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Short Communication

Clonal relation and antimicrobial resistance pattern of extended-spectrum β-lactamase- and AmpC β-lactamase-producing *Enterobacter* spp. isolated from different clinical samples in Tehran, Iran

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Abstract

Introduction: Here, we determined the genes encoding antibiotic resistance enzymes and virulence factors and evaluated the genetic relationship between *Enterobacter* spp. isolated from different clinical samples. **Methods:** A total of 57 clinical isolates of *Enterobacter* spp. were tested for the production of extended-spectrum β-lactamases (ESBLs), carbapenemase, and AmpC using phenotypic and genotypic methods. **Results:** The most common ESBLs and AmpC β-lactamases were bla_{TEM} (63.3%) and bla_{EBC} (57.7%), respectively. The most prevalent virulence gene was *rpos* (87.7%). The random amplified polymorphic DNA (RAPD) patterns of strains were genetically unrelated. **Conclusions:** RAPD polymerase chain reaction analysis revealed high genetic diversity among isolates.

Keywords: *Enterobacter*. ESBL. AmpC. RAPD-PCR.

Enterobacter species may cause severe nosocomial infections, including bloodstream, respiratory tract, and central nervous system infections as well as endocarditis[1,2]. Nosocomial infections caused by these microorganisms have been associated with high rates of mortality and morbidity[1]. *Enterobacter cloacae* and *Enterobacter aerogenes* are the most common species isolated from clinical samples[3]. Several virulence genes are involved in the pathogenesis of these microorganisms[4-7]. Curli fimbria, encoded by *csgBAC*, is an important factor for cell adhesion, aggregation, and biofilm formation in many enterobacteria[4]. In addition, RpoS regulation is known to play an important role in multiple stress conditions such as acid, heat, and oxidative stress, starvation, high osmolarity, and near UV exposure[5]. Another important virulence factor is the type III secretion system encoded by *FliI* that delivers a variety of effectors directly into the cytosol of host as well as aerobactin, encoded by the *iutA*, described as a virulence factor related to iron acquisition from host-binding proteins[6,7]. β-lactam antibiotics, especially third-generation cephalosporins and carbapenems, are used to treat infections cause by several species of *Enterobacter*[1-3]. β-lactamase enzymes, including extended-spectrum β-lactamases (ESBLs) and AmpC, are involved in the mechanism underlying resistance to β-lactam antibiotics in *Enterobacter* spp[1-3]. ESBLs are often encoded by genes located on large plasmids that also carry genes for resistance to other antimicrobial agents such as aminoglycosides and fluoroquinolones[1]. ESBLs are capable of hydrolyzing penicillins, broad-spectrum cephalosporins, and aztreonam, but may not hydrolyze cephamycin, and are inhibited by clavulanic acid. AmpC β-lactamases are usually encoded on the bacterial chromosome and in some cases on the bacterial plasmid (plasmid-mediated AmpC)[3]. In Iran, ESBL production was recently reported in 44.28% of *E. cloacae* isolates[1]. Despite the high incidence of *Enterobacter* spp. infection among Iranian patients, very little is known about the antibiotic resistance pattern, virulence factors, and molecular characteristics of *Enterobacter* spp. isolates. In the current study, the genes encoding antibiotic resistance enzymes and virulence factors were determined and the genetic relationship between *Enterobacter* spp. isolated from different clinical samples was evaluated.

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Bacterial isolates

A total of 57 isolates of *Enterobacter* spp. were obtained from different patients admitted to three teaching hospitals of the Tehran University of Medical Sciences between September 2013 and April 2014. The isolates were collected from various clinical samples, including urine, wounds, tracheal aspirate, and blood. No duplicate isolates from the same patient and no environmental strains were included in this study. All isolates of *Enterobacter* spp. were identified by standard biochemical tests[8].

Susceptibility testing

Antibiotic-containing discs (Mast, UK) were used to determine the susceptibility of *Enterobacter* spp. using the disc diffusion method, as per the Clinical and Laboratory Standards Institute (CLSI) guidelines[9]. The antimicrobial agents used were as follows: aztreonam-amikacin (30µg), amoxicillin-clavulanic acid (20/10µg), cefpodoxime (10µg), cefotaxime (30µg), ceftazidime (30µg), imipenem (10µg), cefepime (30µg), gatifloxacin (5 mg), cefoxitin (30µg), gentamicin (30µg), ciprofloxacin (30µg), levofloxacin (5µg), ertapenem (10µg), and meropenem (10µg). Isolates that showed resistance to at least three classes of antibiotics were defined as multi-drug resistant (MDR) strains[1]. ESBL-producing strains were detected using the combined double-disc test[1]. In addition, organisms were screened for carbapenemase production with the modified Hodge test (MHT)[9]. The minimum inhibitory concentration (MIC) of imipenem was determined by the microbroth dilution method according to CLSI criteria[9]. AmpC overproduction was confirmed according to the method of Kalantar-Neystanaki et al[10].

Detection of β-lactamases and virulence genes

Genomic DNA was extracted by the boiling method[2]. The genes encoding ESBLs (bla_{TEM}, bla_{SHV}, bla_{CTX-M}, and bla_{PER}), AmpC (bla_{ACC}, bla_{FOX}, bla_{MOX}, bla_{DHA}, bla_{CIT}, and bla_{EBC}), and carbapenemase (bla_{IMP}, bla_{VIM}, bla_{NDM}, bla_{KPC}, bla_{GIM}, and bla_{OXA-48}) were targeted by polymerase chain reaction (PCR) using specific primers[10,11]. The detection of seven different virulence genes (*csgA*, *csgB*, *csgD*, *rpos*, *FliI*, *fepA*, and *iutA*) was performed with PCR using the oligonucleotide primers listed in **Table 1**.

Random amplified polymorphic DNA-PCR

For molecular analysis of isolates, random amplified polymorphic DNA (RAPD)-PCR was performed as previously described[12]. In brief, PCR protocol comprised a pre-denaturation step at 95 °C for 5 min, followed by 30 cycles of 60 s at 95 °C, 60 s at 33 °C, and 60 s at 72 °C. A final extension step was performed at 72 °C for 10 min. PCR products were separated by electrophoresis on 1% agarose gels with 0.5× Tris-borate-ethylenediaminetetraacetic acid (EDTA) buffer (TBE buffer). Gels were stained with ethidium bromide and the images were captured using a gel documentation system. Isolates that differed by more than two prominent bands were assigned to different types.

Of 57 isolates, 44 (77.1%) were *E. cloacae* and 13 (22.8%) were *E. aerogenes*. These were cultured from wounds (n = 26), urine (n = 15), blood (n = 8), and other sources (n = 8). Resistance to cefoxitin (84.3%), cefotaxime (49.1%), cefpodoxime (36.8%), and ceftazidime (36.8%) was more prevalent, but only eight (14.1%), seven (12.3%), and six (10.5%) isolates were resistant to imipenem, levofloxacin, and gatifloxacin, respectively. Microbroth dilution method showed that 20 (35.1%) strains were resistant to imipenem. Ten (17.5%) isolates were defined as MDR. The phenotypic test for ESBL, AmpC β-lactamase, and carbapenemase production showed that 30 isolates (22 *E. cloacae* and 8 *E. aerogenes*) produced ESBL, 21 isolates (16 *E. cloacae* and 5 *E. aerogenes*) produced AmpC β-lactamases, and 8 isolates (6 *E. cloacae* and 2 *E. aerogenes*) produced carbapenemases. The phenotypic and genotypic characteristics of ESBL and AmpC-producing isolates of *E. cloacae* and *E. aerogenes* are shown in **Table 2** and **Table 3**, respectively. The genes encoding ESBL, bla_{TEM}, bla_{CTX-M}, and bla_{SHV}, were detected in 19 (63.3%), 19 (63.3%), and 8 (26.6%) isolates, respectively, making them the most prevalent ESBL genes in these isolates. We failed to detect bla_{PER} .

The gene for AmpC, bla_{EBC}, was detected in only 17 (57%) isolates. Another common AmpC-associated gene, bla_{ACC}, was detected in 5 (16.6%) isolates. The genes bla_{CIT} and bla_{DHA} were detected in only 2 (6.6%) and 2 (6.6%) of *E. cloacae* isolates, respectively. The genes bla_{FOX} and bla_{MOX} were not detected. In addition, we failed to detect carbapenemase genes. The most prevalent genes were *rpos* and *fliI* reported in 50 (87.7%) isolates, followed by *csgB*, *csgD*, csgA, *iutA*, and *fepA* observed in 40 (70.2%), 39 (68.4%), 34 (59.6%), 31 (54.4%), and 29 (50.9%) isolates, respectively. *E. cloacae* isolates were grouped into 21 RAPD types, which were designated as type A (two isolates) to S (one isolate each) (**Table 2**). *E. aerogenes* isolates were grouped into seven RAPD types, which were designated as type A (two isolates) to G (one isolate each) (**Table 3**). In the present study, the most prevalent species was *E. cloacae* (77.1%) and its predominance was similar to that reported by Khari et al. and Kanamori et al[2,3]. In recent years, *E. cloacae* is the most common pathogen causing nosocomial infections[1]. In this study, 84.3% of isolates were resistant to cefoxitin. High level resistance to cefoxitin has been previously reported by other investigators[2,3], suggesting that treatment with these drugs should be avoided in *Enterobacter* infections.

Our study revealed that 35.1%, 12.3%, and 10.5% of isolates were resistant to imipenem, levofloxacin, and gatifloxacin, respectively. Previous reports from Iran have shown that the resistance rate of *Enterobacter* isolates to imipenem and gatifloxacin was 2% and 7%, respectively[1]. Our results indicated the significant increase in the resistance to carbapenem and ciprofloxacin, which may be attributed to the inappropriate and widespread use of antibiotics[1]. Of the 30 isolates that were recognized as phenotypically positive for ESBL production in this study, 27 were positive for ESBL genotypes. In the study conducted by Kanamori et al. from Japan, 22 of 364 *Enterobacter* spp. were identified phenotypically positive for ESBL production, but only 11 isolates harbored ESBL genes; ESBL genes were undetected in the remaining 11 isolates[2]. Discrepancy between disc tests and PCR detection results may be associated with the lack of any standardized method

TABLE 1: The oligonucleotide primers used in this study for the amplification of virulence genes.

Gene	Primer sequence (5′ to 3′)	Annealing temperature (°C)	Product size (bp)	Reference
csgA	F- TTCAAAGTGGCAGTTATTGCAG R- TTTTTGCAGCAGATCGATAGAA	56	276	[4]
csgD	F- GAAATTGCATAATATTCAACGTTC R- TTTGTTCAGGATCTCTTTTTCAC	54	385	
csgB	F- TCCTGGGAAACGATGGACAA R- TTACATTACTGGGAGCGCCT	54	193	this study
fliI	F- ATACGGCGCAGTGCGTTAC R- ACCAAAGAGAGGACACAATGC	54	154	this study
rpoS	F- CACTTCACGCTGTTTGGCG R- CGCGAGTTGTCCCATAAACTG	56	273	this study
fepA	F- TCTTTT TTCACCGGCATGGA R- CGTGCGGTGGTCAATATCT	57	572	this study
iutA	F- TGAAACGTTCTCATCTTTGGGTT R- TCG AAGGTTTCATGGTCGGC	56	1117	this study

TABLE 2: Characteristics of *Enterobacter cloacae* isolates.

Isolate ID	Date	Source	Resistance pattern	MDR	ESBL gene	MIC of IMI	AmpC gene	RAPD type
1	11/11/2013	Burn	CTX, CAZ, CPM, CPD, FOX, AUG, IMI, MEM, ETP, AK, GM, CIP, LEV, GAT	+	bla_{TEM}, bla_{CTX-M}	1	bla_{EBC}	D
2	11/11/2013	Burn	CTX, CAZ, CPM, CPD, FOX, AUG, AK, GM	–	bla_{TEM}, bla_{CTX-M}	2	bla_{EBC}	E
3	11/25/2013	Burn	CTX, CAZ, CPM, CPD, FOX, AUG, AK, GM, CIP	+	bla_{TEM}, bla_{SHV}	2	bla_{EBC}	F
4	12/21/2013	Eye	CTX, CAZ, CPM, CPD, FOX, AUG, AK, GM	–	bla_{TEM}	4	bla_{ACC}, bla_{EBC}	G
5	12/29/2013	Respiratory	CTX, CAZ, CPM, CPD, FOX, AUG, AK	–	bla_{TEM}	4	bla_{ACC}, bla_{DHA}	H
6	12/28/2011	Urine	CTX, CAZ, CPM, CPD, FOX, AUG, GM, CIP	+	bla_{TEM}, bla_{CTX-M}, bla_{SHV}	0.25	bla_{DHA}, bla_{EBC}	B
7	12/28/2011	Wound	CTX, FOX, AUG	–	bla_{TEM}, bla_{CTX-M}	2	bla_{ACC}, bla_{EBC}	C

Continue...

TABLE 2: Continuation.

8	1/12/2011	Wound	CTX, FOX, AUG	−	bla_{TEM}	2	bla_{EBC}	I
9	6/1/2012	Wound	CTX, CAZ, CPM, CPD, FOX, AUG, GM	−	bla_{TEM}, bla_{CTX-M}	2	bla_{EBC}	C
10	12/13/2013	Wound	CTX, CAZ, CPM, CPD, FOX, AUG, AK, GM, CIP, LEV	+	bla_{TEM}, bla_{CTX-M}	4	bla_{EBC}, bla_{CIT}	J
11	12/13/2013	Urine	CTX, FOX, AUG	−	bla_{TEM}, bla_{CTX-M}	4	−	K
12	2/25/2014	Urine	CTX, CAZ, CPM, CPD, FOX, AUG, AK, GM	−	bla_{TEM}, bla_{CTX-M}	1	bla_{ACC}, bla_{EBC}	L
13	3/11/2014	Burn	CTX, CAZ, CPM, CPD, FOX, AUG, IMI, MEM, ETP, AK, GM, CIP, LEV, GAT	+	bla_{TEM}, bla_{CTX-M}	64	bla_{EBC}, bla_{CIT}	A
14	3/11/2014	Burn	CTX, CAZ, CPM, CPD, FOX, AUG, IMI, MEM, ETP, AK, GM, CIP, LEV, GAT	+	bla_{TEM}, bla_{CTX-M}	64	bla_{EBC}	A
15	4/22/2014	Urine	CTX, FOX, AUG	−	bla_{CTX-M}	4	bla_{EBC}	M
16	4/25/2014	Respiratory	CTX, CAZ, CPM, CPD, AUG, GM	−	−	4	bla_{EBC}	B
17	4/29/2014	Respiratory	CTX, CAZ, CPM, CPD, FOX, AUG, AK, GM	−	bla_{TEM}, bla_{CTX-M}	4	bla_{EBC}	N
18	5/5/2014	Blood	CTX, CAZ, CPM, CPD, FOX, AUG, IMI, MEM, ETP, AK, GM, CIP, GAT	+	−	16	−	O
19	5/6/2014	Blood	CTX, CAZ, CPM, CPD, FOX, AUG, IMI	−	bla_{TEM}, bla_{CTX-M}, bla_{SHV}	16	bla_{EBC}	P
20	5/7/2014	Urine	FOX, AUG	−	bla_{TEM}	2	−	Q
21	5/7/2014	Urine	FOX, AUG	−	−	8	bla_{EBC}	R
22	5/15/2014	Wound	CTX, CAZ, CPM, CPD, FOX, AUG, IMI, MEM, ETP, GM, CIP, LEV, GAT	+	bla_{SHV}	4		S

CTX: cefotaxime; **CAZ**: ceftazidime; **CPM**: cefepime; **CPD:** cefpodoxime; **FOX**: cefoxitin; **AUG:** amoxicillin-clavulanate; **IMI**: imipenem; **MEM**: meropenem; **ETP**: ertapenem; **AK**: amikacin; **GM**: gentamicin; **CIP**: ciprofloxacin; **LEV**: levofloxacin; **GAT**: gatifloxacin; **MDR**: multi-drug resistant; **ESBL**: extended-spectrum β-lactamase; **MIC**: minimum inhibitory concentration; **RAPD**: random amplified polymorphic DNA.

for the detection of ESBLs in *Enterobacter* spp[2]. In the present survey, 30 (52.6%) *Enterobacter* isolates were found to be ESBL producers. Kanamori et al. also reported that 6% *Enterobacter* spp. were ESBL producers[2]. The high prevalence of ESBL-positive isolates in our study may be associated with the extensive use of third-generation cephalosporins for the treatment of *Enterobacter* infections. It should be noted that 10% (3/30) isolates were ESBL negative and eight isolates that were recognized phenotypically positive for carbapenemase failed to show any carbapenemase-related genes, suggestive of the involvement of other resistance mechanisms. In our study, 26.7% (8/30) of ESBL-positive isolates were MDR. Peymani et al. reported that all ESBL-positive *Enterobacter* isolates were MDR[1]. In our study, bla_{TEM} and bla_{CTX-M} were the most common ESBL resistance genes, which were frequently reported in other countries[2]. In the present study, bla_{EBC} (57.7%)

TABLE 3: Characteristics of *Enterobacter aerogenes* isolates

Isolate ID	Date	Source	Resistance pattern	MDR	ESBL gene	MIC of IMI	AmpC gene	RAPD type
1	2/19/2012	Wound	CTX, AUG	−	bla_{TEM}	2		B
2	2/6/2014	Blood	CTX, CAZ, CPM, CPD, FOX, AUG, IMI, MEM, ETP, AK, CIP	+	bla_{TEM}, bla_{CTX-M}	4		C
3	2/25/2014	Urine	CTX, CAZ, ETP, GM, CIP	−	bla_{SHV}, bla_{CTX-M}	2		D
4	3/3/2014	Urine	CTX, CAZ, CPM, CPD	−	$bla_{SHV,}$ bla_{CTX-M}	0.625		A
5	3/11/2014	Burn	CTX, CAZ, CPD, FOX, GM	−	bla_{CTX-M}	0.5	bla_{EBC}	E
6	4/22/2014	Respiratory	-	−	bla_{CTX-M}	0.25	bla_{ACC}	F
7	5/15/2014	Wound	CTX, CAZ, CPM, CPD, FOX, AUG, IMI, MEM, ETP, GM, CIP, LEV, GAT	+	bla_{SHV}	0.625		A
8	5/25/2014	Urine	-	−	bla_{SHV}, bla_{CTX-M}	2		G

CTX: cefotaxime; **CAZ**: ceftazidime; **CPM**: cefepime; **CPD**: cefpodoxime; **FOX**: cefoxitin; **AUG**: amoxicillin- clavulanate; **IMI**: imipenem; **MEM**: meropenem; **ETP**: ertapenem; **AK:** amikacin; **GM**: gentamicin; **CIP**: ciprofloxacin; **LEV**: levofloxacin; **GAT**: gatifloxacin; **MDR**: multi-drug resistant; **ESBL**: extended-spectrum β-lactamase; **MIC**: minimum inhibitory concentration; **RAPD**: random amplified polymorphic DNA.

was the most common type of AmpC β-lactamase, followed by bla_{ACC} (16.6%). Miró et al. reported that the CMY (78.3%) and DHA (19.5%) families were the most prevalent type of AmpC β-lactamase in 35 hospitals in Spain[13]. However, the prevalence of ESBL and AmpC-producing *Enterobacter* spp. varied among different studies, which may be associated with the differences in the geographical area, type of infection, and settings (hospital or community). Similar to previous reports, we observed the coexistence of ESBL-encoding genes in clinical isolates[1,2]. Several virulence factors have been identified in the pathogenesis of *Enterobacter* spp[4-7]. The majority of isolates (87.7%) carried *rpos* and *fliI*. The high frequency of these genes may indicate that these genes are essential for the development of disease. In contrast to the findings of our study, Krzyminska et al. observed that only 27% of isolates harbored *fliI* (TTSS gene)[6]. In the current study, the frequency of *csgB*, *csgD*, and *csgA* was 70.2%, 68.4%, and 59.6%, respectively, which is lower than that reported in the previous study by Akbari et al. These authors showed that *csgD* and csgA genes were present in 100% and 77.75% of isolates, respectively[14]. The genes *iutA* and *fepA* were found in 54.4% and 50.9% of isolates in our study. Mokracka et al. reported that 49% of *E. cloacae* strains produced aerobactin[15]. However, differences were observed in the frequency of virulence genes reported in different studies; this difference may be associated with the variation in the geographical area, clinical samples, and other factors. RAPD-PCR analysis revealed the significant genetic heterogeneity. In addition, molecular analysis demonstrated that more than 90% (28/30) of ESBL-producing isolates were clonally unrelated, indicating that the reported infections had no relation with clonal outbreak. In conclusion, bla_{TEM}, bla_{CTX-M}, and bla_{EBC} are the most common resistance gene types and more than 50% of isolates harbored virulence genes. RAPD-PCR analysis revealed high genetic diversity among isolates.

Conflict of interests

The authors declare that there is no conflict of interest.

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