Selective and Enhanced Recovery of Group A and B Streptococci from Throat Cultures with Sheep Blood Agar Containing Sulfamethoxazole and Trimethoprim

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Sheep blood agar containing 23.75 μg of sulfamethoxazole and 1.25 μg of trimethoprim (SXT-BA) per ml was compared with conventional sheep blood agar (SBA) for isolating group A and B streptococci from throat cultures. This selective medium allowed much better recovery of group A and B streptococci and suppressed the growth of the normal flora, including "viridans" streptococci. In an initial study of 700 throat cultures, SXT-BA recovered 42% more group A and 49% more group B streptococci than did SBA. When SXT-BA was introduced into the routine microbiology laboratory and used by a number of medical technologists, SXT-BA recovered 28% more group A and 37% more group B streptococci than did SBA. In addition, the selective medium inhibited 83% of the non-group A and B streptococci that were recovered by SBA.

The search for a more sensitive medium for recovery of group A beta-hemolytic streptococci has continued (4, 6, 7, 12-14, 16). In the United States, sheep blood agar (SBA) is recommended and used by most clinical laboratories for primary isolation of these microorganisms. The need for a more sensitive medium for recovery of group A streptococci is essential because clinical assessment and treatment of a sore throat remains so uncertain. Recent studies have shown that selective media incorporating various inhibitors will insure better recovery of these organisms.

Recovery of group A streptococci from agars and broth media has improved through the incorporation of agents such as sodium azide, crystal violet, and antibiotics. These agents have very effectively eliminated the growth of "diphtheroids," neisseria, staphylococci, and gram-negative rods, but they generally have not inhibited the growth of the "viridans" streptococci. Nakamizo and Sato in 1972 (13) succeeded in inhibiting the "viridans" streptococci and other members of the normal flora with an ingenious mixture of chemical agents. They reported that direct plating on SBA missed 5% of the positive cultures that were recovered by direct plating on their selective agar. Vincent et al. (16) incorporated neomycin and nalidixic acid into SBA and showed that 3% of 66 beta-hemolytic group A streptococci were missed by SBA. With a commercially available medium, Freeburg and Buckingham (7) found that neomycin, nalidixic acid, and amphotericin B improved recovery of group A streptococci by inhibiting certain members of the normal flora

that occasionally overgrew and obscured the presence of beta-hemolytic streptococcal colonies. Murray et al. (12), using gentamicin blood agar, detected fewer group A but more non-group A strains on their selective medium than on SBA. These studies have clearly shown that direct plating on selective agar media will improve recovery of group A streptococci by no more than 5% over the number of isolates detected by direct plating on SBA alone. Use of multiple swabs may also help detect more group A streptococci (8 to 12%) than will direct plating on SBA (10).

A recent review pointed out that several investigators, utilizing a variety of broth enrichment methods, demonstrated that 15 to 18% of group A streptococci will not be recovered by direct plating on SBA (6). Direct plating on selective agar media will miss approximately 10% of the beta-hemolytic streptococci that the selective broth methods will detect. Thus, the bacteriologist should do everything possible to maximize isolation of group A streptococci through proper use of selective agars and broths.

This study was done to evaluate a selective medium, SBA incorporating sulfamethoxazole and trimethoprim (SXT-BA), for the isolation of group A and B streptococci. Preliminary studies in our laboratory demonstrated the usefulness of trimethoprim and sulfamethoxazole to selectively reduce the normal flora while allowing growth of group A and B streptococci.

MATERIALS AND METHODS

Conventional SBA was prepared by adding 5 to

7% defibrinated sheep blood to Trypticase soy agar (Difco). SXT-BA was made by the addition of 1.0 ml of a 25-mg/ml stock antibiotic solution to 1.0 liter of conventional SBA. The stock antibiotic solution was prepared by combining equal parts of solution A with solution B. Preparation of solution A involved dissolving 0.125 g of trimethoprim (Burroughs Wellcome Co.) in a few milliliters of 0.1 N HCl and then bringing the volume up to 50 ml with distilled water. Solution B was prepared by dissolving 2.375 g of sulfamethoxazole (Burroughs Wellcome Co.) in a few milliliters of 1.0 N NaOH and then bringing the volume up to 50 ml with distilled water. The stock antibiotic solution was stored in 6.0-ml portions at -30°C and was not sterilized prior to use. The final combined concentration of trimethoprim and sulfamethoxazole in SXT-BA was 25 $\mu\text{g/ml}$.

Each batch of SXT-BA was quality assured by testing for growth of the control organisms, *Streptococcus pneumoniae* (WRAMC 06, Walter Reed Army Medical Center), *S. pyogenes* (ATCC 19615; BACTROL, Difco), and *Proteus vulgaris* (ATCC 13315; BACTROL, Difco). *Streptococcus pyogenes* grew as beta-hemolytic colonies on this medium and served as the positive control for both growth and beta-hemolysis. SXT-BA was prepared weekly and stored at 5 to 10°C in plastic bags until use.

Seven hundred throat cultures were obtained from both in-patients and out-patients at Walter Reed Army Medical Center, Washington, D.C. These cultures were accessioned as they were received from all the clinics and wards that submitted specimens during this study. Approximately 40% of the cultures were obtained from pediatric patients. After these initial 700 cultures were tested to determine the usefulness of SXT-BA to improve recovery of group A and B streptococci, an additional 5,500 throat cultures were inoculated to both media in a similar manner over a period of 5 months. This portion of the study was initiated to determine the ability of the medium to be used effectively by bench technologists in a microbiology laboratory.

Throat cultures were taken with a sterile rayon-tipped swab and transported in a modified Stuart transport medium (Culturette, Marion Health and Safety, Inc.). The specimens were transported at room temperature and processed within 16 h from the collection time.

Throat culture swabs were inoculated onto SBA and then SXT-BA. Inoculated areas were streaked for isolation, and the agar was stabbed several times for observation of subsurface hemolysis. The inoculated plates were incubated for 18 to 24 h at 35°C in an atmosphere of 5 to 9% carbon dioxide in air with a relative humidity of 40 to 60%. After incubation, all plates were examined for the presence of beta-hemolytic colonies.

All beta-hemolytic streptococci were serologically grouped by counterimmunoelectrophoresis and presumptively grouped by the bacitracin-SXT disk susceptibility test described elsewhere (8). To determine whether the antibiotics in the selective medium would alter the outcome of the bacitracin-SXT susceptibility results, 64 group A and B strains were tested for susceptibility to bacitracin and SXT disks on both media. Zones of inhibition were measured to

the nearest millimeter after 18 to 24 h of incubation at 35°C .

RESULTS

Seven hundred throat cultures were plated on both SXT-BA and SBA during an initial 8-week period of study. A total of 165 (24%) cultures contained beta-hemolytic streptococci when both media were used for isolation. Of these, 55 were serotyped as group A, 33 as group B, and 77 as groups other than A or B.

Sixty-five percent of the group A streptococci were isolated from children, but only 33% of the group B and 20% of the other groups were recovered from pediatric patients.

The antibiotics in SXT-BA effectively reduced the normal flora, both qualitatively and quantitatively, when compared with SBA. Sixty-three (9%) of the cultures had luxurious growth of normal flora on SBA but did not grow on SXT-BA. In all other cultures, the density of growth of viridans streptococci, staphylococci, neisseria, diphtheroids, and gram-negative rods on SXT-BA was reduced by 25 to 75%. Growth of yeast was not affected by the selective medium, and, on four occasions, these microorganisms were isolated in pure culture on SXT-BA, whereas they were not observed on SBA. Colonies of group A and B streptococci were smaller on SXT-BA than on SBA, and hemolytic zones around the colonies were proportionately smaller (Fig. 1).

SBA missed 44% (25/55) of the group A streptococci that were recovered by both media (Table 1). Of these twenty-four strains cultured only on SXT-BA, 54% grew as greater than 100 colonies on each plate (Fig. 2). Of the remaining cultures, 33% grew as less than 10 colonies and 13% grew between 10 and 100 colonies. SXT-BA missed only 2% (1/55) of the group A streptococci recovered by both media. When this group A strain was initially isolated, only four beta-hemolytic colonies grew on the primary plate. Of the 30 group A strains that were isolated on both media, 97% had more than 100 colonies on the selective plate, but only 70% of the group A strains isolated on SBA grew as luxuriantly. Overall, SXT-BA recovered 42% more group A streptococci than did SBA. These findings were significant by chi square ($P < 0.025$).

SBA missed 61% (20/33) of the group B streptococci that were recovered with both media used for isolation (Table 1). Twenty percent of the strains that were recovered only on SXT-BA had more than 100 colonies on each plate. Of the remaining cultures, 30% grew with less than 10 colonies and 50% grew between 10 and 100 colonies. SXT-BA missed 12% (4/33) of the

group B streptococci recovered by both media. In general, the colony counts on SXT-BA were always greater than on SBA. Overall, SXT-BA recovered 49% more group B streptococci than did SBA. These findings also were significant by chi square ($P < 0.05$).

The antibiotics present in SXT-BA suppressed the growth of 90% (69/77) of the non-group A and B streptococci, except for eight Lancefield group C strains.

After the first 700 throat cultures were studied, SXT-BA was used in our laboratory along with SBA as a routine medium and was clinically compared with SBA for screening throats for beta-hemolytic streptococci. In this study, 508 group A, 210 group B, and 269 other groupable beta-hemolytic streptococci were isolated from 5,500 throat cultures. Approximately one-half of the streptococcal cultures were presumptively grouped by the bacitracin-SXT test, and the remaining cultures were serologically grouped. In this study, SXT-BA recovered 28% more group A and 37% more group B beta-hemolytic streptococci than did SBA. Only 17% of the 269 non-group A and B streptococci grew on the selective medium. Seven group A strains grew on SBA but not SXT-BA. These strains were all resistant to SXT and grew on SXT-BA upon subculture. The number of observable colonies on SXT-BA was generally greater than the number of colonies on SBA (Table 2).

DISCUSSION

This study clearly demonstrates that SXT-BA improves the isolation of group A and B streptococci significantly. This medium inhibits the growth of most normal flora, including the viridans streptococci, to such an extent that bacterial competition is greatly modified and recovery of *S. pyogenes* is increased.

Compared with SXT-BA, SBA missed a significant number of group A ($P < 0.025$) and group B ($P < 0.05$) streptococci. Although it is well documented in the literature that selective media will improve the recovery of group A and B streptococci (1, 2, 4, 6, 12, 13, 16), selective agars are less efficient than selective broths for this purpose. However, in this study, SXT-BA attained recovery rates comparable to the selective broth enrichment media.

Beta-hemolytic colonies growing on SXT-BA and their zones of hemolysis were always smaller than when grown upon SBA (Fig. 1).

TABLE 1. Number of beta-hemolytic streptococci recovered from SBA and SXT-BA media

Group	SBA only	SXT-BA only	Both media	Total
A	1	24	30	55
B	4	20	9	33
Other ^a	69	1	7	77

^a Other, Beta-hemolytic streptococci other than groups A and B.

TABLE 2. Comparison of the number of beta-hemolytic group A streptococcal colonies recovered on SBA and SXT-BA

Medium	Total cultures	No. of colonies			
		0	1-10	11-100	100+
SBA	508	149 29% ^a	47 9%	148 29%	164 32%
SXT-BA	508	7 1%	56 11%	216 43%	229 45%

^a Percentage of total cultures.

Although hemolytic colonies could usually be subcultured directly on SBA for performance of the bacitracin-SXT test, occasionally subcultures to SXT-BA were necessary to obtain a valid bacitracin-SXT test. Apparently, the normal flora was inadvertently picked and overgrew the beta-hemolytic streptococci on SBA. This observation again demonstrates the inhibitory nature of normal flora upon *S. pyogenes*.

The ecological relationships of "nonpathogenic" bacteria to group A streptococci are both interesting and complex. Sanders in 1969 (15) tested 111 strains of *Staphylococcus epidermidis*, 109 diphtheroids, 100 nonpathogenic neisseria, and 124 viridans streptococci for inhibition of *S. pyogenes*. Only viridans streptococci (47%) were capable of bacterial interference.

In 1975, Bill and Washington (3) described inhibition of group A streptococci by a common throat inhabitant, *S. salivarius*. Jagg et al. (9) recently reviewed the literature concerning bacteriocins of gram-positive bacteria and listed the viridans streptococci as very potent inhibitors of beta-hemolytic streptococci. Included among the causes for inhibition were viridins (bacteriocins of alpha-hemolytic streptococci), toxic metabolic products (e.g., hydrogen peroxide), substrate depletion, creation of unphysiological environments, and prevention

FIG. 1. Group A beta-hemolytic streptococci growing luxuriantly on both isolation media. (A) SBA; (B) SXT-BA.

FIG. 2. Normal throat flora growing on SBA (A). Same throat culture showing group A beta-hemolytic streptococci growing only on SXT-BA (B).

of beta-hemolysis expression by peroxides (5, 9, 15, 16).

Many of the bacterial interactions that occur *in vivo* also occur on plated media. Crowe and Sanders (5) reported that colonization of children with group A streptococci varied according to the degree of bacterial interference that the normal oropharyngeal flora of each child was capable of exerting. Under the selective pressure of colonization inhibitory organisms increased. He suggested that this selective process may be the mechanism whereby adults ultimately become more resistant than children to streptococcal infection. From these studies it would be assumed that the expression of growth and beta-hemolysis of group A streptococci growing among nonpathogenic microorganisms would be influenced by the percentage of the total number of bacteria capable of exerting bacterial interference. To maximize the recovery of *S. pyogenes* from throat cultures, it would be necessary to eliminate as much of the inhibitory flora as possible. Since the viridans streptococci have most often been associated with the inhibition of group A streptococci, selective media should be designed to suppress their growth. This has not been the case, however, primarily because those agents that are capable of inhibiting the viridans streptococci usually inhibit *S. pyogenes*. In this study, SXT antibiotics were shown to be exceptions.

Kaplan (10) in 1972 reported studies in which the degree of positivity of throat cultures was not entirely reliable for identifying the acutely infected individual and differentiating an acutely infected patient from an asymptomatic carrier. Quite possibly the reason that the degree of positivity of cultures has not correlated with the degree of significant clinical illness has been due to the failure of selective broths and agars to adequately grow beta-hemolytic colonies as does SXT-BA.

Beta-hemolytic colonies on SXT-BA were always observed in the primary inoculation areas due to the suppression of so much normal flora (Fig. 2). In comparison, beta-hemolytic colonies were only occasionally isolated in the primary inoculation areas of SBA. These SBA findings have also been observed in our laboratory with selective agar media other than SXT-BA. It becomes quite evident when SXT-BA plates are compared with SBA plates that the observable number of colonies on SBA does not reflect the actual numbers of beta-hemolytic streptococci that were present on the original swab (Fig. 2). The selective plates more reliably present the degree of throat involvement than do SBA plates by reducing the normal flora that tends to overgrow, mask, and/or inhibit the beta-

hemolytic streptococci.

The one group A strain that grew only on SBA presented four observable beta-hemolytic colonies on the primary plate. What was surprising about this isolate was that there were absolutely no beta-hemolytic colonies on SXT-BA. In other cultures, colonies on the selective medium were always equal to and in most instances greater than the number of colonies on SBA. Inoculation of the SBA plates before the selective plates could possibly explain the recovery of this strain only on SBA, especially in view of the finding of others (10) that use of single swabs is far less than optimum for maximum recovery of group A streptococci.

An additional advantage of SXT-BA over other selective media is the inhibition of beta-hemolytic streptococci other than groups A and B. Although non-group A streptococci have been isolated from patients with pharyngitis, only rarely have they been associated with rheumatic fever or acute glomerulonephritis (11). By not recovering these microorganisms, SXT-BA accomplishes two objectives. First, by reducing the number of bacitracin tests to be performed, SXT-BA saves both technical time and laboratory media. Second, by inhibiting many of the bacitracin-susceptible non-group A streptococci, the treatment of non-group A pharyngitis is reduced, and, therefore, the possibility of allergic reactions to penicillin is somewhat lessened. That group B streptococci are isolated on SXT-BA along with group A strains is not considered a disadvantage, as preliminary studies in our laboratory indicate that SXT-BA may be used as a general purpose medium for the isolation of group A and B streptococci from a variety of clinical specimens.

The final study of 5,500 throat cultures demonstrated that SXT-BA can be used by technologists possessing varying bacteriological skills. During this portion of the study it was determined that beta-hemolytic colonies on SXT-BA primary plates should always be subcultured to fresh SXT-BA plates for performance of the bacitracin-SXT test. The normal flora so often overgrew the beta-hemolytic colonies when subcultured to SBA that the bacitracin-SXT test had to be repeated on numerous occasions. It has shown during this study that SXT disk results on the antibiotic-impregnated agar were not altered by increased concentrations of trimethoprim and sulfamethoxazole when compared with results obtained on SBA. Inhibition zones around bacitracin disks on SXT-BA were, on the average, 4 mm greater in diameter than those zones on SBA.

Although the recovery of group A and B

streptococci during the trial study varied somewhat from the initial study of 700 throat cultures, SXT-BA still maintained its superiority over SBA and was comparable to selective broth techniques for the recovery of these streptococci. It was not only shown that SXT-BA improved the recovery of group A and B streptococci, but the selective medium also increased the quantity of colonies observed over those observed on SBA. The seven strains of group A streptococci isolated only on SBA presented less than 10 observable colonies on the non-selective medium. As mentioned previously, these cultures could be accounted for by the inoculation method as well as by the use of single rather than duplicate swabs. All seven of these strains were resistant to SXT by the bacitracin-SXT test and were able to grow on SXT-BA upon subculture.

From the results of this study, SXT-BA is superior to direct plating on SBA for isolating group A and B streptococci from throat cultures. The medium inhibits, to varying extents, all species of normal oropharyngeal flora as well as most beta-hemolytic streptococci not of groups A and B. Complete reliance on SBA as the sole means of recovering group A beta-hemolytic streptococci is far less than optimum, and the use of selective media, such as SXT-BA, to maximize recovery of these pathogens should be utilized in the clinical laboratory.

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