

ENZOOTIC ABORTION OF EWES (ovine chlamydiosis)

SUMMARY

Ovine chlamydiosis, also known as enzootic abortion of ewes (EAE) or ovine enzootic abortion (OEA), is caused by the bacterium Chlamydomphila abortus. Chlamydial abortion typically occurs in the last 2–3 weeks of pregnancy with the appearance of stillborn lambs and inflamed placentas. However, infection can also result in the delivery of full-term stillborn lambs or weak lambs that do not survive longer than 48 hours. Infected ewes can also give birth to healthy lambs and it is not uncommon to observe delivery of a dead and a weak or healthy lamb. There are rarely any predictive signs that abortion is going to occur, although behavioural changes and a vulval discharge can be observed in the last 48 hours of pregnancy.

Diagnosis of enzootic abortion depends on the isolation and identification of the causative agent or detection of the agent or its nucleic acid in the products of abortion or vaginal excretions of freshly aborted females. A humoral antibody response may be detected following abortion. Goats as well as sheep and, less commonly, cattle, pigs, horses and deer, can be affected. Chlamydiosis of small ruminants is a zoonosis and the organism must be handled with biosafety precautions. Pregnant women are particularly at risk.

Identification of the agent: *The basis for a positive diagnosis of infection with C. abortus depends on a history of abortion in sheep or goats (often in late pregnancy), evidence of necrotic placentitis, and the demonstration of large numbers of the organism in stained smears of affected placentae. The still moist fleece of fetuses or vaginal swabs of females that have freshly aborted are also useful. Care is needed to distinguish cotyledonary damage caused by Toxoplasma gondii and, in stained smears, to be aware of the morphological similarities between C. abortus and Coxiella burnetii, the agent of Q fever.*

Chlamydial antigen can be detected by enzyme-linked immunosorbent assay, immunohistochemistry or the fluorescent antibody test, whereas chlamydial DNA can be detected by the polymerase chain reaction or by microarray. Some of these methods are available in commercial kit form.

Chlamydomphila abortus can be isolated only in living cells; thus facilities for growth in chicken embryos or cell cultures, with appropriate biohazard containment, are required.

Serological tests: *A rise in antibody titre to C. abortus, detected by the complement fixation (CF) test, is common after abortion or stillbirth, but this does not occur in every case. Chlamydomphila abortus shares common antigens with C. pecorum and some Gram-negative bacteria, so that the CF test is not wholly specific, nor does it distinguish between responses to vaccination and to infection. Low CF titres need to be interpreted with caution, particularly if these are encountered in individual animals or in flocks with no history of abortion.*

Alternative serological tests have been developed and some commercialised, but none has been sufficiently appraised so far for field use. A delayed hypersensitivity reaction to chlamydial antigen can be elicited in infected sheep, but the procedure is not amenable to routine use.

Requirements for vaccines and diagnostic biologicals: *Inactivated and live vaccines are available that have been reported to prevent abortion and to reduce excretion. They assist in control of the disease but will not eradicate it. Serological screening during the period after parturition helps to identify infected flocks, to which control measures can then be applied.*

A. INTRODUCTION

Ovine chlamydiosis (enzootic abortion of ewes [EAE] or ovine enzootic abortion [OEA]) is caused by the bacterium *Chlamydophila abortus*. Chlamydial abortion in late pregnancy causes serious reproductive wastage in many sheep-rearing areas of the world, particularly where flocks are closely congregated during the parturient period (1, 19). Abortion typically occurs in the last 2–3 weeks of pregnancy with the appearance of stillborn lambs and grossly inflamed placentas. Infection can also result in the delivery of full-term stillborn lambs and weak lambs that generally fail to survive beyond 48 hours. It is also not uncommon in multiple births for an infected ewe to produce one dead lamb and one or more weak or healthy lambs. Infection is generally established in a 'clean' flock through the introduction of infected replacements and results in a small number of abortions in the first year, which is followed by an 'abortion storm' in the second year that can affect up to around 30% of ewes.

Infected animals show no clinical illness prior to abortion, although behavioural changes and a vulval discharge may be observed in ewes within the last 48 hours of pregnancy. Pathogenesis commences around day 90 of gestation coincident with a phase of rapid fetal growth when chlamydial invasion of placentomes produces a progressively diffuse inflammatory response, thrombotic vasculitis and tissue necrosis. Milder changes occur in the fetal liver and lung and, in cases in which placental damage is severe, there may be evidence of hypoxic brain damage (6). Abortion probably results from a combination of impairment of materno-fetal nutrient and gaseous exchange, disruption of hormonal regulation of pregnancy and induced cytokine aggression (10).

Chlamydial abortion also occurs in goats and, less frequently, cattle, pigs, horses and deer may be affected. In sheep, abortion in late pregnancy with expulsion of necrotic fetal membranes are key diagnostic indicators, with care being needed to distinguish the diffuse pattern of necrosis from that caused by *Toxoplasma gondii* (cotyledons only). Distinction from other infectious causes of abortion such as brucellosis (see Chapter 2.7.2), coxiellosis (see Chapter 2.1.12) or other bacterial pathogens (*Campylobacter* [see Chapter 2.9.3], *Listeria* [see Chapter 2.9.7], *Salmonella* [see Chapter 2.9.9]) can be achieved by microscopy and/or culture.

Taxonomically, the family Chlamydiaceae is divided into two genera and nine species based on sequence analysis of the 16s and 23s rRNA genes (12). The genus *Chlamydia* includes *C. trachomatis* (humans), *C. suis* (swine) and *C. muridarum* (mouse and hamster). The genus *Chlamydophila* includes *C. psittaci* (avian), *C. felis* (cat), *C. abortus* (sheep, goat and cattle), *C. caviae* (guinea-pig), the former species *C. pecorum* (sheep and cattle) and *C. pneumoniae* (humans). The two genera and nine species have merit both on the basis of molecular structure and for the purposes of host range and clinical disease. The species show a marked degree of correlation with host range, disease syndrome and virulence, thus assisting in understanding the epidemiology of the various species and serovars affecting mammals and birds. The terms 'chlamydiosis' and 'chlamydia(e)' are used to refer to members of either of the two genera. However, a binomial of the generic and specific names is used when referring to a particular chlamydial species.

Infected females shed vast numbers of infective *C. abortus* at the time of abortion or parturition, particularly in the placenta and uterine discharges (23). Environmental contamination, resulting from this shedding, is considered the primary source of infection for other females. There is also limited evidence to suggest that sheep that have experienced an abortive episode following experimental infection, can also excrete *Chlamydia* from the reproductive tract during subsequent oestrus cycles (23). Human infection may be acquired from infected products of abortion or parturition or from carelessly handled laboratory cultures of the organism, with effects that range from subclinical infection to acute influenza-like illness. Appropriate precautions should be taken when handling cultures and potentially infected tissues (see Chapter 1.1.2 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities). Authenticated cases of human placentitis and abortion caused by *C. abortus* of ovine origin indicate that pregnant women are at special risk and should not be exposed to sources of infection (7, 19).

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

a) Smears

Where the clinical history of the flock and the character of lesions in aborted placentae suggest enzootic abortion, a diagnosis can be attempted by microscopic examination of smears made from affected chorionic villi or adjacent chorion. Several staining procedures are satisfactory, for example, modified Machiavello, Giemsa, *Brucella* differential, or modified Ziehl–Neelsen stains (28). In positive cases stained by the latter method and examined under a high-power microscope, large numbers of small (300 nm) coccoid elementary bodies are seen singly or in clumps stained red against the blue background of cellular debris. Under dark-ground illumination, the elementary bodies are pale green. If placental material is not available, smears may be made from vaginal swabs of females that have aborted within the previous 24 hours, or from the moist

fleece of a freshly aborted or stillborn lamb that has not been cleaned by its mother. In general, such preparations contain fewer organisms than placental smears.

In terms of morphology and staining characteristics, *C. abortus* resembles the rickettsia *Coxiella burnetii*, which, in some circumstances, may provoke abortion and which, in humans, causes Q fever. Care must be taken to differentiate between these two organisms in cases lacking a good history or evidence of chlamydial-induced placental pathology. Antigenic differences between *C. abortus* and *Coxiella burnetii* can be detected serologically. Fluorescent antibody tests (FATs) using a specific antiserum or monoclonal antibody may be used for identification of *C. abortus* in smears.

b) Antigen detection

Several chlamydial genus-level antigen-detection tests are available commercially. A comparative assessment of several such assays, on non-ovine material, indicated that those using enzyme-linked immunosorbent assay (ELISA) methodology were more sensitive than kits employing a FAT (34). Under the test conditions used, a kit that detects chlamydial lipopolysaccharide (LPS) was judged to be the most sensitive of the rapid ELISA-based systems investigated. Though occasionally yielding false-positive results, particularly with avian faecal samples, the kit also gave satisfactory results with ovine placental samples (33). In histopathological sections, antigen detection can be performed using commercially available anti-*Chlamydia* antibodies directed against LPS or MOMP (major outer membrane protein) (5).

c) DNA

Amplification of chlamydial DNA by polymerase chain reaction (PCR) and real time PCR provide alternative approaches for verifying the presence of chlamydiae in biological samples without resorting to culture. PCR is highly sensitive for this purpose, but has the attendant risk of cross-contamination between samples, so appropriate measures must be taken to avoid this happening (see Chapter 1.1.7 Biotechnology in the diagnosis of infectious diseases and vaccine development). Another potential problem is in the production of false negatives resulting from PCR-inhibitory substances in the samples. Methods for discriminating between amplified DNA sequences originating from *C. abortus* and *C. pecorum* have been described (8, 11, 15, 18, 31). Such tests are beginning to be introduced into diagnostic laboratories throughout Europe. Recently, DNA microarray hybridisation assays using the ArrayTube™ platform have been developed and hold much promise for the direct detection and identification of organisms from clinical samples (9, 25).

d) Tissue sections

Intracellular chlamydial inclusions can be demonstrated by Giemsa staining of thin ($\leq 4 \mu\text{m}$) sections taken from target tissues that have been suitably fixed in fluids such as Bouin or Carnoy. More striking results can be obtained by immunological staining procedures. The direct immunoperoxidase method (13) is rapid and simple, while the method with streptavidin–biotin is more complex (29). Electron microscopy can also be performed using negative contrast, to differentiate chlamydiae from *Coxiella burnetii*.

e) Isolation of the agent

Chlamydophila abortus can be isolated in embryonated chicken eggs or in cell culture, the latter being the method of choice for isolation of new strains. The causative agent of chlamydiosis is zoonotic (19) and thus isolation and identification procedures must be carried out under the appropriate containment level as described in Chapter 1.1.2.

Tissue samples, such as diseased cotyledons, placental membranes, fetal lung or liver, or vaginal swabs, that may be subject to any delay before isolation procedures begin, should be maintained in a suitable transport medium in the interim period. The most satisfactory medium is sucrose/phosphate/glutamate or SPG medium (sucrose [74.6 g/litre], KH_2PO_4 [0.512 g/litre], K_2HPO_4 [1.237 g/litre], L-glutamic acid [0.721 g/litre]) supplemented with 10% fetal bovine serum, antibiotic (streptomycin and gentamycin are suitable, but not penicillin), and a fungal inhibitor (27). A tissue:medium ratio of 1:10 is commonly employed. Alternatively, approximately 1 g of tissue is ground with sterile sand in 8 ml of transport medium.

Chicken embryos: Test samples are prepared as 10% suspensions in nutrient broth containing streptomycin (not penicillin) (200 $\mu\text{g/ml}$); 0.2 ml of suspension is inoculated into the yolk sac of 6–8-day old embryos, which are then further incubated at 37°C. Infected embryos die between 4 and 13 days after inoculation. Smears prepared from their vascularised yolk sac membranes reveal large numbers of elementary bodies.

Cell cultures: *Chlamydophila abortus* of ovine origin can be isolated in a variety of cell types, but McCoy, Buffalo Green Monkey (BGM) or baby hamster kidney (BHK) cells are most commonly used. For confirmatory diagnosis, cultured monolayers are suspended in growth medium at a concentration of 2×10^5 cells/ml. Aliquots of 2 ml of the suspension are dispensed into flat-bottomed glass Universal bottles, each containing a single 16 mm cover-slip. Confluent cover-slip monolayers are achieved after incubation for 24 hours at 37°C. The growth medium is removed and replaced by 2 ml of test inoculum, which is then

centrifuged at 2500 **g** for 30 minutes on to the cover-slip monolayer to promote infection. After further incubation for 2–3 days, the cover-slip monolayers are fixed in methanol and stained with Giemsa or according to the method of Gimenez (3, 14). After methanol fixation, infected cultures contain basophilic (Giemsa) or eosinophilic (Gimenez) intracytoplasmic inclusions. Similar procedures are used in culturing *C. abortus* for antigen preparation. FAT techniques can also be used and are equally effective.

Chlamydial activity can be further enhanced by chemical treatment of cultured cells, before or during infection, to favour chlamydial growth. Various substances that have been described for incorporation into the infective inoculum to which cover-slip monolayers are exposed include: cycloheximide (0.5 µg/ml) in the maintenance medium, emetine (1 µg/ml) for 5 minutes before infection, and 5-iodo-2-deoxyuridine (80 µg/ml) for 3 days prior to infection. Unless preconditioned cells are available, the latter isolation procedure requires increased time for successful agent isolation.

2. Serological tests

a) Complement fixation test

Complement fixation (CF) is the most widely used procedure for detecting infection (sheep and goats are generally tested within 3 months of abortion or parturition). The test will also detect evidence of vaccination. Infection is evident principally during active placental infection in the last month of gestation and following the bacteraemia that often accompanies abortion. Consequently, paired sera collected at the time of abortion and again at least 3 weeks later may reveal a rising CF antibody titre that will provide a basis for a retrospective diagnosis. Antigenic cross-reactivity between *C. abortus* and *C. pecorum*, as well as with some Gram-negative bacteria (e.g. *Acinetobacter*), can give rise to low false-positive CF test results. Thus, titres less than 1/32 in individual animals should be considered to be nonspecific for *C. abortus*, although they could also be due to a low grade infection with *C. abortus*. Ambiguous results can be investigated further by western blot analysis using purified elementary bodies (17).

Antigen is prepared from heavily infected yolk sac membranes obtained from chicken embryos that have been inoculated in the same manner as those used to isolate the organism from field material. The preparation of the antigen should be carried out in a biosafety cabinet with the appropriate biosecurity precautions to prevent human infection (see Chapter 1.1.2). Chopped and ground membranes are suspended in phosphate buffer, pH 7.6, at the rate of 2 ml per g membrane. After removal of crude debris, the supernatant fluid is centrifuged at 10,000 **g** for 1 hour at 4°C, the deposit is resuspended in a small volume of saline, and a smear of this is examined to ensure a high yield of chlamydiae. The suspension is held in a boiling water bath for 20 minutes, or is autoclaved, and sodium azide (0.3%) is added as a preservative. Antigen may also be prepared from cell cultures infected with *C. abortus*. Infected monolayers are suspended in phosphate buffer, pH 7.6, and the cells are disrupted by homogenisation or ultrasonication. Gross debris is removed and subsequent procedures are as for the preparation of antigen from infected yolk sacs. In either case, CF tests with standardised complement and antisera will establish the optimal working dilution for each batch of antigen.

b) Other tests

The serological responses to *C. abortus* and *C. pecorum* can be resolved by indirect micro-immunofluorescence, but the procedure is too time-consuming for routine diagnostic purposes. ELISAs developed independently by several research groups have not been adapted for general diagnostic work, partly because of difficulties associated with the use of particulate antigens. However, a novel ELISA that incorporates a stable, solubilised antigen has been used to test experimental and field samples, and has given results that, though lacking species specificity, have a higher sensitivity than the CF test (2, 17). Other tests using monoclonal antibody technology in a competitive ELISA (26) and recombinant antigen technology in indirect ELISAs (20) have been developed and shown to be more sensitive and specific than the CF test in differentiating animals infected with *C. abortus* from those infected with *C. pecorum*. However, these tests are currently mainly used as research tools, and have not been developed commercially. A number of commercially available serological tests have been evaluated and compared with these 'in-house' tests with variable results (17, 32). None of the serological tests that is available can differentiate vaccination titres from those acquired as a result of natural infection (4).

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Currently, two types of vaccine (inactivated and attenuated live vaccines) are available commercially, to be administered intramuscularly or subcutaneously at least 4 weeks before breeding to aid in the prevention of abortion. A multi-component recombinant vaccine against *C. abortus* remains a future goal of chlamydial vaccine research (21).

Inactivated vaccines can be prepared from infected yolk sacs or cell cultures (16) and incorporate whole organisms or fractions of them (30), using the appropriate biosecurity precautions to prevent human infection (see Chapter 1.1.2). Operator care should be observed in handling commercial inactivated vaccines that incorporate mineral oil-based adjuvants, as self-injection can result in severe local inflammation and tissue necrosis. The commercial live, attenuated vaccine is a chemically induced temperature-sensitive mutant strain of the organism that grows at 35°C but not at 39.5°C, the body temperature of sheep (24). This vaccine is supplied lyophilised and must be reconstituted in diluent immediately before administration. Operator care should be observed in handling and administering this live vaccine, particularly by immunocompromised individuals and pregnant women. Importantly, the live vaccine must not be given to animals being treated with antibiotics, particularly tetracyclines.

Both types of vaccine have a role to play in controlling disease, but neither confers absolute protection against challenge or completely reduces the shedding of infective organisms. However, vaccinates exposed to infection do experience significantly lower abortion rates and reduced excretion of chlamydiae for at least two to three lambings after vaccination. It has been claimed that the live vaccine could be an aid to eradication of disease (22).

1. Seed management

a) Characteristics of the seed

One or more ovine abortion isolates that consistently grow productively in the chosen substrate are suitable, and an early passage of the seed stock can be established. Alternatively, an isolate that has been adapted to the chicken embryo by multiple passage (>100) can be used. This permits more of the embryo to be used for vaccine production. Although adaptation to the embryo may diminish the isolate's virulence for sheep, there is no evidence that such change reduces its protective efficacy as an inactivated vaccine.

b) Method of culture

For low passage isolates, the procedures described for the preparation of CF antigen are suitably adapted and amplified for bulk production. Once the final harvest suspension is obtained, an aliquot is removed for titration of its infectivity. The bulk is treated with formalin to a final concentration of 0.4%, and stored until sterility tests confirm complete inactivation.

c) Validation as a vaccine

Before inoculation of large numbers of embryos or cell cultures, the viability and freedom from contamination of seed stock should be verified. It may be convenient to collect the total harvest in separate manageable lots. In this case, the infectivity of an aliquot of each lot should be separately titrated to ensure that each matches the requirements (see Section C.2 below). Store under refrigerated conditions.

2. Method of manufacture

The inactivated harvest is centrifuged and resuspended in phosphate buffered saline containing 0.2% formalin to a volume representing a preinactivation infectivity titre of approximately 10^8 infectious units/ml. Usually, the aqueous suspension is blended with an oil adjuvant, either directly or after precipitation by potassium alum ($\text{AlK}[\text{SO}_4]_2 \cdot 12 \text{H}_2\text{O}$). A preservative, such as 0.01% thiomersal, may also be added.

3. In-process control

The main requirements are to ensure adequate growth of *C. abortus*, avoidance of extraneous infection of the culture substrate, completeness of inactivation and biohazard awareness by process workers.

4. Batch control

Each separate batch of manufactured vaccine should be tested for sterility, safety and potency.

a) Sterility

Tests for sterility and freedom from contamination of biological materials may be found in Chapter 1.1.9.

b) Safety

Subcutaneous inoculation into two or more seronegative sheep of twice the standard dose (usually 1.0 ml) of manufactured vaccine should elicit no systemic reaction, but oil-adjuvant vaccines can cause a nonharmful swelling at the inoculation site.

c) Potency

At present, potency is judged by the occurrence of a serological response in previously unvaccinated sheep given 1 ml of vaccine subcutaneously. Blood samples taken before and 28 days after vaccination are compared. Ultimately, potency has to be judged against experimental challenge or field performance, but no *in vitro* correlation of protective efficacy has yet been established.

d) Duration of immunity

No firm data are available, but revaccination is recommended after 1–3 years, according to the exposure risk.

e) Stability

Vaccine stored under refrigeration (5±3°C) should remain stable for at least 1 year. Before use it should be held at room temperature for 24 hours, and the container should be shaken vigorously immediately before vaccine is withdrawn.

5. Tests on the final product

a) Safety

See Section C.4.b.

b) Potency

See Section C.4.c.

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NB: There are OIE Reference Laboratories for Enzootic abortion of ewes (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).