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# Factors Affecting Antimicrobial Susceptibility of *Fusobacterium* Species

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**Fifteen clinical isolates of *Fusobacterium* species were studied to determine their quality of growth on five agar media, their susceptibility to penicillin, cephalothin, cefoxitin, and cefotaxime, the inoculum effect, and the presence of L forms and beta-lactamase. Wilkins-Chalgren agar supported confluent growth best, but *Fusobacterium nucleatum* exhibited poor growth on all agar media. Most isolates exhibited poor reproducibility of MIC results with repeated agar dilution testing. However, most isolates were susceptible to all antibiotics at the breakpoint concentrations. No inoculum effect was observed, but preparation of an inoculum at a 0.5 McFarland nephelometric standard produced a lower than expected number of CFU ($10^6$ CFU) in some isolates. L forms were frequently seen. No beta-lactamase was found. The variability in MICs seen with beta-lactam antibiotics was not found when clindamycin was tested. MIC studies with *Fusobacterium* spp. may be complicated by poor growth on agar media, poor reproducibility, and small inoculum size.**

*Fusobacterium* species are frequent clinical isolates in anaerobic microbiology laboratories. They are found in specimens obtained from brain abscesses, lung abscesses, oral infections, and abdominal abscesses in humans (9, 11). In addition, they are the etiologic agents of necrobacillosis of cattle and lambs, sheep footrot, and necrotic rhinitis in pigs (2, 11). They can be found in pure culture in some of these diseases and often are found in mixed culture (9). As a group, they are the second most frequently isolated gram-negative anaerobic rod-shaped organisms in our clinical laboratory (species of *Bacteroides* are the most frequently isolated organisms). Despite their clinical importance, *Fusobacterium* spp. have not been evaluated extensively for antimicrobial agent susceptibility.

The most widely accepted method of susceptibility testing in anaerobes is the agar dilution method (7). This method has been proposed as the reference standard method, with Wilkins-Chalgren agar as the medium (17, 21). Collaborative evaluation of this method has been encouraging in terms of reproducibility of results; however, no *Fusobacterium* spp. strains were included in the strains tested (18). MIC data obtained by using the agar dilution technique for *fusobacterium* spp. on various agar media have been reported, but the reliability of results obtained for *Fusobacterium* spp. has not been addressed previously (1, 5, 6, 10, 14–16, 19).

Some investigators have reported difficulty in supporting growth of *Fusobacterium* spp. in broth (3) and agar (15) media during susceptibility testing. Also, the occurrence of beta-lactamase (20) in L forms (C. C. Johnson, H. M. Wexler, and S. M. Finegold, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, C96, p. 316) may affect antimicrobial agent susceptibility in *Fusobacterium* spp. We evaluated the susceptibility to antimicrobial agents of *Fusobacterium* spp. with respect to optimum medium for support of growth, presence of any inoculum effect, reproducibility of agar dilution susceptibility data, presence of beta-lactamase, and presence of L forms.

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## MATERIALS AND METHODS

**Organisms.** The clinical isolates obtained from our clinical laboratories included four *Fusobacterium nucleatum* strains, three *Fusobacterium necrophorum* strains, one *Fusobacterium varium* strain, one *Fusobacterium necrogenes* strain, and six other strains of fusobacteria that produced major amounts of butyric acid and minor amounts of acetic and propionic acids from glucose but did not fit the biochemical profile of a specific species as specified by the *Anaerobe Laboratory Manual* (12). The following *Bacteriodes* strains were used as controls in all experiments: *Bacteriodes fragilis* ATCC 25285 and *Bacteroides thetaiotaomicron* ATCC 29741.

**Media.** The growth characteristics of strains were studied on Wilkins-Chalgren agar, brucella agar, brucella agar supplemented with laked sheep blood, brain heart infusion agar supplemented with yeast extract, vitamin K, hemin, and laked sheep blood, and Columbia blood agar supplemented with laked sheep blood. All media were obtained from Difco Laboratories, Detroit, Mich. Each agar medium was inoculated with *Fusobacterium* sp. cells by using a replicating device of the Steers type (Melrose Machine Shop, Woodlyn, Pa.). After 48 h of incubation at 37°C, the results were noted and recorded as no growth, growth without confluence, or confluent growth. Confluent growth was defined as smooth growth covering the entire area of the inoculum with a density that was the same as the density of the two *Bacteroides* spp. control strains.

**Inoculum.** Three organisms were used to evaluate the effect of inoculum size on the MIC. The number of CFU of each strain in a culture adjusted to a 0.5 McFarland standard was determined by diluting the culture by 10-fold increments ($10^3$ to $10^6$) and evenly spreading a 0.1-ml sample of each dilution on a Wilkins-Chalgren agar plate. The colonies were counted after incubation for 48 h. The number of colonies was then multiplied by the dilution factor to obtain the number of CFU in the original inoculum. In the experiments in which we evaluated the effect of inoculum size on the

TABLE 1. Effect of type of agar on growth of *Fusobacterium* species

| Organisms[a] | No. of strains | Type of agar[b] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | WC | | LBCBA | | LBBHI | | Br | | LBBr | |
| | | %[c] | No.[d] | %[c] | No.[d] | %[c] | No.[d] | %[c] | No.[d] | %[c] | No.[d] |
| *F. nucleatum* | 4 | 77 | 47 | 66 | 56 | 58 | 69 | 64 | 58 | 69 | 55 |
| Other *Fusobacterium* species | 11 | 99 | 111 | 92 | 132 | 91 | 142 | 87 | 101 | 78 | 118 |
| All *Fusobacterium* spp. strains tested | 15 | 92 | 158 | 85 | 188 | 80 | 211 | 79 | 159 | 75 | 173 |

[a] Each isolate represented an approximately equal proportion of the inoculations on each agar.
[b] WC, Wilkins-Chalgren agar; LBCBA, Columbia blood agar containing laked blood; LBBHI, brain heart infusion agar containing laked blood; Br, brucella agar; LBBr, brucella agar containing laked blood.
[c] Percentage of inoculations yielding confluent growth.
[d] Number of inoculations tested.

variability of the MIC, the inoculum delivered to the surface of the antibiotic-containing plates was a $10^3$ or $10^6$ dilution of a culture adjusted to a 0.5 McFarland standard.

**Reproducibility.** MICs for each strain were determined a minimum of two times and a maximum of seven times on Wilkins-Chalgren agar. Each MIC was determined by using the protocol outlined in the proposed reference dilution procedure for antimicrobial agent susceptibility testing of anaerobic bacteria from the National Committee for Clinical Laboratory Standards (17, 18). All inoculations were made in an anaerobic chamber. The two *Bacteroides* spp. strains were processed in exactly the same method as the *Fusobacterium* spp. strains in each MIC test.

The MIC for each strain was the lowest concentration of drug yielding no growth, one discrete colony, or a fine, barely visible haze as determined with the unaided eye. We also determined a second endpoint, namely, the lowest concentration of drug that yielded no growth or one colony; a fine haze was read as evidence of growth.

All 15 organisms were tested against penicillin, cephalothin, cefoxitin, and cefotaxime. Five of the organisms were tested against clindamycin.

**Beta-lactamase.** Each strain was examined for the presence of beta-lactamase by using a nitrocefin solution and PADAC strips (4, 6, 8). Two *B. fragilis* strains were used as controls; one was always strongly positive, and one was never positive. The nitrocefin solution was subsequently used to screen for induced beta-lactamase following exposure of the organisms to cefoxitin at concentrations of 0.1, 1, and 10 μg/ml. In addition, each broth culture was sonicated to disrupt the cells and centrifuged to separate cellular debris from intracellular contents. Nitrocefin was used to screen cellular debris and the intracellular soluble contents of induced and noninduced cultures for beta-lactamase.

**L forms.** During two determinations of MIC, each strain was examined for L-form colony morphology by using Dienes stain (13) at the next four consecutive concentrations of antimicrobial agent above the MIC for that strain. Each strain was evaluated as forming either no variant colonies or L-form variant colonies consisting of fried egg colonies when viewed under ×500 magnification after staining. All of the observations regarding L forms were made on Wilkins-Chalgren agar.

## RESULTS

**Media.** Of the five media tested (Table 1), Wilkins-Chalgren agar supported confluent growth in 126 of 158 inoculations (92%). This was significantly better than all other agar media ($P < 0.05$, chi-square test). Columbia blood agar supplemented with laked sheep blood was next in the percentage of inoculations with confluent growth (159 of 188 inoculations [85%]). The other three agar media fared less well, with 169 occurrences of confluent growth in 211 inoculations (80%) for brain heart infusion agar supplemented with yeast extract, vitamin K, hemin, and laked sheep blood, 125 occurrences in 159 inoculations (79%) for brucella agar, and 130 occurrences in 173 inoculations (75%) for brucella agar supplemented with laked sheep blood. Each agar medium supported the growth of *F. nucleatum* strains less well than the growth of the other species ($P < 0.001$). Each medium yielded confluent growth for all inoculations with both of the *Bacteroides* spp. control strains. Seven *Fusobacterium* spp. strains also yielded confluent growth with all inoculations on each medium.

**Reproducibility.** The MICs for the two control *Bacteroides* spp. strains were within 1 $\log_2$ dilution of the previously published mode MIC for each antimicrobial agent (18) for each MIC determination (27 measurements). In contrast, in concurrent experiments, a broader range of MICs was observed for most *Fusobacterium* spp. strains. Only the one strain of *F. varium* tested was within a 2-$\log_2$ dilution range for all MIC determinations with all antibiotics. The difference from the lowest MIC to the highest MIC for each isolate on multiple determinations varied from 1 to 12 $\log_2$ dilutions for penicillin, from 2 to 9 $\log_2$ dilutions for cephalothin, from 2 to 16 $\log_2$ dilutions for cefoxitin, and from 1 to 9 $\log_2$ dilutions for cefotaxime (Table 2). The median differences from the low MIC to the high MIC for each strain expressed in $\log_2$ dilutions were as follows: penicillin, 4; cephalothin, 5; cefoxitin, 3; and cefotaxime, 4. This variability did not affect antimicrobial agent susceptibility when it was defined by susceptibility at the following breakpoints: penicillin, 12.5 U/ml; cephalothin, 16 μg/ml; cefoxitin, 16 μg/ml; cefotaxime, 16 μg/ml; and clindamycin, 4 μg/ml (6, 10). When these breakpoints were used, no isolate was resistant during any measurement for penicillin or clindamycin, and none varied between susceptible and resistant to cefotaxime. Three

TABLE 2. Variability of susceptibility to antimicrobial agents of 15 isolates of *Fusobacterium* species

| Antimicrobial agent | No. of isolates with the following $\log_2$ dilution differences between the lowest and highest measured MICs | | | | | |
|---|---|---|---|---|---|---|
| | >5 | 5 | 4 | 3 | 2 | 1 |
| Penicillin | 4 | 2 | 3 | 1 | 1 | 4 |
| Cephalothin | 8 | 2 | 2 | 0 | 3 | 0 |
| Cefoxitin | 4 | 1 | 2 | 3 | 5 | 0 |
| Cefotaxime | 3 | 2 | 3 | 2 | 1 | 4 |

isolates were always resistant to the breakpoint concentration of cefotaxime. Two isolates varied between susceptible and resistant on repeated measurements to cephalothin and cefoxitin (Table 3). Those isolates that were less susceptible as defined by the mode of MIC determinations tended to have less difference between the lowest and highest MIC results than those isolates that were more susceptible as judged by their mode of MICs.

The MICs for the five isolates tested against clindamycin revealed less variability overall. The greatest difference from lowest MIC to highest MIC for these isolates was 3 $\log_2$ dilutions. The same isolate had a difference of 14 $\log_2$ dilutions between the lowest and highest cephalothin MIC determinations.

**Inoculum.** Diluting the inoculum by 100 did not alter the MIC by more than 3 dilutions. The isolates which showed wide variability during MIC testing tended to clump when they were grown in broth, although the clumps dispersed when the preparations were mixed with a Vortex mixer. These isolates had fewer CFU in broth adjusted to a 0.5 McFarland nephelometric standard after the use of a Vortex mixer than did isolates with less variability in MIC tests. The average value for the *Bacteriodes* spp. strains was $3 \times 10^8$ CFU/ml, whereas the *Fusobacterium* spp. isolates with large amounts of MIC variability averaged $3 \times 10^6$ CFU/ml and those with little MIC variability averaged $3 \times 10^7$ CFU/ml.

For some MIC determinations, the endpoint seemed to be less obvious than the endpoint for the *Bacteroides* spp. control strains. When a barely visible haze was interpreted as evidence for growth, the MIC increased severalfold. The variability with multiple determinations was even greater when this endpoint was used. The data recorded here were derived by using conventional endpoint criteria.

**Beta-lactamase.** There was no beta-lactamase found in any isolate by the nitrocefin technique or by PADAC strips. In addition, induction by cefoxitin did not reveal a beta-lactamase, nor did disruption of the cells.

**L forms.** Seven strains formed classic L-form fried egg colonies when they were stained with Dienes stain. Only one strain formed these colonies with several different antibiotics, and this strain showed wide variability in its MIC results. However, other strains that formed L forms did not show wide variability in their MIC results.

## DISCUSSION

Most isolates of *Fusobacterium* spp. appear to be susceptible to penicillin, cefoxitin, and clindamycin, which are commonly used to treat anaerobic bacterial infections. A few isolates can be resistant to these drugs and are usually members of rarely isolated species, such as *F. varium* and *Fusobacterium mortiferum* (9). Strains of the more commonly isolated species *F. nucleatum* and *F. necrophorum* are almost invariably susceptible to the drugs mentioned above when they are studied by in vitro testing (19).

TABLE 3. Susceptibility of *Fusobacterium* species to breakpoint concentrations of antimicrobial agents

| Antimicrobial agent | No. of isolates tested | % Of isolates susceptible at breakpoint on three determinations[a] | | |
|---|---|---|---|---|
| | | For lowest MIC determination | For mode MIC determination | For highest MIC determination |
| Penicillin | 15 | 100 | 100 | 100 |
| Cephalothin | 15 | 94 | 80 | 80 |
| Cefoxitin | 15 | 100 | 100 | 87 |
| Cefotaxime | 15 | 80 | 80 | 80 |
| Clindamycin | 5 | 100 | 100 | 100 |

[a] The breakpoints were as follows: penicillin, 12.5 U/ml; cephalothin, 16 μg/ml; cefoxitin, 16 μg/ml; cefotaxime, 16 μg/ml; and clindamycin, 4 μg/ml.

We found that the best medium to support the growth of our clinical isolates of *Fusobacterium* species was Wilkins-Chalgren agar. This medium virtually always supported the growth of *Fusobacterium* species other than *F. nucleatum* and did as well as the other media tested in supporting *F. nucleatum* growth. Still, the growth of *F. nucleatum* on all media was disappointing.

When Wilkins-Chalgren agar medium was used, repeated agar dilution testing of 15 *Fusobacterium* spp. isolates showed that the MICs varied more than 3 $\log_2$ dilutions for many isolates. This variability was only rarely clinically significant since the isolates were usually susceptible to concentrations of the antimicrobial agent that were lower than the breakpoint concentration, even when the MIC results varied greatly in multiple determinations.

The exact reason for the MIC variability is uncertain. The isolates that were most variable in repeated MIC tests tended to form clumps when they were grown in broth media and to have fewer CFU per milliliter when they were diluted to a nephelometric standard than those isolates that were not variable in repeated susceptibility tests. However, the MICs did not change significantly with dilution of the inoculum. Therefore, there does not appear to be a prominent inoculum effect in susceptibility testing of *Fusobacterium* spp. by the agar dilution method.

Seven isolates formed L forms. A few isolates produced L forms spontaneously without the presence of antibiotics in the media. L forms were not observed in isolates grown in the presence of clindamycin. Since L forms were present during growth in media containing beta-lactam antimicrobial agents and were not present with clindamycin, they may account for some of the MIC variability seen with the beta-lactam agents but not with clindamycin. However, this does not account for the MIC variability seen with several strains in which L forms were not documented.

A novel penicillinase has been described from some penicillin-resistant *F. nucleatum* isolates (20). No constitutive, inducible, or cell bound beta-lactamase was found in the strains which we studied.

It is apparent that further studies need to be done on the antimicrobial agent susceptibility of *Fusobacterium* spp. isolates. The factors of poor growth of *Fusobacterium* spp. in most media, low numbers of CFU in inocula prepared to nephelometric standards, and variable results may play a role in determining antimicrobial agent susceptibility by most methods. Since most *Fusobacterium* spp. found resistant by the agar dilution technique have reproducible MIC results and since susceptibility as defined by breakpoint criteria appears to be reproducible, the agar dilution technique is probably acceptable for susceptibility studies of *Fusobacterium* spp. in clinical microbiology laboratories.

## LITERATURE CITED

1. **Bansal, M. B., S. K. Chuah, and H. Thadepalli.** 1981. Susceptibility of intestinal anaerobes to new beta-lactam antibiotics. Chemotherapy (Basel) **30**:237–243.
2. **Blood, D. C.** 1983. Diseases caused by *Fusobacterium* spp., p. 661–672. *In* D. C. Blood, O. M. Radostits, and J. A. Henderson (ed.), Veterinary medicine: a textbook of the diseases of cattle, sheep, pigs, goats, and horses, 6th ed. Bailliere Tindall, London.
3. **Bourgault, A.-M., and F. Lamothe.** 1984. Comparison of anaerobic susceptibility results obtained by two methods of inoculum preparation. J. Clin. Microbiol. **20**:1060–1064.

4. **Bourgault, A.-M., and J. E. Rosenblatt.** 1979. Characterization of anaerobic gram-negative bacilli by using rapid slide tests for β-lactamase production. J. Clin. Microbiol. **9**:654–656.
5. **Chow, A. W., and S. M. Finegold.** 1982. In-vitro activity of ceftizoxime against anaerobic bacteria and comparison with other cephalosporins. J. Antimicrob. Chemother. **10**(Suppl. C):45–50.
6. **Del Bene, V. E., P. J. Carek, J. A. Twitty, and L. J. Burkey.** 1985. In vitro activity of cefbuperazone compared with that of other new β-lactam agents against anaerobic gram-negative bacilli and contribution of β-lactamase to resistance. Antimicrob. Agents Chemother. **27**:817–820.
7. **Finegold, S. M., and R. D. Rolfe.** 1983. Susceptibility testing of anaerobic bacteria. Diagn. Microbiol. Infect. Dis. **1**:33–40.
8. **Gabay, E. L., V. L. Sutter, and S. M. Finegold.** 1981. Rapid beta-lactamase testing in *Bacteroides*. J. Antimicrob. Chemother. **8**:413–416.
9. **George, W. L., B. D. Kirby, V. L. Sutter, D. M. Citron, and S. M. Finegold.** 1981. Gram-negative anaerobic bacilli: their role in infection and patterns of susceptibility to antimicrobial agents. II. Little-known *Fusobacterium* species and miscellaneous genera. Rev. Infect. Dis. **3**:599–626.
10. **Hoffler, U., and G. Pulverer.** 1984. Antibiotic sensitivity testing of anaerobic bacteria by the breakpoint method. Chemotherapy (Basel) **30**:392–397.
11. **Hofstad, T.** 1981. The genus *Fusobacterium*, p. 1464–1467. *In* M. P. Starr (ed.), The prokaryotes: a handbook on habitats, isolation, and identification of bacteria. Springer-Verlag, New York.
12. **Holdeman, L. V., E. P. Cato, and W. E. C. Moore (ed.).** 1977. Anaerobe laboratory manual, 4th ed. Virginia Polytechnic Institute and State University, Blacksburg.
13. **Madoff, S.** 1981. The L-forms of bacteria, p. 2225–2237. *In* M. P. Starr (ed.), The prokaryotes: a handbook on habitats, isolation, and identification of bacteria. Springer-Verlag, New York.
14. **Nord, C. E., and B. Olsson-liljequist.** 1984. Anaerobic bacteria and beta-lactam antibiotics. Scand. J. Infect. Dis. Suppl. **42**:72–82.
15. **Owens, W. E., and S. M. Finegold.** 1983. Comparative in vitro susceptibilities of anaerobic bacteria to cefmenoxime, cefotetan, and *N*-formimidoyl thienamycin. Antimicrob. Agents Chemother. **23**:626–629.
16. **Rolfe, R. D., and S. M. Finegold.** 1982. Comparative in vitro activity of ceftriaxone against anaerobic bacteria. Antimicrob. Agents Chemother. **22**:338–341.
17. **Sutter, V. L.** 1985. Susceptibility testing of anaerobes, p. 988–990. *In* E. H. Lennette, A. Balows, W. J. Hausler, Jr., and H. J. Shadomy (ed.), Manual of clinical microbiology, 4th ed. American Society for Microbiology, Washington, D.C.
18. **Sutter, V. L., A. L. Barry, T. D. Wilkins, and R. J. Zabransky.** 1979. Collaborative evaluation of a proposed reference dilution method of susceptibility testing of anaerobic bacteria. Antimicrob. Agents Chemother. **16**:495–502.
19. **Sutter, V. L., and S. M. Finegold.** 1976. Susceptibility of anaerobic bacteria to 23 antimicrobial agents. Antimicrob. Agents Chemother. **10**:736–752.
20. **Tunér, K., L. Lindqvist, and C. E. Nord.** 1985. Purification and properties of a novel β-lactamase from *Fusobacterium nucleatum*. Antimicrob. Agents Chemother. **27**:943–947.
21. **Wilkins, T. D., and S. Chalgren.** 1976. Medium for use in antibiotic susceptibility testing of anaerobic bacteria. Antimicrob. Agents Chemother. **10**:926–928.