EXTENDED SPECTRUM BETA (B) – LACTAMASES IN CLINICAL ISOLATES OF GRAM-NEGATIVE BACILLI IN AJMAN, UNITED ARAB EMIRATES

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ABSTRACT

Objective: Extended spectrum beta–lactamase (ESBLs) producing strains of *Enterobacteriaceae* and *Pseudomonas* have emerged as a major problem worldwide. ESBLs, a major group of beta–lactamases, are being identified in large numbers along with inducible AmpC beta–lactamases and derepressed mutants. Many clinical laboratories currently test Gram-negative bacilli for ESBL production. However, there is scarcity of information from United Arab Emirates (UAE). This study was conducted to estimate the frequency of ESBLs and to detect different mechanisms of beta–lactam resistance (ESBLs, AmpC beta–lactamases, derepressed mutants, multiple mechanisms) in Gram-negative bacilli in Ajman, UAE.

Materials & Methods: A total of 123 isolates of Gram-negative bacilli were tested for ESBL production. Screening and confirmatory tests for ESBL production were done according to Clinical Laboratory Standard Institute's guidelines. The novel method by "Rodrigues et al" was used to detect different mechanisms of resistance.

Results: Forty-eight (39.1%) isolates showed ESBL production, of which 12(9.8%) were plain ESBL producers and multiple mechanism was exhibited by 36(29.3%). Nine(7.3%) were plain derepressed mutants. Inducible AmpC beta–lactamase production was detected only in *Pseudomonas aeruginosa*(3.3%). The most common ESBL producer was *E.coli* (60.4%), however *Klebsiella* was the most frequent ESBL producer(40%). Forty one percent of ESBL producers were isolated from urine. All the isolates were sensitive to Imipenem.

Conclusion: The occurrence of high frequency of ESBL-producing Gram-negative bacilli in our hospital is alarming and urgent action required to be taken for screening and confirmation of ESBL production along with continued surveillance, judicious use of antimicrobial agents as well as implementation of infection control measures.

Keywords: Beta-lactam resistance, ESBL, Gram-negative bacilli, Enterobacteriaceae, UAE

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INTRODUCTION

β-lactam antibiotics are one of the most frequently prescribed antimicrobial agents worldwide. The emergence of resistance to these antibiotics in the past few decades has resulted in a major clinical crisis[1,2]. The most common cause of bacterial resistance to β-lactam antibiotics is the production of β-lactamase enzymes. Most of the second and third generation penicillins and cephalosporins were specifically designed to resist the hydrolytic action of major β-lactamases. Unfortunately, the battle between antibiotics and bacteria possessing β-lactamase, is far from over. As new classes of β-lactam antibiotics are introduced, bacteria develop novel ways of overcoming these antibiotics[3]. The indiscriminate use of newer antibiotics in the treatment of patients has given an edge to the new variants of β-lactamases, and the major group forming variant is extended spectrum β-lactamase (ESBL). In last few years, there has been an increased incidence and prevalence of ESBLs, enzymes that hydrolyze and cause resistance to oxyimino-cephalosporins and aztreonam[4,5]. It is unclear whether we will be able to keep pace with bacterial genetic changes and be able to effectively treat Gram- negative infections in future.

ESBLs are currently being identified worldwide in large numbers and a significant percentage has been seen in strains of *E.coli* and *K.pneumoniae*. They have also been found in *Pseudomonas aeruginosa,* other *Enterobacteriacae* strains

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like *Enterobacter, Citrobacter, Proteus, Morganella morganii, Serratia marsescens, Shigella dysenteriae* and non- *Enterobacteriaceace* Gram-negative bacilli[4,5].

Production of these enzymes is either chromosomally mediated or plasmid mediated[5,6]. Point amino acid substitution of the classical plasmid mediated β-lactamases like TEM-1, TEM-2 and SHV-1 increases the spectrum of activity from earlier generation β-lactams to 3rd generation cephalosporins and monobactams[7].However, they retain their stability against cephamycins and carbapenems and are inhibited to an extent by β-lactamase inhibitors[6]. Being plasmid mediated these enzymes spread fast amongst various bacteria and are important by infection control, clinical and therapeutic implication[8]. The chromosomally mediated β-lactamase production is mainly through expression of AmpC gene which is either constitutive or inducible[5,9].

Detection of organisms producing ESBLs provides helpful information to the clinicians. Treatment with newer cephalosporins or aztreonam may still result in treatment failure even when the causative organism appears to be susceptible to these antimicrobial agents by routine susceptibility testing[10]. Many clinical laboratories currently test *E. coli* and *Klebsiella* spp. for ESBL production. However, there is scarcity of information on these isolates in United Arab Emirates (UAE). This study was conducted to:

- estimate the frequency of ESBLs in Gram-negative bacilli,
- detect different mechanisms of β–lactam resistance (ESBLs, AmpC beta–lactamases) and
- suggest a simple susceptibility testing protocol for an early presumptive identification of these enzymes in our laboratory.

MATERIALS AND METHODS

The study was conducted by the Department of Microbiology, Gulf Medical University (GMU), Ajman, United Arab Emirates from January 2010 to June 2010. The clinical isolates were obtained both from inpatients and outpatients attending Gulf Medical College Hospital, the teaching hospital of GMU. One hundred and twenty three isolates of Gram-negative bacilli were recovered from different clinical specimens submitted for routine microbiological analysis. This comprised of *E.coli*: 75, *Klebsiella* spp: 25, *Pseudomonas aeruginosa*: 12, *Enterobacter* spp.: 8, *Citrobacter* spp.: 2 and *Proteus mirabilis*: 1. Of the 123 isolates, 71 were cultured from urine alone. Other clinical samples included pus/wound aspirates, sputum, high vaginal swab, blood, and eye and ear secretions. The organisms were identified by standard identification techniques[11]. Confirmation to the species level was done by API 20 E.

Antimicrobial susceptibility of all the isolates was performed for amikacin, amoxicillin-clavulanic acid, cefotaxime, ceftriaxone, cefepime, ceftazidime, ceftazidime- clavulanic acid, cefoxitin, imipenem and aztreonam on Mueller Hinton Agar (MHA) using Modified Kirby-Bauer disk diffusion method as per Clinical Laboratory Standards Institute (CLSI) guidelines[12]. The culture media and antibiotic disks were purchased from Hi-Media, India. *E.coli* ATCC 25922 and *K.pneumoniae* ATCC 700603 were used as controls to validate these susceptibility tests.

Initial Screening Test: Isolates that exhibited resistance to any of the third generation cephalosporins (3GC) were the candidates for ESBL detection. An isolate was suspected to be an ESBL producer if it had the zones of inhibition for cefotaxime (30 μg) ≤ 27mm, ceftazidime (30 μg) ≤ 22mm, ceftriaxone (30 μg) ≤ 25mm and aztreonam (30 μg) ≤ 27mm[12].

Further ESBL detection was carried out by two procedures:

1. Double Disk Synergy Test (DDST)[13]: The DDST was performed as a standard disk diffusion assay on MHA. Disks containing 30μg of ceftazidime and cefotaxime, each were placed 20mm apart (centre to centre) around a disk containing amoxicillin-clavulanic acid (20μg + 10μg). The MHA plate was incubated at 37°C overnight. Enhancement of inhibition zone of any one of the test antibiotics towards amoxicillin-clavulanic acid disk was regarded as presumptive ESBL production and subjected to phenotypic confirmatory test. [Figure].

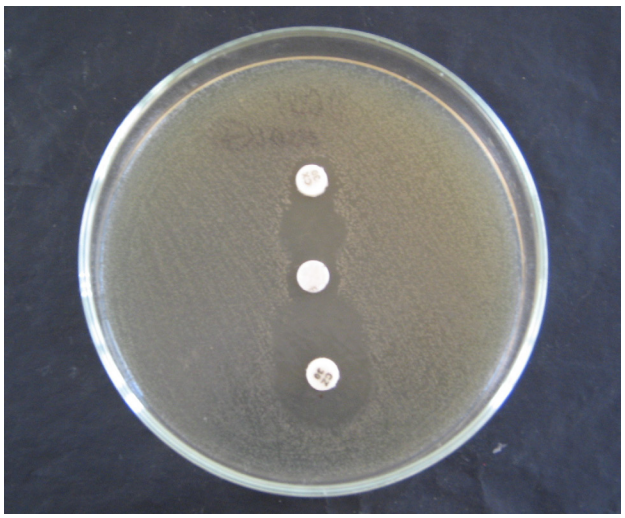

Figure 1: Enhanced zone of inhibition for ceftazidime and cefotaxime on the side of Amoxy-clav indicate ESBL production

2. Phenotypic confirmatory test for ESBL production: This test was performed on MHA by disk-diffusion method as recommended by CLSI. [12] A ≥ 5 mm increase in zone diameter for ceftazidime tested in combination with clavulanic acid versus its zone when tested alone confirms an ESBL producing organism. [Figure 2]

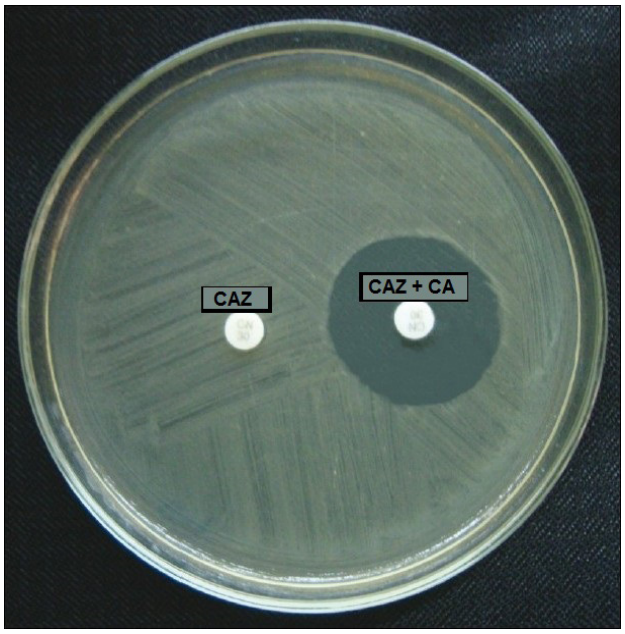

Figure 2: Phenotypic confirmatory test: A ≥ 5 mm increase in zone diameter for ceftazidime in combination with clavulanic acid versus its zone when tested alone confirmed an ESBL producing organism.

The novel method by "Rodrigues et al" to assess ESBL and other mechanisms of production of β-lactam resistance was also studied for each isolate[5,14]. The ceftazidime and ceftazidime-clavulanic acid disks were kept 15 mm apart from each other (center to center). Imipenem, an inducer, was placed in the centre and on either side of it, at a 15 mm distance, were placed ceftazidime and cefotaxime (indicators of induction). In addition, another inducer cefoxitin was placed at 15 mm from cefotaxime (indicator). This was placed opposite to that of ceftazidime -clavulanic acid to avoid any effect of inducible β-lactamase on the zone of inhibition of the latter. The remaining disks were placed as shown in figure 3.

Figure 3: Novel method of placement of antibiotic disc to assess ESBL and AmpC production. Blunting of zone towards

*Inducer indicates inducible AmpC production. Imipenem 1, Cefotaxime 2, Cefoxitin 3, Ceftazidime 4, Ceftazidime-Clavulanic Acid 5, Aztreonam 6, Ceftriaxone 7.

The following criteria were used to categorize isolates exhibiting different mechanisms of resistance[5,12,14]:

ESBL producer:

i) zone diameters for various 3rd generation cephalosporins as mentioned above

ii) susceptible to cefoxitin

iii) increase in zone size with addition of an inhibitor by ≥5 mm

Inducible AmpC producer:

i) blunting of zone towards inducer

ii) no increase in zone size with addition of an inhibitor

iii) susceptible to cefepime

Derepressed mutants:

i) resistant to cefoxitin and cefotaxime

ii) no increase in zone size with addition of an inhibitor

Multiple mechanisms:

i) resistant to cefoxitin

ii) blunting of zone towards inducer

iii) increase in zone size with addition of an inhibitor by ≥5 mm

RESULTS

We observed that ceftazidime was the most effective 3GC for initial screening of ESBL producers. Resistance to aztreonam was seen in 80% of the ESBL producing isolates. (Table 1) ESBL producing strains of *Citrobacter* spp. and *Proteus mirabilis* showed resistance to all 3GC.

Table 1: Screening Test: Resistance of ESBL[a] producing Gram- negative bacilli to 3GC[b] and aztreonam

ESBL positive bacterial strains	No. (n)	Aztreonam n (%)	Cefotaxime n (%)	Ceftazidime n (%)	Ceftriaxone n (%)
E.coli	29	24 (82.8%)	25 (86.2%)	28 (96.5%)	20 (69%)
Klebsiella	10	6 (60%)	7 (70%)	10 (100%)	6 (60%)
Pseudomonas	4	4 (100%)	3 (75%)	4 (100%)	3 (75%)
Enterobacter	3	2 (66.7%)	3 (100%)	3 (100%)	2 (66.7%)

[a]ESBL: extended spectrum beta-lactamase

[b]3GC – 3rd generation cephalosporins

ESBL strains which tested positive by DDST were also found to be positive by the phenotypic confirmatory test. Of the 123 isolates, 48 (39.1%) were ESBL producers, of which 29 (60.4%) were *E.coli*, 10 (20.9%) *Klebsiella* spp., and 4 (8.3%) *Pseudomonas aeruginosa*. The commonest ESBL producers were *E.coli* and *Klebsiella* spp. (Figure 4) but the most frequent ESBL producer was *Klebsiella* spp. (40%) closely followed by *E.coli* (38.7%), *Enterobacter* spp. (37.5%) and *Pseudomonas aeruginosa* (33.3%).

The novel method by "Rodrigues et al" showed that out of 123 isolates, 48 (39.1%) were ESBL producers of which 36 (29.3%) exhibited multiple mechanisms of resistance while remaining 12 (9.8%) were plain ESBL producers. Nine (7.3%) were plain derepressed mutants. Inducible AmpC β-lactamase production was detected only in *Pseudomonas aeruginosa*. Majority of ESBL producers and derepressed mutants belonged to *Enterobacteriaceae*. The percentage of different mechanism of β–lactam resistance is shown in Table 2.

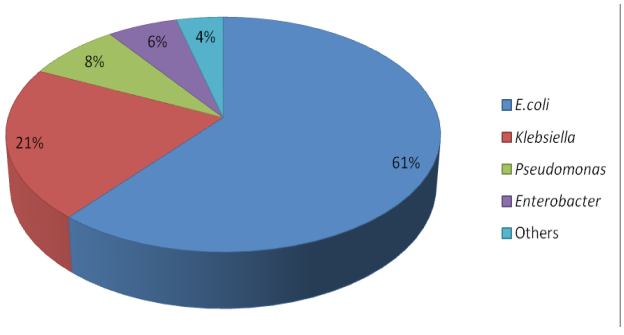

Figure 4: Percentage of Common ESBL producers

Table 2: Percentage of different mechanism of β–lactam resistance

Organism (Isolates)	No. of isolates tested (n)	Plain ESBL n (%)	Multiple Mechanism n (%)	Derepressed n (%)	Inducible n (%)	No resistance n (%)
E.coli	75	7 (9.3%)	22 (29.3%)	4 (5.3%)	-	42 (56.1%)
Klebsiella	25	3 (12%)	7 (28 %)	-	-	15 (60%)
Pseudomonas	12	1 (8.3%)	3 (25%)	2 (16.7%)	4 (33.3%)	10 (16.7%)
Enterobacter	8	-	3 (37.5%)	3 (37.5%)	-	2 (25%)
Citrobacter	2	-	1 (50%)	-	-	1 (50%)
Proteus	1	1 (100%)	-	-	-	-
Total	123	12 (9.8%)	36 (29.3%)	9 (7.3%)	4 (3.3%)	62 (50.3%)

Forty one percent of ESBL producers were isolated from urine whereas 22.3% were isolated from exudates. All ESBL producers and derepressed mutants were sensitive to imipenem. Among non β–lactam antibiotics, 12% of ESBL producing isolates showed resistance to amikacin.

DISCUSSION

This study demonstrates the presence of ESBL-mediated resistance in Gram-negative bacilli causing infections in patients attending our teaching hospital in Ajman, UAE. There have been numerous reports of ESBLs from different parts of the world but limited data is available from UAE[15]. ESBL detection is not commonly carried out in many microbiology units in developing countries and this could be due to lack of awareness or lack of resources and facilities to conduct ESBL identification or both[16]. The high rate of resistance amongst the isolates in our study is a matter of serious concern. Forty eight out of the 123 (39.1%) Gram-negative bacilli were ESBL producers. The prevalence of ESBL producers varies across continents and countries and also within hospitals. A predominance of either *Klebsiella pneumoniae* or *E. coli* has often been reported among the ESBL isolates identified worldwide[2-5,9,14-17].

Previous studies from India have reported ESBL production varying from 6.6% to 91%[3,5,14,16,17]. Studies in Pakistan show a variable rate ranging from 24% to 76%[2,18,19]. A survey of intensive care units in Europe has found that the prevalence of ESBL in *Klebsiella* spp. varied from 3% in Sweden to 34% in Portugal[20]. Higher figures of 30–60% have been reported from the South American countries of Brazil, Venezuela and Colombia[21-23]. In Korea, Japan, Malaysia and Singapore, National Surveys have demonstrated the presence of ESBLs in 5–8% of *E. coli* isolates whereas higher rates of up to 24% has been reported from other Asian countries[10,24]. ESBL production in *Klebsiella* spp. have also been reported to be as low as 5% in Japan and Australia with higher rates of 20–50% in other parts of the continent[10,24]. In UAE, 41% of *E.coli* and *Klebsiella* spp. isolated over 14 months were found to be ESBL producers[15]. In the Arabian Gulf region, 7.5% of *Enterobacteriaceae* and *Pseudomonas* spp. isolated over a one-year period in Kuwait were reported as being ESBL producers using the VITEK 2 system[25] whereas 23.6% of gram-negative bacilli were positive for ESBL production in Iran[26].

In our study, *Klebsiella* spp. was the most frequent ESBL producer followed by *E.coli* which is in concordance with three other studies[15, 25, 27]. *E.coli* was found to be the most frequent ESBL producer followed by *Klebsiella* spp. in studies conducted in India[5, 14] whereas *Enterobacter cloacae* (79%) was the most frequent ESBL producer in another study conducted in Pakistan. A higher rate of ESBL production by *Klebsiella pneumoniae* (55%) was observed in Riyadh[28].

The novel method by "Rodrigues et al" indicated that ESBL producing organisms like *Klebsiella* spp., *E.coli*, *Pseudomonas aeruginosa* and *Enterobacter* spp. showed different mechanisms for

the production of multiple β-lactamases. Similar observations were made in other studies[5,14]

The percentage of plain ESBL producers among *E. coli* (9.3%) and *Klebsiella* spp. (12%) was less frequent when compared to studies conducted by Shobha et al[14] and Varaiya et al[29] but the results have been comparable to the study done by Rodrigues et al[5].

In this study, only 5.3% of *E. coli* and 16.7% of *Pseudomonas* spp. were derepressed mutants while this percentage was much higher in studies conducted by Rodrigues et al, and Shobha et al.[5,14] *Pseudomonas aeruginosa* was the only Gram-negative bacilli that produced inducible AmpC β-lactamases. One-third (33.3%) of *Pseudomonas* isolates showed inducible AmpC β-lactamase production while 26.5% of *Pseudomonas* spp. were inducible AmpC β-lactamase producer in a study done at Mumbai[5].

When the phenotypic confirmatory method was compared to the novel method described by "Rodrigues et al", the latter was found to be better than the former, because it assesses ESBL producers, de-repressed mutants, inducible AmpC β-lactamase production and multiple mechanisms in a single culture plate and it was also easy to perform. Although CLSI also recommends MIC broth micro-dilution for ESBL detection, we used disk diffusion method as it is comparatively simple and also cost effective.

Similar to other reports,[15,30] the majority of *Enterobacteriaceae* isolates were from urine specimens, indicating the need for active screening of urine cultures for ESBL producers. Indeed, the detection of EBSL producers in urine has been described as representing an epidemiologic marker of colonization and potential for transfer between patients[10]. Clinical isolates obtained from other sources were also found to be ESBL producers; hence it is imperative that active screening of *Enterobacteriaceae* isolates from all specimen types should be adopted to address the high prevalence rate of ESBL isolates. Unfortunately, many clinical microbiological laboratories still face significant problems with ESBL screening and identification as ESBL pathogens can present with variations in the in-vitro pattern of resistance to β-lactam agents[15]. Enzymes such as TEM-3 and SHV-2 confer high levels of resistance to cephalosporins, whereas others, namely TEM-7 and TEM-12, confer low levels of resistance, which possibly makes detection of resistance even more difficult with the routinely used antimicrobial susceptibility tests[4,7,8].

The antimicrobial susceptibility test showed that all the isolates including ESBL strains were susceptible to imipenem but 12% of the ESBL producers showed resistance to amikacin. Similar studies conducted in UAE and India[5,14,15] showed 100% susceptibility to amikacin and imipenem. Although amikacin and carbapenems are used only as the reserve drugs for treatment of infection by ESBL producing strains, production of β-lactamases capable of hydrolyzing carbapenems has also been reported mostly in *Enterobacter* spp. and *Serratia* spp[15]. Carbapenem resistance is due to the production of chromosomal and plasmid-mediated cephaloporinases along with decreased permeability of drug through outer membrane. ESBL producers often show cross-resistance with non- β -lactam antibiotics, such as quinolones and aminoglycosides, resulting in limitation of antimicrobial choice. This co-resistance arises probably because these plasmid-mediated enzymes are transferable between bacterial species and are also capable of incorporating genetic material coding for resistance to other antibiotics[4,8].

While this Study was aimed at determining the prevalence of ESBL isolates in our hospital, the need for the molecular characterization of these isolates cannot be neglected. Although we could not carry out the molecular characterization due to lack of facilities and infrastructure, the finding of such resistant isolates in our setting is a matter of concern.

CONCLUSION

Clinical laboratories must be aware of the importance of ESBL and plasmid mediated AmpC β-lactamase production. Although CLSI recommendations exist, they are limited to ESBL producers of *E. coli*, *Klebsiella* spp. and *Proteus mirabilis*. No recommendations exist for ESBL detection and reporting for other organisms, or for the detection of AmpC β-lactamases. Screening methods of ESBL with recommended zone size should be immediately applied to suggest the presence of an ESBL. Large surveys, continued surveillance by clinical microbiology laboratory, judicious use of antimicrobial agents as well as implementation of infection control measures are recommended if the frequency of ESBL isolates is to be reduced in this setting.

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