



## Case report

# An outbreak of chlamydiosis in captive psittacines

Roselene Ecco<sup>1\*</sup>, Ingrid S. Preis<sup>1</sup>, Nelson R. S. Martins<sup>1</sup>,  
Daniel A. R. Vilela<sup>2</sup>, and Hulimangala L. Shivaprasad<sup>3</sup>

<sup>1</sup> Veterinary School, Universidade Federal de Minas Gerais.

<sup>2</sup> Cetas – IBAMA, Belo Horizonte, Minas Gerais- Brasil.

<sup>3</sup> California Animal Health and Food Safety, Laboratory System, University of California, Fresno, CA, USA.

\*Corresponding author: Roselene Ecco, Departamento of Surgery and Clinic, Veterinary School, UFMG, Av. Antônio Carlos, 6627, Belo Horizonte, MG, Brazil. E-mail: [ecco@vet.ufmg.br](mailto:ecco@vet.ufmg.br)

Submitted April 22nd 2009, Accepted June 1st 2009

## Abstract

This paper describes an outbreak of chlamydiosis in psittacines recovered in Minas Gerais, Brazil, from illegal trade in the Brazilian Southeastern Region. Clinically, some birds showed apathy and anorexia or died suddenly without evidence of clinical signs. Necropsy was performed on 15 psittacines; 13 Amazon parrots (*A. aestiva*), one Peach-fronted parakeet (*Aratinga aurea*) and one Scaly-headed parrot (*Pionus maximiliani*). The most visible macroscopic changes were mild to marked hepatomegaly, with many white foci ranging in size from 1 mm to 100 mm that extended in to the parenchyma. Other findings included splenomegaly and fibrinopurulent airsacculitis. Microscopic lesions included multifocal to coalescing foci of necrosis of hepatocytes with infiltration of heterophils and lymphocytes and plasma cells randomly scattered through out. In some parrots there were multiple foci of necrotic and granulomatous hepatitis with biliary duct proliferation. The birds with splenomegaly had loss of normal architecture and infiltration of macrophages mixed with plasma cells, fibrin and heterophils. The PVK (modified Gimenez) and Giemsa stains revealed small round intracytoplasmic bacteria approximately 1µm in diameter suggestive of *Chlamydophila psittaci*, in the cytoplasm of macrophages of spleens and livers. Immunohistochemistry (IHC) using monoclonal antibodies for *C. psittaci* confirmed *Chlamydophila* in the cytoplasm of macrophages of the liver and spleen and in a few other organs of four parrots.

**Key Words:** Psittacines, diseases of birds, Chlamydiosis, *Chlamydophila psittaci*, pathology, immunohistochemistry

## Introduction

Chlamydiosis, also known as psittacosis or parrot fever, is a disease caused by *Chlamydophila (Chlamydia) psittaci*, an obligate, intracellular, gram-negative bacterium, which infects birds and mammals, including humans (1,13). Until 1932, when one human case of the chlamydiosis was reported as being transmitted by infected chickens, it was believed that the origin of the disease was just parrots (25). *Chlamydophila psittaci* have been classified in to seven serotypes (A-F and E / B) that infect different groups of birds, and psittacines have been identified as natural hosts of serotype A (1,8).

The disease has been documented in more than 140 species of wild birds, including 17 orders, among them Psittaciformes, Passeriformes, Anseriformes and Columbiformes (13). Outbreaks have been registered in the USA and Europe, affecting geese and turkeys (1,22,24) and in Brazil, reaching wild birds like Amazon parrot (*Amazona aestiva*) (11,18).

Some birds may demonstrate nonspecific clinical signs of disease such as anorexia, dyspnea, dehydration, diarrhea, weight loss, conjunctivitis, rhinitis and sinusitis (19). The emergence of clinical signs depends on the virulence of the strain, the immune status of the bird, the environment and the presence of other diseases (17).

However, many birds become chronically infected and may not have any clinical signs, and can remain as reservoirs and source of infection for humans and other birds (5,17).

The primary mode of infection is through the respiratory tract, followed by oral route or contact (3,23). Male psittacines feed the female by regurgitation during the incubation of eggs and the food can be contaminated by secretions of the crop, pharynx and nasal cavity (19). The infected birds eliminate the microorganism by nasal and ocular secretions and by feces, which can remain viable for long periods (23).

This paper reports the occurrence of the chlamydiosis in psittacines recovered from illegal commerce and housed in the Center for Triage of Wild Animals (Centro de Triagem de Animais Silvestres - CETAS), IBAMA- Belo Horizonte-MG.

### Case Report

From January through April 2008, the CETAS received 3455 individual birds including 56 adult Amazon parrots. Local authorities had recovered these birds, which were being sold illegally, from different routes or from illegal domestic custody. In the CETAS, the birds were kept in two aviaries in an area of 30 x 40 feet each. The aviaries were enclosed by wire mesh in the front and two sides and by brick wall at the back and the roof. All psittacines received a daily diet of specific ration and fruits. Biosecurity and management were poor due to close contact with other free-living birds, such as pigeons, doves and passerines, by constant arrival of rescued individuals and by overcrowding. Thirty six psittacines died during this period (60 days). Except for a few birds that showed apathy, most of the birds did not exhibit any clinical signs before death. Necropsies were performed on 15 psittacines, including 13 parrots (*A. aestiva*), one Peach-fronted parakeet (*Aratinga aurea*) and one Scaly-headed parrot (*Pionus maximiliani*). All birds were necropsied in the Setor de Patologia Veterinária da UFMG and gross lesions were recorded. Portions of viscera like liver, spleen, heart, kidney, brain, lungs, air sac, pancreas, intestine, etc., were collected, fixed in 10 % neutral buffered formalin, embedded in paraffin, sectioned at 5 micrometers, stained with hematoxylin and eosin (H & E), and examined by bright field microscopy. Selected tissues were also stained by MacCallum Goodpasture Gram, Giemsa, PAS, Ziehl Neelsen Acid fast and PVK (modified Gimenez) according to the methods already described (12). Liver, spleen, brain, and kidney from four birds and heart, intestine, air sac and lungs from four birds were subjected to immunohistochemistry (IHC) to detect *Chlamydomphila psittacci* antigen according to the method previously already described (4). IHC in tissues was based on the visualization of tissue bound *Chlamydomphila* -specific avidin-biotin-labeled antibodies.

Necropsies revealed that most of the birds were in poor bodily condition and many had food in their crops. The most visible macroscopic changes were mild to marked hepatomegaly (15/15) (Figure 1), many with white foci (1 mm to 100 mm) of necrosis that extended in to the parenchyma. Splenomegaly was present (7/10) and the parenchyma was clear red. The air sacs were thickened with fibrinous exudate (10/15). Fibrin deposition was also observed in the pericardium of one bird. One parrot had the liver twice the size of normal, firm, with several dry and white foci and the spleen three times the normal size.

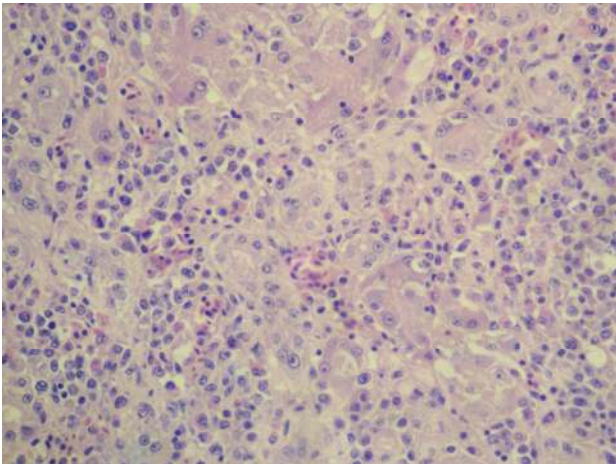
Microscopic lesions were similar in most birds. The main lesions were observed in the liver and in the spleen. In the liver there were multifocal to coalescing foci of lymphoplasmacytic inflammation with loss of hepatocytes randomly scattered though out. Several of these foci contained macrophages and heterophils (Figure 2). Within the cytoplasm of some of these macrophages there were numerous basophilic small coccoid bacteria measuring about one  $\mu\text{m}$  in diameter. Plasma cells were observed in abundance in the liver and spleen of all birds. In some parrots there were multiple foci of necrosis (9/15) and granulomatous hepatitis with biliary duct proliferation (6/15). The birds with splenomegaly had increased numbers of mononuclear phagocytic system cells, loss of normal architecture, fibrin exudation and many macrophages contained basophilic intracytoplasmic coccoid bacteria of approximately one  $\mu\text{m}$  in diameter similar to liver suggestive of *Chlamydomphila psittacci* (Figure 3). PVK stain demonstrated clearly the individual bacteria which stained purple within the cytoplasm of macrophages in the liver and spleen (Figure 4). The bacteria appeared bluish by Giemsa stain (9/15). Two birds had *Chlamydomphila* inclusion-like in the cytoplasm of macrophages, showed also by Giemsa technique stain in liver (Figure 5) and spleen. However, it should be pointed out that formation of inclusion bodies due to *Chlamydomphila* in avian species is very rare. Immunohistochemistry (IHC) using monoclonal antibodies for anti-*C. psittacci* demonstrated *Chlamydomphila* antigen in the cytoplasm of macrophages in the spleen (Figure 6) and liver of three parrots and one king parakeet. In addition, macrophages in the air sac, endocardium of the heart, interstitium of the lung and kidneys were positive for *Chlamydomphila* by IHC (Table 1). Good Pasture Gram and acid fast stains of liver were negative for bacteria, and PAS stain was negative for fungi.

### Discussion

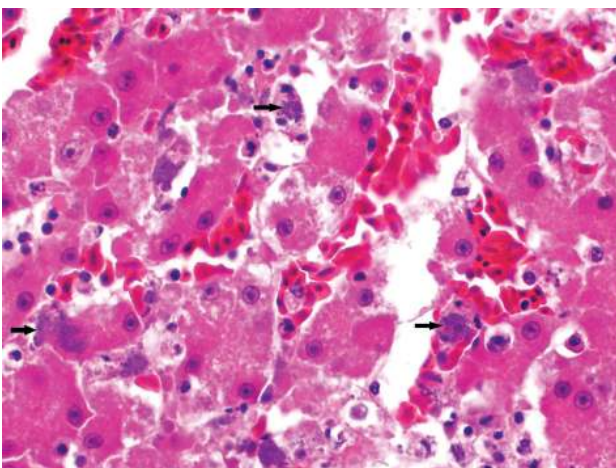
Clinical history, gross and microscopic lesions and demonstration of bacteria in macrophages by histopathology and, confirmation of these bacteria as *Chlamydomphila* by special stains and immunohistochemistry indicated that these birds had chlamydiosis.



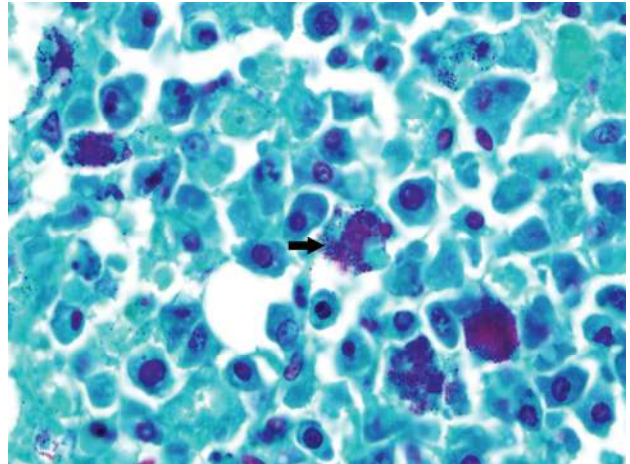
**Figure 1** – Liver, Amazon parrot. Marked hepatomegaly.



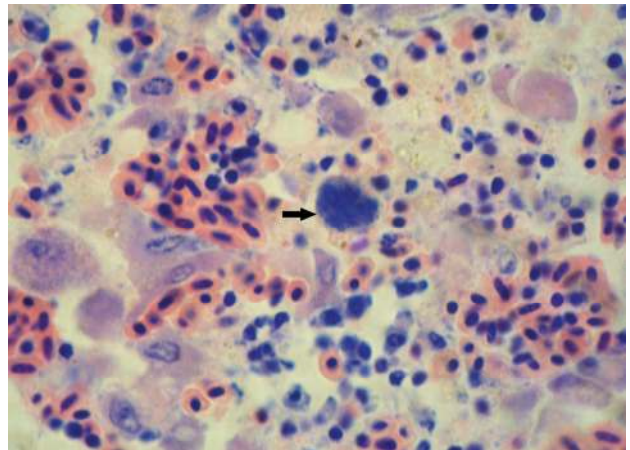
**Figure 2.** Amazon parrot. Photomicrograph of liver, multifocal to coalescing areas of lymphoplasmacytic and heterophilic inflammation and loss of hepatocytes. H&E. Obj. 20.



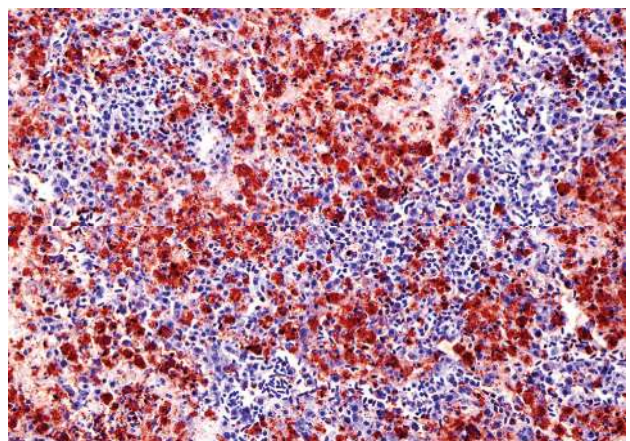
**Figure 3** – Peach-fronted parakeet. Photomicrograph of liver. Macrophages contain basophilic intracytoplasmic coccoid bacteria (*Chlamydophila psittaci*) of approximately one  $\mu\text{m}$  in diameter. H&E. Obj. 40.



**Figure 4.** Peach-fronted parakeet. Photomicrograph of spleen. Individual bacteria (*Chlamydophila psittaci*) stained purple within the cytoplasm of macrophages. PVK stain. Obj. 40.



**Figure 5** – Amazon Parrot. Photomicrograph of liver *Chlamydophila* inclusions in macrophages. Giemsa stain. Obj. 100.



**Figure 6** – Amazon Parrot. Photomicrograph of spleen positive for *Chlamydophila* antigen in the cytoplasm of macrophages demonstrated by immunohistochemistry. Obj. 10.

**Table 1** – Chlamydiosis in Psittacines - distribution of *Chlamydomphila psittaci* in different tissues as identified by immunohistochemistry\*

Bird n°	Liver	Spleen	Heart	Kidney	Lung	Intestine	Brain	Others
01	+++	+++	++	++	++	++	-	
02	+++	+++	-	ND	+	+++	-	Peritoneum. ++
03	+	+	-	-	-	-	-	Peritoneum +
04	++	++	ND	+	-	ND	-	Air sacs +

+ Mild, ++ Moderate, +++ Severe, - Negative, ND: Not determined.

\*Immunohistochemistry employing monoclonal antibodies specific to *Chlamydomphila psittaci*.

Clinically, the birds can have anorexia, weight loss, lethargy, ruffled feathers, watery yellowish-green diarrhea, dehydration, dyspnea, sneezing, mucoid nasal secretions, sinusitis and conjunctivitis (1,5,9). Birds in this report were observed to have only lethargy and weight loss (12/15).

Many birds may be chronically or latently infected or the disease can become only evident after situations of stress such as adverse environmental conditions, inadequate standards of transport and confinement, poor nutrition and immune suppression. These birds often shed *Chlamydomphila psittaci* intermittently and serve as a source of infection for humans and other birds (1,9,14,23). All birds in this study were from the illegal trade and were subjected to transport conditions of discomfort, stress which probably contributed to the condition reported. It is likely that these factors lead to the precipitation of clinical disease in an asymptomatic carrier and subsequent spread to other birds. *Chlamydomphila* infection rates tend to increase with overcrowding (6).

The serovars of *Chlamydomphila psittaci* are classified into six genotypes based on genetic sequencing. The genotype A is endemic in psittaciformes (cockatoos, parrots, parakeets and lorries) and its zoonotic role is well known. The genotype B was identified in pigeons and the genotypes C and D were detected mainly in poultry; turkeys and ducks. The genotype E was isolated from turkeys, ducks, pigeons, ostrich and rheas and the genotype F of American parakeet and turkey (3,9). We did not identify the genotype of *Chlamydomphila* in the birds of this report. Psittaciformes are mainly infected with genotype A, which is extremely virulent, intensively excreted and often cause mortality (9).

At necropsy, lesions suggestive of chlamydiosis in psittacines are hepatomegaly, splenomegaly, enteritis, pericarditis, sinusitis, conjunctivitis and fibrinopurulent air sacculitis (23). However, in most cases, the lesions in these birds are confined to the liver, spleen and air sacs (7,23) coinciding with the lesions observed in parrots of this report. Fibrinous pericarditis was observed in only one bird among these cases. An outbreak in Amazon parrots

reported haemorrhagic enteritis, this was not present in any of the birds examined in this report (18).

The airsacculitis due to Chlamydiosis can be acute, subacute or chronic. Subacute airsacculitis is characterized by fibrin exudation with a few heterophils and macrophages, and many plump, reactive, vacuolated macrophages, some of which can contain *Chlamydomphila*. The chronic form of *Chlamydomphila* airsacculitis is characterized by pyogranulomatous or granulomatous inflammation (7). One bird in this study had severe airsacculitis and IHC demonstrated *Chlamydomphila* organisms in the cytoplasm of macrophages.

Histopathologic lesions of chlamydiosis may vary with the virulence of the strain, route of infection, and host susceptibility (6). Myocarditis and interstitial pneumonia are observed only in turkeys and conjunctivitis and rhinitis, sinusitis and tracheitis occur in waterfowl and pigeons (3,7).

The histological lesions in the liver of psittacines due to *Chlamydomphila* are characterized by dilatation of sinusoids with infiltration of macrophages, lymphocytes and heterophils, in addition to multifocal areas of necrosis (7,23). Similar lesions were observed in the livers of all the birds examined in this study. Foci of coagulation necrosis were observed in 9 birds. Foci of caseous necrosis surrounded by multinucleated giant cells were found in 4 birds. Special stains failed to reveal any bacteria including acid fast bacteria or fungi. In acute, fatal chlamydiosis multiple foci of coagulation necrosis of hepatic parenchyma have been described and the necrosis may also accompany lesions of subacute to chronic illness (7). The necrosis in the liver is probably due to a direct effect of proliferating *Chlamydomphila* organisms within cells (23). Immunohistochemical examinations in parakeets have previously detected the antigen in necrotic areas, hepatocytes, bile duct epithelium and swollen macrophages (20). Moderate to marked bile duct proliferation, as seen here, characterizing chronic infection, probably occur in parrots with a history of recurrent, subacute episodes of illness over a period of many months or even years (7).

The degree of splenomegaly generally evident is quantitatively proportional to the degree of histiocytosis. The severity and extent of histiocytosis may increase to the point that it is diffuse and the normal architecture is obscured and depletion of lymphocytes from the white pulp is common. In many cases, however, the lymphocyte paucity is common and is accompanied by intense plasmacytosis (7), as seen in all spleens in these birds. In one possibly chronically infected parrot in this report the spleen was three times bigger than its normal size.

The observation of the lesions in association with the visualization of the bacteria inside the macrophages is strongly indicative of infection by *Chlamydomphila psittaci* (7). Well-trained eyes can detect organisms morphologically compatible with *Chlamydomphila* bacterial in tissues stained by Giemsa, as seen in most spleens and livers of these psittacines. The definitive diagnosis can be made by isolation (16) or by semi-nested polymerase chain reaction (semi-nested PCR) detection of *Chlamydomphila* DNA from fecal samples and oral pharyngeal and cloacal swabs (10,25). However, the diagnosis of chlamydiosis can be problematic because many birds can be carriers and may harbor subclinical infections and furthermore *Chlamydomphila* organisms are shed intermittently in the feces, a single culture can lead to a false-negative result (4). IHC, using a monoclonal anti-*Chlamydomphila* lipopolysaccharide to diagnose avian chlamydiosis, is a technique that can be used to more accurately diagnose avian chlamydiosis in formalin-fixed, paraffin-embedded tissues (4,5).

For the differential diagnosis, the Pacheco's parrot disease (PPD) caused by a psittacid herpesvirus 1 (PsHV1) and, bacterial infections caused by *Mycobacterium* spp., *Salmonella* spp., *Yersinia pseudotuberculosis*, *Coxiella* spp., etc. should be considered. The gross lesions caused by PPD are characterized by diffuse red mottling of the liver and increase in volume (15). Microscopically, PPD has been characterized by moderate to marked, multifocal acute hepatic necrosis, at the margins of which were hepatocytes with eosinophilic intranuclear inclusions. There is a moderate diffuse infiltrate of plasmacytes and lymphocytes around portal areas. The abundance of inclusion bodies, however, has varied from parrot to parrot, and in some cases the inclusion bodies were either rare or absent entirely. Splenitis, tracheitis and enteritis also were described as features of PPD (15,21). Recommended diagnostic techniques to confirm a diagnosis of Pacheco's disease are histopathology, ultrastructural examination of thin sections of liver (15) or viral isolation and polymerase chain reaction (PCR) (21). Special stains of livers and spleens failed to reveal any bacteria including acid fast bacteria and eosinophilic intranuclear inclusions were not observed.

After the necropsy and diagnosis compatible with chlamydiosis was made, the parrots were treated with tetracycline and transferred to an area where conditions of stress were minimal. This resulted in significant decrease

in the mortality of psittacines. An alert is forwarded against the over use of these drugs should be done due to the risk of developing tetracycline-resistant strains. Owners of birds frequently use tetracycline for any disease, or even prophylactically, possibly unaware of the importance of tetracyclines in human therapy chlamydiosis. Information campaigns on antibiotic use in pet birds with respect to zoonotic agents are urgently required, and worldwide access to antibiotics has to be restricted to veterinarians and medical doctors (9).

This study demonstrates the infection by *Chlamydomphila psittaci* associated with disease and mortality in captive psittacines in Brazil. In many countries, chlamydiosis is a notifiable disease (10). In Brazil, more attention must be given to quarantine measures and to postmortem procedures to ensure rapid diagnosis of the disease (18). The relatively poor control of the disease in birds and the broad spectrum of clinical syndromes in humans infected with *Chlamydomphila psittaci* raise the question if this organism may be a much more frequent pathogen than previously considered (10). On the State of Rio Grande do Sul, Brazil, in 2003, an outbreak of chlamydiosis was reported in a family after contact with parakeets showed respiratory signs. Seven individuals of same family presented pneumonia not responsive to usual antibiotics. Antibodies specific to *Chlamydomphila* were detected by ELISA in all patients. The occurrence was reported to the local health authority (2).

Unfortunately, the illegal bird traffic still occurs in Brazil. To avoid dissemination of *Chlamydomphila psittaci* between birds and to humans, especially when incoming birds have a history of inadequate health management, adequate prophylaxis and control measures are fundamental.

## References

1. ANDERSEN AA., VANROMPAY D. Avian Chlamydiosis (psittacosis, ornithosis). In: SAIF, Y.M et al. (Ed). *Disease of poultry*, 12<sup>th</sup> edition. Iowa: Blackwell Publishers, 2008. p. 971-988.
2. BERCINI M., SCHERMANN MT., RANIERI T., ALMEIDA MAB. Surto intrafamiliar de psitacose. Anais 5<sup>a</sup> Mostra Nacional de Experiências Bem-Sucedidas em Epidemiologia, Prevenção e Controle de Doenças – EXPOEPI. Brasília: Ministério da Saúde, 2006. 136p.
3. EIDSON M. Psittacosis/avian chlamydiosis. *J. Am. Vet. Med. Assoc.*, 2002, 221, 1710-1712.
4. ELDER J., BROWN C. Review of techniques for the diagnosis of *Chlamydia psittaci* infection in psittacine birds. *J. Vet. Diagn. Invest.*, 1999, 11, 539-541.
5. ERBECK DH., NUNN SA. Chlamydiosis in pen-raised bobwhite quail (*Colinus virginianus*) and chukar partridge (*Alectoris chukar*) with high mortality. *Avian Dis.*, 1999, 43, 798-803.

6. FUDGE AM. Avian chlamydiosis. ROSSKOPF WJ., Jr., & WOERPEL, E. W. (eds.). Diseases of Caged and Aviary Birds. Pennsylvania: Williams and Wilkins, 1996, p. 572–585.
7. GRAHAM DL. Histopathologic lesions associated with chlamydiosis in psittacine birds. *J. Am. Vet. Med. Assoc.*, 1989, 195,1571-73.
8. GUSCETTI F., HOOP R., SCHILLER I., CORBOZ L., SYDLER T., POSPISCHIL A. Experimental enteric infection of gnotobiotic piglets with a *Chlamydia psittaci* strain of avian origin. *J. Vet. Med. B Infect. Dis.Vet. Public Health*, 2000, 47, 561-72.
9. HARKINEZHAD T., VERMINNEN K., VANDENBROUCKE-GRAULS CMJE., VANROMPAY D. *Chlamydia psittaci* genotype E/B transmission from African grey parrots to humans. *J. Med. Microbiol.*, 2007, 56, 1097-1100.
10. HEDDEMA ER., VAN HANNEN EJ., DUIM B., de JONGH BM., KAAAN JA., VAN KESSEL R., LUMEIJ JT., VISSER CE., VANDENBROUCKE-GRAULS CMJE. An outbreak of psittacosis due to *Chlamydia psittaci* genotype A in a veterinary teaching hospital. *J. Med. Microbiol.*, 2006, 55, 1571-1575.
11. JOHNSON FW. Chlamydiosis. *Br. Vet. J.*, 1983, 139, 93-101.
12. LUNA LG. Manual of histologic staining methods of the Armed Forces Institutes of Pathology. 3. ed. New York: McGraw-Hill Book Company, 1968: 260p.
13. MIRANDÉ LA., HOWERTH EW., POSTON RP. Chlamydiosis in a red-tailed hawk (*Buteo jamaicensis*). *J. Wildl. Dis.*, 1992, 28, 284-287.
14. MOHAN R. Epidemiologic and laboratory observations of *Chlamydia psittaci* infection in pet birds. *JAVMA*, 1984, 184, 1372-1374.
15. O'TOOLE D., HAVEN T., DRISCOLL M., NUNAMARKER C. An outbreak of Pacheco's disease in an aviary of psittacines. *J. Vet. Diagn. Invest.*, 1992, 4, 203-205.
16. PEARSON JE., GUSTAFSON G.A., SENNE DA., PETERSON L.A. Isolation and identification of *Chlamydia psittaci* from pet birds. *J. Am. Vet. Med. Assoc.*, 1989, 195, 1564-1567.
17. RASO TF., JÚNIOR AB., PINTO AA. Evidence of *Chlamydia psittaci* infection in captive Amazon parrots in Brazil. *J. Zoo. Wildl. Med.*, 2002, 33,118-121.
18. RASO TF., GODOY SN., MILANELO L., de SOUZA CA., MATUSCHIMA ER., ARAÚJO JÚNIOR JP., PINTO AA. An outbreak of chlamydiosis in captive blue-fronted Amazon parrots (*Amazona aestiva*) in Brazil. *J. Zoo. Wildl. Med.*, 2004, 35, 94-96.
19. RASO TF., SEIXAS GH., GUEDES NM., PINTO AA. *Chlamydia psittaci* in free-living Blue-fronted Amazon parrots (*Amazona aestiva*) and Hyacinth macaws (*Anodorhynchus hyacinthinus*) in the Pantanal of Mato Grosso do Sul, Brazil. *Vet. Microbiol.*, 2006, 117, 235-41.
20. SUWA T., TOUCHI A., HIRAI K., ITAKURA C. Pathological studies on chlamydiosis in parakeets (*Psittacula krameri manillensis*). *Avian Path.*, 1990, 19, 355-370.
21. TOMASZEWSKI, E., WILSON, V.G., WIGLE, W.L., PHALEN, D.N. Detection and Heterogeneity of Herpesviruses Causing Pacheco's Disease in Parrots. *J. Clin. Microbiol.*, 2001,39, 533–538.
22. SHIVAPRASAD HL. An unusual outbreak of Chlamydiosis in Commercial Turkeys. Proceedings 7<sup>th</sup> International Symposium on Turkey Diseases. Berlin, Germany. 2008, pp 145.
23. VAN BUUREN CE., DORRESTEIN GM., VAN DIJK JE. *Chlamydia psittaci* infections in birds: a review on the pathogenesis and histopathological features. *Vet. Q.*, 1994, 16, 38-41.
24. VAN LOOCK M., LOOTS K., Van De Zande S., et al. Pathogenic interactions between *Chlamydia psittaci* and avian pneumovirus infections in turkeys. *Vet. Microbiol.*, 2006, 112, 53-63.
25. VERMINNEN K., DUQUENNE BB., de KEUKELEIRE D., DUIM B., PANNEKOEK Y., BRAECKMAN L., VANROMPAY D. Evaluation of a *Chlamydia psittaci* infection diagnostic platform for zoonotic risk assessment. *J. Clin. Microbiol.*, 2008, 46, 281-285.