



Prevalence of AmpC type extended spectrum beta lactamases genes in clinical Samples of *E.coli* Isolated from Poultry and Humans

Elham Farrokhnazar¹, Soheila Moradi Bidhendi², and Sajedeh Karimi³

¹MS.c., Department of Biology, Faculty of Microbiology, Islamic Azad University, Tehran North Branch, Tehran, Iran

²Assistant Professor, Department of Microbiology, Razi Vaccine and Serum Research Institute, Karaj, Iran

³MS c, Faculty of Medicine, Department of Microbiology, Clinical Microbiology Research Center, Ilam University of Medical Sciences, Ilam, Iran
Corresponding Email: s.bidhendi@rvsri.ac.ir

ABSTRACT

Emergence of antibiotic resistance among pathogens, particularly in health centers and hospitals, has become a major public health problem. This study identified AmpC-type beta-lactamase against the antibiotic ceftazidime, cefotaxime and cefpodoxime in *E.coli* isolated from human and poultry and types of producing genes were studied by PCR. In this study, 500 clinical human samples of urine from hospitals of Tehran during 5 months as well as 300 poultry samples were collected and transferred to the microbiology laboratory. Biochemical tests such as TSI, Urea and IMViC were performed on suspected colonies with *E.coli*. To identify ESBL producing strains, beta-lactamase samples were cultured on Mueller-Hinton agar through antimicrobial susceptibility test by disk agar diffusion based on the standard CLSI for initial screening. PCR reactions were done using specific primers CITM, EBCM, DHAM and MOXM to identify the beta-lactamase AmpC. A number of 200 human and 55 poultry *E.coli* samples were screened. In human samples, 105 (52.5%) were resistant and potential producers of ESBL and AmpC; out of those, 102 (51%) produced ESBL and 3 (1.5%) potentially produced AmpC. In the study on 55 *E.coli* isolates from poultry samples based on the results of disk agar diffusion test, 4 (7.2%) samples were resistant and potential producers of ESBL. None of the samples were AmpC producers. Through PCR, 2 human samples (1%) were CITM positive and one sample (0.5%) was DHAM positive. Through the PCR carried out on poultry samples, there were no bands with 4 primers. There was AmpC in human samples; while further studies are required for poultry samples, because poultry significantly contribute in production of food for humans and can be an important source for dissemination of antibiotic resistance. Given the significance of Ampc in providing high levels of beta-lactam antibiotic resistance, particularly third generation cephalosporins which are very common treatments, more extensive research is recommended.

Keywords: AmpC, *E.coli*, spectrum beta, Poultry, Humans

INTRODUCTION

Escherichia coli [*E.coli*] are Gram-negative bacteria belonging to the Enterobacteriaceae family which can be found in diverse environments. These bacteria are part of the normal intestinal flora. *E.coli* is the most common cause of urinary tract infection. *E.coli* is transmitted through fecal-oral route from one person to another. The bacteria can also cause disease in animals such as poultry. This disease is called colibacillosis. Once these bacteria affect humans or poultry, one treatment is antibiotics [1].

The emergence of antibiotic resistance among pathogens, particularly in health centers and hospitals, has become a major public health problem. Bacteria use different strategies to remain immune from the deleterious effects of antibiotics. One of the most important of these mechanisms used by Gram-negative bacteria against beta-lactam antibiotics is to produce beta-lactamase enzymes [2]. Beta-lactamases are enzymes that hydrolyze and disable the beta-lactam ring [3]. The emergence of new antibiotics such as extended-spectrum cephalosporins, aztreonam and their extensive use in treatment of bacterial infectious diseases have led to the emergence of a new class of enzymes called Extended spectrum beta-lactamase [ESBL] [4].

According to Ambler Classification [1980], beta-lactamase enzymes are classified into four original classes as A, B, C and D based on their function [5].

AmpC-type beta-actamase appeared in the late 1970s and was studied [6]. This group of beta lactamases is classified in Class C of the Ambler Classification [5]. Most of these enzymes are cephalosporinases, while can partially hydrolyze other beta-lactams. These enzymes hydrolyze oxy-immunocephalosporins such as ceftazidime, ceftriaxone, cefepime cefpodoxime and monobactams such as aztreonam and cephamycins such as ceftiofur. They are lowly sensitive or simply resistant to carbapenem, while they are not inhibited by ordinary inhibitors such as clavulanates [7].

Resistance was first emerged in organisms such as *Enterobacter Cloacae*, *Citrobacter freundii*, *Serratia marcescens* and *Pseudomonas aeruginosa* due to additional production of chromosomal *AmpC*. This enzyme caused bacterial resistance against 7-alpha-methoxy-cephalosporins and monobactams.

Then, resistance appeared in bacterial species which lacked inducible *AmpC*, such as *Klebsiella pneumonia*, *E.coli*, *Salmonella* spp and *Proteus mirabilis*. It was determined that the resistance was caused by *ESBL*-encoding plasmids [8-10]. This beta-lactamase was first identified and defined in 1983 [11, 12]. According to Ambler Classification, they belong to the class A and D [5]. They generally include beta-lactamases which are able to hydrolyze and disable beta-lactam common antibiotics, such as penicillins, oxy-amino cephalosporins, monobactams and even carbapenems. However, they cannot hydrolyze cephamycins such as ceftiofur which are inhibited by clavulanic acid [11]. Common *ESBL* genes include *OXA*, *CTX-M*, *SHV* and *TEM* [5]. Infections with *ESBL*-producing bacteria through widespread dissemination of these strains, particularly in hospitals, increase the cost of treatment and the length of stay. Currently, *ESBL*-producing *Enterobacteriaceae* is a growing concern throughout the world. In addition, *ESBL*-producing bacteria are frequently resistant to many non-beta-lactam drugs such as quinolones, aminoglycosides, trimethoprim and sulfamethoxazole. This causes many problems in treatment of infections caused by them [12].

Following 7-alpha-methoxy-cephalosporins [ceftiofur and cefotetan] and clinical introduction of beta-lactam inhibiting compounds [clavulanate with amoxicillin or ticarcillin, sulbactam with ampicillin, and tazobactam or piperacillin], Class C beta-lactam encoding plasmids appeared [13]. In the late 1980s, these inducible chromosomal genes appeared on plasmids and transmitted to those organisms which naturally did not express this beta-lactamase such as *Klebsiella* species, *E.coli* or *Salmonella* species and caused the resistance of these organisms to oxy-immuno cephalosporins [7]. Currently, the increasing prevalence of resistance to beta-lactam antibiotics through *AmpC*-type beta-lactamases among *E.coli* strains has become a clinical concern. These organisms can acquire the ability to produce *AmpC*-type beta-lactamase on the plasmid. In addition, they can produce a large amount of chromosomal *AmpC*-type beta-lactamase in some clinical samples, while these enzymes are normally produced in small quantities [14]. This ability is observed in ceftiofur resistant samples, with stronger *AmpC* promoter or mutation in normal attenuated [15, 16].

AmpC genes include *CMY*, *FOX*, *MOX*, *ACT*, *ACC*, *MIR-1*, *DHA*, and *BIL-1* [5]. *CMY-2* is the most common *AmpC* enzyme [17]. *AmpC* genes were named contradictory (5) based on resistance product: cephamycins (*CMY*) ceftiofur (*FOX*), moxalactam (*MOX*).

AmpC gene is normally a part of intrusion, while it is not included in the gene cassette with an affiliated 59-base element. Note that the same *bla_{AmpC}* gene can be incorporated in different frameworks in different plasmids.

This study detects *AmpC*-type beta-lactamase against antibiotic ceftazidime, cefotaxime and cefpodoxime in *E.coli* and examines a variety of *AmpC*-producing genes by PCR method.

MATERIALS AND METHODS

For human studies, 500 clinical samples of urine were collected in five months from June to November 2012, from Imam Khomeini and Amir Alam hospitals as well as Boghrat and Imam Hassan clinics in Tehran and transferred to the microbiology. For poultry samples, 300 cloacal swabs were taken from poultry and transferred to the microbiology of Razi Vaccine and Serum Institute.

The samples were cultured on the Blood Agar medium and differential EMB medium. The plates were incubated at 37°C for 24 hours. Biochemical tests such as TSI, Urea and IMViC were performed on suspected colonies with *E.coli* and differential media were kept at 37°C for 24 hours. Verified samples were kept at -20°C in TSB with glycerol to be used in the later stages (18). To detect beta-lactamase-producing strains, beta-lactamase enzyme samples were cultured on Mueller-Hinton agar primarily through antimicrobial susceptibility test (disk agar diffusion) based on the CLSI standard for initial screening.

In this method, the bacterial suspension was prepared considering the concentration of the half McFarland pipe and completely spread on Mueller-Hinton agar. Then, antibiotic discs acquired from the Mast Company containing 30µg cefotaxime (CTX), 30µg ceftazidime (CAZ) and 30µg cefpodoxime (CPD) were placed onto the medium at least 2cm from each other; after 24 hours of incubation at 37°C, the inhibition zone around each disc was measured by a ruler and compared to the international standards (CLSI) (19). Ceftazidime, cefotaxime and cefpodoxime resistant samples were examined by the combined disk test to verify the presence of *ESBL*. In this test, the 30µg ceftazidime, 10-30µg ceftazidime-clavulanic acid (CAZ-CV), 30µg cefotaxime, 10-30µg cefotaxime-clavulanic acid (CTX-CV), 30µg cefpodoxime, and 10-30µg cefpodoxime-clavulanic acid (CPD-CV) disks, acquired from Mast Company, were placed on Mueller Hinton agar. In each series of experiments, the suspension prepared from 24 hours culture of standard *E.coli* strains (*E.coli* ATCC 25922) and *klebsiella pneumoniae* (*klebsiella pneumonia* ATCC 700603) was used (20). After 24 hours of incubation at 37°C, inhibition zone was measured around the disc containing clavulanic acid as *ESBL* inhibitor compared to the disc lacking clavulanic acid. If the inhibition zone was ≥ 5 mm around the disc containing clavulanic acid compared to the disc lacking clavulanic acid, the strain was considered as *ESBL* producer (21). According to CSLI, the samples in which beta-lactamase was not inhibited by the beta-lactamase inhibitor in the phenotypic verification test were potentially considered as beta-lactamase *AmpC* producers. These samples were also resistant to the discs containing clavulanic acid (22).

Polymerase Chain Reaction

Used buffers included TAE, ethidium bromide and the DNA loading buffer.

Materials: to determine DNA template, the genomic DNA purified from *E.coli* was used in this study. This molecule acts as a template strand for the enzyme *taq*. In addition, 10mM Deoxynucleotide triphosphate (dNTP) was used. The final concentration of each nucleotide was 50mM in PCR. The enzyme DNA Taq polymerase is a polypeptide with DNA polymerase activity 5'→3'. For this enzyme, the optimal temperature is 70°C and optimal pH optimum is 9. DNA Taq polymerase, Metabion Company, was used in this study.

The primers CITM, EBCM, DHAM and MOXM were used to perform polymerase chain reaction (Table 1).

Table 1: designation, sequence, target genes and primers used in this study, and the molecular weight of the PCR product resulting from these primers (23)

primer	DNA(5' TO 3')	Target	Amplicon size
MOXM-F MOXM-R	GCT GCT CAA GGA GCA CAG GAT CAC ATT GAC ATA GGT GTG GTG G	CMY-1,CMY-8 toCMY-11 MOX-1, MOX-2	520
CITM-F CITM-R	TGG CCA GAA CTG ACA GGC AAA TTT CTC CTG AAC GTG GCT GGC	LAT-1 TO LAT-4,CMY-2 TO CMY- 7,BIL-1	462
DHAM-F DHAM- R	AAC TTT CAC AGG TGT GCT GGG T CCG TAC GCA TAC TGG CTT TGC	DHA-1, DHA-2	405
EBCM-F EBCM- R	TCG GTA AAG CCG ATG TTG CGG CTT CCA CTG CGG CTG CCA GTT	MIR-1T, ACT-1	302

To prevent pollution, waste of reagents and increased accuracy, PCR mixture was prepared every day for each sample considering 25µl reaction volume.

PCR reaction was performed in a final volume of 25 μ l. Following materials were added to the Eppendorf tubes (Table 2)

Table 2: The materials needed to perform PCR reaction

PCR mixture	The amount of material
1) PCR water	18 μ l
2) PCR buffer	2.5 μ l
3) dNTPs	1 μ l
4) Forward primer	1 μ l
5) Reverse primer	1 μ l
6) Genomic DNA	1 μ l
7) Taq polymerase enzyme	0.5 μ l

Once the primers were added, vortex was performed. Then, genomic DNA and Taq polymerase enzyme were added and vortex was again performed. The mixture was immediately transferred to PCR device.

PCR reaction was performed using specific primers as detailed in Table 3. PCR mixture was amplified in a thermocycler.

Table 3: thermal and temporal profile of PCR performed in this study

Reaction steps	Heat ($^{\circ}$ C)	Time (s)
Pre-denaturation	94	180
Denaturation	94	30
Annealing	64	30
Extension	72	60
Final Extension	72	420

Finally, the PCR product was electrophoresed using 1% agarose gel.

RESULTS

In this study, 500 human urine samples were collected from Imam Khomeini Hospital, Amir Alam Hospital, Boghrat Clinic and Imam Hassan Clinic. Moreover, 300 poultry samples were taken from Razi Vaccine and Serum Institute. First, samples were cultured on Blood Agar and EMB media and incubated at 37 $^{\circ}$ C for 24 hours. According to the shape and color of colonies on Blood Agar and EMB media as well as biochemical tests, 200 human *E.coli* samples and 55 poultry *E.coli* samples were screened.

Resistance of 200 *E.coli* samples was examined against three antibiotic cefotaxime, ceftazidime and cefpodoxime. The results are shown in Table 4 and Figure 1.

Table 4: Number and percentage of sensitive, moderately sensitive and CAZ, CPD and CTX resistant E.coli in human samples

Antibiotics	Resistant (%)	Moderate (%)	Sensitive (%)
Cefpodoxime	92 (46%)	15 (7.5%)	93 (46.5%)
Ceftazidime	89 (44.5%)	25 (12.5%)	102 (51%)
Cefotaxime	98 (49%)	11 (5.5%)	91 (45.5%)

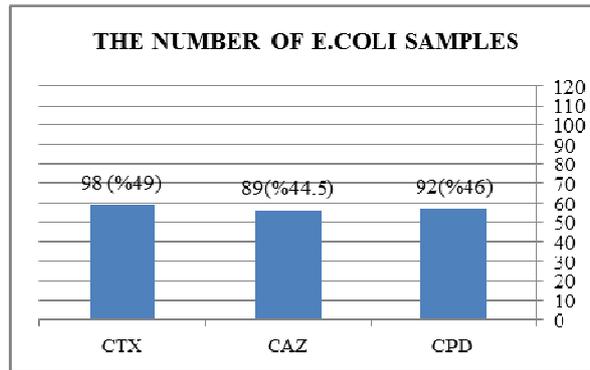


Figure 1: Antibiotic-resistant strains studied in human samples

According to results of disk agar diffusion test, 105 (52.5%) samples were simultaneously resistant to all three antibiotic cefpodoxime, ceftazidime and cefotaxime and potential *ESBL* and *AmpC* producers. The rest 95 strains (47.5%) were ultrasensitive or semi-sensitive to all three antibiotics and considered negative in terms of *ESBL* and *AmpC* production. Verification test was performed using clavulanic acid on 105 samples which were potential *ESBL* and *AmpC* producers; of those, 102 (51%) strains were sensitive to three antibiotics, cefpodoxime-clavulanic acid and ceftazidime-clavulanic acid and cefotaxime-clavulanic acid. These 102 strains were verified for producing *ESBL*. Moreover, 3 (1.5%) strains were resistant to all three antibiotic cefpodoxime-clavulanic acid, ceftazidime-clavulanic acid and cefotaxime-clavulanic acid; these strains were potential producers of *AmpC* (Figure 2, 3 and 4).

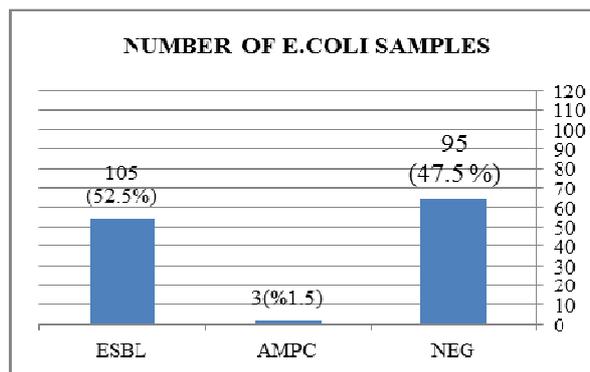


Figure 2: frequency of ESBL and AmpC producing human E.coli samples

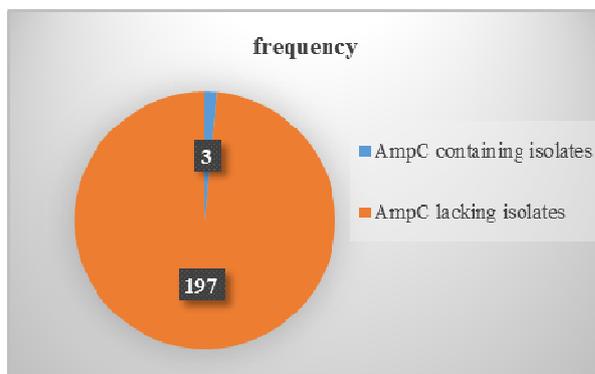


Figure 3: frequency of AmpC-producing human E.coli samples

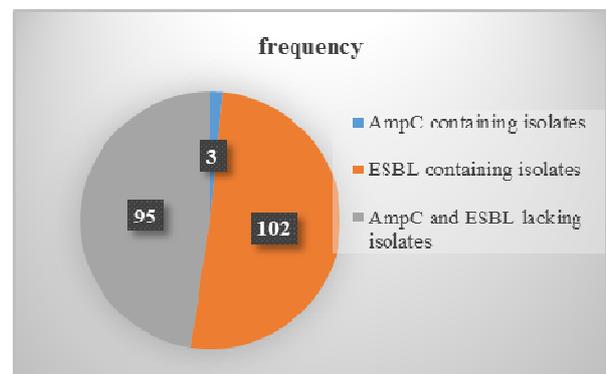


Figure 4: frequency of ESBL- and AmpC-producing human E.coli samples

According to results of disk agar diffusion test, 4 (7.2%) of poultry *E.coli* samples were simultaneously resistant to all three antibiotic cefpodoxime, ceftazidime and cefotaxime and potential *ESBL* and *AmpC* producers. The rest 51 (92%) were sensitive or semi-sensitive to all three antibiotics and considered negative in terms of *ESBL* and *AmpC* production. Verification test was performed using clavulanic acid on 4 samples which were potential *ESBL* and *AmpC* producers; of those, 4 (7.2%) strains were sensitive to three antibiotics, cefpodoxime-clavulanic acid and ceftazidime-clavulanic acid and cefotaxime-clavulanic acid. These 4 strains were verified for producing *ESBL*. None of the strains was resistant to above antibiotics and potential producer of *AmpC* (Figure 5).

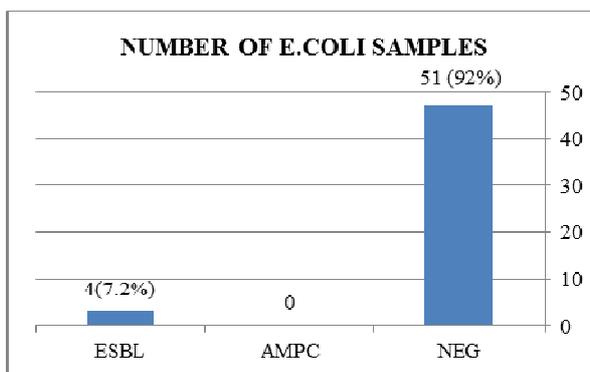


Figure 5: frequency of ESBL and AmpC producing poultry E.coli samples

PCR was carried out on 105 clinical human samples which were beta-lactamase producing candidates. Four primers, CITM, EBCM, DHAM and MOXM were used to find *AmpC*-type beta-lactamase gene. Three (1.5%) of 200 *E.coli* isolates had *AmpC*-type beta-lactamase gene. In PCRs performed with the primer CITM, two of the samples form 480bp bands on the agarose gel; these bands resulted from the primer CITM which targeted the genes *LAT-1*, *LAT-4*, *BIL-1* and *CMY-1* to *CMY-7*. Given that the prevalence of *CMY* genes, particularly *CMY-2*, is higher than other genes, these samples are likely to have one of the *CMY* genes. In PCRs performed with the primer DHAM, one of the samples formed 40bp bands on the agarose gel; these bands resulted from the primer DHAM which targeted the genes *DHA-1* and *DHA-2*. In PCRs conducted with MOX, there was no band. MOXM targeted the genes *CMY-1*, *CMY-8* to *CMY-11* and *MOX-1* and *MOX-2*. In PCRs performed with EBCM, no band was observed. This primer targeted the genes *MIR-1T* and *ACT-1* (Figure 6).

PCR was performed on 4 poultry samples which were beta-lactamase producing candidates. The primers used included CITM, EBCM, DHAM and MOXM for detecting *AmpC*-type genes. In PCRs performed with the primers CITM, EBCM, DHAM and MOXM, no band was observed (Figure 7 and 8).

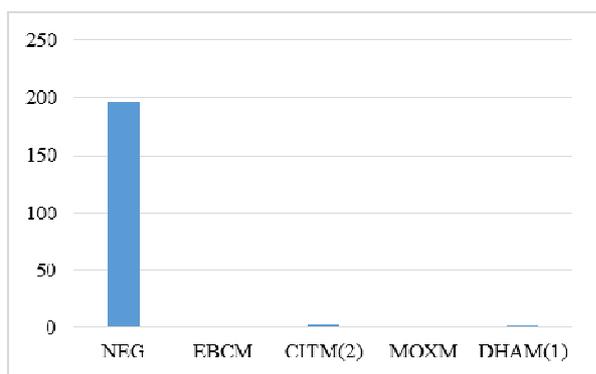


Figure 6: frequency of AmpC-producing human E.coli samples

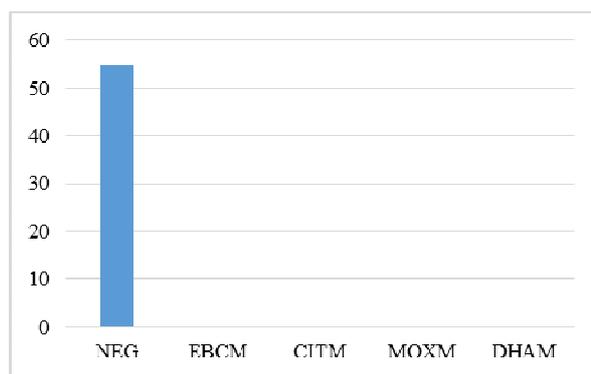


Figure 7: frequency of AmpC-producing poultry E.coli samples



Figure 8: results of electrophoresis from PCR on samples; Column 1: marker size (100bp); Column 2: positive control (*Klebsiella pneumoniae* ATCC700603); Column 3: negative control; Columns 4 and 6: human samples resulting from the primer CITM; Column 7: human samples resulting from the primer DHAM

DISCUSSION

E.coli is the most common cause of urinary tract infection, accounting for nearly 90% of early urinary tract infections in young women. Currently, urinary tract infection (UTI) is one of the most common problems encountered in practice with a wide range of clinical wards dealing with it. Community-acquired and symptomless UTI is due to *E.coli* in most cases; otherwise, it can result from other Enterobacteriaceae, such as *Klebsiella* and *Proteus* (24). In hospitalized UTI patients with symptoms or people with a history of antibiotic use or with a urethral catheter, *E.coli* is still known as the most common cause (25). Beta-lactamase enzymes hydrolyze beta-lactam ring and disable beta-lactam antibiotics (26). Beta-lactam antibiotics are the best choice for treatment of many bacteria. In the past two decades, many new beta-lactam antibiotics have been developed which were specifically resistant to hydrolyzing beta-lactamase enzymes. Despite this new group of antibiotics used to treat patients, new types of beta-lactamase enzymes such as *ESBLs* and *AmpC*-type beta-lactamases have emerged. Gram-negative bacteria with many new beta-lactamases described above could acquire resistance to most beta-lactam antibiotics (27).

In this study, 200 out of 500 human samples and 55 out of 200 poultry samples were *E.coli*. In human samples, 105 (52.5%) strains were resistant and potential *ESBL* and *AmpC* producers; of those, 102 (51%) strains were *ESBL* producers. Moreover, 3 (1.5%) strains were potential *AmpC* producers. In the study on 55 *E.coli* samples isolated from poultry samples based on the results of disk diffusion agar test, 4 (7.2%) strains were resistant and potential producer of *ESBL*. None of the strains was potential *AmpC* producer. In PCR on human samples, 2 (1%) strains were CITM positive and 1 (0.5%) strain was DHAM positive. In PCRs carried out on poultry samples, no band was found with four primers.

Savara et al (2010) showed *ESBL*-producing *E.coli* in 34% of isolates from broilers in Sweden (30). Reviews showed that *ESBL* and *AmpC* beta-lactamase resistance of the samples was higher than broilers in Iran. Dierikx et al (2010) conducted a study on broilers in Netherlands and showed that 80% of isolates were *E.coli* producing antibiotic resistance genes (28).

Their results compared to the current study (7.2% resistance) can be attributed to the number of samples worked which was higher than the number of *E.coli* samples or could be due to a higher incidence of resistant *E.coli* with beta-lactamase genes in the Netherlands compared to Iran. The meat imported can be an important source of *E.coli* with beta-lactamase genes.

Borjesson et al (2011) in Sweden showed that 92% of the meat imported from South America had beta-lactamase producing *E.coli*, which was very high compared with meat imported from Europe (19%). Studying antibiotic resistance in the isolates, they showed that *E.coli* isolates from South America were resistant to a wider range of antibiotics and had a higher variation of beta-lactamase genes (29).

Their results were not consistent with the current study where the prevalence of beta-lactamase genes was 7.2% in Iran, which is lower than the prevalence of these genes in South America (92%) and parts of Europe (19%). This is

due to the difference in the prevalence of beta-lactamase genes in different countries. The results show that the imported meat is an important source of *E.coli* with beta-lactamase genes.

Momtaz *et al* (2012) determined the distribution of antibiotic resistant genes in *E.coli* isolates from commercial chickens in Iran using PCR techniques. Of 360 samples isolated, 57 (15.8%) *E.coli* was isolated. No known genes associated with resistance to streptomycin, Cephalotin and ampicillin was recognized and no *bla CMY* beta-lactamase gene was detected (30).

They isolated 55 (18.3%) *E.coli* from 300 poultry samples. Disk agar diffusion and PCR was used and the results obtained showed the absence of *CMY* gene, which is consistent with the current study.

Mooljunttee *et al* (2010) studied 30 *E.coli* samples obtained from broilers in Thailand. All *E.coli* samples isolated from broilers were resistant to tetracycline, ampicillin and erythromycin, associated with 90%, 93.3% and 73.3% prevalence for the genes *tet(A)*, *bla cmy* and *ere(A)*, respectively. In this study, antibiotic sensitivity tests showed 100% antimicrobial resistance to ampicillin, erythromycin and tetracycline which are often used in Thai broiler industry. This contrasts with zero percent antimicrobial resistance to gentamicin and chloramphenicol which are two types of antimicrobial agents less common in Thailand's poultry industry (31).

The methods used in their research (disk agar diffusion and PCR) were consistent with the methods used in this study; however, their results were inconsistent with the current study. The current study detected *ESBL*-type beta-lactamase gene in 7.2% of samples, while no *AmpC*-type beta-lactamase gene (*bla cmy*) was detected in *E.coli* samples isolated from poultry. This may be due to the fact that the antibiotics related to this resistance are used more in Thailand.

Olusoga *et al* (2013) conducted a study on 114 samples of *Klebsiella* (60 samples) and *E.coli* (54 samples) in an outpatient hospital in southern Nigeria (32). Similar to the current study, they used cephalosporin and those antibiotics with clavulanic acid. They used cephalosporin disks of cefepime and cefpodoxime, while the current study used cefpodoxime, ceftazidime and cefotaxime. Once the initial screening was performed, similar to the current study, PCR was performed. To detect *AmpC* genes, they also used the primers CITM and EBCM to identify 462bp and 302bp bands. In their study, 3 (5.6%) of *E.coli* samples had *AmpC* gene, while the current study found 3 (1.5%) samples with *AmpC* gene. These results are nearly similar.

Shayan *et al* (2013) examined *AmpC* resistance of *E.coli* bacteria to the third-generation of cephalosporins and ceftazidime by PCR with the primers FOXM, MOXM, DHAM, CITM and EBCM. The current study also used the primers MOXM, DHAM, CITM and EBCM. Out of 392 *E.coli* samples, 13 (3.3%) produced *AmpC* genes, which is similar to the current study (3 samples, 1.5%). This represents similar resistance in these two studies. Of these 13 samples, 11 (84%) were CITM positive. In the current study, 2 (66.6%) out of 3 samples were CITM positive; these results are nearly consistent (33).

Coşkun (2012) detected 50 bacteria (42 *E.coli* and 8 *Klebsiella*) resistant to ceftazidime in Turkey (34). Of these, 25 (50%) were CITM positive, while the current study found 2 (1%) samples with CITM positive; this suggests greater prevalence of these bacteria in Turkey. In their study, one sample was detected with EBCM positive and one sample was detected with MOXM positive, while the current study detected no sample with EBCM and MOXM positive. They detected no sample with DHAM positive, while the current study detected one sample with DHAM (34).

Pitout *et al* (2000-2003) isolated 78275 *E.coli* samples from the patients in Canada. Of them, 72756 (93%) were community-acquired and 5519 (7%) were hospital-acquired. They detected 408 (0.7%) ceftazidime resistant *E.coli* samples. According to disk screening test, 369 (96%) of them had *AmpC*-type beta-lactamase gene. PCR tests on ceftazidime resistant samples showed that 125 (34%) of them had *CMY-2* gene and 0.05% had *AmpC*-type beta-lactamase genes among which 125 (0.16 %) had *bla_{cmy-2}* gene (35).

Their results are slightly different from the current study, which may be due to the fact that the beta-lactamase genes increasingly spread among the human population. Their study was conducted in 2000 to 2003, while the current study was conducted in 2013.

Mulvey et al (1999-2000) isolated 29323 *E.coli* strains from 12 hospitals in Canada, among which 234 *E.coli* isolates were resistant to cefoxitin. They also used the disk agar diffusion method; however, they only used cefoxitin disk to detect samples containing *AmpC*-type beta-lactamase. Of the samples resistant to cefoxitin, 25 (10.8%) had *AmpC*-type beta-lactamase with *CMY-2* gene (36). This number is higher than the current study (5.1%), which can be due to the higher number of samples.

Hopkins et al (1995-2003) studied 103 *E.coli* samples resistant to cephalosporins in the England; they found 37 species resistant to cefoxitin, of which 25 (24%) had *AmpC*-type beta-lactamase gene (37). This number is higher than the current study (5.1%) which can be due to differences in years of study.

Gazoli et al (1996) studied 2133 *E.coli* isolates from patients in 10 hospitals in Greece; they found 63 isolates resistant to cefoxitin. PCR found 55 (2.57%) species resistant to cefoxitin with *AmpC*-type beta-lactamase genes (38). Their results are close to the current study.

Similar to the current study, they also used the disk agar diffusion method to detect the samples with *AmpC*-type beta-lactamase gene. However, they only used cefoxitin disk, while the current study used cefotaxime, ceftazidime and cefpodoxime disks plus clavulanic acid. Their results (2.57% *AmpC* genes *LAT-1* and *LAT-4*) were nearly consistent to the current study (1% *AmpC* gene *LAI-4* and *LAI-1*).

Soltan Dallal et al (2010) conducted a study on 500 clinical samples in Tehran and detected 200 *E.coli* bacteria. Of them, 128 (64%) were resistant to cefotaxime and ceftazidime. Using clavulanic acid, 115 (89.8%) strains were candidate for production of *ESBL* and 13 (10.2%) strains were candidate for production of *AmpC*. Of 128 clinical samples, 13 (10.2%) had *AmpC*-type beta-lactamase genes (39).

They used the primers CITM and FOX, while the current study used the primers CITM, EBCM, MOXM and DHAM. Using the primer CITM, the bands were observed in 13 (10.2%) strains, while the current study observed bands in 3 (1.5%) strains. These results are slightly different, which may be due to different hospitals where samples were isolated. They found no band with FOXM, while a band was observed in the current study with DHAM.

Mansouri et al (2009) conducted a study on 154 clinical isolates of *E.coli* in Tehran and showed that 57.15% of them were resistant to all three antibiotics ceftriaxone, ceftazidime and cefepime. Using clavulanic acid, 53.9% of samples were candidate for production of *ESBL* and 3.25% were candidates for *AmpC* production. The current study also used ceftazidime, cefotaxime and cefpodoxime as well as clavulanic acid. In their study, 5.7% of the samples were able to produce *AmpC*, which is consistent with the current study (1.5%). They found 3 (4.3%) band with CITM, 2 (2.2%) band with EBCM and 1 (1.9%) band with DHAM, while the current study found 2 (1%) bands with CITM, 1 (0.5%) band with DHAM and no band with EBCM. Their results are consistent with the current study (40). The results of this study showed that 1.5% prevalence of *AmpC* producing *E.coli* is acceptable in comparison with the results obtained from different parts of the world; however, the problem of antibiotic resistance in *E.coli* and other bacteria is of great importance and requires more attention.

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