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Investigation of meningococcal disease

Meningococci cause a spectrum of disease, ranging from asymptomatic colonisation of the nasopharynx, through virulent bacterial meningitis, to fulminating septicaemia. The organisms also cause septic arthritis, conjunctivitis, and pneumonia. Meningitis is the commonest clinical syndrome seen, occurring predominantly in infants and young children, though teenagers and adults may also be affected, particularly during epidemics. Meningococci vary in their fastidiousness; specimens must be transmitted as rapidly as possible to the laboratory.

Strains with decreased sensitivity to penicillin have been reported from Spain, South Africa, and the United Kingdom[1-3]; a β-lactamase producing strain causing meningitis has also been reported from South Africa.[2] It is therefore imperative that careful sensitivity testing is undertaken on all clinically important isolates.

Meningococci are grouped according to the antigenic nature of the capsular polysaccharide. Groups B and C are currently most prevalent in the United Kingdom while strains of group A are common in Africa and the Middle East. Groups Y and W135 are associated with only a very small percentage of infections. Organisms may be typed and subtyped by variations in the outer membrane proteins (porins). Sensitivity to sulphonamide is a further useful discriminatory test. Changes in the epidemiology of meningococcal disease occur constantly and all clinically important isolates should therefore be sent to a reference laboratory for full identification and sensitivity testing. Meningitis is a notifiable disease, though substantially undernotified at present.[4]

The laboratory is likely to be aware of most local cases. Good liaison with clinical colleagues and with the consultant responsible for communicable disease control enables a clinical diagnosis of meningococcal meningitis to be confirmed as rapidly as possible, with follow up of contacts in the community where necessary.

An increasing proportion of cases of suspected meningococcal infection are being treated with antibiotics by the general practitioner before admission to hospital. This is optimal management and is to be encouraged, but it makes the task of the laboratory somewhat more difficult. Emphasis has been given in this Broadsheet to methods that may assist in the isolation of an organism in these circumstances.

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Specimens

CEREBROSPINAL FLUID

Though about 10% of cases of invasive meningococcal disease have septicaemia without clear evidence of meningitis, a combination of the two conditions is by far the most common presentation. Examination of cerebrospinal fluid (CSF) is likely to provide immediate confirmation of the diagnosis for the clinician in about 90% of cases of meningitis; CSF is also the specimen most likely to yield a meningococcus for sensitivity testing and epidemiological typing.

Cell count

Make a cell count in a Neubauer counting chamber in the routine way. If desired (and if the specimen is of sufficient size) a stain such as Azure A MacNeal, which aids differentiation of white cell nuclei, may be used. Add one drop of stain to 0·5 ml of CSF. Mix and leave for five minutes before charging the counting chamber. Count in the usual way; the dilution factor is not important. A neutrophil leucocytosis is commonly seen with counts varying between a few cells and up to 40 000 cells/mm^3 CSF. Red blood cells are often also present in smaller numbers, even in a specimen collected atraumatically.

Chemistry

A portion of the specimen should be reserved for estimation of total protein and glucose. CSF protein in bacterial meningitis is usually greater than 0·45 g/l. CSF glucose is compared with a blood glucose sample taken at the same time; the normal CSF glucose should be 70% or more of the blood glucose value an relative reduction is usually seen in bacterial meningitis.

Microscopy for organisms

If organisms are abundant in the CSF they may be

seen during the cell count, though this is unusual in meningococcal disease.

One or two loopsful of neat CSF are transferred to a clean microscope slide, fixed, and Gram stained. At the same time a larger aliquot of CSF is centrifuged at 2000 rpm for five minutes. The supernatant is aspirated aseptically to a sterile container and preserved at −25°C. (It may be used for viral culture in the event of bacterial culture proving negative). The deposit after centrifugation is used to make a second Gram film and to inoculate culture plates. Either or both films may be examined according to the concentration of cells in the original specimen.

Careful examination of the Gram film is the most important step in examination of a CSF specimen exhibiting neutrophil pleocytosis. Up to 80% of cases in which a meningococcal aetiology is subsequently confirmed will show Gram negative diplococci if examined carefully. Examination may take half an hour or longer if bacteria are very scanty. Often bacteria are predominantly intracellular with occasional cells showing one to dozens of pairs of diplococci while most polymorphs contain no bacteria at all. If the patient has received antibiotics more than an hour or two before the lumbar puncture was undertaken swollen degenerating pairs of diplococci and a greater proportion of single organisms may be seen. Occasionally it may be difficult or impossible to be certain that some Gram negative, intracellular inclusions are truly bacteria.

Alternative staining techniques, such as Sandiford's modification of Gram's stain in which malachite green is used as a counterstain,[5] or simple staining with methylene blue may be tried, but probably do not add greatly to the chances of providing a definitive answer for the clinician on a difficult specimen.

Antigen detection

If there are polymorphs in the CSF and organisms are not seen after careful examination of a Gram film, meningococcal meningitis is a more likely diagnosis than haemophilus meningitis in children aged between 3 months and 3 years. In these circumstances examination of CSF for the presence of bacterial antigen may be tried. Various methods for the detection of free antigen in CSF are available including latex agglutination (Wellcogen, Wellcome Diagnostics; Directigen, Becton Dickinson), enzyme immunoassay (Pharmacia Diagnostics), and counter-current immunoelectrophoresis (CIE). The limulus amoebocyte assay which detects endotoxin, denoting infection with a Gram negative organism, has also been used.[6] Good comparative trials of the sensitivity and specificity of these methods in meningococcal disease are lacking. Latex agglutination seems to be superior to both CIE and the limulus test in meningitis due to *Haemophilus influenzae* type b,[7] but was less sensitive in our hands than microscopy in meningococcal meningitis due to group B organisms. The group B polysaccharide is a poor antigen and detection systems for the polysaccharide are less sensitive than with groups A, C, Y and W135. There is even less information on the acute examination of other fluids such as blood and urine but they are likely to be less satisfactory owing to the lower concentration of meningococcal antigen.

In general, latex agglutination is the simplest and most convenient of these methods as well as being at least as sensitive as enzyme linked immunosorbent assay (ELISA) and better than CIE. The limulus assay is technically demanding.

Culture

Inoculate blood agar and chocolate agar plates. A drop of centrifuged deposit should be placed on each plate and allowed to dry in before being spread with a loop. This is particularly important in patients who have received parenteral antibiotics before admission to hospital as the area of original inoculation may retain an inhibitory concentration of antibiotic. Plates should be incubated in 5% carbon dioxide at 37°C for 48 hours, though almost all meningococci will grow after overnight incubation. Even though plates have been inoculated in the middle of the night, careful examination with a hand lens the following morning may show developing colonies.

In 5% or more of patients with clinically suspected meningococcal meningitis the organism is grown from a CSF specimen which contains *no cells and in which organisms are not seen* (Cartwright KAV, unpublished observations). CSF protein and glucose are also normal in these cases. Negative CSF findings must not be used to defer antibiotic treatment in a patient with headache and a suspicious rash. A pure lymphocytic cellular response in CSF, normally associated with viral meningitis, is occasionally seen in culture positive meningococcal meningitis (and is not uncommon in meningococcal septicaemia, resulting from meningeal irritation without invasion).

Blood culture

At best, blood cultures are positive in only a third to a half of the untreated patients, despite the presence of a characteristic meningococcal rash often accompanied by positive CSF on microscopy or culture, or both. Despite this surprisingly low figure blood cultures are a mandatory part of the investigation of patients with suspected meningococcal disease—either septicaemia or meningitis. Meningococci will often grow only in the aerobic bottle of a two-bottle blood culture system. Ideally aerobic cultures should be incubated in an atmosphere of 5% carbon dioxide for at least five

days, though most positive results will be obtained within the first 48 hours. In patients who have received parenteral antibiotics before admission to hospital the chances of a positive blood culture are remote.[8 9]

Using the Bactec radiometric blood culture system we have found occasional strains which have failed to use sufficient labelled substrate to give a positive reading but which have survived. We therefore routinely subculture daily the aerobic bottles from patients suspected of having meningococcal disease whether or not they give a positive reading. Isolates obtained from blood cultures should be identified in the same way as for strains from CSF.

Throat swab

Though the isolation of a meningococcus from the throat does not confirm a diagnosis of invasive meningococcal disease, it may be a useful finding in a patient with suggestive symptoms. It offers a good chance to obtain an isolate for sensitivity testing and for epidemiological purposes in two particular situations. The first is when the patient has been given antibiotics before the collection of diagnostic specimens. Blood cultures will almost certainly be negative and even if a lumbar puncture is performed about 30–40% of all cases of invasive meningococcal disease will not yield a positive culture from blood or CSF. The second situation, encountered increasingly often, is when a lumbar puncture is not undertaken. Lumbar puncture is contraindicated in the presence of raised intracranial pressure, usually diagnosed clinically by the finding of papilloedema on fundoscopy. Some clinicians, however, take signs of increasing confusion and disorientation, or progression towards a comatose state, as evidence of raised intracranial pressure and will not undertake a lumbar puncture in these circumstances. A well taken throat swab then assumes great importance. Swabs can be collected most reliably by the medical microbiologist, ensuring correct swabbing technique and rapid plating of the specimen. A postnasal (transoral) swab is preferable[10] but often difficult to obtain in the ill patient and in young children. Because up to 25% of young adults may carry nasopharyngeal meningococci a throat isolate will need to be grouped and typed before the possible relevance of the strain can be assessed.

POSTNASAL (TRANSORAL) SWAB

The patient is asked to open the mouth as wide as possible and the swab is swept over the posterial pharyngeal wall directly behind the uvula and avoiding the tonsillar areas. Fluffy charcoal impregnated swabs are preferred as meningococci do not remain viable on plain swabs for very long; they can be used in conjunction with Stuart's transport medium[11] but immediate plating gives better results. If plain swabs are used the plates should be inoculated immediately after the swab has been taken, although a transport medium that contains charcoal (Amies) achieves acceptable but reduced survival.[10]

PERNASAL SWAB

Pernasal swabs are necessary in small children and also in older children and adults who are so disorientated as to be unable to cooperate with the collection of a postnasal swab. A pernasal swab consisting of a small cotton wool pledget mounted on flexible wire in a blue tube (Medical Wire & Equipment Co., Corsham, Wiltshire) is needed. The child's head should be steadied by one nurse while a second nurse holds the patient's hands. The swab is passed gently via either nasal orifice directly backwards towards the occiput. The posterior nasopharyngeal wall is encountered at a depth of about two inches. The swab is quickly rotated and withdrawn. The procedure is moderately irritant and babies and small children will try to pull the swab out if not prevented from doing so. If possible two swabs should be obtained so that one may be used for virus culture.

Postnasal or pernasal swabs should be plated out immediately on to a selective medium such as Modified New York City medium or GC agar medium to suppress other components of the normal oropharyngeal flora.

Smears from purpuric skin lesions

We do not have personal experience of this technique, rarely used nowadays. Nevertheless, it seems to give positive results in a substantial proportion of cases[12 13] and may permit the demonstration of organisms from patients with negative blood and CSF cultures. The method below is that of Tompkins.[13]

A purpuric skin lesion is pinched between finger and thumb to exclude circulating blood. The lesion is "picked" with a sterile hypodermic needle or fine scalpel blade. More pressure is applied to squeeze out a drop of tissue fluid and blood which is then smeared on a glass slide. Several small smears of 3–4 mm are better than one large one. After fixation in methanol the smears are better stained with Wright's or Giemsa stain than with the Gram stain. Dilute Giemsa stain (one part of stock to 50 parts of buffer at pH 7·2 is recommended for optimal differentiation of bacteria and polymorph nuclei). Organisms stain blue-black and can be detected easily within polymorphs. Extracellular organisms should be ignored as possible contaminants.

Confirmation of identity of presumptive colonies

Meningococci appear as 2–4 mm grey, round entire

colonies after 24–48 hours' incubation. An oxidase test using aqueous 1% tetramethyl-p-phenylenediamine prepared freshly should be performed. A known, oxidase positive, control organism should be used. A Gram film of a colony should show characteristic Gram negative diplococci.

Colonies of meningococci produce γ-glutamyl aminopeptidase as a preformed enzyme which hydrolyses γ-glutamyl p-nitroanilide to release yellow p-nitroaniline. This forms the basis of a commercial Neisseria identification kit (Gonocheck 11—EY Laboratories Inc., San Mateo, California, USA). This kit will differentiate satisfactorily strains of *N lactamica, N gonorrhoeae*, and *N meningitidis*. A second tube containing a chromogenic β lactam is used to detect β lactamase production. Careful determination of the penicillin minimum inhibitory concentration should be considered if there is any indication that the penicillin sensitivity of an isolate from a systemic infection is reduced.

RAPID CARBOHYDRATE UTILISATION TEST (RCUT)

Growth on a medium containing glucose induces carbohydrate degrading enzymes which may be used to confirm the identity of putative meningococcal colonies in a rapid test. (Appendix).

MENINGOCOCCAL REFERENCE LABORATORIES

All strains of meningococci isolated from deep sites (blood, CSF etc) should be sent to a meningococcal reference laboratory (Appendix). This is important to provide epidemiological data on the incidence of meningococcal disease and also to monitor the prevalence of meningococcal serogroups, serotypes, and drug resistance in the United Kingdom. Meningococcal disease is substantially undernotified and the submission of strains to reference laboratories provides invaluable additional data.

SENSITIVITY TESTING

Sensitivity testing presents problems in laboratories which encounter only a few meningococcal isolates each year. Disc sensitivity testing is inherently unreliable. The method below is acceptable for occasional use, but results should be regarded as provisional and should be confirmed as soon as possible by determination of antibiotic minimum inhibitory concentrations, preferably by a reference laboratory.

Oxoid DST agar CM261 with 5% lysed horse blood and Mast antibiotic discs with the following concentrations should be used: penicillin 1 μg; chloramphenicol 10 μg; rifampicin 5 μg; sulphafurazole 5 μg. The Stokes method[14] with a rotary plater is used. A documented, fully sensitive strain of meningococcus is the best control, but if none is available the Oxford staphylococcus may be used instead for testing sensitivity to penicillin, rifampicin, and chloramphenicol. The Oxford staphylococcus is *not* suitable as a control for sulphonamide sensitivity testing and a known sensitive meningococcus must be used.

Low level penicillin resistance will not be detected by this method but β-lactamase production (if and when it occurs in the United Kingdom) should be apparent. Clinically important resistance to the other agents should also be detectable.

GROUP AND SEROTYPE DETERMINATION

Cultures may be grouped by bacterial agglutination using monoclonal or polyclonal anti-polysaccharide antibodies (DMRQC, Difco, or Wellcome). These occasionally yield misleading results. Latex preparations for antigen detection may also be used to obtain a presumptive result. The group may be confirmed and the serotype determined by the reference laboratory.

SEROLOGY

Invasive meningococcal disease is accompanied by an antibody response detectable in a variety of ways using tests with antigens of different specificities. While these tests are not routine, and may only be available at reference laboratories, they may occasionally be of value to confirm the nature of an infection for epidemiological purposes, or to investigate patients thought to have some immunological deficiency.

STORAGE OF ORGANISMS

Meningococci can be stored in the short term on Dorset egg slopes. Strains should be inoculated on to slopes, incubated overnight at 37°C, and then held at 30°C. Viability should be maintained for at least six months. Strains on Dorset egg slopes are suitable for sending through the post. For longer term storage liquid nitrogen is satisfactory; scrape the growth from an overnight non-selective culture plate into Oxoid nutrient broth with 16% added glycerol.

Freeze dried cultures remain viable for 10–15 years.

Appendix

NON-SELECTIVE MEDIA

Blood agar: Columbia agar base with 5% sterile defibrinated horse blood.

Chocolate agar: as for blood agar but with chocolated horse blood.

SELECTIVE MEDIA

A *GC agar*

Oxoid GC agar base: CM367
Oxoid sterile GC Supplement: SR56

Contains yeast autolysate	10·0 g/l
dextrose	1·5 g/l

sodium bicarbonate	0·15 g/l
vancomycin	3·0 mg/l
colistin	7·5 mg/l
nystatin	12500 units/l
trimethoprim	5·0 mg/l

Suspend 36 g of Oxoid GC agar base in 880 ml of distilled water and bring gently to the boil to dissolve the agar. Autoclave at 110°C for 20 minutes. Cool to 50°C. Add 100 ml lysed sterile defibrinated horse blood and two phials of sterile GC supplement reconstituted with 10 ml sterile distilled water to give final concentrations as above. Mix gently to avoid trapping air bubbles.

B *Modified NYC agar*

(a) NYC Base (to make 1000 ml base)

Constituents:

5% lysed horse red blood cells	300 ml
distilled water	330 ml
horse serum	300 ml
yeast extract 0·5% (Difco)	62·5 ml
trimethoprim 10 mg/ml	0·76 ml
vancomycin 10 mg/ml	0·76 ml
colistin 10 mg/ml	1·87 ml
glucose	12·5 g
peptone (Evans)	37·5 g
K_2HPO_4	11·5 g
KH_2PO_4	0·65 g
NaCl	12·5 g
L-glutamine	0·5 g

Method:

Make 5% lysed red cells; clarify with Seitz grade 5 filter. Make bulk of medium in bucket; clarify with Seitz grade 5 filter; add red blood cells.

Reclarify total medium using Seitz grade S9 filter.

Check pH; adjust to 7·2 if necessary.

Sterilise by filtration, such as Carlson Ford 10 litre filter holder with EKS filter pad (No XE 675).

Bottle aseptically in 200 ml volumes.

Prepare test batch of finished medium using final bottle.

Store at −20°C.

(b) NYC Starch Agar (to make 3000 ml)

Distilled water	2800 ml
Davis agar	60 g

Autoclave to dissolve.

Distilled water	200 ml
Starch	10 g

Boil to dissolve.
Mix agar and starch.

Bottle in 300 ml amounts; autoclave at 121°C for 20 minutes.

Store.

To make Modified NYC Agar:
Melt 300 ml starch agar; cool to 65°C.

Mix with 200 ml NYC base.

Pour plates.

Final concentrations of antibiotics:
trimethoprim 3 μg/ml
vancomycin 3 μg/ml
colistin 6 μg/ml

RAPID CARBOHYDRATE UTILISATION TEST

Purity plate

Oxoid GC agar base	100 ml
Vitox solution	1 ml
10% glucose	1 ml

Sugar solutions

10% w/v solutions of lactose, glucose, maltose and sucrose in sterile distilled water prepared in advance and stored as 1 ml volumes in bijoux at −20°C. Use BDH extra pure maltose to avoid trace contamination with glucose. For use distribute 20 μl of 10% sugar solutions into screw-capped phials and store at −20°C.

Buffer solution

0·1 M K_2HPO_4	40 ml
0·1 M KH_2PO_4	12 ml
8% KCl	100 ml
1% aqueous phenol red	10 ml
Sterile distilled water	838 ml

Distribute as 20 ml volumes in universals and store at −20°C, For use distribute in bijoux as 2·5 ml volumes and store at −20°C.

The test

Inoculate a single colony of a suspected meningococcus on to modified GC agar and incubate overnight. Take from the deep freeze the appropriate number of sugars and a bijou of buffer solution. Distribute 0·6 ml of buffer into a labelled clean tube. Make a heavy suspension of the test organism in the buffer (at least a loopful). Dispense 120 μl of suspension into each sugar. Incubate in a 37°C waterbath for four hours. Positive reactions are indicated by development of a yellow colour. Tubes remaining red are negative. Each batch of sugars is controlled with a known meningococcus and gonococcus but control organisms are not used with each individual test. Meningococci give positive reactions with glucose and maltose, negative reactions with lactose and sucrose.

REFERENCE LABORATORIES

England

Public Health Laboratory, Withington Hospital, Manchester M20 8LR

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