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TITLE:
Clinical Epidemiology of Bocavirus, Rhinovirus, Two Polyomaviruses and Four Coronaviruses in HIV-Infected and HIV-Uninfected South African Children

ABSTRACT:
BACKGROUND: Advances in molecular diagnostics have implicated newly-discovered respiratory viruses in the pathogenesis of pneumonia. We aimed to determine the prevalence and clinical characteristics of human bocavirus (hBoV), human rhinovirus (hRV), polyomavirus-WU (WUPyV) and –KI (KIPyV) and human coronaviruses (CoV)-OC43, -NL63, -HKU1 and -229E among children hospitalized with lower respiratory tract infections (LRTI). METHODS: Multiplex real-time reverse-transcriptase polymerase chain reaction was undertaken on archived nasopharyngeal aspirates from HIV-infected and –uninfected children (<2 years age) hospitalized for LRTI, who had been previously investigated for respiratory syncytial virus, human metapneumovirus, parainfluenza I–III, adenovirus and influenza A/B. RESULTS: At least one of these viruses were identified in 274 (53.0%) of 517 and in 509 (54.0%) of 943 LRTI-episodes in HIV-infected and -uninfected children, respectively. Human rhinovirus was the most prevalent in HIV-infected (31.7%) and –uninfected children (32.0%), followed by CoV-OC43 (12.2%) and hBoV (9.5%) in HIV-infected; and by hBoV (13.3%) and WUPyV (11.9%) in HIV-uninfected children. Polyomavirus-KI (8.9% vs. 4.8%; p = 0.002) and CoV-OC43 (12.2% vs. 3.6%; p<0.001) were more prevalent in HIV-infected than –uninfected children. Combined with previously-tested viruses, respiratory viruses were identified in 60.9% of HIV-infected and 78.3% of HIV-uninfected children. The newly tested viruses were detected at high frequency in association with other respiratory viruses, including previously-investigated viruses (22.8% in HIV-infected and 28.5% in HIV–uninfected children).
CONCLUSIONS: We established that combined with previously-investigated viruses, at least one respiratory virus was identified in the majority of HIV-infected and HIV-uninfected children hospitalized for LRTI. The high frequency of viral co-infections illustrates the complexities in attributing causality to specific viruses in the aetiology of LRTI and may indicate a synergetic role of viral co-infections in the pathogenesis of childhood LRTI.

Introduction:
Pneumonia is a leading cause of mortality in children under 5 years age worldwide, including in HIV-infected children [1]–[3]. The aetiology of childhood pneumonia may include infection with bacteria and/or respiratory viruses. Although respiratory viruses are more frequently identified than bacteria in children with pneumonia, this may be biased by lack of availability of sensitive and specific tests for diagnosing bacterial causes of pneumonia [4]. Furthermore, respiratory viral infections may heighten the susceptibility to developing a super-imposed bacterial infection resulting in severe pneumonia [5], [6]. Traditionally, respiratory viruses that have been associated with lower respiratory tract infections (LRTI) include respiratory syncytial virus (RSV), parainfluenza viruses I–III (PIV I–III), influenza viruses A/B and adenovirus. Two human coronaviruses (CoV), OC43 (CoV-OC43) and 229E (CoV-229E) were initially identified as causes of upper respiratory tract infections (URTI) in the 1960s using classical culture methods [7], [8]. More recently, advances in molecular diagnostics have resulted in the discovery of other respiratory viruses which have also been associated with LRTI. Included among these are human metapneumovirus (hMPV) [9], human bocavirus (hBoV) [10], human coronavirus NL63 (CoV-NL63) [11] and HKU1 (CoV-HKU1) [12] and WU and KI polyomaviruses (WUPyV, KIPyV) [13]–[15]. Also, human rhinovirus (hRV), which was previously mainly associated with mild URTI, has increasingly been implicated in having a role in the pathogenesis of LRTI and asthma exacerbations [16], [17].
Due to impaired humoral and cell-mediated immunity, HIV infection in children has been described as a risk factor for severe illness and mortality caused by respiratory-viral associated LRTI, such as RSV, hMPV and influenza virus [18], [19]. There are, however, limited data on the role of other respiratory viruses, including the more recently-discovered viruses which occur as single or co-infecting pathogens in HIV-infected children hospitalized with LRTI, and of these studies, most have small sample sizes [20]–[22].
The aim of this study was to identify the prevalence of hBoV, hRV, WUPyV, KIPyV, CoV-OC43, CoV-NL63, CoV-HKU1 and CoV-229E among HIV-infected and -uninfected children who were hospitalized for LRTI using real-time reverse transcriptase–polymerase chain reaction (RT-PCR). The study-cohort had been previously investigated for RSV, influenza A/B, PIV I-III and adenovirus by immunofluorescence assay and hMPV by nested-PCR as described [5], [23].

Ethics Statement ::: Methods:
The main 9-valent pneumococcal conjugated vaccine (PCV9) efficacy trial and subsequent retrospective analysis of study participants were approved by the Human Research Ethics Committee (Medical) of the University of the Witwatersrand. The main study did not have a clinical trials number since it was undertaken prior to registration of clinical trials having been made mandatory. Signed informed consent was obtained from the parent/legal guardians of all the study participants as part of the main trial. The Ethics Committee did not require additional consent for this study.

Study population ::: Methods:
This was a retrospective study of children who participated in a phase III trial in South Africa which investigated the efficacy of a PCV9 as described [5], [24]. Briefly, 39836 children recruited from March 1998 to October 2000 were randomized (1:1) to receive 3 doses of either PCV9 or placebo. Vaccination occurred at a mean of 6.6±1.2 (± standard deviation), 11.2±2.5, and 15.9±3.9 weeks of age [24]. Hospital-based surveillance for all-cause hospitalization was undertaken at Chris Hani-Baragwanath Hospital, the only public hospital in the study community. Hospitalized children had their signs and symptoms recorded and underwent HIV testing according to the study protocol [24]. Nasopharyngeal aspirates (NPA) were obtained for respiratory viral studies from children hospitalized with LRTI [5], and archived from January 2000 onward. In the present study only NPA collected from 1st February 2000 to 31st January 2002 from children less than 2 years old were analysed. If a child had recurrent LRTI hospitalizations, only NPA collected at least 28 days apart were included in the analysis. Blood cultures were performed with the use of an automated blood-culture system (BacT-Alert, Organon Teknika).

Viral testing ::: Methods:
Total nucleic acids were extracted from archived NPA using a NucliSENS easyMAG platform (bioMerieux), and eluted in a final volume of 60 µl of elution buffer [25]. RNA was reverse transcribed with High Capacity cDNA Reverse Transcriptase (Invitrogen, Life Technologies) and primed with oligo-dT primers (Invitrogen, Life Technologies). Real-time PCR was done in an ABI 7500 RT-PCR system (Applied Biosystems, Life Technologies), reactions were performed in 20 µl using TaqMan Universal PCR Master Mix (Applied Biosystems, Life Technologies) and the primers and probes listed in Table S1.
Five duplex RT-PCR reactions, targeting the 8 respiratory viruses, were developed. Internal controls (the human genes: ribonucleoprotein and glyceraldehyde-3-phosphate dehydrogenase; or the spiked viruses: lambda and Newcastle Disease Virus) were included to check the efficiency of the extraction step and to detect the presence of PCR inhibitors. Positive controls were included in each experiment.

Study-specific definitions ::: Methods:
The clinical definitions used in this study are the same as previously described [5]. Briefly, LRTI was defined as any episode with clinical diagnosis of pneumonia or bronchiolitis done by a study physician. Children with LRTI were categorized as having clinical pneumonia if they had evidence of alveolar consolidation on chest x-ray (CXR-AC) or if they fulfilled the clinical diagnosis of LRTI without wheeze on chest auscultation but had rales and/or bronchial breathing. Children were categorized as having bronchiolitis in the presence of wheezing on chest auscultation and in the absence of documented CXR-AC or bronchial breathing on chest wall auscultation. A clinical diagnosis of WHO severe/very severe pneumonia was made if the child had a cough <14 days in duration and lower chest wall in-drawing and/or any of the following signs and symptoms of severe pneumonia: feeding difficulties, convulsions, central cyanosis, or encephalopathy. The previously developed respiratory index of severity in children (RISC) score was used to compare disease severity between single and multiple viral infections [26].

Statistical analysis ::: Methods:
Demographic, clinical and laboratory characteristics at admission were compared between HIV-infected and –uninfected children, chi-square or Fisher's exact tests were used to compare the distribution of categorical variables and mean or median of continuous variables were compared by two-tailed Student t-test or Mann-Whitney test, respectively. Viral prevalence was compared between HIV groups and clinical and laboratory characteristics were compared between episodes with single and multiple viral detection using multivariate logistic regression models adjusted for

study intervention arm (i.e. whether received PCV9 or placebo), year of sampling, detection of a virus previously-tested and age. The results are presented as adjusted odds ratios (aOR) with 95% confidence intervals (95% CI) and p-values. A p-value<0.05 was considered significant. Analyses were performed using STATA version 11.0 (Statacorp, Texas, USA).

Study population ::: Results:
During the follow-up period included in this analysis, there were a total of 2147 hospitalizations for LRTI, including 2094 (97.5%) in which NPA had been collected. Of the initial collected samples 69.7% were available for RT–PCR analysis. The same proportion of samples were available for RT–PCR from PCV9-recipients (69.2%) and placebo-recipients (70.2%; p = 0.621), from HIV-infected (78.2% vs. 73.1%; p = 0.130) and HIV-uninfected children (65.5% vs. 68.6%; p = 0.218); Table 1. A lower proportion of samples was, however, available for RT-PCR analysis (65.9% [1000/1518]) from the first period (February 2000–January 2001) compared to the second period (February 2001–January 2002) (79.9% [460/576]; p<0.001) of the study.
Children from whom NPA were available for RT–PCR testing compared to those in whom samples were unavailable were older (median age: 10 vs. 8 months; p<0.001), were 1.3-fold less likely to have tested positive for one of the previously-tested respiratory viruses (33.3% vs. 42.3%; p<0.001), had a higher prevalence of cyanosis (11.4% vs. 8.1%; p = 0.025), higher evidence of CXR-AC (26.6% vs. 22.1%; p = 0.041), higher C-reactive protein (CRP) levels (median: 15 vs. 12 mg/l; p = 0.003) and higher procalcitonin (PCT) concentration (median: 0.26 vs. 0.15 ng/ml; p = 0.006); Table S2. There were no other demographic, clinical or laboratory differences observed between the LRTI-episodes with NPA available versus unavailable for RT-PCR.
A total of 517 NPA samples from HIV-infected children were analysed by RT–PCR, including 45.0% from PCV9-recipients and 55.0% from placebo-recipients. Among HIV-uninfected children, 943 specimens were available for RT-PCR analysis, including 49.5% from PCV9-recipients and 50.5% from placebo-recipients. On admission HIV-infected children compared to HIV-uninfected were younger (median age: 9 vs. 11 months; p<0.001), more frequently presented with cyanosis (23.8% vs. 4.6%; p<0.001), had lower mean oxygen saturation (89.8% vs. 92.2%; p<0.001), had a higher median respiratory rate (54 vs. 48 breaths per minute; p<0.001), were more likely to present as pneumonia (88.8% vs. 49.6%; p<0.001) than bronchiolitis, had higher CRP (18 vs. 14 mg/l; p = 0.007) and PCT levels (0.47 vs. 0.17 ng/ml; p<0.001), had a longer hospital stay (4 vs. 1 median days; p<0.001), had a higher case-fatality rate (17.8% vs. 0.95%; p<0.001) and more frequently had bacteraemia (7.8% vs. 2.7; p<0.001); Table 2.
Of the 1460 specimens analysed, all but 13 (1 from HIV-infected and 12 from HIV-uninfected children) were previously tested for hMPV by nested-PCR [23] and 1458 were tested by an immunofluorescence assay for RSV, which if found negative, were further tested for influenza A/B, PIV I–III and adenovirus as described [5].

Prevalence of newly-tested respiratory viruses ::: Results:
Among HIV-infected children, the RT-PCR viral panel was positive in 274 (53.0%) LRTI-episodes for at least one of the newly-tested viruses; Table 3. The prevalence of any of the newly-tested respiratory viruses was similar between PCV9- and placebo-recipients in HIV-infected children, except for WUPyV (11.2% vs. 6.3%; p = 0.047, respectively) and hBoV (12.5% vs. 7.0%; p = 0.034, respectively); Table S3. In HIV-infected children hRV was the most frequently detected virus (31.7%) followed by CoV-OC43 (12.2%), hBoV (9.5%), KIPyV (8.9%), WUPyV (8.5), CoV-NL63 (1.7%) and CoV-HKU1 (1.4%); Table 3. The newly-tested viruses were frequently identified as co-infecting viruses among HIV-infected children, including 49.4% of LRTI-episodes associated with hRV; Table 4. The most common viral co-infections with hRV included KIPyV (14.6%), WUPyV (11.6%), CoV-OC43 and hBoV (11.0%, each); Table 4. Among the 486 children on whom blood culture was done, bacteria were isolated on 38 (7.8%) occasions, 20 (52.6%) of which were associated with concomitant detection of one of the newly-tested viruses and 22 (57.9%) of any of the viruses.
In HIV-uninfected children at least one newly-tested virus was detected in 509 (54.0%) LRTI-episodes, with hRV also being the most common (32.0%), followed by hBoV (13.3%), WUPyV (11.9%), KIPyV (4.8%), CoV-OC43 (3.6%), CoV-NL63 (2.6%), CoV-HKU1 (1.6%) and CoV-229E (0.42%); Table 3. Comparing HIV-uninfected PCV9 and placebo recipients, differences in the prevalence of newly-tested viruses were evident for KIPyV (2.1% vs. 7.4%; p<0.001), CoV-HKU1 (0.21% vs. 2.9%; p = 0.001), CoV-OC43 (2.1% vs. 5.0%; p = 0.017) and hRV (36.2% vs. 27.9%; p = 0.007); Table S3. Of the 302 LRTI-episodes in which hRV was identified, 51.3% had at least one other virus detected, including 13.6% with RSV, 12.3% with WUPyV or hBoV and 2.0% (N = 6)

with both WUPyV and hBoV; Table 5. The prevalence of bacteraemia in HIV-uninfected children among those with blood culture results was 2.7% (N = 24/881) of which 12 (50.0%) occurred in the presence of infection with one of the newly-tested viruses and 15 (62.5%) in presence of any of the studied viruses.
By multivariate analysis, adjusting for PCV-vaccination status, period of collection and age, single infections with a newly-tested virus were more frequent in HIV-infected (30.2%) than HIV-uninfected children (25.5%) (adjusted odds ratio [aOR] 1.3; p = 0.033); Table 3. Also, HIV-infected compared to HIV-uninfected children had a higher prevalence of KIPyV (aOR 2.14; p = 0.002) and CoV-OC43 (aOR 3.67; p<0.001) and a lower prevalence of hBoV (aOR 0.69; p = 0.043) and WUPyV (aOR 0.66; p = 0.035); Table 3. Concurrent bacteraemia and infection with at least one of the newly-tested viruses was more frequent in HIV-infected (7.7%) compared to HIV-uninfected children (2.5%, aOR 3.49; p = 0.001). There were no differences in the frequency of bacteraemia comparing children in whom newly-tested viruses were detected and those without viral detection both in HIV-infected (7.7% vs. 8.0%; p = 0.911) and HIV-uninfected children (2.5% vs. 2.9%; p = 0.713).

Respiratory viruses and clinical manifestations ::: Results:
When compared to LRTI-episodes associated with the identification of only a single virus the detection of multiple viruses in HIV-infected children was significantly associated with a higher frequency of bronchial breathing (aOR 2.11; p = 0.015) and in HIV-uninfected children with a higher prevalence of cyanosis (aOR 2.50; p = 0.008) and wheezing (aOR 1.55; p = 0.006); Table 6. No differences were detected in the severity of LRTI-episodes with single and multiple viral infections using the RISC score previously developed.
In both HIV-infected and –uninfected children with bacteria isolated from blood, there were no significant differences in the clinical and laboratory characteristics between children in whom viruses were detected and children without any virus detected. The same was observed restricting the analysis to Streptococcus pneumoniae isolation (data not shown).

Discussion:
To our knowledge, this study provides the most in-depth analysis of the prevalence of hRV and some of the newly-discovered respiratory viruses in HIV-infected children. Our study identified hRV to be the most frequently detected virus both in HIV-infected and –uninfected children followed by CoV-OC43 in HIV-infected and hBoV in HIV-uninfected. Most cases of hospitalizations associated with single infections overall were noted for hRV and RSV, the rate of co-infections was high in children infected with the other newly-discovered viruses.
Very few viral aetiology studies have been conducted in Africa: in a Mozambican study of virus-associated acute respiratory infections (ARI) in infants with an estimated 3–5% HIV prevalence, the most frequently detected viruses were hRV (26%), influenza (15%) and adenovirus (14%) [27]. A recent study from South Africa on children with ARI requiring medical attention, 61% (N = 383) being HIV-infected or HIV-seropositive, also reported that hRV was the most frequently detected virus (33%) followed by RSV (30%), PIV (8%) and hBoV (6%) [21]. In the South African study hospitalized children with respiratory disease had higher hRV detection rates compared to healthy children (36% vs. 19%; p = 0.047) which may indicate a causality effect of hRV in disease [21]. In Kenya in older children (5–17 years old), despite hRV being the most commonly detected virus (38%) in patients presenting with ARI, its detection rate was similar among asymptomatic controls [28]. Indeed two other studies in Kenya found that with the exception of RSV, viral detection in the nasopharynx did not have a significant association with pneumonia in hospitalized children under 5 years [29] or under 12 years, [30] compared to children both without symptoms of URTI or with respiratory symptoms but not meeting any criteria for pneumonia.
It is uncertain as to what the impact of HIV infection on the duration of shedding of these viruses might be. An analysis of serial respiratory samples from otherwise healthy children detected shedding of CoV-NL63 for up 21 days [31], hRV up to 41 days and of hBoV up to 44 days [32]. Detection of CoV-HKU1 and CoV-229E in respiratory specimens of transplanted children were also reported for at least 38 days and 11 weeks, respectively, possibly suggesting that immunocompromised children may have a prolonged duration of shedding of these viruses [33], [34]. Such prolonged shedding in immunocompromised individuals such as HIV-infected children, may result in a greater frequency of co-incidental identification of these viruses when investigated for respiratory illness, affect the seasonal occurrence of the viruses as well as potentially present a threat to greater nosocomial transmission of these viruses because of higher incidence of hospitalization of HIV-infected children.

The casual relationship between viral DNA detection, development of disease and mixed infections is very complex. In the case of hBoV serological studies have shown that the presence of viral DNA in respiratory samples was not proof of an acute primary infection and that prolonged viral shedding could be an explanation for viral detection at high rates in asymptomatic controls [35]. Enrolment of healthy non-hospitalized children and quantification of viral loads may help to elucidate the problem of co-infections and is currently being under-taken in a multi-centre study on aetiology of pneumonia in children [36]. Contradictory findings on whether infection with multiple viruses contribute to disease severity in hospitalized children have been reported, with some studies reporting an increased severity among children with viral co-infections [21], [37] and others finding decrease severity associated with co-infections [38]. In some studies no differences were observed [39]. We found that multiple viral infections were associated with bronchial breathing in HIV-infected children and with cyanosis and wheezing in HIV-uninfected children. Bacterial co-infections can also increase the severity of viral diseases [40]. Furthermore, in children with invasive pneumococcal disease, superimposed viral co-infections are common and may lead to higher mortality rates [41]. Of the participants in our study from whom bacterial cultures performed, no differences in clinical and laboratory characteristics were observed between children with or without viral infections. Our analysis, however, included PCV9 vaccinees who probably present with less severe LRTI cases since it was shown before that PCV9 vaccination was associated with a decrease in LRTI viral-associated hospitalizations [5], [23]. We detected a high prevalence of bacterial co-infections with the newly-tested viruses especially in HIV-infected children (8%), what may have contributed to the higher C-reactive protein and procalcitonin levels, the longer hospital stay and the higher mortality in HIV-infected children compared to HIV-uninfected.

Our high co-infection detection rates are similar to other molecular detection studies of paediatric respiratory samples, with coronaviruses (40–75%) [42], [43], hBoV (up to 78%) [13], [44], [45] and polyomaviruses (68–79%) [46]–[48]. Our results may, however, have under-estimated the true prevalence of viral co-infections since the previously-studied viruses were tested by immunofluorescence [5] or conventional PCR [23]. Although the sensitivity of the conventional methods is reported to be high, comparative studies have shown that RT-PCR is considerably more sensitive for detection of respiratory viruses [21], [49]. Moreover the specimens tested by immunofluorescence were initially screened for RSV and only if negative were further tested for influenza A/B, PIV I–III and adenovirus [5].

Limitations of our study include that this was a post-hoc analysis and only 70% of specimens collected during the study period were available for further testing [24]. Compared with the specimens available for RT-PCR the specimens unavailable were collected from younger children and were more likely to have tested positive for a previously-studied virus. This selection bias may have also contributed to an under-estimation of the number of co-infections. Along the same line RSV-associated LRTI are more common among younger children [21], [39], for whom we had less available samples increasing our uncertainty on the prevalence of RSV co-infections with the newly-tested viruses.

An equal proportion of PCV9- and placebo-recipients hospitalized with LRTI were investigated in this study and our analyses were adjusted for the initial intervention arm. Further detailed analyses on the effect of PCV9 on the incidence of hospitalization associated with the individual newly-tested viruses will be reported on in future.

Although the development of new diagnostic techniques allows more detailed investigation and has led to the discovery of a number of new putative respiratory pathogens, the role for some of these potential pathogens in respiratory illness remains speculative in the absence of fulfilling Koch's postulates for causality. In the present study we demonstrate that at least one respiratory virus was identified in the majority of HIV-infected and HIV-uninfected children hospitalized for LRTI. The difficulty in attributing disease causality to specific viruses due to the high frequency of viral co-infection suggests a possible synergy among different pathogens during childhood LRTI.