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Research Article

Evaluation of *In-vitro* Efficacy of Beta-lactam/Non-Beta-lactam Antimicrobials against ESBL Genotypes from Wound Infection

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ABSTRACT

Gram-negative isolates with multiple beta-lactamase enzymes often possess gene determinants for resistance to non-beta-lactam antibiotics. The present study evaluates the *in-vitro* efficacy of β lactam/non-β lactam antimicrobials against extended-spectrum beta-lactamase (ESBL) genotypes from wound infection. The minimum inhibitory concentrations (MICs) of antimicrobials against 38 Enterobacteriaceae isolates from wound infection were determined by Vitek 2 ID/AST cards. ESBL genotypes: SHV, TEM, CTX-M, and OXA-10/11 genes were detected by real-time PCR. A correlation was found between ESBL genotypes and its resistance to imipenem and amoxycillin clavulanate that is statistically significant (*p-value* < 0.005). No statistically significant finding was noted among ESBL genotypes which showed resistance to meropenem, amikacin, gentamicin, piperacillin-tazobactam, ciprofloxacin and cotrimoxazole (*p-value* > 0.005). About 85.19% ESBL genotypes showed imipenem and meropenem susceptibility (MIC:- 0.025–1 µg) and to amikacin (MIC:≤ 2–16 µg). In 44.44% of ESBL genotypes showed susceptibility to cefepime (MIC: ≤ 2 µg) and 7.41% showed cefepime MIC of 4 to 8 µg (Susceptible Dose-Dependent). The emergence of carbapenem-resistant Enterobacterales have highlighted the need to assess the *in-vitro* efficacy of non-carbapenem betalactam and non-betalactam therapeutic alternatives to treat ESBL infections. Depending on the MIC of cefepime and susceptibility data of aminoglycosides, cotrimoxazole and fluoroquinolones, these drugs can be considered as carbapenem sparing drug as well as for non bacteremic ESBL therapy.

Introduction

The ability of beta-lactamase families to disseminate on plasmids and other mobile genetic elements across a wide range of gram-negative organisms and their expanded spectrum of activity while a new substrate is introduced to the clinic lead to the evolution of extended-spectrum beta-lactamases (ESBLs).[1] The synthesis of temoniera (TEM ESBL) and sulfhydryl variable (SHV ESBL) lead to increased minimum inhibitory concentration (MIC) value of third-generation cephalosporin, ceftazidime and fourth-generation cephalosporin, cefepime but not of cefotaxime.[2] Most CTX-M ESBL types exhibit high resistance pattern to cefotaxime and ceftriaxone but variable levels of resistance towards cefepime and cefpirome. The MIC of ceftazidime will also raise depending upon CTX-M type exhibited by the particular isolate.[3]

The evolution of resistant strains has compromised the clinical and *in-vitro* efficacy of beta-lactamase inhibitor drugs during the past decade. Derepressed class C β lactamase, overproduction of TEM or production of TEM and OXA (low-affinity inhibitor-resistant enzymes) contribute to beta-lactam/lactamase inhibitor resistance.[4] Against CTX-M Enterobacteriaceae, beta-lactam/lactamase inhibitor combinations remain active *in-vitro* but their use in the treatment is controversial.[5] Clavulanate, sulbactam, and tazobactam in conventional doses are regarded as clinically equivalent at counteracting common beta-lactamases, TEM and SHV. The broad-spectrum

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activity of piperacillin-tazobactam against gram-negative bacterial isolates makes it an important agent for sparing the use of carbapenems which are globally recognised as important last-line antibiotics.[6]

ESBL isolates with multiple beta-lactamase (KPC, TEM & SHV) often possess gene determinants for aminoglycosides and quinolone resistance determinants on the same plasmid.[7] Imipenem and meropenem are frequently used for ESBL Enterobacteriaceae invasive infections.[2] It is important to evaluate the efficacy of non-carbapenem beta-lactams and non-beta-lactam drugs as an alternative therapy for ESBL infections as well as for deescalating carbapenems. Even though beta-lactam/beta-lactamase inhibitor drugs and quinolones are considered as less effective agents when compared to carbapenems for ESBL invasive therapy, their use in non-bacteremic ESBL infection needs to be assessed. Quinolone drugs are recommended as oral option, whenever susceptibility data is available and high-dose cefepime can be effective for ESBLs as a carbapenem-sparing agent. Synergism has been observed with the antibiotic combination of beta-lactam and aminoglycoside for gram-negative infections.[8,9] In clinically stable patients, the use of cotrimoxazole as a carbapenem-sparing alternative therapy against ESBL Enterobacteriaceae have been reported. There are study reports on the *in-vitro* efficacy of cotrimoxazole, fluoroquinolone and aminoglycoside for milder infection caused by ESBL uropathogens.[9] This study was undertaken to evaluate the *in-vitro* efficacy of beta-lactam/non-β-lactam antimicrobials against ESBL genotypes from wound infection.

Materials And Methods

This study was conducted over one year period from March 2021 to February 2022 after obtaining Institutional Ethical Committee approval. About 38 Enterobacteriaceae isolates collected from wound samples were subjected to standard biochemical techniques such as catalase test, oxidase test, mannitol motility medium, triple sugar iron agar test, indole test, citrate utilization test, and urea hydrolysis test. Susceptibility to antimicrobials was tested by the Kirby-Bauer disc diffusion method on Muller Hinton agar using the following discs (Hi Media Laboratories Pvt. Ltd., Mumbai, India): Imipenem (10 µg), meropenem (10 µg), cefoperazone/sulbactam (75/30 µg), piperacillin/tazobactam (100/10 µg), amoxycillin/clavulanate (20/10 µg), amikacin (30 µg), gentamicin (10 µg), cefepime (30 µg), cefotaxime (30 µg), ceftazidime (30 µg), ciprofloxacin (5 µg) and cotrimoxazole (25 µg).

Enterobacteriaceae isolates with zone size ≤ 27 mm for cefotaxime and ≤ 22 mm to ceftazidime were subjected to phenotypic confirmation by Vitek 2 ID / AST gram-negative cards. The MICs for amikacin, gentamicin, ciprofloxacin, imipenem, meropenem, piperacillin/tazobactam, amoxycillin/clavulanate, cotrimoxazole, cefepime, cefotaxime, ceftazidime and ceftriazone were determined by Vitek 2 ID/AST cards and antibiotic susceptibility data was interpreted according to Clinical Laboratory Standard Institute guidelines. [10] ATCC *Escherichia coli* 25922 and ATCC *Klebsiella pneumoniae* 700603 were used as negative and positive controls, respectively.

ESBL genotypes: SHV,TEM,CTX-M ,OXA 10/11 were detected by real time PCR as per manufacturer's instructions. (MBPCR 131/Hi Media Laboratories Pvt. Ltd., Mumbai, India). Final reaction volume of 25 µL was subjected to the following cyclic condition: Initial denaturation for 10 minutes at 95°C followed by denaturation for 15 seconds at 95°C and annealing and extension for 30 seconds at 60°C. The cycle threshold (Ct) value of ≤40 was interpreted as positive for ESBL genes SHV, TEM, CTX-M and OXA-10/11.

Results

Out of 38 isolates studied 23 (60.53%) were *E. coli,* 9 (23.68%) were *K. pneumoniae*, 4 (10.53%) were *Proteus mirabilis*, one each was *Enterobacter aerogenes* (2.63%) and *E. cloaceae* (2.63%). ESBL genotypes were detected in 27 (71.05%) Enterobacteriaceae isolates by PCR. Out of these 27 isolates, 20 were *E. coli*, 6 were *K. pneumoniae* and a single isolate was *E. aerogenes*. CTX-M was detected in 48.15% (13/27) isolates. CTX-M and TEM type were identified in 29.63% of strains (8/27). Presence of multiple ESBL genotypes, SHV, CTX-M & TEM were noted in 22.22% (6/27) isolates.

CTX-M was identified in *12* (60.00%) *E. coli* isolates and a single (100%) isolate of *E. aerogenes*. Presence of CTX M and TEM was noted in 40.00% (8/20) *E. coli* isolates. Co-harbouring of multiple ESBL genotypes, SHV, CTX-M & TEM were detected in 100% (6/6) *K. pneumoniae* isolates. Of 20 ESBL *E. coli*, 12/20 (60.00%) with CTX-M gene and 8/20 (40.00%) isolate with CTX M & TEM genes showed resistance to ceftriaxone, ceftazidime and cefotaxime (MIC:-16–64 µg). 4/12 (33.33%) CTX-M *E. coli* exhibited resistance to cefepime (16–64 µg), 7 (58.33%) CTX-M *E. coli* showed susceptibility to cefepime (≤2 µg) and a single isolate (8.33%) showed a MIC of 4 to 8 µg (Susceptible Dose-Dependent). In 4/8 (50.00%) isolates each of *E. coli* with CTX-M & TEM exhibited resistance (16–64 µg) and susceptibility to cefepime (≤2 µg), respectively.

Five (83.33%) out of six *K. pneumoniae* showed cefepime resistance (16–64 µg) and a single isolate (16.67%) showed a MIC of 4 to 8 µg (Susceptible Dose-Dependent). In 6 (100%) isolates of *K. pneumoniae* with multiple enzymes, CTX M, TEM and SHV genes exhibited resistance to ceftriaxone, ceftazidime and cefotaxime (MIC:-16–64 µg). A single isolate (100%) of *E. aerogenes* with CTX M gene showed resistance to ceftriaxone, ceftazidime and cefotaxime (MIC:-16–64 µg). A single (100%) isolate of *E. aerogenes* with CTX M ESBL type showed susceptibility to cefepime (≤2 µg).

Table 1: MIC of beta-lactam/beta-lactamase inhibitors against ESBL genotypes

Organisms	*MIC*					
	Amoxycillin/Clavulanate			*Piperacillin/Tazobactam*		
	≤2–8 µg (S)	*16 µg (I)*	*≥32 µg (R)*	*≤0.5–16 µg (S)*	*32–64 µg (I)*	*≥128 µg (R)*
No of isolates (27)	d(f) = 6; $p = 0.003$			d(f) = 6; $p = 0.49$		
E. coli (12) (CTX- M)	10 (83.33%)	0 (00.00%)	2 (16.67%)	7 (58.33%)	3 (25.00%)	2 (16.67%)
E.coli (8) CTX-M & TEM	2 (25.00%)	5 (62.5%)	1 (12.5%)	3 (37.50%)	2 (25.00%)	3 (37.50%)
E. aerogenes (1) (CTX- M)	0 (0.00%)	0 (0.00%)	1 (100%)	0 (0.00%)	0 (0.00%)	1 (100%)
K. pneumoniae (6) CTX-M, SHV & TEM	3 (50.00%)	0 (0.00%)	3 (50.00%)	1 (16.67%)	2 (33.33%)	3 (50.00%)

Among the beta-lactam/beta-lactamase inhibitors tested, 7 (25.93%) out of 27 ESBL isolates exhibited resistance to amoxicillin/clavulanic acid (≥32 µg) and 9 (33.33%) isolates were piperacillin/tazobactam resistant (≥128 µg) (Table 1).

In our study, an association was observed between ESBL genotypes studied and its resistance to amoxycillin clavulanate, which is statistically significant (*p-value* < 0.005). No significant finding was noted among ESBL genotypes which showed resistance to piperacillin tazobactam ($p > 0.005$).

Among the aminoglycosides tested, 4 (14.81%) out of 27 isolates were resistant to amikacin (≥ 64 µg) and eleven (40.74%) isolates showed resistance to gentamicin (≥16 µg) (Table 2).

19/20 (95.00%) *E. coli* isolates with ESBL genotype showed susceptibility to amikacin and 12/20 (60.00%) isolates were susceptible to gentamicin. In 3 (50%) out of six *K. pneumoniae* isolates (CTX-M, TEM and SHV) showed susceptibility to amikacin and gentamicin. Among the aminoglycosides studied, no statistically significant finding was observed among ESBL genotypes which showed resistance to amikacin and gentamicin. ($p > 0.005$)

Among the carbapenems tested, 4 (14.81%) out of 27 isolates each showed resistance to imipenem and meropenem (4–16 µg) (Table 3). About 3 (50%) out of six *K. pneumoniae* isolates with multiple enzymes showed resistance to carbapenems. In 19/20 (95.00%) ESBL *E. coli* showed susceptibility to carbapenems. A significant correlation was observed between ESBL isolates and its resistance to imepenem ($p < 0.005$). No significant finding was found among ESBL genotypes which showed resistance to meropenem. ($p > 0.005$)

As 24/27(88.89%) isolates showed resistance to ciprofloxacin (≥4 µg) and 21/27 (77.78%) isolates were resistant to cotrimoxazole (≥320 µg) (Table 4). No significant correlation was noted among ESBL isolates which showed resistance to ciprofloxacin and cotrimoxazole ($p > 0.005$).

Discussion

The *in-vitro* efficacy of antimicrobials against multidrug-resistant Enterobacterales from wound infection have been evaluated in our study. In the present study ESBL CTX-M type was detected in 12 (60%) out of 20 *E. coli* isolates and a single isolate of *E. aerogenes*. The presence of CTX M and TEM was identified in 40% (8/20) *E. coli* isolates. The presence of multiple ESBL genotypes, SHV, CTX-M & TEM were detected in 100% (6/6) *K. pneumoniae* isolates. In a similar study, TEM and CTX-M was reported as the predominant ESBL genotypes in 39.2% *E. coli* and TEM, CTX-M and SHV in 42.6% *K. pneumoniae* isolates.[11]

The activity of β lactam agents against ESBLs vary depending upon TEM, SHV and CTX-M enzyme ESBL subgroups and they have variable β-lactam/β-lactamase inhibitor combinations susceptibility.[12]

In our study 16.67% CTX-M *E.coli* showed resistance

Table 2: MIC of aminoglycosides against ESBL genotypes

Organisms	*MIC*					
	Amikacin			*Gentamicin*		
	≤2–16 µg (S)	*32 µg (I)*	*≥64 µg (R)*	*≤1–4 µg (S)*	*8 µg (I)*	*≥16 µg (R)*
No of isolates (27)	d(f) = 3; $p = 0.049$			d(f) = 3; $p = 0.816$		
E. coli (12) (CTX- M)	11 (91.67%)	0 (0.00%)	1 (8.33%)	7 (58.33%)	0 (0.00%)	5 (41.67%)
E. coli (8) CTX-M & TEM	8 (100%)	0 (0.00%)	0 (0.00%)	5 (62.50%)	0 (0.00%)	3 (37.50%)
E. aerogenes (1) (CTX- M)	1 (100%)	0 (0.00%)	0 (0.00%)	1 (100%)	0 (0.00%)	0 (0.00%)
K. pneumoniae (6) CTX-M, SHV & TEM	3 (50.00%)	0 (0.00%)	3 (50.00%)	3 (50.00%)	0 (0.00%)	3 (50.00%)

Table 3: MIC of carbapenems against ESBL genotypes

Organisms	*MIC*					
	Imipenem			*Meropenem*		
	0.025–1 µg (S)	*2 µg (I)*	*4–16 µg (R)*	*0.025–1 µg (S)*	*2 µg (I)*	*4–16 µg (R)*
No of isolates (27)	d(f) = 6; $p = 0.003$			d(f) = 3; $p = 0.042$		
E. coli (12) (CTX- M)	12 (100%)	0 (0.00%)	0 (0.00%)	12 (100%)	0 (0.00%)	0 (0.00%)
E. coli (8) CTX-M & TEM	7 (87.50%)	0 (0.00%)	1 (12.50%)	7 (87.50%)	0 (0.00%)	1 (12.50%)
E. aerogenes (1) (CTX- M)	1 (100%)	0 (0.00%)	0 (0.00%)	1 (100%)	0 (0.00%)	0 (0.00%)
K. pneumoniae (6) CTX-M, SHV & TEM	3 (50.00%)	0 (0.00%)	3 (50.00%)	3 (50.00%)	0 (0.00%)	3 (50.00%)

to amoxycillin clavulanate (≥32 µg) and piperacillin-tazobactam (≥128 µg) whereas 12.5% CTX-M and TEM *E. coli* showed resistance to amoxycillin clavulanate and 37.50% *E. coli* isolates each with CTX-M and TEM showed resistance to piperacillin-tazobactam. In 3 (50%) out of 6 *K. pneumoniae* isolates with CTX-M, SHV and TEM showed resistance to amoxycillin clavulanate (MIC: ≥32 µg), and piperacillin-tazobactam (MIC : ≥128 µg). In our study, a significant association was observed between ESBL genotypes (CTX-M, TEM, SHV) detected among the isolates and its resistance to amoxycillin clavulanate ($p < 0.005$). No significant correlation was observed among ESBL genotypes that showed resistance to piperacillin-tazobactam ($p > 0.005$). Piperacillin-tazobactam is considered as less effective drug when compared to carbapenems for treating bacteremic ESBL infection. Piperacillin-tazobactam should be considered for therapy in low inoculum infections when MIC is ≤4 mg/L and when used in appropriate doses. The combination of piperacillin-tazobactam with a non-beta-lactam drug such as aminoglycosides or fluoroquinolone increases the susceptibility of ESBL isolates.[8]

In our study 83.33% CTX-M *E. coli* and 62.5% CTX-M and TEM type *E. coli* showed resistance to cotrimoxazole with a MIC of ≥320 µg . A single *E. coli* isolate with CTX-M ESBL type showed resistance to amikacin (MIC:- ≥ 64 µg). In 3 (50%) out of 6 *K. pneumoniae* isolates with multiple enzymes, CTX-M, SHV and TEM showed resistance to Amikacin (MIC ≥ 64 µg). About 100% *K. pneumoniae* isolates with multiple enzymes, CTX-M, SHV and TEM showed resistance to cotrimoxazole with a MIC of ≥320 µg. No significant finding was observed in our study among ESBL isolates which showed resistance to amikacin and cotrimoxazole ($p > 0.005$). The present study showed high susceptibility of ESBL genotypes to amikacin. A study on the *in-vitro* efficacy of non-beta-lactam drugs showed that the combination of amikacin and piperacillin-tazobactam will be a susceptible regime against ESBL isolates when compared with carbapenems. Amikacin can be considered for combination therapy with beta-lactam drugs such as cefepime or piperacillin-tazobactam against ESBL isolates.[8]

Our study showed 91.67% *E. coli* and a single (100%) isolate of *E. aerogenes* with CTX-M enzyme, 87.50% CTX-M and TEM *E. coli* and 83.33% *K. pneumoniae* isolates (multiple enzymes) with ciprofloxacin resistance (MIC:- ≥4 µg). In our study, no significant finding was observed among ESBL isolates, which showed resistance to ciprofloxacin ($p > 0.005$). In a similar study report, 68% multidrug-resistant isolates of *K. pneumoniae* showed CTX-M enzyme, 45% isolates exhibited SHV and TEM enzymes, respectively with the association between coexistence of enzymes CTX-M, SHV and TEM and fluoroquinolone resistance that is statistically significant[13] G. Rajiv Gandhi *et al.* have reported the coexistence of TEM, SHV, CTX-M and OXA type ESBL genotypes among ciprofloxacin-resistant gram-negative bacilli.[14]

Table 4: MIC of ciprofloxacin and cotrimoxazole against ESBL genotypes

Organisms	*MIC*				
	Ciprofloxacin			*Cotrimoxazole*	
	≤0.5–1 µg (S)	*2 µg (I)*	*≥ 4 µg (R)*	*≤20 µg (S)*	*≥ 320 µg (R)*
No of isolates (27)	d(f) = 3; $p = 0.94$			d(f) = 3; $p = 0.09$	
E. coli (12) (CTX- M)	1 (8.33%)	0 (0.00%)	11 (91.67%)	2 (16.67%)	10 (83.33%)
E. coli (8) CTX-M & TEM	1 (12.50%)	0 (0.00%)	7 (87.50%)	3 (37.5(%)	5 (62.5%)
E. aerogenes (1) (CTX- M)	0 (0.00%)	0 (0.00%)	1 (100%)	1 (100%)	0 (0.00%)
K. pneumoniae (6) CTX-M, SHV & TEM	1 (16.67%)	0 (0.00%)	5 (83.33%)	0 (0.00%)	6 (100%)

In our study, 100% CTX-M *E. coli* and, 87.50% CTX-M and TEM type *E. coli* and 50% *K. pneumoniae* isolates with CTX-M, SHV and TEM showed susceptibility to the carbapenems tested with a MIC range of 0.025 to 1 µg. In 3 (50%) out of six *K. pneumoniae* isolates with CTX-M, SHV and TEM showed resistance to imipenem and Meropenem (MIC: 4–16 µg). Haji, S.H *et al.* have reported carbapenem susceptibility among 28.5% *E. coli* and 55.55% *K. pneumoniae* with SHV gene.[15] A significant association was noted between ESBL isolates and its resistance to imepenem ($p < 0.005$) in our study. No significant association was noted in ESBL genotypes, which showed resistance to meropenem. ($p > 0.005$)

In our study 20.00% *E. coli* each with CTX-M enzyme and CTX-M and TEM enzyme, 83.33% *K. pneumoniae* with CTX-M, SHV and TEM were resistant to cefepime (MIC:16-64 µg). A single isolate of *E. aerogenes* with CTX- M gene showed susceptibility to cefepime (≤2 µg). Manandhar S *et al.* have reported significant correlation between existence of CTX-M and OXA enzymes and decreased susceptibility to cefotaxime, ceftriaxone and cefepime. In their study finding, a significant association was observed between multidrug-resistant phenotypes and presence of CTX M and TEM enzymes.[16] In our study, 44.44% ESBL genotypes showed susceptibility to cefepime (MIC: ≤2 µg). Cefepime may be considered as an effective agent against ESBL genotypes if it is test susceptible (MIC:-≤ 2 µg) and when administered in high dose.[2] The cefepime susceptibility rate in combination with non beta-lactam drug was observed to be higher than that of cefepime alone in a study report.[8]

Expanded spectrum beta-lactamases are more prevalent among Enterobacterales, such as *E. coli* and *K. pneumoniae*. It is essential to find non-β-lactam/non-carbapenem beta-lactams as alternatives to bring down the effects associated with overuse of carbapenems. It is still debatable whether beta-lactam/beta-lactam Inhibitor combination can be considered for patients with ESBL Enterobacterales infections.[17] Analysis of clinical study reports has shown the results of *in-vitro* studies on beta-lactams, aminoglycosides, fluoroquinolones and cotrimoxazole synergy is contrary to those of *in-vivo* studies [18]

Conclusion

Carbapenems are a globally important last-line drug to treat severe ESBL infections. With emerging carbapenem resistant Enterobacterales in clinical settings, the evaluation of *in-vitro* efficacy of non-carbapenem beta-lactam and non-beta-lactam drugs helps to assess various therapeutic alternatives for ESBL infections as monotherapy or combination therapy. Our study showed an association between ESBL isolates with genotypes (CTX-M, SHV and TEM) and its resistance to beta-lactam-beta-lactamase inhibitor (Amoxycillin-Clavulanate) and carbapenem (Imipenem) that is statistically significant. In the present study, no significant association was observed among ESBL isolates, which showed resistance to non-beta-lactam drugs: aminoglycosides, fluoroquinolones and cotrimoxazole.

Limitation of The Study

The present study report is limited to isolates obtained from wound infection and the *in-vitro* efficacy of beta-lactam/non- beta-lactam antibiotics against ESBL genotypes. Further study is required to evaluate the efficacy of non-beta-lactam drugs against Amp C and carbapenemase-producing isolates. This study gives an insight to the efficacy of beta-lactam/non- beta-lactam drugs against isolates with ESBL genotypes in a clinical setting and possible therapeutic options for ESBL wound infection.

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