Rhinovirus-C Detection in Children Presenting With Acute Respiratory Infection to Hospital in Brazil

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Human rhinovirus (RV) is a common cause of acute respiratory infection (ARI) in children. We aimed to characterize the clinical and demographic features associated with different RV species detected in children attending hospital with ARI, from low-income families in North-east Brazil. Nasopharyngeal aspirates were collected from 630 children <5 years with ARI. Clinical diagnosis and disease severity were also recorded. Samples were analyzed by multiplex PCR for 18 viral and atypical bacterial pathogens; RV positive samples underwent partial sequencing to determine species and type. RV was the fourth commonest pathogen accounting for 18.7% of pathogens detected. RV was commonly detected in children with bronchiolitis, pneumonia, and asthma/episodic viral wheeze (EVW). Species and type were assigned in 112 cases (73% RV-A; 27% RV-C; 0% RV-B). Generally, there were no differences in clinical or demographic characteristics between those infected with RV-A and RV-C. However, in children with asthma/EVW, RV-C was detected relatively more frequently than RV-A (23% vs. 5%; P = 0.04). Our findings highlight RV as a potentially important pathogen in this setting. Generally, clinical and demographic features were similar in children in whom RV-A and C species were detected. However, RV-C was more frequently found in children with asthma/EVW than RV-A.

INTRODUCTION

Human rhinoviruses (RV) are a major cause of upper respiratory tract infection and acute wheezy episodes in pre-school children and asthmatics [Simons et al., 2005; Renwick et al., 2007; Arden and Mackay, 2010; Gern, 2010]. Traditionally, they have been divided into two species (A & B) and over 100 serotypes. In 2006, a number of novel RV strains were identified with significant variation in the viral protein (VP) regions (VP2, VP4) and 5' non-coding region (5' NCR); these formed a distinct new species that was designated “rhinovirus C” (RV-C) [Arden et al., 2006; Lamson et al., 2006; Arden and Mackay, 2010]. Approximately 60 RV-C types are now recognized based on phylogenetic clustering, and these viruses have been associated with severe acute respiratory infection (ARI), particularly wheezing ARIs, in young children [Lau et al., 2007; Dominguez et al., 2008; Bizzintino et al., 2011] and asthmatics [Lemanske et al., 2005; Miller et al., 2009; Calvo et al., 2010].

Although the role of RV-C infection as a cause of ARI in children is increasingly recognized in the developed world, its disease-causing role in low-income settings is relatively unknown. In this study, we sought to characterize the clinical and demographic features associated with different RV species detected in children attending hospital with ARI from low-income families in North-east Brazil.

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METHODS
Setting and Study Design
This study was undertaken as part of a larger research project investigating the viral and atypical bacterial causes of paediatric ARI in a low-income setting in Brazil [Bezerra et al., 2011; Fawkner-Corbett et al., 2012]. This prospective cross-sectional study was conducted between April 2008 and March 2010 at the Instituto de Medicina Integral Prof Fernando Figueira (IMIP), a large publically funded teaching hospital in Recife, Pernambuco, North-east Brazil. Between one third and one half of the population of Recife lives in relative poverty in urban slums and favelas with IMIP’s paediatric emergency department (ED) primarily providing primary, secondary, and tertiary medical care to these low-income families.

Subjects
All children aged less than 5 years with upper and/or lower respiratory tract manifestations of ARI of less than 7 days duration were eligible for inclusion. A trained research assistant approached consecutive patients with ARI between 7 am and 3 pm (Monday to Friday) whilst they were in the paediatric ED and collected clinical and demographic data from parents/guardians. Baseline observations including temperature, oxygen saturation, respiratory, and pulse rates were recorded at the time of arrival in the ED.

One research assistant collected all nasopharyngeal aspirate (NPA) samples within 4 hr of the child seeing a doctor in the ED using a standardized protocol [Semple et al., 2005].

Ethics Statement
The Ethics Committee at IMIP and the National Research Ethics Office of Brazil approved the study and written informed consent was obtained from parents/guardians prior to enrolment.

Clinical Manifestations of ARI
The clinical outcome and diagnosis for each child were recorded following discharge from the hospital. The discharge diagnosis for each child was made by attending physicians not involved in the study and based on standard clinical criteria. Thus bronchiolitis was diagnosed in children <18 months in whom upper respiratory symptoms preceded lower respiratory symptoms of wheeze, tachypnoea and signs of respiratory distress. Pneumonia was diagnosed in children with fever, tachypnoea, and respiratory distress where focal or diffuse crackles or decreased vesicular sounds were present on auscultation. Most diagnoses of pneumonia were based on clinical criteria alone, but radiographic findings were used in some. A diagnosis of asthma/episodic viral wheeze (EVW) was made in children in whom discreet episodes of wheeze occurred, often in association with a presumed viral upper respiratory tract infection. Upper respiratory tract infections (including group) were diagnosed based on symptoms such as coryza, earache, sore throat, and stridor.

Pathogen Detection
Respiratory samples were stored at −70°C and transported to Liverpool, UK where multiplex real-time PCR (RT-PCR) was undertaken as previously described for 18 viral and atypical bacterial pathogens including human rhinovirus (RV), respiratory syncytial virus (RSV), human metapneumovirus (hMpV), influenza (flu), parainfluenza virus (PIV) 1–4, bocavirus (hBoV), coronavirus (CoV), adenovirus (AdV), Mycoplasma pneumonia (Myc), and Chlamydo- philia pneumonia (Cpp) [Bezerra et al., 2011; Fawkner-Corbett et al., 2012].

RNA samples from children in whom RV had been identified by RT-PCR were reverse transcribed to cDNA. Random primers, AMV-RT, RNAase inhibitor and dNTPs (Promega, Southampton, Hampshire, UK) were added to each sample at 4°C. The RNA samples were reverse transcribed using a thermocycler TC-512 machine (Techne, Stone, Staffordshire, UK) with conditions of 35 min at 42°C, 20 min at 50°C, and finally 5 min at 85°C. All cDNA was then stored at −30°C prior to transportation to Perth, Australia for further analysis [Lee et al., 2007a].

Analysis of Rhinovirus Strain
In Perth, cDNA was amplified using semi-nested PCR primers specific to the 390 bp variable region in the 5’ non-coding region (5’ NCR) of the RV genome, employing primers [Bochkov et al., 2014] and cycling conditions described previously [Bizzintino et al., 2011; Lee et al., 2007a,b]. The Australian Genome Research Facility sequenced the PCR products. RV genotypes were then assigned based on comparisons of the 5’ NCR sequences with those of the 101 classical serotypes as well as the 52 newly identified genotypes by phylogenetic tree analysis using the ClustalX software (Conway Institute, University College Dublin, Dublin, Ireland). Representative samples of each genotype have previously been sequenced at the VP4–VP2 coding region to confirm the species assignment [Lee et al., 2012].

Samples that could not be successfully typed in Perth were sent to Wisconsin for cloning, sequencing, and species assignment as described previously [Lee et al., 2007a,b].

Statistical Analysis
Statistical analysis was performed using SPSS v18.0.0 (SPSS Inc, Chicago, IL). Differences in hospital admissions, clinical presentations and co-infection rates were calculated using the χ² test. Variation between groups was assessed with Kruskal–Wallis test. Differences in
age were calculated using the Mann–Whitney U test with a P-value <0.05 considered significant.

RESULTS
Clinical and Demographic Features of Children With RV Infection

Of 630 children presenting with ARI, initial RT-PCR in Liverpool detected RV in 118 (18.7%). Analysis in Perth/Wisconsin confirmed the RV detection and identified strains in 112 (17.8%). In the 6/118 samples in which RV detection was not confirmed, coxsackievirus was identified in three samples, and poliovirus (1/HEV-C, strain Sabin 1), enterovirus, and no virus at all in the remainder. RV was the fourth most prevalent pathogen detected after RSV (37%), Adenovirus (25%), and Bocavirus (19%) [Fawkner-Corbett et al., 2012]. Of the 112 children (55% male) in whom RV detection was confirmed, the median (range) age was 6 (0–48) months, with 95/112 (85%) being under 18 months of age and 85/112 (76%), 12 months or less. In 62/112 (55%) patients, the RV-associated ARI necessitated hospital admission. Twenty-one children (19%) had a history of low birth weight and 46/112 (41%) had a member of the immediate household who smoked. Bronchiolitis (67/112; 60%) was the most prevalent ARI diagnosis, followed by pneumonia (21%), asthma/EVW (10%), and URTI (5%). Overall no significant clinical or demographical differences were found between children in whom RV was and was not detected (Table I).

Co-detection of other viruses and atypical bacteria was found in 65/112 (58%) RV-positive samples, with the commonest viruses co-detected being AdV (29/65; 45%), hBoV (39%), and RSV (20%). Children in whom RV was detected were significantly more likely to have another pathogen detected (RV+ve, 58%; RV−ve, 39%; χ² = 13.5, P < 0.001). Viral or atypical bacterial co-detection was not associated with particular clinical presentations or severities of disease (data not shown).

Clinical and Demographic Features of Children With RV-C Infection

Of 112 RV samples, RV-A was detected in 82/112 (73%) and RV-C detected in 30/112 (27%); no RV-B was detected. In three samples, two RV-A strains were detected (Supplementary Table S1). These were samples from a 3 month old male (types A20 and A11), an 8 month old male (A47, A44), and an 11 month old male (A78, A47). We identified a novel strain of RV-A (A106), in an 8 month male with multiple co-infections (AdV, PIV, and hBoV), diagnosed with bronchiolitis who required hospital admission for 6 days. There was no significant difference in clinical or demographical data between those infected with RV-A or RV-C (Table I). Levels of co-infection were similar in patients in whom RV-A and RV-C were detected (60% vs. 53%).

Clinical Presentations

RV was commonly detected in children with bronchiolitis, pneumonia and asthma/EVW (Fig. 1). In children with asthma/EVW, RV-C was detected relatively more frequently than RV-A. Thus, 7/30 (23%) children with RV-C infection presented with asthma/EVW, whereas only 4/82 (5%) children with RV-A infection presented in this way (χ² = 8.44, P = 0.04). In children with pneumonia, RV-A was more frequently detected than RV-C (RV-A, 26%; RV-C, 10%), but this did not reach statistical significance (P = 0.059).

Seasonality

The monthly frequency with which RV-A and C strains were detected over the study period is shown in Figure 2. Generally, both strains were found to circulate during most months apart from February–April following the dry season. There was a peak in presentation following the rainy season (July and November).

DISCUSSION

Using genetic amplification techniques, we successfully classified 112 RV samples from pre-school children from predominantly low-income families, presenting with ARI to a children’s hospital in Recife, Brazil. Most RV clinical presentations in this cohort attending hospital were in children less than 1 year with lower respiratory tract symptoms, particularly bronchiolitis, pneumonia, and asthma/EVW. Overall the prevalence of RV-A was three-times that of RV-C, but in children with asthma/EVW, RV-C was detected relatively more frequently than RV-A.

RV accounted for 17.8% of children presenting to hospital with ARI over the 2 year period, the fourth most prevalent pathogen in the study. This is
similar to other epidemiological studies that report the prevalence of RV of 12–26% in ARI [Jartti et al., 2004; Miller et al., 2007; Wang et al., 2010]. In our cohort, RV-C was detected in 4.7% of ARI cases over the 2 years. Another study in Brazil (São Paulo) in 120 children <12 years presenting as outpatients with ARI detected RV in 47% and RV-C in 28% of children [Moreira et al., 2011]. Our study differed in both age groups included (<5 years [most <1 year], vs. <12 years) and setting (ED vs. outpatient). Our cohort was also from a predominantly low income background [Bezerra et al., 2011]. Our finding of 27% of RV samples being RV-C positive is comparable to similar studies in children in Spain and China that report a prevalence of 35% and 36%, respectively [Jin et al., 2009; Calvo et al., 2010]. Although we were surprised not to detect RV-B at all in our cohort, it is often only found at very low levels (0–3%) or in relatively asymptomatic patients [Loens et al., 2006; Lau et al., 2007; Dominguez et al., 2008].

This study supports others suggesting an association between RV-C and severe ARI, particularly wheezing ARI, in young children. Recent in vitro studies have shown that, in contrast to other RV species, RV-C grows optimally at either 34°C or 37°C,

Fig. 1. Relative RV detection frequency in different clinical presentations of ARI in preschool children (URTI, upper respiratory tract infection; Asthma/EVW, asthma/episodic viral wheeze; * = Significant difference, \( P < 0.05 \)).

Fig. 2. Frequency of RV infections each month (April–July shaded to indicate the local “rainy” season).
potentially allowing it to infect the lower respiratory tract as easily as the upper respiratory tract [Ashraf et al., 2013]. It is likely that other factors are also involved, however, given that we found similar rates of infection with RV-C and A in infants with bronchiolitis and a trend towards more RV-A than RV-C in children with pneumonia.

The strengths of this study are that it is one of the largest on RV epidemiology [Onyango et al., 2012], with good quality clinical and demographic data, and samples, collected systematically by one research assistant, from children with both RV-A and C infection. It supports other studies [Mansbach et al., 2008] suggesting that RV might be an important cause of bronchiolitis [Bezerra et al., 2011]. This is also the first study to examine RV seasonality in Brazil. Unlike previous studies in Australia and North America, RV infection appears to occur throughout the year with peaks during the rainy season, a pattern also seen in Trinidad [Matthew et al., 2008]. It may be that the high population density within our cohort results in continual RV transmission throughout the year.

However, despite recruiting a relatively large cohort of children with RV, the number of children with RV-C was only 30 and no children had RV-B. Our cohort was also from one center only and so may not have been representative of other hospitals within Brazil or further afield. We can also not provide an estimate of the precise prevalence of RV infection in this population as data on numbers of children presenting to IMIP with ARI over this time period were not available.

In conclusion we have shown that RV does cause significant lower respiratory disease in young preschool children and that in our cohort, RV-C was more frequently associated with presentations of asthma or epidemics viral wheeze than RV-A.

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