# Chlamydiae – what's new?

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**Abstract**

This paper provides an overview of the current knowledge of chlamydiae. These intracellular microorganisms belonging to the *Chlamydiaceae* family are widely distributed throughout the world. Constant development of culture-independent approaches for characterisation of microbial genomes enables new discoveries in the field of *Chlamydia*. The number of new taxa is continuously increasing as well as the range of hosts. New species and genotypes are constantly being discovered, particularly new avian and reptilian agents, which are discussed in this article. Interestingly, wild animals are the main hosts for new *Chlamydia* species including different species of bird, turtle and snake. The availability of next-generation sequencing opens up a new prospect for research and leads to deeper knowledge of these interesting microorganisms about which much is still to discover.

**Keywords:** chlamydiae, chlamydiosis, NGS, birds, reptiles.

## Introduction

Chlamydiae are Gram-negative bacteria isolated independently by Levinthal, Cole and Lillie in 1929–1930 and described in 1932 by Bedson and Bland (3), although chlamydial disease had been known for centuries before this research formalised the knowledge. A search of the literature reveals that chlamydial-like diseases of the eye appear in ancient Chinese writings (2700 BC) and Egyptian papyri (1555–1553 BC) (18, 61, 76). Initially, chlamydiae were considered to be viruses because of their small size, ability to pass through 0.45 µm diameter filters and biphasic intracellular development cycle. However, due to the presence of both DNA and RNA, the ability to synthesise proteins, lipids, and nucleic acids, and their sensitivity to antibiotics, they were finally classified as bacteria (44). They are distributed worldwide in humans, livestock, and companion and free-living animals (7).

Taxonomically, chlamydiae belong to the order *Chlamydiales* and the family *Chlamydiaceae* comprising one genus, *Chlamydia (C.)*. So far, there have been 14 species described within this genus: *C. abortus*, *C. psittaci*, *C. avium*, *C. gallinacea*, *C. buteonis*, *C. caviae*, *C. felis*, *C. muridarum*, *C. pecorum*, *C. pneumoniae*, *C. poikilothermis*, *C. serpentis*, *C. suis* and *C. trachomatis* (6, 37, 56, 68). *Chlamydia trachomatis* and *C. pneumoniae* predominantly occur in humans, while other *Chlamydia* species cause infection in animals but may also pose a threat to human health in some cases (38). In recent years, due to the striding development of molecular biology techniques including next-generation sequencing (NGS), a pronounced increase in research on *Chlamydiaceae* has been observed. New *Chlamydia* species have mainly been noted in birds and reptiles. However, without isolating the strain and determining its phenotypic characteristics, it is not possible to introduce a new species into the taxonomy (6). Therefore, some strains representing the new *Chlamydia* species still retain their *Candidatus* (*Cand.*) status: *Cand.* C. ibidis identified in sacred ibis in France, *Cand.* C. sanzinia and *Cand.* C. corallus found in snakes, and *Cand.* C. testudinis described in turtles (36, 77, 80, 84).

The last decade's investigation of *Chlamydia* concerned new taxa discovered mainly in wild animals (36, 37, 68, 77, 80, 84). It seems that wildlife has become an excellent niche for the development of *Chlamydia*. The continuous improvement of the next-generation sequencing techniques, as well as the availability of

genome analysis tools independent of strain isolation, open up new prospects for scientists and allow them to deepen their knowledge of these distinctive and interesting microorganisms (27, 85).

The purpose of this article is to present the latest data on *Chlamydia* spp., with particular emphasis on taxa found in birds and reptiles.

## Genome structure

Chlamydial genomes are reduced due to their obligatory intracellular life cycle, hence their size is about 1 Mb and they contain 850–1100 genes (8, 14, 66). This simplification, in contrast to how it was caused in many pathogens with a reduced genome, derives from genome streamlining rather than degradation (43). A characteristic feature of chlamydial genomes is a small number of pseudogenes (2, 45). Up to 14 transcription factors (TFs) have been predicted to regulate gene expression (14, 66). The gene order is highly conserved outside the region of extensive variation between chlamydial genomes located near the replication terminus commonly called the plasticity zone (PZ) (2, 45, 51). The PZs have been described in all the genomes of *Chlamydia* species identified to date and usually range from ~12 kbp to ~86 kbp, with 11 to 48 genes (2, 36, 37, 45, 68, 77, 80, 84). Despite the intracellular parasitism of *Chlamydia* spp., the bacteria have avoided the accumulation of harmful mutations leading to genome degradation as has occurred in many parasitic bacteria. Numerous studies indicate that these mutations can be prevented by homologous recombination among *Chlamydiae* (21, 30, 52). In addition, the genomes are free of destructive mobile elements, excluding the insertion sequence in the genomic island in *C. suis* linked to tetracycline resistance and the remains of insertion sequence-like elements and prophages in several genomes (45, 56, 59, 66, 84). Lack of certain genes encoding key enzymes of the metabolic pathways results in the high dependence of *Chlamydia* on host cells, from which they draw amino acids, nucleotides and cofactors (8). Genome reduction allows significant phenotypic variation; so far 18 species with a wide range of tissue and host tropism have been identified (6, 36, 37, 56, 68, 77, 80, 84). It should be added that the number of new taxa is constantly increasing as well as the range of hosts which can provide them with a suitable environment. The genomes of *Chlamydia* spp. mostly comprise single circular chromosomes but a small amount of genetic material is carried on a plasmid present in the genomes of many *Chlamydia* species, the presence of which is presumed to be associated with the virulence of the strain. Chlamydial plasmids are approximately 7.5 kbp in length with some exceptions, and are circular, highly conserved, and not integrated into the genome (26, 47, 81). They include eight coding sequences and non-coding RNA with unknown functionality (47). Generally, plasmids can be carriers of antibiotic resistance genes, but to date, there has been no evidence presented of antibiotic resistance located on chlamydial plasmids (70). Plasmids were found in the majority of chlamydial genomes: in *C. trachomatis*, *C. psittaci*, *C. pecorum*, *C. avium*, *C. caviae*, *C. felis*, *C. muridarum*, *C. suis*, *C. pneumoniae*, *C. buteonis*, *C. gallinacea*, *C. poikilothermis*, *C. serpentis*, *Cand.* C. corallus, *Cand.* C. sanzinia, and *Cand.* C. testudinis (6, 36, 37, 56, 68, 77, 80, 84). Until recently, it was thought that no plasmid existed in the *C. abortus* genome (47, 70), but surprisingly, the genomes of the recently described avian strains have a plasmid homologous to that found in *C. psittaci* (75, 88). According to the latest data, a plasmid is apparent in the genome of the common bacterial ancestor of all the species, but with time, the plasmids have diverged. It is assumed that because of the high level of conservation across the *Chlamydia* genus (excluding *C. pneumoniae*), there is a strong evolutionary selection for these species retaining their plasmids. Moreover, based on the performed evolutionary analysis it is assumed that the plasmid and the chromosome have co-evolved (70).

## Avian chlamydiosis

Until recently, it was believed that avian chlamydiosis, called psittacosis or ornithosis, was caused only by *C. psittaci* (32, 60, 73). However, researchers latterly described cases of infections in birds with the species *C. avium*, *C. gallinacea*, *C. buteonis* and *Cand.* C. ibidis (37, 56, 57, 58, 84). Moreover, data show that *C. abortus*, *C. pecorum*, *C. trachomatis*, *C. suis* and *C. muridarum* have been found in birds (19, 46). Hence, the World Organisation for Animal Health (OIE) has extended the definition of avian chlamydiosis to indicate that *C. psittaci* is not the only aetiological agent of avian chlamydiosis but various species of *Chlamydia* may also be (86).

*Chlamydia psittaci* is a well-known pathogen and most common in birds. So far, its occurrence has been recorded in at least 464 bird species, including free-living and domestic birds (31). The highest percentage of infections has been noted in pigeons and psittacines. It should be highlighted that *C. psittaci* causes heavy economic losses on poultry farms and in aviaries. Clinical symptoms vary in severity and depend on the species, age of the bird, and the level of virulence of the infecting strain (31, 86). The most frequent clinical signs among pet birds are conjunctivitis, diarrhoea, weight loss, yellowish droppings, sneezing, sinusitis, respiratory distress, biliverdinuria, nasal discharge, lachrymation, and decreased egg production (86). The infection can also be asymptomatic, especially in poultry and older parrots, and therefore birds cannot easily be identified and quarantined because they do not show signs of infection but are active shedders for a long time and can infect other individuals in the flock (31, 48). Infection can lead to systemic disorders and even death in birds. Pneumonia, myocarditis, pericarditis, fibrinous

peritonitis, multifocal necrosis, splenomegaly and hepatomegaly are common in acute infection while these last two symptoms, are frequently observed in chronic infection and may progress to organ rupture (1, 7, 60). Doxycycline and tetracycline are usually the drugs of choice. To avoid relapses, a 45-day therapy duration is recommended (38, 67, 74).

The epidemiological significance of the emerging agents *C. avium* and *C. gallinacea* responsible for the occurrence of avian chlamydiosis has not yet been fully understood (86). According to literature data, *C. avium* is most common in pigeons and parrots (59, 86), but its presence has also been confirmed in mallard in Poland (71). There is no evidence that *C. avium* shows clinical signs in pigeons, however from currently available data it seems likely that it can lead to respiratory disease and/or diarrhoea among psittacine birds and pigeons (59). It should be noted that contrary to the previous prevailing hypothesis, *C. gallinacea* rather than *C. psittaci* is the dominant agent of infection in poultry (19). Its occurrence has been confirmed in flocks of chickens, turkeys and guinea fowl in various countries including Poland (33, 72, 89). Initially, *C. gallinacea* was considered to be a commensal in the gastrointestinal tract of poultry, but Guo *et al*. (19) confirmed its additional presence in cattle, which may suggest the species barrier has been crossed. So far, the consequences of *C. gallinacea* infection in cattle remain unknown. Moreover, no case of infection associated with clinical symptoms in poultry has been described, and only the phenomenon of asymptomatic shedding was reported (19). However, You *et al*. (87) published data describing the negative impact of *C. gallinacea* infection on production in chickens including body weight gains lower by 6.5–11.4%. Monitoring of poultry flocks in Poland confirmed the presence of *Chlamydia* spp. in 15.9% of them. It is salient that *C. gallinacea* was identified in 65.5% of positive samples, whereas *C. psittaci* was recorded in only three flocks of chickens, ducks and geese (10.3%). Interestingly, four flocks of chickens (13.8%) were found to be carriers of *C. abortus*, which is unusual because this species is isolated almost exclusively from mammals (72).

Research shows that different chlamydial agents occur in avians (37, 71, 72) and free-living birds can host unknown and unclassified species. Recently, data on cases of the non-mammalian *C. abortus* strains in free-living mallards and swans were published (71). The structure of their genomes is very similar to *C. abortus* but a plasmid characteristic for its sister species *C. psittaci* is included. *Chlamydia abortus* strains isolated from birds create a separate group and were preliminarily defined as avian *C. abortus* (71, 75). As a result, the taxonomic definition of *C. abortus* has become outdated. Previously, the strains affiliated to *C. abortus* were labelled monophyletic and were only isolated from mammals which aborted (65, 83). Meanwhile, the new group of avian *C. abortus* is represented by three different genotypes G1, G2 and 1V isolated from different species of birds (71, 75, 88). The introduction

of adequately substantiated changes in the taxonomy of the *Chlamydiaceae* family requires further research using next-generation sequencing.

It is widely acknowledged that *C. psittaci* has zoonotic potential (9). Taking into account the growing interest in exotic birds over recent years and the rise in popularity of keeping psittacine birds as pets, it seems the problem of shedding of *C. psittaci* is growing in importance. Cases of *C. psittaci* infections in parrots are found in pet stores, aviaries and private homes in different countries including Poland (48, 74). There are no data concerning pathogenicity or the possibility of transmission of *C. gallinacea* and *C. avium* infection to humans. However, based on a case report of atypical pneumonia in slaughterhouse workers in France who had contact with poultry carcasses positive for *C. gallinacea*, zoonotic potential of *C. gallinacea* cannot be ruled out (35).

Avian chlamydiosis has also been reported in raptors in Europe and North America including red-tailed, Swainson's, ferruginous, and red-shouldered hawks, in which the presence of the new species *C. buteonis* in the conjunctival sac and cloaca was confirmed (4, 28, 37, 41, 62). Based on the cases described so far, it can be assumed that *C. buteonis* causes or contributes to conjunctivitis and/or respiratory symptoms. The route of transmission has not yet been investigated nor confirmed, but it can be mooted that, as with other chlamydial agents, infection occurs by airborne aerosols containing dried faeces or dust. Comparative analysis of the *C. buteonis* genomes and other members of the *Chlamydiaceae* family demonstrated the greatest similarity of the new species to *C. psittaci*. The zoonotic potential of *C. buteonis* has not yet been scrutinised but taking into consideration its phylogenetic relationship with *C. psittaci*, it cannot be excluded (37).

**Chlamydiae in reptiles**

The first case of chlamydiosis in reptiles was described in 1944, when the infection was confirmed in a lizard (82). Subsequently, further individual case reports appeared, but due to diagnostic limitations, species identification was usually impossible. The 21st century has brought intensive development of DNA diagnostic techniques and the execution of retrospective studies which have enabled complete and easier species identification. As a result, the presence of *C. pneumoniae*, the most common species found in humans, was confirmed in reptiles. In recent years, the presence of *Chlamydia* spp. has been noted in many diapsids with or without clinical signs, including pythons, turtles, iguanas and crocodiles (5, 22, 24, 25, 42, 68, 79). Strains displaying similarity to *C. pneumoniae* or *C. caviae* in snakes were also identified, which led to the classification of the new taxa *C. serpentis*, *C. poikilothermis*, *Cand.* C. corallus and *Cand.* C. sanzinia (68, 77, 80). Infections in snakes are very often asymptomatic, but without

proper treatment they can result in death (17, 55). The source of infection for snakes remains unknown (34). Laroucau *et al.* (34) indicate that mite participation in the spread of infection cannot be excluded because before the onset of infection in snakes, mite infestation had been observed in a snake habitat. It is still unknown whether *C. serpentis* has zoonotic potential, but taking into account its phylogenetic relationship with *C. pneumoniae* it cannot be ruled out (15, 34). So far, evidence has not been uncovered of *C. serpentis* transmission or development of symptoms of infection in persons who had direct contact with infected snakes. A new species phylogenetically similar to *C. pecorum* was identified in turtles in Spain, Poland and Germany, but due to the lack of an isolate, it has been designated only as *Cand.* C. testudinis (24, 36, 42). It is possible to identify representatives of this species using a specific real-time PCR targeting the *isp*E gene and its presence was confirmed in cloacal, conjunctival and choanal swabs (36). It can cause or contribute to ocular disease and/or nasal discharge. So far, the route of transmission has not been precisely defined but by analogy with other chlamydial agents' routes, infection may take place through direct contact with fresh faeces or airborne aerosols containing faecal or dust particles.

### Novel diagnostic tools

Initially, evaluation of chlamydial diversity was possible only with application of laborious and expensive culture-dependent methods. In recent years, researchers have developed culture-independent sample preparation methods that can be easily utilised directly on the sample or on genomic material extracted from the sample (78). Development of these methods combined with NGS enabled novel chlamydial taxa to be discovered and characterised and non-cultivable strains to be classified using procedures without strain isolation.

According to data published by Taylor-Brown *et al.* (78), there are non-targeted metagenomic techniques and targeted capture methods. Three non-targeted (meta)genome capture techniques could be used for culture-independent sequencing. The first one is multiple displacement amplification (MDA), which effectively increases the yield of total or target DNA and enables isothermal strand-displacing whole genome amplification (WGA) directly from clinical samples (12, 13, 23, 78) or single cells (39, 50, 78). Depletion–enrichment is another non-targeted metagenomic technique. The method is based on separation of non-methylated microbial DNA from methylated host DNA (16). This approach is useful when the pathogen is unknown, uncultivable, in low abundance or potentially novel (78). It makes exploration of *Chlamydia* biology realisable and offers insights into chlamydial diversity. Recently, novel *Chlamydia* species were discovered in reptiles through this technique (77, 80). The last approach is cell-sorting MDA. Single cells separated on the basis of fluorescence, granularity and size are collected from cryopreserved samples (11, 53, 78). In order to obtain a higher yield of DNA from a single genome, MDA or other WGA methods are required (69, 78). In contrast to the previous method described, cell-sorting MDA allows the genome of a single species to be amplified rather than the entire microbiome. Unfortunately, genomes recovered by this method are often incomplete or highly fragmented (40, 78).

Targeted capture methods are based on knowledge of the pathogen, e.g. through the existence of a reference genome. The available methods are sequence capture, immunomagnetic separation–multiple displacement amplification (IMS-MDA) and multiplexed microdroplet PCR (78). The sequence capture method is based on hybridization of chlamydial DNA away from a complex DNA mixture to capture the chlamydial DNA using biotinylated RNA probes called baits (10). This approach was successfully performed on *C. trachomatis*, clinical specimens of *C. pecorum*, sensitive cell cultures which became no longer cultivable, and chlamydial cell cultures after several passages (9, 10, 20, 54). The subject of the next culture-independent approach, IMS-MDA, was *C. trachomatis* (49). This method uses primary mouse IgG antibodies directed at antigens located on the chlamydial cell surface – lipopolysaccharides – whereas intact chlamydial elementary bodies (EBs) are bound using secondary IgG antibodies conjugated to magnetic beads (49, 63, 64). To remove host DNA, DNase treatment is required prior to DNA extraction from the bound EBs, MDA and sequencing (78). The last method is multiplexed microdroplet PCR using 500 primer pairs designed exploiting the reference genome to generate overlapping 1–1.3 kbp amplicons spanning the selected region (29, 78). So far, it has been used only on *C. trachomatis*. This method is intermediate between WGS and multi-gene sequencing and could be maximised to cover a complete genome. The authors of this approach created specific downstream bioinformatics methods which should be used for genomic analysis (29, 78).

All methods have broad application and individual advantages and limitations which their users need to be aware of. Choosing the most suitable approach might be fundamental to success in sequencing. Taylor-Brown *et al.* (78) presented the features of all of these techniques in depth and their conclusion indicated that non-targeted approaches are the best choice for pathogen discovery and further characterisation of chlamydial diversity, whereas targeted approaches are perfect for diagnostic and epidemiological purposes because of their high sensitivity, lower cost and higher throughput. The advent of these methods definitely improves understanding of chlamydial diversity, biology and phylogeny (78).

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