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Original research

Detection of genes TEM, OXA, SHV and CTX-M in 73 clinical isolates of *Escherichia coli* producers of extended spectrum Beta-lactamases and determination of their susceptibility to antibiotics.

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Abstract

A total of 73 clinical isolates of extended spectrum β- lactamase producing *Escherichia coli* were sampled in North Lebanon. The ESBL resistance was screened using disc diffusion method, while the resistance genes were detected by polymerase chain reaction (PCR). The results marked the high prevalence of gene CTX-M (72 strains), whereas 33 strains carried the gene OXA, 16 strains carried the gene TEM and 3 carried the SHV gene. The majority of the strains carried two or more genes. This study points out the high rate of transmission of these genes among *Escherichia coli* strains in North–Lebanon and shows a reduced sensitivity to quinolones (pipemidic acid(12.1%); ofloxacin (21.9%) and ciprofloxacin (23.9%), in addition, all tested strains were highly susceptible to tigecycline (100%), fosfomycin (98.6%) and imipenem (97.2%).

Key words: ESBLs genes, *E. coli*, antibiotic susceptibility.

Introduction

The *Enterobacteriaceae* is a source of serious threatening diseases worldwide, with the occurrence of *Escherichia coli* (*E. coli*) secondary to Gram positive cocci (1). Beta-lactam antibiotics such as long spectrum cephalosporins and carbapenems are the preferred treatment of enterobacterial infections (1). The genes of extended spectrum beta-lactamases (ESBLs) are encoded by transferable plasmids (2); thus enabling these bacteria to acquire ESBL by different resistance mechanisms. In recent years, the emergence of β-lactamases worldwide, increased the challenge of infections caused by bacteria species carried these enzymes (2). Moreover, the excessive and unregulated use of antibiotics are the fundamental cause in the selection of resistance mechanisms (1). The *Enterobacteriaceae* producers of ESBLs have become a serious problem of public health worldwide since 1995, because of the increased emergence of new variants; especially CTX-M) (3,4). In fact, the majority of the enterobacterial infections caused by ESBL producing organisms are *E. coli* and *K. pneumoniae* strains carrying the CTX-M gene responsible for community infections, especially the urinary tract infections (1, 5).

The aim of this study is to evaluate the susceptibility of the ESBL producing E. coli strains to commonly used antibiotics and to detect the presence of the four common ESBL genes: TEM, OXA, CTX-M and SHV using PCR method.

Material and Methods

Bacterial Strains. A total of 73 clinical strains were isolated between the years 2007 and 2009 in the Microbiology department of the Nini laboratory hospital in Tripoli, North Lebanon. Of these strains, 58 were isolated from urine, 3 from blood, 4 from bronchial fluid, 6 from deep wounds and 2 from peritoneal fluid. The strains were identified by the API 20E system (bioMérieux® France). A strain of *E. coli* CMUL 028 sensitive to all antibiotics (Collection Microbiologie Université Libanaise) was used as control.

The other positive controls used in PCR, were *E.coli* strains obtained from the Collection d'Institut Pasteur: The *CIP 103983* strain is TEM-4 positive, the *CIP 105836* is SHV-1 positive and the *CIP 103982* strain is CTX-M positive. *E.coli* strain « Imp 15 » was used as positive control for the gene OXA-1 (**Table 1**).

TABLE 1. Primers sequences and PCR cycles performed for each tested gene (8).

Primer	Primer sequences	PCR cycles
TEM-1/F TEM-1/R	ATGAGTATTCAACATTTCCG CTGACAGTTACCAATGCTTA	1 cycle of 5 minutes (min) at 96 °C; 35 cycles of [1min at 96oC, 1min at 58 °C, 1min at 72 °C]; 1 cycle of 10min at 72 °C.
SHV-1/F SHV-1/R	GGTTATGCGTTATATTCGCC TTAGCGTTGCCAGTGCTC	1 cycle of 5min at 96 °C; 35 cycles of [1min at 96 °C, 1min at 60 °C, 1min at 72 °C; 1 cycle of 10min at 72 °C
OXA-1/F OXA-1/R	ACACAATACATATCAACTTCGC AGTGTGTTTAGAATGGTGATC	1 cycle 5min at 96 °C; 35 cycles of [1min at 96 °C, 1min at 60oC, 2min at 72 °C]; 1 cycle of 10 min at 72 °C
CTX-MU1 CTX-MU2	ATGTGCAGYACCAGTAARGT TGGGTRAARTARGTSACCAGA	1 cycle of 7 min at 94oC; 35 cycles of [50seconds (sec) at 94 °C, 40sec at 50 °C, 1min at 72 °C]; 1 cycle of 5min at 72 °C
CTX-M1-A2 CTX-M1- B2	CTT CCA GAA TAA GGA ATC CCG TTT CCG CTA TTA CAA	1 cycle of 10min at 94 °C, 30 cycles of [1min at 94 °C, 1min at 48 °C, 1 min at 72 °C], 1 cycle of 7min at 72 °C.

R and Y are variable nucleotides. R: Purine (adenine or guanine). Y: Pyrimidine (thymine or cytosine) (http://www.chem.qmul.ac.uk/iubmb/misc/naseq.html)

Antibiotics Susceptibility

The susceptibility of strains to antibiotics was performed using the disc diffusion method on Müller-Hinton agar following the recommendations of the Comité Antibiogramme, Société Française de Microbiologie (CA-SFM) (www.sfm-microbiologie.org).

The following antibiotic discs were used:

Ampicillin (AMP) (10µg); ticarcillin (TIC) (75 µg); piperacillin (PP) (75µg); amoxicillin – clavulanic acid (AUG) (20/10µg); piperacillin-tazobactam (TZP) (75/10µg); cephalexin (CFL) (30µg); cefuroxime (CXM) (30µg); cefoxitin (FOX) (30µg); cefixime (FIX) (30µg); cefotaxime (CTX) (30µg); ceftazidime (CAZ) (30µg); cefepime (FEP) (30µg); imipenem (IMI) (10µg); tigecycline (TGC) (15µg) ; aztreonam (AZM) (30µg); trimethoprim - sulfamethoxazole (STX) (1,25/23,75µg); colistine (Cs) (50µg); ticarcillin - clavulanique acid (TCC) (75/10µg); gentamicin (GEN) (15µg); tobramicin (TM) (10µg); amikacin (AMK) (30µg); netilmicin (NET) (30µg); nitrofurantoin (FT) (300µg); pipemidic acid (PIP) (20µg); pefloxacin (PEF) (5µg); ciprofloxacin (CIP) (5µg); fosfomycin (FOS) (50 µg) (BioRad®, France).

The ESBL presence was screened using the disc diffusion method on Müller-Hinton agar. The synergy between the clavulanic acid (Beta-lactamases inhibitor) and a third generation cephalosporin: cefotaxime, ceftazidime, cefepime, and aztreonam, was detected following the recommendations of CA-SFM (www.sfm-microbiologie.org). The amoxicillin-clavulanic acid disc was placed in the center of the plate at a distance of 25-30 mm between the cephalosporins and the amoxicillin-clavulanic acid. The clavulanic acid inhibits the production of ESBL by *E. coli* thus forcing sensitivity to cephalosporins upon viewing visible expansion of the sensitivity zone by the side of clavulanic acid disc, forming a mushroom shape confirms the test.

The ESBL genes detection

The detection of gene sequences coding for the TEM, OXA, SHV and CTX-M-type enzymes was performed by using a commercial Plasmid Miniprep kit (GenElute™) (Sigma-Aldrich), following the protocol delivered by the manufacturer. PCR was applied using the ready Mix Kit (REDTaq® ReadyMix™ PCR reaction Mix with $MgCl_2$), Sigma - Aldrich, and the primers used to amplify the above mentioned genes are listed in table I. Sixteen strains show none of these 4 genes. The Isoelectric focusing of these 16 strains was performed with a polyacrylamide gel containing ampholines with a pH range of 3.5 to 10.0, using the Multiphor II flat - bed (Pharmacia Biotech, Uppsala, Sweden); 2 hours of migration at 3000 V, 150 mA, 15W)(6). TEM-1 (pI 5.4), OXA-1 (pI 7.5), CTX-M-14 (PI 7.9) and CTX-M-15 (pI 8.6) were used as standards. Molecular screening of bla_{CTX-M} was performed using the primer of CTX-M1 as shown in the **Table 1.**

Results

The results shown in the **Table 2** revealed a predominance of the CTX-M gene among the strains of *E. coli* isolated in North Lebanon (32 strains). The majority of the *E. coli* strains carried two or more ESBL genes, and only one strain carried all 4 gene types.

TABLE 2. CTX-M Genes distribution among 73 tested strains.

Gene type	No. of strains
CTX-M	32
OXA-1	1
CTX-M + TEM-1	6
CTX-M + OXA-1	22
CTX-M + SHV	2
CTX-M + OXA + TEM	9
CTX-M + OXA + TEM-1 + SHV	1

The number of strains carrying the CTX-M gene was 72 strains. The number of strains carrying OXA-1 was 33 strains. The number of strains carrying only the CTX-M gene (32 strains) and only the OXA-1 gene (1 strain) are shown above.

The isoelectric focusing experiments pointed that 16 strains produced β-lactamases of pI 8.6 in addition to their natural respective β-lactamases. The PCR-based analysis of β-lactamase-encoding genes revealed that all these 16 strains harbored the CTX-M1 gene.

Table 3 shows the results of susceptibility to antibiotics for the 73 tested *E. coli* ESBLs producers. The results shows a low susceptibility of these strains to quinolones (pipemidic acid: 12.5%) and fluoroquinolones (ofloxacin;25% and ciprofloxacin;28%). Two of the isolates were resistant to imipenem; the first one carried 3 resistance genes (OXA, TEM & CTX-M) and the second one carried the CTX-M gene alone.

TABLE 3. Antibiotics Susceptibility of the 73 tested strains.

Antibiotic	% Susceptibility
AMP	0
TIC	0
PP	0
AUG	23.3
TZP	82.2
CFL	0
CXM	0
FOX	82.2
FIX	0
CTX	0
CAZ	0
FEP	0
IMI	97.2
TGC	100
ATM	0
SXT	19.2
CS	100
TCC	23.19
GEN	35.2
TM	23.3
AMK	81.94
NET	60.9
FT	89.7
PIP	12.06
PEF/OF	21.9
CIP	23.9
FOS	98.6

ampicillin (AMP), ticarcillin (TIC), piperacillin (PP), amoxicillin – clavulanic acid (AUG), piperacillin - tazobactam (TZP), cephalexin (CFL), cefuroxime (CXM), cefoxitin (FOX), cefixime (FIX), cefotaxime (CTX), ceftazidime (CAZ), cefepime (FEP), imipenem (IMI), tigecycline (TGC), aztreonam (AZM), trimethoprim - sulfamethoxazole (STX), colistine (Cs), ticarcillin - clavulanic acid (TCC), gentamicin (GEN), tobramicin (TM), amikacin (AMK), netilmicin (NET), nitrofurantoin (FT), pipemidic acid (PIP), pefloxacin (PEF), ciprofloxacin (CIP), fosfomycin (FOS).

The susceptibility rates of these strains to the following antibiotics were: amoxicillin - clavulanic acid: 23.3%; ticarcillin - clavulanic acid: 23.19%; imipenem 97.2%; tazobactam - piperacillin 82.2%; cefoxitin 82.2%; trimethoprim - sulfamethoxazole 19.2%; amikacin 81.94%; gentamicin 35.2%; tobramycin 23.3%; netilmicin 60.9% ; tigecycline 100%; fosfomycin 98.6%; and nitrofurantoin 89.7%.

Discussion

The results of the study confirm the high prevalence of CTX-M gene, while the prevalence of other SHV genes was very low (Table 2). Recently, the ESBL distribution in Europe showed a dramatic increase of CTX-M gene instead of TEM and SHV genes (7). The ESBL production is much less frequent in Europe than in Latin America and Asia, and they are even less frequent in the Pacific than in North America (7).

Recent European studies on *Enterobacteriaceae* have also confirmed the persistence of strains producing TEM and SHV, and the increasing prevalence of strains producing CTX-M (2). Other types of ESBL such as PER, GES, IBC and some OXA types are existing. These genes are mostly detected in Pseudomonas aeruginosa and in Acinetobacter spp.(9). The prevalence of ESBL productions revealed a significant geographical differences, ranging from 0% (Iceland) to less than 1% (Estonia) to 41% for *E. coli* (Romania) and 91% (Romania) for K. pneumonia (7).

In the past several years, the emergence of new variants of ESBL producers, especially CTX-M has suggested the involvement of the co-resistance to other drug classes during endemic condition. This co-resistance is due to the transmission of different types of resistance genes within the same clone. Several studies showed that blaCTX-M genes are commonly found on large plasmids that often carry other genes conferring resistance to other antimicrobial agents including aminoglycosides, fluoroquinolones, chloramphenicols, tetracyclins and others (particularly, blaOXA-1, blaTEM-1, tetA, aac(6')-Ibcr) (9-11). This may explain the high rate of transmission of CTX-M gene among the *E. coli* strains by acquiring R-plasmid, and often the high prevalence of the CTX-M resistance gene is combined with another resistance genes in these strains.

The CTX-M gene predominates in Europe, while in other countries, the ESBL genes are more diverse (2,9). In the United Kingdom, a recent dramatic increase of the ESBL producing strains was observed both in hospitals and in the community, and this increase is attributed to CTX-M-15 (7). In Norway and Portugal, the CTX-M is the ESBL enzyme most frequently found in *E. coli* (12-13). In Italy, the prevalence of *E. coli* producers of ESBL has also increased with a predominance of TEM (45.4%), SHV-12 and the emergence of CTX-M and PER (14). Few of these studies reported the type of ESBL produced by these strains and some showed the presence of TEM, OXA, SHV and the dramatic emergence of CTX-M (10,15,16,17).

The major prevalence of ESBLs was attributed first to K. pneumonia strains which have produced TEM and SHV, and then followed by the emergence of *E. coli* strains producers of CTX-M which become the prevalent type (10,17).

In Turkey, a study conducted by Ozgumus et al. 2007, showed that 15% of *Enterobacteriaceae* isolates are producers of ESBLs. The 2 commonly TEM and SHV genes were detected. Of these strains, 5 were carrying the gene TEM, 12 were carrying the two genes TEM and SHV and 3 were negative for both of the 2 genes (18). Also, Kiratisin et al.2008 in Taïland, have studied 235 strains of *E. coli* producers of ESBLs, and found that 87.3 % of the strains were carriers of the gene CTX-M, 77% were carrying TEM gene, and 3.8% were carriers of the gene SHV. In addition, few strain produced VEB-1 and OXA-10, and non of the isolate produced of PER and GES (19).

The general susceptibility pattern of ESBL producer strains to antibiotics has decreased in recent years in many countries (15,17,20). However, the present study showed high susceptibility patterns to the most effective antibiotics used against *E. coli* producers of ESBLs. In addition, *E.coli* producer of ESBLs have mostly a reduced susceptibility to the trimethoprim - sulfamethoxazole (19.2%), whereas other studies conducted in Bahrain and in Khartoum have shown different results (21,22,23). But on the other site, this study revealed an alarming decrease in the susceptibility of our isolates to the quinolones and the fluoroquinolones (table 3). Similar results are found in a study previously conducted in Lebanon (15), whereas other studies carried out in the Middle East areas have shown higher susceptibility rates of their isolates to fluoroquinolones (23, 24).

It is important to note that 23.3% of our isolates were susceptible to the clavulanic acid associated with the amoxicillin or ticarcillin, whereas this percentage increased significantly in the case of tazobactam associated with the piperacillin (82.2%). Our results show a similarity with a study conducted by Oliver et al. 2002 (25). The susceptibility of our isolates to cefoxitin was 82.2%, which is similar to the results of study performed in Iran by Mehrgan et al. 2008 (24).

With regards to the aminoglycosides, our results showed that amikacin remains the most active drug (81.4%). Kader et al. 2005, have shown similar results in their study conducted in Saudi Arabia (27). The susceptibility of gentamicin and tobramycin was low (35.2%, 23.3%) respectively, in comparison to study carried out in recently in Sudan which has reported higher susceptibility to aminoglycosides (23).

Among the 73 strains of *E. coli* producers of ESBLs, 58 (79.5%) were isolated from urine; which indicates the highly occurrence of these strains in urinary tract infections. A previous study from the Calgary Health Region in Canada, demonstrated that CTX-M - producing *E. coli* is emerging as an important cause of community- onset urinary tract infections (28,29).

It should be noted that the percentage of *E. coli* resistance to ampicillin, aminoglycosides, tetracycline, chloramphenicol and sulphonamides are lower in the industrialized countries than in the developing countries (11), since the overuse and the uncontrolled use of antibiotics and the low standard of personal and community hygiene are favoring the spread of resistant strains between humans in the developing countries.

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This study has also demonstrated that 58/73 (79.5%) of *E. coli* strains which produced ESBLs, were isolated from urine; which indicates the highly occurrence of these strains in urinary tract infections. A previous study from the Calgary Health Region in Canada, demonstrated that CTX-M-producing *E. coli* is emerging as an important cause of community-onset urinary tract infections (27). Pitout, JD et al. 2008, have documented that extended-spectrum ß-lactamase producing *Enterobacteriaceae* are becoming an emerging public-health concern (28).

It should be noted that the percentage of *E. coli* resistance to ampicillin, aminoglycosides, tetracycline, chloramphenicol and sulphonamides is lower in the industrialized countries than in the developing countries (11), since the overuse and the uncontrolled use of antibiotics and the low standard of personal and community hygiene are favoring the spread of resistant strains between humans in the developing countries.

Conclusion

This study confirms the large dissemination of the gene CTX-M among *E. coli* in the North Lebanon, and the results of antibiotic susceptibility revealed a high rates of resistance against the quinolones and the fluoroquinolones which are widely used in treatment of the urinary tract infections in Lebanon. These results should draw the attention of the Lebanese medical authorities to the serious consequences of increasing antimicrobial resistance, therefore, it is import to increase efforts to monitor and control the spread of antimicrobial resistant strains in hospitals and community.

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