DEVELOPMENT OF A MULTIPLEX REAL-TIME PCR FOR DETECTION AND DIFFERENTIATION OF *CHLAMYDIACEAE* SPECIES WHICH ARE PATHOGENIC FOR HUMANS

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ABSTRACT

Objectives: Real-time PCR (RT-PCR) offers many advantages over conventional PCR methods for detection of microbial pathogens. The aim of our study was to develop and evaluate the performance of a multiplex 5'-nuclease-based RT-PCR assay for direct detection and differentiation of *Chlamydia trachomatis* (CTR), *Chlamydophila pneumoniae* (CPN) and zoonotic agents (*Chlamydophila psittaci* (CPS), *Chlamydophila abortus* (CAB) and *Chlamydophila felis* (CFE)) in clinical specimens.

Methods: The 5'-end sequence of the *ompA* gene which is well characterised in all chlamydial species was selected as a PCR target. It was amplified using the family-specific primers CM1 and CM2 (H.Yoshida et al., 1998) on a Rotor-Gene 2000 system (Corbett Research). Three probes containing different fluorescent dyes, JOE, ROX and Cy5 (Biosearch Technologies) were designed to target the signature sequences in the amplified *ompA* region which are highly conserved within CTR, CPN and zoonotic agents and are distinctive between them. The forth FAM-labelled probe was used for the detection of a heterogeneous internal standard (IS). The analytical sensitivity of the assay was determined by testing peripheral blood leukocyte specimens spiked with chlamydial elementary bodies or recombinant plasmids containing *ompA* fragments of the following strains: CTR L2, CPN Kajaani 7, CPS 6BC, CAB B577 and CFE FePn. In addition, a panel of 219 genital swab specimens was used to assess the sensitivity and specificity of CTR detection using RT-PCR in comparison with a commercial PCR assay targeting the cryptic plasmid of this species.

Results: As shown in the table 2, the multiplex RT-PCR was able to detected specifically and reproducibly single DNA copies of each chlamydial species in the presence of IS and excess of human DNA. As compared to monoplex PCRs, multiplexing of four probes did not decrease sensitivity, while no cross-detection between CTR, CPN and zoonotic species was observed. In testing clinical specimens, RT-PCR detected CTR DNA in 44 of the 46 samples that were positive and in one sample that was negative by commercial PCR. The lack of amplification of IS indicated the presence of inhibitors in two samples. Consequently, with commercial test used as a reference, the sensitivity and specificity of RT-PCR were 95.7% and 99.4%, respectively.

Conclusion: The developed method enables rapid, sensitive and specific detection of all members of *Chlamydiaceae* which are pathogenic for humans.

INTRODUCTION

Several species of the family *Chlamydiaceae* are known to be pathogenic for humans. *Chlamydia trachomatis* (CTR) is the most prevalent sexually transmitted bacterial pathogen, with an estimated 90 million new cases occurring each year worldwide (World Health Organization, 2001). It is also known as the causative agent of trachoma still considered to be the most frequent reason of blindness. *Chlamydophila pneumonia* (CPN) is mainly recognized as the respiratory pathogen, either contributed to the development of some chronic diseases (e.g. asthma and atherosclerosis). In addition, three species: *Chlamydophila psittaci* (CPS), *Chlamydophila abortus* (CAB) and *Chlamydophila felis* (CFE), which are mainly pathogenic for animals, have zoonotic potential.

Although some forms of chlamydiosis are associated with a particular organism, different species may be responsible for clinically similar symptoms. Thus a rapid, sensitive and specific method for detection and differentiation of *Chlamydiaceae* species could be crucial for successful diagnostic of chlamydiosis. We aimed our study at the developing of a multiplex 5'-nuclease-based real-time PCR assay for direct detection and differentiation of CTR, CPN and zoonotic agents (CPS, CAB and CFE) in clinical specimens.

MATERIALS AND METHODS

The reference chlamydial strains: *C. trachomatis* L2, *C. suis* (CSU) S45, *C. muridarum* (CMU) MoPn, *C. pneumonia* Kajaani 7, *C. psittaci* 6BC, *C.abortus* B577, KS-93; 250; Rostinovo-70; PP-87, *C. felis* FEPN, *C. caviae* GPIC, *C. pecorum* LW613, 66p130, L71, 1710S, were used as partially purified and heat-inactivated elementary bodies or as isolated DNA.

The PCR reactions were conducted with the family-specific primers CM1 and CM2 (H. Yoshida, 1998) targeting the 5'-end sequence of the *ompA* gene. Three probes containing different fluorescent dyes, JOE, ROX and Cy5 in combination with Black Hole Quenchers BHQ; Biosearch Technologies, USA) were designed to target the signature sequences in the amplified *ompA* region which are highly conserved within CTR, CPN and zoonotic agents and are distinctive between them. The forth FAM-labelled probe was used for the detection of a heterogeneous internal standard (Fig. 1, Table 2).

To detect a possible PCR inhibition by any specimen components we invented a heterogeneous internal standard (IS). The λ phage 341-bp DNA fragment (GenBank Accession J02459 M17233) was amplified with two hybrid primers (λHYB1: 5'-CAG GAT ATC TTG TCT GGC TTA TCG GGA TGA TTA CGG TCC-3' and λHYB2: 5'-CAA GGA TCG CAA GGA TCT CCG CAT GGA TTC TGT CGA CC-3'), containing the phage complimentary sequences at their 3' ends and CM1 and CM2 sequences at the 5' ends. The PCR product was cloned into the pCR2.1 vector (Invitrogen, USA) and the resulting plasmid (pIS3.2) was used as internal standard. Minimal detectible amount of pIS3.2 (600 copies) which was not affecting the amplification of chlamydial DNA was added to each reaction.

Amplification was performed using Ready-To-Go PCR Beads (Amersham Biosciences, USA), so that PCR mixtures (25 µl) contained 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 6 mM MgCl2, 200 µM dNTP's, 1.5 µl AmpliTaq DNA polymerase,12.5 pmoles of each primer, 5 pmoles of each DNA probe, internal standard (600 copies of pIS3.2) and template DNA. Hot-start PCR reactions were cycled as follows: 83ºC for 6 min, 95ºC for 20 s, followed by 50 cycles of 95ºC for 25 s and 68ºC for 40 s in a Rotor-Gene 2000 thermocycler (Corbett Research, Australia).

To evaluate the sensitivity and specificity of the method, chlamydial elementary bodies or recombinant plasmids containing *ompA* fragments of 9 chlamydial strains (CTR L2, CMU MoPn, CSU S45, CPN Kajaani 7, CPS 6BC, CAB B577, CFE FePn, CCA GPIC, and CPE 1710S) were tested in the presence of IS (600 copies) and excess amount of human DNA (200 ng) isolated from peripheral blood lymphocyte specimens. Serial dilutions of the plasmids containing *ompA* fragment of CTR (pGEM-T-CTR), CPN (pGEM-T-CPN) and CPS (pGEM-T-CPS) were subjected to the assay to determine its analytic sensitivity.

The specificity of the assay was tested with the following organisms: *Gardnerella vaginalis, Mycoplasma pneumoniae, Mycoplasma hominis, Enterococcus faecalis, Escherichia coli, Haemophilus influenzae, Neisseria gonorrhoeae, Clostridium perfringens, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus pneumoniae, Micrococcus luteus, Streptococcus pyogenes, Enterobacter cloacae, Klebsiella pneumoniae, Staphylococcus epidermidis, Serratia marcescens, Salmonella typhimurium, Proteus vulgaris, Candida albicans, Candida parapsilosis*.

A batch of 219 genital swab samples was tested to prove the sensitivity and specificity of CTR detection using the real-time PCR in comparison with a commercial PCR assay targeting the cryptic plasmid of chlamydial species.

Table 1. The design of fluorogenic probes

DNA target	Probe	λ_{ex}/ λ_{em}, nm	Number of sequences*	Identity, %
Internal Standard	5' 6-FAM-d(N)$_{23}$-BHQ-1 3'	495/520		
Chlamydia spp.	5' JOE-d(N)$_{25}$-BHQ-1 3'	520/548	83	100
C. trachomatis			63	100
C.muridarum			5	100
C.suis			15	100
C.pneumoniae	5' ROX-d(N)$_{29}$-BHQ-2 3'	575/602	8	100
Zoonotic agents	5' Cy5-d(N)$_{28}$-BHQ-3 3'	649/670	38	100
C. psittaci			21	100
C. abortus			14	100
C. felis			3	100

* The number of *ompA* sequences found in BLAST (http://www.ncbi.nlm.nih.gov/blast)

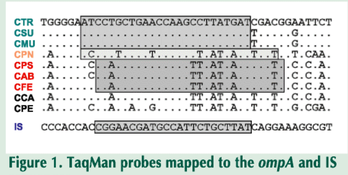

Figure 1. TaqMan probes mapped to the *ompA* and IS sequences
Chlamydial sequences were aligned against *C. trachomatis*. Identical nucleotides are shown by a dot.

RESULTS

We have developed a multiplex 5'-nuclease-based real-time-PCR assay that allows distinctive detection of CTR, CPN and three zoonotic agents (CPS, CAB, CFE) in a single tube. Since the fluorogenic probes targeting the *ompA* sequences of different species were designed to have 2-7 nucleotide differences between each other (Fig. 1), no crossdetection of *Chlamydia* spp., CPN and zoonotic agents was observed (Fig. 2). The JOE-labeled probe detects all species of the genus /Chlamydia/, including CTR, CSU and CMU, though the latter two species are nonpathogenic for humans.

Analytical sensitivity of the assay was evaluated by testing serial dilutions of the plasmids containing *ompA* fragment of CTR, CPN and CPS in the presence of IS and excess amount of human DNA (200 ng). We observed an inverse linear relationship between the logarithm of initial template DNA concentration and the Ct values in the range from 7 to 7 million copies of pGEM-T-CTR and pGEM-T-CPS and from 70 to 7 million copies of pGEM-T-CPN (Fig. 3).

Minimal detectible level for all chlamydial strains was equal to 1-4 genome equivalents.

Multiplex assay with the use of the four probes displayed the same specificity and sensitivity as monoplex PCRs as was revealed by comparison of respective threshold cycle (Ct) values and fluorescence intensities.

There was no unspecific interaction of DNA probes with unrelated organisms found.

When applied to the testing of clinical specimens, real-time PCR detected CTR DNA in 44 of the 46 positive samples and in one sample which was judged to be negative by commercial PCR. The lack of IS amplification suggested the presence of inhibitors in two samples.Consequently, with commercial test used as a reference, the sensitivity and specificity of our assay were 95.7% and 99.4%, respectively.

CONCLUSIONS

The developed method enables rapid, sensitive and specific detection of all members of *Chlamydiaceae* which are pathogenic for humans. The valuable benefit of this assay is the possibility of quantification or semiquantification of template DNA in a sample by comparing its amplification efficacy with the one of IS, even though there is a partial inhibition.

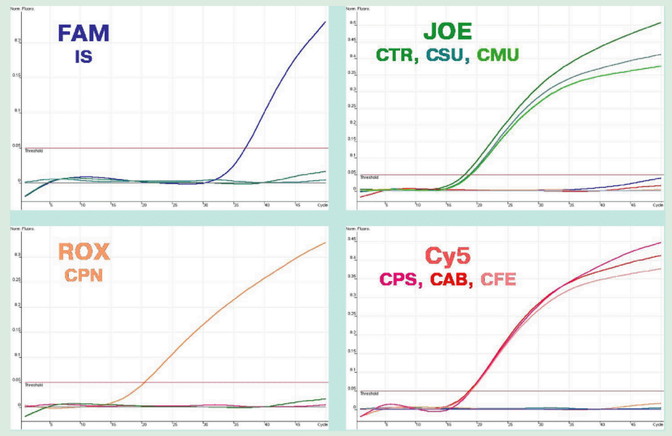

Figure 2. Assessment of the specificity of probes in multiplex reactions
DNA from all chlamydial strains (≈5 mln. genome equivalents) and IS (600 copies) was tested in multiplex assays with four probes. Each probe gave rise to fluorescence only with specific targets.

Table 2. Detection and differentiation of chlamydial species using the four probes

DNA target	FAM-BHQ1 IS	JOE-BHQ1 Chlamydia spp.	ROX-BHQ2 C.pneumoniae	Cy5-BHQ3 Zoonotic agents
Internal Standard	+	–	–	–
C.trachomatis	–	+	–	–
C.muridarum	–	+	–	–
C.suis	–	+	–	–
C.pneumoniae	–	–	+	–
C.psittaci	–	–	–	+
C.abortus	–	–	–	+
C.felis	–	–	–	+
C.caviae	–	–	–	–
C.pecorum	–	–	–	–

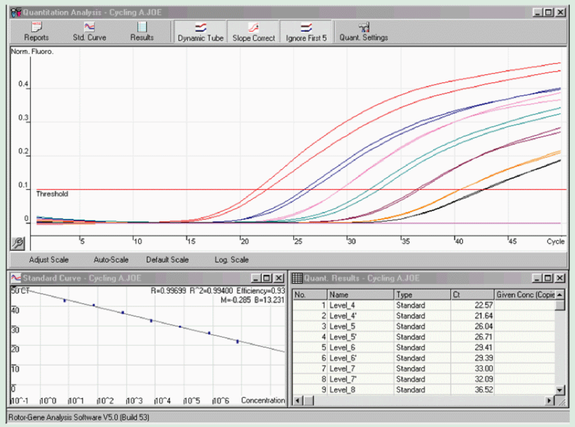

Figure 3. Quantitative analysis of *C. trachomatis* DNA

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