

***Salmonella* genus**

Laboratory case definition

The Public Health Laboratory Network (PHLN) has developed standard case definitions for the diagnosis of key diseases in Australia. This document contains the laboratory case definition for *salmonella*.

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1 PHLN Summary Laboratory Definition

Introduction

The genus *Salmonella* is comprised of two species: *Salmonella bongori* and *Salmonella enterica*. *S. enterica* is further divided into six subspecies: *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV), and *indica* (VI) which include over 2,600 recognised serotypes.

There are two distinct syndromes caused by *Salmonella*:

- Typhoid and paratyphoid fever (enteric fever)
- Salmonellosis (gastroenteritis)

These syndromes will be discussed separately in this document since there are differences in clinical manifestations and laboratory diagnosis.

1.1 Typhoid and Paratyphoid

The causative organism of Typhoid fever is *Salmonella* Typhi and of Paratyphoid fever are *Salmonella* Paratyphi A (SPA), *Salmonella* Paratyphi B (SPB) and *Salmonella* Paratyphi C (SPC).

Typhoid and Paratyphoid are common in the developing world but rare in Australia. In 2018, the number of *S. Typhi* and Paratyphi (A and B) notified to the National Notifiable Diseases Surveillance System (NNDSS) were 175 and 81, respectively². The majority of cases are related to recent travel to endemic areas or contact with travellers⁷.

Typhoid fever typically presents with a sustained debilitating high fever, headache and a serious bloodstream infection without diarrhoea. Illness is milder in young children, where it may manifest as nonspecific fever. Humans are the only reservoir for *S. Typhi*, indicating this serotype has adapted to the human host; asymptomatic carriers have been noted. Typhoid fever typically has a low infectious dose ($<10^3$ organisms) and a long, highly variable incubation period (1 to 6 weeks)³. It is transmitted through person-to-person contact or faeces-contaminated food and water.

Paratyphoid fever manifests as a syndrome similar to typhoid fever.

Serotype Paratyphi B is a diverse serotype that is associated with both paratyphoid fever and gastroenteritis⁴. The two pathovars are typically differentiated on the basis of the ability to ferment tartrate conferred by presence/absence of an underlying genetic mutation. Isolates causing paratyphoid fever are tartrate negative. Isolates causing gastroenteritis are tartrate positive and are referred to as *Salmonella* Paratyphi B variant L(+)-tartrate + or *Salmonella* Paratyphi B variant Java (S. Java)⁵. SPC is very rarely seen in Australia.

1.2 Gastroenteritis/Salmonellosis

Gastroenteritis is caused by salmonellae other than *S. Typhi* and the Paratyphis, which are referred to as non-typhoidal *Salmonella* (NTS).

NTS usually cause intestinal infections (accompanied by diarrhoea, fever, and abdominal cramps) that often last 1 week or longer⁶. Less commonly, extraintestinal NTS infections occur (e.g., bacteraemia, urinary tract infection, or osteomyelitis), especially in immunocompromised persons. Persons of all ages are affected, but the incidence is highest in infants and young children.

In 2017, the number of Salmonellosis cases notified to NNDSS was 16,414; of salmonellosis cases notified to the National Enteric Pathogens Surveillance Scheme (NEPSS), 4049, 133, 105 and 27 were from faeces, urine, blood and unusual sites respectively⁷.

Salmonella is ubiquitous in animal populations, and human illness is usually linked to consumption of contaminated foods. Salmonellosis is also transmitted by direct contact with animals, by water, and occasionally transmitted person-to-person.

2. Laboratory Diagnosis/Tests

2.1 Collection, Transport, and Storage of Specimens

Faeces or a rectal swab, blood, urine, and sterile site swabs collected into an appropriate sterile container are suitable samples for the isolation of *Salmonella* (food and water may also be sent for culture in the event of a suspected outbreak, however, this is beyond the scope of this document).

Faecal specimens should be collected in the early stages of any enteric illness (preferably within 4 days of onset), when pathogens are usually present in high numbers and before antimicrobial therapy has commenced. Whole stools are usually the specimen of choice. Collection of multiple specimens may enhance the recovery rate of *Salmonella*², particularly of *S. Typhi* and *S. Paratyphi*.

Faecal specimens should be transported to the laboratory as soon as possible, preferably in less than 4h³. If delayed longer than 4h keep at 4°C or store at 4°C for up to 24 h before transport⁴. The specimen size should be at least 5g, as bacterial, viral and parasitological examination may all be required.

Rectal swabs should be placed into commercially available transport media (e.g. Cary-Blair, Stuart or Amies) and transported to the laboratory as soon as possible or can be stored at room temperature for 24h⁴.

Ideally, faecal specimens should be examined as soon as they are received in the laboratory but if not processed immediately they should be refrigerated.

2.2 Direct Examination of *Salmonella* species

2.2.1 Macroscopic

Stool specimens are inspected macroscopically and the presence of mucus and/or blood is noted and recorded.

2.2.2 Microscopic

Salmonella cannot be distinguished from other Gram-negative rods by microscopy or staining methods.

2.3 Culture Independent Diagnostic Testing (CIDT)

CIDTs are able to detect the presence of an organism without the need for culture, either via molecular methods, i.e. amplification and detection of *Salmonella* specific target genes (real-time PCR) or by serological enzyme immunoassays (EIA). There are a number of commercial systems available for the detection of *Salmonella* in clinical specimens with the majority of the assays detecting a panel of bacterial and/or viral and parasitic pathogens including *Salmonella*. Molecular based systems include LightMix Multiplex Gastrointestinal Panel (Tib-Molbiol, Genova, Italy and Roche, Mannheim, Germany), xTAG Gastrointestinal Pathogen Panel (Luminex Molecular Diagnostics, Inc., Toronto, Ontario, Canada), ProGastro SSCS (Hologic Gen-Probe, Inc., San Diego, CA), FilmArray Gastrointestinal Panel (BioFire Diagnostics, Salt Lake City, UT), Faecal Pathogen M (AusDiagnostics, Mascot, Australia) and BD MAX Enteric Bacterial Panel (Becton Dickinson and Co., Franklin Lakes, NJ).

CIDTs are often fast, easy to perform and reliable. However, they are generally unable to identify serotypes or genotypes that are used to link cases in an outbreak, or to provide antimicrobial susceptibility for treatment⁵.

Maintaining concurrent, reflexive *Salmonella* culture alongside the use of CIDTs remains of public health importance. Isolation of *Salmonella* is essential for serotyping and subtyping, antimicrobial resistance testing and the further characterisation such as whole genome sequencing required for outbreak/cluster investigations and national surveillance.

2.3.1 Test Sensitivity and Specificity

Published findings from the initial evaluations of two multiplex enteric pathogen panels have been promising with respect to their reported overall sensitivities and specificities compared to conventional methods⁶. Individual sensitivities and specificities of the xTAG panel for *Salmonella* were 82.7 to 92%, respectively, and of the ProGastro SSCS panel for *Salmonella* were each 100%¹².

2.3.2 Predictive Values

Published data for the BD MAX Enteric Bacterial Panel in a North American study (Becton Dickinson and Co., Franklin Lakes, NJ) report PPV of 97.1% and NPV of 99.2% for *Salmonella spp.*⁷.

2.3.3 Test Acceptance

Internal sample process control and external positive and negative control results observed are as expected, and reaction is not inhibited.

2.3.4 Suitable External Controls

Positive control: *Salmonella* positive sample (e.g. stool)

Negative: *Salmonella* negative sample

2.3.5 Validation

CIDTs used to diagnose Salmonellosis for the purpose of patient management must be registered with the Therapeutic Goods Administration (TGA) and comply with in-vitro diagnostic (IVD) validation criteria.

Laboratory verification of IVDs includes testing of positive and negative samples to assess sensitivity and specificity and demonstrate the limit of detection (LOD) meet the manufacturer's specifications.

2.3.6 External Quality Control

(Simulated) Clinical Specimens: Royal College of Pathologists of Australia (RCPA) Quality Assurance Programs Pty. Limited.

2.4 Isolation Methods of *Salmonella* species

Salmonellae are classed as non-fastidious organisms, however, most faecal specimens collected for testing contain a complex community of bacteria. Therefore, it is necessary to use selective media which inhibit growth of many of the commensal bacteria and also differentiate between *Salmonella* and other enteric organisms by their characteristic appearance. In addition, variation between the serovars of *Salmonella enterica* requires the use of more than a single medium.

2.4.1 Enrichment

Maximal recovery of *Salmonella* from faecal/rectal specimens is obtained by using an enrichment broth, although isolation from acutely ill persons is usually possible by direct plating of specimens. Enrichment broths for *Salmonella* are usually highly selective and inhibit certain serotypes of *Salmonella*, particularly *S. Typhi*. The selective enrichment medium most widely used to

isolate *Salmonella* from faecal specimens is selenite enrichment medium (SEL). Specimens that might contain *Salmonella* strains inhibited by SEL, e.g. *S. Paratyphi* A, should be plated directly or cultured in a nonselective enrichment broth (e.g. Gram-negative broth). Inoculate (approximately a pea-size amount of) faeces into selective broth and swirl the swab in broth to emulsify; incubate the broth at 35-37°C.

2.4.2 Faecal Inoculum

Remove a small amount of faeces from the specimen container with a sterile swab and spread the faecal material onto the selective agar plates, to prepare the primary inoculum, covering an area equivalent to a quarter to a third of the surface of the agar. Spread the primary inoculum with a sterile loop to obtain single colonies; incubate the plates at 35-37°C.

Faeces may be diluted 1:10 into an appropriate diluent, e.g. PBS prior to inoculation (if necessary, homogenise suspension using mechanical means e.g. vortex). Transfer one drop onto the selective agar plates and 1mL to the enrichment/selective broth.

It has been shown that dilution significantly reduces the amount of competing flora without compromising low numbers of pathogens¹³.

2.4.3 Plating Media

Many differential plating media are available for isolation of *Salmonella* from faecal specimens and vary from slightly to highly selective (refer to Table 1).

An important principle is to use more than one plating medium, as individual strains may grow poorly on certain media³.

Table 1. Selective Level of Media for *Salmonella* Isolation and H2S detection

Selective Level	Media	Abbreviation	H2S indicator system
Low (Slightly)	MacConkey	MAC	
	Eosin methylene blue	EMB	
Intermediate	Xylose-lysine-desoxycholate	XLD	yes
	Desoxycholate citrate agar	DCA	
	Salmonella Shigella agar	SS	

Selective Level	Media	Abbreviation	H2S indicator system
	Hektoen enteric agar	HE	yes
High	Bismuth sulphite agar*	BSA	yes
	Brilliant green agar	BGA	

*BSA inoculation may be used if enteric fever is suspected.

Chromogenic media are both selective and differential; they inhibit non-target organisms (e.g., using antibiotics or other inhibitors) and utilize synthetic chromogenic enzyme substrates in order to specifically target pathogens based on their enzyme activity which grow as coloured colonies due to their metabolism (usually by hydrolysis) of one or more chromogenic enzyme substrates (refer to Table 2).

Plates and broths for *Salmonella* are incubated aerobically at 35-37°C for 18-24h, except BSA plates are incubated for 48h, and are examined for typical colonies at 18-24h (and 48h for BSA).

A loopful (10µL) of selective enrichment broth medium are plated onto selective agar and incubated 35-37°C for 18-24h.

Plates are examined for typical colonies (refer to Table 2). A representative colony can be selected and MALDI-TOF performed. If MALDI-TOF is not available, a representative colony may be picked using a straight wire and used to inoculate biochemical tests. The straight wire colony pick may be emulsified into 1mL sterile saline and the suspension used as the inoculum for biochemical tests (refer to Section 2.5.3).

Table 2. Appearance of Typical *Salmonella* (and non-*Salmonella*) on Selective Media

XLD	NLF, non-spreading, red colonies with black (H ₂ S producing) centres (non-H ₂ S producing <i>Salmonella</i> are known) <i>Salmonella</i> (typical): differentiated from other organisms as xylose fermenters, and lysine decarboxylation and H ₂ S production <i>Salmonella</i> exhaust the small amount of xylose in the medium and decarboxylate the lysine turning the pH alkaline lactose and sucrose in the medium allows differentiation from coliforms which ferment these turning the pH acid and form yellow opaque colonies <i>S. Typhi</i> : small (trace) amount H ₂ S-production LF <i>Salmonella</i> : often ssp. III and dairy environment isolates SPA (typical): non H ₂ S-producing (rare small to medium amount H ₂ S-production); does not decarboxylate lysine; may not grow or be recognised
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DCA	18h: colourless, slightly opaque and with a central grey dot including <i>S. Typhi</i> at 48h (no dot at 18h) <i>Salmonella</i> : usually dome-shaped; <i>S. Typhi</i> : conical but flatter LF bacteria form pink colonies
BSA	18h: green/clear mucoid, black (H ₂ S-producing) rabbit eye colonies surrounded by a black zone and a characteristic metallic sheen 48h: uniformly black useful for <i>S. Typhi</i> and LF colonies medium has short shelf life use within 3 days of preparation
MAC	NLF colonies (require confirmation: biochemical or other tests) (9)
Salm CA (Oxoid)	magenta (or blue) 1.0mm diameter colonies, smooth and raised
BBL CA Salm	<i>Salmonella</i> : blue-violet or purple colonies. <i>Citrobacter</i> and other coliforms: light blue-green to blue-green colonies. Organisms that do not hydrolyse any of the chromogenic compounds: colourless colonies.
Brilliance Salm Agar	<i>Salmonella</i> : purple colonies; <i>Escherichia coli</i> : inhibited

Salm, *Salmonella*; *CA*, chromogenic agar; *H₂S*, hydrogen sulphide

No single culture medium or combination of media can preclude the necessity for enrichment of stool samples, e.g. in selenite broth, which is essential for detection of low numbers of *Salmonella* and significantly enhances detection¹⁴.

2.4.4 Test Sensitivity

Factors that may affect the recovery of salmonellae include: (i) quality of the specimen, (ii) type of media used, (iii) number of organisms present, (iv) time between the onset of diarrhoea and collection of the sample, (v) time between collection and culture of the specimen and (vi) antibiotic treatment.

Statistical data is not available. Most studies only deal with a known number of specimens, particular media used and then only comment on how each medium fared with regard to isolation of *Salmonella* species. The number of false negatives is not known.

It has been consistently demonstrated that chromogenic media do not offer a superior sensitivity to conventional agars such as XLD agar and Hektoen enteric agar^{14, 15}. Furthermore, in contrast to almost all chromogenic media, such conventional agars offer the opportunity to isolate both *Salmonella* and *Shigella* using a single culture medium.

2.4.5 Test specificity

The isolation of a *Salmonella*-like organism confirmed by both specific biochemical and screening serological techniques or by matrix-assisted laser desorption-ionization-time-of-flight mass spectrometry (MALDI-TOF MS) systems indicate *Salmonella* spp.

The isolation of a *Salmonella* is always significant.

The sole advantage of chromogenic media for *Salmonella* is the significantly higher specificity they offer compared to conventional media; fewer confirmatory tests than with conventional media are required to investigate colonies of other species that may resemble *Salmonella*.

The use of a chromogenic medium after enrichment in selenite broth is an attractive option to target *Salmonella* spp. with high specificity and consequently reduce the number of colonies requiring investigation. There is no clear advantage of any particular chromogenic medium for this purpose¹⁶.

2.4.6 Predictive values

A negative culture does not exclude the diagnosis of salmonellosis.

A positive PCR test cannot differentiate between viable or non-viable organisms, nevertheless, detection of *Salmonella* is significant, particularly where clinical signs and symptoms are suggestive of salmonellosis.

2.4.7 Suitable acceptance criteria

Colonies exhibit typical, therefore acceptable, characteristics on selective media.

2.4.8 Suitable internal controls

A relevant and well-documented quality control program must be implemented for each type and batch of medium used. Incorporation of a control *Salmonella* with each batch of cultures is not recommended due to the risk of cross contamination with the test samples or organisms. Where a control *Salmonella* is used, the serovar selected must be extremely rare e.g. S. Hofit, therefore, identification of this rare serotype should alert the reference laboratory to a possible cross-contamination event.

2.4.9 Suitable test validation criteria

Isolation of a *Salmonella* species, confirmed by both biochemical and serological parameters is the gold standard.

MALDI-TOF systems, PCR and WGS can be used to identify suspect *Salmonella* colonies.

2.4.10 Suitable external QC programme

(Simulated) Clinical Specimens: Royal College of Pathologists of Australia (RCPA) Quality Assurance Programs Pty. Limited.

2.4.11 Special considerations

XLD may be slightly inhibitory to strains of SPA, therefore, a medium other than XLD is recommended for optimal isolation of SPA.

2.5 Identification of *Salmonella* species

There are two levels of *Salmonella* identification:

1. Clinical laboratories identify the *Salmonella* on the basis of colony morphology, biochemical parameters and/or MALDI-TOF MS and may perform preliminary serology.
2. Reference laboratories, including state and Australian reference laboratories, confirm *Salmonella* using biochemical identification, complete serological identification, perform other specific subtyping e.g. *S. Paratyphi* B biotyping, and molecular typing including *S. Typhimurium* MLVA typing and/or whole genome sequencing (refer to Section 2.8).

2.5.1 Screening Procedures

A latex agglutination kit is available for screening for *Salmonella* in SEL (Wellcolex Color *Salmonella*; Remel, Inc., Lenexa, KS). This kit can also be used to screen individual colonies from primary plates. This technique only identifies common *Salmonella* serogroups, it does not give a complete serological identification i.e. serotype. Another latex test (Oxoid *Salmonella* Test Kit, Thermofisher, UK) is also available; this is an agglutination test for the presumptive identification of *Salmonella* spp. It can be used to screen presumptive *Salmonella* colonies isolated on selective agar plates, from both food and clinical samples. The test allows the user to presumptively identify and confirm the presence of *Salmonella* spp. At the same time, other species such *Citrobacter* spp. and *Proteus* spp. can be more rapidly eliminated. The method is not appropriate for the detection of non-motile strains of *Salmonella* spp.

Note: Isolates must be sent to a designated reference laboratory for complete serological identification.

2.5.2 Presumptive Identification of *Salmonella* species

Clinical laboratories may issue a preliminary report of *Salmonella* when an isolate is positive either with *Salmonella* O group antisera or by other phenotypic identification methods.

An isolate is confirmed as *Salmonella* when:

- phenotypic identification has been completed and the specific O serogroup has been determined, or
- the MALDI-TOF MS identification is *Salmonella* sp., or
- specific *Salmonella* PCR is positive.

An isolate is confirmed as *Salmonella* Typhi when:

- biochemical reactions are as expected (Table 3)

- antigenic formula has been determined as O=9 (Group D), Vi and H1=d

An isolate is confirmed as *Salmonella* Paratyphi A when:

- biochemical reactions are as expected (Table 3)
- antigenic formula has been determined as O=2 (Group A), H1=a (and H2-1,5)

2.5.3 Phenotypic Identification

2.5.3.1 Suitable specimen

A pure culture on non-selective solid medium.

2.5.3.2 Biochemical Test Media

Various specific biochemical substrates. Biochemical identification of isolates is performed using pure cultures suspected isolates using:

- traditional selected biochemical tests e.g. TSI for H₂S production
- API 20E plate system (Biomérieux, France)
- Vitek® GN (Gram negative) Identification cards (Biomérieux, France)
 - which rapidly and accurately identify *Salmonella* species. and
 - importantly can differentiate *S. Typhi* and *S. Paratyphi A* from NTS.

Suspect colonies from one of the differential plating media mentioned above can be identified phenotypically as *Salmonella* spp. by use of traditional media in tubes or commercial identification systems. However, *Salmonella* is a diverse group, and phenotypically atypical strains are not uncommon; commercial identifications systems sometimes misidentify *Salmonella*³.

A representative colony is picked using a straight wire used to inoculate biochemical tests. Alternatively, the straight wire colony pick may be emulsified into 1ml sterile saline and the suspension used as the inoculum for biochemical tests.

Suspect colonies may be inoculated onto a screening medium such as Kligler's Iron Agar (KIA) or Triple Sugar Iron Agar (TSI). On KIA or TSI, most *Salmonella* strains produce an alkaline slant, indicating that only glucose is fermented, with gas and H₂S. On these media, *S. Typhi* isolates characteristically produce an alkaline slant but do not produce gas, and only a small (trace) amount of H₂S will be visible at the site of the stab and in the stab line. Also, laboratories may overlook SPA due to its atypical phenotypic profile (H₂S negative and lysine decarboxylase negative; refer to Table 3) which can be confused with that of (inactive) *E. coli*.

Phenotypic identification is commonly combined with serogrouping or serotyping for culture confirmation. Clinical laboratories should consider that an isolate is confirmed as a *Salmonella* when both determination of O serogroup and biochemical identification have been completed³. Preliminary flagella serology such as a poly "H" screening may also be performed.

If the phenotypic traits for a particular isolate are not characteristic of *Salmonella* but *Salmonella* antigens are found, the cultures should be plated to obtain a pure culture, tested with a complete set of phenotypic tests, or forwarded to a reference laboratory.

2.5.4 Identification Using Mass Spectrometry

MALDI-TOF Mass Spectrometry (MS) has emerged as a rapid, accurate means of identifying many bacteria, offering substantial cost and time savings over phenotypic methods. The two main platforms are the Bruker Biotyper (Bruker Daltonics, Germany) and Vitek® MS, (Biomerieux, France).

MALDI-TOF MS appears to identify an isolate as *Salmonella*, and has shown to correctly discriminate *S. Typhi* from other serotypes and to identify some *Salmonella* to the serotype level (possible for at least some serotypes although such applications are still in the research phase)¹⁰.

2.5.5 Molecular identification

Specific PCR assays can be used to confirm *Salmonella* isolates e.g. *invA* gene or WGS and bioinformatic techniques such as k-mer identification can be applied for *Salmonella* identification; furthermore, the read data is available for additional analyses such as *in silico* serotyping, detection of resistance genes/mutations and phylogeny studies.

2.5.6 Test sensitivity

Depends on the biochemical tests performed.

Production of lysine decarboxylase and H₂S, no fermentation of lactose, no production of urease are good parameters to identify a *Salmonella* species.

Most salmonellae are motile¹⁸.

Comparison of sensitivity, specificity and predictive values of each test system as well as between systems is too complex to discuss in this document.

2.5.7 Test specificity

Not all salmonellae exhibit the same biochemical properties. Typical properties and notable exceptions are shown in Table 3.

Table 3. Biochemical properties for differentiation of *Salmonella*: Non-Typhoidal (ssp. *enterica*), Typhi and Paratyphi A

Biochemical Test	Non-typhoidal <i>S. ssp. enterica</i> (I)	<i>S. Typhi</i>	<i>S. Paratyphi A</i>
H ₂ S production	+	+weak (b)	–c
gas production (a)	+	–	+
lysine decarboxylase	+	+	_d
ornithine decarboxylase	+	–	–
dulcitol	+	–	+
lactose	–e	–	–

*Extract from Reference 5.

Comprehensive differential biochemical characteristics of *Salmonella* species and subspecies can be found in References 5 and 6.

Notes:

- 1% glucose or 1% mannitol peptone water
- S. Typhi* may only produce small amounts of H₂S i.e. weak or trace.
- H₂S production may be slow i.e. > 24 hr. Some strains of *S. Typhi* may fail to produce detectable H₂S in KIA or TSI.
- S. Paratyphi A* rarely produce H₂S
- S. Paratyphi A* does not decarboxylate lysine.
- Salmonella* from dairy factory environments may ferment lactose.

In 2018, of 4,609 isolates submitted to an enteric reference laboratory as "*Salmonella* species", 8 (0.002%) isolates were confirmed as NOT *Salmonella* (MDU PHL, unpublished data).

2.5.8 Predictive values

Statistical estimates not available.

An isolate which biochemically resembles a *Salmonella* must be confirmed by serotyping (traditional or molecular) or MALDI-TOF MS.

2.5.9 Suitable test acceptance criteria

An isolate which exhibits biochemical characteristics consistent with documented reactions for *Salmonella* species or using identification systems such as MALDI-TOF MS or Vitek GN (Gram negative) ID card.

2.5.10 Suitable internal controls

Test each batch of biochemical substrate with both positive and negative control strains. Results of all testing recorded and kept for audit purposes.

For MALDI-TOF MS, (i) Vitek MS includes an internal *E. coli* control with each slide and Bruker perform internal controls regularly.

2.5.11 Suitable validation criteria

Correct biochemical reactions exhibited by a standard *Salmonella* strain.

2.5.12 Suitable external QC program

RCPA Quality Assurance Programs Pty Limited.

2.5.13 Special considerations

As indicated in 2.5.3 biochemical variants of salmonellae occur. Even if an isolate is not biochemically typical of *Salmonella*, MALDI-TOF, or serology, may be performed to confirm any suspect isolates. *Salmonella* subspecies can be differentiated using specific biochemical parameters and with genome sequence typing pipelines e.g. *Salmonella In Silico* Typing Resource (SISTR) (refer to Section 2.9).

For accurate results it is imperative to follow the manufacturer's procedure as prescribed, including internal QC.

2.6 Serological Diagnosis of *Salmonella* isolates

2.6.1 Suitable Specimen

Pure bacterial culture.

2.6.2 *Salmonella* Agglutinating Antisera

Verified polyvalent and monospecific *Salmonella* somatic (O serogroup) and flagella (H) antisera. Some Enteric (*Salmonella*) reference laboratories (ERLs) raise their own antisera in rabbits. Prior to use each serum is titred for the specific antigen and for cross reactions to any other *Salmonella* antigens. Alternately, commercial antisera are available for purchase. On arrival or prior to use, purchased antisera must be verified as fit for purpose.

All testing/titres must be recorded and available for audit purposes.

2.6.3 Molecular Serotyping

2.6.3.1 xMAP® Salmonella Serotyping Assay (SSA)

SSA is a multiplex, nucleic acid based assay used to identify ~85% of the common *Salmonella* serotypes (Luminex Corporation, U.S.A.)

2.6.3.2 In silico Serotyping

Salmonella serotyping remains the gold-standard tool for the classification of *Salmonella* isolates²². The increasing use of WGS has led to development of *in silico* tools to replace traditional serotyping methodologies. *In silico* tools for *Salmonella* serotyping include the *Salmonella in silico* Typing Resource (SISTR), SeqSero and the seven gene MLST for serovar prediction^{1, 2, 3}.

2.6.4 Test sensitivity

Most *Salmonella* isolates will serotype, unless an isolate is 'rough' e.g. autoagglutination occurs. Of 1,976 *Salmonella* isolates submitted to an ERL for serotyping, 37 (1.87%) were designated "rough", therefore, a complete serotype could not be determined (MDU PHL, unpublished data).

For non-motile salmonellae, only the somatic antigens can be determined and flagella antigens are not detected.

Three *in silico* *Salmonella* serotyping methods were assessed and compared to traditional serotyping techniques using a set of 813 verified clinical and laboratory isolates²¹. Successful results were obtained for 94.8, 88.2, and 88.3% of the isolates tested using SISTR, SeqSero, and MLST, respectively, indicating most serotypes be suitable for backward compatibility and surveillance systems²¹. Note that not all *in silico* serotypes, approximately 1 in 10, are going to be backward compatible with phenotypic serotypes because WGS cannot split or resolve some serotypes, etc. Therefore, there is some concession in the transition to *in silico* determination of *Salmonella* serotypes.

2.6.5 Test specificity

The statistical data not available.

2.6.6 Predictive values

The statistical data not available.

Failure to completely serotype a strain does not mean it cannot be serotyped. Using a different batch of antisera, using a younger subculture or another isolate from the patient may reveal the *Salmonella* serotype. Occasionally a culture will not give an acceptable serotype. This may indicate that the culture is mixed. The culture must be purified and an individual colony/ies must be tested.

2.6.7 Suitable test acceptance criteria

Identification of both the somatic and flagella antigens which corresponds with a published serotype¹⁹, and the same serotype has been previously isolated from a similar source. Extremely rare or unusual

serotypes e.g. antigenic formula does not match a known serotype, must be verified by the Institute Pasteur (WHO designated International Reference Laboratory for *Salmonella* Serotyping) before a final report on the serotype is issued.

2.6.8 Suitable internal controls

A known *Salmonella* strain should be included in every serotyping test run.

Ideally, the control should be a rare serotype e.g. *Salmonella* Hofit to enable detection potential mix-ups with clinical isolates.

2.6.9 Suitable validation criteria

Unequivocal demonstration of somatic and flagella antigens of recognised serotypes.

2.6.10 Suitable External QC program

National: Australia and New Zealand Enteric Reference Network (ANZERN)

- 10 *Salmonella* isolates for serotyping
- Australian *Salmonella* Reference Centre, SA Pathology on behalf of ANZERN; issued annually

International: WHO External Quality Assurance Scheme (EQAS) Global Food and Waterborne Infections Network (GFN)

- 8 *Salmonella* isolates for serotyping and antimicrobial susceptibility testing; issued annually

2.6.11 *Salmonella* Nomenclature

Serovars belonging to subspecies other than *enterica* are designated by their antigenic structure and their biochemical properties¹⁹. Some of these serotypes were formerly known by serotype names and for the sake of continuity and where the names were familiar the former name is often included in the report. Also, the Roman numeral is often still used for the sake of brevity (refer to Section 1).

2.6.12 Special considerations

Because members of the family *Enterobacteriales* are closely related, antigenic crossover can occur. Therefore, it is imperative that both flagella and somatic antigens are identified, the strain has been confirmed as a *Salmonella* biochemically and serotyping (using quality controlled antisera) or by MALDI-TOF system, by PCR or WGS.

2.7 Culture for *Salmonella* Typhi

Blood culture is probably the single most useful diagnostic procedure for diagnosis of enteric fever. With few exceptions, a positive blood culture has a high predictive value for current enteric fever.

Bone marrow aspirates has repeatedly been shown to be the specimen yielding the most positive cultures. Sampling of marrow is not often performed because it requires sterile equipment, skill and is an invasive, unpleasant procedure with associated risks for patients²³.

Direct identification of *Salmonella* from positive blood cultures using the Bruker MALDI-TOF MS can be performed³.

Isolates suspected as *S. Typhi* should be tested serologically with *Salmonella* antisera versus Vi, O-group D and H-type; *S. Typhi* can also be identified by its unique phenotypic profile (Table 3)³.

2.8 Serodiagnosis

2.8.1 Serologic Tests

Serodiagnostic tests are not used for routine diagnosis of *Salmonella* infections. Serodiagnosis can be helpful in areas where typhoid fever is endemic, but lack of specificity limits its utility. Widal was the most commonly used method for the serodiagnosis of *Salmonella* enteric fever, measures agglutinating antibodies to the O and H antigens of *Salmonella* Typhi. However, it produces false-positive reactions, likely due to cross-reaction with antibodies produced during infections with other *Salmonella* serotypes, as well as false-negative reactions; it does not provide a definitive diagnosis of individual cases of infection.

Other serodiagnostic techniques have been developed for the detection of antibodies to various antigens e.g., outer membrane proteins, lipopolysaccharide, flagellin protein, however these are not routinely used or recommended for the diagnosis of salmonellosis¹⁰. Only the Widal test will be discussed.

2.8.2 Suitable specimen

Appropriately stored, sterile paired sera (acute and convalescent phase).

2.8.3 Test sensitivity

The Widal test has low to moderate sensitivity; 30% of culture-proven cases have negative results.

2.8.4 Test specificity

Normal serum may agglutinate the test suspensions in low dilutions, and no diagnostic significance can be attached these reactions. Non-specific antigens, such as fimbrial antigens, may be present in test suspensions and react with an agglutinin in human serum.

Infections, such as bacteraemia, with other enteric organisms including salmonellae may produce a low titre response to the antigens, particularly "O" antigens, used in the Widal test²⁶. Agglutination with the Widal antigens is not specific, for example *S. Typhi* "O" antigen will agglutinate with antibodies to any group D *Salmonella* and *Salmonella* "H" antigen will agglutinate with antibodies produced against any organism possessing an H = d e.g. *S. Stanley*.

Low titres may be exhibited by patients previously vaccinated with TAB (Typhi, SPA, SPB).

Cirrhosis is associated with non-specific antibody production.

2.8.5 Predictive values

A fourfold rise in relevant O and H antibody titres in paired acute and convalescent serum acquired two weeks apart suggests *S. Typhi* infection.

2.8.6 Suitable test acceptance criteria

All test antigens must react to titre with the positive control antisera; no reaction should be observed with antigen controls.

2.8.7 Suitable internal controls

Standardised antigen suspensions and relevant antisera with documented titres.

2.8.8 Suitable validation criteria

Isolation of a *Salmonella* Typhi.

2.8.9 Suitable external QC program

Not available.

2.8.10 Special considerations

Infections, such as bacteraemia, with other enteric organisms including other *Salmonella* serotypes may produce low titres to the antigens used in the Widal test. Previous typhoid or paratyphoid vaccination may affect the titres, also.

2.9 Reference Laboratories

2.9.1 Subtyping

Provisional *Salmonella* isolates are referred to designated ERLs for confirmation and serotyping. Subtyping methods are applied to further subdivide common serovars to provide useful epidemiological information for public health action.

Subtyping methods include:

- Phage typing for serovars Typhimurium, Enteritidis, Virchow, Hadar and Bovismorbificans
 - Note: the same typing phages and the same techniques are used for the specified phage typing schemes internationally, results between countries are comparable.
 - Typing phage were last issued in 2016, therefore, this method is being phased out and will shortly be unavailable.
- Multilocus Variable tandem repeat Analysis (MLVA) for *S. Typhimurium*.
- Pulsed-field gel electrophoresis performed in outbreak investigations; currently rarely applied.
- Whole Genome Sequencing and analysis of sequence data based on
 - i. single nucleotide polymorphisms of the core genome,
 - ii. core genes, or (iii) other markers³.

2.10 Antimicrobial Susceptibility Testing (AST)

2.10.1 Resistance Trends

A review of antimicrobial data on NTS in two Australian states over 32 years, between 1984 and 2015 showed that, overall, 17% of isolates were non-susceptible to at least one antimicrobial, 4.9% were non-susceptible to ciprofloxacin, and 0.6% were non-susceptible to cefotaxime. Most isolates with clinically relevant AMR profiles were associated with travel, particularly to Southeast Asia²⁸.

Pathology laboratories routinely perform AST on Enteric fever isolates and report susceptibility results to clinicians for patient management. Treatment may be guided by destination of travel. In an analysis of Enteric fever isolates collected from 74 countries or regions between 1985 and 2010, the findings indicated nalidixic acid and multidrug resistance rates are highest in isolates from the Indian subcontinent²⁹. Some countries in South-East Asia, such as Indonesia, had very low rates of resistance; however, this varied across the region²⁹. In late 2016, an extensively drug-resistant (XDR) typhoid clade emerged in Pakistan³⁰. Over the past 2 years, cases have been reported in returned travellers by a number of countries including Australia³¹. Resistance to macrolides, namely azithromycin is rare (MDU PHL personal communication).

2.10.2 National Alert for Critical Antimicrobial Resistance (CAR Alert) (31)

Resistances to nationally agreed last line antimicrobial identified in *Salmonella* and are reported to the National Authority include:

- 3rd Generation Cephalosporin resistance including resistance genes
- 16S methylases
- carbapenemase producers including genes
- colistin including resistance genes.

2.11 Safety Considerations

2.11.1 Primary Hazard

The primary hazard when working with *Salmonella* is accidental risk of ingestion.

2.11.2 Laboratory Acquired Infections

Between 1979 and 2004, 64 cases and 2 deaths due to laboratory acquired *Salmonella* spp. infections were reported in Canada, most of them associated with *S. Typhi*³².

2.11.3 Personal Protective Equipment and Control

In Australia, NTS, *S. Typhi* and *S. Paratyphi* are classed as Risk Group 2 organisms and must be handled in a PC2 (Physical Containment 2) facility.

Work with NTS, *S. Typhi* and *S. Paratyphi* requires the use of standard techniques to minimize the risk to people and the environment³³.

The minimum requirements for safe handling of RG2 organisms includes:

- wear personal protective clothing i.e. lab coat
- wear eye protection, i.e. safety glasses, particularly where there is a known or potential risk of exposure to splashes.
- wear gloves when direct skin contact with infected materials or animals is unavoidable.

Additional personal protective equipment must be used when work procedures result in a significant risk to humans or the environment from the production of infectious aerosols, and a class 2 biological safety cabinet (BSC II) should be used³³.

2.11.4 Spills

Allow aerosols to settle, then, wearing protective clothing, gently cover the spill with absorbent paper towel and apply appropriate disinfectant, starting at the perimeter and working towards the center. Allow sufficient contact time before starting the clean-up³³.

2.11.5 Disposal

All wastes should be decontaminated before disposal either by steam sterilization, incineration or chemical disinfection³³.

3. PHLN Laboratory Case Definition

3.1 Condition Typhoid fever

3.1.1 Tests

Definitive criteria:

1. Isolation of *Salmonella* Typhi

OR

2. Detection of *Salmonella* Typhi specific targets by validated nucleic acid assay for specified sample types.

3.2 Condition

Paratyphoid fever.

3.2.1 Tests

Definitive criteria:

1. Isolation of *Salmonella* Paratyphi A or *Salmonella* Paratyphi B or *Salmonella* Paratyphi C

OR

2. Detection of *Salmonella* Paratyphi A or *Salmonella* Paratyphi B or *Salmonella* Paratyphi C specific targets by validated nucleic acid assay for specified sample types.

3.3 Condition

Salmonellosis

3.3.1 Tests

Definitive criteria:

1. Isolation of a non-typhoidal *Salmonella* species/serovars (i.e. not *S. Typhi*, *S. Paratyphi A* or *S. Paratyphi B* or *S. Paratyphi C*) from faeces (or other sites).

OR

2. Detection of *Salmonella* specific targets by validated nucleic acid assay in faeces (or other specified sample types).

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