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Evaluation of E Test as a Rapid Method for Determining MICs for Nutritionally Variant Streptococci

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E test was evaluated as an alternative rapid and simple method of MIC estimation for nutritionally variant streptococci. E test with various media was compared with conventional broth and plate dilution techniques supplemented with 0.001% (wt/vol) pyridoxal hydrochloride (vitamin B_6). Of the 14 strains tested with E test, isosensitest agar supplemented with 5% defibrinated horse blood and 0.001% pyridoxal HCl, with and without 0.01% cysteine, gave complete agreement within one twofold-dilution titer of the agar reference method and between 93 and 86% agreement within one twofold-dilution titer of the broth reference method. E test MICs with other media were comparable; however, these were considerably more difficult to interpret. Use of Mueller-Hinton and Columbia-based supplemented agar showed hazy growth and double zoning around the endpoint, respectively. The addition of 0.01% (wt/vol) cysteine to media exhibited no significant effect, and incubation in 5% carbon dioxide (CO_2) did not affect MICs.

Symbiotic, thiol dependent, pyridoxal hydrochloride (vitamin B_6) deficient, D-alanine requiring, nutritionally deficient, and satellitizing (5, 9, 14, 16, 21, 25) are characteristic descriptions of streptococci that require nutritional supplements to grow on conventional diagnostic laboratory solid-phase media. These nutritionally variant streptococci (NVS) were thought to be variants of viridans group streptococci but are now recognized as specific entities. Two species have been proposed, *Streptococcus adjacens* and *S. defectivus* (4, 8).

The importance of NVS as a cause of endocarditis was first described in 1961 (9). Further studies especially in the late 1970s and early 1980s identified 5 to 6% of endocarditis patients as infected with NVS (17, 19) and noted that NVS accounted for 10% of all streptococcal endocarditis (27). It is suggested that because of their pleomorphic natures, unusual cell walls, slow growth rates, increased structural abnormalities in the presence of antibiotic (ability to produce L forms) and exopolysaccharide production, conventional antibiotic therapy against them is associated with higher treatment failure and mortality rate than against other streptococci and enterococci (1, 3, 6, 9, 11, 14, 25, 27). Stein and Nelson reviewed 30 cases of NVS infection, describing a 41% failure of therapy (defined as positive blood cultures after 7 days of treatment) and a 17% mortality rate (25). The length of time required to isolate, identify, and determine the penicillin susceptibility of NVS may contribute to the higher treatment failure and mortality rates. E test offers a rapid and efficient method of establishing high levels of penicillin resistance.

Fourteen organisms (12 *S. adjacens* and 2 *S. defectivus* [4]) from endocarditis patient blood cultures ($n = 10$) and mouth swabs of volunteers ($n = 4$) between 1986 and 1993 were obtained from Sydney teaching hospitals. As a control, a known penicillin-susceptible *S. pneumoniae* was included in all testing.

Broth dilution MICs were calculated in duplicate by using Todd-Hewitt broth (12) (Difco Laboratories, Detroit, Mich.) supplemented with millipore-filtered (pore size, 0.22 μm) (Sterile Acrodisc; Gelman Sciences) 0.001% pyridoxal HCl (ICN Biochemical, Aurora, Ohio). Each sterilized tube contained 1 ml of supplemented broth with serially diluted penicillin G (0.008 to 256 μg/ml) and 0.1 ml of NVS bacterial suspension (10^6 CFU/ml of Todd-Hewitt broth from a fresh overnight culture). Tubes were incubated for 18 h at 35.5°C (26), and MICs were estimated at this time.

Agar dilution MICs were calculated in duplicate by using Mueller-Hinton agar (BBL Becton Dickinson) supplemented with 5% lysed horse blood, 0.001% pyridoxal HCl, and penicillin G (concentrations ranging serially from 0.008 to 128 μg/ml). Inocula containing 10^8 CFU/ml were prepared with a Mast inoculating apparatus according to the criteria of Steers et al. (23). Approximately 10^5 CFU/ml was applied to each plate. Two sets of plates were inoculated; one set was incubated at 35.5°C for 18 h in 5% carbon dioxide, and the other set was incubated at 35.5°C in air.

E test MICs were calculated in duplicate with log-phase inocula of 10^8 CFU/ml in tubes containing 5 ml of Mueller-Hinton broth (E test insert instructions). Suspensions were poured onto six different media (Columbia agar [BBL] supplemented with 5% defibrinated horse blood and 0.001% pyridoxal HCl [BAP]; isosensitest agar [Oxoid] supplemented with 5% defibrinated horse blood and 0.001% pyridoxal HCl [BIP]; isosensitest agar supplemented with 5% defibrinated horse blood, 0.001% pyridoxal HCl, and 0.01% cysteine [BIPC]; Mueller-Hinton agar [BBL] supplemented with 5% defibrinated horse blood and 0.001% pyridoxal HCl [BMHP]; Mueller-Hinton agar supplemented with 5% defibrinated horse blood, 0.001% pyridoxal HCl, and 0.01% cysteine [BMHPC]; and Mueller-Hinton agar supplemented with 5% lysed horse blood and 0.001% pyridoxal HCl [LBMHP]), the excess was decanted, and plates were allowed to air dry before the graduated penicillin G E test strip was placed on each plate. Plates were incubated in 5% carbon dioxide at 35.5°C for 18 h.

E test results were compared with the MICs obtained by the conventional agar and broth dilution methods. Comparisons of the results are presented in Tables 1 and 2. E test MICs with BAP, BIP, BIPC, BMHP, BMHPC, and LBMPH showed 86, 100, 100, 93, 93, and 85% agreement, respectively, within one

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TABLE 1. Comparison of NVS E test (penicillin G) MICs determined after 18 h of incubation

Medium	No. of strains	E test MICs within indicated concentration (log_2) of agar dilution MICs (%)							% Agreement ± 1 dilution	% Interpretive errors[a]		
		>−2	−2	−1	0	+1	+2	>+2		Very major	Major	Minor
BAP	14	0	0	0	4	8	2	0	86	0	0	21
BIP	14	0	0	0	7	7	0	0	100	0	0	21
BIPC	14	0	0	0	7	7	0	0	100	0	0	21
BMHP	14	0	0	0	4	9	1	0	93	0	0	21
BMHPC	14	0	0	0	3	10	1	0	93	0	0	28
LBMHP	13	0	0	0	5	6	2	0	85	0	0	28
THB[b]	14	0	0	2	9	2	0	1	93	0	0	7

[a] Very major errors, (number of false-susceptible results/number of resistant isolates) × 100. Major errors, (number of false-resistant results/number of susceptible isolates) × 100. Minor errors, (number of susceptible to intermediate, intermediate to susceptible, resistant to intermediate, or intermediate to resistant results/total number of isolates tested) × 100.

[b] Todd-Hewitt broth supplemented with 0.001% pyridoxal HCl as a control.

twofold-dilution titer of the agar reference method and 79, 86, 93, 86, 86, and 77% agreement, respectively, within one twofold-dilution titer of the broth reference method. The two reference methods showed 93% agreement within one twofold-dilution titer of each other (Tables 1 and 2). Growth in 5% carbon dioxide incubated at 35.5°C for 18 h was superior to growth in air, which in some cases required 48 h. Incubation in carbon dioxide or air made no difference to the accuracy of MIC estimation.

Various media have been reported to support the growth of NVS, although not all are suitable for susceptibility testing (19). In this study, consideration was given to established susceptibility testing media. Supplemented isosensitest base agars gave very good growth in 5% CO_2 incubated at 35.5°C for 18 h, allowing clear sharp endpoints and easy MIC estimations for all isolates. There was moderate growth on supplemented Mueller-Hinton base agars incubated in 5% CO_2 at 35.5°C for 18 h. However, hazy growth around the E test endpoint resulted in difficulties with some MIC estimations. Supplemented Columbia base agar exhibited good growth in 5% CO_2 incubated at 35.5°C for 18 h; even so, MIC estimation was made very difficult because of double zoning around the endpoint.

Vitamin B_6 is not the only limiting nutrient required to sustain NVS growth. Gephart and Washington showed that media containing pyridoxal HCl and no glucose poorly sustained the growth of NVS (10). Holloway and Dankert also noted slower NVS growth in Mueller-Hinton broth than in Todd-Hewitt broth, which contains 2 g of glucose per liter (12). This requirement for glucose may explain the differences in growth observed among the different media. Isosensitest base agar contains 2 g of glucose per liter, whereas Columbia and Mueller-Hinton base agars do not ordinarily contain glucose. Differing MICs with various media may be related to the unusual growth characteristics of NVS, such as the ability to produce L forms. Alternatively, cysteine has been reported to interfere with penicillin activity (12, 15). However, cysteine interference appeared to be negligible at the low concentrations tested in this study.

Even though most MICs were within one twofold dilution, generally the penicillin E test MICs tended to be the same as or one twofold-dilution higher than those determined by the reference methods. There were no very major or major interpretive category errors with penicillin G E test results on the basis of National Committee for Clinical Laboratory Standards streptococcus (not enterococcus) MIC interpretive categories (13, 18). Minor errors varied between 14 and 28% with various media (Tables 1 and 2). The majority of these errors occurred with strains for which the MIC was close to the breakpoint for separating susceptible and intermediate strains (0.12 μg/ml). All of these isolates were classified as penicillin sensitive by the reference methods but as intermediate by E test. The minor errors detected in this study would not have altered treatment decisions.

The appropriate choice of therapy for treating NVS infection, especially endocarditis, has become increasingly complicated with the discovery of penicillin-resistant and -tolerant strains (2, 14). Bosley and Facklam found that 35% of NVS (n = 43) strains were sensitive to penicillin (MIC, ≤0.12 μg/ml), 56% were intermediate (MIC, 0.25 to 2.0 μg/ml), and 9% (4/43) were resistant to penicillin (MIC, ≥4.0 μg/ml) (2, 18). These findings are comparable to the E test MICs in this study; 36% were sensitive, 57% were intermediate, and 7% were resistant to penicillin. The last, an endocarditis isolate,

TABLE 2. Comparison of NVS E test (penicillin G) MICs determined after 18 h of incubation

Medium	No. of strains	E test MICs within indicated concentration (log_2) of broth dilution MICs (%)							% Agreement ± 1 dilution	% Interpretive errors[a]		
		>−2	−2	−1	0	+1	+2	>+2		Very major	Major	Minor
BAP	14	0	0	1	6	4	2	1	79	0	0	14
BIP	14	0	0	1	8	3	2	0	86	0	0	14
BIPC	14	0	0	1	8	4	1	0	93	0	0	14
BMHP	14	0	0	1	7	4	1	1	86	0	0	14
BMHPC	14	0	0	1	6	5	1	1	86	0	0	21
LBMHP	13	0	1	0	7	3	1	1	77	0	0	21
MHA[b]	14	1	0	2	9	2	0	0	93	0	0	7

[a] See Table 1, footnote *a*.

[b] Mueller-Hinton agar supplemented with 5% defibrinated horse blood and 0.001% pyridoxal HCl as a control.

had a MIC of 6 μg/ml. A high level of penicillin resistance in NVS needs to be identified rapidly so that alternate therapy may be considered. Synergistic combination therapy with penicillin and an aminoglycoside is the recommended therapy for both penicillin-sensitive and intermediate NVS strains, but for resistant strains, other combinations, including vancomycin and rifampin, vancomycin and an aminoglycoside (gentamicin, streptomycin, or tobramycin), or vancomycin alone, have also been proposed (1, 3, 4, 6, 7, 11, 12, 20, 22, 24–27).

E test provides a reliable alternative to conventional MIC methods. Its advantages are clearly the ease of use and the savings in labor costs to smaller nonreference diagnostic laboratories; it is also an efficient method for susceptibility testing of all streptococci, including NVS.

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