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Comparison of four multiplex PCR assays for the detection of viral pathogens in respiratory specimens

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Multiplex PCR has become the test of choice for the detection of multiple respiratory viruses in clinical specimens. However, there are few direct comparisons of different PCR assays. This study compares 4 different multiplex PCR assays for the recovery of common respiratory viruses. We tested 213 respiratory specimens using four different multiplex PCR assays: the xTAG respiratory viral panel fast (Abbott Molecular Laboratories), Fast-track Respiratory Pathogen assay (Fast-track Diagnostics), Easyplex respiratory pathogen 12 kit (Ausdiagnostics), and an in-house multiplex real-time PCR assay. The performance of the four assays was very similar, with 93–100% agreement for all comparisons. Other issues, such as through-put, technical requirements and cost, are likely to be as important for making a decision about which of these assays to use given their comparative performance.

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1. Introduction

PCR methodologies for the detection of respiratory pathogens have become commercially available and give a high diagnostic yield, the ability to detect difficult or non-culturable pathogens and provide clinically relevant turn-around times (Bellau-Pujol et al., 2005; Gadsby et al., 2010; Gunson, Collins, and Carman, 2005; Kuypers et al., 2006; Murdoch et al., 2010). The increased sensitivity of PCR over classical methods is well established (Murdoch et al., 2010) while the detection of newly discovered respiratory pathogens, the human bocavirus, (Schenk et al., 2007) and rhinovirus type C (Dominguez et al., 2008), contributes to the improved diagnostic yield. The increasing acceptance of nucleic acid amplification techniques (NAATs) and the development of high through-put assay platforms have changed how the modern diagnostic laboratory operates. For the detection of respiratory viruses, the emphasis has now shifted from cell culture-based methods and viral antigen detection to the use of PCR as standard methodology. (Kuypers et al., 2006; Ganzenmueller et al., 2010; Mahony et al., 2007) The technique also allows quantitation of pathogens (Heim et al., 2003; Jansen et al., 2011; Ward et al., 2004) and PCR amplified products can be used for genetic sequencing (Monto et al., 2006).

Deciding which PCR assay system to select for use in a routine diagnostic service is complex and requires systematic evaluation

of the laboratory's needs and resources. The selection of an assay is dependent on variables such as the range of pathogens detected, expected work-load and workflow, financial resources, clinical requirements, staff expertise and the laboratory's ability to perform assay validations. A variety of commercial and in-house PCR assays are now available that use multiplexing technology which has simplified workflows and significantly reduced processing times (Gunson et al., 2008). Multiplex PCR, as described in this publication, refers to the simultaneous amplification of multiple sequences in a single reaction. Unfortunately, the relative performance of such assays is rarely compared in head-to-head studies.

This study directly compares the performance of three commercial multiplex PCR assays, the xTAG® respiratory viral panel fast (RVP) (Abbott Molecular Laboratories Chicago, USA), Fast-track Diagnostics (FTD) respiratory pathogen (Fast-track, Junglinster Luxembourg), Easyplex® (EP) respiratory pathogen 12 kit (Ausdiagnostics, Sydney, Australia), and an established in-house (IH) multiplex PCR assay.

2. Materials and Methods

2.1. Clinical samples

Respiratory samples sent to Canterbury Health Laboratories (Christchurch, New Zealand) for respiratory pathogen analysis during 2009–2010 were archived at -80°C . Samples ($n=213$) were randomly selected for study without knowledge of prior test results. They included the following specimen types: throat swab

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Table 1
Summary of multiplex PCR specifications.

	xTAG RVP	FTD Respiratory	IH triplex PCR	EP Respiratory
Manufacturer	Abbott molecular	Fast-track diagnostics	Generic	AusDiagnostics
PCR	Conventional PCR	Real-time PCR	Real-time PCR	Multiplex tandem PCR
Detection	Multiplex liquid bead array	Multiplex dye labelled probes	Multiplex dye labelled probes	Singleplex DNA dye
Nucleic acid volume	10 μ L	60 μ L	25 μ L	10 μ L
Post PCR handling	Yes	No	No	No
Automated result calling	Yes	No	No	Yes
Samples per run	96	12	12	6
Turn-around time	4.0 h	2.5 h	2.5 h	2 h
Labour requirements	7.5 min	5.8 min	6.6 min	3.6 min
FDA or CE marked	FDA	CE	None	None

Note: Turn-around time excludes the nucleic acid extraction time

($n=9$), nasal swab ($n=49$), nasopharyngeal/pernasal swab (89), combination throat and nasopharyngeal/pernasal swab ($n=4$), bronchoalveolar lavage ($n=16$), sputum ($n=17$), tracheal aspirate ($n=7$), and upper respiratory swab (site not stated) ($n=22$).

The nasopharyngeal/pernasal flopped swabs (Copan Diagnostics, Brescia, Italy), and throat swabs (Medical Wire, Wiltshire, United Kingdom) were collected in 2.5 mL viral transport media (VTM) (Copan Diagnostics, Brescia, Italy), and stored at 4 °C for 7 days until routine testing was complete. Swabs from non-specified sites that were received in VTM were assumed to be nasopharyngeal/pernasal swabs. Bronchoalveolar lavage, tracheal aspirates and sputum specimens were received in sterile containers and processed by homogenisation of the sample using a 1:1 ratio of sputasol (Oxoid, Cambridge, United Kingdom); additional sputasol was added to ensure sample homogeneity where necessary (up to a 1:2 ratio of sputasol).

The study was approved by the Upper South B Ethics Committee, Christchurch, New Zealand.

2.2. Nucleic acid extraction

Samples were processed for nucleic acid extraction using the M2000sp platform (Abbott Molecular Chicago, USA) and total nucleic acid protocol (m2000-RNADNA-LL-500-70-v62509) as recommended by the manufacturer. Nucleic acids were extracted from 500 μ L of sample and eluted into 70 μ L of elution buffer; this was performed a total of three times to obtain sufficient volumes of nucleic acids for all four PCR assays. The nucleic acid extracts from each sample were pooled and aliquoted into four 96 deep-well plates (Abbott Molecular, Chicago, USA) one for each PCR assay to prevent freeze-thaw damage. Plates were stored at –80 °C until testing.

A “No template control” (molecular grade water) was extracted in each run to monitor for carry-over contamination. An internal control (IC) for the validation of negative PCR results was performed as recommended by each manufacturer: MS2 bacteriophage for xTAG RVP fast and bromomosaic virus (BMV) for the Fast-track Diagnostics kit. The Easyplex used an artificial sequence and the in-house assay (Canterbury Health Laboratories) used an artificial sequence inserted into a plasmid (unpublished data) that is unique and not found in human clinical samples.

2.3. Multiplex in-house PCR assay

The in-house multiplex PCR assay was based on an assay described by Gunson et al. (2005). Notable differences were the updating of the influenza A PCR, (Ward et al., 2004) and the inclusion of a specific probes and primers for the detection of the influenza A(H1N1)pdm 2009 strain (Whiley et al., 2009), human metapneumovirus (Maertzdorf et al., 2004), and adenovirus (Heim et al., 2003). The different targets were multiplexed into 5 pools

of triplex PCR using FAM, VIC, or Cy5 dyes. The 25 μ L PCR reaction mixture contained each primer and probe (concentration for each reaction is available upon request), AgPath-ID™ One-Step 2x RT-PCR buffer and 25x RT-PCR enzyme (Applied Biosystems, Life Technologies Melbourne, Australia), and 5 μ L of nucleic acid extract amplification was performed on an ABI 7500 real-time PCR thermal cycler (Applied Biosystems, Melbourne, Australia). The thermal cycling parameters were: reverse transcriptase (RT) step of 50 °C for 20 min, 95 °C for 10 min followed by 40 PCR cycles of 95 °C for 15 s, and 60 °C for 30 s.

2.4. Commercial multiplex PCR assays

The commercial multiplex PCR assays were performed according to the manufacturers' instructions; multiplex PCR specifications are summarised in Table 1. The FTD respiratory pathogens had 6 triplex RT-PCR pools per sample which required 10 μ L of extracted nucleic acids in each pool. The FTD assay PCR was performed on the ABI 7500 (Applied Biosystems Melbourne, Australia) using the thermal cycling profile recommended by the manufacturer. The EP assay required 5 μ L extracted sample for the respiratory 12 panel and 5 μ L extracted sample for the respiratory pneumonia panel. The EP PCR was performed on the Gene-plex system which includes a liquid pipetting platform and a Rotor-gene 6000 (Ausdiagnostics, Sydney, Australia). The xTAG RVP required 10 μ L extracted sample and the PCR was performed on an GeneAmp 9700 (Applied Biosystems, Melbourne, Australia) thermal cycler using the profile recommended by the manufacturer.

2.5. Data Analysis

Sensitivity and specificity were determined by 2 \times 2 tables (McNemar's test) and percentage agreement and kappa statistics were calculated for comparisons between assays.

3. Results

Two hundred and thirteen respiratory samples were tested by the 4 different assays with only 10 respiratory viral pathogen targets common to all assays; targets that were unique to a single assay were not analysed further. Table 2 summarises the pathogens detected by each assay. A respiratory pathogen was found in 134/213 (62.9%) of samples for FTD assay, 142/213 (66.7%) for the xTAG RVP, 130/213 (61.0%) for IH triplex, and 135/213 (63.4%) for the EP assay.

Although samples were not sequential, the pattern of viral pathogens detected still represented the local epidemiology with influenza A, RSV and picornavirus being the most common (Table 2). The predominant influenza A virus strain detected was A(H1N1)pdm 2009. Only 4 isolates were identified as the A(H1N1) seasonal strain circulating up to mid-2009, and one was A(H3N2).

Table 2
Organisms detected by each assay used in this study.

Sample size 213	RVP	FTD	IH	EP	Consensus
Pan-Influenza A	26	24	25	25	25 (24)
Influenza B	0	0	0	0	0
Parainfluenza virus 1	1	1	1	0	1
Parainfluenza virus 2	2	2	3	4	3 (2)
Parainfluenza virus 3	10	11	13	11	11 (10)
Parainfluenza virus 4	0	0	NT	NT	0
Parainfluenza grouped	13	14	17	15	15 (13)
Human bocavirus	4	12	NT	0	4
Human metapneumovirus	3	4	2	0	3
Respiratory syncytial virus	40	35	36	38	37 (35)
Enterovirus/Parechovirus	NT	4	NT	NT	
Rhinovirus	NT	25	32 ^b	NT	
Picornavirus	44	29 ^a	NT	35	33 (25)
Coronavirus OC43	6	5	7	11	6 (4)
Coronavirus NL63	2	2	2	NT	
Coronavirus 229E	3	3	5	4	3 (3)
Coronavirus HKU1	0	NT	0	NT	
Coronavirus grouped	9	10	14	15	8 (8)
Adenovirus	3	6	4	7	4 (2)
Total detected	142	134	130	135	

NT = not tested as no target available.

Consensus is positive result by more than one assay and () values indicate positive by all 4 assays

^a Enterovirus/Parechovirus and Rhinovirus PCR combined.

^b Rhinovirus only PCR.

The majority of picornaviruses detected were rhinoviruses; the FTD assay was the only assay to differentiate rhinovirus within the picornavirus family.

Analysis of the internal control results, for each assay revealed no PCR inhibition in the FTD, IH PCR or EP assays. However inhibition was detected in 11 samples by MS2 PCR in the xTAG RVP assay. Of these 11 inhibited samples, 5 were sputum and 4 had a pathogen detected which indicates issues with the sample matrix and the MS2 PCR.

Statistical analysis using analytical (percentage) agreement between the 4 different assays showed high agreement rates with a range from 93.0 to 100% agreement. The Kappa values ranged from 0 for bocavirus (FTD vs Easyplex) to 1.00 for influenza A virus (IH vs EP), see Table 3 for results.

All influenza viruses detected were influenza A. Results for parainfluenza viruses 1–3 were pooled (no parainfluenza type 4 was detected), as were the results for the 3 strains of coronavirus detected.

4. Discussion

A high degree of correlation was found between the FTD, xTAG RVP, EP and in-house assays. The agreement between these assays for influenza A, RSV, the parainfluenza viruses, picornaviruses, adenoviruses, HMPV, coronaviruses and human bocavirus ranged between 93.0% and 100%. The highest analytical agreement was for the detection of influenza A and RSV (between 97.7% and 100%). While good agreement was also seen for the detection of the parainfluenza viruses and coronavirus, limited numbers of samples containing these viruses were tested and therefore further evaluation would be useful to confirm these observations.

Variation was seen in the analytical agreement for pathogen detection between individual assays. The EP assay correlated poorly with the other assays for the detection of adenovirus, with Kappa values of 0.39 for xTAG RVP, 0.45 for FTD, and 0.53 for the in-house assay. Seven samples were positive with the EP assay only and it may be inferred that the EP assay was producing false positive adenovirus results in this sample series. A possible explanation is that the EP assay uses a non-specific DNA binding dye with melt curve analysis and therefore poor primer design would produce

Table 3
Comparison of platform performance per target.

Target	Platform	FTD	IH	EP
FA	xTAG	99.0% (0.96)	99.5% (0.98)	99.5% (0.98)
	FTD		99.5% (0.98)	99.5% (0.98)
	IH			100% (1.00)
RSV	xTAG	97.7% (0.92)	99.5% (0.98)	98.6% (0.95)
	FTD		99.5% (0.98)	98.6% (0.95)
	IH			99.1% (0.97)
PIV	xTAG	99.5% (0.96)	98.6% (0.90)	98.1% (0.85)
	FTD		98.6% (0.896)	99.1% (0.95)
	IH			98.1% (0.90)
PV	xTAG	93.0% (0.75)	94.8% (0.86)	93.9% (0.80)
	FTD		94.8% (0.79)	95.3% (0.82)
	IH			97.7% (0.91)
ADV	xTAG	98.6% (0.66)	99.1% (0.80)	97.2% (0.39)
	FTD		99.1% (0.80)	96.7% (0.45)
	IH			97.7% (0.53)
HMPV	xTAG	99.5% (0.86)	99.1% (0.66)	98.6% (0.00)
	FTD		99.1% (0.80)	99.1% (0.66)
	IH			99.1% (0.00)
CV	xTAG	99.5% (0.95)	100% (1.00)	95.3% (0.59)
	FTD		100% (1.00)	95.8% (0.62)
	IH			93.9% (0.52)
BV	xTAG	96.2% (0.95)	NT	92.1% (0.00)
	FTD		NT	94.4% (0.00)
	IH			NT

% = agreement.

() = Kappa value.

FA = influenza A virus.

RSV = respiratory syncytial virus.

PIV = parainfluenza 1–4 virus.

PV = picornavirus.

ADV = adenovirus.

HMPV = human metapneumoniae virus.

CV = coronaviruses.

BV = bocavirus.

NT = no test available.

non-specific PCR products. Similarly, xTAG RVP had only moderate agreement for picornavirus detection with the Kappa values of 0.75 for FTD, 0.8 for EP and 0.86 for the in-house assay, although the in-house assay was restricted to rhinovirus detection only. This difference could be due to the increased sensitivity of the xTAG PCR (which detected picornavirus in 44 samples) or that certain strains were not detected by the primers used in the other assays. More than 150 different rhinoviruses exist and it would be difficult to design primers that can detect all strains with the same PCR efficiencies; Faux et al., has recently shown that optimal rhinovirus detection requires two primer sets (Faux et al., 2011).

The least agreement between assays was seen for human bocavirus with a Kappa value 0.486 for the FTD assay versus xTAG RVP. The Kappa value was zero for the EP versus the xTAG RVP and FTD assays, which did not detect bocavirus in this series of samples. These low values could be due to specificity and sensitivity issues and further investigation is required to establish whether the FTD and xTAG RVP assay produces false positive results or the EP assays produce false negative results due to strain variation.

In our opinion, any of the assays included in this comparative study could be used as a laboratory diagnostic tool for the detection of the most common respiratory pathogens. A limitation of this study was that it was carried out over a period when the (H1N1)pdm09 virus was dominant. Thus we could not comment on the ability of these assays to detect some pathogens e.g. influenza B, HMPV, as they were not present or only detected in small numbers, and therefore further studies are required. We noted that differences do occur in assay detection rates that may reflect the

expected assay variability caused by selection of gene target, analytical sensitivity, differences in equipment and reagents such as enzymes.

In addition to performance data, deciding which assay system to use also requires, information on sample through-put capability, hands-on time, technical skills required, and the range of targets detected. In our comparison, the xTAG RVP assay required 4 h for amplification and analysis however it could process 96 samples including controls in a single run, offering high volume through-put during an 8 h working day (Gadsby et al., 2010). The xTAG RVP is an open system assay that requires the operator to handle PCR product during the product detection step. The FTD and IH PCR each require 2.5 h for amplification and result analysis. They are real-time PCR assays that can be run on three widely used systems. The FTD and IH PCR assay require significant hands-on time to set-up the PCR plates but these steps could be semi-automated using an automated pipetting platform, leading to a reduced labour requirement. The FTD and in-house PCR assays have a moderate sample through-put; each can process 12 samples per run, with up to 4 runs in an eight hour day. Extra capacity could be obtained by using 384 rather than 96 well plates. An advantage of these real-time PCR formats is that they are closed systems and do not require operator manipulation of PCR products as the amplification, product detection, and quantitation steps are all combined in a single reaction. The EP assay is based on the nested PCR principle in which the first round is the RT-PCR which is followed by second round of PCR. This PCR is called multiplex tandem PCR (MT-PCR) (Stanley and Szewczuk, 2005). Six samples can be processed in 2 h, representing a low through-put capacity, although this can be increased by the addition of an extra real time PCR thermal cycler. An advantage of the xTAG RVP and EP systems was that results were automatically generated whereas the FTD and IH assay outputs required complex manual analysis to generate results.

The study samples were collected from April 2009, when the influenza A(H1N1)pdm 2009 virus was first detected in New Zealand and all 4 assays were able to detect influenza A. The xTAG RVP although detecting the influenza A on the matrix gene target was the only assay not to have updated primers for the typing of the new A(H1N1)pdm 2009 variant and an influenza A untypeable result indicated this strain (Ginocchio and St George, 2009). This highlights a major limitation of the commercial assays; updating primer and probe sequences once an assay has been marketed is problematic. In contrast, the in-house assay is flexible in design and can be rapidly modified. Its disadvantage is that standardisation is difficult because there are many variables in the PCR work-flow, all of which can impact on assay performance.

A simple cost analysis was performed to compare the 4 assays based on consumables and labour. The most economical assay, per sample, was the in-house triplex PCR (if set-up and development costs were excluded), then the Easyplex respiratory 12 panel, the FTD respiratory pathogen kit, and finally the xTAG RVP. The cost of these PCR assays maybe higher compared to the classical respiratory pathogen detection procedures, however the ability of these multiplex assays to provide additional pathogen information in a rapid time frame should lead to savings in patient management.

5. Conclusion

The four multiplex PCR assays assessed in this study showed a high degree of correlation for the detection of the common respiratory viruses. The decision on the best assay to use is most likely to be based on other issues, such as sample through-put, staffing levels and staff expertise, clinicians' expectations and overall funding structures.

Conflict of interest

The authors declare no conflicts of interest.

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