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Original Full Article

Detection of *Chlamydia* sp. by fluorescence *in situ* hybridization (FISH) in histologic sections of the liver from exotic and native avian species

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Abstract

Chlamydiosis is a zoonotic disease that affects several animal species. Therefore, reliable detection techniques are essential for efficient control of the disease. Fluorescence *in situ* hybridization (FISH) for *Chlamydia sp.* was applied to 137 formalin-fixed, paraffin-embedded liver sections from native and exotic birds. The samples were divided into two groups: retrospective (*n*=57) and prospective (*n*=80). The probe was designed based on an annealing sequence that targets the Major Outer Membrane Protein coding gene. Livers previously confirmed for *Chlamydia psittaci* by polymerase chain reaction (PCR) and immunohistochemistry (IHC) were used as positive controls. Also, 47 randomly selected samples from the prospective group were submitted to PCR for *Chlamydia psittaci* for confirmation. From all 137 samples, 67% (92/137) were positive for *Chlamydia* sp. through FISH, from which 39% (36/57) and 61% (56/80) were from the retrospective and prospective groups, respectively. From the samples of the prospective group submitted for PCR confirmation, 83% (39/47) of them had a positive correlation with FISH results. When considering the number of targeted microorganisms per 400x field, 42,39% (39/92) of the samples had up to five microorganisms, 14,13% (13/92) had from six to ten, and 43,47% (40/92) of the samples had 11 or more microorganisms per field. FISH is a specific and reliable method to identify *Chlamydia* sp. in histologic sections of the liver, providing an additional tool for detecting avian chlamydiosis.

Keywords: immunohistochemistry, psittacosis, zoonosis, diagnosis.

Introduction

Chlamydiosis is an important zoonosis (19) caused by an obligate intracellular, gram-negative bacterium from the genus *Chlamydia*, from which *C. psittaci* is the most common species affecting birds (8, 16, 24, 34, 35). The clinical diagnosis is complex, and outbreaks are reported worldwide, with significant economic and public health impacts (22, 31).

The gold standard test for its diagnosis is bacterial isolation. However, this technique requires high biosafety levels

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and cell culture (6, 31). PCR is currently the most used test, but false negatives are not rare due to intermittent shedding (31). Additionally, subclinical and asymptomatic infections can present a diagnostic challenge in a clinical routine (23).

Immunohistochemistry (IHC) can be used to diagnose chlamydiosis in formalin-fixed, paraffin-embedded (FFPE) tissues (6,11). This technique is currently the only one available that allows co-localization of the organism within tissue lesions (6). Its limitation, however, resides particularly in samples that were kept for a long time in formalin, which can give false negative results (41). However, this is not the only critical point of the IHC since the use of some chromogens, such as diaminobenzidine (DAB), in histological sections of the liver and spleen, are difficult to distinguish from other pigments such as hemosiderin, biliary pigment, and hematin (6).

Fluorescence *in situ* hybridization (FISH) is a method that allows visualization, quantification, identification, and anatomic distribution of a microorganism in histologic sections through direct observation (5, 28, 29). Previous studies used FISH in cell culture to detect different *Chlamydia* species in humans (30). A similar technique was used to detect *Chlamydia psittaci* in rodents (37). However, despite this technique being recommended for diagnosing *Chlamydia* sp. (14), to our knowledge, no studies describe in detail and evaluate the use of the technique in birds. The data found in the literature mention use through personal communication and non-public data (3). This paper describes using FISH to identify *Chlamydia* sp. in FFPE liver samples from native and exotic birds, with and without clinical manifestation of the disease.

Material and Methods

This research was approved by the Animal Use Ethics Committee from the Federal University of Paraná (UFPR) – Palotina, PR, Brazil, protocol number CEUA/Palotina 04/2019, and by the Sistema de Autorização e Informação em Biodiversidade (SISBIO), Brazil, registration number 70370-1.

Samples

Liver samples from birds with and without clinical suspicion of chlamydiosis were selected from the archive of the Laboratório de Patologia Animal (LPA), UFPR, from 2013-2019 (retrospective group). Furthermore, liver samples from free-ranging rock doves (*Columba livia*) that were collected in the year of 2019 as part of a different study with synanthropic animals were also evaluated (prospective group).

Within the prospective group, the liver samples were routinely processed and stained with Hematoxylin and Eosin (H&E), Giemsa (G), Modified Gimenez (PVK), and processed for FISH (39, 11).

Within the retrospective group, three sections were taken, two were stained with G and PVK stains, and the last was used for FISH. The pathological findings within this group were obtained from the respective pathology reports.

Positive control

Liver histologic sections from a *Columba livia* (Rock dove) and a *Myiopsitta monachus* (Monk parakeet) positive for *Chlamydia psittaci* were used as positive controls. PCR confirmed the former in a previously studied cohort (26), and IHC confirmed the latter.

Negative control

Liver histological sections from a *Columba livia* negative for *Chlamydia psittaci* were used as a negative control by the PCR technique.

Polymerase chain reaction (PCR)

Forty-seven randomly selected samples from the prospective study were submitted to PCR according to a study already published (18). The primers used were CPF-5'GCA AGA CAC TCC TCA AAG CC-3' and CPR-5' CCT TCC CAC ATA GTG CCA TC-3'. DNA extraction was performed with NucleoSpin® Tissue (Macharey-Nagel) extraction kit. PCR was performed in a 50 µl volume containing 24 µl of Taq DNA polymerase (GoTaq® Green Master Mix 2x- Promega), 1 µl of each forward and reverse primers (100 mM) (IDT® Biotechnology), 4 µl of sample, and 20 µl of DNAse free H_2O. A previously known positive sample for *Chlamydia psittaci* was used as the positive control, and DNAse free H_2O was used as the negative control. DNA amplification was performed at 94°C for three minutes, followed by 40 cycles of 94°C for 30 seconds + 50°C for 30 seconds + 72°C for 45 seconds, and a final extension at 72°C for 45 seconds. Electrophoresis was performed in agarose gel 2% at 90 mA for 80 minutes.

FISH probe

The probe was commercially synthesized (Invitrogen®) based on a published study (18). The chosen annealing sequence is from a preserved region of the ompA gene that codifies the Major Outer Membrane Protein (MOMP), which is common to the family Chlamydiaceae. The probe sequence CPF-5'GCA AGA CAC TCC TCA AAG CC-3 was bound to the fluorochrome Alexa Fluor 555 (Thermo Fisher®) and purified by high performance liquid chromatography (HPLC). The chosen sequence had a 100% match to the genus *Chlamydia* using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

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FISH technique

Five µm-thick histological liver sections were deparaffinized and hydrated in serial solutions containing decreasing ethanol concentration. The slides were inserted into coverplates (Thermo Fisher, catalog number 72110017, Waltham, MA, USA) and placed in a coverplate slide rack (Thermo Fisher, catalog number 73310017, Waltham, MA, USA). Each slide received 80 µL of hybridization buffer (100 mM Tris, pH 7.2, 0.9 M of NaCl, and 0.1% of sodium dodecyl sulfate) and 100 µL of a solution containing 200 ng of the probe. The slides were incubated at 37°C for 16 hours. Then, the slides were washed three times with 100 µL of hybridization buffer pre-heated at 45°C, three times with washing solution (100 mM Tris, pH 7.2, and 0.9 M NaCl) pre-heated at 45°C, and finally with ultrapure water for 2 minutes. The slides were placed inside an incubator to dry at 45°C. Coverslips were placed using a glycerol-based liquid mountant (ProLong Diamond Antifade Mountant, Thermo Fisher®) and the slides were immediately read using a fluorescence microscope (Olympus, FSX 100, Japan).

The slides that presented positive hybridization signals were further scored according to the average number of microorganisms present in six 400x fields. The score created by the authors considers that samples with up to five microorganisms labeled with a probe per 400x field on average were considered mild infection (+), between six to ten probe-labeled microorganisms per 400x field were considered moderate infection (++), and more than eleven probe-labeled microorganisms per 400x field were considered severe infection (+++).

Results

Liver samples were evaluated from 137 birds of 22 different species (Table 1) and nine different orders (Accipitriformes, Caprimulgiformes, Cathartiformes, Columbiformes,

Table 1. Orders and species of birds that were positive for *Chlamydia* sp. by fluorescent *in situ* hybridization.

Source	Order	Species	Sample size	Positive
Retrospective study (*n*=57)	Accipitriformes (*n*=5)	*Gampsonyx swainsonii*	2	2
		Rupornis magnirostris	3	2
	Caprimulgiformes (*n*=1)	*Hydropsalis parvula*	1	1
	Cathartiformes (*n*=1)	*Coragyps atratus*	1	1
	Columbiformes (*n*=7)	*Columba livia*	7	6
	Passeriformes (*n*=14)	*Sporophila angolensis*	1	1
		Coryphospingus cucullatus	1	1
		Cyanoloxia brissonii	4	2
		Turdus rufiventris	1	1
		Saltator similis	4	0
		Pitangus sulphuratus	1	0
		Sporophila lineola	1	0
		Euphonia chlorotica	1	0
	Pelecaniformes (*n*=1)	*Tigrisoma lineatum*	1	1
	Psittaciformes (*n*=19)	*Psittacara leucophthalmus*	2	2
		Ara ararauna	1	0
		Amazona aestiva	8	7
		Nymphicus hollandicus	8	4
	Piciformes (*n*=1)	*Colaptes campestris*	1	0
	Strigiformes (*n*=8)	*Athene cunicularia*	2	2
		Megascops choliba	4	2
		Tyto furcata	2	1
Prospective study (n=80)	Columbiformes (*n*=80)	*Columba livia*	80	56

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Passeriformes, Pelecaniformes, Psittaciformes, Piciformes, and Strigiformes). Of those 137 samples, 57 composed the retrospective group, and 80 composed the prospective group.

Only 1.45% (2/137) of the birds had clinical suspicion of chlamydiosis and compatible gross and/or histological lesions. Both were turquoise-fronted Amazon (*Amazona aestiva*) from the retrospective group.

FISH examination revealed 67.15% (92/137) of positivity for *Chlamydia* sp., with 63.15% (36/57) from the retrospective group and 70% (56/80) from the prospective group. The bacterium was visualized within the cytoplasm of hepatocytes. It was characterized by a 0.3-2 µm, bright, strong, round signal (Fig. 1A and 1C) that disappeared under an incompatible light filter (Fig. 1B).

FISH quantitative evaluation showed that 42.39% (39/92) of the liver samples were classified as containing small numbers (+), 14.13% (13/92) with moderate numbers (++), and 43.47% (40/92) with large numbers of microorganisms (+++). Giemsa and PVK stains revealed the presence of the agent in 13.13% (18/137) and 59.12% (81/137) of the cases, respectively. When the identification results were compared between each special stain to FISH, the PVK stain showed a better chance of identifying the agent in the histological sections. Of 92 positive samples by FISH, 81 were also positive using PVK stain, even in cases where FISH labeling was considered mild. On the other hand, G stain was positive in only 13 samples from 91 positive samples by FISH, and all of them had moderate (++, 4/13) or marked (+++, 9/13) infection determined by FISH.

Histologically, the birds that were positive for FISH presented variable degrees of hepatic necrosis associated with lymphoplasmacytic, histiocytic, and heterophilic inflammatory infiltrate (50%, 46/92). Other unspecific histological findings consisted of congestion (8.69%, 8/92), cytoplasmic vacuolation (7.6%, 7/92), cytoplasmic vacuolation with congestion (5.43%, 5/92), cytoplasmic vacuolation with congestion and cholestasis (4.34%, 4/92), cytoplasmic vacuolation with cholestasis (3.26%, 3/92), periportal fibrosis and bile duct hyperplasia (2.17%, 2/92), and congestion and cholestasis (2.17%, 2/92). Histological lesions were absent in 11.95% (11/92) of the cases. The relationship between histological lesions and the quantitative evaluation of microorganisms on FISH slides is described in Table 2.

From 47 samples of the prospective group that were submitted to PCR, 95.75% (45/47) were positive for *Chlamydia psittaci*. From these 47 samples, 82.97% (39/47) were positive by both FISH and PCR, 12.76% (6/47) were positive by PCR and negative by FISH, and 4.25% (2/47) were negative by PCR and positive by FISH. Considering the slides from the prospective group that were positive by FISH (*n*=41), 51.21% (21/41) of the samples had one plus (+) of probe-labeled microorganisms, 19.51% (8/41) had two plus (++)

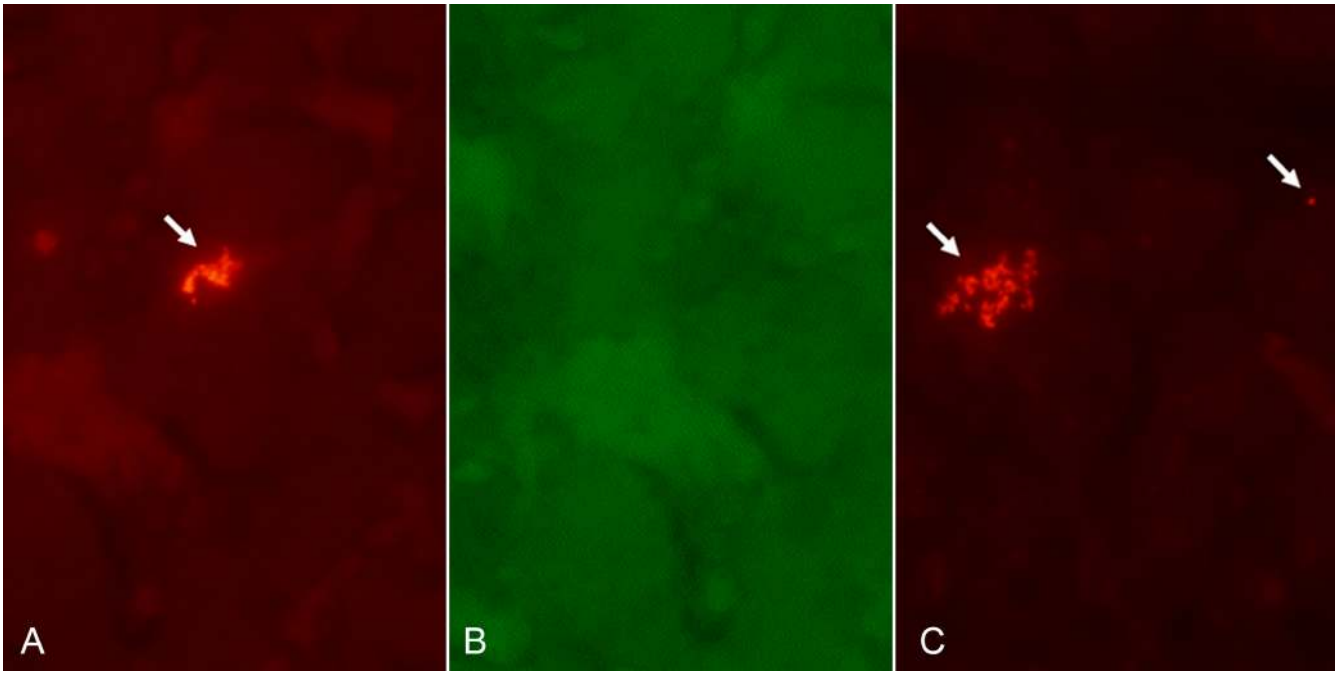

Figure 1. Hybridization labeling in the liver for *Chlamydia sp*. A- Positive hybridization labeling (arrow) in the liver of turquoise-front parrot (*Amazona aestiva*) under the red-light filter. FISH, obj. 40x. B- Counterproof of the liver section shown in Fig. 1A under unmatched green-light filter. FISH, obj. 40x. C- Positive hybridization labeling (arrows) in the liver of roadside hawk (*Rupornis magnirostris*) under the red-light filter. Note the positive hybridization signal within the cytoplasm of a hepatocyte. FISH, obj. 40x.

Table 2. Correlation of histological findings with quantitative positive hybridization signals by fluorescent *in situ* hybridization, including the retrospective and prospective groups.

Lesion	Quantitative evaluation of the presence of the bacterium in FISH slides		
	(+)	(++)	(+++)
Necrotizing, lymphoplasmacytic, histiocytic, and heterophilic hepatitis (*n*= 46)	48% (22/46)	13% (6/46)	39% (18/46)
Congestion (*n*=8)	38% (3/8)	13% (1/8)	50% (4/8)
Cytoplasmic vacuolation (*n*=6)	33% (2/6)	33% (2/6)	33% (2/6)
Cytoplasmic vacuolation, cholestasis, and congestion (*n*=4)	25% (1/4)	25% (1/4)	50% (2/4)
Cholestasis (*n*=5)	40% (2/5)	0%	60% (3/5)
Cytoplasmic vacuolation and congestion (*n*=5)	60% (3/5)	20% (1/5)	20% (1/5)
Cytoplasmic vacuolation and cholestasis (*n*=3)	33% (1/3)	0%	67% (2/3)
Portal fibrosis and bile duct hyperplasia (*n*=2)	50% (1/2)	0%	50% (1/2)
Cholestasis and congestion (*n*=2)	0%	100% (2/2)	0%
No histological changes (*n*=11)	27% (3/11)	9% (1/11)	64% (7/11)
n=92			

(+) up to five positive hybridization signals per 40x field, (++) six to ten positive hybridization signals per 40x field, and (+++) more than eleven positive hybridization signals per 40x field.

of probe-labeled microorganisms, and 29.26% (12/41) had three plus (+++) of probe-labeled microorganisms.

The Orders with higher positivity to *Chlamydia* sp. by FISH in this study were Columbiformes and Psittaciformes, with 67.39% (62/92) and 14.13% (13/92) of the total positive cases, respectively. Strigiformes composed 5.43% (5/92) of the positive cases, the same percentage of Passeriformes (5/92), and 4.34% (4/92) of the total positive cases were Accipritiformes. Birds from the species Pelecaniformes, Cathartiformes, and Caprimulgiformes composed, from each Order, 1.08% (1/92) of the total positive cases.

One specimen of Little nightjar (*Hydropsalis parvula*), Order Caprimulgiformes, and one specimen of Rufescent Tiger-heron (*Tigrisoma lineatum*), Order Pelecaniformes, were positive for *Chlamydia* sp. by FISH. Both species had not reported positivity for chlamydiosis in the literature yet.

Discussion

Pathological findings of chlamydiosis vary, and there are no pathognomonic lesions (32). The animal is sometimes positive, but no lesions or clinical signs are observed (6, 13, 21). In patients with lesions, they are characterized by necrotizing hepatitis and changes in the air sacs, heart, lungs, and spleen (33). In this study, only one percent of the animals had clinical or pathological evidence of chlamydiosis.

Histologically, the liver contains areas of lymphoplasmacytic and histiocytic inflammation, diffuse infiltration of macrophages containing hemosiderin, and bile in the sinusoids (6, 11, 36). Hepatic necrosis and heterophilic infiltrates were observed in 46 birds that were positive for *Chlamydia* detected by FISH, indicating the agent's active role in these areas. Hepatic necrosis is caused by the release of elementary bodies after activating the reticulate body within the cytoplasm of the affected cells. Even though important, hepatic necrosis is not unique to *Chlamydia* sp. In birds, as other bacteria such as *Salmonella* sp., *Streptococcus* sp., and *Staphylococcus* sp. It can also cause similar lesions (36). Because of that, techniques that help in the definitive diagnosis are essential.

In chronic cases, portal fibrosis and bile duct hyperplasia are also described (6, 36). Within the evaluated samples in this study, these findings were present in 2% (3/137) of the samples. Two out of the three birds were positive for *Chlamydia* sp. by FISH, with labeling at the lesion site. This finding suggests the involvement of this agent in cases of hepatic fibrosis in birds.

Unspecific histological findings were observed in 34% (47/137) of the samples, in which the agent was found through FISH in 70% (33/47) of the cases. This data highlights the lack of specificity of the lesions in cases of chlamydiosis, and they can be associated with various diseases. On the other hand, the absence of histologic lesions was observed in 18% (25/137) of the cases, of which 44% (11/25) were positive for chlamydiosis by FISH. The occurrence of positive animals that do not have hepatic lesions is associated with the agent's capacity to cause inapparent infection. It occurs because *Chlamydia* sp. can invade the host cell and become latent, forming a reticulate body. In these cases, disease manifestation can occur under stress or immunosuppression (6, 31).

Even though it is possible, the agent was not observed histologically in any of the cases using only routine HE staining, and histochemical stains can be necessary to

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identify *Chlamydia* sp. (11, 33). However, histochemical stains alone are not enough to reach a definite diagnosis of chlamydiosis, and histochemical staining is a low-sensitivity method for pathogen detection (12). The PVK stain offers better tinctorial contrast to identify *Chlamydia* sp. than the G stain. In our cohort, G stain had a high percentage of false negative results, and staining was usually noted when the number of bacteria in the section was higher.

The degree of labeling by FISH in this research was similar to that obtained in liver sections of mice infected by *C. pneumoniae* (27). Bacteria were observed within the cytoplasm of hepatocytes, with good distinction from the background tissue autofluorescence. Labeling confirmation was performed by switching the red-light filter (compatible filter) to the green-light filter (non-compatible filter). In this research, autofluorescence was observed in red blood cells and tissues rich in elastin and collagen. However, it did not interfere with the visualization of the agent, as the agent's fluorescence was of high intensity.

The annealing sequence of the probe is a well-preserved region of the MOMP codifier gene, common to *Chlamydia* sp. This gene codifies the protein portion that corresponds to 60% of the weight of the bacterium's external membrane (17, 31, 40). The probe size was chosen according to the technique's standard, which easily allowed the identification of the genus. Probes that target preserved regions of the MOMP codifier gene, such as the one used in this experiment, allow the identification of a broader spectrum among the *Chlamydia* species. Compared to PCR, FISH requires a small number of ribosomal RNA copies to detect the bacterium, which gives FISH a higher specificity, even though its sensitivity is low. While PCR sensitivity is high, cross-reaction with other organisms is more common (1).

The great advantage of FISH compared to PCR is that the former can differentiate sick animals from carriers, as the histological lesions and FISH positivity scores correlate with sick or infected animals. Therefore, animals with necrotizing hepatitis had higher FISH labeling scores when compared to other lesions. The greatest disadvantage of FISH, besides the high cost of equipment, is its low applicability in antemortem detection in the clinical routine, as it requires the association with invasive procedures, such as biopsy.

PCR allows quick and sensible identification of *Chlamydia*, regardless of whether the microorganism is viable and can be performed in secretions and excretions (1, 29). In this experiment, the number of positive samples by PCR was higher than the number of positive samples by FISH, which is expected, given the higher sensitivity of PCR. Another factor is that a higher area of hepatic tissue is evaluated in PCR than in FISH, as in FISH, a section of 5 µm thick fraction of liver is evaluated, whereas PCR uses 2 to 5 grams of liver for DNA extraction. However, FISH and PCR positively correlated in 83% (39/47) of the samples. Positivity to the bacterium does not necessarily mean the presence of disease, which can be seen in samples with positive PCR with minimal or no tissue changes, which was observed in 28% (11/39) of positive cases by PCR and FISH. This data highlights the limitation of PCR in associating the presence of an agent with presence of a lesion. Our objective was not to compare the efficiency of both techniques; hence, PCR was used as a reference technique to validate FISH results.

IHC is considered an accurate technique in FFPE samples for post-mortem diagnosis of avian chlamydiosis (6, 11). However, in general, FISH has more specificity and sensibility when compared to IHC (29).

Chlamydiosis is reported in 469 avian species of 30 different orders, and this number is increasing (20). In our investigation, we observed positivity in birds of the species Little nightjar (*Hydropsalis parvula*), Order Caprimulgiformes, and Rufescent tiger-heron (*Tigrisoma lineatum*), Order Pelecaniformes, both of which had not reported positivity for chlamydiosis in the literature yet.

The Columbiformes and Psittaciformes orders have a higher frequency of infection by *Chlamydia* sp. and are considered its main reservoirs (2, 7, 16, 17, 25), which was also observed in this study. The columbiformes birds sampled in this study were birds from the species *Columba livia*, an invasive species for the Brazilian fauna that is present in urban and country areas, present flocking behavior (9), and are considered synanthropic animals (10, 15). These behaviors favor disease dissemination among birds and can be a risk factor for other animals and human health. Approximately 40% of the doves from the prospective group had unspecific or no lesions, which illustrates the difficulty in diagnosing this disease in this species. Consequently, it contributes to underreporting and insufficient epidemiological data. Furthermore, it is important to emphasize this species as a contamination source and reservoir of *Chlamydia* spp. in the environment.

The evaluated birds from the LPA caseload were almost entirely from apprehended wildlife illegal breeders and trades. In a lot of the cases, the birds had a history of malnutrition and overpopulation, which increases the risk of chlamydiosis, as well as other diseases (38). Some evaluated species, such as the Turquoise-fronted Amazon (*Amazona aestiva*), are usually kept as pets and have close contact with humans, increasing concern for zoonotic transmission. From the sampled parrots of this species, 87.5% (7/8) were positive for *Chlamydia* sp. by FISH, of which 28.57% (2/7) had any clinical or pathological manifestation of the disease.

For the Passeriformes birds, 1.35 % (5/369) were positive for *Chlamydia* sp., a similar proportion to the results of another study (21). Even though the number of animals is low, previous studies have shown chlamydiosis as an important disease in birds from this Order, as well as the importance of this Order in disease maintenance and dissemination (4).

Efficient detection methods for chlamydiosis are important to further understand the pathogenesis and zoonotical relevance of this disease. Our findings support that FISH is a specific and sensible technique that allows the identification

of *Chlamydia* sp. in histological sections of the liver from birds and can be used for the diagnosis in carrier birds.

Conflict of Interest

The authors declare no competing interests.

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References

1. Andersen AA, Franson JC. Avian Chlamydiosis. In: Thomas NJ, Hunter B, Atkinson CT, editors. Infectious diseases of wild birds. Iowa: Blackwell Publishing; 2007. p. 303-16.
2. Araujo SAA, Pereira WLA, Silva SP, Cardoso JF, Filho ES, Bernal MKM, Mendes FF, Nunes MRT. Clinical and molecular diagnosis of *Chlamydophila* in captive parrots in Pará State, Brazil. Semina: Ciênc Agrár. 2019;40(6):2603-12.
3. Balsamo G, Maxted AM, Midla JW, Murphy JM, Wohrle R, Edling TM, Fish PH, Flammer K, Hyde D, Kutty PK, Kobayashi M, Helm B, Oiulfstad B, Ritchie BW, Stobierski MG, Ehnert K, Tully Jr TN. Compendium of Measures to Control *Chlamydia psittaci* Infection Among Humans (Psittacosis) and Pet Birds (Avian Chlamydiosis). J Avian Med and Surg. 2017;31(3):262-82.
4. Beckmann KM, Borel N, Pocknell AM, Dagleish MP, Sachse K, John SK, Pospischil A, Cunningham AA, Lawson B. Chlamydiosis in British garden birds (2005–2011): Retrospective diagnosis and *Chlamydia psittaci* genotype determination. Ecohealth. 2014; 11(4):544-63.
5. Bojesen AM, Christensen H, Nielsen OL, Olsen JE, Bisgaard M. Detection of *Gallibacterium* spp. in chickens by fluorescent 16S rRNA *in situ* hybridization. J Clin Microbiol. 2003;41(11):5167-72.
6. Casagrande RA, Machado VR, Souza SO, Watanabe TTN, Sonne L, Pavarini SP, Driemeier D. Diagnóstico imuno-histoquímico e caracterização anatomopatológica de clamidiose em psitacídeos. Pesqui Vet Bras. 2014;9(34):885-90.
7. Chahota R, Ogawa H, Mitsuhashi Y, Ohya K, Yamaguchi T, Fikushi H. Genetic diversity and epizootiology of *Chlamydophila psittaci* prevalent among the captive and feral avian species based on VD2 region of ompA Gene. Microbiol Immunol. 2006;50(9):663-78.
8. Cheong HC, Lee CYQ, Cheok YY, Tan GMY, Looi CY, Wong WF. *Chlamydiaceae*: Diseases in Primary Hosts and Zoonosis. Microorganisms. 2019;7(5):146.
9. Cong W, Huang SY, Zhang XY, Zhou DH, Xu MJ, Zhao Q, Song HQ, Zhu XQ, Qian AD. Seroprevalence of *Chlamydia psittaci* infection in market-sold adult chickens, ducks and pigeons in north-western China. J Med Microbiol. 2013;62(Pt 8):1211-4.
10. Dickx V, Beeckman DSA, Dossche L, Tavernier P, Vanrompay D. *Chlamydophila psittaci* in homing and feral pigeons and zoonotic transmission. J Med Microbiol. 2010;59(Pt 11):1348-53.
11. Ecco R, Preis IS, Martins NRS, Vilela DAR, Shivaprasad HL. An outbreak of chlamydiosis in captive psittacines. Braz J Vet Pathol. 2009;2(2):85-90.
12. Elder J, Brown C. Review of techniques for the diagnosis of *Chlamydia psittaci* infection in psittacine birds. J Vet Diagn Invest. 1999;11(6)2:539-41.
13. Evans EE. Zoonotic diseases of common pet birds: psittacine, passerine, and columbiform species. Vet Clin North Am Exot Anim Pract. 2011;14(3):457-76.
14. Frickmann H, Zautner AE, Moter A, Kikhney J, Hagen RM, Stender H, Poppert S. Fluorescence *in situ* hybridization (FISH) in the microbiological diagnostic routine laboratory: a review. Crit Rev Microbiol. 2017;43(3):263-93.
15. Geigenfeind I, Haag-Wackernagel D. Detection of Chlamydophila psittaci from feral pigeons in environmental samples: problems with currently available techniques. Integr Zool. 2010;5(1):63-9.
16. Guo W, Li J, Kaltenboeck B, Gong J, Fan W, Wang C. *Chlamydia gallinacea*, not *C. psittaci*, is the endemic chlamydial species in chicken (*Gallus gallus*). Sci Rep. 2016;6: 19638.
17. Harkinezhad T, Geens T, Vanrompay D. *Chlamydophila psittaci* infections in birds: a review with emphasis on zoonotic consequences. Vet Microbiol. 200916;135(1-2):68-77.
18. Hewinson RG, Griffiths PC, Bevan BJ, Kirwan SE, Field ME, Woodward MJ, Dawson M. Detection of Chlamydia psittaci DNA in avian clinical samples by polymerase chain reaction. Vet Microbiol. 1997;54(2):155-66.
19. Hulin V, Bernard P, Vorimore F, Aaziz R, Cléva D, Robineau J, Durand B, Angelis L, Siarkou VI, Laroucau K. Assessment of *Chlamydia psittaci* shedding and environmental contamination as potential sources of worker exposure throughout the mule duck breeding process. Appl Environ Microbiol. 2016;82(5):1504-18.
20. Kaleta EF, Taday EMA. Avian host range of *Chlamydophila* spp. based on isolation, antigen detection and serology. Avian Pathol. 2003;32(5):435-62.
21. Krawiec M, Piasecki T, Wieliczko A. Prevalence of *Chlamydia psittaci* and other *Chlamydia* species in wild birds in Poland. Vector Borne Zoonotic Dis. 2015;15(11):652-5.

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DOI: https://10.24070/bjvp.1983-0246.v16i3p159-166

22. Laroucau K, Vorimore F, Aaziz R, Berndt A, Schubert E, Sachse K. Isolation of a new chlamydial agent from infected domestic poultry coincided with cases of atypical pneumonia among slaughterhouse workers in France. Infect Genet Evol. 2009;9(6):1240-7.
23. Leal DC, Negrão VB, Santos F, Raso TF, Barrouin-Melo SM, Franke CR. Ocorrência de *Chlamydophila psittaci* em pombos (*Columba livia*) na cidade de Salvador, Bahia. Arq Bras Med Vet Zootec. 2015;67(3):771-6.
24. Li Z, Liu P, Hou J, Xu G, Zhang J, Lei Y, Lou Z, Liang L, Wen Y, Zhou J. Detection of *Chlamydia psittaci* and *Chlamydia ibidis* in the Endangered Crested Ibis (*Nipponia nippon*). Epidemiol Infect. 2020 Jan 8;148:e1.
25. Madani AS, Peighambari SM. PCR-based diagnosis, molecular characterization and detection of atypical strains of avian *Chlamydia psittaci* in companion and wild birds. Avian Pathol. 2013; 42(1):38-44.
26. Madico G, Quinn TC, Boman, Gaydos CA. Touchdown enzyme time release-PCR for detection and identification of *Chlamydia troschomatis*, *C. pneumoniae*, and *C. psittaci* using the 16S and 16S-23S spacer rRNA genes. J Clin Microbiol. 2000;38(3):1085-93.
27. Marangoni A, Donati M, Cavrini F, Aldini R, Accardo S, Sambri V, Montagnani M, Cevenini R. *Chlamydia pneumoniae* replicates in Kupffer cells in mouse model of liver infection. World J Gastroenterol. 2006;12(40):6453-7.
28. Moter A, Göbel UB. Fluorescence in situ hybridization (FISH) for direct visualization of microorganisms. J Microbiol Methods. 2000;41(2):85-112.
29. Neves SMN, Guedes RMC. Hibridização *in situ* fluorescente: princípios básicos e perspectivas para o diagnóstico de doenças infecciosas em medicina veterinária. Arq Inst Biol. 2012;79(4):627-32.
30. Poppert S, Essig A, Marre R, Wagner M, Horn M. Detection and differentiation of *Chlamydiae* by fluorescence *in situ* hybridization. Appl Environ Microbiol. 2002;68(8):4081-9.
31. Proença LM, Fagliari JJ, Raso TF. Infecção por *C. psittaci*: uma revisão com ênfase em psitacídeos. Cienc Rural. 2011;41(5):841-7.
32. Raso TF. (2009). Clamidiose (Chlamydiosis). In: Júnior AB, Silva EN, Di Fábio J, Sesti L, Zuanaze MAF, editor. Doenças das Aves. 2rd ed. Campinas: Fundação Apinco de Ciência e Tecnologias Avícolas; 2009. p. 553-63.
33. Reavill DR, Dorrestein G. Psittacines, Coliiformes, Musophagiformes, Cuculiformes. In: Terio KA, McAloose D, Leger JS, editors. Pathology of Wildlife and Zoo Animals. London: Academic Press; 2018. p. 775-98.
34. Sachse K, Bavoil PM, Kaltenboeck B, Stephens RS, Kuo C, Rosselló-Móra R, Horn M. Emendation of the family *Chlamydiaceae*: Proposal of a single genus, *Chlamydia*, to include all currently recognized species. Syst Appl Microbiol. 2015,38(2):99-103.
35. Sachse K, Laroucau K, Riege K, Wehner S, Dilcher M, Creasy HH, Weidmann M, Myers G, Vorimore F, Vicari N, Magnino S, Liebler-Tenorio E, Ruettger A, Bavoil PM, Rosselló-Móra R, Marz M. Evidence for the existence of two new members of the family *Chlamydiaceae* and proposal of *Chlamydia avium* sp. nov. and *Chlamydia gallinacea* sp. nov. Syst Appl Microbiol. 2014;37(2):79-88.
36. Schimidt RE, Reavill DR, Phalen DN. Liver. In: Schimidt RE, Reavill DR, Phalen DN, editor. Pathology of pet and aviary birds. Iowa: Wiley-Blackwell; 2015. p. 95-125.
37. Theil D, Hoop R, Herring AJ, Pospischil A. Detection of Chlamydia in formalin-fixed and paraffin-embedded avian tissue by in situ hybridization. A comparison between in situ hybridization and peroxidase-antiperoxidase labelling. Zentralbl Veterinarmed B. 1996;43(6):365-70.
38. Tolba HMN, Abou Elez RMM, Elsohaby I. Risk factors associated with *Chlamydia psittaci* infections in psittacine birds and bird handlers. J Appl Microbiol. 2018;126(2):402-10.
39. Tolosa EMC, Rodrigues JC, Behmer OA, Freitas Neto, AG. Manual de Técnicas Para Histologia Normal e Patológica. São Paulo: Manole; 2003. 341 p.
40. Vanrompay D. Avian Chlamydiosis. In: Swayne DE, editor. Diseases of Poultry. 14th ed. Hoboken: Wiley-Blackwell; 2020. p. 1086-107.
41. Webster JD, Miller MA, DuSold D, Ramos-Vara J. Effects of prolonged formalin fixation on diagnostic immunohistochemistry in domestic animals. J Histochem Cytochem. 2009;57(8):753-61.