Detection of new respiratory viruses in hospitalized infants with bronchiolitis: a three-year prospective study

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Abstract
Aim: We have designed a study with the objective of describing the clinical impact of other viruses different from the respiratory syncytial virus (RSV) in hospitalized infants with bronchiolitis.

Methods: A 3 year prospective study was conducted on infants admitted to the Paediatrics Department of the Severo Ochoa Hospital (Spain). We studied the frequency of 16 respiratory viruses. Clinical characteristics of RSV-only infections were compared with other single agent viral infections. Results: Positive results were confirmed in 275 (86.5%) of the 318 children studied. A single virus was detected in 196 patients and 79 were dual or multiple viral infections. RSV was detected in 61.3% of total bronchiolitis. Rhinovirus (RV) was 17.4% of the identified virus, followed by human bocavirus (HBoV), adenovirus and metapneumovirus (hMPV). Only RV, HBoV and hMPV were significant as single infections. RSV patients were younger than HBoV (p > 0.0001) and hMPV (p = 0.025). Seasonality was clearly different between them. Children with RSV infection needed treatment in the intensive care unit more frequently than others.

Conclusions: In hospitalized infants, RSV was the most frequent agent in bronchiolitis in winter, but other viruses were present in 47% of the patients. RV, HBoV and hMPV had a significant proportion of single infections. Clinical characteristics were similar amongst them, but seasonality was clearly different.

INTRODUCTION
Acute bronchiolitis is the most common lower respiratory tract infection in infants. It is the leading cause of hospitalization of infants under 1 year old, and more than 80% of children hospitalized are under the age of 6 months. Respiratory syncytial virus (RSV) is the most frequent viral pathogen responsible for bronchiolitis worldwide. Outbreaks of RSV at epidemic levels occur yearly at predictable times (1,2).

In the last few years several new respiratory viruses have been detected in infants and children with lower respiratory tract diseases. Human metapneumovirus (hMPV), first described in The Netherlands (3) plays an important role in the paediatric respiratory infections. Most studies have been performed in hospitalized patients and data show that this virus is prevalent amongst patients with acute bronchiolitis or recurrent wheezing (4–6). Human bocavirus (HBoV) was detected in respiratory tract samples of Swedish infants and children (7) and since then, has been detected worldwide (8–10). Previous studies have shown that rhinovirus (HRV) is one of the most frequent agents in paediatric respiratory infections, second only to RSV (11,12).

The specific role of these new viral agents in acute bronchiolitis has been insufficiently investigated, especially in dual or multiple viral detections. The detection of more than one viral pathogen may influence the natural history of acute bronchiolitis. We have designed a prospective study with the objective of describing the clinical impact of the different respiratory viruses detected in hospitalized infants with acute bronchiolitis in Spain. To clarify if non-RSV infections or multiple viral infections have some singular characteristics, clinical and epidemiological features of RSV-single infections have been compared firstly, with the most prevalent respiratory virus-single bronchiolitis in the same population, and secondly with a multiple-viral detections bronchiolitis group.

MATERIALS AND METHODS
Clinical assessment
The study population comprised infants who were diagnosed with acute viral bronchiolitis and were admitted to the secondary public hospital Severo Ochoa (Leganés, Madrid), between September 2005 and August 2008. The study was approved by The Medical Ethics Committee. Informed consent was obtained from parents or legal guardians. All patients were evaluated by an attending physician.

During the hospital stay, and as part of the study, a physician filled out a study-questionnaire with the following variables: age, gender, clinical diagnosis, history of prematurity and underlying chronic diseases, need for oxygen therapy assessed by transcutaneous oxygen saturation, axillary
temperature ≥38°C, presence of infiltrates/atelectasis in radiographs, administration of antibiotic therapy, duration of hospital stay, total white blood cell count, C reactive protein (CRP) serum values and result of blood culture if performed. Oxygen therapy was provided to achieve oxygen saturation ≥94%.

Acute bronchiolitis, was diagnosed as the first episode of acute onset expiratory dyspnea with previous signs of viral respiratory infection – whether or not this was associated to respiratory distress or pneumonia in children under 2 years old. Children with wheezing, breathlessness and obstruction of the airways, in whom similar episodes had previously been diagnosed and treated by a physician, were diagnosed as recurrent wheezing and were excluded.

Virus detection
Specimens from patients consisted of nasopharyngeal aspirates (NPA) taken from each patient at admission (Monday through Friday). NPA from patients admitted Saturday and Sunday were taken next Monday in the morning. Each specimen (one for each patient) was sent for virological investigation to the Influenza and Respiratory Virus Laboratory at the National Microbiology Center (ISCIII, Madrid, Spain). Specimens were processed within 24 h after collection. Upon receipt of NPAs, three aliquots for each were prepared and stored at −70°C. Both, the reception and the NPA sample aliquoting areas were separate from those defined as working areas.

Nucleic acid extraction
Total nucleic acids were extracted from 200 µl aliquots using QIAamp MinElute Virus Spin Kit in the QIAcube automated extractor (Qiagen, Valencia, CA, USA). A total of 100 molecules per tube of an internal control were included in the lysis buffer for checking both the efficiency of acid nucleic extraction and presence of amplification inhibitors.

PCR methods for detection of sixteen respiratory viruses
Three RT-nested PCR assays were performed to detect a total of 16 respiratory viruses. In these assays, reverse transcription (RT) and first round amplification were carried out in a single tube using the Qiagen OneStep RT-PCR kit (Qiagen). Influenza virus A, B and C were detected using previously described primer sets only to amplify influenza viruses in a multiplex PCR assay (13). A second multiplex PCR was used to detect parainfluenza viruses 1–4, human coronaviruses 229E and OC43, enteroviruses and rhinoviruses (14). The presence of RSV-A and B types, hMPV, HBoV and adenoviruses was investigated by a new third multiplex RT-nested PCR (BRQ method) developed specially for this study, based on gene targets previously described in other PCR assays published by our group (13–17). Primer sets for each virus were chosen from nucleotide sequences specific for the fusion protein gen (RSV-A and RSV-B), for the matrix gen (hMPV), for the NP1 and VP1/VP2 genes (HBoV) and for the hexon protein gen (ADV).

Appropriate precautions were implemented to avoid false positive results by carryover contamination. Positive results were confirmed by testing a second aliquot of the sample.

Statistical analysis
Clinical characteristics of acute bronchiolitis associated to RSV were compared with those associated with single infections of HRV, HBoV and hMPV infections. Patients with viral co-infections were also compared with those with a single one. The RSV- single group was compared with the RSV-multiple group. Values were expressed as percentages for discrete variables, or as mean and standard deviation for continuous variables. Clinical characteristics and laboratory variables were compared using the Student t-test, the Mann–Whitney U-test, the χ² test and Fisher’s exact test. A two-sided value of p < 0.05 was considered statistically significant. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) for windows, version 13.0 (SPSS Inc., Chicago, IL, USA).

RESULTS
Patient characteristics and screening of viruses
The study population consisted of 370 hospitalized children under 2 years old, with a clinical diagnosis of acute bronchiolitis. As 52 patients were excluded either because of lack of NPA samples or because they refused to participate, a total of 318 episodes were analysed. Clinical characteristics of the total population studied are described in Table 1. Seven children were admitted in the intensive care unit. Six of them were under 2 months old.

At least one respiratory virus was detected in 275 NPA samples (86.5% of the 318 tested), of which 196, corresponding to 196 different episodes, were single virus infections (71.2%), 79 children presented dual or multiple viral infection (28.7%) and 43 were negative for the 16 respiratory viruses tested. Specific viruses detected and identified in the total population of 318 children are listed in Table 2, in descending order of frequency. RSV was detected in 195 patients (61.3% of total bronchiolitis), but only in 137 patients as a single infection (69.9% of single infections, Table 1).
RSV-single infections group). Five children of seven admitted in the intensive care unit were RSV positive (one co-infection with HRV) and the other two were negative.

Clinical findings associated with the presence of RSV, HRV, HBoV and hMPV

Bronchiolitis in 196 infants was associated to a single virus. RSV was present in 137 of those patients (69.3%). Clinical characteristics of the RSV-single infections compared with the single infections associated to other prevalent viruses detected, HRV (n = 24, 12.2%), HBoV (n = 14, 7.1%) and hMPV (n = 11, 5.6%), are shown in Table 3.

**HBoV vs. RSV**

Patients with HBoV-single infection were significantly older ($p < 0.0001$) than those 137 with RSV infections (290.2 ± 158 vs. 124.9 ± 110 days). Half of the patients with HBoV were under 12 months old (ranging from 67 to 657 days) and half of the patients with RSV infection were under 81 days old (ranging from 6 to 494 days). Table 3 shows age differences amongst groups. Serum CRP was higher in the HBoV group ($p = 0.05$).

**HRV vs. RSV**

With the exception of hypoxia, no significant clinical differences were found between RSV- and the HRV-single infections. Patients with HRV infection required administration of oxygen less frequently than the RSV group (41.7% HRV vs. 78.6% RSV, $p = 0.05$).

**hMPV vs. RSV**

The patients with hMPV-single infections had a higher blood leucocytes count ($p = 0.017$), and CRP was more elevated than in the RSV group ($p = 0.03$). They were also significantly older than the RSV-single group ($p = 0.025$). Half of the patients with hMPV were less than 191 days (around 6 months old; range from 39 to 313 days). No other clinical differences were found between them.

**Monthly distribution of single infections**

RSV-single bronchiolitis was more frequent in winter, especially in December. HBoV cases showed a similar distribution in winter but had a second peak in spring (February and March) $p = 0.023$. Nevertheless, during September all cases were associated to HRV-single infection, although HRV infections occur through all the year. Data are significantly different to RSV ($p < 0.0001$). The highest activity of hMPV was observed from February to March. Furthermore, in March, 56% of the bronchiolitis episodes were associated with hMPV infection. So, hMPV distribution was clearly different to the RSV pattern ($p < 0.0001$). Monthly distribution is expressed in Figure 1.

**Multiple viral infections vs. virus-single infections groups**

No significant clinical differences were found between both groups, with exception of age. Patients with single infections were younger than infants with multiple viral infections (any combination), (158 ± 176 days vs. 214 ± 168 days, $p = 0.017$). Patients with ADV detection had co-infections

### Table 3

| Clinical features                  | RSV (n = 137) | HBoV (n = 14) | RV (n = 24) | hMPV (n = 11) |
|-----------------------------------|--------------|--------------|------------|--------------|
| Male                              | 73 (53.5%)   | 9 (64.3%)    | 15 (62.5) | 8 (72.7)     |
| Prematurity                       | 15 (11)      | 3 (21.4)     | 2 (8.3)   | 1 (9.1)      |
| Temperature >38°C                 | 86 (62.8%)   | 9 (64.3)     | 11 (45.8) | 8 (72.7)     |
| Hypoxia (SatO2 <95%)              | 85 (62.5%)   | 11 (78.6%)   | 5 (41.7)  | 5 (45.5)     |
| Abnormal chest radiograph         | 45 (32.8%)   | 5 (35.7)     | 4 (16.7)  | 2 (18.2)     |
| Antibiotic treatment              | 15 (10.9%)   | 3 (21.4)     | 3 (12.5)  | 3 (27.3)     |
| Highest temperature               | 38.3 ± 0.8   | 38.2 ± 1.1   | 38.2 ± 0.6| 38.8 ± 0.6  |
| Leucocytes (cells/mm³)            | 11 633 ± 4188| 11 295 ± 3480| 11 766 ± 3908| 15 356 ± 3957* |
| Serum C reactive protein          | 30.2 ± 40.5  | 290.2 ± 188  | 228 ± 351 | 177 ± 81.9** |
| Age (days)                        | 124.9 ± 110  | 81           | 248        | 81           |
| Percentile 50                     | 2.9 ± 2.2    | 2.8 ± 1.4    | 2.8 ± 1.4 | 2.8 ± 1.4    |
| Hospital stay (days)              | 5 ± 2.3      | 5.6 ± 2.1    | 4.3 ± 2.4 | 5.3 ± 2.5    |
| Fever duration (days)             | 2.9 ± 2.2    | 4.1 ± 2.2    | 1.8 ± 2.0 | 2.8 ± 1.4    |
| Hypoxia duration (days)           | 2.2 ± 2      | 2.8 ± 1.4    | 2.0 ± 1.6 | 3.2 ± 1.4    |

*RSV = respiratory syncytial virus; HRV = human rhinovirus; HBoV = human bocavirus; hMPV = human metapneumovirus. Significant differences are in presented in bold font. Values in parentheses are in percentage.

* $p = 0.05$, † $p < 0.0001$, ‡ $p = 0.05$, § $p = 0.017$, ¶ $p = 0.03$, ** $p = 0.028$. 

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in 78.6% of the cases, vs. 29.7% of infants with bronchiolitis associated to RSV (OR: 3.2, IC: 1.1–8.9). Only three patients were exceptionally ADV-single infections.

As the above two groups were heterogeneous, the RSV-single infection group was compared with the RSV multiples infection group. Again, the only difference was the age between them (124 ± 110 days for the RSV group vs. 199 ± 172 days for the co-infections group, p = 0.02).

**DISCUSSION**

Bronchiolitis, a viral associated condition, defined as the first episode of acute respiratory wheezing in infants under 2 years old, is a major cause of hospitalizations around the world. In our study, a viral agent was identified in 86.5% of the patients. RSV was the most prevalent virus (61.3% of hospitalized infants), but in 47% of the patients viruses other than RSV were detected and identified. The role of these other respiratory viruses as single or as multiple viral infections in bronchiolitis has not yet been thoroughly studied.

A major strength of this study is the use of three sensitive PCR assays, two of which have been previously published (13–17). For this study a third RT-PCR was optimized using primer sets, partially or completely published by our group, for the specific detection of viruses traditionally involved in bronchiolitis such as RSV, hMPV, ADV and the new HBoV. A very high sensitivity and specificity for a complete range of respiratory viruses over three full calendar years were obtained using these RT-multiplex assays simultaneously. Except for CoV-NL63 and HKU1 and the recently identified polyomavirus KI virus (KIV) and WU virus (WUV), the rest of the respiratory viruses were successfully detected and identified. These technologies allow us to attribute the presence/absence to each of the sixteen different viruses or group of viruses (HRV, ADV). In addition, we studied the viral circulation for three consecutive years, which compensates for annual variations in viral circulation. This fact also explains the high percentage of viral agents identified (86.6%), higher than in other published studies (18).

Multiple viral infections were present in 22.8% of the patients with positive viral detection. It is a very similar percentage to the one found by other authors such as Andreoli (19), Papadopoulos (12) and Jacques (18). The role of viral co-infections is still unclear, and different opinions regarding their severity are found in literature. Whereas Papadopoulos (12) found increased severity in co-infected patients, other authors did not find clinical differences (20). Aberle (21) found small differences in dual and single infections in infants, namely more hypoxia and longer stays in multiple viral infections and Gerna (22) did not find a different hospital stay in RSV or co-infected bronchiolitis. Previously, our group conducted another prospective study in hospitalized infants with respiratory viral co-infections and these patients were associated with no more complications or increased severity than higher fever, prolonged stays in hospital and more frequent use of antibiotics than for single infections (23). In this study, no significant clinical differences were found between both groups, with the exception of age. This result confirms previous data found and it is in agreement with other authors.

Respiratory syncytial virus and HBoV related single bronchiolitis occurred mainly in November and December although HBoV had another epidemic peak in spring. Von Linstow (24), found a similar monthly distribution for HBoV infections in Danish infants, although this cohort was composed by non hospitalized infants under 1 year old. Brie (25), in French children, had similar data to ours. Rhinovirus infections were present throughout all the year, although the highest overall activity was observed during September. During this month only HRV bronchiolitis was detected. Circulation of hMPV was also different to other viruses and was predominant in February and March which is consistent data in the literature (26,27). Bronchiolitis is associated to different viruses in winter, fall or spring and RSV does not appear out of winter in Spain.

Clinical characteristics of bronchiolitis are similar when associated either with RSV or any other virus, but the age of presentation is different. Five patients with RSV infection were admitted in the intensive care unit and no other respiratory virus was co-detected. These severe cases were under 2 months old. Children with RSV were younger than HBoV and hMPV-single infection groups. In previous reports of a large number of infants with hMPV infections, over a long period of time, these data were confirmed (27). HBoV bronchiolitis was present more frequently in older infants than RSV. This is consistent with other authors (28), with very similar results to ours except in the seasonality (more frequent in July, August and September, winter in New Zealand). HBoV was present in 3.5% of bronchiolitis in this series and in 1.4% in our patients. In our series, patients with HRV infections need oxygen less frequently than those with RSV, this being the only difference between these groups. The proportion of HRV infections is quite similar to other epidemiologic studies in which HBoV were not investigated (29).
In conclusion, although RSV was the most frequent agent in patients with diagnosis of bronchiolitis in winter, other viruses were present in 47% of the cases. Clinical characteristics were similar amongst them, with small variations but seasonality was clearly different. HBoV appears mainly in December and spring, hMPV in spring and HRV in fall. Single infections were frequent and support the pathogenic role of these viruses in acute viral bronchiolitis. Dual and multiple detection of different viruses in patients with acute bronchiolitis was obtained in nearly a quarter of the patients without further severe clinical characteristics. Children with RSV single infection needed treatment in the intensive care unit more frequently than others. The multiplex RT-nested PCR described in this study provides a rapid, sensitive and specific approach to diagnose the most important viruses involved in bronchiolitis in one reaction tube, yielding relevant information for patient management and treatment, if applicable.

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