Chapter 7

Q FEVER

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7.1 SUMMARY

Clinical symptoms of Q fever in small ruminants may include pulmonary effects and occasionally ocular problems but the most common manifestation of the disease in sheep and goats is abortion. Q fever is currently one of the most researched causes of abortion. It is caused by a *Rickettsia*, *Coxiella burnetti*. In a previously uncontaminated environment, the presence of *C.burnetti* can lead to abortion in more than 60% of the pregnant females.

Q fever is highly contagious for man. Direct bacteriological diagnosis (staining or immunofluorescence) is used to assure a rapid diagnosis and minimise the risks of contamination. Isolation techniques in embryonated hen's eggs, cell culture or laboratory animals are restricted to highly specialised and well equipped laboratories. The high sensitivity of molecular biological techniques (PCR) which allow detection of the microorganism's genetic material have a certain use in the future. Complement fixation remains the most common test for serological diagnosis. The emergence of new techniques (ELISA), commercially available in kit form, may lead to replacement of the classical techniques (complement fixation, immunofluorescence, agglutination) in due course.

7.2 INTRODUCTION

Derrick et. al. described the first case of Q fever in abattoir workers in Australia in 1937 and shortly afterwards the causal agent was identified as a *Rickettsia* by Burnett. In 1938, Davies & Cox isolated the pathogenic agent from ticks [11].

The relationship between these febrile episodes in man and enzootic abortion in small ruminants was only established after a number of human cases had been reported after which the zoonotic nature of the organism was rapidly confirmed. The importance of sheep and goats as the source of infection for man is now well established.

Natural infection in small ruminants can occasionally be expressed clinically as pulmonary or ocular symptoms but the classical manifestation is abortion. Q fever is currently one of the most studied causes of abortion together with chlamydiosis, salmonellosis and toxoplasmosis. The placenta of the pregnant female is the site of predeliction for C.burnetti, the organism that causes Q fever. Massive placental colonisation generally leads to abortion during the final months of gestation or to the birth of sickly offspring which have little chance of survival. Maternal complications (retained placenta, metritis, infertility) are rare in small ruminants and there are no specific signs at necropsy that assist in the establishment of diagnosis.

A member of the order of Rickettsiales and the family of Rickettsiaceae, the genus of *Coxiella* consists of a single species ; *Coxiella burnetti*. It behaves as a bacteria at the structural level and biochemically but

only multiplies in living cells. The *Coxiella* genus differs from other genera of the family of Rickettsiaceae on account of its high resistance to physicochemical agents. *Coxiella burnetti* can survive in the external environment without necessarily passing through an arthropod vector and can remain viable for long periods of time in, for example, dehydrated faecal material. This is an important factor for its perpetuation in different ecological niches. Furthermore, certain authors have reported a vegetative and/or spore forming form of the organism which may explain the very high resistance of this bacteria to the external environment. *Coxiella burnetti* multiplies preferentially in the reticuloendothelial cells of the host which it infects [2]. The average size is about 0.5µm x 1.0µm, but it may also occur in a coccoid forms of about 0.3mm diameter [8]. The organism can exist in two phases: Phase I, contagious for humans, is the type found naturally in the animal; Phase II predominates in vitro and is far less dangerous for man.

Standard sanitary precautions and hygiene measures must be implemented during each lambing period: disinfection of the premises, clothing and hands, lambs in isolated cases, destruction of placentas.

The use of tetracyclines in cases where *C.burnetti* is diagnosed as being the cause of abortion can prevent or at least limit the occurrence of further abortions. The relatively high cost of the treatment limits its use to cases where most of the females are still pregnant at the time of treatment.

In the vast majority of cases, stringent and regular vaccination remains the best method of preventing abortions from *C.burnetti*. Nevertheless, the excretion of organisms in the foetal membranes and the milk can still occur, so that, although the danger of disease spread to animals and man is reduced it is not eliminated.

In most countries where infection occurs, abortion as a result of *C.burnetti* infection is not subject to any official statutory regulations even though Q fever is recognised as being a zoonotic disease.

7.3 SAMPLES

Diagnosis of abortions resulting from C.burnetti infection should be undertaken on a flock basis not on individual animals. The samples required for direct and indirect diagnosis must be collected from many animals, generally not less than ten, for serological diagnosis.

7.3.1 Direct diagnosis

7.3.1.1 Choice of sample

Samples for bacteriology or isolation in embryonated hen's eggs, in order of preference are:

1. the placenta and cotyledons;
2. vaginal samples;
3. the stomach contents from the aborted foetus.

Vaginal samples, collected within the first 48 hours after the abortion, indicate the general level of infection present in the placenta. Bacteriological analysis of these samples is only necessary when the placenta is absent. The samples tend to be difficult to spread and are not easy to examine microscopically even under favourable conditions. Similarly samples of stomach contents are not ideal as they tend to be low in *Coxiella* and contain insufficient numbers of observable cells. The stomach contents usually contain an uneven population of Rickettsian bodies but are rarely contaminated with bacteria. These samples can be used for the isolation of the organism in embryonated hen's eggs or laboratory animals but are

frequently toxic for cell cultures.

For isolation in cell culture vaginal swabs or samples of discharge give the best results since these are rich in *Coxiella* and bacteriologically suitable.

For isolation in mice or guinea-pigs, all three types of sample can be used; the immune system of the animal will eliminate the main undesirable germs.

Other organs from the aborted foetus (spleen, kidney, liver, lung) tend to be infected at highly variable rates [9].

7.3.1.2 Treatment of samples

a / Bacteriology

The cotyledons are unevenly infected; 5-6 should be recovered and placed in a watertight container. The cotyledons should be rinsed in physiological saline and blotted dry with absorbent paper. For each cotyledon, individual smears should be made on microscope slides for several examinations.

The samples may be stored for several days at 4°C or for some months at -20°C.

b / Isolation

The isolation of *C.burnetti* can be undertaken in embryonated hen's eggs, cell culture or laboratory animals. The latter system of isolation is least used because there are greater risks of human contamination.

1. Grind or emulsify the samples diluted 1/10 (w/v or v/v) in physiological saline containing 5 mg/ml streptomycin and 100 µg/ml gentamycin.

2. Centrifuge for 30 minutes at 3,000 xg at 4°C.

3. Use samples immediately or store at -80°C in the following buffer to assure maximal preservation of the *Coxiella*.

Saccharose	74.62g
KH_2PO_4	0.52g
K_2HPO_4	1.25g
Sodium monosodium glutamate	0.83g
Bovine serum albumin	10.00g
Distilled water	1000ml

Sterilize the buffer by filtration through a 0.22µm membrane.

7.3.2 Indirect diagnosis

7.3.2.1 Choice of sample

Blood samples without anticoagulant should be collected aseptically from about ten of the females that abort. If necessary these samples can be supplemented by samples from animals that are still to lamb or that lamb at term. The dates of abortion and sampling should be carefully noted and each tube correctly identified. This method allows production of a "simulated kinetic profile" of antibody production which is useful when it is not possible for a second later sera sample to be obtained and it allows a more rapid verification of a serological diagnosis.

7.3.2.2 Treatment of samples

1. Remove the blood clots from the samples on arrival in the laboratory.

2. Centrifuge the sera for 5 minutes at 2500 - 3000 xg at 4°C.

In case of further use, serum samples held in capped tubes should be stored in the refrigerator between 4 and 8°C for up to a week or for many months in the freezer at -20°C.

7.4 RISKS TO HUMAN HEALTH

In addition to being a known pathogen in small ruminants, *C.burnetti* can also infect man. Man does not transmit the disease, he only exhibits disease symptoms. The human illness occurs in two main clinical forms :

• An acute infection with an influenza-like syndrome, pyrexia, severe headaches, anorexia, debility, pain behind the eyes and in the muscles. Respiratory difficulties can be accompanied by a dry cough and interstitial pneumonia can be shown to be present by radiological examination [6]. In the majority of cases, hepatic functions are altered. Treatment with tetracyclines leads to complete recovery of the patient in several months.

• A chronic form of the illness can also occur; the organism survives for a very long time in certain organs and ganglia. The effect on the heart may produce a grave prognosis. The manner by which the microorganism escapes the defence mechanisms of the host is still not fully understood; the sporogenic stage described for *C.burnetti* possibly plays a role in this form of infection.

Serological diagnosis is essential and may take the form of indirect immunofluorescence, ELISA and/or complement fixation tests.

Vaccination does not seem to be standard practice in human medicine; inactivated vaccines can induce severe local reactions. Vaccination is nevertheless recommended for workers at risk, particularly those with weak hearts.

There are many possible sources of human infection but infection between humans is rare. The main source of infection is from the foetal membranes of small ruminants, their vaginal secretions and the products of abortion. Contamination through milk containing dried faecal material from ticks has been reported. However, ticks are not a necessary element for the transmission of *C.burnetti*.

Although excretion through the mammary gland is intermittent, ingestion of raw milk or fromage frais can cause infection in humans. The O.M.S. recommend correct pasteurisation of milk (74°C for at least 15 seconds) and a delay of at least two months between the manufacture and consumption of cheese when purchased from an infected farm.

A case of human infection by the aerosol produced from dried manure spread on gardens was recently described. In-depth studies have ascribed a greater importance to aerial infection than to the ingestion of the microorganism from milk [4]. The potential danger that *C.burnetti* presents to pregnant women is also highlighted.

In the laboratory, as outside, abortion products must be treated with all the inherent precautions for the handling and disposal of actual or suspected infectious samples.

7.5 DIRECT DIAGNOSIS

Direct diagnosis of Q fever is undertaken using simple techniques that detect the presence of *C.burnetti* or more complex techniques that identify and/or isolate the organism.

7.5.1 Bacteriology

This easily performed technique is the most important one used in the standard diagnostic laboratory. The rapid production of results allows advice to be given on the introduction of recognised sanitary measures.

Principle

Coxiella burnetti has particular staining properties; it is characteristically Gram negative but is sensitive to basic stains. It also shows a slight resistance to acids which allows its presence to be verified by use of stains such as May-Grunwald-Giemsa [7], STAMP stain (or

modified ZIEHL-NEELSON). The latter staining method is used most frequently (the technique described here is a variation of the method reported in the chapters on Brucellosis and Chlamydiosis).

Materials and reagents

- Microscope with immersion objective x 100.
- Basic fuschin stain.
- Acetic acid.
- Methyl blue.

Procedure

1. Dry the slide in the air.
2. Cover the slide in basic fuschin stain diluted 1 in 5 in distilled water for 10 minutes.
3. Rinse the slide with distilled water.
4. Destain with acetic acid diluted 1 in 30 in distilled water for 15-40 seconds.
5. Counterstain with methyl blue diluted to 1% in distilled water for 15 seconds.
6. Air dry the slide.

Reading and interpretation of results

Read by microscope using an immersion objective. Rickettsian bodies appear red against a blue background. They are located intracellularly but may be dispersed on stained smears. They take the form of coccobacilli or short rods.

Note

• All reagents should be diluted before use.

• The time of destaining in acetic acid depends on the thickness of the smear.

• The same bacteriological staining method is used for the detection of *Brucella* and *Chlamydia*. It is possible that a technician with limited experience will have difficulty in differentiating the three microorganisms. However, the polymorphism of *Coxiella* provides a determining factor for recognition and so helps in the differential diagnosis.

• In all cases, bacteriology must be backed up by a serological analysis to prevent errors in interpretation.

• The presence of rickettsian bodies is always indicative of an infection. In endemic areas, *C. burnetti* must be detected in samples from many animals to confirm that it is the cause of the observed abortions.

• Examination of vaginal samples presents some limitations:

1) the difficulty of making sufficiently thin smears;

2) the time of sample collection, due to the limited duration of excretion.

7.5.2 Immunofluorescence

This technique is more specific than bacteriology but its use is not very widespread due to the absence of commercially available hyperimmune sera or monoclonal antibodies (Mabs). Specialised laboratories may be able to supply these reagents in small quantities to diagnostic laboratories.

Principle

Immunofluorescence allows detection and identification of microorganisms by use of a direct (hyperimmune sera or Mabs labelled with fluorescein isothiocyanate) or indirect (fluorescent anti-immunoglobulin conjugate against hyperimmune sera or Mabs) method.

Materials and reagents

- Fluorescent microscope.
- Acetone.
- Phosphate buffered saline (PBS).
- Hyperimmune sera or Mabs.
- Fluorescent anti-immunoglobulin conjugate against hyperimmune sera or Mabs.

Procedure

1. Fix the slides in acetone at 4°C for 30 to 45 minutes.
2. Incubate with anti-Coxiella antibodies at a predetermined dilution for 30 minutes at 37°C.
3. Rinse slides three times for 5 minutes each time in PBS.
4. Incubate with fluorescent anti-immunoglobulin conjugate at a predetermined dilution for 30 minutes at 37°C.
5. Rinse slides three times for 5 minutes each time in PBS.
6. Cover with a preparation of glycerine buffer and a coverslip.

Reading and interpretation of results

Read by ultraviolet light using a x100 objective. Rickettsian bodies appear fluorescent green.

Note

• In the direct technique, anti-*Coxiella* antibodies are bound directly to the fluorescent isothiocyanate and procedures 4 and 5 do not apply [13].

• Use of the indirect method with hyperimmune sera (rabbit, sheep, goat) produces a reasonable level of sensitivity, but poor storage conditions of the samples and the presence of large numbers of contaminants acting to increase the presence of fluorescently-stained organisms can impede the observation of the *Coxiella*. The use of Mabs in the indirect technique combines specificity, sensitivity and exclusion of contaminating fluorescent organisms.

Techniques describing the detection of the organism using immunohistochemical methods are beginning to appear; the results obtained point to a more and more standard use of these techniques.

7.5.3 Isolation

Direct diagnosis by isolation, theoretically the only way to confirm the existence of Q fever, is only undertaken in specialised laboratories because *C.burnetti* is highly contagious for humans. Personnel involved must be qualified and informed of the risks involved in handling the organism.

7.5.3.1 Isolation in laboratory animals

Principle

The advantage of using the animal model is two fold; it allows the use of a possibly contaminated inoculum and it produces seroconversion as evidence of the infection. The models of choice are the guinea-pig and the mouse.

Procedure

1. Inoculate guinea-pigs (3-5 ml) or mice (0.5-1.0 ml) by the intraperitoneal route. Volumes used should conform to regulatory guidelines.
2. Examine the animals daily and note their temperatures and general condition.
3. Remove blood samples immediately prior to inoculation and about two weeks later by cardiac puncture (guinea-pigs) or retro-orbitally (mice).
4. Analyse sera for the presence of antibodies by complement fixation (see Section 7.6.1).

Reading and interpretation of results

Seroconversion will be detectable two weeks after inoculation.

Note

• The diagnosis is positive if seroconversion, against a specific antigen, is observed in two sera taken at a 15 day interval.

• The spleen of the autopsied animal

can be used to undertake isolation in embryonated hen's eggs or cell cultures.

• Isolation in laboratory animals can be hazardous for the handler. As far as possible, alternative isolation techniques should be used.

7.5.3.2 Isolation in embryonated hen's eggs

Principle

The endodermal cells of the yolk sac membrane of the 7 day old chick embryo are highly sensitive to *C.burnetti*. Multiplication of the *Coxiella* causes death of the embryo. After autopsy, the yolk sac membrane is recovered and examined. The technique is identical to that described in the chapter on Chlamydiosis except that:

1. Eggs in which the embryo dies after the 4th day following inoculation should be held at 4°C for 24 hours as the coxiella will continue to multiply during this period. Embryonic death before the 4th day following inoculation will be due to the inoculation procedure or to other contaminating organisms.

2. To confirm the presence of coxiella, make a smear with a piece of the yolk sac membrane and stain.

Note

• It is best to use logarithmic dilutions of the initial sample to overcome possible contamination by undesirable organisms.

• It is sometimes necessary to undertake blind passages before any mortalities occur.

• This is a highly sensitive method for the isolation of *C.burnetti*. Isolation techniques should only be used to confirm the results of bacteriology when absolutely necessary.

7.5.3.3 Isolation in cell culture

Principle

Coxiella burnetti can be studied in a number of continuous cell culture lines [12]: embryonic chick cells, monkey cells, mice cells and notably the L292 cell line. The principle is the same as that for culture in embryonated hen's eggs.

Materials and reagents

- CO_2 incubator.
- High security laminar flow hood.
- Inversion microscope.
- Light or UV microscope, depending on the staining method used.
- Flasks of cells.
- Maintenance media
- Growth media (see Media I for isolation of *Chlamydia*)
- Tubes or plates for coverslip cell cultures.

Procedure

1. Grow cells in monolayers on coverslips placed in tubes or culture plates.

2. Remove the media and replace with successive logarithmic dilutions of samples.

3. Incubate the cultures for 30 minutes at 37°C to allow adsorption of the inoculum.

4. Remove the inoculum and replace by maintenance media.

5. Incubate cultures for 4 to 7 days at 37°C.

6. Remove the coverslips and stain or examine by immunofluorescence according to the techniques described previously.

Reading of results

Coxiella appear intracellularly as cytoplasmic inclusion bodies.

Note

• Cell culture isolation of *C.burnetti* is

much simpler than isolation in laboratory animals or embryonated hen's eggs although it is less sensitive than the latter technique. The reduced risk of contamination of personnel, however, makes it the preferred method for use.

Molecular biological techniques are playing a more and more important role in detection of pathological infections in small ruminants. Research laboratories have identified specific DNA primers of *C.burnetti* that can be used in the polymerase chain reaction (PCR). These highly sensitive techniques, currently under validation, may soon lead to production of direct diagnostic methods for Q Fever in small ruminants that are accurate, sensitive, rapid and reasonably priced. They will also have important applications in the study of the routes of human infection (milk, manure, etc.).

7.6 INDIRECT DIAGNOSIS

The objective of indirect diagnosis is to determine the latent characteristic or progression of infection through the flock. It is a flock diagnosis which cannot be used for diagnosis in an individual animal. It detects the presence of specific antibodies to *C.burnetti*.

Different techniques have been proposed over the past 20 years but at present, despite some disadvantages, complement fixation is probably used most frequently. It works by combining, in addition to the classical reagents (complement, haemolytic serum, red blood cells), a specific antigen and serial dilutions of the test serum. Serological diagnosis of the flock, essential in small ruminants, avoids the requirement to show a seroconversion. Use of a microtitre plate technique, possibly automated, the reference method of the French Reseau National d'Essais, allows the simultaneous performance of large numbers of analyses [10].

7.6.1 Complement fixation

Principle

The principle of the technique is the same as that described in the chapter on *Brucella*.

Materials and reagents

- Refrigerated centrifuge at 4°C, capable of spinning at 500 xg.
- Mirror for reading plates.
- Phase II antigen, commercially available and containing a mixture of Nine Mile and Henzerling strains of *C.burnetti*. The working dilution is determined following titration.
- Standard positive and negative sera.
- Veronal-calcium-magnesium buffer at pH 7.2.
- Complement: It is preferable to purchase a commercially available reagent, titrated by a reference technique.
- Haemolytic system: Comprising a mixture of equal parts of 2% sheep red blood cells and rabbit anti-sheep haemolytic serum, used after 20 minutes incubation at room temperature.

Preparation of reagents

- Veronal-calcium-magnesium buffer (pH 7.2)

Sodium chloride	8.500 g
Diethylmalonylurea	0.575 g
Sodium diethylmalonylurea	0.185 g
Magnesium chloride ($6H_2O$)	0.168 g
Calcium chloride	0.028 g
Distilled water	1000 ml

Procedure

1. Decomplement 1/10 test sera dilutions for 30 minutes at 60°C.

2. Make doubling dilutions of the sera in microtitre plates using 25µl volumes. Each test sera is diluted from 1/10 to

1/320. To confirm the absence of anticomplement activity in the sera, set up one well of standard serum without antigen for each of the first 4 dilutions (1/10 to 1/80).

3. Add 25µl of antigen to the test sample wells, or 25µl of diluent in the standard serum wells.

4. Add 25µl of complement at working dilution to all wells.

5. Leave plates at 4°C overnight (16 to 18 hours), preventing evaporation.

6. The following day, remove the plates from the refrigerator and bring back to room temperature.

7. Add 25µl haemolytic system to all wells.

8. Gently agitate the plates and incubate for 30 minutes at 37°C, preventing evaporation.

9. Centrifuge the plates at 500 xg and read the results using the mirror.

Reading and interpretation of results

The batch of tests is validated by the results of the standards which are included in each batch of reactions (Table 7.1).

- Antigen standard : 100% haemolysis
- Complement standard :100% haemolysis
- Red blood cell standard : 0% haemolysis
- Titres of positive and of negative sera.

Assess the percentage haemolysis in the reaction wells for samples where the anticomplement activity is expressed by haemolysis below 100% in the corresponding standard wells. The titre of the serum is the lowest dilution showing 50% haemolysis or less. The dilution of 1/80 is taken as the limiting dilution beyond which a diagnosis of abortion or of infection with *C.burnetti* can be estblished. Positive reactions between 1/10 and 1/40 are not significant of recent infection but may relate to a latent infection.

Note

- Q Fever is a zoonosis. Sanitary measures must be introduced immediately titres of above 1/40 are obtained.
- Naturally infected animals mainly produce IgG1, thus complement fixation is a useful technique for flock diagnosis. Sera containing IgG2 and IgM are difficult to detect by this method and can lead to production of false negative results, especially in early infections.
- Animals infected by wild strains of *C.burnetti* may react differently with the two strains used in the manufacture of the commercial antigens (Nine Mile and Henzerling).

These disadvantages underline the utmost importance of flock diagnosis in this abortive disease.

In addition to complement fixation, other techniques which can be used are:

Microagglutination in capillary tubes [5].

Table 7.1: Composition of different controls introduced to each series in complement fixation tests

	Wells		Controls		
	Reagent	**Control**	**Antigen**	**complement**	**Erythrocytes**
Serum	25µl	25µl	0	0	0
Antigen	25µl	0	25µl	0	0
Complement	25µl	25µl	25µl	25µl	0
Diluant	0	25µl	25µl	50µl	75µl

Indirect immunofluorescence can be undertaken using commercially available diagnostic kits for detection of antibodies to the organism in humans (BioMerieux, Diagnostic Pasteur) using the relevant anti-ovine or anti-caprine fluorescently conjugated immunoglobulin. The technique, however, is difficult to use and reading of the results is tedious.

ELISA, a technique in full development for production of marketable reagents in the near future. These reagents, currently under validation, are due to replace the techniques that already exist, with the following advantages: greater sensitivity, automation, simpler to use, convenient to read the results (Boehringer Diagnostic).

7.7 FUTURE WORK

The zoonotic disease, Q Fever, is relatively easy to diagnose in the laboratory by standard direct and indirect techniques, providing that it is always undertaken as a flock diagnosis and not a diagnosis of the individual. However, in-depth study of new methods of diagnosis is still warranted: the ELISA is set to take an important position as an indirect diagnosis and molecular biological techniques such as PCR open attractive prospects within the context of organism identification. It is evident that the perfection of more sensitive and easier to use

Summary of advantages and disadvantages of different laboratory techniques

1. Direct diagnosis

						Needs		
	Sensitivity	**Specificity**	**Easiness**	**Rapidity**	**Cost**	**Qualified personnel**	**Specific equipement**	**Automa-tisation**
Bacterioscopy	±	-	+	+++	-	++	-	-
DIF[a]	+	++	+	++	++	++	++	-
kits	+	++	+	++	+++	++	++	-
ELISA	+	++	+	+	++	+	+	+
kits	+	++	+	+	+++	+	+	+
PCR ou LCR[b]	+++	++	-	+	+++	+++	+++	+
Isolation on egg	+	++	±	-	+	+	-	-
Cell cultures	+	++	±	±	++	++	+	-

a : Direct Immunofluorescence
b : Ligase Chain Reaction

2. Indirect diagnosis

						Needs		
	Sensitivity	**Specificity**	**Easiness**	**Rapidity**	**Cost**	**Qualified personnel**	**Specific equipement**	**Automa-tisation**
Complement fixation	±	+	+++	++	+	+	-	-
Immunofluo-rescence	+++	++	+	+++	++	++	++	-
ELISA	+++	+	+++	++	++	+	+	++
Delayed hypersensitivity	+	++	++	+	+	+	-	-

techniques, utilising Mabs, can allow early and reliable detection of an organism which is hazardous to isolate. It will naturally lead to better control of the infection at a flock level and a decrease in human infections.

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