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Rapid Recognition of Group B Streptococci by Pigment Production and Counterimmunoelectrophoresis

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Streptococci from clinical isolates were studied for their ability to produce pigment in stab cultures in Columbia agar. Serological grouping of these organisms was done by counterimmunoelectrophoresis using Burroughs-Wellcome antisera. In this group of isolates, 66 of the 68 organisms grouped as B by serological testing produced pigment in the Columbia agar stab cultures. Pigment was not produced by any of the other 36 streptococci studied (11 group A, 9 group C, 4 group D, and 12 nongroupable). The use of the Columbia agar stab culture is recommended as a rapid and simple test for recognition of group B streptococci. The counterimmunoelectrophoresis test is also suggested as a convenient, rapid, and sensitive method for grouping the streptococci.

Group B beta-hemolytic streptococci (*Streptococcus agalactiae*) have been implicated recently as a significant cause of neonatal morbidity and mortality. However, a rapid, convenient test for the identification of group B streptococci remains to be developed. Testing for hippurate hydrolysis is recommended as a procedure for the identification of group B streptococci, although other groups, especially D, may occasionally hydrolyze hippurate (4). This test, although quite reliable, may not be convenient for the busy routine laboratory. The CAMP test has been described as useful for the identification of group B streptococci (3). However, this test also is not convenient for the busy routine laboratory.

The groups of streptococci are specifically identified on the basis of their antigenic structure, as described by Lancefield (10). The technique for grouping usually involves the use of capillary tubes, specific antisera, and acid extracts of the organisms. More recently streptococci have been grouped using acid extracts and counterimmunoelectrophoresis (CIE), and it has been demonstrated that this procedure is as specific as the capillary precipitin test but more sensitive (2, 4). It has also been shown that the group-specific antigen of group B streptococci does not need to be extracted with acid to detect it with CIE (7). Inoculation of the organism into Todd-Hewitt broth and incubation for 4 h provides enough antigenic material for the test. This is a simple, rapid method for grouping the streptococci as B.

It was described early in the studies on group B streptococci that some strains produced pigments under certain conditions (1). This finding was neglected for many years and was not developed as a useful laboratory tool. A recent article by Fallon (6) describes the usefulness of pigment formation by group B streptococci growing on Columbia agar for rapid identification of these organisms. Of special importance was Fallon's observation that most of his clinical isolates formed a pigment. We have also been interested in the production of pigment by these organisms and have found the production of pigment by streptococci in stab cultures in Columbia agar tubes to be a reliable test for group B organisms. Our use of stab cultures incubated aerobically provides a simpler test and is therefore more reliable than the use of Columbia agar plates under the special anaerobic conditions as described by Fallon.

In this paper we describe the excellent correlation between pigment production in stab cultures in Columbia agar, hippurate hydrolysis, and grouping of the streptococci by CIE. We recommend the use of Columbia agar stab cultures as a convenient screening test for recognition of streptococci as group B, especially in laboratories not doing serological analyses.

MATERIALS AND METHODS

Organisms. We obtained from the hospital microbiology laboratory of the Dartmouth-Hitchcock Medical Center, over a 3-month period, the streptococci that were isolated and were shown not to be group A or enterococci since they were negative on bile-esculin medium, did not grow in salt broth (6.5% NaCl), and did not react with fluorescent antibody to group A streptococci and were therefore

suspected of being group B. We obtained a few group A streptococci and enterocci for comparison. These organisms came from a variety of sources, including neonates with meningitis or respiratory disease, neonates with no disease, vaginal or cervical swabs from pregnant and nonpregnant women, urine cultures (male and female), and various wounds and ulcers.

Bacitracin sensitivity. Beta-hemolytic streptococci were streaked on a sheep blood agar plate to yield confluent growth. A taxos A disk with 0.04 units of bacitracin (BBL) was placed on the plate. A zone of inhibition greater than 10 mm after 24 h of incubation was considered positive.

Hippurate hydrolysis. Hippurate medium was made up by the directions of Facklam et al. (5). We used brain heart infusion broth (Difco) as the control and added sodium hippurate in a concentration of 10 g/liter of the medium for the hippurate hydrolysis test. The organisms were inoculated directly from the agar plate into 5 ml of the control and 5 ml of the hippurate medium. After 24 h at 37 C we centrifuged the culture, aseptically removed 0.8 ml and placed this in a Kahn tube, and to this added 0.2 ml of 12% ferric chloride. The tubes were shaken and examined for precipitate after 15 min. The cultures were reincubated for another 24 h, the test was repeated, and the cultures were incubated for another 24 h, so that 72 h of culture time was allowed.

Columbia agar. Columbia agar medium was purchased from BBL and prepared according to instructions. The medium was dispensed in a volume of about 7 ml into screw-capped tubes (16 by 100 mm), autoclaved, and stored until use as agar tubes for stab cultures. Organisms to be tested were then stabbed into the deep agar, and the culture was incubated aerobically at 37 C for 24 h. The stab culture was then observed for a yellow to orange pigment. The pigment produced is obvious but is observed slightly better with daylight than with fluorescent lights, as was pointed out by Fallon (6). Some lightly pigmented strains were best observed if the stab was off center and growth occurred near the side of the tube, where interference from the lightly colored agar was minimal.

Antigenic grouping of the streptococci. The organisms were inoculated from the original plate into 1 ml of Todd-Hewitt broth and incubated from 4 to 24 h at 37 C. The culture produced enough antigen for the test in 4 h; however, we found 18 to 24 h to be satisfactory for the test and more convenient for us. The culture was then run in the CIE test against antisera to group B streptococci (7). Those that were negative on this CIE test were then typed by the capillary precipitin test procedure and by CIE (3). For this test the organisms were inoculated into 50 ml of Todd-Hewitt broth and incubated for 18 to 24 h before acid extraction and grouping studies (9, 10).

The antisera purchased from Burroughs-Wellcome worked very well in both the CIE test for group B cultures and with the acid-extracted material from cultures of groups A through D using either the capillary precipitin test or CIE. The group B streptococci actually are better in the CIE test without being acid extracted since some of the antigen is soluble and in the growth medium. The other groups should be acid extracted.

RESULTS

We studied 104 streptococcal isolates that were obtained from 100 individuals. In this group there were 11 group A streptococci, 68 group B, 9 group C, 4 group D, and 12 that were not groupable. The results (Table 1) indicate that of the 68 streptococci we classified as group B, 66 were positive on CIE, hydrolyzed hippurate, and produced pigment in Columbia agar. The other two were positive on CIE and hydrolyzed hippurate but were negative on Columbia agar. Nothing else unusual was noted about these two organisms. One was isolated from the deep ear of a neonate and one was isolated from the urine of a 77-year-old man. All the tests were repeated on these two cultures, and the results were the same.

No organisms other than group B produced pigment in Columbia agar. Two organisms were hippurate positive, did not produce pigment in Columbia agar, and were negative for group B on CIE. One of them was grouped as C and the other as D.

We found that our group B organisms grew well in 24 h and gave a positive hippurate test. We did not call cultures negative until 72 h, as described by Facklam (5), but in our study the 24-h result was reliable as long as there was visible growth of the organism.

It is also of interest that 5/68 of our group B streptococci were sensitive to bacitracin. These could be confused with group A streptococci in laboratories relying solely on bacitracin sensitivity. All five of these produced pigment in Columbia agar. Similarly, 4/68 of our group B streptococci were not beta-hemolytic but produced pigment in Columbia agar.

In undertaking the serological analysis of these cultures we found the Difco antisera (lots 606217 and 586467) to group B streptococci to be unsuitable for the CIE test, but they reacted in some instances with acid-extracted antigen. The antisera to groups A, C, and D were more satisfactory and reacted with the acid-extracted material. The Burroughs-Wellcome antisera were very good in both the CIE test for group B and the capillary tube test or CIE using acid extracts of groups A through D.

All of the organisms studied were isolated from humans. Some individuals were outpatients, some were hospitalized, and some were cultured for routine screening of newborn infants. Most of the organisms were not considered the cause of infection, except in the neonates with meningitis or respiratory disease.

TABLE 1. *Correlation of biochemical tests with antigenic analysis determined by CIE*

Test	No. positive/no. tested in serological group:				
	A	B	C	D	Not A→D
Pigment in Columbia agar	0/11	66/68	0/9	0/4	0/12
Hydrolyze hippurate	0/11	68/68	1/9	1/4	0/12
Sensitive to bacitracin	11/11	5/68	0/9	0/4	0/12

The sources of the group B streptococci are as follows: urine, 6; cervix or vagina, 15; throat, 7; wound, 8; healthy infant survey, 6; newborns, 18. The bulk of the organisms were isolated from the GU tract of adults. In five cases of neonatal sepsis or meningitis we first recognized the organism as group B with the use of pigment production.

DISCUSSION

Infections with group B streptococci are being recognized as an important cause of neonatal morbidity and mortality. Procedures for the prompt recognition of group B beta-hemolytic streptococci in the mother or baby at the time of delivery would help in identifying the infant at risk

We have found the use of the Columbia agar stab tube, for the clinical isolates studied, to be a reliable, simple, and rapid test for group B streptococci. In the other 36 cultures which were not group B we encountered no streptococci producing the pigment. This is in agreement with Fallon's observations (6), although he refers to an article which mentions the production of pigment by a group D organism on different media. The incidence of false negatives (group B streptococci not forming a pigment) was 15% in Fallon's study by his technique (6) and 3% in our study with the stab cultures. This compares favorably with the bacitracin tests for identification of group A streptococci, since it has been shown that 0.5% of group A are resistant to bacitracin (false negatives), whereas 2.6% of group B, 6% of group C, 8% of group G, and 2.2% of the nongroupables are sensitive to bacitracin (false positives) (11). Similarly, a positive hippurate test can be obtained with streptococci other than group B (false positives) (5).

We have found the Columbia agar stab culture more convenient than the hippurate test for screening for group B streptococci in a busy hospital laboratory. The stab can be inoculated directly from the primary blood agar plates at the same time that the test for bacitracin sensitivity is inoculated, and the stab can be read after 20 to 24 h of incubation. The agar is commercially available and simple to prepare and store, and there is no requirement to add reagents when the test is read. Our finding that stab cultures incubated aerobically are optimal for observing pigment formation was fortunate, since it avoids the problems Fallon reported (6) in selecting procedures for producing the correct anaerobic conditions critical for pigment production on the surface of Columbia agar plates. In preliminary studies we were unable to produce these anaerobic conditions reliably in our laboratory and therefore could not document pigment production by these organisms on the surface of Columbia agar plates.

It is important to recognize that a small percentage (3% in our study) of group B streptococci do not produce any pigment. Further studies on human isolates will be needed before the magnitude of this percentage among clinical isolates is known. It will be necessary to test these uncommon nonpigmented organisms by serological studies or by hippurate hydrolysis using the technique described by Facklam et al. for the precipitation of benzoic acid by ferric chloride (5) or by using ninhydrin to detect glycine in the rapid hippurate test (8).

Serological studies for identification of the different streptococcal groups have been in use for many years (9, 10). The use of bacitracin disks, hippurate medium, bile esculin medium, salt broth, and serological studies taken together positively identify most beta-hemolytic streptococci. For grouping the beta-hemolytic streptococci by serological studies, we recommend the CIE procedure as described by others (2, 4), since it requires less antiserum and is more sensitive and easier to read than is the capillary precipitin test. In addition, group B organisms do not need to be acid extracted (7).

We suggest the use of Columbia agar stab culture as a simple addition to the routine laboratory procedure, especially where serological studies are not done for the identification of group B streptococci. As reported by Fallon (6), we found that the intensity of pigment production varies from a deep brick red to a faint yellow-orange. In one strain a faint pigment production was missed by a less experienced observer. Therefore, in introducing this test into a laboratory we recommend the initial use of a negative control consisting of a group A

streptococcus to compare with lightly pigmented group B strains.

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