CHAPTER 2.9.9.

SALMONELLOSIS*

SUMMARY

Salmonellosis is an infectious disease of humans and animals caused by organisms of the two species of Salmonella (Salmonella enterica, and S. bongori). Although primarily intestinal bacteria, salmonellae are widespread in the environment and commonly found in farm effluents, human sewage and in any material subject to faecal contamination. Salmonella organisms are aetiological agents of diarrhoeal and systemic infections in humans, most commonly as secondary contaminantants of food originating from animals and the environment, usually as a consequence of subclinical infection in food animals leading to contamination of meat, eggs, and milk or secondary contamination of fruits and vegetables which have been fertilised or irrigated by faecal wastes. Human salmonellosis is one of the most common and economically important zoonotic diseases. Salmonella organisms may also be found in feedstuffs, causing gastro-intestinal asymptomatic carriage or infectious disease in animals, particularly poultry and pigs. Salmonellosis has been recognised in all countries, but appears to be most prevalent in areas of intensive animal husbandry, especially in pigs and calves and some types of poultry reared in confinement. Reptiles are commonly asymptomatic carriers of Salmonella. Several serovars are host specific (e.g. S. Abortusovis in sheep or S. Typhi in humans) or host adapted (e.g. S. Choleraesuis, S. Dublin).

The disease can affect all species of domestic animals; young animals and pregnant and lactating animals are the most susceptible. Enteric disease is the commonest clinical manifestation, but a wide range of clinical signs, which include acute septicaemia, abortion, arthritis and respiratory disease, may be seen. Many animals, especially pigs and poultry, may also be infected but show no clinical illness. Such animals may be important in relation to the spread of infection between flocks and herds and as sources of food contamination and human infection.

Fowl typhoid and Pullorum disease, poultry diseases caused by Salmonella, are addressed in Chapter 2.3.11 of this Terrestrial Manual.

Identification of the agent: Diagnosis is based on the isolation of the organism either from tissues collected aseptically at necropsy or from faeces, rectal swabs or environmental samples, food products and feedstuffs; prior or current infection of animals by some serovars may also be diagnosed serologically. When infection of the reproductive organs, abortion or conceptus occurs, it is necessary to culture fetal stomach contents, placenta and vaginal swabs and, in the case of poultry, embryonated eggs.

Salmonellae may be isolated using a variety of techniques, which may include pre-enrichment to resuscitate sublethally damaged salmonellae, enrichment media that contain inhibitory substances to suppress competing organisms, and selective plating agars to differentiate salmonellae from other enterobacteria.

Various biochemical, serological and molecular tests can be applied to the pure culture to provide a definitive confirmation of an isolated strain. Salmonellae possess antigens designated somatic (O), flagellar (H) and virulence (Vi), which may be identified by specific typing sera, and the serovar may be determined by reference to the antigenic formulae in the Kauffman–White scheme. Many laboratories may need to send isolates to a reference laboratory to confirm the full serological identity and to determine the phage type and genotype of the strain, where applicable.

Serological tests: Serological tests should be conducted on a statistically representative sample of the population, but are of limited value if vaccination is used. In poultry, the whole blood test is used for rapid diagnosis of S. Pullorum/Gallinarum on the farm, being a relatively reliable diagnostic test under certain circumstances. In the laboratory, the tube agglutination test is the method of choice for export and diagnostic purposes for samples from all species of farm animals. Enzyme-linked immunosorbent assays are available for some serovars and may be used for serological diagnosis and surveillance, especially in poultry and pigs. Vaccination may compromise the diagnostic value of serological tests.
Requirements for vaccines and diagnostic biologicals: Many inactivated vaccines are used against salmonellosis and some live vaccines are available commercially. Due to the low efficacy of inactivated vaccines, oil or alhydrogel adjuvants are used to improve their immunogenic properties. Field efficacy data are often lacking, although laboratory testing may provide a useful indication. Innocuity tests are performed in laboratory animals and, in the case of inactivated vaccines, sterility tests using bacteriological enrichment media are carried out. Further reassurances, such as environmental impact and stability, are necessary for vaccines produced using genetic manipulation. Competitive exclusion may be used to reduce Salmonella infections in poultry and other animal species.

A. INTRODUCTION

According to the latest nomenclature, which reflects recent advances in taxonomy (42), the genus Salmonella consists of only two major species: S. enterica and S. bongori. A third putative species, S. subterranea, has also been proposed following the isolation of a single unusual environmental strain (24, 27, 50, 54). Salmonella enterica is divided into six subspecies, which are distinguishable by certain biochemical characteristics and some of which correspond to the previous subgenera. These subspecies are:

<table>
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<tr>
<th>Original subgenera</th>
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<tr>
<td>Subspecies I</td>
<td>subspecies enterica</td>
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<tr>
<td>Subspecies II</td>
<td>subspecies salamae</td>
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<tr>
<td>Subspecies IIIa</td>
<td>subspecies arizonae</td>
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<td>Subspecies IIIb</td>
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<td>Subspecies IV</td>
<td>subspecies houtenae</td>
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<td>Subspecies VI</td>
<td>subspecies indica</td>
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For the serovars of S. bongori, the symbol V was retained to avoid confusion with the serovar name of S. enterica subsp. enterica. Strains of Salmonella are classified into serovars on the basis of extensive diversity of lipopolysaccharide (LPS) antigens (O) and flagellar protein antigens (H) in accordance with the Kauffmann–White scheme; currently approximately 2500 serovars are recognised (42). This number is constantly being increased. The most common serovars that cause infections in humans and food animals belong to subspecies enterica. The serovars of the other subspecies are more likely to be found in poikilothersmic (cold-blooded) animals and in the environment, but are occasionally associated with human disease. Some serovars of subspecies arizonae and subspecies diarizonae have been associated with disease in turkeys and sheep and others may be carried by free-living or captive reptiles and amphibians.

Names are retained only for subspecies enterica serovars. These names must no longer be italicised. The first letter is a capital letter. In clinical practice the subspecies name does not need to be indicated as only serovars of subspecies enterica bear a name, e.g. Typhimurium, London or Montevideo are serovars of subspecies enterica. The genus Salmonella followed by the serotype name may be used for routine practice (e.g. Salmonella Typhimurium). Most serovars of the other subspecies are designated by an antigenic formula, including subspecies designated by Roman numeral (e.g. Salmonella IV 48:g:z51).

In this chapter, the abbreviated new conventions are followed, i.e. S. Typhimurium rather than the more complete nomenclature S. enterica, subsp. enterica serovar Typhimurium. There are also regular changes to serotype classifications as new evidence on genetic relatedness becomes available, e.g. S. Pullorum is now classified as S. Gallinarum biovar Pullorum (42).

Salmonellosis is an infectious disease of humans and animals caused by organisms of the two species of Salmonella (Salmonella enterica and S. bongori). Although primarily intestinal bacteria, salmonellae are widespread in the environment and may commonly be found in farm effluents, human sewage and in any material subject to faecal contamination. Salmonellosis has been recognised in all countries, but appears to be most prevalent in areas of intensive animal husbandry, especially of poultry or pigs.

The disease can affect all species of domestic animals; young animals and pregnant animals are the most susceptible. Enteric disease, often presenting as a bloody or profuse watery diarrhoea with pyrexia, is the commonest clinical manifestation, but a wide range of clinical signs, which include acute septicaemia, abortion, arthritis, necrosis of extremities and respiratory disease, may be seen. The signs and lesions are not pathognomonic. Many animals, especially poultry and pigs, may also be infected but show no clinical illness (65). Such animals may be important in relation to the spread of infection between flocks and herds and as causes of human food poisoning. In the latter case, this can occur when these animals enter the food chain thus producing contaminated food products (64, 65).
The course of infection, the clinical signs, the post-mortem findings and epidemiological patterns vary according to the serovar and the animal species involved. Some serovars only affect certain hosts, e.g. S. Gallinarum in poultry or S. Choleraesuis in pigs, although most serovars may cause disease in a wide range of animal species (51). Many serovars, including some that are host adapted such as S. Choleraesuis and S. Dublin, have been shown to cause disease in humans, and animal attendants, veterinarians and abattoir workers may be infected directly during the course of their work, as may laboratory personnel.

Disease is usually referred to as salmonellosis, although the term paratyphoid may be used, e.g. swine paratyphoid. In poultry, pullorum disease or bacillary white diarrhoea and fowl typhoid are often used to describe infections caused by S. Pullorum and S. Gallinarum, respectively (51). Fowl typhoid and Pullorum disease are covered in detail in Chapter 2.3.11 of this Terrestrial Manual.

For detailed epidemiological investigations, strain identification is necessary and such investigations have traditionally relied on biochemical and serological methods, phage typing of some serovars, and antibiograms. Genotypic analysis of the organism by use of real time-polymerase chain reaction (RT-PCR) and molecular fingerprinting of DNA has been used to good effect in recent years. Plasmid profile analysis is a quick and relatively easy method to fingerprint strains, and has been used in both human and veterinary medicine to study the spread of Salmonella. This technique has limitations as not all strains of Salmonella harbour plasmids, and plasmids may be readily acquired or may be of similar size but genetically different. The method has however proved to be useful in outbreak investigations to supplement other methods. Alternative genetic techniques, such as pulsed-field gel electrophoresis, AFLP (amplified fragment length polymorphism), VNTR (variable number tandem repeat), SNP (single nucleotide polymorphism) analysis and automated ribotyping, are increasingly used (4, 32, 53, 56). Genotyping is a rapidly expanding field and many new methods have been developed in recent years. It should be remembered that a single method may not work for all isolates and it may be necessary to evaluate a number of different techniques to find a method or combination of methods that is satisfactory and capable of differentiating clones of a particular serovar or phage type (45, 55). The molecular techniques are often more discriminatory and rapid and are replacing the phenotypic methods, such as serotyping and phage typing, for epidemiological investigations in some laboratories. However, these molecular tools are not necessarily available in all laboratories or standardised to give reproducible results in different laboratories and isolates may need to be forwarded to a suitable Reference Laboratory.

Genetic techniques such as micro-array and multiplex PCR analysis aimed at identifying specific serotypes as well as providing additional information on gene content are being developed (18, 19, 44).

The isolation and subsequent identification of salmonellae depend not only on the quality of the sample but also on the culture medium and growth characteristics of the serovar, particularly those adapted to a host species. A comprehensive review of Salmonella infection in domestic animals has recently been published (65).

National schemes have been implemented in many countries to control Salmonella infections in animals in order to protect the consumer. In the European Union, the Zoonoses Directive 2003/99/EC requires the monitoring of breeding flocks of more than 250 birds and hatcheries for S. Enteritidis and S. Typhimurium. In further legislation S. Virchow, S. Infantis and S. Hadar will also be subject to special controls (10). Culture of chicken delivery-box liners and dead or culled chickens is carried out on the day of arrival. At 4 weeks of age and 2 weeks prior to laying, pooled faeces of up to 60 samples, depending on the flock size, are cultured. Subsequently, adults are sampled every 2 weeks. At the hatchery, the meconium or dead-in-shells are cultured every 2 weeks, though there are plans to replace this with on-farm monitoring or monitoring of hatcher basket liners. New EU legislation controlling Salmonella in commercial laying flocks, broilers, turkeys and pigs is also being introduced. Serological monitoring is permitted as an additional measure but can no longer replace bacteriological monitoring in poultry. In Denmark serological monitoring for Salmonella is used for pig herds and commercial laying flocks. Several other countries also now have serological monitoring programmes for finishing herds of pigs using ‘meat-juice samples or serum taken at slaughter.

Salmonella infections of food animals play an important role in public health and particularly in food safety, as food products of animal origin are considered to be the major source of human Salmonella infections (64). Special programmes have been implemented for surveillance of poultry, swine and cattle and include the surveillance of healthy animals that may be subclinical carriers of Salmonella organisms. Cross-contamination during food processing is also monitored as contamination by healthy food handlers can occur (65).

Feed contaminated with Salmonella has been the most common original source of introduction of new strains of Salmonella into livestock production networks, from whence it is further distributed by movement of carrier animals and other routes. In many situations international or national trade in livestock or other animals may be the major threat; feed also may contain less pathogenic ‘environmental’ serovars that may not be a cause of disease or cycles of infection in animals. As feed contamination may be caused by Salmonella serovars of relevance to public health, feedstuffs should be investigated for the presence of salmonellae (65). As feed is milled with ingredients of mixed global origin a wide range of ‘exotic’ Salmonellas may be found in feed. Once established on
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a holding, spread between animals, environmental contamination and farm pests become more important in perpetuating and disseminating the infection.

Samples of food and feed tested for Salmonella should be truly representative. Proper steps should be taken to prevent contamination during transport or storage (20, 21). Because of the large variety of food and feed products there is no single sampling method appropriate to all products. Therefore different methods specific to the product should be used (11, 22).

The World Health Organization provides information on the development of appropriate measures for the prevention and control of food-borne diseases, including Salmonella infections, of humans. The most common vehicles of infection are eggs and egg products, poultry meat and meat from other food animals, and meat products. Contaminated salad crops and spices have also been involved in numerous outbreaks. Salmonella Enteritidis and S. Typhimurium are the most widespread serovars in many European countries (although Salmonella is rare in livestock production, some EU countries have strict control programmes), while S. Typhimurium is the dominant serovar in North America (64).

A Salmonella control policy for public health purposes should cover all stages from 'the stable to the table'. It should include the mandatory reporting of all outbreaks of the disease (13), and animal, food and feed testing (65). Feed monitoring includes sampling of compound feed and other feed materials which are fed unprocessed, and ingredients, as well as sampling during feed processing. World-wide epidemiological investigations should be done to monitor Salmonella transmission and support Salmonella control policies.

Health and hygiene controls at slaughter are essential, and special precautions should also be applied when slaughtering potentially infected herds. Decontamination measures should be implemented during processing. Vaccines are increasingly used to reduce Salmonella in poultry and it is essential that these can be distinguished from field infections for monitoring purposes and to ensure that the vaccines do not spread beyond the vaccinated group of animals (60).

Another essential element in the prophylaxis of human salmonellosis is retailer and consumer education, in particular awareness of safe handling and storage of food, kitchen hygiene and proper cooking to limit the risk of infection.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

The frequency of sampling and the type of samples obtained will depend largely on the objectives of the testing programme (including any statutory requirements), clinical findings, level of detection or precision of prevalence estimates required, cost and availability of sampling resources and laboratory facilities.

Individual samples for bacteriological tests are collected as aseptically as possible and in the case of clinical disease or routine monitoring, samples should be collected before any antibiotic treatment has commenced. Preferably clinical samples are collected during the acute phase of the disease or as soon as possible after death. In the case of flocks of poultry or other avian species, environmental samples, such as naturally pooled faeces, litter and dust or drag or boot swabs from floor surfaces (5, 25), may be the most cost-effective way to identify infected flocks. Precautions should be taken to avoid cross-contamination of samples during collection, transit and at the laboratory. Packages should be kept cool and accompanied by adequate information. For smaller animal species, it may be preferable to submit a representative number of sick or recently dead animals to the laboratory, if that is possible (63). Host-adapted serovars are usually more difficult to isolate from faeces so if these are suspected, infected tissues should be cultured where possible.

Particular attention should be given to the isolation of salmonellae from animals with subclinical infection, as these may only excrete bacteria intermittently and in low numbers. An increased sample size, increased number of samples representing more individuals, combined in some cases with pooling of samples and repeat sampling can provide an increased diagnostic sensitivity. In such situations bacteriological or serological methods should be used to identify infected flocks or herds rather than to identify infected individual animals.

- Culture

There are numerous methods for isolation of Salmonella in use world-wide (9, 14, 17, 29, 46, 63). Some of the more common methods are described below. The culture techniques and media that may work best in a particular diagnostic situation depend on a variety of factors, including the type of Salmonella, source and type of specimens, animal species of origin, experience of the microbiologist, and availability of selective enrichment and selective plating media.
All culture media should be subjected to quality control and must support growth of the suspect organism from a small inoculum. The routine use of a reference strain in parallel with routine samples may lead to cross contamination of samples if careless techniques are used, so a rare serovar with typical growth characteristics that are similar to the highest priority target strains should be used.

The increasing application of external quality assurance programmes has led to greater use of international standard methods, such as ISO 6579:2002; even though this has not been validated for faecal and environmental samples and was intended for foodstuffs and feedingstuffs. In recent years a standard method for detection of *Salmonella* from primary animal production has been developed and evaluated, and an ISO-method is now nearly adopted (35). The core of the standard method is pre-enrichment in buffered peptone water, enrichment on modified semi-solid Rappaport–Vassiliadis (MSRV) and isolation on xylose-lysine-deoxycholate (XLD) and an additional plate medium of choice.

### a) Pre-enrichment media

The number of salmonellae in faeces from asymptomatic animals, environmental samples, animal feed and food is usually low, and it is necessary to use pre-enrichment media, such as buffered peptone water or universal pre-enrichment broth, to assist isolation. This may allow the small numbers of salmonellae, which may otherwise be killed by the toxic effect of enrichment media, to multiply, or it may help to resuscitate salmonellae that have been sublethally damaged, e.g. by freezing, heating, exposure to biocides or desiccation. Pre-enrichment may not be the best method for isolating less vigorous *Salmonella* strains, such as the host-adapted strains, from faeces because of overgrowth by competing organisms during non-selective pre-enrichment.

### b) Enrichment media

Enrichment media are liquid or semi-solid agar media that contain additives that selectively permit salmonellae to grow while inhibiting the growth of other bacteria. Some, however, are also relatively toxic to certain serovars of *Salmonella*, e.g. selenite inhibits *S*. Choleraesuis, and brilliant green is toxic to many strains of *S*. Dublin. Elevated temperatures have also been used to increase the selectivity of enrichment medium, and a temperature of 43°C is used in some laboratories, although this may be inhibitory with some media, e.g. tetraionate and Rappaport–Vassiliadis at 43°C inhibit temperature-sensitive strains, especially *S*. Dublin and 41.5°C is now recommended for incubation of Rappaport–Vassiliadis broth (22). Selective motility enrichment may also be used to increase the sensitivity of *Salmonella* isolation and semi-solid enrichment media, e.g. MSRV or diagnostic semi-solid *Salmonella* medium (DIASALM), may provide greater sensitivity (59). The formulation of the medium, which may vary between suppliers, or even between batches in some cases, temperature and duration of incubation, and the volume of the samples used to inoculate the medium, may all serve to influence the isolation rate, and these variables should always be taken into account. Examples of selective enrichment media are sodium tetraionate, as in Muller–Kaufmann broth, selenite F, selenite cystine, brilliant green broth and Rappaport–Vassiliadis broths, or semi-solid Rappaport–Vassiliadis medium. In some cases it may be advantageous to use more than one selective broth or to culture by both pre-enrichment and direct selective enrichment/direct plating, although often the benefit does not justify the extra cost. Additions such as Ferroxamine E may be added to selective media to enhance isolation of *Salmonella* from iron or nutrient-limited samples such as eggs, water or soil (46) or antibiotics may be added to enhance the isolation of antimicrobial resistant strains.

### c) Selective plating media

These are solid, selective agars that permit differential growth to varying degrees. They inhibit growth of bacteria other than *Salmonella* and give information on some of the principal differential biochemical characteristics – usually nonlactose fermentation and hydrogen sulphide (H₂S) production. The results are read after 24 and 48 hours of culture at 37°C. Salmonellae form characteristic colonies on such media that are usually distinguishable from the colonies of other bacteria on the plate, with the possible exceptions of *Proteus*, *Pseudomonas* and *Citrobacter*. Lactose-fermenting salmonellae may occasionally be isolated and H₂S production may be variable. Such atypical strains may be more effectively detected when semi-solid selective media are used. DIASALM medium is particularly useful in this respect as presumptive confirmation by slide agglutination testing using polyvalent O, H or specific antisera can be carried out on liquid from the growth zone in the plate. *Salmonella* Abortusovis is a slow-growing serovar and it is usual to incubate plates for up to 72 hours and to use the nonselective blood agar. Examples of selective media are brilliant green agar, xylose lysine deoxycholate agar, deoxycholate/citrate agar, Rambach agar, and bismuth sulphite agar, although many more will be found in the literature and media catalogues. A wide range of chromogenic agars are now available. Many of these may aid differentiation of suspect colonies, but must be validated for the sample matrices, culture systems and serovar range used as sensitivity can be poor in some circumstances.

### o Identification of suspect colonies

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Suscept colonies are subcultured onto selective and non-selective agars to ensure that possible contaminants, such as *Proteus* spp., are absent. If there is an abundant pure growth, suspect colonies may be tested by slide agglutination with polyvalent *Salmonella*-typing sera (28). In some cases, the suspect colony may not agglutinate or auto-agglutinate and it is necessary to use biochemical tests to confirm the identity. These tests can be performed with peptone water sugars or commercial systems (such as the Analytical Profile Index [API] system), OBIS test, or composite media (such as triple sugar iron agar [TSI]), can be used to screen organisms (12).

The determination of the O factor(s) and the H antigen(s), and in special circumstances the Vi antigen, is performed by direct slide agglutination or tube agglutination using specific antisera. In the case of biphasic organisms, it is necessary to determine both phases, by the use of phase inversion – this involves passage through semi-solid agar containing antiserum to the known phase. Screening is facilitated by the availability of antisera directed against several factors, which can be pursued further by the use of monovalent typing sera. While many laboratories can identify the more common serotypes, it is necessary to use the facilities of a reference laboratory to confirm the identity of an isolate and possibly to obtain information on the phage type, if there is a scheme available, and genetic profile.

Additional biochemical tests may be necessary to identify some serotype variants, e.g. d-tartrate, which can be used to differentiate *S. Paratyphi* B var. Java from *S. Paratyphi* B. Isolates should also be tested for their sensitivity to a range of antimicrobial agents as there is increasing concern about the emergence of new multiple resistant strains harbouring transferable resistance genes to cephalosporins and fluoroquinolones (26, 43, 61). Live vaccine strains are also commonly identified by antimicrobial resistance markers, biochemical changes such as auxotrophism or roughness.

**o Immunological and nucleic acid recognition methods**

Numerous alternative *Salmonella* detection methods are in use and are commercially available (3, 6, 8, 12, 48, 49, 52, 57, 66). These include electrical conductance/impedance, immunomagnetic separation (IMS), enzyme-linked immunosorbent assay (ELISA), gene probes PCR methods, including nucleic acid sequence based amplification (NASBA) (6) and real time (16, 31, 40) or quantitative PCR (41). Many of these methods have not been validated for faecal and environmental samples and are more suited to analysis of human foodstuffs (39). The rapid methods are usually more expensive than conventional culture, but can be economically viable for screening materials where a low prevalence of contamination is expected or where materials such as feedstuffs are held pending a negative test. An enrichment/IMS method linked with ELISA or PCR can give results within 24 hours. Currently none of the rapid methods has been shown to be suitable for direct detection of *Salmonella* so non-selective or selective enrichment stages are required (37). Typically this introduces more steps and operator time in the detection procedure. For DNA-based methods, inhibition of the PCR reaction by elements of the test sample matrix, especially in the case of faeces, is problematic and requires suitable DNA extraction techniques (23).

Rapid isolation methodologies may also be linked with sophisticated detection systems, such as biosensors (38). There are many variations and developments in rapid methods for *Salmonella* detection, but none has been shown to satisfactorily replace culture in all circumstances. It is therefore not possible to provide details of all the methods in this chapter or to make recommendations, but the review articles cited above will provide further information. Efforts are currently being made to standardise the use of certain rapid methods internationally (30), but there is a considerable amount of work still to be done.

**o Example test procedures for isolation of *Salmonella* from food, feedstuffs, faecal and environmental samples**

i) Add a 10–25 g sample to ×10 volumes of buffered peptone water at ambient temperature. (NB: for many host-adapted serovars and some arizonae serovars, it is preferable to add the sample to selective enrichment medium, such as selenite cysteine broth, and to test tissue samples where possible [including direct plating]; see culture method for *S. Pullorum/Gallinarum* in Chapter 2.3.11 of this *Terrestrial Manual*.)

ii) Incubate buffered peptone water for 16–20 hours at 37°C.

iii) Inoculate 20 ml modified semi-solid Rappaport–Vassiliadis or DIASALM in a Petri dish with 0.2 ml incubated buffered peptone water broth.

iv) Inoculate 10 ml tetraphionate broth with 1 ml incubated buffered peptone water broth.

v) Incubate modified semi-solid Rappaport–Vassiliadis or DIASALM at 41.5°C and tetraphionate broths at 37°C (ensure that a reputable brand of tetraphionate suitable for use at 37°C is used).

vi) After 24 and 48 hours of selective enrichment, plate out modified semi-solid Rappaport–Vassiliadis or DIASALM by taking 1 µl loop of material from the edge of the turbid growth zone and streaking over one plate of Rambach agar or brilliant green agar and one plate of xylose lysine deoxycholate agar plus novobiocin.

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vii) Plate out 10 µl of tetrathionate broth on Rambach agar or brilliant green agar and xylose lysine desoxycholate agar plus novobiocin.

viii) Incubate plates at 37°C for 24 hours.

ix) Check up to five suspect colonies (red/pink with reddening of the media on brilliant green agar, crimson with pale borders or orange/colourless on Rambach agar, red with black centre on xylose lysine desoxycholate agar) using poly ‘O’ and poly ‘H’ (phase 1 and phase 2) antiserum or composite biochemical media.

x) Subculture strongly suspect colonies that do not agglutinate with poly H antiserum on to nonselective media then repeat testing. If a strong poly ‘O’ and poly ‘H’ agglutination can be obtained, this is sufficient for presumptive confirmation. Such isolates can then be serogrouped. If agglutination results are unclear then carry out further biochemical testing using composite media, such as TSI or use ONPG (o-nitrophenyl-beta-d-galactopyranoside) and urea or commercial biochemical tests such as API ID 32 E.

2. Serological tests

○ Serological identification of infected animals, flocks and herds

A number of serological tests have been developed for the diagnosis of Salmonella infections in animals. In poultry, the whole blood test, which uses a stained antigen, and the serum agglutination test (SAT) have been used successfully for over 50 years for the identification of flocks infected with S. Pullorum/Gallinarum. Because S. Enteritidis possesses the same group D somatic antigen as S. Pullorum/Gallinarum, the whole blood test and related tests can be used for the diagnosis of infection but the sensitivity is low. In recent years, other tests, such as the ELISA (2, 58) have been developed for the diagnosis of S. Enteritidis and S. Typhimurium infections in poultry and for other serovars in farm animals. The ELISA has been used effectively to identify serologically S. Dublin carrier cattle and can be applied to bulk milk for screening dairy herds. The mix-ELISA is used in Denmark on serum or tissue fluid released by freezing then thawing muscle samples to detect Salmonella infections in pigs (36). A similar test is used to detect antibodies to S. Enteritidis and S. Typhimurium in egg yolk from commercial laying flocks (13).

Some ELISAs are now in routine use and a number are available commercially and there is a need for standardisation of their use. The purpose of this section is to consider the serological tests that have been fully evaluated and are in routine use for the diagnosis of salmonellosis in animals. Other tests that are still in the development stage will therefore not be considered. New tests for Salmonella diagnosis are developed frequently so an Internet search is often the best means of obtaining current information.

○ Factors affecting serological diagnosis

1. Serological methods should be used to identify infected flocks/herds rather than to identify infected individual animals, although repeated herd tests can be used as an aid to selective culling of chronic carrier animals. Serological tests are normally designed to detect a limited range of Salmonella serovars or serogroups.

2. It is well recognised that some animals with a positive serological response may no longer be infected with Salmonella organisms. Likewise, animals that are actively excreting salmonellae may be serologically negative. Similar considerations may also apply to bacteriological culture methods, and negative faecal culture results may not necessarily indicate that an animal is not infected. However, neither of these situations should be considered as a major problem if enough tests are carried out. Animals that are serologically positive may have ceased to excrete salmonellae although circulating immunoglobulin concentrations may remain high. Other animals on the farm may still be infected. Serologically negative animals may result from a recent infection causing excretion before immunoglobulin production is maximal, or infection with less invasive serotypes. Animals that have been infected recently would, in all probability, eventually be detected serologically by an appropriate monitoring programme throughout the life of the flock/herd.

3. Newborn animals are immunologically immature and do not respond serologically to the somatic LPS antigen until 2–3 weeks of age. They do, however, produce a serological response to the flagellar protein antigens. Cattle may be unresponsive until about 10–12 weeks of age, and sucking pigs may fail to develop an immune response or have an antibody response that reflects maternal immunity. Differential responses involving different antibody classes (IgM, IgA, IgG) can be used in pigs to differentiate recent infection from infection that occurred some time ago, but this is often not useful for herd testing where individuals are usually at different stages of infection. Most tests are based on IgG and raised antibody levels typically appear 1–3 weeks after infection and last 2–3 months.

Chickens may also acquire anti-Salmonella antibodies passively from their parents via the yolk sac; this may indicate an infected parent flock. Mammals can acquire maternally derived antibodies via the colostrum.
4. Immunisation has been used for many years to control certain *Salmonella* infections in farm animals, and if diagnostic serology is to be used, it is necessary to differentiate the vaccine response from that of actual infection. Many live vaccines given orally do not provide a significant serum antibody response in the majority of animals but there may be occasional exceptions. Injectable killed vaccines used for control of *S. Enteritidis* in chickens may produce a very prolonged antibody response. It would be advantageous if a marked live vaccine could be produced which could be differentiated from field challenge by serological testing.

5. The effect of antibiotic therapy on the serological response remains unclear. Some workers found reduced titres following therapy whereas others found no effect. Serology, however, may be a more useful diagnostic technique for salmonellosis than culture if antimicrobial therapy has been used.

6. Approximately 2500 different *Salmonella* serovars exist. Depending on the antigen and test used, serological cross-reactions between different serovars may occur, e.g. *S. Typhimurium*, *S. Pullorum* and *S. Enteritidis*. In some cases cross-reactions may also occur as a result of exposure to organisms other than *Salmonella*.

7. In poultry, egg yolk may be tested for immunoglobulins to *Salmonella*, and eggs may provide a method to screen flocks. This approach is used for monitoring commercial laying flocks in Denmark. In cattle, milk may be tested for anti-*Salmonella* antibodies to screen dairy herds.

8. The use of filter-paper discs for serum collection obviates the necessity to separate serum. The discs also provide long-term storage and reduce transport costs to the laboratory. The sensitivity of the test may be slightly reduced compared with tests carried out on fresh serum.

a) The whole blood test

The whole blood test provides a rapid test for fowl typhoid and pullorum disease that can be used on the farm. The sensitivity of the whole blood test is low and in inexperienced hands false-positive and false-negative results may be recorded.

For a detailed description of the whole blood test, see Chapter 2.3.11 Fowl typhoid and Pullorum disease.

b) Rapid slide agglutination test

Serum (0.02 ml) is mixed with polyvalent crystal-violet-stained antigen (0.02 ml). The tile is rocked gently for 2 minutes, after which the test is read. The test components are stored at 4°C and must have reached room temperature before being used.

Test sera should be free from contamination and haemolysis. It may be helpful to centrifuge serum samples that have been stored for any period of time.

If nonspecific false-positive reactions are suspected, positive/suspicious sera may be retested after heat-inactivation at 56°C for 30 minutes.

c) Serum agglutination test

The SAT is relatively insensitive, and many older animals have low levels of agglutinins in their sera caused by enterobacteria other than *Salmonella*. Single samples are of little diagnostic value except for initial screening on a herd basis. Paired samples are needed as the minimum requirement for confirmation of active infection. The test is relatively inexpensive; the antigens can be readily prepared and expensive equipment is not necessary. The SAT can be adapted to the microtitre format and can be readily used to determine somatic and flagellar titres. It is advisable to use standard sera and other confirmatory methods for quality control of the purity and immunogenicity of SAT antigen preparation(s) that are not dependant on sera produced from those antigens.

o Preparation of somatic antigen

i) Plate out the *Salmonella* culture from the appropriate stock culture onto a blood agar base (BAB) plate, or other suitable medium, for single colony growth. Incubate overnight at 37°C (±2°C).

ii) Select a smooth colony and carry out a slide agglutination test to ensure that the required somatic antigen is present.

iii) Using a sterile loop, inoculate a nutrient agar slope in a universal container from the selected colony.

iv) Incubate the culture for 8–12 hours at 37°C (±2°C).

v) Using a Pasteur pipette, wash off the culture, preferably inside a safety cabinet, with approximately 2 ml of absolute alcohol, and transfer into a sterile universal container.
vi) Leave the antigen for 4–6 hours at room temperature to enable the alcohol to kill the bacteria and detach flagellae.

vii) Spin the universal container in a bench-top centrifuge for 5 minutes at 1000 \text{g}. Pour off the liquid and add enough phenol saline to make the antigen up to an opacity equivalent to Brown’s tube No. 2 (approximately 10^8 colony-forming units/ml) or other appropriate standard.

viii) Carry out standard titration with known serum to ensure that the antigen is positive for the required factor.

ix) Store in a refrigerator at 4°C until required.

o Preparation of flagellar antigens

i) Plate out the appropriate Salmonella stock culture on to a BAB plate, or other appropriate medium. Incubate overnight at 37°C (±2°C).

ii) Passage in semi-solid agar (about 0.3%) in a Craigie’s tube, or other suitable container, to induce optimum expression of the appropriate flagellar antigen. If the serovar is biphasic, H antiserum corresponding to the phase to be suppressed is added to the agar.

iii) Use slide agglutination to check that the Salmonella is in the required phase. If this is correct, inoculate a loop of culture into 20 ml of nutrient broth. Incubate for 12–18 hours at 37°C (±2°C) for optimum growth. (If the phase is incorrect, repassage through semi-solid agar.)

iv) Pipette 250 µl of 40% formaldehyde into the antigen suspension (use gloves and preferably work in a safety cabinet), and leave overnight.

v) Test the antigen by SAT using the appropriate typing serum.

o Test procedure

i) It is easiest to screen the sera at a dilution of 1/20; 0.25 ml of antigen is added to 0.25 ml of serum prediluted to 1/10 in normal saline.

ii) The tests are incubated in a water bath at 50°C for 24 hours in the case of somatic antigens and for 4 hours for the flagellar antigens. The dilution and time of incubation will vary depending on the antisera that is used.

iii) Sera that give a positive reaction are then diluted from 1/20 to 1/320 and retested with the appropriate antigen.

d) Enzyme-linked immunosorbent assays for Salmonella Enteritidis

Two main basic systems are available for detection of IgG (IgY) specific for S. Enteritidis: the indirect ELISA (2) and the competitive ‘sandwich type’ ELISA (58).

The indirect ELISA involves the use of a detecting antigen coated on to the wells of a microtitre plate. After the application of a blocking reagent to reduce nonspecific binding, test samples are applied to the wells. Specifically bound antibody in the sample is detected by an antibody/enzyme conjugate. A variety of antigens, including LPS, flagella, SEF14 fimbriae, outer membrane proteins and cruder whole cell antigen preparations have been used.

The competitive sandwich ELISA employs a specific reagent – a monoclonal antibody (MAB) or polyclonal antibody – for coating antigen to wells. This is then followed by a pure or crude antigen preparation. Test samples are applied followed by conjugated antibody, which will not bind to the antigen if the sample contained specific antibodies. The assay time can be shortened by adding both test sample and conjugate together. MAbs have been prepared for LPS, flagella and SEF14 for S. Enteritidis.

There are advantages and disadvantages to both systems. The indirect assay is simpler and reagents are available for all Salmonella serotypes of chickens, turkeys, ducks and mammalian hosts. The competitive ELISA can be applied to all animal species and in general shows higher specificity. However, reagents are not available commercially for all serotypes. There are also some affinity problems and it may be less sensitive than the indirect assays. In the field, both systems have produced false-positive reactions and in some cases screening with an indirect LPS ELISA may be followed by confirmation with a flagellar competitive ELISA.

Both types of assay may be used with serum, egg yolk or reconstituted dried blood eluted from filter paper discs. A mix-ELISA (or meat-juice ELISA), is used in Denmark and other countries to detect Salmonella infections in pigs (36). This ELISA contains the ‘O’ LPS antigens 1, 4, 5, 6, 7 and 12, from S. Typhimurium and S. Choleraesuis, which enables it to detect serologically 95% of the Salmonella serogroups found in pigs in most European countries. Group D antigens have also been added to some ELISA kits. Serum is used to
screen breeding and multiplying herds, whereas for pigs in the abattoir, the assay is performed on the tissue fluid (‘meat-juice’) that is liberated when a frozen 10 g muscle sample is thawed.

With some ELISAs differentiation can be made between infections produced by Salmonella serotypes from different serogroups. Some cross-reaction occurs between groups B and D and other invasive serovars. There is, however, usually a greater antibody response when LPS from the homologous serotype is used in the ELISA. The optimal method for choosing a ‘cut-off’ absorbance value, above which sera are designated as having come from an S.-Enteritidis-infected flock, without producing an unacceptable level of false-positive tests, has not yet been decided on and agreed upon internationally.

ELISAs are readily adapted to automation and hence to large-scale testing programmes. A major problem is that expensive equipment is necessary and many of the reagents are also expensive. Several commercial ELISA kits for S. Enteritidis, S. Typhimurium and Group B/C mix-ELISAs are available. Ideally these should be validated by international ring trials before adoption for surveillance purposes.

An example of a validated ELISA is the one developed at the OIE Reference Laboratory at VLA Weybridge (see Table in Part 3 of this Terrestrial Manual for address). The requirements are given below.

### Equipment
- Falcon microtest III PVC plates; appropriate pipettes and measuring cylinders; ultrawash microtest plate washer; ELISA plate reader; test filter of 405–410 nm and reference filter of 630 nm.

### Antigen
- Phenol-extracted S. Enteritidis LPS is available commercially (Sigma Cat. No. L6011). This is reconstituted in 1 ml deionised water and stored at –20°C in 100-µl aliquots in phosphate buffered saline (PBS), pH 7.2, at a concentration of 2.5 mg/ml. For use, the antigen should be thawed in coating buffer at the appropriate concentration.
- The LPS antigen can also be prepared by the technique of Westphal & Luderitz (62) and standardised as to its carbohydrate concentration by the method of Gerhardt (15), and adjusted to 1000 µg/ml.

### Serum and conjugate diluent
- Add bovine serum albumin (BSA) (2 g) and Tween 20 (0.05 ml) to PBS (100 ml). (Alternatively, powdered milk [1 g] can replace the BSA.) Store at 4°C and make fresh solutions every week.

### Coating buffer
- add sodium carbonate (1.59 g) and sodium bicarbonate (2.93 g) to deionised water (1 litre) and adjust to pH 9.6. (Alternatively, dissolve one tablet of 0.05 M Sigma carbonate/bicarbonate buffer [Cat. No. C-3041] in deionised water [100 ml].) Store at 4°C and renew every 2 weeks.

### Substrate buffer
- make a 10% (v/v) solution of diethanolamine in deionised water. The diethanolamine should be prewarmed to 37°C before dispensing, and the pH of the solution should be adjusted to pH 9.8 with 1 M hydrochloric acid. Store at 4°C and renew every 2 weeks.

### Enzyme conjugate
- goat anti-chicken immunoglobulin conjugated to alkaline phosphatase (e.g. ICN Immunobiologicals, alternative supply Sigma: A9171) or other species anti-chicken globulin. Store at 4°C diluted in diluent at the appropriate concentration and renew every week.

### Enzyme substrate
- dissolve one tablet of p-nitrophenyl phosphate disodium (5 mg) in substrate buffer (5 ml) no earlier than 30 minutes before dispensing, and store in the dark.

### Standards
- Positive control antiserum prepared by intramuscular inoculation of four 1-week-old specific pathogen free (SPF) chickens with an inoculum containing 10⁶ S. Enteritidis. The serum is subsequently obtained 3–4 weeks later when antibody titres are maximal.
- Negative control serum A from four 1-week-old SPF birds.
- Negative control serum B from 58 1-week-old breeders known to be free from Salmonella infections. Pool the sera and store in 100 µl volumes at –20°C.

### C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Many inactivated vaccines are used against salmonellosis caused by different serovars in various animal species, including a combined S. Enteritidis and S. Typhimurium vaccine for use in poultry. Inactivation is usually achieved
by either heating or the use of formalin and an adjuvant, such as alhydrogel, is usually used. Live vaccines have also been used in a number of countries; these include the semi-rough strains, such as 9R for fowl typhoid and HWS51 for S. Dublin infections (33). Other attenuated vaccines include auxotrophic and ‘metabolic drift’ mutants, which are used to prevent Salmonella infections in farm animals in Germany and for S. Enteritidis and S. Typhimurium in poultry in the United Kingdom. Mutant vaccines attenuated rationally by molecular biological gene-deletion techniques have been developed for poultry and other species; these include aroA mutants and strains with mutations in the genes encoding adenylate cyclase (cya) and the cyclic adenosine monophosphate receptor protein (crp) (7), which is available in the United States of America. In Europe genetically modified organisms are not normally permitted for use as vaccines.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 Principles of veterinary vaccine production. The guidelines given here and in Chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

1. Seed management

a) Characteristics of the seed

For killed or living vaccines, the bacterial strain should be an organism as closely related to currently circulating field strains as possible. It should be carefully chosen from cases of severe clinical disease, and virulence and antigen production should be assessed. It is best to evaluate a panel of potential strains in this way before testing the final selection. The final vaccinal strain should be identified by historical records and characterised by stable phenotypic and/or genetic markers. Living vaccinal strains should be marked by stable characters allowing distinction from wild strains. Markers, such as resistance to antimicrobials, for example rifampicin, may be used. Attenuation of virulence should be stable and preferably obtained by two independent defined mutations. The stability of live vaccine strains can be verified by regular checks using sensitive molecular fingerprinting and micro-array techniques.

b) Method of culture

The seed culture is propagated and maintained using suitable media, of which many have been described (in textbooks) for growth of Salmonella. The media used must not contain serum or animal tissues. Culture may be on solid medium, in Roux flasks, or in liquid medium, in which case large-scale fermentation equipment may be used. Iron limitation or low temperature incubation on a minimal media may enhance LPS antigen production by the vaccine strain.

c) Validation as a vaccine

i) Purity

The vaccine strain must be checked as follows:

- Staining of a smear of bacterial suspension on a glass slide using Gram stain.
- Homogeneity of culture on nonselective media.
- Metabolic requirements as indicated by biochemical tests.
- Detection of markers, and phage type.
- Agglutination with specific antiserum.

ii) Safety

The LD₅₀ (50% lethal dose) or ID₅₀ (50% infectious dose) may be determined in mice. Ten times the field dose of live vaccine or twice the dose for killed vaccines must be given to the target species at the recommended age and by the recommended route. The animals are observed for absence of adverse reactions. Stability and non-reversion to virulence after serial passages in susceptible species should be shown for live vaccines. It is also necessary to consider repeat vaccination. Live vaccine should be shown not to persist for long in vaccinated animals or be transmitted to milk or eggs that may be consumed, and the method of application should not present a hazard to operators.

iii) Efficacy

Laboratory experiments and field trials should be used to show that the vaccine is effective. The laboratory experiments consist of vaccination–challenge tests in the target species at the recommended dose and age. The efficacy data can also be used as the basis for a batch potency test. Field trials are more difficult to undertake with respect to testing efficacy because of difficulties with standardising the challenge and providing appropriate controls.

iv) Environmental aspects
Live vaccine strains should be tested for their ability to persist in the environment and infect non-target species such as rodents and wild birds that are likely to be exposed. Prolonged survival of some live vaccines in faeces and litter may present an unacceptable environmental hazard when the material is removed from the animal houses. Live vaccines should not be used in poultry during lay.

2. Method of manufacture

Vaccine must be made in suitable clean rooms to which only approved personnel have access. Care must be taken to avoid cross-contamination between areas where live organisms are processed and other areas. Contamination from operators and/or the environment must be avoided and vaccine preparation should take place in a separate area from diagnostic culture work. Operators must not work with vaccine whilst ill and must not be subject to immunosuppressive conditions or medications. Personnel must be provided with protective clothing in production areas and in animal rooms.

Seed-lot cultures are prepared from the primary seed-lot, and the number of passages is dependent on the validation of the process. The vaccine may be prepared by inoculation of a suitable medium, such as nutrient broth, with a fresh culture and incubation on a shaker at 37°C for 24 hours, with or without aeration. The organisms are harvested by sedimentation or centrifugation. Alternatively, the organisms may be grown on and harvested from a solid medium, such as nutrient agar. In the case of live vaccines, the suspension is diluted in PBS, pH 7.0, and may be freeze-dried.

The time of inactivation of dead vaccines should be at least 33% more than that taken to reduce the viable number to an undetectable level. The inactivation process must be applied to the whole volume of the vaccine cell harvest.

Preservatives, excipient for lyophilisation, stabiliser for multidose containers or other substances added to or combined with a vaccinal preparation must have no deleterious effect on the immunising potency of the product.

3. In-process control

The following points require attention:

- Visual control of the suspension, homogeneity by Gram stain, culture on nonselective medium.
- Slide agglutination with specific antisera.
- Titration of bacteria by turbidimetry and/or plate count.
- Test of effective inactivation (dead vaccine) by plating on nonselective medium or use of a medium that gives optimum chance of recovery e.g. production medium with neutralisation of the inactivating compound.
- Titration of viable bacteria (living vaccine) before and after lyophilisation.

4. Batch control

a) Sterility

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

b) Safety

A laboratory test in mice that has previously shown a correlation with safety in the target species may be used to determine the LD₅₀ and/or ID₅₀. Each batch should be tested in the target species at the recommended age and route, using at least twice the field dose for killed vaccines and ten times the dose for live vaccines.

c) Potency

Potency is tested using vaccination–challenge assay in mice and/or other species, including (if practicable) the target species and immunological response in target species.

d) Duration of immunity

The duration of immunity is likely to vary considerably between products, vaccination regimes and individual vaccinated animals. Immunity to Salmonella is normally serogroup specific. Consultation among colleagues suggests that most killed vaccines will provide protection for 6 months, while some live vaccines given by injection may elicit stronger immunity, which may persist for 1 year or more. It should be remembered,
however, that a strong challenge such as that associated with continuously occupied farms or infected rodents may overwhelm vaccinal immunity and commercial live vaccines may be attenuated to reduce environmental survival in a way that reduces the immune response. There may also be problems with ensuring effective oral administration with live vaccines or accuracy of injection with killed and live injectable vaccines.

e) Stability
Information is lacking on the stability of killed vaccines. Stability is affected by storage conditions and by the presence of contaminating microorganisms growing in the product. The stability is assessed by potency tests repeated at appropriate time intervals. The stability of live vaccines can be assessed by performing counts of the number of viable organisms repeated at appropriate time intervals.

f) Preservatives
Chemicals with antimicrobial activity, such as thiomersal, phenol or crystal violet, are often included as preservatives in killed bacterial vaccines.

g) Precautions
Certain killed vaccines may occasionally cause abortion in pregnant animals because of their LPS content, and likewise live vaccines should be used with caution in pregnant animals. It is often necessary, however, to vaccinate pregnant animals to provide maternal immunity for their offspring. It may be useful to include endotoxin assay in the safety test programme so that the levels can be compared with those shown to be safe in the double-dose tests. Vaccines may also cause swelling at the site of injection.

5. Tests on the final product

a) Safety
Killed vaccines are assessed in a double-dose test, and live vaccines are assessed in a test using ten times the dose, in the target species.

b) Potency
If possible, the potency test should relate to the efficacy of the vaccine in the target species, and suitable criteria should be applied for passing batches. It may be possible to assess killed vaccines by the O-H antibody response produced, although it should be remembered that serum antibodies are only part of the host’s protective mechanism against Salmonella. Alternatively, the potency of the vaccine may be assessed by its effect on the LD<sub>50</sub> in mice.

D. COMPETITIVE EXCLUSION

Susceptibility to Salmonella infection in poultry can be substantially reduced by spray or oral treatment prior to exposure (ideally in the hatchery) with an anaerobic culture of caecal microflora that inhibits colonisation by Salmonella by occupying intestinal colonisation sites, producing acids and other inhibitory substances and stimulating local and mucosal generalised immune responses. This treatment is widely used in some countries but only used as an aid to decontaminating persistently infected farms in others. It is also useful to minimise perturbation of intestinal flora following antimicrobial treatment or stress and to potentiate the effect of vaccines given subsequently. As with any treatment that reduces the prevalence or numbers of Salmonella organisms excreted by infected groups of animals, there may be some interference with monitoring programmes and the sampling and test sensitivity may have to be adjusted to compensate for this (1, 34, 47).

REFERENCES


Chapter 2.9.9. - Salmonellosis


Chapter 2.9.9. - Salmonellosis


Chapter 2.9.9. - Salmonellosis


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