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Chlamydiosis in Birds, Wild and Domestic Animals

Pathology, Serology, Microbiology, DNA and Antigen Detection

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Contents

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1. Safety Precautions

Staff should be aware that there is a high risk of laboratory infections when working with *Chlamydia*. Aerosols are particularly infective. The avian strains are the most infectious, although human infection has also resulted from contact with diseased cats and sheep. Staff involved with chlamydial work should have serology done prior to commencing work and at yearly intervals thereafter. All work should be performed in a Class II Biohazard cabinet, using proper microbiological technique and by trained staff.

2. Overview of Diagnostic Methods for Chlamydiae

The genus *Chlamydia* consists of four species: *C. trachomatis*, *C. pneumoniae*, *C. psittaci* and *C. pecorum*. Two of these species infect birds and animals, *C. psittaci* and *C. pecorum*. *C. psittaci* has a very wide host range, infecting birds, cattle, dogs, koalas, pigs, goats and horses. The bird strains (psittacosis) also cause severe disease in humans. In addition to the wide host range in *C. psittaci*, it also results in a wide range of disease syndromes, including psittacosis, conjunctivitis, pneumonitis, infertility, abortion, enteric infection, polyarthritis and meningitis. The most recently described species, *C. pecorum*, infects sheep and cattle where it causes abortion, encephalomyelitis, polyarthritis and seminal vesiculitis. In both wild and domestic animals, chlamydial disease can result in significant reductions in fertility and production levels in the herd or population. Of particular importance are chlamydial infections in birds, which not only result in severe flock losses but also represent a significant threat to human health. The term 'chlamydiae' will be used in this document to refer to infections in birds and animals due either to *C. psittaci* or *C. pecorum*.

A positive diagnosis for *Chlamydia* should only be made by identifying the causative organism. However, isolation of chlamydiae is specialised and time consuming. Thus several non-culture methods for detecting dead chlamydial particles have recently been developed. In addition, the detection of chlamydial DNA directly in clinical specimens using the polymerase chain reaction (PCR) has become an accepted diagnostic method for both human and animal chlamydial infections. While each method offers different levels of sensitivity and specificity, the advantages of the rapid antigen detection approach has meant that this is now the most commonly used method for chlamydial diagnosis. Nevertheless, a range of methods are still used by various laboratories to diagnose chlamydial infections. These are listed as follows in general order of acceptability:

(a) detection of chlamydial antigens using fluorescent labelled monoclonal antibody kits;
(b) isolation in eggs or tissue culture;
(c) detection of chlamydial antigens by enzyme-linked immunosorbent assay (ELISA);
(d) serology;
(e) polymerase chain reaction;
(f) microscopic examination of stained smears;
(g) specific pathology.

3. Selection of Tissue Samples and Transport of Specimens

As with any infective agent, selection of the appropriate tissue samples and suitable transport and storage conditions are vital for an accurate diagnosis.

3.1. Selection of Tissue Samples

In avian chlamydiosis, the liver and spleen are the tissues of choice although other tissues, as well as faeces, may be used.

In enzootic ovine abortion and epizootic bovine abortion, the affected cotyledons in the placenta, as well as foetal lung and liver, are the best tissues for diagnosis.

In polyarthritis and sporadic bovine encephalomyelitis, many organs and tissues (including brain) may be expected to yield the organism. Synovial fluid, fibrinous material from tendon sheaths and peritoneal exudates are usually good sources. The conjunctiva should be used for smear or isolation from cases of conjunctivitis while the lungs should be sampled from cases of pneumonia.

For koalas (*Phascolarctos cinereus*) showing signs of keratoconjunctivitis, rhinitis, dirty tail disease, cystitis or infertility, swabs should be taken from the conjunctiva, nasal septum, bladder, ureter, urethra, vagina, uterus, Fallopian tube, ovary or ovarian cysts.

For all isolations and identifications to be successful it is important that the tissue is swabbed vigorously. This may require the use of a local anaesthetic.

3.2. Transport and Storage of Specimens

Tissue samples or swabs should be placed directly into transport medium. A suitable transport and storage medium for chlamydial culture is sucrose phosphate glutamate (SPG) (Spencer and Johnson, 1983). SPG medium is prepared as follows.

3.2.1. Sucrose Phosphate Glutamate Transport and Storage Medium

Sucrose	220 mmol/L
Dipotassium hydrogen phosphate, K_2HPO_4	7 mmol/L
Glutamic acid, $C_6H_9NO_4$	5 mmol/L
Foetal calf serum (FCS)	10%
Gentamicin	100 µg/mL
Vancomycin	100 µg/mL
Amphotericin B	2 µg/mL

Adjust pH to 7.2 and sterilise by filtration. Store at -20°C.

Providing isolations can be made within 24 hours of collection, the specimens may be stored at 4°C. Otherwise they should be immediately frozen and stored at -70°C. Viability is retained for several years at -70°C, but is much less certain at -20°C. For use, the specimens are thawed rapidly at 37°C and held on ice for up to one hour prior to testing.

4. Detection of Chlamydial Antigens Using Commercial Fluorescent Monoclonal Antibody Kits

Several antigen detection kits for the human pathogen, *C. trachomatis*, have recently become commercially available. Some of these use *C. trachomatis*-specific antibodies and are, therefore, of no use for *C. psittaci* detection. Others, however, use antibodies to group specific antigens (lipopolysaccharide) and are suitable for the detection of all the chlamydial species. All kits contain a fluorescein-labelled monoclonal antibody. A smear of the sample is made on a glass slide which is then fixed with acetone (C_3H_6O) or methanol (CH_3OH). This inactivates the *Chlamydia* and makes the rest of the test non-infectious. The antibody is reacted with the slide (15–30 min, 37°C, moist chamber) and binds to any chlamydial antigens which are present (both viable and non-viable). After a series of washings in PBS, the brightly fluorescing, apple green dots (chlamydial elementary bodies) are easily visualised with a fluorescent microscope.

The test is less sensitive than yolk sac inoculation and cell culture, but does not require viable *Chlamydia*, is less technically demanding and produces results in less than one hour, compared to 3–10 days for culture. It is applicable to all chlamydiae. Kits are commercially available for this purpose from several suppliers including:The Boots Company (Imagen), Pacific Diagnostics (Bartels Microscan Chlamydia), Commonwealth Serum Laboratories (Bio Merieux Chlamydia Direct IF), Difco (Chlamydia Direct FA Detection System) and Cellabs (Chlamydia — Cell VET).

5. Isolation in Eggs or Tissue Culture

Chlamydiae can be isolated and grown in the yolk-sac of embryonated hen eggs or in mammalian cell culture. Most laboratories prefer cell culture because it is less tedious, less prone to contamination, more applicable to large sample numbers and results in characteristic inclusion morphology which may be used for typing of chlamydiae strains. Nevertheless, growth in embryonated hens eggs is very sensitive and is applicable to all strains of chlamydiae. As a consequence, many laboratories still use this procedure.

5.1. Isolation in Embryonated Hen Eggs

Six- to eight-day-old embryonated hens eggs should be used. A suspension (0.1–0.5 mL) of the test sample is prepared in SPG transport medium and is used to inoculate the yolk sac. The inoculated eggs are incubated at 35–37°C, not rotated, in a humidified incubator. The eggs should be candled after 48 hours and any deaths (due to trauma) are discarded.

Continue candling daily from day five to day 14. When deaths are observed, prepare an impression smear of the yolk sac and yolk sac stalk, and stain with Macchiavello, Castaneda, or Giemsa stain. Examine under a light microscope (x1000) for characteristic chlamydial elementary bodies.

If no deaths occur on the first passage, at least one blind passage should be done before deciding that the sample is negative.

5.2. Isolation in Mammalian Cell Culture

A range of mammalian cell lines, including McCoy, L, BHK and BGM cells, may be used. The most common procedure employs a monolayer of cells growing on a 13 mm diameter glass coverslip in the bottom of a 5 mL plastic vial. The chlamydial inoculum is centrifuged onto the cell monolayer which is then made non-replicative by the inclusion of cycloheximide in the growth medium. The vials, plus coverslips, are incubated at 37°C in an atmosphere of 5% carbon dioxide for three to seven days. Representative coverslips are removed, stained with Giemsa and examined for characteristic chlamydial inclusions. A recent improvement has seen the use of fluorescein-labelled monoclonal antibody staining in place of the Giemsa step. A detailed isolation protocol using BGM cells is given below.

5.2.1. Isolation of *Chlamydia* for Diagnostic Staining

Small scale culture is done in 5 mL, flat-bottomed polystyrene vials (SCS 501 4H, Bunzl, Seaton, SA, Australia) containing sterile 13 mm diameter glass coverslips. Individual vials, plus coverslips, are seeded with 2×10^5 BGM or McCoy cells, in 1 mL of growth medium (GM = Dulbecco modified Eagle medium containing 10% FCS, 100 µg/mL gentamicin, 100 µg/mL vancomycin and 2 µg/mL amphotericin B, pH 7.2) and incubated overnight at 37°C in 5% carbon dioxide, with caps loosened. This results in a just-confluent layer of cells within 16–20 hours. Aliquots (0.1 mL) of chlamydial inoculum, either undiluted or diluted in SPG transport medium, are added and the vials centrifuged at 2000 *g* for 60 min at 32°C (IEC bench top centrifuge). The centrifuged vials are then placed in a carbon dioxide incubator, the caps loosened, and incubated for a further two hours at 37°C, 5% carbon dioxide.

The supernatant fluid is decanted and replaced with 1 mL of warm, fresh GM containing 0.5 µg/mL cycloheximide, and reincubated at

37°C in 5% carbon dioxide. For cycloheximide-sensitive strains the cycloheximide medium is replaced with normal GM after 24–48 hours incubation. At regular intervals, coverslips are recovered from representative cultures and stained with a fluorescent labelled monoclonal antibody (see 4.). Background cells usually stain brown–red whereas chlamydial inclusions and free elementary bodies fluoresce brightly when examined by fluorescence microscopy. When performed by a qualified person, with access to good, properly transported specimens, cell culture followed by fluorescent antibody staining is still the most sensitive and specific diagnostic approach.

These culture conditions give good growth of all avian, koala conjunctivitis, ovine abortion and ovine polyarthritis strains but it is necessary to modify the conditions to obtain adequate growth of sporadic bovine encephalomyelitis and feline conjunctivitis strains. For these latter strains, the cell monolayer is treated with 20 µg of DEAE dextran/mL for 30 min at 37°C, before the addition of the chlamydial inoculum. In addition, cycloheximide is not included in the GM.

6. Detection of Chlamydial Antigens by Enzyme-linked Immunosorbent Assay

ELISA detection of chlamydial antigens is simply a modification of fluorescent antibody detection. The same genus-specific antibodies described in 4. are used, except that instead of labelling them with a fluorescent dye and examining the slide under a fluorescent microscope, the antibodies are labelled with an enzyme which acts on a suitable substrate to give a colour change. This colour change can either be recorded spectrophotometrically (plate ELISA) or visually by eye (solid phase ELISA). Once again, there are several commercially available kits for ELISA detection of chlamydiae. The most commonly used plate ELISA kits are: Pharmacia (Chlamydia EIA), Kallestad Diagnostics (Pathfinder), Wellcome (Wellcozyme Chlamydia), Carter-Wallace (Visuwell Chlamydia), Syva (Microtrak) and Boots Company (IDEIA). Most ELISA assays follow a basic procedure which usually takes 4–6 hours to obtain a result and is best suited to large batches of samples.

The more recent development in chlamydial diagnosis is the use of rapid, solid phase, visual ELISA assays. These take about 30 min to complete and can be read visually. They have proven to be sufficiently sensitive and specific for the detection of chlamydial infections in birds and koalas (Wood and Timms, 1992). The two most common solid phase ELISAs are: Oxoid (Chlamydia Clearview) and Kodak (Surecell).

7. Serology

Evidence of current or previous chlamydial infections may be provided by detecting specific chlamydial antibodies. A variety of serological tests are available for this purpose [complement fixation (CF), indirect immunofluorescence, ELISA]. These tests merely demonstrate the presence of a previous chlamydial infection. Evidence of a rising titre, using paired sera, is necessary to demonstrate an active infection.

The most commonly used method is the complement fixation test. This test detects antibodies to the chlamydial genus-specific antigen (Dhir *et al.*, 1972) and, therefore, is of use in detecting all chlamydial infections. Suitable antigen for the test can be prepared from infected yolk sac (Topping and Sheppard, 1946) or purchased from Commonwealth Serum Laboratories.

Recent evidence, however, suggests that the CF test is insensitive for particular hosts such as koalas. In these cases, indirect immunofluorescence using the commercially available *Chlamydia psittaci*-spot IF reagent (bioMerieux) is recommended. This reagent is also commonly used by hospitals who wish to determine if human infections are due to *C. trachomatis*/*C. pneumoniae*, or are due to a bird or animal source (*C. psittaci*). Nevertheless, the CF test is still useful, particularly for testing such diverse hosts as birds, humans, cats, sheep, cattle and koalas.

7.1. Complement Fixation Test for Chlamydia

The test uses several reagents (chlamydial group complement fixing antigen and control, haemolysin, guinea pig complement, sensitised sheep red blood cells, diluent, positive control serum and test serum) which may require preparation, standardisation or titration, prior to use in the actual test. It is recommended that the tests and all standardisations be done in 96-well, round-bottomed microtitre plates.

7.1.1. Heat Inactivation of Sera

All sera should be inactivated by heating at 56°C for 30 min.

7.1.2. Standardisation of Haemolysin and Complement

Before using a new batch of either haemolysin or complement, both should be block titrated as follows.

7.1.2.1. Haemolysin dilutions

(a) Set up eight tubes and add 0.4 mL of diluent to tubes 2–8.
(b) Add 0.4 mL of 1:100 haemolysin to tubes 1 and 2 and sequentially dilute from tube 2 to tube 7. Tube 8 remains as a control.
(c) Add 0.4 mL of 3% sheep red blood cells to all tubes.
(d) Mix thoroughly and incubate in a water bath at 37°C for 30 min. Store at 4°C until required.

7.1.2.2. Complement dilutions

(a) Set up eight tubes and add 1.5 mL of diluent to tube 1 and 0.4 mL to tubes 2–8.
(b) Add 50 µL of complement to tube 1, and sequentially transfer 1.2 mL from tube 1 through to tube 7. Tube 8 remains as a control.

7.1.2.3. Block titration

(a) To 64 wells (8 x 8) of a microtitre plate add 50 µL of diluent.
(b) Add 25 µL of each complement dilution to all wells of a vertical column.
(c) Add 25 µL of each sheep red blood cell haemolysin dilution to all wells of a horizontal row.
(d) Mix and incubate at 37°C for 30 min, mixing at 15 min.
(e) Centrifuge and read the plate.

7.1.2.4. Interpretation of results

The optimal sensitising dose of haemolysin is the dilution giving the most lysis with the highest dilution of complement.

The highest complement dilution giving 100% lysis with the optimal sensitising dose of haemolysin is taken as the end point and is equal to one unit of complement. Five units of complement is usually suitable for diagnostic purposes.

7.1.3. Standardisation of Antigen

The quantity of antigen required for use in the test is that quantity which will fix the greatest amount of complement in the presence of the least amount of antibody. This will render the test as sensitive as possible.

7.1.3.1. Antigen dilutions

(a) Set up six tubes and add 0.3 mL of diluent.
(b) Add 0.3 mL of antigen to tube 1 and sequentially transfer 0.3 mL from tube 1 through to tube 6.

7.1.3.2. Antiserum dilutions

(a) Set up six tubes and add 0.3 mL of diluent to tubes 2 through to tube 6.
(b) Prepare a 1:4 dilution of antisera and add 0.3 mL to tube 1, and 0.3 mL to tube 2. Sequentially transfer 0.3 mL from tube 2 through to tube 6.

7.1.3.3. Block titration

(a) Add 25 µL of the appropriate antigen dilutions to all wells of the horizontal rows A–F.
(b) Add 25 µL of the appropriate antiserum dilution to all wells of the vertical columns 1–6.
(c) Add 25 µL of diluent to row G and column 7. These are control rows.
(d) Add 25 µL of complement to all wells.
(e) Mix thoroughly and incubate for one hour at 37°C.
(f) Add 25 µL of sensitised 1.5% red blood cells, mix and incubate at 37°C for 30 min.
(g) Centrifuge the plate and read the results.

7.1.3.4. To read plate

Read the highest antigen–antiserum dilution combination that is not subject to anticomplementary action of either component.

7.1.4. Complement Fixation Test

Each serum should be tested in duplicate, with both positive antigen and negative control antigen. These antigens are usually tested in separate plates, as follows (Fig. 1). Columns 1–10 are for test sera, column 11 is for a known positive control serum and column 12 is for a known negative control serum.

7.1.4.1. Procedure

(a) Add 25 µL of diluent to all rows.
(b) Add 25 µL of heat inactivated serum to rows A and B.
(c) Serially dilute from row B to row H (25 µL transferred).
(d) Add 25 µL of complement fixing antigen to rows B through to row H (plate 1).
(e) Add 25 µL of control antigen to rows B through to row H (plate 2).
(f) Add 25 µL of diluted complement to all rows.
(g) Mix thoroughly, incubate at 37°C for 60 min.
(h) Add 25 µL of 1.5% sensitised red blood cells to all rows.
(i) Mix thoroughly, incubate at 37°C for 30 min, mixing at 15 min.
(j) Centrifuge trays and read the results.

7.1.4.2. Interpretation

A true positive is read when a serum reacts with the positive antigen and not with the control antigen. Non-specific reactions may be seen in some sera which react with both antigen preparations. A reaction of 2+ or greater at a dilution of 1:4 is taken as positive if there is no reaction with the negative control antigen. The complement fixing antibody titre is calculated as the highest dilution of serum in which no more than 50% of the erythrocytes are lysed.

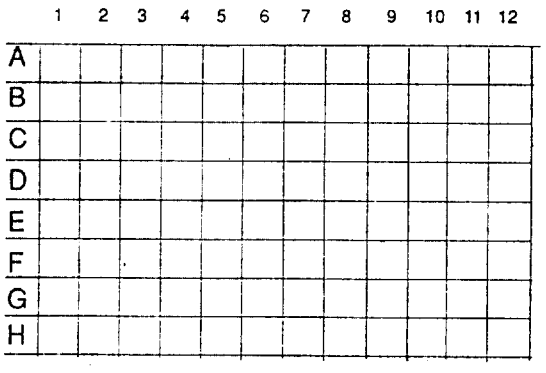

Figure 1. Plate lay out (separate identical plates are used for positive and negative control antigen).

8. Polymerase Chain Reaction

The genetic detection of human chlamydial infections has advanced to the stage where DNA probe assays are now the routine method for diagnosis for some hospital and pathology laboratories. A single DNA probe assay (Gen-Probe) is commercially available for the detection of *C. trachomatis*. However, the target RNA sequence is specific for *C. trachomatis* and, therefore, this assay is not suitable for the other three chlamydial species.

The PCR has advanced from a research tool to the stage where it can now make a significant contribution to chlamydial diagnosis. PCR is exquisitively sensitive and can be designed to be highly specific (Rasmussen and Timms, 1991). As more laboratories incorporate PCR technology for use with other pathogens, it will also become more widely used for the diagnosis of chlamydial infections. PCR is presently used to detect *C. psittaci* infections in birds, koalas, sheep, cattle and cats.

9. Microsopic Examination of Stained Smears

Microscopic examination of histochemically stained specimens of tissue, swab material or exudate is a useful diagnostic procedure during the peak of the chlamydial infection (Meyer and Eddie, 1956). However, the small (0.3 µm) chlamydial elementary bodies lack characteristic morphology, which makes them difficult to detect when they are present in low numbers. In these cases, it is necessary to use an enhancement procedure such as fluorescein-labelled specific antibody (see 4.). A range of histochemical stains, such as Macchiavello, Castaneda, and Giemsa, have been used successfully. The procedure for Macchiavello's stain is given below.

9.1. Macchiavello's Stain (for Rickettsiae and Chlamydia*)*

9.1.1. Preparation of Stain

Basic fuchsin (90% dye content) (0.05 g) dissolved in 100 mL distilled water, buffered at pH 7.2–7.4 with 4 mL of 0.07 mol/L phosphate buffer prepared as follows.

Potassium dihydrogen phosphate, KH_2PO_4	9.078 g
Disodium hydrogen phosphate, $Na_2HPO_4.12H_2O$	11.876 g
Distilled water	1 L

Mix in the proportion of 19 parts of the first to 81 parts of the second to obtain a buffered mixture of pH 7.4.

(a) Prepare an impression smear by touching the piece of tissue to the slide.
(b) Dry in air and fix by gentle heating.
(c) Pour the above staining fluid onto the slide through a coarse filter paper (No. 1). Allow to stand for five minutes.
(d) Rinse very rapidly with 0.5% aqueous citric acid ($C_6H_8O_7$) in a Coplin jar.
(e) Wash quickly and thoroughly with tap water.
(f) Counterstain for 10 s with 1% aqueous methylene blue in a Coplin jar.
(g) Rinse in tap water.
(h) Dry and examine using light microscopy (x1000).

9.1.2. Interpretation

Small (0.3 µm) elementary bodies stain bright red; larger, immature forms take up counterstain and stain blue. Characteristic clusters of several hundred spherical *Chlamydia* staining red and blue (located in the cytoplasm of cells) are most characteristic. Cell nuclei stain deep blue and the cytoplasm stain light blue.

10. Pathology

Although gross pathology and histopathology often give an indication that the cause of the disease is *Chlamydia*, the lesions are not pathognomonic (apart from the demonstration of elementary bodies in the lesions by the staining referred to above, see 9.).

Pathology cannot be regarded as a substitute for direct parasite detection in the diagnosis of the chlamydial diseases.

Lesions which may be caused by *Chlamydia* include encephalomyelitis, polyarthritis, polyserositis, pneumonia, enteritis, hepatitis, nephrosis, splenomegaly, splenitis, placentitis and foetal lesions.

11. Differentation of Chlamydial Species and Strains

There are four species in the genus *Chlamydia:* *C. trachomatis* and *C. pneumoniae* infect humans; *C. psittaci* and *C. pecorum* infect birds, wild and domestic animals (and sometimes humans); *C. pecorum* infects sheep and cattle. During a disease outbreak it may be necessary to confirm an isolate as being *C. psittaci* or perhaps even to assign it to a particular subgroup.

11.1. Differentiation of Chlamydia trachomatis *from other* Chlamydia

The differentiation of *C. trachomatis* from the other chlamydial species is based on two tests.

(a) The presence of glycogen in chlamydial inclusions (determined by staining with iodine):
C. trachomatis = inclusions stain with iodin;. other chlamydial species = inclusions do not stain with iodine.
(b) Sensitivity to sodium sulfadiazine:
C. trachomatis = sensitive to sulfadiazine; other chlamydial species = not sensitive to sulfadiazine.

Procedures for these tests are given by Page (1968). The non-C. *trachomatis* species (C. *psittaci*, C. *pecorum* and C. *pneumoniae*) can only be truly differentiated from each other by DNA homology/sequence analysis.

11.2. *Differentiation of* Chlamydia psittaci *Strains and Subgroups*

Several attempts have been made to subgroup chlamydiae strains using techniques such as: microimmunofluorescence (Schachter *et al.*, 1974; Spears and Storz, 1979*a*; Perez-Martinez and Storz, 1985); biotyping in cell culture (Spears and Storz, 1979*b*); restriction endonuclease analysis (Peterson and de la Maza, 1983; Herring *et al.*, 1987; Timms *et al.*, 1988*a*); DNA probe analysis (Timms *et al.*, 1988*b*, 1988*c*) and PCR analysis (Rasmussen and Timms, 1991). While most of these techniques are only available in research laboratories they have already proved useful in identifying the source, and disease potential to humans, of several chlamydial strains (Herring *et al.*, 1987) .

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