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Prevalence of Class 1 and Class 2 Integrons in Extensively Drug-Resistant *Escherichia Coli*isolated from Iraqi Patients with Diabetic Foot Ulcers in Diyala Province

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Abstract
Diabetic foot infectionis a common and serious problem for all health systems in the world. This study aimed tomolecularlydetect class (1)and class (2) integron genes responsible for antibiotic resistance by using PCR techniquein extensively drug-resistant *Escherichia coli*bacteria isolated from clinical samples of (285) Iraqi patients with diabetic foot ulcers(DFUs) who attended different hospitals in Diyala province/Iraq during the period from July to the end of October /2020. The results showed that bacterial isolates appeared in (250) of these samples. The conventional microbiological methods and VITEK 2 automated system showed that 65 (26%) of the isolates were *Escherichia coli* isolates.For all the (65) pathogenic isolates of *Escherichia coli*, susceptibility testswere performed against 15antimicrobial agents.The results of antibiotic resistance showedthe following:Amoxicillin-Clavulanic acid 100%, cefotaxime 92.3%, piperacillin 84.%, ceftriaxone 78.4%, cefepime 76.6%,ceftazidime75.3%, ciprofloxacin 67.9%, levofloxacin 69.2%,while resistance rate to aminoglycosides was 92.3, 80% and 76.9% for gentamicin, amikacin and tobramycin respectively. Resistance rate foraztreonam was 52.3%, Imipenem 33.8% and Meropenem 43%.However, 20 (30.7%) isolatesof *Escherichia coli* were found to be extensively drug-resistant. Depending on the detection of integrase gene for the investigation of class 1 and class 2 integrons, PCR assay showed that 16(80%) of the extensively drug-resistant *E. coli* isolates were integrase gene positive, which confirms the extremely high dissemination of class 1 integron at the hospitals of Baquba city in Diyala province, while class2 integrase gene was not found. Analysis of class 1 integron variable regions showed the presence of (5) different fragment sizes of approximately 500, 600,700, 800 and 900 bp. On RFLP using restriction enzyme *Alu*I, five different restriction patterns obtained Class1(500bp, 200-300 fragment), Class2 (±600bp, 90-200-300 fragment) Class3 (700bp, 200-500 fragment), Class4 (800bp, 100-200-500 fragment) and Class5(900bp, 100,200,600 fragment).
Keywords: *Escherichia coli*, Diabetic Foot ulcers, antibiotic resistance, PCR, Class 1 and Class 2 Integrons

Introduction

Diabetic foot ulcer (DFU) is a serious and common diabeticcomplication that significantly increases the cost of treatment.[1] the world health organization defined diabetic foot (DF) as a pathologic consequence including infections, ulcerations and/or destructions of deep tissuesrelated to neurologic abnormalities, different degrees of peripheral vascular disease and/or metabolic complication of diabetes in the lower limbs [2]. One of the bacteria in diabetic foot ulcer is *Escherichia coli*.Its long term complication represents a major health problem of high mortality and morbidity rates. According to bacterial culture and molecular approaches, DFU can be colonized with numerous aerobic and anaerobic polymicrobials[3,4].Among several types of bacteria isolated from diabetic foot ulcer patients, *E .coli*is considered one of the most important causes in the last years [5].
Escherichia coli, one of the most widely studied micro-organism worldwide, resides in the gastrointestinal tract of warm-blooded animals and reptiles and is an important pathogen in humans[6].In a world health organization report on antimicrobial resistance surveillance,

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E.coli is listed as one of the 9 bacteria of global concern responsible for common infections in hospitals and communities [7].The rapid dissemination of multidrug-resistant bacteria has become a concern worldwide and complicates the treatment of infections. This phenomenon is a consequence of the bacterialability for acquiring exogenous genes by mobile elements (e.g. conjugative plasmid, transposons & integrons), and has been recognized as a major cause of widespread multidrug resistance pheno- and genotypes[8].Although integrons are not mobile by themselves, they can be transferred horizontally. Resistance integrons are elementscontaining the components of a system for sitespecific recombination, distinguishing, capturing and carrying resistance genes in mobile cassettes. For these reasons, integrons are the major contributors to the spread and maintenance of MDR. [9,10]. Integrons are classified into several classes based on the sequence of their intI genes. Class 1–3 integrons are widely associated with resistance determinants in human clinical isolates. Class 1 integron is primarily associated with capture gene cassettes from a huge pool of resistance genes conferring antibiotic resistance[10]. Moreover, class 1 integron is the most frequently element found in nosocomial and community environments[11].Because the integron system is able to create novel combination of resistance gene, it could be a dynamic force in the MDR bacteria evolution. Moreover, the entire integron element is usuallyfound within other mobile genetic elements like the plasmid & transposon. The integron element containing its gene cassettes is able disseminate horizontally through a bacterial population [12].This study aimed to assess the occurrence of class1& class2 integrase-specific int1 and int2 genes integrons and to investigatethe diversity of their variable regions carried by commensal*E. coli* isolates collected from Iraqi Patients with Diabetic Foot ulcers(DFUs) in Diyala Provence.

Materials and methods

Sample collection

In our study,(285) swab specimens were collected from diabetic foot infection ulcers from Baquba hospital/Central health LabsfromJuly to the end of October /2020.The specimens collected were (21 males and 15 females) with their ages ranging between (35-80)years. A single colony was selected from each primary positive culture on blood agar, MacConkey agar and mannitol salt agar, and identified depending on its morphological and cultural characteristics. The biochemical tests were used to confirm the diagnosis of *E.coli* using the Vitek-2 system [13].

Antimicrobial SusceptibilityTest

For the detection of the sensitivity of 65*E.coli* isolates, 15 antimicrobial discswere used in accordance withthe method of Bauer and Kurby[14].Muller Hinton agar plates were prepared. In (5) ml of normal saline, the isolated colonies were suspended and mixed by vortex, and the turbidity was compared with the standard McFarland solution. On Muller Hinton agar,aliquot of 2µl bacterial suspensionswere placed anddistributed by cotton swabs in three different directions via rotating the plates 60^{o} for each direction. The agar plates were placed upside down at room temperature for few minutes. Antibiotic discs were put on the agar plates and incubated at 37^{o}C overnight. The zone inhibition ruler was used to measure the inhibition zones in (mm) and the results were compared with the National committee for clinical laboratory standards[15].

Any bacterialstrain that resisted the minimum of at least 3 differentclasses of antibiotics was considereda multi-drug resistant(MDR), while any bacterial strain that remained susceptible toonly one or twoclasses of antibiotics wasconsidered anextensive-drugresistant(XDR),whereas any bacterial isolate which resistedall sub classes of antibiotics was considered a pandrugresistant (PDR)[16].

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DNA Extraction

From all bacterial isolates, the genomic DNA was extractedby using the extraction kit of genomic DNA. The purification was based on the manufacturing company guidelines (Promega USA). The DNA concentration was misheard by using Quantus Florometer (Promega, USA). All twenty extensively-drug resistant (XDR) isolates were examinedusingthe standard PCR conventional by applying specific primers for class 1 integrase gene, class 2 integrase gene and variable regions of class 1 integron CS genes as shown in table (1).

Table (1): The primers used fortarget gene detection

Target	Gene	Seq.	Annealing Temp. (C°)	Product Size (bp)
Class 1 integrase gene	*int1*-F *int1*-R	5-CCT CCC GCA CGA TGA TC -3 5'TCC ACG CAT CGT CAG GC -3'	60	280
Class 2 integrase gene	*Int2*-F *Int2*-R	5'- GCAAATGAAGTGCAACGC -3' 5'- ACACGCTTGCTAACGATG -3'	48	466
Variable region of class 1 integron	*In5`CS*-F *In3`CS* -R	5'-GGCATCCAAGCAGCAAG-3' 5'AAGCAGACTTGACCTGA-3'	55	Variable

The PCR conditions started with a thermocycler program according to data shown in table (2). Amplified PCR products were detected by agarose gel electrophoresis.

Table (2): The PCR thermocycler program for *Escherichia coli* target genes

Steps	°C	m:s	Cycle
Initial denaturation	95	05:00	1
Denaturations	95	00:30	30
Annealings	60,48or 55	00:30	30
Extensions	72	01:00	30
Final extensions	72	07:00	1
Holds	10	10:00	1

* Elongation in 72ºC/ 1 minute and final extension 72°C/ 7 min for all genes

The restriction fragment length polymorphism (RFLP)

To confirm that the different bacterial isolates were carrying identicalintegrons, RFLP typing was used to compare amplicons with similar size by using the restriction endonucleases*AluI*. RFLP of the variable region class 1 integronCS gene was performed using the restriction enzyme *AluI* (promega U.S.A) in accordance with the manufacturer's guidelines. In this technique, 2U of the *Alu*I enzyme was used to digest 10 µl of the *CS* gene PCR products and incubated for 4 hours at 37^{o}C in a waterbath.Then the 2%agarose of gel electrophoresis was used to separate the restricted fragments.

Resultsand discussion

From the (150) diabetic patients with foot ulcers,(185) bacterial isolates were taken. The ages of the patients ranged between (35-80) years, and the highest number of diabetic foot ulcers (DFUs) patients was found within the age group (60-65) years.The results showed that*Staphylococcus spp* was the most frequent isolate 76 (30%) followed by *Escherichia coli*

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(26%) then*Enterococcus spp*. (21%)**.** All isolates were identified through morphological, cultural and some biochemical tests by using Vitek-2-GN system. The bacterial antibiotic susceptibilitytesting was also performed. The resistance of the (65) *E. coli* isolates was studied against (15) different antibiotics. The table (3) shows different sensitivity and resistance patterns to the (15) antibiotics.

Table (3): Antibiogram susceptibility of *E. coli* isolatestoward antibiotics

Antibiotics	Resistance Number of isolates n=65	Percentage%
Pipracilin	55	**(84.6%)**
Ticarcilin	60	**(92.3%)**
Amoxicillin/Glavulanic acid	65	**(100%)**
Cefotaxime	60	**(92.3%)**
Ceftriaxone	51	**(78.4%)**
Ceftazidime	49	**(75.3%)**
Cefepime	50	**(76.9%)**
Ciprofloxacin	50	**(76.9%)**
Levofloxacin	45	**(69.2%)**
Gentamycin	60	**(92.3%)**
Amikacin	52	**(76.9%)**
Tobramicin	50	**(80%)**
Azetreonam	34	**(52.3%)**
Impenem	22	**(33.8%)**
Meropenem	28	**(43%)**

The higher resistance was shown tothe β-lactams, aminoglycosides and flouroquinolones. A moderate resistance was shown to monobactams, as (52.3%) of the isolates were resistant to azetreonam, whereascarbapenemes showed the lowest resistance (43%) and (33.8%) for meropenem and imipenem respectively. The bacterial isolates exhibitedthe highest resistance (100%) to Amoxicillin/Glavulanic acid followed by Cefotaxime & Gentamicin (92.3%), Pipracilin (84.6%), Amikacin (80%), Ceftriaxone (78.4%), Cefepime and Ciprofloxacin (76.9%). Resistance to other antibiotics was (75%) to Ceftazidime, (69%) to Levofluxacin. Table (3) showed various antibiotic resistance patterns among the studied isolates. In the current study, 18(27.6%) of isolateswere classified as multidrug sensitive (MDS),27(41.5%) of isolates were multidrug resistant (MDR) because they showed resistance to three antibiotic classes at least. Among these isolates, 20(30.7%)were resistant to 5 or 6 classes of antibiotics meeting criteria for extensively-drug resistant (XDR) organisms.

Astudy by Naqid [17] inKurdistan Region of Iraq revealed that *E. coli*isolates were highly sensitive to eretapenem andimpenem (96.4%) and (97.6%) respectively, but showed (87.8%) resistance to ampicillin. Results in the present study agreed with Al–Zubaidi [18] who stated that the isolates of*E. coli* inDiyala hospitals demonstrated resistance to various antibiotic types, such as floroquinolones and carbapenemsand 50%, 25% of isolates were resistant to Levofloxacin and Imipenem, respectively.

Detection and analysis of integrons

Class 1 integron gene *intI1* was found in 16 (80%) of theextensively drug-resistant *E. coli* isolates (Figure 1,2). No strain was shown to harbor class 2 integrase gene*intI-2*, which confirms the extremely high dissemination of class 1 integron at the hospitals of Baquba in Diyala province. This result agrees with Singh [19] whose PCR results showed that 75% of *Escherichia coli* isolates carried class 1 integron but it differs from the Iranian study

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presented by Halaji *et al*[20]who revealed 47% integron positive *Escherichia coli* isolates.Among the multidrug resistant Gram-(-ve) bacteria, integrons play a key role because of their higher ability to transfer antibiotic resistance genes [21]. In our study, a highly prevalence of integrons was observed; however, intl-1 integrons were found to be much more than intl-2 integrons.

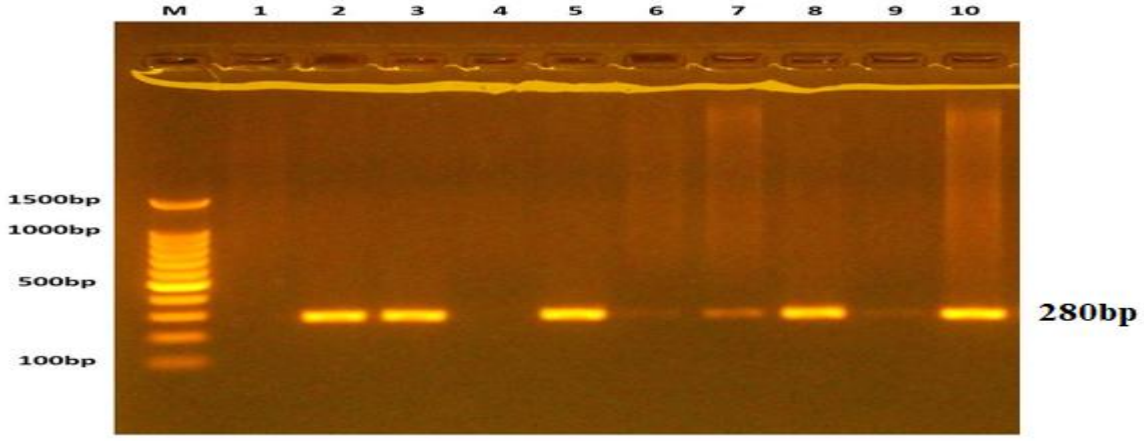
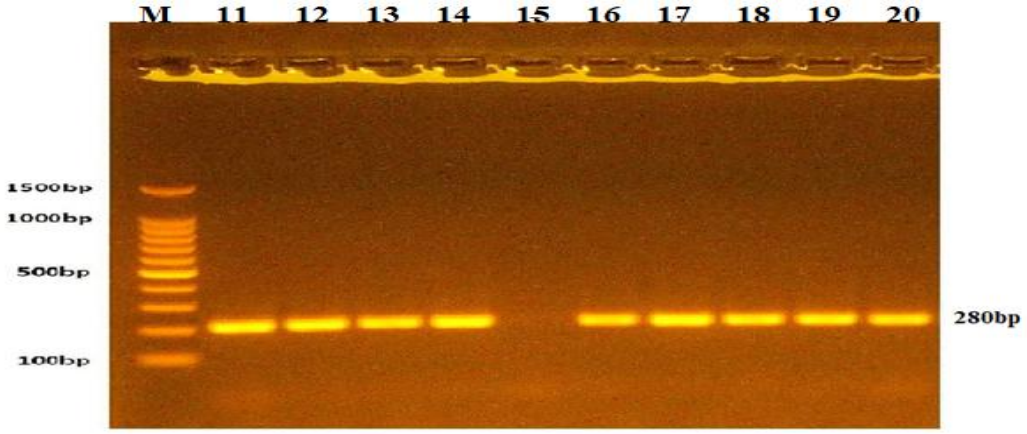


Figure (1):Agarose gel electrophoresis of the amplified PCR productsto detectIntgron class1(IntI1) genes (280bp) run on 1.5%agarose (at 70 volts for 90 minutes), stainingby ethidium bromide, Lane 1-10*Escherichia coli*isolate ;M: Marker DNA ladders (100bp); Lane2,3,5,7,8,9,10 positive to class1 Intgron IntI1 gene.



Figure(2):Agarose gel electrophoresis of amplified PCR productsto detect Intgron class1(IntI1) genes (280bp) run on 1.5%agarose (at 70 volts for 90 minutes), staining with ethidium bromide, lane 11-20 *Escherichia coli*isolates;M: Marker DNA ladder (100bp); Lanes 11,12,13,14,16,17,18,19,20 positive for class1 Intgron(IntI1) gene.

The analysis of class 1-integron variable regionshowed existence of five(5) variable fragment sizes of about 500, 600,700, 800 and 900 bp(Table 4)(Figure 3).

Table (4): Class1 Intgron variable regionsCS gene typing

:lass1 Intgron variable ions typing	**PCR product (pb)**	**No. of isolates & %**

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Type I	500	**2(** 12.5%)
Type II	600	**3** (18.7%)
Type III	700	**8**(50%)
Type IV	800	**2**(12.5%)
Type V	900	**1**(6.25%)

In our study, PCR amplification of *CS* gene of the sixteen extensively drug-resistant *E. coli*isolates (class 1 integron positive) were digested with *Alu*I enzyme and obtained five distinct RFLP patterns with different number of fragments that varied from 1 to 4 with sizes of fragments varied from (90 to 600 bp).On RFLP using restriction enzyme *Alu*I, five different restriction patterns obtained Class1(500bp, 200-300 fragment), Class2 (±600bp, 90-200-300 fragment) Class3 (700bp, 200-500 fragment), Class4 (800bp, 100-200-500 fragment) and Class5 (900bp, 100,200,600) (Figure 3).

The class 1 integron variable regions (CS) gene and PCR–RFLP of the *CS*genotyping is an essentialtechnique to investigate infectious diseaseoutbreaksaiming to explainthe temporal and local increase in infection incidence causedbysome bacterial species [22]. The outbreak strain typingmakes theoutbreak control plans easier, definies thedegree of spreading of bacterial types and clone numberscontributed toinfection transmissions, observingthe reservoir of epidemic cloneand controling the assessment of the control plan,like monitoring vaccinations process effectiveness[23].

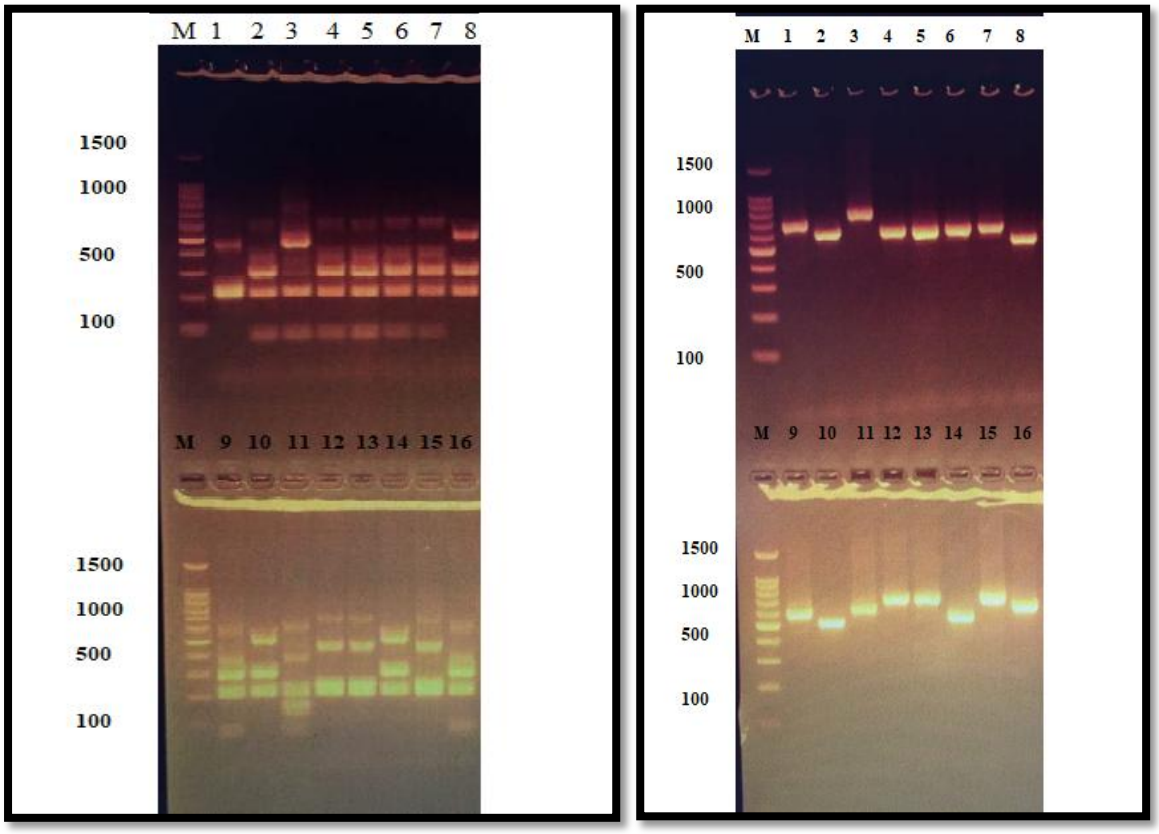

FIGURE 3 :The ethidium bromide stained gel showing the typical banding patternexamined with the uniplex PCR assay, PCR amplification of the class1 Intgron variable regions using primersIn5'CS and In3'CSgene from *Escherichia coli*, with the amplicon size 500-900bp. DNA amplification products were separated by

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electrophoresis in an (2%) agarose gel. The electrophoresis was done at 70 volts for 1.5 hours. The symbol "M" refers to ladder marker, Lane (10,14): *class1 Intgron variable regions*gene type I (500bp), lane(8,9,11) type II (600bp), lane (4,5,6,7,12,13,16)type III(700) , lane (1,15)type IV(800bp),lane 3 type V (900bp), RFLP patterns obtained of class1 Intgron variable regions PCR products digested by*Alu*I endonuclease. Lane (10,14)type1500bp (fragments : 200, 300 bp), lane(8,9,11) typeII 600 pb (fragments: 90,200,300bp),lane(4,5,6,7,12,13,16)typeIII700bp (fragments : 200, 300,400 bp), lane (1,15)typeIV 800bp (fragments :100, 200, 500 bp), lane 3 type V (900bp) (fragments: 100,200,600).

Conclusions

Thecurrent study demonstratedsuperiority of extensively drug-resistantstrainin the isolates of*E. coli*from patients with DFUs. Knowing the isolate's antibiotic susceptibility patterns canhelp todetermine the empirical treatment of diabetic ulcer. Therefore, indiscriminate antibiotic use and chances of resultant antibiotic resistance emergencemay also be decreased.It can be concluded from this study that integrons were prevalent and playedbasic roles in multidrug resistant *E. coli*, which cangive some essential surveillance information representing the antibiotic selective pressures in this specific area. It is important to concentrate on investigating the infection source in order to reduce the emergence of the multi-resistant strains.

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