CHAPTER 3.9.8.

SALMONELLOSIS*[1]

SUMMARY

Description of the disease: *Salmonellosis is an infectious disease of humans and animals caused by bacteria of the genus* Salmonella. *Salmonellae are aetiological agents of diarrhoeal and systemic infections. They often cause subclinical infections and may be shed in large numbers within the faeces of clinical cases and carrier animals resulting in contamination of the environment. Infection in food animals often leads to contamination of meat, eggs, milk and cheese. Salmonellosis is one of the most common and economically important food-borne zoonotic diseases in humans. The disease has been recognised in all countries and non-typhoidal* Salmonella *appears to be most prevalent in areas of intensive animal husbandry, especially in pigs, intensively reared calves and poultry.*

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 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 host-specific Salmonella*, are addressed in chapter 3.3.11 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 or abortion 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 and multiply sublethally damaged salmonellae, enrichment media that contain inhibitory substances to suppress competing organisms, and selective plating agars to differentiate salmonellae from other enterobacteria. Alternative methods such as polymerase chain reaction and immunological detection of Salmonella *antigens can also be used, according to legislative requirements.*

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 White-Kauffmann–LeMinor scheme. Many laboratories may need to send isolates to a reference laboratory. Alternative serotyping methods, including multi-locus sequence typing* (*MLST*)*-based on whole genome sequencing, are increasingly used. Phage-typing schemes are also available for some serovars.*

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 the laboratory, the tube agglutination test is the method of choice for export and diagnostic purposes for samples from all*

1 Although certain diseases caused by *Salmonella* are included in some individual species sections of the OIE List, this chapter covers several species and thus gives a broader description.

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. Salmonella *vaccination may compromise the diagnostic value of serological tests.*

Requirements for vaccines: *Inactivated and live vaccines are available commercially. In the case of the inactivated vaccines, they contain oil or alhydrogel adjuvants to improve their efficacy.*

A. INTRODUCTION

Salmonellosis is an infectious disease of humans and animals, clinically characterised by septicaemia, acute enteritis or chronic enteritis. Animals may be infected without being overtly ill. Salmonellae are primarily intestinal bacteria and may be shed continuously or intermittently within the faeces, resulting in contamination of the environment.

1. Causal pathogen

The genus *Salmonella* consists of only two species: *S. enterica* and *S. bongori* (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 *enterica*
•	Subspecies II	=	subspecies *salamae*
•	Subspecies IIIa	=	subspecies *arizonae*
•	Subspecies IIIb	=	subspecies *diarizonae*
•	Subspecies IV	=	subspecies *houtenae*
•	Subspecies VI	=	subspecies *indica*

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) or O antigens and flagellar protein or H antigens in accordance with the White-Kauffmann–Le Minor scheme; currently more than 2600 serovars are recognised (Issenhuth-Jeanjean *et al.,* 2014). 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 cold-blooded animals and in the environment, but are occasionally associated with human disease. Some serovars of subspecies *arizonae* and subspecies *diarizonae* may cause disease in turkeys and sheep and others may be carried by reptiles and amphibians. In accordance with the White-Kauffmann–Le Minor scheme only serovars of subspecies *enterica* bear a name, e.g. *S. enterica* subsp. *enterica* serovar Enteritidis, or *S. enterica* serovar Enteritidis, or, in short, *S.* Enteritidis. Serovars of other subspecies of *S. enterica* and those of *S. bongori* are designated only by their antigenic formula (e.g. *Salmonella* IV 48:g.z51).

Changes to serovar classification may occur when different O antigens are expressed due to colony form variation or lysogenation by bacteriophage(s) or when different flagellae are produced as a result of phase variation.

2. Description of the disease

The course of infection, the clinical signs, the post-mortem findings and epidemiological patterns vary according to the serovar and the animal species involved. Most serovars can cause disease in a wide range of animal species (Barrow & Methner, 2013), but some serovars are host-specific, e.g. *S.* Typhi in humans and *S.* Abortusovis in sheep. Other serovars are host-adapted e.g. *S.* Choleraesuis in pigs and *S.* Dublin in cattle. Host-specific and host-adapted serovars often cause septicaemic disease. In poultry, the host-specific diseases Pullorum disease or bacillary white diarrhoea and fowl typhoid are used to describe infections caused by *S.* Pullorum and *S.* Gallinarum (Barrow & Neto, 2011), respectively. Fowl typhoid and Pullorum disease are covered in detail in chapter 3.3.11 of this *Terrestrial Manual.*

In humans, young children, the aged and those immunologically compromised are most susceptible to salmonellosis. The disease can affect all species of domestic animals; young animals and pregnant animals are particularly susceptible. A wide range of clinical signs, including acute septicaemia, acute or chronic diarrhoea, respiratory disease, abortion, and arthritis, may be seen. Chicks and poults of less than 1 week of age are highly susceptible to *Salmonella* infection and may occasionally exhibit signs including anorexia, adipsia, depression,

ruffled feathers, huddling together, somnolence, dehydration, white diarrhoea and pasted vents with considerable mortality as a result, but even in young poultry, subclinical infection is most likely. In calves, septicaemic infection with the host-adapted *S*. Dublin serovar occurs mainly at 2–6 weeks of age. The calves are dull, pyrexic, are anorexic, have diarrhoea with blood and mucus in the faeces, may have pneumonia, and often become quickly dehydrated and die if appropriate treatment is not given in a timely manner. In pregnant cows, infection with *S*. Dublin is a common cause of abortion. Pigs infected with the host-adapted *S*. Choleraesuis may show clinical illness, are anorexic, have a high fever, become lethargic and the extremities may appear cyanotic.

Many animals, especially poultry and pigs, may be infected but show no clinical illness (Barrow & Methner, 2013). Such animals may be important in relation to the spread of the infection between flocks and herds. Salmonellosis has been recognised in all countries, but non-typhoidal infection appears to be most prevalent in areas of intensive animal husbandry, especially of poultry or pigs (Barrow & Methner, 2013).

3. Zoonotic risk and biosafety requirements

Human salmonellosis is one of the most common and economically important zoonotic diseases. The Centers for Disease Control and Prevention (CDC) estimates that salmonellosis causes more than 1.2 million illnesses each year in the United States of America, with more than 23,000 hospitalisations and 450 deaths (CDC, 2013). The most common cause of infection with *Salmonella* is eating contaminated foods including raw or under cooked eggs or egg products, meat, poultry, contaminated fresh fruit and vegetables and soft cheeses made from unpasteurised milk.

Salmonella can also be spread to people through contact with infected birds, livestock, reptiles, amphibians, and dogs and cats. These animals may carry the bacteria even when apparently healthy. Many serovars, including some that are host-adapted such as S. Choleraesuis and S. Dublin, have been shown to cause serious disease in humans. Abattoir workers, animal attendants and veterinarians may be infected directly during the course of their work when in contact with infected animals. Laboratory personnel may also acquire the infection if safe working practices are not implemented. To prevent occurrences of laboratory infections, the biosafety and biosecurity measures in veterinary diagnostic and animal facilities should be adhered to (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*).

4. Differential diagnosis

Signs in young birds with generalised salmonellosis closely resemble those seen in Pullorum disease, fowl typhoid and those of other acute septicaemic illnesses caused by a wide variety of bacteria including *Escherichia coli.* In all avian species arthritis caused by *Salmonella* infection may be mistaken for synovitis or bursitis caused by other infections. Septicaemic salmonellosis in pigs caused by *S*. Choleraesuis may be mistaken for hog cholera. Septicaemic salmonellosis in calves may be confused with colibacillosis, although the latter disease occurs usually at a younger age. The acute enteric form of salmonellosis in calves may resemble coccidiosis. Abortions in sheep may be caused not only by *S. abortusovis* but also by *Coxiella burnetii, Chlamydophila abortus, Brucella ovis* or other pathogens.

B. DIAGNOSTIC TECHNIQUES

Table 1. *Test methods available for the diagnosis of salmonellosis and their purpose*

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Agent identification[2]						
***Salmonella* isolation**	+++	+++	+++	+++	+++	n/a
Rapid alternative methods, e.g. PCR	+	+	+	+	+	n/a

2 A combination of agent identification methods applied on the same clinical sample is recommended.

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of immune response						
SAT	++	–	++	–	+	++
ELISA	++	–	+++	+	++	++

Key: +++ = recommended method; ++ = suitable method; + = may be used in some situations, but cost, reliability, or other factors severely limits its application; – = not appropriate for this purpose; n/a = not applicable. Although not all of the tests listed as category +++ or ++ have undergone formal validation, their routine nature and the fact that they have been used widely with satisfactory results, makes them acceptable, although repeated testing may be required in some situations.
PCR = polymerase chain reaction-based tests; SAT = serum agglutination test; ELISA = enzyme-linked immunosorbent assay.

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, import and export regulations, clinical findings, level of detection or precision of prevalence estimates required, cost and availability of sampling resources and laboratory facilities. General guidelines on the collection submission and storage of diagnostic or survey samples, the sample size, the information to be sent with the samples and the packaging and transportation of samples are described in Chapters 1.1.2 and 1.1.3 of this *Terrestrial Manual.*

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. Clinical samples are preferably 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. For smaller animal species, it may be preferable to submit a representative number of sick or recently dead animals to the laboratory (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. As bacteria are usually clustered in faecal samples, a thorough mixing of the sample before culture may also increase the sensitivity of the procedure (Cannon & Nicholls, 2002). Bacteriological and also serological methods may be used to identify infected flocks or herds, rather than to identify infected individual animals.

1.1. Culture

There are numerous methods for isolation and detection of *Salmonella* in use world-wide (Fricker, 1987; Harvey & Price, 1974; Lee *et al.,* 2015; Park *et al.,* 2014; 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; therefore, 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 other markers, such as fluorescence.

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 method has not been

validated for faecal and environmental samples and was intended for foodstuffs and feeding stuffs (ISO, 2002). A standard method for detection of *Salmonella* from primary animal production has been published, and the ISO method has now been adopted (ISO, 2007). The basis of the standard method is pre-enrichment in buffered peptone water, followed by enrichment on modified semi-solid Rappaport–Vassiliadis (MSRV) and isolation on xylose-lysine-deoxycholate (XLD) agar and an additional plate medium of choice. This method has also been shown to be highly effective for animal feed, animal environmental samples and meat products, and is simpler and less expensive than the full ISO method. Diagnostic methods and assays should be validated as described in Chapter 1.1.6 *Principles and methods of validation of diagnostic assays for infectious diseases*.

1.1.1. Pre-enrichment media

The number of Salmonellae in faeces from asymptomatic animals, environmental samples, animal feed and food is usually low, and it is often necessary to use pre-enrichment media, such as buffered peptone water 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, and it may help to resuscitate salmonellae that have been sub-lethally damaged, e.g. by freezing, heating, desiccation, or exposure to biocides or organic acids (Harvey & Price, 1974). For intermittent shedders, it is advantageous to test at least three consecutive faecal samples.

1.1.2. Selective enrichment media

Enrichment media are liquid or semi-solid agar media containing additives that selectively permit salmonellae to grow while inhibiting the growth of other bacteria. Examples of selective enrichment media are tetrathionate, as in Müller–Kauffmann broth, selenite cystine, brilliant green broth, Rappaport–Vassiliadis broth and modified semi-solid Rappaport–Vassiliadis (MRSV) agar. Some additives, 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, with a temperature of 43°C being used in some laboratories, although this may be inhibitory with some media, e.g. tetrathionate. With Rappaport–Vassiliadis at 43°C temperature-sensitive strains, especially *S.* Dublin, are inhibited and 41.5°C is now recommended for incubation of Rappaport–Vassiliadis broth-based media. Selective motility enrichment media such as MSRV or diagnostic semi-solid *Salmonella* medium (DIASALM) are commonly used to increase the sensitivity of the *Salmonella* isolation procedure (Voogt *et al*., 2001). Use of at least two enrichment broths is recommended, with one incubated at 37°C and the other at a suitable higher temperature. 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. Additives, such as ferrioxamine E, may be added to selective enrichment 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.

1.1.3. 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_2S) 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_2S production may be variable. Such atypical strains may be more effectively detected when semi-solid motility media are used. Examples are the modified semi-solid MSRV and DIASALM. The selectivity of these media is based on the motility of the organism, the presence of malachite green dye and novobiocin, and a high concentration of magnesium chloride. The semi-solid medium allows motility to be detected as halos of growth away from the site of inoculation. DIASALM medium is particularly useful for the detection of atypical strains 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. Also, plates such as desoxycholate-citrate agar (DCA), brilliant green agar (BGA) or bismuth-sulphite agar 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 plating media are BGA, XLD agar, DCA, and bismuth-sulphite agar. A wide range of chromogenic agars such as Rambach agar and SMID (*Salmonella* detection and identification agar) 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 targeted as sensitivity can be poor in some circumstances. Certain chromogenic agar media, such as Brilliance or Rapid, may, however, be more efficient for detection of biochemically atypical salmonellae.

1.1.4. 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 3.3.11 *Fowl typhoid and Pullorum disease.*)

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

iii) Inoculate 20 ml MSRV or DIASALM in a Petri dish with 0.1 ml incubated buffered peptone water.

iv) Inoculate 10 ml Müller–Kauffmann tetrathionate broth with 1 ml incubated buffered peptone water broth.

v) Incubate MSRV 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 MSRV or DIASALM by taking 1 µl loop of material from the edge of the turbid growth zone and streaking over one plate of chromogenic agar (e.g. Rambach agar) or BGA plus novobiocin and one plate of XLD agar.

vii) Plate out 10 µl of tetrathionate broth on one plate of chromogenic agar (e.g. Rambach agar) or BGA plus novobiocin and XLD 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 BGA, crimson with pale borders or orange/colourless on Rambach agar, red with black centre (or occasionally translucent red in the case of H_2S negative strains) on XLD agar) biochemically, using composite media such as TSI, LDC and urea, or commercial biochemical tests. Confirm to serogroup level by using poly 'O' and poly 'H' (phase 1 and phase 2) antiserum or composite biochemical media. Sero-grouping alone is not sufficient because of cross-reactions with polyvalent sera, e.g. by *Citrobacter* spp. Multiple biochemical tests can provide confirmation.

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. Biochemically and serologically confirmed isolates can then be submitted to a reference laboratory for serotyping. 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 test kits.

This method differs from ISO 6579:2002 and its Annex D method in that it allows for inoculation of DIASALM in place of MSRV for selective enrichment and uses the chromogenic Rambach agar as its first plating agar. However, it provides the possibility of additional detection by combining elements of broth and semi-solid agar enrichment (Carrique-Mas *et al*., 2009).

1.2. 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. A miniaturised MPN method has been described (ISO, 2012) based on Fravalo *et al.,* (2003). Furthermore quantitative real-time polymerase chain reaction (PCR) methods have also been developed.

1.3. Identification of suspect colonies

Suspect colonies are subcultured onto selective and non-selective agars to ensure the absence of possible contaminants, such as *Proteus* spp. If there is an abundant pure growth, suspect colonies may be tested by slide agglutination with polyvalent *Salmonella*-typing antisera (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 using peptone water sugars or commercial systems 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. MALDI-TOF is also an acceptable method for identification of *Salmonella.*

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. More details on serotyping of *Salmonella* are described in ISO (2014) and by Grimont & Weill (2007) 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, including phage typing, if serovar-specific typing phages are available, and for genetic characterisation.

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 (d-tartrate +) 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 (Figueiredo *et al.,* 2015; Greene *et al.,* 2008). Live vaccine strains are also commonly identified by antimicrobial resistance markers, biochemical changes such as auxotrophism or roughness.

1.4. Immunological and nucleic acid recognition methods

Numerous alternative *Salmonella* detection methods are in use and some are commercially available. These include immunomagnetic separation (IMS) (Park *et al.,* 2011), reverse transcriptase real-time (RT)-PCR (Park *et al.,* 2014), enzyme-linked immunosorbent assay (ELISA) (Barrow, 1992; Wang *et al*., 2015), real time PCR (Malorny *et al*., 2004), quantitative PCR (Piknova *et al*., 2005) and microarray analysis (Porwollik *et al*., 2004; Rasooly & Herold, 2008). These methods may be used to identify specific *Salmonella* serovars (Maurischat *et al.,* 2015a) or to distinguish live vaccine strains from *Salmonella* serovars infecting the flock or herd (Maurischat *et al*., 2015b). Some consist of a combination of methods such as a two-step enrichment and real-time PCR (Krascseniscová *et al.*, 2008). Many of these methods have not been fully validated for faecal and environmental samples, although progress has been made (Eriksson & Aspan, 2007; Malorny & Hoorfar, 2005). These methods are more suited to analysis of human foodstuffs where inhibitors of the PCR reactions are not as problematic as for faeces (Kanki *et al*., 2009), though 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 initial 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 *Salmonella* contamination within 24 hours. As currently none of the rapid methods has been shown to be suitable for direct detection of *Salmonella*, 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 *et al*., 2013). There are many variations and developments in rapid methods for *Salmonella* detection, but none has been shown to satisfactorily replace culture in all circumstances. In contrast, molecular methods for serotyping or subtyping *Salmonella* isolates are increasingly widely used (EFSA, 2013) and some kits using these methods are suitable for use in small laboratories that lack the facilities of a reference laboratory. It is important that kits used have been fully validated in accordance with chapter 1.1.6. Kits should preferably be selected from those listed on the OIE Register (see http://www.oie.int/en/scientific-expertise/registration-of-diagnostic-kits/the-register-of-diagnostic-kits/).

2. Serological tests

2.1. 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 (see Chapter 3.3.11). 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. An ELISA that includes somatic antigens from a mix of serovars ("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[3] and the Netherlands[4].

2.2. 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 countries with a low prevalence of salmonellosis 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. 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 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 or vaccinated 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

3 Statens Serum Institut, Copenhagen, Denmark (www.ssi.dk)
4 GD, Deventer, the Netherlands (www.gddeventer.com)

are difficult to interpret. Injectable killed vaccines used for control of *S.* Enteritidis in chickens may produce a very prolonged antibody response. Live marker vaccines have been produced and can be distinguished from field challenge strains because of their antimicrobial resistances to rifampicin and by real-time PCR assays (Maurischat *et al*., 2015b).

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 2600 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.

2.3. 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 3.3.11.

2.4. 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.

2.5. 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, and *S.* Abortusequi.

2.5.1. 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 10^8 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.

2.5.2. 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, re-passage 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.

2.5.3. 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 pre-diluted 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 are used.

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

2.6. 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 APHA Weybridge (see Table in Part 4 of this *Terrestrial Manual* for address). The requirements are given below.

2.6.1. Equipment

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.

2.6.2. Antigen

i) Phenol-extracted *S.* Enteritidis LPS is available commercially. 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.

2.6.3. 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.

i) 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. Store at 4°C and renew every 2 weeks.

ii) Substrate buffer

Make a 10% (v/v) solution of diethanolamine in deionised water. The diethanolamine should be pre-warmed 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.

iii) Enzyme conjugate

Goat anti-chicken immunoglobulin conjugated to alkaline phosphatase or other species anti-chicken globulin. Store at 4°C diluted in diluent at the appropriate concentration and renew every week.

iv) 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.

2.7. 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

1.1. 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. 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) (Desin *et al.,* 2013; Hassan & Curtiss III, 1997), 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

2.1. Characteristics of the seed

2.1.1. 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 or genetic markers. Live 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.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

i) Sterility and purity

The vaccine strain must be checked as follows:

a) Staining of a smear of bacterial suspension on a glass slide using the Gram stain.

b) Homogeneity of culture on non-selective media.

c) Metabolic requirements as indicated by biochemical tests.

d) Detection of markers, and phage type.

e) Agglutination with specific antiserum.

f) The vaccine culture and any adjuvants, preservatives or other materials must be microbiologically sterile and non-toxic at the concentrations used.

ii) Safety

The LD_{50} (50% lethal dose) or ID_{50} (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.

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 commercial laying flocks during lay.

2.2. Method of manufacture

2.2.1. Procedure

The seed culture is propagated and maintained using suitable media for growth of *Salmonella*. The media used should not contain serum or animal tissues (unless permitted by national regulations). 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 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.

2.2.2. 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.

2.2.3. In-process controls

The following points require attention:

i) Visual control of the suspension, homogeneity by Gram stain, culture on non-selective medium.

ii) Slide agglutination with specific antisera.

iii) Titration of bacteria by turbidimetry or plate count.

iv) 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.

v) Titration of viable bacteria (living vaccine) before and after lyophilisation.

2.2.4. Final product batch tests

i) Sterility/purity

Tests for sterility and freedom from contamination of biological materials intended for veterinary use are found in chapter 1.1.9 of this *Terrestrial Manual*.

ii) 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 is made of tissue reactions at the injection site.

iii) Batch potency

Potency is tested using vaccination–challenge assay in mice 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.

2.3. Requirements for authorisation

2.3.1. 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.

i) 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.

ii) 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.

iii) Environmental consideration

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

2.3.2. Efficacy requirements

i) 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.

ii) 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. It is recommended that live vaccines that contain *Salmonella* serovars that are not endemic in a particular region should not be used for control of other serovars (van Immerseel *et al.,* 2013).

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NB: There are OIE Reference Laboratories for Salmonellosis
(see Table in Part 4 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list:
http://www.oie.int/en/scientific-expertise/reference-laboratories/list-of-laboratories/).
Please contact the OIE Reference Laboratories for any further information on
diagnostic tests, reagents and vaccines for Salmonellosis

NB: FIRST ADOPTED IN 1991 AS SALMONELLOSIS (*S. ABORTUS OVIS* AND *S. EQUI*) AND SALMONELLOSIS (*S. TYPHIMURIUM* AND *S. ENTERITIDIS*); MOST RECENT UPDATES ADOPTED IN 2016.