ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 1978, p. 483-487
0066-4804/78/0014-0463$02.00/0
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Vol. 14, No. 3
Printed in U.S.A.

Antimicrobial Susceptibility of Flavobacteria

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Received for publication 10 May 1978

Antimicrobial susceptibility patterns of 28 clinical isolates of *Flavobacterium* sp. were determined by standard disk diffusion technique and by antimicrobial dilution in agar. Rifampin, clindamycin, trimethoprim-sulfamethoxazole, cefoxitin, and vancomycin are among the antimicrobial agents which may be clinically useful to treat infections caused by flavobacteria. All 28 isolates were resistant to erythromycin with minimal inhibitory concentrations of 32 μg/ml or more. Currently recommended interpretive zones of inhibition by disk diffusion did not reliably predict antimicrobial susceptibility of the 28 flavobacteria isolates when compared with the agar dilution technique, and, therefore, a more direct measurement of minimal inhibitory or bactericidal concentration is recommended.

Flavobacteria uncommonly cause human disease but have been associated with such serious infections as endocarditis (11, 16), meningitis (2, 6, 7), bacteremia (10, 12), and pneumonia (13). Selection of an appropriate chemotherapeutic agent for such infections is often difficult because of the typical resistance of these organisms to antimicrobial agents commonly tested against gram-negative bacilli in the clinical microbiology laboratory. In many previous reports disk diffusion susceptibility testing has been utilized, although interpretive zones of inhibition have not been clearly established for many of the commonly used antimicrobial agents against flavobacteria.

This study reports the antimicrobial susceptibilities of 28 clinical isolates of flavobacteria tested by a standard disk diffusion technique and by a standard agar dilution technique.

(This paper was presented in part to the 17th Interscience Conference on Antimicrobial Agents and Chemotherapy, 12 October 1977, New York, N.Y.)

MATERIALS AND METHODS

Twenty-eight isolates of flavobacteria from clinical specimens (24 from sputum, 2 from urine, 1 from blood, 1 from wound) were collected during a 9-month period, September 1975 through May 1976. Duplicate isolates from the same patient were excluded in all but one instance in which isolates from sputum and urine from a patient occurred at different times and had different antimicrobial susceptibility patterns by disk diffusion.

Flavobacteria were identified in the clinical microbiology laboratory by the following criteria: (i) typical colonial morphology and pigmentation, (ii) Gram stain, (iii) typical reactions on Kligler and lysine iron agar slants (K/N), (iv) production of indole and oxidase, and (v) typical oxidation-fermentation (OF) sugar reactions. If the OF mannitol reaction was positive, the isolate was called *Flavobacterium meningosepticum*. Of 28 strains, 6 were identified as *F. meningosepticum*, and the remainder were identified as *Flavobacterium* species.

Antimicrobial susceptibilities of all isolates concomitantly were determined by two methods: (i) standard Kirby-Bauer disk technique (5, 9) and (ii) antimicrobial agent agar dilution procedure (5, 15). Antimicrobial disks used included: penicillin (10 U), methicillin (5 μg), erythromycin (15 μg), cephalothin (30 μg), tetracycline (30 μg), chloramphenicol (30 μg), ampicillin (10 μg), streptomycin (10 μg), kanamycin (30 μg), gentamicin (10 μg), colistin (10 μg), tobramycin (10 μg), cefamandole (30 μg), amikacin (10 μg), clindamycin (2 μg), vancomycin (30 μg), cefoxitin (30 μg), and rifampin (5 μg). (Cefamandole disks were supplied by Eli Lilly & Co.; cefoxitin disks were supplied by Merck Sharp & Dohme; all other disks were purchased from Pfizer Pharmaceuticals.) Plates were read and zone diameters were recorded after overnight incubation at 35°C.

Antimicrobial dilution agar plates were prepared by standard techniques with Mueller-Hinton agar and the following antimicrobial agents: ampicillin sodium (Bristol Laboratories), cephalothin sodium (Eli Lilly & Co.), cefazolin sodium (Eli Lilly & Co.), cefoxitin sodium (Merck Sharp & Dohme), gentamicin sulfate (Schering Corp.), chloramphenicol base (Parke, Davis & Co.), erythromycin gluceptate (Eli Lilly & Co.), clindamycin hydrochloride (Upjohn), tetracycline hydrochloride (Lederle Laboratories), rifampin (Calbiochem), vancomycin hydrochloride (Eli Lilly & Co.), sulfamethoxazole (SMX; Burroughs Wellcome Co.), trimethoprim-sulfamethoxazole (TMP-SMX; Burroughs Wellcome Co.).

Test organisms were incubated overnight at 30°C in Mueller-Hinton broth, then diluted 1 to 20 with Mueller-Hinton broth, and added to the wells of a Steer replicator. Samples of the final dilutions yielded 1.8×10^6 to 3.5×10^6 colony-forming units (CFU) of flavobacteria per ml, 2.9×10^6 CFU of *Escherichia coli* ATCC 25922 per ml, and 5.7×10^5 CFU of *Staphylococcus aureus* ATCC 25923 per ml.

Plates containing antimicrobial agents were stored at 4°C and used within 72 h of preparation. A Steer replicator was used to inoculate the plates. Inoculated plates were incubated at 30°C for 16 to 18 h, and then read. The minimal inhibitory concentration (MIC) was judged to be the lowest concentration at which complete inhibition occurred for all antimicrobial agents except SMX and TMP-SMX. A fine, barely visible haze or a single colony was disregarded. The end points for SMX and TMP-SMX were more difficult to interpret and were read as the lowest concentration which inhibited 80% or more of the growth as judged by visual examination by one of us (R.C.A.). Quality control organisms, *S. aureus* ATCC 25923 and *E. coli* ATCC 25922, were inoculated onto each test plate simultaneously with the *Flavobacterium* sp. strains.

RESULTS

The agar dilution susceptibilities of 28 strains of *Flavobacterium* sp. to 13 antimicrobial agents are given in Tables 1 and 2. All organisms were resistant to ampicillin, cephalothin, cefazolin, tetracycline, chloramphenicol and SMX. Disk susceptibility testing (performed with all of the above except SMX) confirmed the resistance in all instances. Indeed, ampicillin and cephalothin disks produced no zone of inhibition against any of the 28 strains. The diameter of the zones of inhibition produced by tetracycline disks was less than 14 mm for all isolates, and that for chloramphenicol was less than 12 mm for every organism tested.

All organisms were likewise resistant to gentamicin (MIC > 32 μg/ml). However, by disk susceptibility testing, 2 of these isolates would have been considered susceptible (zone diameter ≥ 15 mm) and 13 would have been considered intermediately susceptible (zone diameter, 13 to 14 mm) to gentamicin.

Sixteen isolates would be considered resistant (zone diameter ≤ 14 mm) and six would be intermediately susceptible (15 to 16 mm) to clindamycin. There was no zone of inhibition around the disk with six of the resistant isolates. All 28 isolates were inhibited by ≤8 μg of clindamycin per ml and the majority (25) were inhibited by ≤4 μg/ml, including 4 of the 5 isolates with no zone of inhibition (Table 3).

Three of the 28 isolates would be considered susceptible to erythromycin by disk diffusion testing (zone diameter ≥ 18 mm), but the MICs

TABLE 2. *Susceptibility of 28 strains of flavobacteria to TMP-SMX*

TMP (μg/ml)	SMX (μg/ml)	Cumulative % of strains inhibited
0.019	0.370	
0.037	0.740	
0.075	1.48	
0.150	2.95	18
0.310	5.90	46
0.620	11.8	79
1.25	23.8	100
2.50	47.5	
5.0	95.0	

TABLE 3. *Susceptibility of 28 flavobacteria isolates to clindamycin—zone diameter around 2-μg disk versus MIC*

Zone diam (mm)[a]	No. of isolates susceptible at MIC (μg/ml) of:		
	<1	2-4	8
≤14 (R)	0	13	3
15-16 (I)	1	5	0
≥17 (S)	4	2	0

[a] R, resistant; I, intermediate; S, susceptible.

TABLE 1. *Susceptibility of 28 strains of flavobacteria to 12 antimicrobial agents*

Agent	No. of isolates	Cumulative % of strains inhibited at concn (μg/ml) of agent:									
		0.25	0.5	1.0	2.0	4.0	8.0	16	32	64	128	>128
Ampicillin	28									18	46	100
Cephalothin	28										39	100
Cefazolin	28										71	100
Cefoxitin	28					10	68	96	96	100		
Vancomycin	28						21	100				
Erythromycin	28								21	43	89	100
Clindamycin	28		4	18	36	89	100					
Tetracycline	28							18	82	100		
Chloramphenicol	28									14	100	
Rifampin	28	18	21	61	100							
Gentamicin	28								11	68	79	100
SMX	28								4	7	96	100

of erythromycin for these strains were 32, 64, and 128 μg/ml. Another 11 isolates would be considered intermediately susceptible by disk testing (zone diameter, 14 to 17 mm), but the MIC of erythromycin for each of these organisms was ≥32 μg/ml). No strains were inhibited by less than 32 μg of erythromycin per ml (Table 4).

Thirteen isolates were determined to be susceptible (zone diameter ≥ 18 mm), 13 were intermediately susceptible (15 to 17 mm), and 2 were resistant (≤14 mm) to cefoxitin by disk testing. For 19 strains the MIC of cefoxitin was ≤8 μg/ml, and for 27 it was ≤16 μg/ml (Table 5). The manufacturer (Merck Sharpe & Dohme, West Point, Pa.) currently suggests that ≤15 μg/ml tentatively be considered the susceptible MIC correlate to an 18-mm zone diameter. Our data suggest that such criteria also apply to the strains of *Flavobacterium* sp. included in this study.

All 28 isolates appear susceptible to vancomycin by disk testing (zone diameter ≥ 12 mm; Table 6); however, for no strains was the MIC of vancomycin <5 μg/ml, as would be characteristic of susceptible gram-positive cocci. In contrast to most gram-negative bacilli, the MIC of vancomycin for all *Flavobacterium* sp. was ≤16 μg/ml.

Large zones of inhibition (14 to 29 mm) were produced by the 5-μg rifampin disk, and agar dilution testing confirmed that all strains were inhibited by ≤2 μg of rifampin per ml.

Agar dilution end points for SMX and TMP-SMX were difficult to read, but the combination demonstrated greater activity than SMX alone and inhibited all 28 isolates at achievable serum levels (Table 2).

TABLE 4. *Susceptibility of 28 flavobacteria isolates to erythromycin—zone diameter around 15-μg disk versus MIC*

Zone diam (mm)[a]	No. of isolates susceptible at MIC (μg/ml) of:				
	≤2	4-16	32	64	≥128
≤13 (R)	0	0	1	1	12
14-17 (I)	0	0	4	4	3
≥18 (S)	0	0	1	1	1

[a] R, Resistant; I, intermediate; S, susceptible.

TABLE 5. *Susceptibility of 28 flavobacteria isolates to cefoxitin—zone diameter around 30-μg disk versus MIC*

Zone diam (mm)[a]	No. of isolates susceptible at MIC (μg/ml) of:			
	≤4	8	16	≥32
≤14 (R)	0	0	1	1
15-17 (I)	0	7	6	0
≥18 (S)	3	9	1	0

[a] R, Resistant; I, intermediate; S, susceptible.

TABLE 6. *Susceptibility of 28 flavobacteria isolates to vancomycin—zone diameter around 30-μg disk versus MIC*

Zone diam (mm)[a]	No. of isolates susceptible at MIC (μg/ml) of:			
	<8	8	16	≥32
≤9 (R)	0	0	0	0
10-11 (I)	0	0	0	0
≥12 (S)	0	6	22	0

[a] R, Resistant; I, intermediate; S, susceptible.

Disk testing alone was performed with penicillin, methicillin, colistin, tobramycin, cefamandole, nitrofurantoin, kanamycin, streptomycin, and amikacin. The 28 strains of *Flavobacterium* sp. were resistant by disk testing to all of these agents except streptomycin and kanamycin. Indeed, no zones of inhibition were produced by disks containing penicillin, methicillin, colistin, or tobramycin. Twenty-four of the isolates were resistant (zones of inhibition ≤ 11 mm), and four were intermediately susceptible (12 to 13 mm) to amikacin. Eighteen isolates were resistant (zone diameter ≤ 11 mm), and 10 were intermediately susceptible (12 to 14 mm) to streptomycin.

Comparison of expected and observed MICs for the quality control organisms revealed only one difference of more than a single dilution (tetracycline versus *E. coli* AATC 25922) which might introduce a bias into the results (Table 7). Such a bias would make the test isolates appear more resistant to tetracycline than they truly are.

Of the 28 *Flavobacterium* sp. isolates, 6 were identified as *F. meningosepticum* and did not differ from the other 22 isolates in susceptibility to any of the antimicrobial agents tested by disk diffusion or by agar dilution.

DISCUSSION

When confronted with a patient in whom one believes a *Flavobacterium* sp. isolate may be producing disease, the clinician turns to the microbiology laboratory to assist him in selecting an antimicrobial agent which is likely to be effective. Many microbiology laboratories still use the Kirby-Bauer disk diffusion test and the associated interpretive zone sizes to provide such assistance despite the current trend toward more widespread use of direct measurement of minimal inhibitory or bactericidal concentrations. The data presented herein strongly suggest that for many antimicrobial agents standard interpre-

TABLE 7. *Experimental and reference MICs: quality control organisms used (E. coli ATCC 25922 and S. aureus ATCC 25923)*

Agent	*E. coli* ATCC 25922		*S. aureus* ATCC 25923	
	Observed	Expected	Observed	Expected
Ampicillin	4	2–4	≤0.25	0.06
Cephalothin	8	8	≤0.25	0.12
Cefazolin	1.0	2	0.5	
Cefoxitin	2		2	
Vancomycin	>128		1.0	1.0
Erythromycin	32	32	≤0.25	0.25
Clindamycin	128	64	≤0.25	0.06
Tetracycline	2	0.5	≤0.25	0.12
Chloramphenicol	8		4	4–8
Rifampin	8		≤0.25	
Gentamicin	0.5	0.25	≤0.25	<0.01

tive zone diameters by disk diffusion testing may not accurately predict antimicrobial susceptibility of flavobacteria and, therefore, should be supplemented with or replaced by a direct measurement of MIC to be of greatest assistance to the clinician.

This investigation was not designed to seek the cause(s) of the observed dissociation between the two methods of susceptibility testing employed; however, we observed that many strains of flavobacteria grew slowly at an incubation temperature of 35°C in Mueller-Hinton broth. Thus, unless the suspension used to inoculate the Mueller-Hinton agar plates is carefully controlled by comparison to a turbidity standard, the inoculum may contain less than the recommended 10^8 organisms per ml. Even when the inoculum is standardized appropriately, overnight incubation of the Mueller-Hinton agar plates of 35 to 37°C may impair growth of many flavobacteria.

Rifampin, clindamycin, TMP-SMX, vancomycin, and cefoxitin appear to be among the antimicrobial agents which may be clinically useful to treat infections caused by flavobacteria. Altmann and Bogokovsky (1) demonstrated susceptibility of 11 strains of *F. meningosepticum* to rifampin (MIC ≤ 1.25 μg/ml), erythromycin (MIC ≤ 10 μg/ml), and novobiocin (MIC ≤ 10 μg/ml) and found the same organisms resistant to all penicillins, cephalosporins, and aminoglycosides tested by a tube dilution method. They did not attempt to correlate the MICs with zones of inhibition by the disk diffusion method. It is of some importance that they as well as others (1, 8) have found isolates of *F. meningosepticum* susceptible to erythromycin by direct measurement of MIC in contrast to the results presented herein. It may be that all the isolates from our institution have acquired or developed resistance to erythromycin by a common mechanism and therefore represent a very biased sample of all *Flavobacterium* sp. isolates. Additional studies from other institutions should resolve this issue. Altmann and Bogokovsky used erythromycin lactobionate for determining MICs, whereas we used erythromycin gluceptate; however, the observed MICs for the quality control organisms argue strongly that the activity of erythromycin gluceptate was as it should be. Coyle-Gilchrist et al. (3) reported susceptibility of 10 strains of *F. meningosepticum* to clindamycin (MIC = 4 μg/ml) by an agar dilution technique. They also found all 10 strains resistant to erythromycin and to the penicillins, cephalosporins, and aminoglycosides tested. Many other investigators have reported susceptibility of flavobacteria based upon disk diffusion testing, but we believe that these results must be viewed as "suspicious" sources of valid or clinically useful information.

Von Graevenitz and Grehn (14) have compared the results of disk diffusion with microtube dilution tests to measure the susceptibility of 20 strains of *Flavobacterium* IIb and found discrepancies similar to those presented herein between the two techniques. They also have concluded that the disk diffusion method may be misleading and recommend that a more direct measurement of MIC be utilized.

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