

The use of multiplex PCR for the diagnosis of viral severe acute respiratory infection in children: a high rate of co-detection during the winter season

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Abstract Respiratory tract infection is a major cause of hospitalization in children. Although most such infections are viral in origin, it is difficult to differentiate bacterial and viral infections, as the clinical symptoms are similar. Multiplex polymerase chain reaction (PCR) methods allow testing for multiple pathogens simultaneously and are, therefore, gaining interest. This prospective case-control study was conducted from October 2013 to February 2014. Nasopharyngeal (NP) and oropharyngeal (throat) swabs were obtained from children admitted with severe acute respiratory infection (SARI) at a tertiary hospital. A control group of 40 asymptomatic children was included. Testing for 16 viruses was done by real-time multiplex PCR. Multiplex PCR detected a viral pathogen in 159/177 (89.9 %) patients admitted with SARI. There was a high rate of co-infection (46.9 %). Dual detections were observed in 64 (36.2 %), triple detections in 17 (9.6 %), and quadruple detections in 2 (1.1 %) of 177 samples. Seventy-eight patients required intensive care unit (ICU) admission, of whom 28 (35.8 %) had co-infection with multiple viruses. AdV, HBoV, HRV, HEV, and HCoV-OC43 were also detected among asymptomatic children. This study confirms the high rate of detection of viral nucleic acids by multiplex PCR among hospitalized children admitted with SARI, as well as the high rate of co-detection of multiple viruses. AdV, HBoV, HRV, HEV, and HCoV-OC43 were also detected in asymp-

tomatic children, resulting in challenges in clinical interpretation. Studies are required to provide quantitative conclusions that will facilitate clinical interpretation and application of the results in the clinical setting.

Introduction

Respiratory tract infection is a major cause of hospitalization in children. Most such infections are of viral origin, but viral infection is often hard to distinguish from bacterial infection [1]. Pathogen-specific clinical symptoms are often lacking [1]. Specific diagnosis, therefore, relies almost entirely on laboratory investigation [2].

Treatment with antibiotics induces the development of antibiotic resistance in bacteria [3] and has a negligible effect on most acute respiratory infections (ARIs), which are generally of viral origin [4]. Nevertheless, antibiotics are frequently prescribed due to a lack of clinically valid diagnostic tests verifying a viral etiology [5].

Sensitive methods, such as quantitative real-time polymerase chain reaction (qPCR) analyses on nasopharyngeal samples, for a number of viruses have been introduced in hospitals as a sensitive diagnostic tool among children with respiratory tract infection [6].

Investigations using specific PCR for individual viruses (“monoplex PCR”) are too time consuming and elaborate for the laboratory, and are usually used to detect influenza and respiratory syncytial virus (RSV). When the viruses targeted by monoplex PCR are negative, no specific etiology is identified to help clinical management of the patient and epidemiological monitoring of infections. Multiplex PCR methods, on the other hand, enable testing for many pathogens in parallel in a single analysis and are, therefore, increasingly gaining importance [1].

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Methodology

This prospective case-control study was conducted during the period from October 2013 to February 2014 (winter season) at Cairo University Pediatric Hospitals (800 beds) and included all children who were hospitalized in intensive care units (ICUs) and hospital wards with severe acute respiratory infections (SARI) according to the World Health Organization (WHO) case definition for SARI [7]. A standardized study protocol was developed to record the demographic characteristics and medical history of the patients, clinical features including symptoms and signs, outcome of the illness, hospital course, and laboratory and radiological findings.

Forty healthy age-matched asymptomatic children with no history of a recent respiratory tract infection during the previous 2 weeks, who were not admitted to the hospital, and who do not have any chronic underlying illness were included as a control group.

Informed verbal consent was obtained from the parents of all the patients and the controls. The study design conformed to the Revised Helsinki Declaration of Bioethics and was approved by the Scientific Ethics Committee of the Department of Pediatrics, Faculty of Medicine of Cairo University.

Sampling and sample processing: nasopharyngeal (NP) and oropharyngeal (throat) swabs were obtained, transported, and preserved on viral transport media. The swabs inside the 15-mL tube were agitated vigorously for 10 s using a vortex mixer to free cells from the swab tip.

Real-time PCR

Viral testing was done by real-time multiplex PCR using Anyplex™ II RV16 Detection (v1.1) (cat. no. RV7G01Y) supplied by Seegene, operated on a CFX96™ Real-Time PCR Detection System (Bio-Rad), which simultaneously detects 16 respiratory viruses [adenovirus (AdV), influenza A virus (Flu A), influenza B virus (Flu B), parainfluenza viruses 1–4 (PIV-1, -2, -3, -4), rhinovirus (HRV), respiratory syncytial virus (RSV), bocavirus (HBoV), metapneumovirus (MPV), coronavirus 229E (CoV 229E), coronavirus NL63 (CoV NL63), coronavirus OC43 (CoV OC43), and enterovirus (HEV)]. Nucleic acid extraction was done automatically using SeePrep12™ Viral (#SPN1004) supplied by NorDiag, using the extraction SeePrep machine (Seegene), as indicated as a nucleic acid extraction option by the manufacturer. Protocol viral NA was operated using 530 µL from the sample to result in an eluted volume of 60 µL. Reverse transcription was done using the cDNA synthesis kit for manual set up cDNA Synthesis Premix (SGRT801) from Seegene. Interpretation of the results was done according to the manufacturer's instructions, in addition to automatic analysis using the Seegene viewer software after exporting the run data to it [8].

Statistical analysis

Data were coded and entered using the statistical package SPSS version 15 (IBM Corp., Armonk, NY, USA). Data were summarized using mean [standard deviation (SD)] or median (range) for quantitative variables, and number and percentage for qualitative variables. Comparison between groups was done using the Chi-square test for qualitative variables, and the Mann–Whitney U-test was used for quantitative variables that were not normally distributed. A *p*-value < 0.05 was considered significant.

Results

The turnaround time for multiplex PCR was 6 h, but the test was done in batches three times per week. Nucleic acids of respiratory viruses causing SARI were detected in 159/177 (89.9 %) patients enrolled in the study.

The demographic and clinical characteristics of the patients are shown in Table 1, and the frequency and monthly distribution of pathogens among the patients are shown in Table 2 and Fig. 1. The median interval between the onset of symptoms and testing in our study was 3 days.

Detection of multiple viruses among the patients

The nucleic acids of more than one virus were detected in 83 patients (46.9 %). Dual detections were observed in 64 (36.2 %), triple detections in 17 (9.6 %), and quadruple detections in 2 (1.1 %) of 177 samples. The frequency of co-detection with each of the studied viruses among the patients is shown in Table 2. Important combinations included RSV with other viruses in 45 patients. Rhinovirus was mostly detected in association with other viruses (64 out of 78 patients positive for HRV). Bocavirus was detected in 16 patients. It was the only virus detected in three of them, while it was detected in association with another virus in 13 patients.

A large proportion of the patients had received antibiotic therapy prior to hospital admission (121 patients, 68.4 %), and almost all the patients received antibiotic therapy during hospital admission (169 patients, 95.5 %).

Seventy-eight patients required ICU admission, of whom 28 (35.8 %) had co-infection with multiple viruses as follows; 24 had co-infection with two viruses, three had co-infection with three viruses, and one patient had co-infection with four viruses. Twenty-one of the patients who were admitted to the ICU had RSV in association with other viruses, 14 of whom had co-infection of RSV and rhinovirus.

Table 1 The demographic and clinical characteristics of the patients

Variable	
Age, months (median, range)	4 (1–35)
Duration of hospital stay, days (median, range)	5 (1–33)
Temperature, °C (median, range)	38.0 (36.0–40.5)
Median interval between onset of symptoms and hospital admission, days (median, range)	3 (1–26)
Respiratory rate, breaths/min (median, range)	50 (30–80)
Gender, <i>n</i> (%)	
Male	94 (53.4)
Female	82 (46.6)
Clinical manifestations	
Tachypnea, <i>n</i> (%)	151 (85.3)
Chest indrawing, <i>n</i> (%)	134 (75.7)
Wheezing, <i>n</i> (%)	108 (61.0)
Grunting, <i>n</i> (%)	64 (36.2)
Refusal of feeding, <i>n</i> (%)	43 (24.3)
Lethargy, <i>n</i> (%)	36 (20.3)
Vomiting, <i>n</i> (%)	31 (17.5)
Nasal flaring, <i>n</i> (%)	23 (13)
Convulsions, <i>n</i> (%)	2 (1.1)
Stridor, <i>n</i> (%)	1 (0.6)
Underlying chronic illness, <i>n</i> (%)	58 (32.8)
Chest X-ray findings (total = 85), <i>n</i> (%)	
Consolidate	46 (54.1)
Bronchopneumonia patches	20 (23.5)
Infiltrate	15 (17.6)
Hyperinflation	13 (15.3)
Lung collapse	2 (2.4)
Effusion	1 (1.7)
Oxygen therapy, <i>n</i> (%)	107 (60.5)
Duration of oxygen therapy, days (median, range)	4 (1–15)
ICU admission, <i>n</i> (%)	78 (44.1)
Duration of ICU admission, days (median, range)	8 (2–41)
Mechanical ventilation, <i>n</i> (%)	35 (19.8)
Duration of mechanical ventilation, days (median, range)	7 (1–36)
Antibiotic therapy during hospital admission, <i>n</i> (%)	169 (95.5)
WBC count, cells/mm ³ (mean, SD)	13,495.83 (8253.15)
Outcome, <i>n</i> (%)	
Discharge	106 (59.9)
Death	16 (9.0)
Transfer	55 (31.1)

Detection of viral pathogens in asymptomatic children

Among the 40 healthy asymptomatic children who were included as a control group, no virus was detected in 12 of them, while a viral pathogen was detected in 28 controls. The only viruses that were detected among the control group were AdV, HBoV, HEV, HCoV-OC43, and HRV, either as a single detection or as a co-detection (Table 3). A dual detection of these

viruses was present in six controls, while triple detections of these viruses were present in four controls. The frequency of detection of these viruses among the patients and the controls is shown in Fig. 2.

The following viruses were not detected at all among the control group, either alone or as a co-detection with other viruses: RSV, hMPV, Flu A, Flu B, hPIV-1-2-3-4, HCoV-NL63, HCoV-229E.

Table 2 The frequency distribution of the pathogens among the patients

Pathogen	Frequency, <i>n</i> (%)	Single detection (<i>n</i>)	Co-detection (<i>n</i>)
RSV	85 (48)	40	45
HRV	78 (44.1)	14	64
AdV	31 (17.5)	3	28
HBoV	16 (9)	3	13
Flu A	11 (6.2)	5	6
hMPV	9 (5.1)	3	6
HEV	8 (4.5)	0	8
PIV-4	6 (3.4)	1	5
PIV-3	5 (2.8)	2	3
Flu B	4 (2.3)	2	2
HCoV-NL63	4 (2.3)	2	2
PIV-1	2 (1.1)	0	2
HCoV-OC43	2 (1.1)	0	2
PIV-2	1 (0.6)	0	1
HCoV-229E	1 (0.6)	1	0

RSV, respiratory syncytial virus; HRV, human rhinovirus; AdV, adenovirus; HBoV, human bocavirus; Flu A, B, influenza virus group A, group B; hMPV, human metapneumovirus; HEV, human enterovirus; PIV-1, -2, -3, -4, parainfluenza virus type 1, 2, 3, 4; HCoV, human coronavirus

Discussion

We used multiplex PCR for the detection of respiratory viruses among patients admitted with SARI. We also assessed the presence of viral nucleic acids by PCR in healthy asymptomatic children in order to better understand the etiologic role of the tested viruses in respiratory disease.

Viral nucleic acids were detected in 89.9 % of patients admitted with severe lower respiratory tract infections. This is in keeping with a previous study that reported the detection of a viral pathogen by PCR in 72.3 % of children aged ≤ 5 years [9], and with Bierbaum et al., who reported viral pathogen detection in children in excess of

Table 3 Frequency of pathogen detection among the control group

Pathogen	Frequency (<i>n</i>)
None	12
AdV	14
HBoV	12
HEV	6
HCoV-OC43	6
HRV	4
Mixed	10

AdV, adenovirus; HBoV, human bocavirus; HEV, human enterovirus; HCoV-OC43, human coronavirus OC43; HRV, human rhinovirus

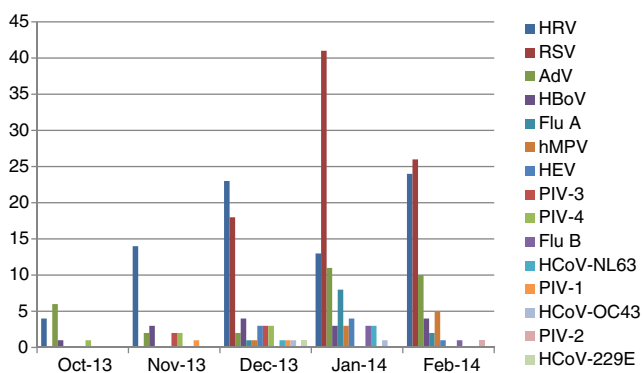
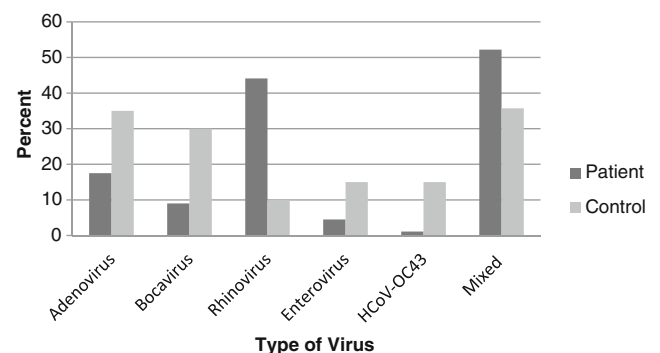
80 %, compared to only 20 % in adults presenting with acute respiratory illness [10].

Similar to previous reports [10, 11], the most frequently detected virus in our study was RSV (48 %).

Flu A was detected in 6.2 % of the samples and Flu B was detected in 2.3 % of the samples. These results are similar to those of Lam et al., who reported a positive rate of 6.3 % for Flu A by PCR and a positive rate of 3.3 % for Flu B [2]. Some 6–12 % of all children use healthcare services every year because of influenza [12]. Additionally, many cases of infection with influenza virus are not diagnosed as such [1]. Early confirmation of influenza viruses is helpful because treatment with neuraminidase inhibitors then becomes an option [1].

HPIVs are important causes of upper respiratory tract illness and lower respiratory tract illness, especially in children [13, 14]. Collectively, the four types of hPIVs were detected in 8 % of the patients with SARI in the current study.

Similar to previous studies, multiplex PCR allowed the detection of HRV, HCoV-OC43, and hMPV, which were not detectable by conventional cell culture isolation or direct detection using immunofluorescence assay. The improvement in the diagnostic yield by adding HRV was confirmed in our study, and also reported previously by Gruteke et al. [15].

**Fig. 1** Monthly distribution of the detected viruses among the patients**Fig. 2** Comparison of the frequency of the viruses detected in patients and controls

Detection of multiple viruses among patients

Multiplex PCR detected a single pathogen in 53.1 %, dual detections in 36.2 %, triple detections in 9.6 %, and quadruple detections in 1.1 % of 177 samples. Similarly, for children aged <18 years, the percentages of single virus, multiple viruses, and no virus detected by the multiplex reverse transcriptase PCR method were 63 %, 14 %, and 23 %, respectively, in the study by Zimmerman et al. [16]. These findings are comparable to previous reports [10, 11].

In view of the fact that respiratory tract infections in children occur in close succession during the winter season, it can be assumed that infections actually overlap. This may provide an explanation for the fact that viral nucleic acids of different viruses in one specimen are found virtually only in children—for example, in 8 % of all specimens analyzed by Bierbaum et al. [17] and 46.9 % of the samples in our study. Co-detections were reported more commonly than single infections in children than older adults (≥ 65 years; $p = 0.01$) [16].

Co-infection of RSV with other respiratory viruses is common and can increase the severity of the disease [18]. In the current study, co-infection with RSV was present in 52.9 % of the patients who tested positive for RSV. This may have increased the severity of the disease, resulting in hospital admission. Moreover, 26.9 % of the patients who were admitted to the ICU had RSV in association with other viruses.

Detection of viral pathogens among asymptomatic children

For a long time, it was assumed that symptomatic infection with respiratory tract viruses was regularly shown by the confirmation of viral nucleic acids in respiratory secretions. However, in recent years, there have been increasing indications that this is not necessarily the case [19]. Data are available from only a few case–control studies that examined to what extent specific respiratory viruses cause disease [9, 20–23].

In the current study, AdV, HBoV, HRV, HEV, and HCoV-OC43 were not only detected in patients admitted with SARI, but were also detected in asymptomatic children.

The clinical importance of bocaviruses remains the subject of controversy. Bocavirus DNA can be confirmed in the respiratory tract of asymptomatic children; a high concentration of these viruses in respiratory specimens seems to be associated with symptoms [19]. Only quantitative monoplex PCR or multiplex PCR can provide information about virus concentration. Bocavirus was detected by PCR in nasopharyngeal swabs samples of children suffering from acute respiratory tract infection in Saudi Arabia [24]. In our study, HBoV was detected among symptomatic children admitted with SARI, as well as in asymptomatic children.

Six different human pathogenic coronavirus species (HCoV) are known to date [1]. As a rule, they cause benign

disorders of the upper respiratory tract [25]. However, after the SARS (severe acute respiratory syndrome) epidemic in 2003 with the SARS CoV, HCoV-NL63 was described in 2004 and HCoVHKU1 in 2005 [21]. The Middle Eastern Respiratory Syndrome (MERS) is caused by MERS-CoV [26].

Human corona viruses (HCoV-NL63, HCoV-OC43, HCoV-229E) were detected in seven patients admitted with SARI. However, HCoV-OC43 was also detected in six asymptomatic children. Adenovirus was the third most frequently detected virus in our study population. Adenovirus DNA is also often found in the respiratory secretions of asymptomatic children [1], and was detected among patients as well as asymptomatic children in our study.

HRVs are frequently found in asymptomatic children [27], but are also detected in patients with symptoms ranging from mild common colds [28] to serious lower respiratory tract disease [29]. Since the development of molecular assays for the detection of HRVs, the detection rate of HRV in patients with respiratory infections has increased to up to 50 % [30]. HRV was only second to RSV in frequency of detection by multiplex PCR in swab samples of children admitted with SARI in our study. However, HRV was also detected in 4/40 controls. HRVs are frequently detected in asymptomatic children, making it difficult to interpret the clinical significance of a positive PCR finding [9]. Moreover, HRVs could be detected until 2–5 weeks after the onset of symptoms [31]. In spite of these facts, Rhedin et al. [9] reported that their data indicate that 39 % [95 % confidence interval (CI): 1 to 62] of acute respiratory infections in their population could be attributed to HRV.

The potentially important rate of excretors of viral nucleic acids of specific pathogens that are not directly associated with the acute illness presents a problem. To date, quantitative conclusions are not reliably established, neither for monoplex PCR nor for multiplex PCR [1].

Rhedin et al. showed that the nucleic acids of RSV, hMPV, and parainfluenza virus can be confirmed almost exclusively in symptomatic children and very rarely in asymptomatic children. Their results indicate that a qPCR finding of RSV, hMPV, or PIV is likely to be causative of disease in children with acute respiratory infection, and that detection of several other viruses, such as HBoV, HRV, AdV, HCoV, and HEV, must be interpreted with caution, due to the high detection rates among healthy children [9]. The detection of AdV, HBoV, HRV, HCoV-OC43, and HEV among asymptomatic children in the current study and the absence of RSV, hMPV, Flu A, Flu B, hPIV-1-2-3-4, HCoV-NL63, and HCoV-229E in these children support these findings.

Whereas the detection of some viruses, such as influenza virus and respiratory syncytial virus (RSV), is clearly predictive for respiratory disease [32–34], the clinical significance upon the detection of several other viruses needs further investigation. The interpretation of a viral detection is complicated by the fact that infections with multiple viruses are

common in children with acute respiratory infection and that many viruses have lately been reported to be found also in asymptomatic children [20].

Most respiratory tract infections in children are of viral origin, especially during the winter season, which, in Egypt, is from November to February. Almost all the patients in our study received antibiotic therapy during hospital admission. The identification of specific viral pathogens will limit the unnecessary use of antibiotics and will facilitate giving specific antiviral therapy when indicated (e.g., neuraminidase inhibitors in confirmed influenza virus infection).

Better understanding of how to interpret viral findings by the use of new technologies is important to improve management decisions, which, in turn, will ameliorate patient outcomes and reduce unnecessary use of antibiotics.

Conclusion

This study confirms the high rate of detection of viral nucleic acids by multiplex PCR among hospitalized children admitted with severe acute respiratory infection, as well as the high rate of detection of multiple viruses. Adenovirus, HBoV, HRV, HEV, and HCoV-OC43 were also detected in asymptomatic controls, resulting in challenges in clinical interpretation. Further studies are required to provide quantitative conclusions that will facilitate clinical interpretation and application of the results in the clinical setting.

Compliance with ethical standards

Funding source None.

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval Informed verbal consent was obtained from the parents of all the patients and the controls. The study design conformed to the Revised Helsinki Declaration of Bioethics and was approved by the Scientific Ethics Committee of the Department of Pediatrics, Faculty of Medicine of Cairo University.

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