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Distribution of Macrolides Resistant Genes among Local Clinical Isolates of *Enterococcus* spp. from Root Canal and Urine Samples

Nabaa Kamil Molan[1*], Alyaa Razooqi Hussein[2]
[1,2]Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq.
Corresponding author *

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Abstract

Gram-positive enterococci are opportunistic and resistant to many antibiotics. This search investigated the prevalence of macrolide antibiotic resistance genes in local enterococcal isolates and its correlation with biofilm formation. We collected 112 clinical samples from the Medical City Hospital, dentists' clinics, and labs in Baghdad from October 2022 to March 2023, which included root canal samples from 50 patients and urine samples from 62 patients with urinary tract infections. The samples were cultured on Pfizer-specific *Enterococcus* media. Twenty-one isolates were identified as *Enterococcus* spp. by biochemical tests and confirmed using the VITEK 2 system. After that, the crystal violet staining method was used to assess enterococci isolates' ability to form biofilms in a polystyrene microtiter, and then molecular detection was done to detect the *ermB* gene. The results revealed that the percentage of enterococcal isolates positive for the *ermB* gene was 87.5% in root canal samples. In urine samples, the percentage of enterococcal isolates with the same gene was 84.6%. All isolates succeeded in forming biofilm; for urine isolates, 77% and 23% of isolates formed moderate and strong biofilm, respectively. While for root canal isolates, 12.5%, 75%, and 12.5% of isolates formed weak, moderate, and strong biofilm, respectively. We conclude in this study that *the ermB* gene was detected in enterococcal isolates from the tooth root canal and urine samples, with a higher prevalence percentage in urine sample isolates than tooth root canal isolates. Finally, the findings demonstrated that there is no connection between this gene's existence and the tested isolates' ability to create biofilms.

Keywords: *Enterococcus* spp., *ermB* gene, macrolide antibiotics, urine samples, root canal samples.

1.Introduction

Enterococci are gram-positive, facultative anaerobic bacteria that can be opportunistic when naturally occurring in the female genital tract and the human intestinal tract. The environment, including water and soil, abundantly harbors enterococci (1). The most common enterococcal species are *E. faecium* and *E. faecalis*, which are responsible for most of the nosocomial infections causing serious conditions such as endocarditis and septicemia (2). Bacterial infections have a severe problem due to the significant effects they have in medical settings and public. The gastrointestinal system, urinary tract, respiratory tract, soft tissue, and skin are the locations of most infections (3). Enterococci are major nosocomial microorganisms that are resistant to a variety of antimicrobial treatments through acquired and intrinsic mechanisms. Inherent resistance is another name for intrinsic resistance.

Chromosome characteristics, naturally encoded in all or some strains of the *Enterococcus* species, trigger this resistance. Specific *Enterococcus* species or groups frequently link intrinsic resistance mechanisms to various antimicrobials, unlike acquired resistance, which is substantially more varied (4). Several popular antibacterial medications are inherently resistant to enterococci. All enterococci exhibit decreased sensitivity to penicillin and ampicillin as well as considerable levels of resistance to the majority of cephalosporins and all semi-synthetic penicillins as a result of the development of low-affinity penicillin-binding proteins. The prevalence of ampicillin resistance in many bacteria does not exclude the therapeutic use of this drug. In reality, ampicillin is still the go-to medication for enterococcal infections that don't exhibit other forms of high-level resistance (5). DNA mutations and the creation of new genetic material are two factors that contribute to enterococci's acquired resistance. Examples of medicines with this resistance include ampicillin, tetracycline, macrolides, aminoglycosides (high levels), chloramphenicol, quinolones, glycopeptides, streptogramins, and even some more contemporary medications like linezolid and daptomycin (6). Without a doubt, the development of resistant bacteria is a result of the widespread usage of these antibiotics [7]. One of the most common resistance mechanisms is 23S rRNA methylation expressed by *erm* genes, which prevent macrolide from binding to ribosomes (8). Efflux pump genes (*msrA, msrC, mefA,* and *mefE*) are engaged in additional mechanisms that remove macrolide-resistant antibiotic compounds from within the bacterium (9). A family of medications known as macrolides is used to control and treat different bacterial infections. Commonly recommended antibiotics for infections, including tonsillitis, sinusitis, and pneumonia, include azithromycin, clarithromycin, and erythromycin (10). *Erm* genes are the more common macrolide resistance determinants. This code is for a single methyltransferase that reacts with certain 23S rRNA subunit residues. This enzyme inhibits erythromycin binding by N6-dimethylating an adenine residue in the 23S rRNA subunit. (11). The presence of erythromycin resistance methylase (*erm*) genes, such as *ermA, ermB,* and *ermC,* is linked to erythromycin resistance in enterococci. The *ermB* gene, which encodes the ribosomal methylase, is the most common *erm* gene among isolates of enterococci that are resistant to erythromycin (12).

Biofilms play a crucial role in the survival of enterococci by their adaptability to a variety of environments and their ease in acquiring mobile genetic elements, such as plasmids, from other bacteria, because of this genetic material exchange (13). The process of biofilm formation involves the aggregation of microbial cells into collectives that adhere to both biological and non-biological surfaces. These collectives are embedded in a self-produced extracellular matrix made of microbial biopolymers like proteins, exopolysaccharides, and extracellular DNA, which creates a unique microenvironment (14). According to (15), biofilm development shields the microbial population from environmental stress. Additionally, the development of biofilms makes it easier for community members to engage and capture resources. As a result, bacteria that form biofilms are physiologically different from bacteria that are free to move about.

The current study aimed to investigate the prevalence of macrolide antibiotic resistance genes in local enterococcal isolates and its correlation with biofilm formation.

2. Materials and Methods

2.1. Bacterial isolation and identification

To learn more about the distribution of macrolide-resistant genes in local isolates of *Enterococcus* spp., urine and root canal samples were gathered from the Medical City

Hospital, dentists' clinics, and labs in Baghdad between October 2022 and March 2023. For root canal samples, sterile paper points and files with infected root canals of 50 patients were collected, while urine samples from 62 individuals with urinary tract infections were centrifuged, supernatant removed, and Pfizer-specific *Enterococcus* media was used for inoculation, and samples were incubated at 37°C for 24 hours. (16). All isolate identifications were done by biochemical tests, including the catalase test, growth in medium containing 6.5% NaCl, and growth at pH 9.6, and confirmed by the Vitek 2 system (17, 18).

2.2.Molecular detection of macrolides resistance gene (*ermB*).

Following the manufacturer's instructions, a DNA genome microextraction kit from Norgen (Canada) was used to extract DNA from purified colonies of bacteria. The amount of the collected DNA was then calculated with Qubit 4 to evaluate the sample quality for more usage. Using the PCR approach, genotyping the *ermB* gene is accurately followed the following:

2.2.1.Selection of primers

The primer listed in **Table 1** was employed for this investigation.

Table 1. The primer and its sequence used in conventional PCR

Gene	Sequence 5′→3′	Size bp	by Reference
ermB	F: GAAATTGGAACAGGTAAAGGG R: CGTTTACTTTGGCGTGTTT	562	Newly designed by Nabu Scientific Foundation

2.2.2. PCR Amplification

At 4°C, the primers, the PCR premix, and extracted DNA were defrosted. In order to ensure that the contents reached the bottom of their tubes, they were also momentarily vortexed. A 25 µl PCR mixture was created by combining 5 µl of PCR premix, 1 µl of each primer (forward and reverse), 3 µl of DNA template, and 15 µl of clean deionized distilled water (19). After quickly mixing the PCR reaction tubes, the DNA was amplified using the thermocycler PCR instrument in line with the PCR protocol **(Table 2).**

Table 2. Program PCR amplification of *ermB* gene

Stage	Temperature °C	Time	
Initial denaturation	94	4 min	
Denaturation	94	40 sec	
Annealing	48	40 sec	30 Cycle
Extension	72	40 sec	
Final Extension	72	5 min	

2.2.3. Agarose Gel Electrophoresis

DNA was identified by electrophoresing a 2% agarose gel for 50 minutes at 75 volts and staining it with RedSafe dye. Additionally, an ultraviolet transilluminator was used to image the agarose gel (20).

2.3. Biofilm formation

The crystal violet staining method was used to assess enterococci isolates' ability to form biofilms in a polystyrene microtiter plate. We assessed the optical density (OD) using a wavelength of 630 nm. After incubation, 200 µl of each bacterial isolate suspension was added to a 96-well plate. We fixed the biofilm by heating it to 60°C and then added a 0.1% w/v crystal violet solution. The biomass of the biofilm exhibited an inverse correlation with the absorbance at 630 nm. **Table 3** provided the calculations for the outcomes (21).

Table 3. Calculation of biofilm formation by Enterococcal isolates

OD value	Biofilm formation
<OD	Non
ODc < ODt < 2ODc	Weak
2ODc < ODt < 4ODc	Moderate
4ODc < ODt	High

3. Results

3.1. Isolation and Identification of *Enterococcus* spp.

Samples were collected with the goal of employing the Gram stain technique to isolate and identify *Enterococcus* spp. We arranged gram-positive cocci on Pfizer *Enterococcus* Selective medium into 2 mm in diameter, grey, spherical colonies with black borders and tips **(Figure 1).** It was obtained twenty-one (21) isolates, selected for further investigation such as molecular technique and biofilm production ability, that have the ability to resist one or more of the macrolide group of antibiotics (unpublished data).

Figure 1. *Enterococcus* spp. on Pfizer Selective medium

3.2. Biofilm formation

Biofilm formation capacity was assessed in *Enterococcus* isolates using 96-well microtiter plates, showing varying capabilities.

3.2.1. Root canal *Enterococcus* spp. biofilm production

Eight isolates from root canal samples that showed high antibiotic resistance were chosen for the detection of biofilm formation. About 12.5% of the tested isolates were weak producers of biofilm, while 75% were moderate, and 12.5% were strong biofilm producers, as shown in **Figure 2** and **Table 4.**

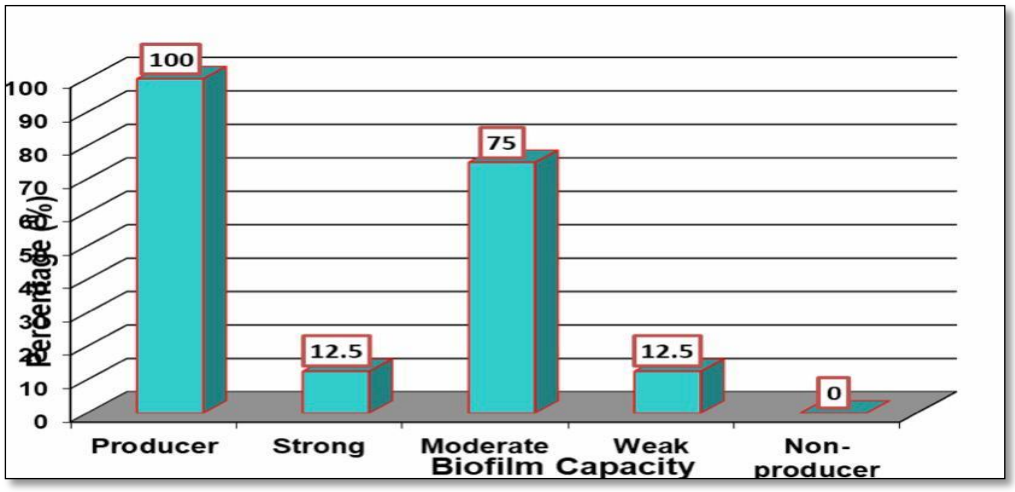

Figure 2. Distribution of *Enterococcus* spp. among weak, moderate and strong groups of biofilm production from root canal isolates

Table 4. Biofilm Capacity for Root canal isolates

Biofilm Capacity for root canal isolates	MTP method n=8 (%)
Producer	8 (100%)
Strong	1(12.5%)
Moderate	6 (75.0%)
Weak	1 (12.5%)
Non-producer	0 (0%)
Chi-Square (χ^2)	12.294 **
P-value	0.0098

** (P≤0.01).

3.2.2. Urine *Enterococcus* spp. biofilm production

Thirteen isolates from urine samples that showed high antibiotic resistance were chosen for the detection of biofilm formation. About 23% of the tested isolates were strong biofilm producers, while 77% were moderately biofilm producers, as shown in figure **(3)** and table **(5).**

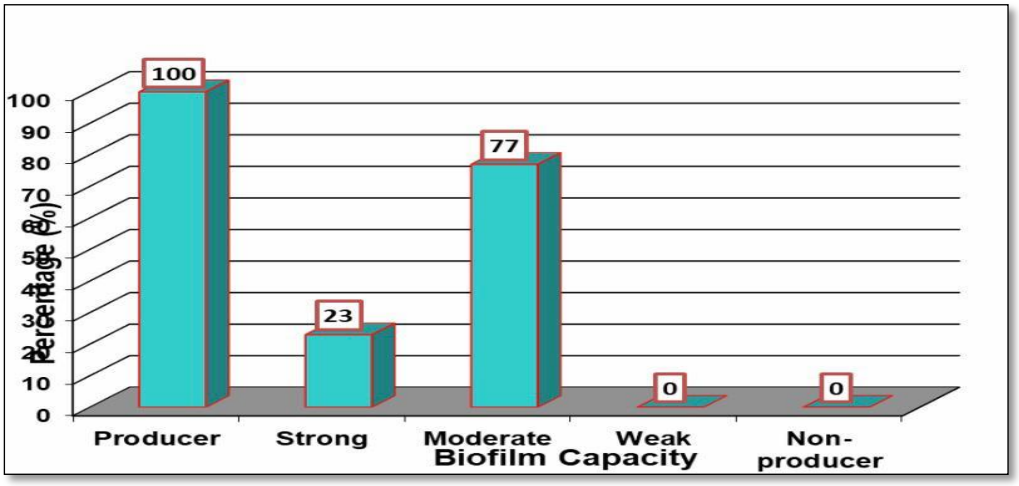

Figure 3. Distribution of *Enterococcus* spp. among weak, moderate and strong groups of biofilm production from urine isolates

Table 5. Biofilm Capacity for Urine isolates

Biofilm Capacity for urine isolates	MTP method n=13(%)
Producer	13 (100%)
Strong	3 (23%)
Moderate	10 (77.0%)
Weak	0 (0%)
Non-producer	0 (0%)
Chi-Square (χ^2)	23.271 **
P-value	0.0001

** (P≤0.01).

3.3. Molecular detection

The results of PCR showed that 18 (85.7%) out of 21 isolates from urine and root canal samples had the *ermB* gene. The positive result of *the ermB* gene was confirmed by 2% agarose gel electrophoresis stained with RedSafe, electrophoresed at 75 volts for 50 minutes. And photographed under an ultraviolet (UV) transilluminator **(Figure 4)**.

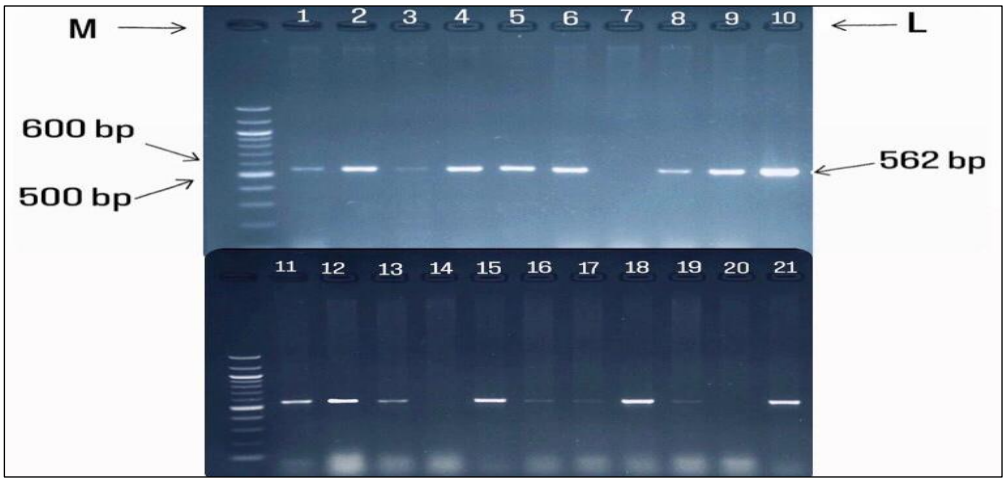

Figure 4. RedSafe-stained PCR amplified products for the *ermB* gene were electrophoresed on an agarose gel at 2% (75 volt/cm) for 50 minutes. Lane M: l500bp Ladder marker. Lane L: isolate NO. with positive bands of 562bp of *ermB* gene as follow:1, 2, 3, 4, 5, 6, 8, 9, 10, 11, 12, 13, 15, 16, 17, 18, 19, 21.

3.3.1 Urine samples

The results of PCR showed that 11 (84.6%) isolates had the *ermB* gene from 13 enterococcal isolates from urine samples **(Table 6).**

Table 6. Percentage distribution of *ermB* gene among *enterococcal* isolates from urine samples

Gene	Positive No. (%)	Negative No. (%)	Total (%)
ermB	11 (84.6)	2 (15.3)	13 (100)
Chi-Square -χ^2	4.865 *		---
(P-value)	(0.0437)		
		* (P≤0.05).	

3.3.2. Root canal samples

The results of PCR showed that seven (87.5%) isolates had the *ermB* gene from eight enterococcal isolates of root canal samples **(Table 7).**

Table 7. Percentage distribution of *ermB* gene among *enterococcal* isolates from root canal samples

Gene	Positive No. (%)	Negative No. (%)	Total (%)
ermB	7 (87.5)	1 (12.5)	8 (100)
Chi-Square - χ^2	4.138 *		----
(P-value)	(0.0497)		
		* (P≤0.05).	

3.4. Correlation between antibiotics resistance and biofilm formation in m Enterococcal clinical isolates

The correlation between *ermB* gene with the category of biofilm formation was investigated; the findings demonstrated that there is no connection between this gene's existence and the tested isolates' ability to create biofilms as shown in **Tables 8, 9** and **10**. The results presented in **Figure 5.**

Table 8. Gene type Biofilm formation Cross-tabulation

			Biofilm formation			Total
			Weak	Moderate	Strong	
ErmB gene	Positive	Count	1	13	4	18
		% of Total	4.8%	61.9%	19.0%	85.7%
	Negative	Count	0	3	0	3
		% of Total	0.0%	14.3%	0.0%	14.3%
	Total	Count	1	16	4	21
		% of Total	4.8%	76.2%	19.0%	100.0%

Table 9. Chi-Square Tests

	Value	df	Asymptotic Significance (2-sided)
Pearson Chi-Square	1.094[a]	2	.579
Likelihood Ratio	1.782	2	.410
Linear-by-Linear Association	.313	1	.576
N of Valid Cases	21		

a. 5 cells (83.3%) have expected count less than 5. The minimum expected count is .14.

Table 10. Correlations between gene type and biofilm formation

Correlations			***ErmB* gene**	**Biofilm formation**
Spearman's rho	*ErmB* gene	Correlation Coefficient	1.000	-.136
		Sig. (2-tailed)	.	.556
		N	21	21
	Biofilm formation	Correlation Coefficient	-.136	1.000
		Sig. (2-tailed)	.556	.
		N	21	21

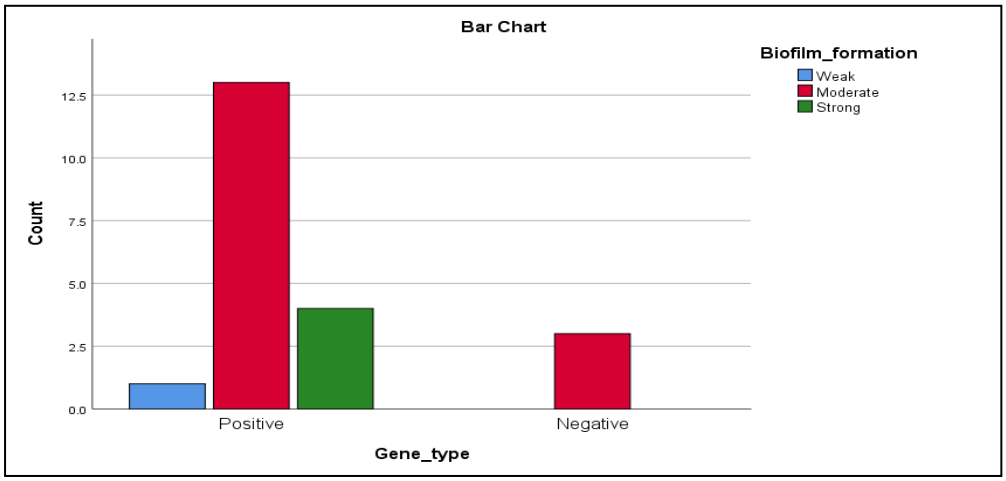

Figure 5. Correlations between *ermB* gene and biofilm formation

4.Discussion

The obtained isolates and results correspond with previous local findings by (22), who also reported macrolide resistance in a significant proportion of *Enterococcus* isolates.

In research by (23), 49% of the isolates were shown to be strong biofilm producers, 42% showed moderate biofilm development, and 9% showed weak or no biofilm, which is different from the results of the current study, while local research by (24) and (25) showed that all isolates were biofilm producers. The local study by Salih revealed that about 23.5% of the tested isolates were weak producers of biofilm, while 47% were moderate and 29.5% were strong biofilm producers. The research makes clear the significance of biofilm growth in bacterial pathogenicity. With the help of biofilms, bacteria may colonize during an illness and develop drug resistance. A well-hydrated matrix consisting of proteins, exopolymeric substances, and nucleic acids released by dead, lysed cells shields biofilm-forming bacteria from immune clearance and antibiotic treatment (26). Both tissue epithelia and body implant devices are susceptible to bacterial biofilm formation. Hospitals may employ more antibiotics and implant devices, which may enhance clinical pathogens with characteristics that encourage the production of biofilms (27).

It was conducted an investigation into the ability of *Enterococcus* spp. from urine samples to form biofilm (28). Out of 22 isolates, the study discovered that 0 (0%), 17 (77.3%), 3

(13.6%), and 2 (9.1%) were classified as high, medium, weak, and non-biofilm formers, respectively. Therefore, this result stands in contrast to the findings of the current study. Another study categorized 24 (32%), 33 (44%), and 18 (24%), respectively, in 75 samples as weakly, moderately, and highly producing biofilms (29). The majority of these moderate values align with the results of the current investigation. Moreover, a study by (30) revealed that 4 strains had no biofilm formation, 17 had weak formation, and 4 had moderate formation.

When compared to the DNA ladder displayed in **Figure 4,** the results of the current investigation revealed that the *ermB* gene band was found at a 562 bp area. The bands appeared distinct, single, and not diffused, with no smear indicative of DNA breakdown.

A global study done by (31) demonstrated that (80.5%) of *Enterococcus* spp. isolated from urine samples were found to have the *ermB* gene. Another study done by (32) demonstrated that (41%) of isolates were found to be positive for the *ermB* gene. Another study done by (33) reported that the *ermB* gene was the most common gene in 56 (77.7%) of all enterococcal urine isolates. Also, (34) reported that the *ermB* gene was detected in 55.14% of all enterococcal isolates. Another study found that of all enterococcal isolates, *ermB* (67.7%) had the highest frequency of erythromycin ribosome methylation (35).

A study by (36) revealed that 47.6% of the isolates (10 of 21) carried the *ermB* gene. Also, the most often occurring gene, according to (37), it was the *ermB* gene. Erythromycin resistance was discovered in 7% of the growing bacteria. It was found that two of the isolates tested positive for the *ermB* gene, one of the most prevalent genes in oral isolates and more prevalent in strains that are highly resistant to the antibiotic erythromycin (38). Anoother study found the *ermB* gene in 24% of all enterococcal isolates in their latest study. Most often, *ermB* expressed erythromycin resistance (39).

The results in **Table 8** demonstrated that the highest percentage of biofilm formation was 61.9% of the moderate type in the case of gene presence, and also when the gene did not appear, the highest percentage of biofilm formation was 14.3% also of the medium type, and this indicates that the appearance of the gene had no role in biofilm formation. According to the results of the chi-square comparison between the types of biofilm **(Table 9),** it was found that there were no significant differences between the three types; weak, medium, and strong.

According to **Table 10,** there is no correlation between the expression of the gene and the formation or type of biofilm.

5.Conclusion

We conclude in this study that *the ermB* gene was detected in enterococcal isolates from the tooth root canal and urine samples, with a higher prevalence percentage in urine sample isolates than tooth root canal isolates. Finally, the findings demonstrated that there is no connection between this gene's existence and the tested isolates' ability to create biofilms.

Conflict of Interest

Conflict of Interest the authors declare that they have no conflicts of interest.

Funding

There is no funding for the article.

Ethical Clearance

The study was conducted following the receipt of participant consent and ethical approval from the Ethics Committee of the Biology Department at the University of Baghdad's College of Science (CSEC/0922/0097) on September 26, 2022. This procedure is in line with the guidelines set forth by the Iraqi Ministry of Health and Environment.

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