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Evaluation of Three Broth Disk Methods for Testing the Susceptibility of Anaerobic Bacteria to Imipenem

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Imipenem is a member of a new class of highly potent β-lactam antibiotics, carbapenems, with an antibacterial spectrum that includes nearly all currently known aerobic and anaerobic bacterial species of clinical significance. Although relatively stable in most standard laboratory media used for antimicrobial susceptibility testing, imipenem undergoes rapid inactivation in thioglycolate broth, a recommended medium for susceptibility testing of anaerobic bacteria by the broth disk method. In the current study, a panel of 36 anaerobic bacteria consisting of 28 clinical isolates and eight quality control strains was used to determine the suitability and accuracy of the broth disk methods with brain heart infusion, Schaedler, and anaerobic broths, in comparison to the reference agar dilution method, for the anaerobic susceptibility testing of imipenem. To achieve single test concentrations of approximately 8, 16, and 64 μg/ml for imipenem, cefoxitin, and piperacillin, respectively, which correspond to the MIC breakpoints of the test drugs, four 10-μg imipenem disks, three 30-μg cefoxitin disks, and three 100-μg piperacillin disks were used in 5 ml of broth. The correlation between the reference agar dilution method and each of the three broth disk elution procedures evaluated was excellent, for imipenem (100% agreement) and somewhat less so for cefoxitin and piperacillin. Therefore, brain heart infusion, Schaedler, and anaerobic broths, but not thioglycolate broth, are suitable for anaerobic susceptibility testing of imipenem by the disk elution method.

The clinical significance of anaerobic infections has gained increasing recognition during the last two decades. The reported decrease in the predictability of the susceptibility patterns of anaerobes to existing antibacterial agents has clearly underscored the need for frequent in vitro susceptibility testing of these microorganisms (10). Of the three currently approved methods of testing, namely, the agar dilution, the broth dilution, and the broth disk elution methods, the disk elution method is the simplest, most cost effective and practical, and therefore most popular, especially among small clinical laboratories (10, 14). This technique involves testing only one concentration of each antibacterial agent, that concentration which corresponds to the expected therapeutic level of the test compound in serum. The broth disk test as first described by Wilkins and Thiel (14) uses prereduced and supplemented brain heart infusion broth (BHI-B). Since this method requires the use of oxygen-free CO_2 or anaerobic chambers, a similar but simpler system that uses thioglycolate broth and aerobic incubation was introduced by Kurzynski and co-workers (5) and is being used by a substantial number of clinical laboratories.

The increased potency and the unusually broad antibacterial spectrum of imipenem (*N*-formimidoyl thienamycin, MK0787) in comparison to those of other β-lactam antibiotics have been well documented (3, 9). Nearly all data presently available on the in vitro activity of imipenem against anaerobic bacteria have been derived by the reference agar dilution method (1, 2, 6, 8). A preliminary investigation in our laboratory has shown that imipenem sustains a substantial loss of in vitro activity in thioglycolate, i.e., more than 90% inactivation in 6 h (unpublished data). In other studies, imipenem (12) and penicillins (7) lost more than 99% of their antibacterial activities after an overnight incubation in Thiol broth. Some of these results could have been predicted on the basis of an earlier communication (4), in which thienamycin was shown to be unstable in the presence of *p*-amino thiols, cysteine, and Tris buffer. The purpose of the current study was to identify a suitable alternative medium to thioglycolate broth for the antibacterial susceptibility testing of imipenem by the broth disk method.

MATERIALS AND METHODS

Bacterial strains. A total of 28 anaerobic clinical isolates recently acquired from several hospitals in the United States and stored at −70°C in 15% skim milk were used in this study. These isolates included three strains each of *Bacteroides fragilis*, *Bacteroides distasonis*, and *Bacteroides thetaiotaomicron*, two strains each of *Bacteroides ovatus* and *Clostridium difficile*, and one strain each of *Bacteroides vulgatus*, *Bacteroides melaninogenicus*, *Fusobacterium mortiferum*, *Fusobacterium nucleatum*, *Clostridium sordellii*, *Clostridium tertium*, *Clostridium butyricum*, *Clostridium perfringens*, *Clostridium sporogenes*, *Propionibacterium acnes*, *Eubacterium lentum*, *Actinomyces naeslundii*, *Veillonella parvula*, *Peptostreptococcus anaerobius*, and *Peptococcus magnus*. Eight quality control and reference strains recommended by the National Committee for Clinical Laboratory Standards (8) also were included: *B. fragilis* ATCC 25285, *B. thetaiotaomicron* ATCC 29741, *B. thetaiotaomicron* ATCC 29742, *B. vulgatus* ATCC 29327, *C. perfringens* ATCC 13124, *Peptococcus magnus* ATCC 29328, *P. variabilis* ATCC 14956, and *Peptococcus asaccharolyticus* ATCC 29743. Before use, all bacterial strains were subcultured on reduced 5% sheep blood agar plates (enriched with hemin and vitamin K_1) at 37°C for 48 h in an anaerobic

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chamber (Forma Scientific, Marietta, Ohio) containing 85% nitrogen, 10% hydrogen, and 5% carbon dioxide.

Antimicrobial agents. Standard laboratory powders and susceptibility disks of imipenem and cefoxitin were supplied by Merck & Co., Inc., Rahway, N.J., and those of piperacillin were supplied by Lederle Laboratories, Pearl River, N.Y. Imipenem was dissolved in 0.01 M phosphate buffer (pH 7.2), and cefoxitin and piperacillin were dissolved in sterile distilled water. Antibiotic solutions were prepared just before use.

Media. Media were purchased from commercial houses and rehydrated according to the instructions of the manufacturers. Since some of the anaerobic media may contain ingredients known or suspected to affect the antibacterial activity of imipenem, it was deemed essential to list the contents of all media used in the current study.

The Wilkins-Chalgren agar (Difco Laboratories, Detroit, Mich.) used for MIC determinations contained (per liter of distilled water) tryptone (10 g), peptone (10 g), yeast extract (5 g), glucose (1 g), sodium chloride (5 g), L-arginine (1 g), sodium pyruvate (1 g), hemin (5 mg), vitamin K_1 (0.5 mg), and agar (15 g). Four liquid media were used: experimental anaerobic broth (Difco), Schaedler broth (BBL Microbiology Systems, Cockeysville, Md.), BHI-B (Difco), and thioglycolate medium-135 without indicator (BBL). The composition of experimental anaerobic broth was essentially identical to that of Wilkins-Chalgren agar without the agar. For this study, both of these media were supplemented with an additional amount of hemin and vitamin K_1 to final concentrations of 10 and 1 μg/ml, respectively. Schaedler broth contained (per liter of distilled water) peptone (11.6 g), yeast extract (5 g), glucose (5.82 g), sodium chloride (1.7 g), dipotassium phosphate (0.82 g), Tris (3 g), hemin (10 mg), and L-cystine (0.4 g). BHI-B contained (per liter of distilled water) calf brain infusion (200 g), beef heart infusion (250 g), Proteose Peptone (10 g; Difco), yeast extract (5 g), glucose (2 g), sodium chloride (5 g), and disodium phosphate (2.5 g). The thioglycolate medium contained (per liter of distilled water) Trypticase Peptone (17 g) (BBL), Phytone Peptone (3 g; BBL), glucose (6 g), sodium chloride (2.5 g), sodium thioglycolate (0.5 g), L-cystine (0.25 g), sodium sulfite (0.1 g), and agar (0.7 g). Each of these three media was supplemented with hemin (5 μg/ml) and vitamin K_1 (0.1 μg/ml). The thioglycolate medium also was supplemented with sodium bicarbonate (1 mg/ml).

Imipenem in thioglycolate. To confirm the alleged inactivation of the antibacterial activity of imipenem in thioglycolate, the susceptibility of a small panel of bacterial strains was determined by both the macrodilution method and the broth disk method, with the thioglycolate and the anaerobic broths. Serial twofold dilutions of the test drug in 5-ml volumes were prepared in each medium. Inocula were prepared by subculturing the test isolates twice in BHI-B and once in the final test medium. One-milliliter portions of an inoculum that had been previously adjusted to a McFarland standard of 0.5 and diluted 1 to 100 with fresh medium were added to each set of tubes. The tubes then were incubated at 37°C under anaerobic conditions for 48 h. The broth disk test was performed as described elsewhere in this report.

Determination of MICs. MICs were determined by the agar dilution method as recommended by the National Committee for Clinical Laboratory Standards (8). Inocula were prepared in supplemented thioglycolate broth standardized to match a McFarland standard of 0.5. A multipoint inoculator capable of delivering 1-μl volumes was used to achieve a final inoculum concentration of approximately 10^5 CFU per spot.

Broth disk test. Tests by the broth disk method involved the use of screw cap tubes (20 by 125 mm) containing 5 ml of each of the following three test media: experimental anaerobic broth, BHI-B, and Schaedler broth. A set of four such culture tubes was required per test medium and per test organism: one tube for each of the three antibacterial agents tested and one tube as an untreated control. An appropriate number of susceptibility disks were added to achieve either a concentration of each antibiotic approximating that attainable in blood under normal therapeutic regimens or the concentration equal to the susceptible MIC breakpoint of the test drug: four 10-μg imipenem disks, three 30-μg cefoxitin disks, and three 100-μg piperacillin disks resulting in final test concentrations of 8, 18, and 60 μg/ml, respectively (A. L. Barry and R. R. Packer, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, C306, p. 287; J. H. Jorgensen, J. S. Redding, and V. Holloway, Program Abstr. 23rd Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 548, 1983). A 30-min elution-diffusion period at room temperature was allowed before inoculation of each tube with a drop of an 18- to 24-h culture of each test organism previously grown in the same medium as the final test medium. Tubes were incubated at 37°C in an anaerobic glove box and examined for turbidity both at the 24- and 48-h marks. Results were recorded at the 48-h mark as − (no growth; susceptible), + (greater than 50% of the turbidity of the untreated control; resistant), or ± (ca. 50% of the turbidity of the growth control; or intermediate).

RESULTS AND DISCUSSION

The data (Table 1) for the broth macrodilution test with *B. fragilis* ATCC 25285, *B. thetaiotaomicron* ATCC 29742, and *C. perfringens* ATCC 13124 show a significant decrease, by at least 64-fold, in the antibacterial activity of imipenem in the thioglycolate medium versus the anaerobic broth. In addition, the qualitative results from the broth disk test with the same media and the same reference strains agree completely with the MIC data. These results suggest that at the level of 0.5 mg/ml or 4 mM normally included in the thioglycolate broth, sodium thioglycolate is capable of causing substantial inactivation of imipenem. This phase of the study clearly confirms the unsuitability of thioglycolate broth for testing the susceptibility of anaerobes to imipenem.

The results of the anaerobic susceptibility testing of imipenem, cefoxitin, and piperacillin by three broth disk methods compared with the reference agar dilution method are

TABLE 1. Comparison[a] of the antibacterial activity of imipenem in anaerobic versus thioglycolate broths

Strain	Anaerobic broth		Thioglycolate broth	
	Macrodilution (μg/ml)	Broth disk	Macrodilution (μg/ml)	Broth disk
B. fragilis ATCC 25285	0.25	S	16	R
B. thetaiotaomicron ATCC 29742	0.125	S	16	R
C. perfringens ATCC 13124	0.125	S	128	R

[a] Comparison is made in qualitative (broth disk test) and quantitative (tube macrodilution test) terms. S, Susceptible (no growth); R, resistant (turbidity greater than 50% of the untreated control).

TABLE 2. Anaerobic susceptibility testing of imipenem: broth disk versus agar dilution method

Organism[a]	Drug[b]	Anaerobic susceptibility in[c]:			
		W-C agar	BHI-B	ABE	SDLB
B. fragilis CLA 77	Imipenem	0.016	−	−	−
	Cefoxitin	4.0	−	−	−
	Piperacillin	1.0	−	−	−
B. fragilis CLA 228	Imipenem	0.125	−	−	−
	Cefoxitin	16.0	−	±	−
	Piperacillin	64.0	+	±	−
B. fragilis CLA 68	Imipenem	64.0	+	+	+
	Cefoxitin	128.0	+	+	+
	Piperacillin	64.0	+	+	+
B. distasonis CLA 71	Imipenem	0.03	−	−	−
	Cefoxitin	16.0	+	±	±
	Piperacillin	32.0	±	−	−
B. distasonis CLA 232	Imipenem	0.5	−	−	−
	Cefoxitin	64.0	+	+	+
	Piperacillin	>128.0	+	+	+
B. distasonis CLA 233	Imipenem	0.5	−	−	−
	Cefoxitin	64.0	+	+	+
	Piperacillin	>128.0	+	+	+
B. ovatus CLA 223	Imipenem	0.06	−	−	−
	Cefoxitin	4.0	−	−	−
	Piperacillin	128.0	+	+	+
B. ovatus CLA 236	Imipenem	0.25	−	−	−
	Cefoxitin	128.0	+	+	+
	Piperacillin	>128.0	+	+	+
B. vulgatus CLA 88	Imipenem	0.016	−	−	−
	Cefoxitin	4.0	−	−	−
	Piperacillin	16.0	−	−	−
B. melaninogenicus CLA 182	Imipenem	≤0.004	−	−	−
	Cefoxitin	0.06	−	−	−
	Piperacillin	0.03	−	−	−
B. thetaiotaomicron CLA 83	Imipenem	0.008	−	−	−
	Cefoxitin	4.0	−	−	−
	Piperacillin	1.0	−	−	−
B. thetaiotaomicron CLA 47	Imipenem	0.5	−	−	−
	Cefoxitin	64.0	+	+	+
	Piperacillin	>128.0	+	±	±
B. thetaiotaomicron CLA 235	Imipenem	0.125	−	−	−
	Cefoxitin	32.0	+	+	±
	Piperacillin	64.0	±	−	−
F. mortiferum CLA 137	Imipenem	0.125	−	−	−
	Cefoxitin	4.0	−	−	−
	Piperacillin	32.0	−	−	−
F. nucleatum CLA 168	Imipenem	≤0.004	−	−	−
	Cefoxitin	1.0	−	−	−
	Piperacillin	0.25	−	−	−
C. difficile CLA 52	Imipenem	2.0	−	−	−
	Cefoxitin	32.0	+	+	+
	Piperacillin	8.0	−	−	−
C. difficile CLA 191	Imipenem	2.0	−	−	−
	Cefoxitin	64.0	+	+	+
	Piperacillin	4.0	−	−	−
C. sordellii CLA 122	Imipenem	0.03	−	−	−
	Cefoxitin	0.25	−	−	−
	Piperacillin	0.06	−	−	−
C. tertium CLA 132	Imipenem	2.0	−	−	−
	Cefoxitin	1.0	−	−	−
	Piperacillin	8.0	−	−	−
C. butyricum CLA 133	Imipenem	0.125	−	−	−
	Cefoxitin	2.0	−	−	−
	Piperacillin	0.5	−	−	−
C. perfringens CLA 144	Imipenem	0.008	−	−	−
	Cefoxitin	0.125	−	−	−
	Piperacillin	0.03	−	−	−
C. sporogenes CLA 176	Imipenem	0.06	−	−	−
	Cefoxitin	0.25	−	−	−
	Piperacillin	0.25	−	−	−
Propionibacterium acnes CLA 135	Imipenem	0.125	−	−	−
	Cefoxitin	2.0	−	−	−
	Piperacillin	32.0	−	−	−
E. lentum CLA 181	Imipenem	≤0.004	−	−	−
	Cefoxitin	1.0	−	−	−
	Piperacillin	0.5	−	−	−
A. naeslundii CLA 224	Imipenem	0.008	−	−	−
	Cefoxitin	≤0.004	−	−	−
	Piperacillin	0.03	−	−	−
V. parvula CLA 130	Imipenem	0.125	−	−	−
	Cefoxitin	4.0	−	−	−
	Piperacillin	32.0	−	−	−
Peptostreptococcus anaerobius CLA 107	Imipenem	≤0.004	−	−	−
	Cefoxitin	0.03	−	−	−
	Piperacillin	0.008	−	−	−
Peptococcus magnus CLA 128	Imipenem	≤0.004	−	−	−
	Cefoxitin	0.03	−	−	−
	Piperacillin	0.25	−	−	−
B. fragilis ATCC 25285	Imipenem	0.06	−	−	−
	Cefoxitin	4.0	−	−	−
	Piperacillin	128.0	+	+	±
B. thetaiotaomicron ATCC 29741	Imipenem	0.06	−	−	−
	Cefoxitin	16.0	±	+	±
	Piperacillin	16.0	−	−	−
B. thetaiotaomicron ATCC 29742	Imipenem	0.125	−	−	−
	Cefoxitin	32.0	+	+	±
	Piperacillin	16.0	−	−	−
B. vulgatus ATCC 29327	Imipenem	0.125	−	−	−
	Cefoxitin	2.0	−	−	−
	Piperacillin	0.5	−	−	−
C. perfringens ATCC 13124	Imipenem	0.03	−	−	−
	Cefoxitin	0.125	−	−	−
	Piperacillin	0.03	−	−	−
Peptococcus magnus ATCC 29328	Imipenem	0.016	−	−	−
	Cefoxitin	0.03	−	−	−
	Piperacillin	0.008	−	−	−

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TABLE 2—*Continued*

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TABLE 2—*Continued*

Organism[a]	Drug[b]	Anaerobic susceptibility in[c]:			
		W-C agar	BHI-B	ABE	SDLB
P. variabilis ATCC 14956	Imipenem	0.016	–	–	–
	Cefoxitin	0.06	–	–	–
	Piperacillin	0.016	–	–	–
Peptococcus asaccharolyticus ATCC 29743	Imipenem	0.008	–	–	–
	Cefoxitin	0.03	–	–	–
	Piperacillin	0.008	–	–	–

[a] CLA, Clinical Laboratory Anaerobic collection of Merck & Co., Inc.
[b] The following MIC breakpoints were used: imipenem, 8 μg/ml; cefoxitin, 16 μg/ml; piperacillin, 64 μg/ml.
[c] Anaerobic susceptibility was measured by the MIC (micrograms per milliliter) for Wilkins-Chalgren agar (W-C agar) and by turbidity for BHI-B, anaerobic broth, experimental (ABE), and Schaedler broth (SDLB). Turbidity: +, turbid-growth (resistant); –, clear-nogrowth (susceptible); ±, 50% of the turbidity of the untreated control (intermediate).

summarized in Table 2. A total of 36 bacterial species consisting of 28 clinical isolates and eight quality control strains were tested. For imipenem and cefoxitin, the MIC results for the quality control strains fell well within the expected ranges (8; R. Zabransky, R. Birk, A. Helstad, P. Murray, J. Emmerman, and V. Sutter, Program Abstr. 23rd Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 168, 1983). Of the 36 bacterial cultures tested, 35 cultures or 97.2% were susceptible to imipenem (MIC, ≤8 μg/ml) by both the agar dilution test and each of the three broth disk methods, suggesting a complete agreement among the results of these tests. Except for *B. fragilis* CLA 68 with an MIC of 64 μg/ml and for three clostridial strains (two *C. difficile* and one *C. tertium*) with MICs of 2 μg/ml, all the remaining test strains had MICs of imipenem of ≤0.5 μg/ml. *B. fragilis* CLA 68 was a special isolate received from Tufts-New England Medical Center, Boston, Mass., and was deliberately included in this study because of its known resistance to a variety of antibiotics, including imipenem. To more accurately evaluate an antibacterial agent by the broth disk method for anaerobic activity, it is necessary to include organisms with MICs near the susceptible and resistant MIC breakpoints of the test drug. For imipenem, inclusion in this study of bacterial isolates with MICs in the range of 4 to 16 μg/ml would have been highly desirable. However, a review of the literature coupled with several personal communications with some investigators in the field who have tested imipenem suggest that such strains still are extremely difficult to find.

The correlation among the results obtained by the various testing procedures was also quite good for cefoxitin: 75% of the bacterial strains tested were susceptible by the agar dilution method and 69.4, 66.7, and 69.4% were susceptible by the broth disk test with using BHI-B, anaerobic broth, and Schaedler broth, respectively. Seven of the nine test cultures shown to be resistant to cefoxitin (MIC, ≥32 μg/ml) by the agar dilution test also were resistant to this drug by all three broth disk methods. The other two cultures *B. thetaiotaomicron* CLA 235 and *B. thetaiotaomicron* ATCC 29742 yielded intermediate results in Schaedler broth. It should be pointed out that since a study such as the one reported here requires a deliberate inclusion of both susceptible and resistant bacterial isolates, the computed rates of susceptible strains, i.e., 66.7 to 75% for cefoxitin, appear somewhat lower than what would be expected under normal conditions, i.e., >80% for cefoxitin (11).

Four of the six strains of anaerobic bacteria shown to be resistant to piperacillin (MIC, ≥128 μg/ml) by the agar dilution method also were resistant to this antimicrobial agent by each of the three broth disk tests. The other two strains *B. fragilis* ATCC 25285 and *B. thetaiotaomicron* CLA 47 yielded resistant or intermediate results by the broth disk method when the anaerobic broth, Schaedler broth, or both were used (Table 2). Of the remaining 30 test strains, only *B. fragilis* CLA 68 was susceptible to piperacillin (MIC, ≤64 μg/ml) by the agar dilution procedure and resistant by all three broth disk test systems. The overall agreement between the agar dilution and the broth disk methods for piperacillin was quite good: 83.3% of the strains were susceptible by the MIC test and 72.2, 77.8, and 80.6% were susceptible by the broth disk method with BHI-B, anaerobic broth, and Schaedler broth, respectively.

Tris at 0.2 M concentration has been shown to inactivate imipenem (4), and Schaedler broth contains 0.025 M Tris. The perfect correlation observed between agar dilution and Schaedler broth disk test results (Table 2) suggests that the antibacterial property of imipenem is not noticeably affected by the low level of Tris normally contained in this medium.

It can be concluded from the data herein presented that BHI-B, Schaedler broth, and anaerobic broth can and should be used instead of thioglycolate broth in the broth disk method for in vitro susceptibility testing of anaerobic bacteria to imipenem. However, unlike the aerobically incubated thioglycolate broth (5), the three broth disk test systems evaluated in this study require, as usual, anaerobic incubation or a variation thereof (13).

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