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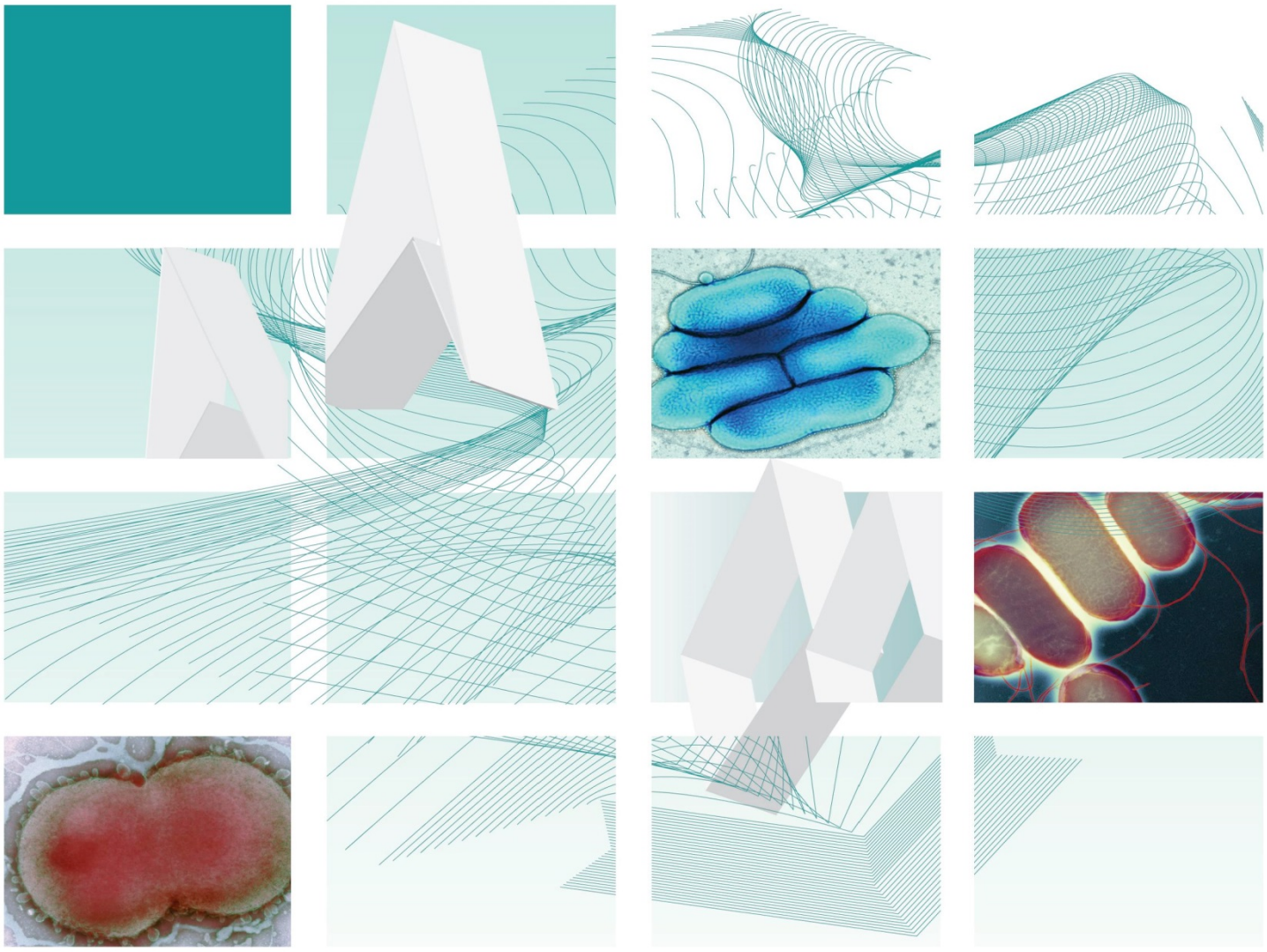
UK Standards for Microbiology Investigations

Identification of Enterobacteriaceae



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Amendment Table

Each 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 No/Date.	6/13.04.15
Issue no. discarded.	3.2
Insert Issue no.	4
Section(s) involved	**Amendment**
Whole document.	Hyperlinks updated to gov.uk.
Page 2.	Updated logos added.
Introduction.	The taxonomy of the family Enterobacteriaceae has been updated. More information has been added to the Characteristics section. The medically important species are mentioned. Section on Principles of Identification has been updated to include the MALDI-TOF.
Technical Information/Limitations.	Addition of information regarding indole test, serotyping, misinterpreted results, commercial identification systems and MALDI-TOF MS.
Safety Considerations.	This section has been updated on handling of organisms and laboratory acquired infections.
Target Organisms.	The section on the Target organisms has been updated and presented clearly.
Identification.	Updates have been done on 3.2, 3.3 and 3.4 to reflect standards in practice. Section 3.4.4 and 3.4.5 have been updated to include MALDI-TOF MS and NAATs with references. Subsection 3.5 has been updated to include the Rapid Molecular Methods.
Identification Flowchart.	Modification of flowchart for identification of Enterobacteriaceae has been done for easy guidance.

Reporting.	Subsections 5.3, 5.5 and 5.6 have been updated to reflect the information required on reporting practice.
Referral.	The addresses of the reference laboratories have been updated.
Whole document.	Document presented in a new format.
References.	Some references updated.

UK Standards for Microbiology Investigations[#]: Scope and Purpose

Users of SMIs

- SMIs are primarily intended as a general resource for practising professionals operating in the field of laboratory medicine and infection specialties in the UK
- SMIs provide clinicians with information about the available test repertoire and the standard of laboratory services they should expect for the investigation of infection in their patients, as well as providing information that aids the electronic ordering of appropriate tests
- SMIs provide commissioners of healthcare services with the appropriateness and standard of microbiology investigations they should be seeking as part of the clinical and public health care package for their population

Background to SMIs

SMIs comprise a collection of recommended algorithms and procedures covering all stages of the investigative process in microbiology from the pre-analytical (clinical syndrome) stage to the analytical (laboratory testing) and post analytical (result interpretation and reporting) stages.

Syndromic algorithms are supported by more detailed documents containing advice on the investigation of specific diseases and infections. Guidance notes cover the clinical background, differential diagnosis, and appropriate investigation of particular clinical conditions. Quality guidance notes describe laboratory processes which underpin quality, for example assay validation.

Standardisation of the diagnostic process through the application of SMIs helps to assure the equivalence of investigation strategies in different laboratories across the UK and is essential for public health surveillance, research and development activities.

Equal Partnership Working

SMIs are developed in equal partnership with PHE, NHS, Royal College of Pathologists and professional societies.

The list of participating societies may be found at https://www.gov.uk/uk-standards-for-microbiology-investigations-smi-quality-and-consistency-in-clinical-laboratories. Inclusion of a logo in an SMI indicates participation of the society in equal partnership and support for the objectives and process of preparing SMIs. Nominees of professional societies are members of the Steering Committee and Working Groups which develop SMIs. The views of nominees cannot be rigorously representative of the members of their nominating organisations nor the corporate views of their organisations. Nominees act as a conduit for two way reporting and dialogue. Representative views are sought through the consultation process.

SMIs are developed, reviewed and updated through a wide consultation process.

[#]Microbiology is used as a generic term to include the two GMC-recognised specialties of Medical Microbiology (which includes Bacteriology, Mycology and Parasitology) and Medical Virology.

Quality Assurance

NICE has accredited the process used by the SMI Working Groups to produce SMIs. The accreditation is applicable to all guidance produced since October 2009. The process for the development of SMIs is certified to ISO 9001:2008.

SMIs represent a good standard of practice to which all clinical and public health microbiology laboratories in the UK are expected to work. SMIs are NICE accredited and represent neither minimum standards of practice nor the highest level of complex laboratory investigation possible. In using SMIs, laboratories should take account of local requirements and undertake additional investigations where appropriate. SMIs help laboratories to meet accreditation requirements by promoting high quality practices which are auditable. SMIs also provide a reference point for method development.

The performance of SMIs depends on competent staff and appropriate quality reagents and equipment. Laboratories should ensure that all commercial and in-house tests have been validated and shown to be fit for purpose. Laboratories should participate in external quality assessment schemes and undertake relevant internal quality control procedures.

Patient and Public Involvement

The SMI Working Groups are committed to patient and public involvement in the development of SMIs. By involving the public, health professionals, scientists and voluntary organisations the resulting SMI will be robust and meet the needs of the user. An opportunity is given to members of the public to contribute to consultations through our open access website.

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The evidence base and microbial taxonomy for the SMI is as complete as possible at the time of issue. Any omissions and new material will be considered at the next review. These standards can only be superseded by revisions of the standard, legislative action, or by NICE accredited guidance.

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Scope of Document

This SMI describes the identification of members of the family Enterobacteriaceae. There are a large number of species included in the family. In diagnostic clinical microbiology laboratories, it is usual to attempt identification by use of biochemical tests. The level of identification depends on the site of infection, the immune status of the host and the need for epidemiological surveillance.

Because of the large number of species involved, this SMI will concentrate on the most common genera and species isolated from clinical specimens. The identification of Enterobacteriaceae can be simplified by taking advantage of the fact that three species comprise 80-95% of all isolates in the clinical setting. These are *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus mirabilis*[1]. The other species can be easily identified using biochemical tests.

This SMI should be used in conjunction with other SMIs.

Introduction

Taxonomy

Taxonomically, the bacterial family Enterobacteriaceae currently has 53 genera (and over 170 named species) and they include *Arsenophonus, Biostraticola, Brenneria, Buchnera, Budvicia, Buttiauxella, Calymmatobacterium, Cedecea, Citrobacter, Cosenzaea, Cronobacter, Dickeya, Edwardsiella, Enterobacter, Erwinia, Escherichia, Ewingella, Gibbsiella, Hafnia, Klebsiella, Kluyvera, Leclercia, Leminorella, Levinea, Lonsdalea, Mangrovibacter, Moellerella, Morganella, Obesumbacterium, Pantoea, Pectobacterium, Phaseolibacter, Photorhabdus, Plesiomonas, Pragia, Proteus, Providencia, Rahnella, Raoultella, Saccharobacter, Salmonella, Samsonia, Serratia, Shigella, Shimwellia, Sodalis, Tatumella, Thorsellia, Trabulsiella, Wigglesworthia, Xenorhabdus, Yersinia and Yokenella.* Of these, 26 genera are known to be associated with infections in humans.

The nomenclature of the Enterobacteriaceae is complicated and has been based on biochemical and antigenic characteristics. Recently, the application of new technologies such as DNA hybridisation has resulted in numerous changes in classification of the Enterobacteriaceae[1]. Many new genera and species have been discovered, some unusual and rare, and many species have also been reclassified to other genera eg the transfer of *Enterobacter sakazakii* to the *Cronobacter sakazakii*[2].

Characteristics

Members of the Enterobacteriaceae are small Gram negative, non-sporing straight rods. Some genera are motile by means of peritrichous flagella except *Tatumella*, *Shigella* and *Klebsiella* species which are non-motile. They are facultatively anaerobic and most species grow well at 37°C, although some species grow better at 25-30°C. They grow well on peptone and meat extract media. Some strains grow on D- glucose as the sole source of carbon and energy, but other strains require vitamins and or amino acids. Acid is produced during the fermentation of D- glucose and other carbohydrates[3,4].

They are oxidase negative and catalase reactions vary among Enterobacteriaceae. Nitrates are reduced to nitrites except by some strains of *Erwinia.* They are distributed worldwide and may be found in soil, water, plants, humans and animals.

Medically Important Genera of the Family Enterobacteriaceae are[5];

Citrobacter species

There are 11 species of which 10 have been recovered from clinical material[6]. They may be found in the faeces of humans and animals as part of the normal flora and grow readily on ordinary media.

Cells are short rods arranged singly, in pairs, or in short chains with rounded ends. They are motile with pertrichous flagella. Colonies are generally grey, smooth and moist although mucoid or rough strains occur. Some strains of *Citrobacter* resemble *Salmonella* species biochemically and agglutinate with *Salmonella* polyvalent antisera, which may lead to misidentification[7].

They are positive for indole, catalase and nitrate reduction tests. Acid and gas is produced from aesculin, arabinose, glucose, galactose, glycerol, inositol, lactose, levulose, maltose, mannitol, mannose, raffinose, rhamnose, salicin, sorbitol, starch, sucrose, trehalose, and xylose.

Enterobacter species

There are 26 species and two subspecies, of which recently, six have been reclassified to other genera. Only 10 have been isolated from clinical material[8]. They grow readily on ordinary media, ferment glucose with the production of acid and gas, and are motile by peritrichous flagella. Some strains with a K antigen possess a capsule. Colonies of *Enterobacter* strains may be slightly mucoid. They are catalase positive and oxidase negative. Nitrates are also reduced. They also ferment glucose and lactose with the production of acid and gas.

Enterobacter has the general characteristics of *Klebsiella* species but can be differentiated because they are motile and ornithine positive.

Enterobacter species are widely distributed in nature. They are found in the soil, water, dairy products, and in the intestines of animals as well as humans.

Escherichia species

There are five species and all are known to cause human disease[9]. Cells are typically rod-shaped, non-spore forming, motile with peritrichous flagella or non-motile, and are about 2.0µm long and 0.25 - 1.0µm in diameter. They are able to grow under aerobic and anaerobic conditions. Optimal growth occurs at 37°C. On MacConkey agar, colonies are either red or colourless and about 2 - 3mm in diameter. They are catalase positive and oxidase negative. Nitrates are also reduced.

The most commonly isolated is *Escherichia coli*, which contains numerous serotypes, some of which are associated with specific diseases. A number of strains of *E. coli* may produce enterotoxins or other virulence factors, including those associated with invasiveness. Some strains are capsulated with a K antigen.

For more information on the identification of *E. coli* O157, refer to ID 22 - Identification of *Escherichia coli* O157.

Hafnia species

The genus *Hafnia* currently has two species[10]. The optimum temperature for growth is 35°C. It grows readily on ordinary media and is generally motile. Motility is more pronounced at 30°C than 37°C[11]. *H. alvei* can resemble non-motile *Salmonella* biochemically, and can agglutinate in polyvalent salmonella antisera.

On moderately selective agars, they typically appear as large, smooth, convex, translucent colonies of 2 - 3 mm in diameter with an entire edge; some may exhibit an irregular border. Some strains of *Hafnia* also produce red or pink colonies on xylose-lysine-desoxycholate agar.

They are oxidase and indole negative and are positive for nitrate reduction and for enterobacterial common antigen. They also produce acid with or without gas from the metabolism of D-glucose and other carbohydrate or carbohydrate-like compounds[12].

Klebsiella species

The genus *Klebsiella* contains six species and three subspecies. There are four species related to humans and they include *K. pneumoniae subspecies pneumoniae, ozaenae,* and *rhinoscleromatis; K. oxytoca; K. granulomatis and K. variicola.* The other two species*, K. singaporensis* and *K. michiganensis* have been isolated from the soil and from a tooth brush holder respectively. The genus consists of over 77 capsular antigens (K antigens), leading to different serogroups[13]. These well-developed polysaccharide capsules give the colonies their characteristic mucoid appearance.

Klebsiella species are non-motile, usually encapsulated rod-shaped bacteria, belonging to the family *Enterobacteriaceae*. These bacteria produce lysine decarboxylase but not ornithine decarboxylase and are generally positive in the Voges-Proskauer test as well as indole and urease tests. They are generally facultatively anaerobic, and range from 0.3 - 1.0mm in width and 0.6 - 6.0mm in length[14].

All strains grow readily on ordinary media. On MacConkey agar, the colonies typically appear large, mucoid, and red, with red pigment usually diffusing into the surrounding agar, indicating fermentation of lactose and acid production.

They can cause bacteraemia and hepatic infections, and have been isolated from a number of unusual infections, including endocarditis, primary gas-containing mediastinal abscess, peritonitis, acute cholecystitis, crepitant myonecrosis, pyomyositis, necrotising fasciitis, psoas muscle abscess, fascial space infections of the head and neck, and septic arthritis[15].

Morganella species

The genus *Morganella* contains two species, and only one is known to cause infections in humans, *Morganella morganii*[16]. *M. morganii* is divided into two sub species on the basis of their abilities to ferment trehalose. On nutrient agar, colonies are 1 - 2mm in diameter, greyish, and opaque, circular, convex, and smooth with entire edges after 24hr at 35°C. Good growth also occurs at 22°C.

They are motile with peritrichous flagella, but some strains do not form flagella above 30°C. *M. morganii* can resemble non-motile *Salmonella* biochemically, and can agglutinate in polyvalent salmonella antisera. They are urease and indole positive as well as oxidase negative. Acid and gas are produced from utilizing glucose. Acid is also produced from mannose, galactose and trehalose[17].

They have been isolated from human clinical specimens (stool, wound, sputum, eye, bile, gastric ulcer, urine)[17].

Plesiomonas shigelloides

The genus *Plesiomonas* contains one specie, *Plesiomonas shigelloides*[18]. Cells are short Gram negative rods. They are generally 0.3 - 1.0µm in width, 0.6-6.0µm in length, motile with lophotrichous polar flagella, non-spore-producing, and facultatively anaerobic. They are able to grow at salt concentrations of 0-5%, at a pH of 4.0-8.0, and at temperatures of 8 - 44°C.

Plesiomonas shigelloides will grow on most enteric media where they produce non-lactose, non-sucrose fermenting colonies. On Inositol Brilliant Green Bile Agar, they have pink colonies and on blood agar, colonies are 2 - 3mm in diameter, large grey, opaque, and convex, β-haemolytic colonies after incubation at 35-37°C for 16 - 24 hours.

They are also oxidase-positive and catalase-positive and can be distinguished from other *Enterobacteriaceae* as it is the only oxidase positive genus in this family.

Plesiomonas is negative for DNAse; this and other biochemical tests (Moeller's lysine, ornithine, and arginine tests and fermentation of meso-inositol) distinguish it from *Aeromonas* species.

Some *Plesiomonas* strains share antigens with *Shigella sonnei*, and cross-reactions with *Shigella* antisera occur[19].

The have been isolated from human clinical specimens - faeces, blood, CSF, wounds, respiratory tract and urine. It has also been isolated from fresh water, freshwater fish, and shellfish and from many types of animals[20].

Proteus species

There are four species of *Proteus*, of which three cause disease (see section 2)[21]. Since it belongs to the family of *Enterobacteriaceae*, general characteristics are applied on this genus. All strains are motile. They may swarm on blood agar, producing concentric zones or an even film. On MacConkey agar, colonies are colourless, flat, often swarm slightly and are 2-3mm in diameter (this is specific to *Proteus vulgaris* and *Proteus mirabilis*). Other species do not swarm. They are resistant to polymyxin B and colistin. Proteus species can resemble non- motile *Salmonella* biochemically, and can agglutinate in polyvalent salmonella antisera.

Proteus species do not ferment lactose, but have shown to be capable lactose fermenters depending on the species in a triple sugar iron (TSI) test. They are also oxidase negative but catalase and nitrate positive. Other specific tests are the urease (which is an essential test to differentiate *Proteus* from *Salmonella*) and phenylalanine deaminase tests. All strains are urease positive.

They have been isolated from human faeces, urine, abdominal, neck, groin, and hip wounds, infected conjunctiva, sacral decubitus, and sputum[22].

Providencia species

The genus *Providencia* was originally established for organisms similar to *Proteus* species that were urease negative. There are eight species within the genus, of which three cause disease (see section 2)[23]. All species are motile. On blood agar and MacConkey agar, colonies are colourless, flat, 2 - 3mm in diameter and do not swarm.

They do not ferment lactose and little gas is produced from fermentable carbohydrates. They do not produce hydrogen sulphide and urea is not hydrolysed. They are resistant to polymyxin B and colistin.

Human isolates of *Providencia* species have been recovered from urine, throat, perineum, axilla, stool, blood, and wound specimens[22].

Salmonella species

For information on *Salmonella* species, refer to ID 24 - Identification of *Salmonella* species.

Serratia species

The genus *Serratia* contains 15 species and three subspecies (but only two are commonly isolated from clinical material)[24]. They are *Serratia liquefaciens* and *Serratia marcescens*, the latter often producing a pigment called prodigiosin, which ranges in colour from dark red to pale pink, depending on the age of the colonies when grown at 20°C. Pigment production is highly variable among species and is dependent on many factors such as species type and incubation time. Non-pigmented colonies resemble other members of *Enterobacteriaceae*. The optimal growth temperature is 37°C but they can also grow in temperatures that range from 5 – 40°C. They are facultative anaerobes. Most of the species are motile and have peritrichous flagella. Cells are rod shaped and 0.5 - 0.8µm x 1.0 - 5.0µm in diameter. Members of the genus characteristically produce three enzymes lipase, DNase and gelatinase. They are also resistant to polymyxin B and colistin and this resistance may be heterogeneous, leading to a target-zone appearance.

They are positive for glucose and sucrose (with gas production) fermentation and nitrate test; and negative for indole, urease and oxidase.

Rare reports have described disease resulting from infection with *Serratia plymuthica, Serratia liquefaciens, Serratia rubidaea, Serratia odorifera,* and *Serratia fonticola*.

Serratia species are found in faeces, wound exudates, respiratory specimen, blood, eye culture, and urine[20].

Serratia marcescens is the type species.

Shigella species

For information on the identification of *Shigella* species, refer to ID 20 - Identification of *Shigella* species.

Yersinia species

For information on the identification of *Yersinia* species, refer to ID 21 - Identification of *Yersinia* Species from Faeces.

Other genera of the family Enterobacteriaceae[25-28]

Other genera of the family reported to have caused infection are listed in section 2.

Principles of Identification

Colonial morphology, Gram stain, oxidase and the use of several biochemical tests identify isolates from clinical material. Enteric pathogens such as *Salmonella* species should be identified biochemically and typed serologically. *Hafnia*, *Morganella* and *Proteus* species can resemble non-motile *Salmonella* biochemically, and can agglutinate in polyvalent Salmonella antisera. Because of the diversity of biochemical

activities, all the reactions of every species are not described in this SMI. Therefore only a few screening tests are included together with results for the more common genera and species.

Careful consideration should be given to isolates that give an unusual identification. All evidence including growth characteristics, cultural morphology and serology should be considered before accepting commercial identification system results.

Full molecular identification using for example, Matrix Assisted Laser Desorption Ionisation Time-of-Flight Mass Spectrometry (MALDI-TOF MS) can be used to identify isolates to species level.

If further identification or confirmation is required, isolates should be sent to the Reference Laboratory

Technical Information/Limitations

Indole Test

Organisms to be tested by the spot indole method must be taken from a tryptophan - containing medium (eg blood agar) and never on MacConkey agar as they have pH indicators and pigmentation of lactose-positive colonies which will make interpretation of colour reaction difficult[29].

Commercial Identification System

With new species being recently established, no commercial system currently includes all species in its database and so when tested with these systems, the final identification with an unacceptable profile or no identification at all may be generated eg *Escherichia albertii* and *Hafnia alvei*[12,30].

Serotyping

For more information on technical limitations on *Shigella* species, refer to ID 20 – Identification of *Shigella* species.

Capsular antigens:

This also applies to *Klebsiella* species where cross-reactions occur among the 77 capsule types and then, individual sera have to be absorbed with the cross-reacting K-antigens. The serotyping procedure for *Klebsiella* is cumbersome because of the time needed to perform the test and is susceptible to subjective interpretations because of weak reactions that are not always easy to interpret. Since the anti-capsule antisera are not commercially available, this technique is practiced mostly in specialized laboratories[14].

Misinterpreted results

When conflicting results are observed, for example; biochemical profile disagrees with serological profile, tests should be repeated from the original plate.

Quality control

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.

MALDI-TOF MS

One of the limitations is the current inability of MALDI-TOF MS to reliably distinguish pathogenic from non-pathogenic *E. coli* isolates, in addition, numerous reports have also described the difficulty encountered when trying to discriminate *E. coli* from *Shigella* species. Differentiation of pathogenic *E. coli* strains from *Shigella* species is challenging because of the close genetic relatedness of the organisms[31].

As a tool for subspecies and serovar typing, MALDI-TOF MS shows significant promise but will require additional studies and modifications to existing protocols before the method can be used as a stand-alone mechanism[31].

1 Safety Considerations[25-28,32-44]

All *Salmonella* Typhi*, Salmonella* Paratyphi A, B and C, *Shigella dysenteriae* type 1, *E. coli* O157 and *Yersinia pestis* are Hazard Group 3 organisms and suspected isolates particularly from systemic infections in returning travellers and in other relevant infections must be handled in a containment level 3 room.

Salmonella species

Most *Salmonella* species are in hazard group 2 with important exceptions including *S*. Typhi and *S.* Paratyphi A, B and C. Work involving these organisms must be performed under containment level 3 conditions.

S. Typhi, *S*. Paratyphi A, B and C cause severe and sometimes fatal disease. Approximately 258 cases and 20 deaths due to laboratory acquired infections have been reported[45,46].

S. Typhi vaccination is available and guidance is given in the Department of Health immunisation policy[47].

Shigella species

Shigella species are highly infective, particularly *S. dysenteriae*[38,39]. As few as 10 viable organisms are required for an infective dose and cause infection[48].

Shigella species have been recently identified to be the most frequently identified agent of laboratory-acquired infections because of their high virulence and low infectious dose. A large number of laboratory acquired infections have been reported. Infection may be acquired through ingestion or accidental parenteral inoculation[49,50].

Escherichia species

Escherichia species are highly infective particularly *E. coli* O157, and as few as 10 viable organisms are required for an infective dose.

It has been identified as one of the enteric pathogens reported to cause laboratory acquired infection, but they are as less common. There are four reported cases of laboratory infections with *E. coli* since 1981[49-52].

Yersinia pestis

Yersinia pestis is highly infective and the infectious dose is unknown. There have been 10 reported laboratory-acquired infections with four deaths[53]. Vaccination is recommended for laboratory personnel who are routinely exposed to live *Y. pestis*.

Refer to current guidance on the safe handling of all organisms documented in this SMI.

Appropriate personal protective equipment (PPE) and techniques designed to minimise exposure of the laboratory workers should be worn and adhered to at all times.

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.

Compliance with postal and transport regulations is essential.

2 Target Organisms

Enterobacteriaceae Reported to have Caused Human Infections[12,14,15,17,22,25-27,30,51,53-65]

Genus	Species
Cedecea	*davisae, lapagei, neteri,* species 3, species 5
Citrobacter	*amalonaticus, braakii, farmeri, freundii, gillenii, koseri, murliniae, sedlakii, werkmanii, youngae*
Cronobacter	*sakazakii (Enterobacter sakazakii) , malonaticus, turicensis, universalis* (previously called *Cronobacter* genomospecies 1)
Edwardsiella	*hoshinae, ictaluri, tarda*
Enterobacter	*aerogenes, amnigenus, asburiae, cancerogenus*, *cloacae, cowanii, gergoviae, hormaechei, kobei, ludwigii,*
Escherichia	*albertii, coli, fergusonii, hermanii, vulneris,*
Ewingella	*americana*
Hafnia	*alvei, paralvei* (previously known as *Hafnia alvei* biogroup 2)
Klebsiella	*granulomatis, oxytoca, pneumoniae* subspecies *ozaenae, pneumoniae, and rhinoscleromatis, variicola*
Kluyvera	*ascorbata, cryocrescens, georgiana*
Leclercia	*adecarboxylata*
Morganella	*morganii subspecies morganii and sibonii*
Pantoea	*agglomerans, dispersa, septica,*
Photorhabdus	*luminescens, asymbiotica* subspecies *australis, asymbiotica*
Plesiomonas	*shigelloides*
Proteus	*mirabilis, penneri, vulgaris*
Providencia	*alcalifaciens, rettgeri, stuartii*
Rahnella	*aquatilis*
Salmonella	*bongori, enterica (>2500 serotypes)* - subspecies *arizonae, diarizonae, enterica, houtenae, indica* and *salamae,* important serotypes (serovars) - Enteritidis, Paratyphi, Typhi, Typhimurium.
Serratia	*fonticola, grimesii, liquefaciens, marcescens, odorifera, plymuthica, proteamaculans, quinovorans, rubidaea*
Shigella	*boydii, dysenteriae, flexneri, sonnei*
Tatumella	*ptyseos*
Yersinia	*bercovieri, enterocolitica, intermedia, frederiksenii, kristensenii, mollaretti, pestis, pseudotuberculosis, rohdei*
Yokenella	*regensburgei*

Other Genera of the Family Enterobacteriaceae that may have been Associated with Human Infections[66-69]

*Citrobacter gillenii (*Formerly *Citrobacter Genomospecies* 10*), Citrobacter murliniae (*Formerly *Citrobacter Genomospecies* 11*), Leminorella grimontii, Leminorella richardii, Moellerella wisconsensis*,

Other genera and species of the Enterobacteriaceae may rarely be associated with human disease.

3 Identification

3.1 Microscopic Appearance

Gram stain (TP 39 - Staining Procedures)

Gram negative rods, some may show bipolar staining (eg *Yersinia* species).

3.2 Primary Isolation Media

Blood agar (BA): 18 - 24hr incubation in 5-10% CO_2 at 35 - 37°C.

MacConkey (MAC) agar: 18 – 24hr incubation in air at 35 - 37°C.

Cystine-lactose-electrolyte deficient (CLED) agar with bromothymol blue (CLED B) or Andrade's indicator (CLED A): 18 – 24hr incubation in air at 35 - 37°C.

Chromogenic media are also available depending on specimen type or specific target organism. Refer to relevant ID SOP. Chromogenic media incubated in air at 35 - 37°C for 18 - 24hr.

3.3 Colonial Appearance

BA– Colonies are 2 - 3mm diameter, low, convex, grey, smooth or mucoid, may be haemolytic or swarming.

MAC– Colonies may appear pink (lactose fermenting) or colourless (lactose non-fermenting), size and shape vary with individual species.

CLED B– Colonies may appear yellow (lactose fermenting) or blue (lactose non-fermenting), size and shape vary with individual species.

CLED A- Colonies may appear pink (lactose fermenting) or green, translucent (lactose non-fermenting), size and shape vary with individual species.

For colonial appearance of specific target organism on chromogenic media, refer to appropriate ID SOP.

Note: Colonies of *Yersinia* species may be smaller than those of other Enterobacteriaceae.

3.4 Test Procedures

3.4.1 Biochemical tests

Oxidase Test (TP 26 - Oxidase Test)

All Enterobacteriaceae are oxidase negative except *Pleisomonas shigelloides*.

Indole Test (TP 19 – Indole Test) - Optional

The indole test determines the ability of an organism to produce indole from the degradation of the amino acid tryptophan. It is an aid in differentiation of the Enterobacteriaceae and other genera.

Klebsiella, Enterobacter, Hafnia and *Serratia* species give variable indole reactions but are usually negative.

Escherichia species are positive for indole except *E. vulneris.*

Carbohydrates Fermentation Test

Lactose fermentation exhibits variable results depending on the genus and species.

3.4.2 Commercial Identification Systems

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

3.4.3 Serotyping

Serotyping is subtyping method based on the immuno-reactivity of various antigens. *Shigella* species are by definition non-motile, as such, only the somatic (O) antigens are utilized for the determination of serotype. Flagellar (H) antigens are not expressed.

The majority of serotypes of *Salmonella* possess two phases of H (flagellar) antigens and serotyping of these species can also be done. Refer to TP 3 - Agglutination Test for *Salmonella* species and TP 32 - Changing the Phase of *Salmonella* for the detailed procedures.

3.4.4 Matrix-Assisted Laser Desorption/Ionisation - Time of Flight (MALDI-TOF) Mass Spectrometry

Matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS), which can be used to analyse the protein composition of a bacterial cell, has emerged as a new technology 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. 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[70].

This has been utilized to aid in both the detection and species-level identification of *Salmonella* species and *Enterobacter cloacae* complex. As a tool for subspecies and serovar typing, MALDI-TOF MS shows significant promise but will require additional studies and modifications to existing protocols before the method can be used as a stand-alone mechanism[31].

This technique has equally been used for the identification of *Pantoea* species but the database would benefit from additional entries being added to further populate it with entries of both environmental and clinical interest[71].

It has also been used successfully for the identification and characterization of the *Proteus mirabilis*, *Plesiomonas shigelloides* and *Enterobacter cloacae* isolates except that an improved MALDI-TOF MS database needs to be compiled in order to be able to determine species-level identifications, eg the *Enterobacter cloacae* complex[31].

MALDI-TOF MS has the ability to accurately discriminate between the two clinically relevant and highly genetically similar organisms with identical 16S rRNA gene sequences, *Y. pestis* and *Y. pseudotuberculosis*[72]. The methods of inactivation used for these pathogenic organisms does not have any influence on the MALDI-TOF MS spectra generated. This has also been used to identify and subtype *Yersinia enterocolitica* isolates[73].

MALDI-TOF MS is able to derive genus- and species-level identifications for *Cronobacter* isolates or identify them as non-*Cronobacter* isolates in the case of non-target strains and to identify different biovars within the *C. sakazakii* species but according to a study by Cetinkaya *et al*., molecular techniques such as 16S rRNA and *fusA* gene sequencing and multilocus sequence typing (MLST) are more reliable mechanisms of *Cronobacter* identification[74-76].

One of the limitations is the current inability of MALDI-TOF MS to reliably distinguish pathogenic from non-pathogenic *E. coli* isolates, in addition, numerous reports have also described the difficulty encountered when trying to discriminate *E. coli* from *Shigella* species. Differentiation of pathogenic *E. coli* strains from *Shigella* species is challenging because of the close genetic relatedness of the organisms and so biochemical and serology testing is essential in identification[31].

3.4.5 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 Enterobacteriaceae - *Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Citrobacter* species*, Enterobacter cloacae* and *Salmonella subspecies I, Salmonella enterica* serovars Typhimurium*,* Typhi and Enteritidis as well as *Salmonella enterica* subspecies *arizonae* and *diarizonae* (rapidly and accurately without the need for serological testing)[77-80].

However, multiplex PCR for *Salmonella* serovars identification may be cumbersome (due to its limitation on specificity) and sometimes lack reproducibility between laboratories because of the specific conditions needed for simultaneous amplification of several regions[78,81].

PCR has been used for the detection of *Y. pestis* among pathogenic *Yersinia* species and other Enterobacteriaceae having antigens common to *Y. pestis*. It is very useful in CBRN (chemical, biological, radiological and nuclear) emergencies and for the surveillance of epidemics[82].

This method has also been used to detect virulence genes of *Yersinia enterocolitica* and *Y. pseudotuberculosis* in human clinical isolates[83].

Commercial real-time PCR systems have been developed and validated for simultaneous detection of bacterial enteric pathogens either directly from faeces without any pre-enrichment or requires overnight pre-enrichment[84-86].

3.5 Further Identification

Rapid Molecular Methods

Most clinical microbiology laboratories still use traditional, culture-based diagnostic techniques for routine detection of bacterial enteric pathogens. These are both time-consuming and laborious and, in addition, have a prolonged delay in the reporting of results. Molecular detection of pathogens has, however, been shown to be faster and more sensitive than traditional culture[84]. Faster diagnostic outputs allow earlier epidemiological investigations and infection control interventions. It is especially important where culture-based pathogen detection is problematic or lacks sensitivity.

Molecular methods have had an enormous impact on the taxonomy of Enterobacteriaceae. Analysis of gene sequences has increased understanding of the phylogenetic relationships of Enterobacteriaceae 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.

A variety of rapid typing methods have been developed for isolates from clinical samples; these include molecular techniques such as Pulsed Field Gel Electrophoresis (PFGE), Multilocus Sequence Typing (MLST), Multiple-Locus Variable-Number Tandem-Repeat Analysis (MVLA), SNP assays and Whole Genome Sequencing (WGS). All of 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.

Pulsed Field Gel Electrophoresis (PFGE)

PFGE detects genetic variation between strains using rare-cutting restriction endonucleases, followed by separation of the resulting large genomic fragments on an agarose gel. PFGE is known to be highly discriminatory and a frequently used 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[87,88].

This has been used successfully to identify and discriminate between species of the family Enterobacteriaceae – for example, it has been used in tracking the source of *Salmonella* infections for different serotypes and is considered the gold standard for *Salmonella* molecular typing and also for typing of *Klebsiella* species[14,89,90].

This has been used successfully to discriminate between *Yersinia enterocolitica* strains and will still be useful for surveillance of the sources and transmission routes of sporadic *Yersinia enterocolitica* strains in future[91].

Multilocus Sequence Typing (MLST)

MLST measures the DNA sequence variations in a set of housekeeping genes directly and characterizes strains by their unique allelic profiles. The principle of MLST is simple: the technique involves PCR amplification followed by DNA sequencing. Nucleotide differences between strains can be checked at a variable number of genes depending on the degree of discrimination desired. The technique is highly discriminatory, as it detects all the nucleotide polymorphisms within a gene rather than

just those non-synonymous changes that alter the electrophoretic mobility of the protein product. One of the advantages of MLST over other molecular typing methods is that sequence data are portable between laboratories and have led to the creation of global databases that allow for exchange of molecular typing data via the Internet[92].

MLST has been extensively used as one of the main typing methods for analysing the genetic relationships within the Enterobacteriaceae population especially the genus *Salmonella*[89,90]. MLST was found to provide better discrimination of *Salmonella* serotype Enteritidis strains than PFGE and accurately differentiate outbreak strains and clones of the *Salmonella* serovars most commonly associated with human disease. It has also been useful for typing non-typhoidal *Salmonella* strains[93].

This has also been used to identify Shiga-toxin producing *E. coli* and *Shigella* species as well as *Klebsiella* species[14,94].

The drawbacks of MLST are the substantial cost and laboratory work required to amplify, determine, and proof read the nucleotide sequence of the target DNA fragments, making the method hardly suitable for routine laboratory testing.

Multiple-Locus Variable Number Tandem Repeat Analysis (MVLA) also known as VNTR

Multiple-Locus Variable number tandem repeat Analysis (MLVA) is a method used to perform molecular typing of particular microorganisms. It utilizes the naturally occurring variation in the number of tandem repeated DNA sequences found in many different loci in the genome of a variety of organisms. The molecular typing profiles are used to study transmission routes, to assess sources of infection and also to assess the impact of human intervention such as vaccination and use of antibiotics on the composition of bacterial populations.

This has been used successfully in the subtyping of *Salmonella enterica* subsp *enterica* serovar Typhimurium, Enteritidis, Typhi, Infantis, Newport, Paratyphi A, Saintpaul, and Gallinarum isolates[89,93,95,96]. The method has proven very useful for detecting and investigating outbreaks, since it has the capacity to differentiate closely related strains. It is technically simple and inexpensive to perform. However, it has no usefulness for serovar assignment or for global phylogenetic studies because the scope of each MLVA is commonly restricted to a unique serovar.

MLVA has been used successfully to identify and discriminate between *Yersinia enterocolitica* strains and it has been found to be a more effective method than PFGE. This method is also less labour intensive and the results from it are easier to analyse. This is also used in outbreak investigations[91].

This has also been used successfully to identify *Shigella* and *Escherichia* strains, suggesting that it could significantly contribute to epidemiological trace-back analysis of *Shigella* infections and pathogenic *Escherichia* outbreaks. It has also been used to genotype *Yersinia pestis*[97].

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, Ion Torrent sequencing, etc. This 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 has been used successfully to explore the genome of *Shigella* species and *E. coli* O157:H7 to identify candidate genes responsible for pathogenesis, and to develop better methods of strain detection and to advance the understanding of the evolution of *E. coli.* With this technique, lateral gene transfer of *E. coli* was discovered and found to be very extensive[48,98].

WGS has also been used to provide important insights into the pathology of *Yersinia enterocolitica* and, more broadly, into the evolution of the genus and other human enteropathogens[99].

This technique has equally been used to characterise *Salmonella enterica* serovar 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[90].

rpoB Single Nucleotide Polymorphism (*rpoB* SNP) assay

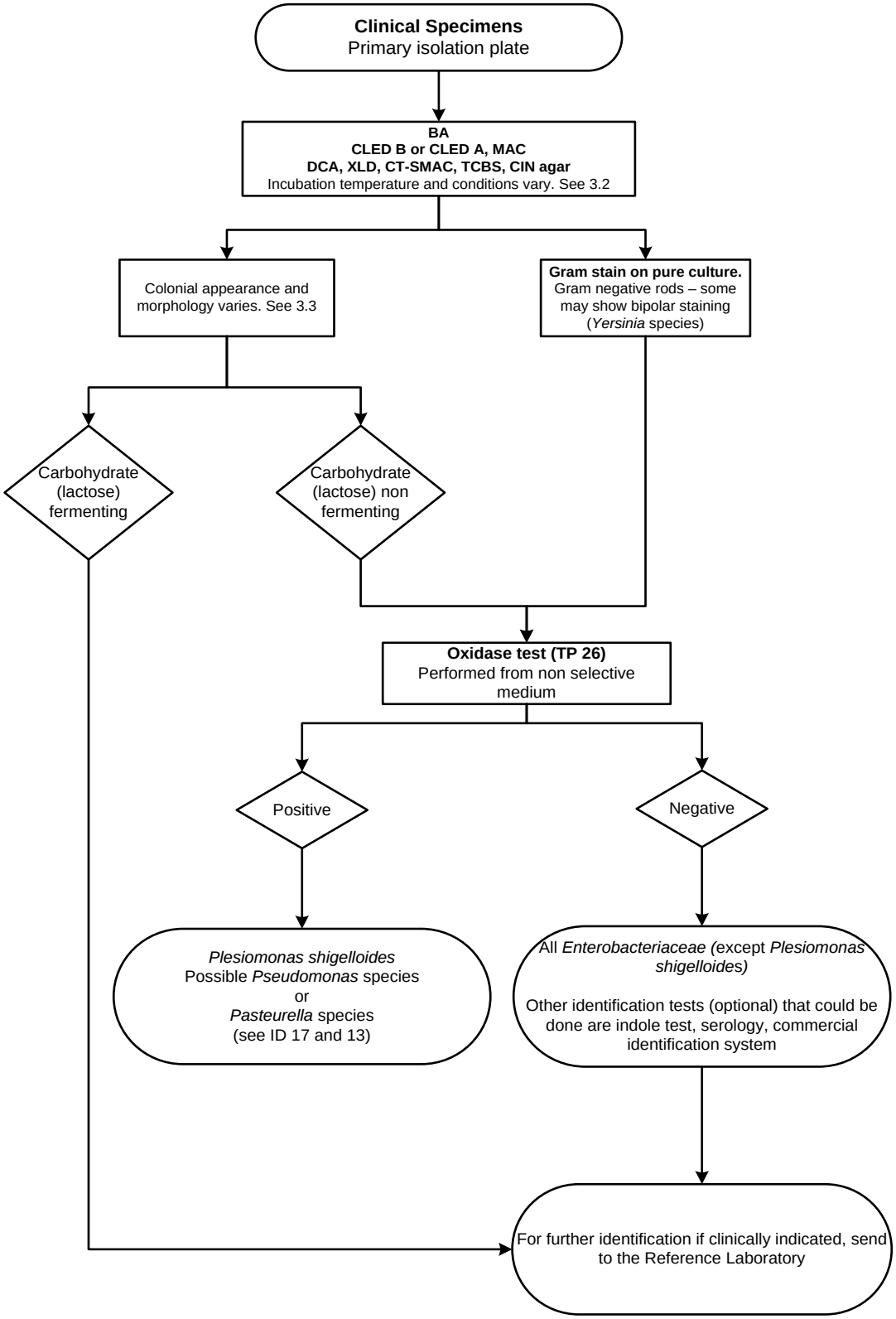
rpoB gene is a single-copy chromosomal gene encoding the RNA polymerase β-subunit. This gene has been previously used in phylogenetic analysis for bacteria species and genus delineation, since it is highly conserved across organisms[100]. However, the 16SrRNA gene has been used widely and its usefulness has been greatly enhanced through the establishment of public domain databases but its sensitivity has been questioned particularly among Enterobacteriaceae and so when the *rpoB* gene was used as an alternative for detection based on a Single Nucleotide Polymorphism, it was found to be more compatible with the currently accepted classification of Enterobacteriaceae and a powerful identification tool which may be useful for universal bacterial identification. This has been used to demonstrate that the genus *Klebsiella* is polyphyletic and to detect *Salmonella enterica* serotype Typhimurium[81].

The distinct advantage of SNP and other nucleotide sequence-based methods over profile-generating methods is that genetic relationships can be established on the basis of discrete data that are directly suitable for biocomputing and statistical analysis[89].

3.6 Storage and Referral

Save the pure isolate on a nutrient agar slope for referral to the Reference Laboratory.

4 Identification of Enterobacteriaceae



The flowchart is for guidance only.

5 Reporting

5.1 Presumptive Identification

If appropriate growth characteristics, colonial appearance, Gram stain on pure culture, oxidase and serological results are demonstrated.

5.2 Confirmation of Identification

Further biochemical tests and/or molecular methods and/or reference laboratory report.

5.3 Medical Microbiologist

Inform the medical microbiologist of presumptive and confirmed *Y. pestis*, *S.* Typhi, *S.* Paratyphi, *Shigella* species, *E. coli* O157 and *Salmonella* species (according to local procedures).

According to local protocols, the medical microbiologist should also be informed if the request bears information relating to infection with *Y. pestis* eg

- Ulceroglandular/pneumonic syndrome
- Septicaemia
- Travelling, hunting, farming, or veterinary work overseas

Information relating to cases of:

- Enterocolitis
- Dysentery
- Septicaemia
- Haemolytic-uraemic syndrome
- Neurological dysfunction or confusional states
- (non-blanching) rash

Presumptive or confirmed agents of enteric fever, dysentery, and enterocolitis should also be relayed to the medical microbiologist, especially if the patient has a history of:

- recent foreign travel
- farming (or visits to farms)
- veterinary or laboratory work
- alcoholism, substance abuse, immunodeficiency or other serious underlying disorder such as cancer

Presumptive and confirmed isolates of Enterobacteriaceae from cases of food poisoning and from investigations of outbreak situations should additionally be reported to the medical microbiologist.

Follow local protocols for reporting to clinician.

5.4 CCDC

Refer to local Memorandum of Understanding**.**

All clinically significant isolates should be notified by the diagnostic laboratories to ensure urgent initiation of proper procedures.

5.5 Public Health England[101]

Refer to current guidelines on CIDSC and COSURV reporting.

Notify all isolates of the following:

E. coli (presumptive [locally-confirmed] VTEC O157 and other possible VTEC strains)

Salmonella species

Shigella species

Yersinia pestis

Urgent oral notification to the Public Health England Centre within 24hr of identification is likely to be necessary to protect human health when presumptive identification is made of the following:

S. Typhi or *S*. Paratyphi

Salmonella species, if a suspected outbreak or a case in a food handler or closed community such as a care home

Shigella species other than *S. sonnei*

S. sonnei, if a suspected outbreak or a case in a food handler or closed community such as a care home

E. coli O157 when presumptive (locally confirmed) at the diagnostic laboratory

Other verocytotoxigenic *E. coli* O157

Yersinia pestis

Confirmatory and typing results should be forwarded to the Public Health England Centre as soon as they are available to expedite appropriate health protection interventions.

5.6 Infection Prevention and Control Team

Inform the infection prevention and control team of presumptive and confirmed isolates of *E. coli* O157, *Yersinia*, *Salmonella* and *Shigella* species.

6 Referrals

6.1 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:

Gastrointestinal Bacteria Reference Unit
Bacteriology Reference Department
Public Health England
61 Colindale Avenue
London
NW9 5EQ

https://www.gov.uk/gbru-reference-and-diagnostic-services

Contact PHE's main switchboard: Tel. +44 (0) 20 8200 4400

England and Wales

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

Scotland

http://www.hps.scot.nhs.uk/reflab/index.aspx

Northern Ireland

http://www.belfasttrust.hscni.net/Laboratory-MortuaryServices.htm

7 Notification to PHE[101,102] or Equivalent in the Devolved Administrations[103-106]

The Health Protection (Notification) regulations 2010 require diagnostic laboratories to notify Public Health England (PHE) when they identify the causative agents that are listed in Schedule 2 of the Regulations. Notifications must be provided in writing, on paper or electronically, within seven days. Urgent cases should be notified orally and as soon as possible, recommended within 24 hours. These should be followed up by written notification within seven days.

For the purposes of the Notification Regulations, the recipient of laboratory notifications is the local PHE Health Protection Team. If a case has already been notified by a registered medical practitioner, the diagnostic laboratory is still required to notify the case if they identify any evidence of an infection caused by a notifiable causative agent.

Notification under the Health Protection (Notification) Regulations 2010 does not replace voluntary reporting to PHE. The vast majority of NHS laboratories voluntarily report a wide range of laboratory diagnoses of causative agents to PHE and many PHE Health protection Teams have agreements with local laboratories for urgent reporting of some infections. This should continue.

Note: The Health Protection Legislation Guidance (2010) includes reporting of Human Immunodeficiency Virus (HIV) & Sexually Transmitted Infections (STIs), Healthcare Associated Infections (HCAIs) and Creutzfeldt–Jakob disease (CJD) under 'Notification Duties of Registered Medical Practitioners': it is not noted under 'Notification Duties of Diagnostic Laboratories'.

https://www.gov.uk/government/organisations/public-health-england/about/our-governance#health-protection-regulations-2010

Other arrangements exist in Scotland[103,104], Wales[105] and Northern Ireland[106].

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