A serum-free medium for testing fermentation reactions in *Neisseria gonorrhoeae*

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SYNOPSIS Gonococcal infections can no longer be considered exclusively urogenital but it is difficult in a busy routine laboratory to identify gonococci from non-genital sites, especially if other commensal neisseria are present. Unfortunately, conventional serum agar sugars sometimes give equivocal results. A fermentation medium is described for the identification of gonococci. On preliminary testing this medium gives rapid and reliable results and should prove to be much more helpful to the routine laboratory than conventional serum agar sugars. Its potential usefulness, as demonstrated by the results of our first 200 examinations, would seem to justify further study in the routine work of other laboratories.

Recent reports show that gonococcal infections can no longer be considered to be exclusively urogenital and the organisms may be found in sites such as the pharynx (Fiumara, Wise, and Many, 1967), tongue (Cowan, 1969), tonsils (Hellgren, 1971; Iqbal, 1971), and skin (Barr and Danielsson, 1971). The standard method used to identify neisseria is the fermentation reactions when tested against glucose, maltose, and sucrose. A disadvantage of the serum agar slopes of these sugars is that many laboratories find difficulty in interpreting the results owing to the occurrence of a non-specific change in the indicator which sometimes develops and often makes the interpretation of the maltose results impossible and, moreover, leads to a complete lack of confidence in the test. The identification of a suspected pathogen is always desirable but this will not be achieved when gonococci are isolated from sites where other neisseria are likely to be found, unless the fermentation tests employed are absolutely reliable. The existence of several alternative media today suggests that none has been found to be completely satisfactory.

Vera (1948) described a medium containing a pancreatic digest of casein which at first appeared to give reliable results, but it was shown later (White and Kellogg, 1965) that on this medium some strains of *N. gonorrhoeae* failed to grow, or grew poorly, whilst others grew but failed to ferment carbohydrate. A more satisfactory medium was developed at the Statens Seruminstitut, Copenhagen, which consisted of human placenta broth-ascitic fluid-

agar, plus carboxylase (Reyn, 1965). A new medium somewhat similar to that of Reyn, to which was added hemin, was developed by Juhlin (1963) and this has proved itself as a useful medium.

Such media may be ideal for a large specialized unit but are less suitable for smaller routine laboratories. They are tedious to prepare and the use of human biological fluids such as serum, hydrocele fluid, or placenta extracts may present the hazard of hepatitis or other infections to the technical staff.

White and Kellogg (1965) described a new medium consisting of a proteose peptone agar to which was added glutamine, cocarboxylase, and ferric nitrate. Despite the good results they reported, the medium is still not in common use. Cocarboxylase, being heat labile, must be added as a sterile solution after the other constituents have been autoclaved. Most workers will have been reluctant to omit the cocarboxylase, as Lankford (1946) claimed that if this was omitted a small percentage of fastidious strains of gonococci would not grow.

Materials and Methods

As Difco GC medium base + 2% defined supplement, consisting of ferric nitrate, L-glutamine, cocarboxylase, and glucose (White and Kellogg, 1965), is routinely used for the growth of *N. gonorrhoeae* in our laboratory, it seemed a reasonable start to see if the incorporation of sugars into this medium would give a satisfactory fermentation medium. A number of workers, including White

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and Kellogg, believed that cocarboxylase was necessary, so we decided to test batches of Difco GC medium base containing only L-glutamine and ferric nitrate, with and without the addition of cocarboxylase. As preliminary results were the same on both media, we decided to omit the cocarboxylase.

MEDIA

Difco GC medium base

Proteose peptone no. 3	..	..	..	15 g
Corn starch	..	..	..	1 g
Di-potassium phosphate	..	..	..	4 g
Potassium di-hydrogen phosphate	..	..	..	1 g
Sodium chloride	..	..	..	5 g
Bacto agar	..	..	..	10 g
Distilled water	..	..	..	1 000 ml

SUPPLEMENT

This is prepared by adding 90 ml of A to 10 ml of B:

A Glutamine solution

Dissolve 1 g of L-glutamine in 90 ml of distilled water.

B Ferric nitrate

Ferric nitrate, 0.05 g, was dissolved in 10 ml of distilled water.

PHENOL RED

0.2% stock solution.

SUGARS

Ten per cent solutions of either glucose, sucrose, or maltose which are sterilized by Seitz filtration.

The fermentation medium was prepared by boiling 36 g of the GC medium base in 970 ml of distilled water and adding, when clear, 20 ml of the supplement and 10 ml (0.2%) of phenol red solution. The pH of the medium was adjusted to 7.6 with N.NaOH, and it was then distributed in 90 ml volumes into 4 oz screw cap bottles, which were autoclaved at 15 lb for 10 minutes. After cooling to 50°C, 10 ml of the appropriate sugar was added aseptically to give a final concentration of 1%. Three ml amounts were dispensed into sterile bijoux bottles and sloped. The sugars were identified by a colour code on the caps.

Thus, the trial medium contained Difco GC base, L-glutamine, ferric nitrate, and the appropriate substrate, ie, glucose, maltose, or sucrose. The addition of vancomycin 3 µ/ml and colomycin 8 µ/ml was considered, but we decided that these are unnecessary as only pure cultures should be used for identification of strains.

BACTERIAL CULTURES

One hundred strains of *N. gonorrhoeae* were examined, 70% of which had been recently isolated from patients attending VD clinics in the Sheffield area, and the remainder being earlier isolates which had been stored in liquid nitrogen for periods ranging from three to 24 months. In addition, we tested three strains of *N. meningitidis* and the following five strains of commensal neisseria obtained from the National Collection of Type Cultures, London: *N. catarrhalis* (3622); *N. flavescens* (C8262); *N. pharyngis* (4591); *N. animalis* (10202); and *N. caviae* (10292).

All bacteria were grown on 5% lysed horse blood agar for 18 hours and purity was checked by Gram staining and a positive oxidase reaction. A slope of new fermentation medium and a standard serum agar slope were heavily inoculated with each test organism and incubated at 37°C in 'candle jars' with the screw caps loose. The fermentation reactions were observed over a period of 48 hours. The advantage of heavy inocula was that positive results could be read as early as eight to 12 hours. However, the medium will give positive results with very small inocula and we obtained typical fermentation reactions after 60 hours with as few as 150 organisms/ml.

Results

Table I shows the comparative results of fermentation reaction of *N. gonorrhoeae* on standard serum agar sugars and the test medium.

Medium	No. of Strains Tested	Time of Incubation (hr)	No. of Strains Interpreted as Showing Acid Production		
			Glucose	Maltose	Sucrose
Serum agar	100	24	79	21	0
		48	100	21	0
Test media	100	24	97	0	0
		48	100	0	0

Table I Comparison of the fermentation reactions of *N. gonorrhoeae* on serum agar sugars and on test media

Table II shows the fermentation reactions of the reference strains of neisseria.

On serum sugar media 11 strains failed to utilize glucose in 24 hours but all were positive after 48 hours; however, 21 strains gave what must be interpreted as positive maltose reactions. All the other neisseria species listed in Table II gave typical sugar fermentation patterns on both media.

Three of the 100 strains of *N. gonorrhoeae* examined failed to utilize glucose on the test medium

Organism		Acid Production after Incubation for 48 Hours (serum agar)			Acid Production after Incubation for 48 Hours (test media)		
		Glucose	Maltose	Sucrose	Glucose	Maltose	Sucrose
<i>N. meningitidis</i>	C453	+	+	-	+	+	-
<i>N. meningitidis</i>	C587	+	+	-	+	+	-
<i>N. meningitidis</i>	C466	+	+	-	+	+	-
<i>N. catarrhalis</i>	(3622)	-	-	-	-	-	-
<i>N. flavescens</i>	C8262	-	-	-	-	-	-
<i>N. pharyngis</i>	(4591)	+	+	d	+	+	d
<i>N. animalis</i>	(10212)	d	-	+	d	-	+
<i>N. caviae</i>	(10293)	-	-	-	-	-	-

Table II Fermentation reactions of *neisseria* species for both serum agar sugars and test media

+ = acid production; - = no acid production; d = doubtful.

after 24 hours' incubation. All, however, fermented glucose after 48 hours. Neither sucrose nor maltose was fermented by any of the test organisms.

Discussion

The need for a simple and consistently reliable medium for testing fermentation reactions in the identification of *neisseria* species has long been recognized. The interpretation of results using serum agar sugars in the identification of gonococci is complicated by a large number of false positive maltose reactions. In this series of experiments we set out to develop a medium which would grow the organisms well but eliminate false fermentation reactions. Subsequently, we have brought it into routine use and examination of a further 100 fresh isolates confirms our hopes of its usefulness.

The results obtained so far indicate that this medium has advantages over serum agar sugars, especially in the interpretation, as the problem of false positive maltose reactions does not appear to arise. Positive reactions may be read after eight hours' incubation and the medium is easier to prepare. One possible disadvantage may be presented by strains of gonococci which require cocarboxylase for growth. So far, we have not encountered any cocarboxylase-sensitive strains, but if any do occur the problem they present could be overcome by adding the appropriate concentration of cocarboxylase directly to the sugar solutions (and subsequently incorporating both into the medium.) The total shelf

life of the medium must be assessed, but is certainly long enough to be useful, and the possibility of using this medium for other fastidious organisms may be considered.

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