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Respiratory Viruses in Children Admitted to Hospital Intensive Care Units: Evaluating the CLART® Pneumovir DNA Array

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Viruses play a significant part in children's respiratory infections, sometimes leading to hospitalization in cases of severe respiratory distress. The aim of this study was to investigate respiratory infections in children treated in a hospital intensive care unit (ICU). Assays were performed using the CLART® Pneumovir DNA array assay (Genomica, Coslada, Madrid, Spain), which makes it possible to detect 11 genus of respiratory viruses simultaneously. During the winter of 2008–2009, 73 respiratory specimens collected from 53 children under 2 years of age and admitted to an ICU were tested. At least one virus was detected in 78% (57/73) of the samples. The virological diagnosis was based on single infections in 65% (37/57) and on multiple infections in 35% (20/57) of cases. The array assay revealed respiratory syncytial virus (RSV) in 73.6% (42/57) of the samples and rhinovirus in 24.6% (14/57), either on their own or in co-infections. All viruses identified in single and multiple infections were tested, taking into account clinical features, risk factors, and severity criteria. Children with no risk factors presented more multiple infections, up to 42% of cases, than children with at least one risk factor. RSV seemed to induce severe symptoms by itself as no difference in intubation needs was observed when RSV was detected on its own or in co-infection. The CLART® Pneumovir DNA array was useful for examining severe viral respiratory infections, when other viruses than those detected by conventional methods could be involved, particularly in an ICU. ***J. Med. Virol. 83:150–155, 2011.*** © 2010 Wiley-Liss, Inc.

KEY WORDS: respiratory viruses; DNA array; pediatric intensive care unit

INTRODUCTION

Acute respiratory diseases are very common in children and a viral etiology can be identified in up to 80% of cases [Mahony, 2008]. These infections can lead to damage to the lower respiratory tract, including bronchitis, bronchiolitis, pneumonia, and bacterial superinfections. In some cases, particularly for children under 2 years of age, hospitalization in an intensive care unit (ICU) may be required in severe cases.

Respiratory syncytial virus (RSV) is the most frequently reported virus in infants admitted to hospital, but many other viral agents can be associated with acute respiratory infections [Mentel et al., 2005; Freymuth et al., 2006]. Rhinovirus (hRV), influenza (Flu) virus, and metapneumovirus (hMPV) are also now well known to be involved in such pathologies, with prevalence of

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Conflict of interest: B.L. declares conflicts of interest with Argène, Biocryst, bioMérieux, GSK, MedImmune, Merck, Novartis, Plasmair, Roche, Sanofi-Pasteur, and Wittycell. E.F., V.E., M.B.D. and F.M. declare conflicts of interest with Argène, bioMérieux, Cepheid, and R-Biopharm. D.F. received research grants from Biomerieux and Merieux Foundation. E.Y. and Y.G. declare no conflict of interest.

Ethics: This study was conducted during the standard diagnosis of virological parameters and did not require any additional samples. For standard diagnoses, typically no approval by the Ethics Committee or informed consent from the parents is required. Nevertheless, at the Hospices Civils de Lyon, diagnosis is performed in compliance with French laws and HCL guidelines and in accordance with the ethical standards of the Declaration of Helsinki.

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around 30%, 10%, and 5%, respectively [Bouscambert-Duchamp et al., 2005; Freymuth et al., 2006]. In pediatric ICU, the prevalence of viruses may differ, with a larger proportion of dual or mixed infections [Paranhos-Baccala et al., 2008].

Viral respiratory diagnosis has traditionally relied on antigen detection and virus isolation. But the lack of sensitivity of antigen detection, the delay in the results with virus isolation and the limitations of detecting only cultivable viruses make the diagnosis of respiratory viruses incomplete. As a consequence, some clinical cases remain virologically negative.

Molecular technology has better sensitivity and the development of multiplex amplifications makes it possible to detect a broader panel of viruses. Adenoviruses (ADV), parainfluenza viruses (PIV), enteroviruses (EV) but also coronaviruses (HCoV) and bocaviruses (HBoV) can now be detected by multiplex assays. These assays are based on different types of technology, such as ligation-dependent probe amplification (MLPA®), dual priming oligonucleotide (DPO) technology, target-specific primer extension (TSPE), or target-specific extension (TSE) [Chun et al., 2007; Mahony et al., 2007; Marshall et al., 2007; Reijans et al., 2008]. Other techniques are based on RT-PCR amplifications followed by microarray analysis, as performed in the present study [Li et al., 2007]. Broadening viral diagnosis in this way may help clinicians to decrease prescriptions of antibiotics, implement early antiviral treatments when available, and prevent virus transmission.

The aim of the present study was to investigate respiratory infections in children admitted to a hospital pediatric ICU. Assays were performed with the CLART® Pneumovir DNA array assay (Genomica, Coslada, Madrid, Spain) which makes it possible to detect 11 genus of respiratory viruses simultaneously. Technical aspects, virological, and clinical data were analyzed.

MATERIALS AND METHODS

Patients and Samples

Seventy-three respiratory samples from 53 children under 2 years of age and admitted to the hospital pediatric ICU in Lyon were collected between December 1, 2008 and March 30, 2009. Some children were sampled more than once but the samples corresponded to distinct episodes of respiratory disease. In most cases, the samples were nasopharyngeal or tracheo-bronchic aspirates. Samples were collected in different clinical contexts: bronchiolitis (30 samples), pneumonia (14 samples), bronchiolitis associated with pneumonia (3 samples). Twenty samples were collected in other clinical contexts including isolated apnea (10 samples), sepsis (1 sample), apnea associated with sepsis (3), convulsions associated with fever (5), and asthma (1). No clinical information was available for 6 samples. Risk factors were considered for each patient. Of the 53 children, 12 were <42 days old (22.6%), 4 were born prematurely (7.5%), 2 had a cardiopathy (3.8%), 1 child had chronic respiratory insufficiency, and 1 child was immunodepressed. Twenty-one children presented more than one risk factor (39.6%) and 12 children had no risk factors at all (22.6%). The severity of the respiratory disease was related to intubation requirements.

Nucleic Acid Extraction

Two hundred microliters of samples were treated with. 10 µl of proteinase K at 20 mg/ml (Proteinase K PCR grade, Roche, Diagnostics GmbH, Mannheim, Germany). RNA was then extracted using the automated NucliSens easyMAG system (Biomerieux, SA, Mercy l'Etoiles, France). Elution of the extracted nucleic acids was performed in 70 µl.

DNA Array Essay

The CLART® Pneumovir DNA array assay (Genomica, Coslada, Madrid, Spain) detects and characterizes the most frequent human viruses causing respiratory symptoms in a total of 8 hr after nucleic acid extraction. The viruses analyzed include: RSV A and B; Flu virus A, B, C; PIV 1, 2, 3, 4A, 4B; hMPV A and B; ADV (33 subtypes), EV (50 subtypes), hRV (65 subtypes); HCoV (subtype 229E); HBoV. This kit is based on the amplification of specific fragments of the viral genome by means of two multiplex RT-PCR or PCR and a subsequent detection by hybridization with specific binding probes. During the 5-hr RT-PCR/PCR amplification, the amplified products were labeled with biotin. Following amplification, hybridization with specific probes immobilized in sites of the micro-array was performed in a second step lasting 1 hr:30 min. After incubation with a streptavidin-peroxidase conjugate, the addition of tetramethylbenzidine (TMB) induced the appearance of an insoluble product which precipitated at the hybridization sites on the micro-array.

Statistical Analysis

Student's *t*-test and Person's chi-squared test were used to assess intergroup differences. Statistical analyses were performed on EpiInfo software (V 3.5.1 CDC). Odds ratio (OR) and 95% confidence interval (CI) were calculated for the likelihood of co-detection and to test the association between multiple infections and risk factors with distress severity. A test was considered to be significant when the *P*-value was <0.05.

RESULTS

Virological Data Obtained With CLART® Pneumovir in a Pediatric Intensive Care Unit

Of the 73 samples collected in this study, 57 samples contained at least one virus (78%; Table I). The virological diagnosis revealed single infections in 65% (37/57) and multiple infections in 35% (20/57) of the cases (Table I). Of the single infections, RSV was found

TABLE I. Virological Results on 73 Respiratory Samples

		Number of positive samples (%) detected by CLART® Pneumovir	
	Virus	Number	%
Positive samples		57	78
Negative samples		16	22
Single infections	RSV	28	49
	RSV A	*20*	*35*
	RSV B	*8*	*14*
	hRV	4	7
	Flu A	0	0
	Flu B	1	1.7
	ADV	1	1.7
	PIV	1	1.7
	hMPV	1	1.7
	HBoV	1	1.7
	Total	*37*	*65*
Multiple infections	RSV A + hRV	4	7
	RSV A + RSV B	3	5.3
	hRV + HBoV	3	5.3
	RSV A + hRV + ADV	2	3.5
	RSVA + ADV	1	1.7
	RSV A + EV	1	1.7
	RSVA + HBoV	1	1.7
	RSV A + RSV B + ADV	1	1.7
	RSVA + hRV + FluA	1	1.7
	FluA + HBoV	1	1.7
	PIV + ADV + HBoV	1	1.7
	HBoV + hMPV	1	1.7
	Total	*20*	*35*

in 49% (28/37) of cases and hRV in 7% (4/37). Other viruses, such as Flu B, ADV, PIV, hMPV, and HBoV were also detected. In multiple infections, RSV was found in co-infection with hRV in 7% of cases, RSV A with RSV B in 5.3% of cases, hRV with HBoV in 5.3% of cases, and RSV A associated with both hRV and ADV in 3.5% of cases. Eight other viral co-infections were also detected as reported in Figure 1. In total, RSV was detected with at least one other virus in 24.3% of cases, hRV in 17.5% of cases, and HBoV in 12% of cases.

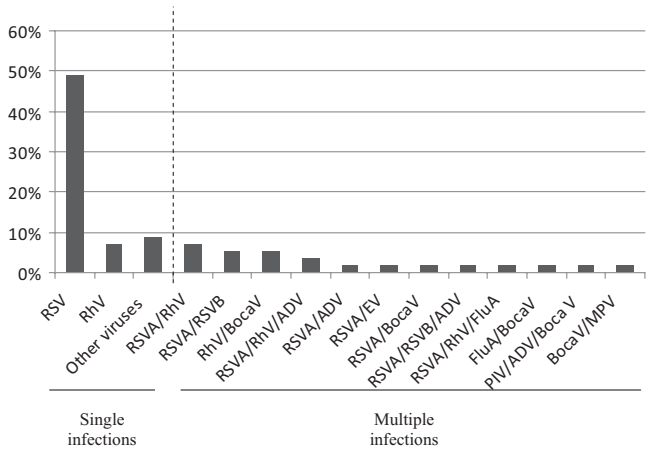

Fig. 1. Distribution of virus detected in single and multiple infections by CLART® pneumovir in pediatric intensive care unit.

Clinical Data

Respiratory syndrome. CLART® Pneumovir DNA array made it possible to diagnose at least one virus in 85% of the bronchiolitis cases (Table II). In 57.6% of the cases, RSV was detected as a single infection. Multiple infections were also detected in around 27% of the bronchiolitis cases. They involved mostly RSV A associated with other viruses (8/33, 24.2%). hRV and HBoV were also detected in co-infections in a proportion of 15% (5/33) and 6% (2/33), respectively.

In the pneumonia cases (17 samples), CLART® Pneumovir DNA array detected viruses in 76.4% of cases (Table II). The implication of RSV in 41.2% (7/17) of the cases was less than in bronchiolitis, with 23.5% (4/17) in single infections and 17.6% (3/17) in multiple infections. Other viruses were involved, such as hRV, Flu B, ADV, and PIV in single infections. Two cases of hRV associated with HBoV were also reported.

In total, RSV was implicated in 81.8% (27/33) of the bronchiolitis cases and in 41.2% (7/17) of the pneumonia cases, either in single or multiple infections. hRV was implicated in 15.2% (5/33) of the bronchiolitis cases and in 29.5% (5/17) of the pneumonia cases.

Risk factors. Of the 53 children, 20 presented one risk factor (37.8%), 21 presented more than one risk factor (39.6%), and 12 children had no risk factors at all (22.6%). For the children with no risk factors, viral factors that could have led to the severe respiratory

TABLE II. Viral Distribution Correlated to Respiratory Syndrome

		Number of positive samples (%) detected by CLART® Pneumovir	
	Virus	Number	%
Bronchiolitis n = 33			
Single infections	RSV	19	57.6
RSV A	*13*		
RSV B	*6*		
hRV	0		
Total	*19*	*57.6*	
Multiple infections	RSV A + hRV	3	9
	RSV A + RSV B	2	6
	RSV A + EV	1	3
	RSV A + HBoV	1	3
	hRV + HBoV	1	3
	RSV A + hRV + ADV	1	3
	Total	*9*	*27.3*
Global positivity rate			*85*
Pneumonia n = 17			
Single infections	RSV	4	23.5
	RSV A	*4*	
	RSV B	*0*	
	hRV	1	5.9
	Flu B	1	5.9
	ADV	1	5.9
	PIV	1	5.9
	Total	*8*	*47.1*
Multiple infections	RSV A + hRV	1	5.9
	RSV A + RSV B	1	5.9
	hRV + HBoV	2	11.8
	RSV A + hRV + ADV	1	5.9
	Total	*5*	*29.5*
Global positivity rate			*76.4*

Three samples were collected in case of bronchiolitis associated to pneumonia.

infection were investigated. Children with no risk factors presented more multiple infections, up to 42% of cases, than children with at least one risk factor, even if the difference was not significant, probably because of the low number of patients (n = 12, $P = 0.23$; Table III).

Severity. Need for intubation was used as a surrogate for the severity of the respiratory distress. Nineteen episodes of respiratory disease required intubation and 52 did not. Information regarding intubation was missing in two cases. There was no difference in the severity of the distress between single and multiple infections ($P = 0.62$). In addition, the detection of RSV in a co-infection did not worsen the respiratory distress, as intubation was required in 23.1% of the cases when RSV was detected alone and in 14.3% of the cases when detected in co-infection with another viral agent. No association was observed between RSV in either single or multiple infections and distress severity ($P = 0.4$). RSV seemed to induce severe symptoms on its own. In RSV negative samples, no difference was observed in the distress severity between single and multiple infections ($P = 0.35$), probably because of the low number of RSV negative samples.

HBoV was detected in 8 clinical cases (1 in single, 7 in multiple infections) with intubation required in 3 cases (Table IV). Interestingly, detection of HBoV resulted in an increased likelihood of co-detecting another virus as reflected by an OR of >1 (95% CI: 3.15–248) $P = 0.0003$. But there was no difference in distress severity between single and multiple infections when HBoV was detected ($P = 0.5$), probably because of the low number of cases (n = 8).

DISCUSSION

Microarray assay was found to be highly sensitive and made it possible to detect viruses that are not detected otherwise, either with the standard multiplex RT-PCR

TABLE III. Risk Factors Associated to Single or Multiple Infections

Risk factors	Single infection	Multiple infection	No virus detected
Age <42 years	67%	20%	13%
Prematurity	48%	15%	27%
Chronical respiratory disease	42%	25%	33%
Cardiopathy	38%	25%	37%
No risk factor	42%	42%	16%

TABLE IV. Clinical Cases Involving HBoVirus

Virus	Clinical data	Ventilation	Risk factor
HBoV	Apnea, convulsions	IV	Age <42 days
HBoV + hRV	Bronchiolitis	NIV	None
HBoV + hRV (n = 2)	Pneumonia	IV	CRD, cardiopathy
HBoV + RSV A	Bronchiolitis, apnea	NIV	Age <42 days
HBoV + influenza A	Apnea	NIV	Prematurity, CRD
HBoV + hMPV	Apnea	NIV	None
HBoV + ADV + PIV	Apnea, convulsions	IV	Age <42 days

CRD, chronical respiratory disease; IV, invasive ventilation; NIV, non-invasive ventilation.

(PIV, ADV, EV, HBoV, hMPV, HCoV) or in culture (HBoV, hMPV, HCoV) and which may be involved in multiple infections. From a practical point of view, the CLART® Pneumovir array assay is easy to use even if technical training is necessary to avoid false positives because of insufficient washing or overexposure of the arrays. Nevertheless, after nucleic acid extraction, the array assay takes 8 hr and is divided into two steps: multiplex RT-PCR followed by hybridization. The main advantages are its high sensitivity and broader detection of respiratory viruses, improving viral diagnosis. However, the detection of multiple infections raises certain questions regarding the pathogenicity of the different viruses. Are all the viruses detected responsible for the clinical signs presented by the patient? These multiple viruses can be successive infections and molecular tests could detect a persistent genome in the absence of virus activity. This array assay is only a qualitative technique. However, quantitation should help to identify the predominant viral agent believed to be responsible for an acute respiratory syndrome.

Different studies have described previously the clinical characteristics of multiple viral infections versus RSV single infection. Multiple infections varied from 17.4% to 30% in children under 2 years of age admitted to hospital [Calvo et al., 2008; Miron et al., 2010] and were associated with higher fever, longer hospitalization, and more frequent use of antibiotics than in the case of infection with RSV alone [Calvo et al., 2008]. Infants with multiple infections are at higher risk of being admitted to a pediatric ICU [Paranhos-Baccala et al., 2008; Richard et al., 2008]. In the present study, multiple infections were detected by arrays in 35% of the positive cases. In bronchiolitis, viral co-infection was found in 27.3% of the cases with a predominance of the RSV and hRV co-infection, as described previously [Richard et al., 2008]. RSV seems to induce severe symptoms on its own. Viral co-infections with RSV effectively did not increase the need for intubation in comparison to infections with RSV as a single agent, as recently reported in a study considering duration of ventilation and length of hospitalization [Marguet et al., 2009]. Interestingly, children admitted to a pediatric ICU with no risk factor were found to present more co-infections than children with risk factors, even if the difference was not significant.

RSV prevalence has previously been detected at around 40% using multiplex RT-PCR in children admitted to the emergency room during winter [Freymuth et al., 2006]. In the population studied, the array assay revealed RSV in 73% of the samples either alone or in co-infection. This prevalence of RSV may be explained by a higher sensitivity of array assay versus RT-PCR, but this point needs to be evaluated in more detail. It may also suggest that RSV may be responsible for a higher risk of hospitalization in a pediatric ICU. The detection of hRV has been greatly improved by molecular techniques [Mahony, 2008]. The prevalence of hRV was previously reported as being around 30–35% with multiplex RT-PCR [Freymuth et al., 2006]. This study reported hRV in 24% of the samples. This could suggest that infections caused by hRV are less represented in pediatric ICU than in other pediatric units.

hMPV is an RNA virus discovered in 2001 and is known to induce symptoms very similar to those caused by RSV [van den Hoogen et al., 2001, 2003]. hMPV outbreaks occur predominantly in the winter and spring months, overlapping or following RSV outbreaks [Mahony, 2008]. However, there can be significant differences in the detection of hMPV across seasons from year to year. Overall prevalence is around 7%, ranging from 2% to 25% [Mahony, 2008; Gaunt et al., 2009]. Only two hMPV infections were detected in this study, probably because the study period missed potential hMPV circulation during the spring season.

HBoV was discovered in 2005 using large-scale molecular viral techniques [Allander et al., 2005]. Prevalence, ranging from 2% to 11% in respiratory samples from symptomatic patients, has been reported worldwide. In most cases, as in this study, HBoV was detected in co-infections with other known viral agents raising the question of its actual pathogenic role [Mahony, 2008]. However, HBoV has been associated with low respiratory infections [Kesebir et al., 2006; Manning et al., 2006; Fry et al., 2007] and acute wheezing [Allander et al., 2007]. Even if HBoV can be found in asymptomatic patients, HBoV genome quantitation could help to identify the implication of HBoV in lower respiratory tract infections, as a high-HBoV load has been reported in patients with lower respiratory tract infections [van de Pol et al., 2009]. In this study, HBoV prevalence was around 14%, higher than the prevalence usually reported [van de Pol et al., 2009], almost certainly because of the differences in detection techniques, patient recruitment and the seasonal study period. Nevertheless, Fry et al. [2007] have previously

reported up to 12% of HBoV in children under 5 years of age with pneumonia.

In conclusion, microarrays have a high level of sensitivity and make broader viral detection possible. However, the relevance of the positive results obtained with this highly sensitive array is not easy to determine, particularly in cases of multiple infections caused by the lack of quantitation. DNA array may be useful regarding severe viral respiratory infections, particularly in pediatric ICU when no viral diagnosis has been established using conventional techniques that detect the most common viral respiratory pathogens.

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