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Comparison of the fast track diagnostics respiratory 21 and Seegene Allplex multiplex polymerase chain reaction assays for the detection of respiratory viruses

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ABSTRACT

Background: Real-time multiplex PCR assays are increasingly used for respiratory virus detection, and offer automated analysis in a closed tube system, but they have the disadvantage of low-throughput due to multiplexing limitations. In this study, the established fast-track respiratory 21 assay (FTD) (fast-track diagnostics, Junglinster Luxembourg) was compared to the new Seegene Allplex assay (Seegene) (Seegene Inc. Seoul, Korea) which offers greater multiplexing as multiple targets can be detected in each fluorescence channel. The Seegene Allplex assay is quicker to perform than previous Seegene respiratory multiplex assays.

Materials and methods: The assays were evaluated using 199 mostly upper respiratory tract samples.

Results: A respiratory pathogen was found in 127/199 (63.8%) of samples by the FTD assay and 123/199 (61.8%) using the Seegene assay. Kappa agreement was between 0.87 and 1 for all targets except human bocavirus and adenovirus.

Conclusion: Although the performance of the assays were similar, the Seegene assay had the advantage of simultaneous detection of two gene targets for each of the common Influenza A subtypes, improved throughput of 30 samples per run and automated result analysis. The FTD assay could only test 17 samples per run but validation for use on several different real-time thermal cyclers made it easier to integrate into an existing laboratory system. Both assays were cost effective compared to in-house multiplex PCR respiratory virus screening.

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Introduction

Viral respiratory infections are a common cause of hospital admissions. Such infections cannot usually be diagnosed from clinical symptoms alone and laboratory testing is required for this purpose. Testing for respiratory viruses in the laboratory is increasingly dependent on molecular genetic assays, such as PCR, which are rapid and highly sensitive. Increasingly, multiplex PCR panels are being used for respiratory virus detection, enabling the simultaneous detection of all significant respiratory viral targets. These are relatively complex to set up and maintain as in-house assays. Consequently, the recent availability of commercial multiplex PCR assays has been very welcome.

We previously compared The Fast-track diagnostics multiplex respiratory PCR assay with two other commercial multiplex PCR assays including the widely used xTAG® respiratory viral panel fast (RVP) (Luminex corporation, U.S.A) [1]. In this study, we found that the performance of the assays was very similar, but the real-time multiplex PCR assays offered the advantage of much simpler automated monitoring for PCR product accumulation in real time at the expense of lower throughput due to reduced multiplexing ability. Here, we compare the performance of the fast-track diagnostics respiratory 21 (FTD) (Fast-Track diagnostics, Junglinster, Luxembourg) with the recently released Seegene Allplex multiplex real-time PCR assay (Seegene) (Seegene Inc. Seoul, Korea). The FTD assay is very similar to the assay evaluated in our original study, [1] except that the original FTD assay did not include detection of Corona virus HKU1 and Influenza A H1N1pdm. The Seegene assay offers greater multiplexing as multiple targets can be detected in each fluorescence channel during the real-time PCR without any post-amplification melting curve analysis and more rapid cycling time than previous Seegene assays [2]. The aim of this study is to determine whether the new Seegene assay technology offers any advantages when compared to the existing FTD assay.

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Materials and methods

During 12–21 September 2015, in the height of the winter respiratory season, 199 consecutive respiratory samples were tested by both FTD and Seegene assays within three days of collection. These were 87 nasopharyngeal swabs, 54 nasal swabs, 6 throat swabs, 3 broncho alveolar lavage samples, 2 nasopharyngeal aspirates, 2 per nasal swabs, 1 tracheal swab, 1 sputum sample and 43 unnamed upper respiratory swab samples. All swab samples were collected in 2.5 mL viral transport media (VTM) (Copan Diagnostics, Brescia, Italy) and stored at 4°C until tested. Broncho alveolar lavage, sputum samples and nasopharyngeal aspirates were processed by homogenisation of the samples in a 1:1 ratio of Sputasol (Oxoid Ltd. Basingstoke, United Kingdom). Additional Sputasol was added to ensure homogeneity as required.

Nucleic acid extraction was performed using the Nuclisens Easymag platform (Biomerieux Australia PTY LTD, Sydney, Australia) and the generic 2.0.1 total nucleic acid protocol as recommended by the manufacturer. For the FTD assay, a total of 400 µL of sample was extracted and nucleic acid was eluted in 65 µL of elution buffer. For the Seegene assay, 200 µL of sample was extracted and nucleic acids were eluted in 100 µL of elution buffer as recommended by Seegene. A 'no template control' (molecular grade water) was extracted in each run to monitor for carry-over contamination. The FTD and Seegene assays both included synthetic ribonucleic acid (RNA) internal controls that were spiked into the Easymag lysis buffer to control for PCR inhibition (false negatives), and to check for efficient extraction of viral nucleic acid. Extracted nucleic acids were used directly in the respective assays and any residual nucleic acids were stored at –80°C in case any confirmatory testing was required.

Regarding nucleic acid amplification, the FTD assay was performed on a Roche 480 real-time PCR Instrument. PCR was performed according to the manufacturers' instructions, except that the reverse transcription step in the thermal cycling profile was extended from 15 to 20 min and the PCR ramp rate was reduced to 2.2°C/s for all steps. The thermal cycling profile change was validated in our laboratory to increase the sensitivity of the assay for the detection of Influenza A virus (Validation data available in request). The FTD assay had five, fourplex RT-PCR pools per sample which required 10 µL of extracted sample in each pool. The FTD assay results were analysed manually according to the manufacturers' instructions. The Seegene assay was performed on the dedicated Bio-Rad CFX96 real-time thermal cycler according to the manufacturers' instructions. The Seegene assay consisted of 3 pools each with six to eightplex reactions per sample. Each pool could be purchased separately. The results were analysed automatically using Seegene software (version 2.1.00.048_beta). Details of the two assays are shown in Table 1.

Where discrepant results occurred between the two assays for a particular virus, both the nucleic acid extracts from the FTD and the Seegene assays were tested in duplicate using an in-house singleplex PCR reference method for that virus. For rhinovirus discrepant testing, a rhinovirus specific assay and a picornavirus assay were performed to include as many rhinovirus genotypes as possible and an enterovirus assay was also performed to exclude any enteroviruses. This approach was used to detect as broad a range of rhinovirus types as possible as no single PCR assay can detect all rhinovirus genotypes [3].

Analysis was restricted to the 15 respiratory viral pathogen targets common to both assays. Per cent

Table 1. Details of the Seegene and Fast-track diagnostics multiplex PCR assays.

Manufacturer	Fast-track diagnostics respiratory 21	Seegene Allplex respiratory
Real-time thermal cyclers validated for the assay	ABI7500 LightCycler 480 Rotor-gene Q Cepheid SmartCycler	Biorad CFX-96
PCR	Real-time PCR	Real-time PCR
Detection	Dye labelled probes	Dye labelled probes
Nucleic acid volume	5 × 10 µL	3 × 8 µL
Post-PCR handling	No	No
Pool 1 targets	Pan-Influenza A, AH1N109pdm, Pan-Influenza B, Rhinovirus	Pan-Influenza A, Influenza AH1N109pdm, Influenza A H1, Influenza A H3, Pan-Influenza B, Respiratory syncytial viruses A and B (separate)
Pool 2 targets	Human coronaviruses, OC43, NL63, 229E, HKU1	Adenovirus, Enterovirus, Parainfluenza viruses 1,2,3,4, Human metapneumovirus
Pool 3 targets	Parainfluenza viruses 2,3,4 and Internal control	Human Bocavirus, Rhinovirus, Human coronaviruses, OC43, NL63, 229E
Pool 4 targets	Parainfluenza 1, Human metapneumovirus, Human Bocavirus, Mycoplasma pneumoniae	–
Pool 5 targets	Respiratory syncytial viruses A and B (combined), Enterovirus, Adenovirus, Parechovirus	–
Automated result analysis	No	Yes
Maximum samples per run	17	30
Turn-around time including nucleic acid extraction	3.0 h	3.5 h

Table 2. Organisms detected by each assay.

	Fast track diagnostics respiratory 21[a]	Seegene Allplex	% Agreement	Kappa agreement	Kappa 95% Confidence Interval
Pan-Influenza A	25	25[a]	100	1	1.000 to 1.000
Influenza B	18	17	98.4	0.901	0.790 to 1.000
Parainfluenza virus 1	0	0	100	1	1.000 to 1.000
Parainfluenza virus 2	2	2	100	1	1.000 to 1.000
Parainfluenza virus 3	13	13	100	1	1.000 to 1.000
Parainfluenza virus 4	1	1	100	1	1.000 to 1.000
Human bocavirus	4	10	94.4	0.265	−0.047 to 0.576
Human metapneumovirus	18	18	100	1	1.000 to 1.000
Respiratory syncytial virus	10	11	99.4	0.950	0.852 to 1.000
Enterovirus	1	1	100	1	1.000 to 1.000
Rhinovirus	34	34	97.5	0.912	0.837 to 0.988
Coronavirus OC43	8	8	99.0	0.870	0.691 to 1.000
Coronavirus NL63	2	2	100	1	1.000 to 1.000
Coronavirus 229E	5	4	99.5	0.886	0.666 to 1.000
Adenovirus	8	4	98.0	0.657	0.345 to 0.970

Note: Fast Track assay also includes Parechovirus and Mycoplasma pneumonia.
[a]One Seegene result was pan-influenza negative but Influenza H3 positive and this was included as a pan-influenza positive sample.

Table 3. Testing of samples with discrepant results by reference in-house molecular methods.

Virus (Method reference in brackets- see references section)	Fast Track/Seegene mPCR results	Number Samples with discordant results	Results of in-house PCR reference testing (Original crossing-point values of sample in brackets)	
			Positive	Negative
Influenza B [11]	Pos/Neg	2	2(32,35)	0
	Neg/Pos	1	1(40)	0
Respiratory syncytial virus [12]	Pos/Neg	0		
	Neg/Pos	1	0	1(42)
Rhinovirus [10,13,16]	Pos/Neg	3	2(33,36)	1(38)
	Neg/Pos	2	2(40,35)	0
Coronavirus OC43 [10]	Pos/Neg	1	0	1(35l)
	Neg/Pos	1	1(36)	0
Coronavirus 229E [10]	Pos/Neg	1	0	1(36)
	Neg/Pos	0		
Human bocavirus [14]	Pos/Neg	2	2(36,36)	0
	Neg/Pos	8	1(42)	7(40–42)
Adenovirus [15]	Pos/Neg	4	2(35,35)	2(37,37)
	Neg/Pos	0		

agreement and Cohen's Kappa coefficient were calculated for comparisons between the two assays.

Results

Overall, a respiratory pathogen was detected in 127 (63.8%) samples by the FTD assay and in 123 (61.8%) samples by the Seegene assay. Kappa agreement was between 0.87 and 1 for all targets except human bocavirus and adenovirus. Table 2 shows the comparative results by viral target. Details of discrepant analysis testing are shown in Table 3.

Discussion

There was overall good agreement between the assays, with ≥98% agreement for all targets, except human bocavirus and adenoviruses. However, it is important to note that there was a low prevalence of detection of some viruses, limiting our ability to assess these targets in detail. Good agreement between FTD and other PCR and multiplex PCR assays including the more widely used Xtag RVP fast assay has previously been seen by ourselves [1], and others [4–6].

The Seegene assay detected more bocavirus positive samples than the FTD assay, and almost all of these additional results were low-level positives that could not be confirmed with our in-house assay. Analytical sensitivities of the Seegene assay and our in-house assay for bocavirus were similar (data not supplied), so it is possible that the Seegene assay results were non-specific although there was no obvious pattern of cross-reactivity with other virus targets in the Seegene assay. It is possible the Seegene assay detected a different range of bocaviruses compared to the other assays. Nine of the twelve bocavirus-positive results were in samples for which other viruses were also detected, and in each case the bocavirus was in a lower concentration than the other viruses. The clinical significance of the positive bocavirus results in this study is difficult to determine, as the virus can be detected at low concentration in asymptomatic patients and there is some evidence that bocavirus is generally pathogenic only when present as a sole pathogen at higher viral loads [7].

Although there was generally good agreement between assays for rhinovirus, there were five discrepant results in the study, 3 detected by FTD only and 2 detected by Seegene only. Four of the results were confirmed with our in-house assays as either rhinoviruses or non-enteroviral picornaviruses. One very low positive FTD assay result could not be confirmed. It is probable that neither assay could detect the same range of rhinoviruses, this is to be expected as no single PCR assay can detect all rhinovirus genotypes [3]. There were four low positive discrepant adenovirus results that were positive only with the FTD assay, two of which were confirmed with the in-house assay. The presence of adenoviral DNA at low concentration is also of uncertain clinical significance in respiratory samples as adenovirus can be detected in asymptomatic patients [8].

Other characteristics of multiplex PCR assays are also important to consider. An important issue with molecular screening for respiratory viruses is changes in the primer and/or probe binding regions due to mutations or the emergence of new strains, this is a particular problem for influenza A virus which has a high mutation rate. Occasionally, influenza A subtypes H3N2 and H1N1pdm have exhibited mutations that can result in false negative results with current pan-influenza A protocols [9]. The simultaneous use of different gene targets in an assay can overcome this issue. The inclusion of influenza A H3 and H1pdm09 subtype gene targets, as well as pan-influenza A, in the Seegene assay gives simultaneous detection of two gene targets for each of the common circulating influenza A viruses, which is an improvement over the FTD assay which detects two gene targets for Influenza A H1N1pdm09 only. The nucleic acid extraction step for the FTD assay utilises a larger volume of sample (400 µL vs. 200 µL) and elutes into a smaller volume (65 µL vs. 100 µL). This more concentrated nucleic acid could potentially improve assay sensitivity. Out of the 25 discrepant samples utilised in this study, only one more positive result was seen with the more concentrated nucleic acid, indicating that it had little effect on assay sensitivity.

The hands-on time was similar for the assays assessed here, however, greater throughput is possible with the Seegene assay due to the greater multiplexing in each reaction pool. Only three pools are required with the Seegene assay compared to five pools in the FTD assay meaning that 30 samples could be analysed in one run compared to 17 samples with the FTD. Another useful feature with the Seegene assay was the automated result calling which reduced the hands-on time and simplified the result analysis. The advantages provided by the FTD assay are that the PCR amplification step is 30 min faster using the Roche Lightcycler 480 thermal cycler and also that the FTD assay has been validated for several different real-time thermal cyclers making it easier to integrate into an existing laboratory system. The FTD assay was considered to be cost effective when compared to our previous in-house multiplex PCR assay [10] which was complex and labour intensive to maintain. The In-house assay was the equivalent of 32USD per sample and the FTD assay was 44USD per sample including all reagents consumables and labour. The Seegene assay is a similar price to the FTD assay.

In conclusion, both assays offer the advantages of real-time multiplex PCR and have similar performance, The Seegene assay had the advantage of automated result calling, although this was only available when using the dedicated Bio-Rad real-time thermal cycler. The Seegene assay also had higher throughput and simultaneously detected two gene targets for each common circulating Influenza A viruses which is important due to their high mutation rate which can lead to detection failures. The FTD assay is validated for use on different real-time thermal cyclers. This work represents an advance in biomedical science because it provides clinical validation for a new real-time PCR technology that gives improved multiplexing allowing higher throughput of respiratory samples and automated analysis of results.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Summary table

What is known about this subject

- Multiplex PCR offers a unique combination of rapidity and high sensitivity when used for respiratory virus screening.
- In-house multiplex PCR methods are complex and difficult to maintain and are being replaced by commercial methods.
- Real time multiplex PCR methods are the most commonly used commercial methods but have low throughput due to multiplexing limitations.

What this paper adds

- The new Seegene assay had equivalent performance to current multiplex PCR methods

- The new Seegene assay had improved multiplexing ability compared to other real-time PCR methods with almost double the throughput compared to the FTD assay
- The Seegene assay offered simultaneous detection of two gene targets for both common influenza A subtypes which is important due to their high mutation rate.

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