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In Vitro Synergism between Cefotaxime and Minocycline against *Vibrio vulnificus*

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We conducted time-kill studies to evaluate the inhibitory activities of either cefotaxime or minocycline alone and the two drugs in combination against a clinical strain of *Vibrio vulnificus*. The MICs of cefotaxime and minocycline were 0.03 and 0.06 μg/ml, respectively. When approximately 5×10^5 CFU of *V. vulnificus* per ml was incubated with cefotaxime at 0.03 or 0.05 μg/ml, the bacterial growth was inhibited during the initial 2 and 8 h, respectively. Thereafter, *V. vulnificus* regrew and the level of growth reached that of the control. Within the dose range of less than five times the MIC, the duration of the inhibitory effect of cefotaxime was proportional to its concentration. When minocycline at 0.015, 0.03, 0.045, and 0.06 μg/ml was used to evaluate the inhibitory effect, a similar trend was observed. Either antibiotic at a concentration of five times the MIC or greater prevented the regrowth of *V. vulnificus* for at least 48 h. When cefotaxime at 0.05 μg/ml and minocycline at 0.045 μg/ml were combined in the same culture, the inhibitory effect against *V. vulnificus* persisted for more than 48 h, with no regrowth noted. The use of a combination of these two antibiotics resulted in the reduction of growth by 6 orders of magnitude compared to the use of either of the two antibiotics alone, and the number of surviving organisms in the presence of the antibiotics combined was approximately 3 orders of magnitude less than that in the starting inoculum. We conclude that cefotaxime and minocycline acted synergistically in inhibiting *V. vulnificus* in vitro.

Vibrio vulnificus is a halophilic gram-negative bacillus recovered from various marine and brackish environments (17). Many cases of *V. vulnificus* infections have been reported in coastal areas of the United States (1, 2, 18) and Taiwan (5, 7, 8). Frequently encountered clinical manifestations of *V. vulnificus* infection include primary septicemia, wound infection, and gastroenteritis (2, 5, 7, 8, 18). Other occasionally reported clinical manifestations include pneumonia (16) and spontaneous bacterial peritonitis (27). Primary septicemia is often complicated by severe soft-tissue infections such as necrotizing fasciitis and myonecrosis coupled with hemorrhagic bullous formation (2, 7, 8, 12, 18, 25, 27, 28). The mortality rate from primary septicemia exceeds 50% (2, 8, 12, 18), and the median interval from the time of hospitalization to death is approximately 2 days (2, 8). Treatment includes antibiotic administration and surgical debridement, when necessary.

Most of the *V. vulnificus* isolates are susceptible in vitro to a variety of antibiotics (1, 3, 13, 15, 16). As a result, the use of various antibiotics, based on the in vitro susceptibility of the organism, has been reported (1, 2, 15, 18). Although studies with a murine model of *V. vulnificus* septicemia induced by intraperitoneal injection of the bacteria showed that tetracycline is superior to cefotaxime (3), our own clinical experiences have indicated that the broad-spectrum cephalosporins may be clinically superior to tetracycline (6–8). The currently recommended treatment for *V. vulnificus* infections has been unsatisfactory. Sanford et al. (23) have proposed the combined use of tetracycline and a broad-spectrum cephalosporin for the treatment of *V. vulnificus* infections.

In order to demonstrate which one of the two antibiotics, cefotaxime or minocycline, is the better in vitro inhibitor of *V. vulnificus* and to ascertain whether or not their use in combination elicits better activity than the use of either antibiotic alone, we conducted a time-kill study to evaluate synergy (4).

MATERIALS AND METHODS

Bacterial strains. Forty-two clinical isolates of *V. vulnificus* recovered from 42 consecutive patients were collected in our institution. These strains were originally isolated from blood, wounds, or bullous fluid. All isolates were identified as *V. vulnificus* by positive tests for cytochrome oxidase, glucose fermentation, citrate utilization, indole production, ornithine decarboxylase, and hydrolysis of *ortho*-nitrophenyl galactoside and by growth in broth plus 3% sodium chloride. The organism was stored at −70°C in Luria-Bertani broth (Difco Laboratories, Detroit, Mich.) before being cultured on nutrient agar (Difco Laboratories) with 3% sodium chloride. Except for 1 isolate, these 42 clinical isolates, as tested by the disk diffusion method, showed no apparent difference in their sensitivities to antibiotics. Isolate *V. vulnificus* 20 was randomly chosen from an initially selected set of three isolates that had no substantive difference in growth characteristics in response to antibiotic treatments. *V. vulnificus* 20 was selected for use throughout the study.

Antimicrobial susceptibility test. The in vitro susceptibilities of the 42 isolates of *V. vulnificus* to the following antibiotics were determined: ampicillin (Boehringer Mannheim GmbH, Mannheim, Germany), ceftriaxone (Hoffmann-La Roche, Nutley, N.J.), ceftazidime (Glaxo, Greenford, England), cefotaxime (Hoechst AG, Frankfurt, Germany), cefoperazone (Pfizer Inc., New York, N.Y.), moxalactam (Shionogi Pharmaceutical Co., Lt., Osaka, Japan), imipenem (Merck Sharp & Dohme, West Point, Pa.), ofloxacin (Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan), minocycline (American Cyanamid Co., Pearl River, N.Y.), and gentamicin (Sigma Chemical Co., St. Louis, Mo.). The MIC of each antibiotic was determined by the agar dilution method with Mueller-Hinton agar (Difco Laboratories). The drugs were incorporated into the agar in serial twofold concentrations, as follows: ampicillin, 0.25 to 32 μg/ml; ceftriaxone, 0.03 to 64 μg/ml; ceftazidime, 0.03 to 32 μg/ml; cefotaxime, 0.03 to 64 μg/ml; cefoperazone, 0.03 to 64 μg/ml; moxalactam, 0.03 to 64 μg/ml; imipenem, 0.03 to 16 μg/ml; ofloxacin, 0.03 to 8 μg/ml; minocycline, 0.06 to 16 μg/ml; and gentamicin, 1 to 16 μg/ml. The bacterial inocula were prepared, and the MIC was defined in accor-

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TABLE 1. Susceptibilities of 42 isolates of *V. vulnificus* to 10 antimicrobial agents

Antimicrobial agent	MIC (μg/ml)		
	50%	90%	Range
Ampicillin	1.0	1.0	0.25–2.0
Ceftriaxone	≤0.03	≤0.03	0.03–0.12
Ceftazidime	1.0	2.0	1.0–32.0
Cefotaxime	≤0.03	0.06	≤0.03–1.0
Cefoperazone	0.06	0.12	≤0.03–2.0
Moxalactam	0.25	0.5	0.25–32.0
Imipenem	0.12	0.12	0.06–0.12
Ofloxacin	0.12	0.12	0.06–8.0
Minocycline	0.06	0.25	0.06–0.25
Gentamicin	2.0	4.0	1.0–8.0

dance with the procedures of the National Committee for Clinical Laboratory Standards (20). Final inocula of approximately 10^4 CFU per spot were applied onto the plates with a Steers replicator. Plates were incubated in ambient air at 35°C for 24 h. MICs were the lowest concentrations of antibiotics giving complete inhibition of visible growth of the organism. *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used in each run as controls for susceptibility testing.

Determination of inhibitory effect of cefotaxime and minocycline, alone and in combination, against *V. vulnificus* in time-kill kinetics. Bacteria were diluted to about 5.0×10^5 CFU/ml in 50 ml of fresh Mueller-Hinton broth in a 250-ml glass conical flask for each of the concentrations of the drugs tested. Minocycline was selected for the time-kill studies because tetracycline, a similar compound, was usually recommended for use in the treatment of *V. vulnificus* infection. Cefotaxime was chosen because our clinical experiences indicated that broad-spectrum cephalosporins were efficacious for the treatment of *V. vulnificus* infection. Various concentrations of cefotaxime and minocycline were prepared, and each drug was placed in a separate flask at the indicated concentrations: for cefotaxime, 0.03, 0.05, 0.075, 0.15, and 0.3 μg/ml; for minocycline, 0.015, 0.03, 0.045, 0.06, 0.3, and 0.6 μg/ml. Each flask was incubated under the conditions mentioned above. Bacterial counts were measured at 0, 2, 4, 6, 8, 12, 24, 30, 36, and 48 h by enumerating the number of colonies from 10-fold serially diluted specimens of 100-μl aliquots plated on nutrient agar (Difco Laboratories). The plates were then incubated overnight under ambient conditions at 35°C. Antibiotic carryover effects were minimized by transferring aliquots of 100 μl and/or using serial dilutions (11, 21, 26); thus, no additional assessments were carried out. To evaluate the synergistic effects of the antibiotics in combination, we purposefully selected the concentration of the individual antibiotic which had only minimal inhibitory activity on the microorganism when the antibiotic was used alone. The usual criterion was that the concentration was one that would inhibit bacterial growth only minimally, usually less than 2 $\log_{10}$ compared with the growth of the control at 48 h. Different concentrations of each antibiotic (all of them at less than five times the MIC) were tested in pairs to determine the effects of the antibiotics in combination. Synergism was defined as a ≥2 $\log_{10}$ reduction in the numbers of CFU per milliliter below the starting inoculum by use of the combination of drugs compared with the reduction in the numbers of CFU per milliliter by use of the more active single constituent after 24 h. For statistical accuracy, the lower limit of the viable counts was set at 30 colonies (300 CFU/ml). This threshold was used in all experiments. All the experiments were performed at least twice for confirmation of the results.

RESULTS

MICs. The MICs of 10 antimicrobial agents for 42 strains of *V. vulnificus* are presented in Table 1. All antibiotics tested showed good in vitro activity against all except one of the isolates (strain 26). The strain was fourfold or greater less sensitive to all of the antibiotics tested. The MICs of cefotaxime and minocycline for strain 20 were 0.03 and 0.06 μg/ml, respectively.

Determination of inhibitory effects of cefotaxime and minocycline, alone and in combination, against *V. vulnificus* in time-kill kinetics. All of the cefotaxime concentrations tested alone were at the MIC or higher, while three of the six concentrations of minocycline tested were below the MIC. Cefotaxime at 0.03 μg/ml elicited an inhibitory effect at 2 h, but thereafter, the microorganism regrew and proliferated to an extent approaching the growth curve for the control at 24 h. The higher

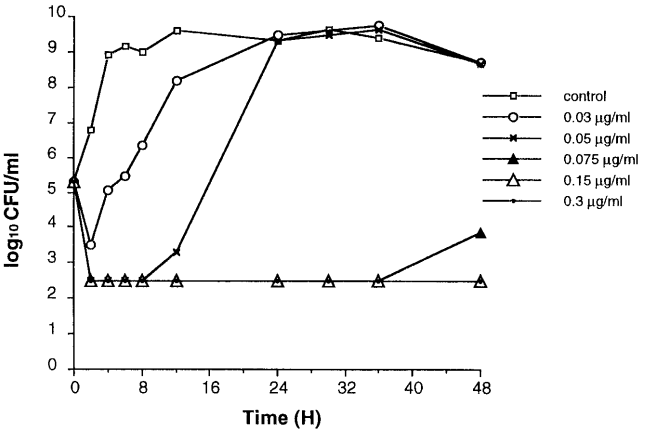

FIG. 1. Inhibition of growth curves for *V. vulnificus* 20 after incubation with different concentrations of cefotaxime at a starting inoculum of 5×10^5 CFU/ml. The cefotaxime MIC was 0.03 μg/ml.

the cefotaxime concentrations below five times the MIC, the earlier, greater, and longer the duration of the inhibitory effect (Fig. 1). Minocycline at 0.015 μg/ml elicited an inhibitory effect during the initial 2 h, but after that bacterial regrowth began and eventually approached the growth curve for the control. The same trend was observed at concentrations of 0.03, 0.045, and 0.06 μg/ml, which were at or below the MIC (Fig. 2). As with cefotaxime, the higher the minocycline concentration, the earlier, the greater, and the longer the duration of the inhibitory effect. However, no regrowth was observed for at least 48 h with cefotaxime at ≥0.15 μg/ml (five times the MIC) or minocycline at ≥0.3 μg/ml (five times the MIC). Cefotaxime at 0.05 μg/ml (5/3 times the MIC) elicited an inhibitory effect during the initial 8 h, and then the bacteria regrew and reached the level of growth of the control at 24 h. Minocycline at 0.045 μg/ml (3/4 times the MIC) showed an inhibitory effect for the initial 24 h but regrew beginning at 24 h, and growth reached that of the control at 36 h. When cefotaxime at 0.05 μg/ml and minocycline at 0.045 μg/ml were combined, a reduction of growth by approximately 3 orders of magnitude compared to that for the starting inoculum and by 6 orders of magnitude compared to that with either of the two antibiotics used alone was observed, and the growth inhibitory effect persisted for up to 48 h (Fig. 3).

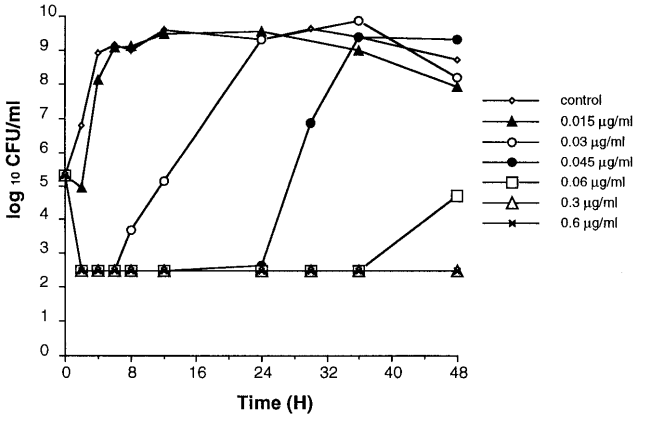

FIG. 2. Inhibition of growth curves for *V. vulnificus* 20 after incubation with different concentrations of minocycline at a starting inoculum of 5×10^5 CFU/ml. The minocycline MIC was 0.06 μg/ml.

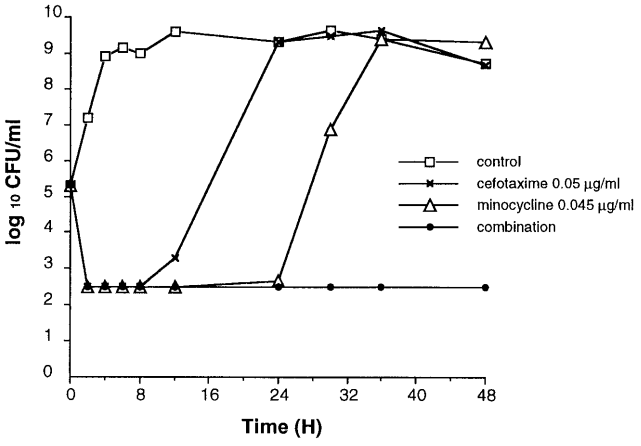

FIG. 3. Inhibition of growth curves for *V. vulnificus* 20 after incubation with either cefotaxime or minocycline alone, or the two in combination, at a starting inoculum of 5×10^5 CFU/ml. MICs were 0.03 μg/ml for cefotaxime and 0.06 μg/ml for minocycline.

DISCUSSION

Our results indicate that *V. vulnificus* is susceptible in vitro to a variety of antimicrobial agents. These results are similar to those reported by Hsueh et al. (13), except that the MICs of cefotaxime, ceftazidime, imipenem, minocycline, and ofloxacin at which 90% of isolates are inhibited (MIC_{90}s) were two to eight times lower than those of Hsueh et al. (13). However, Hsueh et al. (13) tested only 19 clinical isolates from the same geographic areas in Taiwan. French et al. (10) reported that a large proportion of their strains tested were resistant to ampicillin (MIC_{90}s ≥128 μg/ml). This difference can be attributed to variations in the geographic origins of the isolates tested.

At an inoculum size of 5×10^5 CFU/ml and a concentration of less than five times the MIC, the inhibitory effects of each of cefotaxime and minocycline can be demonstrated initially, but regrowth occurs. The bacteria growing back formed smaller colonies, and their growth rates were slower than those of the original bacteria, yet microscopically they showed no morphological changes.

Tetracycline has been recommended as the antimicrobial agent of choice for the treatment of *V. vulnificus* infection. Morris and Tenney (19) stressed the superiority of tetracycline over cefotaxime on the basis of a study with a mouse model conducted by Bowdre et al. (3). Morris and Tenney (19) extrapolated the effectiveness of tetracycline for treating *V. vulnificus* from the experience with *Vibrio cholerae*. Fang (9) advocated the use of tetracycline for the treatment of *V. vulnificus* infections because an antibiotic which could inhibit protein synthesis was thought to be preferable to one which damages the cell wall and causes the release of increased levels of toxic microbial proteins. On the other hand, our clinical experience has indicated that cephalosporins were the better choice for the treatment of *V. vulnificus* infections (6). Jawetz and Gunnison (14) observed that the bacteriostatic antibiotics may antagonize the actions of bactericidal drugs, and this conclusion has been observed as an important general principle of antibiotic therapy since then (22). However, in this study, when cefotaxime at 0.05 μg/ml (5/3 times the MIC) was combined with minocycline at 0.045 μg/ml (3/4 times the MIC), persistent inhibitory effects were noted for at least 48 h. The magnitude of inhibition is consistent with the criteria of synergism. To our knowledge, this is the first report of an in vitro study of the effect of the combination of cefotaxime (a bactericidal drug) and minocycline (a bacteriostatic drug) against *V. vulnificus.*

The present findings have extremely important clinical implications. More than 50% of patients with *V. vulnificus* infections develop severe primary or secondary infections involving soft tissue, manifested as hemorrhagic bullae or necrotizing fasciitis (8, 18). The clinical course of a septicemic patient with *V. vulnificus* infection is fulminant, and more than 50% of such patients die within 48 h of hospitalization (8, 18). The skin manifestations usually develop at the time of admission or within 24 h of hospitalization and are rapidly aggravated (within hours) (8). In the case of severe wound infection, especially necrotizing fasciitis, local swelling, necrosis, vessel occlusion, and vessel thrombosis are present without exception, and these manifestations would seriously compromise the blood supply. A high tissue antibiotic level can hardly be expected in such poorly perfused tissue. The in vitro synergistic effect may be useful in patients in this clinical condition. We are in the process of assessing the applicability of these experimental data in an animal model.

REFERENCES

1. **Blake, P. A., M. H. Merson, R. E. Weaver, D. G. Hollis, and P. C. Heublein.** 1979. Disease caused by a marine *Vibrio*: clinical characteristics and epidemiology. N. Engl. J. Med. **300:**1–5.
2. **Bonner, J. R., A. S. Coker, C. R. Berryman, and H. M. Pollock.** 1983. Spectrum of vibrio infections in a Gulf Coast community. Ann. Intern. Med. **99:**464–469.
3. **Bowdre, J. H., J. H. Hull, and D. M. Cocchetto.** 1983. Antibiotic efficacy against *Vibrio vulnificus* in the mouse: superiority of tetracycline. J. Pharmacol. Exp. Ther. **225:**595–598.
4. **Chalkley, L. J., and H. J. Koornhof.** 1985. Antimicrobial activity of ciprofloxacin against *Pseudomonas aeruginosa*, *Escherichia coli*, and *Staphylococcus aureus* determined by the killing curve method: antibiotic comparisons and synergistic interactions. Antimicrob. Agents Chemother. **28:**331–342.
5. **Chang, J. J., I. S. Sheen, S. M. Peng, P. C. Chen, C. S. Wu, and H. S. Leu.** 1994. *Vibrio vulnificus* infection: report of 8 cases and review of cases in Taiwan. Chang Gung Med. J. **17:**339–346.
6. **Chuang, Y. C.** 1992. Clin. Infect. Dis. **15:**1072. (Letter.)
7. **Chuang, Y. C., C. Young, and C. W. Chen.** 1989. *Vibrio vulnificus* infection. Scand. J. Infect. Dis. **21:**721–726.
8. **Chuang, Y. C., C. Y. Yuan, C. Y. Liu, C. K. Lan, and A. H. M. Huang.** 1992. *Vibrio vulnificus* infection in Taiwan: report of 28 cases and review of clinical manifestations and treatment. Clin. Infect. Dis. **15:**271–276.
9. **Fang, F. C.** 1992. Use of tetracycline for treatment of *Vibrio vulnificus* infections. Clin. Infect. Dis. **15:**1071–1072. (Letter.)
10. **French, G. L., M. L. Woo, Y. W. Hui, and K. Y. Chan.** 1989. Antimicrobial susceptibilities of halophilic vibrios. J. Antimicrob. Chemother. **24:**183–194.
11. **Friedland, I. R., M. Paris, S. Shelton, and G. H. McCracken.** 1994. Time-kill studies of antibiotic combinations against penicillin-resistant and -susceptible *Streptococcus pneumoniae*. J. Antimicrob. Chemother. **34:**231–237.
12. **Howard, R. J., M. E. Pessa, B. H. Brennaman, and R. Ramphal.** 1985. Necrotizing soft-tissue infections caused by marine vibrios. Surgery **98:**126–130.
13. **Hsueh, P. R., J. C. Chang, S. C. Chang, S. W. Ho, and W. C. Hsieh.** 1995. In vitro antimicrobial susceptibility of *Vibrio vulnificus* isolated in Taiwan. Eur. J. Clin. Microbiol. Infect. Dis. **14:**151–153. (Letter.)
14. **Jawetz, E., and J. B. Gunnison.** 1952. An experimental basis of combined antibiotic action. JAMA **150:**693–695.
15. **Jenkins, R. D., and J. M. Johnston.** 1986. Inland presentation of *Vibrio vulnificus* primary septicemia and necrotizing fasciitis. West. J. Med. **144:**78–80.
16. **Kelly, M. T., and D. M. Avery.** 1980. Lactose-positive *Vibrio* in seawater: a cause of pneumonia and septicemia in a drowning victim. J. Clin. Microbiol. **11:**278–280.
17. **Kelly, M. T., F. W. Hickman-Brenner, and J. J. Farmer III.** 1991. *Vibrio*, p. 384–395. *In* A. Balows, W. J. Hausler, Jr., K. L. Herrmann, H. D. Isenberg, and H. J. Shadomy (ed.), Manual of clinical microbiology, 5th ed. American Society for Microbiology, Washington, D.C.
18. **Klontz, K. C., S. Lieb, M. Schreiber, H. T. Janowski, L. M. Baldy, and R. A. Gunn.** 1988. Syndromes of *Vibrio vulnificus* infections: clinical and epidemiologic features in Florida cases, 1981–1987. Ann. Intern. Med. **109:**318–323.
19. **Morris, J. G., Jr, and J. Tenney.** 1985. Antibiotic therapy for *Vibrio vulnificus* infection. JAMA **253:**1121–1122. (Letter.)
20. **National Committee for Clinical Laboratory Standards.** 1994. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically.

Approved standard M7-A2. National Committee for Clinical Laboratory Standards, Villanova, Pa.
21. **Pankuch, G. A., M. R. Jacobs, and P. C. Appelbaum.** 1994. Study of comparative antipneumococcal activities of penicillin G, RP 59500, erythromycin, sparfloxacin, ciprofloxacin, and vancomycin by using time-kill methodology. Antimicrob. Agents Chemother. **38:**2065–2072.
22. **Rahal, J. J., Jr.** 1978. Antibiotic combinations: the clinical relevance of synergy and antagonism. Medicine (Baltimore) **57:**179–195.
23. **Sanford, J. P., D. N. Gilbert, and M. A. Sande.** 1996. Selection of initial empirical antibacterial therapy on clinical grounds, p. 1–45. *In* J. P. Sanford, D. N. Gilbert, and M. A. Sande (ed.), Sanford guide to antimicrobial therapy, 25th ed. Antimicrobial Therapy, Inc., Dallas, Tex.
24. **Tacket, C. O., F. Brenner, and P. A. Blake.** 1984. Clinical features and an epidemiological study of *Vibrio vulnificus* infections. J. Infect. Dis. **149:**558–561.
25. **Tyring, S. K., and P. C. Lee.** 1986. Hemorrhagic bullae associated with *Vibrio vulnificus* septicemia: report of two cases. Arch. Dermatol. **122:**818–820.
26. **Visalli, M. A., M. R. Jacobs, and P. C. Appelbaum.** 1996. MIC and time-kill study of activities of DU-6859a, ciprofloxacin, levofloxacin, sparfloxacin, cefotaxime, imipenem, and vancomycin against nine penicillin-susceptible and -resistant pneumococci. Antimicrob. Agents Chemother. **40:**362–366.
27. **Wongpaitoon, V., B. Sathapatayavongs, R. Prachaktam, S. Bunyaratvej, and S. Kurathong.** 1985. Spontaneous *Vibrio vulnificus* peritonitis and primary sepsis in two patients with alcoholic cirrhosis. Am. J. Gastroenterol. **80:**706–708.
28. **Woo, M. L., W. G. D. Patrick, M. T. P. Simon, and G. L. French.** 1984. Necrotising fasciitis caused by *Vibrio vulnificus*. J. Clin. Pathol. **37:**1301–1304.