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Comparative efficacy of antibiotics in biofilms eradication formed by ESBL and non ESBL producing micro-organisms

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Abstract

Growth of bacterial cells within a biofilm complicate the treatment of infections. Therefore, in the present study biofilm eradication efficacy of (ceftriaxone and sulbactam plus EDTA; CSE1034) was compared with ceftriaxone alone, ceftriaxone plus EDTA and ceftriaxone plus sulbactam against biofilms of ESBL producing *Escherichia coli, Klebsiella pneumoniae* and *Salmonella typhi*. Susceptibility testing of each drug was performed on planktonic and biofilm cells in non ESBL producing and ESBL producing strains according to the recommendations of clinical and laboratory standards institutes guidelines. CSE1034 inhibited the growth of planktonic cells of non ESBL producing strains with minimum inhibitory concentration (MIC) from 0.25 to 1.0 µg/ml; the minimum biofilm eradication concentration (MBEC) values ranged from 8 to 32 µg/ml where as ESBL producing strains MIC values were 2 to 4 times higher and corresponding MBEC values were higher by 4 to 8 times. When biofilms of ESBL producing organisms were treated with the half MBEC of drugs, CSE1034 decreased 3 log of bacteria present in biofilm when compared with ceftriaxone, ceftriaxone plus EDTA and ceftriaxone plus sulbactam. In conclusion, combination of CSE1034 acts synergistically and reduces the MIC and MBEC values, significantly. One dimensional polyacrlamide gel elctrophoresis of extracellular proteins revealed distinct difference in protein expression of the group treated with CSE1034. Hence, CSE1034 at low concentration showed greater efficacy in the eradication of biofilm as compared to other two drugs and could be one of the best choices to eradicate the biofilm infections caused by these organisms as compared to other drugs.

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Key words:
Beta-lactamase; biofilm; minimum inhibitory concentration; minimum bactericidal concentration; minimum biofilm eradication concentration

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Introduction

In recent years there has been an increasing in incidence and prevalence of diseases caused by ESBL (extended spectrum beta-lactamases) producing microbial strains (Thokar et al., 2010).

Beta-lactamases are mostly coded by plasmids and are transferable between different bacterial species; these enzymes have resistance to penicillins, cephalosporins and aztreonam (Philippon et al. 1994; Sturenburg and Mack, 2003; Drawz and Bonomo, 2010). The emergence and spread of resistance is also threatening to create species resistant to all currently available agents. Approximately 20% of *K. pnenumoniae* infections and 31% of *Enterobacter* species infections in intensive care unit in the United States now involve strains not susceptible to 3rd-generation cephalosporins (Paterson and Bonomo, 2005).

Biofilm is an aggregate of microorganisms and can be produced by both ESBL and non-ESBL organisms; cells adhere to each other or to a number of different surfaces, such as natural aquatic and soil environments, living tissues, medical devices and encased in a hydrated polymer matrix (Banin *et al.*, 2006). Metallic cations are essential for microbial adherence, biofilm formation, and bacterial growth (Raad *et al.,* 2008). Biofilms have been found to be involved in a wide variety of microbial infections in the body (approx. 80% of all infections), costing the health care system billions of dollars (Costerton *et al.,* 1995; Mah and OO'toole, 2001).

The treatment of bacterial infections becomes very hard when bacteria grow in biofilm form because gene expression of bacteria in biofilm structure is altered, up to 20% of all bacterial genes are expressed differently leading a better protection against antibiotics compared to free living cells (Whiteley *et al.,* 2001; Stewart *et al.,* 2001; Schuster *et al.,* 2003).

A wide variety of antibiotics are used to treat biofilm infections (Hajdu *et al.,* 2009; Harrison *et al.,* 2005; May *et al.,* 2009). Beta-lactam antibiotics and beta-lactamase inhibitor combinations are not the optimal therapy for chronic infections due to ESBL-producing organisms. Although the inhibitors have significant activity against ESBLs *in vitro*, their clinical effectiveness against serious infections due to ESBL-producing organisms is controversial (Islam, 2009). *E. coli* biofilms are up to 50 times more resistant than planktonic cells to the antibiotics like amikacin, ceftriaxone, and tobramycin (Harrison *et al.,* 2005). The effect of antibiotics on the biofilm of ESBL producing *Salmonella typhimurium (S. typhi)* is little known. Presterl *et al.* (2009) also studied the effect of vancomycin, daptomycin, fosfomycin, tigecycline and ceftriaxone alone and in combination with azithromycin on biofilms. However, these antibiotics are becoming resistant to treat the biofilm infections. None of the antibiotics could reduce the bacterial count of the biofilms when used alone. However, when used in combination azithromycin plus tigecycline, fosfomycin or ceftriaxone at high concentrations have little effect on biofilm density (Presterl *et al.,* 2009). All these antibiotics alone and in combination are being used to treat the infections caused by bacterial biofilm with least success. Therefore, it is required to develop new antibiotic combination which has to be effective against biofilms produced by ESBL organisms.

Present study was carried out to investigate the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) and minimum biofilm eradication concentration (MBEC) against non ESBL producing and ESBL producing strains. Furthermore, biofilms of each ESBL producing strain was treated with half of MBEC of each drug to study the comparative efficacy of EDTA alone, ceftriaxone alone, ceftriaxone plus EDTA, ceftriaxone along with addition of beta-lactamase inhibitor sulbactam (ceftriaxone plus sulbactam) and CSE1034 (ceftriaxone plus sulbactam plus EDTA).

Materials and Methods

Antimicrobial agents

CSE1034 (Ceftriaxone: Sulbactam :: 2:1 with 10 mM EDTA) and ceftriaxone (Rocephin) used in the study were provided by sponsor Venus Pharma

GmbH, Germany and combination of ceftriaxone plus sulbactam (Oframax forte) was procured from Indian market on behalf of sponsor for the study. All the antibiotics CSE1034, ceftriaxone+EDTA and ceftriaxone plus sulbactam were reconstituted with water for injection as stock solutions. Working solutions were prepared in MHB at a concentration of 0 to 1024 µg/ml, and from these working solutions serial two fold dilutions were made in CAMHB in wells of 96-well plate. EDTA (Himedia) was also recontituted with water for injection.

Bacterial strains and culture conditions

A total of three ESBL producing organisms naming *E. coli, K. pneumoniae, and S. typhi* were used as a model microorganisms obtained from clinical settings from Postgraduate Institute of Medical Science (PGIMS), Lucknow, India. Confirmation of ESBL production was carried out by PGIMS, Lucknow using CLSI (clinical and laboratory standards institute) guidelines and BD Diagnostic Sparks MD; and Remel, Leneva, KS. Test disks used for ESBL detection were in the combination of ceftazidime/clavulanic acid (30/10 µg) and cefotaxime/clavulanic acid (30/10 µg). An ESBL producer had a ≥5-mm-zone size difference between the cefotaxime/clavulanic acid or ceftazidime/clavulanic acid disks compared to disks without the clavulanic acid. Three non-ESBL producing strains of same microbes *E. coli* MTCC 739*, K. pneumoniae* MTCC 109*, and S. typhi* MTCC 531 were procured from Institute of Microbial Technology Sector 39-A Chandigarh, India. Bacterial strains were maintained as glycerol stocks and stored at -80° C. Before use, bacterial suspensions were spread onto Mueller-Hinton solid medium (MHSM; Himedia) and incubated at 35 °C for 24 hrs. For each strain, three to five colonies were transferred into 10 ml of cation adjusted Mueller-Hinton broth (CAMHB) and incubated under orbital agitation at 150 rpm for 4 h at 35 °C (Rotary flask shaker) to obtain a planktonic culture in exponential growth phase. This bacterial suspension was used as inoculum at a concentration of 10^6 colony-forming units (cfu/ml). The CAMHB was also used in reaction vessels to initiate biofilm formation. Bacterial counts were done on MHSM. Antibiotic susceptibility screening and recovery of viable biofilm organisms were carried out in MHSM.

MICs and MBCs determination

Susceptibility testing to each drug and EDTA alone was performed on planktonic cultures according to Clinical and Laboratory Standards institutes guidelines, 2003. MICs were performed in 96-well microplates and results were recorded after incubation at 37 °C for 24 hrs. To determine MBC, 5 µL was aspirated from wells where there was no visible growth of planktonic bacterial population in the MIC experiment and spresd onto MHBA plates and incubated overnight at 37°C in incubator. The MBC was read as the lowest antibiotic concentration to kill 99.9% of the initial inoculum (Koneman *et al.,* 1997).

MBECs determination

MBECs determined by a broth macrodilution method in MHSM, as described by CLSI, with some modifications. MBECs were performed when pegs contained approximately 10^5 to 10^6 bacteria growing as a biofilm described by Ceri *et al.* (1999). After development of biofilm on pegs, non-adherent bacteria on the pegs were washed once by immersion in a microtitre plate containing 200 µl of sterile PBS. In another microtitre plate, 200 µl of each antibiotic of 2-fold dilutions (from 1024 µg/ml to 2 µg/ml) were prepared. All samples were run in duplicate and one lane served as a negative control.

The pegged lid with biofilm was then placed onto the microtitre plate containing antibiotics and incubated, for 24 hr at 37°C. The lid with peg was then placed onto a micro plate containing 200 µl of

fresh sterile broth medium. The remaining biofilm was removed from the pegs by ultrasonic disruption for 5 min. This plate was incubated for 24 h at 37°C and the presence of viable bacteria determined by plate counts or turbidity determined at 650 nm in a 96-well plate reader (Molecular Devices; Fisher Scientific, Nepean, Ontario). Growth of bacteria in a particular well indicates regrowth of planktonic bacteria from surviving biofilm. Therefore, MBEC value represents the lowest dilution at which bacteria fail to regrow.

In vitro Biofilms formation

In vitro biofilm model was developed using calgary biofilm device (CBD) (MBEC Biofilm Technologies, Calgary, Alberta) according to described by Ceri *et al.* (1999). The device contains a lid with 96 pegs. The pegs are designed in a way so that channels of the bottom component of the reaction vessel can be fit into the wells of a standard 96-well plate. The bottom of the vessel serves to channel the flow of medium across the pegs to create consistent shear force across all pegs, resulting in the formation of equivalent biofilms at each peg site.

Biofilm of each isolate was developed by taking of 10^6 CFU/ml bacterial inoculum of bacterial strain. Biofilm formation of each strain was carried out at 37°C for 24 hrs and 95% relative humidity on a rocking table such that fluid flowed along the channels of the CBD, generating the required shear force across all pegs. Biofilm formation was determined by viable counts on MHA plates.

Quantification of biofilms

After 24 hrs, pegs from lid were removed, placed in microcentrifuge tubes containing 200 µl of MHB, and sonicated for 5 min on sonicator. Viable counts were determined on MHSM plates. The same procedure was used to control for the numbers of cfu/peg in all antibiotic susceptibility tests prior to exposure to antibiotic.

Staining of biofilm

The pegged lid after treatment was then placed onto the microtitre plate containing 200 µl PBS. Following washing lid was dried under laminar air flow and stained with 4 % crystal violet for 30 min. The excess dye was removed by thoroughly washing with water and air dried under laminar air flow and observed.

Effect of antimicrobial drugs on preformed biofilms

Investigation of the effect of antimicrobial drugs on pre-formed biofilms was performed on the ESBL producing *E. coli, K. pneumoniae* and *S. typhi.* Assessment of drug activity was performed by three independent methods: visual observation of growth; crystal violet staining and enumeration of bacteria before and after treatment of the biofilm with antibiotics. Biofilms were allowed to form as mentioned above. The lid with pegs was then removed, rinsed and placed in contact with various concentration of antimicrobial agents. The sealed plates were incubated at 37 °C for 24 hrs at 30 rpm in an orbital shaker. Microplates were then observed for detection of any visible growth of bacteria detached from the biofilm through the cycle of biofilm formation. Staining was done to observe the persistence of the biofilm. Bacterial count was also done after treatment of different drugs.

SDS-PAGE analysis of extracellular proteins

Following treatment for 24 h, bacterial cells were scraped from each treatment (5000 rpm for 10 min at 15 °C.) The pellet was suspended in 467 µl of TE (pH 8.0) and 33 µl of 10 % SDS and incubated at 37 °C overnight. After incubation, 4 ml of methanol was added and mixed well by shaking and then 1 ml chloroform was added followed by 3 ml of water. The solution was mixed well (7000 rpm for 10 min at 15 °C.)

The upper phase was discarded and 3 ml methanol was added and, vortexed well. Then, solution was centrifuged (7000 rpm 5 min at 15 °C). The precipitate was air dried and dissolved in an appropriate volume of sample solution (0.5M, Tris-HCl [pH 6.8], 5% 2-mercaptoethanol, 10% glycerol, 2% sodium dodecyl sulfate [SDS]). 12% SDS-polyacrylamide gel electrophoresis (PAGE) by the method of Laemmli (1970) and visualized by silver staining (Dunn, 1996).

Results and Discussion

Microbial efficacy of CSE1034, ceftriaxone, ceftriaxone plus EDTA, ceftriaxone plus sulbactam and EDTA alone against planktonic cells of ESBL producing and non ESBL producing *E. coli, K. pneumoniae,* and *S. typhi* was measured by MIC and MBC. The MIC, MBC and MBEC values of non-ESBL organisms are lower than that of the ESBL producing organisms indicating the presence of different intrinsic levels of tolerance in ESBL and non-ESBL strains. MIC of ceftriaxone in non ESBL organisms *E.coli, K. pneumoniae,* and *S. typhi* was 4, 8 and 2 µg/ml which was increased to 64, 128 and 32 respectively in ESBL producing strains. Addition of EDTA to ceftriaxone MIC values reduced two to four times in all ESBL and non ESBL producing organisms respectively. EDTA enhances the penetration of drug into the bacterial cells through opening of porin channels by chelating the divalent ions, thus enhancing susceptibility. Previously, several authors have demonstrated that EDTA enhances penetration of drugs to bacterial cells resulted in increased sensitivity of drugs which in turn decreased MIC (Costerton et al., 1967;Leive 1965; Brown and Richards, 1965). When sulbactam was added to ceftriaxone, MICs values were reduced 2 times in all microbes except ESBL producing E.coli where 4 times reduction in MIC was observed. The addition of beta lactamase inhibitor probably allows slight inactivation of betalactamase enzyme produced by ESBL producing organisms. A number of authors have demonstrated that a reduction in MICs values when sulbactam is combined with beta-lactam antibiotics (Pfaller et al., 2005; Bolivar et al., 1984). **A**ddition of both EDTA and sulbactam to ceftriaxone (CSE1034), MIC was recorded 0.25, 0.125 and 0.25 µg/ml, respectively in non ESBL producing *E.coli, K. pneumoniae,* and *S. typhi* and 4, 32 and 8 µg/ml, respectively for ESBL producing strains, suggesting that EDTA and sulbactam acts synergistically and reduces the MIC values 8 to 16 times in non ESBL and ESBL organisms as compared to that of ceftriaxone alone. Like vise, MBC values of CSE1034 in non ESBL producing *E. coli, K. pneumoniae,* and *S. typhi* in all three drug groups were in the range from 1, 0.25 and 2 µg/ml which was raised to 8, 64 and 16 µg/ml in corresponding ESBL producing strains, indicating higher drug concentrations are required in resistant strains. MBC values of CSE1034 were 16 to 256 times lower than ceftriaxone alone in ESBL producing strains. MIC, MBC and MBEC of EDTA alone ranged from 1000 to >8000 µg/ml, indicating that EDTA does not possess antibacterial activity on its own. (Table 1)**.**

In case of ceftriaxone, there was 32 times increase in MBEC values for non ESBL *E. coli* and *S. typhi* and 256 times increased in MBEC value for *K. pnenumoniae*. However, for ESBL organisms MBEC values of ceftriaxone alone were found >1024µg/ml, indicating failure of ceftriaxone to respond to biofilm eradication. Addition of either EDTA or sulbactam to ceftriaxone could reduced 2 to 4 folds in MBEC for non ESBL organism and ESBL organisms, indicating that individually either of these components fail to make ceftriaxone penetrate the EPS formed by sessile bacteria in biofilm. Earlier many studies have shown that a combination of EDTA is uniquely useful in disrupting the biofilm and synergistically eradicating organisms from the biofilm environment (Evans and Holmes, 1987; Raad and Sherertz, 1994; Rose, 2000).

Table 2 shows viable cell count of microorganisms in biofilm and planktonic cultures of only ESBL producing microbes, suggesting that all bacteria would not form biofilms under standard cultural conditions some still remains in planktonic form. This also, indicates that every organism requires specific physiological environment for biofilm formation.

It has been observed, that when biofilms of ESBL producing *E. coli, K. pneumoniae,* and *S. typhi* were exposed to half of MBEC of each drug for 24 hrs, ceftriaxone could reduce only 0.22, 0.21 and 0.12 logs of *E. coli, K. pneumoniae,* and *S. typhi* present in biofilm, where as after addition of either EDTA alone or sulbactam alone to ceftriaxone no significant log reduction was observed 0.83, 0.78 and 0.69 logs reduction and 0.83, 1.14 and 0.81 logs reduction was noted respectively. This suggests that betalactamase inhibitor, sulbactam and EDTA alone is not effective in disrupting bacterial biofilm and was able to kill some bacterial cells which might be in planktonic form. The reason could be due to the accumulation of relatively large amounts of beta-lactamases within the glycocalyx which inactivates antibiotics.Vidal *et al.* (1997) reported that sulbactam is effective only against the young cells of biofilm but it lost its activity against the old cells of biofilms. On the other hand, treatment of biofilms with CSE1034 (ceftriaxone plus sulbactam plus EDTA) resulting in 3 logs reduction was observed in all the strains. The addition of EDTA with ceftriaxone plus sulbactam is uniquely useful in disrupting the biofilm and synergistically eradicating organisms from the biofilm environment**.** EDTA chelates with divalent ions present in sessile microbial cells and EPS (extracellular polymeric substances) of biofilms thus making the membrane more porous and susceptible for antibiotics. Donlan, (2000) reported that EPS contributes to the antimicrobial resistance properties of biofilms by impeding the mass transport of antibiotics through the biofilm, probably by binding directly to antibiotics. Further it is clearly evident from image that EDTA alone, ceftriaxone alone, has little effect on the eradication of biofilm whereas CSE1034 significantly eradicates the biofilm developed by ESBL producing *E. coli, K. pneumoniae, and S. typhi* obtained from clinical settings due to synergistic action of all three components.

It is known that that protein play an important role in regulation of the biofilm phenotype (Oosthuizenet al., 2002). Different classes of extracellular proteins have been described as part of an adaptive response to a change in the environment (Tjalsma et al., 2000). The extracellular protein profile of all bacterial strains following 24 h treatments were studied. Distinct band differences between the extracellular proteins of cultures grown in the presence of different drugs could be identified. The variability in the expression of extracellular proteins may indicate that the biofilm cells are regulated differentially (Figure 2). Comparison of the extracellular proteins of biofilms grown with different drugs by one-dimensional SDS PAGE analysis showed that the protein profile of the groups treated with CSE1034 is different than that of ceftriaoxone+EDTA and ceftriaxone+sulbactam treated groups. Differences were observed in the expressed proteins but the identity of the proteins was not investigated further in this study. The changes in the protein expression profile probably may resensitize bacteria towards antibiotics.

In conclusion, this work demonstrates combining of ceftriaxone with EDTA and sulbactam significantly reduce the MIC, MBC and MBEC values against selected ESBL producing organisms, however all the drug groups have good efficacy in non ESBL producing microbes. It also shows ceftriaxone alone is not effective in the biofilm eradication of ESBL organisms, however combination of ceftriaxone with either sulbactam or EDTA is slightly more effective than ceftriaxone alone due to EDTA induced

penetration but due to lack of sulbactam ceftriaxone fails to act. Complete biofilm eradication is observed when ceftriaxone was combined with EDTA and sulbactam both in CSE1034. Further , addition of sulbactam and EDTA into ceftriaxone resensitize the bacteria to antibiotics by inhibiting some of the extra-cellular expressed protein. Therefore, CSE1034 could be one of the best choice to eradicate the biofilm caused by these organisms.

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Fig. 1. Quantitative assay for biofilm formation on microtitre plate.

Row A positive control- 1 to 3 *E. coli*; 4 to 6 *K. pnenumoniae*; 7 to 9 *S. typhi*. Row B treated with ceftriaxone- 1 to 3 *E. coli*; 4 to 6 *K. pnenumoniae*; 7 to 9 *S. typhi*. Row C treated with ceftriaxone plus EDTA- 1 to 3 *E. coli*; 4 to 6 *K. pnenumoniae*; 7 to 9 *S. typhi*.Row D treated with CSE1034- 1 to 3 *E. coli*; 4 to 6 *K. pnenumoniae*; 7 to 9 *S. typhi*. Row E treated with ceftriaxone plus sulbactam 1 to 3 *E. coli*; 4 to 6 *K. pnenumoniae*; 7 to 9 *S. typhi*. Row F treated with EDTA 1 to 3 *E. coli*; 4 to 6 *K. pnenumoniae*; 7 to 9 *S. typhi*.

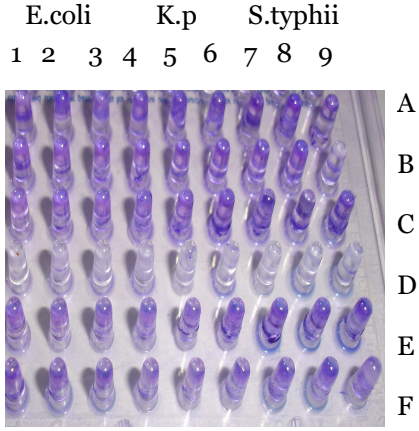

Figure 2: Extracellular protein profile of ESBL producing *K. pneumoniae* treated with (A) Ceftriaxone (B) ceftriaxone +EDTA (C) ceftriaxone+sulbactam (D) CSE1034. Other organisms also expressed the same pattern of protein profile (data not shown).

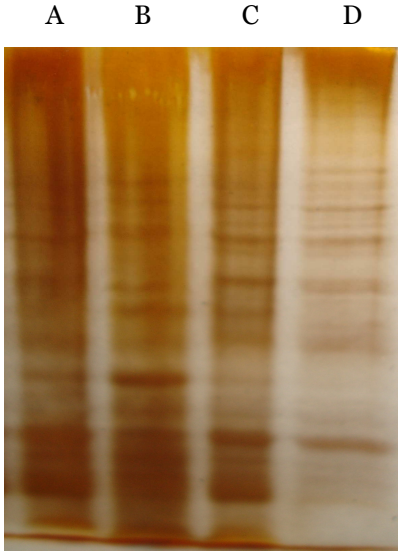

Table 1. Sensitivity of planktonic and sessile (biofilm) bacteria to different antibiotics

Name of drugs	Names of Tests	*E.coli*		*K.pneumoniae*		*S.typhii*	
		Non-ESBL (MTCC-739)	ESBL	Non-ESBL (MTCC 109)	ESBL	Non-ESBL (MTCC 531)	ESBL
CSE1034	MIC	0.25	4	0.125	32	0.25	8
	MBC	1	8	0.25	64	2.0	16
	MBEC	4	64	1	128	8	64
Ceftriaxone	MIC	4	64	8	128	2.0	32
	MBC	16	256	32	256	16.0	128
	MBEC	128	>1024	256	>1024	256	>1024
Ceftriaxone+EDTA	MIC	1	32	2	64	0.5	16
	MBC	2	128	16	128	8	32
	MBEC	32	512	64	>1024	128	512
Ceftriaxone+Sulbactam	MIC	2	16	4	64	1.0	16
	MBC	4.0	64	8	128	8.0	64
	MBEC	32	512	128	>1024	64	>1024
EDTA	MIC	2	>8	4	>8	1	>8
	MBC	>8	>8	>8	>8	>8	>8
	MBEC	>8	>8	>8	>8	>8	>8

Table 2. Viable count of sessile (biofilm) and planktonic bacteria in ESBL producing microbes

Name of organism	Time (h)	Biofilm concentration (cfu/peg)[a]	Planktonic concentration (cfu/well)[b]
E.coli	24	$6.2x10^6±1.7$	$3.5x10^8±1.6$
K.pneumoniae	24	$4.5x10^6±2.1$	$2.7x10^9±1.4$
S.typhii	24	$3.2x10^5±1.2$	$4.3x10^7±1.8$

a, represents the mean number of sessile bacteria present on each peg
b, represents the mean number of planktonic bacteria growing in the trough of the CBD at the same time the peg was sampled

Table 3. Quantification of peg biofilm before and after exposure to drugs

Biofilm concentration cfu/ml									
Name of ESBL producing organism	**Pre-antibiotic exposure**	**After antibiotic exposure**							
	$6.2x10^6±1.7$ (6.79±0.23)[a]	CSE1034	Log reduction	Ceftriaxone	Log reduction	Ceftriaxone+EDTA	Log reduction	Ceftriaxone+Sulbactam	Log reduction
E.coli	$4.5x10^6±2.1$ (6.65±0.32)[a]	$2.6x\ 10^3±1.5$ (3.41±0.17)[a]	3.35±0.06	$3.8x10^6±2.1$ (6.57±0.32)[a]	0.22±0.10	$9.2x10^5±1.4$ (5.96±0.140)[a]	0.83±0.09	$7.3x10^5±1.6$ (5.86±0.2)[a]	0.93±0.03
K.pneumoniae	$3.2x10^5±1.6$ (5.50±0.20)[a]	$3.4x\ 10^3±1.6$ (3.53±0.20)[a]	3.12±0.12	$2.8x10^6±1.5$ (6.44±0.17)[a]	0.21±0.15	$7.5x10^5±1.6$ (5.87±0.20)[a]	0.78±0.12	$3.3x10^5±1.6$ (5.51±0.20)[a]	1.14±0.12
S.typhii		$3.4x10^2±1.1$ (2.53±0.04)[a]	2.97±0.16	$2.4x10^5±1.2$ (5.3±0.08)[a]	0.12±0.12	$6.6x10^4±1.1$ (4.81±0.041)[a]	0.69±0.16	$4.8x10^4±1.5$ (4.68±0.17)[a]	0.82±0.03

[a] log cfu/ml. The antibacterial drugs used are equal to MBEC. When biofilms of these organisms were treated with EDTA alone non significant reduction of bacteria present in biofilm was observed.

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