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Antibacterial Activity of Ciprofloxacin and Trimethoprim, Alone and in Combination, Against *Vibrio cholerae* O1 Biotype El Tor Serotype Ogawa Isolates

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Abstract

In this communication, the ciprofloxacin-trimethoprim (Cp-Tm) combination showed synergistic (Fractional Inhibitory Concentration, FIC index 0.399) and additive (FIC index 0.665–0.83) effects against *Vibrio cholerae* O1 biotype El Tor serotype Ogawa isolates having Cp MICs 10 μg/ml and Cp 0.66 μg/ml, respectively, following agar dilution checkerboard method. The time-kill study results demonstrated synergy between Cp and Tm against both groups of isolates providing 2.04 $\log_{10}$ (for strain with Cp MIC 0.66 μg/ml) and 3.12 $\log_{10}$ (for strain with Cp MIC 10 μg/ml) decreases in CFU/ml between the combination and its most active compound. Thus, the findings of the present study suggest an introduction of Cp-Tm combination treatment regimen against drug resistant cholera and this in turn will help in combating the drug resistance of *V. cholerae* O1 biotype El Tor serotype Ogawa.

Key words: Antibacterial activity, ciprofloxacin, fractional inhibitory concentration index, trimethoprim

Introduction

The *Vibrio cholerae*, both O1 and O139, are the causative agents of a potentially epidemic and life-threatening disease, cholera. Antibiotic therapy is a useful adjunct to fluid replacement in the treatment of cholera; tetracycline (T) has been the mainstay of cholera therapy, and the other effective alternative antimicrobials used in treating cholera included furazolidone, erythromycin, cotrimoxazole: a trimethoprim-sulfamethoxazole (Tm-Sm) combination, chloramphenicol and ampicillin. But, due to rampant and haphazard use of the antibiotics in the treatment of *V. cholerae* (O1 and O139) infection there were rapid emergence of multidrug resistant (MDR) strains (Garg *et al*., 2000; Samal *et al*., 2001; Sengupta *et al*., 2000), and the fact is considered an issue of great global significance for public health.

In the era of MDR cholera, a high degree of sensitivity of *V. cholerae* to fluoroquinolones, cephalosporins and azithromycin have been reported, and thus these agents were considered as the alternative treatment regimen for MDR cholera (Bhattacharya *et al*., 2003; Das and Gupta, 2005; Taneja *et al*., 2005). Conversely, there are reports on the emergence of fluoroquinolone (including ciprofloxacin; Cp)-resistant *V. cholerae* isolates from different parts of the globe (Garg *et al*., 2001; Saha *et al*., 2005). In addition, the high cost of the other alternative drugs is the limitation as well as the major disadvantage to their usage particularly in the developing country like India. Therefore, the present study has been undertaken to evaluate the *in vitro* efficacy of Cp in combination with Tm against *V. cholerae* O1 biotype El Tor serotype Ogawa isolates from cholera patient in and around Kolkata, India, in order to make Cp-Tm combination as the cost effective treatment regimen for MDR cholera.

Experimental

Materials and Methods

Bacterial strain. A total of 10 isolates of *Vibrio cholerae* O1 biotype El Tor serotype Ogawa from rectal swabs from cholera patients in and around Kolkata were employed for the present study. The *Salmonella*

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enterica serovar Typhi D1/01 laboratory strain was used as the control (Mandal *et al*., 2004a).

Bacterial inocula preparation. The bacterial isolates were grown in Mueller-Hinton broth (Hi-Media, Mumbai, India) and incubated at 35°C for 24 h. Each of the liquid cultures was diluted serially, and inocula were adjusted, by colony count technique, to approximately 10^4 CFU/spot for agar dilution and agar dilution checkerboard techniques, and 5×10^5 CFU/ml for time kill studies (Mandal *et al*., 2003a).

Determination of minimum inhibitory concentration. The minimum inhibitory concentration (MIC) values of Cp and Tm (Hi-Media, Mumbai, India) for the *Vibrio cholerae* O1 biotype El Tor serotype Ogawa isolates were determined by agar dilution method, using antibiotic concentration ranging from 0.25 to 25 µg/ml and 25–250 µg/ml, respectively. The method is according to the criteria of the National Committee for Clinical Laboratory Standards (NCCLS, 1997), and the methodology in details is described earlier (Mandal *et al*., 2003b).

Combined antibacterial activity. The combined antibacterial activities of Cp and Tm against the isolates were determined by agar dilution checkerboard method (Krogstad and Moellering, 1980), employing 8×8 template system, with antibiotic concentration 0.25–66 µg/ml and 0.011–0.72 µg/ml, respectively, for Tm and Cp. The method in details is described in our earlier publications (Mandal *et al*., 2004a; NCCLS, 1997). Synergy, additive and antagonism, if any, were defined as Fractional Inhibitory Concentration (FIC) index ≤0.5, >0.5–4 and >4, respectively (Leclercq *et al*., 1991).

Time-kill curve. Time-kill studies, following standard method published earlier (NCCLS, 1997), using antibiotic concentration ¼ × MIC, alone and in combination were carried out for two representative *Vibrio cholerae* O1 biotype El Tor serotype Ogawa strains: S19 (Cp MIC 0.66 µg/ml) and S2 (Cp MIC 10 µg/ml), for which, based upon the FIC index, the combined effects were respectively synergistic and additive. Synergism was defined as a ≥2 $\log_{10}$ decrease in CFU/ml between the combination and its most active compound (Leclercq *et al*., 1991).

Statistical analysis. The χ^2-test was employed to compare the antibacterial potentiality of Cp alone and Cp in combination with Tm, and that of Tm alone and Tm in combination with Cp. A p value of ≤0.001 was considered significant.

Results and Discussion

The MICs and FICs of Cp and Tm, and the FIC indices for 10 isolates of *Vibrio cholerae* O1 biotype El Tor serotype Ogawa are represented in Table I. Out of 10 isolates, 6 (60 %) had Cp MICs 0.66 µg/ml, and the other 4 (40 %) had Cp MICs 10 µg/ml. All the 10 (100 %) isolates were resistant to Tm showing MICs 75–200 µg/ml. The FICs of Cp and Tm ranged 0.33–0.66 µg/ml and 25–33 µg/ml, respectively. The FIC index was 0.399 for 4 isolates showing Cp and Tm MICs 10 µg/ml and 75 µg/ml, respectively. The 6 isolates (Cp MIC 0.66 µg/ml) that showed higher Tm MICs (100–200 µg/ml) had FIC indices 0.665–0.83.

Table 1
Effect of ciprofloxacin (Cp)-trimethoprim (Tm) combination against *V. cholerae* O1 biotype El Tor serotype Ogawa isolates (n = number of isolates)

n (%)	MIC (µg/ml)		FIC (µg/ml)		FIC Index
	Cp	Tm[4]	Cp	Tm[4]	
4 (40%)	10[1]	75	0.66[1]	25	0.399
2 (20%)	0.66[2]	100	0.33[2]	33	0.83
4 (40%)	0.66[3]	200	0.33[3]	33	0.665

MIC, Minimum inhibitory concentration; FIC – Fractional Inhibitory Concentration. [1] $p<0.001$ in between Cp MICs and Cp FICs, [2] $p<0.95$ in between Cp MICs and Cp FICs, [3] $p<0.95$ in between Cp MICs and Cp FICs, [4] $p<0.001$ in between Tm MICs and Tm FICs. n; Number of isolates.

The killing activities of Cp and Tm, in presence of sub inhibitory concentration (¼ × MIC) of each of the antibiotics, alone or in combination are represented in Fig. 1 and Fig. 2. The Cp-Tm combination showed growth inhibitory effect on both the strains, *V. cholerae* S19 and *V. cholerae* S2, after 3 h of incubation, while after 24 h the bacterial cell counts were reduced by 2.42 $\log_{10}$ CFU/ml and 2.508 $\log_{10}$ CFU/ml, respectively, compared to the initial, 5×10^5 CFU/ml, inocula. The Cp and Tm, when used alone, did not show bactericidal activity against *V. cholerae* S19 and *V. cholerae* S2 strains; the cell counts due to the action of Cp and Tm after 24 h were recorded as 4.46 $\log_{10}$ CFU/ml and 4.49 $\log_{10}$ CFU/ml respectively (for *V. cholerae* S19 strain), and 5.79 $\log_{10}$ CFU/ml and 5.628 $\log_{10}$ CFU/ml respectively, for *V. cholerae* S2 strain.

Huovinen *et al.* (1992) first reported the synergistic effect of Cp-Tm combination against clinical bacterial strains such as *Escherichia coli*, *Staphylococcus aureus* and *Streptococcus*. Genedani *et al.* (1983) and Bertolini *et al.* (1980) reported synergistic activity of Tm in combination with oxolinic acid and nalidixic acid against gram negative bacteria including *E. coli*, and against gram positive cocci. Mandal *et al.* (2004a) showed a marked synergy between Cp and Tm against Tm-resistant *Salmonella enterica* serovar Typhi showing reduced susceptibility to Cp, which in combination with some other antibiotics such as gentamicin and cefazolin (Cz) showed synergistic effect, and additive effect with amoxicillin and Cz on MDR *S. enterica* serovar Typhi (Mandal *et al.*, 2004a; Mandal *et al.*, 2003b; Mandal *et al.*, 2004b)

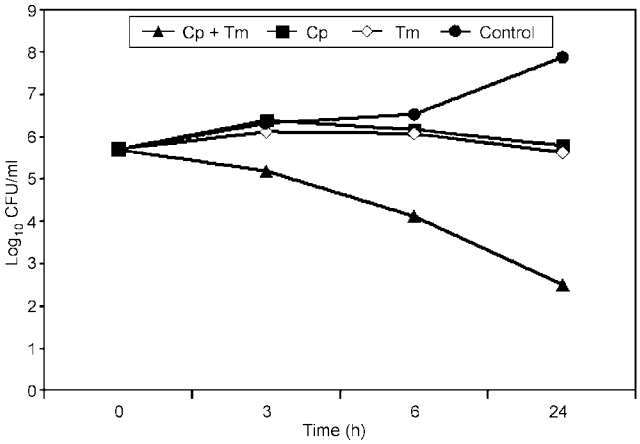

Fig. 1. Time-kill curves for *V. cholerae* O1 biotype El Tor serotype Ogawa S19 strain using ciprofloxacin (Cp) and trimethoprim (Tm). The Cp MIC of S19 is 10 µg/ml. The control was antibiotic-free medium.

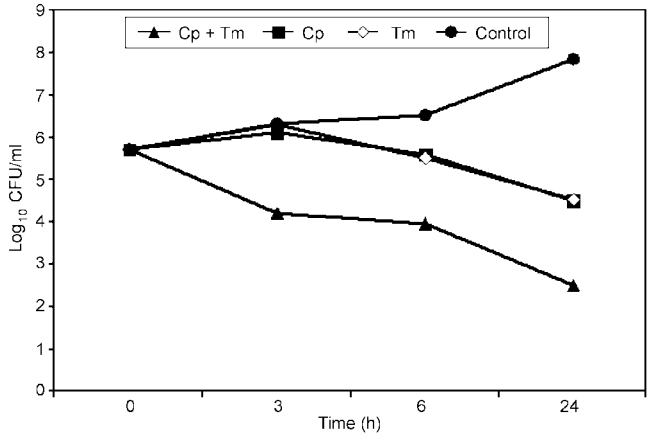

Fig. 2. Time-kill curves for *V. cholerae* O1 biotype El Tor serotype Ogawa S2 strain using ciprofloxacin (Cp) and trimethoprim (Tm). The Cp MIC of S2 is 0.66 µg/ml. The control was antibiotic-free medium.

In the present study, Cp in combination with Tm had synergistic effect (FIC index 0.399) on *V. cholerae* O1 biotype El Tor serotype Ogawa isolates having Cp MIC 10 µg/ml, while additive activity (FIC indices 0.665 – 0.83) was noticed against the isolates having Cp MIC 0.66 µg/ml, by checkerboard agar dilution method, and there were significant differences in between MICs and FICs of Cp for isolates with Cp MIC 10 µg/ml ($p<0.001$), and in between MICs and FICs of Tm for all isolates ($p<0.001$). Following time-kill studies, Cp-Tm combination showed synergistic effects on *V. cholerae* O1 biotype El Tor serotype Ogawa strains having high Cp MIC (10 µg/ml) as well as low MIC for Cp (0.66 µg/ml). The decrease in bacterial cell counts by 2.04 $\log_{10}$ CFU/ml for *V. cholerae* S19 (Cp MIC 0.66 µg/ml) strain and by 3.12 $\log_{10}$ CFU/ml for *V. cholerae* S2 (Cp MIC 10 µg/ml) strain due to the action of Cp-Tm combination compared to the cell count due to the action of its most active component (here, Cp for S19 and Tm for S2 strains), after 24 of incubation at 35°C, supported this view, and significant differences in killing were found between Cp-Tm combination and Cp alone ($p<0.001$), and between Cp-Tm combination and Tm alone ($p<0.001$) for the isolates. Moreover, the combination (Cp-Tm) had bactericidal effect on *V. cholerae* S19 as well as *V. cholerae* S2 strain; the reduction of cell counts respectively by 3.278 $\log_{10}$ CFU/ml and 3.19 $\log_{10}$ CFU/ml (after 24 h incubation at 35°C) in the presence of Cp-Tm combination established the fact.

Bertolini *et al.* (1980) reported the incidence of suppression of antibiotic resistance and the enhancement of antibacterial activity of quinolon-Tm combination, and Mandal *et al.* (2004a) expressed similar view using CP-Tm combination against *S. enterica* serovar Typhi. In the present study, synergistic and additive effect of the antibiotic combination in terms of FIC index, respectively on Cp-resistant (MIC 10 µg/ml) and Cp-sensitive (MIC 0.66 µg/ml) isolates of *Vibrio cholerae* O1 biotype El Tor serotype Ogawa, and synergism between Cp and Tm against both categories of *Vibrio cholerae* O1 biotype El Tor serotype Ogawa isolates by time-kill studies indicated the Tm-mediated suppression of Cp-resistant and *vice versa.* The earlier authors (Craig and Vogelman, 1987; Vogelman and Craig., 1986; Westh *et al.*, 1992): reported the system that demonstrates both the rate and extent of bacterial killing (kill kinetics) providing more accurate description of antimicrobial activity than does the MIC. Thus, the strong *in vitro* antibacterial activity of Cp-Tm combination against *Vibrio cholerae* O1 biotype El Tor serotype Ogawa showing resistance to Cp and Tm suggests that Cp in combination with Tm could be the potential treatment regimen against drug-resistant cholera.

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