

SECTION 3.3.

AVES

CHAPTER 3.3.1.

AVIAN CHLAMYDIOSIS

SUMMARY

Description and importance of the disease: Avian chlamydiosis (AC) is caused by a Chlamydia species in birds. The taxonomy of the family Chlamydiaceae was recently revisited. The genus Chlamydia currently includes 11 recognised species, and among them C. psittaci, C. avium and C. gallinacea have been isolated from birds.

Outbreaks of AC in psittacine birds and domestic poultry farms cause considerable economic damage. The infection can lead to systemic and occasionally fatal disease in birds. The clinical signs are generally nonspecific and vary greatly in severity, depending on the species and age of the bird and the virulence of the Chlamydia strain, but respiratory distress is mostly involved. Many birds, especially older psittacine birds and poultry, may show no clinical signs; nevertheless, they may often shed the agent for extended periods.

Special laboratory handling as determined by biological risk analysis (see Chapter 1.1.4 Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities) is recommended and even obligatory in many countries because avian chlamydial strains can cause serious illness (pneumonia) and death in humans when left untreated.

Identification of the agent: The preferred method for the identification of AC is no longer isolation of the organism. Considering the time involved, the need for high-quality samples, the fact that some strains will never grow in vitro and the hazard to laboratory personnel, nucleic acid amplification tests (NAATs) are currently recommended for quick, sensitive and specific diagnosis. These methods include conventional and real-time polymerase chain reaction (PCR), DNA microarray-based detection and DNA sequencing. Isolation, cytological staining of smears of exudate or faeces, and of impression smears of tissues, immunohistochemical staining of cytological and histological preparations and antigen-capture enzyme-linked immunosorbent assays (ELISA) can be used if NAATs are not available.

Serological tests: 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 antichlamydial antibodies. In most bird species, there is a high background rate of antichlamydial antibodies. Thus, to determine if a single bird is infected, serology should always be used in conjunction with gene or antigen detection, or paired sera should be examined. 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 seroconversion. Treatment with antibiotics may also delay or diminish the antibody response.

The main serological methods that are used for detecting chlamydial antibodies are: (1) various methods of elementary body agglutination (EBA), (2) the complement fixation test and (3) ELISA. ELISA is highly sensitive and specific when using recombinant proteins/peptides as antigen targets and it detects IgM, IgG and IgA.

Requirements for vaccines: There are no commercial vaccines available for chlamydiosis control in poultry.

A. INTRODUCTION

Avian chlamydiosis (AC) is caused by infection with a *Chlamydia* species in birds. In 2015, the taxonomy of the family *Chlamydiaceae* was revisited by Sachse et al. (Sachse et al., 2015). The genus *Chlamydia* currently includes 11 recognised species, namely *C. abortus* (sheep, goats, cattle), *C. caviae* (guinea-pigs), *C. felis* (cats), *C. muridarum* (mouse, hamster), *C. psittaci* (birds and others), *C. pecorum* (sheep, cattle), *C. pneumonia* (human and others), *C. suis* (swine), *C. trachomatis* (human) and two recently established species isolated from birds, *C. avium* and *C. gallinacea* (Sachse et al., 2014). While most of these organisms are highly host specific, *C. pneumonia* and *C. psittaci* have a broader host range. The latter has been reported to occur not only in birds and humans, but also in cattle, sheep, swine, horses and other animals.

Until very recently, *C. psittaci* was considered to be the sole causative agent of the disease in birds. Originally called psittacosis, the term ornithosis was introduced later to differentiate the disease in domestic and wild fowl from the disease in psittacine birds. The two syndromes are currently considered to be the same (Andersen & Vanrompay, 2008). Their earlier separation was based on the assumption that in humans, ornithosis was a milder disease than psittacosis. However, it should be noted that the disease in humans contracted from turkeys and ducks is often as severe as that contracted from psittacine birds.

Infection of birds with *C. psittaci* is common all over the world and has been found in about 465 avian species (Kaleta & Taday, 2003). Outbreaks of AC in psittacine birds and domestic poultry farms cause considerable economic damage. The infection can lead to systemic and occasionally fatal disease in birds. The clinical signs are generally nonspecific and vary greatly in severity, depending on the species and age of the bird and the chlamydia strain. AC can produce lethargy, hyperthermia, abnormal excretions, nasal and eye discharges, and reduced egg production. Mortality rates will vary greatly. In pet birds, the most frequent clinical signs are conjunctivitis, anorexia and weight loss, diarrhoea, yellowish droppings, sinusitis, biliverdinuria, nasal discharge, sneezing, lachrymation and respiratory distress. Many birds, especially older psittacine birds and poultry, may show no clinical signs; nevertheless, they may often shed the agent for extended periods. Necropsy of affected birds will often reveal multifocal hepatic necrosis, spleen and liver enlargement, fibrinous airsacculitis, pericarditis and peritonitis (Andersen & Vanrompay, 2008; Vanrompay, 2013). Histological lesions are suggestive of infection but are non-pathognomonic unless there are identifiable chlamydiae present.

Until recently, nine different genotypes based on the *ompA* gene coding for the major outer membrane protein (MOMP) were distinguished within *C. psittaci* strains. Seven of these genotypes are thought to occur predominantly in a particular order or class of Aves and two in non-avian hosts, i.e. genotype A in psittacine birds, B in pigeons, C in ducks and geese, D in turkeys, E in pigeons, ducks and others, E/B in ducks, turkeys and pigeons, F in parakeets, WC in cattle, and M56 in rodents. Most of the avian genotypes have also been identified sporadically in isolates from cases of zoonotic transmission to humans, particularly A, B and E/B (Hedderma et al., 2006; Vanrompay et al., 2007). Meanwhile, subgroups for three of the more heterogeneous genotypes have been introduced, i.e. A-VS1, A-6BC, A-8455, EB-E30, EB-859, EB-KKCP, D-NJ1, D-9N, and provisional genotypes to cover the strains that were previously non-typable have been suggested (Sachse et al., 2008).

Antibiotics are the only current means of control. *Chlamydia psittaci* is susceptible to a number of antibiotics: the drug of choice varies from country to country. Chlortetracycline, doxycycline, and other tetracyclines are the most commonly used. Treatment needs to be maintained for extended periods of time. For pet birds, 45 days is often recommended (Vanrompay, 2013).

Evidence suggests that other chlamydial species, such as *C. abortus*, *C. pecorum*, *C. trachomatis*, *C. suis* and *C. muridarum* can also be harboured by birds (Guo et al., 2016; Pantchev et al., 2009), as well as by the avian species *C. avium* and *C. gallinacea* described by Sachse et al., in 2014. Their epidemiological importance is still unclear, however *C. avium* and *C. gallinacea* appear to be quite widespread in pigeons and psittacines or poultry birds, respectively. The pathogenicity of these two newly introduced species has yet to be systematically investigated. In surveys reported to date, no clinical signs have been observed in chickens carrying *C. gallinacea* (Guo et al., 2016; Laroucau et al., 2009), nor in most of the *C. avium* carriers among pigeons. However, it seems likely from currently available data that *C. avium* is able to cause respiratory disease in parrots and pigeons (Sachse et al., 2014). It is now recommended to do a differential diagnosis and use diagnostic methods that are capable of differentiating between

C. psittaci and the other species that can be hosted by birds. To date only molecular methods can make this distinction.

1. Zoonotic risk and biosafety requirements

The strains of avian chlamydiae can infect humans and should be handled with appropriate biosafety and containment procedures (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*). Risk assessment and management are essential when performing diagnosis of AC. Adequate information, communication, and health surveillance by an occupational physician are recommended.

Most infections occur through inhalation of infectious aerosols. While the disease from psittacine birds is best known, the infection in poultry is of particular concern as transmission to humans is common during handling and slaughter of the birds (Dickx et al., 2010; Lagae et al., 2014). Post-mortem examinations of infected birds and handling of cultures should be done in certified Class II laminar flow hoods whenever possible or with proper protective equipment. Appropriate zoonotic agent decontamination procedures should be followed because human infection can result from transient exposures. The incubation period is usually 5–14 days; however, longer incubation periods are known. Human infections vary from inapparent to severe systemic disease with interstitial pneumonia and encephalitis. The disease is rarely fatal in properly treated patients; therefore, awareness of the danger and early diagnosis are important. Infected humans typically develop headache, chills, malaise and myalgia, with or without signs of respiratory involvement. Pulmonary involvement is common. Auscultatory findings, however, may appear to be normal or to underestimate the extent of involvement. Diagnosis can be difficult and in the past was usually established through testing paired sera for antibodies to chlamydia by the complement fixation test (CFT). However, some patients remain seronegative though hospitalised with psittacosis; serology is therefore being replaced by nucleic acid amplification tests (NAATs), which also allow the bird source to be traced. In humans, tetracycline, doxycycline, or azithromycin are usually the drugs of choice unless contraindicated. The length of treatment will vary with the drug, but should be continued for at least 14 days for tetracycline.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of avian chlamydiosis and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Identification of the agent						
Conventional PCR	–	–	–	++	+	–
Real-time PCR	–	–	–	+++	++	–
DNA microarray	–	–	–	++	+	–
Cytological staining	–	–	–	+	–	–
Isolation in cell culture or embryonated eggs	–	–	–	++	+	–
IHC on fixed tissue	–	–	–	++	–	–

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of immune response						
CFT	+	+	-	+	+	-
ELISA	++	+	-	+	++	-

Key: +++ = recommended for this purpose; ++ recommended but has limitations;

+ = suitable in very limited circumstances; - = not appropriate for this purpose.

PCR = polymerase chain reaction; IHC = immunohistochemistry; CFT = complement fixation test;

ELISA = enzyme-linked immunosorbent assay.

1. Identification of the agent

The preferred method for the identification of AC is no longer isolation of the organism. Considering the time involved, the need for high-quality samples, the fact that some strains will never grow *in vitro* and the hazard to laboratory personnel, NAATs are currently recommended for quick, sensitive and specific diagnosis. These include conventional and real-time polymerase chain reaction (PCR), DNA microarray-based detection and DNA sequencing. Isolation, cytological staining of smears of exudate or faeces, and of impression smears of tissues, immunohistochemical staining of cytological and histological preparations and antigen-capture enzyme-linked immunosorbent assays (ELISA) can be used if NAATs are not available.

The samples to be collected will depend on the disease signs in evidence. Specimens from acute cases should include inflammatory or fibrinous exudate in or around organs that display lesions, ocular and nasal exudates, impression smears of liver, whole blood and tissue samples from kidney, lung, pericardium, spleen, and liver. In cases with diarrhoea, colon contents or excrement should be used. In live birds, the preferred samples are pharyngeal and nasal swabs. Intestinal excrement, cloacal swabs, conjunctival scrapings, and peritoneal exudate can also be taken.

1.1. Molecular methods – detection of nucleic acids

Chlamydia psittaci can be identified and sub-typed using: (1) species-specific conventional PCR; (2) real-time PCR (reviewed in Sachse et al., 2009); (3) *ompA*-sequencing (Sachse et al., 2008); (4) multi-locus sequence typing (MLST) (Pannekoek et al., 2010); and (5) DNA microarray (Sachse et al., 2005, 2008).

As mentioned above, *C. psittaci* is not the only chlamydial agent encountered in birds. The new chlamydial agents described by Sachse et al. in 2014 have to be taken into consideration when a given avian sample is positive in a general chlamydial test, e.g. *Chlamydiaceae*-specific PCR or immunohistochemistry, but negative in a species-specific test for *C. psittaci*. In such a case, partial or complete sequencing of the *ompA* gene and the rRNA operon or alternative species-specific PCR assays will reveal the identity of the strain. The occurrence of *Chlamydia* strains that are phylogenetically in between *C. psittaci* and *C. abortus* has also been described (Van Loock et al., 2003; Pannekoek et al., 2010), and should likewise be considered as a possible differential diagnosis.

Reagents designed to stabilise the DNA should be considered when a delay in processing the sample is anticipated (DeGraves et al., 2003). DNA samples can be prepared using inexpensive reagents or commercially available kits.

1.1.1. Conventional polymerase chain reaction

PCR techniques have been replacing isolation for the detection of chlamydiae from animal tissue. Infection risks to laboratory staff are avoided by inactivation of the sample prior to testing. The sensitivity of conventional PCR assays will usually exceed that of isolation. Current conventional PCR tests for detection of *C. psittaci* target the 16S–23S rRNA or the *ompA* gene (reviewed in Sachse et al., 2009). Sensitivity and specificity vary on sample preparation and PCR test, but are considered inferior when compared with quantitative real-time PCR assays. Sensitivity is

increased by targeting a relatively short DNA segment or using a nested procedure. However, there is the risk of contamination if extreme care is not taken when manipulating the reactions (see Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals*).

1.1.2. Real-time PCR

Real-time PCR has become the preferred method in diagnostic laboratories for its rapidity, high throughput, potential for quantification and ease of standardisation (Sachse et al., 2009). This technology requires a fluorescent-labelled probe and special equipment, which increases costs. Its sensitivity can be equivalent to that of the nested system, but contamination problems and labour are reduced as it is based on one reaction in a closed system.

A hierarchical approach is recommended for the detection and identification of *C. psittaci* DNA. Such an approach includes a *Chlamydiaceae*-specific screening PCR based on the sequences of 23S-rRNA in positive cases (DeGraves et al., 2003; Ehricht et al., 2006; Everett et al., 1999), followed by a *C. psittaci*-specific PCR assay based on sequences of the outer membrane protein (*ompA*) (Pantchev et al., 2009) or of the *incA* gene (Ménard et al., 2006). Minor groove binding (MGB) probes are used to rule out cross-reactions with *C. abortus*.

The protocol of the *ompA*-based *C. psittaci*-specific assay (Pantchev et al., 2009) is given in more detail below. The assay is conducted as a duplex amplification that includes an internal amplification control (IAC). A detection limit of 2 inclusion-forming units per reaction mix was determined.

- i) The *C. psittaci*-specific oligonucleotides are primers CppOMP1-F (5'-CAC-TAT-GTG-GGA-AGG-TGC-TTC-A-3') and CppOMP1-R (5'-CTG-CGC-GGA-TGC-TAA-TGG-3'), as well as MGB[®] probe CppOMP1-S (FAM-CGC-TAC-TTG-GTG-TGA-C-TAMRA). The IAC system includes primers EGFP1-F (GAC-CAC-TAC-CAG-CAG-AAC-AC) and EGFP10-R (CTT-GTA-CAG-CTC-GTC-CAT-GC), as well as TaqMan probe EGFP-HEX (HEX-AGC-ACC-CAG-TCC-GCC-CTG-AGC-A-BHQ1). Plasmid IC2 (available commercially) serves as the IAC template.
- ii) The amplification is conducted in 96-well microtitre plates on an Mx3000P thermocycler or comparable equipment. Each 25-µl reaction contains 12.5 µl of 2 × universal real-time PCR Master Mix. The final concentration is 0.8 µM for each *C. psittaci* primer, 0.4 µM for each IAC primer, and 0.2 µM for each probe.
- iii) IAC template DNA (500 copies) is added to each reaction before the final volume is made up with water.
- iv) The following cycling parameters are used: initial heating cycle at 95°C for 10 minutes (single denaturation step), 45 cycles of 95°C for 15 seconds and 60°C for 1 minute (annealing and extension).
- v) The cycle threshold value ($C_t = C_q$ quantification cycle) automatically calculated by the software should be used. C_q values of 35 or lower are considered as positive. C_q values higher than 35 should be treated with caution as they may represent cross-reaction with related microorganisms. In such cases, the samples should be re-examined, preferentially by alternative tests using different genomic targets (Ménard et al., 2006; Opota et al., 2015). Samples can also be re-tested in the real-time PCR. In this case, only repeatedly positive samples are judged as true positive.

Other *ompA*-based real-time PCR protocols were developed to differentiate between genotypes of *C. psittaci* (Geens et al. 2005, Heddema et al., 2015). The latter PCR is also validated for use on human samples in case of a zoonotic infection and thus helpful to trace chains of zoonotic transmission.

Real-time PCR protocols are available for the specific detection of *C. avium* (Zocevic et al., 2013) and *C. gallinacea* (Laroucau et al., 2015) species.

1.1.3. DNA microarray

DNA microarray technology was shown to be a powerful tool in the diagnosis of chlamydial infections (Sachse et al., 2005). The assay for detection and identification of *Chlamydiaceae* spp. is based on PCR amplification of the 23S rRNA gene and subsequent identification of *C. psittaci* and the other avian agents *C. avium* and *C. gallinacea* by hybridisation with species-specific probes. It has been validated and proved suitable for routine diagnosis (Borel et al., 2008). This methodological approach enables detection of mixed chlamydial infections and identification of unexpected chlamydial species directly from clinical samples. An extended version of the DNA microarray allows for *ompA*-based genotyping of *C. psittaci* strains and clinical samples (Sachse et al., 2008).

1.2. Direct visualisation – cytological staining

Chlamydiae can be detected in smears of cloacal or conjunctival swabs and in impression smears of tissues (lung, liver, spleen, kidney and airsacs if enough material is available) by cytological staining such as Giemsa, Giménez, modified Giménez, Ziehl–Neelsen and Macchiavello's stains (Campbell et al., 2015). The modified Giménez technique is most often used (Andersen & Vanrompay, 2008). However, none of the stains specifically detects chlamydia. They are all less sensitive than antibody-based antigen detection methods or specific NAATs. Therefore, use of a cytological staining is losing popularity.

1.2.1. Modified Giménez staining

i) Reagents

a) Solution 1

Distilled H₂O (450.0 ml) and phenol (5.0 ml) added to basic fuchsin (2.5 g) and 95% ethanol (50.0 ml). Incubate at 37°C for 48 hours. Filter and store in the dark at room temperature.

b) Solution 2

Na₂HPO₄ (11.65 g); Na₂HPO₄.H₂O (2.47 g); distilled H₂O, pH 7.5 (to 1.0 litre).

c) Solution 3

Solution 1 (20.0 ml); and solution 2 (25.0 ml). Let stand for 10 minutes, filter and use.

d) Solution 4

0.5% citric acid.

e) Solution 5

Fast green (0.2 g); distilled H₂O (100.0 ml); and glacial acetic acid (0.2 ml).

f) Solution 6

Solution 5 (20.0 ml); and distilled H₂O (50.0 ml).

ii) Procedure for smears

a) Fix in methanol for 5 minutes.

b) Stain in solution 3 for 10 minutes and rinse in tap water.

c) Counterstain in solution 6 for 2 minutes.

d) Rinse in tap water and air-dry.

iii) Procedure for paraffin sections

a) Deparaffinise and hydrate with distilled H₂O.

b) Stain in solution 3 for 10 minutes and rinse in tap water.

c) Dip in solution 4 until no more red runs out of the section. Rinse in tap water.

- d) Counterstain in Solution 6 for 20 dips.
- e) Dip in two changes of 95% alcohol, for five dips each. Dehydrate, clear, and mount.

Note: a shorter procedure with “ready to use” carbol fuchsin (1/10 in distilled water), acetic acid (0.1%) and malachite green counterstain (0.8%) is also available (Vanrompay et al., 1992). Chlamydiae will appear red against a green background.

1.3. Isolation in cell culture

1.3.1. Treatment of samples for isolation

Proper handling of clinical samples is necessary to prevent loss of infectivity of chlamydiae during shipping and storage. A special medium consisting of sucrose/phosphate/glutamate (SPG) was developed for rickettsiae and has proven to be satisfactory for transport of chlamydial field samples. The medium as recommended for chlamydiae consists of SPG buffer: sucrose (74.6 g/litre); KH₂PO₄ (0.52 g/litre); K₂HPO₄ (1.25 g/litre); L-glutamic acid (0.92 g/litre), and bovine serum albumin – fraction V (1 g/litre), which can be sterilised by filtering. Added to this are streptomycin, vancomycin, (25–100 µg/ml), amphotericin B and gentamicin (50 µg/ml each). The addition of antibiotics reduces the effect of contamination, even when samples are shipped at ambient temperatures. The organism remains viable for several days even in the absence of refrigerative storage. This medium can also be used as a laboratory diluent and for freezing of chlamydiae.

Contaminated samples must be pre-treated before being used to inoculate cell cultures. There are three basic methods: treatment with antibiotics, treatment with antibiotics together with low-speed centrifugation (Andersen & Vanrompay, 2008), and treatment with antibiotics with filtration (Andersen & Vanrompay, 2008). A number of antibiotics that do not inhibit chlamydia can be used. Samples are homogenised in phosphate buffered saline (PBS), pH 7.2, containing a maximum of the following: streptomycin, vancomycin (100 µg/ml each), and gentamicin (50–200 µg/ml). Amphotericin B or nystatin (50 µg/ml each) can be added to control yeast and fungal growth. Penicillin, tetracycline and chloramphenicol should be avoided as these inhibit the growth of chlamydiae. In some cases, for example porcine faecal samples, treatment with penicillin G (500 IE/ml) can be useful.

When contamination is light, samples should be homogenised in the antibiotic solution prior to inoculation into tissue cultures. Samples are often left to stand in the antibiotic solution for 24 hours at 5°C before inoculation. Heavily contaminated samples, such as faecal samples, should be homogenised in antibiotics and then centrifuged at 500 **g** for 20 minutes. The surface layer and the bottom layer are discarded. The supernatant fluid is collected and re-centrifuged. The final supernatant fluid is used for inoculation. Samples should be passed through a filter of 450–800 µm average pore size if contamination persists.

Cell cultures are the most convenient method for the isolation of *C. psittaci*. The most common cell lines are buffalo green monkey (BGM), McCoy, HeLa, African green monkey kidney (Vero), and L cells (Vanrompay et al., 1992). The cells are grown as monolayers using standard tissue culture media containing 5–10% fetal calf serum and antibiotics that are not inhibitory to chlamydia (as described previously).

When selecting cell culture equipment, it is important to remember that:

- i) Chlamydiae can be identified by direct or indirect immunofluorescence or some other appropriate staining technique;
- ii) The inoculum is usually centrifuged on to the monolayer to enhance its infectivity;
- iii) The sample may need to be blind passaged at 4–5 days to increase sensitivity of isolation;
- iv) The sample will need to be examined from two to three times during any one passage; and
- v) Chlamydiae can be infectious to humans.

Small flat-bottomed vials, such as 1-dram (3.7 ml, 15 × 45 mm) shell vials or bottles containing cover-slips that are 12 mm in diameter, will meet these requirements. A number of vials, often four to six, are inoculated with each sample to permit fixing and staining at various intervals, and to permit repassaging of apparently negative samples 6 days after inoculation. When testing multiple samples, 24-well multiwell dishes can also be used as they have a labour-saving advantage. However, it should be noted that cross-contamination between samples can be a problem.

Chlamydiae can be isolated from cells that are replicating normally, but the use of non-replicating cells is preferable as these may provide increased nutrients for the growth of chlamydiae. Suppressed cells can also be observed for longer periods. Host cell division can be suppressed by cytotoxic chemicals, such as cycloheximide, which can be added to the medium at the rate of 0.5–2.0 µg/ml at the time of inoculation of the monolayer (Andersen & Vanrompay, 2008). A similar cytostatic effect that will enhance the growth of most chlamydial strains is reached by the use of serum-free tissue culture medium.

Attachment of chlamydia to cells is increased by centrifuging the inoculum on to the monolayer at 2000–3500 *g* for 30–90 minutes at 37°C. After a 2-hour incubation period at 37°C and 5% CO₂, the inoculum is removed and replaced with serum-free or cycloheximide-containing tissue culture medium, and cultures are incubated at 37–39°C. Cultures must be examined for chlamydiae at regular intervals using an appropriate staining method. This is usually done on day 2 or 3, as well as on day 4 or 5. Cultures that appear to be negative at the fifth day are harvested and repassaged. When repassaging chlamydiae, cells and culture media should be passaged without using freeze–thawing to disrupt cells, as this will destroy the chlamydiae.

Before staining the cultures, the medium is first removed, the cultures are washed with PBS and fixed with acetone or methanol for 2–10 minutes. The fixation time will depend on the tissue culture vessel used. As acetone will soften most plastics, the use of a mixture of 50% acetone and 50% methyl alcohol may be preferable. A number of staining methods can be employed to demonstrate chlamydial inclusions. The preferred method is direct immunofluorescence (Andersen & Vanrompay, 2008). A chlamydial fluorescein-conjugated antiserum is applied to the infected cells and incubated in a humid chamber for 30 minutes at 37°C. The cover-slips are then washed three times with PBS, mounted immediately, and examined. Chlamydial inclusions fluoresce in green. Commercial conjugate preparations using monoclonal antibodies (MAbs) are available and are highly specific. Conjugates may also be prepared from polyclonal sera, but it is important to obtain specific, high-titred antisera. Polyclonal antisera can be prepared in rabbits, guinea-pigs, sheep or goats. Conjugates are then prepared using standard techniques (Andersen & Vanrompay, 2008). Chlamydial inclusions can also be demonstrated by indirect fluorescent antibody and immunoperoxidase techniques (Andersen & Vanrompay, 2008). Direct staining can be done with Gimenez (see Section B.1.2.1), Giemsa, Ziehl–Neelsen, or Macchiavello's stains. Except for immunofluorescence, all these stains have the advantage that standard light microscopes can be used.

1.4. Isolation in embryonated eggs

Chicken embryos are still used for the primary isolation of chlamydiae. Samples should be handled and pre-treated with antibiotics as described in Section B.1.3. The standard inoculation procedure is to inject up to 0.5 ml of inoculum into the yolk sac of a specific pathogen free 6–7-day-old embryo (Andersen & Vanrompay, 2008). The eggs are then incubated in a humid atmosphere at 39°C, rather than at 37°C, as multiplication of chlamydiae is greatly increased at the higher temperature. Replication of the organism usually causes the death of the embryo within 3–10 days. If no deaths occur, two additional blind passages are usually made before designating any sample as negative. Chlamydial infections will give rise to a typical vascular congestion of the yolk sac membranes. These are harvested and homogenised as a 20% (w/v) suspension in SPG buffer, and can be frozen to preserve the strain, or inoculated into eggs or on to cell cultures.

The organism can be identified by preparing an antigen from an infected yolk sac and testing it by direct staining of smears using appropriate stains or by using the antigen in a serological test. Cell culture monolayers can be inoculated with the yolk sac suspension and examined by direct immunofluorescence 48–72 hours later for the presence of chlamydial inclusions. Typical inclusions are intracytoplasmic round or hat-

shaped bodies. With some virulent strains, the inclusions rapidly break up and the chlamydial antigen is dispersed throughout the cytoplasm.

1.5. Antigen detection

1.5.1. Immunohistochemical staining

Immunohistochemical staining can be used to detect chlamydiae in cytological and histological preparations and is an indispensable tool to show the association of chlamydial agents and pathological lesions in tissues. The technique is more sensitive and specific than histochemical staining. Antigen detection can be performed using commercially available anti-*Chlamydia* antibodies directed against LPS or MOMP (major outer membrane protein).

The selection of the primary antibody is very important. Both polyclonal and monoclonal antibodies have been used. Because formalin affects chlamydial antigens, it is recommended that polyclonal antibodies be made to purified formalin-inactivated chlamydiae. The chlamydial strain used is not important, as the antibodies will be reactive mainly to the group-specific antigens. MAbs should also be selected for reactions to formalin-fixed chlamydiae. A pool of group-reactive MAbs can be used.

1.5.2. Enzyme-linked immunosorbent assays

The ELISA has been extensively promoted in kit format for use in the diagnosis of human chlamydiosis. These test kits detect the lipopolysaccharide (LPS) antigen (group reactive) and will detect all species of *Chlamydiaceae*. A number of these kits have been tested for use in detecting chlamydiae in birds (Vanrompay et al., 1994), but none of the kits has been licensed for detection of *C. psittaci*. One problem with some of these tests is that the chlamydial LPS shares some epitopes with other Gram-negative bacteria, and these epitopes can cross-react, resulting in a high number of false-positive results. This problem has been reduced or eliminated in more recently developed kits by careful selection of the MAbs used. These kits, however, still lack sensitivity because a few hundred organisms are still needed to give a positive reaction. Most diagnosticians believe that a diagnosis of AC can be made when a strong positive ELISA reaction is obtained from birds with signs of psittacosis. Because of the number of false-positive results, a positive in an individual bird without signs of disease is not considered to be significant, but indicates the need for further testing using different methods.

2. Serological tests

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 antichlamydial antibodies. In most bird species, there is a high background rate of antichlamydial antibodies in birds. 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 (Vanrompay, 2013). 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 seroconversion. Treatment with antibiotics also may delay 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), (2) the CFT and (3) ELISA.

EBA detects primarily IgM antibodies and thus can detect early infections. A negative result does not guarantee that a bird is free of infection because the sensitivity of the test is rather low. The direct CFT detects avian IgG but not IgM, so recent infections can be ‘missed’. Its disadvantages are that: (1) the test antigens are not commercially available, (2) the test cannot 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) it is fairly laborious when there is a large number of samples to be tested. The modified CFT is more sensitive but has the same disadvantages as the CFT.

The CFT is more and more being replaced by highly sensitive and specific ELISAs based on the use of recombinant proteins (Verminnen et al., 2006) or peptide antigens (Sachse et al., 2009). ELISAs can detect avian IgM, IgG and IgA as long as the correct isotype-specific conjugate is used.

2.1. Modified direct complement fixation test for *Chlamydia*

For AC, a modified direct CFT method is used. This method differs from the direct CFT in that normal, unheated chicken serum from chickens without chlamydial antibody is added to the complement dilution. The normal serum increases the sensitivity of the CFT procedure so that it can be used to test sera from avian species whose antibodies do not normally fix guinea-pig complement.

2.1.1. Test procedure

- i) Serum to test is heat-inactivated at 60°C for 30 minutes prior to use.
- ii) Serum is then diluted 1/5 in veronal (barbiturate) buffer saline (VBS)
- iii) Twofold dilutions of the diluted serum are prepared in 96-well round bottom microtitre plates.
- iv) Guinea-pig complement is diluted in VBS prior to the addition of the antigen and 2 complement haemolytic units are used.
- v) Sera complemented with 5 % of fresh chicken serum, complement and antigen are reacted in the plates and incubated for 1 hour at 37°C (and alternate acceptable procedure is overnight incubation at 4°C).
- vi) A 2–4% suspension of sensitised washed red blood cells is added.
- vii) Plates are incubated for 30 minutes at 37°C, and the centrifuged for 5 minutes at 600 *g*.

When using commercially available CFT antigens and ready-to-use CFT reagents, the manufacturers' instructions should be applied.

Recommended controls to verify test conditions:

- i) Positive control: a control serum that gives a positive reaction;
- ii) Negative control serum: a control serum that gives a negative reaction;
- iii) Anti-complementary control (serum control): diluent + inactivated test serum + complement + haemolytic system;
- iv) Antigen control: diluent + antigen + complement + haemolytic system;
- v) Haemolytic system control: diluent + haemolytic system;
- vi) Complement control: diluent + complement titration + antigen + haemolytic system.
- vii) The absence of anti-complementary activity must be checked for each serum; anticomplementary sera must be excluded from analyses.

A sample that produces 100%haemolysis at the 1/5 dilution is negative and a sample that produces 25–100% haemolysis is positive.

2.1.2. CFT antigen preparation

The simplest methods start with the growth of chlamydiae in cell culture. The two methods described below produce antigens that can be used in the micro-CFT. The procedures are quite similar: both include the growth of chlamydiae in cell culture, the inactivation of the chlamydiae, partial purification of the antigen, mechanical disruption, and dilution into the appropriate buffer. The method selected will depend on the equipment available.

The first procedure (Grimes, 1985) starts with the chlamydiae and cell culture debris harvested when cytopathic effects are noted. The culture is inactivated by the addition of phenol to a final concentration of 1.0%, incubated for 24 hours at 37°C, and concentrated by centrifugation at 10,000 *g* for 1 hour. The sediment is reconstituted to 10% of the original volume using VBS, pH 7.2, containing 1.0% phenol and 1.0% glycerol.

The sediment is then homogenised in an omnimixer at top speed for three 1-minute periods while cooled in ice water. The homogenate is centrifuged for 15 minutes at 100 *g* to remove debris.

Some procedures suggest heating the antigen for 30 minutes in a boiling water bath at this time. The supernatant is saved and diluted to the desired concentration.

In the second procedure for the production of antigen for the CFT (Bracewell & Bevan, 1986), antigen is prepared from L cells infected with a psittacine strain. The cell culture medium is discarded, and the cells are heated for 40 minutes at 56°C. The cells are lysed in distilled water, the chlamydiae are disrupted by ultrasonication, and then made isotonic in VBS. The antigen is tested against a standard sheep convalescent serum and used at 2 units in the micro-CFT.

There are a number of procedures for preparing the antigen from infected yolk sacs, some of which are quite elaborate. However, with the following procedure it is relatively easy to prepare a crude infected yolk sac antigen that works well in the modified direct CFT. An egg-adapted strain of *Chlamydia* is used to inoculate 6–7-day-old embryonated chicken eggs via the yolk sac. The yolk sacs are harvested from embryos that die between 3 and 7 days post-inoculation. The yolk-sac harvest is diluted 1/3 in PBS, Tris buffer, or cell culture medium, and then autoclaved for 20 minutes. The suspension is cooled and then homogenised thoroughly. The use of a high-speed tissue homogeniser for 3–5 minutes is recommended. After homogenisation, phenol is added to make a final concentration of 0.5% phenol (prepare a 5% phenol stock solution and add 1 ml for every 9 ml of antigen). The antigen preparation is prepared, held for 3 days, and then the supernatant is used after centrifugation for 20 minutes at 1000 *g*. The antigen can be stored for long periods of time at 4°C.

2.2. Recombinant major outer membrane protein ELISA

The recombinant major outer membrane protein (MOMP) ELISA (Verminnen et al., 2006) can be performed on chicken and turkey sera pretreated with kaolin to remove background activity. MOMP-specific antibody titres are determined using a standard ELISA protocol and micro-well plates coated with recombinant MOMP. Serum, diluted 1/100, is added to the coated wells. Recombinant MOMP is produced in pcDNA4::MOMPHis transfected COS7 cells (Vanrompay et al., 1999). Briefly, COS7 cells are cultured in Dulbecco's modified Eagle's medium supplemented with 3.7 g of sodium bicarbonate/litre, 1 mM L-glutamine, and 10% fetal calf serum. Transfection with plasmid DNA is performed by the diethylaminoethyl dextran method. Forty-eight hours post-transfection recombinant MOMP production is monitored by an indirect immunofluorescence staining using serovar and genus-specific MAbs. His-tag labelled recombinant MOMP is purified by affinity chromatography and the protein concentration is determined by the bicinchoninic acid protein assay. For the determination of antibody titres, 1/2000 and 1/4000 dilutions of biotinylated anti-chicken/turkey IgG (H+L) antibody and peroxidase-conjugated streptavidin are used, respectively. The results are positive if the absorbance exceeds the cut-off value of the mean of the negative control sera plus three times the standard deviation.

2.3. Other tests

Other tests include the agar gel immunodiffusion test, the latex agglutination (LA) test, the EBA test (Grimes & Arizmendi, 1996) and the micro-immunofluorescence test (MIFT). Immunodiffusion is less sensitive than the CFT. The LA test will detect antibodies to *C. psittaci*, and is easy and rapid to perform (Grimes et al., 1993). Latex beads are coated with purified chlamydial antigen, mixed thoroughly with the test serum on a glass plate, and rotated for 2 minutes to enhance agglutination. The test is read against a dark background. Sera giving positive reactions should be retested with uncoated beads to eliminate possible nonspecific agglutination. The LA and direct CFTs correlate in 72.5% of tests with paired sera. The LA test has a sensitivity of 39.1% and a specificity of 98.8% relative to the direct CFT (Grimes et al., 1993). The test detects both IgM and IgG, but it is best at detecting IgM. It has been suggested for use in detecting recent or active infections. The EBA test detects only IgM, and it is indicative of a current infection. The MIFT is rapid and easy to perform; however, fluorescence-conjugated anti-species sera are not always available.

C. REQUIREMENTS FOR VACCINES

To date, no commercial vaccines against avian chlamydiosis are available, although vaccination with a recombinant DNA plasmid containing the *C. psittaci* *ompA* gene provided significant (partial) protection in experimentally infected specified pathogen free (SPF) turkeys (Verminnen et al., 2010) and budgerigars (Harkinezhad & Schautteet,

2009). DNA vaccination has the advantage that it can be used in the presence of maternal antibodies (Van Loock et al., 2004) and the antigen is processed in the same way as during a natural infection, resulting in humoral and cell-mediated immune responses.

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NB: There are WOAH Reference Laboratories for avian chlamydiosis (please consult the WOAH Web site:
<https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3>).

Please contact the WOAH Reference Laboratories for any further information on
diagnostic tests and reagents for avian chlamydiosis

NB: FIRST ADOPTED IN 1990. MOST RECENT UPDATES ADOPTED IN 2018.