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Benefits and Drawbacks of Molecular Techniques for Diagnosis of Viral Respiratory Infections. Experience With Two Multiplex PCR Assays

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Molecular techniques have represented a major step forward in the diagnosis of viral respiratory infections. They are considered highly sensitive and specific compared to conventional techniques. In this study two nucleic acid amplification tests (NAATs) were compared to conventional methods (immunofluorescence and viral culture). The aim of this work was to discuss the clinical interpretation of the results obtained by NAATs on the basis of the two-decade experience of our group and the literature. Eighty nasopharyngeal aspirates were collected from children under six years attended for acute respiratory illness at the pediatric emergency room of a third level Hospital. Both NAATs tested (Seeplex® and Clart®) showed an overall higher performance regarding sensitivity (76% and 90%, respectively). Compared to Seeplex®, the Clart® system tripled the number of multiple detections (8 by Seeplex® vs. 25 by Clart®). In some specimens both NAATs detected different viruses. Given these discrepancies and the fact that detection of viral nucleic acids is not necessarily related to the current clinical syndrome, the interpretation of molecular results may not always be so straightforward. The pros and cons of NAATs should always be taken into account when giving a result. ***J. Med. Virol. 88:45–50, 2016***.

KEY WORDS: multiplex PCR; respiratory virus; viral isolation; immunofluorescence

INTRODUCTION

Respiratory tract infections are the most frequent cause of illness in children, and most of them are of viral etiology [van de Pol et al., 2007; Mahony, 2008; Raymond et al., 2009; Vallieres and Renaud, 2013]. The most common respiratory viruses are *Influenza A virus* (FLUAV), *Influenza B virus* (FLUBV), *Human parainfluenzavirus* 1–4 (PIV-1-4), *Human respiratory syncitial virus* (RSV), *Human metapneumovirus* (hMPV), *Enteroviruses* (EV), *Rhinoviruses* (RV), *Human coronaviruses* (HCoV), *Human bocaviruses* (HBoV), and *Human adenoviruses* (AdV). As infections caused by respiratory viruses show similar clinical manifestations, microbiological studies must be performed to identify the etiology [Caliendo, 2011; Pagarolas and Sune, 2014].

Diagnosis of viral respiratory infections is traditionally performed using conventional methods based on viral culture (VC), or antigen detection such as immunofluorescence assays (IF). VC remains the gold-standard but it requires trained personnel, results may take a few days, and not all viruses can multiply in culture. IF techniques are quick and they allow quality control of samples but they need big expertise for interpretation. Additionally, they are not available for all viruses and lack sensitivity for others [Ginocchio and McAdam, 2011].

Nucleic acid amplification tests (NAATs) have become an alternative that offers high sensitivity and specificity when compared to conventional methods [Elnifro et al., 2000; Mahony, 2008; Ginocchio and McAdam, 2011]. Numerous tests have been developed using multiplex PCR to detect several viruses, including those that are not detected either by VC or by immunoassays. Although PCR-based techniques were

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initially limited by the few types of viruses that could be detected in a single test [Liolios et al., 2001; Coiras et al., 2004; Bellau-Pujol et al., 2005; Freymuth et al., 2006], numerous multiplex PCR tests have been developed, allowing more than 10 viruses to be detected in a single assay. Amplicon detection can be performed by hybridization using microarrays [Henrickson et al., 2007; Raymond et al., 2009; Cannon et al., 2010; Renois et al., 2010; Frobert et al., 2011; Culebras et al., 2013; Pillet et al., 2013] or semiconductor-based DNA microspheres [Mahony et al., 2007]. Several respiratory virus panels (RVP) have been commercialized, such as xTAG® RVP from Luminex® [Mahony et al., 2007; Pabbaraju et al., 2008; Gadsby et al., 2010; Balada-Llasat et al., 2011; Kim et al., 2013; Pillet et al., 2013], Multicode PLx RVP from Eragen [Balada-Llasat et al., 2011], and Resplex® II from Qiagen [Li et al., 2007; Balada-Llasat et al., 2011; Pillet et al., 2013]. Other NAATs are the multiplex ligation-dependent probe amplification system (MLPA) [Reijans et al., 2008; Bruijnesteijn van Coppenraet et al., 2010; Pillet et al., 2013] and the dual priming oligonucleotide (DPO) system [Roh et al., 2008; Bruijnesteijn van Coppenraet et al., 2010; Zhang et al., 2012; Cho et al., 2013; Kim et al., 2013; Pillet et al., 2013]. PCR-based systems generate a diagnostic result within only one working day, but implementation in a laboratory routine may not be cost-effective.

This study assessed two multiplex PCR-based techniques commercially available, namely Clart® and Seeplex®. Both NAATs have been studied previously [Roh et al., 2008; Bruijnesteijn van Coppenraet et al., 2010; Renois et al., 2010; Frobert et al., 2011; Zhang et al., 2012; Cho et al., 2013; Culebras et al., 2013; Kim et al., 2013; Pillet et al., 2013] and, in the present work, they were compared with each other and also with two conventional methods for the diagnosis of viral respiratory infections. The aim of this work was to discuss the clinical interpretation of the results obtained by NAATs on the basis of our two-decade experience on conventional methods. The benefits and pitfalls of molecular techniques are also discussed.

MATERIALS AND METHODS

Samples

Eighty nasopharyngeal aspirates were collected from children under six years attended for acute respiratory illness at the pediatric emergency room of a third level Hospital, from October to November 2008 and from February to March 2009. Consecutive specimens, with more than 25 cells/field by microscopic observation and sufficient residual volume (300 μL), were selected. These specimens were processed within the same working day or stored at +4°C during the weekends (maximum 72 hr). Epidemiological data were collected.

Viral Isolation and Antigen Detection

Samples were assessed using D3 DFA Metapneumovirus Identification Kit (Diagnostics Hybrids, Athens, OH), RSV Direct IF (bioMérieux, Marcy-l'Etoile, France), and Respiratory Panel 1 Viral Screening & Identification IFA kit (Millipore, Light Diagnostics, Temecula, CA) to detect AdV, FLUAV, FLUBV, PIV-1,2,3 and RSV according to the manufacturer's instructions. For viral isolation, these samples were cultured using six cell lines: MRC5, A549, HEP2, MDCK, LLCMK2, and RD (Vircell S.L Santa Fe, Granada, Spain). All cell cultures were examined daily for cytopathic effect during two weeks. Definitive identification was assessed by IF.

Nucleic Acid Detection

Nucleic acid was extracted from 200 μL of clinical samples using EZ1 Virus Mini Kit v2.0 and Bio Robot EZ-1 (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The extracts were stored at −80°C until use. Molecular viral detection was performed using two NAATs: Seeplex® RV12 ACE Detection Kit (Seegene, Seoul, South Korea) and Clart® Pneumovir Kit (Genomica, Madrid, Spain), following the manufacturer's instructions.

The Seeplex® method is based on the DPO system and amplicon detection is performed by fully automated capillary electrophoresis separation (MultiNA, ShimadzuBiotech, Kyoto, Japan). This method provides results in about six hours. The Clart® system is based on microarray technology and analysis of the resulting hybridization pattern is performed using optical equipment (Clondiag Chip Technologies, Jena, Germany). This method provides results in about 9 hr. Both assays detect FLUAV, FLUBV, AdV, hMPV, HCoV-229E, PIV-1, PIV-2, PIV-3, RSVa, RSVb, and RV. Seeplex® also detects HCoV-NL63 and HCoV-OC43, and differentiates between species of RV (A and B). Clart® also detects HBoV, *Influenza C virus* (FLUCV), EV, PIV-4 A/B, and differentiates between hMPVa and hMPVb. Both assays included an internal control to detect amplification inhibition. Both NAATs gave qualitative results only.

Data Analysis

Epidemiological data, such as age, sex, date of sample collection and clinical diagnosis, were collected. Statistical analyses were performed using the Vassar Stats website (http://vassarstats.net/).

In this work a result was considered clinically relevant or true positive when a respiratory virus was detected by IF and/or VC regardless of NAATs results or by both NAATs (criterion of positivity).

RESULTS

Overall Viral Detection

The range of viruses detected differed depending on the virus and the method used (Table I). Of 80

samples included in this study, viral detection was obtained in 37, 40, and 62 samples by IF/VC, Seeplex® and Clart®, respectively. Either NAAT yielded a positive result in 40 samples that were negative by IF/VC. Only three samples (4%) were negative by all the techniques assessed (Table I).

Seeplex® and Clart® detected 11 and 57 viruses more than IF/VC, respectively. RVs were the viruses most frequently detected by PCR although only two were recovered by IF/VC (Table I). Multiple detection was obtained in 30 samples by NAATs while IF/VC did not achieve multiple recovery in any case. Inhibition was observed in 19 samples, 10 by Seeplex® (12.5%) and 11 by Clart® (14%); two of these samples were inhibited by both methods.

Comparison of Results Obtained By IF/VC and NAATs

According to the established criterion of positivity, 46 (57.5%) samples were considered positive for a respiratory virus; 36 by IF/VC regardless of NAAT results and 10 by two NAATs only. Viral detection was coincident between IF/VC and both NAATs in 20 samples out of 36. Coincident results were found between IF/VC and only one NAAT in 12 samples (one by Seeplex® and 11 by Clart®). Three samples had a positive viral detection by IF/VC but were negative by NAATs. Another positive sample was inhibited. In three positive samples by IF/VC, additional viruses were detected by both Seeplex® and Clart®. RV were detected in all samples positive only by both NAATs (n = 10), one of them having an additional virus (AdV). Overall, 50 significant viruses were detected. Since EVs were only detected by one NAAT, they were not included in further calculations. Considering the remaining significant viruses, the relative sensitivity of IF/VC, Seeplex® and Clart® was 70% (32/46), 76% (35/46), and 89% (41/46), respectively (Table II).

Multiple Viral Detection

NAATs achieved a high rate of multiple virus detection (37.5%; 30/80), corresponding to 10% (8/80) by Seeplex® and 31% (25/80) by Clart®. Regarding viruses identified only by one technique, Clart® detected HBoVs in 16 samples and 13 of them (81%) were multiple detections. Seeplex® detected HCoV-OC43 in five samples corresponding to multiple detections in three cases (60%). Following the criterion of positivity previously established, only four samples were considered to have real multiple infections (8.7%; 4/46) (Table III).

Clinical Analysis

Regarding clinical and demographic analysis, no relevant data were obtained. From the total number of samples assessed, 39 and 41 specimens belonged to patients with upper and lower respiratory tract infections (URTI and LRTI), respectively. The viral distribution did not follow a definite pattern although RSV and AdV were predominantly detected in patients with LRTI and URTI, respectively. No statistically significant association was found between viruses detected and each clinical group.

DISCUSSION

The main finding in the present study was that both NAATs used to detect respiratory viruses yielded different results. As expected, NAATs detected more viruses than the conventional techniques. The question remains, however, as to whether this extra sensitivity provides more reliable information about the etiology of the infection.

TABLE I. Total of Viruses Detected By Each Technique Considering All 80 Samples Analysed

Virus	IF/VC	Seeplex®	Clart®
RSV	11[a]	7	12
AdV	13[b]	14	13
EV	4	ND	10
hMPV	4	1	7
RV	2	17	27
PIV-3	2	4	4
FLUBV	0	0	3
HCoV-OC43	ND	5	ND
HBoV	ND	ND	16
PIV-4	ND	ND	1
FLUCV	ND	ND	1
Human herpesvirus 5	1	ND	ND
Total of virus detected	37	48	94
Total positive samples	37	40	62
Total negative samples	43	30	7
Total inhibited samples	ND	10	11
Total of samples with multiple viruses	0	8	25

ND, unable to be detected by the corresponding technique.
[a]IF and VC were positive in seven cases, and only IF in four cases.
[b]IF and VC were positive in one case, and only VC in 12 cases.

TABLE II. Total of Viruses Detected By Each Technique Considering the Criterion of Positivity

Virus	Total	IF/VC	Seeplex®	Clart®
RSV	11	11	7	10
AdV	14	13	11	12
PIV-3	2	2	2	2
hMPV	4	4	1	3
RV	15	2	14	14
EV	4	4	ND	4
Multiple detection	4	0	2	4
Total of virus detected	50	36	35	45
Sensitivity[a]	—	70%	76%	89%

ND, unable to be detected by the corresponding technique.
[a]A total of 46 viruses were considered after exclusion of EV.

TABLE III. All Samples With Multiple Viral Detection. Real Multiple Infections According to the Criterion of Positivity Are Shown in Bold

No	IF/VC	Seeplex®	Clart®
1	RSV	RSV	RSV + HBoV
2	NEG	RV + HCoV-OC43	RV
8	NEG	RV	RV + HBoV + PIV-3
10	NEG	RV	RV + HBoV
13	NEG	Inhibited sample	HBoV + EV + PIV-4
15	**PIV-3**	**PIV-3 + RV**	**PIV-3 + RV**
17	NEG	PIV-3 + RV	Inhibited sample
18	**EV**	**RV**	**RV + EV**
20	AdV	AdV	AdV + RV
21	RSV	RSV	RSV + HBoV
22	RSV	RSV	RSV + HBoV
23	**EV**	**RV**	**RV + EV**
26	PIV-3	PIV-3	PIV-3 + HBoV
27	**NEG**	**AdV + RV**	**AdV + RV +** RSV
29	NEG	NEG	AdV + RV
30	RSV	NEG	RSV + HBoV
36	EV	NEG	EV + RV + HBoV
37	RSV	NEG	RSV + EV + RV + HBoV
38	RSV	NEG	RSV + EV + RV
43	AdV	AdV	AdV + HBoV
47	AdV	AdV	AdV + FLUCV
51	AdV	AdV + RV	AdV
52	AdV	AdV + HCoV-OC43	AdV
55	hMPV	hMPV + HCoV-OC43	hMPV + RV
60	AdV	AdV	AdV + HBoV
63	AdV	AdV + RV	AdV
65	AdV	AdV	AdV + hMPV
67	NEG	Inhibited sample	EV + FLUBV
69	NEG	NEG	hMPV + HBoV
79	EV	NEG	EV + hMPV

Clart® had the highest detection rate and results agreed with conventional results more often than those by Seeplex®. This could be due to the different range of viruses covered by each technique. Although both NAATs have been investigated before, the methods used as gold standard were different [Roh et al., 2008; Bruijnesteijn van Coppenraet et al., 2010; Frobert et al., 2011; Zhang et al., 2012; Cho et al., 2013; Culebras et al., 2013; Kim et al., 2013; Pillet et al., 2013]. An important drawback in comparing NAATs is therefore the lack of a real gold standard.

Both conventional and PCR-based methods yielded similar results for RSV and ADV. For RSV, IF was more sensitive than VC and equally sensitive to the molecular methods. For AdV, isolation in cell cultures is usually required because of the poor sensitivity of IF and the number of AdV isolates coincided with those detected by molecular methods. These findings coincided with the experience of our group over the last two decades and have been reported by other authors [Dunn et al., 2004; Mahony, 2008; Doan et al., 2012; Cho et al., 2013].

Molecular techniques have been the key to diagnosing a large range of new viruses incapable of multiplying in VC, such as HCoV and HBoV, and the new species of RV. Considering those viruses detected only by PCR, about 72% corresponded to RV, hMPV, HCoV-OC43, PIV-4, FLUCV, and HBoV. As in previous studies, both NAATs largely increased the number of RV detected [Freymuth et al., 2006; Li et al., 2007; Mahony et al., 2007; Bruijnesteijn van Coppenraet et al., 2010; Cho et al., 2013].

NAATs do not always give a result. Inhibition of the amplification reaction is a drawback, mainly because of the specimen itself or the extraction protocol. Both NAATs tested yielded inhibited results. The fact that most inhibited samples differed between the two techniques, despite the extract being the same, suggests that the methods were probably the main cause of PCR inhibition.

The failure of NAATs to give a result may also be because of the lack of a specific target. This is not only because the target is not included in the reaction, as in the case of *Human herpesvirus 5*, but also because of the variability of the respiratory viruses. Therefore laboratories, especially those that rely only on NAATs, need to constantly review the viruses included in the assays they use [Ogilvie, 2001].

NAATs have created a new scenario in the diagnosis of viral respiratory infections: the high yield of multiple viral detections [Kuypers et al., 2006; Madhi and Klugman, 2006; Leland and Ginocchio, 2007]. Compared to Seeplex®, the Clart® system tripled the number of multiple detections; differences in their design, particularly concerning the primers and the amplification conditions, may contribute to this disparity. The high rate of multiple detection, which may be explained because of the pediatric population, is in agreement with other authors [Roh et al., 2008; Kim et al., 2009; Bruijnesteijn van Coppenraet et al., 2010; Frobert et al., 2011; Zhang et al., 2012].

So an important remaining question is the clinical relevance of the results obtained by NAATs. Covering a broad range of etiological agents, these quick techniques may be especially useful in immunosuppressed or critically ill patients although their clinical impact is not yet well established [Garbino et al., 2009; Schnell et al., 2012]. However, as NAATs detect viral nucleic acids regardless of the presence of viral antigens or infective viruses [Pagarolas and Sune,

2014], a positive result can reflect either a present infection or a past infection [Elnifro et al., 2000; Mahony, 2008; Ginocchio and McAdam, 2011; Jansen et al., 2011]. Although the viral load may be useful to predict the correlation of a virus and an infectious process it is not well-established for respiratory viral infections [Kuypers et al., 2006; Debiaggi et al., 2012].

The prevalence of specific viruses based on NAATs differs between symptomatic and asymptomatic children [Debiaggi et al., 2012; Buller, 2013]. Some viruses, such as RSV, are usually associated with clinical illness and should be considered as the etiological agent in a patient with respiratory symptoms. In contrast, viruses such as RV, HCoV, and HBoV seem to be frequently found in asymptomatic children [Debiaggi et al., 2012; Meriluoto et al., 2012] although more studies on this issue are required.

The management of patients requires reporting results within a clinically relevant time-scale; microbiology laboratories should take this into consideration when choosing a diagnostic test. NAATs are faster than culture, which requires two days minimum; both NAATs tested had a turn around time of six and nine hours. However, most NAATs are not faster than IF or immunochromatography. Rapid antigen detection tests of certain respiratory viruses provide a result with very high specificity within 15–30 min [Mandell et al., 2007]. In the epidemic situation, the use of these techniques as the first step to detect viruses such as influenza virus or RSV may accelerate results and bring simplicity to the laboratory. Only the negative results would require further analysis [Buller, 2013]. Another important point is that NAATs and antigen detection techniques only detect the specific viruses under investigation; other viruses that may be related to the clinical process will not be detected.

The combination of several methods in the diagnosis of viral respiratory infections is still a great option. However, not all laboratories have the facilities or the personnel required to perform a wide range of techniques, such as antigen detection tests, virus isolation and molecular tests. NAATs are particularly useful in detecting new viruses and in giving a quick result. However, the interpretation of a molecular test is not always straightforward. The clinical virologist must consider the pros and cons of the technique used and the clinical impact of the result.

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