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

Chlamydia psittaci is the causative agent of avian chlamydiosis, 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.*, 2001). 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 *omp*1 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 CP-3, 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′-AAGGTCTCCTCCCATAATTGAG-3′) and CA-CAB537-AS (5′-GAACGGTGCT-TTTTATTGGAAA-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′-CGAAACCTTA-ACCTCAGGAA-3′ and IncA-IntAS 5′-GAGTACCTACAGATTTGAAA-3′). The *incA* sequences of GR9, VS1, 225, NJ1 and CP3 strains were submitted to GenBank and assigned 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 phylogenic 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′-GCCATCATGC-TTGTTTCGTTT-3′ and R1-incA-Cpsi 5′-CGGCGTGCCACTTGAGA-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-TCATTGTCATTATGGTGATTCAGGA-NFQ-MGB). The MGB (minor groove binder) TaqMan probe was synthesized

```
C. psittaci  GR9    TGATTGCCATCATGCTTGTTTCGTTTGTCATTGTCATTATGGTGATTCAGGATAGCACCCCCTCTCAAGTGGCACGCCGTATGA
C. psittaci  225    TGATTGCCATCATGCTTGTTTCGTTTGTCATTGTCATTATGGTGATTCAGGATAGCACCCCCTCTCAAGTGGCACGCCGTATGA
C. psittaci  VS1    TGATTGCCATCATGCTTGTTTCGTTTGTCATTGTCATTATGGTGATTCAGGATAGCACCCCCTCTCAAGTGGCACGCCGTATGA
C. psittaci  NJ1    TGATTGCCATCATGCTTGTTTCGTTTGTCATTGTCATTATGGTGATTCAGGATAGCACCCCCTCTCAAGTCGCACGCCGTATGA
C. psittaci  CP3    TGATTGCCATCATGCTTGTTTCGTTTGTCATTGTCATTATGGTGATTCAGGATAGCACTCCCTCTCAAGTGGCACGCCGTATGA

C. abortus   S26/3  TGATTGCCATTATGCTCGTTTCGTTTGTCATTGTCATTATGGTCATTCAGGATAGCACACCTTCTCAAGTGGCGCGTCGAATGA

Consensus           TGATTGCCATcATGCTtGTTTCGTTTGTCATTGTCATTATGGTgATTCAGGATAGCAC.CCcTCTCAAGTgGCaCGcCGtATGA
```

Fig. 1. Representation of the nucleotide sequence from the *incA* gene of five *C. psittaci* strains and one 'C. *abortus*' strain, S26/3, and location of the primers and probe used for real-time PCR amplification. The sequences are shown from nucleotide 305 up to nucleotide 388. Primers are underlined and the probe is boxed. Mismatches are indicated in bold.

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 i.e. very close to −3·32 corresponding to the maximum efficiency. The regression curve was linear from 10^6 to 1 bacterial genome for a reaction volume of 25 µl.

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
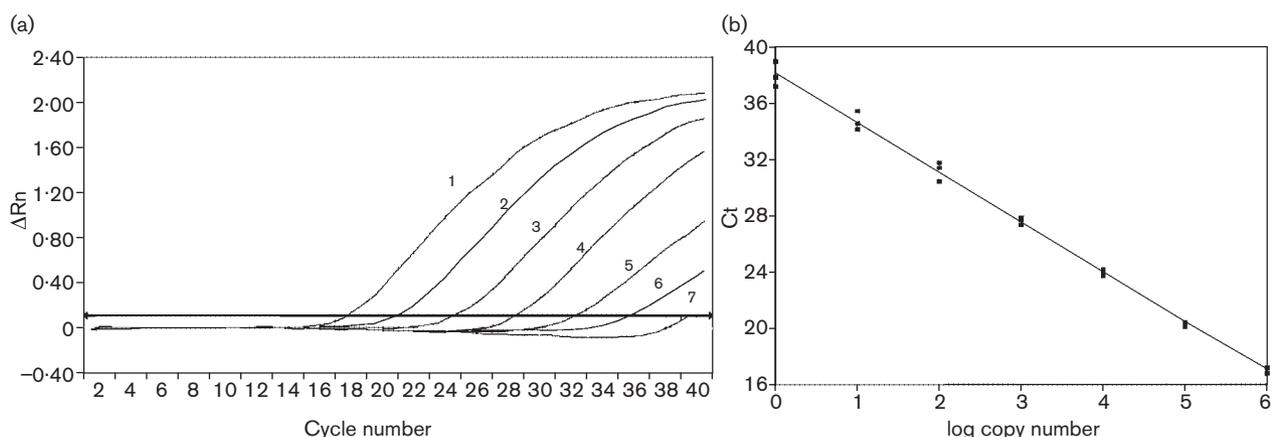

Fig. 2. Amplification plots and deduced standard curve. (a) Example of amplification plots from 1 to 7 generated from 10^6, 10^5, 10^4, 10^3, 10^2, 10 and 1 bacteria, respectively. The PCR cycle numbers are indicated on the *x* axis, and the corresponding ΔRn (magnitude of the signal generated by the specified set of PCR conditions) are given on the *y* axis. Fluorescence = f(cycles). (b) A standard curve was generated from each sample tested in triplicate. The copy numbers of *C. psittaci* genomic DNA used for the standard curve are indicated on the *x* axis, and the corresponding Ct (fractional cycle number at which the fluorescence passes the threshold) values are given on the *y* axis. Ct = f(log copy number). $y = -3·524 \times \log(x) + 38·12$. The $r2$ value from the linear regression in this assay is 0·99.

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.

Acknowledgements

Armelle Ménard,[1] Maithé Clerc,[2] Agathe Subtil,[3] Francis Mégraud,[1] Christiane Bébéar[2] and Bertille de Barbeyrac[2]

[1,2]INSERM ERI 10 Laboratoire de Bactériologie[1] and Centre National de Référence des Chlamydia, Laboratoire de Bactériologie[2], Université Victor Segalen Bordeaux 2, Bordeaux, France
[3]Unité de Biologie des Interactions Cellulaires, Institut Pasteur, CNRS URA 2582, Paris, France

Correspondence: Bertille de Barbeyrac (Bertille.de.Barbeyrac@u-bordeaux2.fr)

Bannantine, J. P., Stamm, W. E., Suchland, R. J. & Rockey, D. D. (1998). *Chlamydia trachomatis* IncA is localized to the inclusion membrane and is recognized by antisera from infected humans and primates. *Infect Immun* **66**, 6017–6021.

Corpet, F. (1988). Multiple sequence alignment with hierarchical clustering. *Nucleic Acids Res* **16**, 10881–10890.

DeGraves, F. J., Gao, D., Hehnen, H. R., Schlapp, T. & Kaltenboeck, B. (2003). Quantitative detection of *Chlamydia psittaci* and *C. pecorum* by high-sensitivity real-time PCR reveals high prevalence of vaginal infection in cattle. *J Clin Microbiol* **41**, 1726–1729.

Delevoye, C., Nilges, M., Dautry-Varsat, A. & Subtil, A. (2004). Conservation of the biochemical properties of IncA from *Chlamydia trachomatis* and *Chlamydia caviae*: oligomerization of IncA mediates interaction between facing membranes. *J Biol Chem* **279**, 46896–46906.

Everett, K. D., Bush, R. M. & Andersen, A. A. (1999). Emended description of the order Chlamydiales, proposal of Parachlamydiaceae fam. nov. and Simkaniaceae fam. nov., each containing one monotypic genus, revised taxonomy of the family *Chlamydiaceae*, including a new genus and five new species, and standards for the identification of organisms. *Int J Syst Bacteriol* **49**, 415–440.

Huang, J., DeGraves, F. J., Gao, D., Feng, P., Schlapp, T. & Kaltenboeck, B. (2001). Quantitative detection of *Chlamydia* spp. by fluorescent PCR in the LightCycler. *Biotechniques* **30**, 150–157.

Lederer, P. & Muller, R. (1999). Ornithosis – studies in correlation with an outbreak. *Gesundheitswesen* **61**, 614–619 (in German).

Maegawa, N., Emoto, T., Mori, H., Yamaguchi, D., Fujinaga, T., Tezuka, N., Sakai, N., Ohtsuka, N. & Fukuse, T. (2001). Two cases of *Chlamydia psittaci* infection occurring in employees of the same pet shop. *Nihon Kokyuki Gakkai Zasshi* **39**, 753–757 (in Japanese).

Martin, J. L. & Cross, G. F. (1997). Comparison of the *omp* I gene of *Chlamydia psittaci* between isolates in Victorian koalas and other animal species. *Aust Vet J* **75**, 579–582.

Scieux, C., Grimont, F., Regnault, B., Bianchi, A., Kowalski, S. & Grimont, P. A. (1993). Molecular typing of *Chlamydia trachomatis* by random amplification of polymorphic DNA. *Res Microbiol* **144**, 395–404.

Stubbs, R., Dralle, W. & Williams, J. (1989). Psittacosis pneumonia. *J Tenn Med Assoc* **82**, 189–190.

Sudler, C., Hoelzle, L. E., Schiller, I. & Hoop, R. K. (2004). Molecular characterisation of chlamydial isolates from birds. *Vet Microbiol* **98**, 235–241.