Chapter 5

CHLAMYDIOSIS

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5.1 SUMMARY

Chlamydia psittaci induces a range of pathological symptoms in small ruminants. Abortion is the most common clinical symptom, one that poses a risk to human health particularly in pregnant women and causes important economic losses. Diagnosis is confirmed by the detection of bacteria in smears or impressions of the placenta combined with serological analysis of at least ten sera samples, by complement fixation or ELISA. These techniques lack sensitivity and specificity but are the only methods available in a large number of laboratories. More specific techniques, such as direct detection of chlamydia in vaginal swabs using commercial kits designed for diagnosis of human infections, are expensive. Isolation of *Chlamydia* and detection by PCR are restricted to the research domain or very specialised laboratories. The production of antigens specific to abortigenic *Chlamydia*, utilised for serological diagnosis in veterinary laboratories, has improved diagnosis considerably.

5.2 INTRODUCTION

Chlamydiosis is a frequent cause of infectious abortion in numerous sheep and goat farming regions. First identified in 1950 in Edinburgh, Scotland by Stamp, it is caused by *Chlamydia psittaci*, a very common micro-organism. *Chlamydia* can be isolated from most domestic and wild animals and virtually all avian species.

Chlamydia psittaci is one of three species of the genus *Chlamydia* from the family of Chlamydiaceae of the order of Chlamydiales. It is composed of a heterogeneous group of strains that infect mammals, birds and marsupials. The creation of a fourth species, *C.pecorum*, to differentiate porcine and ruminant strains, has been proposed. *Chlamydia pecorum* corresponds to serotype 2 strains of *C.psittaci* which are responsible for pneumonia, arthritis and conjunctivitis, as well as to strains isolated from inapparent intestinal infections.

Chlamydia trachomatis and *C.pneumoniae* are human pathogens. Recently porcine strains showing characteristics of *C.trachomatis* have been isolated from abortions, vaginal infections and pneumonias. In the same way equine respiratory strains may form new serotypes of *C.pneumoniae*.

Chlamydia are small bacteria. They multiply in the cytoplasm of eukaryotic cells in a unique cycle of development. During the cycle, a resistant infectious form, the elementary bodies (EB) alternate with a metabolically active non-infectious form, the reticulate bodies (RB). RB's do not survive outside the cells whilst EB's are capable of surviving in the extra-cellular fluid but are rapidly inactivated.

Man can be infected by *C.psittaci* : psittacosis, a serious and sometimes fatal pneumonia, is a chlamydial zoonosis of avian origin. Abortion with severe complications caused by *C.psittaci* has been described in

young women working with lambing flocks affected by abortive chlamydiosis.

In small ruminants *C.psittaci* causes abortion, epididymitis, orchitis, pneumonia and arthritis. Healthy animals can sometimes carry *Chlamydia* which they excrete in their faeces but *Chlamydia* can also be isolated from the faeces of lambs suffering from enteritis.

The economic losses from chlamydial abortion are severe. In a newly infected flock about one third of pregnant ewes and more than 60% of pregnant goats may abort. This high rate of abortion is observed for two or three years after which the disease takes on a cyclic nature: abortion affects less than 10% of pregnant females for several years until a new outbreak when all primiparous ewes abort.

The disease manifests itself in the way of an abortion, production of premature.

Lambs, or birth of weak young at term that quickly fall ill or have difficulty in rising. In the majority of cases abortion occurs in the final month of gestation without any previous clinical symptoms. Sometimes vaginal bleeding is observed a few days before abortion, more frequently in goats than in sheep. The foetus does not exhibit any specific macroscopic lesions. Depending on the time between death and its expulsion, the foetus may be more or less autolysed. Lambs born close to term are generally healthy but may be covered in a frothy light brown material and show subcutaneous oedema, petechiae on the tongue, in the buccal cavity and on the hooves and have exudates, with or without blood, in the pleural cavity and peritoneum.

Aborted ewes usually recover rapidly and placental retention is rare. Ewes may contract metritis which can be fatal in a very small number of cases. Complications such as arthritis, pneumonia, retained placenta and metritis are more common in goats.

Abortion generally produces a high level of immunity: it is exceptional for a female to abort twice.

Females that abort play a key role in the transmission of infection. Massive amounts of *Chlamydia* are excreted with the placenta and foetal fluids. Susceptibility to infection varies in relation to physiological status. Sheep and goats at less than 100 days of gestation are more susceptible than those that are barren or at the end of gestation. Those that abort early in the lambing season are an important source of contamination for the rest of the flock. However, in intensive lambing conditions, contamination at the time of lambing can lead to abortions during the following gestation.

In sheep, excretion basically occurs at the time of abortion and for at least the following two days although *Chlamydia* can also be excreted in smaller amounts in the urine, faeces and milk for a number of days after abortion. Goats excrete large quantities of chlamydia in vaginal fluids from more than two weeks before abortion to more than two weeks afterwards. This may explain the higher incidence of abortion in newly infected goat herds.

The infection is retained in the flock, or is transmitted to other flocks by replacement stock, born to infected mothers, which abort during their first gestation. These young sheep and goats provide a reservoir of infection which may go undetected during screening since their antibody titres are generally low and not detectable by the currently available serological diagnostic tests.

The role of the male in the venereal transmission of the disease has still to be

clarified. However, experimentation on venereal transmission indicates that genital infection in the males may result in male sterility and infertility or metritis rather than abortion in females.

The role of inapparent intestinal chlamydial infections in the epidemiology of abortigenic infections has not been sufficiently investigated due to lack of epidemiological markers.

Control and preventive measures to limit the spread of the disease :

- avoid introducing animals of unknown health status to the flock;
- isolate females about to abort or lamb;
- collect placentas and dead lambs.

Such measures are more effective with sheep than with goats as sheep very rarely excrete chlamydia before abortion.

Chlamydia are sensitive to antibiotics and injection with tetracycline is an effective preventive measure. However, antibiotic treatment regimes (two intramuscular injections of tetracycline at a rate of 20 mg/kg at 105 and 120 days of gestation) whilst limiting the number of abortions do not prevent the excretion of chlamydia at birth or control the level of infection in the flock.

Killed adjuvant vaccines give similar results to antibiotic treatment which is important from the epidemiological point of view. The systematic use of this type of vaccine can result in selection of chlamydial strains against which the vaccine is not as effective. To date one live vaccine containing a temperature sensitive mutant obtained by mutation of an abortigenic wild strain of *Chlamydia* has been produced. This vaccine prevents abortions and the excretion of *Chlamydia*.

5.3 SAMPLES

5.3.1 Direct diagnosis

5.3.1.1 Bacteriological

The best sample for bacteriology is a smear or impression of placental cotyledons showing lesions. When placental material is not available, a smear can be

Table 5.1 Sample suitability for different methods of analysis

	Placenta	Vaginal Swab	Aborted Foetus: Fluids			Blood
			Stomach	Pleural	Peritoneal	
Bacteriology	+++	±	±	±	±	-
Antigen detection						
Immunofluorescence	+++	+	+	+	+	-
ELISA	±	+++	+	+	+	-
PCR	±	+++	±	±	±	-
Chlamydial isolation						
Eggs	+	+++	+	+	+	-
Cell culture	+	+++	-	-	-	-
Serology						
Complement fixation	-	-	-	-	-	+++
ELISA	-	-	-	-	-	+++
Immunmofluorscence	-	-	-	-	-	+++

+++ : highly suitable sample ; + : sample can be used but may not give the best results ; ± : samples difficult to use

made using the stomach contents of the aborted foetus or from a vaginal swab collected within 24 hours following abortion in sheep or three days following abortion in goats. Vaginal secretions should be sampled by swabbing the walls and base of the vaginal cavity (Table 5.1).

5.3.1.2 Antigen detection

Immunofluorescence can be used to demonstrate the presence of *Chlamydia* or their antigens in the smears prepared for bacteriological examination (Table 5.1).

Chlamydial antigen can also be detected by ELISA carried out on vaginal swab samples, collected as described previously, with the swab being placed in a small volume of transport medium. The suspension obtained should be transferred to a sterile plastic tube, hermetically sealed and transported to the laboratory.

Transport medium

The following transport medium ensures maximum survival of *Chlamydia* :

Sucrose	75g
KH_2PO_4	0.52g
Na_2HPO_4	1.22g
Glutamic acid	0.72g

Dissolve all ingredients in deionised water and make up to 1 litre, adjust the pH to 7.4-7.6 and sterilise by filtration through a 0.22µm sterile filter.

5.3.1.3 DNA analysis : PCR

The identification of chlamydial genes by PCR can be carried out on vaginal swab samples collected in the same way as samples analysed for the presence of chlamydial antigens (Table 5.1).

5.3.1.4 Isolation of Chlamydia

Isolation of *Chlamydia* is rarely undertaken in veterinary medicine. Chlamydial organisms are very fragile and rarely survive in the external environment, their intracellular multiplication requires uncontaminated samples which are often difficult to obtain.

Samples must be prolific in *Chlamydia* and must not be contaminated with other micro-organisms or cytotoxins which will effect the survival of the *Chlamydia*. Vaginal swabs taken for antigen detection are the best samples to use for chlamydial isolation.

The placenta is often heavily infected although the level of infection varies considerably from one cotyledon to another, which can easily result in an incorrect diagnosis. Vaginal swabs indicate an average infection of the placenta but can give a negative result when cotyledons are highly positive. However, they provide better samples for bacteriological analysis since they are easier and safer to handle, are rarely cytotoxic and can easily be resampled from the transport medium.

If the aborted foetus is well conserved, stomach, pleural and peritoneal fluids which are often very rich in *Chlamydia* and free of other contaminants, can be sampled. However, these samples may be cytotoxic and cannot be used for chlamydial isolation in embryonated hens eggs.

5.3.2 Indirect diagnosis

Blood samples should be collected sterilely using evacuated, dry tubes, and the blood clot separated quickly from the serum to avoid microbiological contaminants and haemolysis which will affect the results of analysis. The antibody titre is often highest three weeks after abortion and it is best to take two samples at a three week interval from several animals that have aborted during the preceding weeks.

5.4 RISKS TO HUMAN HEALTH

Samples must be collected and handled with the same precautions as all biological materials that are potentially dangerous to human health because:

- *Chlamydia* isolated from ruminants are contagious to humans. For a long time they have been considered as minor pathogens, similar to avian strains, even though they can induce mild to severe pneumonia's and Girand has, for a long time, highlighted the risk that they present to pregnant females. The latter was only taken seriously after a description by British investigators of abortion with severe complications in pregnant human females who had been involved with lambing of contaminated ewes. One of these women actually died as a result of the infection. Initially, it was thought that this isolated case resulted from the selection of a particularly virulent strain by use of a live vaccine, but the description of the case is similar to others described in France where this problem is a lot more common and very underestimated.
- Samples can also be contaminated with other abortive organisms pathogenic to humans: *Coxiella burnetti*, *Brucella melitensis*.

5.5 DIRECT DIAGNOSIS

5.5.1 Bacteriological

A number of staining methods have been developed to detect *Chlamydia* in biological samples. These techniques are quick and can be undertaken easily in most laboratories but their interpretation is often tricky. Their major limitation is their level of sensitivity and specificity but stained slides keep well and can be sent to a specialised laboratory for confirmation.

Principle

Chlamydia in impressions or smears of the organs, stained by the Stamp, Gimenez, Machiavello or Giemsa method and examined by microscope at high magnification, appear as bright coccoid structures either individually or in groups (the technique described here is a variation of the method reported in the chapter on Brucellosis).

5.5.1.1 Staining method of stamp

Materials and reagents

- Microscope, 10x eyepiece, immersion objective 100/1.25.
- Basic Fuchsin solution.
- 0.3% acetic acid.
- 1% malachite green in deionised water (counterstain).

Procedure (Figure 5.1)

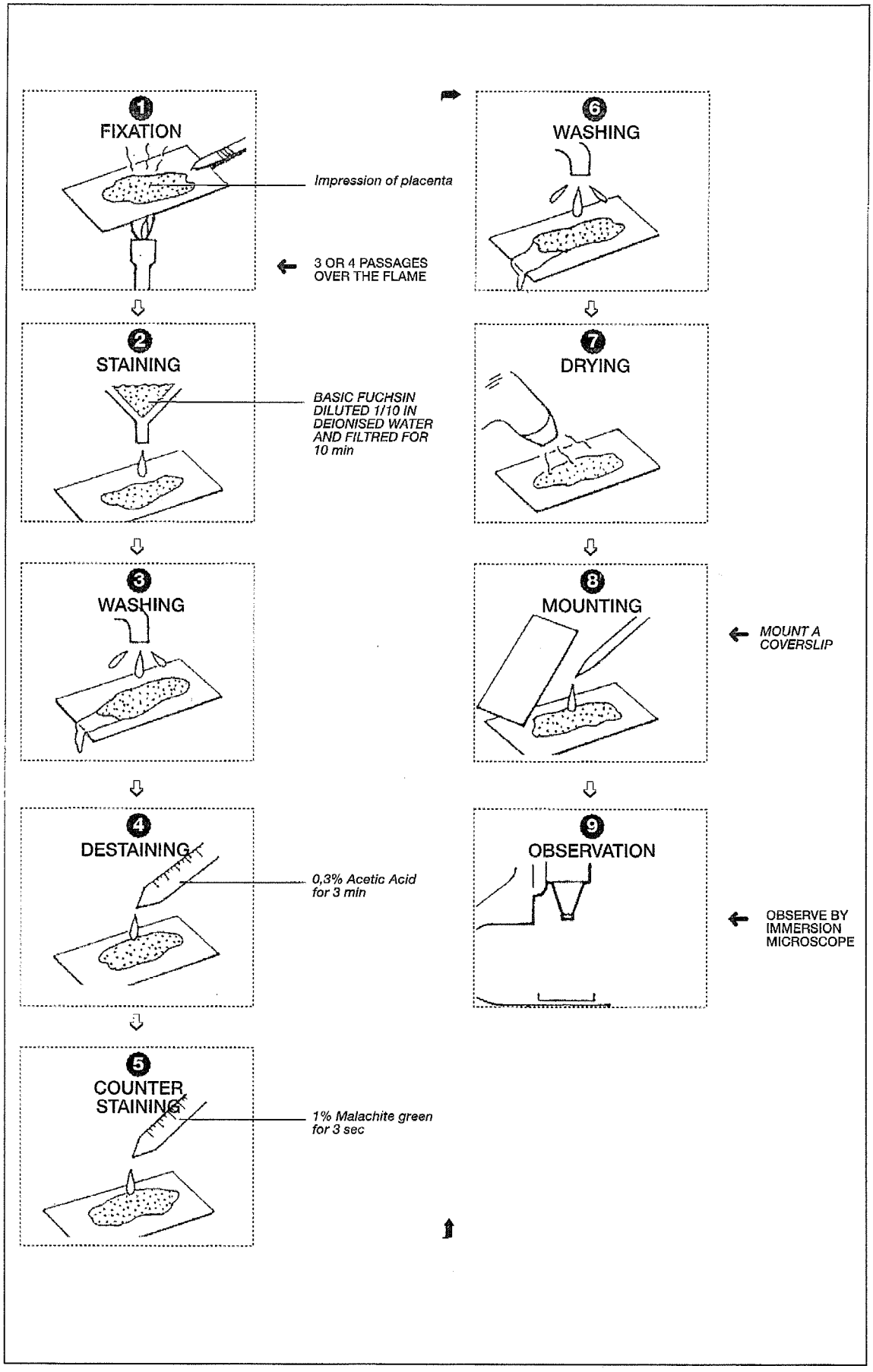
1. Fix the slide over a flame.
2. Leave for 10 minutes in a solution of basic Fuchsin freshly diluted 1 in 10 in deionised water and filtered.
3. Rinse with tap water.
4. De-stain in 0.3% acetic acid for 30 seconds.
5. Counterstain with malachite green solution for 30 seconds.
6. Rinse with tap water.
7. Dry, mount and observe by immersion microscopy.

Reading of results

Elementary bodies appear as small bright red bodies against a blue-green background.

Note

- The diluted solution of basic Fuchsin should not be kept for longer than one week. Filter directly onto the slide (Figure 5.1) because the presence



5.1 : Bacteriological detection. Staining of Chlamydia by the stamp method

of grains or small crystals of the stain can give false positives.

- The time of destaining in acetic acid and counterstaining in malachite green will vary depending on the thickness of the smear.
- Reading by microscope is lengthy, difficult and requires an experienced person who can differentiate *Chlamydia* from *Coxiella* and *Brucella*.
- In all cases bacteriological analysis should be undertaken alongside serological analysis to avoid interpretative errors.

Preparation of solutions

- Solution of Basic Fuchsin
- Solution a: 1.5g of basic Fuchsin ground in a mortar dissolved in 15ml of 95% ethanol.

Filter and allow to stand for 12 to 24 hours.

- Solution b: 2g of phenol dissolved in 50ml of double distilled water freshly boiled and cooled away from draught.
- Mix 10ml of Solution a with Solution b.
- Make up to 100ml with double distilled water in a volumetric flask.
- Store in a wide necked bottle.

5.5.2 Antigen detection methods

New methods, based on detection of chlamydial antigen, that are specific and more sensitive than bacteriological methods are being developed. They require use of more sophisticated equipment and are more expensive to carry out.

Principle

Chlamydial antigens can be detected in samples by use of chlamydial antibodies which are themselves detected by immunoglobulin conjugates labelled with fluorescien isothiocyanate (immunofluorescence) or alkaline phosphatase (ELISA).

Diagnostic kits for detection of *C.trachomatis* infection in humans are commercially available and can be used to detect infections by *C.psittaci* cusing antibodies raised against specific antigens [1, 16]. Although they are expensive, they can be useful in laboratories not regularly involved in chlamydial research or for confirmation of doubtful bacteriological results.

5.5.2.1 Direct immunofluorescence

Immunofluorescent analysis can be carried out using smears prepared for bacteriological analysis by means of commercially available kits (Chlamydia Direct IF, bioMerieux, France; Chlamydia DETECT, Eurobio, France; IMAGEN™ DAKO Diagnostics Ltd, UK; Micro Tract, Syva CA, USA, etc) according to the manufacturers instructions or, alternatively, using serum from the laboratory according to the following method :

Materials and reagents

- Fluorescent microscope.
- Monoclonal antibodies or serum raised against chlamydia (positive sheep or rabbit serum
- see method of immunisation of rabbits described for the ELISA test).
- Immunoglobulin serum conjugate raised in mice, labelled with fluorescien isothiocyanate.
- 150mM NaCl in deionised water.
- Evan's blue.

Procedure

1. Dry the slide for 30 minutes at 37°C.
2. Fix in acetone for 20 minutes at room temperature.
3. Dry again for 30 minutes at 37°C.
4. Incubate for 30 minutes at 37°C with the monoclonal antibody.

5. Rinse once using deionised water then twice for 10 minutes in NaCl solution.

6. Incubate for 30 minutes at 37°C in the dark with the appropriate dilution of conjugate containing 0.01% Evan's blue.

7. Examine by fluorescent microscope.

Reading of results

Chlamydia appear as small, bright, yellow-green bodies, individually or in groups.

Note

• The reading of the results by microscope is lengthy and difficult but it is not possible to confuse *Chlamydia* with other bacteria.

• When the level of chlamydial infection is low, diagnosis may be confounded by the presence of non-specific fluorescence. To aid the interpretation of the results, non-lysed infected cells containing fluorescent cytoplasmic inclusion bodies should be sought.

• This test can be undertaken on sections of placental tissue where the chlamydia appear as inclusion bodies in the cell cytoplasm and are much easier to see than the extracellular *Chlamydia* [1].

5.5.2.2 ELISA

This test is carried out using vaginal swab samples by means of commercially available kits (*Chlamydia* IDENT, Eurobio, France; Chlamydiazyme", Abbott Diagnostic Products IL, USA; Clearview, Unipath Ltd, UK; IDEIA, Novo BioLabs, UK; Microtrak EIA, Syva CA, USA; Wellcozyme *Chlamydia*, Wellcome, etc) following the manufacturers instructions or by the following method [12] :

Materials and reagents

- Microtitre plates.
- Rotary shaker.
- ELISA plate reader.
- 20mM Phosphate buffered saline (PBS), pH 7.3.
- Tween 20.
- NaOH.
- Ammonium sulphate.
- Dialysis tubing.
- 0.1M Bicarbonate buffer, pH 9.8.
- Antibodies raised against *Chlamydia* for antigen capture.
- Antibodies raised against *Chlamydia* for antigen detection.
- Immunoglobulin conjugate raised in rabbits or mice (depending on availability) marked with alkaline phosphatase.
- Substrate: r-Nitrophenyl phosphate disodium (Sigma Chemical Co, USA) diluted to 1mg/ml in diethanolamine buffer, pH 10.2.

Procedure

1. Absorb 200µl of the optimum dilution of the capture antibody in bicarbonate buffer onto each well of the microtitre plate for 3 hours at 37°C, shaking at 200 cycles/minute.

2. Wash the plates three times with deionised water.

3. Dry the plates for 2 hours at 37°C. Plates can be used immediately or stored at 4°C for up to one month.

4. Heat the vaginal swabs for 30 minutes at 100°C following addition of NaOH to a final concentration of 0.1N.

5. Make two dilutions of each sample of 1 in 5 and 1 in 10 in PBS containing 0.05% Tween 20.

6. Add 100µl aliquots of the different sample dilutions to wells coated with capture antibody and incubate for 3 hours at 37°C shaking at 200 cycles/minute. Include a negative control

sample (a swab collected from an uninfected ewe) and a positive control sample (prepared from the negative swab sample with the addition of a known quantity of *Chlamydia*), treated and diluted by the same methods as the samples in each set of samples.

7. Rinse the plates four times with deionised water and wash three times for 5 minutes each wash in PBS with 0.05% Tween 20.

8. Add 100µl of the optimal dilution of the detection antibody in PBS with 0.05% Tween 20 to wells and incubate for one hour at 37°C shaking at 200 cycles/minute.

9. Wash the plates as previously described.

10.Add 100µl of conjugate diluted according to the manufacturers instructions and incubate for 1 hour at 37°C shaking at 200 cycles/minute.

11.Wash the plates as previously described.

12.Add 100µl of substrate and incubate for 1 hour at 37°C or until an OD of <0.2 is obtained in the negative control.

13.Stop the reaction by addition of NaOH.

Reading of results

Read the plates at an OD of 405nm. A sample is positive if the OD is >0.4.

Note

• The plates may also be sensitised by leaving overnight at 4°C and they can be stored, unwashed, at this temperature for up to one month.

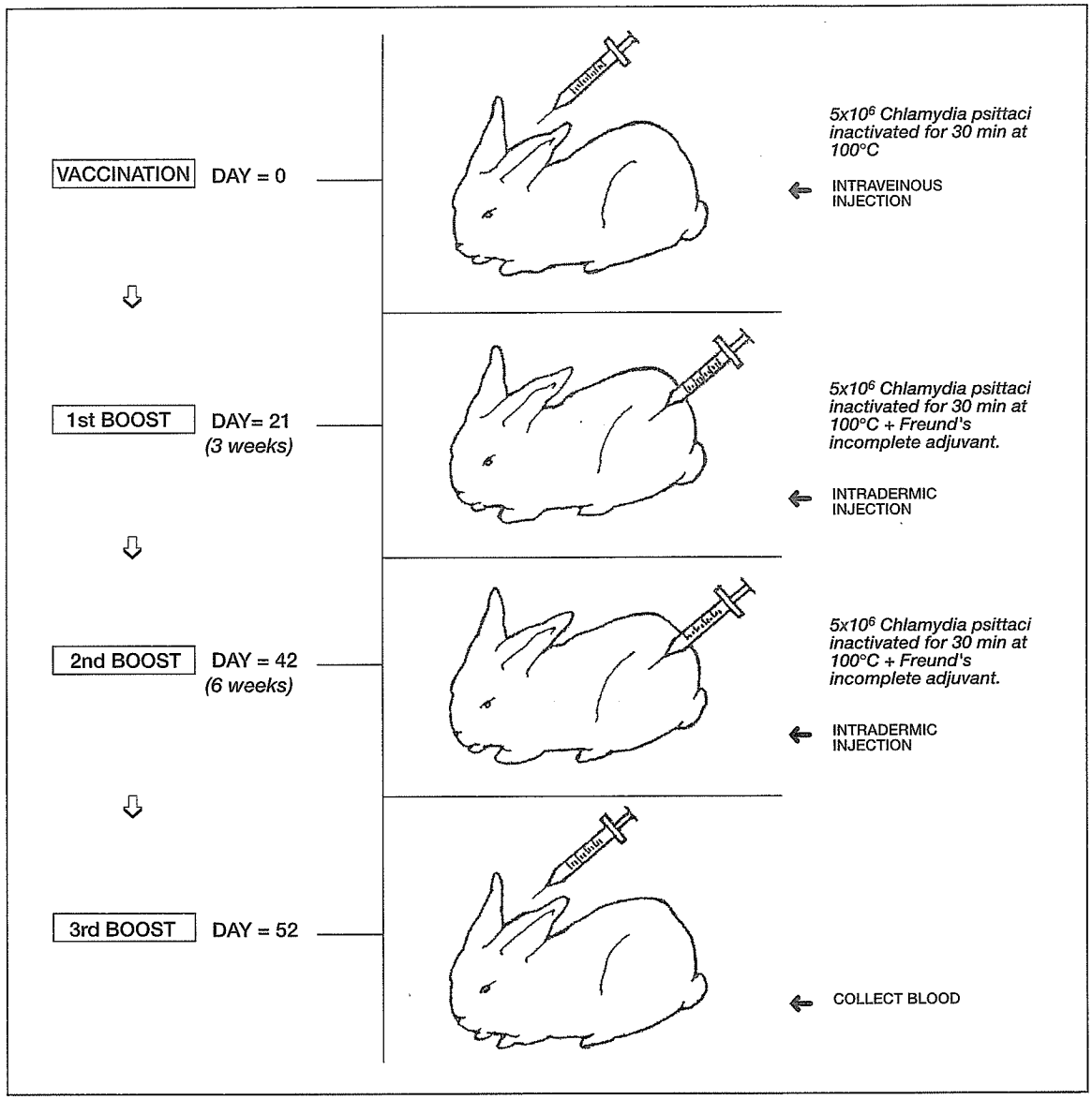
Preparation of reagents

- Capture Antibodies. Capture antibodies are prepared from sera taken from a positive ewe. Precipitate the immunoglobulins by addition of ammonium sulphate to a 50% concentration. Resuspend the precipitate containing the chlamydial antibodies in 40% ammonium sulphate [14], dialyse overnight at 4°C against PBS then dilute 1/1 (v/v) in glycerol and store at -20°C. The optimal dilution for use is determined by using a known quantity of *Chlamydia* diluted on a vaginal swab taken from an uninfected ewe.

- Detection Antibodies. (Figure 5.2). Monoclonal antibodies or polyclonal antibodies are obtained by immunisation of a rabbit by intravenous injection of 5 x 10^6 *C.psittaci* inactivated for 30 minutes at 100°C, followed by two repeat doses given intradermally at three week intervals, using the same quantity of inactivated *Chlamydia* prepared in Freud's incomplete adjuvant. Collect blood 10 days after the final dose. The optimal dilution for use is determined by a direct ELISA reaction using plates sensitised for one night at 37°C with 10 - 10^6 *C.psittaci* diluted in bicarbonate buffer.

5.5.3 DNA analysis

It is possible to detect chlamydial DNA in vaginal swab samples by hybridisation with a DNA probe, or better by amplification of DNA by PCR (polymerase chain reaction) [4, 15] or by a variation of PCR, LCR (ligase chain reaction) [3]. PCR and LCR are used in human medicine for diagnosis of chlamydial infections and are considered to be the most sensitive diagnostic methods available. Some of the diagnostic kits available can be used in veterinary medicine because they work by priming the oligonucleotides of the genes for the major outer membrane proteins (MOMP) common to all type of *Chlamydia*. However, these techniques require expensive apparatus and reagents



5.2 : Chlamydial antigen detection by ELISA
Rabbit immunisation protocol for production of antichlamydial antibodies

as well as experienced personnel working with very strict precautions and usually in isolated locations to prevent the occurrence of false positives.

It is unlikely that routine application of these techniques will occur in many veterinary laboratories in the near future but such methods may be applied regularly in laboratories familiar with molecular biological techniques.

5.5.4 Isolation of *Chlamydia*

Isolation of *Chlamydia* can be carried out :

- in embryonated hen's eggs, an old and lengthy technique but one that allows easy recovery of the chlamydial strains and preparation of antigens ;
- in cell culture by staining of the inclusion bodies, a very quick but expensive technique.
- by the technique of plaque lysis, a long and expensive method but one which allows cloning of strains [8].

5.5.4.1 Embryonated hen's eggs

Principle

Chlamydia are inoculated into the yolk sac of embryonated eggs at 6-8 days of age. They multiply there and cause the

death of the embryo after 5-12 days depending on the number of *Chlamydia* inoculated. The yolk sac membranes from eggs that die from the 7th day after inoculation are very rich in *Chlamydia* and can be used to isolate the strain or to prepare antigens.

Materials and reagents

- Egg incubator and candler.
- Physiological saline and sterile sand.
- Antibiotics: gentamycin (gentalline) or streptomycin and amphotericin B (fungizone).

Procedure (Figure 5.3)

1. Incubate the embryonated hen's eggs at 38°C in a humid atmosphere (50 - 70% humidity). Candle to verify their vitality and mark on each the position of the air sac and the embryo.
2. Dilute the samples 1 in 2 then by further 10-fold dilutions (1/20, 1/200, 1/2000) with the physiological saline containing antibiotics (500µg/ml gentamycin or 200µg/ml streptomycin and 0.5µg/ml amphotericin B).
3. Inject 0.2ml of the different sample dilutions into the yolk sac of each of 3 to 5 eggs.
4. Incubate the eggs and candle twice daily.
5. Examine eggs dying from the 5th day after inoculation. Discard those which die before the 5th day as these deaths result from the inoculation or from external contaminants. Embryos infected with *Chlamydia* have cyanotic feet and claws (Figure 5.4, page 93). The yolk sac membrane is generally thin and red and the yolk is more fluid than in an uninfected embryonated egg of the same age.
6. Take an impression smear from a part of the yolk sac membrane and stain for detection of *Chlamydia*.
7. Check for the absence of any contaminating bacteria by culturing a sample on blood agar.
8. Remove and sterilely grind the rest of the membrane in a mortar with 2ml physiological saline and some Fontainebleau sand, then centrifuge at 125 xg for 20 minutes to remove the sand, gross cellular debris and fat.
9. Aliquot the supernatant containing the *Chlamydia* and store at -70°C or in liquid nitrogen to conserve the strain, or as a stock solution at -20°C for preparation of antigens.

Note

• It is preferable to use white eggs (making candling easier) produced under specific pathogen free (SPF) conditions to prevent mycoplasmal and viral contamination.

5.5.4.2 Cell culture method

Principle

A monolayer of McCoy L929 or Hela 229 cells is covered with sample then incubated for 48 hours at 37°C. The presence of *Chlamydia* is determined by staining the cytoplasmic inclusion bodies using methylene blue, Giemsa or acridine orange or by immunofluorescence.

Materials and reagents

- CO_2 incubator.
- Inversion microscope or fluorescent inversion microscope depending on the staining method.
- Cell culture flasks.
- Cell culture microtitre plates.
- Culture medium suitable for chlamydial multiplication (Medium 2).
- PBS - DEAE: 100µg/ml diethylaminoethyl dextran in 20mM PBS, pH 7.2 - 7.4