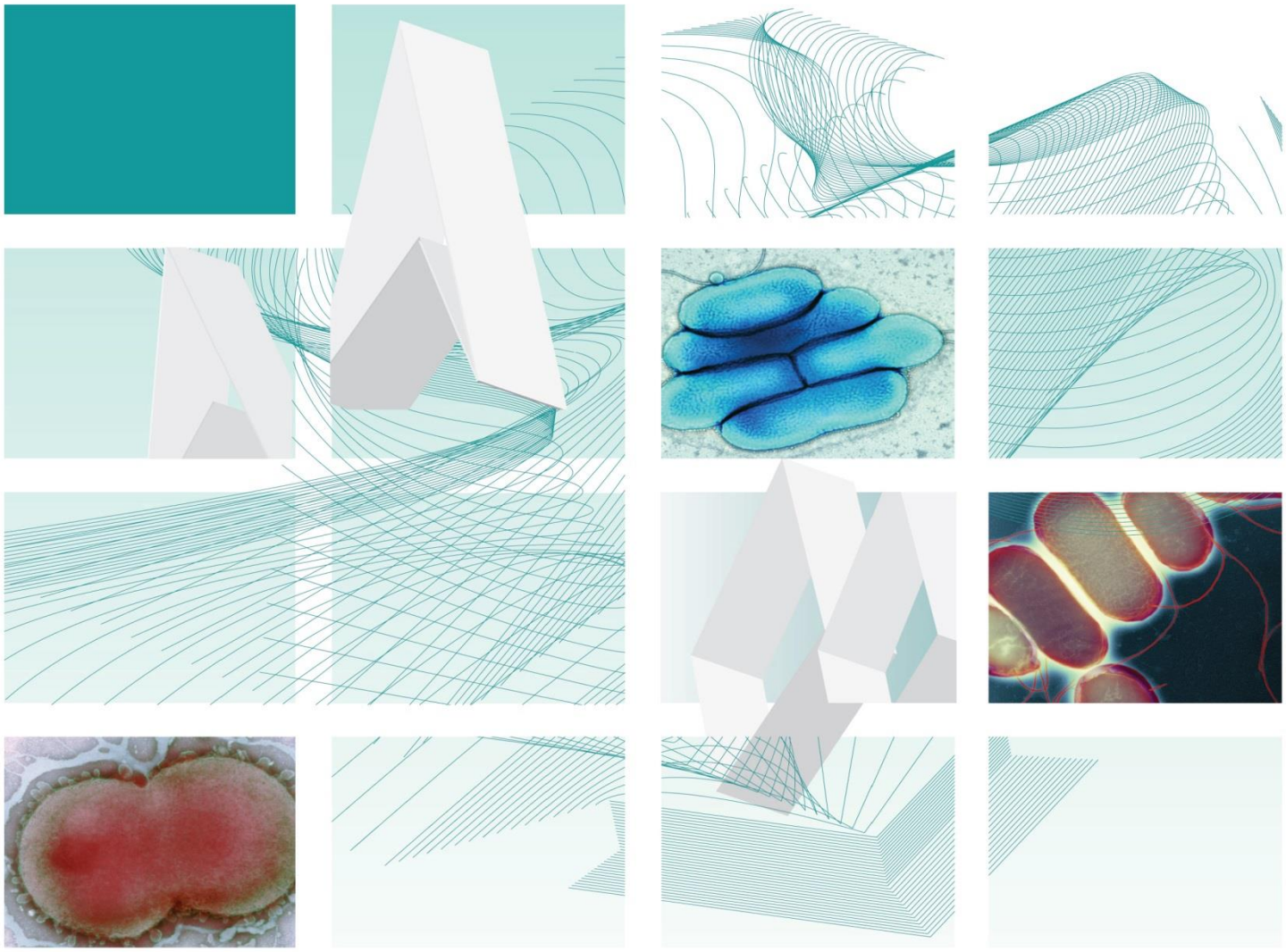
UK Standards for Microbiology Investigations

Identification of *Salmonella* species



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Contents

Amendment table

Each UK SMI method has an individual record of amendments. The current amendments are listed on this page. The amendment history is available from standards@phe.gov.uk.

New or revised documents should be controlled within the laboratory in accordance with the local quality management system.

Amendment number/date	7/12.03.21
Issue number discarded	3
Insert issue number	4
Anticipated next review date*	12.03.24
Section(s) involved	**Amendment**
Whole document	Document has been transferred to a new template.
Section 4.1 Taxonomy/characteristics	Updated with current number of infections and cases
Section 8.3 Colonial appearance	Updated with additional media
Appendix 1	Flowchart updated with identification by automated methods.
References	References reviewed and updated.

*Reviews can be extended up to five years subject to resources available.

1 General information

View general information related to UK SMIs.

2 Scientific information

View scientific information related to UK SMIs.

3 Scope of document

This UK SMI describes the identification of *Salmonella* species. *Salmonella* is a bacterial pathogen that can be isolated from faeces, blood, bone marrow, bile, urine, food, animal feed and environmental materials. Ingestion of contaminated food and water can cause foodborne infections, including gastroenteritis, typhoid fever, paratyphoid fever or even death in humans. For more information on gastroenteritis refer to the document UK SMI S 7: gastroenteritis. *Salmonella* species detected by PCR from stool or sterile sites must be cultured to determine the species, serotype and antimicrobial susceptibility profile.

This UK SMI includes routine culture and biochemical test methods for the identification of microorganisms. Some biochemical tests may not be done routinely in laboratory except in cases where confirmation by an alternative technique is required or automated methods are not available. Laboratories are implementing rapid techniques such as whole genome sequencing (WGS), matrix-assisted laser desorption/ionisation time of flight mass spectrometry (MALDI-TOF), Nucleic acid amplification tests (NAATs) and molecular methods for the identification of *Salmonella* species.

This UK SMI should be used in conjunction with other relevant UK SMIs.

4 Introduction

4.1 Taxonomy/characteristics

Salmonella genus is part of the *Enterobacterales* family. They are classified and identified into serotypes according to the White-Kauffmann-Le Minor scheme. The *Salmonella* genus is split into two species: *Salmonella enterica* and *Salmonella bongori. Salmonella enterica is split into six subspecies: Salmonella enterica* subsp. *enterica*, *Salmonella enterica* subsp. *salamae*, *Salmonella enterica* subsp*. arizonae*, *Salmonella enterica* subsp. *diarizonae*, *Salmonella enterica* subsp. *houtenae*, and *Salmonella enterica* subsp. *indica*[1]*.* There are more than 2,600 *Salmonella* serotypes that have been described and reported. New serotypes are being discovered, adding to the complexity of this large bacterial population. Classification of *Salmonella* into serotypes is important because the information can be used in epidemiological investigations and outbreak cases[2,3].

Serotyping is based on the O antigens and the H flagellar by reaction with specific antisera[2]. The O antigens determines what group the *Salmonella* isolate belongs to[1]. These antigens occur on the surface of the outer membrane and are determined by the specific sugar sequences on the cell surface. The serogroups are: O2 (A), O4 (B), O8 (C2-C3), O9 (D1), O9,46 (D2), O9,46,27 (D3), O3,10 (E1-E3) and O1,3,19 (E4)[4].

The H antigen defines the serovar or serotype of a *Salmonella* isolate. Many serotypes of *Salmonella* possess two phases of H (flagellar) antigens - phase 1, phase 2 or may have both phases simultaneously. If agglutination is obtained with only one phase, the organism may be induced to change to the other phase[5].

For example, the antigenic formula for *Salmonella* Virchow is 6,7,14:r:1,2. The strain has O antigen factors 6,7 and 14; the flagellar H antigen r (first phase) and the flagellar H antigens 1, 2 (second phase). Strains of *Salmonella* Typhi may produce Vi antigen, which is an acidic polysaccharide layer outside the cell wall also known as a surface antigen. When fully developed, it renders the bacteria agglutinable with Vi antiserum and not agglutinable with “O” antiserum. Antigens similar to Vi may also be found in some strains of *Salmonella* Typhi*, Salmonella* Paratyphi C and in few strains of *Salmonella* Dublin[3,4].

Some serovars share the same antigenic formula, therefore further testing is required for confirmation of species. For example, *Salmonella* Choleraesuis, *Salmonella* Paratyphi C and *Salmonella* Typhisuis have the same antigenic formula. *Salmonella* Dublin has a similar antigenic formula to *Salmonella* Enteriditis[2,6].

All *Salmonella* serotypes can cause disease in humans. Some serotypes are host-specific such as *Salmonella* Dublin in cattle and *Salmonella* Choleraesuis in pigs. Other serovars such as *Salmonella* Typhi, Salmonella Sendai and *Salmonella* Paratyphi A, B and C are host specific for humans. They are the most common causes of enteric fever in humans, transmitted by contaminated food and water. Most serotypes can affect different hosts, causing gastroenteritis. Most cases do not require treatment but it can be more severe in the young, elderly or patients with weak immunity[7].

Cells are rod-shaped, non-spore-forming, and predominantly motile (except serovar Gallinarum) by means of peritrichous flagella with diameters of around 0.7 to 1.5µm and lengths of 2 to 5µm with a few exceptions. On blood agar, colonies are 2 to 3mm in diameter. Colonies are generally lactose non-fermenters. They obtain their energy from oxidation and reduction reactions using organic sources and are facultative anaerobes. They produce acid from glucose usually with the production of gas and are oxidase negative. Most *Salmonella* are aerogenic except *Salmonella* Typhi, which never produces gas. Anaerogenic variants of normally gas-producing *Salmonella* serovars may occur. This is particularly common with serovar Dublin. Most *Salmonella* produce hydrogen sulphide except for some strains of *Salmonella* Choleraesuis and most strains of *Salmonella* Paratyphi A. They are identified with a combination of serological (agglutination with specific antisera), biochemical tests and molecular methods[3]. Isolates (at least one per patient if multiple isolates from various specimens available) should be sent to the reference laboratory for confirmation of species (refer to section 10).

Salmonella enterica subspecies *enterica* is divided into typhoidal and non-typhoidal serovars. Non-typhoidal include *Salmonella* Enteritidis and *Salmonella* Typhimurium, which have broad host specificity[8]. In the UK it was reported that 70% of *Salmonella* infections were caused by *Salmonella* Enteritidis[9].

PHE in England, Wales and Northern Ireland have reported 306 laboratory-confirmed symptomatic cases of *Salmonella* Typhi and *Salmonella* Paratyphi in 2017. For the most current information, refer to https://www.gov.uk/government/publications/typhoid-and-paratyphoid-laboratory-confirmed-cases-in-england-wales-and-northern-ireland.

Salmonella Typhi*,* and *Salmonella* Paratyphi A, B & C cause severe and sometimes fatal disease. The infectious dose varies with the serotype. Laboratory acquired infections have been reported. In 1974, 258 cases and 20 deaths due to laboratory-acquired typhoid fever were reported. There have been reports of laboratory acquired infections related to non-typhoidal *Salmonella.* Between 2012 and 2016 there were reports of *Salmonella* Typhimurium laboratory acquired infection. One case was traced back to a university teaching hospital[10].It is estimated that non typhoidal *Salmonella* cause 93.8 million enteric infections globally and 155, 000 deaths annually. It is the third most common cause of bacterial gastroenteritis in the UK[11].

In the UK it is estimated that over 38,000 community cases of salmonellosis occur annually[12]. In 2016, 8630 cases of non-typhoidal *Salmonella* and 2356 cases of *Salmonella* Enteritidis were reported in England and Wales[13].

5 Technical information/limitations

5.1 Quality control

If using commercially manufactured antisera, check suitability of use for all methods. Each new lot or shipment of antisera/commercial identification systems should be tested and validated for positive and negative reactivity using known control strains, ensuring it is fit for purpose. Laboratories must follow manufacturer's instructions when using these products.

5.2 Agglutination test

Salmonella species should agglutinate with Polyvalent O antiserum. Some serotypes for example *Salmonella* Typhi may produce a Vi antigen, which can prevent agglutination with Polyvalent O antiserum. Not all O serotypes are included in Polyvalent O antisera. Where results are inconclusive, it may be necessary to perform additional biochemical tests[5,14].

For slide agglutinations, growth on solid media is not optimal for the formation of flagella. False negative results may be obtained with H antisera. Inoculation of the pure culture to a wet nutrient agar slope will aid flagellum formation. Refer to UK SMI TP 3 agglutination test.

6 Safety considerations[15-32]

Most *Salmonella* species are in Hazard Group 2 with important exceptions including *Salmonella* Typhi, *Salmonella* Choleraesuis and *Salmonella* Paratyphi A, B & C which are Hazard Group 3 organisms[21].

Typhoid vaccine is indicated for active immunisation against typhoid fever and is recommended for laboratory personnel who may handle *Salmonella* Typhi in the course of their work [33]. Refer to current guidance on the safe handling of all organisms documented in this UK SMI.

Appropriate personal protective equipment (PPE) and techniques designed to minimise exposure of the laboratory workers should be worn and adhered to at all time. The most effective method for preventing laboratory-acquired infections is the adoption of safe working practices. Laboratory procedures that give rise to infectious

aerosols must be conducted in a microbiological safety cabinet. The above guidance should be supplemented with local COSHH and risk assessments.

7 Target organisms

7.1 Most common serotypes of *Salmonella* isolated and known to cause infections in humans

Salmonella Enteritidis (1,9,12:g,m:-), *Salmonella* Typhimurium (1,4,5,12:i:1,2), *Salmonella* Virchow (6,7:r:1,2), *Salmonella* Hadar (6,8:z10:e, n, x), *Salmonella* Heidelberg (1,4,5,12:r:1,2), *Salmonella* Newport (6,8:e,h:1,2), *Salmonella* Infantis (6,7:r:1,5), *Salmonella* Agona (4,12:f,g,s:-), *Salmonella* Paratyphi A (1,2,12:a:1,5), *Salmonella* Paratyphi B (1,4,5,12:b:1,2), *Salmonella* Paratyphi C (6,7,Vi:c:1,5) and *Salmonella* Typhi (9,12,Vi:d:-)

8 Identification

8.1 Microscopic appearance

Gram stain TP 39 - Staining procedures

Gram negative rods

8.2 Primary isolation media [3,34]

Blood agar incubated in 5 to 10% CO_2 and in air at 35 to 37°C for 18 to 24hr.

Cystine-lactose-electrolyte deficient (CLED) agar incubated in air at 35 to 37°C for 18 to 24hr.

Xylose-lysine-desoxycholate agar (XLD) agar incubated in air at 35 to 37°C for 18 to 24hr.

Desoxycholate citrate (DCA) agar incubated in air at 35 to 37°C for 18 to 24hr.

Brilliant Green agar (BGA) incubated in air at 35 to 37°C for 18 to 24hr.

Bismuth sulphite agar (BSA) incubated at 35 to 37°C for 48hr but examined for typical colonies at 18 to 24hr.

Other commercial validated media may be used.

8.3 Colonial appearance [3,34]

Blood agar - colonies are moist and 2 to 3mm in diameter.

CLED agar - *Salmonella* species are non-lactose fermenters.

XLD agar – colonies are red, and usually with a black centre (some serotypes such as *Salmonella* Paratyphi A and *Salmonella* Typhi may not produce a black centre).

DCA agar – contains lactose, neutral red and selective agent desoxycholate and ferric ammonium citrate which indicates H_2S production. Positive colonies are colourless, and usually with a black centre (some serotypes for example *Salmonella* Paratyphi A and *Salmonella* Typhi may not produce a black centre).

Brilliant green agar (BGA agar) – contains lactose, phenol red and selective agent brilliant green. Positive colonies appear as red to pink, 1 to 3mm in diameter, surrounded by brilliant red zones in the agar.

Salmonella-Shigella agar (SS agar) – contains lactose, neutral red and selective agents are brilliant green and bile salts. Ferric citrate indicates H_2S production. Colonies are colourless with a black centre.

MacConkey agar (MAC) contains lactose, neutral red, selective inhibitors crystal violet and bile salts. Colonies appear red indicating lactose positive. *Salmonella* species are non-lactose fermenters and are transparent or colourless.

Hektoen enteric medium (HE) contains lactose, sucrose, salicin, bromothymol blue, Andrade's pH indicator, ferric citrate and sodium desoxycholate. Colonies appear blue green with a black centre indicating H_2S production. Colonies that do not ferment the three sugars are indicators of *Salmonella*.

Eosin methylene blue (EMB).

Bismuth sulphite agar (BSA) indicator of H_2S production.

8.4 Test procedures

8.4.1 Agglutination

Agglutination test for *Salmonella* species (TP 3 - Agglutination test for *Salmonella* species)

The following limited ranges of antisera are adequate for routine use:

Polyvalent O

Single factor O (2, 4, 6:7, 8, 9, 3:10)

Polyvalent H

Rapid H sera (RSD 1, 2, 3)

Polyvalent H phase 2 (1-7)

Single factor H (a, b, c, d, e, g, i, r)

Vi antisera

Changing the Phase of *Salmonella* (TP 32 - Changing the phase of *Salmonella*) – optional

Cultures that are not expressed in one phase upon primary culture, may be switched to the other phase using a Craigie's tube or another medium to enhance motility before the H antigens can be detected. This method allows organisms that are motile to be separated from non-motile organisms[5].

8.4.2 Biochemical tests

Urease TP 36 - Urease test

Salmonella species do not produce urease

Oxidase TP 26 - Oxidase test

Salmonella species are oxidase negative

Indole Test TP19 – Indole test

Salmonella species are indole negative

Commercial identification systems

Many rapid confirmation and identification methods have been developed for *Salmonella* and a large number have been developed into commercial products.

Biochemical confirmation can be accomplished using commercial identification systems.

Rapid immunological identification and confirmation tests based on latex agglutination, enzyme immunoassay (EIA) and enzyme-linked immunosorbent assay (ELISA) have been developed for *Salmonella*, and simple-to-use lateral flow test strips using immunochromatographic technology have also been developed into commercial products by a number of manufacturers.

Laboratories should follow manufacturer's instructions. Rapid tests and kits should be validated and be shown to be fit for purpose prior to use.

8.4.3 Protein based methods

Matrix-assisted laser desorption/ionisation time of flight mass spectrometry (MALDI-TOF MS)

MALDI-TOF MS can be used to analyse the protein composition of a bacterial cell and is used by some laboratories for species identification. This has been shown to be a rapid and powerful tool because of its reproducibility, speed and sensitivity of analysis. The advantage of MALDI-TOF as compared with other identification methods is that the results of the analysis are available within a few hours rather than several days.

Firstly, a culture is incubated 18 to 24hr then processed using MALDI-TOF and the results are available within minutes. The speed and the simplicity of sample preparation and result acquisition associated with minimal consumable costs make this method well suited for routine and high-throughput use[35]. The limitations of using MALDI-TOF is that it cannot differentiate to species level, so additional testing is required. Similar species can be incorrectly identified because of the lack of sufficient spectra in the database. In the case of food and environmental samples culture is still required for bacterial colonies as *Salmonella* cannot be directly detected[36].

8.4.4 Molecular Methods

Molecular methods have had an enormous impact on the taxonomy of *Salmonella*. Analysis of gene sequences has increased understanding of the phylogenetic relationships of *Salmonella* and related organisms; and has resulted in the recognition of numerous new species. Molecular techniques have made identification of many species more rapid and precise than is possible with phenotypic techniques.

Nucleic acid amplification tests (NAATs)

PCR is usually considered to be a good method for bacterial detection as it is simple, rapid, sensitive and specific. The basis for PCR diagnostic applications in microbiology is the detection of infectious agents and the discrimination of non-pathogenic from pathogenic strains by virtue of specific genes. However, it does have limitations. Although the 16S rRNA gene is generally targeted for the design of species-specific

PCR primers for identification, designing primers is difficult when the sequences of the homologous genes have high similarity.

This has been used successfully in the identification of *Salmonella* subspecies I, *Salmonella* Typhimurium, Typhi and Enteritidis as well as *Salmonella arizonae* and *diarizonae* (rapidly and accurately without the need for serological testing)[37-40].

Multiplex PCR for serovar identification may sometimes be complex and lack reproducibility between laboratories because of the specific conditions needed for simultaneous amplification of several regions. However, more evaluation needs to be done with varieties of *Salmonella* serovars in conjunction with other laboratories in order to demonstrate accuracy of Salmonella identification in epidemiological and taxonomical studies[38,41]. There is however a PCR developed and validated to distinguish non-typhoidal *Salmonella* (NTS) from typhoidal *Salmonella* and can identify enteric fever pathogens to a serovar level including *Salmonella* Typhi, *Salmonella* Paratyphi A, B or C [42].

Whole genome sequencing (WGS)

This is also known as “full genome sequencing, complete genome sequencing, or entire genome sequencing”. It is a laboratory process that determines the complete DNA sequence of an organism's genome at a single time. There are several high-throughput techniques that are available and used to sequence an entire genome such as pyrosequencing, nanopore technology, IIlumina sequencing and Ion Torrent sequencing. The sequencing method holds great promise for rapid, accurate, and comprehensive identification of bacterial transmission pathways in hospital and community settings, with concomitant reductions in infections, morbidity, and costs.

This technique has equally been used to characterise *Salmonella* Typhi and to discover its recently acquired genes, such as those encoding the Vi antigen, by horizontal transfer events and it has provided new insights into how this pathogen has evolved to cause invasive disease in humans[43].

WGS is routinely used in the reference laboratory for identification, surveillance and monitoring of *Salmonella* isolates[44]. The method has been adopted by laboratories due to reduced costs and can detect bacterial pathogens rapidly compared to other methods. This is particularly important in outbreak situations. The WGS databases are expected to grow with emerging outbreaks that can be analysed to obtain actionable information. The method is used for cluster detection and can link previous isolates at every geographical level [45], hence it is essential that *Salmonella* isolates are referred to the reference laboratory for identification and typing.

8.5 Further identification– subtyping of strains

A variety of subtyping methods have been developed for isolates from clinical, food, environmental and animal samples. All these approaches enable subtyping of unrelated strains, but do so with different accuracy, discriminatory power, and reproducibility.

However, some of these methods remain accessible to reference laboratories only and are difficult to implement for routine bacterial identification in a clinical laboratory. Below is a summary of subtyping methods in order of discriminatory power.

rpoB Single nucleotide polymorphism (rpoB SNP) assay

rpoB gene is a single-copy chromosomal gene encoding the RNA polymerase β-subunit. This gene has been previously used in phylogenetic analysis for bacterial species and genus delineation, since it is highly conserved across organisms[46]. This method has been used to detect *Salmonella* Typhimurium[41].

Multilocus sequence typing (MLST)

MLST measures the DNA sequence variations in a set of housekeeping genes and characterizes strains by their unique allelic profiles. The principle of MLST is simple: the technique involves PCR amplification followed by DNA sequencing[43,47]. In *Salmonella,* MLST has been used to replace traditional serotyping and is routinely used in the reference laboratory from genomic data[48]. Since MLST is at the discriminatory level of serovar, additional fine typing methods are required to differentiate outbreak strains and clones[44].

Phage typing

Phage typing has proven to be an important tool for strain characterisation and the results obtained have been used in surveillance, source attribution and outbreak investigations. This technique has also been used successfully in the characterisation of several clusters of *Salmonella* Typhimurium[49]. This method has been phased out with the introduction of genomic methods.

Pulsed field gel electrophoresis (PFGE)

PFGE[50] uses restriction enzymes, which recognise specific restriction sites along the genomic DNA. The large genomic fragments are separated in a flat agarose gel. PFGE is known to be highly discriminatory technique for outbreak investigations and has gained broad application in characterizing epidemiologically related isolates. However, the stability of PFGE may be insufficient for reliable application in long-term epidemiological studies. However, due to its time-consuming nature (30hr or longer to perform) and its requirement for special equipment, PFGE is not used widely outside the reference laboratories[47].

PFGE has been used successfully to identify and discriminate between species of the family *Enterobacterales.* For example, it has been used in tracking the source of *Salmonella* infections for different serotypes and was considered the gold standard for *Salmonella* molecular typing before the introduction of MLVA typing and whole genome sequencing[43,47].

Multiple-locus variable number tandem repeat analysis (MLVA) also known as “VNTR”

MLVA is a method used to perform molecular typing of particular microorganisms. This has been used successfully in the subtyping of *Salmonella* Typhimurium, Enteritidis, Typhi, Infantis, Newport, Paratyphi A, Saintpaul, and Gallinarum isolates[47].

Whole genome sequencing (WGS)

As described in section 8.4.4, WGS is a highly robust and reproducible method that has multiple applications. For strain discrimination, isolates can be compared using Single Nucleotide Polymorphism (SNP) of the core genome[51]. The reference laboratory routinely performs this analysis of common Salmonella serovars for surveillance[44]. The alternative method is using core genome multilocus sequence typing (cgMLST) which is an allele based method of 3,002 genes and shown to be

useful in analysing *Salmonella* transmission[52 53]. Both SNP and cgMLST methods can be used for phylogenetic analysis of strain to distinguish clusters.

8.6 Storage and referral

If required, save the pure isolate on nutrient agar slopes for referral to the reference laboratory.

9 Reporting

9.1 Infection Specialist

According to local protocols inform the infection specialist of all positive cultures of *Salmonella* species presumptive or confirmed.

Inform the infection specialist of a presumptive or confirmed *Salmonella* species when the request bears relevant information for example,

- pyrexia/fever of unknown origin (PUO, FUO)
- sepsis
- enterocolitis, especially with ulceration and possible perforation of the bowel
- rose spots
- history of substance abuse, prosthetic devices including stents, alcoholism, immunodeficiency or other serious underlying disorder, such as cancer or persons receiving treatment for cancer, inducing neutropenia and/or mucositis
- laboratory work
- food handler
- investigation of outbreaks or carrier state

Follow local protocols for reporting to clinician.

9.2 Preliminary identification

If appropriate growth characteristics, colonial appearance, urease and serology results are demonstrated.

9.3 Confirmation of identification

For confirmation and identification please see Specialist and Reference microbiology: laboratory tests and services page on GOV.UK for reference laboratory user manuals and request forms.

9.4 Health Protection Team (HPT)

Refer to local agreements in devolved administrations.

9.5 Public Health England[54]

Refer to current guidelines on Second Generation Surveillance System (SGSS) reporting.

9.6 Infection prevention and control team

Inform the infection prevention and control team of presumptive and confirmed isolates of *Salmonella* species.

10 Referral to reference laboratories

For information on the tests offered, turnaround times, transport procedure and the other requirements of the reference laboratory see the Specialist and reference microbiology: laboratory tests and services page on GOV.UK for user manuals and request forms

Organisms with unusual or unexpected resistance, or associated with a laboratory or clinical problem, or an anomaly that requires investigation should be sent to the appropriate reference laboratory.

Contact appropriate devolved national reference laboratory for information on the tests available, turnaround times, transport procedure and any other requirements for sample submission:

England and Wales

https://www.gov.uk/specialist-and-reference-microbiology-laboratory-tests-and-services

Scotland

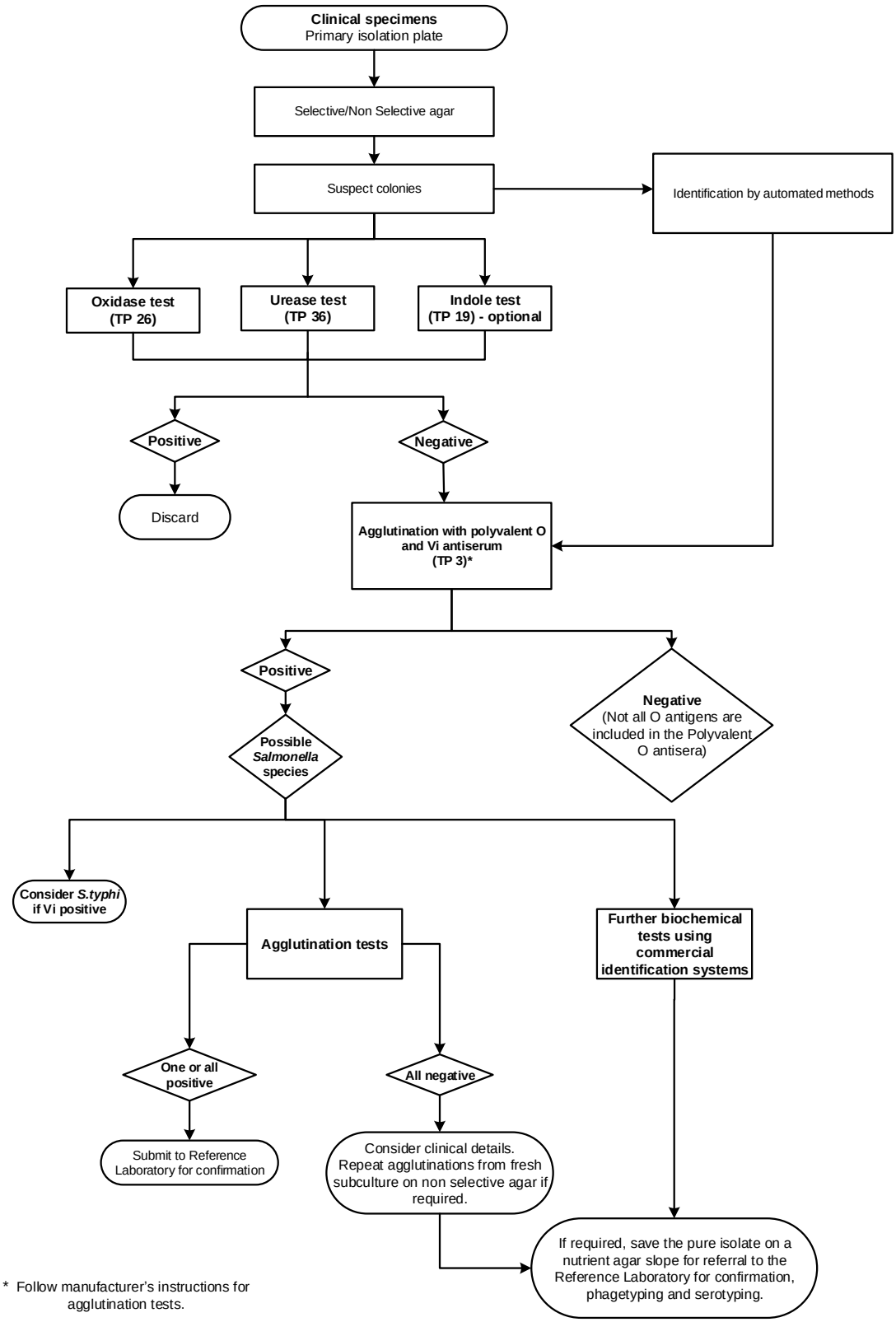
https://www.hps.scot.nhs.uk/a-to-z-of-topics/reference-laboratories/

Northern Ireland

http://www.publichealth.hscni.net/directorate-public-health/health-protection

Appendix 1: Identification of *Salmonella* species

A text description of this algorithm is provided with this document.



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For information on the reference gradings given, refer to the scientific information.

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states: "The design must allow easy handling and, where necessary, reduce as far as possible contamination of, and leakage from, the device during use and, in the case of specimen receptacles, the risk of contamination of the specimen. The manufacturing processes must be appropriate for these purposes". 1998. **++**

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