Chapter 24

Avian Chlamydiosis

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a Introduction

b Definition and Synonyms

Avian chlamydiosis is caused by the bacterium *Chlamydia psittaci*. Avian chlamydiosis is a respiratory disease, usually systemic and occasionally fatal. Today, *C. psittaci* has been demonstrated in about 465 bird species spanning 30 bird orders (61). This chapter primarily covers avian chlamydiosis as it occurs in birds raised commercially for meat and egg production. It should be noted that the disease in pet birds is quite similar, and the disease characteristics, transmission, and diagnosis are essentially the same. An update of the disease and control procedures for chlamydiosis in companion birds was published (104) and is on the American Veterinary Medical Association (AVMA) and Centers for Disease Control and Prevention (CDC) Web sites.

Chlamydia psittaci can be transmitted to humans. The disease in birds and humans originally was called psittacosis or parrot fever (80) as it was first recognized in psittacine birds and in

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Table 2. *Chlamydiaceae* taxonomy (adapted from Schautteet and Vanrompay, 2011)

	Chlamydial taxonomy in the late 1990s		**Chlamydial taxonomy used in the twenty-first century (Everett *et al*., 1999)**				**Chlamydial taxonomy used on the twenty-first century (Stephens *et al*., 2009)**	
Order	*Chlamydiales*		*Chlamydiales*				*Chlamydiales*	
Family	*Chlamydiaceae*		*Chlamydiaceae*, *Simkaniaceae*, *Parachlamydiaceae*, *Waddliaceae*				*Chlamydiaceae*, *Simkaniaceae*, *Parachlamydiaceae*, *Waddliaceae*	
Genus	*Chlamydia*		*Chlamydia*		*Chlamydophila*		*Chlamydia*	
Species	*C. trachomatis*	Trachoma biovar	*C. trachomatis*	Trachoma biovar			*C. trachomatis*	Trachoma biovar
		LGV biovar		LGV biovar				LGV biovar
		Murine biovar	*C. muridarum*				*C. muridarum*	
		Porcine biovar	***C. suis***				***C. suis***	
	C. pneumoniae	Human biovar			***C. pneumoniae***	TWAR biovar	***C. pneumoniae***	TWAR biovar
		Koala biovar				Koala biovar		Koala biovar
		Equine biovar				Equine biovar		Equine biovar
	C. psittaci	Avian subtype			***C. psittaci***		***C. psittaci***	
		Abortion subtype			***C. abortus***		***C. abortus***	
		Feline subtype			*C. felis*		*C. felis*	
		Guinea-pig subtype			*C. caviae*		*C. caviae*	
	C. pecorum				***C. pecorum***		***C. pecorum***	

humans associated with psittacine birds. Ornithosis was a term introduced in 1941 by Meyer (76, 77) to differentiate the disease in or contracted from domestic and wild fowl from the disease in or contracted from psittacine birds. However, the two syndromes are actually the same (84). Nowadays, we speak of chlamydiosis in birds and psittacosis in humans.

b Public Health Significance

Chlamydia psittaci can infect humans and precautions should be taken when handling infected birds or contaminated materials. Most infections are through inhalation of infectious aerosols; thus, poultry processing plant employees are especially at risk, as are farm workers, veterinarians, poultry inspectors and taxidermists (26-28, 35, 42, 64, 120, 138).

In humans, the incubation period is usually 5-14 days; however, longer periods are known (104). Infections vary from inapparent to severe systemic disease with pneumonia (14). Secondary spread among humans rarely occurs. Because the disease is rarely fatal in properly treated patients, awareness of the danger and early diagnosis are important.

a History

The first description of a psittacosis outbreak dates from

1879 and was described by Jacob Ritter, linking the disease to pet parrots and finches (94). Historical aspects of chlamydia-related diseases in animals and humans are reviewed by Andersen and Vanrompay (1) and more recently by Pospischil (92).

a Etiology

b Classification

The members of the family *Chlamydiaceae*, order *Chlamydiales* are obligate, intracellular gram-negative bacteria. In 1999, Everett et al., (33) proposed a reassignment from the single genus *Chlamydia* into two genera, *Chlamydia* and *Chlamydophila*, based on clustering analyses of the 16S rRNA and 23S rRNA genes (Table 24.1). However, recent comparative genome analyses are consistent with the conclusion that host-divergent strains of chlamydia are biologically and ecologically closely related. The previous taxonomic separation of the genus based on ribosomal sequences is neither consistent with the natural history of the organism revealed by genome comparisons, nor widely used by the chlamydia research community eleven years after its introduction. Consequently, it was proposed to reunite the *Chlamydiaceae* into a single genus, *Chlamydia* (63) (Table 24.1). Accordingly, the chlamydia nomenclature is used in this chapter.

b Morphology and Staining

The three morphologically distinct forms of *Chlamydia* are

termed elementary body (EB), reticulate body (RB), and intermediate body (IB). The EB (Fig. 24.1) is a small, electron-dense, spherical body, about 0.2–0.3 mm in diameter. The EB is the infectious form of the organism, which attaches to the target epithelial cell and gains entry. The EBs have a highly electron-dense nucleoid, located at the periphery of the EB and clearly separated from an electron-dense cytoplasm. Following entry into the host cell, the EB expands in size to form the RB, which is the intracellular metabolically active form. The RB measures approximately 0.5–2.0 mm in diameter (Fig. 24.2). The RB divides by binary fission and thereafter matures into new EBs. During this maturation, morphologically intermediate forms (IB), measuring about 0.3–1.0 mm in diameter, can be observed. The IB has a central electron-dense core with radially arranged individual nucleoid fibers surrounding the core. Cytoplasmic granules are tightly packed at the periphery of the IB and are separated from the core by a translucent zone.

The Gram stain is of no practical value in identifying chlamydiae as the presence of peptidoglycan in the chlamydial cell envelope has yet to be established although the genes encoding the enzymes for peptidoglycan biosynthesis are present (108). Chlamydiae are large enough to be seen with a light microscope using special optics or selective stains. Chlamydiae can be observed in swab smears or touch impressions of infected

tissues by staining them with Castaneda, Giemsa, Giménez (40), modified Giménez, Machiavello, or Stamp methods (2). Chlamydiae appear dark purple with Giemsa; blue with Castaneda; and red with Machiavello, Giménez, modified Giménez and Stamp stains against contrasting backgrounds. The modified Giménez method (136) is most often used.

b Biochemical Properties

The RB is the intracellular, metabolically active form. Although DNA and RNA are both found in the EB and the RB, the ratio of RNA to DNA is greater in the RB. The RBs synthesize their own DNA, RNA, and protein, but some of their metabolic capabilities are limited when compared with free-living, colonizing bacteria. For example, they cannot complete the pentose cycle and do not use pyruvate by way of the tricarboxylic acid cycle. They can, however, catabolize pyruvic, aspartic, and glutamic acids, generating CO_2 and 2- and 4-carbon residues.

b Susceptibility to Chemical and Physical Agents

The survival of microorganisms in aerosols depends on five different factors: relative humidity (RH), temperature, level of oxygen, presence of UV radiation, and constituents of the aerosol and of air (116). The degree to which these factors influence the survival of microorganisms in aerosols depends

strongly on the type of microorganism and the time it has to spend in the aerosol. In general, the following rule applies. Gram-negative bacteria survive the best at low temperatures and RH. Thus, *C. psittaci* remains viable at low temperatures and is resistant to desiccation. The bacterium is highly susceptible to repeated freeze-thawing cycles and is destroyed within 3 minutes when exposed to UV-light.

Chlamydiae are highly susceptible to chemicals that affect their lipid content or the integrity of their cell walls. Even in a milieu of tissue debris they are inactivated rapidly by surface-active compounds, such as quaternary ammonium compounds and lipid solvents (104). Infectivity is destroyed within minutes, by exposure to common disinfectants such as benzalkonium chloride, alcoholic iodine solution, 70% ethanol, 3% hydrogen peroxide, and silver nitrate; but they are resistant to cresol compounds and lime. Dilute suspensions (20%) of infectious tissue homogenates are inactivated by incubation for 5 minutes at 56°C, 48 hours at 37°C, 12 days at 22°C, and 50 days at 4°C (88).

b Antigenic Structure and Toxins

The cysteine-rich major outer membrane protein (MOMP) is well studied. It has a molecular weight of 40 kDa and represents approximately 60% of the weight of the outer membrane. The MOMP

of *C. psittaci* is an immunodominant protein, and there is considerable evidence that antibodies to surface-accessible epitopes of MOMP have a protective role in immunity to chlamydial infection (23). The outer membrane protein A (*ompA*) gene (formerly referred to as *omp1* gene) encodes the MOMP. The *ompA* gene contains 5 conserved- and four variable-sequence regions, VS1 to VS4, which encode for the variable-protein domains VDI to VDIV. VDI, VDII, and VDIV especially protrude from the *C. psittaci* membrane. Epitope mapping has shown the presence of genus- and species-specific antigenic determinants within the conserved regions. However, species-specific antigenic determinants have also been found in the most conserved parts of VDIV. Serovar-specific antigenic determinants are located within VDI and VDII. Monoclonal antibodies to the highly immunoaccessible serovar-specific epitopes on the MOMP can passively neutralize chlamydial pathogenicity and infectivity. Monoclonal antibodies to genus-, species-, or serovar-specific epitopes on the MOMP are excellent tools for specific chlamydial diagnosis.

A chlamydial cysteine-rich heat shock protein 60 (hsp60 or GroEL) has been described that is cross-reactive with other gram-negative bacteria such as *Escherichia coli*, *Neisseria gonorrhoeae*, and *Coxiella burnetii* (144). Thus, the presence of cross-reactive epitopes on the chlamydial outer membrane should

be kept in mind when choosing or interpreting a specific diagnostic test.

Other chlamydia proteins under study are OmcA (EnvA or Omp3), OmcB (EnvB or Omp2), Hc1, RpoB', RpoB, polymorphic outer membrane proteins (pmps), PorB (OmpB), Omp85, elongation factor Tu (EF-Tu/TufA), DnaK (hsp70), and OprB (19, 62, 79, 81, 107, 114).

The chlamydial lipopolysaccharide (LPS) is also an essential constituent of the outer membrane and, like the MOMP, represents one of the major surface-exposed antigens of chlamydiae in both the EB and the RB. It has a molecular weight of 10 kDa and is chemically and serologically related to the LPS of gram-negative *Enterobacteriaceae*. In fact, the chlamydial LPS contains several antigenic determinants cross-reacting with the LPS of enterobacterial Re mutants of *Salmonella* species and *Acinetobacter calcoaceticus* (20, 83). However, the chlamydial LPS contains in its saccharide moiety, a trisaccharide of 3-deoxy-D-manno-2-octulosonic acid (Kdo) of the sequence αKdo(2→8)-αKdo-(2→4)-αKdo. This antigenic epitope is shared only by all members of the genus *Chlamydia* and, thus, represents a *Chlamydiaceae*-specific antigen useful for specific diagnosis (21).

A putative chlamydial cytotoxin has been described (16, 22) in all chlamydia genomes studied so far (103, 106), including

the *C. psittaci* 6BC and Cal10 strains (48). So far, extensive comparisons among *C. psittaci* clinical isolates have not been made, nor has the actual function of the *C. psittaci* putative cytotoxin or its cellular localization been established with certainty.

b Strain Classification

c Antigenicity

All *Chlamydiaceae* are recognized by monoclonal antibodies (MAbs) that detect the LPS αKdo(2→8)-αKdo-(2→4)-αKdo. *Chlamydia* species have a common antigenic epitope in variable segment 4 of the MOMP: NPTI, TLNPTI, LNPTIA, or LNPTI. *C. psittaci* strains are recognized by serovar-specific monoclonal antibodies. The 8 known *C. psittaci* serovars (A to F, M56, and WC) can be distinguished by use of a panel of serovar-specific monoclonal antibodies in a micro-immunofluorescence test (3, 6, 134). However, serotyping is currently rarely performed as the serovar-specific MAbs are not provided by a commercial supplier, and as serotyping appears less discriminatory compared to the newly developed molecular characterization methods (66, 89, 125).

c Genetic, molecular

Chlamydia species are mostly distinguished by analysis of: i) full-length 16S and 23S rDNAs, ii) the 16S-23S intergenic

spacer (*rrn* spacer), iii) signature sequences in the 16S and 23S ribosomal genes (33, 34), iv) the *Chlamydiaceae* RNase P RNA gene (*rnpB*) encoding a ribonucleoprotein complex that removes 5' leader sequences from tRNA precursors during tRNA biosynthesis (54), or of v) the outer membrane protein A (*ompA*) gene encoding the *Chlamydia* major outer membrane protein (MOMP). PCR-RFLP, nested (multiplex) PCR, TaqMan-based real-time PCR, micro arrays, PCR followed by high resolution melt (HRM) curve analysis of the amplified 16S rRNA gene, as well as gene sequencing has been described for identifying *Chlamydia* species in livestock (34, 36, 74, 96, 99).

Genotyping of *C. psittaci* by *AluI* restriction fragment length polymorphism (RFLP) analysis of the *ompA* gene was introduced in 1995, as a more sensitive, more reliable sub-typing method compared to *C. psittaci* serotyping (102). RFLP analysis of the *ompA* gene of all known *C. psittaci* serovars revealed corresponding restriction patterns or genotypes (5, 127). More recently, *ompA* genotyping by real-time PCR using genotype-specific probes has been described, allowing the detection of an additional variant described as the avian *C. psittaci* genotype E/B (36, 37). A few years ago, a genotyping micro array was introduced allowing the identification of all currently known avian and mammalian *C. psittaci* genotypes (98). Genotyping is very convenient, as it is a rapid, powerful

technique that can be used directly on clinical samples in any veterinary clinical laboratory.

Some avian *ompA* genotypes appear to occur more often in a specific order of birds. Genotype A for instance, is endemic among psittacine birds (*Psittacidae*) but is has also been found in turkeys, ducks, pigeons and *Passeriformes*. Genotype B is endemic in pigeons (*Columbiformes*) but can also infect chickens, turkeys, ducks, *Psittacidae* and *Passeriformes* (6). Waterfowl (*Anseriformes*), like ducks and geese most frequently seem to be infected with genotype C. Genotype C has also been detected in chickens and pigeons (29, 145). Genotype D strains are most often associated with turkeys, but genotype D strains can also infect pigeons. More recently, genotype D has also been detected in chickens (28). Genotype E, also known as Cal-10, MP, or MN was first isolated during an outbreak of pneumonia in humans during the early 1930s. Later on, genotype E isolates were obtained from a variety of bird species including, turkeys, pigeons, ducks, ostriches, and rheas. Genotype F is represented by the psittacine isolates VS225, Prk Daruma, 84/2334 (125) and 10433-MA, but has also been isolated on a Belgian turkey farm (123). Genotype E/B is often found in ducks, but it has also been detected in parrots (52), in turkeys (118) and in pigeons (38). The mammalian M56 and WC genotypes were isolated during an outbreak in muskrats and hares and during an outbreak of

enteritis in cattle, respectively. All genotypes should be considered to be readily transmissible to humans.

Occasional detections of *C. abortus* (68, 109) and so far non-classified chlamydial agents (65), as well as genetic evidence on intermediate strains between *C. psittaci* and *C. abortus* (125) suggest that the spectrum of *Chlamydiaceae spp.* encountered in birds is not confined to a single species.

Recently, Pannekoek *et al.*, (98), used multi-locus sequence typing (MLST) for studying the population structure of *C. psittaci* and *C. abortus,* as Van Loock *et al.*, (125) showed that *ompA* sequencing and even sequencing of the *rrn* spacer (33, 34) can not always distinguish *C. psittaci* from *C. abortus*. The obtained MLST scheme was based on the partial sequences of seven housekeeping genes *enoA*, *fumC*, *gatA*, *gidA*, *hemN*, *hflX* and *oppA*, representative for the whole genome sequence. MLST of *C. psittaci* strains resulted in 11 unique sequence types (STs). MLST was extremely useful for distinguishing the phylogenetic highly related species *C. psittaci* and *C. abortus*. Interestingly, according to MLST, the *ompA* genotype F strain 84/2334 appears to be a *C. abortus* strain instead of a *C. psittaci* strain (89).

According to Wang *et al.*, (140), high-resolution genotyping within one chlamydia species can be achieved by multilocus variable-number tandem-repeat (VNTR) analysis (MLVA) in

combination with *ompA* sequencing (MLVA-*ompA*). This method is highly accurate for distinguishing closely related strains within one *Chlamydia* species. VNTR analysis was used for exploring the diversity of *C. psittaci*. For *C. psittaci*, 20 selected genetic loci were initially tested on 9 avian reference strains including representatives of all major *ompA* genotypes (A to F and E/B). Thereafter, 8 loci were retained for a more complete study performed on over 150 *C. psittaci* isolates from different bird species and geographical origins. The MLVA system provides an additional level of discrimination within the *C. psittaci* species, with 20 distinct patterns identified to date (66). MLVA could provide the high-resolution needed for local epidemiology and accurate contact tracing in case zoonosis contracted from poultry. However, current MLVA (and MLST) methods are still easier to perform on culturable samples or clinical samples of *C. psittaci* infected poultry with a high bacterial DNA load.

c Pathogenicity

Chlamydia psittaci strains isolated from birds fall into two general categories: 1) Highly virulent strains that cause acute epidemics in which 5–30% of affected birds die and 2) less virulent strains that cause slowly progressive epidemics. Strains of both high and low virulence appear to have equal

ability to spread rapidly through a flock, as evidenced by serologic test results. Highly virulent strains are isolated most often from turkeys and occasionally from ducks, chickens, pigeons and clinically normal wild birds.

The isolates serotyped from some of the earlier outbreaks with high mortality have been of serotype D (Andersen, 1991b; Vanrompay et al., 1993b). More recently, highly virulent strains have been characterized in European turkeys, ducks and chickens. A highly virulent Genotype D strain was isolated on a French chicken farm (Vanrompay et al., 2012, unpublished results) as well as a highly virulent not yet characterized strain on another French chicken farm (65). Highly virulent strains cause rapidly fatal disease in natural and experimental hosts with lesions characterized by extensive vascular congestion and inflammation of vital organs. Highly virulent strains have a broad spectrum of pathogenicity for laboratory animals and can cause serious human infections (some fatal) in poultry handlers and laboratory research workers. Strains of low virulence cause slowly progressive epidemics with a mortality rate of less than 5% when uncomplicated by secondary bacterial or parasitic infection. Strains of this category are routinely isolated from pigeons and ducks and occasionally from turkeys, sparrows, and other wild birds. The turkey isolates from outbreaks with low mortality have been of genotype B or E. Birds infected with

these strains usually do not develop the severe vascular damage typical in birds infected with the virulent strains, nor do they have the severe clinical signs (115).

c Virulence factors

The initial event in the infectious process begins with attachment of *C. psittaci* EBs to microvilli at the apical surface of a susceptible columnar epithelial cell (58) (Fig. 24.1). The EB travels down the microvillus and locates in indentions of the eukaryotic plasma membrane, some of which resemble coated pits. The bases of micropilli represent areas of active transport of extracellular materials into the cells and, therefore, might assist rapid and efficient entry of EBs. After 1–3 hours, the EBs are internalized in invaginations of the plasma membrane. Uptake of *C. psittaci* is an endocytic mechanism, involving microfilament-dependent and/or independent processes. The *C. psittaci* containing endocytic vesicles or vacuoles escape interaction with lysosomes and proceed in about 8–12 hours to the nuclear area, where EBs are converted to RBs. Conversion to RBs primarily involves reduction of disulfide bond cross-linking among the outer membrane proteins altering the EB cell wall. Synthesis of DNA, RNA, and proteins is initiated, permitting growth and binary fission of the RBs. Binary fission is characterized by the appearance of typical “hour-glass”

profiles inside the vacuole (Fig. 24.2). The enlarging vacuole is also termed an "inclusion." *C. psittaci* microorganisms do not always remain within the inclusion throughout their intracellular development. In some cases and in apparent correlation with high virulence of strains, the inclusion membrane seems to degrade during the active multiplication, liberating the bacteria into the cytoplasm of the host cell (128). About 30 hours after internalization of the EB, the first RBs are reorganized into newly formed EBs. At about 48–50 hours, the developing chlamydial inclusion may contain anywhere from 100–500 progeny, depending on the characteristics of the *C. psittaci* strain (Fig. 24.3). With most *C. psittaci* strains, the host cell has undergone severe degenerative changes, and microorganisms are released by lysis (Fig. 24.4). Exocytosis of the inclusion, followed by a "healing" or closing of the open-cavern structures where the inclusion had existed, has been reported (128). Persistent infections may occur with aberrant non-replicating RBs remaining inside the host cell cytoplasm.

Since long, unique rosette-like structures and projections have been observed on the surface of both EBs and RBs of *C. psittaci* strain Mn (73). Shortly after the first description of a Type III secretion system (T3SS) in *C. caviae* (GPIC strain)(59), Bavoil and Hsia speculated that Matsumoto's projections are in fact functional T3SSs, injecting chlamydial

virulence proteins into the host cell cytoplasm (10). A detailed description of the *C. psittaci* T3SS and the role of the chlamydial T3SS in virulence can be found in recent papers (13, 15, 18, 91).

Outer virulence factors under study are the polymorphic membrane proteins (pmps) (114). The pmps belong to the family of autotransporters. Although specific roles in pathogenesis have been described for some pmps, including adhesion their role in the growth and development of *Chlamydiaceae* is still unclear (78, 113).

Chlamydiaceae can also engage in a long-term relationship with the host cell, a phenomenon known as persistence, in which no visible growth of the chlamydial organisms can be observed. The normal developmental cycle can be interrupted by a number of conditions and agents, such as antibiotics, nutrient deprivation, or immune factors, interferon-gamma (IFN-γ) in particular (41). This is generally accompanied by the development of relatively small inclusions, enlarged pleiotrophic aberrant RBs and inhomogeneity of the inclusions. Aberrant RBs accumulate chromosomes, but genes for cell division are no longer expressed. Once the stress-inducing factor is removed, aberrant RBs revert to normal RBs, complete the developmental cycle and generate infectious EBs.

a Pathobiology and Epidemiology

b Incidence and Distribution

Avian chlamydiosis occurs worldwide, with the incidence and distribution varying greatly with the species of bird and the genotype of the chlamydial organism. The economic losses in poultry and the human infections usually follow a sporadic pattern. Antibiotics are been used extensively to control the spread of the disease in birds and to reduce the risk to humans.

The disease pattern in turkeys has changed. Historically, most outbreaks were explosive and occurred in free-ranging birds (45, 51). These severe respiratory disease outbreaks were attributed to genotype D. Chlamydia was thought introduced from the outside. Nowadays, genotype D and sometimes genotype A is found in confinement turkeys when death losses are high, whereas genotype B is less virulent (6, 97). Introduction through vertical transmission (70, 141), or through infected hatchlings (27) might occur. Recent studies on turkeys in Belgium and France showed that highly and low virulent strains (genotypes A, B, D, E, E/B and F) are widely distributed in commercial turkeys and may be endemic. Chlamydia is part of the turkey respiratory disease complex (123). Infection of turkeys with *C. psittaci* also seems to predispose the animals to amore severe clinical outcome of a simultaneous or subsequent infection with *E. coli*, APV and/or ORT (121-123)

Information on the incidence and epidemiology of chlamydia in domestic ducks is more limited. In the United States, it has not been a significant problem although infections occur. *Chlamydia psittaci* infections in ducks seem to occur more often in Australia, China and Europe (9, 24, 49, 57, 69, 143). In Europe, the number of outbreaks, in ducks seems to be increasing (64). Former European isolates have been characterized as genotype C (134). More recently, outbreaks in French ducks were associated with human disease. Human samples and duck isolates exhibited the same PCR-RFLP restriction pattern, which appeared to be an intermediate between genotypes A and B. Analysis of ompA gene sequences and comparison to those of the type strains showed that the isolates could not be strictly assigned to any of the generally accepted *C. psittaci* genotypes. Further analysis by MLVA of the PCR-positive human samples revealed two distinct patterns, which were related to previously isolated *C. psittaci* duck strains (64).

Reports on *C. psittaci* outbreaks on chicken farms or reports on zoonotic transmissions linked to contact with *C. psittaci*-infected chickens are extremely rare. It could be the case that chickens rarely become infected and/or that the strains infecting chickens are less virulent, presenting a minor risk for humans. Recently, we investigated the occurrence of *C. psittaci* by performing a retrospective study with 300 serum

samples collected in 2005 from 10 randomly selected chicken breeder, broiler, and layer farms in Belgium. Sera were examined by a recombinant MOMP-based enzyme-linked immunosorbent assay (ELISA)(139). We obtained 98, 95, and 95% seropositive results for layers, broilers, and breeders, respectively (28). Seropositive birds were found on all farms. Therefore, the statement that *C. psittaci* infections occur less frequently in chickens is not true. Recently, highly virulent genotype D strains (Vanrompay *et al*., 2012, unpublished results) as well as less virulent genotypes B, C and E/B strain have been found in chickens in Belgium, China, France and Germany (35, 65, 146; Vanrompay *et al*., 2012, unpublished results).

In 2008, three cases of atypical pneumonia in individuals working at a French poultry slaughterhouse processing guinea fowl, ducks and especially chickens prompted an epidemiological survey in 10 poultry farms that had supplied birds. Using a *Chlamydiaceae*-specific real-time PCR assay, chlamydial agents were detected in 14 of 25 investigated flocks. The one duck flock being investigated was positive (20% of the tested animals positive). Additionally, 12 of 18 (67%) and 1 of 6 (17%) of the examined chicken and guinea fowl flocks were PCR positive, respectively. Positivity for the chicken flocks ranged from 10 to 100%. For the positive guinea flow flock, 12.5% of the tested animals were positive. Rather unexpectedly, *Chlamydia psittaci*

was identified only in the positive duck flock, whereas ArrayTube DNA microarray testing indicated the presence of a new chlamydia agent in all other examined French poultry flocks. Further examination is needed to define the exact taxonomic status of this agent found in chickens and guinea fowl (65).

Strains occurring naturally in birds are distinct from those in mammals (5, 6, 86). Attempts to infect birds with mammalian strains usually resulted in aborted or asymptomatic infections (60, 115).

b Natural and Experimental Hosts

Chlamydiae or chlamydial antibodies have been found in at least 465 bird species (61). Common reservoirs of chlamydiae include wild and feral birds such as sea gulls, ducks, Canada geese, herons, egrets, pigeons, blackbirds, grackles, house sparrows, and killdeer, all of which freely intermingle with domestic birds. Highly virulent strains of *C. psittaci* can be carried by and excreted in large numbers without any apparent effect on these hosts.

Experimental hosts of avian chlamydiae can include virtually any species of bird. Mammalian laboratory hosts used for avian chlamydiae are principally mice and occasionally guinea pigs. Mice and guinea pigs are the naturally hosts for *C. muridarum* and *C. caviae*, respectively. Investigators using these animals

should determine the chlamydial status of the breeding stock. Rabbits are refractory to clinical disease caused by avian chlamydiae, but they may be used to produce polyclonal antibodies.

Younger domestic birds generally are more susceptible than older birds to infection, clinical disease, and mortality. However, maternal antibodies might protect against respiratory disease outbreaks on the farm. Infection in old turkeys, such as spent breeder hens, can go unnoticed unless birds are subjected to stressful conditions such as shipment to market on crowded trucks. Turkey toms may have a higher mortality rate than turkey hens (117).

b Transmission, Carriers, and Vectors

Transmission of *C. psittaci* primarily occurs from one infected bird to another susceptible bird in close proximity. The agent is excreted in faeces and nasal discharges. Faecal shedding occurs intermittently and can be activated through stress caused by nutritional deficiencies, prolonged transport, overcrowding, chilling, breeding, egg laying, treatment or handling. Bacterial excretion periods during natural infection can vary depending on virulence of the strain, infection dose and host immune status. However, shedding may occur for several months. Transmission of chlamydiae occurs mainly through

inhalation of contaminated material and, sometimes, ingestion. Large numbers of C. psittaci cells can be found in respiratory tract exudate and faecal material of infected birds. The importance of the respiratory exudate has become more apparent. In turkeys, the lateral nasal glands become infected early and remain infected for more than 60 days. Choanal/oropharyngeal swabs are more consistent for isolation of the agent than faecal swabs, especially during early stages of infection. Direct aerosol transmission through aerosolization of respiratory exudate must be considered as the primary method of transmission

Avian species, including domestic poultry sharing aquatic or moist soil habitats with wild infected aquatic birds may become infected via contaminated water. Granivorous birds like pigeons, doves, pheasants and house sparrows may become infected by dust inhalation in faeces contaminated barnyards and grain storage sites. The consumption of infected carcasses may transmit C. psittaci to host species that are predators or scavengers of other birds.

Transmission of *C. psittaci* in the nest is possible. In many species, such as *Columbiformes*, cormorants, egrets, and herons, transmission from parent to young may occur through feeding, by regurgitation, while contamination of the nesting site with infective exudates or faeces may be important in other species, such as snow geese, gulls and shorebirds. Furthermore, C.

psittaci can be transmitted from bird to bird by blood-sucking ectoparasites such as lice, mites and flies or, less commonly, through bites or wounds. Transmission of *C. psittaci* by arthropod vectors would be facilitated in the nest environment. Mites from turkey nests can contain chlamydiae (30), and during an epidemic in turkeys in South Carolina, simulid flies were suspected as a possible method of transfer (85).

Vertical transmission has been demonstrated in turkeys, chickens, ducks, parakeets, seagulls and snow geese, although the frequency appears to be fairly low (70, 141). However, it could serve as a route to introduce chlamydiae into a poultry flock.

Chlamydia psittaci can be introduced into poultry through the wild bird population. Contaminated feed or equipment can also be a source of infection, and feed should therefore be protected from wild birds. Careful cleaning of equipment being used in several barns during one and the same production round is extremely important as *C. psittaci* can survive in faeces and bedding for up to thirty days (52).

b Incubation Period, Clinical Signs, Morbidity and Mortality, Pathology and Pathogenesis

Turkeys

In 1959, Page, (87) was the first to examine the pathogenesis

of a *C. psittaci* infection in commercial turkeys after inoculation by aerosol or by the oral route with a genotype D strain. In 1995, Vanrompay *et al.*, (129) studied the pathogenesis of *C. psittaci* genotype A, B, and D strains in specific-pathogen-free turkeys kept in negative pressure isolation units by immunodetection, which allowed the precise determination of the tissue and cell tropism. In this study, turkeys were aerosol infected as this most likely represents the natural route of infection as demonstrated by Page (87). From these studies, the following pathogenic sequence of events can be deduced for all three genotypes investigated. In turkeys infected by aerosol, the primary site of replication is the upper respiratory tract, where epithelial cells become infected. Subsequently, epithelial cells of the lower respiratory tract and macrophages throughout the respiratory tract become infected. Then, intense replication occurs in the respiratory tract. At the same time, chlamydiae can be demonstrated in plasma and monocytes, indicating septicemia, and chlamydiae appear in epithelial cells and macrophages of various tissues throughout the body.

In turkeys, an experimental infection with a genotype B strain induced much milder clinical signs and lesions than infection with genotype A or D strains (132). Genotype B strain had a longer incubation period, took longer to reach maximum

titers in the tissues, and had shorter periods during which the organism was found in the tissues.

The incubation period of chlamydiosis in naturally infected birds varies, depending upon the number of chlamydiae inhaled, the virulence or pathogenicity of the infecting strain for that host species and host immunogenetics. Experimentally, definitive disease signs in young turkeys receiving a virulent strain may be evident in 5–10 days. In birds naturally exposed to smaller doses or in older birds, the period may be longer. Strains of lower virulence, which cause less severe signs, may have longer incubation periods. Clinical signs may not be noticeable until 2–8 weeks after exposure.

Signs of chlamydiosis in turkeys infected with virulent strains are cachexia, anorexia, elevated body temperature, conjunctivitis, and respiratory distress. The birds excrete yellow-green, gelatinous droppings. Egg production of severely affected hens declines rapidly to 10–20% and may temporarily cease or remain at a very low rate until complete recovery. Disease signs in a flock infected with strains of low virulence are usually anorexia and loose, green droppings in some birds, with less effect on egg production.

At the peak of disease in a flock infected with a virulent strain, 50–80% of the birds will show clinical signs, and morbidity from less virulent strains is only 5–20%. Mortality

caused by virulent chlamydia ranges from 10–30% and is only 1–4% with less virulent strains.

The less virulent strains cause gross lesions, which are similar to those caused by virulent strains, only less severe and extensive. In overwhelming infections with virulent strains, lungs show diffuse congestion, and the pleural cavity may contain fibrinous exudate. In fatal cases, a dark transudate may fill the thoracic cavity. The pericardial membrane is thickened, congested, and coated with fibrinous exudate. The heart may be enlarged, and its surface may be covered with thick fibrin plaques or encrusted with yellowish, flaky exudate (Fig. 24.5). The liver is enlarged and discolored and may be coated with thick fibrin. Air sacs are thickened and heavily coated with fibrinous exudate (Fig. 24.6). The spleen is enlarged, dark, and soft and may be covered with gray-white spots representing areas of focal cellular proliferation. The peritoneal serosa and mesentery show vascular congestion and may be coated with foamy, white fibrinous exudate. All of these exudates contain large numbers of mononuclear cells in which numerous micro colonies of chlamydial RBs may be seen. Fibrinous exudates, found on all organs and tissues of the thoracic and peritoneal cavities, reflect vascular damage as well as increasing inflammatory response caused by the continued multiplication of the organisms. In birds that survive infection with a strain of low

virulence, the lungs may not be seriously affected. However, multiplication of organisms on the epicardium may result in the formation of one or more fibrin plaques.

Vanrompay *et al.* (130), examined histopathologic changes in four groups of 20 specific-pathogen-free (SPF) turkeys, kept in isolation units and inoculated by the natural route of infection (aerosol). Turkeys were experimentally infected with strain 84/55 (*C. psittaci* genotype A), isolated from a parakeet, strain 92.1293 from a turkey (*C. psittaci* genotype D), the Texas Turkey strain (*C. psittaci* genotype D), or strain 89/1326 (*C. psittaci* genotype B) from a pigeon. All four strains proved to be pathogenic for SPF turkeys. Turkeys showed conjunctivitis, sinusitis, rhinitis, keratitis, pericarditis (Fig. 24.5), pneumonia, airsacculitis (Fig. 24.6), hepatosplenomegaly, enteritis, congestion of the kidneys, and congestion of the ovaries or testes. There were epithelial erosions and fibrin deposit in the conjunctivae (Fig 24.7), corneal ulceration, bronchopneumonia (Fig. 24.8), fibrinous necrotizing airsacculitis (Fig. 24.9), fibrinous pericarditis, interstitial nephritis, peritonitis, and catarrhal enteritis. The type and distribution of the lesions was similar for genotypes A and D. The lesions produced after the genotype A infection, however, appeared more severe. For genotype B, in comparison with both other genotypes, no lesions were observed in the small

intestine, the pancreas, ovary, and testis.

Controlled dual infections in SPF turkeys demonstrated the pathogenic interplay between *C. psittaci*, the avian metapneumovirus (aMPV: formerly named the turkey rhinotracheitis virus or TRT virus), and *E. coli*. *Escherichia coli* is a predisposing factor for the outcome of a *C. psittaci* infection. In its turn, *E. coli* can increase the severity of a *C. psittaci* infection and can reactivate a latent *C. psittaci* infection (121). An aMPV infection during the acute phase of a *C. psittaci* infection aggravates the severity of clinical signs, macroscopic lesions, pharyngeal aMPV excretion, and histological tracheal lesions. However, no clear interaction was established after aMPV infection in latently *C. psittaci*-infected turkeys (122).

Chickens

Epidemiologic evidence formerly indicated that chickens are relatively resistant to disease caused by *C. psittaci*. Acute infection progressing to disease and mortality only occurred in young birds, and the incidence of actual epidemics was very low. Most natural infections in chickens were believed to be inapparent and transient. However, *C. psittaci* strains isolated from turkeys did cause similar pathology and mortality in chickens as in turkeys (111, 112).

Recently, highly virulent *C. psittaci* strains have been

isolated from the lungs of diseased chickens raised in Belgium, France, Germany and China (35, 65, 146; Vanrompay *et al*., 2012, unpublished results). The strains obtained from Belgian and French farms were genotype D and could successfully be used to reproduce the disease in experimentally infected SPF chickens (Vanrompay et al., 2012, unpublished results). Chickens showed conjunctivitis, rhinitis, pneumonia, fibrinous airsacculitis, fibrinous pericarditis and hepatosplenomegaly. Mortality was observed.

Ducks and Geese

Chlamydiosis in domestic ducks is important both economically and as a public health hazard in especially, Australia, China and Europe (8, 9, 17, 24, 49, 50, 57, 64, 67, 69, 72, 82, 142). Chlamydiosis in ducks is usually a severe, debilitating, often fatal disease in which young ducks develop trembling, imbalanced gait, and cachexia. They become anorexic with green, watery intestinal contents. They develop a serous to purulent discharge from the eyes and nostrils causing the feathers on the head to become encrusted with exudate. As the disease progresses, the ducks become emaciated and die in convulsions. Morbidity ranges from 10–80%, and mortality varies from 0–30% depending on age and the presence of concurrent infections. Recently, severe outbreaks associated with human disease were reported in France

(65).

Incidental to studies of chlamydiosis in ducks, several investigators have observed *C. psittaci* antibodies or the disease in geese and have isolated *C. psittaci* from diseased tissues (1). Clinical disease and necropsy findings were similar to those in ducks.

Pigeons

The incubation period for chlamydiosis in pigeons is not known. Infection is endemic and is believed perpetuated primarily by a parent-to-nestling transmission cycle (39, 101).

Signs of uncomplicated chlamydiosis in pigeons are variable, but those that develop acute disease are anorexic, unthrifty, and diarrhetic (71). Some develop conjunctivitis, swollen eyelids, and rhinitis. Respiratory difficulty is accompanied by rattling sounds. As disease progresses, birds become weak and emaciated. Recovered birds become carriers without signs of disease. Some birds progress through an infection showing no signs or, at the most, transient diarrhea before becoming carriers. Salmonellosis or trichomoniasis exacerbates the illness in chlamydia-infected carrier birds, inducing signs and lesions of acute disease. Serologic surveys indicate that infection rates in pigeons of 30–90%, and active infection rates, as measured by isolation, of 20% are common (90, 101).

Gross lesions of uncomplicated chlamydiosis in pigeons are fibrinous exudates on thickened air sacs, the peritoneal serosa, and occasionally the epicardium. The liver is usually swollen, soft, and discolored. The spleen may be enlarged, soft, and dark. Greater than normal amounts of urates are seen in cloacal contents if catarrhal enteritis occurs. In less severe infections, only the liver or air sacs are involved. Some heavily infected shedders have no lesions (87, 90).

Pheasants, Quail, Guinea fowl, Peacocks and Partridges

Chlamydiosis has been reported in farm-raised pheasants, quail, peacocks, guinea fowl and partridges all over the world (32). The clinical signs and lesions are similar to those seen in other birds. Morbidity and mortality can be very high, especially in young birds. Human infections have been reported (31, 45, 65, 75, 110, 142).

b Immunity

Natural immunity to chlamydia is generally poor and short-lived. As birds become older, however, they become more resistant to clinical disease, even though infection may occur. Indeed, some birds, notably pigeons, are refractory to disease-producing infection even with highly virulent strains.

The immune response to *Chlamydia* is a coordinated event where innate immune cells, B cells and T cells act in concert and

where each of these immune effectors have roles in recognizing different stages of the infection. To date, chlamydial vaccine studies suggest that the ideal, efficacious vaccine should induce systemic CD4+ T helper (Th) 1 responses, but also a humoral response as B cells are important antigen presenting cells in the activation of antigen-specific memory Th2 cells (95).

Less is known on innate immune detection of *Chlamydia* (93). Beeckman *et al.*, (12) determined the cytokine responses following *C. psittaci* infection of avian monocytes/macrophages. High IL-10 and no TGF-β4 responses were observed at 4h post inoculation. This could induce macrophage deactivation and NF-κB suppression and thereby, could dampen innate immunity and promote *C. psittaci* survival in macrophages.

a Diagnosis

b Specimen Collection and storage of samples

Laboratory diagnosis of avian chlamydiosis usually includes isolation and/or identification of the organism from the host. Isolation requires inoculation of cell cultures, embryonated SPF chicken eggs or mice with living *C. psittaci*. Specimens should be collected aseptically, as contaminant bacteria may interfere with isolation of the chlamydiae.

Proper handling of clinical samples is necessary to prevent loss of infectivity. If specimens are used to inoculate animals or cell cultures immediately, most diluents will be adequate; however, if the specimen is to be shipped or stored, a special diluent should be used. A diluent consisting of sucrose–phosphate–glutamate (SPG) was developed for *Rickettsiae* and has proven satisfactory for transport of chlamydial field samples (105). The recommended medium for chlamydiae consists of SPG buffer (sucrose, 74.6 g/liter; KH_2PO_4, 0.512 g/liter; K_2HPO_4, 1.237 g/liter; and L-glutamic acid, 0.721 g/liter), which can be sterilized by autoclaving. To this is added fetal calf serum (10%), vancomycin and streptomycin (100 mg/ml), and nystatin and gentamicin (50 mg/ml). The antibiotics reduce the effect of contamination even when samples are shipped at ambient temperatures. This medium also serves as a laboratory diluent and a medium for freezing of chlamydiae.

For isolation, the following samples should be preferably collected: pharyngeal/choanal slit swabs in live birds (4, 129). Cloacal swabs or fresh faeces are less optimal as chlamydial shedding is intermittent. In dead birds, lungs, spleen, liver, thickened exudate-coated air sacs and free exudates can be sampled. The samples should not be frozen if they can be processed in 2–3 days. Otherwise they should be stored at -80°C.

The same tissues can be collected for antigen detection,

PCR and other molecular tests. If the DNA extraction will not be done immediately, it may be beneficial to collect the specimen in a DNA stabilization buffer (25).

The stability of chlamydiae during storage depends upon the material in which it is contained. Chlamydiae in tissue specimens or yolk-sac suspension can be preserved indefinitely by storage at -80° C (1). Note that chlamydiae harvested from cell culture require special media during freezing. A satisfactory method for freezing them is to replace the cell culture medium with SPG buffer before freezing, as the organism is highly susceptible to the presence of sodium ions in the medium (105).

b Microscopic examination of specimens

c Cytological and Immunohistochemical staining

Chlamydiae can be detected in smears of exudates and faeces and in impression smears of tissues by cytological stainings as mentioned formerly. However, none of the stainings specifically detects chlamydia and they are all less sensitive than antibody-based antigen detection methods or specific nucleic acid amplification techniques (NAATs) (7, 136). Therefore, the use of a cytological staining is loosing popularity.

Nowadays, imunohistochemistry (115) is not very often used for routine diagnosis. It is more in use for research or

retrospective examination of samples for chlamydiae, if necessary.

b Antigen Enzyme-linked immunosorbent assays

A number of manufacturers produced enzyme-linked immunosorbent assay (ELISA) kits for detection of *Chlamydia trachomatis* in humans. These test kits detect *Cp. psittaci* as they react to the lipopolysaccharide (LPS) or family (*Chlamydiales*) antigen. ELISA is rapid, does not require a high level of expertise, and is safe for the technician because the sample can be inactivated. A number of the test kits have been evaluated for use in avian samples (133), although none of the kits are licensed for *C. psittaci*. These kits detect the lipopolysaccharide antigen which gave false positives as the chlamydial LPS shares epitopes with the LPS of other gram-negative bacteria. The more recently developed ELISA's use more specific monoclonal antibodies. However, these kits still lack sensitivity: a few hundred organisms are needed for a positive reaction. The general rule with these tests is that a diagnosis of chlamydiosis can be made when a strong positive reaction is seen along with clinical signs of chlamydiosis.

b Isolation

c Preparation of inoculum

Tissue specimens, fecal samples, and swabs are routinely used as samples for the isolation of *Chlamydia*. Before the inoculation samples must be treated properly. The processing of the samples is similar for inoculation of cell cultures or embryonated eggs. A number of laboratories are using SPG buffer, or transport medium (105) as diluent. These diluents have the advantage of stabilizing the agent during refrigeration or freezing of the samples. Penicillin and tetracyclines should be avoided, as they will inhibit the growth of *C. psittaci*.

A standard procedure is to prepare 20% tissue suspensions in diluent. The suspensions are centrifuged (2790 x g, 4°C) for 10 min. The supernatants are collected and 10 µl/ml streptomycin sulphate (streptomycin sulphate 1% w/v) and 20 µl/ml vancomycin (vancomycin 0.5 w/v) are added. If needed 0.1% amphotericin B can be added. After one hour of incubation at room temperature the suspensions are centrifugated for 30 min (2790 x g, 4°C) and supernatants are immediately used for inoculation or stored at -80°C until use. Swabs are shaken for 1 hour at 4°C a rocking platform, centrifuged (2790 x g, 4°C) and the supernatant immediately used or stored at -80°C until use. Faecal samples, although not preferably used for diagnosis because of the intermittent chlamydia shedding and the risk for false negatives, are processed as follows. A 20% suspension is made in diluent and the suspension is shaken for 1 hour (4°C) on a

rocking platform. Afterwards, the suspension is sonicated for 10 min using an ultrasonic water bath. The supernatant is collected and 10 µl/ml streptomycin sulphate (streptomycin sulphate 1% w/v), 20 µl/ml vancomycin (vancomycin 0.5 w/v) and 0.1% amphotericin B are added. The suspension is incubated at room temperature (1 hour) and subsequently centrifuged (2790 x g, 4°C) for 30 min. The supernatant is collected and thereafter ultracentrifugated (45,000 x g, 4°C, 45 min). The supernatant is discarded and the remaining chlamydia pellet is resuspended in dileunt and immediately used for inoculation or stored at -80°C until use.

c Cell cultures and SPF embryonated chicken eggs

Cell cultures are the most common and convenient method for the isolation of *C. psittaci.* The most commonly used cell lines are BGM, Vero, McCoy, HeLa, and L-929, although a number of other cell cultures, like chickens embryo fibroblasts can be used. A study showed BGM to be the most sensitive, with Vero and L-929 listed as satisfactory (136). Standard cell culture medium is used, containing 5%–10% fetal calf serum and antibiotics like vancomycin, streptomycin and amphotericin B as they do not inhibit the growth of *C. psittaci*.

The culture equipment must be suitable for the following procedures: i) identification by fluorescent antibody (FA) or

another appropriate technique of staining of the infected monolayer, ii) centrifugation (500-1500 x g for 60 to 90 min)of the inoculum onto the monolayer at 37°C to enhance infectivity (eventually in the presence of DEAE), iii) possible blind passages at 3 or 6 days post inoculation to increase sensitivity of isolation, iv) examination of the sample 2 to 3 times during passage, v) protection of humans against possible infection. Small flat-bottomed vials (1-dram shell vials) or bottles, with 12-mm diameter glass coverslips, will meet these requirements and are often used because the cell culture monolayer can be grown directly on the coverslip. Several vials are inoculated with each sample to permit fixing and staining at various times and to permit passages of negative samples after 6 days of incubation.

Chlamydiae can be isolated from cells that are replicating normally. Most diagnosticians, however, prefer to use non-replicating host cells for two reasons: i) to provide increased nutrients for the replication of chlamydiae, and ii) because non-replicating cells can be maintained for longer periods for observation. Host-cell replication is suppressed most commonly using cycloheximide (0.5-2.0 mg/ml). Incubation is usually at 37–39 °C, depending on the cell culture used. Disruption of the monolayer by freeze-thawing should be avoided, as it can destroy *C. psittaci*.

Some laboratories still use chicken embryos for primary isolation of chlamydiae. The standard procedure is to inject up to 0.3 ml of inoculum into the yolk sacs of 6-day-old embryos (136). Replication of chlamydia usually will cause the death of the embryo within 5–12 days after inoculation. If no deaths occur, two additional blind passages are usually made before calling the sample negative. Chlamydia infection typically causes vascular congestion of the yolk-sac membranes, which are harvested and homogenized as a 20% yolk-sac suspension. This suspension can be frozen (-80˚C) to preserve the strain or inoculated into other eggs if needed or into cell culture monolayers. *C. psittaci* is usually identified by immunofluorescence staining of yolk-sac impression smears.

c Staining cell monolayers or yolk-sac impression smears

The preferred method for fixing of the monolayer is to remove the medium, wash once with PBS, and fix with cold acetone for 10 min (-20˚C). If the cell culture vessel is plastic, the monolayer can be fixed with a mixture of 50% acetone and 50% methyl alcohol or with 100% methyl alcohol. The preferred method for staining is the fluorescence method. With the fluorescence method, the fluorescein-conjugated anti-chlamydia serum is applied to the glass slide and incubated for minimum 30 min at 37˚ C. The slides are then washed with PBS and aqua dest, air-

dried, and mounted to be monitored by use of a fluorescence microscope. Commercially fluorescently labeled monoclonal antibodies are available. Conjugates also can be prepared from polyclonal sera from rabbits, guinea pigs, sheep, or goats. The fluorescein-labeled conjugate is than prepared using standard conjugation techniques.

b nucleic acid amplification techniques

The possibilities of diagnostic detection of *Chlamydia psittaci* are considerably improved with the introduction of molecular methods, which permit direct identification from clinical specimens and accelerates diagnosis from several days to a single working day. Tests published in the literature mainly utilize two different genomic target regions for amplification, namely the ribosomal RNA gene region (34, 53, 55, 56, 74).

The sensitivity of the PCR largely depends on the amount and quality of the extracted DNA. Optimal extraction of nucleic acids from a wide range of clinical samples is one of the most under appreciated, but nevertheless challenging and important steps. Proper extraction must efficiently release nucleic acids from samples, remove PCR inhibitors, avert the degradation of nucleic acids, and ensure adequate concentration of the nucleic acids after extraction. This is relatively easy achievable for

viral and mammalian amplification targets, but much more difficult for nucleic acid targets of bacteria. Several in-house-made and commercial DNA extraction methods have been described (25, 100). The use of commercial kits can be recommended especially when dealing with PCR inhibitors. They contain a special buffer reagent for lyses of the bacterial and eukaryotic host cells mostly based on a guanidine- detergent lysing solution. Simultaneous disruption of cells and inactivation of nucleases is achieved by lysis in strong denaturing reagents, for instance 4M guanidinium isothiocyanate or 4M guanidinium-hydrochloride.

Tissue sample homogenization in cell culture medium, PBS, or Tris buffered saline and/or freeze-thaw cycles of samples lead to rapid loss of chlamydial target DNA. Specimens that contain low target numbers before freezing often become negative after cryostorage. The preservation of DNA from degradation is critically important. DeGraves *et al.*, (25) advise to collect all specimens for PCR analysis in a DNA stabilization reagent. Such reagents are commercially available; for instance the RNA/DNA Stabilization Reagent for Blood/Bone Marrow® from Roche Applied Science.

Nested PCR procedures are more sensitive than traditional PCR and in most cases are also more sensitive than real-time PCR. However, they are more time-consuming. False positives can more

easily occur due to post-PCR carry over. Two highly sensitive nested PCR assays have been developed to detect *C. psittaci* (100, 124). Recently, a SYBR Green-based real-time PCR was developed targeting the rDNA ribosomal spacer of *C. psittaci* (37).

Sachse *et al.*, (99) developed a DNA microarray-based detection and identification method for *Chlamydia spp*. The test uses the ArrayTube platform technology (CLONDIAG® chip technologies). The company has also developed an *ompA*-based *C. psittaci* genotyping microarray (31).

b Serology

Serology alone is not particularly useful in diagnosing a current chlamydial infection in birds because of the high prevalence of this infection in birds and the long-term, up to several months, persistence of anti-chlamydial antibodies. In most bird species, there is a high background rate of anti-chlamydial antibodies, and until we have more information on the disease pattern in certain bird species in relation to direct identification of the bacteria and serology, we are unable to comment on the real significance of antibody titers obtained. Thus, to determine if a single bird is infected, serology should always be used in conjunction with antigen or gene detection or paired sera should be examined. However, obligatory examination

of paired sera removes serology from immediate clinical relevance. A positive test is evidence that the bird was infected by the bacterium but does not necessarily indicate an active infection. False negative results can occur in birds with acute infections that are sampled before sero-conversion. Treatment with antibiotics may also delay and/or diminish the antibody response. The main serological methods that are being used for detecting chlamydial antibodies are: 1) various methods of elementary body agglutination (EBA) (47), 2) the complement fixation test (CF) (47), 3) an indirect (micro) immunofluorescence (MIF) test and 4) several commercial ELISAs.

The EBA detects primarily IgM antibodies and thus can detect early infections (43-45). A negative result does not guarantee that a bird is free of infection as the sensitivity of the test is rather low. The direct complement fixation (DCF) test detects avian IgG but not IgM. Disadvantages are: 1) test antigens commercially unavailable, 2) the test can not be used for testing sera from avian species whose immunoglobulins do not fix complement, 3) it is only relatively sensitive, 4) it cannot be used to differentiate between IgG and IgM antibodies, and 5) fairly laborious when there is a large number of samples to be tested. The modified DCF test is more sensitive but has the same disadvantages as the DCF test (45, 46). The indirect IF test detects all immunoglobulin isotypes and is, as is the MIF test,

widely used to detect *C. trachomatis*, *C. pneumoniae* and *C. psittaci* antibodies in human sera. The MIF test appears to be more sensitive than the complement fixation tests. Some years ago a large number of commercially available ELISAs were evaluated for demonstrating *C. psittaci* antibodies in birds. All of these ELISAs were highly sensitive but showed low specificity, as they were mainly based on the use of whole chlamydial organisms, LPS, or chlamydial outer membrane fractions of LPS and lipoglycoprotein nature (1). More recently, peptide-based ELISA systems, or ELISAs using recombinant LPS, have become commercially available for the specific detection of *C. trachomatis*, *C.pneumoniae* and *C.abortus* antibodies (Medac, Savyon, Labsystems), (1). These tests performed as well as the MIF assay, but are less time-consuming, less expensive, and easier to perform. In the future this principle might also be useful in the serodiagnosis of *C. psittaci* infections. At present, an ELISA using recombinant MOMP of *C. psittaci* has already been described (139) for testing avian sera. The test detects antibodies against all *Chlamydia* species.

b Differential Diagnosis

Suspected chlamydiosis may have to be differentiated from pasteurellosis, particularly in turkeys, in which some signs and lesions may be similar. Pasteurellosis can be ruled out by

appropriate culture procedures. Because of some similar signs and lesions, *Ornithobacterium rhinotracheale* infections and mycoplasmosis may need to be ruled out in turkeys and chickens suspected of having chlamydiosis. That can be accomplished by culturing and serologic testing. Colibacillosis may mimic chlamydiosis to some extent; it can be excluded by the use of the appropriate coliform culturing procedures. Avian influenza or aMPV may have to be ruled out in suspected chlamydiosis by virus isolation attempts and by serologic testing.

a Intervention Strategies

b Management Procedures

Ideally birds should be reared in confinement without any contact with potentially contaminated equipment or premises. Contact with potential reservoirs or vectors such as pet birds, rodents, arthropods, and wild and feral birds should also be prevented. General sanitation must be practiced diligently. Movement of people should be restricted so that visitors do not have free access to premises holding birds. This is easier to accomplish if birds are confined in houses and if the 'all-in-all-out' principle is used on the farm.

Recently, *C. psittaci* bioaerosol monitoring came available and this could be used for monitoring the infection pressure in the poultry industry (117, 119).

b Vaccination

Commercial chlamydia vaccines for poultry are not available.

Protective immunity to *Chlamydiaceae* is believed to be effected primarily through the action of CD4+ T helper type 1 (Th1) lymphocytes, CD8+ T lymphocytes, mononuclear phagocytes, and cytokines secreted by these cells. In addition, the role of local antibodies in mucosal secretions is not to be underestimated. The only protective chlamydial antigen that has been unambiguously identified is the major outer membrane protein (MOMP). However, attempts at chlamydial vaccine development based on a subunit design using MOMP have generally failed, probably because the immunogens did not induce the protective cellular and humoral immune responses elicited by native bacterial epitopes. In light of the current knowledge on protective chlamydial immunity, plasmid DNA expressing the MOMP of *C. psittaci* has been tested for its ability to raise a protective immune response in SPF turkeys against challenge with *C. psittaci* strains (126, 137). Effective priming of T cell memory and significant reduction in clinical signs, lesions, bacterial excretion and *C. psittaci* replication in tissues was observed.

b Prevention by means of ovotransferrin

Recently, ovotransferrin (natural anti-microbial protein) was

successfully used to reduce clinical signs, lesions, excretion, and chlamydia replication in experimentally infected SPF turkeys (120). Next, *C. psittaci* infection pressure on a turkey broiler farm was significantly reduced by administering ovotransferrin aorosols. Ovotransferrin significantly reduced respiratory disease, mortality and antibiotic use on the farm (117).

b Treatment

Chlamydiosis treatment for poultry is not changed over the years (1, 104, 131). The drug of choice varies from country to country. Tetracylines are the drugs of choise; chlortetracycline and doxycycline are most often used. Enrofloxacine (fluoroquinolone antibiotic) can be used. Turkeys can be treated with chlortetracycline (CTC) at a concentration of 400 g/ton of pelleted feed. Care must be taken so that heat produced during the pelleting process does not destroy CTC and lower the concentration below an effective level. The CTC-medicated feed must be given for 2 weeks and then replaced by non-medicated feed for 2 days prior to the birds being slaughtered for meat for human consumption. Calcium supplement should not be added to CTC-medicated pellets because calcium ions chelate CTC and diminish its effectiveness. Turkeys can also be treated with doxycycline hyclate formulations for drinking water (doxycycline hyclate 500mg/g; dose of 20 mg/kg BW per day) for 5 days or

enrofloxacin (100 mg/ml) at a daily dose of 10 mg of enrofloxacin per kg of body weight, in water for 3 to 5 days and in case of mixed or chronic infections for 5 to 10 days. Medicated drinking water should be replaced every 24 hours. It is recommended that all turkeys on an infected premise be treated.

Essentially the same treatment methods are used to treat other fowl infected with *C. psittaci*. In other birds, salmonellosis may often be a complicating factor so it may be necessary to use a combination of antibiotics. Treatment may not be effective in eliminating the carrier state. Additional periods of treatment may be needed, especially when birds are kept for several weeks.

b State Regulations

Because regulations may vary from state to state, the appropriate public health and/or animal health agencies should be consulted as necessary. In many countries, psittacosis (humans) and even chlamydiosis in poultry are notifiable diseases and must be reported within 48 h.

According to USDA regulations, movement of poultry, carcasses, or offal from any premise is prohibited where the existence of chlamydiosis has been proved by isolation of a chlamydial agent. The Animal and Plant Health Inspection Service

of the USDA and the U.S. Department of Health and Human Services forbid interstate movement of birds from infected flocks, but there is no restriction on movement of eggs from such flocks.

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Figures

24.1. Buffalo green monkey (BGM) cell culture, 1 hour after inoculation with the *C. psittaci* Texas Turkey genotype D strain showing an elementary body (EB) attached to the side of a host

cell microvillus.

24.2. Buffalo green monkey (BGM) cell culture, 18 hours after inoculation with a *C. psittaci* genotype B strain (89/1326). Note the vacuole near the nucleus (N) with an early and late stage of division of a reticulate body (R). Note the "hour-glass" profile (arrow H).

24.3. BGM cell culture, 52 hours after inoculation with a C. psittaci genotype D strain (92/1293), isolated from diseased turkeys. Note the large inclusion and the elementary bodies (EB) apparently "escaping" from the inclusion. Also notice the mitochondria (M) lining the inclusion.

24.4. BGM microcarrier culture, 50 hours post inoculation with the *C. psittaci* Texas Turkey genotype D strain showing lysis of an infected BGM cell. The cell is sloughing of the microcarrier (MC). Note the presence of elementary bodies (E), reticulate bodies (R) and intermediate bodies (I).

24.5. Turkey experimentally infected (aerosol) with a C. psittaci genotype A strain (84/55) isolated from the lungs of a budgerigar. Note the presence of serous fluid together with fibrin in the pericardial sac.

24.6. Turkey experimentally infected (aerosol) with a C. psittaci genotype A strain (84/55). Note the thickened abdominal airsac totally covered with fibrin cloths (arrow).

24.7. Hematoxylin and eosin staining of experimentally infected turkeys. Conjunctiva with infiltration of lymphocytes and heterophils together with epithelial vacuolization and hyperplasia (x172).

24.8. Hematoxylin and eosin staining of experimentally infected turkeys. Congested lung with infiltration of lymphocytes (arrow A) and dilated bronchi and parabronchi (arrow B) (x69).

24.9. Hematoxylin and eosin staining of experimentally infected turkeys. Fibrinous necrotizing airsacculitis (x172).