Development of a real-time PCR for the detection of *Chlamydia psittaci*

*Chlamydia psittaci* is the causative agent of avian chlamydioidosis, and zoonotic infection can result in severe pneumonia and other serious health problems (Stubbs et al., 1989). Even if this infection remains exceptional in humans, psittacosis can be considered as an emerging occupational disease (Lederer & Muller, 1999; Maegawa et al., 1989). Direct detection of *C. psittaci* by cell culture is hazardous and requires a level 3 laboratory, given its contagiousness; currently, there is no other direct technique marketed. However, the interpretation of serodiagnosis is difficult because of cross-reactions with other species of *Chlamydia* and the high prevalence of *Chlamydia pneumoniae* in the general population. Therefore, molecular biology techniques represent invaluable tools for the diagnosis of this infection. The aim of this study was to develop a TaqMan PCR assay to detect *C. psittaci* by using the specific *incA* (inclusion membrane protein A) gene as a target. Since 1990, numerous standard PCR techniques have been developed for the detection and identification of *C. psittaci*. Most of them target major outer-membrane protein (MOMP) genes (Martin & Cross, 1997; Sudler et al., 2004), as it is difficult to differentiate between species of *Chlamydia* based on the 16S rDNA. Recently several formats of real-time PCR have also been proposed for the identification and/or quantification of *Chlamydia* spp. They are usually very sensitive and rapid, and they avoid the possibility of contamination with PCR products since all of the steps occur in the same tube and no post-experimental handling is necessary. DeGraves et al. (2003) described a FRET (fluorescence resonance energy transfer)-PCR targeting the 23S rRNA gene that allows the identification of *Chlamydia* species. Two real-time PCRs targeting the *omp1* gene and using FRET or SYBR Green chemistries have been developed (Huang et al., 2001). No real-time PCR using TaqMan chemistry has been described to date. In this study, we used TaqMan methodology to develop a PCR for the detection of *C. psittaci*, in order to employ only one probe instead of two in FRET chemistry, and to shorten the size of amplicon, thus optimizing PCR efficiency. *IncA* is a protein synthesized and secreted onto the inclusion membrane by bacteria (Bannantine et al., 1998). It was first identified in *Chlamydia caviae* strain GPIC, and homologues of the gene were found in all the chlamydial genomes sequenced. All *IncA* proteins share the same organization, but in spite of the structural conservation, there is little similarity in the sequences of *IncA* between different species (Delevoye et al., 2004). This observation prompted us to design *C. psittaci*-specific primers and probe based on the *incA* gene.

Several *Chlamydia* species such as *C. pneumoniae* TWAR strain AR39 (ATCC 53592), *Chlamydia trachomatis* strain D/UW-3/Cx (ATCC VR-885), *C. caviae* strain GPIC, *Chlamydia abortus* strains AV2 and AV7, *Chlamydia pecorum* strains IB2 and IB5, and *C. psittaci* strains GR9, VS1, 225, NJ1 and CP3, kindly supplied by A. Rodolakis (INRA, Roumilly, France), were used in this study. They were cultured on appropriate cells in minimal essential medium supplemented with 1 mM glutamine and 5% fetal bovine serum. Genomic DNA was extracted using a Magna Pure LC DNA isolation kit I and a Magna Pure LC isolation station (Roche Applied Science). DNA was stored at −20°C.

First, we sequenced the *incA* gene from different *C. psittaci* strains. Because the chlamydial strain sequenced that is closest to *C. psittaci* is *C. abortus*, PCR primers were designed in the genes flanking the *C. abortus* *incA* gene (CAB536, according to *C. abortus* annotation, GenBank accession number CR848038): CA-CAB535-S (5′-AAGGTTCTCCCTCAATTGAG-3′) and CA-CAB537-AS (5′-GAACGGTGCT-TTATATGGA-3′). The products of PCR amplification of genomic DNA from five different strains of *C. psittaci* were purified using MicroSpin S-400 HR columns (Amersham Pharmacia). Sequencing of these products was achieved on both strands using the initial CA-CAB535-S and CA-CAB537-AS primers, and internal primers designed within the sequences (IncA-Int2 5′-CGAAAACCTTACCTCAGGAA-3′ and IncA-IntAS 5′-GAGTACCTACAGATTTGAAA-3′). The *incA* sequences of GR9, VS1, 225, NJ1 and CP3 strains were submitted to GenBank and accession numbers DQ117471–DQ117475, respectively.

Alignment of *incA* genes from *C. abortus*, *C. caviae* and *C. trachomatis* with the five newly sequenced *incA* genes of *C. psittaci* showed that *C. psittaci* and *C. abortus* *incA* genes were the most closely related, and that *C. trachomatis* and *C. caviae* were more distant from each other (data not shown); this is in agreement with phylogenetic trees based on 16S rRNA and 23S rRNA genes (Everett et al., 1999).

Second, we designed TaqMan primers and a probe for PCR. The five *C. psittaci* *incA* genes and the *C. abortus* *incA* gene were aligned using the multiple sequence alignment with hierarchical clustering program (Corpet, 1988), http://prodes.toulouse.inra.fr/multalin/multalin.html, and a search was carried out to identify conserved regions specific for *C. psittaci*. Primers and probes were designed using the Primer Express software package (Applied Biosystems) and were synthesized by Eurogentec. The primers (F1-incA-Cpsi 5′-GGCATCATGCTTTTCTTCTT-3′ and R1-incA-Cpsi 5′-GGGCGTGCACATTGGA-3′) were designed to amplify a 74 bp region on the *C. psittaci incA* gene (Fig. 1). The probe was designed to match a sequence conserved in the five *C. psittaci* sequences but with one mismatch with the *C. abortus* sequence to ensure specificity (Cpsi-incA-NM, FAM-TGATTGTCCATTGTTGATCAGA-NFQ-MGB). The MGB (minor groove binder) TaqMan probe was synthesized.
with the reporter dye FAM (6-carboxy-fluorescein) covalently linked to the 5' ends, and the non-fluorescent quencher, NFQ, at the 3' ends, which were phosphorylated to prevent probe extension. The primers and probe were tested on DNA extracted from the five C. psittaci strains, five other Chlamydia species, and from diverse bacterial species commonly found in human specimens, including Gram-negative bacteria (Escherichia coli, Salmonella enteritidis, Salmonella typhimurium, Neisseria meningitidis, Haemophilus influenzae, Haemophilus parainfluenzae, Bordetella pertussis, Bordetella parapertussis, Bordetella holmesii) and Gram-positive and related bacteria (Mycoplasma pneumoniae, Mycoplasma genitalium, Streptococcus pneumoniae, Staphylococcus aureus). The TaqMan real-time PCR amplification and hybridization reactions were carried out in a final volume of 25 μl containing 12.5 μl TaqMan Universal PCR master mix, 0.3 μM each primer, 0.2 μM labelled probe Cpsi-incA-FT and 5 μl purified DNA in a ABI PRISM 7000 thermocycler (Applied Biosystems). DNA was amplified using the following cycling parameters: heating at 95°C for 10 min, followed by 40 cycles of a two-stage temperature profile of 95°C for 15 s and 60°C for 1 min. Only DNA extracted from C. psittaci led to amplification, demonstrating the specificity of the method.

To evaluate sensitivity, infectious bacteria, called elementary bodies, were purified (Scieux et al., 1993), and quantification was performed by diluting the bacterial suspension in PBS and counting the elementary bodies after staining with the direct fluorescent monoclonal antichlamydia antibody (IMAGEN Chlamydia antibody; J2L Elitech). Serial dilutions of the extracted DNA from bacterial suspensions were tested using this assay (Fig. 2). The slope of the regression curve was $-3.524 \times \log(x) + 38.12$. The r2 value from the linear regression in this assay is 0.99.

This PCR was applied to two clinical samples (broncho-alveolar lavages) from patients presenting severe respiratory infections and being positive by serology. One patient worked in a poultry abattoir and the other patient was a parakeet breeder. These two samples were positive by TaqMan PCR. In conclusion, we have...
developed a new real-time PCR for the identification of *C. psittaci* that we believe is the first to be applied successfully to human samples. This method is rapid, sensitive and specific, and can be easily implemented in a setting where a real-time TaqMan-PCR apparatus is available.

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