

In vitro antimicrobial effect of cefazolin and cefotaxime combined with minocycline against *Vibrio cholerae* non-O1 non-O139

Bo-An Su¹, Hung-Jen Tang¹, Yin-Yi Wang¹, Yung-Ching Liu², Wen-Chien Ko³, Cheng-Yi Liu⁴, Yin-Ching Chuang¹, and the Surveillance of Multicenter Antimicrobial Resistance in Taiwan (SMART) Program

¹Department of Medicine and Medical Research, Chi Mei Medical Center, Tainan; ²Department of Medicine, Kaohsiung Veterans General Hospital, Kaohsiung; ³Department of Medicine, National Cheng-Kung University Hospital, Tainan; and ⁴Taipei Veterans General Hospital, Taipei, Taiwan

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The most common clinical manifestation of *Vibrio cholerae* non-O1 non-O139 is gastroenteritis. This vibron may also cause bacteremia, soft tissue infection, and other extraintestinal invasive disease, especially in immunocompromised patients. This study evaluated the current status of antimicrobial resistance in clinical isolates of *V. cholerae* non-O1 non-O139 in Taiwan as part of the SMART (Surveillance from Multicenter Antimicrobial Resistance in Taiwan) program. Minimal inhibitory concentrations (MICs) of 9 antimicrobial agents were determined by the agar dilution method. All of the isolates were susceptible to minocycline (MIC at which 90% of the isolates were inhibited [MIC₉₀], 0.12 µg/mL), cefotaxime (MIC₉₀, 0.06 µg/mL), lomefloxacin (MIC₉₀, 0.12 µg/mL), levofloxacin (MIC₉₀, 0.03 µg/mL), ciprofloxacin (MIC₉₀, 0.03 µg/mL), moxifloxacin (MIC₉₀, 0.06 µg/mL), sparfloxacin (MIC₉₀, 0.06 µg/mL), gatifloxacin (MIC₉₀, 0.03 µg/mL), and cefazolin (MIC₉₀, 8 µg/mL). We conducted time-kill studies to evaluate the inhibitory activities of either cefazolin or minocycline alone or in combination against *V. cholerae* non-O1 non-O139 (Vc2). We also evaluated the inhibitory activity of cefazolin or cefotaxime combined with minocycline. The individual MICs of cefazolin, cefotaxime, and minocycline were 4 µg/mL, 0.0075 µg/mL, and 0.12 µg/mL, respectively, when approximately 5 × 10⁵ colony-forming units/mL of *V. cholerae* non-O1 non-O139 was incubated. Bacterial growth was inhibited initially but resumed later when cefazolin, cefotaxime, or minocycline was used alone. When cefazolin or cefotaxime was combined with minocycline, *V. cholerae* non-O1 non-O139 was inhibited over 48 h and no regrowth was noted. We conclude that the combination of cefazolin or cefotaxime with minocycline has a synergistic inhibitory effect on *V. cholerae* non-O1 non-O139 in vitro.

Key words: Combined antibiotics, microbial sensitivity tests, *Vibrio cholerae* non-O1, vibrio infections

Cholera is a clinical epidemiologic syndrome characterized by a severe watery diarrhea caused by *Vibrio cholerae* (usually of serogroup O1), which colonizes the small intestine and produces an enterotoxin, the cholera toxin [1]. The hallmark of clinical cholera is the development of severe dehydration with signs of hypovolemic shock usually requiring resuscitation and intravenous fluids [2]. The genus *Vibrio* contains several species, of which *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* are the most important pathogens in humans [3-5]. *V. cholerae* is a curved Gram-negative bacillus varying in size (from 1 to 3 µm in length and 0.5 to 0.8 µm in diameter)

that belongs to the family of *Vibrionaceae* and shares common characteristics with the family of *Enterobacteriaceae*. A natural inhabitant of the aquatic environment. *V. cholerae* is classified on the basis of its somatic antigens (O antigens) into serovars or serogroups, and there are at least 155 known serogroups [5,6]. *V. cholerae* O1 was the cause of the first 7 cholera epidemics since 1817 [7-11]. The causative strain of a cholera epidemic in late 1992 was *V. cholerae* non-O1 strain, which was later serogrouped as O139 [12]. The outbreak of severe clinical cholera due to *V. cholerae* O-139 alerted us to the importance of *V. cholerae* serogroups other than O1 and O139. The other strains are defined as *V. cholerae* non-O1 non-139; the great majority of them do not produce cholera toxin and are not associated with epidemics of diarrhea

Corresponding author: Yin-Ching Chuang, Chi Mei Medical Center, No. 901 Jung Hua Rd., Yung Kang City, Tainan 710, Taiwan.
E-mail: chuangkenneth@hotmail.com

[13]. These strains are occasionally isolated from cases of diarrhea (non-epidemic) and from a variety of non-intestinal infections, including wounds and infections of the ear, lung, genitourinary tract, and central nervous system [3,13-15]. The majority of known cases have occurred in immunocompromised patients, particularly those with hematologic malignancy or cirrhosis of the liver [13,16]. The recommended antibiotic treatment for *Vibrio cholerae* is tetracycline [17,18]. However, the resistance rate has increased recently [19-21]. Resistance to other antibiotics including sulfamethoxazole-trimethoprim, chloramphenicol, and furazolidone has also been reported [21-23]. Establishing the appropriate regimen to treat *V. cholerae* is a pressing requirement. Using our experience in the treatment of *V. vulnificus* infections [24-27], we conducted time-kill studies to evaluate the inhibitory activity of first- and third-generation cephalosporins combined with minocycline against *V. cholerae* non-O1 non-O139.

Materials and Methods

Bacterial isolates

Clinical isolates of *V. cholerae* non-O1 non-O139 were collected from 4 medical centers in Taiwan: Chi Mei Medical Center, National Cheng Kung University Hospital, Kaohsiung Veterans General Hospital, and Taipei Veterans General Hospital. In 2001, 22 strains were originally isolated from blood (8 strains), wound (2 strains), bile (1 strain), pus (2 strains), and stool (9 strains). All isolates were identified as *V. cholerae* non-O1 non-O139 by conventional biochemical methods, as described previously [28]. 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, MI, USA).

Determination of minimal inhibitory concentrations for *V. cholerae* non-O1 non-O139

The minimal inhibitory concentrations (MICs) for the following antibiotics were determined by the agar dilution method as previously described [29]: cefotaxime (Hoechst AG, Frankfurt, Germany), cefazolin (Sigma-Aldrich Co., St. Louis, MO, USA), minocycline (American Cyanamid Co., Pearl River, NY, USA), moxifloxacin (Bayer AG, Frankfurt, Germany), gatifloxacin (Bristol-Myers Squibb, Humacao, Australia), sparfloxacin (Dainippon Pharmaceutical Co., Osaka, Japan), levofloxacin (Daiichi Pharmaceutical

Co., Tokyo, Japan), ciprofloxacin (Bayer AG, Frankfurt, Germany), and lomefloxacin (Shionogi Pharmaceutical Co., Osaka, Japan). The drugs were incorporated into the agar using serial 2-fold dilutions, with final concentrations ranging as follows: minocycline, 0.06 to 0.12 $\mu\text{g}/\text{mL}$; cefotaxime, 0.00375 to 0.06 $\mu\text{g}/\text{mL}$; cefazolin, 0.5 to 8 $\mu\text{g}/\text{mL}$; lomefloxacin, 0.015 to 0.5 $\mu\text{g}/\text{mL}$; levofloxacin, 0.00375 to 0.12 $\mu\text{g}/\text{mL}$; ciprofloxacin, 0.00375 to 0.06 $\mu\text{g}/\text{mL}$; moxifloxacin, 0.0075 to 0.25 $\mu\text{g}/\text{mL}$; sparfloxacin, 0.00375 to 0.25 $\mu\text{g}/\text{mL}$; and gatifloxacin, 0.00375 to 0.12 $\mu\text{g}/\text{mL}$. The fluoroquinolone powder was dissolved in 0.05 M NaOH and minocycline in 0.1 M NaOH; cefotaxime and cefazolin were dissolved in sterile water, and then diluted with sterile water to the required test concentration. Approximately 1×10^4 colony-forming units (CFU) of *V. cholerae* non-O1 non-O139 per spot were applied onto the plates, which were then incubated at 37°C for 24 h. *E. coli* (American Type Culture Collection [ATCC] No. 25922) was used as control.

Inhibitory effects of cefotaxime or cefazolin combined with minocycline time-kill studies

The *V. cholerae* non-O1 non-O139 isolates Vc2, originally isolated from blood at Chi Mei Medical Center, were selected for the time-kill studies. Bacteria were diluted to approximately 5×10^5 CFU/mL in 25 mL of fresh Mueller-Hinton broth in a 125-mL glass conical flask. Each flask was incubated at 37°C . A 100- μL aliquot from each 10-fold serial dilution was plated on nutrient agar (Difco Laboratories), and bacteria were counted at 0, 2, 4, 6, 8, 12, 24, 30, 36, and 48 h by enumerating the colonies. The lower limit of detection was set at 10 colonies (100 CFU/mL). All experiments were performed at least twice to confirm the results.

Results

The results of antimicrobial susceptibility testing of 22 *V. cholerae* non-O1 non-O139 isolates are shown in Table 1. The MIC of minocycline, cefotaxime, cefazolin, and all of the fluoroquinolones except cefazolin was ≤ 0.12 $\mu\text{g}/\text{mL}$ for 90% of the isolates tested (MIC_{90}). The MIC_{90} of cefazolin was 8 $\mu\text{g}/\text{mL}$. The MIC of minocycline, cefotaxime, lomefloxacin, levofloxacin, ciprofloxacin, moxifloxacin, sparfloxacin, gatifloxacin, and cefazolin for the Vc2 (an isolate from the blood of a patient with sepsis) was 0.12, 0.0075, 0.015, 0.0075,

Table 1. Susceptibility of 22 isolates of *Vibrio cholerae* non-O1 non-O139 to 9 antimicrobial agents

Antimicrobial agent	MIC ($\mu\text{g/mL}$)			
	MIC ₅₀	MIC ₉₀	Range	MIC for Vc2 isolate
Minocycline	0.12	0.12	0.06-0.12	0.12
Cefotaxime	0.0075	0.06	0.00375-0.06	0.0075
Lomefloxacin	0.015	0.12	0.015-0.5	0.015
Levofloxacin	0.0075	0.03	0.00375-0.12	0.0075
Ciprofloxacin	0.00375	0.03	0.00375-0.06	0.00375
Moxifloxacin	0.015	0.06	0.0075-0.25	0.015
Sparfloxacin	0.00375	0.06	0.00375-0.25	0.0075
Gatifloxacin	0.0075	0.03	0.00375-0.12	0.0075
Cefazolin	4	8	0.5-8.00	4

Abbreviations: MIC = minimal inhibitory concentration; MIC₅₀ = MIC at which 50% of isolates were inhibited; MIC₉₀ = MIC at which 90% of isolates were inhibited

0.00375, 0.015, 0.0075, 0.0075, and 4 $\mu\text{g/mL}$, respectively.

When Vc2 (initial inoculum of 4.7×10^5 CFU/mL) was incubated with minocycline or cefazolin alone, or both, at a concentration equal to their respective MICs, bacterial growth was inhibited temporarily for 12 h, at most, and the bacteria later re-grew (Fig. 1). However, the combination of minocycline and cefazolin demonstrated an enhanced inhibitory effect on Vc2. When a subinhibitory concentration of minocycline (equal to the MIC) and a high concentration of

cefotaxime (8 times the MIC) were combined, the inhibitory effect on *V. cholerae* non-O1 non-O139 persisted for more than 48 h with no regrowth noted (Fig. 2).

Discussion

Tetracycline is recommended as the drug of choice for the treatment of vibrio infections. However, levels of tetracycline resistance in East Africa, Bangladesh, and parts of India have become high, and therefore

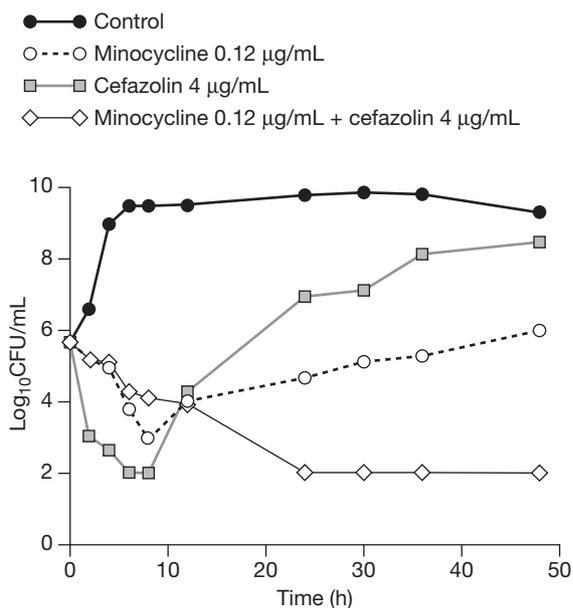


Fig. 1. Time-kill curves of Vc2 isolate after incubation with minocycline alone, cefazolin alone, or a combination of minocycline and cefazolin. Vc2 initial inoculum was 4.7×10^5 colony-forming units (CFU)/mL. Each point represents the mean value of duplicate experiments.

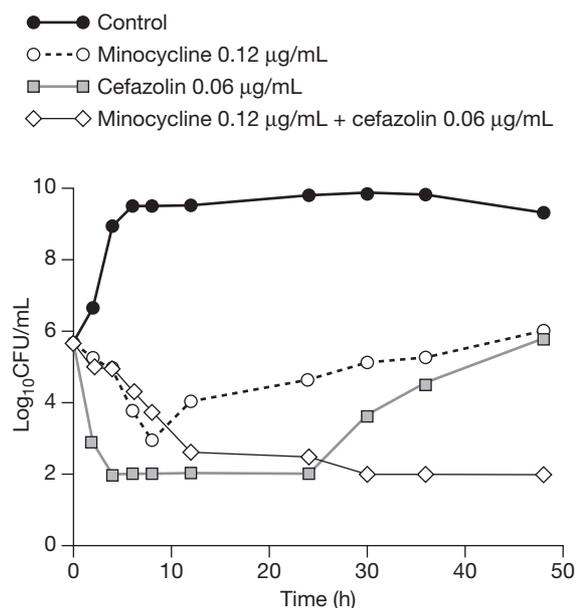


Fig. 2. Time-kill curves of Vc2 isolate after incubation with minocycline alone, cefotaxime alone, or a combination of minocycline and cefotaxime. Vc2 initial inoculum was 4.7×10^5 colony-forming units (CFU)/mL. Each point represents the mean value of duplicate experiments.

V. cholerae isolated from these areas should be considered tetracycline-resistant until results of susceptibility testing are available [19-21]. Also, widespread use of tetracycline prophylaxis has been associated with the rapid development of antimicrobial resistance [19, 30], and tetracycline is inappropriate for the treatment of children. Resistance to other antimicrobial agents including sulfamethoxazole-trimethoprim, chloramphenicol, streptomycin, and furazolidone has also been reported.

Non-O1 non-O139 *V. cholerae* appears to behave clinically and biologically like *V. vulnificus* in many aspects [31]. Both organisms cause invasive soft tissue infections and bacteremia associated with high rates of mortality. They are susceptible to many antibiotics and preferably grow in warm temperatures. Infections caused by either pathogen occur commonly during hot seasons. Risk factors for these vibrio infections are similar (exposure to seawater and ingestion of raw seafood, particularly among immunocompromised patients such as those with hematologic malignancy or cirrhosis [32-35]). Minocycline combined with cefotaxime had been shown to be appropriate for treating *V. vulnificus* infections in vitro and in a mouse model [24,27]. Clinical experience also supports the use of minocycline + cefotaxime for severe *V. vulnificus* infections. Thus, we evaluated inhibitory activities of cephalosporins combined with minocycline against *V. cholerae* non-O1 non-O139.

We found herein that *V. cholerae* non-O1 non-O139 is susceptible to all quinolone-like antibiotics (low MICs). With an inoculum size of *V. cholerae* of 4.7×10^5 CFU/mL, an inhibitory effect of each of cefazolin and minocycline (at concentrations equal to their MICs) can be demonstrated for the first 8 h, but bacterial regrowth is noted later. Combination of minocycline and cefazolin (at concentrations equal to their MICs) inhibits the growth of *V. cholerae* for more than 48 h, and no regrowth is noted. On the other hand, with an inoculum size of *V. cholerae* of 4.7×10^5 CFU/mL, cefotaxime (at MIC concentration) can inhibit bacterial growth for more than 20 h, but regrowth is noted after 24 h. The combination of minocycline and cefotaxime is not synergistic when cefotaxime's concentration is below 8 times the MIC according to our previous results. The combination of minocycline + cefotaxime (at concentrations 8 times the MIC) and the combination of cefazolin + minocycline showed similar efficacy on *V. cholerae*. According to a previous study, a bacteriostatic antibiotic may antagonize the action of a bactericidal drug [36]. However, our results revealed that the

combinations of cefazolin + minocycline and ceftoaxime + minocycline were synergistic. Therefore, the combination of cefazolin + minocycline may be useful clinically for the treatment of *V. cholerae* non-O1 non-O139 infections. However, in vitro susceptibility to antimicrobial agents does not always correlate with in vivo activity. For instance, despite the in vitro susceptibility of *V. cholerae* to ampicillin, this antibiotic appears to have minimal efficacy in the treatment of cholera. Therefore, the in vitro synergistic effect of cefazolin + minocycline and of ceftoaxime + minocycline may not translate into in vivo activity. Thus, testing these antibiotics combinations in animal models is needed in order to predict their suitability for clinical use.

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