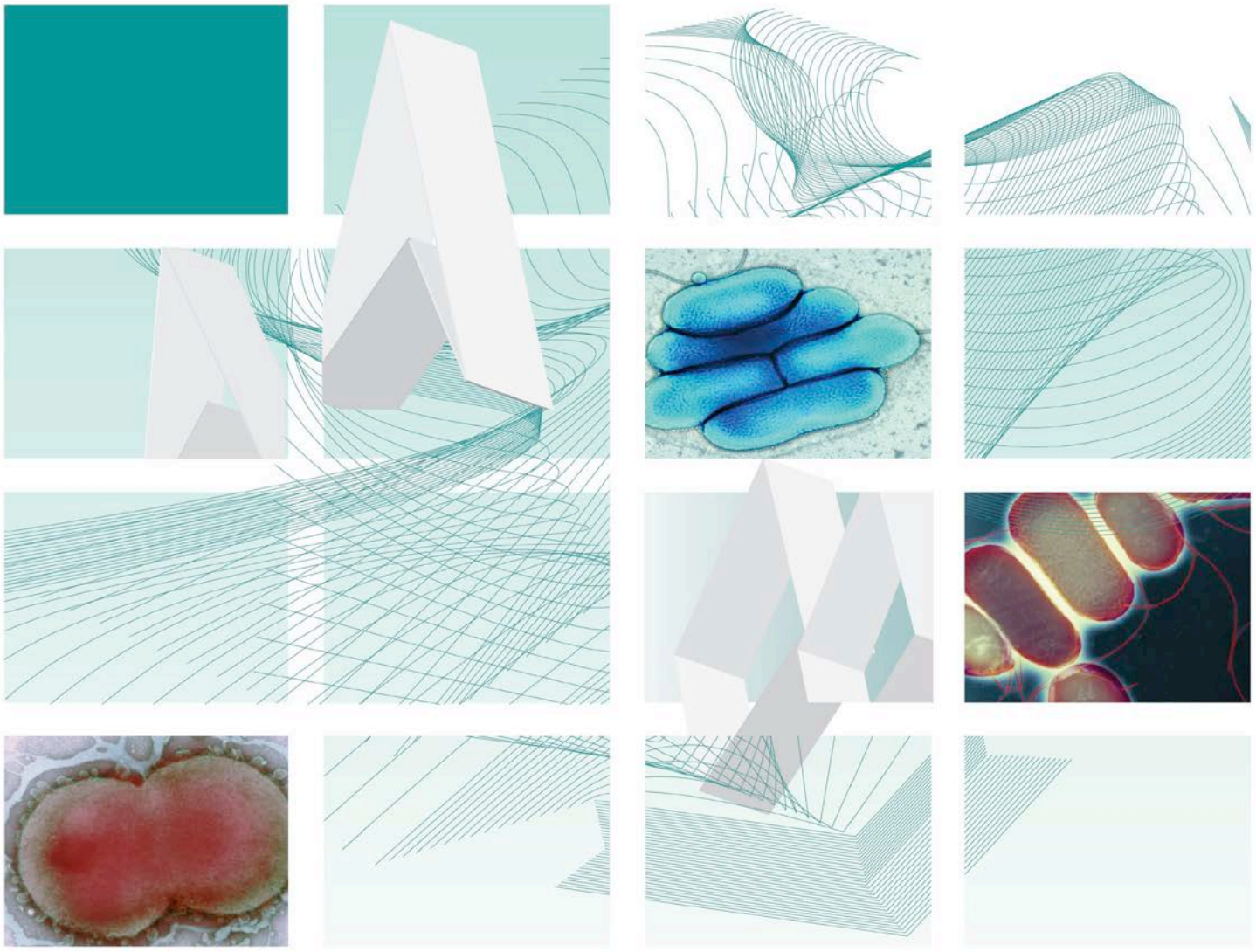
Public Health England

NHS

UK Standards for Microbiology Investigations

Identification of *Clostridium* species



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Acknowledgments

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UK Standards for Microbiology Investigations are produced in association with:

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Contents

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.	6/12.01.15
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.
Whole document.	Document presented in a new format. Reorganisation of some text. Edited for clarity. Information regarding *Clostridium difficile* updated. Test procedures updated. Removal of Reference Laboratory contact details.
Scope of document.	The scope has been updated to include webpage link for B 10 document.
Introduction.	The taxonomy of *Clostridium* species has been updated. More information has been added to the Characteristics section. The medically important species are mentioned and their characteristics described. Use of up-to-date references. Section on Principles of Identification has been updated to inform the users of the appropriate reference laboratory to send *Clostridium* species for further identification.
Technical Information/Limitations.	Addition of information regarding Gram stain, antibiotic susceptibility, sporulation and commercial identification systems has been described and referenced.
Safety considerations.	Update on Laboratory-acquired infections with

	references.
Target Organisms.	The section on the Target organisms has been updated and presented clearly. References have been updated.
Identification.	Amendments have been done on 3.1, 3.2, 3.3 and 3.4 have been updated to reflect standards in practice. Subsection 3.5 has been updated to include the Rapid Molecular Methods. 3.6 has been rephrased and informs users to refer to appropriate laboratory user manual for referrals.
Identification Flowchart.	Modification of flowchart for identification of species has been made for easy guidance.
Reporting.	Subsection 5.2 and 5.6 has been updated to reflect reporting practice.
Referral.	The address of the reference laboratories has been updated.
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.

Information Governance and Equality

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The development of SMIs are subject to PHE Equality objectives https://www.gov.uk/government/organisations/public-health-england/about/equality-and-diversity. The SMI Working Groups are committed to achieving the equality objectives by effective consultation with members of the public, partners, stakeholders and specialist interest groups.

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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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Suggested Citation for this Document

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Scope of Document

This SMI describes the identification of *Clostridium* species.

There are many species of clostridia which may be found naturally in the environment and animal faeces. Only species associated with human infection will be discussed in this SMI.

For more information on *Clostridium difficile*, refer to B 10 - Investigation of Faecal Specimens for *Clostridium difficile*.

This SMI should be used in conjunction with other SMIs.

Introduction

Taxonomy

The genus *Clostridium* belongs to the family *Clostridiaceae* and it currently contains 203 species and 5 subspecies, with only a few species being pathogenic to humans. Of these species, 21 have been reclassified to other genera, 5 have been reclassified within the genus and 1 has been de-accessioned[1].

In 1994 the heterogeneity of this species was confirmed by 16S rRNA gene sequencing[2]. This has been reaffirmed by the work of Yutin *et al* that 16S rRNA and ribosomal protein sequences are better indicators of evolutionary proximity than phenotypic traits. This genus like several others has undergone a number of revisions with the increasing availability of genomic data. An analysis of proteins from a number of members of this genus suggested another revision[3]. The main findings from the proposal suggested that:

- The *Selenomonas-Megasphaera-Sporomusa* group are still members of the genus *Clostridium*
- *Clostridium difficile* and its close relatives are placed within the family *Peptostreptococcaceae*. Under this proposal, the species *Clostridium difficile* would become *Peptoclostridium difficile*
- Members of the family *Ruminococcaceae* belong to the genus *Clostridium*
- It was also proposed to create six new genera to accommodate the 78 validly described species that fell outside the family *Clostridiaceae*. These genera are: *Erysipelatoclostridium, Gottschalkia, Lachnoclostridium, Peptoclostridium, Ruminiclostridium* and *Tyzzerella*

The type species is *Clostridium butyricum*.

Characteristics

Clostridium are phylogenetically heterogeneous and are Gram positive but can decolourise easily and appear Gram negative or Gram variable, spore formers and non-spore formers, rods and cocci and anaerobic and non-anaerobic bacteria[4].

Medically significant Clostridium strains tend to be Gram positive rods (some are Gram variable), 0.3 – 2.0 x 1.5 – 20.0µm which are often arranged in pairs or short chains, with rounded or sometimes pointed or square ends. They are commonly pleomorphic and vary considerably in their oxygen tolerance. Some species such as *Clostridium novyi* type A and *Clostridium haemolyticum* may require extended

incubation on pre-reduced or freshly prepared plates and total handling in an anaerobic chamber. Conversely, *Clostridium tertium, Clostridium histolyticum* and *Clostridium carnis* are aerotolerant and will form colonies on blood agar plates incubated in an atmosphere of air with 5-10% added CO_2[5].

Virtually all of the members of the genus, except *Clostridium perfringens*, are motile with peritrichous flagellae and form oval or spherical endospores that may distend the cell. They may be saccharolytic or proteolytic and are usually catalase negative. Many species produce potent exotoxins.

Toxins of *Clostridium* species

Clinically significant *Clostridium* species produce a variety of toxins. It is the production of these toxins which leads to the distinctive clinical features of the diseases they cause, eg tetanus and botulism result from the production of neurotoxins that are amongst the most lethal substances known to man[6]. Clostridial toxins are biologically active proteins that are antigenic in nature and can therefore be neutralised with specific antisera.

Detection of particular toxins directly from some clinical samples may render the isolation of the organism unnecessary for primary investigation eg *C. difficile* (refer to B 10 - Investigation of Faecal Specimens for *Clostridium difficile*). Culture is required for typing (outbreaks and incidents) and susceptibility testing.

Clostridium perfringens is the most commonly isolated *Clostridium* species. Five types (A-E) may be distinguished by the combinations of major lethal toxins they produce[5].

Clostridium tetani produces two exotoxins, tetanolysin and tetanospasmin – Tetanolysin causes lysis of RBCs and serves no known benefit to *C. tetani* infections while tetanospasmin is a neurotoxin that causes the clinical manifestations of tetanus[6].

Clostridium botulinum also produces neurotoxins (which are the most potent natural poisons known) that cause botulism, a disease characterized by a symmetrical, descending paralysis[7]. There are seven toxin types (A-G), man is susceptible to type A, B, E, F and G toxins; types A, B, C and D cause intoxication in animals. Although less common, bivalent strains that express two different toxin types exist and are designated by the predominant toxin produced. Strains of *C. baratii* and *C. butyricum* have been implicated as causative agents of botulism as they also produce the types F and E respectively[6,8]. *C. argentinense* (formerly *C. botulinum* type G) produce botulinum neurotoxin.

Clostridium difficile is the most common toxigenic *Clostridium* species. They produce two potent exotoxins namely – Toxin A (enterotoxin) and toxin B (cytotoxic activity)[6].

Clostridium novyi comes in three types, labelled A, B, and a non-pathogenic type C distinguished by the range of toxins they produce. The toxins are designated by Greek letters[6].

Clostridium sordellii produces three toxins in common with non-pathogenic *C. bifermentans* namely; a lecithinase, an oxygen-labile haemolysin and a fibrinolysin. It also has a major lethal toxin referred to as beta-toxin that distinguishes it from *C. bifermentans*. This beta-toxin actually contains two toxins: lethal toxin (LT) and haemorrhagic toxin (HT)[6].

Other *Clostridium* species produce similar toxins to that produced by *C. perfringens*.

The medically important species are:

Clostridium perfringens

They are non-motile straight-sided gram-variable rods with blunt ends that occur singly or in pairs, 0.6 – 2.4µm wide by 1.3 – 19.0µm long, and rarely produce spores. They grow vigorously at temperatures between 20 and 50°C, with an optimum of 45°C for most strains. On blood agar, large discrete colonies are produced after overnight incubation. They may be flat and rough-edged or smooth and domed, and either non-haemolytic or with a narrow zone of complete haemolysis inside a larger zone of partial haemolysis. Haemolysis is more pronounced on sheep blood agar than on horse blood agar[9]. They are positive for lecithinase, nitrate, and fermentation of sugars but negative for lipase, indole and urease tests.

Clostridium tetani

Cells are 0.5 – 1.7 by 2.1 – 18.1µm and often possess terminal endospores that give a "drumstick" appearance. Cells in culture older than 24hr may appear Gram negative. They are also motile by peritrichous flagella. The optimal growth temperature is 37°C and little or no growth takes place at 25 or 42°C.

Growth may appear as a film rather than discrete colonies because of swarming due to the vigorous motility after 48hr incubation. On blood agar, the colonies are flat, translucent, and grey with a matte surface, showing a zone of β- haemolysis and are 4 to 6mm in diameter. Colonies have irregular and rhizoid margins. They are negative for fermentation of sugars, lecithinase, lipase, urease, nitrate reduction tests but give variable results for indole test.

Clostridium botulinum

Cells are gram variable bacilli that show profuse sub-terminal and free spores. The proteolytic types A, B and F initially produce discrete rhizoidal colonies that spread and coalesce. Haemolysis is variable, but the odour is strong and redolent of rotten eggs due to production of H2S. They are positive for lipase but negative for indole and urease tests. They give variable test results for lecithinase reaction.

They have been isolated from clinical samples such as – faeces, wounds, tissue, and pus as well as from foods.

Clostridium difficile

Cells are motile rods, with dimensions of 0.5 -1.9 by 3.0 – 16.9µm, which forms oval sub-terminal spores and show optimum growth on blood agar at human body temperatures in the absence of oxygen. Colonies of *C. difficile* are 4 - 6mm in diameter, irregular, raised, opaque, and grey-white after 48hr incubation. They may be isolated from faecal specimens using cycloserine cefoxitin fructose agar (CCFA) or cycloserine cefoxitin egg yolk agar (CCEY). They ferment sugars but are negative for lecithinase, lipase and indole tests.

Refer to B 10 - Investigation of Faecal Specimens for *Clostridium difficile* for further information.

Clostridium novyi

Cells are motile, gram variable rods with occasional sub-terminal spores. Cell dimensions are 0.5 – 1.6 by 1.6 – 18µm except for *C. novyi* type B, which are larger, 1.1 – 2.5 by 3.3 – 22.5µm. Isolating and identifying *C. novyi* is difficult due to its extreme anaerobic nature. Because of their fastidious nature and difficulty in culturing, they require the presence of thiols for growth[10]. Growth is stimulated by fermentable

carbohydrates, serum or peptic digest of blood. On blood agar after overnight incubation anaerobically, colonies appear as small, flat, rough or rhizoidal, translucent, haemolytic colonies with a spreading edge and 1 - 5mm in diameter and after incubation for 48–72hr, colonies will often coalesce to give a fine spreading growth that may cover the entire plate, often with a marked β-haemolysis so as to make the blood agar plate completely transparent. There is poor growth in nutrient broth or cooked meat broth. They ferment glucose and liquefy gelatin. Proteolytic activity is variable. They are positive for lecithinase and lipase reactions but give variable results for indole test.

C. novyi type A is usually unreactive in commercial anaerobe identification kits and commonly is not identified by this approach. *C. novyi* type B has different phenotypic characteristics and can be distinguished by its biochemical reactions[9].

Clostridium sordellii

Colonies are large, grey-white and irregular, sometimes with a “fern-leaf” edge. They produce indole and lecithinase as well as ferment sugars. They are also urease positive, which differentiates them from *C. bifermentans*, generally regarded as a non-pathogen.

Clostridium septicum

Cells are gram variable rods with numerous sub-terminal spores. On blood agar, they grow rapidly and usually produce a thick haemolytic swarming growth. In culture, it has no characteristic odour. They are negative for lecithinase, lipase, indole and urease tests.

They are easily recognised by use of commercially available identification kits. The most common source of *C. septicum* isolates seen in recent years has been from blood cultures from patients with malignancies of the colon or caecum[9].

Principles of Identification

Clues to the identity of certain pathogenic species may be obtained by observing characteristics such as colonial appearance, Gram stain appearance and the presence or absence of β-haemolysis. Other phenotypic tests may also be applied to obtain a presumptive identification[11]. It is important to ensure the culture is pure, as the fine spreading growth of some *Clostridium* species may mask contaminating organisms.

If confirmation of *Clostridium* species is required, isolates should be referred to the Anaerobe Reference Unit, Public Health Wales, Cardiff.

If *C. difficile* confirmation is required, refer to B 10 - Investigation of Faecal Specimens for *Clostridium difficile*.

If *C. botulinum* is suspected, samples of patient’s serum, faeces and implicated foodstuff should be referred directly to the Foodborne Pathogens Reference Section, Colindale.

Technical Information/Limitations

Antibiotic susceptibility

Reduced susceptibility of *C. difficile* to metronidazole has been demonstrated[12].

Sporulation

Several species of *Clostridium*, including *C. carnis, C. histolyticum* and *C. tertium* can grow, but not sporulate, in air[5].

Gram stain

It is important to ensure the culture is pure, as the fine spreading growth of some *Clostridium* species may mask contaminating organisms.

There can be failure to determine the Gram reaction correctly (many anaerobes over decolourise and appear Gram negative). For example, Gram negatively staining *Clostridium* species, especially *C. clostridioforme*, can be misidentified as *Bacteroides*[11].

Commercial identification Systems

The use of commercially available anaerobe identification kits alone may not give an accurate identification eg *C. novyi* type A is usually unreactive in commercial anaerobe identification kits and commonly is not identified by this approach. *C. novyi* type B has different phenotypic characteristics and can be distinguished by its biochemical reactions[9].

1 Safety Considerations[13-29]

Hazard Group 2 organisms

Laboratory acquired infections have been reported[30,31].

Refer to current guidance on the safe handling of all Hazard Group 2 organisms documented in this SMI.

Laboratory procedures that give rise to infectious aerosols must be conducted in a microbiological safety cabinet[21].

The above guidance should be supplemented with local COSHH and risk assessments.

Compliance with postal and transport regulations is essential.

2 Target Organisms

Clostridium species Reported to have Caused Human Disease[5,11]

C. perfringens, C. septicum, C. novyii type A, C. sordellii, C. tetani, C. difficile, C. botulinum, C. butyricum, C. baratii*, C. tertium, C. histolyticum*

Non-pathogenic *Clostridium* species Commonly Isolated that may have Caused Human Infections[5,32,33]

C. sporogenes, C. ramosum. C. innocuum, C. paraputrificum, C. cadaveris, C. bifermentans, C. fallax, C. clostridioforme

3 Identification

3.1 Microscopic Appearance

(TP 39 - Staining Procedures)

Gram stain

Clostridium species are Gram positive rods, which may possess a single endospore. Some species may be Gram variable.

Spore stain

This is used to determine the shape and position of the spore (phase contrast microscopy is an alternative option).

C. perfringens	Oval, subterminal

Note: *C. perfringens* spores are rarely seen in vivo or usual in vitro conditions. They do not sporulate on normal agar media. *C. perfringens* also have non-spore forming strains.

C. botulinum	Oval, subterminal
C. difficile	Oval, subterminal
C. novyi	Oval, central or subterminal
C. sordellii	Oval, subterminal

C. septicum	Oval, subterminal
C. tetani	Round, terminal (giving a drumstick appearance)

3.2 Primary Isolation Media

Agar containing blood incubated anaerobically at 35-37°C for 40–48hr.

Egg Yolk agar incubated anaerobically at 35-37°C for 16-24hr.

If culturing for toxigenic *C. difficile*, Cycloserine Cefoxitin Fructose agar (CCFA) or Cefoxitin Cycloserine Egg Yolk agar (CCEY) should also be inoculated and incubated anaerobically at 35-37°C for 24 - 48hr. The antibiotics cycloserine and cefoxitin inhibit the growth of most bacteria other than *C. difficile*[11].

3.3 Colonial Appearance

Colonial appearance varies with species and brief descriptions of the most common species are given here:

Organism	**Characteristics of growth on agar containing blood after anaerobic incubation at 35–37°C for 40–48hr**
C. botulinum/sporogenes	Large (3mm), irregularly circular, smooth, greyish, translucent with a fibrillar edge that may spread. Most strains are β-haemolytic; produces lipase.
C. difficile	Glossy, grey, circular colonies with a rough edge; fluoresce green-yellow under long wavelength UV light (360 nm ± 20nm). They are usually non-haemolytic, with a characteristic farmyard smell.
C. novyi	Raised, circular colonies, which become flattened and irregular in old cultures. Colonies tend to fuse forming a spreading growth with a double zone of β-haemolysis. Type A produces lecithinase and lipase.
C. perfringens	Large, smooth, regular convex colonies, but may be rough and flat with an irregular edge. Usually has a double zone of β-haemolysis; produces lecithinase.
C. septicum	Usually produce a thick swarming growth with a narrow zone of β-haemolysis.
C. sordellii/bifermentans	Grey-white, convex, circular colonies with crenated edges, which may spread. They may be β-haemolytic; produce lecithinase; indole positive.
C. tetani	Fine swarming growth (may be difficult to see) which may appear β-haemolytic.
Other *Clostridium* species	Colonial appearances vary, but may produce a spreading growth which may or may not be β-haemolytic.

3.4 Test Procedures

The following tests can be used to differentiate between *Clostridium* species. If clinically indicated, refer to the appropriate Anaerobe Reference Unit for further identification.

3.4.1 Biochemical tests

Nagler test TP 22 - Nagler Test

The nagler test determines the ability of a microorganism to produce the enzyme lecithinase. Lecithinase producing organisms are identified by a zone of opalescence surrounding individual colonies on egg yolk agar. *C. perfringens* lecithinase is inhibited by the antitoxin *C. perfringens* type A.

Clostridium baratii, Clostridium absonum, Clostridium bifermentans, Clostridium sordelli and *Clostridium novyi* also produce lecithinase. *C. sordelli* and *C. bifermentans* produce enzymes that are also closely related to *C. perfringens* alpha toxin (lecithinase) and can produce a partial cross-reaction[34].

A Nagler positive result is indicated by lecithinase production and inhibition due to antitoxin.

Note: In recent years, popularity of the Nagler test has declined because the antitoxin has not been widely available. An alternative to the Nagler test used in some laboratories is the reverse CAMP test.

Reverse CAMP test

Reverse CAMP test can be used for differentiation of *C. perfringens* from other *Clostridium* species. Alpha toxin producing *C. perfringens* and group B, β-haemolytic streptococci grow in a characteristic pattern on blood agar; however care must be taken to ensure pure cultures are used[35].

Indole test TP 19 - Indole Test

The indole test determines the ability of an organism to produce indole from the degradation of the amino acid tryptophan.

Anaerobes, particularly *Clostridium* species, form indole but can rapidly break it down as it is produced; therefore, false negative reactions may occur[36].

C. novyi A strains give variable indole test results but are usually indole negative.

Lipase test

The lipase test determines the ability of microorganisms to produce the enzyme lipase that catalyses the hydrolysis of triglycerides and diglycerides to fatty acids and glycerol. This is shown by the iridescent sheen on and surrounding colonies on plate medium. This aids in differentiation of *Clostridium* species.

C. botulinum, C. sporogenes, C. novyi A, C. ghonii and *C. cochlearium* produce lipase. *C. leptum* give variable lipase reactions but are usually lipase negative.

Urease test TP 36 - Urease Test

The urease test is used to determine the ability of an organism to split urea, through the production of the enzyme urease.

C. sordellii are urease positive which distinguishes it from *C. bifermentans*, which it resembles and are urease negative.

3.4.2 Commercial identification Systems

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

3.4.3 Matrix-Assisted Laser Desorption/Ionisation - Time of Flight (MALDI-TOF)

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[37].

This has been used for the identification of *Clostridium* species especially to discern different ribotypes among isolates of *C. difficile*. However, an extensive database is essential to identify species and closely related strains reliably and available databases needs to be optimised[38,39].

Other limitations to the use of this technique is the presence of spores of *Clostridium* species and so younger cultures are now used to minimize spectral interference[39].

3.4.4 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 *Clostridium* species eg *C. perfringens, C. botulinum, C. baratii and C. butyricum, C. novyi, C. difficile*[40-44].

3.5 Further Identification

Rapid Methods

A variety of current rapid typing methods have been developed for isolates from clinical samples; these include molecular techniques such as Pulsed- Field Gel Electrophoresis (PFGE), Fluorescent Amplified Fragment Length Polymorphism (AFLP),16S rDNA gene sequencing, PCR- restriction fragment length Polymorphism (PCR-RFLP), Microarray analysis, Multiple-Locus Variable-Number Tandem-Repeat Analysis (MVLA), and even whole-genome sequencing (WGS). All of these approaches enable subtyping of 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.

Fluorescent Amplified Fragment Length Polymorphism (AFLP)

Fluorescent Amplified Fragment Length Polymorphism is a high-resolution whole genome methodology used as a tool for rapid and cost-effective analysis of genetic diversity within bacterial genomes. It is useful for a broad range of applications such as identification and subtyping of microorganisms from clinical samples, for

identification of outbreak genotypes, for studies of micro and macro-variation, and for population genetics.

FAFLP has numerous advantages over other DNA fingerprinting techniques because it assesses the whole genome for both conserved and rapidly evolving sequences in a relatively unbiased way. The number of fragments obtained for comparative purposes between isolates is significantly greater than pulsed-field gel electrophoresis (PFGE), thus making it more discriminatory than PFGE and the FAFLP results are highly reproducible due to stringent PCR cycling parameters.

This relatively fast method can be applied to different clostridia and used for the generation of identification libraries. Libraries of AFLP profiles of well-defined *Clostridium* strains provide a valuable additional tool in the identification of *Clostridium* species.

This technique has been used to genotype *C. botulinum, C. difficile*, *C. novyi* and *C. perfringens*[45-48]. It has also been used to differentiate between *C. bifermentans* and *C. sordellii* strains (which closely resemble phenotypically) and between strains of *C. perfringens*[49].

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. However, the stability of PFGE may be insufficient for reliable application in long-term epidemiological studies. Due to its time-consuming nature (30hr or longer to perform) and its requirement for special equipment and the interpretation of its results often being subjective, PFGE is not used widely outside reference laboratories[50,51].

PFGE is considered a very useful tool for molecular epidemiological analysis of proteolytic *C. botulinum* types A and B as it enabled discrimination between them but this has not been very successful with non-proteolytic *C. botulinum*[52]. It has been used for typing *C. difficile* although a considerable proportion of strains are non-typable by this technique due to degradation of the DNA during the procedure; making un-interpretable gel smears or likely spore formation[53].

PFGE has also been used to establish *C. perfringens* as the etiological agent in food-borne outbreaks and to reveal its wide genetic diversity from different sources[54].

16S rDNA gene sequencing analysis

A genotypic identification method, 16S rDNA gene sequencing is used for phylogenetic studies and has subsequently been found to be capable of re-classifying bacteria into completely new species, or even genera. It has also been used to describe new species that have never been successfully cultured.

This has been used to differentiate between *Clostridium* species eg *C. novyi* type A and *C. botulinum* type C that are closely related[9].

Microarrays

DNA microarray technology can provide detailed, clinically relevant information on the isolate by detecting the presence or absence of a large number of virulence-associated genes simultaneously in a single assay; however, their clinical value has

been limited by a complicated methodology that is unsuitable for routine use in diagnostic microbiology laboratories.

This technique has been used and it demonstrates the high-throughput detection and identification of pathogenic *Clostridium* species and it has advantages over the conventional traditional methods. This has also been particularly useful in efficiently and specifically identifying all *Clostridium* species present in a mixed bacterial population. The high-throughput feature of this technique is very useful in the detection and analysis of outbreak strains and for epidemiologic studies of *Clostridium* infections[7].

Multiple-Locus Variable Number Tandem Repeat Analysis (MVLA)

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 typing of *C. perfringens* and newly emerging variants of *C. difficile* [55,56].

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.

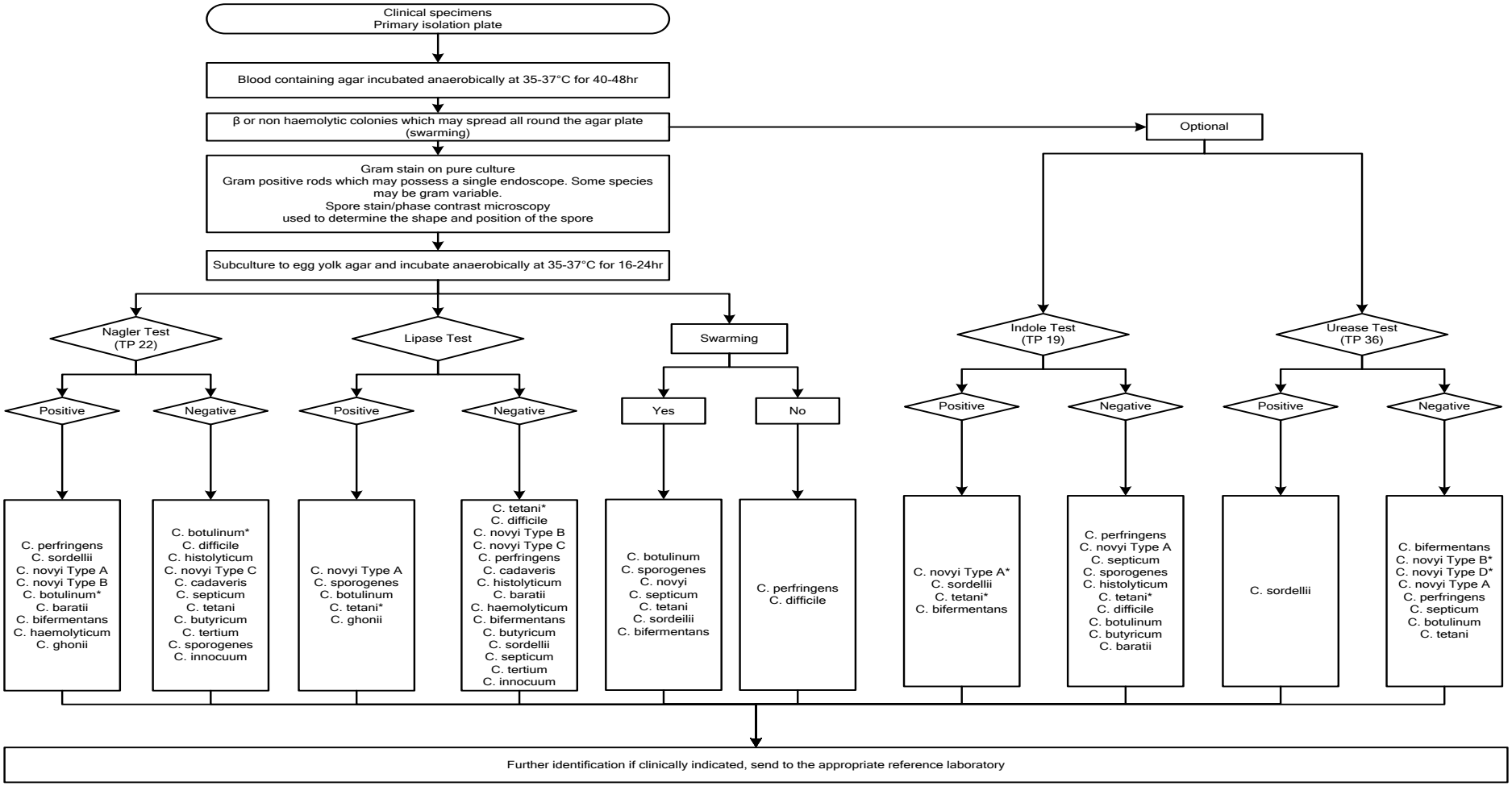
This has been used successfully to explore the phylogeny, horizontal gene transfer, recombination, and micro and macroevolution of the major hospital-acquired pathogen, *C. difficile* as well as proteolytic *C. botulinum* and *C. perfringens*[57-59].

3.6 Storage and Referral

If further identification is required, refer to the appropriate reference laboratory user manual for details on referral.

Frozen storage (-20°C) of toxin positive faecal samples is recommended for retrospective culture should the need for further investigation arise[60,61].

4 Identification of *Clostridium* species



* These give variable test results

The flowchart is for guidance only.

5 Reporting

5.1 Presumptive Identification

If appropriate growth characteristics, colonial appearances and Gram stain of the culture are demonstrated and the isolate is metronidazole susceptible.

5.2 Confirmation of Identification

Following identification processes as outlined in this document and/or Reference Laboratory report.

5.3 Medical Microbiologist

Inform the medical microbiologist of all positive cultures from normally sterile sites.

According to local protocols, the medical microbiologist should also be informed of a presumptive and confirmed *Clostridium* species. When the request card bears relevant information eg:

- Cases of trauma, penetrating injury, compound fracture or retained foreign body, or known injecting drug abuse (especially heroin)
- Septic abortion
- Suspicion of clostridial myonecrosis, (necrotising) myofasciitis, surgical wound infection (especially in cases with occlusive peripheral vascular disease and/or diabetes mellitus)
- Other serious medical conditions eg alcohol or substance abuse, immunodeficiency, cancer, or persons receiving treatment for cancer (including neutropenia and/or mucositis)
- Food poisoning (especially involving descending paralysis with cranial nerve involvement) and/or consumption of unusual or imported foods (suspicion of botulism)
- Investigation of outbreaks
- Pseudomembranous colitis or antibiotic related diarrhoea
- Suspicion of tetanus

Follow local protocols for reporting to clinician.

5.4 CCDC

Refer to local Memorandum of Understanding.

5.5 Public Health England[62]

Refer to current guidelines on CIDSC and COSURV reporting.

5.6 Infection Prevention and Control Team

Inform the infection prevention and control team of presumptive and confirmed isolates of *C. botulinum* and *C. difficile* according to local protocols.

6 Referrals

6.1 Reference Laboratory

For information on the tests offered, turnaround times, transport procedure and the other requirements of the reference laboratory refer to the appropriate reference laboratory.

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

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

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

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

Northern Ireland

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

7 Notification to PHE[62,63] or Equivalent in the Devolved Administrations[64-67]

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[64,65], Wales[66] and Northern Ireland[67].

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