Laboratory Studies with a Selective Enterococcus Medium

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Lancefield group D streptococci are involved with appreciable frequency in a variety of infectious processes. The presumptive recognition of these bacteria on initial culturing of clinical specimens is an objective not attained readily by selective media available in the clinical laboratory. Selective Enterococcus agar was evaluated with emphasis on its ability to sequester enterococci from specimens with many microbial components. In addition, the sensitivity of this new agar was compared with Trypticase Soy agar containing sheep blood and Mitis Salivarius agar. All enterococci isolated from clinical material were classified in accordance with accepted biochemical and immunochromical criteria. The enterococci grew on the new medium as distinctive colonies surrounded by a black zone. Only Listeria monocytogenes presented similar colonial morphology after 48 hr. Most other bacteria did not grow at all or appeared markedly different. The sensitivity of the new agar was of the same order of magnitude as on blood or Mitis-Salivarius agars, but its selectivity was superior.

The group D streptococci are involved with appreciable frequency in a variety of infectious disease processes among which wound, urinary tract, and nosocomially acquired diseases are especially prominent. These streptococci, which include the enterococci as well as Streptococcus faecium var. casseliflavus, S. bovis, and S. equinus, have established themselves as not uncommon components of the microbiota of body surfaces and membranes regarded as unlikely domiciles for these bacteria in the not-so-distant past. Several confirmatory media have been available commercially. However, workers in the clinical microbiology laboratory have not been provided a selective medium which sequesters enterococci from the multifarious microbial components present in some clinical specimens or which leads to the recognition of these streptococci in a battery of primary isolation media, an objective in keeping with the intent of clinical microbiologists to report as rapidly as possible to the clinician the presence of microorganisms of potential consequence in the diagnosis and treatment of their patients. Recently, we have had the opportunity to study a newly formulated medium which combines selectivity with sensitivity for the enterococci. This report is a summary of our findings.

MATERIALS AND METHODS

Media. The medium under study, Pfizer Selective Enterococcus medium (PSE), is constituted as follows (grams/liter): Pfizer peptone C, 17.0; Pfizer peptone D, 3.0; Pfizer yeast extract, 5.0; Pfizer bacteriological bile, 10.0; NaCl, 5.0; sodium citrate, 1.0; esculin, 1.0; ferric ammonium citrate, 0.5; NaN₃, 0.25. For use as a solid substrate, 15.0 g of Pfizer agar per liter is present; the broth is identical to the base.

The following other media were used in this study: Mitis Salivarius agar (Difco; MS); Trypticase Soy agar (BBL; TS) as the base to which washed sheep erythrocytes were added to a concentration of 5% (BP); Litmus milk (BBL); Brain Heart Infusion broth (BBL; BHI) with and without 6.5% NaCl; gelatin (BBL); and Cysteine Trypticase Agar base (BBL; CTA) with Taxos (BBL) carbohydrate discs, especially sorbitol and mannitol, but also trehalose, lactose, sucrose, inulin, and raffinose. Streptococcal grouping sera with controls (BBL) were used to establish the presence of group D polysaccharide.

Microorganisms. Besides the 250 group D streptococci isolated from clinical material, 8 strains were supplied through the courtesy of M. D. Moody, National Communicable Disease Center, Atlanta, Ga. These represented the following species: S. faecalis, S. faecalis var. liquefaciens, S. faecalis var. zymogenes, S. faecium, S. faecium var. casseliflavus (1), and an organism with the biochemical and physiological characteristics of S. equinus. The following microorganisms were tested on the three primary isolation media (MS, BP, PSE): Shigella sp., 2;
Salmonella sp., 4; Providencia sp., 2; Pseudomonas aeruginosa, 3; P. stutzeri, 1; P. putida, 1; P. maltophilia, 1; P. alcaligenes, 1; P. pseudomallei, 1; Serratia marcescens, 1; Flavobacterium sp., 1; Klebsiella sp., 1; Escherichia coli, 2; Proteus morganii, 2; P. mirabilis, 3; Acridobacter sp., 1; Aeromonas hydrophilia, 1; Alcaligenes faecalis, 1; A. odorans, 1; Staphylococcus aureus, 3; S. epidermidis, 3; Sarcina lutea, 1; Streptococcus pyogenes, 3; S. pneumoniae, 3; viridans streptococci (both S. salivarius and S. mitis groups), 20; Neisseria sp., 3; Corynebacterium sp., 3; Listeria monocytogenes, 5; Candida albicans, 1.

**Procedures.** The investigation consisted of three phases employing the following procedures. (i) To establish the sensitivity of PSE, 18-hr BHI broth cultures of enterococci and other group D streptococci were diluted one million-fold to contain 50 to 100 colony-forming units per 0.1 ml. This volume was delivered to PSE, BP, and MS plates, streaked out with a bacteriological loop, and incubated at 35 C for 18 hr; then the colonies were counted. These experiments, like all others in this study, were performed in duplicate.

(ii) The selectivity of the medium was tested by inoculating PSE, MS, and BP with 0.1 ml of 1:10,000 dilution of an 18-hr BHI broth culture. All of the various microorganisms listed earlier were used in this manner. After overnight incubation at 35 C, the media were inspected for growth, colony morphology, action on indicators, etc.

(iii) The performance of PSE agar was checked in the clinical laboratory situation. The medium was included in the battery of primary isolation media which consist of BP and MS; already part of the study, as well as Staphylococcus 110 agar (BBL), Eosin Methylene Blue agar (BBL), and various broths. These media constitute the core media for the screening of all specimens received from patients. Additional media are added in many instances, but the aforementioned are almost always represented in the microbiological analysis. Colonies on PSE or MS agar displaying reactions or morphology consistent with presumptive identification as enterococci were tested further by a series of tests suggested by M. D. Moody (personal communication). These tests included restudying the organism from the original PSE or MS agar on BP, MS, and PSE, action on Litmus milk, growth in 6.5% NaCl, growth in BHI broth after heating at 60 C for 30 min, fermentation of mannitol and sorbitol, and grouping with antisera by the Lancefield method. Only rarely were additional tests required to identify the various enterococcal species.

The survey of organisms isolated from clinical sources also provided the opportunity to examine the capability of PSE broth. Colonies suspected of belonging to group D streptococci on the basis of their morphology on MS agar or BP were suspended in 2.0 ml of the broth. When heavy suspensions were made, esculin hydrolysis ensued within 2 hr. A light inoculum required additional time to register the reaction.

**RESULTS**

All enterococci and related streptococci grew on PSE agar within 18 to 24 hr as round, entire, convex, translucent to whitish colonies, 1 to 2 mm in diameter, surrounded by dark brown- to black-appearing iron salts of hydrolyzed esculin. No other microorganism tested approached this appearance. Of the 86 other protista representing 29 microbial species which were tested on this medium, only Staphylococcus aureus and S. epidermidis grew invariably as very small (less than 1 mm in diameter) white-gray colonies which exerted no action on the esculin. In the area of heavy inoculum, Corynebacterium and viridans streptococci appeared as one or two tiny colonies; one to two colonies of Proteus mirabilis also grew out in the area of densest inoculum application. Candida albicans and Aeromonas hydrophilia appeared as extremely small white dots on the agar. The only bacterium which consistently blackened the medium around its growth other than the enterococci was Listeria monocytogenes. After 18 to 24 hr, the colonies of this organism were discernible only by the reddish to black-brown zones of hydrolysis surrounding pinpoint colonies. After 48 hr of incubation, the colonies of L. monocytogenes achieved diameters of about 1 mm; they were round, entire, and convex and contained considerable whitish-gray pigment. One can also not escape the impression that even after 48 hr the listeriae do not attain the same marked degree of esculin splitting displayed by the enterococci in the short incubation period.

The ability of PSE agar to permit uninhibited growth of group D streptococci was demonstrated by the recovery of approximately the same number of bacterial colonies on this agar as on BP and MS. The 40 different representatives of various group D streptococcal species never showed a significant difference in number whether growing on selective media or on the blood plate.

Enterococci and other group D streptococci recognized by typical colonial morphology and black zones of hydrolysis were recovered from 600 clinical specimens of various anatomical origins 250 times with PSE agar. Colonies, readily identified as enterococci by virtue of their appearance on MS agar, were encountered 219 times from the same specimens. However, on 23 occasions, overgrowth of enterococci occurred with Proteus sp. or Pseudomonas. Recognition of enterococci on BP inoculated simultaneously with all of the other media was difficult with mixed cultures especially when other streptococci were present; there were several instances when other components of the microbiota in the lesion being analyzed completely obscured the presence of enterococci on the blood-agar plate. Only when original broths were subcultured on PSE or MS did the enterococcal constituents become evident. To assess the ability of these media to recover enterococci from specimens where their presence
might have some significance, the number of stools screened was limited to 25 of the 600 tested. PSE proves especially helpful in detecting enterococci in postoperative wounds and blood cultures as well as in exudates from various sites. Neither MS, BP, or any of the other media yielded enterococci on primary culture or on subculture which were not detected on PSE. The group D streptococci were speciated presumptively as shown in Fig. 1, based on the scheme of classification devised by Moody (personal communication). Although this cursory classification scheme will not satisfy the taxonomic purists nor detect atypically reacting streptococci, it can serve as a guide in the clinical laboratory. Confirmed in addition by precipitin reaction with group D antisera, the streptococci isolated from patients consisted of 98 representatives of S. faecalis, 76 of S. faecalis var. liquefaciens, 37 of S. faecalis var. zymogenes, 7 of S. durans, 28 of S. faecium, and 6 of S. bovis. No representative of S. equinus was present among the streptococci isolated from clinical material. Twenty-three of the S. faecium displayed characteristics suggesting their designation as S. faecium var. casseliflavus. Since this organism has not been reported from clinical sources and would require confirmatory tests beyond the scope and need of the clinical microbiology laboratory, these bacteria were considered as atypically reacting S. faecium.

**DISCUSSION**

It hardly seems necessary to underline that of all gram-positive amphibiota bacteria the group D streptococci have gained a position which warrants attention, especially for their role in the institutional environment. Although the staphylococci have not exactly surrendered their significant contribution to complicating a variety of diseases and procedures, their singular lead as causative agents in nosocomial disease has been challenged seriously during the past few years by various gram-negative rods, with the enterococci occupying a minor but definite place in the microbial populations of hospital-acquired infectious complications. The rapid recognition of their presence in clinical specimens has depended, to date, on careful observation of colonial morphology on blood-enriched media such as Mitis Salivarius agar. Usually, even their presumptive identification requires additional confirmatory tests which exploit specific physiological and biochemical attributes of these bacteria. PSE broth can fulfill this requirement in the desirable short time of 2 hr. PSE agar enables the clinical microbiologist to include a specific selective medium into the primary screening of clinical specimens and permits the identification of the enterococcal group with the same speed achieved for staphylococci, the family Enterobacteriaceae, viridans streptococci, etc., by selective enrichment modalities. The combination of esculin and a rather low concentration of bile in the presence of azide permits the selection of enterococci and other group D streptococci by typical colonial morphology and visible evidence of esculin hydrolysis which is not achieved by any other bacterium tested or by components in the microbiota of clinical specimens. Most gram-negative rods are suppressed completely; the few Proteus species grow only when present in very large numbers and then as very small gray colonies. The staphylococci and occasional viridans streptococci are easily distinguished from the enterococci by pigmentation, colonial size, and the absence of the phenolic iron complex. The ability of PSE to detect Listeria presumptively in 24 hr is an advantage rather than a discredit for this medium. The size of the colony and the intensity of the background indicator of esculin hydrolysis do not allow confusion between Listeria and the enterococci. Detection of Listeria in lesions where they were not suspected should prove helpful to the clinician as well as the clinical microbiologist. The identification of a microorganism as an enterococcus is often considered sufficient for the purposes of clinical microbiology. The ease of recognition of group D streptococci provided by PSE agar ought to persuade practitioners of clinical microbiology to speculate enterococci by biochemical and physiological studies as outlined by Shattuck (2) and advocated by Moody (personal communication). Undoubtedly, a better appreciation of enterococci and their role in health and disease is made possible by this specific selective enrichment medium.
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LITERATURE CITED

1. Mundt, J. O., and W. F. Graham. 1968. Streptococcus faecium var. casseliflavus, nov. var. J. Bacteriol. 95:2005–2009.
2. Shattuck, P. M. F. 1964. Enterococci, p. 303–319. In J. C. Ayres, A. A. Kraft, H. E. Snyder, and H. W. Walker (ed.), Chemical and biological hazards in food. Iowa University Press, Ames, Iowa.