

NATIONAL STANDARD METHOD

IDENTIFICATION OF ANAEROBIC GRAM-NEGATIVE RODS

BSOP ID 25

Issued by Standards Unit, Department for Evaluations, Standards and Training
Centre for Infections







IDENTIFICATION OF ANAEROBIC GRAM-NEGATIVE RODS

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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.

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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.

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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 conditions¹.

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.

Please note the references are now formatted using Reference Manager software. If you alter or delete text without Reference Manager installed on your computer, the references will not be updated automatically.

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AMENDMENT PROCEDURE

Controlled document reference	BSOP ID 25
Controlled document title	Identification Of Anaerobic Gram-Negative Rods

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.

Amendment no./ Date	Issue no. Discarded	Insert Issue no.	Page	Section(s) involved	Amendment

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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 old². 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*^{3,4}.

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 throats⁵. 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-dependent⁶.

***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-positive⁷.

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 production⁶.

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***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 lecithinase 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.

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1 SAFETY CONSIDERATIONS⁸⁻¹⁸

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 ORGANISMS¹⁹

***Bacteroides fragilis* group reported to have caused human infection**

<i>B. fragilis</i>	<i>B. ovatus</i>
<i>B. caccae</i>	<i>B. stercoralis</i>
<i>B. distasonis</i>	<i>B. thetaiotaomicron</i>
<i>B. eggerthii</i>	<i>B. uniformis</i>
<i>B. merdae</i>	<i>B. vulgatus</i>

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

<i>B. capillosus</i>	<i>B. pyogenes</i>
<i>B. coagulans</i>	<i>B. splanchnicus</i>
<i>B. forsythus</i>	<i>B. tectum</i>
<i>B. putredinis</i>	<i>B. ureolyticus</i>

***Fusobacterium* species reported to have caused human infection**

<i>F. alocis</i>	<i>F. nucleatum</i> subspecies <i>fusiforme</i>
<i>F. gonidiaformans</i>	<i>F. nucleatum</i> subspecies <i>nucleatum</i>
<i>F. mortiferum</i>	<i>F. nucleatum</i> subspecies <i>polymorphum</i>
<i>F. naviforme</i>	<i>F. nucleatum</i> subspecies <i>vincentii</i>
<i>F. necrogenes</i>	<i>F. periodonticum</i>
<i>F. necrophorum</i>	<i>F. russii</i>
<i>F. necrophorum</i> subspecies <i>funduliforme</i>	<i>F. sulci</i>
<i>F. necrophorum</i> subspecies <i>necrophorum</i>	<i>F. ulcerans</i>
<i>F. nucleatum</i>	

***Porphyromonas* species reported to have caused human infection**

<i>P. asaccharolytica</i>	<i>P. endodontalis</i>
<i>P. catoniae</i>	<i>P. gingivalis</i>

***Prevotella* species reported to have caused human infection**

<i>P. bivia</i>	<i>P. heparinolytica</i>
<i>P. buccae</i>	<i>P. intermedia</i> *
<i>P. corporis</i> *	<i>P. loescheii</i> *
<i>P. dentalis</i>	<i>P. melaninogenica</i> *
<i>P. denticola</i> *	<i>P. nigrescens</i> *
<i>P. disiens</i>	<i>P. oris</i>
<i>P. enoeca</i>	<i>P. tanneriae</i> *

* Pigmented species

Other species may be associated with human disease

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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

Genus	Characteristics of growth on fastidious anaerobe agar after anaerobic incubation at 35°C - 37°C
<i>Bacteroides</i>	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
<i>Fusobacterium</i>	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; <i>F. necrophorum</i> may be beta-haemolytic. Indole positive, fluorescent yellow-green under long wave UV light.
<i>Porphyromonas</i>	<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
<i>Prevotella</i>	Colonies are similar to those of <i>Bacteroides</i> species, except some species are pigmented (may be pale brown to black). Most pigmented species are haemolytic

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.

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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.

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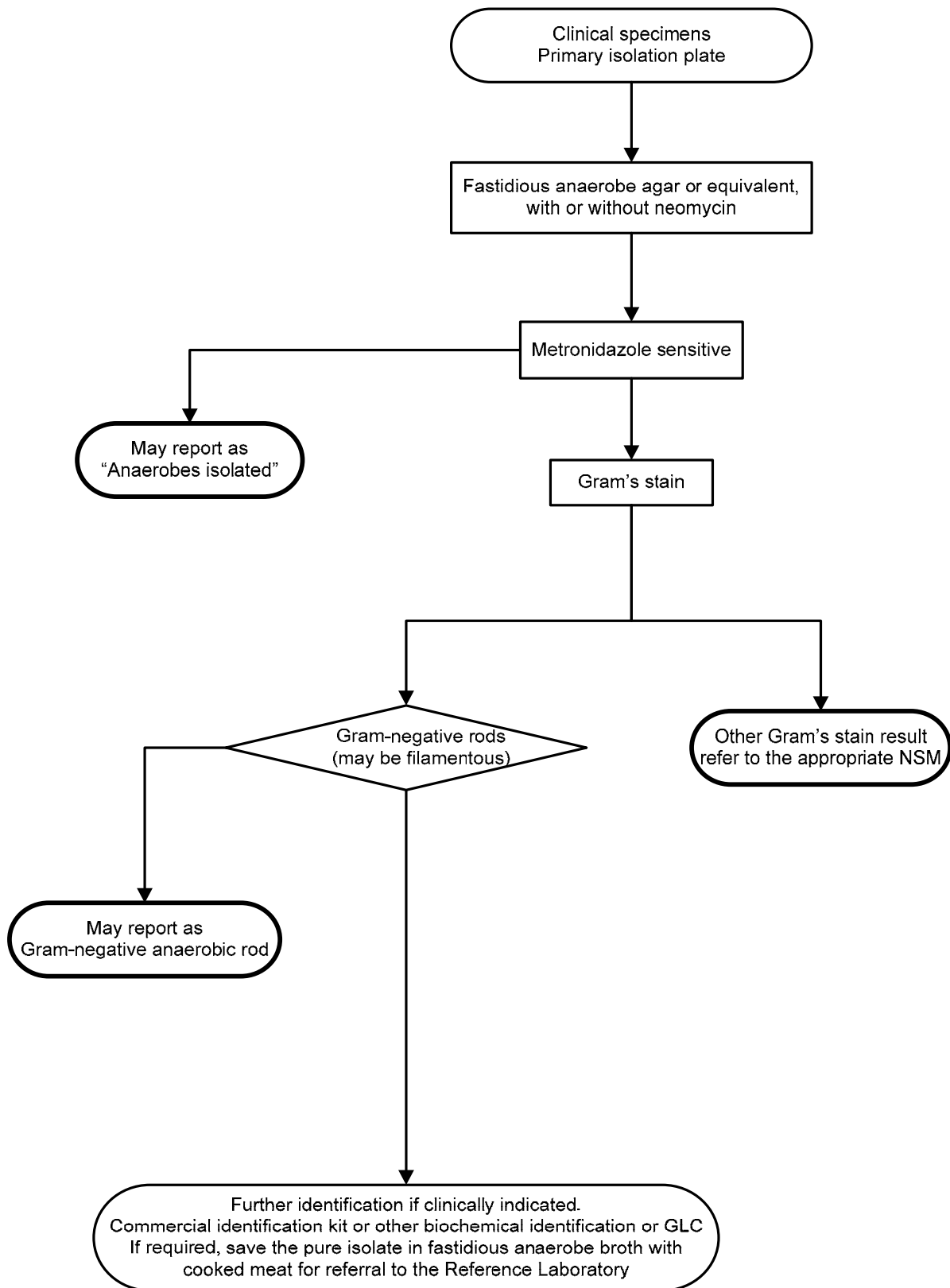
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4 FLOW CHART - PRESUMPTIVE IDENTIFICATION OF ANAEROBIC GRAM-NEGATIVE RODS



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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 INFECTIONS²⁰

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/ar/default.htm>

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7 ACKNOWLEDGEMENTS AND CONTACTS

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