

EPIDEMIOLOGICAL STUDY OF *CHLAMYDOPHILA PSITTACI* IN PET BIRDS IN CROATIA

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A total of 411 samples from birds of different species originating from all counties of the Republic of Croatia have been tested for the presence of Chlamydophila psittaci. The sampling was conducted in pet stores, breeders' aviaries, in a specialized bird clinic and in zoos. The testing included 177 parrots, 169 pigeons, 58 canaries and 7 finches. For the detection of specific C. psittaci antigen a commercial ELISA kit was used- IDEIA™ PCE Chlamydia (DAKO Cytomation Ltd., United Kingdom). The samples that were non-specifically positive or doubtful in the ELISA test (a total of 26 samples) were analyzed also by means of polymerase chain reaction (PCR). Diagnostic ELISA method found a total of 17.03% birds positive for chlamydiosis, and after additional testing by PCR a total of 12.65% positive ones were found. According to bird species, the most frequently positive ones were canaries and pigeons (15.52% and 13.02%), and according to the sampling location most of the positive birds were found in pet stores (16.52%), but a high percentage of positive samples were also found in breeders' aviaries (11.76%).

The average positive result for chlamydiosis in 12.65% of tested birds is alarming and it confirms the importance of monitoring bird health and of prescribed legal regulations when it comes to chlamydial diseases, as well as education of persons involved in breeding, keeping or selling birds.

Key words: Chlamydophila psittaci, ELISA, epidemiology, PCR, pet birds

INTRODUCTION

Infection caused by *Chlamydophila psittaci* (*C. psittaci*) can be transmitted from birds to humans, and therefore represents an important public health problem. Clinically, in birds the disease manifests in its acute, inapparent or sub-clinical form, but also as a chronic disease, and frequently also as an

asymptomatic infection, which represents additional danger (Andersen et al. 1997; Andersen and Vanrompay 2003; Sudler et al. 2004). The studies implemented in the Republic of Croatia so far prove the frequent presence of bacteria of the *Chlamydophila* genus in birds. They can be found especially frequently in urban pigeons (Prukner-Radovčić et al. 2005b; Magnino et al. 2009), but the presence of *Chlamydophila* genera in the Republic of Croatia has also been proven in domestic poultry, in turkeys and chickens (Prukner-Radovčić et al. 2005a; Prukner-Radovčić et al. 2006a; Prukner-Radovčić et al. 2006b). An increased number of humans affected by chlamydiosis in the Republic of Croatia in 2007 instigated the research of the presence of chlamydiosis in birds found in direct vicinity of humans (Aleraj 2008, personal communication, Croatian Institute of Public Health, Department of Infectious Diseases Epidemiology).

The investigation was focused on birds mostly kept as pets (parrots, finches and canaries) and pigeons held and bred as pets or for sport. Samples were taken from birds brought for examination to the Bird Clinic at the Faculty of Veterinary Medicine, University of Zagreb, as well as at breeders' aviaries and in pet stores. Some birds were tested in zoos, while in quarantine during import. The samples were collected from the whole territory of the Republic of Croatia, where the disease is controlled in accordance with the law (Official Gazete 7/10 and 14/10).

This obligate intracellular bacterium presents a complex problem in diagnostics. Because of the zoonotic potential of *C. psittaci*, it is important to make a rapid, definitive diagnosis in birds. Detection of specific chlamydial antigens has several advantages over isolation techniques and serologic tests. Most commercially available tests use a monoclonal antibody (MAb) against the genus-specific epitope on the chlamydial lipopolysaccharides (LPS), like enzyme-linked immunosorbent assay (ELISA) test. Since many authors found the ELISA test appropriate for diagnostics of chlamydiosis (Gerbermann, 1997; Andersen, 1998), we also opted for ELISA in this research. However, this method sometimes yields non-specific or doubtfully positive results (Bevan and Davies, 1987). Therefore, when doubtful results occurred, samples were additionally tested by means of conventional PCR.

MATERIALS AND METHODS

Birds

The research included the analysis of a total of 411 samples (faeces, cloacal swab, and the so-called triple swabs: from conjunctiva, pharynx and cloaca) from parrots, pigeons, canaries and finches. Samples were sent to the Laboratory for chlamydia infections, in accordance with the legal regulations currently in force in the Republic of Croatia. The analysis involved 177 parrots, 169 pigeons, 58 canaries and 7 finches. The samples were collected in the Town of Zagreb and in all 20 counties of the Republic of Croatia. Sampling was performed in pet stores, breeder's aviaries, in the specialized Bird Clinic of the Faculty of Veterinary Medicine in Zagreb, and in zoos.

Detection of specific chlamydial antigens by ELISA test

The 411 samples that had been collected were analyzed by enzyme-linked immunosorbent assay (ELISA) for the detection of the *Chlamydia* genus-specific antigen. The specific antigen was detected by commercially available enzyme immunoassay IDEIA™ PCE *Chlamydia* Test (DAKO, Hamburg, Germany or OXOID, UK), in accordance with the manufacturer's instructions. The optical density was measured at 490 nm within one or two minutes in the Labsystems iEMS Reader MF (Labsystems, Finland) after immunological reaction of bonding of the antigen from the sample with specific monoclonal antibodies adsorbed onto a microtiter plate. If it was a faecal sample, the calculation procedure was modified (Gebermann and Janeczek 1991; Gebermann 1997; Prukner-Radovčić et al. 2005b).

Detection of specific chlamydial DNA by PCR

Samples which showed a non-specific reaction (a total of 26), were additionally tested by PCR method. The total DNA was isolated from the samples using DNeasy® Blood & Tissue Kit (Qiagen, USA), according to manufacturer's instructions. By conventional PCR reaction 264 base pairs DNA sequence of 5'-non-translated region of *ompA* gene of bacterium *C. psittaci* was amplified using specific primers (Hewison et al. 1997). The PCR reaction was performed using GoTaq Flexi DNA Polymerase (Promega, USA) kit on GenAmp PCR System 2400 device (Applied Biosystems, USA). Following amplification, 10 µL of PCR products were separated by electrophoresis on 2% agarose gel stained with ethidium bromide and visualised under UV light.

RESULTS AND DISCUSSION

Analysis of a total of 411 samples originating from different bird species using the ELISA procedure showed that as many as 70 were positive for chlamydiosis (17.03%). The number and percentage of analyzed samples and those positive for the presence of bacterium *C. psittaci*, as well as their origin according to the sampling location and bird species are presented in Table 1.

Table 1. Results of testing for chlamydiosis in birds using the ELISA method (n=411)

Sampling location	Bird species				Total
	Pigeons	Parrots	Canaries	Finches	
Breeders	154/38 (24.68)*	48/2 (4.17)	35/5 (14.29)	1/0 (0)	238/45 (18.91)
Stores	1/0 (0)	87/16 (18.39)	21/4 (19.05)	6/0 (0)	115/20 (17.39)
Clinic	9/0 (0)	35/3 (8.57)	2/0 (0)	0	46/3 (6.52)
ZOO	5/0 (0)	7/1 (14.29)	0	0	12/1 (8.33)
Total	169/39 (23.08)	177/22 (12.43)	58/9 (15.52)	7/0 (0)	411/70 (17.03)

*number of analyzed/number of positive (% of positive)

In order to determine the highest risk of an outbreak of infection, the samples were sorted not only according to bird species, but also according to sampling location. From breeders' aviaries a total of 238 samples were analyzed, 115 samples were from pet stores, 46 samples originated from birds brought to the clinic by their owners, and 12 samples were from birds in zoos. Most frequently positive were birds from breeders (18.91%), followed by those from pet stores (17.39%), while only three samples were positive from birds brought for treatment in the clinic (6.52%), whereas only one sample was tested positive from zoos (8.33%).

According to the bird species the most frequently positive samples were from pigeons, where 39 tested positive (23.08%); from parrots 12.43% were positive, and from canaries 15.52% were positive. None of the seven samples taken from finches tested positive.

ELISA tests are relatively easy to use and have good specificity. Although these tests successfully demonstrate the presence of chlamydial organisms, they generally do not allow the identification of the respective species, serotype or subtype involved. Positive results obtained by ELISA test are considered relevant if the bird also showed clinical symptoms. But, due to the fact that LPS antigens of some other bacteria can cross-react with monoclonal antibodies specific for chlamydial LPS and cause false positive results, precaution should be taken when a high positive titer occurs (Gerlach 1997; Andersen 1998). That is why 26 samples that yielded doubtfully positive results (higher test values than the positive control) by means of ELISA were retested by PCR method, as well. The results are presented in Table 2.

Table 2. Result of PCR testing (n=26)

Bird species	Doubtful positive samples yielded by ELISA assay	PCR positive	
		Number	%
Pigeon	18	1	5.55
Parrot	4	3	75.00
Canary	4	4	100
Total	26	8	30.77

The presented results show that out of 18 pigeon samples which reacted non-specifically positive in the ELISA method, only one sample (5.55%) tested positive for the presence of chlamydia by means of PCR procedure. Contrary to that result, a high percentage of samples originating from parrots and canaries (75% and 100%) also tested positive by PCR method. This shows that only some of the results from the ELISA assay were rightfully doubted: primarily when the samples were from pigeons and only in one case when the sample originated from parrots. The results for samples analyzed by PCR are considered more reliable due to the specificity of the test. As previously mentioned, the commercially available ELISA kit detects chlamydial LPS antigen which shares

some epitopes with other gram-negative bacteria, so some authors believe that they can cross-react by yielding a false positive result (Bevan and Davies 1987; Andersen 1998). This was probably the case in these samples too; namely, it is well known that faeces samples from parrots and canaries only exceptionally contain gram-negative bacteria, whereas in pigeons their occurrence is much more frequent (Gerlach 1994; Vogel et al. 1994). Taking into consideration the results of re-testing by PCR procedure, the total number of birds positive for chlamydia would be somewhat smaller; instead of a total of 70 positive birds (17.03%), it turns out that only 52 were in fact positive, which is a total of 12.65%. According to bird species, the number of chlamydia-positive results after retesting by PCR method in pigeons is 13.02% instead of 23.08%, in parrots 11.86% instead of 12.43%, whereas in canaries the result remains unchanged with 15.52% positives. According to the sampling location the most frequently positive were birds from pet stores (16.52%) followed by those from breeders (11.76%).

The conducted research shows that in Croatia there were as many as 17.03%, i.e. 12.65% birds positive for chlamydiosis. Regardless of which result we take as valid, the one yielded by ELISA assay or the other additionally corrected by PCR procedure, both are quite worrying, and they confirm the importance of monitoring the health of birds, especially in pet stores and in breeders' aviaries. By analyzing the samples from different bird species we discovered the presence of *C. psittaci* bacterium in all counties of the Republic of Croatia. The occurrence of chlamydiosis in so high percentage in birds, which are in the close contact with humans, proves the importance of reliable diagnostics for this disease, as an important prerequisite for the protection of the human health, especially of owners and breeders of pet birds. Since 2001 chlamydiosis in the Republic of Croatia has been listed among infectious and parasitic animal diseases which are prevented, detected and controlled by the prescribed legal regulations. Legal regulations for systematic control of chlamydiosis are the most acceptable solution for prevention, because the presented data shows that the awareness of controlling the disease among breeders and owners is still on a very low level. Additional education of breeders and pet bird traders also proved to be a necessity. The PCR procedure turned out to be the method of choice when it comes to clarify non-specific or in any other way doubtful results of other diagnostic procedures. But, nonetheless, it is our opinion that ELISA procedure remains the method of choice for those laboratories for which other methods of diagnostics of *Chlamydophila*, like PCR or RealTime PCR, are unavailable.

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EPIDEMIOLOŠKO ISPITIVANJE *CHLAMYDOPHILA PSITTACI* KOD DOMAĆIH PTICA U HRVATSKOJ

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SADRŽAJ

Na prisutnost bakterija roda *Chlamydophila* ukupno je pretraženo 411 uzoraka porijeklom od ptica različitih vrsta pristiglih iz svih županija Republike Hrvatske. Uzorkovanje je izvršeno u trgovinama ptica, kod uzgajivača, u specijaliziranoj ambulanti za ptice Veterinarskog fakulteta u Zagrebu, te u zoo vrtovima. Pretraženo je 177 papiga, 169 golubova, 58 kanarinaca, te 7 zeba. Za dokaz specifičnog antigena bakterije *C. psittaci* korišten je komercijalni ELISA kit - IDEIA™ PCE Chlamydia (DAKO Cytomation Ltd., United Kingdom). Uzorci koji su ELISA testom bili nespecifično pozitivni ili sumnjivi (ukupno 26 uzoraka) pretraženi su i lančanom reakcijom polimeraze (PCR). ELISA kitom nađeno je sveukupno 17,03% ptica pozitivnih na klamidiozu, dok ih je PCR metodom 12,65% bilo pozitivnih. Prema vrstama ptica, najčešće su pozitivni bili kanarinci (15,52%) i golubovi (13,02%), a prema mjestu uzorkovanja najviše je bilo pozitivnih ptica u trgovinama kućnih ljubimaca (16,52%), no vrlo visok postotak pozitivnih uzoraka nađen je i kod uzgajivača (11,76%).

Prosječno pozitivan nalaz na klamidiozu u 12,65% ptica zabrinjavajući je, te se potvrdila važnost monitoringa zdravlja ptica i propisana zakonska regulativa vezana uz klamidije, kao i edukacija osoba koje se bave uzgojem, držanjem ili prodajom ptica.

