Chlamydial infections in birds and animals

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Summary

Chlamydiae are important pathogens of a range of birds and animals including sheep, cattle, pigs, cats, goats, koalas, other Australian marsupials, amphibians and reptiles. Recent changes to chlamydial taxonomy has seen the proposal of two genera, *Chlamydia/Chlamydophila* with nine species – *C trachomatis*, *C muridarum*, *C suis*, *C psittaci*, *C pecorum*, *C abortus*, *C caveae*, *C felis* and *C pneumoniae* – eight of which can infect and cause disease in animals.

Avian chlamydiosis affects a wide range of psittacines as well as ducks and turkeys. Infected birds may seem healthy but shed chlamydiae in respiratory exudates as well as in their droppings. Chlamydial disease is quite common in sheep, cattle and pigs in many countries of the world (notably Europe and USA), but disease levels are much lower in Australia and New Zealand. Nevertheless, chlamydial polyarthritis of lambs, abortion in sheep and sporadic bovine encephalomyelitis in cattle have been reported in Australia. While porcine infections with *Chlamydia/Chlamydophila* are common in Europe, they have not been reported in Australia or New Zealand. Cats are also infected with *Chlamydia/Chlamydophila* (*C felis*) where it primarily causes conjunctivitis. Most populations of koalas are infected with *Chlamydia/Chlamydophila*. *C pecorum* is the most common chlamydial species infecting koalas and is thought to be responsible for most of the clinical disease, although *C pneumoniae* is also widespread in many populations. In koalas, chlamydial infection can result in keratoconjunctivitis, genital tract infection (both males and females) leading to ovarian cysts in the females, urinary tract infection (both males and females) leading to urinary incontinence and the so-called 'wet bottom' disease, and respiratory infection leading to pneumonia in some cases.

A wide range of test methods have been and still are used to diagnose chlamydial infections, including (a) microscopic examination of stained smears, (b) specific lesions, (c) isolation of the agent in embryonated hens eggs or in eukaryotic cell lines, (d) antibody detection, by indirect immunofluorescence, indirect ELISA or complement fixation test (CFT), (e) antigen detection, by microimmunofluorescence, plate-based ELISAs or solid-phase ELISAs, and (f) polymerase chain reaction (PCR). All methods have their uses in specific circumstances. The older methods of microscopic examination of stained smears or of specific lesions are used much less frequently now, as they are technically demanding and often imprecise. Serology is still a convenient and commonly used technique, with the CFT being the most commonly used serological assay, even though it is acknowledged to have a sensitivity that is well below 100%. Cell culture in eukaryotic cell monolayers is a very useful technique if further work, such as speciation, is required; however

the technique is technically demanding and has demanding transport requirements. As a result, cell culture is restricted to a few laboratories. Antigen detection using either plate-based ELISAs or solid-phase ELISAs has several advantages over cell culture, although the sensitivities of these assays are again less than 100% and have been reported to vary between hosts. One commercially available solid-phase ELISA is the Clearview® *Chlamydia* assay, which detects the Chlamydiale*s* family-specific LPS antigen. While not as sensitive as some other immunoassays, this assay is rapid and technically easy to perform, allowing a veterinarian or diagnostic laboratory to perform a rapid screen prior to subsequent PCR testing. PCR is both sensitive and specific and also enables subsequent speciation and sub-typing by RFLP analysis, High Resolution Melt (HRM) analysis or by sequencing of the PCR product, and as a consequence, is becoming the diagnostic test of choice.

Part 1 - Diagnostic overview

The rapid and accurate diagnosis of chlamydial infections is an essential aspect of reducing the burden of disease caused by these organisms. There are two main approaches to the diagnosis of chlamydial infections in birds and animals. The first is the use of serology to detect the presence of anti-chlamydial antibodies in serum, while the second is the direct detection of the organism in tissues or swab samples. The test that is used depends directly on the type of specimen that is submitted to the diagnostic laboratory and on the range of laboratory tests available. This document provides an overview of the range of diagnostic tests currently available, including antibody detection, growth of viable *Chlamydia* in hens eggs or in cell monolayers, use of stains for the direct detection of antigens in tissue sections, use of immunoassays for the detection of chlamydial antigens in swab samples or tissues, and the use of PCR to detect chlamydial DNA.

Aetiology, clinical signs and epidemiology

Taxonomy and nomenclature : The family Chlamydiaceae consists of a diverse group of obligate intracellular bacterial pathogens that infect a wide range of birds and animals, as well as humans.[1] Chlamydiae have been through several nomenclature changes over the last 40 years and are currently in a state of uncertainty. Chlamydial strains were originally classified into two species: (i) *Chlamydia trachomatis,* which infects humans, causing diseases such as conjunctivitis leading to trachoma and sexually transmitted disease leading to infertility, and (ii) *Chlamydia psittaci,* which infects birds and animals, resulting in conditions such as psittacosis, abortion, arthritis, conjunctivitis, infertility and respiratory conditions.[2] The development of DNA-based analysis in the 1980s led to the recognition of two additional species, (a) *Chlamydia pneumoniae,* which primarily infects humans[3] and (b) *Chlamydia pecorum,* which infects ruminants.[4]

In 1999, a re-organisation of the order *Chlamydiales* into two genera, *Chlamydia* and *Chlamydophila*, based on a range of phenotypic, bio-ecological and genotypic data, was proposed.[5] At present, therefore, the family Chlamydiaceae comprises three species in the genus *Chlamydia* (*C trachomatis* – humans, *C muridarum* – mice and *C suis* – pigs) and six species in the genus *Chlamydophila* (*C psittaci* – birds and humans; *C pecorum* – sheep, cattle, koalas; *C abortus* – sheep, cattle; *C caviae* – guinea pigs; *C felis* – cats; *C pneumoniae* – humans, marsupials, reptiles) (Table 1). While most chlamydiologists accept this division into nine species as being logical and useful, the community is split over the proposal for two genera[6] and as a result both genus-level terminologies are in common use. Many veterinary chlamydiologists have adopted the new 'two genus' terminology, mainly because it places most of the veterinary species in the *Chlamydophila* genus, but there is still no universal usage of the term *Chlamydophila*.

A sub-committee of the International Committee on Systematics of Prokaryotes was formed in 2009 to re-examine the taxonomy and nomenclature issues; however for the rest of this ANZSDP '*C*' will be used to represent both *Chlamydia* and *Chlamydophila,* to avoid further confusion.

Table 1. Taxonomy of Chlamydiae

Systematics	Major natural host	Pathogenesis
Order Chlamydiales		
Family I: Chlamydiaceae		
Genus I : *Chlamydia*		
C trachomatis	Humans	Ocular, urogenital infections
C muridarum	Rodents	Respiratory, ocular, urogenital infections
C suis	Pigs	Enteritis
Genus II : *Chlamydophila*		
C psittaci	Birds, humans	Avian chlamydiosis
C abortus	Ruminants	Abortion
C pecorum	Cattle, sheep, koalas	Enteritis, abortion, polyarthritis, ocular, and/or urogenital infections
C felis	Cats	Ocular, respiratory infections
C caviae	Guinea pigs	Ocular, urogenital infections
C pneumoniae	Humans, horses, koalas	Respiratory, ocular, urogenital infections

Birds: Avian infections are not only the most common but also probably the most important non-human chlamydial infections worldwide. *C psittaci* is a highly pathogenic species that causes avian chlamydiosis, which is also referred to as psittacosis in psittacines and ornithosis in other bird species. It infects a wide range of species and has been reported in pigeons and psittacines. While published Australian and New Zealand data are sparse, infection levels in birds are thought to be between 10 and 15%. Transmission to humans, which occurs frequently, can lead to severe cases of respiratory disease. In addition to psittacine birds, domestic poultry, pigeons and wild birds can also be infected with virulent strains and can serve as a source of human infection. Infected birds may seem healthy, but can shed *C psittaci* organisms in their excreta, including in nasal secretions. Transmission to humans can occur when the agent is inhaled from bird secretions, desiccated

droppings or dander. The clinical presentation of psittacosis in humans may include fever, malaise, headache, myalgia and chills. Untreated psittacosis has a reported case-fatality rate of 15-20% making early rapid and accurate diagnosis imperative. Psittacosis is most commonly reported among people in close contact with domestic birds, such as bird owners, poultry farmers (although not in Australia or New Zealand to date), veterinarians and workers within pet shops and poultry processing plants.[7] Additional information on avian chlamydiosis is available at http://www.oie.int/eng/normes/mmanual/2008/pdf/2.03.01_AVIAN_CHLAMYD.pdf

Sheep, goats, cattle and pigs : Chlamydial disease in sheep, cattle and pigs is commonly reported in the UK and Europe. In these countries, chlamydial infections commonly cause abortions in sheep (*C abortus*), enteritis, pneumonia, conjunctivitis, pericarditis, perinatal mortality and reproductive disorders in pigs (*C suis*), enteric infections, polyarthritis, conjunctivitis, pneumonia, metritis and encephalomyelitis in sheep and cattle (*C pecorum*).[8] In comparison with these high rates of infection and disease in the UK and Europe, infection levels in Australia are much lower, although they do occur. Outbreaks of ovine polyarthritis have been reported in NSW[9] and these were attributed to chlamydial infection, based on consistently rising or high CFT titres. Affected lambs were depressed, pyrexic and markedly lame. While the species of *Chlamydia* involved is usually not determined, as yet *C abortus* has not been detected in Australia, whereas *C pecorum* has been detected and linked to sporadic ovine abortions and small outbreaks, again in NSW.[10] Late-term abortions in a flock of Dorper sheep at Narrabri in NSW were diagnosed as *C pecorum* based on cell culture and PCR analysis. Histological examination of tissues from these aborted foetuses revealed inflammation and necrosis of blood vessels in the placenta. The reason for these differences between Australia and USA/UK is unknown, but the differences in farming practices may be one explanation, as these sheep are often housed in sheds or barns or on fields of lush pasture in the UK, whereas in Australia and New Zealand they are usually reared on expanses of grassland. These differences make transmission between sheep more likely in the UK and less likely in Australia and New Zealand. It is also possible though that the more virulent strains of *C abortus* are apparently not present in Australasia.

Chlamydial arthritis is a systemic infection in sheep with localisation in various organs, especially the serous membranes. Infection probably starts in the alimentary tract and transmission is probably via the oral-faecal route. Chlamydial polyarthritis occurs in all countries, including Australia, although it is seen at much higher rates in Europe. It usually affects lambs less than 6 months of age, although one outbreak in Queensland involved 13-month-old rams.[11] Rapidly growing lambs on irrigated pasture may be more susceptible than similar lambs on dry pasture.[12] While it is not known how infection spreads, chlamydiae have often been demonstrated in the faeces of normal sheep.[13] The other major chlamydial disease of sheep is abortion although it has only been reported with uncertainty, and infrequently in Australia.[14] It usually affects ewes during their first or second pregnancy as abortion late in pregnancy and dead or live lambs born prematurely or dead lambs born at term. Enzootic abortion has not been reported in New Zealand where active and passive surveillance is undertaken for *C abortus*. For additional information see http://www.oie.int/eng/normes/mmanual/2008/pdf/2.07.07_ENZ_ABOR.pdf

An infection of cattle, which may be reasonably widespread, occasionally causes sporadic bovine encephalomyelitis as overt disease. Clinical signs may include a transient fever, inappetence, depression and death, but can be vague and inapparent. The mode of transmission and the epidemiology in cattle, and the susceptibility of humans are unknown.

Koalas : *Chlamydia/Chlamydophila* is the most common infection in koalas. It is responsible for four major disease syndromes: (i) keratoconjunctivitis, (ii) genital tract infection, (iii) urinary tract

infection, and (iv) respiratory tract infection.[15] Most free-ranging populations are infected although the level of clinical disease varies considerably. It is widely accepted that host or environmental factors that lead to increased stress levels for the animal result in more severe signs of clinical disease. These stress factors can include (a) lack of adequate food trees, (b) encroachment by urban sprawl and housing developments, (c) roads and domestic dogs, and (d) infection with the koala retrovirus. Koalas are infected, not with *C psittaci* as previously thought, but with *C pecorum*, the commonest infection, which is responsible for most clinical disease; *C pneumoniae*, which can cause respiratory infections and pneumonia; and novel chlamydiales, whose role in causing disease is unknown. Many koalas in zoos or sanctuaries are also infected with *Chlamydia/Chlamydophila*, although the level of infection (as assessed by PCR) is usually very low and obvious signs of clinical disease are usually absent as a result of good management practices.

Other hosts : Chlamydial infections have been reported in several other animal species in Australia[16] including cats (conjunctivitis[17]), bandicoots (conjunctivitis[18]), goats[19] and recently in crocodiles[20]. While infection levels are often low with no clinical signs of disease, any significant change in environmental conditions can result in clinical chlamydiosis occurring.

Part 2 – Diagnostic test methods

Safety precautions

Staff should be aware that there is a risk of laboratory infections when working with *Chlamydia/Chlamydophila*. The highest risk is associated with handling samples from birds that may have *C psittaci* infections. Aerosols are particularly infective. While the avian strains are the most infective, there are reports of people becoming infected with strains from sheep with abortion or cats with conjunctivitis. It is recommended that all handling of samples, but definitely avian samples, should be performed in a Class II Biological Safety cabinet, using proper microbiological technique and by adequately trained staff.

Background to the tests available

A wide range of test methods have been and still are used to diagnose chlamydial infections, including (a) microscopic examination of stained smears; (b) specific pathology; (c) isolation of the causal agent in embryonated hens eggs or in eukaryotic cell lines; (d) antibody detection, by indirect immunofluorescence, indirect ELISA or complement fixation test; (e) antigen detection, by microimmunofluorescence, plate-based ELISAs or solid-phase ELISAs; and (f) PCR. All methods have uses in specific circumstances. The older methods of microscopic examination of stained smears, or specific pathology are much less frequently used now as they are technically demanding and often imprecise. Serological tests are still a convenient and commonly used, with the CFT being the most commonly used test, even though it is acknowledged to have a sensitivity significantly less than 100%. Cell culture in eukaryotic cell monolayers (for example, McCoy cell line) is a very useful technique if further work, such as speciation, is required; however the technique is technically demanding and the sample must be kept chilled at 0-4°C for no longer than 24 hours from time of collection to receipt by the diagnostic laboratory. To enable the best chance of success with cell culture isolation of *Chlamydia*, it is essential to collect fresh tissue samples or to process swabs freshly collected from *animals suspected of being infected with Chlamydiae.* As a result, cell culture is restricted to a few laboratories. Antigen detection using either plate-based ELISAs or solid-phase ELISAs has several advantages over cell culture, although the sensitivities are again less than 100% and have been reported to vary between hosts. One commercially available solid-phase ELISA is the

Clearview® *Chlamydia* assay, which detects the Chlamydiaceae family-specific lipopolysaccharide (LPS) antigen. While not as sensitive as some other immunoassays, and definitely less sensitive than PCR, this assay is rapid and technically easy to perform, allowing a veterinarian or diagnostic laboratory to perform a rapid screen prior to subsequent PCR testing on suitable samples from certain species, especially when PCR is not immediately available. PCR is both sensitive and specific and also enables subsequent speciation and sub-typing e.g. by sequencing of the PCR product. It is becoming the diagnostic test of choice.

Antibody detection

The use of serological tests to detect antibodies to *Chlamydia/Chlamydophila* can be useful in making a diagnosis. However, if animals have had an infection some time previously, then this will complicate the interpretation. Equally, collection of serum samples too early in an infection will not enable an antibody response to have developed sufficiently. It is preferable therefore to collect any serum sample at or just after the peak of infection. Infected animals make antibodies against a range of chlamydial antigens and the antibodies can be detected by one of three tests; (a) indirect immunofluorescence test (MIF), (b) indirect ELISA, or (c) CFT. Serological tests have several advantages over some of the more specific and sensitive tests mentioned later, including (a) a blood sample is all that is required, making sample collection and transport relatively straight forward; (b) the presence of antibodies to chlamydial antigens can be used to indicate current or recently past infections, making the timing of the collection of the sample less critical; and (c) the technology required to conduct the tests is usually readily available in most laboratories. Serological tests do, however, have some disadvantages, such as (a) they require species-specific reagents, for example, anti-species Ig, which might be available for some hosts, such as sheep and cattle, but are not readily or commercially available for others, such as koalas; and (b) they require batches of purified chlamydial antigen to be available. Very few laboratories have the facilities to prepare such antigen. There is also the issue of having separate antigens for each chlamydial species available because cross-reactivity is sometimes unreliable. Nevertheless, the most commonly used serological assay is the CFT. Despite general acknowledgement that the CFT is considerably less than 100% sensitive, it is still used and hence the procedure is described below. A range of ELISA tests have also been reported[21] and these target whole chlamydial organisms, LPS, or the chlamydial major outer membrane protein (MOMP). The Immunocomb test (Biogal) is also used by some avian specialists. While these have produced useful results in the hands of the groups that developed them, they have not become widely used as yet.

Diagnosis of *Chlamydia/Chlamydophila* by the Complement Fixation Test (CFT)

A detailed description of the chlamydial CFT test procedure is given in http://www.oie.int/eng/normes/mmanual/2008/pdf/2.03.01_AVIAN_CHLAMYD.pdf, which should be consulted if more details are required. Nevertheless, a reasonably detailed procedure is provided here.

Principle of the test

The CFT detects antibodies to the chlamydial genus-specific antigen and therefore is of use in detecting all chlamydial infections. Suitable antigen for the test can be prepared from infected yolk sac. The CFT is used with some animal species including birds, humans, cats, sheep and cattle, but may be insensitive for other species including koalas.

Reagents and materials

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 standardisation be performed in 96-well, round-bottomed microtitre plates. All sera should be inactivated by heating at 56°C for 30 min.

Test procedure

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

Haemolysin dilutions

- Set up 8 tubes and add 0.4 mL of diluent to tubes 2-8.
- Add 0.4 mL of 1:100 haemolysin to tubes 1 and 2 and sequentially dilute in two-fold steps from tube 2 to tube 7. Tube 8 remains as a control.
- Add 0.4 mL of 3% sheep red blood cells to all tubes.

Mix thoroughly and incubate in a water bath at 37°C for 30 min. Store at 4°C until required.

Complement dilutions

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

Block titration

- To 64 wells (8 x 8) of a microtitre plate, add 50 µL of diluent.
- Add 25 µL of each complement dilution to all wells of a vertical column_
- Add 25 µL of each sheep red blood cell haemolysin dilution to all wells of a horizontal row.
- Mix and incubate at 37°C for 30 min, mixing at 15 min.
- Centrifuge at 400 *g* for 5 min and read the plate.

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.

Standardisation of Antigen

The quantity of antigen required for use in the test is that quantity that 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.

Antigen dilutions

- Set up 6 tubes and add 0.3 mL of diluent to each tube.
- Add 0.3 mL of antigen to tube 1 and sequentially transfer 0.3 mL from tube 1 to tube 6.

Antiserum dilutions

- Set up 6 tubes and add 0.3 mL of diluent to tubes 2 through to tube 6.
- 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 to tube 6.

Block titration -

- Add 25 µL of the appropriate antigen dilutions to all wells of the horizontal rows A-F.

- Add 25 µL of the appropriate antiserum dilution to all wells of the vertical columns 1-6.
- Add 25 µL of diluent to row G and column 7. These are control rows.
- Add 25 µL of complement to all wells.
- Mix thoroughly and incubate at 37° C for 60 min.
- Add 25 µL of sensitised 1.5% red blood cells, mix and incubate at 37°C for 30 min.
- Centrifuge the plate at 400 *g* for 5 min and read the results.

To read the plate
Read the highest antigen-antiserum dilution combination that is not subject to anticomplementary action of either component.

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: 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.

Procedure
- Add 25 µL of diluent to all rows.
- Add 25 µL of heat-inactivated serum to rows A and B.
- Serially dilute from row B to row H (25 µL transferred).
- Add 25 µL of complement fixing antigen to rows B through to row H (plate 1).
- Add 25 µL of control antigen to rows B through to row H (plate 2).
- Add 25 µL of diluted complement to all rows.
- Mix thoroughly, incubate at 37°C for 60 min.
- Add 25 µL of 1.5% sensitised red blood cells to all rows.
- Mix thoroughly, incubate at 37°C for 30 min, mixing at 15 min.
- Centrifuge trays at 400 *g* for 5 min and read the results.

Interpretation of results : 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 that 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.

Diagnosis of Chlamydia/Chlamydophila by growth in embryonated hens eggs or in cell monolayers

Growth of the organism has long been considered to be the method of choice as it confirms the presence of the organism in affected tissue and enables future analysis of the strain involved. Few laboratories in Australia have the capability to perform chlamydial cell culture as the technique is technically demanding. As with the diagnosis of human *Chlamydia trachomatis*, the use of eggs and cell culture has been largely replaced by PCR. Nevertheless, the procedures for the in vitro culture of *Chlamydia/Chlamydophila* in either embryonated hens eggs or eukaryotic cell monolayers are provided for those laboratories that may have the facilities and expertise.

Principle of the test

Chlamydiae can be isolated and grown in the yolk sac of embryonated hen eggs or in cultured mammalian cells. 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, some laboratories still use this procedure.

Isolation in embryonated hen eggs

Test procedure : Six 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 inoculated into 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 h and any deaths (due to trauma) are discarded.
Continue candling daily from day 3 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 the presence of characteristic chlamydial elementary bodies, which are round, and 0.3 to 0.5 μm in diameter.
If no deaths occur on the first passage, at least one blind passage should be done before deciding that the sample is negative.

Isolation in mammalian cell culture

A range of mammalian cell lines, including McCoy, L, BHK and Buffalo Green Monkey (BGM) cells, may be used. The isolation rate of some strains of chlamydiae can be improved by centrifuging the inoculum onto the cell monolayer. The most common procedure employs a monolayer of cells growing on a coverslip in the bottom of a 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 containing the coverslips are incubated at 37°C in an atmosphere of 5% carbon dioxide in air for 3-7 days. Representative coverslips are removed, stained with Giemsa or preferably fluorescein-labelled monoclonal antibody and examined for characteristic chlamydial inclusions.

Reagents and media
(i) Cell line – A range of eukaryotic cell lines can be used although BGM and McCoy cell lines are the most broadly useful and sensitive.
(ii) Growth medium (GM) – Dulbecco's modified Eagle medium containing 10% foetal calf serum, 100 μg/mL gentamicin, 100 μg/mL vancomycin and 2 μg/mL amphotericin B, pH 7.2.0
(iii) Transport and dilution medium – SPG is the preferred medium for any dilutions and transport of viable chlamydiae. It contains sucrose (74.6 g/L), KH_2PO_4 (0.51 g/L), K_2HPO_4 (1.2 g/L), L-glutamic acid (0.72 g/L) plus 10% foetal calf serum.
(iv) *Chlamydia/Chlamydophila*-specific fluorescent monoclonal antibody – A *Chlamydia/ Chlamydophila*-specific fluorescent monoclonal antibody that targets the chlamydiales LPS antigen is available from Cellabs Pty Ltd, Sydney.

Test procedure
Small-scale culture is done in 5 mL, flat-bottomed polystyrene vials containing sterile 13 mm diameter glass coverslips. Individual vials, plus coverslips, are seeded with 2 x 10^5 BGM or McCoy cells, in 1 mL of GM and incubated overnight at 37°C in 5% carbon dioxide, with caps loosened. This results in a just-confluent layer of cells within 16-24 h. Samples (0.1 mL) of chlamydial inoculum, either undiluted or diluted in SPG transport medium, are added and the vials centrifuged at 2000 *g* at 32°C for 1 h. The centrifuged vials are then placed in an incubator in an atmosphere of 5% carbon dioxide in air, the caps loosened, and incubated at 37°C for a further 2 h.

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 h incubation. At regular intervals, coverslips are removed from representative cultures and stained with a fluorescent-labelled monoclonal antibody. Background cells 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.

Antigen detection

The presence of chlamydial antigen in tissue or swab samples is a convenient way of making a diagnosis. Samples may be in the form of either (a) tissues, which may be fresh, formalin-fixed or formalin-fixed and paraffin-embedded; or (b) swabs collected from various anatomical sites.

Smears for the identification of antigens in tissue sections

Where chlamydial infections are already suspected, the collection and staining of smears can be a useful method of rapid preliminary diagnosis. In the case of chlamydial infections in sheep, smears can be prepared from placental membranes and cotyledons or swabs can be taken from the vagina at the time of abortion or from arthritic joint fluid. For *C psittaci* in birds, smears can be prepared from faeces, cloacal swabs or conjunctival scrapings. In the case of samples from necropsy of any infected animal, impression smears can be prepared from liver, spleen, kidney, lung, etc. A range of staining techniques have been used, including modified Gimenez or Giemsa, but the commercially available fluorescent-labelled Chlamydiaceae-specific antiserum is usually found to be more sensitive and specific.

Immunoassays

Most of the commercially available antigen detection tests that have been developed over the past 20 years use antigens (usually MOMP) that make them specific for the human *Chlamydia*, *C trachomatis*. Some, however, use the family-specific LPS antigen, which makes them suitable for diagnosing all chlamydiae, including veterinary species. These immunoassays include
(a) direct fluorescent antibody (DFA) tests, for example, IMAGEN, Celltech; *Chlamydia*-Direct IF, BioMerieux; and Vet-IF, Cell Labs;
(b) plate-based ELISAs, for example, Chlamydiazyme, Abbott; IDEIA, Dako; IDEIA PCE, Dako; Pathfinder, Kallestad; and *Chlamydia*-EIA, Pharmacia; and
(c) solid-phase ELISAs , for example, Clearview® *Chlamydia*, Unipath; and Surecell, Kodak.
There are several advantages of using immunoassays over cell culture for diagnosing infections:
(i) shorter time to complete the test, (ii) less requirements on laboratory equipment and reagents, and (iii) they are not dependent on viability and hence they do not require rigorous sampling and transport conditions. While the specificity of these assays is generally considered to be good, they vary in their sensitivity levels.[21] For example, while several reports suggest that the Clearview® *Chlamydia* assay has good sensitivity for some animals, for example, koalas[22], other reports suggest that it can result in some false positives and false negatives with avian cloacal specimens.[23] Nevertheless, these solid-phase immunoassays do have practical use in veterinary situations and the Clearview® *Chlamydia* solid phase assay is described in more detail below.

Diagnosis of *Chlamydia/Chlamydophila* by the Clearview® *Chlamydia* solid-phase ELISA assay

Principle of the test

The Clearview® *Chlamydia* assay detects the Chlamydialies LPS antigen. This antigen is present in all Chlamydiales strains and is highly conserved, enabling it to be used with all animal specimens. The assay is a solid-phase ELISA assay, which uses anti-*Chlamydia*-specific LPS antibodies bound to a solid support. The sample is heated at 80°C, which releases the soluble chlamydial LPS antigen (if present) into solution, which then binds to the surface-bound anti-LPS antibodies. Conjugate and substrate then bind and the result is a coloured line on the specially designed microscope slide-like device. The Clearview® *Chlamydia* test kit is described in Part 3 of this ANZSDP.

Reagents and materials

The commercially available Clearview® *Chlamydia* kit contains its own collection swab (provided sterile within a plastic sheath) as well as the testing kit. A workstation is also available from the manufacturer of the kit: this is a convenient 80°C heating block with tube holder. It is possible to use a normal laboratory heating block or even an 80°C water bath in place of the commercially available workstation.

Test procedure

The test can be performed on any type of sample by swabbing the area to be tested. Resist collecting too much debris on the swab as this may cause some false readings. The test is very simple and consists of 4 steps:

(a) add 0.6 mL extraction fluid to the extraction tube; add the sample swab and mix for 5 sec,

(b) place the tube containing the swab in the heating block/workstation and heat at 80°C for 10 min,

(c) remove the tube from the heating block/workstation and remove the swab, squeezing off the fluid in the process. Allow the tube plus solution to cool at room temperature for at least 5 min,

(d) remove a solid-phase ELISA 'slide-shaped' device from its foil pack and add 5 drops of cooled specimen to the Sample Window.

A positive result is indicated by a coloured line appearing in the Test Window within 15 min. Exercise caution if reading the test at longer periods.

The assay has a Control Window (positive control) and this window should develop a coloured line within the 15 minute test period for the assay to be valid. The test is semi-quantitative with more intense lines indicating stronger positive samples.

While the assay is at least ten-fold less sensitive than PCR, it is very rapid (30 min to perform) and easy to perform even in the field. It therefore has some value as a preliminary assay to screen selected animals showing more severe clinical signs, with subsequent PCR follow-up.

Quality control aspects

This is a quality controlled, commercially available test kit so validation has been done, although primarily for human samples. The kit contains an internal positive control, which is automatically run for each test sample, ensuring adequate quality control.

Interpretation of results

Interpretation is relatively straightforward. It is preferable to read the test within the prescribed 15 min as leaving the test to develop for longer periods may result in 'positive' lines eventually coming up with all samples. Negative results are usually completely clear; hence faint lines probably represent a weak positive result. The Clearview® *Chlamydia* assay has been shown to be reasonably sensitive and specific for some hosts, for example, koalas[22], but has resulted in some false positives and false negatives with certain sample types, for example, cloacal swabs, in other hosts, for

example, birds.[23] A comparison of the Clearview® *Chlamydia* assay with eight other antigen detection kits for the diagnosis of urogenital infections in koalas showed that the Clearview® *Chlamydia* assay performed the best of all nine antigen detection kits tested and was able to detect as few as 13 to 130 chlamydial particles in a serial dilution assay.[22] When evaluated with clinical swab samples from female koalas, the assay has a sensitivity of 91% and a specificity of 100%. The Clearview® *Chlamydia* assay also out-performed cell culture, presumably because of loss of infectivity during transport of the swab samples from the field to the laboratory. The assay has varying performance results with difference animals and it should not be used, for example, for analysis of avian faecal samples.

Diagnosis of Chlamydia/Chlamydophila by PCR

PCR is probably now the preferred method for diagnosis because of its reliability, high sensitivity, and specificity. The method also enables subsequent speciation and typing of isolates. In addition, this method enables the use of non-viable specimens, thus reducing the need to transport the specimens to the laboratory on ice within 24 hours, as is required for cell culture diagnosis. A large number of PCR assays that have been published report a range of sensitivities and target genes.[21] The most common amplification targets are 16S rRNA, 23S rRNA and ompA. An example of a 16S rRNA – 23S rRNA PCR assay is provided below.

Principle of the test

The most commonly used family-wide PCR assay for the Chlamydiaceae targets the 16SrRNA gene. A PCR assay that targets the conserved 16SrRNA-23SrRNA region of the genome is preferred as this ensures that all Chlamydiales infections will be detected. While the type of host infected gives some idea of the likely chlamydial species involved, this is not always the case. Omp1 (MOMP) has been widely used as a target gene for PCR, but it is highly variable, making selection of conserved PCR primers difficult. For this reason, the 16SrRNA-23SrRNA region is the target of choice for veterinary *Chlamydia/Chlamydophila* diagnosis. Everett *et al*[5] designed a set of PCR primers that should amplify all known (and presumably yet to be characterised) strains of Chlamydiales. Most laboratories report good success with this primer set.

Reagents and materials

PCR primers : 16SIGF 5'CGGCGTGGATGAGGCAT3' and 16SIGR 5'TCAGTCCCAGTGTTGGC3'.[5] All other PCR reagents are standard.

Test procedure

A 298 bp fragment of the 16S rRNA gene is PCR-amplified with the primer set: 16SIGF 5'CGGCGTGGATGAGGCAT3' and 16SIGR 5'TCAGTCCCAGTGTTGGC3'.[5] PCR reactions are performed in a final volume of 25 µL, including 2.5 µL 10x PCR reaction buffer (Roche), 0.5 µL PCR nucleotide mix (Roche), 1.0 µL of each (10 µM) primer, 0.1 µL of 5U/µl Taq DNA polymerase (Roche), 1.0 µL of template and PCR grade water to a final volume of 25 µL. Amplification conditions consist of an initial denaturation at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 56°C, 1 min at 72°C, and a final extension for 10 min at 72°C. PCR products are separated by electrophoresis and visualised by ethidium bromide (10 µg/mL) staining of a 2% Tris Borate EDTA (TBE) gel.

Quality control aspects

Always include two types of controls in each PCR run: (a) a negative control containing all reagents except water in place of the sample, and (b) a positive control, which is a cell culture positive or

DNA sample of a known *Chlamydia/Chlamydophila* strain. While a high concentration of DNA in the positive control can be used to produce a strong PCR band, it is preferable to use a low concentration to test the limits of sensitivity of each PCR run. Some clinical samples may contain PCR inhibitors. In this case, either dilute the sample 1/2 or even 1/10 and re-run the PCR, or 'spike' the sample with a small amount of known positive and re-run the PCR. It is preferable to include an inhibition control in each run, testing at least 10% of any new batch of samples for PCR inhibitory factors.

Interpretation of results
The test is invalid if the negative control does not give a negative result.
True positive samples should have a single band of the expected size (298 bp).
If there are numerous bands on the gel, including one of the expected size, this should be interpreted cautiously.

Other PCR assays
While the 16SrRNA PCR assay of Everett et al[5] is probably the assay of choice, a range of other sensitive and validated PCR assays have been described and can be used in particular situations.[21,24,25,26]

Part 3 - Approved reagents and kits

The Clearview® *Chlamydia* kit is available from Inverness Medical via http://www.invernessmedicalpd.com/poc/products/clr_chlamydia.html. The kit contains its own sterile collection swab within a plastic sheath in addition to the testing kit. A workstation is also available from the manufacturer of the kit: this is a convenient 80°C heating block with tube holder. It is possible to use a normal laboratory heating block or even an 80°C water bath in place of the commercially available workstation.

Chlamydia/Chlamydophila-specific fluorescent monoclonal antibody
A *Chlamydia/Chlamydophila*-specific fluorescent monoclonal antibody that targets the chlamydiales LPS antigen is available from Cellabs Pty Ltd, Sydney (www.cellabs.com.au).

References

1. Longbotton D, Coulter L. Animal chlamydiosis and zoonotic implications. *J Comp Path* 2003;128:217-244.
2. Page L. Proposal for the recognition of two species in the genus *Chlamydia*, Jones, Rake and Stearns, 1945. *Int J Syst Bact* 1968;18:51-66.
3. Grayston J, Kuo T, Campbell LA, Wang SP. *Chlamydia pneumoniae* sp. Nov. for *Chlamydia* sp. Strain TWAR. *Int J Syst Bact* 1989;39:88-90.
4. Fukushi H, Hirai K. Proposal of *Chlamydia pecorum* sp. nov. for *Chlamydia* strains derived from ruminants. *Int J Syst Bact* 1982;42:306-308.
5. Everett K, Bush R, Andersen A. Emended description of the Order Chlamydiales, proposal of Parachlamydiacese fam. Nov. and Simkaniaceae fam. nov., each containing one monotypic genus, revised taxonomy of the family Chlamydiaceae, including a new genus and five new species, and standards for the identification of organisms. *Int J Syst*

Bact 1999;49:415-440.
6. Schachter J, Stephens R, Timms P et al. Radical changes to chlamydial taxonomy are not necessary just yet. *Int J Syst Evol Micro* 2001;51:251-253.
7. Yung AP, Grayson M. Psittacosis – A review of 135 cases. *Med J Aust* 1998;148:228-233.
8. Longbotton D. Chlamydial infections of domestic ruminants and swine : new nomenclature and new knowledge. *Vet J* 2004;168:9-11.
9. Polyarthritis in lambs. *New South Wales Animal Health Surveillance.* 2004/3:3. www.dpi.nsw.gov.au/__data/assets/pdf_file/0013/113161/ahs-04-3.pdf
10. Abortion in sheep. *New South Wales Animal Health Surveillance.* 2008/1:2. www.dpi.nsw.gov.au/__data/assets/pdf_file/0016/231703/AHNews-Mar08.pdf
11. Tammemagi L, Simmons G. Psittacosis lymphogranuloma polyarthritis of sheep in Queensland, Australia. *Aust Vet J* 1968;44:585.
12. Aitken I. Chlamydial abortion. In: *Diseases of sheep*, 3rd edn, Martin W Aitken I; eds Blackwell Scientific Ltd, Oxford, UK, 2000:81-86.
13. St George T. The isolation of chlamydiae from faeces of sheep in Australia. *Aust Vet J* 1970;47:74.
14. Rofe J. Suspected enzootic virus abortion of ewes. *Aust Vet J* 1967;43:117-118.
15. Brown S, Girjes A, Lavin M, Timms P, Woolcock J. Chlamydial disease in koalas. *Aust Vet J* 1987;64:346-349.
16. Bodetti T, Jacobsen E, Wan C et al. Molecular evidence to support the expansion of the host range of *Chlamydophila pneumoniae* to include reptiles as well as humans, horses, koalas and amphibians. *Syst Appl Micro* 2002;25:146-152.
17. Sykes J, Anderson G, Studdert V, Browning G. Prevalence of feline *Chlamydia psittaci* and Feline Herpesvirus 1 in cats with upper respiratory tract disease. *J Vet Int Med* 2008;13:153-162.
18. Warren K, Swan R, Bodetti T et al. Ocular Chlamydiales infections in Western Barred bandicoots (*Permeles Bougainville*) in Western Australia. *J Zoo Wild Med* 2002;36:100-102.
19. Mackereth GF, Stanislawek W. First isolation of *Chlamydophila pecorum* in New Zealand. *Surveillance* 2002;29:17-18.
20. Condon K, Oakey J. Detection of Chlamydiaceae DNA in veterinary specimens using a family specific PCR. *Lett Appl Micro* 2007;45:121-127.
21. Sachse K, Hotzel H. Detection and differentiation of Chlamydiae by nested PCR. In: *PCR Detection of Microbial Pathogens*, Sachse K Frey J. eds Humana Press, New Jersey, USA 2003;123-136.
22. Wood M, Timms P. Comparison of nine antigen detection kits for diagnosis of urogenital infections due to *Chlamydia psittaci* in koalas. *J Clin Micro* 1992;30:3200-3205.
23. Vanrompay D, van Nerom A, Ducatelle R et al. Evaluation of five immunoassays for detection of *Chlamydia psittaci* in cloacal and conjunctival specimens from turkeys. J Clin Micro 1994;32:1470-1474.
24. Everett K, Andersen A. Identification of nine species of Chlamydiaceae using PCR-RFLP. *Int J Syst Bact* 1999;49:803-813.
25. Geens T, Dewitte A, Boon N, Vanrompay D. Development of a *Chlamydophila psittaci* species-specific and genotype-specific Real-Time PCR. *Vet Res* 2005;36:1-11.
26. Hewinson R, Griffiths P, Bevan B et al. Detection of *Chlamydia psittaci* DNA in avian clinical samples by polymerase chain reaction. *Vet Micro* 1997;54:155-166.