Preface

Neisseria meningitidis is a major cause of morbidity and mortality in childhood in industrialized nations and the organism is responsible for epidemics of disease in Africa and in Asia. Because of its public health impact, meningococcal disease is of interest and importance to clinicians, clinical microbiologists, public health physicians, epidemiologists, and research scientists. In *Meningococcal Disease* we have brought together a series of review and methods-based chapters that provide essential information for diagnosis in the clinical microbiology laboratory, isolate characterization, clinical management, and control of meningococcal disease.

New vaccine initiatives are expected in many countries over the next few years, to control both epidemic and endemic disease, and it is hoped that the information available in this book on surveillance and characterization of meningococcal isolates will be of use in the planning of vaccine strategies. A companion text on *Meningococcal Vaccines* is also available from Humana Press and includes overview chapters and detailed methods for the design and evaluation of meningococcal vaccines.

We are grateful to the many contributors to this text who have provided a series of state-of-the-art chapters for inclusion in this book. We are grateful to the series editor, John Walker, and the staff of Humana Press, for inviting our participation as book editors. We acknowledge the contribution of the many clinicians, microbiologists, and scientists who continue to make a major contribution to the diagnosis, management, surveillance, and control of meningococcal disease around the world.

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Isolation, Culture, and Identification of Meningococci from Clinical Specimens

Per Olcén and Hans Fredlund

1. Introduction

Humans are the only natural reservoir for meningococci. The appropriate specimens that should be taken for isolation of meningococci are dependent on the clinical question. The most appropriate specimen and/or laboratory techniques for microbiological diagnosis in an acutely sick patient with suspected invasive disease like meningitis/septicemia ***(1)*** may be quite different from those required for diagnosis of the cause of a local infection in eye, upper respiratory tract, lower respiratory tract, or urogenital tract, or for the study of the carrier state of healthy persons.

Culture still forms the backbone of diagnosis in spite of major improvements in nonculture diagnostic methods (*see* Chapters 3–5), the latter being especially valuable when cultures are "falsely" negative. This can occur for a number of reasons, most often owing to antibiotic treatment before culture, but might also be related to transport media and isolation media. Necropsy tissues and fluids are also particularly difficult ***(2,3)***.

Culture is very important because the availability of an isolate growing in the laboratory will allow species designation, antibiotic-susceptibility testing (*see* Chapter 6), and characterization of an isolate for public-health and epidemiological purposes (*see* Chapters 8–22). An evident factor of importance is also that almost every microbiological laboratory can perform cultures for meningococci.

2. Materials (for Diagnostic Sampling Procedures)

In patients with suspected invasive meningococcal disease, it is logical to take cultures from the suspected primary site of infection (throat/nasophar-

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ynx), and sites of septic metastasis (e.g. cerebrospinal fluid [CSF], joint fluid, etc.) in conjunction with blood cultures. Other superficial/local sights should also be considered if clinical signs and symptoms are suggestive (e.g., skin scrapings or aspirate from petechiae or purpuric rash, conjunctiva, middle-ear fluid, secretions from sinuses, sputum, urogenital).

2.1. The Referral Note Accompanying the Sample(s)

Recognition or suspicion of meningococcal disease in the clinical setting requires laboratory confirmation whenever possible as this information can be critical for managing the individual patient, outbreak management, epidemiological purposes, and for vaccine evaluation. Providing the laboratory with appropriate information can aid this process. Besides basic information (patient identification, sample, date, and sender) the clinical data, tests requested, and diagnostic questions can be crucial in directing the optimal handling and reporting of the specimen in the laboratory.

For throat and nasopharyngeal cultures, it is mandatory to request explicitly culture for meningococci. This is most important because many bacterial colonies of the normal flora look the same as meningococci, which can be in a minority. The inclusion of selective culture medium is therefore necessary.

It is also important to inform the laboratory if antibiotics have been given prior to sampling and if any antibiotic treatment is planned, because this will direct appropriate antibacterial-susceptibility testing. The clinicians' contact details should always be available so that information can be directed to the appropriate individual.

2.2. Blood Cultures

A number of blood-culture systems with different indicator systems are in general use. Most of them utilize bottles containing culture media into which the blood is inoculated ***(4,5)***. The manufacturer's instructions should be followed for the use of these blood-culture systems. For meningococci, media with higher concentrations of sodium polyanethol sulfonate (SPS) should be avoided. Any blood-culturing system must be evaluated for its ability to support growth of fastidious bacteria like meningococci.

Detailed descriptions of the procedures for collection of blood for culture is outlined in laboratory methods published by the Centers for Disease Control and Prevention (CDC) in Atlanta, and the World Health Organization (WHO) in Geneva ***(6,7)***. The following general points should be noted:

1. The concentration of meningococci in blood can be low, less than 1 cfu/mL (*see* **ref. *8***). It is therefore important that the cultured blood volume is as large as possible. For smaller children, 1–3 mL is sufficient, whereas 5–10 mL should be recommended from adults.

2. The concentration of meningococci in blood is probably not constant over time. It is therefore recommended that two blood cultures are performed to increase the likelihood of catching live meningococci.
3. In critically ill patients, it is only feasible to take one blood culture, preferably prior to antibiotics. In benign recurrent meningococcaemia blood cultures may have to be repeated several times, preferably at the early phase of chills and fever in order to obtain a positive result.
4. Inoculated blood culture bottles are kept at room temperature until delivery (as fast as possible) to the laboratory.
5. If certified incubators are available at clinics outside the laboratory, the bottles may be kept at 35–37°C to start the growth process before delivery to the laboratory.

2.3. Cerebrospinal Fluid

In patients with signs/symptoms suggesting meningitis/meningoencephalitis, a lumbar puncture is usually performed ***(9)***, providing that there are no absolute contraindications, such as signs of raised intracranial pressure, substantial hemodynamic instability, or known coagulopathy. The concentration of meningococci in CSF varies considerably between patients from 0 up to 10^7 cfu/mL ***(10)***. When antibiotics have been given intravenously for treatment, it can be assumed that meningococci stay alive somewhat longer in CSF than in blood. As a result, lumbar puncture might reasonably be deferred for a few hours until the patient has been fully assessed and contraindications to lumbar puncture excluded. The following general points should be noted:

1. CSF is collected in 3–4 sterile tubes preferably with ≥ 1 mL CSF/tube.
2. Culture bottles can also be inoculated with CSF at the bedside.
3. Examinations are performed for CSF white blood cells, the proportion of polymorphonuclear/mononuclear white blood cells, glucose, protein, lactate (1 tube); microbiological diagnosis (2 tubes) and 1 extra tube, just in case.
4. Transport should be as rapid as possible to the laboratory (minimize "needle to laboratory time") with the sample at room temperature.
5. Trans-isolate (TI) medium ***(11)*** was designed to allow survival of sensitive bacteria in ambient temperature even in tropical settings for long times. In this medium, meningococci can stay alive for weeks after inoculation with infected CSF, thus allowing safe transport from remote areas to diagnostic laboratories far away ***(6,7)***.

2.4. Throat and Nasopharynx

The optimal place from which to take a swab for culture of meningococci in patients and healthy carriers is not known. With good selective culture media, however, it is clear that carriers with or without local symptoms carry meningococci on the tonsils more often than in the nasopharynx with sample taken via the nasal route ***(12)***. Antibiotic activity is decreased on the membranes of

the throat and perhaps the upper respiratory tract and meningococci can subsequently survive there ***(13)*** for some hours ***(14)*** in spite of effective treatment of invasive meningococcal disease with high doses of parenteral antibiotics. For this reason, throat cultures should be routinely performed for all cases with suspected meningococcal disease ***(14)***. General observations include:

1. The swab used must be proved to be nontoxic to *Neisseria gonorrhoeae* and meningococci and is often provided with charcoal as the absorbing material for toxic substances.
2. The charcoal destroys most of the quality of direct microscopy (DM) and should subsequently not be used if this is requested.
3. If a swab has to be transported it must be in a high-quality reduced medium, such as different variants of Stuart transport medium ***(15)***, kept and transported at room temperature.
4. In scientific/epidemiological studies of healthy carriers, when it is important to find almost all carriers, direct inoculation on culture media at the bedside and immediate incubation (at least placed in a CO_2 atmosphere), e.g., in a candle jar, is recommended.
5. It has been calculated that 90% of the material on a swab is lost by just putting it in a transport medium ***(12)***. Subsequently, up to 40% of meningococcal carriers can turn out culture-negative when using Stuart transport medium if the sample is kept at room temperature for 24 h before inoculation of culture media, as compared to direct inoculation ***(12)***.
6. It is also well known that taking more than one culture from the same site gives additional yield in the case of hemolytic streptococci ***(16)***. It would be surprising if the situation was different for meningococci.
7. In some studies, it is important to know if several strains of meningococci are carried at the same time (*see* Chapter 19).

2.5. Maculopapular Skin Lesions: Petechiae, Echymoses

Meningococci are well known for their propensity for hematogenous spread, with adhesion/trapping in the periphery, and damage of vessel walls. This is most noticeable in the skin where maculopapular eruptions without pustulation and/or extravasation of blood will give the characteristic picture ranging from single petechiae to extensive cutaneous bleeding. Differential diagnosis concerning the hemorrhagic skin lesions differs from place to place and over time, but disseminated streptococcal disease, measles, hemorrhagic viral diseases, conditions causing thrombocytopaenia, coagulopathies, and vasculitis should always be considered. In patients with dark skin, the manifestations can be difficult to detect and the conjunctiva, oral cavity, hand palms, and foot soles may be the only locations where these lesions can be seen.

Meningococci can be isolated from fresh skin lesions. A high diagnostic sensitivity is reached by direct immunofluorescence (IFL) ***(3)***. Owing to lack

of high quality and commercially available IFL conjugates, this method has only rarely reached the status of a routine diagnostic procedure. The number of preserved meningococci is fairly low and Gram staining/methylene blue staining may be used ***(17,18)***. The use of acridine orange (AO) staining ***(19)*** has not been evaluated. Culture from lesions can be helpful. After scraping away the outer epidermis, if possible without causing bleeding, a swab is taken preferably with direct inoculation of culture media. Alternatively, if the lesion is deeper, aspiration may be used with a fine-gauge needle ***(17)***.

2.6. Joint Fluid

Arthritis, usually of a big joint, sometimes results from the systemic spread of meningococci giving signs/symptoms in the acute phase. A so called "reactive arthritis" (sterile) can also be seen after a few days of treatment. In these cases, a diagnostic aspiration of the affected joint is recommended with further handling undertaken as for CSF. Joint fluid could be inoculated into bottles at the bedside, but it is also important to keep some of the fluid in a sterile tube for diagnosis at the laboratory as direct microscopy after Gram and AO staining should be done on the fresh material.

2.7. Other Samples

For other body fluids, the principles are the same as for joint fluid. In cases when very small volumes are aspirated, it is suggested that the material is directly inoculated into a blood-culture bottle. This procedure can include aspiration and reinstallation of a few mL of the broth from the bottle in order to wash out aspirated material from the inside of the needle.

Diagnostic cultures from urethra, cervix and rectum are often taken with a request for *N. gonorrhoeae*. Single patients harbor meningococci ***(20)***, with or without symptoms, probably encountered from the throat (compare gonococci in throat cultures). Meningococci can cause lower respiratory-tract infections and may constitute approx 1% of community-acquired pneumonia in Western countries ***(21,22)***. On rare occasions, meningococci can be isolated from almost any site ***(23)***.

3. Methods for Laboratory Diagnostic Procedures

3.1 Culture Media for Meningococci

Chocolate agar is a rich non-selective medium that is generally used for demanding aerobic bacteria like meningococci, gonococci, and Haemophilus species ***(24,25)***. A formula that is used in accredited clinical diagnostic laboratories has the following constituents: 36 g GC II agar base, 10 g haemoglobin powder, 100 mL horse serum, 10 mL IsoVitalex enrichment, and 900 mL of high-quality water.

This medium can be modified to be fairly selective for meningococci (and gonococci) by addition of a mixture of antibiotics like vancosin - colistin - nystatin (VCN Inhibitor, 10 mL/L of agar). Some laboratories also add trimethoprim. For a detailed presentation of different media, see the CDC/WHO protocols ***(6,7)***.

3.2. Identification of Meningococci by Culture

Plate cultures are inspected after overnight incubation at 35–37°C in humid 5% CO_2 and after 2 d. The broth cultures are inspected daily for turbidity indicating growth or according to the specific suggestions from the manufacturer. If bacterial growth is suspected DM after Gram staining is conducted and two drops of the broth spread on culture media (*see* **Subheading 3.1.**).

Meningococcal colonies are smooth and nonpigmented and, after 18–24 h incubation, 1–2 mm in diameter. From a nonsterile site, the size is dependent on the presence of other competing bacteria. The colonies look the same on chocolate agar medium (nonselective) and selective medium including antibiotics and have a distinctive smell.

Suspected colonies are tested for fast oxidase activity and those giving positive results subjected to Gram staining and microscopy for Gram-negative diplococci. Colonies suspected to be meningococci are subcultured and biochemically tested for degradation of glucose and maltose without degradation of fructose and lactose (ONPG-test) by in house prepared test-plates or commercially available testkits like Rapid NH or api NH. Reference strains for control of all the reactions must always be included. A rapid system which does not require growth but utilizes the preformed enzymes in a heavy suspension can also be used ***(26)***. In this system, the individual high-quality sugars are kept in buffer with a pH-indicator. Ready made mixtures can be kept frozen in a mictrotiter format, thus facilitating practical use. Some meningococcal isolates do not degrade maltose in the system used, thus behaving like gonococci ***(27)***. In inexperienced hands, these isolates can then be wrongly identified as *N. gonorrhoeae*, a diagnosis that may have serious consequences. On rare occasions, degradation of glucose can be weak or absent.

For problem isolates, additional tests have to be performed, including assays for meningococcal antigens like serogroup/type/subtype, biological requirements, genogroup/type/subtype, or additional genetic methods. Reference laboratories provide essential support in these situations.

3.3. Sensitivity Testing for Antibiotics

Sensitivity testing for antibiotics used for treatment of patients and for prophylaxis (prophylactic treatment) of proven or suspected meningococcal carri-

ers at risk should be performed (*see* Chapter 6). Commonly tested antibiotics are penicillin G, ampicillin, a cephalosporin such as cefotaxime or ceftriaxone, chloramphenicol, rifampicin, and a quinolone. The E-test (AB Biodisk) has proven itself to give reliable MIC values ***(28)*** providing a high-quality medium is used and an experienced technician performs the test. Sulphonamide is seldom used these days for treatment or prophylaxis, but sensitivity/resistance (breakpoint 10 mg/L) is an additional characteristic of an isolate that is used for epidemiological purposes. Tests for β-lactamases with, for example, a chromogen cephalosporin test ***(29)*** should be performed, in spite of the fact that less than 10 such strains have been reported so far in the world ***(30)***.

3.4. Grouping of Meningococci

Serogrouping or genogrouping ***(31–33)*** should be done as soon as possible because this provides valuable information concerning the risk of clusters of cases and the possibility of the use of meningococcal vaccines as a prophylactic tool (*see* Chapter 9). Uncommon groups also indicate possible immune defects, including complement deficiencies, in the host, which can be of importance in the short as well as long perspective.

3.5. Blood Culture

A great number of systems for blood culture are available ***(4,5)***. In a European survey among reference laboratories, the Bactec and the BactAlert systems were predominantly used (European Monitoring Group on Meningococci, 1998). In the laboratory, blood cultures are incubated at 35–37°C for 7–10 d. If there are indications of bacterial growth, a bottle is opened and material taken for DM by Gram stain (*see* below) and eventually AO staining ***(19)***. Tests for meningococcal antigens/DNA can be used on blood-culture material to strengthen the meningococcal suspicion when typical diplococci are seen and also if clinical suspicion is high despite negative cultures (*see* Chapter 4).

One drop of blood-culture material is inoculated on chocolate agar, spread, and incubated at 35–37°C in 5% CO_2-enriched humid atmosphere. The plates are inspected after overnight incubation at 35–37°C in humid 5% CO_2 and after 2 d. In situations with high clinical suspicion of meningococcal bacteremia, the inoculated bottles can be subcultured as mentioned on chocolate medium after 2–4 and 7–10 d despite lack of "signs" of bacterial activity.

3.6. Cerebrospinal Fluid

DM and culture are the main methods used. They are sometimes supplemented by specific nonculture tests, either immediately after receiving the CSF at the laboratory, or when the cultures are negative after 2–3 d in spite of per-

Table 1
Protocol for Laboratory Processing of CSF Samples

Clear CSF (centrifuge if < 1 mL at 600*g* for 10 min)	Turbid CSF (no centrifugation)
1. Microscopy: make two slides for a. Gram staining b. Acridine orange-staining	1. Microscopy make 3–4 slides for a. Gram staining b. Acridine orange-staining c. Slide for teaching/extra
2. Culture a. Chocolate agar b. Anaerobic blood-agar plate for anaerobic incubation c. Broth inoculation	2. Culture a. Chocolate agar b. Anaerobic blood-agar plate for anaerobic incubation c. Broth inoculation d. Consider direct inoculation for antibiotic sensitivity testing e. Consider optochine test (on blood-agar medium) when suspecting pneumococci
Consider antigen detection Consider DNA detection	Consider antigen detection Consider DNA detection

sistent suspicion of meningococcal disease. Some of these methods are described in other chapters of this book and comprise antigen-detection methods including latex- and co-agglutination techniques ***(10)***; direct immunofluorescence with specific conjugates ***(3,10)***; enzyme immunoassays ***(34,35)***; and DNA amplification methods like PCR for different target sequences like the 16S rRNA gene ***(36–38)*** and the *ctrA* gene. A protocol for the laboratory processing of CSF samples is shown in **Table 1**.

3.6.1. Direct Microscopy

1. Apply a drop of CSF on each of the clean microscope slides.
2. Let the drops air dry.
3. Fix by heating in a bunsen burner flame from below.
4. Mark the sample area with a wax crayon or by engraving.
5. Apply Gram and AO stains according to local protocols. Gram staining can be performed as follows:
   a. Flood the slide with crystal violet.
   b. After 1 min wash the slide with water.
   c. Flood the slide with Lugol's iodine.
   d. After 1 min decolorize the slide with 95% alcohol.

e. Wash the slide immediately with water.
f. Counter-stain for at least 15 s with carbol-fuchsin.
g. Wash with water.

The stains may be purchased commercially or prepared according to CDC/WHO ***(6,7)*** or the Clinical Microbiology Procedures Handbook ***(24)***, alt. Manual of Clinical Microbiology ***(25)***.

5. Dry the slides by using filter paper outside the sample area and air dry.
6. Read the Gram-stained slide ×1000 in a high-quality, clean light microscope and the AO-stained slide in a high-quality, clean, and optimally adjusted fluorescent microscope ×400–1000.

AO stain is commercially available and staining is performed by flooding the fixed slide with the solution and washing after 2 min with water. After drying, the slide can be read at a magnification of ×400–1000.

In 60% or more of untreated cases of meningococcal meningitis, Gram-negative diplococci of Neisserial shape can be seen extracellularly and also phagocytosed in neutrophile granulocytes, thus suggesting the diagnosis ***(10)***.

With AO staining, the detection level (expressed as bacterial concentration) can be judged to be 10 times lower as compared to Gram stain, which can be calculated to give a diagnostic sensitivity of at least 70%. AO staining is more easy to read (compared to Gram staining) because Gram-negative bacteria give low contrast to the red-stained debris/protein material commonly seen in meningitis.

The less time there is between LP and slide-making, the better the quality of DM slides. This fact can be used to secure sample quality in field situations by making the slides (without staining) bedside just after LP.

3.6.2. Culture

Culture is performed by placing two drops (about 100 μL) on a high-quality, rich, nonselective solid-agar medium like chocolate agar, spreading the plate, then incubating at 35–37°C in humid 5% CO_2. A candle jar ***(39)*** in 35–37°C is another way to create acceptable incubation conditions in laboratories without CO_2 incubators. Enrichment is achieved by inoculating a ~200 μL of CSF in blood-culture bottles (with nutrient additive owing to lack of the blood) or a broth medium like Müller-Hinton broth for 7–10 d. Just as with blood cultures, blind sub-cultures onto agar could be performed at intervals. It is wise to try to keep some original CSF in the refrigerator/freezer for any further diagnostic procedures.

3.7. Joint Fluid

Owing to high concentration of white blood cells (polymorphonuclear leukocytes dominating) and high protein levels, the Gram-stained samples can be difficult to interpret, especially for Gram-negative bacteria. In these cases,

staining with methylene blue ***(25)*** can be superior owing to less denaturation of the material. The ability of meningococci to stay intact for a while intracellularly after phagocytosis can be helpful in the interpretation. AO staining gives an easier picture than Gram staining and with typical diplococci side by side it is easy to determine the presence of pathogenic Neisseria (do not forget *N. gonorrhoeae*). Culture is performed as for CSF (*see* **Subheading 3.6.**). Joint fluid can, if necessary, be studied further with nonculture methods (*see* Chapters 3 and 4).

3.8. Urogenital Samples

The culture media for gonococci readily allow meningococci to multiply and can cause confusion, both species being rapidly oxidase positive Gram-negative diplococci ***(20)***. Growth characteristics (bigger colonies) and species diagnostic tests (sugar degradation or agglutination/co-agglutination tests) will in most cases give clear-cut results, but further characterization is sometimes needed with serological, biological, or genetic methods.

3.9. Reporting of Clinical Isolates

In many countries, meningococcal isolates from normally sterile sites should be reported from the diagnostic laboratories to a National Health Authority and the strains sent for further characterization to a National Reference Laboratory in order to get reliable epidemiological data.

3.10. Storage Meningococcal Isolates

It is often useful to preserve the strains of meningococci in the diagnostic clinical laboratory at either –70°C or freeze-dried for any additional examination in the near or far future ***(6,7)***. A reliable medium for storage for many years in –70°C has the following composition: 30.0 g Trypticase soy broth, 3.0 g yeast extract, 0.5 g agar No. 2, 700 mL water (RO), 300 mL horse serum. Mix the first four items. Adjust pH to 7.5 with 2 *M* NaOH. Sterilize at 121°C for 15 min. Allow to cool to +50°C in water bath. Add horse serum and mix. Check pH 7.50 ± 0.1. Dispense in sterile tubes, 2 mL/tube.

3.11. Selective Media

Because of the possibility of meningococcal infection, it is always a good strategy for culture diagnosis to include a very rich nonselective culture medium, such as chocolate agar, for most clinical samples. A high-quality, selective medium like VCN(T) (*see* **Subheading 3.1.**) should be included for culture concerning pathogenic Neisseria from normally non sterile sites and when mixed infections can be suspected. This includes necropsy material.

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