

Comparison of the Conventional Multiplex RT–PCR, Real Time RT–PCR and Luminex xTAG[®] RVP Fast Assay for the Detection of Respiratory Viruses

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Detection of respiratory viruses using polymerase chain reaction (PCR) is sensitive, specific and cost effective, having huge potential for patient management. In this study, the performance of an in-house developed conventional multiplex RT–PCR (mRT–PCR), real time RT–PCR (rtRT–PCR) and Luminex xTAG[®] RVP fast assay (Luminex Diagnostics, Toronto, Canada) for the detection of respiratory viruses was compared. A total 310 respiratory clinical specimens predominantly from pediatric patients, referred for diagnosis of influenza A/H1N1pdm09 from August 2009 to March 2011 were tested to determine performance characteristic of the three methods. A total 193 (62.2%) samples were detected positive for one or more viruses by mRT–PCR, 175 (56.4%) samples by real time monoplex RT–PCR, and 138 (44.5%) samples by xTAG[®] RVP fast assay. The overall sensitivity of mRT–PCR was 96.9% (95% CI: 93.5, 98.8), rtRT–PCR 87.9% (95% CI: 82.5, 92.1) and xTAG[®] RVP fast was 68.3% (95% CI: 61.4, 74.6). Rhinovirus was detected most commonly followed by respiratory syncytial virus group B and influenza A/H1N1pdm09. The monoplex real time RT–PCR and in-house developed mRT–PCR are more sensitive, specific and cost effective than the xTAG[®] RVP fast assay. *J. Med. Virol.* **88:51–57, 2016.**

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KEY WORDS: respiratory viruses; RT–PCR; xTAG[®]; RVP assay

INTRODUCTION

Respiratory viral infections are the main cause of morbidity and mortality in developing countries [Nair et al., 2010; Walker et al., 2013]. Accurate and rapid identification of the etiological agent is important for patient management and control of respiratory disease outbreaks [Garbino et al., 2004].

The clinical presentation of respiratory infections caused by various viral pathogens can be very similar; hence, etiological diagnosis is important [Coiras et al., 2004]. Also, there may be a possibility of co-infections occurring in pediatric and immunocompromised individuals.

Respiratory viral infections have traditionally been diagnosed in the laboratory by culture of respiratory specimens and direct fluorescent assay (DFA) [Ginocchio, 2007]. Nucleic acid amplification tests (NAAT) have been developed for classical as well as newly emerging respiratory viruses and have been shown to be more sensitive and time efficient than DFA or culture. However, testing for all respiratory viral targets using individual PCRs is expensive and laborious. A conventional multiplex RT–PCR would be a very useful tool in resource limited settings. Therefore, multiplex PCR methods were developed with the aim of detecting different viruses simultaneously [Coiras et al., 2004; Bellau-Pujol et al., 2005; Kim et al., 2009; Choudhary et al., 2013a].

The introduction of multiplex real time RT–PCR assays has increased the efficiency of routine molecular diagnosis of respiratory viruses and has been shown to be cost effective [Gunson et al., 2005; Mahony et al., 2009; Auburn et al., 2011; Jansen et al., 2011]. The use of specific labeled probes ensures easy interpretation when used in a multiplex format. However, multiplexing of real-time RT–PCR may reduce the assay sensitivity and is limited due

Grant sponsor: Indian Council of Medical Research (ICMR)

Conflicts of interest: The authors declare that they have no conflict of interest.

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Accepted 11 June 2015

DOI 10.1002/jmv.24299

Published online in Wiley Online Library (wileyonlinelibrary.com).

to availability of few commercially available fluorophores and instrumentation [Fox, 2007].

A multiplex PCR followed by detection of products by suspension microarray is one of the most promising approaches for simultaneous detection of multiple respiratory viruses [Kronic et al., 2007; Lee et al., 2007; Mahony et al., 2007; Merante et al., 2007; Pabbaraju et al., 2008]. The xTAG respiratory virus panel (RVP) fast assay (Luminex Molecular Diagnostics Inc., Toronto, Canada) is a CE marked commercially available kit based on suspension microarray technology and enables the detection of large number of viruses in a single reaction [Kronic et al., 2007; Merante et al., 2007]. RVP fast assay has a simple protocol and shorter turnaround time than the original RVP assay and detects 19 different viruses or subtypes: influenza A (subtype: H1, H3), influenza B, respiratory syncytial virus A (RSV-A), RSV-B, parainfluenza virus 1 (PIV-1), PIV-2, PIV-3, PIV-4, human metapneumovirus, coronaviruses 229E, NL63, OC43, HKU1, rhinovirus/enterovirus (RhV/EV), adenovirus, and human bocavirus. Recent studies show that the RVP assay is more sensitive and specific compared with culture and antigen detection [Wong et al., 2009; Gadsby et al., 2010]. However a major disadvantage of RVP fast assay is the cost of the kit and the instrument. In this study, we compared the performance of an in-house developed conventional multiplex RT-PCR, monoplex real time RT-PCR and xTAG RVP fast assay for the detection of multiple respiratory viruses and their subtypes in clinical specimens.

MATERIALS AND METHODS

Clinical Specimens

Respiratory specimens (nasal/throat swab, n = 310) mainly from pediatric patients less than 5 years of age (253/310) referred to the National Institute of Virology, Pune from August 2009 to March 2011 for diagnosis of influenza A/H1N1pdm09 were included in this study. All the samples were pre-screened for influenza A/H1N1pdm09 at the time of collection and were stored at -70°C . This was a retrospective study and approved by institutional ethical committee. Mean age of patients was 3.8 years. The study comprised 192 males (61.9%) and 118 females (38.1%).

Nucleic Acid Extraction

Total nucleic acid (DNA and RNA) extraction was performed using QIAamp nucleic acid extraction kit (Cat No 57704) (Qiagen, Germany) according to the manufacturer's instructions. Two hundred microlitres of clinical sample was spiked with 20 μl MS2 (Internal control for RVP fast assay, provided by manufacturer) and eluted in 50 μl elution buffer. All the three tests were performed in parallel.

RT-PCR

Conventional multiplex RT-PCR and monoplex real time RT-PCR were carried out as described by Choudhary et al., [2013a]. mRT-PCR was carried out in three tubes/sets covering 18 viruses/ subtypes (set 1: influenza A, subtype seasonal H1, H3 and pandemic H1, influenza B; set 2: RSV A and B, HMPV, PIV 1-3; set 3: PIV-4, coronavirus OC43/HKU1, coronavirus 229E/NL63, rhinovirus/enterovirus). Adenovirus and bocavirus were not tested by mRT-PCR and real time RT-PCR.

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 according to the protocol provided by Centers for Disease Control and Prevention, USA (CDC). For RSV-A and B, PIV-1, -2, -3, coronavirus OC43, 229E, NL63, and rhinovirus, the rRT-PCR assay was performed as described by Gunson et al., 2005; for HMPV by Maertzdorf et al., 2004 and for PIV-4 as described on the following website www3.applied-biosystems.com/cms/groups/mcb/cms/088565.pdf.

Luminex xTAG[®] RVP Fast Assay

The Luminex xTAG[®] RVP fast assay is a CE certified kit and detects influenza A (including subtypes H1 and H3), influenza B, RSV A and B, HMPV, PIV 1-4, coronavirus OC43, 229E, NL63, HKU1, rhinovirus/enterovirus, adenovirus, and bocavirus. The xTAG[®] RVP fast assay kit cannot differentiate between rhinovirus and enterovirus. The xTAG[®] RVP fast assay kit detects influenza A only and do not have marker to subtype influenza A in H1N1pdm09. In addition, the RNA bacteriophage MS2 was used as an internal extraction control and DNA bacteriophage lambda was used as an amplification and assay performance control. If the MS2 or lambda controls failed in the RVP fast assay, the test was repeated to obtain a valid result. A one step single tube multiplex RT-PCR was performed according to the xTAG RVP fast assay product insert instructions. PCR amplification was performed on a GeneAmp PCR System 9700 (Applied Biosystems, Foster City) using the following cycling parameters: 1 preheating step (cDNA synthesis) at 50°C for 20 min, 1 denaturing cycle at 95°C for 15 min, followed by 34 amplification cycles at 95°C for 30 sec, 59°C for 30 sec, and 72°C for 30 sec, ending with 1 cycle at 72°C for 2 min and a hold at 4°C until ready for use. All reagents, the xTAG RVP fast bead mix, reporter buffer, the xTAG streptavidin and phycoerythrin (SA-PE) conjugate were vortexed before use. A 1:75 dilution of SA-PE was prepared in xTAG reporter buffer. Each hybridization reaction contained 20 μl of bead mix, 2 μl of amplified DNA and 75 μl of SA-PE in a 96-well plate. The plate was incubated for 20 min at 45°C followed by analysis on the Luminex 200 instrument using XPONENT[®] software and results were analyzed on TDAS[®] software.

Discordant and Co-Infection Analysis

A true positive (gold standard) was defined as being positive by more than one test used or any specimen that was positive by only one of the tests with further confirmation by a sequencing method. True agreement was defined as at least one virus being the same in at least two methods and if third method detected more than one virus, only this additional virus was confirmed with sequencing. For sequencing, RSV glycoprotein gene [Choudhary et al., 2013b], metapneumovirus nucleoprotein gene [Choudhary et al., 2014], parainfluenza virus hemagglutinin gene, rhinovirus 5'-untranslated region and coronavirus OC43 surface glycoprotein gene were sequenced using in-house developed unpublished primers. Viruses not included in the mRT-PCR and rtRT-PCR i.e., adenovirus and bocavirus, which were identified by RVP fast assay, were not considered as a discordant and also not confirmed with sequencing.

Statistical Analysis

Statistical analysis was carried out using the PASW Statistics 18 software package. The sensitivity and specificity were also computed for all the three tests.

RESULTS

The archived retrospective clinical specimens were selected to compare the sensitivities of the assays. A total 310 samples were tested in this study and results are summarized in Table I. According to the gold standard, a total 202 samples were found positive. Three samples were found positive for adenovirus by xTAG RVP fast assay only, therefore sensitivity calculation for mRT-PCR and rtRT-PCR, 199 samples were considered as true positives. Of the 193 (62.2%) samples detected positive by mRT-PCR, 179 samples were positive for single virus and

14 (7.8%) for two viruses. Of the 175 (56.4%) samples detected positive by rtRT-PCR, 167 samples were positive for a single virus and eight (4.5%) samples showed two viruses. xTAG[®] RVP fast assay detected 138 (44.5%) samples as positive where 123 samples were positive for one virus, 13 for two virus and two samples for three viruses respectively as shown in Table II. The overall sensitivity of the mRT-PCR was 96.9% (95% CI: 93.5, 98.8), for rtRT-PCR was 87.9% (95% CI: 82.5, 92.1) and for xTAG[®] RVP fast assay 68.3% (95% CI: 61.4, 74.6). Overall specificities of all the three tests were 100%.

Performance of the Three Assays for Individual Targets and Discordant Analysis: Influenza A and B

Thirty five known influenza A positive samples were tested in this study (35/310 or 11.2%). All the 35 were subtyped as H1N1pdm09 virus according to the rtRT-PCR protocol and mRT-PCR. The xTAG[®] RVP fast kit detects influenza A only and do not have a marker to subtype influenza A in H1N1pdm09. The xTAG[®] RVP fast kit detected 19 positive for influenza A only and 16 false negative for influenza A. Sensitivity and specificity for influenza A virus was 100% (95% CI: 89.9, 100) for rtRT-PCR and mRT-PCR. Sensitivity for RVP fast assay was 54.2% (95% CI: 36.6, 71.1). Of the five samples positive for influenza B by the rtRT-PCR and mRT-PCR, RVP fast assay detected only one specimen as positive. 100% concordance was observed between the mRT-PCR and rtRT-PCRs for influenza A/H1N1pdm09 and influenza B. Sensitivity of RVP fast assay for influenza B was 20% (95% CI: 3.3, 71.1).

Rhinovirus and Enterovirus

A total of 75 (24.1%), 75 (24.1%), and 57 (18.3%) samples were detected positive for rhinovirus/

TABLE I. Performance Comparison of Individual Targets in Each Test

Virus	No. true positive specimens	Real time RT-PCR		Multiplex RT-PCR		Luminex xTAG	
		No. detected	% Sensitivity (95% CI)	No. detected	% Sensitivity (95% CI)	No. detected	% Sensitivity (95% CI)
Rhinovirus/Enterovirus	86	57*	66.2 (55.2, 76.1)	75	87.2 (78.2, 93.4)	75	87.2 (78.2, 93.4)
RSV-B	38	37	97.3 (86.1, 99.5)	37	97.3 (86.1, 99.5)	18	47.3 (30.9, 64.1)
H1N1pdm09 ^a	35	35	100 (89.9, 100)	35	100 (89.9, 100)	19	54.2 (36.6, 71.1)
PIV-3	18	17	94.44 (72.6, 99)	18	100 (81.3, 100)	8	44.4 (21.5, 69.2)
RSV-A	15	15	100 (78, 100)	15	100 (78, 100)	2	13.3 (2, 40.4)
HMPV	7	5	71.4 (29.2, 95.4)	7	100 (58.9, 100)	6	85.7 (42.2, 97.6)
Influenza B	5	5	100 (47.9, 100)	5	100 (47.9, 100)	1	20 (3.3, 71.1)
Corona OC43	6	6	100 (54, 100)	4	66.6 (22.6, 94.6)	6	100 (54, 100)
PIV-4	9	4	44.4 (13.9, 78.6)	9	100 (66.2, 100)	7	77.7 (40, 96.5)
PIV-1	2	2	100 (19.2, 100)	2	100 (19.2, 100)	1	50 (8.1, 91.83)
Total sample positive	199	175		193		138	

*Real time RT-PCR detect only Rhinovirus.

^aThe xTAG[®] RVP fast kit detects influenza A only and do not have marker to subtype influenza A in H1N1pdm09.

TABLE II. Combinations of Multiple Viruses Identified and Number of Instances Were Detected by Each Assay

Viral combination	No. of co-infections detected by			No. true positive
	rtRT-PCR	mRT-PCR	xTAG RVP	
H1N1pdm09 ^c + Rhino	1	3	2	6
H1N1pdm09 + RSV-A	1	1	0	1
RSV-A + Rhino	2	1	0	2
RSV-B + Rhino	1	3	2	4
RSV-B + PIV-3	1	1	1	1
RSV-B + Boca ^a	ND ^a	ND ^a	1	1
PIV-1 + Rhino	0	1	0	1
PIV-3 + Rhino	1	1	0	1
PIV-3 + CoronaOC43	1	0	0	1
PIV-3 + Adeno ^b	ND ^b	ND ^b	1	1
PIV-3 + Boca ^a	ND ^a	ND ^a	0	1
PIV-4 + Rhino	0	1	1	1
HMPV + Rhino	0	2	2	2
Rhino + Boca ^a	ND ^a	ND ^a	3	4
H1N1pdm09 + Rhino + Boca ^a	ND ^a	ND ^a	2	2
Total	8	14	15	29

^aND: Boca virus Not Determined.

^bND: Adenovirus Not Determined.

^cInf A: H1N1pdm09.

enterovirus by mRT-PCR, RVP fast assay and rtRT-PCR respectively. The sensitivity for rhinovirus/enterovirus detection was 87.2% (95% CI: 78.2, 93.4) in mRT-PCR and RVP fast assay and 66.2% (95% CI: 55.2, 76.1) in rtRT-PCR. Ten samples were detected positive by RVP fast assay only and 1 by mRT-PCR and rtRT-PCR respectively which were confirmed as rhinovirus by sequencing. A total of 48 samples positive for rhinovirus/enterovirus were sequenced for 5'-UTR region. 42/48 (87.5%) samples were confirmed as rhinovirus and 6 (12.5%) as enterovirus by sequencing.

RSV

For RSV-B, 37 (11.9%) samples were detected positive by both rtRT-PCR and mRT-PCR while RVP fast assay detected only 18 (5.8%). Sensitivity of rtRT-PCR and mRT-PCR for RSV-B was 97.3% (95% CI: 86.1, 99.5) and for RVP fast assay was 47.3% (95% CI: 30.9, 64.1). One sample negative by both rtRT-PCR and mRT-PCR but positive by RVP fast assay was confirmed by sequencing to be positive for RSV-B. Of the twenty samples negative by RVP fast assay for RSV-B, eight samples were randomly selected for sequencing and confirmed as RSV-B. For RSV-A, 15 (4.8%) samples were detected positive by both rtRT-PCR and mRT-PCR and only two by RVP fast assay. Out of the 13 samples negative by RVP fast assay, nine randomly selected samples were confirmed as RSV-A positive by sequencing. 100% concordance was observed between rtRT-PCR and mRT-PCR for RSV A and B.

PIV

Two samples were detected positive for PIV-1 by both rtRT-PCR and mRT-PCR but only one was

positive by RVP fast assay. The sample negative by RVP fast assay was confirmed to be PIV-1 positive by sequencing. A total 18 (5.8%) and 17 (5.4%) samples were detected positive for PIV-3 by mRT-PCR and rtRT-PCR respectively and only eight by RVP fast assay. Sensitivity of mRT-PCR for PIV-3 was 100% (95% CI: 81.3, 100), rtRT-PCR was 94.4% (95% CI: 72.6, 99) and RVP fast assay was 44.4% (95% CI: 21.5, 69.2). Out of 10 samples negative for PIV-3 by RVP fast assay, nine samples were positive by both rtRT-PCR and mRT-PCR. One sample positive for PIV-3 only by mRT-PCR was confirmed by sequencing. Out of nine samples positive for PIV-4, seven samples were positive by both mRT-PCR and RVP fast assay. Four samples were positive for PIV-4 by rtRT-PCR. Two samples negative by both rtRT-PCR and RVP fast assay but positive by mRT-PCR were confirmed as PIV-4 by sequencing.

HMPV

For human metapneumovirus, seven, six and five samples were detected positive by mRT-PCR, RVP fast and rtRT-PCR respectively. Two samples negative by rtRT-PCR and one by RVP fast assay were confirmed to be HMPV positive by sequencing.

hCoV

Coronavirus OC43 was detected in six samples by both rtRT-PCR and RVP fast assay. Only four samples were detected as OC43 positive by mRT-PCR. Two specimens negative by mRT-PCR were confirmed to be coronavirus OC43 positive by sequencing. Adenovirus and bocavirus were not included in sensitivity calculation since we did not test for the two viruses by mRT-PCR and rtRT-PCR. None of the samples were found to be positive for

seasonal influenza A/H3N2, PIV-2, coronavirus-NL63, 229E, and HKU1.

Cost and Workflow

One kit of xTAG RVP fast assay contains reagents for 96 tests. Additionally, external controls need to be included in each run, and for a full plate, more than one control is recommended. Reagents for the nucleic acid extraction step are purchased separately. The cost of testing the 18 viruses discussed in the present study for one sample by mRT-PCR, rtRT-PCR, and RVP fast assay was US\$ 27, US\$ 45, and US\$ 92 respectively and this includes only reagents cost excluding labor cost.

The overall duration of the xTAG RVP fast assay was 5 to 6 hr for 16 samples including one positive and one negative control (90 min for RNA extraction, 15 to 20 min for PCR setup, 150 min for RT-PCR, 15 to 20 min for hybridization setup, 45 min for hybridization, and 10 to 15 min for reading on Luminex instrument). The overall duration of the real time RT-PCR assay was 8 to 9 hr for 16 samples including one positive and one negative control (90 min for RNA extraction, 60 to 70 min for PCR setup, 6 hr to run the RT-PCR thrice on one instrument). The overall duration of the mRT-PCR assay was 6 to 7 hr for 16 samples including one positive and one negative control (90 min for RNA extraction, 30 to 40 min for PCR setup, 3 hr to run the RT-PCR and 60 to 90 min for gel run.

DISCUSSION

Traditional methods of detection and identification of respiratory viruses like cell culture and immunofluorescence are labor intensive, slow and are dependent on specimen quality. Nucleic acid amplification tests (NAAT) have shown increased sensitivity and quicker turnaround time compared to non-amplification tests [Arens et al., 2010]. The high detection rates of NAATs are due to the inclusion of a larger number of viral targets with increased sensitivity allowing detection of low copy numbers of different viruses [Mahony et al., 2007; Pabbaraju et al., 2011]. Finally, accurate diagnosis will optimize antiviral treatment and implementation of infection control and public health measures. The xTAG[®] respiratory virus panel assay is based on suspension microarray technology which enables the detection of a large number of targets in a single reactions [Kronic et al., 2007; Merante et al., 2007]. The RVP fast assay provides a considerable reduction in time and less steps of post PCR manipulation from the first version of the assay [Merante et al., 2007].

The xTAG[®] RVP assay has been shown to offer results comparable to or superior to those of culture/DFA and nucleic acid tests for the diagnosis of respiratory viral infections [Mahony et al., 2007; Pabbaraju et al., 2008; Jokela et al., 2012]. The xTAG RVP assay has shown the best sensitivity to common

viral targets when compared to the ResPlex II and MultiCode-PLx assays when compared with culture [Balada-Llasat et al., 2011]. The xTAG RVP assay has been also used successfully for the detection of etiological agents in outbreaks of respiratory illness [Wong et al., 2009].

In this study, the performance of the in-house developed conventional multiplex RT-PCR, with monoplex real time RT-PCR and Luminex xTAG[®] RVP fast assay was assessed by the retrospective testing of 310 respiratory clinical samples originally submitted for the diagnosis of influenza A/H1N1pdm09 virus. The overall sensitivity was 96.9%, 87.9%, and 68.3% for mRT-PCR, rtRT-PCR, and xTAG[®] RVP fast assay respectively. Since bocavirus and adenovirus were not tested by multiplex RT-PCR and real time RT-PCR hence excluded for sensitivity calculation for these tests. In this study, we found that the xTAG[®] RVP fast assay is less sensitive than mRT-PCR and rtRT-PCR. The overall RVP fast assay sensitivity was 78.8% reported by Gadsby et. al. [2010], 77.5% for all the targets reported by Pabbaraju et. al. [2011], 87.2% reported by Kim et. al. [2013] while 33% reported by Raymaekers et. al. [2011]. Pabbaraju et. al. [2008] reported RVP assay sensitivity and specificity as 91.2% and 99.7% respectively when their in-house NAATs were considered as a gold standard. In another study, RVP fast assay showed a sensitivity and specificity of 100% and 91% when compared with culture, while MultiCode-PLx showed 89% and 87% and ResPlex II showed 89% and 94%, respectively [Balada-Llasat et al., 2011]. Popowitch et al. [2013] reported sensitivity and specificity for xTAG RVP fast as 84.4% and 99.9% respectively. In the present study, a lower sensitivity of RVP fast assay was observed compared to that reported in other studies. This may be due to the fact that in this study we have compared the RVP fast assay against well standardized and robust molecular assays using clinical samples whereas most of the other studies have compared the RVP fast assay against cultures/DFA using isolates. Another reason may be the sequence variation in the primer-probe binding regions between viruses circulating in India and other parts of the world.

In a recent study by Synlab in Germany (<http://www.luminexcorp.com/prod/groups/public/documents/lmncorp/p104-escv-2012-synlab-rvp.pdf>) [30], comparisons were made between their homebrew light cyclers assay, RVP fast v2, and RVP fast v2 'extended amplification'. The RVP fast v2 assay performed well with Quality Control for Molecular Diagnostic (QCMD) samples which had Ct values lower than 30. However for samples with Ct values higher than 30, a considerable drop in sensitivity was observed. To remedy this, PCR cycles were increased by 3 cycles (extended amplification) to increase sensitivity. The protocol for xTAG RVP fast assay was not modified in the present study since it is a CE marked kit.

Considering the entire detection spectrum, the present study detected 62.2% samples positive by mRT-PCR, 56.4% by rtRT-PCR and 44.5% by xTAG[®] RVP fast assay. Similar findings have been reported in previous studies using the RVP fast assay [Gadsby et al., 2010; Babady et al., 2012] or the original RVP assay [Mahony et al., 2007; Pabbaraju et al., 2008; Gharabaghi et al., 2011]. The 7.8% co-infections were detected by mRT-PCR, 4.5% by rtRT-PCR and 10.8% by RVP fast assay as shown in Table II. Similar co-infections were reported by others for RVP assay [Mahony et al., 2007; Pabbaraju et al., 2008; Gadsby et al., 2010; Jokela et al., 2012].

The RVP fast assay mainly failed to detect influenza A, B, RSV A, B, and PIV-3 viruses. Concordance between mRT-PCR and rtRT-PCR for influenza A/H1N1pdm09, influenza B, RSV A and B was 100%. Influenza A samples missed by RVP fast assay had MFI (Median Fluorescent Intensity) values ranging between 150 and 300. The cut off value to consider positive for influenza A target was 300 MFI. It was observed that the discordant results of the RVP negative samples were associated with relatively high Ct values (mean Ct in RT-PCR 34). The limitation of xTAG RVP fast assay was that it does not subtype influenza A into H1N1pdm09 and the kit was not upgraded since the emergence of H1N1pdm09. Upgrading of the xTAG RVP fast assay to accommodate the subtyping of influenza A into H1N1pdm09 virus might increase the diagnostic value of the kit. The lower sensitivity of influenza B was also reported by Pabbaraju et al. [2011] and suggested sequence variation in the hemagglutinin gene of Flu B in the primer binding region. Gharabaghi et al. [2011] reported influenza B sensitivity 64.9% for RVP fast assay. The sensitivity of RVP fast assay for RSV-A, RSV-B, and PIV-3 was 13.3%, 47.3%, and 44.4% respectively. Gharabaghi et al. [2011] reported RSV-A sensitivity 85.5% and RSV-B 98.3%. Similar reports were published by other groups [Gadsby et al., 2010; Rand et al., 2011; Kim et al., 2013; Popowitch et al., 2013]. Low sensitivity of RSV in RVP assay attributed to the high Ct value in real time RT-PCR and may be due to the genetic variation of the virus. Pabbaraju et al. [2008] also observed that their discordant RSVs were of low titer as well. The lower sensitivity in RVP fast assay for PIV-3 was also reported [Gharabaghi et al., 2011; Babady et al., 2012].

HMPV, rhinovirus and PIV-4 were detected with lower sensitivity by rtRT-PCR when compared with mRT-PCR. RVP fast assay and mRT-PCR have combined rhinovirus and enterovirus targets, these viruses clubbed together as RhV/EV to perform sensitivity of the tests. Real time RT-PCR sensitivity for rhinovirus was 66.2%, mRT-PCR and RVP fast assay 87.2% respectively. The reduced sensitivity may be because both mRT-PCR and RVP fast assay detect rhinovirus and enterovirus while rtRT-PCR specifically detected only rhinovirus. Sensitivity of

RhV/EV in RVP fast assay was reported 93% by Popowitch et al. [2013], 89.6% by Gadsby et al., [2010]. A total 48 rhinovirus/enterovirus positive samples were sequenced, out of which 42 (87.5%) were confirmed as rhinovirus and six (12.5%) as enterovirus by sequencing. Similar findings were also reported by others [Pabbaraju et al., 2008; Gadsby et al., 2010]. A relatively lower number of specimens were positive for coronaviruses, parainfluenza and metapneumovirus in our sample set. Two coronavirus OC43 positive samples missed by mRT-PCR had Ct values of 33 and 35 in the rtRT-PCR. A significant limitation in the present study was the absence of influenza A (H3N2) to evaluate the kit performance for detection of it. Therefore, a larger number of positive specimens are required in order to fully assess the sensitivity of the RVP fast assay for all targets.

In addition to sensitivity and specificity, ease of use, required time and cost are important factors to consider when choosing a multiplex PCR. Each assay evaluated in this study had advantages and disadvantages. The cost of testing the 18 viruses discussed in the present study for one sample by mRT-PCR, rtRT-PCR and RVP-fast assay was US\$ 27, US\$ 45, and US\$ 92 respectively which include only reagents and consumables. The overall duration of the RVP fast assay was 5 to 6 hr, for real time RT-PCR was 8 to 9 hr and mRT-PCR was 6 to 7 hr. Similar observation for workflow and cost were reported for RVP fast assay by Rand et al. [2011] and Babady et al. [2012]. Luminex xTAG RVP fast assay has the advantage of strict quality control and manufacturing practices which minimizes errors during testing of the samples. Luminex xTAG RVP assay is an open PCR system and post amplification products are manipulated (hybridization), increasing the risk of potential laboratory contamination [Kim et al., 2013]. mRT-PCR has disadvantage because it requires the visual inspection of products in gel and have a potential for amplicon contamination. Real time RT-PCR offers decreased contamination risk. More technical skill is required to perform xTAG RVP fast assay and rtRT-PCR than mRT-PCR. Limitation of the present study was that we have not tested prospective samples.

In summary, the in-house developed mRT-PCR and rtRT-PCR showed better sensitivity, specificity and is more cost effective compared to the RVP fast assay. Results of this study indicate that implementation of the in-house developed mRT-PCR for testing of respiratory samples will improve our rate of diagnosis of respiratory tract infections and can be used in resource limited settings.

ACKNOWLEDGMENTS

The authors would like to thank the Director, Dr. DT Mourya, for his support. We would like to

acknowledge Archana, Kunal, Minal and the technical team of the influenza group for their help.

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