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Original article

Development of a *Chlamydophila psittaci* species-specific and genotype-specific real-time PCR

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Abstract – A *Chlamydophila psittaci* species-specific real-time PCR targeting the rDNA ribosomal spacer was developed as well as a genotype-specific real-time PCR targeting the *Cp. psittaci* outer membrane protein A (*ompA*) gene. The SYBR Green-based species-specific real-time PCR detected *Cp. psittaci* genotypes A to F, and the recently discovered E/B genotype. The genotype-specific real-time PCR could easily distinguish genotypes C, D, F by use of TaqMan probes. Genotypes A, B and E could not be distinguished from each other by simply using TaqMan probes. For this purpose, non-fluorescent competitor oligonucleotides, had to be used next to the TaqMan probes. Genotype E/B could only be detected by use of a minor groove binder (MGB) probe. Both real-time PCR assays allowed reproducible, sensitive (10 rDNA or *ompA* copies/µL DNA extract) and specific detection of *Cp. psittaci* DNA. The genotype-specific real-time PCR was compared to *ompA* sequencing and *ompA* restriction fragment length polymorphism (RFLP) analysis using five *Cp. psittaci* field isolates (99, 61/8, 7344/2, 8615/1 and 7778B15) each consisting of two different genotypes. The currently developed real-time PCR assays were used in a case study on a veterinary school and a turkey farm. In the veterinary school, *Cp. psittaci* genotypes D, E/B and F infection were detected in all five groups of turkeys, and one veterinarian who was taking care of all these turkeys. On the turkey farm, the presence of two *Cp. psittaci* genotype B infection waves was demonstrated in one randomly selected turkey, the first wave at the age of 6 weeks, and the second at the age of 12 weeks.

***Chlamydophila psittaci* / real-time PCR / species-specific / genotype-specific / diagnosis**

1. INTRODUCTION

Chlamydiaceae are Gram-negative obligate intracellular bacteria replicating in mucosal epithelial cells and macrophages, causing disease in birds, humans, other mammals and marsupials. *Chlamydophila psittaci* (formerly *Chlamydia psittaci*) is a respiratory avian pathogen able to cause zoonotic disease in humans. Human psittacosis mostly originates from exposure to infected psittacines, pigeons or poultry, mainly turkeys [11] or ducks [14, 18]. Symptoms in man vary from asymptomatic to severe systemic disease [3].

Cp. psittaci in birds and man still represents a diagnostic challenge. Isolation is

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Table I. *Cp. psittaci* strains used for developing species- and genotype-specific primers and probes as well as inhibition control plasmids.

Strain	Reference	Country	Host	Genotype	Control plasmid
90/1051	[9]	Belgium	*Amazona* sp.	A	pGemT::CpPsGAS
41A12	[9]	Belgium	*Meleagris gallopavo*	B	pGemT::CpPsGBS
GD	[15]	Gemany	*Anas platyrhyncos*	C	pGemT::CpPsGCS
7344/2	[9]	Italy	*Columba livia*	D	pGemT::CpPsGDS
3759/2	[9]	Italy	*Columba livia*	E	pGemT::CpPsGES pGemT::CpPsSS
7778B15	[9]	Belgium	*Meleagris gallopavo*	F	pGemT::CpPsGFS
WS/RT/E30	[9]	Germany	*Anas platyrhyncos*	E/B	pGemT::CpPsGE/BS

labour-intensive, relatively insensitive and not without danger. In birds, serology is used and is nowadays mostly performed by an enzyme linked immunosorbent assay [6, 21]. However the interpretation of the results is often difficult since most birds have pre-existing antibodies from previous exposures, and antibodies can persist for up to several months. In man, the complement binding assay (CBA) is often used. However, the CBA cannot distinguish *Cp. psittaci* specific antibodies from antibodies against other chlamydial human pathogens, like *Cp. pneumoniae* and *Chlamydia trachomatis*. As a result, the CBA is more and more often replaced by the micro-immunofluorescence (MIF) test. The MIF test distinguishes all chlamydial species and measures IgG as well as IgM titres, allowing the detection of recent infections. However, early antibiotic treatment can interfere with antibody formation and in some patients, antibody responses can develop rather slowly. Moreover, obligatory examination of paired sera removes serology from immediate clinical relevance. Therefore, several commercial antigen detection methods were developed for both birds and man but they are either insensitive and/or less specific [21, 25]. Due to these shortcomings, nucleic acid amplification methods have been designed. However, currently described polymerase chain reaction (PCR) assays use labour-intensive and/or insensitive post PCR detection methods and are not quantitative or only semi-quantitative. In addition, genotyping still needs to be performed by *ompA* restriction fragment length polymorphism (RFLP) analysis and *ompA* sequencing, and both techniques often require bacterial culture, since amplification of full length *ompA* can mostly not be carried out directly from clinical specimens.

The present study describes the development of a *Cp. psittaci* species-specific real-time PCR. Additionally, we describe the development of a genotype-specific real-time PCR allowing the identification of all avian *Cp. psittaci* genotypes including the recently discovered new E/B genotype [9]. The performance of the genotype-specific real-time PCR was compared to *ompA* RFLP analysis and *ompA* sequencing. The species- and genotype-specific real-time PCR assays were used in a case study in a veterinary school and on a turkey farm.

2. MATERIALS AND METHODS

2.1. Bacterial cultures

Cp. psittaci genotypes A to F plus E/B strains 90/1051, 41A12, GD, 7344/2, 3759/2, 7778B15 and WS/RT/E30 (Tab. I) were used for the development of the species- and genotype-specific real-time PCR assays.

Bacteria were grown in cycloheximide treated Buffalo Green Monkey (BGM) cells as described previously [26]. For each strain, an infected monolayer of 300 cm^2 was disrupted by freezing and thawing, followed by ultrasonic treatment for 1 min in a tabletop sonicator (Bransonic 12, BIOMEDevice, San Pablo, CA, USA). A two-hundred millilitre cell culture harvest was centrifuged for 10 min (1 000 × *g*, 4 °C) and subsequently concentrated by ultracentrifugation for 1 h (45 000 × *g*, 4 °C). Bacteria were resuspended in 2 mL sucrose phosphate glutamate buffer (SPG, 218 mM sucrose, 38 mM KH_2PO_4, 7 mM K_2HPO_4, 5 mM L-glutamic acid) and stored at –80 °C until use.

2.2. Preparation of genomic DNA

Genomic DNA for real-time PCR assays was prepared as described by Wilson et al. [34]. DNA samples were further purified by extracting them twice with 200 µL phenol-chlorophorm (1:1). Precipitation was performed (1 h, –80 °C) by adding 20 µL sodiumacetate (3M) and 400 µL of 100% ethanol. The pellets obtained following centrifugation (20 min, 4 °C, 16 060 × *g*) were washed for 5 min with 500 µL of 70% ethanol (4 °C, 16 060 × *g*) and were finally resuspended in 30 µL bidest.

2.3. Species-specific primers and inhibition control plasmid

Published ribosomal spacer sequences of all chlamydial species [7] were aligned using ClustalX software (default settings) [22]. *Cp. psittaci* species-specific forward (CpPsSSfor) and reverse (CpPsSSrev) primers (Isogen Life Sciences, Maarssen, The Netherlands) were designed using Primer Express Software (Applied Biosystems, Foster City, CA, USA). Primer specificity was checked by BLAST [1].

Subsequently, the rDNA of genotypes A to E/B (Tab. I) was PCR amplified using 35 cycles of 95 °C for 20 s, 63 °C for 20 s and 72 °C for 30 s. PCR products were purified using Qiagen spin columns (Westburg, Leusden, The Netherlands) and cloned into pGem®-T (Promega, Madison, WI, USA) following the manufacturer's protocol. Sequence analyses were performed by the VIB Genetic Service Facility (University of Antwerp, Antwerp, Belgium) using vector associated T7 and SP6 priming sites. Sequence alignment using ClustalX software [22] allowed us to select a *Cp. psittaci* species-specific inhibition control plasmid (pGemT::CpPsSS, Tab. I).

2.4. Species-specific real-time PCR

Real-time PCR was performed with the LightCycler 2.0 Instrument (Roche, Applied Science, Penzberg, Germany) using the LightCycler FastStart DNA MasterPLUS SYBR Green I kit and LightCycler Capillaries. The reaction mixture (20 µL) was prepared according to the manufacturer's protocol: 11 µL PCR grade water, 2 µL of primer mixture (300 nM CpPsSSfor and CpPsSSrev), 2 µL 10× Master Mix, 5 µL of DNA template. The cycling conditions were as follows: 50 cycles of 95 °C for 10 s, 63 °C for 10 s and 72 °C for 8 s. All default program settings were used. Standard graphs of the Cycle threshold (Ct) values, obtained by testing tenfold serial dilutions (10^8 to 10^1) of the purified species-specific inhibition control plasmid, were used for quantification. Ct-values were automatically converted into initial template quantities (N_0) using the LightCycler Software 4.0. DNA from clinical samples was always tested in the presence of control plasmid (50 copies/µL) to check for PCR inhibitors.

2.5. Genotype-specific primers, probes and inhibition control plasmids

The *ompA* gene of the genotypes A to F and E/B reference strains (Tab. I) was amplified as described previously [32]. *OmpA* genes were cloned in pGEM®-T and sequenced by the VIB Genetic Service Facility (Antwerp, Belgium). Plasmids were used as

inhibition controls in the genotype-specific real-time PCR. Genotypes A to E/B specific primers (e.g. *Cp. psittaci* Genotype A-Specific forward and reverse primer; CpPsGASfor and CpPsGASrev), (Invitrogen, Carlsbad, CA, USA) as well as genotype-specific TaqMan probes (e.g. CpPsGAS-pro) (Applied Biosystems, Foster City, CA, USA) were designed from aligned variable segments of presently sequenced *ompA* genes using Primer Express Software (Applied Biosystems). Specificities were checked using BLAST [1].

2.6. Genotype-specific real-time PCR

Genotype-specific probes (Applied Biosystems) were 5' and 3' labelled with the reporter dye 6-carboxyfluorescein (FAM) and the quencher dye carboxytetramethylrhodamine (TAMRA), respectively. Genotype-specific reactions were performed with the LightCycler 2.0 instrument in LightCycler Capillaries, using the LightCycler FastStart DNA MasterPLUS Hybridisation Probes kit, in a total reaction volume of 20 µL (9 µL PCR grade water, 2 µL of primer (300 nM) / probe (300 nM) / competitor (50 nM or 150 nM) mixture, 4 µL 5× Master Mix, 5 µL of DNA template). Cycling conditions were as follows: 95 °C for 10 min and subsequently 50 cycles of 95 °C for 10 s followed by 63 °C (genotype B) or 60 °C (all other genotypes), for 20 s. Genotypes A, B and E could only be distinguished by using competitor oligonucleotides enhancing the TaqMan probe specificity. The detection of the E/B genotype was made possible using a minor groove binder (MGB) probe (Applied Biosystems) [17]. Standard Ct-value graphs obtained from testing serial dilutions of purified control plasmids (10^8 to 10^1) were used for quantification. Clinical samples from case studies were tested in the presence of genotype-specific control plasmids (50 *ompA* copies/µL) in order to check for PCR inhibitors. Ct-values for clinical samples were plotted against standard graphs and *Cp. psittaci* genotypes present in clinical samples (N_0) were quantified using the LightCycler 4.0 software.

2.7. Analytical sensitivity and specificity

The analytical sensitivity of both PCR assays was evaluated using tenfold serial dilutions (10^8 to 10^1 copies/µL) of all inhibition control plasmids. The specificity was evaluated using (1) genomic DNA from *Cp. pneumoniae*, *Cp. felis*, *Cp. caviae*, *Cp. abortus*, *Cp. psittaci*, *C. muridarum* and *C. trachomatis* as well as (2) DNA from other bacterial species commonly found in the avian or human respiratory tract and (3) genomic DNA from avian and human respiratory tissue (Tab. II).

2.8. Genotype-specific real-time PCR compared to *ompA* RFLP analysis and *ompA* sequencing

We previously sequenced the *ompA* genes of 21 European *Cp. psittaci* field isolates, revealing the presence of five mixed-genotype infections [9]. Five field isolates (99, 61/8, 7344/2, 8615/1 and 7778B15), each consisting of two different genotypes, were selected in order to compare the performances of the genotype-specific real-time PCR, *ompA* RFLP analysis and *ompA* sequencing. The latter two tests were performed as described elsewhere [32].

2.9. Case studies

2.9.1. In a veterinary school

Five groups of five conventional turkeys were brought to the Faculty of Veterinary Medicine (Ghent University) to become experimentally infected with *Ornithobacterium rhinotracheale* (ORT). However, the birds started to die, even before being infected, showing severe respiratory disease. Routine bacteriology and virology revealed no pathogens. Therefore, all remaining animals were sampled using one pharyngeal

Table II. Organisms of non-chlamydial origin used for specificity testing.

Human pathogens		Avian pathogens
Acinetobacter baumannii	*Proteus mirabilis*	*Acinetobacter* sp.
Bacteroides fragilis	*Proteus vulgaris*	*Aspergillus flavus*
Bordetella bronchiseptica	*Salmonella* sp.	*Candida albicans*
Citrobacter braakii	*Serratia marescens*	*Enterococcus faecelis*
Citrobacter freundii	*Staphylococcus aureus*	*Escherichia coli*
Corynebacterium urealyticum	*Stenotrophomonas*	*Klebsiella* sp.
Enterobacter aerogenes	*maltophilia*	*Mycobacterium avium*
Enterococcus sp.	*Streptococcus agalactiae*	*Mycoplasma gallisepticum*
Escherichia coli	*Streptococcus nilleri*	*Mycoplasma meleagridis*
Haemophilus influenza	*Streptococcus pyogenes*	*Ornithobacterium rhinotracheale*
Hafnia alvei		*Pasteurella* sp.
Klebsiella oxytoca		*Proteus mirabilis*
Klebsiella pneumoniae		*Pseudomonas* sp.
Legionella pneumophila		*Salmonella enteritidis*
Moraxella catarrhalis		*Salmonella gallinarum*
Morganella morganii		*Salmonella pullorum*
Mycoplasma pneumoniae		*Staphylococcus* sp.
Pneumococcus sp.		*Streptococcus* sp.
Propionibacterium sp.		*Xanthomonas maltophila*

swab for each group. Being aware of having a possible *Cp. psittaci* infection, the responsible veterinarian was sampled twice, providing a pharyngeal swab on the same day (t1) and a second one two weeks later (t2). All swabs were used for isolation in BGM cells as described previously [32] as well as for the species- and genotype-specific real-time PCR.

2.9.2. On a turkey farm

The occurrence of *Cp. psittaci* on a Belgian turkey farm was examined from production onset until slaughter at 15 weeks of age. Seroconversion demonstrated the presence of a first *Cp. psittaci* infection when the turkeys were 3 to 6 weeks of age, and a second one when they were 8 to 12 weeks of age [24]. Sequencing revealed the presence of the *Cp. psittaci* genotype B during both infection waves [9]. Weighted pharyngeal swabs from one randomly selected turkey at the age of 3, 6, 8, 12 and 15 weeks were used for the genotype B specific real-time PCR in order to confirm *ompA* sequencing results.

3. RESULTS

3.1. Species-specific primers and inhibition control plasmid

Cp. psittaci species-specific forward (CpPsSSfor) and reverse (CpPsSSrev) primers generated a PCR product of 151 bp (Tab. III). Aligment of rDNA of genotypes A to E/B allowed us to choose a *Cp. psittaci* species-specific internal inhibition control plasmid designated pGemT::CpPsSS (Tab. I).

Table III. Primers, probes and competitors.

Oligonucleotide	Sequence (5'-3')	Position[a]	Specificity
CpPsSSfor	TTATTAAGAGCTATTGGTGGATGCC	1 822	*Cp. psittaci*
CpPsSSrev	AACGTATAATGGTAGATGATTAATCTACCG	1 972	
CpPsGASfor	GGTTTTCAGCTGCAAGCTCAA	488	Genotype A
CpPsGASpro	CTACCGATCTTCCAACGCAACTTCCTAACG	512	
CpPsGAScomB	CTACCGATCTTCCAATGCAACTTCCTAACG[b]	512	
CpPsGASrev	CCACAACACCTTGGGTAATGC	565	
CpPsGBSfor	AATAGGGTTTTCAGCTACCAACTCAA	483	Genotype B
CpPsGBSpro	TCTACCGATCTTCCAATGCAACTTCCTAACGTA	511	
CpPsGBScomA	TCTACCGATCTTCCAACGCAACTTCCTAACGTA	511	
CpPsGBScomE+E/B	TCTACCGAGCTTCCAATGCAACTTCCTAACGTA	511	
CpPsGBSrev	CCACAACACCTTGGGTAATGC	565	
CpPsGCSfor	GCATCGCTCAACCTAAATTGG	929	Genotype C
CpPsGCSpro	TCTGCTGTTATGAACTTGACCACATGGAACC	952	
CpPsGCSrev	ATTGTGGCTTCCCCTAAAAGG	1 009	
CpPsGDSfor	AACCACTTGGAACCCAACACTTT	969	Genotype D
CpPsGDSpro	AGGAAAGGCCACAACTGTCGACGG	993	
CpPsGDSrev	CGAAGCAAGTTGTAAGAAGTCAGAGTAA	1 062	
CpPsGESfor	CCAAGCCTTCTAGGATCAAGGA	982	Genotype E
CpPsGESpro	TACTTTGCCCAATAATGGTGGTAAGGATGTTCTATC	1 005	
CpPsGEScomA+B	TGCTTTGCCCAATAATAGTGGTAAGGATGTTCTATC	1 005	
CpPsGEScomE/B	TGCTTTGCCCAATAATGCTGGTAAGGATGTTCTATC	1 005	
CpPsGESrev	CGAAGCAATTTGCAAGACATCA	1 062	
CpPsGFSfor	GCAACTTTTGATGCTGACTCTATCC	904	Genotype F
CpPsGFSpro	CATCGCTCAACCTAAATTAGCCGCTGC	930	
CpPsGFSrev	GTTCCATGTGGTCAAGTTCAAAAC	981	
CpPsGE/BSfor	CCAAGCCTTCTAGGATCAACCA	982	Genotype E/B
CpPsGE/BSpro	TGCTTTGCCCAATAATGCTG[c]	1 005	
CpPsGE/BScomA+B	TGCTTTGCCCAATAATAGTG	1 005	
CpPsGE/BScomE	TACTTTGCCCAATAATGGTG	1 005	
CpPsGE/BSrev	TGCAAGACATCAGATAGAACATCCTT	1 052	

[a] Binding position of the 5' end on the reference strain.
[b] Nucleotide mismatches between the competitor and the probe are marked in grey.
[c] MGB probe.

3.2. Species-specific PCR

The species-specific PCR detected all known *Cp. psittaci* genotypes, including the recently discovered E/B genotype. Amplification of genomic DNA extracts of all *Cp. psittaci* genotypes resulted in the expected PCR products with amplification curves exceeding the 0.02 threshold before cycle 23. The species-specific PCR was able to detect 10 rDNA copies/µL DNA extract.

The species-specificity test revealed a Ct-value of 20 for *Cp. psittaci* with a product melting temperature of 79.3 °C. Amplification of *Cp. pneumoniae*, *Cp. felis*, *Cp. abortus*, *C. muridarum* and *C. trachomatis* rDNA generated no reaction products. However, amplification of *Cp. caviae* rDNA generated a fluorescent signal at a Ct-value above 37. The amplified rDNA showed a melting temperature of 79.0 °C instead of 79.3 °C. Sequencing revealed a *Cp. caviae*-specific rDNA sequence of 151 bp. The *Cp. psittaci* species-specific primers did not react with other pathogens commonly found in the avian and human respiratory tract (Tab. II), neither with DNA from avian or human respiratory tissue.

3.3. Genotype-specific primers, probes and inhibition control plasmids

The cloned *ompA* genes from *Cp. psittaci* genotypes A to E/B revealed sequences of 1 065 to 1 098 bp depending on the genotype (GenBank accession numbers AY762608, AY762609, AY762610, AY762611, AY762612 and AY762613). Sequences of the determined genotype-specific primers generating amplicons of 71 to 85 bp as well as from the genotype-specific TaqMan probes are listed in Table III. The detection of the E/B genotype was only possible using a minor groove binding (MGB) probe and genotypes A, B and E could only be distinguished from each other using competitor oligonucleotides next to the TaqMan probes (Tab. III).

3.4. Genotype-specific PCR

We developed a genotype-specific real-time PCR detecting all 7 known avian *Cp. psittaci* genotypes. Genotype C-, D-, F- and E/B- specific probes specifically reacted with the corresponding genotypes and were therefore easily distinguishable. However, the genotype A-, B- and E- specific TaqMan probes not only reacted with the corresponding genotype, but also with the two other genotypes. Adding 50 mM of competitors to the PCR reaction mixture in combination with the use of a cycling temperature of 63 °C for the genotype B specific reaction, could solve the problem (Tab. III). For the genotype B specific reaction competitor CpPsGBScomA had to be added. Genotype A specific detection required CpPsGAScomB, while specific detection of genotype E required CpPsGEScomA, CpPsGAScomB and CpPsGEScomE/B. The detection limit of the genotype-specific real-time PCR was 10 *ompA* copies/µL DNA extract. Standard curves made using 10^8 to 10^1 copies/µL showed almost ideal slopes around –3.3 with correlation coefficients > 98.5%. The *Cp. psittaci* genotype-specific primers did not react with other pathogens commonly found in the avian and human respiratory tract (Tab. II) neither with DNA from avian or human respiratory tissue.

3.5. Genotype-specific real-time PCR compared to *ompA* RFLP and *ompA* sequencing

Five *Cp. psittaci* field isolates, each consisting of a mixture of 2 different genotypes were used to compare the developed genotype-specific real-time PCR with the overall used *ompA* RFLP genotyping method and *ompA* sequencing. All 10 genotypes, present in those 5 field isolates, could be detected by the genotype-specific real-time PCR, including the recently discovered E/B genotype (Tab. IV). *OmpA* RFLP incorrectly characterised E/B genotype as genotype E.

3.6. Case studies

3.6.1. In a veterinary school

Cp. psittaci was isolated from all examined samples, which was confirmed by the species-specific real-time PCR detecting *Cp. psittaci* rDNA in all pharyngeal swabs. Ribosomal DNA copies/µL DNA extract in

Table IV. Comparison of the genotype-specific real-time PCR with *ompA* RFLP analysis using 5 isolates each consisting of 2 different genotypes as determined with *ompA* sequencing.

Isolate	*OmpA* sequencing	*OmpA* RFLP	Real-time PCR No.
99	A	A	918
	E/B	E	4 020
61/8	A	A	111 000
	E/B	E	422 000
7344/2	B	B	5 160
	D	D	1 840
8615/1	B	B	9 000
	E/B	E	11 200
7778B15	B	B	3 660
	F	F	2 900

turkey groups taken at t1 varied from 790 to 1 772 while the human sample taken at t1 and t2 showed 2 160 and 710 rDNA copies/µL DNA extract, respectively. Genotype-specific real-time PCR revealed the presence of genotypes D, F and E/B in all turkey groups as well as in the veterinarian. Turkeys as well as the veterinarian showed the highest amount of *ompA* copies/µL DNA extract for genotype E/B (up to 13 000 for both turkeys and the veterinarian) followed by genotype F (up to 7 000 for turkeys and up to 2 000 for the veterinarian) and genotype D (up to 1 000 for turkeys and up to 2 000 for the veterinarian). The veterinarian showed no clinical signs at both sampling time points.

3.6.2. On a turkey farm

DNA extracts from five different pharyngeal swabs (weeks 3, 6, 8, 12 and 15) of one randomly selected turkey, shown previously to be infected with genotype B, were tested in the genotype-specific real-time PCR. Seroconversions, demonstrated elsewhere when turkeys were 6 weeks of age and 12 weeks of age, were correlated to a two- to threefold increase in *Cp. psittaci* genotype B *ompA* DNA/µL DNA extract.

4. DISCUSSION

Real-time technology offers the possibility to automatically combine amplification, specific hybridisation and detection in one single test, allowing specific and sensitive gene quantification with a minimal contamination risk. The technique has been used to detect *Cp. pneumoniae* [10, 16, 23, 33], *C. trachomatis* [19], *Cp. felis* [12, 13, 20] and *Cp. pecorum* [5]. The present study is the first to demonstrate the use of real-time PCR to specifically detect *Cp. psittaci* as well as its presently known genotypes. In the currently developed real-time PCR, kits with the AmpErase® UNG system incorporated were used to prevent post PCR carry over, and constructed species- or genotype-specific plasmids were applied in the assays as internal inhibition controls. The present data show our species-specific and genotype-specific real-time PCR assays to be potentially useful for testing both avian (chlamydiosis) and human (psittacosis) specimens. The species-specific real-time PCR could be suitable for diagnosing psittacosis in man, by distinguishing the human pathogens *C. trachomatis* and *Cp. pneumoniae* from the zoonotic agents *Cp. felis*, *Cp. abortus* and *Cp. psittaci*. The genotype-specific PCR could be used for epidemiological research in both birds and humans. However, further research is needed.

The specificity of all our primers was checked by alignment with sequences deposited in public databases revealing only perfect matches with *Cp. psittaci* DNA. Only avian chlamydial DNA could be amplified with the exception of *Cp. caviae* DNA, generating a *Cp. caviae*-specific amplicon in the *Cp. psittaci* species-specific real-time PCR. However, the *Cp. caviae* amplicon could easily be distinguished from the *Cp. psittaci* species-specific amplicon since the Ct-value of the latter amplification was remarkably lower (20 against > 37) and the melting temperature of the non-specific amplicon was 0.3 °C higher than for the *Cp. psittaci* specific amplicon. Moreover, *Cp. caviae* is normally not present in birds

and humans since it only infects guinea pigs and therefore does not compromise the use of the currently developed *Cp. psittaci* species-specific real-time PCR in birds and humans.

Cp. psittaci comprises 9 known *ompA* genotypes (A to F, E/B, WC and M56). All of them, except for genotype E/B can be identified using *ompA* RFLP analysis [30] or *ompA* sequencing [4]. Genotype E/B can only be detected using *ompA* sequencing since it generates the same restriction pattern as genotype E [9]. Genotypes A, B and D differ in pathogenicity for turkeys [28, 29] but the pathogenic significance of avian genotypes for different host species, including man has to be investigated further. The presently developed genotype-specific real-time PCR, allowing the detection of the newly discovered E/B genotype by the use of an MGB probe, could contribute to this research. MGB probes are shorter than normal TaqMan probes and show a higher specificity for single base mismatches. Moreover, their fluorescence quenching is higher, resulting in increased sensitivity [17]. We used competing oligonucleotides to distinguish genotypes A, B and E. Competitors have already been used in Fluorescence In Situ Hybridisation (FISH), blocking non-specific probe binding sites on contaminating DNA and as such increasing the accessibility of the target DNA for the labelled probes [8]. However, the use of these non-fluorescent helper oligonucleotides is innovative in real-time technology. The genotype-specific real-time PCR was certainly more rapid, easier to perform and more sensitive than *ompA* RFLP and *ompA* sequencing since the latter two typing methods often required bacterial culture. Moreover, all genotypes present in the 5 "mixed infection" samples could be detected. Infections with two different *Cp. psittaci* strains have already been demonstrated in the past by Vanrompay et al., detecting two different *ompA* serovars in 4 of 105 examined bird infections [27, 30]. The genotype-specific real-time PCR demonstrated the presence of two different genotypes in 5 of the 21 examined birds, which is remarkably high as compared to serotyping, indicating that genotype-specific real-time PCR is more suitable for epidemiological examinations.

Relative copy numbers could be determined in both PCR assays by using standard curves for the target of interest. The analytical sensitivity of the species-specific and genotype-specific real-time PCR was 10 rDNA or *ompA* copies/µL DNA extract.

The species- and genotype-specific real-time PCR assays were successfully used in two different case studies. In the first case study, turkeys were infected with three different genotypes showing the highest amount of genotype E/B organisms in each swab followed by genotype F and D, respectively. Genotype E/B has mainly been detected in ducks but also in two pigeons and one parrot. Clinical data on the ducks and the parrot were not available. The pigeons showed no clinical signs [9]. This is the first time genotype E/B has been discovered in turkeys. So far, genotype F has only been discovered in one American parakeet (strain VS225) and one Belgian turkey (strain 7778B15) [2, 9]. Genotype D has often been isolated from turkeys being responsible for either severe or mild respiratory signs [31]. As for the turkeys, the veterinarian also had the highest amount of genotype E/B organisms present in each pharyngeal swab. Accurate quantification in swabs taken at different time points was rather difficult since the swabs were not weighted as in the case study on the turkey farm and primers for human house keeping genes were not included. The veterinarian showed no clinical signs at sampling times, which can be normal regarding the incubation time of approximately two weeks and in the following weeks and months, the veterinarian experienced only the usual "common cold". This is the first report on *Cp. psittaci* genotype D, E/B and F infection in man. In the second case study, the genotype B infection on the farm could be proven and the number of *ompA* copies/µL of DNA extract corresponded remarkably

well to the observed seroconversion, indicative of the presence of replicating bacteria.

The present results show the potential usefulness of the *Cp. psittaci* species-specific and genotype-specific real-time PCR in both the veterinary and human clinic. However, technical performances of both assays can be further improved by including primers for avian and human house keeping genes allowing a more accurate quantification of bacterial DNA. Quantification is especially useful when monitoring an infection or antibiotic treatment. In addition, the genotype-specific real-time PCR could be adapted to a more cost-effective multiplex assay.

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