CORRELATION BETWEEN ANTIBIOTIC RESISTANCE PHENOTYPES WITH MOBILE GENETIC ELEMENTS (INTEGRON CLASS I) IN UROPATHOGENIC *E. coli*

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ABSTRACT

This study was aimed to determine the relationship between Integron class I and multidrug resistance in Uropathogenic *E. coli*. A total of 302 urine samples were used in this study. 139 Uropathogenic *E coli* were collected from hospitalized patients. Then, using biochemical testing and the Vitek II compact system, all of the isolates were diagnosed and confirmed. All *E. coli* isolates were tested using a disc diffusion procedure to detect their susceptibility to 23 types of antibiotics from different classes, and the result showed that these bacteria were highly resistant to most used antibiotics especially, Cefotaxime, Ampicillin, Piperacillin, Trimethoprim- sulfamethoxazole ,Cefepime and Ceftazidime, while the most effective antibiotics were Colistin and Tigecyclin. Also the results show different resistance patterns in which 111 (79.86%) isolate showed multidrug resistance MDR, Extensively drug resistance XDR 18 isolate (12.94%), while Pandrug resistance PDR 3 (2.15%). PCR was used to detect the presence of integron class I in MDR isolates ,which was found in 87 of isolates with a 483 bp amplification product. The resolved PCR products were sequenced in both directions (forward and reverse), and the NCBI BLASTn engine revealed 100% sequence similarity between the sequenced samples and the planned reference target sequences. These findings revealed that *E. coli* strains were highly resistant to common antibiotics; however, given the high prevalence of integron class I among these isolates, we can deduce that antibiotic resistance genes were most likely carried on mobile genetic elements (integron) in these isolates.

Keywords: Nucleic acids sequencing, PCR, MDR, XDR, PDR.

الارتباط بين الأنماط المظهرية لمقاومة المضادات الحيوية مع العناصر الوراثية المتنقلة (انتكرون الصنف الاول) في الإشريكية القولونية المسببة لأمراض الجهاز البولي

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المستخلص

هدفت هذه الدراسة إلى تحديد العلاقة بين انتكرون الصنف الأول ومقاومة الأدوية المتعددة في الإشريكية القولونية المسببة لأمراض الجهاز البولي. تم استخدام إجمالي 302 عينة بول في هذه الدراسة، وجمعت 139 عينة من الإشريكية القولونية الم من المرضى في المستشفى. بعد ذلك ، باستخدام الاختبارات البيوكيميائية ونظام Vitek II ، تم تشخيص جميع العزلات وتأكيدها. تم اختبار جميع عزلات الإشريكية القولونية باستخدام طريقة الانتشار القرصي لاكتشاف مدى حساسيتها ل23 نوعاً من المضادات الحيوية من فئات مختلفة ، وأظهرت النتائج أن هذه البكتيريا كانت شديدة المقاومة لمعظم المضادات الحيوية المستخدمة خاصة سيفوتاكسيم ، أمبيسيلين ، بيبراسيلين ، تريميثوبريم– سلفاميثوكسازول ، سيفيبيم والسفتازديم . في حين أن المضادات الحيوية الأكثر فعالية كانت الكولستين و التيكاسيكلين .كما أظهرت النتائج أنماط مقاومة مختلفة حيث كانت 111 عزلة مقاومة للأدوية المتعددة ، 18 كانت XDR ، و 3 عزلة PDR تم استخدام تفاعل البلمرة المتسلسل للكشف عن تردد الصنف الاول من الانتكرون في العزلات المقاومة للادوية المتعددة، حيث وجد في 87 منها والتي اظهرت ناتج تضخيم قدره 483 زوج قاعدي. تم تحديد تسلسل منتجات PCR في كلا الاتجاهين (إلى الأمام و الخلف) ، وكشف محرك NCBI BLASTn عن تشابه تسلسلي بنسبة 100 ٪ بين العينات المتسلسلة والتسلسل المرجعي الهدف. أظهرت هذه النتائج أن سلالات الإشريكية القولونية كانت شديدة المقاومة للمضادات الحيوية الشائعة. ومع ذلك ، نظرًا لارتفاع معدل وجود الصنف الاول من Integron بين هذه السلالات ، يمكننا أن نستنتج أن جينات مقاومة المضادات الحيوية من المحتمل أن تكون محمولة على العناصر الوراثية المتنقلة (Integron) في هذه السلالات.

كلمات مفتاحية: تسلسل الحوامض النووية, تفاعل البوليمريز المتسلسل, PCR, MDR, XDR, PDR.

Received:11/5/2022, Accepted:7/8/2022

INTRUDCTION

Multidrug resistance is a severe health problem among dangerous bacteria like *Escherichia coli* and it has been related to increasing mortality and morbidity all around the world (18,20). Despite the fact that antibiotic use is definitely crucial in the selection of bacterial resistance, the ease with which resistance genes can spread has been a driving force in the rapid evolution of antimicrobials resistance across a broad spectrum of bacteria. *E. coli* can be found in the intestines and feces of warm-blooded mammals and reptiles (1, 8). It is responsible for 80% of community acquired UTI. In human and veterinary medicine around the world, the resistance of *E. coli* to multiple antimicrobials has become a major concern (15). Despite being innately sensitive to nearly most therapeutically relevant antibiotics treatments, *E. coli* has a significant capacity for resistance gene accumulation, which is mostly accomplished through horizontal gene transfer (19). Horizontal gene transfer helped *E. coli* to develop by allowing it to acquire phage, plasmids, and DNA segments. Virulent uropathogenic isolates are more likely than commensal strains to have specific fimbriae, toxins, and iron receptors (12). Antibiotic resistance has steadily increased among pathogenic and commensal bacteria, posing a global health issue (21). The majority of the antibiotic resistance problems have been caused by mobile genetic determinants such as transposons, plasmids and Integrons (3). Integrons are genetic elements that have a role in the spread resistance to antimicrobials by horizontal transmission (17). Integrons are genetic determinants that are capable of acquiring, expressing, and exchanging resistance genes through a site-specific recombination mechanism (16). The goal of this study is to look at antibiotic resistance in clinical Uropathogenic *E. coli* isolates and see if there's a link between Integron class I and resistance.

MATERIALS AND METHODS

Collection of samples, isolation of UPEC, and identification: This study was authorized by the Baghdad University of Science's ethical committee (CSEC/0921/0047) From October 2020 to April 2021Approximately 302 urine samples from patients admitted to the medical city Hospital were placed in sterile tubes and quickly sent to the laboratory for culture. *E. coli* was identified by cultivating on MacConky agar, Eosin methyl blue (EMB) agar, and biochemical assays (Indol test, Oxidase test, Catalase test, Methyl-Red, and Vogas-Broskaor tests), and the results of identification were validated by utilizing the API 20 E and Vitek 2 systems.

Antibiotic susceptibility of Uropathogenic *E. coli* :This test was carried out using the disk diffusion method, as recommended by CLSI (2020) (4). This study involved 23 antibiotics, with more than one antibiotic from each class being used (Ampicillin 10 µg, Piperacillin 1001 µg, Amoxicillin-clavulanate 301 µg, Aztreonam 30µg, Cefotaxime 301 µg, Cefoxitin 301 µg, Cefepime 30 µg, Ceftriaxone 30 µg, Ceftazidime 30 µg, Imipenem 10 µg, Meropenem 10 µg, Ciprofloxacin 5µg, Levofloxacin 5µg, Nalidixic acid 301 µg, Gentamicine 101 µg, Amikacin 301 µg, Azithromycin 15 µg, Tetracycline 30 µg, Trimethoprim-1 sulfamethoxazole 1.251\ 23.75 µg, Chloramphenicol 301 µg, Nitrofurantoin 300 µg, Colistin 10µg and Tigecyclin 15µg). The results were interpreted using CLSI guidelines (2020) (4). =

Extraction of DNA

E. coli isolates had their DNA extracted with the WizPrep gDNA Mini Kit company (Korea) (2). This kit take advantage of silica membrane technology to complete the arduous procedures and provides an easy, dependable technique to extract DNA with high precision and less time.

PCR for Integron class I detection

The detection was carried out by amplifying the *int1* gene in a DNA template isolated from *E. coli* and using a reaction mixture with end volume of 25µl. The primer sequence utilized in this study is as in Table 1 (14). The PCR reaction was carried out with mixing the components that was listed in Table 2. to make mixture 25 µl. In a Pioneer/Korea MyGenie 96/384 Gradient Thermal Block, the PCR reaction was carried out. The reaction of PCR was performed in number of cycles under thermal condition controlling as in Table 3.

Table 1. Design of primers which utilized in this study

Primer name	Sequence	Product size (bp)
IntI1 F	GGTCAAGGATCTGGATTTCG	483
IntI1 R	ACATGCGTGTAAATCATCGTC	

Table 2. Components and volumes of PCR reaction solutions

Component	Volume (µl)
Go Taq Green master mix	12.5
Forward primer	1
Reverse primer	1
DNA	5
Nuclease free water	5.5
Total volume	25

Table 3. The PCR program design

Steps	ºC	Time	Cycle
Initial Denaturation	95	5 min.	1
Denaturation	95	30 sec.	
Annealing	62	30 sec.	35
Extension	72	1 min.	
Final extension	72	5 min.	1

Nucleic acids sequencing of PCR products

The resolved PCR products were sequenced commercially in both directions (forward and reverse) according to the sequencing company's instruction manuals (Macrogen Inc. Geumchen, Seoul, South Korea). Only clear chromatographs from ABI (Applied Biosystem) sequence files were examined further, verifying that the annotation and variances were not due to PCR or sequencing errors. The virtual locations and other details of the obtained PCR fragments were identified by comparing the observed nucleic acid sequences of a local sample with the retrieved nucleic acid sequences.

Data analysis and interpretation

Using BioEdit Sequence Alignment Editor Software Version 7.1 (DNASTAR, Madison, WI, USA), the sequencing results of the targeted sample's PCR products were edited, aligned, and assessed as long as they matched appropriate sequences in the reference database). Each sequenced sample's detected differences were numbered in PCR amplicons and their matching positions within the referring genome. The nucleic acids found in PCR amplicons were numbered, as well as their locations within the reference genome. SnapGene Viewer ver. 4.0.4 (https://www.snapg ene.com) was used to annotate each discovered variant within the bacterium sequences. For each studied sample, the identified changes were deposited in the NCBI-bankit database under a unique accession number.

Nucleic acid changes translation into amino acid residues: The amino acid sequences of the protein encoded by the *Intg I* gene were obtained from` the NCBI server (http://www.ncbi.nlm.nih.gov). There were 337 amino acid residues in this protein. By using the Expasy online software (http://web.expasy.org/translate/), the detected nucleic acid variations in the coding parts were translated into a reading frame matching the relevant amino acid residues in the generated protein. Using the BioEdit server's "align" script, multiple amino acid sequence alignment was performed between the reference amino acid sequences and their observed mutant counterpart.

Comprehensive phylogenetic tree construction: This research used a complete tree to figure out what effect might exist of the detected single nucleic acid substitution on the phylogenetic positioning of the investigated sample. Using the NCBI-BLASTn server, the detected variations were matched to their homologous reference sequence neighbors (24). Then, using the Interactive Tree Of Life (ITOL)(https:\\itol.embl.de) as an online tool for the display,manipulation,and annotation of phylogenetic treeand other trees was used. A full inclusive tree was generated, including the observed variant, using the neighbour-joining approach and visualized as a circular cladogram (9).

Analytical statistics of the results

A statistical analysis is carried out. SPSS is a statistical package for the social sciences (version 25). For independence and goodness of fit, the Chi-square (χ^2) test was employed; P≤1 0.05 was regarded statistically significant, and P ≤ 10.01 was considered Statistically significant high (22).

RESULTS AND DISCUSSION

In the current study, 87 urine samples showed no growth, while 215 showed positive growth, with 139 (64.65%) being identified as *E. coli* isolates by producing off-white or beige in color with a shiny texture on Blood Agar, On MacConkey agar, pink to dark pink, dry, and donut-shaped, surrounded by a dark pink region of precipitated bile salts, and metallic

green sheen on Eosin methylene blue (EMB). Biochemical tests (IMVC), API 20, and the VITIC system confirmed the results.

Results of antibiotics susceptibility

The resistance to routinely used antimicrobials against urine isolates acquired from Medical City Hospital in Iraq is shows in Tables 4 and 5. For each antimicrobial agent, Table 4 displays the percentage of susceptible, intermediate, and resistance isolates, while Table 5 shows multidrug resistance. E. coli demonstrated a significant level of resistance to Cefotaxime, Ampicillin, Piperacillin, Trimethoprim- sulfamethoxazole, Cefepime, Ceftazidime at percentage (100, 98.6, 96.4, 95.7, 94.2 , 91.4%) respectively. Followed by Ceftriaxone, Aztreonam, Amoxicillin-clavulanate, Azithromycin, Cefoxitin, Nalidixic acid, Levofloxacin, Nitrofurantoin, Tetracycline, Gentamicin, Ciprofloxacin and Chloramphenical at (89.9, 88.4, 84.9, 77.7, 73.4, 72.7, 71.2, 63.3, 61.2, 53.2, 51.1 and 41%) respectively. Tigecyclin, Colistin, Amikacin, Meropenem, and Imipenem were the most effective medications against these isolates that showed a high sensitivity to it at (3.6, 12.9, 25.2, 25.2 and 30.9) respectively. Out of 139 UPEC isolates, 111 (79.85%) isolate were multidrug resistance MDR which have at least one antimicrobial drug resistance in three or more antimicrobial categories, and 18 (12.94 %) were In all but two or fewer antimicrobial groups, XDR isolates were resistant to at least one agent in all but two or fewer antimicrobial catigories (i.e. bacterial isolates remain susceptible to only one or two categories), while 3 (2.15%) were PDR in which isolates exhibited non-susceptibility to all antimicrobial drugs in all categories (10). Some of antibiotics assessment results in the current research were quite similar to those obtained by Manal, *et al*., (2018) in which the goal of their study was to investigate the frequency of integron class 2 and resistance among 301 fecal *E. coli* isolates from healthy individuals (1-80 years), also to determine the association of Integron class 2 with antibiotic resistance (11), as well as a study conducted by Zainab Jaber et al. (23). There are a number of reasons why these isolates are extremely resistant to most antibiotics, but the presence of mobile genetic elements (Integrons) may be the most important (5, 6, 24).

Table 4. The result of antibiotics susceptibility test.

	Resistance		Intermediate		Sensitive		*P-value*
	No.	%	No.	%	No.	%	
Ampicillin	**137**	**98.6**	**0**	**0**	**2**	**1.4**	**<0.0001****
Piperacillin	**134**	**96.4**	**3**	**2.2**	**2**	**1.4**	**<0.0001****
Amoxicillin-clavulanate	**118**	**84.9**	**3**	**2.2**	**18**	**12.9**	**<0.0001****
Aztreonam	**123**	**88.4**	**8**	**5.8**	**8**	**5.8**	**<0.0001****
Cefotaxime	**139**	**100**	**0**	**0**	**0**	**0**	**<0.0001****
Cefoxitin	**102**	**73.4**	**8**	**5.8**	**29**	**20.9**	**<0.0001****
Cefepime	**131**	**94.2**	**2**	**1.4**	**6**	**4.4**	**<0.0001****
Ceftriaxone	**125**	**89.9**	**5**	**3.6**	**9**	**6.5**	**<0.0001****
Ceftazidime	**127**	**91.4**	**3**	**2.2**	**9**	**6.5**	**<0.0001****
Imipenem	**43**	**30.9**	**5**	**3.6**	**91**	**65.5**	**<0.0001****
Meropenem	**35**	**25.2**	**1**	**0.7**	**103**	**74.1**	**<0.0001****
Ciprofloxacin	**71**	**51.1**	**14**	**10.1**	**54**	**38.8**	**<0.0001****
Levofloxacin	**99**	**71.2**	**13**	**9.4**	**27**	**19.4**	**<0.0001****
Nalidixic acid	**101**	**72.7**	**14**	**10.1**	**24**	**17.3**	**<0.0001****
Gentamicin	**74**	**53.2**	**11**	**7.9**	**54**	**38.8**	**<0.0001****
Amikacin	**35**	**25.2**	**9**	**6.5**	**95**	**68.3**	**<0.0001****
Azithromycin	**108**	**77.7**	**20**	**14.4**	**11**	**7.9**	**<0.0001****
Tetracycline	**85**	**61.2**	**10**	**7.2**	**44**	**31.7**	**<0.0001****
Tigecyclin	**5**	**3.6**	**0**	**0**	**134**	**96.4**	**<0.0001****
Trimethopri sulfamethoxazole m-	**133**	**95.7**	**0**	**0**	**6**	**4.3**	**<0.0001****
Chloramphenicol	**57**	**41**	**8**	**5.8**	**74**	**53.2**	**<0.0001****
Nitrofurantoin	**88**	**63.3**	**6**	**4.3**	**45**	**32.4**	**<0.0001****
Colistin	**18**	**12.9**	**0**	**0**	**121**	**87.1**	**<0.0001****

**** Significant at p < 0.01, data provided as Chi-square (2) goodness of fit.**

Table 5. The end outcome of Multi- drug resistant

Number of antibiotics Resistance (Antimicrobial Class)	No.	%	*P-value*
3	2	1.4	<0.0001**
4	6	4.3	
5	20	14.4	
6	1	0.7	
7	4	2.9	
8	16	11.5	
9	22	15.8	
10	34	24.5	
11	12	8.6	
12	4	2.9	
13	18	12.9	
Total	139	100	

**** Significant at p < 0.01, data provided as Chi-square (2) goodness of fit**

Result of Integron class I and its association with antimicrobial resistance: Class 1 integron was found in 87 (78.4%) of the 111 multidrug resistance isolates, with an amplification product of 483 bp (Figure 1). The prevalence of antibiotic resistance for class I integron isolates revealed a substantial correlation between1 the existence of mobile1 genetic element (integron) and resistance to the majority of antibiotics studied, as shown in Table (6); Ampicillin 78.4%, Piperacillin 78.4%, Amoxicillin-clavulanate 70.3%, Aztreonam 73.9%, Cefotaxime 78.4%, Cefoxitin 58.6%, Cefepime 78.4%, Ceftriaxone 75.7%, Ceftazidime 76.6%, Imipenem 34.2%, Meropenem 27.9%, Ciprofloxacin 55.9%, Levofloxacin75.7%, Nalidixic acid 72.1%, Gentamicin 53.2 %, Amikacin 27.9%, Azithromycin 76.6%, Tetracycline 59.5%, Trimethoprim-sulfamethoxazole 78.4%, Chloramphenicol 43.2% and Nitrofurantoin 64%. While very low association of Integron class I with resistance to Colistin and Tigecycline at percentage 1 % respectively. The presence of Integron class I at this frequency explains why *E. coli* isolates are able to have great resistance to diverse antibiotic classes. MDR is encoded by resistance genes clustered in integrons, which are potentially mobile genetic components that are thought to be involved in MDR transmission (6).The current Integron class I results were similar to those reported by Mohammad Kargar et al (14) who discovered Integron class I in 78.26% of clinical *E. coli* isolates While the prevalence of class I integron was lower in prior investigations than in the current study (7, 13).

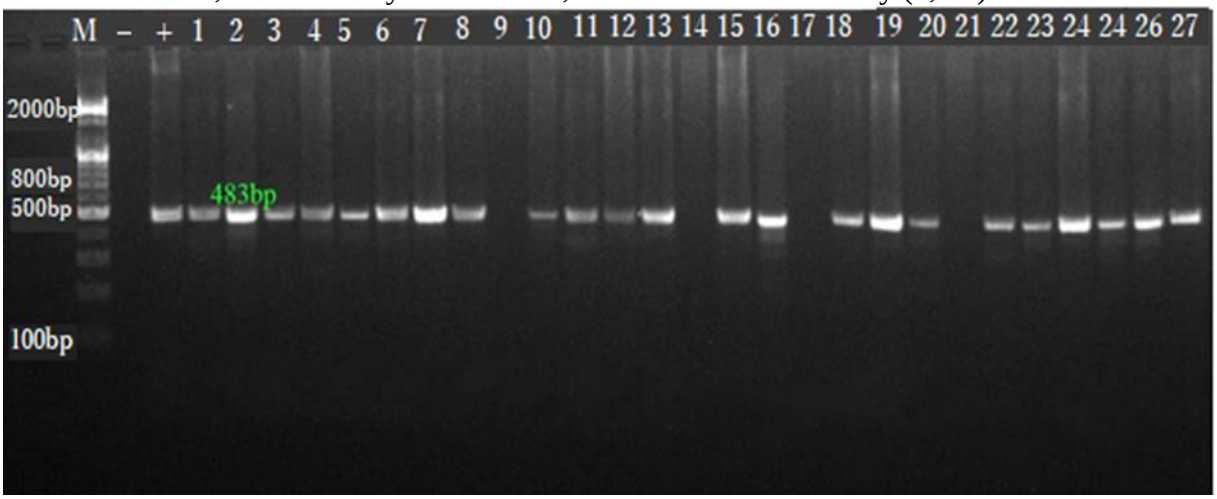

Figure 1. PCR amplified products from E. coli isolates extracted DNA on Agarose gel, M: Marker DNA (100-2000 bp), Lane 1 control negative , Lane 2 control positive , Lane 1 to 8, 10 to 13, 14 and 15, 18,'19 and 20, 22 to 27 reflect positive results

Table 6. Distribution of antibiotics resistance in clinical *E coli* isolates, based on the existence or absence of Integron class I

Antibiotics	Int. class I				*P-value*
	Present		Absent		
	No.	%	No.	%	
Ampicillin	87	78.4	24	21.6	<0.0001**
Piperacillin	87	78.4	24	21.6	<0.0001**
Amoxicillin-clavulanate	78	70.3	12	10.8	<0.0001**
Aztreonam	82	73.9	19	17.1	<0.0001**
Cefotaxime	87	78.4	24	21.6	<0.0001**
Cefoxitin	65	58.6	11	9.9	<0.0001**
Cefepime	87	78.4	22	19.8	<0.0001**
Ceftriaxone	84	75.7	22	19.8	<0.0001**
Ceftazidime	85	76.6	20	18	<0.0001**
Imipenem	38	34.2	5	4.5	<0.0001**
Meropenem	31	27.9	4	3.6	<0.0001**
Ciprofloxacin	62	55.9	9	8.1	<0.0001**
Levofloxacin	84	75.7	15	13.5	<0.0001**
Nalidixic acid	80	72.1	21	18.9	<0.0001**
Gentamicin	59	53.2	15	13.5	<0.0001**
Amikacin	31	27.9	4	3.6	<0.0001**
Azithromycin	85	76.6	23	20.7	<0.0001**
Tetracycline	66	59.5	19	17.1	<0.0001**
Tigecyclin	4	3.6	1	0.9	0.18^{NS}
Trimethopri sulfamethoxazole	87	78.4	20	18	<0.0001**
Chloramphenicol	48	43.2	9	8.1	<0.0001**
Nitrofurantoin	71	64	17	15.3	<0.0001**
Colistin	17	15.3	1	0.9	0.0001**

Chi-square (2) goodness of fit is used to present the data. NS Non-significant. ** Significant at P < 0.01; * significant at P < 0.05

Sequencing results

The current investigation includes three samples which were screened to partially amplify *INTI1* sequences of the infecting bacterial sequences (three PDR isolates). As a result, the *INTI1* sequences can be employed for discrimination among various types of *E. coli* due to the possible roles of these sequences in adapting variable diversity in different environmental situations. After performing NCBI blastn for their PCR amplicons, the sequencing reactions revealed the exact identity of these sequences (25). The NCBI BLASTn engine found 100 percent sequence similarity between the sequenced samples and the targeted reference sequences for the 127 bp amplicons. The precise locations and other features of the obtained PCR fragments were discovered by comparing the observed nucleic acid sequences of these studied samples with the retrieved nucleic acid sequences (GenBank acc. CP091927.1). The overall length of the targeted locus was determined in the NCBI server, as well as the start and end positions of the targeted locus inside the most similar bacterial target (Fig. 2).

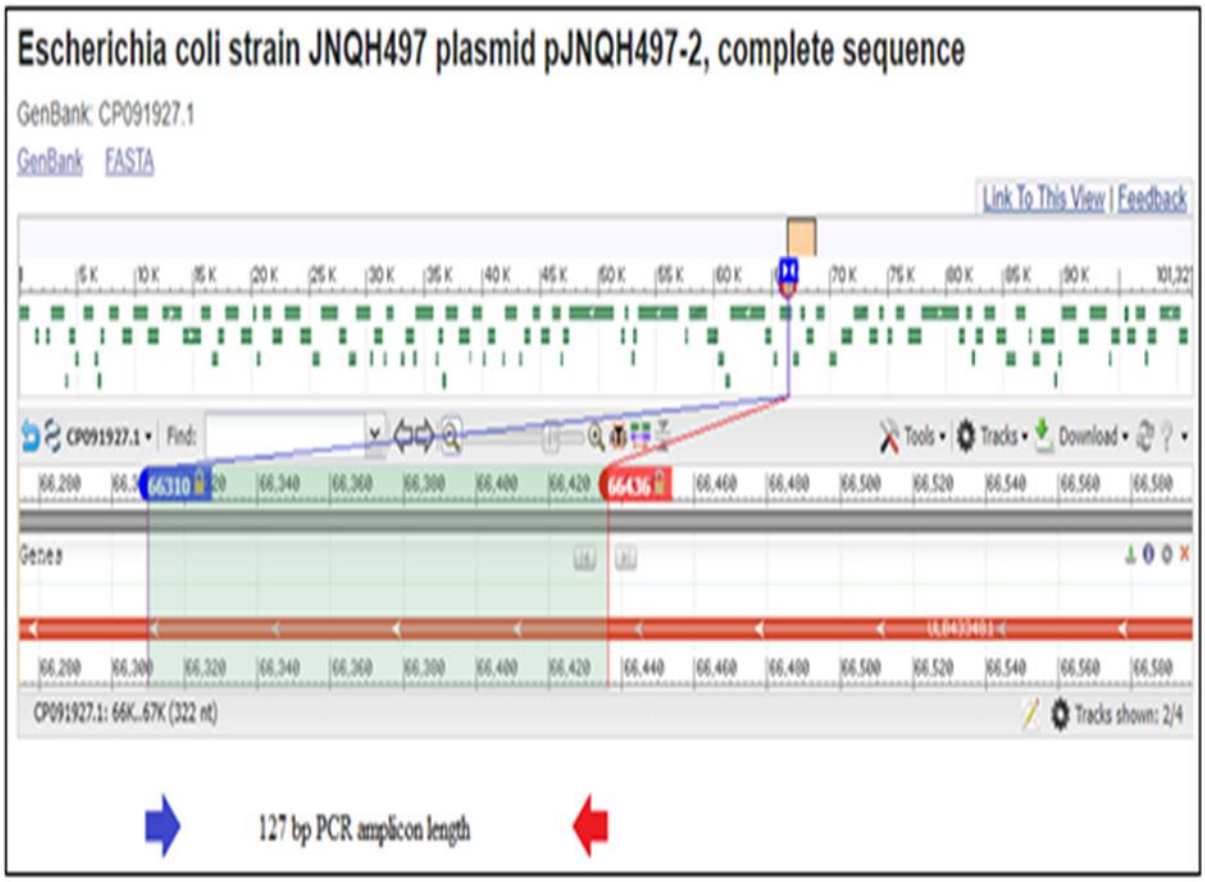

Fig. 2. The exact location of the 127 bp amplicons inside *E. coli* genomic sequences covered a portion of the *INTI1* regions (GenBank acc. no. CP091927.1). This amplicon's starting point is indicated by the blue arrow, while its endpoint is indicated by the red arrow

The features of the 127 bp amplicons' sequences were emphasized after locating them inside the genomic sequences of the studied bacterial sequences, and the overall length of the amplified fragments was also determined (Table 7).

Table 7. The length and position of the 127 bp PCR amplicons utilized to amplify a portion of the INTI sequences inside E. coli genomic sequences (GenBank acc. no. CP091927.11).

Amplicons	Reference locus sequence (5′ - 3′)	Length
The amplified *IntI1* fragment.	***AGGATGCGAACCACTTCATCCGGGGTCAGCACCACCGGCAA GCGCCGCGACGGCCGAGGTCTTCCGATCTCCTGAAGCCAGG GCAGATCCGTGCACAGCACCTTGCCGTAGAAGAACAGCAAGG CCG****	**127 bp**

*** refers to the forward strand that is oriented forward.**

**** refers to the reverse strand that is put in the opposite direction of the complement strand**

The alignment results of the 127 bp samples revealed no identifiable nucleic acid variation when compared to the most similar referring reference nucleic acid sequences (Fig. 3). Sequencing reactions revealed that no variation was detected in the investigated samples in comparison with the *INTI1* referring sequences (GenBank acc. no. CP091927.11).

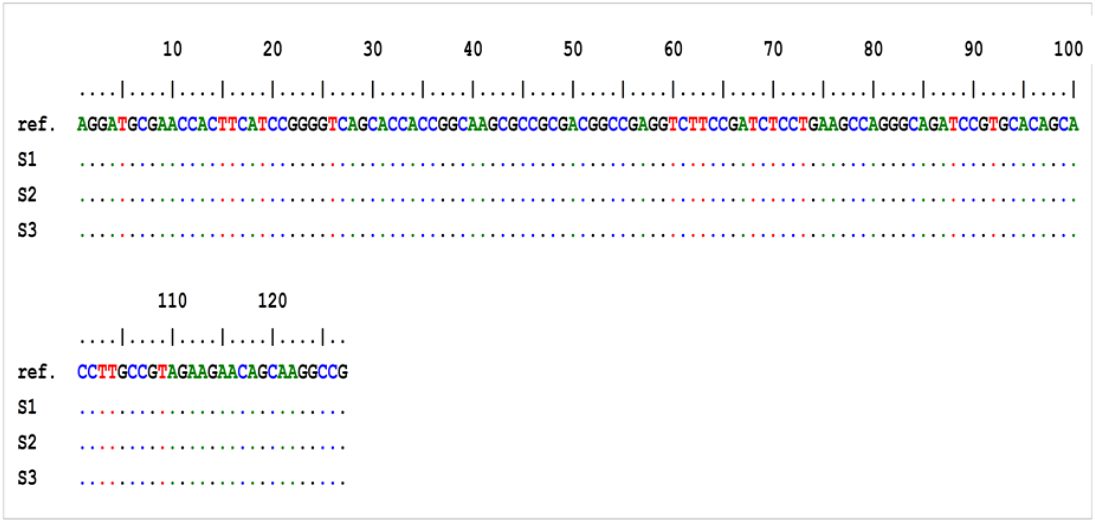

Fig. 3. Three samples' nucleic acid sequences were aligned with their respective reference sequences of the INTI1 sequences' 127 bp amplicon. The letters "S," followed by a number, relate to the examined *E. coli* sequences, while the sign "ref" refers to the NCBI referencing sequence

The observed nucleic acid variation was further analyzed to identify their accurate positioning within the entire class 1 integron integrase IntI1 protein. *The Expasy translate suite* was used to convert all nucleic acid sequences in the S1–S3 samples to their matching amino acid sequences. When these amino acid sequences were aligned with their references, it was observed that these samples occupied 41 amino acid residues in the class 1 integron integrase *IntI1* protein (Fig. 4a). Results showed that these amino acid sequences were positioned between the 85th to 125th amino acid residues in the entire protein (Fig. 4b).

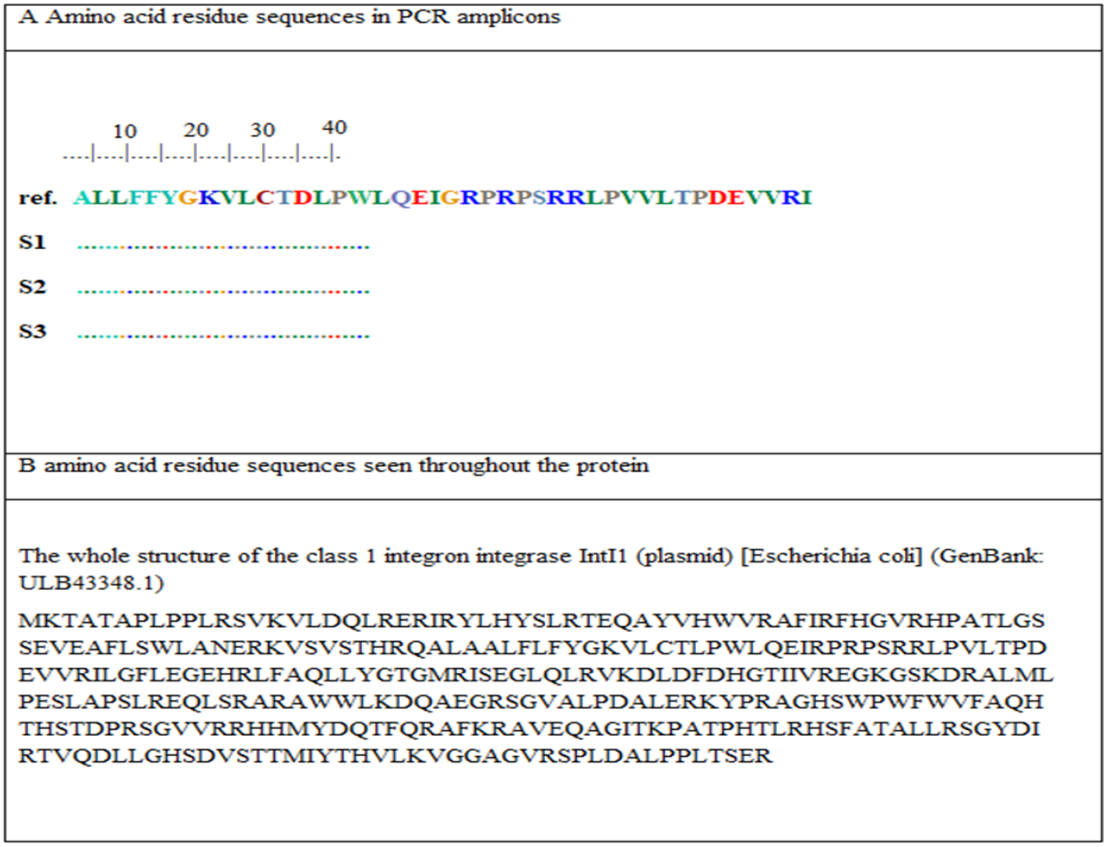

Fig. 4.a. Within the examined *E. coli*, amino acid residues alignment of the amplified segments of the INTI1-encoded protein. A) Amino acid residues are marked in the amplified 127 bp locus according to their appropriate locations. B) The amino acid residues are marked in the protein according to their relative locations. The grey highlights relate to the *INTI1*-encoded enzyme's amplified area

A thorough phylogenetic tree was constructed in the current research based on nucleic acid variations detected in the amplified 127 bp of the *INTI1* sequences amplicons to provide a phylogenetic comprehension of the real distances between our studied sample and its relevant sequences. This phylogenetic tree included the currently analyzed sample as well as other *E. coli* relative nucleic acid sequences. Our researched samples were included alongside relative sequences within this tree, resulting in only one type of organism of incorporated sequences within the cladogram, which is *E. coli.* In this complete tree, there were a total of 46 aligned nucleic acid sequences. Within the E. coli sequences, the integrated samples were clustered into two major phylogenetic clades in the cladogram. The most intriguing finding in our study of bacterial isolates was a link between their location and the E. coli genomes that surrounded them. The major clade in which the investigated S1 – S3 sample was incorporated was made of a total of thirty-nine sequences (Fig. 4). All S1 to S3 samples were suited in the immediate vicinity to all incorporated reference sequences within this clade. Such a sort of positioning of these samples was may be due to the absence of any nucleic acid variation compared to the *INTI1* gene's amplified fragment's reference sequences. However, these samples were suited beside many sequences that were deposited from the USA (such as GenBank acc. no. CP054232.1, CP054828.1. and CP054343.1), China (GenBank acc. no. CP055256.1, CP055254.1, and CP055251.1), and Myanmar (GenBank acc. no. AP023228.1). Therefore, the American / Asian sources of these biological samples were determined. Thus, these samples were suited in this clade alongside these sequences without any tilt. This sort of positioning indicated no possible variability potential for the amplified fragment with respect to the original positioning these bacteria samples occupied within this cladogram. However, the Asian - American sources of the investigated S1 – S3 samples were confirmed by other neighboring sequences in the same major clade. However, the aggregation of all sequences of the major clade in extremely small phylogenetic distances refers to the presence of an extremely close correlation of the phylogenetic distribution among the observed major clade. In addition to the major clade, another clade was found with distinct phylogenetic distances within the same tree. This clade was assigned as a minor clade. Within this clade, seven *strains of variable *E. coli* sequences were incorporated. It was found that the *E. coli* sequence of this clade was originated from several Asian, African sources. This is due to the presence two sequence from China (GenBank acc. no. CP034789.1 and MN086777.1), Hong Kong (GenBank acc. no. CP055020.1 and CP019276.1), India (GenBank acc. no. CP034254.1), Myanmar (GenBank acc. no. AP023238.1), and Ghana (GenBank acc. no. CP029631.1). However, our investigated samples did not reside in this clade and occupied distinct phylogenetic positions apart from this minor clade. Because it explained the actual neighbour -joining-based positioning in such reported variances, This tree's current observation has confirmed sequence reactions. Majority of the incorporated organisms were found to have belonged to various strains isolated from variable biological sources. The use of *INTI1* sequences in this work has provided additional evidence for the presence of exact identification of the true identity of these bacterium species. The analyzed bacteria samples were determined to occupy one position within the cladogram's included main clade, with no phylogenetic distances. This suggested that the bacterium sequences of both strains shared a complete genetic similarity. This data necessitates utilization of a larger fragment to incorporate more potential genetic variations and more consequent phylogenetic differences. However, This comprehensive tree based on *INTI1* sequences has supplied an all-encompassing tool for this gene fragment's strong ability to efficiently detect bacterium identity using *INTI1*-based sequences. This, in turn, demonstrates the ability of the currently used *INTI1* sequence- specific primers to accurately define E. coli bacteria studied and their phylogenetic locations. As a result, the *INTI1* sequence-based fragments were used to demonstrate the great ability of such genetic fragments to efficiently detect bacterium

sequences. This, in turn, adds to the evidence that the currently used mitochondrial sequence-specific primers can accurately define the *E. coli* strain under investigation (9).

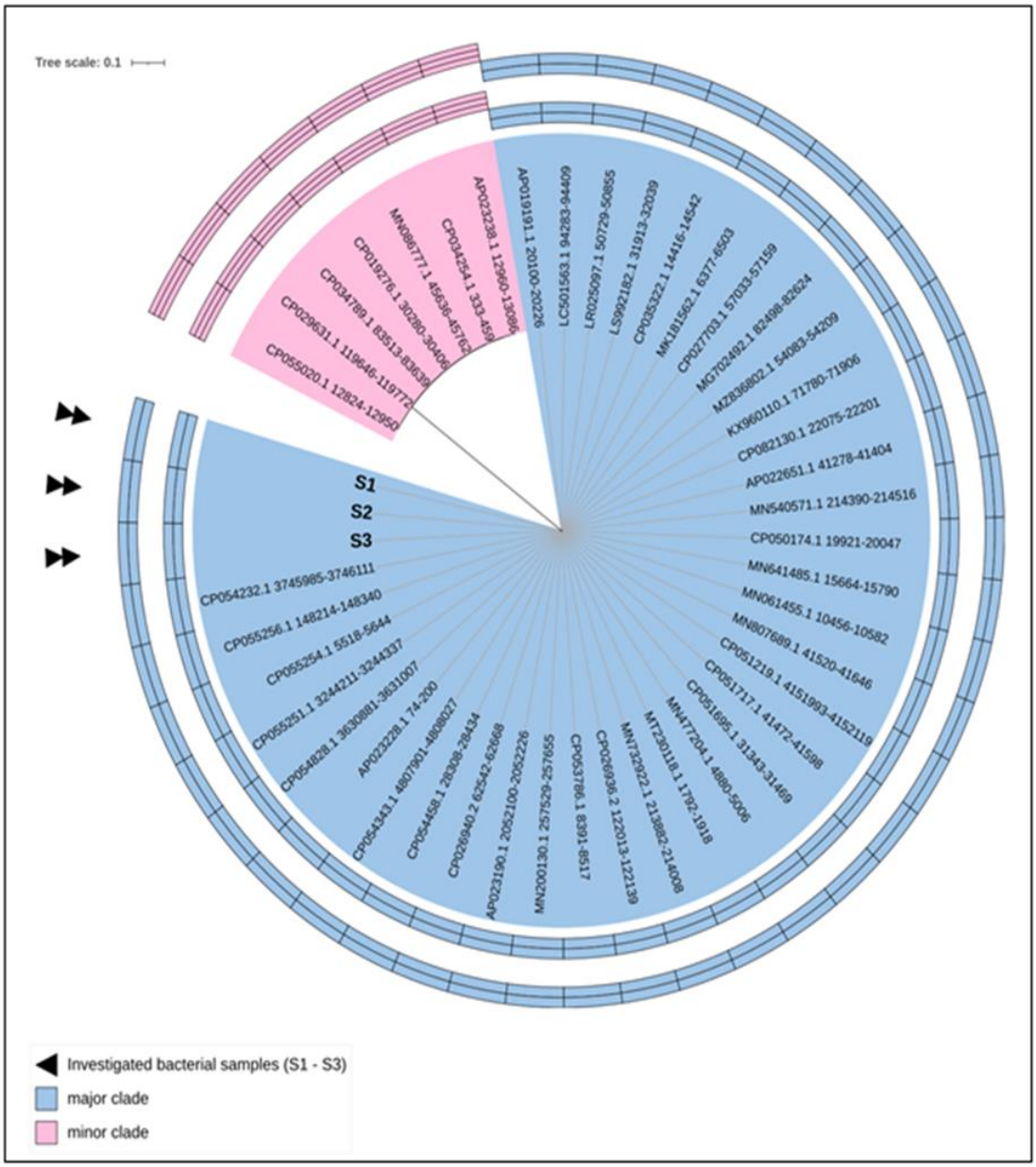

Fig. 4.b. The evolutionary tree of genetic variations of the *INTI1* sequence fragment in *E. coli*, as represented by a cladogram. The studied bacterium variations are represented by the black-colored triangle. All of the numbers referred to each referencing species' Gen Bank entry number. The number "0.1" at the top of the tree denotes the extent to which the organisms' scales differ. classified by comprehensive tree. The letter "S," followed by a number, refers to the *E. coli* sequences that have been studied

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