Serological evidence of *Chlamydia psittaci* in psittacines in three zoological parks in Portugal

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Abstract

Chlamydia psittaci is an agent that causes ornithosis or psittacosis, which can infect homing and wild birds, mammalian animals and humans. Since this disease is an important zoonosis that is fatal and distributed worldwide, it is important to know its occurrence. This study aimed to survey the seropositivity of *Chlamydia psittaci* in three psittacine collections in three zoos in Portugal. In this study, 112 blood samples of the psittacine belonging to Order *Psitaciformes* (encompassing 31 species from 14 genera) were used. These samples were tested using a commercial ELISA kit (Immunocomb®, Biogal). The serological examination of psittacine samples using ELISA showed that 54 were positive (48.2%; 95% confidence interval, CI: 39.0-57.4%). The genus *Ara* exhibited significantly higher seropositivity than other genera ($P<0.05$). Based on the serological data from this study, we demonstrate that antibodies against *Chlamydia psittaci* are circulating in the blood of these tested animals. Since psittacosis is a public health concern, zoonotic issues of these results should be considered.

Key words: *seropositivity; Chlamydia psittaci; psittacines; Portugal*

Introduction

Chlamydia psittaci is part of the genus *Chlamydophila* (Everett et al., 1999). Avian chlamydiosis, commonly named ornithosis or psittacosis (Ciftci et al., 2008; Beeckman and Vanrompay 2009; Fraeyman et al., 2010) is an infectious

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disease that affects human (Marti and Jelocnik, 2022), and domestic animals and birds (Vanrompay et al., 2005; Pennycott et al., 2009; de Lima et al., 2011; Čengić et al., 2019; Akter et al., 2021), with the *Psittacidae* as one of the most affected families (Maluping et al., 2007). There are many records of its zoonotic condition (Smith et al., 2005; Berk et al., 2008; Gaede et al., 2008; Dickx et al., 2010; Tolba et al., 2019; Marti and Jelocnik, 2022). The agent can be found on bird feathers, in excrement and blood, in animals presenting or not presenting clinical signs of disease (Fenga et al., 2008; Geigenfeind and Haag-Wackernagel 2010; Chereau et al., 2018). Psittacines very often become infected but show no signs of disease before they are stressed (Ciftci et al., 2008; Beeckman and Vanrompay 2009; Harkinezhad et al., 2009; Magnino et al., 2009). Reports on pigeon *Chlamydia* infections are vast (Dickx et al., 2010; Vazquez et al., 2010), making these animals a serious carrier of the disease. Infection studies of turkeys showing a rate of 5-40% mortality without treatment and ducks with ranges up to 30% are evidence of an economic and health problem (Laroucau et al., 2009).

The diagnosis of this disease could be made by various laboratory techniques. Antibodies against *Chlamydia psittaci* can be detected by the complement fixation test (CFT), as the main laboratory diagnosis for psittacosis (Griffiths et al., 1996) and Enzyme-linked Immunosorbent Assay (ELISA) test that detect immunoglobulin G (IgG) against *Chlamydia psittaci* (Raso et al., 2002; Maluping et al., 2007). Molecular methods, like PCR, are also useful tools for diagnosing chlamydiosis (Chan et al., 2000; de Barbeyrac et al., 2000; Darwin et al., 2002; Petrovay and Balla 2008; Van Droogenbroeck et al., 2009; Murao et al., 2010).

Material and Methods

The sample population included a total of 112 psittacines from three different zoo collections (A, B, and C) in Portugal. In collection A, birds are kept in different sizes cages, some with a glass barrier between visitors and birds in the observation standing point. Psittacines are confined to the same area in the zoo, although in different cages, sorted by species or number of individuals. In collection B, animals are kept in non-similar types of cages that differ in their size, style, and material. Psittacines are distributed through eleven enclosures arranged side-by-side and divided by a solid wall. In collection C, the birds are kept in different places throughout the zoo and psittacines are caged in gigantic-sized cages. Outside, birds can contact all animals from all collections as these are placed in an outdoor environment.

All birds appeared to be healthy upon physical examination, and were more than 6 months old. All animals from the three sites were placed, conditioned and handled in accordance with the Portuguese and European Union Animal Welfare Standards. All procedures involving live animals were conducted following the national legislation and the Council of European Convention ETS 123 on the Protection of Animals Used for Experimental and Other Scientific Purposes (L358/1, 24 November 1986). The study received ethical approval from the Ethics Commission of the University of Trás-os-Montes e Alto Douro (Doc34-CE-2020).

During a year, with the bird manually restrained, blood was drawn from either the brachial or the jugular vein. About 0.1 mL of blood was drawn using sterile 1 mL syringes and a 25-gauge needle. Serum was separated after clotting by centrifugation at 200 *g* for 10 minutes and frozen at -20°C until use.

An ELISA test kit specific for avian species and for *Chlamydia psittaci* bacteria (Immunocomb® Biogal, Kibbutz Galed, Israel), which is based on a rapid competitive enzyme-linked immunosorbent assay technique and detects immunoglobulin G (IgG) against *Chlamydia psittaci* in avian species was used. The technique was performed following the manufacturer's instructions. The results were read by comparing the shade of grey of the test result with that on the combscale card supplied by the kit manufacturer. Combscale is a 1-6 classification, being samples from 1-2 considered negative for the test. The positive scale has two gradations: the scale of 3-4 is considered positive and 5–6 as highly positive.

Data obtained in this ELISA test were processed using the statistical program SPSS® (version 20.0 for Windows Vista). The Chi-square test ($\chi 2$) was used for statistical analysis of the association between independent and dependent variables. The probability level of $P<0.05$ was considered statistically significant.

Results

In this study, a total of 112 animals from 31 different species of psittacines were examined from three different zoo collections in Portugal. All animals were in good conditions at the time of sampling. Fifteen (13.4%) birds were adults and in 97 (86.6%) animals it was not possible to determine the age, although all psittacines were more than 6 months old, based on the evaluation of conformation, size, and feather coloration.

The species with the highest number of animals included in this study was *Psittacus erithacus* (African Grey Parrot, 16.1%), followed by *Ara ararauna* (Blue-and-yellow Macaw, 11.6%) and *Amazona aestiva* (Blue-fronted Amazon, 8.9%). In terms of genus, most of the animals belong to the genus *Amazona* (33.9%). Twenty-five percent of processed samples were from the genus *Ara* and 17.9% from the genus *Psittacus*. The site of origin of animals was also considered, with half from Collection A (50.9%; *n*=57), 35.7% (*n*=40) from Collection B, and 13.4% (*n*=15) from Collection C.

The serological examination of psittacines samples using ELISA showed that 54 (48.2%; 95% confidence interval, CI: 39.0-7.4%) tested positive. The species *Ara ararauna* (20.4%) have the highest number of positive samples, followed by *Ara chloroptera* (9.3%). Other species such as *Ara macao*, *Amazona amazonica* and *Psittacus erithacus* also presented high positivity (7.4%). Differences found in positivity between the species were statistically significant.

In seven species, all animals sampled presented antibodies: *Ara ambigua* (*n*=1), *Ara chloroptera* (*n*=5), *Ara macao* (*n*=4), *Amazona tucumana* (*n*=1), *Amazona vinacea* (*n*=1), *Psittacula krameri* (*n*=2), *Propyrrhura couloni* (*n*=2). In 16 species (53.1%), the occurrence was 50% or higher: *Ara ararauna* (*n*=13), *Ara militaris* (n=3), *Amazona farinosa* (*n*=2), *Amazona amazonica* (*n*=6), *Amazona autumnalis* (*n*=2), *Aprosmictus erythropterus* (*n*=2), *Coracopsis vasa* (*n*=2), *Aratinga acuticaudata* (*n*=3), *Eos bornea* (*n*=2). The genus *Ara* (42.6%) presented the highest positivity to the test, followed by the genus *Amazona* (29.6%). Differences between genera were statistically significant. Psittacines from the genus *Polytelis, Poicephalus, Eclectus,* and *Cyanoliseus* all tested negative. When only birds from the genus *Ara* were considered, the incidence was 82.1%. For *Psittacula* this was 66.7%, for *Aprosmictus, Coracopsis, Aratinga,* and *Eos* 50% each, and for *Amazona* 42.1%. The two *Propyrrhura* birds were tested in this study were both positive. The distribution of the positive results and occurrence by genus is presented in Table 1. Differences between species were statistically significant.

Table 1. Distribution of frequency, positive results and occurrence by genus.

Genus	Number of samples	Relative frequency (%)	Positive Results in ELISA test (*n*; %)	Frequency of positive results (%)
Ara	28	25.0	23 (42.6)	82.1
Amazona	38	33.9	16 (29.6)	42.1
Eclectus	2	1.8	0 (0.0)	0.0
Aprosmictus	2	1.8	1 (1.9)	50.0
Cyanoliseus	1	0.9	0 (0.0)	0.0
Coracopsis	2	1.8	1 (1.9)	50.0
Poicephalus	2	1.8	0 (0.0)	0.0
Psittacula	3	2.7	2 (3.7)	66.7
Polytelis	1	0.9	0 (0.0)	0.0
Psittacus	20	17.9	4 (7.4)	20.0
Propyrrhura	2	1.8	2 (3.7)	100
Aratinga	6	5.4	3 (5.6)	50.0
Eos	2	1.8	1 (1.9)	50.0
Nymphicus	3	2.7	1 (1.9)	33.3
Total	**112**	**100**	**54 (100)**	**–**

In total, 33 (29.5%) birds had a score on the Immunocomb test scale of 3-4 (positive), and 21 (18.8%) had a score of 5–6 and are considered to be highly positive. Differences in scores by genus were statistically significant.

The occurrence of positivity was not influenced by the months of sample collection. Furthermore, the frequency of positive results for *Chlamydia psittaci* from the three different collections was not influenced by the origin of the sample (P=0.602).

Discussion

In this study, the ELISA test kit was the laboratory diagnosis used to detect the presence of IgG against *Chlamydia psittaci* in psittacines in three zoos in Portugal and to confirm the presence of *Chlamydia psittaci* antibodies among captive psittacines in these collections. Vasquez et al. (2010), indicate a 52.6% prevalence in feral pigeons (*Columba livia*) in Madrid, using PCR (Vazquez et al., 2010). In a study performed with varying methods in crocodiles and chickens, a 30% value was reported (Robertson et al., 2010). Another PCR study performed on wild birds in Poland detected 7.3% positive tests for chlamydia DNA (Krawiec et al., 2015). A study performed in Arizona, USA, detected antibodies in 76% lovebirds and 7% sympatric birds (Dusek et al., 2018), and in another study *Chlamydia psittaci* DNA was detected in 52.5% psittacine birds (Tolba et al., 2019). Our ELISA test kit was previously used in a study in psittacines and raptors in the Philippines and found a seroprevalence of 25% for psittacosis; however, the prevalence in psittacines was slightly higher (Maluping et al., 2007). Psittacines as potential carriers of *Chlamydia psittaci* have been

proven in countries other than Portugal (Smith et al., 2005; Vanrompay et al., 2007), and there is a lack of epidemiological knowledge concerning this agent in the studied bird species in our country.

Although Complement Fixation Test is the most used laboratory diagnostic test for *Chlamydia psittaci* according to guidelines of the Office International des Epizooties (World Organization for Animal Health - OIE), the ELISA test is useful for performing screening and to determine seroprevalence or seropositivity of this disease (Maluping et al., 2007). ELISA was used in this study because it is standardized for the bacteria, is considerably faster and has a lower price than the Complement Fixation Test, allowing us to achieve the objectives to screen for the seropositivity of *Chlamydia psittaci.* The ELISA test used is designed to provide information about previous exposure to bacteria. Specific *Chlamydia psittaci* IgG antibodies can be found up to 15 days after exposure to the antigen (Huang et al., 1999). This fact suggests the possibility of false-negative results as some animals could be going through an acute state of the disease and showing IGM instead of IgG in the blood. Poor humoral antibody responses produced to certain chlamydial infections may not be detected as a few hundred organisms are needed for a positivity result (Griffiths et al., 1996; Harkinezhad et al., 2009).

In general, all lodging facilities allowing closer contact between individuals enhance the risk of infection (Maluping et al., 2007). It was observed that cages of psittacines in all three collections were placed adjacent to one another. In some, birds have direct contact with the next one and in others, many birds are placed together in a single enclosure. All cages are located in an external environment, and therefore animals such as pigeons are capable of contact with infected psittacines and may also become infected or vice-versa (Magnino et al., 2009; Tolba et al., 2019).

The risk of infection after close contact with carrier birds is increased in any circumstance when the human/bird interface is narrower and this reason is considered a public health concern (Raso et al., 2002; Maluping et al., 2007; Raso et al., 2010). This disease increases morbidity and mortality of infected animals, which involves great economic losses, as some psittacines are known to be very high price animals. As psittacosis is a zoonosis and the detection of *Chlamydia psittaci* was high in asymptomatic birds, this creates a potential risk of infection in other animals, bird owners, workers, and tourists, making it an important public health issue (Maluping et al., 2007; Dickx et al., 2010; Raso et al., 2010; Chereau et al., 2018; Tolba et al., 2019).

Conclusions

This is the first study to analyse *Chlamydia psittaci* seropositivity using the ELISA test in psittacines in Portugal, as test that should be performed as initial screening for the detection of this disease due to its feasibility and simplicity. The positivity results of 48.2% are within the range of those described in other reports in other countries; however, more studies should be performed in other parts of the country to complement this work to give more definitive conclusions on the prevalence of this disease in Portugal.

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Serološki dokaz *Chlamydia psittaci* u papiga u tri zoološka parka u Portugalu

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Chlamydia psittaci uzročnik je ornitoze (psitakoze) koja može inficirati i domaće i divlje ptice, sisavce, ali i ljude. S obzirom da je ova bolest sveprisutna zoonoza koja može biti fatalna, a rasprostranjena je diljem svijeta, važno ju je znati prepoznati kada se pojavi. Cilj je ovog rada bio ispitati seropozitivnost na *Chlamydia psittaci* u tri populacije papiga koje pripadaju trima zoološkim parkovima u Portugalu. U ovoj studiji rabljeno je 112 uzoraka krvi papiga iz reda *Psitaciformes* (31 različita vrsta iz 14 različitih rodova); uzorci su podvrgnuti komercijalnom ELISA testu (Immunocomb®, Biogal). Serološko ispitivanje uzoraka papiga uporabom ELISA testa pokazalo je da ih je 54 (48,2 %; 95 % interval pouzdanosti, CI: 39,0 %-57,4 %) bilo pozitivno. Rod *Ara* pokazao je značajno veću seropozitivnost od ostalih rodova (P<0,05). Na temelju seroloških podataka iz ove studije, dokazali smo da protutijela za *Chlamydia psittaci* cirkuliraju u krvi testiranih životinja. S obzirom da psitakoza predstavlja javnozdravstveni problem, potrebno je razmotriti zoonotska pitanja naših rezultata.

Ključne riječi: *seropozitivnost, Chlamydia psittaci, papige, Portugal*