Performance of the Luminex xTAG Respiratory Viral Panel Fast in a clinical laboratory setting

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A B S T R A C T

The aim of the study was to develop a real-time RT-PCR for the detection of enteroviruses (EVs) and rhinoviruses (RVs) and to assess the performance of the xTAG RVP Fast assay in comparison to a direct fluorescent assay (DFA), a real-time RT-PCR assay for the detection of respiratory syncytial virus (RSV) and human metapneumovirus (hMPV), and the EV/RV RT-PCR assay developed in this study. The performance of the RVP Fast assay was assessed in the analysis of 373 nasopharyngeal samples. For the viruses of the DFA panel, detection rates of 27.6% and 23.8% were obtained by RVP and DFA, respectively, in analysis of a set of 297 samples collected in 2009–2010. These results show statistically significant superiority of the RVP Fast assay ($P = 0.049$). For RSV, hMPV, EV, and RV, detection rates of 48.0% and 45.2% were achieved by RVP and RT-PCR, respectively. For individual targets, increased detection of EV/RV ($P = 0.043$) and decreased detection of influenza A virus ($P = 0.004$) by RVP in comparison to real-time RT-PCR was observed. The results of the present study imply the need to adjust the InfA component of the RVP Fast assay to also cover the InfA(H1N1) 2009 virus.

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1. Introduction

Beyond the common viral respiratory pathogens, numerous infectious agents have been recognized during the last decade, including human metapneumovirus (hMPV), coronaviruses (CoVs) HKU1 and NL63, severe acute respiratory syndrome CoV (SARS-CoV), human bocavirus (hBoV), and avian influenza virus H5N1. The nonspecific symptoms of respiratory infections and overlapping seasons of the various viruses have forced virology laboratories to shift towards molecular assays. These enable rapid and cost-effective detection of potential viral pathogens by offering short turn-around time, sensitivity and specificity superior to those of direct fluorescent assay (DFA) or culture, and a potential for multiplexing.

Real-time polymerase chain reaction (PCR) assays employing fluorogenic probes offer remarkable benefits, such as short turn-around time and the nonnecessity for handling amplified products. However, multiplexing of real-time PCR assays is limited by the spectral overlap of fluorescent labels and loss of sensitivity associated with the multitude of oligonucleotides, factors that restrict the number of targets to be differentiated.

A feasible approach to broaden the detection spectrum is to employ postamplification detection in a solid phase (Li et al., 2007; Quan et al., 2007; Raymond et al., 2009; Takahashi et al., 2008) or a suspension array format (Lee et al., 2007; Li et al., 2007; Mahony et al., 2007; Marshall et al., 2007; Merante et al., 2007; Nolte et al., 2007; Pabbaraju et al., 2008). The xTAG respiratory virus panel (RVP) assay is a suspension array based on a multiplex PCR amplification. In the RVP Fast assay, sequential PCR, an exonuclease-phosphatase reaction, and target-specific primer extension steps of the original RVP assay (Merante et al., 2007) have been replaced by a PCR amplification using biotin-labeled primers followed by hybridization of the amplicons to fluorescent beads with specific antitag sequences. The biotin label of the amplicons enables attachment of a reporter molecule, streptavidin-R-phycoerythrin. During the detection phase, the beads are identified and the signal from phycoerythrin is measured as an indicator of the specific amplification product present. The RVP Fast assay detects influenza A and B viruses (InfA and InfB), respiratory syncytial viruses A and B (RSV-A and RSV-B), parainfluenzaviruses 1–4 (PIV1–4), adenovirus (AdV), hMPV, CoVs 229E, OC43, NL63, and HKU1, enterovirus/rhinovirus (EV/RV), and hBoV. Additionally, the assay allows subtyping of InfA viruses to prepandemic seasonal H1 or H3 viruses. In this study, the performance of the RVP Fast assay is assessed in comparison to DFA, a real-time RT-PCR assay for detection of RSV and hMPV (Jokela et al., 2010) and a real-time EV and RV RT-PCR assay first described in the present study.

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2. Materials and methods

2.1. Clinical samples and quality controls

Of the samples sent to HUSLAB, Helsinki University Central Hospital for routine virus diagnostics between December 2009 and April 2010, 284 nasopharyngeal aspirates or swabs and 13 bronchoalveolar lavage samples from pediatric and adult patients with respiratory symptoms were available for the present study. An additional 42 respiratory samples that were positive for InfA by real-time RT-PCR (Rönkkö et al., 2011; Ward et al., 2004), and 34 samples positive for either InfB or PIV3 by DFA, were analyzed in evaluation of the RVP Fast assay. These samples were collected in 2010 and 2011. Furthermore, 14 validation samples of the NATrol Respiratory Validation Panel 2 (NATRVP-2) Global Panel (Zeptometrix Corp., Buffalo, NY, USA) containing InfA H1N1, InfA H3N2, InfB, RSV-A, RSV-B, PIV1–3, AdV-7A, EV, RV, CoVs 229E and OC43, and SARS-CoV were analyzed with the RVP Fast assay. In addition, 20 quality assessment samples of the 2009 and 2010 EV and parechovirus RNA External Quality Assessment (EQA) Program (Quality Control for Molecular Diagnostics (QCMD), Glasgow, Scotland, UK) containing EV71, echoviruses (ECs) 11, 16, and 30, coxsackie A virus 9 (CAV9), CAV16, coxsackie B virus 3 (CBV3), and poliovirus type 3 (PV3), as well as 19 samples containing CAV21, RV8, RV16, RV42, RV72, and RV90 and CoVs 229E, OC43, and NL63 from the 2009 and 2010 RV and CoV RNA EQA Program of the same organizer were analyzed, using the real-time RT-PCR assay for EVs and RVs developed in this study.

2.2. Direct fluorescent assay (DFA)

Cells from clinical samples were concentrated by centrifugation and applied on a multiwell slide, dried, and fixed in acetone. The slide was tested for AdV, InfA, InfB, RSV, and PIV1–3, using the Light Diagnostics™ Respiratory DFA Viral Screening and Identification Kit (Millipore, Billerica, MA, USA) according to the manufacturer's instructions. Only samples with sufficient numbers of cells for analysis were included in the study.

2.3. RVP Fast assay

Total nucleic acid from samples stored at −70 °C was isolated by MagNA Pure robot (Roche Diagnostics, Basel, Switzerland) and the extracts were immediately subjected to the RVP Fast assay (Luminex Molecular Diagnostics Inc., Toronto, Canada). An internal positive control (Escherichia coli phage MS2) provided by the manufacturer was added to the samples before extraction. An aliquot of the extracts was stored at −70 °C for later testing for EV and RV by the real-time RT-PCR described below.

The RVP Fast assay was performed according to the manufacturer's instructions. A positive run control (bacteriophage lambda DNA) was used in each run to monitor assay performance. The resulting median fluorescence intensities (MFIs) were analyzed by the Luminex 100 IS system and TDAS RVP Fast version 2.00 (Luminex).

2.4. In-house real-time RT-PCRs

Real-time RT-PCR for the detection of RSV and hMPV was performed simultaneously with the RVP Fast assay, as described previously (Jokela et al., 2010). For the detection of EV and RV, three primers and two probes from the highly conserved 5′-untranslated regions of the viral genomes were designed: EV-F1 GACATGGTGYGAAGAGTCTATTGAG, RV-F1 AGGTGTGAAGAGCCCCGTGT, picorna-R GAAACACGGACACC-CAAAGTAGT, EV-probe Fam–CGGCCCCTGAATGCGGCTAATCC and

RV-probe Hex–CCGGCCCCTGAATGYGGCTAACCT. Furthermore, the primers EV-F2 GACATGGTGYGAAGAGTCTATTGAGCT (300 nM) and RV-F2 AAGGTGTGAAGAGCCCCGTGT (400 nM) were used in the reanalysis of clinical samples with discordant results. The reaction conditions of the EV/RV RT-PCR were as described previously (Jokela et al., 2010), except for the duplex RT-PCR, in which a concentration of 4.0 mM manganese acetate and 2 U of AmpliTaq Gold polymerase (Roche) were used. Modified primer concentrations were used for EV-F1 (300 nM), RV-F1 (400 nM), and picorna-R (700 nM). The optimal concentrations for the EV and RV probes were 150 nM and 200 nM, respectively. Optimization of the assay was performed, using RNA transcripts of EC11 and RV1B clones (Jokela et al., 2005). Analysis of clinical samples was performed in three reactions: one for EV PCR, one for RV PCR, and one for duplex PCR.

A precision study of EV/RV RT-PCR was performed, using dilution series of the RNA transcripts corresponding to 1×10^3, 1×10^4, 1×10^5, 1×10^7, and 1×10^9 genome equivalents per reaction. Intra-assay variability was evaluated by running five parallel reactions of the dilution series on one plate and interassay variability by testing one dilution series on four consecutive days.

2.5. Statistics

Concordance of the results obtained for the clinical samples by the RVP Fast assay and DFA as well as RVP Fast and RT-PCR was examined by McNemar's test using SPSS/PASW statistical program package (version 18; IBM-SPSS, Armonk, NY, USA), and statistical significance was set at $P < 0.05$.

3. Results

3.1. Performance of the real-time RT-PCR for EVs and RVs

Sensitivities of 10 and 50 RNA transcripts, respectively, were achieved for EC11 and RV1B in separate RT-PCR reactions, and 50 and 100 RNA transcripts for EC11 and RV1B, corresponding to 10^3 copies per mL of sample in the duplex assay. In the precision study, the mean intra-assay coefficients of variation (CV) of the cycle threshold (Ct) values for EC11 and RV1B were 1.52% and 1.00%, and mean interassay CV were 1.48% and 3.60%, respectively.

In the analysis of the EV and parechovirus quality assessment samples, 18 samples were positive for EV and two samples remained negative, consistent with the results reported by the organizer. The RT-PCR for the samples of the RV and CoV programs detected all RV types except RV72 at all dilutions provided. No signal was detected in the negative control or samples containing CoVs. One quality assessment sample containing RV42 gave a positive signal with the EV probe. Furthermore, samples containing EC11, EC16, EC30, EV71, CBV3, CAV9, CAV16, CAV21, and PV3 were positive with both probes in duplex RT-PCR. Therefore, the RT-PCR assay was considered incapable of distinguishing between the two picornavirus genera and, similar to the RVP Fast assay, a positive result was considered indicative of EV or RV.

3.2. Performance of the RVP Fast assay

All viruses present in the NATRVP-2 Global Panel were detected in the analysis, except SARS-CoV, which is not detected by the RVP Fast assay. In all, 12 clinical samples failed to give a valid result for the MS2 internal control in initial testing. Valid results for these samples were not obtained by repeating the post-amplification steps; however, extracting nucleic acids anew and repeating the analysis gave valid results for nine of the samples. The remaining three samples with no valid results in the RVP analysis were excluded from further analysis, and therefore the results of the

2009–2010 sample set below are described for the remaining 294 samples. No failures were detected for the positive run control.

3.3. Comparison of the RVP Fast assay with DFA and RT-PCR in analysis of clinical samples

For the viruses covered by the DFA panel, a significant difference in the overall detection rate between the RVP Fast assay and DFA was detected ($P = 0.049$), since 81 samples (27.6%) and 70 samples (23.8%) of the 2009–2010 sample set were positive for at least one virus by the RVP Fast assay and DFA, respectively. Considering the positive results for all viral targets included, a detection rate of 60.9% (179/294 samples) by the RVP Fast was observed, due to the broader detection spectrum provided by the assay.

In the analysis of the 2009–2010 sample set, 141 samples (48.0%) were positive with RSV, hMPV, EV or RV by the RVP Fast assay and 133 samples (45.2%) by the RT-PCR assays. All RT-PCR-positive samples were positive in both single and duplex reactions. Coinfections were identified in 26 specimens. The RVP Fast assay detected 19 dual infections and two triple infections, and RT-PCR detected an additional five coinfections. The results of the RVP Fast assay for individual targets in comparison to DFA and PCR are provided in Table 1. Furthermore, the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for the RVP Fast assay, using DFA as the gold standard, are provided, except for EV/RV and hMPV, for which PCR was used as the gold standard. For RSV and InfA, the calculations were done separately, using both gold standards.

A total of 61 samples (20.7%) were positive for RSV in the 2009–2010 sample set, 12 of which were coinfections. Of the 57 RVP-positive samples (39 RSV-A and 18 RSV-B), 52 samples were also positive in DFA and RT-PCR (mean Ct 23.1) and one sample not tested in DFA turned positive in RT-PCR (Ct 20.6). Discordant results were obtained for a total of eight samples. Four of the samples were positive in RVP, two of which were also positive in RT-PCR (mean Ct 37.0), and two samples remained negative in RT-PCR and DFA. Of the four RVP-negative samples, two were positive in DFA and RT-PCR (mean Ct 22.0) and two were positive only in RT-PCR (mean Ct 36.0). RSV detection rates of 19.4%, 18.4% and 20.1% were obtained in RVP, DFA, and RT-PCR, respectively. In statistical analysis, no significant difference in detection of RSV was found between the RVP Fast assay and DFA ($P = 0.289$) or RVP and RT-PCR ($P = 0.687$).

The performance of the RVP Fast assay in detecting EV, RV, and hMPV, in the 2009–2010 sample set was assessed in comparison to the RT-PCR assays. In all, 55 samples were positive for EV/RV by the RVP Fast assay and RT-PCR. Six of the samples were PCR-negative in the initial analysis, but were EV-positive in reanalysis, using the EV-F2 primer. In addition to the 18 RT-PCR-negative samples, discordant results were obtained for seven RVP-negative samples (Table 1). Four RVP-negative samples turned out to be EV/RV-positive in RT-PCR (mean Ct 34.6). Additionally, RT-PCR detected EV/RV in two samples positive for RSV and in one sample positive for AdV by the RVP Fast assay (mean Ct 30.4). EV/RV detection rates of 24.8% and 21.1% were obtained by the RVP Fast assay and RT-PCR, respectively. The difference in detection rates of EV/RV between the RVP Fast assay and RT-PCR was statistically significant ($P = 0.043$).

In all, 12 samples of the sample set collected in 2009–2010 were positive for hMPV, 11 of which were positive in RVP and RT-PCR (mean Ct 32.2). One sample was weakly positive for hMPV in RT-PCR (Ct 44.0), and was hMPV-negative but positive for AdV and hBoV in the RVP Fast assay. The detection rates for hMPV in RVP and RT-PCR were 3.7% and 4.1%, respectively.

The RVP Fast assay detected InfA in four samples of the 2009–2010 sample set, but was unable to type the virus in all samples. One of the RVP-positive samples remained negative in DFA. Three out of the four positive samples were available for analysis

Table 1
Performance of the RVP Fast assay in the analysis of clinical samples in comparison to DFA and RT-PCR results.

Virus	RVP+ DFA+	RVP+ DFA−	RVP− DFA+	RVP− DFA−	RVP+ PCR+	RVP+ PCR−	RVP− PCR+	RVP− PCR−	Performance compared to DFA				Performance compared to PCR			
									Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
RSV	52	5[a]	2	269	55	2	4	233	96.3	98.2	91.2	99.3	93.2	99.1	96.5	98.3
EV/RV	-	-	-	-	55	18	7	214	-	-	-	-	88.7	92.2	75.3	96.8
hMPV	-	-	-	-	11	0	1	282	-	-	-	-	91.7	100.0	100.0	99.6
InfA	3	1	0	324	28	0	9	8	100.0	99.7	75.0	100.0	75.7	100.0	100.0	47.1
InfB	20	0	2	306	-	-	-	-	90.9	100.0	100.0	99.4	-	-	-	-
AdV	4	5	1	318	-	-	-	-	80.0	98.5	44.4	99.7	-	-	-	-
PIV 1	2	2[b]	1	323	-	-	-	-	66.7	99.4	50.0	99.7	-	-	-	-
PIV 2	2	2	0	324	-	-	-	-	100.0	99.4	50.0	100.0	-	-	-	-
PIV 3	17	0	1	310	-	-	-	-	94.4	100.0	100.0	99.7	-	-	-	-

[a] One sample not tested in DFA.
[b] One sample positive for PIV2 in DFA.

by real-time RT-PCR (Rönkkö et al., 2011), and all tested positive for InfA(H1N1) 2009. To further assess the detection of InfA by the RVP Fast assay, 15 samples positive for seasonal InfA, 19 samples positive for InfA(H1N1) 2009 and eight samples negative by real-time RT-PCR, were analyzed by the RVP Fast assay. Concordant results were obtained for the negative samples, whereas two of the samples PCR-positive for seasonal InfA (mean Ct 37.2) remained negative by RVP. Moreover, the RVP Fast assay was unable to type one of the positive samples. Furthermore, only 12 (63.2%) of the 19 samples positive for InfA(H1N1) 2009 by RT-PCR were positive in the RVP Fast assay. These samples missed by the assay had a mean Ct of 32.8 in RT-PCR. The figures described above result in 76.2% concordance of the results, and the difference between the results was statistically significant in McNemar's test ($P = 0.004$).

No InfB was found in the sample set collected in 2009–2010. To assess the performance of the RVP Fast assay in detection of InfB, 22 additional samples positive for the virus in DFA were analyzed by the assay. Two of the samples remained negative by RVP, resulting in 90.9% concordance of the results by the two assays.

The RVP Fast assay detected nine samples positive for AdV and five of these remained negative in DFA. Additionally, one RVP-negative sample appeared positive in DFA. In all, 12 of the 2009–2010 samples were positive for PIV1–3. Eleven of these were positive in the RVP Fast assay, seven of which were also positive by DFA. One sample was positive for PIV1 only by DFA. PIV4 was not detected in the sample set. Due to the low prevalence of PIV in the sample set, 12 additional samples positive for PIV3 by DFA were analyzed in the RVP Fast assay; positive results were obtained for 11 of the samples.

Of the viruses with no other detection measures available in the Helsinki University Central Hospital laboratory, the RVP Fast assay detected CoVs HKU1 in 15 samples (5.1% detection rate), 229E in eight samples (2.7% detection rate), and OC43 in three samples (1.0% detection rate) of the 2009–2010 sample set. CoV NL63 was not detected, while 14 samples (4.8%) were positive for hBoV.

4. Discussion

Multiplex PCR with detection of amplification products in suspension microarray is a recent diagnostic advancement among efforts to identify respiratory viruses in a rapid and cost-effective manner. In this study, the performance of a suspension microarray-based RVP Fast assay in the analysis of 373 nasopharyngeal samples was assessed. The results of the RVP Fast assay were compared with those of DFA as well as a real-time RSV and hMPV RT-PCR assay (Jokela et al., 2010) and a real-time EV/RV RT-PCR assay first described in this study. The sensitivity of the RVP Fast assay for PIV4, CoVs and hBoV could not be assessed in this study design, because no previous measures for detection of these viruses in the testing algorithm of the Helsinki University Central Hospital laboratory existed.

The RVP Fast assay was rapid, simple to perform, and required relatively little hands-on time. Together with the large amount of data produced in one analysis, these are all desired features in routine diagnostics. Notably, 3.2% of the samples (12/373) were reanalyzed due to the invalid control calls in initial RVP analysis. Together the results of MS2 RNA and lambda phage DNA showed that the failures of the assay were due to faulty nucleic acid extraction or RT reaction (Merante et al., 2007). Indeed, repeated testing including the RT-PCR step produced valid results in most cases and after reanalysis a total of 99.2% of all samples (370/373) produced valid results in the RVP analysis.

Analysis of the 2009–2010 sample set resulted in a remarkable increase in the overall detection rate of respiratory viruses by the RVP Fast assay over DFA for those viruses currently covered by the DFA panel. Considering the entire detection spectrum of the RVP Fast assay, a detection rate of 60.9% was achieved. Similar findings have also been reported in previous studies, using the RVP Fast assay (Gadsby et al., 2010) or the original RVP assay (Gharabaghi et al., 2011; Mahony et al., 2007). The 7.1% prevalence of co-infections as detected in the present study by the RVP Fast assay is similar to the prevalences shown by others (Gadsby et al., 2010; Mahony et al., 2007; Pabbaraju et al., 2008). High Ct values of false RVP-negative samples have been reported for the RVP Fast assay (Gadsby et al., 2010) and the original RVP assay (Mahony et al., 2007). Similarly, it was observed, that the discordant results of the RVP-negative samples was associated with relatively low viral load (mean Ct in RT-PCR 34.0), except when the target missed by RVP was InfA(H1N1) 2009.

For RSV, hMPV, EV, and RV, an increase in the overall detection rate by the RVP Fast assay over RT-PCR was observed. In the detection of EV/RV, a statistically significant difference was shown as the detection rate of the RVP Fast assay increased over that of RT-PCR. This implies that the in-house RT-PCR assay may not have been able to detect all EVs and RVs, although additional freeze/thaw cycles may also have influenced the results. The EV/RV RT-PCR was also unable to distinguish between the two picornavirus genera, but nonetheless a positive result indicated a representative of either genus.

An InfA finding that is unsubtypeable by the RVP Fast assay is highly indicative of the 2009 pandemic InfA(H1N1) (Ginocchio and St George, 2009; Vinikoor et al., 2009). Indeed, the three InfA-positive samples that were tested by real-time RT-PCR were all positive for the novel InfA (Rönkkö et al., 2011). Since all four samples dated back to December 2009, they were all likely to contain the 2009 InfA(H1N1). However, as the performance of the RVP Fast assay in detecting InfA was further assessed, a significant decrease in the detection rate of the RVP Fast assay, compared with that of the real-time RT-PCR assays utilized, was observed. Indeed, the results of the study suggest adjustment of the InfA component of the assay to also cover the novel InfA(H1N1) 2009 virus. Although the low sensitivity of the RVP Fast assay in detection of InfB has been described previously (Gharabaghi et al., 2011), the few discordant findings of the present study cannot be generalized for overall performance of the assay.

Results obtained with the RVP Fast assay show reliable detection of PIV, but the low prevalence of PIV1–2-positive samples in our study limits interpretation of the results. Since low sensitivity of the RVP Fast assay to PIV1–3 has been described previously (Gharabaghi et al., 2011), a greater number of positive samples would have been essential to assess the assay performance in detection of these targets.

Due to the low sensitivity of the DFA, the contemporary basis of respiratory virus diagnosis in the Helsinki University Central Hospital laboratory, negative test results cannot be interpreted as ruling out a viral infection, and thus higher sensitivity is desired. Implementing the RVP Fast assay into daily diagnosis of respiratory infections would result in negative findings with more clinical relevance, since a negative result of a reliably performed RVP analysis may be considered as absence of a spectrum of pathogens in the sample. Evaluation of the RVP Fast assay demonstrated that adopting a PCR-based multiplex assay results in a remarkable increase in overall viral detection rate of respiratory viruses, compared with conventional methods. The relatively high prevalence of EV/RV, CoVs, hMPV, and hBoV in our study population also emphasizes the need for a broader detection spectrum of respiratory virus diagnostics than what is achievable by the DFA. Moreover, a cost analysis study (Mahony et al., 2009) suggested that substantial savings may be achieved with testing by the xTAG RVP test alone, with most of the savings resulting from shortened lengths of hospital stays. However, since batching of samples for the RVP analysis

retards the test results, the DFA is still the method of choice when PCR-based testing is too slow to support clinical decisions. Broad-spectrum RVP analysis will result in samples with two or more viral pathogens identified. Although the clinical relevance of such findings is currently not clear, it has been suggested that coinfection with more than one virus may result in greater disease severity and longer hospital stays (Cilla et al., 2008; Esper et al., 2011; Paranhos-Baccalã et al., 2008). With multiplex testing becoming more common, our understanding of the epidemiology of respiratory viruses may be improved further and the clinical importance of mixed infections systematically studied.

Conflict of interest

Acknowledgments

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