CHAPTER 3.3.11.

FOWL TYPHOID AND PULLORUM DISEASE

SUMMARY

Description and importance of the disease*: Pullorum disease of chickens is a bacterial infection caused by* Salmonella enterica *subspecies* enterica *serovar Gallinarum biovar Pullorum* (Salmonella *Pullorum*)[1]*. At this time the serovar is referred to as Gallinarum in some parts of the world and Pullorum in others; in this chapter the serovar will be referred to as Gallinarum or Pullorum according to the biovar under discussion as this is more meaningful from a clinical and epidemiological perspective.*

In its acute form, Pullorum disease is almost exclusively a septicaemic disease of young chickens. However, the organism may also be associated with disease in turkey poults and may be carried subclinically or lead to reduced egg production and hatchability, plus a range of atypical signs in older birds. Ovarian transmission is a major route by which the organism can spread. Game birds and 'backyard' poultry flocks may act as reservoirs of infection, and wild birds may act as vectors for the organism and as such are important in the epidemiology of the disease.

Fowl typhoid in chickens and turkeys is caused by S. *Gallinarum biovar Gallinarum and is more often observed in the later growing period and in mature stock. Disease is often characterised by rapid spread with high morbidity and acute or subacute mortality. Red mites may be involved in the transmission of disease and persistence in poultry houses*

Clinical signs in chicks and poults include anorexia, diarrhoea, dehydration, weakness and death. In mature birds, Pullorum disease is less severe but decreased egg production, poor hatchability and some increased mortality may occur. Fowl typhoid is a more acute septicaemic condition which mainly affects mature birds and may be particularly severe in commercial laying flocks.

Identification of the agent: *Samples should not be taken from birds or eggs that have recently been treated with antimicrobial drugs. Swabs or aseptically collected samples from infected tissues, or intestinal and cloacal contents should be used for diagnostic testing. Other materials that may be sampled include eggs, embryos, faecal droppings and hatcher debris, especially fluff, dust and broken eggshells and chick box linings. Samples of tissues such as caecal tonsils, liver, gall bladder and spleen from infected birds are preferable to faecal and environmental samples. Tissue samples should be inoculated into non-selective and selective enrichment broths and on selective agar medium, such as brilliant green agar, as soon as possible after collection. In case of delay, samples should be stored at 4°C. Typical colonies can be identified by serological and biochemical tests. Molecular approaches can also be used to identify and differentiate* S. *Gallinarum and* S. *Pullorum. Final serological confirmation of suspect isolates can normally only be completed in a* Salmonella *Reference Typing Laboratory.*

Serological tests: *These are satisfactory for identifying the presence and estimating the prevalence of infection within a flock. The test used in the field is the rapid whole blood plate agglutination test. This test is unreliable in turkeys and ducks as many uninfected birds may give positive reactions. In the laboratory a serum agglutination test is used, either as a rapid plate test or as a tube test. These can be applied as macro- or microagglutination tests, though the latter may be more likely to give false-positive results with turkey sera. Any positive reactors should be confirmed as being infected by culture at post-mortem examination. Enzyme-linked immunosorbent assays have been reported but no commercial test is available.*

1 See the note in Chapter 3.9.8 *Salmonellosis* for the principles followed concerning the nomenclature of *Salmonella*.

The use of vaccines to control S. *Enteritidis or* S. *Gallinarum infections in chickens may cause problems in the interpretation of serological results.*

***Requirements for vaccines*:** *Live and inactivated vaccines are available for fowl typhoid in some countries. The most commonly used vaccine is a commercial live vaccine derived from the stable rough strain of S. Gallinarum known as '9R'.*

A. INTRODUCTION

Fowl typhoid and pullorum disease, caused by *Salmonella enterica* subspecies *enterica* serovars Gallinarum biovars Gallinarum and Pullorum, respectively, are widely distributed throughout the world, especially in developing countries (Barbour *et al.,* 2015) where increasing antimicrobial resistance in these strains has also become a problem (Parvej *et al*., 2016). They have been eradicated from commercial poultry in many developed countries of Western Europe, the United States of America (USA), Canada, Australia and Japan. The move towards fee-range production in many countries may increase the risk of infection (Vielitz, 2016), but many outbreaks involve intensively housed laying hen or breeding flocks. In the USA and the United Kingdom the serovar is referred to as Pullorum (Hitchner, 2004), even though the strains are now considered to be the same serovar that is derived from *S*. Enteritidis by gene deletion events (Thomson *et al.*, 2008). In this chapter the terms serovar Gallinarum or Pullorum will be used, as this more usefully distinguishes the two biovars that cause clearly distinct clinical syndromes and are therefore epidemiologically different. *Salmonella* Gallinarum recurred in some European countries in the first decade of the 21st century (Ivanics *et al.,* 2008). *Salmonella* Pullorum remains as a constant reservoir in wild and game birds (Barrow *et al.*, 2012; Shivaprasad *et al*., 2013).

Salmonellosis caused by *Salmonella bongori* or subspecies of *Salmonella enterica* is covered in Chapter 3.9.8 *Salmonellosis*.

Clinical signs of fowl typhoid are typical of a septicaemic condition in poultry and include increased mortality and poor quality in chicks hatched from infected eggs. Older birds show signs of anaemia, depression, laboured breathing and diarrhoea causing adherence of faeces to the vent. The highest mortality in pullorum disease occurs in birds of 2–3 weeks of age. In older birds disease may be mild or inapparent. In breeding and laying flocks susceptibility is increased at the point of lay (Wigley *et al.,* 2005), but reduced egg production and hatchability may be the only signs of pullorum disease. Trans-ovarian infection resulting in infection of the egg and hatched chicks or poults is one of the most important transmission routes for both diseases (Haider *et al.,* 2014).

Post-mortem signs of pullorum disease in newly hatched chicks are those of peritonitis with generalised congestion of tissues and an inflamed unabsorbed yolk sac. Longer standing infections commonly lead to typhlitis with development of necrotic caecal casts and small necrotic foci in the liver, lungs and other viscera. Small lesions in the liver and spleen of Pullorum-infected birds may show a 'white spot' appearance that is not seen with Gallinarum; however, this lesion is not pathognomic. These *Salmonella* are very poor at colonisation and survival in the gastrointestinal tract is often indicative of later stages of clinical disease. Adult birds may develop misshapen, discoloured and/or shrunken ovaries with follicles attached by pedunculated fibrous stalks. Variant strains of *S*. Pullorum do not normally cause clinical disease or may result in mild, nonspecific signs but may lead to seroconversion.

In fowl typhoid, as well as generalised signs of septicaemia, the liver is usually enlarged, dark and friable with a distinctive coppery bronze sheen that may only develop after exposure to air. The bone marrow is also often dark brown. Although clinical signs and post-mortem findings of pullorum disease and fowl typhoid may be highly suggestive of the conditions, they are not sufficiently distinct from other causes of septicaemia to be pathognomic. It is therefore necessary to confirm disease by isolation of the organisms. Serological tests can be used to establish the presence of the disease in a flock.

1. Zoonotic risk and biosafety requirements

Salmonella Gallinarum and *S.* Pullorum are host adapted to avian species (Eswarappa *et al.,* 2009) and are considered to pose a minimal zoonotic risk (Shivaprasad, 2000). Although the genome is adapted to a non-intestinal environment and has lost flagella genes to help evade host immune responses (Lopes *et al*., 2016), it is continually evolving, which could theoretically widen the host range in future (Liu *et al*., 2002). Non-typhoidal *Salmonella* serovars should be handled with appropriate biosafety and containment procedures as determined by biological risk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*).

B. DIAGNOSTIC TECHNIQUES

Table 1. *Test methods available for the diagnosis of fowl typhoid and Pullorum disease 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						
Bacterial isolation	+++	+++	+++	+++	+++	n/a
Rapid alternative methods e.g. PCR	+	+	+	+	+	n/a
Detection of immune response						
WBT	++	_	+++	_	+	++
RSA	++	_	++	_	+	++
SAT	++	_	++	_	++	+++
ELISA	+	_	+	_	+	++

Key: +++ = recommended method, validated for the purpose shown; ++ = suitable method but may need further validation;
+ = may be used in some situations, but cost, reliability, or other factors severely limits its application;
– = not appropriate for this purpose; n/a = purpose not applicable.
PCR = polymerase chain reaction-based tests; WBT = whole blood agglutination test; RSA = rapid slide agglutination test;
SAT = serum agglutination test; ELISA = enzyme-linked immunosorbent assay.

1. Identification of the agent

1.1. Bacteriological culture methods

In the acute stages of disease, the agent of both diseases can be recovered from almost all organs, tissues and faeces. In older birds that have become carriers, *S.* Pullorum is most commonly recovered from the ova and oviduct; and it is recovered only occasionally from other organs and tissues, including the alimentary tract. In the acute phase of fowl typhoid the organism is also widely distributed, but in carrier birds, the organism is found most often in the liver, spleen and reproductive tract, and occasionally in the caecal tonsils.

Salmonella Pullorum and *S.* Gallinarum belong to the White-Kauffmann–Le Minor scheme serogroup D, along with *S.* Enteritidis, which is closely related (Grimont & Weill, 2007). The organisms are Gram-negative non-sporogenic rods 1.0–2.5 µm in length and 0.3–1.5 µm in width. They are considered to be non-motile under normal conditions but inducement of flagellar proteins and motility has been shown in some strains of *S*. Pullorum when grown in special media (Holt & Chaubal, 1997).

For optimal recovery of the organisms, the birds being sampled should not have been treated with antimicrobial drugs for approximately 2–3 weeks previously.

Samples may be obtained from live birds, preferably after identifying them as highly sero-positive birds. Fresh or freshly chilled carcasses, egg materials, fresh faeces, or any contaminated materials from housing, incubators or transport boxes may also be taken, but faecal and environmental samples often fail to reveal the presence of the organism because of inconsistent shedding and the poor sensitivity of

bacteriological detection methods[2]. Swabs may be taken from the cloaca of sick live birds but post-mortem tissues are preferable. Samples from visibly abnormal tissues are preferred, but aseptically gathered samples can also be taken from the spleen, liver, gall-bladder, kidneys, lungs, heart, ova, testes, alimentary tract or joint lesions. The preferred tissues for routine investigation are liver, ileo–caecal junction and ovaries/oviduct. The surface is seared with a hot spatula and a sample is obtained by inserting a sterile cotton swab or sterile loop through the heat-sterilised surface. The demonstration of infection in serological reactor birds that are apparently normal may, in some cases, require the culture of large volumes of homogenised tissues as well as direct swabbing. Tissue pools may be made from tissues collected from a number of birds, and, for routine testing, five ileo-caecal junction samples may be pooled. Larger numbers of aseptically collected non-intestinal samples can be pooled, but for practical purposes, composite samples of liver, spleen and ovary from five birds are often tested.

When floor litter, faecal material or hatchery material such as hatcher basket liners is sampled, it should be remembered that *S.* Pullorum and *S.* Gallinarum in low numbers associated with subclinically infected carrier birds are more difficult to isolate from faecal and environmental samples than other salmonellae and it is always preferable to culture sick or recently deceased birds or 'dead in shell' embryos. Red mites associated with poultry that are infected with *S*. Gallinarum often contain the organism after feeding and can be cultured. These samples should be cultured by direct inoculation of a selective enrichment broth such as selenite cysteine or selenite F, followed by plating on selective media such as brilliant green agar (Parmar & Davies, 2007; Proux *et al.*, 2002).

Both *S.* Pullorum and *S.* Gallinarum grow well in pure culture on non-selective media, but selective and enrichment media have been described that contain substances to inhibit the growth of extraneous organisms. *Salmonella* Pullorum may grow slowly and produce very small colonies on selective media so incubation of plates for 48 hours is recommended. The efficiency of recovery of *Salmonella* varies according to circumstances, and experience in the use of a medium is an important but unquantifiable factor. Some complex media may have an inhibitory effect on these organisms, so that it is advisable to use both non-selective and selective media for isolation from tissues. Both solid media and broths can be employed. As the toxic properties of selective media may vary, it is preferable to monitor these by comparing growth of control cultures on both types of medium. The inhibitory media should grow at least 75% of the colonies of the corresponding non-inhibitory medium (Ellis *et al.*, 1976; Mallinsen & Snoeyenbos, 1989)

All the media mentioned below are examples of commonly used media, but there are many others found to be equally satisfactory and it is recommended that the most suitable products are validated locally in relation to strains that are circulating in a particular region.

Non-inhibitory media include nutrient agar and blood agar, on which colonies are seen to be smooth, translucent, slightly raised, and about 1–2 mm in diameter. *Salmonella* Gallinarum grows more rapidly than *S.* Pullorum and produces larger colonies with a distinctive smell resembling that of seminal fluid on most media. Broths include buffered peptone water and nutrient and meat infusion broths or universal pre-enrichment broth.

1.1.1. Selective media

i) MacConkey agar

MacConkey agar is inhibitory to non-enteric organisms; it differentiates lactose fermenters (pink colonies) from non-lactose fermenters (colourless colonies). NaCl is omitted to limit the spread of *Proteus* colonies. *Salmonella* colonies are smooth and colourless. *Salmonella* Pullorum produces smaller colonies than other salmonellae. MacConkey is the agar of choice for direct plating from tissues.

ii) Xylose lysine deoxycholate agar

This agar is inhibitory to non-enteric organisms. *Salmonella* Pullorum grows sparsely as small red translucent colonies. *S.* Gallinarum colonies are small, dome-shaped, and may have a central black spot due to H_2S production, but this reaction may be delayed or variable.

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

iii) Brilliant green agar

Brilliant green agar (BGA) is inhibitory to coliforms and most *Proteus* strains; useful for distinguishing enteric organism colonies. Salmonellae form low, convex, pale red, translucent colonies of 1–3 mm in diameter, similar to *Citrobacter*. *Proteus* forms pin-point colonies, *Pseudomonas aeruginosa* appears as small red colonies, and lactose fermenters are green. *Salmonella* Pullorum produces smaller more pale colonies than other salmonellae. BGA is the agar of choice following enrichment, but low numbers of *Salmonella* can be easily overgrown by competing organisms when BGA is used for faecal and environmental samples.

iv) Brilliant green sulphapyridine agar

This agar is inhibitory to coliforms and *Proteus* strains. The sulphapyridine is added to stabilise selectivity in the presence of egg materials. *Salmonella* Pullorum produces small colonies.

Salmonella Pullorum and Gallinarum grow poorly and do not produce typical colonies on some newer chromogenic agars such as Rambach agar, but more recently developed chromogenic media with a wider detection range could be evaluated for suitability.

1.1.2. Liquid enrichment and selective media

i) Selenite cysteine and F broths

These broths are inhibitory to coliforms but not *Proteus* and are improved by addition of brilliant green. Loss of activity after 24 hours limits its use. Selenite cysteine broth is more stable and less inhibitory than selenite F broth, so is normally preferable (e.g. for hatchery-based monitoring of hatcher basket liners or meconium) except in the case of fresh faecal samples from mature birds where highly competitive flora may be present. Although selenite-based broths are considered to be preferable for isolation of *S.* Pullorum and *S.* Gallinarum from faeces by direct enrichment (Shivaprasad *et al.*, 2013), if there are difficulties with issues of toxicity or shelf life in particular laboratories the other enrichment broths mentioned below may be used. Most of these other broths are however designed to be used following a non-selective enrichment stage and *S*. Gallinarum and *S*. Pullorum are readily overgrown by competitor organisms in non-selective faecal culture resulting in false-negative tests. Direct selective enrichment is therefore recommended for faeces and intestinal or environmental samples. Non-selective enrichment may give better results for tissues obtained by aseptic post-mortem where there should be no competing organisms (Mallinson & Snoeyenbos, 1989).

ii) Tetrathionate/brilliant green broth

This broth is inhibitory to coliforms and *Proteus*, but may also inhibit some strains of *S.* Pullorum/Gallinarum.

iii) Rappaport–Vassiliadis soya (RVS) peptone broth

This broth is normally only used for selective enrichment following pre-enrichment but is more stable than selenite broth; use 1 part inoculum to 100 parts medium. *Salmonella* Pullorum and Gallinarum are more likely to be overgrown by other organisms during pre-enrichment of faeces or intestinal contents than salmonellae that are not host-adapted so direct enrichment with RVS may also be attempted.

1.2. Recovery of salmonellae

The methods for recovering *S.* Pullorum and *S.* Gallinarum vary according to the origin of the samples. Although their isolation from cloacal swabs and faeces may be unrewarding, examination of tissues taken at post-mortem is usually more successful. The methods are described.

1.2.1. Cloacal swabs and fresh faeces from live birds

Swabs dipped in nutrient broth are suitable; small swabs being used for young chickens. The swabs are streaked on selective media, and placed in enrichment broth. The plates and the broth are incubated at 37°C. Higher temperatures may be used with some broths, e.g. 41.5°C for Rappaport–Vassiliadis (RVS). Subcultures are made on to selective media after 24 and 48 hours.

1.2.2. Gall-bladder contents

Swabs of gall-bladder contents are streaked on to non-selective and selective agars and placed in inhibitory and non-inhibitory broths, followed by incubation at 37°C and subculture on to selective agar after 24 and 48 hours.

1.2.3. Organs and tissues

Swabs or segments of tissues are taken in an aseptic manner from individual tissues and lesions and cultured on to non-selective and selective media, and into similar non-selective and selective broths. These are incubated at 37°C and subcultured on to selective agar after 24 and 48 hours. Parallel incubation at a higher temperature, e.g. 40°C, can also be used to enhance the overall isolation rate. Carrier birds: larger amounts of material may be required to identify the carrier birds. The ovary and oviduct are the tissues of choice for *S*. Pullorum, and the liver, gall-bladder and caecal tonsils as well as ovary and oviduct should be tested for *S*. Gallinarum. In practice it is usually best to pool samples from a variety of tissues including the spleen, but intestinal tissues should not be pooled with other tissues. Tissues are homogenised in a small volume of broth and directly plated out. Approximately 10 ml of homogenate is also added to 100 ml of non-selective enrichment broth (e.g. buffered peptone water) and selective enrichment broth (e.g. selenite cysteine broth or brilliant green broth), and incubated at 37°C. These broths are subcultured on to non-selective and selective agar after 24 hours.

1.2.4. Alimentary canal, including the caecal tonsils and intestinal contents

After grinding or homogenisation in a small volume of broth, 10 ml of the homogenate is incubated in 100 ml of selective enrichment broth at 37°C. In general, better isolation is achieved with selenite cysteine broth.

1.2.5. Eggshells

The broken eggshells are placed in a tenfold volume of enrichment broth (e.g. selenite cysteine broth). The broth is incubated at 37°C and subcultured on to selective agar after 24 and 48 hours.

1.2.6. Egg contents

Aseptically taken contents of fresh eggs are homogenised and mixed with 200 ml of buffered peptone water or nutrient broth, incubated at 37°C, and subcultured on to non-selective and selective agar after 24 and 48 hours. Incubated eggs, whether infertile or containing small embryos, can be similarly treated.

1.2.7. Embryos

Homogenised viscera and swabs from the yolk sacs of well-developed embryos may be streaked on to non-selective and selective agar, one swab being placed in 10 ml of both non-selective and enrichment broth (e.g. selenite cysteine broth or brilliant green broth). Incubation is carried out at 37°C, and subcultures are made on to non-selective and selective agars after 24 and 48 hours.

1.2.8. Environmental samples

These include hatcher fluff, debris and macerated egg/chick waste samples and chick box liners or floor faecal or litter samples; 25 g is mixed with 225 ml of enrichment broth (e.g. selenite cysteine broth, brilliant green broth), incubated at 37°C, and subcultured on to selective agar after 24 and 48 hours.

Polymerase chain reaction (PCR) based tests may also be used to confirm the serovar, or its vaccinal status, but have not been fully validated internationally (Batista *et al.,* 2016; Kang *et al.,* 2012; Soria *et al.,* 2012).

1.3. Confirmatory procedures

Typical *S.* Gallinarum colonies on non-selective media are round, translucent, glistening, domed, smooth, and 1–2 mm in diameter after 24–48 hours' incubation. *Salmonella* Pullorum colonies are slightly smaller and translucent. On selective media their appearance varies with the medium, but suspect colonies can be investigated serologically by testing for 'O'9 somatic antigens, observing for motility and testing biochemically.

After incubation for 20–24 hours, the plates should be examined carefully for the presence of typical *S.* Pullorum and *S.* Gallinarum colonies. The plates should be re-incubated for a further 24 hours and examined again. For biochemical and serological confirmation from each plate, five typical or suspect colonies should be chosen for further examination. If there are fewer than five typical or suspect colonies, all of them should be tested. Selected colonies should be inoculated onto the surface of nutrient agar, in a manner that allows the growth of separate colonies. For biochemical confirmation, only pure cultures taken from non-selective media should be used. The following media should be inoculated using an inoculating loop: triple sugar iron (TSI) agar; lysine iron agar (or l-lysine decarboxylation medium); urea agar according to Christensen; tryptone/tryptophan medium for indole reaction; glucose with an inverted Durham tube for acid and gas production; dulcitol, maltose, ornithine decarboxylation medium and semisolid agar, for motility. The reactions shown in Table 2 occur.

Identification kits are commercially available for Enterobacteriaceae. Molecular tests using ribotyping techniques and PCR have been developed in research laboratories (Kang *et al.,* 2012), and can be used for confirmation and differentiation between *S.* Gallinarum and *S.* Pullorum.

For serological confirmation to serogroup level, colonies from non-selective media (nutrient or blood agar) are used. The first stage is elimination of autoagglutinable strains. For this, material taken from a single colony of pure culture is transferred to a glass slide and mixed with a drop of sterile saline. The slide is rocked gently or the drop stirred with a loop for 30–60 seconds and observed for agglutination against a dark background, preferably with the aid of a magnifying glass or dissecting microscope. If the bacteria have clumped into more or less distinctive units, the strain is considered to be autoagglutinable and must not be submitted for the following tests. If the bacterial sample is recognised as non-autoagglutinable, it is tested with a polyvalent 'O' (A–G) antiserum. For this purpose, the material from a single colony is dispersed in the drop of polyvalent 'O' antiserum on the glass slide to obtain a homogenous and turbid suspension. After gently rocking for 30–60 seconds, the reaction is observed against a dark background for agglutination. Alternatively the slide agglutination test may be carried out with smaller volumes of suspension under a dissecting microscope. In this case a portion of the colony to be checked is added to a loopful of saline on the microscope slide to produce a light suspension to check for autoagglutination ('rough strains'). If no agglutination takes place, one or two loops of antisera are added, the drop stirred with a loop and observed for agglutination. *Salmonella* Pullorum and *S.* Gallinarum should agglutinate with polyvalent 'O' antisera but not with polyvalent flagella (poly 'H' phase 1 and phase 2) antisera. If the reaction is positive, the single colony is tested further in the same manner using group-specific sera for *S.* Pullorum and *S.* Gallinarum serovar ('O'9 antiserum). After serogrouping, isolates may be sent to a reference laboratory for serotyping.

Table 2. *Biochemical investigation of* Salmonella *Pullorum and* S. *Gallinarum*

	Salmonella Pullorum	*Salmonella* Gallinarum
TSI glucose (acid formation)	+	+
TSI glucose (gas formation)	v	–
TSI lactose	–	–
TSI saccharose	–	–
TSI hydrogen sulphide	v	v
Gas from glucose (medium with Durham tube)	+	–
Urea hydrolysis	–	–
Lysine decarboxylation	+	+
Ornithine decarboxylation	+	–
Maltose fermentation	– or late +	+
Dulcitol	–	+
Motility	–	–

+ = 90% or more positive reaction within 1 or 2 days; – = No reaction (90% or more); v = Variable reactions.

It is also possible to confirm and differentiate *S.* Gallinarum by specific PCR (Kang *et al.,* 2012).

1.4. Test procedure for culture of visceral, faecal, intestinal and environmental samples for *S.* Pullorum and *S.* Gallinarum

i) Where possible, begin laboratory procedures on the same day as samples are collected.

ii) Homogenise the material as much as possible by manual mixing, gentle macerating or stomaching with a small volume of sterile saline if the material is dry.

iii) Stir the mixture with a small rectal swab or loop and streak thickly on to one-quarter of a brilliant green agar plate. (Swabs from uncontaminated tissues sampled in an aseptic manner can also be streaked on to blood agar.)

iv) From this deposit of material on the plate, streak the rest of the plate to obtain individual colonies.

v) Add 5–25 g of the homogenised sample to freshly made selenite cysteine broth (see note on liquid enrichment and selective media above) to make a 1:10 sample to broth ratio. Shake or stir to disperse the sample in the broth.

vi) Incubate the brilliant green agar plates and selenite cysteine broth at 37°C for 24 hours.

vii) Examine the plate after 24 hours' culture. Carry out agglutination tests on up to five suspect colonies with polyvalent 'O' (A–G) antisera and polyvalent H (phase 1 and phase 2) antisera. If agglutination is unclear subculture suspect colonies on to nutrient agar or blood agar and repeat tests after 24 hours' incubation of those media.

viii) If poly 'O' is positive then check with 'O'9 antiserum. If 'O'9 is positive and poly 'H' is negative, this is indicative of the possible presence of *S.* Pullorum or *S.* Gallinarum.

ix) If there are no positive colonies on the brilliant green agar plate, streak out a 10 µl loop of incubated selenite cysteine broth onto brilliant green agar as in step iv above.

xi) Incubate the brilliant green agar plates at 37°C for 24 hours and re-incubate the previous (negative) brilliant green agar plates and the selenite cysteine broths for a further 24 hours.

xi) Repeat examination of plates as in step vii above.

xii) If plates are still negative, re-plate from selenite cysteine broth and incubate brilliant green agar plate, that was inoculated in step ix, for a further 24 hours and examine as in step vii above.

xiii) Confirm *S.* Pullorum and *S.* Gallinarum using biochemical tests as shown in Table 2. Isolates can be sent to a *Salmonella* reference laboratory for confirmation of serotype and for further molecular typing if required for epidemiological purposes.

1.5. Molecular epidemiology

Standard molecular 'fingerprinting' techniques used for *Salmonella*, such as plasmid profile analysis, pulsed field gel electrophoresis PCR-restriction fragment length polymorphism (RFLP) or ribotyping can be used for investigating outbreaks of *S.* Pullorum or *S.* Gallinarum. It is often necessary to use combinations of such methods and different restriction enzyme combinations to obtain maximum discrimination because of a high level of clonality. The most effective techniques may also vary by country because of the nature of circulating clones in that region. High throughput whole genome sequencing has also been applied to *S*. Gallinarum, but is not yet available or economically viable in all countries (De Carli *et al*., 2016).

2. Serological tests

Serological tests are best applied as a flock test as results for individual birds will vary according to the stage of infection. It is therefore necessary to take sufficient individual samples to determine infection in the flock. The number of samples will depend on the expected prevalence and level of confidence desired (see Chapter 1.1.2 *Collection, submission and storage of diagnostic specimens* and Chapter 1.1.3 *Transport of biological materials*). If the test is to be used for detecting individual infected birds for culling, it should be repeated at least twice and preferably until the whole flock has given at least two negative tests.

The tests that are most readily applied include rapid whole blood agglutination, rapid serum agglutination (RST), tube agglutination and micro-agglutination (USDA, 1996). Other invasive *Salmonella* such as *S.* Enteritidis and *S.* Typhimurium or use of vaccination may lead to false-positive results in serological tests for *S.* Pullorum.

Both *S.* Pullorum and *S.* Gallinarum possess 'O' antigens 9 and 12 and may also possess O antigen 1 (Brooks *et al.,* 2008). However, in the case of *S.* Pullorum, there is a variation in the ratio of 12_1, 12_2 and 12_3; the standard strain contains more 12_3 than 12_2, while the reverse is true of the variant form. Intermediate forms also exist. (There appears to be no such form variation in the case of *S.* Gallinarum.) As this variation occurs, it is necessary

to use a polyvalent antigen in immunodiagnostic tests. The same antigen is used to detect both *S.* Pullorum and *S.* Gallinarum, but detection of the latter may be relatively poor (Proux *et al.,* 2002).

2.1. Rapid whole blood agglutination test

The rapid whole blood agglutination test can be used under field conditions for detecting both *S.* Pullorum and *S.* Gallinarum, and the reactors can be identified immediately. However, it is not reliable in turkeys or ducks as the test results in a significant proportion of false-positive results. Sera can be screened by rapid slide agglutination test and positive reactions confirmed by the more specific tube agglutination test. Chickens can be tested at any age, although some authorities specify a minimum age of 4 months (Shivaprasad *et al.*, 2013; USDA, 1996) and positive results from chicks less than 4 weeks of age may be due to maternal antibodies.

2.1.1. Preparation of stained antigen for the rapid whole blood or rapid serum agglutination test

Incubate one standard form strain of *S.* Pullorum (antigenic structure 9, 12_1, 12_3) and one variant form (antigenic structure 9, 12_1, 12_2) at 37°C and harvest separately until final mixing for the complete antigen.

Sow strains on to separate agar slopes, incubate at 37°C for 24 hours, emulsify growth with sterile normal saline and spread an inoculum over an agar plate to produce easily selected discrete colonies. For this the plates are incubated for 48 hours, a number of colonies are marked out and each is tested for agglutination on a slide with 1/500 acriflavine in saline. Smooth-phase colonies do not produce agglutination. Pick off typical colonies that do not produce any agglutination, seed on to agar slopes, and incubate for 24 hours. Emulsify the growth in saline and evenly distribute 2 ml over the surface of the medium (200 ml) in a Roux or similar flask. Incubate the flasks for 60 hours.

For harvesting the bacterial growth, flood the surface of each flask with approximately 10 ml sterile buffered formol saline, pH 6.5 (8.5 g/litre sodium chloride, 10 ml/litre neutral formalin, 4 ml/litre 0.5 M sodium phosphate: made up to 1 litre with distilled water, pH adjusted to 6.5 using 1 M orthophosphoric acid or 1 M sodium hydroxide), to give dense cell suspensions. Add 12–15 sterile glass beads of 3–5 mm diameter and rock the flasks until all the culture is in even suspension; leave in a vertical position for at least 15 minutes. Check the morphology and purity of the suspensions by preparing and examining Gram-stained films. Bulk the suspension from each flask containing the same strains. To each 100 ml of suspension, add 200 ml of absolute alcohol. Shake the mixture and allow to stand for 36 hours, or until precipitation is complete. Check the agglutinability of the standard and variant precipitate by first centrifuging a sample to separate the alcohol, which is removed, dilute with normal saline and test with a known positive and negative serum. If satisfactory, remove the clear supernatant alcohol (centrifugation at 2000 ***g*** for 10 minutes may be helpful for precipitation), and add sufficient phosphate buffered saline (PBS) containing 10% (v/v) glycerol to standardise the density to 75 × No. 1 Wellcome opacity tube (or 50 × tube No. 1.0 on the McFarland scale). Add equal volumes of standard and variant strains, and add 1% (v/v) of 3% (w/v) alcoholic crystal violet solution to the final mixture, and allow to stand for 48 hours at room temperature. Store in a tightly closed container at 0–4°C for up to 6 months. To assess safety, carry out a culture test on blood agar to confirm non-viability of the unwashed antigen before standardisation. Each bottle of antigen must be tested after alcoholic precipitation and before standardisation against standard titre antisera for *S.* Pullorum and *S.* Gallinarum, and against a negative serum. If possible, also test with known positive and negative serum and blood from positive and negative chickens.

Stained antigen products for the whole blood plate agglutination test are available commercially, and although there seem to be some slight differences in their sensitivity (Gast, 1997), it is unlikely that poultry flocks infected with the different variants of *S.* Pullorum would be missed.

2.1.2. Test procedure

i) Use a clean white tile marked into squares of about 3 × 3 cm. If a tile with 3 × 4 squares is used, up to 12 blood samples can be tested at the same time.

ii) Place 1 drop (about 0.02 ml) of crystal-violet-stained antigen in the centre of each square.

iii) Obtain a sample of fresh whole blood. This is conveniently done by making a stab of a wing vein using a needle with a triangular point.

iv) Place an equal size drop of fresh whole blood next to a drop of antigen.

v) Mix the drops of antigen and blood using a fine glass rod, which is wiped clean between samples.

vi) Use a gentle rocking motion to keep the drops agitated for up to 2 minutes. Several tests may be carried out simultaneously on the same tile, but the drops should not be allowed to dry out during this time. In very warm conditions, a smaller number of larger drops per plate may be required to avoid drying out.

vii) A positive reaction is indicated by easily visible clumping of the antigen within 2 minutes.

viii) A negative reaction is indicated by absence of clumping of the antigen within 2 minutes.

ix) Include known positive and negative control sera on each testing occasion, using them in the same way as the blood.

x) On completion of a set of tests, the tile is washed and dried, ready for further use.

In the absence of positive reactions, any inconclusive reactions can only be interpreted in the light of the previous *Salmonella* testing history of the flock. Where there are positive reactors, any doubtful reactor should be regarded as positive. Also, recently infected birds may not show a typical positive reaction until they are retested after 3–4 weeks.

2.2. Rapid serum agglutination test

The RST is performed in the same manner, except that serum is substituted for whole blood. For export test purposes an initial screening of sera by RST followed by confirmation of positives by the tube agglutination test is the optimal approach. Ideally serum samples tested by any method should be tested within 72 hours of collection as nonspecific reactions may increase in older samples. Fresh samples can be frozen for later testing if a delay is unavoidable.

2.3. Tube agglutination test

Fresh serum from chickens, turkeys or other birds is used at an initial dilution of 1/25, obtained by mixing 0.04 ml of serum with 1.0 ml of antigen[3]. Positive and negative control sera are included in each test. The antigen is prepared from unstained *S.* Pullorum or *S.* Gallinarum cultures diluted to a concentration of No. 1 on the McFarland scale (as described above). The mixture is incubated at 37 or 50°C for 18–24 hours before reading. A positive reaction consists of a granular white deposit with a clear supernatant fluid; a negative reaction shows uniform turbidity. Samples positive at a dilution of 1/25 are retested at a higher range of dilutions and a titre of 1/50 is usually considered to be positive, although this figure seems to vary in the literature. In many cases a single dilution of 1/50 is used but this may fail to detect some flock infections if only small numbers of samples are taken.

2.4. Micro-agglutination test

This resembles the tube agglutination test, but requires much smaller volumes of reagents. The test is performed in microtest plates. Sera are first diluted by adding 10 µl of serum to 90 µl of normal saline, and then adding 100 µl of previously standardised stained microtest antigen to give a final dilution of 1/20. By titrating the serum in doubling dilutions and adding an equal volume of standardised stained antigen, an end-point (titre) can be obtained. The plates are sealed and incubated at 37°C for 18–24 or 48 hours. A positive reaction consists of a fine diffuse precipitation, whereas a negative reaction shows a button-like precipitate. Titres of 1/40 are usually considered to be positive but this test is more liable to produce false-positive results with turkey sera.

Other serological tests include micro-antiglobulin (Coombs), immunodiffusion, haemagglutination and enzyme-linked immunosorbent assay (ELISA).

ELISA techniques have been described for detecting antibodies to *S.* Pullorum and *S.* Gallinarum (Oliviera *et al.,* 2004). The indirect ELISA using lipopolysaccharide antigen is likely to be the most sensitive and specific serological flock test for *Salmonella*, including *S.* Gallinarum and *S.* Pullorum. It is relatively easy to perform with serum or yolk, and can be used for quantifying the titre of antibody (Barrow, 1992; 1994). No commercial ELISA kits for *S.* Pullorum and *S.* Gallinarum are currently available, but an indication of likely infection can often be obtained using a lipopolysaccharide (LPS)-based commercial ELISA for *S*. Enteritidis; these tests have not been validated for this purpose.

3 For preparation of small volumes of somatic antigens see chapter 3.9.8.

C. REQUIREMENTS FOR VACCINES

1. Background

1.1. Rationale and intended use of the product

Although both live and inactivated vaccines have been prepared for use against *S.* Gallinarum (Paiva *et al.,* 2009), the vaccine most widely used is made from the rough 9R strain (Harbourne *et al.*, 1963; Revolledo & Ferreira, 2012). It is normally only employed in chickens. The number of viable organisms per dose is important; these organisms can survive in vaccinated birds for many months and may be transmitted through the egg (and perhaps from bird to bird). Vaccination may reduce flock losses, but will not prevent infection with field strains. In addition, vaccination with 9R may sometimes precipitate high mortality in infected birds (Silva *et al.*, 1981), and may stimulate the production of transient antibodies. It is usual to vaccinate at 8 weeks and again at 16 weeks of age. Antimicrobials should be avoided before and after vaccination.

Currently available vaccines, however, have only a minor role to play in the control of fowl typhoid as they offer short-lived protection against clinical disease and limited or variable protection against infection. Autogenous or locally produced vaccines can also be used to control clinical disease, but care must be taken to avoid strain instability leading to reversion to virulence (Okamoto *et al.*, 2010; van Immerseel *et al.,* 2013). Control can best be achieved by biosecurity, hygiene, good management, monitoring and removal of infected flocks, although the routine vaccination against *S*. Enteritidis and *S*. Typhimurium that is carried out in breeding and laying hen flocks in many countries may be partially protective against introduction of *S*. Gallinarum (Lee, 2015). Commercially available 9R vaccines have been used for reduction of *S.* Enteritidis in laying flocks in some countries but may be prohibited or are not commercially available in some countries where fowl typhoid is not present (Lee *et al.*, 2005). Even in countries with fowl typhoid, use of vaccine may complicate control as it does not prevent infection, only reduce clinical disease and allow production to continue from infected flocks. It is therefore preferable to aim for eradication of the organism rather than acceptance of on-going disease, but this is often not economically viable in large multi-age holdings as eradication of red mites is necessary to ensure continued freedom from infection (Wales *et al.,* 2010).

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 in 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. It is recommended that a validated commercial vaccine is used unless there is no alternative because of the need to maintain quality and avoid risk associated with reversion to virulence. Live vaccines must also be bacteriologically distinguishable from field strains or surveillance and control programmes may be compromised. Observations from some countries suggest that it is not always straightforward to distinguish between *S*. Gallinarum vaccines and field strains (van Immerseel *et al.,* 2013). There will inevitably be some interference with serological monitoring for *S*. Gallinarum and potential interference with serological monitoring for *S*. Enteritidis, unless a stepwise approach is used in which a sensitive LPS-based ELISA is used to test for antibodies to O9 antigens and positive sera are further tested with a flagella antigen ELISA, which will give a negative reaction in cases of *S*. Gallinarum infection (Shivaprasad *et al.,* 2013). Recent work on the molecular mechanisms of infection should lead to the development of improved vaccines in future (Barrow *et al.,* 2012).

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 and/or genetic markers, preferably using whole genome sequencing. Live vaccinal strains should be marked by stable characters allowing easy 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 whole genome sequencing.

Live fowl typhoid vaccine is a suspension of suitably attenuated living organisms of a rough strain of *S.* Gallinarum, e.g. 9R. The organisms in the vaccine give the biochemical reactions characteristic of *S.* Gallinarum. Colonies of a 24-hour culture prepared from the vaccine on nutrient agar plates are rough when examined by the acriflavine slide test. The culture should not produce any smooth colonies or contain the somatic antigens characteristic of the smooth forms of *S.* Gallinarum.

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 Gram stain.

b) Homogeneity of culture on non-selective media.

c) Metabolic requirements as indicated by biochemical tests.

d) Detection of phenotypic and/or genetic markers.

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 chickens 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 periods in vaccinated animals or be transmitted to meat or eggs that may be consumed, and the method of application should not present a hazard to operators. In the case of *S*. Gallinarum vaccine at least six healthy, susceptible (preferably specific pathogen free [SPF]) chickens, 8–16 weeks of age, are each injected subcutaneously with a ten-fold dose of vaccine, and are observed for at least 7 days; no local or systemic reaction should develop.

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. In the case of *S*. Gallinarum 9R vaccine or similar vaccines, at least fifteen healthy chickens, 8–16 weeks of age, of a brown layer hybrid breed, and taken from a stock that is free from *S*. Pullorum infection, are each injected subcutaneously with a quantity of vaccine corresponding to one field dose, i.e. 5×10^7 viable organisms. After an interval of 21–28 days, the vaccinated chickens and an equal number of similar unvaccinated chickens are deprived of food for approximately 18 hours. The chickens are then challenged by oral administration of 1 ml of a broth suspension containing 5×10^7 organisms of a virulent strain of *S*. Gallinarum mixed with 300 mg of a powder consisting of chalk (40%), light kaolin (43%) and magnesium trisilicate (17%). All the chickens are observed for 14–21 days. The vaccine passes the test if at the end of this period the number of surviving vaccinated chickens that show no macroscopic lesions of fowl typhoid at post-mortem exceeds by eight or more the number of similarly defined control chickens.

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, litter or dust 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 minimal media may enhance LPS antigen production by the vaccine strain. In the case of *S.* Gallinarum (9R), the vaccine may be prepared by inoculation of a suitable medium, such as peptone broth, with a fresh culture of *S.* Gallinarum (9R) and incubation at 37°C for 24 hours, with agitation. 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 either case, the suspension is diluted in PBS solution, pH 7.0, and may be freeze-dried. The dose used per bird is between 5×10^6 and 5×10^7 organisms.

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 multi-dose 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 and/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 may be made of tissue reactions at the injection site.

Safety tests in target animals are not required by many regulatory authorities for the release of each batch. Where required, standard procedures are generally conducted using fewer animals than are used in the safety tests required for the relevant regulatory approval.

iii) Batch potency

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

2.3. Requirements for authorisation

2.3.1. Safety requirements

Certain killed vaccines may occasionally cause reactions in vaccinated animals because of their LPS content or the adjuvant used, and likewise live vaccines should be used with caution in animals that are not completely healthy at the time of vaccination. It is often necessary, however, to vaccinate flocks in the face of clinical fowl typhoid. 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 shed by vaccinated animals. As *S*. Gallinarum and *S*. Pullorum are host specific, non-target species are less of a concern.

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 whole genome sequencing. 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). Special care should also be taken to ensure that attenuated vaccines are not incompletely attenuated or contaminated with seed organisms.

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. The vaccine should provide protection throughout the laying period, and this can be measured by potency (efficacy) tests at

stages during lay. A booster dose during lay may be required, but live vaccines should not be used during lay in flocks providing eggs for human consumption.

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. Orally administered vaccines may produce more variable protection, particularly in the case of labile vaccines that are administered in drinking water. Vaccination of day old chicks by coarse spray may be beneficial where there is early challenge, and programmes that combine live and killed vaccines may provide superior protection. It should be remembered however, that a strong challenge such as that associated with continually occupied farms or infected wild birds and mite populations 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 accuracy of injection with killed and live injectable vaccines. The *Salmonella* vaccines are intended to limit the extent of clinical disease in poultry, and also to reduce the risk of introduction of infection to flocks. 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 and injected 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 flocks but can increase the threshold for infection, reduce the level of shedding 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.

2.3.3. 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.

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NB: FIRST ADOPTED IN 1991; MOST RECENT UPDATES ADOPTED IN 2018.