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New real-time PCR tests for species-specific detection of *Chlamydophila psittaci* and *Chlamydophila abortus* from tissue samples

Alexandra Pantchev [a], Reinhard Sting [a], Rolf Bauerfeind [b], Judith Tyczka [c], Konrad Sachse [d,*]

[a] *Chemisches und Veterinäruntersuchungsamt Stuttgart, 70736 Fellbach, Fellbach, Germany*
[b] *Institut für Hygiene und Infektionskrankheiten der Tiere, Universität Giessen, 35392 Giessen, Germany*
[c] *Chemisches und Veterinäruntersuchungsamt Karlsruhe, 76187 Karlsruhe, Germany*
[d] *Friedrich-Loeffler-Institut Jena, Nationales Referenzlabor für Psittakose, 07743 Jena, Germany*

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Abstract

Chlamydophila psittaci and *Chlamydophila abortus* are the causative agents of avian chlamydiosis (psittacosis) and ovine enzootic abortion, respectively. Both pathogens are known to possess zoonotic potential. Due to their close genetic relatedness, direct and rapid species identification is difficult. In the present study, new real-time PCR assays are reported for both species. The tests are based on highly specific probes targeting the *omp*A gene region and were conducted as duplex PCRs including an internal amplification control.

The *Cp. psittaci* assay successfully passed a proficiency test at national level. Examination of field samples revealed *Cp. psittaci* as the dominating species in birds, but also *Cp. abortus* in a few psittacines. Real-time PCR assays for species-specific detection of *Cp. psittaci* and *Cp. abortus* are suited for routine diagnosis, which renders them important tools for the recognition of outbreaks of psittacosis and ovine enzootic abortion.

Keywords: *Chlamydophila psittaci*; *Chlamydophila abortus*; Birds; Real-time PCR; Tissue samples

Introduction

Chlamydophila psittaci is a highly pathogenic bacterial agent causing avian chlamydiosis, which is also referred to as psittacosis in psittacines or ornithosis in other bird species. Chlamydial infection was shown to be present in more than 460 avian species of 30 orders (Kaleta and Taday, 2003). Zoonotic transmission to humans can lead to severe cases of respiratory disease (Vanrompay et al., 2007; Gaede et al., in press). Parrots and parakeets are particularly known to harbour *Cp. psittaci* strains that are highly virulent in humans. In addition to psittacine birds, domestic poultry, pigeons and wild birds can also be infected by virulent *Cp. psittaci* strains and can serve as source of human infection.

On account of the severity and potentially fatal outcome of human psittacosis, Germany and other countries introduced special legislation regulating notification and control of *Cp. psittaci* infections in birds. According to the current psittacosis regulation in Germany, species-specific identification of *Cp. psittaci* is required to diagnose psittacosis, which implies that mere detection of chlamydiae at the genus or family level is no longer sufficient. Recent studies providing evidence of the occurrence of chlamydial species other than *Cp. psittaci* in birds are also highly relevant. Of particular interest was the detection of *Cp. abortus* in turkeys (Sting et al., 2006), a budgerigar and two oriental white stork samples (Chahota et al., 2006). This microorganism, formerly classified as serotype 1 of *Chlamydia psittaci*, is better known as the agent of ovine enzootic abortion and was recently reclassified as a separate species of the *Chlamydiaceae* (Everett et al., 1999).

* Corresponding author. Tel.: +49 3641 804334; fax: +49 3641 804228.
E-mail address: konrad.sachse@fli.bund.de (K. Sachse).

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Because of the close genetic relatedness between *Cp. psittaci* and *Cp. abortus*, direct species identification remains a challenge. Currently, polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) and *omp*A gene sequencing are available, but these procedures are only conducted at specialised laboratories due to labour requirement and cost (Sachse and Grossmann, 2002).

The main aim of the present work was to design a real-time PCR assay to allow rapid, sensitive and specific detection of *Cp. psittaci* in a single protocol, thus offering a new test suitable for routine diagnostic purposes. We also aimed to develop a real-time PCR assay for identification of the closely related species *Cp. abortus*.

Materials and methods

Chlamydial strains

The following chlamydial strains (provided by the Institute for Hygiene and Infectious Diseases of Animals, University of Giessen) were used as reference material (identified by RFLP according to Everett and Andersen, 1999) for determination of specificity of the *Cp. psittaci*- and *Cp. abortus*-specific real-time PCR assays: Z 1904/82 (*Cp. psittaci* isolated from a budgerigar in Germany in 1982), Z 178/02 (*Cp.* abortus, sheep, Germany, 2002), 1215/86 (*Cp. abortus*, cattle, Germany, 1986), Z 114/02 (*Chlamydophila pecorum*, cattle, Germany, 2002), Z 3202-II/91 (*Chlamydophila felis*, cat, Germany, 1991), 432 (*Chlamydophila caviae*, guinea pig, Germany, year unknown), Z 359/02 (*Chlamydia suis*, pig, Germany, 2002).

The Friedrich-Loeffler-Institut Jena, provided two chlamydial strains for determination of sensitivity and specificity, namely, C5/98 (*Cp. psittaci*, calf, Germany, 1980) and C10/98 (*Cp. abortus*, sheep, Germany, 1993), and 11 strains for the national proficiency test, i.e. DC5 (*Cp. psittaci*, cattle, Germany,1998), DC9 (*Chlamydophila pneumoniae*, frog, Central African Republic, 2000), DC10 (*Chlamydophila trachomatis* D, ATCC VR-885, human), DC11 (*Cp. psittaci*, pig, Germany, 2001), DC14 (*Cp. psittaci*, cattle, Germany, 2001), DC15 (*Cp. psittaci*, cattle, Germany, 2001), DC20 (*C. suis*, pig, Germany, 2002), DC24 (*Cp. psittaci*, cattle, Germany, 2001), DC47 (*Cp. pecorum*, cattle, Germany, 2004), C12/98 (*Cp. psittaci*, cattle, Turkey, 1978) and C18/98 (*Cp. abortus*, B577, ATCC VR-656, sheep). The identity of these strains was determined using RFLP according to Everett and Andersen (1999) and DNA microarray testing according to Sachse et al. (2005).

Bacteria were cultivated in buffalo-green-monkey-kidney (BGM) cells using standard methodology (Sting et al., 2006). For quantitation, the chlamydial suspension in question was diluted and inoculated onto BGM monolayers grown on cover slips. At 48 h p.i., monolayers were fixed with methanol and stained with an anti-LPS *Chlamydiaceae*-specific fluorescein isothiocyanate-labelled antibody using the IMAGEN Chlamydia Kit (DakoCytomation). Cells containing inclusions were visualised using a BX 51 M fluorescence microscope (Olympus) at 400- and 1000-fold magnification. The number of inclusions was counted in 20 random fields and calculated as inclusion-forming units (IFU) per millilitre of the chlamydial suspension.

Non-chlamydial bacterial strains

Bacterial strains obtained from the American Type Culture Collection (ATCC) and the German Collection of Microorganisms and Cell Cultures (DSMZ), as well as field strains isolated from clinical material were selected for specificity testing. Field isolates were identified using standard biochemical tests as described by Quinn et al. (1994) or the API system tests (Apareil Pour Identification System, BioMérieux). These were:

ATCC strains: *Staphylococcus aureus* (ATCC 25923), *Campylobacter jejuni* subsp. *jejuni* (ATCC 49943), *Klebsiella pneumoniae* (ATCC 10031)

DSMZ strains: *Pseudomonas aeruginosa*, (DSM 50071), *Escherichia coli* (DSM 85799); *Salmonella enterica* subsp. *enterica* serovar Typhimurium (DSM 5569), *Enterococcus faecalis* (DSM 20478)

Field isolates: *Pasteurella multocida*, *Bordetella bronchiseptica*, *Streptococcus equi* spp. *equi*, *Streptococcus equi* subsp. *zooepidemicus*, *Proteus vulgaris*, *Arcanobacterium pyogenes*, *Haemophilus parasuis*, and *Actinobacillus pleuropneumoniae*.

Chlamydiaceae field samples

All *Chlamydiaceae* specimens were provided as direct DNA extracts from swabs and faecal samples. The original samples had been collected from parrots ($n = 35$) and parakeets ($n = 42$) with clinical signs or evidence of psittacosis in 2004–2006. Samples testing positive in a *Chlamydiaceae*-specific real-time PCR according to Ehricht et al. (2006) were re-tested using the species-specific *Cp. psittaci* and *Cp. abortus* real-time PCR assays described in this study. Fifty DNA specimens were kindly provided by VetMedLabor (IDEXX). Additionally, 27 DNA samples that were submitted to our laboratory for chlamydia diagnosis were also included in this study.

Preparation of genomic DNA from bacteria

DNA extraction from purified chlamydial suspensions was performed using the QIAamp MinElute Virus Spin Kit (Qiagen), following the manufacturer's instructions. For specificity testing, five colonies each of other bacterial species (see above) were suspended in 500 μL sterile PCR water (Sigma) and boiled at 99 °C for 10 min. After cooling and centrifugation at 15,000 *g* for 10 min, the supernatant was used for PCR.

Primers and probes

Sequences of the *omp*A, 16S and 23S rRNA genes and the intergenic spacer of all chlamydial species were obtained from the public database GenBank of the National Centre of Biotechnology Information (NCBI)[1] and aligned using Clustal W[2] and Boxshade 3.21[3]. The alignment revealed that the *omp*A gene offered the most promising segments for unambiguous discrimination among chlamydial species by real-time PCR. These segments were pasted into the Primer Express 2.0 Software (Applied Biosystems) to select primer and probe sequences. In accordance with the program's instructions, melting temperatures (T_m) were kept between 58 and 60 °C for the primers, 65 and 67 °C for the *Cp. psittaci* probe, and 68 and 70 °C for the *Cp. abortus* probe. While specific detection of *Cp. abortus* could be assured by a conventional TaqMan probe, it was necessary to design a TaqMan minor groove binding (MGB) probe for unambiguous identification of *Cp. psittac*i to cope with the high similarity between target sequences of *Cp. psittaci* and *Cp. abortus*. Sequences of primers and probes are given in Table 1.

All oligonucleotides were synthesised by Applied Biosystems and tested at concentrations of 0.3, 0.6 or 0.9 μM (final concentration of primers) and 0.1, 0.2 or 0.4 μM (final concentration of probes), respectively.

Real-time PCR

Real-time PCR was performed in microtitre plates (Abgene) as a duplex PCR using the 2 × TaqMan Universal PCR Master Mix supplemented with ROX (Applied Biosystems) including an internal amplification control (IAC). The IAC system was developed by Hoffmann et al. (2006) and included 0.3 μM of both primer EGFP1-F (GAC CAC TAC CAG CAG AAC AC), and primer EGFP10-R (CTT GTA CAG CTC GTC CAT GC), as well as 0.2 μM of the TaqMan probe EGFP-HEX (HEX-AGC ACC CAG TCC GCC CTG AGC A-BHQ1), respectively.

[1] See: http://www.ncbi.nlm.nih.gov/
[2] See: http://clustalw.genome.ad.jp/
[3] See: www.ch.embnet.org/software/BOX_form.html