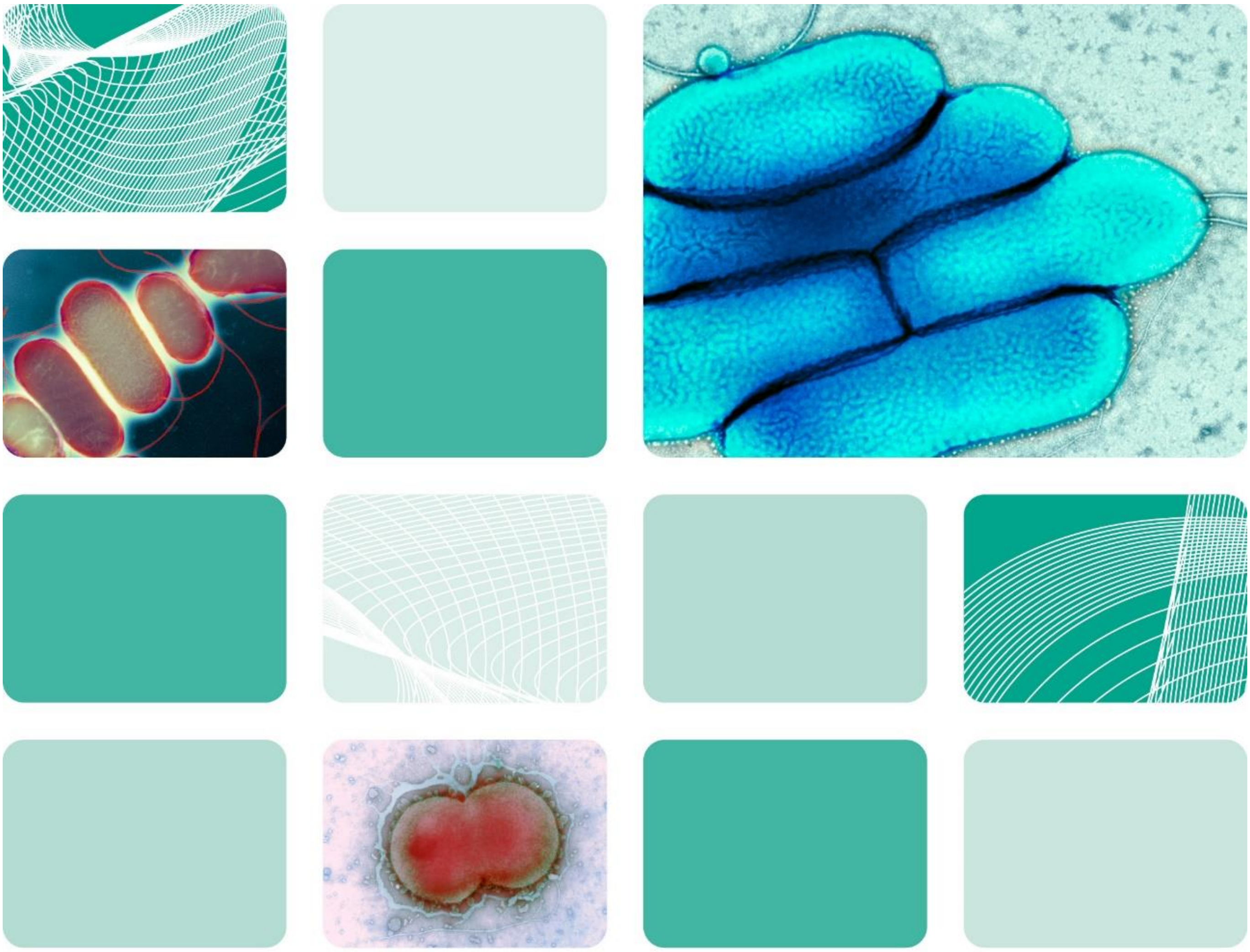
UK Standards for Microbiology Investigations

Identification of anaerobic Gram negative rods



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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.

UK SMIs are produced in association with:

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Contents

Amendment table

Each UK SMI document has an individual record of amendments. The amendments are listed on this page. The amendment history is available from standards@ukhsa.gov.uk.

Any alterations to this document should be controlled in accordance with the local document control process.

Amendment number/date	6/18.09.25
Issue number discarded	2
Insert issue number	2.1
Section(s) involved	**Amendment**
Whole document.	**This is an administrative point change.** **The content of this UK SMI document has not changed.** **The last scientific and clinical review was conducted on 29/06/2015.** Hyperlinks throughout document updated to Royal College of Pathologists website. Public Health England replaced with UK Health Security Agency throughout the document, including the updated Royal Coat of Arms Partner organisation logos updated. Broken links to devolved administrations replaced. References to NICE accreditation removed. Scope and Purpose replaced with General and Scientific information to align with current UK SMI template. ‘Public Health Responsibilities of Diagnostic Laboratories’ section added.

Amendment No/Date.	5/29.06.15
Issue no. discarded.	1.4
Insert Issue no.	2
Section(s) involved	**Amendment**

Whole document.	Hyperlinks updated to gov.uk.
Page 2.	Updated logos added.
Introduction.	The taxonomy of Anaerobic Gram Negative Rods has been updated. More information has been added to the Characteristics section. The medically important species are mentioned.
Technical information/limitations.	Addition of information regarding Gram stain, Agar media, metronidazole susceptibility, commercial identification systems and MALDI-TOF MS.
Target organisms.	The section on the Target organisms has been updated and presented clearly for all the organisms.
Identification.	Updates have been done on 3.2, 3.3 and 3.4 to reflect standards in practice. Section 3.4.2 and 3.4.3 has been updated to include MALDI-TOF MS and NAATs with references. Subsection 3.5 has been updated to include the Rapid Molecular Methods.
Identification flowchart.	Modification of flowchart for identification of Anaerobic Gram negative rods has been done for easy guidance.
Referral.	The addresses of the reference laboratories have been updated.
References.	Some references updated.

*Reviews can be extended up to 5 years where appropriate

1 General information

View general information related to UK SMIs.

2 Scientific information

View scientific information related to UK SMIs.

3 Scope of document

This UK SMI describes the characterisation of non-sporing, non-branching, Gram negative anaerobic bacteria.

Anaerobic spore-forming organisms are described in UK SMI ID 8 - Identification of Clostridium species, UK SMI ID 15 – Identification of anaerobic Actinomyces species and UK SMI ID 10 - Identification of aerobic Actinomycetes.

Anaerobic cocci can be found in UK SMI ID 14 – Identification of anaerobic cocci.

This UK SMI should be used in conjunction with other UK SMIs.

4 Introduction

4.1 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[1]. 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*[2,3].

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.* Other genera that have been associated with infections in humans are, *Parabacteriodes, Odoribacter, Tannerella, Alloprevotella* and *Mitsuokella*[1].

4.2 Characteristics

Bacteroides species

There are currently 44 validly published species. Twenty seven of which are from humans despite few taxonomic changes having occurred in the genus; new species described and some former species moved to other genera[2].

Bacteroides species belong to the family Bacteroidaceae and are short 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. They are catalase variable but usually negative and do not reduce nitrates. They also give variable test results for indole production. They do not produce pigment.

Their optimal growth temperature is 35-37°C. On FAA plate, colonies appear as mostly non-haemolytic, circular, low convex, smooth, semi-opaque grey, often moist or even mucoid and are 1-3mm diameter.

Bacteroides fragilis is the most commonly isolated species from clinical samples. Other highly relevant species in human infections are *Bacteroides ovatus* and *Bacteroides thetaiotamicron*[1].

They have been isolated from blood, ulcers, abscesses, bronchial secretions, bone, intra-abdominal infections, inflamed appendix and the head[4].

Fusobacterium species

There are currently 14 validly published species and 10 of which have been isolated in humans[5].

Fusobacterium species are rods which may be spindle-shaped eg *Fusobacterium nucleatum* or pleomorphic eg *Fusobacterium necrophorum*. They exhibit irregular staining. 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[6].

Their optimal growth temperature is 35-37°C. 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; *F. necrophorum* may be beta-haemolytic. *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[7].They are indole positive and fluoresce under UV light and produce lipase on egg yolk agar.

They have been isolated in root canal infections, dentoalveolar abscesses and spreading odontogenic infections. They have also been found in extraoral infections and abscesses in a wide range of body sites – blood, brain, chest, heart, lung, liver, appendix, abdomen, genitourinary tract, etc. as well as infected human bite lesions[1].

Porphyromonas species

There are currently 15 validly published species and 7 of which have been isolated in humans[8].

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

Most *Porphyromonas* species isolated from humans are catalase negative whereas those from animals are catalase positive[9].

Their optimal growth temperature is 35-37°C. On FAA plate, colonies are 1.0mm diameter, smooth, shiny and grey after 48hr incubation. Dark brown or black pigment develops after 3-7 days caused by protoheme production. Growth may be enhanced by “satellitism” around colonies of other organisms eg staphylococci.

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[7].

Prevotella species

There are currently 48 validly published species; 39 of which have been isolated in humans[10]. The genus *Prevotella* is composed of mainly saccharolytic, pigmented or non-pigmented species that were previously classified as *Bacteroides*, and these are usually pleomorphic.

Their optimal growth temperature is 35-37°C. On FAA plate, colonies are similar to those of *Bacteroides* species, except some species are pigmented (may be pale brown to black). Most pigmented species are haemolytic. 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.

They give variable results on catalase test but are usually negative.

They have been isolated from nearly all oral infections, infected human bite lesions, genital tract infections, urine, blood, etc[1].

4.3 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 stain reaction and are usually sensitive to a 5µg 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 rapid molecular methods, 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.

5 Technical information/limitations

Gram stain

There can be failure to determine the Gram reaction correctly (many anaerobes over decolourise and appear Gram negative). For example, *Clostridium* species that appear Gram negative on staining, especially *C. clostridioforme*, can be misidentified as *Bacteriodes*[11].

Agar media

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.

Susceptibility to metronidazole

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* group 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[4,12].

Commercial identification systems

Identification may be attempted using commercial kits but their results are not always reliable. These rapid easy to use systems have been used successfully for fast growing and biochemically reactive anaerobes such as *B. fragilis* group organisms. However, some of these kits have incorrectly identified a number of clinically relevant species such as *F. necrophorum, P. intermedia* and *P. melaninogenica* as well as not being able to identify new species that are not in the system’s limited database[1,13].

Another disadvantage of the automated commercial identification systems is its inability to differentiate between closely related species such as, *F. nucleatum* and *F. necrophorum*[14].

MALDI-TOF MS

This technique has been successful as an aid in both the detection and species-level identification of *Bacteroides* species – *B. fragilis* group. However, database expansion could aid in the accurate species-level identification of *Bacteroides* species and perhaps enhance MALDI-TOF MS performance such that more discriminatory types of analysis could be performed, such as grouping of subspecies typing and antibiotic resistance determination among clinical isolates[13,15].

6 Safety considerations[16-32]

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

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.

7 Target organisms[1,4,6,33-36]

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

*B. cellulosilyticus, **B. fragilis**, **B. ovatus**, B. caccae, B. stercoralis, **B. thetaiotaomicron,** B. eggerthii, B. uniformis, B. vulgatus, B. clarus, B. coprocola, B. coprophilus, B. dorei, B. faecis, B. finegoldii, B. fluxus, B. galacturonicus, B. intestinalis, B. massiliensis, B. nordii, B. oleiciplenus, B. pectinophilus, B. plebius, B. salyersiae, B. xylanisolvens, B. pyogenes*

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

B. coagulans

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

*F. gonidiaformans, **F. nucleatum** - F. nucleatum, subspecies fusiforme, F. nucleatum subspecies nucleatum, F. nucleatum subspecies polymorphum, F. nucleatum subspecies vincentii, F. mortiferum, F. necrogenes, F. naviforme, F. periodonticum, **F. necrophorum** - F. necrophorum subspecies funduliforme, F. necrophorum subspecies necrophorum, F. russii, F. ulcerans, F. varium*

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

*P. asaccharolytica, P. bennonis, **P. endodontalis**, P. catoniae, **P. gingivalis**, P. somerae, P. uenonis*

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

P. amnii, P. aurantiaca, P. baroniae, P. bergensis, P. buccae, P. buccalis, P. bivia, P. dentalis, P. denticola, P. disiens, P. enoeca, P. heparinolytica, P. intermedia*, P. copri, P. corporis*, P. fusca, P. histicola, P. jejuni, P. loescheii*, P. maculosa, P. marshii, P. micans, P. multiformis, P. multisaccharivorax, P. nanceiensis, **P. melaninogenica***, P. nigrescens*, P. oris, P. pallens, P. pleuritidis, P. oralis, P. oulorum, P. saccharolytica, P. salivae, P. scopos, P. shahii, P. stercorea, P. timonensis, P. veroralis*

Other reclassified species that have been associated with human disease –

Parabacteriodes distasonis, Parabacteriodes goldsteinii, Parabacteriodes gordonii, Parabacteroides johnsonii, Parabacteriodes merdae, Tannerella forsythia, Mitsuokella multacida, Odoribacter splanchinus, Filifactor alocis, Eubacterium sulci, Alloprevotella tannerae, Faecalibacterium prausnitzii*

* Pigmented species

8 Identification

8.1 Microscopic appearance

Gram stain

(UK SMI TP 39 - Staining procedures)

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

Fusobacterium species are Gram negative rods with unique cell morphology, highly variable in length and width, and they may have pointed ends. *F. nucleatum* usually exhibits long, spindle-shaped cells with tapered ends and is indole positive while *F. mortiferum* and *F. necrophorum* have highly pleomorphic cells, with or without swollen areas and large bodies and are indole and lipase positive.

8.2 Primary isolation media

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

Note: some species of organisms such as *Porphyromonas* may require longer incubation[1].

8.3 Colonial appearance

Genus	**Characteristics of growth on fastidious anaerobe agar after anaerobic incubation at 35-37°C**
Bacteroides	Colonies are 1-3mm 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.
Fusobacterium	Colonial appearance is variable, but most are 1-3mm diameter, with an irregular or dentate edge. They vary from translucent to granular and opaque; *F. necrophorum* may be beta-haemolytic. Indole positive, fluorescent yellow-green under long wave UV light.
Porphyromonas	Colonies are <1.0mm diameter after 48hr 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.
Prevotella	Colonies are similar to those of *Bacteroides* species, except some species are pigmented (may be pale brown to black). Most pigmented species are haemolytic.

8.4 Test procedures

8.4.1 Biochemical tests

Susceptibility to metronidazole

Isolate shows a zone of inhibition to metronidazole 5µg discs 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* group are becoming resistant to metronidazole, and these organisms will be missed by such an approach[4,12]. Colonies of suspected *Bacteroides* species resistant to metronidazole should be referred to the Anaerobe Reference Laboratory for confirmation.

AnIdent ring/discs

Follow manufacturer’s instructions.

Aesculin hydrolysis test

(UK SMI TP 2 - Aesculin hydrolysis test)

This may be used as a presumptive identification test for *Bacteroides fragilis* group as well as for differentiation of *Fusobacterium* species.

Bacteriodes species are aesculin positive and *Fusobacterium* species are aesculin negative apart from *F. mortiferum* and *F. necrogenes*.

Catalase test

(UK SMI TP 8 - Catalase test)

Bacteriodes and *Prevotella* species give variable catalase reactions but are usually negative while *Fusobacterium* and *Porphyromonas* species are catalase negative.

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 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.

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

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

8.4.2 Matrix-assisted laser desorption/ionisation time of flight mass spectrometry (MALDI-TOF MS)

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 MS 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[37].

This technique has been successful as an aid in both the detection and species-level identification of *Bacteroides* species – *B. fragilis* group. However, database expansion could aid in the accurate species-level identification of *Bacteroides* species and perhaps enhance MALDI-TOF MS performance such that more discriminatory types of analysis could be performed, such as grouping of subspecies typing and antibiotic resistance determination among clinical isolates[13,15].

8.4.3 Nucleic acid amplification tests (NAATs)

PCR is usually considered to be a good method for bacterial detection as it is simple, rapid, sensitive and specific. The basis for PCR diagnostic applications in microbiology is the detection of infectious agents and the discrimination of non-pathogenic from pathogenic strains by virtue of specific genes. However, it does have limitations. Although the 16S rRNA gene is generally targeted for the design of species-specific PCR primers for identification, designing primers is difficult when the sequences of the homologous genes have high similarity.

This rapid method has been used successfully for the identification of *Bacteriodes fragilis* group species[38].

8.5 Further identification

Rapid molecular methods

Molecular methods have had an enormous impact on the taxonomy of Gram negative anaerobic bacteria. Analysis of gene sequences has increased understanding of the phylogenetic relationships of anaerobes and related organisms; and has resulted in the recognition of numerous new species. Molecular techniques have made identification of many species more rapid and precise than is possible with phenotypic techniques.

A variety of rapid typing methods have been developed for isolates from clinical samples; these include molecular techniques such as Polymerase Chain Reaction-Restriction Fragment Length Polymorphism Analysis (PCR-RFLP), Multilocus Sequence Typing (MLST), *rpoB* rDNA sequencing and Whole Genome Sequencing (WGS). 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.

rpoB rDNA sequencing

This method is being increasingly used for the identification of anaerobic bacteria, because sequencing of the gene is faster and more accurate than biochemical testing and, notably, independent of growth characteristics. *rpoB* genes are used as they have a greater resolving power than those for 16S genes, and because *rpoB* exists in the genome as a single gene, it is considered to have a faster evolutionary rate than 16S, and also, being a protein-coding gene, possesses fewer indel regions.

This has been successfully used for distinguishing *F. nucleatum* and *F. periodonticum*, and for oral isolates versus those isolated from intestinal biopsies[1,39].

However, sequencing, as a routine method may not be feasible for many clinical laboratories.

Polymerase chain reaction-restriction fragment length polymorphism analysis (PCR-RFLP)

PCR amplification, followed by restriction digest analysis, is a simple technique that has been applied to species identification and could be used to analyse resistance genes. Restriction fragment length polymorphism analysis of amplified small subunit rRNA gene (16S rDNA PCR-RFLP) has been shown to be a rapid, accurate, and effective method for the identification of clinically important anaerobes – for example, clostridia and actinomycetes.

This has been used successfully for improved identification of *Bacteroides* species and the detection of metronidazole (MTZ) resistance determinants[40]. It has also been used for the differentiation of *P. intermedia* and *P. nigrescens*[41].

Its advantages are its reliability, rapidity and accuracy. Another added advantage is that the method is inexpensive.

Multilocus sequence typing (MLST)

MLST measures the DNA sequence variations in a set of housekeeping genes directly and characterizes strains by their unique allelic profiles. The principle of MLST is simple: the technique involves PCR amplification followed by DNA sequencing. Nucleotide differences between strains can be checked at a variable number of genes depending on the degree of discrimination desired. The technique is highly discriminatory, as it detects all the nucleotide polymorphisms within a gene rather than just those non-synonymous changes that alter the electrophoretic mobility of the protein product. One of the advantages of MLST over other molecular typing methods is that sequence data are portable between laboratories and have led to the creation of global databases that allow for exchange of molecular typing data via the Internet[42].

The drawbacks of MLST are the substantial cost and laboratory work required to amplify, determine, and proofread the nucleotide sequence of the target DNA fragments, making the method hardly suitable for routine laboratory testing.

This has demonstrated to be a valuable technique for the identification and classification of species of the genus *Bacteriodes*[43].

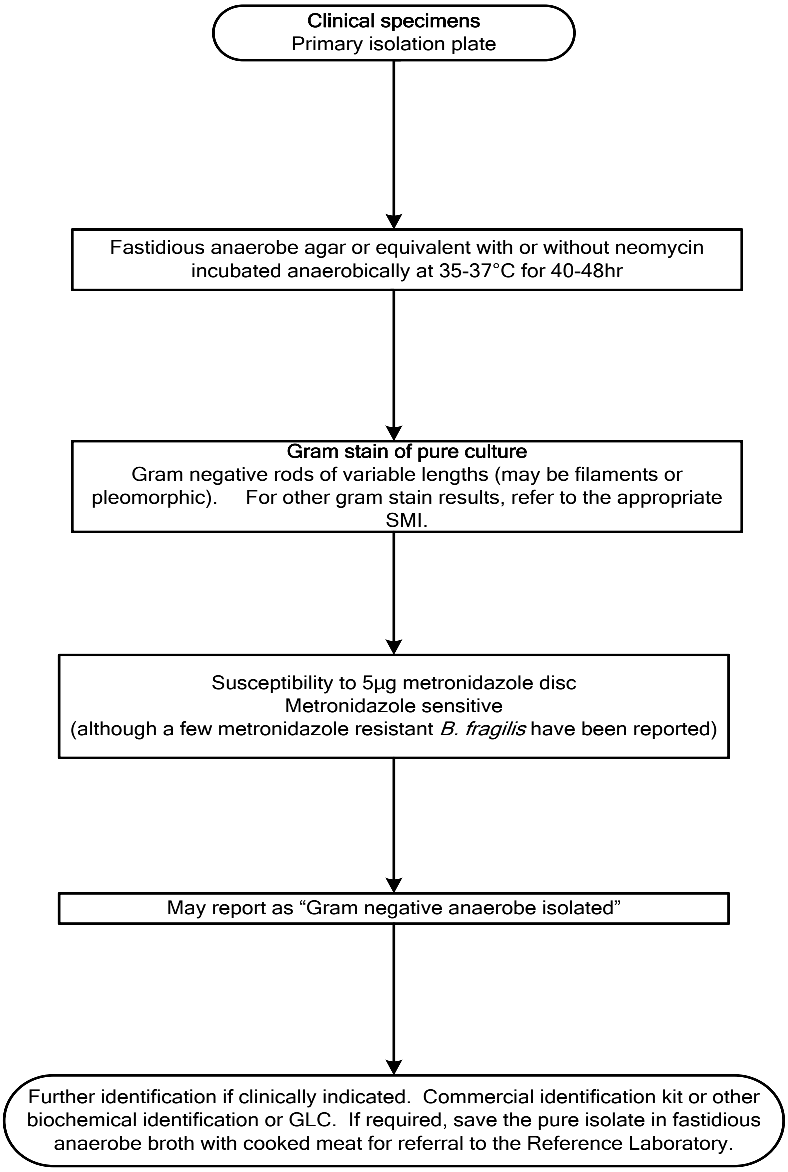
Other more specialized tests

Gas-Liquid Chromatography of metabolic end products.

8.6 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.

Algorithm: Presumptive identification of anaerobic gram negative rods



The flowchart is for guidance only.

9 Reporting

9.1 Presumptive identification

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

9.2 Confirmation of identification

Further biochemical tests and/or molecular methods and/or reference laboratory report.

9.3 Medical microbiologist

Inform the medical microbiologist of presumptive or confirmed non-sporing anaerobes when the request 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 Lemièrre’s Syndrome (post anginal sepsis, often with jugular suppurative thrombophlebitis and haematogenous pulmonary abscesses)

Follow local protocols for reporting to clinician.

9.4 CCDC

Refer to local Memorandum of Understanding.

9.5 UK Health Security Agency[44]

Refer to current guidelines on CIDSC and COSURV reporting.

9.6 Infection prevention and control team

N/A

10 Referral to reference laboratories

For information on the tests offered, turnaround times, transport procedure and the other requirements of the reference laboratory see user manuals and request forms

Contact appropriate reference laboratory for information on the tests available, turnaround times, transport procedure and any other requirements for sample submission:

England

Wales

Scotland

Northern Ireland

Note: In case of sending away to laboratories for processing, ensure that specimen is placed in appropriate package and transported accordingly.

11 Public Health responsibilities of diagnostic laboratories

Diagnostic laboratories have public health responsibility as part of their duties. Amongst these are additional local testing, or referral, to further characterise the organism, as required, primarily for public health purposes e.g. routine cryptosporidium detection; serotyping or microbial subtyping; and a duty to refer appropriate specimens and isolates of public health importance to a reference laboratory.

Diagnostic laboratory outputs inform public health intervention, and surveillance data is required to develop policy and guidance, forming an essential component of healthcare. It is recognised that additional testing and referral of samples may entail some costs that has to be borne by the laboratory but in certain jurisdictions these costs are covered centrally.

Diagnostic laboratories should be mindful of the impact of laboratory investigations on public health and consider requests from the reference laboratories for specimen referral or enhanced information.

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An explanation of the reference assessment used is available in the scientific information section on the UK SMI website.

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