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Rapid Identification and Antimicrobial Susceptibility Testing of Gram-Negative Bacilli from Blood Cultures by the AutoMicrobic System

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A procedure was developed which allows direct identification and antimicrobial susceptibility testing of fermentative and nonfermentative gram-negative bacilli from positive blood cultures. A 10-ml sample was removed from turbid blood culture bottles, and the bacteria were washed and concentrated by centrifugation. The bacterial pellet was used to inoculate an *Enterobacteriaceae* Plus Identification Card and a Gram-Negative General Susceptibility Card of the AutoMicrobic system. Results with these cards were compared with results obtained with standard techniques for 196 blood cultures seeded with recent clinical isolates. Identification of most cultures was available in 8 h, whereas the antimicrobial susceptibility results were available in an average of 4.7 h for all organisms. Direct identification was correct for 95% of the cultures, whereas the antimicrobial susceptibility data had an average agreement of 87% with 3.8% very major and 1.4% major errors. In using this procedure it was possible to provide accurate preliminary identification and results of antimicrobial susceptibility tests for gram-negative bacilli on the same day that a blood culture was determined to be positive.

The importance of rapid diagnosis of bacteremia requires that the earliest possible identification and antimicrobial susceptibility testing of blood culture isolates be a primary responsibility of the clinical microbiology laboratory. Recently there has been considerable interest in rapid blood culture techniques. Systems have been developed which allow more rapid detection of positive blood cultures through measurement of the metabolism of ^{14}C-labeled substrates (16), the decrease in electrical impedance (11), or an increase in electrical potential (7). Procedures to rapidly identify bacteria in positive blood cultures include inoculation of various media and substrates (5, 19), inoculation of commercially available identification systems (2-4), and performing counterimmunoelectrophoresis of cultures for detection of bacterial antigens (20). These identification techniques have been shown to be accurate in identifying a variety of bacteria. However, they have certain shortcomings. For example, some techniques are not truly rapid, requiring overnight incubation, and others are time-consuming or limited in the types of bacteria they can identify. Rapid susceptibility testing by direct inoculation of Kirby-Bauer plates has been reported. Although this method is quite accurate, it does require overnight incubation (6, 10, 13).

The AutoMicrobic system (Vitek Systems Inc., Hazelwood, Mo.) is an automated system designed to perform identification, enumeration, and antibiotic susceptibility testing of a variety of medically significant bacteria (1, 15). Both *Enterobacteriaceae* and selected nonfermentative gram-negative bacilli are accurately identified by the *Enterobacteriaceae* Plus Identification Card (EBC+) (8). The Gram-Negative General Susceptibility Card (GSC) contains various concentrations of 13 antibiotics and provides Kirby-Bauer-type susceptibility data for aerobic or facultatively anaerobic gram-negative bacilli and group D enterococci (9). This paper describes a procedure for the inoculation of both the EBC+ and the GSC, using as the inoculum the bacteria obtained directly from blood culture bottles. To test the feasibility and accuracy of this procedure, simulated positive blood cultures were processed, and the results were compared with those obtained with standard laboratory techniques. This procedure permitted both rapid and accurate identification as well as antimicro-

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bial susceptibility test results of gram-negative bacilli from such cultures.

MATERIALS AND METHODS

Seeded blood cultures. Organisms used in this study were members of the families *Enterobacteriaceae* and *Vibrionaceae* or were nonfermentative gram-negative rods isolated from a variety of patient specimens by the Clinical Microbiology Laboratory of the University of California-Los Angeles Hospital and Clinics. Blood culture bottles (90 ml of Trypticase [BBL Microbiology Systems, Cockeysville, Md.] soy broth with 0.05% sodium polyanetholsulfonate; Clinical Standards Laboratories, Carson, Calif.) that had been originally inoculated with 10 ml of a patient's blood and had subsequently remained negative for bacterial growth for a period of 8 days were each inoculated with a single colony of a pure culture of the organism to be tested. All seeded blood cultures were incubated at 35°C for 18 to 24 h.

Preparation of inoculum. A 10-ml sample of broth was removed aseptically from each turbid blood culture bottle and added to a 13-ml sterile plastic centrifuge tube (Corning Glass Works, Corning, N.Y.). All tubes were centrifuged for 1 min at 3,000 rpm (full speed) in an International table-top centrifuge containing a fixed-angle rotor to pellet the erythrocytes. The bacteria contained in the supernatant were pelleted by centrifugation for an additional 3 min at 3,000 rpm, washed by the addition of 10 ml of 0.086 M NaCl (saline; AMS Diluent), and again pelleted by centrifugation at 3,000 rpm for 3 min. The supernatant was discarded, and the pellet was brought to a density approximating a McFarland no. 1 standard by adding the appropriate amount of saline.

The sample injector attached to the EBC+ received 1.8 ml of the standardized organism suspension, whereas the sample injector attached to the GSC was inoculated with 0.01 ml of this bacterial suspension mixed with 1.8 ml of saline. All cards were filled in the AutoMicrobic system filling module and then placed in the incubator-reader module. The oxidase reaction was not recorded on the EBC+ for any organism because of the difficulty in obtaining a valid oxidase reaction from the washed cells. Identification and antimicrobial susceptibility test results were printed automatically when the determinations were complete and were compared with the results determined by standard methods used in the laboratory.

Standard techniques. Members of the families *Enterobacteriaceae* and *Vibrionaceae* were routinely identified by the use of the API 20E system (Analytab Products, Plainview, N.Y.). The nonfermenters were identified by a combination of test substrates that included API 20E, Oxi-Ferm (Roche Diagnostics, Nutley, N.J.), and the use of acetamide and cetramide media. Antimicrobial susceptibility tests were performed by the agar dilution technique (18).

RESULTS

Identification by the EBC+. A total of 196 seeded blood cultures were processed for rapid identification. The EBC+ correctly identified 187 (95%) of the organisms (Table 1). Identifications were available in 8 h for 98% of the *Enterobacteriaceae* and 54% of the nonfermenters, and the remaining results were made available in 13 h. Nine identification errors occurred, and the reason(s) for each of the errors is presented in Table 2. Three of the organisms tested (*Flavobacterium odoratum*, CDC group IIK-1, and *Vibrio alginolyticus*) are not currently programmed in the AutoMicrobic system computer. These organisms were used in the study primarily to test the EBC+ identification capability when confronted with nonprogrammed organisms. Identification of *V. alginolyticus* was correct to the genus level, whereas the other two organisms were identified as *Acinetobacter calcoaceticus* subsp. *lwoffii* due to growth only in the control well.

Five organisms (*Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Klebsiella pneumoniae*, *Citrobacter freundii*, and *Enterobacter cloaceae*) were misidentified due to insufficient growth. In all cases the control well showed positive growth, but the biochemical wells necessary for correct identification showed no growth. The two nonfermenters were identified

TABLE 1. *Identification of gram-negative bacilli from seeded blood cultures by the AutoMicrobic System EBC+*

Strain	No. tested	No. (%) identified correctly
Escherichia coli	76	76 (100)
Klebsiella pneumoniae[a]	27	25 (93)
Klebsiella ozaenae	3	3 (100)
Enterobacter cloacae	6	5 (83)
Enterobacter aerogenes	6	6 (100)
Citrobacter freundii	8	7 (88)
Citrobacter diversus	1	1 (100)
Serratia marcescens	17	17 (100)
Proteus vulgaris	1	1 (100)
Proteus mirabilis	8	8 (100)
Morganella morganii	6	6 (100)
Shigella sonnei	4	4 (100)
Pseudomonas aeruginosa	26	25 (96)
Pseudomonas maltophilia	1	1 (100)
Pseudomonas fluorescens	1	0 (0)
Fusobacteirum odoratum[b]	1	0 (0)
CDC group IIK-1[b]	1	0 (0)
Vibrio alginolyticus[b]	1	0 (0)
Acinetobacter calcoaceticus subsp. *anitratus*	2	2 (100)
Total	196	187 (95%)

[a] One strain of *Klebsiella oxytoca* was included; EBC+ does not differentiate *K. pneumoniae* from *K. oxytoca*.

[b] Organisms not programmed for identification by EBC+ card.

TABLE 2. *Analysis of identification discrepancies*

Strain	EBC+ identification (% probability)	Reason for discrepancy
Pseudomonas aeruginosa	*Acinetobacter calcoaceticus* subsp. *lwoffii* (99%)	Insufficient growth; acetamide (−), arginine (−)
Klebsiella pneumoniae	*Shigella dysenteriae* (89%)	Insufficient growth
K. pneumoniae	*Enterobacter aerogenes* (99%)	Ornithine decarboxylase (+)
Citrobacter freundii	*Enterobacter agglomerans* (52%) *C. freundii* (47%)	H_2S (−), plant indican (−)
Pseudomonas fluorescens	*A. calcoaceticus* subsp. *lwoffii* (84%) *Pseudomonas cepacia* (15%)	Oxidase (−), arginine (−)
Fusobacterium odoratum	*A. calcoaceticus* subsp. *lwoffii* (99%)	Organism not programmed
Vibrio alginolyticus	*Vibrio cholerae* (41%) *Vibrio parahemolyticus* (27%)	Organism not programmed
CDC group IIK-1	*A. calcoaceticus* subsp. *lwoffii* (99%)	Organism not programmed
Enterobacter cloacae	*Enterobacter sakazakii* (81%) *E. cloacae* (18%)	Sorbitol (−), rhamnose (−)

TABLE 3. *Concentrations of antibiotics used to determine antibiotic susceptibility breakpoints*

Antibiotic	Agar dilution MIC[a] breakpoint (μg/ml) for:			GSC[b] breakpoint (μg/ml) for:		
	Sensitive	Indeterminate	Resistant	Sensitive	Indeterminate	Resistant
Amikacin	≤8	16–32	>32	<8	>8–<32	>32
Ampicillin	≤8	16	>16[c]	<8	>8–<32	>32
Carbenicillin (*Pseudomonas* spp. only)	≤256		>256	≤256		>256
Cefamandole	≤8	16	>16[c]	<8	>8–<32	>32
Cephalothin	≤8	16	>16[c]	<8	>8–<32	>32
Chloramphenicol	≤8	16	>16[c]	>8	>8–<32	>32
Gentamicin	≤4	8	>8	<4	>4–<8	>8
Kanamycin	≤8	16	>16[c]	<8	>8–<32	>32
Tetracycline	≤8[c]	16	>16	<4	>4–<16	>16
Tobramycin	≤4	8	>8	<4	>4–<8	>8
Trimethoprim-sulfamethoxazole	<2/40	4/80	>4/80[c]	<2/40	>2/40–<8/160	>8/160

[a] MIC, Minimal inhibitory concentration.
[b] GSC also contains carbenicillin at 16 and 32 μg/ml, cefoxitin at 8 and 32 μg/ml, and nitrofurantoin at 32 and 128 μg/ml.
[c] Breakpoint does not match GSC.

as *A. calcoaceticus* subsp. *lwoffii*, and one strain of *K. pneumoniae* was identified as *Shigella dysenteriae*. For two organisms, *C. freundii* and *E. cloacae*, correct identification was given as the second choice by the AutoMicrobic system. One organism (*K. pneumoniae*) was identified incorrectly because of a false-positive ornithine decarboxylase reaction.

Antimicrobial susceptibilities. The respective concentrations of antimicrobial agents used in the agar dilution test and the GSC breakpoint criteria defining susceptible, indeterminant, and resistant are listed in Table 3. It should be noted that the concentrations of antibiotics used in the GSC did not exactly correspond with the expanded scale of dilutions used routinely in our laboratory with the agar dilution test. It should be further noted that although the GSC contains carbenicillin concentrations for non-*Pseudomonas* spp., cefoxitin, and nitrofurantoin, these agents were not compared in this study since these antibiotics are not routinely employed in this manner in our laboratory.

Results obtained from the GSC were available after an average incubation of 4.4 h for the *Enterobacteriaceae* and 7.4 h for the nonfermenters, with the average time for all organisms being 4.7 h. Eight (4.6%) of the *Enterobacteriaceae* and 13 (40%) of the nonfermenters, or 11% of the total organisms tested, did not have sufficient growth to allow a GSC result after 8 h of incubation.

Agreements and discrepancies between the GSC and agar dilution test results were classified as follows: agreements, very major errors, major errors, and minor errors (17). The percentage of agreement and errors for each drug tested is presented in Table 4. Of the 175 organisms

TABLE 4. *Antimicrobial susceptibility correlation*[a]

Drug	Agreement (%)	Errors (%)		
		Very major	Major	Minor
Amikacin	91	1.1	0	8.0
Ampicillin[b]	73	6.8	1.2	20.0[c]
Carbenicillin (*Pseudomonas* spp. only)[d]	74	5.3	21.0	0
Cefamandole	85	8.5	2.8	4.0
Cephalothin[b]	82	4.3	2.5	11.0
Chloramphenicol	91	3.4	1.7	10.0
Gentamicin	89	2.3	0.6	7.9
Kanamycin	85	4.5	0	10.0[c]
Tetracycline	86	2.8	2.8	8.5
Tobramycin	93	1.2	2.3	3.5
Trimethoprim-sulfamethoxazole	95	2.9	0	1.7
Average	87	3.8	1.4	8.5

[a] 175 strains.
[b] *Enterobacter* spp. not included.
[c] Number of minor errors increased due to unmatched resistance breakpoints.
[d] 19 *Pseudomonas* spp. tested; results not included in average.

tested, the average agreement was 87% with 3.8% very major, 1.4% major, and 8.5% minor errors. Amikacin, gentamicin, tobramycin, and trimethoprim-sulfamethoxazole had the highest correlations, with ≥89% agreement and ≤3.5% combined major and very major errors.

Very major errors were relatively frequent for ampicillin, cefamandole, cephalothin, and kanamycin. These errors were partially due to strains of *Serratia marcescens* which caused 45%, 67%, and 25% of the very major errors for ampicillin, cefamandole, and kanamycin, respectively. Except for carbenicillin, the major errors were all less than 3% for each drug. When tested only against the 19 strains of *Pseudomonas*, carbenicillin had a high percentage of errors, with the very major errors being caused by a single strain of *Pseudomonas*.

As noted earlier for several of the antimicrobial agents, the breakpoints used in this study were not exactly the same because of the respective antibiotic concentrations employed in the agar dilution technique and GSC. Because of this, the ampicillin, cefamandole, cephalothin, chloramphenicol, kanamycin, and trimethoprim-sulfamethoxazole resistance breakpoints were one doubling dilution higher for the GSC (Table 3). Although this difference could have led to an increase in the number of minor errors due to organisms being classified as resistant by the agar dilution method and indeterminate by the GSC, this did not seem to be a significant factor. Indeed, only with ampicillin and kanamycin was this type of minor error a contributing factor, accounting for 85% and 47% of the total minor errors for each drug, respectively. For tetracycline the susceptible breakpoint was one dilution lower for the GSC as compared with the agar dilution technique. Although this difference could have led to an agar dilution-sensitive/GSC-indeterminate situation, only 7% of the minor errors were due to this type of error.

DISCUSSION

In this report we have described a procedure which permits both rapid and accurate identification and antimicrobial susceptibility testing of gram-negative bacilli obtained from seeded blood cultures. Seeded blood cultures were employed as a means of obtaining a more varied selection of organisms to be tested as well as offering a controlled situation in which many positive cultures could be analyzed in a relatively short period of time. Moreover, preliminary data in our laboratory on a limited number of positive blood cultures obtained from patients (which actually preceded this study) indicated that this method showed a great deal of promise.

The technique developed for preparation of the inocula from the blood culture bottle requires three short centrifugation steps and results in separation of the erythrocytes, removal of growth and inhibitory factors, and adjustment of the inoculum to a density matching a McFarland no. 1 standard. Although this type of inoculum preparation is not new for rapid processing of blood in the laboratory, the use of a single automated system which provides both rapid identification and antibiotic susceptibility test results represents a reduction in the time and effort necessary to provide complete and accurate preliminary results from positive blood cultures.

The rapidity of the method is dependent upon the AutoMicrobic system. In this study the great majority of organisms tested were identified by the EBC+ after 8 h of incubation, and for all organisms susceptibility results were available in an average of 4.7 h. Since these results were available within 1 working day, the method presented here can be classified as a true "rapid" method, as distinguished from "direct" methods which use an inoculum from positive blood cultures but require overnight incubation.

Accuracy of identification by the EBC+ as used in this study (95%) was comparable to the accuracy (97.6%) reported with the *Enterobacteriaceae* Biochemical Card (a precursor of the EBC+ with identical *Enterobacteriaceae* identification capabilities) in an earlier collaborative study (8). This observation gives strong indication that the technique used to prepare the

inoculum from blood culture is effective in removing any substances which may interfere with the biochemical reactions taking place in the EBC+. Moreover, the high level of accuracy obtained in this study demonstrates once again the efficacy of using a direct inoculum from a positive blood culture for preliminary identification purposes. In our hands, this level of identification correlated well with the accuracy obtained from final identification by standard techniques. Furthermore, ongoing preliminary experiments being carried out in our laboratory indicate that this centrifugation technique for inocula preparation works equally well in the identification of gram-negative bacteria from positive blood cultures received in our laboratory. This is due to the fact that blood cultures showing visible growth usually contain a relatively high concentration of bacteria. In addition, cultures visibly positive but containing a lower than normal concentration of bacteria also can be processed by this technique. By using relatively large volumes of blood culture for centrifugation, one can be sure an adequate bacterial pellet will be obtained.

Unlike the seeded cultures used in this study, approximately 5% to 16% of positive blood cultures have been reported to be polymicrobic (12, 14). Use of any technique which relies on a direct inoculum from blood culture could lead to errors in identification where mixed cultures are involved. In our laboratory, mixed blood cultures represent 7% of the total number of positive cultures, of which 4.5% contained gram-negative bacilli (unpublished data). The majority of these polymicrobic bacteremias contained both gram-positive cocci and gram-negative bacilli, and the remaining cultures (28%) contained two or more genera of gram-negative bacilli. Although it is this latter type of mixed culture that gives us the most concern, they accounted only for slightly more than 1% of all positive blood cultures (unpublished data).

Actual practice dictates that all positive blood cultures be Gram stained. This practice obviously lends itself to the technique presented here, not only to determine whether gram-negative bacilli are present but also to determine those blood cultures that may be polymicrobic. These latter cultures, therefore, would be excluded from processing by this rapid method. Although results to date with this method have been gratifying, we feel that a subculture to a blood agar plate must be routinely performed to rule out the possibility of a mixed culture. Because of this possibility, the results obtained from this technique, however accurate, are considered preliminary until the culture is definitively identified by standard methods.

Results obtained with the use of the GSC indicated that approximately 89% of the organisms used in this study could be adequately tested. Of interest, however, is that 40% of the nonfermenters showed insufficient growth with this card. Although the reason for this is unclear, it may be due to the dual effect of low inoculum density combined with a long lag period which characterized some of the nonfermenters employed in this study. To overcome this situation, it may be necessary to increase the inoculum size, particularly in those situations where nonfermenters may be isolated.

One could argue about the validity of our results, since the concentrations of some of the antibiotics used with the agar dilution test did not exactly correspond to those of the GSC. However, we chose to compare systems, that is, to compare a rapid automated system approach with one that is used on a day-to-day basis in our laboratory. Therefore, no attempt was made to alter antibiotic concentrations to coincide with those incorporated in the GSC. It should be noted that a collaborative study that involved several laboratories has recently reported on the GSC and how well it compared to standardized disk diffusion and broth microdilution methods (C. Thornsberry, H. D. Isenberg, T. L. Gavan, A. Barry, and P. Jones, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 20th, New Orleans, La., abstr. no. 388, 1980). This study achieved between 87 and 90% agreement, depending upon their interpretative criteria.

Overall, the rate of agreement between the GSC and the agar dilution procedure as used in our laboratory was quite good. However, there was a relatively large number of very major errors (3.8%). This was partially due to problems encountered with strains of *S. marcescens* giving false susceptible results with ampicillin, cefamandole, and kanamycin. The manufacturer's instructions in the use of the GSC indicate that *Enterobacter* spp. may give a high rate of false susceptibility readings against ampicillin and cephalothin, and this observation has been verified in our laboratory. (For this reason, we felt justified in excluding these organism-drug combinations from the rest of the drug comparisons made during this study.) However, it came somewhat as a surprise to us to learn that our strains of *S. marcescens* showed a considerable number of false susceptible determinations against ampicillin, cefamandole, and kanamycin which resulted in a relatively large number of very major errors for these three drugs. On the other hand, there was a high level of agreement for amikacin, chloramphenicol, tobramycin, and trimetho-

prim-sulfamethoxazole, with >90% agreement, and for gentamicin, kanamycin, and tetracycline, with ≥85% agreement. With the exception of the errors that were caused by *Pseudomonas* spp. with carbenicillin, *Enterobacter* spp. with ampicillin and cephalothin, and *S. marcescens* with ampicillin, cefamandole, and kanamycin, the GSC as used in this study provided accurate and very rapid antibiotic susceptibility results for the majority of cultures tested.

In conclusion, a method is presented that requires very little preparatory time in the rapid identification and antibiotic susceptibility testing of gram-negative bacilli from blood cultures. This method is currently under evaluation in the processing of blood cultures in our laboratory.

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