

UK Standards for Microbiology Investigations

Identification of *Clostridium* species



Acknowledgments

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Users of SMIs

Three groups of users have been identified for whom SMIs are especially relevant:

* SMIs are primarily intended as a general resource for practising professionals in the field operating in the field of laboratory medicine in the UK. Specialist advice should be obtained where necessary.
* SMIs provide clinicians with information about the standard of laboratory services they should expect for the investigation of infection in their patients and the documents provide information that aids the electronic ordering of appropriate tests from hospital wards.
* SMIs also provide commissioners of healthcare services with the 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 essential laboratory methodologies which underpin quality, for example assay validation, quality assurance, and understanding uncertainty of measurement.

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 interventions, surveillance, and research and development activities. SMIs align advice on testing strategies with the UK diagnostic and public health agendas.

Involvement of Professional Organisations

The development of SMIs is undertaken within PHE in partnership with the NHS, Public Health Wales and with professional organisations.

The list of participating organisations may be found at <http://www.hpa.org.uk/SMI/Partnerships>. Inclusion of an organisation’s logo in an SMI implies support for the objectives and process of preparing SMIs. Representatives of professional organisations are members of the steering committee and working groups which develop SMIs, although the views of participants are not necessarily those of the entire organisation they represent.

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Quality Assurance

The process for the development of SMIs is certified to ISO 9001:2008.

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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 well referenced 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. SMIs should be used in conjunction with other SMIs.

UK microbiology laboratories that do not use SMIs should be able to demonstrate at least equivalence in their testing methodologies.

The performance of SMIs depends on well trained staff and the quality of reagents and equipment used. 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.

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Microbial taxonomy is up to date at the time of full review.

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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.

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| 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. |
| References. | Some references updated. |

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| References. | References reviewed and updated. |

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.

This SMI should be used in conjunction with other SMIs.

Introduction

Taxonomy

The genus *Clostridium* belongs to the family *Clostridiaceae* and itcurrently 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-accessioned1.

In 1994 the heterogeneity of this species was confirmed by 16S rRNA gene sequencing2. 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 a number 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 of *Clostridium* species

*Clostridium* are phylogenetically heterogeneous and genus now includes both Gram positive and Gram negative bacteria, spore formers and non spore formers, rods and cocci and anaerobic and non-anaerobic bacteria4.

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* are 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 CO25.

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 man6. 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*](http://www.hpa.org.uk/smi/pdf/bacteriology)). 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 produce5.

*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 tetanus6.

*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 respectively6,8. *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 letters6.

*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 agar9. 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.

***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 growth10. 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 reactions9.

***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 caecum9.

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 identification11. 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*](http://www.hpa.org.uk/smi/pdf/bacteriology).

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 demonstrated12.

**Sporulation**

Several species of *Clostridium*, including *C. carnis, C. histolyticum* and *C. tertium* can grow, but not sporulate, in air13.

**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). Eg Gram negatively staining *Clostridium* species, especially *C. clostridioforme*, can be misidentified as *Bacteriodes*11.

**Commercial identification kit**

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 reactions9.

1 Safety Considerations14-22

Hazard Group 2 organisms

Laboratory acquired infections have been reported23,24.

Refer to current guidanceon 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 cabinet16.

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 disease5,11

*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 infections13,25,26

*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](http://www.hpa.org.uk/SMI/pdf/Testprocedures))

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 UV light. 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 s*pecies | 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.

Nagler test [TP 22 - Nagler Test](http://www.hpa.org.uk/SMI/pdf/Testprocedures)

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-reaction27.

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 called 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 used28.

Indole test [TP 19 - Indole Test](http://www.hpa.org.uk/smi/pdf/testprocedures)

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 occur29.

*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](http://www.hpa.org.uk/smi/pdf/testprocedures)

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.

**Commercial identification kit**

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

3.5 Further Identification

**Rapid Methods**

A variety of current rapid identification and sensitivity methods have been developed for isolates from clinical samples; these include molecular techniques such as Real-time Polymerase Chain reaction (PCR), 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), Matrix Assisted Laser Desorption Ionisation Time-of-Flight (MALDI-TOF) 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, these methods remain accessible to reference laboratories only and are difficult to implement for routine bacterial identification in a clinical laboratory.

**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 use30.

This has been used for the identification of *Clostridium* species however, an extensive database is essential to identify species reliably and available databases needs to be optimised31.

**Real-time Polymerase Chain reaction (RT-PCR)**

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*32-36.

**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*37*, C. difficile*38, *C. novyi*39 and *C. perfringens*40. (It has equally being used to differentiate between *C. bifermentans* and *C. sordellii* strains (which closely resemble phenotypically) and between strains of *C. perfringens*41.

**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 laboratories42,43.

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*44. 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 smears45 or likely spore formation.

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 sources46.

**16S rDNA gene sequencing analysis**

A genotypic identification method, 16S rRNA 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 for to differentiate between *Clostridium* species eg between *C. novyi* type Aand *C. botulinum* type C that are closely related9.

**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* infections7.

**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*47,48.

**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, IIIumina 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*49 as well as proteolytic *C. botulinum* and *C. perfringens*50,51.

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 arise52,53.

4 Identification of *Clostridium* species Flowchart



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 England54

Refer to current guidelines on CDSC and COSURV reporting.

5.6 Infection Control Team

Inform the infection 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.

<http://www.hpa.org.uk/ProductsServices/InfectiousDiseases/LaboratoriesAndReferenceFacilities>

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1. # UK Standards for Microbiology Investigations were formerly known as National Standard Methods.

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. [↑](#footnote-ref-1)