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Detection of Metallo-β-Lactamases and their association with integrons among Multidrug Resistant Clinical Isolates of *Escherichia coli*

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Abstract : One hundred and thirty clinical samples of urine, swab (burn, wounds and vaginal) and stool were collected from individuals of both genders and different ages from deferent hospitals and laboratories in Babylon province, during the period of November 2015 to February 2016.

Forty-two *Escherichia coli* isolates were recovered from the study and their identification was confirmed by routine biochemical tests and Vitek2 System. The results revealed resistance to antibiotics at percentages as follows: AMP: 95.24%; ATM: 61.9%; CAZ: 66.66%; GEN: 57.14%; ENO: 42.86%; FEP: 64.28%; IMP: 26.19%; KM: 49%; MEM: 21.43% and TET: 66.66%, while the percentage to multidrug resistance was 79 %. The metallo- β -lactamases enzymes were 45.45% and 57.57% from MDR isolates by CDDT and DDST methods respectively, while the results of production the metallo- β -lactamases enzymes were 69.69% from MDR isolates by E-test IMP. PCR analysis showed the presence of *int11* in (78.78%), *int12* (24.24%), and *int13* (0) from MDR isolates, while the genes that related to antibiotics resistance were *imp* (66.66%), *spm* (15.15%) and *vim* (36.36%) among MDR isolates. **Keywords** : *Escherichia coli*, MDR, Intgrons, MBLs genes.

Introduction

Escherichia coli is a common pathogen linked with community-acquired as well as nosocomial infections. The bacterium causes diseases in intestinal and extra-intestinal environments via virulence factors that are acquired through horizontal gene transfer, genetic recombination and natural selection ¹. Urinary tract infection (UTI) represents one of the major nosocomial infections commonly caused by *Escherichia coli*. UTIs account for 90% of community acquired and 50% of nosocomial UTIs. Additionally, it causes of enteritis, neonatal meningitis, abdominal sepsis, and septicemia².

The emergence and wide dissemination of *E. coli* strains showing resistance to broad-spectrum of antimicrobial agents has been reported. Multi-drug resistance (MDR) among clinical isolates of bacteria such as *E. coli* pathotypes, is major healthcare problem and is associated with increased morbidity and mortality worldwide³. Although classically acquired and spread through chromosomal mutations, resistance genes can be disseminated by extrachromosomal elements acquired from other bacteria. These include different types of mobile DNA segments, such as plasmids, transposons, and integrons⁴. Integrons are one of the mobile genetic elements which can carry genes of resistance to different antibiotics, which contain integrase gene, two

conserve areas of *sull* and *intl*, and one variable area of gene cassettes. A gene cassette includes an open reading frame and at the 3'-end, a recombination site *attC*. Integration or excision of cassettes occurs by a site-specific recombination mechanism catalyzed by the integrase⁵. The aim of this study is the detection of association of metallo- (3-lactamases and integron with multi drug resistance in clinical isolates *E. coli*.

Materials and methods

Isolation and Identification of E. coli

A total of 130 clinical samples from different clinical sources (urine, swab and stool) collected from two public hospital in Babylon/ Iraq during November 2015 to February 2016. Forty-two *Escherichia coli* isolates were recovered from the study and their identification was confirmed by routine biochemical tests and Vitek2 System.

Antimicrobial susceptibility test

Susceptibility of all the isolates to 10 antibiotics was determined by the disk diffusion method, as recommended by National Committee for Clinical Laboratory Standards (CLSI, 2014). The antibiotic discs used (Bioanalyse /Turkey) were Ampicillin 10 μ g (AMP), Aztreonam 30 μ g (ATM), Ceftazidime 30 μ g (CAZ), Gentamicin 10 μ g (GEN), Enoxacin 10 μ g (ENO), Cefepime 30 μ g (FEP), Imipenem 10 μ g (IMP), Kanamycin 30 μ g (KM), Meropenem 10 μ g (MEM) and Tetracycline 30 μ g (TET).

Phenotypic Detection of MBL production

a) Combined Double Disc Test (CDDT)

Two 10 **u**g imipenem discs (one impregnated with 10 **u**l of 0.5M EDTA) were placed on the Muller Hinton (MH) agar medium inoculated with test organism standardized with 0.5 McFarland standards. After overnight incubation at 37°C, an increase in zone diameter of > 7 mm around the imipenem- EDTA disk compared to that of the imipenem disk alone were considered positive for MBL production, ⁶.

b) Double Disc Synergy Test (DDST)

The test was performed as described by Saderi *et al.*, ⁷ by inoculating the tested organism onto MHA plate as recommended by ⁸. A 10 μ g imipenem disk and a blank filter paper disc 6 mm in diameter were placed 10 mm apart from edge to edge, then, 10 μ l of 0.5 M EDTA solution was applied to the blank disk, after 18 hours of incubation at 37°C. The presence of a synergistic inhibitory zone was considered as MBL positive.

c) E-Test

Tested colonies from overnight culture were suspended with 0.85% of normal saline (NaCl) to a turbidity of 0.5 McFarland s standard as recommended by kumar *et al*, ⁹. A sterile cotton swab was used to produce a uniform layer on a Mueller-Hinton agar plate. Once dried, an E-test MBL strip (Liofilchem/ Italy) was applied on the plate which was incubated at 37°C for 16 to 20 h. E-test demonstrated enhanced MIC of imipenem in the presence of EDTA IMI D with IMI / IMI D of > 8 for MBL activity.

Genotypic Detection of MBL and Integrases Genes

Isolation of Genomic DNA

DNA extraction from the selected bacterial isolates was performed by the Gentra puregene Bact. /kit (Qiagen/ UAS) according to the instructions of the manufacturer. Concentrations of DNA were measured by using Nano Drop-spectrophotometer.

PCR detection of MBLS and integrases genes

The specific designed primers were ordered for synthesis from AccuOligo / Bioneer /Korea. Table 1. Amplify the entire sequence of metallo- β -lactamases genes *imp*, *vim*, and *spm* by multiplex-PCR technique and integrase genes *int11*, *int12*, and *int13*. The PCR reaction was performed in a total volume 25 µl contain 10 p

mole/µl of each primer, 12.5 µl Taq Green Master Mix 2X (Promega/USA) and 200ng genomic DNA. The reaction mixture was amplified in a GTC thermal cycler (Cleaver Scientific, UK). PCR amplification for the detection of MBLs genes was carried out under following condition: initial denaturation was carried out at 94°C for 5 min and the target DNA was amplified in 36 cycles. Subsequently, each cycle consisted of denaturation at 94°C for 30 sec, followed by annealing at 52°C for 40 sec. Elongation was carried out at 72°C and the extension time at 1 min. The final extension step was performed at 72°C for 5 min and the holding temperature was 4 min ¹⁰. For amplification of the of integrase genes *int1* and *int1*, the annealing temperature was increased to 55°C for 45 sec., whereas for *int1* gene the annealing temperature was 57°C for 45 sec.¹¹.

Primer Name	Primer Sequence 5' - 3'	Product Size / bp
imp	F-GGA ATA GAG TGG CTT AAT TCT C	188
	R-CCA AAC CAC TAG GTT ATC T	
vim	F- GAT GGT GTT TGG TCG CAT A	390
	R- CGA ATG CGC AGC ACC AG	
spm	F-AAA ATC TGG GTA CGC AAA CG	271
	R-ACA TTA TCC GCT GGA ACA GG	
Mil	F - GCA TCC TCG GTT TTC TGG R-	457
	GGT GTG GCG GGC TTC GTG	
MI2	F-CAC GGA TAT GCG ACA AAA AGG T	789
	R-GTA GCA AAC GAG TGA CGA AAT G	
MB	F- ATC TGC CAA ACC TGA CTG	922
	R-CGA ATG CCC CAA CAA CTC	

Table 1: Primers used for PCR amplification of MBL genes and integrons genes

Gel Electrophoresis

The amplified PCR products were checked for the expected size on 1.5 and 2 % (w/v) agarose gel and visualized after staining with ethidium bromide under ultraviolet. A DNA molecular weight marker (Promega / USA) was used to measure the weight of the fragments .

Statistical Analysis:

SPSS version 23.0 statistical software package was used for statistical analysis by using Chi-square test for all obtained results p value < 0.05 was considered statistically significant ¹².

Results and Discussion

Isolation of E.coli

Out of 130 clinical samples, 42 *E. coli* isolates (32.3 %) were isolated. The isolates were purified and characterized by morphological and biochemical tests according to the methods of MacFaddin ¹³, and Vitek2 System. The differences in percentages of clinical samples that were positive and negative for *E. coli* isolation were significant (x^2 at/? < 0.05) as presented in table 2.

Source of samples	f Total No. (%) of positive case		No. (%) of negative case	<i>P</i> -value	
UTI	77	32 (24.6)	45 (34.6)	0.044*	
Swab	41	7 (5.4)	34 (26.2)	0.000*	
Stool	12	3 (2.3)	9 (6.9)	0.000*	
Total %	130	42 (32.3)	88 (67.7)		

Table 2: Number and percentages of positive and negative clinical samples for *E. coli* isolation

 χ^2 significance at (p < 0.05)

Alhetar *et al.*, ¹⁴ demonstrated that the highest rate of *E. coli* isolation was from urine (40%) from a public hospital in Malaysia.

Figure 1 shows percentage of *E. coli* isolates from UTI 32(76.2%), swab 7(16.7%) and stool 3(7.1%).



Figurer 1: Number and percentage of E. coli isolates from different samples

The high incidence (76.2%) of *E. coli* isolated from UTI recorded in this study could be due to the virulent nature of the organism, which gives it the ability to overcome body defense mechanisms, and resistance to antibiotics. On the other hand, these results disagreed with Kariuki *et al.*, ¹⁵, who reported a low recovery rate of *E. coli* accounted for 36% from UTIs.

Antimicrobial susceptibility test

Susceptibility test was done for all the 42 *E. coli* isolates against 10 antibiotics. The results indicated that the various levels of susceptibilities to different antibiotics among isolates could be recorded. The results are summarized in figure 2.



Figure 2: Antibiotics susceptibility of 42 *E. coli* isolates. AMP: Ampicillin, ATM: Aztreonam, CAZ: Ceftazidime, GEN: Gentamicin, ENO: Enoxacin, FEP: Cefepime, IMP: Imipenem, KM: Kanamycin, MEM: Meropenem, TET: Tetracycline.

The present study showed that E. coli isolates were resistant to ampicillin (95.24%), Ceftazidime (66.66%), Tetracycline (66.66%), Cefepime (64.28%), Aztreonam (61.90%), Gentamicin (57.14%), Kanamycin (52.38%), Enoxacin (42.86%), Imipenem (26.19%), and Meropenem (21.43%). The highest level of sensitivity was observed against Meropenem (57.14%). Several studies reported results similar to the present study as they reported 95.7% and 96% resistance of E. coli isolates to ampicillin respectively ^{16, 17}. Resistance of E. coli isolates to Ceftazidime, and Tetracycline reported in this study were similar to studies done by Adwan et al,¹⁰in Palestine, respectively. The resistance to third generation cephalosporins is caused mainly by mutations in the common group of class A (3-lactamases ¹⁸. The percentages of resistance to fourth generation cephalosporins (Cefepime) and monobactam (Aztreonam) were 64.28%, 61.90% respectively. The result of this study was agreement with the finding of ¹⁹, who reported 68.4% resistance of \mathbf{f} coli isolates to Aztreonam. These results are comparable to other reports where 78.9% and 59.6% E coli isolates exhibited resistance to Cefepime ¹⁹²⁰ respectively. The rate of resistance to aminoglycoside: Gentamicin (57.14%) and Kanamycin (52.38%) in this study was nearly similar to that recorded earlier ²⁰ which was (61.5%) resistant to Gentamicin, but higher than that recorded by Chigor et al,²¹, who reported all the E. coli isolates were susceptible to Gentamicin. Lower resistance rates were observed for carbapeneme: Imipenem and Meropenem in the present study were 26.19% and 21.43% respectively. The rate of resistance to Imipenem was nearly similar to that recorded earlier ²², but lower than that recorded by Adwan et al, ¹⁰, who reported 44.39% of the isolate were resistance to Imipenem. While greatly higher than that recorded by Al-Hilali, (9.6%)²⁰. However, Al-Hilali, ²⁰, and Adwan *et al*, ¹⁰have reported that 25% and 38% of $\mathbf{\pounds}$ coli isolates were resistant to Meropenem respectively.

The various percentages of resistance in different part of the world are due to differences in the prevalence of antibiotic consumption in each country ²³. In Saudi Arabia is particularly relevant as it has a large expatriate population, mainly from South and East Asian countries where antimicrobial resistance is prevalent, including to carbapenems ²⁴.

DAgata, ²⁵ defined multi-drug resistance as resistance to 3 or more antimicrobial classes. He found that, multidrug resistant Gram-negative bacilli increased from 0.2% to 4% for *E. coli* ,1% to 16% for *Pseudomonas aeruginosa*, 4% to 13% for *Enterobacter* spp., and 0.5% to 17% for *Klebsiella* spp., the most common pattern of multi-drug resistance was co-resistance to quinolones, third generation cephalosporins, and aminoglycosides. The current study revealed that of the 42 isolates tested, MDR *E. coli* were present in 33(79%) were significantly higher (p < 0.05) than other sensitive isolates 9 (21%), figure 3.



Figure 3: Frequency of MDR *E coli* isolates.

Previous studies in Iraq, concerning the (82.6% and 93.7%) of *E. coli* isolates were observed as MDR ^x, ²⁰ respectively. Whereas in Tabriz, northern west of Iran, 84.2% of *E. coli* were MDR²⁷. The emergence of **£** *coli* isolates with different MDR phenotypes, involving co-resistance to three or more unrelated families of antimicrobial agents, has been previously reported and is considered a serious health concern²⁸. Moreover, the increased resistance to antibiotics are multi-factorial and may be due to lack of proper policy to antibiotics usage and transfer of resistance genes by transportation tools such as plasmids, bacteriophage, and integrons.

Phenotypic Detection of MBL production

All MDR *E. coli* isolates were subjected to three phenotypic confirmatory screening tests for metallo-plactamases (MBLs) production; combined double disk test (CDDT), double disk synergy test (DDST), and Epsilo meter-test (E-test). The results indicated that the CDDT 15 (45.45%) of isolates that were positive showed an increase in the zone diameter around the IMP - EDTA disk, whereas (DDST) method revealed that 19 (57.57%) of isolates showed positive extension of zone towards the impregnated EDTA disk. Twenty-three (69.69%) of isolates were positive for E-test.

No. (%) of MDR	No. (%) of MBL Producer Isolates Detected By			
E. coli Isolate	CDDT	DDST	E – test	
33 (100%)	15 (45.45%)	19 (57.57%)	23 (69.69%)	

Table 2: Numbers and percentages of E. coli isolates that produced and non-product MBL

In CCDT test, an increase in zone diameter of > 7 mm around the imipenem- EDTA disk compared to that of the imipenem disk alone were considered positive for MBL production. Double-disk synergy test (DDST) with synergistic zone of inhibition surrounding imipenem - EDTA disk indicating MBL activity. Whereas in E-test, MBL imipenem / imipenem- EDTA, E-test demonstrated enhanced MIC of imipenem in the presence of EDTA IMI / IMD of > 8 for MBL activity, as show in figure 4.



Figure 4: Phenotypic tests to detect MBL production. (a) Combined disk test (CDDT) showing enhanced inhibition zone of >7mm around IMP + EDTA disk indicating MBL positivity. (b) Double-disk synergy test (DDST) with synergistic zone of inhibition surrounding IMP and EDTA disks indicating MBL activity. (c) MBL IMI / IMD E-test demonstrating enhanced MIC of imipenem in the presence of IMI / IMD of >8 for MBL activity.

MBLs can hydrolyze most β - lactams except for monobactams and confer a broad- spectrum β - lactam resistance to the bacterial host, which is not reversible by conventional therapeutic β - lactamase inhibitors²⁹. Several phenotypic methods are available for the detection of MBL-producing bacteria. All these methods are based on the ability of metal chelatons, such as EDTA and thiol-based compound to inhibit the activity of MBLs. These tests include: CDDT, DDST, Hodge test, and MBL-E test³⁰. Results of the current study using the E-test revealed higher prevalence rate of MBL-producers in comparison to the CDDT, DDST, as also previously described by Walsh *et al*, ³⁰, ³¹. On the other hand, this is unlike Fam *et al*., ³², who demonstrated that the CDDT and DDST were superior to E-test for screening MBL production in enterobacteriaceae.

Genotypic Detection of MBL and Integrases Genes

Detection of MBL Genes

Among the 33 MDR isolates of *E. coli*, 22 (66.66%) had PCR products corresponding to bla_{IMP} gene fragment, and 12 (36.36%) of the bla_{VIM} . In five isolates (15.15%) only bla_{SPM} gene was found, as show in figures (5 and 6).



Figure 5: Occurrence of metallo-β-Lactamase genes: *imp, spm* and *vim*



Figure 6: Triplex PCR amplification with specific primers for *imp, spm* and *vim* genes product. Lane M, 100 -bp DNA marker. Lanes (E 2, 3, and 5) positive isolates with *imp gene*, lanes (E6, 7 and 8) positive isolates with *spm* and *vim* genes, lanes (E1, and 4) negative isolates with *imp, spm, and vim* genes and lanes (E6, 7 and 8) negative isolates with *imp genes*, on 1.5% agarose at 70 volt for 2 hrs.

Adwan *et al.*,¹⁰ found that the prevalence of MBLs among *E. coli* in North of Palestine using multiplex PCR technique was 87.4%. They reported that bla_{SPM} MBL was the most common in *E. coli* isolates, and this result is different from results of the present study in that it shows bla_{SPM} gene at lower percentage among MBLs genes. In other countries, the prevalence of MBL producers *E. coli* ranged from 1.7%-45.2% ^{33,34}.

Fluit and Schmitz, ⁵reported that multi-drug resistance encoded by resistance genes clustered in integrons, which are potentially mobile genetic elements, considered to be involved in the transfer of MDR.

The high prevalence of MBLs producers among *E. coli* isolates may be due to several risk factors such as long term exposure to antibiotics in hospitals, prolonged hospitalization, incorrect therapy, nursing home residency, severe illness, catheterization and movement of health staff in the hospital leading to dissemination of these pathogens throughout the hospital 35 .

Detection of Integrases Genes

Out of 33 MDR isolates the presence genes encodes (integrases) to class 1 integron was detected in 26 (78.78%) of isolates showing amplification product of 457 bp, figure 7.



Figure 7: PCR amplification with specific primers for *int11* gene in clinical isolates of *E. coli*. Lane M, 100 - bp DNA marker. Lanes (E1, 4, 5, 6, 7, 8, 9,10,11,12,13, and 14) positive isolates, lanes (E2, 3, 15, 16, 17, 18, and 19) negative isolates on 2 % agarose at 70 volt for 2 hrs.

Whereas presence the genes encodes (integrases) to class 2 integron was detected in 8 (24.24%) of clinical isolate of *E. coli* with amplification product of 789 bp, as show in figure 8.



Figure 8: PCR amplification with specific primers for *int12* gene in clinical isolates of *E. coli*. Lane M, 100 - bp DNA marker. Lanes (E3, 4, and 7) positive isolates, lanes (E1, 2, 5 and 6) negative isolates, on 2 % agarose at 70 volt for 2 hrs.

All other *E. coli* isolates were negative for class 3 integrase as in figure 9.



Figure 9: PCR amplification with specific primers for *int13* gene in clinical isolates of *E. coli*. Lane M, 100 - bp DNA marker. All *E. coli* isolates show negative, on 2 % agarose at 70 volt for 2 hrs.



Figure (10) shows that class1 integron was the principle integron class in the study isolates.

Figure 10: Occurrence of integron classes; intI1, intI2, and intI3

Integrons are mobile genetic elements thought to play an important role in the dissemination and accumulation of resistance genes in bacteria³⁶. Integrons are usually located within transposons or conjugative plasmids.

In previous study, 65% of isolates harbored class 1 integron³⁷, whereas in another study class 1 integron was found in 49% of uropathogenic isolates³⁸. In another study class 1 integron was detected in 41% of uropathogenic isolates³⁹. Only one isolate was positive for class 2 integron all were negative for class 3 integron³⁷.

Association between Integrons and Antibiotic Susceptibility

The association between integrons and antibiotics resistance were investigated. The current study shows that the presence of an integron was significantly associated with 33 MDR isolates at (p < 0.05), table 3.

Antibiotics	Integron 1 – positive isolates (n=26) (P. %)	Integron 1 – negative isolates (n=7) (R %)	P- value	Integron 2 – positive isolates (n=8) (R %)	Integron 2 - negative isolates (n=25) (P. %)	P- value	% Resistance of total
AMP	100	100	1	100	100	1	100
ATM	80.8	71.4	0.000*	75	80	0.211	78.8
CAZ	84.6	85.7	0.842	87.5	84	0.391	84.8
GEN	73.1	71.4	0.723	87.5	68	0.000*	72.7
ENO	50	71.4	0.000*	50	56	0.227	54.5
FEP	80.8	85.7	0.179	87.5	80	0.073	81.8
IMP	34.6	28.6	0.157	50	28	0.000*	33.3
КМ	69.2	57.1	0.016*	75	72	0.504	66.7
MEM	34.6	0.0	0.000*	25	28	0.504	27.3
TET	80.8	57.1	0.000*	75	76	0.815	75.8

Table 3: Association between resistance to antibiotics and presence of integron

 χ^2 was significant at (p < 0.05)

The presence of integrons in clinical pathogenic *E coli* isolates is highly related to multi drug resistance. The present study revealed the strong association between integron class 1 and resistance to Ampicillin, Aztreonam, Enoxacin, Kanamycin, Meropenem, and Tetracycline. While association between integron class 2 and resistance to Ampicillin, Gentamycin, and Imipenem, were listed in above table 3. Lee *et al*, ⁴⁰ reported that horizontal transfer of integron-carrying elements plays a dominant role in the development of multi resistance by enterobacteriaceae.

Maurine *et al*, ⁴¹ found a significant (p > 0.0001) relation between multi drug resistance and integrons in enterobacteriaceae. Previous studies characterizing integrons in clinical isolates showed that inserted gene cassettes predominantly confer resistance to trimethoprim and aminoglycosides in addition to resistance to chloramphenicol, and erythromycin ⁴²⁴³. As well as, Lee *et al*, ⁴⁰, found the presence of integron is significantly associated with multidrug resistance in commensal *E. coli* isolates.

Conclusion

In this clinical microbiologic study, most *E. coli* isolates containing class 1 integron gene were multi drug resistant but all isolates were negative for class 3 integron. There is a high association between integrons and metallo (3-lactamase genes (*imp*) especially class 1 integron in most MDR *E. coli* isolates. Prudent use of antibiotics can be helpful in preventing transmission of resistance genes through integrons.

Conflict of Interests

The authors have declared no conflict of interests.

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