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Human bocavirus infection as a cause of severe acute respiratory tract infection in children

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Abstract

In 2005 human bocavirus (HBoV) was discovered in respiratory tract samples of children. The role of HBoV as the single causative agent for respiratory tract infections remains unclear. Detection of HBoV in children with respiratory disease is frequently in combination with other viruses or bacteria. We set up an algorithm to study whether HBoV alone can cause severe acute respiratory tract infection (SARI) in children. The algorithm was developed to exclude cases with no other likely cause than HBoV for the need for admission to the paediatric intensive care unit (PICU) with SARI. We searched for other viruses by next-generation sequencing (NGS) in these cases and studied their HBoV viral loads. To benchmark our algorithm, the same was applied to respiratory syncytial virus (RSV)-positive patients. From our total group of 990 patients who tested positive for a respiratory virus by means of RT-PCR, HBoV and RSV were detected in 178 and 366 children admitted to our hospital. Forty-nine HBoV-positive patients and 72 RSV-positive patients were admitted to the PICU. We found seven single HBoV-infected cases with SARI admitted to PICU (7/49, 14%). They had no other detectable virus by NGS. They had much higher HBoV loads than other patients positive for HBoV. We identified 14 RSV-infected SARI patients with a single RSV infection (14/72, 19%). We conclude that our study provides strong support that HBoV can cause SARI in children in the absence of viral and bacterial co-infections.

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Keywords: Human bocavirus, intensive care, paediatrics, severe acute respiratory tract infection, next-generation sequencing (NGS)

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Introduction

In the past decade, many unknown viruses have been identified using novel molecular pathogen discovery techniques [1]. After detection, it is not always clear if these newly discovered viruses are able to cause disease. For some viruses it remains

difficult to rule out simultaneous infections in the patient with other known or yet unknown pathogens. Human bocavirus (HBoV) is a clear example of such a newly discovered virus.

Human bocavirus was discovered in 2005 in respiratory tract samples from children suffering acute respiratory tract infections [2]. Currently, four genotypes have been described (HBoV1–4) [3]. Based on seroprevalence studies, HBoV-specific antibodies in adults range from 64 to 95%, indicating the high number of HBoV encounters [4]. HBoV infection in children has been associated with respiratory tract infections (HBoV1–2) and gastrointestinal disease (HBoV2–4) [3]. HBoV infection results often in a mild self-limiting respiratory tract infection and may even be asymptomatic [3,5]. Many children

shed HBoV in the respiratory tract for prolonged periods, making interpretation of a positive test result in a patient difficult [6]. Nonetheless, a few case reports describe HBoV as the cause of severe acute respiratory tract infection (SARI) in children requiring intensive care. Although the role of other pathogens was addressed, co-infections were not excluded using a structured method [3,7–14]. Altogether, this has resulted in the on-going debate of whether HBoV as the single causative agent can cause disease.

To address this issue, we set up an algorithm to exclude cases with no other likely cause than HBoV for the need for intensive care. We searched for overlooked viruses by next-generation sequencing (NGS) in these cases and studied their HBoV viral loads. To benchmark this method, the same algorithm was applied to patients with respiratory syncytial virus (RSV) infection. As RSV is generally considered to be a well-established paediatric pathogen that can cause SARI in children [15–17].

Materials and methods

Patient and sample selection

We conducted a retrospective cohort study during 5 consecutive years from April 2007 through March 2012. We selected paediatric patients (<18 years) admitted to the Paediatric Intensive Care Unit (PICU) of Erasmus MC-Sophia, which is a tertiary paediatric referral centre. This is the sole PICU for a region with a general population of ~4 million and an annual birth cohort of ~47 000 children [18].

We selected respiratory tract samples of these patients that tested positive with real-time RT-PCRs for respiratory viral diagnostics. We used the electronic laboratory information management system of the Viroscience Department to obtain all test results. Samples included nasal washings, sputum, throat swabs and bronchoalveolar lavages, which were obtained during routine clinical practice. We only used the first available sample of each patient. A viral pathogen was defined if a cycle threshold value (Ct-value) <40 was detected for adenoviruses, coronaviruses (OC43, 229E and NL63), HBoV1–4, human metapneumovirus, influenza A and B viruses, parainfluenza viruses 1–4, RSV and/or rhinoviruses [19]. The Ct-value represents the number of cycles required for the fluorescent signal to cross the threshold (exceeds background level) and is inversely related to viral load.

Algorithm

All HBoV RT-PCR-positive cases, with or without co-infections, were originally included. Next, four selection steps were applied: first, we selected all PICU-admitted patients, as these

TABLE 1. Baseline characteristics of selected human bocavirus and respiratory syncytial virus RT-PCR-positive patients admitted to the paediatric intensive care unit at the Erasmus MC-Sophia from 2007 to 2012

Characteristics	HBoV-positive PICU cases (n = 7)	RSV-positive PICU cases (n = 17)
Age, median (min–max, IQR)		
Months	24 (4–31, 14.3–31.3)	2 (0–39, 0–7.5)
Years	2 (0.3–2.62, 1.19–2.60)	0.18 (0.02–3.26, 0.06–0.68)
Female, n (%)	4 (57)	5 (29)
Male, n (%)	3 (43)	12 (71)
Reason for admission, n (%)		
Respiratory failure	3 (43)	16 (94)
ECMO indication due to respiratory failure	1 (14)	—
Bronchiolitis/PSA	3 (43)	—
ALTE with ARTI	—	1 (6)
Clinical diagnosis at admittance, n (%)		
URTI	—	1 (6)
LRTI	1 (14)	1 (6)
BHR/PSA	4 (57)	—
ARDS	1 (14)	—
Severe atelectasis with ARTI	1 (14)	—
Bronchiolitis	—	15 (88)
Medical history, n (%)		
None	3 (43)	6 (35)
Pulmonary disease	3 (43)	2 (12)
GSA <37 weeks	—	2 (12)
GSA <37 weeks and pulmonary disease	1 (14)	—
Cardiac disease	—	1 (6)
Congenital anatomical malformations	—	4 (24)
Macrosome or dysmature	—	2 (12)
Laboratory testing, median (min–max, IQR)		
CRP (mg/L)	8 (1–36, 5–28)	8 (2–22, 4–11.5)
WBC count ($\times 10^9/L$)	13.6 (8.1–27, 9.3–20)	13.5 (8.7–33, 10.5–15)
Sputum obtained and start antibiotics, n (%)		
≤ 12 hours before sputum obtained	1 (14)	3 (17)
> 12 hours after sputum obtained	4 (57)	4 (24)
Sputum not obtained, n (%)	2 (28)	10 (59)
Respiratory support, n (%)		
Supplemental	1 (14)	7 (41)
Invasive	5 (72)	10 (59)
ECMO	1 (14)	—
PICU admission duration (days) median (min–max)	4 (2–7)	4 (2–29)
Survival, n (%)	7 (100)	17 (100)
Viral metagenomics, n (%)		
HBoV as sole viral pathogen detected	7 (100)	—
RSV as sole viral pathogen detected	—	14(82) ^a

Abbreviations: ALTE, acute life-threatening event; ARDS, acute respiratory distress syndrome; ARTI, acute respiratory tract infection; BHR, bronchial hyperreactivity; CRP, C-reactive protein; ECMO, extracorporeal membrane oxygenation; GSA, gestational age; HBoV, human bocavirus; LRTI, lower respiratory tract infection; PICU, paediatric intensive care unit; PSA, paediatric status asthmaticus; RSV, respiratory syncytial virus; URTI, upper respiratory tract infection; WBC, white blood cells.

^aOne sample could not be processed, one tested negative for viruses, one tested positive for rhinovirus.

patients are critically ill and key for testing our hypothesis. Second, patients with a viral co-detection upon RT-PCR were excluded. Third, selected clinical data were extracted from electronic medical files of the patients. Only patients with SARI as the reason for admission were included (see Table 1 and Supplementary material, Table S1). Fourth, absence of bacterial co-infections was considered likely if C-reactive protein (CRP) levels were ≤ 40 mg/L upon admission and bacterial cultures

tested negative, such as sputum, blood or cerebrospinal fluid cultures. We defined a sputum culture negative if ten or fewer bacteria per ocular field were present in Gram staining, if no respiratory pathogenic bacteria were cultured, and/or when commensal bacterial growth was $\leq 2/4$. Sputa containing fewer than ten epithelial cells per ocular field (10×10 magnification) were considered quality sputa. Sputa containing ten or more epithelial cells per ocular field were only considered quality sputa if the leucocyte-to-epithelial cell ratio was ≥ 10 and more than six bacteria with the same morphology were present. Absence of a sputum culture was not a reason for exclusion, as sputum is not easily obtained from children. A reason for exclusion was if other bacterial cultures tested positive, such as a cerebrospinal fluid cultures or blood cultures.

Lastly, selected samples were tested for *Mycoplasma pneumoniae* using RT-PCR and only negative samples were used for NGS. Confirmatory serology detecting antibodies against HBoV or RSV was not performed, as serum samples were not collected routinely from these patients.

Three investigators independently reviewed the previously described selection steps and disagreement was resolved by consensus (FM, JVK and PF).

Next-generation sequencing

After applying the algorithm, samples were analysed for the presence of known and unknown viruses. We used a viral metagenomics procedure as previously described, which includes random RNA and DNA amplification in combination with NGS (454 Life Sciences[®], Branford, CT, USA) [20,21]. In brief, the amplicons of random RNA and DNA amplification were pooled and purified, after which rapid library preparation, emulsion PCR and NGS were performed. Iterative exhaustive assembly of sequences was applied according to our virus discovery pipeline written in the python programming language (PYTHON 2.7). An absolute minimum of two reads was required for a distinct hit to be reported. Hits positive for endogenous retroviruses or anelloviruses were excluded from analysis. Presence of genome material for bovine viruses was discarded because bovine reagents were used. Sequences were deposited in GenBank and given accession numbers (see Supplementary material; Supplementary data).

Phylogenetic analysis of HBoV

We performed phylogenetic analyses to study whether a specific HBoV subtype could be linked to a more virulent HBoV infection resulting in PICU admission. We used the VP1/VP2 genes for sequence analysis, after which a phylogenetic tree was reconstructed. Samples tested were HBoV RT-PCR-positive selected cases for NGS and selected controls. Controls were samples of patients with Ct-value < 30 , which is the minimum requirement to sequence HBoV in this method. These control

samples were obtained from children whether or not they were admitted to PICU, without SARI and with co-infections. Two samples were obtained from children born in hospital and admitted for more than 90 days and were called nosocomially infected controls. All samples were amplified and sequenced as described previously [22]. Phylogenetic analyses were conducted with MEGA version 5.

HBoV Ct-values in respiratory tract samples

To study the role of HBoV Ct-values in patients with SARI, we compared median Ct-values in three groups of patients. Groups were defined as: all hospitalized HBoV patients, all PICU-admitted patients with HBoV and viral co-detection, and selected PICU-admitted patients with a single HBoV infection.

Validation method using RSV infections

To validate our algorithm we applied the exact same selection steps to RSV-positive samples of patients admitted to PICU. We chose RSV for validation because its role in children with SARI is well-established compared with other pathogens and we therefore expected that RSV-related SARI admissions would remain to be identifiable after using our algorithm [15–17].

Statistical analysis

Data were analysed using SPSS version 20.0 (IBM, SPSS, Chicago, IL, USA) and GRAPHPAD PRISM version 6 (GraphPad, San Diego, CA, USA). For continuous data, medians, interquartile range (IQR), lower and upper IQR were calculated. For the assessment of HBoV viral loads, median Ct-values were compared using the Mann–Whitney *U* test. A *p* value < 0.05 was taken as threshold of statistical significance.

Ethics considerations

The Medical Ethical Committee of the Erasmus MC approved this study (MEC 2013-221). Informed consent was waived because this is a retrospective cohort study. Data were stored anonymously and cannot be retraced to individual patients.

Results

Patient and sample inclusion

In total 990 paediatric patients with a median age of 0.82 years (IQR 3.13), were identified for whom respiratory viral diagnostics were performed. Of those, 178 patients (18%) were HBoV RT-PCR-positive. PICU admission was necessary for 49 HBoV cases (28%), of which 20/49 were positive for HBoV alone (41%) and 29/49 were positive for HBoV and other viruses (59%) (see Supplementary material, Table S2). Subsequently, clinical charts were reviewed and only patients

admitted to PICU because of SARI were selected (11/20, 55%). Next, samples of patients with CRP levels ≤ 40 mg/L upon admission were included (8/11, 73%). Lastly, cases with negative blood, liquor and sputum cultures (if available) and negative RT-PCR test results for *M. pneumoniae* were selected. Sputa were obtained from intubated children. A total of 7/8 cases (88%) were considered with a single HBoV infection resulting in SARI and their samples were tested with NGS (see Fig. 1).

Next-generation sequencing of HBoV cases

The seven identified respiratory tract samples were subjected to NGS and near full-length genomes were obtained in most samples (see Supplementary material, Table S3). No other viral pathogens besides HBoV were detected with NGS in seven cases selected with our algorithm (2.8–44.3% of total number of analysed reads).

Clinical data from children with HBoV

The median age of the seven children with HBoV was 24 months (min 4.4, max 31.4, IQR 15.7–28) and four patients

were female (4/7, 57%). One patient was 4 months old upon admission and was born prematurely with a gestational age of 27 weeks and was previously diagnosed with bronchopulmonary dysplasia. Because of the small sample size we referred from further comparative statistical testing. The length of symptoms before PICU admission was 1–7 days. Upon PICU admission CRP levels with a median of 8 mg/L (min 1, max 36, IQR 5–28) were tested within a median PICU stay of 2.5 h (min 0, max 6 h). For 6/7 children follow-up CRP levels were obtained. In two children there was an increase in CRP, none of these CRP levels were ≥ 80 mg/L (see Table 2). For patient 2, CRP levels increased after initiation of extracorporeal membrane oxygenation (from 18 to 58 mg/L, respectively). For the other patient, negative bacterial sputum samples were obtained in the absence of antibiotics at day 1 of the PICU stay. Three of the seven patients (43%) with a single HBoV infection had no pre-existing medical history. All seven patients received antibiotics during admission and required supplemental oxygen. Mechanical ventilation was performed in five patients (71%) and extracorporeal membrane oxygenation treatment was needed

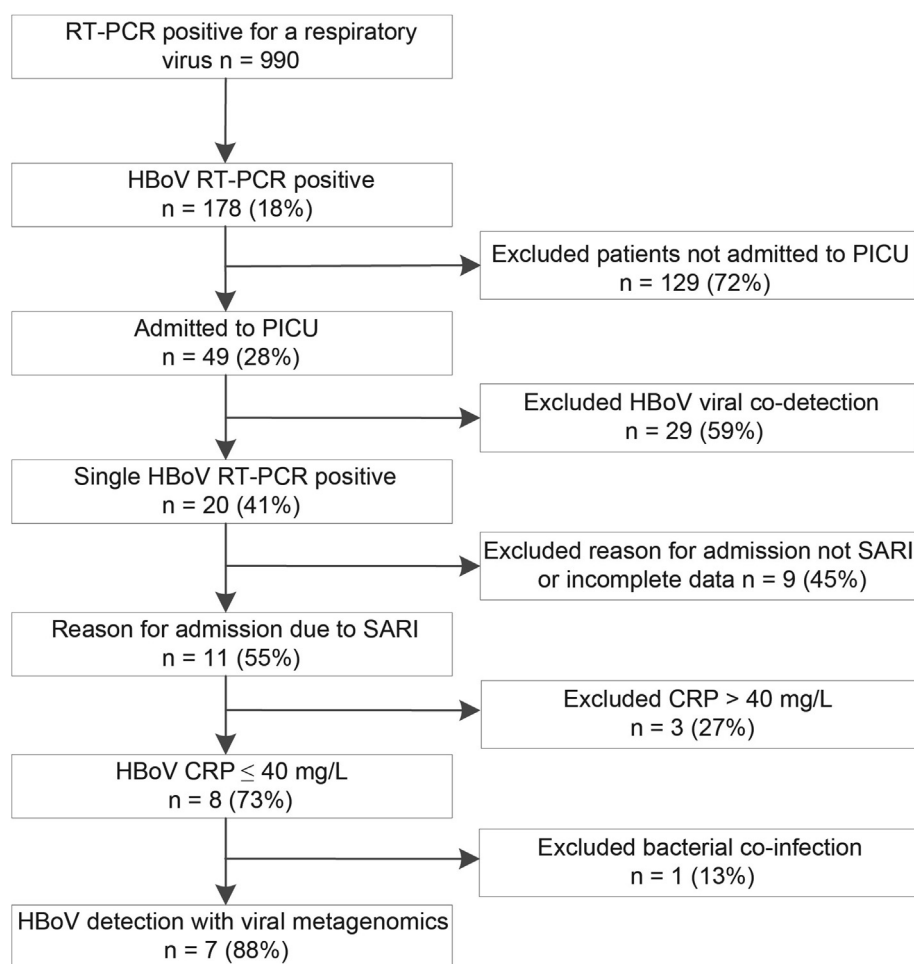


FIG. 1. Flowchart for patient selection of human bocavirus RT-PCR-positive patients admitted to the Erasmus MC-Sophia from 2007 to 2012.

TABLE 2. Overview non-structured follow-up C-reactive protein levels obtained during paediatric intensive care unit stay

CRP levels (mg/L)	PICU stay (days)						
	1	1.5	2	2.5	3	3.5	4
Patient 1	5	—	—	—	—	—	—
Patient 2	36	18	55	58	41	—	—
Patient 3	8	—	4	—	—	—	—
Patient 4	11	9	—	5	—	—	—
Patient 5	28	—	79	—	66	—	10
Patient 6	7	—	1	—	—	—	—
Patient 7	1	—	5	—	6	—	4

—, represents missing data.

in one patient (14%). All patients survived. The median duration of PICU admission was 4 days (IQR 2–7 days) (see Table 1).

Phylogenetic analysis of HBoV

To study whether a specific subtype of HBoV was associated with severe disease, phylogenetic analyses were performed. The VP1/VP2 region of HBoV was successfully amplified in all seven selected cases (100%) and 40 controls (87%) (see Supplementary material, Fig. S1).

BLAST and phylogenetic analyses showed that all HBoV strains of cases and controls were closely related to a total of two reference genotypes corresponding to HBoV genotype I (Stockholm: ST1 and ST2) [22].

HBoV Ct-values in respiratory tract samples

We compared the median HBoV Ct-values to study whether a low Ct-value resulted in severe disease and if viral co-detection resulted in higher Ct-values. We found significantly lower Ct-values in the seven-selected HBoV-positive cases (median Ct-value 15.6, IQR 11.6–16.3) compared with all HBoV-positive patients admitted to hospital (median Ct-value 33.9, IQR 11.3–36.8) ($p < 0.0001$) (see Fig. 2).

Furthermore, HBoV Ct-values were significantly lower compared with 29 PICU patients with HBoV and viral co-detection (median Ct-value 28.6, IQR 13.6–35.45) ($p = 0.001$) (see Fig. 2).

Validation method using RSV infections

Of the 990 paediatric patients, 366 tested positive for RSV on RT-PCR (37%). PICU admission was required in 72/366 of RSV cases (20%). After applying our selection steps, 17/24 RSV RT-PCR-positive samples were tested with NGS (71%). Of these, 15/17 samples yielded results (88%). One sample could not be processed; another had a limited number of reads. In all samples, RSV was detected (0.01–31.7% of total number of analysed reads). In addition, in one sample a rhinovirus was co-

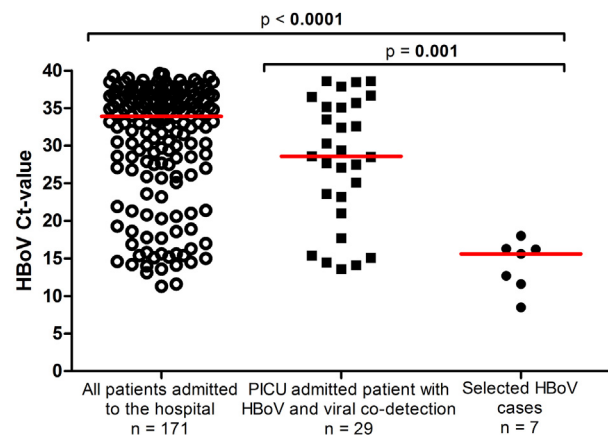


FIG. 2. Comparison between median Ct-values of human bocavirus (HBoV) RT-PCR-positive respiratory tract samples of paediatric patient admitted to the Erasmus MC-Sophia from 2007 to 2012; all hospital-admitted paediatric patients versus patients admitted to the paediatric intensive care unit (PICU) with HBoV and viral co-detection versus PICU-admitted patients with a single HBoV infection. Horizontal bars represent group medians.

detected (1.17% reads; Fig. 3 and see Supplementary material, Table S4). Overall, in 14/72 SARI PICU patients RSV was found to be the sole pathogen (19%).

Discussion

We here present a study on the relation between a recently discovered viral pathogen—HBoV—and SARI in children. We analysed HBoV RT-PCR-positive samples from paediatric patients in whom viral and bacterial co-infections were considered unlikely after a rigid selection process. Pivotal to our approach is the addition of NGS to the clinical and conventional laboratory data. Based on the algorithm and NGS, we showed that a single HBoV infection caused SARI in seven patients requiring PICU admission. Which is in concordance with case reports on severely ill children with HBoV infection [7–14]. Although, in these reports viral and bacterial co-infections were described, they were not structurally assessed and NGS was not performed to detect overlooked viruses [7–14].

By adding NGS to our selection method, we bypassed the limitation of testing with RT-PCR alone, which relies on the use of selected primers for suspected pathogens. NGS allows detection of variant viruses that would otherwise escape detection [23,24]. It is specifically designed to detect viruses and is optimized to decrease the number of other nucleic acids from host and bacteria. We showed previously that the

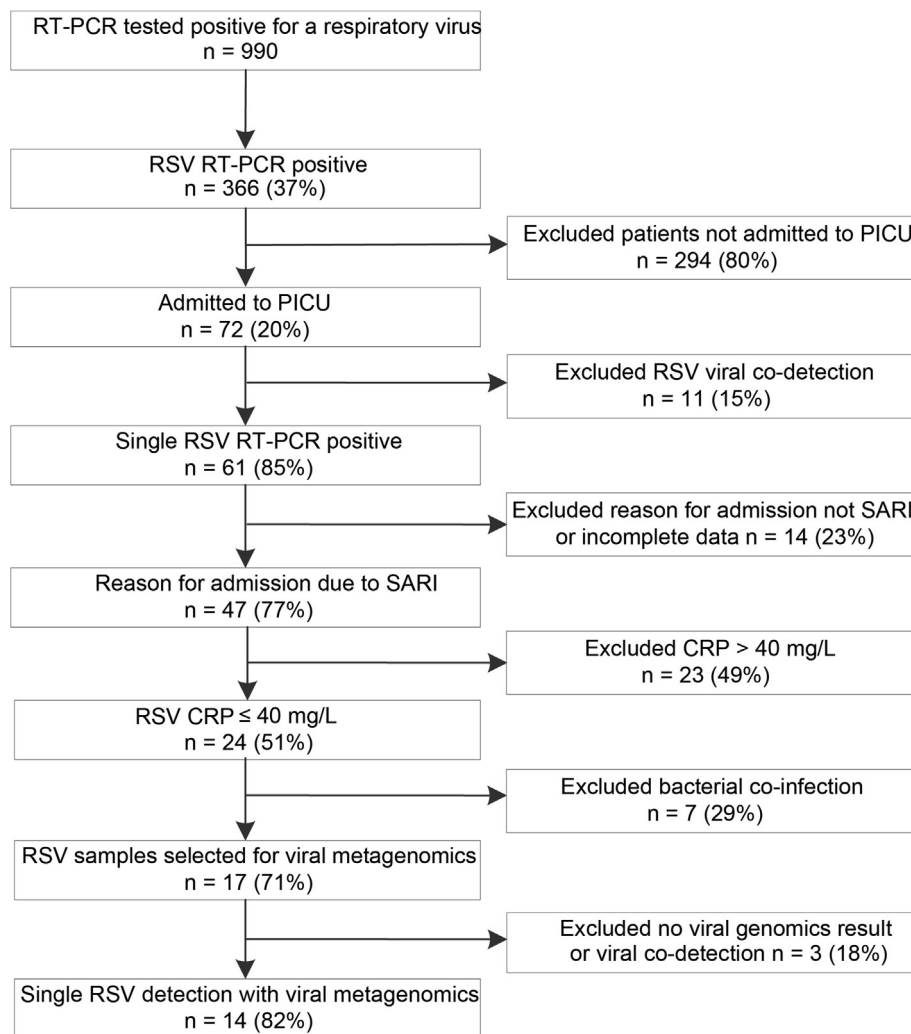


FIG. 3. Flowchart for patient selection of respiratory syncytial virus RT-PCR-positive patients admitted to the Erasmus MC-Sophia from 2007 to 2012.

sensitivity of detection with this method is approaching that of RT-PCRs in respiratory specimens [25]. The routine clinical use of NGS alone is still in development, but it may well be that it will replace RT-PCR in the future [24].

In addition to the NGS findings, we also studied the difference between median HBoV Ct-values of selected cases and other patients with HBoV detection. We found a significantly lower median Ct-value in the selected cases than in patients who were also admitted to PICU, but who had viral co-detection. This may indicate that a single recent HBoV infection is associated with a higher viral load than in combination with other viruses, as suggested previously by other studies [13,26,27]. Based on the age of the patients and the low Ct-values we assume that the severe HBoV infection was a consequence of a primary infection [13]. Furthermore, we compared the median Ct-values of selected cases with patients not admitted to PICU with HBoV and found that the Ct-values

of the selected cases were also significantly lower, suggesting a direct relation between disease severity and viral load as reported by others [10,13,27]. Interestingly, others have also found a relation between Ct-values, gender and underlying disease. Unfortunately our sample size was too small to confirm or refute these findings [13].

In order to benchmark our algorithm that was used to exclude patients with viral and bacterial co-infections, we also applied it to patients with RSV infection. We only found 14 cases with RSV as the single causative agent for SARI. Considering the incidence of RSV-associated hospitalizations, this is lower than expected [17]. We were able to benchmark our algorithm, although it seems to be very stringent and results in an underestimation of the true HBoV epidemiology. It is therefore of note that we did not set out to estimate the true burden of disease of HBoV, but to establish whether HBoV by itself can cause SARI. Our study is subject to several limitations.

It may be argued that the retrospective nature of this study and its inherent limitations may have resulted in a sample bias. Blood samples for HBoV serology or PCR were not obtained, because these are not routinely tested at our hospital. Furthermore, not all bacteriology samples were obtained, as more invasive procedures would have had to be carried out to fully rule out the role of bacteria in lower respiratory tract infections. These are currently not routinely practiced in critically ill children for ethical and technical reasons. As an alternative we set strict CRP levels to our inclusion criteria. Based on previously published data we set a lower cut-off for bacterial co-infections (40 mg/L) than the usually applied 80 mg/L, to minimize the contribution of bacterial infections to the observed disease. In addition, during non-structured follow up, none of the patients showed an increase in CRP levels >80 mg/L. Still, in two cases a moderate increase was observed and it should be noted that despite these low CRP levels, bacterial involvement cannot be fully ruled out based on CRP levels alone [28–30]. Other biomarkers such as pro-calcitonin and interleukins could have been used to differentiate between bacterial and viral infections, but these were not carried out in our hospital [30,31].

Based on our findings we conclude that a single HBoV infection can cause SARI in children in the absence of viral and bacterial co-infection.

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Transparency declaration

PF and AO participate in the IRIS trial sponsored by Hoffmann-La Roche, Inc. AO is a part-time employee of Viroclinics Biosciences BV, which performs contract research for pharmaceutical companies.

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Appendix A. Supplementary data

Supplementary data related to this article can be found online at <http://dx.doi.org/10.1016/j.cmi.2015.06.014>.

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