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## Development of a multiplex one step RT-PCR that detects eighteen respiratory viruses in clinical specimens and comparison with real time RT-PCR

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Rapid and accurate diagnosis of viral respiratory infections is crucial for patient management. Multiplex reverse transcriptase polymerase chain reaction (mRT-PCR) is used increasingly to diagnose respiratory infections and has shown to be more sensitive than viral culture and antigen detection. Objective of the present study was to develop a one-step mRT-PCR that could detect 18 respiratory viruses in three sets. The method was compared with real time RT-PCR (rRT-PCR) for its sensitivity and specificity. Clinical specimens from 843 pediatric patients with respiratory symptoms were used in the study. 503 (59.7%) samples were detected positive by mRT-PCR. Of these 462 (54.8%) exhibited presence of a single pathogen and 41 (4.9%) had multiple pathogens. rRT-PCR detected 439 (52.1%) positive samples, where 419 (49.7%) exhibited one virus and 20 (2.4%) showed co-infections. Concordance between mRT-PCR and rRT-PCR was 91.9% and kappa correlation 0.837. Sensitivity and specificity of mRT-PCR were 99.5% and 83.7% while that of rRT-PCR was 86.9% and 99.4% respectively. Rhinovirus (17.2%) was the most frequently detected virus followed by respiratory syncytial virus B (15.4%), H1N1pdm09 (8.54%), parainfluenza virus-3 (5.8%) and metapneumovirus (5.2%). In conclusion, mRT-PCR is a rapid, cost effective, specific and highly sensitive method for detection of respiratory viruses.

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### 1. Introduction

Respiratory viral disease is a serious public health problem globally and has social and economic impact. Respiratory viruses cause disease that ranges from mild respiratory illness to fatal pneumonia and contribute significantly to morbidity and mortality worldwide (Williams et al., 2002). Clinical presentations of respiratory viral infections are very similar to each other and etiological diagnosis based on clinical parameters is difficult. Rapid diagnosis is essential for prompt patient management and public health action.

Diagnosis of viral respiratory infections has been based on the use of conventional methods such as isolation by cell culture and antigen detection. Although these methods are effective and often complementary, they have certain limitations. Cell culture is considered to be the “gold standard” for virus detection, but it is too

laborious and time consuming. Antigen detection is insufficiently sensitive and/or specific.

Sensitivity and specificity of respiratory virus detection have improved considerably with the advent of nucleic acid amplification tests (NATs; Bellau-Pujol et al., 2005; Fox, 2007; Freymuth et al., 2006; Liolios et al., 2001; Vabret et al., 2000; Weinberg et al., 2004). To enable a rapid response to a potential outbreak, it is desirable to have fast, accurate and comprehensive diagnostic methods that are capable of detecting simultaneously and subtyping viruses. However, it is very expensive to use virus specific PCR assays. Several groups have developed multiplex RT-PCRs to identify respiratory viruses in clinical samples (Bellau-Pujol et al., 2005; Bharaj et al., 2009; Brittain-Long et al., 2010; Coiras et al., 2004; Gunson et al., 2005; Jansen et al., 2011; Kim et al., 2009; Lee et al., 2007; Mahony et al., 2007; Pabbaraju et al., 2008; Wang et al., 2009). The preference of one test over the other depends on its specificity, sensitivity and turnaround time as well as cost in resource limited settings.

The purpose of this study was to develop a one step mRT-PCR that could detect respiratory viruses including influenza A viruses, H1N1pdm09, seasonal H1N1, H3N2, influenza B viruses, respiratory syncytial virus (RSV) A and B, human metapneumovirus (HMPV), parainfluenza viruses (PIV) 1, 2, 3, 4, rhinovirus, enterovirus, corona

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viruses OC43, 229E, NL63 and HKU1 in three sets in human clinical samples and to compare it with rRT-PCR.

## 2. Materials and methods

### 2.1. Primer design

Primers used in this assay were designed using dual priming oligonucleotide (DPO) technology which allows specific detection of viruses without any non specific amplification (Chun et al., 2007). Conserved regions of target genes were chosen to design the forward and reverse primers. For mRT-PCR, different viral genes with their PCR product sizes are given in Table 1. Primer sequences can be provided on request. The above mRT-PCR primers were obtained from Sigma (Bangalore, India). Multiplex RT PCRs were carried out in 3 tubes (sets) (set 1: influenza A, subtype seasonal H1, H3 and pandemic H1 (2009), influenza B; set 2: RSV-A, RSV-B, HMPV, PIV-1, -2, -3; and set 3: PIV-4, corona virus OC43/HKU1, corona virus 229E/NL63, rhinovirus/enterovirus). The mRT-PCR did not differentiate between corona virus OC43 and HKU1; 229E and NL63; and between rhinovirus and enterovirus. Monoplex one step real time RT PCRs (rRT-PCR) for influenza A, subtype seasonal H1, H3, pandemic H1 (2009), influenza B and internal control *RnaseP* were carried out as per protocol provided by Centers for Disease Control and Prevention, USA (CDC) (CDC protocol, 2009). For RSV-A and B, PIV-1, -2, -3, corona virus OC43, 229E, NL63 and rhinovirus, the rRT-PCR assay was performed as described (Gunson et al., 2005), for HMPV (Maertzdorf et al., 2004) and for PIV-4 as described on the following website [www3.appliedbiosystems.com/cms/groups/mcb/cms.088565.pdf](http://www3.appliedbiosystems.com/cms/groups/mcb/cms.088565.pdf). For rRT-PCR, all influenza, RSV-A, rhinovirus, PIV-1, and coronavirus NL63 TaqMan probes were labelled with FAM (fluorescein amidite); RSV-B, PIV-2, -3, -4, coronavirus OC43 and 229E were labelled with VIC. Real time PCR primers and TaqMan probes were obtained from Applied Biosystems, USA.

### 2.2. Viral isolates and clinical samples

Tissue culture grown viruses used as positive controls for influenza B, seasonal influenza A H1N1, H3N2, H1N1pdm09, RSV-A and B, HMPV, PIV-1, -2, -3 and enterovirus. Clinical samples positive for PIV-4, rhinovirus, human corona virus OC43/HKU1 and 229E/NL63 were confirmed by sequencing and used to check primers cross reactivity. Nasal, nasopharyngeal or throat swab specimens of ILI (influenza like illness)/SARI (severe acute respiratory illness) patients collected from July 2009 to August 2011 and referred to National Institute of Virology by different hospitals in Pune for diagnosis of H1N1pdm09 were used in this study. The

**Table 1**  
Different viral targets with product size.

Set	Virus	Gene	Size
Set 1	Influenza A	Matrix	450 bp
	Influenza B	Matrix	515 bp
	InfAH1 (seasonal)	Haemagglutinin	190 bp
	H1N1pdm09	Haemagglutinin	599 bp
	InfAH3	Haemagglutinin	303 bp
Set 2	RSV-A	Fusion	248 bp
	RSV-B	Fusion	316 bp
	HMPV	Fusion	471 bp
	PIV-1	Nucleoprotein	400 bp
	PIV-2	Nucleoprotein	636 bp
	PIV-3	Nucleoprotein	192 bp
	PIV-4	Nucleoprotein	539 bp
Set 3	Rhino/enterovirus	5'-UTR	230 bp
	HCoVOC43/HKU1	Membrane	343 bp
	HCoV229E/NL63	Spike glycoprotein	148 bp

study was approved by the institutional ethics committee. A total of 843 clinical specimens of pediatric patients were tested by conventional mRT-PCR and monoplex rRT-PCR. One hundred and fourteen influenza positive samples (27 seasonal H3N2, 27 seasonal H1N1, 29 H1N1pdm09 and 31 influenza B) were used for validation of the multiplex assay set 1.

### 2.3. RNA extraction

RNA was extracted using the MagMax automated RNA extractor as per manufacturer's instructions. Fifty microlitres of clinical sample was used for RNA extraction and finally eluted in 50  $\mu$ l of elution buffer.

### 2.4. Conventional multiplex RT-PCR

Amplification for conventional mRT-PCR was carried out on GeneAmp PCR System 9700 using Invitrogen Superscript III one step RT-PCR kit. Master mix for mRT-PCR comprised of 25  $\mu$ l 2 $\times$  buffer, 2  $\mu$ l enzyme mix, 20  $\mu$ M of forward and reverse primer each and 19  $\mu$ l of RNA template to make a 50  $\mu$ l reaction. Negative and positive controls were run with each experiment. Thermal cycling conditions were 50  $^{\circ}$ C for 30 min for reverse transcription, initial denaturation at 94  $^{\circ}$ C for 10 min, 45 cycles of three steps – 15 s at 94  $^{\circ}$ C, 60 s at 55  $^{\circ}$ C, 45 s at 68  $^{\circ}$ C, and final extension at 68  $^{\circ}$ C for 5 min. 10  $\mu$ l of PCR products were mixed with gel loading dye and run on a 2% agarose gel containing ethidium bromide and visualized on a UV transilluminator.

### 2.5. Real time RT-PCR

Real time RT-PCR assay was performed on Applied Biosystems' ABI 7500 machine using Invitrogen Superscript III one step qRT-PCR kit. A typical 25  $\mu$ l PCR reaction comprised of 10  $\mu$ M of each forward and reverse primer, 5  $\mu$ M of TaqMan probe, 12.5  $\mu$ l 2 $\times$  buffer, 0.5  $\mu$ l Superscript III enzyme and 5  $\mu$ l RNA template. Thermal cycling conditions for rRT-PCR were 50  $^{\circ}$ C for 30 min for reverse transcription, initial denaturation at 94  $^{\circ}$ C for 5 min, 45 cycles of 15 s at 94  $^{\circ}$ C, 45 s at 55  $^{\circ}$ C.

### 2.6. Specificity and analytical sensitivity of conventional mRT-PCR

The specificity of the multiplex PCR assay was evaluated by cross reaction tests with known viral isolates and different panels of sequence confirmed known clinical respiratory samples as reference material. The assay set 1 was also tested on influenza A H5N1, H1N1, H9N2, and newcastle disease virus procured from National Institute of Virology's virus repository and WHO (World Health Organization) influenza panel for QA/QC.

To analyze sensitivity, known positive samples were subjected to monoplex PCR. The forward primer was tagged with T7 promoter and by gel electrophoresis in a 1% agarose gel, bands of expected size were observed. These bands were extracted from agarose gel using Qiagen gel extraction kit (Qiagen, Germany). Purified PCR product was quantified using a Nanoplex nanophotometer (Implant, Germany). *In vitro* transcription was carried out using RiboMax T7 *in vitro* transcription system from Promega as per manufacturer's instructions (Promega, USA). Further, RNA was purified using Qiagen's RNA extraction kit and estimated using the Nanoplex nanophotometer. RNA copy number was calculated and 10 fold serial dilutions of the *in vitro* RNA were performed. Copy number referred to the number of copies of the target gene used to ascertain the limit of detection of the assay. The concentrations tested for each target in set 1 were 10<sup>6</sup> to 10 copies and in sets 2 and 3 were 10<sup>7</sup> copies to 1 copy of template RNA per reaction.

The limit of detection (LOD) was defined as the lowest concentration at which each target could be detected consistently. PCR products were detected by gel electrophoresis. Positive controls were prepared by pooling *in vitro* RNA of each set.

### 2.7. Gene sequencing

Initial PCR was set up using monoplex primers for the relevant target gene with the same mastermix composition and thermal cycling conditions as described for the mRT-PCR. PCR product was purified using Qiagen's PCR purification kit according to the manufacturer's instructions. The purified product was used to set up cycle sequencing reaction with each sequencing primer as follows: mastermix: 5× sequencing buffer – 2 µl, cycle sequencing reaction mix RR100 – 2 µl, primer – 1 µl, DDW – 3 µl and template – 2 µl. Thermal cycling conditions: 95 °C for 5 min; 25 cycles of 94 °C – 15 s, 50 °C for 10 s and 60 °C for 4 min. The above cycle sequencing product was purified using Qiagen's DyeEx cycle sequencing purification kit as per manufacturer's instructions. Applied Biosystems 3730xl 96 capillary sequencer was used for sequencing and results were analyzed using sequencing analysis 5.2 software. The sequences were confirmed using NCBI BLAST.

### 2.8. Statistical analyses

Statistical analysis was carried out using PASW Statistics 18 software. The agreement between two diagnostic tests was calculated by means of concordance and kappa statistics. The sensitivity and specificity of one test with reference to the other and vice versa were also computed.

## 3. Results

### 3.1. Specificity and sensitivity of mRT-PCR

Specificity of the mRT-PCR assay was evaluated by cross reaction tests against known respiratory virus isolates/positive samples and a WHO QA/QC panel for influenza viruses showed no cross reactivity amongst different viruses. A product of the expected size was obtained for each viral target in the presence of all the primers of the respective multiplex PCR and the specific products could be distinguished on agarose gel electrophoresis based on their size. For confirmation of the observed bands, sequencing of the amplified PCR products was carried out. For validation of set 1 of the multiplex assay, 114 respiratory clinical specimens previously positive for influenza viruses by rRT-PCR were used and results were 100% concordant. Co-infection was observed in one sample where both seasonal influenza A/H1N1 and A/H3N2 detected, this

was confirmed by sequencing of the hemagglutinin (HA) and neuraminidase (NA) genes. Set 1 of the mRT-PCR detected specifically only H1N1pdm09, seasonal H1N1, H3N2 and all other influenza-A viruses were detected as a universal influenza-A product of 450 bp. In the WHO Influenza panel, 3 samples exhibited bands corresponding to influenza-A only. These samples were confirmed as influenza A H5N1 by sequencing the matrix gene.

Analytical sensitivity of the conventional multiplex assay was determined by testing 10 fold serial dilutions of the quantified RNA transcripts of each target. Set 1 detected 100 RNA copies per reaction for each target. Set 2 detected RSV-A at 10 copies per reaction; HMPV, PIV-1, -2 and -3 at 100 copies per reaction and RSV-B at 1000 RNA copies per reaction. Set 3 detected enterovirus at 1 copy per reaction; PIV4, rhinovirus and corona OC43/HKU1, 229E/NL63 at 1000 RNA copies per reaction in the mRT-PCR. The experiments were repeated three times using all three sets to obtain consistent limits of detection for each virus in the mRT-PCR

### 3.2. Performance of multiplex RT-PCR against real time RT-PCR

Performance of mRT-PCR was evaluated using clinical samples and its sensitivity and specificity were compared with rRT-PCRs. Of the 843 samples tested, 503 (59.7%) samples were detected positive by mRT-PCR which included 462 (54.8%) single infections and 41 (4.9%) co-infections. Real time RT-PCR detected 439 (52.1%) positive samples of which 419 (49.7%) were single infection and 20 (2.4%) were co-infections (supplementary Table 1).

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jviromet.2012.12.017>.

Overall, concordance between both the methods was 91.9% and kappa correlation was 0.837. Sensitivity and specificity of mRT-PCR were 99.5% and 83.7% respectively considering rRT-PCR as the "gold standard". Conversely sensitivity and specificity of rRT-PCR were 86.9% and 99.4% respectively considering mRT-PCR as the "gold standard" (Table 2). For seasonal H3N2, H1N1pdm09, influenza B, RSV-A and B and PIV-2, 100% concordance were observed using both the methods. The sensitivity and specificity for rhinovirus using mRT-PCR were 96.6 and 92.2% and rRT-PCR were 59.3% and 99.6% respectively. Thirty seven out of 59 samples positive for rhinovirus/enterovirus in mRT-PCR but negative by rRT-PCR were selected randomly and sequenced for the 5'-UTR region. Sequencing result confirmed 34 samples as rhinovirus (91.9%) and 3 as enterovirus (8.1%).

For HMPV, sensitivity and specificity were 100% and 97.6% for mRT-PCR while the sensitivity and specificity were 54.6% and 100% respectively for rRT-PCR. The 20 samples positive in the mRT-PCR, but negative by rRT-PCR was confirmed to be positive for HMPV

**Table 2**  
Performance of real time RT-PCR and multiplex RT-PCR.

Virus	No. of samples with result real time RT-PCR vs. multiplex RT-PCR				Concordance (%)	Kappa	rRT-PCR performance with mRT-PCR as gold standard		mRT-PCR performance with RT-PCR as gold standard	
	+/+	+/-	-/+	-/-			Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)
Overall	437	2	66	338	91.93	0.837	86.88	99.41	99.54	83.66
Rhino/Entero	86	3	59	695	92.65	0.695	59.31	99.57	96.63	92.18
H1N1pdm09	72	0	0	771	100.00	1.000	100.00	100.00	100.00	100.00
H3N2	16	0	0	827	100.00	1.000	100.00	100.00	100.00	100.00
Influenza B	22	0	0	821	100.00	1.000	100.00	100.00	100.00	100.00
RSV-A	29	0	0	814	100.00	1.000	100.00	100.00	100.00	100.00
RSV-B	130	0	0	713	100.00	1.000	100.00	100.00	100.00	100.00
HMPV	24	0	20	799	97.63	0.695	54.55	100.00	100.00	97.56
PIV-1	9	0	1	833	99.88	0.947	90.00	100.00	100.00	99.88
PIV-2	6	0	0	837	100.00	1.000	100.00	100.00	100.00	100.00
PIV-3	44	0	5	794	99.41	0.943	89.80	100.00	100.00	99.37
PIV-4	5	0	5	833	99.41	0.664	50.00	100.00	100.00	99.40
Corona OC43	8	4	5	826	98.93	0.635	61.54	99.52	66.67	99.40



by sequencing. Sensitivity and specificity by mRT-PCR were 100% and 99.4% for PIV-3 and those for rRT-PCR were 89.8% and 100% respectively. Among PIV-3 positive clinical samples, approximately 15% samples showed amplification bands corresponding to PIV-1 and PIV-2 which were tested further using monoplex primers. They were negative for PIV-1 and PIV-2 indicating that the bands were nonspecific. For PIV-4, the sensitivity and specificity were 50% and 100% for rRT-PCR respectively. Four out of the 5 samples negative by rRT-PCR were sequenced and found to be PIV-4. Randomly selected 8 corona virus OC43/HKU1 positive samples were sequenced and confirmed as corona virus OC43.

Rhinovirus [145/843 (17.2%)] was the respiratory virus detected most frequently followed by RSV-B in 130 samples (15.4%), H1N1pdm09 in 72/843 (8.5%), PIV-3 in 49 samples (5.8%) and HMPV in 44 samples (5.2%). Corona virus 229E was detected in 4 samples by rRT-PCR. As shown in supplementary Table 1, rhinovirus was detected most frequently in co-infection followed by RSV. All co-infections were confirmed by performing monoplex PCRs with their respective primers.

#### 4. Discussion

Major respiratory viral pathogens recorded globally are RSV, influenza virus, metapneumovirus, parainfluenza virus, rhinovirus, enterovirus, adenovirus and bocavirus. A number of studies have attempted for development and evaluation of multiplex PCRs for detection of various respiratory viruses (Auburn et al., 2011; Bellau-Pujol et al., 2005; Boonsuk et al., 2008; Brittain-Long et al., 2010; Coiras et al., 2004; Jansen et al., 2011; Kim et al., 2009; Mahony et al., 2007; Suwannakarn et al., 2008; Wang et al., 2009). This is the first study using single step mRT-PCR for the detection of 18 respiratory viruses from clinical specimens.

For the conventional mRT-PCR, conserved regions of genes were selected (Table 1). Each set in the assay produced distinctively sized PCR products which could be visualized and easily differentiated by agarose gel electrophoresis. The only limitation of this multiplex PCR is lack of an internal control to check quality of the samples.

The mRT-PCR is sensitive as determined by testing 10 fold serial dilutions of quantified RNA transcripts of each target. Multiplex PCR set 1 detected 100 RNA copies/reaction for each target which is comparable with multiplex PCR developed by Kim et al. (2009). An influenza typing kit available commercially, the Seeplex A/B typing kit has a sensitivity and specificity of 83.7% and 100% in detecting H1N1pdm09 when compared to the CDC real time RT-PCR (Choi et al., 2010) while sensitivity and specificity of our mRT-PCR was 100%, showing excellent concordance with CDC's real time RT-PCR.

Multiplex RT-PCR detected 503 (59.7%) samples positive for one or more of the respiratory viruses listed above and real time PCR detected 439 (52.1%) samples as positive (supplementary Table 1). This low detection rate by rRT-PCR was mainly due to the low sensitivity of detecting rhinovirus and metapneumovirus. Previously, detection rates between 43% and 63% have been reported (Brittain-Long et al., 2010; Broor et al., 2007; Coiras et al., 2004; Kim et al., 2009; Mahony et al., 2007; Maitreyi et al., 2000; Yeolekar et al., 2008). Co-infections have been described previously in approximately 5–20% of infected patients in different studies (Brittain-Long et al., 2010; Coiras et al., 2004; Kim et al., 2009; Mahony et al., 2007; Wang et al., 2010).

One of the biggest advantages of mRT-PCR is its ability to detect co-infections which are often undetected in viral culture with immunofluorescence detection. Several cases of co-infection were detected using both the PCR methods. Multiplex RT-PCR detected 41 (4.9%) samples with co-infection including 3 samples in which 3 viruses were detected whereas rRT-PCR detected 20 (2.4%) samples with co-infection. One case of co-infection with influenza A/H1N1

and A/H3N2 viruses was also detected. Co-infection with different influenza viruses occurs naturally and plays an important role in evolution, epidemiology and pathogenicity of the virus.

Concordance between both the methods was 100% for seasonal H1N1, H3N2, H1N1pdm09, influenza B, PIV-2, RSV-A and B. Lower detection rates by rRT-PCR were due to the lower sensitivity of this system for rhinovirus and HMPV. This may be due to sequence variation in primer probe binding regions in Indian subtypes. Frequency of PIV-1, PIV-2, PIV-4 and coronavirus was low; a larger number of samples are required to better assess their clinical sensitivity and specificity.

The multiplex RT-PCR scores over the monoplex rRT-PCR in a resource limited settings. The cost of mRT-PCR for the 18 viruses discussed here is approximately US\$ 27 whereas for the real time PCRs it is US\$ 45. Moreover, the real time PCR machines are very expensive when compared to a conventional thermal cycler.

#### 5. Conclusion

The one step multiplex RT-PCR assay developed in the present study provides a simple, rapid and cost effective method for the detection of 18 different respiratory viruses from human clinical specimens. The mRT-PCR is as sensitive and as specific as rRT-PCR based assay. This mRT-PCR assay can be used for respiratory virus surveillance as well as diagnosis.

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