First Report of Multidrug-Resistant ESBL-Producing Urinary Escherichia coli in Jordan

L. F. Nimri¹* and B. A. Azaizeh¹

¹Department of Medical Laboratory Sciences, Jordan University of Science & Technology, Irbid 22110, Jordan.

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ABSTRACT

Aims: To detect ESBL-producing E. coli isolates and the genes underlying their resistance to β-lactams and sulfamethoxazole.

Place and Duration of Study: Department of Medical Laboratory Sciences, Jordan University of Science & Technology, Irbid 22110, Jordan, between January 2009 and December 2010.

Methodology: The blaCTX-M, blaTEM, blaSHV genes and sul genes were tested for in 165 urinary E. coli isolates by PCR assays. The association between the presence of gene and the antibiotic resistant was analyzed.

Results: Multidrug resistance was detected among the isolates. Eighty-three (50.3%) of the isolates were ESBL-producing, 67(80.7%) had either blaCTX-M or blaTEM, or both and none had blaSHV gene, sul2 (41.2%) was prevalent (21.8%) isolates had two sul genes, while (1.8%) isolates had three sul genes. A significant association (p ≤ 0.05) was found between blaCTX-M, or blaTEM and resistance to several antibiotics e.g., cefixime. Several (29.9%) of the ESBL-producing strains harbored multiple ESBL genes and had high resistance to various antibiotic classes.

Conclusion: The results revealed a high-level of sul2 and blaCTX-M positive ESBL isolates among other β-lactam resistant genes circulating in the community. These findings indicate that these patients are more likely to have ineffective initial empirical antimicrobial therapy.

Keywords: Extended-spectrum β-lactamase; multidrug resistance; urinary Escherichia coli; bla/sul genes.
1. INTRODUCTION

During the past decade, drug resistance in Enterobacteriaceae has increased dramatically worldwide. This increase has been caused mainly by an increased prevalence of extended-spectrum β-lactamase (ESBL)–producing Enterobacteriaceae (Cantón et al., 2008). The production of β-lactamase by many bacteria that breaks the β-lactam ring, rendering the antibiotic ineffective is the single most prevalent mechanism responsible for resistance to β-lactams among clinical isolates of the family Enterobacteriaceae (Sanders and Sanders, 1992).

Extended-spectrum β-lactamases (ESBLs) are plasmid-mediated rapidly evolving groups of β-lactamases, capable of hydrolyzing third-generation cephalosporins and aztreonam yet are inhibited by clavulanic acid (Paterson and Bonomo, 2005). In typical circumstances, ESBLs result from mutations in the TEM-1, TEM-2, or SHV-1 genes that alter the amino acid configuration around the active site of these β-lactamases. These mutations extend the spectrum of β-lactam antibiotics susceptible to hydrolysis by these enzymes. CTX-Ms type β-lactamases are cefotaximases that usually hydrolyze cefotaxime rather than ceftazidime, however; point mutations can extend their target spectrum to ceftazidime (Ruppé et al., 2009). The ESBLs are frequently encoded by genes located on plasmids, which frequently carry genes encoding resistance to other drug classes (e.g. amino glycosides) and their presence carries tremendous clinical significance. An increasing number of ESBLs not of TEM or SHV lineage have recently been described. Therefore, antibiotics options in the treatment of ESBL producing organisms are extremely limited.

Trimethoprim-Sulfamethoxazole (TMP-SMX) has been the first line empirical treatment for more than 30 years for community-acquired urinary tract infections (UTIs) (Warren et al., 1999). The combination of both TMP-SMX in treating UTIs is more effective than either of them individually, because it inhibits successive steps in the foliate synthesis pathway (Masters et al., 2003).

Usually SMX resistance is encoded by the plasmid born sul genes that encode dihydropteroate synthetase, which are not inhibited by the drug. Unfortunately, the prevalence of E. coli resistant strains to TMP-SMX has increased during the past decade in many parts of the United States and other countries (Gniadkowski, 2001). Members of the family Enterobacteriaceae commonly express plasmid-encoded β-lactamases (e.g., TEM-1, TEM-2, and SHV-1 enzymes) (Livermore, 1995), which confer resistance to penicillin’s, but not to expanded spectrum cephalosporin.

The objectives of this study were to assess the antibiotics resistance of urinary E. coli isolates by conventional antibiotics testing methods, and to determine the prevalence of blaCTX-M, blaTEM, blaSHV and three sul genes underlying resistance to β-lactams and TMP-SMX by PCR assays.

2. MATERIALS AND METHODS

2.1 Clinical Isolates

E. coli isolates were collected from 165 outpatients (January 2009-December 2009) diagnosed of having UTI in the University Hospital (KAUH), and a Health Center, in Irbid, Jordan. These were community outpatients whom had no or only limited recent hospital
contact. The age range of patients was 18-75 years; male to female ratio was 1:1. Quantitative cultures on blood and MacConkey agar were done, a UTI was defined by >10^4 leukocytes/ml of urine and >10^5 CFU/ml of urine.

The study protocol was reviewed and approved by the Institutional Review Boards.

2.2 Identification of *E. coli* Strains

The identification of *E. coli* was done on selected lactose fermenting colonies growing on MacConkey agar using the RapID™ ONE system (Remel, USA). Isolates were stored in brain heart infusion (BHI) broth containing 25% glycerol at 4ºC.

2.3 Antibiotic Susceptibility Testing

The Kirby-Bauer disc diffusion method on Mueller-Hinton agar was used (CLSI 2010 guidelines). Isolates were tested against 16 antimicrobial agents commonly used to treat UTI: amikacin, amoxicillin/clavulanic acid (augmentin), cefixime, cefuroxime, cefotetan, piperacillin, levofloxacin, nitrofurantoin, piperacillin/clavulanic acid, imipenem, nalidixic acid, norfloxacin, ciprofloxacin, gentamicin, tetracycline and sulfamethoxazole/trimethoprim (cotrimoxazole) (MASTRING™-S™, Bootle, UK).

2.4 Screening for and Confirmation of ESBLs

The initial screening for the ESBLs isolates was performed by measuring the diameter of inhibition zone around ceftazidime (30 µg), and cefotaxim (30 µg), on Mueller-Hinton media. A positive result was considered to be suspicious for the presence of ESBLs. The positive isolates were tested by phenotypic confirmatory testing using ceftazidime (30 µg) (Oxoid Limited, Hampshire, England), versus ceftazidime/clavulanic acid (30/10 µg) and cefotaxime (30 µg) versus cefotaxime/clavulanic acid (30/10 µg) as issued by CLSI (2010).

*E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 were used as negative and positive controls respectively.

2.5 Identification of ESBLs Genes

DNA was prepared from five colonies of each *E. coli* isolate suspended by vortexing in one ml DNase- and RNase-free water and boiled for 10 min. Supernatant containing template DNA was stored at -20ºC till tested.

The ESBL (blaCTX-M, blaTEM, blaSHV) encoding genes (Ruppé et al., 2009) and the sulfamethoxazole resistance (*sul1*, *sul2*, *sul3*) genes were detected by PCR as previously described (Koljal et al., 2009).

2.6 Statistical Analysis

Data were analyzed with Chi square test to determine relationships between the MIC distributions of antibiotic agents and antibiotic resistance genes using the statistical software package SPSS for Windows (SPSS Inc, Chicago, IL) and a p value of ≤ 0.05 was considered statistically significant.
3. RESULTS

3.1 Antibiotics Susceptibility Testing

Disc-diffusion susceptibility testing indicated high and variable resistance of the isolates to most of the antibiotics, with the highest recorded for the antibiotics that have been used for long time such as tetracycline (67.9%) and trimethoprim- sulfamethoxazole (64.8%). The lowest resistance was recorded for amikacin (3.0%), and nitrofurantion (4.2%) (Fig. 1).

![Antibiotics Susceptibility Testing](image)

**Fig. 1.** Antibiotics Susceptibility Testing for all *E. coli* isolates by the disc diffusion method.

*S*: Susceptible; *I*: Intermediate; *R*: Resistant.

3.2 ESBL Confirmatory Test

Out of the 165 isolates, 83(50.3%) were ESBL-producing isolates, 67(80.7%) of these had at least one ESBL gene (either *blaCTX-M* or *blaTEM*, or both), 16(19.3%) isolates didn’t have any of the three ESBL genes, and *blaSHV* was not detected in any of the isolates. Out of the 67 isolates 47(70.1%) had either *blaCTX-M* (28 isolate), or *blaTEM* gene (19 isolates) (Fig. 2), while 20(29.9%) isolates had both *blaCTX-M* and *blaTEM* genes.

Out of the 83 having ESBL phenotype, 78(94%) were resistant to cefotaxime and ceftazidime, 4(4.8%) were resistant only to cefotaxime (CTX), while 1(1.2%) isolate was resistant only to ceftazidime. These results indicate a significant association (*p* ≤ 0.05) between the ESBL phenotype and the resistance to cefotaxime and ceftazidime antibiotics (Table 1).
Fig. 2. *blaCTX-M* gene PCR product (550 bp). M: 100 bp DNA marker; PC, NC: positive and negative controls; lanes 1-4: *E. coli* isolates positive for the *blaCTX-M* gene

Table 1. ESBL phenotype test results in association with resistant to cefotaxime (CTX) and ceftazidime (CAZ)

| ESBL phenotype |CTX (%) |CAZ (%) |P value |
|---|---|---|---|
|Positive (n=83) |82 (98.8)|1 (1.2)|79 (95.2)|4 (4.7)|0.000|
|Negative (n=82) |0 (0.0)|82 (100)|0 (0.0)|82 (100)|0.000|

3.3 The ESBL and sul Genes

The presence of one or more of the ESBL (*blaCTX-M, blaTEM*) genes, and the sulfamethoxazole resistance genes (*sul1, sul2, sul3*) were detected in all the 165 isolates (Table 2, Fig. 3). The *sul1* gene was detected in 51(30.9%) of the bacterial isolates, *sul2* gene was detected in 68(41.2%), while the *sul3* gene was detected in 12(7.3%) of the *E. coli* isolates. Out of the 165 isolates 50(30.3%) isolates had only one *sul* gene, 36(21.8%) isolates had two *sul* genes and 3(1.8%) isolates had all three genes.

3.4. The ESBL Genes and the Antibiotic Susceptibility Pattern

A significant association (p ≤ 0.05) was found between the *blaCTX-M* gene and resistance to 13 antibiotics but not to cefotetan, imipenem, nitrofurantoin, amikacin, or piperacillin-clavulanic acid. The *blaTEM* gene was significantly associated with resistance to: ceftazidime, cefixime, gentamicin, nalidixic acid, piperacillin, tetracycline, TMP-SMX, and cefotaxime (Table 2).
Table 2. Distribution (%) of bla/sul resistance genes among 165 urinary E. coli isolates

| Gene   | The main antibiotics the genes confer resistance to                                                                 | Number Present (%) | Number Absent (%) |
|--------|------------------------------------------------------------------------------------------------------------------------|--------------------|-------------------|
| bla\(_{CTX-M}\) | Augmentin, ceftazidime, cefixime, ciprofloxacin, cefotaxime, cefuroxime, gentamicin, levofloxacin, nalidixic acid, norfloxacin, pipercillin, tetracycline, trimethoprim-sulfamethoxazole | 48 (29.1)          | 117 (70.9)        |
| \(\text{bla}_{TEM}\) | Ceftazidime, cefixime, gentamicin, nalidixic acid, pipercillin, tetracycline, trimethoprim-sulfamethoxazole, cefotaxime | 39 (23.6)          | 126 (76.4)        |
| \(\text{bla}_{SHV}\) | None                                                                                                           | 0 (0.0)            | 165 (100)         |
| sul\(1\) | Augmentin, cefixime, cefuroxime, ciprofloxacin, cefotaxime, cefuroxime, gentamicin, levofloxacin, nalidixic acid, norfloxacin, pipercillin, tetracycline, trimethoprim-sulfamethoxazole | 51 (30.9)          | 114 (69.1)        |
| sul\(2\) | Cefixime, cefuroxime, gentamicin, nalidixic acid, pipercillin, cefotaxime, cefotaxime, trimethoprim-sulfamethoxazole | 68 (41.2)          | 97 (58.8)         |
| sul\(3\) | Tetracycline                                                                                                    | 12 (7.3)           | 153 (92.7)        |

3.5. The sul genes

Out of 165 isolates, 89(53.9%) isolates were positive for one or more of the sul genes (Fig. 3), while 76(46.1%) had none of the genes. A significant association (\(p \leq 0.05\)) was found between the sul\(1\) gene and the resistance to: augmentin, cefixime, cefuroxime, ciprofloxacin, ceftazidime, cefotaxime, gentamicin, levofloxacin, nalidixic acid, norfloxacin, pipercillin, tetracycline and TMP-SMX, but not the other antibiotics. The sul\(2\) gene was significantly associated with resistance to cefixime, cefuroxime, gentamicin, nalidixic acid, pipercillin, ceftazidime, cefotaxime, and TMP-SMX.

Fig. 3. sul\(1\) gene PCR product (fragment size 667 bp). Lane M: 100 bp DNA marker; lane PC: positive control, K. pneumoniae ATCC 700603; lane NC: negative control; lanes 1 and 2: E. coli isolates positive for the gene
Results also revealed a significant association between the **suB** gene and resistance to only tetracycline. In addition, a significant association ($p \leq 0.05$) was found between the presence of multiple genes (2-5 genes) and resistance to augmentin, cefuroxime, cefixime, ciprofloxacin, gentamicin, levofloxacin, nalidixic acid, norfloxacin, piperacillin, tetracycline, TMP-SMX, ceftazidime, cefotaxime.

4. DISCUSSION

In Jordan as in some other countries, the inappropriate prescription of antibiotics by some physicians and irresponsible consumption by patients caused by the widespread access to antibiotics often increases the antibiotics resistance. The purchase of antibiotics without a prescription in Jordan was previously reported as (46%) either via self-medication (23.2%) or pharmacist recommendation (23.1%) (Al-Bakri et al., 2005). In Central and South American countries, Philippines, some European countries and many developing countries around the world, antibiotics can be purchased without prescription (Borg and Siciluna, 2002; Lansang et al., 1990). A study in Philippine, reported that purchases without prescription were made in 66.3% of 1608 transactions; most of these were aminopenicillins or penicillins G or V (40.0%) (Lansang et al., 1990). In the United States, some antibiotics such as amoxicillin, ampicillin, tetracycline, cephalaxin, metronidazole and erythromycin can be bought either from a pet store, an ethnic market/convenience store, the Internet, or from Mexico (http://www.coreynahman.com/antibiotics.html).

Extended-spectrum β-lactamase-producing *Enterobacteriaceae* are an emerging public-health concern (Pitout and Laupland, 2008), because the ESBL-encoding isolates often co-express resistance to fluoroquinolones, TMP-SMX, tetracycline and aminoglycosides, thus are often classified as multidrug-resistant pathogens (Paterson and Bonomo, 2005; Morosini et al., 2006).

The current study is the first in Jordan to characterize the extended-spectrum β-lactamase-producing urinary *E. coli* isolates. The results demonstrated high resistance of the isolates against many antibiotics with the maximum resistance recorded to antibiotics that have been extensively in use for a long time such as tetracycline (67.9%) and TMP-SMX (64.8%). The extraordinary genetic capacities of microbes have benefited from man’s overuse of antibiotics by means of horizontal gene transmission to develop multiple mechanisms of resistance as the results of many years of unremitting selection pressure from human applications of antibiotics (Davis and Davis, 2010).

High resistance was also recorded to piperacillin (63.6%), nalidixic Acid (61.2%) and to the 2nd, 3rd generations of cephalosporins including cefuroxime (56.4%), cefixime (53.9%), cefotaxime (49.7%), ceftazidime (47.9%) and other antibiotics, indicating an increased resistance to β-lactam antibiotics among the tested isolates. The same isolates showed low resistance to amikacin (3.0%), imipenem (4.8%), nitrofurantoin (4.2%), and piperacillin-clavulanic acid (5.5%). It is also noticed that almost half of the tested *E. coli* isolates were resistant to fluoroquinolones and their precursors including nalidixic acid, levofloxacin, ciprofloxacin and norfloxacin. Amikacin and carbapenems such as imipenem had the widest spectrum of activity against the *E. coli* isolates. These findings are in agreement with earlier studies of urinary *E. coli* reporting on high resistance to tetracycline ranging (83%-86%), TMP-SMX (48%-77%), and also moderate to high rates of resistance to other commonly prescribed antibiotics in Jordan such as cefuroxime and norfloxacin (Abu-Elteen et al., 2000; Abu Shaqura, 2000).
A study of community-acquired UTI in a rural area in Northern Jordan reported lower resistance of the *E. coli* isolates to ciprofloxacin (10.8%), TMP-SMX (46.8%), nalidixic acid (38.5%), tetracycline (30.7%) and to nitrofurantoin (10%) (Nimri and Batchoun, 2010). The differences in the results could be explained by differences in the strains circulating in the community and in the prescribed drugs.

The results of the current study compared to studies conducted in Jordan and other countries varied depending on the antibiotics tested. In a study of community-acquired UTIs in Jordan, where *E. coli* accounted for (82%) of the isolates reported higher resistance to tetracycline (86%), augmentin (45%), and lower resistance to TMP-SMX (48%) (Abu Shaqura, 2000). The difference could be related to the presence of other members of the *Enterobacteriaceae* in their study affecting the overall antibiotics resistance pattern. A study of uropathogenic *E. coli* isolates from inpatients, and outpatients was in agreement with our results for resistance to norfloxacin (39%), while relatively higher resistance to TMP-SMX (77%), tetracycline (74%), gentamicin (48%) and nitrofurantoin (24%), and lower resistance to nalidixic acid (47%) was reported (Shehabi et al., 2004). The higher antibiotics resistance could be explained by the difference in the patient group included in the study, since hospital admission was reported as a risk factor for acquiring high resistant strains (Calbo et al., 2006). We compared our results with those reported in the pocket chart antibiogram of 1550 isolates of *E. coli* issued by the infection control unit at King Abdullah University Hospital for the years 2005-2009 for both inpatients and outpatients. The resistance reported for the outpatients was close to the findings of the current study for: amoxacilin-clavulanic acid (63%), cefuroxime (42%), ciprofloxacin (41%), gentamicin (28%), imipenem (2%), piperacillin-clavulanic acid (11%), but was a little higher for amikacin (7%), and lower for cefotaxime (28%), cefazidime (29%). These differences could be explained by the different clinical source (blood, ear, or wound) of *E. coli* isolates evaluated by the hospital since not all were of urinary origin, while the current study included only urinary isolates.

A previous study (Bindayna et al., 2010) reported higher (43/84, 51%) urinary *E. coli* ESBL producers harboring both *bla*CTX-M, and *bla*TEM, and the *bla*SHV gene detected in one of their isolates was not detected in our isolates. The difference in results might be due to the *K. pneumoniae* included in 16% of their study.

The emergence of ESBLs has been tied to the overuse and misuse of third-generation cephalosporins, and other antimicrobials. However, the emergence of one ESBL variant over another at a given medical center might be a result of several factors, with antibiotic use as a contributing, but not necessarily a determining, factor. The diverse nature of ESBLs, with different enzymes having substrate preferences among the oxyimino-β-lactams, means that selection pressure should favor those ESBLs that are highly active against β-lactams, and are currently used at a center (Gniadkowski, 2001). The presence of a single ESBL variant in different centers may be attributed mostly to the dissemination of an ESBL gene-carrying plasmid.

The results of a previous study in UK, where only 38.3% were urinary *E. coli*, reported similar resistance to piperacillin and amikacin, but lower resistance to tetracycline, trimethoprim-sulfamethoxazole, nalidixic acid, ciprofloxacin, gentamicin, augmentin, and imipenem (Woodford et al., 2004). Lower resistance of uropathogenic *E. coli* was reported in Canada to TMP-SMX (18.9%), nitrofurantoin (0.1%), ciprofloxacin (1.2%), and (44%) to TMP-SMX in Canada and Europe (Blahna et al., 2006). Notably, factors leading to antibiotic resistance and strains reported vary based on the geographical area, the patients’ group, antibiotic prescribing regime, self-medication practices, as well as epidemiologic, and clinical
parameters of the target populations that play a major role in increasing resistance (Gniadkowski, 2001; Cohen, 1997).

5. CONCLUSION

The results revealed a high-level of sul2, and blaCTX-M positive ESBL isolates circulating in the community. The trend of multidrug resistance profile associated with carriage of blaCTX-M gene, and other genes is a cause for concern (Bradford, 2001). The results of the phenotypic confirmatory test support the findings that resistance to cefotaxime and ceftazidime works as a good indicator for the ESBL producing E. coli isolates. The resistance to those antibiotics was restored when both antibiotics were used in combination with clavulanic acid, known to be β-lactamase inhibitor. These findings indicate that these patients are more likely to have ineffective initial empirical antimicrobial therapy. Thus, more rapid diagnostic testing of ESBL-producing bacteria, and the possible modification of guidelines for community-UTIs are required.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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