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Research Article

Respiratory Virus Multiplex RT-PCR Assay Sensitivities and Influence Factors in Hospitalized Children with Lower Respiratory Tract Infections*

Jikui Deng[1,3], Zhuoya Ma[1], Wenbo Huang[2], Chengrong Li[1], Heping Wang[1], Yuejie Zheng[1], Rong Zhou[2]✉ and Yi-Wei Tang[3]✉

1. Shenzhen Children's Hospital, Chongqing Medical University, Shenzhen 518026, China;
2. State Key Laboratory of Respiratory Diseases, Guangzhou Medical University, Guangzhou, Guangdong 510230, China;
3. Department of Laboratory Medicine, Memorial Sloan-Kettering Cancer Center, New York, NY 10065, USA

Multiplex RT-PCR assays have been widely used tools for detection and differentiation of a panel of respiratory viral pathogens. In this study, we evaluated the Qiagen ResPlex II V2.0 kit and explored factors influencing its sensitivity. Nasopharyngeal swab (NPS) specimens were prospectively collected from pediatric inpatients with lower respiratory tract infections at the time of admission in the Shenzhen Children's Hospital from May 2009 to April 2010. Total nucleic acids were extracted using the EZ1 system (Qiagen, Germany) and 17 respiratory viruses and genotypes including influenza A virus (FluA), FluB, parainfluenza virus 1 (PIV1), PIV2, PIV3, PIV4, respiratory syncytial virus (RSV), human metapneumovirus (hMPV), rhinoviruses (RhV), enteroviruses (EnV), human bocaviruses (hBoV), adenoviruses (AdV), four coronaviruses (229E, OC43, NL63 and HKU1), and FluA 2009 pandemic H1N1(H1N1-p) were detected and identified by the ResPlex II kit. In parallel, 16 real-time TaqMan quantitative RT-PCR assays were used to quantitatively detect each virus except for RhV. Influenza and parainfluenza viral cultures were also performed. Among the total 438 NPS specimens collected during the study period, one or more viral pathogens were detected in 274 (62.6%) and 201(45.9%) specimens by monoplex TaqMan RT-PCR and multiplex ResPlex, respectively. When results from monoplex PCR or cell culture were used as the reference standard, the multiplex PCR possessed specificities of 92.9-100.0%. The sensitivity of multiplex PCR for PIV3, hMPV, PIV1 and BoV were 73.1%, 70%, 66.7% and 55.6%, respectively, while low sensitivities (11.1%-40.0%) were observed for FluA, EnV, OC43, RSV and H1N1. Among the seven viruses/genotypes detected with higher frequencies, multiplex PCR sensitivities were correlated significantly with viral loads determined by the TaqMan RT-PCR in FluA, H1N1-p and RSV (p=0.011-0.000). The Qiagen ResPlex II multiplex RT-PCR kit possesses excellent specificity for simultaneous detection of 17 viral pathogens in NPS specimens in pediatric inpatients at the time of admission. The sensitivity of multiplex RT-PCR was influenced by viral loads, specimen process methods, primer and probe design and amplification condition.

Multiplex RT-PCR; Respiratory viral loads; Cell culture; Lower respiratory tract infection

Lower respiratory tract infections (LRTIs) are the most frequent cause of hospitalization among children worldwide (Ahn K M, et al., 1999; Garbino J, et al., 2004; Ruuskanen O, et al., 2011; Sung C C, et al., 2011; Thompson W W, et al., 2003; van Woensel J B, et al., 2003). A large proportion of LRTIs are caused by respiratory viruses including influenza virus A (FluA) and B (FluB), parainfluenza viruses 1-4 (PIV1-4), respiratory syncytial virus (RSV), rhinoviruses (RhV), enteroviruses (EnV), and adenoviruses (AdV) (Juven T, et al., 2000; Legg J P,

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✉ Corresponding authors.
Rong Zhou: Phone: +86-20-83337750,
Fax: +86-20-83062729, Email: zhou3218@yahoo.com.
Yi-Wei Tang: Phone: +1-212-639-8181,
Fax: +1-646-422-2061, Email: tangy@mskcc.org

et al., 2005; Weigl J A, et al., 2005). Over the past decade, the viral pathogen list has been expanded to several newly discovered viruses including human metapneumovirus (hMPV) (van den Hoogen B G, et al., 2001), some coronaviruses (NL63, HKU1, SARS) (Pyrc K, et al., 2007), human bocaviruses (hBoV) (Allander T, et al., 2005), and pandemic influenza A/H1N1 2009 virus (H1N1-p) (Anonymous, 2009). Various viruses with different shedding levels may result in a large disease severity range from common bronchitis to fatal pneumonia (Li C C, et al., 2010; Martin E T, et al., 2012; Takeyama A, et al., 2012; Torres J P, et al., 2010).

Rapid and accurate etiologic diagnosis of LRTIs is essential to patient management. Several multiplex RT-PCR-based devices are commercially available for detection and differentiation of a panel of respiratory viral pathogens (Zhang S, et al., 2011). They are being increasingly used in the clinical setting as a cornerstone technique in the clinical virology laboratory as they possess sensitivities greater than rapid viral antigen testing and test turnaround time shorter than standard respiratory virus culture (Balada-Llasat J M, et al., 2011; Kim S R, et al., 2009; Rand K H, et al., 2011; Schindera C, et al., 2010). Varying sensitivities have been reported between different devices for specific viral pathogens in different studies (Balada-Llasat J M, et al., 2011; Kim S R, et al., 2009; Rand K H, et al., 2011; Schindera C, et al., 2010). In this study, we evaluated the ResPlex II V2.0 kit (Qiagen, Germany), which uses a target enriched multiplexing RT-PCR amplification coupled with a suspension array detection, for detection and identification of a panel of respiratory specimens in pediatric inpatients with LRTIs. Clinical accuracy of the ResPlex II assay was validated on a panel of prospectively collected consecutive nasopharyngeal swab (NPS) specimens in comparison to viral culture and a monoplex real-time TaqMan RT-PCR. We also correlated the ResPlex II assay sensitivity with viral loads determined by the quantitative, monoplex real-time TaqMan RT-PCR.

MATERIALS AND METHODS

Study population and samples

Patients recruited in this study were pediatric inpatients aged less than 14 years old with clinical diagnosis of LRTIs in Shenzhen Children's Hospital as part of routine clinical care at Division of Respiratory Disease between May 2009 and April 2010. Samples used in this study were NPS specimens collected by an eSwab (482C, Copan Diagnostics, Inc., Murrietta, CA) prospectively from these patients at the time of admission. Consecutive NPS specimens were collected and included except for low volumes after routine diagnostic tests were performed. Each sample was divided into three 1 mL aliquots and stored immediately in -80 °C. The Shenzhen Children's Hospital Institutional Review Board (IRB) classified this study as non-human research without clinical history review; the study was exempted from IRB approval and informed consent requirements were waived.

Viral culture

One NPS aliquot was used to perform influenza and parainfluenza viral cultures. Specimens were inoculated into MDCK (Madin-Darby Canine Kidney) cells in 96-well microplates (Corning, USA) using enhanced cell culture with fluorescent antibody detection for primary viral isolation of influenza viruses A and B. Plates were incubated at 37 °C in 5% CO_2 and inspected daily after inoculation for the presence of cytopathic effect (Yang Z F, et al., 2010). Hemagglutination assays (HA) using MDCK cell culture supernatants 2-3 days post-infection were performed for confirmation of FluA infections (Yang Z F, et al., 2010). All cultures were screened at 5 to 7 days post-inoculation by direct immunofluorescence using Imagen reagents (Chemicon, USA). In cultures with positive cytopathic effect or HA results, direct immunofluorescence was performed to identify the types of influenza viruses (Landry M L, et al., 2000).

Monoplex real-time TaqMan RT-PCR

One NPS aliquot was sent to the State Key Laboratory of Respiratory Diseases of the Guangzhou Medical University where several quantitative monoplex real-time TaqMan RT-PCR assays were performed as previously described (Liu W K, et al., 2011). Pathogens detected individually by this platform included FluA, H1N1-p, FluB, PIV1, PIV2, PIV3, PIV4, RSV, hMPV, EnV, hBoV, AdV, four coronaviruses (229E, OC43, NL63 and HKU1) as well as two atypical bacterial pathogens (*Mycoplasma pneumonia* and *Chlamydia pneumonia*). Total nucleic acids from NPS were extracted using a QIAamp MinElute virus Kit (Qiagen, Germany), in accordance with the manufacturer's protocol and previously described (Sefers S E, et al., 2006). Primers and the probe were synthesized by TaKaRa (Dalian, China). One step RT-PCR reaction buffer (PrimeScript One Step RT-PCR Kit Ver.2) was also purchased from TaKaRa. Amplification was conducted using 10 pmol of primers, 3 pmol of probe and 5 μL

specimen extract in a final volume of 25 μL on the ABI-7500 real-time PCR instrument (Applied Biosystems, Foster City, CA). Cycling conditions included an initial incubation at 50 °C for 30 min, 94 °C for 2 min, followed by 40 cycles of 94 °C for 10 sec and 55 °C for 35 sec (Liu W K, et al., 2011). The amplified gene target sequence was inserted into the pMD18-T vector (TaKaRa) and used as a positive control for quantification analysis. Sensitivity of the PCR assay was calculated to be 10 copies of plasmid DNA using positive control plasmid diluted titrations. Five standards covering viral loads from 10-100,000 copies/mL were included to generate a quantification standard curve for absolute viral load determination of each tested NPS specimen.

ResPlex II Panel v2.0 (ResPlex)

One NPS aliquot was used for a panel of respiratory viral pathogen detection by the ResPlex II Panel (Qiagen, Germany) (Li H, et al., 2007). Version 2.0 covers viruses and types of FluA, FluB, RSV, PIV1, PIV2, PIV3, PIV4, hMPV, coxsackieviruses/echoviruses, RhV, human bocaviruses, adenoviruses B and E, and four coronaviruses (NL63, HKU1, 229E, and OC43). A newer version (ResPlex II plus panel PRE) was used later in the study which also includes FluA 2009 pandemic H1N1 (H1N1-p). Total nucleic acids were extracted from 200 μL of NPS specimen using the Qiagen Q-card EZ1 virus mini kit (Cat. No.955314) in an EZ1 Extraction System and eluted in 50 μL of water. The RT-PCR amplification was performed on the ABI-7500 real-time PCR System with 10 μL of extract included in each reaction. The amplification product was then detected and identified by using a suspension bead array for multiplex hybridization in the LiquiChip 200 Workstation with the QIAplex MDD Software (Qiagen, Germany). Controls were included on each test run including a positive control (*in vitro* transcribed RNA corresponding to a human genomic DNA sequence), an internal control to check for viral RNA isolation and PCR inhibition, and a sample control to detect traces of human genomic DNA present in each specimen. A positive result was determined using a cut-off median fluorescence intensity (MFI) of 250 or the mean plus 2 standard deviations of the negative controls (Gharabaghi F, et al., 2011).

Statistical analysis

The combined results of the viral culture and monoplex real time RT-PCR was used as a reference standard. A sample was determined to be positive for the tested virus when viral culture or real time RT-PCR was positive. For comparison of qualitative categorical data, χ^2 test or Fisher's exact test and Mantel-Haenszel χ^2 test were used wherever appropriate. A $p<0.05$ was considered statistically significant. Trend analysis and rank test was used to determine the correlations between ResPlex assay sensitivities and viral loads.

Table 1. The comparison of ResPlex (R) and monoplex real-time TaqMan RT-PCR (M) for 16 respiratory viruses *

Virus	No. detected				Sensitivity	Specificity	PPV	NPV
	M+R+	M+R-	M-R+	M-R-	(%)	(%)	(%)	(%)
RSV	46	92	6	294	33.3	98	88.5	76.2
FluA	4	32	2	400	11.1	99.5	66.7	92.6
hMPV	21	9	3	405	70	99.3	87.5	97.8
PIV3	19	7	5	407	73.1	98.9	79.2	98.4
H1N1-p**	10	15	0	289	40	100	100	95.1
PIV1	12	6	3	417	66.7	99.3	80	98.6
hBoV	10	8	0	420	55.6	100	100	98.1
OC43	4	11	1	422	26.7	99.8	80	97.5
EnV	2	12	30	394	14.3	92.9	6.3	97
ADV	4	3	0	431	57.1	100	100	99.3
NL63	3	3	1	431	50	99.8	75	99.3
229E	3	2	1	432	60	99.8	75	99.5
FluB	2	2	0	434	50	100	100	99.5
PIV4	2	1	1	434	66.7	99.8	66.7	99.8
PIV2	0	3	0	435	NA	100	NA	99.3
HKU1	0	0	0	438	NA	NA	NA	NA

* RhV was not evaluated as it was not detected by the monoplex real-time RT-PCR. PPV, positive predictive value; NPV, negative predictive value. ** The Resplex II Panel v2.0 was replaced by a ResPlex II Plus Panel PRE to enhance H1N1-P coverage during the study. A total of 314 samples were tested by the ResPlex II Plus Panel PRE version.

Table 2. Relationship of sensitivity of ResPlex PCR and viral loads determined by monoplex PCR

Monoplex copies/mL	No. of ResPlex-PCR positive/No. of Monoplex PCR positive						
	RSV (%)	FluA (%)	hMPV (%)	PIV3 (%)	H1N1-p (%)	PIV1 (%)	hBoV (%)
$\geqslant 10^6$	6/9 (66.7)	1/1 (100)	3/4 (75.0)	2/3 (66.7)	4/5 (80.0)	0/0 (0)	9/11 (81.8)
1-9.9×10^5	34/45 (75.5)	2/5 (40.0)	10/14 (71.4)	8/8 (100.0)	5/6 (83.3)	5/5 (100.0)	1/3 (33.3)
1-9.9×10^4	5/38 (13.2)	1/7 (14.3)	6/9 (66.7)	4/5 (80.0)	1/6 (16.7)	6/7 (85.7)	0/2 (0)
1-9.9×10^3	1/33 (3.0)	0/12 (0)	2/3 (66.7)	2/4 (50.0)	0/6 (0)	1/3 (33.3)	0/1 (0)
1-9.9×10^2	0/13 (0)	0/11 (0)	0/0 (0)	3/6 (50.0)	0/2 (0)	0/3 (0)	0/1 (0)
Negative	6/300 (2.0)	2/402 (0.5)	3/408 (0.7)	5/412 (1.2)	0/289 (0)	3/420 (0.7)	0/420 (0)
z	-6.859	-5.119	-1.267	-1.011	-2.538	-1.068	-1.511
p	0.000	0.000	0.205	0.312	0.011	0.285	0.131

RESULTS

A total of 438 qualified NPS specimens collected during a one full year study period were included in the final testing and analysis. The male ratio was 77.6%. The mean age of patients was 1.4 years, ranging from one month to 11 years. Among the total specimens tested, 263 (60.0%), 134 (30.6%) and 41 (9.4%) were from children of <1, 1-3 and >3 years old, respectively.

Among the total 438 NPS specimens, one or more viral pathogens were detected in 274 (62.6%) and 201(45.9%) specimens by monoplex TaqMan RT-PCR and multiplex ResPlex RT-PCR, respectively. Viral culture was positive for 56 (12.8%) specimens including 17 FluA, 3 FluB, 14 PIV1, and 22 PIV3. Among these culture positive specimens, monoplex real-time TaqMan RT-PCR results were fully concordant while ResPlex results were negative for 13 specimens including six FluA, four PIV1 and three PIV3. When results from monoplex RT-PCR or cell culture were used as the reference standard, the multiplex PCR possessed specificities ranging from 92.9% to 100.0% for the viruses tested. The sensitivities of multiplex PCR varied with high sensitivity observed for PIV3, hMPV, PIV1 and hBoV (55.6-73.1%) and low sensitivity for FluA, EnV, OC43, RSV and H1N1-p (11.1-40.0%) (Table 1).

Rhinoviruses were detected from 36 specimens (8.3%) by the ResPlex assay including 22 rhinovirus alone and 14 co-detected with other viruses including RhV/EnV (n=10), RhV/RSV (n=2), RhV/hMPV (n=1), and RhV/229E (n=1). Since rhinoviruses were not detected by the monoplex TaqMan RT-PCR, these data were not further analyzed.

We further correlated viral load information determined by the TaqMan RT-PCR with the ResPlex diagnostic sensitivities for seven viruses/genotypes detected with higher frequencies (positive rate >4% detected by monoplex RT-PCR). The ResPlex multiplex PCR sensitivities correlated significantly with viral loads for RSV, FluA and H1N1-p (p=0.011-0.000). Significant correlation was not observed for PIV1, PIV3, hBoV, and hMPV (p>0.05) (Table 2).

During the study, a new version of ResPlex, ResPlex II Plus Panel PRE, was manufactured to increase coverage of H1N1-p. There were 124 and 314 specimens which were tested by ResPlex II Panel and ResPlex II Plus Panel PRE, respectively (Table 1). For the seven non-H1N1-p viruses/genotypes detected with higher frequencies, the sensitivities dropped (range from 3.6% to 43.7%) for five viruses including FluA, RSV, hBoV, PIV1 and PIV3. The

Table 3. Sensitivity of two kinds of ResPlex II kit for common viruses *

Virus	Resplex II Panel No. of specimens				Sensitivity (%)	ResPlex II Plus Panel PRE No. of specimens				Sensitivity (%)	χ^2 value	*P* value
	M+R+	M+R-	M-R+	M-R-		M+R+	M+R-	M-R+	M-R-			
RSV	14	25	3	82	35.9	32	67	3	212	32.3	0.161	0.688
FluA	2	2	2	118	50.0	2	30	0	282	6.3	6.891	0.053
hMPV	4	4	2	114	50.0	17	5	1	291	77.3	2.078	0.195
PIV3	6	0	2	116	100.0	13	7	3	291	65.0	2.874	0.146
PIV1	3	0	2	119	100.0	9	6	1	298	60.0	1.80	0.515
hBoV	5	1	0	118	83.3	5	7	0	302	41.7	2.813	0.152

* R, ResPlex II Panel; M, monoplex real-time TaqMan RT-PCR.

sensitivity increased only for hMPV (Table 3). None of these sensitivity differences reached statistical significance by χ^2 or Fisher's exact test ($p>0.05$). When the Mantel-Haenszel χ^2 test was used for the combined strata analysis, no significant difference was observed ($\chi^2_{M-H}=2.676$, $p=0.102$).

DISCUSSION

Several multiplex RT-PCR-based devices have been commercially available for detection and identification of a panel of viral pathogens causing LRTIs. We evaluated one of the platforms (ResPlex II), which combines a target enriched multiplexing RT-PCR amplification and a suspension array detection (Balada-Llasat J M, et al., 2011; Li H, et al., 2007), using prospectively collected NPS specimens from pediatric inpatient patients with clinical diagnosis of LRTIs. The ResPlex II multiplex RT-PCR kit possessed excellent specificity and varied sensitivities ranging from 11.1 to 73.1%, but which was relatively lower than two recently reported studies on children populations (Forman M S, et al., 2012; Hayden R T, et al., 2012).

Significantly lower sensitivities by the ResPlex II assay were observed especially for FluA, EnV, OC43, RSV and H1N1-p. We observed the following factors which might explain the discrepancies between our and previously reported studies. First, based on the patient populations and reference standards, the test sensitivities could vary significantly. Mak G et al (Mak G C, et al., 2011) reported the sensitivity of ResPlex II for FluA and H1N1-p were only 31.8% and 42.9% when monoplex RT-PCR was used for comparison. However in previous studies in which virus culture or DFA was used as the main reference standard, the sensitivities of ResPlex II were observed to be from 84%-100% (Balada-Llasat J M, et al., 2011; Li H, et al., 2007). In our pediatric inpatients with lower respiratory tract infections at the time of admission, the monoplex RT-PCR detected significantly more Flu and PIV than in cell culture. Secondly, different extractions vary significantly in quality and quantity of nucleic acid recovery. In our study, nucleic acids were extracted using the Qiagen EZ-1 system for the ResPlex II, a system with reported low quantity and quality of nucleic acid recovery (Tang Y W, et al., 2005; Yang G, et al., 2011). Finally, the multiplex PCR condition requires careful optimization; any addition or modification of primers/probes may significantly alter the amplification efficiency. In our study, when the target of H1N1-p was added, the sensitivities of ResPlex II plus panel PRE assay dropped for most of the viral targets, especially for FluA detection.

Previous studies revealed that the detection of FluA (H1N1) viruses correlated with the time after symptom onset and viral load (Cheng P K, et al., 2010). In our study, among the seven viruses/genotypes detected with higher frequencies, ResPlex II sensitivities correlated significantly with viral loads determined by the TaqMan RT-PCR in RSV, FluA and H1N1-p. In contrast, no viral load correlation was observed in PIV3, hMPV, PIV1 and hBoV. Since viral load did not correlate with lower sensitivities for some of the targets, the PCR amplification and hybridization efficiencies may also play a role. It is reasonable that multiplex PCR sensitivities decreased with decreasing viral load of target virus. In the ResPlex II, primers and probes were designed from the F genome of hMPV in which significant sequence variations were revealed by the recent finding of four geno/ subtypes (Mackay I M, et al., 2006). Whether the poor correlation between viral load and PCR sensitivity in hMPV is related to the primer/probe design merits further investigation. Varied analytical sensitivities were noticed in other viral targets in a recent study in immunocompromised children (Hayden R T, et al., 2012).

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