Prevalence *Escherichia coli*, *Klebsiella* and *Enterobacter* Species and AmpC-producing Enterobacteriaceae in Clinical Specimens of Hospitals Affiliated to Babol University of Medical Sciences, Iran using Phenotypic and Molecular Methods

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**ABSTRACT**

**Background and Aim:** AmpC-producing bacteria are a severe threat to treating infectious diseases caused by gram-negative bacteria. The actual prevalence of these bacteria is not clearly determined as there is no reliable diagnostic method available to detect them. Therefore, this study was performed to determine the frequency of *Escherichia coli*, *Klebsiella*, and *Enterobacter* species producing AmpC among clinical samples by phenotypic and molecular methods.

**Materials and Methods:** In this study, 163 bacteria of Enterobacteriaceae species isolated from different clinical samples in 2018 were examined. Suspected isolates of producing pAmpC were identified using cefoxitin disk (FOX) and disk diffusion method. Three confirmatory phenotypic methods were performed to identify pAmpC production, and *blaDHA*, *blaFOX*, *blaMOX* genes were searched using a molecular method for all bacteria. Specificity and sensitivity of phenotypic tests were obtained compared to the presence of *blaDHA* gene.

**Results:** Of 163 bacteria, 80 (49.1%) isolates were resistant to FOX, and 21 (12.9%) carried the *blaDHA* gene. Among the bacteria carrying the gene, 5 (6%) isolates were sensitive to FOX. 49 (61.3%) FOX-resistant bacteria were positive in one of the chromosomal and/or plasmid phenotypic tests. The highest specificity and sensitivity were observed in the AmpC disk (90.8%) and CAM (42.7%) methods, respectively.

**Conclusion:** It seems phenotypic methods are more successful in distinguishing true negatives (higher specificity). Also, sensitivity to cefoxitin is not a criterion for not producing the enzyme AmpC. For this reason, it is recommended that national monitoring be performed to identify the genes of AmpC producing bacteria.

**Keywords:** AmpC, *blaDHA*, *Enterobacter*, *Escherichia coli*, *Klebsiella*, PCR

1. Introduction

The production of beta-lactamase enzymes is one of the most important mechanisms of resistance to beta-lactam antibiotics in bacteria. These enzymes are divided into 4 groups based on biochemical and substrate properties. AmpC beta-lactamas develop resistance to broad-spectrum beta-lactams other than carbapenems (1). If a mutation in the purine base is associated with the production of the enzyme AmpC, it also leads to resistance to carbapenems in bacteria (2, 3). Bacteria that produce the enzyme AmpC are a new threat, and their spread is recognized as an increasing problem in the treatment of diseases.
caused by Enterobacteriaceae (3). These bacteria are also affected by beta-lactamase inhibitors such as clavulanic acid (4).

The genomes that produce beta-lactamase enzymes, such as Class C β-lactamases or AmpC enzymes, are located on the chromosomes and plasmids of bacteria (2). Plasmid types are derived from chromosomal genes and have a structure and function similar to the chromosome type (6). Chromosomal beta-lactamases are mainly found in Enterobacter, Serachia and Citrobacter species and are not present in Klebsiella species (4, 5). Also, genes encoding plasmid AmpC (pAmpC) can cause a wide range of nosocomial and community-acquired infections. Beta-lactamase enzymes (class C in Ambler group) are classified into 5 groups, including EBC variants, CIT variants, FOX and MOX variants, DHA variants, and ACC variants (2). These 5 groups are responsible for developing resistance to most beta-lactams (7). Unlike chromosomal type, plasmid type is not inducible and is found in bacteria such as Escherichia coli, Klebsiella pneumoniae, and Proteus mirabilis (8). The plasmid type is often associated with other types of resistance, such as ESBLs, quinolone resistance, and aminoglycosides. Thus, infections caused by them lead to increased mortality (2, 9).

The genes that produce pAmpC enzymes are transmissible between different species, and their prevalence may be higher than previously thought (10). Although the detection of AmpC-producing bacteria is essential for clinical and epidemiological purposes, a reliable method for their detection has not yet been recommended in the CLSI or EUCAST antimicrobial susceptibility testing guidelines (11). Since no valid and simple diagnostic method is available to study and identify the bacteria that produce this enzyme, their exact prevalence is unknown (10). Some phenotypic tests are available to confirm the production of AmpC, but these tests are also unable to differentiate plasmid-type enzymes from chromosomomes (12, 13).

In different studies, different phenotypic methods, including the use of inhibitory compounds such as boronic acid, have been described to indicate the presence of pAmpC (1). On the other hand, due to the lack of a standard phenotypic protocol approved by CLSI for AmpC breeders (12), genotyping methods are considered the gold standard (8). Various studies performed in many countries (Saudi Arabia, Taiwan, Korea, North and South America) aiming to identify the producers of this enzyme have revealed differences in the genes producing the enzyme AmpC in different geographical areas (14).

It is noteworthy that AmpC-producing bacteria also interfere with the standard ESBL-type beta-lactamase-identifying test. In this way, these bacteria are positive in the initial stage of detection or screening for the production of ESBL enzyme, but their confirmatory test is negative. On the other hand, AmpC beta-lactamases are also associated with the phenomenon of multidrug resistance (MDR) (15). Therefore, identifying them to limit the spread of MDR organisms effectively controls nosocomial and community-acquired infections. It is also necessary for accurate identification of ESBL-producing strains and epidemiological studies of AmpC group producers.

Due to the small number of reports on the prevalence of AmpC-producing bacteria in Mazandaran province, Iran, this study was conducted to investigate the prevalence of E. coli, Klebsiella species, and class B or AmpC-producing Enterobacteria using Cefoxitin disk. Phenotypic methods were used to confirm the identification, and multiplex PCR was used to evaluate the frequency of blaDHA, blaFOX, and blaMOX genes.

2.Materials and Methods

Sample Collection and Antimicrobial Susceptibility Testing

In this cross-sectional descriptive study, 163 bacterial samples of Enterobacteriaceae (35 Enterobacteria spp, 55 Klebsiella spp, and 73 E. coli) were isolated from different clinical samples (urine, feces, sputum, fluids, and blood) from patients admitted to hospitals of the Babol University of Medical Sciences in 2018. The samples were randomly selected from Enterobacteriaceae positive culture samples.

Antibiotic Susceptibility Test

Antibiogram or antibiotic susceptibility test was carried out using beta-lactam family antibiotics including Cefotaxime (CTX30μg), Ceftazidime (CAZ 30 μg), Cefepime 30 μg (CPD), Ertapenem (ETP 10 μg), and Aztreonam (including the family AZM 30g, GM Ciprofloxacin 5μg (CIP) and the family of Sulfonamides namely Trimethoprim-sulfamethoxazole (23.75μg TMP1.25μg / SXZ) (MastDisk, UK) by standard disk diffusion method on Mueller Disk, India) (16).

In this study, isolates resistant to at least three antibiotics (at least one antibiotic from each family) were considered MDR (10).

Also, using cefoxitin disk (FOX), the isolates suspected of producing pAmpC with a growth inhibition diameter of less than or equal to 18 mm were identified (6).
Methods for Detecting Plasmid AmpC (pAmpC) Production

Cefoxitin Agar Medium (CAM) Method

Preparation of Enzymatic Extract from Bacteria

The 0.5 McFarland turbidity suspension was prepared from freshly grown bacteria on blood agar, and 25 μL of it was added to 6 mL of TSB medium. The samples were then incubated at 35°C for 4 hours. Next, the bacterial suspensions were centrifuged at 3000 rpm for 15 minutes. The supernatant was discarded, and about 1 mL of the precipitate was stored at -20°C for enzyme extraction. Freezing-thawing was performed on them 7 times (17, 18).

Then McFarland suspension equivalent to 0.5 E. coli ATCC25922 standard strain was cultured on MHA medium containing 6 μg/mL cefoxitin (TAB / 0.4 mg, Mast Diagnostics, UK, 325304) by the spread plate method. After that, a well with a diameter of 5 mm was created by observing sterile conditions in the center of the agar, and 30 μL of the bacterial extract was added to the well. The plate was incubated at 35°C for 24 hours. The growth of E. coli bacteria around the well was considered to represent a pAmpC-producing isolate, which can be seen in Figure 1 (19, 20).

Aminophenyl Boronic Acid Method (APBA)

First, 90 mg of aminophenyl boronic acid (Sigma-Aldrich: MKBH1495V) was dissolved in 3 mL of dimethyl sulfoxide (DMSO) Sigma-Aldrich: D8418) and then 3 mL of sterile distilled water was added. From this solution, 20 μL was added to the ceftazidime disk (CAZ). CAZ disks with and without 3-APBA were then placed on an MHA medium containing the lawn culture of each test bacteria. After incubation at 35°C for 24 h, an increase in growth inhibition diameter of 5 mm or more around the disk containing 3-APBA was considered a pAmpC-producing bacterium. A positive culture sample is shown in Figure 2 (21, 22).

Figure 1. Positive CAM test in MHA culture medium with bacterial enzyme extract in the well of the culture center (20).

Figure 2. Positive APBA test with CAZ disks with and without 3-APBA on MHA medium (22).

Disk Method AmpC or Modification Disk Method

First, the spread plate was prepared from a suspension equivalent to 0.5 McFarland turbidity by E. coli ATCC 25922 on an MHA medium. A 30 microgram Fox disk and a blank disk were placed adjacent to the above medium. Also, 10 μL of the enzyme extract of the test bacterium was added to the blank disk. After incubation at 37°C for 18 to 24 hours, the inhibition zone around Fox disc was considered the production of pAmpC (Figure 3) (23).

Figure 3. The AmpC disk method is shown in the Figure. On the left is a positive test with an inhibition zone around the FOX disk. On the right, there is an inhibition without any deformation around the disks.
Identification of Chromosomal or Induced AmpC (iAmpC: Inducible AmpC)

Mast Disk (UK) was used to induce chromosomal AmpC production as an inducer of enzyme production. A suspension (with a turbidity of 0.5 McFarland standard) of the studied bacterium was cultured using a spread plate method in the MHA medium. Then, discs containing imipenem, cefotaxime (CTX) alone, and cefotaxime containing 3APBA (60 g / L DMSO) were cultured. After incubation at 35°C for 24 h, a change in the growth inhibition zone shape around the CTX disk and no change in the growth inhibition shape around the CTX + 3APBA disk was considered the production of chromosomal AmpC (Figure 4).

Figure 4. Chromosomal AmpC production: right, positive test with change in the growth inhibition zone shape around CAZ disk alone, left, negative test without halo deformation around CAZ disk alone (15).

Molecular Analysis

DNA Extraction and Molecular Analysis of \textit{blaDHA}, \textit{blaFOX}, \textit{blaMOX} Genes

A suspension of the bacteria was prepared in sterile physiological serum, and then the DNA of the bacteria was extracted using Cinnagen Co., Iran, according to its instructions. The frequency of \textit{blaDHA}, \textit{blaFOX}, and \textit{blaMOX} genes was then evaluated using specific primer pairs in samples by the Multiplex-PCR technique. (24). The sequence of primers is demonstrated in Table 1.

The following schedule was used to amplify \textit{blaDHA}, \textit{blaFOX}, and \textit{blaMOX} genes: the initial denaturation phase at 94°C for 10 minutes, 30 cycles at 94°C for 40 seconds, 60 seconds at 55°C, for one minute at 72°C, and the final phase of elongation at 72°C for 7 minutes. The PCR product was evaluated on 2% agarose gel by electrophoresis and assessed by a gel documentation system (Vilber, Lourmat, France).

The amplified products were examined by Bioneer (South Korea) for sequencing genes using forward primers. Sequencing results were evaluated by Chromas software (V.2.6.4), and then blast analysis was performed (www.ncbi.nlm.nih.gov).

Statistical Analysis

Data were analyzed using SPSS 22 software (SPSS Inc., Chicago, Ill., USA). Descriptive statistical tests were used to obtain the prevalence of AmpC producers in the studied bacteria. Also, the sensitivity and specificity of phenotypic methods were calculated compared to the molecular method.

Table 1. Specific primers for the study of AmpC genes

| Genes   | Sequence of primers (5'-3')                                                                 | Product length (bp) | Reference |
|---------|------------------------------------------------------------------------------------------|---------------------|-----------|
| \textit{blaDHA} | F:TGA TGG CAC AGC AGG ATA TTC R:GCT TTG ACT CTT TCG GTA TTC G                           | 997                 | 24        |
| \textit{blaMOX}   | F:GCA ACA ACG ACA ATC CAT CCT R:GGG ATA GGC GTA ACT CTC CCA A                           | 895                 | 24        |
| \textit{blaFOX}    | F:CTA CAG TGC GGG TGG TTT R: CTA TTT GCC GCC AGG TGA                                   | 162                 | 24        |
3. Results

The antibiotic susceptibility of 163 samples was evaluated. The highest resistance was to cefpodoxime, and the lowest resistance to ertapenem in 111 (68.1%) and 20 (12.3%) isolates, respectively. Also, 66 samples (40.5%) were multidrug-resistant (10 Enterobacter isolates, 18 Klebsiella isolates, and 38 E. coli isolates).

Of the total bacteria (49.1%), 80 isolates were resistant to FOX or suspected of producing plasmid AmpC. Of these, 49 isolates (61.3%) were positive for plasmid or chromosomal AmpC phenotypic tests, and 7 (14.3%) isolates of these positive phenotype bacteria carried the blaDHA gene. Of 83 FOX-sensitive bacteria, 18 (21.7%) isolates were positive for at least one of the plasmids or chromosomal AmpC phenotypic tests, and 5 isolates (6%) of these positive phenotypic bacteria carried the blaDHA gene (Table 2). Also, molecular analysis of the frequency of resistance genes showed that 21 (12.9%) bacteria had the blaDHA gene, and out of 12 samples (9.61), 13 isolates were resistant to FOX.

All Klebsiella isolates tested negative for the chromosomal phenotype (iAmpC), while 11 Enterobacter isolates tested positive (Table 3).

Table 2. Distribution of AmpC-producing bacteria based on phenotypic tests and PCR

| Bacteria               | E. coli | Klebsiella spp. | Enterobacter spp. | Total |
|------------------------|---------|----------------|-------------------|-------|
|                        | Negative | Positive (*) | Negative | Positive (*) | Negative | Positive (*) |       |
| Fox resistant          | 8 (3)    | 23 (6)        | 13           | 7 (1)        | 10 (3)    | 19          | 80 (13) |
| Sensitive to FOX       | 29 (2)   | 13 (4)        | 31 (1)       | 4 (1)        | 5         | 1           | 83 (8)  |
| Total                  | 37 (5)   | 36 (10)       | 44 (1)       | 11 (2)       | 15 (3)    | 20          | 163 (21) |

(*): Isolates that tested positive for at least one phenotypic test.
The numbers in parentheses in the Table indicate the number of bacteria carrying the blaDHA gene.

Table 3. Types of phenotypic tests (plasmid and chromosomal) positive for bacteria

| Phenotypic test | Bacteria | CAM (n) | APBA (n) | AmpC disk (n) | iAmpC (n) |
|-----------------|----------|---------|----------|---------------|-----------|
|                 | Enterobacter | 14      | 4        | 2             | 11        |
|                 | Klebsiella   | 11      | 0        | 1             | 0         |
|                 | E. coli      | 31      | 18       | 12            | 3         |
|                 | Total        | 56      | 22       | 14            | 14        |

Note: Some bacteria tested positive in more than one test.

In comparison between different plasmid phenotypic methods with the presence of the blaDHA gene, the highest specificity was related to the AmpC Disk method (90.8%), and the highest sensitivity was associated with CAM (42.7%). Also, the specificity and sensitivity of the chromosomal method regarding this gene were 91.55% and 9.5%, respectively (Table 4).

Table 4. Comparison of sensitivity, specificity, and diagnostic value with the presence of blaDHA gene of each of the plasmid and chromosomal AmpC phenotypic methods

| Phenotypic diagnostic test | Specificity (%) | Sensitivity (%) | Diagnostic value |
|---------------------------|-----------------|----------------|------------------|
| CAM                       | 66.7            | 42.9           | 1.5              |
| APBA                      | 86.6            | 28.7           | 2.1              |
| AmpC disk                 | 90.8            | 9.5            | 1.01             |
| iAmpC                     | 91.55           | 9.5            | 1.1              |
4. Discussion

In the present study, the prevalence of bacteria suspected of producing AmpC based on the FOX resistance method was 49 (49.1%). Also, using phenotypic methods, the number of AmpC-producing bacteria was 31 isolates of Enterobacter (19%), 12 isolates of Klebsiella (7.4%), and 62 isolates of E. coli. The blaFOX and blaMOX genes were not observed in the studied bacteria, while the blaDHA gene was reported in 3 Enterobacteriaceae (1.8%), 3 Klebsiella (1.8%), and 15 E. coli (9.2%).

In 2017, Qanavati et al. reported a prevalence of suspected Pneumoniae producing AmpC, 52% based on cefoxitin resistance test and 52% based on confirmatory phenotypic methods. Also, in these bacteria, the presence of blaDHA and blaFOX genes were 7.8% and 0.98%, respectively (25). In another study by the same researcher in 2018, on Enterobacteria isolated from different clinical specimens, 84.3% of the isolates were reported to be resistant to cefoxitin and (6.6%) had two bacteria carrying blaDHA genes. Still, blaFOX and blaMOX genes were not observed (26).

In a study in Qazvin in 2021, Robatjazi et al. found that 18.4% of E. coli bacteria and 30.4% of K. pneumoniae were resistant to cefoxitin; the blaDHA gene was identified in 8.7% and 6.34% of the isolates, respectively (27). The observed differences between the present study and other studies may be due to differences in the studied populations in different geographical areas.

According to the CLSI protocol, cefoxitin-resistant isolates (FOX) can be considered susceptible strains to pAmpC production. In the present study, 55% of cefoxitin-resistant isolates tested positive for at least one of the plasmid phenotypic tests, and the blaDHA gene was reported in 15% of them.

In other studies, bacteria carrying different pAmpC genes responded differently to cefoxitin (resistance or susceptibility). In Egypt (2018), 28.5% of cefoxitin-resistant bacteria had blaDHA, blaMOX, and blaCIT genes (12). But in Malaysia (2016), 64% of cefoxitin-resistant bacteria did not have blaFOX, blaMOX, blaMIR / blaACT, blaDHA genes (6). Cefoxitin resistance is a sensitive but not distinct method for isolating pAmpC-producing bacteria. Because its mechanism is to reduce the permeability of the outer membrane of bacteria, it can lead to resistance in the presence of a group of carbapenemase enzymes (10). In the present study, it was observed that 19.2% of cefoxitin-sensitive bacteria were positive in at least one of the plasmid phenotypic tests, and 18.7% of them had the blaDHA gene. It seems that cefoxitin sensitivity is not a sign of not producing pAmpC.

Based on the observations of Reuland et al., the bacteria carrying the ACC-1bla gene were sensitive to cefoxitin, thus supposed to be negative for pAmpC production by this method. This is a critical point as this gene was identified in bacteria in studies in several European countries and caused many problems in Garches in France (28).

Another finding of the present study was the lack of production of the chromosomal AmpC enzyme in Klebsiella. According to Dutch national guidelines, the chromosomal type AmpC (induction) is generally predominant in bacteria such as Enterobacter species. Still, chromosomal type (induction) is unusual or does not exist in Klebsiella and E. coli species (10).

Due to the lack of reliable laboratory diagnostic methods and the low specificity of existing phenotypic methods for pAmpC detection, the exact prevalence of β-lactamase generators is not available (6). In the present study, among the three plasmid phenotypic methods for detecting these generators concerning the blaDHA gene, the AmpCDisk method exhibits the highest specificity (90.8%), and the CAM method demonstrates the highest sensitivity (42.9%). Also, the diagnostic value (DOR) of the APBA (boronic acid) method (1/2) to separate AmpC generators is more than the other two methods. The iAmpC detection method (induction also in the detection of true negative cases (91.5%)) is much more successful than the real positive (9.5%) and has an acceptable diagnostic value (more than one) in the detection of chromosomal generators of the AmpC enzyme.

In other studies, to select the appropriate phenotypic method for the identification of this enzyme in the family Enterobacteriaceae, in Egypt in 2014 the boronic acid method (14), and in 2018 the CC-DDS method (12), in Turkey (2013), the boronic acid method (29), and in New Delhi (2018), the use of FOX disk (4) were the successful techniques in separating AmpC generators.

Comparing the above studies, it seems that the presence of different genes is effective in the quality of diagnosis of confirmatory phenotypic methods, and in general, these methods can better detect actual negative cases (specificity higher than 90%).

In recent years, excessive use of antibiotics in humans and animals globally, especially in developing countries, has contributed to the development of MDR bacteria (30, 31).

Another finding of this study is the high frequency of positive MDR bacteria (40.5%). Also, in a study by Uzunović et al. on the same bacteria used in this study, 10 isolates (6.1%) (out of 163 bacteria) carried the blaDHA gene simultaneously with the blaCTX gene,
and all of them were MDR. In the study of Uzunovic (2009-2010) in Bosnia and Herzegovina, this concurrence was 45.4% [32]. Considering the increasing growth of MDR bacteria in recent years and the evolution of plasmid-dependent blaDHA genes in Enterobacteriaceae isolates (87.5% of carriers of MIR / DHA genes in Malaysia), investigating AmpC plasmid genes acting as repositories in the emergence of antibiotic resistance and controlling infections in hospitals (6) seem necessary.

Due to the unavailability of simple and high-quality diagnostic methods, many pAmpC and iAmpC-producing organisms remain unknown. A standard antibiogram should be performed to prevent the growth of this type of bacteria in the world.

5. Conclusion

The present study's findings indicated the presence of AmpC-producing isolates in the city of Babol, Iran. Due to the presence of the blaDHA gene even in FOX-sensitive isolates, the use of molecular methods to identify AmpC-producing bacteria in this region is essential.

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Authors’ Contribution

Ms. Shahandeh and Ms. Sedighiyan were involved in collecting samples, conducting experiments, analyzing the results, and writing the article. Ms. Kalantari has been involved in analyzing the results and correcting the article.

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Conflict of Interest

There is no conflict of interest between the authors.

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