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PREVALENCE OF CLASS 1, CLASS 2, CLASS 3 INTEGRONS IN ANTIBIOTIC RESISTANT UROPATHOGENIC *ESCHERICHERIA COLI* ISOLATES

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Uropathogenic Escherichia coli, class 1 integron, multidrug resistant strains, plasmid-borne

Abstract

Background: Uropathogenic Escherichia coli (UPEC) isolates were resistant to commonly used antibiotics and multi-drug resistant strains are emerging in hospital settings. Integron (IGN) are genetic elements containing integrase gene, attl site and gene cassettes which carry multiple antibiotic resistant genes. Although IGNs themselves are not mobile, the integrase enzyme excises and integrates the gene cassettes from and into the IGN. Class 1 integrons have been extensively studied because these were most prevalent among clinical isolates.

Objectives: To investigate the prevalence of Class 1, Class 2 and Class 3 integrons in drug resistant UPEC isolates.

Methods: In this study, UPEC isolates were determined for the antibiotic susceptibility patterns to ten antibiotics and the drug resistant isolates were investigated for the class 1, class 2 and class 3 IGNs by polymerase chain reaction methods.

Results: Out of drug resistant isolates, 25 isolates were observed to have class 1 IGN while both class 2 and 3 IGNs were negative in all isolates. Three isolates were resistant to 7 antibiotics including cefotaxime, ciprofloxacin, gentamycin and co-trimethoxazole. IGNs from these isolates were plasmid-borne.

Conclusion: The isolates which were resistant to these four antibiotics had possibility of sequence type (ST) 131 which is the pandemic clone which carries multiple drug resistant genes. As a consequence, this clonal group gives rise to treatment failure and fatal complication. As a further recommendation, it is necessary to proceed to multi-locus sequence typing to investigate the possibility of ST131 which will also be valuable information for the clinician

Introduction

The widespread use of antibiotics as well as transfer of drug resistance determinants mediated by plasmids, transposons and gene cassettes in IGNs caused rapid transmission of drug resistance in bacterial pathogens. The genetic organizations of IGNs are quite diverse with the presence of variable no. of drug resistance genes in their gene cassettes.[1]

An IGN is defined as a genetic element that encodes integrase that mediates site-specific recombination events, possesses a site, *attI*, at which gene cassettes, can be integrated by site-specific recombination and gene cassettes. Although IGNs themselves are not mobile, the integrase enzyme excises and integrates the gene cassettes from and into the IGN.[2, 3]

Gene cassettes are discrete genetic elements that may exist as free non-replicating DNA molecules when moving from one genetic site to another. Gene cassettes normally contain a single gene and a 59 base element that functions as a specific recombination site. Accordingly, the cassettes are 500–1000 bp in length. The IGN has a promoter which expressed the genes carried on gene cassettes.[2]

IGN can be classified into two types depending upon their nature and genetic constitution. Resistance Integrons (RIs) and Super-integrons (SIs) are the two types. RIs contain cassettes in which genes encoding antibiotic resistance and disinfectant resistance are present and these are located on chromosomes or plasmids. However, SIs contain cassettes carrying genes for various functions. These are located on the chromosomes and the size is large in comparison to RI.[2]

There are five classes of IGN and the first three classes are RI while class 4 and 5 are SI. The most prevalent IGN is class I and it is present in gram-negative bacteria including *Escherichia coli*. However, *Mycobacterium fortuitum, Corynebacterium glutamicum and Enterococcus faecalis* are gram-positive bacteria that contain class 1 IGN. Class 1 IGNs have been extensively studied because these were most prevalent among clinical isolates as well as commensals out of three classes. IGNs contain three parts: two conserved platforms flanking a variable middle region. An *integrase (intI)* gene, an attachment site (attI) and a promoter (Pc) are essential ones in the first conserved platform. The variable region contains one or more gene cassettes. After gene cassettes carrying antibiotic resistant genes were integrated in this variable region, their expressions are under the control of Pc promoter. The 5´conserved segment bears the recombination and expression loci while the 3´ conserved segment contains two genes, *qacEdelta* 1 and *sulI*. [4 - 13]

Class 2 IGN is located on Tn7 transposon while there is a gene cassette that follows *intl* gene and *Salmonella* and *Shigella* species are known to have this RI. The amino-acid sequences of IntI1 and IntI2 are less than 50% homologous. The properties of the *IntI3* from class 3 integrons are similar to those of the *inti1*. In terms of nucleotide sequences, *inti3* was 59% homology to *intl1* while *intI1*, *intI2* and *intI3* were 57 to 96% similar in pairwise comparisons. Class 3 IGN was present in *Serratia marcescens*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Enterobacter cloacae*.[14 -20]

Vibrio cholerae was well known to have SI on their chromosome together with other *Vibrio* species. The gene cassettes captured in SI contains more than 100 genes and these include antibiotic resistant genes while the gene cassettes on RI have 10 or more genes. [3, 21]

Integrase genes from Class 4 IGN (*inti4*) from different strains of *Vibrio cholerae* are nearly identical. Similarly, *intI5* gene sequence of Class 5 integrons , encoding the V. mimicus SI integrase is 75% identical to the sequence for *inti4*. [21-23]

In this study, UPEC isolates were determined for the antibiotic susceptibility patterns to ten antibiotics and the drug resistant isolates were investigated for the class 1, class 2 and class 3 IGNs. In addition, plasmids were extracted from three UPEC isolates which were highly resistant to antibiotics and investigation was performed whether IGNs were present in these plasmids or not.

Materials and Methods

Bacterial strains and culture media

One hundred and sixty-two *Escherichia coli* isolates of UPEC from Hospital Queen Elizabeth and Hospital Papar, Sabah, East Malaysia having significant bacteriuria were investigated for antibiotic susceptibility patterns and prevalence of integrons in this study. Culture media used are nutrient agar, MacConkey agar, tryptic soy broth (BD Co. Ltd.) and novobiocin mEC broth (Eiken Co.Ltd).

Study of significant bacteriuria

One ml of urine sample was diluted to 10^{-6} dilution and 50µl of each dilution was inoculated on to nutrient agar by micropipette. The agar plates were incubated at 37^{0}C for 18hrs every time. The significant bacteriuria was determined by counting the colonies on the plates which had 30–300 colonies. To get the bacterial count, the colony count on the plates was multiplied by dilution factor.

Identification of *Escherichia coli* in urine samples
One hundred µl of urine was inoculated into 1 ml of Novobiocin mEC broth and incubated for 6hrs at 37˚C. This bacterial suspension was subcultured onto MacConkey agar by streak inoculation method and incubated at 37˚C for 18hrs. The next day, lactose fermenting colonies on the plates were confirmed for *E.coli* by IMViC tests.

Stocking of *E.coli* isolates
Isolates showing typical biochemical test for *E. coli* were stocked on nutrient agar slant and 70 µl of overnight broth culture and 30µl of glycerol were mixed to prepare glycerol stock. Nutrient agar slant were stocked at 4˚C and glycerol stocks were stored at -80˚C freezer.

Disc Diffusion Method
The antibiotic discs used in this study were co-trimethoxazole (SXM), tetracycline (TE), ciprofloxacin (CIP), gentamycin (GM), chloramphenicol (CAM), ampicillin (AMP), cefotaxime (CTX), amikacin (AK), ceftazidine (CAZ) and levofloxacin (LVX). The stocked UPEC isolates were cultured on MacConkey agar and disc diffusion method was performed on Mueller–Hinton agar plates, according to Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2012) to test antibiotic susceptibility.[24]

Determination of Minimum Inhibitory Concentration
This procedure was performed using the agar dilution method as described by the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2012). The minimum inhibitory concentration (MIC) is defined as the lowest antimicrobial concentration able to totally inhibit bacterial growth. *Escherichia coli* ATCC 25922 was used as a negative control for both disc diffusion method and MIC study.[24]

PCR for class 1, class 2 and class 3 IGNs
The primers used in this study were shown in table 1. The bacterial isolates were inoculated in 3 ml of Luria-Bertani broth and incubated at 37˚C for 18 hrs. The bacterial DNA was extracted by boiling method. [26,27]
Amplification was performed in a total of 50 µL PCR mixture with 2 µL of template DNA, 0.4 mM primers, 1x PCR buffer, 0.2 mM dNTPs, 1 mM $MgCl_2$ and 1.25 U of *Taq* polymerase. The PCR conditions were 94°C for 5 min; 30 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min. A final extension was performed at 72°C for 10 min. The positive control was the isolate which PCR product was sequenced in both direction and the DNA sequence was homologous with the sequence from the database. Negative control was the EC22592 which has no IGN. Amplification was performed using Applied Biosystems Thermocycler. The sizes of PCR amplicons were checked by 1.5% TAE agarose gel electrophoresis and Alpha Imager® HP System gel documentation apparatus after the gel was stained with ethidium bromide.

Extraction of plasmids from highly multidrug resistant UPEC isolates
Two ml of overnight broth culture were centrifuged at 13,000 rpm for 10 min and supernatant was removed. The pellet was suspended in 100 µl of buffer containing 50 mM of glucose, 10 mM EDTA, 10mM Tris-HCl pH 8.0 by vortexing. Two hundred µl of lysis buffer containing 0.2M NaOH and 1% sodium dodecyl sulphate was added and incubated at room temperature for 5 min. Addition of 150 µl 7.5M ammonium acetate and chloroform and the solution was mixed by inversion. The tube was chilled on ice for 10 min and then centrifuged at 13,000 rpm for 10 min. The supernatant was transferred to precipitating solution which consists of 30% polyethylene glycol with 1.5 M NaCl and mixing was done by inversion. Chilling was done for 15 min and the plasmid DNA was precipitated by centrifugation at 13,000 rpm for 10 min. The supernatant was discarded and pellet was suspended in TE solution.[28]

Detection of IGNs in plasmids by PCR
PCR was performed by the same procedure as above except 0.5 µl volume of plasmid DNA was used as the template.

Table. 1 Oligonucleotide primers used in this study.

Target gene	Sequence of Primers	Expected size of PCR products
Class 1 integron	F: 5'-CAGTGGACATAAGCCTGTTC-3' R: 5'-CCCGAGGCATAGACTGTA-3'	160 bp (25)
Class 2 integron	F: 5'-CACGGATATGCGACAAAAAGGT-3' R: 5'-GATGACAACGAGTGACGAAATG-3'	788 bp (25)
Class 3 integron	F: 5'-GCCTCCGGCAGCGACTTTCAG-3' R: 5'-ACGGATCTGCCAAACCTGACT-3'	979 bp (25)

Results

One hundred and sixty-two UPEC isolates were tested for antibiotic susceptibility patterns with ten antibiotics mentioned in the method. Out of 162 isolates, 139, 45, 40 were resistant to ampicillin, tetracycline and co-trimethoxazole, respectively. The isolates which are resistant to antibiotic in disc-diffusion method were confirmed by MIC determination.

Out of multidrug-resistant isolates, 25 isolates were observed to have class 1 integron while both class 2 and 3 integrons were negative in all the isolates. Six isolates which was positive for class 1 integron with PCR were shown in figure 1.

Nine patterns of antibiotic resistance were observed in class 1 integron positive 25 isolates and it was shown in table 2. Three UPEC isolates which were resistant to 7 out of 10 antibiotics and these isolates were also mentioned in table 2.

IGNs were usually plasmid borne so that plasmid extraction was done and large sized plasmids (megaplasmid) more than 20 kb were observed. By means of Class 1 IGN PCR, it was found that these IGNs from 3 multidrug resistant isolates were plasmid borne IGNs (figure 2).

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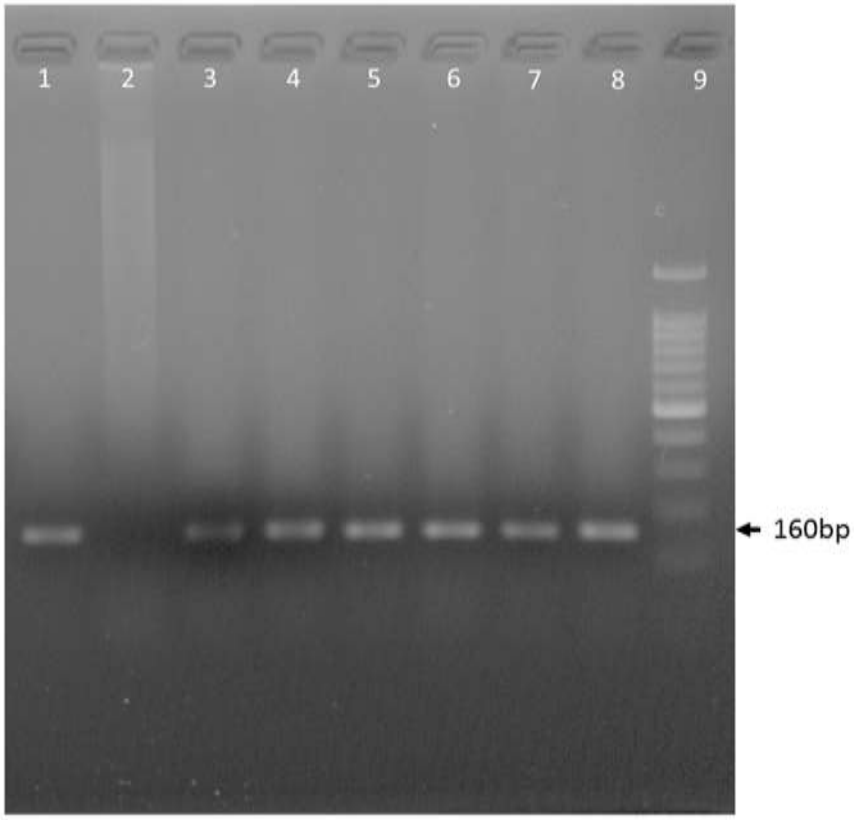

Fig. 1 Gel electrophoresis picture of PCR products of polymerase chain reaction for Class 1 integron Lane 1 is positive control, Lane 2 is negative control, Lane 3 to 8 are PCR products of Class 1 integron from UPEC isolates and Lane 9 is 100 bp molecular marker.

Table 2. Antibiotic susceptibility pattern of 25 UPEC isolates positive for class 1 integron.

Isolate no.	SMX	TE	CIP	GM	CAM	AMP	CTX	AK	CAZ	LVX
EC271**	R	R	R	R	S	R	R	S	S	R
EC067, EC070**	R	R	R	R	S	R	R	S	R	S
EC256, EC257	R	R	S	R	S	R	R	S	S	S
EC088, EC160, EC161	R	R	S	S	R	R	S	S	S	S
EC091	R	R	S	S	S	R	S	S	R	S
EC065, EC097, EC109, EC110	R	R	S	S	S	R	S	S	S	S
EC207, EC237, EC240	R	S	S	S	R	R	S	S	S	S
EC186, EC187, EC225, EC274, ECQ88, ECQ99	R	S	S	S	S	R	S	S	S	S
EC051, EC064, EC193	S	S	S	S	S	R	S	S	S	S

*****The upper two rows with three isolates were resistant to 7 out of 10 antibiotics.***

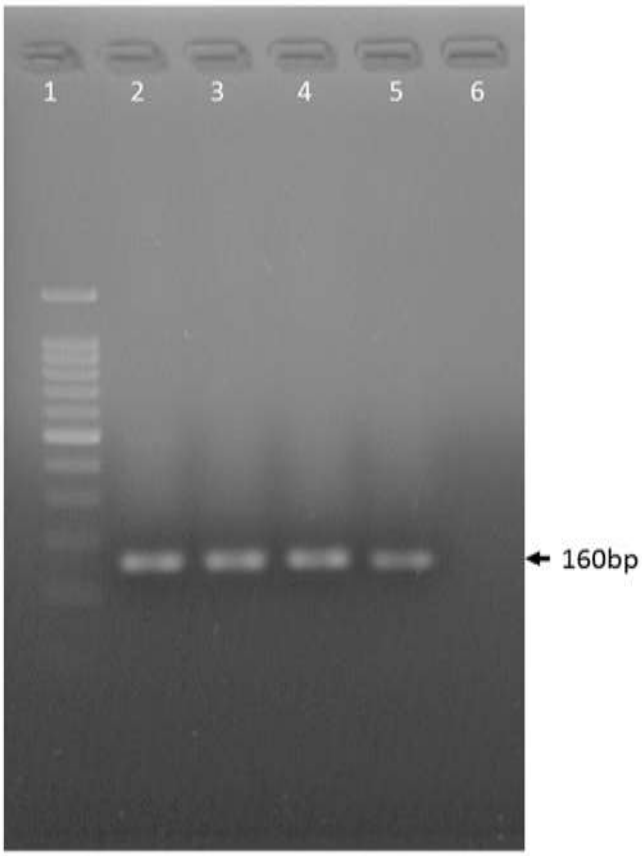

Fig. 2 Gel electrophoresis picture of PCR products of polymerase chain reaction for Class 1 integron from megaplasmid of three multidrug resistant isolates. Lane 2 to 4 are PCR products of plasmids extract from UPEC EC067, EC070, EC271 isolates, Lane 5 is positive control and Lane 6 is negative control while Lane 1 is 100 bp molecular marker.

Discussion

IGNs are capable of collecting of promoterless gene cassettes through the actions of specialized site-specific recombination enzymes; intI. IGNs operate as fully equipped site-specific recombination systems which are located on other mobile genetic elements such as transposons and plasmids to horizontally transfer resistance encoding genes between bacterial species commonly within the *Enterobacteriaceae* family.[29]

The class 2 integrase *intl*2 is, has an internal stop codon in its open reading frame and consequently non-functional. The integration and excision of gene cassettes need the presence of another type of *intl*. There is frequently presence of *intl1* in the same isolates as class 2 IGNs. A small number of different gene cassettes have been observed in class 2 IGNs because of the non-functional *IntI* .[30] Recombination between attC and secondary integration sites is also catalysed by *intl3*, but not as strong as *IntI1* .[31] These were the reasons why class 2 and 3 IGNs were negative in this study.

In the previous data on Class 1 integron prevalence, out of 100 UPEC isolates, 22 were positive for class 1 IGN in northern Iran. In 22 *E. coli* isolates positive for IGN class1 gene, resistance against co-trimethoxazole, cefixime and ciprofloxacin antibiotics were 100%, 95.45% and 90.90%. In this study, out of 25 isolates positive for class 1 IGN, co-trimethoxazole, cefotaxime and ciprofloxacin resistant isolates were 80%, 25% and 12% indicating that multidrug resistance to antibiotics currently used for UTI were relatively uncommon in Sabah State.[32]

In Aleppo, Syria where trimethoprim-resistant UPEC isolates were tested for IGN class 1 and 54.6% were positive with presence of *dhfr* gene in some isolates. Because the researchers have used trimethoprim-resistant UPEC

isolates, the study design was different and it is impossible to compare with this study. However, the study in Sabah indicates that UPEC isolates were still sensitive to antibiotics commonly used for UTI.[33]
The important finding in this study was that no isolates were resistant to amikacin and this finding will be valuable information for the clinical microbiologist for the choice of antibiotics in the management of UTI. In this study, three UPEC isolates were resistant to 7 out of 10 antibiotics. Because these isolates were positive for IGN, it was confirmed in this study these were plasmid borne. It has the possibility that some of the drug resistant genes were carried on gene cassettes present on class 1 IGNs and the IGNs were further transferred into these isolates by plasmid mediated conjugation.

On the other hand, this study highlights the possibility of presence of UPEC isolates which have class 1 integron could be sequence type (ST) 131 which is the pandemic clone which carries multiple drug resistant genes as well as many virulence factors. In addition, as a consequence, this clonal group gives rise to treatment failure with ultimate recurrent UTI and fatal complication. *E. coli* ST131 was identified as a clone with the characteristic of having the CTX-M-15 extended-spectrum β-lactamase (ESBL) resistance. [34-36] In the next 2-3 years, indicated *E. coli* ST131 has also been highly associated with fluoroquinolone, aminoglycosides and trimethoprim-sulfamethoxazole (TMP-SMX) resistance.[37-39] In this study, isolate no. EC67, EC70 and EC271 were at least resistant to cefotaxime, ciprofloxacin, gentamycin and TMP-SMX indicating that it is necessary for us to investigate multi-locus sequencing typing (MLST) to find possibility of ST131. This finding will also be important information for the clinician.

Conclusion
Class 1 IGN were uncommon when compared with previous studies which were undertaken in other countries. UPEC isolates were still sensitive to ciprofloxacin and third generation cephalosporin indicating that antibiotic restriction policy was followed by most of the physicians in the hospitals of Sabah.

Recommendation
Three UPEC isolates in this study were resistant to four antibiotics named cefotaxime, fluoroquinolone, aminoglycosides and TMP-SMX, which is the proposed characteristic of ST131 clone of *E. coli*. Because these pandemic clones may cause treatment failure in the hospital settings, it is essential for the molecular epidemiologist to do MLST for UPEC isolates of Sabah.

Conflict of Interest
The authors declare no conflict of interest.

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