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Multiplex real-time PCR for detection of respiratory tract infections

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Abstract

Background: Broad diagnostics of respiratory infection by molecular assays has not yet won acceptance due to technical difficulties and high costs.
Objectives: To evaluate clinical applicability of multiplex real-time PCR.
Study design: An assay targeting influenza virus A (IfA) and B (IfB), parainfluenza 1-3 (PIV), human metapneumovirus (MPV), respiratory syncytial virus (RSV), rhinovirus (RV), enterovirus (EV), adenovirus (AdV), human coronaviruses (229E, OC43, NL63), M. pneumoniae and Ch. pneumoniae was developed and run daily on consecutive clinical nasopharyngeal swab samples.
Results: An etiology was identified in 48% of the 954 samples, with IfA in 25%, RV in 20%, MPV in 10% and M. pneumoniae in 10% of the positive. By a rational procedure costs could be reduced and the customer price set relatively low (€33 per sample).
Conclusion: Streamlined testing and cost limitation is achievable and probably critical for implementation of a broad molecular diagnostics of respiratory infections.

Keywords: Molecular diagnostics; Respiratory infection; Real-time PCR; Taqman

1. Introduction

Community acquired respiratory tract infections are frequent and constitute an important cause of morbidity in Sweden and other European countries. A significant part of these infections are caused by viral pathogens (Creer et al., 2006; Diaz et al., 2007). It is often difficult for the clinician to distinguish between viral and bacterial aetiologies, and this may result in overuse of antibiotics (Lundborg et al., 2002). Diagnosis of viral respiratory tract infections using viral culture, antigen detection or serology is either too slow or too insensitive to be applicable in clinical practice (Gunson et al., 2005). The more reliable, specific and sensitive PCR methods have not yet won acceptance as the first choice for diagnosis owing to the high cost when several agents are targeted. Recently, multiplex assays that detect a large number of viral agents have been described. Some of these are based on traditional PCR (Bellau-Pujol et al., 2005; Gruteke et al., 2004; Liolios et al., 2001; Puppe et al., 2004), others on real-time PCR (Gunson et al., 2005; Kuypers et al., 2006; Templeton et al., 2004; Watzinger et al., 2004) or PCR combined with Luminex liquid chip hybridization and identification (Li et al., 2007).

2. Methods

We developed a real-time PCR procedure, based on automated specimen extraction and multiplex amplification, at a relatively low cost (€33). Primers and hydrolysis probes were obtained from the literature or developed in our laboratory. The following agents were analysed: influenza virus A (IfA) and B (IfB), parainfluenza 1-3 (PIV), human metapneumovirus (MPV), respiratory syncytial virus (RSV), rhinovirus (RV), enterovirus (EV), adenovirus (AdV), human coronaviruses 229E, OC43 and NL63. In addition, M. pneumoniae and Ch. pneumoniae were included in the panel.

Nucleic acid from 100 μL of specimen was extracted into an elution volume of 100 μL by a Magnapure LC robot

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(Roche Molecular Systems, Mannheim, Germany), using the Total Nucleic Acid protocol, and was amplified in an ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA). Amplification was then carried out in 50 μL reaction volumes, including 10 μL of sample preparation and 25 μL of one-step RT-PCR master mix from Applied Biosystems (this was exchanged to a master mix from Invitrogen (Carlsbad, CA) in the later part of the study period to improve sensitivity). After a reverse transcription step at 46 °C for 30 min followed by 10 min of denaturation at 95 °C, 45 cycles of two-step PCR was performed (15 s at 95 °C, 60 s at 58 °C). Each sample was amplified in 5 parallel reactions, each containing primers and probes specific for 3 targets (mixtures A–E), as described in Table 1. Multiplexing tended to hamper the performance of the amplification significantly for many combinations, and therefore the reagent mixtures were carefully evaluated: combinations were only accepted if the Ct value in multiplex was no more than 2 cycles higher than when detection was carried out in separate reactions. The performance was evaluated using pUC57 plasmids with inserts

Table 1
Primers and probes used in multiplex real-time PCR run in five reaction mixtures, A-E

Mix	Primer/Probe	Sequence	Origin	Reporter
A	PIV-1_F	GTTGTCAATGTCTTAATTCGTATCAATAATT	Watzinger et al. (2004)	
A	PIV-1_R	GTAGCCTMCCTTCGGCACCTAA	Watzinger et al. (2004)	
A	PI V-1_ Probe	TAGGCCAAAGATTGTTGTCGAGACTATTCCAA	Watzinger et al. (2004)	FAM-TAMRA
A	PIV-2_F	GCATTTCCAATCTTCAGGACTATGA	Watzinger et al. (2004)	
A	PIV-2_R	ACCTCCTGGTATAGCAGTGACTGAAC	Watzinger et al. (2004)	
A	PIV-2_Probe	CCATTTACCTAAGTGATGGAATCAATCGCAAA	Watzinger et al. (2004)	FAM-TAMRA
A	PIV-3_F	To be published elsewhere	[a]	
A	PIV-3_R	To be published elsewhere	[a]	
A	PIV-3_Probe	To be published elsewhere	[a]	FAM-BHQ
B	IfB_F	AAATACGGTGGATTAAATAAAAGCAA	[a]	
B	IfB_R	CCAGCAATAGCTCCGAAGAAA	[a]	
B	IfB_Probe	CACCCATATTGGGCAATTTCCTATGGC	[a]	JOE-TAMRA
B	RSV_F	GCAAATATGGAAACATACGTGAACA	This publ	
B	RSV_R	GCACCCATATTGTWAGTGATGCA	This publ	
B	RSV_Probe	CTTCACGAAGGCTCCACATACACAGCWG	This publ	FAM-BHQ
B	NL63_F	ACGTACTTCTATTATGAAGCATGATATTAA	Gunson et al. (2005)	
B	NL63_R	AGCAGATCTAATGTTATACTTAAAACTACG	Gunson et al. (2005)	
B	NL63_P	ATTGCCAAGGCTCCTAAACGTACAGGTGTT	Gunson et al. (2005)	HEX-BHQ1
C	MPV_F	ATGTCTCTTCAAGGGATTCACCT	This publ	
C	MPV_R	AMAGYGTTATTTCTTGTTGCAATGATGA	This publ	
C	MPV_Probe	CATGCTATATTAAAAGAGTCTCARTAC	This publ	FAM-MGB
C	Ch. pneumoniae_F	CAAGGGCTATAAAGGCGTTGCT	This publ	
C	Ch. pneumoniae_R	ATGGTCGCAGACTTTGTTCCA	This publ	
C	Ch. pneumoniae_P	TCCCCTTGCCAACAGACGCTGG	This publ	CY5-BHQ
C	EV_F	CATGGTGYGAAGAGTCTATTGAGCTA	This publ	
C	EV_R	GGACACCCAAAGTAGTCGGTTC	This publ	
C	EV_probe	CGGCCCCTGAATGCGGCTAATC	This publ	HEX-BHQ1
D	IfA_F	AAGACCAATCCTGTCACCTCTGA	Ward et al. (2004)	
D	IfA_R	CAAAGCGTCTACGCTGCAGTCC	Ward et al. (2004)	
D	IfA_Probe	TTTGTGTTCACGCTCACCGT	Ward et al. (2004)	FAM-MGB
D	AdV_F	GCCACGGTGGGGTTTCTAAACTT	Heim et al. (2003)	
D	AdV_R	GCCCCAGTGGTCTTACATGCACATC	Heim et al. (2003)	
D	AdV_Probe	TGCACCAGACCCGGGCTCAGGTACTCCGA	Heim et al. (2003)	HEX-BHQ1
D	OC43_F	CGATGAGGCTATTCCGACTAGGT	Gunson et al. (2005)	
D	OC43_R	CCTTCCTGAGCCTTCAATATAGTAACC	Gunson et al. (2005)	
D	OC43_Probe	TCCGCCTGGCACGGTACTCCCT	Gunson et al. (2005)	CY5-BHQ2
E	RV_F1	GGTGTGAAGAGCCSCRTGTGCT	This publication	
E	RV_F2	GGTGTGAAGACTCGCATGTGCT	This publication	
E	RV_F3	GGGTGYGAAGAGYCTANTGTGCT	This publication	
E	RV_R3	GGACACCCAAAGTAGTYGGTYC	This publication	
E	RV_probe	CCGGCCCTGAATGYGGCTAAYC	This publication	FAM-BHQ1
E	Mycoplasma_F	GGAATCCCAATGCACAAGAACA	This publication	
E	Mycoplasma_R	GCTTTGGTCAACACATCAACCTT	This publication	
E	Mycoplasma_Probe	GCCTTGAAGGCTGGGTTTGCGCTA	This publication	CY5-BHQ
E	229E_F	CAGTCAAATGGGCTGATGCA	Gunson et al. (2005)	
E	229E_R	AAAGGGCTATAAAGAGAATAAGGTATTCT	Gunson et al. (2005)	
E	229E_P	CCCTGACGACCACGTTGTGGTTCA	Gunson et al. (2005)	HEX-BHQ1

[a] Personal communication from Dr Lars Nielsen, Copenhagen. FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine; JOE, dichloro-2′,7′-dimethoxy-6-carboxyfluorescein; HEX, hexachlorofluorescein; CY5, Cyanine-5; BHQ, black-hole quencher; MGB, minor groove binder.

of the targeted viral or bacterial sequences, synthesized by by GenScript Corp. (Piscataway, NJ).

3. Results

During the period from October 2006 through March 2007, 954 specimens were collected from patients of all ages with symptoms of respiratory tract infections who had been admitted to hospital clinics as well as health care centers in the Göteborg region in Western Sweden (catchments area of approximately 600,000 inhabitants). Both in-patients and out-patients were included. The specimens were obtained from either oropharynx or nasopharynx by rotating swabs. The specimens were submitted for diagnosis to the virology laboratory at Sahlgrenska University Hospital.

Out of the 954 specimens analysed 457 (48%) were positive for one ore more agents. The following agents were detected in order of frequency (n, % of positives): IfA ($n=114$, 25%), RV ($n=93$, 20%), MPV ($n=46$, 10%), RSV ($n=41$, 9%), OC43 ($n=33$, 7%), Ad ($n=33$, 7%), NL63 ($n=26$, 6%), PIV ($n=19$, 4%), IfB ($n=4$, 1%), and EV ($n=1$). M. pneumoniae was the most frequent bacterium ($n=44$, 10%) whereas Ch. pneumoniae was found only in 2 specimens. Thirty-one specimens were positive for two agents, and one specimen was positive for 3 agents. The procedure was run every working day and results from samples arriving in the morning could be delivered during the same day to the clinician. The positivity rate (48%) is comparable with previous reports with similar study subjects. The rate of RSV and IfB were remarkably low, probably because these infections had appeared in extensive epidemics the preceding year.

By targeting multiple agents in each reaction well, a broad range of aetiologies could be investigated in each run. The cost per sample could be kept low, since the procedure was streamlined and a reasonable number of samples arrived to the laboratory each day. The cost for was divided on reagents for nucleic acid extraction (€3), reagents for real-time PCR (€11), personnel (€8), laboratory space (€3), instrument cost (€1), and overhead (€5), yielding a customer price of €33.

4. Discussion

We believe that a combination of low cost, high accuracy and prompt result delivery is the key to achieving a wide clinical use of molecular diagnostics of respiratory infections. We have also noted that inclusion of bacterial agents has promoted the willingness from clinicians to use our service, probably because Mycoplasma pneumoniae or Ch. pneumoniae infections may require antibiotic treatment. Viral detection may support the clinician's decision to avoid antibiotics, or may prompt antiviral treatment such as oseltamivir (against influenza) to the patient or exposed family members. Identifying the aetiology also allows the clinician to better inform the patient about the prognosis and ways of limiting the spread to other individuals. We therefore believe that an expanded use of molecular diagnostics of respiratory infections is of great clinical value. It should, of course, be kept in mind that viral agents detected by highly sensitive assays might reflect asymptomatic infection rather that being the cause of the present illness, and that an identified viral agent does not exclude concomitant bacterial infection. To some extent, the probability that a given agent constitutes an aetiological agent should be related to the Ct value: The lower Ct value, the higher the probability, and this may also be of relevance for the interpretation of double infections. Further studies of respiratory infection aetiologies in different patient categories, and of the clinical utility of this and similar multiplex assays, need to be carried out.

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