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Short Communication

Effect of Selected Antibiotics on Biofilm Formed by *Salmonella Enterica* Serovars Typhi and Paratyphi

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Salmonella enterica **serovars Typhi and Paratyphi are the causative agents of typhoid and paratyphoid, respectively, in human.** ***Salmonella*** **is able to form biofilms whereby members are resistant and persistent in both host and non-host environments. In the present study the effect of the antibiotics, Azithromycin, Imipenem, Ceftriaxone and Cefixime, on planktonic and biofilm phase clinical isolates of** ***Salmonella enterica*** **serovars Typhi (n = 30) and Paratyphi A (n= 07) was investigated. MIC (Minimum Inhibitory Concentration) and MBC (Minimum Bactericidal Concentration) of planktonic phase bacteria were determined and compared with MRC (Minimum Re-growth Concentration) and MBEC (Minimum Biofilm Eradication Concentration) of biofilm population. The present study indicates that, with the exception of Azithromycin, a considerably higher concentration is needed for all other antibiotics investigated to inhibit growth of test isolates in the biofilm phase.**

We conclude that the requirement of Azithromycin at sub-MIC concentration to inhibit/kill ***Salmonella*** **in biofilm is of particular significance in that it can be employed for the eradication of** ***Salmonella*** **spp. biofilms.**

Keywords: Biofilm, ***Salmonella*****, antibiotics**

Introduction

Biofilm is a complex, sessile community of microorganisms that usually remains attached to a surface or as an extracellular matrix in aggregates[1]. *Salmonella* enterica serovars Typhi and Paratyphi are the causative agents of typhoid and paratyphoid fever, respectively. They can exist both as planktonic cells and as sessile or multicellular forms or biofilm states. It is important to form biofilm because it helps bacteria to survive against extreme environments like disinfectants and chemical, physical, and mechanical stresses[2-4]. In addition, bacterial biofilm are more resistant to antibiotics and also against the host immune defence than the planktonic phase bacteria[5,6]. For some antibiotics, the concentration required to kill biofilm bacteria may be greater than a thousand times that required to kill planktonic bacteria of exactly the same strain[7-8]. During the last decade, it has become increasingly clear that bacteria, including foodborne pathogens such as *Salmonella enterica*, grow predominantly as biofilms in most of their natural habitats, rather than in planktonic mode[9].

In the present study, we investigated the influence of Imipenem, Cefixime, Ceftriaxone and Azithromycin on planktonic and biofilm forming cells of *Salmonella enterica* serovars Typhi and Paratyphi obtained from clinical sources.

Materials and methods

Bacteria

In the present study, 30 *S. enterica* serovar Typhi (*S*. Typhi) and 7 *S. enterica* serovar Paratyphi (*S*. Paratyphi) isolated from clinical samples and stored in the laboratory repository were studied. Clinical samples were previously identified by agglutination test by using *Salmonella enterica* serovars Typhi and *Salmonella enterica* serovars Paratyphi specific antisera.

Determination of MIC

McFarland 0.5 turbidity standards were prepared as per the standard guidelines described by the Clinical and Laboratory Standards Institute[10]. McFarland standards were stored in the dark at room temperature (22 $^{R"}$C to 25 $^{R"}$C). Before use, the standards were shaken well, mixing the fine white precipitate of barium sulfate in the tube.

Bacteria inoculated into Mueller Hinton Broth (MHB) within sterile 4ml vials were incubated at 37 $^{R"}$C for 4 hrs. The turbidity of actively growing cultures in fresh MHB was adjusted to obtain the turbidity of McFarland 0.5 standard. From this culture, bacteria were patched with sterile tips on to Mueller Hinton Agar (MHA) containing different concentrations of antibiotics with a numbered grid line attached on the bottom of each plate. The plates were then incubated at 37°C for 24 hrs. After 24 hrs the plates were observed for the presence or absence of growth in presence of different concentration of antibiotics. The minimum concentration of antibiotic that inhibited visible growth was interpreted as the MIC.

MRC determination assay

MRC (minimum re-growth concentration) is defined as the minimum antibiotic concentration (mg/ml) which inhibits re-

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growth of the cells from biofilm phase. MRC was determined according to a procedure described previously[11]. Biofilm was produced in 96 well microtiter plate containing 190 µl fresh ATM (Adherence Test Media) inoculated with 10 µl bacterial culture from TSB (Trypticase soy broth). The microtiter plate was then incubated at 37°C for 72 hrs. Stock solutions of antibiotics were prepared. After biofilm production, wells containing bacterial cultures were removed from the media and washed with 1X phosphate buffered saline or PBS buffer three times under aseptic condition. The microtiter plate was kept in an inverted position for 5 min to dry the plate. Volumes of 200 µl of appropriate dilutions of antibiotic in Muller Hinton Broth were prepared from stock solution and transferred into the wells with established biofilms. For each sample antibiotic dilution in each concentration was run in triplicate. A negative control (control of microtiter plate sterility; only diluted antibiotics) were included in all experiments. The plates were incubated for 24 hr at 37°C. OD values were obtained by measuring turbidity at 600 nm in a 96-well plate reader (Epoch, USA)[12].

MBEC determination assay

MBEC (Minimal biofilm eradication concentration) was defined as the lowest concentration of antibiotic required to eradicate biofilm phase bacteria. For MBEC determination each well containing antibiotic treated biofilm was washed three times with 1X PBS buffer under aseptic conditions and filled with 10 µl 0.1% BPW. The biofilm layers were collected by scraping the biofilm area with sterile cotton swabs and vigorously mixed in the wells to release biofilm forming cells. The cotton swabs were patched on XLD agar plates containing without antibiotic. Then the plates were incubated at 37°C for 24 hours.

Results

Minimum Inhibitory, Bactericidal, Regrowth and Biofilm Eradication Concentrations of S. enterica Typhi and Paratyphi: Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of Imipenem, Ceftriaxone, Cefixime and Azithromycin for the isolates of *Salmonella enterica* serovar Typhi and Paratyphi were determined by broth dilution method according to Clinical and laboratory standard institute (CLSI) guidelines[10] and following the protocol of Andrews, 2001[13]. Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC), Minimum Regrowth Concentrations (MRC) and Minimum Biofilm Eradication Concentrations (MBEC) of Imipenem, Ceftriaxone, Cefixime and Azithromycin were determined (Tables 1 and 2).

From Tables 1 and 2, MIC values ranged for Azithromycin between 32-128 µg/ ml with MBC ranging between 64-256 µg/ ml. The MRC values were 0.4- 1.6 µg/ ml and the MBEC values were 0.8- 3.2 µg/ ml. The thirty-seven samples of *S. enterica* serovars Typhi (81.081%) and *S. enterica* serovars Paratyphi (18.918%) exhibited MBEC values ½ that of the MIC value and MRC value ¼ that of the MIC value for Azythromycin. In contrast, ² - lactam antibiotics (Imipenem, Ceftriaxone and Cefixime) showed MIC values ranging 8 µg/ ml for Imipenem and 32 µg/ ml for Ceftriaxone and Cefixime. Besides, MBC values ranged between 16-64 µg/ml (16 µg/ml for Imipenem and 64 µg/ml for Ceftriaxone and Cefixime). A total of thirty-seven samples indicated MRC values of 200-800 µg/ml (200-800 µg/ml for Imipenem and 320 µg/ml for Ceftriaxone and Cefixime) and MBEC values of 400- 1600 µg/ml (400- >1200 µg/ml for Imipenem and 1600 µg/ml for Ceftriaxone and Cefixime).

Table 1. *Comparison among MIC, MRC, MBC and MBEC of different antibiotics against clinical isolates of Salmonella enterica serovar* Typhi

Name of Antibiotics	MIC (µg/ml)	MRC (µg/ml)	MRC/MIC (µg/ml)	MBC (µg/ml)	MBEC (µg/ml)	MBEC/MBC (µg/ml)
Imipenem	8	200-800	25-100	16	400-1200	25-75
Ceftriaxone	32	320	10	64	1600	25
Cefiime	32	320	10	64	1600	25
Azithromycin	32-128	0.4-1.6	0.0125	64-256	0.8-3.2	0.0125

MIC=Minimum inhibitory concentration; MBC=Minimum bactericidal concentration; MRC =Minimum Re-growth Concentration; MBEC=Minimum Biofilm Eradication Concentration

Table 2.*Comparison among MIC, MRC, MBC and MBEC of different antibiotics against clinical isolates of Salmonella enterica serovar* Paratyphi

Name of Antibiotics	MIC (µg/ml)	MRC (µg/ml)	MRC/MIC (µg/ml)	MBC (µg/ml)	MBEC (µg/ml)	MBEC/MBC (µg/ml)
Imipenem	8	200-800	25-100	16	400-Ã1200	25-75
Ceftriaxone	32	320	10	64	1600	25
Cefiime	32	320	10	64	1600	25
Azithromycin	64-128	0.8-1.6	0.01	128-256	1.6-3.2	0.01

MIC=Minimum inhibitory concentration; MBC=Minimum bactericidal concentration; MRC =Minimum Re-growth Concentration; MBEC=Minimum Biofilm Eradication Concentration

Discussion

Salmonellae are recognized worldwide as major zoonotic pathogens for both humans and animals. Thus, biofilm production acts as a key adaptive strategy adopted by *S. enterica* serovars Typhi in order to allow microbial persistence, sustaining an increased resistance against antibiotics[14]. A biofilm lifestyle affords bacteria a 10 to 1,000-fold increase in antibiotic resistance compared to their planktonic counterparts[8]. Biofilms can form on medical implants[15], leading to increased morbidity and mortality of affected individuals. The present study has demonstrated a clear difference in antibiotic susceptibility between planktonic populations and biofilm populations of *S.* Typhi and *S.* Paratyphi. Results were obtained by MRC (Minimum Re-growth Concentration) and (Minimum Biofilm Eradication Concentration) assays. MBEC assays were developed for rapid and reproducible antimicrobial susceptibility testing for bacterial biofilms in the anticipation that the MBEC would be more reliable for selection of clinically effective antibiotics[12]. MRC and MBEC methods make it possible to study the influence of different concentrations of antibiotics on biofilm phase bacteria. It can also help in testing microorganisms whose MIC determination do not provide clinically relevant information.

The present study showed that, the macrolide antibiotic Azithromycin (AZM) is effective to biofilm phase bacteria of *Salmonella* Typhi and *Salmonella* Paratyphi at a sub-MIC level. Similarly, it was detected that, the XDR *Salmonella* Typhi strain is only suscep-tible to azithromycin and carbapenems[16]. A previous report indicated that Azithromycin was effective at sub-MIC levels against *Porphyromonas gingivalis* biofilms and could be used for future clinical treatment for oral biofilm infections[17] which is similar to our finding. Similarly, Azithromycin is also a potential inhibitor of *Staphylococcus xylosus* biofilm formation by altering protein expression[18].

In a study[19], Azithromycin significantly inhibited the production of alginic acid from the mucoid strain at e" 1/256 MIC, and the production of exopolysaccharides from the nonmucoid strain at e" 1/16 MIC. These findings suggest that Azithromycin inhibits biofilm formation by *Pseudomonas aeruginosa* at concentrations well below the MIC. All of these reports are very similar to our finding that; 1/2MIC of Azithromycin can remove *Salmonella* Typhi and *Salmonella* Paratyphi biofilms. In contrast, the present study showed that the antibiotic from ²-lactam group, Imipenem, Ceftriaxone, Cefixime, were able to kill planktonic phase bacteria at lower concentrations that were not effective in killing biofilm bacteria. According to a previous report, because of poor penetration into cells ²-Lactams have been recognized ineffective against microbes growing inside mammalian cells. However, Cefixime, a ²-Lactam antibiotic has been proven to be clinically effective against typhoid fever[20]. After being combined with Imipenem, sodium houttuyfonate showed a greater effect against biofilms[21]. They also found that, the most effective way to eradicate the more resistant biofilm-like microcolonies was by the daptomycin/doxycycline/ceftriaxone triple drug combination without pulse dosing[22]. The present study concludes that higher concentrations of beta lactam antibiotics are required to eradicate *Salmonella* biofilms. In contrast, it is also demonstrated the efficacy of Azithromycin to inhibit or eradicate *Salmonella* Typhi or Paratyphi at sub-inhibitory concentrations. Azithromycin is likely to be useful for the treatment of diseases caused by *Salmonella enterica* serovars Typhi and Paratyphi biofilms. However, the mechanism of action of Azithromycin against *Salmonella enterica* serovars Typhi and Paratyphi biofilms remains a subject for future studies. Our study shows promise in clinical or environmental cases where removal of biofilm is needed. It is also important to realize that MIC and/or MBC values may actually not be suitable indications for the amounts of antibiotics needed for biofilm bacteria.

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