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Respiratory viruses in children hospitalized for acute lower respiratory tract infection in Ghana

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Abstract

Background: Acute respiratory tract infections are one of the major causes of morbidity and mortality among young children in developing countries. Information on the viral aetiology of acute respiratory infections in developing countries is very limited. The study was done to identify viruses associated with acute lower respiratory tract infection among children less than 5 years.

Method: Nasopharyngeal samples and blood cultures were collected from children less than 5 years who have been hospitalized for acute lower respiratory tract infection. Viruses and bacteria were identified using Reverse Transcriptase Real-Time Polymerase Chain Reaction and conventional biochemical techniques.

Results: Out of 128 patients recruited, 33(25.88%, 95%CI: 18.5% to 34.2%) were positive for one or more viruses. Respiratory Syncytial Virus (RSV) was detected in 18(14.1%, 95%CI: 8.5% to 21.3%) patients followed by Adenoviruses (AdV) in 13(10.2%, 95%CI: 5.5% to 16.7%), Parainfluenza (PIV type: 1, 2, 3) in 4(3.1%, 95%CI: 0.9% to 7.8%) and influenza B viruses in 1(0.8%, 95%CI: 0.0 to 4.3). Concomitant viral and bacterial co-infection occurred in two patients. There were no detectable significant differences in the clinical signs, symptoms and severity for the various pathogens isolated. A total of 61.1% (22/36) of positive viruses were detected during the rainy season and Respiratory Syncytial Virus was the most predominant.

Conclusion: The study has demonstrated an important burden of respiratory viruses as major causes of childhood acute respiratory infection in a tertiary health institution in Ghana. The data addresses a need for more studies on viral associated respiratory tract infection.

Keywords: Respiratory Viruses, Hospitalized children, Real-Time PCR

Background

Acute respiratory infections (ARI) are one of the major causes of morbidity and mortality in young children throughout the world especially in developing countries [1,2]. Data from WHO estimated the burden of ARI at 94,037,000 disability-adjusted life years (DALYs) and 3.9 million deaths in 2001 [3]. Similar report from a meta-analysis study demonstrates that throughout the world 1.9 million (95% CI 1.6-2.2 million) children died from ARI in 2000, 70% of them in Africa and Southeast Asia [2]. A further systematic analysis also estimated 1.575

million (uncertainty range: 1.046 million - 1.874 million) deaths of children worldwide in 2008 as due to ARI [4].

Majority of acute lower respiratory tract infections (ALRTI) in developed countries have been reported to be often due to viral pathogens of which most common are RSV, PIV, influenza viruses, Adv, human Coronaviruses and Bocaviruses [5-7]. On the contrary, information on these viruses in developing countries is limited probably due to paucity of modern diagnostic molecular techniques. These infections are therefore treated unsuccessfully with antibiotics based on suspicion of bacterial causes [8].

Apart from the public health concern of nosocomial infections that are associated with viral respiratory infections [9,10], significant costs derived from long duration of hospitalization and several healthcare visits could also

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aggravate the poor socio-economic status and increased child mortality in developing countries including Ghana.

Lessons from the outbreak of the Severe Acute Respiratory Syndrome (SARS) epidemics which resulted in the death of 776 individuals [11] and the recent emergence of a novel swine flu pandemic emphasize the risk posed by respiratory viral infections in humans [12].

This study was done to determine the burden of respiratory viruses among children hospitalized at the Komfo Anokye Teaching Hospital for acute lower respiratory illness using the Real Time Polymerase Chain Reaction (RT-PCR).

Methodology

Study design

This was a hospital based cross-sectional study of children less or equal to five years and hospitalized for acute lower respiratory tract illness.

Study site

The study was performed at the children's ward of the Komfo Anokye Teaching Hospital (KATH), Ghana from January to December 2008. KATH is approximately a thousand bed tertiary medical facility located in Ashanti Region, Kumasi, the confluence of the transportation network in the central part of Ghana. Its position makes it the most accessible tertiary and referral medical facility in Ghana attending to a population of over 4.4 million in the Ashanti region and beyond [13].

Sampling

To have a year round incidence of the various viral agents, recruitment was done throughout the year 2008. Screening and recruitment were started at the beginning of every week till two or three patients were recruited. After a maximum of three patients have been obtained it was suspended till the beginning of the next week. This cycle was repeated till the maximum of 11 patients were recruited for each month.

At the recruitment station all patients arriving at the unit were screened but only those less than five completed years were recruited. Patients recruited into the study should have features of severe pneumonia or very severe pneumonia as defined by the WHO-IMCI (World Health Organisation Integrated Management of Childhood Illness) protocol [14,15]. Severe pneumonia was said to be present if the child has a history of cough and/or difficult breathing of less than 3 weeks duration, with lower chest wall recession. Severe pneumonia in addition to cyanosis and/or inability to feed or drink was classified as very severe pneumonia. A child was recruited into the study only after the guardian or parents had consented after the objectives of the study had been explained in English or the local dialect. A standardized case record

form was then used to record the history of the illness as well as the presenting clinical features of pneumonia.

Five (5 ml) of blood sample was then taken into 25 ml of Brain Heart Infusion Broth (BHIB) and quickly sent to the bacteriological laboratory for culture. Nasopharyngeal specimens were also taken using the nasopharyngeal flocked swab (Copan, Italy). The swab was gently inserted up the nostril towards the pharynx until resistance was felt and then rotated 3 times to obtain epithelial cells. It was then withdrawn and put into 2.5 ml phosphate buffered saline (X1). The samples were transported on ice to the laboratory within few hours of collection. They were then vortexed, transferred into 1.5 ml eppendorf tubes (Eppendorf, Germany) and kept at -80°C for Polymerase Chain Reaction (PCR). Both samples were taken by experienced prior trained nurses of the children ward. Children were excluded if both samples could not be obtained for any reason.

Viral identification

Viral nucleic acids were extracted from samples using QIAamp viral mini kit (Qiagen, Germany) according to the manufacturers' instruction [16]. RT-PCR amplification was performed for the detection of Adv, RSV, Influenza A (Flu A), Influenza B (Flu B), Parainfluenza 1 (PIV 1), Parainfluenza 2 (PIV 2) and Parainfluenza 3 (PIV 3) using the light cycler version 1.5 (Roche, Germany). Primers highly specific to each of the viruses were used (Table 1). The reaction mixture for RNA viruses was made up of 5 μl of RNA extract, 1 μl deoxynucleotide (dNTP) triphosphate (10 mM) (Qiagen), 1 μl Bovine Serum Albumin (BSA)(1 mg/ml)(Qiagen), 12.5 μl Qiagen One Step RT-PCR Buffer x5 (containing 12.5 mM MgCl_2) (Qiagen), 1 μl Qiagen One Step Enzyme Mix, 1 μl each of sense and antisense primer(10 μM each), 0.5 μl Probe and 2.0 μl of RNase free water. DNA virus reaction mixture was made up of 5 μl PCR buffer (10X), 1 μl of dNTP mix (10 mM each), 1 μl of MgCl_2 (50 mM), 0.5 μl of Hot Star Taq DNA Polymerase, 0.5 μl of probe, 1 μl of BSA (1 mg/ml) and 1 μl each of sense and antisense primers.

The PCR conditions for PIV, RSV and Influenza viruses consisted of an initial reverse transcription at 20 minutes for 50°C , Taq DNA polymerase activation at 95°C followed by forty five (45) cycles of denaturation at 15 seconds for 95°C and annealing at 15 seconds for 58°C . The initial reverse transcription step was omitted in the case of Adv.

Controls and standards

For each batch of tests that were run, RNase free water (Qiagen, Germany) was used as negative control and *in vitro* transcribed gene of the various viruses was used to determine the detection limit of the assay as positive control. The *in vitro* transcripts were prepared in our

Table 1 Target genes and primer sequences for respiratory pathogens

Virus	Target gene	Function	Oligonucleotide sequence
RSV ^a (A/B)	Matrix gene [17]	Forward primer	5'-GGAAACATACGTGAACAAGCTTCA
		Reverse primer A	5'-CATCGTCTTTTTCTAAGACATTGTATTGA
		Reverse primer B	5'-TCATCATCTTTTTCTAGAACATTGTACTGA
		Probe	6FAM-TGTGTATGTGGAGCCTT- MGBNFQ
Adenovirus	Hexon gene [18]	Forward primer	5'-GCCACGGTGGGGTTTCTAAACTT
		Reverse primer	5'-GCCCCAGTGGTCTTACATGCACAT
		Probe	6FAM-TGCCACCAGACCCGGGCTCAGTACTCCGA-TAMRA
PIV I ^b	Polymerase gene [19]	Forward primer	5'-ACAGATGAAATTTTCAAGTGCTACTTTAGT
		Reverse primer	5'-GCCTCTTTTAATGCCATATTATCATTAGA
		Probe	6FAM-ATGTAATAAATCGACTCGCT- MGBNFQ
PIV II ^c	Polymerase gene [19]	Forward primer	5'-TGCATGTTTTATAACTACTGATCTTGCTAA
		Reverse primer	5'-GTTTCGAGCAAAATGGATTATGGT
		Probe	6FAM-ACTGTCTTCAATGGAGATAT- MGBNFQ
PIV III ^d	Matrix gene [19]	Forward primer	5'-TGCTGTTCGATGCCAACAA
		Reverse primer	5'-ATTTTATGCTCCTATCTAGTGAAGACA
		Probe	6FAM-TTGCTCTTGCTCCTCA- MGBNFQ
Influenza A	Matrix gene [20]	Forward primer A1	5'-GGACTGCAGCGTAGACGCTT
		Forward primer A2	5'-CATCCTGTTGTATATGAGGCCCAT
		Reverse primer	5'-CATTCTGTTGTATATGAGGCCCAT
		Probe	6FAM-CTCAGTATTCTGCTGGTGCCTTGCCA- MGBNFQ
Influenza B	Hemagglutinin (HA)gene [20]	Forward primer	5'-AAATACGGTGGATTAATAAAGCAA
		Reverse primer	5'-CCAGCAATAGCTCCGAAGAAA
		Probe	6FAM-CACCCATATTGGGCAATTCCTATGGC- MGBNFQ

RSV^a, Respiratory syncytial virus; PIV I^b, Parainfluenza virus type 1; PIV II^c, Parainfluenza virus type 2, PIV III^d, Parainfluenza virus type 3.

collaborative institution (Bonn Institute of Virology, Bonn, Germany) and transported to Ghana via cooling chain. The *in vitro* transcripts were prepared by amplifying the genes of interest of the viruses with specific set of primers. The products were ligated to the PCR2.1 vector using the TOPO TA cloning kit (Invitogen Corp, Carlsbad, CA) and transformed into competent E.coli cells. After overnight incubation, desirable colonies were selected and purified using Qiamp Spin-Miniprep Kit (Qiagen, Germany). PCR was performed on the purified product and RNA transcripts were then synthesized from the lowest dilution (with clear band) using Ambion Megascripts T3 kit (Invitogen corp, Calsbad, CA). The final products were purified using Rneasy Mini Kit (Qiagen, Germany) and the concentrations of the transcripts were determined using a spectrophotometer (Themoscientific, U.S.A). The copy numbers of the RNA transcripts were determined following the method of Fronhoffs [21]. Ten-fold dilutions of the RNA transcripts were prepared in Carrier RNA-RNase free water (310 µg of Carrier RNA in 310 ml of RNase free water) and tested. The detection limit was determined as the last dilution after which all other replicates gave negative results. The detection limits were Respiratory Syncytial

Virus (RSV): 20 copies/µl, PIV 1: 1 - 78 copies/µl, PIV 2: 400 copies/µl, PIV 3: 30 copies/µl, Influenza A: 120 copies/µl and Influenza B: 480 copies/µl.

To determine the specificities, our assays were tested on other different viruses and they turn out negative.

Bacteria identification

Bacterial isolates were identified using conventional biochemical methods including urease and indole production, citrate utilization, hydrogen sulphide, gas production and fermentation of sugars. The biochemical media used included Simon's Citrate medium, Urea and Triple Sugar Iron agar (TSI). Coagulase tests were performed for all staphylococci organisms.

Statistical analysis

Data obtained was double entered into a spreadsheet database prepared with Microsoft[®] Excel. It was then compared and cleaned for abnormal wrongful entries. Statistical analysis was done using STATA SE statistical software version 11.2(Texas, USA) after the data had been imported. Categorical variables such as age groups and their association with respiratory agents were analyzed using the Fischer's exact test. Continuous variables

were expressed as medians with their inter-quartile ranges. A non-parametric K-sample test on the equality of medians was used to evaluate the differences in the medians of the various subgroups of the continuous variables. For all analysis done, a p-value of less than 0.05 was considered statistically significant.

Ethical approval

The study protocol was approved by the Committee for Human Research, Publications and Ethics (CHRPE) of KATH and School of Medical Sciences, KNUST, Kumasi, Ashanti region, Ghana.

Results

A total of 128 patients were recruited over the period of January to December 2008. The median age of the patients was 12 months (IQR: 6-24 months) and the number of males was 81(63.3%). Sixty one (47.7%) had severe pneumonia with the rest diagnosed as very severe pneumonia. At least one respiratory virus was detected in 33 (25.7%, 95%CI: 18.5% to 34.2%) children. Multiple viral infections were detected in 2 patients. One had an RSV and PIV 1 co-infection while the other had a combination of RSV, Influenza B and Adenovirus co-infection. Bacteria was isolated from only 12 (9.4%, 95%CI: 4.9 to 15.8%) patients with 10 of these being *Staphylococcus aureus*. The other two were *Klebsiella* species and *Coliform*. RSV and *Staphylococcus aureus* coinfection was also identified in two patients.

Six (20.0%) out of 30 children less than six months of age were found to be positive for viral infection (Table 2). The highest number of children infected were between 6 months and 2 years, the proportions infected for the various age groups were however not significantly different.

Clinical presentation of patients were compared to general pathogens identified (Table 3) and the individual viral pathogens (Table 4). Generally, there were no detectable significant differences in the clinical presentation as well as the gender and duration of illness of patients for the various pathogens isolated.

The study also investigated the seasonal variation of viruses. RSV infections were detected throughout the year however the peak infection rate occurred during the minor rainy seasons (October) (Figure 1). Adenovirus infections on the other hand had two main peaks occurring in the month of April and October.

Discussion

Viral agents play an important role in acute lower respiratory infections and may herald the onset of pneumonia caused by secondary bacterial infections [22]. Although information on the causes of respiratory illness in tropical countries is very scanty, available data indicates about one-third of the cases of respiratory tract infections are due to viruses [23].

The present study identified viruses in 25.8% of patients hospitalized for ALRTI with RSV being the most predominant. The overall prevalence is comparable to previous studies done in other developing countries [24] and the predominance of RSV is in accordance with the assertion that this virus is the single most frequent lower respiratory tract pathogen in infants and young children worldwide [25-27].

Adenoviruses, the second highest viruses detected in this study have been reported to be responsible for 5-10% of lower respiratory tract infections with the highest rate occurring in younger children [28-31]. Our study similarly recorded a 10.2% detection rate of Adenoviruses however there was no statistical difference in the age specific prevalence. This could possibly be due to the few number of younger children (less than one year) enrolled in this study.

This study generally recorded higher cases of tachypnoea, chest recessions and very severe pneumonia in RSV compared to Adenovirus infected patients but the differences were not statistically significant. Although similar clinical presentations have been found to be associated with RSV [32,33], our small sample size could account for our inability to detect these differences.

Table 2 Association between age groups and respiratory virus infection

	Age (months)			p-value
	≤ 5 (n = 30)	6 to 23 (n = 59)	24 to 60 (n = 39)	
Infections				
Patients infected with at least one virus, no.(%)	6(20.0)	18(30.5)	9(23.1)	0.549
Respiratory syncytial virus, no (%)	4(13.3)	9(15.3)	5(12.8)	1.000
Adenovirus, no. (%)	2(6.7)	8(13.6)	3(7.7)	0.596
Parainfluenza 1, no. (%)	0 (0.0)	1(1.7)	1 (2.6)	1.000
Parainfluenza 3, no. (%)	0 (0.0)	2(3.4)	1(2.6)	0.798
Influenza B, no.(%)	0 (0.0)	1(1.7)	0 (0.0)	1.000

Table 3 Distribution of clinical presentation and microbial isolation

Presentation	Microbial Isolation				Total (n = 128)	*p-value
	None (n = 85)	Virus (n = 31)	Bacteria (n = 10)	Bacteria & Viruses (n = 2)		
Age(months) median(IQR)	12(5-25)	11(6-19)	17(8-24)	48(36-60)	128(100%)	0.282
Temperature median(IQR)	38(37.8-38.5)	38(37.7-38.6)	37.8(37.4-38.0)	37.8(37.4-38)	128(100%)	0.134
Duration of illness (days) median(IQR)	4 (3-6)	4(3-5)	6.5(4-14)	4.5(4-5)	4 (3-6)	0.187
Sex (male) n(%)	54(63.3)	20(64.5)	5(50.0)	2(100.0)	81(63.3)	0.668
Tachypnoea n (%)	61(71.8)	18(58.1)	6(60.0)	1(50.0)	86(67.2)	0.382
Chest recessions n (%)	80(94.1)	29(93.6)	9(90.0)	2(100.0)	120(93.4)	0.751
Wheeze n (%)	31(36.5)	14(45.2)	3(30.0)	2(100.0)	50(39.1)	0.268
Cyanosis n (%)	3(3.4)	3(5.5)	0 (0.0)	1(50.0)	7(5.3)	0.07
Poor feeding n (%)	48(56.5)	10(32.3)	3(30.0)	0	61(47.7)	0.029
Diarrhea n (%)	14(16.5)	4(12.9)	3(30.0)	0	21(16.4)	0.606
Vomiting n (%)	25(29.4)	8(25.8)	1(10.0)	1(50.0)	35(27.3)	0.461
Severe Pneumonia n (%)	35(41.2)	18(58.1)	7(70.0)	1(50.0)	61(47.7)	0.142
Very severe Pneumonia n (%)	50(58.8)	13(41.9)	3(30.0)	1(50.0)	67(52.3)	0.142

* p-values determined assuming the null hypothesis that the proportions of the clinical presentations are uniform among the various subgroups (i.e. none, viral, bacterial or bacterial and viral infections) for categorical variables or their medians for continuous clinical variables

Among patients who were poorly fed, those without viral or bacterial infections were found to be higher ($p = 0.03$). Poor feeding and malnutrition has generally been associated with less risk for respiratory viral infection in developing countries [34-36] however the possibility of increased mortality could occur especially when bacterial infections are involved. Further case controlled studies are therefore needed in developing countries to investigate this observation

There have been fewer studies of PIV infections in developing countries and most of them do not differentiate the

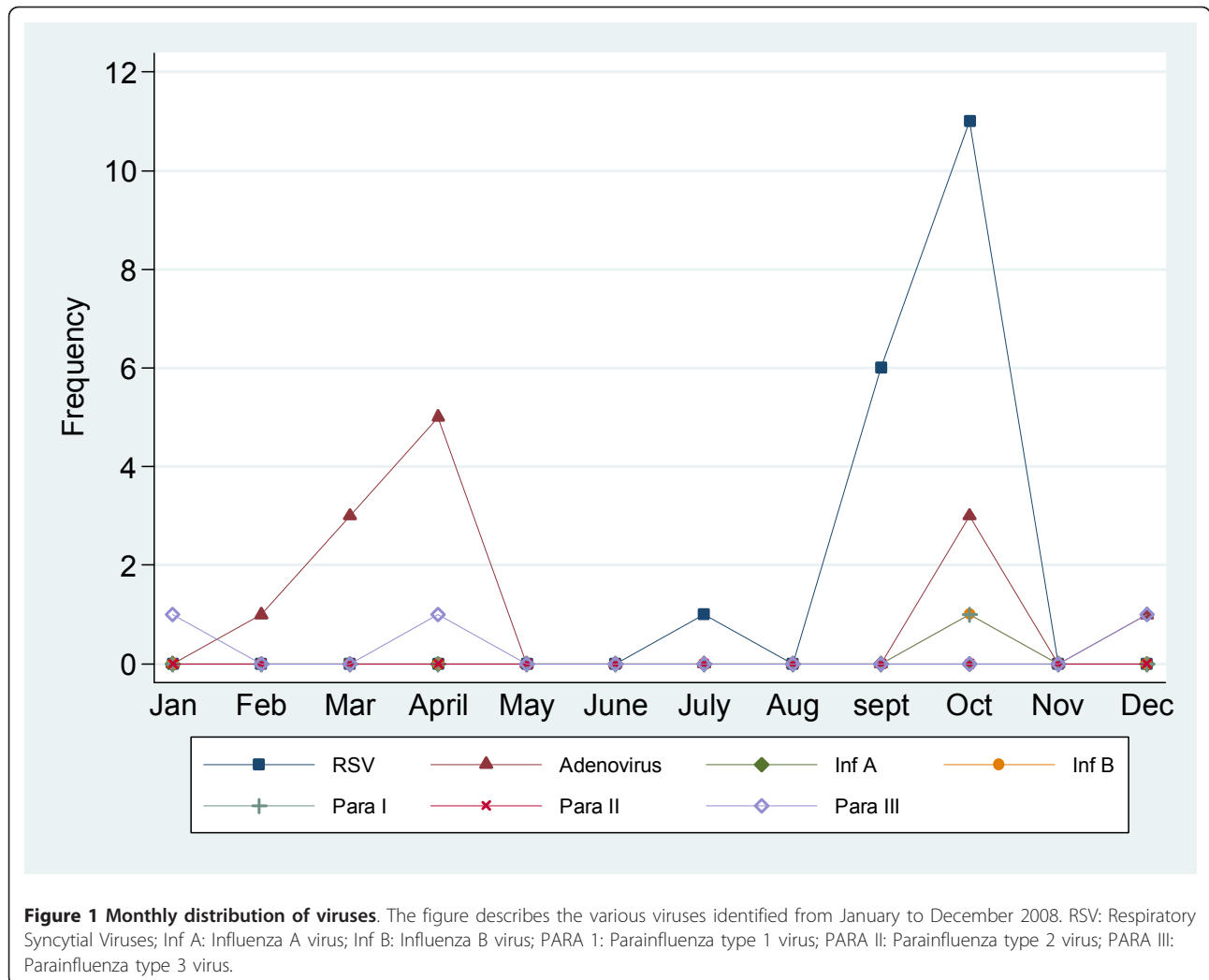
subtypes of the viruses. Our study recorded a 3.1% prevalence of PIV infections with the predominant type being PIV-3. Similar hospital based studies have been reported in other developing countries [37-40]. PIV infections have been strongly associated with croup [41,42]. The present study recorded no case of croup among PIV patients.

Viral associated bacteremia was detected in two patients (1.7%). Low rate of secondary bacterial infections has generally been reported in developing countries with the isolation rate varying between 2 and 10% [43-45]. Studies in some developed countries however

Table 4 Clinical presentations of patients infected with individual respiratory viruses

Clinical Presentation	Respiratory Viruses				
	RSV (n = 18)	Adv (n = 13)	PIV-1 (n = 1)	PIV-3 (n = 3)	Flu B (n = 1)
Temperature median(IQR)	38(37.7-38.2)	37.9(37.4-38.5)	37.5(37.5-37.5)	38.6(38.2-39)	38.8(38.8-38.8)
Duration of illness median(IQR)	4(3-5)	3 ((2-6)	4(4-4)	3((2-4)	6(6-6)
Sex (male) n(%)	13(72.2)	9(69.2)	1(100.0)	1(33.3)	1(100)
Tachypnoea n(%)	11(61.1)	7(53.9)	0	2(66.7)	1(100)
Chest recessions n(%)	17(94.4)	12(92.3)	1(100.0)	3(100)	1(100)
Wheeze n(%)	9(50.0)	8(61.5)	0	3(100)	1(100)
Cyanosis n(%)	1(5.6)	3 (23.1) ^a	0	3(100)	1(100)
Poor feeding n(%)	8(44.4)	1(7.7) ^b	0	1(33.3)	0
Diarrhea n(%)	1(5.6)	1(7.7)	0	3(100)	0
Vomiting n(%)	6 (33.3)	1(7.7)	0	3(100)	0
Severe Pneumonia n(%)	9(50.0)	9(69.2)	1(100.0)	3(100)	1(100)
Very severe Pneumonia n (%)	9(50.0)	4(30.8)	0	3(100)	0

RSV; Respiratory syncytial virus; PIV-I, Parainfluenza virus type 1, PIV-III; Parainfluenza virus type 3, Adv; Adenovirus, Flu B; Influenza B. Parainfluenza type 2 and Influenza A were not detected in clinical samples. ^aP = 0.03 vs patients having cyanosis and infected with adenovirus. ^bP = 0.02 vs patients who were poorly fed with adenovirus infection.



reported the converse [46,47] probably due to the higher sensitivity of the bacteria identification techniques used. The blood culture identification system used in this study may be limited in the detection of bacteria associated with lower respiratory tract infection. Perhaps the detection rate could have increased if the present study had identified bacterial pathogens from nasopharyngeal aspirates or washings as reported by Hishinki et al. [47].

The isolation of *Staphylococcus aureus* in an RSV positive patient in the present study was similar to studies reported by Berman et al., [48] and Cherian et al., [43]. In their case, the bacterium was associated with fatality in the children.

The present study observed that all subjects enrolled in this study were treated empirically with antibiotics in accordance with the paediatric emergency treatment protocol for managing severe and very severe pneumonia. Similar occurrences have been reported where patients were treated unsuccessfully with multiple antibiotics [8].

Policy makers may therefore consider reviewing the clinical algorithm for patient management as the economic cost both to the health service and the patient's household derived from the use of antibiotics could be enormous.

The period of this study experienced major rainfalls in the months of May to July and minor rainfalls in the month of September to October. The month of October recorded the highest detection of viral infections (57.6%; 19/33) with the commonest being RSV. This phenomenon has been reported in other developing countries [43,49]. The occurrence of RSV infections in the rainy and cold season could be due to overcrowding as a result of populations staying more to their homes. More studies are however needed to define the seasonality of respiratory viruses in tropical countries.

Our study had some limitations which include underestimation of the overall prevalence of respiratory viruses since we did not test for coronaviruses, human

metapneumovirus, bocaviruses and rhinoviruses which have also been reported in hospitalized patients. Also the negative results recorded for Influenza A and PIV 2 and the low prevalence of influenza B in our samples could be due to the low sensitivity of the assay for these viruses. The limit of detections of Influenza A, Influenza B and PIV 2 are 120 copies/μl, 480 copies/μl and 400 copies/μl respectively as such viral copies below these limits of detections could be missed by our assay.

Conclusion

This study has demonstrated that respiratory viruses are associated with a considerable number of hospital admissions in Ghana. Hospitalized children presenting with symptoms of ALRTI may not necessarily need treatment with antibiotics but rather antiviral drug options could be explored. The importance of further cross-sectional and longitudinal studies of viral-associated respiratory infections among out-patients and healthy populations cannot be over emphasized. This will contribute immensely to overcoming the paucity of data on the importance of respiratory associated viruses to the burden of disease and the morbidity and mortality of under five children.

Abbreviations

Adv: Adenoviruses; ALRTI: Acute lower Respiratory Tract Infection; ARI: Acute Respiratory Tract Infection; BHIB: Brain Heart Infusion Broth; BSA: Bovine Serum Albumin; CHRPE: Committee for Human Research Publications and Ethics; DALYs: Disability Adjusted Life Years; Flu A: Influenza A; Flu B: Influenza B; KATH: Komfo Anokye Teaching Hospital; KNUST: Kwame Nkrumah University Of Science and Technology; PCR: Polymerase Chain Reaction; PIV: Parainfluenza Virus; RSV: Respiratory Syncytial Virus; SARS: Severe Acute Respiratory Syndrome; TSI: Triple Sugar Iron agar; WHO-IMCI: (World Health Organisation Integrated Management of Childhood Illness).

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Authors' contributions

YAA, AA and BN co-worked on the data collection and contributed to laboratory analysis of the samples. SBN performed statistical analysis of the data and contributed to writing of the manuscript. TBK planned, initiated the study and contributed to writing of the manuscript. OW carried out the laboratory analysis of the samples and contributed to writing of the manuscript and interpretation of the data. All authors have read and approved the manuscript.

Competing interests

The authors declare that they have no competing interests.

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