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Virus Type and Genomic Load in Acute Bronchiolitis: Severity and Treatment Response With Inhaled Adrenaline

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Background. Acute bronchiolitis frequently causes infant hospitalization. Studies on different viruses or viral genomic load and disease severity or treatment effect have had conflicting results. We aimed to investigate whether the presence or concentration of individual or multiple viruses were associated with disease severity in acute bronchiolitis and to evaluate whether detected viruses modified the response to inhaled racemic adrenaline.

Methods. Nasopharyngeal aspirates were collected from 363 infants with acute bronchiolitis in a randomized, controlled trial that compared inhaled racemic adrenaline versus saline. Virus genome was identified and quantified by polymerase chain reaction analyses. Severity was assessed on the basis of the length of stay and the use of supportive care.

Results. Respiratory syncytial virus (83%) and human rhinovirus (34%) were most commonly detected. Seven other viruses were present in 8%–15% of the patients. Two or more viruses (maximum, 7) were detected in 61% of the infants. Virus type or coinfection was not associated with disease severity. A high genomic load of respiratory syncytial virus was associated with a longer length of stay and with an increased frequency of oxygen and ventilatory support use. Treatment effect of inhaled adrenaline was not modified by virus type, load or coinfection.

Discussion. In infants hospitalized with acute bronchiolitis, disease severity was not associated with specific viruses or the total number of viruses detected. A high RSV genomic load was associated with more-severe disease.

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Keywords. bronchiolitis; respiratory syncytial virus; human rhinovirus; racemic adrenaline; infant.

Acute bronchiolitis in infants is a major health burden worldwide, closely associated with seasonal epidemics of respiratory syncytial virus (RSV) infection [1, 2]. Detection of >1 virus has been reported in up to 40% of the infants [3]. Modern techniques yield increased detection rates of RSV and of other viruses that have a less certain role in the causation of acute bronchiolitis [4].

Treatment of acute bronchiolitis in hospitalized infants is generally supportive [5], although bronchodilators, including inhaled adrenaline, are commonly used [6, 7]. We recently showed in 404 infants that treatment with inhaled racemic adrenaline was not superior to inhaled isotonic saline [8]. Longitudinal studies have shown that many but not all children with acute bronchiolitis develop asthma later [9] and that, in children hospitalized for obstructive airways disease, human rhinovirus (HRV) is associated with a greater risk than RSV for later development of asthma [10, 11]. However, it is not clear whether HRV represents a marker of predisposition to obstructive airways disease or has a causative role. Studies to assess whether viral etiology may modify the effect of treatment with inhaled bronchodilators have been requested [12] but not reported.

Attempts have been made to link the presence of different viruses with the severity of disease, with conflicting results in regard to RSV [12–16] and HRV, including subtypes [17, 18]. RSV has been associated with increased disease severity in some [12–14] but not all [3, 15] studies. Studies have shown higher [15], unchanged [16], or lower [12, 18–20] clinical severity in patients who tested positive for HRV, compared with those who tested negative HRV. The presence of the recently discovered [21] HRV type C strains has been associated with more-severe obstructive airway disease in young children with an acute lower airway infection [22, 23]. However, results of studies focusing on infants with bronchiolitis have been unclear on this association [18, 20].

Studies of the viral genomic load in nasopharyngeal aspirates have shown a positive relationship with disease severity, as a higher concentration of RSV in nasopharyngeal aspirates has been associated with more-severe disease [3, 24–28]. The same association has not been found for HRV [29].

The significance of coinfections, found in 9%–41% of patients, may have major clinical impact on the guidelines for isolation of hospitalized patients. However, studies have shown conflicting association with disease severity [3, 19, 30]. Brand

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et al [3] examined 142 samples for 15 different viruses, finding >1 virus in 41% but no association with disease severity. In contrast, Richard et al [30] found that coinfections (occurring in 24% of subjects and including RSV, HRV, and 6 other viruses) increased the risk of pediatric intensive care unit admission by 2.7-fold. The aims of the present study were to investigate whether the presence or concentration of individual or multiple viruses were associated with disease severity in acute bronchiolitis and to evaluate whether detected viruses modified the response to inhaled racemic adrenaline.

SUBJECTS AND METHODS

Study Design

The Bronchiolitis ALL-SE study was a multicenter, randomized, double-blinded, factorial-designed clinical trial comparing the effect of inhaled racemic adrenaline versus saline and 2 inhalation strategies (on demand vs fixed schedule; Figure 1) in infants in Norway in 2 consecutive winter seasons from January 2010 through May 2011 [8]. Inclusion criteria were age of <12 months and clinical signs of moderate-to-severe bronchiolitis. An overall clinical score of ≥4 (on a scale of 0–10, with higher scores indicating more-severe illness; Supplementary Table 1) were used as inclusion criteria, indicating moderate-to-severe illness. The exclusion criteria were the presence of any serious cardiac, immunologic, neurologic, or oncologic disease or any serious pulmonary disease other than bronchiolitis; >1 previous episode of obstructive airway disease; symptoms of disease of the lower airway (eg, coughing) for >4 weeks; and any glucocorticoid therapy in the preceding four weeks.

Patients were enrolled at all hours, with enrollment limited occasionally by the overall capacity of the acute ward. The study was approved by the regional committees for medical and health research ethics and by the Norwegian Medicines Agency and is registered in the Norwegian Biobank Registry. Written informed consent was obtained from a parent of each child before the start of therapy. The study was audited by the Norwegian Medicines Agency in 2011. The trial was registered at ClincialTrials.gov (NCT00817466) and EudraCT (2009-012667-34).

Subjects

The Bronchiolitis ALL-SE study enrolled 404 infants from 8 participating hospitals in southeast Norway with moderate-to-severe acute bronchiolitis [8]. A total of 62% were male, and the mean age was 4.2 months. Of these 404 infants, the present study evaluated 363 with a nasopharyngeal aspirate available. The median length of stay (LOS) was 67.1 hours (95% confidence interval [CI], 58.4–71.3 hours). Baseline characteristics were comparable between treatment groups among patients in the present study (Table 1) and between patients with and those without nasopharyngeal aspirates available.

Methods

Details on randomization and study medication are described elsewhere [8].

The use of supportive care was recorded daily and verified in manual patient record reviews.

The LOS was defined as the time from the first study inhalation until discharge from the hospital, as recorded in the medical record for each patient [8]. Most children were discharged between 8:00 AM and 11:00 PM.

Nasopharyngeal aspirates were collected using a standardized procedure performed by trained pediatric nurses at study

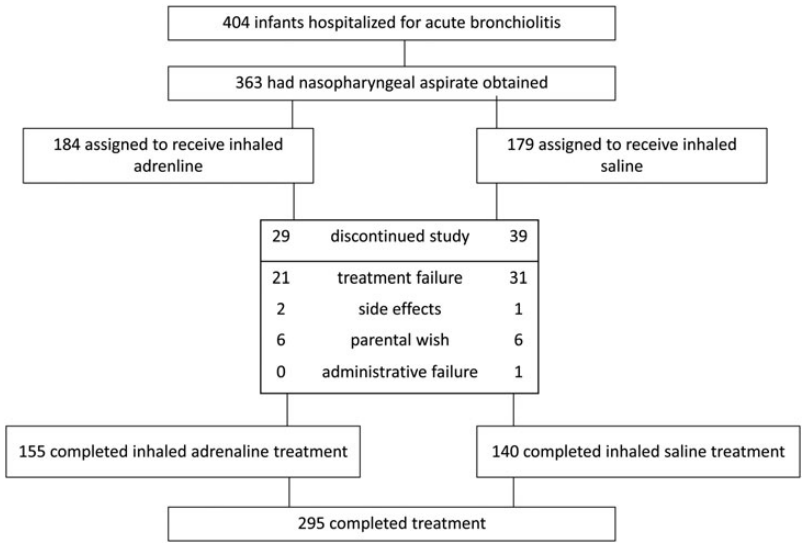

Figure 1. Randomization of the study patients. For 1 patient, the study medication was discontinued because of administrative failure (ie, the supply of study medication was insufficient).

Table 1. Baseline Characteristics of 363 Infants With Acute Bronchiolitis, by Treatment Group

Characteristic	Inhaled Racemic Adrenaline (n = 184)	Inhaled Saline (n = 179)
Male sex	62.0	58.1
Age, mo	4.2 (3.7–4.6)	4.2 (3.8–4.6)
White race among parents		
Father	92.5	90.9
Mother	90.8	91.7
Atopic eczema	9.9	10.8
Allergy[a]	1.8	1.8
1 previous BO	25.8	30.1
Respiratory symptoms persisting for >1 wk[b]	12.1	14.7
Asthma in ≥1 parent	22.6	25.9
Rhinoconjunctivitis in ≥1 parent	30.5	32.1
Clinical score[c]	4.9 (4.8–5.1)	4.9 (4.7–5.0)
SpO_2, %	96.0 (95.5–96.5)	96.0 (95.5–96.5)
Respiratory rate, breaths/min	53.3 (51.6–55.0)	54.0 (52.3–55.7)
Heart rate, beats/min	154.6 (151.9–157.3)	151.5 (148.7–154.3)

Data are % of patients or mean value (95% confidence interval).

Abbreviations: BO, bronchial obstruction; SpO_2, oxyhemoglobin saturation.

[a] Defined as parental report of previously diagnosed allergy in interview with physician at inclusion.

[b] Defined as coughing, rattling, or respiratory distress.

[c] A clinical score of ≥4 (on a range of 0 to 10, with 0 being the best score) was required for study inclusion.

inclusion, using a tracheal suction set (Unomedical, Lejre, Denmark); were immediately frozen at −20°C; and were transferred without melting to the central storage facility (temperature, −78°C) at Oslo University Hospital within 4 weeks. Each sample was melted and separated into 2 portions, one of which was transported on dry ice for batch analysis at the Department of Allergy, University of Athens. Several participating hospitals performed additional nasopharyngeal sampling and viral analyses by polymerase chain reaction (PCR) and immunoassay as part of their local routines.

Virus nucleic acids were isolated using the QIAamp Viral RNA Mini Kit (Qiagen, Limburg, Netherlands) and carrier RNA (Qiagen) for increased isolation yield of small sequences. Reverse-transcription PCR (RT-PCR) was performed using SuperScript II reverse transcriptase (Invitrogen, Life Technologies, Carlsbad, California) with a starting volume of 10 μL of genetic material in a 20-μL final reaction volume with default reaction conditions (Invitrogen).

Amplification of viral target sequences was performed using dual-priming oligonucleotide and real amplicon amplification technology (Magicplex RV Panel Real-time Test, Seegene, Eschborn, Germany) [31]. This assay detects influenza A virus (including subtypes H5N1 and H1N1), influenza B virus, RSV subtypes A and B, metapneumovirus, adenovirus (all of species B, C, and E and some of species A, D, and F), coronavirus (species 229E, NL63, and OC43), HRV (species A, B, and C), human bocavirus (species 1, 2, 3, and 4), and human parainfluenza virus (species 1, 2, 3, and 4). Samples testing positive for HRV were subsequently subtyped for HRV species A, B, and C, based on the PCR-based assay published by Wisdom et al [32] The assay includes 3 internal controls: a nucleic acid isolation and RT amplification control against the human RNAse P sequence and 2 virus detection controls (one positive and one negative). PCR reactions were performed on the Rotorgene Q 6plex Real-time PCR platform (Qiagen), and results were analyzed using the Seegene Viewer for real-time instruments (Seegene).

Results of real-time PCR analysis were considered positive when the accumulation of fluorescent signal crosses the cycle threshold (Ct); that is, the signal strength required for a detection to be identified. In the assay that we used, for a sample to be considered positive for a specific virus, the cycle threshold for that virus should be crossed before the 20th cycle of amplification, as determined by the manufacturer.

Our protocol included 2 normalization steps. In the first step, we evaluated the RNA isolation and RT-PCR efficiencies. A total of 95% of the RNA/RT-PCR control Ct values followed a normal distribution over a very small range of Ct values (2–3 cycles). Samples with poor RNA isolation/RT-PCR amplification efficiencies (high Ct values) were selected and excluded from the analysis as outliers of the bench protocol (21 samples), since they could heavily bias the clustering procedure. In the second step, we normalized virus-specific Ct values against the EPC (defined as the extraction and PCR controls minus the virus positive control), using the equation $\Delta Ct = Ct_{target} - Ct_{EPC}$, thus defining the change in Ct and allowing comparison of the same viral sequences between different samples. Samples with low ΔCt values represent PCR analyses with a high genomic load for the specific target viral sequence, and those with high ΔCt values represent a low genomic load for the specific target viral sequence. Because the Ct values represent very different actual numbers of microbes for the different virus types, semiquantitative categorization into tertiles or quartiles is common [28]. However, because the different viruses may show a variety of Ct patterns, researcher-driven categorization may be arbitrary and introduce cutoffs that do not harmonize with the natural distribution of viral concentrations. We therefore chose a data-driven approach that used cluster analyses to improve the classification of viral genomic load.

Based on the PCR Ct values (0–20), the algorithm iteratively estimated the cluster means and assigns each sample to the cluster whose mean value was closest to this particular sample. After all of the samples were assigned to clusters, the cluster centers were recomputed until no center changed appreciably or the maximum number of iterations (10) was reached. A preset number of clusters were set to a maximum 5.

Complementary analyses on all outcomes were performed on samples positive versus those negative for the highest-concentration

clusters only. In the present article, analyses were done for high-concentration clusters versus all other clusters (Supplementary Figure 1). The clusters are hereafter referred to as genomic loads, and analyses were performed for high genomic loads versus all other genomic loads.

Outcomes

Disease severity was assessed by the LOS and the level of supportive care, categorized as no supportive care, use of oxygen and/or nasogastric tube feeding, or use of ventilatory support, as previously published by Brand et al [3].

The outcome for modification of treatment effect by the presence of virus was given by the interaction between the presence of virus (RSV, HRV, or multiple viruses) and randomization group (inhaled racemic adrenaline or saline) on LOS.

Statistical Analyses

Continuous data are presented as mean values (95% CIs), and categorical data are presented as numbers and percentages. Categorical data were analyzed with the use of the Pearson χ^2 test. Because data on LOS and level of supportive care had a nonnormal distribution, comparisons between groups were analyzed with the use of a robust, 2-sample *t* test and the Huber M estimator, with 95% CIs. This method was also used to analyze the association between virus subgroups and the level of supportive care as an ordinal outcome (possible scores, 0, 1, 2, and 3). The Holm sequentially rejective multiple test procedure was used [33].

Analysis of viral etiology as an effect modifier of treatment was performed by interaction analyses in a robust linear regression model for the main outcome (LOS).

The level of significance was set at an α of 0.05. Analyses were performed with the use of SPSS, version 21.1 (cluster analyses), and Stata software, version 13.1.

RESULTS

One or more viruses were detected in 91% of patients, with RSV (detected in 83%) and HRV (detected in 34%) detected most frequently. Detection of other airway viruses ranged in frequency from 8%, for influenza B virus, to 15%, for adenovirus and coronavirus. Two or more viruses were detected in 61% of patients, and ≥3 viruses were detected in 30%, with up to 7 viruses detected in a single patient (Figure 2). RSV accounted for 82% of monoinfections (89 of 108). Additional details are available in Supplementary Figure 2 and Supplementary Table 2. Infants with RSV monoinfection were significantly younger and infants with HRV monoinfection significantly older than infants infected with other viruses (Supplementary Table 1).

One or more viruses were found at a high genomic load in 70% of the patients. RSV was found at a high genomic load in 55% of the study population; HRV, in 6%; and other viruses, in 1%–12% (Supplementary Figure 1). Coinfection with viruses at high genomic loads was observed in 18% of patients; ≥3 viruses were detected at high loads in 3%, and, in 1 patient, 4 viruses at high genomic loads were simultaneously detected. RSV accounted for 78% (145 of 186) of monoinfections at high genomic loads. See Supplementary Figure 1 and Supplementary Table 3 for details.

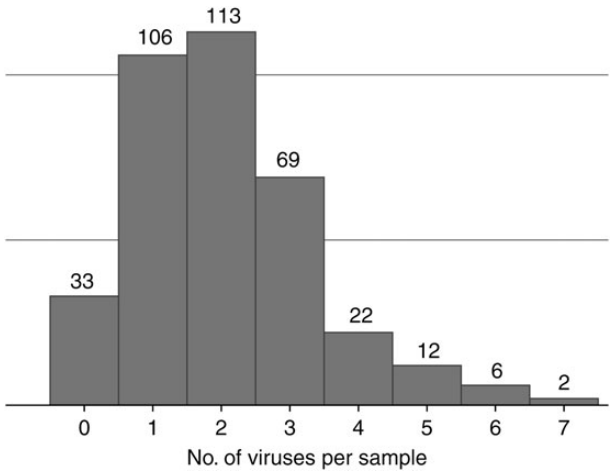

Figure 2. Frequencies of patients, by number of viruses present simultaneously.

Neither LOS nor level of supportive care was associated with the presence of RSV, HRV A/B, HRV C, influenza A virus, influenza B virus, human parainfluenza virus, adenovirus, or bocavirus (Supplementary Table 2). Although LOS was significantly longer in the presence of coronavirus (17.1 hours; 95% CI, 1.2–33.0 hours; $P = .04$) and shorter with the detection of human metapneumovirus (−19.3 hours; 95% CI, −36.0 to −2.5; $P = .02$; Figure 3), the results were no longer statistically significant after adjustment for multiple testing.

However, the presence of RSV at a high viral genomic load was associated with the severity of disease in terms of longer LOS and higher level of supportive care (defined as significantly more use of oxygen and ventilatory support; Supplementary Table 3). No other virus classified with a high genomic load was associated with the severity of disease.

Neither the presence of coinfections in general, the specific combination of HRV and RSV, nor having 1 virus only (ie, monoinfection) were associated with disease severity (Supplementary Table 2 and Figure 3). However, in the analyses of high genomic load only, monoinfection of RSV was associated with an increased level of supportive care (Supplementary Table 3).

Treatment response of inhaled racemic adrenaline in terms of LOS was not modified by the presence of RSV, the presence of HRV, or the presence of ≥2 viruses (all $P > .40$) in regular or high viral genomic load analyses (Supplementary Figure 3).

DISCUSSION

In infants with acute bronchiolitis, we found no association between the presence of 0, 1, or >1 virus (ie, RSV and HRV, RSV

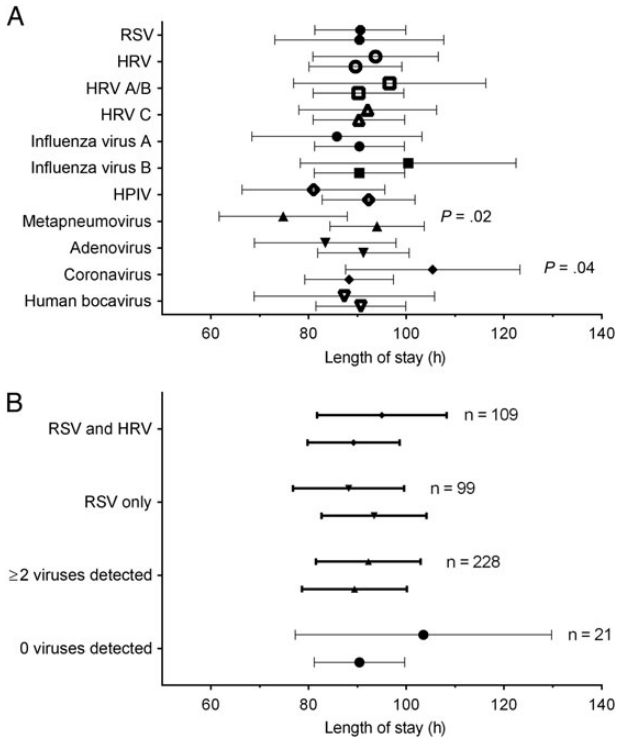

Figure 3. Mean length of stay for 363 infants with acute bronchiolitis, by individual viruses detected (*A*) and viral subgroup (*B*). In panel *A*, pairwise comparisons are shown for a positive (top) versus a negative (corresponding below) result of polymerase chain reaction analysis for each virus. In panel *B*, the patients are stratified by the presence (top) or not (below) of viral subgroups. The estimated mean length of stay was adjusted for age in a robust linear regression model. The associations between type/subgroup of virus and length of stay were no longer statistically significant after adjustment for multiple comparisons. Abbreviations: HPIV, human parainfluenza virus; HRV, human rhinovirus; RSV, respiratory syncytial virus.

only, no virus, or ≥2 viruses) and disease severity after adjustment for multiple testing. A high genomic viral load for RSV only, but not for any of the other viruses or combinations thereof was associated with increased LOS and more use of oxygen and ventilatory support. We found no associations between the presence of single or multiple airways viruses, including RSV and HRV, and the treatment effect of inhaled racemic adrenaline versus saline.

The high rate of virus detection (91%), including RSV in 83% of patients and coinfections in 61%, is in line with a recent study from Finland (86% detection rate) of children with bronchiolitis [34] but is higher than in other studies [3, 19, 30], with coinfection in up to 41% of subjects in studies by Miron et al [1] and Bamberger et al [13]. Interestingly, the Finnish study with a high rate of detection of at least 1 virus reported only 15% of children with >1 virus [34], whereas in the same multicenter study, which also included 16 US study sites, RSV was detected in 67% of 2615 children <2 years of age with severe bronchiolitis, with additional viruses detected in 31% [28]. Further, approximately half of the prematurely born infants aged <6 months of age in Brazil with lower respiratory tract infection and 1 virus identified had coinfection, most commonly with RSV and HRV [35]. Similar to Brand et al [3], but in contrast to Marguet et al [19], we found that infants with RSV monoinfection were significantly younger than infants with coinfection. However, unlike Marguet et al [19], we found that infants with HRV monoinfection were significantly older than infants with all other detected viruses except bocavirus. These apparent discrepancies may be related to study design, as the mean age was lower (2.4 months), and infants with previous wheeze were excluded in the latter study [19].

Coinfection with up to 7 viruses, as well as detection of ≥3 viruses in 30% of enrolled children, have to our knowledge not previously been published. The differences in virus detection may be explained in part by modern and highly sensitive virus detection techniques, a relatively homogenous study population of moderate-to-severely ill infants with a strict definition of acute bronchiolitis, and use of a structured nasopharyngeal aspirate procedure performed by trained and experienced personnel. Also, a large proportion of young Norwegian children (77% aged 1–2 years and 96% aged 3–5 years) attend day care centers [36], which might increase the incidence and morbidity of respiratory virus infections [37] that are subsequently transmitted to their infant siblings.

Our analyses indicated a longer LOS with coronavirus and a shorter LOS with metapneumovirus, in line with a previous report [19]. However, we found no significant association between type of virus or coinfection (with 2–7 viruses) and disease severity or LOS, after adjustment for multiple testing in the present study. This is in contrast to previous reports of studies with small sample sizes [30, 38], as well as in a study of 61 premature infants with severe lower respiratory tract infection in Brazil [35], in which RSV combined with HRV was associated with increased LOS, compared with RSV alone. Our findings are, however, in line with those from a recent, large study that found no association between disease severity and coinfection among 31% of infants infected with RSV and another virus [28]. The clinical relevance of the presence of viruses known to be pathogenic, including influenza virus [39], adenovirus [40], human bocavirus [41], coronavirus [42], human parainfluenza virus [43], and metapneumovirus [44], found in significant rates in our study population (37–56 patients [10%–15%]) are not clear.

Our novel approach with the use of cluster analyses to increase specificity showed that a high genomic RSV load, but not a high HRV load, was associated with an increased severity of disease, in line with previous reports [3, 24–27, 29] and a recent report that included >2000 children and used a tertile approach to categorize genomic RSV load [28]. Cluster analysis has the advantage that it can take into account the pattern of genomic load individually for each virus. The presence of a high genomic load of RSV and HRV in combination was also associated with significantly increased LOS, in line with the findings in premature infants in Brazil. However, we were not

able to investigate whether this was due to coinfection or mainly related to the high genomic load.

In the present study, the percentage of children with >1 virus detected (ie, coinfection) decreased from 61% to 18% when considering viruses detected at a high genomic load only. Identification of viruses does not necessarily indicate a causal association with bronchiolitis, as suggested by the present study. However, the very high rate of RSV infection, the very low rates of monoinfections with viruses other than RSV, and the lack of an association between HRV (A/B or C) or multiple viruses detected and disease severity support the dominating role of RSV in acute bronchiolitis, which is further supported by the association between high concentrations of RSV and disease severity. Although similar rates of HRV detection in young children have been found regardless of the presence of a symptomatic airway infection, as Yoshida et al recently showed in a comparison of hospitalized patients (mean age, 1.5 years) that found HRV in 35% of controls versus 29% of patients and RSV in 3% of controls versus 39% of patients [4], the role of HRV C has been unclear. Bizzintino et al found an association between HRV C and severity of acute asthma [23] in children >2 years of age, while Cox et al [45] found an increased number of hospital admissions among 197 children aged <5 years (mean age, 31.0 months). We were not able to identify sufficiently powered studies of hospitalized infants that assessed disease severity.

The viral genomic load should be compared to the incidence of obstructive airways disease later during follow-up. Nevertheless, we found no significant impact of viral genomic load on the treatment effect of inhaled racemic adrenaline.

The present study found no evidence for an effect modification of inhaled racemic adrenaline by detection of RSV, HRV, or multiple viruses, neither in regular or high-specificity analyses. This is in line with our previous report that showed no effect of inhaled racemic adrenaline in the whole study population [8]. Mansbach et al [12] suggested in 2010 that future clinical trials should categorize results by infectious pathogen, including HRV, because such information was lacking. Our negative result does not support the use of viral etiology as a guide for initiation of bronchodilator therapy.

The present study population was insufficient for robust subgroup analyses for all types of viruses. However, it is likely that analyses of the major viruses, RSV and HRV, are sufficiently large to indicate the lack of clinically relevant associations.

Although interaction tests are considered appropriate for detecting effect modification, it is necessary to increase the size of a study by a factor of about 4 to detect an interaction effect of the same magnitude as a postulated main effect [46]. However, the study population in the present study is large, and we have a high detection rate of the major viruses. Nevertheless, the lack of significant interaction must be interpreted with caution.

In acute bronchiolitis in infants, coinfecting respiratory tract viruses were found in 61% of patients, with up to 7 viruses simultaneously detected. Disease severity was not associated with the identification of virus or with the total number of viruses detected, but a high genomic load of RSV was associated with a longer LOS and more use of oxygen and ventilatory support. Neither the presence of viruses nor the viral genomic load modified the response to inhaled adrenaline therapy.

Supplementary Data

Supplementary materials are available at http://jid.oxfordjournals.org. Consisting of data provided by the author to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the author, so questions or comments should be addressed to the author.

Notes

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H. O. S. conceptualized and designed the study, coordinated and supervised data collection, analyzed data, drafted the initial manuscript, and approved the final manuscript as submitted. S. M. and N. G. P. performed the viral analyses and interpretation of data, reviewed and revised the manuscript, and approved the final manuscript as submitted. P. M. performed the statistical analyses and interpretation of data, reviewed and revised the manuscript, and approved the final manuscript as submitted. K.-H. C. conceptualized and designed the study, analyzed data, reviewed and revised the manuscript, and approved the final manuscript as submitted. K. C. L. C. has been the principle investigator of the study, conceptualized and designed the study, analyzed data, reviewed and revised the manuscript, and approved the final manuscript as submitted. All authors have full insight into all data.

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