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CORRELATION AMONG CRISPR-CAS SYSTEMS AND ANTIBIOTIC RESISTANCE *BLA*CTX-M GENE IN UPEC

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Abstract

The treatment of patients with infections caused by organisms producing beta-lactamases especially is often associated with many problems due to the spread of antibiotic resistance. *bla*CTX-M, of plasmid origin, as an ESBL producing gene, plays an important role in the development of antibiotic resistance. The CRISPR/Cas system, as a new immune system in bacteria, causes adaptive resistance to foreign genetic elements. This study investigated the distribution of CRISPR-associated *cas*1,2,3 genes and their association with the presence of *bla*CTX-M gene in *Escherichia coli* isolated from urinary tract infections. Uropathogenic *E. coli* samples (UPEC) collected from several Mazandaran and Tehran's hospitals. Detection of antibiotic resistance and beta-lactamase phenotypic was performed using the disk diffusion method. After DNA extraction from isolates, PCR was performed for *cas*1,2,3 and *ctx*-M genes with specific primer. Results showed that out of 106 UPEC isolates, 28.3% isolates were detected as ESBL- producing. The findings of this study showed 35.9% of isolates carried *bla*CTX-M gene and 61.76% of isolates have simultaneous *cas*1 and *cas*3 gene. Overall, there was no significant relationship between the presence of *cas*1,2,3 genes and beta-lactamase phenotype activity. Also, no correlation was in presence of *cas* genes and antibiotic resistance. The results showed no significant relation was between the presence of CRISPR-associated *cas* and *ctx*-M genes. So the CRISPR system is not a suitable solution for the deletion of betalactamase resistance antibiotic.

Keywords: CRISPR-associated *(cas)* genes, Uropathogenic *E. coli*, UPEC, *bla*CTX-M, Antibiotic resistance.

INTRODUCTION

A form of adaptive immunity in bacteria is the CRISPR system that defends the bacteria by degradation from foreign genetic DNA. CRISPR (Clustered Regularly Inter-Spaced Short Palindromic Repeat), is part of prokaryotes chromosomal DNA or Plasmids. this system is approximately found in 45% of bacteria and 84% of archaea. CRISPR activity requires the presence of a set of genes associated with CRISPR-Cas and encoding proteins that are essential for the immune response (1, 2).

Protecting of bacteria was done in three-step: the first stage is the spacer acquisition, the second stage is the expression of CRISPR RNA, the final stage is DNA interference. In fact, the CRISPR system consists of three parts: *cas* genes, leader sequence, and CRISPR locus. Based on the existence of *cas* genes, the CRISPR system is classified into several types. *cas* genes encode Cas proteins that are involved in various immune processes. CRISPR – Cas system is classified in to three types I, II, and III, that have several subtypes. type I is divided into six subtypes (A–F) that encode *cas*1, *cas*2, *cas*3 and *cas*5 gene. Type I is common in most bacteria and archaea. Type II is the simplest system with four genes: *cas*1, *cas*2, *cas*9, and *cas*4. Type II is observed in bacteria. Type III is classified in to type III-A and type III-B that encodes *cas*6 and *cas*10 genes. This system is most commonly in archaea and sometimes in bacteria (3, 4). Each Cas protein has the specific activity, Cas1 acts as an endonuclease and with Housekeeping proteins are involved in DNA repair and recombination (5). Cas2 has endoribonuclease activity and doxy ribonuclease activity (6). Cas3 has the function of DNA helicase and endonuclease (7). *Escherichia coli* is one of the first bacteria, which studied to investigate the molecular mechanisms of the CRISPR system (10). *E. coli* as a pathogen of urinary tract infections, that produce of Beta-Lactamase Enzymes and antibiotic resistance that caused many financial problems for treating patients. Continuous contacts of these bacteria to many Beta-lactamase causes mutation and produces different beta-lactamases and even their activity expands against new beta-lactam antibiotics. Beta-lactamases enzyme is the hydrolysis of the beta-lactam ring of the antibiotics. Extended-spectrum β-lactamase (ESBL) enzymes have broad activity against most beta-lactams such as penicillins, cephalosporins, aztreonam, and rarely carbapenems (11, 12). CTX-M (Cefotaxime Hydrolyzing Capabilities), SHV (Sulfhydryl variable) and TEM (Temoneria) are classified into four major groups A, B, C, and D based on function. These enzymes are present in A group (13, 14). CTX-M-was initially found in *E. coli* but It is also found in other members of the Enterobacteriaceae and has a worldwide distribution. This enzyme is often on IncFII plasmids and is associated with the IS26 genetic element. CTX-M produced from cefotaxime hydrolysis. Based on the amino acid sequences, CTX-M can be divided into five groups 1, 2, 8, 9 and 25 (15). The origin of CTX-M enzymes is different from TEM and SHV. SHV-ESBLs and TEM-ESBLs occur by the substitution of amino acids in their parent enzymes, whereas CTX-M-ESBLs are transferred from other bacteria by plasmid or transposon conjugation through horizontal gene transfer (16).

Antibiotic resistance is the most important in public health. Infection rates of resistant pathogens are increased, so the need for new approaches seems urgent to fight the antimicrobial resistance. One of these approaches is the CRISPR-Cas system. There is still a trial for researching these methods in combat with antimicrobial-resistant pathogens. Therefore, the aim of this study was the obtained relationship of *cas*1, *cas*2, *cas*3 and *ctx*-M beta-lactamase genes presence in UPEC isolates.

2. Materials and Methods:

2.1. Test Microorganisms

A total of 106 uropathogenic *E. coli* isolates were chosen based on their clinical importance and obtained from various infections samples of patients between January 2018 to December 2019, which attended to several Mazandaran and Tehran hospitals. Routine laboratory procedures which included macroscopic morphology and biochemical tests including Gram staining, Indole test, Methyl Red test, Voges-Proskauer test, and Citrate utilization test were done on clinical isolates to confirm their identity. Enterobacteriaceae detection kit (Padtan Teb) was used also for the identification of uropathogenic *E. coli* strains that were identified.by EMB,agar.

2.2. Antibiotic susceptibility and resistance test for uropathogenic *E. coli* isolates

Antibiotic susceptibility was identified via the paper disc diffusion method (Disc diffusion assay)on Mueller-Hinton agar according clinical and laboratory standards antibiotics included ceftriaxone (CRO:30 µg), nalidixic Acid (NA :30µg), ciprofloxacin (CP: 5µg), sulfamethoxazole trimethoprim (SXT), cefepim (PEP :30µg), ceftazidime (CAZ: 30 µg), cefotaxime (CTX :30 µg), norfloxacin (NOR:-10µg), cefixime (CFM:5µg), gentamycin (GM:-10µg), nitrofurantoin (FM :300 µg) and ampicillin (AM 10 µg). Mueller-Hinton sterile agar plates were cultured with indicator bacterial strains (10^5 CFU) and kept at 37 °C for 3 hours. Then discs were placed on the surface of Mueller-Hilton agar plates. for quality assurance control experiments were carried out under similar conditions by using *E. coli* ATCC 25922. The sensitivities of the microorganism species to the antibiotics were determined by measuring the sizes of inhibitory zones after 18 to 24 hours of incubation at 37 °C on the agar surface around the disks, and values <5 mm were considered as not active against microorganisms. The diameter of the inhibition zones was measured by using a ruler and interpreted according to the criteria recommended by the CLSI ˙2017 ˙(17).

2.4. Beta-lactamase activity assay by phenotypic confirmatory (PCT)

Beta-lactamase activity was determined by using the disk diffusion method. Antibiotics comprised cefotaxime (ctx:30µg), cefotaxime with clavulanic acid (ctx:10µg), ceftazidim (caz:30µg), ceftazidim with clavulanic acid (caz:10µg). Mueller Hinton agar plates containing 1×10^7 CFU/ml of each of the test organisms. The plates incubated at 37 °C for 24 h. *E. coli* ATCC 25922 was used as a positive control. After incubation, the diameter of inhibitory zones formed was measured and recorded.

2.5. PCR amplification for *ctx*-M gene

Bacterial DNA was extracted from all uropathogenic *E. coli* isolates by kit manual (PZP, Iran). Primers were based on the *ctx*-M gene of UPEC (18) .PCR-amplification was done for the *ctx*-M gene according to (Ojdana et al, 2014) . A 20 µL reaction was prepared which contained 12.5 µL mastermix Redgel (Amplicon), 1µL of each primer FW (5'--SCVATGTGCAGYACCAGTAA-3') and RW (5'- ACCAGAAYVAGCGGBGC-3'), 2.5 µL DNA template and 4 µL distilled water. The thermal profile was as follows: initial denaturation at 94 °C for 3 min; followed by 35 cycles at 94 °C for 30s, 55 °C for 30 s, 72 °C for 45 s; and then a final step at 72 °C for 10 min. PCR products was detected on 1.5% agarose gel after electrophoresis. These gels were estimated by LED transilluminator (Bio Intellectica, Canada).

2.6. PCR amplification for *cas* 1,2,3 gene bands

The extracted DNA was used for doing PCR. Primers were designed according by Macrogen company, Korea (Table 1). PCR reactions were performed by Eppendorf Thermocycler, in 20µl volume containing like *ctx*-M. Run thermal condition for *cas*1/ *cas*3 as follows: initial denaturation at 95 °C for 5 min; 35 cycles as subsequent at 95°C for the 30s, 55°C for 30s, 72°C for 30s; and then a final extension at 72°C for 5 min. Amplification for *cas*2 involved an initial denaturation at 95 °C for 5 min followed by 35 cycles of denaturation at 94 °C, 40 s), annealing at 54 °C, the 30s, extension at 72°C, 45s and a final extension step 72°C, 5 min. The amplified DNA was determined by gel electrophoresis. Then, the gels were stained and assessed using an LED transilluminator. After that, 25 µL of PCR products for each gene were sent for sequencing, (Macrogen, South Korea). Chromas software version 1.45 was used to analyze the sequences. Following that, obtained sequences were BLASTed in The National Center for Biotechnology Information (NCBI) database.

2.7. Nucleotide sequence accession numbers

The nucleotide sequences have the following GenBank accession numbers: MT885230 for *cas*1, MW044948 for *cas*2 and MT980728 for *cas*3.

2.8. Statistical analysis

Statistical analysis was performed with Stata Version 14.0 (IBM, Armonk, NY, USA) statistic software. Chi-square (χ^2) test and Fisher's exact test were used to assess the categorical variables. A p-value less than 0.05 was recognized as significant.

Table 1. primers used for PCR reaction in this study

Gene	Primer Sequence	PCR Product Size (bp)	Primer Concentration (pmol)
*cas*1	F GG GTT GCC TCG CTG CTA TT R GTT GTG AAT CGC TGC ATC AGT G	198	10
*cas*2	F GCG CTT ACG TGG ACG GCT C R ACT CGG TAT TGG TCG CCC AGG	157	10
*cas*3	F CCT GAC TTA CCG CCG ATT CGA TAC R AAC ATA ATA ATC TGC GGC TTC TGG G	387	10

3. Result

3.1. detection of *ctx*-M and *cas*1-3 gene bands in UPEC

Presence of *ctx*-M and *cas*1-3 gene bands in UPEC isolates showed in Fig1 and Fig2. Our results showed that 35.9 % of all isolates had *ctx*-M gene. The frequency of *cas*1, 2 and *cas*3 genes in *ctx*-M positive isolate were 35.29%, 37.25% and 40.62%, respectively.

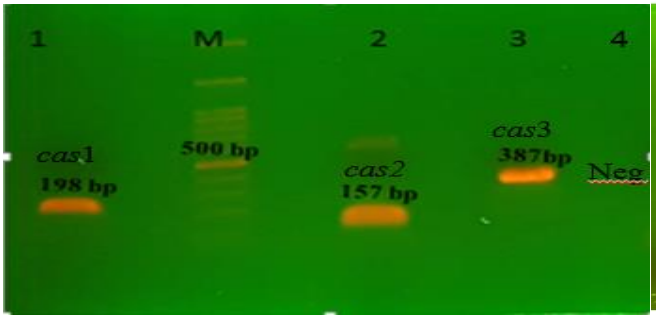

Figure1

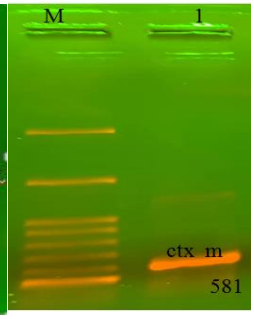

Figure2

Figure1. PCR products for *cas*1-3 genes. line 1. *cas*1, line 2. *cas*2, line3. *cas*3, line 4. Negative control, M: 1000bp ladder

Figure2. PCR products for *ctx*-M, Line1. *ctx*-M. M: 1000bp ladder

3.2. The antimicrobial activity tests

Among all isolates, 66/04% of isolates were susceptible to antibiotic and 33/96% were resistance to one or more antibiotics. The degree of resistance results for isolates by different antibiotics according to disc diffusion method were in order to ampicillin (93.3%), naldixic Acid (50%), sulfamethoxazole trimethoprim(43.33), ceftriaxon(32%), ceftazidime (26.4%), cefotaxime(25.5%), ciprofloxacin(23.34%), cefixime(12.2%) and cefepim (7.6%). The highest antibiotic resistance was related to ampicillin and the least resistance was to cefepim.

3.3. Beta lactamase detection by using disk diffusion methods

Between 106 isolates, 28.3 % showed beta-lactamase activity. The frequency of the *ctx*-M genes in ESBL-producing strains with beta-lactamase phenotype was more than other strains. The beta-lactamase phenotype has no statistically significant relation in the presence of the *cas*

genes. The results of these correlations in this study shown in table 2.

Table 2. Frequency of *cas* 1-3 genes in antibiotic resistance, beta lactamase phenotype and *ctx*-M gene.

genes	Antibiotic resistance(N:106) p-value		Beta lactamase phenotype(N:106) p-value		*ctx*-M gene p-value	
cas 1	34(33.33%)	0.49	29(28.4%)	1	37(36.3%)	1
cas 2	19(37.25%)	0.49	16(31.4%)	0.49	19(37.2%)	0.77
cas 3	21(32/81%)	0.76	18(28.12%)	0.96	25(39.1%)	0.39

3.4. Relation between *cas* genes and antibiotic resistance

The spreading of *cas*1-3 genes in UPEC isolates analyzed by PCR reaction and statistical analysis. Each *cas* genes represented different behavior against different the antibiotic. The frequency of antibiotic-resistant isolates and statistically significant relation between *cas* genes and antibiotic resistance were shown in Table 2. A high frequency of resistance was detected among isolates that have *cas*2 gene (37.25%).

3.5. Investigation *ctx*-M and *cas* gene relation

The presence of *ctx*-M and *cas* genes was analyzed in UPEC isolates. After that correlation between *ctx*-M and *cas* genes was investigated. There was no statistically significant correlation between presence of *cas*1- 3 genes with *ctx*-M presence. The results were shown in table 2.

3.6. Prevalence of $bla_{\text{CTX-M}}$ gene in uropathogenic *E. coli* and relation on ESBL production isolates

Figure 3, represents the frequency of ESBL genes among UPEC isolates. Overall, it was observed that 35.9% of the isolates carried $bla_{\text{CTX-M}}$ genes. In the evaluation of ESBL genotypes by PCR, it was showed that there was no statistically significant relation between the peresence of *bla*ctx-M gene and ESBL phenotype in UPEC isolates.

(Table 3).

Table 3. Distribution of β-lactamase gene patterns in different ESBL phenotypes of UPEC isolates

β-lactamase gene	ESBL phenotype	Number (%)	p-value
CTX-M	ESBL positive	3 (10)	**0.38**
	ESBL negative	4 (5)	

Discussion

where gentamicin was used. UTI *E. coli* was resistant in a patient with a history of prematurity, nephro-urological malformations with other chronic Serious threat to medicine is antimicrobial resistance (AMR). They cause infections untreatable. The development of new antibiotics can't solve the resistance problem and researchers are looking for a viable answer to infectious bacteria resistance to beta-lactam antibiotics (19). scientific proven CRISPR-Cas can remove AMR genes from bacterial populations. This research obtained, discuss the field of CRISPR associated *cas* genes and their relation with antibiotic resistance and *ctx*-M gene. An accepted method for detecting of antibiotic resistant bacteria is disk diffusion according to the CLSI standard. All the antibiotics used in this study had a significant bactericidal effect on the UPEC isolates except norfloxacin, gentamicin, and nitrofurantoin. This observation is contrary to Another study (Salas-Mera et al., 2017) that showed UTI *E. coli* is norfloxacin-resistant but sensitive to nitrofurantoin (20). Thirty isolates (28.3%) in this study were detected as ESBL- producing, however, a previous study in Iran (Khalili et al., 2018) and China (Liu et al, 2015) detected that more UPEC isolates were ESBL- positive (28,29). Results of this study Showed there was no statistically significant relationship between the presence of *bla*ctx-M gene and the ESBL phenotype in UPEC isolates but according to recent studies, there is a direct relationship between Beta-lactamase mRNA expression and antibiotic resistance (21). A significant increase in Beta-lactamases gene expression levels are present in resistant strains compared to susceptible strains and their possible role in the pathogenesis of the disease has been proposed and suggested that increased expression of β-lactamase mRNAs and their pathogenicity is due to multifactorial parameters that cause phenotypes and genotype changes (22). In this research *ctx*-M was a mid-presence in isolates but a study in Tehran hospital (Seyed javadi et al., 2016) showed a high frequency of *ctx*-M gene inpatient (23).

By examining the beta-lactamase phenotype, some isolates showed resistance to antibiotics and the frequency of *ctx*-M beta-lactamase gene was higher than in non-ESBL strains. Other research showed (Fam et al., 2011) ESBL phenotypic activity in *E. coli* strains has a relation with the presence of *bla*CTX-M gene (24). In this study, 61.76% of isolates with *cas*1 had *cas*3 gene simultaneously, Also the other study (Wang et al., 2020) identified *cas*1 and *cas*3 genes in samples of *Klebsiella pneumoniae*, that almost all isolates that were positive for *cas*1 had the *cas*3 gene (30). Overall result showed, there is no significant relationship between the presence of *cas*1-3 genes and presence *ctx*-M beta-lactamase genes and antibiotic resistance. Touchon et al., also reported no meaningful connotation between the presence/absence of the *cas* genes and the antibiotic resistance in *E. coli* (27). Whereas (Wang et al, 2020) acknowledged that the presence of CISPR/Cas system can partially prevent the distribution of drug resistance genes in Klebsiella pneumoniae (30). The other studies used phagemid to transport CRISPR-Cas9 constructs programmed to target AMR genes on plasmids, which successfully removed these plasmids from *E. coli*. Furthermore, to kill bacteria with AMR genes in the chromosome CRISPR-Cas9 constructs were used the other study showed CRISPR-Cas9 construct can destroy foreign virulence and AMR genes too. Thus bacteria re-sensitive to antibiotics (25). *Shewanella algae* have increased resistance to carbapenem. The researcher knocked out resistance genes by using CRISPR/Cas9 and *rec*E/*rec*T recombinase in *S. algae* (26). In our study, 106 UPEC resistance isolates investigated presence *cas* 1–3 genes. The frequencies of these genes were *cas*2 < *cas*3 < *cas*1 in all isolate. The sample frequency based on resistance antibiotic was *cas*3 < *cas*1 < *cas*2. Phenotype beta lactamase frequency was *cas*3 < *cas*1 < *cas*2 and the frequency of *ctx*-M gene was *cas*1 < *cas*2 < *cas*3. These results showed, there is no significant relationship between the presence of CRISPR associated *cas* genes and presence *ctx*-M beta-lactamase gene and antibiotic resistance in uropathogenic *E. coli* strains.

Conclusion

From this study, it can be concluded that the presence of *cas* genes as a main component of CRISPR/Cas system can have different effects on the presence or absence of *bla*CTX-M, and the lack of a statistically significant relationship between the presence and absence of *cas* genes and the beta-lactamase gene may be due to changes in the expression of CRISPR/Cas system in UPEC isolates in vitro. However, further studies are needed to prove the relationship between the presence of *cas* genes and the reduction of beta-lactamase resistance genes.

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