Emergence of CMY-2- and DHA-1-type AmpC β-lactamases in *Enterobacter cloacae* isolated from several hospitals of Qazvin and Tehran, Iran

Amir Peymani, Taghi Naserpour-Farivar*, Moein Yeylagh-Beygi, Shahin Bolori

Cellular and Molecular Research Center, Qazvin University of Medical Sciences, Qazvin, Iran

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ABSTRACT

**Background and Objectives:** The emergence of plasmid-mediated AmpC (pAmpC) β-lactamases conferring resistance to third-generation cephalosporins has become a major clinical concern worldwide. The aims of this study were to determine the prevalence of pAmpC-producing *E. cloacae* isolates and typing of them in Qazvin and Tehran provinces, Iran.

**Materials and Methods:** A total of 120 cefoxitin non-susceptible isolates of *E. cloacae* were obtained from educational hospitals of Qazvin and Tehran, Iran. Bacterial identification was performed by standard laboratory methods and API 20E strips. Susceptibility to cefoxitin was determined by Kirby-Bauer disk diffusion method. PCR and sequencing were employed to detect pAmpC families’ genes (ACC, FOX, MOX, DHA, CIT and EBC) and the clonal relatedness of pAmpC-positive isolates was evaluated by enterobacterial repetitive intergenic consensus (ERIC)-PCR method.

**Results:** In total, 20 (16.7%) isolates of *E. cloacae* were positive for presence of pAmpC genes among those *bla*<sub>DHA-1</sub> (14.2%) was the most common gene followed by *bla*<sub>CMY-2</sub> (2.5%). Results of ERIC-PCR showed that the prevalence of DHA-1 and CMY-2-producing *E. cloacae* isolates was not due to clonal outbreaks.

**Conclusion:** In present study, we showed the first emergence of DHA-1 and CMY-2 types of pAmpC-producing *E. cloacae* isolates in Iran. The appearance of pAmpC should be considered as a warning for the implementation of appropriate infection control and therapeutic policies in order to prevent the dissemination of these resistant organisms in our hospital settings.

Keywords: *Enterobacter cloacae*, PAmpC, ERIC-PCR

INTRODUCTION

*Enterobacter cloacae* is a prevalent opportunistic pathogen which is associated with nosocomial infection in hospital settings (1). The most common causing infections of this organism are the urinary tract, lower respiratory tract, skin and soft tissue, and central nervous system infections (2). The β-lactams are one of the most prescribed choices against bacterial infections (3). β-lactamase production is the major β-lactam resistance mechanism in Gram-negative bacteria, such as *Enterobacteriaceae* and *Pseudomonas aeruginosa* (4). In recent years, emerging of newer β-lactamase enzymes, including extended-spectrum β-lactamases (ESBLs) and AmpC β-lactamases complicates the process of therapy and limits treatment options (4, 5). The extensive and inappropriate use of β-lactam antibiotics are associated with the appearance of these resistant determinants, especially when third-generation cephalosporins are used to treat serious infections (6).

AmpC β-lactamase production is one of the mechanisms of resistance to β-lactam antibiotics in Gram
negative bacteria conferring resistance to a wide variety of β-lactam antibiotics including 7α-methoxy-cephalosporins (cefoxitin or cefotetan), oximinocephalosporins (cefotaxime, cefazidime, and ceftriaxone), and monobactams (aztreonam) (7, 8). Resistance due to pAmpC enzymes is less common than extended spectrum β-lactamases (ESBLs) production, however they have rapidly become a leading clinical concern due to their self-transmissibility which permits their spread among different bacteria (9-11).

AmpC β-lactamases are not inhibited by clavulanic acid, sulbactam and tazobactam which generally inhibit the ESBL production (12-14). Although pAmpC enzymes most often found in nosocomial isolates of Escherichia coli and Klebsiella pneumoniae, but there are few published reports regarding occurrence of these enzymes in other genera of Enterobacteriaceae (15).

ACC, FOX, MOX, DHA, CIT and EBC are most commonly reported pAmpC genotypes, among those CMY-2-like enzymes are the most prevalent in clinical enterobacterial isolates, although the DHA-like β-lactamases have also spread extensively (11). These enzymes confer a resistance pattern similar to the overproduction of chromosomal AmpC β-lactamases, which may involve all β-lactam antibiotics except for carbapenems and cefepime (16).

Detection of pAmpC β-lactamase remains a challenge in clinical microbiology laboratories because there are no guidelines of Clinical and Laboratory Standards Institute (CLSI) for phenotypic detection of AmpC-producing organisms. Although several phenotypic tests are now available for detection of pAmpC among clinical isolates of Enterobacteriaceae but none of these tests are standardized and they are unreliable and unsuitable, resulting in misreporting and treatment failures (8, 10). In addition, phenotypic tests do not differentiate between chromosomal and pAmpC genes (17). However, molecular characterization is considered as the gold standard method (8). There was no report on prevalence of pAmpC β-lactamases in Enterobacter spp. in Iran. In this study, we describe the prevalence of pAmpC-encoding genes in clinically E. cloacae isolates collected from two distinct provinces of Iran.

MATERIALS AND METHODS

Study design and Bacterial isolates. During a 16-month period from August 2012 to December 2013, a total of 120 non-duplicate cefoxitin-resistant clinical isolates of E. cloacae were collected from different clinical samples of patients admitted to several teaching hospitals of Qazvin, and Tehran. The isolates were collected from various clinical specimens including urine, wound, sputum, bronchoalveolar lavage (BAL), trachea, blood, and cerebrospinal fluid. Isolates were obtained from patients admitted to intensive care units, internal medicine, infectious diseases, neurology, surgery, and orthopedic wards. Forty-eight patients (40%) were females and seventy two (60%) were males aged between 18 and 85 years with a mean of 49.3±17.4 years. Written informed consent was obtained from all subjects enrolled in this study. Isolates were identified using conventional laboratory techniques and confirmed by the API 20 E (bioMérieux, France). They were stored at -70°C in trypticase soy broth containing 20% glycerol and sub-cultured twice prior to testing.

In order to initial screen of pAmpC production, the antimicrobial susceptibility of isolates was determined using Kirby-Bauer disc diffusion method against cefoxitin (30μg) as instructed by CLSI (18). Additionally, susceptibility to imipenem (10μg), and meropenem (10μg) was determined. Antibiotic discs were purchased from Mast (Mast Diagnostics Group Ltd, Merseyside, UK). E. coli ATCC 25922 and Pseudomonas aeruginosa ATCC 27853 were used as the quality control strains in antimicrobial susceptibility testing.

Multiplex PCR and sequencing of pAmpC-encoding genes. Plasmid DNA from cefoxitin resistant E. cloacae isolates was extracted using extraction kit (Bioneer, Seoul, Korea). All cefoxitin-non susceptible isolates (n=120), as putative AmpC producers, were tested by multiplex PCR assay to identify six family-specific AmpC genes including MOX, FOX, EBC, ACC, DHA and CIT as previously described by Pérez-Pérez et al. (Table 1) (15). Amplification reactions were prepared in a total volume of 50 µl. Each reaction contained 20 mMTris-HCl (pH 8.4); 50 mM KCl; 0.2 mM each deoxynucleoside triphosphate; 1.5 mM MgCl2; 0.6 µM primers and 1.25 U of Taq DNA polymerase. Template DNA (2 µl) was added to 48 µl of the master. PCR amplification was performed in a thermocycler (Applied Biosystems, USA) as follows: 94°C for 3 min and 35 cycles of 1 min at 94°C, 1 min at specific annealing temperature for each primer.
and 1 min at 64 °C. A final extension step of 10 min at 72 °C was performed. PCR products were electrophoresed on a 1% agarose gel at 100 V and then were stained with the ethidium bromide solution and finally visualized in a gel documentation system (UVtec). The purified PCR products were sequenced by the Macrogen Company (South Korea) and sequence alignment and analysis were performed online using

**Table 1.** Primers used for amplification of pAmpC genes in this study

| Genes          | Sequence (5’ to 3’)                                                                 | References |
|----------------|--------------------------------------------------------------------------------------|------------|
| MOX-1, MOX-2, CMY-1, CMY-8 to CMY-11 | F-GCTGCTCAAGGAGCACAGGAT R-CAACATTGACATAGGTGGTGC | 15         |
| LAT-1 to LAT-4, CMY-2 to CMY-7, BIL-1 | F-TGGCCAGAAGCTGACAGGCAA R-TTTCCTCTGAAACGTGGTGC | 15         |
| DHA-1, DHA-2   | F-AACTTTCACAGGTTGTTGCTGGT R-CCCTTACGCTATCAGGCT TTG | 15         |
| ACC            | F-AAACAGCCTCAGCAGGCCGTAA R-CTCAGCCTCAGCAGGCCGTAA | 15         |
| MIR-1T ACT-1   | F-TCGGTAAAGCCGATGTTGCG R-CTCAGCCTCAGCAGGCCGTAA | 15         |
| FOX-1 to FOX-5b| F-AACAAGGGGTTACAGGGAGATG R-CAAAGGCGTAACCCGATG | 15         |
| ERIC-PCR       | F-ATGTAAGCTCCTGGGGGTGTAC R-AAAGAAGTACGGGAGTAC | 19         |

**Table 2.** Case histories and characteristics of the 20 pAmpC-producing E. cloacae isolates collected from Qazvin and Tehran hospitals

| Isolates | City     | Age (yr)/gender | Ward     | Source | Susceptibility to IMI | Susceptibility to MEM | pAmpC gene |
|----------|----------|-----------------|----------|--------|-----------------------|------------------------|------------|
| En.c 2   | Qazvin   | 46/female       | ICU      | Tracheal | S                      | S                     | -          |
| En.c 5   | Qazvin   | 58/male         | ICU      | Urine   | R                      | R                     | -          |
| En.c 18  | Qazvin   | 29/male         | ICU      | Urine   | R                      | S                     | -          |
| En.c 20  | Tehran   | 35/female       | ICU      | Wound   | S                      | S                     | -          |
| En.c 22  | Tehran   | 39/female       | ICU      | Wound   | S                      | S                     | +          |
| En.c 34  | Tehran   | 48/male         | Internal | Wound   | S                      | S                     | -          |
| En.c 36  | Tehran   | 38/female       | Internal | Wound   | S                      | S                     | -          |
| En.c 37  | Tehran   | 65/male         | ICU      | Urine   | S                      | S                     | -          |
| En.c 39  | Tehran   | 50/male         | ICU      | Urine   | S                      | S                     | -          |
| En.c 40  | Tehran   | 23/male         | ICU      | Sputum  | S                      | S                     | -          |
| En.c 43  | Tehran   | 45/male         | ICU      | Wound   | S                      | S                     | +          |
| En.c 55  | Tehran   | 48/female       | ICU      | Wound   | S                      | S                     | +          |
| En.c 56  | Qazvin   | 28/male         | ICU      | Urine   | S                      | S                     | +          |
| En.c 73  | Tehran   | 71/female       | ICU      | Blood   | S                      | S                     | +          |
| En.c 74  | Tehran   | 63/male         | Infectious | Tracheal | S                      | R                     | -          |
| En.c 92  | Tehran   | 23/male         | Internal | Urine   | S                      | S                     | +          |
| En.c 97  | Tehran   | 59/male         | Internal | Wound   | S                      | S                     | -          |
| En.c 101 | Tehran   | 32/male         | Internal | Wound   | S                      | S                     | -          |
| En.c 112 | Qazvin   | 38/female       | ICU      | Urine   | S                      | S                     | -          |
| En.c 120 | Qazvin   | 59/female       | Surgery  | BAL     | R                      | R                     | -          |

BAL: bronchoalveolar lavage; ICU: Intensive Care Unit; IMI; imipenem, MEM; meropenem; S: Susceptible; R: Resistance
the BLAST program of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov).

**ERIC-PCR analysis.** The epidemiological relationships of pAmpC-producing *E. cloacae* isolates were analyzed by ERIC-PCR as previously described. Cycling conditions were as follows: denaturation at 94 °C for 1 sec, annealing at 52 °C for 10 sec, and extension at 72 °C for 35 sec for 30 cycles, followed by a final extension at 72 °C for 4 min. The resulting products were analyzed on 1.5% agarose gels. Fingerprints were compared visually, and the patterns differing by two or more bands were classified as different (19).

**Statistical analysis.** Statistical analysis was performed for descriptive statistics, including frequencies, cross tabulation of microbiological, clinical, and demographic characteristics using the computer software program SPSS version 16.

**RESULTS**

During the study period, 120 cefoxitin non-susceptible clinical isolates *E. cloacae* were recovered from different clinical specimens including urine (44 isolates; 36.7%), wound (29 isolates; 24.2%), blood (18 isolates; 15%), trachea (16 isolates; 13.3%), sputum (6 isolates; 5%), bronchoalveolar lavage (5 isolates; 4.2%), and cerebrospinal fluid (2 isolates; 1.7%). Isolates were obtained from patients admitted to the intensive care units (52-43.3%), internal medicine (29-24.2%), infectious diseases (14-11.7%), neurology (6-5%), surgery (10-8.3%), and orthopaedic (9-7.5%) wards. In total, 19 (15.8%) and 14 (11.7%) isolates (6-5%), surgery (10-8.3%), and orthopaedic (9-7.5%) were mostly admitted to ICU (13-10.8%) and the internal medicine (5-4.2%) wards.

**DISCUSSION**

Emergence of pAmpC enzymes among Gram-negative bacteria especially *E. cloacae* is an important clinical concern which contributes to high rates of morbidity and mortality in hospital settings, in particular among ICU patients (11). AmpC β-lactamases are either plasmid or chromosomal mediated. In the late 1980s, plasmid-borne AmpC cephalosporinases were detected on plasmids which facilitate their spread between the Enterobacteriaceae (10, 20). Detection of pAmpC-producing organisms is important to ensure effective therapeutic intervention and optimal clinical outcomes (8). In Iran, AmpC detection is not routinely done in most laboratories. Up to date, there are rare reports to related incidence of plasmid-mediated AmpC in *Enterobacter* spp. worldwide. To the best of our knowledge, this is the first report of emergence of pAmpC in clinical isolates of *E. cloacae* in Iran.

Based on our findings, the plasmid encoded AmpC genes were found in 20 (16.7%) isolates by PCR. The prevalence rate found in our study is lower than those reported by Wang and al. from China (21), and Yusuf et al. from Nigeria (22) in which 35.06% and 18.8% of *E. cloacae* isolates were found to be AmpC producer, respectively. Plasmid AmpC β-lactamases have been increasingly reported among other members of Enterobacteriaceae. Yamasaki et al. reported that 0.12% of *E. coli*, 0.13% of *K. pneumoniae*, 0.17% of *K. oxytoca* and 0.08% of *P. mirabilis* isolates in Japan were positive for pAmpC production (23). Li et al. from China reported that 4.29% *K. pneumoniae*, 1.91% of *E. coli*, and 3.03% of *K. oxytoca* were found to be positive for pAmpC (24).

In Turkey, Yilmaz et al. using PCR reported that 10.9% of *E. coli* and 3.6% of *K. pneumoniae* isolates were AmpC producers (25). In Egypt, Nevine Fam et al. reported that 28.3% of *E. coli*, *Klebsiella* spp. and *P. mirabilis* isolates were positive for AmpC production (10). In the another study from Egypt, 16.8% of cefoxitin resistant *E. coli*, *K. pneumoniae*, and *P. mirabilis* isolates were positive for the presence of pAmpC genes (17). Tan et al. reported that pAmpC was pres-
ent in 26% of E. coli, Klebsiella spp., and P. mirabilis isolates in Singapore (26). These findings indicate that the rate of plasmid mediated AmpC β-lactamases is increasing among Enterobacteriaceae worldwide. We previously reported that 53 (44.2%) of E. cloacae isolates were found to be extended-spectrum β-lactamase (ESBL) producers (6). It seems that, inappropriate and extensive use of broad spectrum antibiotics can contribute to the emergence of these resistant isolates in our hospital settings. Our study indicated that most pAmpC-producing E. cloacae isolates were frequently collected from the patients admitted to intensive care units. The ICU stay, exposure to third-generation cephalosporins, and use of invasive procedures such as urinary catheterization appear to predispose these patients to infections with these resistant organisms.

In the current study, 83.3% of cefoxitin non-susceptible E. cloacae isolates were negative for the presence of pAmpC, suggesting that other mechanisms might alternatively be contributed to the resistance, most importantly, the overexpression of chromosomal ampC, known to confer resistance to oxyimino-cephalosporins in this organism, as well as impermeability or reduced expression of outer membrane proteins, and alteration in the expression of efflux pumps (27, 28).

In the current study, 17 (14.2%) isolates of E. cloacae carried blaDMH1, and 3 (2.5%) isolates carried blaCMY2 gene. No blaACC, blaFOX, blaMOX, or blaEBF families’ genes were detected. To the best of our knowledge, this is first report of blaCMY2 and blaDMH1 genes in clinical isolates of E. cloacae in Iran. In a report from Iran, Tajbakhsh et al. reported the emergence of blaCMY2 in Shigella somai phase II isolates (29). In Europe, Mata et al. from Spain reported that CMY-2 type was predominantly found in E. coli (70.7%) and Proteus mirabilis (95%) isolates whereas DHA-1 type was more frequent in K. pneumoniae (62.5%) followed by CMY-2 type (30). In Africa, Nevine Fam and et al. from Egypt reported that CMY-2 was the most prevalent gene (70.5%) followed by DHA-1 (23.5%) and CMY-4 (5.8%) in Enterobacterial clinical isolates (10). In Asian countries, Song and et al. from Korea reported that DHA-1 (66.6%) was the most common type of AmpC β-lactamase followed by CMY-2 (20%), CMY-10 (6.6%) and CMY-11 (6.6%) (31). Yamasaki and et al. from Japan reported that 69.0%, 20.7%, 6.9%, and 3.4% of pAmpC-producing enterobacterial species carried CMY-2, DHA-1, CMY-8, and MOX-1 genes, respectively (23). Li et al. from China reported that 96.7% and 3.3% pAmpC-producing K. pneumoniae isolates carried blaDMH1 and blaCMY2 genes whereas 52.18% and 47.8% of E. coli isolates were positive for the presence of blaCMY2 and blaDMH1 genes, respectively (24). Tan et al. from Singapore reported that CMY-like enzymes detected predominantly in E. coli and DHA-like enzymes were frequently found in K. pneumoniae (26).

Since the pAmpC-producing isolates typically exhibit resistance to several classes of β-lactam antibiotics so carbapenem are used as a therapeutic of choice for treating of serious infections caused by these organisms (4). However, we encountered a considerable rate of carbapenem resistance among E. cloacae isolates in this study; 19 (15.8%) and 14 (11.7%) isolates were non-susceptible to imipenem and meropenem, respectively which would have more clinical impact if these strains become more prevalent in the future.

In this study, ERIC-PCR analysis showed all blaCMY-positive isolates displayed different genotypic patterns and blaDMH-positive isolates presented ten different types; suggesting that the dissemination of these isolates was not due to a clonal outbreak. It could be explained by the fact that the isolates in the present study were collected from seven hospitals in Tehran, and five hospitals in Qazvin; two different locations in Iran.

In conclusion, the results of this study showed the emergence of blaCMY5 and blaDMH1 genes among the clinical isolates of E. cloacae in Iran. The emergence and spreading of these resistant determinates in our clinical settings emphasizes on the necessity for establishing tactful policies for infection control and antibiotic therapy.

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