NATIONAL STANDARD METHOD

IDENTIFICATION OF
BACILLUS SPECIES

BSOP ID 9
Issued by Standards Unit, Evaluations and Standards Laboratory
Centre for Infections

This SOP should be used in conjunction with the series of other SOPs from the Health Protection Agency
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Suggested citation for this document:
Each National Standard Method has an individual record of amendments. The current amendments are listed on this page. The amendment history is available from standards@hpa.org.uk.

On issue of revised or new pages each controlled document should be updated by the copyholder in the laboratory.

<table>
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<th>Amendment Number/ Date</th>
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<th>Insert Issue no.</th>
<th>Page</th>
<th>Section(s) involved</th>
<th>Amendment</th>
</tr>
</thead>
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<td>12</td>
<td>6 Referrals</td>
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<td>References</td>
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IDENTIFICATION OF BACILLUS SPECIES

SCOPE OF DOCUMENT
This National Standard Method (NSM) 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.

INTRODUCTION

Taxonomy
The genus Bacillus currently comprises in excess of 60 species, commonly found in the environment and as laboratory contaminants.

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 and are very resistant to adverse conditions. Sporulation is not repressed by exposure to air. Bacillus species can be broadly divided in three groups based on the morphology of the spore and sporangium. The groups are:

Group 1 - Gram-positive, produce central or terminal ellipsoidal or cylindrical spores that do not distend the sporangium
Group 2 - Gram-variable with ellipsoidal spores and swollen sporangia
Group 3 - Gram-variable, sporangia swollen with terminal or subterminal spores

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 CO₂ concentration. Alternatively a small volume of sterile defibrinated horse blood may be inoculated and incubated for 6 - 18 hours. Colonies of capsulate B. anthracis appear mucoid and the capsule can be seen by the use of McFadyean’s polychrome methylene blue. Avirulent strains may occur which do not produce a capsule or toxin and these may be misidentified as Bacillus cereus.

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.

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 incubated for at least 2 days. 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. Significant isolates should be referred to the Reference Laboratory for confirmation of identity and toxin testing.
Species differentiation of the genus is complex and, in some instances in a routine laboratory, a combination of Gram stain and colonial appearance may be regarded as sufficient indication of a *Bacillus* species being present in a clinical specimen.

If *B. anthracis* is suspected, specimens should be referred directly to the Reference Laboratory. This organism is described on the HPA website – [http://www.hpa.org.uk/infections/topics_az/deliberate_release/Anthrax/LaboratoryPictures.asp?Source=Professional&Agent=Anthrax&Document=LaboratoryPictures](http://www.hpa.org.uk/infections/topics_az/deliberate_release/Anthrax/LaboratoryPictures.asp?Source=Professional&Agent=Anthrax&Document=LaboratoryPictures)

**TECHNICAL INFORMATION**

N/A
1 SAFETY CONSIDERATIONS

Bacillus anthracis is a Hazard Group 3 organism - all work on suspected isolates must be performed in a microbiological safety cabinet in a Containment Level 3 room.

If B. anthracis is suspected clinically, refer specimens directly to the Reference Laboratory.

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

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

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

Compliance with postal and transport regulations is essential.

2 TARGET ORGANISMS

Bacillus species reported to have caused human infection

Group 1 - Gram-positive, produce central or terminal ellipsoidal or cylindrical spores which do not distend the sporangium.
- Bacillus anthracis
- Bacillus cereus
- Bacillus megaterium
- Bacillus mycoides
- Bacillus thuringiensis

Group 2 - Gram-variable with ellipsoidal, central or sub-terminal spores and swollen sporangia.
- Bacillus alvei
- Bacillus brevis
- Bacillus circulans
- Bacillus coagulans
- Bacillus licheniformis
- Bacillus macerans
- Bacillus pumilus
- Bacillus subtilis

Group 3 - Gram-variable with spherical, terminal or sub-terminal spores and swollen sporangia.
- Bacillus sphaericus

Other species may rarely be associated with human infection.
3 IDENTIFICATION

3.1 MICROSCOPIC APPEARANCE

Gram stain
Large Gram-positive rods (which may have a single endospore). Some species may be Gram-variable.

McFadyean’s stain
Use to stain the capsule of *B. anthracis*.

Giemsa’s stain
Use to stain the capsule of *B. anthracis*.

NB capsules are only normally seen if *B. anthracis* is growing in blood serum or is present in very fresh tissue samples

3.2 PRIMARY ISOLATION MEDIA

Blood agar incubated in air/CO\(_2\) at 35°C - 37°C for 24 – 48 h.

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

<table>
<thead>
<tr>
<th>Organism</th>
<th>Haemolysis</th>
<th>Characteristics of growth on horse blood agar or PEMBA after incubation at 35°C – 37°C for 18 - 24h</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. anthracis</em></td>
<td>non (may occasionally be weakly haemolytic)</td>
<td>Blood agar - Colonies are flat and irregular, 2 – 5 mm in diameter, grey/white in colour with a ground glass appearance</td>
</tr>
<tr>
<td><em>B. cereus</em> group</td>
<td>β</td>
<td>Blood agar - Colonial appearance is similar to that of <em>B. anthracis</em> although <em>B. cereus</em> colonies are cream to white and <em>B. mycoides</em> are rhizoid or hairy looking PEMBA - Colonies are crenated, 5 mm diameter, turquoise to peacock blue with a zone of egg yolk precipitation</td>
</tr>
<tr>
<td>Other <em>Bacillus</em> species</td>
<td>β</td>
<td>Blood agar - Colonies are large (2 - 7 mm) with a frosted-glass appearance, but may become opaque. Colour varies. Some species may produce mucoid or smooth colonies PEMBA - <em>B. thuringiensis</em> forms similar colonies to <em>B. cereus</em></td>
</tr>
</tbody>
</table>

3.4 TEST PROCEDURES

Lecithinase production (see BSOP 22 - Nagler Test)
Inoculate an egg yolk agar plate and incubate at 35°C – 37°C for 18 - 24 h then examine for a zone of egg yolk precipitation. *B. anthracis, B. cereus, B. thuringiensis* and *B. mycoides* are positive.

Motility (see BSOP 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**

Use to differentiate *B. cereus* from *B. thuringiensis*. After growth on sporulation agar or on nutrient agar for at least 48 h, *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**

<table>
<thead>
<tr>
<th>Lecithinase</th>
<th>Motility</th>
<th>Penicillin susceptibility</th>
<th>Crystal formation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus anthracis</em></td>
<td>+*</td>
<td>-</td>
<td>S</td>
</tr>
<tr>
<td><em>Bacillus cereus</em></td>
<td>+</td>
<td>+</td>
<td>R</td>
</tr>
<tr>
<td><em>Bacillus megaterium</em></td>
<td>-</td>
<td>+</td>
<td>R</td>
</tr>
<tr>
<td><em>Bacillus mycoides</em></td>
<td>+</td>
<td>-</td>
<td>R</td>
</tr>
<tr>
<td><em>Bacillus thuringiensis</em></td>
<td>+</td>
<td>+</td>
<td>R</td>
</tr>
<tr>
<td><em>Bacillus alvei</em></td>
<td>-</td>
<td>+</td>
<td>R</td>
</tr>
<tr>
<td><em>Bacillus brevis</em></td>
<td>-</td>
<td>+</td>
<td>R</td>
</tr>
<tr>
<td><em>Bacillus circulans</em></td>
<td>-</td>
<td>+</td>
<td>R</td>
</tr>
<tr>
<td><em>Bacillus coagulans</em></td>
<td>-</td>
<td>+</td>
<td>R</td>
</tr>
<tr>
<td><em>Bacillus licheniformis</em></td>
<td>-</td>
<td>+</td>
<td>R</td>
</tr>
<tr>
<td><em>Bacillus macerans</em></td>
<td>-</td>
<td>+</td>
<td>R</td>
</tr>
<tr>
<td><em>Bacillus pumilus</em></td>
<td>-</td>
<td>+</td>
<td>R</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>-</td>
<td>+</td>
<td>R</td>
</tr>
<tr>
<td><em>Bacillus sphaericus</em></td>
<td>-</td>
<td>+</td>
<td>R</td>
</tr>
</tbody>
</table>

* *B. anthracis* may produce narrow lecithinase zones and colony may need to be scraped away to see reaction.

### 3.5 FURTHER IDENTIFICATION

N/A

### 3.6 STORAGE AND REFERRAL

Save the pure isolate on a nutrient agar slope for referral to the Reference Laboratory.
4 IDENTIFICATION OF BACILLUS – FLOW CHART

Clinical specimen
Primary isolation plate

Blood agar

B. anthracis/bacillus group – flat, greyish/brown color, ground glass appearance, 2-5 mm diameter. B. mycoides – smooth, with spreading colony. Other Bacillus sp. 2-5 mm diameter, smooth, mucoid, crenated – generally β haemolytic.

PEMBA

B. cereus, B. thuringiensis
5 mm diameter, turquoise to peacock blue, with zone of precipitation

Non-β haemolytic colonies should be sent immediately to the reference lab for B. anthracis PCR and further characterisation.

Gram stain of pure culture
Gram-positive rods (may have single endospore)
Some species may be Gram-variable

Lecithinase

Negative

Positive

Motility

Send to the reference lab for B. anthracis PCR and further characterisation

Penicillin susceptibility

Sensitive

Resistant

Presumptive B. anthracis

Presumptive B. mycoides

Presumptive B. cereus or B. thuringiensis

Further identification if clinically indicated
Refer to the Reference Laboratory
If required, save the pure isolate on a nutrient agar slope

IDENTIFICATION OF BACILLUS SPECIES
Issue no: 2.1 Issue date: 14.09.07 Issued by: Standards Unit, Evaluations and Standards Laboratory Page no: 10 of 15
BSOP ID 912.1
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5 REPORTING

5.1 PRESumptIVE IDENTIFICATION
If appropriate growth characteristics, colonial appearance and Gram’s stain of the culture, are demonstrated.

5.2 CONFIRMATION OF IDENTIFICATION
Following lecithinase activity, motility, penicillin susceptibility and commercial identification kit 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

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 outbreaks

Follow local protocols for reporting to the patient’s clinicians

5.4 CCDC
Refer to local Memorandum of Understanding.

5.5 CENTRE FOR INFECTIONS
Refer to current guidelines on CDSC and COSURV reporting.

5.6 INFECTION CONTROL STAFF
Inform the relevant infection control team of presumed or confirmed isolates of *B. anthracis*. 

*IDENTIFICATION OF BACILLUS SPECIES*
Issue no: 2.1 Issue date: 14.09.07 Issued by: Standards Unit, Evaluations and Standards Laboratory Page no: 11 of 15
BSOP ID 9i2.1
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6 REFERRALS

6.1 REFERENCE LABORATORIES

For information on the tests offered, turn around times, transport procedure and the other requirements of the reference laboratory refer to:

*Bacillus anthracis*
Special Pathogens Reference Unit
Centre for Emergency Preparedness and Response
Porton Down
Salisbury
Wilts
SP5 OJG
Telephone +44 (0) 1980 612100
http://www.hpa.org.uk/cepr/specialpathogens/default.htm

*Bacillus cereus* and other *Bacillus* species
Food Hygiene Laboratory
Centre for Infections
Health Protection Agency
61 Colindale Avenue
London
NW9 5HT
Contact Centre for Infections main switchboard: Tel. +44 (0) 20 8200 4400
http://www.hpa.org.uk/cfi/dgi/downloads.htm
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The National Standard Methods are issued by Standards Unit, Evaluations and Standards Laboratory, Centre for Infections, Health Protection Agency London.

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Issue no: 2.1 Issue date: 14.09.07 Issued by: Standards Unit, Evaluations and Standards Laboratory Page no: 14 of 15

BSOP ID 92.1

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