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Detection and genotyping of human respiratory viruses in clinical specimens from children with acute respiratory tract infections

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ABSTRACT

Respiratory virus infections are a major health concern and represent the primary cause of testing consultation and hospitalization for young children. The application of nucleic acid amplification technology, particularly multiplex PCR coupled with fluidic or fixed microarrays, provides an important new approach for the detection of multiple respiratory viruses in a single test. The aim of this study was to analyze respiratory samples from children with acute respiratory tract infection (ARTI) using a commercial array-based method (CLART® PneumoVir Genomica, Coslada, Spain). These tests were used to identify viruses in 281 nasopharyngeal samples obtained from children affected by ARTI. Samples were obtained form October 2008 to April 2009. Viruses were identified in 80% of the studied ARTI providing useful information on clinical features and epidemiology of specific agents affecting children in cold months. Multiple viral infections were found in 33.45% of the specimens.

Key words: Pediatric, ARTI, Respiratory viruses.

Detección y genotipado de virus respiratorios humanos en muestras clínicas de niños con infección respiratoria aguda

RESUMEN

Las infecciones por virus respiratorios representan la primera causa de consulta y hospitalización en la población pediátrica. El empleo de técnicas moleculares, principalmente aquellas basadas en PCR múltiple acoplada a detección por microarrays, supone un importante avance para la detección de varios de estos virus en un único ensayo.

El objetivo de este estudio ha sido el análisis de muestras respiratorias procedentes de niños con infección respiratoria aguda (ARTI) mediante un método comercial (CLART® PneumoVir). Este método se basa en la amplificación y detección por microarrays de los 17 virus humanos más frecuentes en este tipo de patología. El ensayo se ha llevado a cabo en 281 muestras nasofaríngeas provinientes de niños con ARTI que acudieron al Hospital Clínico San Carlos de Madrid entre Octubre del 2008 y Abril del 2009. El 80% de las muestras estudiadas presentaron un resultado positivo para, al menos, uno de los 17 virus analizados proporcionando una valiosa información sobre las características clínicas y epidemiológicas de los agentes específicos que afectan a la población pediátrica en los meses fríos. Gracias a la técnica empleada pudieron detectarse infecciones múltiples en el 33,45% de las muestras.

Palabras clave: Virus respiratorios, infección respiratoria aguda.

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INTRODUCTION

Acute respiratory infection is an extremely common disease and the type of infection varies according to factors such as age, environment, and comorbid condition. More than 200 different causal viruses have been described. Wheezing episodes are a common cause of hospitalization in infants and young children in developed countries and a major cause of death in developing countries. Direct diagnosis of viral respiratory infections was previously based on conventional methods such as isolation by cell culture and antigen detection. Nevertheless, even when these methods were combined, some samples remained negative despite clinical or epidemiological evidence of viral respiratory infection[1-4].

Detection can be improved using molecular biology techniques. Numerous studies have developed and evaluated detection and typing methods based on polymerase chain reaction (PCR) and reverse transcriptase (RT)-PCR[5-7].

Methods based on consensus PCR and reverse hybridization of PCR products provide high sensitivity and extensive typing information, including identification of multiple infections. CLART® PneumoVir (Genomica, Coslada, Spain), an assay based on amplification and array hybridization for the detec-

tion and genotyping of human respiratory viruses in routine clinical specimens, uses a combination of two multiplex RT-PCR tests followed by primer extension and microarray hybridization (more details at www.genomica.es). This assay makes it possible to detect simple or mixed infections with 17 different human respiratory viruses: bocavirus (HBoV); coronavirus (HCoV); enterovirus (echovirus); rhinoviruses (HRV); influenza viruses (Flu) A, B and C; human metapneumoviruses (HMPV) A and B, human respiratory syncytial viruses (HRSV) A and B, parainfluenza viruses (PIV) type 1, 2, 3, 4A and 4B and adenoviruses (ADV).

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METHODS

We investigated the presence of 17 different human viruses in 281 respiratory samples obtained from 253 children who were hospitalized for acute wheezing episodes between October 2008 and April 2009. Seventy-five of these samples had been analyzed by rapid immunoassay for HRSV (TRU RSV, Meridian Bioscience, Inc Cincinnati, Ohio USA, 60 samples) and influenza virus (Clearview Exact Influenza A&B, 15 samples) according to the manufacturers´ recommendations. A nasopharyngeal sample was obtained on admission using a standardized procedure and sent immediately (less than 15 minutes after extraction) to the microbiology laboratory where it was placed in viral transport medium. Rapid immunoassay was performed immediately and the samples obtained for PCR assays were stored in tubes at -70ºC before processing.

Human respiratory viruses were detected and identified using the CLART® PneumoVir assay in three steps, according to the manufacturer's protocol. Briefly, nucleic acids from samples were obtained with automatic extraction in an EasyMAG system (Biomerieux Marcy l´Etoile. France) and samples were eluted in 25μl of elution buffer. A specific 120-330 bp fragment of the viral genome was then amplified using RT-PCR. RNA/DNA was amplified by PCR together with an internal control used to exclude inhibition. RT-PCR is carried out in two different ready-to-use amplification tubes. During the process, amplified products are labeled with biotin. Following amplification, they hybridize with their respective specific probes that are immobilized at different sites in the array. Incubation with streptavidinperoxidase generates an insoluble product that precipitates at the hybridization sites in the array. Hybridization occurred in a low-density microarray containing triplicate DNA probes specific to the respiratory viruses studied.

The study received ethical approval from Hospital Clinico San Carlos.

Table 1 **Viruses detected in 281 samples obtained from patients with ARTI during a 6-month study period.**

Virus(es)	No (%) of samples with virus	No (%) of samples with virus as sole agent
Rhinovirus	26 (9.25)	21 (7.47)
Respiratory syncytial virus A	82 (29.2)	46 (16)
Respiratory syncytial virus B	38 (13.5)	12 (4.3)
Enteroviruses	27 (9.6)	9 (3.2)
Human Bocavirus	42 (14.94)	8 (2.85)
Adenovirus	13 (4.6)	3 (1)
Human metapneumovirus A	18 (6.4)	7 (2.5)
Human metapneumovirus B	16 (5.7)	8 (2.8)
Parainfluenza virus types 1	0 (0)	0 (0)
Parainfluenza virus types 2	0 (0)	0 (0)
Parainfluenza virus types 3	7 (2.5)	3 (1)
Parainfluenza virus types 4	1 (0.35)	0 (0)
Influenza A virus	16 (5.7)	4 (1.4)
Influenza B virus	8 (2.8)	5 (1.8)
Influenza C virus	3 (1)	0 (0)
Human coronavirus	2 (0.7)	0
Samples with ≥2 viruses	94 (33.45)	-
Samples with ≥ 1 virus	225 (80)	-
Negative samples	56 (20)	-

Table 2 Distribution of infections by age group.

Age group (No; %)	Predominant viruses (No. of positive samples)	Virus detected; [Nº/(%)] None	One virus	Coinfections 2 viruses	Coinfections >2 viruses
<1 year (120; 42.2%)	HRSVA (51)	23 (19.2)	53 (44.2)	34 (28.3)	10 (8.3)
	HRSVB (21)				
1-3 years (93; 33%)	HRSVA (24)	13 (14)	43 (46.2)	31 (33.3)	6 (6.45)
	BOCAVIRUS (19)				
3-5 years (33; 11.8%)	HRV (11)	7 (21.2)	19 (57.6)	5 (15.1)	2 (6.1)
	HRSVA (8)				
>5 years (35; 12.5%)	HRV (6)	13 (37.1)	16 (45.7)	6 (17.2)	0
	ENTEROVIRUS (4)				

RESULTS

Of the patients from whom these specimens were collected, 142 (56%) were male and 246 (97.2%) were <5 years old. The mean age of the study population was 2 years (range 1 month to 15 years). Twenty-four infants had to be admitted several times during the study period (21 children, 2 admissions; 3 children, 3 admissions). The samples obtained were considered as different because the time between them was more than 20 days and viruses detected at each admission were different.

Of the 281 samples, 225 were positive for one or more viruses. HRSVA (n=82; 29.2%) was the most prevalent virus, followed by bocavirus (n=42; 15%), enterovirus (n=27, 9.6%) and HRV (n=26; 9.25%) (table 1).

Sixteen (5.7%) of the samples were positive for the influenza A virus. Fifteen were also analyzed using the rapid test. The results for the Clearview system were consistent with the microarray results in 12 samples: 7 were positive by both techniques, 5 were negative by both techniques and 3 were array-positive and ClearView negative. Similar results were obtained for HRSVA (60 samples analyzed, 37 positive results by both techniques, 12 negative by both methods and 11 array-positive and rapid tests negative).

We found few HCoV and PIV4 and no PIV1 or PIV2. Multiple viral infections were found in 33.45% of the specimens. Most were coinfections with only two different viruses although in 15 and three samples we detected three and four viruses respectively. The distribution of single viral infections and coinfections was similar in all the age groups studied (table 2). No monthly variation was noted for most of the viruses analyzed. Influenza B virus was detected almost throughout the study period. Influenza A virus was detected only in December and January, although relatively low numbers of influenza viruses were detected during the study period.

Most of patients included in the study presented similar clinical signs. All 253 children had fever and wheezing. Apart from fever, cough and runny nose were also common.

Influenza was the virus most represented in patients with high fever. RSV A/B and HRV were significant pathogens associated with bronchiolitis and pneumonia. Human bocavirus was the most frequent coinfecting virus, and this coinfection did not affect appreciably disease severity. No significant differences were found in clinical signs between either the co- and mono-infection groups.

Viruses were identified in 80% of the acute respiratory infections studied and provided useful information on the clinical features and epidemiology of specific agents affecting children during the cold months.

DISCUSSION

The number of positive samples was higher than in other studies[8, 9]. This may be due to the low sensitivity of some classic detection methods, the number of viruses tested in our study and the fact that some respiratory viruses were not systematically sought (e.g. PIV-4, influenza C virus and HCoV). Detection of several viruses is considerably enhanced by the use of molecular biology techniques[2,4]. This could explain the greater quantity of viruses that were found using the array.

The CLART® PneumoVir was able to detect 34 (12 %) HMPV. HMPV has been reported to be responsible for 5%-7% of viral respiratory tract infections in hospitalized children worldwide[10-12].

Human bocavirus samples were similar to those detected in other studies[13-15]. A large proportion of the cases were mixed infections with other viruses, however, in 8 samples (2.85%), human bocavirus was the only virus detected (table 1). We found more than one virus in 33.45% of our specimens. It is likely that mixed infections play a greater role in disease than previously thought.

As different viral respiratory infections have similar symptoms, a diagnostic tool able to detect multiple infections in small quantities of clinical specimens could prove extremely useful. In that sense, our analysis of clinical samples has clearly proven to be capable of simultaneously detecting the presence of 17 viruses.

The similar clinical presentations of patients infected by various respiratory viruses and some bacterial make etiological diagnoses difficult when decisions are based only on physical symptoms. This is one important reason for the over-prescription of unnecessary antibiotics to children presenting with fever at Pediatrict Department. Proper diagnoses are important to cohort patients on admission and implement appropriate infection control measures. Also can modify days of hospital stay and appear associated with better antibiotic stewardship.

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