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Direct Inoculation Procedure for the Rapid Classification of Bacteria from Blood Culture

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A procedure was developed for 4-h identification of bacteria from blood culture. From a turbid blood culture bottle a 10- to 15-ml aliquot was drawn off and centrifuged. The pellet was utilized to inoculate a series of enzymatic and physiologic reactions. Three hundred eight positive blood cultures were tested including 222 strains of *Enterobacteriaceae*, 40 strains of facultative gram-positive cocci, 26 strains of anaerobes, and 20 assorted strains. There was over 96.5% agreement between 4-h and conventional methods with the *Enterobacteriaceae*, 98% with facultative gram-positive cocci, 100% with anaerobes, and 99% with the assorted strains.

There is little debate that all organisms in positive blood cultures must be identified. Modern microbiology has amply proven the relative virulence of organisms formerly deemed strict saprophytes. It is no longer considered acceptable to empirically dismiss a blood culture isolate as a contaminant until it has been identified and the patient's clinical situation evaluated. Although technology exists for the accurate, rapid identification of bacteria, little has been accomplished in the application of these tools to the blood culture. To obviate the 18- to 24-h delay inherent in the performance of most biochemical reactions, a 4-h direct inoculation procedure, following the Wasilauskas and Ellner (12) guidelines, was developed. The method does not require elaborate equipment, materials, or techniques and, as such, is designed for any microbiology laboratory, regardless of size.

MATERIALS AND METHODS

Blood culture. Blood cultures were drawn by the attending physician with 5 ml of blood directly inoculated into a 50-ml Vacutainer (BBL, Cockeysville, Md.). For each venipuncture two, Vacutainers were utilized. The medium consisted of a thioglycolate and Trypticase soy broth during the first half of the study and Columbia broth and Columbia broth with cysteine during the second half. Appropriate accessory factors were included in each bottle. Incubation was at 36 C. All bottles were visually observed each day; blind subcultures were routinely performed after 24 to 48 h.

Inoculum preparation. When growth was noted, 10 to 15 ml of medium were removed above the blood cell layer with a 22-gauge needle attached to a 20-ml syringe. The medium was placed in a sterile tube and centrifuged for 15 min at 4,000 rpm. The supernatant was discarded and the pellet was utilized as the inoculum. Routine procedures, consisting of aerobic and anaerobic subcultures and 18- to 24-h biochemical tests, were simultaneously performed. If growth was found only by blind subculture, one to three colonies served as the inoculum. In all cases Gram stains were made.

Enzymatic and physiologic tests. Depending upon the Gram stain of the culture various tests were performed (Table 1). If the culture appeared mixed, or contained fungi, alternative procedures were employed. The techniques used in the performance of tests were as follows: cytochrome oxidase, phenylalanine deaminase, nitrate reduction, acetoin production (Voges-Proskauer), indole production, urease production, lysine decarboxylase, H_2S production, esculin hydrolysis, ornithine decarboxylase, and *o*-nitrophenyl-β-D-galactopyranoside hydrolysis were determined utilizing PathoTec strips (General Diagnostics, Morris Plains, N.J.). The centrifuged pellet was suspended in distilled water and inoculated for each strip per directions from the manufacturer.

Gelatin hydrolysis was determined utilizing Key gelatin strips (Key Scientific Co., Los Angeles, Calif.) or X-ray film with an inoculum density equal to that used for the PathoTec strips. Removal of the gelatin coat from the supporting base with gentle shaking constituted a positive test.

Anaerobiasis was ascertained by heavily inoculating a 5% sheep chocolate agar plate and incubating it under 5 to 7% CO_2 at 36 C. Growth was visible for aerobes and facultative anaerobes in 4 h; no growth was visible with anaerobes.

Esculin hydrolysis in the presence of bile (Pfizer Diagnostics, Clifton, N. J.) was determined by adding several loopfuls of inoculum to the liquid medium. Growth in the presence of 6.5% NaCl was determined in the same way.

Bile solubility was determined by immersing

TABLE 1. *Enzymatic and physiologic tests performed*

Determinants	Cocci	Bacilli
Gram positive	BEM[a]	Hanging drop motility
	Growth in 6.5% NaCl	Aerobic growth on agar
	Bile solubility	Catalase production
	Bacitracin sensitivity	BEM[a]
	Catalase production	Lecithinase production
	Anti-pneumococcal omni serum[b]	Lactose fermentation
	Aerobic growth on agar	Anti-*Listeria* agglutination[b]
Gram negative	Cytochrome oxidase	Cytochrome oxidase
	Anti-meningococcal agglutination	Phenylalanine deaminase
	Aerobic growth on agar	Nitrate reduction
		Acetoin production
		Indole production
		Malonate utilization
		Urease production
		Lysine decarboxylase
		H_2S production
		Esculin hydrolysis
		Ornithine decarboxylase
		ONPG[a] hydrolysis
		Gelatin hydrolysis
		Hanging drop motility
		Aerobic growth on agar
		Anti-*Haemophilus* CEP[b]

[a] BEM, Esculin hydrolysis in the presence of bile; ONPG, *o*-nitrophenyl-β-D-galactopyranoside.
[b] If warranted based on biochemical characteristics. CEP, Counterelectrophoresis.

several loopfuls of inoculum in 0.5 ml of a 2% solution of sodium desoxycholate. A second tube, containing 0.5 ml of 0.85 M NaCl, was used as a control. Clearing, evidence of a positive test, was generally seen within 1 h.

The presence of catalase was determined by emulsifying a small amount of inoculum in a 3% solution of hydrogen peroxide on a standard microscope slide. A positive strain produced immediate bubbles.

Bacitracin sensitivity was determined by heavily inoculating a 5% sheep blood agar plate, placing a bacitracin disk (0.02 U) on the surface, and incubating under 5 to 7% CO_2. A zone of inhibition was considered positive.

Lecithinase production was determined for clostridium-like organisms by heavily inoculating a lecithin-lactose plate (BBL). Lactose fermentation could also be determined for clostridia utilizing this procedure.

Motility was determined by making a hanging drop preparation directly from the turbid blood bottle. The motility test was repeated after 4 h of incubation from the Trypticase soy broth utilized to inoculate the sensitivity plates.

Miscellaneous examinations included the utilization of anti-meningococcal, anti-hemophilus, anti-pneumococcus (omni serum), and anti-listeria antisera. Agglutination or counterimmunoelectrophoresis were performed when indicated by morphological and physiological information.

RESULTS

Comparisons were made both of the individual tests and of microorganism identification for all strains (Table 2). All biochemical procedures were run in parallel with established tests (2–5). Overall, with the *Enterobacteriaceae*, a 96.5% agreement on an individual test basis was seen. As shown in Table 3, however, there were variations in individual tests. Consistent with other reports excellent results were obtained with the PathoTec system (1, 8, 9, 11) even though the manufacturer's inoculation instructions were not followed. There was 100% agreement with cytochrome oxidase and *o*-nitrophenyl-β-D-galactopyranoside and over 98% agreement with phenylalanine deaminase, nitrate reduction, acetoin production, indole production, malonate utilization, and ornithine decarboxylase and standard methods. Lysine decarboxylase agreed within 97%. There was 95% agreement with the strip detection of H_2S and the triple sugar iron agar detection of H_2S. The H_2S strip appeared somewhat oversensitive with 10 of 11 disagreements occurring due to false positives. There was only 90% agreement with the urease strips and the Christensen slants. Of 22 discrepant reactions 19 were false negatives. In no case was a *Proteus* or other strong urease producer missed. Of 19 false negatives 16 were due to *Klebsiella* species. At 90% correspondence esculin hydrolysis showed the worst agreement. There were seven false positives, distributed among *Escherichia, Proteus,* and *Enterobacter,* and 15 false negatives,

TABLE 2. *Strains studied by the 4-h procedure*[a]

Strain	No.	Strain	No.
Escherichia coli	104	*Aeromonas hydrophila*	1
Klebsiella pneumoniae	48	*Acinetobacter calcoaceticus*	1
K. ozaenae	2	*Staphylococcus aureus*	6
Enterobacter cloacae	12	*S. epidermidis*	11
E. aerogenes	4	Enterococcus group	5
E. agglomerans	2	Group D, non-enterococcus	1
Proteus mirabilis	16	*Streptococcus pneumoniae*	8
P. morganii	8	*Streptomyces viridans* group	7
P. rettgeri	8	Other *Streptococcus* (groups B and C)	2
Providencia stuartii	1	*Bacteroides* sp.	12
P. alcalifaciens	3	*Clostridium perfringens*	2
Serratia liquefaciens	4	*C. sordellii*	1
S. marcescens	2	*Peptostreptococcus* sp.	2
S. rubidaea	1	*Peptococcus* sp.	2
Citrobacter freundii	3	*Propionibacterium acnes*	7
C. diversus	2	*Listeria monocytogenes*	2
Salmonella D and C_2	2	*Corynebacterium* sp.	3
Pseudomonas aeruginosa	10	*Neisseria meningitidis*	2
P. cepacia	1	*Haemophilus influenzae*	1
P. maltophilia	1		

[a] Gram-positive organisms were incorporated into the study sometime after gram-negative organisms, hence their numbers may be smaller in proportion. All organisms were not identified to species by the 4-h test system. Additional conventional tests were utilized for complete classification.

TABLE 3. *Accuracy of the individual test procedure*[a]

Test	No. of tests performed	Agreement with conventional (%)
Cytochrome oxidase	249	100.0
Phenylalanine deaminase	222	99.5
Nitrate reduction	222	98.5
Acetoin production	222	98.5
Indole production	222	98.5
Malonate utilization	222	98.5
Urease production	222	90.0
Lysine S/B decarboxylase	222	97.0
H_2S production	222	95.0
Esculin hydrolysis	222	90.0
Ornithine S/B decarboxylase	222	99.5
ONPG[b] hydrolysis	222	100.0
Gelatin hydrolysis	222	99.5
Hanging drop motility	264	99.5
Aerobic growth on agar	308	98.5
BEM[b]	59	100.0
Growth in 6.5% NaCl	44	98.0
Bile solubility	44	100.0
Bacitracin sensitivity	44	98.0
Catalase production	59	100.0
Serological procedures	13	100.0
Lecithinase production	15	100.0
Lactose fermentation	15	100.0

[a] Figures rounded to the nearest 0.5%. Contains those tests confirmed by conventional procedures.

[b] ONPG, *o*-Nitrophenyl-β-D-galactopyranoside. BEM, Esculin hydrolysis in the presence of bile.

12 occurring among the *Klebsiella*. There was over 99% agreement with gelatin hydrolysis with either the Key gelatin strip or X-ray film. Only one strain gave a false-negative reaction with the hanging drop motility, a correspondence of over 99%.

There were no false positives utilizing growth on chocolate agar as a screening method for the detection of anaerobes. There were six false negatives, all *Pseudomonas*, for a skewed overall agreement rate of 98%. Since the one strain of *Pseudomonas maltophilia* isolated did grow on chocolate agar, and all six strains of *Pseudomonas* responsible for the false-negative reactions were oxidase positive, there was no confusion concerning the aerobes and anaerobes.

Of the tests employed in the identification of the facultative gram-positive cocci, there was 100% agreement with esculin hydrolysis, bile solubility, and catalase tests. There was one false negative with growth in 6.5% NaCl and one false positive with bacitracin sensitivity, for an overall agreement of 98% in each of these cases.

In this series only two strains of *N. meningitidis*, one strain of *H. influenzae*, and one strain of *L. monocytogenes* was isolated. Each was serogrouped within 4 h either by agglutination or counterimmunoelectrophoresis. All eight strains of *S. pneumoniae* were identified in 20 min by quellung with omni serum. The two strains of *C. perfringens* were both lecithinase production and lactose fermentation positive. Such small numbers do not permit statistical interpretation.

For the purposes of this study it was considered sufficient, within the 4-h framework, to establish an organism as a strict anaerobe with an accompanying morphological description since antimicrobial therapy correlates quite well with these data. Because not all tests used in this study were utilized in this laboratory for identification of anaerobes, not all were run in parallel. There was, however, over 95% agreement with the nitrate reduction, gelatin hydrolysis, *o*-nitrophenyl-β-D-galactopyranoside, indole, esculin hydrolysis, and acetoin production. These results were in agreement with those of Schreckenberger and Blazevic (10), who used bacterial colonies to inoculate PathoTec strips.

Because not all enzymatic and physiologic reactions weigh equally, there were considerably fewer incorrect classifications than false biochemical reactions. Of 308 strains employed in this series, 12 were falsely identified for a 96% agreement with conventional methodology. Of these, nine were misidentified across genus lines, whereas three were misidentified as to species (Table 4).

DISCUSSION

The identification of a bacterial blood culture isolate provides critical information for choice of antimicrobial therapy. Often, there is considerable delay in the acquisition of this information. Bacterial identification technology is such that this delay is avoidable. Wasilauskas and Ellner (12) presented a system that is readily adoptable to the microbiology laboratory. Kocka and Morello (6) used the Inolex Enteric I card to correctly identify enterobacteria directly from blood culture.

A 4-h test largely depends upon the inherent enzymatic content of the bacterial inoculum (7). There is limited time for the induction of enzymes necessary for many reactions. When a sufficient mass of an enzyme is inately present, growth of the culture is unnecessary; only the initial inoculum density assumes importance. Utilizing this principle it is possible to rapidly identify even fastidious, slowly growing organisms. The only limitation is governed by the enzyme-substrate ratio necessary, in conjunction with the indicator system, to bring about a visible reaction. Although blood cultures yield a rather small inoculum mass, the minimum enzymatic content is apparently achieved and the procedure is efficacious for a wide spectrum of microorganisms.

The method itself required only a modest extension of procedures generally used in the clinical microbiology laboratory. Relatively few limitations were experienced with the procedure. Within the number of enzymatic and physiologic reactions used, all groups of bacteria were accurately identified. In this study *Staphylococcus* was not speciated in 4 h; preliminary results indicated better correlation with a 6-h coagulase test. Although it was not generally possible to speciate the anaerobes with this system, it was possible to classify them by morphology, Gram stain, growth characteristics, and a limited number of biochemical reactions. This provided useful information in 4 h since the sensitivity of anaerobes to penicillin, chloramphenicol, and clindamycin show good correlation with morphological and Gram-staining characteristics.

Knowing the pattern of sensitivity of the various species in this institution, and knowing the inherent antibiograms of the various genera, the 4-h procedure practically assisted in the choice of antibiotics. With rapid sensitivity procedures available rapid identification should be provided. In addition, the 4-h procedure provided valuable information for the elucidation of the patient's clinical syndrome.

TABLE 4. *Misidentifications based on the 4-h test*

Final identification	No. of isolates misidentified	4-h identification	Biochemical[a] disagreement
Escherichia coli	2	*Klebsiella pneumoniae*	Mal+
E. coli	1	*E. hafniae*	Ind−
Enterobacter agglomerans	1	*E. coli*	Lys+
Proteus mirabilis	1	*E. agglomerans*	H_2S-, NO_3-
E. cloacae	2	*E. hafniae*	Lys+
E. aerogenes	1	*E. agglomerans*	Lys−
E. aerogenes	1	*Serratia* sp.	Mal−
Proteus rettgeri	1	*Providencia* sp.	Ur−
Serratia sp.	1	*E. hafniae*	Esc−, VP−
Enterococcus group	1	Group D, non-enterococcus	6.5% NaCl−

[a] Biochemical disagreements listed indicate the false reactions observed in 4 h of incubation.

Four-hour tests other than those described here can be developed. The PathoTec system, based on the inherent enzyme principle, proved an excellent basic choice for bacteria both within and outside the family *Enterobacteriaceae*. The greater than 96.5% accuracy in the classification of bacteria from blood cultures within 4 h, coupled with its relative ease of performance, proved a useful procedure in the routine clinical microbiology laboratory.

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