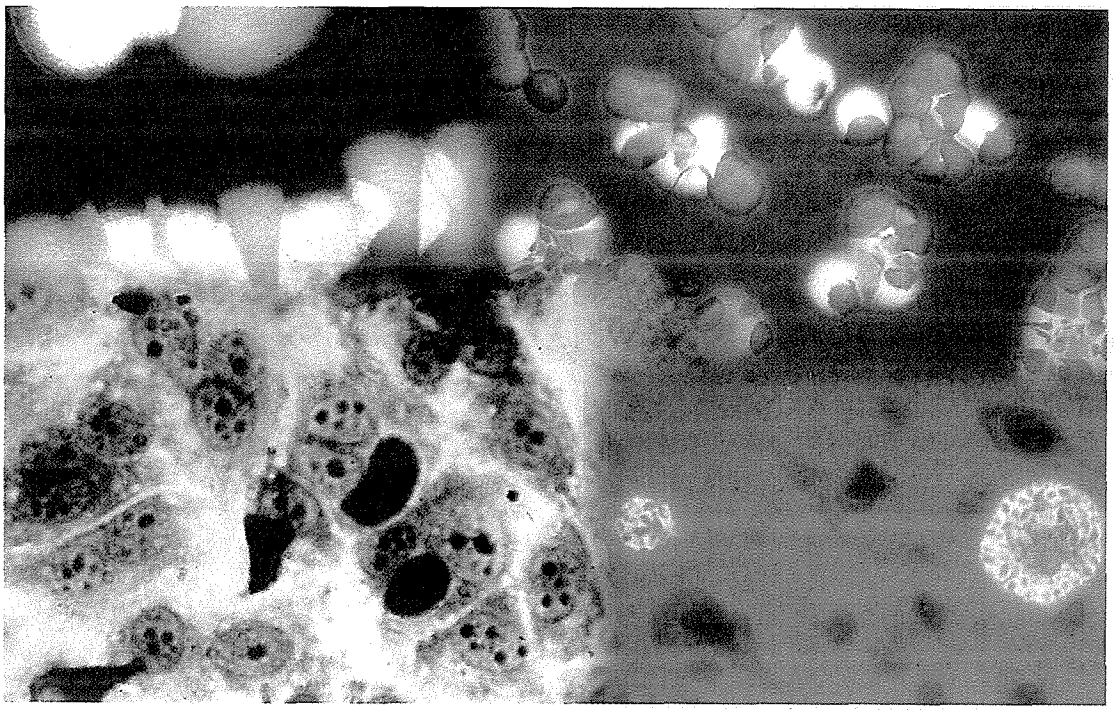
# MANUAL FOR LABORATORY DIAGNOSIS OF INFECTIOUS ABORTIONS IN SMALL RUMINANTS



**By :**

**A. RODOLAKIS**
INSTITUT NATIONAL DE LA RECHERCHE AGRONOMIQUE (INRA)
TOURS, FRANCE

**P. NETTLETON**
MOREDUN RESEARCH INSTITUTE
EDINBURGH, UK

**and**

**A. BENKIRANE**
Animal Health Service, AGAH, FAO

**FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS**
**Rome, 1998**

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# LIST OF AUTHORS

**B.J.H. Barnard**, Onderstepoort Veterinary Institute, Onderstepoort, South Africa.

**Abdelali Benkirane**, Animal Production and Health Division, Animal Health Service, FAO, Rome.

**Jocelyn Brebner**, Moredun Research Institute, 408 Gilmerton Road, Edinburgh EH17 7JH, Scotland.

**David Buxton**, Moredun Research Institute, 408 Gilmerton Road, Edinburgh EH17 7JH, Scotland.

**Michel Calamel**, CNEVA Sophia Antipolis, Laboratoire de Pathologie des Petits Ruminants et des Abeilles, "Les Templiers" 105, route des Chappes, -06410 BIOT, France.

**William Donachie**, Moredun Research Institute, 408 Gilmerton Road, Edinburgh EH17 7JH, Scotland.

**Barbara Dufour**, CNEVA Direction Generale, 23 Av General de Gualle BP19 94701, Maisons-Alfort, Cedex, France.

**William A. Ellis**, Veterinary Sciences Division, Stoney Road, Stormont, Belfast BT4 3SD, Northern Ireland.

**M. Grayon**, Pathologie Infectieuse et d'Immunologie, INRA, 37380 Nouzilly, France.

**Maurice Lambert**, CNEVA Sophia Antipolis, Laboratoire de Pathologie des Petits Ruminants et des Abeilles, "Les Templiers" 105, route des Chappes, -06410 BIOT, France.

**Christiane Le Louedec**, Pathologie Infectieuse et d'Immunologie, INRA, 37380 Nouzilly, France.

**J.C. Low**, SACVS Edinburgh, Bush Estate, Penicuik, Midlothian EH26 0QE, Scotland.

**S.J. McDowell**, Veterinary Sciences Division, Stoney Road, Stormont, Belfast BT4 3SD, Northern Ireland.

**Francois Mouton**, CNEVA Direction Generale, 23 Av General de Gualle BP19 94701, Maisons-Alfort, Cedex, France.

**Peter F. Nettleton**, Moredun Research Institute, 408 Gilmerton Road, Edinburgh EH17 7JH, Scotland.

**P. Pardon**, Pathologie Infectieuse et Immunologie, INRA, 37380 Nouzilly, France.
**Michel Pepin**, CNEVA Sophia Antipolis, Laboratoire de Pathologie des Petits Ruminants et des Abeilles, "Les Templiers" 105, route des Chappes, -06410 BIOT, France.

**Michel Plommet**, Pathologie Infectieuse et Immunologie, INRA, 37380 Nouzilly, France.

**Hugh W. Reid**, Moredun Research Institute, 408 Gilmerton Road, Edinburgh EH17 7JH, Scotland.

**Annie Rodolakis**, Pathologie Infectieuse et Immunologie, INRA, 37380 Nouzilly, France.

**Pierre Russo**, CNEVA Sophia Antipolis, Laboratoire de Pathologie des Petits Ruminants et des Abeilles, "Les Templiers" 105, route des Chappes, -06410 BIOT, France.

**Richard Sanchis**, CNEVA Sophia Antipolis, Laboratoire de Pathologie des Petits Ruminants et des Abeilles, "Les Templiers" 105, route des Chappes, -06410 BIOT, France.

**Armel Souriau**, Pathologie Infectieuse et Immunologie, INRA, 37380 Nouzilly, France.

**J.M. Verger**, Pathologie Infectieuse et Immunologie, INRA, 37380 Nouzilly, France.

**Christian Vitu**, CNEVA Sophia Antipolis, Laboratoire de Pathologie des Petits Ruminants et des Abeilles, "Les Templiers" 105, route des Chappes, -06410 BIOT, France.

# PREFACE

Due to their wide adaptability to climatic conditions sheep and goats are raised worldwide. This industry is often the only possible deal in arid, poor soils inadequate to grow crops or to practice more lucrative livestock such as dairy farming. It provides a subsistence means and an occupation for a large proportion of rural populations being utilized at the same time as a treasury which is supplied by selling the flocks' offspring.

Any misfunctioning of the replacement within the flock can have a dramatic effect in terms of financial disponibility, sometimes resulting in rural exodus in developing countries.

Causes of abortion in small ruminants may be of a genetic, nutritional/toxic or more often of infectious nature. In the event of an abortion outbreak occurring in a given flock, it is necessary to immediately identify its specific cause and this will help in the decision-making to take control measures. Given that in most cases the clinical signs are not characteristic, laboratory diagnosis plays an important role in this process.

This is the reason why we are proposing this manual for the use of veterinarians, biologists and technicians dealing with veterinary diagnosis. This tool is of practical use for daily work on abortions of small ruminants and will hopefully improve the capability of diagnosticians, especially those working in developing countries where access to special documentation might be difficult.

**Yves CHENEAU**
**Head of Animal Health Service**
**Animal Production and Health**
**Division**

## Foreword

In order to carry out the techniques decribed in this manual the following standard basic equipment of a diagnostic laboratory must be available:

- glassware : pipettes, tubes, microscope slides, etc....
- micropipettes
- centrifuges
- incubators
- water baths
- microscope...

Only specific equipment and materials will be mentioned for each technique.

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# Chapter 1

# THE IMPORTANCE OF ABORTIONS IN SMALL RUMINANTS

C. LE LOUEDEC, M. PLOMMET

Sheep and goats were among the first species of animals to be domesticated by man for the production of meat, milk, leather and fibres. Since then other small ruminants species, such as llamas, domestic deer and wapitis, have been domesticated. However, despite some recent attempts to adapt these latter species to new habitats (such as the rearing of llamas in the South of France), their utilisation by man remains geographically limited. In contrast, sheep and goats are reared throughout the world from the polar circle to the equator, evidence that these two species possess great adaptability to different climatic conditions. They are the most common species of small ruminants worldwide. It has, however, proved to be very difficult to accurately assess the total global population of small ruminants because their numbers fluctuate enormously. The number of animals in a flock can increase or decrease depending on the climatic conditions: a period of drought can reduce a flock to virtually nil; in contrast, when there is an overabundance of forage, flock sizes increase because of the low mortality rate in the young.

*Table 1.1 : Distribution of sheep and goats throughout the world*

| Geographical Area | 1979-1981 | 1992 | 1993 | 1994 |
|---|---|---|---|---|
| **Sheep** | | | | |
| Asia | 316 162 | 341 381 | 336 104 | 340 102 |
| Africa | 180 465 | 206 843 | 205 785 | 208 845 |
| South Sea Islands | 202 272 | 200 779 | 189 116 | 182 758 |
| Europe | 123 288 | 138 834 | 133 776 | 130 692 |
| South America | 102 944 | 101 540 | 92 876 | 94 054 |
| North & Central America | 21 073 | 16 270 | 16 168 | 17 529 |
| **Total** | **1 088 794** | **1 133 372** | **1 096 049** | **1 086 661** |
| **Goats** | | | | |
| Asia | 268 239 | 351 369 | 359 866 | 373 005 |
| Africa | 138 228 | 168 452 | 171 468 | 176 088 |
| South Sea Islands | 415 | 1 144 | 986 | 1 007 |
| Europe | 11 781 | 15 242 | 14 905 | 14 809 |
| South America | 18 538 | 22 593 | 22 679 | 22 819 |
| North & Central America | 13368 | 15 470 | 15 726 | 14 944 |
| **Total** | **456 380** | **581 317** | **592 874** | **609 488** |

*Source: FAO Production Yearbook 1994; 48: 192-194*

Additionally some owners tend to underestimate numbers in their flocks to avoid taxes and taxation, making estimations even more inaccurate.

FAO is attempting to establish accurate world statistics by collecting data as "non-official information" or "FAO estimations" from a maximum number of information sources. To date data has only been collected from 70 countries but shows that estimated number of small ruminants in Africa and Asia represents more than 50% of the world total of sheep and more than 90% of the world total of goats (Table 1.1).

Whether the purpose of rearing sheep or goats is production of milk, wool or meat, the process is dependent on the ability of the animals to reproduce successfully. Consequently any decrease in the total number of reproductive animals in a flock will cause important economic losses that could close the operation.

Failure of reproduction, that is the failure of the dam to produce live, viable young, is referred to as infertility when temporary and sterility when permanent. Reproductive failure results from two main sets of factors: non-infectious and infectious factors. Non-infectious factors include genetic, immunological, endocrine, nutritional and toxic causes. Apart from non-infectious nutritional causes, infectious causes tend to be easier to control.

In addition to heavy economic losses, the infectious diseases responsible for reproductive problems in sheep and goats can affect the health of humans associated with the animals since many of the disease-causing infectious organisms are transmissible to man.

## 1.1 THE ECONOMIC IMPORTANCE OF SMALL RUMINANTS

According to the geographical area, the economic reasons for keeping small ruminants can differ and may be:

- in response to the need for food;
- to serve as a safe investment;
- as the basis of an industry.

Small ruminants supply food to small communities in the form of milk and meat.

Milk can be consumed directly after purchase or after processing by one of a range of treatments which result in different levels of preservation and edible products of varying nature: it may be processed into cheese (fromage frais, soft cheeses, hard cheeses) or butter, used as fermented milk (yoghurt, "Doughe" in the Middle East, "Leben" or "Ajran" in the Far East) or caramelised milk ("dulces" in Central America, "cajeta" in Mexico, "geitost" in Norway, "Kurt" in central Asia). During the course of many infectious diseases such as brucellosis, tuberculosis, Q fever, listeriosis, massive excretion of infectious organisms occurs in the milk. Consumption of raw milk, milk that has been insufficiently pasteurised, or certain products produced from contaminated milk are therefore the origin of many diseases in man.

Ovine and caprine meat is a major source of protein for many human communities. The small size of sheep and goats allows the meat to be consumed by a limited sized group of humans without need to preserve the carcass. During certain religious festivals, such as "Aid El Kebir", the slaughtered animal is divided between the family and their neighbours and the meat and offal of the animal is

completely consumed. The meat from infected animals may contain infectious organisms. Certain offal, for example the liver and the mammary gland, concentrate such organisms and their consumption can be the origin of diseases in man even though the meat is generally cooked before being eaten.

In certain countries, small ruminants can be considered as a "liquid asset" in case of need. They require less investment than other ruminants since the risk of financial loss is much lower if an animal should disappear. The capital return is faster because of early sexual maturity (from 7 months) and the resultant short interval between generations (of the order of one year).

Certain small ruminant rearing methods are aimed at specific production (meat, wool, milk). The main objective of rearing sheep in Australia is for the supply of wool for the textile industry. Goat hair in Cashmere and Tibet is also used by the textile industry: mixed with wool it is manufactured into light, warm fabrics and in a similar way fine wool is combined with the long fleece of the alpaca.

The meat of small ruminants is the origin of the meat industry. Certain abattoirs specialise in the preparation and packaging of meat from small ruminants. Sheep from New Zealand are exported for their meat as carcases. To export meat it is imperative that the sanitary conditions demanded by the exporting country are respected.

In the French Cevennes, sheep supply the milk used by the "Roquefort" cheese industry. Goats and sheep around the Mediterranean supply the raw material for the local cheese industry, for example for the production of Greek "Feta". The recent increase in export of these cheeses from the area of production has resulted in a massive expansion of the original "cottage" industry. Yoghurts made from goat and sheep milk have started to appear commercially on a large scale. Treatment of large quantities of milk requires a high level of vigilance particularly regarding the sanitary aspects of the supplying flocks.

The hides of sheep and goats are used in the leather industry for the manufacture of clothing, shoes and leather goods (bags, cases, gloves). During the handling of hides from contaminated animals, aerosols formed can be the origin of diseases in tannery workers (for example, Q fever).

## 1.2 METHODS OF REARING AROUND THE WORLD

The method of rearing is the combination of the practises used to ensure the upkeep of the animals so that they achieve the performance expected by the owner. Methods of rearing differ according to the climatic area in which the small ruminants are found. The availability of food is the main determinant of both the rearing method and the sizes of flocks. The actual method used depends on the technical means and the labour available and the best way these can be adapted to the prevailing environment. Adapting to the situation creates different approaches to the fight against diseases.

Different rearing methods vary in the degree of control of the animals, their reproduction and of the environment. The economic consequences and the risks of disease transmission between animals, and between animals and man differ according to the rearing method. Different rearing methods can either

control or aid the transmission of infection. Four types of small ruminant rearing methods exist in the world: tethering, two types of extensive rearing (nomadic and seasonal movement) and intensive rearing.

### 1.2.1 Rearing by tethering

This is a rearing practise undertaken in the countries of South-East Asia, Central America and Africa.

#### *1.2.1.1 The environment*

In the villages, small ruminants often wander freely but during periods of land cultivation they are tied up to prevent wandering (a posted animal) and to control the areas in which they graze. The animals are tied by a cord of two to three metres in length to a tree or a post sunk into the ground. They are moved each day and are grazed by the sides of the roads, on fallow land or on natural paths. The grazing can be supplemented by the feeding of domestic waste (for example, potato peelings) and/or small quantities of cereals or derived products.

The animals are watered when they return to the village. At night the animals are assembled in the village or the immediate proximity but they remain tethered.

#### *1.2.1.2 The flocks*

In this rearing method, each owner has a small number of tethered animals (between one and ten), often less than five. Such flocks tend to consist of family groups. Increases in and/or replenishment of the flock is carried out by retaining the young born to the flock or by acquisition of females either by purchase, if the owner has the means, or by exchange with, for example, poultry.

#### *1.2.1.3 Labour*

The size of the animals combined with low total numbers minimises handling and allows rapid adaptability to the available resources. The quantity of work involved in this rearing method is small in comparison with other jobs such as the growing of cereals. The care of the goats and sheep requires little training in techniques and therefore demands little specialised labour (in comparison with grazing animals), often met in the form of unpaid family labour (women, children). In Africa, small ruminants are allowed a certain autonomy by the chief of the village.

#### *1.2.1.4 Reproduction*

Reproduction will be affected by the rearing practises in force at the time, for example, whether ewes are tethered or wandering, the number of ewes the males have already covered. Only certain owners in the village keep entire males (one or two), the other owners can obtain them on loan from the breeders or they can simply release their females in the direction of the best males in the village making any control of reproduction relatively difficult.

#### *1.2.1.5 Consequences for transmission of infections*

The close proximity of the small ruminants and humans is often the origin of zoonoses acquired by direct contact with sick animals or by removal of contaminated products from the animals.

### 1.2.2 Nomadism

This rearing method predominates in the Middle East and North Africa.

#### *1.2.2.1 The environment*

This rearing method necessitates adaptation to a difficult environment and,

above all, requires a good knowledge of the surrounding area in which the flock is moved. The animals graze on large areas of marginal ground unsuitable for cultivation. They are moved according to the climatic conditions. In the Near East, the low humidity level has led to the traditional system of exploitation of lands with sparse vegetation of poor quality. During the dry season the animals are maintained close to watering places.

#### *1.2.2.2 The flocks*

In this situation, small ruminants constitute the principal source of subsistence for the rearers and the size of the flocks is therefore usually larger than in flocks reared by tethering. A nomadic flock can contain about fifty head in suburban areas but can increase to more than 200 animals in rural areas. Very often a flock will belong to a nomadic human tribe and move with it. Reproduction is usually sufficient to assure the replenishment of the flock.

#### *1.2.2.3 Labour*

The life of the owning community of the flock depends directly on this flock and close observation of the flock is therefore assured by members of the group, generally unpaid, including children.

#### *1.2.2.4 Reproduction*

The nomadic flock is mainly composed of females with entire males present in limited numbers. With the exception of choosing the males to be maintained as entire, the rearers have no other means of intervention in the control of reproduction.

#### *1.2.2.5 Consequences of transmission of infections*

The spread of infectious diseases in this rearing method occurs through direct contact with infected animals or indirectly by contamination of the environment, for example, of watering points where successive groups of animals go to drink. In addition, the close contact between humans and the animals aids transmission to man.

### 1.2.3 Seasonal movement or semi-nomadic

This method of rearing is used in five continents.

#### *1.2.3.1 The environment*

Seasonal movement is characterised by alternation according to the season between a settled period and the use of variable areas of grazing. During the moving period a shepherd has the responsibility for animals belonging to several owners (collective flocks). Seasonal movement can take many forms according to the geographical location :

- In mountain regions alternation occurs between enclosed winter grazing in the plains and valleys which are not cultivated during this season and extensive summer grazing on the pastures of the mountains that are clear of snow at this time.
- In desert regions winter grazing takes place in the desert and summer grazing close to the oases and cultivated areas.

Seasonal movement can also be affected by sanitary conditions: grazing on the plains and valleys may be abandoned because of flooding during the humid season but also because of the risk of diseases (glossina, parasites). The movement of flocks from one region to another often follows the same route from year to year but can vary according to the availability of food. Grazing areas are usually collective lands which belong to tribes or families. This rearing method

exploits the forage resources of natural paths or of fallow land with residues of cultivation, and allows manuring of the land especially where animals are assembled at night.

### *1.2.3.2 The flocks*

The flocks are collective during seasonal movement but can be divided into individual groups again during the settled period. The replenishment of individual groups either occurs by acquisition of animals from outside the group or by the maintenance of young which are selected according to certain criteria with the remainder being sold.

### *1.2.3.3 Labour*

The flocks are moved under the responsibility of one or a group of shepherds who have a good knowledge of both the environment and the animals. They are paid by the owners when they return the animals at the end of the seasonal movement.

### *1.2.3.4 Reproduction*

Characterised by movement and management of the often collective flocks managed completely in the open air. This rearing method does not allow any control of reproduction unless the sexes are segregated.

### *1.2.3.5 Consequences of transmission of infection*

The mixing of flocks during seasonal movement aids the transmission of infectious diseases.

## 1.2.4 Intensive rearing

Intensive rearing is characterised by the number of animals raised per unit area and by the control of food and reproduction. This rearing method is predominant in Europe and North America.

### *1.2.4.1 The environment*

The animals are held in a restricted space with provision of feed. Intensive rearing can be:

- outdoors;
- partly housed / partly outdoors;
- permanently housed.

The method of using forage resources is therefore:

- solely by grazing;
- by grazing and distribution;
- solely by distribution.

This rearing method uses techniques of intensive production and use of forages.

### *1.2.4.2 The flocks*

In this type of rearing, the breeder chooses his animals according to the available forage resources and the type of production (meat, milk). He may either manage his natural forage resources using animals adapted to the environment or he may create a new forage environment using more productive animals with the flock held under artificial living conditions (housing usually with mechanisation of food distribution). In intensive rearing the flocks are almost exclusively comprised of females with very few selected males retained for mating.

### *1.2.4.3 Labour*

The intensive rearing method leads to specialised production often combined with other forms of animal or vegetable production. Specialisation may be very restricted, for example, certain rearers may only carry out breeding whilst others may only fatten lambs. Such specialisation can lead to the creation of

chains of production, such as those which exist in Britain, in which individual participants may be "cottage" industries, semi-industrial or wholly industrial. Intensive rearing can therefore be considered as the partnership of two sectors:

- a "plant sector" for production of primary feed materials;
- and a "animal sector" for transformation of these primary materials to milk, wool, hide and especially to meat.

Such rearing methods require shepherds to have good technical knowledge and may demand heavy and expensive equipment. For this reason the labour for intensive rearing is highly specialised with shepherds skilled in one or other field.

### *1.2.4.4 Reproduction*

According to the chosen specialisation, meat, milk or wool, rearers select their reproductive animals not only according to appearance but also according to performance criteria such as fertility, proliferacy and lamb survival rates. To replenish the flock, rearers retain young from females that are fertile and prolific, giving birth to at least two lambs or kids, that produce viable offspring, and that are good milkers and mothers. Reproduction can be controlled at the level of both the females and the males. This control can be variable:

- selected males can be released into the flock without any control: the young are born of unknown paternity;
- a group of ewes remain with the same ram for the duration of mating: the paternity of the offspring is known, therefore selection is very effective;
- the ewes are artificially inseminated which allows complete control of reproduction as breeding stock are chosen on precise criteria. Thereafter the pregnant ewes are grouped together.

### *1.2.4.5 Consequences for transmission of infection*

The concentration of a large number of animals into a limited space aids the transmission of infections and exposure to certain diseases, but it also allows for better surveillance and sanitary conditions. Artificial insemination can allow control of transmission of infections from the male to the female. Similarly, synchronisation of ovulation can aid the control of abortive diseases: the females can be grouped together in batches according to their stage of gestation. If abortions occur at the end of gestation, excretion of abortive organisms does not occur in the vicinity of more susceptible animals at the beginning of gestation.

## 1.3 THE IMPORTANCE OF ABORTIONS AMONG THE DISEASES OF SMALL RUMINANTS

It is very difficult to assess the incidence of any disease among small ruminants. From published material it should be possible to ascertain if a specific disease could be encountered in a given country at a given time and this type of information does exist in certain official documents (the zoo-sanitary situation in the member countries of the International Office of Epizootics, Annual of Animal Health edited by FAO/WHO/OIE). However, it is impossible to know the incidence of a given disease for at least two reasons:

- Firstly, there is the problem of the quality of diagnosis of a disease. Before diagnosing the cause of an abortion, the clinical signs must be observed. In small

ruminants such observations are easy if reproduction is controlled but this is not the case in extensive rearing (tethering, nomadic, seasonal movement). For satisfactory confirmation of abortion it must be known which females are pregnant and the date that each one began its gestation. When there is no control of reproduction abortions will only be detected at the end of gestation when the foetus is large enough, previous abortions will pass unnoticed and will not be distinguishable from a low fertility rate. The precision of diagnosing abortion is therefore directly related to the rearing method.

- Secondly, even if the cause of abortion is correctly diagnosed, the owner may be reluctant to impart this information.

### 1.3.1 Importance of research work on small ruminants

Is it possible to use the published articles on the diseases of small ruminants to estimate the incidence of a disease? Awareness of the existence of a disease always originates in an article, scientific or not. A large number of articles illustrates the importance that a disease has in the community. It is therefore possible to estimate the importance which specialists give to ovine abortions by consulting the reference database. In the largest four biological databases the mean number of references per annum is of the order of 20 for Agris, 40 for Biosis and Medline and 80 for CAB Abstracts (Table 1.2). Three-quarters of the references deal with at least one infectious abortive disease. Articles dealing with brucellosis and chlamydiosis are the most numerous (always more than 50 percent of references in each database) then come articles on salmonellosis, toxoplasmosis, Q fever, infections by Campylobacter and listeriosis (Table 1.3).

## 1.4 PATHOLOGY OF ABORTIVE DISEASES OF SMALL RUMINANTS

The placenta, the foetal organ in contact with maternal endometrium at the level of the cotyledons on the placentome, assures :

1. exchange of nutrients;
2. hormone balance;
3. immunological competence;
4. protection against possible infective organisms, micro-organisms in particular.

If one of these functions, normally controlled with high precision, is altered it may result in premature expulsion of the foetus. The foetus may be live or dead, infected or uninfected. In sheep rearing consideration is given to the flock rather than the individual. "Abortive disease" - without inference of the cause - is said to occur when the threshold of abortions exceeds 1% since below this level the causal factors are uncertain.

Abortions can be classified, in a somewhat academic way according to the principal cause, into three categories :

1. genetic (malformations);
2. nutritional and/or toxic;
3. infectious.

Infectious abortion occurs most frequently and is the subject of the current work. However, initial mention will be made of the other causes to avoid any error in the preliminary careful diagnostic examination being systematically directed towards an infectious cause.

### 1.4.1 Genetic causes

Malformations of the foetus leading to its death or premature expulsion can be due to abnormalities of the genome.

*Table 1.2 :Number of references concerning abortive diseases of small ruminants presented in the four largest bibliographic databases*

| Period of Publication | Agris March 1974-1996 | Biosis August 1969-1996 | Cab Abstracts July 1972-1996 | Medline October 1966-1996 |
|---|---|---|---|---|
| 1976-1980 | 107 | 211 | 577 | 204 |
| 1981-1985 | 134 | 241 | 599 | 248 |
| 1986-1990 | 142 | 313 | 625 | 281 |
| 1991-1995 | 110 | 260 | 386 | 216 |
| *Total number of references selected* | *504* | *1274* | *2628* | *1352* |

*Source of information : Dialog, Knight-Ridder information, Mountain View*
*Selection criteria for references: (ovin? + sheep? + ewe? + lamb + lambs + ram + rams + caprin? + goat?)*abort?*

*Table 1.3 : Distribution of references concerning abortive diseases of small ruminants according to the nature of the infectious organism.*

| Infectious Disease | Agris March 1974-1996 | Biosis August 1969-1996 | Cab Abstracts July 1972-1996 | Medline October1966-1996 |
|---|---|---|---|---|
| *Total number of references selected* | **504** | **1274** | **2628** | **1352** |
| Brucellosis | 86 | 432 | 646 | 167 |
| Chlamydiosis | 136 | 163 | 552 | 132 |
| Salmonellosis | 76 | 140 | 351 | 50 |
| Toxoplasmosis | 54 | 91 | 276 | 46 |
| Q Fever | 26 | 20 | 110 | 26 |
| Campylobacter Infections | 28 | 40 | 174 | 21 |
| Listeriosis | 18 | 35 | 132 | 12 |
| Border Disease | 7 | 20 | 55 | 15 |
| Leptospirosis | 11 | 12 | 91 | 7 |
| Rift Valley Fever | 5 | 16 | 43 | 12 |
| Yersiniosis | 5 | 12 | 30 | 11 |
| Mycoplasmosis | 7 | 7 | 39 | 3 |
| Corynebacteriosis | 2 | 16 | 32 | 6 |
| Mycosis | 3 | 2 | 29 | 1 |
| Others | 148 | 214 | 717 | 412 |

*Source of information: Dialog, Knight-Ridder information, Mountain View*
*Selection criteria for references: (ovin? + sheep? + ewe? + lamb + lambs + ram + rams + caprin? + goat?)*abort?*

*One reference may refer to more than one infectious organism and may appear in more than one database therefore rows and columns cannot be summated*

These can result either from random accidents, which occur rarely during meiotic fusion or the first stages of cellular division, or from mutations that tend to be transmitted by the male. When these latter abnormalities occur at a high frequency, the ram appears to be infertile or of low fertility; this is easy to detect in rearing systems where reproduction is controlled but difficult to identify in extensive rearing.

Before making a diagnosis of male genetic low fertility, the absence of a genital infection by, for example *Brucella ovis* or *Chlamydia psittaci*, should be assured. These organisms cause low fertility in males and are transmissible to the female.

### 1.4.2 Nutritional and toxic causes

The live foetus is nutritionally dependent on its mother and is subjected to her deficiencies and toxicities. If there is already a severe deficiency in the dam it will be reflected during the course of gestation. Low fertility, on the other hand, may occur as a result of numerous deficiencies including energy, protein, vitamins, minerals, but is only one of many symptoms of such deficiencies. The foetus is poorly protected against toxicities: poorly equipped for detoxification and/or excretion of dangerous products, it accumulates these substances to which the dam is subjected until, in most cases, definitive, lethal lesions and abortion occur. The placenta can, in the same way, suffer major vascular disruption from toxic, endotoxic or anaphylactic shock which can affect the dam and lead to the death of the foetus. It occurs when there is:

1. Direct intoxication, from the chemical products used in agriculture or industry, by consumption of toxic vegetation or food contaminated by mycotoxins.

2. Indirect intoxication, secondary to a general bacterial or viral infection, particularly when a strong febrile reaction occurs due to bacterial endotoxin.

### 1.4.3 Infectious causes

Infection can reach the placenta basically by two routes:

1. the ascending route via the vagina, cervix and uterus;

2. the descending blood route, by far the most important route of abortive disease.

In the ascending infection, an external contaminant crosses the antimicrobial barrier of the cervix, for example during insemination or obstetric handling, then settles in the endometrium, frequently causing infertility and/or early abortion, or it may directly invade the membranes then the placenta and the foetus.

In the descending infection, during the course of the bacteraemic or viraemic phase of a general infection, the trophoblastic cells of the placenta - certain of which are active "macrophage-like" - capture some of the circulating bacteria [2, 4]. These foetal cells without doubt have the capacity to destroy a large number of invading organisms but they are not immunologically equipped to oppose those organisms that are "facultative or obligatory intracellular parasites" [11]. On the contrary, the placenta offers, by virtue of its blood supply and numerous metabolic activities, the ideal physiological and nutritional conditions for the rapid multiplication of bacteria, allowing the breakdown of all lines of defence.

The capture of circulating bacteria, however, is not obligatory. Contrary to the accepted theory there is no particular "tropism" of bacteria towards the pla-

centa, capture tending to occur randomly, by chance and in relation to the level and duration of the bacteraemia. Placental capture occurs less frequently, under normal conditions, than phagocytosis by the purifying organs of the body, the liver and spleen. Under controlled experimental conditions it has been shown that each of the 10-12 placenta in the uterus of a mouse can act as an independent unit [3] and that, up to a point, a single captured bacteria is sufficient to infect an individual placenta [4]. These results can be transposed to ruminants where the cotyledons seem to act independently of each other. It is not exceptional to find uninfected cotyledons alongside infected ones on the same placenta or even neighbouring cotyledons infected by different organisms. This leads to the conclusion that initial colonisation in ruminants occurs in the same way as in the mouse: a very small number of accidentally captured bacteria are sufficient to "colonise" a cotyledon where, in the absence of any maternal immunity, they multiply rapidly to excessive levels - up to $10^{12}$ *B.abortus* per g of cotyledon in bovines [1] - causing serious lesions and abortion.

### 1.4.4 Immunity in abortive diseases

Maternal immunity can work at two levels, systemic and local, and can partially or totally protect the foetus. In general, from results and observations in mice, cattle and sheep infected with Brucella, one can predict that the following process occurs :

1. Initial access of bacteria to the placenta can be limited by the strong reduction in the level and duration of the bacteraemic phase, principally by the action of opsonic antibodies.

2. Immunity can then pass over the placental barrier - probably at the inflammatory centre - in the form of maternal immune antibodies and lymphocytes [5], by mechanisms that are currently poorly understood.

3. If totally protected, the foetus will be born at term from an infected dam but will not necessarily be protected from contracting infection post-partum.

4. If partially protected, the foetus can be born prematurely or at term, infected or uninfected, with many placental cotyledons infected to varying degrees. The foetus born infected can, according to the causal organism, survive and make a full recovery with or without after effects or residual congenital infection, then becoming a possible source of subsequent recontamination for the flock.

According to the organism, the level of contamination and the age and stage of gestation at the time of contamination, the primary bacterial infection can be eliminated and leave a solid and lasting natural systemic immunity or, after a possibly abortive acute phase, persist in an inapparent chronic state. Immunity in this latter case is sufficient to protect the placenta during a new cycle of infection or on recontamination. This is why, according to current theory, the ewe generally only aborts once from a given infection, usually during the first gestation.

Understanding the mechanisms of immunity has justified the long-practised natural preventive method whereby immature lambs are placed in contact with (infected) adults at the time of birth. This technique certainly reduces the frequency of abortion, but can also be responsible for the persistence of infection in a flock. The technique has been effectively replaced today by prevention using live attenuated vaccines.

### 1.4.5 Abortive infections ; epidemiology ; hygiene

Besides epidemiological traits specific to individual infectious organisms, "abortive diseases" resulting from infections have two major features in common :

1. Genital excretion, the principal, but not the only, source of contamination.

2. The ovine species whose (a) gregarious behaviour and (b) often confined cohabitation with other susceptible species of animals, contains and/or spreads the potential of infection.

These features are the basis of the epidemiological cycle of infection within and between flocks.

In a theoretical breeding situation where ewes have no contact with other vectorial species (e.g. horses, cattle, camels, dogs) and lambing takes place in accommodation with strict application of the rules of isolation and hygiene, abortive disease will not exist. In contrast, the epidemiological reality in sheep breeding is the opposite. In seasonal movement and nomadic rearing, for example, in order to exploit forage resources animals must be moved and come into contact with other flocks thereby creating suitable conditions for the maintenance and spread of abortive disease. Moreover, it is common for many types of infection to coexist in the same flock, sometimes in the same animal.

The personnel in charge of diagnosis and prevention of the diseases should be aware of the basic theories of the epidemiology of abortive disease - the role of the birth, vector species and containment, coexistence of many infections - as well as the features specific to each organism; in particular, resistance and/or multiplication in the external environment (*Brucella, Coxiella, Salmonella, Listeria*) and the role of intermediary hosts (*Toxoplasma* and cats).

Finally it must be re-emphasised that many of these infections (*Brucella, Coxiella, Listeria*) can seriously affect humans who become contaminated during contact with animals, for example, during obstetrical handling or by consumption of infected products (milk or fromage frais, raw meat or offal). The pregnant human female is particularly susceptible to several of the small ruminant diseases causing abortion and/or perinatal foetal infections (*Listeria, Toxoplasma, Brucella, Chlamydia, Coxiella*). They must avoid contact with infected animals at all costs since these organisms can cause fatal or seriously disabling human diseases. While humans commonly contract infections from animals they are epidemiological end points and rarely transmit the diseases back to animals except in the absence of basic hygiene.

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# Chapter 2

# METHODS OF SAMPLE SELECTION AND COLLECTION

B. DUFOUR, F. MOUTOU & A. RODOLAKIS

## 2.1 SUMMARY

In presenting methods of sample selection and sample collection, the words are defined in absolute, relative precision and accuracy. Random samples are differentiated from empirical samples and some simple rules of sampling are proposed and explained. The main types of samples to be collected are described together with the precautions that should be taken during their handling and transportation.

## 2.2 INTRODUCTION

During the study of the epidemiology of animal diseases, comprehensive prophylactic campaigns or investigations may be undertaken involving large numbers of livestock in one region or even one country, with one or more species being sampled. Under such conditions, the results obtained from analysis of the samples can, with some allowances, be directly exploited and interpreted particularly if the samples have been collected under the best possible conditions.

Unfortunately this situation does not always occur. For reasons of cost and lack of personnel and materials, sampling is often restricted to only one part of the population, which represents a sub-sample of the whole. It is then necessary to question how, under what conditions and with what precautions, the results obtained from analysis of these samples represent the original population.

In the first instance it is necessary to know if a subsample in fact represents the original population. To answer this question a certain number of rules must be followed and these are presented in this chapter, together with recommendations for carrying out good sampling techniques. However, it is first necessary

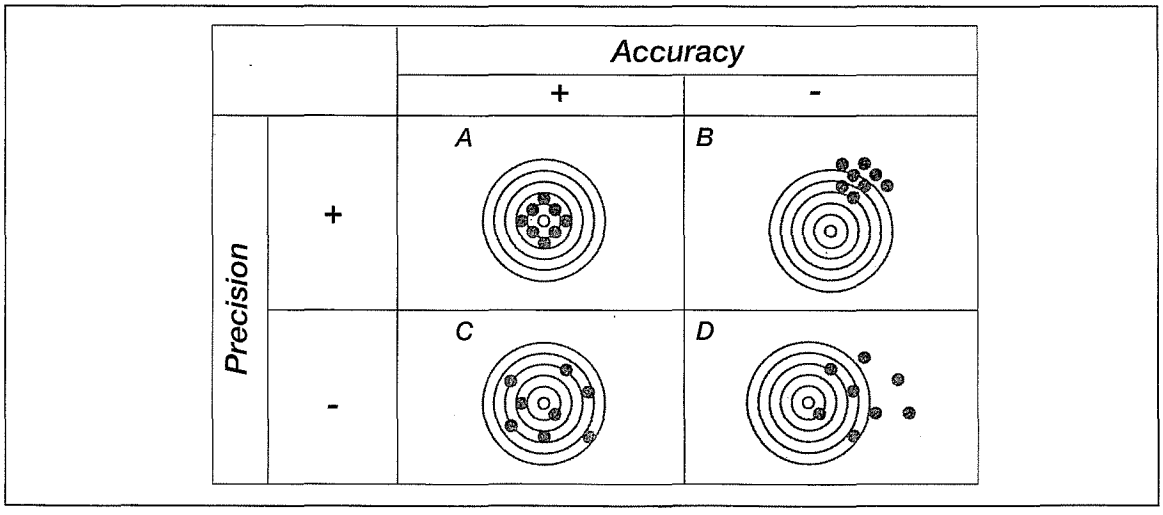


*Figure 2.1 : Accuracy and precision*

to define some of the terms commonly used in this context.

## 2.3 METHODS OF SAMPLE SELECTION

### 2.3.1 Sample quality

#### *2.3.1.1 Definitions*

Two important terms associated with sample selection are accuracy and precision. Accuracy is the quality of the agreement between the measured value and the true value, whilst precision is the measure of deviation of the values of a repeated measurement from the mean value. These two parameters are illustrated in Figure 2.1. Both can change independently of the other which results in four possible scenarios. The situation in diagram A corresponds to the desired accurate and precise result. In diagram B, the result is still precise but it has become inaccurate. This type of result could be obtained from a measuring device on which the zero has been poorly calibrated. In the situation in diagram C, precision has not been obtained but the accuracy, on average, is good. The variation around the mean of the tested value is important, but the true value is effectively included in this large spread. Finally, the last situation in the figure, diagram D, is the one to be avoided at all costs. The result is imprecise and inaccurate and allows absolutely no conclusion to be drawn.

The most common problem is to identify where the risk is located since this cannot always be known in advance. There are two ways to tackle these two risks. To obtain good accuracy, one must select the individuals - animals or flocks - to make up the sample randomly. When this process is effective the pitfall of inaccuracy can be avoided. There is actually little chance that all the individuals would possess the same difference from the mean. To increase the precision the size of the sample should be enlarged so that the range within which the real test value is located will be reduced.

Experience in the field has shown that the selection of individuals to be sampled poses a number of serious problems. A complete list of the flocks, farms, their addresses and sometimes additional assurance that the owners of the animals agree to participate in the investigation needs to be prepared. As the number of samples increases, financial constraints or the capacity of the analytical laboratory to handle the samples will quickly become obvious and impose limitations.

#### *2.3.1.2 Measure of accuracy*

If the local agricultural statistics are available, the selection of animals can be undertaken automatically using tables of random numbers or any similar method. Random number tables contain series of totally independent numbers which can be used to select the livestock or the animals to be sampled from the population, after the latter have been given a unique numerical identifier. A number in the table can then be linked directly with the number of an individual to be sampled.

Computers can also be used to generate random numbers and to select animals to be sampled. For example, if one wishes to choose 20 livestock from a total of 100, one numbers the livestock from 1 to 100 and asks the computer to choose 20 numbers between 1 and 100.

### *2.3.1.3 Absolute precision, relative precision*

The absolute precision of a test corresponds to the size of the confidence interval, i.e. to the quantity that is added or subtracted from the mean to obtain the limits of the confidence interval. The relative precision is equal to the absolute precision divided by the value of the estimated proportion.

In a population of 500 individuals where 10%, that is fifty, are infected, selection of a sample of ten individuals will not always give nine healthy individuals and one infected one. Of the ten individuals chosen none may be infected or in another case two, or very rarely three or more may be infected. The estimated percentage of infected animals in the original population will thus be estimated with a certain margin of error. Different selections of samples of ten individuals will each result in a different estimation of the disease prevalence; each of these estimations can be calculated, along with a certain confidence interval in which the true value will be found. The confidence interval can be adjusted to ensure that the probability of the true value being contained within it is equal to any given percentage, the most frequently used value being 95%. Other values could be used, dependent on the scientist's minimum tolerable probability of success. In this case, the confidence interval is approximately + two standard deviations each side of the mean. The precision will increase, and hence the confidence interval will decrease in size as the number of individuals included in the sampling increases and when the value of the disease prevalence in the population approaches 50%, moving away from the extreme values (for a fixed sampling size). Tables of percentage confidence intervals exist for a maximal tolerable probability of success, most often 95%. For high levels of the disease prevalence, the confidence interval will increase in absolute value; the absolute precision then diminishes but the relative precision improves. If the confidence interval is two times the value of the standard deviation about the mean given by the samples, the absolute precision corresponds to the standard deviation and the relative precision to the ratio of the standard deviation to the disease prevalence. If the disease prevalence is calculated as 10% with an absolute precision of 2% (that is 10 + 2%), the relative precision must be 2/10, i.e. 20%, which is ten times less precise.

Finally, it is acknowledged that in some cases the sampled population may be made up of animals from different flocks. The standard of sampling may then differ and the sampling objectives will not necessarily be the same. In one case the proportion of infected animals is being sought in order to identify an infected flock, in another case the proportion of infected flocks is being sought to identify an endemic region. An extreme possibility would be to sample livestock herds in a region then to sample animals within certain of these herds.

### *2.3.1.4 Objectives*

Two questions commonly arise in relation to the objectives of sampling:

* How many individuals must be sampled to detect at least one infected animal or, alternatively, how many individuals must be sampled to confirm a population as uninfected (having a rate of infection below a pre-established threshold) with a tolerable probability of success?

* How can one calculate the minimum or necessary number of individuals to confirm the prevalence of the disease looked for in the sampled number, and how does one choose the level of precision and a maximal tolerable probability (of success)?

The first question corresponds to a calculation of probability. To obtain a result, the level of disease prevalence below which the population is considered as uninfected must be set and the required probability of success specified, usually as 95%. If P is the prevalence of the infective agent in the defined sample, P can also be regarded as the probability of selecting an infected animal from the population and therefore 1-P as that of selecting an uninfected animal. In a sample of n individuals, the probability that none of the animals is infected is thus $(1-P)^n$. The probability that at least one animal is infected is $1-(1-P)^n$, which can then be calculated at 95%, or even 99%. If $C = 1-(1-P)^n$, solving this equation for n yields,

$$n = \frac{\log (1-C)}{\log (1-P)}.$$

To resolve this equation the confidence level, C, must be set, usually at 95%, the maximal tolerable probability (of success). The equation assumes that the size of the sample is small in relation to that of the population, which only actually occurs with numbers greater than about 500, and if the disease prevalence is not too low. Very often, the size of the total population necessitates a correction. The are statistical tables wich show the size of the sample to be taken in relation to the size of the flock and of the disease prevalence for a maximal tolerable probability of success of 95%. It should be noted that the table also incorporates the level of the sensitivity and specificity of the analytical test performed. If in a given population of 400 individuals there is a disease prevalence of at least 1%, according to the table 210 individuals must be selected and must be negative to establish that the population is uninfected with only a 5% risk of being wrong.

To answer the second question, that is the calculation of the minimum number of individuals that must be sampled to calculate the prevalence of an infective agent in a population, it is necessary to have some idea of the prevalence, P, being assessed and to set the desired absolute precision, i, and the minimal tolerable probability of success. In the case of a very large population, the size of the sample, n, is :

$$n = \frac{\varepsilon^2 P(1-P)}{i^2}$$

where e represents the reduced deviation corresponding to the maximum probability of success. In the case of a population which is small relative to the sample, the value of n must be corrected. There is statistical table which show the number of individuals to be selected for sampling as a function of the desired relative precision, the size of the population and the estimate of the disease prevalence. The maximal tolerable probability of failure is 5%.

### 2.3.2 Different types of sampling

A simple definition of a sample is that it is a subset of a population and as a small part of the whole it must represent certain characteristics of the whole. In selecting a sample, the main risks lie in the possible inclusion of bias which will alter the accuracy and precision. Unfortunately experience has shown that it is very difficult to obtain an unbiased sample. It is therefore important to know where bias is most likely to occur. Bias will vary according to the situation and must be taken into account during the interpretation of the results. Some examples of sample selection are as follows :

### *2.3.2.1 Empirical sample*

An empirical sample, also known as a blind sample, must be distinguished from other sample types. With empirical samples there is no system or a predetermined plan for sample selection and this type of sample should be avoided whenever possible. There is no way of knowing what bias is introduced in this sampling method but, because there is no selection process, accuracy will be poor. In a flock the first 10 animals captured would be sampled whilst in a region samples would be collected from the first 10 farms along the road. A random sample is always better.

### *2.3.2.2 Random sample*

In this situation the sample is composed of selected individuals each with a certain probability, known to the selector, that the sample is representative of the population. Many of the alternative methods available combine knowledge of the population and the studied disease but financial constraints will always affect the choice of method applied.

* Simple random samples. Each individual in a population has the same probability of being selected. The population is homogenous with respect to the considered disease and there is no known influence of factors such as age, sex or farming method.

* Stratified random sample. In this case, factors such as age, sex or the type of breeding are known to have an effect. The population is stratified by, for example, age to create sub-populations of 0-1 years, 1-3 years and more than 3 years. A sample is then randomly selected but the number in each age category is represented by the same percentage as exists in the whole population.

* Clustered random sample. The selected units are of groups of individuals, for example, of livestock, even groups of livestock. This type of sample must not be confused with the flock sample where each basic unit is a flock. With a clustered random sample the unit can be part of a flock, the flock or many flocks. The flock sample is a specific case of cluster sample, also known as a group sample.

* Systematic random sample. This is a practical method of obtaining a random sample which utilises the rule of systematic selection. If 10% of a flock must be sampled and all the animals can be caught individually, every tenth animal that passes the gate of the sheepfold or enclosure is selected and sampled.

### *2.3.2.3 Choice of method*

Ideally the selection method chosen will be the one that is easiest to carry out. However, to provide a good sample it is necessary to have a good knowledge of the disease being studied, the farming conditions and the region where the sampling is taking place so that all the foreseeable bias can be identified. It is better to have a modest sample where all the bias is known than to try to obtain a very sophisticated sample where few of the parameters are controlled. In the field, it is also necessary to know who will do the sampling, who will analyse the samples and to remain very pragmatic.

## 2.4 SAMPLES

To make a valid diagnosis, samples must be collected correctly, at the right time.

Samples must be clearly labelled and transported to the laboratory as quickly as possible, having been cooled and packaged in a waterproof, insulated container

holding sufficient absorbent material to avoid any loss of liquid in the case of accidental damage. It must always be borne in mind that these biological products are potentially hazardous to humans and therefore any possible leakage from the package must be prevented during transportation. Cool boxes should be used to conserve a low temperature.

All samples must be accompanied by documentation detailing the name and address of the sender, the analyses required and all pertinent information about the abortions. This documentation must be placed in a plastic bag attached to the outside of the package.

Packages must be visibly labelled "Pathological Samples, Fragile, Handle With Caution".

If the fresh tissues are not to be analysed immediately on arrival at the laboratory, they must be stored frozen at -70°C. Fixed tissues must be preserved in a fixative for some days or weeks before being treated.

### 2.4.1 The placenta

The placenta, when available and not too soiled, is the best sample for the isolation of the majority of the abortive agents. It can also be used for detection of organisms by staining of impression smears or histological sections. Since the entire placenta is difficult and hazardous to handle on arrival at the laboratory, it is preferable to sample cotyledons. Where possible those showing visible lesions should be collected since the degree of infection often varies from one cotyledon to another.

For isolation, 5 or 6 cotyledons together with their associated intercotyledonary membranes should be placed in a sterile container and transported to the laboratory. If the cotyledons are soiled they can be washed beforehand with sterile physiological saline. For isolation:

1. of viruses, cotyledons should be placed in viral transport medium (VTM: see Chapter on Border Disease);
2. of *Campylobacter*, cotyledons should be placed in FPB/glycerol medium (see Chapter 8);
3. of leptospires, cotyledons should be placed in 100ml of 1% bovine serum albumen (BSA) diluent (see Chapter 12).

For histology, sections 0.5cm thick should be taken from other cotyledons showing lesions. These should be placed in glass bottles containing fixative (0.85g NaCl dissolved in 90ml water to which is added 10ml of formol) in a ratio of 10 volumes of fixative to 1 volume of tissue.

For bacteriology, smears should be made by application of cotyledons showing lesions to slides.

### 2.4.2 Vaginal swabs

Vaginal secretions sampled immediately after abortion by swabbing also provide good samples for isolation of abortive organisms. They are not usually as heavily infected as the cotyledons, but they reflect moderate infection of the placenta. Correctly sampled they are more "appropriate" bacteriologically and less hazardous to the handler. Samples should be collected as soon as possible after abortion. Vaginal excretion, often abundant during the first few days, can decrease rapidly or become intermittent making testing more inaccurate if insufficient numbers of samples are collected.

The swab is made up of a metal wire about 15cm in length, covered with cotton wool at one end. A glass cylinder about 10cm in length and 8mm internal diameter surrounds the speculum and avoids contamination of the cotton wool when the swab is introduced into the

vagina. The whole device should be sterilized by autoclaving in a test tube (Figure 2.2). If unavailable, commercial sterile swabs can be used but these are generally a little short and the cotton wool swab is a little small.

The swab should be sent to the laboratory as it is or preferably in an appropriate transport medium according to the organism to be detected (*Chlamydia*, *Coxiella*, viruses, *Campylobacter*, etc.).

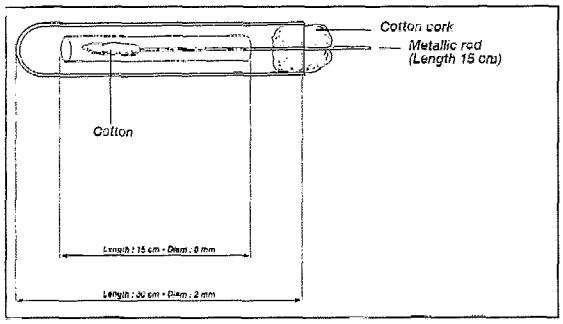


*Figure 2.2 : Vaginal swab*

### 2.4.3 Tissues from aborted foetuses and newly dead lambs

The tissues (spleen, liver, kidneys, brain, spinal cord, etc.) must be sampled aseptically as soon as possible after abortion or death. Samples taken must be placed :

- in sterile bottles for isolation or for antigen detection. Samples should be placed in VTM for isolation of viruses, FBP/glycerol for isolation of *Campylobacter* or 1% BSA diluent for isolation of leptospires, as for the placenta ;
- in a glass bottle containing at least 10 volumes of appropriate fixative for histology.

When it is not too autolysed the entire brain should be removed from the foetus (Figure 2.3).

### 2.4.4 Foetal fluids

The stomach contents, heart blood and peritoneal and pleural fluids can be sampled if the foetus is not too autolysed. These fluids should be sampled aseptically as quickly as possible after abortion using a sterile syringe.

### 2.4.5 Milk

Colostrum and milk from the two quarters should be sampled aseptically (disinfect the teats and discard the first two jets of milk) for isolation of the abortive agent or antibody detection.

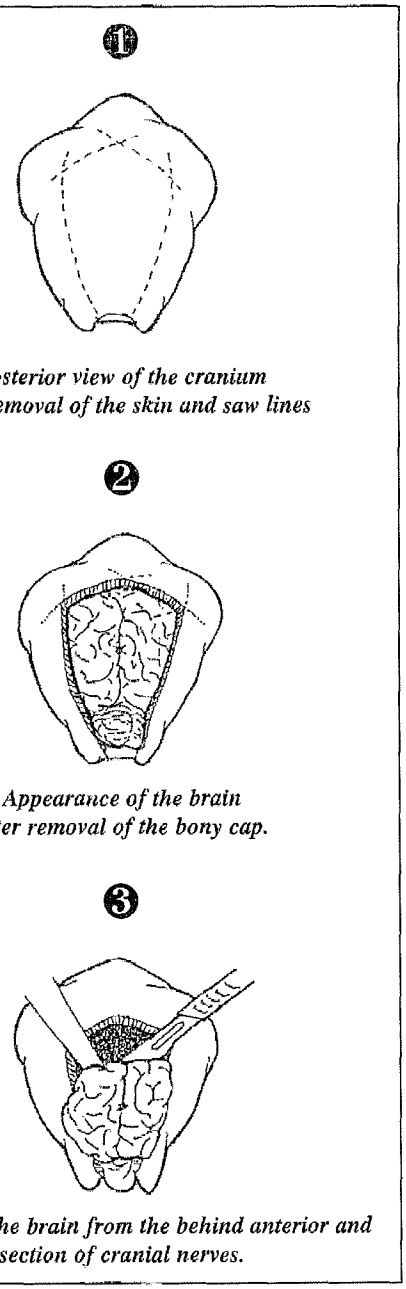


*Figure 2.3 : Sample of the brain.*

### 2.4.6 Blood

Samples of blood from ewes that have aborted are the best samples to take at the time of abortion for the isolation of organisms.

Two samples of blood should be taken from each animal in evacuated sterile tubes (e.g. Vacutainer), one tube without anticoagulant for antibody detection, the other with heparin for isolation of organisms.

A second sample of blood should be collected 2 to 3 weeks later in a clean tube without anticoagulant to detect any seroconversion.

When possible, precolostral blood from new born lambs should be sampled at the same time as those taken from the aborted ewes to detect any antibodies to Akabane virus or Border Disease virus.

Blood samples should be collected from about ten females that have aborted. If this number of samples are not collected at the time of intervention, it is possible to make up numbers by collecting samples from females that have not aborted providing that the actual status of the animals is recorded.

If testing is carried out some time after abortions have taken place and if the animals that aborted cannot be identified precisely, samples must be taken from a representative number of adult females or at least twenty of the flock.

The rate of serum antibody production may decrease rapidly depending on the infectious organism involved or the serological technique employed. Blood samples should therefore be collected less than eight weeks after the time of abortion or lambing. If samples are collected beyond this time testing will not always allow a distinction to be made between a latent infection or recent vaccination and the infection responsible for the observed abortions.

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# Chapter 3

# DIFFERENTIAL DIAGNOSIS OF INFECTIOUS ABORTION IN SMALL RUMINANTS

R. SANCHIS, P. RUSSO, M. CALAMEL & M. PEPIN

## 3.1 SUMMARY

Infectious abortion in small ruminants can be bacterial, viral or parasitological in origin. The relative importance of the different aetiological agents depends on the local epidemiological conditions. Multiple infections often occur.

Direct diagnosis involves the isolation and identification of the pathogenic agents present in samples from the aborted foetus, the placenta or vaginal secretions. A selection of techniques can be applied to samples to detect the endemic infectious agents. These techniques include direct examination and cultural methods adapted to the nature of the infectious agents being detected.

Indirect diagnosis, carried out on serum samples taken from the aborted female, uses serological techniques to detect specific antibodies to the abortive agents that occur most frequently in the considered region. It is necessary to analyse a number of samples to confirm the nature of the infection and establish the enzootic character.

Determination of the cause of infectious abortion can only be achieved by differential diagnosis. The number and type of analyses undertaken depends on the nature of the infectious agents studied with regard to the local epidemiological situation and the nature of the samples recovered. The analytical capability of the laboratory and financial and sanitary criteria mean that studies are sometimes limited, in the first instance, to the diagnosis of the main abortive diseases of bacterial origin. Other infections are only studied in the second instance. Current development of new techniques will probably allow the production of standard analytical techniques better adapted to differing epidemiological situations in the near future.

## 3.2 INTRODUCTION

Infectious abortion usually occurs during the last three months of gestation. Abortions are generally of an enzootic nature and several potentially abortive diseases are able to co-exist. These diseases are caused by bacteria, viruses or parasites which colonise the genital region particularly during gestation. They cause premature expulsion of the foetus and sometimes, depending on the aetiology, illness and even death of the dam. The gross pathology at necropsy is rarely indicative of a precise aetiology, diagnosis is essentially differential, and tests must be carried out to distinguish latent infections from the advanced infection responsible for the observed abortions.

Direct diagnosis, undertaken on samples of the products of the abortion, is sometimes sufficiently conclusive to allow the immediate establishment of sanitary measures or treatments designed to limit the clinical and economic consequences of the concurrent infection. Indirect diagnosis, often undertaken

after the abortion, allows implementation of prophylactic measures, sanitary or vaccinal, relevant to the epidemiological conditions and the local legislation concerning the different infections.

## 3.3 SAMPLES

In addition to the identification of the cause of infectious abortions, diagnosis should establish the enzootic nature of the disease. Analyses for the main abortive diseases should therefore be undertaken on several samples by direct and indirect methods. Samples should include several aborted foetuses and their associated membranes (Figure 3.1).

Most of the pathogenic agents can be effectively isolated from the organs of aborted foetuses but some agents can be detected more easily in the placenta. Vaginal samples (swabs, tampons, scrapings) can also be used but they must be collected as soon as possible after abortion [10].

## 3.4 RISKS TO HUMAN HEALTH

To prevent the risk of human infection, samples taken from abortions must be handled with all the precautions necessary to protect personnel and to avoid contamination of the environment.

## 3.5 DIRECT DIAGNOSIS

Direct diagnosis involves the application of a group of methods designed to simultaneously detect the potentially abortive infectious agents that occur most frequently in a given region (Figure 3.2). These techniques include direct examinations and culture methods adapted to the nature of the samples and the infectious agents. In non-specialised analytical laboratories investigations are often limited to detection of the most common bacterial causes of abortion.

Virological, histological and parasitological investigations are generally only undertaken in more specialised laboratories when such agents are strongly suspected of being involved.

### 3.5.1 Bacteriological examination

Under certain conditions simple bacteriological testing is all that is required to detect some of the major causes of abortion (rickettsiosis, campylobacterosis, etc.). Confirmation of a speculative diagnosis by isolation of the organism in culture can lead to the rapid introduction of medicinal or sanitary measures to control the infection. Identification of the species of bacteria isolated is achieved by application of a number of differential staining methods although, in recent years, more specific immunohistological and immunoenzymatic techniques have been introduced.

#### *3.5.1.1 Placenta*

Smears from cotyledons should be mounted on grease-free slides. Since the cotyledons are unlikely to be uniformly infected it is best to undertake a single analysis on as many cotyledons as possible (5 to 10) rather than take multiple samples from a single cotyledon.

A smear of the vaginal sample should be made in the absence of the placenta.

*Note*

• If neither the placenta nor a vaginal sample have been collected it is sometimes possible to obtain a smear from the mucus of the aborted foetus or to take a scraping from the umbilical cord. Testing of these samples is, however, generally less effective.

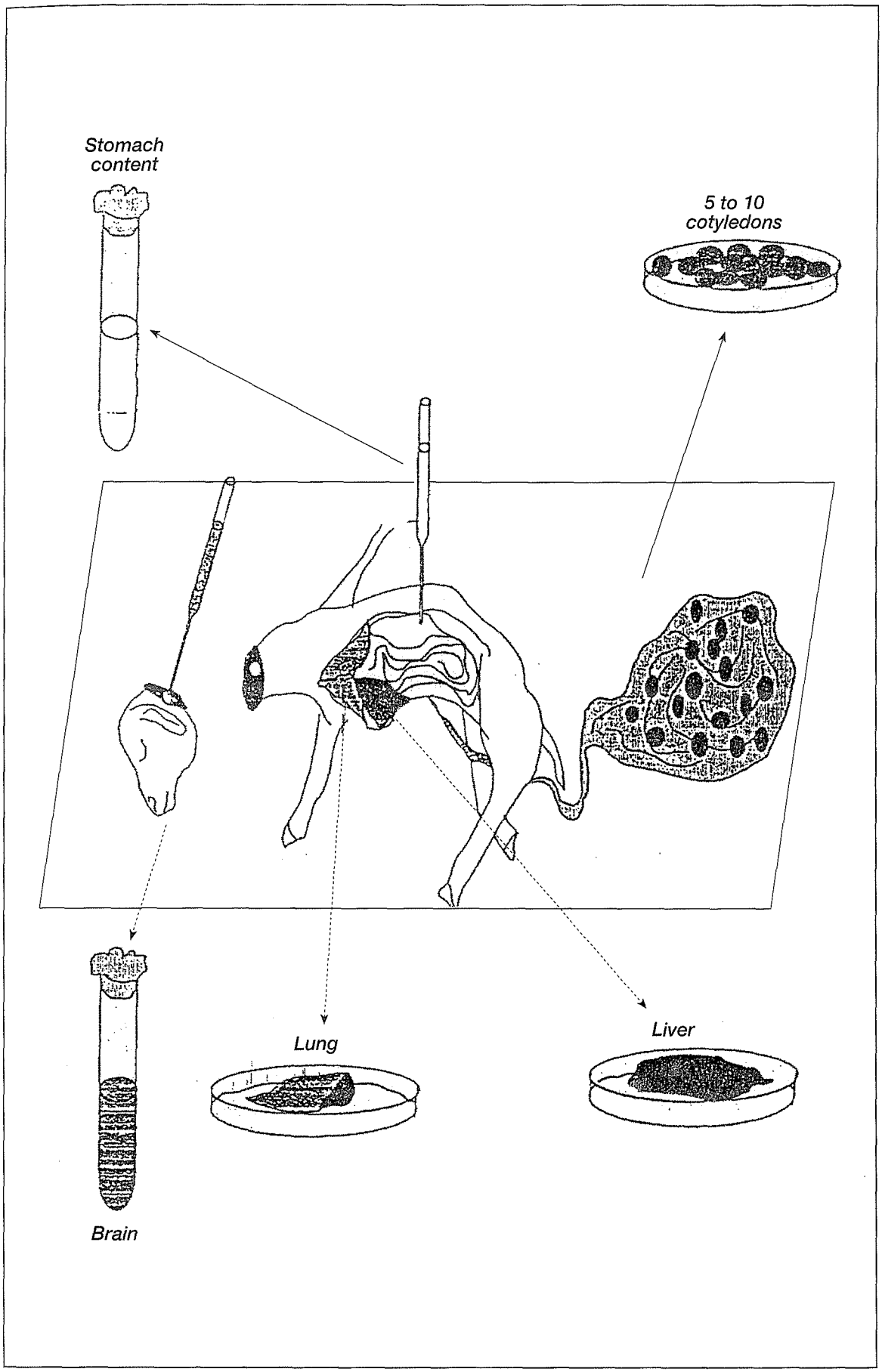


*Figure 3.1 : Bacteriological diagonsis of infectious abortions. Sample collection.*

### *3.5.1.2 Stomach contents*

Fresh samples of the stomach contents of the aborted foetus can be examined directly on a slide against a black background or by phase contrast to show the presence and characteristic motility of certain infectious agents (for example, *Campylobacter*). Two or three smears should also be made on grease-free slides, one of which should be reserved for immunoenzymatic analysis.

### *3.5.1.3 Staining*

Sections of cotyledons and smears should be submitted for differential staining. For example, the Stamp stain is used to distinguish *Brucella, Chlamydia* and *Coxiella* (Figure 3.3, page 89) ; the Vago stain, or more simply the Gram stain, counterstained with fuchsin allows detection of *Campylobacter* in smears taken from the stomach contents. Other stains can be applied (Koster, Machiavello, May Grunwald Giemsa, etc) provided that the results can be interpreted with confidence.

Immunoenzymatic or gene amplification (PCR) techniques can be applied to all types of samples. They will probably eventually provide an alternative to the previously described techniques which could lead to direct and more specific control of a number of infectious agents.

## 3.5.2 Culture methods

### *3.5.2.1 Foetal organs*

Foetal organs (e.g. brain, liver) and the stomach contents should be systematically seeded into non-specific nutrient media (e.g. nutrient agar or broth, Tryptose soya). These media allow the isolation of common bacteria (enterobacteria or others) which cause abortion under certain conditions, as well as more specifically abortive bacteria that have simple culturing requirements and are sufficiently abundant in the products of abortion (*Salmonella, Listeria*). The seeding into specific or selective media allows the culture of

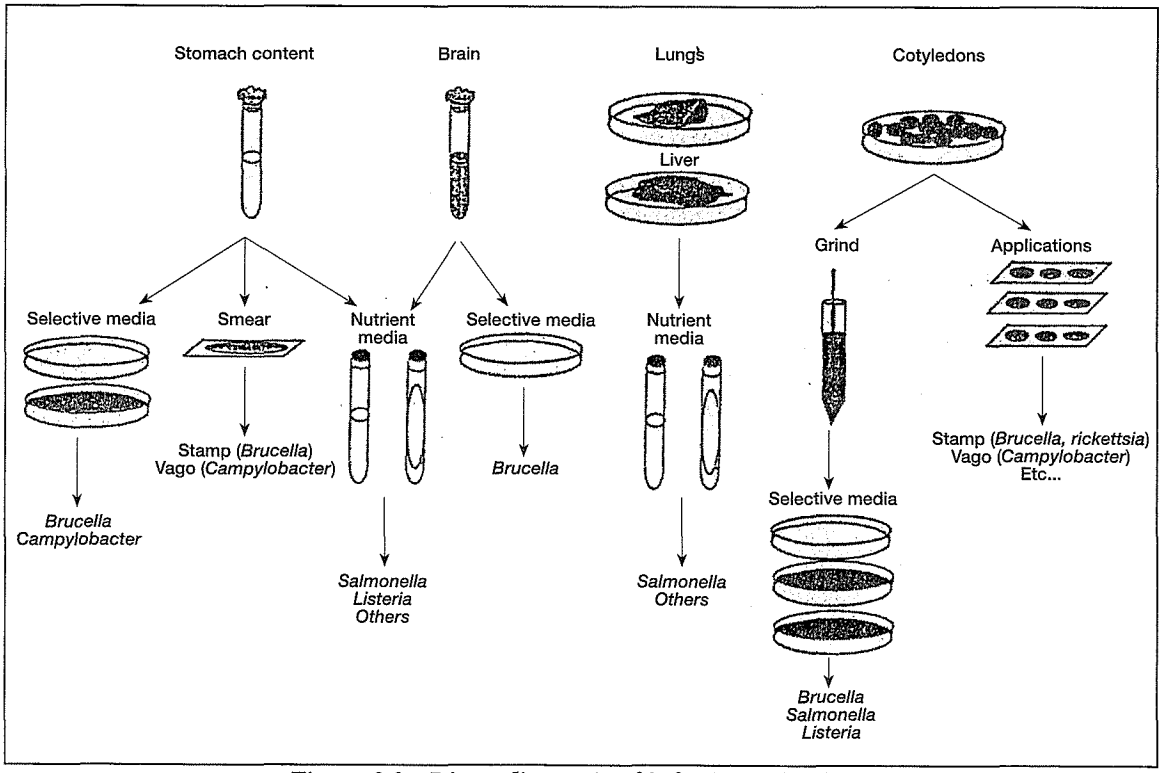


*Figure 3.2 : Direct diagnosis of infectious abortions. Analysis of samples*

more fastidious abortive bacteria (*Brucella, Campylobacter, Leptospira, Yersinia*).

### *3.5.2.2 Placenta*

Ground samples of cotyledons or vaginal samples, usually more heavily infected than the foetal organs, should be seeded into selective media for the detection of the more common abortive bacteria (*Brucella, Salmonella, Listeria,* etc.). These samples are usually sufficiently infected to produce abundant cultures and do not require the use of enrichment media. Enrichment media may also allow the isolation of bacteria of environmental origin (for example *Listeria*) and so lead to erroneous results.

It is possible to detect two potentially abortive agents in the same sample in areas of multiple infections (for example: isolation of *Salmonella* or of *Brucella* and detection of *Chlamydia*). The overall flock diagnosis is then employed to identify the agent responsible for the observed abortions, the presence of the other perhaps indicating a latent infection.

Testing in sensitive cell cultures (Border disease, rickettsia), histological and immunohistological examination (*Toxoplasma*, mycosis), immunoenzymatic [1, 4, 7] or other [12] techniques are not usually undertaken systematically in routine diagnosis. Such methods are only undertaken when the involvement of a specific agent is strongly suspected or when previous tests have not identified the cause of the abortions. Some of these infections are most easily detected by serodiagnosis, often more rapidly and at lower cost.

## 3.5.3 Interpretation of results and limitations

The proposed steps allow detection of the main bacterial abortive diseases by use of simple techniques. The effectiveness of the testing, however, depends on the quality of the samples. The relevance of such testing is highest when certain bacterial diseases predominate (brucellosis, salmonellosis, listeriosis, etc.) and decreases considerably when samples are restricted (for example in the absence of the placenta) or when viral (pestiviruses) or parasitological (toxoplasmosis) infections predominate [8, 13]. Taking these factors into consideration, a positive result is always proof of the cause of infection but a negative result does not prove an agent was not involved. Detection of an abortive agent in a single sample does not necessarily confirm the cause of the observed enzootic disease; all individual results must be backed up by flock diagnosis or a serological test which provides confirmation of the enzootic nature of the infection and after other causes of abortion have been ruled out in preceding tests.

It is therefore imperative that the analytical report includes a statement of the effectiveness of the different tests and gives the details of their respective results (Table 3.1).

*Table 3.1 : Types of analytical reports of direct diagnosis of infections abortions*

| |
|---|
| ***Direct examination*** : Stamp stain : **positive** (presence of rickettsia bodies)<br>**Cultures** : Test for *Salmonella* : negative<br>Test for *Listeria* : negative<br>Test for *Campylobacter* : negative<br>Test for *Brucella* : negative<br>**Interpretation : Chlamydiosis suspected** |
| ***Direct examination*** : Stamp stain : **negative**<br>**Cultures** : Test for *Salmonella* : negative<br>Test for *Campylobacter* : negative<br>Test for *Brucella* : negative<br>Test for *Listeria* : positive<br>***Listeria monocytogenes*** : isolated from the brain of the aborted fœtus<br>**Interpretation : *Listeriosis*** |

## 3.6 INDIRECT DIAGNOSIS

Serological diagnosis is used either to confirm the findings of direct diagnosis or is carried out as an independent analysis after abortions have taken place. It consists of techniques intended to simultaneously detect specific antibodies to the main abortive agents.

### 3.6.1 Principle

The serological diagnosis of infectious abortions is inevitably a comparative diagnosis applied to the flock. In the Mediterranean region, for example, simultaneous testing should be undertaken for brucellosis, salmonellosis, chlamydiosis and Q fever. Other analyses are only then undertaken if these initial tests prove negative or for identification of the source of infection.

The choice of techniques used depends on the nature of the infectious agent, on the techniques that can be undertaken in the laboratory and on the local legislation regarding the handling of contagious organisms. Traditional techniques such as seroagglutination, complement fixation or immunofluorescence recommended by the O.I.E. [9] generally give satisfactory results in the serodiagnosis of abortions. The only requirement demanded of the test used is that it gives quantitative results. In areas of multiple infection, serodiagnosis is the only effective way to differentiate between a vaccination or latent infection and the infection responsible for the observed abortions. Abortions or low productivity frequently produce an anamnestic response which is expressed by an overall boost in the rate of serum antibody production [14]. The interpretation depends on a comparative analysis of the results obtained with different antigens.

### 3.6.2 Interpretation of results and limitations

To interpret serological results, details of the effectiveness of the tests carried out and the individual results as titres or as dilution factors of the sera tested must be included in the report (Table 3.2). Depending on the criteria adopted, the results of the analyses can be interpreted at two levels.

- Firstly, for serodiagnosis of infectious abortion and if samples are taken less than 8 weeks after the abortions :

*Table 3.2 : Example of the report of serodiagnosis of infectious abortions*

| Sera | Brucellosis (1) | Chlamydiosis (1) | Q fever (1) | Salmonellosis (2) | Toxoplasmosis (3) |
|---|---|---|---|---|---|
| 1 | 0 | 1/10 | 0 | **1/160** | 0 |
| 2 | 0 | 1/20 | 0 | **1/1280** | 0 |
| 3 | 0 | 1/10 | 1/5 | **1/5120** | 0 |
| 4 | 0 | 0 | 0 | **0** | 0 |
| 5 | 0 | 0 | 1/10 | **1/10240** | 0 |
| 6 | 0 | 1/20 | 1/10 | **1/640** | 0 |
| 7 | 0 | 1/10 | 0 | **1/1280** | 0 |
| 8 | 0 | 0 | 0 | **1/2560** | 0 |
| 9 | 0 | 0 | 1/20 | **1/2560** | 0 |
| 10 | 0 | 1/40 | 0 | **1/640** | 0 |

*1: Complement fixation test* *2 : Seroagglutination test* *3 : ELISA*

***The most probable cause of the observed abortion is the organism for which the greatest number of sera present antibody levels higher than the threshold level for the method used.***

- Secondly, during sero-epidemiological investigations, interpretation can be made in terms of flock infection. The best time for samples to be taken is during the period 4 to 8 weeks after low productivity.

***The flock is considered to have a latent infection (or recent vaccination) for the disease when steady antibody levels higher than the treshold for the methods used are recorder.***
***It is considered that no contact with the pathogens has occurred when all sera tested give results below the threshold.***

It is considered that no contact with the pathogens has occurred when all sera tested give results below the threshold.

In the example in Table 3.2, the abortions may be attributed to a *Salmonella* infection; the flock is latently infected with *Chlamydia* and Q fever; the flock is presumed to have had no contact with *Brucella* and *Toxoplasma* (the uninfected status can only be confirmed by two successful tests undertaken on all animals in the flock).

## 3.7 CONCLUSIONS AND FUTURE WORK

The cause of infectious abortion can only be ascertained by careful differential diagnosis. From a good knowledge of the main local pathogens, which allows screening to be limited, a group of analyses can be undertaken which, in most cases, can be carried out in non-specialised analytical laboratories.

Direct diagnosis allows detection of most of the major bacteriological causes of abortion. Indirect diagnosis, used alone or as confirmation of direct diagnosis, allows establishment of the enzootic nature of infections and determination of

*Table 3.3 : Comparative summary of diagnostic methods for determination of infectious abortions*

| Infectious abortions | Direct diagnosis | | Indirect diagnosis |
|---|---|---|---|
| | Direct examination | Culture | Serological testing |
| Brucellosis | + | + | + |
| Salmonellosis | - | + | + |
| Listeriosis | - | + | (+) [a] |
| Campylobacterosis | + | + | (+) [a] |
| Chlamydiosis | + | (+) [c] | + |
| Q fever | + | (+) [c] | + |
| Leptospirosis | + | | (+) [a] |
| Other bacteria | - | + | (-) [b] |
| Toxoplasmosis | (+) [b] | - | + |
| Viral diseases | - | (+) [c] | (+) [d] |

*(a) : following confirmation as the source of infection*
*(b) : immunoenzymatic or immunohistological techniques*
*(c) : sensitive cell culture or immunoenzymatic techniques*
*(d) : seroneutralisation in sensitive cell cultures*

certain non-bacteriological infections (Table 3.3). Serological tests can also be applied to heart blood, pleural or peritoneal fluids of the aborted foetus. However, the foetus will not have developed the ability to produce antibodies if the infection is very severe or occurs very early in gestation and a negative result in such cases would not exclude that organism as the cause of infection.

Development of immunoenzymatic techniques may allow future use of a technique similar to that of the ELISA for all indirect diagnosis, providing that there is a sufficiently consistent system of interpretation of results. Advancement of knowledge in this area in the last few years and, in particular, the emergence and expansion of immunohistological techniques and those of gene amplification may allow the production of future diagnostic techniques which are more rapid, more specific and better adapted to different local epidemiological situations.

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# Chapter 4

# BRUCELLOSIS

J.M. VERGER & M. GRAYON

## 4.1 SUMMARY

Brucellosis in sheep, caused by *Brucella melitensis*, is essentially an abortive illness of the ewe. It is highly contagious and hazardous to man. Direct diagnosis involves the isolation of the bacteria on a selectively adapted medium, followed by identification by phenotyping tests (lysotyping, biochemical and serotyping) or by molecular methods (PCR-RFLP). Indirect diagnosis is undertaken using serological tests (Rose Bengal and complement fixation) and allergen tests (delayed hypersensitivity to *Brucella*).

## 4.2 INTRODUCTION

Brucellosis, caused by *Brucella melitensis*, is a contagious infection which affects the reticulo-endothelial systems and reproductive organs of small ruminants. *Brucella melitensis* has a predilection for the pregnant uterus and the mammary gland. The main clinical manifestation is abortion in ewes and goats and less commonly epididymitis in rams and billy-goats. The cyclic aspect of abortive storms, interrupted by a short or long silent phase, is characteristic of the development of *B.melitensis* infection within a flock.

The products of a past infection or the infected material associated with them are nearly always the source of transmission. Excretion of bacteria in milk is a danger to humans but does not appear to be a source of inter-animal infection except perhaps via the hands of the milker. The most common route of infection is via the naso-pharynx but entry through the skin is possible particularly through abrasions. Having crossed the mucus or cutaneous barrier, the *Brucella* passes via the lymphatic system to the nearest lymph nodes, usually those of the head. In the resistant animal, the bacteria are killed by macrophages, cellular components of the immune system, aided by certain antibodies and activated by T lymphocytes. In the susceptible animal, on the other hand, the bacteria multiply in phagocytic cells, kill and lyse them and go on to infect new cells. Bacteraemia results although *Brucella* rarely occur in large numbers in the general circulation. If the ewe or goat is pregnant, the bacteria usually invade the uterus and multiply in the placenta. Eventually they infect the foetus and cause a partial infection, with or without abortion, which releases large quantities of *Brucella* into the environment. During chronic infection certain lymph nodes are automatically infected whether or not the animal is pregnant. The mammary gland is a common target organ and provides a favourable environment for the persistence of infection and *B.melitensis* excretion in the milk. In the male, the infection preferentially affects the genital system - the epididymis, testicles, seminal vesicles - with frequent excretion of the bacteria in the sperm and occasionally clinical expression as epididymitis.

Brucellosis in small ruminants is controlled by prophylaxis and/or medication. Prophylactic methods alone, based

on the detection and slaughter of animals which react positively in immunological tests, is only effective in regions where the prevalence of the infection is low and contagion can be well controlled. The method is ineffective in regions of high infection where positive animals must first be treated by a preliminary phase of vaccination. This reduces the prevalence rate to an acceptable level to allow the introduction of a combined approach of vaccination associated with culling of infected animals (medical-sanitary). Finally, vaccination is omitted and any infected animals are identified and culled until the disease is eradicated.

The live vaccine, Rev. 1, is produced by a laboratory induced mutation of *B.melitensis* and has been the basis of the anti-Brucella vaccine for sheep and goats for many years. The vaccine is normally administered subcutaneously at a dose rate of 1 x $10^9$ viable bacteria. Under these conditions a serological response is induced which lasts longer in older animals than in young ones. This limits the vaccines usefulness in young animals, although antibodies do persist until an adult age in 1-2% of young animals. The procedure of conjunctival administration [5, 6], which limits the spread of the vaccinal bacteria to the lymph nodes in the cervical region, induces a solid immunity whilst limiting the serological reaction to a period usually no longer than four months. This process therefore renders the use of the Rev 1 vaccine compatible with prophylactic sanitary measures and allows quicker eradication of the infection.

The infectious disease, known as *contagious ram epididymitis*, involves another aetiological agent, *Brucella ovis*, a naturally "rough" species with exclusive pathogenicity for the sheep under natural conditions. Ewes are sensitive to *B.ovis* but infection is usually inapparent and very rarely results in abortions or foetal deaths. In the latter case it is difficult to differentiate between *B.ovis* and occasional "rough" varieties of *B.melitensis*.

## 4.3 SAMPLES

The samples recommended for bacteriological examination, the optimal sampling time and the conditions of transportation

*Table 4.1 : Recommended samples for bacteriological examination of the aborted female*

| | **Recommended samples** | **Sampling time** | **Transport and storage conditions** |
|---|---|---|---|
| Live animal | - Vaginal swab<br>- milk (about 20 ml for each quarter) | Preferably during the 15 days after birth or abortion | All samples should be packaged in waterproof containers and maintained at 4°C if analysis is undertaken winthin 48 hours or at -20°C if analysed later.<br>For transportation it is best to use insulated packaging, conform to safety standards and keep journey times to a minimum |
| Dead animal | - lymph nodes (retropharyngeal,sub maxillary, retromammary<br>- uterus<br>- liver<br>- mammary gland | From the carcass at the autopsy | |
| Aborted fœtus | - stomach fluid<br>- liver<br>- lung | As soon as possible after the abortion | |

are summarised in Table 4.1. Samples must be collected as aseptically as possible. For milk samples, the teats must be disinfected and dried, the first two milk jets discarded and the following milk collected in a sterile container avoiding any contact with the hands. Table 4.1 does not include samples to be taken from the male since these are not of direct concern in the present context (if necessary, see Alton et al [1]).

Blood samples for serological examination should be collected in silicone coated, sterile tubes (e.g. Vacutainers) and removed to the laboratory as quickly as possible.

### 4.3.1 Treatment of samples for bacteriological examination

Whatever the sample type, bacterial multiplication in a selective medium will increase the chances of isolating *Brucella.*

#### *4.3.1.1 Milk*

1. Centrifuge approximately 10ml of milk, taken from the two quarters of the udder and mixed together, at 3000 - 5000 rpm for 15 minutes.
2. Spread the cream over half of the surface of a plate selective medium.
3. Empty the intermediate liquid into a receptacle containing disinfectant, spread the precipitate over the other half of the medium and incubate the plates at 37°C.

#### *4.3.1.2 Vaginal swabs*

Remove the swab from its protective sheaf, smear the sample onto the surface of a plate of selective medium and incubate the plates at 37°C.

#### *4.3.1.3 Tissues (organs and lymph nodes)*

Any system of maceration may be used but a "stomacher" (Seward Medical, London, UK) which permits disruption of the tissue in watertight plastic bags is especially recommended.

1. Dip the sample rapidly into 95% ethanol, flaming as a precaution, and place in a sterile Petri dish then cut up the sample.
2. Trim the tissue if necessary and chop coarsely using scissors.
3. Grind the tissue.
4. Take a sample of the ground tissue using a flamed and cooled spatula and spread over the surface of the selective medium. Incubate the plates at 37°C.

#### *4.3.1.4 Stomach fluid*

Stomach fluid is the best sample to take from the aborted foetus. Remove a sample using a platinum loop or a syringe and spread over the surface of the selective media.

## 4.4 RISKS TO HUMAN HEALTH

*Brucella* is classified in risk group III [2], which necessitates strict protective measures during handling: working in class L3 containment areas or, if unavailable, in a conventional laboratory equipped with a laminar flow hood. In all circumstances it is essential to use good bacteriological practices, namely:

- Wear gloves during handling of samples.
- Never pipette directly by mouth.
- Avoid producing aerosols.
- Disinfect the work surfaces after all manipulations.
- Decontaminate re-useable materials by autoclaving, and incinerate waste.
- Wash hands with a disinfectant soap.
- For more details refer to the OMS Manual [2] or to the paper of Alton et al [1].

## 4.5 DIRECT DIAGNOSIS

Direct diagnosis of brucellosis is achieved by isolation and identification of the responsible micro-organism using bacteriological tests which determine the phenotypic characteristics of the bacteria. *Brucella* can also be detected using molecular tests which take account of all the characteristics of the genome.

### 4.5.1 Bacterial detection

#### *4.5.1.1 Bacteriological*

***Principle***

This technique consists of demonstrating the presence of the bacteria in smears or histological sections of tissues by specific staining. The Stamp stain is the classical method used for the bacteriological identification of *Brucella*.

***Materials and Reagents***

- Staining boat.
- Immersion microscope.
- Ziehl Fuchsin stain (stock solution: dissolve 1g of basic fuchsin in 10ml of absolute alcohol and add 90ml of 5% phenol solution).
- 0.5% acetic acid.
- 1% methylene blue.

***Procedure***

1. Take a smear or impression of sample and fix by flaming.
2. Stain with Ziehl Fuchsin diluted 1 in 10, for 10 minutes.
3. Wash under running water.
4. Destain with 0.5% acetic acid for a maximum of 30 seconds.
5. Wash under running water.
6. Counterstain with a 1% methylene blue solution for 20 seconds.
7. Wash under running water and dry.

***Reading and interpretation of results***

*Brucella* appear as red bodies on a blue background, isolated or in groups, usually intracellularly. A positive result is not necessarily confirmation of infection. This stain is subject to errors due to excess or lack of organisms and the result, whether positive or negative, must always be confirmed by culture.

#### *4.5.1.2 Bacterial isolation*

***a / By selective media***

The selective medium recommended for the isolation of *Brucella* is Farrell's medium [4] prepared as follows :

- To one litre of basal media BAB (Blood-Agar-Base No 2, Oxoid) or BMB (Brucella-Medium-Base, Oxoid), cooled to 50°C, add 5% sterile horse serum plus the following antibiotics :

| | |
|---|---|
| - cyclohexamide | 100 mg |
| - bacitracin | 25000 units |
| - polymixin B sulphate | 5000 units |
| - vancomycin | 20 mg |
| - nalidixic acid | 5 mg |
| - nystatin | 100000 units |

- Pour into Petri dishes.
- Incubate overnight at 37°C then store at 4°C. Under these conditions the dishes are stable for up to one month.
- Oxoid retail the antibiotic supplement (ref. SR 83) ready prepared in flasks containing the necessary quantities for 500ml of base media.

***b / By inoculation in laboratory animals***

Farrell's medium provides an efficient method of isolation and it is rarely necessary to resort to inoculation of guinea-pigs or mice except in cases where the sample is highly contaminated (for more details consult Alton et al [1]).

## 4.5.2 Identification of the *Brucella* genus

On Farrell's medium, colonies of *Brucella* are visible after 2 to 3 days incubation. After five days the colonies are translucent, convex, light blue, with regular edges and a diameter of 0.5-1.0mm. If the sample is heavily infected a dark blue coating of culture is apparent after 48 hours.

### *4.5.2.1 Microscopic examination*

Brucella are small coccobacillus organisms (0.6-1.5µm x 0.5-0.7µm), immobile, Gram-negative, usually occurring singly but sometimes in pairs or groups. They do not have a cell wall and do not show bipolar staining.

### *4.5.2.2 Simple bacteriological tests for identification of the Brucella genus*

| Positive Tests | Negative Tests |
|---|---|
| - Strict aerobes | - Haemolysis |
| - Catalase | - Acidification of glucose |
| - Oxydase * | - Indole |
| - Reduction of nitrates to nitrites* | - Citrate |
| - Urease* | - Methyl red and Voges Proskauer |

** B. ovis gives negative results to these tests.*

The urease test for Brucella is best undertaken on Christensen medium which has the following composition :

- Peptone 1 g
- Sodium chloride (NaCl) 5 g
- Potassium phosphate ($KH_2PO_4$) 2 g
- Phenol red 0.0012 g
- Dextrose 1 g
- Agar 20 g
- Distilled water 1 litre
- Adjust to pH 6.8 and aliquot prior to sterilisation.
- Add urea (20% solution sterilised by filtration) to a final concentration of 2% before pouring the medium into Petri dishes. For effective growth cultures seeded onto Christensens medium should come from a solid culture. A positive reaction is indicated by a change in the colour of the medium around the edge of the culture from yellow to a fairly bright red, the rate of production depending on the Brucella strain (1 to 15 minutes).

### *4.5.2.3 Control of the purity and morphological typing of colonies*

Colonies suspected of being *Brucella* after the previous tests should be seeded onto plates of a non-selective base medium to confirm the purity of the strain and the morphological type of the colonies prior to continued identification.

Commercially available media based on Tryptic soya, solid (TSA) or liquid (TSB), are highly suitable for culture of *Brucella* : BBL Tryptose, Difco Bacto-tryptose, Gibco Tryptose agar, Oxoid Tryptose agar or bioMerieux Tryptose agar. 5% serum must be added for growth of *B.ovis*.

#### *a / Colony observations without staining*

Colonies should be observed by oblique transillumination using the apparatus shown in Figure 4.1. The apparatus comprises a light source (1) the rays from which converge on a plain mirror (3) and are returned, following a 45°

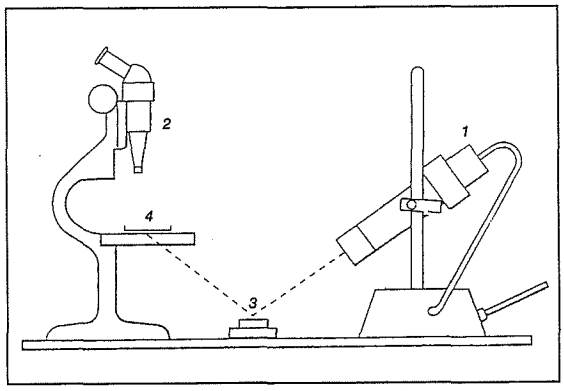


*Figure 4.1 : Apparatus for observing colonies of Brucella*

turn, to the plate placed on the support of the stereo microscope (2).

This set up allows the purity of the strain and the morphological type of the colonies to be assessed: smooth colonies are blue, iridescent and finely textured, rough colonies are more yellow and more granular in appearance (Figure 4.2, page 90).

***b / Colony observations after staining with crystal violet***

This staining method aids differentiation of the two morphological types, rough and smooth.

The stock solution of crystal violet is prepared as follows:

- dissolve :

• 2g of crystal violet in 20ml absolute alcohol;

• 0.8g of ammonium oxalate in 80ml distilled water;

- mix and store this stock solution for up to three months at 4°C.

- At time of use :

1. Dilute the solution 1 in 40 in distilled water.

2. Flood the plates to be examined and allow to react for 15-20 seconds.

3. Remove the surplus stain into a disinfectant with a Pasteur pipette and observe the plates by stereo microscope.

Smooth colonies will not be stained and will appear yellow on a violet background. Rough colonies will be stained violet-red (Figure 4.3, page 90).

Colonies of *B.melitensis* isolated from samples are usually of the smooth type whereas *B.ovis* is a naturally rough species.

***Note***

The Crystal violet does not affect the viability of the *Brucella* in the minutes following staining, allowing colonies to be subcultured to determine their type.

## 4.5.3 Determination of species

Differentiation of *B.melitensis* species within the *Brucella* genus is achieved by two classical tests: sensitivity to bacteriophages and determination of the metabolic oxidation profile by manometric methods.

### *4.5.3.1 Sensitivity to bacteriophages*

A number of phages have been described in the literature. Three of them are particularly well adapted for differential diagnosis of *B.ovis* and *B.melitensis* :

• Tb phage, isolated by Tbilisi in the USSR, lytolytic for smooth *B.abortus* ;

• Iz phage, lytolytic for all species of smooth *Brucella* (therefore *B.melitensis*);

• R/C phage, lytolytic for most of the rough *Brucella*.

These bacteriophages, although not commercially available, can be obtained from the FAO/WHO Collaborating Centre for Reference and Research on Brucellosis, CVL, Weybridge, New Haw, Surrey KT15 3NB, UK. They are used in the test at a standard working concentration (SWC).

***a / Determination of the SWC***

***Materials and reagents***

- Tubes containing 4.5ml TSB media.

***Procedure***

1. Remove a 24 hour culture of the strain of *Brucella* under investigation from TSA agar and make a suspension (titre of approximately $10^9$ cells per ml) in physiological saline.

2. Seed a tube of TSA agar by the appropriate technique (e.g. flooding, double coating) to obtain a homogeneous cover of culture and allow to dry for a few minutes.

3. For each run prepare a series of 10-fold dilution's of the test phage in TSB media.

4. Using a Pasteur or calibrated pipette, place a drop (20µl) of each dilution of the phage without touching onto the surface of the agar.

5. Once the drops have been absorbed, incubate the tubes at 37°C, with or without $CO_2$, according to the requirements of the strain.

***Reading and interpretation of results***

Read after 24 hours incubation or after 48 hours for strains that grow more slowly. The SWC is the highest dilution which gives a complete lysis (Figure 4.4, page 90).

When using a calibrated pipette, an estimation of the titre of the phage suspension can be made by counting the number of plaques of lysis at one of the lower dilutions and multiplying this by the dilution factor.

*Note*

• Handling of the phages must be undertaken with the greatest of care to avoid the formation of aerosols which may cause contamination of the *Brucella* cultures. The pipette must be held upright when depositing the drops to avoid spraying phages over the cultures.

• The diluted phages can be stored at 4°C for up to one month but the stock suspensions must be frozen.

***b / Lysodiagnosis***

The combined use of the three bacteriophages, at SWC, allows *B.melitensis* to be differentiated from the other smooth *Brucella* and from *B.ovis*.

***Materials and reagents***

- Sterile swabs.
- Plates of TSA agar plus 5% sterile horse serum.

***Procedure***

1. Remove a 24 hour culture of the strain to be tested from TSA agar and make up suspensions of similar concentrations in 0.5% sterile physiological saline.

2. Soak a sterile swab in each suspension and make streaks, one for each of the test phages, across the surface of the agar plates, without resoaking the swab (it is possible to make up to five separate streaks of culture on one plate). Allow to dry for a few minutes.

3. Using a Pasteur pipette, place a spot of the SWC of each phage in the middle of the streaks without touching the surface of the agar.

4. Once the spots have been absorbed incubate the dishes at 37°C, with or without $CO_2$, according to the conditions required for each strain.

***Reading and interpretation of results***

Read after 24 hours incubation or 48 hours for strains which grow more slowly. A positive result is indicated by the absence of culture around the position the phage was deposited.

*B.melitensis* is only lysed by the Iz phage and *B.ovis* by the R/C phage (Figure 4.5, page 90).

*Note*

The quality of interpretation of the test depends on:

- Inclusion of 5% serum in the base media, preventing the lypholytic activity of the R/C phage.
- The homogenicity of the morphological type, smooth or rough, of the strain of *Brucella* submitted for testing.
- The systematic inclusion of standard strains representative of the different lysotypic profiles.

### *4.5.3.2 Metabolic oxidation*

This technique, based on the ability of suspensions of non-proliferating cells of Brucella to utilise oxygen in the presence of certain substrates (amino acids, sugars), allows determination of specific metabolic profiles relating to the morphological type of the strain (smooth or rough). This method, which is hazardous to perform, requires specific materials (Warburg apparatus) and highly competent personnel. The following laboratories are able to carry out this test on request, but samples must only be submitted by previous arrangement :

- FAO/WHO Collaborating Centre for Reference and Research on Brucellosis, CVL, Weybridge, New Haw, Surrey KT15 3NB, UK.
- United States Department of Agriculture, National Veterinary Service Laboratories, P.O. Box 844, Ames, IA, 50010, USA.
- INRA, Laboratoire des Brucella, Laboratoire de Pathologie Infectieuse et d'Immunologie, 37380 Nouzilly, France.

### *4.5.3.3 Molecular identification of B.melitensis and B.ovis*

A number of tests using molecular biological techniques are proposed today for identification and typing of *Brucella*. One which distinguishes *B.ovis* and *B.melitensis* from other *Brucella* is presented here. The identification of bacteria by molecular biological techniques requires initial extraction of the genomic DNA.

#### *a / DNA extraction*

The proposed technique, adapted for *Brucella*, allows recovery of 100µl of good quality DNA containing approximately 1mg/ml.

***Principle***

The DNA is extracted in three main stages: (1) Lysis of the bacterial cells, (2) purification by a series of phenol-chloroform extraction cycles, (3) precipitation by frozen ethanol.

***Materials and reagents***

- Refrigerated centrifuge with rotors suitable for 10ml tubes and microtubes (Eppendorf).
- Desiccator or Speedvac.
- Electrophoresis apparatus.
- TENa buffer (50mM Tris, 50mM EDTA, 100mM NaCl, pH 8.0).
- 10% Sodium dodecyl sulphate (SDS).
- Proteinase K (20mg/ml) stored frozen at -20°C.
- 5M sodium perchlorate.
- TE buffer (10mM Tris, 1mM EDTA, pH 8.0).
- Agarose.
- Cold ethanol (frozen at -20°C).
- DNA marker of known molecular mass.

***Procedure (Figure 4.6)***

- Lysis of Bacterial Cells

1. Remove a 24 hour culture from the surface of TSA agar and suspend in 3ml sterile distilled water and centrifuge at 4500 rpm at 4°C for 10 minutes (in 10ml tubes).
2. Remove the supernatant and add 567µl of TENa buffer.
3. Resuspend the pellet (by vortex) and transfer to a microtube.
4. Add 30µl of SDS and 3µl of Proteinase K.
5. Mix gently by rotation of the tube and incubate for up to one hour at 37°C.

- Deproteination and Purification

6. Add 600µl of phenol and shake vigorously until a homogeneous white suspension is obtained.
7. Centrifuge at 13500 rpm at 4°C for 20 minutes.

8.Transfer 500µl of the upper aqueous phase into a new microtube.

9. Add 80µl of sodium perchlorate and *600µl of chloroform, shake.

10. Centrifuge at 5000 rpm at 4°C for 10 minutes.

11. Gently remove 500µl of the aqueous phase into a new microtube.

12. If necessary repeat from * until all trace of the interface disappears.

- Precipitation With Ethanol and Titration of DNA

13. Add 1ml cold ethanol and mix by gently turning the tube (filaments of DNA appear rapidly).

14. Centrifuge at 13500 rpm at 0°C for 10 minutes.

15. Remove the ethanol by turning the tube upside-down and leave to drain for a few seconds on absorbent paper.

16. Dry the precipitate by Speedvac for 5-10 minutes or in a disiccator.

17. Add 100µl of TE buffer to the precipitate and leave the DNA to re-enter the solution overnight at 37°C.

18. Determine the approximate concentration of DNA of an aliquot by electrophoresis on agarose gel in comparison with a marker of known molecular mass.

***Note***

- The first part of the extraction, up to the addition of phenol, must be undertaken using normal bacteriological precautions as described earlier. Phenol and chloroform must be handled under a hood.
- The DNA can be stored for up to one month at 4°C or frozen.

### *b / Restriction analysis of gene amplification product coding for the 25kDa Membrane protein*

Molecular typing of these bacteria is based on the restriction analysis (RFLP) of the "Polymerase Chain Reaction" (PCR) amplification products of certain genes of *Brucella* [3]. The PCR-RFLP of the gene for the 25kDa outer membrane protein (OMP) of *Brucella* is particularly useful for the differential diagnosis of *B.melitensis* and *B.ovis*.

***Principle***

The aim of PCR is to exponentially amplify a specific DNA fragment. The different stages consist of denaturation at a high temperature of a fragment of double stranded DNA, hybridisation of each strand containing the region of interest which is to be amplified using a specific primer, and finally synthesis of the complementary sequence by DNA polymerisation. After n cycles the number of amplified fragments is $2^n$.

Cleavage of the amplified product by an endonuclease allows production of restriction fragments of different lengths, correlated with the degree of polymorphism of the gene (RFLP).

***Materials and reagents***

- Thermocycler.
- Microtubes for PCR.
- Electrophoresis apparatus.
- UV light box (254nm).
- Agarose.
- Restriction endonuclease EcoRV.
- Molecular weight markers.
- DNA genome of *Brucella* + standard heterologous DNA.
- Primers:
  - 25A (5'-GGACCGCGCAAAACG-TAATT-3').
  - 25B (5'-ACCGGATGCCTGAAAT-CCTT-3').
- PCR kit including: Taq polymerase, dNTP, buffer ...

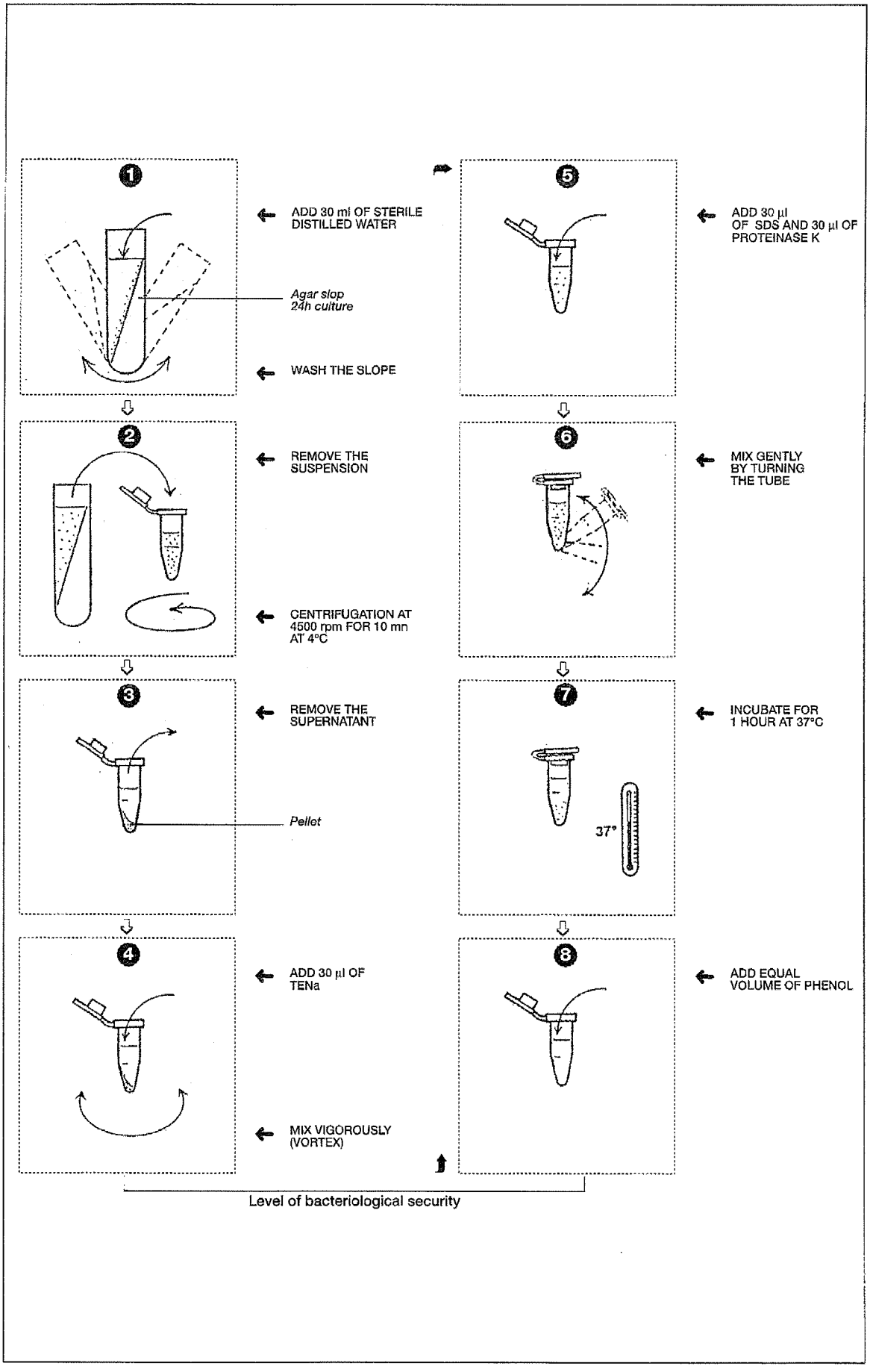


*Figure 4.6 : Protocol for the extraction of Brucella DNA*

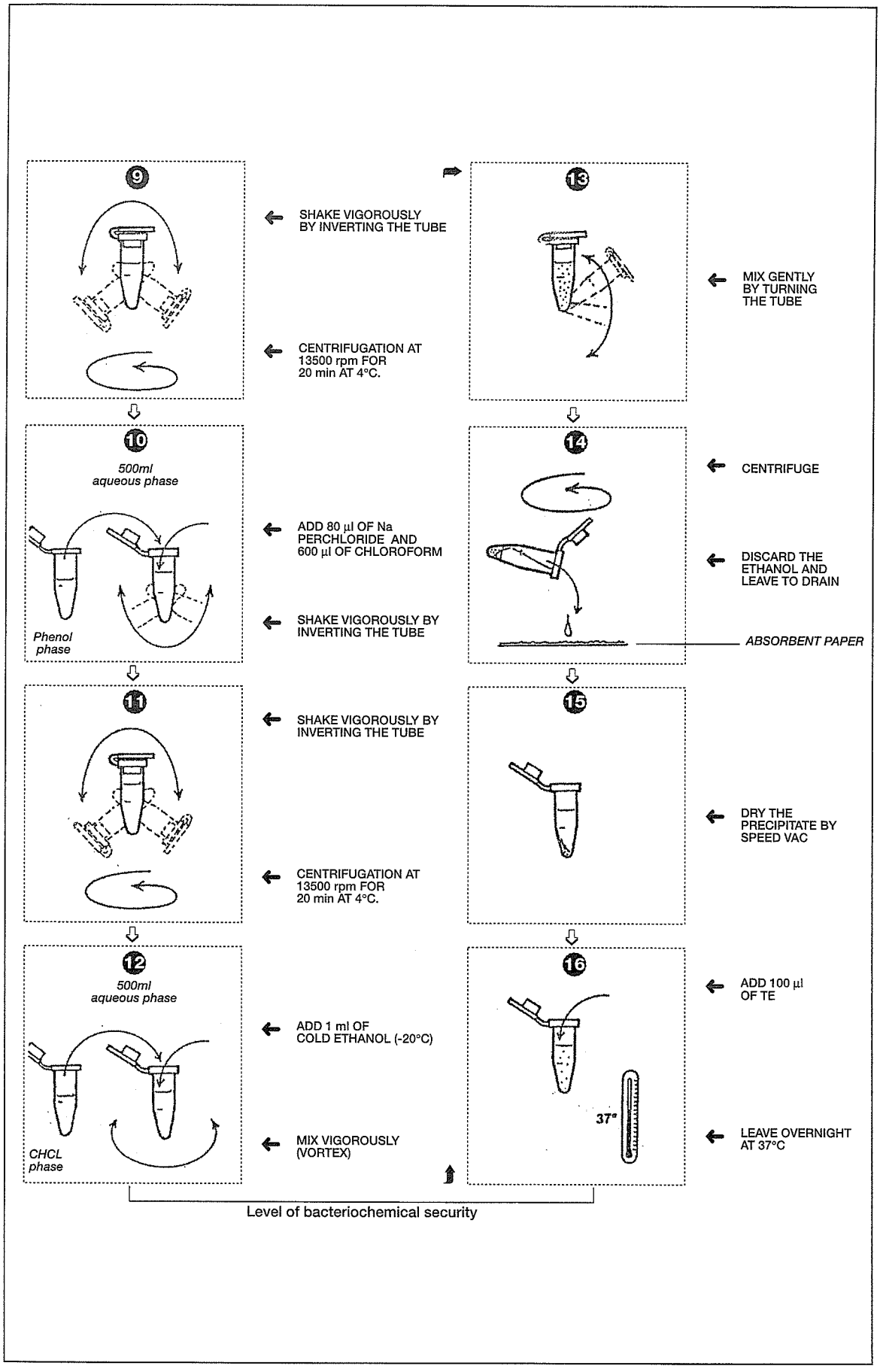


*Figure 4.6 : (continued)*

***Procedure***

The general conditions for PCR are adapted according to the type of thermocycler and the PCR kit used.

For an optimal amplification of the 25kDa OMP gene, under the specific conditions, the concentrations of reagents and the temperature are:

- for a total reaction volume of 100µl per tube :

-1µM of each primer;

- 100ng of *Brucella* genome DNA or standard heterogeneous DNA;

- 2.5 units of Taq polymerase;

- the temperature cycles for the denaturation, hybridisation and extension stages, respectively are 95°C, 58°C and 70°C although these temperatures can vary with the type of thermocycler used. The following programme is therefore given as an example:

| | |
|---|---|
| - First cycle: | 95°C for 5 min |
| | 58°C for 2 min |
| | 70°C for 3 min |
| - Following 30 cycles: | 95°C for 1 min |
| | 58°C for 2 min |
| | 70°C for 3 min |
| - Final cycle: | 95°C for 1 min |
| | 58°C for 2 min |
| | 70°C for 10 min |

Approximately 5µl of each PCR product is subjected to electrophoresis on agarose gel in order to check the specificity and quality of amplification.

These products are then digested with EcoRV, following the method recommended by the supplier, and subjected to electrophoresis on agarose gel to allow assessment of the restriction profiles.

***Reading and interpretation of results***

The results are illustrated in Figure 4.7, page 91.

Migration of the PCR products on the agarose gel allows differentiation of *B.ovis* from other *Brucella* since it has the simplest profile as a result of the deletion of the 36 bp.

The results of restriction analysis confirm this observation and also show the absence of the EcoRV site for *B.melitensis*.

***Note***

• Precautions must be taken (location, materials) when carrying out this technique to avoid any contaminants that may introduce foreign DNA. It is therefore advisable to undertake each amplification alongside two standards :

- one tube containing all the reagents with the exception of the DNA (contamination control);

- one tube in which the *Brucella* DNA is replaced by a heterologous DNA (to control the specificity of the primers).

• Neither of these standards should show any traces of amplification in the zone of the 25kDa OMP gene.

### 4.5.4 Biotype determination

The differentiation of biotype of the six main species of *Brucella* is achieved by four tests: requirement for $CO_2$, production of $H_2S$, growth in the presence of stains and agglutination by monospecific sera.

#### *4.5.4.1 Requirement for $CO_2$*

This test determines the absolute requirement of the developing culture for $CO_2$. It must be carried out as soon as possible after isolation, at the time of transfer from selective medium to non-selective medium for purity control.

***Procedure***

1. Emulsify a few colonies of the strain to be tested in 0.5ml of sterile physiological saline.

2. Undertake isolation on four plates of TSA media.

3. Incubate two plates in an incubator at 37°C with normal atmosphere and the other two plates in an incubator at 37°C with the atmosphere enriched with 5-10% $CO_2$.

***Reading and interpretation of results***

*B.melitensis* strains do not require $CO_2$ for their growth and will therefore be present on all four plates. *B.ovis*, which requires $CO_2$, will only be present on the plates incubated in the presence of $CO_2$ and supplemented with 5% serum.

### *4.5.4.2 Production of $H_2S$*

For *Brucella*, production of $H_2S$ is determined using strips of paper impregnated with lead acetate.

***Principle***

When a strain produces hydrogen sulphide, the sulphur combines with the lead to form a black precipitate of lead acetate in the strips.

***Materials and reagents***

- Strips of paper impregnated with basic lead acetate (Prolab, code 26 488 261) prepared as follows:
  - Soak a sheet of filter paper in a 10% solution of lead acetate and leave to dry at room temperature.
  - Cut strips approximately 12 x 1 cm and store in the dark without sterilisation.

***Procedure***

1. Prepare a BAB gel slope.
2. Introduce a strip of lead acetate impregnated paper into the tube avoiding contact with the gel and secure the end of the strip between the bung and the side of the tube.
3. Incubate at 37°C, with or without $CO_2$ according to the requirements of the strain.

***Reading and interpretation of results***

Read after 24 hours incubation. The paper will remain white with strains of *B.melitensis* and *B.ovis* that do not produce $H_2S$.

***Note***

- It is recommended that BAB medium is used as opposed to TSA medium because the latter is likely to have a high concentration of sulphur containing amino acids which may give "false positives".

### *4.5.4.3 Growth in the presence of stains*

The different Brucella types are sensitive or resistant to thionin or to basic Fuchsin incorporated directly into the base media.

***Materials and reagents***

- Sterile swabs ;
- BAB medium in 100ml flasks ;
- Staining solutions (0.1%) prepared as follows :
  - dissolve 0.1g of basic Fuchsin in 100ml distilled water
  - dissolve 0.1g of thionin in 100ml distilled water
  - sterilise these two solutions using a steamer or in a boiling water bath for one hour and store at 4°C for 3-4 months.

***Procedure***

1. Twenty-four hours before performing the test:
   - Remove samples of the test and reference strains from the surface of TSA agar and incubate at 37°C, with or without $CO_2$ according to the requirements of the strain.
   - Place 2ml of the made up staining-solutions (20µg/ml) and 98ml of BAB medium plus 5% horse serum in the stai-

ning plates, alongside a plate of the same base medium without staining solution. Leave overnight at 37°C.

2. On the following day, prepare the suspensions and seed the plates by streaking in the same way as for the lysotyping tests.

3. Incubate the dishes preferably in an atmosphere of 10% $CO_2$.

***Reading and interpretation of results***

Read after 3-4 days incubation. All strains of *B.melitensis,* whatever their biotype, will grow in the presence of the thionin and Fuchsin, but *B.ovis* will not grow in the presence of thionin (Figure 4.8 page 91).

***Note***

It is necessary to include during each test run:

- representative standards of the different growth profiles (media control);
- the seeding, under the same conditions, of the plate of medium without staining solutions (strain viability control).

### *4.5.4.4 Agglutination with monospecific sera*

This test is used to determine the *B.melitensis* types. It is the only test that differentiates the three biotypes of this species.

***Principle***

All smooth *Brucella*, independent of species, possess two determinant surface antigens, A and M, distributed in different proportions according to the strain. Characterisation of the three possible antigen profiles lies in a simple plate agglutination test using specific sera of A and M antigens. A-dominant strains are only agglutinated by anti-A sera, M-dominant strains by anti-M sera and strains containing notable quantities of both epitopes by both sera. The methods of preparation of these sera are described by Alton et al [1] but it is also possible to obtain sera for comparison from the FAO/WHO Centre of Reference at Weybridge, mentioned previously.

***Materials and reagents***

- Glass or wooden rods.
- Monospecific anti-A and anti-M sera, tested against representative strains with the three surface antigen profiles and, if necessary, diluted in 5% phenol in physiological saline to give the agglutination patterns relating to these profiles. The working dilutions can be stored at 4°C for up to one month but must not be frozen.

***Procedure***

1. Place a spot of each of the diluted anti-A and anti-M sera on a slide in a Petri dish.

2. Using a loop, sample a few colonies of the test strain and place by the side of each spot.

3. With two different rods make a homogeneous bacterial suspension in each spot of serum.

4. Close the lid of the Petri dish and holding firmly gently rock to aid the mixing of the agglutinants.

***Reading and interpretation of results***

Only agglutinations appearing within one minute are considered as positive. The three biotypes of *B.melitensis* are uniquely distinguished on the basis of their agglutination: A-, M+ (biotype 1), A+, M- (biotype 2) and A+, M+ (biotype 3). B.ovis is not agglutinated by anti-A or anti-M sera but possesses an antigenic determinant common to all rough *Brucella.* A serum prepared against this determinant (see Alton et al [1]) agglutinates *B.ovis* but also rough varieties of *B. melitensis.*

*Note*

• All new batches of sera must be tested alongside representative strains of all three types of agglutination.

### 4.5.5 Differentiation between the Rev. 1 vaccinal strain and wild strains of *B.melitensis*

The Rev. 1 vaccinal strain is a modified biotype 1 strain of *B.melitensis*. In exceptional cases where *Brucella* is suspected of having caused abortion in vaccinated animals it is useful to be able to differentiate between wild strains of *B.melitensis* and the vaccinal strain. Differential diagnosis is basically achieved by a growth test in the presence of two antibiotics, penicillin (5 IU/ml) and streptomycin (2.5 µg/ml), on TSA media. The test cultures are prepared and seeded by streaking in the same way as for the lysotyping tests and for growth in the presence of stains. Results are read after 48 hours incubation. In contrast to biotype 1 wild strains of *B.melitensis*, the Rev 1 strain is sensitive to penicillin and resistant to streptomycin.

## 4.6 INDIRECT DIAGNOSIS : IMMUNOLOGICAL TESTS

In the live animal, bacteriological examination is not always practical. Diagnosis of brucellosis in individuals and groups of small ruminants is achieved by three main immunological tests: the Rose Bengal agglutination test, complement fixation and the allergen test.

### 4.6.1 Antigenic buffer test (Rose Bengal)

*Principle*

An antigenic buffer at acidic pH (3.65), stained with Rose Bengal, is used as the basis of a simple and rapid agglutination test carried out on a tile and is very effective for identifying brucellosis in individuals within a group.

*Materials and reagents*

- White tiles (ceramic or plastic).
- Glass or wooden rods (tooth-picks).
- Shaker with rocking motion (approximately 30 rocks per minute).
- Antigen stained with Rose Bengal, available from commercial suppliers (Rhône-Mérieux, Commonwealth Serum Laboratories, Central Veterinary Laboratory) or prepared and standardised in the laboratory according to the method described by Alton et al [1]. It can be stored at 4°C but not frozen.
- Standard positive and negative sera.

*Procedure*

1. Bring the sera (test and standards) and the quantity of antigen necessary for tests being undertaken that day up to room temperature.
2. Place a spot (25 or 30µl) of each sera for testing on a white tile.
3. Mix the antigen thoroughly then place a spot of antigen, of equal volume, alongside each spot of serum (Figure 4.9, page 92 ).
4. Carefully mix each serum-antigen pair, using a clean rod for each sample, until a circular area of approximately 2cm diameter is obtained (Figure 4.10, page 92).
5. Rock the tile gently for 4 minutes, either manually or using a shaker with a rocking motion.

*Reading and interpretation of results*

Any agglutination visible to the naked eye after 4 minutes is considered as positive. Agglutination appearing after this delay is not considered positive.

*Note*

• The test is less sensitive if the serum and antigen are mixed immediately after removal from the refrigerator. The stock antigen, on the other hand, must be stored at 4°C to prevent deterioration.

### 4.6.2 Complement fixation

The complement fixation test (CFT) is unanimously acknowledged to be the most specific and sensitive technique for individual diagnosis of brucellosis in sheep and goats.

***Principle***

Complement is a complex protein that is able to attach itself to antigen-antibody complexes and induce cellular lysis in an standardised system. In the first stage of the CFT, the *Brucella* antigen and the test serum are mixed with fresh guinea-pig (complement) serum. If the test serum contains antibodies against *Brucella*, the complement becomes attached. In the second stage, sensitised red blood cells from sheep (i.e. mixed with antibodies raised against sheep erythrocytes or haemolysin) are added. If all the complement has been fixed during the first stage, no haemolysis occurs which signifies that the test serum contains antibodies. The reaction is positive. The presence of haemolysis indicates that the complement has not attached during the first stage because the serum does not contain antibodies against *Brucella*. The reaction is negative.

***Materials and reagents***

- 96 well microtitre plates (round bottomed wells).
- 12 head mechanical diluter to dispense 25µl volumes or multichannel pipette such as "Titertek" (Flow Laboratories) with disposable plastic tips for single use.
- Plate shaker.
- Centrifuge suitable for plates.
- Mirror for reading results.
- Antigen.
- Complement.
- Haemolytic serum.
- Sheep red blood cells.
- Veronal-Calcium-Magnesium buffer.
- Standard negative and positive sera of known titre.

***Procedure***

The four reagents used to measure the antibody titre of the test sera (the antigen, red blood cells, haemolytic serum or haemolysin and the complement) should be standardised before the test is carried out. Dilute antigen with veronal buffer according to the manufacturers instructions or following titration by the method of Alton et al [1]. The haemolytic complex is usually prepared from a 3% suspension of fresh sheep red blood cells mixed with an equal volume of rabbit serum raised against sheep red blood cells, at 2 to 5 times the minimum concentration required to produce 100% haemolysis in the presence of the complement. Titrate the complement separately in the presence [7] or in the absence [1] of antigen to determine the quantity of complement necessary to produce 50% lysis of a suspension of sensitised red blood cells. This quantity is known as the 50% haemolytic unit ($HU_{50}$) and 5 $HU_{50}$ are used for the test.

The test proper, presented below, is undertaken in 96 well microtitre plates (round bottomed).

- Stage One

1. Place 50µl of each test serum in the first well (A) of each of the 12 rows of the required number of plates (Figure 4. 11).
2. Similarly, place 50µl of each of

the two standards, positive and negative, in the first wells (A) of rows 1 and 2 on the standards plate (Figure 4.11).

3. Cover the wells with an adhesive strip and inactivate the sera in a water bath at 62°C for 30 minutes.

4. Remove the adhesive strip and add 25µl of veronal buffer to each well except those in the first column (A) which already contain the serum.

5. Dilute the serum by repeated doubling dilutions using a 12 head mechanical diluter or a multichannel pipette (25µl) until the last wells (H) of the plate when the dilution will be 1:128.

6. Add 25µl of veronal buffer to the second well (B) of each row (anti-complement control for each serum).

7. Add 25µl of antigen, freshly diluted with veronal buffer (according to the manufacturers instructions or the result of titration by the method of Alton et al [1]), from the third (C) to the last (H) wells of each row.

8. Add 25µl of complement containing five 50% haemolytic units ($HU_{50}$) in each well except the first wells (A) in each row.

9. Complete the standards plate (antigen, complement and haemolytic complex controls) as follows (Figure 4.11):

• Antigen Control (wells A and B of row 3)

- 25µl of veronal buffer
- 25µl of antigen
- 25µl of complement containing 5 HU50

• Complement Control (wells C, D, E and F of row 3)

- 50µl of veronal buffer
- 25µl of complement containing ($HU_{50}$)

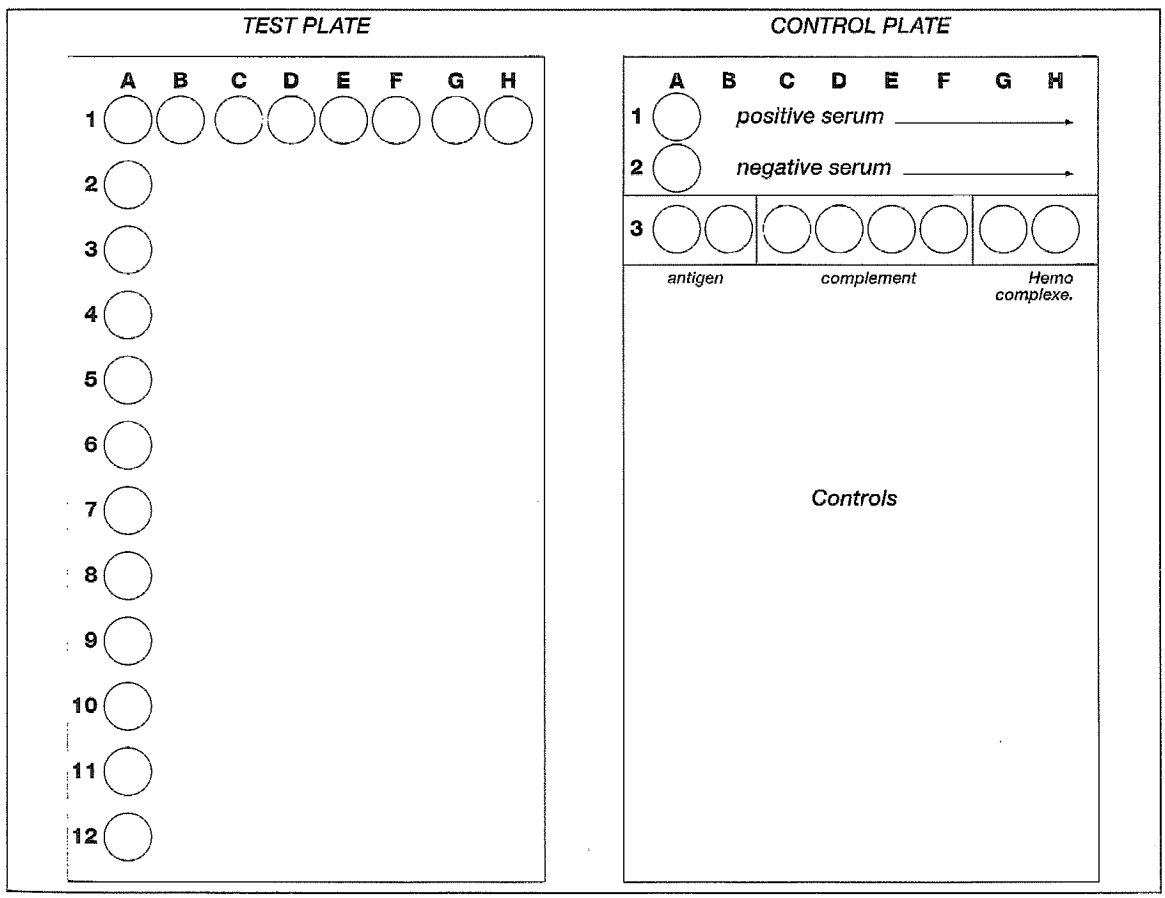


*Figure 4.11 : Complement fixation test : Lay-out for a test plate and a control plate*

• Haemolytic Complex Control (wells G and H of row 3)

- 75µl of veronal buffer

10. Gently tap each plate to mix the reagents, re-cover with an adhesive strip and place in an incubator at 37°C for 30 minutes (fixation in the warm) or at 4°C for 14 to 18 hours (fixation in the cold).

- Stage Two

11. Prepare the haemolytic complex by mixing equal volumes of a 3% red blood cell suspension and a haemolytic serum diluted in veronal buffer (according to manufacturers instructions or by the method of Alton et al [1]). Leave the mixture shaking at room temperature.

12. On removal of the plates from the incubator or refrigerator, add 25µl of haemolytic complex to wells B to H on the test plates and into all wells on the standards plate except for wells A in rows 1 and 2.

13. Re-cover the plates with an adhesive strip and incubate at 37°C for 30 minutes on a shaker (to prevent precipitation of red blood cells at the bottom of the wells).

14. On removal from the incubator, centrifuge the plates at 2000 rpm for 10 minutes at 4°C or, if a suitable centrifuge is not available, place the plates in a refrigerator for up to 2 hours to allow precipitation of all non-lysed erythrocytes.

## ***Reading and interpretation of results (Figure 4.12, page 92)***

Read the results from underneath the plates with the aid of a magnifying mirror or, if a mirror is not available, from above with the plate placed on a white light box. By either method the different controls, under satisfactory conditions, will show the following: Complete haemolysis (100%) in the antigen control and the complement control wells and absence of haemolysis in the haemolytic complex control wells. The results for each test serum are then scored from 0 (negative reaction) to 4+. With complete fixation (4+), relating to an absence of lysis, a button of red blood cells will be visible in the bottom of the well with a clear, colourless supernatant liquid. In the case of a negative reaction (score of 0) with complete lysis, no button of red blood cells will be visible and the liquid in each well will be clear and coloured by haemoglobin. The intermediate degrees of incomplete haemolysis are scored 1+, 2+ and 3+. The B well (anti-complement control) must show a complete haemolysis otherwise the result for the corresponding serum is not valid.

The serum titre is taken as the highest dilution showing a reaction of 1+ (or more), that is 25% (or more) fixation. A titre of 1:4 (or more) is regarded as positive or borderline. A titre of 1:8 is always considered as positive. The titre of a serum can also be expressed in International Units of Complement Fixation (IUCF) by comparison with anti-abortus international standard serum (available from CVL, Weybridge, UK) or a national equivalent. This standard serum, which is assigned an arbitary value of 1000 IUCF/ml, is used to establish the conversion factor which allows expression of a IUCF titre for an unknown serum using a given technique. If the titre (50% fixation or inhibition of haemolysis) of the standard serum is equal to 200, the IUCF/ml value of the unknown serum is given by the formula : 1000/200 x titre of unknown serum. The EC consider all sera with a titre equivalent to 20 IUCF as positive.

*Note*

• The reagents for complement fixation; antigens, complement, haemolytic serum, sheep red blood cells, veronal-calcium-magnesium buffer are available from commercial suppliers (BioMerieux) or can be prepared and standardised in the laboratory according to the protocols described by Alton et al [1].

• Complement fixation is a complex test which requires very careful preparation and standardisation of reagents. In particular, the complement, which is highly labile, must be titrated for each series of tests, preferably in tubes rather than on plates for the best determination of ($HU_{50}$) units. Each new batch of haemolytic serum must be systematically titrated.

• Anti-complementary reactions are not uncommon for sheep. Inactivation of the sera at 62°C and/or cold fixation allow a reduction of this phenomenon although this type of fixation increases the frequency and intensity of the prozone phenomenon. It is necessary therefore to take into account these characteristics when choosing the temperature of fixation.

### 4.6.3 Allergen test

The allergen test, together with serological diagnosis, increases the chances of detecting infection especially in flocks where the extent of *Brucella* infection is not clear. This test never gives false positive results and a positive result in a single animal always signals the presence of *Brucella* infection in the flock. On the other hand, all infected animals do not necessarily give positive results, making the test more applicable for identifying the stage of brucellosis infection in the flock than as a diagnosis of infection in individual sheep and goats. An important practical advantage of this test is that the results must be read and interpreted directly by the tester, which favours their co-operation in implementing prophylactic measures for control of brucellosis.

***Principle***

The allergen test is based on a delayed hypersensitivity reaction in animals inoculated with cytoplasmic proteins of rough and smooth *Brucella*. The initial allergens were prepared from filtrates of old boiled cultures and had the drawback of inducing antibody production and sensitisation of the animal. They have now been replaced with an allergen, devoid of LPS-S, the brucelline-INRA, prepared from part of one rough strain of *B.melitensis*, which is available commercially (Brucellergene OCB, Rhône-Mérieux).

***Materials and reagents***

- Syringe and needle for intramuscular injection.
- Callipers.
- Brucellergene OCB (Rhône-Mérieux).

***Procedure***

In sheep and goats the palpebral route is generally used for convenience of injection of the allergen and for assistance in the reading of the results. The recommended volume of Brucellergene OCB is 0.1ml, injected intradermally into the lower eyelid.

***Reading and interpretation of results***

The reaction is assessed 48 hours after injection, by comparison with the other eye. Any visible or palpable reaction on the test eyelid is considered positive. Intensity is assessed by the degree of oedema of the eyelid which generally causes a large swelling under the eye and is usually accompanied by lacrimation (Figure 4.13, page 93).

*Note*

- To avoid production of antibodies or sensitisation of the animal, it is essential that the allergen is of highest quality and contains no LPS-S.
- The allergen test cannot be used in flocks immunised with the Rev 1 vaccine as this induces a long lasting (2 years or more) hypersensitivity.

## 4.7 FUTURE WORK

The current techniques of direct (bacteriological) and indirect (immunological) diagnosis of brucellosis generally give satisfactory results when they are undertaken with rigorous control of the environment, reagents and procedures. However, the emergence of new biotypes of *Brucella*, particularly in marine mammals highlights the limitations of the present methods of identification and typing of the strains which do not always allow clear distinction between the different types of *Brucella* responsible for brucellosis in animals and man. The development of a simple system of molecular typing of *Brucella* is therefore necessary for the direct diagnosis of brucellosis and techniques studying the polymorphism of the genome (RFLP, PCR-RFLP) look promising for the future. In the area of indirect diagnosis, the strong association between serological (RB and CFT) and allergen tests usually allows efficient identification of infected flocks. Immunoenzymatic (ELISA) tests have been developed for many years and are used successfully on a wide scale in certain countries for the diagnosis of bovine brucellosis. Their application to diagnosis of ovine brucellosis would allow improved sensitivity and specificity of identification in comparison to those from RB and CFT but further work is still required in this area particularly in relation to the standardisation of reagents.

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# Chapter 5

# CHLAMYDIOSIS

A. RODOLAKIS & A. SOURIAU

## 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)*

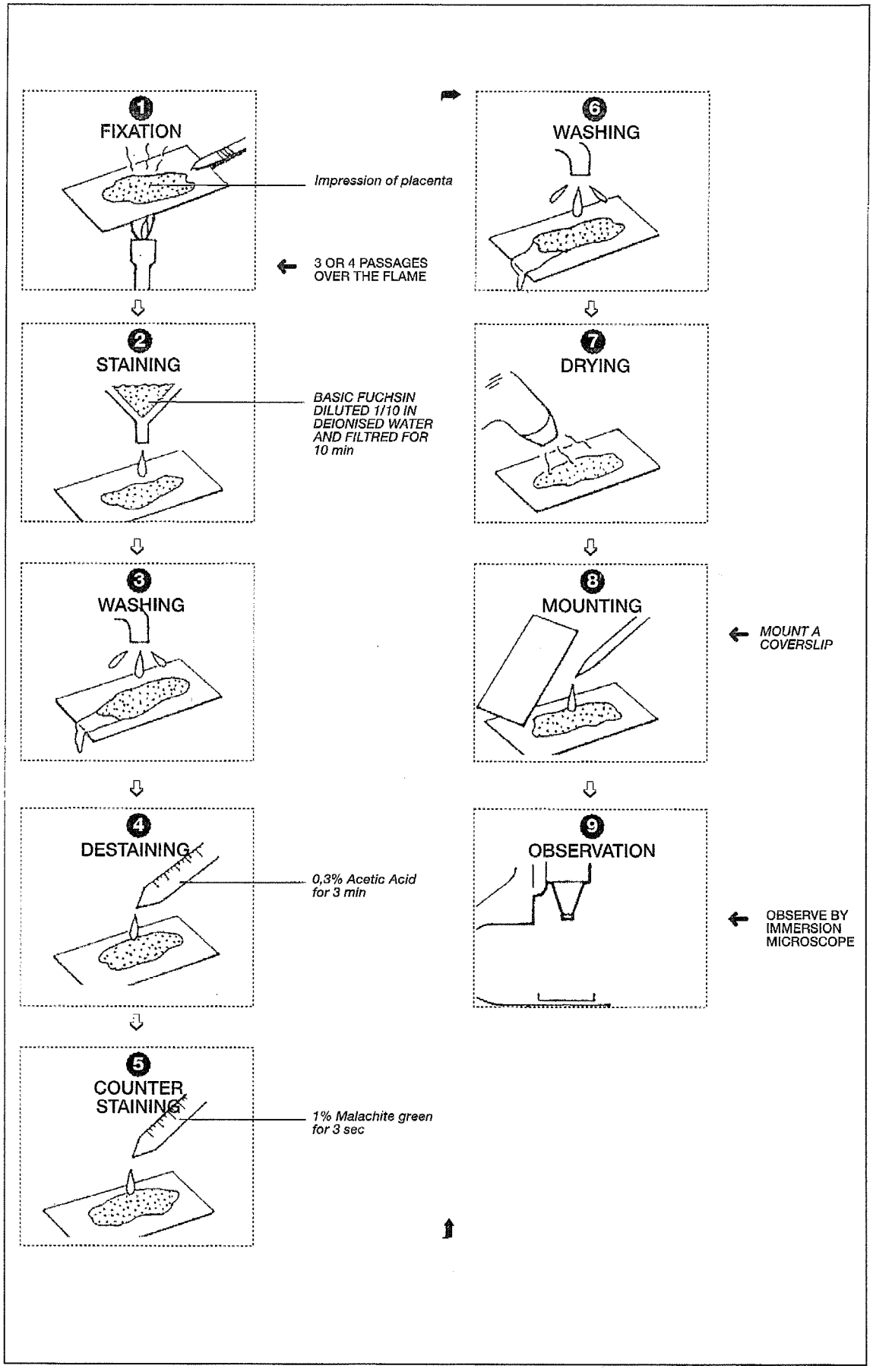
1. Fix the slide over a flame.
2. Leave for 10 minutes in a solution of basic Fuchsin freshly diluted 1 in 10 in deionised water and filtered.
3. Rinse with tap water.
4. De-stain in 0.3% acetic acid for 30 seconds.
5. Counterstain with malachite green solution for 30 seconds.
6. Rinse with tap water.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

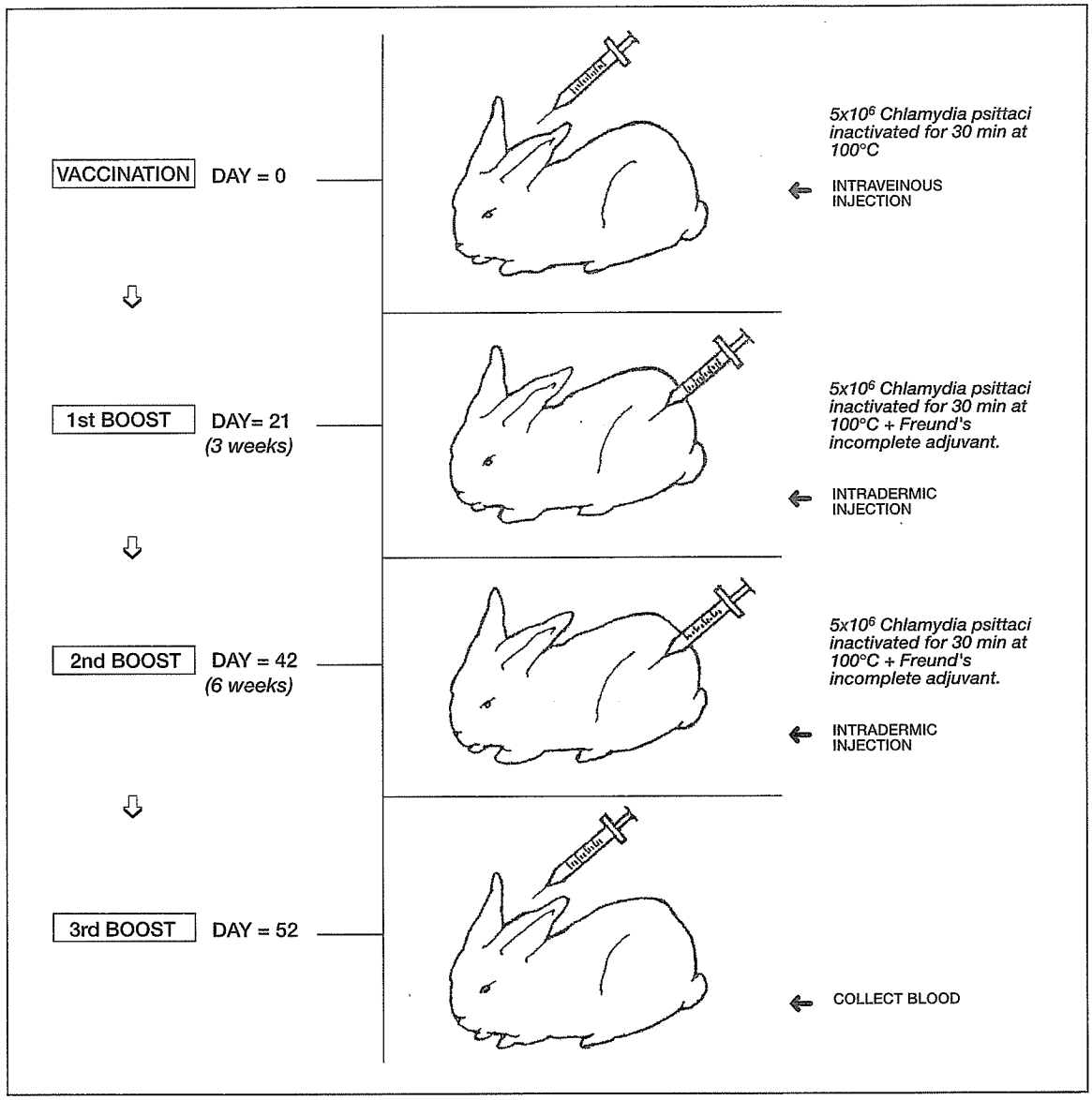
***Preparation of reagents***

- 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.

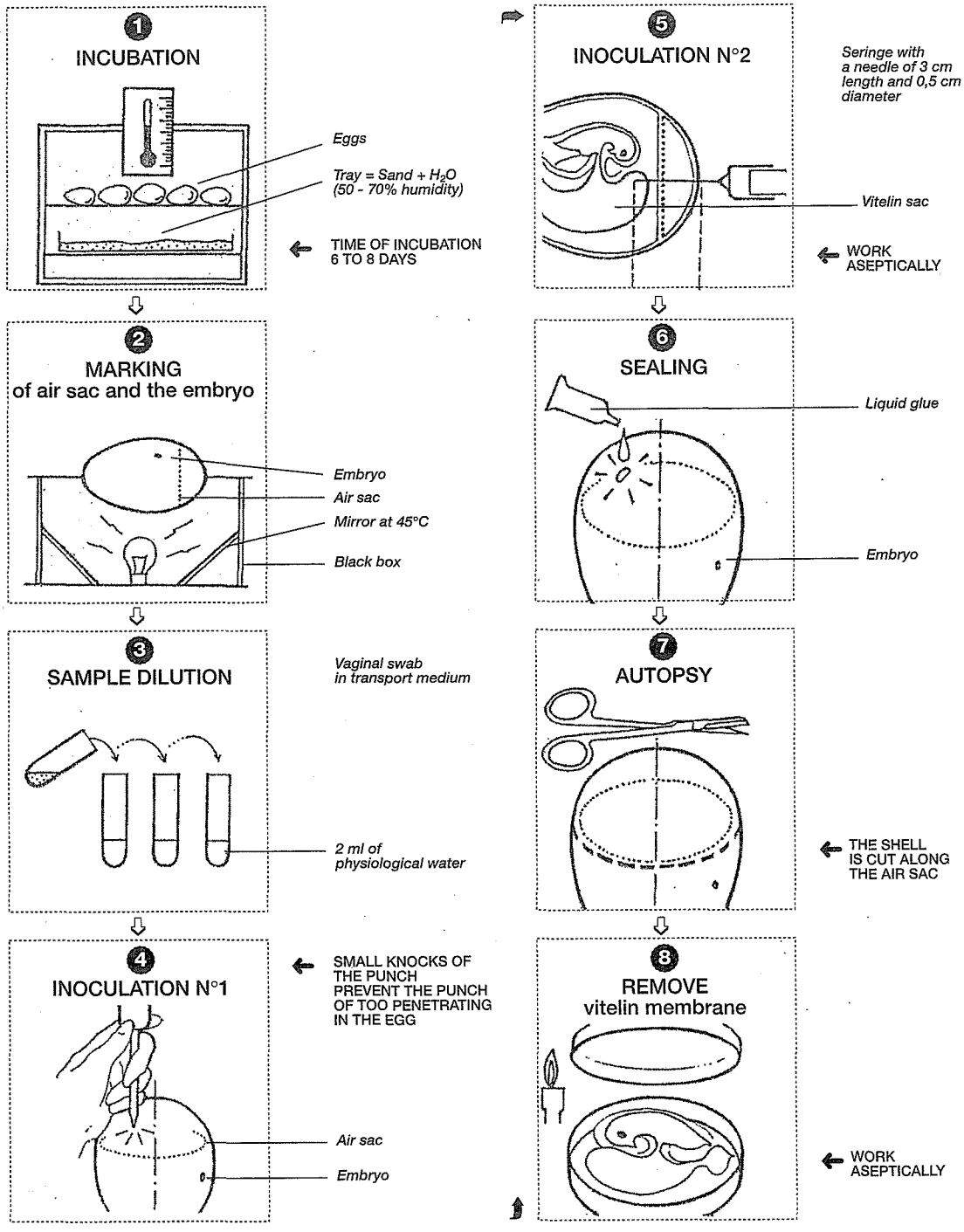
### *5.5.4.2 Cell culture method*

***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




*5.3 : Isolation of Chlamydia in embryonated hen's egg. Egg inoculation protocol*

- Acridine orange stain :
  - Acridine orange
  - Absolute alcohol
  - Glacial acetic acid
  - Citric acid
  - $Na_2HPO_4$.

***Procedure***

1. Culture the McCoy cells in monolayers in Medium 1.
2. Trypsinise once cells are confluent.
3. Suspend the cells in Medium 1 at a concentration of 3.5 x $10^5$ cells/ml.
4. Place 200µl aliquots of this suspension in 96 wells of a microtitre plate and incubate overnight at 37°C in a humid atmosphere containing 5% $CO_2$.
5. Remove the Medium 1 and replace with the vaginal swab samples diluted 1 in 2, 1 in 20 or 1 in 200 in PBS-DEAE (two wells per dilution).
6. Incubate the plates for 1 hour at 37°C.
7. Remove the inoculum and replace with 200µl of Medium 2.
8. Incubate the plates for 30-48 hours at 37°C in a humid atmosphere containing 5% CO2.
9. Fix and stain the plates by:

• May-Grunwald Giemsa (Figure 5.5, page 94)

• or acridine orange:

1. Fix the cultures without drying for 10 minutes in a 2:1 (v/v) mixture of ethanol:acetic acid. The fixed cultures can be stored if necessary in 80% alcohol.
2. Wash the cultures twice for 2 minutes each wash in McIlvain buffer.
3. Stain for 4 minutes with 0.01% acridine orange.
4. Wash the cultures twice for 2 minutes each wash in McIlvain buffer as before.

***Reading of results***

Examine the slides by microscope in McIlvain buffer by UV light. Chlamydial inclusion bodies, rich in DNA, appear orange at the side of the cell nucleus which is stained green (Figure 5.6, page 94).

• Either by immunofluoresence as described in 5.5.2.1 except the cultures are not air-dried but are fixed in ethanol for 10 minutes before reaction with antibodies. The *Chlamydia* than appear as yellow- green cytoplasmic inclusion bodies (Figure 5.7, page 94).

• Or by ELISA according to the same immunofluorescence method but using a conjugate coupled to alkaline phosphatase and a mixed 5-bromo-4-chloro-3-indoyl phosphate (BCIP) and nitro blue tetrazolium (NBT) substrate (Figure 5.8, page 95).

***Note***

• Centrifugation of the samples on the cells at 1400 xg for one hour helps chlamydial penetration of the cells and therefore increases the number of visible cytoplasmic inclusion bodies.

• Addition of 1µg/ml of cyclohexamide to Medium 2 blocks the division of eukaryotic cells and helps the reading of the results: the cells and chlamydial inclusion bodies appear larger (Figure 5.7, page 94).

***Preparation of reagents***

Cell Maintenance Medium (Medium 1) : Eagles Minimal Essential Medium (MEM) supplemented with 1% (v/v) of vitamins (100x concentrate vitamins for Eagles MEM), 1% (v/v) 200mM glutamine, 2g/l $NaHCO_3$ and 5% foetal calf-serum.

Cell Growth Medium (Medium 2): Same composition as Medium 1 but containing 10% foetal calf serum and antibiotics : 20µg/ml gentamycin, 25µg/ml vancomycin and 5µg/ml amphotericin B.

Fixative for Acridine Orange Stain :
Prepare immediately before use :
absolute alcohol 2 volumes
glacial acetic acid 1 volume
Acridine Orange :
Stock solution: 1% in distilled water. This can be kept for several months at 4°C in the dark. The solution should be discarded if heavy precipitation occurs.
Working solution: dilute the stock solution to 1% with McIlvain buffer.
McIlvain buffer.
Solution 1 : 0.1M citric acid
Solution 2 : 0.2M $Na_2HPO_4$
Mix 12.9ml of solution 1 with 7.1ml of solution 2 to obtain 20ml of buffer, pH 3.8.

#### *5.5.4.3 General notes about the isolation of Chlamydia*

Certain strains of *Chlamydia* are sensitive to penicillin and it should therefore not be used. Gentamycin is more expensive than streptomycin but it remains active for longer.

### 5.5.5 Comparison of the different direct diagnostic techniques

The different methods of direct diagnosis are compared in a large number of publications concerned with the diagnosis of human infections with *C.trachomatis* and in a much smaller number of reports concerned with the diagnosis of *C.psittaci* infections in ruminants. However, in all accounts PCR and LCR have been found to be the most sensitive methods allowing detection of the greatest numbers of infected individuals. On the other hand, differences in sensitivity have been noted in both human and veterinary medicine when comparing the isolation of chlamydia in cell culture with immunofluorescent detection or ELISA: isolation in cell culture is more sensitive than the other techniques in human medicine but less sensitive in veterinary medicine. This is probably due to the fragility of the *Chlamydia* and differences in the sampling conditions. In human medicine the patients go directly to the laboratory for sampling or samples are sent very quickly to the analytical laboratory under the best conditions which maximises the survival chances of the *Chlamydia.*

## 5.6 INDIRECT DIAGNOSIS

The diagnosis of abortive chlamydiosis is usually undertaken by detecting chlamydial antibodies in the blood of the animal.

The complement fixation reaction is usually used. It is an easy method to perform but does present some drawbacks :

The antigen used may also detect antibodies raised against intestinal *Chlamydia* present in most ruminants. This has resulted in standardisation of the method and fixing of a threshold value (1/80) to separate animals considered as positives.

Even taking this threshold into account, the complement fixation reaction cannot be used for individual diagnosis and it does not allow detection of infection in the young or in males. It should preferably be carried out 3 weeks to one month after abortion or time of low production (time of maximum antibody response).

Other techniques (ELISA, immunofluorescence) can be used. They present the same drawbacks as complement fixation as they use the same type of antigen although problems with anti-complementary sera do not occur. They are more expensive methods and require specific pieces of equipment.

### 5.6.1 Complement fixation

Breakdown in the supply of commercial complement fixation antigens for the diagnosis of abortigenic *Chlamydia* frequently occurs. Antigens sold for the diagnosis of avian

*Chlamydia* can be used but it is also possible to prepare a stock antigen by the following method :

### *5.6.1.1 Antigen preparation (Figure 5.9)*

Antigen can be prepared from infected yolk sac membranes, placental material or cell cultures.

***Principle***

The antigen used is a lipopolysaccharide antigen extracted from bacterial bodies using ether.

***Materials and reagents***

- Diethyl ether.
- Sterile physiological saline.

***Procedure***

1. Place the preparations of cell culture derived *Chlamydia* or ground samples of yolk sac membrane or placental material in a boiling water bath for 30 minutes.
2. Allow to cool and add 10 volumes of cold diethyl ether.
3. Mix vigorously, then leave overnight at 4°C with gentle shaking with the flask positioned to avoid any evaporation of the ether.
4. The cellular debris will settle in the bottom of the flask. The ether will become clear yellow.
5. Remove the ether with a pipette and evaporate in a water bath at 37°C or under vacuum.
6. Resuspend the residue in a small volume of physiological saline.
7. Determine the antigen titre by the complement fixation reaction method, using a positive control serum at a constant dilution and various dilutions of the antigen. One unit of antigen is the highest dilution of antigen which gives 100% complement fixation.

***Note***

• The volume of physiological saline in the initial chlamydial suspension must be sufficient otherwise coagulation may occur during boiling at 100°C.

• The temperature during extraction in ether can be higher than 4°C but if the temperature rises too high evaporation may extend beyond the ether phase and reduce the yield.

### *5.6.1.2 Complement fixation reaction [9]*

Antibody titre is determined according to the cold incubation micromethod of Kolmer, following the technique described in the chapter on Brucellosis with the following differences:

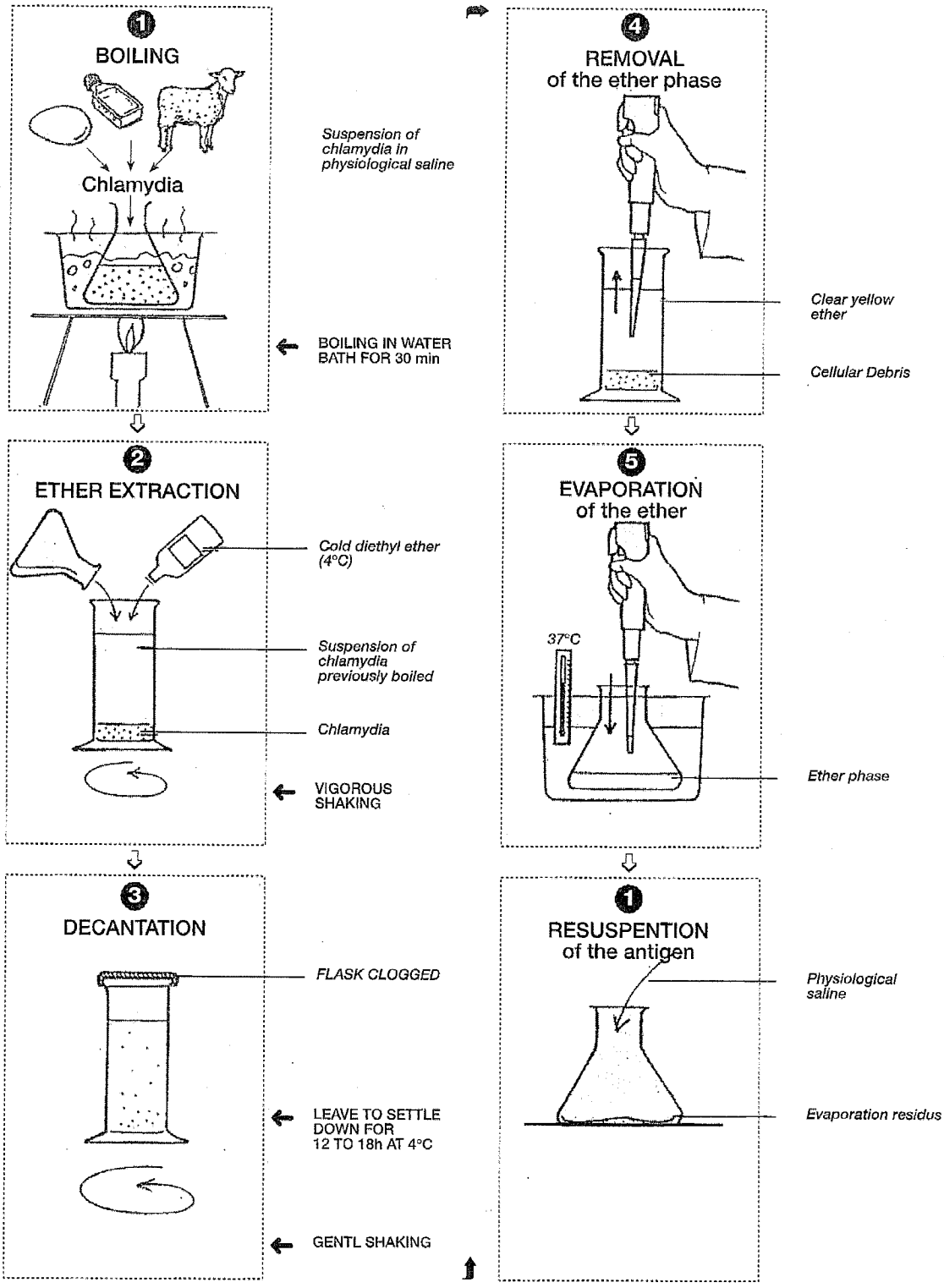
- Dilute test sera 1/5 in veronal buffer and decomplement in tubes at 58.5°C for 30 minutes.
- Decomplement positive and negative control sera in the same way.
- Chlamydial antigen contains 4 units.
- Guinea-pig complement is diluted in veronal buffer to contain 2 units at 100% haemolysis.

***Procedure***

1. Add 50µl of the decomplemented serum to 50µl of veronal buffer in the first row of wells on the microtitre plate then make doubling dilutions in successive rows. Dilutions will range from 1 in 10 to 1 in 640 over seven rows of wells.
2. Add 25µl of antigen and 25µl of complement.
3. Seal the plates, agitate and incubate overnight at 4°C.
4. Add 25µl of sensitised red blood cells (RBC) and agitate the plates. Read after 30 minutes incubation at 37°C.

***Note***

• To avoid anticomplementary activity




*5.9 : Complement fixation. Antigen preparation protocol*

the blood cells must be separated from the sera as quickly as possible.

• The following standards should be included in each batch of tests :

- for each serum, an anticomplement standard prepared by a 1 in 10 dilution of the serum sample plus the complement but without the antigen, in the 8th well of the plate ;
- a positive serum ;
- a negative serum ;
- an antigen standard without addition of serum ;
- a complement standard without addition of serum or antigen ;
- a RBC standard without addition of RBC in veronal buffer.

Replace any reagents omitted from the wells with the same volume of veronal buffer.

The results are highly reproducible when the same source of RBC is used. It is best to use cell samples from the same sheep between 3 and 10 days after sampling.

• Decomplementation of the serum:

- at 60°C decreases the sensitivity of the reaction;
- at 56°C increases the number of anticomplementary sera.

## 5.6.2 ELISA

***Principle***

Chlamydial antibodies are detected in test sera by means of sheep or goat immunoglobulin conjugates labelled with alkaline phosphatase or peroxide, which react with the antibodies when they are bound to the chlamydial antigens which coat the bottom of the wells of the ELISA plates.

Kits are available for the diagnosis of human chlamydial infections. Although the antigens can be used to detect antibodies raised against other types of antigens, the human immunoglobulin conjugates do not react with ovine and caprine immunoglobulins. Until the commercialisation of specific kits, the following protocol can be used :

### *5.6.2.1 Antigen preparation*

Semi-purified elementary bodies or antigen for complement fixation can be used but the best results are obtained using lysed semi-purified *Chlamydia.*

***Materials and reagents***

- Centrifuge suitable for Eppendorf tubes.
- Tris KCl buffer, pH 7.5.
- Renografine (Radioselectan, Schering, France).
- Extraction buffer, pH 8.8.
- Triton X100.

***Procedure***

1. Freeze and thaw *Chlamydia* (approximately 5 x $10^8$ pfu/ml) recovered from cell culture or yolk sac membrane preparations three times at -70°C, then sonicate for 15 second (optional) to free the maximum number of EB's.
2. Centrifuge the suspension for 10 minutes at 300 xg to precipitate the cellular debris.
3. Prepare cushions of 1.2ml 40% Renografine in Tris KCl in Eppendorf tubes.
4. Place a 200µl volume of sample on the surface of the cushion of Renografine in each Eppendorf tube.
5. Centrifuge the tubes for 2 hours at 17600 xg at 4°C.
6. The precipitated pellet containing the semi-purified *Chlamydia* can be stored at -20°C or processed to obtain lysed *Chlamydia.*
7. In the latter case, resuspend the pellet in each tube in 200µl of extraction buffer. Homogenise then sonicate to help

resuspension of the plug.

8. Incubate the tubes for 2 hours at 37°C to ensure good solubilisation of the proteins.

9. Add Triton X100 to a final concentration of 1% (v/v).

10. Incubate the suspension for 15 minutes at 37°C to stop the action of the SDS.

11. Determine the antigen titre in an ELISA using a positive serum.

***Preparation of reagents***

- Tris KCl Buffer, pH 7.5

| | |
|---|---|
| Tris hydroxymethyl aminomethane | 2.42g |
| KCl | 11.2g |
| Deionised water | 1000ml |

Adjust to pH 7.5 with 1M HCl.

- Extraction Buffer, pH 8.8

| | |
|---|---|
| NaCl | 0.2mM |
| Tris-HCl | 0.1M |
| EDTA | 4 mM |
| SDS | 0.2% (v/v) |
| β-mercaptoethanol | 0.5% (v/v) |

### *5.6.2.2 ELISA test*

***Materials and reagents***

- ELISA plate reader.
- Microtitre plates.
- 0.1M bicarbonate buffer, pH 9.6.
- TBS buffer, pH 7.5.
- Tween 20.
- NaOH.
- Skimmed milk powder.
- Ovine or caprine immunoglobulin conjugate labelled with alkaline phosphatase.
- ρ-Nitrophenyl phosphate disodium substrate (Sigma Chemical Co, USA) diluted to 1mg/ml in diethanolamine buffer pH 10.2.

***Procedure***

1. Sensitise the plates overnight at 4°C with 100µl of the optimal antigen dilution (determined at the time of the titration) in bicarbonate buffer.

2. Remove the excess liquid by vigorously shaking the plates.

3. Wash the plates 4 times in demineralised water and dry.

4. Add 150µl of TBS containing 2% skimmed milk to each well to saturate remaining free sites and incubate for 30 minutes at 37°C with shaking.

5. Remove the excess liquid as before.

6. Rinse the plates 4 times with deionised water and wash 3 times for 5 minutes each wash with TBS containing 0.05% Tween 20 (v/v).

7. Add 100µl of the test serum dilutions (in TBS/2% milk) to each well. Add a standard negative serum and a standard positive serum to each set of tests.

8. Incubate for 90 minutes at 37°C with shaking.

9. Remove the excess liquid and wash the plates as in (6).

10. Add 100µl of the conjugate diluted in TBS/2% milk to each well and incubate for 90 minutes at 37°C with shaking.

11. Remove the excess liquid and wash the plates as in (6).

12. Add the substrate and incubate for 90 minutes at 37°C or until an OD of <0.2 is obtained in the negative standard.

13. Stop the reaction by addition of 1N NaOH.

***Reading of results***

Read the plates at 405nm. A sample is considered to be positive if the OD is >0.4.

***Preparation of reagents***

- TBS Buffer, pH 7.5

| | |
|---|---|
| Tris hydroxymethyl aminomethane | 2.42 g |
| NaCl | 29.24 g |
| Demineralised water | 1000 ml |

Adjust to pH 7.5 with 1M HCl.

### 5.6.3 Immunofluorescence

*Principle*

Chlamydial antibodies are detected in test sera by means of ovine or caprine immunoglobulin conjugates labelled with fluorescein isothiocyanate, which react with the antibodies that are bound to the chlamydial antigens which cover the wells of teflon coated slides.

For the same reason as before, kits for the diagnosis of human infections cannot be used but slides coated with ten samples of *Chlamydia psittaci* are commercially available (*Chlamydia* spot, IF bioMerieux, France) or can be prepared in the following way:

*Materials and reagents*

- Fluorescent microscope.
- 10 well teflon coated slides without Chlamydia coating (Figure 5.10, page 95).
- Ovine or caprine immunoglobulin fluorescent conjugate.
- PBS.

*Antigen preparation*

1. Place *Chlamydia* cultured in eggs in the wells of the teflon coated slide. Dry for 30 minutes at 37°C and fix in acetone for 20 minutes at room temperature.
2. Dry the slides again.
3. Store at -20°C until required for use.

*Procedure*

1. Thaw the slides.
2. Dilute the test sera by 10-fold dilutions with PBS together with the standard positive sample.
3. Place the sera and the standard negative dilutions in the wells and incubate for 30 minutes at 37°C in a humid atmosphere.
4. Rinse the slides twice in deionised water and twice for 10 minutes in PBS.
5. Add the conjugate and incubate for 30 minutes at 37°C in a humid atmosphere.
6. Wash the slides as previously, mount and examine.

*Note*

- *Chlamydia* cultured in cells detach more easily from the slides than chlamydia cultured in eggs.
- The ease of interpretation of the slide results is directly related to the concentration of *Chlamydia* in the samples.
- Teflon coated slides with 3 x 8 wells (L24C5T # XES 230, CML France Nemours) allow the use of multichannel pipettes.

### 5.6.4 Delayed hypersensitivity [9]

A delayed hypersensitivity skin test can be undertaken in goats by intradermal injection of purified *Chlamydia* cultured in eggs or in cells. The reaction is easily visible 72 hours after injection. Although this test gives fewer questionable results than complement fixation it cannot be used as an individual diagnosis. In sheep the test can be undertaken on the lower eyelid but is a lot more difficult to assess (see Figure 4.13 in the chapter on Brucellosis).

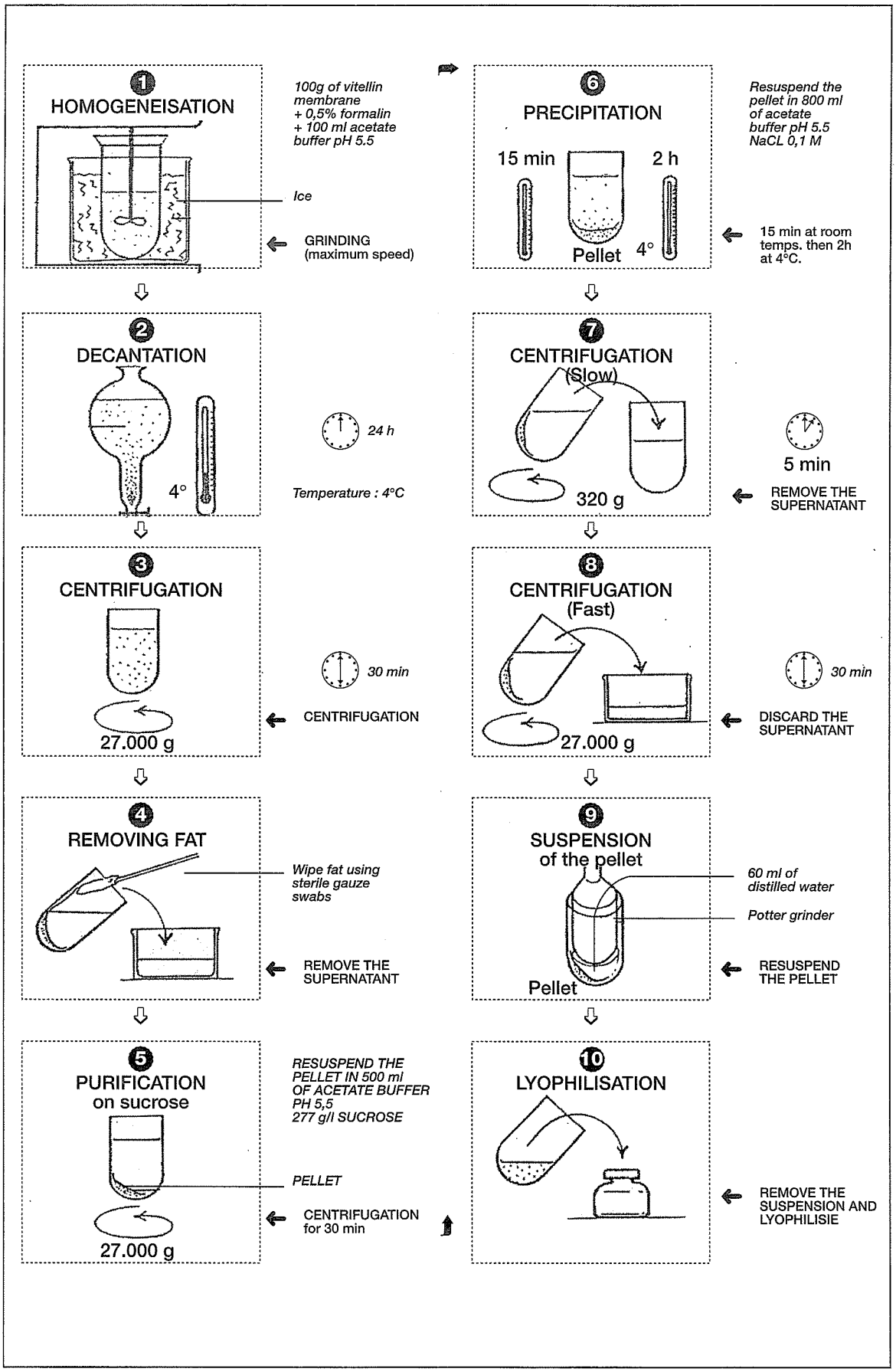
*Principle*

Delayed hypersensitivity detects a cell mediated response in the animal after intradermal injection of purified antigen.

#### *5.6.4.1 Antigen preparation*

*Materials and reagents*

- Grinder (Omni-Mixer, Sorvall).
- Potter grinder.
- Lyophiliser.
- Acetate buffer, pH 5.5.
- NaCl.
- Sucrose.




*5.11 : Delayed hypersensitivity reaction*
*Antigen preparation protocol*

*Procedure (Figure 5.11)*

1. Grind 100g of infected yolk sac membrane in 0.5% formalin and 100ml of acetate buffer, pH 5.5 1M NaCl, in 250ml pots cooled in ice with the grinder (Omni-Mixer, Sorvall) at maximum speed.

2. Reduce the volume of the suspension to 100ml and allow to settle for 24 hours at 4°C.

3. Remove the gross debris and centrifuge the suspension at 27000 xg for 30 minutes.

4. Remove the supernatant. Wipe any fat from the walls of the centrifuge tubes using sterile gauze swabs and resuspend the pellet in 500ml of acetate buffer, 2M NaCl, 277g/l sucrose.

5. Centrifuge the suspension at 27000 xg for 30 minutes.

6. Resuspend the pellet in 800ml of acetate buffer, pH 5.5, 0.1M NaCl. Allow to stand for 15 minutes at room temperature to initiate precipitation, then leave for 2 hours at 4°C.

7. Centrifuge the suspension at 320 xg for 30 minutes.

8. Remove the supernatant and centrifuge at 27000 xg for 30 minutes.

9. Resuspend the pellet in 60ml of distilled water.

10. Homogenise the suspension using the Potter grinder and centrifuge at 320 xg for 5 minutes in conical bottomed tubes.

11. Remove the supernatant with a pipette and lyophilise.

12. Check the antigen by injecting 100µg of the allergen dissolved in pyrogen-free physiological saline intradermally into a guinea-pig at a previously shaven site. Read the result after about 72 hours (Figure 5.12, page 95).

*Note*

• Antigen can also be prepared using *Chlamydia* cultured in cells [9].

### *5.6.4.2 Test method*

*Procedure*

1. Inoculate 100µl of allergen dissolved in 0.1ml pyrogen-free physiological saline intradermally in the neck of goats or the lower eyelid of sheep.

2. Examine the reactions 72 hours after injection and assess their intensity visually and by palpation in comparison to the other eyelid of the sheep or the other side of the neck of the goat.

*Note*

• When the injection is given in the neck the thickness of the skin can be measured with callipers.

• The period of 72 hours from injection to assessment appears to be necessary to detect maximum positive responses. Weak reactions are never visible before 48 hours and can be non-detectable by 96 hours.

## 5.7 TRENDS AND FUTURE WORK

Diagnosis of abortigenic chlamydia is still usually undertaken using either the complement fixation reaction alone or in association with bacteriological examination of part of the placenta. These two techniques lack specificity and neither permit individual diagnosis, nor do they predict animals at risk from abortion.

Improved diagnostic specificity and sensitivity may be obtained by :

• Replacement of bacteriological assessment by detection of *Chlamydia* by immunofluorescence or better by PCR with specific emphasis on abortive strains.

• Use of an antigen specific to abortive strains of *C.psittaci* for serological diagnosis [13].

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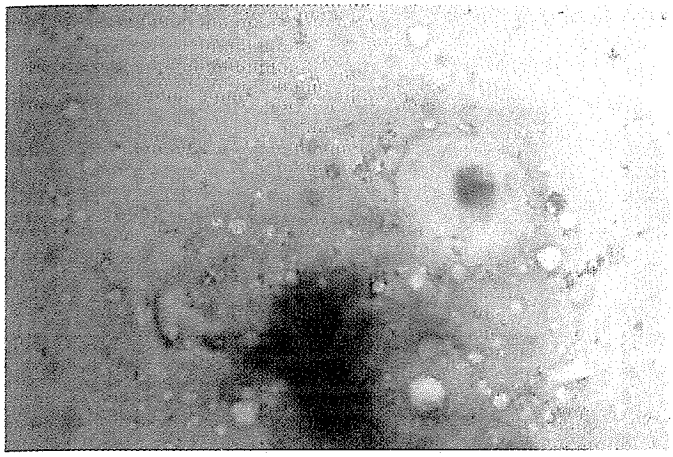

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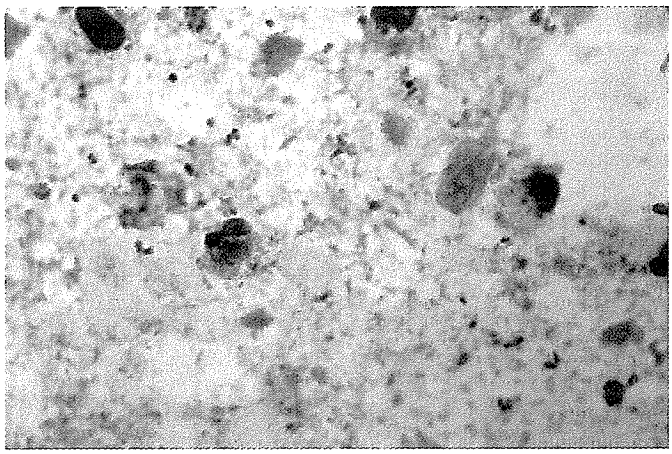

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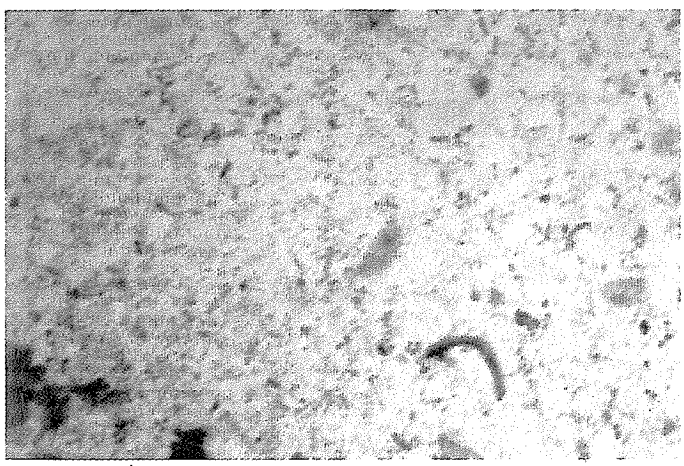

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**Figure 3.3 :**
Bacteriological examination by stamp stain of
(a) The stomach content affected by Brucellosis
(b) an impression of a cotyledon infected with *Chlamydia psittaci*
(c) or by *Coxiella burnetti*

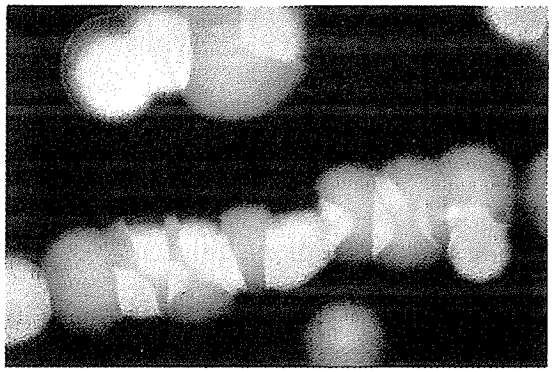


**Figure 4.2 :**
Smooth (blue) and rough (yellow) morphological types of *Brucella* colonies

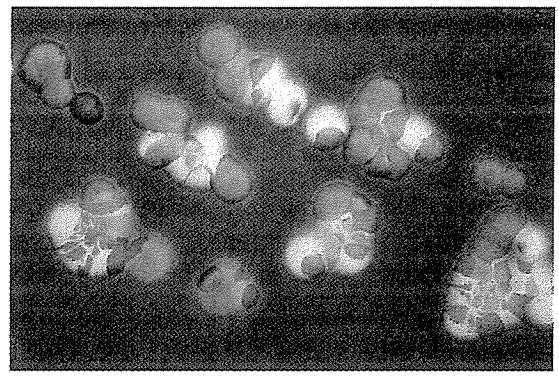
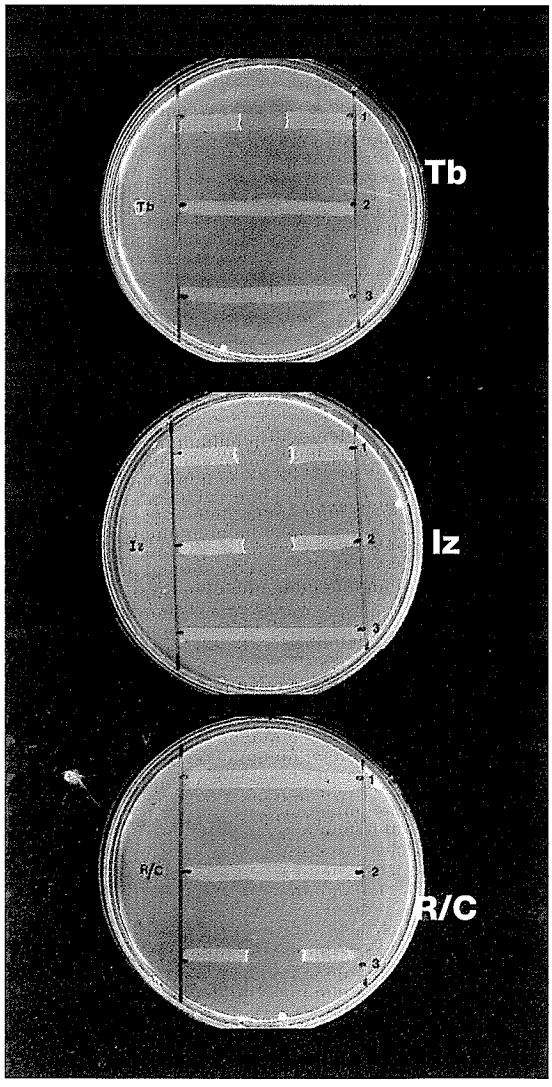


**Figure 4.3 :**
*Brucella* colonies stained by cristal violet



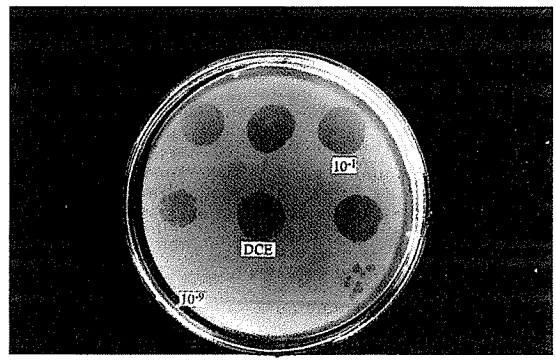




**Figure 4.4 :**
Determination of SWC in the example presented, the SWC corresponds to the $10^{-5}$ dilution

**Figure 4.5 :**
Lysotyping of *Brucella* by brucellophages Tb, Iz and R/C.
1. *B. abortus 554.*
2. *B. melitensis 16 M.*
3. *B. suis 63/290.*

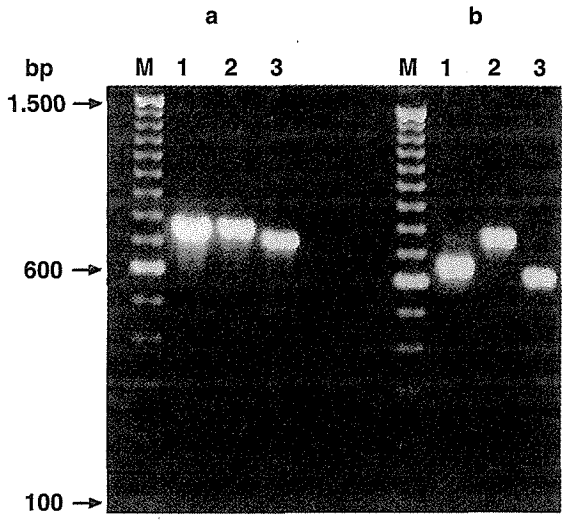



**Figure 4.7 :**
Migration profiles of the amplification products of the gene coding the 25KDa OMP of *B.abortus* 554(1), *B.melitensis* 16M (2), and *B.ovis* 63/290 (3), uncut (a) and cut by EcoRV(b). M: Molecular marker (100bp DNA ladder, Gibco). 1.5% agarose gel.

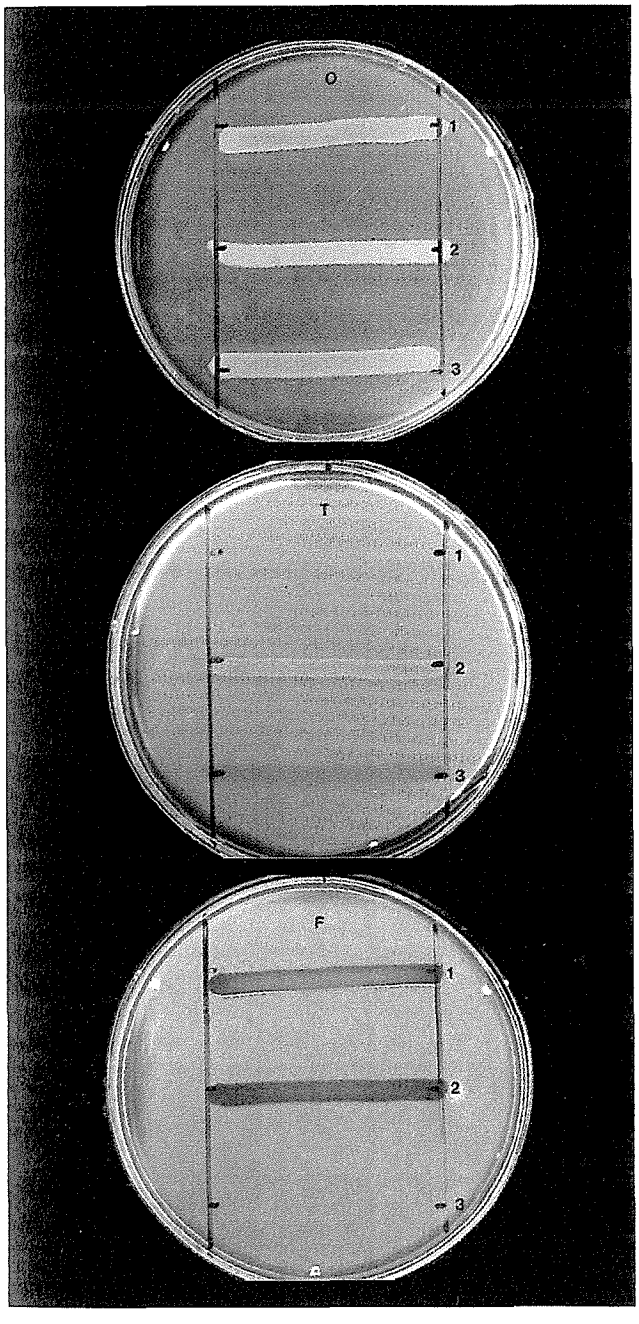



**Figure 4.8 :**
Growth of *B. abortus* 554 (1), *B. melitensis* 16 M (2) and *B. ovis* 63/290 (3) in the presence of thionin (T) and basic fuchsin (F) at a concentration of 20 μg/ml of base medium.

0 = standard without stain.

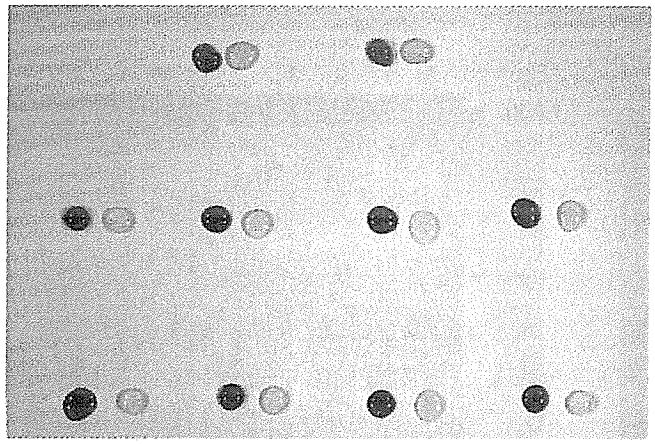


**Figure 4.9 :**
Antigenic buffer test (Rose Bengal) : place spots of antigen and serum on the tile.

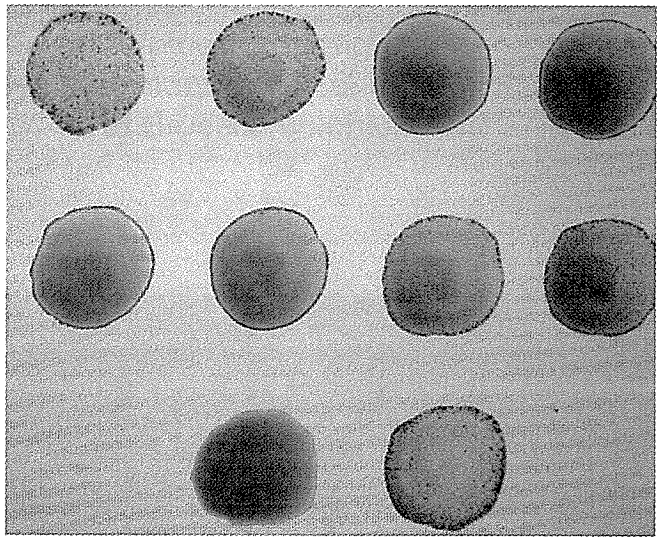


**Figure 4.10 :**
Antigenic buffer test (Rose Bengal) : examples of positive and negative reactions.

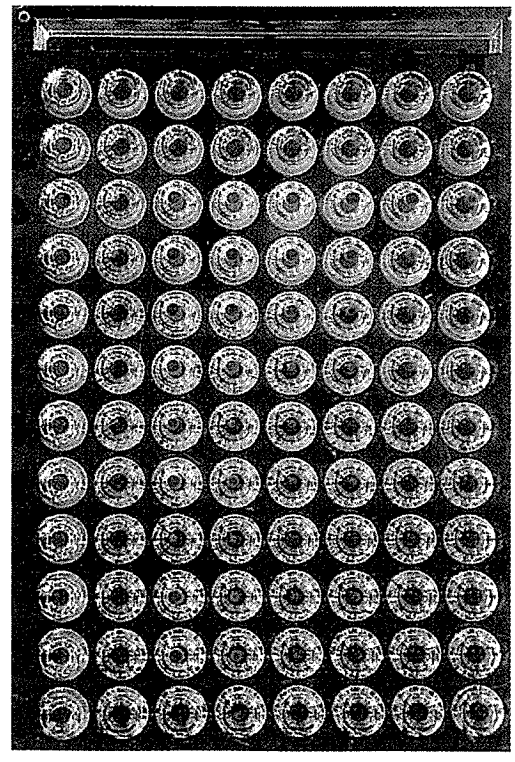


**Figure 4.12 :**
Complement fixation test : examples of positive and negative reactions.

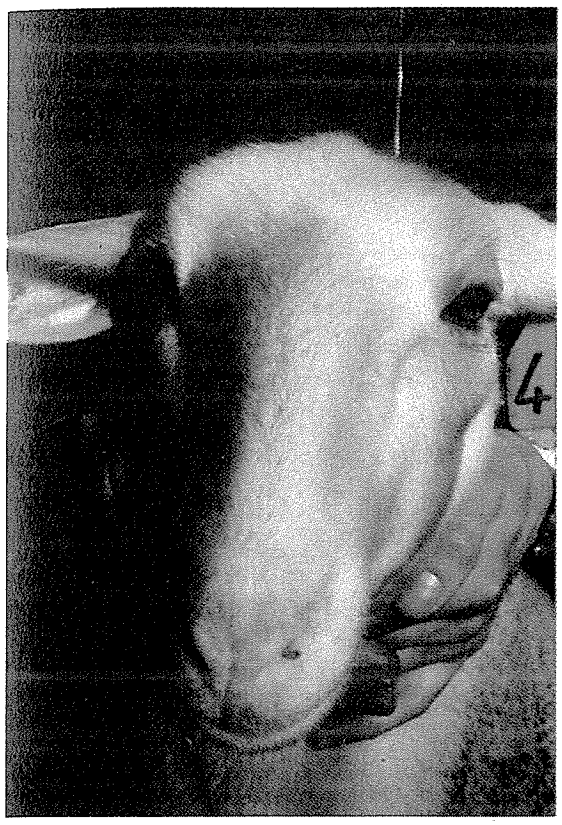
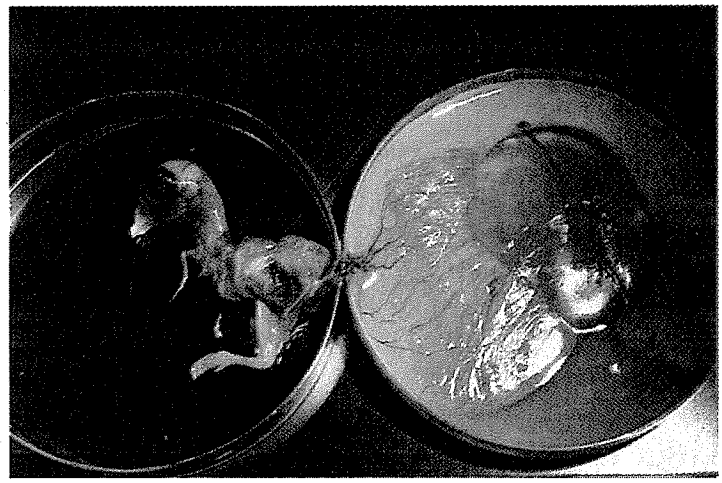


**Figure 4.13 :**
Appearance of an allergic reaction in sheep, 48 hours after inoculation of the brucelline into the lower eyelid.



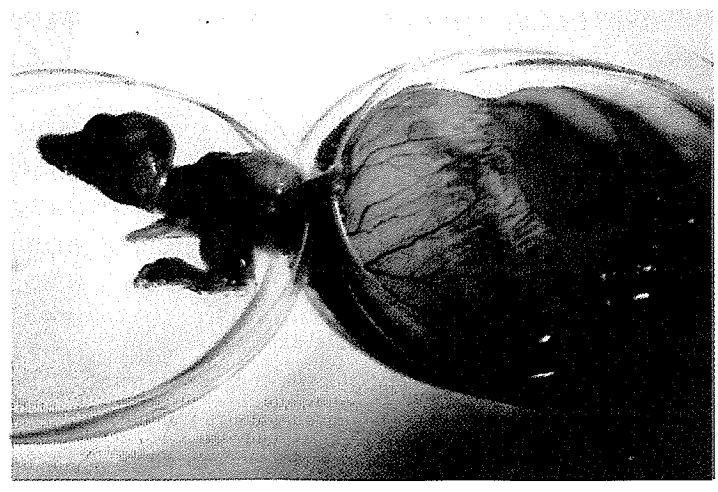


**Figure 5.4:**
Isolation of *Chlamydia* in embryonated hen's eggs. Appearance of embryos at autopsy : (A) normal embryo, (B) embryo that died as a result of chlamydial infection. The feet and claws of the embryo are cyanotic, the yolk sac membrane is congested.

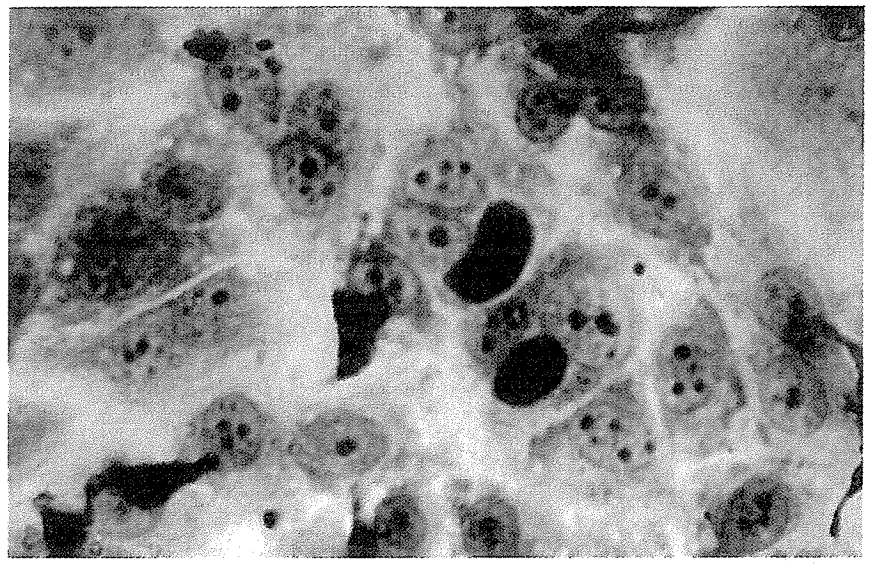


**Figure 5.5 :**
Isolation of *Chlamydia* in cell culture. Layer of cells stained by May-Grunwald Giemsa.

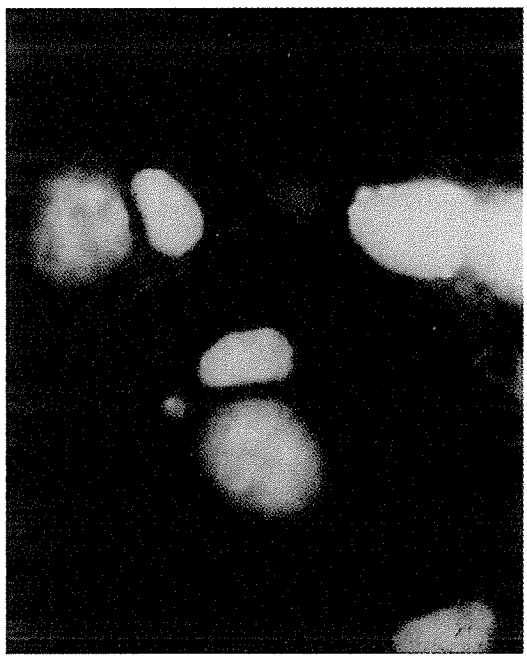


**Figure 5.6 :**
Isolation of *Chlamydia* in cell culture. Layer of cells stained by Acridine orange 48 hours after infection with *C.psittaci*. Inclusion bodies containing *Chlamydia* appear red and the nuclei of the cells green. If left for 72 hours after infection, the inclusion bodies containing *Chlamydia* turn green and are difficult to distinguish from the cell nuclei.

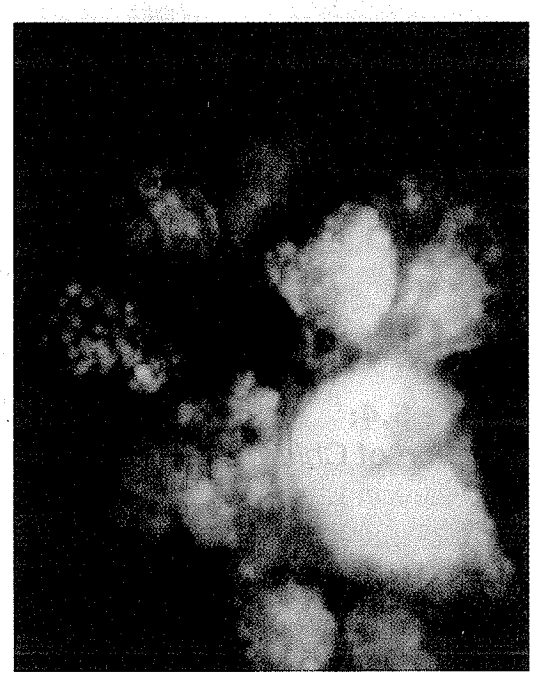


**Figure 5.7 :**
Isolation of *Chlamydia* in cell culture. Acridine orange stain of a culture of McCoy cells treated with 1 μg/ml cyclohexamide 72 hours after infection with *C.psittaci.* Large yellow inclusion bodies can be observed. Some orange inclusion bodies are also visible but the earlier the cells are stained after infection (minimum of 24 hours otherwise the inclusion are too small), the more orange colouration is formed.

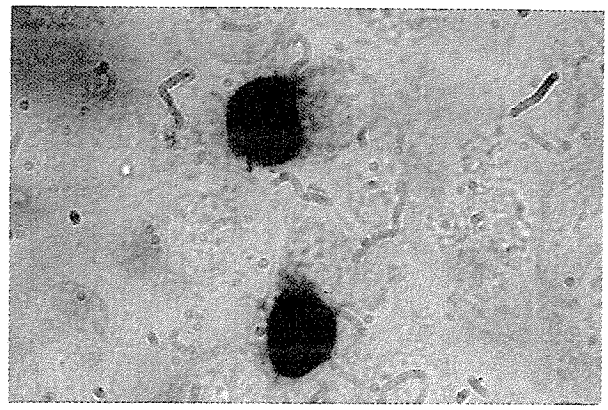


**Figure 5.8 :**
Isolation of *Chlamydia* in cell culture. Layer of cells stained by ELISA-immunophosphatase alkaline-BCIP-NBT, taken from cell culture 48 hours after infection with *C. psittaci*. Inclusion bodies are stained black. This staining method has the advantage of being specific, not requiring a fluorescent microscope and will keep better than immunofluorescence.

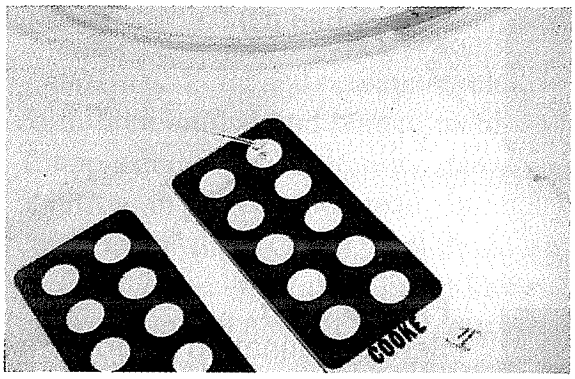


**Figure 5.10 :**
Immunofluorescence. Teflon coated slides suitable for coating with chlamydial antigen.

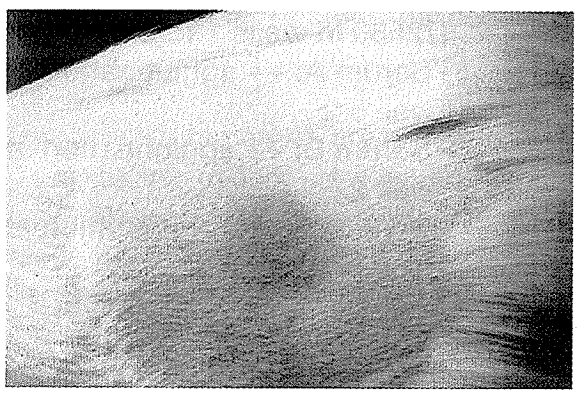


**Figure 5.12 :**
Delayed hypersensitivity reaction. Appearance of a positive reaction (A) on a guinea-pig, the reaction has similar appearance as that on the neck of a goat (B).

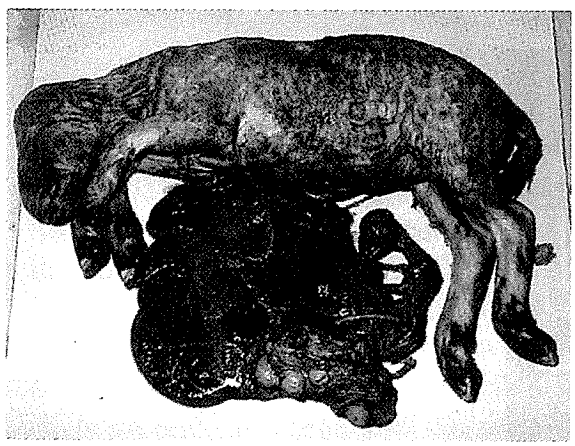


**Figure 6.1 :** Placenta from a case of *Toxoplasma* abortion in a ewe. (Note the characteristic white spots on the cotyledons denoting foci of necrosis and the normal appearance of the intercotyledonary membrane.)

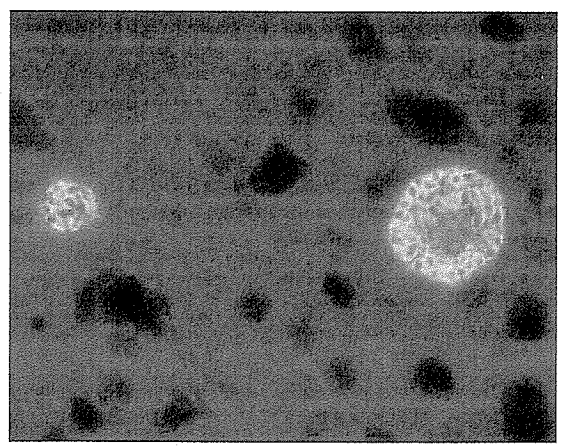


**Figure 6.2 :**
*Toxoplasma gondii* tissue cysts filled with bradyzoites, in the brain of a mouse.

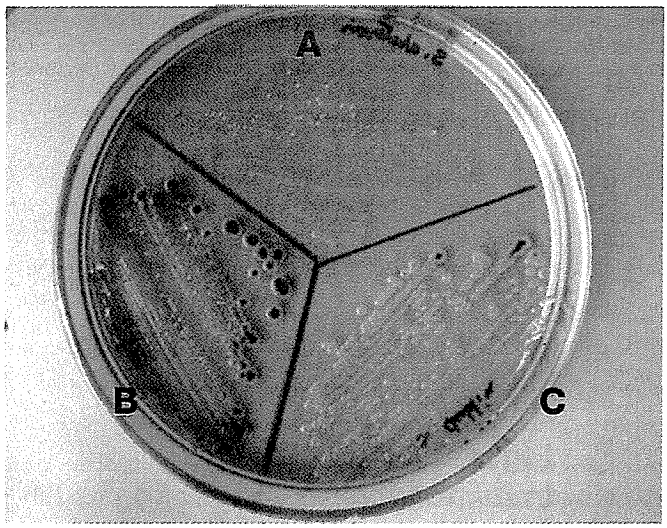



**Figure 9.2 :**
Cultures of different *Salmonella* serotypes:
- *S. abortus ovis (A)*
- *S. typhimurium (B)*
- *S. dublin (C)*

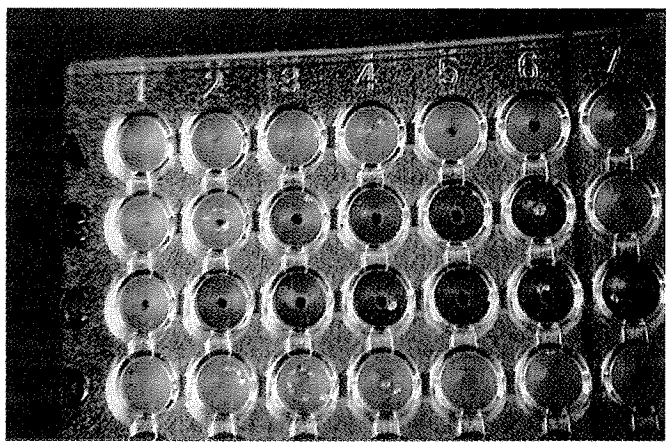



**Figure 9.4 :**
Seroagglutination microtechnique. Sera are diluted between 1/80 and 1/2560 in wells 1 to 6.
- Serum A: ++ agglutination at 1/1280 well 5
- Serum B: ++ agglutination at 1/160, well 2
- Serum C: ++ agglutination at 1/80, well 1
- Serum A: agglutination at > 1/2560, well 6

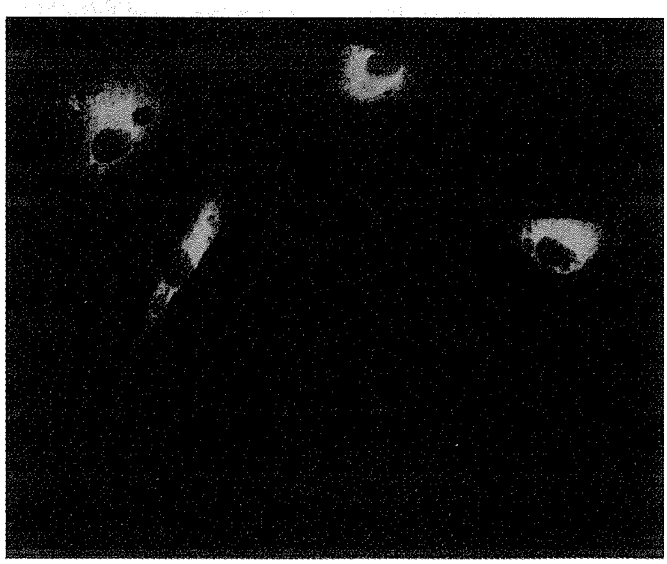


**Figure 13.1 :**
Cells infected with Border disease virus and stained by IFT.

# Chapter 6

# TOXOPLASMOSIS

D. BUXTON & J.BREBNER

## 6.1 SUMMARY

Toxoplasmosis, a zoonotic infection caused by the protozoan parasite, *Toxoplasma gondii*, has social and economic relevance to both animal and human medicine. Toxoplasmosis is a serious cause of abortion in sheep and goats in many countries of the world. Direct diagnosis of *Toxoplasma* infection is limited to isolation by inoculation of infected tissues into laboratory mice and identification of the organism in sections of placental cotyledons and/or brain of aborted foetuses. However, due to the irregular and sparse distribution of organisms in tissues the use of indirect diagnostic tools such as gross pathology, histopathology and serology are essential in the demonstration of infection.

## 6.2 INTRODUCTION

*Toxoplasma gondii* is a cyst-forming coccidian parasite of domestic ruminants world-wide. The parasite is classified in the phylum Apicomplexa, class Sporozoasida, order Eucoccidiorida and family Sarcocystidae.

The vast majority of natural *Toxoplasma* infections in domestic animals are subclinical. Clinical signs, when present, are generally vague and non-specific and may include a period of fever, anorexia, respiratory distress and sometimes diarrhoea. Central nervous system disorders are rarely recorded. *T.gondii* infection, however, is a major cause of ovine and caprine abortion and perinatal mortality.

The life cycle of *T.gondii* can be divided into two parts: an asexual cycle in intermediate hosts and a sexual cycle, confined to the enteroepitheal cells of the feline definitive host, which results in the production of oocysts. In the asexual cycle there are two developmental stages, the rapidly multiplying tachyzoite (syn. endozoite, trophozoite) and the slowly multiplying bradyzoite (syn. cystozoite). Tachyzoites actively penetrate host cells, particularly those of the reticuloendothelial system where within a parasitophorous vacuole they multiply until the cell ruptures to release organisms locally and into the bloodstream to parasitise further cells. This process continues until the host dies, or more usually it develops immunity, extracellular parasites are then eliminated, intracellular multiplication slows, tissue cysts containing bradyzoites develop and a chronic or persistent infection is established. These microscopic cysts are found most frequently in brain and skeletal muscle and represent the quiescent stage of the parasite within the host. In some species, such as sheep, goats, pigs and man, cysts may remain for the rest of the life of the individual, while in others such as cattle, water buffalo and deer the host may eventually become clear of infection.

The sexual cycle is initiated when a non-immune cat ingests infective *T. gondii* either in the form of tissue cysts, tachyzoites or oocysts. The most impor-

tant sources of feline infection are birds and rodents persistently infected with tissue cysts. Gastro-intestinal proteolytic enzymes release bradyzoites from tissue cysts to penetrate the epithelial cells of the cat's small intestine, where subsequent sexual reproduction results in the formation of oocysts, which are released into the gut lumen to be excreted in the faeces. The prepatent period after ingestion of tissue cysts is 3-6 days and vast numbers of oocysts may be shed continuously in the faeces from this time until up to 14 days after infection. Following sporulation in the open over the next one to five days, depending upon aeration and temperature, the oocysts become infective. They are very resistant and may remain infective in the environment for a year or more. Sporulated oocysts are 11 x 13 mm in diameter and contain four sporozoites in each of two sporocysts.

The major source of *Toxoplasma* infection for susceptible herbivores is feed and water contaminated with sporulated oocysts. Fifty grams of infected cat faeces may contain as many as ten million oocysts. If, in a hypothetical situation this was evenly distributed throughout ten tonnes of concentrated animal feed, then each kilogram could contain between five and twenty-five sheep-infective doses. Similarly, pasture spread with manure and bedding from farm buildings where cats live may be a source of infection for sheep.

To limit environmental contamination by oocysts the number of cats capable of becoming infected and shedding oocysts should be reduced. Since these tend to be young animals, breeding should be controlled to favour a small healthy population of mature animals. Feed should also be kept covered to prevent its contamination by cat faeces.

Abortions and neonatal mortality occur when sheep and goats suffer a primary infection during pregnancy. Within four days of ingestion of sporulated oocysts by susceptible pregnant sheep, organisms can be found in the mesenteric lymph nodes, where they multiply causing marked lymph node enlargement, sometimes with focal necrosis. Around the fifth day tachyzoites are released to cause a parasitaemia which may last until the twelfth day. Coinciding with the parasitaemia the ewe displays a febrile response which can exceed 41°C around day six or seven. The cessation of the parasitaemia coincides with the onset of an effective maternal immune response. With the exception of the gravid uterus, the infection then persists as bradyzoites within tissue cysts.

In pregnant ruminants the gravid uterus is an "immunologically privileged" site. On the uterine side maternal immunological responses are locally suppressed, while the ability of the foetus, with its placenta, to recognise and respond to a pathogen such as *T.gondii* commences during the first half of gestation and develops for the remainder of pregnancy, so that foetal lambs become immunocompetent by the time they are born. During maternal parasitaemia, tachyzoites are able to parasitise the caruncular septa, invade the adjacent trophoblast cells of the foetal villi, and from there the rest of the foetus, between five to ten days after the onset of parasitaemia.

In a typical outbreak, disease first becomes apparent when ewes lamb a few days early with a significant proportion of lambs, although outwardly quite normal, being stillborn and some accompanied by a "mummified" foetus. In addition a proportion of lambs born alive are weak and

die within the first few days of life despite careful nursing. The ewe remains clinically normal. However, the real effect of infection in susceptible pregnant ewes depends upon the stage of gestation at which infection occurs. Infection in early pregnancy, when there is a minimal foetal immune response, rapidly results in foetal death followed by foetal resorption with the ewes ending up barren. In naturally-occurring outbreaks of toxoplasmic abortion aborted foetuses are seldom detected before the fourth month of gestation. Infection between about 70 and 120 days presents the clinical picture described while ewes which become infected in late pregnancy would be expected to produce infected but clinically normal lambs. The parasite does not cause abortion in sheep in subsequent pregnancies following toxoplasma abortion or if ewes are first infected whilst not pregnant. The key to preventing uterine infection is to prevent parasitaemia occurring during pregnancy.

No killed *Toxoplasma* vaccines are currently available for use in animals or man. However, a vaccine containing a live attenuated strain of *T.gondii* (S48) is available in New Zealand (Toxovax; MAF) and some European countries (Toxovax; Intervet/Mycofarm) for use in sheep. Live tachyzoites are injected into sheep where they induce a short-lived infection. Thus, while sheep are protected they do not appear to be left persistently infected, so avoiding the potential public health hazard of meat persistently infected with a vaccinal strain of *T.gondii* being available for human consumption. Correctly used the vaccine is very effective, its disadvantages are that is has a very short shelf-life and if not handled with care it could be a hazard to those using it or handling meat from animals killed soon after vaccination.

The ionophore, monensin, which has significant anti-toxoplasma activity in sheep, may be used to control infection in countries where legislation permits its use. The anticoccidial drug, decoquinate, has also recently been shown to significantly reduce the effect of *Toxoplasma* oocysts ingested by pregnant sheep. Both monensin and decoquinate work best if they are already being fed to susceptible ewes at the time they first encounter infection. They have not been shown to be so effective as chemotherapeutic agents. Administration of certain drug combinations such as pyrimethamine and sulphamezathine after infection has become established in the placenta and foetus may be effective. However, since the first abortions in a flock occur several weeks after infection, the practical benefit of chemotherapy is limited in many instances.

## 6.3 SAMPLES

Diagnosis of *Toxoplasma* infection is generally carried out using one or more of the following samples:

- Placental material
- Brains from aborted foetuses
- Foetal fluids
- Blood :Blood from aborted ewes · Pre-colostral blood from live lambs

### 6.3.1 Isolation

*Toxoplasma gondii* can be isolated from placental cotyledons, the brain, pleural and peritoneal fluids and heart blood from aborted foetuses that are not too autolysed. Samples should be collected as soon as possible after abortion and expulsion of the placenta as described in Chapter 2. If not analysed immediately, samples should be stored frozen at -20°C.

### 6.3.2 Histology

Five or six cotyledons and the entire brain from aborted foetuses that are not too autolysed should be aseptically sampled and placed in a glass sample jar containing a suitable fixative as described in Chapter 2. In the absence of secondary fixation tissues are best left for at least two weeks before further processing.

### 6.3.3 Antibody detection

Blood samples from aborted ewes are best collected at the time of abortion when antibody titres to *Toxoplasma gondii* would be expected to be raised. Precolostral blood samples from lambs born weak or lambs born alongside aborted foetuses can sometimes aid a diagnosis, however, there are practical difficulties in obtaining such samples.

Blood samples for serological examination should preferably be collected in sterile, evacuated glass tubes (e.g. Vacutainers) and removed to the laboratory as quickly as possible. Samples should be centrifuged for 10 minutes at 3,000 xg, sera removed from the clot and either analysed immediately or stored frozen at -20°C.

## 6.4 RISKS TO HUMAN HEALTH

*Toxoplasma* is classified as a category 1 pathogen. Samples must be collected and handled with the same precautions as all biological materials that are potentially dangerous to human health. Laboratory work should be undertaken in type L3 containment areas or, if unavailable, in a conventional laboratory equipped with a laminar flow hood.

*Toxoplasma* can present a serious threat to the unborn child if the mother becomes infected for the first time during pregnancy. Maternal infection acquired early and transmitted to the foetus usually results in spontaneous abortion, stillbirth or severe disease of the unborn child. The greatest risk of foetal damage occurs following maternal infection between 10 and 24 weeks of pregnancy. Maternal infection in later pregnancy most commonly results in subclinically- infected infants, although they may subsequently display severe symptoms such as mental retardation and visual handicap, the latter as a result of a progressive chorioretinitis.

Other people at risk of developing clinical illness include those who are immunosuppressed, such as tissue transplant patients and victims of AIDS, patients receiving chemotherapy for cancer and individuals with certain tumours of the lymphoid system. For such vulnerable groups, infection with *Toxoplasma* can prove life-threatening.

Emphasis in the laboratory should be placed on educating staff of the potential risks of accidental infection with this zoonotic agent. Pregnant women and other personnel knowingly in any other "at risk" category should not be allowed to handle any materials possibly infected with *Toxoplasma*. Ideally non-pregnant female staff should be screened twice yearly for antibody status to *Toxoplasma*. Pregnant women should not assist with lambing ewes or kidding goats or the care of newborn lambs or kids not only because of the risk of toxoplasmosis but also because sheep and goats may carry other zoonotic infections such as *Chlamydia psittaci* and *Coxiella burnetti*.

In the event of accidental inoculation or ingestion of potentially infective material personnel should be referred to medical experts who may opt to treat with chemotherapeutic drugs such as a course of pyrimethamine and sulphonamide.

## 6.5 DIRECT DIAGNOSIS

A definitive diagnosis of toxoplasmosis as a cause of abortion and neonatal mortality in sheep and goats is generally based on a combination of the identification of distinct macroscopic and histological changes and the outcome of serological tests on the ewe or foetus and/or results of isolation tests. The actual techniques used in any specific case depends upon the availability of the different types of samples.

### 6.5.1 Gross pathology of placenta and aborted foetuses

The most characteristic macroscopic changes following Toxoplasma abortion in sheep and goats are found in the placenta. The cotyledons are usually bright to dark red and speckled with white foci of necrosis 2 to 3 mm in diameter. These foci may be sparse or so numerous that they can become confluent and on cut section they can be seen to occur on any plane. Cotyledons with macroscopic changes can be found alongside apparently normal ones in the same placenta. The intercotyledonary allanto-chorion appears normal (Figure 6.1, page 95).

Visible changes in lambs can vary, the most obvious being the mummified foetus, a small chocolate brown miniature of a lamb, often with its own small grey-brown placenta. Foetuses dying later in gestation are born in various stages of decomposition often with clear to bloody subcutaneous oedema and a variable amount of clear to bloodstained fluid, sometimes flecked with strands of fibrin, in body cavities. However, while these latter changes indicate an intrauterine infection they are not specific to *Toxoplasma* infection.

### 6.5.2 Histopathological examination of tissues

#### *6.5.2.1 Simple staining*

***Principle***

This technique relies on showing the presence of histopathological changes in tissue sections following haematoxylin and eosin staining. In rare cases organisms of *T.gondii* can also be visualised.

N.B. The chemical concentrations and timings of the various processes involved in fixing and staining samples for histopathological examination will vary according to the availability of equipment and chemicals in the laboratory undertaking the work. Many laboratories will have their own standard methods and the method described is given as one example.

***Materials and reagents***

- Microtome
- Ethanol
- Toluene
- Xylene
- Paraffin wax.

***Preparation of histological tissue sections***

1. Prior to processing, samples of placenta must have been fixed for a minimum period of one week and brains for a minimum of two weeks. More rapid fixation can be achieved using Bouin's fixative either on its own or as a secondary fixative.

2. Remove tissue from fixative, blot dry and trim into suitably sized blocks no more than 4mm thick.

3. Dehydrate tissue blocks by gently agitating them in an alcohol series (80% ethanol for 45 minutes, 94% ethanol for 60 minutes, 99% ethanol for 3 x 60 minutes).

4. Clear blocks by immersion in a mixture of equal parts of 99% ethanol/toluene for 2 x 90 minutes then toluene alone for 2 x 60 minutes.

5. Embed blocks in paraffin wax by immersing in molten wax for 2 x 120 minutes.

6. Trim the resultant blocks and cut sections of uniform thickness between 4 and 6mm with a microtome.

7. Mount sections on glass slides pretreated with 2% egg albumin or equivalent to bind the section to the glass.

***Staining***

1. Immerse slides in xylene for 2 x 10 minutes to remove wax.

2. Hydrate tissue through a graded alcohol series (99% ethanol for 2 x 2 minutes, 90% ethanol for 2 minutes, 70% ethanol for 2 minutes).

3. Rinse slides thoroughly under running tap water.

4. Immerse slides in haematoxylin stain for 5-15 minutes to stain the cell nuclei.

5. Rinse slides thoroughly under running tap water.

6. Immerse slides in Scotts Tap Water Substitute (STWS) for 3 minutes.

7. Examine under a microscope: if the background is blue differentiate by immersing in 1% HCl in 70% alcohol to clear to grey; when the background is grey continue to step 8.

8. Rinse slides thoroughly under running tap water and re-immerse in STWS for 3 minutes.

9. Rinse slides thoroughly under running tap water.

10. Immerse slides in eosin solution for 3 minutes.

11. Rinse slides thoroughly under running tap water.

12. Dehydrate and clear slides through a graded alcohol series and xylene (70% ethanol for 2 minutes, 90% ethanol for 2 minutes, 99% ethanol for 3 x 2 minutes, xylene for 2 x 2 minutes).

13. Mount coverslip over stained tissue section and examine under a microscope.

***Reading and interpretation of results***

Cell nuclei will appear blue; red blood cells, red; muscle, connective tissue and cell cytoplasm, varying shades of pink. *Toxoplasma* will appear light blue.

Well-preserved samples of placental cotyledons can reveal moderate oedema of the mesenchyme of the foetal villi with a diffuse hypercellularity due to the presence of large mononuclear cells. In addition there can be foci of swollen trophoblastic epithelium in foetal villi which can progress to necrosis and desquamation. The larger affected areas give rise to foci of coagulative necrosis which may become mineralised with time. Sometimes small numbers of intracellular and extracellular toxoplasms are visible, usually on the periphery of a necrotic area or in a villus which is in the early stages of infection. The organisms appear ovoid, 1-4µm long, with nuclei which are moderately basophilic and located centrally or towards the blunt end [1].

In the foetal brain both primary and secondary lesions develop. Glial foci, typically surrounding a necrotic and sometimes mineralised centre, often associated with a mild lymphoid meningitis, represent a foetal immune response following direct damage by local parasite multiplication. Toxoplasms are only rarely found, usually at the periphery of the lesions. Focal leukomalacia is also common and is thought to be due to foetal anoxia in late gestation caused by advanced necrosis in the placentome preventing sufficient oxygen transfer from

mother to foetus. These foci occur most commonly in cerebral white matter cores and the corpus striatum but sometimes also in the cerebellar white matter [1].

***Preparation of reagents***

- Bouin's Fixative

| | |
|---|---|
| Saturated aqueous picric acid | 75ml |
| 40% formaldehyde | 25ml |
| Glacial acetic acid | 5ml |

- Haematoxylin Solution

| | |
|---|---|
| Haematoxylin | 1g |
| Sodium iodinate | 0.2g |
| Potassium alum | 50g |
| Chloral hydrate | 50g |
| Citric acid | 1g |
| Distilled water | 1 litre |

Dissolve the haematoxylin in the water with the aid of gentle heat then add the sodium iodinate and alum and shake at intervals to effect the solution of alum. Add citric acid and chloral hydrate and mix until dissolved. The solution remains for several months.

- Scott's Tap Water Substitute

| | |
|---|---|
| Sodium bicarbonate | 3.5g |
| Magnesium sulphate | 20g |
| Tap water | 1 litre |
| Thymol | 1 crystal |

Dissolve salts in the water and add thymol.

- Eosin Solution

| | |
|---|---|
| Eosin | 1g |
| Distilled water | 100ml |
| Thymol | 1 crystal |

Dissolve eosin in water and add thymol.

## 6.5.3 Detection of *Toxoplasma gondii*

The most direct and established method of demonstrating *Toxoplasma* infection in cases of abortion is to transmit the infection from aborted material to laboratory mice [2]. The method, however, is slow and does not distinguish between acute and latent infections. *Toxoplasma* can be grown in virtually any mammalian cell line. However, although more rapid than mouse inoculation, cell culture is rarely used for routine diagnosis. An alternative, much more rapid but less sensitive, method of isolation is the direct demonstration of *T.gondii* tissue cysts, by centrifugation of lamb brain homogenate on a discontinuous density gradient of 30% and 90% colloidal silica solution [3].

Immunohistochemical techniques allowing visualisation of intact *T.gondii* and its antigenic residues in tissue sections of aborted materials are recognised as convenient and sensitive methods of demonstrating *Toxoplasma* infection. Such methods have the advantage of detecting *Toxoplasma* antigen even in severely decomposed tissues.

Techniques such as the polymerase chain reaction (PCR) for identifying and amplifying specific lengths of DNA have been developed for *T.gondii*. PCR and related techniques involving nucleic acid probes still need to be evaluated as diagnostic tools in veterinary protozoology but may become important techniques in the future.

### *6.5.3.1 Mouse inoculation*

***Principle***

Isolation of *T.gondii* from dead lambs and foetal membranes are best made by inoculation of laboratory mice. The choice of material for inoculation is dictated by the quality of the material available, the best being the cotyledons and foetal brain.

***Materials and reagents***

- Equipment suitable for aseptic maceration of tissues e.g. a "stomacher" (Seward Laboratory, London, UK)
- Laboratory mice e.g. Swiss white
- Sterile phosphate buffered saline (PBS)
- Antibiotics: Penicillin
  Streptomycin
- Giemsa stain.

***Procedure***

1. Aseptically remove 2-5g of placental material or brain tissue from the dead lamb.
2. Homogenise the tissue in an equal volume of sterile PBS with added antibiotics (100 IU/ml penicillin and 745 IU/ml streptomycin) in a "stomacher" or other suitable macerating equipment. Brain tissue may be effectively macerated by passing it through a 16 gauge needle 10 times by means of a syringe.
3. Inoculate each of 3 *Toxoplasma*-free mice intraperitoneally with 0.5ml of the homogenate.
4. Six to 8 weeks after inoculation, kill the mice and remove brains. Blood should also be recovered from the mice at this stage and serum separated and stored at -20°C.
5. Homogenise each mouse brain with an equal volume of sterile PBS in a "stomacher" or by passing through a 16 gauge needle 10 times by means of a syringe.
6. Place drops of the suspensions on a suitable number of slides.
7. Dry and stain with Giemsa, dehydrate and mount under a coverslip.
8. Examine slides under a microscope.

***Reading and interpretation of results***

Tissue cysts appear as circular structures measuring 5 to 50µm filled with blue-staining crescent shaped bradyzoites.

Failure to demonstrate cysts does not rule out a positive diagnosis. Serum from the mice must be analysed for the presence of antibodies to *Toxoplasma* and if this analysis is also negative, *Toxoplasma* infection can be ruled out.

***Preparation of reagents***

- Giemsa Stain

| | |
|---|---|
| Giemsa powder | 4g |
| Glycerol | 250ml |
| Methanol | 250ml |

Stock Solution: Dissolve giemsa powder in glycerol at 60°C with regular agitation. Add methanol, mix thoroughly and leave to stand for 7 days. Filter before use.

Working Solution: Dilute 4ml of stock solution in 96ml buffered distilled water (pH 6.8).

### *6.5.3.2 Immunohistochemical methods*

In order to overcome problems of visualisation of *Toxoplasma* organisms in histological sections, immunohistochemical labelling methods detecting *T.gondii* antigens have become increasingly important. Two immunoperoxidase techniques are regularly used. The ABC indirect immunoperoxidase method is available commercially in kit form (VECTASTAIN" Elite ABC Kit, Vector Laboratories, USA) combined with an appropriate primary antibody. Alternatively the peroxidase anti-peroxidase (PAP) technique [4] can be carried out as follows:

***Principle***

The PAP method relies on the use of a primary antibody specific for the antigen in question, a "PAP complex" comprised of the enzyme peroxidase and an antibody against peroxidase, and a "link" (secondary) antibody capable of binding to both the primary antibody and to the PAP complex. The ABC and PAP method both allow visualisation of

*T.gondii* organisms as well as parasitic intra- and extracellular antigenic residues even in quite severely decomposed and necrotic tissues.

***Materials and reagents***

- Microscope.
- Staining boxes and slides.
- Xylene.
- Ethanol.
- Tris-buffered saline solution (TBSS).
- Hydrogen peroxide.
- Non-immune porcine serum (Dakopatts, Copenhagen, Denmark).
- Rabbit anti-Toxoplasma serum.
- Porcine anti-rabbit immunoglobulin (PAP) (Dakopatts, Copenhagen, Denmark).
- Substrate : Diaminobenzidine (dissolve 24mg diaminobenzidine-tetrahydrochloride in 40ml TBSS buffer and add 0.4ml 3% hydrogen peroxide immediately before use).
- Haematoxylin stain.

***Procedure***

All incubations should be carried out at room temperature in a humidity chamber.

1. Fix and cut tissue sections as described in Section 6.5.2.1.
2. Dewax sections by immersing slides in xylene for 2 x 2 minutes.
3. Rehydrate tissues through an alcohol series (99% ethanol for 2 minutes, 90% ethanol for 2 minutes, 70% ethanol for 3 x 2 minutes).
4. Block endogenous peroxidase activity by immersing slides in 3% hydrogen peroxide in Tris-buffered saline solution (TBSS) for 15 minutes.
5. Incubate the tissue sections for 30 minutes with 1:10 dilution of normal non-immune porcine serum.
6. Remove the excess liquid and incubate for 45 minutes with the pre-determined optimal dilution of primary rabbit anti-Toxoplasma serum.
7. Rinse slides 3 times with TBSS.
8. Remove the excess liquid and incubate for 30 minutes with a 1:20 dilution of PAP.
9. Rinse slides 3 times with TBSS.
10. Incubate slides for 30 minutes with a 1:100 dilution of PAP.
11. Rinse slides 3 times with TBSS.
12. Place slides in substrate bath containing diaminobenzidine solution and monitor the positive control wells for development of specific brown staining (up to 8 minutes).
13. Rinse slides 3 times with TBSS.
14. Counterstain slides with haematoxylin.
15. Examine tissue sections microscopically.

***Reading and interpretation of results***

*Toxoplasma* tachyzoites appear as brown, round or crescent-shaped organisms, approximately 2 x 5µm. Tachyzoites are usually situated intracellularly, singly or in clusters of 4-10 organisms. The parasite cell wall tends to be distinctly stained. Bradyzoites are similar in appearance to tachyzoites but will be seen within tissue cysts in muscle or brain (Figure. 6.2, page 95).

### *6.5.3.3 DNA analysis*

Techniques such as the polymerase chain reaction (PCR) for identifying and amplifying specific lengths of DNA have recently been developed for T.gondii. Methods are based on the amplification of either the P30 or the B1 gene of *T.gondii* and have been used for the detection of *Toxoplasma* in various clinical specimens collected from infected humans. Limited studies have also been carried out using ovine samples and the methods have been successfully applied

to aborted placental material, brain and peritoneal fluid from aborted foetuses and lymph, blood and lymph nodes from artificially infected ewes [5]. Detection of *T.gondii* by amplification of the B1 gene has been shown to be more sensitive than by the P30 gene. This may be due to the repetitive nature of the B1 gene of which 25-50 copies are present in the genome of *T.gondii* whereas there is only a single copy of the longer P30 gene. However, further comparative investigations into the efficacy of P30 and B1 PCR in a full range of clinical materials are required before any conclusion can be made about the use of either target gene for positive detection of *T.gondii* and the use of such a method in routine diagnosis.

### 6.5.4 Serological methods

Serology is an important tool in the diagnosis of ovine and caprine *Toxoplasma* abortion. The presence of specific antibodies in serum or tissue fluid from stillborn lambs or kids or in precolostral serum from live lambs or kids indicates uterine infection. However, high *Toxoplasma* antibody titres in sera taken from ewes within a few weeks of abortion or production of stillborn lambs or kids can only suggest toxoplasmosis as titres remain relatively high for long periods after initial infection. Serology is very useful as a way of assessing the degree of exposure to infection in a given group of animals. Many serological methods for the diagnosis of toxoplasmosis have been established over the years. Most have been developed for detection of *Toxoplasma* infection in humans and are now commercially available in kit form. Many of the techniques are readily modified for use in the detection of *Toxoplasma* infection in other species including sheep and goats but are not produced as commercially available kits. The methods include :

a) Dye test of Sabin and Feldman (DT)
b) Complement Fixation test (CFT)
c) Indirect Fluorescent Antibody test (IFAT)
d) Direct Agglutination test (DAT)
e) Latex Agglutination test (LAT)
f) Indirect Haemagglutination test (IHA)
g) ELISA
h) Radioimmunoassay (RIA)
i) Carbon Immunoassay (CIA)
j) Diffusion-in-gel ELISA (DIG-ELISA)

#### *6.5.4.1 Comparison of serological methods*

The first methods to be developed were the DT of Sabin and Feldman and the CFT. The DT is sensitive and specific and is traditionally regarded as being the definitive test for *T.gondii* antibody detection. However, it has the drawbacks of being expensive and time consuming, requiring live *Toxoplasma* tachyzoites as antigen, and has now been replaced in many laboratories by less demanding methods.

The IFAT gives titres comparable with the DT and is extensively used for the analysis of ovine and caprine sera. It is a safer technique than the DT for the operator as it uses killed tachyzoites but a fluorescent microscope and skilled staff are required for the reading and interpretation of results, which renders the IFAT suitable primarily for well equipped central laboratories. Antigen for use in IFAT analysis for sheep and goats is commercially available from BioMerieux, France.

The IHA and LAT tests are easy to perform and both are commercially available in kit form (Tox HA Test; Wellcome Diagnostics, UK: Toxoreagent Kit; Eiken Chemical Co., Japan distributed by Diamed Diagnostics, Bootle, UK). IHA and LAT tests do not require species-specific antisera or conjugates and since they are available in kit form they have become popular for serodiagnosis of ovine toxoplasmosis. However, antibodies detected by IHA and LAT may appear later in infection than those detected by tests such as DT and IFAT and their sensitivity may therefore be low especially in acute infections.

ELISA for *T.gondii* antibodies has been adapted for use in most domestic animals including sheep and goats and modified methods have been developed for the detection of *Toxoplasma* antigen in body fluids. The ELISA is readily automated and therefore suitable for handling large numbers of test sera and can also distinguish IgG and IgM antibodies.

The RIA, CIA and DIG-ELISA techniques are less commonly used for *Toxoplasma* antibody detection in animals.

### *6.5.4.2 Indirect fluorescents antibody test (IFAT)*

#### *Principle*

Antibodies to *T.gondii* present in test samples are bound to antigen fixed on microscope slides. Immunoglobulin bound in this way is detected by subsequent binding of fluorescently labelled antiglobulin [6].

Glass microscope slides with 10 printed circular areas covered with RH strain of *T.gondii* may be obtained from Gull Laboratories, Salt Lake City, USA. Alternatively, lyophilised formalin treated antigen slides can be purchased from BioMerieux, France.

#### *Materials and reagents*

- Humidity chambers.
- Fluorescent microscope.
- Antigen slides.
- Fluorescein isothiocyanate (FITC) conjugated rabbit anti-sheep immunoglobulin (Dakopatts, Denmark).
- Standard positive and negative sera.

#### *Procedure*

1. Dilute test and standard positive and negative sera in PBS (pH 7.6) 1:10 and subsequently in twofold steps.
2. Add one drop of each serum dilution to prepared antigen wells on microscope slides.
3. Incubate slides at room temperature in a moist chamber for 30 minutes.
4. Wash slides twice using PBS (pH 7.6) and air dry.
5. Cover antigen with a suitable dilution of FITC-labelled rabbit anti-sheep IgG preparation and incubate at room temperature in a moist chamber for 30 minutes.
6. Wash slides twice using PBS (pH 7.6) and air dry.
7. Cover antigen using a coverslip and buffered glycerol (pH 8 - 9).
8. Read slides by fluorescent microscope at a final magnification of between 200 and 400.

#### *Reading and interpretation of results*

A sample dilution is considered to be positive when at least 50% of the tachyzoites show a bright unbroken peripheral fluorescence. However a titre is not normally considered to be positive in adult sheep and goats unless it is equal to or greater than 1/160 while in foetal samples a titre of 1/40 or more is likely to represent a specific response to the parasite.

### *6.5.4.3 ELISA*

***Principle***

Test sera are reacted with *T.gondii* antigen-sensitised wells of a microtitre plate. Bound antibody is then detected by addition of an enzyme-linked antiglobulin followed by an assay of the enzyme reaction with its substrate [7].

A commercially available kit (Toxoelisa; Microbiological Associate Bioproducts, USA) for use in the diagnosis of human *Toxoplasma* infections can be used for ovine samples by replacing the anti-human IgG conjugate with an alkaline-phosphatase labelled rabbit, anti-sheep IgG (Kirkgaad and Perry Laboratories Inc., USA) or antigen coated plates can be obtained from BioMerieux, France and the test carried out as follows :

***Materials and reagents***

- ELISA plate reader.
- *Toxoplasma* antigen coated microtitre plates (BioMérieux, France).
- Phosphate buffered saline (PBS).
- Tween 20.
- Horseradish peroxidase (HRP) conjugated rabbit-anti-sheep immunoglobulin (Dakopatts, Denmark).
- Substrate: 1 mg p-nitrophenylphosphate per ml diethanolamine buffer, pH 9.8, containing 0.5 mmol 2M $MgCl_2$.
- Standard positive and negative sera.

***Procedure***

1. Dilute test and standard sera samples 1 in 400 with PBS with 0.05% Tween 20.
2. Add 100µl of diluted serum to each well and incubate at room temperature for 2 hours.
3. Wash the plates three times each for 3 minutes in PBS with 0.05% Tween 20 and shake dry.
4. Add 100µl of the HRP conjugated rabbit anti-sheep immunoglobulin diluted to a suitable concentration in PBS-Tween 20 to each well and incubate at room temperature for 3 hours.
5. Wash the plates three times each of 3 minutes in PBS with 0.05% Tween 20 and shake dry.
6. Add 100µl of substrate to each well and leave for 30 minutes.
7. Stop the reaction by addition of 50µl 2M sodium hydroxide to each well.
8. Read the optical density (O.D.) of each well at 400nm.

***Reading and interpretation of results***

Antibody can be expressed either as a titre or as a percentage of a standard positive serum. A sample of Toxoplasma-positive serum, which consistently produces an O.D. in the region of 1.0 is used at different dilutions in an ELISA to construct a standard curve. The O.D. at a dilution of 1 in 500 is read and given the arbitrary value of 100% and all subsequent O.D. values within the run are expressed as a percentage of this with the equation :

$$\frac{\text{(Experimental O.D.) - (Negative antigen O.D.)}}{\text{(Standard O.D.)-(Negative antigen O.D.)}} \times \frac{100}{1}$$

## 6.6 FUTURE WORK

The diagnosis of toxoplasmosis is well established with numerous diverse immunological techniques suitable for many situations. To standardise and further increase their specificity, defined antigens and antisera are required. In the future, early diagnosis of toxoplasma infection in sheep and goats with developing placental pathology could allow the employment of chemoprophylactic and chemotherapeutic regimes. The detec-

tion of parasite DNA by nucleic acid probe techniques such as the PCR also needs to be evaluated. Their specificity and high sensitivity will ensure them an important role in veterinary diagnosis.

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# Chapter 7

# Q FEVER

P. RUSSO

## 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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# Chapter 8

# CAMPYLOBACTERIOSIS

W. DONACHIE

## 8.1 SUMMARY

Campylobacteriosis is a highly contagious and economically significant disease in sheep, most often caused by *Campylobacter fetus subspecies fetus* and *C.jejuni*. It is characterised by abortion, stillbirths, premature births, weak lambs and occasional ewe deaths due to metritis. A similar disease is recognised in goats but herd outbreaks have only rarely been reported. *Campylobacter* organisms are widespread and many animals carry them in their intestines and excrete organisms in their faeces.

A presumptive diagnosis can be made by a history of widespread abortion and typical lesions of the foetus and placenta but this should be confirmed by isolation and identification of the organism. Microscopical demonstration of typical *Campylobacter* organisms in aborted materials by specific staining techniques can also be useful in diagnosis. Although serological tests are available for the detection of antibodies against *Campylobacter* these are not used in diagnosis of abortive campylobacteriosis as antibodies are frequently found in the sera of healthy animals with no history of *Campylobacter* infections.

## 8.2 INTRODUCTION

Vibrionic abortion in sheep was first described in 1913 and has since been reported in many countries. The causal organism was originally assigned to the genus *Vibrio*, hence the name vibriosis, but is now in the genus *Campylobacter*. Eleven species of *Campylobacter* are currently recognised but abortion in sheep and goats is caused by infection with only one or other of two catalase-positive campylobacters *Campylobacter fetus subspecies fetus* (subsequently referred to as *C. fetus*) or *C. jejuni* (see Figure 8.1). Campylobacters are Gram-negative, non-sporing, curved or spiral rods with characteristic "S" or "seagull" shape, 0.2-0.9 μm wide and 0.5-5.0 μm long. They are microaerophilic and grow best in an atmosphere of 5% oxygen, 10% carbon dioxide and 85% hydrogen or nitrogen. *Campylobacter* organisms are motile by means of single polar flagellum at one or both ends and have a characteristic darting, corkscrew motion.

The clinical sign of an active infection in pregnant sheep is abortion during late pregnancy. In field out breaks many ewes have a mild diarrhoea before the first abortions occur. Lambs may be carried to full term but are born dead or in a weak condition. Usually there is no indication of the impending abortion, but a few ewes may show a prior vaginal discharge. Recovery of the ewe is prompt and fer-tility in subsequent breeding seasons usually good. Occasionally, abortion is complicated by metritis and subsequent death of the ewe. *Campylobacter* abortion occurs in all breeds of sheep and all ages of ewes. Within flocks the abortion rate varies from 5 to 50% although rates as high as 80 and 90%

have been reported. Experimental evidence suggests that abortions are commoner if infection is acquired beyond the twelfth week of pregnancy and that abortion occurs between 7 and 25 days after infection, usually during the last 8 weeks of pregnancy. Both *C. fetus* and *C. jejuni* have been shown to cause abortion in goats, the clinical symptoms being similar to those in sheep. However, reported cases of field outbreaks of *Campylobacter* abortion in goats are limited.

Campylobacteriosis in sheep is transmitted orally. It occurs in many closed flocks and the source of infection is not always clear. Sheep can carry *C.fetus* and *C. jejuni* in their intestines and excrete the organisms in the faeces. It is therefore likely that some outbreaks are due to the contact of pregnant sheep with a faecal excretor which may have been introduced into a flock. The appearance of the disease in closed flocks has been attributed to contamination of pastures by wildlife vectors, as sparrows, carrion crows and magpies have been found to be intestinal carriers. After the first abortion, infection is further spread as a result of contamination of pastures from the products of this abortion and by direct contact with foetal membranes and foetuses.

The disease is self-limiting in that it is unusual for abortions to occur in a self-contained flock the year after an outbreak of abortion. Ewes which are in contact with abortions but which do not themselves abort, and non-pregnant females, do not abort at subsequent pregnancies. This development of immunity to abortion can be used to ensure that an outbreak is confined to one lambing season. Ewes which abort

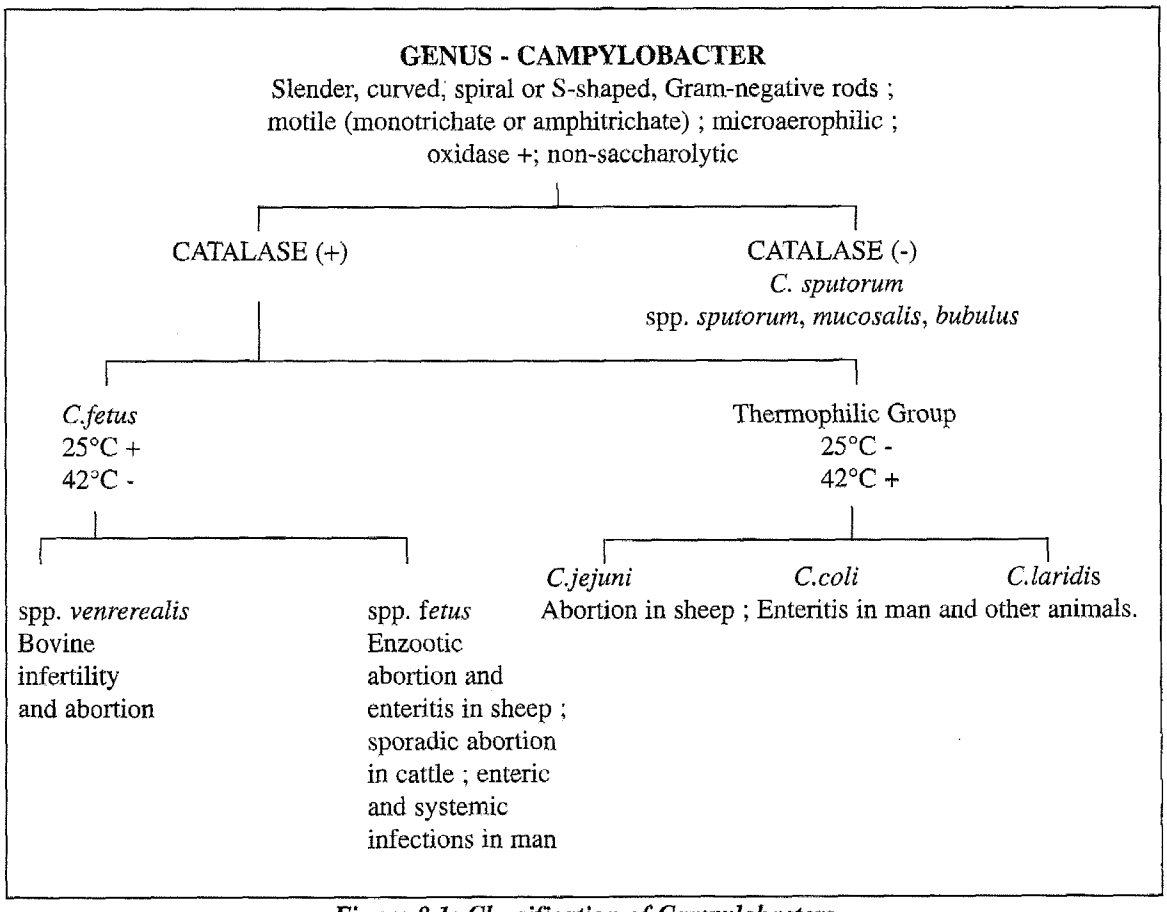


*Figure 8.1: Classification of Campylobacters*

should not be removed from the flock and potential additions to the breeding flock should be mixed with the infected flock. However, hygienic measures should be taken to prevent spread of infection from foetal membranes and aborted foetuses to adjacent flocks and to possible wild-life vectors. Although Campylobacters die rapidly on exposure to air, heat and desiccation, in moist conditions the organism can remain viable for several days, such as in vaginal discharge on pasture or bedding.

Treatment with penicillin and streptomycin and concurrent vaccination of in-contact ewes after the first abortion has prevented further abortions but there is no evidence that therapy alone is useful. Vaccination with disrupted, formalinized cells of *C. fetus* subspecies intestinalis in oil adjuvant has been shown to protect against experimental challenge. Also vaccination of ewes, after abortion in an experimentally-infected contact ewe, has been shown to reduce abortions. This suggests that vaccination in the face of an outbreak might be of value. The value of routine vaccination of commercial flock will depend on the prevalence of the disease. There are a number of commercial *Campylobacter* vaccines available for use in sheep in the USA but the efficacy of some of them is in doubt.

## 8.3 SAMPLES

Campylobacteriosis can only be confirmed by isolation and identification of the specific aetiological agent. Samples of choice are the aborted placenta, foetus and vaginal excretions. Samples should be fresh and submitted in a suitable transport medium such as FBP/glycerol and kept cool and moist during transportation to the laboratory. Stomach contents from the aborted foetus is also suitable for the isolation of *Campylobacter* because it can be sampled aseptically with a syringe.

Histopathological examination of aborted tissue samples is also advised for positive diagnosis. Small representative sections of tissue of no more than 0.5cm thickness should be taken as soon as possible after abortion or death and placed in a suitable fixative. Fixed tissues are best left for at least two days before further processing.

***Preparation of reagents***

- FBP/Glycerol Medium

Nutrient broth No. 2 (Oxoid, Basingstoke, UK).

0.12% w/v agar (Difco).

15% glycerol.

0.1% w/v yeast extract (Oxoid, Basingstoke, UK).

Double strength FBP enrichment supplement (Oxoid, Basingstoke, UK).

Dissolve the nutrient broth, agar and glycerol by gentle heat. Autoclave at 121°C for 15 minutes and allow to cool to 50-55°C in a water bath. Add the FBP enrichment supplement aseptically and mix gently. Dispense 4.0ml aliquots into sterile Bijou bottles.

## 8.4 RISKS TO HUMAN HEALTH

Campylobacters, particularly *C.jejuni*, have been recognised as important human pathogens which cause both acute bacterial gastroenteritis and extraintestinal infections. Individuals of all ages may be infected but infections are most frequent in young children and young adults. *Campylobacter jejuni* infections usually occur following ingestion of improperly handled or improperly cooked food, pri-

marily poultry products.

Due to the zoonotic potential of Campylobacters all materials from aborted ewes and does should be handled with the same precautions as any biological materials that are potentially dangerous to human health. Infection of pregnant women with *Campylobacter* can lead to abortion therefore pregnant women should not assist with lambing ewes and kidding goats nor be allowed to handle potentially infected materials in the laboratory.

## 8.5 DIRECT DIAGNOSIS

The placental and foetal lesions associated with *Campylobacter* abortion are specific and provide good evidence of infection by this organism. However, there is wide variation in the lesions and it is necessary to have bacteriological confirmation of the diagnosis.

### 8.5.1 Gross pathology of the placenta and foetus

The principal lesion in *Campylobacter* abortion is a placentitis. The placenta is oedematous and the cotyledons are pale and necrotic and separate easily from the caruncles.

In some aborted foetuses, most frequently those close to term, the liver is enlarged and haemorrhagic and shows typical gray, necrotic foci varying from 1-3 cm in diameter. Usually the foetus is oedematous and the body cavities contain a blood-stained serous fluid. There may also be a mild to moderate fibrinopurulent bronchopneumonia.

### 8.5.2 Isolation of *Campylobacter*

***Principle***

*Campylobacter* usually grow on most non-selective culture media especially when blood is added. A wide range of selective media have also been developed providing increased sensitivity and specificity of isolation mainly in response to the requirement to detect Campylobacters in human faeces. The majority of these media have been developed for isolation of *C. jejuni* and are rarely suitable for the isolation of *C. fetus*, therefore having limited application to veterinary samples. 5% blood agar is still a suitable media for culture of both *C. fetus* and *C. jejuni* from samples collected from aborted ewes in which *Campylobacter* numbers are high and contamination with other bacteria is minimal. However, a pre-culture membrane filtration stage is recommended to minimise any contaminants. Optimum atmospheric growth conditions (5% oxygen, 10% Carbon dioxide, 85% hydrogen or nitrogen) can be obtained by the use of commercially available gas-generating kits specifically for *Campylobacters* (Oxoid Ltd, Hampshire, UK) in conjunction with standard anaerobic jars, use of a Tri-Gas incubator or evacuation and replacement of an anaerobic incubator with the approximate gas mixture.

***Materials and reagents***

- 5% sheep or horse blood agar plates.
- 0.65 µ millipore membrane filters (Sartorius, Gottingen, Germany).
- Gas jars.
- Gas packs (Oxoid gas generating pack for Campylobacters, Basingstoke, UK).

***Procedure***

1. Centrifuge macerated tissue samples or foetal stomach contents at 100 xg for 10 minutes. Aseptically remove the supernatant and incubate at 37°C for 1 hour.

2. Place a 0.65 μ membrane filter on the surface of each isolating agar plate

3. Place between five and ten drops of the incubated supernatant fluid onto the center of each filter and incubate at 37°C for 1 hour.

4. Remove and discard the filters and spread filtrates over the agar surface. Allow plates to dry.

5. Incubate the plates in an atmosphere of 5% $O_2$, 10% $CO_2$, 85% $H_2$ or $N_2$ at 37°C for up to 72 hours.

3. Examine by a plate microscope for typical colonies of *Campylobacter* species after 48 and 72 hours incubation.

***Reading and interpretation of results***

Following culturing on blood containing solid media at 37°C, colonies will vary in appearance from small, round and convex ones in the case of the *C. fetus* group to fairly large mucoid colonies with *C. jejuni / C. coli*. Occasionally, *C. jejuni* and *C. coli* produce large and small colonies co-existing on the same plate, which when mature often become tanned. Strains of *C. jejuni* can develop a metallic sheen over the culture surface. Generally coccoid forms of *Campylobacters* are invariably due to long incubation and may be considered degenerative and non-viable. However, *C.jejuni* cultures frequently produce coccoid forms as early as 48 hours.

Haemolysis is not observed on blood agar.

***Preparation of reagents***

- Blood agar

Columbia blood agar base (Oxoid-Unipath or Difco).

5% sterile defibrinated sheep or horse blood.

Suspend blood base agar according to manufacturers instructions in distilled water and heat to boiling. Autoclave at 15 psi for 15 minutes. Bring to 45-50°C and aseptically add 5% sterile sheep or horse blood. Mix well and pour into sterile Petri dishes. Place in plate drier for a short period and store at 4°C wrapped or in sealed boxes.

### 8.5.3 Identification of *Campylobacter* species

Colonies that appear to be *Campylobacter* species on agar plates can be confirmed as such and catalase positive and negative strains differentiated using three simple tests :

1. Gram stain.
2. Oxidase reaction.
3. Catalase reaction.

*C. fetus* and *C.jejuni* can be distinguished from other *Campylobacter* species on the basis of further simple biochemical reactions:

1. Growth temperature studies.
2. Antibiotic sensitivity.
3. Production of hydrogen sulphide ($H_2S$).
4. Hippurate hydrolysis.

The expected result profiles for these tests for the various species of *Campylobacter* of interest are shown in Table 8.1.

#### *8.5.3.1 Gram stain*

***Principle***

This technique consists of showing, by specific staining, the presence of the bacteria on smears taken from the cotyledons and stomach contents of a freshly-aborted foetus. *Campylobacter* cells are often difficult to see on conventional Gram staining and it is recommended that strong (Zeihl-Neelsen's) carbol fuschin be used.

***Materials and reagents***

- Ammonium oxalate-crystal violet solution (mix 2ml of a 10% alcoholic

*Table 8.1 : Biotyping of catalase positive Campylobacters*

| Test | *C.fetus* spp *fetus* | *C. fetus spp venerealis* | *C. jejuni* biotype 1 | *C. jejuni* biotype 2 | *C. coli* | *C. laridis* |
|---|---|---|---|---|---|---|
| Catalase | + | + | + | + | + | + |
| Relative size | Medium | Long | Short | Short | Short | |
| Swarming on moist media | - | - | + | + | ± | |
| Rapid coccal formation | - | - | + | + | - | - |
| Growth at 25°C | + | + | - | - | - | - |
| Growth at 42°C | - | - | + | + | + | + |
| Naladixic acid sensitivity | R | R | S | S | S | R |
| Cephalothin sensitivity | S | S | R | R | R | R |
| $H_2S$ production | - | - | - | + | - | + (slow) |
| Hippurate hydrolysis | - | - | + | + | - | - |

***R : resistant*** ***S : sensitive***

solution of crystal violet, 18ml of distilled water and 80ml of a 1% aqueous solution of ammonium oxalate).

- Gram's iodine solution (dissolve 2g of potassium iodide in 100ml of distilled water and add 1g of iodine).
- 95% ethanol.
- Counterstain (15 parts 1% neutral red: 1 part carbol fuchsin).

***Procedure***

1. Remove colonies from a 48 or 72 hour culture and smear on a microscope slide. Fix by flame.
2. Stain with ammonium oxalate-crystal violet solution for 1 minute.
3. Rinse slide with water.
4. Stain with Gram's iodine solution for 1 minute.
5. Rinse slide with water.
6. Rapidly apply 95% ethanol until no further colour appears to flow from the sample.
7. Wash slide with water.
8. Apply counterstain for 30 seconds.
9. Dry slide and examine microscopically.

***Reading and interpretation of results***

Gram-positive organisms appear blue-black, Gram-negative organisms appear red.

Campylobacter cells are short Gram-negative rods and have a distinctive curved or spiral appearance.

***Preparation of reagents***

- Carbol Fuchsin

| | |
|---|---|
| Basic fuchsin | 40g |
| Phenol | 80g |
| Absolute alcohol | 200ml |
| Distilled water | 1 litre |

Dissolve the basic fuchsin in the alcohol and the phenol in the water. Mix the two solutions thoroughly and leave to stand at room temperature overnight. Filter through wet paper before use.

***Caution*** : phenol is an extremely hazardous reagent and precautions should be taken during assay.

### 8.5.3.2 Oxidase test

***Principle***

Certain bacteria possess "oxidases" that are involved in electron transfer from electron donors. If the redox dye tetra-methyl-p-phenylene diamine is used as electron acceptor, this will be reduced, and the reduced dye has a deep blue colour.

***Materials and reagents***

- Platinum loop.
- 1% tetra-methyl-p-phenylene diamine hydrochloride (aqueous solution).

***Procedure***

1. Place some dye solution onto a filter paper.
2. Using a platinum loop remove a representative colony from a 48 or 72 hour culture and smear onto the still wet dye.

***Reading and interpretation of results***

A deep purple colour will form within 10 seconds with oxidase positive cultures.

### 8.5.3.3 Catalase test

***Principle***

This test demonstrates the presence of the enzyme catalase which catalyses the release of oxygen from hydrogen peroxide.

***Materials and reagents***

- 3% hydrogen peroxide.

***Procedure***

1. Remove a representative colony from a 48 or 72 hour culture and place on a microscope slide.
2. Place a drop of 3% hydrogen peroxide on the colony and mix gently.

***Reading and interpretation of results***

Bubbling indicates a positive catalase test.

***Note***

• Care should be taken when removing colonies from blood containing media to prevent removal of any media with the sample as this may result in a false positive catalase reaction.

### 8.5.3.4 Growth temperature and antibiotic sensitivity tests

***Materials and reagents***

- 5% sheep or horse blood agar plates.
- Gas jars.
- Gas packs (Oxoid gas generating pack for Campylobacters, Basingstoke, UK).
- Naladixic acid and cephalothin disks (30µg).

***Procedure***

1. Make a single streak of sample colonies removed from 72 hour cultures across the surface of three blood agar plates.
2. Incubate one of the plates at 25°C and one at 42°C in an atmosphere of 5% $O_2$, 10% $CO_2$, 85% $H_2$ or $N_2$ for up to 72 hours.
3. Place the naladixic acid and cephalothin disks one at each end of the streak of inocula on the third plate. Incubate this plate at 37°C in an atmosphere of 5% $O_2$, 10% $CO_2$, 85% $H_2$ or $N_2$ for up to 72 hours
4. Examine all plates for typical colonies of *Campylobacter* species after 48 and 72 hours incubation.

***Reading and interpretation of results***

*Campylobacter fetus* species will grow at 25°C and 37°C but not at 42°C.

Thermophilic species of *Campylobacter* (including *C.jejuni*) will grow at 37°C and 42°C but not at 25°C.

Isolates susceptible to either antibiotics, naladixic acid or cephalothin, will show a zone of inhibition around the relevant disk.

### *8.5.3.5 Production of $H_2S$*

***Principle***

Production of $H_2S$ is shown by a technique using the *Campylobacter* enrichment supplement (FBP; Oxoid, Hampshire, UK) which contains ferrous sulphate ($FeSO_4.7H_2O$), sodium metabisulphate and sodium pyruvate each at a concentration of 2.5 mg/ml. When a strain produces hydrogen sulphide, the sulphur combines with the iron to form a black precipitate of iron acetate.

***Materials and Reagents***

- FBP/Glycerol medium (see section on differential diagnosis of infectious abortion in small ruminants).

***Procedure***

1. Prepare the FBP/glycerol medium.
2. Heavily inoculate the medium with colonies removed from a 48 or 72 hour culture.
3. Incubate at room temperature for several hours in the dark.

***Reading and interpretation of results***

Production of $H_2S$ will be indicated by the formation of black precipitate in the medium.

### *8.5.3.6 Hippurate hydrolysis*

***Principle***

When hippurate is hydrolysed, glycine and benzoic acid are formed. A ninhydrin tube test can be used to detect glycine production or gas-liquid chromatography (GLC) can be used to detect benzoic acid. The ninhydrin test is simple to conduct but can occasionally be difficult to interpret whereas GLC is more reproducible but is technically more difficult and requires specialised equipment. One variation of the ninhydrin tube test is described [1].

***Materials and reagents***

- Sodium hippurate solution (1% v/w in distilled water).
- Ninhydrin solution (3.5% ninhydrin in a 1:1 mixture of acetone and butanol).

***Procedure***

1. Remove a large loopful of 24 or 48 hour inoculum grown on thioglycolate agar containing 5% sheep blood or brucella agar containing 5% sheep blood and emulsify with 0.4ml of sodium hippurate solution in a suitable tube.
2. Incubate for 2 hours at 37°C in a water bath.
3. After 2 hours add 0.2ml ninhydrin solution with a pipette down the side of the tube to form an overlay.
4. Incubate for 10 minutes at 37°C in air and then examine tube by eye.

***Reading and interpretation of results***

Production of a deep purple colouration indicates a positive reaction. No colour or a weak colour reaction are considered negative.

## 8.5.4 Further typing methods

A number of commercial systems have been developed as an aid to identifying selected *Campylobacter* species to genus level. Two latex agglutination assays (Meritec Campy jcl, Meridian Diagnostics, Ohio, USA [2] ; Campyslide, BBL Microbiological Systems, Cockeysville, USA [3]) can detect *C. jejuni* and *C. coli* but

cannot differentiate between them. Many typing systems have been devised to study epidemiology of *Campylobacter* infections; they vary in complexity and ability to discriminate between strains. These methods include serotyping, biotyping, bacteriocin sensitivity, detection of preformed enzymes, auxotyping, lectin binding, phage typing and multilocus enzyme electrophoresis. The most frequently used system is serotyping which tends to be performed in only a few reference laboratories because of the time and expense needed to maintain quality serotyping antisera. Commercially available heat-labile serotyping reagents are generally of poor quality.

### 8.5.5 DNA analysis

The requirement for rapid and sensitive methods of detection of *Campylobacter* in human faeces following outbreaks of food poisoning has led to the development of molecular-based methods such as restriction endonuclease analysis, ribotyping and polymerase chain reaction (PCR), in particular for the thermophilic *Campylobacter* species. A DNA probe directed against Campylobacter RNA sequences is available commercially (Accuprobe, Gen-Probe Inc, California, USA) for detection of *C. jejuni*, *C. coli* and *C.laridis* [4]. The probe has been shown to be 100% sensitive but also hybridised with some strains of *C. hyointestinalis*. At present such methods may be useful for confirming *Campylobacter* species if other tests are not conclusive. Investigations into the use of PCR techniques to detect and differentiate different strains of Campylobacters have also been reported [5].

The use of such techniques in veterinary diagnosis and development of probes specific to *C. fetus* have still to be evaluated.

## 8.6 INDIRECT DIAGNOSIS

### 8.6.1 Histopathology

***Principle***

A suitable method for histopathological preparation of tissue samples (placenta and foetal liver) and haematoxylin and eosin staining is described in the chapter on Toxoplasmosis.

Immunohistochemical labeling methods can also be applied to histological tissue sections and smears of foetal stomach contents to detect *Campylobacter* antigens using the techniques described in the chapter on *Toxoplasmosis*. The ABC indirect immunoperoxidase method is available commercially in kit form (VECTASTAIN® ABC Kit 4001, Vector Laboratories, USA) combined with an appropriate primary antibody. Alternatively the peroxidase anti-peroxidase (PAP) technique can be carried out as described in pages 104-105 using suitable primary and binding antibodies.

***Reading and interpretation of results***

Histologically, changes found in the placental tissue include large areas of necrosis in the chorionic villi, arteriolitis and thrombosis in the hiliar zone of the placentomes, abundant necrotic debris and high numbers of leukocytes within the stroma. Bacterial antigen can be detected within the placental trophoblasts lining most chorionic villi and within the subadjacent stroma, vascular endothelium and lumens.

The histological appearance of lesions in the foetal liver are of multiple, focal areas of necrosis.

## 8.7 FUTURE WORK

Abortion resulting from infection by *C. fetus* or *C. jejuni* in sheep and goats can only be confirmed by isolation and identification of the causal organism in aborted tissues and vaginal secretions. Campylobacters can be difficult to isolate and current techniques of isolation and identification are not ideal. Recent interest in improving techniques has mainly centered on *C. jejuni* and other species pathogenic to humans rather than *C. fetus*. Improvements to techniques to date therefore have limited application to veterinary samples. However, application of such improvements to veterinary materials will hopefully lead to :

- Improvements in the sensitivity and specificity of isolation media.
- Simplification of biochemical tests for species identification such as the ninhydrin test.
- Introduction of other typing systems such as serotyping, biotyping, bacteriocin sensitivity, detection of preformed enzymes, auxotyping, lectin binding, phage typing and multilocus enzyme electrophoresis.
- Application and expansion of molecular biological techniques such as PCR.

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# Chapter 9

# SALMONELLOSIS

R. SANCHIS & P. PARDON

## 9.1 SUMMARY

Infection with the ubiquitous serotypes of *Salmonella enteritica* can cause a range of clinical symptoms in small ruminants including septicaemia, enteritis or abortions. These symptoms, which depend on the physiological status of the animals and the epidemiological conditions, however, are rarely enzootic in nature.

In contrast, abortion occurs as the main manifestations of infection with the specific subspecies of *Salmonella enterica*, enterica type abortusovis which is a host-specific serotype of sheep. Abortion resulting from infection with this serotype is always endemic in nature and can affect up to 60% of pregnant ewes in a flock.

Direct diagnosis of abortive salmonellosis is undertaken on the products of abortion using standard bacteriological techniques, followed by identification of characteristic antigens specific to the causal serotype. Seroagglutination still remains the most common method of indirect diagnosis. Serodiagnosis, using an antigen prepared from the serotype being detected, is a flock diagnosis intended to confirm the source of infection and to establish the endemic nature of the disease.

## 9.2 INTRODUCTION

Abortions resulting from Salmonellosis in small ruminants are caused by *Salmonella enteritica* subspecies enterica which is a sub-type I of *S. enterica*. *Salmonella enterica* is one of two species of the *Salmonella* genus and is divided into six sub-types according to biochemical and genetic criteria. Within these subtypes there are more than 2,000 serotypes classified into groups by means of their major O antigens.

The development of infection, clinical signs and the epidemiology vary according to serotype (Table 9.1). Abortion is the main manifestation of infection with *Salmonella enterica* subspecies enterica type abortusovis (referred to as S.abortusovis), a serotype that almost exclusively affects ovines [1, 2]. Infection with this serotype is always endemic in nature and can affect up to 60% of the pregnant ewes in a flock. In contrast, the acute form of disease caused by other serotypes which is transmissible to humans and other species of animals, generally causes septicaemia

*Table 9.1 : Three examples of Salmonella infections*

| | Hots | | | | Clinical signs | | | | | Evolution |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Adults | | Youngs | | | |
| Serotype | Humans | Bovine | Small ruminants | Other species | Abortion | Enteritis | Enteritis | Pneumonia | Septicemia | |
| *S typhimurium* | ++ | +++ | ++ | ++ | + | +++ | ++ | ++ | ++ | Sporadic |
| *S dublin* | + | +++ | + | + | +++ | + | ++ | ++ | ++ | Enzootic |
| *S abortusovis* | - | - | +++* | - | +++ | - | - | ++ | ++ | Enzootic |

* : *mainly sheep*

and normally affects young animals. The enteric or abortive subacute form mainly affects adults and tends to occur sporadically in small numbers of animals with abortion generally constituting only one of the clinical symptoms.

Abortion resulting from *S. abortusovis* infection generally occurs in the second half of gestation, usually without any faecal excretion of the organism, although earlier abortions may occur. The economic consequences of infection depend on the time abortion occurs and any complications that may follow, for example, retained placentas can lead to fatal septicaemia with accompanying faecal excretion [13].

Experimental work has shown that infection via the mucus membrane can lead to early colonisation of the drainage lymph nodes. This is followed by a phase of transient dissemination during which no clinical signs are observed. Finally colonisation of the genital tract, which is most susceptible after the third month of gestation, takes place [8, 13]. The resulting abortions usually occur during the third trimester of gestation and generally a large number of ewes are affected. Infection can also lead to stillbirths or to pulmonary infections in lambs of one to three months old.

*Salmonella abortusovis* has been isolated mainly in Europe, western Asia and South America. The main source of contamination is from the products of abortion, with all parts of the uterus being infected. Excretion of the organism in milk or faeces is usually irregular and only sustained during periods of septicaemia accompanying retained placentas [13]. The exact modes of transmission, apart from by contact between adults, are not well known. Venereal transmission, probably of little importance, cannot be excluded and similarly prenatal and perinatal transmission with survival of the organism in an asymptomatic carrier until sexual maturity may occur.

Survival of *Salmonella* in the external environment is generally better than that of other enterobacteria. The ubiquitous serotypes, able to infect other species of animals, frequently contaminate water courses, vegetables and produce of animal origin whereas any *S. abortusovis* present is rarely isolated.

Antibiotic treatment must take account of a number of resistances that have appeared, notably in the ubiquitous serotypes of *Salmonella*. An antibiotic resistance test must be carried out before starting any treatment regime. However, treatment of all supposedly infected animals is expensive and often not very effective.

Specific and systematic vaccination to prevent sporadic *Salmonella* infections caused by the ubiquitous serotypes is not profitable for the farmer. Inactivated and adjuvant vaccines which require many successive injections have a limited efficacy against *S. abortusovis* infections. Use of a live attenuated vaccine [9] allows more satisfactory control of infection and of the clinical and economic consequences.

*Salmonella* infections resulting in abortions are not subject to any statutory regulations in contrast to others such as those relating to *Salmonella* contamination of products destined for food and those concerned essentially with the ubiquitous serotypes.

## 9.3 SAMPLES

Diagnosis of abortions due to *Salmonella* is a flock diagnosis and is an inherent component of the differential diagnosis of infectious abortions. Samples for both direct and indirect diagnosis must be collected from several animals that have aborted. However, the

two types of diagnosis are rarely undertaken simultaneously, either for reasons associated with the breeding conditions of small ruminants or for economic considerations.

### 9.3.1 Direct diagnosis

Direct diagnosis involves the isolation of the bacteria therefore samples should be collected as aseptically as possible.

#### *9.3.1.1 Choice of sample*

All products of abortion will be heavily infected and suitable for isolation of *Salmonella*. The aborted foetus provides the best sample material but it is useful if the placenta or vaginal samples are also collected.

#### *9.3.1.2 Treatment of samples*

***Aborted foetus***

Any organs from the aborted foetus can be used for isolation of Salmonella. The brain and the stomach contents provide the best samples since they are generally heavily infected and can remain protected from external contamination for long periods. Following autopsy of the aborted foetus, samples of organs (liver, lung, spleen), brain (samples from the occipital lobe) or the stomach contents should be placed in sterile containers.

***Placenta***

Since the cotyledons are unlikely to be evenly infected it is best to sample several (5 to 10). If the cotyledons are heavily soiled they should be washed carefully in physiological saline and dried on absorbent paper. Pieces of the sampled cotyledons should be ground in a similar volume of sterile physiological saline prior to bacteriological examination.

***Vaginal samples***

It is not generally necessary to undertake any preparation of vaginal samples. However, if samples are dry or not very abundant, a suspension of the swab or vaginal mucus can be made in 0.5-1.0ml of sterile physiological saline before cultural methods are undertaken.

If samples are required for other uses, they can be stored for several days at 4°C or for several weeks at -20°C without any major effect on the isolation of *Salmonella*.

### 9.3.2 Indirect diagnosis

Blood samples should be collected aseptically in tubes containing no anticoagulant during the 8 weeks following the time of abortion or poor lambing because of the kinetics of antibody production (Figure 9.1). The best time to undertake testing is during the first 4 weeks.

To satisfy the requirements of flock diagnosis, samples must be collected from 5 to 10 females that have aborted. If this number of samples are not obtained at the time of intervention, it is possible to make up the numbers by collecting samples from animals which lamb at term. If the diagnosis is carried out after the abortions or if the animals that aborted are not identified, samples must be taken from a representative number or at least twenty adult females in the flock.

#### *9.3.2.1 Treatment of samples*

In the laboratory, after removal of the blood clot, the sera should be centrifuged (5 minutes at 500 xg). If analyses are not undertaken immediately, capped tubes of sera can be held for 5 to 8 days under refrigeration (4 to 8°C) or frozen (-20°C) for several weeks without any noticeable fall in antibody titre.

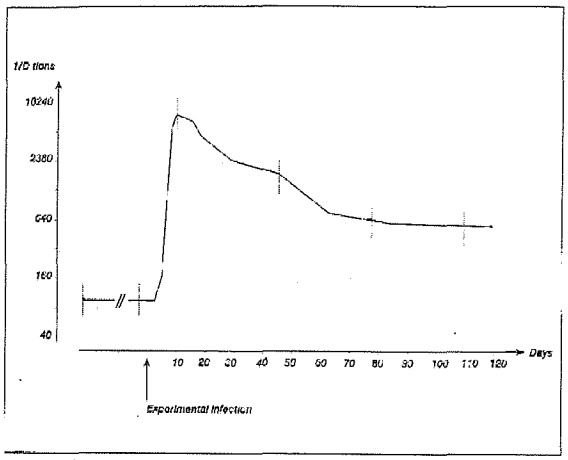


*Figure 9.1 : Antibody kinetics after experimental infection of sheep with Salmonella abortusovis (seroagglutination microtechnique)*

## 9.4 RISKS TO HUMAN HEALTH

Small ruminants can be asymptomatic carriers of a number of the ubiquitous serotypes of *Salmonella* [4]. The main sources of contamination are faeces, vaginal excretions, products of abortion and, less frequently, milk. Infected animals can contaminate the environment directly as well as personnel involved in lambing. Food products of animal origin contaminated as a result of infection or indirectly during the course of handling can cause food poisoning which can pose public health problems.

*Salmonella abortusovis* is a highly host-specific serotype considered to be naturally non-pathogenic for humans and human infection has never been reported even in endemic areas. In the laboratory, however, the products of abortion must be treated with all the precautions applicable to handling and disposal of potentially infectious materials.

## 9.5 DIRECT DIAGNOSIS

Direct diagnosis involves the isolation and identification of *Salmonella* in the products of abortion. The number of cultures set up depends on the nature and the quality of the available samples. To maximise efficiency of diagnosis at least two organs from the aborted foetus (brain and stomach contents or others) and the placenta or a vaginal sample should be analysed.

### 9.5.1 Isolation

***Principle***

*Salmonella enterica* subspecies enterica constitute the majority of strains isolated from man and warm-blooded animals. Specific culture media are not required for isolation and there are no differential staining methods applicable to identification of *Salmonella* species. *Salmonella* are small, Gram negative bacillus which occur in sufficient abundance in the products of abortion to avoid any use of enrichment media. Selective media can be used for culture and presumptive identification of *Salmonella* in order to limit interference from Proteus and development of Gram positive bacteria. The most frequently used selective media is "*Salmonella-Shigella*" (SS).

***Materials and reagents***

- Grinder.
- Standard nutrient media.

***Procedure***

1. Culture samples of organs from the aborted foetus, removed by nicking with a Pasteur pipette after cauterisation of the surface, by streaking on nutrient agar or placing in 10ml of nutrient broth.

2. Make one streaked culture on selective media (for example, SS) using a ground sample of pieces of cotyledons.

3. Culture vaginal mucus by streaking on the selective media. Vaginal swabs can also be applied directly to a Petri dish.

***Reading of results***

After 18 to 24 hours incubation at 37°C, the ubiquitous *Salmonella* appear as colonies of 2-4 mm in diameter. On SS media these colonies are white (absence of lactose breakdown) and have a black centre (production of $H_2S$) (Figure 9.2, B and C, page 96). Subculture isolated colonies onto nutrient agar. If problems arise in isolation (overgrowth by contaminants or cultures negative on agar) further isolation can be attempted using nutrient broth.

Colonies of *S. abortusovis* are small (sometimes < 1 mm) and often occur individually: small colonies may coexist with other much larger ones and can be separated by subculturing. On selective media, the colonies are very small presenting no distinguishing characteristics (even a black centre) and can easily be confused with contaminants (Figure 9.2, A, page 96).

***Preparation of reagents***

- Nutrient Broth

| | |
|---|---|
| Peptone | 5g |
| Yeast extract | 2g |
| Meat extract | 1g |
| NaCl | 5g |
| Demineralised water | 1 litre |

Adjust pH to 7.4.

- Nutrient Agar

Add 15g of agar to the previous preparation.

Sterilise for 20 minutes at 120°C

- Selective *Salmonella*-Shigella Media

| | |
|---|---|
| Peptone | 5g |
| Meat extract | 5g |
| Bile salts | 8.5g |
| Sodium citrate | 10g |
| Sodium thiosulphate | 8.5g |
| Ferrous citrate | 1g |
| Lactose | 10g |
| Neutral red | 0.025g |
| Agar | 15g |
| Brilliant green | 0.00033g |
| Demineralised water | 1 litre |

Adjust pH to 7.0. Sterilise for 20 minutes at 120°C.

***Note***

• Because of the particular characteristics of *S. abortusovis*, it is sometimes best to incubate for 48 hours to assist the subcultures.

## 9.5.2 Identification

### 9.5.2.1 Biochemical characteristics

***Principle***

Isolated *Salmonella* can be identified by standard biochemical tests used for the diagnosis of enterobacteria.

The classical media for identification of enterobacteria (peptone water with phenol red and others) are generally rarely used in routine diagnosis having been replaced by complex commercially available media (Kligler-Hajna, Mannitol-mobility-nitrate, Urea-indole, etc.) which allow a range of identifications to be carried out.

***Procedure***

1. Check the purity of the culture by Gram stain.
2. Culture isolates grown on nutrient media on the identification media.

***Reading of results***

Read the results of the identifying biochemical characteristics [3] after overnight incubation at 37°C (Table 9.2).

*Table 9.2 : Principle characteristics for identification of Salmonella enterica subspecies enterica*

| | **Ubiquitous serotypes** | ***S.abortusovis*** |
|---|---|---|
| β–galactosidase (ONPG Test) | - | - |
| Production of acid from : Lactose | - | - |
| Dulcitol | + | ±[a] |
| Mannitol | + | + |
| Utilisation of malonate | - | - |
| Production of : Gas from sucrose | + | -[b] |
| Hydrogen sulphide | + | -[b] |
| Indole | - | - |
| Growth in the presence of KCN | - | - |
| Hydrolysis of gelatin | - | - |
| Methyl red | + | + |
| Vogues Proskauer reaction | - | - |
| Culture on Simmons citrate | + | - |
| Presence of a urease | - | - |
| Reduction of nitrate to nitrite | - | - |

*a : Variable, b : Negative or poor expession after 24 hours.*

***Note***

• In endemic areas, *S. abortus ovis* represents more than 95% of the Salmonella strains recovered from sheep. The other auxotrophic strains, for which the pathogen is confined to a particular host, multiply more slowly [1, 11]. Degradation of sucrose, production of gas and reduction of nitrate to nitrite are often poor in 24 hour cultures and may lead to errors in identification. As a precaution it is sometimes necessary to test for biochemical characteristics after 48 hours incubation and to avoid the use of rapid systems of identification.

### *9.5.2.2 Determination of serotypes*

***Principle***

Serotypes are differentiated either on the basis of their membrane antigens (O antigens) or their flagella antigens (H antigens) or both. Identification is undertaken by slide agglutination according to the KAUFFMANN-WHITE system [3].

Precise identification of strains (serotype, biotype, lysotype) is usually only carried out in reference laboratories although a presumptive diagnosis of the principal abortive serotypes can be reached in an analytical laboratory. Commercial producers (for example, Sanofi-diagnostic Pasteur, Marnes-la-Coquetter, France or Difco Laboratories, Detroit MI, USA) retail a mixture of sera produced from the major antigens of each group which can be used for initial differentiation. The principal abortive serotypes can then be identified using monospecific sera (Table 9.3).

***Materials and reagents***

- Glass or wooden rods.
- Mixed sera.
- Monospecific anti-O and anti-H sera.

*Table 9.3 Antigenic identification of several potentially abortive serotypes of Salmonella*

| Serotypes | Antigens Somatic "O" | Flagella Antigens "H" | |
|---|---|---|---|
| | | Phase 1 | Phase 2 |
| *S. abortusovis* | 4, 12 | (c) | 1, 6 |
| *S. typhimurium* | 1, 4, (5), 12 | i | 1, 2 |
| *S. dublin** | 1, 9, 12, (Vi) | g, p | - |
| *S. enteritidis* | 1, 9, 12 | g, m | (1, 7) |

*1 : prone to phagic conversion ; ( ) : may be absent ; (*) : monophasic serotype.*

***Procedure***

1. Place a drop of each of the mixed sera on a slide.
2. Using a loop, remove isolated colonies of the type to be tested from the surface of the isolation agar or from an identification media and place next to each drop of serum.
3. Using a different rod for each, mix the bacteria with each drop of specific serum.

***Reading of results***

Agglutination is generally rapid and clearly visible.

Repeat the procedure for samples that react with the mixed sera using each of the monospecific serum.

Somatic (O) agglutinations are usually "granular" and difficult to discern. Flagella (H) agglutinations are more flocculent and easier to discern.

***Note***

• Two biotypes of *S. abortusovis* exist [11]. One of these is always in phase 2 (Table 9.3), does not ferment dulcitol and can present problems of precise identification.

### 9.5.3 Interpretation of results

Isolation of *Salmonella* by the described methods is always indicative of an infection. However, *Salmonella* isolation from a single abortion case is not necessarily proof of the cause of the abortion outbreak, particularly in areas of multiple infections. In such areas, *Salmonella* and another abortive agent may be simultaneously isolated from the same farm and even from the same animal. The endemic nature of the infection must therefore be established in many samples or confirmed by serological testing.

When vaginal samples are the only samples available, negative results must be interpreted with care since vaginal excretion is intermittent and occurs for a limited period after abortion [6, 7, 13]. To maximise reliability of the results, many samples should be collected on the day following abortions. Any positive results are significant of infection.

## 9.6 INDIRECT DIAGNOSIS

The aim of indirect diagnosis is to establish the endemic nature of infection. It involves the quantitative measurement of specific antibodies raised against *Salmonella* infection. The reference technique is the slow seroagglutination test [10].

In sporadic infections caused by the ubiquitous serotypes, testing is not generally undertaken to confirm the cause of infection after the responsible serotype has been

identified. Serodiagnosis of salmonellosis is only undertaken in a systematic fashion in endemic areas of *S. abortusovis* infection.

## 9.6.1 Seroagglutination test

***Principle***

The seroagglutination test involves the combining of an inactivated antigen with increasing dilutions of the test sera. The analysis is usually limited to testing of anti-H agglutinins which provide an earlier and consistent result and are more specific than the anti-O agglutinins [3, 7, 10, 13]. Group diagnosis necessitates sampling of animals that have aborted on different dates but it removes the requirement to detect seroconversion. One technique using microtitre plates with a stained antigen [14] allows large groups of samples to be analysed simultaneously making the testing more convenient and reading of the results easier.

### *9.6.1.1 Antigen preparation*

The antigen and a standard positive serum are usually commercially available from National Laboratories (for example, CNEVA, Sophia Antipolis, France). They can also be prepared from a local strain of *S. abortusovis* expressing the two phases of flagella agglutination for the serotype [14].

***Materials and reagents***

- 0.5% triphenyltetrazolium chloride in distilled water.
- Formalin.
- Physiological saline.
- Nutrient broth.

***Procedure***

1. Seed 500ml of media with 50ml of a 24 hour culture.
2. Incubate at 37°C for 6 hours.
3. Add 25ml of the 0.5% triphenyltetrazolium chloride solution.
4. Incubate at 37°C. A red precipitate should appear in less than 2 hours.
5. Add formalin to a final concentration of 0.5%.
6. Incubate overnight at 37°C.
7. Centrifuge for 30 minutes at 4000 xg.
8. Resuspend the precipitate in 10 volumes of physiological saline containing 0.5% formalin.
9. Repeat steps 7 and 8.
10. Titrate each batch of antigen against a standard positive serum.

### *9.6.1.2 Agglutination test*

***Materials and reagents***

- Haemolysis tubes (5ml) and carrier.
- Microtitre plates (round bottom wells).
- Adhesive film for microtitre plates or humidity chamber.
- Mirror for reading plates.
- Diluent: Physiological saline: 9% NaCl (w/v) in distilled water.
- Colour labelled antigen of known titre.
- Negative control sera.
- Standard positive sera or secondary standard of known titre.

***Procedure (Figure 9.3)***

1. Prepare initial 1/20 dilutions (50µl/950µl) of the test sera and the control sera in haemolysis tubes.
2. Place 50µl of the 1/20 serum dilution in the first well.
3. Add 50µl of physiological saline to all the wells.
4. Make doubling dilutions of the sera by transferring 50µl from the first well to the next and then continuing until the

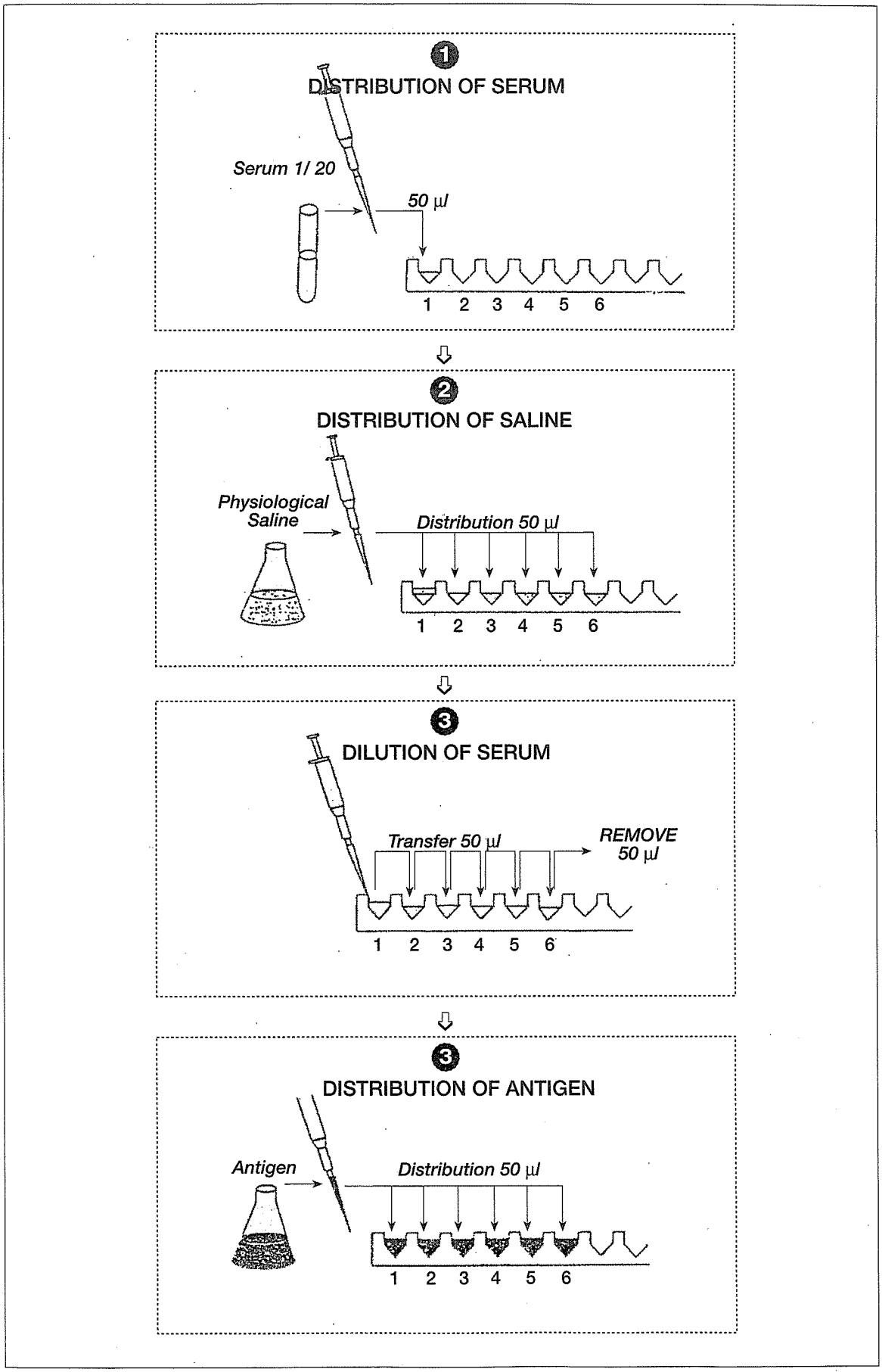


*Figure 9.3 : Seroagglutination microtechnique. Reaction method*

sixth well. Discard the final 50µl. In a series of 6 wells the serum dilutions will now range from 1/40 to 1/1280.

5. Rinse or change the tip on the micropipette between each serum sample.

6. Mix the concentrated antigen solution and prepare a diluted solution according to the titre (5 ml for each plate).

7. Add 50µl of diluted antigen to all wells. The sera are then diluted between 1/80 and 1/2560.

8. Gently agitate the plates.

9. Incubate the plates overnight at 37°C under conditions that prevent evaporation (cover with adhesive film or incubate in a humidity chamber).

***Reading of results***

At the end of the incubation examine the wells with a reading mirror. Total agglutination (100%) is expressed by the deposition of a pink layer in the bottom of the wells, the absence of agglutination by the deposition of a dark red round disc in the bottom of the wells (Figure 9.4, page 96). The amount of agglutination in the test sera wells is assessed by comparison with the agglutination in the positive and negative controls. The titre of each test serum is the lowest dilution showing less than 50% agglutination.

*Note*

• The test can be undertaken in haemolysis tubes with reagent volumes increased to 0.5ml and the same system of reading and interpretation of the results.

### 9.6.2 Interpretation of results

Interpretation of the results is as follows:

POSITIVE : titre ≥ 1/640
SUSPECT : titre = 1/320
NEGATIVE : titre < 1/320

In cases of *S. abortusovis* infection, animals that aborted and were sampled during the weeks that followed the abortions should have titres > 1/640.

Titres observed around 1/640 for some animals only suggests a previous vaccination, an old infection or a more recent infection by a serotype similar to *S.abortusovis*.

Beyond 8 weeks after abortions or low productivity, titres decrease rapidly and the results must be interpreted with care.

## 9.7 FUTURE WORK

The described techniques allow the diagnosis of advanced *Salmonella* infections but are poorly adapted for diagnosis of latent infections and for the detection of asymptomatic carriers.

The application of immunoenzymatic techniques to the detection of *Salmonella* infection has not been very successful mainly due to a lack of specificity resulting from the large common antigens present within the family of enterobacteria. It is probable, however, that the current development of powerful molecular biological techniques will, in time, lead to new methods of direct and indirect diagnosis.

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# Chapter 10

# LISTERIOSIS

J.C. LOW

## 10.1 SUMMARY

*Listeric* infections, caused by micro-organisms of the genus *Listeria*, occur world-wide and in a variety of animals including man. The organism is widespread in the environment, in foodstuffs and many animals shed small numbers in their faeces. Two species, *Listeria monocytogenes* and *Listeria ivanovii* are pathogenic. The former causing encephalitis, abortion and septicaemia in a wide range of animal species, the latter is responsible for abortion and perinatal death in sheep, goats and occasionally cattle.

Direct diagnosis of listeric abortion is by isolation of the organism from abortive material on non-selective or selective media. Histological examination may be necessary for conclusive diagnosis since the organism may occasionally be isolated from normal placentas resulting from faecal contamination. Although serological tests are available for the detection of antibodies against *Listeria*, these are not used in the diagnosis of abortive listeriosis as antibodies are frequently found in the sera from healthy animals with no histories of listeric infections and many of the assays suffer from a lack of specificity.

## 10.2 INTRODUCTION

The bacterium now known as *Listeria monocytogenes* was first isolated from an epidemic disease of rabbits and guinea-pigs in a laboratory animal breeding unit in 1926 and causes infectious disease in both animals and man. Infection is truly widespread, having been recorded in more than forty mammalian species as well as in fowls, fish, crustaceans, flies and ticks and in countries in six continents. Listeriosis is of major veterinary importance in cattle, sheep and goats with encephalitis and uterine infections being most frequently identified.

The genus *Listeria*, formerly thought to contain only one species, *Listeria monocytogenes*, has now been expanded and contains : *L. monocytogenes* sensu stricto, *L. ivanovii*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, and *L. murrayi*. Clinical infections are caused primarily by *L. monocytogenes* though *L. ivanovii* is also pathogenic, being particularly associated with abortion in ruminants. The remaining species are regarded as non-pathogenic. *Listeria monocytogenes* can be divided into 16 serovars [1] on the basis of somatic and flagella antigens with serovars 4b, 1/2a and 1/2b most commonly isolated from disease problems. Virulent strains produce a haemolysin, listeriolysin O, which is a major virulence factor and enables intracellular multiplication within macrophages, monocytes and other cells.

*Listeria monocytogenes* is a small Gram positive, non-sporing rod, 1-2μm in length and 0.5μm wide, with bacteria often lying parallel to each other in palisades, and thread like forms present in some cultures. The optimum temperature range is 30 to 37°C but growth occurs between 3 and 45°C and *Listeria* are

among the few pathogenic bacteria that grow readily at 4°C. The organism multiplies in aerobic or microaerophilic conditions at pH values as high as 9.6. Growth is absent or scant in complete anaerobic conditions and multiplication is inhibited by pH values lower than 4.5. The bacterial colonies are small, smooth, slightly flattened and milky white by reflected light. When illuminated by obliquely transmitted light the colonies exhibit a characteristic blue/green colour. Organisms grown at 37°C show little or no motility but a characteristic tumbling motility can be demonstrated by incubation of cultures at room temperature with motile organisms commonly possessing many peritrichious flagellae.

Clinical disease in animals occurs mainly in the northern and southern latitudes and is much less commonly recognised in tropical and subtropical climates. Encephalitis, abortion and septicaemia are the most prevalent manifestations in sheep and goats. Though it is rare for an outbreak of encephalitis and abortion to occur simultaneously these may follow each other in a single flock or herd and septicaemia may occur alongside encephalitis or abortion. Occasionally mastitis, spinal myelitis or keratoconjunctivitis result from listeric infections.

Listeric abortion caused by *L. monocytogenes* occurs in ruminants and many other species of domestic animal. *Listeria ivanovii* is also recorded as a cause of abortion in sheep, goats and cattle but occurs less frequently than *L. monocytogenes*. Cases of listeric abortion in sheep and goats are usually sporadic although in exceptional circumstances up to 50 percent of a flock have been affected. Occasional massive outbreaks of septicaemia involving pregnant ewes have been described with clinically affected animals being pyrexic showing profuse diarrhoea and subsequently aborting. Experimentally, oral infection does not consistently produce abortion but the gravid uterus is highly susceptible to infection and abortion is readily produced by the intravenous injection of pregnant ewes with *L.monocytogenes* or *L. ivanovii*. In the pregnant animal invasion of the placenta and foetus occurs within 24 hours of the onset of bacteraemia and, following oedema and necrosis of the placenta, abortion arises 5-10 days post-infection. In sheep and goats abortion is seen from the 12th week of pregnancy onwards and there is a blood-stained vaginal discharge for several days. Infection in late pregnancy results in stillbirths or the delivery of young which develop a fatal septicaemia. Aborted foetuses are rarely mummified but may be autolysed. In most cases ewes or nanny goats make uneventful spontaneous recoveries and other forms of infection are unlikely to occur. On some farms listeric abortion may recur each year.

The natural habitat for all *Listeria* species including *L. monocytogenes*, is the environment and the organism is widespread having been isolated from surface soils, decaying vegetation, pasture herbage, silage, sewage sludge, animal slurry, factory effluent and surface and river waters. The organism has been shown to persist for up to 7 months on dry straw, up to months in damp soil and for more than 2 years in dry soil and faeces. In most animal species including ruminants *L.monocytogenes* can occasionally be isolated from the faeces, nasal secretions and milk of healthy animals. Factors that are believed to predispose the host to clinical disease include :

- factors that cause a lowering of the host animal's resistance

1. the stress of late pregnancy and parturition;

2. poor nutritional state;

3. sudden changes of weather to very cold and wet;

- or factors that increase the infection pressure of the organism, such as massive multiplication of *L. monocytogenes* in feed or the environment.

Although the organism is ubiquitous, listeric infections have been particularly associated with silage feeding. Much of this association is attributable to the high bacterial numbers which may be present in poorly preserved silage (>$10^7$ *L. monocytogenes* colony forming units (cfu) per kilogram). Abortive disease in sheep and goats can occur within 6-13 days of introduction to silage. It is unclear to what extent infection of other animals occurs following abortion but in the majority of instances abortions are sporadic and major outbreaks are associated with heavily contaminated foodstuffs.

Practically all common antibiotics, except cephalosporins, are active against *L.monocytogenes* in vitro. However, in vivo, a low efficiency may be expected and is probably in part due to the organism's intracellular location. Experimentally, ampicillin, amoxicillin are most active. Tetracycline and chloramphenicol though widely used are reportedly not the therapeutic agents of choice. Though antimicrobial resistance in clinical isolates is rare, resistance to chloramphenicol, erythromycin, streptomycin and tetracycline has been recorded.

Most attempts to produce a satisfactory vaccine have been unsuccessful, but a live attenuated vaccine has been in use in Norway for several years and is reported to reduce the annual incidence of listeric encephalitis from 4% to 1.5% in field trials. A commercial killed vaccine is available for the control of the disease in some other countries. However, the results of field trials for each of these vaccines are equivocal and no experimental model is available to test their efficacy. Since listeriosis is a disease of complex aetiology, further investigation into mechanisms of immunity and their role in the pathogenesis of disease are necessary before effective vaccines can be developed.

Although there is potential for zoonotic transmission of *Listeria* it would appear that the majority of human exposure to the organism, and risk for disease, results from contamination of foods during processing and from the particular ability of *Listeria* to grow at refrigerator temperatures. There is therefore no reason for statutory control measures to be taken to protect consumers following an outbreak of listeric abortion, though stock handlers should adopt routine hygiene precautions.

## 10.3 SAMPLES

Listeriosis can only be confirmed by isolation and identification of the specific aetiological agent. Samples of choice are the aborted placenta, foetus and vaginal excretions. Histopathological examination of aborted tissue samples is also advised for positive diagnosis.

### 10.3.1 Isolation of *Listeria*

Samples of placenta, the liver and spleen of the aborted foetus should be collected aseptically and placed in a suitable sterile container.

Stomach contents should be removed from the foetus as soon as possible after abortion using a sterile needle and either a sterile syringe or a sterile, evacuated glass tube (e.g. Vacutainer).

Vaginal swabs must be collected

within 48 hours after abortion. Immediately after collection the swabs should be placed in their protective sheaf and transported to the laboratory.

On arrival in the laboratory the samples should be cultured immediately or stored at 4°C.

### 10.3.2 Histopathology

Small representative samples of the placenta and the liver and spleen from the aborted foetus, of no more than 0.5cm thickness, should be collected as quickly as possible after abortion or death and placed in a glass sample jar containing a suitable fixative such as formol saline (0.85g NaCl dissolved in 10ml 40% formaldehyde and 90ml of water), with a ratio of fixative to tissue of at least 10:1 (v/v). The tissues should be fixed for at least two days before further processing.

## 10.4 RISKS TO HUMAN HEALTH

Despite the apparently low invasiveness of *L. monocytogenes* all suspect material should be handled with caution. Aborted foetuses and necropsy of septicaemic animals present the greatest hazard and such materials should only be cultured in properly equipped laboratories under the control of a skilled microbiologist. Personnel have rarely developed fatal meningitis, septicaemia and papular exanthema on the arms after handling aborted material. However, low-grade infection in man may be more common than suspected and, in pregnant women may lead to death of the foetus and for this reason pregnant women should not work with known cultures of *Listeria*.

## 10.5 DIRECT DIAGNOSIS

### 10.5.1 Gross pathology of placenta and aborted foetuses

Placental lesions are pin-point, yellowish, necrotic foci involving the tips of the cotyledonary villi with a focal to diffuse intercotyledonary placentitis covered in a red/brown exudate.

The foetus often shows few lesions because of advanced autolysis but miliary necrotic foci with pin-point greyish, white nodules are occasionally visible throughout the liver and spleen. There may also be small abomasal erosions and yellow-orange meconium.

### 10.5.2 Isolation of *Listeria*

***Principle***

*Listeria* usually grows on most non-selective culture media especially when blood is added. A wide range of selective media have also been developed providing increased sensitivity and specificity of isolation mainly in response to the requirement to detect low numbers of *Listeria* in human foodstuffs. Of these media LPM agar, Oxford agar, Modified Oxford agar and PALCAM agar have particular application to veterinary samples and are commercially available (Oxoid, Unipath Ltd., UK). 5% sheep or horse blood agar is still a suitable media for culture of *Listeria* from samples collected from aborted ewes and nanny goats in which *Listeria* numbers are high and contamination with other bacteria is minimal.

***Materials and reagents***

- 5% sheep or horse blood agar plates.

***Procedure***

1. Inoculate 0.1ml of tissue or foetal stomach contents onto the surface of the isolation medium. Smear vaginal swab

samples directly onto the surface.

2. Incubate at 37°C for up to 48 hours.

3. Examine by a plate microscope for typical colonies of *Listeria* species after 24 and 48 hours incubation.

***Reading and interpretation of results***

Following overnight culturing on blood containing solid media at 37°C, the smooth form of *Listeria* give rise to colonies 1-2mm in diameter which are round, translucent and slightly raised, have an entire margin and a watery consistency, and are easily emulisfiable.

*Listeria monocytogenes*, *L .ivanovii* and *L. seeligeri* are the only haemolytic species and colonies of these species grown on blood agar are surrounded by variable zones of haemolysis. Colonies of *L. monocytogenes* are surrounded by a narrow zone of b-haemolysis, those of *L. ivanovii* are surrounded by a wide indistinct zone of haemolysis and an inner clear haemolytic zone. *Listeria seeligeri* cultures produce a faint narrow zone of haemolysis.

***Note***

• The selective media often contain indicators to aid the identification of *Listeria species*. However, it is recommended that multiple suspect colonies are subcultured on blood agar plates to allow an examination for haemolysis and prior to further identification.

• Isolation from tissue held at 4°C for several weeks and recultured at intervals, "cold enrichment", has been historically used for the isolation of *Listeria*. The technique is particularly appropriate where bacterial numbers are low but has largely been superseded by modern isolation methods. It is unlikely to be relevant to the diagnosis of listeric abortion.

***Preparation of reagents***

- Blood agar

The method of preparation of blood agar is described in the chapter on Campylobacteriosis.

## 10.5.3 Identification of *Listeria* species

Colonies that appear to be *Listeria* species on blood agar plates can be confirmed as such using three simple tests :

1. Gram stain.
2. Motility at room temperature.
3. Catalase reaction.

*Listeria monocytogenes* and *L. ivanovii* can be distinguished from other *Listeria* species on the basis of the CAMP test and other simple biochemical reactions (Table 10.1)[2]. Recently a rapid ten test strip identification system for *Listeria* species differentiation has been developed [3] and is commercially available (Api *Listeria*, BioMérieux, France).

*Table 10.1 : Identification of Listeria species*

| Character | *Listeria monocytogenes* | *Listeria ivanovii* | *Listeria seeligeri* |
|---|---|---|---|
| β-Haemolysis on blood agar | + | ++ | + |
| CAMP test with *Staphylococcus aureus* | + | - | - |
| CAMP test with *Rhodococcus equi* | + | + | - |
| Acid produced from: | | | |
| L-rhamnose | + | - | - |
| D-xylose | - | + | + |
| a- methyl D-mannoside | + | - | ± |

*++Strongly positive reaction ; + Positive reaction ; - Negative reaction ; ± Variable.*

### *10.5.3.1 Gram stain*

The Gram stain should be carried out by the method described in the chapter on Campylobacteriosis using colonies of *Listeria* removed from 24 or 48 hour cultures.

***Reading and interpretation of results***

Gram-positive organisms appear blue-black, Gram-negative organisms appear red.

*Listeria* cells are short Gram-positive rods and have a distinctive appearance and disposition often occurring as regimented pairs or rows adjacent to each other.

***Note***

• *Listeria* species are relatively easy to over-decolourise in comparison to other Gram positive bacteria. The cellular morphology of isolates direct from selective media are often irregular or bizarre.

### *10.5.3.2 Motility test*

***Materials and reagents***

- Bijoux bottles containing sterile broth (brain heart infusion or Nutrient broth n°. 2).
- Paraffin wax or "Blue-tac".

***Procedure***

1. Remove colonies from a 24 hour culture and inoculate into two bottles of broth.
2. Incubate for 4-6 hours, one bottle at room temperature and the other at 37°C.
3. Place single drops of the resultant broths on the underside of coverslips which are raised above a microscope slide on bridges of paraffin wax or "blue-tac" (hanging drop technique).
4. Examine cultures microscopically.

***Reading and interpretation of results***

A tumbling, rotating motility in cultures incubated at room temperature but not in cultures incubated at 37°C is characteristic of *Listeria* species. If motility is not seen at room temperature re-examine both cultures at 18 hours before discarding as negative.

***Note***

• Non-motile strains have been identified on rare occasions.

***Preparation of reagents***

- Brain Heart Infusion Broth

Add brain heart infusion (Oxoid-Unipath or Difco) according to manufacturer's directions to distilled water. Dispense 2.5ml volumes in sterile bijoux and autoclave at 15 psi for 15 minutes. Store at 4°C.

- Nutrient Broth N° 2

Add nutrient broth base n°2 (Oxoid-Unipath or Difco) according to manufacturer's directions to distilled water. Dispense 2.5ml volumes in sterile bijoux and autoclave at 15 psi for 15 minutes. Store at 4°C.

### *10.5.3.3 Catalase test*

The catalase test should be carried out by the method described in the chapter on Campylobacteriosis using a single drop from a 4-6 hour culture of *Listeria.*

***Reading and interpretation of results***

Bubbling indicates a positive catalase test. *Listeria* species are all catalase positive.

False positive catalase reactions may occur if a colony is taken from a medium containing blood.

The test may be safely performed using inoculated medium described in 10.5.3.2.

### *10.5.3.4 CAMP test (Christie-Atkins-Munch-Peterson)*

***Principle***

The CAMP test is used to differentiate between the three haemolytic species of *Listeria* on the basis of an enhancement of haemolysis that occurs when *L. monocytogenes* is cultured on blood agar plates alongside *Staphylococcus aureus* (NCTC 1803) and when *L. ivanovii* is cultured alongside *Rhodococcus equi* (NCTC 1621) [2, 4].

***Materials and reagents***

- 5% Sheep blood agar plates (Oxoid, Hampshire, UK).
- Standard cultures of *Staphylococcus aureus* (NCTC 1803) and *Rhodococcus equi* (NCTC 1621).
- Control cultures of *L. monocytogenes*, *L. ivanovii* and *L .seeligeri.*

***Procedure***

1. Prepare sheep blood agar plates by pouring a thin layer of 5% v/v blood agar made with washed sheep cells onto the surface of nutrient broth agar plates. Allow to set and dry before use.
2. Make parallel streaks of cultures of *S. aureus* and *R. equi* across each sheep blood agar plate.
3. Streak the test cultures at right angles to the *S.aureus* and *R.equi* leaving a minimum of 1-2mm between the cultures.
4. Incubate plates at 37°C overnight.
5. A separate sheep blood agar plate prepared using the known control cultures is recommended.

***Reading and interpretation of results***

Results are recorded as positive when an enhanced zone of haemolysis occurs between the test and standard culture. Table 10.2 shows the results obtained with the haemolytic species *L. monocytogenes* and *L. ivanovii* and the non-haemolytic species *L. innocua.*

*Table 10.2 : CAMP reaction for L. monocytogenes, L. ivanovii and L. innocua.*

| | *Rhodococcus equi* | *Staphylococcus aureus* |
|---|---|---|
| *L. monocytogenes* | - | + |
| *L. ivanovii* | + | - |
| *L. innocua* | - | - |

***Note***

- If using a commercial control strain of *L. monocytogenes*, strain NCTC 7973 should be used and not strain ATCC 15313 since the latter does not exhibit beta-haemolysis.
- Enhancement of the haemolysis of *L. monocytogenes* with *R. equi* may be apparent.

### *10.5.3.5 Further species identification criteria*

In addition to the properties outlined in Table 10.1, all *Listeria* species produce acid within 24 hours from: amygdalin, cellobiose, esculin, fructose, glucose, mannose and salicin. Acid production from galactose, lactose, melezitose, alpha-methyl-D-mannoside, rhamnose, sorbitol, starch, sucrose, trehalose and xylose is variable both between species and in some cases within species. Acid is not produced from adonitol, dulcitol, erythritol, inulin, raffinose, sorbose, arabinose, or melibiose. Aesculin is hydrolysed. Litmus milk is acidified and slowly decolourised. The methyl-red reaction and Voges-Prokauer test are positive. The oxidase test is negative. There is no production of indole or urease and no growth in citrate. $H_2S$ is not formed, and ornithine, lysine, glutamic acid and arginine decarboxylases are not produced, nor is an arginine dihydrolase present. Phosphatase is produced. Methylene blue is decolourised.

Tributyrinase is not formed. Lecithinase (phospholipase C) is produced, and hydrolysis of Tweens 20, 40, 60 and 80 takes place slowly.

*Note*

• When testing for the production of acid from carbohydrates a variety of basal media and pH indicators have been used. A peptone water medium with phenol red as indicator is recommended and where possible all carbohydrates should be sterilized by filtration and not autoclaving.

### 10.5.4 DNA analysis

The requirement for rapid and sensitive methods of detection of *L. monocytogenes* in human foods combined with recent advances in the genetic characterisation of the organism has led to the development of specific gene probes. Polymerase chain reaction (PCR) technology has been applied to these probes and PCR-based assays developed for detection of genes encoding haemolysin, a pleotrophic regulatory factor, phospholipases, invasin associated proteins, internalin, delayed type hypersensitivity proteins and rRNA sequences of *L. monocytogenes*. Such assays have proved to be rapid, sensitive and specific when carried out on pure cultures of *L. monocytogenes* but their effectiveness when applied to contaminated materials is influenced by the type of sample, the numbers of organisms and the presence of other microflora. Preliminary selective enrichment steps are advised and also eliminate the possibility of amplification of DNA from dead cells. Specific primers have subsequently been developed for *L. innocua*, a set of primers for L.ivanovii, *L. seeligeri* and *L. welshimeri*, and a set of primers for all members of the genus *Listeria* [5].

The use of such techniques in veterinary diagnosis has still to be evaluated.

### 10.5.5 Non-cultural methods of detection

In conjunction with the continued refinement of enrichment broths and selective media to isolate *Listeria* species many non-cultural methods including: nucleic acid hybridisation, immunochemical and flow cytometric techniques are being developed. Commercially available kits covering all these technologies are being marketed for use in the detection of *Listeria* in human foods. However, the suitability of such techniques and kits for use in the veterinary field have still to be evaluated. Indeed, many are directed towards genus specific rather than *L. monocytogenes* specific detection and are thus not strictly relevant to veterinary work.

## 10.6 INDIRECT DIAGNOSIS

### 10.6.1 Histopathology

***Principle***

A suitable method for histopathological preparation of tissue samples and haematoxylin and eosin staining is described in the chapter on Toxoplasmosis. Gram staining should also be carried out on dewaxed, hydrated samples as described in the chapter on Campylobacteriosis.

***Reading and interpretation of results***

Histologically, the foci found in the placental tissue show coagulative necrosis and infiltration to variable degrees by macrophages and neutrophils. Gram staining should demonstrate the presence of large numbers of Gram positive bacterial rods in affected areas.

The histological appearance of lesions in the foetal liver and spleen are of multiple, focal areas of necrosis with invasion by polymorphs and mononuclear cells. Gram staining should demonstrate the presence of

large numbers of organisms consistent with the appearance of *Listeria* in the lesions.

## 10.7 FUTURE WORK

There have been major advances in recent years in the understanding of *Listeria*, its taxonomy and also a molecular definition of its mechanism of virulence. However, despite advances there remain major gaps in the understanding of the epidemiology and pathogenesis of listeric infections in farm animals. Improved isolation techniques and methods of bacterial typing have been used by a number of investigators but these studies are by no means complete since the diversity and ubiquitous nature of the organism require detailed study to determine the ecological niches in which strains may thrive and mechanisms by which the organism may spread.

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# Chapter 11

# LEPTOSPIROSIS

W.A. ELLIS & S.J. MCDOWELL

## 11.1 SUMMARY

Leptospirosis is a common zoonotic disease affecting most mammals throughout the world. It causes economic losses in cattle and swine production due to abortion, stillbirth, infertility, decreased milk production and death. Sheep and goats have been considered resistant to clinical leptospirosis but recent findings indicate they may be susceptible in late pregnancy with resulting abortion, stillbirth, birth of weakly lambs and kids and agalactia in ewes and does.

In cases of abortion, stillbirth or birth of weakly lambs and kids, direct diagnosis is based on the isolation of the organism from, or its demonstration by immunofluorescence, in the foetus and placenta. However, since leptospires are fastidious, slow growing organisms, most practical diagnostic attempts concentrate on indirect serological techniques. The most commonly used technique is the microscopic agglutination test (MAT).

## 11.2 INTRODUCTION

Leptospirosis is a zoonotic disease caused by members of the genus *Leptospira*. Relative to cattle and pigs, sheep and goats have always been considered resistant to leptospiral infections. While that view is still generally true, it is apparent that, under certain intensive management systems, leptospirosis can cause overt clinical disease in sheep during late gestation and in the immediate post-partum period. This can cause considerable loss to individual farmers on such management regimes.

Leptospires are thin, helical, motile, Gram negative organisms, which are often hooked at one or both ends. In a suitable liquid environment, they spin constantly on their long axis. They range in length from about 10-20μm, with an amplitude of approximately 0.1-0.15μm and a wavelength of about 0.5μm. Under adverse nutritional conditions, leptospires may be greatly elongated, while under conditions such as high salt concentration-sageing culture or in tissues, leptospires may form coccoid forms of about 1.5-2μm. They divide by binary fission.

The taxonomy of leptospires is in a period of change and this has caused considerable confusion. Until recently a single genus *Leptospira* was recognised in the family Leptospiraceae. Two groupings were recognised within the genus - those which are found in animal species (the parasitic strains) and in water (the saprophytic strains). These two groupings, which were referred to as the interrogans and biflixa complexes, can be differentiated by their growth requirements and biochemical reactions. Only the parasitic strains are of medical and veterinary interest. For taxonomic purposes and as an aid to epidemiological studies the parasitic leptospires were divided into serogroups on the basis of antigenic relationships as determined by cross agglutination reactions and further subdivided into serovars by agglutination-absorption patterns. There are some 23 serogroups recognised

containing approximately 212 serovars.

The advent of genetic typing methods has provided rapid, reproducible typing protocols. The current recommendations on the taxonomy of leptospires [1] recognise eight species of pathogenic leptospires within the family Leptospiraceae. There are *Leptospira* interrogans, *L. borgpetersenii*, *L. inadai*, *L. kirschneri*, *L. noguchii*, *L. meyer*, *L. weilli*, and *L. santarosai*.

The species definition is based on a level of DNA-DNA homology of at least 70% and < 5% divergence in DNA relatedness. Taxonomy at the subspecies level continues to be based on serovars but other valid methods which give comparable results to conventional serotyping can be used for their identification. Such methods include monoclonal antibody agglutination profiles, factor analysis, analyses in which restriction fragment length polymorphisms or rRNA gene restriction patterns are used in pulsed field gel electrophoresis analyses. The term "type" is used to indicate strain differences at the subserovar level [1].

The epidemiology of leptospirosis in domestic animals is potentially very complicated because animals can be infected by any of the pathogenic serovars. However, only a small number of serovars are endemic in any particular region or country. Furthermore, leptospirosis is a disease which shows a natural nidality and each serovar tends to be maintained in specific maintenance hosts. Therefore, in any region an animal species will be infected by serovars maintained either in the same species or in other animal species maintained in the same area. The relative importance of these incidental infections is determined by the opportunities for contact and transmission of leptospires from other species to the target host species provided by prevailing social, management and environmental factors.

There is consistent serological evidence that hardjo infection is the major infection found in sheep worldwide. In some countries there is evidence that sheep may maintain this serovar without the presence of the established maintenance host - cattle. It has also been shown that transmission of hardjo can occur between sheep grazed together under experimental conditions and also that infected sheep urine can experimentally infect calves. However, despite widespread infection in England and Wales field investigations found no evidence to suggest that the prevalence of hardjo infection in sheep was dependent on broad sheep population parameters.

Seroprevalence data indicates that other leptospiral infections in sheep are uncommon and are incidental. The serovars involved in these incidental infections vary with the geographical region and reflect the common serovars found in other domestic and wild animals in those regions. Only the Pomona, Grippotyphosa, Australis and Ballum serogroups [2] have been implicated in incidental clinical infections in sheep. These are acquired from other hosts and there is no evidence that sheep populations are capable of maintaining them, for example, the purported transmission of pomona from pigs to sheep.

There is no cultural or serological evidence to suggest that goats act as maintenance hosts for leptospires. High seroprevalences have been reported in some countries but the predominant infecting serovar varies suggesting an incidental pattern of infection.

The vast majority of leptospiral infections are sub-clinical. Two groups of animals are most likely to experience clinical infections:

1. The very young animal infected by an incidental infection. This may give rise to severe illness characterised by jaundice, haematuria, haemaglobinuria, evidence of renal damage, meningitis and can be fatal. Such acute clinical events are very occasionally seen in older animals. Sporadic outbreaks of acute clinical disease have occasionally been reported in both sheep and goats.

2. Sexually mature lactating and/or pregnant females infected by either incidental or host maintained leptopsires. This can result in a) agalactia in sheep or b) reproductive wastage.

• Agalactia. An agalactia similar to that seen in cows has been observed in hardjo-infected sheep in Northern Ireland. Recently lambed ewes suddenly go off their milk giving rise to lamb deaths due to starvation. Udders of affected ewes feel soft, not hard as in mastitis and ewes return to milk after 3 to 4 days without treatment.

• Reproductive wastage in sheep. Reproductive wastage in sheep is seen as late-term abortion, stillbirth and the birth of weak lambs. In a study in Northern Ireland, approximately 17 per cent of 872 aborted lambs examined between 1981 and 1987 were positive for leptospires on immunofluorescent examination. Culture data indicated that in the majority of these the infecting strain was serovar hardjo while a small number were due to either pomona, ballum or bratislava [2]. In Scotland, it has been reported [3] that 7 out of 170 aborted sheep foetuses had antibody to serovar hardjo, while in a South Dakota study [4] 8 per cent of aborted foetuses had antibody to leptospires. Serovar pomona has also been implicated in abortions in Spain [5] and Albania [4] while grippotyphosa was deemed to be the cause of abortions in Hungary [6].

It has been observed [2] that hardjo abortion was rarely found in extensively managed flocks. It was largely confined to ewes which were bred in an extensive system and subsequently bought as replacements for intensively managed flocks with high stocking rates and where ewes are frequently housed indoors. Reproductive wastage and agalactia were seen in these groups in their first lambing season following introduction, but not in subsequent years.

• Reproductive wastage in the goat. There are few reports of leptospirosis causing reproductive wastage in goats. A report from Spain [5] attributed 2.7 per cent of goat abortions to leptospirosis - mainly pomona. In Israel an outbreak of acute grippotyphosa infection in goats has been described [7], a sequel to which was a high incidence of abortion. Abortion has also been reported in goats in Guyana [8] and in India [9].

• The main pathological changes are essentially the same in all species, with the primary lesion being damage to the membranes of the endothelial cells of small blood vessels.

In acute leptospirosis there are no pathognomonic gross changes. Many of the features recorded after death from leptospirosis are those which would occur in death from renal failure from other causes, accompanied by jaundice in some cases. There may be petechial or echymotic haemorrhages in the skin, conjunctivae, mucosal and serosal surfaces, in the subcutaneous tissues and fat and in the endocardium. The thoracic and peritoneal cavities may contain blood stained yellow fluid. There may be subpleural haemorrhages. Splenomegaly is occasionally a feature. The liver may be enlarged and tense or pale or yellow. There may be disruption of cord and lobular structure, which is sometimes accompanied by centri-

lobular necrosis. The liver cells become irregular, swollen and degenerate. The most significant lesions are in the kidneys, which may be swollen, and yellow-green in jaundiced patients, with subsurface haemorrhages. The constant histological feature is interstitial nephritis, and tubular necrosis. Haemorrhages are apparent particularly in the medulla.

In chronic leptospirosis in animals, lesions are confined to the kidneys and consist of scattered small grey/white foci, often surrounded by a ring of hyperaemia. Microscopic examination shows these lesions to be a progressive focal interstitial nephritis. The interstitial leucocytic infiltrations, which consist mainly of lymphocytes, macrophages and plasma cells, may be extensive in some areas. Focal damage may also involve glomeruli and renal tubules. Some affected glomeruli are swollen, some atrophic, and others are replaced by fibrosis. The Bowman's capsule may be thickened, containing eosinophilic granular material. Tubular changes involve atrophy, hyperplasia and presence of necrotic debris in the lumen in some areas. Occasionally, petechial haemorrhages may be present in interstitial spaces.

With the probable exception of hardjo infection in sheep, sheep and goats are always incidental hosts for infections maintained by other animals, thus interruption of transmission from these hosts to sheep and goats is the critical factor in the control. The following are key elements in achieving this and as many as are appropriate to the situation should be included in a strategy:

1) Identify the sources of infection: where transmission is occurring and what are the host species.

2) Control infection in the host species, if possible.

3) Control of rodent carriers e.g. rat control programs.

4) Reduce contact with carrier animals e.g. don't mix sheep and pigs.

5) Exclusion from known contaminated environments.

6) Vaccination. This option has been widely used in the former USSR for the control of leptospirosis in both sheep and goats. Vaccination with 1/4 a cattle dose of hardjo vaccine is the easiest method for controlling hardjo infection in sheep.

## 11.3 SAMPLES

All samples must be collected as aseptically as possible and transferred to the laboratory as quickly as possible, preferably maintained at a temperature of 4°C.

### 11.3.1 Direct diagnosis

#### *11.3.1.1 Isolation*

Isolation is best carried out on samples of tissue taken from the aborted foetus (kidney, liver, lung etc.), foetal fluids (aqueous humour, thoracic, peritoneal, pericardial etc.) and the placenta. Samples should be collected from the aborted foetus as quickly as possible after expulsion from the dam since the numbers of viable leptospires is likely to decrease rapidly as autolysis proceeds making recovery in culture more difficult.

For tissues, a 5-10g sample or 5-6 cotyledons from the placenta should be recovered aseptically and placed in a sterile container holding 100ml of 1% bovine serum albumin (BSA) diluent (1g BSA per 100ml 0.005M phosphate buffer [87 mg/l $KH_2PO_4$ + 664 mg/l $Na_2HPO_4$] containing 200µg/ml 5-fluorouracil). On arrival at the laboratory the tissue samples should be homogenised, one part tissue to

nine parts 1% BSA diluent, using a suitable blender or laboratory "stomacher". Samples must be cultured immediately.

Foetal fluids should be collected aseptically by use of a sterile syringe and needle or an evacuated sterile glass tube and transferred to the laboratory in a suitable sterile container.

#### *11.3.1.2 Demonstration of leptospire presence*

Immunofluorescence can be used to detect the presence of leptospires in tissue homogenate smears and cryostat sections of aborted foetal tissues and the placenta.

Tissue samples for cryostat sections may be stored frozen at -70°C.

### 11.3.2 Indirect diagnosis

Serological analysis using the microscopic agglutination test (MAT) should be undertaken on sera recovered from aborted ewes. Blood samples should be collected from as many ewes as possible (minimum of 10) that have aborted as soon as possible after abortion. Samples of blood from the aborted foetuses may also contain antibodies to leptospires if infection occurred during the immunocompetent phase of development and should be collected for screening where possible. All blood samples should be collected in sterile, glass blood tubes containing no anticoagulant (e.g. Vacutainer). On arrival at the laboratory, blood samples should be centrifuged for 10 minutes at 3,000 xg and the sera removed from the clot. If not analysed immediately, sera can be stored frozen at -20°C.

## 11.4 RISKS TO HUMAN HEALTH

Leptospirosis is an occupational zoonosis of those who work in agriculture, meat processing and laboratory diagnosis. People must be made aware of the risks involved in handling both animals and potentially infected materials from the animal. Those working with potentially infected animals or materials must:

- use water-proof clothing to give a barrier to infection;
- be stringent with personnel hygiene;
- disinfect all areas and equipment after contact with potentially infected animals or materials;
- dispose of all infected material by incineration or sterilization.

## 11.5 DIRECT DIAGNOSIS

Direct diagnosis is based on the isolation of leptospires from, or their demonstration in the internal organs (such as kidney, liver, lung) and body fluids (aqueous humour, blood, cerebrospinal, thoracic and peritoneal fluid) of the aborted foetus and/or the placenta which provides a definitive diagnosis of leptospiral abortion and probable chronic infection of its mother.

### 11.5.1 Isolation

Isolation by bacteriological culture is expensive, difficult and time consuming (4-6 months may be required for a conclusive result) and is normally only conducted at specialist laboratories. Isolation is, however, the most sensitive method of demonstrating the presence of leptospires, providing that antibiotic residues are not present, that tissue autolysis is not advanced and that tissues for culture have been stored at a suitable temperature (4°C) since collection. Identification of isolated leptospires to serogroup level can be undertaken by cross agglutination reactions but these test are only available at large diagnostic laboratories and reference centres.

***Principle***

Culture should be carried out in semi-solid (0.1 to 0.2 per cent agar) bovine serum albumin medium: either Ellinghausen-McMullough-Johnson-Harris medium (EMJH) which is commercially available (Difco) or Tween 80/40 medium [10]. These should be supplemented by a small amount of fresh rabbit serum (0.4 to 2 per cent). Contamination may be controlled by the use of a variety of selective agents, e.g. 5-fluorouracil (100-400 µg/ml), nalidixic acid (10-20 µg/ml), fosfomycin (400 µg/ml) and a mixture of rifamycin (10 µg/ml), polymixin (0.2 µg/ml), neomycin (2 µg/ml), 5-fluorouracil (250 µg/ml), bacitracin (40 µg/ml) and actidione (100 µg/ml). The use of selective agents will reduce the chance of isolation where there are only small numbers of viable leptospires. The most useful technique to use is the dilution culture technique in which many aliquots of medium are used. This technique is time consuming and is not really a practical consideration for the routine diagnostic laboratory. It has, however, been shown to be very useful in culturing leptospires from aborted foetuses, even those in advanced states of autolysis.

***Materials and reagents***

- 1% BSA diluent (1g BSA per 100ml 0.005M phosphate buffer [87 mg/l $KH_2PO_4$ + 664 mg/l $Na_2HPO_4$]).
- Modified semi-solid (0.1 to 0.2% agar) bovine serum albumin medium (EMJH or Tween 80/40 medium).
- Positive control cultures.
- Microscope suitable for darkground examination.

***Procedure***

1. Make ten-fold dilutions of samples of homogenised tissue or foetal fluids in 1% BSA diluent to cover the range $10^{-1}$ to $10^{-4}$.
2. Inoculate 50µl aliquots of each of the sample dilutions and positive control cultures into six 7.5ml volumes of culture medium in plastic tissue culture tubes.
3. Incubate cultures at 29°C for at least 12 weeks.
4. Examine cultures by darkground microscopy every 1-2 weeks.

***Interpretation of results***

Organisms with typical leptospiral morphology can be seen in positive samples.

***Note***

• Visualisation of very small numbers of leptospires present in positive cultures can be helped by diluting drops of semi solid culture media with liquid medium to give a flatter, less dense preparation for microscopic examination and to make movement of the organisms more discernible.

## 11.5.2 Demonstration of leptospires

Leptospires do not stain satisfactorily with the aniline dyes and silver staining techniques lack sensitivity and specificity. Dark-ground microscopy of foetal fluids has been used in the diagnosis of leptospirosis abortion and can be useful if conducted by an experienced diagnostician. However, many tissue artefacts can be mistakenly identified as leptospires. The demonstration of leptospires by immunochemical staining techniques is more suited to most laboratory situations although these tests are 'number of organism dependent', lack the sensitivity of culture and require access to antiserum to the specific serovar(s) being detected. The immunochemical methods which have been used for diagnosis include immunofluorescence, immunoperoxidase, avidin-biotin and immunogold techniques.

### *11.5.2.1 Immunofluorescence*

Immunofluorescence has been the most widely used immunochemical technique and has the advantage of giving the best contrast between the leptospires and the tissue background. This is particularly important since leptospires are small and filamentous which makes them difficult to differetiate from some connective tissue elements and cilia. The technique, however, requires a long inoculation regime in rabbits to produce the good quality polyclonal antisera required,full details of which have been given by Ellis et al (1982) [11]. With suitable antisera the following method can be carried out on foetal tissue homogenates:

***Materials and reagents***

- Fluorescent microscope equipped for transmitted and incident light investigations.
- Acetone.
- Conjugate: Fluorescein isothiocyanate (FITC) labelled rabbit antiserum of known titre.
- 0.1M Phosphate buffer (pH 7.4).
- Eriochrome black solution (1:60 aqueous solution).
- Buffered glycerol solution (pH 8.0).

***Procedure***

1. Make smears of tissue homogenates on microscope slides and air dry.
2. Fix smears in acetone at 4°C for 10 minutes and air dry. Smears can be stored frozen at -20°C for up to two weeks if not analysed immediately.
3. Cover smears with diluted conjugate and incubate at 37°C for 45 minutes.
4. Wash smears in 0.1M phosphate buffer for two 20 minute periods.
5. Counterstain for 10 seconds in eriochrome black solution.
6. Rinse smears rapidly three times in phosphate buffer.
7. Mount in buffered glycerol solution.
8. Examine samples by microscope.

***Interpretation of results***

Fluorescing organisms with typical leptospiral morphology can be seen in positive samples.

***Note***

- Immunofluorescent techniques can also be undertaken on cryostat sections cut in a freezing microtome from blocks of foetal kidney, lung, liver, brain and cotyledon tissue which has been snap frozen in isopentane and liquid nitrogen.
- It is generally easier to see distinct organisms in tissue homogenate smears than in cryostat sections but care must be taken to prevent smears being washed off glass slides during staining.

## 11.6 INDIRECT DIAGNOSIS

Indirect serological testing is the most widely used method for diagnosis of leptospirosis. Leptospiral antibodies appear within a few days of the onset of acute infection and persist for weeks or months. Abortions may occur any time between 1 and 12 weeks after infection, the actual period varying with the causal serovar. Blood samples taken from ewes at the time of, or just after, abortion are likely to have either high or declining titres. It is therefore critical that diagnosis is carried out on a herd basis rather than in the single animal to confirm *Leptospira* as the cause of the observed abortions. The presence of antibody in foetal serum is diagnostic of leptospiral abortion and has been shown to be useful in the investigation of sheep abortions.

### 11.6.1 Microscopic Agglutination Test (MAT)

Among the various serological tests which have been described, the microscopic agglutination test (MAT) using live antigens is generally accepted as the reference test against which all other serological tests are evaluated. The test can, however, be time-consuming and present a degree of risk to laboratory workers due to its use of live antigens. Formalin-killed antigens have been used by some laboratories but titres tend to be lower and cross-reactivity higher compared to the results obtained with the use of live antigens.

***Principle***

The MAT is used for the detection of agglutinating antibodies. It involves the use of live antigen derived from an undiluted culture which has been sub-cultured at least 5 days before use. The MAT is largely serogroup specific and therefore for optimum sensitivity representative strains of all the serogroups known to exist in the country or preferably, strains representing all the known serogroups should be used.

N.B. All manipulations of live leptospires or material which may contain live leptospires should, when ever possible, be carried out in a safety cabinet. The microscope stage and controls must be swabbed with 70% alcohol after use.

***Materials and reagents***

- U-well microtitre plates.
- Dark ground microscope (dry x 200 magnification).
- Physiological saline (0.85 % NaCl (w/v)).
- Positive control sera of known titre.
- Negative control sera.

***Procedure***

1. Make 1 in 12.5 dilutions of serum in sterile physiological saline.
2. Place 100µl volumes of each sample in the first row of the microtitre plate.
3. A positive and negative control sample should be included in each test batch.
4. Make serial four fold dilutions of each sample and controls in subsequent rows of the plate.
5. Add 100 µl of live antigen to each well.
6. Incubate plates at 29°C for 2 hours.
7. Determine the antibody level by dry examination of drops taken from individual wells and placed on a microscope slide.

***Interpretation of the results***

The degree of agglutination is assessed in terms of the proportion of free leptospires present, e.g., at 100% (++++) agglutination there are no free leptospires visible, at 50% (++) agglutination about half the leptospires remain free when compared with a negative control. The titre of a serum sample is taken as the highest dilution in which there is agglutination of 50% or more of the organisms.

***Note***

• Culture used as antigens in the MAT should be actively motile, free from contamination and at the late log phase of growth with no sign of breakdown. Cultures are incubated at 29°C but if on microscopic examination, a culture appears to be growing more quickly than required, it may be held at room temperature in the dark.

• Despite its universal use and numerous attempts by various investigators to standardise the MAT, it is difficult to obtain consistent results between laboratories. There are varying opinions as to what is a diagnostic titre and no one single titre can be considered diagnostic for all cases.

## 11.7 FUTURE WORK

Currently detection of leptospires as the cause of abortion in small ruminants is undertaken by a combination of culture isolation, demonstration by immunofluorescence and serology. None of these techniques provides a definitive diagnosis and all tend to be time consuming, expensive and inconvenient for use in routine diagnosis in non-specialised laboratories. Future improvements in diagnosis may include:

1) Improved media for culture.

2) Improved ELISA techniques for more rapid, sensitive, specific and automated serological analysis. Attempts have been made to develop an ELISA technique to detect leptospiral antibodies and although the method has been found to have technical advantages over the MAT it still presents the problem that cross-reactivity among heterologous serovars is greater than in the MAT.

3) Application of gene probe and polymerase chain reaction (PCR) techniques. A variety of PCR methodologies for the detection of leptospires have been published [e.g. 12] but these have not gained acceptance as diagnostic tests due to problems with inhibitors interfering with test sensitivity.

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# Chapter 12

# OTHER BACTERIAL CAUSES OF ABORTION

M. PEPIN, R. SANCHIS, M. CALAMEL,
M. LAMBERT, P. RUSSO & C. VITU

## 12.1 SUMMARY

A large number of bacteria can cause occasional abortion either by colonising the placenta during a septicaemia phase or when the immune defences of the dam are low. When repeated abortions cannot be attributed to any of the major abortive organisms, the presence of other bacteria can be investigated using the techniques presented briefly in this chapter.

## 12.2 INTRODUCTION

Apart from the major pathogenic bacteria responsible for abortion in small ruminants (*Chlamydia*, *Coxiella*, *Brucella*, *Listeria*, *Campylobacter*, *Salmonella* abortusovis and *Leptospira*), other bacteria can occasionally be implicated in abortion outbreaks (Table 12.1).

These occasional abortions can arise in two, not unrelated, circumstances:

1) Abortions that arise from a systemic bacterial infection; in essence, all bacteria that occur in sheep and goats can potentially, at the time of gestation and during a period of septicaemia, colonise the placenta and foetus and cause abortion.

2) Abortions caused by opportunistic organisms which take advantage of a lowering of immune defences in the sheep or goat.

Eventhough the importance of these outbreaks remains limited and they are usually confined to a particular geographical area, it is acknowledged that certain of these "minor" pathological agents could become important in the future (the concept of "new diseases"). The relative importance of these secondary infections has been assessed in two studies, one in the USA with 1784 cases [4] and one in France with 1614 cases [7]. These studies showed that "minor" pathogens caused 24% and 5%, respectively, of occasional abortions (i.e. abortions not attributed to the major pathogenic agents) out of the infectious abortions diagnosed. These figures probably under-estimate the real percentage of occasional abortions due to "minor" pathogens since the cause of 56% and 57%, respectively, of the abortions was

***Table 12.1 : List, in order of decreasing importance, of the minor pathogenic agents responsible for abortions in small ruminants***

| Minor pathogenic agent | Reference |
|---|---|
| *Actinomyces pyogenes* | [4, 7] |
| *Yersinia* | [2, 4, 7, 12, 14] |
| *Y.enterocolitica* | |
| *Y.pseudotuberculosis* | |
| *Pasteurella* | [4, 7] |
| *P.haemolytica* | |
| *P.multocida* | |
| *Escherichia coli* | [4] |
| *Bacillus sp* | [4] |
| *Flexispira rappini* | [3 - 5] |
| *Pseudomonas sp* | [4] |
| *Fusobacterium nucleatum* | [4] |
| *Steptococcus sp* | [4] |
| *Staphylococcus sp* | [4] |
| *Clostridium sp* | [4] |
| *Enterobacter sp* | [4, 13] |
| *Histophilus ovis* | [6] |
| *Erysipelothrix rhusiopathiae* | [7] |
| *Corynebacterium sp* | [4] |

not established. These results also illustrate the large variability in the causes of abortion in small ruminants, whether due to major or minor pathological agents, between countries [4, 7] and within the same country [7, 10].

## 12.3 ABORTIONS DUE TO ENTEROBACTERIACEAE

### 12.3.1 Bacteria of the *Yersinia genus*

Bacteria of the *Yersinia* genus, family Enterobacteriaceae, have been associated with abortions in sheep. Three species have been identified, *Yersinia enterocolitica*, *Yersinia intermedia* and *Yersinia pseudotuberculosis*. In general, bacteria of the *Yersinia* genus are associated with intestinal infections of ungulates but they can infect a large number of other species including man (particularly *Y.enterocolitica*).

In cases of suspected infection with *Yersinia*, the bacteria can be detected directly in all sample types by use of selective media (CIN, Oxoid). Colonies grown on CIN media can be rapidly identified as *Yersinia* by the urease test and acid production from the carbohydrates, cellobiose, melibiose, rhamnose and saccharose [11]. Yersinia can also be isolated and identified using conventional agar (blood and McConkey: the bacteria are McConkey positive and lactose negative) or after enrichment at 4°C (the sample is placed in a phosphate buffer [0.1 M], held under refrigeration for 3 weeks and tested for Yersinia each week on McConkey or CIN media). Identification can then be undertaken using conventional biochemical tests or by using identifying kits such as AP120E (BioMerieux) [9].

Abortions caused by *Y.enterocolitica* or *Y.intermedia* occur at the end of gestation. The affected foetus shows no distinctive external signs but the internal organs show signs of a general bacterial infection. One study carried out with 120 isolates of *Y.enterocolitica* and *Y.intermedia*, recovered at the time of abortions, showed that the isolates that could be typed belonged to serogroups O:6, 30 and 7 [1]. The ability of these isolates to cause abortion was verified at the time by experimental infections [1, 2]. It should be noted that *Y.enterocolitica* possesses a common antigen with *Brucella* resulting in cross reactions during indirect serological diagnosis [9].

*Yersinia pseudotuberculosis* can cause abortion in sheep and goats, probably as a result of an initial intestinal infection. Placental cotyledons may have opaque off-white centres associated with suppurative microscopic lesions and the aborted dam may develop a suppurative pneumonia [12, 14].

### 12.3.2 Enterobacter cloacae and other enterobacteria

*Enterobacter cloacae*, a lactose positive bacteria, was originally described in abortive episodes in goats. Abortion occurs towards the third month of gestation and is associated with the expulsion of the foetus from which the causal agent can be isolated in pure culture [13].

Other enterobacteria, in particular *Escherichia coli* and *Salmonellae* other than *Salmonella abortusovis*, can be isolated at the time of abortion in small ruminants, generally following a febrile illness.

## 12.4 ABORTIONS DUE TO ACTINOMYCES PYOGENES

*Actinomyces pyogenes*, previously *Corynebacterium pyogenes*, is the cause of a number of suppurative infections in sheep and goats (mastitis, navel-ill,

arthritis). This bacteria can be isolated at the time of abortion, mainly in sheep [4, 7]; it can be cultured aerobically or in an atmosphere of 5-10% $CO_2$ on blood agar. After 48 hours very small colonies about 1mm diameter are visible surrounded by a narrow zone of haemolysis and on microscopic examination curved, Gram positive bacillus are seen. Identification of *A.pyogenes* can be confirmed by a positive CAMP test with *Staphylococcus aureus* [9].

## 12.5 ABORTIONS DUE TO OTHER BACTERIA

- *Pasteurella* (*Pasteurella haemolytica and P.multocida*), responsible for respiratory infections (acute or subacute, non-progressive pneumonias), have been recovered at the time of abortion in sheep and goats in a number of cases; such abortions illustrate the concept of opportunistic bacterial infection [4, 7].

- *Histophilus ovis* is a Gram negative, facultative anaerobic coccobacillus, close to the genus *Haemophilus*, belonging to the family of Pasteurellacae. This bacteria has been associated with a number of ovine pathologies (mastitis, epidydimitis, synovitis, meningoencephalitis) and has recently been identified as a cause of abortion in sheep [6]. *Histophilus ovis* grows slowly in small colonies on blood and chocolate agar incubated in an atmosphere of 10% $CO_2$. Identification of the bacteria, oxidase positive, catalase negative, nitrite positive, indole positive, can be made using the AP150CH kit (BioMerieux) [6].

- Flexispira rappini is an anaerobic, flagellated bacteria, recognised as a possible cause of abortion in small ruminants since 1984 [5]. This bacteria resembles *Campylobacter* and can be detected by culture on blood agar in an anaerobic atmosphere (80% nitrogen, 10% hydrogen and 10% $CO_2$) after about 7 days incubation. In recent published cases the proportion of abortions that had occurred by the end of gestation remained low: 2-4% [3, 5].

- Other bacteria have very occasionally been identified as the cause of abortion in small ruminants (Table 12.1), following recovery in pure cultures from aborted foetuses or placental samples.

## 12.6 BACTERIA RESPONSIBLE FOR IMPORTANT DISEASES IN SMALL RUMINANTS THAT DO NOT CAUSE ABORTION

It is important to note that several bacteria cause important diseases in ovines and caprines that do not lead to abortions except in very exceptional cases during general illnesses. Amongst these bacteria and the corresponding illness, it is necessary to mention :

- *Corynebacterium pseudotuberculosis*, the causal agent of caseous lymphadentitis [8];

- infections due to anaerobic bacteria of the genus *Clostridium*, responsible for enterotoxaemias, gaseous gangrene, tetanus ;

- *Dilechobacter* (*Bacteroides*) *nodosus*, the causal agent of footrot ;

- Mycobacteria : *Mycobacterium tuberculosis* (tuberculosis) and *Mycobacterium paratuberculosis* (paratuberculosis).

These non-abortive infections are the result of bacteria that have pathogenic characteristics or a specific tropism.

## 12.7 CONCLUSION

The difficulty of diagnosing abortions due to the less important bacteria in small ruminants is linked to the number of possible causal agents. To minimise the amount of testing involved, it is best to analyse samples for the main pathogenic agents responsible for abortions. Only if the results are negative (and before abortions are repeated), should tests for the bacteria of lesser importance be carried out (see Chapter 2 on differential diagnosis of abortions in small ruminants).

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# Chapter 13

# BORDER DISEASE

P. F. NETTLETON & J. BREBNER

## 13.1 SUMMARY

Border disease (BD), caused by the pestivirus Border Disease virus (BDV), is a congenital disease of sheep characterised by barren ewes, abortions, stillbirths and the birth of small weak lambs. Affected lambs can show tremor of skeletal muscles, abnormal body conformation, hairy fleeces (so-called "hairy-shaker" or "fuzzy" lambs), defective myelination of the central nervous system, inferior growth and a variable degree of skeletal deformity. The virus produces similar symptoms in goats but disease outbreaks are rarely reported. Although BDV is principally a cause of congenital disease it can also cause acute and persistent infections. BDV spreads naturally among sheep and goats by the oronasal route and by vertical transmission. Sheep and goats may also be infected following close contact with cattle excreting the closely related bovine virus diarrhoea virus (BVDV).

The diagnosis of BD presents little difficulty if typical "hairy shaker" lambs are born although laboratory confirmation is advisable. Histological examination of the central nervous system can confirm BD but should be supported by virus isolation from blood and tissues or the demonstration of viral components by specific immunological or reverse transcriptase-polymerase chain reaction methods.

## 13.2 INTRODUCTION

Border Disease (BD) is a congenital virus disease of sheep first reported in 1959 from the border region of England and Wales. It has subsequently been reported from most major sheep-producing countries of the world. Prevalence rates vary from 5% to 50% between countries and from region-to-region within countries. The disease occurs naturally primarily in sheep and occasionally in goats.

BD virus (BDV) is a pestivirus in the genus Flaviviridae and is closely related to classical swine fever and BVDV. Nearly all isolates of BDV are non-cytopathogenic (ncp) in cell culture although occasional cytopathic viruses have been isolated. There are no defined serotypes but virus isolates exhibit considerable antigenic diversity and three distinct antigenic groups have been identified.

Healthy newborn and adult sheep exposed to BDV experience only mild or inapparent disease. The main clinical signs of BD are seen following the infection of pregnant ewes. While the initial maternal infection is subclinical or mild the consequences for the foetus are serious. Foetal death may occur at any stage of pregnancy but is commoner in foetuses infected early in gestation. Small dead foetuses may be resorbed or their abortion pass unnoticed since the ewes continue to feed well and show no sign of discomfort. As lambing time approaches, the abortion of larger foetuses, stillbirths and the premature births of small, weak lambs is seen.

During lambing, an excessive number of barren ewes may become apparent but it is the diseased live lambs that present the main clinical features characteristic of BD. The clinical signs exhibited by BD lambs are very variable and depend on the breed of sheep, the virulence of the virus and the time at which infection was introduced into the flock. Affected lambs are usually small and weak, many being unable to stand. Nervous signs and fleece changes are often apparent. The nervous symptoms of BD are its most characteristic feature. The tremor can vary from violent rhythmic contractions of the muscles of the hind legs and back to barely detectable fine trembling of the head, ears and tail. Fleece abnormalities are most obvious in smooth-coated breeds which have hairy fleeces especially on the neck and back. Abnormal brown or black pigmentation of the fleece may also be seen in BD-affected lambs. Deformities of the skeleton include abnormally shortened long bones, a reduction in crown-rump length and the long axis of the skull which results in lambs appearing more compact and short-legged than normal.

With careful nursing a proportion of BD lambs can be reared although deaths may occur at any age. The nervous symptoms gradually decline and can have disappeared by 3 to 6 months of age. Weakness, swaying of the hind-quarters together with fine trembling of the head may reappear at times of stress. Affected lambs often grow slowly and under normal field conditions many will die before or around weaning time. Occasionally this is the first presenting sign of disease when losses at lambing time have been low and no lambs with obvious symptoms of BD have been born.

Some foetal infections occurring around mid-gestation can result in lambs with severe nervous signs, locomotor disturbances and abnormal skeletons. Such lambs have lesions of cerebellar hypoplasia and dysplasia, hydranencephaly and porencephaly resulting from necrotising inflammation. The severe destructive lesions appear to be immune mediated, and lambs with such lesions frequently have high concentrations of serum antibody to BDV. Most lambs infected in late gestation are normal and healthy and are born free of virus but with BDV antibody. Some such lambs, however, can be stillborn or weak and may die in early life.

In sheep foetuses infected before the onset of immune competence viral replication is uncontrolled and 50% foetal death is common. In lambs surviving infection in early gestation, virus is widespread in all organs. Such lambs appear to be tolerant to the virus and have a persistent infection usually for life. Typically, there is no inflammatory reaction and the most characteristic pathological changes are in the CNS and skin. At all levels in the CNS there is a deficiency of myelin which is responsible for the nervous symptoms. In the skin there is an increased size of primary wool follicles and fewer secondary wool follicles causing the hairy or coarse fleece.

Some viraemic sheep survive to sexual maturity and are used for breeding. Lambs born to these infected dams are always persistently viraemic. Rams that are persistently infected usually have poor quality, highly infective semen and reduced fertility. Persistently viraemic sheep are a continual source of infectious virus to other animals, excreting the virus in nasal secretions, saliva, urine and faeces. Viraemia is readily detectable at any time except in the first two months of life when virus is masked by colostral antibody and in animals older than 4 years old some of which develop low

levels of anti-BDV antibody.

In goats, foetal death is the major outcome of infection of the pregnant doe and infections prior to 60 days of gestation almost invariably result in reproductive failure. Persistently infected "shaker" kids and clinically normal kids are born with infections around 60 days of gestation but are very rare. The caprine foetus develops immune competence against pestiviruses between 80 and 100 days of gestation.

There is no standard vaccine for BDV but a commercial killed whole virus vaccine has been produced. Ideally such a vaccine should be able to be administered to females before breeding to prevent transplacental infection. The use of BVDV vaccines has been advocated but this would be irresponsible given current knowledge on the antigenic diversity of BD viruses. BD viruses have contaminated several modified live veterinary vaccines produced in sheep cells or containing sheep serum. This potential hazard should be recognised by manufacturers of biological products.

Control strategy lies in attempting to engender a flock immunity and to avoid exposing sheep or goats to infection during pregnancy. Persistently infected animals are a continuous source of infection and are best identified and culled. However, persistently infected animals can be run with the flock when it is not pregnant, particularly with replacement stock, in an attempt to produce infection and immunity prior to gestation. Close herding for at least 3 weeks, preferably indoors, is necessary for BDV to spread effectively and exposure should stop two months before the start of the breeding season when the persistently infected animals should be removed. In most flocks a serious outbreak of disease is followed by minor disease in subsequent years, the flock developing immunity in the initial outbreak. In flocks that are free from infection consideration should be given to blood testing all brought-in replacements to ensure none are persistently infected.

## 13.3 SAMPLES

The diagnosis of BD on clinical grounds presents little difficulty when typical "hairy-shaker" lambs are born, but laboratory confirmation is frequently necessary to distinguish BD from sway-back, hypothermia, "daft-lamb" disease, bacterial meningo-encephalitis and focal symmetrical encephalomalacia. Since placentas and foetuses aborted due to BD infection have no distinguishing characteristics, laboratory confirmation is required to differentiate BD from other known causes of abortion. The specimens required by the laboratory to confirm BD are summarised in Table 13.1.

### 13.3.1 Virus isolation

#### *13.3.1.1 Placental and foetal tissues*

Five or six placental cotyledons and the associated intercotyledonary membranes and tissues from aborted foetuses and newly dead "hairy shaker" lambs should be collected aseptically and placed in a suitable sterile container holding virus transport medium (VTM). On arrival at the laboratory samples should be frozen at -70°C or treated as follows:

1. Macerate tissue in VTM by a suitable method e.g. a "stomacher" to form a homogeneous suspension.
2. Centrifuge the suspension at 3,000 xg for 10 minutes.
3. Pass the supernatant through a sterile 0.45μ filter and retain the filtrate.
4. If not analysed immediately store the filtrate at -70°C.

*Table 13.1 : Samples to be collected for the laboratory confirmation of Border Disease as the cause of abortion in small ruminants*

| Clinical Signs | Diagnostic Technique | Samples From: | |
|---|---|---|---|
| | | Live Animal | Dead Animal |
| Abortion | Virus Isolation | Heparinised blood from the dam | Thyroid, kidney, spleen, brain, placenta in VTM |
| | Antigen detection | Heparinised blood from the dam | Thyroid, kidney, spleen, brain, placenta |
| | Histology | -- | Brain and spinal cord in fixative |
| | Serology | Blood from the dam | Foetal fluids |
| "Hairy shaker", Weak Poorly , Thriving or Scouring Lambs | Virus Isolation | Heparinised blood from the lamb and dam | Thyroid, kidney, spleen, brain, gut, lymph nodes in VTM |
| | Antigen detection | Heparinised blood from the lamb and dam | Thyroid, kidney, spleen,, brain, gut, lymph nodes |
| | Histology | | Brain and spinal cord in fixative |
| | Serology | Blood from the lamb and dam | |

### *13.3.1.2 Blood samples*

Blood samples can be used for virus isolation or can be analysed for the presence of BD antigens or antibodies.

Blood samples from aborted ewes are best collected at the time of abortion. Pre-colostral blood samples from "hairy-shaker", weak, poorly thriving or scouring lambs should be collected, although, there are practical difficulties in obtaining such samples.

Two blood samples should be collected from each animal, one in a sterile, evacuated glass blood tube containing no anti-coagulant for antibody detection and one in a sterile, evacuated, heparinised glass blood tube (e.g. Vacutainers) for virus isolation and antigen detection. On arrival at the laboratory, heparinised blood samples should be processed as follows to recover leukocytes:

1. Centrifuge samples at 3,000 xg for 10 minutes. Plasma may be retained for serological analysis.

2. Remove the buffy coat with minimum amounts of plasma and erythrocytes and divide equally between:

a) 1 ml of serum-free maintenance media (see Section 13.5.1) for virus isolation

b) 1ml of Tris-ammonium chloride for antigen detection by ELISA.

3. Following addition of Tris-ammonium chloride, rotate tubes gently for 20-30 seconds to lyse any contaminating erythrocytes.

4. Pellet the leukocytes by centrifugation in a microcentrifuge (11,600 xg) for

15 seconds.

5. Discard the supernatant and re-suspend the pellet in 1.5ml phosphate buffered saline (PBS).

6. Repeat centrifugation and washing with PBS.

7. Resuspend final pellet in 1.5ml PBS containing 1% Nonidet P-40 and agitate at room temperature for 2 hours.

8. Analyse immediately or store samples at -70°C until required.

### 13.3.2 Antigen detection

Antigen detection can be undertaken on placental samples and samples of tissues from aborted foetuses or newly dead "hairy shaker" lambs collected aseptically and placed in sterile containers or on leukocytes recovered from heparinised blood samples as described above.

### 13.3.3 Histological examination

Samples of brain and spinal cord should be placed in a glass sample jar containing a suitable fixative such as calcium formol saline (0.85g sodium chloride and 1.00g calcium chloride dissolved in a mixture of 10ml 40% formaldehyde and 90ml water) with a ratio of fixative to tissue of at least 10:1 (v/v). Fixed tissues should be left for at least four weeks prior to further processing.

### 13.3.4 Antibody detection

#### *13.3.4.1 Blood samples*

Clotted blood samples should be centrifuged at 3,000 xg for 10 minutes and serum removed. If not analysed immediately serum should be stored at 4°C for processing within 7 days or at -20°C for later testing.

#### *13.3.4.2 Foetal fluids*

Stomach contents, heart blood, pleural and peritoneal fluids can be removed from aborted foetuses that are not too autolysed. The fluids should be aseptically removed from the foetus as soon as possible after abortion using a sterile syringe and placed in a suitable sterile container. On arrival at the laboratory, samples should be centrifuged at 3,000 xg for 15 minutes and the supernatants removed and either analysed immediately or stored frozen at -20°C.

***Preparation of reagents***

- Virus Transport Media

Hanks balanced salt solution (see Section 13.5.1)

1% bovine serum albumin (BSA)

| | |
|---|---|
| Penicillin G | 300 IU/ml |
| Streptomycin sulphate | 300µg/ml |
| Polymixin B sulphate | 150 IU/ml |

- Tris Ammonium Chloride

| | |
|---|---|
| Ammonium chloride | 8.3g |
| Tris | 20.6g |
| Distilled water | 1.9litres |

Dissolve ammonium chloride in 1 litre of water (0.16M). Dissolve Tris in 900mls of water (0.17M) and adjust pH to 7.65 with 1M hydrochloric acid.

Working solution: Combine 90mls of 0.16M ammonium chloride and 10mls of 0.17M Tris solution, mix thoroughly and adjust pH to 7.2 with 1M hydrochloric acid.

## 13.4 RISKS TO HUMAN HEALTH

There are no known human health risks associated with pestiviruses.

## 13.5 DIRECT DIAGNOSIS

Confirmation that an abortion or stillbirth is due to BDV is often difficult to establish. The most sensitive proven method for identifying BDV is still virus isolation achieved by growing the virus in cell culture. Direct detection ELISA

and PCR methods are also used.

*N.B. : It is essential that laboratories undertaking virus isolation have a guaranteed supply of pestivirus-free susceptible cells and foetal bovine or equivalent serum which contains no antipestivirus activity and no contaminating virus.*

## 13.5.1 Virus isolation

### *Principle*

Border Disease virus can be isolated from blood, placental material and foetal or lamb tissues in a number of primary or secondary ovine cell cultures (e.g. kidney, testes, lung). Ovine cell lines for BDV growth are rare and unobtainable. Semi-continuous cell lines derived from foetal lamb muscle (FLM) or sheep choroid plexus can be useful but different lines vary considerably in their susceptibility to virus. Detection of viral growth in cell cultures is undertaken using immune-labelling methods.

There are many variations in virus isolation procedures. All should be optimised for maximum sensitivity using reference virus preparation and, whenever possible, recent BDV field isolates. A practical tube isolation procedure with detection by indirect immunofluorescence is outlined below:

### *Materials and reagents*

- Test tubes cultures with sub-confluent or newly confluent monolayers of a suitable primary ovine cell line e.g. foetal lamb kidney.
- UV microscope.
- Reference cytopathic and non-cytopathic BDV strains.
- Maintenance media.
- Growth media.
- Hanks BSS.
- Cold acetone.
- Phosphate buffered saline (PBS).
- BDV antiserum (Moredun Scientific Ltd., UK).
- Conjugate: Donkey anti-sheep IgG conjugated with fluorescein isothiocyanate (FITC).

### *Procedure*

1. Wash test tube cultures with sub-confluent or newly confluent monolayers of susceptible ovine cells at least twice with Hanks BSS to remove growth medium.
2. Inoculate duplicate tubes with 200µl of each test sample.
3. Make suitable dilutions of the reference viruses in maintenance media and inoculate quadruplicate tubes with 200µl of each dilution. Adsorb for 2 hours at 37°C.
4. Wash cultures with 2ml medium, discard and add 1ml of culture maintenance medium.
5. Incubate for 5 to 7 days at 37°C. Examine microscopically on a daily basis and record evidence of cytopathic effect.
6. Freeze tubes at -70°C, and then thaw and passage as before to fresh tube cultures containing cells growing on coverslips.
7. Remove coverslips three days later and fix in cold acetone at 4°C for 15 minutes.
8. Remove coverslips from acetone and place on a staining tray.
9. Add 100µl of BDV antiserum to each coverslip and incubate at 37°C for 30 minutes in a moist atmosphere.
10. Wash coverslips three times in warm PBS for 10 minutes each wash and allow to air dry.
11. Add 100µl of FITC conjugate to each coverslip and incubate at 37°C for 30 minutes in a moist atmosphere.
12. Wash coverslips three times in warm PBS for 10 minutes each wash and allow to air dry.

13. Mount coverslips with cells facing downwards on microscope slides using buffered glycerol.

14. Examine coverslips under UV microscope.

***Reading and interpretation of results***

BDV is confirmed by the presence of diffuse cytoplasmic fluorescence characteristic of pestiviruses (Figure 13.1, page 96).

***Preparation of reagents***

- Growth Media

Medium E199 (Northumbria Biological Ltd, UK)

10% Foetal bovine serum

- Maintenance Media

Medium E199 (Northumbria Biological Ltd, UK)

2% Foetal bovine serum

| | |
|---|---|
| Penicillin G | 100 IU/ml |
| Streptomycin sulphate | 100 µg/ml |

- Hanks BSS

| | |
|---|---|
| Sodium chloride (NaCl) | 40g |
| Potassium chloride (KCl) | 2g |
| Calcium chloride ($CaCl_2.2H_2O$) | 0.93g |
| Magnesium chloride ($MgCl_2.6H_2O$) | 0.5g |
| Magnesium sulphate ($MgSO_4.7H_2O$) | 0.5g |
| Sodium hydrogen phosphate ($Na_2HPO_4.12H_2O$) | 0.76g |
| Potassium dihydrogen phosphate ($KH_2PO_4$) | 0.3g |

Dissolve salts in 5 litres of distilled water, mix thoroughly and sterilise by filtration.

- Buffered Glycerol

| | |
|---|---|
| Sodium hydrogen carbonate ($NaHCO_3$) | 0.286g |
| Sodium carbonate ($Na_2CO_3$) | 0.064g |
| Distilled water | 40ml |
| Glycerol | 360ml |

***Note***

- Alternatively, frozen and thawed cultures can be added to cells growing on chamber slides and stained by IFT as above.
- Immunoperoxidase staining can also be used on coverslips or chamber slides as well as microtitre plates (see method under Virus Neutralisation test, Section 13.6.2.1).
- Frozen and thawed cultures can also be tested in an antigen detection ELISA system (see below).

## 13.5.2 ELISA for antigen detection

The ELISA test for antigen detection is most commonly employed to identify persistently infected viraemic sheep using washed, detergent-lysed blood leukocytes. The sensitivity is close to that of virus isolation and it is a practical method for screening high numbers of bloods. As with virus isolation, high levels of colostral antibody can mask persistent viraemia. The ELISA test is more effective than virus isolation in the presence of antibody, but may give false negative results in viraemic lambs younger than two months old. As well as for testing leukocytes the antigen ELISA can also be used on tissue suspensions, especially spleen, from suspected PI sheep and, as an alternative to IF and IP methods, on cell cultures.

Several pestivirus ELISA methods have been published and a number of commercial kits are now available for detecting BVDV in cattle. While some of these may be suitable for use in sheep further evaluation is required.

***Principle***

The ELISA for pestivirus antigen detection described is a double monoclonal antibody (mab) capture system

which can be used for sheep and cattle samples [1]. Two capture mabs are bound to wells in microtitre plates and two other mabs conjugated to horse radish peroxidase serve as detector mabs.

***Materials and reagents***

- 96 well microtitre plates.
- ELISA plate reader.
- Carbonate buffer, pH 9.6.
- Capture mabs (VPM20 and 13G4; Moredun Scientific Ltd., Scotland).
- Phosphate buffered saline (PBS).
- Tween 20.
- Horse serum free of anti-pestivirus activity.
- Positive control antigen (Moredun Scientific Ltd., Scotland).
- Conjugate: Detection mabs VPM22 and VPM12 conjugated to horse radish peroxidase (HRP) (Moredun Scientific Ltd., Scotland).
- Orthophenylene diamine (OPD) substrate.
- 2.5M sulphuric acid.

***Procedure***

1. Add 100µl of a mixed suspension of the capture mab's each diluted to 1mg/ml with carbonate buffer to all wells of the required number of 96 well microtitre plates. Seal plates and incubate overnight at 4°C (or for one hour at 37°C).

2. Wash plates 3 times with PBS with 0.05% Tween 20 (PBST) and blot dry.

3. Add 100µl of PBST containing 10% horse serum (PBSTH) to all wells. Seal plates and incubate for 1 hour at 37°C.

4. Wash plates 3 times with PBST and blot dry.

5. Add 100µl of each test sample or positive control antigen diluted in PBSTH to plate wells as appropriate.

6. Seal plates and incubate for 2 hours at 37°C or overnight at 4°C.

7. Wash plates 3 times with PBST and blot dry.

8. Dilute the HRP conjugated detection mabs in PBSTH and add 100µl to all wells of the plates.

9. Seal plates and incubate for 1 hour at 37°C.

10. Wash plates 3 times with PBST and blot dry.

11. Add 100µl of activated OPD substrate to all wells and incubate at room temperature for 15 minutes.

12. Add 50µl of 2.5M sulphuric acid to all wells.

13. Read plates using an ELISA plate reader at 492nm.

***Reading and interpretation of results***

Calculate the mean optical densities (OD) of the two control dilutions and of each test sample. Samples with OD's greater than the highest control are positive, samples with OD's less than the lower control are negative, samples with OD's between the two controls are inconclusive and should be retested.

***Preparation of reagents***

- Carbonate Buffer

| | |
|---|---|
| 0.2M sodium carbonate ($Na_2CO_3$) | 7.9ml |
| 0.2M sodium hydrogen carbonate ($NaHCO_3$) | 17.1ml |
| Deionised water | 75.0ml |

Add solutions to distilled water and mix thoroughly. Adjust the pH to 9.6 using 0.2M $NaHCO_3$.

- Orthophenylene Diamine Substrate

| | |
|---|---|
| 0.1M citric acid | 24.3ml |
| 0.2M sodium hydrogen phosphate ($Na_2HPO_4$) | 25.7ml |
| Deionised water | 50.0ml |
| Orthophenylene diamine (OPD) | 0.08g |

Add citric acid and $Na_2HPO_4$ to the

deionised water and mix thoroughly. Adjust the pH to 5.0 with 0.1M citric acid and then add OPD. Immediately before use activate by addition of 40ml of hydrogen peroxide per 50ml substrate buffer.

### 13.5.3 Nucleic acid detection

Ovine pestiviruses can be detected by the reverse transcriptase-polymerase chain reaction (RT-PCR) using primers which also detect pestiviruses from other species [2]. While RT-PCR has not yet been evaluated for diagnostic purposes in sheep it is likely to be of future value. The detection of viral RNA in foetal tissues may yet be an important application since other methods are insensitive. The exquisite sensitivity of RT-PCR makes it a valuable tool for detecting low level virus contaminations as in cell culture constituents or vaccines

In-situ hybridisation has also been shown to be a sensitive method for detecting BDV but it has been used more for research than diagnostic purposes [3].

## 13.6 INDIRECT DIAGNOSIS

### 13.6.1 Histopathology of the brain and spinal cord

*Principle*

A suitable method for preparation and staining of tissues for histopathological examination is described in the chapter on Toxoplasmosis. For diagnosis of BD specific myelin stains such as luxol fast blue can be used as follows:

*Materials and reagents*

- Luxol fast blue stain.
- Ethanol (99%, 95%, 90%, 70% solutions).
- Xylene.
- Saturated lithium carbonate.
- Neutral red stain.

*Procedure*

1. Immerse slides holding processed tissue sections in xylene for 2x10 minutes to remove wax.
2. Hydrate tissues through a graded alcohol series (e.g. 99% ethanol for 2 x 2 minutes, 90% ethanol for 2 minutes, 70% ethanol for 2 minutes).
3. Rinse slides thoroughly under running tap water.
4. Immerse slides in luxol fast blue stain (1% solution in ethanol) overnight at 60°C.
5. Rinse slides in 95% ethanol then in distilled water.
6. Immerse slides in saturated lithium carbonate for 5 seconds.
7. Examine under microscope: if there is clear differentiation between blue stained white matter and colourless grey matter continue to step 8; if not, immerse slides in 70% alcohol until there is a clear differentiation between the tissues.
8. Rinse slides thoroughly under running tap water.
9. Wash slides in distilled water.
10. Immerse slides in 1% neutral red counterstain (1% in distilled water) for 3 minutes.
11. Examine under microscope: if there is clear differentiation between blue stained white matter and red stained grey matter continue to step 12; if not, immerse slides in 95% alcohol until there is a clear differentiation between the tissues.
12. Rinse slides thoroughly under running tap water.
13. Dehydrate and clear slides through a graded alcohol series and xylene (e.g. 70% ethanol for 2 minutes, 90% ethanol for 2 minutes, 99% ethanol for 3 x 2 minutes, xylene for 2 x 2

minutes).

14. Mount coverslips over the stained tissue sections and examine under a microscope.

***Reading and interpretation of results***

The most characteristic feature of BD is a deficiency of stainable myelin.

## 13.6.2 ANTIBODY DETECTION

Antibody to BDV can be detected in sheep sera using virus neutralisation (VN) or an ELISA. Control positive and negative reference sera must be included in every test. These should give results within predetermined limits for the test to be considered valid. Single sera from several sheep can be tested to determine the prevalence of BDV in a flock, region or country. For diagnosis, however, acute and convalescent sera are the best samples for confirming acute BDV infection. Bleeds from one animal should always be tested alongside each other on the same plate.

### *13.6.2.1 Virus neutralisation test*

***Principle***

The cytopathic Moredun strain of BDV can be used for the VN test with semicontinuous cells such as FLM. An outline protocol is given below.

***Materials and reagents***

- Cell culture grade flat-bottomed 96 well microtitre plates.
- $CO_2$ incubator.
- Suitable cells diluted to 2 x $10^5$ cells/ml.
- Growth media (see Section 135.1).
- Cytopathic BDV (Moredun strain).
- Control positive and negative sera.

***Procedure***

1. Heat inactivate test sera for 30 minutes at 56°C.

2. It is usual to screen all test sera initially at a dilution of 1/4 and then to titrate out positive sera. To screen sera a minimum of four wells per sample is required: two wells to measure neutralisation activity and two wells to verify the absence of serum toxicity to the cells. To titrate positive sera, starting from a dilution of 1/4, make serial twofold dilutions of the test sera in cell culture media in 96 well microtitre plates using two wells for each sample. The lowest serum dilution of each sample should be added to two additional wells to act as a toxicity control.

3. Use 25µl volumes of the diluted serum in each well and add 25µl of media to each of the lower two control wells.

4. Add 25µl of media containing 100 TCID50 of virus to each of the test wells and 25µl of media to each of the wells for toxicity control. Include control positive and negative sera and a virus titration in every test.

5. Seal plates with non-toxic plate sealers or lids and incubate at 37°C for 1 hour.

6. Add 100µl of a cell suspension with a count of 2 x $10^5$ cells/ml to every well.

7. Seal the plates or incubate in a moist chamber in 5% $CO_2$ for four days at 37°C.

8. Examine the wells microscopically for cytopathic effect.

***Reading and interpretation of results***

The VN titre for each serum is the dilution at which the virus is neutralised in 50% of the wells. This can be calculated by the Spearman-Karber method [5]. A seronegative animal will show no neutralisation at the lowest dilution.

*Note*

• Cytopathic effects in the control wells of the test sera will be due to toxicity. Further dilution of toxic sera can be attempted but it may not be possible to obtain reliable results with occasional sera.

• The choice of test virus is difficult due to antigenic diversity among pestiviruses [6]. Reference strains of cytopathic BVD viruses can be used. Oregon C24V results correlate better with Moredun BDV than results with the NADL strain. No single strain is ideal. A local strain which gives the highest antibody titre with a range of positive sheep sera should be used.

• The VN test can also be used with non-cytopathic viruses when the following immunoperoxidase staining system is used after stage 7 above.

### *13.6.2.2 Imunoperoxydase staining*

*Materials and reagents*

- 95% acetone in distilled water (held at -20°C overnight).
- Phosphate buffered saline (PBS).
- BDV antiserum.
- Tween 80.
- Conjugate: anti-sheep IgG, peroxidase conjugated (Sigma/ICN-flow/Dako).
- 3-amino-9-ethyl carbazole (AEC) substrate.

*Procedure*

1. Remove culture medium and wash cells gently with warm PBS.
2. Air dry cells and cool plates to 4°C.
3. Fix cells by quickly adding 95% acetone (in water) previously cooled to -20°C to all wells. Hold plates at -20°C for 30 minutes. Do not stack plates or allow them to warm or etching of the plastic may occur.
4. Remove acetone and dry plates quickly in a cool environment.
5. Wash plates with PBS and blot dry. Fixed plates can be stored at -20°C before staining.
6. Add 50µl of BDV antiserum to all wells at a predetermined dilution in buffered saline with 1% Tween 80 (PBST). Incubate at 37°C for 30 min in a moist atmosphere.
7. Empty plates and wash three times with PBST.
8. Drain wells and add 50µl of conjugate at a predetermined dilution in PBST to all wells. Incubate for 30 minutes at 37°C in a moist atmosphere.
9. Empty plates and wash three times with PBST.
10. Drain wells and add 50µl of activated AEC substrate.
11. Incubate plates at room temperature and monitor known virus positive control wells for development of specific red-brown cytoplasmic staining (5-10 minutes). When staining is complete remove substrate carefully and wash wells thoroughly with tap water. Leave tap water in wells and examine microscopically for virus containing wells.

*Reading and interpretation of results*

The VN titre is calculated as above using the Spearman-Karber method [5].

*Preparation of reagents*

- 3-Amino-9-Ethyl Carbazole Substrate

Stock solution: 0.1g AEC dissolved in 15ml dimethyl formamide.

Working solution: add 0.3ml stock to 5µl 0.05M acetate buffer pH 5.0.

Activate immediately before use by addition of 25µl 30% $H_2O_2$.

***NB*** *: This solution is toxic and should be handled with adequate precautions.*

### *13.6.2.3 ELISA*

***Principle***

A monoclonal antibody capture ELISA for measuring BDV antibodies is described. Two pan pestivirus mabs detecting different epitopes on the immunodominant non-structural NS 2/3 protein are used to capture detergent-lysed cell culture-grown antigen. The results correlate qualitatively with the VN test [7].

***a / Antigen preparation***

***Materials and reagents***

- 225cm$^3$ flasks FLM cells.
- Cytopathic BDV (Moredun strain).
- Maintenance media.
- Foetal bovine serum (FBS).
- Phosphate buffered saline (PBS).
- Nonidet P40.

***Procedure***

1. Use eight x 225cm$^2$ flasks of newly confluent FLM cells, four controls and four infected. Wash flasks and infect four with a 0.01-0.1 m.o.i. of Moredun cytopathic BDV. Adsorb virus for 2 hours at 37°C.

2. Add maintenance media containing 2% FBS and incubate cultures for 4-5 days until cytopathic effect is obvious.

3. Pool four control flask supernatants and separately pool four infected flask supernatants. Centrifuge at 3000xg for 15 minutes to pellet cells.

4. Discard supernatants and retain cell pellets.

5. Wash flasks with 50ml PBS and repeat centrifugation as above.

6. Pool all control cell pellets in 8ml PBS containing 1% nonidet P40 and return 2ml to each control flask to lyse remaining attached cells.

7. Repeat for infected cells.

8. Keep flasks at 4°C for at least 2 hours agitating the small volume of fluid on the cells vigorously every 1/2 hour to ensure total cell detachment.

9. Centrifuge control and infected antigen at 12,000xg for 5 minutes to remove cell debris.

10. Store supernatant antigens at -70°C in small aliquots.

***b / ELISA test***

***Materials and reagents***

- 96 well ELISA grade microtitre plates.
- ELISA plate reader.
- Capture monoclonal antibodies (Moredun Scientific Ltd., Scotland).
- Bicarbonate buffer (see Section 13.5.2).
- Phosphate buffered saline with 1% Tween 80 (PBST).
- Horse serum.
- Anti-ovine IgG peroxidase conjugate.
- Orthophenylene diamine (OPD) substrate.
- 2.5M Sulphuric acid.

***Procedure***

1. Dilute the two mabs to a pre-determined dilution (commonly 1/4000 ascites) in 0.05M bicarbonate buffer, pH9.6. Coat all wells of a suitable ELISA grade microtitre plate (e.g. Nunc maxisorb, Greiner 129b) overnight at 4°C using 100µl volumes per well.

2. Wash 3 times in PBST, then add 100ml of a blocking solution of PBST + 10% horse sera (PBSTH) to all wells and incubate at 37°C for 1 hour.

3. Dilute the antigen to a pre-determined dilution in PBSTH and coat alternate rows of wells with 100µl of virus and control antigens for 1 hour at 37°C.

4. Wash plates 3 times in PBST.

5. Dilute test sera 1/50 in PBSTH and add 100µl of each sera to duplicate virus and duplicate control wells. Incubate for

one hour at 37°C.

6. Wash plates 3 times in PBST.

7. Dilute anti-ovine IgG peroxidase conjugate to a pre-determined dilution in PBSTH and add 100µl to all wells. Incubate for 1 hour at 37°C.

8. Wash plates 3 times in PBST.

9. Add 100µl of a suitable activated enzyme substrate, such as ortho-phenylene diamine noting the manufacturer's toxicity warning.

10. After colour development stop the reaction with 50µl of 2.5M sulphuric acid.

11. Read the optical density (OD) on an ELISA plate reader.

***Reading and interpretation of results***

The mean value of the 2 control wells is subtracted from the mean value of the 2 virus wells to give the corrected OD (CODs) for each serum. Results are expressed as CODs with reference to CODs of known positive and negative sera. Alternatively, ELISA titres can be extrapolated from a standard curve of a dilution series of a known positive reference serum.

## 13.7 FUTURE WORK

While reliance on cell culture methods is essential for evaluation of most methods direct detection systems, especially those using PCR, will continue their development.

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# Chapter 14

# ARTHROPOD-BORNE DISEASES

B. J. H. BARNARD

## 14.1 SUMMARY

Rift Valley Fever (RVF), Wesselsbron disease (WSL), Bluetongue (BT) and Akabane disease (AKA) are arthropod-borne viral diseases of sheep and goats, transmitted primarily by mosquitoes and biting midges, that can result in abortion, neonatal death and/or congenital malformations when the pregnant animal is infected. The outcome of infection by RVF, WSL, BT and AKA viruses may differ from inapparent to severe. The main factors responsible for this variation are the stage of gestation at the time of infection, viral characteristics and susceptibility of the ewe.

The unequivocal diagnoses of RVF, WSL, BT or AKA virus as a cause of abortion in sheep and goats is not easy. The climatological conditions and clinical manifestations combined with virus isolation, histopathological changes in specific tissues, viral antigen detection and presence of antibodies in the sera of the dam or precolostral sera from affected young have varying relevance in the diagnosis of involvement of each of the four viruses in abortion.

## 14.2 INTRODUCTION

Known arthropod-borne viral diseases most likely to result in abortions, neonatal deaths and/or congenital malformations in sheep are Rift Valley Fever (RVF), Wesselsbron disease (WSL), Bluetongue (BT) and Akabane disease (AKA). Strictly speaking BT and AKA are not single disease entities. Bluetongue includes 24 serotypes all of which are interrelated in a complex network of cross-relationships but often with insufficient cross-protection to prevent infections by other serotypes. Akabane virus on the other hand is a single member of the Simbu group. Several members of this group may produce similar defects in the foetus. Akabane virus is nevertheless by far the best studied and probably the most pathogenic member. Other members of the Simbu group which have been associated with natural disease are Aino and Tinaro and the list of viruses involved in this syndrome will probably grow. On rare occasions other Flavi viruses like Banzi and West Nile, members of the Palyam-serogroup, may cause sporadic abortions and congenital defects. It is quite possible that other yet unidentified arthropod-borne viruses may also be responsible for abnormalities in sheep.

Wild-type RVF viruses referred to as hepato-, viscero- or pantropic viruses usually cause a peracute or acute zoonotic disease of domestic ruminants in Africa. In sheep and goats there is a high mortality rate in lambs and kids and affected ewes and nanny goats usually abort. In fully susceptible flocks natural infections cause abortion storms of foetuses at all stages of pregnancy. Livers of infected animals are enlarged, soft and friable with small necrotic foci and are, in lambs and kids, almost invariably accompanied by generalized haemorrhages. Icterus may be evident. The peripheral and visceral lymph nodes are enlarged, oedematus and may show petechia.

The clinical signs of WSL in adult sheep and lambs are very similar to those

of RVF but icterus is more prominent. Subclinical infections in adult sheep are common and WSL normally occurs sporadically with a much lower mortality rate particularly in adult sheep. Congenital malformation accompanied by arthrogryposis has been reported in a few outbreaks but are mostly restricted to pregnant ewes which have been vaccinated with neurotropic attenuated live vaccines. The occurrence of WSL under conditions which favour the occurrence of RVF are a probable reason for the infrequent diagnosis of WSL.

Bluetongue particularly affects sheep and the virus causes haemorrhages, ulceration, cyanosis, coronitis, laminitis, oedema of the head and neck and torticollis. Mortality can vary from 2 to 30 percent. Abortions and congenital deformities in sheep as a result of BT are extremely rare but have been produced under experimental conditions and following vaccination of ewes during pregnancy.

Akabane disease produces clinically inapparent infections in adult sheep. In pregnant ewes, however, marked teratology, particularly of the foetal nervous system may result. The physical effects are usually only observed with the birth of affected lambs. Abortion and stillbirth are other, variable manifestations of the infection.

As these diseases are insect transmitted they have a seasonal occurrence affected by the distribution, seasonal activity and abundance of insect vectors. Mosquitoes are proven vectors of RVF and WSL, and distinct species act as the most important vector in a particular region. *Culicoides* species are the only proven vector of BT and both mosquitoes and biting midges have been incriminated as vectors of AKA. It has also been shown that AKA virus can multiply in *C.brevitarsis* under experimental conditions. The isolation of all four viruses from a wide variety of other arthropods does not indicate their importance as vectors of the virus but provides evidence of the presence of the virus.

Rift Valley Fever and WSL are African diseases. In Eastern and Southern Africa where RVF occurs as epidemics in relatively dry countries, outbreaks have always been associated with above average rainfall and in irregular cycles of 5 - 20 years and even longer. In endemic areas, where insect vectors are present continuously, the disease occurs more regularly and indications are that WSL follows a similar pattern. The occurrence of RVF in Western and Northern Africa is probably the result of the movement of infected persons or the transportation of infected slaughter animals to markets in these areas.

Bluetongue, on the other hand, is present in subtropical and tropical areas of Africa, the Middle East, the Americas and Australia and can occur regularly every year although it has a higher prevalence in wetter years. The continuous presence of BT is most likely due to the involvement of dung breeding Culicoides species which are much less dependent on wet seasons for their presence.

Most of Africa, Asia (excluding Russia) and Australia may be regarded as endemic for AKA virus and, in all probability, many of its endemic relatives. The American continent (except for the presence of Cache Valley virus in Texas), Papua-New Guinea and the island countries of the Pacific are free of infection.

The incidence of RVF, WSL and BT increases in summer and reaches a peak in late summer to early autumn. This pattern correlates closely with the increase and abundance of insect vectors. After the first frost in winter both insect vec-

tors and disease usually disappear. However, abnormal lambs may be born up to 3 months after disappearance of the vectors. In warmer climates where insect vectors occur continuously, seasonality may not be a common feature of these diseases. It is also clear that under normal conditions these diseases cannot occur in the absence of the insect vector. Consequently the introduction of animals from infected countries into vector free areas or in winter, when the vector populations are insignificant, may be a relatively safe procedure. However, global warming may result in the enlargement of regions where vectors can survive and this may expand the areas where arthropod-borne diseases can occur.

Based on the success of diagnosis, arthropod-borne viruses causing abortion or malformations can be divided into two groups. Rift Valley Fever virus usually produces non-characteristic signs in individual animals, but causes a high mortality rate in lambs and is accompanied by abortion in ewes at all stages of pregnancy. The virus can usually be isolated from the blood of the ewe at the time of abortion and almost invariably from tissues of the aborted foetus. On the other hand, isolation of WSL, which rarely, and AKA, which never, produces clinical signs in adult non-pregnant sheep is mostly unsuccessful. It is widely accepted that BT virus does not cause abortions and the isolation of this virus from an aborted foetus is very unlikely.

General recommendations made to farmers concerning the control of arthropod-borne infections of livestock include housing of sheep at night when mosquitoes and midges are most active and moving stock away from low-lying, poorly-drained areas to high, wind-swept grazing where biting arthropods are usually less prevalent. Where *Culicoides* species are involved, the use of insecticides, lavicides and sterilization of males by irradiation can help control vector populations. Keeping cattle in close proximity to sheep has also been reported to be effective, apparently because *C.imicola* (the most prevalent vector in South Africa) has a preference for cattle. Such methods are usually impractical, instituted too late and, at best, palliative in the face of viral epidemics where immunization remains the only effective way to protect livestock.

Two vaccines are currently available for immunization against RVF. A modified live vaccine that uses the Smithburn strain of RVF is available for use in South Africa and Kenya. The vaccine induces durable immunity in sheep six to seven days after a single inoculation. In a proportion of pregnant animals it may cause abortions or teratology of the foetus and hydrops amnii and prolonged gestation in the dam. A formalin-inactivated cell culture vaccine is available in South Africa and Egypt which has proved safe to use even in pregnant animals. The vaccine, however, is expensive to produce and only induces a short-lived immunity so that administration of regular booster doses is necessary to ensure adequate protection. An attenuated live vaccine is available for use against WSL in non-pregnant sheep and goats. The immunity induced following vaccination is life-long so revaccination is not recommended.

Prophylactic immunization of sheep against BT is complicated by the existence of multiple serotypes. Three live attenuated pentavalent vaccines have been developed in South Africa and they are administered to sheep at three-weekly intervals and repeated annually. After two or three annual immunizations most sheep are immune to all serotypes in the vaccine.

There are indications of temporary infertility in both ewes and rams vaccinated for the first time which necessitates immunization well before or after the mating season. Immunization of pregnant ewes can result in brain defects in foetuses ruling out vaccination during pregnancy.

A formalin-inactivated vaccine is available in Japan for use against AKA and a similar vaccine will soon be commercially available for use in Australia.

## 14.3 SAMPLES

Proper collection and handling of samples is highly important to the successful recovery of any virus which might be present. Samples should be taken with sterile precautions to prevent contamination with bacteria. Post-mortem materials should be taken with care to avoid cross-contamination of tissues and it is recommended that separate sets of sterile instruments are used for recovery of each tissue.

Fresh non-fixed tissue samples should be treated as described in the chapter on Border Disease.

Pre-colostral blood samples from newborn lambs should be collected when possible together with blood samples from aborted ewes for antibody detection. Samples should be taken into sterile, evacuated glass blood (e.g. Vacutainers) and treated as described in the chapter on Border Disease.

## 14.4 RISKS TO HUMAN HEALTH

In man, both RVF and WSL can cause an influenza-like syndrome, which may be lethal in the case of RVF and is caused by the handling of infected material and through transmission by mosquito vectors. It is advisable to protect people likely to handle RVF infected animals or products by wearing of protective clothing and vaccination where available.

## 14.5 DIAGNOSIS

The available methods of diagnosis, both direct and indirect, and their applicability for each of the four diseases is summarised in Table 14.1. In the case of RVF, WSL and AKA the history and epidemiology of the disease, including a relatively high rate of abortion, foetal abnormalities and/or mortality in newborn lambs or kids, as well as climatological conditions which favour the occurrence of large populations of suitable vectors, should warn of the possible presence of these viruses. Viral isolation and detection, histopathological changes in specific tissues and detection of viral antigens are the main diagnostic methods but their relevance varies for each of the viruses. A variety of methods are also available to detect antibodies to each of the viruses in sera. Sera collected from aborted ewes or nanny goats has limited diagnostic value: sera that contains no antibodies to a specific virus can establish that the virus was not the cause of abortion, but the presence of antibodies does not confirm viral involvement in the abortion as it cannot be proven that infection occurred during pregnancy. Where lambs and kids are born live, antibody determination in precolostral blood samples can be used, in conjunction with other methods, to help confirm viral infection as the cause.

### 14.5.1 Rift Valley fever

Histopathological lesions in the liver of aborted lambs and kids leave

little room for doubt about the diagnosis of RVF. The virus can be readily isolated from the aborted placenta and foetal tissues by intracerebral or intraperitoneal injection of infected materials into suckling mice and/or cell culture with detection by immunofluorescence (IF). Viral antigen can frequently be detected rapidly in impression smears of infected tissues by IF, in tissue suspensions by complement fixation (CF) and immunodiffusion and in tissue sections by immunoperoxidase staining. The presence of antibodies to RVF in sera can be determined by a number of techniques (Table 14.1).

*Table 14.1 : Summary of diagnostic methods and their applicability for the detection of arthropod-borne diseases as the cause of abortion in small ruminants*

| | Rift Valley Fever | Wesselsbron Disease [a] | Bluetongue [b] | Akabane Disease [c] |
|---|---|---|---|---|
| **Virus Isolation from:** | | | | |
| Placenta | + | - | + | - |
| Foetal tissue | + | - | + | + |
| **Virus Isolation by :** | | | | |
| Mice inoculation | + | (+) | + | + |
| Cell culture | + | (+) | + | + |
| Embryonated Hens eggs | (+) | - | + | (+) |
| Histopatholgy | Foetal liver | Foetal liver | - | Foetal brain |
| **Antigen Detection in Fixed Tissues** | + | + | + | + |
| **DNA Probes/PCR** | + | - | + | - |
| **Antibody Detection by :** | | | | |
| Virus Neutralisation Test | + | + | + | + |
| Serum Neutralisation Test | + | + | + | - |
| ELISA | + | + | + | + |
| Indirect / Direct | | | | |
| Haemagglutination Test | + | + | + | + |
| Complement Fixation Test | + | + | + | + |
| Indirect / Direct Fluorescent | | | | |
| Antibody Test | + | - | - | - |
| Gel Diffusion | + | - | + | - |

*a - Wesselsbron virus has only ever been isolated from experimental infections, never from field samples.*

*b - Bluetongue virus has only been shown to produce abortion in sheep under experimental conditions or following vaccination of ewes during pregnancy. Tests, however, may be relevant when young are born with congenital defects.*

*c - Akabane virus has rarely been isolated from aborted foetuses although placental material has occasionally been shown to contain the virus.*

### 14.5.2 Wesselsbron disease

Wesselsbron virus has never been isolated from an aborted foetus in the field although it has been isolated from foetuses aborted from experimentally infected pregnant ewes. The macroscopic and microscopic lesions in the liver of aborted foetuses are often sufficient to make a diagnosis of WSL. The presence of antibodies to WSL in sera can be determined by haemagglutination inhibition (HAI), CF and virus neutralisation test.

### 14.5.3 Bluetongue

Since infection of pregnant ewes with BT virus produces foetal abnormalities rather than abortion, clinical signs and lesions in the affected animals are significant in diagnosis. Bluetongue virus can be isolated from clarified tissue suspensions of affected young most readily by intravascular inoculation of 10-12 day old embryonated hen's eggs followed by passage in suckling mice brain or cell cultures with detection by CF or IF. An indirect peroxidase-antiperoxidase (PAP) test has been used to demonstrate BT virus in sections of the chorio-allantoic membrane as has an avidin-biotin amplified staining procedure to demonstrate BT virus antigens in tissue samples from infected foetuses. The presence of antibodies to BT in sera can be determined CF, gel immunodiffusion and ELISA. Type specific antibodies are best detected by means of virus neutralisation tests, such as plaque reduction.

### 14.5.4 Akabane disease

Lambs which develop hydranencephaly (HE) or arthrogryposis (AG) or both as a result of intra-uterine infection with AKA virus may be aborted, stillborn or delivered alive at term. Culture of tissues from affected animals for virus is usually negative although placental material may contain virus which can be isolated in suckling mice and/or cell culture. Diagnosis therefore requires that parts of the brain affected are determined macroscopically and microscopically. Precolostral serum collected from affected animals can be used to detect antibodies to AKA virus by virus neutralisation test, agar-gel diffusion or ELISA and provide strong evidence that the affected animal was infected in utero.

## 14.6 DIRECT DIAGNOSIS

### 14.6.1 Virus isolation

Demonstration of virus in placental material or aborted foetuses is the best way of diagnosing RVF, BT and AKA viruses as the cause of abortion or congenital malformation. Virus isolation is best carried out in suckling mice inoculated intracerebally or in a variety of cell cultures including VERO, BHK 21, CER as well as primary foetal calf and lamb kidney and testis cells. Viruses are detected by tests such as immunofluorescence, ELISA, or complement fixation. For isolation of BT increased sensitivity can be achieved by intravenous inoculation of 10-12 day old embryonated hen's eggs with blood or clarified tissue suspensions. After a single passage in eggs the virus can be readily cultivated in cell cultures or in mouse brain. Virus neutralization tests are commonly used to distinguish between serotypes of BT.

*N.B. It is essential that laboratories undertaking virus isolation have a guaranteed supply of virus-free susceptible cells and foetal bovine or equivalent serum which contains no antivirus activity and no contaminating virus.*

### *14.6.1.1 Preliminary virus isolation*

#### *a / Mouse inoculation*

***Principle***

Sample materials are inoculated intracerebrally into 1-2 day old suckling mice. Any virus present will multiply and cause death of the mice. The brains are removed from dead mice and used to inoculate susceptible cell cultures as described in Section 14.6.1.2.

***Materials and reagents***

- 1-2 day old suckling mice.
- Maintenance media (p.190)

***Procedure***

1. Inoculate 20µl volumes of each sample intracerebrally into each of at least 5 suckling mice.
2. Observe the mice at least twice daily for at least 10 days.
3. Aseptically remove the brains from any mice that die.
4. Homogenise brains in maintenance media to give a 10% suspension (w/v).
5. Inoculate cell monolayers with the resultant homogenate or store homogenate at 4°C for up to 2 weeks, at -20°C for between 2 weeks and 3 months and at -70°C for period of longer than 3 months.

***Note***

• When only one or two mice die or there are no deaths, surviving mice should be killed, their brains removed, homogenised and a second passage undertaken in infant mice.

#### *b / Virus isolation in embryonated hen's eggs*

***Principle***

Samples are inoculated intravenously into embryonated hen's eggs at 10-12 days of age. Any viruses will multiply there and cause the death of the embryo after 2-5 days depending on the number of viral particles inoculated. The embryos in the dead eggs, recovered on the 4th day after inoculation are very rich in virus and are used to inoculate susceptible cell cultures as described in Section 14.6.1.2.

***Materials and reagents***

- Specific pathogen free embryonated hen's eggs.
- Egg incubator and candler.
- Phosphate buffered saline (PBS).
- Antibiotics: gentamycin (gentalline) or streptomycin and amphotericin B (fungizone).

***Procedure***

1. Incubate the embryonated hen's eggs at 38°C in a humid atmosphere (50 - 70% humidity) until 10-12 days of age. Candle to verify their vitality which is indicated in each by the air sac and the embryo.
2. Dilute the samples 1 in 10 with PBS containing antibiotics (500µg/ml gentamycin or 200µg/ml streptomycin and 0.5µg/ml amphotericin B).
3. Inject 0.1ml of the neat and 1 in 10 dilution of samples intravenously into each of 3 to 5 eggs.
4. Incubate the eggs at 34°C and candle daily.
5. Aseptically harvest embryos dying from the 2nd day after inoculation.
6. Homogenise each embryo with 5ml of PBS, then centrifuge at 900 xg for 10 minutes.
7. Remove the supernatant and either inoculate cell monolayers or aliquot and store at 4°C for up to 2 weeks, at -20°C for between 2 weeks and 3 months and at -70°C for period of longer than 3 months.

### *14.6.1.2 Tissue culture isolation and detection*

Viral isolation can be undertaken following the method described in the chapter on Border Disease using the relevant reference virus strain, viral antiserum, a rabbit or mouse IgG conjugated with fluorescein isothiocyanate (FITC) and the following cell maintenance media :

Eagles minimum essential medium (MEM)
10% tryptose phosphate broth (TPB)
2% foetal bovine serum (FBS)

| | |
|---|---|
| Penicillin G | 200 IU/ml |
| Streptomycin sulphate | 100 µg/ml |
| Kanamycin | 100 µg/ml |
| Fungizone | 2.5 µg/ml |

***Reading and interpretation of results***

Viral presence is confirmed by the presence of fluorescence characteristic of virus.

## 14.7 INDIRECT DIAGNOSIS

### 14.7.1 Histopathology

***Principle***

A suitable method for histopathological preparation of tissue samples and haematoxylin and eosin (H & E) staining is described in the chapter on Toxoplasmosis. For diagnosis of RVF and WSL virus infections characteristic changes occur in the liver whereas in AKA virus infections changes in the brain and spinal cord are the most useful diagnostic tool. In the latter case specific myelin stains such as Luxol fast blue can be used as described in the chapter on Border Disease.

### *14.7.1.1 Rift valley fever*

Histopathological examination of the liver reveals primary foci of necrosis comprising dense aggregates of cytoplasmic and nuclear debris, some fibrin, and a few neutrophils and macrophages which can be discerned against a background of parenchyma reduced by nuclear pyknosis, karyorrhexis and cytolysis, with only narrow rims of degenerated hepatocytes remaining reasonably intact close to portal triads. Destruction of hepatocytes may be so marked that most of the normal architecture of the organ is lost, giving the liver almost the appearance of lung tissue. Intensely acidophilic cytoplasmic bodies which resemble the Councilman bodies of yellow fever are common, and rod-shaped or oval eosinophilic intranuclear inclusions are found in about 50% of affected livers. Mineralisation of necrotic hepatocytes may be evident as small purplish-blue cytoplasmic granules in H & E stained sections in approximately 60% of lambs while bile casts are present in about 30% of livers.

### *14.7.1.2 Wesselsbron disease*

Histopathological examination of the liver reveals mild to extensive necrosis of the parenchyma; individual or small groups of necrotic hepatocytes are scattered throughout the lobules. Apart from the necrotic hepatocytes, most other liver cells reveal cloudy swelling and hydropic degeneration. Other changes in the liver include mild to moderate Kupffer cell proliferation, cholestasis, mild to moderate proliferation of bile ductules, and infiltration of mononuclear cells, particularly histiocytes, in the portal triads. Intranuclear inclusion bodies, which stain eosinophilically in H & E tissue sections and which are inconsistent in shape and size, are present in some necrotic hepatocytes.

### *14.7.1.3 Akabane disease*

The pathogenic effects of AKA virus on the developing foetus depend on the gestational age at the time of infection and may vary from arthrogryposis (AG) and hydranencephaly (HE) to porencephaly (PE) and micrencephaly (ME). The extent of damage to the brain of infected lambs can vary within and between brains and between regions of the brain.

In lambs exhibiting AG and HE the skull tends to appear normal but most of the forebrain is replaced by membranous sacs of liquid and structures remaining are not recognisable. The midbrain, cerebellum and medulla oblongata may be reduced in size and the meninges may consist of fused membranes with focal areas of necrotic debris and fibrous thickening. In the medulla of the thymus Hassall's corpuscles may be prominent and contain much keratin and eosinophilic hyaline material. The cortex may be narrow and sparsely populated with thymocytes. The spinal cord may be reduced in size with marked reduction in the extent of myelination of the ventral cord and reduction in ventral cord neurons many of which exhibit degenerative changes. Small mineralised plaques may be present beneath the meninges of the spinal cord.

In lambs exhibiting PE there may be large liquid filled cavities in the subcortical areas of the cerebellum and numerous small to large areas of caviation and small malacic foci present in the cerebral cortex. The rest of the brain and spinal cord tend to appear normal.

Lambs with ME have small heads with abnormally thick skull bones and a reduced cranial cavity. The brain is markedly smaller than in similar uninfected lambs due to a reduction in the size of the cerebral hemispheres. Sections of the brain show few changes but there may be decrease in the parenchyma of the cortex of the cerebellum but no degenerative changes.

## 14.7.2 Detection of viral antigen

Immunohistochemical labeling techniques, such as immunoperoxidase and peroxidase anti-peroxidase (PAP) staining methods, can also be applied to histological tissue sections to detect RVF, WSL and BT antigens. The PAP technique can be carried out as described in the chapter on Toxoplasmosis using suitable primary and binding antibodies.

## 14.7.3 Detection of viral antibodies

Serological diagnosis is of limited value in the case of RVF, WSL and BT but it is currently the only way to assist in the diagnosis of AKA in which serological and circumstantial evidence and the exclusion of other causes must be employed. The only evidence of specific infection in an abnormal lamb is the presence of specific antibody in serum collected before the lamb has received colostrum. It may also be possible to demonstrate antibodies against the causative virus in the pre-colostral serum of normal newborn lambs born in the same period as abnormal lambs as not all infected lambs will necessarily develop clinical signs.

The presence of IgG antibodies indicates the presence of virus but is poor evidence of recent infection. Where tests for IgM antibodies are available these should be the serological test of choice to establish recent infection. Virus neutralisation tests are the most specific serological test available.

## 14.7.4 Virus Neutralisation test

The virus neutralisation test described in the chapter on Border Disease can be used to diagnose AKA using a suitable cytopa-

thic AKA virus, suitable control positive and negative sera and the growth media given below with the following changes :

1. Add virus at a concentration of 200 TCID50 per 25µl.

2. Following the addition of cells, incubate plates for up to seven days.

- Growth Media

Eagles minimum essential medium (MEM)

10% tryptose phosphate broth (TPB)

10% foetal bovine serum (FBS)

***Reading and interpretation of results***

The VN titre for each serum is the dilution at which the virus is neutralised in 50% of the wells. This can be calculated by the Spearman-Karber method. A seronegative animal will show no neutralisation at the lowest dilution.

## 14.8 FUTURE WORK

In recent years, genomic probes have been developed. The sensitivity of probe assays is generally lower than that of most other methods and their use is limited to well equipped laboratories. However, the advent of the polymerase chain reaction (PCR) and its application to transcription-based in vitro gene amplification has led to the development of highly sensitive assays using non-radioactive probes. This rapid method is relatively expensive and needs further investigation and/or modification before it can be used routinely for diagnostic purposes. Demonstration of viral antigen by PCR in cases of abortion must be evaluated cautiously as this sensitive technique can detect virus antigens produced long before the actual infection that precipitated the abortion and may lead to a false positive result.

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# Chapter 15

# NAIROBI SHEEP DISEASE

H.W. REID

## 15.1 SUMMARY

Nairobi sheep disease (NSD) is a tick-transmitted virus infection of sheep and goats. The disease is characterized by a marked febrile response followed by profuse watery diarrhoea that often becomes haemorrhagic and frequent abortion in pregnant animals. The course of disease may be peracute, acute, mild or inapparent, with a case mortality frequently over 80 percent.

As with other arthropod-transmitted viral infections, the unequivocal diagnosis of NSD is not easy. Nairobi sheep disease virus can be isolated from aborted materials and tissues of adult animals by inoculation either into infant mice or into baby hamster kidney cells with identification by immunofluorescence. In recovered animals the detection of serum antibody by indirect immunofluorescence, indirect haemagglutination or enzyme-linked immunoabsorbent tests will indicate previous infection with NSD.

## 15.2 INTRODUCTION

The virus of NSD belongs to the Nairovirus genus within the family of arthropod-transmitted viruses known as the Bunyaviridae. Nairoviruses have distinct structural and antigenic characteristics and can be subdivided into six serogroups. The serogroup to which NSD virus belongs also contains Dugbe and Ganjam viruses isolated from ticks in Nigeria and India, respectively. The virus measures 90 to 100 nm in diameter and contains RNA, and all isolates appear to be antigenically identical.

Nairobi sheep disease was first described in Kenya in 1910 and it was subsequent established that the disease was caused by a virus transmitted by Rhipicephalus appendiculatus ticks that had previously engorged on sheep reacting to infection. Following the recognition of NSD in the area around Nairobi, its distribution within Kenya was found to correspond generally to that of *R.appendiculatus*. However, other ticks can transmit the virus and it has been isolated from *Amblyomma variegatum* and *R. pulchellus*, suggesting that the epidemiology of NSD may be complex. The disease has now been identified in Uganda, Northern Somalia, and probably Zaire and Ruanda. There is serological evidence that it is present in Botswana, Ethiopia, Tanzania and South Africa.

Natural transmission between sheep occurs only be tick bite and it has been concluded that NSD is maintained in a sheep-tick-sheep cycle. Both transtadial and transovarial transmission have been demonstrated in the tick vector. The virus is shed in the urine and faeces but the disease is not spread by contact. Clinical signs in animals reared in areas infested with infected ticks are usually not observed. In contrast, the response of naive sheep moved to endemic areas is dramatic.

Following exposure to infected ticks,

the incubation period is four to six days. Pyrexia of sudden onset is the first sign, with temperature rising rapidly to 40 to 42°C. Affected animals are dull and anorexic, and there may be a mucous nasal discharge. Shortly thereafter, watery green faeces are voided and may become haemorrhagic. Distress and constant straining become evident, and the nasal discharge often becomes blood-tinged. The external genitalia of ewes swell, and pregnant animals frequently abort. Death may follow within 24 hours of the onset of signs but can be delayed for as long as six days. It is generally considered that the prognosis is poor if diarrhoea develops.

All breeds of sheep and goats are susceptible. The mortality rate of African sheep tends to be high, even up to 80%, whereas that of European breeds is seldom more than 50%. Goats appear to be less susceptible, with a mortality rate of 10%, but losses in the field have been reported to reach 88%.

At postmortem examination the hindquarters are usually soiled with faeces and the nostrils caked with blood-tinged nasal discharge. There is general lymphadenosis and haemorrhages occur throughout the alimentary and respiratory tracts in all sheep except those dying peracutely. The genital tract, particularly of pregnant ewes, is oedematous and hyperemic, and the mucous membranes are catarrhal. Foetuses exhibit dermal haemorrhages. The only histological changes that are consistently present and considered to be of diagnostic value are a severe glomerulotubular nephritis associated with hyaline and epithelial casts and a generalized vascular congestion of the kidney that is most marked around the glomeruli.

Virus serially passaged by intracerebral inoculation of mice or in tissue culture loses pathogenicity for sheep and has been used as attenuated live virus vaccines. However, the degree of attenuation is accompanied by a loss of immunogenicity. Thus, owing to the variability in susceptibility of different breeds of sheep, none of these vaccines has proved generally acceptable. An inactivated vaccine prepared from virus propagated in baby hamster kidney cells and then formalinized and precipitated with methanol produced a high level of resistance to laboratory challenge but has not yet been assessed in the field.

Nairobi sheep disease virus can infect man and is related to a hazardous viral zoonosis of man known as Crimean-Congo haemorrhagic fever.

## 15.3 SAMPLES AND DIAGNOSIS

The clinical and postmortem picture cannot be relied on to provide a specific diagnosis although the occurrence of the clinical symptoms described above and death of adult animals at the same time as abortion can rule out many other common abortive agents. Confirmation depends on laboratory tests which should be undertaken on both aborted materials and tissues (including spleen , mesenteric lymph nodes and liver) from ewes dying at the time of or soon after abortion. Since the virus is heat sensitive samples must be collected promptly after abortion or death and sent to the laboratory as quickly as possible on ice and also in phosphate buffered glycerol solution at pH 7.2.

In the laboratory virus may be detected in tissue homogenates by intracerebral inoculation of suckling mice or in baby hamster kidney cell line (BHK 21)

cultures in which it produces characteristic perinuclear inclusion bodies. Tissue culture combined with detection by direct or indirect immunofluorescent staining provides the most rapid and certain means of detection [1]. Reliance on viral detection by cytopathic effects in tissue culture is less sensitive as generally isolates must be serially passaged two to five times before cytopathic effects are detected. The presence of antigen in tissue sections or impression smears of material from infected sheep can be carried out by direct or indirect immunofluorescent techniques. In recovered animals the detection of serum antibody by indirect immunofluorescence, indirect haemagglutination or enzyme-linked immunoabsorbent tests will indicate previous infection with NSD. The relevant methodologies are described in full in the chapter on Arthropod-Bourne Diseases.

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Whether under intensive, semi-extensive or extensive farming system sheep and goat flocks suffer heavy losses from abortive infections throughout the world. Brucellosis due to Brucella melitensis is known to be widespread and well established in most countries of the Mediterranean region and it is also constituting a worisome emerging problem in other geographical regions such as southern Africa, Central Europe or North Asia. However, this infection is not the only important cause of abortions. Indeed, even when the incidence of the disease has been successfully reduced in countries where it occurs, the abortion rate in small ruminants may be only slightly affected.
In these countries, as in others, Chlamydiosis, Toxoplasmosis, Q fever and other viral and bacterial diseases require a better knowledge of their epidemiology and an effort of prevention sustained with a view to maintain their impact at an acceptable level.
This work strives to contribute to fill the gaps with this respect especially in developing countries.

**Edited By :**

Espace Vétérinaire

**Résidence Al Mouahidine, Imm. Youssef I, Appt. 1, Bd de la Grande Ceinture**
**Hay Mohammadi, 20250 - Casablanca**