

## CHAPTER 2.9.9.

# SALMONELLOSIS\*

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### 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 contaminants 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 that 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 animal feedstuffs, causing subclinical gastro-intestinal 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 subclinical 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 in pigs and S. Dublin in cattle).*

*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 that 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 results are not always indicative of active infection. 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:** Many inactivated vaccines are used against salmonellosis and some live vaccines are available commercially. Owing 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 (Grimont & Weill, 2007), 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 (Shelobolina *et al.*, 2004), but more recent unpublished data suggest that this organism does not actually belong in the genus *Salmonella* (Grimont & Weill, 2007). *Salmonella enterica* is divided into six subspecies, which are distinguishable by certain biochemical characteristics and susceptibility to lysis by bacteriophage Felix O1. These subspecies are:

•	Original subgenera	=	Current nomenclature
•	Subspecies I	=	subspecies <i>enterica</i>
•	Subspecies II	=	subspecies <i>salamae</i>
•	Subspecies IIIa	=	subspecies <i>arizonae</i>
•	Subspecies IIIb	=	subspecies <i>diarizonae</i>
•	Subspecies IV	=	subspecies <i>houtenae</i>
•	Subspecies VI	=	subspecies <i>indica</i>

For the serovars of *S. bongori*, the symbol V was retained to avoid confusion with the serovar names 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 over 2500 serovars are recognised (Grimont & Weill, 2007). 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 poikilothermic (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). Serovars of the other subspecies are designated by an antigenic formula, including subspecies designated by Roman numerical (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 serovar classifications as new evidence on genetic relatedness becomes available, e.g. *S. Pullorum* is now classified as *S. Gallinarum* biovar Pullorum and *S. Thomasville* is designated as an antigenic variant of *S. Orion* (Grimont & Weill, 2007).

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 present 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 (Wray & Wray, eds, 2000). 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. Wild animals such as badgers and some types of birds may carry specific strains of *Salmonella* (Wray & Wray, eds, 2000).

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. Abortusovis* in sheep, although most serovars may cause disease in a wide range of animal species (Snoeyenbos, 1994). Many serovars, including some that are host adapted such as *S. Choleraesuis* and *S. Dublin*, have been shown to cause serious 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 (Snoeyenbos, 1994). 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. 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 PFGE (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 and MLST (multi-locus sequence typing) can be used to investigate evolutionary pathways. Full sequencing is increasingly used because low cost automated methods have been developed but analysis of results is complex (Boxrud *et al.*, 2007; Foley *et al.*, 2007; Threlfall & Frost, 1990; Torpdahl *et al.*, 2007). 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. 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. There may also be problems with interpretation of minor genetic variations identified by highly discriminatory techniques and it is usually necessary to consider such results in the context of specific epidemiological data.

Genetic techniques such as micro-array and multiplex PCR analysis aimed at identifying specific serotypes as well as providing additional information on virulence and antimicrobial resistance gene content have been developed but are not yet fully validated for routine diagnostic use (Batchelor *et al.*, 2008; Herrera-Leon *et al.*, 2004; Porwollik *et al.*, 2004; Wattiau *et al.*, 2008).

The isolation and subsequent identification of salmonellae depends 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 is available (Wray & Wray, eds, 2000). Increasing resistance to important antimicrobials used for human therapy, e.g. cephalosporins and fluoroquinolones, related to use in primary production in some parts of the world, as well as increasing multiple resistance, often linked with virulence determinants, is of increasing concern (Garcia-Fernandez *et al.*, 2009; Su *et al.*, 2008).

National schemes have been implemented in many countries to control *Salmonella* infections in animals in order to protect the consumer. In the European Union (EU), 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* and slaughter of positive flocks (EU, 2003). In further legislation *S. Virchow*, *S. Infantis* and *S. Hadar* are also subject to special controls (EU, 2003). Culture of day-old 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, two pairs of boot swabs or pooled faeces of up to 60 samples are cultured. Subsequently, adults are sampled every 2 weeks. At the hatchery, hatcher basket liners, broken egg shells or swabs from hatchers or hatcher baskets are taken or flocks can be monitored at the holding by taking five pairs of boot swabs per flock. New EU legislation controlling *Salmonella* in commercial laying flocks, broilers, turkeys and pigs has been or 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. Baseline surveys have been carried out to determine the flock prevalence of *Salmonella* in laying hens, broiler flocks, breeding and fattening turkey flocks, individual pigs at slaughter, herds of breeding pigs and contamination of broiler carcasses at slaughter (European Food Safety Authority [EFSA], 2009).

Standardised monitoring and control programmes have been defined for all categories of chicken and turkey flocks. Monitoring of breeding flocks is as described above and commercial meat flocks are monitored by boot swabs. In the case of cage laying flocks, naturally pooled faeces samples are collected and additional dust samples are used for annual official monitoring of laying flocks by the competent authority. Operators are offered the option of a voluntary confirmatory test, which may be five faecal samples and two dust samples, post-mortem culture of carcasses and ovary/oviduct of 3000 birds or culture of 4000 whole eggs in pools of 40. Eggs from laying flocks where *S. Enteritidis* or *S. Typhimurium* is confirmed may not be sold as fresh table eggs. Details of these complex control programmes can be found on the DG SANCO website (European Commission, 2009).

*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. 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 (World Health Organization [WHO], 1994).

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 occasionally be caused by *Salmonella* serovars of relevance to public health, feedstuffs should be investigated for the presence of salmonellae (Anon, 2008). As feed is milled with ingredients of mixed global origin a wide range of 'exotic' salmonellas, including some serovars that are normally associated with reptiles, may be found in feed. Once established on a holding or integration, spread between animals, environmental contamination and farm pests becomes 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 (International Organization for Standardization (ISO), 1996; 1999). 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 and it is usually more effective to carry out process monitoring than mass testing of individual feed ingredients and finished feed (Anon, 2008).

The WHO 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, sprouted seeds, milk products 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 in some EU countries because of their very strict control programmes). *Salmonella* Typhimurium is the dominant serovar in North America (Saeed *et al.*, 1999; Wray & Wray, eds, 2000). Recently a monophasic variant of *S. Typhimurium* (S.4,5,12:i:-DT193 with resistance to ampicillin, streptomycin, sulphonamides and tetracycline) has emerged in pigs and caused outbreaks of salmonellosis in humans in several countries worldwide, and there are several other emergent monophasic S.4,5,12:i:- and S.4,12:i:- strains with varying resistance patterns that have been recognised in various animal species and humans in many countries in recent years (Soyer *et al.*, 2009).

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, and animal, food and feed testing (WHO, 1994). 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. In the EU, new regulations will require withdrawal from the market of

batches of meat where absence of *Salmonella* in 25 g of material cannot be demonstrated. Measures should be implemented during processing to limit contamination, but chemical decontamination is not currently permitted in the EU (European Commission, 2009). 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 persist long-term or spread beyond the vaccinated group of animals (Wallis, 2004).

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. A new ISO standard is being prepared to provide guidance on sampling methods for primary production and preparation of samples in the laboratory.

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 (Carrique-Mas & Davies, 2008), 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 (WHO, 1994). 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 may also 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 (Ellis *et al.*, 1976; Fricker, 1987; Gerhardt, 1981; Harvey & Price, 1974; Reissbrodt, 1995). 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 *Salmonella* serovar, 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 in the presence of a relevant sample matrix. 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. It is also possible to use strains with antimicrobial resistance or green fluorescent protein markers.

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 (ISO 6579:2002 Annex D) has now been adopted (ISO, 2002). 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. This method has also been shown to be highly effective for animal feed and meat products, and is simpler and less expensive than the full ISO method.

#### 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 usually 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 selective enrichment media, to multiply, or it may help to resuscitate salmonellae that have been sub-lethally damaged, e.g. by freezing, heating, exposure to biocides, organic acids, bacteriocins, phage 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. tetrathionate 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-based media. Selective motility enrichment may also be used to increase the sensitivity of *Salmonella* isolation and semi-solid enrichment media, e.g. MSR/V or diagnostic semi-solid *Salmonella* medium (DIASALM), may provide greater sensitivity (Voogt *et al.*, 2001). 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 tetrathionate, as in Müller–Kaufman broth, selenite F, selenite cysteine, 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 Ferrioxamine E may be added to selective media to enhance isolation of *Salmonella* from iron or nutrient-limited samples such as eggs, water or soil (Reissbrodt, 1995) or antibiotics such as novobiocin may be added to suppress most Gram-positive organisms or other Gram-negative bacteria such as *Proteus*. Specific antibiotics can be added to enhance the isolation of antimicrobial resistant *Salmonella* 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 non-lactose fermentation and hydrogen sulphide (H<sub>2</sub>S) 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*, *Citrobacter* and *Hafnia*. Lactose-fermenting salmonellae may occasionally be isolated and H<sub>2</sub>S 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 and plates such as desoxycholate-citrate agar (DCA) or bismuth-sulphite agar (BS) can be used, but these are subject to a higher frequency of false-positive colonies. *Salmonella Abortusovis* is a slow-growing serovar and it is usual to incubate plates for up to 72 hours and to use the non-selective blood agar. Examples of selective media are brilliant green agar, xylose lysine desoxycholate agar, deoxycholate/citrate agar, and bismuth sulphite agar, although many more will be found in the literature and media catalogues. A wide range of chromogenic agars such as Rambach agar, SMID, ASAP, 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.

- **Quantification methods**

*Salmonella* from infected tissues can be enumerated by direct plating, but most probable number (MPN) techniques are necessary for faecal, feed or environmental samples. Recently a miniaturised MPN method has been adopted as the preferred method in the EU (Fravalo *et al.*, 2003).

- **Identification of suspect colonies**

Suspect 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 (Ellis *et al.*, 1976). 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

(Ewing, 1986). It is particularly important to ensure that *Salmonella* cultures used for determination of antimicrobial resistance are not mixed with other organisms such as *Pseudomonas* that are more likely to be multi-resistant.

The determination of the O factor(s) and the H antigen(s), and in special circumstances the Vi antigen (present in *S. Typhi*, *S. Paratyphi C* and *S. Dublin*), 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 serovars, it is usually 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. Colonial form variation may occur in certain serogroup C2 serovars in which there is variable expression of minor antigens in different single colony picks from the same strain. This may result in apparent recovery of two different serovars (e.g. *S. Hadar* and *S. Istanbul* or *S. Newport* and *S. Bardo*) from the same isolate (Popoff, 2001).

Additional biochemical tests may be necessary to identify some serovar variants, e.g. d-tartrate fermentation, 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 (Greene *et al.*, 2008; Weill *et al.*, 2004). Live vaccine strains are also commonly identified by antimicrobial resistance markers, biochemical changes such as auxotrophism or roughness.

- **Immunological and nucleic acid recognition methods**

Numerous alternative *Salmonella* detection methods are in use and are commercially available (AOAC [Association of Official Analytical Chemists] International, 2009; Blackburn, 1993; De Boer & Beumer, 1999; Ewing, 1986; Ricke *et al.*, 1998; Rijpens & Herman, 2002; Swaminathan & Feng, 1994). These include electrical conductance/impedance, immunomagnetic separation (IMS), enzyme-linked immunosorbent assay (ELISA), gene probe PCR methods, real time PCR (Malorny *et al.*, 2004), quantitative PCR (Piknova *et al.*, 2005) or microarray analysis (Rasooly & Herold, 2008). Many of these methods have not been fully validated for faecal and environmental samples, although progress has been made (Jensen & Hoorfar, 2003; Malorny & Hoorfar, 2005), and are more suited to analysis of human foodstuffs where inhibitors of the PCR reactions are not so problematic (Kanki *et al.*, 2009) although there is a role for rapid methods in test and release of batches of *Salmonella*-free animal feedstuffs. 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 identify most contamination within 24 hours but faecal and environmental samples can be problematic for rapid methods. 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 (Oliveira *et al.*, 2003). 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 and controls to detect inhibition, which may reduce the sensitivity of the test in some cases (Jensen & Hoorfar, 2003). Rapid isolation methodologies may also be linked with sophisticated detection systems, such as biosensors (Olsen *et al.*, 2003). 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.

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

- i) Add a 10–25 g sample to ×10 volume 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 0.1 ml incubated buffered peptone water.
- iv) Inoculate 10 ml tetrathionate broth with 1 ml incubated buffered peptone water broth.
- v) Incubate modified semi-solid Rappaport–Vassiliadis or DIASALM at 41.5°C and tetrathionate broths at 37°C (ensure that a reputable brand of tetrathionate 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 plus novobiocin and one plate of xylose lysine desoxycholate agar .
- vii) Plate out 10 µl of tetrathionate broth on Rambach agar or brilliant green agar plus novobiocin and xylose lysine desoxycholate agar.
- 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 (or occasionally translucent red in the case of H<sub>2</sub>S negative strains) 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 antisera on to non-selective 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.

This method differs from ISO 6579:2002 and its Annex D method, but provides the possibility of additional detection by combining elements of broth and semi-solid agar enrichment.

## 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* and is thought to originate from it (Thomson *et al.*, 2008), the whole blood test and related tests can be used for the diagnosis of *S. Enteritidis* infection, but the sensitivity is low. In recent years, other tests, such as the ELISA (Barrow, 1994) 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, Germany, the Netherlands, the United Kingdom, and some other countries on serum or tissue fluid released by freezing then thawing muscle samples to detect *Salmonella* infections in pigs (Nielsen *et al.*, 1998). A similar test can be used to detect antibodies to *S. Enteritidis* and *S. Typhimurium* in egg yolk from unvaccinated commercial laying flocks.

Some ELISAs are now in routine use and a number are available commercially. There is a need for standardisation of their use and to this end panels of control sera are available commercially from Denmark<sup>1</sup> and the Netherlands<sup>2</sup>. 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 and in low prevalence countries specificity issues mean that most positive results will be false. Animals that are actively excreting salmonellae may be serologically negative in the early stages of disease and some individual infected animals never seroconvert. 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

1 Statens Serum Institut, Copenhagen, Denmark ([www.ssi.dk](http://www.ssi.dk))

2 GD, Deventer, the Netherlands ([www.gddeventer.com](http://www.gddeventer.com))

immunoglobulin production is maximal, or infection with less invasive serovars. 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 but there are often cost limitations to the application of effective monitoring programmes.

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 provoke a significant serum antibody response in the majority of animals but there may be occasional exceptions that are difficult to interpret. 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. Over 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. This method has been used for identification of exposure to various *Salmonella* serovars, e.g. *S. Typhimurium*, *S. Enteritidis*, *S. Dublin*, *S. diarizonae* in turkeys, *S. Abortusequi*.

- **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 *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<sup>8</sup> 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.

- **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.

- **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 antiserum 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 and the competitive 'sandwich type' ELISA (Barrow, 1994).

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 crude 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* serovars 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 serovars. 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. This combination has been used to differentiate *S. Enteritidis* field infection from a vaccinal response to *S. Gallinarum* 9R vaccine, which lacks flagellar antigens.

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 (Nielsen *et al.*, 1998). 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 up to 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 usually 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* serovars 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 serovar 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**

- i) 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.
- ii) The LPS antigen can also be prepared by the technique of Westphal & Luderitz (1954) and standardised as to its carbohydrate concentration by the method of Gerhardt (1981), 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**

- i) Positive control antiserum prepared by intramuscular inoculation of four 1-week-old specific pathogen free (SPF) chickens with an inoculum containing  $10^6$  *S. Enteritidis*. The serum is subsequently obtained 3–4 weeks later when antibody titres are maximal.
- ii) Negative control serum A from four 1-week-old SPF birds.
- iii) 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

### 1. Background

#### a) Rationale and intended use of the product

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 or mineral oil, 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 (Mastroeni *et al.*, 2001). 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 and other countries. 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*) (Cooper, 1994), 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. Most vaccines are produced as highly industrial commercial processes and are regulated by national veterinary medicines licensing authorities. Smaller quantities of emergency herd vaccines or autogenous vaccines are produced by private laboratories but each production has to be specifically licensed.

### 2. Outline of production and minimum requirements for conventional vaccines

#### a) Characteristics of the seed

- i) Biological characteristics

For killed or live 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 or auxotrophism, 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.

- ii) Quality criteria (sterility, purity, freedom from extraneous agents)

*Sterility and 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 non-selective media.

- Metabolic requirements as indicated by biochemical tests.
- Detection of markers, and phage type.
- Agglutination with specific antiserum.
- The vaccine culture and any adjuvants, preservatives or other materials must be microbiologically sterile and non-toxic at the concentrations used.

#### *Safety*

The LD<sub>50</sub> (50% lethal dose) or ID<sub>50</sub> (50% infectious dose) may be determined in mice or preferably signs of more mild adverse reactions should be checked in the target species. 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.

#### *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.

#### *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 commercial laying flocks during lay.

### **b) Method of manufacture**

#### *i) Procedure*

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.

Vaccine must be produced 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.

#### *ii) Requirements for substrates and media*

All chemicals and growth media used should be guaranteed to be fit for purpose and checked by the use of suitable controls.

iii) In-process controls

The following points require attention:

- Visual control of the suspension, homogeneity by Gram stain, culture on non-selective medium.
- Slide agglutination with specific antisera.
- Titration of bacteria by turbidimetry and/or plate count.
- Test of effective inactivation (killed vaccine) by plating on non-selective 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.

iv) Final product batch tests

*Sterility/purity*

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

*Safety*

A laboratory test that has previously shown a correlation with safety in the target species may be used to determine the absence of deleterious effects on vaccinated animals. 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. Observations are made on any adverse effects on the demeanour and health of the vaccinated animals and an assessment may be made of tissue reactions at the injection site.

*Batch 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. Many *Salmonella* vaccines are intended for use in poultry so these should be used for potency and safety tests.

**c) Requirements for authorisation**

i) Safety requirements

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, particularly if an oil-emulsion adjuvant is used.

*Target and non-target animal safety*

Killed vaccines are assessed in a double-dose test, and live vaccines are assessed in a test using ten times the dose, ideally in the target species. Live vaccines should be proven to be harmless in relevant non-target species that could be exposed to vaccine excreted by vaccinated animals.

*Reversion to virulence for attenuated/live vaccines*

Live vaccines shall be shown in replication tests in target species to not revert to virulent strains during a suitably large number of replications. Mutations, especially undefined mutations, should be shown to be stable and checks on stability can be made by molecular fingerprinting methods or sequencing. Although the risk is small it is wise not to use live vaccines in a country where the organism in the vaccine has been eradicated.

*Environmental consideration*

Live vaccines should not be able to replicate in the environment or persist for more than a short period.

ii) Efficacy requirements

*For animal production*

The duration of immunity is likely to vary considerably between products, vaccination regimes and individual vaccinated animals. Immunity to *Salmonella* is normally serovar or serogroup specific. Consultation among colleagues suggests that most killed vaccines will provide some 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. The *Salmonella* vaccines are intended to limit the extent of clinical disease in the case of ruminants, pigs and *S. Gallinarum* in poultry. 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 challenged vaccinated animals compared quantitatively and statistically with unvaccinated controls.

*For control and eradication*

Vaccines for *Salmonella* are not capable of eradicating infection from herds or flocks but can increase the threshold for infection, reduce the level of excretion of the organism and reduce vertical transmission in poultry that results in contamination of hatching or table eggs. Vaccination is therefore an aid to other eradication and control measures such as culling, all in-all out production, biosecurity and farm hygiene.

iii) 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. Chemicals with antimicrobial activity, such as thiomersal, phenol or crystal violet, are often included as preservatives in killed bacterial vaccines. 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, and genotyping tests to identify genetic changes during fermentation production.

### 3. Vaccines based on biotechnology

a) Vaccines available and their advantages

None.

b) Special requirements for biotechnological vaccines, if any

None.

## D. COMPETITIVE EXCLUSION

Susceptibility to *Salmonella* infection in poultry can sometimes 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, including vaccination or organic acid treatment, that may reduce 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 (Mead, 2000; Revollo *et al.*, 2006).

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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](http://www.oie.int)).