

Comparison of the Luminex Respiratory Virus Panel Fast Assay with In-House Real-Time PCR for Respiratory Viral Infection Diagnosis[∇]

Naomi J. Gadsby,¹ Alison Hardie,¹ Eric C. J. Claas,² and Kate E. Templeton^{1*}

Specialist Virology Centre, Royal Infirmary of Edinburgh, Edinburgh, United Kingdom,¹ and Department of Medical Microbiology, Center of Infectious Diseases, Leiden University Medical Center, Leiden, Netherlands²

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The Luminex xTAG Respiratory Virus Panel (RVP) assay has been shown to offer improved diagnostic sensitivity over traditional viral culture methods and to have a sensitivity comparable to those of individual real-time nucleic acid tests for respiratory viruses. The objective of this retrospective study was to test a new, streamlined version of this assay, the RVP Fast assay, which requires considerably less run time and operator involvement. The study compared the performance of the RVP Fast assay with those of viral culture, a direct fluorescent assay (DFA), and a panel of single and multiplex real-time PCRs in the testing of 286 respiratory specimens submitted to the Edinburgh Specialist Virology Centre for routine diagnosis of viral infection between December 2007 and February 2009. At least one respiratory viral infection was detected in 13.6% of specimens by culture and DFA combined, in 49.7% by real-time PCR, and in 46.2% by the RVP Fast assay. The sensitivity and specificity of the RVP Fast assay compared to the results of real-time PCR as the gold standard were 78.8% and 99.6%, respectively. Real-time PCR-positive specimens missed by the RVP Fast assay generally had low viral loads or were positive for adenovirus. Additionally, a small number of specimens were positive by the RVP Fast assay but were not detected by real-time PCR. For some viral targets, only a small number of positive results were found in our sample set using either method; therefore, the sensitivity of detection of the RVP Fast assay for individual targets could be investigated further with a greater number of virus-positive specimens.

Viral infections of the respiratory tract have traditionally been diagnosed in the laboratory by culture of respiratory specimens and direct fluorescent assay (DFA). However, the availability of real-time PCR has allowed us to detect respiratory viruses with greater sensitivity and shorter turnaround times (12). In recent years, a number of new respiratory viruses have been identified, so we must now consider a wider range of viruses in our diagnoses (see, e.g., references 1 and 14). However, the number of fluorophores that can be differentiated in a multiplex real-time PCR assay limits the number of viral targets that can be detected.

One solution is to screen each specimen with several different multiplex real-time PCRs to cover a large number of viruses (4). An alternative, the xTAG respiratory virus panel (RVP) assay (Luminex Molecular Diagnostics Inc., Toronto, Canada), is based on suspension microarray technology, which enables the detection of a large number of targets in a single reaction (6, 9). The xTAG RVP assay has been shown to offer results comparable or superior to those of culture/DFA and nucleic acid tests for the diagnosis of respiratory viral infections (7, 10). Recently, the RVP assay has been used successfully for the detection of etiological agents in outbreaks of respiratory illness (3, 15).

The latest version of this test, the RVP Fast assay, has a

simpler protocol and a shorter turnaround time than the original assay but still detects 19 different viral and subtype targets: influenza A virus (with additional subtyping: H1, H3, and H5), influenza B virus, respiratory syncytial virus A (RSV-A), RSV-B, parainfluenza virus 1 (PIV-1), PIV-2, PIV-3, PIV-4, adenovirus, human metapneumovirus, coronaviruses 229E, NL63, OC43, and HKU1, enterovirus/rhinovirus (EV/RhV), and human bocavirus. Here we compare the performance of the RVP Fast assay with those of culture/DFA and in-house real-time PCR assays, using respiratory specimens collected for routine viral testing.

MATERIALS AND METHODS

Clinical specimens. Specimens selected were those submitted for virological culture to the Specialist Virology Centre (SVC) at the Edinburgh Royal Infirmary between December 2007 and February 2009. All bronchoalveolar lavage (BAL) specimens received are cultured as routine procedure, along with certain nasopharyngeal secretion (NPS) specimens where clinically indicated. A total of 286 specimens were used for this study: 151 BAL and 135 NPS specimens. The age of patients ranged from <1 to 84 years; the median age was 5 years. These specimens are part of an anonymized archive of respiratory specimens held by the SVC under the approval of the NHS Lothian Regional Ethics Committee.

Culture and DFA. Respiratory specimens were suspended in viral transport medium (VTM) and were inoculated into cultures of MRC-5 fibroblasts. Cell cultures were incubated at 33°C and were checked twice a week for cytopathic effects; negative cultures were discarded after 1 month. DFA was also performed on Cytospin preparations of most specimens. Depending on the time of year, any number of the following were tested using fluorescent monoclonal antibodies (Dako, Ely, United Kingdom): influenza A virus, influenza B virus, PIV, and RSV.

Real-time PCR. Total nucleic acid was extracted from specimens suspended in VTM by using the BioRobot MDx automated system (Qiagen Ltd., Crawley, United Kingdom), and three multiplex real-time PCRs were carried out on each extract using the ABI real-time system, model 7500 (Applied Biosystems, Warrington, United Kingdom), as part of routine testing. The first multiplex real-

* Corresponding author. Mailing address: Specialist Virology Centre, Department of Laboratory Medicine, Royal Infirmary of Edinburgh, Little France Crescent, Edinburgh, EH16 4SA, United Kingdom. Phone: 0044 131 242 6015. Fax: 0044 131 242 6008. E-mail: kate.templeton@luht.scot.nhs.uk.

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time PCR tested for influenza A virus, influenza B virus, and RSV; the second, for PIV-1, -2, and -3; the third, for adenovirus and *Mycoplasma pneumoniae*. These assays were developed in-house and/or adapted from published methods (5, 12). Nucleic acid extracts for routine multiplex respiratory real-time PCR were not stored; therefore, original specimens in VTM were retrieved from storage at -70°C , and nucleic acids were extracted anew for real-time PCR testing for additional targets. Total nucleic acid extraction was carried out using the NucliSENS easyMAG automated system (bioMérieux, Basingstoke, United Kingdom) rather than the BioRobot MDx system in order to minimize disruption to the routine work flow. These extracts were then tested by individual real-time PCRs for PIV-4, human metapneumovirus, coronaviruses 229E, NL63, OC43, and HKU1, rhinovirus, enterovirus/rhinovirus, and human bocavirus. These were not part of the routine testing service at the time of this study but had been developed in-house and/or adapted from published methods later (2, 11–13; also unpublished results).

RVP Fast assay. Original specimens frozen in VTM were retrieved from storage at -70°C , and total nucleic acids were extracted using the NucliSENS easyMAG automated system (bioMérieux, Basingstoke, United Kingdom). These extracts were then tested using the RVP Fast assay according to the manufacturer's instructions, in a 96-well plate format (Luminex Molecular Diagnostics Inc., Toronto, Canada). The RVP Fast assay comprised a single multiplex PCR with labeled primers, followed by a single-step hybridization of PCR products to the fluorescent bead array and incubation with reporter reagents. The plate was then analyzed using the xMAP 100 IS instrument (Luminex Molecular Diagnostics Inc., Toronto, Canada), and the median fluorescent intensity (MFI) was determined. An MFI value above the threshold level determined by the manufacturer for a particular target indicated a positive result for that target.

The RVP Fast assay simultaneously detects influenza A virus (subtyped as either H1, H3, or H5), influenza B virus, RSV-A and -B, PIV-1, -2, -3, and -4, adenovirus, human metapneumovirus, coronaviruses 229E, NL63, OC43, and HKU1, enterovirus/rhinovirus, and human bocavirus. The assay also tests an internal positive control added to each specimen at the extraction stage (*Escherichia coli* phage MS2 RNA) and a positive run control that is added to each plate (bacteriophage lambda DNA).

The RVP Fast method provides a considerable reduction in time and specimen manipulation from the first version of the assay (9). The original version of the assay comprised sequential PCR, exonuclease-phosphatase reaction, and target-specific primer extension (TSPE) steps, which have been replaced by a single multiplex PCR in the RVP Fast assay. In addition, the individual bead hybridization and reporter incubation steps from the previous version of the assay have been combined into a single step in the new assay format.

Statistics. Data were compared by Pearson's chi-square test using Minitab, version 15.1.0.0 (Minitab Inc., 2006), and *P* was deemed significant at the 0.05 level.

RESULTS

Viral culture and DFA. Of the 286 specimens submitted for culture, 21 (7.3%) were positive for a respiratory virus; 13 cultures were incomplete due to contamination; and 12 had no culture result recorded. The respiratory viruses isolated were 16 RSVs, 4 adenoviruses, and 1 rhinovirus. A further 4 samples were positive for cytomegalovirus (CMV), and 3 were positive for herpes simplex virus type 1 (HSV-1). Additionally, the majority of these specimens were tested by DFA for at least one virus, as determined by the seasonal incidence. Using this approach, 27 RSV- and 3 influenza A virus-positive specimens were identified, but no specimens were found positive for influenza B virus or PIV. Eleven specimens were found positive for RSV both by culture and by DFA, and one specimen was found to have a dual infection with adenovirus and influenza A virus. Therefore, by combining the DFA and culture results, a total of 39 specimens were found to be positive for at least one respiratory virus (13.6%).

Real-time PCR and the RVP Fast assay. All 286 specimens were tested both by real-time PCR and by the RVP Fast assay. Multiplex PCRs for (i) influenza A virus, influenza B virus, and

TABLE 1. Numbers of single and multiple infections detected by the RVP Fast assay compared to those detected by real-time PCR

No. of viral targets	No. (%) of specimens with a positive result by:	
	RVP	PCR
0	154 (53.8)	144 (50.3)
1	98 (34.3)	99 (34.6)
2	28 (9.8)	35 (12.2)
3	6 (2.1)	3 (1.0)
4	0 (0.0)	5 (1.7)

RSV, (ii) PIV-1 to -3, and (iii) adenovirus and *M. pneumoniae* were carried out at the time of specimen submission as part of routine testing. Specimens were then stored (for approximately 1 to 17 months), and nucleic acids were later extracted anew for testing by the RVP Fast assay and by individual real-time PCR.

No significant difference was found between the RVP Fast assay and real-time PCR in the overall rate of detection of single or mixed viral infections (*P*, 0.403 and 0.270, respectively). At least one virus was detected in 142 (49.7%) of the specimens tested by the panel of real-time PCR assays and in 132 (46.2%) of the specimens tested by the RVP Fast assay (Table 1). Mixed infections were identified in 34 (11.9%) of the specimens by the RVP Fast assay and in 43 (15.0%) by real-time PCR. Through real-time PCR and the RVP assay, five quadruple infections and six triple infections were identified. The five quadruple infections each comprised rhinovirus/enterovirus and human bocavirus, plus two of the following: RSV, adenovirus, influenza A virus, and PIV-3. *M. pneumoniae* was detected in one specimen by real-time PCR, but this specimen was excluded from comparative analysis, because the equivalent test is not present in the RVP Fast assay.

The RVP Fast assay detected 156 of the 198 infections detected by real-time PCR, a significantly lower detection rate (*P*, 0.032) (Table 2). The sensitivity and specificity of the RVP assay, compared to the results of real-time PCR as the gold standard, were 78.8% and 99.6%, respectively. With the exception of adenovirus, the PCR-positive specimens missed by the RVP assay tended to have higher threshold cycle (C_T) values than those detected by both assays, although for several targets, the total numbers of specimens found positive by either method were small. The RVP Fast assay detected an additional 16 infections over those found by real-time PCR (mainly picornaviruses); therefore, the overall virus detection rates of the two methods on this sample set were not significantly different (*P*, 0.171). Furthermore, the RVP assay simultaneously subtyped the influenza A virus- and RSV-positive specimens.

For influenza A virus, 11 specimens were found to be positive by both the RVP Fast assay and real-time PCR; these had a mean C_T of 23.9 (range, 16.1 to 38.9). Additionally, the RVP Fast assay subtyped the positive specimens as 2 H1 and 9 H3 viruses. No H5 influenza virus was detected. Five real-time PCR-positive specimens were found to be negative by the RVP Fast assay; these had a mean C_T of 35.9 (range, 33.9 to 37.0).

TABLE 2. Performance of the RVP assay for respiratory virus identification relative to those of routine multiplex and single real-time PCR assays

Virus ^a	No. of specimens with the following result:				Performance of the RVP Fast assay with real-time PCR as the gold standard	
	PCR ⁺ RVP ⁺	PCR ⁺ RVP ⁻	PCR ⁻ RVP ⁺	PCR ⁻ RVP ⁻	Sensitivity (%)	Specificity (%)
Influenza A virus*	11	5	0	270	68.8	100.0
Influenza B virus*	4	0	0	282	100.0	100.0
RSV*	36	2	2	246	94.7	99.2
PIV-1*	0	0	0	286	100.0	100.0
PIV-2*	0	0	0	286	100.0	100.0
PIV-3*	1	2	0	283	33.3	100.0
PIV-4	1	0	0	285	100.0	100.0
Adenovirus*	13	8	0	265	61.9	100.0
Coronavirus 229E	0	0	0	286	100.0	100.0
Coronavirus NL63	2	0	0	284	100.0	100.0
Coronavirus OC43	7	0	2	277	100.0	99.3
Coronavirus HKU1	1	0	0	285	100.0	100.0
RhV/EV	60	7	12	207	89.6	94.5
hBoV	12	17	0	257	41.4	100.0
hMPV	8	1	0	277	88.9	100.0

^a hBoV, human bocavirus; hMPV, human metapneumovirus. Viruses marked with asterisks were tested by multiplex real-time PCR assays; the rest were tested by single real-time PCR assays.

Four specimens were positive for influenza B virus by both the RVP Fast assay and real-time PCR.

For RSV, 36 specimens were positive by both the RVP Fast assay and real-time PCR; these had a mean C_T of 26.5 (range, 19.5 to 38.6). The two real-time PCR-positive specimens that were negative by the RVP Fast assay had C_T values of 37.0 and 41.8. The RVP Fast assay picked up a further 2 RSV-positive specimens and additionally subtyped all the positive specimens as RSV-A (n , 27) or RSV-B (n , 11).

No specimens were found to be positive for PIV-1 or PIV-2 by either assay. For PIV-3, one specimen was positive by both the RVP Fast assay and real-time PCR (C_T , 28.4) and two specimens were positive only by real-time PCR (C_T s, 37.9 and 31.3). One specimen was identified as PIV-4 positive by both the RVP Fast assay and real-time PCR (C_T , 36.0).

For adenovirus, 13 specimens were positive by both the RVP Fast assay and real-time PCR; these had a mean C_T of 30.1 (range, 16.6 to 36.9). However, 8 real-time PCR-positive specimens that were negative by the RVP Fast assay had a similar mean C_T , 32.6 (range, 17.3 to 38.8). As part of our routine testing protocol, the multiplex real-time PCR assay for adenovirus also includes *M. pneumoniae*. One *M. pneumoniae*-positive specimen was identified in this study, but since this target is not present in the RVP assay, no comparison could be made.

For the coronaviruses, two specimens were found to be positive for NL63, one for HKU1, and 7 for OC43 by both the RVP Fast assay and real-time PCR. The RVP Fast assay picked up an additional two OC43-positive specimens that had tested negative by real-time PCR. No specimens were found to be positive for 229E by either test.

For picornavirus detection, 60 specimens were positive by both the RVP and real-time PCR assays; by use of the rhinovirus-specific real-time PCR, 52 (86.7%) of these were found to have rhinoviruses. This implies that the remaining 8 (13.3%)

PCR-positive specimens were infected with enteroviruses. Seven specimens were positive only by RhV/EV and RhV real-time PCR; these had a mean C_T of 41.1 (range, 35.6 to 44.5). An additional 12 specimens were positive by the RVP Fast assay but negative by both the RhV/EV and RhV real-time PCR assays.

For human bocavirus, 12 specimens were positive by both the RVP Fast assay and real-time PCR; these had a mean C_T of 30.8 (range, 16.1 to 37.8), whereas the 17 specimens positive only by real-time PCR had a mean C_T of 40.4 (range, 33.8 to 45.3). For human metapneumovirus, 8 specimens were positive by both the RVP Fast assay and real-time PCR, with a mean C_T of 29.5 (range, 20.2 to 39.5). The one specimen positive only by real-time PCR had a C_T of 31.6.

DISCUSSION

Here we assessed the performance of the RVP Fast assay by the retrospective testing of 286 respiratory specimens originally submitted for routine viral culture, DFA, and real-time multiplex PCR testing. The RVP Fast assay results were also compared to those of a panel of in-house single real-time PCR assays, performed on the same specimen set, for a more comprehensive analysis.

We found the RVP Fast assay to be rapid and straightforward to perform. Several different multiplex real-time PCRs would need to be performed on the same specimen, with associated labor, reagent, and development costs, in order to provide a level of viral identification and subtype information equivalent to that offered by the RVP assay. Recently, a cost analysis carried out on a pediatric population showed the RVP assay to be a less costly option than DFA and shell vial culture (8), although no cost comparison between multiplex PCRs and the RVP assay has been carried out yet.

The RVP Fast assay, like the panel of real-time PCRs, was a more sensitive method of viral detection than our routine culture and DFA. Within our specimen set, the rate of positive specimens increased from 13.6% with culture/DFA to 46.2% and 49.7% with the RVP Fast assay and PCR, respectively. This improvement in the detection rate over that of DFA/culture was also found with the previous version of the RVP assay (7).

Direct comparison of the RVP Fast assay with our real-time PCR panel showed an overall sensitivity of 78.8%, reflecting a range of sensitivities across the targets. Unfortunately for our study, a relatively low number of specimens in our sample set were positive for influenza viruses, parainfluenza viruses, coronaviruses, and human metapneumovirus. Therefore, a study involving larger numbers of positive specimens will be required in order to fully assess the sensitivity of the assay for all targets. With the exception of adenovirus, most discordant results were associated with C_T values of >35 in the real-time PCR, indicating low viral loads. For example, human bocavirus PCR-positive samples missed by the RVP assay had a mean C_T of 40 (although the clinical significance of detecting this virus at such levels is uncertain). Reduced detection in samples with high C_T values is consistent with the findings for the previous version of the RVP assay (10).

Discordant results for adenovirus compared to those of a nucleic acid test were found with the previous version of the

RVP assay (10) and may be due to suboptimal primer binding in particular adenovirus serotypes. Adenovirus-positive specimens missed by the RVP Fast assay had a wide range of C_T values by real-time PCR, similar to those for specimens that were actually found positive by the assay, indicating that low viral loads are unlikely to be the cause of the discrepancy. Sequencing of the adenovirus hexon gene primer targets in those discordant samples may provide an explanation for the low sensitivity for this target, since it is possible that there are primer mismatches.

The RVP Fast assay, as confirmed by the panel of real-time PCR assays, revealed that almost half of the respiratory specimens tested were positive for at least 1 viral infection and that viral coinfections were frequent. Similar observations have also been made in studies using the original RVP assay (7, 10). The routine use of extensive testing by such methods as the RVP Fast assay should make large studies of the clinical relevance of single and multiple respiratory virus infections more feasible.

In conclusion, the RVP Fast assay performed well in comparison to culture/DFA for the detection of respiratory viral infections and rapidly provided a large amount of useful diagnostic information. The sensitivity of the RVP Fast assay compared to the results of the real-time PCR gold standard was generally good, with the exception of adenovirus detection and the detection of some targets in specimens with low viral loads. These issues may be clarified with further investigation.

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