

NATIONAL STANDARD METHOD

IDENTIFICATION OF *NEISSERIA* SPECIES

BSOP ID 6

Issued by Standards Unit, Evaluations and Standards Laboratory
Centre for Infections



Association of Medical Microbiologists
Association of Medical Microbiologists
Association of Medical Microbiologists



IDENTIFICATION OF NEISSERIA SPECIES

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Suggested citation for this document:

Health Protection Agency (2007). Identification of *Neisseria* species. National Standard Method NSM BSOP ID 6 Issue 2.1. http://www.hpa-standardmethods.org.uk/pdf_sops.asp.

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AMENDMENT PROCEDURE

Controlled document reference	BSOP ID 6
Controlled document title	Identification of <i>Neisseria</i> species

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 Number/ Date	Issue no. Discarded	Insert Issue no.	Page	Section(s) involved	Amendment
5/ 11/09/07	2	2.1	1	Front Page	Northern Ireland logo added
			All	All	PDF links inserted to cross- reference NSM documents
			5	Introduction	Technical information added and Taxonomy
			13	4	Flowchart in Visio format
			15	Referrals	Amended and link added

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IDENTIFICATION OF *NEISSERIA* SPECIES

SCOPE OF DOCUMENT

This National Standard Method (NSM) describes the identification of pathogenic *Neisseria* species isolated from clinical specimens and their differentiation from non-pathogenic *Neisseria* species and the related genera of *Moraxella* and *Kingella*. The identification of these two genera is covered in [BSOPID 11 - Identification of Moraxella Species and Morphologically Similar Organisms](#)

INTRODUCTION

Taxonomy

The genus *Neisseria* belongs to the family Neisseriaceae. The genus consists of *N. gonorrhoeae* and *N. Meningitidis*, which are of clinical significance, and several non-pathogenic species.

Characteristics of *Neisseria* and related organisms²

Neisseria species³

Neisseria species are obligate human pathogens with no other natural host. They are Gram-negative cocci, 0.6 - 1.0 µm in diameter, occurring singly but more often in pairs with adjacent sides flattened. They are non-motile and flagella are absent. Some species produce a greenish-yellow carotenoid pigment and may be nutritionally fastidious and haemolytic. The optimum growth temperature is 35°C - 37°C. *Neisseria* are oxidase-positive and catalase-positive (except *Neisseria elongata*). All except *Neisseria gonorrhoeae* and *Neisseria canis* reduce nitrite.

The clinically important species of *Neisseria* species (*Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Neisseria lactamica*, *Neisseria cinerea* and *Moraxella catarrhalis*) are relatively easy to identify from the non pathogenic *Neisseria*. *N. gonorrhoeae* and *N. meningitidis* are the two main pathogens of the group. The other species of *Neisseria* such as *N. lactamica*, *N. cinerea* and *M. catarrhalis* are generally considered commensals, but have been implicated as causes of infection in patients who are immuno-compromised.

Moraxella species

Moraxella species may be rods or cocci. The rods are often very short and plump, resembling a coccus shape and are usually 1.0 - 1.5 x 1.5 - 2.5 µm in size. Cells occur in pairs and short chains with one plane of division. The cocci are smaller, 0.6 - 1.0 µm in diameter and occur as single cells or in pairs with adjacent sides flattened. Differing planes of division sometimes result in tetrads. Cells may be capsulate. *Moraxella* species are Gram-negative with a tendency to resist decolourisation. Flagella are absent. Some strains may grow weakly under anaerobic conditions. Most species except *Moraxella osloensis* are nutritionally fastidious. The optimum growth temperature is 33°C - 35°C. *Moraxella* species are usually catalase-positive and no acid is produced from carbohydrates. All *Moraxella* species are oxidase-positive.

Kingella species

Kingella species are straight rods, 1.0 µm in length, often in pairs or short chains. Endospores are not formed. Cells are Gram-negative but there is a tendency to resist decolourisation. They are non-motile. Growth is aerobic or facultatively anaerobic and the optimum growth temperature is 33°C - 37°C. Two types of colony are produced on blood agar: a spreading, corroding type with twitching motility; and a smooth convex type, which does not show twitching motility. *Kingella* are oxidase-positive, but may give a weak or negative reaction with tetramethyl-p-phenylenediamine. They are catalase-negative and urease-negative. Glucose and some other carbohydrates are utilised with acid production. *Kingella* may be mis-identified as *Neisseria* because they are Gram-negative rods which are often arranged in pairs, oxidase positive and may grow on GC selective agar.

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Principles of identification

Isolates from primary culture are identified by Gram stain, oxidase and by at least two of the following identification principles: carbohydrate utilisation, detection of preformed enzymes or reactivity with immunological reagents.

TECHNICAL INFORMATION

N/A

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1 SAFETY CONSIDERATIONS⁴⁻¹⁴

Hazard Group 2 organisms

Suspected isolates of *N. meningitidis* should always be handled in a microbiological safety cabinet.

N. meningitidis is in Hazard Group 2 although in some cases the nature of the work may dictate full Containment Level 3 conditions.

N. meningitidis causes severe and sometimes fatal disease. Laboratory acquired infections have been reported. The organism infects primarily by the respiratory route. An effective vaccine is available for some meningococcal serogroups.

Laboratory procedures that give rise to infectious aerosols must be conducted in a microbiological safety cabinet, isolator or be otherwise suitably contained.

Refer to current guidance on the safe handling of all Hazard Group 2 organisms documented in this NSM.

The above guidance should be supplemented with local COSHH and risk assessments. Compliance with postal and transport regulations is essential.

2 TARGET ORGANISMS

The main *Neisseria* species reported to have caused human infection

N. gonorrhoeae
N. meningitidis
N. lactamica
N. sicca

Other species may be associated with human diseases

N. subflava
N. cinerea,
N. canis,
N. elongata subspecies *nitroreducens*
N. mucosa.

Species which may be misidentified as *Neisseria* species

*Moraxella catarrhalis**
*Kingella denitrificans**

Moraxella species which have been isolated from clinical specimens

*M. atlantae**
*M. catarrhalis**
*M. lacunata**
*M. nonliquefaciens**
*M. osloensis**
M. phenylpyruvica

Kingella species which have been isolated from clinical specimens

*K. denitrificans**
K. indologenes
*K. kingae**

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Asaccharolytic *Neisseria* species which may be misidentified as *N. gonorrhoeae* or *N. meningitidis*

*N. canis**
N. caviae
*N. cinerea**
N. cuniculi
*N. elongate**
N. flavescens
*N. ovis**

*Species reported to have caused human infection

3 IDENTIFICATION

3.1 MICROSCOPIC APPEARANCE

Gram stain (see [BSOFTP 39 - Staining Procedures](#))

Neisseria species are Gram-negative cocci arranged in pairs with long axes parallel.

3.2 PRIMARY ISOLATION MEDIA

GC selective agar incubated for up to 48 hours in 5 - 10% CO₂ at 35°C - 37°C. For maximum isolation rates plates should be left for 72 hours if possible.

GC selective agar usually consists of GC agar base supplemented with lysed or chocolatised horse blood with or without the addition of VitoX or IsoVitaleX. Antibiotic cocktails used for selection contain vancomycin or lincomycin, colistin, trimethoprim, and nystatin or amphotericin.

Whole Blood agar/heated blood (chocolate) incubated for 18 - 48 hours in 5 - 10% CO₂ at 35°C - 37°C. These media usually consist of Columbia agar base supplemented with 5% horse blood or chocolatised horse blood.

3.3 COLONIAL APPEARANCE

The species of *Neisseria* (which sometimes grow on non-selective medium) are usually pigmented and opaque. However, both *N. gonorrhoeae* and *N. meningitidis* form smooth, round, moist, uniform grey/brown colonies with a greenish colour underneath on primary isolation medium. *N. gonorrhoeae* may grow poorly on blood agar when the medium is very fresh or the number of bacteria present in the sample is especially high. As with all bacterial culture, the quality of the sample determines the quality of the isolates.

3.4 TEST PROCEDURES

Oxidase test (see [BSOFTP 26 - Oxidase Test](#))

Oxidase-positive: *Neisseria* species and *Kingella* species and *M. catarrhalis*

3.5 FURTHER IDENTIFICATION

Neisseria species can be differentiated from similar organisms by biochemical and other tests. At least two principles of identification should be used as there are very few taxonomic differences between members of the genus and therefore definitive identification can prove problematic.

3.5.1 NEISSERIA GONORRHOEAE^{15,16}

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N. gonorrhoeae is sexually transmitted, primarily causing infection of the anogenital tract and is always considered a pathogen. This contrasts with *N. meningitidis* which colonises the upper respiratory tract as a commensal and occasionally invades to cause systemic disease.

Neisseria have a typical Gram-negative envelope, which consists of a cytoplasmic membrane, a thin layer of peptidoglycan and an outer membrane. Many of the major antigens of the cell envelope are shared between *N. gonorrhoeae* and *N. meningitidis*, with the exception of the capsule which is never expressed by *N. gonorrhoeae* but, when expressed by *N. meningitidis*, enhances survival in the blood.

Identification of *N. gonorrhoeae*

There are two approaches that can be taken to confirm the identity of *N. gonorrhoeae* and eliminate other *Neisseria* species.

1. The use of gonococcal specific antibodies, which confirms *N. gonorrhoeae* alone.
2. The use of carbohydrate utilization tests¹⁷, with or without the detection of preformed enzymes such as the aminopeptidases and β -galactosidase, which will give the full speciation of the organism.

Identification tests available:

Carbohydrate utilisation

Traditionally identity has been confirmed by detecting the acidification of glucose-containing media, but not those containing maltose, sucrose or lactose. This is an oxidative and not a fermentative process. It is important that the basal medium is carbohydrate-free (if serum sugars are used, the serum should be checked for maltase activity). The inoculated plates or bottles are incubated in 5 - 10% CO₂ for twenty four hours with the caps loosened and are then allowed to stand on the bench for 30 min to allow any acidification due to dissolved CO₂ to dissipate. There are disadvantages to this method in that it is slow and requires a heavy, pure growth of gonococci. Some meningococci metabolise maltose slowly and may require at least two days for acidification of the conventional test system, and some gonococci can be slow to utilize glucose. Several commercial systems are available for the rapid detection of carbohydrate utilisation.

Preformed enzymes

Detection of aminopeptidases, gamma-glutamyl transferase and proline iminopeptidase together with β -galactosidase, with chromogenic substrates allows identification to species level. Reagents are available as commercial kits. This can be a useful alternative to the approaches above, but should only be used on strains isolated on selective media, as certain non-pathogenic *Neisseria* give similar reactions to those that are given by *N. gonorrhoeae*. However, *N. gonorrhoeae* that have a mutation in the proline iminopeptidase gene and therefore appear negative for this enzyme are prevalent in England and Wales and kits that detect solely the production of aminopeptidases should not be used alone^{18,19}.

Carbohydrate and preformed enzymes combined

Many of the commercial kits that test for carbohydrate utilization also include aminopeptidases. *N. gonorrhoeae* that are proline iminopeptidase negative will give anomalous results with these kits and should be confirmed with an immunological reagent.

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Immunological

Identification by immunological means can be achieved using antibodies linked to fluorescein or a staphylococcal protein A or latex. These commercially available reagents contain a mixture of monoclonal antibodies raised to specific epitopes on the major outer membrane protein, Por. Because the reagents contain a mixture of antibodies rather than a single antibody to a cross-reactive epitope, false negative reactions do occur, although uncommonly. Because the mixtures themselves are different occasional isolates occur that give a false negative with one, but are positive with another reagent.

Approaches to identification of *N. gonorrhoeae*

Identification should be achieved by a combination of test procedures which both identify the organism and exclude other *Neisseria* species. *N. gonorrhoeae* is usually isolated from high risk patients, where it is only necessary to perform presumptive identification followed by a single confirmatory test. However, in low risk patients and in child and sexual abuse (medicolegal) cases it is necessary to use more than one confirmatory test.

Presumptive identification

There are four minimum criteria that all isolates of *N. gonorrhoeae* should meet:

1. Growth on media selective for pathogenic *Neisseria* species.

Note: If the *Neisseria gonorrhoeae* strain in question is sensitive to vancomycin it will fail to grow on this medium.

2. Appropriate colonial morphology on such media.
3. Typical Gram stain morphology (Gram negative diplococci).
4. Oxidase-positive.

- **High Risk: Patients attending for sexual health care such as GUM patients (high prevalence populations)**

Patients considered to be high risk should have both genital samples and non genital samples (rectal and pharyngeal) tested. One, preferably two, additional tests are required for the confirmation of isolates from genital samples where the Gram stain and oxidase on the specimen has given a presumptive diagnosis of infection with *N. gonorrhoeae*. These should be either biochemical or immunological. Any isolates that give a negative result with an immunological test should be tested in addition with a biochemical test that detects carbohydrate utilisation with/without aminopeptidases to eliminate the possibility of an aminopeptidase negative *N. gonorrhoeae*.

- **Low Risk: Patients attending primary care (low prevalence populations)**²⁰⁻²²

It is recommended that for isolates from patients considered low risk (but with out medico-legal implications) two additional tests should be used for confirmation following presumptive identification. These should be biochemical and immunological. Biochemical kits should not include those that detect aminopeptidases **alone**, but can be those kits that include both carbohydrates and aminopeptidases.

- **Medicolegal : Child or sexual abuse**^{21,22}

In Medicolegal cases the Sexually Transmitted Bacteria Reference Laboratory (STBRL) recommend that isolates should be identified by satisfying the minimum criteria and **three** additional tests; biochemical, immunological and molecular (molecular tests should be referred to STBRL).

In medicolegal patients, sufficient specific tests must be undertaken to ensure, as far as possible, that the identification is robust enough to withstand close scrutiny in a court of law.

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Identification by means of at least one or two confirmatory tests should be carried out at the initial laboratory. This should be followed by confirmation of the identification by the Reference Laboratory as a sensible additional precaution. Isolates should be stored in a viable state at least until a final report is received from the Reference Laboratory in case posted isolates die or are lost. If results are to be used in forensic evidence the 'chain of evidence' must be shown to be intact. The chain of evidence is a formal record of the specimen's progress from point of collection of the sample to the issue of the final report. This should include a fully documented (time, date, place and signatures) chain of persons handling the sample and performing and interpreting the tests, and in addition it should record the conditions of the specimen's storage.

Molecular methods carried out at STBRL include in-house methods to detect *N. gonorrhoeae* specific DNA by amplification of *cppB* and *ompIII* genes for identification and genotyping by means of NG-MAST²³⁻²⁵.

Susceptibility testing²⁶

Most laboratories perform susceptibility testing on all isolates of *N. gonorrhoeae* by means of disc testing following the British Society of Antimicrobial Chemotherapy (BSAC) guidelines. It is recommended that each antimicrobial agent used for therapy should be tested. It should be noted that if nalidixic acid is used to screen for ciprofloxacin resistant isolates that the occasional nalidixic acid susceptible, ciprofloxacin resistant isolate of *N. gonorrhoeae* has been detected. An alternative method, which is useful for laboratories testing only a few isolates, is the E-test method using ciprofloxacin.

Any isolates that show decreased susceptibility to the third generation cephalosporins, ceftriaxone or cefixime or azithromycin should be referred to STBRL for confirmation.

3.5.2 NEISSERIA MENINGITIDIS

For information on screening for Meningococci see [BSOP 51 - Screening for Meningocci](#).

Growth of *Neisseria meningitidis* from a normally sterile site such as CSF (cerebrospinal fluid) or blood is considered definitive for the diagnosis of meningococcal disease²⁷. At case presentation it is generally recommended that two samples are taken, one for microbiological culture and one for PCR (DNA-based detection) where meningococcal disease is suspected. Samples of CSF received in microbiology laboratories from suspected cases of meningitis are routinely tested for cell counts (and differential counts) often followed by Gram staining to achieve rapid confirmation. The lack of sensitivity of microscopy and an increase in the preadmission administration of antibiotics reduce the chances of positive microscopy or culture. This has increased the importance of DNA-based detection methods such as PCR. Latex agglutination, although useful in some cases, is not as sensitive as PCR assay for the confirmation and serogroup characterisation of meningococci. The HPA Meningococcal Reference Unit offers a PCR testing service to confirm meningococcal disease case and determine accurate serogroup characterisation to inform England and Wales epidemiology. DNA-based assays also allow further molecular subtyping if required.

Invasive disease with *Neisseria meningitidis* is generally associated with specific serogroups. Meningococcal strains are carried asymptotically in the oro- and nasopharynx and are often isolated from urogenital sites in both men and women. Apart from resistance to sulfonamides (not used in therapy), meningococci remain susceptible to the antibiotics classically used for treatment and chemoprophylaxis. Since 1985 a decrease in susceptibility to penicillin has been observed due to changes in penicillin protein binding sites. Reduced susceptibility is ascribed to organisms with penicillin MICs ≥ 0.1 mg/L: care should be taken not to infer resistance because meningococci are clinically responsive to the therapeutic levels attained in patients. Some extremely rare strains of meningococci have been identified as harbouring beta-lactamase producing plasmids. Resistance to rifampicin is sometimes observed following chemoprophylaxis of case contacts and this may occasionally be observed in secondary case clusters. Due to the nature of the infection it is very important that laboratories obtain accurate resistance data both for treatment and epidemiological purposes.

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This is best achieved locally by use of E-tests²⁸. Staff need to be trained in their use for valid results to be obtained. It is also expected that all isolates will have undergone wider disc sensitivity testing to confirm susceptibility to locally used therapeutic agents.

Once an isolate has been identified using the method outlined in 3.0, confirmation of the isolate is made in the following way;

- Biochemical testing kit. It is important to note that a number of glucose and maltose negative meningococci have been reported¹⁷
- Rapid biochemical commercial kit
- Characterisation where it is required to serogroup level would normally involve a commercial latex kit or slide agglutination reagents. The latex agglutination kits are designed for direct use on CSF or serum, but will also work for cultures. Slide agglutinating sera are for use on cultures only. Heated clinical samples or formalin treated suspensions of cultures should be processed within microbiological safety cabinets to reduce aerosols

Differentiation of *N. meningitidis* from similar phenotypes

N. meningitidis can be identified by acid production from glucose and maltose but not from lactose or sucrose, and by the production of gamma-glutamylaminotransferase. Maltose-negative strains of *N. meningitidis* have been described and may be differentiated from *N. gonorrhoeae* by their ability to produce gamma-glutamylaminotransferase. Glucose negative variants of *N. meningitidis* may also be observed.

3.5.3 OTHER *NEISSERIA* SPECIES

These can be identified by use of commercially available kits that have been validated. The accuracy of these kits has not been fully determined for species other than *N. gonorrhoeae* and *N. meningitidis* and therefore all results obtained should be treated with caution.

3.6 STORAGE AND REFERRAL

Short term storage – isolates should be kept in a viable state on heated blood (chocolate) agar slopes.

Long term storage – isolates should be frozen at -20°C to -80°C.

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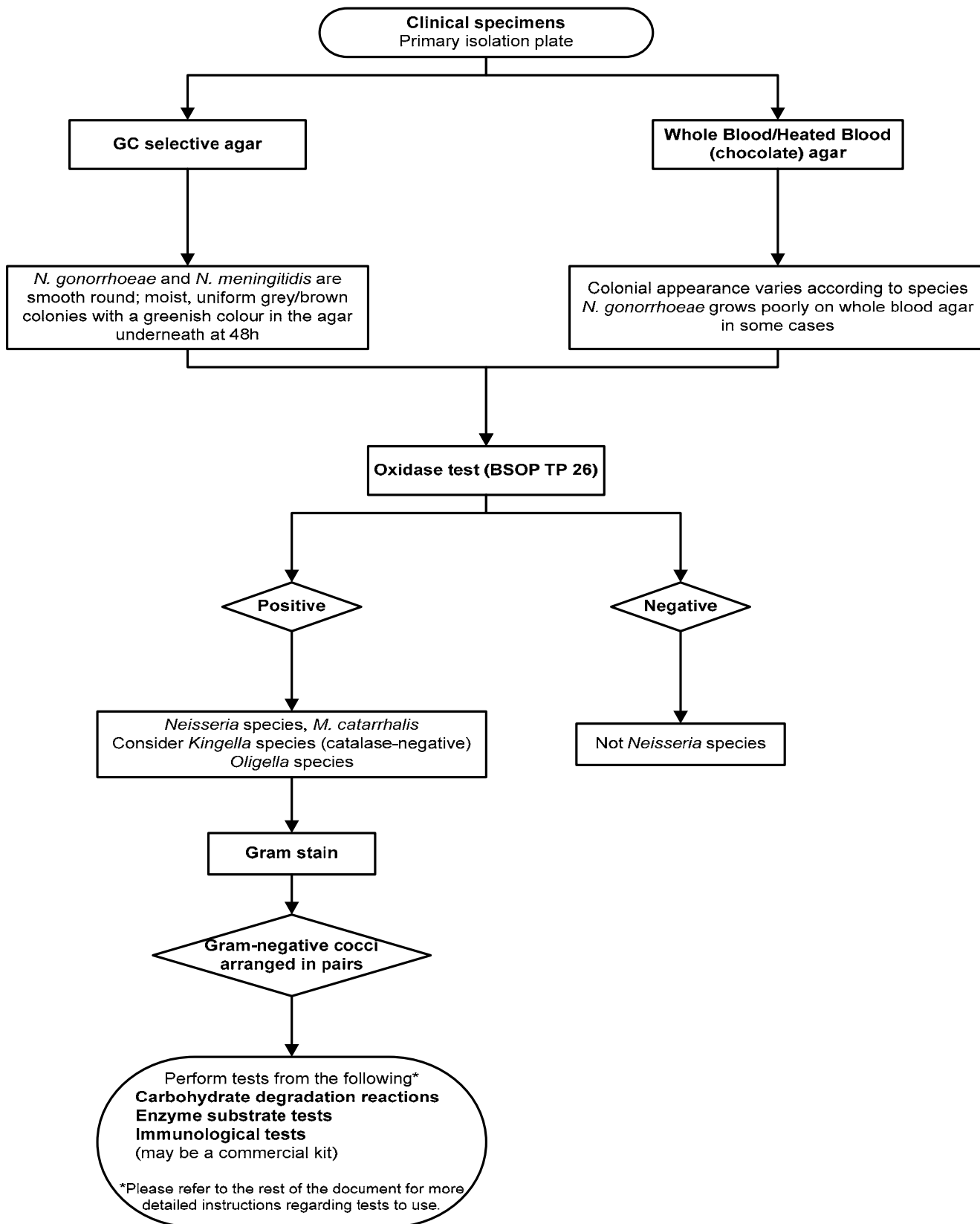
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4 IDENTIFICATION OF *NEISSERIA* SPECIES - FLOW CHART



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5 REPORTING

5.1 PRESUMPTIVE IDENTIFICATION

N. gonorrhoeae

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

N. meningitidis

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

There are four minimum criteria that all isolates of *Neisseria* should meet:

1. Growth on media selective for pathogenic *Neisseria* species.

Note: If the *Neisseria gonorrhoeae* strain in question is sensitive to vancomycin it will fail to grow on this medium.

2. Appropriate colonial morphology on such media.
3. Exhibit typical Gram stain morphology (Gram-negative diplococci).
4. Oxidase-positive.

5.2 CONFIRMATION OF IDENTIFICATION

Using biochemical/immunological results following identification processes as outlined in this document and/or Reference Laboratory report.

5.3 MEDICAL MICROBIOLOGIST

Inform the medical microbiologist of all presumptive and confirmed *N. meningitidis* isolates, and of all *Neisseria* species isolated from normally sterile sites, or in cases of invasive infection.

The medical microbiologist should also be informed if the request card bears relevant information eg:

- Cases of meningitis, septicaemia (especially with purpuric rash)
- Investigation of *N. meningitidis* outbreak, or of the carrier state

Inform the medical microbiologist of all presumptive and confirmed *N. gonorrhoeae* isolates, and of all *Neisseria* species from:

- Minors
- Cases of sexual assault, rape or abuse
- All persons not known to be attending a Genitourinary Medicine clinic
- Extragenital sites (eg throat, anorectum because special care is indicated with identification procedures)

Follow local protocols for reporting to clinician

5.4 CCDC

Refer to local Memorandum of Understanding.

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5.5 CENTRE FOR INFECTIONS²⁹

Refer to current guidelines on CDSC and COSURV reporting.

5.6 INFECTION CONTROL STAFF

Inform the infection control team of presumptive and confirmed isolates of *N. meningitidis*

6 REFERRALS

6.1 REFERENCE LABORATORY

Sexually Transmitted Bacteria Reference Laboratory (STBRL)
Health Protection Agency
Centre for Infections
61 Colindale Avenue
London
NW9 5HT
Tel.+44 (0) 20 8327 6464
<http://www.hpa.org.uk/cfi/stbrl/default.htm>

Meningococcal Reference Unit (MRU)
Manchester Medical Microbiology Partnership
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IDENTIFICATION OF NEISSERIA SPECIES

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7 ACKNOWLEDGMENTS 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). We would like to thank Professor Cathy Ison of the Sexually Transmitted Bacteria Reference Laboratory (STBRL) and Stephen Gray of the Meningococcal Reference Unit (MRU) for their significant contributions. 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 also acknowledged.

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

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