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Formulation of a multiplex-reverse-transcription-polymerase-chain-reaction–based screening protocol to facilitate rapid clinical diagnosis of respiratory tract infections

Key Messages

1. Use of multiplex polymerase chain reaction (PCR) assays can improve the diagnostic yield in terms of overall sensitivity and spectrum of detection for respiratory tract infections.
2. The broad coverage and rapid turn-around achieved by multiplex PCR allows prompt measures in response to serious respiratory tract infections.
3. Implementation of multiplex PCR testing to routine diagnostics is recommended.

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Introduction

Respiratory tract infection accounts for a major proportion of illnesses seen in hospital settings. The outbreak of severe acute respiratory syndrome (SARS) and experience with avian influenza underscore the importance of prompt diagnosis for prompt initiation of specific measures, including isolation, contact tracing, and other public health measures. A wide range of viruses and bacteria can cause respiratory infections with presentations almost indistinguishable from SARS and avian influenza, especially in the initial stage when the patient first presents to hospital. Confirming the diagnosis of 'less severe' infections is equally important from the perspective of excluding serious conditions. Given the albeit small chance of co-infection, an ideal diagnostic approach should have the capacity of identifying multiple pathogens at the same time.

The ultimate goal of this study was to improve the performance and efficiency of laboratory diagnosis for acute respiratory tract infections. We therefore set out to devise, optimise, and evaluate a rapid diagnostic scheme for the simultaneous detection of common causative agents responsible for human respiratory tract infections.

Methods

This study was conducted from February 2005 to April 2006. Multiplex polymerase chain reaction (PCR) assays were designed to detect influenza A H1N1, H3N2 and H5N1; influenza B; parainfluenza 1, 2, 3, and 4; respiratory syncytial virus A and B; rhinovirus, enteroviruses, human coronavirus OC43, 229E and SARS-CoV; human metapneumovirus; *Mycoplasma pneumoniae*; *Chlamydia pneumoniae; Legionella;* and adenovirus.

Primer design

Multiple consensus regions of each organism were determined. Sequences of 10-20 representative strains of each pathogen were downloaded and aligned using Clustal X (http://bips.u-strasbg.fr/en/Documentation/ClustalX/) to verify sequence variability, and to select potential regions for primer design. Primer sets producing amplicon sizes that could be differentiated by agarose gel electrophoresis were identified for further evaluation and optimisation.

Multiplex polymerase chain reaction assay design and optimisation

The optimisation was first conducted using cell culture grown preparations, or when not available, clinical specimens known to contain the target agents as templates. For enteroviruses, the commonly encountered serotypes including Coxsackie A9, B1, B2, B3 and B5, Echo 7, 11, 30, EV71, and Polio 1 were included in all the evaluation processes. Different combinations of primer concentrations (range, 0.01-1.0 μM) for each were evaluated. During the optimisation experiments, a cocktail of 4 to 5 pairs of primers were used throughout, so as to better assess primer-primer interactions, which are often

the most critical technical issues to resolve. Templates containing single as well as mixtures of target organisms were used to assess detection of single as well as multiple infections. The potential for cross-amplification on human DNA/RNA carried in clinical samples was assessed by testing the primer cocktails against 50 negative clinical samples. This approach was also used to identify primer pairs for monoplex confirmatory PCR assays.

Thermal cycler selection

To provide the shortest possible turn-around time, a 'fast' thermal cycler (Applied Biosystems Fast PCR machine, US) was used. When coupled with the DNA polymerase contained in the Fast PCR Master Mix (Applied Biosystems GeneAmp, US), a 35-cycle PCR assay can be completed within 35 minutes, compared to approximately 180 minutes for ordinary cyclers. All multiplex PCR assays were optimised to fulfil the manufacturer's recommendation that a two-step cycling with annealing at 64°C be used. As for the confirmatory PCR assays, a three-step cycling on an ordinary thermal cycler using the hot start T*aq* polymerase (HotStarT*aq*, Qiagen, Germany) was used; as these conditions allow a more flexible choice of primers.

Field evaluation with clinical specimens

To evaluate the performance of the multiplex PCR assays when applied to routinely collected clinical specimens, 303 nasopharyngeal aspirate samples collected from patients who were admitted to the Prince of Wales Hospital for suspected respiratory tract infections were subjected to the multiplex PCR and routine isolation.

Nucleic acid extraction

All 50 nasopharyngeal aspirates selected for extraction evaluation were confirmed positive for respiratory viruses by routine isolation. The results of end-point dilution testing indicated that the QIAamp MinElute Virus Spin Kit (Qiagen, Germany) that extracts both viral RNA and DNA in combination was most effective. This kit was used to extract RNA/DNA throughout the study.

Reverse transcription

The Superscript III Reverse Transcriptase (Invitrogen, US) was chosen based on previous experience, and further optimised for this study. Briefly, the reaction was carried out in a 20-μL reaction mix containing 10 units of reverse transcriptase, 4 units of RNase OUT, 0.5 mM dNTP, 0.5 mM DTT, 2.5 ng random primers, and 8 μL of the extracted preparation derived from the clinical specimen. The reaction mix was subjected to thermal conditions of 65°C for 5 minutes, 4°C for 1 minute, 25°C for 5 minutes, 50°C for 50 minutes, and finally 37°C for 20 minutes.

Multiplex polymerase chain reaction

The optimal primer combinations were as follows. Group 1 comprised influenza A and B group–specific and subtype H1N1, H3N2, H5N1-specific primers; group 2 comprised parainfluenza 1, 2, 3 and 4; group 3 comprised respiratory syncytial virus A and B, rhinovirus, and enterovirus; group 4 comprised human coronavirus OC43, 229E and SARS-CoV, and human metapneumovirus; and group 5 comprised *M pneumoniae*, *C pneumoniae, Legionella* and adenovirus.

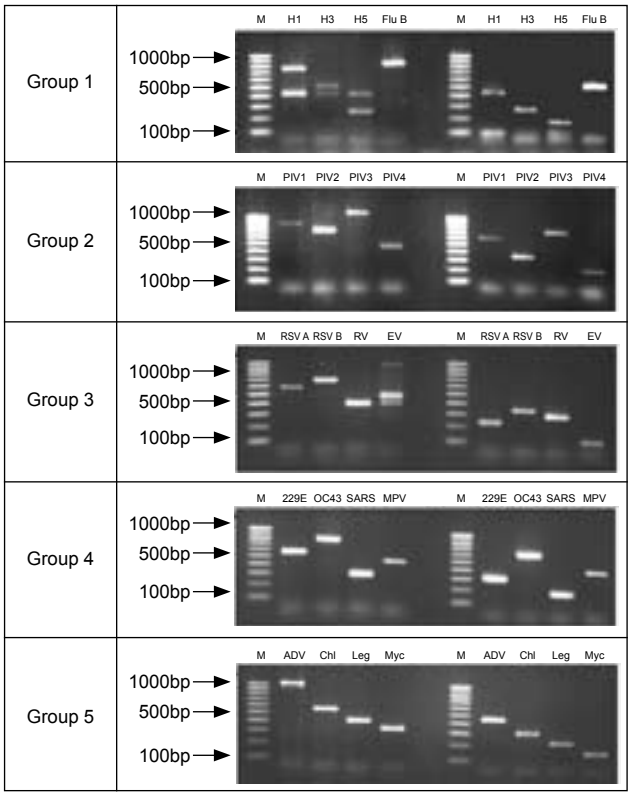

Fig. Agarose gel electrophoresis of multiplex polymerase chain reaction products

M denotes marker, Flu influenza, PIV parainfluenza, RSV respiratory syncytial virus, RV rhinovirus, EV Enterovirus, MPV metapneumovirus, ADV adenovirus, Chl *Chlamydia pneumoniae*, Leg *Legionella*, and Myc *Mycoplasma pneumoniae*

All multiplex PCR assays were conducted using the GeneAmp Fast PCR Master Mix (Applied Biosystems, US) in a 20-μL reaction. Two microlitres of the cDNA preparation were used as templates for the first round of PCR for groups 1 to 4, whereas 8 μL of the extracted preparation was used for group 5. An 0.2-μL aliquot of first-round PCR product was used as template for the second round.

The thermal cycling was carried with the Applied Biosystems Fast PCR machine (Applied Biosystems, US) with an initial denaturation at 95°C for 10 seconds, then 30 cycles of denaturation at 95°C for 1 second and annealing/extension at 64°C for 40 seconds, followed by a final extension at 72°C for 1 minute. The cycling conditions were the same for groups 1 to 4, whereas 35 cycles of denaturation at 95°C for 5 seconds and annealing/extension at 64°C for 40 seconds were used for group 5.

Organism identification

Results of PCR were analysed by electrophoresis using 1.5% agarose with ethidium bromide staining. The identity of amplification products was determined by their sizes (Fig).

Table. Multiplex polymerase chain reaction (PCR) assays compared with conventional isolation

Organism	No. (%) of positive specimen (n=303)		P value*
	Nested multiplex PCR	Conventional isolation	
Any infection	147 (48.5)	61 (20.1)	<0.001
Single infection	140 (46.2)	61 (20.1)	<0.001
Influenza A	19 (6.3)	15 (5.0)	0.480
Influenza A H1	17 (5.6)	NA†	
Influenza A H3	2 (0.7)	NA	
Influenza A H5	0	NA	
Influenza B	10 (3.3)	9 (3.0)	0.820
Parainfluenza virus type 1	19 (6.3)	14 (4.6)	0.371
Parainfluenza virus type 2	6 (2.0)	1 (0.3)	0.123
Parainfluenza virus type 3	3 (1.0)	2 (0.7)	1.0
Parainfluenza virus type 4	2 (0.7)	0	0.499
Respiratory syncytial virus	8 (2.7)	5 (1.7)	0.400
Respiratory syncytial virus group A	5 (1.7)	NA	
Respiratory syncytial virus group B	3 (1.0)	NA	
Rhinovirus	16 (5.3)	NA	
Enterovirus	3 (1.0)	0	0.249
Human coronavirus OC43	16 (5.3)	NA	
Human coronavirus 229E	3 (1.0)	NA	
SARS-CoV	0	0	1.0
Human metapneumovirus	15 (5.0)	NA	
Mycoplasma pneumoniae	5 (1.7)	NA	
Legionella	0	NA	
Chlamydia pneumoniae	0	NA	
Adenovirus	15 (5.0)	15 (5.0)	1.0
Coinfection	7 (2.3)	0	0.015
Influenza A and *Mycoplasma pneumoniae*	1 (0.3)	NA	
Influenza A H1 and *Chlamydia pneumoniae*	1 (0.3)	NA	
Influenza A H3 and human coronavirus 229E	1 (0.3)	NA	
Influenza A H3 and parainfluenza virus type 2	1 (0.3)	T2‡	
Human metapneumovirus and *Mycoplasma pneumoniae*	2 (0.7)	NA	
Human metapneumovirus and parainfluenza virus type 4	1 (0.3)	NA	

* By Chi squared test or Fisher's exact test as appropriate

† NA denotes organisms not isolated/differentiated by conventional isolation

‡ Parainfluenza virus type 2 isolated

Results

Sensitivity and specificity

The nested multiplex PCR assays were found to be 100- to 1000-fold more sensitive than conventional tube culture. The detection limit of group 5 multiplex PCR for *Legionella* and *M pneumoniae* were $2.3x10^3$ and $6.7x10^4$ colony forming units/mL, respectively. The evaluation using 50 clinical specimens containing known organisms did not reveal any cross-amplification.

Field specimen evaluation

Study subjects and specimens

A total of 303 nasopharyngeal aspirate specimens were collected, with 235 from paediatric patients aged 1 month to 5 years (mean, 2 years). The other 68 were from elderly patients aged from 65 to 107 years old (mean, 65 years).

Performance of multiplex polymerase chain reaction versus isolation

Of the 303 specimens, 61 (20%) were positive by conventional virus isolation with 15 influenza A, 15 adenovirus, 14 parainfluenza virus type 1, 9 influenza B, 5 respiratory syncytial virus, 2 parainfluenza virus type 3, and 1 parainfluenza virus type 2. No coinfection was found by conventional virus isolation. All these 61 isolation-positive specimens were also found to be positive by multiplex PCR, and with the corresponding detected viruses (Table). Altogether, 147 specimens were positive by the nested multiplex PCR. The positivity rate was significantly higher than that of conventional isolation (48.5% vs 20.1%, P<0.001; Table). Of the 140 single infections detected by multiplex PCR, 55 were not detected by conventional isolation. Of the seven (2.3%) coinfections revealed by multiplex PCR, six were negative by conventional virus isolation. As for the cultivable organisms, no statistically significant difference in positivity rates between multiplex PCR and conventional isolation was observed (Table). Although a clear agarose gel electrophoresis result was obtained for all positive specimens, for the purpose of this study, all multiplex PCR positive results were confirmed by separate PCR testing using alternative primer pairs.

Discussion

While molecular techniques provide superior analytical sensitivity to conventional isolation, this gain may not be reflected in clinical sensitivity. In settings where clinical specimens are collected and maintained in good quality, the amount of virus present may well be enough for detection by 'less sensitive' conventional methods. Our data are in line with this. Despite there being a higher sensitivity for multiplex PCR, the difference was not statistically significant when the cultivable viruses were compared.

Our finding that for multiplex PCR the overall positivity rate was double that of conventional isolation, was due to the broad spectrum of detection offered by the former. Previous studies targeting as many as nine different respiratory pathogens have been reported.[1] The current study included 17 respiratory pathogens and provided the widest spectrum ever reported. The gain in positivity rate was mainly attributable to the inclusion of rhinovirus, human coronavirus OC43, and human metapneumovirus. All these viruses are not detected by conventional isolation. The improvement in diagnostic yield by adding rhinovirus has also been reported by Gruteke et al.[2] Given that these 'trivial' respiratory viruses can cause severe illnesses, they should be included in multiplex assays. With the rapid PCR system established, the entire testing process can be completed on the same day. Such rapid turn-around is critical in the investigation of urgent outbreaks, and according to some studies, also has the potential to decrease overall hospital costs.[3,4] In our group 1 multiplex PCR, we incorporated specific primers for influenza A subtypes H1, H3, and H5, so that rapid differentiation between H5 and non-H5 influenza can be achieved. The assay also included a consensus of primers for influenza A, which could allow the detection of non-H1/H3/H5 subtypes that occasionally cause human infections.

In conclusion, the multiplex PCR assays developed in this study improved the diagnostic yield in terms of sensitivity and spectrum of coverage for respiratory infections. The assay has a rapid turn-around time, with results becoming available in one day. Overall cost reduction may justify routine use of these broad-cover, rapid, molecular diagnostic assays.

Acknowledgements

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