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Detection of structure Integron and identification of *sul*1 and *qac*EΔ1 genes of multidrug-resistant *Escherichia coli* isolated from children with acute diarrhea in Baqubah city

Ali Shakir Al-Ezee[1], Hadi R. Rasheed Al-Taai [2], Ali Jaffar Saleem [3]
[1,3] Department of Biology / College of Education for Pure Sciences / University of Diyala
[2]Department of Biology / College of Science / University of Diyala

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Abstract

Diarrhea is a common infection in Iraq, often caused by *Escherichia coli*. It is crucial to study the resistance genes of *E. coli* in diarrhea patients and their relationship to Integron genes. The study collected 367 stool samples from patients at the Batul Women's and Obstetric Hospital, Children, and Civil Laboratories between 15/11/2022 and 1/2/2023. *Escherichia coli* was diagnosed with chemical tests, and a confirmation of its diagnosis was obtained using the type gene. This technique is considered to be a confirmed diagnosis of *E. coli*. A total of 50 isolates were accurately and definitively diagnosed as belonging to the bacteria *Escherichia coli*. The results of the test for the sensitivity of *E. coli* to antibiotics (Ampicillin, Amoxicillin, clavulanate, Ceftazidime, Cefotaxime, Aztreonam, Tobramycin) Amikacin, Azithromycin, Doxycycline, Tigecycline, Ciprofloxacin, Levofloxacin, Imipenem, Trimethoprim-sulfamethoxazole, Sulfonamides) were as follows (96%, 94%, 88%, 92%, 68%, 26%, 56%, 52%, 60%, 0%, 26%, 28%, 14%, 74%, 68%) respectively. Integron Class I was detected in 64%, with 23 isolates containing the gene *sul*1 and 29 isolates containing the *qac*EΔ1. The percentage of the *sul*1 gene in the isolates carrying the integron only was 67.64%. The percentage of the *qac*EΔ1 gene was 85.29%. Molecular detection of the integron class I structure for regions involved in the integrin structure (*gene cassette Class 5, CS, 3, CS, ORFend, Sul1B, and F12R-Orf513-lnk13*) and the results of molecular detection of the above regions showed that they were all present in all isolates.

Corresponding Author:

Ali Shakir Al-Ezee
Department of biology, College of Education for Pure Science University of Diyala
Baqubah City, Diyala Governorate, Iraq.
Email: ali.shakir@uodiyala.edu.iq

1. INTRODUCTION

Diarrheagenic *E. coli* (DEC) represents the most common bacterial pathogen worldwide and often contributes to enteric diseases caused by food-borne pathogens. DEC generally causes watery diarrhea, which in some cases can progress to more severe disease, which is associated with a range of virulence factors. It lives naturally in normal flora and is well-suited for biochemical and genetic research. The study of *E.coli* bacteria has become apparent due to their prevalence and ease of isolation and the development of molecular biology techniques [1]. One of the advantages of *E. coli* is understanding the systems that bacteria have evolved to fight antibiotics, so this can help us understand the relationship between these microorganisms and the occurrence of antibiotic resistance in humans [2]. Antibiotic-resistant bacteria pose a significant global threat to human health [3]. Tetracycline inhibits protein synthesis. Bacteria enter via an energy-dependent mechanism and bind inversely to the bacteria's 30S ribosome subunits. This method prevents aminoacyl-tRNA from reaching the RNA-ribosome complex, thus preventing the production of bacterial polypeptides [4].

Trimethoprim resistance is due to the acquisition of DNA that determines DHFR, which reduces trimethoprim inhibition by enzymes [5] [29]. Fluoroquinolones target DNA gyrase and topoisomerase IV bacterial enzymes, stabilizing the enzyme-DNA complex. This leads to the division of DNA into both strands and ultimately causes cell death, making it a very effective way to kill bacteria [6]. Integrons are segments of mobile DNA that can capture genes, especially those expressing antibiotic resistance, through site-specific recombination [7]. While they cannot be transmitted to other bacteria independently, they are usually associated with transposons and plasmids. Plasmid integrons containing antibiotic-resistance genes can spread to other bacteria through conjugation [8]. Integrons class I and II appear more closely associated with antibiotic resistance [9]. Integron class I play a significant role in antibiotic resistance by expressing various resistance genes. These Integrons are often integrated into plasmids and transposons, which allows for their horizontal transfer into multiple pathogens. Integrons Class I are the most common clinical isolates of Enterobacteriaceae [10].

2. MATERIAL AND METHODS:

Discharge specimens (Stool) were collected from patients admitted to Al-Batool Hospital from 15/11/2022 to 1/2/2023. The specimens were cultured directly in the center of McConkey agar and aerated at 37 °C for 24 hours [11]. Standard antibiotic tablets were used in [12] used in the current study.

2.1 DNA Extraction

E.coli DNA was extracted from bacteria using a boiling process[13]. *E.coli* was diagnosed using a specialist initiator *trp*A [14].

2.2 Primers used in the Study

Table 1. primers used in the study

Gene	Nucleotide sequence (5′–3′)	Product size (bp)	References
*trp*A	F: CGGCGATAAAGACATCTTCAC	489	[15]
	R: GCAACGCGGCCTGGCGGAAG		
intl	F: CCTCCCGCACGATGATC	280	[16]
	R: TCCACGCATCGTCAGGC		
*sul*1	F: CGGCGTGGGCTACCTGAACG	433	[17]
	R: GCCGATCGCGTGAAGTTCCG		
*qac*EΔ1	F: ATCGCAATAGTTGGCGAAGT	250	
	R: GAAGCTTTTGCCCATGAAGC		
Class 1 gene cassette	F: GGCATCCAAGCAGCAAGC	Variable	
	R: AAGCAGACTTGACCTGAT		
5′ CS 3′ CS	GGCATCCAAGCAGCAAGC	Variable	
	AAGCAGACTTGACCTGAT		
ORFend Sul1B	CCGTTAAGCTCTTATGTGGG	Variable	
	GCAAGGCGGAAACCCGCGCC		
F12R Orf513-lnk13	AAACCAGCATGGTTGGCTAC	Variable	
	CACCCTGCAAACCTTGCCAGG		

2.3 PCR Interaction Steps

Table 2. PCR interaction steps

PCR interaction steps		*trp*A	*int*l	*sul*1	*qac*EΔ1	3CS-5CS	*Orfend-sulB*	*gene cassette*	*orf513-Ink13-F12R*	Time
		Temperature								
One cycle Initial denaturation		95°C	95°C	95°C	95°C	95°C	95°C	95°C	95°C	5min
40 Cycle	denaturation	95°C	95°C	95°C	95°C	95°C	95°C	95°C	95°C	45Sec
	Annealing	68°C	58°C	67°C	58°C	58°C	65°C	58°C	63°C	45Sec
	Extension	72°C	72°C	72°C	72°C	72°C	72°C	72°C	72°C	1min
One cycle Final Extension		72°C	72°C	72°C	72°C	72°C	72°C	72°C	72°C	10min

2.4 PCR Master Mix

Attended the PCR interaction mix using Go Taq ® G2 Green Master Mix provided by Promega, following the instructions outlined in the table below:

Table 3. PCR master mix

PCR master mix	Volume
Go Taq ® G2 Green Master Mix	12.5 µL
DNA template	5 µL
Forward primer	1.5 µL
Reverse primer	1.5 µL
Free nuclease Water	4.5 µL
Total	25 µL

2.5 Determine the Sequences of the Integron, *qac*EΔ1, and *sul*1

Four isolates (4-6-9-26) ' PCR reaction products were sequenced for the Integron, *qac*EΔ1, and *sul*1 genes using gene-specific primers sent to the Macrogen company in South Korea. By comparing these sequences with reference sequences on the NCBI website, we could identify the exact locations and details of the amplification fragments for each gene.

2.6 Dendogram Analysis

The genetic relationship between all bacterial isolates under study was found using the Dendogram analysis scheme, converting the results that appeared to the description table. If the result was positive, the number (1) was placed, and if the result was negative, the number (0) was placed. The data was then entered into the Software Past program using the Dice option to obtain the cluster analysis scheme.

3. RESULTS AND DISCUSSION

3.1 Molecular Diagnosis of *Escherichia coli*

Escherichia coli was identified using the molecular detection of the *trp*A gene, providing a confirmed diagnosis of *E. coli*. This finding echoes the study of [31] in Dohuk Governorate, where 349 (87.2%) positive specimens of *E. coli* bacteria were obtained. Similarly, it is consistent with the study of [18] in Baghdad Governorate, which reported a positivity rate of 66.23%. However, the results of this study differed from 'the study of [19] in Babil Governorate, where the positivity rate was 30.66%. Figure (1)

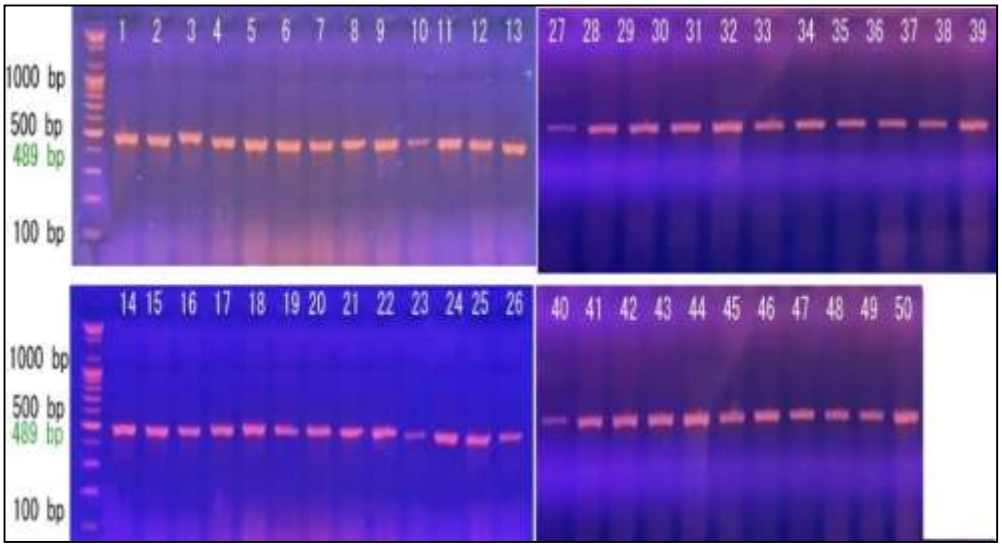

Figure 1. represents the electrophoresis of the PCR reaction product to detect the *trp*A gene of *E. coli*

3.2 Testing the Sensitivity of *E. coli* to Antibiotics

The results shown in Table (4) showed that the antibiotics Ampicillin, Amoxicillin-clavulanate, Ceftazidime, Cefotaxime, Aztreonam, Tobramycin, Amikacin, Azithromycin, Doxycycline, Tigecycline, Ciprofloxacin, Levofloxacin, Imipenem, Trimethoprim-sulfamethoxazole, Sulfonamides had a resistance rate of (96%, 94%, 88%, 92%, 68%, 26%, 56%, 52%, 60%, 0%, 26%, 28%, 14%, 74%, 68%) respectively.

The use of antibiotics to treat diarrheal *Escherichia coli*, especially in children, is limited by the increase in antibiotic resistance [30]. Therefore, routine testing for antibiotic sensitivity, identification of effective antibiotics, and identification of the pathogens of resistant bacteria should be carried out to limit the increase in bacterial resistance to antibiotics [20]. The high rate of antibiotic resistance can be explained by individuals using antibiotics without a prescription [14]. This may be a reflection of how these drugs are used and misused in the community. This finding is because, outside the hospital setting, people can easily take many antibiotics from any pharmacy without a prescription. Furthermore, the lack of oversight and the indiscriminate importation of drugs from reliable sources have been an additional cause of the spread of resistance [21]. Antibiotic resistance in *E. coli* is mostly due to genes located on conjugative plasmids through conjugation, transformation, or transfer. Alternatively, resistance genes may be located on the bacterial chromosome and jump to resistance plasmids through transposition. Transposons carry multiple antibiotic resistance genes; some contain more than one resistance gene. Antibiotics have played a crucial role in treating and preventing common diseases. Still, the rules of evolution and natural selection and their misuse have led to an alarming increase in antibiotic-resistant use worldwide [22].

Each bacterial species can exhibit different levels of antibiotic resistance, which can vary due to the prevalence of specific resistant bacteria or differences in the use of antibiotics [23]. The indiscriminate use of antibiotics leads to the development of bacterial resistance. In healthcare settings like hospitals, repeated antibiotic exposure and the transfer of resistance genes between bacteria can increase resistance levels [24]. Resistance mechanisms can include changes in the cell wall or intracellular transport systems. Integrons form strains resistant to multiple antibiotics, making infections harder to treat. Understanding the role of integrons in antibiotic resistance can help develop better strategies to combat bacterial resistance [25].

3.3 Multiple Drug Resistance

The results showed that 48 96% of the isolates had multiple drug resistance (MDR) characteristics. They ranged from 3 to 14 antibiotics, and no isolate showed sensitivity to all antibiotics used in the study, which confirms the presence of multiple resistance among the local isolates. As for the isolates with extensive drug resistance (XDR), there were 10 isolates, representing 20% Table (4). The results showed that most isolates showed multiple resistance to the antibiotics tested.

Table 4. Multiple antibiotic resistance of the isolates under study

Isolated	%	Resistant	Number of antibiotic
E.12, E.50	4%	do not have	2
E.9, E.19	4%	MDR	3
E.1, E.17, E.48	6%	MDR	4
E.3, E.11, E.15, E.29, E.31	10%	MDR	5
E.4, E.8, E.16, E.47	8%	MDR	6
E.2, E.5, E.20, E.21, E.22, E.27	12%	MDR	7
E.13, E.23, E.24, E.28, E.30, E.37,E.40, E.45	18%	MDR	8
E.33, E.35, E.42, E.43, E.46, E.49	12%	MDR	9
E.34, E.28, E.39, E.44	8%	MDR	10
E.7, E.10, E.14, E.36, E.41	8%	XDR	11
E.6, E.18, E.32	6%	XDR	12
E.25, E.26	4%	XDR	13

3.4 Dendogram Analysis of the Resistance of *E. Coli*

The antibiotic susceptibility test results of the current study are analyzed using the *E. coli* dendrogram, shown in Figure (2). It included two main groups A and B, which were similar by 37%, as group A included two isolates, namely 1 and 5, as the two isolates were similar in their resistance to antibiotics (AMK, AMP, and polymyxin B) and were sensitive to antibiotics (OTX, IMP, CIP, AZM, DOX, TOB, ATM, CTX, AMP, TIG, and AMC). Group B included 48 isolates and was divided into two groups. The first group, 1B, included isolate No. 11, which was characterized by its resistance to antibiotics (DOX, AZM, ATM, AMC, and AMP) and its sensitivity to (OTX, IPM, LVX, CIP, AMK, TOB, CTX, CAZ). The second group, 2B, included 47 isolates and was divided into two sections. The first section included six isolates (2, 12, 17, 19, 31, and 48) resistant to antibiotics (AMC and Polymyxin B). It is also sensitive to antibiotics (OTX, IMP, LVX, CIP, DOX, AZM, TOP, and TIG). The second section included 41 isolates, divided into two sections. The first section included 5 isolates (2, 15, 16, 29, 47) that showed resistance to antibiotics (OTX, CTX, AMC, and AMP) and similarity in their sensitivity to antibiotics (LVX, CIP, DOX, TOP, and TIG). The second section included 35 isolates that varied in their resistance pattern and sensitivity to the antibiotics under study. It included 11 isolated that differed in sensitivity and resistance to antibiotics. Eight isolates were unique in their resistance and sensitivity to antibiotics, with a similarity rate of 0.675%. The isolates of the first and second groups show high resistance to antibiotics, including MDR and XDR patterns, as they are resistant to two or more of the antibiotics under study.

3.5 Determine the Integron genes, *qac*EΔ1 gene, and *sul*1 gene.

The results showed that the percentage of isolates carrying the integron class I was 64%, as bands of 280 base pairs appeared, indicating a positive test, Figure (3). This result was consistent with detecting the integron class I in the study of [26] at a rate of 55.83%. It was also consistent with the study of [27] as its percentage was 69%.

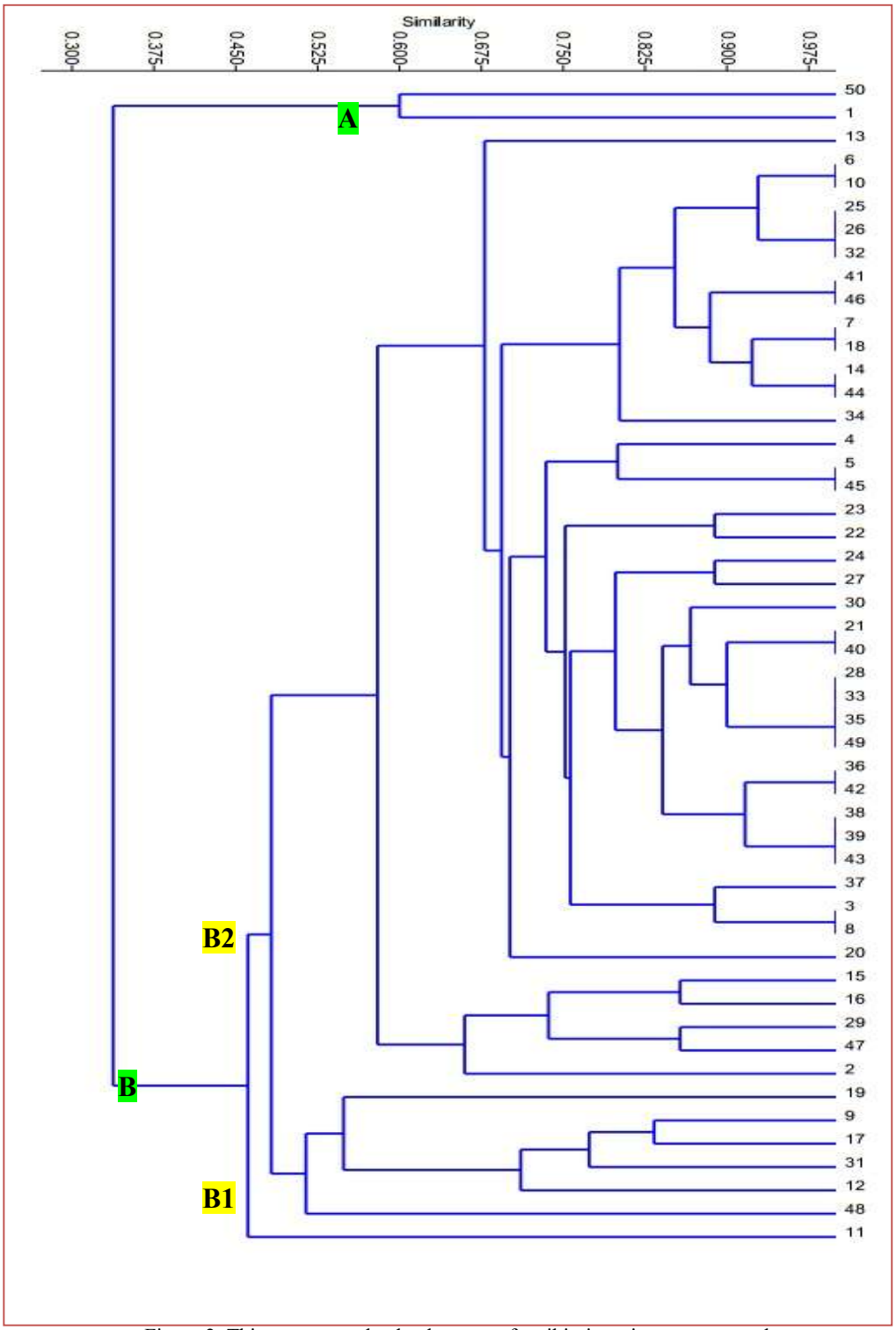

Figure 2. This represents the dendrogram of antibiotic resistance test results

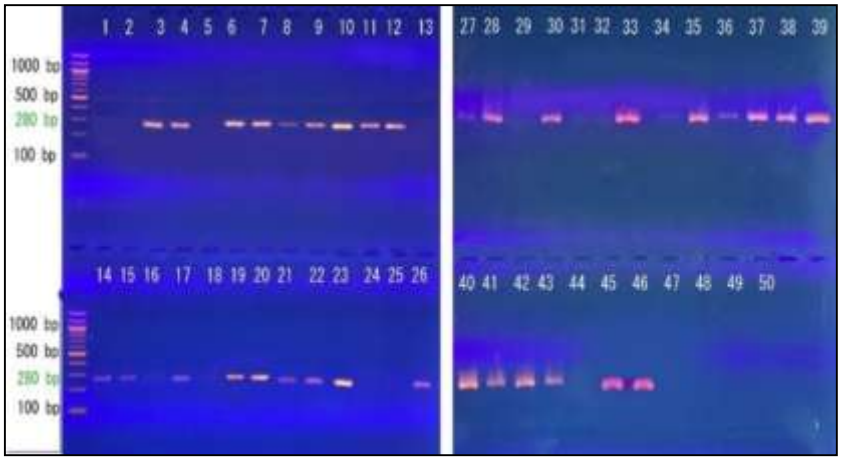

Figure 3. represents the electrophoresis of the PCR reaction product todetect the Integron Class I of *E. coli*

3.6 Integrons and Antibiotic Resistance

The results of the current study showed that 38 isolates carried integrons of the three classes or one or two classes of them, and they were distributed in different proportions. They were associated with antibiotic resistance, while 12 isolates did not carry any class of integrons and were resistant in different proportions. Table (5). Antibiotic resistance genes in the conserved or variable section of integrons or the inclusion of resistance genes in the same transitional elements that carry integrons may explain this [28].

Table 5. represents the distribution of resistance to antibiotics containing integrons class I

Antibiotic	% resistance	Faond *Int*I and resistance
Ampicillin	(%96) 48	(%94) 32
Amoxicillin-clavulanate	(%94) 47	(%91.1) 31
Ceftazidime	(%90) 45	(%85.29) 29
Cefotaxime	(%92) 46	(%88.2) 30
Aztreonam	(%72) 36	(%76.4) 26
Tobramycin	(%54) 27	(%29.4) 10
Amikacin	(%67.2)37	(52.9%) 18
Azithromycin	(%52) 26	(%47) 16
Doxycycline	(%60) 30	(%64.7) 22
Tigecycline	(%0) 0	0
Ciprofloxacin	(%26) 13	(%23.5) 8
Levofloxacin	(%28) 14	(%23.5) 8
Imipenem	(%14) 7	(%8.82) 3
Trimethoprim-sulfamethoxazole	(%74) 37	(%73.5) 25
Sulfonamides	(%68) 34	(%79.4)27

The present study reached the results of detecting antibiotic resistance genes such as the *sul*1 gene, which showed the reaction product with a length of 433 base pairs as incorrect Figure (4), and *qac*EΔ1 gene, which showed the reaction product with a length of 250 base pairs as in Figure (5). The percentage of *sul*1 gene presence in the isolates carrying integron was 67.64%. The *qac*EΔ1 gene was found to be 85.29% in isolates carrying the integron class I. The isolates carrying the integron class I was divided into three groups based on the presence of the *sul*1 gene and the *qac*EΔ1 gene. The first group included 20 isolates containing the *sul*1 and *qac*EΔ1 genes together at a rate of 58.8%. The second group included 3 isolates, at a rate of 8.82%, containing only the *sul*1 gene and not the *qac*EΔ1 gene. The third group was 9 isolates, representing 26.47%, containing both the *qac*EΔ1 gene and the *sul*1 gene. The results showed that two isolates (29, 33) within the fourth group carried the integron but did not carry the resistance genes, representing 5.88%. see Table (6).

Table 6. Bacterial groups are divided based on the presence of integrons and resistance genes.

groups	Isolate	%	Structure integron
1	-41-39-38-23-22-21-19-17-15-14-12-10-7-6-4-3 46-45-44-42	58.8%	Integron + *sul*1 + *qac*EΔ1
2	40-34-28	8.82%	Integron + *sul*1
3	43-37-35-30-26-20-11-9	26.47%	Integron + *qac*EΔ1
4	29-33	5.88%	Integron

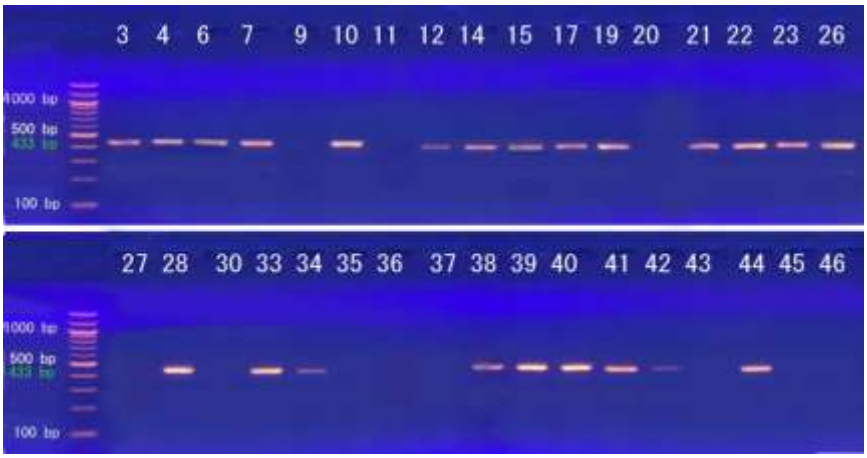

Figure 4. represents the electrophoresis of the PCR reaction product to detect the *sul*1 gene of *E. coli*

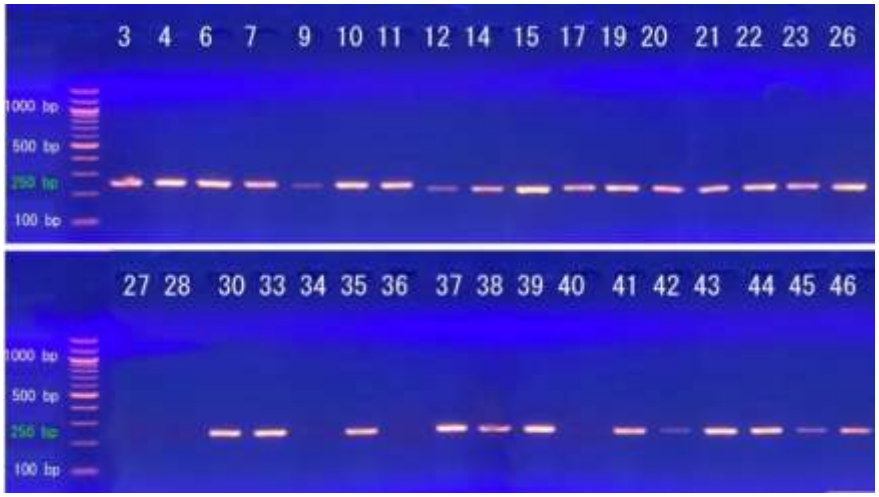

Figure 5. represents the electrophoresis of the PCR reaction product to detect the gene *qac*EΔ1 of *E.coli*

The difference in rates of occurrence of genes carried on integron class I is due to the horizontal transfer of antibiotic resistance genes, resulting in multiple options for resistance. The *qac*EΔ1 gene contributes to bacteria's resistance to disinfectants, while the sul1 gene contributes to resistance to sulfonamide antibiotics. Both genes can be transferred between bacteria through plasmids, leading to increased resistance spread in different environments.

3.7 Detection of Structure Integron

The current study, through molecular detection of the composition of the integron class I, reached structural regions in 26 isolates. The interaction product of the 5'CS region with the 3'CS region, which are variable regions, plays an important role in integrating the new cassette genes. They work together to support the process of integrating genes effectively and stabilizing the genetic composition of the integron. They provide specific DNA sequences recognized by integrase enzymes to integrate the cassette genes into the correct location, Figure (6).

The cassette gene was detected, and the interaction product showed packages, which are variable regions that play an important role in regulating gene expression and environmental adaptation of bacteria, enhancing the integron's flexibility and ability to adapt to surrounding conditions. Thanks to the cassette gene, the integron can change rapidly and provide new features to bacteria, contributing to their evolution and genetic diversity. The interaction product of orfend with sulB, which are variable regions, orfend and *Sul*1B are linked through their role in integron assembly. orfend helps regulate and manage genes within the integron and affects the stability and expression of genes such as *Sul*1B. In contrast, *Sul*1B is a cassette gene that provides sulfonamide resistance and integrates into the conserved segments' integron. The relationship between orfend and *Sul*1B enhances the effectiveness of the integron as a flexible gene complex that can respond to antibiotics and adapt to environmental stresses. Figure (7) shows the product of the interaction of orf513 with Ink13, which are variable regions and genetic elements that can be linked together by regulating gene expression and stability of the integron. orf513 may affect the function of the integron, while Ink13 has a regulatory role that affects how orf513 functions. The relationship between them contributes to the regulation and balance of genes, which enhances the ability of the integron to function effectively and adapt to environmental stresses Figure (8).

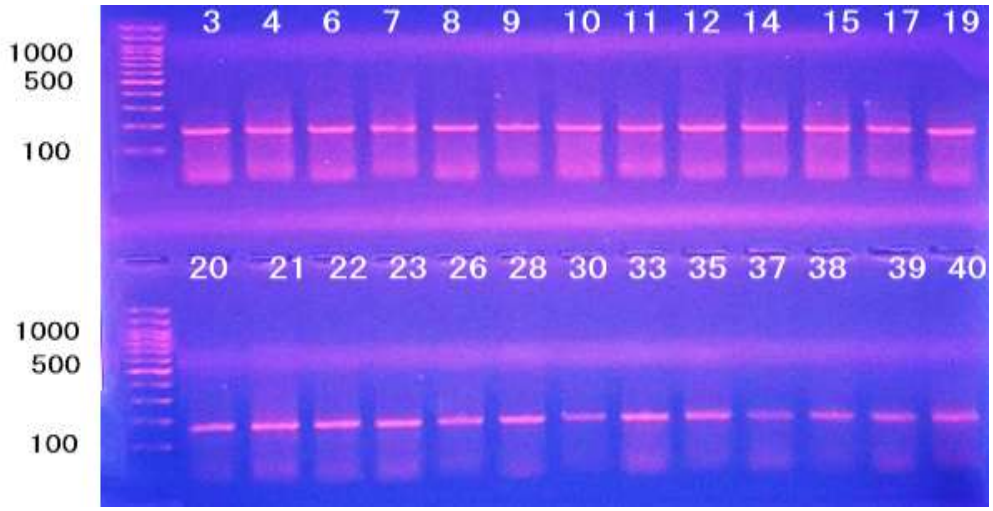

Figure 6. represents the electrophoresis of the PCR reaction product to detect the (3, CS-5, CS) of *E.coli*

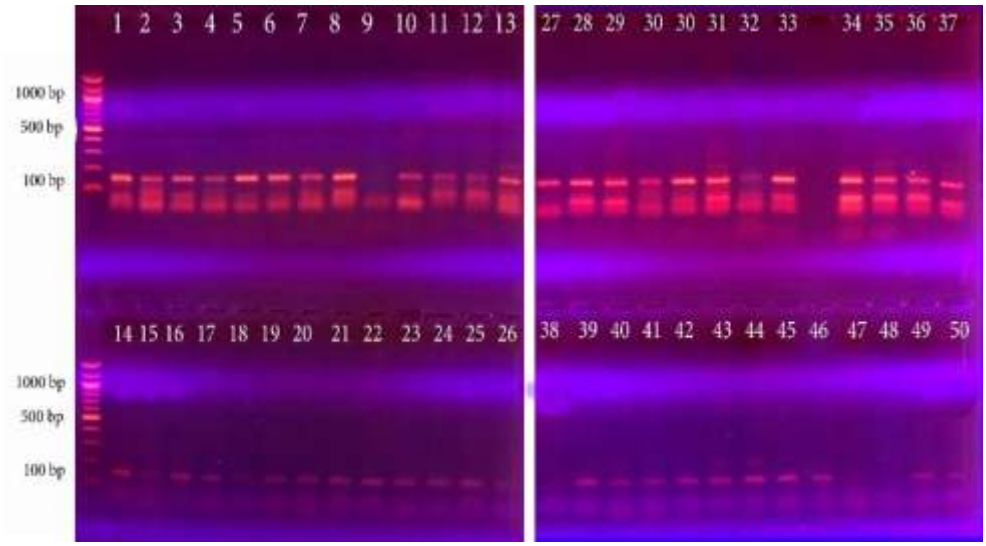

Figure 7. represents the electrophoresis of the PCR reaction product to detect the (*gene cassette*)of *E.coli*

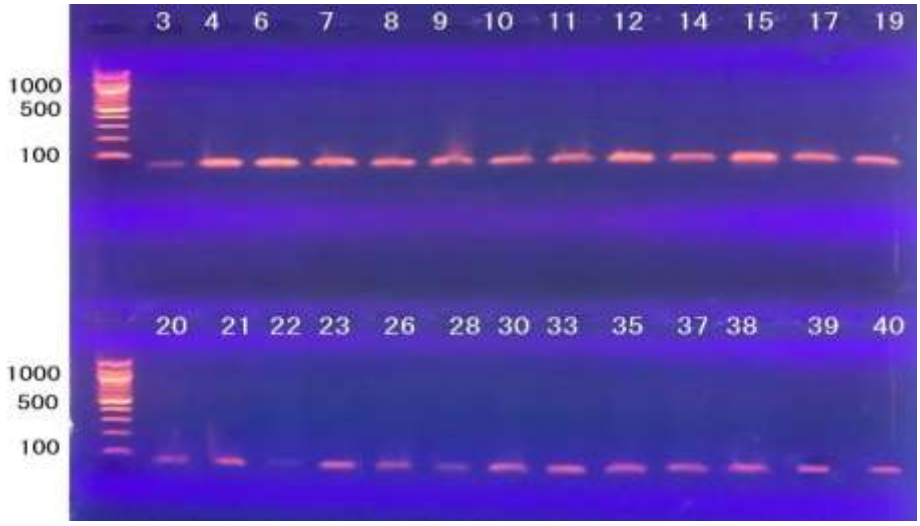

Figure 8. represents the electrophoresis of the PCR reaction product to detect the (*F12R–orf513-Ink13*) of *E.coli*

3.8 Determine the Sequences of the Integron, *qac*EΔ1, and *sul*1

The Integron gene sequence was analyzed by designing the structure and identifying the regions where the *qac*EΔ1 and *sul*1 genes are located within the Integron structure. Figure (9).

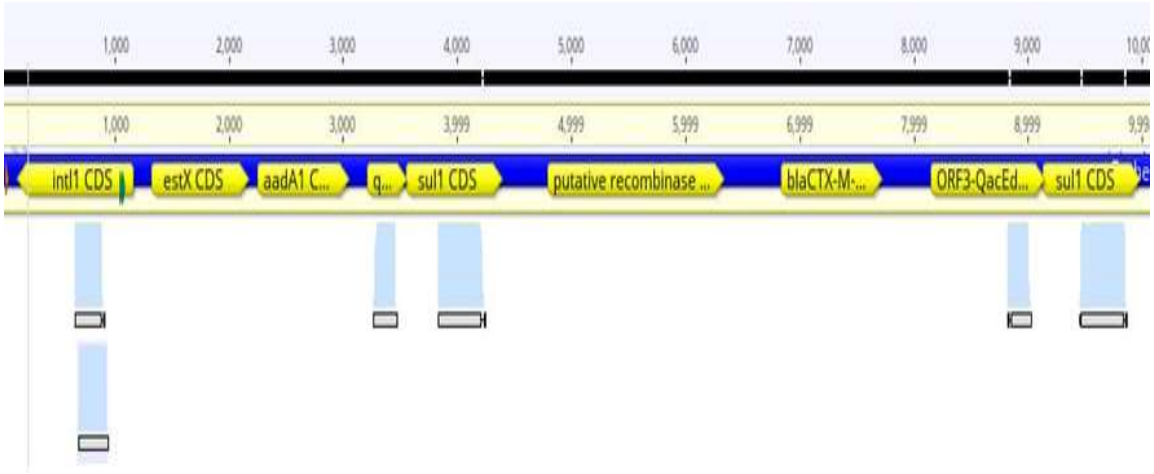

Figure 9. Exact location of the installation of the enlarged enquire that partially covers the region with the presence of *qac*EΔ1 and *sul*1 genes, respectively, in the genomic sequences of *E coli* bacteria with the global sequence of isolation (DQ 125241)

The analysis of Integron gene sequences involves studying the molecular structure and locating genes of interest, such as *qac*EΔ1 and sul1, which play a role in antibiotic resistance. An Integron is a genetic unit containing essential elements that regulate the conversion of genes between different states. A typical Integron structure includes the Integrase Gene (*int*I), which encodes the Integrase enzyme allowing genes to be inserted and removed from the Integron, the *att*C site where genes are inserted, and the Gene Cassette containing insertable genes, including resistance genes. *qac*EΔ1 encodes a protein that acts as an antagonist to microbicides such as chlorhexidine and QACs (Quaternary Ammonium Compounds) and often appears in Integrons as a substance resistant to these compounds. sul1 encodes the sulfonamide enzyme that resists sulfonic compounds by disrupting their effect. When analyzing Integron sequences and identifying gene locations, the DNA sequence must be analyzed using molecular biology tools such as advanced gene sequencing and biological data analysis. Sequence alignment programs like BLAST can identify gene locations within the full sequence. Once the gene locations are identified, it is possible to determine how the Integron structure affects the gene's function and its resistance.

This analysis is crucial for understanding how antibiotic resistance spreads and providing solutions to combat this phenomenon by targeting genes and their related mechanisms.

4. CONCLUSIONS

Escherichia coli is the most common in children with diarrhea. Molecular diagnosis using specialized primers is an accurate method for diagnosing these bacteria. Antibiotic use, local conditions, testing methods, specimen size, and genetic mutations can all affect the level of antibiotic resistance in bacteria. *E.coli* isolates contain Integrons Class I. Its composition includes the antibiotic resistance genes *qac*EΔ1 and *sul*1. It is used for detection by a specialized primer that targets the specific sequence for diagnosis. The presence of Integrons Class I in *Escherichia coli* indicates the spread of multiple types of resistance to different antibiotics.

Some bacteria may not be susceptible to strong antibiotic activity in their natural environment, so they may not require integrons carrying resistance genes. Instead, they may use different mechanisms to resist antibiotics, such as changing the target site, the permeability barrier or efflux pumps, and improving detoxification mechanisms. Bacteria can also acquire antibiotic resistance through genetic mutations or chemical modifications of the antibiotics.

5. RECOMMENDATIONS

Investigate the genes responsible for the spread of multiple antibiotic resistance in local bacterial isolates. Also, conduct a study on other causes of diarrhea in children from the Enterobacteriaceae family and other parasitic species. Other molecular techniques such as real-time PCR can be used to detect the expression of antibiotic-resistance genes in Enterobacteriaceae bacteria that cause diarrhea in children. Additionally, local genotype isolates of *Escherichia coli* were genotyped using other methods. Continuously monitor *Escherichia coli* isolates causing acute diarrhea in children to track the spread of multiple resistance, which poses a risk to public health.

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BIOGRAPHIES OF AUTHORS

	Dr. Ali Shakir Al-Ezee holds a PhD in microbiology and is a lecturer in the Department of biology, College of Education for Pure Sciences, University of Diyala. Email: ali.shakir@uodiyala.edu.iq
	Professor Dr. Hadi R. Rasheed Al-Taai , Department of biology; College of sciences; University of Diyala. Email : hadialtaai@uodiyala.edu.iq
	Ali Jaffar Saleem, PhD. Professor of Molecular Microbiology. Department of biology; College of education for pure sciences; University of Diyala. Email : alijaffar3@yahoo.com