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Rapid identification of pathogenic species of Neisseria by carbohydrate degradation tests

Importance of glucose in media used for preparation of inocula

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SUMMARY Pathogenic species of *Neisseria* were identified more readily by carbohydrate degradation tests when 0·5% glucose was used in media from which inocula for the test were obtained. This improved the performance of both non-growth and growth-dependent methods for these tests. One of the three techniques used a non-nutrient buffered salt solution and depended on the presence of preformed enzymes. This test was more accurate and rapid than the two growth-dependent techniques.

Introduction

Neisseria species, both gonococcal and non-gonococcal, are frequently cultured from genital and extra-genital sites.[1] It is therefore necessary to identify and distinguish isolates of clinical importance. The laboratory identification of these strains is commonly performed by determining their patterns of carbohydrate utilisation, the sugars being degraded by enzymes produced during growth of the organisms in a nutrient medium. These growth-dependent methods, such as those based on cystine-trypticase agar, have well-recognised problems on account of the slow growth of *Neisseria gonorrhoeae* and the occurrence of auxotrophic strains of this species which require nutritional supplements.[2] Despite their deficiencies, these techniques are still recommended in recent[3] and authoritative[4] publications.

Rapid carbohydrate utilisation tests, introduced by Kellogg and Turner,[5] use a heavy inoculum of the organism to be tested, which is harvested after growth on a nutrient medium and suspended in a non-nutrient buffer solution. These techniques rely on enzymes formed during growth on the nutrient medium to degrade the carbohydrates. Several modifications of the original method have been described,[6 7] and this type of test is now widely used because it is quick and overcomes some of the problems of the growth-dependent tests.

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A further problem with carbohydrate utilisation tests arises because the neisseriae metabolise glucose by aerobic means only and produce only small amounts of lactic and acetic acid.[8] However, the enzymes of neisseriae responsible for degradation of glucose are inducible,[9] although whether or not this effect applies to carbohydrate utilisation tests has not been reported. Young *et al*[7] speculated that the absence of glucose in Thayer-Martin medium was one reason why sugar reactions were not reproducible when inocula used for the reactions were derived from this medium. Subsequently Young[10] reported that a glucose content of 0·5% in New York City medium interfered with the interpretation of carbohydrate utilisation test results when performed with inocula harvested from these plates. Other reports evaluating carbohydrate utilisation tests have not specifically mentioned the effect of glucose in culture media used to prepare inocula for sugar degradation tests. Some of these media incorporated glucose,[1 11-14] whereas in other studies the glucose content or even the medium itself was not specified.[3-6 15]

In this study, the effects of incorporating glucose in media used to prepare inocula for carbohydrate utilisation tests were evaluated. The reactions obtained with inocula harvested from culture plates prepared with and without glucose were compared using both growth-dependent tests and rapid carbohydrate utilisation techniques.

Materials and methods

MEDIA

Media used for preparation of inocula (purity plates)

were prepared from GC agar (Difco) with 8% saponin-lysed defibrinated horse blood (CSL). This medium contains no glucose and was used without added carbohydrate and with added glucose at final concentrations of 0·25%, 0·5%, and 1%.

Liquid media used to determine carbohydrate utilisation patterns were:

(1) RM (rapid microcarbohydrate utilisation technique) broth[16] containing per 100 ml of distilled water Casamino acids (Difco) 2 g; L-cysteine hydrochloride (Calbiochem) 0·03 g; sodium sulphate (Baker) 0·03 g; neopeptone (Difco) 2·5 g; and phenol red 0·01 g. The broth was sterilised by filtration and the final pH adjusted to pH 7·4.

(2) BS (buffered salt)[6] solution containing per 100 ml of distilled water K_2HPO_4 0·04 g; KH_2PO_4 0·01 g; KCl 0·8 g; and phenol red 0·01 g. The solution was sterilised by autoclaving and the final pH adjusted to 7·4.

(3) CT broth ("Minitek" cystine-trypticase *Neisseria* broth, Baltimore Biologic Laboratories) was modified by the addition of 0·075 ml of a 2% solution of phenol red per ml of broth.

Each of the liquid media was dispensed in 1·2 ml volumes and stored at 4°C. Glucose, lactose, and sucrose (British Drug Houses) were prepared as 10% solutions and sterilised by filtration. Maltose, free of contaminating glucose (Merck), was similarly prepared.

ORGANISMS

Fifty-four strains of *Neisseria* were used, including 48 of *N gonorrhoeae* (three stock strains and 45 fresh isolates) together with stock cultures of *N meningitidis* (three), *N lactamica* (two), and *N sicca* (one).

All 54 strains were used in experiments with all three liquid media to compare the effects of inocula derived from purity plates with and without 1% added glucose. Eighteen of the strains of freshly isolated gonococci and the six non-gonococcal *Neisseria* strains were used in separate experiments in BS broth to compare the effects of inocula derived from purity plates containing 0·25%, 0·5%, and 1% glucose.

PROCEDURE

The organisms were grown overnight on the appropriate purity plates at 37°C in an atmosphere of 5% CO_2 in air. A heavy inoculum—equivalent to 5×10^9 organisms per ml as determined by surface viable counts—was introduced into 1·2 ml of the broth under test. These inoculated broths were then dispensed in 0·1 ml volumes into five separate wells in clear plastic microtitre plates (Kayline) sterilised by ethylene oxide. To each of four wells 0·025 ml of the appropriate carbohydrate was added. No carbohydrate was added to the fifth well, which served as a control. The plates were gently agitated to ensure proper mixing and reactions recorded after one, two, four, and 18 hours' incubation at 37°C. Reactions were read as follows: yellow coloration indicated positive reaction; red coloration, negative reaction; and orange/yellow or orange coloration, intermediate reaction. The time taken to record a definite positive reaction was noted for each strain.

Results

All three broths produced the expected results more rapidly for both gonococcal and non-gonococcal species of *Neisseria* when inocula were prepared from cultures grown in the presence of glucose (table I).

TABLE I *Carbohydrate reactions in three broth systems of 54 Neisseria species grown on media with and without 1% glucose*

	No of strains grown on different media and time (hrs) taken to produce expected result							
	Without glucose				*With glucose*			
Species and broth system	*1*	*2*	*4*	*18*	*1*	*2*	*4*	*18*
N gonorrhoeae (48 strains)								
Test broth								
Buffered salt	34	44	48	48	44	44	48	48
RM	0	11	38	48	3	32	42	48
Cystine-trypticase	0	2	32	42	7	18	46	48
Other Neisseria (6 strains)*								
Test broth								
Buffered salt	0	6	6	6	6	6	6	6
RM	0	0	4	6	1	5	6	6
Cystine-trypticase	0	0	2	5	1	6	6	6

*Three strains of *N meningitidis*, two of *N lactamica*, and one of *N sicca*

The most noticeable improvement in performance was in the CT broth. Although 47 of 54 strains produced the expected result after 18 hours' incubation when the inocula were prepared from media without glucose, all 54 strains gave positive results after 18 hours' incubation with inocula prepared from glucose-containing media. With the RM broth 11 isolates gave positive results after two hours' incubation with inocula from non-glucose containing media, whereas 37 strains produced positive results after the same time with inocula from glucose-containing media. The BS broth gave the most rapid result; all strains gave positive results within four hours' incubation. When inocula were prepared from glucose-containing media 50 of the 54 strains tested produced a positive result within one hour.

The optimum concentration of glucose added to the basal medium was 0·5% (table II). The amount of acid formed in culture plates before inoculation into the test broths caused a slight colour change in the indicator system of the control tube when growth from plates with 1% glucose was added. When growth from the lowest concentration, 0·25%, was used, some strains produced slower reactions in the BS broth. With the intermediate concentration of glucose added, all expected reactions had occurred within two hours with the 24 strains of *Neisseria* tested, and no colour change occurred in the control well.

TABLE II *Carbohydrate reactions in buffered salt broth of 24 Neisseria strains grown on media with differing glucose content*

	No of strains grown on media containing glucose and time (hrs) taken to give positive result								
	0·25%			*0·5%*			*1%*		
Species	*1*	*2*	*4*	*1*	*2*	*4*	*1*	*2*	*4*
N gonorrhoeae (18 strains)	11	17	18	16	18	18	18	18	18
Other *Neisseria** (6 strains)	5	6	6	6	6	6	6	6	6

**Three strains of N meningitidis,* two of *N lactamica,* and one of *N sicca*

Discussion

The laboratory identification of species of *Neisseria* by carbohydrate utilisation tests is easier when inocula for the tests are obtained from glucose-containing media. The performance of three methods for determining sugar reactions of *Neisseria* species was improved when such inocula were used. The three methods depended for their performance either on growth of the organisms in the broth (CT) or on degradation of the carbohydrate by preformed enzymes (BS) or on a combination of both (RM). With the non-growth-dependent BS broth, the speed of the reaction was increased and 50 of 54 strains tested gave the expected result after one hour's incubation when grown on glucose-containing media before inoculation into the test broth. The two other broths also produced faster reactions when such inocula were used; in addition, CT broth gave positive reactions where inocula from glucose-free media gave negative results.

Reports on the performance of carbohydrate utilisation tests for the identification of *Neisseria* species do not consider the effects of glucose in media from which inocula for the tests are obtained, even though enzymes of neisseriae involved in the metabolism of glucose[9] are induced and alkaline reactions are produced as a result of deamination of amino acids by these organisms when grown on glucose-deficient media.[1] Young *et al*[7] reported that Thayer-Martin medium was not ideal for obtaining inocula for carbohydrate utilisation tests and suggested that absence of glucose was a contributing factor to this deficiency. Young[10] recommended that the glucose content of New York City medium used for both isolation of gonococci and preparation of inocula for confirmatory carbohydrate degradation tests should be reduced, pointing out that excess acid production during growth interfered with this type of test. This effect was observed in this study when 1% glucose was incorporated into the medium; but when the concentration was halved the effect was abolished without the efficiency of the test being reduced.

Thus, we recommend that inocula used for carbohydrate utilisation tests should be taken from media containing 0·5% glucose. Identification of *Neisseria* species was accurately and rapidly performed by establishing sugar degradation patterns using a buffered salt solution. This method, which uses preformed enzymes, was superior to the two other systems, both of which required growth of the organism to produce a positive result. Methods for performing carbohydrate utilisation tests should be compared under optimal conditions, which include incorporation of suitable amounts of glucose in media used to prepare inocula for the tests.

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