Human Rhinovirus is the Most Commonly Identified Respiratory Virus in Children Hospitalized with Pneumonia from Mozambique

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Abstract

OBJECTIVES: To describe the prevalence of human rhinovirus (RV) species in children hospitalized with pneumonia in Manhiça Mozambique and the associations between RV species and demographic, clinical, and laboratory features.

METHODS: Nasopharyngeal aspirates were collected from children 0-10 years of age (n=277) presenting to the Manhiça District Hospital in Mozambique with clinical pneumonia. Blood samples were collected for HIV and malaria testing, blood culture and full blood counts, and a chest X-ray was performed. A panel of common respiratory viruses was investigated using two independent multiplex RT-PCR assays with primers specific for each virus and viral type. RV species and genotypes were identified by semi-nested PCR assays, sequencing and phylogenetic tree analyses.

RESULTS: At least one respiratory virus was identified in 206 (74.4%) children hospitalized with clinical pneumonia. RV was the most common virus identified in both HIV-infected (17/38, 44.7%) and HIV-uninfected (74/237, 31.2%; p=0.100) children. RV-A was the most common RV species identified (47/275, 17.0%), followed by RV-C (35/275, 12.6%) and RV-B (8/275, 2.9%). Clinical presentation of the different RV species was similar and overlapping, with no particular species being associated with specific clinical features.

CONCLUSIONS: RV-A and RV-C were the most common respiratory viruses identified in children hospitalised with clinical pneumonia in Manhiça. Clinical presentation of RV-A and RV-C was similar and overlapping.

Introduction

Acute lower respiratory infections (ALRI) such as pneumonia account for an estimated 1.3 million deaths each year in children under 5 years of age, 43% of which occur in sub-Saharan Africa.(1) Management and prevention efforts against pneumonia in developing countries

have traditionally focused on bacterial pathogens. The introduction of effective conjugate vaccines globally has led to decreasing trends in bacterial pneumonia and a subsequent increased interest in the role of virus-associated ALRI. Recent advances in molecular diagnostics such as PCR have also led to the discovery of new viruses and viral species, highlighting the prominence of viruses in respiratory disease. Respiratory viruses are widely acknowledged to be the most common cause of both upper and lower respiratory tract infections in the developed world.(2) However, advanced diagnostics are largely limited in the developing world and studies including a comprehensive range of viral pathogens are scarce in many African countries. The majority of respiratory viral aetiological studies in Africa have relied on traditional cell culture and serological methods with PCR data only becoming available in recent years. Data beyond respiratory syncytial virus (RSV) are still scarce and aetiological studies of ALRI in African children published as recently as 2013 did not screen for common respiratory viruses such as RV, coronavirus and bocavirus(3), or were unable to distinguish RV from enterovirus.(4)

RV is the most common cause of childhood respiratory infections worldwide and responsible for almost two-thirds of cases of the common cold(5) as well as lower respiratory tract infections, including pneumonia and bronchiolitis. The identification of RV-C as the third RV species, first reported in 2006, led to several investigations on the prevalence of RV species, conducted predominantly in developed countries. The majority of these studies on children hospitalized with ALRI or asthma found that RV-C was the most prevalent RV species and was often associated with more severe illness.(6-13) While there have been previous reports on the overall prevalence of RV in children with acute respiratory infections or pneumonia in Mozambique, none have specifically investigated RV species.(14, 15) In the two Mozambican studies of respiratory viral prevalence, RV was the most commonly identified

virus, identified in 26% and 24% of children with acute respiratory infections and pneumonia, respectively. Only three other studies have investigated the prevalence of RV species in African children.(16-18) While these studies confirmed the importance of RV in African children, they were inconclusive with respect to the role of RV species in ALRI in African children.

Much of our understanding on the viral aetiology of childhood pneumonia in Africa is based on studies conducted prior to the HIV epidemic that has engulfed many African countries. Evidence suggests that HIV-infection is now driving both the frequency and outcome of pneumonia and pneumonia is the leading cause of morbidity and mortality in HIV-infected children.(19) A recent study from South Africa reported that a respiratory virus was identified in the majority of both HIV-infected and HIV-uninfected children, with RV being the most frequently identified virus.(20) No studies have investigated RV species among HIV-infected children.

Given the limitations of routine microbiology facilities in the majority of African countries, most clinicians rely on examination of clinical features to determine the probable aetiology of ALRI in children. Clinical features, which are often indistinguishable for different respiratory viruses, (21, 22) have not been comprehensively investigated in childhood ALRI in Africa. A few studies from Africa and the Middle East found that RV-C was associated with wheezing.(12, 18) The clinical relevance of viral co-infections is also not well established and there is conflicting evidence regarding the association between multiple viral identifications and disease severity. Some studies have reported an association between viral co-infections and disease severity (23-29) while others have reported no differences in disease severity between single and multiple viral infections.(30, 31)

The aim of this study was to describe the prevalence of respiratory viruses, in particular RV and RV species, and the association of RV species with HIV-status, clinical features and seasonality in children with clinical pneumonia from Manhiça, Mozambique.

Materials and Methods

Study Setting and Design

This study was conducted by the Manhiça Health Research Centre (*Centro de Investigação em Saúde da Manhiça*, CISM) at the Manhiça District Hospital (MDH), a public hospital in Southern Mozambique. The Manhiça district in Southern Mozambique has an estimated population of 143,000. The area has a subtropical climate with two distinct seasons; a warm and rainy season between November and April and cool and dry season during the rest of the year. The HIV prevalence among newborns has been estimated between 2.9 and 8%.(15). A demographic surveillance system (DSS) including 500km^2 surrounding the area, developed by CISM, has been running since 1996, and covers a population of around 92,000 inhabitants. Each individual living within the DSS area is issued a unique permanent identification number and information on vital events are collected during routine household visits.(32) Further characteristics of the DSS and study area are described elsewhere.(32)

Children were recruited as part of a larger project aiming to investigate the underlying aetiology of children with respiratory symptoms. Between September 2010 and April 2013, 277 children 0-10 years of age presenting to the Manhiça District Hospital with fever (or a history of fever (>37.5ºC axillary temperature) in the preceding 24 hours) and clinical pneumonia, according to the WHO definition (increased respiratory rate and cough and/or difficulty in breathing), and sick enough to warrant hospital admission, were recruited into

the study. Increased respiratory rate was defined according to Integrated Management of Childhood (IMCI) guidelines: ≥ 60 breaths per minute in children ≤ 2 months, ≥ 50 in 2-12 months, ≥ 40 in 1-5 years, and ≥ 30 in 5-10 years). Exclusion criteria included prior enrolment in this study, use of antibiotics or antimalarial drugs during the preceding two weeks, history of cough for more than two weeks duration, active tuberculosis or history of direct contact with a documented tuberculosis case, and children with an oxyhaemoglobin saturation of less than 85% on examination on admission, as a proxy for possible *Pneumocystis jirovecii* infection.

Clinical and questionnaire data and samples were obtained from the enrolled cases on the day of recruitment. A NPA was collected from each child by a trained study health assistant. HIV testing was routinely conducted, and all patients underwent chest X-ray and extensive clinical and laboratorial screening, including malaria testing, blood culture and full blood counts.

Written informed consent was obtained from parents or guardians prior to participation and the study was approved by the University of Western Australia Human Research Ethics Committee, the Ethics Committee of the Hospital Clínic (Barcelona, Spain) and the Comité Nacional de Bioética para a Saúde (Maputo, Mozambique), prior to commencement.

Laboratory methods

Virus detection

NPAs were stored at -80ºC in Manhiça, Mozambique until shipped to the study laboratories, on dry ice, for processing. Identification of common respiratory viruses (adenovirus, RSV, bocavirus, coronavirus, parainfluenza viruses, influenza viruses and metapneumovirus) was carried out using two independent multiplex RT-PCR assays with primers specific for each

virus and viral type. RV identification and genotyping was based on a published molecular method to determine RV genotypes and to differentiate closely related enteroviruses from RV.(33) Viral RNA was first extracted from a 240µl volume of NPAs using the QIAGEN QIAamp Viral RNA Mini Kit (Spin protocol) and reverse transcribed to cDNA. This was used for the PCR amplification of a 260-bp variable sequence in the 5′ non-coding region of the RV genome using in-house designed primers. PCR products were then sequenced commercially by the Australian Genome Research Facility. Genotypes were assigned based on comparisons of the 5' non-coding region sequences with those of 101 classical serotypes as well as 52 newly identified genotypes using 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.(34, 35)

HIV-specific procedures

Recruited study children were referred for HIV counselling and testing, which required, for study purposes, an additional parental consent. HIV-1 serodiagnosis was performed using a sequential testing algorithm with two rapid HIV-1 antibody tests (Determine® and Unigold®). HIV-infection was confirmed when necessary by an HIV-1 DNA Amplicor test (version 1.5, Roche Molecular Systems, Inc., Branchburg, NJ). Children identified as HIV positive were followed-up according to national guidelines.

Statistical Analyses

Demographic and clinical features (categorical variables) associated with viral identification were examined using Chi-squared (x^2) or Fisher's exact tests. Continuous variables were analysed using variance (ANOVA) models (adjusting for age) and presented as means with

standard deviation. Variables that were not normally distributed were logarithm-transformed and presented as means. Statistical analyses were performed using SPSS version 22.0 (SPSS Inc., Chicago, ILL, USA) and a p-value <0.05 was considered statistically significant.

Results

Population Demographics

Two hundred and seventy-seven cases (51.6% male) enrolled between September 2010 and April 2013 were included in this analysis. The mean age of the study population was 20.7 months. A total of 38 (13.7%) children were HIV-infected. HIV- infected children were older (mean age 27.8 months, 95% CI: 19.4-36.2) than HIV-uninfected children (mean age 19.5 months, 95% CI: 17.0-22.0; p=0.021). There was no difference in gender between HIV-infected and HIV-uninfected children. Of the 277 cases with WHO-defined clinical pneumonia, 31 (11.2%) had a co-infection with malaria. 22/277 (7.9%) cases had a positive blood culture, with pneumococcus, *Haemophilus influenzae type b* and Non-typhoidal Salmonella being the three major causes of invasive bacterial disease. Chest X-rays showed alveolar condensations compatible with bacterial pneumonia (WHO "primary end-point pneumonia").(36)

Respiratory Viral Identification

NPAs from 277 children were available for identification of respiratory viruses. At least one respiratory virus was identified in 206 (74.4%) children. Of the 206 children with at least one respiratory virus identified, 139 (67.5%) had a single virus infection while co-infection of 2, 3 or 4 viruses was identified in 51 (24.6%), 13 (6.3%) and 3 (1.5%) children respectively. RV was the most common respiratory virus, identified in 92 (33.2%) children, followed by adenovirus (19.1%) and RSV (15.5%; Figure 1). RSV-positive children (mean age 8.9

months, SD 3.0) were younger than RSV-negative children (mean age 13.4 months, SD 2.9 p=0.022). Adenovirus-positive children (mean age 18.6 months, SD 2.6) were older than adenovirus-negative children (mean age 11.5 months, SD 3.0). There were no other age or gender differences between children with and without any particular virus. Adjusting for age effects, there were no significant differences in the frequency of any viral pathogen between HIV-infected and HIV-uninfected children (Figure 1).

Clinical features in children with each respiratory virus identified are shown in Supplementary Table 1. There were no differences between demographic characteristics, past morbidities and co-morbidities or current hospitalization (Table 1), clinical features or laboratory and microbiology findings (Table 2) between RV-positive and RV-negative children. Of all respiratory viruses, RSV was positively associated with the most number of clinical features including wheeze, oxygen saturation, lower chest wall indrawing, nasal flare and deep breathing

RV Species and Genotypes

Ninety RV-positive NPAs were successfully genotyped, of which 47 (52.2%) were RV-A, 8(8.9%) were RV-B and 35(38.9%) were RV-C respectively. Of the 47 RV-A specimens, 45 were assigned to one of 24 known genotypes while two specimens could not be assigned as the sequences were equally related to two genotypes. Of the eight RV-B specimens, seven were assigned to one of four genotypes while one was not assigned. All 35 RV-C specimens were assigned to one of 16 genotypes. No single genotype was identified more than five times in this population, with only one RV-A genotype and one RV-C genotype being identified five times. There was no difference in the distribution of RV species between HIV-infected and HIV-uninfected children (p= 0.765).

There were no differences between demographic characteristics, past morbidities and co-morbidities or current hospitalization (Table 3), clinical features or laboratory and microbiology findings (Table 4) between RV-A and RV-C. RV-B was excluded from analyses due to low numbers.

RV Co-Infections with other Respiratory Viruses

Of the 92 RV- positive children, 35 (38%) had co-infections with one other respiratory virus, 11 had co-infections with two other respiratory viruses and three had co-infections with three other respiratory viruses. The most common RV co-infections were with adenovirus (21.7%) and RSV (15.2%). There were no differences in clinical features between children with a single virus identification compared with children who had multiple viruses identified. RV-C infected children were more likely to have co-infection with metapneumovirus than RV-A or RV-B infected children (20.0% in RV-C vs. 4.3% in RV-A and 0% in RV-B; ($p=0.039$)). There were no other differences in RV species and viral co-infections (data not shown).

Seasonality

One hundred and eighty four (66.4%) children were recruited during the warm and wet season (November to April) and 93 (33.6%) during the cool and dry season (May to October). The monthly distribution of RV species in comparison to RSV is shown in Figure 2. Overall, RV showed seasonal variation ($p=0.023$) and was less frequent from June to August (with the exception of May). RSV showed strong seasonality ($p<0.001$), being most prevalent between January and May with 70% of all RSV identification occurring during February and March. When we classified the months into two seasons; children were more likely to be RSV-positive during the warm and wet than cool and dry season (20.7% vs.

5.4%; p<0.01) and more likely to be RV-C positive (18.5% vs. 9.8%, p=0.042) and enterovirus-positive (9.7% vs. 1.1%, p<0.01) during the cool than the warm season. There were no other significant seasonal or monthly patterns for any other respiratory virus.

Discussion

Consistent with previous reports from Mozambique, RV was the most common respiratory virus identified in Mozambican children from Manhiça with clinical pneumonia.(14, 15) RV-A was the most commonly identified RV species followed by RV-C and RV-B. Only three studies have investigated RV species in children with respiratory illness in Africa. Consistent with our findings, two studies of children with ALRI from Kenya and Burundi reported that RV-A was the most common species identified, followed by RV-C and RV-B.(17, 18) In contrast, a study from South Africa investigated acute wheezing illness in young children(16) and reported RV-C as the most common RV species. Other studies of children hospitalized with asthma or wheezing, predominantly from developed countries, have also found RV-C to be the most frequently identified species.(10-12, 37) Several RV genotypes from each species were identified suggesting that a large number of genotypes are circulating in the community and that no single RV genotype predominates at any given time. Hence, our findings support the majority of African studies that report RV-A to be more common than RV-C in children with ALRI.

A respiratory virus was identified in almost three quarters of children hospitalized with clinical pneumonia, which is higher than previous results from Mozambique(14, 15) but comparable with results from other similar settings.(38, 39) We also identified higher prevalence of viral co-infections (24.2%) than the previous reports from Mozambique which may be due to screening with a larger panel of respiratory viruses as well as more sensitive

molecular techniques.(14, 15) However, comparable rates have been reported in paediatric populations outside Mozambique.(40) Given the high rate of viral identifications in children hospitalized with ALRI, our findings support current literature on the importance of respiratory viruses in the pathogenesis of ALRI. However, like the majority of viral aetiology investigations, our study was limited by the use of NPAs to identify respiratory viruses, which may not be entirely representative of respiratory viruses in the lower airway. Further investigations using lower airway samples as well as the inclusion of a contemporaneous control group may facilitate better understanding of the role of viruses in clinical pneumonia.

This is the first study to describe the prevalence of RV species among HIV-infected and HIV-uninfected children in Mozambique with WHO-defined clinical pneumonia. Thirty eight (13.7%) cases were HIV-infected, of which 26 (68.4%) had at least one respiratory virus. Contrary to our hypothesis, respiratory virus (including RV species) identification was not more common among HIV-infected than HIV-uninfected children. However, previous studies from Africa have reported increased viral identification rates among HIV-infected children.(15, 41) Madhi *et al* reported that viral identification among HIV-infected children varied according to respiratory virus and was lowest for RSV.(41) There are limited viral data on HIV-infected children with ALRI and, like ours, most of these studies were limited to relatively small sample sizes and were unable to draw conclusions about the role of HIV. Further investigations including a larger HIV-positive cohort are needed.

Overall, we did not find RV-A or RV-C to be associated with any particular clinical feature. Other studies have reported that overall, RV identification in children with ALRI was associated with unique clinical characteristics such as wheeze(42) and atopic dermatitis.(43) We also did not observe any differences in associated clinical features between the three RV

species. However, this could be due to the small numbers within each RV species, particularly in the RV-B group, as well as our study population representing a moderate to severe subset of all respiratory infections. In concordance with our findings, Luchsinger *et al* did not observe any differences between RV species according to clinical features or severity of illness in infants in Chile with ALRI.(44) We also investigated associations between respiratory viruses and clinical features. Of all other respiratory viruses, RSV was positively associated with the most number of clinical features including wheeze, oxygen saturation, lower chest wall indrawing, nasal flare and deep breathing. Though not significant, we observed that children identified only with RSV were slightly younger than those with RV (15.4 months vs. 22.6 months; p=0.120), which may partly explain differences in clinical severity observed. Similarly, other studies have also reported that RSV was associated with more severe disease than RV.(44, 45) However, these studies investigated children with bronchiolitis rather than pneumonia and may also be confounded by age.

Two-thirds of children were recruited during the warm and wet months. This is consistent with previous studies from Mozambique that reported an increase in the number of outpatient visits associated with malaria(46) and lower respiratory infection(47) during the rainy months of the year. Furthermore, O'Callaghan *et al s*howed that viral respiratory infections contributed to the high burden of hospital visits during these months.(14) RV was prevalent throughout the year, with no significant differences between the seasons. Although we observed slightly lower RV prevalence during the cool and dry season, particularly from June to August, this was not significant and possibly due to population size. Our findings are supported by previous investigations of RV species in Africa, which found no seasonality patterns for RV or RV species identification.(3, 17) In contrast to RV, RSV did show clear seasonal patterns and was most prevalent during the warm and wet season. This finding has

been supported by previous studies from Mozambique(48) and Ghana(3) that reported RSV epidemics during the rainy season. In contrast, other studies from Burkina Faso,(49) Senegal(50) and South Africa(48) found RSV infections peaked in the dry season.

The main strengths of this study are the long recruitment period (over two and a half years) and the inclusion of both HIV-infected and HIV-uninfected children. This study also has a few limitations. Firstly, we identified respiratory viruses in NPAs. Although nasopharyngeal identification of viruses has been associated with lower respiratory tract infections, it also occurs among healthy, asymptomatic individuals. Since the mechanisms that lead to lower respiratory infection remain poorly understood, viral identification in the upper airway may not be entirely representative of that of the lower airway. Furthermore, since we are unable to differentiate asymptomatic infection from clinical (symptomatic) infection using molecular methods of detection, a virus-positive NPA suggests but does not prove causation.

Secondly, our study population is comprised of a group of moderate-severe ALRI cases admitted to a hospital and fulfilling a strict pre-defined set of clinical criteria for pneumonia as defined by WHO guidelines. Hence, our findings are not necessarily representative of the overall ALRI population and do not include children with isolated symptoms such as wheeze. Thirdly, our study did not include a contemporaneous control group to compare the prevalence of respiratory viruses between sick and healthy children. A useful control group may include children from the community without respiratory illness as well as children with an upper respiratory illness not severe enough to present to hospital. Nonetheless, this study provides important data on the prevalence of RV species in children with WHO-defined clinical pneumonia in Mozambique.

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