Detection of respiratory viruses by real-time polymerase chain reaction in outpatients with acute respiratory infection

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Viruses are the major contributors to the morbidity and mortality of upper and lower acute respiratory infections (ARIs) for all age groups. The aim of this study was to determine the frequencies for a large range of respiratory viruses using a sensitive molecular detection technique in specimens from outpatients of all ages with ARIs. Nasopharyngeal aspirates were obtained from 162 individuals between August 2007-August 2009. Twenty-three pathogenic respiratory agents, 18 respiratory viruses and five bacteria were investigated using multiplex real-time reverse transcriptase polymerase chain reaction (RT-PCR) and indirect immunofluorescence assay (IIF). Through IIF, 33 (20.4%) specimens with respiratory virus were recognised, with influenza virus representing over half of the positive samples. Through a multiplex real-time RT-PCR assay, 88 (54.3%) positive samples were detected; the most prevalent respiratory viral pathogens were influenza, human rhinovirus and respiratory syncytial virus (RSV). Six cases of viral co-detection were observed, mainly involving RSV. The use of multiplex real-time RT-PCR increased the viral detection by 33.9% and revealed a larger number of respiratory viruses implicated in ARI cases, including the most recently described respiratory viruses [human bocavirus, human metapneumovirus, influenza A (H1N1) pdm09 virus, human coronavirus (HCoV) NL63 and HCoV HKU1].

Key words: respiratory infections - real-time PCR - multiplex - viruses

Acute respiratory infections (ARIs) are the main causes of morbidity and mortality worldwide, especially in children during the first years of life (Osterhaus 2008).

The viruses most commonly associated with ARIs are influenza viruses A and B (Inf A and Inf B), respiratory syncytial virus (RSV), parainfluenza viruses 1-4 (PIV 1- 4), human adenovirus (HAdV), human rhinovirus (HRV), human coronavirus (HCoV) and enterovirus (EV) (Arruda et al. 2006). Since 2001, new viruses have been detected in ARI cases, such as human metapneumovirus (hMPV) (van den Hoogen et al. 2001); two new types of HCoV, HKU1 and NL63 (van der Hoek et al. 2004, Woo et al. 2005) and human bocavirus (HBoV) (Allander et al. 2005).

The diagnosis of respiratory viruses based on virus isolation, detection of antigens or serology is too time-consuming and, in some cases, has low sensitivity (Gunson et al. 2005). Therefore, molecular diagnostic methods using "in-house" or commercially available techniques are alternatives to obtain faster results and higher sensitivity and specificity. These molecular methods can potentially reduce the length of hospitalisation and unnecessary treatment costs; furthermore, they can also contribute to nosocomial infection control programs and can help to guide therapy (Barenfanger et al. 2000, Mahony 2008, Brittain-Long et al. 2010).

The aim of the present study was to determine the frequencies of a range of respiratory pathogens using the Fast-Track Diagnostics Respiratory Pathogens 21 PLUS (FTDRP 21 plus) multiplex reverse transcriptase polymerase chain reaction (RT-PCR) assay in patients of all ages with ARIs treated in emergency rooms or primary care units.

SUBJECTS, MATERIALS AND METHODS

This descriptive study was developed in Vitória, Southeast Brazil, from a set of biological samples obtained over a two-year period (August 2007-August 2009) as a part of the Respiratory Virus Surveillance Program of the Ministry of Health, Brazil. Nasopharyngeal aspirates (NPAs) were collected from patients of all ages who were attended at either emergency rooms or primary care units. The patients presented with fever (temperature equal to or greater than 38ºC) and runny nose, in addition to one or more of the following symptoms: cough, myalgia, nasal congestion, headache, sore throat and earache, within five days of the symptom onset. The nasopharyngeal aspiration procedure is a part of the routine of the Respiratory Virus Surveillance Program.

All samples were tested by indirect immunofluorescence assay (IIF) and FTDRP 21 plus multiplex real-time RT-PCR assay.

Seven respiratory viruses (RSV, PIV 1-3, Inf A, Inf B and HAdV) were screened in all specimens by IIF using

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the Respiratory Panel 1 Viral Screening & Identification Kit™ (Chemicon International, Millipore, USA) according to the manufacturer's instructions.

Twenty-three respiratory pathogens, including 18 human respiratory viruses and five bacterial species, were tested using the multiplex protocol. Total nucleic acid was extracted using the MagNA Pure LC automated extraction system (Roche, Switzerland). Nucleic acid was extracted from 200 µL of each NPA sample and was eluted to a final volume of 100 µL following the MagNA Pure LC Total Nucleic Acid Isolation v.12.0 (Roche) protocol. An internal RNA virus control, Brome Mosaic Virus (Fast-track Diagnostics, Luxembourg), was introduced into the lysis buffer for each specimen to monitor the sample extraction and reverse transcription. Multiplex real-time RT-PCR was performed using the FTDRP 21 plus according to the manufacturer's instructions (Fast-track Diagnostics). The reaction volume for each test was 25 µL, made up of 10 µL of nucleic acid and 15 µL of buffer/enzyme mix from the AgPath-ID™ One-Step RT-PCR kit (Ambion, Life Technologies, USA). Amplification was performed in the ABI 7500 real-time PCR system thermocycler (Applied Biosystems, USA) and the following cycling conditions were used: 15 min at 50ºC, 10 min at 95ºC and 40 cycles of 8 s at 95ºC and 34 s at 60ºC. The fluorescence reading was taken in the 60ºC/34 s step in each cycle and the threshold cycle (C_t) values were determined by manual adjustment. Each sample was amplified in six parallel reactions, which contained primers and probes for four different targets, detecting viruses and pathogenic bacteria, in addition to the internal control of the reaction. The positive and negative virus plasmid controls provided in the kit were included in all runs to monitor the assay performance.

Exploratory analysis of the data was performed using the SPSS software package v.17.01 (SPSS Inc, USA).

Ethics - The Research Ethical Committee of the Centre for Health Sciences of Federal University of Espírito Santo approved this study, with the registration CEP-093/07.

RESULTS

Here, we present the results of patients who met all inclusion criteria and whose samples were analysed by both methods proposed in this study. One hundred sixty-two outpatients (79 male and 83 female) were included. The study consisted of both adult (n = 46) and paediatric (n = 116) individuals. The paediatric age group consisted of individuals zero-19 years of age, including children and adolescents, according to the World Health Organization criteria. The median age of the entire cohort was seven years (range 1 month to 75 years). Children under nine years of age accounted for 66.7% (108/162) of the samples analysed. All patients had recent (≤ 5 days) symptoms.

IIF detected 33 positive specimens (20.4%). The influenza virus was the most common etiologic agent detected (23/33). Inf A and Inf B corresponded to 8% (13/162) and 6.2% (10/162) of the samples, respectively. The IIF technique detected only single infections. Eighty-eight (54.3%) specimens were positive for one or more respiratory viruses by multiplex real-time RT-PCR (Table I). The influenza virus (15.4%), HRV (8%) and RSV (7.4%) were the viruses most frequently detected and accounted for one-third of the positive samples.

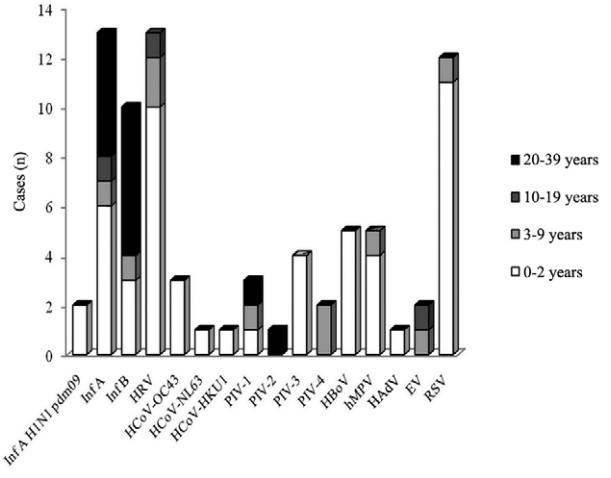
Regarding the patient age groups, the influenza viruses were more frequently identified in young adults (20-39 years of age). All viruses were identified in infants (0-2 years old), with the exception of PIV 2, PIV 4 and EV. RSV and HRV were the most prevalent. Most of the samples positive for RSV (91.6%) and HRV (77%) were identified in infants. No virus was identified in subjects above 40 years old (Figure).

The following bacteria were detected: *Streptococcus pneumoniae* [n = 43 (26.3%)], *Staphylococcus aureus* [n = 20 (12.3%)] and *Haemophilus influenzae* [n = 2 (1.2%)]; *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* were not detected (Table I).

Six specimens (3.7%) were positive for two viruses. All co-detections occurred in children under two years of age and RSV accounted for half of those cases (Table II). No co-detections involved three or more respiratory viruses. Bacterial pathogens were identified in 27 co-detection cases, with at least one infectious agent from the viral or bacterial pathogen groups (Table II).

DISCUSSION

The laboratory diagnosis of viral respiratory tract infections is usually accomplished through conventional techniques, such as culture or antigen detection tests, with limitations related to the delay of the results and the availability of monoclonal antibodies for newly identified viruses (Mahony et al. 2007). Multiplex PCR assays have become an important tool for the identification of etiologic agents in ARIs (Dabisch-Ruthe et al. 2012, Sanghavi et al. 2012). As described recently, the com-



Distribution of respiratory virus of outpatients by age groups identified in nasopharyngeal aspirate by multiplex real-time reverse transcriptase polymerase chain reaction and indirect immunofluorescence assay in Vitória, Southeast Brazil, 2007-2009. EV: enterovirus; HAdV: human adenovirus; HBoV: human bocavirus; HCoV: human coronavirus; hMPV: human metapneumovirus; HRV: human rhinovirus; Inf: influenza virus; Inf A H1N1 pdm09: Inf A H1N1 pandemic; PIV: parainfluenza virus; RSV: respiratory syncytial virus.

TABLE I

Respiratory pathogens identified in nasopharyngeal aspirate of outpatients by multiplex real-time reverse transcriptase polymerase chain reaction (RT-PCR) and indirect immunofluorescence (IIF) assay in Vitória, Southeast Brazil, 2007-2009

	Positive by			
	Paediatric[a]	Adult	Paediatric[a]	Adult
Pathogens	IIF n (%)		Multiplex real-time RT-PCR n (%)	
Viral agents				
Inf A H1N1 pdm09	NA	NA	2 (1.2)	0 (0)
Inf A	5 (3.2)	8 (4.8)	5 (3.2)	8 (4.8)
Inf B	5 (3.1)	5 (3.1)	5 (3.1)	5 (3.1)
HRV	NA	NA	12 (6.75)	2 (1.25)
HCoV-229E	NA	NA	0 (0)	0 (0)
HCoV-OC43	NA	NA	2 (1.2)	1 (0.6)
HCoV-NL63	NA	NA	1 (0.6)	0 (0)
HCoV-HKU1	NA	NA	1 (0.6)	0 (0)
PIV-1	2 (1.2)	0 (0)	2 (1.2)	1 (0.6)
PIV-2	0 (0)	0 (0)	0 (0)	1 (0.6)
PIV-3	2 (1.2)	0 (0)	3 (1.8)	1 (0.6)
PIV-4	NA	NA	2 (1.2)	0 (0)
HBoV	NA	NA	5 (3.1)	0 (0)
hMPV A/B	NA	NA	4 (2.5)	1 (0.6)
HAdV	0 (0)	0 (0)	1 (0.6)	0 (0)
EV	NA	NA	2 (1.2)	0 (0)
HPeV	NA	NA	0 (0)	0 (0)
RSV A/B	6 (3.7)	0 (0)	12 (7.4)	0 (0)
Bacterial agents				
Streptococcus pneumoniae	NA	NA	40 (24.5)	3 (1.8)
Staphylococcus aureus	NA	NA	16 (9.9)	4 (2.4)
Haemophilus influenzae	NA	NA	2 (1.2)	0 (0)
Chlamydophila pneumoniae	NA	NA	0 (0)	0 (0)
Mycoplasma pneumoniae	NA	NA	0 (0)	0 (0)

a: zero-19 years of age; EV: enterovirus; HAdV: human adenovirus; HBoV: human bocavirus; HCoV: human coronavirus; hMPV: human metapneumovirus; HPeV: human parechovirus; HRV: human rhinovirus; Inf: influenza virus; Inf A H1N1 pdm09: Inf A H1N1 pandemic; NA: not applicable; PIV: parainfluenza virus; RSV: respiratory syncytial virus.

mercial multiplex FTDRP real-time RT-PCR assay has enhanced the diagnosis of ARIs, with simultaneous detection of 16 respiratory viruses (Sakthivel et al. 2012). The new version of the FTDRP assay used in this study has expanded the capacity for detecting pathogens (18 viral and 5 bacterial species) and has thus increased the diagnostic potential of the test.

More than half (66.7%) of the study population were children under nine years of age, which is representative of the occurrence of ARIs in the community. The paediatric age group is the main group affected by ARIs, which occur three-eight times a year in infants and young children, with incidences varying inversely to age (Bryce et al. 2005, Bezerra et al. 2011).

The viral agents most frequently identified were influenza viruses, including Inf A (H1N1) pdm09 (2 cases in patients with uncomplicated respiratory infection), followed by HRV and RSV. Since April 2009, the Inf A (H1N1) pdm09 virus has spread worldwide. Given that the country's health authorities only declared sustainable transmission of the new influenza virus in Brazil (MS 2009) on 16 July 2009, we assume that the observed low frequency of Inf A (H1N1) pdm09 is related to the endpoint of the present study, in August 2009.

The frequency of influenza virus infection observed in this study (15.4%) is consistent with previously published rates varying from 2-26% of ARI cases in studies in South, Southeast and Northeast Brazil (Arruda et al. 1991, Bellei et al. 2008). Although wide inter-regional variations should be expected in a large country with different climates, the variation may also be due to study design differences. All viruses screened in this study were

TABLE II

Co-detection of all respiratory pathogens identified in nasopharyngeal aspirate of outpatients by multiplex real-time reverse transcriptase polymerase chain reaction (RT-PCR) in Vitória, Southeast Brazil, 2007-2009

Viruses and/or bacteria	n (%)
RSV A/B + Inf A	1 (0.6)
RSV A/B + HCoV-NL63	1 (0.6)
RSV A/B + HBoV	1 (0.6)
RSV A/B + *Streptococcus pneumoniae*	1 (0.6)
RSV A/B + *S. pneumoniae* + *Staphylococcus aureus*	1 (0.6)
RSV A/B + Inf A + *S. pneumoniae* + *Haemophilus influenzae*	1 (0.6)
HRV + PIV-3	1 (0.6)
HRV + HBoV	1 (0.6)
HRV + *S. pneumoniae*	4 (2.5)
HRV + PIV-3 + *S. pneumoniae*	1 (0.6)
hMPV A/B + HCoV-OC43	1 (0.6)
hMPV A/B + *S. pneumoniae*	2 (1.25)
hMPV A/B + *S. pneumoniae* + *S. aureus*	1 (0.6)
Inf A + *S. aureus*	1 (0.6)
Inf A + Inf A H1N1 pdm09 + *S. aureus*	2 (1.25)
Inf B + *S. pneumoniae*	3 (1.8)
Inf B + *S. aureus*	1 (0.6)
PIV-1 + *S. pneumoniae*	2 (1.25)
PIV-3 + *S. pneumoniae*	2 (1.25)
PIV-4 + *S. pneumoniae*	1 (0.6)
PIV-4 + *H. influenzae*	1 (0.6)
EV + *S. pneumoniae*	1 (0.6)
S. pneumoniae + *S. aureus*	2 (1.25)

EV: enterovirus; HBoV: human bocavirus; HCoV: human coronavirus; hMPV: human metapneumovirus; HRV: human rhinovirus; Inf: influenza virus; Inf A H1N1 pdm09: Inf A H1N1 pandemic; PIV: parainfluenza virus; RSV: respiratory syncytial virus.

more frequently identified in children, with the exception of influenza viruses, which were more often identified in adults (8.6%) than in paediatric patients (6.8%), as has been documented in a recent study (Pelat et al. 2013).

The second most prevalent viral agent was HRV (8%): 77% of HRV-positive samples were identified in children under two years of age. This finding highlights the importance of this virus in infants, as HRV has been associated with recurrent respiratory illnesses and wheezing in this age group (Jartti et al. 2008). Other picornaviruses may be etiologic agents of ARIs; however, HRV is the main virus detected in cases of the common cold (Pitkäranta & Hayden 1998). In a multicentre study, rhinoviruses were often associated with ARIs, even during the peak influenza season (Caruso et al. 2007). In Brazil, according to Arruda et al. (1991), HRV was the most frequent viral agent (46%) detected in children with ARIs.

The frequency of RSV detection (7.4%) was similar to that detected by Raboni et al. (2011), who reported a rate of 8% using multiplex RT-PCR for 14 respiratory viruses. Eleven (91.6%) of the RSV cases in this study were detected in children under two years of age. RSV antibodies are found in virtually all adults and children older than three years of age. At one year of age, 25-50% of children have antibodies against RSV, demonstrating the high frequency of this infection at a young age (Domachowske & Rosenberg 1999). In keeping with this observation, the frequency of RSV in the youngest age group in our study was the highest among all ages (91.6%). However, the study was conducted with patients of widely variable ages: only two-thirds were children under nine and only 40% of the studied patients were children under two years of age. Hence, it is reasonable to consider that the observed relatively low frequency of RSV could be attributed, at least in part, to the underrepresentation of patients of very young ages.

The frequency of HBoV was 3.1% and all cases occurred in children under two years of age. This virus has been frequently identified in this age group, although its pathogenicity is not yet well defined (Longtin et al. 2008, Lüsebrink et al. 2009). Recently, a higher HBoV-positive rate has been documented in inpatients when compared with outpatients or patients attended in emergency departments, suggesting a significant role of this virus in the pathogenesis of ARIs (Xu et al. 2012).

The use of multiplex real-time RT-PCR increased the overall viral detection rate to 54.3%, compared to 20.4% in the IFF test. This difference is likely due to the

high sensitivity of virus genome detection (Lassaunière et al. 2010). In addition, 47.9% of the samples that were positive by multiplex real-time RT-PCR corresponded to respiratory viruses that were not tested for using the IIF kit, such as HRV, HCoV, hMPV, HBoV and EV. This finding confirms the importance of using panels that identify multiple viral agents causing ARIs.

In this study, there were six cases (3.7%) of viral co-detection, most commonly with RSV and HRV. The real clinical significance of these infections has not yet been fully elucidated. In a recent Brazilian study, co-detection between RSV and HRV increased both the length of hospitalisation and the time of supplemental oxygen use in children with lower respiratory tract infections, suggesting the possibility of co-infection among these viruses and not only co-detection (da Silva et al. 2013). All cases of co-detection observed in our study occurred in children under two years old, the age group in which ARI cases are more frequent. It is quite possible that residual nucleic acids from sequential viral infections are detected simultaneously by multiplex PCR with high sensitivity (Lesley 2012). Additionally, lymphoid tissues may serve as a reservoir of respiratory viruses in asymptomatic individuals and may be involved in the transmission of these viruses to the community (Proenca-Modena et al. 2012).

The likelihood of a pathogen to be the aetiologic agent of a given infection could be related to the C_t value in the real-time PCR reaction. Lower C_t values suggest higher loads of the pathogen detected, which may in turn suggest aetiology (Brittain-Long et al. 2008). In this study, the C_t values were well below the cut-off value for positivity proposed by the manufacturer.

The overall bacterial detection rate was 40% and *S. pneumoniae* was the most frequent agent identified. Despite the vaccination program, *S. pneumoniae* is one of the main causes of pneumonia mortality (responsible for at least 18% of severe episodes and 33% of deaths worldwide) (Walker et al. 2013). Bacteria were co-detected with viruses in 25 specimens. The potentially pathogenic bacteria detected in this study could reflect transient microbiota or a nasopharyngeal flora and may not be associated with ARIs; however, it is well known that viral infections within the respiratory tract predispose the individual to bacterial infections, notably through the disruption of the respiratory mucosal epithelium (Bakaletz 1995). Atypical bacteria (*M. pneumoniae* and *C. pneumonia*) were not detected in the studied population.

In Brazil and other countries, the respiratory virus surveillance programs use conventional diagnostic techniques, such as IIF. A large proportion of biological samples remain negative, even with clinical evidence of respiratory infection. A molecular technique such as real-time PCR can be an important tool, increasing the detection capacity of a large number of respiratory pathogens. Moreover, this type of technique can contribute to the correct indication of antiviral medications, can avoid unnecessary use of antibiotics and can promote the adoption of appropriate hospital-control measures.

The findings of this study show that the application of PCR assays in a real-time multiplex format for respiratory pathogens considerably increases the pathogen detection rate when compared to conventional methods, highlighting the role of influenza virus in ARI cases in patients of all ages, in addition to reporting the circulation of recently described respiratory viruses (Inf A H1N1 pdm09, hMPV, HBoV, HCoV-NL63 and HCoV-HKU1).

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