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Vol. 8(19), pp. 1974-1981, 7 May, 2014
DOI: 10.5897/AJMR2014.6670
Article Number: C401B6244478
ISSN 1996-0808
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http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Phenotypic and genotypic identification of extended spectrum β-lactamases (ESBLs) among clinical isolates of *Escherichia coli*

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Received 28 January, 2014; Accepted 7 April, 2014

Extended spectrum β-lactamases (ESBLs) are emerging, diverse group of plasmid - associated antibiotic resistant enzymes that are presenting a public health concern. ESBLs were detected in *Escherichia coli* by performing phenotypic tests on 18 out of 35 *E. coli* isolates recovered from urine samples of patients with urinary tract infections in three Eygpt hospitals and identified as positive ESBLs according to CLSI screening criteria. Results of phenotypic confirmatory tests revealed that, broth microdilution test, combination disc test and double disc synergy test confirmed 14(78%), 16(89%) and 16(89 %), respectively of *E. coli* isolates as positive ESBLs. Genotypic screening using PCR was performed by testing for $bla_{CTX\text{-}M}$, bla_{TEM} and bla_{SHV} enzymes. Sixteen phenotypic ESBL isolates were positive for β-lactamase genes. Ten isolates produced both $bla_{CTX\text{-}M}$ and bla_{TEM}, and 6 isolates produced $bla_{CTX\text{-}M}$ only, while bla_{SHV} was not detected in any isolate. The sensitivity and specificity of combination disc and broth microdilution tests compared to PCR were 100%, while double disc synergy test showed sensitivity and specificity of 87.5 and 100% respectively. ESBL isolates were found to have multi-drug resistance pattern. No correlation could be made between type of ESBL and antimicrobial susceptibility profile of the isolate.

Key words: Extended spectrum β-lactamase, *bla* (TEM), *bla* (CTX-M), urinary tract infection.

INTRODUCTION

The prevalance of Extended spectrum beta-lactamase producing strains of Enterobacteriaceae are increasing globally. Recently, reports from different countries showed increasing number of β-lactamases of various types; OXA, CTX-M, TEM and SHV-derived (Tschudin-Sutter et al., 2012). The situation in developing countries is more serious due to inappropriate use of antibiotics, lack of routine susceptibility testing of clinical isolates, thus leading to poor treatment outcome and dissemination of different resistance genes in the community (Ama´bile-Cuevas, 2010). Occurrence of resistance mechanisms may also have an epidemiological impact since these bacteriae are not only the cause of outbreaks but have become endemic in many hospitals throughout

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the world (Taneja and Sharma, 2008).

The guidelines of phenotypic detection for ESBLs are based on the principle that most ESBLs hydrolyze the third-generation of cephalosporins although they are inhibited by clavulanate (Paterson and Bonomo, 2005). The Clinical Laboratory Standards Institute guidelines (CLSI) for ESBL detection in *E. coli*, *Klebsiella* spp. and *Proteus mirabilis* recommend initial screening with either 8 mg/L of cefpodoxime, 1 mg/L each of cefotaxime, ceftazidime, ceftriaxone, or aztreonam, followed by confirmatory tests such as, the double disc synergy test, the combination disc method, and specific E-test ESBL strips with both cefotaxime and ceftazidime in combination with clavulanate at a concentration of 4 µg/ml (CLSI, 2009a).

The genotypic tests for the detection of ESBLs primarily consist of polymerase chain reaction (PCR) amplification of the bla_{TEM} and bla_{SHV} genes with oligonucleotide primers. PCR may be followed by sequencing for characterization of the enzyme. Alternative approaches to sequencing include restriction fragment length polymorphism (RFLP), restriction site insertion PCR, ligase chain reaction, PCR with single-strand conformational polymorphism, and real-time PCR (Arlet et al., 1995; M'Zali et al., 1996; Kim and Lee, 2000; Chanawong et al., 2001).

Urinary tract infections (UTI) are prevalent worldwide (Gonzalez and Schaeffer, 1999). They may be caused by members of Enterobacteriaceae with *E. coli* presenting 70-95% of the causes of UTI, followed by *Klebsiella* spp (Behzadi et al., 2010). On the other hand, *Pseudomonas aeruginosa* play a major role in complicated and catheter - associated UTI (Tielen et al., 2013). Antibiotic resistance in uropathogens is increasing and is the cause for failure of treatment of complicated and uncomplicated UTI. Treatment of UTI caused by ESBL producing bacteria is challenging since ESBLs mediate resistance to extended spectrum third-generation cephalosporins and monobactams used for treatment (Ena et al., 2006). The recommended antibiotic treatment for such infections is carbapenems (Paterson and Bonomo, 2005). However, the use of piperacillin/tazobactam combination, colistin and fosfomycin was also reported to have a successful outcome comparable to that of carbapenems (Tumbarello et al*.,* 2006; Kanj and Kanafani, 2011).

This study is aimed to investigate ESBLs in *E. coli* as clinical isolates by both phenotypic and genotypic methods with evaluating their antimicrobial susceptibility profiles.

MATERIALS AND METHODS

Bacterial strains

A total of 35 *E. coli* isolates were recovered from 130 bacterial isolates obtained from the urine sample of patients clinically diagnosed as urinary tract infection and were collected at the Clinical Microbiology Laboratories of Shebeen El kom, Theodor Bilharz, and Kasr Al Ainy hospitals in Egypt. Urine samples showed bacterial colony count ≥10^3 CFU/ml upon culture. All isolates were obtained after getting consent from patients. The study was carried out according to the guidelines of the Ethics committee of Cairo University. Isolates were identified by standard microbiological and biochemical tests including; culture on MacConkey agar (Oxoid, UK), Eosin methyllene blue agar (Difco USA), Chrom agar for *E. coli* (Oxoid, UK), indole, methyl red, Voges-Proskauer and citrate test (Oxoid, UK). They were then confirmed using API 20-E kit (Biomerieux, France). For long time preservation, all isolates were maintained in 25% glycerol in Luria-Bertani broth (LB broth) at -70°C.*E. coli* strain ATCC 25922 was used as a quality control strain for antibiotic susceptibility test. *Klebsiella pneumoniae* strain ATCC 700603 (bla_{SHV-18}) was used as a quality control strain for confirmatory tests of ESBLs.

ESBLs screening test

All isolates were screened for the presence of ESBLs by selecting the isolates showing reduced susceptibilities to cefotaxime and/or ceftazidime (MIC ≥ 2 µg/ml) (Tofteland et al., 2007). Cefotaxime sodium and ceftazidimepentahydrate (EL Nasr pharmaceuticls Co, Egypt) were used to prepare stock solution (using water for injecttion as a diluent) at a concentration of 200 µg/ml. The test was performed using agar dilution susceptibility testing according to the protocol of CLSI guidelines (CLSI, 2009a).

ESBL phenotypic tests

Double disc diffusion synergy test (DDS)

Test was performed according to a modified version of the Jarlier double-disc synergy method (Tofteland et al., 2007). Cefotaxime, ceftazidime, cefepime and aztreonamdiscs (Oxoid,UK) were placed around an amoxicillin/clavulanic acid disc at a distance of 25 mm (center to center). A keyhole phenomenon was regarded as positive for ESBL production.

Combination Disc test (CD)

Test was carried out using discs of both ceftazidime 30 µg, ceftazidime-clavulanic acid 30/10 µg and cefotaxime 30 µg, cefotaxime-clavulanic acid 30/10 µg. Using a freshly prepared stock solution of clavulanic acid at concentration of 1000 µg/ml. Ten µl of clavulanic acid stock solution (as potassium clavulanate, Sigma Aldrich) was added to ceftazidime (30 µg) and cefotaxime (30 µg) discs within one hour before they were applied to the plates, allowing about 30 min for the clavulanic acid to be absorbed and the discs to be dried enough for application. Discs were used immediately after preparation or discarded. Results were interpreted according to the standards established by the CLSI (2003). An increase in the zone diameter by ≥ 5-mm for either antimicrobial agent tested in combination with clavulanic acid versus its zone when tested alone indicated the presence of an ESBL positive isolate.

Broth microdilution method (BMD)

Test was performed according to CLSI guidelines (CLSI, 2009b). The used antibiotics were cefotaxime and ceftazidime alone and in combination with clavulanic acid. Antibiotic/clavulanic acid was prepared by adding 4 µl of clavulanic acid stock solution (1000 µg/mL) to each antibiotic dilution. A decrease in the minimal inhibitory concentration (MIC) for either cefotaxime or ceftazidime by ≥ 3 two-fold dilutions when tested in combination with clavulanic acid indicated the presence of an ESBL isolate. The MIC was the lowest concentration of drug at which the microorganism tested did not demonstrate visible growth.

Table 1. PCR mixture (25 µl reaction).

Component	Volume (µl)
10× Dream Taq buffer (Fermentas, EU)	2.5
dNTP Mix, 10 mM (Fermentas, EU)	0.5
Forward primer (10 pM)	1.5
Reverse primer (10 pM)	1.5
Dream Taq polymerase (5 u/ µl) (Fermentas, EU)	0.2
Bacterial DNA	2
Nuclease free water	Up to 25

Table 2. Primers used in PCR.

Amplicon	Primers sequence (5'-3')	Product size (bp)
bla TEM	ATG AGT ATT CAA CAT TTC CG CCA ATG CTT AAT CAG TGA GG	858
*bla*SHV	ATG CGT TAT ATT CGC CTG TG AGC GTT GCC AGT GCT CGA TC	859
*bla*CTX-M	SCS ATG TGC AGY ACC AGT AA ACC AGA AYV AGC GGB GC	581

Genotypic identification of ESBLs

Rapid extraction of total genomic DNA was carried by suspending from 1-5 bacterial colonies in 100 µl of nuclease free water. Suspension was heated at 100°C for 10 min. After centrifugation at 9 000 Xg for 30 s, 2 µl of supernatant were used as template in a 25 µl PCR reaction (Nunes et al., 1999).

PCR for bla_{CTX-M}, bla_{TEM} and bla_{SHV}

PCR reactions were carried out in thermalcycler (Techne FTGRAD2D, UK) using amplification protocol consisting of 30 cycles with an initial denaturation at 94°C for 5 min and final extension at 72°C for 4 min. Each cycle consisted of denaturation at 94°C for 1 min, annealing for 1 min at 53°C for bla_{SHV}, 47.9 °C for bla_{TEM}or at 52°C for bla_{CTX-M} and final extension at 72°C for 1 min. *Klebsiella pneumoniae* strain ATCC 700603 (bla_{SHV-18}) was used as a positive control for bla_{SHV} in PCR. PCR reaction mixture is listed in Table 1. Primers used for detection of bla_{CTX-M},bla_{TEM} *and* $bla_{SHV}$with the amplicon size are listed in Table 2 (Tofteland et al., 2007).

Antimicrobial susceptibility pattern of positive ESBLs isolates

The susceptibility pattern of isolates was determined by disc diffusion susceptibility test according to CLSI guidelines (CLSI, 2009a) using the following antibiotic discs (Oxoid, UK): amoxicillin (AML 10 µg), amoxicillin/clavulanic acid (AMC 20 µg/10 µg), piperacillin (PRL 100 µg), cephalexin (CL 30 µg), cefuroxime (CXM 30 µg), cefoxitin (FOX 30 µg), ceftriaxone (CRO 30 µg), cefoperazone (CFP 30 µg), cefotaxime (CTX 30 µg), ceftazidime (CAZ 30 µg), cefepime (FEP 30 µg), aztereonam (ATM 30 µg), imipenem (IPM 10 µg), meropenem (MEM 10 µg), amikacin (AK 30 µg), Doxycycline (DO 30 µg), ciprofloxacin (CF 5 µg), gentamicin (G 10 µg), Cefoperazone/sulbactam (SCF 10 µg/5 µg) and sulphamethoxazole/trimethoprim 19:1(SXT 25 µg).The susceptibility pattern was determined using the CLSI interpretation chart.

Statistical analysis

Sensitivity and specificity of phenotypic confirmatory tests compared to PCR were calculated using VassarStats website (http://www.Vassarstats.net).

RESULTS

In this study, 35 *E. coli* isolates were obtained from 130 bacterial isolates recovered from urine samples of patients with urinary tract infections. All isolates were screened for ESBLs according to CLSI (2009) screening criteria. Eighteen isolates were identified as ESBL by screening test. In order to confirm the presence of ESBLs, three phenotypic confirmatory tests namely: DDS, BMD and CD were performed. DDS, BMD and CD detected 14 (78%), 16 (89%) and 16 (89%) isolates respectively as ESBL- producing *E. coli*. Figures 1 and 2 show representative photos of DDS and CD test.

Genotypic characterization of the isolates showed that all the 16 *E. coli* isolates carried genes coding for ESBLs. All isolates had bla_{CTX-M} gene but not the bla_{SHV} gene, while 62.5% of the isolates carried bla_{TEM} gene(Figures 3-5).

The results of BMD and CD in the current study indicated 100% sensitivity and specificity (95% CI=75% to 100%) compared to PCR. On the other hand, DDS showed sensitivity and specificity of 87.5% (95% CI=60% to 97%) and 100% (95% CI=19.7% to 100%) respectively.

All ESBL isolates were resistant to amoxicillin, cephalexin, cefuroxime, cefotaxime, cefoperazone, ceftriaxone,

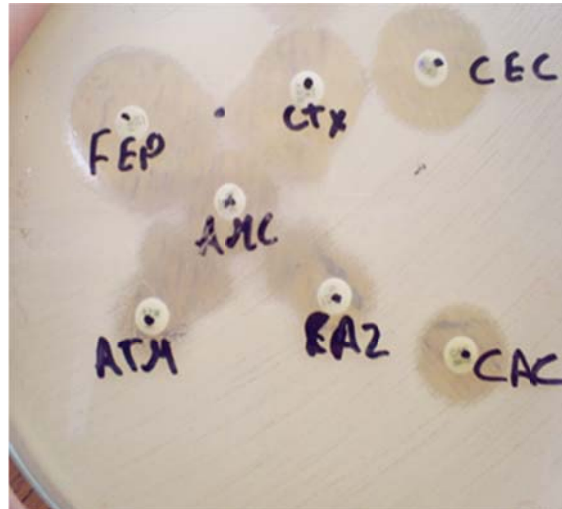

Figure 1. Representative photo of Double Disc Synergy test (DDS). Synergy between cefotaxime (CTX), ceftazidime (CAZ), Aztreonam (ATM) and cefepime (FEP) with amoxicillin/clavulanic acid (AMC) in the center showing keyhole phenomenon

Figure 2. Representative photo of the combination disc test (CD) showing difference in diameters of inhibition zones of cefotaxime (CTX)/ cefotaxime- clavulanic acid (CTX/C) and ceftazidime (CAZ)/ceftazidime-clavulanic acid(CAZ/C).

aztreonam (except 1 isolate was intermediate) and piperacillin. Percentage resistance to ciprofloxacin, cefepime, gentamycin and doxycycline was 62, 81, 56 and 68%, respectively. On the other hand, the isolates showed high susceptibility to imipenem, meropenem, cefoperazone/ sulbactam, and amikacin. Results of the phenotypic and genotypic characterization of the 16 *E. coli* isolates are summarized in (Table 3).

DISCUSSION

In this study, the incidence of ESBL production in *E. coli* isolates causing UTI was investigated. The prevalence of *E. coli* in urine culture was 27%. Among 18 isolates identified as ESBLs by screening test, 16 (89%) were confirmed by 2 phenotypic methods. BMD and CD methods were able to detect more ESBL-producing *E. coli* than DDS. This was in agreement with the previous finding of Tenover et al. (2003) who showed that the BMD method succeeded in detecting some ESBL-producing *E. coli* isolates which other methods failed in detecting. In this study, BMD and CD showed sensitivity and specificity of 100%. This observation was also supported by Taneja and Sharma, (2008) who reported that phenotypic confirmatory tests are highly sensitive and specific compared to genotypic methods in all

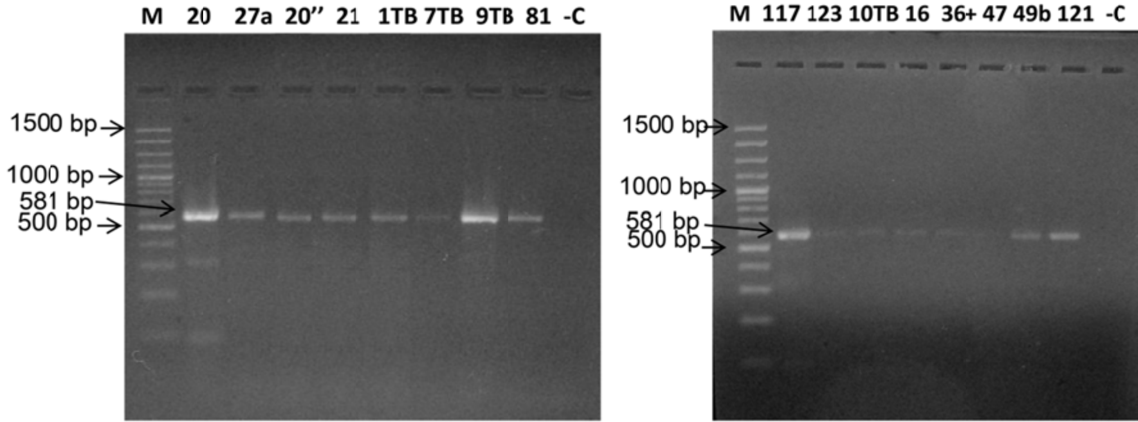

Figure 3. Photograph of agarose gel showing the PCR products of *bla* CTX-M gene of 16 *Escherichia coli* isolates. Lane (M): 100 bp DNA ladder, last lane (-c): negative control.

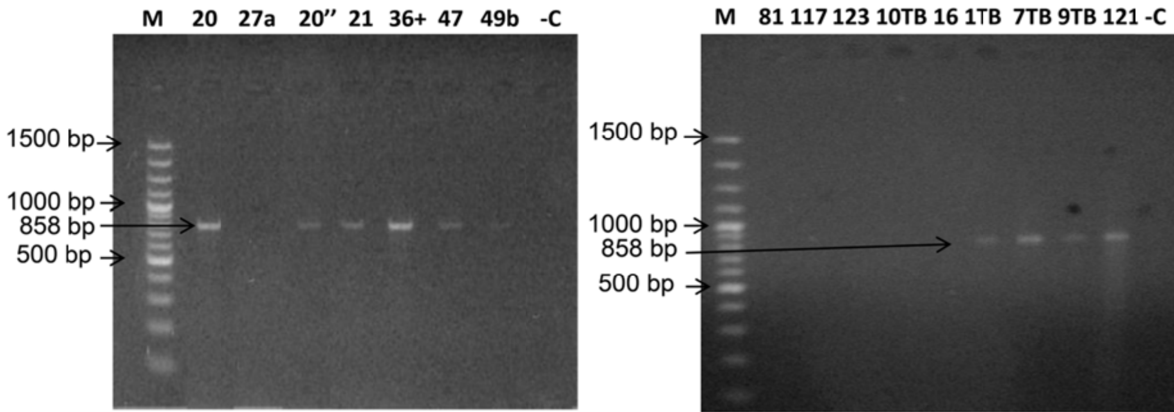

Figure 4. Photograph of agarose gel showing the PCR products of *bla* TEM gene of 16 *Escherichia coli* isolates. lane (M): 100 bp DNA ladder, last lane (-c): negative control.

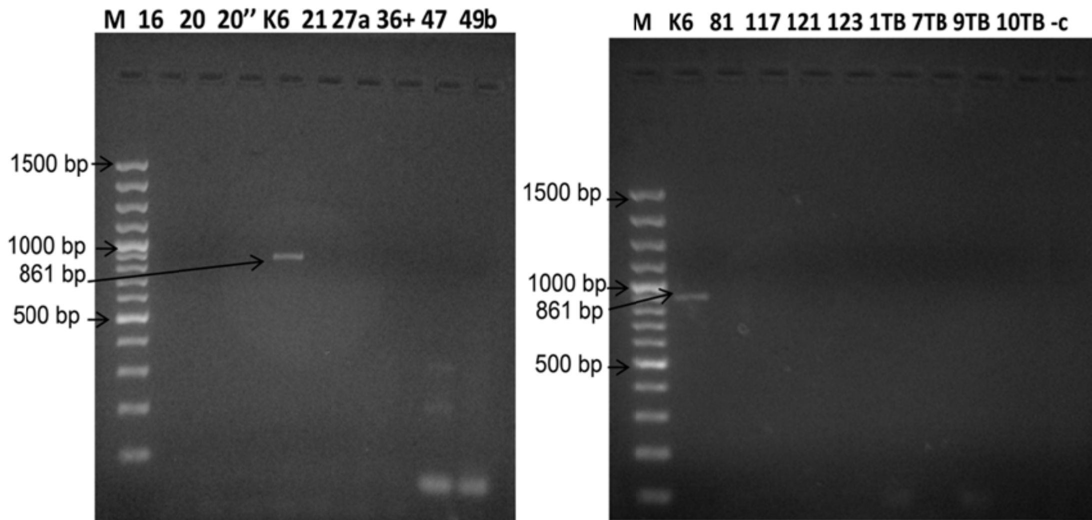

Figure 5. Photograph of agarose gel showing the PCR products of *bla*SHV gene of 16 *Escherichia coli* isolates. lane (M): 100 bp DNA ladder,lane (K6): positive control, last lane (-c): negative control.

Table 3. Phenotypic and genotypic characterization of the 16 *Escherichia coli* isolates.

Isolates number	Antimicrobial susceptibility pattern																				PCR		
	AMC	IPM	CTX	CAZ	FOX	ATM	FEP	CRO	AML	CXM	CL	AK	DO	CF	PRL	G	SXT	SCF	CFP	MEM	CTX-M	TEM	SHV
16	I	S	R	I	S	R	I	R	R	R	R	S	I	R	R	R	R	S	R	S	(+)	(-)	(-)
20	R	S	R	I	S	R	R	R	R	R	R	S	S	R	R	R	I	I	R	S	(+)	(+)	(-)
20"	I	S	R	R	S	R	R	R	R	R	R	S	S	R	R	I	S	I	R	S	(+)	(+)	(-)
21	I	S	R	I	S	R	I	R	R	R	R	S	R	S	R	S	R	S	R	S	(+)	(+)	(-)
27a	R	S	R	I	S	R	R	R	R	R	R	S	R	S	R	R	R	I	R	S	(+)	(-)	(-)
36+	I	S	R	R	S	R	R	R	R	R	R	I	R	R	R	R	R	I	R	S	(+)	(+)	(-)
47	R	S	R	R	S	R	R	R	R	R	R	S	S	R	R	R	R	I	R	S	(+)	(+)	(-)
49b	I	S	R	R	S	R	R	R	R	R	R	S	R	S	R	S	R	I	R	S	(+)	(+)	(-)
81	I	S	R	R	S	R	R	R	R	R	R	I	R	S	R	R	S	S	R	S	(+)	(-)	(-)
117	R	S	R	R	S	R	R	R	R	R	R	S	S	R	R	S	S	S	R	S	(+)	(-)	(-)
121	I	S	R	I	S	R	R	R	R	R	R	S	R	S	R	S	R	I	R	S	(+)	(+)	(-)
123	R	S	R	I	S	R	R	R	R	R	R	I	R	S	R	S	S	S	R	S	(+)	(-)	(-)
1TB	R	S	R	R	I	R	R	R	R	R	R	I	R	R	R	R	R	S	R	S	(+)	(+)	(-)
7TB	R	S	R	R	S	R	R	R	R	R	R	S	R	R	R	R	R	I	R	S	(+)	(+)	(-)
9TB	I	S	R	R	S	I	I	R	R	R	R	S	R	R	R	S	R	I	R	S	(+)	(+)	(-)
10TB	R	S	R	R	S	R	R	R	R	R	R	S	R	R	R	R	R	S	R	S	(+)	(-)	(-)

R (resistant), I (intermediate), S (susceptible), (CTX) cefotaxime, (CAZ) ceftazidime. Amikacin (AK), Amoxicillin (AML), Amoxicillin/clavulanic acid (AMC), Aztreonam (ATM), Cefepime (FEP), Cefoperazone (CFP), Cefoperazone/sulbactam 2:1 (SCF), Cefotaxime (CTX), Cefoxitin (FOX),Ceftazidime (CAZ), Ceftriaxone (CRO), Cefuroxime sodium (CXM), Cephalexin (CL), Ciprofloxacin (CF), Doxycycline (DO), Gentamicin (G), Imipenem (IPM), Meropenem (MEM), Piperacillin (PRL), Sulphamethoxazole/trimethoprim 19:1 (SXT), + (positive), - (negative).

instance except in presence of AmpC-type β –lactamases in addition to ESBLs. In this case, AmpC-type β-lactamases resist inhibition by clavulanate and mask the synergistic effect of clavulanate and cephalosporins against ESBLs (Paterson and Bonoma, 2005). The rate of ESBL-producing *E. coli* as indicated by both phenotypic and genotypic tests was 46%. This rate is not representative of the whole country, and was lower than that observed in a study of UTI in China, where the rate of ESBL-producing *E. coli* was 60.9% during 2011-2012 (Lai et al., 2014). However a rate of 91% was observed for ESBL-producing *E. coli* isolated from urine culture in 11 Spanish hospitals from February 2002 to May 2003 (Rodríguez-Baño et al., 2008).

Genotypic characterization of the isolates revealed that, the prevalence of CTX-M, TEM and SHV genes were 100, 62.5 and 0%, respectively. This finding was in accordance with Rossolini et al. (2008) who reported that, CTX-M enzymes are the most common type of ESBL found in micro-organisms in most areas of the world. The most widely distributed CTX-M enzyme is CTX-M-15 which was detected in Europe, some countries in Asia, Africa, North America, South America and Australia (Pitout, 2010). In addition, genes encoding CTX-Mβ-lactamases have been associated with ISEcp1, ISCR1 insertion elements which are located on conjugative plasmids and which play an important role in the expression and continuous spread of these β-lactamases (Poirel et al., 2003). In contrast to infections caused by TEM- and SHV- derived enzymes, which are limited to nosocomial type, *E. coli*- producing CTX- M enzyme is likely to cause both community- onset and nosocomial infections (Laupland et al., 2008).

The high resistance towards oxyimino-cephalosporins and aztereonam in addition to the lower resistance pattern towards cephalosporins in combination with β-lactamase inhibitors like cefoperazone/sulbactam are consistent with the characteristics of bacteria producing ESBLs (Pitout, 2010). It is worth mentioning that, no ESBL isolate was

resistant to imipenem or meropenem. In this study, all isolates showed multi-drug resistance pattern. In that context, Paterson (2000) reported that, resistance due to ESBLs may not indicate only resistance towards extended spectrum cephalosporins and monobactam antibiotics but also confers resistance towards floroquinolones and aminoglycosides. This cross resistance may be due to localization of the genes coding for ESBLs on plasmids which may have other genes coding resistance towards floroquinolones, aminoglycosides, trimethoprim, sulphonamides, tetracyclines and/or chloramphenicol.

Correlating the antimicrobial susceptibility pattern of the isolates with their genotypic characters revealed that there is no precise association between the type of ESBL and susceptibility to different antibiotics, since the presence of both CTX-M and TEM enzymes did not confer increased resistance to β - lactam antibiotics. These results could be justified by the findings of Spanu et al. (2002) who reported that, susceptibility is multifactorial, depending on ESBL, substrate specificity, production of additional β-lactamases and changes of the outer membrane permeability.

Conclusion

Out of 35 *E. coli* isolates from urine samples of patients with urinary tract infections, 16 were identified as ESBLs by both phenotypic and genotypic methods. Identified ESBLs were either of CTX-M and TEM types or CTX-M alone. All isolates were multi drug resistant. No difference in antibiotic susceptibility pattern was observed between isolates carrying either CTX-M alone or a combination of CTX-M and TEM genes.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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