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Multiple simultaneous viral infections in infants with acute respiratory tract infections in Spain

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Abstract

Background: The clinical significance of the presence of more than one type of virus in the respiratory specimens of children with respiratory infections is not clear.

Objectives: To describe the clinical characteristics of multiple viral infections versus single infection by respiratory syncytial virus (RSV) in hospitalized infants.

Study design: This is a prospective study conducted in all infants under 2 years of age admitted for acute respiratory infection (September 2000–June 2003) in a secondary teaching hospital. Virological diagnosis was made by two different multiplex reverse transcription-nested polymerase chain reaction (RT-PCR) assays in nasopharyngeal aspirates. We describe the clinical characteristics of the patients with multiple viral infections and compare them to a group of 86 randomly selected patients infected only with RSV.

Results: 749 specimens taken were analyzed. Respiratory viruses were detected in 65.9% of the samples. 86 children had multiple viral infections (17.4% of all positive specimens). The most frequent clinical diagnosis in this group was recurrent wheezing in 44% and bronchiolitis in 52%. Fever was significantly more frequent ($p < 0.001$), hospital stays were longer ($p = 0.05$), and antibiotic treatment was used more ($p = 0.03$) in infants with multiple viral infections than in the RSV-infected group.

Conclusions: Multiple viral infections are frequent in hospitalized children with respiratory tract disease (17.4%). Multiple viral infections are linked to higher fever, longer hospital stays and more frequent use of antibiotics than in the case of infants with single RSV infections.

Keywords: Multiple viral infections; Nasopharyngeal aspirates; Hospitalized children; Respiratory syncytial virus; Multiplex polymerase chain reaction

1. Introduction

Acute lower respiratory tract infections (ALRI) are common diseases among children. Viral pathogens play an important role in infants who present with ALRI (Jennings et al., 2004; Iwane et al., 2004). Respiratory syncytial virus (RSV) is the most important viral pathogen in infancy. In addition, children with RSV infections are also exposed to a variety of other viruses with a similar seasonal pattern, mainly during the winter months, such as influenza, parainfluenza, rhinoviruses and human metapneumovirus (hMPV). Despite the fact that numerous studies have revealed that some ALRI pediatric patients become simultaneously infected with multiple respiratory viruses, most studies fail to discuss the issue in depth. Whereas some of these papers show a worse ALRI prognosis with multiple viral infections (Tristram et al., 1988; Semple et al., 2005), others reveal a very similar prognosis relative to ALRI with a single virus (Kellogg, 1991; Huang et al., 1998; Subbarao et al., 1989).

Over the past few years, several groups, including ours, have described multiplex reverse transcription-nested polymerase chain reaction (RT-PCR) assays that are capable of simultaneously detecting and identifying different respira-

Abbreviations: ALRI, acute lower respiratory tract infections; RSV, respiratory syncytial virus; RT-PCR, reverse transcription-nested polymerase chain reaction; hMPV, human metapneumovirus.

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tory viruses (Gröndahl et al., 1999; Weigl et al., 2000; Ong et al., 2001; Legg et al., 2005).

A prospective study was undertaken to determine both the prevalence of multiple viral respiratory infections in hospitalized infants with ALRI and the effect of the detection of multiple viruses on the severity of disease. Since RSV is the most frequently identified pathogen in this population, patients infected by two or more viruses were described and compared to patients infected only by RSV.

2. Patients and methods

2.1. Study population

This is a substudy of an ongoing prospective investigation on respiratory tract infections in children under 2 years of age, funded by the Spanish Health Research Fund. The study was conducted at the Severo Ochoa Hospital Pediatrics Department in Madrid, Spain. Children under 2 years of age were recruited who were consecutively admitted to our hospital for acute respiratory infections from September/2000 to June/2003. Informed consent was obtained from each child's parents or legal guardians, and the study was approved by the Ethics Committee of the hospital.

2.2. Virological study

Nasopharyngeal aspirate (NPA) specimens were taken from each patient upon admission (from Monday to Friday), and were sent in viral transport medium (MEM, Gibco-BRL, Life Technologies, Paisley, Scotland; penicillin 200 U/ml, and streptomycin 200 μg/ml, BioWhittaker, MA; mycostatin 200 U/ml, Sigma; bovine serum albumin 0.25%, Merck, Darmstadt, Germany) for virological testing at the Influenza and Respiratory Virus Laboratory (National Centre of Microbiology, ISCIII, Madrid, Spain). Specimens were processed within 24 h of collection. Upon receipt of NPA, samples were aliquoted in duplicates at 200 μl and one of them immediately processed. The second aliquot and the remaining specimen were stored at −80 °C. Nucleic acids were extracted from the first aliquot of each NPA specimen using a modified method previously published (Casas et al., 1995). Specimens were screened for the presence of 15 different respiratory viruses as previously published. Briefly, two multiplex reverse transcription-nested polymerase chain reaction (RT-PCR) assays were used. The first assay was designed for the detection of specific viral genomes belonging to respiratory syncytial virus types A and B, adenoviruses and influenza types A, B and C (Corias et al., 2003). The second assay was designed to detect parainfluenza viruses (types 1–4) and human coronavirus (229E and OC43), as well as to detect enterovirus and rhinovirus polyprotein genes (Corias et al., 2004). Detection of human metapneumovirus was performed using two independent RT-nested PCR assays which spanned different genes: the matrix protein (M) and the viral polymerase (L), as described elsewhere (López-Huertas et al., 2005).

2.3. Confirmation of PCR results

The reception area and the NPA sample aliquoting area were kept separate from the working areas. Results were considered positive when concordant PCR results were obtained in two different aliquots. Each positive sample in the first batch of test samples was extracted and amplified twice. If a positive result remained 'unconfirmed' after this protocol was followed, the result was reported as negative.

2.4. Clinical assessment and statistical analysis

Upon enrollment, 2 pediatricians in charge of the daily clinical follow-up of the patients used standardized written questionnaires for systematic recording of each patient's demographic and clinical characteristics.

Clinical characteristics of dual or multiple infections were compared with those associated with the most frequent single virus infection: RSV. A random sample of 86 hospitalized infants aged <2 years and documented as RSV-infected was selected from the hospitalized RSV-infected infants taken from the same population and seasons as those children diagnosed with coinfections. Furthermore, patients RSV-infected were compared too with patients with coinfection of RSV and one or more additional viruses.

2.5. Statistical analysis

Values are expressed as percentages for discrete variables, or as mean and standard deviation for continuous variables. Clinical characteristics and laboratory variables are compared using the Student's t test, the Mann–Whitney U test, the χ^2 test, and Fisher's exact test. A two-sided value of $p < 0.05$ is considered statistically significant. All analyses are age-adjusted. Furthermore, a multivariate backward stepwise logistic regression model is used to calculate the adjusted odds ratio (OR) with 95% confidence intervals, in order to estimate the association between different factors and coinfections. Variables with a p-value <0.1 are introduced in the regression model. These variables are removed if the p-value of the change in the −2 log likelihood is >0.05 when the variable is removed. All analyses are performed with the Statistical Package for the Social Sciences (SPSS), Version 11.0.

3. Results

3.1. Study population and clinical features associated with multiple viral infections

The study population included 749 hospitalized children under 24 months of age with ALRI. At least one respira-

Table 1
Viral agents identified in children with multiple infections ($n = 86$)

Number of patients	
2 viral agents	70
3 viral agents	12
4 viral agents	4
Number of isolates ($n = 192$)	
RSV	68 (35.4%)
Adenovirus	37 (19.3%)
Rhinovirus	26 (13.5%)
HMPV	19 (9.8%)
Influenza A	16 (8.3%)
Influenza C	3 (1.5%)
Parainfluenza	8 (4.1%) (type 1: 3, type 2: 2, type 3: 1, type 4: 2)
Enterovirus	9 (4.6%)
Coronavirus	1
Cytomegalovirus	1
Rotavirus (feces)	4 (2%)
More frequent double infections	
RSV + adenovirus	10
RSV + enterovirus	9
RSV + HMPV	8
RSV + influenza	8
RSV + rhinovirus	7
HMPV + adenovirus	13

RSV: Respiratory syncytial virus; HMPV: human metapneumovirus.

tory virus was detected in 494 specimens (65.9%). RSV was detected in 76.1% of all positive cases.

Two or more viral agents were identified in 86 patients (Table 1; 17.4% of all positive specimens). There were three and four different viruses identified in twelve and three patients, respectively. Four of these children presented rotavirus in their feces. RSV was the most frequently isolated agent, followed by adenovirus and rhinovirus.

Clinical data from the patients with multiple viral infections are shown in Table 2. Two children were admitted to the intensive care unit due to severe respiratory deficiency.

3.2. Comparison to the RSV-positive control group

Clinical data for patients with simultaneous multiple viral infections was compared to that of 86 RSV-infected patients. No significant differences were found between the groups regarding sex, the need for oxygen therapy, the presence of infiltrate/atelectases in chest X-rays, concomitant diseases or admission in the intensive care unit. Bacterial cultures were performed on demand from the attending clinician. These analysis were performed in 13.9% and 16.2% of the coinfection and RSV groups respectively. They were all negative with the exception of a patient from the VRS group who presented *S. pneumoniae*, without radiological infiltrate.

In the univariate analysis, patients with multiple viral infections had a higher incidence of fever (80.2% vs. 54.7%; $p = 0.0001$), a longer length of hospitalization (6 days vs. 4.8 days; $p = 0.05$), and received antibiotic treatment more frequently (26.7% vs. 14%; $p = 0.03$), compared to the RSV-infected control group. Patients with multiple infections were older (7.2 months vs. 6.2 months; $p = 0.012$) and were diagnosed with recurrent wheezing more frequently (44.2% vs. 24.4%; $p = 0.01$) than those in the RSV-infected group. White blood cell count and C-reactive protein values were found to be different between the groups, as shown in Table 2.

Differences between patients RSV alone infected and patients with coinfection of RSV and one or more additional viruses were similar to previously described. Coinfected group had higher incidence of fever (82.3% vs. 54.7%; $p < 0.001$; OR 2.36 (1.35–4.12)), received antibiotic treatment more frequently (27.4% vs. 14%; $p = 0.042$; OR 2.3 (1.05–2.27)) and had a longer length oh hospitalization (6 days vs. 4.8 days; $p = 0.057$). White blood cells were higher in coinfected group ($p = 0.012$).

The variables included in Table 2 with a p-value <0.10 were entered into a logistic regression model. This analysis shows that after adjustment, coinfections were associated more frequently with higher fever (OR: 2.90; CI95% 1.45, 5.94) and longer hospital stays (OR: 1.12; CI95% 1.0, 1.23) than single RSV infections. Age adjusted OR was 1.02 (CI95% 1.0, 1.04). Antibiotic treatment and clinical diagnosis did not reach statistical significance.

4. Discussion

In the literature, dual viral infection is found in 5–20% of infected patients (Huang et al., 1998; Weigl et al., 2000; Ong et al., 2001; Brandt et al., 1986; Aberle et al., 2005), while multiple viral infections with three or more agents are considered anecdotal (Jennings et al., 2004; Huang et al., 1998). These rates of coinfection are similar to those found in this series (17.4% of all positive samples), although the rate was higher when sensitive PCR techniques were used which allow for the detection of a larger number of viruses (Subbarao et al., 1989; Legg et al., 2005; Brouard et al., 2000).

The introduction of multiple PCR techniques has become a major breakthrough for the diagnosis of respiratory viral infections, and has revealed some highly frequent infections that previously remained under-diagnosed, including those caused by rhinovirus and human metapneumovirus, both of which are difficult to develop in cell culture (Billaud et al., 2003; Kotaniemi-Syrjänen et al., 2003; Van Den Hoogen et al., 2003; Xepapadaki et al., 2004) The situation is similar for multiple viral infections, where multiplex PCR techniques have shown a high incidence of viral coinfections. These techniques have been validated by various research groups, reaching very high levels of sensitivity and specificity of about 95% (Jennings et al., 2004; Gröndahl et al., 1999). Our techniques have also been validated and published as a highly useful alternative in establishing virological diagnoses for respiratory infections (Corias et al., 2003, 2004; López-Huertas et al., 2005).

This series presents distinct clinical characteristics for groups with multiple infections and single RSV infection. Fever was more frequent in patients with multiple viral infec-

Table 2
Comparison between patients with multiple viral infections and the simple RSV control group

	Multiple infections $N=86$	VRS $N=86$	p	OR
Age (days)	262.7 ± 213	188.4 ± 166	0.01	
<5.7 months	36 (41.9%)	50 (58.1%)	0.03	0.72 (0.53–0.97)
Male	53 (61.6%)	51 (59.3%)	NS	
Temperature ≥38 °C	69 (80.2%)	47 (54.7%)	0,001	1.95 (1.28–2.95)
Hypoxia (sat. O_2 < 94%)	61 (70.9%)	53 (61,6%)	NS	
Length of hospital stay (days)	6 ± 5.4	4.88 ± 2.24	0.05	
Infiltrate/atelectasis	30 (34.9%)	31 (36%)	NS	
Prematurity	12 (14%)	9 (10.5%)	NS	
Underlying diseases	4 (4.6%)	4(4.6%)	NS	
Antibiotic treatment	23 (26.7%)	12 (14%)	0.037	1.4 (1.05–1.93)
Recurrent wheeze	38 (45.8%)	22 (25.6%)	1.61	0.42 (1.1–2.36)
Bronchiolitis	45 (54.2%)	61 (74.4%)	0.007	0.65 (0.49–0.88)
Serum CRP	33 ± 38	55 ± 108	0.02	
White blood cell/mm^3	13872 ± 6348	12047 ± 4746	0.03	

RSV: Respiratory syncytial virus; OR: odds ratio; CRP: C reactive protein; NS: non significative. Underlying diseases: 2 cardiac diseases (myocardiopathy and interauricular communication) and 2 chronic pulmonary diseases in multiples viruses group. 3 interauricular communications and 1 sickle cell anaemia in RSV group.

tions, and this likely contributes to the more frequent use of antibiotic treatment and longer duration of hospitalization. In fact, the need for oxygen therapy, the frequency of radiological infiltrates and the rates of admission to the intensive care unit were similar in both groups. Although analytical differences between the two groups were detected, their clinical meaning remains unclear.

In the majority of studies, no clinical differences were found among patients with respiratory infections caused either by a single viral agent or by two or more viral agents, either for hospitalized patients (Huang et al., 1998; Brouard et al., 2000) or for ambulatory children (Legg et al., 2005). Subbarao et al. (1989) published a retrospective study of 2415 patients (both hospitalized and ambulatory) with respiratory infections, and found 666 children with RSV infection and 51 patients (7.6%) with double infections. A clinical severity score was performed, and no differences were found between these groups. These authors did not use PCR techniques, which may explain the low rate of coinfection. In a prospective study using PCR techniques on samples from infants admitted for bronchiolitis, Brouard et al. (2000) found that up to 27% of patients had coinfections. Again, no clinical differences were found between the groups. Legg et al. (2005) conducted a study in infants with respiratory infection that were receiving outpatient treatment. This study also used PCR techniques and obtained a 20% rate of double infections; moreover, there was not an increased risk of hospitalization or clinical severity. Special attention should be paid to Semple et al. (2005) which suggests that coinfection with RSV and hMPV is linked to a greater risk of requiring mechanical ventilation than simple RSV infection (18 patients vs. 7 patients) in patients admitted to the intensive care unit. However, the authors were unable to find such a difference between simple (RSV) or dual (RSV and hMPV) infections in patients admitted to general wards classified either as moderate (i.e., requiring admission to the hospital without oxygen therapy) or as severe (requiring oxygen therapy). Our population is probably similar to this last group of patients admitted to general wards, and is not comparable to infants admitted to intensive care unit for mechanical ventilation. Further studies with series of infants with hMPV infection have been unable to confirm these results. In previous studies conducted by our group, which involved more than 1000 infants admitted to the hospital over 5 epidemic seasons, no differences were found (García et al., 2006; García-García et al., 2006).

A very interesting study by Aberle et al. (2005) included a significant number of infants with dual infections, and assessed the clinical course of the disease. The clinical symptoms in patients with dual infections included more frequent fever, more hypoxia (patients with RSV infections) and a longer hospital stay. Specific clinical features of RSV infections were dominant when this virus was present. Our series is very similar to this series, except for the presence of hypoxia. hMPV infections were not assessed in Aberle's study.

The major strength of this study is its prospective design for studying multiple viral infections using validated multiplex RT-PCR techniques that detect most traditionally identified respiratory viruses, such as RSV, Influenza, adenoviruses, parainfluenzaviruses, enteroviruses and rhinoviruses. This study was retrospective for the detection of hMPV, discovered in 2003, and no data was produce for the newly describe coronaviruses NL63 and HKU-1, and the human bocavirus. In general, the major limitation of PCR techniques is that the presence of nucleic acid of a viral pathogen is not in itself evidence of an active infection. Due to its high sensitivity, PCR might detect low amounts of viral genome from past infections. This could eventually explain the very few clinical differences between simple and multiple infections since most of them would be simple ones with rests of previous infections. This limitation is difficult to overcome at the moment since most of these viruses are difficult to grow in a cell culture. The development and validation of

quantitative PCR, as well as its correlation with the clinical symptoms, might become a useful tool to clarify the actual role of multiple infections. This is one of the main goals of our research group at this moment.

The inclusion of PCR-based diagnostic strategies might be of great help in identifying the true relevance of multiple viral infections, as well as the characteristics and severity associated with this condition. In our experience, multiple viral infections are associated with high fever, prolonged stays and more frequent use of antibiotics in hospitalized infants.

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