British Microbiology Research Journal
16(3): 1-7, 2016, Article no.BMRJ.27892
ISSN: 2231-0886, NLM ID: 101608140

SCIENCEDOMAIN *international*
www.sciencedomain.org

Extended Spectrum β-lactamases in Clinical Isolates of *Escherichia coli* and *Enterobacter cloacae* Collected from Nablus District - Palestine

Motasem Al-Masri[1*], Nael Abu-Hasan[1] and Maha M. Jouhari[2]

[1]Department of Biology and Biotechnology, An-Najah National University, Nablus, West Bank, Palestine.
[2]Rafidia Hospital, Clinical Laboratory, Nablus, West Bank, Palestine.

Authors' contributions

This work was carried out in collaboration between all authors. Authors MAM and NAH made study design, samples' collection, experiments, statistical analysis as well as writing the manuscript. Author MMJ participated in samples' collection. All authors read and approved the final manuscript

Article Information

DOI: 10.9734/BMRJ/2016/27892
Editor(s):
(1) Laleh Naraghi, Plant Disease Research Department, Iranian Research Institute of Plant Protection, Tehran, Iran.
(2) Birke Bartosch, Laboratoire de Physiopathologie Moléculaire et Nouveaux Traitements des Hépatites Virales, INSERM U871, Bio-optisis 27, Chemin des Peupliers, 69570 Dardilly, France.
Reviewers:
(1) Theocharis Koufakis, University of Thessaly, Larissa, Greece.
(2) Muhammad Yusha'u, Bayero University Kano, Nigeria.
Complete Peer review History: http://www.sciencedomain.org/review-history/15775

Short Research Article

Received 23rd June 2016
Accepted 5th August 2016
Published 13th August 2016

ABSTRACT

Aim: To determine the prevalence of extended-spectrum β-lactamases (ESBLs) among clinical isolates of *Escherichia coli* (*E. coli*) and *Enterobacter cloacae* (*E. cloacae*) in Nablus district.
Methodology: In this prospective study carried out at An-Najah National University, a total of 161 bacterial isolates were collected during a12-month period in Nablus district in Palestine. To detect ESBLs, the isolates were examined by combination disc method. PCR was used to detect *bla*CTX-M, *bla*TEM and *bla*SHV genes in 32 representative ESBL-producer *E. coli* isolates.
Results: Using combination disc method, ESBL enzymes were detected in 73 out 153 (47.7%) *E. coli* and in 1 out of 8 (12.5%) *E. cloacae* isolates. No significant association of ESBL-producer *E. coli* was observed with types of collected specimens, gender, hospital ward, outpatient, or medical source. Among 32 representative *E. coli* ESBL-positive, *bla*CTX-M, *bla*TEM and *bla*SHV genes were detected in 30 (93.8%), 2 (6.3%) and 1 (3.1%), respectively. Two new variants of ESBLs (PALTEM137b and PALSHV-2a') were identified. A unique *E. cloacae* isolate expressing inducible

**Corresponding author: E-mail: motasemm@najah.edu;*

class C B-lactamase was also detected.
Conclusions: In Nablus region, high frequencies of ESBLs were found among *E. coli* bacteria isolated from outpatients and inpatients. *bla*CTX-M is the predominant gene among ESBL producers. New variants of ESBLs were found.

Keywords: E. coli; blaCTX-M; blaSHV; blaTEM; ESBL.

1. INTRODUCTION

Increased prevalence of extended-spectrum β-lactamases (ESBLs) among *Enterobacteriaceae* caused a dramatic increase in drug resistance worldwide [1]. ESBL enzymes have the ability to hydrolyze β-lactam antibiotics containing an oxyimino group (third generation cephalosporins and aztreonam) and are inhibited by β-lactamase inhibitors such as clavulanic acid and tazobactam [2]. ESBLs are found predominantly in Klebsiella species and *Escherichia coli* (*E. coli*), but have been described in other species as well, including Enterobacter, Serratia, Citrobacter, Proteus and Salmonella [2,3]. There are several types of ESBLs, including *bla*TEM, *bla*SHV, and *bla*CTX-M [2,3,4]. *bla*SHV and *bla*TEM types were reported to be evolved from parent enzymes (e.g., *bla*TEM-1, -2 and *bla*SHV-1) due to point mutations around the active site of the β-lactamases. *bla*CTX-M type seems to be mobilized from environmental bacteria [2,5,6].

Despite the high prevalence of ESBL-producing bacteria in many parts of the world, little information is known in our region. Hence, it becomes necessary to assess the status of ESBLs-mediated drug resistance in clinical isolates of *E. coli* and *Enterobacter cloacae* (*E. cloacae*).

2. METHODOLOGY

2.1 Bacterial Isolates

Bacterial isolates primarily identified to be *E. coli* or *E. cloacae* were obtained from three clinical settings: Rafidia Hospital, Nablus Specialty Hospital and New Technology Laboratory (private laboratory). The isolates were collected from September 2011 to October 2012. The isolates were recovered from different types of clinical specimens and only one positive culture per patient was included. Data regarding gender, age, admitting hospital, ward, date of admission, specimen's collection date and specimen type was collected in a specially designed questionnaire. Hospital associated infection was defined as occurrence of infection 48 hours or more after hospital admission.

Identification of bacterial isolates was confirmed by colony morphology on MacConkey and biochemical tests including: triple sugar iron agar reaction, motility, indole, citrate and urea. In addition, chromogenic reaction on Uriselect media (BIO-RAD, France) was also used.

2.2 Phenotypic Detection of ESBLs

2.2.1 ESBL screening using combination disc method

All of 161 collected isolates were examined for ESBL production by combination disc method as described by [7]. Briefly, antibiotic disks containing a combination of cephalosporin plus clavulanic acid were used in conjunction with a corresponding cephalosporin disk alone. The following antibiotic disks were used: ceftazidime (CAZ 30 µg), ceftazidime plus clavulanic acid (CAZ/CA 30/10 µg), cefotaxime (CTX 30 µg) and cefotaxime plus clavulanic acid (CTX/CA 30/10 µg). Interpretation of the results was carried out according to Clinical and Laboratory Standards Institute (CLSI) guidelines [7] and ≥ 5 mm increase in the diameter of the zone of inhibition for the CAZ/CLA- and/or CTX/CLA-containing disk(s) versus the corresponding CAZ or CTX disk was considered positive for ESBL.

2.2.2 ESBL detection using double disc synergy test

To confirm the results of combination disc method and/or determine which method of ESBL detection was more sensitive, all collected *E. cloacae* (8 isolates) and 106 randomly-selected *E. coli* isolates were examined by double disk-approximation method for detection of ESBL production as described by Coudron et al. [8]. Ceftazadime (30 µg) and cefotaxime (30 µg) disks were placed close (20 mm) to an amoxicillin-clavulanic (20/10 µg) disk on a Mueller-Hinton agar plate inoculated with the tested organism. Enhancement of the zone of

inhibition or the so-called ghost zone between clavulanate containing disk and ceftazadime and/or cefotaxime disks indicated the presence of an ESBL. *E. coli* ATCC 25922 bacterial strain was used as negative control of ESBL production in both phenotypic tests.

All antibiotic's disks were obtained from OXOID (UK).

2.3 Detection of Resistant Genes by PCR

Randomly-selected 32 representative isolates of *E. coli* possessing ESBLs were examined by PCR for the detection of *bla*TEM, *bla*SHV and *bla*CTX-M genes. The DNA sequences of the primers and their annealing temperatures were obtained from [9,10,11]. DNA templates were prepared by a simple boiling method. Ready Mix™ (Sigma-Aldrich, USA) was used. Forward and reverse primers (10 pmol each) were added to 50 µl of the reaction mixture. PCR conditions were optimized as follows: 5 min at 94°C, 35 cycles (1 min at 94°C, 1 min at primer-specific annealing temperature, and 1 min at 72°C), and 7 min at 72°C. The primer-specific annealing temperature of *bla*CTX-M, *bla*TEM, and *bla*SHV primers were 60, 55, and 68°C, respectively. The resulting PCR products were analyzed in a 1.5% agarose gel. To confirm identification of *bla*TEM and *bla*SHV types of ESBLs, their PCR products were sequenced. DNA sequencing was made using forward and reverse primers. Sequencing was carried out by the center of genetics at Bethlehem University-Palestine. Each sequence of *bla*TEM and *bla*SHV genes was identified by comparing it with known ESBL sequences available in the GenBank databases by multiple sequence alignment. Programs available at National Center for Biotechnology Information (NCBI) were used for multiple sequence alignment.

2.4 Statistical Analysis

Frequencies of ESBL producers in different variables were analyzed using SPSS 14.0 (SPSS Inc, Chicago, IL, USA). Fisher's exact and Chi-square tests were used. A *P*-value < 0.05 was considered statistically significant.

3. RESULTS

During a 12-month period, a total of 153 *E. coli* and 8 *E. cloacae* isolates were obtained by culturing specimens from 161 patients (103 females and 58 males), as shown in Table 1. The patients' age ranged from 1 day to 90 years (mean = 23.5±24 years). *E. coli* isolates of female origin were represented by 64%. The majority of *E. coli* bacterial isolates (120 isolates) were recovered from urine samples (Table 2). The remainder were from wound swaps (20 isolates), blood (4), vaginal swabs (4), rectal swabs (2), ear swab (1), perianal swab (1) and pleural fluid (1). On the other hand, *E. cloacae* was isolated from urine (4 isolates), wound swabs (3) and nasal swab (1) (Table 2). *E. coli* infections seem to be more common in pediatric (19%) and emergency (7.8%) wards.

Combination disc method detected ESBL production in 73 out of 153 (47.7%) *E. coli* isolates. A total of 106 out of the 153 *E. coli* isolates were also examined by double disk-approximation method, which detected ESBL

Table 1. Distribution of ESBL-producing clinical isolates collected from different sources

Variable	*Escherichia coli*		*Enterobacter cloacae*	
	No. isolates	**ESBL +ve No. (%)**	**No. isolates**	**ESBL +ve No. (%)**
Source[a]				
New Technology Lab.	27	14 (51.9)	2	0
Nablus Specialty Hospital	17	6(35.3)	0	0
Rafidia Hospital	109	53(48.6)	6	1(16.7)
Gender[a]				
Females	98	48(49)	5	1(20)
Males	55	25(45.5)	3	0
Inpatient[a]	74	33 (44.6)	4	1(25)
Outpatient	79	40(50.6)	4	0

[a]New Technology Lab. versus (vs.) Nablus specialty hospital, P= 0.283;
New Technology Lab. vs. Rafidia Hospital, P = 0.764; Nablus specialty hospital vs. Rafidia Hospital, P =0.306
Females vs. males, P = 0,675; In patients vs. outpatients, P = 0.455

Table 2. Distribution of ESBL-producing bacterial isolates among different clinical specimens

Specimen[a]	*Escherichia coli*		*Enterobacter cloacae*	
	No. isolates (%)	ESBL +ve No.	No. isolates (%)	ESBL +ve No.
Urine	120	60(50)	4	1(25)
Wound swab	20	7(35)	3	0
Blood	4	2(50)	0	0
Vaginal swab	4	1(25)	0	0
Rectal swab	2	2(100)	0	0
Ear swab	1	1(100)	0	0
Perianal swab	1	0	0	0
Pleural fluid	1	0	0	0
Nasal swab	0	0	1	0

[a]*Statistical analysis of specimens with isolates' number ≥ 2: urine versus (vs.) wound swab, P= 0.214; urine vs. blood, P = 0.691; urine vs. vaginal swab, P =0.322; urine vs. rectal swab, P = 0.256; wound swab vs. blood, P = 0.486; wound swab vs. vaginal swab, P = 0.593; wound swab vs. rectal swab, P = 0.156; blood vs. vaginal swab, P = 0.500 ; blood vs. rectal swab, P = 0.400; vaginal swab vs. rectal swab, P = 0.200*

production in 28 (26.4%) isolates (combination disc method detected 43 cases). Only 1 out the 8 (12.5%) *E. cloacae* isolates was an ESBL-producer detected by both ESBL phenotypic detection methods.

DNA from randomly-selected representative *E. coli* ESBL producers (32 isolates) was subjected to PCR using *bla*TEM, *bla*SHV and *bla*CTX-M specific primers. All of the 32 isolates possessing ESBL enzymes detected by combination disc method showed 1 or 2 amplified genes. Among the 32 isolates, *bla*CTX-M gene was the most common, as it was detected in 30 (93.8%) isolates. On the other hand, *bla*TEM and *bla*SHV genes (confirmed by nucleotide sequence) were detected in 2 (6.3%) and 1 (3.1%) isolates, respectively. The *bla*CTX-M gene was detected in association with *bla*TEM gene in 1 (3.1%) isolate.

Sequence alignments of the amplified *bla*TEM fragments (839 and 856 nucleotides) showed that the sequence of one isolate was identical to that of *bla*TEM-104 gene (GenBank No. AF516719), while the sequence of another isolate was similar to *bla*TEM-137 gene (GenBank No. AM286274.1). The differences were 4 silent mutations at nucleotides 18 (C→T), 138 (G→A), 709 (A→G) and 710 (G→A). The sequence of this isolate (PALTEM137b) was registered in GenBank database under accession number KP686109. In another isolate, the sequence alignment of the amplified *bla*SHV fragment (894 nucleotides) was similar to *bla*SHV-2a gene sequence (GenBank No. X98102.1). The differences were two silent mutations at nucleotides 475 (A→G) and 793 (G→C). DNA sequence of the isolate of the present study (PALSHV-2a') have been submitted to GenBank under the accession number KP686110.

Tables 1 and 2 show the distribution of ESBL producers in different clinical settings, patients and specimens. Percentages of 51.9%, 48.6% and 35.3% of ESBL *E. coli* producers were found among isolates collected from New Technology Laboratory, Rafeida Hospital and Nablus Specialty Hospital, respectively. Variations in frequency were statistically insignificant. The frequency of ESBL producers among *E. coli* isolated form urine was 50%, blood 50%, vaginal swab 25% and wound swabs 35%. In addition, all the *E. coli* isolates recovered from rectal (2 isolates) and ear (1 isolate) swabs were ESBL producers. Comparison of frequencies of ESBL producer *E. coli* isolated from different specimens showed no significant differences.

The frequency of ESBL producers among pediatric (≤ 14 years old) patients (50%) was insignificantly higher (P=0.649) than that among adults (45.8%). Among wards with clinical specimens' number ≥ 5, the highest percentage of isolation of ESBL-producing *E. coli* was in the gynecology ward (66.7%), followed by neonates (60%), emergency (58.8%), ICU (50%), pediatric (44.8%), and urology (36.4%). Variations in frequencies were not statistically significant. The frequency of ESBL producer isolates in outpatients (50.6%) was slightly higher than that found among inpatients (44.6%), however, these variations were statistically insignificant (P = 0.455).

A unique *E. cloacae* isolate expressing amoxicillin-induced resistance was found. On disc approximation test, a blunting in the inhibition zone around ceftazidime at amoxicillin-clavulanic edge was observed. Replacement of amoxicillin-clavulanic acid with amoxicillin gave the same result. In addition, amoxicillin was able to induce resistance to cefuroxime, aztereonam, ceftriaxone and cefotaxime. Furthermore, cefotaxime and cefotaxime-clavulanic disks showed similar results, indicating clavulanic acid had no effect.

4. DISCUSSION

In the present study, combination disc method detected ESBL production in 47.7% of *E. coli* isolates. A previous study [12] carried out during 2009-2010 in Jordan had reported a close prevalence of ESBL production in *E. coli* (50.3%). A slightly lower ESBL prevalence of 41% was reported among inpatients in the United Arab Emirates [13]. On the other hand, a lower prevalence (13.51%) of ESBL producers was found among urinary *E. coli* isolates collected in Nepal during 2011-2012 [14]. Regional variations in prevalence of ESBL-producing *E. coli* are expected to occur and time factor should be taken into consideration. Out of the 153 *E. coli* isolates, 106 were examines by double disk-approximation method, which detected ESBL production in 28 (26.4%) of tested isolates (combination disc method was positive in 43 isolates). These results showed sensitivity limitation of the double disk-approximation method.

Among the examined isolates, *bla*CTX-M gene was the most common (93.8%). In line with our results, CTX-M β-lactamase was common in most areas of the world [15,16]. In a study in Greece, the prevalence of *bla*CTX-M enzyme was 87% among ESBL producers in a tertiary care hospital [17].

In the current study, sequence alignments of the amplified *bla*TEM and *bla*SHV fragments showed very close sequences to previously described ESBLs' DNA sequences with few silent mutations. Detection of silent mutation and not other types of mutations (e.g., missense mutations) may be explained by the fact that conformation of any ESBL enzyme is very important for its activity and consequently, the survival of bacteria under the selective pressure of B-lactams antibiotics. Occurrence of silent mutations will not harm the bacteria, as they do not affect the final conformation of the enzyme, as other types of mutations do.

No significant association of ESBL producer *E. coli* was observed with any of the various types of collected specimens. Slightly higher frequency of ESBL producer isolates was found among outpatients (50.6%) compared to inpatients (44.6%). Such findings show the widespread nature of ESBLs among *E. coli* bacteria isolated in our region.

The use of intravenous devices, urinary catheters, ventilators, higher selective pressure of antibiotics, and others in inpatients increase the probability of nosocomial infections by ESBL producers [18]. Among inpatient cases, 11 (in Rafidia hospital: 2 in ICU, 4 in Pediatric, 3 in Urology, and 2 in Neonates ward) were suspected to acquire nosocomial infections. All cases were ceftazidime resistant and 8 of these isolates were confirmed ESBL producers. These findings support the theory that nosocomial infections are risk factors that contribute to the acquisition of ESBL-producing microorganisms. On the basis of ESBLs' genotypes, hospital ward, and resistance profiles, the 8 patients appeared to have acquired the nosocomial infection from different sources.

In a study in Mexico, 31 (35.6%) *E. cloacae* isolates were identified as ESBL producers out of 87 isolates [19]. In the current study, only one (12.5%) *E. cloacae* isolates was an ESBL producer detected by both ESBL phenotypic detection methods. However, a unique *E. cloacae* strain expressing amoxicillin-induced resistance was found, where amoxicillin induced resistance in this strain to ceftazidime, cefuroxime, aztereonam, ceftriaxone and cefotaxime. Most likely, this isolate possessed inducible AmpC B-lactamase [20,21]. This unique strain can be induced to express resistance to commonly-used antibiotics in Nablus region. The situation may be further complicated by the risk of conversion of inducible to constitutive mode, which means resistance to a wide range of cephalosporins.

5. CONCLUSION

A relatively high ESBL-producing *E. coli* isolation rate was found in Nablus district. CTX-M appears to be the most common genotype among ESBL producers. We report a unique *E. cloacae* isolate that expressed amoxicillin-inducible AmpC B-lactamase. There is an urgent need to adopt

appropriate control measures to reduce the prevalence of ESBL and inducible AmpC B-lactamase.

ETHICAL APPROVAL

The study protocol was approved by deans of An-Najah National University, Palestinian ministry of health, and directors of participating clinical settings. In addition, patients or their parents accepted to include their bacterial cultures in the current research.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Coque TM, Baquero F, Canton R. Increasing prevalence of ESBL producing *Enterobacteriaceae* in Europe. Euro. Surveill. 2008;13:19-29.
2. Bradford P. Extended-spectrum β-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. Clin. Microbiol. Rev. 2001;14:933-51.
3. Minarini LA, Gales AC, Palazzo IC, Darini AL. Prevalence of community-occurring extended spectrum beta-lactamase-producing Enterobacteriaceae in Brazil. Curr. Microbiol. 2007;54(5):335-41.
4. Cantón R, Coque T. The CTX-M beta-lactamase pandemic. Curr. Opin. Microbiol. 2006;9(5):466-75.
5. Paterson D, Bonomo R. Extended-spectrum B-lactamases: a clinical update. Clin. Microbiol. Rev. 2005;18:657–86.
6. Hawkey P, Jones A. The changing epidemiology of resistance. J. Antimicrob. Chemother. 2009;64(Suppl 1):i3–10.
7. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fourth Informational Supplement. M100-S24 2014.
8. Coudron Ph, Moland E, Sanders C. Occurrence and detection of extended-spectrum b-lactamases in members of the family *Enterobacteriaceae* at a veterans medical center: Seek and you may find. J. Clin. Micro. 1997;7:2593–2597.
9. Arpin C, Dubois V, Coulange L, André C, Fischer I, Noury P, et al. Extended-spectrum ß-lactamase-producing *Enterobacteriaceae* in community and private health care centers. Antimicrob. Agents Chemother. 2003;47:3506-14.
10. De Gheldre Y, Avesani V, Berhin C, Delméé M, Glupczynsi Y. Evaluation of Oxoid combination discs for detection of extended-spectrum ß-lactamases. J. Antimicrob. Chemother. 2003;52:591-7.
11. Saladin M, Cao VT, Lambert T, Herrmann J, Donay J, Ould-Hocine Z, et al. Diversity of CTX-M ß-lactamases and their promoter regions from *Enterobacteriaceae* isolated in three Parisian hospitals. FEMS Microbiology Letters. 2009;161-8.
12. Nimri L, AzaizehA B. First report of multidrug-resistant ESBL producing urinary *Escherichia coli* in Jordan. Brit. Microb. Res. J. 2012;2(2):71-81.
13. Al-Zarouni M, Senok A, Rashid F, Al-Jesmi S, Panigrahi D. Prevalence and antimicrobial susceptibility pattern of extended-spectrum beta-lactamase-producing *Enterobacteriaceae* in the United Arab Emirates. Med. Princ. Pract. 2008;17:32-36.
14. Chander A, Shrestha CD. Prevalence of extended spectrum beta lactamase producing Escherichia coli and *Klebsiella pneumoniae* urinary isolates in a tertiary care hospital in Kathmandu, Nepal. BMC Research Notes. 2013;6:487.
15. Rossolini GM, D'Andrea MM, Mugnaioli C. The spread of CTX-M-type extended spectrum β-lactamases. Clin. Microbiol. Infect. 2008;14:33–41.
16. Zhao WH, Hu Z. Epidemiology and genetics of CTX-M extended-spectrum β-lactamases in Gram-negative bacteria. Critical Reviews in Microbiology. 2013;39(1):79–101
17. Pournaras S, Ikonomidis A, Kristo I, Tsakris A, Maniatis AN. CTX-M enzymes are the most common extended-spectrum β-lactamases among *Escherichia coli* in a tertiary Greek hospital. J. Antimicrob. Chemother. 2008;54: 574-5.
18. Goyal AKN, Prasad P, Gupta S, Ghoshal U, Ayyagari A. Extended spectrum β-lactamases in *Escherichia coli* & *Klebsiella pneumoniae* & associated risk factors. Indian. J. Med. Res. 2009;129:695-700.
19. Garza-Gonza´ lez E, Ibarra SIM, Llaca-Dı´az JM, Gonzalez GM. Molecular

characterization and antimicrobial susceptibility of extended-spectrum b-lactamase producing Enterobacteriaceae isolates at a tertiary care centre in Monterrey, Mexico. J. Med. Micro. 2011;60:84–90.

20. Stapleton P, Shannon K, Phillips I. The ability of b-lactam antibiotics to select mutants with derepressed beta-lactamase synthesis from *Citrobacter freundii*. J. Antimicrob. Chemother. 1995;36:483–496.

21. Thean TY, Yong Ng NLS, He J, Koh TH, Hsu LY. Evaluation of screening methods to detect plasmid-mediated AmpC in *Escherichia coli*, *Klebsiella pneumoniae*, and *Proteus mirabilis*. Antimicrob. Chemother. 2009;53:146-149.

Peer-review history:
The peer review history for this paper can be accessed here:
http://sciencedomain.org/review-history/15775