Research Article

BASJVETRES

BASRAH JOURNAL OF VETERINARY RESEARCH, 2024, 23(4):181-195.
https://bjvr.uobasrah.edu.iq/

Integrons Detection in Antimicrobial resistance *Escherichia coli* Isolated from Clinical and Environmental Sources in Basrah Province

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DOI: https://doi.org/10.23975/bjvr.2024.154422.1149

Received: 1 October 2024 Accepted: 11 November 2024.

Abstract

The study's goal was to find out what kind of integrons and how many there were in anti-drug-resistant (AMR) Escherichia coli found in samples from people with clinical and subclinical mastitis and from the environment in Basrah province. The results revealed a high resistance to penicillin 23(100%), erythromycin 19(82.6%), tetracycline 21(91.3%), and gentamycin 12(52.17%). Also, they showed a significant association (P≤0.05) between antibiotic resistance and the *E. coli* that isolated from mastitis and subclinical mastitis cases. Furthermore, The results of integrons detection was revealed that 8(34.78%) of the total isolates contained IntI1 (491bp in size), 5((21.73%) contained IntI2, and 2 (8.69%) contained IntI3 (788 and 600bp) respectively. The research found a strong link (P ≤ 0.05) between two types of Escherichia coli (IntI1 and IntI2) and AMR Escherichia coli that were found in clinical and environmental samples. Based on the above findings, the development of several integrons linked to antimicrobial resistance in pathogenic bacteria present in dairy farms may complicate antibiotic treatments and endanger public health.

Keywords*:* Integrons, Mastitis, AMR *Escherichia coli*.

Introduction

Mastitis and subclinical mastitis (SCM) have a major economic impact and can infect healthy cows, posing challenges to achieving milk self-sufficiency (1-5). Conditions require immediate entrance and treatment. Antimicrobial therapy is the primary treatment for bacterial infections. However, the increase in AMR strains has reduced the efficacy of these drugs. *Escherichia coli* is an intestinal bacterium that is present in the environment. It may cause foodborne illness in humans and predisposes animals to intramammary infections. According to recent studies, *E.*

coli is a common clinical isolate, but emerging antibiotic resistance makes therapy hard (6). Unfortunately, antibiotic resistance in agricultural and farm animals is widespread in Iraq, as it is in many other countries. Furthermore, evidence has indicated that resistance genes may spread between bacterial populations, regardless of antibiotic usage patterns, and the development of AMR strains is currently believed to be primarily attributed to integrons, which are essential for the emergence and propagation of genes that confer resistance to antibiotics in this bacterial species (7). Numerous studies have reported and conducted the important role of integrons as mobile genetic components in the spreading of antibiotic resistance (8-10).

Integrons are the ancient structures that have mediated the development of bacteria through the acquisition, elaboration, disposal, and utilization of gene cassette reading frameworks (11). A family of genetic components known as an integron can catch gene cassettes. Based on the integrase intI gene sequence, integrons were categorized into three major classes (12). The most prevalent type of integrons, class 1 integrons, have been found in clinical isolates of Gram-negative bacteria on multiple occasions (13).In Enterobacterales, class 2 integrons are less numerous and diverse (14). They are connected to transposons that are members of the Tn7 family (15). Class 3 integrons are extremely uncommon and have only been found in a few species, such as *Serratia marcescens* (16). One of the main issues in health care is the AMR phenomenon. AMR in intestinal bacteria such as *E. coli* is known to be associated with integrons. The incidence of integrons in AMR *Escherichia* coli isolates worldwide has been the subject of several investigations. These investigations discovered a strong correlation between the existence of integrons and resistance to antibiotics (17). However, our region of study lacks sufficient data regarding the distribution of integrons and their correlation with AMR in isolates of *E. coli* that were mainly recovered from animals. So, the present study aims to identify the occurrence of three types of integrons in AMR *E. coli* that were detected from local cases of clinical, and subclinical mastitis and from environmental samples in Basrah, southern Iraq.

Materials and Methods

Sampling and Bacterial Isolation: Ninety samples were collected from various regions in Basrah province south of Iraq from September to December of 2023. The distribution of the samples was as follows: Milk specimens (30) from mastitis and (30) from subclinical cows. The final (30) samples were assembled from local water supply, desalination plants, animal stripes, and drainage water. In the clinical mastitis sample, the signs were observed on examined animals, including udder enlargement, anomalies, heat, hardness, redness, or soreness; the milk seemed watery and had clots or pus in it. Alternatively, in the case of subclinical mastitis, the milk samples are examined by (CMT) prior to collection. All milk samples were taken from the cows after washing the cows' udders to remove dirt, bole, and grime

and drying them with a clean cloth; then a piece of cotton that had been 70% alcohol-moistened was used for sterilization, and 10 ml of milk is collected in a sterile tube. The collected samples were then immediately transported under cold and strict sterilized conditions to the Central Laboratory of Microbiology and identify *E. coli* using a conventional approach (18).

All collected samples were inoculated into the Brain-Heart Infusion (BHI) broth and incubated for 24 hours at 37ºC. Subsequently, loopfuls of broth cultures were inoculated on MacConkey sorbitol agar MSA and MacConkey agar and incubated for 24 hours at 37ºC. The pink lactose fermenter colonies were picked and subcultured on Eosin methylene-blue agar (EMB), followed by incubation for 24 to 48 hours at 37°C. The colonies with a metallic sheen appearance proceeded to the Gram staining step; the VITEK 2 (bioMérieux) was used to confirm and identify the *E. coli.*

Extraction of DNA and PCR investigation: The bacterial DNA was directly extracted by using a boiling technique. Five probable bacteria colonies were collected from the heart infusion agar plate and placed in 1.5ml Eppendorf tubes with 500 µl of sterile distilled water. The organisms were then lysed by boiling the tubes at 100 degrees Celsius for 10 minutes. Centrifugation was employed to extract the DNA after heating for 15 minutes at 12,000 rpm. PCR was carried out for the molecular identification step, and an oligonucleotide primer specific to the *E. coli* species was utilized for detection (Table 1). For PCR, the reaction mixture was composed of 5µl of PCR PreMix from Bioneer, 5µl of DNA, and 1µl of each primer, and for adjusting the final volume, the nucleus-free water was added. The thermocycler program was included: An initial denaturation step at 94°C was accomplished in 4 mins, then proceeded with 30 cycles comprising of another denaturation step at 94°C for 45 seconds, then 60 seconds at 57°C for annealing, and at 72°C for 60 seconds as an elongation step. The PCR condition was processed with a final elongation was conducted for 10 mins at 72°C, and 1.5% agarose gel electrophoresis was used to determine the size of the target PCR product (21). The gel electrophoresis results were then compared with 100bp DNA a GeneRuler (Promega) under UV transilluminator.

PCR-Based Integron Detection: The positive DNA samples were used to detect the presence of integrons by PCR. The reaction mixture was composed of 5µl of PCR PreMix from Bioneer, 5µl of DNA, and 1µl of each primer, and for adjusting the final volume, the nucleus-free water was added. The thermocycler was programmed and included.An initial denaturation step at 94°C which accomplished in 4 mins, then proceeded with 30 cycles comprising of another denaturation step at 94°C for 45 seconds, then 30 seconds at 60°C for annealing to detect the *IntI*1, *IntI*2, and *IntI*3. The elongation stage lasted 1:30 minutes at 72°C and was followed by 10 minutes at the same temperature. The PCR amplicons were separated using electrophoresis on a 1.5% agarose gel.

Table 1: The oligonucleotide primers that were used throughout the study.

Primers	Nucleotides sequence (5'-3')	Size of amplicons (bp)	References
***Eco* 2083**	GCTTGACACTGAACATTGAG	662	19
***Eco* 2745**	GCACTTATCTCTTCCGCATT		
***IntI*1**	GGTCAAGGATCTGGATTT CG	491	
	ACATGCGTGTAAATCATC GTC		
***IntI*2**	CACGGATATGCGACAAAA AGG	788	20
	TGTAGCAAACGAGTGACGA AATG		
***IntI*3**	AGTGGGTGGCGA ATG AGTG	600	
	TGTTCTTGTATCGGC AGGTG		

Antimicrobial susceptibility assessment: The disk diffusion technique was used for the antibiotic susceptibility test results (22). The inoculum was made up by inoculating the normal saline solution with colonies of *E. coli* that overnight culture on an EMB agar plate. The suspension was vortexed and adjusted to a turbidity of 0.5 McFarland standard solution. Subsequently, a sterilizedd swab was dipped in adjusted suspension of bacteria and spread onto the Muller-Hinton Agarbacteria suspension plate and spread onto the Muller-Hinton Agar plate. The antibiotic selection discs were subsequently affixed to the petri dish's surface and then incubated at 37°C for 20hr. The antimicrobial susceptibility result was assessed by measuring the inhibition zone's diameter using a ruler. The susceptible susceptibility and resistance results were categorized based on the authorized criteria (23).

Bio-statistical analysis: The SPSS (version 23.0) was used to analyze the collected data statistically. Frequency data were compared using the T-test and Chi-square ($\chi 2$) test. P-values less than 0.05 were regarded as statistically significant.

Results

***Escherichia coli* Isolation and Identification**: The lactose fermenter *E. coli* colonies on MacConkey sorbitol agar were bright pink with a red halo appearance (Figure 1). Of the 90 collected samples, 27(30%) were *E. coli* presumable colonies, with a distinctive shiny metallic appearance on EMB agar (Table 2 and Figure 1). Furthermore, the Gram-stained step was performed on all the suspicious colonies, and the smear that was noticed on all the suspicious colonies had a characteristic of red short rod *E. coli* cells. Additionally, the PCR technique was used to confirm the identification, and the results revealed that 23(25.55%) isolates were positive for *E. coli* (Table 2). In this table, the variation in *E. coli* incidence in clinical and subclinical mastitis besides the environmental samples was statistically considerable ($p=<0.001$). Furthermore, distinct bands with 662bp size were observed on the agarose gel following electrophoresis, as illustrated in Figure 2.

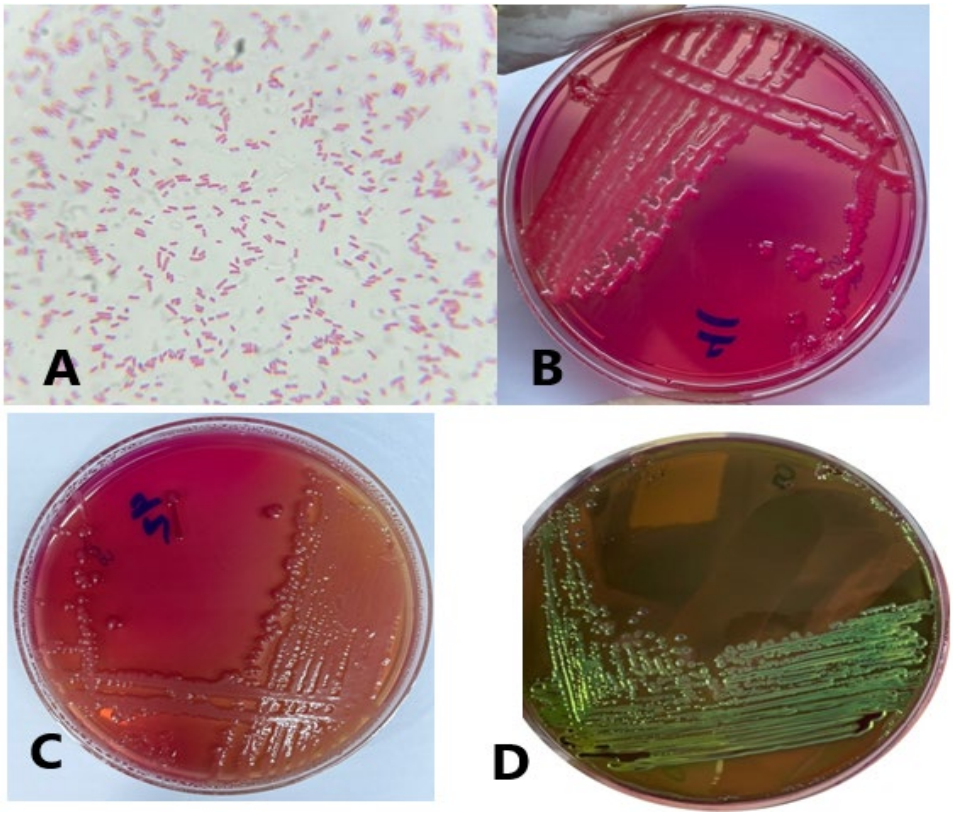

Figure 1: Primary Identification of *E. coli.* A: gram stain *E. coli* under light microscope (1000X). B: Red-pink colonies of *E. coli* on MacConkey agar. C: *E. coli* colonies on SMAC that were thought to be lactose fermenters were brilliant pink colonies with a crimson halo. D: colonies of *E. coli* on EMB seemed to have darkness center large shining with a characteristic a metallic greenish appearance.

Table 2: Isolation of *E. coli* from different sources based on culture media, VITEK 2 System, and PCR.

Source of samples	Examined Samples no= 60	Culturing no+ (%)	VITEK 2 no+ (%)	PCR no+ (%)
Clinical mastitis	30	11(36.66%)	10(33.33%)	10(33.33%)
Subclinical mastitis	30	9(30%)	8(26.66%	8(26.66%)
Environment [1]	30	7(23.33%)	5(16.66%)	5(16.66%)
Total	90	27(30%)	23(25.55%)	23(25.55%)
P value[2]		**<0.001**	**<0.001**	**<0.001**

[1] **Environment samples were collected from local water resources, animal stripes, drainage water, and wastewater desalination plants.[2]Indicate the presence of the significance at (P≤0.05).**

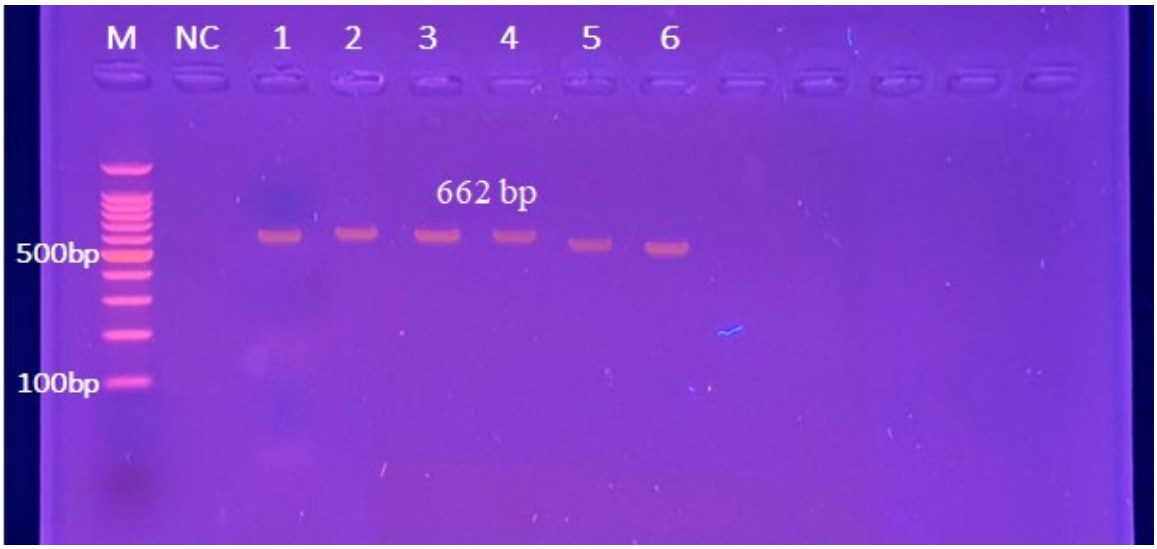

Figure 2 : Predictable PCR amplification results of species- specific gene (662 bp) for *E. coli* isolate. M: molecular-marker (100bp), NC: a negative control sample. Lanes 1-6: positive samples.

Antimicrobial susceptibility assessment

Table 3 and Figure 3 clarify the resistance frequency rate of each antibiotic used in the study. This table is revealed a high resistance to penicillin 23(100%) erythromycin 19(82.6%), tetracycline 21(91.3%) and gentamycin 12(52.17%). Also, the results have shown a significant association (P≤0.05) between antibiotic resistance and the *E. coli* isolated from mastitis and subclinical mastitis cases. In contrast, non-significant (P=0.488) occurrence between the susceptibility to antibiotics and isolates from environmental samples. Additionally, the results of antibiotic susceptibility based on inhibition zone were revealed in Figure 4.

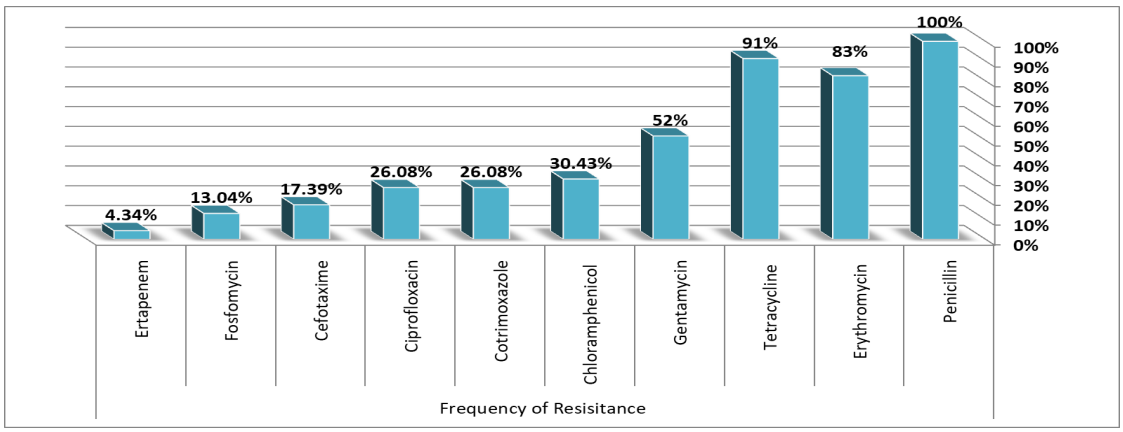

Figure 3: The resistance frequency rate of each antibiotic used in the study against the *E. coli* isolates

Table 3: Resistance of *E. coli* isolates to different antibiotics used in the study.

Antibiotics	Mastitis no=10	Subclinical mastitis No=8	Others No=5	Total No=23
Penicillin	10(100%)	8(100%)	5(100%)	23(100%)
Erythromycin	10(100%)	6(75%)	3(60%)	19(82.6%)
Tetracycline	9(90%)	8(100%)	4(80%)	21(91.3%)
Gentamycin	4(40%)	6(75%)	2(40%)	12(52.17%)
Chloramphenicol	4(40%)	1(12.5%)	2(40%)	7(30.43%)
Cotrimoxazole	4(40%)	1(12.5%)	1(20%)	6(26.08%)
Cefotaxime	4(40%)	1(12.5%)	1(20%)	6(26.08%)
Ciprofloxacin	2(20%)	2(25%)	0(0.0%)	4(17.39%)
Fosfomycin	2(20%)	0(0.0%)	1(20%)	3(13.04%)
Ertapenem	0(0.0%)	0(0.0%)	1(20%)	1(4.34%)
P value	**0.045**	**0.017**	**0.488 ns[3]**	**<0.001**

[1]Other samples were collected from a local water supply, desalination plants, animal stripes, and drainage water.[2]Indicate significant presence at (P≤0.05). [3]ns: indicate non-significant.

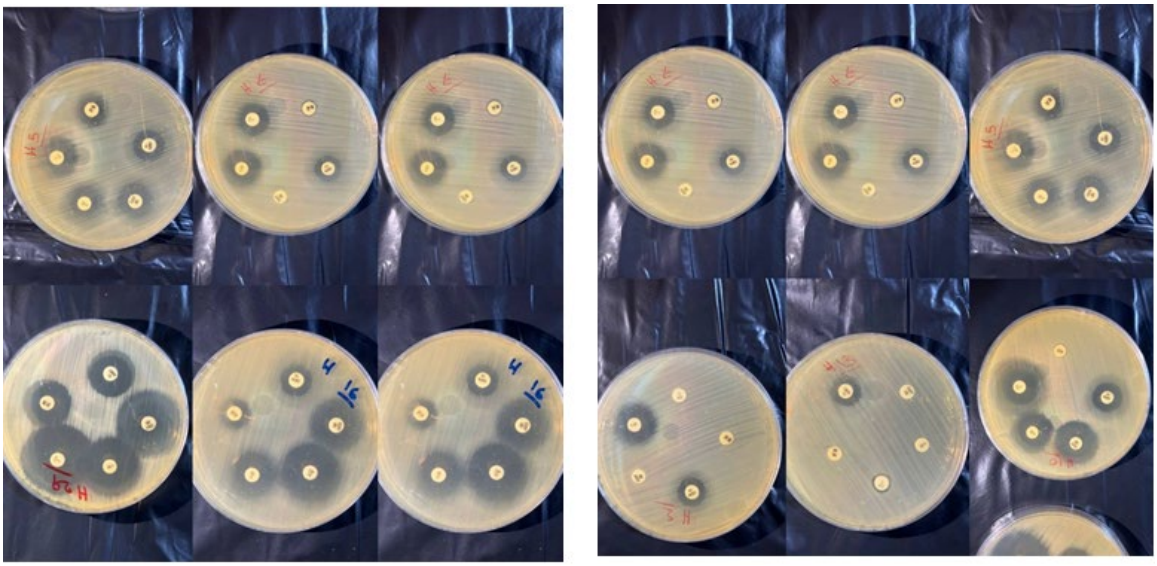

Figure 4: The antimicrobial susceptibility result of local *E. coli* isolates

PCR-Based Integron Detection According to Table 4 results, there is a significant difference (P≤0.05) in the distribution of *intI*1 and *intI*2 across the different sample sources. Additionally, the tables also revealed that 8 isolates (34.78%) possess *intI* 1, while 5 isolates (21.73%) have *intI* 2 (788bp), and only 2 isolates (8.69%) contain *intI* 3. Furthermore, the expected PCR bands for *intI*1, *intI*2, and *intI* 3 have an average size of 491bp, 788bp, and 600bp, respectively (Figures 5,6, and 7).

Table 4: Percentage of *Int1*, *Int2,* and *Int3* in AMR *E. coli* isolates by PCR.

Source of samples	No. of AMR *E.coli*	*IntI*1 no(%)	*IntI*2 no(%)	*IntI*3 no(%)
Clinical mastitis	10	4 (40%)	3 (30%)	1 (10%)
Subclinical mastitis	8	2(25%)	1 (12.5%)	1 (12.5%)
Other*	5	2(40%)	1(20%)	0
Total	23	8(34.78%)	5(21.73%)	2(8.69%)
P value		**<0.001**	**<0.001**	**-**

[1]**Other samples were collected from a local water supply, desalination plants, animal stripes and drainage water.**[2]**Indicate significant presence at (P≤0.05).**

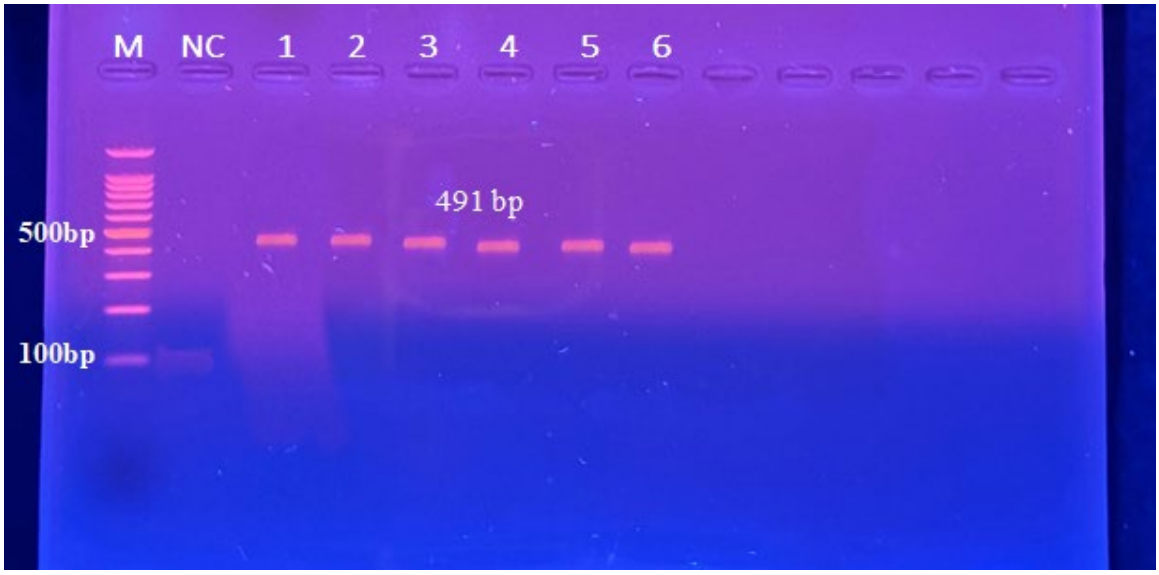

Figure 5: PCR amplification of the *IntI*1 gene (491 bp) in an *E.coli* isolates. M: a molecular marker (100 bp); NC: a negative control sample. Lanes 1-6 contain positive samples.

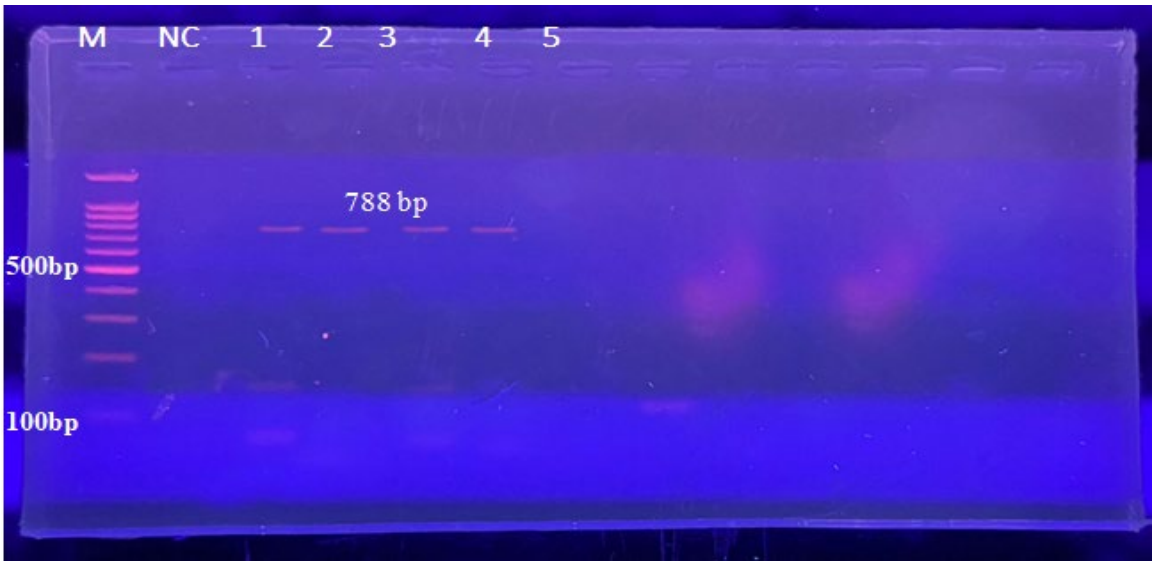

Figure 6: PCR amplification of the *IntI* 2 gene (788 bp) in an *E.coli* isolates. M: a molecular marker (100bp), NC: a negative control sample . Lanes 1-4: positive samples.

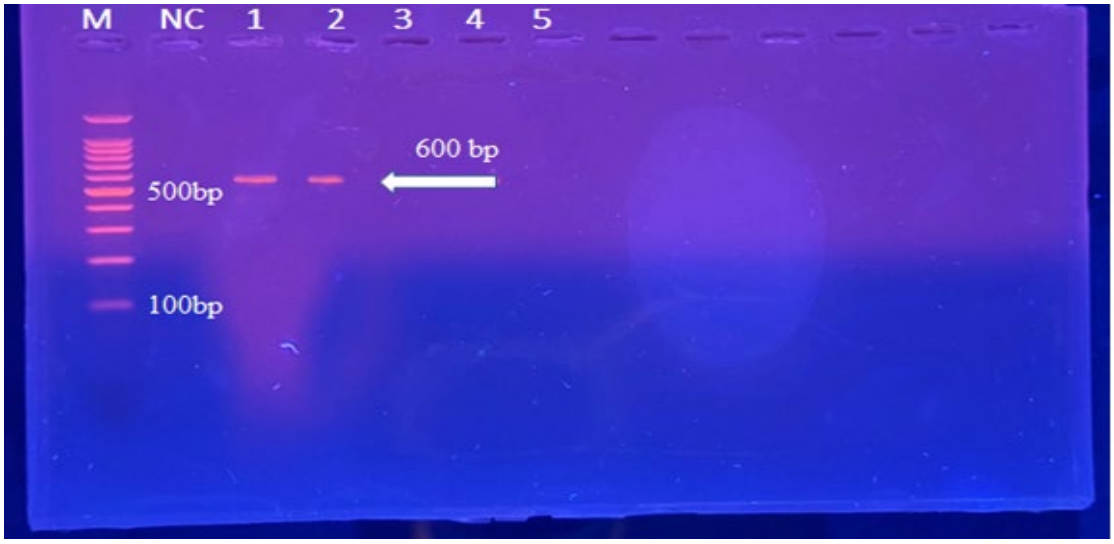

Figure 7: PCR amplification of the *IntI* 3 gene (600 bp) in an *E. coli* isolates. M: a molecular marker (100bp), NC: a negative control sample, Lanes 1-2: positive samples.

Discussion

Clinical and subclinical mastitis, one of the most common infections in cows, poses a significant economic concern in the global dairy trades. The routine screening for antimicrobial resistance (AMR) *E. coli* isolates originating from farms of dairy animals is essential for both animal husbandry practices and public welfare considerations. (24). In the present study, 23(25.55%) of the local isolates of *E. coli* were screened for antibiotic resistance phenotypes and their genetic integrons organization. The antimicrobial activity results showed 100% resistance to penicillin against all tested isolates with high significant resistance (p=0.001) to erythromycin, tetracycline, and gentamycin. These data provide another remarkable example of how antibiotic resistance spreads in bacterial populations. (25), for example, in their study, they indicated a substantial resistance of 100% to ampicillin, amoxicillin, tetracycline, and ceftazidime, except for ciprofloxacin, which had 100% sensitivity in isolates of *E. coli* from cows with subclinical mastitis in Bangladesh. Consistently, Bag and his colleagues (26), discovered positive associations in 2021 between isolates of *E. coli* that recovered from mastitis cases and resistance to tetracycline, amoxicillin, ampicillin, amikacin, and gentamicin. In addition, My et al., (27) recently revealed that AMR *E. coli* gathered from mastitis clinic samples had substantial antibacterial properties against ampicillin, amoxicillin, and tetracycline. In general, the percentage of rats of AMR isolates from clinical mastitis samples was previously reported to be 79.5%, 84.2%, and 38.4% in Jordan, Iran, and China, respectively (28, 29, and 30). Similarly, in the present study, we also reported negligible antimicrobial susceptibility results to ciprofloxacin, ertapenem and Fosfomycin, and this is might be due to their rare use in cattle.

Moreover, our results have shown a significant association ($P \leq 0.05$) between antibiotic resistance and *E. coli* isolated from mastitis and subclinical mastitis cases however non-significant (P=0.488) occurrence between the susceptibility to antibiotics and the isolates of *E. coli* that recovered from environmental samples. These findings with (31), who stated the significant existence of AMR *E. coli* in the environment and their connection with subclinical mastitis cases. Furthermore, the non-significant occurrence among antibiotics susceptibility and *E. coli* isolates obtained from drainage water samples, animal stripes, desalination plants, and local water supplies could be attributed to the low number of *E. coli* that recovered from these samples, and thus the chance of finding a particular susceptibility is irrelevant. On the other hand, all AMR *E. coli* isolates were screened for integron types and frequency, 8(34.78%) of these isolates were found to have *intI*1. Additionally, 5(21.73%) and 2(8.69%) of isolates harbor *intI* 2 and *intI* 3, respectively.

The correlation investigation showed that the frequency of integrons in these isolates was strongly related to the AMR *E. coli* isolated from mastitis and subclinical mastitis (<0.001). This finding was similar to a study by (32), who reported

*intI*1 in 30 (83.33%) of *β*-lactams resistance isolates of *E. coli* that recovered from bovine mastitis. Additionally, Wang *et al*., (33) analyzed integron gene cassettes in multidrug-resistant *E. coli* strains isolated from bovine mastitis. The study found no evidence of class II or class III integrons; however, 33 isolates, representing 56.90%, tested positive for the *intI*1 gene. Furthermore, correlation analysis indicated a strong link between the presence of integrons in these isolates and their resistance profiles, suggesting that integrons significantly contribute to the dissemination and spreading of antimicrobial-resistant strains (33). A study conducted in Tunisia by (34) found 7 out of 14 E. coli strains exhibiting antimicrobial resistance, isolated from cases of bovine mastitis. However, class 2 integrons were absent in this particular collection. Alternatively, in our study, no integron belonging to *intI*3 was observed in *E. coli* isolated from local water supply, desalination plants, animal stripes, and drainage water, and thus the result is consistent with the findings (35). However, these variations might be attributed to the restricted number of samples collected from the environment. As a result, future research may require more samples to study these discrepancies. Finally, it is challenging to understand the relationship between antimicrobial resistance bacteria and specific integron gene cassettes because of the complicated phylogenetic background of the bacteria, which is influenced by several variables including the bacterial species, host, environmental niche, virulence, and resistance mechanisms.

Conclusion

Based on the above findings, the development of several integrons linked to antimicrobial resistance in pathogenic bacteria present in dairy farms may complicate antibiotic treatments and endanger public health.

Acknowledgment

The authors are grateful for the laboratory facilities offered by the College of Veterinary Medicine at the University of Basrah, Iraq.

Conflicts of interest

The authors declare that there is no conflict of interest.

Ethical Clearance

This work is approved by The Research Ethical Committee.

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الكشف عن المدمجات في بكتريا الإشريكية القولونية المقاومة للمضادات الحيوية المعزولة من مصادر سريرية وبيئية في محافظة البصرة

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الخلاصة

هدفت الدراسة إلى تمييز نوع وعدد المدمجات الموجودة في الإشريكية القولونية المضادة للأدوية (AMR) والتي تم عزلها من العينات السريرية والتحت السريرية لالتهاب الضرع ومن العينات البيئية في محافظة البصرة. حيث كشفت النتائج عن مقاومة عالية للبنسلين 23 (100٪), إريثروميسين 19 (82.6٪) ، التتراسيكلين 21 (91.3٪) والجنتاميسين 12 (52.17٪) وكما أظهرت النتائج ارتباطا معنويا ($P\leq0.05$) بين مقاومة المضادات الحيوية والإشريكية القولونية المعزولة من التهاب الضرع وحالات التهاب الضرع تحت الإكلينيكي .كما كشفت نتائج فحص المدمجات أن 8(34.78٪) من إجمالي العزلات تحتوي على المدمج الاول (حجم 491زوج قاعدي) ، و 5 (21.73٪) تحتوي على المدمج الثاني و 2 (8.69٪) تحتوي على المدمج الثالث بحجم(788 و 600زوج قاعدي) على التوالي. وبينت الدراسة وجود ارتباط معنوي ($P\leq0.05$) بين (المدمج الاول والمدمج الثاني) وجراثيم الإشريكية القولونية المضادة للأدوية (AMR)المعزولة من العينات السريرية والبيئية. وعلية يمكن الاستنتاج بان تطور العديد من المدمجات الوراثية المرتبطة بمقاومة مضادات الميكروبات في البكتيريا المسببة للأمراض الموجودة في مزارع الألبان قد يعقد العلاج بالمضادات الحيوية ويعرض الصحة العامة للخطر.

الكلمات المفتاحية: المدمجات الوراثية ,التهاب الضرع ,الاشريكية القولونية المقاومة للمضادات الحيوية.