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# New Mutations in GyrA Gene of Escherichia Coli Isolated form Iraqi Patients

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**Abstract**

The present study included a collecting of 165 specimens form different sources, 93 isolates were identified as Escherichia coli depending on morphological and biochemical tests in addition to automated systems such as VITEK 2 and api 20E. All isolates under study developed high resistance toward cefotaxime, ceftazidime, ceftriaxone, and ciprofloxacin estimated by minimum inhibitory concentration. Stool and wound specimens characterized by harbouring the highest resistant isolates in a percentage reached 100% against antibiotics under study. Insignificant differences were found between isolates collected from males and females. Upon using disk displacement method to detect extended spectrum beta lactamases (ESBL), it was found that 37.73% of isolates were ESBL producers. However, stool and urine specimens harboured the highest percentage of producers. The present study findings revealed a presence of efflux pump in 77.5% of tested isolates using cartwheel method. Nevertheless, PCR technique exposed about 85% of isolates harboured acrAB efflux pump gene. Moreover, the results of this study illustrated that all isolates contained gyrA and parC using PCR technique. Accordingly, three isolates were chosen for nucleotide alignment study; one of them is sensitive and the other two are resistant to ciprofloxacin (acrAB free). The results showed a presence of stable and frequent three mutations in the resistant isolates (14 and 3). A transition mutation found in the site 4808116 in which guanine is replaced by adenine. Furthermore, two transversion mutations were located in 4804221 and 4808222 sites. Additionally, a hot spot was detected in the site 4808109.

**Keywords:** Gyra, Escherichia Coli, Ciprofloxacin.

# طفرات جديدة في جين gyrA لبكتريا الايشيريشية القولونية المعزولة من مرضى عراقيين

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**الخلاصـــة**

تضمنت الدراسة الحالية جمع 165 عينة سريرية من مختلف المصادر شملت الادرار و الخروج و الجروح و الحروق. شخصت 93 عزلة من بكتريا الايشريشية القولونية اعتمادا على الفحوصات المظهرية والبايوكيميائية واستعمال ابي 20 ونظام الفايتك اظهرت العزلات مقاومة عالية لمضادات السيفوتاكسيم

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والسفتازيديم السفترياكسون والسبروفلوكساسين وذلك بطريقة حساب التركيز المثبط الادنى وتميزت عينات الخروج والجروح احتوائها على العزلات الاكثر مقاومة بنسبة (100%) للمضادات الحياتية قيد الدراسة . ولم تظهر فروق معنوية بين نسب العزل في الاناث والذكور. باتباع طريقة استبدال الاقراص للتحري عن انزيمات البيتالاكتاميز موسعة الطيف تبين ان 37,73% من العزلات كانت منتجة للانزيم وتميزت عينتا الادرار والخروج بإيواء النسبة الاكبر من العزلات المنتجة . اوضحت نتائج الدراسة الحالية وجود مضخة الدفق في 77,5 % من مجموع العزلات المختبرة وذلك باستعمال طريقة عجلة العربة في حين كشفت تقنية تفاعل البلمرة المتسلسل عن وجود جين مضخة الدفق *acrAB* في 85% من العزلات المختبره . كذلك اظهرت نتائج هذه الدراسة وجود جينات *gyrA* و *parC* في جميع العزلات التي انتخبت لهذا الغرض وانتخبت ثلاث عزلات منها الاولى حساسة لمضاد السبروفلوكساسين واثنان مقاومه له وخالية من جين *acrAB* لدراسة التطابق تسلسل النيوكليتيدات والتي اظهرت ثلاث طفرات ثابتة ومتكرره في العزلتين 14و38 المقاومتين للسبروفلوكساسين حيث تظهر طفرة الاستبدال المكافي في الموقع 4808116 الذي يتحول فية الكوانين الى ادنين كما ظهرت طفرتين من نوع الاستبدال غير المكافي في الموقعين 4808221 و 4808222 . فضلا عن ذلك ظهرت طفرة نقطة ساخنة (Hot spot) واحدة ثابتة في الموقع 4808109.

## Introduction

*Escherichia coli* causes many infections in the human body, including infections of wounds, eye, skin, urinary tract, otitis media and bacteremia [1] The reason for the difficulty of treating injuries to these bacteria is the resistance to antibiotics [2].

Due to unexpected higher resistance to antibiotics as a result of physiological and genetic changes that evolved as a result of the selective pressures and antibiotic abuse over the past decades has led to the development of multidrug resistant pathogenic bacteria [3]. Furthermore, possessing such species of bacteria a high potential for the acquisition of resistance determinants [4].

This multidrug resistance becomes a focus of global health concern, especially after the emergence of the high increase in resistance against third-generation cephalosporins. Resistant *E. coli* because of its high resistance and this has become the whole world problem and there is a global interest to increase antibiotic life and resistance [5].

Extended spectrum beta lactamases (ESβLs) were discovered in Europe in 1980, they were extracted for the first time in 1983 from the bacterium *Klebsiella pneumoniae* [6]. Plasmids encoding ESβLs genes making it easier to transfer among different bacterial species [7]. ESβLs resist penicillin and to the first, second and third generation of Cephalosporins. However, they are inhibited by beta lactamase inhibitors such as clavulanic acid [8].

Resistance to fluoroquinolones was noted in most isolates that had at least one *gyrA* mutation, or several indicating extra *gyrA* and/or *parC* mutations. These findings suggest that, in colonization in the clinical setting, the first step in the evolution of fluoroquinolone-resistant *E. coli* is a *gyrA* mutation with subsequent steps, likely including additional *gyrA* or *parC* mutations and/or enhanced efflux pumps due to overexpression of *acrAB* genes [9].

The present work aimed to investigate the ciprofloxacin resistance in local isolates of *E. coli* according to responsible genes (*agrA*, *parC* and *acrAB*).

## Materials and Methods

One hundred and sixty five specimens, including urine, burn, wounds, and stool were collected from patients visiting Yarmouk Hospital, Medical City, Al-Karama and west of Erbil, for the period from October 2015 until the February 2016.

After the collection, specimens were cultured on MacConkey agar and all plates were incubated at 37°C for 24 hours. Thereafter, pink or red colonies were recultured on Eosin Methylene Blue agar. Those which have green metalic sheen were chosen for the identification by morphological and cultural tests included, Motility test, Oxidase test, catalase, IMViC tests according to [10]. While api 20E and VITEK2 were used to confirm the identification.

### Minimum inhibitory concentration (MIC)

Microdilution method were adopted in order to estimate the MIC for 53 isolates, an isolate was considered as resistant, intermediate, or sensitive in accordance to breakpoints of CLSI [11]. Concentrations ranging from 0.5 to 2048 µg/ ml were tested.

**β-Lactamase production test**

Disc Replacement Method was employed for the detection of isolates producing β-Lactamase according to [12].

**Detection of efflux pump**

Cartwheel method was used for the detection of efflux pumps in *E. coli* isolates following the method described by [13]. In brief:

Trypton soy agar (HiMedia, India) were prepared according to the manufacturer's instructions have been added 1.5 mg / ml of Ethidium Bromide, poured in Petri plates on the same day and stored away from light. Bacterial suspension (equilibrated with McFarland 0.5) was planted radially starting from the middle of the plate to the periphery forming a cartwheel style. All plates were incubated at 37°C for 18 hours; afterward, growth was examined under UV light. Appearance of fluorescence indicating absence of efflux pump and vice versa.

**Extraction and purification of genomic DNA**

Geneaid Genomic DNA extraction kit was used to extract 40 bacterial isolates out of 53 *E. coli* isolates. Purity and concentration were assayed using Nanodrop instrument.

**Multiplex Polymerase chain reaction technique [PCR] assay**

Multiplex PCR was accomplished to amplify two ciprofloxacin resistance genes including: *gyrA and parC*. Moreover, efflux pump gene (*acrAB*) was amplified as well. Multiplex and uniplex PCR were optimized using the PCR premix (Accupower, bioneer-Korea).

Amplification was achieved in a programmable Thermal cycler (Eppendorf Master cycler Gradient PCR, Germany) using 20 mL PCR Premix (Bioneer, Korea) containing 200 mM of each desoxynucleotide triphosphate, 1.5 U of Taq DNA polymerase, buffer (20 mM Tris-HCl pH8.4, 50 mM KCl) and 3 mM of $MgCl_2$. The following primers were used: *gyrA* F (50-AAATCTGCCCGTGTCGTTGGT-30) and *gyrA* R (50 GCCATACCTACGGCGATACC 30) (0.2 mM each), to amplify a 344 bp fragment specific of *gyrA* gene; *parC* F (50-GTATGCGATGTCTGAACT -30) and *parC* R (50-TTCGGTGTAACGCATTGC-30) (0.4 mM each) [14] to amplify a 229 bp fragment specific for *parC* gene; *acrAB* F (50- ATGAACAAAAACAGAGG -30) and *acrAB* R (50- TTTCAACGGCAGTTTTCG -30) (0.4 mM each) to amplify a 495 bp fragment of the *acrAB* gene [15].

Cycle conditions for *agrA* and *parC* amplification were: denaturation for 2 min at 95°C, followed by 35 cycles of 93°C for 30 sec., 55°C for 1 min and 72°C for 1 min, and a final extension at 72°C for 5 min. However, for *acrAB* were: denaturation for 5 min at 94°C, followed by 30 cycles of 94°C for on min., 52°C for 1 min and 72°C for 1 min, and a final extension at 72°C for 5 min. Multiplex PCR products for *gyrA*, *parC*, and *acrAB* were detected by agarose gel electrophoresis 2% and 1%, respectively.

**Sequencing of PCR products**

PCR products were subjected to direct sequencing (Macrogen, Korea). DNA sequences were analysed and similarity searches were carried out with the Basic Local Alignment Search Tool [BLAST] in National Centre for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov).

**Statistical Analysis**

ANOVA, Correlation coefficient, Qi square, and Least significant difference were carried out to compare the means. Differences considered significant when $P < 0.05$.

**Results and Discussion**

Approximately, 56.36% (93 isolates) out of 165 isolates were identified as *E. coli* in accordance the morphological, cultural, and biochemical results.

**Minimum inhibitory concentration**

Regarding the results presented in Table- 1, *E. coli* isolates developed high resistance reached 100, 98.11, 90.57, and 98.11% to cefotaxime, ceftazidime, ceftriaxone, and ciprofloxacin, respectively. While these isolates did not show intermediate resistance to the previously mentioned antibiotics except ciprofloxacin reached 1.89%. Nevertheless, about 1.89% and 9.43% of the isolates showed sensitivity to ceftazidime and ceftriaxone, respectively.

**Table 1**-Percentages* of *E. coli* antibiotic susceptibility.

| Susceptibility % | Ciprofloxacin | Ceftriaxone | ceftazidime | Cefotaxime |
|---|---|---|---|---|
| Sensitive | 0 | 9.43 | 1.89 | 0 |
| Intermediate resistant | 1.89 | 0 | 0 | 0 |
| Resistant | 98.11 | 90.57 | 98.11 | 100 |

*Percentages were calculated out of 53 isolates

**The productivity of beta lactamase form *E. coli* isolates**

It was observed that about 37.73% of *E. coli* isolates were beta lactamase producer. Such finding did not agree with those of Hammadi [16] as he reported that 80% of his isolates were beta lactamase producer. Such differences could be attributed to sample size as well as the specimen source. Furthermore, geographical differences might affect the results as well.

Results presented in Table- 2 pointed to isolates resistant to the antibiotics under study at high rates except ciprofloxacin which was at low percentage (84.22%). However, the isolates did not show any intermediate resistance in exception of ciprofloxacin, which amounted to 1.89%. Moreover, this is what [17] as well as [18] have mentioned as the beta-expanded spectrum beta lactamases have the ability to break down most of third generation cephalosporins and fluoroquinolones as well.

**Table 2**-Percentage of *E. coli* susceptibility to antibiotics in accordance beta lactamase production.

| Susceptibility% | Ciprofloxacin | | ceftriaxone | | ceftazidime | | Cefotaxime | |
|---|---|---|---|---|---|---|---|---|
| | producer | Non producer | Producer | Non producer | Producer | Non producer | producer | **Non producer** |
| Sensitive | 15.78 | 0 | 0 | 9.43 | 0 | 1.89 | 0 | 0 |
| Intermediate resistant | 0 | 1.89 | 0 | 0 | 0 | 0 | 0 | 0 |
| Resistant | 84.22 | 98.11 | 100 | 90.57 | 100 | 98.11 | 100 | 100 |

*Percentages were calculated out of 53 isolates

On the other hand, the present results did not agree with a local study of Al- [19] as they found that the percentage of resistant isolates reached approximately 31%. Also, the findings of the current study did not agree with those of [16], who stated that the level of resistance for the third generation of cephalosporins was as low as 47%.

**Phenotypic detection the presence of the stream pumps between isolates of the study:**

About 40 *E. coli* isolates were tested for the presence of efflux pump using cartwheel method. The results showed that 31 isolates (77.5%) of the total isolates gave positive results Figure-1. Martins *et al.* [20] stated that the cartwheel method is one of the simplest and important methods to detect the bacterial efflux pumps. Furthermore, it is quite easy to perform, it does not require sophisticated equipment, in addition to that, it considered a cost effective method since it gives results within 24 hours.

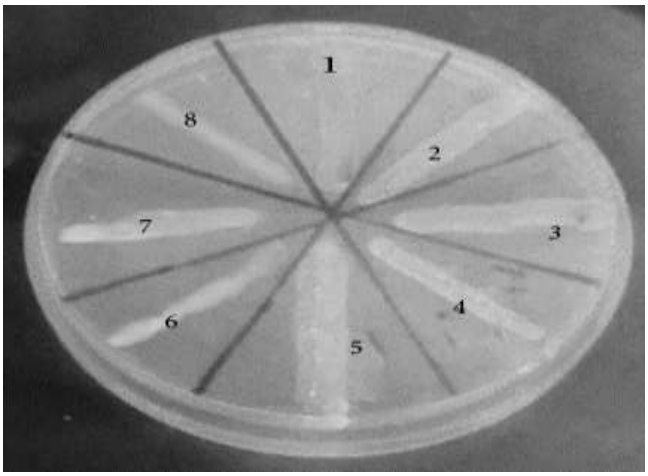


**Figure 1-** Cartwheel method to detect efflux pump. Results are visualized under UV light.

**Molecular study**

**Extracting DNA from *E. coli* isolates**

It shows through the results, the DNA of isolates under study was pure as it depicted in Figure- 2. Nevertheless, purity ranged from 1.44 to 2.12; while the concentration fluctuated from 61.7 to 370 ng/µl.

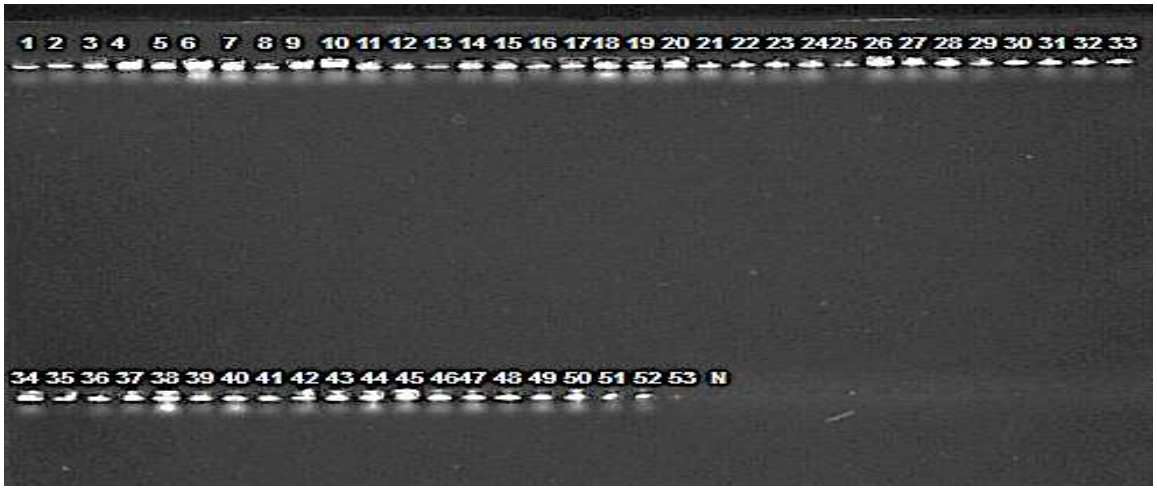


**Figure 2-** Gel electrophoresis on 1% agarose of *E. coli* DNA. Lane M and N represent ladder (100 kb) and negative control, respectively. Lanes 1- 53 represent *E. coli* DNA.

**Detection of *acrAB***

It was observed by the results shown in Figure- 3, a presence of efflux pump gene *acrAB* in 34 isolates, when the molecular size around 495 base pair. This gene plays an important role in expel antibiotics out of the cell [21] and is located in the outer membrane to bacterial cell, and constitute an important part in the efflux pumps to be a three-parts efflux pumps in the case of the loss of some pumps no longer be effective [22].

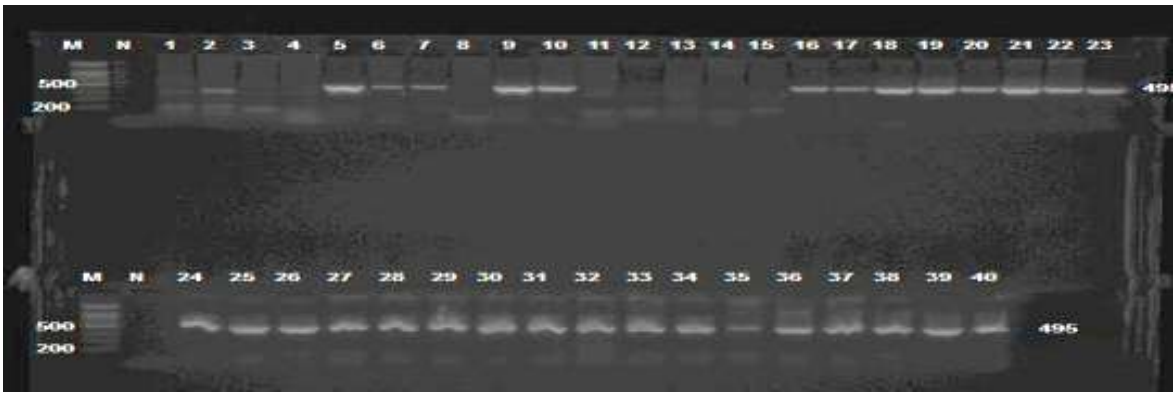


**Figure 3-** Gel electrophoresis on 1% agarose of *acrAB* amplification (495 pb). Lane M and N represent ladder (100 kb) and negative control, respectively. Lanes 1- 40 represent *E. coli* isolates.

The results of the current study did not agree with what [15] found that all Ciprofloxacin resistant isolates harboured *acrAB* gene in *Klebsiella pneumoniae.* It is worthy to note that there are isolates developed positive results by cartwheel method; whereas they lack *acrAB*. On contrary, some isolates showed negative results by cartwheel method and they have the gene in question.

Perhaps the reason is due to the fact that the principle of cartwheel method relies on fluorescence and that the diffusion of ethidium bromide through the cytoplasmic membrane and then accumulate in the bacterial cell. The bacterial efflux pumps are able to distinguish substrates and be able to expulsion to the external medium. These systems depend on the temperature, pH, and calcium availability [20]. In another words, that the efflux pump has the ability to expel the ethidium bromide outside the cell and thus does not show fluorescence. Here it is possible that the bacteria being tolerable and resistant to certain concentrations of ethidium bromide; therefore, will not expel the dye and thus develop fluorescence. Or as noted by [20] that fluorescence might be affected by the permeability of the ethidium bromide. Nevertheless, the efflux pump is not acrAB type.

**Detection of *gyrA* and *parC***

After gel electrophoresis, a presence of one band for each gene in all isolates have been developed Figure-4 .

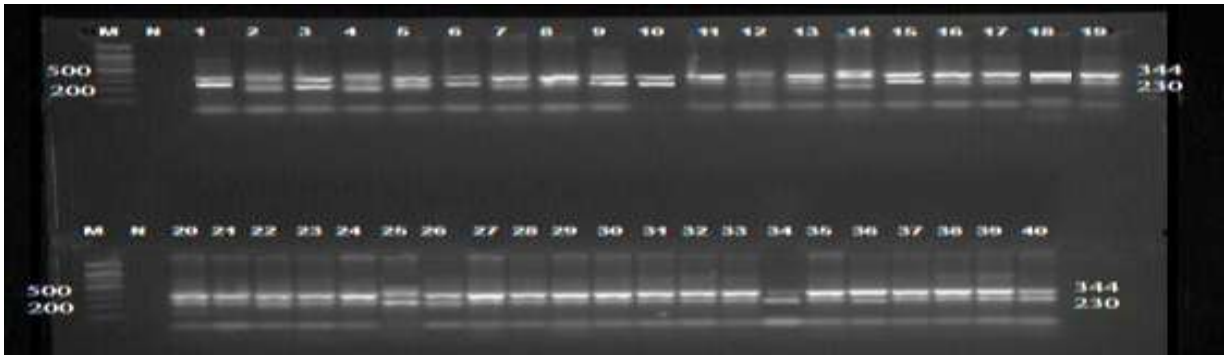


**Figure 4-** Gel electrophoresis on 2% agarose of *gyrA* [344 pb] and *parC* [230 pb] amplification. Lane M and N represent ladder [100 kb] and negative control, respectively. Lanes 1- 40 represent *E. coli* isolates.

Upon this basis two ciprofloxacin resistant isolates were chosen for further studies; first one has no *acrAB* [isolate No. 14]. While the other one [isolate No. 38] contained all three genes. A third isolate [isolate No. 3] sensitive to Ciprofloxacin was elected as well.

The results nucleotide sequence alignment was performed for all three isolates and the results revealed no change in the sequence of isolate No. 3; which confirms the sensitivity of this isolate for ciprofloxacin.

Regarding isolates No. 14 and 38, alignment results show three repeated and stable mutants, where the transition mutations appears at site 4808116. While at the sites 4808221 and 4808222 tow transversion mutations have been developed. Moreover, one mutation appeared at site 4808109 was

stable yet variable for both isolates; which shows that this site is a hot spot for mutations. These mutations have been recorded in the global gene NCBI site ([https://www.ncbi.nlm.nih.gov/nuccore/LC200513) as in Supplement No. 1.

Depending on the above results, congruence can be stated that there are 34mutations occurring in isolate 14 in the *gyrA* of which were 10 transition and 17 transversion of non-equivalent and 4 insertion and 3 deletion mutations Table- 3.

Generally, 21 mutations were found in *parC* of the isolate No. 14, included While we had a 21 for a mutation of the gene itself *parC* isolation [14] included a 6 transition, 8 transversion, 6 insertion and 1 deletion mutations Table- 4.

**Table 3-** Mutation in *gyrA* of isolate No. 14.

| Transition mutations | | | | |
|---|---|---|---|---|
| type | Site | Mutation | Id | Gene |
| Transtion | 4807950 | T → C | 1 | *gyrA* F4 |
| Transtion | 4808116 | G → A | 2 | |
| Transtion | 4808107 | G → A | 3 | *gyrA* R4 |
| Transtion | 4808031 | G → A | 4 | |
| Transtion | 4807987 | G → A | 5 | |
| Transtion | 4807966 | G → A | 6 | |
| Transtion | 4807962 | G → A | 7 | |
| Transtion | 4807916 | G → A | 8 | |
| Transtion | 4807902 | G → A | 9 | |
| Transtion | 4807892 | G → A | 10 | |
| Transversion mutation | | | | |
| type | Site | Mutation | Id | Gene |
| Transversion | 4800221 | G → T | 1 | *gyrA* F4 |
| Transversion | 4800222 | T → A | 2 | |
| Transversion | 4800224 | G → C | 3 | |
| Transversion | 4808109 | G → C | 4 | *gyrA* R4 |
| Transversion | 4808095 | G → T | 5 | |
| Transversion | 4808090 | T → G | 6 | |
| Transversion | 4808019 | G → T | 7 | |
| Transversion | 4807999 | G → C | 8 | |
| Transversion | 4807973 | G → C | 9 | |
| Transversion | 4807958 | G → T | 10 | |
| Transversion | 4807951 | T → A | 11 | |
| Transversion | 4807942 | G → T | 12 | |
| Transversion | 4807940 | G → T | 13 | |
| Transversion | 4807933 | T → G | 14 | |
| Transversion | 4807910 | G → T | 15 | |
| Transversion | 4807907 | A → C | 16 | |
| Transversion | 4806898 | A → C | 17 | |
| Insertion and deletion mutations | | | | |
| type | Site | Mutation | Id | Gene |
| Insertion | 4807937 | T | 1 | *gyrA* F4 |
| Insertion | 4807980 | T | 2 | |
| Deletion | 4807952 | A | 3 | |
| Deletion | 4807184 | A | 4 | *gyrA* R4 |
| Insertion | 4807146 | C | 5 | |
| Insertion | 4807105 | A | 6 | |
| Deletion | 4807043 | A | 7 | |

**Table 4-** Mutation in *parC* of isolate No. 14.

| Transition mutations | | | | |
|---|---|---|---|---|
| type | Site | Mutation | Id | gene |
| Transtion | 266 | G → A | 1 | R 1*parC* |
| Transtion | 275 | A → G | 2 | |
| Transtion | 278 | G → A | 3 | |
| Transtion | 281 | T → C | 4 | |
| Transtion | 303 | G → A | 5 | |
| Transtion | 304 | T → C | 6 | |
| Transversion mutation | | | | |
| type | Site | Mutation | Id | gene |
| Transversion | 258 | G → C | 1 | R 1*parC* |
| Transversion | 269 | G → C | 2 | |
| Transversion | 272 | C → A | 3 | |
| Transversion | 289 | G → T | 4 | |
| Transversion | 293 | G → T | 5 | |
| Transversion | 299 | G → C | 6 | |
| Transversion | 305 | A → C | 7 | |
| Transversion | 311 | C → A | 8 | |
| Insertion and deletion mutations | | | | |
| type | Site | Mutation | Id | gene |
| Insertion | 251 | C | 1 | *ParC R 1* |
| Insertion | 253 | C | 2 | |
| Deletion | 262 | T | 3 | |
| Insertion | 312 | A | 4 | |
| Insertion | 312 | T | 5 | |
| Insertion | 312 | T | 6 | |
| Insertion | 312 | T | 7 | |

While the overall mutations occurred in isolate No. 38 concerning *gyrA* were 7 mutations distributed as 2 transition, 3 transversion and one deletion mutations. However, there is no insertion mutation was noticed as it summarized in Table- 5. In regard to the *parC* for the same isolate (No. 38) has included 16 mutations of which, 7 transition, 5 transversion, 1 insertion and 3 deletion mutations Table- 6.

It should be noted that mutations affecting a specific gene through the creation changes in the structure of the gene and its work; which can lead to influence gene expression, being lead to substitution of amino acids and thus in protein, it can be replaced an amino acid with another very similar in terms of chemical characteristics, and in this case, the protein product is working normally; sometimes replacement of amino acids in a region of the protein does not affect a significant impact in the secondary protein composition or function. There are also amino acids mediated by more than one codon, which could lead to no change in the translation despite a presence of change in the codon [23].

**Table 5-** Mutation in *gyrA* of isolate No. 38.

| Transition mutations | | | | |
|---|---|---|---|---|
| type | Site | Mutation | Id | gene |
| Transtion | 4807941 | C → T | 1 | *gyrA* F 5 |
| Transtion | 4808116 | G → A | 2 | |
| Transversion mutation | | | | |
| type | Site | Mutation | Id | gene |
| Transversion | 4808221 | G → T | 1 | *gyrA* F 5 |
| Transversion | 4808222 | T → A | 2 | |
| Transversion | 4808235 | C → A | 3 | |
| Transversion | 4808108 | G → T | 4 | *gyrA* R 5 |
| Insertion and deletion mutations | | | | |
| type | Site | Mutation | Id | gene |
| Deletion | 4808184 | A | 1 | *gyrA* R 5 |

**Table 6**- Mutation in *parC* of isolate No. 38.

| Transition mutations | | | | |
|---|---|---|---|---|
| type | Site | Mutation | Id | gene |
| Transtion | 124 | A → G | 1 | *ParC F* 5 |
| Transtion | 336 | G → A | 2 | |
| Transtion | 390 | T → C | 3 | |
| Transtion | 286 | C → T | 4 | *ParC R* 5 |
| Transtion | 280 | C → T | 5 | |
| Transtion | 279 | C → T | 6 | |
| Transtion | 273 | C → T | 7 | |
| Transversion mutation | | | | |
| type | Site | Mutation | Id | gene |
| Transversion | 333 | G → C | 1 | *ParC F* 5 |
| Transversion | 346 | C → G | 2 | |
| Transversion | 353 | G → T | 3 | |
| Transversion | 387 | T → A | 4 | |
| Transversion | 104 | C → G | 5 | *ParC R 5* |
| Insertion and deletion mutations | | | | |
| type | Site | Mutation | Id | gene |
| Deletion | 288 | C | 1 | *ParC R 5* |
| Deletion | 271 | A | 2 | |
| Insertion | 248 | A | 3 | |
| Deletion | 117 | C | 4 | |

In conclusion, PCR technique is more reliable than cartwheel method in detecting efflux pump. New mutations were found in local isolates of *E. coli* at sites 4808116, 4808221 and 4808222. Moreover, one mutation appeared at site 4808109 was stable yet variable, could be considered as a hot spot for mutations.

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