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## BACKGROUND & OBJECTIVES

The majority of acute respiratory tract infections are frequently caused by respiratory viruses that generate similar clinical symptoms making the proper diagnosis problematic. Because of their sensitivity and specificity nucleic acid amplification assays are emerging more and more as the preferred methods of diagnostic testing. These assays offer significant advantages in sensitivity over antigen detection methods and in most cases over conventional culture methods. The objectives of this study were:

- To compare the effectiveness of three commercial multiplex RT-PCR Viral Respiratory Panel (VRP) assays with direct immunofluorescence assay (DFA) and culture for the detection of common respiratory viruses in children.
- To assess the value of commercial multiplex RT-PCR assays for detection of swine-origin 2009 A (H1N1) influenza virus.

## METHODS

### 1) Viral Respiratory Panel (VRP) assays study

**Specimens:** A total of 750 nasopharyngeal swabs, from November 2007 to April 2008 and from January 2009 to March 2009, were randomly selected from children with respiratory symptoms. Specimens from 2007/2008 (n = 600) were aliquoted and stored at -80°C until nucleic acid extraction, whereas specimens from 2009 (n = 150) were submitted to nucleic acid extraction before being frozen at -80°C.

**Nucleic acid extraction:** Nucleic acid was extracted from 400 µl of specimen using the combination of biorobot M48 workstation (Qiagen)/MagAttract Virus Mini M48 kit (Qiagen) and eluted in 100 µl of elution buffer. Each extracted nucleic acid was aliquoted into five aliquots of 20µl and stored at -80°C until use.

The extracted nucleic acid was tested by three commercial multiplex assays: Resplex II Panel v2.0 (Qiagen), Seeplex RV15 (Seegene) and xTAG RVP (Luminex Molecular Diagnostics). A wide range of viruses were detected and identified simultaneously (TABLE 1). Each assay had its own internal control (IC) included to rule out any PCR inhibition. The IC must be amplified in a negative specimen to exclude inhibition.

### 2) Swine-origin 2009 A (H1N1) influenza virus study

**Specimens:** Seventy five nasopharyngeal swabs, from May 2009 to December 2009, were selected from children with respiratory symptoms; 49 specimens from proven swine influenza by reference single target PCR, and 26 negative specimens.

**Nucleic acid extraction:** Nucleic acid was extracted from 400 µl of fresh specimens using the combination of biorobot M48 workstation (Qiagen)/MagAttract Virus Mini M48 kit (Qiagen) or easyMAG (bioMerieux) and eluted in 100 µl of elution buffer. Each extracted nucleic acid was stored at -80°C for PCR at a later date. The extracted nucleic acid was tested by three commercial multiplex assays: Astra fluA (Astra Diagnostics), Resplex II Panel Plus PRE (Qiagen), Seeplex® Influenza A/B Subtyping (Seegene). Targets are listed in TABLE 2. Each assay had its own internal control (IC) included to rule out any potential amplification inhibition.

TABLE 1. Technology and targets of different multiplex assays for all respiratory viruses

Assay	Resplex II v2.0	Seeplex RV15	xTAG RVP
<b>Targets</b>	INF (A, B) - PIV(1, 2, 3, 4) - hMPV - RSV (A, B) - ADV - BocV - CoV (OC43, HKU1, 229E, NL63) - Coxsackieviruses/Echovirus - Rhinovirus	INF (A, B) - PIV (1, 2, 3, 4) - hMPV - RSV (A, B) - ADV - BocV - CoV (OC43/HKU1, 229E/NL63) - Enteroviruses - Rhinovirus	INFA (H1, H3, H5) - INFB - PIV (1, 2, 3, 4) - hMPV - RSV (A, B) - ADV - BocV - CoV (OC43, HKU1, 229E, NL63, SARS), Enterovirus/Rhinovirus
<b>Technology</b>	End-point RT-PCR Microspheres-based detection	End-point RT-PCR Dual priming oligo (DPO)	End-point RT-PCR Microspheres-based detection
<b>Equipment</b>	Luminex 100/200 System	Gel electrophoresis, Lab901, MultiNA	Luminex 100/200 System

TABLE 2. Technology and targets of different multiplex assays for swine-origin 2009 A (H1N1) influenza virus

Assay	Resplex II Panel Plus	Seeplex® Influenza A/B Subtyping	Astra Influenza Screen & Type RT- kit 1.0
<b>Targets</b>	INFA (seasonal A, swine H1N1) - INFB - PIV (1, 2, 3, 4) - hMPV - RSV (A, B) - ADV - BocV - CoV (OC43, HKU1, 229E, NL63) - Coxsackieviruses/Echovirus - Rhinovirus	INFA (swine H1N1, seasonal H1, H3) - INFB	INFA (swine H1N1, seasonal INFA)
<b>Technology</b>	End-point RT-PCR Microspheres-based detection	End-point RT-PCR Dual priming oligo (DPO)	Real-time RT-PCR
<b>Equipment</b>	Luminex 100/200 System	Gel electrophoresis, Lab901, MultiNA	Prism 7500, Light Cycler 480, Rotorgene 3000 & 6000

## RESULTS

Test characteristics were evaluated using a composite gold standard for a true positive, defined as: any positive viral isolation or at least two other assays (DFA or PCR) positive. A false positive was defined as any single positive result, except for viral isolation.

## 1) Detection of various respiratory viruses:

TABLE 3. Sensitivity of DFA, culture and three commercial multiplex PCR assays for detection of respiratory viruses from positive and negative specimens

Target	DFA	Culture	Resplex II v2.0	Seeplex RV15	xTAG RVP
INFA	76.7%	60.3%	96.9%	96.9%	96.9%
INFB	78.4%	75.0%	100%	100%	100%
PIV (combined 1, 2, 3, 4)	72.4%	61.5%	82.9%	97.6%	85.4%
hMPV	68.6%	43.3%	82.0%	97.4%	97.4%
RSV (combined A, B)	93.5%	84.9%	83.4%	99.3%	88.2%
ADV	38.1%	44.4%	71.4%	100%	85.7%
BocV	-	-	75.0%	100%	-
CoV (combined OC43, HKU1)	-	-	92.6%	100%	48.1%
CoV (combined 229E, NL63)	-	-	100%	100%	88.2%
Enteroviruses/Rhinovirus	-	-	96.6%	78.0%	93.7%

TABLE 4. Prevalence of enteroviruses, rhinovirus and enteroviruses/rhinovirus using three commercial multiplex PCR assays

Target	Resplex II v2.0	Seeplex RV15	xTAG RVP
Enteroviruses	21.2% (159/750)	0.7% (5/750)	-
Rhinovirus	11.2% (84/750)	17.3% (130/750)	-
Enteroviruses/Rhinovirus(*)	-	-	25.3% (190/750)

(\*) Enteroviruses/Rhinovirus constitute a "single" target and cannot be differentiated with xTAG RVP assay

TABLE 5. Specificity of DFA, culture and three commercial multiplex PCR assays for detection of respiratory viruses from positive and negative specimens

Target	DFA	Culture	Resplex II v2.0	Seeplex RV15	xTAG RVP
INFA	99.7%	100%	100%	98.8%	100%
INFB	99.8%	100%	100%	100%	100%
PIV (combined 1, 2, 3, 4)	99.8%	100%	100%	99.0%	99.6%
hMPV	99.4%	100%	100%	99.7%	99.7%
RSV (combined A, B)	99.6%	100%	100%	97.7%	100%
ADV	100%	100%	99.9%	98.1%	99.9%
BocV	-	-	100%	100%	-
CoV (combined OC43, HKU1)	-	-	100%	99.3%	99.9%
CoV (combined 229E, NL63)	-	-	100%	98.8%	99.9%
Enteroviruses/Rhinovirus	-	-	99.3%	99.1%	96.0%

## 2) Detection of swine-origin 2009 A (H1N1) influenza virus:

TABLE 6. Sensitivity of DFA and three commercial multiplex PCR assays for detection of swine-origin 2009 A (H1N1) influenza virus from positive specimens.

Target	DFA	Astra Influenza Screen & Type	Resplex II Plus PRE	Seeplex Influenza A/B Subtyping
Swine INFA-H1N1 (n=49)	69.6%	100%	91.8%	95.9%

## DISCUSSION & CONCLUSIONS

Multiplex assays offered enhanced sensitivity over DFA and culture in the detection of viral respiratory pathogens in children. The DFA assay was able to detect without differentiating all influenza A subtypes including swine-origin H1N1 (TABLES 3 and 6). For some targets the sensitivity of detection increased from 38.1% in DFA to 100% in Seeplex (2.6 times increase) for ADV and from 43.3% in culture to 97.4% in Seeplex and xTAG RVP (2.2 times increase) for hMPV. Variability in the sensitivity for specific targets was observed among the different molecular assays. Specificity was excellent for most targets and for all assays. Multiplex testing offers rapid, comprehensive viral respiratory detection in a single test with high sensitivity and specificity. Moreover, newer targets can be added to multiplex assays, which, combined with high throughput analysis capabilities, make these assays extremely versatile, sensitive, cost effective and time saving diagnostic tools.

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