Laboratory Diagnosis of Gonococcal Infections *

ALICE REYN, M.D.¹

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INTRODUCTION

The genus Neisseria comprises Gram-negative, aerobic or facultatively anaerobic cocci, usually but not invariably arranged in pairs. The size is about 0.8µ by 0.6µ. They often grow poorly on ordinary media and they are frequently pathogenic. Few carbohydrates are fermented, indole is not produced and nitrates are not reduced. With a few exceptions, catalase and oxidase are abundantly produced; some species are haemolytic. Neisseria are non-motile.

The most important members of the genus Neisseria are N. gonorrhoeae and N. meningitidis; these organisms have been described more thoroughly than the other members of the group, such as N. catarrhalis, N. flavescens, N. sicca and N. flava (subspecies I-III). The classification of the Neisseria is far from being complete and a taxonomic study of this group is needed.

N. gonorrhoeae (gonococcus) is the etiological agent of "gonorrhoea", whereas N. meningitidis (meningococcus) is the organism causing what is commonly called "epidemic cerebrospinal meningitis". The other less pathogenic or non-pathogenic members of the genus are most frequently found in the respiratory tract and occasionally in the genital regions.

In this paper, attention will be paid to the laboratory identification of the gonococcus and reference will be made to the other members of the genus only in connexion with differential diagnostic problems which may be of forensic significance, especially in the diagnosis of gonorrhoea.

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COLLECTION AND HANDLING OF SPECIMENS

GENERAL REMARKS

The material will most frequently be discharge collected from the male or female urethra and the cervix; specimens can also be taken from the rectal mucosa, prostate, vagina, conjunctiva, Bartholin's glands, Skene's ducts, joints, blood, spinal fluid and sediment from centrifuged urine.

The material can be examined by direct microscopy and by culture; for culture immediate plating is preferable, but in most cases this is impossible. In females, where the microscope findings are very unreliable, it is recommended that both culture and microscopy be used. In male contacts (symptomless carriers) the culture is also of great importance. Culture is also much better as a test of cure than is the simple microscope examination of a slide. The method of transport of the infected material plays a decisive role, especially when the transportation time exceeds a few hours and the temperature is high. When specimens are taken for both microscopy and culture the specimen taken first should be used for the culture because it is likely to contain more material than the second specimen (Reyn et al., 1960).

Generally, wooden applicators covered with cotton wool are used to collect the material for microscope and bacteriological examination. However, some kinds of wood are known to be toxic to the gonococcus and the use of aluminium wires has therefore been recommended (Beakly, 1957). The toxic effect of the swabs can be counteracted by boiling in buffer and impregnating with charcoal (Moffett et al., 1948); this procedure appears to act also upon the toxic fatty acids demonstrable in certain batches of agar (Ley & Mueller, 1946). Many different types of collection outfit have been described. In this paper, the only method described in detail is the Stuart method (Stuart, 1946; Stuart et al., 1954; Stuart, 1956) modified by Reyn et al. (1960) and by Ringertz (1960). The Stuart medium is a non-nutrient, reducing medium in which the oxygen tension can be checked by observing the change in the colour of the medium from colourless (reduced) to blue (oxidized). It should only be used in association with charcoal-impregnated swabs, which counteract toxic substances. Immediate plating is possible only where laboratory facilities are available on the spot; in other cases plating is necessarily delayed for several hours or even several days. If posting has to be delayed the specimens should be kept in the refrigerator or in some other cool place until they are despatched. Transportation in temperate or hot climates for more than a few hours favours the growth of contaminants at the expense of the gonococci. Selective inhibition of the contaminants during transportation by means of dyes (methylen blue, crystal violet, Nile blue A, etc.) or other chemical substances (thallium acetate) or by antibiotics has been proved to be of little value (Peizer & Steffen, 1947; Lagergren & Ochterlony, 1948; Bang, 1952). The lower limits of concentrations toxic to the contaminants are very close to those toxic to the gonococci. However, when sampling conditions are very poor or when the patients are from a low social level the addition of inhibitors to the primary isolation medium may be indicated. An exception to the general rule is polymyxin B which completely inhibits the growth of coliform bacilli without harming the majority of gonococcal strains (Crookes & Stuart, 1959). However, at the same time better conditions are provided for other contaminants, especially diphtheroids and Gram-positive rods.1 In hot climates it is recommended that specimens be transported in vacuum bottles at temperatures between 0°C and 4°C.

The sampling technique is of great importance but this is not the place for a detailed description of the techniques for taking samples from the various sites of gonorrhoeal infection.2 Unnecessary contamination should be avoided and the sample should be as "rich" as possible in order to compensate for loss during transportation. It is stressed that sterile vaginal specula should be used for collecting specimens from the cervix; some lubricants are toxic and should be avoided. The cervical plug, if present, should be removed before the swab is taken. The applicator should be inserted about 1 cm into the cervix.

1 Recently, Thayer & Martin (1964) have proposed a selective medium which contains, in addition to 25 IU of polymyxin B, 10 μg of ristocetin per ml. Ristocetin inhibits many Gram-positive micro-organisms. In the author's laboratory, preliminary experiments with a selective medium of similar composition have given very promising results.

2 For further details see US Public Health Service (1962); Carpenter (1963).
METHOD OF TRANSPORTATION

The method of transportation described below was introduced by Stuart (Stuart, 1946; Stuart et al., 1954; Stuart, 1956) and modified by Reyn et al. (1960). See also Ringertz (1960).

The modified Stuart device consists of:

1. 15-mm × 150-mm tubes containing a 10-cm high column of solid Stuart’s medium (for composition see page 465). The labelled tubes are tightly closed with hydrophobic cotton-wool plugs and the date of preparation is stamped on the labels.

2. Cardboard tube containers.

3. Resistant, special coloured paper envelopes marked “Neisseria Department, Statens Seruminstitut, København”.

4. Charcoal-impregnated, sterile wooden applicators.

5. Forms on which to enter the patient’s data.

The Stuart’s medium is dispensed while hot into the sterile cotton-wool-plugged tubes. After preparation, the tubes contain a 10-cm column of medium, which should be colourless except for the uppermost half a centimetre, which will be blue owing to oxidation of the methylene blue. Oxidation will proceed during storage, and completely oxidized medium is no better than broth or saline. Tubes with less than 3 cm of colourless medium left in the bottom must be regarded as useless. However, the medium can be reduced by heating in a water-bath at 100°C for half an hour, leaving the stoppers in the tubes, and subsequent cooling in cold water. This process performed once does not impair the medium. When stored in a cool place and prepared with a controlled batch of thioglycolic acid the tubes will keep for at least two months.

On exposure to light, methylene blue is irreversibly converted into a colourless compound, hence the tubes should be protected from light during storage.

For sampling, good quality absorbent cotton-wool and wooden swab sticks are used. After sampling, the swabs should be inserted deep into
the labelled tubes of media without breaking the sticks; the stoppers should be put in, leaving the upper ends of the sticks projecting about 1 cm above the edge of the tube as shown in Fig. 1. When posted, the specimens should be placed in cardboard containers (Fig. 2), which are then inserted in special envelopes and they should be kept in a cool place if posting is delayed.

BACTERIOLOGICAL EXAMINATION

MICROSCOPY AND GRAM-STAINING TECHNIQUE

The most primitive laboratory method is the microscope examination of a stained smear of discharge. To a great extent the success of this method depends upon the way in which the smear is collected and prepared. Though apparently simple, the method of preparation nevertheless deserves close attention; it should be borne in mind that charcoal-impregnated swabs should be avoided in the preparation of smears. Smears should be made as thin and as even as possible. Before posting or staining, the smears should be exposed either to air-drying and gentle flame-fixation or to air-drying plus methanol fixation for not less than two minutes and preferably for 10 minutes. Fixation with methanol is much better than flame-fixation in the case of protein-rich smears from patients with a florid gonorrhoea. The general rule must be that the diagnosis of gonorrhoea by microscopy is made only after careful Gram-staining; the latter may be performed after previous staining with 1% methylene blue, provided the immersion oil has been completely removed by means of xylol. The Gram-staining technique may be any one of the usually recommended methods, but it is important to be familiar with the method in question and to control the various ingredients for precipitation and infection.

Methylene blue without subsequent Gram-staining is still used even by specialists, who believe that by this means they are able to distinguish between N. gonorrhoeae, staphylococci, Escherichia coli and other Gram-negative, more or less well-defined, short, plump rods. This is impossible. Even after Gram-staining it may be impossible, although this method is more reliable than simple methylene blue staining. The reliability of the Gram procedure depends to a great extent on the staining technique; it is very difficult to obtain the right degree of decolorization, especially when many slides of varying thickness are stained at the same time. It is emphasized that old staphylococcus cultures are very easily decolorized and that short Gram-negative rods (E. coli, etc.) may also be mistaken for gonococci (Reyn, 1951). Badly performed Gram-staining is no better than staining with methylene blue.

At the Statens Serum Institut in Copenhagen, the fixed smears of discharge or of watery suspensions of pure cultures are (1) stained with 0.2% aniline crystal violet for one minute, (2) treated with Lugol’s iodine solution for one minute, (3) decolorized with 96% ethanol for half a minute, (4) rinsed in running tap water for about five seconds, (5) counterstained with 0.1% phenolized fuchsin for one minute, (6) rinsed in running tap water for about five seconds and (7) dried with blotting paper. (For the composition of the reagents, see page 466.)

Classically, in acute male gonorrhoea, large numbers of polymuclear leucocytes containing Gram-negative, coffee-bean-shaped diplococci are observed. In some cases, only a few of the leucocytes show one or two typical intracellular pairs and in other cases all the cocci are extracellular. In chronic male and female gonorrhoea, it is often impossible to find any typical gonococcus pairs, especially in the cervical secretion.

Stained films of pure cultures reveal the gonococci as roundish cocci, typically but not invariably arranged in pairs. After 18 to 24 hours’ growth, the size is uniform and the stain is taken up quite evenly. After 48 hours’ growth, size and staining are more variable and lysis is often observed.

Cellular morphology is progressively altered by contact with penicillin, both in vivo and in vitro. Smears of exudate taken during the first four or five hours of penicillin therapy show gonococcal forms that progressively enlarge and decrease in stainability until none can be detected. Similar morphologic changes are observed during chloromycetin therapy but not during sulfonamide treatment.

In conclusion it is emphasized that it is impossible to diagnose gonorrhoea with certainty by microscopy alone. Clinical evidence and history must also be taken into account, but even then there remains a wide margin of error.
CULTURE AND ISOLATION

Most strains of gonococcus do not grow in veal or beef broth or on simple broth-agar plates. Sensitivity to inhibition, rather than complexity of nutritional requirements may account for much of the fastidiousness of the gonococcus. The beneficial effect of the addition of charcoal, starch, albumin, serum or ascitic fluid to the medium is generally ascribed to a detoxifying action on inhibitors present in the agar or in the peptone, but fresh serum and ascitic fluid also contain factors essential for the growth of certain strains. In semi-solid broth-agar, most laboratory strains grow well in a layer just below the surface. The pH of the medium should be adjusted to between 7.2 and 7.6. Synthetic media have been described (Welton & Scherp, 1944; Welton et al., 1944; Gould et al., 1944; Hill, 1948; Hill et al., 1948, 1949), but these are not suited for primary isolation of N. gonorrhoeae.

The CO₂ tension is of decisive importance for successful primary isolation and a certain degree of moisture is also favourable to growth (McLeod et al., 1934; McLeod, 1947; Reymann, 1941, 1944; Mueller et al., 1942; Hill et al., 1948; Morton, 1945; Griffin & Racker, 1956; Griffin & Rieder, 1957). It is impossible at the present time to prescribe the ideal gonococcus medium, one of the difficulties apparently being that the growth requirements and resistance to toxic substances vary from one strain to another (Lankford & Snell, 1943; Lankford et al., 1943; Gould, 1944; Lankford & Skaggs, 1946). Numerous types of "enriched" media have been described, most of which contain a broth-agar base supplemented with heated blood (or haemoglobin), and/or yeast extract, serum, ascitic fluid and albumin. A chemical supplement, containing glucose, glutamine and cocarboxylase was described by Lankford (1950).

The choice of medium depends upon the local situation; in larger laboratories with easy access to fresh blood (horse, beef, rabbit or sheep) and ascitic fluid and with centralized media production facilities it is natural and relatively cheap to use these products as they are. In smaller laboratories, it is practical to use the commercially available dehydrated complete media or dehydrated media bases.¹ Dehydrated media are sometimes more costly than fresh media, but the cost should be considered in relation to other relevant factors, such as number of technicians and laboratory facilities. However, the dehydrated media are not always quite as good as the fresh media and further experiments are needed. The addition of inhibitors (dyes or antibiotics) to the culture media is not generally recommended (Gould, 1944; Gould et al., 1944; Hill et al., 1948). A high yield of positive cultures may be obtained by using two or more plates, one (or more) containing a selective agent and one without it.

In the gonococcus laboratory of the Statens Seruminstitut a soft chocolate ascitic-fluid-agar medium was formerly used for primary isolation. Recently, the ascitic fluid has been replaced by a combination of yeast and liver autolysates and the heated blood by 1% haemoglobin (Meller & Reyn, 1965).² This so-called HYL (Haemoglobin-Yeast-Liver) medium is also a "chocolate" medium and for convenience both media are described in detail in a later section (page 463). The turbid, brownish medium is dispensed into aluminium Petri dishes 9 cm in diameter and about 1.5 cm deep, or into similar dishes made of plastic and purchased in a sterile condition; the layer of medium should be not less than 5 mm. Polymyxin B sulfate is added and under certain conditions nystatin is also added.

Inoculation and incubation of primary cultures

Immediately upon arrival the swabs are streaked on one half of a plate, care being taken to transfer as much material as possible to the plate.³ The inoculum is spread as shown in Fig. 3 by means of two separate platinum wires (gauge 0.7 mm) about 8 cm long with closed loops 2-3 mm in length. All plates should be examined for surface contamination before use. The result of the spreading is shown in Fig. 4.

The inoculated plates are incubated for about 24 hours at 35°-36°C in closed glass or plastic jars containing a moist atmosphere with about 8% of CO₂ (see Fig. 5).

The glass jars have a capacity of about 4.5 litres and have cut collars which fit into metal lids; between the collar and the lid a ring of rubber is inserted. The plates are placed on a perforated

¹ Bacto-G C Medium Base enriched with haemoglobin and Bacto-Supplement B or C may well be used (see Difco Manual of Dehydrated Culture Media and Reagents for Microbiological and Clinical Laboratory Procedures, 9th ed., Detroit, Mich., Difco Laboratories, 1953, p. 122). Bacto-Supplement C is the same as Bacto-Supplement B but without the addition of glucose.

² See the article on page 471 of this issue.

³ Before inoculation urines, spinal and synovial fluids are centrifuged. Blood cultures are made by introducing 5 ml of blood into flasks containing ox-heart broth enriched with 1% of glucose and 25% of ascitic fluid.
FIG. 3
DIAGRAM ILLUSTRATING METHOD OF INOCULATION

1: Streaking with swab.
2 & 3: Spreading with bent platinum needles.

FIG. 4
GROWTH OF NEISSERIA GONORRHOEAE AND STAPHYLOCOCCUS ALBUS AFTER 48 HOURS' INCUBATION

This photograph demonstrates the effect of the spreading method illustrated in Fig. 3. The organisms were inoculated on a "chocolate" ascitic medium in an aluminium Petri dish, shown natural size.
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FIG. 5
JARS USED FOR INCUBATION OF INOCULATED PLATES

Left: Glass jar, metal lid, clamp and rubber ring. Inside the jar is a perforated metal support with a tube containing sulfuric acid; pellets of sodium bicarbonate can be seen on the bottom.
Right: Plastic jar with lid open. Beside it is the plastic rest with tube containing sulfuric acid.

support holding a tube containing 7 ml of molar H₂SO₄; a calibrated spoon containing about 1.5 g of NaHCO₃ is put in the bottom of each jar. The lid is closed by means of a screw clamp; it is fitted with a valve. After closing the lid with the valve open, the jar is tilted to allow the H₂SO₄ to combine with the NaHCO₃; when the CO₂ evolved has pushed out the superfluous air, the valve is closed.

The chemical process that takes place in the jar is as follows:

\[ 2 \text{NaHCO}_3 + \text{H}_2\text{SO}_4 = \text{Na}_2\text{SO}_4 + 2 \text{H}_2\text{O} + 2 \text{CO}_2. \]

From this equation the amounts of H₂SO₄ and NaHCO₃ can be calculated for other volumes and other concentrations (1 gram molecule = 22.4 litres). It is most practical to use a constant amount of H₂SO₄, which will determine the amount of CO₂, provided an excess of NaHCO₃ is used. The amount should be calculated making allowance for the fact that the jar will normally be full of plates.

A CO₂ atmosphere can also be obtained by the “candlelight method” (Ferguson, 1945) or from an iron tank containing a mixture of compressed air and CO₂. However, in the author’s laboratory it was found, for some unknown reason, that the CO₂ supplied from iron tanks (especially when dispensed into stainless steel containers instead of glass jars) was less favourable to the growth than the CO₂ supplied from H₂SO₄ + NaHCO₃. If the CO₂ is supplied in one of the ways mentioned above, a humified atmosphere should be obtained by means of moistened cotton-wool pads.

Recently, acid-resistant polyvinyl chloride jars have been used. They are much lighter than the glass jars, they do not break, and it is not necessary to close their smoothly fitting lids by means of screw clamps. The arrangement for filling with CO₂ is the same as that used in the glass jars. The lids (without valves) are put on just after the CO₂ has
been produced by mixing the H₂SO₄ with the NaHCO₃.

After 18-24 hours the plates are removed from the jars. Usually, the plates are inspected in artificial light with the naked eye. The typical round colonies are 0.5 to 1 mm in diameter; they are convex, greyish and glistening, with smooth edges; on further incubation the colonies increase in size and develop a roughened surface with crenated edges. It is impossible to give an exact description of the colonies because their appearance varies with the medium, and particularly with the strain. The size and appearance vary greatly with the age of the culture, the degree of crowding, and with the colony type. If the plate is crowded with large numbers of colonies or contaminants, the colonies will be small; if the colonies are well isolated they should be large. The size may vary from less than 1 mm to about 5 mm in diameter. The consistency of the growth is often viscid; this is most pronounced after 48 hours' growth. In laboratories in which the identification of gonococci is a rare event it is recommended that control strains should be kept for comparison. In the author's laboratory, about 80% of the total number of positive cultures are detected after 18-24 hours. However, the longer the transportation time the later will the colonies grow out, resulting in delayed detection.

If the growth of gonococcus-like colonies is abundant, one drop of oxidase reagent (page 466) is added; if the colonies quickly turn deep purple and if the colour is maintained for more than half a minute the reaction is considered positive and a Gram-stained smear is prepared. Colonies of other micro-organisms may also react with the oxidase reagent. Most of these are easily distinguished from gonococcal colonies, but notably species of Alkaligenes and Haemophilus and also of other Neisseria may be impossible to distinguish on the basis of colony form and oxidase reaction. If the growth is scarce, the oxidase reagent can be used by application of a few drops on a filter paper which is streaked with small amounts of growth. In this way several cultures can be tested at a time on one piece of paper.

Subcultures are made from all plates from which Gram-negative diplococci or cocci are found on microscopy. If there is heavy contamination typical colonies are picked out under a low-power stereo-

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1 Recently, Kellogg et al. (1963) observed four morphologically distinct clonal types in N. gonorrhoeae cultures. Virulence in man was found to be linked to clonal type 1.
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Dry-ice/alcohol bath at −70°C and stored in a dry-ice chest until needed. The frozen suspension is thawed in a water-bath at 37°C and promptly streaked on plates for recovery.¹

Fermentation Tests

In laboratories with some experience in the identification of gonococci and meningococci it suffices to use fermentation plates of glucose and maltose only, because these sugars will permit the differentiation between N. gonorrhoeae, N. meningitidis and N. catarrhalis, provided the colonies are typically non-chromogenic (Wilson & Miles, 1955; Breed et al., 1957). It is preferable to use a solid medium on which contamination can be directly observed. In the Neisseria Department of the Statens Serum Institut, translucent fermentation plates are used; they are rather heavily inoculated, and 6-8 strains are tested on one plate. The plates are incubated for about 24 hours in CO₂ jars, then removed and left on the table for about half an hour with the lids open in order to allow the CO₂ to escape from the medium. The fermentation medium hitherto used by the author is described on page 464. Juhlin (1963) has recently introduced a medium that allows growth from a very small inoculum, and very few strains fail to grow on it or to ferment glucose.

If there is typical acid production the inoculated areas of the glucose plates turn yellow; however, the intensity of the acid production varies and strains with weak or absent acid formation are occasionally found. In typical cases, the maltose plates turn purple on the growth areas. In order to detect contamination, Gram-stained smears should be prepared from the fermentation plates in all doubtful cases. If the growth is weak or the acid formation is not very marked, it may help to incubate the glucose plate for another 24 hours.

For the fermentation tests it is important that the growth should be well supported; the use of ascitic fluid from patients treated with antibiotics should be avoided. In the author’s laboratory some batches of phenol red have been found inhibitory. Another source of error is the use of ascitic fluid from patients with uncontrolled diabetes.

Neisseria other than gonococci are occasionally isolated from the genito-urinary tract; the incidence depends on how much attention is paid to their isolation. Most of these strains are easily distinguished from true gonococci by their cultural and biological characteristics (see accompanying table).

Formerly, 1-2% of the “positive” gonococcus-like cultures examined in this laboratory showed an absence of glucose fermentation in spite of satisfactory growth on the fermentation medium. This was the situation until June 1957. At that time the number of non-fermenting, atypical strains showed a sudden increase. Not only was glucose fermentation absent or weak, but the growth was poor on the routine medium used for primary isolation. It is emphasized that freeze-dried strains isolated before June 1957 grew well on the “old” media and showed typical fermentation. Serological tests indicated that these atypical strains could be considered as true gonococci. However, as soon as the media were changed by using beef-heart broth instead of beef-meat broth in the medium for primary isolation and subculture, and placenta or beef-heart broth instead of beef-meat broth in the fermentation medium, both growth and fermentation of the “atypical” strains became typical. This event is reported to demonstrate the great variability of the growth requirements of gonococci (Reyn & Bentzon, 1961). The frequency of non-fermenting strains is now below 0.05%.

Polymorphous Gram-negative rods, growing in gonococcus-like colonies and with a fermentation pattern similar to that of gonococci may be a source of error. In most cases, however, the oxidase-reaction will be negative. Growth at 22°C on unenriched medium will rule out N. gonorrhoeae. On careful microscope examination of successive 16-18 hours’ pure subcultures it will be found that either long rods will gradually predominate or the single elements will appear oval and be orientated in a pattern different from the typical coffee-bean pairing. Large cocci or diplococci, which are evenly and deeply stained by the counter stain, require special attention. When it is found that the isolated micro-organism shows a high degree of penicillin resistance one should be even more suspicious. Further study of this group—or of these groups—of micro-organisms is needed with a view to developing an improved classification. Several authors have called attention to these sources of error which play a comparatively greater role in smaller laboratories or in localities where these micro-organisms are relatively frequent (De Bord, 1939, 1943; Aiken et al., 1956; Cary et al., 1958; Gangarosa & Cary, 1960; Sampson et al., 1961; Svihus et al., 1961).

¹ For further details see US Public Health Service (1962).
CULTURAL AND BIOLOGICAL CHARACTERISTICS OF DIFFERENT SPECIES OF NEISSERIA

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<th>Growth on plain agar</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>dextrose maltose sucrose laevulose mannitol lactose</td>
<td>at 22°C at 37°C</td>
</tr>
<tr>
<td>N. gonorrhoeae</td>
<td>Both more or less greyish white. <em>N. gonorrhoeae</em> often more viacid than <em>N. meningitidis</em> which is</td>
<td>+ - - - - - + + + + + + +</td>
<td></td>
</tr>
<tr>
<td></td>
<td>easily emulsified. <em>N. meningitidis</em> colonies larger than <em>N. gonorrhoeae</em> colonies, both variable in size.</td>
<td></td>
<td>+ + + + + + + + + + + + + + + + + + + + + + +</td>
</tr>
<tr>
<td>N. meningitidis</td>
<td>Colonies variable, may be smooth and translucent or hard, opaque and irregular.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N. catarrhalis</td>
<td>Colonies golden yellow, glistening, similar to <em>N. meningitidis</em>.</td>
<td></td>
<td></td>
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<tr>
<td>N. flavescens</td>
<td>Colonies vary from greenish yellow to slightly yellow.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N. sicca</td>
<td>Colonies usually hard, wrinkled, opaque with greyish yellow pigment.</td>
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* Further information can be found in Wilson & Miles (1955), Breed et al. (1957) and Cruickshank (1960).
+ = acid formation or growth varying, preponderantly positive.
± = acid formation or growth varying, preponderantly negative.

DIAGNOSTIC CRITERIA

As a general rule, a culture isolated from the genito-urinary tract may be considered and reported as a gonococcus, provided the following criteria have been met:

1. the colony morphology is typical;
2. the colony is oxidase-positive;
3. microscope examination of a pure culture shows typical Gram-negative diplococci;
4. only glucose is fermented.

Cultures that fulfil criteria 1 to 3 but do not ferment glucose or any of the sugars listed in the above table may be identified as "gonococcus-like" if no growth is obtained at 22°C.

It is stressed that gonorrhoea cannot be excluded unless repeated cultures show negative results.

REPORTING OF RESULTS

Reports should contain all the identification marks given on the forms accompanying the samples. Missing information or errors should be noted. Furthermore, notice should be taken of whether the transportation tubes are beyond their expiry dates or if swabs other than the charcoal-impregnated applicators have been used. In some cases, it may be convenient to report the transportation time. It is recommended that copies be kept of all reports and that a system of checking be set up to ensure that all extra remarks are given on both the reports and the copies.

Cultures showing no growth of gonococci after 48 hours' incubation should be reported as "negative" or "no growth of gonococci".

Cultures fulfilling the diagnostic criteria given above should be reported as "positive" or "growth of gonococci". Sometimes it is necessary to specify that Gram-negative oxidase-positive diplococci were found but that the strain was lost on subculture, or that growth of "gonococcus-like" micro-organisms with atypical fermentation was observed. In such cases, the report should contain a suggestion that the test be repeated. If it takes more than five days to obtain a pure culture for the fermentation tests, a preliminary report should be made and care should be taken to ensure that the final report is also given.

In addition to "negative" and "positive" findings it is absolutely necessary to report "overgrown" or "unsatisfactory" cultures. A suggestion that the test be repeated should be made as well. Cultures
should be designated as "overgrown" when it is
evident that any gonococci that might be present
would have had no chance to develop in the presence
of the overgrowing micro-organisms, which in turn
may originate either from the patient or from the
medium. If overgrowing micro-organisms show
confluent growth in the fourth quadrant of the plate
it is obvious that the plate is "overgrown", but it is
sometimes very difficult to distinguish between
"overgrowth" and "no growth". Each laboratory
must develop its own style of reporting, balancing
the risk of reporting too many "false negative"
results against that of reporting too many "over-
grown" cultures. In general, overgrowth by Proteus,
E. coli or yeast originates from the patient, and this is
also true of most of the cases in which B. subtilis is
observed. Apart from spreading the swab material,
very little can be done to prevent overgrowth of
contaminating bacteria without damaging the
gonococci, except for the addition to the plates of
polymyxin B and perhaps also of ristocetin. Most of
the substances that have hitherto been shown to be
inhibitory to the swarming of Proteus are too toxic
to N. gonorrhoeae and therefore useless. PNPG/1
forms the only exception (Jungfer, 1958). Two
plates are used, one with the addition to the medium
of 60 μg of PNPG/1 per ml and one without PNPG/1.

In most laboratories it will be impossible to deter-
mine in detail the nature of the overgrowing organ-
isms. It is, however, recommended that it should be
specified if Proteus is encountered. If not specified,
the repeated reporting of "overgrown" cultures
may discredit the method. In certain periods,
overgrowth by soil bacteria or by moulds, both originat-
ing from contamination during the preparation of
the plates, may destroy the cultures. Such over-
growth may be effectively inhibited by the addition
of nystatin (for details, see page 466).

SEROLOGICAL EXAMINATION

GENERAL REMARKS

Serological examination can be used in two ways,
either to classify a culture or to test a patient's serum
for antibodies. Neither of these procedures is much
used in the diagnosis of gonorrhoea. In the majority
of cases the bacteriological examination suffices.
Antibody is rarely present in acute cases. Serologi-
cal testing has a certain diagnostic value in chronic
infections (pelvic inflammations, prostatitis,
arthritis, etc.) and when bacteriological examination
is inconvenient or impossible. However, serological
methods are not quite specific and antibodies tend
to persist for many years in spite of clinical cure.

Despite much effort, serological investigation of
the gonococcus has not been very rewarding. On
culture, the gonococci undoubtedly change their
serological characteristics quite readily and partly or
completely lose their surface antigens; this makes
serological classification difficult (Uroma, 1943;
Reyn, 1947, 1949a, b, c; Chanarin, 1954; Wilson,
1954). Antigenic substances of different chemical
specificity are responsible for the antibodies formed
in gonorrhoeal infections. Antigens of poly-
saccharidal, lipopolysaccharidal and proteinic nature
have been prepared, but further research is necessary
(Boor & Miller, 1934, 1939, 1944; Mutermilch &
Grimberg, 1935; Dmitriw & Demidova, 1935;
Casper, 1937; Tauber, 1959, 1960; Tauber & Russell,
common to all gonococci has been identified by
Reyn (1947, 1949a, c); several type-specific (or strain-
specific) antigenic substances (both thermostable and
thermolaible) were also demonstrated. Using agglu-
tination, Wilson (1954) demonstrated eight heat-
able antigens, four of which behaved as group
antigens. In addition to the complex antigenic
pattern, cross-reactions with other Neisseria are
often found; this applies mainly to N. meningitidis
and N. catarrhalis. In addition some strains of
Pasteurella multocida show cross-reactions. The
cross-reactions depend on antigenic substances
closely related to thermostable antigens (Reyn, 1947,
1949a; Wilson, 1956).

Simple slide or tube agglutination tests are not very
useful since many strains are auto-agglutinable when
suspended in saline. A number of precipitation and
flocculation methods have been described (Saint-
Prix, 1950a, b); however, the only serological method
in common use is the complement-fixation reaction
(Cohn, 1937; Le Minor, 1948; Price, 1949; Car-
penter, 1963). Further references to serological
methods will be found in the review by Scherp
(1955). A fluorescent antibody technique (Deacon et
A. REYN

Al., 1959, 1960; Deacon, 1961; Harris et al., 1961) has been worked out for the identification of N. gonorrhoeae in smears. However, this technique requires that the worker be familiar with the use of the ultraviolet light microscope and that he should have a basic understanding of the principles governing fluorescent antibody techniques. Thus, this very promising method cannot as yet be recommended outside highly qualified laboratories, especially as certain specificity and sensitivity problems still remain to be solved (Danielsson, 1963; Lind, 1963). There have, however, been recent indications that the specificity problems are being overcome.1

The relative efficiencies of the culture method and the fluorescent gonococcal antibody technique have been found to vary from one laboratory to another; for instance, one laboratory has claimed a superiority of 12% for the culture method while another has claimed a superiority of more than 100% for the so-called "delayed fluorescent-antibody test" (Price, 1964). This variation is most probably due to lack of uniformity in performing the conventional culture method.

A rapid staining technique has been described by Kellogg & Deacon (1964). Very recently, Hess et al. (1965) have described how the immuno-fluorescent technique can also be used indirectly as a means of detecting antigonococcal antibody in human sera. Finally, Reising & Kellogg (1964) have proposed a precipitin test for the detection of gonococcal antibodies. Preliminary findings with this test indicate that the virulent gonococcus contains a protein antigen which is species specific. However, the test has not yet been evaluated in large-scale experiments with respect to false positive and false negative results.

**GONOCoccus COMPLEMENT-Fixation Reaction**

The following method was developed at the Statens Seruminstitut, Copenhagen (Kristensen, 1930; Reyn, 1947, 1949a), the procedure being as follows:

The antigen is prepared from a mixture of about 30 fresh strains and seven serologically "known" stock strains.2 The strains are grown on an ascitic agar medium of the same composition as the fermentation medium but without sugar, phenol red and co-carboxylase. Eighteen-hour cultures are harvested in physiological saline and suspended to a density of about $2 \times 10^{10}$ organisms per ml; from each of the suspensions aliquots are taken out and submitted to microscopic and serological examination before the various suspensions are mixed to form the polyvalent antigen. The antigen is dispensed in 1-ml or 2-ml amounts and heated to 56°C for 20 minutes; it is now ready for use and can be stored at $-10°C$ for several years. The harvested, unheated material is stored at about $-10°C$ until the results of the microscopic and serological tests are available. The following criteria should be fulfilled before the suspensions are mixed: (1) no contamination, (2) no anti-complementary effect, (3) a "standard" degree of reaction with pools of positive sera, (4) negative reaction with a pool of negative sera.

To obtain an estimate of the anti-complementary effect, the individual suspensions are tested in complement-fixation experiments with a constant dose of antigen and two or three different complement concentrations; suspensions only slightly anti-complementary are included in the antigen. A suitable antigen concentration is usually obtained by dilution of the stock antigen to give about $5 \times 10^7$ organisms per test-tube. The antigen is diluted in Sorensen's phosphate buffer, pH 7.38. The total volume is 0.3 ml and the quantity of serum in the first tube is 0.025 ml, in the second 0.025 ml diluted 1 in 3, in the third 0.025 ml diluted 1 in 9 and so on. The 100% haemolytic unit of complement is determined in the presence of antigen and one unit is added per test-tube.

Fixation is allowed to take place for three-quarters of an hour at room temperature and for three-quarters of an hour at 37°C in a water-bath; 0.2 ml of a 2.5% suspension of sensitized sheep-blood cells (three haemolytic doses) are then added to the tubes which are re-incubated at 37°C for an additional hour. The degrees of haemolysis corresponding to the different serum dilutions are estimated by comparison with a scale of haemolysis prepared from tubes with complete haemolysis. The results are given as degrees of potency; these are calculated after the method given by Kristensen (1930), the degree of potency being defined as that value of $n$ which when substituted in the formula $a = 0.025 \times 3^{-n/3}$ gives a value for $a$ exactly equal to the volume of serum containing enough antibody to give a minimum reaction, i.e., 60% haemolysis. Further details are given by Reyn (1949a).

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1 US Public Health Service (1962); Brown et al. (1963); Moore et al. (1963); Shapiro & Lentz (1963); Lind (1964); Price (1964).

2 Available in a freeze-dried state from the Neisseria Department, Statens Seruminstitut, Copenhagen, Denmark.
The day-to-day variations in the test are counteracted by means of daily titration of six control sera for each of which an average "standard" value has been determined. The average amount by which the titres of the control sera differ from their standard values is the correction to be applied to the values for the other sera.

Before a new antigen is accepted for use the following comparisons with the old one are made:

(1) The approximate titre of the new antigen is determined in preliminary experiments. As a rule, a fair degree of similarity between the results with the new and the old antigen can be obtained after three to four days on which 10 positive sera of varying potency are tested quantitatively against different dilutions (three per day) of the new antigen.

(2) Having determined the approximate titre, the two antigens are used in parallel titration of about 1000 sera picked out at random among the sera sent in for gonococcus complement-fixation. This will give a comparison of about 75-100 sera that react positively with either or both of the antigens.

The new antigen is accepted for use if the average serum titre difference between the two antigens does not exceed ± 0.3 degrees of potency (±0.048 log₁₀ values). If a difference in the same direction is regularly observed for several days, another antigen dilution is used and the experiments are started again.

In the Neisseria Department of the Statens Seruminstitut, a new antigen is made once a year. Care should be taken to avoid a gradual change in sensitivity as a result of repeatedly accepting antigens with a positive (or negative) difference.

(3) One thousand sera from blood donors (without clinical or serological evidence of syphilis) are tested with the "final" antigen titre. The number of positive reactions should preferably not exceed five per thousand.

The above procedure is related to the average sensitivity or strength of the antigen; another question is whether the standard deviation of the differences should also be taken into consideration. The standard deviation of repeated titration of the same antigen is estimated to be about 0.7 degrees of potency (0.111 log₁₀ values) and it is proposed that the standard deviation of the difference between two different antigens should not exceed 1.4 degrees of potency (0.223 log₁₀ values). This would mean that antigens giving differences greater than ± 2.8 degrees of potency in more than 5% of the sera would not be accepted.

The sensitivity of the complement-fixation reaction described is so adjusted that only about 30% of female cases with uncomplicated, acute gonorrhoea and about 90% of cases with complicated, chronic gonorrhoea (male and female) show positive results.

In spite of clinical cure the reaction may remain positive for several years and in the individual case it is difficult to judge the specificity of a positive reaction. The diagnostic value of a sero-reaction depends on its sensitivity and specificity under the special local conditions under which it is intended to be used. If the incidence of previous and present infection is high, many apparently non-specific reactions will be found and the diagnostic value in the individual case will be small. If, however, the incidence of gonorrhoea is low and confined to certain patient categories, the diagnostic value is greater. A survey of positive reactions obtained in a medical ward in Copenhagen shows a certain trend for the conditions in Denmark (Bang & Krag, 1942). It was found that 7.2% of the males and 6.2% of the females were positive; by dividing the material into groups according to the history and the clinical signs of gonorrhoal infection it was found that the degree of specificity increased with increasing strength of the reaction. In 63.4% of the sera showing one degree of potency (serum concentration \( \leq 1 : 12 \)) and in 84.7% of the sera showing three degrees of potency (serum concentration \( \leq 1 : 36 \)) either the corresponding patients were known to be suffering from gonorrhoea or their case histories made it highly probable that they had previously suffered from this disease.

Strong cross-reactions are usually found in patients with meningococcus infection. Positive reactions have frequently been observed in patients suffering from chronic bronchitis especially in those with bronchiectasis, possibly due to infection with \( N. \) catarrhalis or other \( N. \) Neisseria species sharing antigens with \( N. \) gonorrhoeae.
DRUG-SENSITIVITY DETERMINATION

Until recently, determination of the sensitivity to antibiotics was not very much used in connexion with routine culture of gonococci. However, the number of strains of gonococcus showing decreased sensitivity to antibiotics makes the development of a recommended reference method desirable. The use of reference strains would be a further aid in making the comparison of results obtained in different laboratories more meaningful. The WHO Expert Committee on Antibiotics in its second report (1961) stated that “a standard method of test is required” and that “stocks of lyophilized cultures should be kept, in order that future direct comparisons may be possible between strains of gonococcus isolated at different times”. Under the auspices of WHO a group of laboratory experts is now doing the preparatory work for the establishment of such a reference method. The preliminary results of this study are referred to in the first report of the WHO Expert Committee on Gonococcal Infections (1963). The aim of this work is to agree on a procedure in which the inhibiting concentration of antibiotic is measured by a plate dilution method.

Carefully controlled diffusion tests might be preferred for local use. This conception is supported by the results obtained in a recent collaborative study of different methods by Denmark, Finland, Norway and Sweden (Reyn et al., 1963). The results obtained in this study indicate the possibility of converting a diffusion result read in millimetres to a concentration obtained by a dilution method.

The great majority of cases are cured by penicillin, streptomycin or tetracycline; however, it has been demonstrated both in vitro and in vivo that the penicillin sensitivity is decreasing (Jensen, personal communication, 1957; Cradock-Watson et al., 1958; Curtis & Wilkinson, 1958; King, 1958; Scamberg et al., 1958; Reyn et al., 1958; Hirsch & Finland, 1960; Cole et al., 1961; Reyn, 1961; Thayer et al., 1961; Ringertz, 1961; Gjessing & Ødegaard, 1962; Rantasalo et al., 1962). Completely resistant strains have not been isolated from patients. However, about 25% of strains isolated in Denmark since 1957 need more than 0.225 μg of penicillin per ml for complete in vitro inhibition and this is only two to three times the average serum concentration obtained after 300 000 units of aqueous procaine penicillin G. Geographical differences may, of course, be encountered (Reyn, 1962, 1963). Streptomycin-resistant strains have been isolated and an increased failure rate of streptomycin treatment has been reported by Alergant (1958), Reyn (1961), Durel et al. (1961) and Roiron et al. (1961).

A tablet method and a plate dilution method for sensitivity determination of penicillin, streptomycin and tetracycline were described by Reyn et al. in 1958, and a plate dilution method was described in more detail by Reyn et al. in 1963. These methods can easily be applied to the determination of sensitivity to other antibiotics. The tablets should be chosen so as to give zones of a suitable size (Lund et al., 1951; Lund, 1953). The concentrations in the solid medium used in the dilution method should be so adjusted that the change from full to no growth for the majority of the strains is likely to occur in the middle of the series of concentrations employed. If, for some reason, the agar cup technique, the disc method, the gradient plate technique, or tube dilution methods with fluid or semi-fluid medium are found more convenient, they may well be used. However, regardless of the method it is important to know something about its variation under the local conditions.

Direct sensitivity determination from swabs is possible only in a few cases and it is preferable to use pure cultures from which the size of the inoculum can be controlled. In order to avoid selection, not less than 10 colonies (if possible) should be used for the preparation of the pure cultures.

MEDIA AND REAGENTS

PREPARATION OF BROTH

Containers used in the preparation of broth, etc. should be of stainless steel, glass or some other inert material. It is practical to prepare stock batches of broth-agar base for later use. Suitable amounts may be kept for several months at low temperatures. Erlenmeyer flasks are suitable for this purpose.

Broth formula: chopped meat, 500 g; tap water,
1000 ml; peptone, 10-15 g; \( \text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O} \), 2 g; and \( \text{NaCl} \), 3 g.

The "meat" can be veal or beef-heart or human placenta tissue (see below), which should be freed from fat and tendinous tissue. After cutting and chopping, the meat is mixed with half the water and placed overnight in the cold. Next morning it is boiled for 15 minutes and the soup is collected, using a sieve. The meat is boiled again for 10 minutes together with the other half of the water and the two soups are then combined. After addition of peptone and salts the broth is heated to boiling, during which the pH is adjusted to about 8.1 by means of 5N NaOH. The broth should boil for not less than 10 minutes and for at least 5 minutes after the last addition of sodium hydroxide. By means of distilled water the original volume (plus an extra 5% to counteract the autoclaving effect) is restored. The broth is cleared by paper filtration, the agar \(^2\) melted in the hot broth and boiled for 20-25 minutes. The hot broth-agar is then filtered by suction and dispensed in Erlenmeyer flasks. It is sterilized by autoclaving at 120°C for 20 minutes or by heating for 20 minutes on a boiling water-bath ("steaming") on three days in succession. After sterilization the pH should be about 7.2.

If the broth is prepared for later use it should be sterilized by Seitz filtration and kept in the cold in Erlenmeyer flasks until the agar is added, followed by autoclaving as described above.

Autoclaving is more deleterious than "steaming" and it also has a more acidifying effect than has "steaming".

Placenta broth is prepared from fresh human placenta (no antiseptics) which are freed of the umbilical cords and the coverings. They are cut into pieces and rinsed in cold tap water.

After coarse chopping in a machine, two litres of tap water are added per kg, the crude broth being kept at about +4°C until the following day, when it is boil for 15 minutes. The placenta tissue is separated from the broth and the peptone and salts are added with subsequent boiling for 15 minutes; the pH is adjusted to about 8.1. After autoclaving the pH should be about 7.2.

CHOCOLATE ASCITIC-FLUID-agar

This is prepared by the addition of horse blood and ascitic fluid to a broth-agar base consisting of 2.4% of Danish AKI agar in beef-heart broth with 1% of peptone ("Orthana" special), 0.3% of NaCl and 0.2% of \( \text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O} \).

The base is dispensed in 540-ml amounts in Erlenmeyer flasks and kept cool; before further preparation it is melted and kept at 80°C. 60 ml of defibrinated horse blood are added. The mixture is kept at 80°C for 15 minutes and shaken frequently; it is cooled to 56°C and mixed with 300 ml of sterile ascitic fluid \(^3\) (30%), which is brought to 56°C before mixing. The ascitic fluid causes a rise in pH which should be compensated for by adding 0.5N HCl until the pH is about 7.1-7.2. It is false economy to pour plates too thinly. Immediately afterwards, plates 4-5 mm thick are poured into 9-cm wide aluminium or plastic Petri dishes. If ascitic fluid is difficult to obtain the amount can be reduced to 150 ml (15%) without appreciable loss in the quality of the medium. It is also possible to replace the ascitic fluid entirely by 100 ml (10%) of sterile horse serum. Plates should be stored in the refrigerator and should be used within 10 days to 2 weeks.

The finished medium is a soft agar and should be handled and inoculated with care.

The surface moisture may be removed without undue danger of contamination by inverting the plate in the incubator, lifting the bottom out of the top, and resting it on the edge of the top (agar surface always downwards) until all droplets of moisture on the agar surface have disappeared. The excessive moisture produced by condensation and by hysteresis of the agar is neither necessary nor desirable for growth of the gonococcus, provided the

\(^1\) The precise quantity will depend on the brand and quality.

\(^2\) The exact percentage depends on the batch and brand of agar; the agars should be soft. At the Statens Serum-Institut, Danish AKI agar is used in the chocolate ascitic-fluid medium. Selected batches of Japanese agar Kobe I are employed in the fermentation medium. Preparations with AKI agar do not need to be filtered before autoclaving. The AKI agar is not a true agar; it resembles carrageen which is rich in methyl groups. It solidifies at about 45°C (in the concentration used) whereas Japanese agar, for example, solidifies at about 30°C. The pH also varies when different brands of agar are used.

\(^3\) It is important to exclude the presence of antibiotics in the ascitic fluid. When derived from diabetic patients it should be examined for glucose, especially when used in the fermentation medium. Seitz filtration is necessary unless the technique of collection is absolutely safe. In fermentation tests and in experiments to determine the sensitivity to antibiotics, it is advisable to test the ascitic fluid before use; if possible, fluid from the same patient should be used for long periods. Ascitic fluid and other biological materials are expensive and difficult to procure. Recent experiments indicate that simple autolysates prepared from fresh brewer's yeast and pig liver may replace the ascitic fluid, see "HYL medium", page 464.
plates are incubated in a moist atmosphere. However, drying should not be continued until the surface becomes wrinkled by lines of stress; this indicates that the agar is losing water, and should always be avoided.

While mixing and pouring the medium, care should be taken to avoid the promotion of excessive amounts of foam and bubbles. If bubbles do find their way into the plates, they may be removed by flashing a Bunsen flame over the agar surface before it has solidified.

**HYL**\(^1\) MEDIUM

**Haemoglobin**

Powdered ERG.B6 (Riedel-De Haën AG, Seelze-Hannover) is mixed with a small amount of demineralized water to make a paste after which sufficient demineralized water is added while stirring to give a 6% solution. (This will be clear and the pH will be 7.9.) The solution is poured into screw-cap bottles and the screw-caps tightly closed. It is then heated in a steam-bath for 10 minutes and autoclaved for 15 minutes at a pressure of 0.6 kg/cm\(^2\). When heated in tightly closed bottles and stored at 4°C the haemoglobin remains as an opalescent solution for several months.

**Yeast autolysate**\(^2\)

Fresh brewer’s yeast (extremely flocculent bottom yeast)\(^2\) is obtained from a brewery. Water is added in excess and after some time the supernatant is discarded. After two more washings with water, the suspensions being neutralized each time by means of a bicarbonate solution to remove the resin substances contained in the hops, the suspended yeast is centrifuged and subsequently mixed with an equal amount of water. For every 2.5 litres of suspension 80 ml of ethyl acetate are added and the mixture covered by a tight lid and heated for one hour in a 40°C water-bath. For practical reasons, the mixture is then moved into an incubator at 37°C until the next morning, when the pH is adjusted to about 7.4 using about 17-20 ml of 5N NaOH per litre. The suspension is centrifuged and the supernatant yeast autolysate sterilized by filtration, first through a membrane filter S. S. Göttingen 1118 and then through a membrane filter 1120. It is dispensed in sterile bottles and stored at 4°C.

**Liver autolysate**\(^4\)

1 kg of pork liver is chopped three times and 700 ml of distilled water are added. The mixture is stirred and placed in a 50°C water-bath with subsequent slow slow heating to 75°C; this temperature is maintained for one hour during which the preparation is constantly stirred. After centrifugation, it is sterilized by filtration, using the same filters specified above. The pH is adjusted to 7.4-7.6.

**Formula for preparation of 100 ml of medium**

66 ml of broth-agar base prepared as described under *Chocolate ascitic-fluid-agar*, 15 ml of sterile 6% haemoglobin, 3 ml of a mixture of one part of yeast autolysate and two parts of liver autolysate, distilled water to 100 ml. The brand of agar used should fulfill certain requirements; in particular, it should be non-toxic when used in an amount resulting in a suitable gel strength. A method of checking the gel strength has been published by Fulthorpe (1951); recently, the method was simplified by Møller. To measure the toxicity, comparative experiments should be done, using gonococcal strains with differing growth requirements. Both countings of the viable units and measurements of the colony sizes should be performed. The AKI agar, which in our hands has shown a low toxicity to *N. gonorrhoeae*, has a higher melting-point than have Japanese and Spanish agars. Thus, an AKI agar base must be kept at about 70°C when mixed with the heat-labile enriching substances.

A Spanish agar (brand unknown as yet) has also been shown to be non-toxic and to give a suitable gel strength when used in a final concentration of about 0.65%.

The equipment for the preparation of the medium is simple and the ingredients needed for its preparation are easily obtainable. The constituents of the medium have not yet been analysed and defined chemically. It is possible that further analysis will reveal a close similarity to the media previously described by Lankford and co-workers.

**FERMENTATION MEDIA**

1. **Danish fermentation medium**\(^5\)

*Broth-agar base: 2.2% of agar in human placenta broth prepared with 1% of peptone and 0.5% of

\(^{1}\) Haemoglobin, Yeast, Liver.

\(^{2}\) Society for General Microbiology (1958).

\(^{3}\) If it is impossible to obtain an extremely flocculent bottom yeast, lager beer type, it will be necessary to use centrifugation at this point.

\(^{4}\) Møller, Åke, personal communication, 1961.

\(^{5}\) As developed at the Statens Serum Institut, Copenhagen.
NaCl. No phosphate buffer is added (the pH is adjusted to 7.1-7.2). The quality of the peptone is highly important and should be especially tested. The base is dispensed in 600-ml amounts and mixed with 300 ml of phenol red 1:500, 9 g of sugar (glucose or maltose) and 9 ml of 0.1% co-carboxylase.¹

The sugar is dissolved in a minimum of water and either heated to 100°C for 10-15 minutes or sterilized by filtration. The co-carboxylase (Hoffmann-La Roche) is dissolved in sterile water and, surprisingly, it can be added without sterilizing. 4 g of phenol red (Merck) are dissolved in 160 ml of 0.1N NaOH at 37°C with shaking. The volume is brought up to 2000 ml with distilled water and the solution is sterilized by autoclaving at 120°C for 20 minutes. The constituents are mixed at about 50°C, the pH is adjusted to about 7.4 with 5N HCl; by the following day it will have risen to about 7.8, which is suitable for the incubation in a CO₂ atmosphere. The medium is poured into glass or plastic Petri dishes in a 4-5-mm layer.

2. HAP medium

This is a new solid fermentation medium enriched with haemin, recently introduced by Juhlin (1963). It is somewhat more complex than the Danish medium, but has given very good results and is now used in the Neisseria Department of the Statens Seruminstitut. The method of preparation is as follows:

**Placenta infusion:** Fresh human placentas are ground after removal of membranes and umbilical cords. The amount thus obtained is weighed and mixed with twice as much distilled water and kept at 4°C during the night. The next day the mixture is boiled for 20 minutes and paper-filtered, yielding a clear fluid which is dispensed into Erlenmeyer flasks and autoclaved at 120°C for 20 minutes.

**Broth-agar base:** Placenta infusion, 200 ml; sodium chloride (3% solution in placenta infusion), 60 ml; disodium hydrogen phosphate dihydrate (2% solution in placenta infusion), 60 ml; Proteose Peptone No. 3 (Difco)² (10% solution in placenta infusion), 60 ml; Sodium Alginate, BPC,³ 0.6 g; Bacto Agar (Difco)³ 6.0 g. These ingredients are mixed in each of four flasks and are then autoclaved at 120°C for 20 minutes. The broth-agar base is cooled to 55°C.

**Ascitic fluid:** Each fresh batch is sterilized by filtration (Seitz filter EKS2) and compared with earlier batches as regards growth-stimulating effect on strains of *N. gonorrhoeae, Streptococcus pyogenes, Haemophilus influenzae, and Diplococcus pneumoniae.*

**Phenol-red (Merck):** A 0.4% solution in distilled water is autoclaved at 120°C for 20 minutes.

**Haemin, bovine** (twice recrystallized) (Sigma): A 0.2% solution is prepared by dispersing 0.15 g of haemin crystals in 75 ml of distilled water and adding 1.5 ml of 3N sodium hydroxide. The suspension is shaken well until the crystals have dissolved completely and a perfectly clear, dark-green solution is obtained. This solution is then sterilized by filtration (Seitz filter EKS2).

**Sugar:** A 20% solution of dextrose, maltose, sucrose, or laevulose in placenta infusion. The solution is sterilized by filtration (Seitz filter EKS2).

**HAP medium:** Broth-agar base, 380 ml; ascitic fluid (preheated to 52°C), 180 ml; phenol-red (Merck) (0.4% water solution), 6 ml; haemin (Sigma) (0.2% water solution), 10 ml; sugar (20% solution in placenta infusion), 30 ml. The sterile ingredients are mixed at 52-55°C and the pH is adjusted to 7.7 with 5N sodium hydroxide. The medium is poured into plastic Petri dishes, filling each one to a depth of 4 mm.

Co-carboxylase (Bérolase, Hoffmann-La Roche) is dissolved in a buffer consisting of 1.0 g of sodium acetate, 0.2 g of sodium hydroxide and 100 ml of sterile water to make a 0.1% solution. Of this solution, 6 ml are added to the HAP medium at the time when the broth-agar base, the ascitic fluid, and the other ingredients are mixed, giving a final concentration of about 0.001% of co-carboxylase.

**STUART’S MEDIUM WITH SOLID AGAR**

**Formula**

Agar, 16 g;⁴ sodium glycerophosphate, 20% by volume, 50 ml; thioglycolic acid, 80%, 0.95 ml;

¹ This comparatively high amount of co-carboxylase was found to be needed by Bang (personal communication, 1952), who at that time did not know the results obtained by Lankford & Skaggs (1946).


⁴ The precise quantity of agar will depend on the brand used and on the batch.
CaCl₂, 10%, 1 ml; methylene blue, 0.1 %, 2 ml; and distilled water to 1000 ml. It is recommended that all distilled water be passed through an ion-exchange-resin column to remove free chlorine (Crookes & Stuart, 1959). A 1.5% "water base" is stored in 950-ml amounts. The base is melted and the ingredients are added with subsequent adjustment to pH 7.4 by the addition of 5N NaOH. When hot, the medium is dispensed in sterile hydrophobic cotton-wool-plugged 15-mm × 150-mm tubes forming a column 10 cm high. The tubes are steamed at 100°C for one hour, and then cooled in cold water for 15 minutes (see also page 451).¹ The tubes should be stored in a cool dark place until required. Ordinarily, they will keep (i.e., will still contain a colourless layer more than 3 cm high) for at least two months, provided that the thioglycolic acid fulfils the criteria laid down in the United States Pharmacopoea, vol. 15, p. 1070.

**Preparation of swabs**

For sampling, good quality absorbent cotton-wool and wooden swab sticks (see page 451) are used. The swabs should be carefully prepared, being neither too small nor too thick, and the cotton-wool should fit smoothly to the end of the applicators. Prepared swabs are boiled in Sørensen’s phosphate buffer solution at pH 7.4 and immediately afterwards dipped into a 1% suspension of powdered charcoal in water. After impregnation, the applicators are dried, packed in two layers of paper, and sterilized by autoclaving at 125°C for 20 minutes.

**Oxidase reagent**

A stock suspension is prepared by the addition of 100 ml of concentrated ethanol to 1 g of dry, powdered tetramethyl paraphenylenediamine hydrochloride. The substance is hydrophilic and should be kept in sealed bottles or in a desiccator over P₂O₅. The reagent kills the gonococci in about 30 minutes. The dimethyl compound (p-amino-dimethylaniline monohydrochloride) is cheaper but far more toxic; in addition it is more difficult to observe the colour change to red instead of purple, especially when "chocolate" plates are used.

Every morning, a fresh reagent is prepared by mixing one volume of the stock suspension with two volumes of distilled water. When fresh, it has a very light purple colour (almost colourless); it is slowly oxidized in air, turning a deeper and deeper bluish purple until after several hours it is completely oxidized, and is then useless. The gonococci produce an indophenol oxidase which enhances the oxidation of the reagent, so that on the culture plates the reaction takes place in about a minute instead of over the course of several hours.

**Polymyxin B**

This reagent is used for the inhibition of coliform bacilli.

A 0.1 % stock solution of polymyxin B sulfate (Pfizer) is prepared with sterile distilled water. The unit/weight relation differs from one batch to the other. The stock solutions keep for at least two weeks at about +4°C. The final concentration used in the medium for primary isolation is 25 IU per ml.

*Note:* Gonococcus strains are very rarely susceptible to this drug (Crookes & Stuart, 1959).

**Nystatin**

Nystatin ⁴ is used to inhibit the growth of moulds. Stock solution: 200 ml of 70% ethanol plus 4 drops of 1% sodium citrate are used to dissolve the content of a vial containing 500 000 units of nystatin. The mixture is shaken for 30-60 minutes with sterile glass beads, resulting in a turbid suspension which is dispensed in suitable amounts for later use. This suspension keeps for about two months at −20°C. 1 ml is added per 100 ml of medium giving a final nystatin concentration of 25 IU per ml and an ethanol concentration of 0.7%. This is used in all plates in periods when moulds are disturbing. If a concentration of 25 IU per ml is insufficient, 40-50 IU per ml may be added without any damaging effect.

**Reagents to be used in the Gram-staining technique** ³

0.2% aniline crystal violet

Solution A: 0.2 g of crystal violet is dissolved in 10 ml of 96% ethanol.

¹ In some cases, it may be found convenient after cooling to remove the cotton-wool plugs at a time, soak them in a hot mixture of 9 parts of vaseline and 1 part of solid paraffin (melting point 50°C-60°C) and replace them in the tubes. In this way, evaporation will be minimized—for example, when the tubes are heated in order to reduce the medium (see also page 450).

³ As developed at the Statens Seruminstitut, Copenhagen.

⁴ Mycostatin (Squibb) has been used in the Neisseria Department of the Statens Seruminstitut, Copenhagen.
Solution B: 3.2 g of aniline are dissolved in 90 ml of distilled water, vigorously shaken for 15 minutes and filtered through wet filter paper.

Solutions A and B are then mixed. The final pH is about 7.5. The reagent keeps for 3-4 weeks at 18°-25°C.

Lugol's iodine solution. 1 g of iodine and 2 g of potassium iodate are dissolved in 300 ml of distilled water; the pH will be about 4.8.

0.1% phenolized fuchsine. 1 g of basic fuchsin and 10 ml of 96% ethanol are mixed with 5 ml of liquid phenol (Danish Pharmacopoeia, 1948) and added to 90 ml of distilled water. Before use, this stock solution is diluted 1:9 with distilled water to give a pH of about 4.8.

96% ethanol.

Annex

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REFERENCES

Bang, J. (1952) Acta derm.-venereol. (Stockh.), 32, Suppl. 29, p. 34
Boor, A. K. & Miller, C. P. (1939) Arch. Path. (Chicago), 28, 764
Chanarin, I. (1954) J. Hyg. (Lond.), 52, 425
Dmitriw & Demidova (1935) Derm. Wschr., p. 131 (abstract)
Ferguson, W. M. (1945) Amer. J. Syph., 29, 19
Fulthorpe, A. J. (1951) J. Hyg., 49, 127
King, A. I. (1958) Lancet, 1, 651
Kristensen, M. (1930) Acta path. microbiol. scand., 7, 87
Lankford, C. E. & Skaggis, P. K. (1946) Arch. Biochem. 9, 265
Lind, I. (1964) In: Proceedings of the Sixteenth Scandinavian Congress of Pathology and Microbiology, Universitetsforlaget, Oslo
Lund, E. (1953) Acta path. microbiol. scand., 33, 278
Morton, H. E. (1945) J. Bact., 50, 589
Reymann, F. (1941) Dyrkning af Gonokokker som diagnos- tisk Metode ved Knidegonorrhoe, Copenhagen (Thesis)
Reymann, F. (1944) Acta derm.-venereol. (Stockh.), 24, 130
Reyn, A. (1949a) Acta path. microbiol. scand., 26, 51
Reyn, A. (1949b) Acta path. microbiol. scand., 26, 234
Reyn, A. (1949c) Acta path. microbiol. scand., 26, 262
Reyn, A. (1951) Acta derm.-venereol. (Stockh.), 31, 1
Reyn, A. (1963) Acta derm.-venereol. (Stockh.), 43, 380
Thayer, J. D. & Martin, J. E., jr (1964) Publ. Hlth Rep. (Wash.), 79, 49
Uroma, E. (1943) Acta derm.-venereol. (Stockh.), 23, Suppl. 9, p. 1