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Journal of Clinical Virology 125 (2020) 104288

Contents lists available at ScienceDirect

Journal of Clinical Virology

journal homepage: www.elsevier.com/locate/jcv

A prospective case-control study on the association of Rhinovirus nasopharyngeal viral load and viremia in South African children hospitalized with severe pneumonia

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ARTICLE INFO

Keywords:
Rhinovirus
Molecular epidemiology
Viral load
Viremia
Pneumonia
Children

ABSTRACT

Rhinovirus (RV) role in pathogenesis of severe childhood disease remains controversial. We aimed to explore the association between RV molecular subtyping, nasopharyngeal viral loads and viremia with childhood pneumonia. Nasopharyngeal and blood samples from cases and controls were tested for RV and the 5′ non-coding region sequenced. The cases compared to controls had a similar prevalence of RV detection in the nasopharynx (23 % vs. 22 %, $P = 0.66$), similar RV species distribution (A, B, C = 44 %, 8%, 44 % vs. 48 %, 7%, 38 %; respectively; $P = 0.66$) and similar viral load (4.0 and 3.7 log10 copies/mL, $P = 0.062$). However, RV-viremia was 4.01-fold (aOR 95 % CI: 1.26–12.78) more prevalent among cases (7%) than controls (2%), $P = 0.019$. Furthermore, among cases and controls RV-C was more commonly associated with viremia (14 % and 4%, $P = 0.023$), than RV-A (2% and 1%; $P = 0.529$). Thus RV-viremia could be used as a measure for attributing causality to RV in children hospitalized for pneumonia.

1. Introduction

Rhinovirus (RV) has been widely associated with the common cold; however, RV has also been detected in children with severe respiratory disease [1–7]. However attributing causality of illness to RV is complicated since it is also routinely detected in asymptomatic individuals [8].

RV's are single stranded positive-sense non-enveloped ribonucleic acid viruses with ≈ 7.2 kb genome. Presently more than 100 types have been classified into 3 species - RV-A: 74 known types, RV-B: 25 types, and most recently classified RV-C with > 50 types [6,9–11]. The vast diversity of RV types may account for the differences in clinical phenotypes between sick and asymptomatic individuals [12]. Further, studies on children with respiratory tract infection (RTI) have investigated the association of RV viral load and presence of RV-viremia as markers of disease severity [13,14]. These studies, however, did not enroll asymptomatic controls, which limited any inferences on the association of RV-viremia to disease status or as a marker of disease severity.

We hypothesize that RV-viremia could provide evidence for the etiological role of RV in severe lower respiratory tract infection in infants and children.

2. Materials and methods

2.1. Case and control definitions

This study was undertaken in Soweto, South Africa, from August 2011-August 2013. Details on enrolment of cases and controls in the Pneumonia Etiology Research for Child Health (PERCH) study have been described elsewhere in great detail [15,16]. Briefly, pneumonia cases were children aged 1–59 months hospitalized with World Health Organization (WHO)-defined pneumonia (according to the pre-2013 definitions) [17,18]. Prior to enrolment, cases received a bronchodilator challenge in order to exclude cases with hyper-reactive airway. Controls were enrolled from the same community as cases and were matched 1:1 to cases on age-group frequency and HIV-status. The community controls

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https://doi.org/10.1016/j.jcv.2020.104288
Received 27 November 2019; Received in revised form 30 January 2020; Accepted 10 February 2020

were invited to present at the day-clinic for sample collection and clinical evaluation. The controls could have signs and symptoms of respiratory illness as long as they did not fulfil the criteria for severe pneumonia.

2.2. Specimen collection and laboratory testing

Upon enrolment, flocked nasopharyngeal (NP) swab (Flexible minitip, Copan®, USA), rayon oropharyngeal (OP) swab specimens and blood specimens were collected from cases and controls. The swabs were placed in the same vial containing 3 mL of Universal Transport Media (Copan®) and kept at 4 − 8 °C for a maximum of 24 h, and then archived at −70 °C until tested. Total nucleic acids (NA) were extracted from the swabs using the NucliSens EasyMag extraction system as per manufactures' instructions (bioMérieux, Marcy l'Etoile, France) and were tested by multiplex PCR for evidence of 33 pathogens including RV (FTD-Resp33, Fast-track Diagnostics, Sliema, Malta) [19]. Standard curves were used to calculate pathogen load from PCR cycle threshold values.

Other investigations included blood culture on cases using the BacT/Alert microbial system(Organon Teknika, Durham, NC). Further, induced sputum samples were collected for all cases and tested on the FTD-Resp33 kit; however, the results did not add any additional information beyond that of the NP/OP specimens (substantial *kappa* concordance = 0.65; $P < 0.001$); thus they were not included in this analysis nor the aetiology analysis of the entire PERCH study [20]. The NP/OP specimens also had the analytical advantage of being available for both cases and controls.

2.3. Determination of RV molecular subtyping

All RV positive NP/OP samples were analyzed using single round PCR assays targeting a 390bp area in the 5′ non-coding region of the RV genome as previously described [21,22]. The primer sequences were DK001 forward (5′- CAAGCACTTCTGTTTCC - 3′) and reverse primer DK004 (5′ – CACGGACACCCAAAGTAGT – 3′) using Promega Access RT according to manufacturers' instructions (Promega, Belgium). PCR amplicons were sequenced bidirectionally using the BigDye Terminator V3.1 sequencing kit (Applied BioSystem, Foster City, CA) using the same primers.

The sequences were analyzed and aligned using the ClustalW algorithm implemented in Geneious 4.7.6 [23], and the resultant consensus sequence was compared with reference RV sequences using the nucleotide-nucleotide BLAST algorithm (http://www.ncbi.nlm.nih.gov) from GenBank in order to identify the RV species (A, B and C). Phylogenetic trees were constructed using neighbor-joining methods using Kimura's 2-parameter technique with bootstrap values estimated with 1000 bootstrap replications [24] with evolutionary analysis conducted in MEGA-6 [25].

2.4. Detection of RV-viremia

The blood samples of all cases and controls testing positive for RV in their NP/OP samples were tested for the presence of RV-viremia using the same primers (DK001 and DK004) and methods as those used for determining the RV molecular subtyping in the respiratory samples. These primers have been validated in previous studies of RV-viremia, and were found to be highly sensitive in blood samples [22]. In addition, blood from 70 cases and 70 controls negative for RV in NP/OP samples, were tested for the presence of RV-viremia. Total NA were extracted from archived blood samples using the NucliSens EasyMag robot(bioMérieux, France) using the blood specific extraction protocol as per manufacturers' instructions.

2.5. Statistical analysis

PCR quantifications were $\log_{10}$ transformed. Chi-squared and Wilcoxon tests were used to analyze the demographic characteristics of cases and controls. Binary, multinomial logistic regression and odds ratio analyses were used to model the prevalence of RV in the study population. We initially performed a univariate analysis that included age categories, sex, HIV infection and exposure, socio-economic status, severity of pneumonia diagnosis, presence of fever and hypoxia, chest radiographic findings and case fatality ratios as independent variables. Additionally, we also performed univariate analysis for markers of likely bacterial co-infection, presence of different respiratory viruses detected by the FTD33 assay and RV-species. Independent variables identified with a P-value < 0.2 in the univariate analysis were included in the multivariate models. All statistical analyses and reverse cumulative plots were performed using STATA Version 12.1(College Station, TX, USA) and a two-sided P-value < 0.05 was considered statistically significant. The sequences for all RV-positive samples have been deposited in GenBank(MK858576-MK858936).

3. Results

3.1. RV subtyping among cases and controls

A total of 920 cases and 964 controls were enrolled, of whom 23 % (n = 210) and 22 % (n = 212) respectively($P = 0.66$) had RV identified on NP/OP samples by PCR. Further, cases and controls with RV infection were similar with regard to median age, HIV-positivity, and RV load; furthermore, there was no discernible NP/OP density threshold for differentiating RV-positive cases from controls on reverse cumulative plot analyses. RV-positive cases compared to RV-positive controls were however more likely to be male (55 % vs. 46 %, adjusted $P = 0.027$) and malnourished (8% vs. 2%, adjusted $P = 0.019$); Table 1. Additionally, the RV-associated cases were 1.95-fold (aOR 95 %CI: 1.28–2.97) more likely to have any respiratory virus co-infection compared to controls (45 % vs. 31 %, $P = 0.001$), specifically with RSV (16 % vs. 2%, adjusted $P < 0.001$); Table 1.

The 5′ NCR of the RV genome was successfully amplified in 99 % (n = 207) and 96 % (n = 213) of case and control NP/OP samples, respectively. The samples that failed to amplify had late cycle threshold values > 35 during FTD analysis, indicating a low density of RV. Furthermore, sequencing analysis established that 4% (n = 9) and 7% (n = 14; $P = 0.262$) of samples among cases and controls, respectively, were in fact enteroviruses and thus were excluded from further analysis. The proportional distribution of RV species did not differ between cases compared to controls; Table 1 and Fig. 1.

3.2. RV-viremia

Overall, 7% (n = 13) of cases and 2% (n = 4) of controls were identified as having RV-viremia (adjusted $P = 0.019$). Furthermore, RV-C viremia was 4.43-fold (aOR 95 %CI: 1.22–16.04) more prevalent among RV-C positive cases (12 %, n = 11) than RV-C positive controls (4%, n = 3), adjusted $P = 0.023$. The positivity rate of RV-viremia among cases differed between RV species, being highest for RV-C (12 %, n = 11), lower for RV-A (2%, n = 2; $P = 0.025$) and not identified for any of the RV-B cases (0%); Table 2. For cases and controls with viremia, the RV species detected in blood was identical to the respiratory species and RV-viremia was not detected in any of the cases or controls testing negative for RV in their NP/OP swabs.

The majority of the RV-viremia cases were more than 1 year of age (mean age of 14 months), with no viremia detected in cases < 6 months of age compared to a mean age of 6 months in the viremia negative RV-associated cases ($P = 0.001$). Further, RV-associated viremic cases were less likely to be hospitalized for ≥5 days compared to non-viremic cases (23 % vs. 62 %, $P = 0.006$). The presence of viremia among cases was not associated with any of the other features of more severe disease; Table 3.

3.3. Nasopharyngeal RV load

The NP/OP viral load in RV-associated pneumonia cases did not differ by any clinical or demographic characteristics, except the presence of fever (3.82 vs. 4.25 $\log_{10}$ copies/mL, adjusted $P = 0.009$).

Table 1
Demographics of RV-positive cases and RV-positive community controls.

Characteristics, n (%)	N	RV + cases (n = 210)	RV + controls (n = 212)	Unadjusted *P*-value	aOR (95 % CI)	Adjusted *P*-value
Age (month), median (IQR)	422	6.5 (3–14)	7 (4–13)	0.090		0.129
-1-6 months	172	93 (44)	79 (37)			
-7-12 months	131	58 (28)	73 (34)	0.240		0.227
> 12 months	119	59 (28)	60 (28)			
Male	422	115 (55)	98 (46)	0.080	**1.58 (1.05–2.38)**	**0.027**
HIV+	421	28 (13)	18 (8)	0.175	1.65 (0.86–3.15)	0.130
HEU[a]	421	67 (32)	52 (25)	0.155	1.40 (0.89–2.19)	0.148
Never breast fed	422	64 (30)	75 (35)	0.284	0.75 (0.49–1.16)	0.201
Under weight[b]	416	17 (8)	5 (2)	**0.008**	**3.58 (1.23–10.42)**	**0.019**
Day Care attendance	418	28 (13)	31 (15)	0.930	0.87 (0.48–1.60)	0.655
Smoker in household	421	81 (39)	66 (31)	0.157	1.39 (0.90–2.13)	0.134
Birth weight, Median (IQR)	405	3 (2.6–3.4)	3 (2.6–3.3)	0.694		0.769
RV genotyping:						
RV-A	188	91 (44)	97 (48)			
RV-B	30	16 (8)	14 (7)			
RV-C	169	91 (44)	78 (38)	0.499		0.661
Un-typeable	12	3 (1)	9 (4)			
Enterovirus	23	9 (4)	14 (7)			
RV load, mean (SD)[c]	422	4.0 (0.98)	3.7 (0.94)	0.060		0.062
Viral co-detections in the nasopharynx[d]**:**						
RV Mixed-infection[e]	422	100 (48)	66 (31)	**0.003**	**1.99 (1.32–3.01)**	**0.002**
RSV	422	33 (16)	5 (2)	**< 0.001**	**8.26 (3.07–22.10)**	**< 0.001**
AdV	422	22 (10)	24 (11)	0.781	1.11 (0.58–2.14)	0.738
HMPV	422	7 (3)	5 (2)	0.547	1.36 (0.42–4.47)	0.608
HBoV	422	31 (15)	24 (11)	0.294	1.50 (0.81–2.81)	0.195
Infl A-C	422	2 (1)	2 (1)	0.992	0.98 (0.13–7.13)	0.983
PIV	422	12 (6)	7 (3)	0.238	1.73 (0.66–4.52)	0.262
HCoV	422	15 (7)	16 (8)	0.874	0.96 (0.46–2.02)	0.920

Abbreviations – RV: Rhinovirus; n: number; OR: Odds ratio; aOR: Adjusted odds ratio; CI: Confidence interval; SD: Standard deviation; IQR: Interquartile range; HIV: Human immunodeficiency virus; HEU: HIV exposed uninfected; RSV: respiratory syncytial virus; AdV: adenovirus; HMPV: Human metapneumonia virus; HBoV: Human bocavirus; Infl: influenza virus; PIV: parainfluenza virus; HCoV: Human coronavirus. *P*-values from Chi-square and Wilcoxon tests - logistic regression models adjusted for confounding variates (< 0.2 in univariate analysis) where applicable; odds ratios could not be calculated for variables with zero observations. *All characteristics are expressed as n (%); unless otherwise stated in the table.

[a] HEU defined as HIV-uninfected although HIV-exposed *in utero* or postnatally. Undetectable viral load, HIV seronegative in the child with a positive maternal history of HIV infection. Positive maternal HIV status based on self-report was accepted, except for seronegative children < 7 months of age where documented positive maternal HIV status was required.

[b] Underweight defined as weight for age < -2SD of the mean age-sex specific WHO reference.

[c] RV load in the nasopharynx, expressed as log10 copies/mL.

[d] Any co-infection with other detected respiratory virus including cases with > 2 co-infecting viruses.

[e] RV was the only respiratory virus detected in the nasopharynx.

Similarly, RV load among community controls also did not differ by any clinical or demographic characteristics. RV load was, however, higher among controls with symptoms of RTI compared to asymptomatic controls (4.48 vs. 3.77 $\log_{10}$ copies/mL, $P = 0.041$); Table 4.

Regardless of the small number of viremic positives, among children with RV-associated pneumonia, the presence of viremia was associated with higher RV load compared to non-viremia cases (4.67 vs. 3.90 $\log_{10}$ copies/mL, $P = 0.028$). This difference was mainly driven by the RV-C species (4.72 vs. 3.87 $\log_{10}$ copies/mL, $P = 0.016$), whilst not evident for RV-A ($P = 0.765$). Similarly, among RV-associated controls, the viral load was higher in the presence of viremia compared to the non-viremic controls (4.83 vs. 3.79 $\log_{10}$ copies/mL, $P = 0.018$); Table 4.

Furthermore, there was a discernible NP/OP density threshold of $\geq 4\log_{10}$ copies/mL for differentiating RV-viremia participants from viremia negative participants on reverse cumulative plot analyses; Fig. 2A, B and C, with 93 % of viremic cases and 100 % of viraemic controls compared to only 37 % ($P < 0.001$) of non-viraemic cases and 38 % ($P = 0.012$) of non-viraemic controls having a NP/OP viral load $\geq 4\log_{10}$ copies/mL. Furthermore, the same association of a higher percentage of viremic compared to non-viremic cases having NP/OP viral loads of $\geq 4\log_{10}$ copies/mL was observed for infection by RV-C pneumonia cases (100 % vs. 37 %, $P < 0.001$) and among the RV-C controls (100 %, vs. 42 %, $P = 0.048$); Fig. 2E and F. There was no difference in the RV load between viremic cases (4.67 $\log_{10}$ copies/mL) compared to viremic controls (4.83 $\log_{10}$ copies/mL), $P = 0.285$.

4. Discussion

In this case-control study of WHO-defined pneumonia etiology in children under-5 years of age living in South Africa, the prevalence of RV detection by PCR on nasopharyngeal samples did not differ between cases (23 %) and controls (22 %). Furthermore, the molecular subtyping of the RV-positive study participants was similar between cases and controls, thus highlighting the need for additional techniques for determining the true etiological role of RV in disease. We did, however, observe a 4-fold difference in RV-viremia between these two groups (7% vs. 2% in cases and controls, respectively) regardless of the small sample size. These findings could indicate an attributable role of RV infection in the pathogenesis of pneumonia, albeit lower than would have been imputed solely based on RV nasopharyngeal positivity in cases. Thus the focus on identification of RV on nasopharyngeal samples and in the absence of adequate control selection might falsely attributed a greater role of RV to the pathogenesis of pneumonia.

Over-attribution of pneumonia etiology to RV could, however, be partly mitigated by the use of RV-viremia detection, or nasopharyngeal RV load density as a relative proxy for RV viraemia, with a threshold density of $\geq 4\log_{10}$ copies/mL significantly discriminating between RV-viremic and non-viraemic cases. Additionally, the detection rates of RV-C viremia were higher than both RV-A and RV-B; in fact, no RV-B viremia cases were detected which is in line with other studies which have also failed to detect viremia due to RV-B [13,14,22,26]. This could

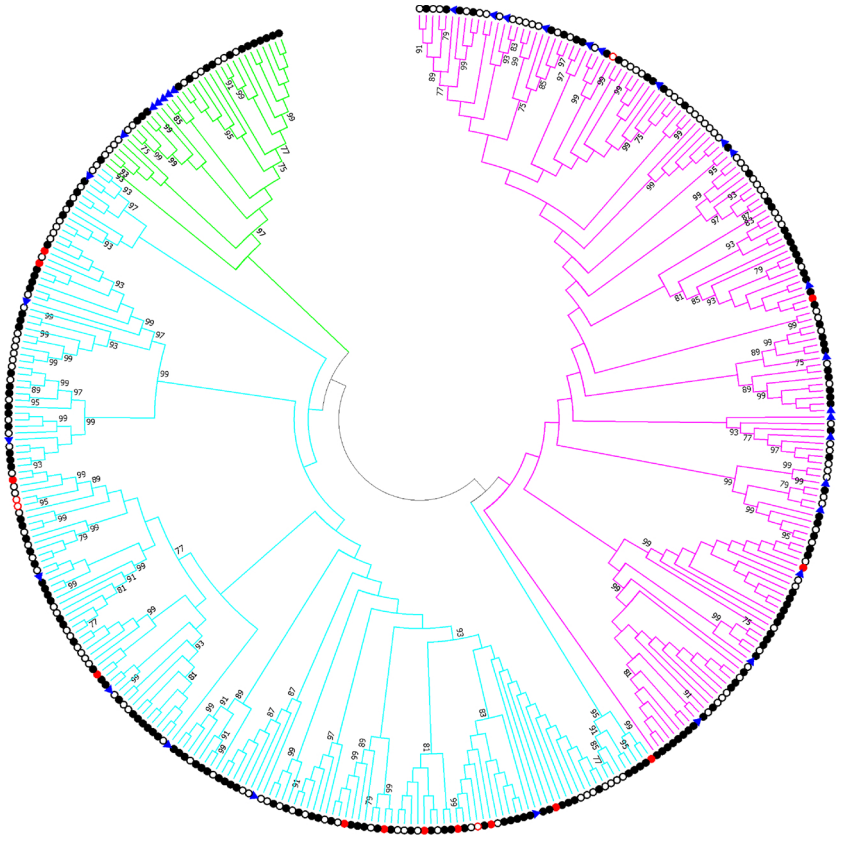

Fig. 1. A phylogenetic analysis of RV sequences. Sequences with closed circles denote types identified in NP/OP case samples (●) and sequences with open circles denote types identified in NP/OP control samples (O). Red, closed circles (●) denote types identified in viremia cases and red, open circles (○) denoted types identified in control with viremia. Closed triangles (▲) denote reference strains from GenBank. RV-A types are indicated by purple branches, RV-B types are indicated by green branches and RV-C are indicated by light blue branches. Bootstrap values after 1000 replicates are shown next to the branches, values < 70 % have been omitted from the tree. The phylogenetic tree is drawn to scale and the branch lengths are in relation to the lengths of those used to infer the tree (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

be related to the much lower prevalence of RV-B in the population (9% among cases and 8% among controls), but strongly supports evidence to the hypothesis that RV-B might have lower pathogenicity than RV-A and RV-C [27–29].

The correlation between viremia and high viral loads found in this study concurs with a previous Italian study. They postulated that high viral loads were a prerequisite for viremia, and that viremia was associated with more severe disease [13]. However, in our study, no positive correlations were found between RV nasopharyngeal viral loads and RV-viremia with markers for more severe disease. In fact those with RV-viremia had shorter hospital stays compared to the non-viremia cases. All of the children in this study were hospitalized with severe or very-severe pneumonia whereas the Italian study was conducted in children with both upper and lower respiratory tract which could account for the differences seen in the association of RV-viremia with more severe disease outcomes. Further; the RV-viremia detection rate in our study (7%) was lower than reported in previous studies (11 %–12 %) [13,14,22], although all of the cited studies were conducted in children less than 14 years of age hospitalized with upper or lower RTI; whereas, we only enrolled children 1–59 months of age hospitalized with pneumonia. In a study from Greece [14], 11 % of hospitalized RV nasopharyngeal positive children had RV-viremia, the majority (70 %) of the RV-viremia positive cases presented with asthma exacerbations, whilst no RV-viremia cases were detected among children hospitalized with RV-associated pneumonia. Similarly, in the Philippines study [22], the majority (73 %) of the RV-viremia positive cases were in children presenting with wheezing disease. Thus the low viremia detection rates reported in our study could be related to cases receiving a bronchodilator challenge prior to enrolment, which sought to exclude children with responsive hyperactive airway disease from being enrolled.

Importantly, none of the above-mentioned studies enrolled controls. In our study, viremia and high RV loads was seen among both cases and controls indicating the need for inclusion of controls in epidemiology studies on the role of RV in the pathogenesis of pneumonia. Of the four controls positive for RV-viremia, one had an upper RTI and the remaining three were asymptomatic at the time of sampling. However, RV-viremia has been described to be mainly detected during the early stages of disease symptomatology [14], and the viral load of RV was substantially higher among our controls with RV-viremia. Thus the three RV-viremia positive controls could have been in the incubation period of illness at the time of sampling. One of the limitations of our study was that controls were not systematically followed up post sampling, hence we were unable to confirm that these children did not subsequently develop pneumonia that required hospitalization. A Finnish case-control study which enrolled asymptomatic controls in addition to cases with acute expiratory wheezing, observed that 38 % of the RV positive controls developed respiratory symptoms within the week following sampling [30]. Nevertheless, the detection of RV-viremia

Table 2
RV molecular subtyping by sample type and viremia status in RV-associated pneumonia cases.

	Cases (n = 198)	Viremia- (n = 185)	Viremia+ (n = 13)	P-value	Controls (n = 189)	Viremia- (n = 185)	Viremia+ (n = 4)	P-value
RV-A	91	89 (48)	2 (15)		96	95 (51)	1 (25)	
RV-B	16	16 (9)	0	**0.001**	14	14 (8)	0	0.384
RV-C	91	80 (43)	11 (85)		79	76 (41)	3 (75)	

Abbreviations: RV: rhinovirus, n: number. *All variables are expressed as n (%) where % refers to proportion of species within the column; *P*-values calculated using Fisher's exact test and *t*-test where necessary.

Table 3
Characteristics and outcomes of children hospitalized with RV-associated pneumonia by viremia status.

Characteristics, n (%)*	Viremia (n = 13)	No viremia (n = 185)	Unadjusted *P*-value	aOR (95 % CI)	Adjusted *P*-value
Age in months, median (IQR)	14 (10–16)	6 (3–12)	**0.002**		**0.001**
-1-6 months	0	89 (48)			
-7-12 months	6 (46)	47 (25)	**0.005**		**0.011**
> 12 months	7 (54)	49 (26)			
Male	7 (54)	81 (44)	0.480	2.16 (0.65–7.18)	0.208
HIV +	0	28 (15)	0.130		0.269
HEU[a]	6 (46)	59 (31)	0.290	1.78 (0.54–5.87)	0.347
Never breast fed	6 (46)	55 (30)	0.215	2.38 (0.71–7.99)	0.158
Under weight[b]	0	17 (9)	0.253		0.505
Day care attendance	4 (31)	21 (11)	0.202	2.78 (0.66–11.72)	0.163
Smoker in household	5 (38)	71 (38)	0.965	0.80 (0.23–2.75)	0.723
Premature birth[c]	2 (15)	34 (18)	0.619	0.61 (0.12–3.14)	0.558
Birth weight, mean (SD)	3 (2.8–3.1)	2.9 (2.7–3)	0.775		0.687
Clinical Features:					
Very severe pneumonia	7 (54)	65 (35)	0.175	2.50 (0.76–8.24)	0.132
Chest radiograph abnormal[d]	6 (46)	93 (53)	0.611	0.71 (0.21–2.43)	0.590
Supplementary Oxygen therapy	12 (92)	171 (92)	0.987	1.11 (0.12–10.25)	0.925
Hypoxia[e]	9 (69)	137 (75)	0.653	0.95 (0.26–3.50)	0.941
Tachycardia[f]	9 (69)	87 (47)	0.126	2.14 (0.59–7.83)	0.249
Tachypnea[g]	12 (92)	147 (81)	0.300	2.24 (0.25–19.77)	0.467
Fever	7 (54)	130 (70)	0.223	0.58 (0.17–1.95)	0.385
Wheezing	8 (62)	67 (37)	0.064	2.77 (0.87–8.81)	0.084
Cough	13 (100)	157 (85)	0.552		0.864
Central cyanosis	0	1 (1)	0.790		0.181
Hospital stay ≥5 days	3 (23)	114 (62)	**0.006**	**0.21 (0.05-0.94)**	**0.041**
Case fatality ratio	0	11 (7)	0.382		0.756
Laboratory markers:					
Leukocytosis[h]	9 (69)	92 (50)	0.174	2.50 (0.70–8.99)	0.159
CRP ≥40 mg/L[i]	4 (31)	45 (24)	0.603	2.03 (0.53–7.72)	0.301
Blood culture positive	0	5 (3)	0.548	0.56 (0.38–4.88)	0.196
RV mono-infection[j]	7 (54)	99 (54)	0.981	1.22 (0.35–4.32)	0.754
RV genotyping:					
RV-A	2 (15 %)	89 (48 %)			
RV-B	0	16 (9%)	**0.001**		**0.001**
RV-C	11 (85 %)	80 (43 %)			

Abbreviations – n: number; NP: Nasopharyngeal; OR: Odds ratio; aOR: Adjusted odds ratio; CI: Confidence interval; SD: Standard deviation; IQR: Inter quartile range; RV: Rhinovirus. *P*-values from Chi-squared and Wilcoxon tests, logistic regression models adjusted for confounding variates (< 0.2 in univariate analysis) where applicable, Odds ratio could not be calculated for continuous variables or variables with no observations, thus cells left blank.

* All characteristics are expressed as n (%); unless otherwise stated in the table.

[a] HEU defined as undetectable viral load, HIV seronegative in the child with a positive maternal HIV history. Positive maternal HIV status based on self-report was accepted, except for seronegative children < 7 months of age where documented positive maternal HIV infection status was required.

[b] Underweight defined as weight for age < -2SD of the mean age-sex specific WHO reference.

[c] Defined as primary end point pneumonia and/or infiltrates.

[d] Hypoxic defined 1) a room air pulse-oximetry reading indicated oxygen saturation < 90 %, or 2) supplemental oxygen requirement in a child without a recorded room air saturation.

[e] Tachycardia defined as heart rate > 160 beats/minute if aged < 11 months, > 150 beats/minute if aged 12–35 months, > 140 beats/minute if aged 36–59 months.

[f] Tachypnea defined as respiratory rate > 60 breaths/minute if aged < 2 months, > 50 breaths/minute if aged 2–12 months, > 40 breaths/minute if aged > 12 month.

[g] Fever defined as body temperature ≥38 °C.

[h] Leukocytosis defined as white blood cell count > 15 000 cells/uL if age < 12 months, > 13 000 cells/uL if age ≥12 months.

[i] CRP defined as levels ≥40 mg/L are considered to potentially indicate bacterial infection.

[j] RV was the only virus detected in the nasopharynx.

among community controls, even at the very low prevalence seen in our study was unexpected, and further highlights the challenges of defining the etiological role of RV in the pathogenesis of pneumonia.

Study limitations included the cross-sectional study design which precluded us from studying the viral load over time and in relation to the onset of disease. Further, almost 50 % of cases had more than one virus detected thus we were unable to determine which virus or combination of viruses were the cause of the disease episode as there is no gold standard when it comes to diagnosing pneumonia. Additionally, the small sample size of RV-viremia positive cases and controls could have limited our power to discern clinical severity differences. Future studies will need to look at characterizing the RV molecular epidemiology and presence of viremia at the remaining 6 PERCH sites in Africa and Southeast Asia. Further, the RV typing method used in this study targeted the non-coding region (NCR) which has been shown to have a higher sensitivity for typing in clinical samples [21,22];

Table 4
Nasopharyngeal RV load by demographics and clinical characteristics in RV-associated cases and controls.

	Cases			Control		
	n = 198	Mean NP RV load (log_{10} copies/mL (SD))	*P*-value	n = 189	Mean NP RV viral load (log_{10} copies/mL (SD))	*P*-value
Age						
1-6 months	89	3.80 (0.89)		71	3.79 (0.82)	
6-12 months	53	4.06 (1.03)	0.148	66	3.96 (0.89)	0.508
> 12 months	56	4.04 (1.04)		52	3.65 (1.12)	
Gender						
Male	110	3.8 (0.95)		83	3.89 (0.96)	
Female	88	4.02 (1.01)	0.312	106	3.75 (0.92)	0.336
HIV						
Negative	170	3.99 (0.99)		173	3.83 (0.88)	
Positive	28	3.65 (0.82)	0.071	15	3.82 (0.95)	0.848
HEU[a]						
Negative	133	3.90		143	3.67 (1.04)	
Positive	65	4.04 (0.93)	0.413	46	3.86 (0.90)	0.226
Diagnosis						
Severe pneumonia	126	3.86 (0.96)				
Very severe pneumonia	72	4.08 (0.99)	0.123			
Asymptomatic control				178	3.77 (0.94)	
RTI control				11	4.48 (0.63)	**0.041**
Chest radiograph[b]						
Normal	88	3.88 (0.93)				
Abnormal	99	4.00 (1.01)	0.384			
Hypoxia[c]						
Yes	146	3.85 (0.95)				
No	50	3.98 0.99)	0.420			
Supplementary Oxygen therapy						
Yes	183	3.95 (0.98)				
No	15	3.81 (0.97)	0.601			
Mechanical ventilation						
Yes	10	3.90 (0.94)				
No	188	3.94 (0.98)	0.886			
Wheeze						
Yes	75	3.95 (0.98)				
No	121	3.93 (0.97)	0.938			
Fever[d]						
Yes	137	3.82 (0.85)				
No	61	4.25 (1.12)	**0.009**			
Tachypnea[e]						
Yes	159	3.99 (0.98)		10	8.40 (8.35)	
No	36	3.67 (0.95)	0.073	159	3.83 (0.97)	0.588
Tachycardia[f]						
Yes	96	4.02 (1.02)				
No	101	3.86 (0.94)	0.250			
Leucocytosis[g]						
Yes	101	4.01 0.89)				
No	97	3.87 (1.06)	0.292			
Hospital stay						
> 5 days	81	3.98 (1.04)				
< 5 days	117	3.90 (0.93)	0.447			
Case fatalities						
Yes	7	4.18 (0.97)				
No	192	3.93 (1.06)	0.503			
RV mono-infection[h]	65	3.90 (0.97)		60	3.82 (0.97)	
RV mixed infection	133	3.96 (0.98)	0.701	129	3.81 (0.93)	0.964
Type of RV species						
-A	91	3.96 0.97)		96	3.75 (0.90)	
-B	16	3.57 (0.70)		14	3.43 (0.82)	
-C	91	3.99 (1.01)	0.271	79	3.95 (0.99)	0.877
RV-viremia						
Yes	13	4.57 (0.73)		4	4.83 (0.83)	
No	185	3.90 (0.98)	**0.028**	185	3.79 (0.93)	**0.018**
RV-A						
Viremia present	2	3.75 (1.7)		1	5.83 (-)	
Viremia absent	89	3.96 (0.9)	0.765	95	3.73 (0.88)	NP

(*continued on next page*)

Table 4 (*continued*)

	Cases			Control		
	n = 198	Mean NP RV load (log_{10} copies/mL (SD))	*P*-value	n = 189	Mean NP RV viral load (log_{10} copies/mL (SD))	*P*-value
RV-C						
Viremia present	11	4.72 (0.45)		3	4.50 (0.60)	
Viremia absent	80	3.87 (1.04)	**0.016**	76	3.93 (0.99)	0.181

Abbreviations: RV: Rhinovirus; n: number; SD: Standard deviation; HIV: Human immunodeficiency virus; HEU: HIV exposed uninfected; RTI: Respiratory tract infection. *P*-values from Chi-square and Wilcoxon tests - logistic regression models adjusted for confounding variates (< 0.2 in univariate analysis) where applicable.

[a] HEU defined as undetectable viral load, HIV seronegative in the child with a positive maternal HIV history. Positive maternal HIV status based on self-report was accepted, except for seronegative children < 7 months of age where documented positive maternal HIV status was required.

[b] Abnormal chest radiographs defined as primary end point pneumonia and/or infiltrates.

[c] Hypoxic defined as 1) a room air pulse-oximetry reading < 90 %, or 2) requirement for supplemental oxygen in a child with no recorded room air saturation.

[d] Fever defined as body temperature > 38 °C.

[e] Tachypnea defined as respiratory rate > 60 breaths/minute if aged < 2 months, > 50 breaths/minute if aged 2–12 months, > 40 breaths/minute if aged > 12 months.

[f] Tachycardia defined as heart rate > 160 beats/minute if aged < 12 months, > 150 beats/minute if aged 12–35 months, or > 140 beats/minute if aged 36–59 months.

[g] Leukocytosis defined as white blood cell count > 15 000 cells/uL if age < 12 months, or > 13 000 cells/uL if age ≥12 months.

[h] RV was the only virus detected in the nasopharynx.

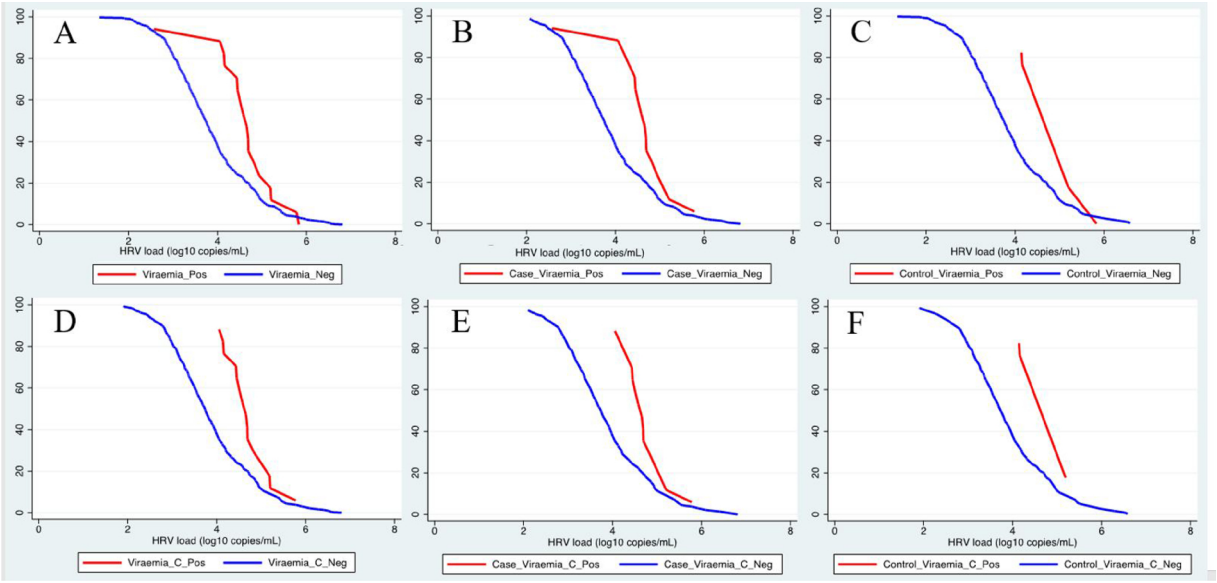

Fig. 2. Reverse cumulative plots of NP/OP RV load in Panel A.) all RV-infected viremic (n = 17) and non-viremic participants (n = 370), Panel B.) cases positive for viremia (n = 13) vs. non-viremic cases (n = 185), Panel C.) community controls positive for viremia (n = 4) vs. non-viremic controls (n = 185), Panel D.) all RV-C viremic (n = 14) and non-viremic participants (n = 156), Panel E.) RV-C viremic cases (n = 11) compared to RV-C non-viremic cases (n = 80), Panel F.) RV-C viremic (n = 3) community controls compared to RV-C associated non-viremic controls (n = 76).

however, the NCR is less conserved thus sequencing of the conserved capsid region to confirm the RV-C typing would be beneficial in order to study the importance of RV species in severe disease in greater detail.

In conclusion, RV-viremia was significantly more prevalent among children hospitalized with pneumonia; albeit at very low prevalence. Thus suggesting that RV-viremia could be used as a measure for attributing a causal role for RV in severe childhood disease; however, the clinical utility of RV-viremia detection during severe disease episodes is limited.

Author contributions

V.L.B conceived and designed the study as well as led analysis and interpretation and drafted manuscript. S.A.M, E.A.S and D.P.M assisted with interpretation of results and drafting of manuscript. V.L.B, D.P.M, A.M, E.A.S and S.A.M supervised study conduct. V.L.B, P.M, A.M and D.P.M were involved in study conduct, data collection, and/or data management. All authors reviewed and approved the manuscript. V.L.B, E.A.S and S.A.M had final responsibility for the decision to submit for publication.

Funding

This work was supported by The Bill & Melinda Gates Foundation grant to the International Vaccine Access Center, Department of International Health, Johns Hopkins Bloomberg School of Public Health (grant number 48968).

Disclaimer

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention, the US Department of Health and Human Services, or the US government.

CRediT authorship contribution statement

Vicky L. Baillie: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing. **David P. Moore:** Data curation, Investigation, Project administration, Resources, Software, Supervision, Writing - review & editing. **Azwifarwi Mathunjwa:** Project administration, Resources, Software, Supervision, Writing - review & editing. **Palesa Morailane:** Resources, Writing - review & editing. **Eric A.F. Simões:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing - review & editing. **Shabir A. Madhi:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Writing - review & editing.

Declaration of Competing Interest

S. A. M. has received honoraria for advisory board participation from Bill & Melinda Gates Foundation, Pfizer, Medimmune, and Novartis and institutional grants from GSK, Novartis, Pfizer, Minervax, and Bill & Melinda Gates Foundation and has served on speakers bureau for Sanofi Pasteur and GSK. All other authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

Acknowledgments

Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland: Katherine L. O'Brien (PI), Orin S. Levine (Former PI, current affiliation Bill & Melinda Gates Foundation, Seattle, Washington), Maria Deloria Knoll (co-PI), Daniel R. Feikin (joint affiliation with Centers for Disease Control and Prevention, Atlanta, Georgia), Andrea N. DeLuca, Amanda J. Driscoll, Nicholas Fancourt, Wei Fu, Meredith Haddix, Laura L. Hammitt, Melissa M. Higdon, E. Wangeci Kagucia, Ruth A. Karron, Mengying Li, Daniel E. Park, Christine Prosperi, Qiyuan Shi, Zhenke Wu, Scott L. Zeger; **The Emmes Corporation, Rockville, Maryland:** Nora L. Watson, **Nuffield Department of Clinical Medicine, University of Oxford, United Kingdom**: Jane Crawley; **University of Otago, Christchurch, New Zealand**: David R. Murdoch; **Canterbury Health Laboratories, Christchurch, New Zealand:** Trevor P. Anderson, Joanne Mitchell.

We acknowledge the work of all PERCH Contributors who were involved in data collection at the local sites and central laboratories, members of the PERCH Chest Radiograph Reading Panel, and Shalika Jayawardena and Rose Watt from Canterbury Health Laboratories. Finally, we offer sincere thanks to the patients and families who participated in the study.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jcv.2020.104288.

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