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行政院衛生署疾病管制局九十一年度科技研究發展計畫

# 創傷弧菌致病因子基因選殖、致病機轉及快速檢驗

# 研 究 報 告

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＊本研究報告僅供參考，不代表衛生署疾病管制局意見＊

# 目　　錄

## 一、摘要：

前言：創傷弧菌會引起嚴重的敗血症及快速進展的壞死性肌膜炎，而導致病患快速死亡。過去 in vitro 及 in vivo 的研究顯示 minocycline 及 cefotaxime 有協同作用，然而新一代 Fluoroquinolones（FQ），近年來被廣泛使用，且其口服吸收良好。有口服及針劑等劑型，可以作 sequential therapy 以減少醫療成本，因此本研究評估 FQ 對於創傷弧菌的抗菌效果。

材料與方法：（1）由奇美醫學中心及成功大學附設醫院的臨床菌株共 46 株作 minocycline、cefotaxime 及新一代 FQ 之 MIC。（2）以 Time-kill study 方法來評估 cefotaxime-minocycline、及 6 種新一代 FQ 對創傷弧菌的抗菌效果。（3）以動物模式評估新一代 FQ 對創傷弧菌感染小白鼠之療效

結果：（1）所有測試的 FQ 均有很好的抗菌效果，$MIC_{90}$ 均在 0.03-0.12 μg/ml 之間。（2）Time-kill study 顯示各種 FQ 在 MIC 2 倍以下之濃度以下就可以抑制細菌生長達 48 小時。（3）動物實驗顯示在 $1.5\times10^{7}$CFU 感染劑量下，以 cefotaxime-minocycline 及 moxifloxacin 治療之存活率分別為 87.5％及 91‰ 與對照組有顯著之差距（$p<0.001$, by log Rank test）。

結論：單一使用新一代 FQ 對創傷弧菌感染動物模式的療效與合併 cefotaxime-minocycline 治療之效果相同。

中文關鍵詞(至少三個)：創傷弧菌、致病機轉、抗生素療法、敗血性休克、傷口感染

**ABSTRACT**

MICs of 6 fluoroquinolones as well as minocycline and cefotaxime against 46 clinical isolates of *Vibrio vulnificus* were determined by the agar dilution method. All had good antibacterial activities against all isolates with $MIC_{90}$s varying between 0.03 and 0.06 μg/ml. $MIC_{90}$ of lomefloxacin, on the other hand, was 0.12 (g/ml. Time-kill studies were conducted with these agents against a clinical strain of *V. vulnificus* VV5823. When approximately $5 \times 10^5$ CFU/ml of *V. vulnificus* were incubated with any one of the above-mentioned six fluoroquinolones at concentrations of 2 × MIC, there was an inhibitory effect against *V. vulnificus* that persisted for more than 48 h with no noted regrowth. The efficacy of the fluoroquinolones was further evaluated in vivo in the mouse model of experimental *V. vulnificus* infection, and compared to combination therapy with cefotaxime plus minocycline. With the inoculum of $1.5 \times 10^7$ CFU, 28 (87.5%) of 32 mice in the combined cefotaxime-minocycline group survived, 29 (91%) of the 32 mice survived in the moxifloxacin-treated group while none of the 32 mice in the control group did. With the inoculum of $3.5 \times 10^7$ CFU, survival among groups of 15 mice treated with levofloxacin (13 of 15), moxifloxacin (10), gatifloxacin (10), sparfloxacin (11), ciprofloxacin (12) and lomefloxacin (10) was not statistically significant, while none of 15 mice treated with saline survived. The authors concluded that the newer fluoroquinolones as single agents are equally effective as combined cefotaxime-minocycline in inhibiting *V. vulnificus* both in vitro and in vivo.

Keyword：*Vibrio vulnificus*、pathogenesis、Anti-microbial therapy、septic shock、wound infection

## INTRODUCTION

*Vibrio vulnificus* is a halophilic gram-negative bacillus recovered from estuarine and seawaters (18). Many cases of *V. vulnificus* infections have been reported from the coastal areas of the United States (1, 2, 19), Asia (4-6, 29) and Europe (11, 22). The high prevalence of hepatitis B infections in areas such as Taiwan may also contribute to the high incidence of severe *V. vulnificus* infections. *Vibrio vulnificus* characteristically produces three discernible syndromes (2, 4, 5, 25, 30): primary sepsis, wound infection, and gastrointestinal illness. The mortality rate is up to 55 % in septic patients and 25 % in those with wound infections (5).

Most of the *V. vulnificus* isolates are susceptible in vitro to a variety of antibiotics (1, 3, 15-17). Tetracycline has been recommended as antimicrobial agent of choice for the treatment of *V. vulnificus* infection by extrapolating the effectiveness of tetracycline for *V. cholerae* infections. More recently, our in vitro study showed a synergistic effect of cefotaxime and minocycline against *V. vulnificus* (7). A further in vivo study showed that combined therapy with cefotaxime and minocycline is more advantangeous than single drug regimens with these agents for the treatment of severe experimental murine *V. vulnificus* infection (10). Ciprofloxacin has also been used successfully for the treament of *V. vulnificus* wound infection (21). In general, the newer fluoroquinolones developed over the past few years have greater potency, a broader spectrum of antimicrobial activity, greater in vitro efficacy against resistant organisms, and a better safety profile than other antimicrobial agents. Moreover, step-down therapy, a cost-saving alternative, has been claimed advantageous. For this reason, the antibacterial activity of the new fluoroquinolones against *V.*

*vulnificus* was evaluated both in vitro and in vivo in comparison with cefotaxime-minocycline in the current study.

## MATERIALS AND METHODS

**Determination of minimal inhibitory concentrations (MIC) of cefotaxime, minocycline and six newer fluoroquinolones against 46 clinical isolates of *V. vulnificus***. Clinical isolates of *V. vulnificus* were collected from Chi Mei Foundation Medical Center, National Cheng Kung University Hospital, and the National Taiwan University Hospital. These strains were originally isolated from blood, wound or bullous fluid. All isolates were identified as *V. vulnificus* by conventional methods as described previously (7). The organisms were stored at –70°C in Protect Bacterial Preservers (Technical Service Consultants Limited, Lancashire, England) before being cultured on Luria Bertani agar (Difco Laboratories, Detroit, Mich.). *Vibrio vulnificus* VV5823, originally isolated from a septicemic patient from National Cheng Kung University Hospital, was arbitrarily selected for both the time-kill and in vivo studies. MIC of the following antibiotics was determined by the agar dilution method as previously described (27): cefotaxime (Hoechst AG, Frankfurt, Germany), minocycline (American Cyanamid Co., Pearl River, NY), moxifloxacin (Bayer AG, Frankfurt, Germany), gatifloxacin (Bristol-Myers Squibb, Humacao, Australia), sparfloxacin (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan), levofloxacin (Daiichi Pharmaceutical Co., Ltd, Tokyo, Japan), ciprofloxacin (Bayer AG, Frankfurt, Germany) and lomefloxacin (Shionogi Pharmaceutical Co., Ltd., Osaka, Japan). The drugs were incorporated into the agar in serial twofold

concentrations as follows: minocycline, 0.03-128 μg/ml; ciprofloxacin, 0.03-16 μg/ml; lomefloxacin, 0.03-16 μg/ml; moxifloxacin, 0.03-64 μg/ml; gatifloxacin, 0.03-128 μg/ml; cefotaxime, 0.03-64 μg/ml; sparfloxacin, 0.03-16 μg/ml; and levofloxacin, 0.03-16 μg/ml. The fluoroquinolone powder was dissolved in 0.05 M NaOH solution and diluted with sterile water to the required test concentration. The minocycline powder was dissolved in 0.1 M NaOH solution instead, while the cefotaxime was dissolved in sterile water to the required test concentration. The bacterial inocula were prepared and MIC was defined as previously described (7), except that final inocula of approximately $1 \times 10^4$ CFU per spot of inoculum were applied onto the plates, and were incubated at 37℃ for 24 h. *Eshcerichia coli* ATCC 25922 was used in each run as controls for susceptibility testing.

**Determination of inhibitory effect of combined cefotaxime-minocycline and six newer fluoroquinolones against *V. vulnificus* by time-kill studies**. Bacterial concentrations were diluted to around $5.0 \times 10^5$ CFU/ml in 25 ml of fresh Mueller-Hinton broth. This was done in a 125-ml glass conical flask each. Varying concentrations of cefotaxime, minocycline, and six newer fluoroquinolones were prepared and placed in flasks: for cefotaxime 0.03 μg/ml and minocycline 0.03 μg/ml, for moxifloxacin 0.015, 0.03, 0.06, 0.075, 0.09, and 0.12 μg/ml, for gatifloxacin 0.015, 0.03, 0.06, 0.075, 0.09, and 0.12 μg/ml, for sparfloxacin 0.015, 0.03, 0.06, 0.075, 0.09, and 0.12 μg/ml, for levofloxacin 0.075, 0.015, 0.03, 0.06, 0.075, and 0.09 μg/ml, for ciprofloxacin 0.015, 0.03, 0.045, 0.06, 0.075, and 0.09 μg/ml, for lomefloxacin 0.06, 0.09, 0.12, 0.18, 0.25,

and 0.36 μg/ml. Each flask was incubated under the aforementioned conditions. Duplicate samples were removed for determination of CFUs specified time intervals as described previously (7), except that Luria-Bertani agar plates were applied and incubated at 37℃ overnight. All the experiments were performed at least twice for confirmation of the results.

**In vivo efficacy of combined cefotaxime-minocycline and six newer fluoroquinolones in experimental *V. vulnificus* infection in mice.** The marketed parenteral form of cefotaxime, minocycline and ciprofloxacin used in vivo experiments were provided by Hoechst, Taiwan Co., Ltd., Lederle, Parenterals, Inc. Puerto Rico, and Bayer AG, Frankfurt, Germany respectively. Parenteral forms of moxifloxacin, levofloxacin, gatifloxacin, sprafloxacin and lomefloxacin were not available in Taiwan, so their standard powders were diluted to the desired concentration for the experiments. Antibiotics were freshly diluted in sterile 0.85% saline in the morning when the experiment was conducted and delivered in sterile disposable plastic syringes.

The clinical isolate of *V. vulnificus* VV5823 was used throughout the study. The bacterial inocula were prepared as previously described (10). Female inbred BALB/c mice (Animal Center, National Science Council, Taipei, Taiwan) weighing 20 g (5-6-week-old) on the average were used throughout the study. An inoculum size of $10^7$ CFU was chosen for the animal experiments because large inoculum size was proved to be more discriminatory in our previous report for evaluation the efficacy of the treament regimens (10). In experiment 1, $1.5 \times 10^7$ CFU of *V. vulnificus* were injected s.c. over the right thigh of each mouse.

There were three groups including control, combined cefotaxime-minocycline, and moxifloxacin-treated groups, with 32 mice in each group. Cefotaxime, minocycline or moxifloxacin was given i.p. in a 0.1-ml volume, beginning 2 h after the animal was infected. The dose of antibiotics was determined according to the recommendation of the pharmaceutical company, i.e. 30 mg/kg of cefotaxime every 6 h, and a loading dose of 4 mg/kg followed by a maintenance dose of 2 mg/kg of minocycline every 12 h. The dose of moxifloxacin was as follows: loading dose of 16 mg per kg of body weight followed by a maintenance dose of 8 mg every 24 h. Control animals received 0.1 ml sterile 0.85% saline every 6 h. Antibiotics were given for a total of 42 h. The numbers of surviving mice were recorded at 6-h intervals after the initial treatment and ended at 120 h. For humanitarian reasons, animals were euthanized when they were moribund even though they were still breathing. In experiment 2, the experimental design was identical except that inocula of $3.5 \times 10^7$ CFU of *V. vulnificus* VV5853 were used and animals were treated for a total of 36 h. There were seven groups of 15 mice each, including six groups treated with fluoroquinolones and a saline-treated control group. The doses of the newer fluoroquinolones were as follows: a loading dose of 16 mg of moxifloxacin, levofloxacin and gatifloxacin per kg of body weight followed by a maintenance dose of 8 mg every 24 h and a loading dose of 10 mg of sparfloxacin; 16 mg ciprofloxacin, 8 mg lomefloxacin, per kg followed by a maintenance dose of 5, 8, 4 mg per kg, respectively, every 12 h. The antibiotics were given for a total of 36 h. The animal experiments have complied with all relevant national guidelines of the Republic of China and Chi Mei Foundation Medical Center Animal Use Policy.

## RESULTS

**MIC values.** All antibiotics tested showed good in vitro activity against all isolates. The $MIC_{90}$s of levofloxacin and ciprofloxacin were 0.03 μg/ml and those of minocycline, cefotaxime, moxifloxacin, sparfloxacin and gatifloxacin were 0.06 μg/ml. Lomefloxacin, on the other hand, was 0.12 μg/ml. The MICs of strain VV5853 for minocycline, cefotaxime, moxifloxacin, gatifloxacin, sparfloxacin, levofloxacin, ciprofloxacin and lomefloxacin were 0.06, 0.06, 0.06, 0.03, 0.06, 0.03, 0.03 and 0.12 μg/ml, respectively.

**Determination of inhibitory effect of combined cefotaxime-minocycline, and six newer fluoroquinolones against *V. vulnificus* in time-kill kinetics.** When approximately $5 \times 10^5$ CFU/ml of *V. vulnificus* were incubated with gatifloxacin, moxifloxacin, ciprofloxacin, sparfloxacin, and levofloxacin at concentrations of MIC, the bacterial growth was inhibited during the initial 6, 8, 8, 12 and 36 h, respectively, and thereafter, *V. vulnificus* regrew (Fig. 1A). When subinhibitory concentrations of cefotaxime 0.03 μg/ml (1/2 × MIC) and minocycline 0.03 μg/ml (1/2 × MIC) were combined in the same culture, the inhibitory effect against *V. vulnificus* persisted for more than 48 h with no regrowth noted (Fig. 1B). When moxifloxacin was used at the concentration of 0.075 μg/ml (5/4 × MIC) (Fig. 1B), gatifloxacin 0.06 μg/ml (2 × MIC) (data not shown), sparfloxacin 0.09 μg/ml (5/4 × MIC), levofloxacin 0.045 μg/ml (3/2 × MIC), ciprofloxacin 0.06 μg/ml (2 × MIC), lomefloxacin 0.12 μg/ml (1 × MIC) (Fig. 1A), the inhibitory effect against *V. vulnificus* persisted for more than 48 h with no regrowth noted. The MIC and MBC were equivalent for sparfloxacin, levofloxacin and lomefloxacin (Fig. 1A).

**In vivo study.** In experiment 1, with an inoculum of $1.5 \times 10^7$ CFU, all the mice in the control group died within 12 h (Fig. 2A). The survival rates recorded at the end of the experiment were 87.5% and 91% for the combined minocycline-cefotaxime group and moxifloxacin-treated group, respectively. Both antibiotic-treated groups had significant higher survival rates than that of the saline-treated group ($p<0.001$, by log-rank test), while the difference between the two antibiotic-treated groups was insignificant. In experiment 2, with the inoculum of $3.5 \times 10^7$ CFU and antibiotic treatment for 36 h rather than 42 h, survival rates among mice treated with the fluoroquinolones (13, 10, 10, 11, 12, and 10 out of 15 mice in each group for levofloxacin-_moxifloxacin, gatifloxacin, sparfloxacin, ciprofloxacin, and lomefloxacin, respectively) were significantly higher than the saline-treated control group (0 of 15) ($p<0.01$, log-rank test), but not significantly different from each other (Fig. 2B).

## DISCUSSION

The results show that minocycline, cefotaxime and a variety of newer fluoroquinolones have good in vitro activities against all the clinical isolates of *V. vulnificus*. The $MIC_{90}$ were as low as 0.03 μg/ml. In the time-kill studies, there was no significant difference in antibacterial effects among the six newer fluoroquinolones. At concentration less /equal to 2 × MIC, the inhibitory effects of all the newer fluoroquinolones persisted for more than 48 h with no regrowth noted. These findings indicate that the fluoroquinolones are generally cidal, with a very small MBC/MIC ratio. These inhibitory effects are as effective as combined cefotaxime-minocycline, which has shown to have synergistic effect against *V. vulnifiucs* in the previous study (7). The in vivo study shows that newer fluoroquinolones alone has the same efficacy as that of combined cefotaxime-minocycline in the treatment of severe experimental murine *V. vulnificus* infection. Based on the time-kill results, it would appear that levofloxacin is the most active. This also appears to be the case in the in vivo study, although the differences among the different fluoroquinolones are not statistically significant.

Because of the sporadic occurrence of *V. vulnificus* infections, there are virtually no radonmized clinical trials to determine which antibiotic is most effective for treatment. Morris et al. (24-25) stressed the superiority of tetracycline over cefotaxime based on the study of a mouse model conducted by Bowdre et al. (3). Fang (12) advocated using tetracycline to treat *V. vulnificus* because an antibiotic, which inhibits protein synthesis, was thought to be preferable to one, which damages the cell wall and may cause the release of an

increased level of toxic microbial proteins. On the other hand, the authors' clinical experiences suggest that the third generation cephalosporins may be superior to tetracycline for *V. vulnificus* infections (5, 6). A previous in vitro study showed the synergistic effect of cefotaxime and minocycline against *V. vulnificus* (7). A further in vivo study showed that combined therapy with cefotaxime and minocycline was more efficacious than single drug therapy with these antibiotics for the treatment of severe experimental murine *V. vulnificus* infection (10).

The mouse model of *V. vulnificus* infection used in the current study was previously shown to cause necrotizing fasciitis, bacteremia and death within 24 h, mimicking *V. vulnificus* bacteremia in humans (8). *V. vulnificus* can produce mutiple extracellular cytolytic or cytotoxic toxins and enzymes that are associated with extensive tissue damage and may play a major role in the development of sepsis (8-9, 14, 20, 23, 28). More than 50% of cases of *V. vulnificus* infections develop either primary or secondary severe soft tissue involvement manifesting as hemorrhagic bullae or necrotizing fasciitis (5, 19). The clinical course of a septicemic patient with *V. vulnificus* is fulminant and over 50% of such patients die within 48 h of hospitalization (5, 19). The skin manifestations usually develop at the time of admission or within 24 h of hospitalization. This condition could aggravate rapidly within hours (5). In the case of severe wound infection, especially in necrotizing fasciitis, widespread obliterative vasculitis and vascular necrosis are the major features of the skin lesion, which could seriously compromise the blood supply. Antibiotic, with good tissue penetration ability, would be urgently needed in these clinical situations. Muller et al. showed that moxifloxacin was promsing in the treatment

of skin and soft tissue infections. This is because its concentrations attained in the interstitial space fluid in humans and in skin blister fluid following single dose of 400 mg exceeded the values for the $MIC_{90}$ of most clinical isolates (27). The unique site of action and good tissue penetration abilities of newer fluoroquinolones may relate to the efficacy of their clinical use. In view of the difference in pharmacokinetic parameters between mice and humans, whether or not all the_results of animal model studies could be extrapolated in clinical situations is an important question that has yet to be answered.

Taken together, in addition to combined cefotaxime-minocycline, the newer fluoroquinolones, such as levofloxacin, are potentially useful as monotherapy for severe *V. vulnificus* soft tissue infections. Further clinical trials with these agents for human *V. vulnificus* infection are warranted.

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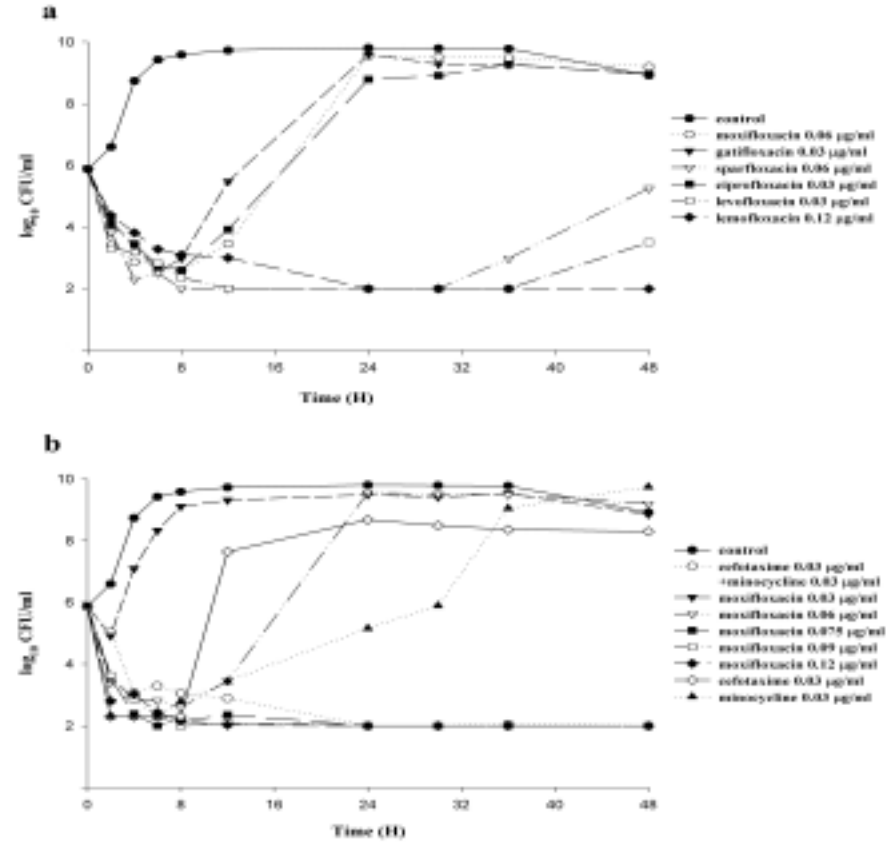
## FIGURE LEGENDS

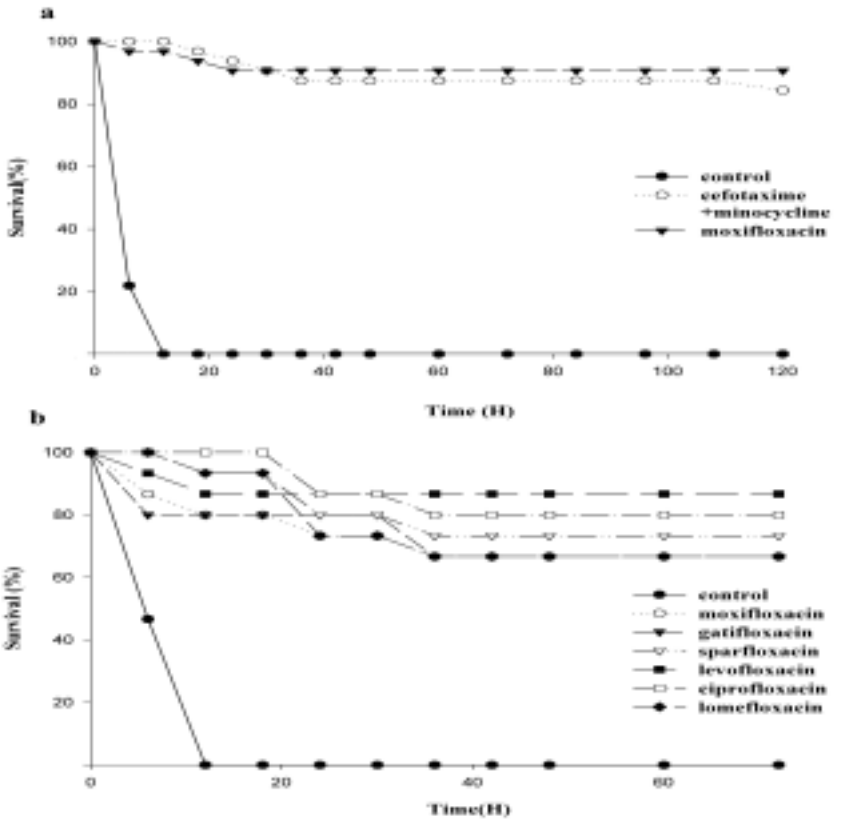
Fig. 1A. Inhibiton of growth curves of *V. vulnificus* VV5823 after incubation with different fluoroquinolones at concentration of MIC with the inoculum size of $5 \times 10^5$ CFU/ml. The lower limit of detection was set at 10 colonies (100 CFU/ml).

Fig. 1B. Inhibiton of growth curves of *V. vulnificus* VV5823 after incubation with minocycline, cefotaxime alone, combined cefotaxime-minocycline, or different concentrations of moxifloxacin, with the inoculum size of $5 \times 10^5$ CFU/ml. MICs were 0.06 μg/ml for cefotaxime, minocycline and moxifloxacin.

Fig. 2A. Survival rates of mice s.c. injected with $1.5 \times 10^7$ CFU *V. vulnificus* following combined cefotaxime-minocycline, moxifloxacin and saline treament.(n=32) The difference between moxifloxacin- and saline-treated groups and that between combined cefotaxime-minocycline and saline-treated groups were significant ($p<0.001$) by log-rank test, while that between combined cefotaxime-minocycline and moxifloxacin-treated groups was not significant.

Fig. 2B. With the inoculum of $3.5 \times 10^7$ CFU and antibiotic treatment for 36 h rather than 42 h, survival rates among mice treated with the fluoroquinolones were significantly higher than the saline-treated control group ($p<0.01$, log-rank test), but not significantly different from each other. (n=15)


a
b
Time (H)
control
moxifloxacin 0.06 μg/ml
gatifloxacin 0.03 μg/ml
sparfloxacin 0.06 μg/ml
ciprofloxacin 0.03 μg/ml
levofloxacin 0.03 μg/ml
lomefloxacin 0.12 μg/ml
cefotaxime 0.03 μg/ml
+minocycline 0.03 μg/ml
moxifloxacin 0.03 μg/ml
moxifloxacin 0.06 μg/ml
moxifloxacin 0.075 μg/ml
moxifloxacin 0.09 μg/ml
moxifloxacin 0.12 μg/ml
cefotaxime 0.03 μg/ml
minocycline 0.03 μg/ml


a
b
Survival(%)
Time (H)
control
cefotaxime
+minocycline
moxifloxacin
gatifloxacin
sparfloxacin
levofloxacin
ciprofloxacin
lomefloxacin