Evaluation of custom multiplex real-time RT-PCR in comparison to fast-track diagnostics respiratory 21 pathogens kit for detection of multiple respiratory viruses

Bharti Malhotra*, M. Anjaneya Swamy, P. V. Janardhan Reddy, Neeraj Kumar and Jitendra Kumar Tiwari

Abstract

Background: Severe acute respiratory infections in children can be fatal, rapid identification of the causative agent and timely treatment can be life-saving. Multiplex real-time RT-PCR helps in simultaneous detection of multiple viruses saving cost, time and labour. Commercially available multiplex real-time RT-PCR kits are very expensive. Therefore the aim of the present study was to develop a cost-effective multiplex real-time RT-PCR for the detection of 18 respiratory viruses and compare it with an in-vitro diagnostics approved Fast Track Diagnostic Respiratory Pathogens 21 Kit (FTD).

Methods: Nasopharyngeal aspirates and throat swabs were collected and processed for extraction of nucleic acid using an automated extraction system and multiplex real-time RT-PCR was performed using the FTD kit and a custom assay on 356 samples.

Results: Custom and FTD assays detected one or more respiratory viruses in 268 (75.29%) and 262 (73.60%) samples respectively. The concordance between the custom assay and the FTD assay was 100% for HCoV OC43, HCoV 229E, HPIV-1, HPIV-2, HBoV, HPeV, Flu A, and Influenza A(H1N1)pdm09 and 94.66 – 99.71% for the remaining viruses; Flu B (99.71%), HRV (99.71%), HPIV-3 (98.87%), HPIV-4 (99.43%), HCoV NL63 (99.71%), HMPV A/B (99.71%), RSV A/B (94.66%), EV (98.31%), HCoV HKU1 (99.71%), HAdV (99.71%). Major discrepancy was observed for RSV A/B, which was over-detected in 18 samples by the custom assay as compared to the FTD assay. The custom assay was much cheaper than the FTD assay and the time taken was only 29 min more.

Conclusion: The custom primer and probe mix was found to be comparable to the FTD assay with good concordance but was much cheaper and the time taken for reporting was only 29 min more. The low cost custom multiplex RT-PCR can be a useful alternative to the costly FTD kit for rapid identification of viral aetiology in resource limited settings.

Keywords: Multiplex real-time RT-PCR, Custom assay, FTD assay, Concordance

Background

Severe acute respiratory infections (SARI) are one of the major causes of illness and death worldwide and are the third most common cause of death among children [1]. Acute respiratory infections (ARI) cause more deaths in children < 5 years with most cases reported from India (43 million), China (21 million), Pakistan (10 million), Bangladesh, Indonesia and Nigeria (56 million) [2]. Respiratory infections can be caused by many viruses, both DNA and RNA. These include the Respiratory Syncytial Virus (RSV), human Parainfluenza Virus (HPIV), Influenza A Virus (Flu A), Influenza B Virus (Flu B), human Adenovirus (HAdV), human Coronavirus (HCoV), human Rhinovirus (HRV), human Metapneumovirus (HMPV) and human Bocavirus (HBoV) [3]. A new wave of viral diagnosis was established with the development of Polymerase Chain Reaction (PCR) techniques in the 1990s [4]. PCR is more sensitive and rapid than conventional methods for detection of respiratory viruses. Different
respiratory viruses present with similar signs and symptoms and can’t be differentiated symptomatically or clinically. Tests capable of rapid simultaneous identification of various viruses at the same time can help expedite initiation of appropriate therapy. Uniplex RT-PCR requires individual amplification of each virus under study which is expensive, time consuming and laborious [5]. To overcome this, multiplex real-time PCRs targeting the detection of multiple pathogens simultaneously have been developed commercially but they are very expensive. There is a need to develop cheaper systems for rapid simultaneous identification of various viruses. The present study compares custom real-time multiplex PCR primers and probes for the simultaneous detection of 18 respiratory viruses with an in-vitro diagnostics (IVD) approved fast track diagnostics (FTD) kit.

Methods

Patient inclusion criteria
Children with SARI, admitted in J. K. Lone Hospital, a pediatric hospital attached to Sawai Man Singh (SMS) Medical College Jaipur were enrolled in the study and tested for respiratory viruses with prior consent of the parent/guardian. Duration of the study was 27 months i.e. between September, 2012 to December, 2014. Children enrolled were ≤ 5 years of age, presenting with fever, cough, sore throat, nasal catarrh, shortness of breath, bronchiolitis, pneumonia, and wheezing.

Patient exclusion criteria
Samples were not collected from patients with chronic respiratory ailments; non-consenting caregivers, with history of hospitalization in the preceding 14 days, not admitted in hospital and children aged > 5 years.

Sample collection and transportation
A total of 356 nasopharyngeal aspirate and throat swab samples were collected from patients with SARI by a trained technician using a sterile nylon flocked swab and placed in viral transport medium (VTM), labelled and transported on ice at the earliest to Advanced research lab (ICMR Grade-1 Virology Lab) of SMS Medical college Jaipur for further processing and storage of the samples. The study was approved by the institutional ethics committee.

Nucleic acid extraction
Viral nucleic acid from samples was extracted using an EasyMAG (Biomeurex) automated extractor according to the manufacturer’s instructions. Briefly, the extraction was done from 400 µl homogenised sample which was added to 1500 µl lysis buffer and was incubated for 10 min off board. The samples were loaded into the EasyMAG and 100 µl of magnetic silica was added to each sample and mixed well. Finally, the nucleic acid was eluted in a volume of 110 µl of which 50 µl was used for the FTD assay and 54 µl for the custom assay.

Multiplex real -time RT-PCR (FTD ASSAY)
The multiplex real-time PCR FTD assay was performed on an ABI 7500 Fast instrument (Life Technologies, USA) as per the manufacturer’s instructions using an AgPath-ID™ One-Step RT-PCR kit (Ambion) with the FTD Respiratory pathogens 21 kit (Fast Track Diagnosis, Luxembourg) for the detection of 18 viruses using five tubes containing primer and probe mix for different viruses; Tube-1 [Influenza A (Flu A), Influenza A subtype H1N1 (Pandemic H1N1), human Rhinovirus (HRV), Influenza B (Flu B)], Tube-2 [human Coronavirus NL63 (HCoV-NL63), 229E (HCoV 229E), OC43 (HCoV-OC43), and HKU1 (HCoV HKU1)], Tube-3 [human Parainfluenza viruses, 2, 3, and 4 (HPIV- 2, 3 and 4) & IC], Tube-4 [human Parainfluenza viruses-1, Mycoplasma pneumoniae (M.pneu), human Bocavirus (HBoV), human Metapneumovirus (HMPV A/B)] and Tube-5 [Respiratory Syncytial virus (RSVA/B), human Adenovirus (HAdV), Enterovirus (EV), human Parechovirus (HPeV)]. The multiplex real time RT-PCR thermal profile for the FTD kit was as follows; 50 °C for 15 min, 95 °C for 10 min, 40 cycles of 95 °C for 8 s, 60 °C for 34 s, whereas the thermal profile for the custom assay was set at 50 °C for 30 min, 95 °C for 10 min, 45 cycles of 95 °C for 15 s, 55 °C for 30 s.

Standardisation of uniplex real-time PCR (Custom assay)
Initial standardisations were done for uniplex real-time PCR using the FTD positive samples as controls. These positive samples included the following viruses namely, HRV, HAdV, Flu A, Pandemic H1N1, Flu B, HPIV-1, HPIV-2, HPIV-3, HPIV-4, RSVA/B, HMPV A/B, HPeV, EV, HCoV-OC43, HCoV-NL63, HCoV 229E, HCoV HKU1, (HBoV). AgPath (Ambion) one step RT-PCR master mix was used for the amplification and detection of viral nucleic acid. Briefly, each reaction was performed in a 20 µl volume which consisted of 12.5 µl of buffer, 1 µl of enzyme, 2 picomoles of each primer (corresponding to each virus), 2 picomoles of probe (corresponding to each virus) (synthesised by Life Technologies) (Table 1), 5 µl of extracted nucleic acid of the positive control/sample and made to a final volume of 20 µl with nuclease free water. The thermal cycling profile for the uniplex RT-PCR was 50 °C for 30 min (1 cycle), 95 °C for 10 min (1 cycle) followed by 90 °C for 15 s and 55 °C for 30 s (45 cycles). Specimens were considered positive when the Ct value was < 35.

Standardisation of multiplex real-time RT-PCR (Custom assay)
Uniplex PCR was followed by multiplex real-time RT-PCR standardisation. In multiplex PCR, each reaction
| VIRUS        | Forward primer (5’ - 3’)                                                                 | Reverse primer (5’ - 3’)                                                                 | Probe (5’ - 3’)                                                                 | References                          |
|-------------|------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------|--------------------------------------|
| **Panel-1** |                                                                                          |                                                                                          |                                                                               |                                      |
| Flu B       | GAGACACAAATTGCCTACCTGCTT                                                               | TTCTTCCACCCAGAACCAAC                                                                      | ^aFAM – AGAAGATGAGAAGGCA AAG CAGA ACTAGC                                      | Esposito et al., 2010 [17]            |
| HCoV 229E   | CAGTCAAAATGGCGGTGATGCA                                                                | AAAGGGCTATAAAGAGATAAAGGTATTCT                                                            | ^bVIC – CCCGTAGCACCGGTCTGGTCCA                                                 | Hammit et al., 2011 [6]              |
| HCoV OC43   | CGATGAGGCTATTCGCCAGCTAGGT                                                            | CCTTCCTGAGCTCTCAATAAGTAGAACC                                                            | ^cNED – TCCGCCTGAGCGTTACTCTCT                                                  | Hammit et al., 2011 [6]              |
| **Panel-2** |                                                                                          |                                                                                          |                                                                               |                                      |
| HPV-4       | CAGAYAACATACATCGGCTTACAAA                                                              | TGGATCTATGACTGCGCAAARA                                                                     | ^aFAM – CCMATACAAGCTCGAATAATYCAAGGTCG                                      | Hammit et al., 2011 [6]              |
| HPV-1       | GTGATTTAAACCGGTAATTTCGCA                                                              | CCGGAGTCTGTAGTTCCAGAGGGA                                                                | ^bVIC – ACCTATGACATCAAGGCA                                                    | Hammit et al., 2011 [6]              |
| HPV-3       | CCAGGGGTATATTAATGATGTAAGGGA                                                           | CCGGGGACGCCAGTTGAGT                                                                     | ^cNED – TGCACTGACAGCTCAAGGCAA                                                   | Hammit et al., 2011 [6]              |
| **Panel-3** |                                                                                          |                                                                                          |                                                                               |                                      |
| Influenza A(H1N1)pdm09 | GTGCTATAAAACACCGCCTTCA                                                                 | CGGGATATCTCTTATATCTGGRG                                                                | ^aFAM – CAGAATATACATCCGGGCAATGGAAN                                                | WHO, 2009 [22]                       |
| HRV         | TGGACCGGGTTGTAAGGAC                                                                  | CAAAGTATCGGCTCCCATCC                                                                    | ^bVIC – TCCGGGCGCCCTGGAATG                                                     | Hammit et al., 2011 [6]              |
| HPMV-2      | ATGAAAACCATATATGATGTAAGGGA                                                            | CCGGGGATCTGTAGTTCCAGAGGGA                                                                | ^cNED – TCCGGCAAAAGGCA                                                         | Hammit et al., 2011 [6]              |
| **Panel-4** |                                                                                          |                                                                                          |                                                                               |                                      |
| RSV A/B     | GGAAACATACGTCGAACAAGGTCCTCA                                                            | RSV-A: CATGCTGGTTTTCTTCAAGACATGTGTA                                                    | ^aFAM – TGCTGATGTCGGAACCT                                                       | Kwofie et al., 2012 [10]             |
| HCoV NL63   | ACGTACTTCTTTATTGAAAGCATGATATTAA                                                        | AGCGAGTCTAAATGTTACATAAAACCTGAGG                                                       | ^bVIC – ATTTGCAAGGCTCTCTAAGGTTG                                                  | Hammit et al., 2011 [6]              |
| HCoV HKU1   | AGTCCCCATTGCTTGGAGTA                                                                  | CCGGGGATCTGTAGTTCCAGAGGGA                                                                | ^cNED – CCGGCCTTCTGAAGCAA                                                      | Cui et al., 2011 [11]                |
| **Panel-5** |                                                                                          |                                                                                          |                                                                               |                                      |
| EV          | CCGTGATGCGCCTTAATCC                                                                   | ATTGTCACCAATAACGCAA                                                                     | ^aFAM: AACGGACTACTTTTGTTGACTCTGTTTC                                              | Wolffs et al., 2011 [23]             |
| HPeV        | GTACAGACSWGCTCTGCGGCCAAAAG                                                             | GGGGCGGCGRTGCAGATCCAGYGT                                                                 | ^bVIC – CCTTGGCTACCTCGGAACTCTCC                                                  | Nix et al., 2008 [12]                |
| HBoV        | TGGACAGACACCGCYTAGTGTGTT                                                             | CTGTCCCCCGCAAGTACA                                                                     | ^cNED – CCGGGATTGCGGGAACCTGCAA                                                   | Sanghvi et al., 2012 [22]            |
| **Panel-6** |                                                                                          |                                                                                          |                                                                               |                                      |
| Flu A       | GACCRATCTTCTGACTACCCCTCTGAC                                                           | AGGCGATTTYGAGCAAACGKCTCTA                                                               | ^aFAM – TGCACTCTTGGGACTCGGAGG                                                   | WHO, 2009 [22]                       |
| HAdV        | GCGCGAGCGTGTCTTATTCCTAGACCTGAC                                                        | GGCACAGGTTGGGTTTTCTAAACTT                                                             | ^bVIC – TCCGGACACCGGCTCAGTCTCCGA                                                  | Hammit et al., 2011 [6]              |
| HMPV A/B    | CATCAGGTAATATCCGCAAAATACG                                                             | GTGAGTATAAAGGCGGACTCTAAGATAAGAAR                                                        | ^cNED – TCCACCCAGACGACACC                                                       | Sanghvi et al., 2012 [13]            |

**NOTE:** The lower limit for the detection of HBoV- 1 DNA copy/ml, HMPV- 30 Rna copies/ml, HPeV- 10^3 (cell culture infective dose) CCID_{50} - 10^5 CCID_{50}, RSV A/B - 2×10^4 copies/μl, HCoV HKU1- 5×10^3 copies/ml, Flu B- 2.2 Log_{10} (viral particles) VP/μl, and Influenza A(H1N1)pdm09 - 2×10^1 to 2×copies/μl
^aFAM - Detection wavelength - 518 nm; ^bVIC detection wavelength - 554 nm; ^cNED detection wavelength 575 nm
^dAll the probes were having (non fluorescence quencher) NFQ as quencher at 3’ end
was targeted for the simultaneous detection of three different viruses in a single reaction tube. Each reaction mix consisted of respective forward and reverse primers, and probes each labelled with a different fluorescent dye (FAM, VIC and NED) specific to each of the three viruses. Tube-1 [Influenza B (Flu B), human Coronavirus 229E (HCoV 229E), OC43 (HCoV-OC43)], Tube-2 [human Parainfluenza viruses, 1, 3, and 4 (HPIV-1, 3 and 4)], Tube-3 [Influenza A subtype H1N1 (Pandemic H1N1), human Rhinovirus (HRV), human Parainfluenza viruses-1 (HPIV-1)], Tube-4 [Respiratory Syncytial virus (RSV/A/B)], human Coronavirus NL63 (HCoV-NL63), HKU1 (HCoV HKU1)], Tube-5 [Enterovirus (EV), human Parechovirus (HPeV), human Bocavirus (HBoV)], Tube-6 [Influenza A (Flu A), human Adenovirus (HAdV), human Metapneumovirus (HMPV A/B)]. FTD positive samples were considered as standard. AgPAth (Ambion) one step RT-PCR master mix was used for the detection of respective viral nucleic acids as mentioned above in a reaction volume of 25 μl but with 9 μl of nucleic acid, 4 picomoles of each primer and 2 picomoles of probe. The thermal profile for the multiplex real-time PCR was as described above. Specimens were considered positive when the Ct value was < 35. After standardisation of multiplex PCR the same protocol was used for screening patient samples. Rnase P was used as an internal control in a separate uniplex RT PCR assay. Samples negative for Rnase P were not included in the study.

**Results**

A total of 356 samples were tested by both assays. Custom and FTD assays detected one or more respiratory viruses in 268 (75.29 %) and 262 (73.60 %) samples respectively (Table 2).

No significant differences were seen in the number of samples positive for each virus by the custom assay as compared to the FTD assay except with RSV A/B which was over detected in 18 samples and one sample being under detected by the custom assay as compared to the FTD assay. Further, to completely assess the results of these 18 discordant RSV/A/B samples, testing was repeated using RSV A and RSV B specific primer and probe mix in uniplex real time RT-PCR as published previously [6]. All 18 samples were found to be positive for RSV B (Table 3).

### Table 2

| Number of viruses detected | Custom assay | FTD assay |
|---------------------------|--------------|-----------|
| 0                         | 88 (24.71 %) | 94 (26.40 %) |
| 1                         | 197 (55.33 %) | 203 (57.02 %) |
| 2                         | 65 (18.25 %) | 55 (15.44 %) |
| 3                         | 6 (1.68 %) | 4 (1.12 %) |

One hundred percent concordance was observed between the custom assay and the FTD assay for eight viruses; HCoV OC43, HCoV 229E, HPIV-1, HPIV-2, HBoV, HPeV, Flu A, and Influenza A(H1N1)pdm09 while it varied from 94.66 to 99.71 % for the remaining ten viruses; Flu B, HRV, HPIV-3, HPIV-4, HCoV NL63, HMPV A/B, RSV A/B, EV, HCoV HKU1, HAdV. (Table 4).

Low concordance was observed between the two assays for RSV A/B (94.66 %) and EV (98.31 %).

The discordant results of the custom assay were seen in 19 co-infection samples, 13 single infection samples and four negative samples as compared to the FTD assay, and the discordance was predominant in the co-infected samples as compared to single infection samples (Table 5).

Comparisons between the custom assay and the FTD assay were made based on the different parameters listed in Table 6. Most of the findings between the custom assay and the FTD assay were similar except for the cost incurred for screening 18 respiratory viruses. In this regard, the custom assay was found to be more economical than the commercial FTD assay.
The present study was performed to compare a custom multiplex assay and an FTD multiplex assay by testing of 356 respiratory samples obtained from children with SARI admitted in J K lone paediatric hospital Jaipur. In the present study, the concordance between the custom assay and the FTD assay was found to be 100% for Flu A, Influenza A(H1N1)pdm09, HCoV OC43, HCoV 229E, HPIV-1, HPIV-2, HBoV, and HPeV. Similarly Chen et al., [7] reported a concordance of 99.60% for Flu A and Influenza A(H1N1)pdm09 when comparing a multiplex PCR assay with a uniplex assay.

The concordance between the two assays varied from 94.66 to 99.71% for the remaining ten viruses; Flu B (99.71%), HPIV-3 (99.71%), HPIV-4 (99.43%), HCoV NL63 (99.71%), HMPV A/B (99.71%), RSV A/B (94.66%), HCoV HKU1 (99.71%), HAdV (99.71%), HRV (99.71%), EV (98.31%). Similar findings have been observed in earlier studies for Flu B (98.25 to 99.42%), HPIV-3 (96.53 to 99.30%), HPIV-4 (97.10%), HCoV NL63 (95.95 to 100.0%), HMPV A/B (99.65 to 100.0%), RSV A/B (93.06 to 98.60%), HCoV HKU1 (98.84 to 100.0%), HAdV (97.20 to 100.0%) [8, 9]. Concordance for EV in the present study was different from an earlier study (93.00%) [8]. The difference in concordance obtained in different studies may be due to the different primer binding regions or may be due to different methodologies employed by various studies. The number of samples positive for HCoV

### Table 5 Discordan results of custom and FTD assays

| Custom assay         | FTD assay      | Number of samples |
|----------------------|----------------|------------------|
| RSV A/B              | NEGATIVE       | 07               |
| HBoV + RSV A/B       | HBoV           | 02               |
| HRV + RSV A/B        | HRV            | 02               |
| EV + RSV A/B         | EV             | 01               |
| HMPV A/B + RSV A/B + Flu B | HMPV A/B + Flu B | 01           |
| Influenza A(H1N1)pdm09 + RSV A/B + HBoV | Influenza A(H1N1)pdm09 + HBoV | 01           |
| Flu B + RSV A/B      | Flu B          | 01               |
| HPV-3 + RSV A/B      | HPV-3          | 01               |
| HPV-3 + HRV          | HRV            | 01               |
| HBoV + HPV-3         | HBoV           | 01               |
| *HPV-4 + HRV*        | *HPV-3 + HRV*  | 01               |
| EV                   | NEGATIVE       | 02               |
| NEGATIVE             | EV             | 01               |
| HBoV                 | HBoV + EV      | 01               |
| HCoV OC43            | HCoV OC43+ HCoV HKU1 | 01           |
| HRV + HAdV           | HRV            | 01               |
| HPIV-2 + Flu B       | HPIV-2         | 01               |
| HPIV-3               | NEGATIVE       | 01               |
| NEGATIVE             | HCoV NL63      | 01               |
| HPIV-3               | HPIV-3 + HMPV/A/B | 01           |
| Influenza A(H1N1)pdm09 + EV | Influenza A(H1N1)pdm09 | 01       |
| HRV + RSV A/B        | HRV            | 01               |
| NEGATIVE             | RSV A/B        | 01               |
| HRV + EV             | HRV            | 01               |

*This sample is counted as variation of co-infection on both the sides*

### Table 6 Comparison of custom and FTD assay in regard to sample testing

| Viral Pathogens | Custom assay | FTD assay |
|----------------|--------------|-----------|
| Flu A, Influenza A(H1N1)pdm09, Flu B | Flu A, Influenza A(H1N1)pdm09, Flu B |
| HPIV - 1, 2, 3, 4 | HPIV - 1, 2, 3, 4 |
| HCoV OC43, 229E, NL63, HKU1, RSV A/B | HCoV OC43, 229E, NL63, HKU1, RSV A/B |
| HPIV A/B | HMPV A/B |
| EV | EV |
| HPeV | HPeV |
| HRV | HRV |
| HAdV | HAdV |
| HBoV | HBoV |
| – | M. pneu |

| Sample volume | 200 µl | 200 µl |
| Elution volume | 55 µl | 55 µl |
| PCR volume | 25 µl | 25 µl |
| Principle of detection | Fluorescence probe based | Fluorescence probe based |
| Hands on time | 50 min | 50 min |
| Time to result | 3.5 h | 3.01 h |
| Total cost per sample | INR 1500 | INR 4300 |
| Ease of Handling | + | + |
| Equipment | NucliSENS EasyMAG, ABI 7500 DX Fast | NucliSENS EasyMAG, ABI 7500 DX Fast |
| No. of PCR tubes used per sample | 6 | 5 |
| Primers & probes | Custom assay (ABI) | FTD |
| Number of targets/tube | 3 | 4 |

**Discussion**

The present study was performed to compare a custom multiplex assay and an FTD multiplex assay by testing of 356 respiratory samples obtained from children with SARI admitted in J K lone paediatric hospital Jaipur.

In the present study, the concordance between the custom assay and the FTD assay was found to be 100% for Flu A, Influenza A(H1N1)pdm09, HCoV OC43, HCoV 229E, HPIV-1, HPIV-2, HBoV, and HPeV. Similarly Chen et al., [7] reported a concordance of 99.60% for Flu A and Influenza A(H1N1)pdm09 when comparing a multiplex PCR assay with a uniplex assay.

The concordance between the two assays varied from 94.66 to 99.71% for the remaining ten viruses; Flu B (99.71%), HPIV-3 (99.71%), HPIV-4 (99.43%), HCoV NL63 (99.71%), HMPV A/B (99.71%), RSV A/B (94.66%), HCoV HKU1 (99.71%), HAdV (99.71%), HRV (99.71%), EV (98.31%). Similar findings have been observed in earlier studies for Flu B (98.25 to 99.42%), HPIV-3 (96.53 to 99.30%), HPIV-4 (97.10%), HCoV NL63 (95.95 to 100.0%), HMPV A/B (99.65 to 100.0%), RSV A/B (93.06 to 98.60%), HCoV HKU1 (98.84 to 100.0%), HAdV (97.20 to 100.0%) [8, 9]. Concordance for EV in the present study was different from an earlier study (93.00%) [8]. The difference in concordance obtained in different studies may be due to the different primer binding regions or may be due to different methodologies employed by various studies. The number of samples positive for HCoV
The detection limit of the custom assay (Table 7) ranged from 1 DNA copy/ml to $2 \times 10^4$ copies/ml [7, 10–14]. The detection limit of the FTD assay for different viruses was $10^2$ copies/ml for FluA, HPIV-2, HMPV and HCoV OC43; $10^3$ copies/ml for FluB, HCoV HKU1, HPIV-1, HBoV, HPIV-3, HCoV NL63, RSV, HAdV, EV, and HPeV; and $10^4$ copies/ml for HRV, HCoV 229E and HPIV-4 [15].

In the present study RSV A/B was the most predominant virus detected by both the custom and FTD assays with positivity in 84 (23.60 %) and 67 (18.82 %) samples respectively and concordance of 94.66 %. This finding is different when compared with other studies [8, 16] where comparisons were made between multiplex PCRs in which RSV was the second most predominant virus detected [16].

The major discrepancy in the present study was found with RSV A/B. The discrepancy in 18 samples which were over detected by the custom assay was resolved by RSV A and RSV B typing. The RSV typing results for the discrepant samples showed that all 18 samples were RSV B. Further all samples positive for RSV A/B by the FTD assay were also subjected to RSV typing which indicated RSV A in 13 (19.40 %) samples, RSV B in 3 (4.50 %) and RSV A & RSV B dual infections in 4 (5.97 %) samples.

During the process of standardisation of the custom assay 3 μl of viral nucleic acid (positive control) was used for each virus including 4 picomoles of primers and 2 picomoles of probes. Each panel consisted of 3 viruses. In total 9 μl of viral nucleic acid was used for each panel. While the FTD assay used 10 μl of nucleic acid in each tube with primers and probes for 4 viruses, the concentration of primer and probe was not disclosed by FTD. In total 4 μl more of viral nucleic acid was used in the custom assay compared to the FTD assay which may have increased the sensitivity/detection of different viruses in the custom assay.

Initially during the process of standardisation of the custom assay, different primer and probe concentrations were tried and the PCR was run for 45 cycles as per the protocol followed by various authors. Although data was analysed using PCRs run for 35 and 40 cycles, best results were achieved using a Ct value of 35 for both the FTD assay and the custom assay. Accordingly, a Ct value of <35 was considered as positive for both assays as per the FTD kit. With the custom assay being run for 40 cycles this reduces the custom assay run time by 8 min, thereby making it only 21 min longer than the FTD assay.

Comparisons were made between various aspects of the custom and the FTD assays (Table 6). No major differences were observed between the two assays except in the cost incurred for both assays. Similar comparisons were also done in an earlier study [21] where three multiplex PCRs were compared. The turn-around time of the custom assay was 29 min more as compared to the FTD assay. But both the assays reported the results on the same day. The excess time of 29 min taken by the custom assay as compared to the FTD assay may not greatly interfere with the treatment process. However, the custom assay was much more economical costing INR 1500/- per sample for screening 18 respiratory viruses compared to the commercial FTD assay which was expensive costing INR 4300/- per sample. This assay may prove to be highly cost effective in resource limited settings like ours. However the limitation of our study was that some of the viruses showed low positivity as a result it is difficult to assess the concordance accurately. Larger numbers of positive samples need to be tested to evaluate the concordance of these less prevalent viruses.

### Table 7 Detection limits of different respiratory viruses by custom assay and FTD kit

| Name of the virus | Custom assay detection limit | FTD assay detection limit |
|-------------------|-------------------------------|---------------------------|
| HBoV              | 1 DNA copy/ml [13]            | $10^3$ copies/ml          |
| HMPV              | 30 RNA copies/ml [13]         | $10^3$ copies/ml          |
| HPeV              | $10^3$ CCID$_{50}$ - $10^4$ CCID$_{50}$ [12] | $10^3$ copies/ml |
| RSV A/B           | $2 \times 10^4$ copies/ml [10] | $10^3$ copies/ml |
| HCoV HKU1         | $5 \times 10^3$ copies/ml [11] | $10^3$ copies/ml |
| Flu B             | $2.2 \log_{10}$ VP/ml [14]   | $10^3$ copies/ml         |
| Influenza A(H1N1)pdm09 | $2 \times 10^1$ to $2 \times 10^2$ copies/ml [7] | $10^3$ copies/ml |

**Conclusion**

This study reported a high prevalence of respiratory viruses in children ≤5 years using a custom assay and an FTD assay. Good concordance was observed for all the viruses between both assays except for RSV A/B. However larger numbers of positive samples need to be tested for thorough evaluation of less prevalent viruses.
The custom primer and probe mix was much more economical than the commercial FTD kit. Our study suggests that this custom multiplex real-time RT-PCR can be used for simultaneous and rapid detection of multiple viruses in resource limited settings. This will help prevent unnecessary use of antibiotics and permit timely initiation of supportive therapy/antiviral drugs if available.

Abbreviations
ARI, acute respiratory infections; CCID, cell culture infective dose; EV, enterovirus; Flu A, influenza A; Flu B, influenza B; FTD, fast track diagnostic; HAdV, human adenovirus; HBoV, human bocavirus; HCoV 229E, human coronaviruses 229E; HCoV HKU1, human coronaviruses HKU1; HCoV NL63, human coronaviruses NL63; HCoV OC43, human coronaviruses OC43; HMPV A/B, human metapneumovirus; HPEV, human parechovirus; HPV-1, 2, 3 and 4, human para influenza viruses 1, 2, 3, and 4; HRV, human rhinovirus; IVD, in-vitro diagnostics; M, pneum, mycoplasma pneumoniae; NFO, non fluorescence quencher; Pandemic: H1N1, influenza A subtype H1N1; PCR, polymerase chain reaction; RSVA/B, respiratory syncytial virus; SAR, severe acute respiratory infections; SARS, Sawa Man Singh; VP, viral particles; VT M, viral transport medium

Acknowledgements
Authors acknowledge the financial support from Indian Council of Medical Research to BM for setting up ICMR Grade-I Viral Research and diagnostic Laboratory and Senior Research Fellowship to MAS.

Authors’ contributions
BM participated in conception and design, experimental studies, analysis and interpretation of data, drafting the manuscript and revising it critically for important intellectual content, final approval of the version to be published. JKT participated in conception and design, drafting the manuscript and revising it critically for important intellectual content, final approval of the version to be published. NK participated in conception and design, drafting the manuscript and revising it critically for important intellectual content, final approval of the version to be published. JKT participated in conception and design, drafting the manuscript and revising it critically for important intellectual content, final approval of the version to be published.

Competing interests
The authors declare that they have no competing interests.

Received: 26 September 2015 Accepted: 24 May 2016
Published online: 06 June 2016

References
1. Kaplan NM, Dove W, Abu-Zeid AF, Shamoon HE, Abd-Eldayem SA, Hart CA. Human Bocavirus: Infection among Children, Jordan. Emerg Infect Dis. 2006;12:1418–20. doi:10.3201/eid1209.060417.
2. Williams BG, Gouws E, Boschi-Pinto C, Bryce J, Dye C. Estimates of worldwide distribution of child deaths from acute respiratory infections. Lancet Infect Dis. 2002;2:25–32. doi:10.1016/S1473-3099(01)00170-0.
3. Albuquerque MCM, Varella RB, Santos N. Acute respiratory viral infections in children in Rio de Janeiro and Teresopolis, Brazil. Rev Inst Med Trop Sao Paulo. 2012;54:249–55. doi:10.1590/S0046-2228201200500003.
4. Jartti L, Langen H, Soderlund-Venermo M, Ruorjaksen T, Ruoskanen J, Jartti T. New Respiratory Viruses and the Elderly. Open Respir Med J. 2011;5:61–9.
5. Bharaj P, Sullender WM, Kabra SK, Mani K, Cherian J, Tyagi V, et al. Respiratory viral infections detected by multiplex PCR among pediatric patients with lower respiratory tract infections seen at an urban hospital in Delhi from 2005 to 2007. Virol J. 2009;6:89. doi:10.1186/1743-422X-6-89.
6. Hammitt LL, Kaungu S, Welch S, Bett A, Onyango CO, Gunson RN, et al. Added value of an oropharyngeal swab in detection of viruses in children hospitalized with lower respiratory tract infection. J Clin Microbiol. 2011;49:2318–20.
7. Chen Y, Cui D, Zheng S, Yang S, Tong J, Yang D, et al. Simultaneous detection of influenza A, influenza B, and respiratory syncytial viruses and subtyping of influenza A H3N2 Virus and H1N1 Virus in multplex real time PCR. J Clinic Microbiol. 2011;49:1653–6.
8. Gadsby NJ, Hardie A, Claas ECJ, Templeton NE. Comparison of the luminex respiratory virus panel fast assay with in-house real-time PCR for respiratory viral infection diagnosis. J Clin Microbiol. 2010;48:2213–6.
9. Babady NE, Mpad P, Stiles J, Brennan C, Li H, Shuptar S, et al. Comparison of the luminex xTAG RVP fast assay and the idaho technology filmarray RP assay for detection of respiratory viruses in pediatric patients at a cancer hospital. J Clin Microbiol. 2012;50:2282–8.
10. Kwofill TB, Anane YA, Nkurnum B, Annan A, Nguah SB, Owusu M. Respiratory viruses in children hospitalized for acute lower respiratory tract infection in Ghana. Virol J. 2011;29:78. doi:10.1186/1743-422X-9-78.
11. Cui L, Zhang C, Zhang T, Lu RJ, Xie ZD, Zhang LL, Liu CY, Zhou WM, Ruan L, Ma XJ, Tan WJ. Human Coronavirus HCoV-NL63 and HCoV-HKU1 in hospitalized children with acute respiratory infections in Beijing, China. Adv Virol. 2011; Article ID 129134. doi:10.1155/2011/129134.
12. Nix WA, Maher K, Johansson ES, Niklasson B, Lindberg AM, Pallanich MA, et al. Detection of all known parechoviruses by real-time PCR? J Clin Microbiol. 2008;46:2519–24.
13. Sanghavi SK, Bullotta A, Husain S, Rinaldo CR. Clinical evaluation of multiplex real-time PCR panels for rapid detection of respiratory viral infections. J Med Virol. 2012;84:162–9.
14. Dabisch-Ruthe M, Vollmer T, Adams O, Knabbe C, Dreier J. Comparison of commercial and Fastertrack diagnostic kits for respiratory viruses. J Med Virol. 2011;83:1922–6.
15. FTD. Validation - FTD Respiratory pathogens 21. Luxembourg: Fast Track Diagnostics; 2014.
16. Gharabaghi F, Hawan A, Dresw SJ, Richardson SE. Evaluation of multiple commercial molecular and conventional diagnostic assays for the detection of respiratory viruses in children. Clin Microbiol Infect. 2011;17:1900–6.
17. Esposito S, Molteni CG, Daleno C, Valzano A, Tagliajue C, Galeone C, et al. Collection by trained pediatricians or parents of mid-turbinate nasal flocked swabs for the detection of influenza viruses in childhood. Virol J. 2010;7:85. doi:10.1186/1743-422X-7-85.
18. Singh AK, Jain A, Jain B, Singh KP, Dangi T, Mohan M, et al. Viral aetiology of acute lower respiratory tract illness in hospitalized paediatric patients of a tertiary hospital. One year prospective study. Indian J Med Microbiol. 2014;32:13–8.
19. Lu X, Chittagangipich M, Olsen SJ, Mackay IM, Soyos TP, Fry AM, Erdman DD, Real-Time PCR assays for Detection of Bocavirus in Human Specimens. J Clin Microbiol. 2006;44:2331–5.
20. Gunson RN, Carman WF. During the summer 2009 outbreak of ‘swine flu’ in Scotland what respiratory pathogens were diagnosed as H1N1/2009 BM. Infect Dis. 2011;11:192. doi:10.1186/1471-2334-11-192.
21. Dabisch-Ruthe M, Vollmer T, Adams O, Knabbe C, Dreier J. Comparison of three multiplex PCR assays for the detection of respiratory viral infections: Evaluation of xTAG respiratory virus panel fast assay, Respifinder 19 assay and Respifinder SMART 22 assay. BMC Infect Dis. 2012;12:163. doi:10.1186/1743-422X-12-163.
22. WHO. CDC protocol of real-time RTPCR for influenza A (H1N1). 2009. (Available from http://www.who.int/csr/resources/publications/swineflu/cdcRealtimeRTPCR_SwineH1Assay-2009_20090630.pdf). Accessed on 05/07/2012.
23. Wolfs FFG, Bruggeman CA, van Well GTJ, van Loo IJM. Replacing traditional diagnostics of fecal viral pathogens by a comprehensive panel of real-time PCRs. J Clin Microbiol. 2011;49:1926–31.