

SYNERGY OF A NOVEL ANTIBIOTIC ADJUVANT ENTITY AGAINST MULTI DRUG RESISTANT *ENTEROBACTERIACEAE*

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ABSTRACT

In the present investigation, we investigated the in vitro interaction of ceftriaxone plus sulbactam with disodium edetate, a Non Antibiotic Adjuvant (NAA) against selected clinical isolates and in vitro susceptibility studies were also performed. The isolates were tested against a range of ratios of ceftriaxone and sulbactam using a microdilution checkerboard method. Having determined the appropriate ratios of ceftriaxone plus sulbactam, effect of various concentration of disodium edetate were also studied using the microdilution checkerboard method. All the results were analysed with the Fractional Inhibitory Concentration (FIC) indices. Susceptibility studies were carried out according to the Clinical and Laboratory Standards Institute (CLSI) methods. Results of this study demonstrated that 2:1 ratio of ceftriaxone and sulbactam was the more synergistic with FIC index values 0.4281, 0.4023, 0.4124 and 0.4325 for *E. coli*, *A. baumannii*, *P. aeruginosa* and *K. pneumoniae*. The synergicity of ceftriaxone and sulbactam was enhanced significantly with increasing concentration of disodium edetate and produced the lowest FIC index (<0.2) at 10 mM of disodium edetate in all positive controls as well as clinical isolates. Further, the synergy between ceftriaxone plus sulbactam with disodium edetate (Elores) was confirmed by broth dilution, time kill curve and agar diffusion methods. In broth dilution method, Elores (ceftriaxone+ sulbactam+ disodium edetate) produced 4 to 5 fold lower MIC when compared with ceftriaxone plus sulbactam. Approximately 10^4 log of killing reduction was observed with synergistic ratio of Elores in time kill curve study. This study suggest that Elores could be an alternative regimen in combating antibiotic resistance among multi drug resistant *Enterobacteriaceae*.

Keywords: Clinical Isolates, *Enterobacteriaceae*, FIC Index, Synergy

1. INTRODUCTION

Increasing resistance to 3rd generation cephalosporins particularly due to extended spectrum beta lactamase production has become a major concern especially among *Enterobacteriaceae* that cause nosocomial infections (Rawat and Nair, 2010). Approximately 20% of *Klebsiella pneumoniae* infections and 31% of Enterobacter species infections in intensive care unit in the United States now involve strains not susceptible to 3rd-generation cephalosporins.

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Salmonella species also getting resistant to expanded-spectrum cephalosporins have been reported in several countries, including Argentina, Turkey, Algeria, Saudi Arabia, Greece, Tunisia and France (Dutil *et al.*, 2010). In addition, most of the bacteria, responsible for community-acquired infections have developed resistance to many antibacterial agents particularly beta-lactams which are being used in over 50% of all systemic antibiotics (Acevedo *et al.*, 2009). Besides that, several evidences pointed towards the development of resistance to extended-spectrum cephalosporins in bacteria isolated

from patients with nosocomial infections (Rawat and Nair, 2010). An increasing number of reports have indicated the steady rise in resistance for ceftriaxone (Unemo *et al.*, 2010; Ohnishi *et al.*, 2011). In addition, aminoglycosides, fluoroquinolones and carbapenems have usually been used for the treatment of infections caused by *Enterobacteriaceae* (Howard *et al.*, 2012; Tam *et al.*, 2010). However, in recent years, these organisms have been reported to be resistant to these commonly used antimicrobial agents worldwide (Chaudhary and Payasi, 2012; Memish *et al.*, 2012; Muthusamy and Boppe, 2012). Acquisition or expression of Metallo- β -Lactamases (MBLs), Extended-Spectrum β -Lactamases (ESBLs), decreased permeability, overexpression of efflux pump are thought to be the main factors contributing to antibiotic resistance development (Chaudhary and Payasi, 2012; Zavascki *et al.*, 2010; Karthika *et al.*, 2009).

The treatment of infections caused by these microorganisms impose a major challenge to health care system due to failure of monotherapy and lacking of effective regimens. Combination antibiotics have been used frequently in clinical practice, but not all of them work synergistically.

Considering the above background, a team of Venus Medicine Research Centre (VMRC), India has developed a novel Antibiotic Adjuvant Entity (AAE) combination of ceftriaxone with a beta lactamase inhibitor sulbactam and a nonantibiotic adjuvant disodium edetate naming Elores. This AAE can be used successfully for the therapy of infections caused by resistant organisms.

The checkerboard titration method was used to test synergy of various ratios of ceftriaxone and sulbactam against selected clinical isolates and results have been presented in term of the Fractional Inhibitory Concentration Index (FICI). The present study was aimed to differentiate the performance of product on Extended-Spectrum Beta-Lactamase (ESBL), Metallo-Beta-Lactamase (MBL) and efflux positive strains. We investigated the in vitro interactions between ceftriaxone and sulbactam with a Non Antibiotic Adjuvant (NAA) disodium edetate using a checkerboard method. Further, the effect of different concentrations of disodium edetate on the double combination of ceftriaxone and sulbactam was studied in detail to determine whether the apparent synergistic interaction between ceftriaxone and sulbactam is enhanced or diminished by the addition of disodium edetate. Furthermore, we studied the in vitro susceptibilities of these isolates to combinations of ceftriaxone and sulbactam and disodium edetate by the use of broth dilution, disk diffusion and time-kill methods.

2. MATERIALS AND METHODS

2.1. Clinical Isolates Collection and Their Identification

A total of 140 clinical isolates 35 of each *E. coli*, *K. pneumoniae*, *A. baumannii* and *P. aeruginosa* were included in the study. The re-identification of clinical isolates were done according to standard microbiological procedures (Khan *et al.*, 2011). *Escherichia coli* ATCC-35218, *K. pneumoniae* ATCC BAA-2146 and *P. aeruginosa* K1455 were included in the study as positive controls. The clinical isolates were obtained from clinical isolate bank of Venus Medicine Research Centre, Baddi and Baba Farid Medical College, Faridkot, Punjab, India, where clinical isolates are preserved. Each of these bacterial cultures were grown and adjusted to 0.5 MacFarland standard.

2.2. ESBL and MBL Characterization

All these isolates were subjected to ESBL and MBL characterization as previously described (CLSI, 2011; Yong *et al.*, 2002).

2.3. Efflux Pump Characterization

All the isolates positive with ESBL and MBL were further subjected for identification of AcrAB-tolC, mexAB-oprM and AdeABC efflux pumps using the methods described earlier (Chaudhary *et al.*, 2012a; Chaudhary and Payasi, 2012; Lopes and Amyes, 2013).

2.4. Fractional Inhibitory Concentration (FIC) Study

In vitro drug interaction was determined by the checkerboard method as described by Wijayanti *et al.* (2010) and results were analyzed with the FIC indices. For each ratio, a two-dimensional checkerboard with twofold dilutions was used for the study. Growth control wells containing medium were included in each plate. Each test was performed in triplicate. The concentration of antibiotics needed to inhibit growth was recorded. The following formula was used to calculate FIC:

$$FIC = \frac{\text{MIC of drug in combination}}{\text{MIC of drug alone}}$$

The FIC index (Σ FIC) calculated as the sum of each FIC, was interpreted as follows: Synergy is defined as an FIC index of ≤ 0.5 . Antagonism is defined as an FIC index

of ≥ 2 . An indifferent/additive effect is defined as an FIC index of >0.5 to 2 or a micro dilution decrease of 1 dilution in the MIC of the one or the other drug or no change in the MIC of either of the drugs.

2.5. Effect of Non-Antibiotic Adjuvant (NAA) on Double Combinations

Effect of NAA on Σ FIC of double combinations, ceftriaxone plus sulbactam was also conducted using checkerboard method (Wijayanti *et al.*, 2010) in the absence and presence of increasing concentration of disodium edetate in all positive controls as well as clinical isolates.

2.6. Determination of Minimum Inhibitory Concentration (MIC)

MICs were determined by broth dilution method following the guidelines of the CLSI (2011) using cation-adjusted Mueller-Hinton Broth (MHB) [Hi-Media, India]. MIC was defined as the lowest concentration of antibiotic that completely inhibited the growth of the organism as detected with the naked eye.

2.7. Determination of Antimicrobial Susceptibility Test (AST)

AST was determined according to the cup plate method described by Chaudhary *et al.* (2012b). The cups were made in the agar plate using a sterile cork borer (6.5 mm). Then, 30 μ L of the drug preparation Elores (ceftriaxone+sulbactam+disodium edetate (30:15 μ g), ceftriaxone+sulbactam (30:15 μ g) and ceftriaxone (30 μ g) were placed into the wells using a micro-pipette and allowed the plates to incubate at 37°C for 18 h in the upright position. After incubation the zone of inhibition around the wells was measured in mm (millimeter), averaged and the mean values were recorded.

2.8. Determination of Time Kill Curve (TKC)

TKC study was performed according to CLSI (2011) guidelines. Twice the MIC of ceftriaxone, ceftriaxone+sulbactam and Elores (ceftriaxone+sulbactam+disodium edetate) was used for this study. For TKC study, two randomly selected clinical isolate of each *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *A. baumannii* and all positive controls were used. Overnight grown bacterial suspension was diluted to approximately 10^6 to 10^7 cfu mL⁻¹ in MHB containing antibiotics or no antibiotics. The samples were removed at 2, 4, 6, 8, 10 and 12 h and were diluted and plated on MHA. The agar plates were incubated at 37°C for 24 h and colony forming unit (cfu) were counted.

3. RESULTS

3.1. Clinical Isolate Identification and Characterization

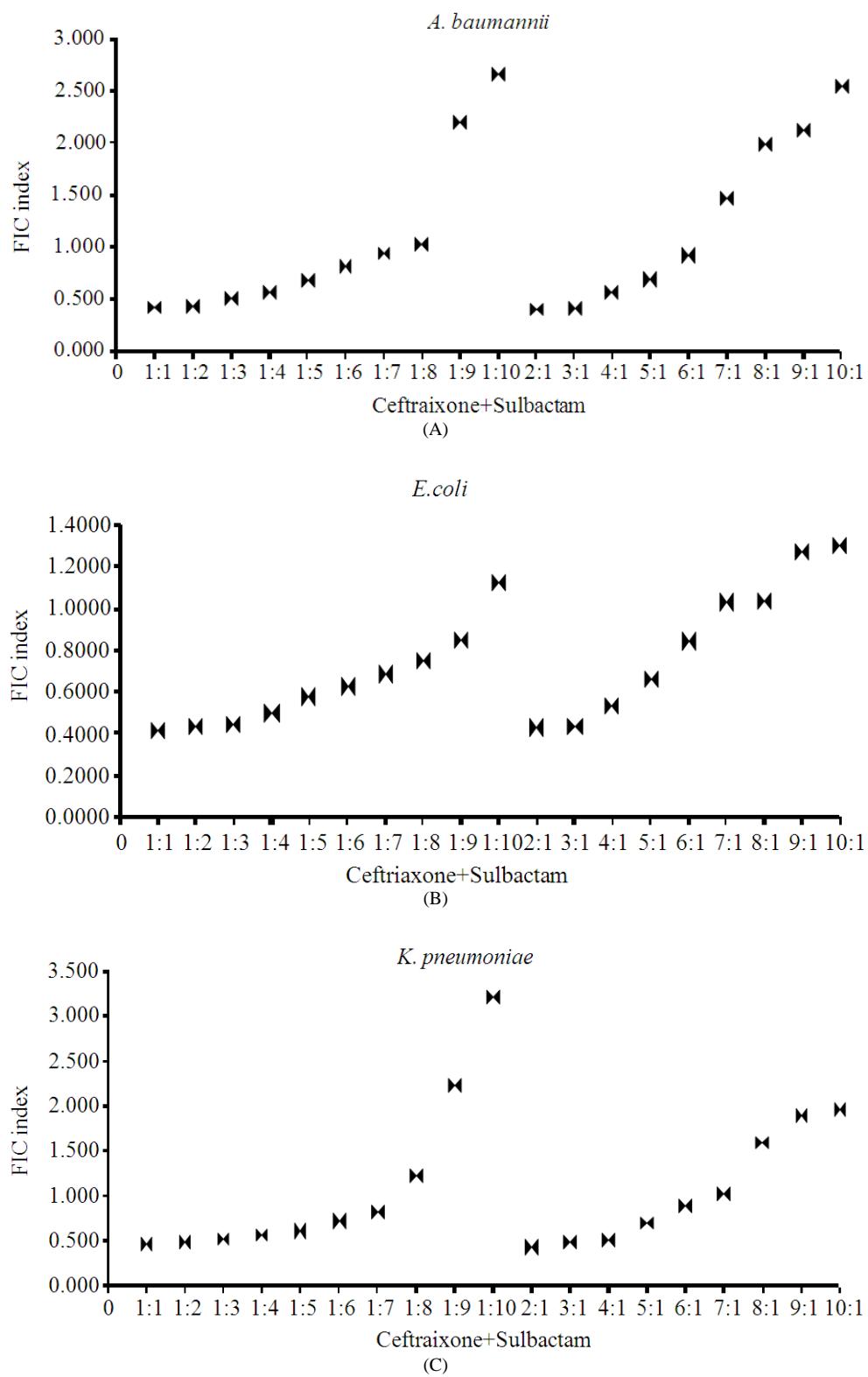
All of the clinical isolates obtained from isolate banks were identified as *A. baumannii*, *E. coli*, *P. aeruginosa* and *K. pneumoniae* based on their morphological and biochemical characterization. Out of the 140 isolates, 60 were found to be ESBL positive (*A. baumannii* 11, *E. coli* 15, *P. aeruginosa* 16, *K. pneumoniae* 18) and 47 were MBL positive (*A. baumannii* 5, *E. coli* 12, *P. aeruginosa* 9, *K. pneumoniae* 11). Out of these ESBL positive isolates, 27 isolates were efflux positive [*A. baumannii* 7 (*AdeABC* positive), *E. coli* 8 (*AcrABC-tolC* positive), *P. aeruginosa* 5 (*mexABoprM* positive), *K. pneumoniae* 7 (*AcrAB-tolC* positive)]. Similarly among MBL positive isolates, 14 were efflux positive [*A. baumannii* 3 (*AdeABC* positive), *E. coli* 5 (*AcrABC-tolC* positive), *P. aeruginosa* 3 (*mexABoprM* positive), *K. pneumoniae* 4 (*AcrAB-tolC* positive)].

3.2. FIC Study

Figure 1 summarizes the results of the FIC index analysis of the various ratios of ceftriaxone and sulbactam tested against *E. coli*, *A. baumannii*, *P. aeruginosa* and *K. pneumoniae*. The results demonstrated that 2:1 ratio of ceftriaxone and sulbactam was the most synergistic. Further increasing the ratio of either ceftriaxone or sulbactam synergistic activity was either lost or no further potentiation was observed. This study was conducted in all selected clinical isolates as well as positive controls and synergistic activity was noted at 2:1 ratio of ceftriaxone and sulbactam. The results of one clinical isolate of each *E. coli*, *A. baumannii*, *P. aeruginosa* and *K. pneumoniae* positive with both MBL and efflux is presented here only.

3.3. Effect of NAA on Double Combinations

Effect of NAA, disodium edetate on FIC indices of double combinations ceftriaxone plus sulbactam in the absence and presence of increasing concentration of disodium edetate was done in all 140 isolates of *E. coli*, *A. baumannii*, *P. aeruginosa* and *K. pneumoniae* and positive controls. Results of this study showed that Σ FIC decreased with increasing concentration of disodium edetate and maximum decrease was found 10 mM of disodium edetate. Further increasing the concentration of disodium edetate Σ FIC remained constant. The FIC analysis for four selected clinical isolates which were used for FIC study are presented in **Fig. 2**.



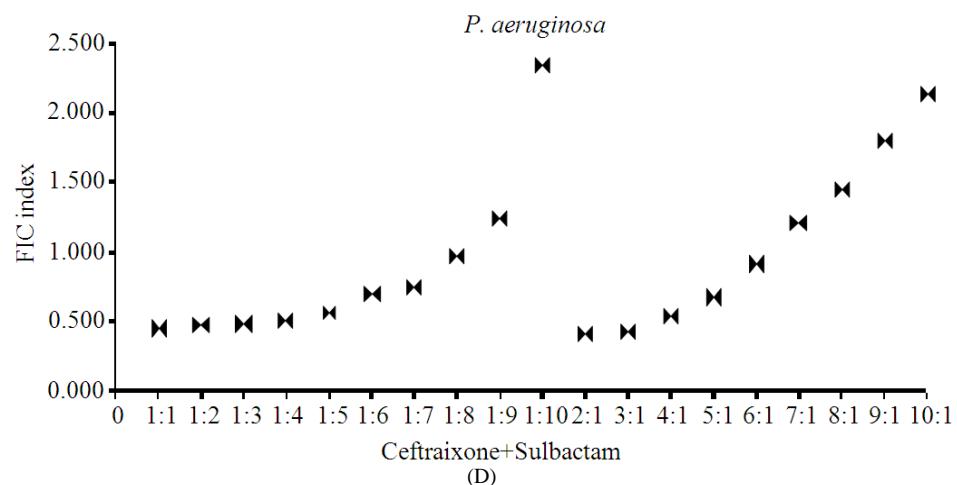
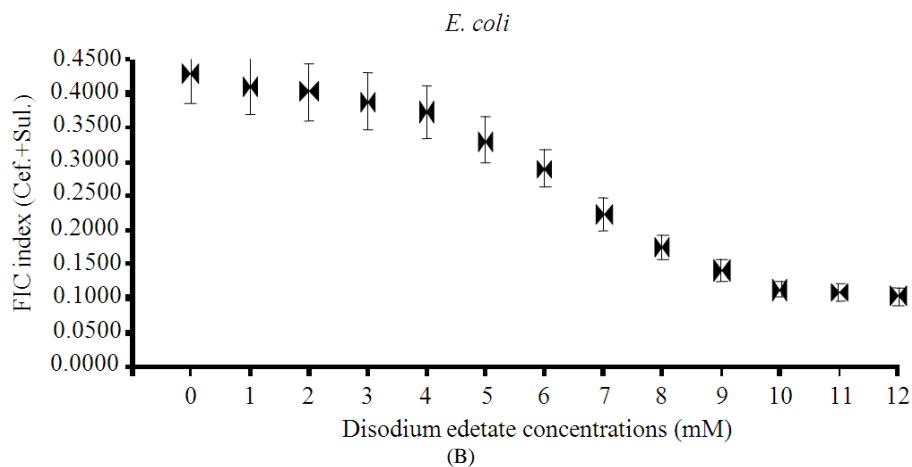
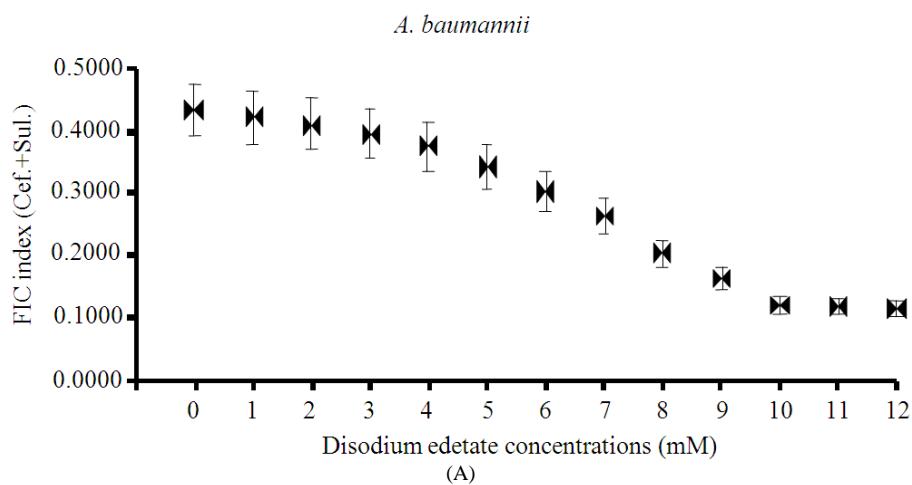


Fig. 1. FIC indices at various ratios of ceftriaxone and sulbactam against MBL positive clinical isolates with efflux. FIC indices of ceftriaxone and sulbactam in the presence of increasing concentration of sulbactam (1:1 to 1:10) and then ceftriaxone (2:1 to 10:1). (A) *A. baumannii* (B) *E. coli* (C) *K. pneumoniae* (D) *P. aeruginosa*. FIC index synergistic when value is ≤ 0.5



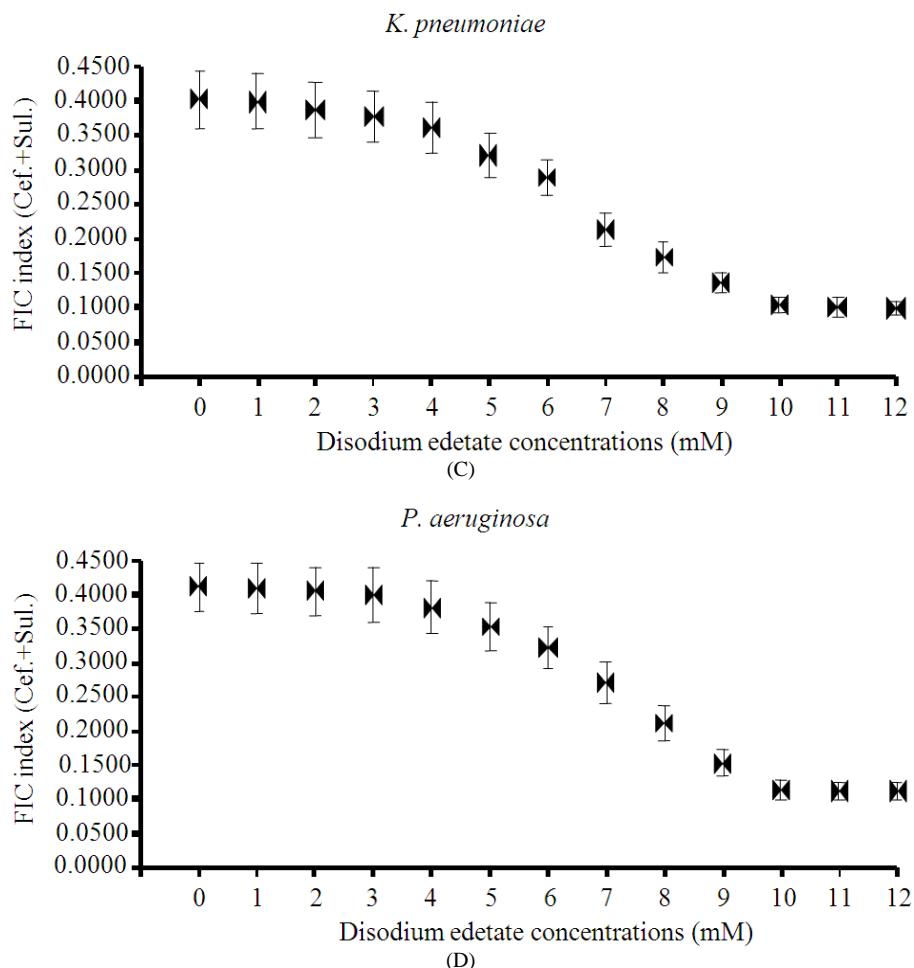


Fig. 2. Effect of disodium edetate on the combination of ceftriaxone and sulbactam against MBL positive clinical isolates with efflux. FIC index of ceftriaxone and sulbactam in the presence of increasing concentration of disodium edetate (A) *A. baumannii* (B) *E. coli* (C) *K. pneumoniae* (D) *P. aeruginosa*. FIC index synergistic when value is ≤ 0.5

From Σ FIC analysis of all clinical isolates, FICI_{\min} and FICI_{\max} were calculated and results are presented in **Fig. 3**. The FICI_{\min} and FICI_{\max} were significantly lower equal to less than 0.5, which indicates the presence of synergistic interactions among the three combinations.

3.4. MIC

Synergism between ceftriaxone and sulbactam along with NAA was also performed by a broth dilution method against selected clinical isolates and positive isolates. The MICs for positive controls ranged $2-4 \mu\text{g mL}^{-1}$ for ceftriaxone+sulbactam+disodium edetate (Elores), whereas it was ranged between $512-1024$ and $256-512$ for ceftriaxone and ceftriaxone+sulbactam, respectively (**Table 1**). MICs for Elores were $4-32 \mu\text{g mL}^{-1}$ for clinical isolates

of *A. baumannii* and $4-16 \mu\text{g mL}^{-1}$ for each of *E. coli*, *K. pneumoniae* and *P. aeruginosa* positive with ESBL. MICs for Elores to MBL positive isolates of *A. baumannii* and *E. coli* were $2-16$ and $1-8 \mu\text{g mL}^{-1}$, respectively whereas it was ranged $2-8 \mu\text{g mL}^{-1}$ for *K. pneumoniae* and *P. aeruginosa* isolates. Similarly, MICs for Elores against efflux positive isolates were $2-16 \mu\text{g mL}^{-1}$ for each of *A. baumannii*, *K. pneumoniae* and *P. aeruginosa* and $1-8 \mu\text{g mL}^{-1}$ for *E. coli*. Contrary to this, ceftriaxone MICs were >1024 to all the isolates except *E. coli* and *K. pneumoniae* positive with efflux. Ceftriaxone+sulbactam demonstrated MICs values 4-6 fold higher than Elores in all isolates (**Table 1**). MIC studies were also conducted using other ratios (1:1, 1:2, 3:1 and 4:1) of ceftriaxone plus sulbactam but significant results were obtained only with 2:1 ratio.

3.5. AST

Synergism of ceftriaxone and sulbactam against *A. baumannii*, *E. coli*, *K. pneumoniae* and *P. aeruginosa* were also demonstrated by a cup-plate agar diffusion method. For positive controls of *E. coli*, *K. pneumoniae* and *P. aeruginosa* inoculated onto a MHA plate containing Elores produced a ≥ 5 mm enhanced zone of inhibition 25.78 ± 1.4 , 26.24 ± 1.8 and 25.53 ± 1.6 mm, respectively compared to ceftriaxone alone and ceftriaxone plus sulbactam, indicating enhanced synergistic activity between the ceftriaxone and sulbactam in presence of non antibiotic adjuvant disodium edetate (Table 1). Similarly for clinical isolates of *A. baumannii*, *E. coli*, *K. pneumoniae* and *P. aeruginosa* positive with ESBL, MBL and efflux, ceftriaxone plus sulbactam with disodium edetate combination (Elores) produced a greater zone of inhibition ≥ 5 mm when compared with other two groups (Table 1). AST studies were also conducted using other ratios (1:1, 1:2, 3:1 and 4:1) of ceftriaxone and sulbactam but did not show significant synergy (data not shown).

3.6. TKC

TKC study was performed on all clinical as well as positive controls and results are presented only for one clinical isolate of each of *A. baumannii*, *E. coli*, *K. pneumoniae* and *P. aeruginosa* positive with both MBL and efflux. Synergy was defined as $\geq 10^3$ log of killing compared to the starting inoculum. Results of TKC demonstrated an enhancement of killing of selected organisms in the presence of AAE ceftriaxone +sulbactam in a ratio of 2:1 with non antibiotic adjuvant disodium edetate, in comparison to ceftriaxone alone and ceftriaxone plus sulbactam. After 12 h of incubation Elores exhibited approximately 10^4 log reduction in *A. baumannii*, *E. coli*, *K. pneumoniae* and *P. aeruginosa* positive with both MBL and efflux whereas when ceftriaxone was tested alone against these isolates no killing was observed at any time point and regrowth appeared after 4 h with ceftriaxone plus sulbactam (Fig. 4). TKC studies were also conducted using other ratios (1:1, 1:2, 3:1 and 4:1) of ceftriaxone and sulbactam with non antibiotic adjuvant disodium edetate, but significant results were obtained only with 2:1 ratio.

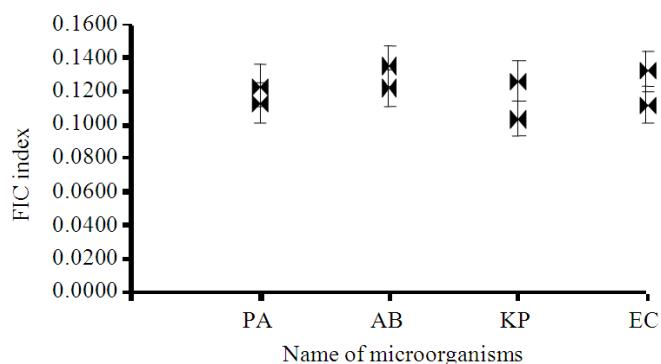


Fig. 3. FICmin and FICmax of the combination of ceftriaxone, disodium edetate and vancomycin against clinical isolates

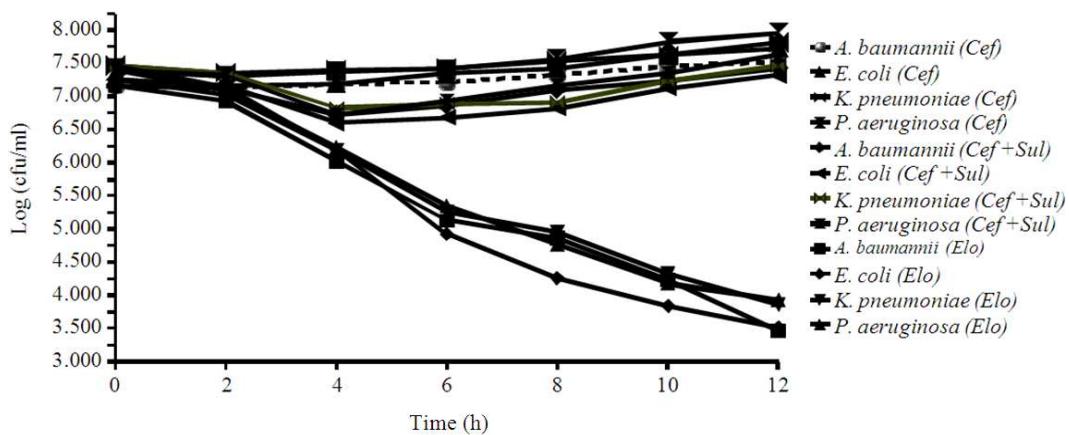


Fig. 4. Time-kill curves for *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *A. baumannii* isolates positive with both MBL and efflux pump

Table 1. AST and MIC of antibacterial agents against selected clinical isolates

Strain no.		Ceftriaxon	Zone of Inhibition (mm)		MIC ($\mu\text{g/mL}$)		
			Ceftriaxone		Ceftriaxone + sulbactam	Ceftriaxone +disodium edetate	Ceftriaxone +sulbactam
			Ceftriaxone	+sulbactam			
<i>E.coli</i> ATCC-35218	ESBL positive	NZ	20.37 \pm 1.2	25.78 \pm 1.4	>1024	512	4
<i>K. pneumonia</i> ATCC BAA-2146	MBL positive	NZ	13.26 \pm 1.2	26.24 \pm 1.8	>1024	512	2
<i>P. aeruginosa</i> MexA- MexB-OprM K1455	Efflux positive	11.25 \pm	17.38 \pm 1.5	25.53 \pm 1.6	512	256	2
<i>A. baumannii</i>	ESBL	8.26 \pm 1.6	10.43 \pm 1.1	24.13 \pm 1.6	>1024	16 - 256	4 - 32
	MBL	7.13 \pm 1.9	11.56 \pm 1.3	26.43 \pm 1.5	>1024	128-1024	2 - 16
	Efflux	8.46 \pm 1.1	11.84 \pm 1.5	25.57 \pm 1.8	>1024	32-512	2 - 16
<i>E. coli</i>	ESBL	8.21 \pm 1.4	10.43 \pm 1.3	26.44 \pm 1.1	>1024	64-512	4-16
	MBL	7.26 \pm 1.1	9.65 \pm 1.7	27.23 \pm 1.3	>1024	128-1024	1-8
	Efflux	8.43 \pm 1.5	10.13 \pm 1.4	25.38 \pm 1.5	512-1024	64-512	1-8
<i>K. pneumoniae</i>	ESBL	8.58 \pm 1.7	9.49 \pm 1.4	24.32 \pm 1.2	>1024	32 - 256	4-16
	MBL	6.96 \pm 1.3	9.57 \pm 1.1	27.32 \pm 1.4	>1024	128-1024	2 - 8
	Efflux	7.63 \pm 1.2	10.47 \pm 1.2	25.23 \pm 1.4	128-1024	64-512	2-16
<i>P. aeruginosa</i>	ESBL	7.46 \pm 1.1	9.88 \pm 1.6	23.23 \pm 1.3	>1024	32 - 512	4-16
	MBL	7.67 \pm 1.3	8.11 \pm 1.5	25.64 \pm 1.6	>1024	128-1024	2-8
	Efflux	6.81 \pm 1.8	10.51 \pm 1.8	24.57 \pm 1.1	>1024	64-512	2-16

NZ = No Zone

4. DISCUSSION

Indiscriminate use of antibiotics, poor patient compliance and improper infection control practices has led to emergence of multi drug resistant strains which transfer resistance through plasmids and confer resistance to commonly used cephaloropporin antibiotics. Combination therapy has been reported to be beneficial for the treatment of infections which fail to respond to single drug therapy because of lacking of efficacy or rapid emergence of resistance (Deresinski, 2009; Kumar *et al.*, 2010). Although there are several data on the inter action of antibacterial agents against gram-positive and gram-negative organisms (Deveci *et al.*, 2012), there is no report on the interaction between ceftriaxone and sulbactam and disodium edetate. Ceftriaxone in combination with sulbactam and disodium edetate show a greater susceptibility against resistant organisms as combination of trio, acting by different mechanisms, is used for the treatment of MDR bacterial infections.

The FIC index is the most commonly used method to determine the interaction between antibacterial drugs. The significant synergy was obtained at ratio 2:1 of ceftriaxone and sulbactam, which enhanced with increasing the concentration of disodium edetate and maximum synergy was found at 10 mM of disodium edetateA, suggesting synergistic activity of ceftriaxone + sulbactam + disodium edetate. This AAE was synergistic for both positive controls as well as selected clinical isolates positive with ESBL, MBL and efflux. Earlier it was demonstrated that ceftriaxone monotherapy is ineffective in the treatment ESBL but when it was

combined with sulbactam and disodium edetate synergy was enhanced significantly (Chaudhary *et al.*, 2012c). Deveci *et al.* (2012) studied the combinations of sulbactam with ceftriaxone, ceftazidime_and gentamicin against *A. baumannii* and observed synergy among these. The synergistic interaction between ceftriaxone plus sulbactam with disodium edetate was also demonstrated in animal model where combination therapy resulted in faster recovery in animals (infected with pneumonia) treated with combination compared with mono therapy (Dwivedi *et al.*, 2012). Moreover, the synergistic interaction between ceftriaxone plus sulbactam with disodium edetate was also proven through clinical trials in patients suffering from Lower Respiratory Tract Infections (LTRIs), Urinary Tract Infections (UTIs), skin and skin structure infections (SSSIs) and Bone and Joint Infections (BJIs) (Chaudhary and Payasi, 2013a; 2013b).

The synergistic activity of ceftriaxone plus sulbactam with disodium edetate was also reported in efflux positive isolates of *E. coli* and *P. aeruginosa* (Chaudhary *et al.*, 2012a; Chaudhary and Payasi, 2012). In addition, TKC, broth dilution, agar diffusion studies also carried out against all clinical isolates and indicated synergy between the ceftriaxone and sulbactam in a ratio of 2:1 with disodium edetate. Earlier, the synergistic activity of ceftriaxone with moxifloxacin was studied and found to be synergistic (Zakaria *et al.*, 2012).

5. CONCLUSION

The current study revealed that novel AAE a combination of ceftriaxone plus sulbactam with

disodium edetate could be the effective solution against the infections caused by *A. baumannii*, *E. coli*, *K. pneumoniae* and *P. aeruginosa* microorganisms positive with ESBL, MBL and efflux rather than searching for new antibiotics for treatment of infections caused by these organisms.

6. ACKNOWLEDGEMENT

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