Synergistic Effect of Combination Antibiotics against Multidrug-Resistant *Salmonella enterica* serovar *Typhi*

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ABSTRACT: Background: Infection caused by multidrug-resistant (MDR) *Salmonella enterica* serovar *typhi* (*S. typhi*) is a major health problem in low- and middle-income countries (LMICs). *S. typhi* has been reported to be resistant to fluoroquinolones and cephalosporins. The Objective of this study is to find out the anti-microbial activity of combination antibiotics against resistant *S. typhi*. First, single antibiotic disks of ciprofloxacin, imipenem and vancomycin with the concentrations of 20, 40, 60 and 80 µg/ml were prepared and applied against sensitive *S. typhi* to confirm its susceptibility. Later, a resistant strain of *Shigella flexneri* was treated with single antibiotic (ciprofloxacin, vancomycin and imipenem) at the highest concentration (80 µg/ml) to determine its resistant behavior by measuring the zones of inhibition obtained from the disc diffusion assay. Co-culture was performed between the sensitive and resistant strains to develop the resistant strain of *S. typhi*. Combinations of antibiotics were used for susceptibility testing against the newly resistant strain of *S. typhi* by using Kirby–Bauer disk diffusion method. Experiments were carried out in triplicates and the average reading was recorded. The study showed that different concentrations of the combination of vancomycin and imipenem (20, 40, 60, 80 µg/ml) exhibited 18, 20, 24- and 29-mm respective zone of inhibition (ZOI) against *S. typhi*. A combination of ciprofloxacin and imipenem also exhibited optimum ZOI. It was observed in this study that a single antibiotic treatment did not show any activity against newly resistant strains of *S. typhi*. The combination therapy can be used as a beneficial treatment approach in multi-drug resistant S. Typhi infections.

Key words: Drug resistance, *Salmonella enterica* serovar *typhi*, *Shigella flexneri*, synergistic effects, zone of inhibition.

INTRODUCTION

Infection due to *Salmonella enterica* serovar *typhi* (*S. typhi*) is a major health concern, especially in low- and middle-income countries (LMICs). Out of approximately 12-18 million cases of typhoid fever globally per annum, an estimated 130000 deaths occur, primarily in LMICs.[1-3] Bangladesh is a typhoid endemic country where *S. typhi* is one of the main causes of morbidity and mortality.[4] The situation become exacerbated when the organism shows resistant against clinically important antibiotics.[5] Although ampicillin, trimethoprim-sulfamethoxazole and chloramphenicol were considered as the first-line agents against typhoid fever during early 1970s, emergence of multi drug resistant (MDR) strains reported in late 1980s from multiple countries was a definitive set-back to antibiotic therapy.[6,7] In response to the MDR *S. typhi*, ciprofloxacin was considered an alternative drug for the treatment of typhoid fever.[6]

In a comparison study of phenotypic and world geodetic system (WGS)-derived antimicrobial resistance (AMR) profiles, 25% of *S. typhi* isolates were found to be MDR with 10.8% resistant and 73.5% showing decreased susceptibility to the

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ciprofloxacin that belongs to fluoroquinolone group.[8] Reduced susceptibility to fluoroquinolones, which is mostly associated with nalidixic acid resistance, followed by resistance towards the azalide antimicrobial (azithromycin) and the third generation cephalosporin (ceftriaxone) have been reported in *S. typh*i in the US, Canada, UK, Germany, Philippines, India and Bangladesh.[3,5]

A study carried out by Chiou *et al.,* 2014 revealed that among 38 isolates of *S. typhi* from Bangladesh, 82% and 40% were found resistant against nalidixic acid and ciprofloxacin respectively. These isolates were also found resistant against ampicillin (68.4%), chloramphenicol (57.9), streptomycin (60.5%) and sulfamethoxazole (68.4%).[9] Antibiotic susceptibility pattern was also observed against *Salmonella enterica* serovars isolates in hospital in northern India. The study showed the recurrence of susceptibility of the isolates to conventional antibiotics, however, a significant increase in resistance was observed to fluoroquinolones.[10] A retrospective study in culture positive *S. typhi* patients in Bangladesh showed that 28.3% isolates were MDR. Nalidixic acids resistance and ciprofloxacin intermediate sensitivity were also observed in more than 90% of the isolates. However, all *S. typhi* isolates were sensitive to third generation cephalosporins.[4] Unfettered use of antimicrobials is an aggressive attitude, resulting in the growing MDR trend.[11] As such, available antimicrobial agents to treat the MDR strains should be prioritized.[12] Propolis extracts (a natural product made by bees) have been shown to exert a synergistic effect with antibiotics against many bacterial species, including *S. typhi*.[13] Many strategies, including the development of new antimicrobial agents, the revival of old antibiotics, combination therapy, and the optimal use of antimicrobial agents, have been proposed to fight or delay resistance as multi-drug resistant organisms.[14] A recent study evaluated the relationship between the emergence of antibiotic heteroresistance and the interactions between antibiotics used in combination therapies for *Salmonella enterica* serovar *typhi*. The study demonstrated that combination antibiotic regimens can both prevent the emergence of cross-resistance to several antibiotic classes and effectively boost antimicrobial efficacy against resistant *Salmonella* species.[15] It has been shown that single antibiotic treatment may not be helpful against the resistant strains; determining the synergistic effects of different antimicrobials against MDR strains would be beneficial in combating the infections. The combinations of antibiotics may provide a useful strategy against antibiotic resistance.[16] Also, synergistic antibiotic combinations can provide greater efficacy at lower doses. Therefore, this study has been carried out to find out the synergistic activity of anti-microbial agents against the resistant strains of *S. typhi*.

MATERIALS AND METHODS

The study was carried out at the Microbiology Laboratory of the Department of Pharmacy, East West University. The co-culture was performed by incorporating *S. flexneri* with the sensitive strain of *S typhi* targeting a horizontal gene transfer mechanism. *S. typhi* strain ATCC 19430 and *S flexneri* strain ATCC 29903 were grown onto two agar plates, namely *Salmonella Shigella* (SS) agar (himedia, india) and xylose lysine deoxycholate (XLD) agar (Himedia, India) respectively. The plates were incubated at 37ºC for 18-24 h. Both organisms showed their typical colony-forming unit in their respective culture plates. The antibiotic susceptibility test was performed using three antibiotics (vancomycin, imipenem and ciprofloxacin) against the two pathogens by using the Kirby-Baurer disk diffusion method. After obtaining the resistant and susceptible strains, co-culture of the two organisms was carried out by using SS agar since both the pathogens grow on SS agar. From the co-culture, colonies of *S. typhi* were again sub-cultured on SS agar plate. Kirby–Bauer disk diffusion was performed for antibiotic sensitivity testing using Mueller– Hinton agar (Himedia, India) according to the Clinical & Laboratory Standards Institute (CLSI) recommendation.[17] Antibiotic stock solutions (ciprofloxacin, imipenem and vancomycin) were

prepared according to Vineetha *et.al,* 2015 and the *S. typh*i was made resistant through co-culture with *Shigella flexneri*.[18]

First, 20-25 ml agar media per petri dish was prepared as test plates and cooled for inoculating pure bacteria *S. typhi*. The antibiotic disks were prepared using Whatman filter paper no. 1 (Whatman / GE Healthcare Companies, United Kingdom), and the antibiotic (ciprofloxacin, imipenem and vancomycin) stock solution was prepared according to Vineetha *et.al,* 2015.[15] Single antibiotic disks of ciprofloxacin, imipenem and vancomycin with concentrations of 20, 40, 60 and 80 µg/ml were applied against sensitive *S. typhi* to determine the susceptibility. The resistant strain, *Shigella flexneri* was treated with single antibiotic (ciprofloxacin, vancomycin, imipenem) at their highest concentration (80 µg/ml) to confirm the resistance. Ciprofloxacin and vancomycin were used in combination with imipenem at 1:1 ratio. The concentrations of antibiotics used were 20, 40, 60 and 80 µg/ml in susceptibility testing against resistant (MDR) *S. typhi*. After incubation, the antimicrobial activities of the test materials were determined by measuring the diameters of the zone of inhibition (ZOI). Experiments were carried out in triplicates and the mean of the readings was recorded. Zone diameters were measured, and interpretations were made according to the CLSI recommendation guidelines. [17]

RESULTS AND DISCUSSION

`Before the co-culture, *S. typhi* was sensitive to different concentrations of ciprofloxacin and imipenem (40, 60 and 80 µg/ml). Vancomycin also inhibited *S. typhi* at concentrations of 60 and 80 µg/ml. (Table 1). However, *S. flexneri* did not show any zone of inhibition when treated with a single antibiotic (ciprofloxacin, vancomycin or imipenem) at the highest concentration of 80 µg/ml. After co-culturing, no satisfactory ZOI with single antibiotics was obtained for the resistant *S. typhi* strain (Table 1).

Table 1. Treatment of *Salmonella enterica* serovar *typhi* with different concentrations of single antibiotic (Ciprofloxacin, Vancomycin, Imipenem).

Antibiotics	Antibiotic concentrations (µg/ml)	Zone diameter (mm)	
		S. typhi (Before co-culture)	*S. typhi* (After co-culture)
Ciprofloxacin	20	20	0
	40	24	0
	60	25	3
	80	27	5
Vancomycin	20	19	0
	40	20	0
	60	21	0
	80	23	4
Imipenem	20	21	0
	40	23	0
	60	26	4
	80	27	9

Using multiple antimicrobial agents instead of single showed satisfactory results against the resistant strains of *S. typhi* (Table 2). For the combination of vancomycin and imipenem (20, 40, 60 and 80 µg/ml), the ZOIs observed were 18, 20, 24 and 29 mm respectively. Similarly, ZOIs were obtained 19, 21,

25 and 32 mm respectively when treated with the combination of ciprofloxacin and imipenem (Table 2). Therefore, we observed a synergistic effect imipenem when combined with vancomycin & ciprofloxacin against the resistant *Salmonella enterica* serovar typhi.

Table 2. Treatment of resistant *Salmonella enterica* serovar typhi with different concentrations of combinations antibiotics (Vancomycin + Imipenem and Ciprofloxacin + Imipenem)

Antibiotic concentrations (µg/ml)	ZOI (mm)				
	Vancomycin (Van)	Ciprofloxacin (Cipro)	Imipenem (Imi)	Vancomycin+ Imipenem*	Ciprofloxacin+ Imipenem**
20	0	0	0	18	19
40	2	4	5	20	21
60	6	8	10	24	25
80	9	9	10	29	32

ZOI=Zone of inhibition, *Vancomycin:Imipenem=1:1, **Ciprofloxacin:Imipenem=1:1

In our study, a standard zone of inhibition (ZOI) was observed when resistant *S. typhi* was treated with combination of vancomycin and imipenem. Combination of ciprofloxacin and imipenem also exhibited optimum ZOI against the resistant *S. typhi*. However, the resistant strain was not susceptible to any of the antimicrobials alone. Compared to the treatment with the single imipenem (80 µg/ml), combination of imipenem, either with vancomycin or ciprofloxacin, exhibited approximately a 3-fold increase in ZOI against the resistant strain. This corroborates the findings of Kim DM *et al.*, 2010, where the authors demonstrated *in vitro* synergistic effect of various combinations of antimicrobial agents (ciprofloxacin, cefotaxime and azithromycin) against nalidixic acid-resistant *S. typhi* (NARST). Significant synergistic effects were observed when a combination of ciprofloxacin (0.012–0.375 µg/ml) and cefotaxime (0.063-0.125 µg/ml) was used against NARST strains when compared with the single antibiotic treatment. A combination of fluoroquinolone and β-lactam may improve the efficacy when compared with fluoroquinolone alone to treat patients with typhoid fever.[19] Another study investigated a possible synergistic effect of extracts of propolis and different antibiotics (amoxicillin, ampicillin and cefalexin) against *S. typhi*.[13] Recent studies of combination antibiotics against resistant strains were observed. Antibiotic synergism was detected from antimicrobial peptides and several antibiotics like gentamicin, vancomycin, azithromycin, amoxicillin against bacterial strains of *S. aureus, P. aeruginosa, A. baumannii and E. coli*.[20]

Typhoid is a common disease in both developing and developed countries. The previously efficacious antibiotics have become ineffective in the treatment of typhoid. Furthermore, inappropriate use of antibiotics has also contributed to the resistance prevalence. Prevention and treatment of infections are now threatened due to antimicrobial resistance caused by bacteria and other pathogens.[21] The situation is getting worse due to increasing multidrug resistance, moreover, it is difficult to formulate a new drug for the treatment of infection caused by *S. typhi*. We obtained the optimum ZOI from the different combinations of antimicrobial agents against the resistant pathogens. As antibiotic resistance is increasing day by day, combination antimicrobials with the existing antibiotics may have greater values to inhibit MDR pathogens. This study only focuses on the susceptibility of combination antibiotics to resistant strains that were created in our lab using the co-culture approach. We assumed that the horizontal gene transfer method was used to create the new resistant pathogen from the original resistant one, but we did not examine the genetic pattern of the produced resistant pathogen.

The development of antibiotic resistance in bacteria has been declared as a matter of global health concern by the World Health Organization.[17] In Bangladesh resistance to nalidixic acid has been observed against *Salmonella* isolates.[4] Abuse and overuse of antibiotics may have increased the incidence of antibiotic resistance in Bangladesh.[21] Here *in-vitro* experiment with the *Salmonella enterica* serovar typhi strain clearly demonstrated that combinations of antibiotics should be opted out as better options for treatment of *S. typhi* infections caused by the MDR strain, instead of using single antibacterial agents as supported by the previous studies. Peptidoglycan polymerization process is prevented by vancomycin from reaching the transglycosylation stage, which weakens the cell wall and damages the underlying cell membrane. Imipenem, like other β-lactam antibiotics, binds to penicillin binding proteins (PBPs), disrupts bacterial cell wall synthesis, and causes the death of susceptible microorganisms. Imipenem is very resistant to hydrolysis by most β-lactamases. On the other hand, ciprofloxacin antibiotics target bacterial DNA gyrase and topoisomerase IV, hence inhibiting the growth of bacteria. Combining different antibiotics produces a synergistic impact. To achieve the therapeutic effect, each of the combination antibiotics uses a different mechanism of action. Antibiotic combination, therefore, shows synergistic action in treating infections brought on by microorganisms that are resistant to antibiotics.

Further investigations should be carried out to finalize the effects of the combination of antibiotics against the resistant strains.

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CONFLICT OF INTEREST

There are no conflicts of interest.

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