

RESEARCH ARTICLE

Effect of subinhibitory concentration of cefetoxime on biofilm formation

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ABSTRACT

Objectives: In the present study, we have analyzed ESBL-producing *S. typhi*'s capability in forming a significant amount of biofilm on plastic and glass surface, and the influence of cefetoxime on biofilm development at subinhibitory (Sub-MIC) concentration.

Methods: Nine strains of cefetoxime-mediated ESBL-producing *S. typhi* were used in the study. *S. typhi* formed biofilm on plastic and glass materials; it was demonstrated using micro titre plate (MTP) and standard test tube methods. Comparative study of the influence of cefetoxime on biofilm formation in its MIC (128 µg/ml) and at sub-MIC (64 µg/ml) was demonstrated by microtitre plate method. The biofilm production was observed in SEM images, statistical analysis (ANOVA) showed significant increase in cell surface and volume due to the influence of Cefetoxime.

Results: Of the nine selected isolates, two *S. typhi* strains, namely BST 51 and BST 130, produced relatively strong biofilm in the presence of cefetoxime at sub-MIC level (64 µg/ml), comparatively weak biofilm formation at MIC level (128 µg/ml). Typical morphological changes were observed in cefetoxime-resistant strains, *S. typhi* BST 51 and BST 130, in comparison to cefetoxime-sensitive strain *S. typhi* BST 63 used as a control. We found an increase in surface and volume of a cell in response to cefetoxime and statistical data (ANOVA) proved that resistant strains were significantly different from control strains.

Conclusion: The above study clearly shows that cefetoxime at sub-MIC level efficiently induces biofilm formation and promotes changes in morphology of the cell. *J Microbiol Infect Dis 2017; 7(2): 67-75*

Keywords: *Salmonella typhi*, Biofilm, Cefetoxime, Subinhibitory concentration (Sub-MIC), MIC, SEM

INTRODUCTION

Salmonella typhi is an enteropathogen causative agent of typhoid fever, which is transmitted through contaminated water, food and feces. Typhoid is majorly endemic in developing countries and adversely affects the local inhabitants and travelers [1-4]. The emergence of multidrug-resistant (MDR) Extended-spectrum β-lactamase (ESBL)-producing *S. typhi* and its ability to form biofilm is posing a challenge to the field of medicine. The production of ESBLs by *S. typhi* aggravates the problem leading to the emergence of cephalosporin-resistant *S. typhi* causing serious concern in the selection of antibiotic for the treatment of typhoid [5].

Bacteria have developed biofilm production as an extravagant defense mechanism to make them highly pathogenic and facilitate their

sequestration from the immune system of the host. Biofilm is an extracellular amorphous matrix produced by the bacteria and is made up of extracellular polymeric substance (EPS) and adheres to a substance enhancing the resistance power against antibacterial agents by shielding the embedded bacterial cells within the matrix [6]. Biofilm matrix is selectively permeable resulting in the retention of antibacterial agents like antibiotics outside the cell membrane itself making them inactive. Reports suggest that 50% of nosocomial infections are associated with biofilm. However, the significance of biofilm is in relation to infections, which are chronic and difficult to treat [7,8]. The gradual development of resistance against higher-line antibiotics, ESBL production, and biofilm formation are a major concern to the field of science and medicine. The sub inhibitory concentrations

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Received: 20 July 2016, Accepted: 12 February 2017

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(sub-MICs) of antibiotics impact the ultra-structure and antigenicity of microorganisms, and also their adherence to epithelial cells [9]. However, other studies show that sub-MICs of antibiotics play a significant role in gene mutation, lead to the hypermutable state, induce various gene transfer processes such as transposition and conjugation [10] and promote enzyme-catalyzed functions [11]. Moreover, in *S. typhi*, the sub-MICs of cefetoxime affect bacterial cell functions like cell wall synthesis and induce biofilm formation [12].

Scanning electron microscopy (SEM) with its high resolution and magnification is widely used to image Biofilm and is employed to evaluate notable morphological modifications made by bacterial cells in response to antibiotics. SEM evaluates the structural morphology of biofilm and gives a conclusive measurement of extensive biofilm formation and of bacterial cells camouflaged in an amorphous matrix [13].

Our primary aim in the present study is to investigate and quantify persistent biofilm formation by ESBL-producing *S. typhi* clinical isolates on plastic and glass surfaces and to characterize the impact of cefetoxime at a MIC and sub-MIC concentration in it and deduce the changes in cell morphology as well as in biofilm formation through SEM study.

METHODS

Bacterial strains and selection of ESBL-producing *Salmonella typhi*

The strains required for the above study were isolated from clinical Widal-positive blood samples collected from the Governmental Hospital, Kalaburagi, Karnataka, India. The samples were processed for *S. typhi* isolation following standard isolation protocols and were screened by biochemical tests and further confirmed genotypically by 16S rDNA sequencing. Subsequently, antibiotic susceptibility test was carried out to confirm drug resistance pattern by Kirby-Bauer disc diffusion method as per standard CLSI guidelines. ESBL production was screened using phenotypic detection methods as per Drieux et al, [14]; and CLSI guidelines [15]; it was also confirmed by PCR amplification of *bla*CTX-M2 and *bla*CTX-M9 genes. Nine *S. typhi* strains were selected for the study based on their drug resistance

pattern and ESBL production to assess biofilm formation.

Determination of MIC to cefetoxime

MIC value to cefetoxime was determined by conventional agar dilution method and MIC. The stock solution of cefetoxime (1 mg/ml) was prepared using cefetoxime sodium salt (Himedia Laboratories, Mumbai) and diluted as per the standard protocol of Andrews [16]. MIC and sub-MIC value of cefetoxime were interpreted according to the guidelines of clinical laboratory standard institute (CLSI 2012) [15].

Biofilm formation assay of *S. typhi* isolates

Test tube method

The formation of biofilm on the glass surface was detected by a simple test tube method and estimated by spectrophotometer assay according to Christensen et al, [17]. The potential biofilm formation was assayed with 3 ml each of four different supplements in four sets of test tubes with LB broth, LB + 1% glucose, LB + cefetoxime (128 µg/ml), LB + 1% glucose + cefetoxime (128 µg/ml), LB + 1% glucose + cefetoxime (64 µg/ml) evenly distributed to each set of test tubes and incubated at 37 °C at different time intervals -24, 48, and 72 h. After incubation, the growth medium was discarded. Each tube was washed with phosphate buffer saline (PBS) to eliminate the unbound bacteria. To evaluate biofilm formation, the remaining attached bacteria were fixed with 3 ml of 99% methanol, and the tubes were emptied after 15 min and left to dry. The attached film was stained for 5 min with 1% crystal violet. Excess stain was rinsed by placing the tubes under running tap water. The tubes were air-dried and the dye attached to the cells was dissolved in methanol: acetic acid: distilled water (4:1:1). The optical density (OD) of each tube was determined at 570 nm. Isolates which show O.D at 570 nm and above (≥ 0.5) were considered as strong biofilm producers, those between ≥ 0.2 and < 0.5 were considered moderate and those < 0.2 were weak or no biofilm producers.

Microtitre plate method

The formation of biofilm was studied as per the method of Stepanovic et al, [18,19], using sterile 96-well flat-bottomed polystyrene microplate

employing four supplements, LB, LB + 1% glucose, LB + cefetoxime (128 µg/ml), and LB + 1% glucose + cefetoxime (128 µg/ml i.e MIC of cefetoxime), LB + 1% glucose + cefetoxime (64 µg/ml, Sub-MIC of cefetoxime). Two hundred µl of each supplement was evenly distributed into each well in triplicate and each row of wells was seeded with 10 µl (0.2 O.D) of test organisms with the final row as a control. They were incubated at 24, 48, and 72 h at 37 °C and the post-incubation period wells were gently washed with 200 µl of PBS (pH 7.4), air-dried in an inverted position and then stained with 200 µl of 2% crystal violet solution for 10 min. After staining, plates were washed twice with PBS (pH 7.4) to drain the excess stain. The biofilm was quantitatively analyzed by adding 200 µl of methanol: acetic acid: distilled water (4:1:1) to each well. Absorbance was taken at 570 nm using microtitre plate reader (BioRad, iMark Microplate reader Sigma-Aldrich, Japan) and the results were recorded.

SEM analysis

SEM is a fundamental technique to determine notable changes in cell morphology in the presence of high concentration of antibiotic and deduce significant biofilm formation by bacteria on the material surface [20,21]. Two ESBL-producing *S. typhi* strains, namely, BST 51 and BST 130, were selected for the comparative study potential biofilm formation in the presence of cefetoxime (64 µg/ml, Sub-MIC), cefetoxime (128 µg/ml, MIC). Biofilm assay was done on 24-well polystyrene culture plates (microtitre plates) containing glass coverslips placed at the bottom of wells. Above procedure was repeated (Microtitre plate method) and incubated at 37°C for 48 h. The microtitre plates were rinsed twice with PBS, fixed with 2% (w/v) glutaraldehyde and incubated overnight at 4 °C. Specimens were dehydrated with a series of ethanol solutions in the range of 30-100% of absolute ethanol and were later dried to critical point, biofilm layered coverslip was removed and coated with gold and examined with an S-200C SEM [22].

Two cefetoxime resistant isolates grown in LB media containing cefetoxime antibiotic and one sensitive *S. typhi* isolates were subjected to the study. Standard SEM fixation protocol was followed and subsequently, changes in cell

morphology among cefetoxime resistant *S. typhi* and sensitive *S. typhi* was determined by measuring the length and width of the cell and subsequently by calculating its total volume and surface area to determine the significant changes in cell morphology using the following equations:

$$V (\mu\text{m}^3) = \pi r^2 h$$

$$A (\mu\text{m}^2) = 2\pi r^2 + 2\pi r h$$

Where r is the radius and h, the length of the cylindrical cells. The average cellular volume and surface area were calculated by using 10 individual bacterial cells per population. Values were expressed as a mean ± standard error of the mean (SEM) by ANOVA using Excel 2007. Differences between control (cefetoxime-sensitive strain) and cefetoxime-resistant strain cells were considered significant at p<0.05 level.

RESULTS

Screening and selection of cefetoxime-resistant strains

Nine MDR ESBL-producing *S. typhi* isolates, resistant to cefetoxime and exhibiting the highest MIC level to it in the range 128 µg/ml, were selected for the present study. Potential ESBL production was phenotypically detected in all the nine strains based on the zone of inhibition to cefetoxime alone and increased it to >5 mm in cefetoxime + clavulanic acid combination. The results have been interpreted as per CLSI standard chart and genotypically confirmed with PCR amplification of *bla*CTX-M2 (accession no. KT277101) and *bla*CTX-M9 (accession no. KT277102) genes responsible for Cefetoxime-mediated ESBL production.

Biofilm formation

Standard test tube method

Standard test tube method was used to study efficient biofilm formation by *S. typhi* on the glass surface. The relative adherence of crystal violet stain on the surface of the test tubes shows positivity for biofilm formation (Figure 1). With LB as a supplement, 03 (33.33%), 02 (22.22%) and 02 (22.22%) strains have shown strong biofilm formation with incubation periods of 24, 48 and 72 h, respectively, 04 (44.44%), 03 (33.33%) and 01 (11.11%) were moderate biofilm producers with similar incubation periods,

and 02 (22.22%), 04 (44.44%), and 06 (66.66%) strains were weak or no biofilm producers.



Figure 1. Biofilm formation by *S. typhi* on glass surface

LB with glucose as a supplement, 04 (44.44%), 05 (55.55%) and 02 (22.22%) strains have shown strong biofilm formation, 02 (22.22%), 01 (11.11%) and 03 (33.33%) strains were moderate, and 03 (33.33%), 03 (33.33%) and 04 (44.44%) strains were weak or no biofilm producers. LB with Cefetoxime antibiotic as a supplement, 01 (11.11%), 02 (22.22%) and 02 (22.22%) strains have shown strong biofilm formation and no strains were moderate biofilm producers; 08 (88.88%), 07 (77.77%) and 07 (77.77%) strains were weak or no biofilm producers with incubation periods of 24, 48 and 72 h respectively. LB with cefetoxime and glucose as an extra supplement resulted in the similar biofilm formation as like LB with cefetoxime alone (Table 1).

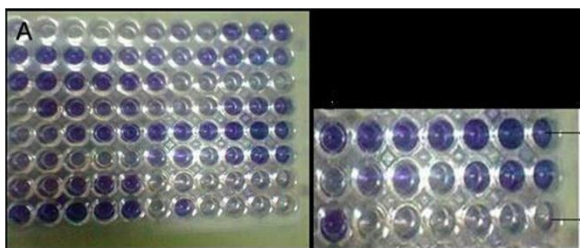


Figure 2. Biofilm formation by *S. typhi* on plastic surface.

Microtitre plate method

Experimental studies of biofilm formation by microtitre plate method on a plastic surface with similar supplements and respective incubation periods have however shown disparity. Nine cefetoxime-mediated ESBL-producing *S. typhi* strains were selected for the study. Adherence

of crystal violet stain in wells indicates positive biofilm formation (Figure 2). Of the nine strains, two, namely, *S. typhi* BST 51 (accession no. KR537431) and *S. typhi* BST 130 (accession no. KR537433), have shown relatively high biofilm formation in the presence of cefetoxime at a subinhibitory concentration (64 µg/ml).

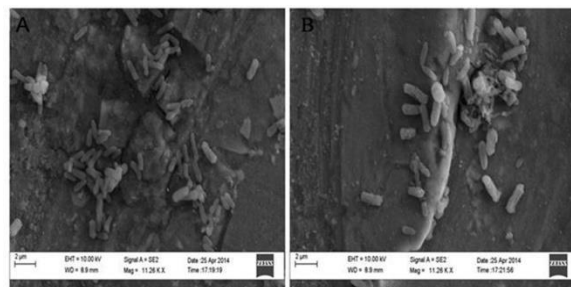


Figure 3. Scanning electron microscope (SEM) Images in presence of cefetoxime (MIC 128µg/ml). (A, B) Biofilm formation by *S. typhi* BST 130.

Relative influence of biofilm formation was studied with two variable parameters like supplements with combinations, and 24, 48 and 72 h incubation periods, respectively. With only LB as a supplement, we found 02 (22.22%) strains showing strong biofilm formation at 24, 48 and 72 h incubation periods. 03 (33.33%) and 05 (55.55%) strains have shown moderate biofilm formation at 24 and 48 h incubation period, respectively, but no strains have shown moderate biofilm formation at 72 h incubation period; in contrast, 04 (44.44%), 03 (33.33) and 07 (77.77%) strains have shown weak or no biofilm formation at 24, 48 and 72 h incubation periods. LB with 1% glucose as a supplement, 02 (22.22%) strains has shown strong biofilm formation at all respective incubation periods. 03 (33.33%) strains have shown moderate biofilm formation at 24 and 48 h incubation periods, and only 01 (11.11%) strain has shown it at 72 h incubation period. Two isolates have shown significantly weak biofilm formation LB with a cefetoxime antibiotic at MIC value (128 µg/ml) at 48 h. On the contrary, cefetoxime at a subinhibitory concentration (64 µg/ml) with 01 (11.11%) strain has shown strong biofilm formation at 24 h incubation period and 02 (22.22%) strains at 48 and 72 h, respectively. No relative change in biofilm formation was observed among the strains when 1% glucose was added as an extra supplement to LB + antibiotic combination. We found the same pattern of biofilm formation among the strains in

LB + antibiotic + 1% glucose with respect to LB + antibiotic supplements (Table 2).

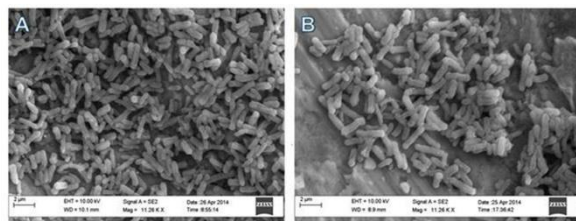


Figure 4: Scanning Electron Microscope (SEM) Images in presence of cefetoxime (Sub-MIC 64 µg/ml) A) Biofilm formation by *S. typhi* BST 51. B) Biofilm formation by *S. typhi* BST 130 SEM analysis

Significant strong biofilm formation was observed in SEM images of both *S. typhi* BST 51 and BST 130 in the presence of cefetoxime (64 µg/ml) at a subinhibitory concentration (Figure 4). Comparatively, we observed relatively weak biofilm formation from BST 51 and BST 130 *S. typhi* isolates in the influence of cefetoxime (128 µg/ml) at MIC level (Figure 3). Bacterial cells encapsulated in exopolysaccharide matrix were observed. The statistical analysis (ANOVA) provided evidence of a difference between cell surface-to-volume ratio

in cefetoxime-resistant strains compared to the cefetoxime-sensitive control strain (Table 3). The significant increase in cell size was observed, shows that surface and volume of resistant cells increased considerably in response to cefetoxime and are significantly different from control at $P < 0.05$ (Figure 5) (Table 3).

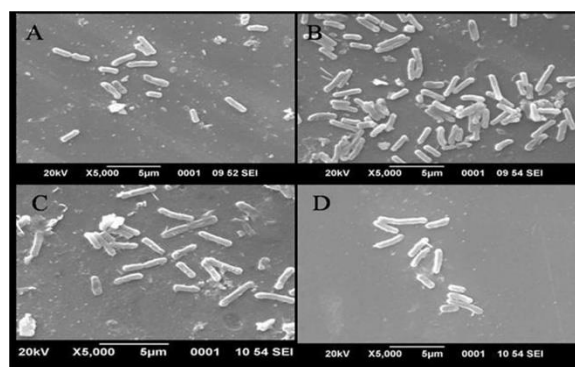


Figure 5: Scanning electron microscopic images A) BST 63 cefetoxime Sensitive *S. typhi* (Control). B) BST 51 cefetoxime resistant *S. typhi*. C & D) BST 130 cefetoxime resistant *S. typhi*.

Table 1. Estimation of biofilm production by *S. typhi* isolates on glass surface.

Incubation period combination	Biofilm	24 h	48 h	72 h
LB	Strong	03	02	02
	Moderate	04	03	01
	Weak (No biofilm)	02	04	06
LB+Glucose	Strong	04	05	02
	Moderate	02	01	03
	Weak (No biofilm)	03	03	04
LB+Cefetoxime	Strong	01	02	02
	Moderate	00	00	00
	Weak (No biofilm)	08	07	07
LB+Glucose+Cefetoxime	Strong	02	02	02
	Moderate	00	00	00
	Weak (No biofilm)	07	07	07

Table 2. Estimation of biofilm production by *S. typhi* isolates on plastic surface.

Incubation period combination	Biofilm	24 h	48 h	72 h
LB	Strong	02	02	02
	Moderate	03	05	00
	Weak (No biofilm)	04	03	07
LB+Glucose	Strong	02	02	02
	Moderate	03	03	01
	Weak (No biofilm)	06	04	06
LB+Cefetoxime	Strong	01	02	02
	Moderate	00	00	00
	Weak (No biofilm)	08	07	07
LB+Glucose+Cefetoxime	Strong	01	02	02
	Moderate	00	00	00
	Weak (No biofilm)	08	07	07

Table 3. The Mean value of cell sizes of the cell with influence of Cefetoxime (64 µg/ml).

Variables	Cell length	Cell width	Radius
BST 63 (Control)	2.211 ± 0.42	0.625 ± 0.079	0.3125 ± 0.030
BST 51	2.326 ± 0.41	0.6804 ± 0.051	0.3423 ± 0.023
BST 130	2.915 ± 0.61	0.6652 ± 0.082	0.3325 ± 0.041

SEM scanning electron microscope, v/v volume in volume

Statistical analysis was performed using ANOVA two-factor with replication. Significantly different from control at * P<0.05

Surface area, Volume were calculated using formulas as described in "Materials and methods"

Each mean value of average 10 cells size based on the scanning electron microscopy photos

DISCUSSION

Salmonella typhi is a major pathogen responsible for approximately 600,000 deaths (3.75% death rate with respect to total illnesses) and 16 million illnesses annually as per a study conducted in 1996 [23, 24]. Over the years, the incidence of typhoid fever decreased gradually in Chile [25-27], Egypt [28], India [29, 30], and the Soviet Union [31]. However, studies conducted in 2000 estimated 21.7 million illnesses but fortunately the death rate has declined sharply with only 217,000 (1% of total illnesses) notable death cases reported globally.

However, now a new issue has cropped up, that is, multidrug resistance to *Salmonella* and to clinically important antimicrobial agents such as fluoroquinolones and third-generation cephalosporins [32,33]. Recent reports mention of the emergence of ESBL-producing *S. typhi* in Poland; Szych et al, [34] and Nepal; Pokharel et al, [5]. It is widely known that *Salmonella* sp. forms a biofilm on surfaces like plastics, glass, and metals under standard laboratory conditions [35-37]. According to Stepanovic et al, [19] 23% of *Salmonella* sp. effectively form biofilms. Some reports suggest the relative influence of antibiotics that effectively induce biofilm formation at sub-MIC level. Majtan et al, [11] have reported of the interference of cefetoxime at sub-MIC level contributing to significant biofilm formation in *Salmonella typhimurium*. In fact, cefetoxime induces biomass production in gram-negative bacteria [38]; several studies show a significant increase in polysaccharide synthesis and biofilm formation in response to antibiotics [39].

In the present study, we have investigated the capability of *S. typhi* clinical isolates to form biofilm on plastic and glass surfaces by microtitre plate and standard test tube methods, respectively, and the relative impact of cefetoxime at MIC (128 µg/ml) and Sub-MIC (64

µg/ml) on biofilm formation in ESBL-producing *S. typhi*. Comparative results of the methods show that select *S. typhi* isolates produced biofilm on plastic and glass surfaces (Tables 1 and 2). In contrast, of the nine *S. typhi* strains, two, namely, *S. typhi* BST 51 (accession no. KR537431) and *S. typhi* BST 103 (accession no. KR537433), have strongly demonstrated biofilm formation in the presence of cefetoxime (64 µg/ml) at sub-MIC but biofilm formation significantly dropped at MIC level (128 µg/ml) relatively forming weak biofilm. Several studies have reported antibiotic sub-MIC-induced biofilm formation in *P. aeruginosa*, *K. pneumonia*, *E.coli*, and *S. aureus* [40-43]. Significant biofilm formation in *P. aeruginosa* was demonstrated in response to tobramycin by Hoffman et al, [40]. SEM images show encapsulated cells i.e. exopolysaccharide indicates significant strong biofilm formation in presence cefetoxime Sub-MIC level (Figure 4) but relatively weak biofilm formation at MIC level (Figure 3) were compared. There is a notable variation in the morphology of cefetoxime-resistant *S. typhi* in comparison with cefetoxime-sensitive *S. typhi*. Statistical analysis (ANOVA) also showed relative changes in cell morphology in resistant and control strains with significant differences in surface and volume of the cell (Table 3). This study clearly demonstrates the relative influence of cefetoxime on cell morphology with clear evidence of a relative increase in cell size of cefetoxime resistant strains with respect to cefetoxime sensitive strain.

In conclusion, the significant outcome of the present study is that ESBL-producing *S. typhi* efficiently produced biofilm on plastic and glass surfaces. Cefetoxime (64 µg/ml) at sub-MIC level influence efficient biofilm formation in *S. typhi* isolates. In contrary, we observed less impact of cefetoxime at MIC level (128 µg/ml). In fact, the relative morphological changes in *S.*

typhi isolates in response to cefetoxime and cefetoxime-resistant strains were significantly different from control at $p < 0.05$.

ACKNOWLEDGEMENT

Declaration of conflicting interests: The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Financial Disclosure: No financial support was received.

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