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Original Scientific Article

DETECTION OF *CHLAMYDIA PSITTACI* IN CAGE BIRDS IN SLOVENIA BY REAL-TIME PCR

Cvetka Marhold[1], Brigita Slavec[1], Karine Laroucau[2], Fabien Vorimore[2], Jožko Račnik[1], Marko Zadravec[1], Darja Keše[3], Uroš Krapež[1], Alenka Dovč[1]*

[1]Institute for Health Care of Poultry, Veterinary Faculty, Gerbičeva 60, 1000 Ljubljana, Slovenia; [2]Bacterial Zoonoses Unit, French Agency for Food, Environmental & Occupational Health Safety (Anses), Maisons-Alfort, France; [3]Institute of Microbiology and Immunology, Faculty of Medicine, Zaloška 4, 1000 Ljubljana, Slovenia

*Corresponding author, E-mail: alenka.dovc@vf.uni-lj.si

Summary: Avian chlamydiosis is a zoonotic disease of birds caused by the bacterium *Chlamydia psittaci*. The highest infection rates are found in psittacine birds (Psittacidae) which are the most common cage birds. *C. psittaci* causes infections of the conjunctiva, respiratory tract, and digestive tract, with or without clinical signs in birds. Infected birds can shed chlamydiae through respiratory tract excretions and in faeces. Transmission of *C. psittaci* primarily occurs by close contact of infected bird to the susceptible bird or human.

To determine the prevalence of *C. psittaci* in cage birds in Slovenia, oropharyngeal and cloacal swabs from 125 cage birds were examined by *Chlamydiaceae*-specific real-time polymerase chain reaction (real-time PCR). Two lovebirds (2/12) and a budgerigar (1/44) were positive for *Chlamydiaceae* by real-time PCR and were also positive for *C. psittaci* by an *omp*A-based real-time PCR assay specific for *C. psittaci*. Multiple loci variable number of tandem repeats analysis (MLVA) identified *C. psittaci* of genotype A in the positive budgerigar and *C. psittaci* of genotype B in the two positive lovebirds. The infected birds had no significant clinical signs of avian chlamydiosis on clinical examination. Using real-time PCR, the study showed a low prevalence (2.4 %) of *C. psittaci* in Slovenia.

Key words: *Chlamydia psittaci*; real-time PCR; MLVA; cage birds; Slovenia

Introduction

Chlamydia psittaci (*C. psittaci*) is a member of the *Chlamydiaceae* family and is an obligatory intracellular, gram-negative bacterium, which can infect many avian species as well as a wide range of mammalian hosts (1). The disease caused by *C. psittaci* is designated avian chlamydiosis, originally termed psittacosis or parrot fever in psittacine birds and ornithosis in other bird species (2, 3). The clinical disease depends on the chlamydial strains and avian host. Symptoms in the affected bird can include respiratory distress, keratoconjunctivitis, sinusitis, mucopurulent nasal discharge, diarrhea (yellow-green droppings are common), polyuria, dullness and affection of the central nervous system. Asymptomatic infections can occur with any strain of *C. psittaci*. A large number of chlamydiae cells can be found in respiratory secretions and faecal material of infected birds. Transmission of *C. psittaci* predominantly occurs through inhalation of contaminated material and sometimes through ingestion, from an infected bird to a susceptible bird. Humans can become infected by inhaling an organism shed by infected birds, mouth-to-beak contact or by handling the

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plumage and tissues of infected birds. In humans *C. psittaci* most often causes a respiratory infection and fever (3, 4). Person-to-person transmission of avian chlamydiosis is possible, but it is believed to be rare (5, 6, 7, 8).

Avian chlamydiosis occurs world-wide. To date, *C. psittaci* infections have occurred in at least 467 bird species, belonging to 30 different bird orders (9). The highest infection rates are found in psittacine birds (Psittacidae) and pigeons (Columbiformes) (4). Psittacidae represent major reservoirs of chlamydiae, especially under captive conditions. Psittacine cage birds should therefore be considered to be potential reservoirs of *C. psittaci* (4, 9).

Diagnosis of chlamydial infection in birds is still a considerable challenge. Clinical changes are not pathognomonical and persistent infections can also occur. The infection can only therefore be confirmed by direct identification of the agent or indirectly by detection of specific antibodies. PCR is currently the method of choice for diagnosis of chlamydial infection. Specific and sensitive PCR methods to detect *C. psittaci ompA* gene have been developed (2, 10, 11).

Serotyping of *C. psittaci* isolates, which was previously performed with a panel of serovar-specific monoclonal antibodies in an immunoflurescence test, allowed their classification into six avian serovars (A through F) (12, 13, 14). Serovar A is repeatedly isolated from psittacine birds, serovar B from pigeons and psittacine birds, serovar C from ducks and geese, serovar D from turkeys, serovar E from pigeons and ratites, and serovar F from psittacine birds and turkeys (13, 14, 15). All serovars appear to be transmissible to humans (4). Analysis of the gene encoding outer membrane protein A (*omp*A) is most often used to characterize avian *C. psittaci* strains into genotypes (16). Nine genotypes of *C. psittaci* are currently accepted (A-F, E/B, WC and M56) (2, 16, 17). Using comparison of serotyping results and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) patterns, Vanrompay et al. (12) were able to demonstrate that serovars have genetic equivalents in the corresponding genotypes (16). Other molecular tools, such as microarray analysis, multiple loci variable number of tandem repeats analysis (MLVA) and multilocus sequence typing (MLST) are also used for genotyping *C. psittaci* (16, 18, 19).

The aim of our study was therefore to establish the excretion rate of *C. psittaci* in oropharyngeal and cloacal swabs from cage birds by real-time PCR in Slovenia. Samples were first verified as *Chlamydiaceae* and positive samples for *C. psittaci* were then detected and genotyped.

Material and methods

Clinical examination

Cage birds were clinically examined at the Department for Birds, Small Mammals and Reptiles of the Institute for Health Care of Poultry, Veterinary Faculty in Ljubljana. Anamnesis was taken and different diagnostic procedures (microscopic examination of faeces and crop swabs, x-ray, molecular investigation to avian bornavirus and circovirus) were performed (data not shown) in some birds after agreement had been obtained from the owners of the birds.

Clinical samples

Oropharyngeal and cloacal swabs were taken during clinical examination of 125 cage birds sampled randomly, in 2010 and 2011. The order, family, genus and species of the examined birds are given in Table 1. Samples were collected with Dacron swab and stored in one millilitre of 2-sucrose-phosphate based transport medium (2SP) at -20°C until processing.

DNA extraction

Total DNA was extracted with a QIAamp® Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's blood and body fluid spin protocol. Each swab was vortexed vigorously before DNA extraction. Two hundred microliters of each sample were used as starting material for the DNA extraction. Finally, DNA was eluted with 100 µl of AE buffer and stored at -20°C until examination.

Detection of chlamydiae DNA by real-time PCR

Generic real-time PCR was used for the amplification of the partial 23S RNA gene of *Chlamydiaceae*. For the amplification, the following primers Ch23S-F (5'-CTG AAA CCA GTA GCT TAT AAG CGG T-3') and Ch23S-R (5'-ACC TCG CCG

TTT AAC TTA ACT CC-3'), and probe Ch23S-p (FAM-5'-CTC ATC ATG CAA AAG GCA CGC CG-3'-TAMRA) were used (20). The reaction mix contained 4.0 µl of nuclease-free water (Promega, Madison, WI, USA), 7.5 µl of 2x QuantiFast probe PCR master mix with ROX, and 0.6 µl of each primer (0.4 µM), 0.3 µl of specific probe and 2 µl of extracted DNA. The reaction was performed by ABI 7300 thermocycler (Applied Biosystems, Foster City, CA, USA). The cycling profile included initial denaturation at 95°C for 3 min, followed by 50 cycles of denaturation at 95°C for 5 s and 60°C for 30 s. The cycle threshold (Ct value) was calculated automatically. All positive samples were tested in two repeats before they were further examined by the primers CppsOMP1-F (5'-CAC TAT GTG GGA AGG TGC TTC A-3'), CppsOMP1-R (5'-CTG CGC GGA TGC TAA TGG-3') and probe CppsOMP1-S (FAM-5'-CGC TAC TTG GTG TGA C-3'-TAMRA) corresponding to the region of the *omp*A gene specific for *C. psittaci* (21). The real-time PCR reaction mix and cycling conditions were the same as for the 23S RNA gene.

DNA-based characterization - multiple loci variable number of tandem repeats analysis (MLVA)

C. psittaci positive samples were examined by MLVA. For the variable number of tandem repeats (VNTR) amplifications, PCR was performed in a total volume of 15 µl containing 2 µl of sample DNA, 1.5 µl 10 x PCR reaction buffer, 0.1 µl (1 U) of Hot start Taq DNA polymerase (Qiagen), 0.15 µl (200 µM) of each deoxynucleotide triphosphate, and 1.5 µl (0.3 µM) of each flanking primer (18).

The initial denaturation step at 95°C for 15 min was followed by 40 cycles consisting of denaturation at 95°C for 30 s, annealing at 56°C for 45 s, and elongation at 72°C for 45 s. The final extension step was at 72°C for 10 min. Nine microliters of amplification product were loaded onto a 4 % standard agarose gel. Gels stained with EZ-Vision® (Interchim, Montluçon, France) were visualized under UV light and photographed. A 100 bp ladder marker was used (Mbi, Euromedex, Souffelweyersheim, France).

Amplicon sizes were determined manually by a recently described methodology (18). The resulting data were compared with known MLVA types (10) for determining the genotype of *C. psittaci*.

Statistical analyses

All statistical analyses were performed with GraphPad Prism 6.0 software (GraphPad Prism 6.0 software, San Diego, California, USA). Fisher's exact test was used to calculate a p-value with statistical significance considered at $p < 0.05$.

Results

A total of 125 cage birds were sampled in 2010 and 2011 during clinical examination (Table 1). Ninety-seven birds (77.6 %) had various clinical problems. Fifteen birds (12 %; quail, two cockatiel, two African grey parrot, Senegal parrot, two Amazon parrot, Superb parrot, four budgerigars and two Hill myna) from among them had a suspected clinical signs of having *C. psittaci*. While the other 28 birds (22.4 %; two cockatiels, one amazon parrot, nine lovebirds, twelve budgerigars, two canaries, one zebra finch and one java sparrow) were apparently healthy (data not shown).

Three cage birds, two lovebirds and a budgerigar, were positive for *Chlamydiaceae* by real-time PCR for the detection of the 23S RNA gene of *Chlamydiaceae* and were also positive for *C. psittaci* by real-time PCR for the detection of the *omp*A gene specific for *C. psittaci*. The two positive samples from lovebirds showed MLVA types (ST) identical to ST 10 and the positive sample from a budgerigar was identical to ST 12. ST 10 is characteristically equivalent to genotype B, whereas ST 12 is equated with genotype A. Detailed information about the results of different diagnostic methods and clinical signs of positive cage birds are given in Table 2.

In order to determine whether budgerigars and lovebirds are more susceptible to the infection with *C. psittaci* in a given sample, the Fisher chi - squared test was used. There was statistically significant presence of *C.psittaci* for lovebirds with $p = 0{,}028$, but no for budgerigars ($p = 1$).

Discussion

C. psittaci is a very important bacterial pathogen in veterinary and human medicine. It can cause a clinical disease called avian chlamydiosis in many avian and mammalian species. Birds,

Table 1: Order, family, genus and species of examined birds. The total number of examined birds and the number of birds that tested positive for *C. psittaci* are shown

	Order	Family	Genus	Species	Birds examined (No)	*C. psittaci* positive birds (No)
Fowl (n=1)	Galliformes	Phasianidae	*Coturnix*	Quail*	1	0
Parrots (n=112)	Psittaciformes	Cacatuidae	*Nymphicus*	Cockatiel	17	0
			Eolophus	Galah	4	0
		Psittacidae	*Psittacus*	African grey parrot	9	0
			Poicephalus	Senegal parrot	1	0
			Pionites	Black-headed parrot	1	0
			Amazona	Amazon parrot	7	0
			Cyanoliseus	Burrowing parrot	1	0
			Aratinga	Sun parakeet	2	0
			Nandayus	Nanday parakeet	1	0
			Eclectus	Eclectus parrot	1	0
			Alisterus	Australian king parrot	1	0
			Polytelis	Superb parrot	2	0
			Psittacula	Ring-necked parakeet	6	0
				Alexandrine parakeet	2	0
			Agapornis	Lovebird	12	2
			Melopsittacus	Budgerigar	44	1
			Cyanoramphus	Yellow-crowned parakeet	1	0
Songbirds (n=12)	Passeriformes	Sturnidae	*Gracula*	Hill myna	2	0
		Fringillidae	*Serinus*	Canary	6	0
		Estrildidae	*Taeniopygia*	Zebra finch	3	0
			Padda	Java sparrow	1	0
Total No					125	3

*kept as a pet birds

Table 2: Details of positive chlamydiae test results of cage birds with clinical signs and results of parallel diagnostic investigation from Slovenia

Place of origin	Bird species	Real-Time PCR *Chlamydiaceae* (Ø Ct value)		Real-Time PCR *C. psittaci* (Ø Ct value)		MLVA Pattern	Genotype of *C. psittaci*	Clinical signs	Results of parallel diagnostic investigation
		OS	CS	OS	CS				
Ljubljana	Lovebird	33.3	32.97	33.94	33.44	10	B	Depression, weight loss, anorexia, abdominal mass.	nd
Ljubljana	Lovebird	neg	30.2	neg	33.34	10	B	With no clinical signs.	nd
Ljubljana	Budgerigar	34.75	27.02	neg	30.25	12	A	Progressive weight loss.	*Macrorahbdus ornithogaster* found in faeces.

OS oropharyngeal swab; *CS* cloacal swab; *neg* negative; *nd* not done

especially under captive conditions, are the major reservoir of *C. psittaci* (10). Most chlamydia infected cage birds are parrots, especially African grey parrots, Amazon parrots, lovebirds, cockatiels and budgerigars (4, 22). The results of our study, in which the three *C. psittaci* positive birds (two lovebirds and a budgerigar) belong to Psittaciformes, are in agreement with this. The birds examined in our study were from the orders Galliformes (0.8 %), Psittaciformes (89.6 %) and Passeriformes (9.6 %). In general, Psittaciformes are the most frequently investigated for chlamydial infections (9). It is still not known whether Psittaciformes are more susceptible to *C. psittaci* than other bird species. It is possible that the clinical signs of avian chlamydiosis are more easily noticed in these birds and infection therefore more often diagnosed (9, 23, 24).

Avian chlamydiosis can also occur in Passeriformes, especially in canaries and finches, but the infection is rarely diagnosed (4, 9, 22, 25). Passeriformes is the largest bird order and contains more than 4000 species (9). Nevertheless, chlamydia has been detected in only 2 % of song birds in comparison to a 45 % prevalence of chlamydial infections in parrot birds (9). Based on our experience, it is very likely that parrots are the largest group among cage birds. For example, during the period of the present study, 399 cage birds (80.7 % parrots and 7.8 % song birds) had the first clinical examination at the Department for Birds, Small Mammals and Reptiles.

In our study, 125 cage birds were sampled during clinical examination, in order to detect chlamydia infections by real-time PCR diagnostic methods. Oropharyngeal and cloacal swabs were taken from each bird, because infected birds can shed chlamydiae through respiratory tract excretions and in faeces (3, 4). Birds were sampled before any antibiotic treatment because antibiotic drugs can prevent shedding of chlamydiae (27). Serological monitoring was not done because the aim of our study was to establish the excretion rate of *C. psittaci* in cage birds through oropharyngeal and cloacal secretions.

C. psittaci infections in birds may range from subclinical to infections with diverse clinical signs, such as lethargy, anorexia, ruffled feathers, ocular or nasal discharge, conjunctivitis and greenish faeces (3, 4). Among the tested birds, twenty-eight (22.4 %) showed no clinical signs, while the other 97 birds (77.6 %) had a variety of clinical signs. In a budgerigar that showed progressive weight loss and was positive for *C. psittaci*, *Macrorhabdus ornithogaster* was also found by parallel diagnostic investigation. Only one of the two lovebirds that were positive for *C. psittaci* showed clinical signs not typical for infection with *C. psittaci.* The other bird appeared clinically healthy (Table 2). Anamnesis revealed that the lovebirds had been in close contact before they were tested. In the lovebird with clinical signs, *C. psittaci* was detected in oropharyngeal and cloacal swabs, while in the clinically healthy lovebird *C. psittaci* was detected only in the cloacal swab. The explanation of this may be that the shedding of *C. psittaci* occurs intermittently and healthy carrier birds might not excrete bacteria for extended periods (5).

Three cage birds (2.4 %) were positive for *Chlamydiaceae* and *C. psittaci* by real-time PCR. Oropharyngeal and cloacal swabs were positive for *C. psittaci* in the lovebird with clinical signs, while in the other two birds, the clinically healthy lovebird and the budgerigar with progressive weight loss, only cloacal swabs were positive. In the budgerigar, the oropharyngeal swab was positive for *Chlamydiaceae* but negative for *C. psittaci*. The reason for this may be that the amount of investigated DNA of *C. psittaci* was under the detection limit of the method.

Real-time PCR is one of the favored methods for the detection of DNA of *Chlamydiaceae* as well as for further detection of *C. psittaci* DNA. The method proved to be rapid and have good specificity and sensitivity as reported by Geens et al., Ehricht et al. and Pantchev et al. (2, 20, 21). The prevalence of *C. psittaci* in cage birds of different species detected by real-time PCR described by Ehricht et al. and Pantchev et al. (20, 21) was, in our study, 2.4 % (3 of 125). One of the reasons for the low prevalence of *C. psittaci* infections detected in the present study may be in randomly sampled cage birds as only 12 % (15 of 125) of them have had clinical signs that could be related to *C. psittaci* infection and no sampled birds have had history of previous infections with *C. psittaci.* The prevalence of the infected birds detected in the present study also correlates well with the results of previous serological monitoring of *C. psittaci* infection in breeding flocks of parrots, canaries and finches (25, 26). Antibodies were detected in 6.2 % of parrots, 0.8 % of canaries and 0.9 % of finches breeding flocks in the period from 1991 to 2001 (25, 26). In our study, most

of the sampled birds (112 of 125) were parrots, of which three (2.7%) were positive for *C. psittaci* DNA. In canaries (4.8% of sampled birds) and in finches (3.2% of sampled birds) no infections with *C. psittaci* were detected.

The *C. psittaci* detected in cloacal swabs samples from the two lovebirds and the budgerigar was typed by the MLVA method. *C. psittaci* of genotype A was detected in the budgerigar and *C. psittaci* of genotype B was detected in both lovebirds. These results correlate with previous findings, whereby genotypes A and B have been predominantly found in psittacine birds (18, 28). Surprisingly, only genotype A was detected by MLVA in samples from lovebirds in a recent study (18).

Even though the statistical analyses showed that lovebirds are more susceptible to the infection with *C. psittaci* in a given sample it should be mentioned that both positive lovebirds were from the same household. In this case, it is very likely that this was the main reason for the infection of both birds rather than the susceptibility of the lovebirds as a species.

For an avian practitioner, *C. psittaci* infections in cage birds, especially in the absence of clinical signs, can be difficult to diagnose. Generally, avian chlamydiosis should be suspected in any parrot that shows respiratory or gastrointestinal or neurological signs or has been in contact with infected birds. Real-time PCR is a diagnostic tool that can be used to determine infection by *C. psittaci*. In addition, the MLVA method can be used for typing the microorganism. If possible, serological analysis should be still used to complement the molecular diagnosis. The infected birds should be correctly treated and the transmission of the pathogen to susceptible animals and to humans should be prevented.

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UGOTAVLJANJE BAKTERIJE *CHLAMYDIA PSITTACI* PRI SOBNIH PTICAH V SLOVENIJI S PCR V REALNEM ČASU

C. Marhold, B. Slavec, K. Laroucau, F. Vorimore, J. Račnik, M. Zadravec, D. Keše, U. Krapež, A. Dovč

Povzetek: Aviarna klamidioza je obolenje ptic, ki jo povzroča bakterija *Chlamydia psittaci*. Okužbe s to bakterijo najpogosteje ugotovimo pri papigah (Psittacidae), ki so hkrati tudi najštevilčnejše predstavnice sobnih ptic. Okužba ptic s *C. psittaci* zelo pogosto poteka brez kliničnih znakov. Pri obolelih pticah pa lahko opazimo apatičnost, neješčnost, našopirjenost, očesni in nosni izcedek ter vodene temnozelene iztrebke. Okužene ptice izločajo povzročitelja najpogosteje v presledkih z očesnimi in nosnimi izcedki ter z iztrebki. Prenos okužbe s *C. psittaci* je neposreden in je možen med različnimi vrstami ptic in tudi s ptic na ljudi.
Za ugotavljanje prisotnosti *C. psittaci* pri sobnih pticah v Sloveniji smo odvzeli brise žrela in kljunske votline ter brise kloake pri 125 pticah. Vzorce smo najprej pregledali na prisotnost bakterij iz družine Chlamydiaceae z metodo PCR v realnem času. Okužbo s *C. psittaci* smo dokazali pri dveh agapornisih (2/12) in eni skobčevki (1/44). Za ugotavljanje okužb s *C. psittaci* smo uporabljali PCR v realnem času, specifičen za gen *omp*A. S hkratno analizo večjega števila lokusov z variabilnim številom tandenskih ponovitev (MLVA) smo pri skobčevki določili *C. psittaci*, genotip A, pri obeh agapornisih pa *C. psittaci*, genotip B. Z metodo PCR v realnem času smo ugotovili nizko prevalenco (2,4 %) okužb s *C. psittaci* pri sobnih pticah v Sloveniji.

Ključne besede: *Chlamydia psittaci*; PCR v realnem času; MLVA; sobne ptice; Slovenija