Phylogenetic analyses of *Chlamydia psittaci ompA* gene sequences from captive *Amazona aestiva* (Aves: Psittaciformes) with hepatic disease

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Summary

Chlamydia psittaci was detected in 152 (72%) Amazon parrots (*Amazona aestiva*) out of a population of 212 that died during 2009–2011 in a wildlife rescue and rehabilitation centre in Minas Gerais, Brazil, following rescue from illegal wildlife trafficking. The macroscopic changes were hepatomegaly with multifocal white foci visible at the serosal surfaces of the liver, and extending into the parenchyma, and splenomegaly. The microscopic lesions in the liver included multifocal to coalescing miliary necrosis of hepatocytes with

infiltration by heterophils, lymphocytes and plasma cells. In the spleen, loss of the normal architecture and infiltration by macrophages and plasma cells were observed. Stained tissue sections (Gimenez) revealed small round clusters suggestive of *C. psittaci* (reticulate bodies) in the cytoplasm of macrophages from the liver and spleen. Nine sequences of segments of the *ompA* gene, obtained from different individuals, were randomly selected for sequencing. The phylogenetic analyses showed that all strains clustered with genotype A, the genotype most virulent for birds. This genotype is involved in mortality of psittacines, is easily transmitted in captivity and represents a problem for successful rehabilitation. The results indicate the necessity to improve biosecurity in triage and to provide individual personal protection for professionals and caretakers.

Keywords

Amazon parrot – Chlamydiosis – Fatal hepatitis – Genotype A – Hepatomegaly – Phylogeny.

Résumé

Chlamydia psittaci a été détectée chez 152 (72 %) perroquets amazoniens (*Amazona aestiva*) sur une total de 212 individus morts dans un centre de sauvetage et réhabilitation de la faune sauvage à Minas Gerais, au Brésil, entre 2009 et 2011 après avoir été interceptés et prélevés hors de la mouvance du trafic illegal d'animaux sauvages Les modifications macroscopiques étaient une hépatomégalie avec des foyers blancs multifocaux visibles sur les surfaces séreuses du foie et s'étendant dans le parenchyme et une splénomégalie. Les lésions microscopiques dans le foie comprenaient une nécrose miliaire à coalescente des hépatocytes avec infiltration d'hétérophiles, de lymphocytes et de plasmocytes. Dans la rate, la perte de l'architecture normale et l'infiltration de macrophages et de plasmocytes ont été observés. Des coupes de tissus colorées (Gimenez) ont révélé de petites grappes rondes évoquant *C. psittaci* (corps réticulés) dans le cytoplasme des macrophages du foie et de la rate. Neuf produits segmentés d'une partie du gène codant pour la protéine *ompA*, obtenus de différents individus, ont été sélectionnés de manière aléatoire pour

le séquençage. Les analyses phylogénétiques ont montré que toutes les souches se regroupaient avec le génotype A, le génotype le plus virulent pour les oiseaux, impliqué dans la mortalité des psittacidés et plus facilement transmissible en captivité, ce qui représente un risque pour une réhabilitation réussie. Les résultats indiquent la nécessité d'améliorer la biosécurité lors de la manipulation de animaux en centres de soins et recommandent une protection individuelle des professionnels et des gardiens.

Mots clés

Chlamydiose – Génotype A – Hépatite mortelle – Hépatomégalie – Perroquet Amazone – Phylogénie.

Resumen

Chlamydia psittaci se detectó en 152 (72%) loros amazónicos (*Amazona aestiva*), de una población de 212 que murieron en triaje durante 2009–2011 en un centro de rehabilitación de vida silvestre en Minas Gerais, Brasil, llegaron de aprehensiones de tráfico. Los cambios macroscópicos fueron hepatomegalia con focos blancos multifocales visibles en las superficies serosas del hígado, que se extienden hacia el parénquima y esplenomegalia. Las lesiones microscópicas en el hígado incluían necrosis miliar multifocal a coalescente de hepatocitos con infiltración de heterófilos, linfocitos y células plasmáticas. En el bazo, se observó la pérdida de la arquitectura normal y la infiltración de macrófagos y células plasmáticas. Secciones de tejido teñidas (Giménez) revelaron pequeños grupos redondos que sugieren *C. psittaci* (cuerpos reticulados) en el citoplasma de macrófagos del hígado y el bazo. Los productos de nueve segmentos de parte del gen que codifica la proteína *ompA*, obtenidos de diferentes individuos, se seleccionaron al azar para la secuenciación. Los análisis filogenéticos mostraron que todas las cepas se agrupan con el genotipo A, el genotipo más virulento para las aves, involucrado en la mortalidad de psitácidos y se transmiten más fácilmente en cautiverio, y representan un riesgo para una rehabilitación exitosa. Los resultados indican la necesidad de

mejorar la bioseguridad en el triaje y se recomienda la protección personal individual para profesionales y cuidadores.

Palabras clave

Clamidiosis – Filogenia – Genotipo A – Hepatitis fatal – Hepatomegalia – Loro del Amazonas.

Introduction

Chlamydia psittaci is an obligatory intracellular bacterium maintained in nature mainly by subclinical infection in avian species of the orders Columbiformes, Galliformes and Passeriformes (1). However, both the role of avian species in the epidemiology and the zoonotic potential of *C. psittaci* have been underestimated (2). Several months of infection may be required for the manifestation of clinical signs in psittacine species; the disease is characterised by lethargy, tremor, rough plumage, nasal discharge, conjunctivitis, dyspnoea and diarrhoea (3). There are currently 11 species of *Chlamydia*, and most are capable of systemic infection of multiple host species, although they are mainly associated with asymptomatic infection and a carrier state (4).

Avian *C. psittaci* strains have been classified into at least 15 genotypes, A to F, C/B, I, J, 1V, 6N, MatI16, R54, YP84 and CPX0308; the non-avian genotypes are WC and M56 (5, 6). Genotype A is endemic in psittacines, B in pigeons, C and D have been detected in non-psittacine birds, E in pigeons, ratites, ducks and turkeys, and occasionally in humans, and F has been obtained from a variety of avian hosts, such as an American parakeet (strain VS225) (7). The WC genotype has been found in Wolfsen cattle (Wolfsen Land and Cattle Company, Los Banos, California, United States of America [USA]) and M56 in muskrats (*Ondatra zibethicus*) (3). Although *C. psittaci* genotypes appear to be host species specific, the phylogenetic tree of *C. psittaci* does not support host-specificity, suggesting host species jumps (8, 9). Therefore, all genotypes should be considered to be readily transmissible to humans and to be able to cause human disease (10).

The zoonotic significance of *C. psittaci* may be underestimated by professional healthcare workers and the general public. This is partly attributable to the poor definition of human cases, because diagnosis is based on serological testing using the micro-immunofluorescence test (MIF), which is cross-reactive and insensitive when compared with enzyme-linked immunosorbent assay (ELISA), or the complement fixation test (CFT), which is also cross-reactive and less sensitive even than MIF (10). It is considered that the criteria defining human cases of psittacosis should be adapted to include nucleic-acid amplification tests (NAATs), which are *C. psittaci*-specific and highly sensitive (3). Based on a specific and sensitive diagnosis using NAATs, a reduction of the impact of psittacosis, whether human or avian, could be achieved through the administration of tetracyclines or macrolides. However, because the use of antibiotics is widespread in the poultry and pet bird industries, the potential emergence of antibiotic-resistant and zoonotic field strains should be taken into consideration (2).

Previous studies in Brazil have used polymerase chain reaction (PCR) for the major outer membrane protein (*omp*) gene on cloacal swab samples (11). This has demonstrated the natural occurrence of *C. psittaci* in nestling free-living *Amazona aestiva* (6.3%), although the birds were negative on serology using CFT, and in 26.7% of nestling macaws, with a few (4.8%) positive for antibodies. However, only one hyacinth macaw was positive by both PCR and serology. The results demonstrated the presence of *C. psittaci* in wild psittacine populations of Miranda (20.2360° S, 56.3751° W) and Aquidauana (20.4653° S, 55.7851° W) in the Pantanal (Mato Grosso do Sul, Brazil), although it was not associated with clinical disease. Several free-living avian species in two protected areas of the Federal District of Brazil were shown to be positive for *C. psittaci* (using PCR for the *omp* gene) in cloacal swab and blood samples, with a prevalence of 83% among all species evaluated, representative of 19 avian families, although they had no clinical signs (12). In 2008, an outbreak of chlamydiosis was reported in psittacines of the species *A. aestiva*, *Aratinga aurea* and *Pionus maximiliani* kept at the same premises as described in this paper (13). Given the frequency of seizures from the

illegal trade in wild avian species, the rescue centre is very busy, especially with parrots. Genetic analysis of *C. psittaci* strains circulating in such environments is needed for the evaluation of risks to other species, including humans. The aim of this study was to characterise, using partial genetic analysis, *C. psittaci* strains infecting Amazon parrots that died in the rescue centre of Belo Horizonte (Brazil) following rescue from wild animal trafficking.

Materials and methods

Amazona aestiva parrots (n = 212) that died in the rescue centre (Belo Horizonte, Brazil) during 2009–2011 were tested for *C. psittaci* and 152 were shown to be positive. The post-mortem evaluations revealed similar lesions, and nine individuals were selected randomly for further molecular and histological evaluations. Organ samples (liver, lung and spleen) were collected and frozen or fixed in formalin. Tissue samples (liver, lung and spleen) from the nine parrots were fixed in 10% neutral buffered formalin and embedded in paraffin wax. Sections (4 µm thick) were stained with haematoxylin and eosin or by modified Gimenez staining for the detection of inclusion bodies (mostly reticular bodies). The histopathological evaluations were performed using light microscopy (Olympus CBB, Biolab, São Paulo, Brazil). For the molecular analyses, tissue samples of lungs, spleen or liver from the nine selected parrots were pooled and stored in ethanol at –20°C until processing.

Template DNA was extracted in a solution containing 6 M sodium iodide, 13 mM ethylenediaminetetraacetic acid (EDTA) and 0.5% *N*-lauroylsarcosine-sodium, pH 8.0, according to a previously described procedure (14). The PCR procedures for diagnosis were based on partial amplification of a 418-base pair (bp) *ompA* gene sequence, with primers VD1-f ACTACGGAGATTATGTTTTCGATCGTGT and VD2-r CGTGCACCYACGCTCCAAGA (3) (Thermo, São Paulo, Brazil). Alternatively, primers Cp-F TGCAAGACACTCCTCAAAG and Cp-R AGGTTCTGATAGCGGGAC were used to amplify a fragment of 1378 bp, to yield a larger product of the *ompA* gene for sequencing (15) (Thermo, São Paulo, Brazil). The PCR reagents

consisted of 1 U Taq DNA polymerase, 1.5 mM $MgCl_2$, 200 µM dinucleotide triphosphates (dNTP) and 1× PCR buffer (Thermo, São Paulo, Brazil). The amplification reaction was performed in a thermocycler (MaxyGene, Axygen) using the following thermal cycles: 94°C for 4 min, followed by 40 cycles of 94°C for 1 min and 59°C for 1 min, and a final extension at 72°C for 2 min. As a positive control, *C. psittaci* DNA was extracted from a commercial vaccine (Felis Quantum 4, Intervet Schering-Plough, Cruzeiro, SP, Brazil) using the extraction procedure described above. As a negative control, all the reagents except template DNA were used. A reaction control was performed by determining avian sex, as described previously (16). The DNA sequences were determined by direct sequencing of PCR products; reactions were carried out using a Big Dye Terminator sequencing kit (Applied Biosystems, Foster City, California, USA), according to the manufacturer's instructions. The thermal cycles were set up at 96°C for 4 min, followed by25 cycles at 95°C for 15 s, 50°C for 10 s and 60°C for 4 min. Reaction products were precipitated with sodium acetate in 95% ethanol and centrifuged (12,000 × *g*/15 min); the pellet was washed with 75% ethanol and dried at room temperature. Sequencing was carried out on a 3100 DNA analyser (Applied Biosystems, Foster City, California, USA). The PCR products were evaluated by electrophoresis in agarose gels as revealed by Gel Red® (Biotium, Fremont, California, USA).

The sequences obtained were analysed for quality (using the quality value); the data were pair-wise aligned and edited using the Sequence Analyser (Applied Biosystems, Foster City, California, USA) software. Alignment and calculation of similarity index p-distance were performed with MEGA 7.0 (17, 18).

A phylogenetic tree was constructed using the neighbour-joining algorithm; comparison of *C. psittaci ompA* gene sequences was based on the Tamura three-parameter model (19) with 1,000 bootstrap replicates applied in MEGA 7.0. The following sequences available in the GenBank database, given as accession number (strain), were used: AF269281.1 (MN Zhang); AB284059.1 (CP0315); EU856024.1 (SP04); EU856033.1 (SP13); EU856023.1 (SP03); HM450409.1

(KMZ07); CP002807.1 (08DC60); X56980.1 (6BC); AF269265.1 (CP3); AY762609.1 (41A12); AY762613.1 (WS/RT/E30); AY762611.1 (3759/2); X12647.1 (EAE A22/M); AF269264.1 (MNOs); AF269263.1 (MNRh); AF269266.1 (NJ1) AF269267.1 (TT3); EU048338.1 (WC); AY762610.1 (7344/2); AF269260.1 (CT1); AF269261.1 (GD); AY762612.1 (7778B15); AF269259.1 (VS225); and AF269282.1 (GPIC).

Results

Out of 212 specimens of *A. aestiva* evaluated post mortem, 72% (152) were shown to be positive for the *C. psittaci* partial *ompA* gene. Sequences of the *ompA* gene from nine *C. psittaci* strains found in *A. aestiva* were obtained and shown to have 99–100% nucleotide identity, with each other and with strains CP0315 (AB284059.1), SP03 (EU856023.1), SP04 (EU856024.1) and SP13 (EU856033.1) from birds, and strains MN_Zhang (AF269281.1) and 08DC60 (CP002807.1) obtained from cases of human psittacosis (20, 21). The phylogenetic analyses showed that all the local strains were grouped together and with *C. psittaci* strains assigned to genotype A (Fig. 1). The macroscopic lesions comprised hepatomegaly with white spots in the parenchyma and splenomegaly with congestion; the lungs showed areas of congestion and increased density. The principal histopathological lesions were multifocal lymphoplasmacytic liver foci (Fig. 2), necrotic hepatitis (Fig. 3), cytoplasmic inclusions in macrophages and splenic cytoplasmic inclusions (Fig. 4). Several of the disease foci in liver and spleen contained macrophages and heterophils, and small basophilic coccoid organisms indicative of *Chlamydia* were observed within some of the macrophages. Plasma cells were observed in abundance in the liver and spleen of all birds. Stained liver and spleen smears (Gimenez) revealed cytoplasmic inclusions suggestive of reticular bodies of *Chlamydia* (Fig. 4). Most lung samples were inadequately conserved for histopathology, owing to significant autolysis, although all had gross changes indicative of pneumonia, such as bronchial exudates.

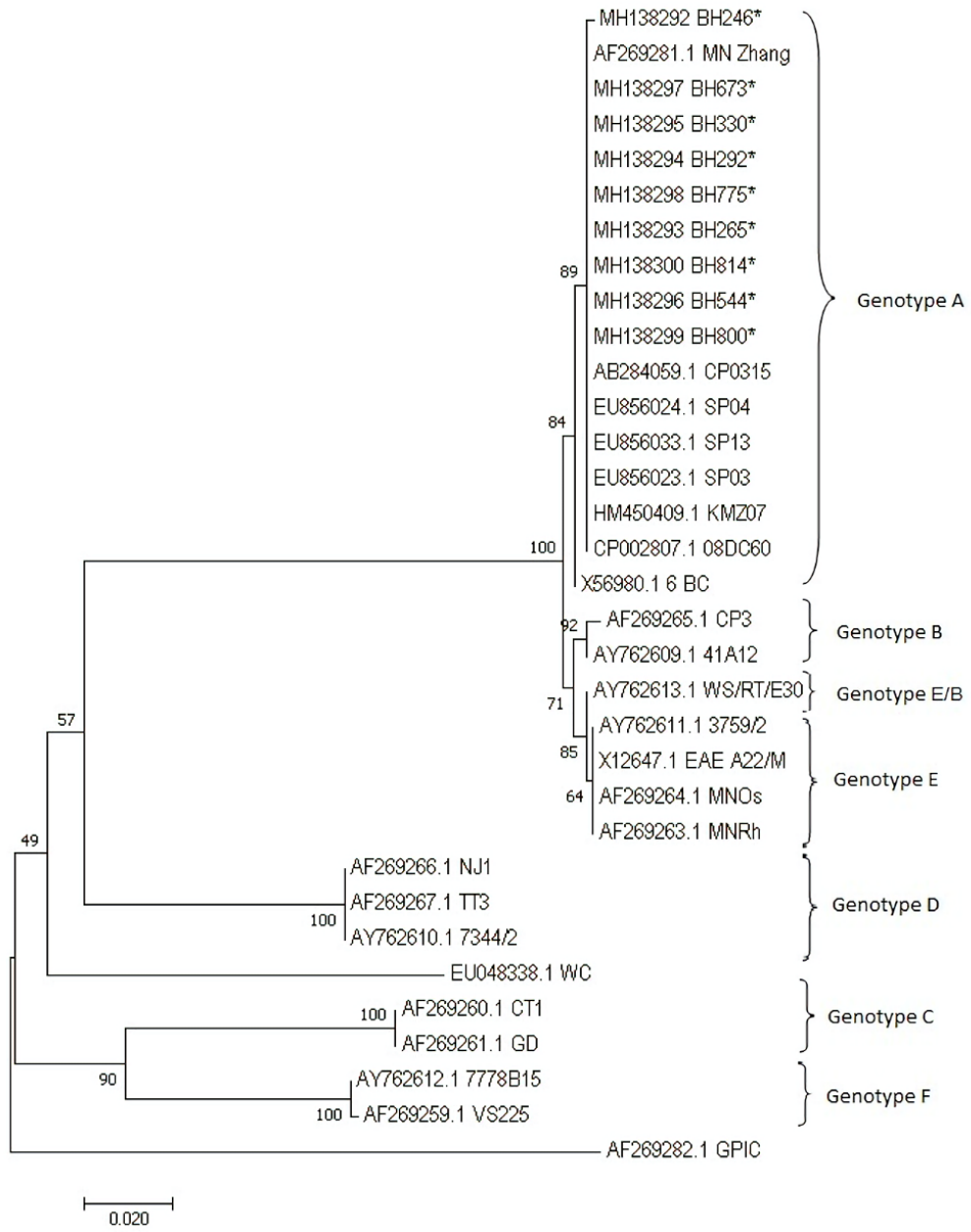

Fig. 1

Phylogenetic tree of *Chlamydia psittaci ompA* gene sequences

Phylogenetic tree constructed with *C. psittaci ompA* gene sequences determined in this study, using the neighbour-joining method based on the Tamura 3-parameter model (20), with 1,000 bootstrap replicates applied in MEGA 7.0, compared with sequences available in the GenBank database (GenBank accession number indicated). *Chlamydia caviae* GPIC (AF269282) was used as an out-group. All strains of this study (*) were grouped with genotype A strains, including MN_Zhang (AF269281.1) and 08DC60 (CP002807.1) described in human psittacosis, and avian strains CP0315 (AB284059.1), SP03 (EU856023.1), SP04 (EU856024.1) and SP13 (EU856033.1).

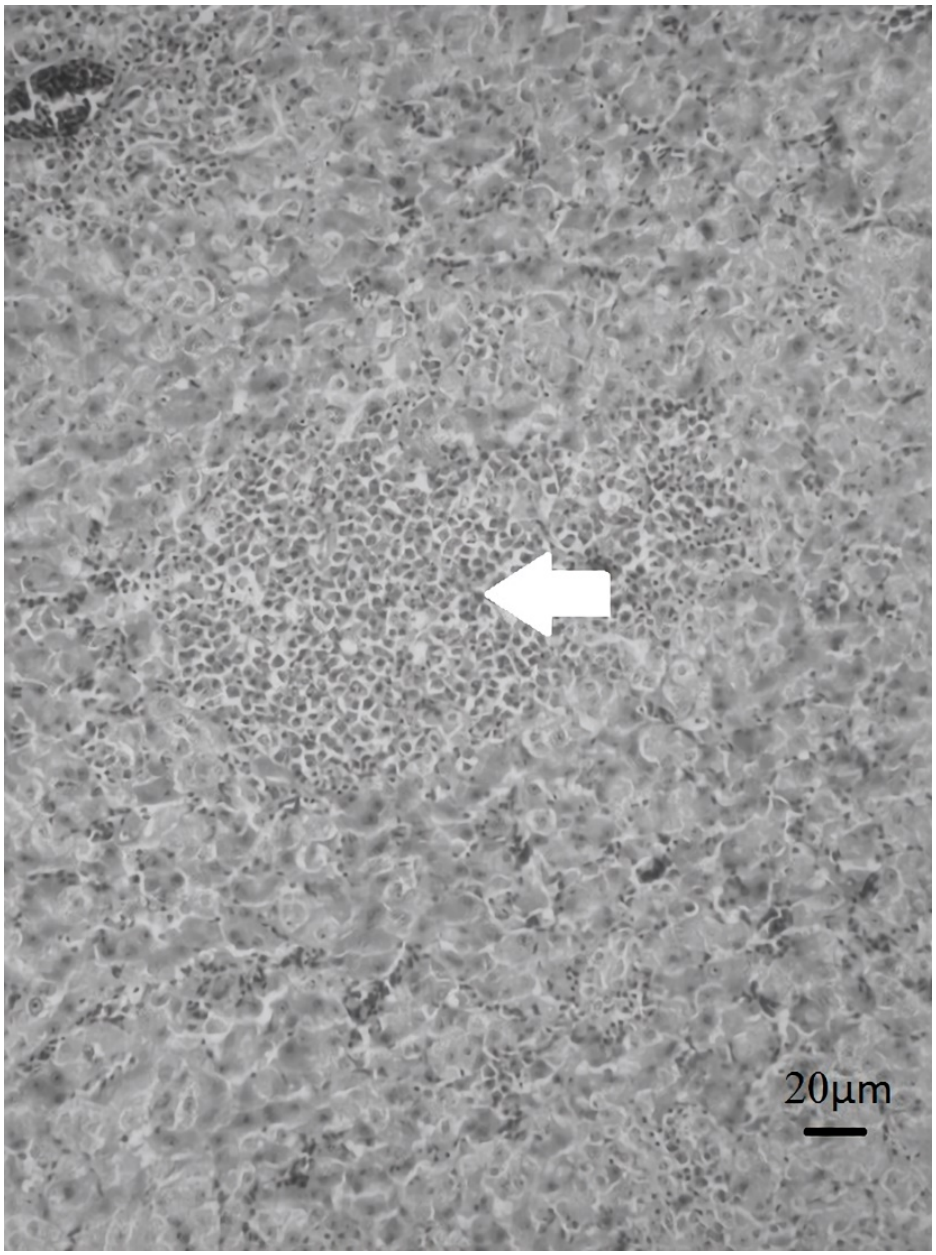

Fig. 2
Amazona aestiva **liver, showing hepatic lymphoplasmacytic infiltrate (arrow). (Haematoxylin and eosin [HE] – 100×)**

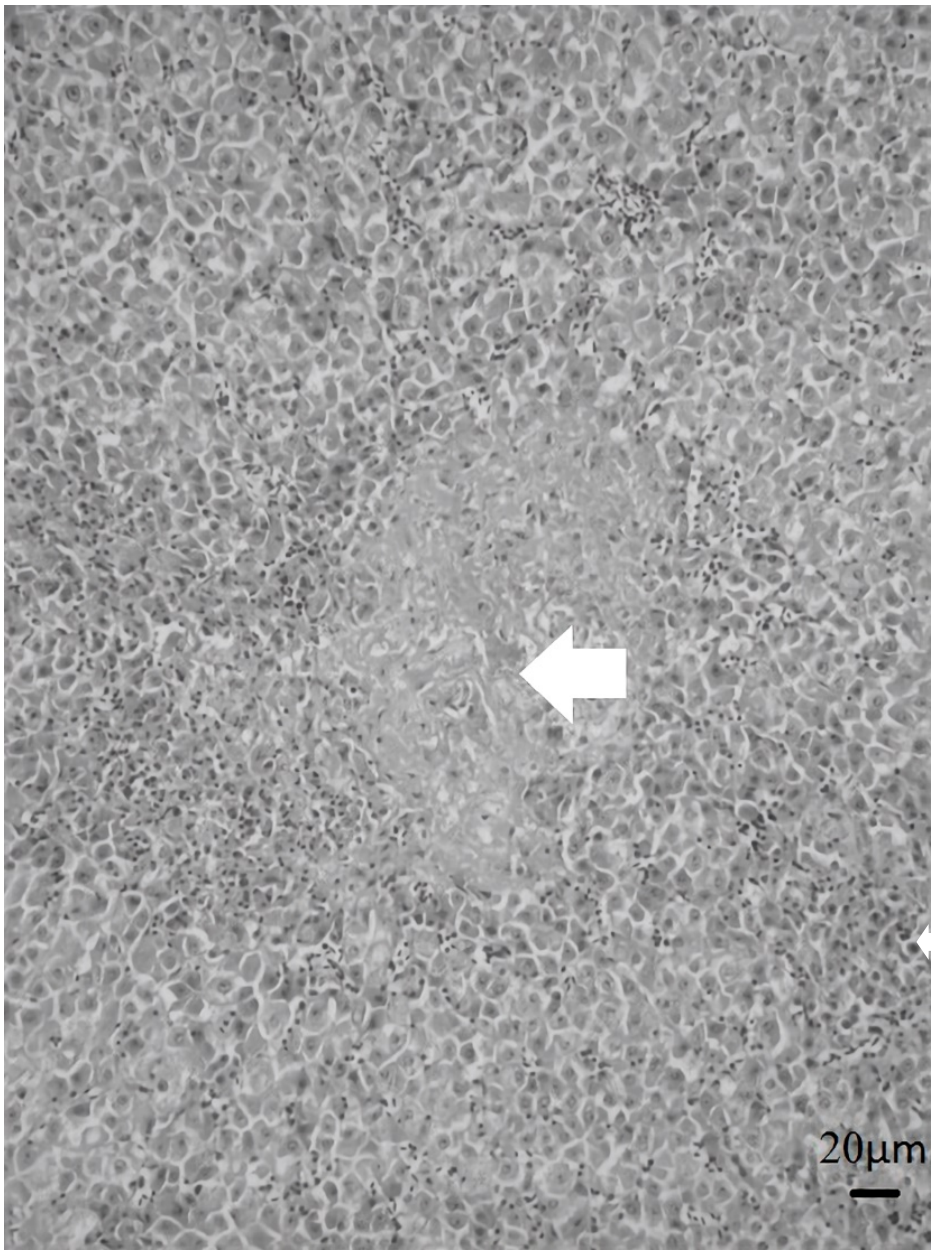

Fig. 3
Amazona aestiva **liver, showing hepatic necrosis (arrow). (HE – 100×)**

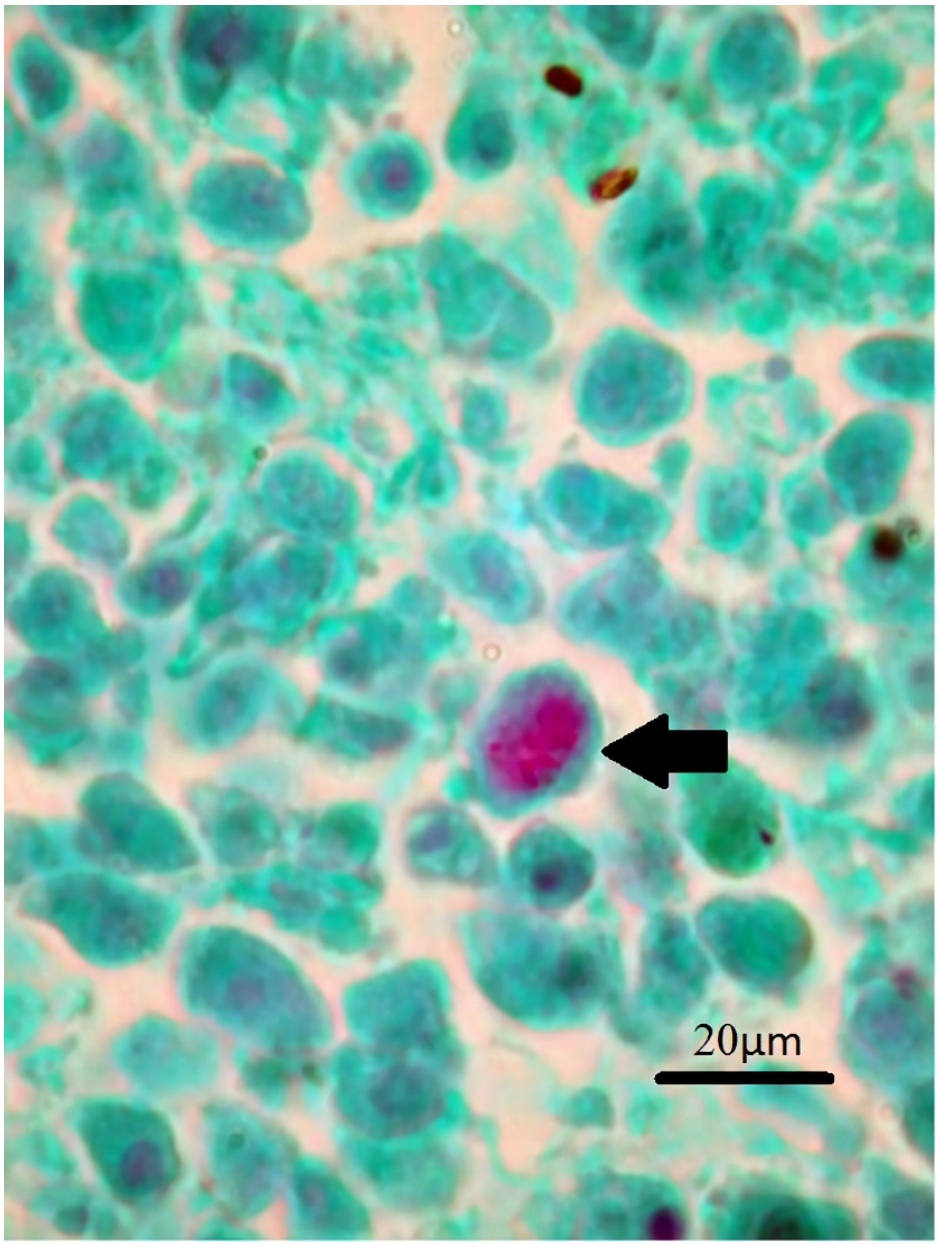

Fig. 4
***Amazona aestiva* spleen, showing cytoplasmic inclusion bodies (arrow). (Gimenez – 400×)**

Discussion

Considering the large number of specimens evaluated post mortem (n = 212) during the study period, the high transmissibility and the extended captivity, for a few individuals of up to two years, genotype A *C. psittaci* may have been disseminated during triage and become

widespread in the facility (22, 23, 24). Birds were housed together or in proximity and became sick, dying within a three-year span (2009–2011).

The phylogeny of Chlamydiaceae, inferred from genomic data, reveals segregation according to their biological properties, with differences associated with host range, tissue tropism and clinical signs. The tree topology of *Chlamydia* is separated into two groups, when based on 16S ribosomal ribonucleic acid (rRNA), with the *C. psittaci* group comprising four species (*C. psittaci*, *C. abortus*, *C. felis* and *C. caviae*) (25, 26). The characterisation of European *C. psittaci* based on the *ompA* gene restriction fragment polymorphism revealed the new genotype E/B in several countries and led to a recommendation for the genotyping of strains (5). Initial PCR-based assays have focused on the single-copy gene *ompA*, which encodes the major outer membrane protein. Analysis of the polymorphism of *ompA* gene sequences has enabled the identification of variants of *C. psittaci* and 15 avian genotypes have already been established (6).

The results of this study suggest the possibility that all 152 infected *A. aestiva* parrots investigated, as they were housed together, were infected with genotype A *C. psittaci*, because no other genotype was detected among the nine samples sequenced. All nine selected strains were successfully sequenced directly from the clinical samples, avoiding genetic changes which could be associated with *in vitro* culture adaptation. Previous reports on genotyping *C. psittaci* strains directly from clinical samples have described difficulties, possibly due to small DNA concentrations in the sample (24, 25). The results indicate a high homology of strains characterised as genotype A, which may be associated with the high transmissibility and pathogenicity observed, as described previously for strains of this genotype (3, 9, 22). The density, proximity and extended duration of housing of the psittacines during rehabilitation may have had a role in the predominance of the genotype. The *ompA* sequences of all strains evaluated showed no sequence dissimilarity or genetic distance.

The phylogenetic tree contained two major clusters (Fig. 1). Each cluster was composed of three sub-clusters. One sub-cluster corresponded to genotype A, which included all nine strains found in *A. aestiva* described herein; strains within this subgroup were virtually identical. The genetic identity of strains characterised in the present study may be associated with the housing and management conditions and their origin in a single host species (*A. aestiva*), potentially leading to similar biological properties, including pathogenesis (27). Strains of genotype B and genotype E formed separate subgroups. In addition, the phylogenetic analysis indicated that there is a close relationship among genotypes A, B and E, but greater genetic distances between genotypes C, D and F. Similar to our findings, other studies have also found genetic conservation in the *ompA* gene of genotype A. Faecal samples from 1,150 animals in five Japanese zoos were evaluated for *C. psittaci*, showing positivity in 3.9% of mammals, 7.2% of birds and 8.1% of reptiles (23). Phylogenetic analysis of 18 Japanese isolates based on the VD2 region of *ompA* showed that nine strains were clustered separately with *C. psittaci* genotype A isolates (designated cluster I), six were grouped with *C. pneumoniae* isolates and three clustered into a distinct branch (23). In Germany, evaluation of *ompA* gene sequences enabled grouping of *C. pneumoniae*, *C. abortus*, *C. pecorum*, *C. felis* and *C. caviae* strains into a single branch and revealed a minimum of 12 clades for *C. psittaci*, of which five included the recognised genotypes C, D, F, M56 and WC. The closely related genotypes A, B, E and E/B of *C. psittaci* were grouped in the ABE cluster. The percentage identity among sequences of the *ompA* gene reached 99.4% within clades B, E and E/B, and 98% for the cluster (2, 5, 24). In Poland, 88.2% of *C. psittaci* strains from pigeons were characterised as genotype B and 11.8% as genotype E (28). In the Republic of Korea, studies have supported the hypothesis that geographical distance results in genetic diversity in *Chlamydia*. Despite high (99%) similarity of 16S rRNA sequences of *C. gallinacea*, the *ompA* gene sequence diversity enabled the geographical separation of strains (29). The *ompA* gene is considered to be subjected to greater evolutionary selection pressure than the rRNA genes. The high percentage identity found among the

local strains of the present study may be associated with the single host species and the restricted geographical area.

The post-mortem findings, including macroscopic and microscopic lesions and the demonstration of potentially infected macrophages in the spleen and liver, and the molecular detection of the *ompA* gene support the diagnosis of infection by *Chlamydia*. Although it was impossible to process most of the lung samples for histopathology, because of significant autolysis, all had macroscopic changes indicative of pneumonia, such as bronchial exudate. It was also not possible to evaluate the cytoplasmic inclusions by immunolabelling for *C. psittaci*.

All the birds in this study were rescued from the illegal trade in wildlife and subjected to a constant stress which could, associated with infection, have resulted in mortality. Stress is an important factor that may lead to the manifestation of clinical disease in asymptomatic carriers of *C. psittaci*, with subsequent increase in shedding and transmission to other birds, as described for genotype A (30).

Conclusion

This study demonstrated infection by *C. psittaci* genotype A associated with hepatic lesions and mortality in captive *A. aestiva* parrots rescued from trafficking in Brazil. The individuals selected for further evaluation represented a larger population of more than 200 parrots that died in a rescue centre and were evaluated post mortem, all showing similar lesions, which may indicate a more widespread problem. The occurrence of *C. psittaci* genotype A in psittacines at a rehabilitation centre necessitates the implementation of biosecurity and quarantine measures to reduce infection during captivity. However, information is lacking regarding the genotypes of *C. psittaci* in free-living wild psittacines, which would enable better understanding of its epidemiology. The molecular methodology employed herein is simple and feasible for most laboratories and will enable the generation of further genetic information on local *C. psittaci* strains.

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