

SECTION 2.3.

AVES

CHAPTER 2.3.1.

AVIAN CHLAMYDIOSIS

SUMMARY

Avian chlamydiosis (AC) is caused by the bacterium Chlamydophila psittaci. AC occurring in humans and all birds was originally called psittacosis, but later the term ornithosis was introduced to identify the disease contracted from or occurring in domestic and wildfowl, while the name of the disease contracted from or occurring in psittacine birds remained psittacosis. These diseases are similar when contracted by humans. The genus Chlamydia was divided into two, Chlamydia and Chlamydophila. A proposal to re-combine them into the single genus Chlamydia is under consideration but has not been adopted for this chapter. The avian strains of Chlamydophila psittaci include at least fifteen genotypes, some of which correlate with the avian species from which they are usually isolated. Chlamydiosis as it occurs naturally in mammalian species and not contracted from avian species is caused by different species of the organism.

Depending on the virulence of the chlamydial strain and the avian host defence, chlamydiae cause pericarditis, conjunctivitis, sinusitis, airsacculitis, pneumonia, lateral nasal adenitis, peritonitis, hepatitis, and splenitis. Generalised infections result in fever, anorexia, lethargy, diarrhoea, and occasionally shock and death. Special laboratory handling (biosafety level 3) is recommended because avian chlamydial strains can cause serious illness and possibly death in humans. While the disease in psittacine birds is best known, the infection in ducks and turkeys is of particular concern as transmission to humans is common during handling and slaughter of the birds. The diagnosis of AC requires the isolation and identification of the organism, the demonstration of chlamydiae in tissues, or the demonstration of a four-fold increase in specific humoral antibody, as well as typical clinical signs.

Identification of the agent: *Isolation of chlamydiae requires the inoculation of embryonated eggs or cell cultures and testing for chlamydiae by cytochemical stains or immunohistochemical methods. The direct inoculation of samples into cell cultures is preferable as they are as sensitive for the isolation of most avian strains of chlamydiae as are chicken embryos. The cell cultures are then stained by immunofluorescence or by other appropriate stains at appropriate times to demonstrate the presence of inclusions.*

Histochemical staining of impression smears from the liver, heart, and spleen are commonly made. The technique gives a rapid diagnosis, but requires some experience.

Enzyme-linked immunosorbent assays (ELISAs) developed for detecting Chlamydophila trachomatis antigen in humans have been used for diagnosing chlamydiae in birds. Many of the earlier tests were developed using monoclonal or polyclonal antisera against lipopolysaccharide epitopes, some of which were shared with other Gram-negative bacteria. Their use when screening individual birds is questionable, as they lack sensitivity and specificity.

Molecular tools (conventional and real-time polymerase chain reaction, restriction length polymorphism, DNA microarray or sequencing) and immunohistochemical staining of histological sections are now widely used in diagnostic laboratories. All of them are rapid and do not require the live agent. The current PCR tests target the ompA gene or the ribosomal RNA genes (16S or 23S). Validated and standardised protocols of both species-specific and family-specific assays are

available. Nested and real-time PCRs can be as sensitive as isolation. There has been an increase in the use of immunohistochemical staining of histological sections because of the recent development and availability of automated staining equipment.

Serological tests: The standard serological test for chlamydial antibodies is the complement fixation (CF) test. The modified direct CF test can be used with most sera. The antigen is a group-reactive lipopolysaccharide antigen present in all strains. The occurrence of high CF titres in the majority of individuals in a flock with clinical signs is presumptive evidence of active infection. The demonstration of a four-fold increase in titre in an individual bird is considered to be diagnostic of a current infection.

Other serological tests, such as the ELISA, latex agglutination, elementary body agglutination, micro-immunofluorescence, and the agar gel immunodiffusion tests can be used. These tests are of value in specific cases and may replace the CF test; however, comparisons of reliability and reproducibility are not yet available.

Requirements for vaccines: There are no commercial vaccines available for chlamydiosis control in poultry. Antibiotics are the only current means of control. *Chlamydophila psittaci* is susceptible to a number of antibiotics. The drug of choice varies from country to country.

A. INTRODUCTION

Avian chlamydiosis (AC) is caused by the bacterium *Chlamydophila psittaci*. The disease in birds was originally called psittacosis, but 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, 2003). 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 *Chlamydophila 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 (European Commission, 2002). 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 strain of chlamydia. 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 (Mohan, 1984). Many birds, especially older psittacine birds, 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, 2003; Vanrompay *et al.*, 1995). Histological lesions are suggestive of infection but are non-pathognomonic unless there are identifiable chlamydiae present.

The taxonomy of the family *Chlamydiaceae* is currently under consideration (Kuo *et al.*, 2011) but for the purpose of this chapter the subdivision into two genera *Chlamydia* and *Chlamydophila* has been retained (Everett *et al.*, 1999a). The genus *Chlamydia* includes *C. trachomatis* (human), *C. suis* (swine), *C. muridarum* (mouse, hamster), *C. psittaci* (birds and others), *C. felis* (cats), *C. abortus* (sheep, goats, cattle), *C. caviae* (guinea-pigs), and *C. pecorum* (sheep, cattle) and *C. pneumonia* (human and others). 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 (Sachse *et al.*, 2009a).

The avian strains associated with AC belong to the species *Chlamydophila psittaci*. Until recently, nine different genotypes based on the *ompA* gene coding for the major outer membrane protein (MOMP) were distinguished. Seven of these “classical serovars” are thought to predominantly occur 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 (Gaede *et al.*, 2008; Heddema *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 six new provisional genotypes to cover the strains that were previously non-typable have been suggested, i.e. 1V, 6N, Mat116, R54, YP84, and CPX0308, thus bringing the total number of genotypes to 15 (Sachse *et al.*, 2008).

Recent evidence suggests that *C. psittaci* is not the only chlamydial agent occurring in birds (Gaede *et al.*, 2008; Laroucau *et al.*, 2009). The taxonomic classification of these new agents within or outside the family *Chlamydiaceae* has yet to be defined and their epidemiological importance is still unclear. They seem to be quite widespread in ducks, chickens and pigeons, and some strains appear to act as facultative pathogens. It is important to use diagnostic methods that are capable of differentiating between these organisms and *C. psittaci*.

Antibiotics are the only current means of control. *Chlamydophila 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 (Smith *et al.*, 2005; Vanrompay *et al.*, 1995).

1. Zoonotic risk and biosafety requirements

The strains of avian chlamydiae can infect humans and should be handled carefully, at biosafety level 3 (Smith *et al.*, 2005) as outlined in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*. Most infections occur through inhalation of infectious aerosols. While the disease from psittacine birds is best known, the infection in ducks and turkeys is of particular concern as transmission to humans is common during handling and slaughter of the birds (Dickx *et al.*, 2010). Post-mortem examinations of infected birds and handling of cultures should be done in laminar flow hoods 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 is usually established through testing paired sera for antibodies to chlamydia by the complement fixation (CF) test. 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

1. Identification of the agent

The preferred method for the identification of AC is the isolation and identification of the organism. Because of the time involved, the need for high-quality samples, and the hazard to laboratory personnel, other techniques are often used. These include histochemical staining of smears of exudate and faeces, and impression smears of tissues, immunohistochemical staining of cytological and histological preparations, antigen-capture enzyme-linked immunosorbent assays (ELISA), conventional and real-time polymerase chain reaction (PCR), PCR-RFLP (restriction fragment length polymorphism), DNA microarray-based detection and DNA sequencing.

1.1. Collection and treatment of samples for isolation

The samples to be collected will depend on the disease signs in evidence. They must be taken aseptically. Contaminant bacteria may interfere with the isolation of the chlamydiae. 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 cultured. In live birds, the preferred samples are pharyngeal and nasal swabs (Andersen, 1996). Intestinal excrement, cloacal swabs, conjunctival scrapings, and peritoneal exudate can also be taken.

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 (Spencer & Johnson, 1983) consists of SPG buffer: sucrose (74.6 g/litre); KH_2PO_4 (0.512 g/litre); K_2HPO_4 (1.237 g/litre); and L-glutamic acid (0.721 g/litre), which can be sterilised by autoclaving or filtering. Added to this are fetal calf serum (10%), vancomycin, kanamycin, and streptomycin (200–500 µg/ml), amphotericin B and gentamicin (50 µg/ml). The addition of antibiotics reduces the effect of contamination, even when samples are shipped at ambient temperatures. In the absence of refrigerative storage, the organism remains viable for up to 30 days and at 4°C for up to 34 days (Spencer & Johnson, 1983). 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 animals or cell cultures. There are three basic methods: treatment with antibiotics (Bevan *et al.*, 1978), treatment with antibiotics together with low-speed centrifugation (Andersen & Vanrompay, 2003; 2005), and treatment with antibiotics with filtration (Andersen & Vanrompay, 2005; Bevan *et al.*, 1978). 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 (1 mg/ml), vancomycin (1 mg/ml), and kanamycin (1 mg/ml). Gentamicin (200 µg/ml) can be used. Amphotericin B (50 µg/ml) can be added to control yeast and fungal growth. Penicillin, tetracycline and chloramphenicol should be avoided as these inhibit the growth of chlamydiae.

When contamination is light, samples should be homogenised in an antibiotic solution prior to inoculation into chicken embryos or 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 recentrifuged. 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.

1.2. Isolation in cell culture

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 (Bevan *et al.*, 1978). 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, 96-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 either by irradiation or, more commonly, by cytotoxic chemicals. The latter include 5-iodo-2-deoxyuridine, cyto-cholasin B, cycloheximide, and emetine hydrochloride. Cycloheximide is the most commonly used and 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, 2003; 2005; Bevan *et al.*, 1978). Emetine is removed after treatment and replaced by medium. The monolayer is first treated for 5 minutes with emetine (0.5 µg/ml), after which the emetine is removed and replaced with culture medium; the monolayer is then ready for use. The growth of most chlamydial strains will be enhanced by the treatment of the monolayer by one of these drugs.

Attachment of chlamydia to cells is increased by centrifuging the inoculum on to the monolayer at 500–1500 g for 30–90 minutes at 37°C. The inoculum is removed and replaced with tissue culture medium containing a cell-division inhibitor, and then 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 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, 2005; Bevan *et al.*, 1978; Moore & Petrak, 1985). 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, air-dried, mounted, and examined. Chlamydial inclusions fluoresce a green colour. 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. Sheep and goats are excellent sources because of the volume and high titres that are readily obtained following infection. Conjugates are then prepared using standard techniques (Andersen & Vanrompay, 2003; 2005).

Chlamydial inclusions can also be demonstrated by indirect fluorescent antibody and immunoperoxidase techniques (Andersen & Vanrompay, 2005; Page, 1974). Direct staining can be done with Gimenez, Giemsa, Ziehl–Neelsen, or Macchiavello's stains. Except for immunofluorescence, all these stains have the advantage that standard light microscopes can be used.

1.3. Isolation in eggs

Chicken embryos are still used for the primary isolation of chlamydiae. The standard 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, 2003; 2005). 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.

1.4. Differentiating among species/strains

Chlamydophila psittaci can be identified using PCR-RFLP (Everett & Andersen, 1999) or species-specific conventional PCR (Messmer *et al.*, 1997; Sachse & Hotzel, 2003; Van Loock *et al.*, 2005), or real-time PCR (Everett *et al.*, 1999b; Geens *et al.*, 2005; Pantchev *et al.*, 2009; reviewed in Sachse *et al.*, 2009b). A DNA microarray assay was shown to differentiate among all nine species of the family *Chlamydiaceae* (Sachse *et al.*, 2005).

Serotyping in its classical form (Andersen, 1991; 1997) is only rarely conducted because the serovar-specific monoclonal antibodies are not available from a commercial supplier. Instead, genotyping is a practical alternative because i) the classical genotypes A–F are equivalent to the corresponding serotypes, and ii) nine of the more recently defined genotypes cannot be characterised by serotyping because of the absence of specific antibodies. Genotypes A–F can be identified using PCR-RFLP (Vanrompay *et al.*, 1997). A DNA microarray procedure, which was shown to differentiate among all currently known genotypes (Sachse *et al.*, 2008; 2009a), can be used for genotyping of *C. psittaci* strains from cell culture and tissue samples. In addition, all genotypes can be identified by complete sequencing of the *ompA* gene.

As mentioned above, *C. psittaci* is not the only chlamydial agent encountered in birds. The recently described new chlamydial agents (Gaede *et al.*, 2008; Laroucau *et al.*, 2009) 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 16–23S rRNA operon will reveal the identity of the strain. The occurrence of *C. abortus* in birds is a rare event, but should also be considered as a possible differential diagnosis.

1.5. Histochemical staining

Giemsa, Gimenez, Ziehl–Neelsen and Macchiavello's stains are commonly used to detect chlamydiae in impression smears of liver and spleen. The following modified Gimenez technique is used by several laboratories (Andersen & Vanrompay, 2005):

1.5.1. Modified Gimenez technique or (Pierce-van der Kamp) stain

- 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 is as follows
 - 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 is as follows
 - 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.

Chlamydiae will appear red against a green background.

1.6. Immunohistochemical staining

Immunohistochemical staining can be used to detect chlamydiae in cytological and histological preparations. The technique is more sensitive than histochemical staining, but some experience is necessary as cross-reactions with some bacteria and fungi require that morphology must be considered.

Most widely used immunohistochemical staining procedures can be adapted to give satisfactory results. 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 mainly to the group-reactive antigens. MAbs should also be selected for reactions to formalin-fixed chlamydiae. A pool of group-reactive MAbs can be used.

1.7. 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.

1.8. DNA detection systems

1.8.1. 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 and specificity will usually exceed that of isolation. Current PCR tests for detection of *C. psittaci* target the *ompA* gene or the 16S–23S rRNA gene (Everett *et al.*, 1999b; Geens *et al.*, 2005; Messmer *et al.*, 1997; Pantchev *et al.*, 2009; Sachse & Hotzel, 2003; Van Loock *et al.*, 2005; and reviewed in Sachse *et al.*, 2009b). The sensitivity and specificity varies on sample preparation and the PCR test. 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 using commercially available kits (Andersen & Vanrompay, 2005). Sensitivity is increased by targeting a relatively short DNA segment, using a nested procedure or using real-time PCR techniques. The nested PCR can equal isolation in sensitivity and specificity (Messmer *et al.*, 1997; Sachse & Hotzel, 2003; Van Loock *et al.*, 2005). However, the risk of contamination is increased if extreme care is not taken when manipulating the reactions (see Chapter 1.1.6 *Principles and methods of validation of diagnostic assays for infectious diseases*). In the last few years, real-time PCR has become the preferred method in diagnostic laboratories for its rapidity, high throughput and ease of standardisation (Sachse *et al.*, 2009b). 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 (Everett *et al.*, 1999b; Geens *et al.*, 2005; Pantchev *et al.*, 2009).

1.8.2. Real-time PCR procedure (Pantchev *et al.*, 2009)

This 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 for this assay.

- i) The *C. psittaci*-specific oligonucleotides are primers CppsOMP1-F (5'-CAC-TAT-GTG-GGA-AGG-TGC-TTC-A-3') and CppsOMP1-R (5'-CTG-CGC-GGA-TGC-TAA-TGG-3'), as well as MGB[®] probe CppsOMP1-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 serves as the ICA template¹.
- ii) The amplification is conducted in 96-well microtitre plates on an Mx3000P thermocycler or comparable equipment. Each 25- μ l reaction contains 12.5 μ l of 2 \times TaqMan Universal PCR Master Mix supplemented with ROX² or a comparable product. The final concentration is 0.9 μ M for each *C. psittaci* primer, 0.3 μ M for each IAC primer, and 0.2 μ M for each probe.

¹ Available from Intype IC-DNA, Labordiagnostik Leipzig, Germany.

² Available from Applied Biosystems.

- iii) IAC template DNA (0.5 µl containing 10^4 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 (Ct) automatically calculated by the software should be used. Ct values higher than 36 should be treated with caution as they may represent cross-reaction with related microorganisms. In such cases, the respective samples should be re-examined by an alternative test.

1.8.3. DNA microarray

DNA microarray technology was recently 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. includes identification of *C. psittaci*. It has been validated and proved suitable for routine diagnosis (Borel *et al.*, 2008). Its sensitivity is comparable to that of real-time PCR and specificity is even higher because sample DNA is hybridised to 36 specific oligonucleotide probes. This methodological approach enables detection of mixed chlamydial infections and identification of unexpected chlamydial species directly from clinical samples.

2. Serological tests

2.1. Modified direct complement fixation test for *Chlamydophila*

The following is a widely used modified direct CF test for the detection of antibody. The reagents are relatively easy to prepare and standardise. There are other CF tests; each has advantages. The modified direct CF test is performed in 96-well round-bottom multiwell dishes. Incubation steps are usually done by floating the plates in a 37°C water bath. The chlamydial antigen can be prepared from either infected yolk sacs or cell culture preparations. The modified direct CF test differs from the direct CF test 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 CF 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) Dilution of sera

Figure 1 gives a suggested pattern for performing the test in round-bottom, 96-well multiwell dishes. All sera must be heat-inactivated at 60°C for 30 minutes prior to use. The sera are diluted in Veronal (barbiturate) buffer saline (VBS) as shown in Figure 1. The dilutions are made in the multiwell dish by adding 100 µl of VBS to each well of rows A and E, and then adding 25 µl of the undiluted sera, positive serum, or negative serum to each of three wells. This gives a starting dilution of 1/5. Then, 25 µl of VBS is added to each well in row B through to D and row F through to H. Twofold dilutions are made, using a 25 µl micropipette, from row A through to D and row E through to H. Appropriate volumes are discarded from the starting and finishing rows to give 25 µl per well. Diluters are rinsed twice in distilled H₂O and once in VBS between each serum.

	Serum #1			Serum #2			Serum #3			Serum #4		
	Antigens: +Ag VBS -Ag			+Ag VBS -Ag			+Ag VBS -Ag			+Ag VBS -Ag		
	1	2	3	4	5	6	7	8	9	10	11	12
serum dilutions	1/5	A	⊗	⊗	⊗	⊗	⊗	⊗	⊗	⊗	⊗	⊗
	1/10	B	⊗	⊗	⊗	⊗	⊗	⊗	⊗	⊗	⊗	⊗
	1/20	C	⊗	⊗	⊗	⊗	⊗	⊗	⊗	⊗	⊗	⊗
	1/40	D	⊗	⊗	⊗	⊗	⊗	⊗	⊗	⊗	⊗	⊗
	1/5	E	⊗	⊗	⊗	⊗	⊗	⊗	⊗	⊗	⊗	⊗
	1/10	F	⊗	⊗	⊗	⊗	⊗	⊗	⊗	⊗	⊗	⊗
	1/20	G	⊗	⊗	⊗	⊗	⊗	⊗	⊗	⊗	⊗	⊗
	1/40	H	⊗	⊗	⊗	⊗	⊗	⊗	⊗	⊗	⊗	⊗
	Serum #5			Serum #6			+ Control			– Control		

Fig. 1. Suggested test pattern for the modified direct complement fixation test when using 96-well dishes.

ii) *Addition of antigen*

To each well in columns 1, 4, 7, and 10, add 25 µl of positive chlamydial antigen. In columns 2, 5, 8, and 11, add 25 µl of VBS (anticomplementary control wells), and in columns 3, 6, 9, and 12, add 25 µl of negative antigen (normal yolk sac or cell culture prepared the same as the chlamydial antigen). The chlamydial antigens are stored undiluted at 4°C and diluted to proper concentration in VBS prior to use.

iii) *Addition of complement*

Complement (C') is stored at –70°C and should be thawed and diluted in VBS prior to the addition of the antigen. Fresh chicken serum is added before diluting the C' to give a 5% concentration in the complement. Dilutions of C' are made as in previous tests or from titrations. C' should be allowed to stand in an ice bath to stabilise for 15 minutes. The diluted C' should be stored at 4°C following stabilisation and should be used within 2 hours: 50 µl of the C' is added to each well immediately following the addition of the antigens. The plates are incubated uncovered in a 37°C water bath for 2 hours.

iv) *Addition of sheep red blood cells*

Mix 4% standardised sheep red blood cells (SRBCs) with an equal volume of VBS. To this add an equal volume of haemolysin diluted in VBS. The final dilution is incubated in a 37°C water bath for 15 minutes to sensitise the SRBCs. To each well add 50 µl of sensitised SRBCs. The plates are then incubated for 1 hour in a 37°C water bath. The plates can be centrifuged at 400 *g* for 5 minutes before reading or they can be refrigerated at 4°C overnight prior to reading.

v) *Interpretation of the results*

The wells are often scored 1+, 2+, 3+, or 4+ corresponding to reduction of haemolysis of 25, 50, 75, or 100%. A positive reaction is 2+ or higher, which is equivalent to 50% or less lysis of the SRBCs. This indicates that the C' was fixed by antibody prior to the addition of the SRBCs. Negative wells are indicated by the complete lysis of the cells: the C' remains unbound and reacts with the SRBCs and the haemolysin to produce lysis of the SRBCs.

Invalid tests occur when the serum is anticomplementary and a positive reaction occurs in the dilution with VBS as the antigen. Nonspecific serum reactions give positive reactions in both the positive and negative wells.

2.1.2. Reagents

i) *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-CF test. 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; Grimes *et al.*, 1970) 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 CF test (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-CF test.

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 CF test. 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.

ii) Preparation of sensitised SRBCs

Defibrinated SRBCs are preserved by mixing in an equal volume of Alsever's solution. These can be stored at 4°C for up to 4 weeks. Wash 25 ml of the stock SRBCs with 25 ml of VBS. Centrifuge at 400 *g* for 10 minutes. Aspirate off the VBS and resuspend in 50 ml of VBS. Repeat the wash a total of three times. Following the final wash, dilute the SRBCs at a ratio of 2.2 ml of packed SRBCs to 98 ml of VBS. The SRBCs can then be standardised by optical density: mix 1 ml of the diluted, washed SRBCs with 14 ml distilled H₂O, determine the absorbance using a spectrophotometer, and standardise to 0.25 at a wave length of 550 nm. The reading obtained can be used in the following formula to determine the dilution needed:

$$\text{Final volume of SRBCs} = \frac{(\text{absorbance reading} \times \text{current volume})}{0.25 \text{ desired absorbance}}$$

The SRBCs are sensitised by rapidly adding an equal volume of VBS containing the appropriate dilution of haemolysin (dilution determined by titration). Incubate at 37°C for 15 minutes prior to use.

iii) Veronal buffer saline

VBS is prepared as a 5 × stock solution and diluted 1/5 with distilled H₂O prior to use. The following formula makes 4 litres. To distilled water add sodium barbital (7.5 g); barbital H₂O (dissolve in boiling H₂O) (11.5 g); MgSO₄·7 H₂O (4.056 g); NaCl (170.0 g); and CaCl₂ (0.078 g). Add distilled H₂O to make to 4 litres.

iv) Complement titration

Complement (C') is unstable and will deteriorate if improperly handled. Normally it should be kept frozen at –70°C in aliquots that are used at one time to eliminate refreezing. To obtain the desired working concentration (2 units per test well) first add 5% normal chicken serum for the modification to enhance sensitivity as described earlier. Then estimate a starting point based on previous lots. A good starting point is a dilution of 1/30 after the chicken serum has been added. Set up a series of tubes with various amounts of complement in VBS. The VBS should contain the antigen to be used in the reaction and take into account any anticomplementary properties of the antigen. A common method is to dilute 0.10 ml C' + 0.90 ml VBS; 0.12 ml complement + 0.88 ml VBS, etc. through 0.25 ml C' + 0.75 ml VBS. Incubate the tubes for 2 hours in a 37°C water bath. Add 0.5 ml of sensitised SRBCs to each tube. Incubate for 1 additional hour in the 37°C water bath. The highest dilution giving complete haemolysis equals 1 unit. Twice that amount equals 2 units. The following formula can be used to obtain 2 units/0.05 ml:

$$x = (di) (v)/2dh$$

where:

x = reciprocal of C' dilution desired to yield 2 units C'/well
 di = reciprocal of C' initial dilution used in titration (1/30)
 v = volume of diluted C' to be added
 dh = twice the volume of C' giving complete haemolysis in titration

v) *Titration of haemolysin*

Haemolysin can be obtained from commercial sources. It must be standardised by titration. The following procedure is recommended:

Prepare a 1/100 dilution of the stock haemolysin in VBS. From this, prepare 1/300, 1/400, and 1/500 dilutions in tubes. From each of these dilutions, make 0.5 ml of twofold dilutions in VBS for a block titration.

To determine haemolysin concentration, add the following to 0.5 ml of each dilution: 0.5 ml of C' at 1/30 dilution, 0.5 ml of unsensitised SRBCs at 0.25 optical density, and 1.5 ml of VBS. Incubate for 1 hour at 37°C, and then centrifuge at 400 *g* for 5 minutes. One unit of the haemolysin is the dilution that gives complete lysis of the SRBCs. The haemolysin solution is prepared in VBS at the dilution containing 2 units of haemolysin. This is then added to an equal volume of SRBCs at the proper concentration.

vi) *Titration of antigen and positive control serum*

In order to standardise the CF test, it is also necessary to have titres of both the antigen and the positive control serum. If the titre is known for either the positive serum or antigen, the titre of the other component can be determined by performing the CF test using dilutions of the component being titred. If titres of both the positive serum and antigen are unknown, a block titration (chequerboard) can be used to determine the limiting dilutions of both the antigen and the antibody where haemolysis starts. It is very critical to obtain these titres accurately.

For both the antigen and the positive control serum, 4 units are used. A unit is the highest dilution that will give a positive test. That is, if a dilution of 1/160 gives a positive test, then a 1/40 dilution has 4 units and is used for the test.

Complement-fixing antibodies usually appear within 7–10 days of infection. For a positive diagnosis, a four-fold rise in CF antibody titre is required. A presumptive diagnosis by serological tests on a flock can only be made if typical clinical signs are present and a majority of the birds have antibody titres of >1/64.

2.2. Other tests

Other serological tests have been developed, but their specificity has not yet been sufficiently evaluated. The ELISA for group-specific chlamydial antibodies is more rapid and sensitive than the CF test; it can be automated. Evaluations of ELISAs for the detection of antibodies to both *C. trachomatis* and *C. psittaci* indicate that the tests are highly sensitive but lack specificity. New tests are being developed that use peptides or recombinant antigens which may correct the specificity problem (Sachse *et al.*, 2009b).

Other tests include the agar gel immunodiffusion test (Page, 1974), the latex agglutination (LA) test, the elementary body agglutination (EBA) test (Grimes & Arizmendi, 1996; Grimes *et al.*, 1994) and the micro-immunofluorescence test (MIFT). Immunodiffusion is less sensitive than the CF test. 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 CF tests 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 CF test (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

There are no commercial vaccines available for chlamydiosis in poultry. Attempts to produce a vaccine have met with limited success, and most have been based on bacterins produced by formalin inactivation of concentrated suspensions of chlamydiae. There is evidence that immunity involves cell-mediated immune responses (Beeckman, & Vanrompay, 2010; Smith *et al.*, 2005), but vaccine manufacture has not been directed towards reactions of this type.

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NB: There is an OIE Reference Laboratory for avian chlamydiosis
(see Table in Part 4 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list:
<http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/>).
Please contact the OIE Reference Laboratories for any further information on
diagnostic tests and reagents for avian chlamydiosis