Development of an internally controlled real-time PCR assay for detection of *Chlamydophila psittaci* in the LightCycler 2.0 system

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**ABSTRACT**

A real-time PCR assay with a DNA purification and inhibition control (internal control; IC) was developed to detect *Chlamydophila psittaci* DNA in human clinical samples. Novel *C. psittaci*-specific primers targeting the *ompA* gene were developed. The IC DNA contained the same primer-binding sites and had the same length and nucleotide content as the *C. psittaci* DNA amplicon, but had a shuffled probe-binding region. The lower limit of detection was 80 target copies/PCR, corresponding to 6250 copies/mL in a clinical sample. Specificity was tested using reference strains of 30 bacterial species. No amplification was observed from any of these samples. Respiratory samples from eight patients were positive with this PCR. Six of these patients were confirmed as positive for *C. psittaci* with serological testing. Two patients had increasing antibody titres, but did not fulfil criteria proposed previously for serologically proven *Chlamydia* spp. infection. The real-time PCR described in this paper is a sensitive, specific and rapid method to detect *C. psittaci* DNA in human clinical respiratory samples.

**Keywords** *Chlamydophila psittaci*, diagnosis, inhibition control, PCR, psittacosis, respiratory samples

**Original Submission:** 19 May 2005;  **Revised Submission:** 9 September 2005;  **Accepted:** 5 November 2005

**Clin Microbiol Infect** 2006; 12: 571–575

**INTRODUCTION**

*Chlamydompha psittaci* (formerly *Chlamydia psittaci*) is an obligate intracellular microorganism that causes psittacosis in humans. Psittacosis is characterised by fever, chills, headache, dyspnoea and cough [1]. The chest X-ray often shows an infiltrate. The disease is acquired through contact with infected birds, bird droppings or feather dust [2]. In 2003, 15 and 27 cases were reported in the USA and The Netherlands, respectively [3,4]. The diagnosis of *C. psittaci* infection can be made by culture, serology or DNA detection. Culture is time-consuming and requires extensive safety precautions. Laboratory-associated infections are well-known [5], and *C. psittaci* should therefore be handled under Biosafety Level 3 conditions [6]. The reference standard for diagnosing psittacosis is the measurement of a four-fold increase in serum antibodies using microimmunofluorescence [2], although this test does not appear to be as species-specific as claimed by the manufacturer [2,7]. Furthermore, the test is difficult to interpret and needs convalescent sera. Most often, serological testing provides only a retrospective diagnosis.

A diagnostic PCR has the potential to overcome the above problems and, in addition, can help clinicians to target antibiotic treatment and to expedite outbreak management. Although PCR assays that detect *C. psittaci* have been described previously, they have lacked an internal control (IC) to monitor DNA purification and possible inhibition of the amplification reaction, and were not developed for a real-time PCR format [8–10]. The need for a real-time assay to detect *C. psittaci* in humans has been emphasised previously [11]. Therefore, the present study aimed to develop a real-time PCR assay with an IC to detect *C. psittaci* in clinical samples.

**MATERIALS AND METHODS**

**Respiratory specimens**

Eight respiratory specimens (four sputum, one bronchoalveolar lavage (BAL) fluid, three throat swabs) from eight individuals were investigated. The BAL fluid from one of these eight
patients had already been shown to be positive for C. psittaci DNA in a PCR assay conducted elsewhere [11]. Ten respiratory specimens (six throat washes and four sputum specimens) from ten patients with respiratory infections caused by other (non-chlamydial) bacteria or viruses were also tested: respiratory syncytial virus (n = 2); parainfluenza virus (n = 3); enterovirus (n = 1); Staphylococcus aureus (n = 1); Enterobacter cloacae (n = 1); and Haemophilus influenzae (n = 2). These pathogens were detected by standard culture procedures or direct immunofluorescence. As pigeons represent one of the main reservoirs of C. psittaci, a pigeon breeder provided nine nose swabs obtained from nine pigeons with nasal discharge that was possibly caused by C. psittaci infection.

**Bacterial strains**

Thirty ATCC or quality control assessment strains, including related members of the Chlamydiaceae, were used for specificity experiments (Table 1). Escherichia coli (E. coli One shot; Invitrogen, Breda, The Netherlands) cells were used for propagation of cloned plasmid constructs. Genomic C. psittaci DNA was purified from the C. psittaci Orni strain isolated from a human case of psittacosis [12,13], and was used for construction of a C. psittaci DNA control. C. psittaci 6BC (ATCC VR-125), Chlamyphila abortus (C18/98), Chlamyphila felis (02DC0026) and Chlamyphila caviae (GPC strain) (kindly provided by D. Vanrompay, Ghent University, Belgium) were also tested.

**DNA extraction**

Sputum samples (eight volumes) were first diluted with 0.1 volume acetylcysteine solution (50 mg/mL) and 0.1 volume bacterial lysis buffer (10 mM Tris-HCl, pH 8.3, 1 mM EDTA (TE buffer) containing SDS 1% w/v, Tween-20 5% w/v, sarcosyl 5% w/v). Thorough mixing and liquefaction was performed in 50-mL sterile tubes containing c. 20 washed and autoclaved glass beads (Emergeo, Landsmeer, The Netherlands). After mixing, the tubes were incubated at room temperature for ≥30 min. Once liquefied, 190 μL of sputum and 10 μL (c. 80 copies/PCR) of IC solution (see below) was subjected to Boom extraction [14]. The pigeon and human throat swabs, 200 μL of the human throat washes or BAL fluid, or 100 μL of the bacterial suspensions, were suspended directly in 900 μL of L6 lysis buffer [14] without pre-treatment. DNA was eluted in 100 μL of TE.

**Primers and probes**

Primers were designed to amplify a conserved region of the C. psittaci ompA gene. All known C. psittaci ompA gene sequences present in the GenBank database were included in this design. The primers used for amplification were CPSittF (5’-CGCTCTCTCTCTCTAAACC; 171 nucleotide (nt) 411–429) and CPSittR (5’-AGCACCTTCCCTCCTATG; nt 474–492). Nucleotide numbering was derived from the C. psittaci 6BC ompA gene (GeneBank accession number X56980). The TaqMan probes used for detection of the C. psittaci and IC amplicons were, respectively, CPSitt Probe (5’-FAM-AGGGAACCCAG-GTCCTTCTCTCTCTAAACC; 75 nucleotide (nt) 441–450) and CPSitt IC Probe (5’-VIC-TCGGTATCTGACGTACGTTGGAGCCTA) (PE Applied Biosystems, Warrington, UK). This primer pair amplifies an 82-bp DNA fragment of the C. psittaci ompA gene, as well as the IC, which has the same length and nucleotide content as the C. psittaci DNA amplicon, but a shuffled probe-binding region.

**Construction of the C. psittaci DNA control**

C. psittaci DNA was purified from the C. psittaci Orni strain and an amplicon was generated using the CPSitt primer pair. The amplicon was cloned into PCR 2.1 plasmid DNA (Invitrogen) to create pPsittWT, which was then propagated in E. coli and purified using the Wizard Plus Miniprep isolation kit (Promega, Leiden, The Netherlands) and purified using the Wizard Plus Miniprep isolation kit (Promega, Leiden, The Netherlands). The sequence of the DNA insert in pPsittWT was checked by dideoxynucleotide sequencing (BigDye Terminator v.1.1; PE Applied Biosystems, Warrington, UK). This primer pair amplifies an 82-bp DNA fragment of the C. psittaci ompA gene, as well as the IC, which has the same length and nucleotide content as the C. psittaci DNA amplicon, but a shuffled probe-binding region.

**Construction of the internal control**

The IC was constructed using two oligonucleotides: IC-CPSitt-1 (5’-CGCTCTCTCTCTCTAAACC; 75 nucleotide (nt) 441–450) and IC-CPSitt-2 (5’-AGCACCTTCCCTCCTATG; nt 474–492). The sequence of the DNA insert in pPsittWT was checked by dideoxynucleotide sequencing (BigDye Terminator v.1.1; PE Applied Biosystems, Warrington, UK). The concentration and purity of the isolated plasmid construct were measured with a spectrophotometer at 260 and 280 nm, respectively, and the construct was then stored in TE at 20°C.

**Dilution series of pPsittWT and IC**

Serial dilutions of pPsittWT and IC were prepared in a stabilising lysis buffer (5.25 M GuSCN, 50 mM Tris-HCl, 1 mM EDTA, 0.5% Sarkosyl, 1% Tween-20, 0.1% SDS, pH 8.3). 100 μL aliquots were mixed with 900 μL of the stabilising lysis buffer containing 10 μL of L6 lysis buffer. The mixture was incubated at room temperature for 30 min, and 100 μL aliquots were subjected to Boom extraction [14]. The DNA was eluted in 100 μL of TE.
pH 6.4, 20 mM EDTA) supplemented with calf thymus DNA 20 ng/µL, and were then stored at −20°C. Extraction of the dilution series, corresponding to 80, 40, 20 and 10 copies/PCR, was performed six times, in a background of 190 µL of C. psittaci DNA-negative pooled and liquefied sputum by the Boom procedure [14].

Real-time PCR assay

Reactions were performed in the LightCycler 2.0 system (Roche Diagnostics, Penzberg, Germany) using two TaqMan probes. The final reaction volume (20 µL) contained 8 µL of eluate, 2 µL of 10× LightCycler Faststart DNA Master Hybridisation Probes Mix (Roche Diagnostics), 0.2 µL uracil- N-glycosylase (PE Applied Biosystems) and final concentrations of 0.3 µM each probe, 0.7 µM each primer and 4.5 mM MgCl₂. The real-time PCR steps comprised 50°C for 10 min, 95°C for 10 min, 49 cycles of 95°C for 10 s, 62°C for 5 s and 72°C for 10 s, and finally, 30°C for 30 s. Fluorescence values for the FAM and VIC probe signal, used for detection of C. psittaci DNA or IC, were detected in channel 530 and 560, respectively. A colour compensation file was used, according to the manufacturer’s instructions, to prevent crosstalk of the two fluorescent probe signals.

Serological diagnosis

An ELISA (Chlamydia IgG/A/M rELISA; Medac Diagnostika, Hamburg, Germany) was used for the serological diagnosis of C. psittaci infections. Serological diagnosis in acute-phase and convalescent sera is usually based on a three-fold rise in the titre, or a two-fold increase in the IgG titre in combination with a two-fold or greater change in the IgM titre, or a two-fold increase in the IgG titre in combination with a two-fold increase in the IgA titre [10,17].

RESULTS

Optimisation of the real-time PCR for use with the TaqMan probes

During the LightCycler assay, an accumulation of amplicon is indicated by an increase in fluorescence emitted by the FAM-reporter dye after hydrolysis of the TaqMan probe. Initially, electrophoresis analysis of the amplicons indicated quantities that were disproportionately greater than the fluorescence signal detected (data not shown). However, changing the temperature transition rate from 20°C/s to 1°C/s during the annealing step allowed optimal binding of the TaqMan probes and adequate fluorescence signals, as shown by the lower limit of detection (see below).

Determination of the lower limit of detection of the C. psittaci real-time PCR

In a background of C. psittaci-negative pooled sputum, it was possible to detect 80 copies/PCR of pPsittWT or IC (Table 2). The lowest detection limit for pPsittWT and IC was ten copies/PCR (1/6 and 3/6 experiments positive, respectively). In the presence of 80 copies of IC, 80 copies of pPsittWT were always detected. This suggests that there is no significant competition between pPsittWT and IC when ≥80 copies of pPsittWT are present in a clinical sample (data not shown). Detection of 80 copies/PCR corresponds to a minimum sensitivity of 6250 copies/mL in a sputum sample.

Specificity of the real-time PCR assay

No amplification was observed when DNA of 30 bacterial species, including related members of the Chlamydiaceae (Table 1), was tested in the PCR. All IC signals were positive. Although this PCR was not developed as a quantitative test, the mean Ct (crossing point) value for the IC signal with these samples was 34.1 (SD 1.3) cycles. DNA from the avian type strain C. psittaci 68c (ATCC VR-125), C. abortus (C18/98), C. felis (02DC0026) and C. caviae (GPIC strain) was amplified, as expected based on sequence homology [16].

Respiratory specimens

Four sputa, one BAL fluid and three throat swabs, tested in six separate runs, were positive by real-time PCR (mean Ct values of 27.2–35.9 cycles, SD 0.3–1.5). There was no clear association between the Ct values obtained and the different respiratory samples. Six of the eight cases were confirmed serologically by ELISA; of the two remaining cases, one had positive IgA and IgM titres that did not change, and a two-fold rise in IgG serum antibodies, while the other had a two-fold rise in IgA, together with increasing, but not doubled, serum IgG. Three of the nine pigeon

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<th>Copies/PCR</th>
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aData shown indicate the number of positive samples vs. the number of samples tested.

bFor all the copy numbers presented, 100% extraction and PCR efficiency was assumed.

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The ten respiratory samples from patients with other respiratory infections were negative. All PCR-negative samples were IC-positive.

DISCUSSION

The real-time PCR described in the present study appears to be a sensitive and specific format for diagnosing psittacosis. This is the first report of an internally controlled real-time PCR assay to detect \( C. psittaci \) DNA in the LightCycler 2.0 system using two \( Taq \)Man probes. The IC monitored the process of nucleic acid purification and amplification for each individual sample. After liquefaction of sputum samples and subsequent Boom extraction, it was possible to reliably detect 80 copies/PCR of pPsitWT or IC. The specificity of the real-time PCR assay was confirmed with a set of bacterial species, including related members of the Chlamydiaceae, and with respiratory samples from patients with evidence of other respiratory infections.

In the recently revised taxonomic classification, \( C. psittaci \) has been subdivided into four \( Chlamydiaphila \) spp., namely \( C. abortus \), \( C. psittaci \), \( C. felis \) and \( C. caviae \) [16,19]. This classification included newly available sequence data and showed the relatedness of \( Chlamydiaphila \) spp. from typical hosts (e.g., cats, birds and guinea-pigs). The new classification is based mainly on minor sequence differences in the 16S rRNA gene, 23S rRNA gene and internal ribosomal spacer region [16]. The \( C. psittaci \) primers and probes designed during the present study also amplified and detected these other three species, but because of sequence homology in the \( ompA \) gene, it was not possible to design primers that could distinguish these four species in a LightCycler assay. However, for clinical purposes, this is not important, since all four are considered to be potentially infectious for humans [20,21]. If required, sequence analysis of the \( ompA \) gene can be performed for strain or serovar speciation [22].

The incidence of confirmed cases of psittacosis is low [3,4], but may be underestimated, as accurate methods for the diagnosis of psittacosis are not always available. In addition, many patients with this disease are unable to produce sputum and receive broad-spectrum antibiotic treatment without invasive sampling. Therefore, the number of samples available for clinical evaluation of the PCR was limited. In the present study, \( C. psittaci \) DNA was detected in eight respiratory samples obtained from eight patients (c. 30% (8/27) of the annual reported cases in The Netherlands) [3]. One of these samples had been shown previously to be PCR-positive with a different primer set [8,11], and six cases were confirmed serologically (the two remaining cases showed increasing titres only of specific IgG).

In general, serological tests for the diagnosis of \( C. psittaci \) infection are hampered by lack of sensitivity and specificity (genus and/or species) and poor reproducibility [7,23–25]. The two PCR-positive, but serologically negative, cases in the present study highlight this problem. A PCR false-positive result is unlikely, as the specificity of the assay was confirmed, but it is always difficult to validate a newly developed PCR against a poor reference standard. Although the positive pigeon samples were not tested against a recognised standard method, these samples were tested as highly suspected animal reservoirs. The detection of \( C. psittaci \) DNA in three of nine pigeons with a possible clinical picture of \( C. psittaci \) infection is highly suggestive of true disease.

In conclusion, this sensitive and specific real-time PCR assay can generate results in a few hours, thereby avoiding the wait for serological confirmation and by-passing the need for culture, which is particularly important for the rapid detection and management of psittacosis outbreaks [5,26]. The assay detects \( C. psittaci \) DNA in human respiratory specimens, and is a valuable addition to the diagnostic tools available for patients suspected of having psittacosis. The test will also help clinicians to target antibiotic treatment and can expedite outbreak management. The inclusion of an IC prevents the occurrence of false-negative PCR results.

ACKNOWLEDGEMENTS

We thank H. C. J. G. Peters, pigeon breeder, for collecting the nine pigeon nasal swabs, N. E. Vrede for the construction of the internal control, and D. Vanrompay (Ghent University, Belgium) for providing the \( C. abortus \), \( C. caviae \) and \( C. felis \) strains.

REFERENCES


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