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Research article

PHENOTYPIC AND MOLECULAR CHARACTERIZATION OF ANTIBIOTICS RESISTANCE *E. CLOACAE* ISOLATES

Abdalnabi J Abid* .Alaa H Alcharrakh** .HalaHusien*

*Biology Dept. College of Science for Women, Babylon university –Iraq
**Microbiology Dept. College of Medicine, Babylon University
Corresponding author: dr_almamory59@yahoo.com

ABSTRACT: The present study aimed detecting and characterizing of β-lactamases producing *E.cloacae* isolated from different clinical sources in Hilla hospitals using phenotypic and molecular methods. A total of 308 samples were collected from two major hospitals at Hilla Province from October 2013 to April 2014. All isolates were tested biochemically, it was found that only 15 isolates from all isolates were belonging to *Enterobactercloacae*. All *E. cloacae* isolates were primarily screened for β-lactams resistance. Antibiotic susceptibility and minimum inhibitory concentration tests were performed using disk diffusion and agar dilution methods, respectively. The molecular study documented a widespread of *Amp C* genes among isolates of *E. cloacae* isolatesrepresented by 6/15(40%) positive isolates for *Amp C* primers. PCR assay revealed that prevalence rate of *bla*-$_{TEM}$ gene among tested isolates was 9(60%). followed by the *bla*-$_{OXA}$ gene was detected only in 3(20%).While *bla*-$_{VEB}$ gene and *bla*-$_{SHV}$ gene was not detected in any of the isolates. Some virulence factors of bacteria were also studied, and the results showed that all bacterial strains have capsule ,the results also also detected biofilm formation among isolates and the results revealed that 13(86%)of the isolates are biofilm former.

Key words: *E.cloacae*, ESBL, *bla.TEM*, *bla-OXA*, MIC.

INTRODUCTION

E.cloacae, a gram-negative bacterium, belongs to the family Enterobacteriaceae. It is rod –shaped bacterium, non-spore forming and facultatively anaerobic(Nishijima *et al*., 1993).The bacterium comprises part of the normal flora of the gastrointestinal tract of 40%–80% of the human population and is widely distributed in the environment(Paterson *et al*., 2005). *E. cloacae* is well - recognized as community and nosocomial pathogen that cause significant infections after the host immune system has been weakened by other infections or injury, and sometimes as a primary pathogen mainly due to its ability to develop resistance to antibiotics (Neto *et al*., 2003).*E.cloacae* causes a wide spectrum of infections involving the urinary tract, lower respiratory tract, skin and soft tissue, biliary tract, wounds, intravenous catheters, and the central nervous system infection (Karam and Heffer, 2000). Most isolates of the *E .cloacae* complex are intrinsically resistant to amoxicillin, ampicillin, amoxicillin–clavulanate, first-generation cephalosporins and cefoxitin owing to the production of constitutive AmpC β-lactamase (Stock *et al.,* 2001). Resistance to β-lactam antibiotics has been a problem throughout the history of the usage of these antimicrobial agents. The production of ß-lactamase is the most frequently encountered mechanism of bacterial resistance to ß-lactam antibiotics. Resistance to these agents has been frequently observed in various strains of *Enterobacter cloacae* that possess the ability to produce elevated levels of β-lactamases (Gootz*et al*., 1982). ESBL are mostly plasmid mediated enzymes capable of hydrolyzing and inactivating a wide variety of β-lactam antibiotics, including different types of penicillins and cephalosporins(Lautenbach*et al*.,2001). ESBLs have emerged as an important mechanism of resistance to β-lactam antibiotics in Gram negative bacteria, mostly in Enterobacteriaceae (Mendelson*etal*., 2005).Extended-spectrum β-lactamases (ESBLs) and carbapenemases have been reported to be widespread in E. cloacae (Bush 2010).

MATERIALS AND METHODS

Sample collection

A total of 308 clinical samples were collected from October 2013 to April 2014. Bacteria were isolated from a variety of sources, including blood , urine , burn, stool, ear, eye, skin and nosefrom patients visited/or admitted to two main hospitals; Hilla Teaching Hospital and Mergan teaching hospitals.

The specimens were transported quickly by sterile transport swabs to the department of bacteriology laboratory and each specimen was inoculated using direct method of inoculation on a selective media namely MacConkey agar and Blood agar, then inoculated at 37°Cfor 18-24 hours.

Bacterial growth and biochemical identification

Identification of *E. cloacae* was first done by the bacteriological methods including colonial morphology, Grams stain, and other biochemical testsCharacteristics of *E.cloacae* were subjected to biochemical tests for identification.

Production of β-Lactamase enzymes

All bacterial isolates thatresisted toβ-lactam antibiotics were tested for their ability to produce β-lactamase using nitrocefin disks.

Antibiotic Susceptibility Assay

MICs of *Enterobacter cloacae* Isolates

The two-fold agar dilution susceptibility method was used for determination of MICs of β-lactam antibiotics. Appropriate dilutions of β-lactam antibiotic solutions were prepared, in which one part of the antimicrobial solution was added to nine parts of liquid Muller-Hinton agar. The prepared dilutions of β-lactam solutions were added to the molten Muller-Hinton agar media that have been allowed to equilibrate in a water bath to 45-50°C.

The agar and antimicrobial solution mixed thoroughly and the mixture poured into petri-dishes, the agar was allowed to solidify at room temperature. A standardized inoculum for agar dilution method was prepared by growing bacteria to the turbidity of 0.5 McFarland standard. The 0.5 McFarland suspensions were diluted 1:10 in sterile normal saline. The agar plates were marked for orientation of the inoculum spots.1-µL aliquot of each inoculum was applied to the agar surface with standardized loop. Antibiotic free media were used as negative controls and inoculated. The inoculated plates were allowed to stand at room temperature (for no more than 30 minutes) until the moisture in the inoculum spots was absorbed by the agar. The plates were inverted and incubated at 37 °C for 16 to 20 hr.

To determine agar dilution break points, the plates were placed on a dark surface, and the MIC was recorded as the lowest concentration of the antimicrobial agent that completely inhibits growth (disregarding a single colony or a faint haze caused by the inoculums) or that concentration (in µg/ml) at which no more than two colonies were detected

Extended-Spectrum β-Lactamase Production

Initial Screening for ESBL Production

All bacterial isolates that were β-lactamase producing were tested for ESBL production by initial screen test. The isolate would be considered potential ESBL producer, if the inhibition zone of ceftazidime (30µg) disks was ≤ 22 mm (CLSI, 2014).

Confirmatory Test

All the β-lactamase-producing isolates were tested for confirmatory ESBL production as follows:

Disk Combination Test (Recommended by CLSI, 2014)

The phenotypic confirmation of potential ESBL-producing isolates was performed by using disk diffusion method. Cefotaxime alone and in combination with clavulanic acid, Ceftazidime alone and in combination with clavulanic acid were tested. Inhibition zone of ≥ 5 mm increase in diameter for antibiotic tested in combination with clavulanic acid versus its zone when tested alone confirms an ESBL producing isolate**.**

DNA Extraction and Purification

Plasmid DNA Extraction and Purification

A single colony of cultivated bacteria, which had been incubated overnight, transfer to 2 ml of sterile nutrient broth and incubate at 37 ◦Cfor 18-20 hours .The DNA extracted and purified using High-Speed mini DNA plasmid extraction kit (GeneaidBiotech, Korae) according to manufacture instructions.Plasmid DNAwas used to detect *TEM, SHV, OXA,* and *VEB*

Detection of*bla* Genes by Polymerase Chain Reaction

Monoplex PCR Mixture

The DNA extract (Total DNA and Plasmid) of *E.cloacae* isolates were subjected to *bla* genes by using Bioneer Monoplex PCR kit protocol depending on manufacturer's instruction .Single reaction (final reaction volume 20 µl) as in table 1. All PCR components were assembled in PCR tube and mixed on ice bag under sterile condition.

Table 1: Protocols of monoplex PCR reaction mixture volumes

PCR reaction mixture	Bioneer protocol (final volume 20µl)
Master mix	5 µl
Primer forward (10µM)	2 µl
Primer reverse (10µM)	2 µl
DNA template	5 µl
PCR grade water	6 µl

Monoplex PCR Thermocycling Conditions

The PCR tubes were placed on the PCR machine and the right PCR cycling program parameters conditions were installed as in Table 2.

Table 2: Programs of monoplex PCR thermocycling conditions

Monoplex gene	Temperature (c) / Time					Cycle number
	Initial denaturatio	Cycling condition			Final extensi	
		Denaturation	Annealing	Extension		
bla-TEM	96/5 min	96/1 min	58/1 min	72/1 min	72/10 min	35
bla-SHV	96/5 min	96/1 min	60/1 min	72/1 min	72/10 min	35
bla-OXA	96/5 min	96/1 min	60/1 min	72/1 min	72/10 min	35
bla-VEB	95/3 min	95/30 sec	64.4/30sec	72/1 min	72/3 min	35

RESULTS

Bacterial Isolates

In this study, 308 clinical samples were collected from a variety of clinical sources. Samples were then subjected for culturing on selective media to isolate *Enterobactercloacae*. A total of 308 clinical samples, only 239 samples (77.59%) showed positive cultures, whereas no growth was seen in the other (69) samples.

Among 239 culture positive samples, only 15 isolates belonged to *Enterobacter cloacae*, and 279 isolates belonged to other different genera of bacteria.

Identification of *E. cloacae* was first made by the bacteriological methods including colonial morphology, Grams stain, and other biochemical tests. Characteristics of *Enterobacter cloacae* were subjected to biochemical tests for identification.

Antibiogram Profile by Disk Diffusion Method

The susceptibility of 15 *E. cloacae* isolates against 18 selected antibiotics was studied to determine the pattern of isolates resistance to various antibiotics depending on disk diffusion method.

The results in figure represent the antibiogram profile of the isolates, indicate that isolates varied in their susceptibility to the antibiotics. All isolates were highly resistant (100%) to ampicillin. It was found that 86% of the isolates were resistant piperacillin. The rate of resistance to cephalosporins was 100% to cepholothin where as 60% of the isolates were resistant to cefoxitin. The percentages of resistance to third generation cephalosporins were as follows: 53%, ceftazidime, 66 % cefotaxime. Additionally, 6% of the isolates exhibited resistance to the fourth generation cephalosporin, cefepime. Resistance to monobactam antibiotics was moderate where 26% of isolates being resistant to aztreonam.

The lowest resistance rate was found against carbapenems. Resistance to carbapeneme antibiotics (represented by imipenem) and aminoglycoside, gentamicin, and tobramycin were 1(6%) isolates. The resistance rate of isolates to the remaining antibiotics was as follows: chloramphenicol 60%, tetracycline %, naldixic acid 26% and trimethoprim-sulfaamethoxazole 100% .

MIC determination

The results of this study indicated that all the isolates were highly resistant to ampicillin with concentration reached beyond the break point values. The MICs valueof ampicillin for most tested *E.cloacae*.3 (20%) isolates was 32µg/ml while the MIC of 12(80%) isolate was 128µg/ml.

The results presented in table 4-6 evaluate that the MIC of ceftazidime range from 0.004 to 128 µg/ml, 8(53%) had a maximum MIC value 128 µg/ml;6(40%) isolates with MIC value reached to 64 µg/ml,and one isolate (6%) with MIC rechead to 16 µg/ml. The results in table 4-6 also indicated that MIC values of cefotaxime were ranged from 0.004 to 128µg/ml. 10(66%) isolates able to grow in concentration 64 µg/ml. Also 3 (20%) exhibited MICs of 32 µg/ml, while only 2(13%) isolates was able to grow in concentration value 16µg/ml. On the other hand, (16%) of the isolates were resistant to imipenem an MIC range from 8 to 16 µg/ml, While other isolates were susceptible to imipenem a MIC ranged between 0.5-1 µg/ml.

Detection of β-Lactamase Producing Isolates

The presence of β-lactamase in theβ-lactam resistant *E.cloacae* isolates was examined by the nitrocefin disk method. The results revealed that among the 15 isolates tested, 12(80%) produced β-lactamase, by changing color of the nitrocefin disk from yellow to reddish-orange within a range from a few seconds to 15 minutes (figure 1) .

Figure 1: β-lactamase detection by nitrocefin disk method; isolates of *E. cloacae*exhibited positive test (red disks).

Production of Extended-Spectrum β-Lactamases

E.cloacae resistant to β-lactam antibioticsaresuspected to be highly producers of ESBLs; therefore, all were subjected to ESBLs production test. Performance of the test isolates in the ESBL initial screen disk test was assessed using ceftazidime disks. According to the CLSI (2014) the isolate is considered to be a potential ESBL producer, if the inhibition zone of ceftazidime disks (30 µg) was ≤ 22 mm. The study found that (53%) of the 15 *E.clocae*. isolates were ESBL positive during the initial screening using ceftazidime disk (Figure 3), which considered as suspected of ESBL producing *E.cloacae*. The detection of ESBL producing isolates was performed using disk combination method. In this method ceftazidime and cefotaxime disks were combined with clavulanic acid and compared to ceftazidim and cefotaxime disks each alone. The isolate was considered ESBL producer, when the inhibition zone of combined disks was more than or equal to 5 mm increased than the inhibition zone of disk alone (Figure 4-). The results in this regard were as follows that out of the 15*E.cloacae* isolates β-lactamase producers, 11(73%) exhibited zones enhancement with clavulanic acid, confirming their ESBL production.

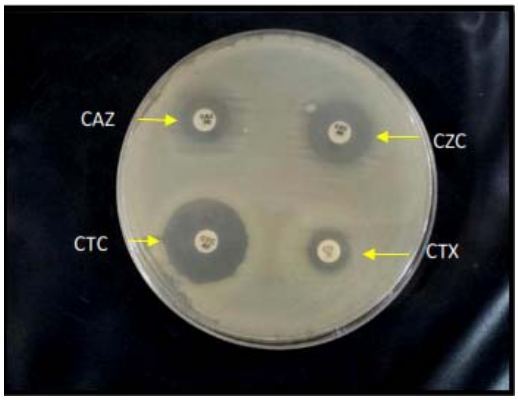

Figure2: Disk combination test exhibiting positive ESBL *E.cloacae* isolate with a significant inhibition zone difference (>5 mm) between ceftazidime-clavulante (CZC), ceftazidime (CAZ) on above side and cefotaxim-clavulante (CTC) and cefotaxime (CTX) disks alone on below side respectively. Plate was incubated at 37°C for 24hr.

Molecular Screening for ESBL Production

All *E.cloacae* isolates were tested at molecular level for their ability to produce ESBLs enzymes. PCR technique has been used to screen and detect ESBLs genes carrying plasmid.DNA of all isolates with specific forward and reverse primers. The lengths of amplification genes are described in paragraph …..this could be illustrated as follows:

Molecular Characterization of the TEM, SHV, OXA and VEB genes

This molecular method was used to detect the most common four kinds of ESBLs; bla_{TEM}, $bla_{SHV, blaOXA}$ and $bla_{VEB.}$ For this purpose, four types of specific primers were used with the help of PCR and electrophoresis system. Gene *bla*-$_{TEM}$ was the most recovered gene from all isolates of *E.cloacae.*9 (60%), (figure3). The *bla*-$_{OXA}$ was detected only in 3 (8%), (Figure 6). On the other hand, all tested isolates did not possess the *bla*-$_{SHV}$ gene and *bla*-$_{VEB}$ gen.

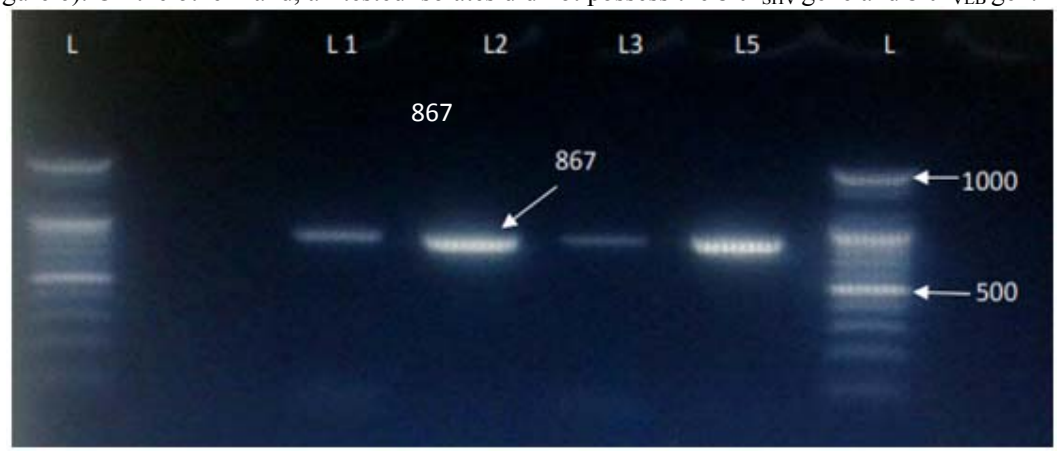

Figure 3: Ethidium bromide-stained agarose gel of PCR amplified products from extracted plasmid DNA of *E.cloacae* isolates and amplified with primer *bla*-$_{TEM}$ forward and *bla*-$_{TEM}$ reverse .The electrophoresis was performed at 70 volt for 1.5-2 hr. lane (L),DNAmolecular size marker(100bp ladder). Lanes L2, L3, L4, and L5 show positive results with *bla*-$_{TEM}$ gene (867bp).

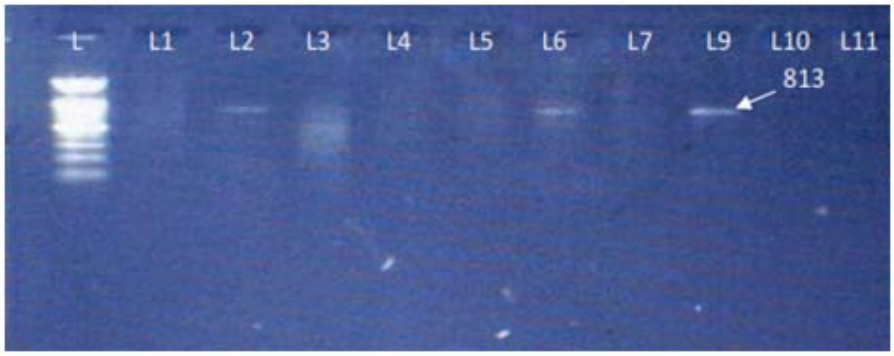

Figure 4: Ethidium bromide-stained agarose gel of PCR amplified products from extracted plasmid DNA of *E.cloacae* isolates and amplified with primer *bla*-$_{OXA}$ forward and *bla*-$_{OXA}$ reverse.The electrophoresis was performed at 70 volt for 1.5-2 hr. lane(L), DNA molecular size marker (100 bpladdar). Lanes (L2, L6, and L8) show positive results with *bla*-$_{OXA}$ gene (813 bp).

DISCUSSION

Bacterial Isolation:

In this investigation, a total number of 308 clinical samples were subjected to bacteriological examination for detecting and isolating *E. cloacae*. Fifteen isolates belonged to *E. cloacae* (5.37%) were recovered from all samples. The percentage of infection with *E. cloacae* in the present study was similar with a study conducted in Poland from November 2003 to January 2004 which revealed that *E. cloacae* represented 5.3% of all Enterobacteriaceae clinical isolates (Empel *et al*., 2008). Hafeez *et al*. (2009) showed that the frequency of *E. clocae* among various clinical species was 4.6%.

On the other hand, in a study carried out in Taiwan, the percentage of isolation of *E. cloacae* from patients was 3% (Lau *et al*., 2004) while Helander and Helen (2005) have pointed that the rate of *E. cloacae* isolation was 2.1 %. In another study, Keller *et al*. (1998) showed that a total number of 54 *E. cloacae* strains were isolated from different clinical specimens.

The isolation rate of *E. cloacae*varies from one study to another and this is affected by several factors such as extensive use and miss-use of the antibiotics, the environmental conditions of hospital (Kartali *et al*., 2002).

Enterobacter species have spilled over into the community occasionally infecting otherwise well individuals causing several infections (Sanders and Sanders, 1997). However, in this study other types of bacteria were isolated. This result was closer to the result obtained by Hafeez *et al*. (2009)

Determination of Minimum Inhibitory Concentrations (MICs)

Determination of the MIC for four β-lactam antibiotics (ampicillin, cefotaxime, ceftazidime and imipenem) was done as complementary test to verify resistance level of isolates towards their substrates.An isolate was characterized as resistant, if the MIC equal or greater than the breakpoint, MIC determined according to CLSI (2014).

The results of this study indicated that most *E. cloacae* isolates were highly resistant to ampicillin with concentration reached beyond the break point values. The MICs values of ampicillin for most tested isolates were≥128µg/ml.

Enterobacter spp., produce AmpC beta-lactamases causing intrinsic resistance to ampicillin (Susić, 2004).

This rate of resistance was in agreement with that reported in a local study conducted by Al-Jobouri (1997) who found that all Enterobacteriaceae isolates were resistant to ampicillin and amoxicillin.

The results presented in table 4-6 indicate that MIC values of ceftazidime range from 16 to 128µg/ml. The level of resistance to ceftazidime was closer to that reported by other studies, Schlesinger *et al*., (2005) found that the MICs of ceftazidime for *E. cloacae* ranged between 16 to ≥64 µg/ml, while they were lower than that found in China by Jiang *et al*. (2005), who found that MIC range was 3 to ≥256 µg/ml.

The results also indicated that the MIC value of cefotaxime for tested isolates ranged from 16 to 64µg/ml. Schlesinger *et al*.(2005) showed that the MIC of cefotaxime for *E. cloacae* ranged from 2≥64µg/ml. In another study by Laura *et al*. (1997) who had stated the MIC of cefotaxime for *E. cloacae* ranged from 2 to ≥256 µg/ml. Overproduction of β-lactamase has led to resistance to many β-lactams, including cefotaxime and ceftazidime, in many species of Enterobacteriaceae. However, the impact of this mechanism of resistance on therapy can be limited by avoiding the use of certain cephalosporins, thereby reducing the chance of selecting highly resistant bacteria, and by good infection control procedures limiting the spread of the bacteria in the hospital environment. New plasmid-mediated β-lactamases, ESBLs, confer resistance to most β-lactams except cephamycins and Carbapenems (Laura *et al*., 1997).

In the present investigation, it was found that imipenem retained good in vitro activity against the majority of *E. cloacae* isolates with a modal MIC ranged 0.5 to 16 µg/ml. This investigation revealed that 13% of the isolates were resistant to imipenem. Overall, the imipenem was the most efficient antibiotic against all the tested isolates. *E. cloacae* can produce the broad spectrum-beta-lactamase and the Amp C enzyme which lead to serious drug resistance, thus carbapenems are choice for the treatment of serious infections caused by ESBL- and Amp C-positive enterobacteriaceae (Courpon-Claudinon *et al*., 2011; Jacoby, 2009).

Kim and Lim (2005), found that among the clinical isolates of *Enterobacter* spp*, Citrobacterfreundii,* and *S. marcescens.* The MIC to imipenem range was 1 to ≥16 µg/ml. The imipenem was the most efficient antibiotic against all the tested isolates, Chan*et al*. (2007) found the MIC of imipenem in *E. cloacae* was 0.25µg/ml.

Screening for β-Lactam Resistant Isolates

The frequency of β-lactam resistance was evaluated whenthe isolates were primarily screened for resistance using ampicillin and amoxicillin (Bush *et al*., 1995).

The results showed that all the 15 *E. cloacae* isolates (100%) were resistant to both ampicillin and amoxicillin. All these isolates were able to grow normally in the final concentrations of 50-100 µg/ml of these two antibiotics. Such high percentage may be due to frequently use of β-lactam antibiotics by patients.

Generally resistance to beta-lactam antibiotics in Gram-negative bacteria can be due to four mechanisms: production of ß-lactamase, decreased affinity of the target penicillin-binding proteins (PBPs) or by pump-mediated resistance, decreased permeability of the drug into the cell (Forbes *et al*., 2007). Reduced permeability through porin losing may reduce the steady state of periplasmic drug concentrations and thereby reduces PBP inactivation. Therefore, decreased permeability may act synergistically with the expression of β-lactamases or active efflux to confer higher levels of β-lactam resistance (Livermore and Woodford, 2000).

In *E. cloacae*, both β-lactamase and outer membrane proteins are significant determinants of the antibiotic susceptibility of the organism. Alterations in β-lactamase affect susceptibility to β-lactam antibiotics only, whereas alterations in outer membrane proteins affect susceptibility to a variety of unrelated antibiotics (Werner *et al*., 1985).
Development of antibiotic resistance is often related to the overuse, and misuse of the antibiotic prescribed. However, β-lactam resistance mostly associated with transmissible plasmids can be transferred between different bacterial species among hospital isolates (Carattoli, 2008).

β-Lactamase Producing *E. cloacae* Isolates

To determine the susceptibility of specific microorganisms to β-lactam drugs should be examine their ability to produce β-lactamase. The results show 12 (80%) of isolates were positive by nitrocefin disk method. Nitrocefin disk is a more sensitive technique is measuring β-Lactamase activity with a chromogenic cephalosporin, usually nitrocephin (O'Callaghan *et al.,* 1972). Each disc can be used to detect bacteria which produce b-lactamase in sufficient quantity to convert the yellow coloured disc red. In addition, for many β-Lactamases, nitrocefin is the substrate that is most readily hydrolyzed by the enzyme. This property makes it often the most sensitive detection system and it provides a very rapid and continent method for detection of β-Lactamases.

Production of Extended Spectrum β-Lactamases

The initial screening for reduced susceptibility to third generation cephalosporins and aztreonam was made by the standard Kirby-Bauer disk diffusion method. The isolate was considered positive for screening test when the zone diameter of any of the indicators met the CLSI criteria (CLSI, 2014) and additional phenotypic tests are mandatory in order to ascertain the production of ESBL.
The results of this study revealed that resistance to screened agents (cefotaxime, aztreonam, and ceftazidime) was at relatively high rates. These rates are closer with the result reported by Al-Sehlawi (2012) who revealed that the antimicrobial susceptibility profile against ceftazidime gave higher resistance percentage 66.9% than other third generation cephalosporins like cefotaxime 65.4%, and aztreonam 60%. Ali *et al*. (2004) revealed a high frequency 79% of ESBL-producing *E.cloacae*among clinical isolates recovered from Military Hospital. Other study reported 50% ESBL positive *E. cloacae* (Jabeen*et al*., 2005).
The identification of ESBL producers is a major challenge for the clinical microbiology laboratory, due to the affinity of ESBL-producing isolates to the different substrates is variable and makes their detection difficult. Additionally, some ESBL isolates may appear susceptible to a third generation cephalosporins *in vitro* (Aggarwal and Chaudhary, 2004; Hadi, 2008).
The β-lactam resistance mediated by ESBL is difficult to detect, therefore the Clinical Laboratory Standards Institute (2014) recommended more than one confirmatory test for ESBL detection.The results shows that 11/15 (73%), *E. cloacae* isolates were detected as ESBL producers by the diskcombination. This result was closer with those displayed by Amin *et al*. (2013) who found that 75.75% of *E. cloacae* isolates were ESBLs producer.
The Jarlier disk approximation or double disk synergy (DDS) was the first detection test described in 1980's (Jarlier*et al*., 1988). The efficacy of β-lactam group of antibiotics is reduced due to the production of β-lactamases by the resistant bacterial strains. Therefore, search for their inhibitors was initiated to protect the antibiotic activity in vivo against β-lactam resistant pathogens. DDS test remains a reliable, convenient and inexpensive method of screening for ESBLs. However, the interpretation of the test is quite subjective. Sensitivity may be reduced when ESBL activity is very low leading to wide inhibition zones around the cephalosporin and aztreonam (Vercauteren *et al.*, 1997).

Molecular Characterization of the ESBL Genes:

The prevalence of ESBL-producing *E. cloacae* was divers worldwide increased length of hospital stay, prior administration of any antibiotics, the use of central venous and arterial catheters, increased severity of illness, and higher hospital cost were often noted in patients infected by ESBL producing pathogens (Jacoby andMunoz-Price, 2005).
This study was designed to investigate the presence of genes coding for ESBLs among 15 β-lactam resistant *E. cloacae* isolates. Notable, the detection of the presence of *bla*-$_{TEM}$, *bla*-$_{SHV}$, *bla*-$_{OXA}$, *bla*-$_{VEB}$, genes was performed with monoplex PCR assay.
(Figure 5) showed that the gene *bla*-$_{TEM}$ was detected present in 9 (60%) isolates.
ESBLs among the isolates of *Enterobacter*spp, *S. marcescens,* and *C. freundii,* have been described from several countries worldwide and become more and more prevalent (Ferreira *et al*., 2011).
In a study in Turkey, to evaluate the roles of *bla*-$_{TEM}$ genes in clinical isolates of *E.cloacae* using PCR. In another study in Brazil the frequency of the *bla* TEM gene was 52.7% (Abreu *et al*., 2013).

In Spain, *bla*-$_{TEM}$ gene had low prevalence 31% of ESBL –producer isolates (Machado *et al*., 2005). A review study by Bradford (2001), reported that up to 90% of Ampicillin resistance in *E. coli* is due to the production of TEM-1. This enzyme has the ability to hydrolyze Penicillins and early cephalosporins such as cephalothin and cephaloridine. The genes encoding TEM-1 and TEM-2 β- lactamases are carried by transposons, as are the genes encoding some TEM- type ESBLs. In hospital outbreak one type of ESBL often predominates. Particular TEM-type ESBL varieties seem to have a fixed geographical distribution (Jacoby and Munoz-Price, 2005).

Among the 15 *E. cloacae* isolates, the molecular analysis of ESBL genes revealed that the*bla*-$_{OXA}$ was detected in only 3/15 (20%) isolates.

Bhattacharjee *et al*. (2007) reported that out of 163 ESBL-positive Enterobacteriacae isolated from different clinical specimens in India, only one isolate harbored the OXA-10 gene.

However, in a study of Dimou *et al*. (2012) which indicated for identification of the *bla*-$_{OXA-48}$ gene in *E. cloacae*, 2 isolates harboured a classical *bla*$_{OXA-48}$ gene.

Colom*et al*. (2003) reported among 51 amoxicillin-clavulanate resistant *E. coli* isolates only one isolate harboured a *bla* (OXA-1) gene.

Some OXA-type β-Lactamases have carbapenemase activity, augmented in clinical isolates by additional resistance machanisms, such as impermeability or efflux (Jacoby and Munoza-Price, 2005).

The present study revealed that no *bla*-$_{SHV}$ gene was identified in all tested isolates, which could be either due to the absence of *bla*-$_{SHV}$ gene or the presence of other subtype of gene that could not be targeted by the primers used in this study. Similar results conducted by Lu *et al*. (2010), in China, and Mendonca *et al.* (2007) in Portugal, reported that no strain carried the *bla*- $_{SHV}$ gene confirms results of the present study.

Among the 15 *E.cloacae* isolates, the molecular analysis of ESBL genes revealed that no *bla*-$_{VEB}$ gene was identified in all tested isolates, In Najaf, Al-Shara (2013) found that *bla*-$_{VEB}$ gene was not detected among 36 carbapenem-resistant *P. aeruginosa* isolates.

In another study in Turkey, reported that the*bla*-$_{VEB}$ was detected in only one (1.6%) isolate among *E. coli* isolates. This enzyme is class A β-lactamase and was named VEB-1(for Vietnamase extended-spectrum β-lactamase). The latter confers high-level resistance to amoxicillin, ticarcillin, piperacillin, cefotaxime, ceftazidime, and aztreonam, which is inhibited by clavulanate (Poirel *et al.,* 1999). Many risk factors may play important role in the increasing frequency of ESBLs include, long hospital stays, prolonged stays in intensive care unit (ICU), increased severity of illness, the use of urinary catheter, prior administration of anoxyimino–β-lactam antibiotic and prior administration of any antibiotic (Jacoby and Munoz-Price, 2005).

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