Detection of viral acute lower respiratory tract infection in hospitalized infants using real-time PCR

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Abstract *Introduction:* Acute lower respiratory tract infection in children causes significant morbidity in the developing countries. Documentation of virus infection using PCR and clinical characteristics of patients affected with viral pneumonia are reviewed in this study.

Methods: 51 children less than three years admitted to the Pediatric Hospital, Cairo University with viral pneumonia were included. All patients had undergone nasopharyngeal aspirate for PCR viral detection.

Results: A total of 51 cases were enrolled in the study, of which 7 cases were negative while 44 children were positive for viruses. The most common respiratory virus was Rhinovirus in 32 patients (72.2%), then parainfluenza virus (PIV) in 12 (27.3%), of which subtypes PIV1 were 2 (4.5%), PIV3 were 5 (11.4%) and PIV4 were 5 (11.4%) cases. The third common viruses were respiratory syncytial virus (RSV) in 9 (20.5%) cases of which 3 (6.8%) were RSVA and 6 (13.6%) were RSVB and adenovirus in 9 cases (20.5%). Boca virus was found in 8 (18.2%) patients, corona virus 2 (4.5%) patients, H1N1 2 (4.5%) patients, enterovirus 2 patients (4.5%) and human metapneumovirus in one case (2.3%). Influenza B and PIV2 were not detected. Coinfection was found in 28 (63.7%). Mortality occurred in 12 (23.5%). There was no significant relation between virus type or coinfection with disease severity.

Conclusions: RV was the most commonly detected virus in children under 3 years admitted with acute lower respiratory tract infections. Coinfection was present in the majority of our patients; however it was not related significantly to parameters of disease severity.

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Peer review under responsibility of Egyptian Pediatric Association Gazette.

Introduction

Viral pathogens account for a large proportion of community acquired pneumonia cases.[1] It is estimated to cause 30–70% of the cases of pneumonia.[2,3]

For children with acute lower respiratory tract infection (ALRTI) treated as outpatients, a virological diagnosis is often not sought, since the diagnosis does not lead to specific therapy. Most respiratory viruses can cause ALRTI of variable severities.[4]

The conundrum of diagnosing viral pneumonia arises from the lack of understanding of the viral etiology in clinical setting. In addition, collecting the appropriate sample from infants and young children is challenging owing to the difficulty in collecting these samples in this age group. It is still difficult to differentiate between viral and bacterial pneumonia using non-specific indirect methods such as blood culture or diagnostic serology.[5] Moreover the use of conventional methods of viral detection as serology and viral culture has lead to underestimation of the role of viruses as a cause of ALRTI.[6]

The use of PCR has gained a lot of attention recently as it permits identification of more than one virus in the same respiratory specimen that has previously been difficult to culture. In the past decade, new respiratory viruses causing ALRTI had been discovered, the emergence of the severe acute respiratory syndrome (SARS) by coronavirus, and the avian influenza type A (H5N1) pandemics are recent examples.[6,7] The present study aims at investigating the epidemiology of viral infection using multiplex real time PCR, and exploring the clinical spectrum of the affected children in relation to viral type, during the winter season in hospitalized children with viral pneumonia.

Material and methods

This prospective and descriptive study was conducted at Cairo University Pediatric Hospital. 51 Children under 3 years of age from October 2013 to March 2014 were included in the study.

Children enrolled had clinical picture of viral ALRTI diagnosed as bronchiolitis or pneumonia that developed few days before the admission to the hospital. Nasopharyngeal aspirates were sent to Cairo University molecular laboratory.

Definitions

Bronchiolitis was characterized by low-grade/absent fever, runny nose, progressing to cough, tachypnea, hyperinflation, and chest indrawing, though wheezes are the hallmark of the disease.[8]

Pneumonia diagnosis was based on clinical and radiological criteria. Children with moderate to high fever, breathing difficulty, crackles, and infiltrates on lung fields on chest X-ray were diagnosed as pneumonia.[9]

Severe pneumonia according to the WHO relied on the presence of any of the following signs: working, nasal flaring, intercostal recession, grunting, difficulty during breast feeding or drinking, persistent vomiting, altered sensorium or convulsions.[10]

Subjects

All children enrolled in the study were subjected to full history taking and clinical examination regarding age, sex, residence, history of cough, sputum production, fever, wheezing, chronic disease, and history of repeated admission to hospital.

Chest X ray (CXR) was done to all cases revealing either hyperinflation in bronchiolitis or pneumonic patches in pneumonia. CRP, total leucocytic count (TLC) and nasopharyngeal aspirate were done to all patients with ALRTI. Appropriate management strategies were applied as required. All patients were followed regarding the need for intensive care admission and mechanical ventilation was used to assess the severity, while improvement or death was used to evaluate the outcome.

Exclusion criteria were patients who developed hospital associated pneumonia after 48 h of hospital admission, children with empyema or lung abscess, and children with history of previous hospital admission with pneumonia within the last 30 days.

Specimen collection for viral analysis

Nasopharyngeal swabs were simply and efficiently collected using flocked swabs (Copan Diagnostics, Italy). Samples were transported in 3 ml of viral transport media (VTM), and were stored at −80 c before testing.

Nucleic acid extraction and internal control (IC)

Nucleic acids were extracted from 500 μl of specimens using SEEPREP 12TM Viral NA Kit (Nordiag, Norway) for automated purification system, according to manufacturer's instructions. Ten microliter of the IC was added to each specimen before the nucleic acid extraction. 10 μl of proteinase K (20 mg/ml) was added to each sample tube. The final elution volume of each sample was 60 μl.

Reverse transcription

The cDNAs were synthesized from extracted RNAs using the cDNA Synthesis Premix (Seegene, Seoul, Korea) for manual set up user, according to manufacturer's instructions. Respiratory virus detection kit A and B (Anyplex TM II RV 16 Seegene, Korea) were used according to the manufacturers' instructions.

Briefly, the assay was conducted in a final volume of 20 μl containing 12 μl of PCR Mastermix and 8 μl of cDNA. PCR Mastermix consists of 5 μl of 4xRV16 A TOM or B TOM, 5 μl of 4× Anyplex PCR Mastermix (with UDG), and 2 μl of RNase free water. For negative control, use 8 μl of RNase-free water instead of sample nucleic acid. There were 2 positive control tubes: RV16 PC 1 and RV16 PC 2. For set A, one tube with RV16 PC 1 and the other with RV16 PC 2 were used. For B set, one tube with RV16 PC 1 and the other with RV16 PC 2 were used. TOCE assay was conducted using the CFX96 real-time PCR detection system (Bio-Rad, CA) under the following conditions: denaturation at 95 °C for 15 min and 50 cycles at 94 °C for 30 s, 60 °C for 1 min, and 72 °C for 30 s. The fluorophores used were FAM, HEX, Cal Red 610 and Quasar 670. After reaction, Catcher Melting Temperature Analysis (CMTA) was performed by cooling the reaction mixture to 55 °C, holding at 55 °C for 30 s, and heating from 55 °C to 85 °C. The fluorescence was measured continuously during the temperature rise, while the

Table I Melting temperature ranges of targets.

| Channel | A set | | B set | |
	Target	T_m (°C)[a]	Target	T_m (°C)
FAM	PIV4	64–68.5	hMPV	64–68.5
	ADV	77–81.5	BoV	77–81.5
HEX	PIV1	62.5–68	CoV229E	62.5–67.5
	PIV2	70–74	CoV NL63	70–74
	PIV3	77.5–81.5	CoV OC43	77.5–81.5
R610	INF A	61.5–66.5	RSVA	62.5–67.5
	INF B	69–73	RSV B	69.5–73.5
	RhV	75.5–80.5	EV	77–81
Q670	IC[b]	63.5–68.5	IC	63.5–68.5

Abbreviations: RV, rhinovirus; RSV, respiratory syncytial virus; PIV, parainfluenza virus; BoV, boca virus; CV, corona virus; AdV, adenovirus; CV, coronavirus; hMPV, human metapneumovirus; EV, enterovirus.
[a] T_m, melting temperature.
[b] IC, internal control.

melting peaks were derived from the initial fluorescence (F) versus temperature (T) curves. The melting-temperature analysis was done by Seegene viewer software.[11]

Interpretation of the results depended on: which tube A or B, which fluorophore (FAM, HEX, Cal Red 610 or Quasar 670), and the melting temperature. (Table I)

The 2 isolates that tested positive for influenza A were further subjected to influenza A characterization according to CDC protocol.[12]

Ethical considerations

The aim and nature of the study was explained for each parent before inclusion. An informed written consent was obtained from parents or caregivers before enrollment. The study design conformed to the requirements of latest revision of Helsinki Declaration of Bioethics (2008). The Scientific Research Committee of Pediatrics Department-Faculty of Medicine – Cairo University revised and approved the study design.

Statistical methods

Data were coded and entered using the statistical package SPSS version 21. Data were summarized using mean, standard deviation, median, minimum and maximum for quantitative variables and frequencies (number of cases) and relative frequencies (percentages) for categorical variables. Comparisons between quantitative variables were done using the non-parametrical Mann-Whitney test. For comparing categorical data, Chi square (χ^2) test was performed. Fishers' test was used instead when the expected frequency is less than 5. *P*-values less than 0.05 were considered as statistically significant.

Results

A total of 51 cases were enrolled in the study meeting the inclusion criteria, of which 7 cases were negative for viruses by PCR, while 44 children were positive for virus. Epidemiological data and co-morbid diseases were recorded in Table II.

Table II Demographic and clinical data of the patient.

Age (months)	Range 1.0–30
(mean + SD)	(9 + 7.29)
Sex (Patient n (%))	
(female)	20 (45.5%)
Male	24 (54.5%)
Length of hospital stay	Range 1–45 days
(mean + SD)	(11.89 + 8.04)
Residence	(Patient n (%))
Greater Cairo	34 (77.3%)
Lower Egypt	6 (13.6%)
Upper Egypt	10 (9.1%)
Diagnosis	
Bronchiolitis	11 (25%)
Bronchopneumonia	33 (75%)
Comorbid diseases	
Cardiac diseases	9 (20.5%)
GERD[a]	1 (2.3%)
Neurologic problems	1 (2.3%)
Place of admission	
PICU[b]	32 (72.7%)
Ward	12 (27.3%)
Mechanical ventilation	22 (50.0%)
Outcome	
Recovery	33 (75%)
Death	11 (25%)
CRP[c]	
Negative	30 (68.2%)
Positive	14 (31.8%)
TLC[d]	
Normal	23 (52.3%)
High	20 (45.5%)
Low	1 (2.3%)
Co-infection	
Single	16 (36.4%)
Dual	23 (52.3%)
Triple	5 (11.4%)

[a] GERD: gastroesophageal reflux disease.
[b] PICU: pediatric intensive care unit.
[c] CRP: C reactive protein.
[d] TLC: total leucocytic count.

Of the 44 cases 32 (72.7%) needed PICU admission due to severe respiratory symptoms and 12 (27.3%) were admitted in hospital ward. All cases needed supplemental oxygen, while 22 (50.0%) were severe enough requiring mechanical ventilation. Of the positive cases, mortality occurred in 12 cases (23.5%). (Table II).

The prevalence of respiratory viruses is shown in (Fig. 1) 12 respiratory viruses were isolated. The most common respiratory virus detected was Rhinovirus (RV) in 32 patients (72.2%), then parainfluenza virus (PIV) in 12 (27.3%), of which subtypes PIV1 were 2 (4.5%) PIV3 were 5 (11.4%) and PIV4 were 5 (11.4%) cases. The third common viruses were respiratory syncytial virus (RSV) in 9 (20.5%) cases of which 3 (6.8%) were RSVA and 6 (13.6%) were RSVB and Adenovirus in 9 cases (20.5%). Boca virus was found in 8 (18.2%) patients, corona virus 2 (4.5%) patients, influenza A virus 2 (4.5%), enterovirus 2 patients (4.5%) and finally human metapneumovirus 1 case (2.3%). The 2 cases that tested positive for influenza A virus were further subjected to influenza A sequencing and characterization and were
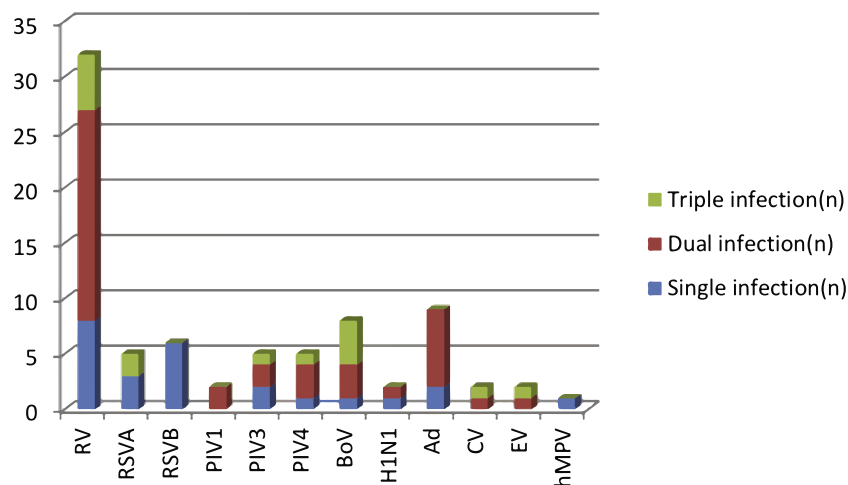
Figure 1   Prevalence of respiratory viruses.

characterized as H1N1 (2.3%). Influenza B and PIV2 were not detected in any specimens.

Among the positive cases the most common single infection was RV present in 8 (18.18%). PIV single infection was detected in 3 (6.8%) cases, adenovirus in 2 (4.5%) cases, boca virus in 1 (2.3%) case, H1N1 in 1 (2.3%) case and finally human metapneumovirus single infection in 1 (2.3%) case.

Single infection was found in 16 (36.4%) cases, while coinfection was found in 28 (63.7%) cases, of which dual infection occurred in 23 (52.3%) cases and triple infection in 5 (11.4%) cases.

There was no statistical significance between virus type, coinfection and comorbidity with length of hospital stay, PICU admission, mechanical ventilation or outcome of the patients. There was no significant association between coinfection (p value 0.501) and length of hospital stay, PICU stay (p value 0.487), MV (p value 0.531), nor outcome (p value 1.000).

Again there was no significant relation between comorbidity and length of hospital stay (p value 0.644), PICU admission (p value 0.457), MV (p value 0.728), nor outcome (p value 0.701).

There was no correlation between each virus type and parameters of disease severity (Table. III) as length of hospital stay, PICU admission, MV and death as outcome.

Discussion

The World Health Organization estimates that ≈2 million children die each year from ALRTI, and most live in developing countries.[13]

The development of sensitive molecular diagnostic assays has increased the yield of virus detection in comparison to other conventional methods. Multiplex PCR is used increasingly to diagnose respiratory infections. A study performed on children with acute respiratory illness analyzed the viral detection capacities of culture, direct immunofluorescence, and multiplex PCR found PCR to be the most sensitive method detecting a viral cause in 91.5% of children in their nasal samples.[14]

Multiplex PCR enabled laboratorians to detect a panel of viruses simultaneously while reducing hands on time and with

Table III   Relation between each virus type and diseases severity parameters.

Virus type	Length of hospital stay	p value	PICU	p value	MV	p value	Death	p value
RV[a]	12.25 ± 8.28	0.451	22 (68.8%)	0.461	15 (68.2)	0.498	7 (63.3%)	0.457
RSV[b]	15.33 ± 12.81	0.35	8 (25.0%)	0.405	6 (27.3%)	0.457	2 (18.2%)	1
RSVA	9 ± 6.56	0.559	2 (6.2%)	1	2 (9.1%)	1	1 (9.1%)	1
RSVB	18.50 ± 14.46	0.127	6 (18.8%)	0.167	4 (18.2%)	0.664	1 (9.1%)	1
PIV[c]	11.5 ± 4.21	0.653	7 (21.9%)	0.259	5 (22.7%)	0.498	2 (18.2%)	0.698
PIV1	6.5 ± 4.95	0.27	0 (0%)	0.07	0 (0%)	0.488	0 (0%)	1
PIV3	14.40 ± 3.21	0.159	5 (15.6%)	0.301	3 (13.6%)	1	0 (0%)	0.309
PIV4	10.60 ± 2.88	0.956	2 (6.2%)	0.116	2 (9.1%)	1	2 (18.2%)	0.589
Boca	14.62 ± 13.09	0.749	5 (15.6%)	0.663	3 (13.6%)	0.698	2 (18.2%)	1
Corona	10.50 ± 0.71	0.932	2 (6.2%)	1	2 (9.1%)	0.488	1 (9.1%)	0.442
H1N1	16.00 ± 19.80	1	2 (6.2%)	1	2 (9.1%)	0.488	1 (9.1)	0.442
Adenovirus	9.22 ± 6.59	0.166	6 (18.8%)	0.687	5 (22.7%)	1	3 (27.3%)	0.669
Enterovirus	13 ± 2.83	0.516	2 (6.2%)	1	1 (4.5%)	1	0 (0%)	1
Human metapneumovirus	14.00 ± 0	0.636	1 (3.1%)	1	0 (0%)	1	0 (0%)	1

[a] RV: rhinovirus.
[b] RSV: respiratory syncytial virus.
[c] PIV: para influenza virus.

greater analytical sensitivity.[15] There are several multiplex assays evaluated for detection of respiratory viruses. Several studies have demonstrated the advantages of Anyplexll RV16 detection assay.[11,15,16]

We studied children attending our hospital and emergency department during the respiratory season for the occurrence of viral ALRTI. In this study 33 (75%) cases were diagnosed with pneumonia, while 11 (25%) cases were diagnosed with bronchiolitis though differentiating between those two conditions is difficult as they exhibit less specific clinical picture.[17]

In the present study 7 cases were negative for viral infections that may be due to other viruses not used in the study or atypical pneumonia.

The causes of ALRTI in children under 5 years have been studied in dissimilar terrains and population. RSV, RV, hMP, and PIV have been shown to prevail, with co-existence of multiple infections with more than one viral pathogen occurring in 4–33% of children.[18]

RSV infections were the most frequently encountered infections responsible for hospitalization among infants and were responsible for about 3.4 million hospital admissions of children less than 5 years worldwide.[19]

Although RSV is well recognized as the main agent associated with severe ALRTIs, recent data indicate that other viruses may play a significant role in these clinical outcomes, RV seems to be of particular interest, as the most prevalent virus in respiratory illnesses even in the first years of life, being associated with severe acute bronchiolitis.[20] The high frequency of RV co-infection with RSV has been observed previously.[21–23]

RV in our study was the most commonly encountered virus accounting for 72.2% (32/44) of cases in single or mixed infection. PIV virus was the second most common responsible for 12 cases (27.3%), while the third common viruses were RSV/ Ad infecting 9 cases (20.5%). Whether RV per se or associated with other viruses showed significant correlation with case severity cannot be assessed in this study.

Another one-year-prospective study conducted at Cairo University Children's Hospital enrolled 450 children under the age of 5 years referred to outpatient and emergency department to the same hospital as ours, during a 1 year period, and viral detection was done using direct fluorescence essay, real time PCR and shell viral culture found that RSV was the most common virus responsible for hospitalization for viral ALRTI (63.3%) followed by PIV in 38 (12.2%), hMPV in 34 (10.9%) and adenovirus in 26 (8.4%) patients, but this study did not include RV detection by PCR.[24]

The results of our study were similar to a study conducted in hospitalized patients admitted with viral pneumonia in South Africa where the majority of their patients were children under 5 years of age, showing that among patients aged ⩽1 year, the most common virus was rhinovirus (985 of 3157 [31.2%]), followed by RSV (845 of 3157).[25] Another study found that rhinovirus was present in 18% of asymptomatic children and in >30% of children hospitalized with severe acute respiratory infection (SARI), and concluded that it might suggest a possible role in disease severity.[26]

The proportional contribution of influenza viruses in ALRTI in children was referred to in this work. During the study period there was a major fear of swine flu infection (H1N1) that raised public health alert for fear of fatal influenza pneumonia. Though influenza causes fever without localization with lack of respiratory symptoms, it makes these children not eligible for the study. In our study only 2 cases with H1N1 were detected out of 44 cases, those infected had an underlying congenital heart disease and required intensive care admission and mechanical ventilation but with no case fatality.

The overall burden of influenza to ALRTI in other studies was in accordance with our study. A 3-year prospective study of children younger than 24 months hospitalized with a febrile respiratory illness found only 3 patients out of 201 children with influenza infection.[27] A study from Australia found that the bulk of influenza-like illness in children less than 2 years during the 2009 H1N1 influenza pandemic was actually caused by RSV.[28]

A meta-analysis of 9 studies in which influenza virus was lab confirmed, estimated that the burden of influenza virus results in 3.0% of hospitalized ALRTI in children.[29]

The impact on severity of respiratory infections by PCR viral co-detections was the subject of many studies. Some studies contrasting to our study have shown a positive association between viral co-detection and worse clinical outcomes.[30] In a study conducted on 749 infants less than 2 years of age found that coinfection occurred in 86 children (17.4% of positive samples) and that coinfection was associated with more fever, longer hospital stays and more frequent use of antibiotics.

In this work single infection was found in 16 (36.4%) cases, while multiple infections were found in 63.7% of cases, of which dual infection occurred in 23 (52.3%) cases and triple infection in 5 (11.4%) cases, a coinfection rate in most studies is between 17% and 36% (24) (Caroline et al., 2012). Greensill et al.[31] studied 30 infants with bronchiolitis requiring mechanical ventilation and reported a 70% co-infection rate among hMPV and RSV.

Another study on hospitalized infant less than 1 year concluded that the presence of dual viral infections, (RSV and RV) increased the risk of PICU admission of infant with bronchiolitis.[32]

We need to perform a population based study in Egypt to evaluate the viral load of ARLTI in children and draw epidemiologic map of the country. Also we need to take into account the cost effectiveness of some vaccine preventable viral illnesses in our developing country, to redirect our financial resources to the most prevalent viruses in the region.

There were some limitations in our study as the small sample of our studied patients was limited by financial issues as the study was self-funded, single institution search and not a population-based study, and also it was limited to one season. Another limitation was the lack of surveillance over a prolonged period of time, which could have biased our results in case of an outbreak of a specific virus in a given year.

Conclusions

We found that RV was the most commonly detected virus in hospitalized children under 3 years of age with ALRTI. We could also detect simultaneous infections in the majority of patients which did not affect short term outcome as case fatality and length of hospital stay.

Authors' contribution

Abdel Latif (MD) and M. Erfan (MD) initiated the study idea and design and helped in the analysis and interpretation of data.

Meligy B. (MD) drafted the original manuscript and was responsible for publication.

Sayed A. (MD), and Kadry D. (MD) conducted the laboratory part of the study. Kamal (MD) collected data and performed the clinical part of the study. All authors contributed to interpretation of data and critically revised the final manuscript.

Conflict of interest

None declared.

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