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Psittacosis

João Morais, Ana Cláudia Coelho and
Maria dos Anjos Pires

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1. Introduction

Chlamydophila psittaci (Bacteria kingdom, Chlamydiae phylum, Chlamydiae class, Chlamydiales order, Chlamydiaceae family) is part of the genus *Chlamydophila*, where *Cp. abortus*, *Cp. caviae*, *Cp. felis*, *Cp. pecorum* and *Cp. pneumoniae* are the other five species of bacteria [1].

Avian chlamydiosis induced by this Gram-negative obligate intracellular bacteria [2] is traditionally known as ornithosis or psittacosis [3-5]. It is an infectious disease capable of infecting domestic and wild birds [6,7], being the Psittacidae family where most occurrences are reported [8].

Chlamydophila psittaci infects primarily birds [9], but mammals, including humans, are also susceptible of infection [10-11]. In fact, zoonotic status of this organism is largely described and emphasizes the scientific reports worldwide [12-15].

Chlamydophila psittaci can be found in bird feathers, excrements and blood, whether or not these animals are showing clinical signs of disease [16,17].

Psittacosis can be transmitted by vertical and horizontal via [2]. The agent is excreted on faeces and ingested from the food or inhaled via aerosols [13]. At the lungs of newly infected animals, the organism gets an infecting status becoming capable to replicate and causing clinical signs of disease [18,19].

There are three morphologically distinct forms of *Chlamydophila*:

- The elementary body, which is small, spherical, of about 0.2-0.3 mm in diameter;
- The reticular body, wider, of about 0.5-2.0 mm, which is able to replicate by binary division;
- And the intermediate body, with 0.3-2.0 mm in diameter, seen in infected host cells [18,20].

Parrots are often infected animals but they show no signs of disease unless they are stressed out [21], and thus can be a source of contamination to other birds and mammals, so as the man [3,4,22]. Infections in turkeys have been described long ago, with a mortality range of 5-40% without any treatment [23], and more recently in ducks, with a mortality of 30%, which was highlighted as an economic problem and health impairment [24]. The mortality rate in humans is low if treatment is appropriate. Therefore, setting up a quick prognosis is essential [20,23].

Clinical symptoms are very extensive in number and vary depending on the serotype of the bacteria and the animal affected. General infections may cause fever, anorexia, lethargy, diarrhoea and sometimes shock or even death. Chlamydiosis in psittacines is mostly chronic and cause conjunctivitis, enteritis, sacculitis, pneumonitis, hepatomegaly and droppings can range from green to yellow-green in colour [20]. Clinical signs in humans range from unapparent to severe systemic disease with interstitial pneumonia and encephalitis [12,20]. Signs of disease are headache, chills, malaise and myalgia with possible respiratory involvement. Infection of pigeons by *Chlamydophila* is vast and well reported [15]. These animals are a major spreader of the disease, and present clinical signs such as conjunctivitis, blepharitis and rhinitis [20]. *Chlamydophila psittaci* antibodies can be detected by several laboratory methodologies. The complement fixation test (CFT) is the most frequently used test [25,26]. Other serological tests such as PCR (polymerase chain reaction) and ELISA are used as well for its diagnosis [8,22,27].

Psittacosis or "parrot fever" was documented for first time in 1879, when Jakob Ritter described an epidemic and unusual pneumonia, associated with exposure to tropical birds in seven Swiss individuals causing flu-like symptoms and pneumonia [28]. The term "psittacosis" (from the Latin word for parrot - *psittacus*) was firstly used by Morange, in 1895, when transmission of an infectious agent to humans from parrots was notorious [29-30].

Investigations of chlamydiosis began in 1907 when microorganisms within intracytoplasmic vacuoles in conjunctival scraping cells from humans with trachoma were found. Trachoma is a chronic infectious disease of the conjunctiva and cornea [18] a disease well known since ancient civilizations [31]. These organisms were named *Chlamydozoa*, from the characteristic shape of the mantle or "*chlamys*" in Greek [32].

A psittacosis outbreak occurred during the winters of 1929 and 1930 in Europe and in the United States. The causative agent of psittacosis was isolated from birds and infected humans and the source of contamination traced to parrots of the genus *Amazona*, originated in South America [33]. At the same time, a microorganism causing lymphogranuloma venereum (LGV) was isolated in humans [34]. Until then, it was believed avian psittacosis was restricted to *Psittaciformes* birds [18]. However, a number of studies demonstrated a much higher number of species concerned or potentially infected by this microorganism. In fact, Meyer and Eddie, in 1932, described a case of transmission of human psittacosis from domestic fowl [35] and Haagen and Mauer, six years later, reported an infection in fulmar (*Fulmarus glacialis*). Pinkerton and Swank, in 1940, proved the existence of this agent in domestic pigeons, as Wolins, in 1948, described the same disease in ducks [33]. Later studies of this matter enhanced the idea that psittacosis was not restricted to parrots, reporting infections in humans by contact with other affected birds [18,36].

Later, Everett and collaborators (1999), proposed a new classification, where the *Chlamydomphila* genus would contain six species: *Chlamydomphila pneumoniae*, *Chlamydomphila pecorum*, *Chlamydomphila abortus*, *Chlamydomphila felis*, *Chlamydomphila caviae* and *Chlamydomphila psittaci* [1]. This taxonomic classification was accepted and is still used worldwide [17].

The aim of this study was to discuss phylogeny, epidemiology, clinical signs, pathology, diagnostic techniques, treatment, prevention and public health concerns in psittacosis with special attention due to *Chlamydomphila psittaci* infection.

2. Phylogeny and biology of Chlamydiaceae family

Bacteria kingdom consists of different phyla, including the Chlamydiae, holding the class with the same name. The order Chlamydiales belongs to the class Chlamydiae and the family Chlamydiaceae to the order described. The genus *Chlamydomphila* belongs to the family Chlamydiaceae and accordingly with the National Center For Biotechnology Information Taxonomy Browser (www.ncbi.nlm.nih.gov), it has six species of bacteria [1]. The Chlamydiae genus contains three species: *C. trachomatis*, *C. suis* and *C. muridarum* [1].

In the genus *Chlamydomphila*, *Chlamydomphila abortus* is a bacterium whose disease is severe, especially in small ruminants [37], but it is also described as an important zoonotic disease [38-40].

Chlamydomphila pneumoniae has been primarily described as an infection in humans but later has been reported in other animals as well, such as mammals, marsupials, reptiles and amphibians [41-44]. The transmission of *Cp. pneumoniae* among animals and humans was not yet described; however, Myers and collaborators proved that human could be infected from animal isolated bacteria following adaptation to the new host [44].

Chlamydomphila caviae was found in animals such as rabbits, guinea pigs, horses, cats and dogs [45-48]. In 2006, it was reported the identification of this bacteria in humans, raising the possibility that it has a zoonotic potential, which has not been yet clarified [45].

Chlamydomphila felis affects cats mainly under one year old and is very often associated with conjunctivitis [49-52]. Although there is evidence that this bacterium can cause keratoconjunctivitis in humans, there is still little evidence that can create systemic disease or severe pneumonia in the man [53].

Chlamydomphila pecorum affects several small and large mammals such as ruminants, swine and koalas [54-59], but little or no studies have been published to date that can prove human infection from this agent.

Chlamydomphila psittaci is the causative agent of Psittacosis and is capable of infecting domestic and wild birds [6, 60], but also reptiles [61] and mammals such as man [10,11]. This bacterium is definitely an important zoonosis and such potential has been widely reported across the years [12-15].

All species of the genera *Chlamydophila* and *Chlamydiae* have one or more serotypes, whose sequence are known [62-63] or are still under intense study [64].

a. *Chlamydophila psittaci*

Chlamydophila psittaci is negative to Gram test bacterium [2, 49, 57], having a cytoplasmic membrane and an outer three-layer membrane [65-66], with a significant cell wall fraction insoluble to ionic detergents [67]. In bacteria of the genus *Chlamydophila*, this portion is referred to as COMC (*Chlamydia* outer membrane complex), which is composed of MOMP (major outer membrane protein) and other small proteins [68]. MOMP is composed of cysteine-rich proteins and takes approximately 60% of the total weight of the outer membrane [69]. This constituent is important to maintain the integrity and rigidity of the bacteria, and the main antigen to host immune system [70].

b. *Chlamydophila psittaci* biology

Chlamydophila bacteria have three distinct forms during its life cycle: elementary body (EB), reticular body (RB) and intermediate body (IB) [19,20, 71].

The EB is small, electro-dense, spherical and about 0.2 to 0.3 μm in diameter [18,20]. The outer membrane is composed of proteins, lipids, lipopolysaccharides and proteins. However, unlike the Gram-negative bacteria, this species is devoid of muramic acid [72]. This is an active form of the bacterium capable of binding with the host cell allowing to reach its inside [19]. This first form is characterized by a highly electro-dense nucleoid [71, 73], located in the periphery and clearly separated from the cytoplasm [19, 71]. The Hc1 histone maintains the chromatin highly condensed [74]. Within the cell, the elementary body increases in size to form the reticular body, which is the metabolic active intracellular form of the organism [19, 20]. At this stage, the chromatin is dispersed, since the bacteria begin a process of transcription [20, 75].

The RB is of about 0.5 to 2.0 μm in diameter and its inner and outer membranes are relatively nearby, thereby reducing the virtual space between them [18, 20]. This form of the bacterium has the ability to split by binary fission, resulting in new RB [19, 65]. During this process, intermediate bodies can be observed within the host cell, measuring between 0.3 and 1.0 μm in diameter [18, 20].

This third form of the bacterium (IB) has a very specific presentation with an electro-dense core nucleoid surrounded by fibres dispersed radially [65, 73]. In the periphery, there exists an agglomerate of cytoplasmic granules, separated from the core by a translucent area [71]. When the replication process is over, several elementary forms are observed, which can be somewhat condensed, depending on their conformations [20]. The less condensed are more immature form, have further fibrous elements in the granular cytoplasm, bearing for the electro-dense nucleoid, which will become progressively highly condensed [71]. The nucleoid is close to the inner membrane [20].

The mature elementary bodies are condensed, having a homogeneous oval shaped nucleoid, irregular or an elongated, separated from the cytoplasmic organisms by a well visible electro-transparent area [20, 75].

Some studies have shown the existence of hemispherical-shaped projections of the cytoplasm on the surface of the elementary bodies and ridge-shaped projections of the reticular forms for this bacterium [76-78]. These projections range from the bacteria to the surface of the membrane inclusion and it is speculated that can possess pores of type III secretion [32].

c. Pathogenesis

Infection of *Chlamydophila psittaci* begins with the attachment of the elementary body to the host cell and subsequent parasite-mediated endocytosis [79]. The bacterium binds to the cell membrane receptor of the host cell, often associated with a cytoplasm protein known as clathrin [20]. This connection stimulates the cell to emit pseudopods; these increase in size until all bacteria have been surrounded, forming a vacuole delimited by clathrin, referred to as inclusion [18, 80]. In the vacuole, the bacterium has a biphasic cycle (Figure 1), alternating between states of elementary body and reticular body [19].

Once inside the vacuole, the EB are redistributed from the periphery of the cell and join in the region of the Golgi apparatus, which corresponds to the MTOC (Microtubule Organizing Centre) [19, 80]. The non-acid inclusions are firstly too small, allowing to avoid its fusion with lysosomes, and are formed by endocytic constituents of the plasma membrane of the host cell such as proteins and lipids [19, 80]. All of these conditions lead to a more efficient survival and infection by the bacterium [20].

On the first hours, the elementary bodies initiate to differentiate into reticular bodies. These forms of *Chlamydophila* are not infectious, have a non-condensed nucleoid and a greater size than EB [19, 65]. From eight to ten hours of cycle, the reticular bodies start to replicate by binary fission, remaining in contact with the inclusion membrane [20, 65]. In this moment, it's possible to see numerous mitochondria surrounding the inclusion [18, 20], possibly due to protein motors function as kinesins [81]. *Cp. psittaci* may not be able to generate as much energy in the form of ATP as other species of the genus, so a deeper association with the host cell mitochondria is necessary for the bacteria to recruit the necessary ATP by an alternative mechanism [20]. The migration of nutrients from the host cell into the inclusion is essential. Amino acids and nucleotides cross the inclusion membrane [20, 82]. Since the vacuole is not in the vicinity of lysosomes leads to the assumption that the releasing of nutrients by fusion with endocytic vesicles is unlikely [20]. It is known that exocytic vesicles containing sphingomyelin fuse with the membrane of the inclusion, delivering nutrients and lipids to the bacteria [80]. It was demonstrated that the inclusion membrane is permeable to small molecules of a molecular weight ranging from 100-520 Da [83,84]. After releasing the nutrients into the lumen of the bacterium by passive diffusion, membrane-specific carriers/transporters located in the membrane would facilitate the entry of new nutrients and would be indispensable for the entry of molecules larger than 520 Da, but such carriers have not been yet described [20].

Throughout the cycle, the size of the vesicles increases, as they accumulate growing numbers of bacteria inside [20, 84]. The surface of the inclusion membrane also increases, while it intercepts biosynthetic transport pathways of the host cell membrane and acquires the ability to fuse with a subset of Golgi derived vesicles [20, 84]. Within 20 hours of cycle, reticular

bodies continue their replication and each body can give rise to a thousand of new bacteria [20]. As this process occurs, the inclusion becomes increasingly overloaded with bacteria and as such they are obligated to leave the inclusion [19, 20]. This may be the reason for the reticular bodies to turn into intermediate bodies and new infectious elementary bodies, which occurs about 36 hours after infection [19]. During the final stage, at 50 hours of cycle, the host cell and the inclusion undergo a process of lysis or, very often, the elementary bodies are released by reverse endocytosis [20], which leaves the host cell intact, allowing persistence of a chronic and silent infection [19].

Figure 1. Chlamydia life cycle. Infection begins with the attachment of the elementary bodies (EB) to the surface of target epithelial cells. These cells promote a pseudopod formation to engulf the EB. Inside the cytoplasm this bacterium inhibits the fusion of the vesicle with the cell lysosomes. The nascent inclusion is accompanied by the transition from EBs to reticulate bodies (RB). Late in the cycle, RBs replicate by binary fission to generate both RBs and intermediate bodies (IB). At this stage, antigenic proteins are exposed into the cell surface. An elongated, aberrant RB could be formed at this time with an arrest on chlamydia cycle originating a persistent infection, or continuing the cycle. The various intracytoplasmic inclusions with bacterium inside, can also be fused in this phase, and the agent develop into intermediate bodies (IB), before DNA condensation and RB transformation into a newly EB. The mature inclusion increases in size with EB formation, until becoming infectious and released into the extracellular space to continue a new intracellular cycle. N – nucleus; G – Golgi apparatus; EB – elementary bodies; RB – reticulate bodies; IB – intermediate bodies.

After treatment with cytokines, antibiotics or restriction to particular nutrients [19, 85], this cycle may undergo a modification with the emergence of “persistent” bacteria [85-86]. This kind of bacterium does not complete its continuing transformation from reticular bodies to infectious elementary form. Instead, it remain with low metabolic activity [19]. These RB are morphologically aberrant, appearing with a dilated oval shape within inclusions of small size [20, 85-86]. Excessive accumulation of chromosomes is due to the continuous DNA replication of the bacteria that lack the ability to divide [20]. These persistent forms of *Cp. psittaci* are associated with chronic infections, and it has been shown that aberrant reticular bodies can quickly develop into normal forms and subsequently into infective elementary bodies [85-86]. The way this bacteria model passes through such transformations between aberrant forms and normal forms of reticular bodies is not yet completely understood [75, 87-88].

3. Epidemiology

Parrots were the first animals linked to *Cp. psittaci* in 1929-1930 and 1930-1938 when they were confirmed to be the source of outbreaks of psittacosis [33]. However, thereafter it became clear that this disease was not confined to these birds. In 1939, the agent was isolated from two pigeons in South Africa and later two new cases of psittacosis arose in citizens who contacted with pigeons in the U.S. [89]. Ducks and turkeys are given as possible sources of infection since the early fifties, although in the seventies there was a large decline in the prevalence of bacteria, but without ever ceasing [20]. Confirmation of new U.S. cases of chlamydiosis in turkeys in the following decade [20] and in Europe, already in the nineties [90,91], proved the continuing problematic status of this disease. In the last thirty years it was reported several cases in humans who have contracted the disease through direct contact with ducks [92-95] and more recently the number of these cases increased dramatically [96].

Studies on *Chlamydothila's* prevalence increased and psittacosis was classified as an endemic disease in Belgium [97-98] and other European countries, such as France and Germany [97].

Psittacines and pigeons are the most disturbing cases, being the range of prevalence within the first, 16% to 81% and mortality frequently above 50% [99-101]. Other studies have shown that parrots are the largest sources of *Cp. psittaci*, especially when they are in captivity [102-103].

The wild pigeons have a broad seropositivity that ranges from 12.5% up to 95.6%. These data were obtained from studies conducted from 1966 to 2005 [20,104-105]. The seropositivity in this species is alarming, once they live in urban and rural areas throughout the world, in close contact with human [60]. Carrier pigeons are reported as having a lower seropositivity than wild pigeons, between 35.9 and 60% [20].

Kaleta and Taday (2003) reported that seabirds are more often infected with *Cp. psittaci* than other birds like chickens, quails and pheasants, but virtually all species of birds can

contract the disease, even without apparent symptoms, such as for instance, the cranes and seagull. Psittacosis in arthropods can be detected, however without initiation of infection in these animals [106].

Although the horizontal transmission is the most common way of infection [24], the vertical transmission was also described [18].

Chlamydophila psittaci are most excreted in the faeces [21, 107], nasal [12] and oral discharges of infected animals [108]. Sareyyupoglu and collaborators (2007) indicates that excretion of the bacteria may occur intermittently in sub-clinical infections for a long time, being activated in situations of stress such as transportation and handling [21] or nutritional restrictions and egg laying [108]. Bacteria are found in the powder of feathers [106], in excrements, secretions and respiratory exudates from infected animals, and when dry, become spread in the air [20-21] being the aerosol transmission extremely feasible [109]. The contact of non infected animals with bacteria or its proximity to animals with *Cp. psittaci* gain greater importance, being this an essential criteria for the development of new infections [22].

Animals become infected by ingestion [110] or inhalation of the bacteria [13, 60], and the isolation of this agent is much more substantial from choanal and throat swabs collection rather than from faeces collection [111], especially in the early stages of the disease [20]. Thus, contamination by aerosol exudates must be considered the primary form of infection [89].

Birds who share contaminated water are also susceptible of infection [18], as well as predators eating carcasses contaminated with *Cp. psittaci* [108]. The nest environment is also very susceptible to disease transmission, since this is a place where are deposited loads debris that may contain multiple bacteria [20]. Granivorous animals, such as pigeons or pheasants, that are often found in corrals or stables contaminated with faeces, and also grain storage areas can become infected by inhalation of dust from grain or from aerosols of faeces [18, 20].

Ectoparasites such as fleas, mites and lice may also serve as vectors for the transmission of disease from animal to animal [112].

4. Clinical signs and pathology

After 4 hours of infection via aerosol, bacteria can be found in the respiratory system of the animal [113].

This disease may be acute, sub-acute, chronic or subclinical [113], being the last one found when the animal is showing no signs of disease, and elimination of the bacteria can occur intermittently due to stress [21], inadequate nutrition or other diseases [108]. These animals are persistently infected and named as “source of infection” to other animals [20]. The acute form, in turn, is a generalized form, affecting all the organs of the animal [113].

General clinical signs of disease and highlights of the most common within the species are listed on table 1, being generally flu-like symptoms, CNS disorders, pericarditis, sacculitis and occasionally shock and death, signs of infection [18, 20,113].

Clinical signs	
Overall	Difficulty breathing, fever, lethargy, anorexia, ruffled feathers, diarrhoea, oral and nasal discharges, decreased egg laying, polyuria, pericarditis, sacculitis, pneumonia, lateral nasal adenitis, peritonitis, hepatitis and splenitis, occasionally shock and death.
Species	
Psittacines	Anorexia, diarrhoea, difficulty breathing, sinusitis, conjunctivitis, yellowish droppings and, perhaps, CNS disorders.
Pigeons	Signs appear only when there is another competitor disease. Acute Infection - anorexia, diarrhoea, conjunctivitis, rhinitis, swollen eyelids and a decrease in flight performance. Chronic Infection - lameness, stiff neck, opisthotonos, tremor and convulsions.
Turkeys	D serotype of the bacteria - Anorexia, cachexia, diarrhoea gelatinous yellow-green, low egg production, conjunctivitis, sinusitis, sneezing and mortality between 10 and 30%. B serotype of the bacteria - Anorexia and green manure
Ducks	It affects mostly the young ones. Agitation, unsteady gait, conjunctivitis, serous to purulent nasal discharge and depression.
Chickens	Blindness, anorexia and occasionally death.

Table 1. General clinical signs of disease (adapted from [18,20,113].

Chlamydophila psittaci post-mortem lesions are not specific enough to be able to differentiate this disease from other systemic diseases [113]. The severity of injury depends on several factors including the virulence of serotype, the susceptibility and age of the host, the form and time of exposure and the presence of concurrent diseases [18].

In most cases of psittacosis, lesions are limited to three structures: spleen, liver and air sacs [113]. Table 2 summarizes the post-mortem lesions in these specific organs and points additional possible lesions of *Cp. psittaci* found in animals [18, 108,113].

Spleen	Size: Increased (splenomegaly).
	Colour: blackish, sometimes with greyish white necrotic foci and petechial haemorrhages.
	Consistency: soft.
	Perivascular sheaths of macrophages in the arterioles transforming the architecture of the organ.
	Increased macrophages numbers and decreased number of lymphocytes.
Liver	Size: Increased (hepatomegaly).
	Colour: Colour ranging from yellow to green.
	Consistency: friable.
	Acute infection – often the presence of a multifocal necrosis is the only sign of disease, and the other organs show no alterations
	Subacute or chronic infections - hyperplasia of the biliary ducts and sinus histiocytosis with mononuclear cell and heterophils infiltrate.
	Increased activity of Kupffer cells, stimulated by hemosiderin accumulation.
Air Sacs	
	Acute and subacute infections – changes in thickness of the membranes that were covered with fibrinous or fibrinopurulent exudate.
	Chronic infections - presence of pyogranulomatous infections or diffuse granulomas on serous surfaces.
Intestine	Lymphocytic enteritis.
Kidney	Acute necrosis and nephritis with inflammatory infiltrate of mixed type.
Injuries and/or changes in other organs	Infectious pericarditis, myocarditis, adenitis and peritonitis.
	Pneumonia although not frequent is possible.
	Inflammation of adrenals and gonads sporadically found.
	Brain injuries are rare.
	Medium increase in granulocytic cells series in spinal cord.

Table 2. Post-mortem lesions in specific organs (Adapted from [18,108,113]).

5. Diagnostic techniques

There are several available methods for the diagnosis of *Chlamydophila psittaci*, including the agent direct visualization with a specific staining technique, isolation of the agent followed by agent identification, detection of bacteria specific antigen or genes in samples and, finally, serology tests that identify antibodies against this organism [20,89].

a. Harvesting and storage of samples

Sampling should be carried out aseptically to avoid contaminating bacteria [89]. At necropsy, the main structures to isolate this bacterium are the air sacs, spleen, pericardium, heart, areas of hyperemia of the intestine, liver and kidney. On live animals the choanal, oropharyngeal and cloacal swabs are the principal material for *Chlamydophila psittaci* search [52,89,111]. Other samples can be taken from blood, conjunctiva and peritoneal exudates [20].

The samples for subsequent isolation of *Cp. psittaci* must be handled carefully to prevent the loss of infectivity of bacteria during transport and handling, but also due to its high zoonotic potential [89]. Thus, the safety of the operator must be respected (use of gloves, gown and mask) and the samples and swabs are placed in SPG (sucrose phosphate glutamate) transport medium, which is also suitable for bacteria of the *Rickettsia* genus [89, 114]. SPG is formed according to Table 3 [115].

Sucrose	74.6 g / L
K ₂ HPO ₄	1.237 g / L
Glutamic Acid	0.721 g / L
Fetal calf serum	10%
Streptomycin	100 µg / mL
Vancomycin	100 µg / mL
Nystatin	50 µg / mL
Gentamicin	50 µg / mL

Table 3. Composition of SPG [115].

SPG is also used for samples dilution and freezing, if they will be processed with a delay of four or more days after harvest; otherwise samples should not be frozen [89, 114]. Freezing of material must be performed within the first 24 hours, in a stabilized 7.2 pH phosphate-buffered saline medium and kept at a minimum temperature of -20 ° C [89, 114]. Spencer and Johnson (1983) report that *Cp. psittaci* can survive in SPG to just over 30 days at 4 ° C, but their infectivity decreased by 1.55% [115]. Detection of *Cp. psittaci* in the blood, liver, spleen and kidney is only possible two whole days after, whereas in faeces is only possible beyond the 72nd hour [113].

b. *Chlamydophila psittaci* identification

Giemsa cytological staining

Chlamydophila psittaci can be identified by direct observation of the organism using different staining techniques such as the Giemsa, Stamp and Gimenez stainings [116]. Giemsa staining could be performed from faeces, exudates, or liver and spleen cytologies [20]. Although cytological staining can be useful and quick, it is less sensitive and less specific than immunochemical staining or molecular detection methods [18, 20]. With Giemsa staining the methanol fixed material can be observed under the microscope. Inside the cells a basophilic inclusion composed by EB and IB located near the nucleus is found in infected cells [117]. Hayashi visualized bacteria using this technique, once isolated from organs such as liver, spleen, kidney, heart and intestine [118]. This technique is a powerful tool in the *Chlamydia* diagnosis, but it requires a vast experience from the observer.

Gimenez staining

This staining is often used, although it is not specific to this bacterium and has reduced sensitivity. Other agents beyond *Chlamydophila* (for instance, *Coxiella burnetii*, *Helicobacter pylori*) are stained red with Gimenez staining in contrast to background in light green [119].

This staining technique is based on carbol-fuchsin (basic fuchsin) reactions contrasting the background with malachite green [120]. The Gimenez staining technique is considered as a rapid diagnostic for the detection of *Cp. psittaci* on dead birds [121]. Some authors compared the use of DGMB (dark-ground methylene blue) staining with MZN (modified Ziehl-Neelsen) and DGG (Giemsa dark-ground) using infected goat foetal membranes, concluding that DGMB is a more specific staining for *Chlamydia* elementary bodies than MZN and DGG [123]. Woodland and colleagues (1982) developed a new staining technique for inclusions of *Chlamydia psittaci* and *Chlamydia trachomatis* and concluded that it was significantly better sensitivity than Giemsa staining. This technique consists on using a methyl green staining with neutral red and washing at pH 5.0 [124].

c. Immunohistochemistry

Immunohistochemistry is a method based on specificity of immune complex formation by the specific antibodies union with their specific antigen, being the IgG much more frequently used in comparison to IgM [20]. Nevertheless, a variability in Immunohistochemistry methods exists, and the choice of appropriated variations depends mainly on the available equipment and characteristics of existing antibody, since virtually all techniques on paraffin sections are suitable for detection of *Cp. psittaci* [20]. This technique is more sensitive than routine histochemical staining; however, the user experience is essential to the identification of the bacteria. The body morphology of the agent must be remembered to avoid false positives in situations of cross-reactions with other bacteria and fungi [125]. In some cases, hemosiderin can lead an inexperienced user into errors, directing him into an incorrect positive diagnosis. Antigen positive tissues and sections stained with hematoxylin-eosin must be used as control [20]. The antibody used in this method could be polyclonal [126] or monoclonal for the genus of the family *Chlamydophila* [127]. Some antibodies detect the specific epitope of the family Chlamydiaceae, located in the LPS of the bacteria [128]. Other authors reported the degree of sensitivity of this technique with peroxidase-antiperoxidase in the identification of *Chlamydia* and point out the positive samples to Chlamydiae when unapparent to the hematoxylin-eosin technique [129].

d. Serological Tests

Serological tests are still widely used, yet these are not particularly useful for the disease diagnosis on birds, since they show a high prevalence of bacteria [106] and *Chlamydophila psittaci* antibodies are maintained in circulation for months or even years. Due to lack of information and studies on the direct identification of bacteria by serological tests in some birds, these tests are still viewed with some uncertainty concerning the interpretation of the results [20]. A positive diagnosis does not mean active infection, but confirms that the animal had contact with the bacteria in the past [12, 114]. Further, in case of acute infections, these tests easily lead to false negative results since the serum sample may be carried out before the seroconversion [18], which is necessary to the method efficiency. Another reason for false negatives is the treatment of animals with antibiotics that reduce or delay the antibody response [12]. The serological methods most usually used include the methods of elementary body agglutination (EBA), the complement fixation test (CF), the indirect immunofluorescence (MIF) and commercial ELISA tests [22, 114, 130].

Methods of elementary body agglutination (EBA)

EBA detects IgM [12], therefore diagnosing recent (acute) cases of infection [121]. A negative result does not mean, however, that the animal is not infected, having this technique a very reduced sensitivity [20].

Complement fixation test (CF)

Although CF is the most used serological method [26, 89, 114, 130], it has several disadvantages in the use in psittacines because the immunoglobulins do not fixate the complement [131-132]. In such cases, an indirect test for complement fixation using the spot-*Chlamydo-phila psittaci* IF (bioMérieux, Basingstoke, Hants) reagent [89] or other serological test such as MIF should be used [114]. This test has also low sensitivity [114] and the technique is very laborious, being discarded whenever multiple samples need to be tested at same time [20].

Micro-indirect immunofluorescence (MIF)

This method detects all isotopes of immunoglobulin produced against the genus *Chlamydo-phila*. Thus, it is widely used in the detection of antibodies against *C. trachomatis*, *Cp. psittaci* and *Cp. pneumoniae* serotypes in serum or plasma [20]. The indirect immunofluorescence test seems to be more sensitive than the complement fixation test and histochemical techniques, albeit it shows cross-reactivity with other chlamydial species [20].

Direct fluorescent antibody (FA)

The preferably staining method to detect the *Chlamydo-phila* is the direct FA [89,133]. In this method, an anti-chlamydia fluorescein-conjugated is applied to the smear [134] following the incubation for 30 minutes at 37 ° C [20]. Thereafter, the slides are washed up with PBS and distilled water, dried on air and finally mounted with a resinous compound like Entellan® [20]. The inclusions of the bacteria show a bright green under ultraviolet microscopy [96].

ELISA technique

The primary purpose of a great number of commercial ELISA developed in the last 25 years was for *Chlamydia trachomatis* in humans, but such tests are also suitable for diagnosing *Chlamydo-phila psittaci*, as they are specific to the LPS antigen of all species of the family Chlamydiales [1,27,135-136]. Commercial ELISA tests were, over the years, widely inquired and several studies about the low specificity and high sensitivity of these methods came up [20], with the first dramatically reducing in situations where the prevalence of the bacterium is low and hence where there is a low number of detected bodies [136]. In fact, it takes hundreds of bacteria so that the results are positive [20]. One of the major disadvantages of this technique is the possible occurrence of false positives, since have cross-reaction with other bacterias (such *Acinetobacter calcoaceticus*, *Escherichia coli*, *Staphylococcus aureus* and *Klebsiella pneumoniae*) have been demonstrated in humans [34,137]. Nevertheless, the development and improvement of these commercial tests increased with the use of monoclonal antibodies and advanced blockers methods [20, 136] that improve the specificity and reduced the false positive cases. There are already available specific commercial ELISA tests for *C. trachomatis*, *Cp. pneumonia*, *Cp. abortus* antibodies [132,138-139], and for *Chlamydo-phila psittaci's* as well [101]. These methods are as reliable as the

indirect immunofluorescence tests, but are faster, easier to perform and less expensive [20], as well as more sensitive than the complement fixation technique, easier to standardize and, above all, more suitable for large epidemiological studies [22,101]. They also do not depend on the viability of the elementary bodies, or of soluble antigens in secretions [136]. An ELISA based on a recombinant ELISA (rMOMP ELISA) was developed and tested, giving a sensitivity and specificity of 100% in cases of psittacosis [18,140].

In our opinion, Immunocomb® (Biogal, Kibbutz Galed, Israel) commercial kit is nowadays the most suitable ELISA test available, as it has a nearly 100% sensibility and specificity [101]. This performance is enjoyable, but the labourer must understand that the choice for the most suitable laboratorial test depends on each case (Figure 2).

Figure 2. Commercial kit Immunocomb® (Biogal, Kibbutz Galed, Israel) ELISA test, with the reference sample (>) and examples of strong positive animals (*).

e. Isolation of *Cp. psittaci*

Isolation of bacteria may be performed from tissue and faecal specimens or scrapings [145]. A 20% to 40% homogenized suspension sample is prepared using diluents such as phosphate buffer (PBS) at pH 7.2 and the culture media [20,146]. When samples are inoculated within 24 hours after harvested and not frozen, these solvents are used with antibiotics [20]. Whenever samples are chilled or frozen, a transport media such as SPG or Bovarnick's should be used as stabilization agent [15,147].

Before cell cultures or animal inoculation, potentially contaminated samples must be treated by three possible methods: with antibiotics [133], followed or not by low speed centrifugation [148] or filtration [133,149]. The sample is afterwards submitted to a standard procedure, where it is homogenized in diluent containing 1 mg/ml streptomycin, 1mg/ml of vancomycin and 1mg/ml kanamycin [20]. It's possible to select other antibiotics, however penicillin, tetracycline and chloramphenicol should be avoided, since they inhibit the growth of these bacteria [148]. If the sample is slightly contaminated, before the inoculation into the cell culture or on the test an-

imal, samples must be homogenized in an antibiotic solution and remain there for 24 hours [150]. In case of deep contamination of samples, such as faecal samples, they must be homogenized in an antibiotic solution and then subjected to centrifugation at 1000-2000x g for 30 min [148]. Thereafter the upper and lower layers must be discarded and the supernatant inoculated into the culture fluid cells or laboratory animals. In case of persistent contamination, the sample should be subjected to filtration pores of 450 to 800 micrometre [148].

f. Cell culture and embryonated SPF eggs

The cell culture is the method of choice used to isolate *Chlamydophila psittaci* [7]. Although other cultures can be used, the most frequently used are the BGM (*Buffalo green monkey*), McCoy, Vero, HeLa or L-929 [24]. In a 1992, a study reports that, within these, BGM has the highest sensitivity [151].

Bacteria can be isolated from cells that have its normal cell cycle of replication, but stable cell lines without reproduction are more satisfactory, since they provide additional nutrients for the *Cp. psittaci* replication and are much easier to observe [148]. For that, suppression of the cells is made by irradiation or by cytotoxic chemicals such as 5-iodo-2-deoxiodine [149], cytolasine, B, cycloheximide, cycloheximide and emetine hydrochloride (0.5 - 2.0mg/ml) [148]. The type of material used will mainly depend on the available material in the laboratory [20].

To increase the binding of bacteria with cells, after the inoculation of the bacterium into the cell line a centrifugation (500-1500x g) for 30-90 minutes is needed [150]. This union will be stronger when incubation is performed at 35°C to 39°C [152]. Cultures are checked for bacteria on days 2, 3, 4 and 5 [148].

Some laboratories use chicken embryos to isolate the organism [96, 153], usually injecting up to 0.3 ml of inoculum in the yolk sac of 6 days embryos [89]. Thereafter, bacterium replicates, which leads to the death of the embryo within a maximum period of 12 days. In case of failure, two additional inoculations should be made before considering the *Cp. psittaci* sample as negative [20].

Usually, the organism causes vascular congestion in the yolk sac membranes, which is homogenized in a 20% suspension membrane [20]. This suspension may be frozen in order to preserve bacteria or inoculated into new eggs or cell culture monolayers [148]. The identification of inclusions is done through cytological or immunohistochemical methods [152], like the indirect fluorescent antibody technique, the immunoperoxidase technique [129], or by histochemical stainings [134] such as Gimenez and Macchiavello based-stains, allowing the visualization of the bacteria [20].

Fixation of monolayer cells was made with acetone for 2 to 10 minutes, after transport media has been removed and washed with PBS [154-155]. If the support is made of plastic material, alcohol should be used instead of acetone for fixation [156].

g. Polymerase chain reaction (PCR)

Molecular methods such as PCR allow the direct detection of *Chlamydophila psittaci* from clinical specimens within one day [157]. In literature, the amplification is made from the ribosomal RNA gene region [128, 158, 159] and the gene encoding the antigen, known as omp1 or ompA [132, 160].

The quality and quantity of extracted DNA is essential for the sensitivity of the test [20]. The efficient extraction of DNA samples and removal of PCR inhibitors ensure the proper functioning of the method [20]. The efficient extraction of DNA is much more difficult in bacteria, when compared to viruses, since bacteria possessing outer membranes are very resistant to destruction [20].

There are several methods of commercial DNA extraction capable of extracting *Chlamydophila* DNA. The test QIAamp® DNA Mini Kit (Qiagen) was reported as being the one with best results for pharyngeal scrapings, whereas the test High Pure PCR Template Preparation Kit® (Roche) was the top performer for faecal and cloacal samples [20].

These commercial tests are suitable when working with PCR inhibitors [20]. They also contain a particular reagent for the bacterial and eukaryotic cells lysis. One of these reagents is GIT (Guanidine IsoThiocyanate) [20].

The cellular RNA is digested with an RNase and then the lysate is centrifuged in a mini-column, where the DNA is joined to a solid phase that can be modified by silica hydroxyapatite or special filters membranes. Then, the elution takes place by immersing DNA of high purity and free of PCR inhibitors [20]. Cold storage leads to a rapid loss of DNA from organisms, so samples with low levels of target DNA often become negative. To avoid this problem, DNA stabilizers are used in PCR analysis [161]. Some reagents are available commercially as the RNA/DNA Stabilization Reagent for Blood/Bone Marrow® from Roche Applied Science [161,162].

In 2005, a PCR assay proved sensitive enough to detect *Chlamydophila psittaci* in samples from birds [97]. Sachse and colleagues (2005) detected the bacteria based on the amplification, resulting on a specific *ompA* product for the genus, followed by a second amplification using a specific primer for the genus and a specific primer for the species. At the end, it is created a specific amplicon for *Chlamydophila psittaci* [20, 163].

The PCR results are visualized by electrophoresis and the sensitivity of PCR-EIA was set at 0.1 IFU (infection forming unit) [97].

These tests are in progress and recently new types of PCR emerged. One such case is the SYBR Green-based real-time PCR that targets the rDNA spacer of *Chlamydophila psittaci* [20]. This test detects 10rDNA copies/ml of extracted DNA and all *ompA* genotypes of the bacteria [20].

h. DNA microarray-based detection

Sachse and colleagues (2005) developed a method to identify *Chlamydia* and *Chlamydophila* spp. species. This test was developed through the platform ArrayTube (CLONDIAG® chip technologies) and it obtained specific hybridization patterns for the species in all organisms of the Chlamydiaceae family. Thus, this study proved this test is a viable alternative for the identification of ambiguous *Chlamydia* cell culture, and a possibility for detection of these microorganisms from tissues [163].

Table 4 below summarizes the advantages and disadvantages of some methods for diagnosis of *Chlamydophila psittaci* [20].

Diagnostic methods	Advantages	Inconveniences
Cytological staining	<ul style="list-style-type: none"> - easy - cheap - quick - no sophisticated equipment needed - dead and live bacteria can be demonstrated 	<ul style="list-style-type: none"> - non-specific - less sensitive - non-automated - interpretation by experienced person
Immunocytochemistry	<ul style="list-style-type: none"> - more sensitive and specific than cytology - easy - quick - dead and live bacteria can be demonstrated 	<ul style="list-style-type: none"> - cross-reaction with other bacteria (when MAb is against LPS) - interpretation by experienced person - more expensive - fluorescence microscope required - non-automated
Immunohistochemistry	<ul style="list-style-type: none"> - automation possible - detection in the morphological context 	<ul style="list-style-type: none"> - more labour intensive than immunocytochemistry - histology laboratory required - MAb detecting <i>Cp.psittaci</i>-specific antigen in formalin-fixed samples needed - more time consuming than immunocytochemistry - more expensive
Antigen-ELISA	<ul style="list-style-type: none"> - quick - multiple samples can be tested at once - quantification - easy - dead and live bacteria can be demonstrated 	<ul style="list-style-type: none"> - commercial kits often insensitive - non-specific if the target is LPS or Hsp60 - ELISA reader needed
Culture	<ul style="list-style-type: none"> - propagation for further investigations - more specific than direct antigen detection - direct evidence of live bacteria - quantification of live bacteria 	<ul style="list-style-type: none"> - transport and storage of samples is critical - BSL3 laboratory - time consuming - expensive - labour intensive - trained personnel required - not all strains can be cultured
Molecular diagnosis (PCR, Micro array)	<ul style="list-style-type: none"> - highly sensitive - highly specific - quick - automation possible - multiple samples can be tested at once - possibility of direct typing on clinical samples - can be quantitative - can detect live and dead bacteria 	<ul style="list-style-type: none"> - expensive - specialized equipment needed - trained personnel needed

Diagnostic methods	Advantages	Inconveniences
Serology: antibody ELISA	- easy	- convalescent sera (retrospective diagnosis)
	- quick	- not a proof that the organism is still present
	- multiple samples can be tested at once	- tests detecting antibodies against LPS, hsp60 or whole organisms are non-specific
	- quantification is possible	- less sensitive than molecular diagnosis
	- automation possible	- ELISA reader needed
	- valuable for epidemiological research	

Table 4. Advantages and disadvantages of some methods for diagnosis of *Chlamydophila psittaci* (Adapted from [20]).

It is our opinion that there’s no better diagnostic test for *Chlamydophila psittaci* but a more suitable or adequate to each case instead, for different biologic material sampling and laboratory conditions. If the goal is to define *Chlamydophila*’s prevalence in a large group of animals, PCR should be the first choice, although more reliable tests imply more expensive tests. If the investment had limitations, an ELISA test can be the most adequate choice for the diagnosis. If the group of animals is just of some units, then cultured cells can be a possibility if the proper laboratory conditions are available.

6. Treatment

Treatment of psittacosis is performed with medication, being tetracycline the drug of choice [12, 148]. In cases developing in pregnant women or in children under the age of 9 years, the use of tetracycline or doxycycline is contraindicated, being the use of erythromycin the most suitable. Treatment of the patient should be made for at least 14 consecutive days using the drug of choice [164]. In birds, tetracycline or doxycycline should be given over a period of 7 weeks in the feed or on medicated seed [165].

7. Prevention

a. Vaccines

Today there are still no vaccines available against avian chlamydiosis [148]. Attempts on DNA vaccines have reduced signs of disease, injuries and excretion of bacteria. However, a complete protection of individuals remains distant [98]. Currently, treatments and strategies for reducing contamination of these bacteria are the best way to control the disease [12].

b. Disinfection

Chlamydophila psittaci can survive up to 30 days in faeces and cage materials or beds, so regular cleaning of equipment and places where animals are infected is essential [127]. Bacteria of the family *Chlamydiae* are quite sensitive to chemical that affecting the lipid contents or the integrity of the cell walls. Most disinfectants used as detergents inactivate *Cp. psittaci* bacteria, such as a 1 to 1000 ammonium quaternary, 1 to 100 chlorophenol, 70% isopropyl alcohol and 1% lysol [12, 113].

8. Public health concerns

Chlamydophila psittaci is a zoonosis, hence it is an organism capable of producing infection in humans [14, 24, 166]. Man is mainly infected by the dispersed bacteria in the inhaled air, after faeces, urine or secretions of the respiratory system of infected animals dry [21]. The direct contact with infected animals may also lead to infection [4], so that the post mortem examination and manipulation of cultures should be done properly, using appropriate safety equipment and the use of flow laminar chambers [23].

This disease has particular significance in public health, since parrots are common pets in our houses, even in schools or nursing places [148]. The risk of contracting the disease increases with the contact with these animals, but also in day-to-day activities, for example mowing the lawn, shrubs or others without a suitable container for this purpose, exposing individuals to the bacteria [16, 167]. Psittacosis can also be transmitted from person to person, however this process is believed to be uncommon [167-168].

The incubation period is usually between 5-14 days [12]. In humans, signs of psittacosis can vary greatly, ranging from cases where they are completely unapparent to situations where it can be found signs of systemic disease with severe interstitial pneumonia and encephalitis [12, 170]. Infected humans may develop headaches, chills, discomfort and myalgia [12]. Respiratory involvement is common and a number of cases have been documented [171-174]. This disease is, however, rarely fatal if patients are provided with proper treatment, so an awareness of the dangers of psittacosis and rapid evaluation of the case are vital [12, 123].

Chlamydophila psittaci was associated with ocular lymphoma [174], however, this subject is still matter of debate and disagreement and conflicting reports have been published [175,176]. The true greatness of this disease is very far from knowing, since cases where psittacosis is diagnosed and individuals have severe signs of disease are only a tiny fraction of the total occurrences. Cases where the disease is less severe, unnoticed or misdiagnosed infections due to similar symptoms with other respiratory pathogens or asymptomatic infections remain in the shadow of *Cp. psittaci* infection in humans [103, 162].

9. Conclusion

Psittacosis is a disease that is virtually throughout the world and is a zoonosis. This fact must be present in all healthcare professionals, being veterinarians or doctors.

Psittacines are the most commonly affected animals and this fact must be an alert for veterinarians to pay special caution during its manipulation. Owners and handlers of exotic birds should as well learn how to prevent this disease, as they are a potential risk group for infection.

The number of infected animals by *Chlamydophila psittaci* is underestimated [106] and new cases of outbreaks continue to appear worldwide, some of which often die facing this disease [20].

A more feasible, fast and easy diagnostic method and universally accorded is yet to be implemented.

Altogether, control of this disease should be stricter and information about its maleficence worldwide known.

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Author details

João Morais, Ana Cláudia Coelho and Maria dos Anjos Pires

CECAV –Univ. of Trás-os-Montes and Alto Douro, Portugal

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