Laboratory and Field Evaluation of Selective Media for Isolation of Group B Streptococci

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Problems encountered with currently recommended selective media for group B streptococci (GBS) (selective broth medium and CNA agar) prompted a search for alternative culture methods in ongoing epidemiological studies. Previously recommended inhibitory agents were tested in vitro. Gentamicin, alone or in combination with nalidixic acid, proved inhibitory for many GBS strains. Among other agents tested, polymyxin was most complementary to the gram-negative spectrum of nalidixic acid, without compromising GBS growth. Crystal violet provided the simplest, most economical staphylococcal inhibitor. Broth and agar media, constituted with these three agents and designated NPC, were evaluated in vitro and in field studies. This investigation represents the first direct comparison of broth media containing inhibitory agents for the preferential isolation of GBS. In maternal colonization studies, NPC broth proved superior to Todd-Hewitt broth containing nalidixic acid and gentamicin at concentrations employed in the previously described selective broth medium (95% versus 59% recovery). Our comparisons were done without added sheep blood since GBS grow well in Todd-Hewitt broth. NPC broth proved more sensitive than NPC agar for detecting GBS colonization in newborns. The NPC agar medium was useful for further purification of broth cultures and quantitative culture techniques.

A selective medium for bacterial epidemiological studies must prevent or minimize growth of unwanted species without compromising the recovery of desired organisms. Most media used for the isolation of group B streptococci (GBS) have been identical to, or adapted from, media useful for isolating other streptococci, notably members of group A (7, 10, 12, 14). All except Pike's broth were blood agar incorporating two or more inhibitors. The medium devised by Ellner et al. (7), containing colistin and nalidixic acid and available commercially as "CNA agar," has been widely used. A selective broth medium (SBM) recommended specifically for isolation of GBS was prepared by Baker and co-workers by the addition of nalidixic acid and gentamicin to Todd-Hewitt blood broth (1).

In an epidemiological study of newborns in an intensive care nursery, we found CNA agar and SBM to yield similar recovery rates of GBS (L. Eisenfeld, G. E. Cassady, and H. C. Dillon, Jr., Clin. Res. 26:79A). Overgrowth of other organisms, especially *Staphylococcus aureus*, proved a problem with SBM. Alternative combinations of inhibitors suitable for use in a broth medium for extensive GBS epidemiological studies were therefore explored. These results are now reported.

MATERIALS AND METHODS

Effect of inhibitory agents on bacterial growth in vitro. Bacterial strains. Reference strains for each of the five major GBS serotypes, kindly provided by Hazel Wilkinson (Center for Disease Control, Atlanta, Ga.), and 27 clinical isolates of all serotypes from our own collection were used. A total of 101 additional wild strains of GBS, representing all serotypes, were examined in in vitro studies evaluating a selective agar medium. Gram-negative species, representing flora of the gastrointestinal and genitourinary tracts, enterococci, and staphylococci were obtained from the Children's Hospital bacteriology laboratory and from ongoing epidemiological studies. These included 12 strains each of coagulase-positive and coagulase-negative staphylococci, 5 strains of enterococci, and 1 each of *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Enterobacter aerogenes*, *Proteus vulgaris*, *Acinetobacter calcoaceticus* subsp. *lwoffi* (*Mima polymorpha*), *A. calcoaceticus* subsp. *amitratus* (*Herellea vaginicola*), *Serratia marcescens*, and *Pseudomonas aeruginosa*.

Inhibition of log-phase growth by antimicrobial agents was determined by methods similar to those described by Brown and Garrett (3). Inhibitory agents tested were nalidixic acid (15 µg/ml; Winthrop), gentamicin (1 to 8 µg/ml; Schering), kanamycin (30 µg/ml; Bristol), neomycin (30 µg/ml; Squibb), polymyxin (1 to 15 µg/ml; Burroughs Wellcome), trimethoprim (30 to 50 µg/ml; Hoffmann-LaRoche), and crystal vi-

olet (0.025 to 10 $\mu\text{g/ml}$; BACTO, BANCO, Harleco). Additional experiments were done comparing two lots of gentamicin (Schering, lots no. 5AMK220 and 7AMK31). Todd-Hewitt broth (Difco) was prepared with the addition of inhibitory agents and dispensed in 3-ml volumes in culture tubes (13 by 100 mm); identical tubes containing only Todd-Hewitt broth served as controls. Two drops of overnight broth culture of each strain was added to test and control tubes; the optical density at 590 nm in a Bausch and Lomb Spectronic 20 spectrophotometer was 0.001 at inoculation. Tubes were incubated at 37°C, and optical density was read at 1, 2, 3, 4, 5, 6, and 18 h; observed growth rates were maximal between 3 and 4 h. The growth rate constant, k , was calculated from the apparent first-order expression, $\ln N - \ln N_0 = kt$, where N_0 is the initial optical density (at 3 h), N is the optical density at time t (4 h), and t is 1 h. Evidence for GBS inhibition by either antibiotic or dye incorporated into the medium was defined by a growth rate less than half that in the control broth containing no antibiotic. Viability of GBS after incubation in the inhibitory broths for 18 h was determined by subculture of test and control broth cultures onto sheep blood agar plates. Inhibition was defined as failure to recover GBS from the broth medium.

Based on results with individual inhibitory agents, the combination of nalidixic acid, polymyxin, and crystal violet was chosen for further evaluation by incorporating them into broth and agar. Blood agar medium (5% sheep blood, tryptic soy agar base, Inolox) was tested with varying concentrations and combinations of nalidixic acid (15 $\mu\text{g/ml}$), polymyxin (1 to 15 $\mu\text{g/ml}$), and crystal violet (0.125 to 15 $\mu\text{g/ml}$). A total of 101 GBS strains and 38 strains of the other species listed were plated with a multipoint inoculator; plates were incubated aerobically at 37°C for 18 h, then examined for growth or inhibition of growth.

The definitive composition of media used in field studies was as follows: NAG broth, nalidixic acid (15 $\mu\text{g/ml}$) and gentamicin (8 $\mu\text{g/ml}$) in Todd-Hewitt broth; NPC broth, nalidixic acid (15 $\mu\text{g/ml}$), polymyxin (1 $\mu\text{g/ml}$), and crystal violet (0.1 $\mu\text{g/ml}$) in Todd-Hewitt broth; NPC agar, nalidixic acid (15 $\mu\text{g/ml}$), polymyxin (10 $\mu\text{g/ml}$), and crystal violet (1 $\mu\text{g/ml}$) in tryptic soy agar base (Inolox) with 5% sheep blood. The concentrations of polymyxin and crystal violet required for effective inhibition of unwanted species in agar medium were 10-fold higher than those used in broth. The broth designated NAG was identical to SBM in antibiotic concentration (15 μg of nalidixic acid and 8 μg of gentamicin per ml, lot no. 5AMK220). Sheep blood was not required.

Field evaluation of selective media for GBS. Selective media were evaluated in epidemiological studies of GBS colonization in mothers and infants examined at Cooper Green Hospital, within the University of Alabama Medical Center Complex, in Birmingham. NPC and NAG broths were compared by examining their suitability for the primary isolation of GBS from maternal vaginal cultures. Duplicate vaginal cultures were obtained immediately after delivery from 400 postpartum patients, using cotton-tipped swabs. Swabs were placed in silica gel packets (Carter, Rice, Storrs, and Bement) and processed in the labo-

ratory within 72 h. NPC and NAG broth culture tubes were inoculated in tandem with the paired swabs. The swabs remained in broth during incubation for 18 h at 37°C, after which a sample of each broth culture was subcultured onto both sheep blood agar and NPC agar. Culture plates were streaked, then incubated aerobically for 18 h at 37°C, after which they were examined for GBS as described below.

NPC broth and NPC agar were compared by examining the respective recovery rates of GBS achieved after inoculating these two forms of selective media with cultures taken from newborn infants shortly after birth. In this investigation, cultures from three or four sites (external ear, throat, anus, and umbilicus) were taken from 248 infants. A single cotton-tipped swab was used for culturing a given site; a total of 949 cultures were taken, then placed in silica gel packets as described above. For processing, swabs were first inoculated directly onto NPC agar, then placed into NPC broth. Both NPC agar and NPC broth cultures were incubated for 18 h at 37°C. Samples of broth culture were subcultured onto sheep blood agar plates, incubated overnight at 37°C, and examined for GBS as described below.

Streptococci producing beta-hemolytic colonies were further examined for CAMP reactions (4) and identified serologically as previously described (5) using grouping and typing antisera from the Center for Disease Control, Atlanta, Ga. Nonhemolytic streptococcal colonies with colonial morphology considered typical of GBS were examined for CAMP reactions and confirmed as GBS by serological methods as above.

RESULTS

Preliminary testing of GBS for in vitro susceptibility to gentamicin at 8 $\mu\text{g/ml}$ revealed definite delay of log-phase growth. In a representative experiment (Table 1), gentamicin at 8 $\mu\text{g/ml}$ depressed the mean growth rate; 2/8 were inhibited during log-phase growth, as defined above, but all eventually grew by 18 h of incubation. At 4 $\mu\text{g/ml}$ this lot of gentamicin (5AMK220) was not inhibitory to GBS. A different lot, however (7AMK31), was markedly inhibitory at the same concentration, inhibiting all eight strains at 4 h and killing all but one strain (Table 2); this gentamicin lot was set aside. Although gentamicin inhibited most gram-negative species at 8 $\mu\text{g/ml}$, it failed to prevent growth of *Proteus*, *Serratia*, and most staphylococci; further reduction in concentration resulted in increased growth of unwanted species. In evaluating the various inhibitory agents of potential value for use in a selective culture medium for GBS, it was evident that no single agent effectively eliminated all gram-negative species; none inhibited enterococci, and only crystal violet was active against staphylococci. Nalidixic acid (15 $\mu\text{g/ml}$) allowed normal GBS growth but did not inhibit *Pseudomonas* or

TABLE 1. Growth of GBS^a in Todd-Hewitt broth containing gentamicin

Expt.	Gentamicin lot no.	Concn. ($\mu\text{g/ml}$)	Mean growth rate ($k \pm \text{SD}$) ^b	<i>P</i>	No. of strains inhibited ^c at:	
					4 h	18 h
A	5AMK220	8	0.66 $\pm$ 0.275	0.001 ^d	2/8	0/8
	5AMK220	4	1.08 $\pm$ 0.270	NS ^e	0/8	0/8
	Control ^f		1.09 $\pm$ 0.184		0/8	0/8
B	7AMK31	4	0.06 $\pm$ 0.162	<0.001 ^e	8/8	7/8
	5AMK220	4	0.87 $\pm$ 0.198		0/8	0/8
	Control		1.07 $\pm$ 0.105		0/8	0/8

^a Five stock and three wild strains.^b SD, Standard deviation.^c Inhibition was defined, at 4 h, by a growth rate less than half that in control broth; at 18 h, by failure to grow on subculture.^d Experiment versus control, Student's *t* test.^e NS, Not significant.^f Control, Todd-Hewitt broth without gentamicin.^g Lot 7AMK31 versus lot 5AMK220, Student's *t* test.

Klebsiella. Although permitting GBS growth, neomycin and kanamycin (30 $\mu\text{g/ml}$) were not suitable for combination with nalidixic acid because they failed to eliminate *Pseudomonas*. Trimethoprim (30 to 50 $\mu\text{g/ml}$) inhibited GBS, while allowing *Pseudomonas* and *Proteus* to grow. Polymyxin did not inhibit GBS at 2 $\mu\text{g/ml}$ but was effective against *Pseudomonas* and *Klebsiella* as low as 1 $\mu\text{g/ml}$; polymyxin was thus chosen for use with nalidixic acid. The third component of NPC, crystal violet, was mildly inhibitory to GBS at 0.25 $\mu\text{g/ml}$ but greatly reduced growth of staphylococci at 0.1 $\mu\text{g/ml}$. Although crystal violet from three different manufacturers varied somewhat in activity against GBS at concentrations above 0.25 $\mu\text{g/ml}$, consistently good growth has been obtained using 0.1 $\mu\text{g/ml}$. Several new lots of nalidixic acid and polymyxin were examined prior to accepting them for routine laboratory use, all with satisfactory results.

In vitro studies of NAG and NPC broths, constituted as for field use, are depicted in Table 2. All but four strains tested were recent clinical isolates. Growth rate was reduced, and 18 of 24 strains were inhibited in log phase in NAG broth. Growth of one strain was completely inhibited. NPC broth adequately supported growth of all GBS strains; none were inhibited at 4 h, although the mean growth rate was slightly reduced. NPC agar supported excellent growth of all 101 GBS strains examined in in vitro tests.

Although NAG broth was shown to inhibit GBS in vitro, direct field comparisons with NPC broth were deemed necessary to demonstrate possible differences in GBS recovery under conditions of epidemiological study. GBS were isolated from vaginal cultures obtained at delivery in 76 of the 400 postpartum patients examined;

TABLE 2. Growth of GBS in NAG and NPC broth

Medium	Mean growth rate ($k \pm \text{SD}$) ^a	<i>P</i> ^b	No. of strains inhibited at:	
			4 h	18 h
NAG broth	0.39 $\pm$ 0.231	<0.001	18/24	1/24
Control	0.92 $\pm$ 0.203		0/24	0/24
NPC broth ^c	0.85 $\pm$ 0.148	<0.001	0/26	0/26
Control	0.95 $\pm$ 0.162		0/26	0/26

^a SD, Standard deviation.^b Experimental versus control by Student's *t* test.^c This collection includes the 24 strains tested with NAG broth; four were stock strains (Ib, Ic, II, and III), and the remainder were wild strains.

72 of these 76 GBS strains (95%) were recovered in NPC broth, compared to only 46 of 76 (59%) in NAG broth. NPC broth thus proved superior for the primary isolation of GBS ($P < 0.001$ by the chi-square test). In this same study, subcultures of both broth media were plated onto sheep blood agar and NPC agar. All GBS strains isolated on plain blood agar grew well on NPC, confirming results of in vitro NPC agar testing. In addition, NPC agar virtually eliminated common contaminating species surviving in either broth medium.

In the study comparing NPC broth and NPC agar for isolation of GBS from newborn infants, 28 of 248 newborn infants had GBS recovered from one or more culture sites. Of the 28 culture-positive infants, 27 were detected by the use of the broth medium, compared to only 12 of 28 with the agar medium. There were 949 multiple-site cultures taken in all; of the 62 positive for GBS, 30 (48%) were recovered in NPC agar, whereas 61 (98%) were recovered in NPC broth. The sensitivity of the broth medium for primary

isolation of GBS was thus proved to be twice that of the agar medium ($P < 0.001$ by the chi-square test).

DISCUSSION

In considering the requirements of selective culture media for ongoing epidemiological studies of GBS infections, we found it essential to inhibit not only gram-negative organisms but also staphylococci, a common "unwanted species" in both maternal and neonatal culture samples. Neither SBM nor CNA agar proved entirely satisfactory (Eisenfeld et al., Clin. Res. 26: 79A). Broth was preferred because of convenience and economy, but critical comparisons of broth media with differing inhibitors for use in the preferential recovery of GBS have not been reported. We examined alternative methods and have drawn upon the experience of others in choosing suitable inhibitory agents. Crystal violet, used in selective media for both group A and group B streptococcal epidemiological work for many years (6, 9, 12), provided a simple and economical inhibitor for staphylococci. Nalidixic acid is also of proven value (1, 10, 14). In vitro studies with large numbers of GBS strains confirmed a lack of inhibitory effect for this agent in the recommended concentration (15 $\mu\text{g}/\text{ml}$) in either broth or agar. Our selection of polymyxin was based both on the recognized usefulness of this agent and colistin in selective streptococcal media (7, 10, 12, 14), and on the in vitro experiences now reported. While either gentamicin or polymyxin complemented the gram-negative spectrum of nalidixic acid, the inhibitory effect of gentamicin on GBS strains clearly contradicted its use. Variability among different lots of gentamicin in terms of inhibiting GBS at those concentrations needed to eliminate unwanted species further compromised the potential of this agent as an inhibitor. The advantages of polymyxin, noted on the basis of in vitro experiments, were confirmed in the direct comparison of those broths designated NAG and NPC. This comparison was done without added sheep blood, which is not required for growth of GBS in Todd-Hewitt broth. The addition of blood adds to time and expense of preparation and precludes simple visual or spectrophotometric monitoring of bacterial growth.

NPC agar clearly resembles CNA agar, but incorporates crystal violet to inhibit staphylococci. We feel this is of particular importance in epidemiological studies of infants, among whom staphylococcal colonization may rapidly occur. As anticipated, NPC broth was clearly more sensitive in identifying infants found to harbor

GBS shortly after birth. Other field comparisons have found higher recovery rates with selective broths than with either plain sheep blood agar (11) or other selective solid media (2, 8). The findings of Ross (13) offer a partial explanation for such methodological differences. Swabs with known numbers of group A streptococci inoculated directly onto solid media were found to release quantitatively only 5 to 10% of the organisms. Moistening and squeezing swabs increased streptococcal recovery to about 50%. These findings may be of relevance when considering quantitative aspects of GBS colonization.

Continuing experience with methods developed in this investigation reveals NPC broth to be a reliable medium for the primary isolation of GBS from various maternal or newborn culture sites. In our hands it is superior to a broth medium containing concentrations of nalidixic acid and gentamicin identical to that incorporated into SBM. NPC blood agar is a useful selective medium for purification of subcultures and quantitation of GBS.

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