Chapter 9

SALMONELLOSIS

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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.

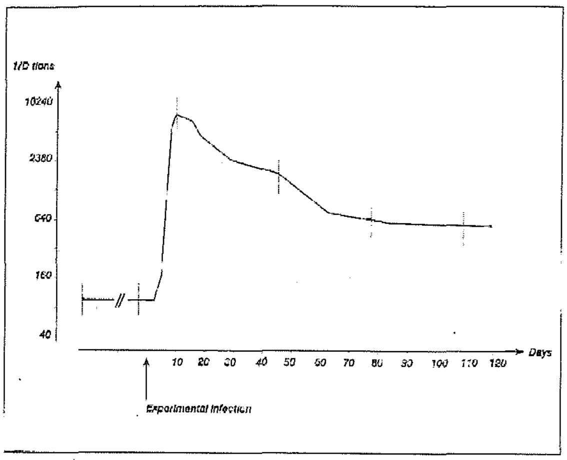

Figure 9.1 : Antibody kinetics after experimental infection of sheep with Salmonella abortusovis (seroagglutination microtechnique)

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 co-exist 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

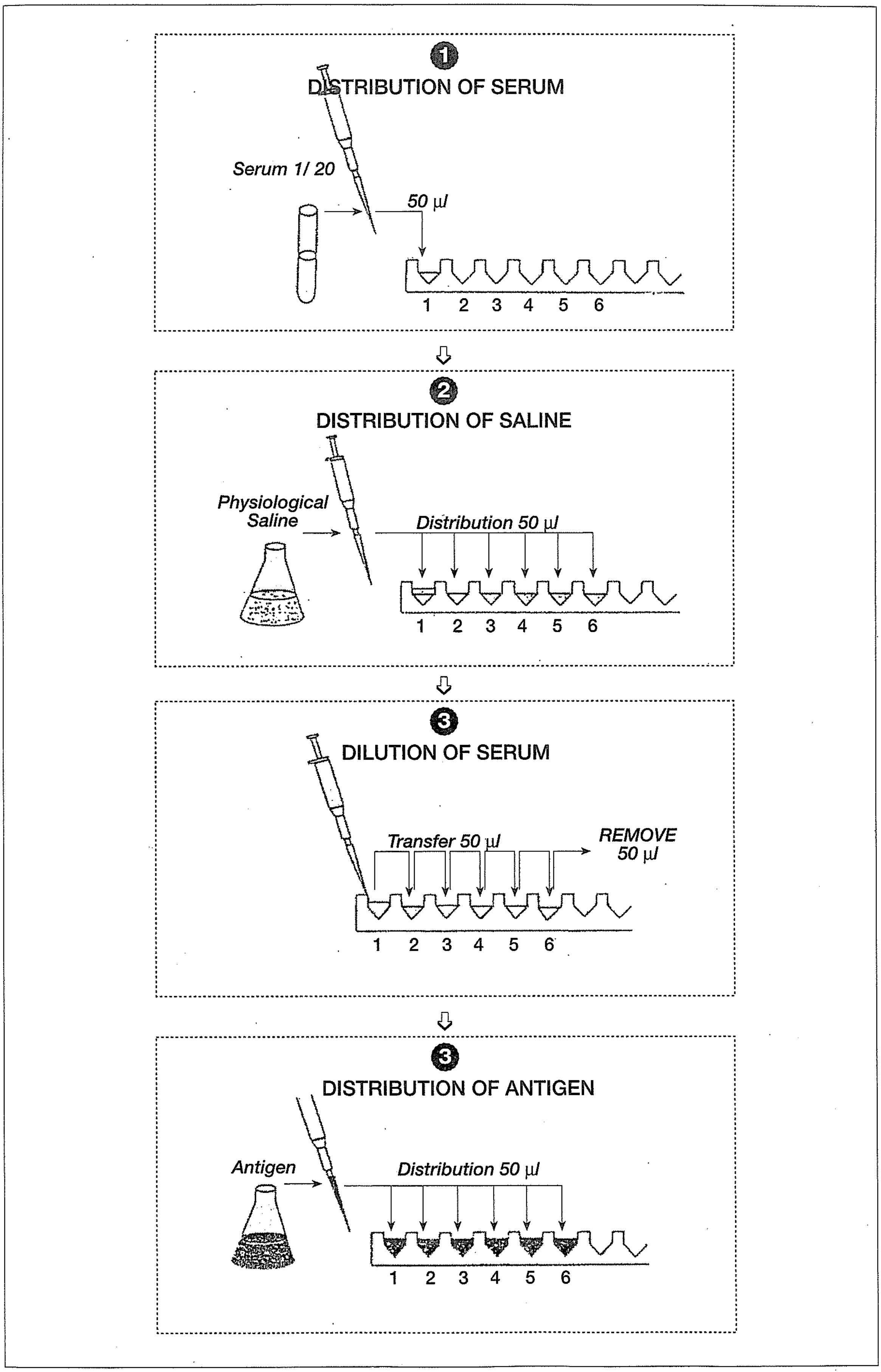

Figure 9.3 : Seroagglutination microtechnique. Reaction method

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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