4)</b>	<b>67 (41.0)</b>	Nil
PIV4	NA	0	Nil



H RV	27 (16.4)	54 (32.7)	<0.001 <sup>d</sup>
EV	NA	3 (1.8)	Nil
HCOV OC43	NA	4 (2.4)	Nil
HCOV 229E	NA	0	Nil
HBoV	NA	6 (3.6)	Nil

<sup>a</sup> including DFA and SVC and conventional cell culture; NA: not available; <sup>b</sup> No of positive samples detected by conventional but not by molecular assay; <sup>c</sup>No of positive samples detected by molecular but not immunological assay; <sup>d</sup> McNemar test; <sup>e</sup> Paired-samples t-test;

More samples detected positive using molecular methods, 158 (95.8%) as compared to conventional methods (69.1%) ( $p < 0.001$ , McNemar’s test).

They resulted in 69 (41.9%), 63 (38.2%) and 36 (21.9%) more positive samples compared with DFA, DFA plus SVC and DFA plus SVC plus conventional cell culture methods, respectively. On the other hand, molecular assays were able to detect 91 more viruses ( $p < 0.001$ , Paired-samples t-test).

**Monthly Distribution of Viral Infections**

The monthly distribution of cases with respiratory tract viruses is shown in Figure 1. During the study period, a continuously persisting activity was seen for RSV, HRV, HAdV, and HMPV. Incidence of Influenza A form July to September, and peaked in August and plateaued from September onwards. HRV and RSV peaked in October, an increase of cases seen after the cease of influenza from September onwards. For the viruses with low incidence, no distinct pattern is seen.

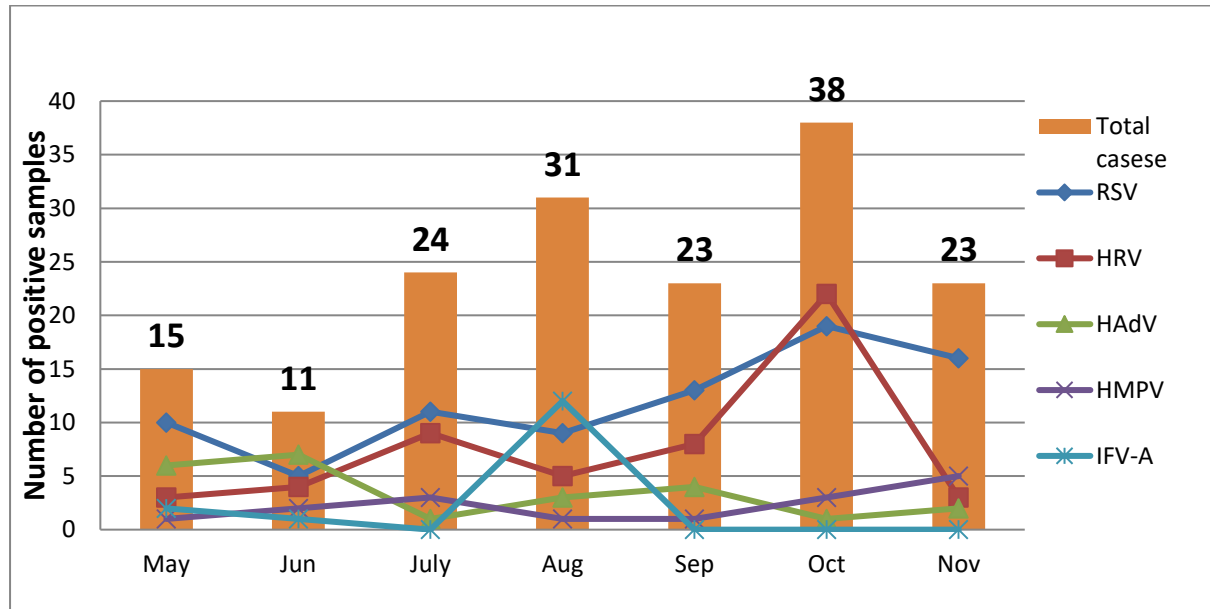


Figure 1: Monthly distribution of HRV, RSV, HMPV, IFV-A and HAdV infections

**Discussion**

Recent developments in molecular diagnosis and discovery of new viruses have renewed the interest in epidemiology of respiratory viruses. Due to the differences in the sensitivities of the

diagnostic assays, evaluation of exact contribution of each virus in epidemiology is difficult.<sup>13</sup> Regional determination of the epidemiology of specific viral infections will improve the treatment



guidelines by doctors.<sup>14</sup> The main goal of the present study was to determine the epidemiology of approximately complete panel of respiratory viruses among hospitalized children with ALRTIs in Sokoto. Our study confirmed the high prevalence of respiratory viruses among hospitalized children.<sup>15-18</sup> A high positivity rate was achieved since highly sensitive nested PCR was applied for a broad spectrum of the viruses. NPA samples from 158 (95.8%) patients were found positive for single and/or multiple viruses. About two-thirds of the samples were infected with single virus as compared with almost one-third infected with multiple viruses. In the present study, the performance of conventional diagnostic methods and molecular methods was also evaluated among samples. The results are similar with other studies<sup>19-23</sup> and it established the superiority of molecular methods over conventional methods for the detection of respiratory viruses among children hospitalized with ALRTIs.

In this study, a wide range of respiratory viruses including RSV-A, and B, IFV-A, PIV1, PIV2, PIV3, HMPV, and HRV-A and C, EV, HAdV, HBoV, and HCoV-OC43 were detected among patients. RSV was the main virus detected in fifty percent of samples. HRV was the second most prevalent virus and infected one-third of patients followed by HAdV (14.5%), HMPV (9.6%) and IFVA (9.1%). PIVs and HBoV were detected in 4.8 % and 3.6 % of the cases, respectively. Previously published findings have frequently confirmed RSV to be the major viral pathogen associated with LRTI in children.<sup>24-27</sup> The results further support that RSV infection is a frequent cause of hospitalization among children in tropical and developing countries.<sup>28, 29</sup>

Significant burden of HRV infection (the second most) was found in this study. One-third of patients were infected with HRV; similar infection rate was found in other studies<sup>30-37</sup> HRV was the second virus only to RSV as a cause of LRTIs; the result is consistent with other studies.<sup>38-40</sup> Approximately one-thirds of HRV infections were connected with other viruses (18/54; 33%). The high prevalence of HRV among hospitalized

children in this study suggests that diagnosis of the virus should be routinely included among hospitalized children with ALRTIs.

HAdV was the third (14.5%) most common detected virus in this study. Adenoviruses are responsible for 3.6- 13% of all LRTIs occurring in infants and children.<sup>41-44</sup> The present findings seem to be consistent with the detection rate of 14% among hospitalized children with ALRTIs in Argentina.<sup>45</sup> This high detection rate is especially important in developing countries with high prevalence of measles and malnutrition.

HMPV was detected as the fourth most prevalent virus with an infection rate of 10%; which is consistent with 5.3-13% detection rate among otherwise healthy children hospitalized with LRTIs in several other studies.<sup>46-49</sup> It demonstrated for the first time that HMPV could be an important cause of LRTI among children in Sokoto. The positivity rate is also comparable with the recent study among children hospitalized with ALRTI in subtropical Brazil (11.4%).<sup>50</sup> The prevalence of HBoV was between 1.5%<sup>51</sup> to 19%<sup>52</sup> among children with respiratory infections. In the current study, HBoV was detected in 6 of 165 nasopharyngeal aspirates giving a prevalence of 3.6%, which is the first report in the Sokoto. The detection rate was comparable to that (3.4%) reported in France<sup>53,54</sup> and Sweden (3.1%).<sup>52</sup> However our result was less than that reported (8.0%) in Singapore.<sup>55</sup>

Sensitive multiplex PCR assay will give interesting information about the presence of multiple infections with their epidemiological and clinical effects.<sup>56</sup> In the current study, all the multiple infections were diagnosed exclusively using PCR, which further support the superiority of molecular methods over conventional methods for the detection of multiple infections.<sup>57, 58</sup> Evaluation of the relative importance of each coexisting agent may play an important role in understanding of etiopathogenesis of these viruses.<sup>59</sup> Identification of all infectious agents is especially important among at-risk patients such as those with immunocompromised disorders for appropriate antiviral therapy.<sup>60</sup> In this study, multiple viral infections were found in 28% of



patients and usually were combination of RSV with HAdV and/or HRV. The co-detection rate is similar to the one found by Calvo (2010)<sup>39</sup> (23%), Belau-Pujol (2005)<sup>56</sup> (23%), Richard (2008)<sup>18</sup> (24.4%), van de Pol (2006)<sup>58</sup> (35%). A relatively high incidence of coinfections found in this study may be explained by broad panel of viruses investigated. This supports the notion that a high prevalence of multiple infections is common involving HRV, HAdV, HCoV and HMPV.<sup>58</sup> It is surprising that a high diversity of virus combinations was found in the samples. This occurrence may be due in part to the continuous activity of all the surveyed viruses during the study period with no distinct seasonality. Coinfection with RSV and HAdV was the most common infection. The high proportion of HAdV infections (87.5%) was co-infected with other viruses in this study which is comparable with (89%) HAdV coinfection in the study by Calvo (2010).<sup>39</sup> The second most prevalent combination was related to RSV and HRV (24%). The combination of RSV and HRV as a main double infection has been reported by the other studies.<sup>34, 61</sup> High incidence of RSV coupled with HRV infection could be explained by the substantial overlapping of monthly distribution observed for these two viruses during the study period. Quantification of the viruses in the samples may better help to understand the etiological role of each detected virus.<sup>57</sup> Study of the clinical features is required to further clarify the disease severity of the mixed infections.<sup>61</sup>

### Conclusion

A wide range of respiratory viruses was confirmed in this study and molecular diagnostic methods facilitate the detection of all the viruses. Molecular methods increased the detection rate by up to 27% as compared with conventional methods. High detection rate of HRV showed its association with severe LRTI and hospitalization. Our study also demonstrated that HMPV and HBoV can be important causes of hospitalization among Sokoto children. Yearly variation in the incidence of respiratory viruses may influence their association with ALRTIs, therefore our six

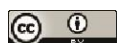
months' study should be considered as an instant picture of viral ALRTIs among paediatric inpatients.

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