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E Test versus Agar Dilution for Antimicrobial Susceptibility Testing of Viridans Group Streptococci

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Viridans group streptococci (VGS) are commonly isolated from the blood of hospitalized patients. The E test represents a convenient method for determining the MICs for VGS, but for this purpose it has not been well validated against reference methods. In this study, 180 unselected VGS isolates were identified to a species level, and the MICs of penicillin, cefuroxime, cefotaxime, and vancomycin were determined by both agar dilution and the E test. Available data regarding demographic and laboratory variables for each VGS bacteremic episode were collected, the significance of each VGS isolate was assessed, and the associations between and among laboratory and clinical variables were investigated. Among all VGS isolates, 68.3% (median of three runs) were found to be fully susceptible to penicillin by agar dilution. The E test and agar dilution showed average agreements (within ±1 dilution) of 92.2% for penicillin, 95.7% for cefuroxime 91.3% for cefotaxime, and 86.7% for vancomycin. Agreements over serial E tests and serial agar dilutions were excellent for β-lactam agents (intraclass correlation coefficients, >0.9) but less impressive for vancomycin. Very major error rates for the E test were ≤0.7%, and combined major and minor error rates were within acceptable limits for all antimicrobial agents tested. Lysis-centrifugation culture methods were more often associated with clinically insignificant VGS isolates; otherwise, no associations between clinical and laboratory variables were noted.

Viridans group streptococci (VGS) are frequently isolated from cultures of blood from hospitalized patients. Notwithstanding the fact that up to three-quarters of these isolates may be deemed inconsequential (25, 31), VGS bacteremias may be a marker for or the cause of serious systemic illness. VGS clearly play a role in the etiology of infective endocarditis (28, 33) and are associated with shock and respiratory distress syndromes in febrile neutropenic populations (2, 8, 19, 24, 29). In these settings, a relative or absolute impairment of the host's response to infection makes the prompt institution of effective antimicrobial therapy imperative. Penicillin has been the cornerstone of therapy for VGS infections, and VGS susceptibility to penicillins was once nearly uniform (4). However, it is increasingly apparent that resistance to β-lactams and to other antimicrobial agents is evolving in VGS, as it has for other gram-positive bacterial pathogens (1, 7, 9, 12, 20, 23). In this context, the rapid determination of a VGS isolate's susceptibility to penicillin and alternate antimicrobial agents becomes a clinical priority. Acknowledging that reference methods (21, 22) for VGS susceptibility testing can be cumbersome and time-consuming to perform, a simpler method of proven accuracy and reliability would be useful.

The E test provides a rapid and convenient means for determining MICs for a variety of microbe-antimicrobial agent combinations. This method has compared favorably with reference methods in measuring antimicrobial MICs for other gram-positive organisms (6, 10, 11, 15, 26, 32), but to date no such standardization has been achieved for VGS. The aims of this study were to compare the E-test and agar dilution MIC methods of susceptibility testing for penicillin, cefuroxime, cefotaxime, and vancomycin and to examine the clinical, laboratory, and demographic characteristics attributable to a large population of unselected VGS isolates from normally sterile body sites.

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MATERIALS AND METHODS

Two hundred unselected VGS isolates (1 isolate from cerebrospinal fluid and the others from blood) stocked in skim milk at −70°C at two large teaching hospitals in Winnipeg, Manitoba, Canada, between January 1991 and December 1995 were thawed, plated on tryptic soy agar supplemented with 5% sheep erythrocytes, and incubated in 5% carbon dioxide at 35°C. Three serial passages ensured adequate growth prior to any testing. The isolates were identified to the species level with the API 20 Rapid Strep system (Bio-Merieux, St. Laurent, Quebec, Canada) in accordance with the manufacturer's instructions (3a). Supplemental tests were used where indicated. Data for isolates for which the species could not be reliably determined or which did not grow well enough to permit complete antimicrobial susceptibility testing were not included in the data analysis.

Agar dilution and E-test MICs were determined with Mueller-Hinton agar supplemented with 5% sheep erythrocytes and by using the incubation conditions described above. Agar dilution media and inoculum preparation, specimen plating, and MIC interpretations were performed in accordance with published guidelines (22). E-test inoculum preparation and plating, strip application, and subsequent MIC determinations were carried out in accordance with the manufacturer's instructions. Two independent observers (J.K. and S.H.) interpreted the agar dilution and E-test MICs. While a specific blinding protocol was not used, neither observer had knowledge of the other's measurements prior to making his or her own MIC determinations. Discrepancies were resolved by consensus. Insufficient growth for MIC determination by either agar dilution or the E test at 24 h warranted an additional 24 h of incubation. E-test and agar dilution MICs were determined in parallel, and any isolate used in the study was tested three times (times 1, 2, and 3) by each method. The antimicrobial agents tested were penicillin, cefuroxime, cefotaxime, and vancomycin. The breakpoints used to define susceptible, resistant, and intermediate categories for each antimicrobial agent were those recommended by the National Committee for Clinical Laboratory Standards (NCCLS) (22). The NCCLS guidelines provide a single breakpoint that defines a "susceptible" category for vancomycin susceptibility testing of VGS.

Patient medical record numbers were obtained, and the following data were collected for discrete bacteremic episodes: patient age and sex, principal medical diagnosis, presence or absence of neutropenia (absolute neutrophil count, less than 500/mm^3) at the time of specimen collection, exposure to antimicrobial agents within 7 days prior to the index clinical specimen, source hospital for the clinical specimen, and whether the blood sample was processed routinely or in a Wampole Isolator lysis-centrifugation system (Oxoid Canada Inc., Nepean, Ontario, Canada). The latter system was still in regular use at hospital B in the early years of our study period. Multiple bacteremias in a single patient were consid-

TABLE 1. MIC_{90}s by Etest and agar dilution for all VGS and streptococcal species[a]

Isolate	MIC_{90} (μg/ml)							
	Penicillin		Cefuroxime		Cefotaxime		Vancomycin	
	ET	AD	ET	AD	ET	AD	ET	AD
All VGS (n = 180)	0.75	1	1.5	1	0.5	0.5	1	0.5
S. oralis (n = 60)	0.38	0.25	2	1	0.5	0.5	1	0.5
S. mitis I (n = 44)	3	2	3	2	1.5	1	1	0.5
S. mitis II (n = 10)	0.5	0.5	0.5	2	0.5	0.5	1	0.5
S. sanguis (n = 43)	0.75	0.5	0.5	0.5	0.25	0.25	1	0.5
S. salivarius (n = 18)	0.5	0.5	0.75	0.5	0.75	0.5	1	0.5

[a] Species of which there were 10 or more isolates. ET, E test; AD, agar dilution.

ered discrete if they were separated by more than 48 h with negative blood cultures in the interim or if they occurred during separate hospitalizations. For each episode, the clinical significance of the isolate was determined according to the following criteria: (i) significant, two or more cultures of specimens from different sites positive for the same organism, a single positive culture with clinical or echocardiographic evidence of endocarditis, or a single positive culture for a febrile neutropenic patient and no other documented source of infection; (ii) indeterminate, a single positive culture with a compatible clinical syndrome (fever and leukocytosis) that did not meet the other criteria for significant or one of two (or more) cultures positive with a compatible clinical syndrome that responded to specific antimicrobial therapy; and (iii) not significant, a single positive culture or one of several cultures positive for other "skin contaminants" (diphtheroids, coagulase-negative staphylococci, *Propionibacterium* spp.) or one of several cultures positive without a compatible clinical syndrome.

The E-test and agar dilution results were analyzed for their agreement, correlation, and concordance. Agreement was defined as $MIC_{ET} = MIC_{AD} \pm$ a single twofold dilution, where MIC_{ET} is the MIC obtained by the E test and MIC_{AD} is the MIC obtained by the agar dilution method. Interclass correlation coefficients were used to characterize the agreement between E-test and agar dilution MICs, and intraclass correlation coefficients were used to estimate the consistency of MIC determinations for individual VGS isolates over serial E tests and serial agar dilutions: coefficients of 0.90 were taken to represent an excellent correlation between or among tests. Concordance was defined as the assignment of an isolate by the E test and agar dilution to the same category of susceptible, intermediate, or resistant; it was assessed both as a proportion (percent concordance) of all comparisons for each time and by using a kappa statistical method, in which a κ value of ≥0.75 indicates excellent concordance. Kappa statistics were also used to assess concordance over serial E tests and agar dilutions. When the E test and agar dilution did not assign the same susceptibility category to an isolate, the discrepancy was categorized as follows: very major error, susceptible by the E test and resistant by agar dilution; major error, resistant by the E test and susceptible by agar dilution; and minor error, all other mismatches of E-test and agar dilution susceptibilities. The denominator for the calculation of very major error rates was the total number of isolates classified by agar dilution as resistant.

By a chi-square test, the clinical and laboratory variables outlined above were assessed for their association with the species of viridans group bacteria isolated, the clinical significance of the isolate, and its susceptibility to penicillin by agar dilution.

RESULTS

Of the 200 VGS isolates processed, 20 could not be used for MIC comparisons: 7 showed insufficient growth or could not be identified reliably and 13 did not have complete antimicrobial susceptibility test results as a consequence of poor growth on one or more of the runs. Of the remaining 180 isolates, 21 (11.7%) required more than 24 h of growth (maximum, 48 h) on one or more of the test runs in order to reliably determine the antimicrobial MICs. Included in this group were *Streptococcus oralis* (n = 7), *Streptococcus sanguis* (n = 7), *Streptococcus mitis* (n = 3), *Streptococcus mutans* (n = 2), and *Streptococcus salivarius* (n = 2) isolates. *Streptococcus milleri* group bacteria are routinely distinguished from other viridans group streptococci at our laboratories and are stocked separately. Consequently, the group is not well represented in this study, although two isolates of alpha-hemolytic *Streptococcus intermedius* were found in our sample.

Table 1 illustrates the species distribution and the apparently comparable E-test and agar dilution MICs at which 90% of isolates are inhibited (MIC_{90}s) for each group of organisms and each antimicrobial agent; shown are the medians of the three MIC_{90}s determined by each test method. Table 2 lists the proportion of organisms classified as susceptible, resistant, and of intermediate susceptibility to each antimicrobial agent. The values given represent the median percent susceptible, resistant, or intermediate for three reference standard (agar dilution) tests.

There was good agreement between E-test and agar dilution MICs, as indicated in Table 3. The correlation coefficients presented in Table 4 also suggest that the agreement between E-test and agar dilution MICs is excellent, with interclass correlation coefficients of >0.90 in most cases. Vancomycin MICs were consistently overestimated by the E-test method: for only 6 of 540 test pairs (1%) was the E-test MIC less than the agar dilution MIC, and for only 82 comparisons (15%) was the E-test MIC MIC equal to the agar dilution. MIC. All isolates were susceptible to vancomycin by agar dilution testing, but for 12 isolates E-test MICs were 1.5 μg/ml (susceptible is an MIC

TABLE 2. Proportion of organisms fully susceptible, fully resistant, and of intermediate susceptibility to each antimicrobial agent

Susceptibility category and isolate	Proportion (%) of organisms[a]			
	Penicillin	Cefuroxime	Cefotaxime	Vancomycin
Fully susceptible				
All VGS (n = 180)	68.3	82.8	93.3	100
S. oralis (n = 60)	68.3	76.7	93.3	100
S. mitis I (n = 44)	63.6	77.3	86.4	100
S. mitis II (n = 10)	70.0	80.0	90.0	100
S. sanguis (n = 43)	65.1	95.3	97.7	100
S. salivarius (n = 18)	72.2	88.9	94.4	100
Intermediate susceptibility				
All VGS (n = 180)	26.1	7.2	2.3	0
S. oralis (n = 60)	25.0	13.3	0	0
S. mitis I (n = 44)	27.2	9.1	4.5	0
S. mitis II (n = 10)	30.0	0	10.0	0
S. sanguis (n = 43)	34.9	2.4	0	0
S. salivarius (n = 18)	16.7	5.6	5.6	0
Fully resistant				
All VGS (n = 180)	5.6	8.3	4.4	0
S. oralis (n = 60)	6.7	10.0	6.7	0
S. mitis I (n = 44)	9.1	15.9	9.1	0
S. mitis II (n = 10)	0	20.0	0	0
S. sanguis (n = 43)	0	4.7	0	0
S. salivarius (n = 18)	11.1	0	0	0

[a] Data are medians of three agar dilution runs.

TABLE 3. Percent agreement between E-test and agar dilution methods

Antimicrobial agent	% Agreement			
	Time 1	Time 2	Time 3	Avg
Penicillin	92.2	96.6	87.7	92.2
Cefuroxime	95.0	97.8	94.4	95.7
Cefotaxime	86.0	95.5	92.3	91.3
Vancomycin	89.9	80.4	89.9	86.7

of ≤1 μg/ml) on one occasion each. For these isolates that were "not susceptible" the corresponding agar dilution MICs were 0.5 μg/ml (9 isolates), 0.25 μg/ml (1 isolate), and 1.0 mg/ml (2 isolates). The agreement over serial E tests and serial agar dilutions for most antimicrobial agents tested was good (Table 5), although not as good as the interclass correlation. Again, vancomycin testing appeared to be the exception, with poor intraclass correlation coefficients by either MIC method.

On the basis of the categorization of the isolates as susceptible, intermediate, or resistant, rates of concordance and very major, major, and minor errors were determined by using simple proportions (percent) and the κ statistic measure of agreement. As outlined in Table 6, concordance rates approached 90% or better, and rates of all error types were within acceptable ranges. Table 7 shows that the concordance between the E test and agar dilution for penicillin, cefuroxime, and cefotaxime was excellent ($\kappa \geq 0.75$) and that the concordance over consecutive E tests and consecutive agar dilutions was again slightly less than that of the E-test or agar dilution pairs.

Medical records were available for 135 discrete bacteremic episodes that occurred in 132 patients and that represented episodes caused by 150 of the 180 isolates tested by the E test and agar dilution. The excess of isolates over bacteremic episodes resulted from the routine stocking of different VGS morphotypes from a single culture and concurrent retrieval of isolates from multiple sites from febrile neutropenic patients. Pairs of organisms from the same bacteremic episode were of the same species by the API 20 Rapid Strep test and had the same median agar dilution susceptibility to penicillin in only two cases (accounting for 4 of 150 isolates). Twenty-nine percent of our isolates were categorized as clinically significant, 39% were not significant, and 32% were of indeterminate significance. There were no statistically significant associations between the VGS species or median agar dilution susceptibilities to penicillin and any of the clinical parameters studied. The Isolator culture method was associated with the classification of isolates as clinically insignificant (chi-square test, $P < 0.001$); otherwise, there were no associations between the perceived clinical significance of an isolate and any of the other study parameters, including antimicrobial susceptibility.

TABLE 5. Test of agreement for serial MICs determined by either E test or agar dilution

Antimicrobial agent	Method[a]	Intraclass correlation coefficient (±95% confidence interval)
Penicillin	ET	0.80 (0.75–0.84)
	AD	0.83 (0.79–0.87)
Cefuroxime	ET	0.89 (0.85–0.91)
	AD	0.85 (0.81–0.88)
Cefotaxime	ET	0.86 (0.81–0.89)
	AD	0.84 (0.80–0.88)
Vancomycin	ET	0.25 (0.15–0.35)
	AD	0.15 (0.05–0.24)

[a] ET, E test; AD, agar dilution.

DISCUSSION

The E test's reliability versus those of reference methods of susceptibility testing has been established for pneumococci (11, 18, 26) and nutritionally variant streptococci (10), and the test has been applied to direct antimicrobial susceptibility testing of blood culture isolates (14); however, the present study represents the first standardization of this newer technology against reference methods for MIC determinations with VGS. In his appraisal of the criteria for choosing an antimicrobial susceptibility testing system, Jorgensen (16) suggests that the system under evaluation should have the following characteristics: 90% of the test MICs should be within ±1 twofold dilution of the MIC obtained by the reference method (agreement), very major errors should occur in <3% of all comparisons for isolates shown to be resistant by the reference method, and combined major and minor error rates should be <7%. The criterion for agreement was clearly met for tests with penicillin, cefuroxime, and cefotaxime, regardless of which definition was used. The agreement for vancomycin susceptibility testing was not as good as those for other antimicrobial agents, reflecting the fact that for a large proportion of isolates E-test MICs of vancomycin were 1.5 dilutions greater than the corresponding agar dilution MICs. A study comparing the E test and the broth macrodilution test of vancomycin susceptibility for *Streptococcus pneumoniae* showed the same consistent overestimation of vancomycin MICs by the E test (13). The reasons for this discrepancy are not clear; however, given the close agreement of the two MIC methods in the aforementioned study for the testing of a *Staphylococcus aureus* control strain, the differences would appear to be more related to the organism tested than the tests themselves.

An interclass correlation analysis confirmed that the E test and agar dilution are in excellent agreement for β-lactam MIC determinations, generating large positive correlation coefficients (>0.90) with narrow confidence intervals. The mean

TABLE 4. Test of agreement between serial pairs of E-test and agar dilution MICs

Antimicrobial agent	Interclass correlation coefficients (±95% confidence interval)			
	Time 1	Time 2	Time 3	Mean
Penicillin	0.86 (0.82–0.90)	0.96 (0.95–0.97)	0.89 (0.86–0.92)	0.94 (0.92–0.95)
Cefuroxime	0.94 (0.92–0.95)	0.96 (0.95–0.97)	0.93 (0.91–0.95)	0.93 (0.90–0.95)
Cefotaxime	0.90 (0.87–0.93)	0.95 (0.93–0.96)	0.94 (0.91–0.95)	0.97 (0.96–0.98)
Vancomycin	0.19 (0.05–0.33)	0.21 (0.06–0.34)	0.18 (0.04–0.32)	0.57 (0.42–0.68)

TABLE 6. Rates of concordance and very major errors, major errors, and minor errors for times 1 to 3

Antimicrobial agent	% Concordance (avg)	No. of errors[a]		
		VME	ME	MN
Penicillin	88.3/93.3/87.2[b] (89.6)	0/0/0	0.6/0/0	11.1/6.7/12.8
Cefuroxime	91.1/95.0/92.2 (94.8)	0.7/0/0	0/0/0.6	8.3/5.0/7.2
Cefotaxime	93.3/96.7/98.3 (96.1)	0/0/0	0.6/0/0	6.1/3.3/1.7
Vancomycin	100/100/100 (100)[c]	0/0/0	0/0/0	0/0/0

[a] VME, very major errors; ME, major errors; MN, minor errors.
[b] Data are for time 1/time 2/time 3.
[c] Data for 12 isolates for which E-test MICs were 1.5 μg/ml for one of the comparison times were included in this category.

interclass correlation coefficients in Table 4 were calculated by comparing the mean of three E-test values for each isolate to the mean of three agar dilution values. Comparison of means reduces the sample variability, leading to an apparent improvement in correlation over the values at the individual times. With respect to vancomycin susceptibility testing and interclass correlation, the limited range of vancomycin MICs generated by the two test methods (clustering predominantly in the 0.5- to 1.5-μg/ml range) made it difficult to demonstrate a close correlation by this method, and lower correlation coefficients with wider confidence intervals are the result. Intraclass correlation coefficients were uniformly lower than interclass coefficients, suggesting that the physical conditions at each time (medium and inoculum preparation, plating and strip application, incubation conditions, and the growth characteristics of individual isolates) may play a greater role in the reproducibility of MIC determinations for VGS than the test method used. For vancomycin susceptibility testing, the limited range of MICs generated did not permit the calculation of a valid kappa statistic.

In our study, the bulk of the isolates were identified as *S. oralis*, *S. mitis*, or *S. sanguis*. The comparison from one study to the next of data related to VGS species determination is problematic. In particular, studies of VGS endocarditis and neutropenic sepsis have used a variety of classification systems over the past two decades, including older versions of the API 20 Strep system. The results of these studies may not be comparable to our own because they may not distinguish a particular species of VGS. For example, in two studies of neutropenic sepsis from 1993 (20) and 1994 (3), the principal VGS isolate identified by reference methods was *S. oralis*, yet this organism was not identified by the API system used in either study. A recent study of the antimicrobial susceptibilities of 352 unselected VGS isolates from across the United States showed a predominance of *S. mitis* isolates and no *S. oralis* isolates (9); again, a different version of the API product or database may have been used.

TABLE 7. Test of agreement for susceptibility classification by E test and agar dilution by κ method

Antimicrobial agent	κ statistic (±95% confidence interval)		
	Interclass method[a]	Intraclass method[b]	
		Agar dilution	E test
Penicillin	0.81 (0.72–0.88)	0.75 (0.68–0.82)	0.72 (0.64–0.78)
Cefuroxime	0.79 (0.73–0.84)	0.73 (0.68–0.78)	0.72 (0.69–0.81)
Cefotaxime	0.75 (0.70–0.80)	0.79 (0.74–0.84)	0.73 (0.68–0.79)
Vancomycin	—[c]	—	—

[a] Compares E test to agar dilution.
[b] Compares consecutive E tests and agar dilutions.
[c] —, Comparison could not be made because all isolates were sensitive.

It is not entirely surprising that we were unable to demonstrate a relationship between specific clinical and demographic variables and individual species of VGS. Studies which have shown a predominance of *S. oralis* or *S. mitis* isolates in VGS sepsis in neutropenic patients (2, 3, 4, 8, 20) or *S. sanguis* isolates in infective endocarditis (5, 28, 33) have started with highly selected demographic groups and as such had greater power (either statistically or intuitively) to show differences in the relative contributions of individual species to their respective disease processes. Our unselected sample included isolates from patients with a wide variety of clinical syndromes and laboratory findings, and the numbers in each group may not have been large enough to demonstrate specific associations. The proportion of VGS isolates categorized as significant, indeterminate, or not significant in our study is in keeping with the proportions generated by other studies of unselected VGS populations (25, 30, 31). That the use of a lysis-centrifugation culture method is associated with a higher rate of clinically insignificant isolates has been demonstrated previously (17, 27).

Conclusions. When VGS isolates are tested, the E test is a reliable method for the determination of the MICs of penicillin, cefuroxime, and cefotaxime. The utility of the E test for the vancomycin MIC determination in this context is debatable: susceptibility breakpoints do not define categories other than sensitive or not sensitive, and a simpler and less expensive method of susceptibility testing (e.g., disk diffusion, followed by MIC determination for isolates showing equivocal results) may be more appropriate.

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