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Co-occurrence of Extended-Spectrum Beta-Lactamases in Isolated *Enterobacter* spp. From Patients Specimens

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Abstract

Background: Clinical significance of Extended-Spectrum Beta-Lactamases (ESBLs) production in *Enterobacter* spp. has not well been established.
Objectives: This study was conducted to investigate the prevalence of ESBLs produced by *Enterobacter* spp. in clinical isolates.
Materials and Methods: This descriptive cross-sectional study was performed during May 2010 to April 2012 in the city of Sanandaj, Kurdistan province, Iran. We did not include and directly contact the patient population, yet had access to two thousand patient specimens (urine, wound, respiratory tube, blood, cerebrospinal fluid and stool), which were collected from patients that had referred to various departments of two government hospitals of Toohid and Besat. As a result, 118 *Enterobacter* spp. isolates were identified and considered. The Clinical and laboratory standard institute (CLSI) Combined Disk Test (CDT) and polymerase chain reaction (PCR) were applied for detecting *Enterobacter* spp. Data were analyzed using the SPSS 11.5 software, Chi-square (x^2) test and a Kappa coefficient (κ) ($P < 0.05$).
Results: Out of 118 *Enterobacter* spp. isolates, 31.36% were *Enterobacter aerogenes* (*E. aerogenes*), 20.34% *Enterobacter agglomerans* (*E. agglomerans*), 12.71% *Enterobacter cloacae* (E. cloacae), and 33.90% were other *Enterobacter* spp. All 118 (100%) *Enterobacter* isolates produced ESBLs. In the detection of ESBLs, CDT and PCR results were similar to each other and all 118 *Enterobacter* spp. were ESBLs producers ($\kappa = 1$).
Conclusions: According to the results, most of the *Enterobacter* spp. produced ESBLs and were Cefotaxime-M (CTX-M) enzyme carriers. Guidelines and appropriate use of antibiotics are necessary to avoid the production of ESBLs.

Keywords: Extended-Spectrum Beta-Lactamases, Clinical Specimens, *Enterobacter* spp

1. Background

Enterobacter spp. such as *Salmonella* spp., *Yersinia* spp., *Escherichia coli* (*E. coli*), *Enterobacter agglomerans* (*E. agglomerans*), *Enterobacter cloacae* (*E. cloacae*), and *Klebsiella* spp. are members of the large family of *Enterobacteriaceae* (1). These organisms may cause a wide spectrum of healthcare-associated infections (HAIs), including urinary tract infections (UTIs), as well as wounds, lungs, abdominal cavity, surgical sites and soft tissues infections (2). Some strains of these bacteria can be extremely resistant against treatment with antibiotics, because *Enterobacter* spp. can produce extended-spectrum beta-lactamase (ESBLs) enzymes (3). These enzymes make bacteria resistant to most beta-lactam antibiotics, such as penicillins, cephalosporins, monobactam and aztreonam (4). The most commonly encountered ESBLs are derived from the Sulfhydryl variable (SHV), Temoneira (TEM), cefotaxime-M (CTX-M) and Oxacillinase (OXA) enzyme families (4-6). Many researchers have shown the production of ESBLs by *Enterobacter* spp. in specimens taken from patients. Ramazanzadeh et al. in Iran used the phenotypic test and polymerase chain reaction (PCR) and reported that 96 Gram-negative bacilli including *E. coli*, *Enterobacter* spp., *Klebsiella* spp., *Pseudomonas aeruginosa* (*P.aeruginosa*), *Salmonella* spp. etc. were isolated from blood cultures and among those 96 isolates, 20.83% produced ESBLs (4). In another study, Aminzadeh et al. in Iran demonstrated that from 292 gram-negative species that were isolated at a microbiology laboratory from patients' specimens, 41.8% of the isolates were detected as ESBLs-producers by the disk diffusion method (7). Ferreira et al. in Brazil used the E-test and PCR based on 16S rRNA protocol, and reported that during July 2007 to August 2008, 17

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gram-negative bacteria including *E. coli*, *Serratia* spp. and *B. cepacia* were isolated from patient specimens, and some of the isolates carried bla_{TEM}, $bla_{CTX\text{-}M}$, bla_{OXA}, bla_{SHV} genes and were resistant to tetracycline, ciprofloxacin, cefoxitin, chloramphenicol, amikacin and cefepime (8).

2. Objectives

In view of the fact that ESBLs increase the rates of treatment failure and death, and given the importance of the above-mentioned materials, the aim of this study was the detection of ESBLs produced by *Enterobacter* spp. from specimens of patients using the combined disk test (CDT) and PCR.

3. Materials and Methods

3.1. Study Population and Specimen Types

This descriptive cross-sectional study was conducted at the faculty of medicine, Kurdistan University of Medical sciences, Sanandaj, Iran (Kurdistan province) during May 2010 to April 2012. In this censuses, we did not include and did not directly contact the patient population, but patient specimens that were collected previously were studied, thus in this study, we had no inclusion and exclusion criteria, sample size formula, expected power, sampling frame and missing value, because all samples that were taken from all patients who had been admitted to the hospital over these years were interred. Overall, two hundred patient specimens including urine (800 samples), wound (200 samples), respiratory tube (125 samples), blood (520 samples), cerebrospinal fluid (100 samples) and stool (255 samples) were collected from patients who were referred to different parts of Toohid and Besat hospitals. Samples were collected from different wards as follows: 345 samples from emergency, 450 samples from intensive care unit (ICU), 200 samples from internal, 125 samples from neonatal, 150 samples from orthopedic, 100 samples from pediatric, 220 samples from surgery, 100 samples from various wards' surfaces, and 310 samples from the outpatients. Toohid and Besat are general government referral hospitals, one with 18 sections and 475 beds, and the other with 22 sections and 400 beds, respectively. This study was approved by the review boards of the ethics committee of the Kurdistan University of Medical Sciences (ethics code: MUK.REC.1394.371-1).

3.2. Microbiological Methods and Enterobacter spp. Detection

In this study, consecutive non-duplicate isolates of *Enterobacter* spp. were detected. For *Enterobacter* detection at the genus level, all samples were routinely cultured on MacConkey and Eosin-methylene blue (EMB) agar (Merck, Germany) plates and were then placed in an incubator at 37°C for 24 hours. The Indole, Methyl Red (MR), Voges-Proskauer (VP) and Simmons' Citrate (IMViC) tests (Merck, Germany) were applied to identify the coliform group (4). Subsequently, isolates were identified using standard biochemical and microbiological tests such as Gram-stain, oxidase test and catalase test. In addition, in order to assess the movement of bacteria, samples were incubated in Sulfur-Indole-Motility (SIM) agar (Merck, Germany), a semi-solid motility test medium. To detect *Enterobacter* at the species level, lysine, arginine and ornithine decarboxylase tests were applied (9).

3.3. Detection of Extended-Spectrum Beta-Lactamases Production Using the Combined Disk Test

Extended-Spectrum Beta-Lactamases production was detected using CDT. The presence of ESBLs was assayed using the following antibiotic disks (MAST, UK): cefotaxime (30 μg), cefotaxime/clavulanic acid (30/10 μg), ceftazidime (30 μg), and ceftazidime/clavulanic acid (30/10 μg). Also, the *E. coli* ATCC 25922 strain (Pasteur Institute, Iran) was used as the positive control (4, 10, 11). At first, a 0.5-McFarland turbidity standard was prepared using the bacteria, and then it was cultured on Mueller-Hinton agar (MHA) (Merck, Germany). Subsequently, antibiotic disks were placed on MHA at distance intervals of 20 mm, and then the mediums were incubated at 37°C for 24 hours. The production of ESBLs by the strain was confirmed, if the inhibition zone diameter of each antibiotic combined with clavulanic acid was $\geq$ 5 mm larger than that of antibiotic alone. This test was performed for all *Enterobacter* isolates resistant to at least two of the applied antibiotics. In addition, the effects of inactive agents against the resistant bacteria in combination with each other were determined in this test (12).

3.4. Polymerase Chain Reaction Assay

The template DNA was prepared according to the following process: a cell pellet from 1.5 mL of overnight culture on MHA was resuspended in 500 μL of Tris-EDTA (Ethylene Diamine Tetraacetic Acid) (TE) (10 mM Tris, 1 mM EDTA, pH 8.0). After centrifugation (4.000 rpm for five minutes) and boiling for 10 minutes (at 100°C), the solution was centrifuged again (4.000 rpm for five minutes). Next, the obtained supernatant was used for PCR. The SHV, TEM, CTX and OXA-1 and OXA-2 primers (Cinna Clon, Iran) were used during this step. The PCR reaction was performed according to the manufacturer's instructions (CinnaGene, Iran). In this study, *E. coli* strains of ATCC 25922 and ATCC 35218 were used as positive and negative controls, respectively (Pasteur Institute, Iran) (Table 1) (4).

Table 1. Primers and Conditions of the Polymerase Chain Reaction (4)

Primer	PCR Primers (5 to 3)	Expecial Size, bp	PCR Conditions	PCR Product
SHV		928	94°c, 5 min, 35 cycles of 94°c, 1 min, 58°C, 1 min, 72°C, 1 min	SHV-1,-2,-5,-7,-11,-12,-18,-26,-32,-33,-38,-44,-46,-49
SHV-F	GGGTTATTCTTATTTGTCGC			
SHV-R	TTAGCGTTGCCAGTGCTC			
TEM		1080	94°C, 5 min, 35 cycles of 94°C, 1 min, 58°C, 1 min, 72°C, 1 min	TEM-1,-52,-71,-104,-105,-138,-151,-152
TEM-F	ATAAAATTCTTGAAGACGAAA			
TEM-R	GACAGTTACCAATGCTTAATCA			
CTX		759	94°C, 5 min, 35 cycles of 94°C, 45 s, 58°C, 45 s, 72°C, 1 min	CTX-M-1,-3,-12,-15,-22,-30,-32,-33,-38,-52,-57,-58,-60,-61
CTX-M-F	ACGCTGTTGTTAGGAAGTG			
CTX-M-R	TTGAGGCTGGGTGAAGT			
OXA-1		813	94°C, 5 min, 35 cycles of 94°C, 1 min, 58°C, 1 min, 72°C, 1 min	OXA-1,–4,-30,-31,-47
OXA-1-F	ACACAATACATATCAACTTCGC			
OXA-1-R	AGTGTGTTTAGAATGGTGGTGATC			
OXA-2		814	94°C, 5 min, 35 cycles of 94°C, 45 s, 61°C, 45 s, 72°C, 1 min	OXA-2,-3,-15,-21,-32
OXA-2-F	TTCAAGCCAAAGGCACGATAG			
OXA-2-R	TCCGAGTTGACTGCCGGGTTC			

3.5. Statistical Analysis

Data were entered into the SPSS 11.5 software program (SPSS Inc., Chicago, IL) and analyzed using the Chi-square (x^2) test. Also Kappa coefficient and validity assessment were performed. All differences, in which the probability of the null hypothesis was $P < 0.050$, were considered significant.

4. Results

4.1. Microbiological Methods and Enterobacter spp. Detection Results

During the two years of study, we detected 118 *Enterobacter* isolates in 2000 clinical specimens obtained from the two mentioned hospitals. *Enterobacter* spp. were found in the following hospital wards: one in emergency, 50 in the ICU, nine in internal, two in neonatal, one in orthopedic, 22 in pediatric, seven in surgery, t in different wards' surfaces and 24 in outpatients. In addition, the 118 *Enterobacter* spp. isolates composed of 37 *Enterobacter aerogenes* (*E. aerogenes*), 24 *Enterobacter agglomerans* (*E. agglomerans*), 17 *Enterobacter cloacae* (*E. cloacae*) and 40 other *Enterobacter* spp. (Table 2).

4.2. Combined Disk Test Results

During the study period, CDT phenotypic test showed that all 118 (100%) *Enterobacter* isolates were ESBLs producers.

4.3. Polymerase Chain Reaction Assay Results

All the 118 ESBLs-positive isolates that were detected by the CDT were also ESBLs-positive by the PCR. In most cases, ESBLs were detected simultaneously in *Enterobacter* spp. isolates. In addition, the following compounds were detected simultaneously in different numbers of isolates as follows: CTX-M was detected in 33 isolates, CTX-M, OXA- in three, TEM, SHV and CTX-M in 15, TEM, SHV, CTX-M and OXA-1 in nine, TEM, SHV, CTX-M, OXA-1 and OXA-2 in one, and TEM, SHV, CTX-M and OXA-2 in 14 isolates (Table 3).

4.4. Statistical Analysis Results

There was no statistically significant difference between both PCR and CDT methods ($P > 0.050$). In addition, it can be observed that when comparing CDT with PCR, the Kappa coefficient between both methods, indicated a good level of agreement ($\kappa = 1$).

Table 2. Frequency Percentages (N; Number of Bacteria) of Isolated *Enterobacter* spp. Based on Wards

Wards	*Enterobacter* spp. Isolates				
	E. aerogenes	*E. agglomerans*	*E. cloacae*	Other *Enterobacter* spp.	Total
Emergency	0.00 (0)	0.00 (0)	0.00 (0)	0.85 (1)	0.85 (1)
ICU	15.25(18)	6.78 (8)	6.78 (10)	11.86 (14)	42.37 (50)
Internal	0.85 (1)	0.85 (1)	0.00 (0)	5.93 (7)	7.63 (9)
Neonatal	0.85 (1)	0.85 (1)	0.00 (0)	0.00 (0)	1.69 (2)
Orthopedic	0.85 (1)	0.00 (0)	0.00 (0)	0.00 (0)	0.85 (1)
Pediatric	6.78 (8)	4.24 (5)	0.85 (1)	6.78 (8)	18.64 (22)
Surgery	0.85 (1)	0.85 (1)	0.00 (0)	4.24 (5)	5.93 (7)
Wards Surface	0.85 (1)	0.00 (0)	0.85 (1)	0.00 (0)	1.69 (2)
Outpatients	5.08 (6)	6.78 (8)	4.24 (5)	4.24 (5)	20.34 (24)
Total	31.36 (37)	20.34 (24)	14.40 (17)	33.90 (40)	100 (118)

Table 3. Frequency Percentages (N; ESBLs Enzymes) in Isolated *Enterobacter* spp. Using the Polymerase Chain Reaction

ESBLs Type	*Enterobacter* spp. Isolates				
	E. aerogenes	*E. agglomerans*	*E. cloacae*	*Enterobacter* Spp.	Total
CTX-M	24.32 (9)	0.00 (0)	52.94 (9)	37.50 (15)	15.38 (33)
CTX-M,OXA-1[a]	8.10 (3)	0.00 (0)	0.00 (0)	0.00 (0)	15.38 (3)
TEM,SHV,CTX-M[a]	40.54 (15)	0.00 (0)	0.00 (0)	0.00 (0)	38.46 (15)
TEM,SHV,CTX-M,OXA-1[a]	0.00 (0)	37.50 (9)	0.00 (0)	0.00 (0)	7.69 (9)
TEM,SHV,CTX-M,OXA-1,OXA-2[a]	2.70 (1)	0.00 (0)	0.00 (0)	0.00 (0)	15.38 (1)
TEM,SHV,CTX-M,OXA-2[a]	0.00 (0)	37.50 (9)	0.00 (0)	12.50 (5)	7.69 (14)
Total	75.67 (28)	75 (18)	52.94 (9)	50 (20)	100 (75)

[a]Detected simultaneously in *Enterobacter* spp. Isolates.

5. Discussion

Members of the *Enterobacteriaceae* family and most of the *Enterobacter* species may obtain resistance to expanded-spectrum cephalosporins by producing chromosomal ß-lactamases and plasmid-mediated ESBLs genes. Extended-Spectrum Beta-Lactamases had this ability to hydrolyze all three generations of cephalosporins and aztreonam, but were inhibited by clavulanic acid (5, 13). The results of our study showed that 118 *Enterobacter* isolates including *E. aerogenes* (31.36%), *E. agglomerans* (20.34%), *E. cloacae* (14.40%) and other *Enterobacter* spp. (33.90%) were isolated from 2000 clinical specimens from different wards of the two mentioned hospitals; the number of isolates obtained from the ICU was more than other sections. Manzur et al. in Spain using microbiological methods identified seven patients in the CT (Cardiothoracic)-ICU with ESBLs-producing *E. cloacae* during July to September 2005. Also in Manzur's study, PCR showed that two isolates of *E. cloacae* carried SHV, and three carried two ESBLs, SHV and CTX-M-9, simultaneously (5). *Enterobacter cloacae* in our study solely carried CTX-M (52.94%) and 6.78% of this strain was isolated from the ICU. The ESBL-producing isolates' outbreak in hospitals is related to different risk factors such as use of antimicrobials and indwelling catheters (urinary catheters and tracheal tubes) and person-to-person transmission, and since these genes are located on plasmids, they can easily spread in hospital environments. Different studies have reported the need for more precaution in use of antibiotics and the contorting rate of antibiotic resistance in ICU and neonatal care units (14-16). Varkey et al. in India reported that out of 361 isolated bacteria from blood samples, including *E. coli*, *K. pneumoniae* and *E. cloacae*, 250 isolates were found to be ESBLs-producing organisms. The TEM type ESBL producers were found in 75% of *E. coli*, 67% of *K. pneumonia* and 89% of *E. cloacae*; the SHV gene was

confirmed in 66% of E. coli, 55% of *Klebsiella pneumoniae* (*K. pneumonia*) and 18% of *E. cloacae*. Also, 71% of CTX-M genes were carried by *E. coli*, 85% by *K. pneumoniae* and 3% by *E. cloacae* (15). Our study focused on detection of *Enterobacter* spp. in urine, wound, respiratory tube, cerebrospinal fluid, stool and blood samples. All *Enterobacter* spp. that were detected in these samples, were positive for ESBLs. Blood systems are immensely important for human health and need special consideration. The ESBL-producing *Enterobacteriaceae* families are associated with high mortality, especially in the blood systems and can exceed 50% in some study populations (17). In a study conducted by Markovska et al. in Bulgaria, 42 ESBLs-producing isolates of *E. aerogenes*, *E. cloacae*, *E. agglomerans*, and *Serratia marcescens* (*S. marcescens*), were collected from a University Hospital in Varna, Bulgaria. Polymerase chain reaction and sequencing showed that most enzyme types were CTX-M-3 (64%). The CTX-M-3 was detected in *E. aerogenes* (100%) and *S. marcescens* (83%). SHV-12, CTX-M-3 and CTX-M-15 were found among *E. cloacae* isolates with frequency of 50%, 35% and 45%, respectively (18). Also in our research, the most commonly found type of enzyme was CTX-M, and 33 (15.38%) of the isolates had only CTX-M type of enzyme. On the other hand, ESBLs in most of the cases were detected simultaneously in all isolates. The following compounds were found at certain percentages of the isolates: TEM, SHV and CTX-M at 38.46%, CTX-M and OXA-1 at 15.38%, TEM, SHV, CTX-M, OXA-1 and OXA-2 at 15.38%, TEM, SHV, CTX-M and OXA-1 at 7.69%, and TEM, SHV, CTX-M and OXA-2 at 7.69%. Studies have shown that most of the ESBLs are mutants of the TEM and the SHV enzymes, but CTX-M type beta-lactamases have become more important and also CTX-M genes are the predominant ESBLs genes (19). Poulou et al. from Greece examined 162 genotypically confirmed ESBLs-positive *Enterobacteriaceae* isolates with PCR and sequencing analyses. In Poulou's study, standard CLSI ESBLs-confirmatory test showed that 106 of the 162 ESBLs-producers were positive for ESBLs (sensitivity, 65.4%) and had false-positive results for four of the 139 non-ESBLs producers (specificity, 97.1%). The modified CLSI ESBLs-confirmatory test detected 158 of 162 isolates to be ESBLs producers (sensitivity, 97.5%) and showed no false-positive results for non-ESBLs producers (specificity, 100%) (20). In a study from Iran, Hosain Zadegan et al. using double disk synergy method reported that 23 (23.6%) of 225 total isolated gram-negative bacilli were ESBLs positive (21). In our study, all *Enterobacter* isolates that were ESBLs positive in the double-disk synergy (DDS) test were also positive in the PCR. Plasmids with ESBLs genes are located on carry genes related to antimicrobial resistance. This can limit the chemotherapeutic options for ESBLs-producing pathogens and facilitate the inter- and intra-species dissemination of ESBLs. Therefore, phenotypic detection of ESBLs among *Enterobacteriaceae* species is important for epidemiological purposes as well as for limiting the spread of resistance mechanisms (20). The CLSI recommends phenotypic confirmatory tests such as CDT and double-disk synergy test (DDST) for detecting the production of ESBLs in *Enterobacteriaceae* (22). These tests are easy to perform and low cost and they accurately detect ESBLs-producing *Enterobacteriaceae*. However, phenotypic tests are not able to distinguish between ESBLs enzymes such as SHV, TEM and CTX-M types. Molecular assays such as PCR are fast, have specificity and accuracy, and provide accurate results for identifying and distinguishing ESBLs genes (22-24). In our study, strong points included two-year survey on patients, large number of patient specimens, valid diagnostic criteria and ESBLs detection, accordance with the CLSI, easy implementation and interpretation of CDT and PCR. On the other hand, possible contaminations in the laboratory that may have caused false results, and lack of access to patient records were the weaknesses and limitations of this study. Overall, *Enterobacter* species are one of the causes of nosocomial infections and can spread in hospitals, especially in wards like the ICU that need more consideration. Detection of ESBLs-producing *Enterobacter* strains in a clinical laboratory is important and policies of antibiotic treatment in hospitals and communities should be in ways that stop the development of ESBLs-producing strains.

5.1. Conclusions

The results of our study demonstrated that relatively large numbers of hospital samples were contaminated with *Enterobacter* spp. and the majority of these isolates were CTX-M carriers. Since the frequency of ESBLs-producing strains among clinical isolates is increasing, this matter needs more epidemiological studies to report the details of nosocomial spread and community-acquired infections of these bacteria. Also powerful infection control programs should be designed and put into action to prevent the dissemination of these resistant isolates throughout the hospitals.

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Footnotes

Authors' Contribution: Rashid Ramazanzadeh supervised the study, participated in designing and conducting it, and in the preparation of the manuscript. Hasan Hosainzadegan, Samaneh Rouhi and Pegah Shakib collected the data and helped with the writing and editing of the manuscript. Bijan Nouri was the statistician for this study.

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