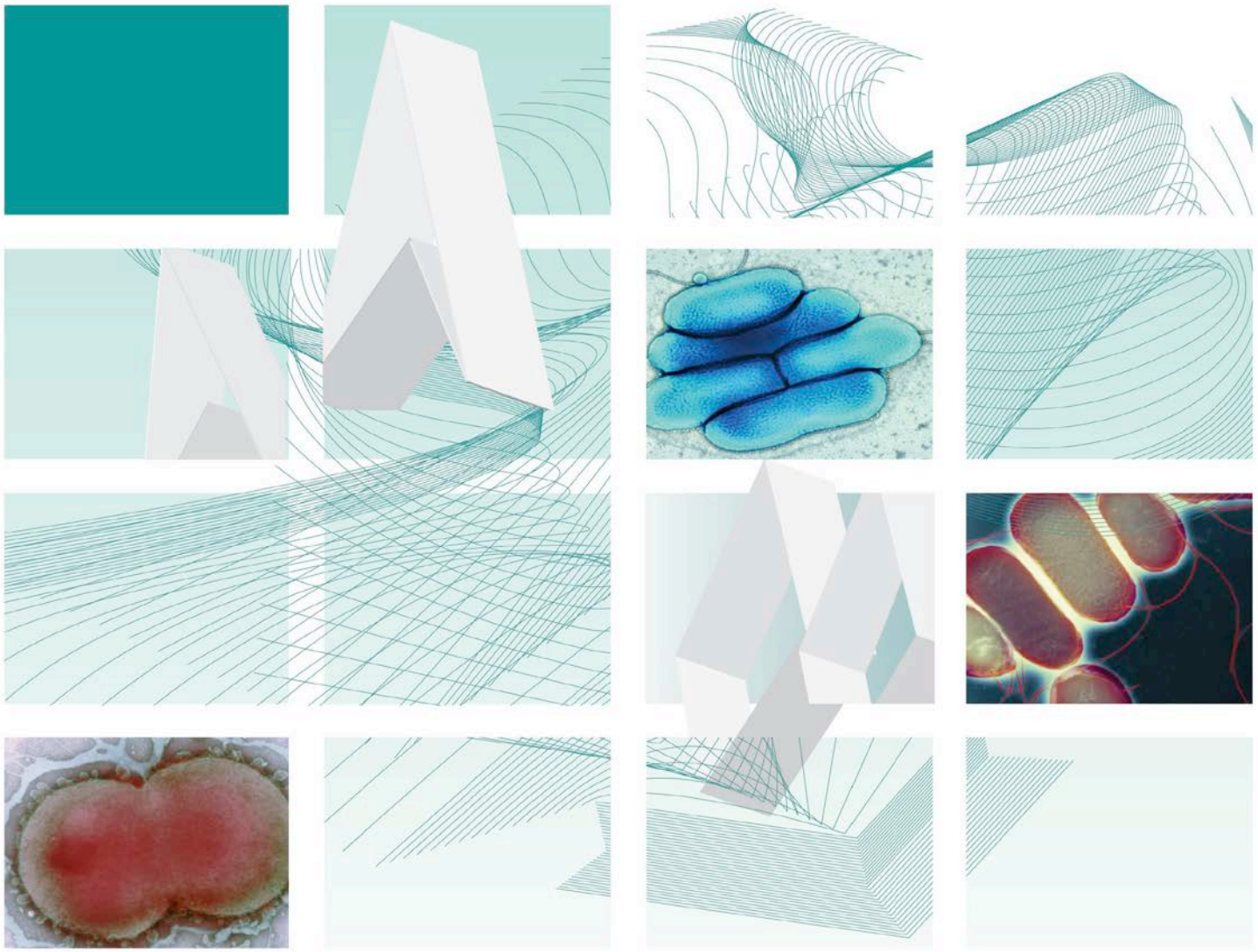
Public Health England

NHS

UK Standards for Microbiology Investigations

Identification of *Bacillus* species



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Acknowledgments

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The contributions of many individuals in clinical, specialist and reference laboratories who have provided information and comments during the development of this document are acknowledged. We are grateful to the Medical Editors for editing the medical content.

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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.	10/24.02.15
Issue no. discarded.	2.3
Insert Issue no.	3
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 *Bacillus anthracis* updated. Test procedures updated. Updated contact details of Reference Laboratories.
Scope of document.	The scope has been updated to include webpage link for B 37 document. Information regarding *Bacillus anthracis* updated.
Introduction.	The taxonomy of *Bacillus* species has been updated. More information has been added to the Characteristics section. The medically important species have been grouped and their characteristics described. Use of up-to-date references. Section on Principles of Identification has been amended for clarity.
Technical Information/Limitations.	Addition of information regarding rapid methods (MALDI-TOF) and commercial identification systems has been described and referenced.
Safety considerations.	Update on Laboratory-acquired infection with references.

	More information on the handling of *B. anthracis* has also been mentioned in this section.
Target Organisms.	The section on the Target organisms has been updated and presented clearly. References have been updated.
Identification.	Amendments and updates have been done on 3.1, 3.2, 3.3 and 3.4 have been updated to reflect standards in practice. The table in 3.4 has been amended and updated. Subsection 3.5 has been updated to include the Rapid Molecular Methods. Section 3.6 has been rephrased to refer to appropriate laboratory user manual for referrals.
Identification Flowchart.	Modification of flowchart for identification of species has been done for easy guidance.
Reporting.	Subsections 5.1 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.

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Suggested Citation for this Document

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Scope of Document

This SMI describes the identification of *Bacillus* species. The organisms described in this document are those which may be isolated from clinical material, although not all have been shown to cause human disease.

If *B. anthracis* is suspected clinically, refer specimens directly to the appropriate Reference Laboratory without doing any further work/manipulations.

Details on potential outbreak or bioterrorism can be found in B 37- Investigation of Blood Cultures (for Organisms other than *Mycobacterium* species).

This SMI should be used in conjunction with other SMIs.

Introduction

Taxonomy

This genus is one of the largest and most ubiquitous, and has gained notoriety with taxonomists for its extreme phenotypic diversity and heterogeneity. The genus *Bacillus* currently comprises 268 species and 7 subspecies although a few of these have been assigned to other genera, commonly found in the environment and as laboratory contaminants but a few of the species have been known to cause infections in humans[1,2].

Two *Bacillus* species are considered medically significant: *B. anthracis*, which causes anthrax, and *B. cereus*, which causes a foodborne illness similar to that of *Staphylococcus.*

Characteristics

Bacillus species are Gram positive rods often arranged in pairs or chains with rounded or square ends and usually have a single endospore. The endospores are generally oval or sometimes round or cylindrical and are very resistant to adverse conditions. Sporulation is not repressed by exposure to air[3]. Traditionally, *Bacillus* species was broadly divided in to three groups based on the morphology of the spore and sporangium[4,5]. The groups are:

- Group 1 – Gram positive, produce central or terminal, ellipsoidal or cylindrical spores that do not distend the sporangium: *Bacillus anthracis, Bacillus cereus, Bacillus mycoides, Bacillus thuringiensis* and *Bacillus megaterium*
- Group 2 – Gram variable with ellipsoidal spores and swollen sporangia: *Bacillus pumilus, Bacillus subtilis, Bacillus circulans, Bacillus coagulans and Bacillus licheniformis. Bacillus alvei, Bacillus brevis* and *Bacillus macerans* belonged to this group but have since been re-classified to other genera[6]
- Group 3 – Gram variable, sporangia swollen with terminal or subterminal spores: *Bacillus sphaericus*

In recent years, there has been a taxonomic development in two selected groups of the genus *Bacillus*[7]. They are called the *B. subtilis* group and the *B. cereus* group.

Bacillus cereus group

The *Bacillus cereus* group include *B. anthracis, B. cereus, B. mycoides, B. pseudomycoides B. thuringiensis* and *B. weihenstephanensis*. Most recently a cluster of thermophilic strains of clinical origin was proposed and to be named Bacillus cytotoxicus[8]. These strains are still referred to as *B. cereus* subsp. cytotoxis in the literature and public databases pending official approval of the new species designation.

This group are easily distinguished from other members of the aerobic endospore-forming bacteria but are difficult to distinguish from each other. Cells of these organisms are wider than 1µm, sporangia are not swollen, and spores are ellipsoidal. They are in principle mesophilic and neutrophilic and are placed in 16S rRNA/DNA group 1. Classical features to distinguish this group from all other aerobic endospore-forming bacteria are their inability to produce acid from mannitol and their production of lecithinase. Within the group, phenotypic differentiation is difficult. Two species (*B. cereus* and *B. thuringiensis*) are usually motile and three species (*B. cereus, B. thuringiensis,* and *B. mycoides*) are described as being haemolytic and penicillin resistant. *B. anthracis* is exclusively lysed by the gamma phage.

Many *Bacillus* species are haemolytic, a useful characteristic in differentiating them from *B. anthracis* (which is non-haemolytic). They are aerobic or facultatively anaerobic and most species are motile (a notable exception is *Bacillus anthracis*) by peritrichous flagella. Most species are oxidase positive, which may lead to confusion with *Pseudomonas* species, especially if the *Bacillus* species are poorly stained. They are usually catalase positive and metabolise carbohydrates by fermentation. *B. anthracis* is almost invariably sensitive to penicillin whereas other species are generally resistant[9].

Bacillus anthracis

If *B*. *anthracis* is suspected, specimens should be referred directly to the appropriate Reference Laboratory without doing any further work/manipulations. This organism is described on the PHE website. This section is included for information only.

B. anthracis is an endospore-forming, rod-shaped bacterium, with a width of 1-1.5µm and a length of 3-10µm in size[10]. It can be grown in an ordinary nutrient medium under aerobic or anaerobic conditions[3]. It bears close genotypical and phenotypical resemblance to *Bacillus cereus* and *Bacillus thuringiensis*. All three species share cellular dimensions and morphology. All form oval spores located centrally in an unswollen sporangium. *B. anthracis* spores in particular are highly resilient, surviving extremes of temperature, low-nutrient environments, and harsh chemical treatment over decades or centuries.

Unlike the other members of the *B. cereus* group, *B. anthracis* is non-motile and non-haemolytic on horse (or sheep's) blood agar, grows at 37°C, and forms typical grey/white flat colonies with bee's eye appearance (that is, oval, slightly granular but not dry, about 2- 5mm in diameter) with irregular edges, which are characteristically tacky on teasing with a loop[10]. The edges of *B. anthracis* are often described as 'medusa head', but this is a character that can be found throughout the *B. cereus* group[5].

Spores do not form in host tissues unless the infected body fluids are exposed to air. When nutrients are exhausted, resistant spores form that can survive in soil for

decades. These spores then germinate when exposed to a nutrient rich environment, such as the tissues or blood of an animal or human host[10].

Virulent strains of *B. anthracis* produce a characteristic polypeptide capsule, which can be demonstrated by culture on a medium containing 0.7% bicarbonate which is incubated overnight in an atmosphere with a raised CO_2 concentration. Alternatively a small volume of sterile defibrinated horse blood may be inoculated and incubated for 6 – 18hr. Colonies of capsulate *B. anthracis* appear mucoid and the capsule can be seen by the use of McFadyean's polychrome methylene blue[11,12]. Avirulent strains may occur which do not produce a capsule or toxin and these may be misidentified as *Bacillus cereus*.

Bacillus cereus

Bacillus cereus is 1 x 3-4µm in size. They present as straight or slightly curved slender bacilli with square ends singly or in short chains. They are facultative anaerobes, and like other members of the genus *Bacillus* can produce protective endospores. Capsules are not formed, but spore and sporangial morphology are similar to those of *B. anthracis*[5]. They are motile by means of peritrichous flagella and exhibit two types of motility including swimming and swarming, depending on the environment and are resistant to lysis by gamma-phage. On blood agar plate, they appear as weakly or strongly β-haemolytic large flat or slightly convex, irregular, dull grey colonies with a slight green tinge and are about 2-5mm in diameter[4]. In some instances, smooth colonies develop either alone or in the midst of rough colonies[13]. They grow optimally at temperatures between 5°C and 50°C, and are capable of adapting to a wide range of environmental conditions[4].

They are positive for metabolising carbohydrates, proteins and amino acids and can reduce nitrates to nitrites. In anaerobic respiration, *B. cereus* utilizes fermentation to generate energy. Classical features to distinguish the group 1 (which includes, *Bacillus anthracis, Bacillus cereus, Bacillus megaterium, Bacillus mycoides* and *Bacillus thuringiensis*) from the other groups are their inability to produce acid from mannitol and their production of lecithinase[7].

B. cereus is resistant to penicillin and gamma phage and this distinguishes it from *B. anthracis*[4].

B. thuringiensis is very similar to *B. cereus* but can be differentiated by the presence of crystal formation[5].

Strains of *B. weihenstephanensis* may carry genes coding for endotoxins generally associated with *Bacillus cereus*[14].

Some strains of *B. cereus* are harmful to humans and cause foodborne illness, while other strains can be beneficial as probiotics for animals.

Bacillus subtilis group

(They include *B. subtilis* subsp. *subtilis*, *B. subtilis* subsp. *spizizenii*, *B. mojavensis, B. vallismortis, B. clausii, B. atrophaeus, B. amyloliquefaciens, B. licheniformis, B. sonorensis, B. firmus, B. lentus* and *B. sporothermodurans*)

The *B. subtilis* group are closely related and are not easily distinguishable. Cells of these organisms are less than 1µm wide, sporangia are not swollen, and spores are ellipsoidal. They are in general mesophilic with regard to temperature and neutrophilic with respect to pH for growth, while often being tolerant to higher pH levels. All

members of the group are placed in 16S rRNA/DNA group 1. Application of the classical phenotypic tests for the differentiation of *Bacillus* species indicates that only for some of them have clearly discriminating features been determined. For others, phenotypic discrimination is weak, such as for *B. atrophaeus*, where pigment formation on tyrosine medium was described to differ from *B. subtilis* from which it is otherwise not distinguishable[7].

The two subspecies of *B. subtilis* (*B. subtilis* subsp. *subtilis* and *B. subtilis* subsp. *spizizenii*), *B. mojavensis,* and *B. vallismortis* are not distinguishable phenotypically so far. The same is true for *B. licheniformis* and *B. sonorensis*. All species can be differentiated on the genetic level and it is to be expected that when genotypic analyses are applied to a wider range of strains of the classical species mentioned above, additional genospecies will be detected. *B. clausii* is, strictly speaking, not a member of the *B. subtilis* group; however, it is listed here for the sake of completeness, because a number of strains previously classified as *B. subtilis* and used as probioticum have recently been reclassified as *B. clausii*[7]. Even more loosely attached to this group are the species *B. firmus, B. lentus,* and *B. sporothermodurans*, which are clearly distinguishable from the other species.

Principles of Identification

Isolates from primary culture on non-selective agar are identified by colonial appearance and the presence or absence of β-haemolysis. On selective agar such as Polymixin egg yolk mannitol bromothymol blue agar (PEMBA) *B. cereus* (which is mannitol negative and hydrolyses lecithin) produces characteristic blue colonies with a zone of precipitation. *Bacillus thuringiensis* produces a similar reaction. *B. cereus,* unlike *B. thuringiensis,* does not produce cuboid or diamond shaped parasporal crystals in cultures on sporulation agar or nutrient agar. The crystals are demonstrated with phase contrast microscopy or staining with malachite green. Care must be taken to distinguish *B. cereus* from other organisms such as *Staphylococcus aureus, Serratia marcescens* and *Proteus vulgaris* which also grow on PEMBA. These colonies can be differentiated from *B. cereus* by colonial morphology and colour. They also produce an egg yolk clearing reaction in contrast to the precipitate produced by *B. cereus*. Identification is verified by Gram stain, lecithinase activity, motility, penicillin susceptibility and biochemistry.

Clinically significant isolates including isolates from sterile sites and from stool specimens in cases of gastroenteritis should be referred to the Reference Laboratory for further confirmation.

Any specimens where *B. anthracis* is expected to be present, isolates of suspected *B. anthracis* or presumptive *B. cereus* which are identified on PEMBA plate / MALDI-TOF and are non-haemolytic should be directly referred to Rare and Imported Pathogens Laboratory (RIPL), Porton Down.

Species differentiation of the genus is complex and, in some instances in a routine laboratory, a combination of Gram stain and colonial appearance, growth on PEMBA or MALDI-TOF may be regarded as sufficient indication of a *Bacillus* species being present in a clinical specimen.

Technical Information/Limitations

Commercial Identification Systems

At the time of writing, some commercial kits may give unreliable results with the identification of *Bacillus* species resulting in poor discrimination between closely related species (*B. cereus*/*B. thuringiensis*/*B. mycoides*) and so supplementary tests are recommended for discrimination; or misidentification, (where the species identified by the identification systems are discordant with the reference identification) or unidentified[15].

MALDI-TOF MS

Rapid detection of *B. anthracis* may be challenging because of its great genetic similarity to other species of the *B. cereus* group and the difficulties of phenotypic differentiation of *B. cereus* group members. However, MALDI-TOF MS method has been found to be useful in the rapid and reliable identification of vegetative cells of the causative agent of anthrax, *Bacillus anthracis,* as long as they are prepared under standardized conditions and inactivated according to a recently developed MS-compatible inactivation protocol for highly pathogenic microorganisms. The technique has also been used in the accurate classification of *Bacillus cereus* group as well as non-*Bacillus cereus* group, especially for differentiating *B. subtilis* and *B. cereus* from *Bacillus amyloliquefaciens* and *Bacillus thuringiensis*, respectively[16-18].

MALDI-TOF has not been very useful in the distinguishing of *B. licheniformis and B. sonorensis* as they are closely related and share more phenotypic traits with each other than with any other taxon[19]. Further studies are still required to test this technology with a large collection of *Bacillus* of diverse origins.

1 Safety Considerations[20-36]

Bacillus anthracis is a Hazard Group 3 organism.

If *B. anthracis* is suspected clinically, refer specimens directly to RIPL, Porton Down without doing any further work/manipulations.

Details on potential outbreak or bioterrorism can be found in B 37- Investigation of Blood Cultures (for Organisms other than *Mycobacterium* species).

B. anthracis causes severe and sometimes fatal disease. A laboratory acquired infection has been reported[37].

In case of suspected *B. anthracis*, all laboratory procedures should be performed, by experienced scientists, in a Containment Level 3 facility using a Class 1 protective safety cabinet. Chain-of-evidence documentation should accompany specimens. Under these circumstances, there is no indication for antibiotic prophylaxis for laboratory staff unless there is an inoculation injury or a spillage releasing aerosols containing spores. Vaccination is only indicated for laboratory staff routinely working with the organism[38,39].

All Laboratory procedures that give rise to infectious aerosols must be conducted in a microbiological safety cabinet[28].

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

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

Compliance with postal and transport regulations is essential.

2 Target Organisms

Bacillus species Reported to have Caused Human Infection[5,11,13,40]

Note: If *B. anthracis* is suspected, specimens should be referred directly to the appropriate Reference Laboratory.

***Bacillus cereus* group -** *Bacillus anthracis, Bacillus cereus, Bacillus thuringiensis*

***Bacillus subtilis* group -** *Bacillus circulans, Bacillus coagulans, Bacillus licheniformis, Bacillus pumilus, Bacillus subtilis*

Other *Bacillus* species associated with infections in humans - *Bacillus sphaericus*

Other species may rarely be associated with human infection.

3 Identification

3.1 Microscopic Appearance

(TP 39 - Staining Procedures)

Gram stain

Large Gram positive rods, often in pairs or chains with rounded or square ends (which may have a single endospore). Some species may be Gram variable.

B.anthracis appears as encapsulated large Gram positive rods (box car shaped) in short chains.

McFadyean stain

Use to stain the capsule of *B. anthracis.*

Giemsa stain

Use to stain the capsule of *B. anthracis*. Capsules are only normally seen if *B. anthracis* is growing in blood serum or is present in very fresh tissue samples.

Spore stain

Use to stain the spores of *Bacillus* species. Spores will be light green and vegetative cell walls will pick up the counterstain safranin. The position of the spore in the cell differs with different species.

Note: Older cultures should be used when performing spore stain because they are lacking in nutrients and in competitive living environment.

3.2 Primary Isolation Media

Blood agar incubated in air/CO_2 at 35°C-37°C for 24 – 48hr.

Polymyxin, egg yolk, mannitol, bromothymol blue agar (PEMBA) – optional.

3.3 Colonial Appearance

Colonial appearance varies with species and a brief description is given here.

Note: If *B. anthracis* is suspected, specimens should be referred directly to the appropriate Reference Laboratory without doing any further work/manipulations.

Organism	**Haemolysis**	**Characteristics of growth on horse blood agar or PEMBA after incubation at 35°C – 37°C for 18 – 24hr**
B. anthracis	Non-haemolytic (may occasionally be weakly haemolytic)	Blood agar - Colonies are flat and irregular, 2 – 5mm in diameter, grey/white in colour with a ground glass appearance. Colonies show a tenacity that allows them to be pulled up and stay upright on teasing with a loop. PEMBA - These can be misidentified as *B. cereus* on PEMBA (*B. anthracis* is a *B. cereus* with a plasmid).
B. cereus group *(B. cereus, B. mycoides, B. pseudomycoides, B. thuringiensis, B. weihenstephanensis*)	β- haemolytic	Blood agar - Colonial appearance is similar to that of *B. anthracis* although *B. cereus* colonies both cream to white or grey and have a slight green tinge and *B. mycoides* are rhizoid or hairy looking adherent colonies which spread over the entire agar and cover the entire surface of the medium in 48 hours. PEMBA - Colonies are crenated, 5mm diameter, turquoise to peacock blue with a zone of egg yolk precipitation after 18-24hr incubation.
Bacillus subtilis group	β- haemolytic	Blood agar - Colonies are large (2 - 7mm) with a frosted-glass appearance, but may become opaque. Colour varies. Variable colonial morphology - some species may produce mucoid or smooth or raised wrinkly colonies. PEMBA – Colonies are cream to light yellow with no zone of egg

		yolk precipitation.

3.4 Test Procedures

3.4.1 Biochemical tests

Lecithinase production (TP 22 - Nagler Test)

Inoculate an egg yolk agar plate and incubate at 35°C – 37° C for 18 – 24hr, then examine for a zone of egg yolk precipitation. *B. anthracis, B. cereus*, *B. thuringiensis* and *B. mycoides* are positive.

Motility (TP 21 - Motility Test)

All *Bacillus* species are motile with the exception of *B. anthracis* and *B. mycoides.*

Penicillin susceptibility

All *Bacillus* species, with the exception of *B. anthracis,* are generally resistant to penicillin as determined by E-Test.

Crystal formation

This is used to differentiate *B. cereus* from *B. thuringiensis.* After growth on sporulation agar or on nutrient agar for at least 48hr, *B. thuringiensis* produces cuboid or diamond shaped parasporal crystals. These are demonstrated with phase contrast microscopy or staining with malachite green.

Summary of test results

Note: If *B. anthracis* is suspected, specimens should be referred directly RIPL, Porton Down without doing any further work/manipulations.

species	Lecithinase	Motility	Penicillin susceptibility	Crystal formation
Bacillus anthracis	+*	-	S	-
Bacillus cereus	+	+	R	-
Bacillus megaterium	-	+	R	-
Bacillus mycoides	+	-	R	-
Bacillus thuringiensis	+	+	R	+
Bacillus circulans	-	+	R	-
Bacillus coagulans	-	+	R	-
Bacillus licheniformis	-	+	R	-
Bacillus pumilus	-	+	R	-
Bacillus subtilis	-	+	R	-
Bacillus sphaericus	-	+	R	-
* *B. anthracis* may produce narrow lecithinase zones and colony may need to be scraped away to see reaction.				

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 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[41].

MALDI-TOF MS method has been found to be useful in the rapid and reliable identification of vegetative cells of the causative agent of anthrax, *Bacillus anthracis*, *Bacillus cereus* group as well as non-*Bacillus cereus* group[16,17]. This work by Lasch was particularly noteworthy because phenotypic tests and sequence analysis of the 16S rRNA gene could not reliably differentiate members of the *Bacillus cereus* group[42]. This technique has also been found to be a good complementary approach to 16S rRNA sequencing and even a more powerful tool in the accurate classification of *Bacillus* species, especially for differentiating *B. subtilis* and *B. cereus* from *Bacillus amyloliquefaciens* and *Bacillus thuringiensis*, respectively[18].

MALDI-TOF has not been very useful in the distinguishing of *B. licheniformis* and *B.* sonorensis as they are closely related and share more phenotypic traits with each other than with any other taxon[19].

However, further studies are still required to test this technology with a large collection of *Bacillus* of diverse origins.

Any presumptive *B. cereus* identified on MALDI TOF can be sent for confirmation to a reference laboratory.

3.4.4 Nucleic Acid Amplification Tests (NAATs)

PCR is usually considered to be a good method for bacterial detection as it is simple, sensitive and specific. 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.

There are many different PCRs for the different groups (*B. cereus* and *B. subtilis* groups) and their target genes and depending on clinical details, the appropriate PCR will be performed[43-45]. For example, in the case of *Bacillus cereus* group, a rapid PCR technique was developed based on the unique conserved sequence of the *motB* gene (encoding flagellar motor protein) from *B. cereus, B. thuringiensis* and *B. anthracis*[43]. The primers designed for this PCR are group specific and does not detect other *Bacillus* or non-*Bacillus* species which is one of its limitations.

3.5 Further Identification

Reflecting the new methods of analysis, the classification of different species into a variety of bacterial taxa has been continuously modified in a very dynamic fashion. One lineage that suitably illustrates the disagreement between molecular and phenotypic/ecological methods of classification in *Bacillus* is the *B. cereus* group. This group, also called *B. cereus sensu lato*, contains six very closely related species according to current taxonomy: *B. cereus B. thuringiensis, B. anthracis, B. mycoides, B. pseudomycoides,* and *B. weihenstephanensis*. Over the past century or so, these six species were described as individual species of the *Bacillus* genus using pathogenic host range, colony morphology and metabolic properties as distinguishing criteria, along with motility, resistance to penicillin and sensitivity to gamma phage. However, molecular methods have since shown that the species boundaries between members of this group are difficult to define, forcing us to rethink our current descriptions of these and other *Bacillus* species[46].

Rapid Methods

A variety of rapid identification and sensitivity methods have been developed for isolates from clinical samples; these include molecular techniques such as Pulsed Field Gel Electrophoresis (PFGE), Multilocus Sequence Typing (MLST), and 16S rRNA gene sequencing. 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.

16S rRNA gene sequencing

16S rRNA gene sequences has been useful in phylogenetic studies at the genus level, its use has been questioned in the case of closely related species groups such as *Bacillus*, where insufficient divergence in 16S rDNA prevented the resolution of strain and species relationships[46]. Subsequent use of housekeeping genes that are essential and therefore not lost from genomes, but that evolve more quickly than 16S rDNA, has proven to be useful for taxonomic classification[47]. Although such approaches are useful for single isolates studied intensively in the laboratory, 16S rDNA remains the gold standard for environmental sequencing projects due to its ubiquity and ease of amplification from divergent species[48].

One lineage that suitably illustrates the disagreement between molecular and phenotypic/ecological methods of classification in *Bacillus* is the *B. cereus* group. This group, also called *B. cereus sensu lato,* contains six very closely related species according to current taxonomy: *B. cereus, B. thuringiensis, B. anthracis, B. mycoides, B. pseudomycoides*, and *B. weihenstephanensis*[46,49].

However, significant improvements could be made by the addition of ecological data as in the case of the *B. cereus* group. For example, sequencing of 16S rDNA or other conserved loci can be used for initial clustering and identification of closely related strains/species, which could then be followed by the more in-depth genomic characterization of interesting groups of strains. Such a study has recently been done in *B. subtilis* using microarray and sequencing technologies and this has uncovered a great deal of genomic diversity within this group of closely related *B. subtilis* strains[50-52].

Multilocus Sequence Typing (MLST)

Multilocus sequence typing (MLST) is a tool that is widely used for phylogenetic typing of bacteria. MLST is based on PCR amplification and sequencing of internal fragments of a number (usually 6 or 7) of essential or housekeeping genes spread around the bacterial chromosome. MLST has been extensively used as the main typing method for analysing the genetic relationships within the whole *B. cereus* group population.

This method reinforces the fact that the *B. cereus* group constitutes a coherent population in which the members are unified by the presence of ubiquitous and specific genetic elements whose genomic locations and sequences allow no distinction between the various species of the group and that this population is dynamic[53]. This is done by use of a developed database (called the 'SuperCAT') that compiles and integrates all MLST data from all the 5 schemes[54].

Pulsed Field Gel Electrophoresis (PFGE)

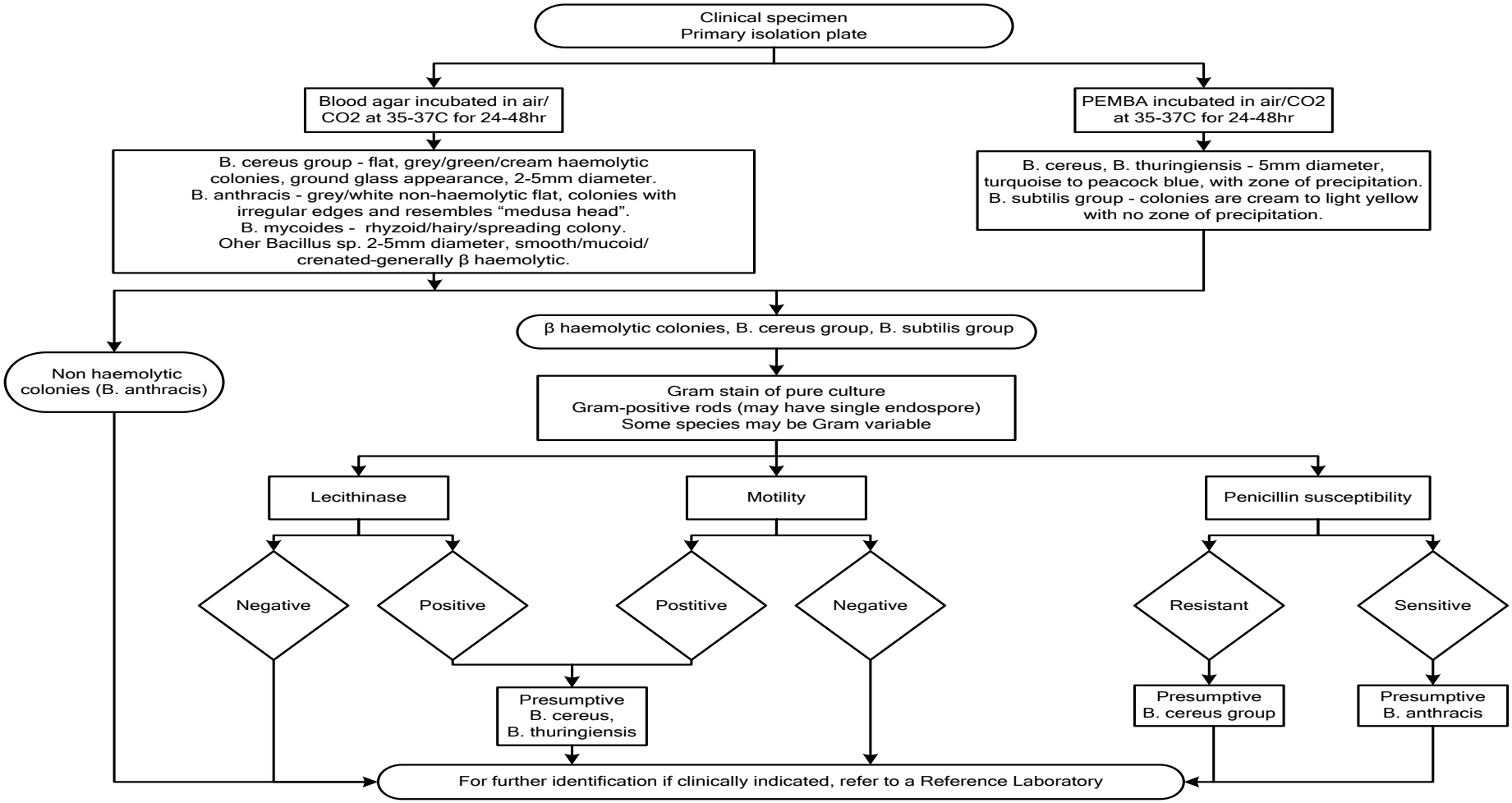
PFGE detects genetic variation between strains using rare-cutting restriction endonucleases, followed by separation of the resulting large genomic fragments on an agarose gel. PFGE is known to be highly discriminatory and a frequently used technique for outbreak investigations. However, the stability of PFGE may be insufficient for reliable application in long-term epidemiological studies. 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[55,56].

This has been used successfully to discriminate *Bacillus anthracis* from *B. cereus* and *B. thuringiensis* by using the PFGE profiles from *Not*I digestion, after a few modifications to the PFGE procedure to facilitate complete lysis[57,58].

3.6 Storage and Referral

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

4 Identification of *Bacillus* species



The flowchart is for guidance only

5 Reporting

5.1 Presumptive Identification

If appropriate growth characteristics, colonial appearance and Gram stain of the culture, are demonstrated.

5.2 Confirmation of Identification

Following lecithinase activity, motility, penicillin susceptibility and crystal formation results and/or the Reference Laboratory report.

5.3 Medical Microbiologist

Inform the medical microbiologist of all positive cultures from specimens from normally sterile sites and of all isolates of presumed and confirmed *Bacillus anthracis.*

According to local protocols, the medical microbiologist should be informed when the request card bears relevant information which suggests anthrax among the differential diagnoses.

- Ulcerating skin lesions with a black eschar
- Fulminating pneumonia (especially with widening of the mediastinum on X-ray) and in outbreaks of the same)
- Circumstances predisposing to infection with *B. anthracis* eg farming, horticulture, veterinary, dockyard, tannery, woollen textile or medical laboratory work
- Deliberate release
- Injecting drug users

The medical microbiologist should also be informed of other *Bacillus* species (other than *B. anthracis*), presumed or confirmed in accordance with local protocol, when the request form bears relevant additional information for example:

- Penetrating injury, compound fracture or retained foreign body
- Infection of an indwelling medical devices, such as prosthetic valves, pacemaker, CSF shunt or peritoneal or vascular catheter
- Food poisoning
- Investigation of a possible outbreak

Follow local protocols for reporting to the patient's clinicians.

5.4 CCDC

Refer to local Memorandum of Understanding.

5.5 Public Health England[59]

Refer to current guidelines on CIDSC and COSURV reporting.

5.6 Infection Prevention and Control Team

Inform the relevant infection prevention and control team of presumed or confirmed isolates of *B. anthracis* according to local protocols.

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:

Bacillus anthracis

Rare and Imported Pathogens Laboratory
Public Health England
Porton Down
Salisbury
Wiltshire
SP4 0JG
United Kingdom
Telephone +44 (0) 1980 612100

https://www.gov.uk/government/collections/rare-and-imported-pathogens-laboratory-ripl

***Bacillus cereus* and other *Bacillus* species**

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

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

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

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[59,60] or Equivalent in the Devolved Administrations[61-64]

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[61,62], Wales[63] and Northern Ireland[64].

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