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# Role of integrons in multi drug resistant extended-spectrum β-lactamase-producing *enterobacteriaceae*

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**The prevalence of antimicrobial resistant bacterial pathogens has become a major public health concern. Extended-spectrum ß-lactamase (ESBL) production in the members of the family *Enterobacteriaceae* can confer resistance to extended spectrum cephalosporins such as azetronam and penicillins. Integrons are genetic structures capable of capturing and excising gene cassettes, which usually encode antimicrobial drug resistance determinants. Although integrons are not self mobilizable, they are usually found in association with transposons and are often located on plasmids, facilitating their mobility. This study aimed to know the usefulness of integron as an indicator of the potential dissemination of multidrug resistant *Enterobacteriaceae* in hospital environment and considerations regarding antimicrobial policy. This study was conducted in Benha University Hospital. 100 clinical samples from different clinical departments were included in the study in order to separate nosocomial *Enterobacteriaceae* and detect the integrons by polymerase chain reaction sequence specific primer (PCR-SSP). Integrons were found only in ESBL group. Incidence of class 1 integron was high in our *Enterobacteriaceae* isolates especially *Klebsiellae* and *E. coli* which were the most frequent organisms in the study. There is association between integrons and multi drug resistance especially to aminoglycosides and tetracyclins.**

**Key words:**Extended-spectrum β-Lactamase (ESBL), integrons, drug resistant, *Enterobacteriaceae*, PCR-SSP.

## INTRODUCTION

Members of Enterobacteriaceae family are important hospital and community-acquired pathogens that are naturally susceptible to extended-spectrum cephalosporins (ESCs). However, strains resistant to these antibiotics mediated by extended spectrum β-lactamases (ESBLs) have now spread worldwide. ESBLs contain several types of β-lactamases, including sulfhydryl variable (SHV), temoneira (TEM), cefotaxime (CTX-M) and oxacillin (OXA) (Rawat and Nair, 2010). Dissemination of antibiotic resistance genes by horizontal transfer has led to the rapid emergence of antibiotic resistance among clinical isolates. In the 1980s, genetic elements termed integrons were identified. At least eight classes of integrons, with different integron genes, have

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**Abbreviations: ESBL**-Extended-spectrum ß-lactamase, **ESCs**-Extended-spectrum cephalosporins, **TEM**-Temoneira, **CTX-M**-Cefotaxime hydrolyzing capabilities, **OXA**-Oxacillin hydrolyzing capabilities, **SHV**-Sulfhydryl variable, **MBL**-Metallo-β-lactamase

been described (Yao et al., 2007). Among the different integron families, class 1 integrons are found to be most prevalent in drug-resistant bacteria (Ahangarzadeh et al., 2012). Class 1 integrons are mobile DNA elements with a specific structure consisting of two conserved segments flanking a central region containing “cassettes” that usually code for resistance to specific antimicrobials. The 5’-conserved segment contains the integrase gene (IntI1), a promoter region and the IntI1-specific integration site attI1. The 3’-conserved segment usually contains a combination of the three genes qacEΔ1 (antiseptic resistance), sulI (resistance to sulfonamides) and an open reading frame (orf5) of unknown function. Between the two conserved segments, the central variable region can contain from zero to multiple cassettes (Wu et al., 2012). The acquisition of resistance genes in bacteria is often facilitated by integrons. The presence of integrons among clinical *Enterobacteriaceae* isolates might account for multiple-antibiotic resistance (Léon et al., 2010). This study aimed to find out the association between multi-drug resistance with ESBL producing *Enterobacteriaceae* and integron genes . Also to clarify the prevelance of multi drug resistant *Enterobacteriaceae* with related integron genes in hospital environment and considerations regarding anti microbial policy.

## MATERIALS AND METHODS

This is a cross sectional observational study that was performed at Benha University Hospital Beha city, Quliubyia, Egypt during one-year period, beginning from August 2011 till July 2012. One hundred clinical specimens from patients were collected from out- and inpatients departments. To eliminate duplicates, only one sample was taken per patient and per site. Full history was taken to differentiate nosocomial from community acquired infections. Nosocomial infection was defined as any infection that occurred more than 48 h after admission of the patient to hospital. Patients who had infection on admission were excluded (Randrianirina et al., 2010). This study followed the protocol approved by the local institutional review board. Written consent was obtained from every patient included in the study.

### Sampling

Samples were blood, sputum, pus, urine and body fluids. History and clinical data were taken form each patient. Clinical specimens were collected before antimicrobial treatments were given to the patient. To target the units at high risk of nosocomial infections, ICU, dialysis unit, surgery unit and internal medicine unit were included in the study.

### Culturivation

The specimens were culttivated at 37°C overnight on blood, MacConkey, chocolate media (Oxoid, UK). For blood cultures Bactec 9050 bottles Becton Dickinson (BD) were used. Bacterial isolates were identified using standard microbiological methods (colony morphology, gram stain, biochemical reactions, DNASE test and oxidase using Microbact strips (API), and catalase test (chemical). All chemicals, strips and diccs were from Oxoid,UK.

The susceptibility of bacterial isolates was tested using manual Kirby Bauer disc diffusion method; Gram negative isolates resistant to 3rd generation cepholosporins were screened for resistance mechanism (ESBL) by using phenotypic confirmatory test (ESBL screening). Cefpirome/Clavulanic acid Oxoid Combination Disc Oxoid, UK

### Phenotypic confirmatory testing

Antibiotic sensitivity testing was done using the standard disc diffusion method according to CLSI (2011). Pure isolate broth suspension was compared with the turbidity standard and density of the test suspension was adjusted to be equal to 0.5; tube contains McFarland. Suspension of the isolates were inoculated on Mueller-Hinton agar (Rawat and Nair, 2010).

### ESBL Screening

According to CLSI (2011) the inhibition zone diameters around Cefotaxime (CTX, 30 µg), Ceftazidime (CAZ, 30 µg) discs with and without Clavulanic Acid (CA, 10 µg) were compared after 16 to 18 h of incubation at 35°C. A five mm or more increase in the inhibition zone diameter of either CTX, and CAZ discs in the presence of Clavulanic Acid (CA) compared to the inhibition zone diameter around CTX, CAZ alone, respectively was considered to be a positive result for ESBL production. The isolates were stored at -20°C in nutrient broth containing glycerol. Genomic DNA was extracted from 400 µL nutrient broth.

### Integron Detection Analysis Using Polymerase Chain Reaction Sequence Specific Primer (PCR-SSP)

#### *Genomic DNA Extraction*

The DNA was extracted by using Genomic DNA purification kits (Ferments, Germany) following the manufacturer instructions. The extracted DNA concentration was confirmed through measurement by UV Spectrophotometer. Readings were taken at wave lengths of 260 and 280 nm. Concentration of DNA sample was measured: = 50 ug $mL^{-1}$× A260 × dilution factor (Alhusseini et al., 2012).

### Amplification of Integrons DNA using PCR-SSP

In rapid cycler PCR (G-Storm Thermal cycler, England) 10 µL from each sample of extracted DNA were used in singleplex-PCR for HBV genomic DNA using PCR master mix kit (Qiagen Gmbh, Hilden, Germany) according to the manufacturer’s instructions. Amplification was performed using the following primer sets provided by Operon, inc Huntsville, Alabama Germany. IntI1-F 5’-CCTCCCGCACGATGA -3 ‘IntI1-R:5’- TCCACGCATCGTCAG -3’for Integron1, IntI2 F: 5’- GCAAACGCAAGCATTCATTA -3’ IntI2 R: 5’-ACGGATATGCGACAAAAAGG-3’ for Integron 2 and forward primer 5’- ACAGACCGAGAAGGCTTATG-3’, reverse primer 5’-ACAGACCGAGAAGGCTTATG-3’ for Integron3. According to the manufacturer instructions, the applied amplification program was 95°C for 5 min as initial denaturation then cycling for 40 cycle (95°C) 30 s, (48-52°C) 30 s and 72°C 1 min). The final extension was 72°C for 2 min.

### Post-PCR processing and analysis of the amplified products

The amplified DNA was analyzed by electrophoresis. About 10 Ml of each reaction mixture and 1000 Base Pair (BP) ladder (Molecular

**Table 1.** Distribution of the organisms in the studied groups.

| Organism | Group | | Total |
|---|---|---|---|
| | Study group (ESBL) | Control group | |
| | Count/ % within group | | |
| *Klebsiella pneumonia* | 13 (61.9%) | 3 (42.9%) | 16 (57.1%) |
| *E. coli* | 4 (19.0%) | 2 (28.6%) | 6 (21.4%) |
| *Proteus* | 2 (9.5%) | 1 (14.3%) | 3 (10.7%) |
| *Citrobacter* | 1 (4.8%) | 0 (0%) | 1 (3.6%) |
| *Enterobacter* | 1 (4.8%) | 1 (14.3%) | 2 (7.1%) |
| Total | 21 (100.0%) | 7 (100.0%) | 28 (100.0%) |

$X^2 = 1.7$; P = 0.8

**Table 2.** Distribution of the studied groups in different departments.

| Section | Group | | Total |
|---|---|---|---|
| | Study group (ESBL) | Control group | |
| | Count (%within group) | | |
| Neonatal ICU | 2 (9.5%) | 2 (28.6%) | 4(14.3%) |
| Pediatric ICU | 2 (9.5%) | 0 (0%) | 2 (7.1%) |
| ICU | 10 (47.6%) | 1 (14.3%) | 11(39.3%) |
| GIT | 2 (9.5%) | 2 (28.6%) | 4(14.3%) |
| Chest | 5 (23.8%) | 1 (14.3%) | 6 (21.4%) |
| Dialysis | 0 (0%) | 1 (14.3%) | 1 (3.6%) |
| Total | 21 (100.0%) | 7 (100.0%) | 28 (100.0%) |

$X^2 = 8.04$; P = 0.15

weight marker) was separated on 2% agarose gel containing 0.3 µg $mL^{-1}$ of ethidium bromide. The bands were visualized using UV Tranilluminator (254 nm) and photographed using a digital camera 8 mega pixel. The image was transferred to be analyzed by computer software (Alpha In no Tech Gel Documentation System).

### Statistical analysis

Statistical analysis was undertaken using SPSS computer Software (SPSS Version 16 for Microsoft Windows, SPSS Inc., Chicago) and the Microsoft office Excel 2007. Quantitative data are expressed in terms of mean, standard deviation and qualitative data were expressed in number and percent, and appropriate statistical tests were used (ANOVA "f" test and correlation co-efficient "r" test). ROC curve analysis was done to determine the diagnostic power of each test. Results were considered to be statistically significant at $p<0.05$.

## RESULTS

From the 100 clinical samples included in this study, 70 samples showed significant bacterial growth, of them 32 isolates were Gram positive and 38 were Gram negative bacilli. Gram negative bacilli were further investigated to get 28 nosocomial *Enterobacteriaceae* strains. They were collected from ICU, chest, dialysis and internal medicine units. Different samples were collected from blood, respiratory tract, urinary tract and body fluid in descending order of frequency. From the 28 gram negative *Enterobacteriaceae* 21 (75%) ESBL positive isolates were detected and 7 (25%) were non ESBL producer (control). 18 (64%) were from males and10 (35.7%) were from females.

The most frequently isolated pathogens from different units are *Klebsiella* species and *E. coli*; 57.1% and 21.4% respectively. They also were the most frequent in ESBL group representing 61.9% and 19% respectively (Table 1). The highest percentages of the studied groups were present in ICU (47.6%) followed by chest department (23.8%) as described in Table 2.

There was a significant association between integron 1 presence and resistance to aminoglycosides (gentamycin and amikacin) with P value 0.023-0.014; cephalosporins and tetracyclin had P value of 0.001.

Most of the isolates were highly resistant to gentamicin, cefotaxime, ceftazidime, amoxicillin, azetronam, cefoxitin, chloramphinicol, sulfamethoxazole, cefpodoxime and amikacin. Most of the isolates showed resistance or decreased susceptibility (intermediate resistance) to ESCs. Although most of the isolates were multiresistant (resistant to more than two classes of antibiotics), the majority remained susceptible to imipenem, cefotaxime with clavulinic acid, and ceftazidime with clavulinic acid.

## DISCUSSION

Antibiotic resistance has been noted in Benha University Hospital. The third generation cephalosporins had been the treatment of choice for serious infections but unfortunately, resistance to these antibiotics has become a usual problem, leading to eventual treatment failure, forcing clinicians to resort to the use of more expensive and/or toxic therapeutic remedies, in addition to a diagnostic laboratory challenge in screening and control of this phenomenon.

The unrecognized widespread presence of integrons-containing Gram negative bacteria, both within hospitals and in the community poses a serious threat of the spread of antibiotic resistance (Roy et al., 2011). Data from the U.S. National Healthcare Safety Network indicate that Gram-negative bacteria were responsible for more than 30% of hospital-acquired infections and these bacteria predominate in cases of ventilator-associated pneumonia (47%) and urinary tract infections (45%) (Peleg and Hooper, 2010).

Infection and colonization with ESBL producing organisms are usually hospital-acquired especially in Intensive Care Units (ICUs) (Oberoi et al., 2013). Other hospital units that are at increased risk include surgical wards, pediatrics and neonatology, reha-bilitation units and oncology wards (Pasricha et al., 2013). Community clinics and nursing homes have also been identified as a potential reservoir (Hanson et al., 2008).

In our research, the isolates of *K. pneumoniae* were the most common among the ESBL-producing isolates (Table 1). Relatively different results were obtained by Monsef and Eghbalian (2010), where they found that the most common *Enterobacteriaceae* were *E. coli* (66.7%), followed by *Klebsiellae* spp. (10.5%). In the study of Basavaraj et al. (2011), isolates of *K. pneumoniae* (46.4%) were the most common ESBL producers, followed by the isolates of *E. coli* (31.7%) and others. In a study conducted by Biswas et al. (2013), it was found that extended spectrum β-lactamase was detected in 32.40% of *E. coli* and 40.32% of species isolates. Urine, pus and respiratory samples were common source of extended spectrum β-lactamase producers and resistance rate of these organisms to third generation cephalosporin was more than 30 to 40%.

From data described in Tables 3 to 5, it was revealed that class I integron was the principal integron class in the study isolates (Figure 1). Many studies pointed out some important facts regarding the prevalence of integrons among nosocomial pathogens. The high rates (approx. 60%) of integron prevalence particularly of the class I integron in the *E. coli* and *Klebsiella* spp. concur with previous studies in other geographical regions including Europe, Northern America and Asia (Rao et al., 2006; Su et al., 2006; Yao et al., 2007; Bhattacharjee et al., 2010). In all these studies, integrons were significantly associated with the resis-tance to multiple classes of antibacterial compounds.

The prevalence of class I integron in clinical *E. coli* strains ranged from 43 to 49% in countries from Europe and Northern America (Rao et al., 2006) The prevalence of integrons was 70% in *Klebsiella* isolates in a USA study (Rao et al., 2006) however, two studies in Asia reported even higher frequency of occurrence of integrons among ESBL-positive *K. pneumoniae* (>90%) in China (Yao et al., 2007) and India (Bhattacharjee et al., 2010).

A question was addressed about the association between the presence of integrons and different kinds of β-lactamases, especially ESBL. The answer of this question could reveal much of the mechanism of how β-lactamases could be horizontally transferred, in the hospital environment.

In our study, we found an association between ESBLs and the presence of integrons especially type I (Tables 6, 7 and Figure 2). This is in agreement with the results of Ahangarzadeh et al. (2012) in Iran which shows that the presence of class I integron and not class II integron, was significantly associated with multi drug resistance and production of ESBLs in *K. pneumoniae* isolates. They also observed a significant association between the presence of integrons and resistance to some antimicrobial agents tested.

A positive association was recorded between the presence of the *int*I1 (the phenotypic gene) and resistance to gentamicin, tetracycline, ceftazidime, cephalothin, chloramphenicol and nalidixic acid. A positive association was also observed between the presence of the *int*I II gene and resistance to tetracycline. Fazeli et al. (2012) referred to that there is an association between integrons of Enterobacterial bacteria such as *E. coli* and the *Klebsiella* spp. and different classes of β-lactamase including AmpC-type cephalosporinases, metallo-β-lactamases and extended-spectrum β-lactamases).

A Spanish study found no association between integron carriage and β-lactam resistance in ESBL-producing *E. coli* strains unless strains contained metallo-β-lactamases (Machado et al., 2005). Multidrug resistance in the *Enterobacteriaceae* has been linked with the carriage of integrons. In particular, aminoglycosides resistances are significantly associated with integron carriage in the Enterobacteriaceae (Nigro et al., 2013).

In our study, integrons were statistically tested as detectors (or predictors) for multi-drug resistance (combined resistance to 3rd generation cephalosporins, tetracycline and aminoglycosides) (Tables 6 and 7). Significant association was observed between integron I and the resistance to aminoglycosides (gentamicin, amikacin and tetracycline. In a study made by Karczmarczyk et al. (2011), class I and II integrons were found to contain trimethoprim (*dfr*) and streptomycin (*aad*) resistance encoding genes, which are frequently reported in *E. coli* isolates recovered from various sources, including human, animal and environ-mental samples. Their extensive dissemination could be attributed

**Table 3.** Integron 1 in different isolated ESBL producing pathogens.

| Organism | Integrin 1 | | Total |
|---|---|---|---|
| | Negative | positive | |
| | Count (%) within integrin 1 | | |
| *Klebsiella pneumoniae* | 6 (54.5%) | 10 (58.8%) | 16 (57.1%) |
| *E. coli* | 2 (18.2%) | 4 (23.5%) | 6 (21.4%) |
| *Proteus* | 1 (9.1%) | 2 (11.8%) | 3 (10.7%) |
| *Citrobacter* | 1 (9.1%) | 0. (0%) | 1 (3.6%) |
| *Enterobacter* | 1 (9.1%) | 1 (5.9%) | 2 (7.1%) |
| Total | 11 (100.0%) | 17 (100.0%) | 28 (100.0%) |

$X^2$ = 1.8; P = 0.77. Integron 1 is more frequent, especially among *klebseilla pneumoniae (*58.8%) and *E. coli* (23.5%). It was absent in control group.

**Table 4.** Integron 2 in different isolated ESBL producing pathogens.

| Organism | Integron 2 | | Total |
|---|---|---|---|
| | Negative | positive | |
| | Count (%) | | |
| *klebseilla pneumoniae* | 14 (60.9%) | 2 (40.0%) | 16 (57.1%) |
| *E. coli* | 5 (21.7%) | 1 (20.0%) | 6 (21.4%) |
| *Proteus* | 2 (8.7%) | 1 (20.0%) | 3 (10.7%) |
| *Citrobacter* | 0 (.0%) | 1 (20.0%) | 1 (3.6%) |
| *enterobacter* | 2 (8.7%) | 0 (.0%) | 2 (7.1%) |
| Total | 23 (100.0%) | 5 (100.0%) | 28 (100.0%) |

$X^2$ = 5.8; P = 0.21. Integron 2 is represented by2 isolates *Klebsiella pneumoniae* (40%), 1 *E. coli* (20%), 1 *Citrobacter* (20%) and 1 isolate *Proteus* (20%).

**Table 5.** Integron 3 in different isolated ESBL producing pathogens.

| Organism | Integrin 3 | | Total |
|---|---|---|---|
| | Negative | Positive | |
| *Klebsiella pneumoniae* | 14 (58.3%) | 2 (50.0%) | 16 (57.1%) |
| *E. coli* | 5 (20.8%) | 1 (25.0%) | 6 (21.4%) |
| *Proteus* | 3 (12.5%) | 0 (.0%) | 3 (10.7%) |
| *Citrobacter* | 0 (0.0%) | 1 (25.0%) | 1 (3.6%) |
| *Enterobacter* | 2 (8.3%) | 0 (0.0%) | 2 (7.1%) |
| Total | 24 (100.0%) | 4 (100.0%) | 28 (100.0%) |

$X^2$= 6.9; p=0.14. Integron3 is present in *Klebsiella pneumoniae* 2 isolates (50%), 1 *E. coli* (25%) and 1 *Citrobacter* (25%).

to their association with integrons and plasmids (Kadlec and Schwarz, 2008).

The association of class 1 integrons with resistance in the studied isolates suggests that integrons facilitate the spread of antimicrobial drug resistance. Integron-positive isolates were more likely to be multi-resistant than integron-negative isolates. Multi-resistant integrons are considered to be important contributors to the development of antibiotic resistance among Gram negative bacteria ( Rawat and Nair 2010). In an Iranian study conducted by Japoni et al. (2011), they found that the association between multi-drug resistance to norfloxacin, ceftazidime, gentamicin, ciprofloxacin, cefepime and amikacin and the presence of integrons was statistically significant.

Positive association of integrons with multidrug resistance was found by Kor et al. (2013) study. They

**Table 6.** Distribution of integrons in the studied groups.

| Integrons | Group | | Total | X2 | p |
|---|---|---|---|---|---|
| | ESBL group | Control group | | | |
| Integron 1 | | | | 11.2 | 0.0001 |
| Negative | 4 (19%) | 7 (100%) | 11 (39.3%) | | |
| Positive | 17 (81%) | 0 (0%) | 17 (60.7%) | | |
| Integron 2 | | | | 2.3 | 0.15 |
| Negative | 16 (76.2%) | 7 (100%) | 23 (82.1%) | | |
| Positive | 5 (23.8%) | 0 (0%) | 5 (17.9%) | | |
| Integron 3 | | | | 1.6 | 0.21 |
| Negative | 17 (81%) | 7 (100%) | 24 (85.7%) | | |
| Positive | 4 (19%) | 0 (0%) | 4 (14.3%) | | |

**Table 7.** Performance of integron in diagnosis of drug resistance.

| P | 95% CI (AUC) | AUC | NPV | PPV | Specificity | Sensitivity | Variable |
|---|---|---|---|---|---|---|---|
| 0.002* | 0.79-1.0 | 0.91 | 63.60% | 100% | 100% | 81.0% | Integron 1 |
| 0.35 | 0.4-0.84 | 0.62 | 30.40% | 100% | 100% | 23.8% | Integron 2 |
| 0.46 | 0.37-0.82 | 0.60 | 29.20% | 100% | 100% | 19.0% | Integron 3 |

PPV= Positive predictive value; NPV = negative predictive value; AUC = area under the curve.

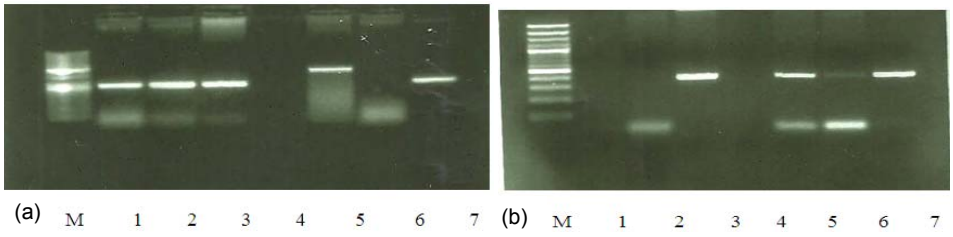


**Figure 1.** Gel electrophoresis of the amplified products of the targeted genes: (a) indicate bands for integron 1 in lanes 1,2,3,7 and integron 3 in lane 5 . (b) indicates bands of integron 2 in lanes 3,5,6,7; M: 100bp DNA marker. Lane 4 in both gel indicate -ve control.

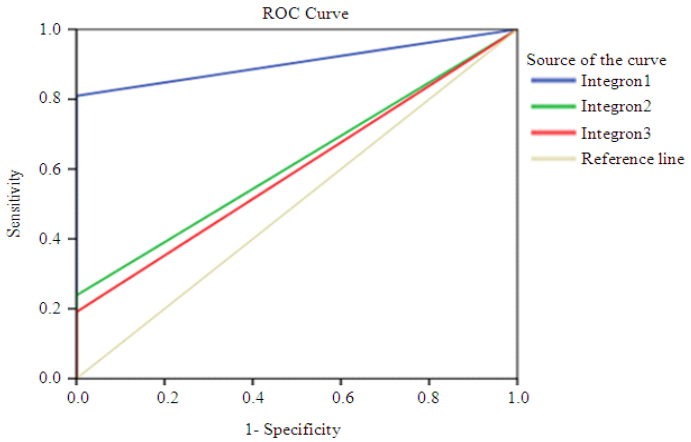


**Figure 2.** Roc curve Total area is 1.0, the yellow line is the reference line, it divides the area into 2 halves. 95% CI of AUC = confidence interval = it is an interval at which the investigator is 95% confident that the true AUC lies. If includes 0.5 = non significant.

stated that class 1 integrons were the most dominant class identified (45.6 %). Three isolates were shown to contain class 2 integrons (2.0%) and No class 3 integrons were detected in their study (Kor et al., 2013).

## Conclusion

Most of integron positive isolates were highly resistant to gentamycin, cefotaxime, ceftazidime, amoxicillin, azetronam, cefoxitin, chloramphenicol, sulfamethoxazole, cefpodoxime and amikacin. Integrons were found only in ESBL group and Integron I is the main integron in our isolates. An association between integron and multi drug resistance especially to aminoglycosides and tetracycline has been found in this study.

## Conflict of Interests

The author(s) have not declared any conflict of interests.

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