IDENTIFICATION OF ANAEROBIC GRAM-NEGATIVE RODS

BSOP ID 25

Issued by Standards Unit, Department for Evaluations, Standards and Training
Centre for Infections
STATUS OF NATIONAL STANDARD METHODS

National Standard Methods, which include standard operating procedures (SOPs), algorithms and guidance notes, promote high quality practices and help to assure the comparability of diagnostic information obtained in different laboratories. This in turn facilitates standardisation of surveillance underpinned by research, development and audit and promotes public health and patient confidence in their healthcare services. The methods are well referenced and represent a good minimum standard for clinical and public health microbiology. However, in using National Standard Methods, laboratories should take account of local requirements and may need to undertake additional investigations. The methods also provide a reference point for method development.

National Standard Methods are developed, reviewed and updated through an open and wide consultation process where the views of all participants are considered and the resulting documents reflect the majority agreement of contributors.

Representatives of several professional organisations, including those whose logos appear on the front cover, are members of the working groups which develop National Standard Methods. Inclusion of an organisation’s logo on the front cover implies support for the objectives and process of preparing standard methods. The representatives participate in the development of the National Standard Methods but their views are not necessarily those of the entire organisation of which they are a member. The current list of participating organisations can be obtained by emailing standards@hpa.org.uk.

The performance of standard methods depends on the quality of reagents, equipment, commercial and in-house test procedures. Laboratories should ensure that these have been validated and shown to be fit for purpose. Internal and external quality assurance procedures should also be in place.

Whereas every care has been taken in the preparation of this publication, the Health Protection Agency or any supporting organisation cannot be responsible for the accuracy of any statement or representation made or the consequences arising from the use of or alteration to any information contained in it. These procedures are intended solely as a general resource for practising professionals in the field, operating in the UK, and specialist advice should be obtained where necessary. If you make any changes to this publication, it must be made clear where changes have been made to the original document. The Health Protection Agency (HPA) should at all times be acknowledged.

The HPA is an independent organisation dedicated to protecting people’s health. It brings together the expertise formerly in a number of official organisations. More information about the HPA can be found at www.hpa.org.uk.

The HPA aims to be a fully Caldicott compliant organisation. It seeks to take every possible precaution to prevent unauthorised disclosure of patient details and to ensure that patient-related records are kept under secure conditions.

More details can be found on the website at www.evaluations-standards.org.uk. Contributions to the development of the documents can be made by contacting standards@hpa.org.uk.

The reader is informed that all taxonomy in this document was correct at time of issue.

Suggested citation for this document:
AMENDMENT PROCEDURE

<table>
<thead>
<tr>
<th>Controlled reference</th>
<th>document title</th>
<th>BSOP ID 25</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Identification Of Anaerobic Gram-Negative Rods</td>
<td></td>
</tr>
</tbody>
</table>

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>
<thead>
<tr>
<th>Amendment no./ Date</th>
<th>Issue no. Discarded</th>
<th>Insert Issue no.</th>
<th>Page</th>
<th>Section(s) involved</th>
<th>Amendment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
IDENTIFICATION OF ANAEROBIC GRAM-NEGATIVE RODS

SCOPE OF DOCUMENT

This National Standard Method (NSM) describes the characterisation of non-sporing, non-branching, Gram-negative anaerobic bacteria.

Anaerobic sporing organisms are described in BSOPID 8 - Identification of Clostridium species, BSOP ID 15 – Identification anaerobic Actinomyces species and BSOPID 10 - Identification of aerobic actinomycetes cover the identification of actinomycetes. Anaerobic cocci can be found in BSOP ID 14 – Identification of anaerobic cocci.

INTRODUCTION

Taxonomy

The taxonomy of the anaerobic bacteria is in a state of continuous change due to the constant addition of new species and the reclassification of the old. An example of this would be the genus Bacteroides. This genus previously included most of the saccharolytic pigmented species that are now included in the genus Prevotella and the asaccharolytic species which have been assigned to the genus Porphyromonas.

There are more than 20 genera of anaerobic Gram-negative rods. The most common human isolates belong to the genera Bacteroides, Fusobacterium, Porphyromonas and Prevotella.

Characteristics

Bacteroides species

Bacteroides species are rod shaped organisms that vary in size, many of them are pleomorphic and show terminal or central swellings, vacuoles or filaments. Bacteroides are bile resistant, aesculin positive and carbohydrate fermenters. Bacteroides fragilis is the most commonly isolated species from clinical samples.

Fusobacterium species

Fusobacterium species are rods which may be spindle-shaped eg Fusobacterium nucleatum or pleomorphic eg Fusobacterium necrophorum. These two species are the most commonly isolated from human clinical material. F. necrophorum is a cause of serious infections (necrobacillosis or Lemièrre’s disease) commonly diagnosed in young adults and also a cause of recurrent sore throats. It can be recognised by production of characteristic cream-yellow colonies that are indole positive and fluoresce under UV light and produce lipase on egg yolk agar.

Fusobacterium species that are grown on fastidious anaerobe agar (FAA) containing blood may fluoresce yellow-green (chartreuse) when exposed to long wave (365 nm) ultraviolet light. This phenomenon is medium-dependent.

Porphyromonas species

The genus Porphyromonas includes asaccharolytic, catalase-negative species of human and animal origin. They are short rods (0.5 - 0.8 x 1.0 - 3.0 µm) and are bile sensitive.

Most Porphyromonas species isolated from humans are catalase-negative whereas those from animals are catalase-positive.

Some Porphyromonas species may fluoresce brick red when exposed to long wave (365 nm) ultraviolet light and can produce a pigment (buff to tan to black) when grown on blood-containing media which is due to porphyrin production.
**Prevotella species**

The genus *Prevotella* is composed of mainly saccharolytic, pigmented or non-pigmented species that were previously classified as *Bacteroides*, and these are usually pleomorphic.

Young cultures of *Prevotella* species may fluoresce brick red when exposed to long wave (365 nm) ultraviolet light, and this may fade to a tan or black pigment when grown on blood-containing media for extended periods.

**Principles of identification**

Colonies are usually isolated on FAA (or equivalent) or blood agar and incubated anaerobically. Colonies can be characterised according to colonial morphology and Gram’s stain reaction and are usually sensitive to a 5 µg metronidazole disc. Some species may require longer than 48 hours incubation to grow. Identification tends to be undertaken only if clinically indicated. Further identification tests include fluorescence under long wave UV light (365 nm), pigment production, indole production, bile tolerance, glucose fermentation, and lecinthinase and/or lipase activity on egg yolk agar. Classification of many anaerobes to species or even genus level requires additional biochemical tests or metabolic end product analysis by GLC. Identification may be attempted using commercial kits but their results are not always reliable. Identification of clinically significant or unusual organisms may be carried out by the Anaerobe Reference Laboratory, Cardiff. Clinical specimens for anaerobic culture should be cultured on a selective medium such as neomycin agar in addition to a non-selective fastidious anaerobe blood agar.

**TECHNICAL INFORMATION/LIMITATIONS**

Neomycin agar is used to inhibit the growth of facultative organisms in a mixed culture, but in certain instances because of the inhibitory aspects of the neomycin some anaerobes may also not grow.

In the clinical diagnostic laboratory, susceptibility to metronidazole is frequently used as an indicator of any anaerobe being present in a clinical specimen. However, an increasing number of metronidazole resistant anaerobes such as *Bacteroides fragilis* are being recorded and these organisms may be missed by such an approach. It is important to consider anaerobes regardless of metronidazole susceptibility in certain clinical specimens or situations where anaerobes are suspected.
1 SAFETY CONSIDERATIONS

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

*Bacteroides fragilis* group reported to have caused human infection

<table>
<thead>
<tr>
<th>B. fragilis</th>
<th>B. ovatus</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. caccae</td>
<td>B. stercoralis</td>
</tr>
<tr>
<td>B. distasonis</td>
<td>B. thetaiotaomicron</td>
</tr>
<tr>
<td>B. eggerthii</td>
<td>B. uniformis</td>
</tr>
<tr>
<td>B. merdae</td>
<td>B. vulgatus</td>
</tr>
</tbody>
</table>

*Bacteroides* species (taxonomic position uncertain) reported to have caused human infection

<table>
<thead>
<tr>
<th>B. capillosus</th>
<th>B. pyogenes</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. coagulans</td>
<td>B. splanchnicus</td>
</tr>
<tr>
<td>B. forsythus</td>
<td>B. tectum</td>
</tr>
<tr>
<td>B. putredinis</td>
<td>B. ureolyticus</td>
</tr>
</tbody>
</table>

*Fusobacterium* species reported to have caused human infection

<table>
<thead>
<tr>
<th>F. alocis</th>
<th>F. nucleatum subspecies fusiforme</th>
</tr>
</thead>
<tbody>
<tr>
<td>F. gonidiaformans</td>
<td>F. nucleatum subspecies nucleatum</td>
</tr>
<tr>
<td>F. mortiferum</td>
<td>F. nucleatum subspecies polymorphum</td>
</tr>
<tr>
<td>F. naviforme</td>
<td>F. nucleatum subspecies vincentii</td>
</tr>
<tr>
<td>F. necrogenes</td>
<td>F. periodonticum</td>
</tr>
<tr>
<td>F. necrophorum</td>
<td>F. russii</td>
</tr>
<tr>
<td>F. necrophorum subspecies funduliforme</td>
<td>F. sulci</td>
</tr>
<tr>
<td>F. necrophorum subspecies necrophorum</td>
<td>F. ulcerans</td>
</tr>
<tr>
<td>F. nucleatum</td>
<td></td>
</tr>
</tbody>
</table>

*Porphyromonas* species reported to have caused human infection

<table>
<thead>
<tr>
<th>P. asaccharolytica</th>
<th>P. endodontalis</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. catoniae</td>
<td>P. gingivalis</td>
</tr>
</tbody>
</table>

*Prevotella* species reported to have caused human infection

<table>
<thead>
<tr>
<th>P. bivia</th>
<th>P. heparinolytica</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. buccae</td>
<td>P. intermedia*</td>
</tr>
<tr>
<td>P. corporis*</td>
<td>P. loescheii*</td>
</tr>
<tr>
<td>P. dentalis</td>
<td>P. melaninogenica*</td>
</tr>
<tr>
<td>P. denticola*</td>
<td>P. nigrescens*</td>
</tr>
<tr>
<td>P. disiens</td>
<td>P. oris</td>
</tr>
<tr>
<td>P. enoeca</td>
<td>P. tannerae*</td>
</tr>
</tbody>
</table>

* Pigmented species

Other species may be associated with human disease
3 IDENTIFICATION

3.1 MICROSCOPIC APPEARANCE

Gram's stain (BSOPTP 39 - Staining Procedures)

*Bacteroides, Porphyromonas* and *Prevotella* species are small, Gram-negative rods of variable length.

*Fusobacterium* species are Gram-negative rods, highly variable in length and width, and they may have pointed ends. *F. nucleatum* is a slim filamentous rod usually with pointed ends and is indole positive. *F. necrophorum* is a pleomorphic rod that produces indole and lipase on egg yolk agar.

3.2 PRIMARY ISOLATION MEDIA

Fastidious anaerobe agar or equivalent (with or without neomycin – some anaerobic organisms may be inhibited by neomycin) incubated for 40 – 48 h anaerobically at 35°C - 37°C.

**Note:** some species may require longer incubation.

3.3 COLONIAL APPEARANCE

<table>
<thead>
<tr>
<th>Genus</th>
<th>Characteristics of growth on fastidious anaerobe agar after anaerobic incubation at 35°C - 37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteroides</td>
<td>Colonies are 1 - 3 mm diameter, circular, low convex, smooth, semi-opaque grey and are often moist or even mucoid. Mostly non-haemolytic and resistant to an ox-bile disc</td>
</tr>
<tr>
<td>Fusobacterium</td>
<td>Colonial appearance is variable, but most are 1-3 mm diameter, with an irregular or dentate edge. They vary from translucent to granular and opaque; <em>F. necrophorum</em> may be beta-haemolytic. Indole positive, fluorescent yellow-green under long wave UV light.</td>
</tr>
<tr>
<td>Porphyromonas</td>
<td>&lt;1.0 mm diameter after 48 h incubation, smooth, shiny and grey. Dark brown or black pigment develops after 3 - 7 days. Growth may be enhanced by “satellitism” around colonies of other organisms eg staphylococci</td>
</tr>
<tr>
<td>Prevotella</td>
<td>Colonies are similar to those of <em>Bacteroides</em> species, except some species are pigmented (may be pale brown to black). Most pigmented species are haemolytic</td>
</tr>
</tbody>
</table>

3.4 TEST PROCEDURES

**Metronidazole**

Isolate shows a zone of inhibition to metronidazole 5 µg disc after anaerobic incubation on a suitable agar medium.

**Note:** In the clinical diagnostic laboratory, susceptibility to metronidazole is frequently regarded as sufficient indicator of an anaerobe being present in a given specimen. Some anaerobes eg *B. fragilis* are becoming resistant to metronidazole, and these organisms will be missed by such an approach. Colonies suspected of being *Bacteroides* species resistant to metronidazole should be checked for lack of growth in air and CO₂ and referred to the Anaerobe Reference Laboratory for confirmation.
AnIdent ring/discs
Follow manufacturers instructions

Bile tolerance
Catalase (BSOPTP 8 - Catalase test)

Nitrate

3.5 FURTHER IDENTIFICATION

Fluorescence under long wavelength UV light (365 nm)
Porphyromonas and Prevotella species may fluoresce orange to brick red, Fusobacterium species may fluoresce yellow-green (chartreuse) and Bacteroides species generally do not fluoresce.

Lipase/Lecithinase production
Production of lipase or lecithinase may be used to differentiate F. necrophorum (lipase positive) from F. nucleatum (lipase negative).

Commercial identification kit
Results should be interpreted with caution in conjunction with other test results.

Glucose fermentation may be used to differentiate Prevotella species from Porphyromonas species.

Other more specialized tests
Gas-Liquid Chromatography of metabolic end products, 16S rDNA sequencing or Amplified Ribosomal DNA Restriction Analysis (ARDRA).

3.6 STORAGE AND REFERRAL
If required, for short term storage save the pure isolate in fastidious anaerobe broth with cooked meat for referral to the Reference Laboratory. Isolates may also be referred on swabs in transport media. For long term storage cultures should be frozen at -70°C in a suitable cryogenic medium.
4 FLOW CHART - PRESUMPTIVE IDENTIFICATION OF ANAEROBIC GRAM-NEGATIVE RODS

Clinical specimens
Primary isolation plate

Fastidious anaerobe agar or equivalent, with or without neomycin

Metronidazole sensitive

May report as “Anaerobes isolated”

Gram’s stain

May report as Gram-negative anaerobic rod

Gram-negative rods (may be filamentous)

Other Gram’s stain result refer to the appropriate NSM

Further identification if clinically indicated:
Commercial identification kit or other biochemical identification or GLC
If required, save the pure isolate in fastidious anaerobe broth with cooked meat for referral to the Reference Laboratory
5 REPORTING

5.1 PRESUMPTIVE IDENTIFICATION
If appropriate growth characteristics, colonial appearance, Gram’s stain of the culture are demonstrated and the isolate is metronidazole susceptible.

5.2 CONFIRMATION OF IDENTIFICATION
Following commercial identification kit results and/or the Reference Laboratory report.

5.3 MEDICAL MICROBIOLOGIST
Inform the medical microbiologist of presumptive or confirmed non-sporing anaerobes when the request card bears relevant information eg:
- Septicaemia/bacteraemia
- Empyemas, surgical wound infection, abscess formation (especially cerebral, intraperitoneal, lung, liver or splenic abscesses)
- Puerperal sepsis
- Myofasciitis (necrotising)
- Suspicion of Lemière’s Syndrome (post anginal sepsis, often with jugular suppurative thrombophlebitis and haematogenous pulmonary abscesses)

Follow local protocols for reporting to clinician

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
N/A

6 REFERRALS

6.1 REFERENCE LABORATORY
Anaerobe Reference Laboratory
NPHS Microbiology Cardiff
University Hospital of Wales
Heath Park
Cardiff CF14 4XW

Telephone +44 (0) 29 2074 2171 or 2378

http://www.hpa.org.uk/cfi/arl/default.htm
ACKNOWLEDGEMENTS AND CONTACTS

This National Standard Method has been developed, reviewed and revised by the National Standard Methods Working Group for Clinical Bacteriology (http://www.hpa-standardmethods.org.uk/wg_bacteriology.asp). The contributions of many individuals in clinical bacteriology laboratories and specialist organisations who have provided information and comment during the development of this document, and final editing by the Medical Editor are acknowledged.

The National Standard Methods are issued by Standards Unit, Department for Evaluations, Standards and Training, Centre for Infections, Health Protection Agency, London.

For further information please contact us at:

Standards Unit
Department for Evaluations, Standards and Training
Centre for Infections
Health Protection Agency
Colindale
London
NW9 5EQ

E-mail: standards@hpa.org.uk
REFERENCES


