

МЕЖДУНАРОДНЫЙ НАУЧНО-ПРАКТИЧЕСКИЙ ЖУРНАЛ

# ЛАБОРАТОРНАЯ ДИАГНОСТИКА

Восточная  
Европа

Laboratory Diagnostics Eastern Europe

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Beginning in the Renaissance, European nobility often used it to make rings bearing family crests and signet rings.

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## Linkage between MicroRNA-125b1 Expression and Fibroblast Growth Factor Receptor 2 (FGFR2) Serum Levels in Patients with Psoriasis

**Conflict of interest:** nothing to declare.

**Authors' contribution:** Moayed Ali Dawood – conceptualization, data curation, investigation, software, visualization, writing – original draft and writing – review & editing; Basima Qasim Hasan AlSaadi – conceptualization, method, project administration, resources, software, visualization, writing – original draft and writing – review & editing.

**Ethics statement.** The study was approved by the Ethical Committee of the Institute of Genetic Engineering and Biotechnology for Postgraduate Studies, University of Baghdad, and conducted in accordance with the Declaration of Helsinki. The article is published in author's edition.

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### Abstract

**Introduction.** Psoriasis is a chronic immune-mediated skin disease where microRNAs may modulate keratinocyte proliferation and inflammatory pathways. MiRNA-125b1 has been reported to target fibroblast growth factor receptor-2 (FGFR2), but their systemic association in psoriasis remains unclear.

**Purpose.** To quantifying peripheral miRNA-125b1 and serum FGFR2 levels in patients with chronic plaque psoriasis versus healthy controls.

**Materials and methods.** This case-control study involved 60 Iraqi patients with psoriasis (31 males, 29 females) and 60 apparently healthy controls (30 males, 30 females) who were admitted to the Dermatology Clinic at Al-Yarmouk Teaching Hospital, Baghdad, from January to May 2025. Whole-blood miRNA-125b1 expression was measured by quantitative real-time PCR ( $2^{-\Delta\Delta C_t}$  method), and serum FGFR2 levels were assessed by ELISA.

**Results.** Psoriatic patients had downregulated miRNA-125b1 expression and significantly elevated FGFR2 levels compared to controls, and altered miRNA-125b1 expression was strongly associated with psoriasis status. FGFR2 showed fair diagnostic performance, whereas the correlation between miRNA-125b1 and FGFR2 was weakly negative in patients. These results indicate an altered miRNA-125b1/FGFR2 axis involved in the pathogenesis of psoriasis as well as the potential biomarker utility.

**Conclusion.** Psoriasis in Iraqi individuals correlates with significant downregulation of miRNA-125b1 expression and elevated serum FGFR2 levels, implicating impaired miRNA-125b1/FGFR2 axis in the mechanism of the disease.

**Keywords:** psoriasis, miRNA-125b1, FGFR2, microRNA, qRT-PCR



## ■ INTRODUCTION

Psoriasis is a chronic immune-mediated inflammatory skin disease characterized by epidermal hyperplasia, dermal inflammatory infiltrates, and systemic comorbidities such as psoriatic arthritis and metabolic syndrome [1]. Growing evidence is found to suggest that epigenetic mechanisms, especially microRNAs (miRNAs), contribute in regulating keratinocyte proliferation, differentiation, and cutaneous inflammation mediating psoriasis [2, 3]. Of these miRNAs, miR-125b1 encoded by the MIR125B1 gene is commonly reported to be downregulated in psoriatic skin and blood. Altered circulating miR-125b1 levels have been linked to disease severity and implicate miR-125b1 as a blood biomarker and a diagnostic miRNA panel. Experimental data have further correlated miR-125b1 axis with fibroblast growth factor receptor-2 (FGFR2) signaling in keratinocytes, showing an inverse relationship between miR-125b expression and FGFR2 levels and demonstrating that modulation of this pathway affects keratinocyte proliferation [4]. Psoriasis has been shown to be associated with systemic immune activation; in Iraqi female patients, Khazem and Ibraheem found significant alterations in serum IL-36 and IL-10 levels compared with healthy women [5]. Other local data indicate that EOS gene expression and serum IL-6 levels are indicative of systemic inflammation in Iraqi psoriasis populations [6]. In addition, polymorphism of the FAS gene (rs1800682) and also changes in FAS serum levels have been correlated w/a susceptibility to psoriasis, which may indicate contribution of abnormal apoptotic signal transduction in the disease mechanism of this population [7].

## ■ PURPOSE OF THE STUDY

To quantifying peripheral miRNA-125b1 and serum FGFR2 levels in patients with chronic plaque psoriasis versus healthy controls.

## ■ MATERIALS AND METHODS

### **Study Design**

The sixty Iraqi patients with chronic psoriasis (31 males and 29 females) and sixty healthy controls (30 males and 30 females) were included. All participants were recruited at the Dermatology Clinic of Al-Yarmouk Teaching Hospital, where sample collection was performed under standard conditions.

### **Inclusion Criteria**

1. Clinically confirmed diagnosis of chronic psoriasis.
2. Disease duration  $\geq 6$  months.
3. Age  $> 18$  years.
4. Both sexes included.

### **Exclusion Criteria**

1. Concomitant autoimmune or chronic inflammatory diseases.
2. Current or past malignancy.
3. Pregnant or lactating women.

### **Samples of Blood Preparation**

For each subject, 5 mL venous blood was gathered aseptically: 3 mL were allowed to clot at room temperature for 30 min, centrifuged at 3000 rpm for 10 min, and the resulting

serum was aliquoted and stored at –20 °C for FGFR2 analysis; the remaining 2 mL were reserved for RNA extraction and stored at –20 °C until processing.

**RNA Extraction and cDNA Synthesis**

Total RNA was extracted from whole blood using the Trizol reagent method (GENEzol™ TriRNA Pure Kit, Taiwan). RNA purity was verified using the QuantiFluor® RNA System. cDNA was synthesized using a miRNA-specific reverse transcription kit (25 °C for 5 min; 50 °C for 15 min; 95 °C for 5 min).

**Primer Design**

The primers used in the experiment were synthesized by Macrogen and delivered in a lyophilized form. These lyophilized primers were reconstituted in 300 µl of nuclease free water to reach a final concentration of 100 pmol/µl. A working solution of primers was prepared by combining 10 µl of the reconstituted primer with 90 µl of nuclease free water to reach a concentration of 10 pmol/µl. The primers and the working solutions were stored frozen at –20 °C until used (Table 1).

**Table 1**  
**Oligonucleotide primers that were used for miRNA-125b1 amplification in this study**

| Primer  | Sequence (5'→3')     | Product size (bp) | Tm (°C) | Designed<br>in this study |
|---------|----------------------|-------------------|---------|---------------------------|
| Forward | CGCGTCCCTGAGACCCTAAC | 70                | 60      |                           |
| Reverse | AGTGCAGGGTCCGAGGTATT |                   |         |                           |

**Quantitative Real-Time PCR (qRT-PCR)**

It was performed using the qTOWER<sup>3</sup> Analytik Jena (Germany). The PCR conditions were 55 °C for 10 minutes, then 40 cycles at 95 °C for 10 seconds and 60 °C for 30 seconds. Relative expression was determined using the 2<sup>Δ-ΔCt</sup> method (Table 2).

**Table 2**  
**Components of the reaction mixture for amplification for miRNA-125b1**

| Component                                | Volume (µl) | Final concentration |
|------------------------------------------|-------------|---------------------|
| 2 × miRNA Universal SYBR qPCR Master Mix | 10          | 1X                  |
| Forward primer (10 pmol/µl)              | 0.4         | 0.4 µM              |
| Reverse primer (10 pmol/µl)              | 0.4         | 0.4 µM              |
| Template cDNA                            | Variable    | 20 ng/ul            |
| Nuclease-free water                      | To 20       |                     |

The thermocycler was set up and operated in accordance with the protocol detailed in Table 3.

**Table 3**  
**Amplification program of qRT-PCR for target gene (miRNA-125b1) and housekeeping gene (U6)**

| Cycle step            | Temperature (°C) | Time       | Cycles |
|-----------------------|------------------|------------|--------|
| Reverse Transcription | 55               | 10 minutes | 1      |
| Initial Denaturation  | 95               | 1 minute   | 1      |
| Denaturation          | 95               | 10 seconds | 40     |
| Extension             | 60               | 30 seconds |        |
| Melt Curve            | 60–95            | Various    | 1      |



**Serum Level Estimation for Fibroblast Growth Factor Receptor-2 (FGFR2)**

Serum FGFR2 levels were measured using a commercial sandwich ELISA kit (FGFR2 ELISA Kit, Bioassay Technology Laboratory, Shanghai, China) according to the manufacturer’s instructions.

**Statistical Analysis**

The categorical variables, such as sex and family history, were examined using Chi-square ( $\chi^2$ ) tests to identify associations between groups. Continuous variables were expressed as mean  $\pm$  standard deviation (SD). An independent samples t-test was used to assess comparisons of the patients versus controls. A Pearson’s correlation coefficient (r) was calculated to evaluate relationships among continuous variables. Receiver Operating Characteristic (ROC) curve analyses were conducted to study diagnostic performance by tabulating an area under the curve (AUC), optimal cutoff, sensitivity, and specificity. Statistical significance was defined as  $p < 0.05$ . All analyses were performed with SPSS version 26.

**■ RESULTS**

**Distribution According to Sex**

The sex distribution was balanced between the control and patient groups. Males constituted 50.0% (n=30) of the control group and 51.7% (n=31) of the patient group, while females constituted 50.0% (n=30) and 48.3% (n=29), respectively. The Chi-Square test revealed no statistically significant difference in sex distribution between the groups ( $p=0.855$ ). As shown in Table 4.

**Table 4**  
**Sex Distribution Across Study Groups**

| Group    | Male, n (%) | Female, n (%) | Total, n |
|----------|-------------|---------------|----------|
| Control  | 30 (50.0)   | 30 (50.0)     | 60       |
| Patients | 31 (51.7)   | 29 (48.3)     | 60       |
| Overall  | 61 (50.8)   | 59 (49.2)     | 120      |

**Quantitative Real-Time PCR (qRT-PCR) of miRNA-125b1**

The gene expression of miRNA-125b1 was downregulated in the patient group, showing an approximately 0.54-fold decrease compared with the control group (Mean  $2^{\Delta-\Delta Ct}$ : patients = 0.561 vs. controls = 1.035;  $\Delta\Delta Ct = +1.122$ ). The relative expression ( $2^{\Delta-\Delta Ct}$ ) of miRNA-125b1 indicates reduced transcriptional activity in the patient group when normalized to U6 as a reference gene (Table 5, Figure 1A and 1B).

**Table 5**  
**Expression Levels of miRNA-125b1 in Study Groups ( $2^{\Delta-\Delta Ct}$  method)**

| Group      | Mean Ct (GOI: miRNA-125b1) | Mean Ct (HKG: U6) | Mean $\Delta Ct$ (GOI – HKG) | Mean $\Delta\Delta Ct$ | Mean $2^{\Delta-\Delta Ct}$ | Experimental group/control group | Fold change of gene |
|------------|----------------------------|-------------------|------------------------------|------------------------|-----------------------------|----------------------------------|---------------------|
| Control    | 28.1560                    | 19.9800           | 8.1760                       | 0.0000                 | 1.035                       | 1.0000                           | 1.0 (reference)     |
| Patient(s) | 29.5635                    | 20.2655           | 9.2980                       | +1.1220                | 0.561                       | 0.542                            | ↓ 0.54-fold (down)  |

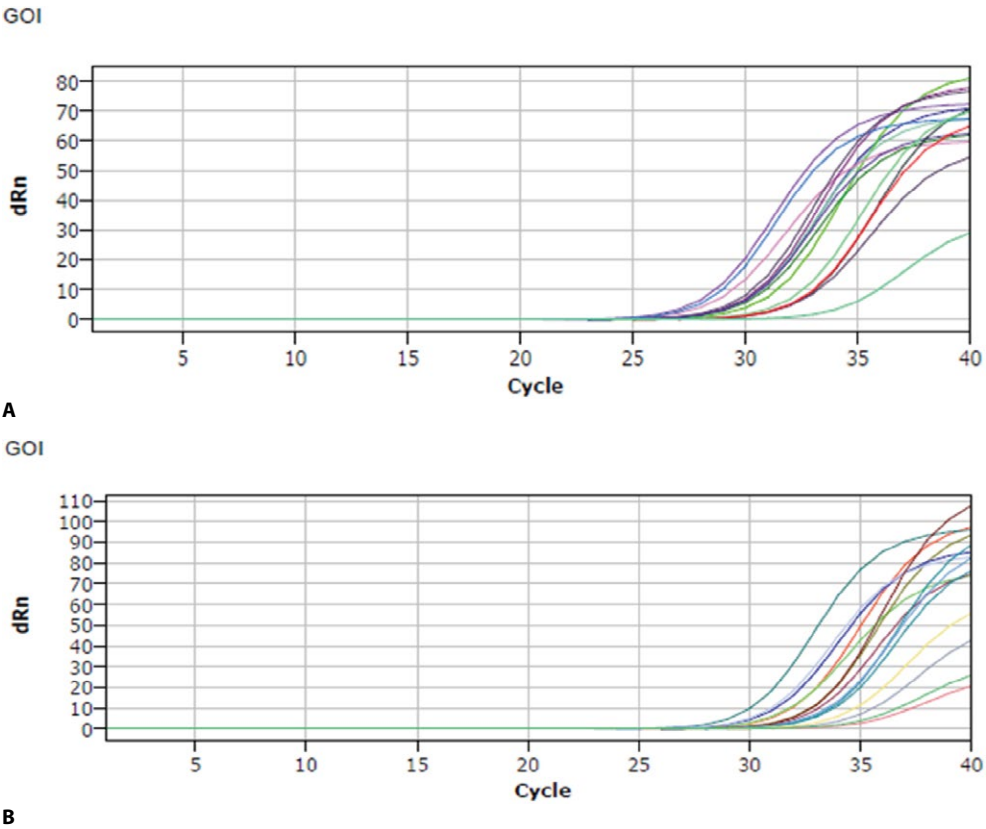


Fig. 1. qRT-PCR amplification plot: A – for the endogenous control (U6); B – for miRNA-125b1

**The Serum Concentration of Fibroblast Growth Factor Receptor 2 (FGFR2)**

The concentration of FGFR2 was significantly higher in the patient group ( $119.91 \pm 30.95$  ng/L) compared to the control group ( $78.09 \pm 35.83$  ng/L). This difference was highly statistically significant ( $p=0.0001$ ), as shown in Table 6.

**Table 6**  
**Comparison of FGFR2 levels between study groups**

|                               | Study groups | Mean   | ±SD   | ±SE  | t-test | p-value |
|-------------------------------|--------------|--------|-------|------|--------|---------|
| Concentration of FGFR2 (ng/L) | Control      | 78.09  | 35.83 | 4.63 | 6.709  | 0.0001  |
|                               | Patients     | 119.91 | 30.95 | 4.00 |        |         |

**Predictive Value of FGFR2 Based on ROC Analysis**

FGFR2 showed significant predictive validity. FGFR2 had a higher Area Under the Curve (AUC=0.722). cut-off of <110.77 ng/L showed 72% sensitivity and 82% specificity. As reported in Table 7 and Figure 2.

**Table 7**  
**The validity of FGFR2 as a marker in the study groups for predicting disease**

| Test Result Variable(s) | Optimal cut-off point | AUC   | Sensitivity % | Specificity % | P. value |
|-------------------------|-----------------------|-------|---------------|---------------|----------|
| FGFR2                   | <110.76923            | 0.722 | 72            | 82            | 0.000    |

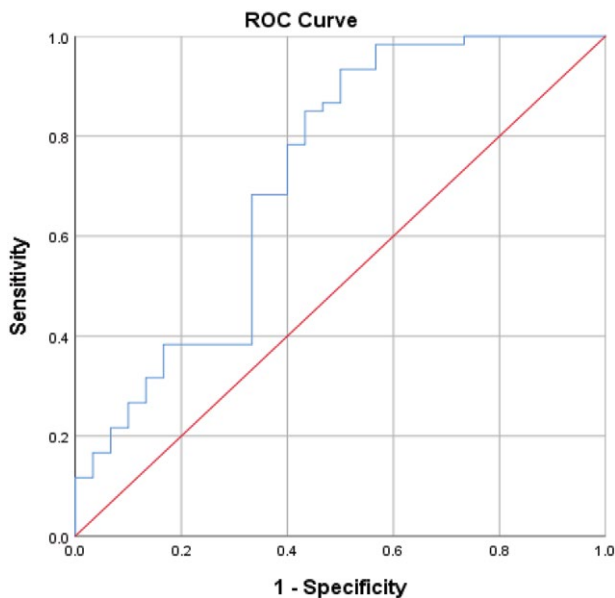


**Fig. 2.** ROC curve of FGFR2

**Correlations Between miRNA-125b1 and FGFR2 in Patient and Control Groups**

In the patient group (n=60), statistically significant negative correlation was found between miRNA-125b1 expression and serum FGFR2 concentrations ( $r=-0.287$ ,  $p=0.026$ ). In the control group, significant negative correlation with FGFR2 ( $r=-0.490$ ,  $p<0.001$ ). All p-values are two-tailed with  $\alpha=0.05$  (Table 8).

**Table 8**  
**Correlations between miRNA-125b1 and biomarkers in patient and control groups**

| Parameters            | Patients r | P. value | Control r | P. value |
|-----------------------|------------|----------|-----------|----------|
| miRNA-125b1 vs. FGFR2 | -0.287*    | 0.026    | -0.490*   | 0.000    |

Notes: \*  $p<0.05$ , r – Pearson correlation.

**■ DISCUSSION**

This pattern is in accordance with extensive epidemiological researches suggesting that psoriasis occurs in men and women with approximately similar frequency, with only minor age- or region-dependent deviations from a 1:1 male-to-female ratio [8–10]. Recent Iraqi data from a Baghdad cohort showed that IL-36A gene expression is significantly

up-regulated in psoriatic patients of both sexes, with no difference between males and females, supporting the need to evaluate immunogenetic markers in sex-balanced Iraqi population [11]. Although sex-dependent differences with respect to immune response have been documented in chronic inflammatory diseases [12], an even sex ratio in this cohort affords a greater internal validity of the study by avoiding potential sex-biased molecular and cytokine profiles.

This observation is consistent with earlier findings that miR-125 family members (MIR125B1/2) are negative regulators (negative regulators of keratinocyte proliferation) by targeting FGFR2 directly, as previously reported in psoriatic epidermis [13]. Reviews of psoriatic microRNAs also identify miR-125b as one of the major down-regulated miRNAs that drive epidermal hyperplasia and chronic inflammation [14]. Additionally, clinical data have also demonstrated lower miR-125b in skin and serum of psoriatic patients, with lower levels associated with higher PASI scores, suggesting utility as a disease-activity biomarker [15]. On a local level, Iraqi data on miR-146a dysregulation in rheumatoid arthritis also show that the circulating miRNAs act as informative immunoinflammatory biomarkers in Iraqi populations [16], which also reinforces the applicability of miRNA-125b1 as a potential biomarker of interest in this psoriatic population.

This result is consistent with prevailing ideas that growth factors of the FGF family and their receptors participate in keratinocyte hyperproliferation, neoangiogenesis and the metabolic comorbidities of psoriasis [17]. In these studies, the increased circulating FGF ligands have been clinically described across cohorts: Yürekli et al. indicated that serum FGF2 levels in psoriatic patients are high and correlated positively with PASI score [18], whereas Kiluk et al. and Okan et al. showed that FGF21 and FGF23 levels are raised in psoriasis and associated with cardiometabolic and inflammatory burden [19, 20]. This observation highlights increased FGFR2 receptor levels and reflects that not only FGF ligands but also their receptors are upregulated, potentially amplifying downstream signalling in skin and systemic tissues. Produal data from Iraqi patients confirmed that psoriasis is associated with a wide elevation of pro-inflammatory mediators: Kareem and Al-Thwani found significantly elevated serum IL-17, IL-18 and IL-22 in Iraqi psoriatic patients, and speculated that these cytokines be crucial indices of the disease [21]. Collectively, they emphasize that systemic activation of inflammatory cytokines and the FGF/FGFR2 pathway is a hallmark of psoriasis in both international and local contexts.

Similar responses have been observed for numerous inflammatory bi-omarkers in psoriasis; Al-Saba et al. reported that serum CHI3L1 had AUC values in the "good" range and outperformed CRP for distinguishing psoriatic patients from controls [22], while visfatin demonstrated superior dis-criminative effectiveness in a recent case-control study [23]. Inconsistent with the concept that circulating immune mediators, including FGFR2, provide clinically relevant predic-tive information in psoriatic disease.

This pattern is consistent with a functional miR-125b1–FGFR2 regulatory axis seen in vi-vo, in which lower miRNA-125b1 expression is correlated with higher cir-culating FGFR2, comparable with recently published epigenomic data that demonstrate that miR-125b downregulation promotes keratinocyte hyperproliferation via upregulation of FGFR2 [24]. In contrast to control, the decreased correlation coefficient of patient group vs controls indicates that with psoriatic in-flammation, additional regulatory pathways (e.g. cytokine-driven signalling or epigenetic changes) may partially uncouple miRNA-125b1 from tightly controlled FGFR2 expression and other miRNA-125b–regulated pathways



such as USP2–NF- $\kappa$ B signalling [25]. These results align with systems-level analyses that reveal miRNA-125b1 to be one of the main down-regulated epidermal miRNAs seen in psoriasis, and both FGFR2 and related growth-factor pathways to be up-regulated in lesional keratinocytes and fibroblasts [26]. In an Iraqi case–control cohort from Baghdad, Joodi and Al-Saadi found that serum soluble FASL and IL-21 levels are significantly higher in psoriatic patients than in healthy controls and tend to rise with increasing disease severity, supporting the concept that immune-gene dysregulation is mirrored by changes in circulating protein biomarkers [27]. Similar immunogenetic dysregulation has also been reported in Iraqi cohorts from Baghdad; Mahmood et al. showed IL-36A overexpression and enrichment of functional IL-36A promoter polymorphisms in psoriatic patients, indicating that genetic variants in inflammatory pathways are reflected at the cytokine-expression level [28]. Coupled, the correlation data provide clinical validation for a miRNA-125b1–FGFR2 regulatory interaction and imply that disruption of this axis is merely one component of broader gene–protein dysregulatory network in psoriasis.

## ■ CONCLUSION

Psoriasis in Iraqi individuals correlates with significant downregulation of miRNA-125b1 expression and elevated serum FGFR2 levels, implicating impaired miRNA-125b1/FGFR2 axis in the mechanism of the disease. FGFR2 showed fair diagnostic value as a supportive serum biomarker, but larger, more comprehensive longitudinal and functional studies are required to determine the clinical and therapeutic significance of these markers in psoriasis.

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# Regulation of Human Integrin Beta-1 Gene Expression by CUP Genes in *Pseudomonas Aeruginosa*

**Conflict of interest:** nothing to declare.

**Authors' contribution:** Farah Ghassan Jawad – conceptualization, data curation, investigation, methodology, project administration, resources, software, writing – original draft and writing – review & editing; Asmaa Salih Almohaidi – conceptualization, data curation, investigation, methodology, supervision, validation, visualization, writing – original draft and writing – review & editing.

**Ethics statement.** The planned study was approved by the Department of Biology Sciences, College of Science for Women, University of Baghdad, Baghdad, Iraq, in October 2024. The team work declare that all experiments were carried out by applicable standards and regulations.

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## Abstract

**Introduction.** *Pseudomonas aeruginosa* is a prevalent opportunistic pathogen associated with chronic hospital infections due to its remarkable ability to form biofilms and resistance.

**Purpose.** To evaluate the correlation between the expression of integrin  $\beta 1$  receptors in human clinical samples and the expression of cup genes (cupA, cupB, cupC, and cupD) in *P. aeruginosa* along with their roles in drug resistance and biofilm development.

**Materials and methods.** The study included 15 control samples from healthy individuals and 22 clinical samples from patients with wound infections (skin swaps and blood). After identifying *P. aeruginosa* using culture techniques and molecular, for diagnosis, the GyrB gene was used amplified by PCR, the isolates' antibiotic susceptibility and biofilm intensity were assessed. To employed quantitative polymerase chain reaction, evaluate the gene expression for cupABCD and integrin beta one.

**Results.** The expression of cupA and cupB genes in *P. aeruginosa* revealed a downregulation of gene expression by 0.48 and 0.75-fold, respectively, while it was upregulation of expression in the Cup C and Cup D genes was 1.89 and 1.95-fold, respectively. As for the human integrin beta one gene, it was 1.60-fold in the group of patients infected with *P. aeruginosa* compared to the group of healthy individuals. Regarding the gene expression of the study genes, it was found that the gene expression of the cup B gene was up regulated in all biofilm phenotypes, as it recorded 20.49, 28.49 and 20.6 folds, for the strong, medium and weak biofilm, respectively. As for integrin beta one gene, it recorded fluctuated expression in all biofilm phenotypes, and the upregulation was recorded in the weak biofilm phenotype (11.39 folds).

**Conclusion.** These findings imply the possibility of therapeutic targeting to lessen persistent *P. aeruginosa* infections and emphasize the role goblet genes play in fostering virulence and resistance within biofilms.

**Keywords:** *Pseudomonas aeruginosa*, cup genes, integrin  $\beta 1$ , ITGB1, Biofilm, RT-QPCR

## ■ INTRODUCTION

*Pseudomonas aeruginosa* A rod-shaped, Gram-negative. It is an opportunistic pathogen [1], particularly in people with immunocompromised conditions such as burn wounds, cystic fibrosis, and extended hospital stays [2]. Biofilms promote the chronicity of disease by providing defense against environmental stressors [3]. Initial reversible adhesion, irreversible attachment, maturation, and eventual spreading are all stages in the multi-step process of biofilm growth. Maturation of Biofilms [4]. Pili proteins, peripheral chaperones, and ushers that aid in the assembly of sticky pili are among the coding modules of the cupA, BCD genes that are responsible of the production of fimbriae on the surface of *P. aeruginosa* [5]. Four cup gene clusters – cupA1-5, cupB1-6, cupC1-3, and cupD1-5 – have been found in *P. aeruginosa*. These clusters encode the chaperones, pili modules, and the outer membrane protein and usher protein [6]. The chaperones are encoded by cups A2, A5, B2, B4, and C2, while the usher proteins are encoded by cups A3, B3, and C3. The cupD1-5. The main Fimbria subunit, the chaperone, and the outer membrane usher are encoded by cupD1, cupD2, and cupD3, respectively, in this group. An anticipated adhesion and a secondary are encoded by cupD4 and cupD5 [7, 8]. Integrins are an adhesion receptor between cells and the extracellular matrix (ECM), the membrane protein integrin is essential for cell contact with its surroundings, Together, its two subunits,  $\alpha$  and  $\beta$ , form a receptor that sends signals throughout the cell, affecting processes like cell migration, cytoskeletal remodeling, and cell cycle regulation [9, 10]. The integrin beta one (ITGB1) gene produces the integrin protein's  $\beta 1$  subunit, a membrane receptor essential to cell adhesion and interaction with the extracellular matrix (ECM). Its numerous receptors by binding to various  $\alpha$  subunits, like  $\alpha 5 \beta 1$  that binds to fibronectin [11]. Numerous biological processes, such as cell migration, proliferation, and survival, it also has a significant impact on wound healing, immunity, when it comes to bacterial infections [12]. ITGB1 is a two-edged sword. On the one hand, ITGB1 plays a crucial role in cell adhesion and communication mechanisms, which aid in tissue regeneration and wound healing [13]. However, it poses a risk when *P. aeruginosa* uses ITGB1 to improve its adherence to host cells, which makes it easier for the bacteria to colonize afflicted tissues. As a crucial receptor in the interaction between cells and the extracellular matrix (ECM) [14]. Treatment of infections becomes more complicated due to the interplay between ITGB1 and bacterial virulence factors [15].

## ■ PURPOSE OF THE STUDY

To evaluate the correlation between the expression of integrin  $\beta 1$  receptors in human clinical samples and the expression of cup genes (cupA, cupB, cupC, and cupD) in *P. aeruginosa* along with their roles in drug resistance and biofilm development.

## ■ MATERIALS AND METHODS

### **Isolation and Molecular Identification of *Pseudomonas Aeruginosa***

Over six months, from October 2024 to March 2025, the sample included patients with various clinical conditions (burns, wounds, DFU, and blood samples). Samples were also collected from the Specialized Burns Center, Martyr Ghazi Al-Hariri Hospital in Baghdad. Bacteria were isolated and identified from skin swabs using bacteriological techniques, biochemical tests, and molecular diagnostics using *gyrB*, resulting in the discovery



of 26 *P. aeruginosa* isolate. DNA was extracted using the Easy Taq® Bacteria Genomic DNA Kit (Transgene Biotech/China) for molecular diagnosis. Bacteria were cultivated in 1.5 µl Eppendorf tubes and centrifuged at 14,000–16,000 × g for one minute. The top DNA extract was discarded during cell harvesting. All The primers listed in Table 1. The total volume for PCR amplification was 25 µl, which included 1 ml of each primer (10 pmol/1 µl), 12.5 µl of Master Mix, 4 µl of DNA, and up to 4.5 µl of nuclease-free water. After five minutes of target DNA amplification at 94 °C, 35 cycles of 30 seconds each at 94 °C, 30 seconds at the optimal annealing temperature of 66 °C, and 30 seconds at 72 °C were performed. After that, another 10-minute cycle at 72 °C was completed. PCR products were detected using gel electrophoresis.

### Antibiotic Susceptibility Testing

The Kirby-Bauer method was used to measure antibiotic activity by comparing each of the 26 bacterial isolates' susceptibility to seven different antimicrobial drugs. The approach involved using Mueller-Hinton agar [16]. the bacterial suspension, some bacterial colonies from the overnight culture were added to 5 milliliters of normal saline with a McFarland turbidity adjustment of 0.5 ( $1.5 \times 10^8$  CFU/ml). The following medications were used: amikacin, ticracillin, ceftazidime, imipenem, and levofloxacin. Resistance (R) and sensitivity (S) were assessed using the manual instructions and (CLSI) guidelines.

### Biofilm Formation Assay

Each isolate was grown for 24 hours at 37 °C in brain-heart infusion broth supplemented with 1% glucose. Following the transfer of 100 microliters of bacterial growth Then the method was followed in [17]. The absorbance of the stained biofilm was measured at 580 nm with an ELISA reader (Biorad, UK).

$OD_i \leq OD_c$  = negative;  $OD_c < OD_i < 2 \times OD_c$  = weakly positive;  $2 \times OD_c < OD_i < 4 \times OD_c$  = moderately positive  $4 \times OD_c < OD_i$  = strongly positive.

### Primers Design

By used the website <https://www.ncbi.nlm.nih.gov/tools/primer-blast> to (CupB, Integrin Beta one) for RT-PCR, which designed for this study. These primers are listed in Table 1, along with CupA, CupC, CupD, fwrpoD, Gyr B, and Gapdh [18–20]. These primers approached by Macrogen and were lyophilized. They were spun down, dissolved in free DNase, RNase water according to the manufacturer's instructions to make a stock solution with a final concentration of 100 pmol/µL, and then were gently mixed.

### Quantitative Expression of the CupABCD Gene in *P. Aeruginosa* and ITGB1 Using RT-qPCR

Twenty-two clinical isolates of *P. aeruginosa* were cultured in brain-heart infusion broth at 37 °C for 24hr. bacteria cells were harvested by centrifugation at 12,000 rpm for 3 min. total RNA was then extracted from the bacteria pellets using TransZol Up Plus Kit (TRANS/China) Following the manufacturer's recommendations. To avoid contamination with DNA, total RNA was combined with the DNase enzyme and promptly kept at –20 °C. *Pseudomonas aeruginosa* samples were exposed to RT-qPCR in order to determine gene expression and standardize data using the fwrpoD RNA polymerase sigma factor for normalization. Regarding blood samples obtained from the same *P. aeruginosa*-infected

**Table 1**  
**Primers used in this study**

| Primer                                    | Primer sequence                                      | Templet length bp | Annealing Tm (°C) | Reference                 |
|-------------------------------------------|------------------------------------------------------|-------------------|-------------------|---------------------------|
| <b>Bacteria PCR detection</b>             |                                                      |                   |                   |                           |
| Gyr B                                     | F /CCTGACCATCCGTCGCCACAAC<br>R/ CGCAGCAGGATGCCGACGCC | 220               | 66                | [18]                      |
| <b>Bacteria RT-qPCR expression Primer</b> |                                                      |                   |                   |                           |
| Cup B                                     | F/ CATCGCCTTCGATCAGGTCA<br>R/ GGGATCTTCGTGGTGTGGT    | 129               | 58                | Designed by second author |
| CupA                                      | F/ GCGGCAAACACTATCACATTC<br>R/ AACAGGGTGGTGAAATGCTC  | 212               | 58                | [13]                      |
| Cup C                                     | F/ AGGCTGATAATGGGGCAGA<br>R/ CGACGCAGATTTTGTGGA      | 179               | 58                |                           |
| CUP D                                     | F/ CGTCTATCGCAACCAGCA<br>R/ CGTCGGGCACTTCGTC         | 152               | 57                |                           |
| fwrpoD RNA polymerase sigma factor        | F/ACAAGATCCGCAAGGTACTGAAG<br>R/ATCGCCCAGGTGCGAAT     | 180               | 60                | [19]                      |
| <b>Human RT-qPCR expression Primer</b>    |                                                      |                   |                   |                           |
| Integri n Beta one exon 2                 | F/ GAGAAGTGCTGTCCTGGGAG<br>R/ CTTCTACCGGTACTGGGTGC   | 98                | 59                | Designed by second author |
| GAPDH                                     | F/GAAATCCCATCACCATCTT<br>R/AAATGAGCCCCAGCCTTCT       | 165               | 59                | [20]                      |

individuals, to create the ITGB1 gene’s expression and normalize the findings to the GAPDH gene.

**Complementary DNA (cDNA) Synthesis**

To test the cDNA synthesis, genomic DNA was taken out following the steps given by the Easy Script® One-Step gDNA removal and cDNA Synthesis Super Mix (TRANS/China) kit. following the instruction.

**Quantitative Real-Time Polymerase Chain Reaction**

Trans Start® Top Green qPCR SuperMix from TRANS Gene biotech / China was used to initiate the reverse transcriptase polymerase chain reaction RT-qPCR for ITGB1and 2xEasyTaq® PCR SuperMix for bacteria samples, The settings employed for RT-PCR were as follows: initial denaturation at 94 °C for 10 seconds, annealing at (cup A, cup C 56 °C), (cup B, cup D 56 °C), and (ITGB1, 60 °C) for 15 secs, followed by extension at 72 °C for 20 sec. then determined for both the experimental and control groups the differences between the starting gene threshold cycles (CT) and the mean reference gene threshold



cycles (CT). Inconsistencies between the two sets of samples (test and control) were used to determine the CT index. A G-STORM/UK apparatus was used to conduct real-time PCR assays for the targets in order to carry out amplification utilizing reverse transcriptase polymerase chain reaction (RT-qPCR).

### Statistical Analysis

By using the SPSS Statistics Software V 17.0 (SPSS Inc., Chicago, IL, USA). Student's t-test, LSD-Least, and Chi-square test was used to significant compare between percentage (0.05 and 0.01 probability). Estimate of correlation coefficient between variables in this study [21].

## ■ RESULTS

### Bacterial Isolates and Identification

One hundred and twenty specimens were collected from different sources, isolated from different hospitals in Baghdad between October 2024 and March 2025. 26 confirmatory strains of *Pseudomonas aeruginosa* was identified after bacteriological detection. All 26 isolates were cultured, Biochemical tests were performed according to [22], the presence of 26 bacterial isolates was confirmed by Molecular detection was achieved using the *gyrB* gene with a band size of 220 bp, as in Figure 1.

### Antimicrobial Sensitivity and Biofilm Assay Testing

As show in Table 2, Antimicrobial susceptibility testing of *Pseudomonas aeruginosa* for 26 isolates showed a high level of resistance to seven antibiotics. The highest resistance rate was recorded for Ticarcillin/clavulanic acid (65.38%), followed by Ceftazidime (57.69%), Imipenem (50%), Aztreonam and piperacillin (46.15%). In contrast, the lowest resistance rate (38.46%) was shown for amikacin and 26.92% for levofloxacin. Figure 2 shows two samples of *P. aeruginosa* grown on MH agar, showing a resistant isolate and an isolate susceptible to antibiotics.

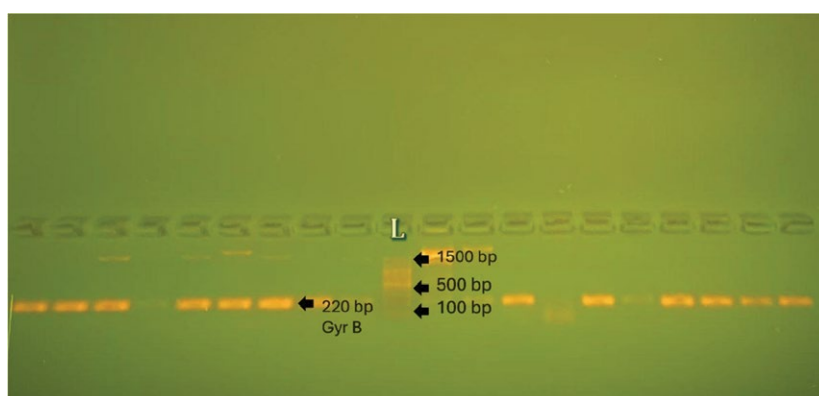


Fig. 1. Gel electrophoresis of 220 bp *Gyr B* (1% agarose, at 100 V, 60 min). Imaged under UV light

**Table 2**  
**List the result of antibiotic disks**

| No.   | Antibiotic disk                    | *S No. %    | **R No. %   |
|-------|------------------------------------|-------------|-------------|
| 1     | Amikacin 10 (µg)                   | (16) 61.53% | (10) 38.46% |
| 2     | Azetreonam 30 (µg)                 | (14) 53.84% | (12) 46.15% |
| 3     | Ceftazidime 30 (µg)                | (11) 42.30% | (15) 57.69% |
| 4     | Imipenem 10 (µg)                   | (13) 50%    | (13) 50%    |
| 5     | Levofloxacin 5 (µg)                | (19) 73.07% | (7) 26.92%  |
| 6     | Piperacillin 100 (µg)              | (14) 53.84% | (12) 46.15% |
| 7     | Ticarillin/clavulanic acid 10 (µg) | (9) 34.61%  | (17) 65.38% |
| Total |                                    | 26          |             |

Notes: \* S – Susceptible, \*\* R – resistant.

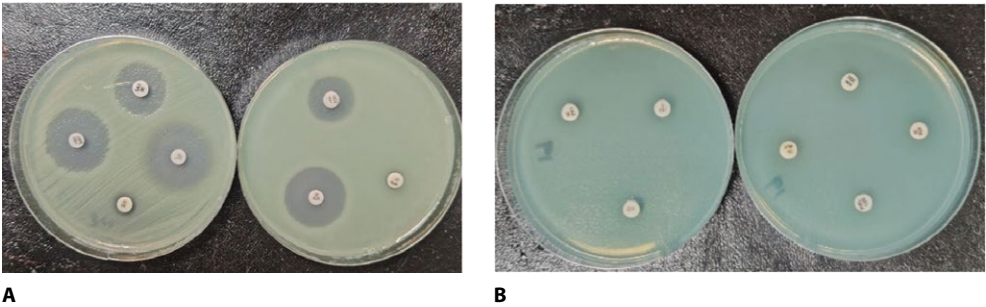


**Fig. 2. A – shows a Pseudomonas aeruginosa isolate susceptible to most antibiotics. B – shows an isolate resistant to all antibiotics tested**

The ability of *P. aeruginosa* isolates to produce biofilms was examined. only one isolate was non-forming for biofilm, and one produced weak biofilm. The percentage of strong biofilm forming was 13 (50%( and the percentage of moderate biofilm forming was 11 (42.30%), Table 3 shows the proportions.

**Table 3**  
**Biofilm formation phenotype in different isolates**

| Pattern of biofilm producers |                |         |
|------------------------------|----------------|---------|
| Biofilm producer             | no. of samples | no. (%) |
| Non-producer                 | 1              | 3.84%   |
| Weak producer                | 1              | 3.84%   |
| Moderate producer            | 11             | 42.30%  |
| Strong producer              | 13             | 50%     |
| total                        | 26             | 100%    |

The results showed a clear correlation between the strength of biofilm formation and antibiotic resistance patterns in *Pseudomonas aeruginosa* isolates. Statistical analyses revealed a highly significant correlation between biofilm intensity and resistance pattern



( $P=0.0084$ ). Among the isolates classified as having strong biofilm formation, 13 (30.77%) were multidrug-resistant (MDR), and 5 (38.46%) were susceptible (S). As for the isolates with moderate biofilm formation, there were 11 isolates, the percentage of sensitive isolates was 4 (36.36%) and MDR 7 (63.64%), and only one isolate was weak and MDR 1 (100%), and the non-biofilm producing isolates only one was sensitive (S) 1 (100%). As shown in Table 4, the relationship between the intensity of biofilm formation and antibiotic resistance patterns.

**Table 4**  
**Relationship between biofilm and AST\* results in the patient groups**

| Biofilm results  | No of Isolates | Antibiotics result |            |
|------------------|----------------|--------------------|------------|
|                  |                | Susceptible        | *** MDR    |
| Strong           | 13             | 5 (38.46%)         | 8 (30.77%) |
| Moderate         | 11             | 4 (36.36%)         | 7 (63.64%) |
| Weak             | 1              | 0 (0.00%)          | 1 (100%)   |
| None             | 1              | 1 (100%)           | 0 (0.00%)  |
| Total            | 26             | 10                 | 16         |
| P-value          | 0.0084**       |                    |            |
| ** $P \leq 0.01$ |                |                    |            |

Notes: \* AST – Antibiotics Susceptible test, \*\*\* MDR – melty drug resistant.

### Gene Expression Analysis

The Gene expression analysis results using the RT-qPCR and livak method were applied to measure the target genes. According to [23], genes are considered differentially expressed if they exhibit a fold change of less than one. Five genes, CUP A B C D, and *fwrpoD* RNA polymerase sigma factor for normalizing as in Figure 3.

Expression of CupA and CupB showed a downregulation level, with a fold change of approximately 0.48 and 0.75 respectively. In contrast, CupC and CupD showed up regulation levels in gene expression. The fold change for CupC reached 1.89 fold, while CupD also recorded the up regulation in expression fold value among the studied genes, at 1.95 fold, indicating a significant increase in transcription of this genes in pathogenic isolates as show in Table 5.

Gene expression of the human ITGB1 gene, the results analysis using the qRT-PCR and livak method, as in figure 4.

Gene expression analysis of the ITGB1, measuring the target genes after normalizing the data to the housekeeping gene GAPDH, revealed an upregulate in gene expression levels in the group of patients infected with *Pseudomonas aeruginosa*, by 1.60-fold, compared to the control group, representing a 60% upregulation in expression levels, as in Table 6.

Figure 5 shows the fold change in expression of genes in this study associated with biofilm formation, classified according to their biofilm-forming ability. The cupA gene recorded upregulation in the weak phenotype (21.11-fold), followed by the moderate (8.37-fold) and strong (4.14-fold), while remaining actual downregulation in the nonforming (0.07-fold). The cupB gene showed upregulation expression in the strong (20.49-fold) and moderate (28.49-fold) phenotype, with no expression in the non-forming biofilm.

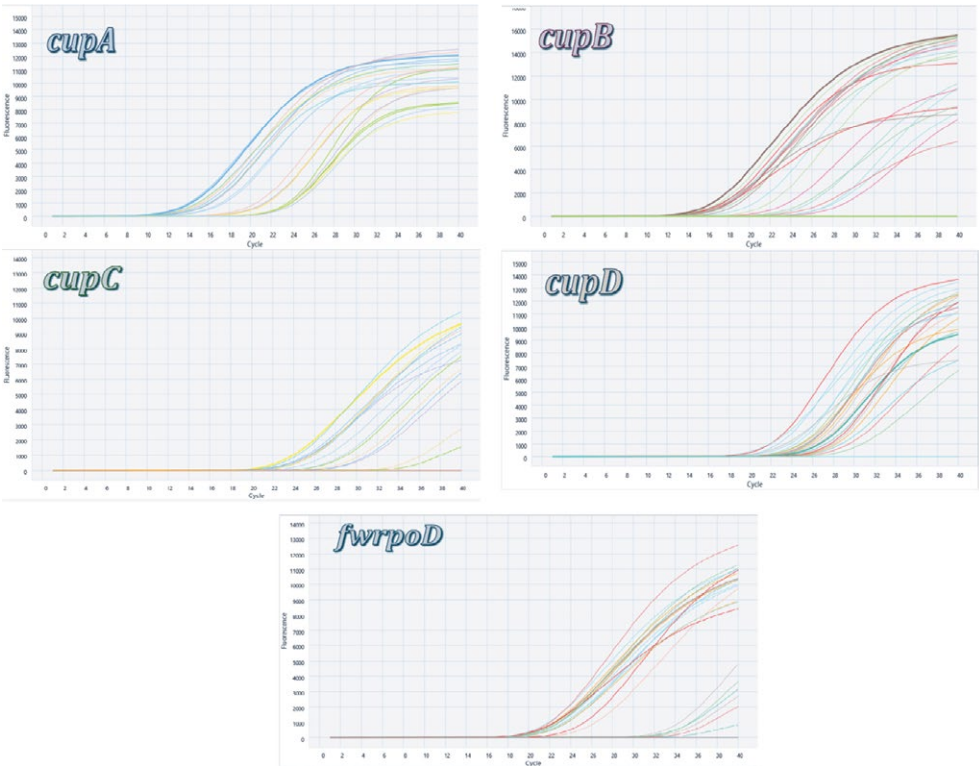


**Fig. 3.** Data from Real Time PCR and the information used to calculate *Pseudomonas aeruginosa* CT for Cup A B C D genes and fwrpoD RNA polymerase sigma factor

**Table 5**  
**Fold of CupABCD expression depending on 2<sup>-ΔΔct</sup> method**

| Study group | Mean of cup ct | Mean of fwrpoD ct | Mean of Δct | Mean of Δct calibrator | ΔΔct  | 2 <sup>-ΔΔct</sup> | Excremental group / control group | Fold of gene expression |
|-------------|----------------|-------------------|-------------|------------------------|-------|--------------------|-----------------------------------|-------------------------|
| CupA        | 20.30          | 23.17             | -2.87       | -2.92                  | 0.05  | 5.28               | 5.28/10.79                        | 0.48                    |
| CupA con.   | 0.15           | 23.31             | -5.96       | -2.92                  | -3.25 | 10.79              | 10.79/10.79                       | 1                       |
| CupB        | 20.97          | 23.08             | -2.11       | 0.04                   | -2.15 | 23.26              | 23.26/30.71                       | 0.75                    |
| CupB con.   | 20.83          | 23.31             | -2.48       | 0.04                   | -2.52 | 30.71              | 30.71/30.71                       | 1                       |
| Cup C       | 26.86          | 23.13             | 3.73        | 4.83                   | 3.83  | 4.82               | 4.38/2.31                         | 1.89                    |
| CupC con.   | 27.51          | 23.31             | 4.2         | 4.83                   | -0.63 | 2.31               | 2.31/2.31                         | 1                       |
| Cup D       | 26.45          | 23.08             | 3.37        | 3.51                   | -0.15 | 2.75               | 2.75/1.41                         | 1.95                    |
| CupD con.   | 26.49          | 23.31             | 3.18        | 3.51                   | -0.34 | 1.41               | 1.41/1.41                         | 1                       |

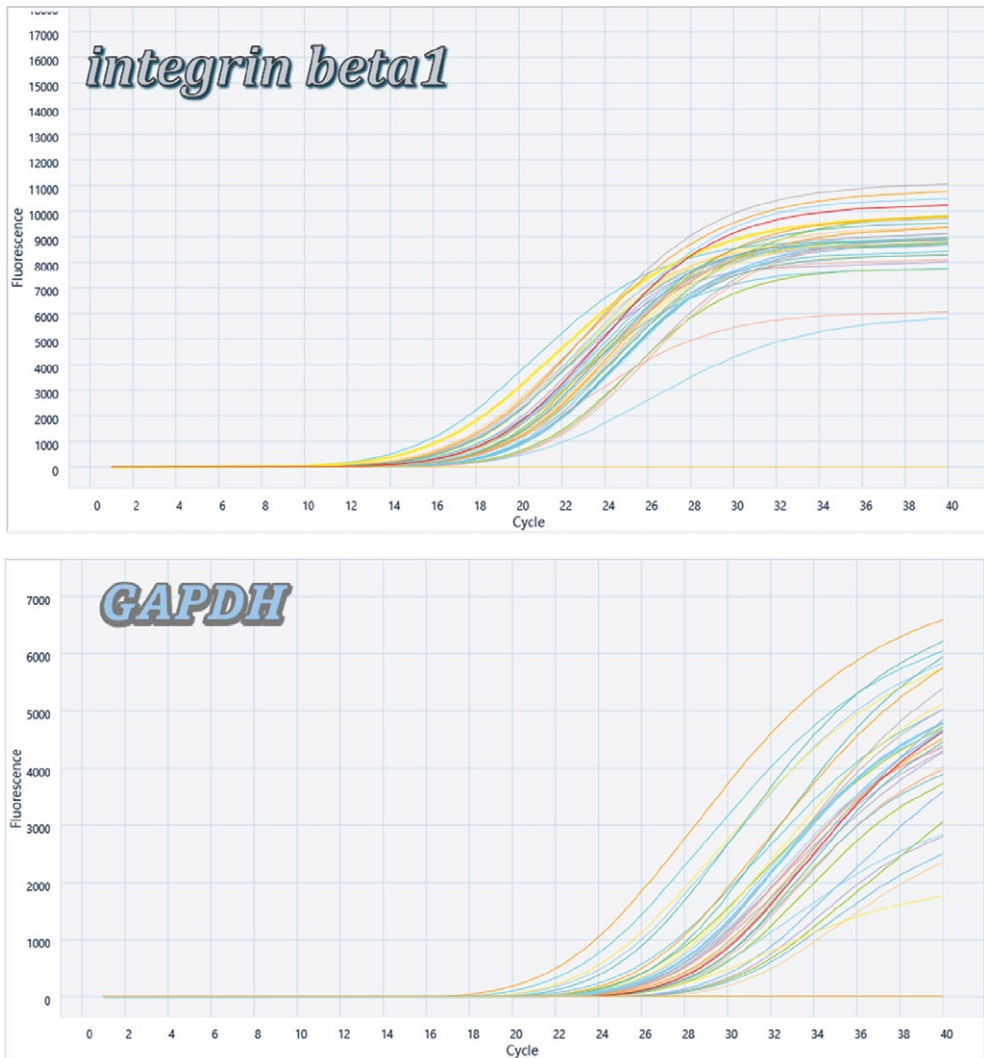


**Fig. 4.** Data from Real Time PCR and the information used to calculate CT for human ITGB1&GAPDH genes

For the cupC gene, have upregulation in expression was observed with decreasing biofilm forming ability, reaching 9.25-fold in the weak biofilm forming in competition with (4.72-fold) in the non-forming biofilm. While the cupD gene recorded the upregulation in expression in the moderate group (4.21-fold). With downexpression in other phenotypes. While the human ITGB1 gene had fluctuating expression in the biofilm formation phenotypes, the gene expression had up regulation was recorded in the weak biofilm phenotype, which was 11.39-fold.

**Table 6**  
**Fold of human ITGB1 expression depending on 2<sup>ΔΔct</sup> method**

| Study group   | Mean of ITGB1 ct | Mean of GAPDH ct | Mean of Δct | Mean of Δct calibrator | ΔΔct  | 2 <sup>Δ-ΔΔct</sup> | Excremental group / control group | Fold of gene expression |
|---------------|------------------|------------------|-------------|------------------------|-------|---------------------|-----------------------------------|-------------------------|
| Patient group | 18.53            | 28.11            | -9.58       | -8.59                  | -0.99 | 3.99                | 3.99/2.49                         | 1.60                    |
| Control group | 19.37            | 28.54            | -9.17       | -8.59                  | -0.58 | 2.49                | 2.49/2.49                         | 1                       |

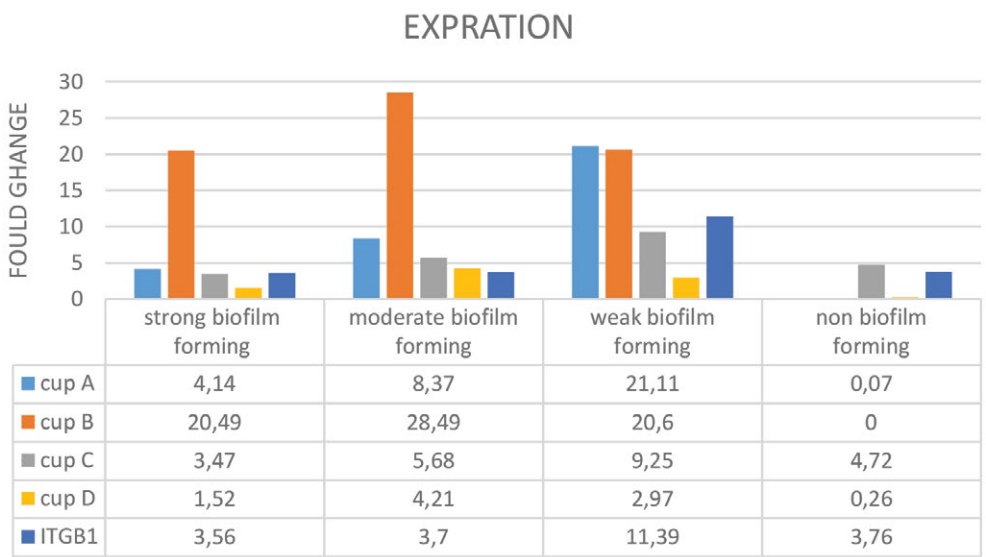


**Fig. 5. The fold change gene expiration of the cup genes and ITGB1 used in this study for biofilm-forming groups**

■ **DISCUSSION**

In most bacteria, the gyrB gene is present for its important function in catabolism and metabolism, and is transmitted horizontally like the 16S rRNA. The GyrB gene encodes the B subunit of DNA gyrase, which represents a type II topoisomerase that cleaves two DNA molecules.it is an essential and conserved gene, gyrase functions in several cellular processes such as transcription, DNA replication, and recombination [22–24]. This supports the results of the 26 isolates positive for PCR testing using the gyrB gene, this finding of corroborate to what mention in the study [25]. Who established that all isolates of P. aeruginosa possessed the gyrB gene.

The susceptibility profile of P. aeruginosa isolates to seven antimicrobial agents. The findings are consistent with the findings of recorded in other study [26] who concluded that these isolates are multidrug-resistant (MDR), posing a significant therapeutic challenge. Ceftazidime, amikacin, piperacillin, and levofloxacin are commonly used antibiotics in the empirical treatment of Pseudomonas infections; however, local investigations conducted in Iraq found significant rates of resistance to these medications [27, 28].



The findings indicate a substantial association between clinical isolates' ability to form biofilms and their multiple antibiotic resistance ( $P=0.0084$ ), with some bacteria exhibiting features that are both multidrug-resistant (MDR). This lends credence to the notion that *P. aeruginosa*'s biofilm is a critical defense mechanism that aids in its resistance. In contrast to weak or non-producing isolates, this study found that isolates capable of forming a strong or moderate biofilm have higher rates of resistance. The extracellular matrix (EPS) of biofilm, operates as a physical barrier, preventing many antibiotics from accessing the membrane's depths, as demonstrated by [29]. Other study which illustrated that biofilms promote the transfer of resistance genes among microbial communities and provide protection against antibiotics through the formation of chemical and physical barriers [30].

The study [31], identifies several potential factors contributing to the downregulation of *cupA* gene expression in strong and moderate biofilms. The *cupA* system is considered to affect the production of biofilms early on and independently of type IVP Pili. The expression of the exopolysaccharide protein Pel, which is located in the biofilm matrix, stops *cupA* from working since it is stopped after biofilm formation. as well as *MvaT* and *MvaU* are strong negative regulators that make sure *cupA* is only expressed in certain situations. The upregulation in of *cupA* in weak biofilms may result from its expression not only during the first stages of biofilm formation and primary adhesion but also during adherence to abiotic surfaces. The findings indicated that upregulation of *cupB* genes correlated with strong and moderate biofilm development. The intermediate expression of the *cupC* gene, aligning with the findings of [6]. which indicated that both the *CupB* and *CupC* systems are crucial in this process, particularly via bacterial aggregation and microcolony formation, also that not having the *cupB3* or *cupC3* genes could make it harder for biofilms to form worldwide regulatory element [32] work encourages the synthesis of the *CupB* and *CupC* pili, which are essential parts of the biofilm matrix, as well as the exopolysaccharide Pel. This clarifies how *CupB* and *CupC* pili work together with exopolysaccharides like (alginate, Pel, and Psl). This clarifies why, the *CupC* gene is downregulated and the *CupB* gene is not expressed. the expression of *cupD* gene remains comparatively low in strong, moderate, and weak biofilms. This is consistent with the findings of [33]. Consequently, *cupD* gene expression is minimal across all biofilm phenotypes due to stringent negative regulation and the necessity for specific, often absent environmental signals.

ITGB1 gene showed up regulation and fluctuated in expression across all phenotypes of biofilm formation, especially in the weak biofilm (11.39-fold). This consistent with the results of [34] The study's findings indicate that elevated gene expression of ITGB1 in patients, as a physiological response to tissue repair, may heighten the risk of bacterial colonization and infection development, particularly from *P. aeruginosa*, which is known to adhere to ITGB1, and intermediate biofilm-forming bacterial patterns associated with wound colonization, considering the three-dimensional interplay of inflammation, infection, and healing, precise regulation of this gene's expression may represent a critical axis that concur with [10]. *P. aeruginosa* adheres to ITGB1 subunit on host epithelial cells through several mechanisms, one key pathway is mediated by the bacterial protein PiliY1, which contains an RGD is a short a.a motif that directly binds to integrin, including beta1, acts as a cell adhesion sequence facilitating bacterial attachment to host cells [15]. The *P. aeruginosa* lectin is a fucose-specific lectin named LecB, which clears integrins from the surface of cells at the wound margin and blocks cell migration and wound healing

in a dose-dependent manner [34]. The bacterial lectin LecB binds directly to glycosylated ITGB1 on the basolateral membrane, causing their internalization and disrupting cell adhesion, to help the bacteria move beneath epithelial cells [35]. The bacterium has evolved ways of manipulating host epithelial cell polarity to promote its infection [36].

## ■ CONCLUSION

The CUP A gene expression, the upregulation is in the weak biofilm phenotype. The Cup C and Cup D genes, gene expression is relatively down regulation. As for the human integrin beta one gene, it recorded fluctuated expression in all biofilm phenotypes, and the upregulation is recorded in the weak biofilm phenotype. These findings imply the possibility of therapeutic targeting to lessen persistent *P. aeruginosa* infections and emphasize the role goblet genes play in fostering virulence and resistance within biofilms.

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## Relationship and Gene Expression between *fsr*-QS Genes, Gelatinase Enzyme, and Biofilm Formation in *Enterococcus Faecalis*

**Conflict of interest:** nothing to declare.

**Authors' contribution:** Waad Khalil Hameed – conceptualization, data curation, investigation, method, project administration, resources, writing – original draft and writing – review & editing; Majid Shaii Hamdalla – conceptualization, data curation, validation, visualization, writing – original draft and writing – review & editing; Ashwak Bassim Al-Hashimy – conceptualization, data curation, validation, visualization, writing – original draft and writing – review & editing.

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### Abstract

**Introduction.** *Enterococcus faecalis* is a significant cause of hospital-acquired infection (HAI) through mechanisms that enhance pathogenicity, known as virulence factors, such as the *gelE* gene, which plays a role in biofilm formation and is regulated by a quorum-sensing system.

**Purpose.** To detect virulence genes in *E. faecalis*, assess gelatinase production and biofilm formation, analyze the relationship between *fsr* genes and these factors in patients with urinary tract infections, and evaluate the level of *gelE* gene expression in the presence or absence of *fsrA* gene.

**Materials and methods.** About 150 urine samples were collected from patients aged 24-58 diagnosed with UTI. Both Culture methods and the VITEK-2 system were employed to identify *Enterococcus* spp. Polymerase chain reaction (PCR) was performed to confirm *E. faecalis* using housekeeping genes 16S rRNA and *ddl* *E. faecalis*, then molecular detection of *fsrA*, *fsrB*, and *gelE* genes. Biofilm formation was measured using the crystal violet technique, and gelatinase activity was assessed by stabbing gelatin tubes. For gene expression, use one-step qRT-PCR using housekeeping gene 16S rRNA as a reference by *syber green*.

**Results.** Showed that 28 (18.6%) isolates were positive for *E. faecalis* and 16S rRNA, *ddl* genes detected in all *E. faecalis* isolates at 100%. The *fsrA* gene was detected in 85.71% and 100% for *fsrB*, *gelE*, respectively. The detection phenotype showed that all isolates formed biofilm with different intensities: 16 (57.14%) were strong biofilm producers, 10 (35.71%) moderate, and 2 (7.14%) weak. About gelatin hydrolysis, a phenotypic test revealed (85.71%) positive results. In the absence of *fsrA* gene isolates, the fold change for *gelE* at (0.474) indicates a decrease in gene expression.

**Conclusion.** Our study reveals a regulatory relationship between the *fsr*-QS system and the *gelE* gene, biofilm formation.

**Keywords:** *E. faecalis*, *fsrA*, *fsrB*, *gelE*, Biofilm formation, gelatinase, UTI



## ■ INTRODUCTION

*Enterococcus faecalis* is a causative agent of UTIs worldwide, including in Iraq, despite being a commensal bacterium of the human intestine. It can cause different diseases due to its ability to produce various virulence factors [1]. One of their virulence factors, gelatinase (gelE). The gelatinase enzyme, encoded by the gelE gene, is an extracellular metalloprotease that hydrolyses host proteins, including gelatin, collagen, and haemoglobin, which also play important roles in pathogenic potential and biofilm formation [2, 3]. Biofilms are defined as biotic or abiotic surface-attached microbial consortia that play a major role in the pathogenicity of UTIs and have multiple stages, such as initial reversible attachment, production of an extracellular polymeric matrix (EPM) including proteins, polysaccharides, and nucleic acids, and irreversible attachment [4]. Biofilms promote bacterial growth, reproduction, and enhance bacterial viability by allowing for the exchange of nutrients and signaling molecules, aid in bacterial adhesion and localization in specific settings, also optimize bacterial resource utilization and adaptation [5].

The signaling molecules, known as pheromones, are released by the quorum-sensing system that induces an intercellular response among members of bacterial communities [6]. The *fsr* locus contains four genes: *fsrD*, *fsrB*, *fsrC*, and *fsrA*. The *fsrD* gene encodes for the propeptide that is processed to produce the gelatinase biosynthesis-activating pheromone (GBAP) with the help of the *fsrB* gene following its extracellular release [7]. The histidine kinase *fsrC* gene, located on the surface of *E. faecalis*, senses the local accumulation of GBAP and then activates the response regulator *fsrA* gene by phosphorylation. This in turn, activates the expression of the gelE gene within the *fsr* locus [7, 8].

## ■ PURPOSE OF THE STUDY

To detect virulence genes in *E. faecalis*, assess gelatinase production and biofilm formation, analyze the relationship between *fsr* genes and these factors in patients with urinary tract infections, and evaluate the level of gelE gene expression in the presence or absence of *fsrA* gene.

## ■ MATERIALS AND METHODS

### **Isolation and Identification of Enterococcus Bacteria**

Urine samples were collected from patients with clinically diagnosed UTI in three Baghdad hospitals (Ibn-Balady, Al Yarmouk, Medical City) between January and March 2025. Midstream urine was obtained in sterile screw-cap containers after withholding antibiotics for 3–4 days, labeled (sex, number, date), and mixed on receipt.

One aliquot was examined microscopically for pyuria, and another was cultured quantitatively with a calibrated loop on blood agar, bile esculin agar, and HiCrome™ UTI agar, then incubated at 37 °C for 24 h. Growth was evaluated, and isolates initially identified by colony morphology and Gram stain. After that, Gram-positive cocci were identified based on their ability to degrade esculin. Suspected enterococci were confirmed using the VITEK-2 system, re-culture on HiCrome UTI agar, and molecular methods.

### **Phenotypic Detection of Enterococcal Virulence Factors**

#### **Gelatin Hydrolysis**

Nutrient gelatin was prepared as described in [9]. *E. faecalis* isolates were inoculated and incubated at 37 °C for 24–27 h, then refrigerated at 4 °C for 30 min. Tubes that

remained liquid were considered gelatinase positive, whereas re-solidified tubes were considered negative.

**Biofilm Formation**

Biofilm formation by *E. faecalis* was assessed using the microtiter plate assay. Overnight cultures in brain heart infusion broth at 37 °C were diluted in tryptic soy broth with 1% glucose and adjusted to 0.5 McFarland. For each isolate, 200 µl were dispensed in triplicate into U-bottom 96-well plates and incubated aerobically at 37 °C for 24 h. Wells were washed twice with distilled water, fixed with 200 µl absolute methanol for 20 min, stained with 0.1% crystal violet for 15 min, washed 2–3 times, and air-dried for about 30 min. Bound dye was solubilized with 33% acid, and OD at 630 nm was measured using an ELISA reader. The mean OD of the sterile medium was subtracted from test values, and an OD cutoff (ODc) was used to classify biofilm-forming isolates.

**DNA Extraction**

Genomic DNA was extracted from *E. faecalis* isolates using the DNA extraction kit (Presto™ Mini) according to the manufacturer’s instructions. and concentration was measured with a Quantus fluorometer after mixing 1 µl DNA with 200 µl dye, using fluorescence to calculate concentration [10].

**Preparation of Primers**

Utilizing specific primers prepared in accordance with the manufacturer’s instructions (Macrogen, Korea). The primers were utilized for the detection of the following genes: 16S rRNA, *ddl*, *fsrA*, *fsrB*, *gelE* *faecalis*, as detailed in Table 1.

The PCR reaction component for the amplification of the (16S rRNA, *ddl*, *fsrA*, *fsrB*, *gelE*) genes was achieved using the following mixtures in the Table 2.

**Identification of *Enterococcus Faecalis* by PCR**

In conventional PCR, two genes were used to identify *E. faecalis* (16S rRNA and *ddl* *E. faecalis*). Optimization of the PCR program for amplification of (16S rRNA and *ddl* *E. faecalis*) genes was accomplished after several trials; thus, the following program was adopted for *E. faecalis* isolates (Table 3).

**Table 1**  
**Primer Sequences and Product Sizes for the Studied Genes**

| Target Genes |   | Nucleotide Sequence (5′—3′) | Size Product Bp | Reference |
|--------------|---|-----------------------------|-----------------|-----------|
| 16S rRNA     | F | GAAGGGGAGTGAAATAGATCC       | 290             | [11]      |
| 16S rRNA     | R | GGGGACGTTCAGTTACTAACGT      |                 |           |
| ddl          | F | ATCAAGTACAGTTAGTCTT         | 941             | [12]      |
| ddl          | R | ACGATTCAAAGCTAACTG          |                 |           |
| fsrA         | F | CGTCCGTCTCTCATAGTTA         | 474             | [13]      |
| fsrA         | R | GCAGGATTGAGGTTGCTAA         |                 |           |
| fsrB         | F | TAATCTAGGCTTAGTCCAC         | 428             |           |
| fsrB         | R | CTAAATGGCTCTGTCGTCTAG       |                 |           |
| gelE         | F | CGTGAAGAAGTTACTCTGAC        | 704             |           |
| gelE         | R | GGTATTGAGTTATGAGGGGC        |                 |           |



**Table 2**  
**The PCR Reaction Components Used in This Study**

| Component           | 25 µl (final volume) |
|---------------------|----------------------|
| Master Mix          | 12.5µl               |
| Forward Primer      | 10 picomol/µl (1 µl) |
| Reverse Primer      | 10 picomol/µl (1 µl) |
| DNA                 | 2 ml                 |
| Nuclease-Free Water | 8.5 ml               |
| Total Volume        | 25 ml                |

### Molecular Detection of *gelE*, *fsrA*, and *fsrB* Genes

After identifying the genes of *E faecalis*, *fsrA*, *fsrB*, and *gelE* genes were amplified using PCR, and then electrophoresis was used to determine the presence of the target genes (Table 4).

### Gene Expression Analysis Using the qRT-PCR Technique

The target gene (*gelE*) and the housekeeping gene (16S rRNA) were amplified using qRT-PCR. The gene expression level of the *gelE* gene was measured in isolates containing the *fsrA* gene and compared to those without it (*fsrA* as a control), where the amount of amplified output indicates the degree of gene expression.

### RNA Extraction

A special kit was used to extract RNA from bacterial isolates (GENEzol™ TriRNA Pure Kit) according to the manufacturer's instructions.

**Table 3**  
**Optimal PCR Program for Detection of 16S rRNA, *ddl E. faecalis* genes by Conventional PCR**

| Genes                   | Step                 | Tm (°C) | Time    | No. of cycle |
|-------------------------|----------------------|---------|---------|--------------|
| 16S rRNA                | Initial Denaturation | 95      | 5 min   | 1 cycle      |
|                         | Denaturation         | 95      | 1 min   | 30 cycle     |
|                         | Annealing            | 60      | 1 min   |              |
|                         | Extension            | 72      | 1 min   |              |
|                         | Final extension      | 72      | 5 min   | 1 cycle      |
| <i>ddl E faecalis</i> , | Initial Denaturation | 95      | 2 min   | 1 cycle      |
|                         | Denaturation         | 95      | 35 sec  | 30 cycle     |
|                         | Annealing            | 55      | 30 sec  |              |
|                         | Extension            | 72      | 100 ses |              |
|                         | Final extension      | 72      | 5 min   | 1 cycle      |

**Table 4**  
**Optimal PCR Program for Detection of *fsrA*, *fsrB*, and *gelE* Genes by Conventional PCR**

| Genes                                     | Step                 | Tm (°C) | Time    | No of cycle |
|-------------------------------------------|----------------------|---------|---------|-------------|
| <i>fsrA</i><br><i>fsrB</i><br><i>gelE</i> | Initial Denaturation | 95      | 2 min   | 1 cycle     |
|                                           | Denaturation         | 95      | 35 sec  | 30 cycle    |
|                                           | Annealing            | 58      | 30 sec  |             |
|                                           | Extension            | 72      | 100 ses |             |
|                                           | Final extension      | 72      | 5 min   | 1 cycle     |

Preparation of Primers

Primers used for qRT-PCR are listed in Table 5, gelE 300pb, newly designed for gene expression using Geneious Prime (Table 5).

Table 5  
Primer sequences used in qRT-PCR in this study

| Target Genes |   | Nucleotide Sequence (5'—3') | Size Product Bp | Reference      |
|--------------|---|-----------------------------|-----------------|----------------|
| gelE         | F | AGAAAAGCAAACGGAAGGCG        | 300             | Study designed |
| gelE         | R | ACCAATGATACGGGGTCACC        |                 |                |

One-Step Quantitative Real-Time PCR Assay (qRT-PCR)  
Reaction Setup and Thermal Cycling Protocol

The components of the RT-PCR mixtures are listed in the table below. the housekeeping gene 16S rRNA was used as an internal control (Table 6).

Table 6  
Shows the components of qRT-PCR

| Component                                 | Volume    | Final concentration |
|-------------------------------------------|-----------|---------------------|
| Luna Universal One-Step Reaction Mix (2X) | 10        | 1X                  |
| Luna WarmStart® RT Enzyme Mix (20X)       | 1         | 1X                  |
| Forward primer (10 pmol/μl)               | 0.8       | 0.4 μM              |
| Reverse primer (10 pmol/μl)               | 0.8       | 0.4 μM              |
| Template RNA                              | Available | 20 ng/ul            |
| Nuclease-free water                       | To 20     |                     |

Quantitative Real-Time PCR (qRT-PCR)

All reaction components were thawed at room temperature, mixed, and combined in a sterile PCR tube. After adding the RNA template, the tubes were sealed, spun to remove bubbles, and placed in the thermocycler according to the specified program (Table 7).

Table 7  
Program used in the study

| Cycle step            | Temperature (°C) | Time    | Cycle |
|-----------------------|------------------|---------|-------|
| Reverse Transcription | 55               | 10 min  | 1     |
| Initial Denaturation  | 95               | 1 min   | 1     |
| Denaturation          | 95               | 10 sec  | 40    |
| Extension             | 60               | 30 sec  |       |
| Melt curve            | 60-95            | Various | 1     |

Data Analysis of qRT-PCR

Gene expression levels were measured as fold changes using the ΔΔCT method, which calculates the difference in threshold cycle values (ΔCT) between target and reference genes for each sample Schmittgen and Livak [14].



## ■ RESULTS

One hundred and fifty (150) urine samples were collected from patients of different ages 24–58, Among them, 57 were males, and 93 were females, respectively. A total of 150 urine samples, 28 of which were positive for *E. faecalis*, were identified according to characteristics by using selective and differential media, the VITEK-2 system, and the isolate was confirmed using molecular methods.

### Isolation and Identification of *Enterococcus Faecalis*

Following the activation of pathological samples in BHI broth, bacterial growth resulted in turbidity. *Enterococcus* was isolated on blood agar, characterized by white to grey colonies with a sticky texture, predominantly  $\gamma$ -hemolytic [15].

Subsequent culturing on selective media, bile esculin agar, and chromogenic UTI agar yielded round colonies with convex edges. On bile esculin agar, a black halo formed due to the hydrolysis of esculin, while colonies on chromogenic UTI agar displayed blue due to  $\beta$ -glucosidase activity. The VITEK-2 compact system diagnosed *E. faecalis* in the respective isolates grown on blood agar (Table 8).

**Table 8**  
Shows the percentage of *E. Faecalis* isolates identified using different approaches

| The test                 | No. of isolates | %     |
|--------------------------|-----------------|-------|
| Grown on selective media | 47              | 31.33 |
| VITEK-2 system           | 28              | 18.67 |
| Chromogenic media        | 28              | 18.67 |

### Characterization of *Enterococcal* Virulence Factors

#### Gelatin Hydrolysis

Gelatin hydrolysis is a classical assay used to assess the ability of bacteria to produce gelatinases that degrade the protein gelatin into soluble peptides and amino acids. All clinical *E. faecalis* isolates were tested for gelatinase activity. 85.71% were capable of forming gelatinase.

#### Biofilm Formation

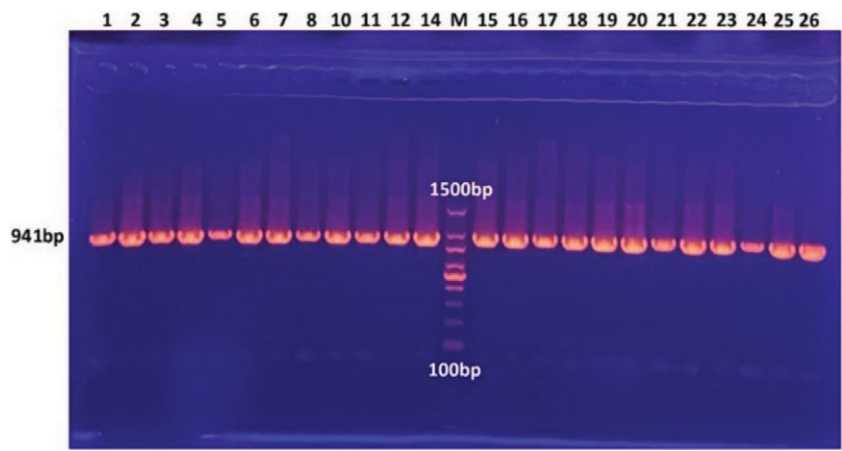
Using a microtiter plate method, all *E. faecalis* isolates demonstrated the ability to form biofilms 100%. but at varying levels, ranging from strong to moderate – weak. Strong biofilm-forming isolates constituted 16 (57.14%) of the isolates, while moderate biofilm-forming isolates constituted 10 (35.71%) biofilm-forming isolates and weak biofilm-forming isolates constituted 2 (7.14%). Biofilm classification followed the method by Stepanović et al. [16], calculating the Optical Density cut-off (OD<sub>c</sub>) as the mean OD of the negative control plus three standard deviations. Results were categorized into three groups of biofilm producers: strong (Table 9).

### Molecular Detection of *Enterococcus Faecalis* by Conventional PCR

The polymerase chain reaction (PCR) technique was used to identify *E. faecalis* using specific primers prepared according to the manufacturer's instructions (Macrogen, Korea). The target gene was detected using gel electrophoresis. The result showed the presence of 16S rRNA and *ddl* genes in all bacterial isolates at 100%. The figure shows the *ddl* gene.

**Table 9**  
**This shows the Classification of biofilm formation**

| Mean OD values              | Adherence  | Biofilm formation |
|-----------------------------|------------|-------------------|
| ≤ODc                        | None       | 0                 |
| ODc < OD ≤ 2ODc             | Weak       | 2 (7.14%)         |
| 2ODc < OD ≤ 4ODc            | Moderately | 10 (35.71%)       |
| 4ODc < OD                   | Strong     | 16 (57.14%)       |
| Total                       |            | 28                |
|                             |            | 10.57             |
| P-value                     |            | 0.005             |
| (P≤0.01) Highly Significant |            |                   |



**Gel electrophoresis of the *ddl* gene of *E.faecalis*, line 1–26 showing a specific band at 941 bp (80 volts for 1 hour).using 1% agarose gel and visualized under UV light after ethidium bromide staining. Lane M: DNA ladder; Lan**

Then detection of the *fsrA* and *fsrB*, *gelE* genes was performed using the conventional PCR. The result showed that the band *fsrA* gene was detected at 474 bp in 24 isolates (85.7%), while the bands *fsrB* and *gelE* were present in all 28 isolates (100%).

**The Relationship Between *fsrA*, *fsrB*, *gelE*, Gelatin Hydrolysis, and Biofilm Intensities**

The relationship between virulence determinants and phenotypic traits is fundamental to understanding the pathogenicity of *E. faecalis*, as shown in Table 10.

**Quantitative Real-Time-PCR  
Transcriptional Analysis by Quantitative Real-Time PCR**

Real-time quantitative PCR (RT-qPCR) was employed to assess the expression of the *gelE* gene, using the housekeeping gene 16S rRNA for reference. This process included the detection of double-stranded DNA, including cDNA, via the fluorescent dye SYBR green. The cycle threshold (Ct) value was measured, with higher Ct values indicating lower gene expression and lower Ct values indicating higher gene expression [14].

**Table 10****Shows the relationship between *fsrA*, *fsrB*, and *gelE* with phenotypic traits**

| No    | <i>fsrA</i> | <i>fsrB</i> | <i>gelE</i> | Biofilm Intensity | Gelatine hydrolysis |
|-------|-------------|-------------|-------------|-------------------|---------------------|
| E.f1  | –           | +           | +           | Weak              | –                   |
| E.f2  | +           | +           | +           | Strong            | +                   |
| E.f3  | +           | +           | +           | Moderate          | +                   |
| E.f4  | –           | +           | +           | Moderate          | –                   |
| E.f5  | +           | +           | +           | Weak              | +                   |
| E.f6  | +           | +           | +           | Strong            | +                   |
| E.f7  | +           | +           | +           | Strong            | +                   |
| E.F8  | –           | +           | +           | Strong            | –                   |
| E.f9  | +           | +           | +           | Strong            | +                   |
| E.F10 | +           | +           | +           | Strong            | +                   |
| E.F11 | +           | +           | +           | Strong            | +                   |
| E.F12 | +           | +           | +           | Strong            | +                   |
| E.F13 | –           | +           | +           | Strong            | –                   |
| E.F14 | +           | +           | +           | Strong            | +                   |
| E.F15 | +           | +           | +           | Strong            | +                   |
| E.F16 | +           | +           | +           | Strong            | +                   |
| E.F17 | +           | +           | +           | Strong            | +                   |
| E.F18 | +           | +           | +           | Moderate          | +                   |
| E.F19 | +           | +           | +           | Moderate          | +                   |
| E.F20 | +           | +           | +           | Strong            | +                   |
| E.F21 | +           | +           | +           | Moderate          | +                   |
| E.F22 | +           | +           | +           | Moderate          | +                   |
| E.F23 | +           | +           | +           | Moderate          | +                   |
| E.F24 | +           | +           | +           | Moderate          | +                   |
| E.F25 | +           | +           | +           | Moderate          | +                   |
| E.F26 | +           | +           | +           | Moderate          | +                   |
| E.F27 | +           | +           | +           | Strong            | +                   |
| E.F28 | +           | +           | +           | Strong            | +                   |

**Table 11****Shows the isolates which selected for gene expression**

| No   | <i>fsrA</i> | <i>fsrB</i> | <i>gelE</i> | Biofilm Intensity | Gelatin hydrolysis |
|------|-------------|-------------|-------------|-------------------|--------------------|
| E.f1 | –           | +           | +           | Weak              | –                  |
| E.f3 | +           | +           | +           | Moderate          | +                  |
| E.f4 | –           | +           | +           | Moderate          | –                  |
| E.F8 | –           | +           | +           | Strong            | –                  |
| E.f9 | +           | +           | +           | Strong            | +                  |

**Table 12**  
**Gene Expression for *gelE* with and without *fsrA***

| First group contains <i>fsr</i> A (3,9) for <i>gelE</i> isolate |      |             |             |                 | Second group without <i>fsrA</i> (1,4,8) for the <i>gelE</i> isolate |       |             |             |                 | $\Delta\Delta Ct$ | Fold change       |
|-----------------------------------------------------------------|------|-------------|-------------|-----------------|----------------------------------------------------------------------|-------|-------------|-------------|-----------------|-------------------|-------------------|
| ID                                                              | Hg   | <i>gelE</i> | $\Delta Ct$ | Avg $\Delta Ct$ | ID                                                                   | Hg    | <i>gelE</i> | $\Delta Ct$ | Avg $\Delta Ct$ |                   |                   |
| 3                                                               | 10   | 26.86       | 16.86       | 17.66           | 1                                                                    | 10.2  | 28.87       | 18.67       | 18.73           | 1.07              | 0.474<br>Decrease |
| 9                                                               | 7.71 | 26.17       | 18.46       |                 | 4                                                                    | 11.55 | 27.95       | 16.4        |                 |                   |                   |
|                                                                 |      |             |             |                 | 8                                                                    | 9.2   | 30.34       | 21.14       |                 |                   |                   |

Notes: ID = Isolate code, Hg = housekeeping gene, Avg  $\Delta Ct$  = average  $\Delta Ct$

Five isolates in the table 11 were selected for gene expression (*gelE*) based on the presence or absence of the *fsrA* gene and based on biofilm density, gelatin activity, as detailed in the table 12.

■ DISCUSSION

A total of 150 urine samples were collected from Baghdad hospitals. All samples were cultured on bile esculin agar, and selected isolates matched the selective/differential properties of the medium, resulting in 47 suspected *E. faecalis* isolates. These bacterial isolates were tested using the VITEK-2 compact system, and 28 isolates were positive for *E. faecalis* and excluded other bacterial species that share some characteristics, such as *Enterococcus* spp. and Group D *Streptococcus*. For further confirmation, the isolates were cultured on chromogenic UTI agar, and the result compatible with the VITEK-2 compact system was obtained. The percentage of *E. faecalis* isolated in our study was 18.6%, which is nearly identical to the results of a study in Iraq by Mazhar et al. [17] and Al-jmor [18] showed that the percentage of *E. faecalis* isolated from urine was 22.63% and 20.6%, respectively. The result by Abdullah [19] was 11.0%; While other Iraqi studies showed higher percentages, such as Kadhem and Flayyih [20] was 46.6%, which is far from our study. These differences in isolate percentages may stem from variations in sample size, urine collection sites, and diagnostic procedures used across the studies.

The gelatin degradation results obtained in our study showed results somewhat similar to those of Yang et al. [21] (73.3%). However, they differed significantly from those of Al-Hilali et al. [22] and Hashim et al. [23], which reached 30% and 22% respectively.

Regarding the biofilm results, this study is consistent with that of Shahfeh et al. [24], who found that 80% of *E. faecalis* isolates were strong biofilm-forming, 12% moderate, and 8% weak. In contrast, Khodair [25] reported 13.63% strong, 54.54% moderate, and 31.81% weak biofilm formation, which differs from the current results. Another study by Ghareeb [26] identified 28.12% strong, 34.37% moderate, and 37.5% weak biofilm production, highlighting significant variability in biofilm production compared to the current study results.

At the molecular level, the current findings are identical to those of Azhar and Hussein [27] and Hadi et al. [28], who detected *Enterococcus* using 16S rRNA, as well as those of Al-Halaby et al. [29]; Dhahir [30]; Auhim et al. [31], who utilized the *ddl* gene for molecular detection of *E. faecalis*.The results showed that the band *fsrA* gene was found at 474 bp in 24 (85.71%) of the *E. faecalis* isolates. This percentage differs from that reported by



Hashem et al. [23] and Abdel-Rahman et al. [32], which were (42%) and (26%), respectively, indicating significant variation in the distribution of the *fsrA* gene among different studies and diverse clinical settings.

This may be attributed to the limited number of studies specifically focusing on the distribution of the *fsrA* gene in UTIs of *E. faecalis*. While the result showed that the band *fsrB* gene was detected at 428 bp, present in all isolates 28(100%). The current study's findings matched those of Stpien-Pysniak et al. [33], who showed that *fsrB* was present in 100% of *E. faecalis* isolates. However, the findings of a research by Gök et al. [34] showed that the *fsrB* gene was present in 11.8% of *E. faecalis* isolates, which contradicts our findings.

The result showed that the band *gelE* gene was detected in a 704 bp in 28 (100%) of *E. faecalis* isolates that contained the *gelE* gene, identical to studies by Hashem et al. [13], who showed that 100% of *gelE* gene. Another study by Coskun [35] found that the *gelE* gene was present in 22.8% of the isolates, which contradicts our findings.

To illustrate the relationship between genes and phenotypic traits such as gelatinase degradation and biofilm density, the *fsrB* gene is essential for the bacterial quorum-sensing system, processing the GBAP peptide post-release. The *fsrB* gene is more prevalent than *fsrA*, with no reports of *fsrA* existing without *fsrB*, which aligns with our study [13]. The presence of the *gelE* gene in all isolates (genotype) does not necessarily imply functional expression of gelatinase (phenotype). The gene may be present but not functionally expressed [36]. This is what we observed in our study: the *gelE* gene was present in 100% of cases, while gelatinase was present in 85.71% of cases. This is consistent with the study by Hashem et al. [13], who found that detecting *gelE* in *E. faecalis* was not sufficient to predict gelatinase activity.

Many studies have linked the presence of the *gelE* gene to biofilm formation, meaning that its presence gives a genetic predisposition to biofilm formation, and this is what studies have proven by Hancock and Perego. [37], Saffari et al. [38] and Pirbonyeh et al. [39], and this is consistent with our study, which shows the association between the presence of genes )genotype( level associated with biofilm formation.

As for gelatin enzyme, it is present in most isolates with the exception of four isolates (1, 4, 8, 13) that also lack the *fsrA* gene. This suggests a role for the *fsrA* gene in gelatinase expression. This is consistent with a study that indicated that loss of expression of the virulence factor was associated with the loss of one or more of the regulatory *fsr* operon genes, despite the presence of the *gelE* gene, indicating that a complete *fsr* operon is required for a *gelE*-positive phenotype [40].

Isolate No. 5, despite possessing *fsrA*, *fsrB*, *gelE*, and gelatinase, appears to have a weak biofilm. This may be due to these genes making bacteria more likely to form biofilms, but they are not enough to make a strong biofilm on their own.

In the gene expression, we observed a significant decrease in *gelE* gene expression in isolates lacking the *fsrA* gene, with a fold change of 0.47. This decrease demonstrates that the regulatory component of the *fsrA* gene is crucial for maximizing *gelE* transcription. This supports that the quorum-sensing system of the *fsr* gene positively regulates *gelE* expression. This suggests that the FSR system genes may be autorganized and that they regulate the gene expression of both *gelE* and *sprE*. Any defect or deficiency in one of the system's components (*fsrA*, *fsrB*, *fsrC*, *gelE*, or *sprE*) leads to a loss of gene expression. This is consistent with the study by Qin et al. [41] and Ashraf et al. [7].

## ■ CONCLUSION

Our study reveals a regulatory relationship between the *fsr*-QS system and the *gelE* gene, highlighting the necessity of both *fsrA* and *fsrB* genes for effective gelatinase production and biofilm development, instead of depending only on the *gelE* gene. These results support the concept that the FSR system plays a critical role in regulating virulence factors associated with *E. faecalis* biofilm formation and proteolytic activity.

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# Evaluation of in Vitro Biological Potency of Glycolipid against Some Indicator Pathogens Isolated from Urinary Tract

**Conflict of interest:** nothing to declare.

**Authors' contribution:** Oulan Alaauldeen Hussien – conceptualization, data curation, investigation, method, project administration, resources, software, writing – original draft and writing – review & editing; Nadhem Hasan Haydar – conceptualization, data curation, investigation, supervision, validation, visualization, writing – original draft and writing – review & editing.

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## Abstract

**Introduction.** Glycolipid-biosurfactant (BS) produced by *Lactobacillus plantarum* O91, a strain isolated from vaginal swabs of healthy Iraqi women. Glycolipid biosurfactants synthesized by LAB have attracted increasing scientific interest due to their antimicrobial and antibiofilm activities, as well as their potential applications in medical and biotechnological sectors.

**Purpose.** To optimize, characterize and biologically evaluate a glycolipid biosurfactant (BS) produced by *Lactobacillus plantarum* O91.

**Materials and methods.** Production parameters were optimized by evaluating different nutritional and environmental conditions. Lactose (1.5%) and meat extract (2%) were identified as the most efficient carbon and nitrogen sources, respectively, while pH 5 supported the highest emulsification activity. Following production, the biosurfactant was purified and examined using FTIR spectroscopy, which revealed characteristic aliphatic and polysaccharide-associated functional groups confirming its glycolipid structure. Antimicrobial and antibiofilm activities were assessed by testing different BS concentrations (100–200 mg/mL) against *Candida albicans*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli*. Biofilm inhibition assays were also performed

**Results.** The purified biosurfactant demonstrated clear FTIR spectral signatures consistent with glycolipid compounds. Biologically, it exhibited broad-spectrum antimicrobial activity, producing inhibition zones that ranged from moderate to high across the tested pathogens. The glycolipid significantly disrupted preformed biofilms and reduced microbial adhesion, with *S. aureus* showing the highest sensitivity in both assays. Collectively, these findings indicate that glycolipid biosurfactants produced by *L. plantarum* O91 represent promising natural antimicrobial and anti-biofilm agents with potential applications in medical, pharmaceutical, and biotechnological fields.

**Conclusion.** This study confirms *Lactobacillus plantarum* O91 as a potent glycolipid biosurfactant producer with strong antimicrobial and antibiofilm activities. Optimized



conditions (lactose, meat extract, pH 5, 72 h) maximized yield, highlighting its potential as a sustainable biomedical alternative to conventional therapies.

**Keywords:** Lactobacillus, optimization, GL, antimicrobial, anti-biofilm

## ■ INTRODUCTION

Lactobacillus species are non-spore-forming, Gram-positive coccobacilli or rods that generally inhabit carbohydrate-rich environments. They constitute a major component of the normal microbiota of the oral cavity, gastrointestinal tract, and female genital tract, and many strains are recognized for their probiotic capacity. Beyond their traditional roles, several Lactobacillus isolates have recently gained attention for their capability to synthesize a diverse array of functional lipids [1]. These molecules of lipids have variety of complimentary mechanisms that participate to their actions of antibacterials. Depending on [2], their parts of hydrophobics have the action to indicate themselves into bilayers of phospholipid of microbial cells, declining stability of membranes and enhancing intracellular leakage of materials. Lipopeptides have the action to additional interrupt ion transference ways and membrane-linked enzymatic molecules, therefore weakening vigorous processes of metabolites [3]. Reactive oxygen species (ROS), which result in oxidative harm to biological macromolecules, can also be formed via some types of lipids. In addition to these impacts, lipids of biosurfactants have essential anti-adhesive actions that avoid pathogenic organisms like *E. coli* and *Candida albicans* from attaching into epithelial cells. Specific molecules of biosurfactants have the action to raise the efficiency of conventional antimicrobial medicines. Their efficiencies toward biofilms of microbial cells are other aspects of their significances [4]. In addition, biofilms – complicated communities enclosed in extracellular polymeric substances (EPS) – can aggregated on surfaces of abiotic and biotic substances, consisting of stainless steel and therapeutic apparatuses. They are highly linked with persistent infectious illnesses, raised resistances toward antimicrobials, and declined susceptibility toward disinfectants [5]. Their resilience exists essential complications in both industrial and clinical applications. Probiotic-derived BSs have become attractive alternates in light of the global raise of antimicrobial resistances and the inadequacies of traditional treatment approaches [6]. According to [7, 8], BSs from Lactobacillus species are biocompatible, biodegradable, and amphiphilic molecules that can damage adhesion of bacterial cells, weaken matrices of EPS, and decline the improvement of mature biofilms. Lactobacillus-derived BSs effectively avoid formation and development of biofilms through an amount of clinically essential infectious illnesses, drawing attention to their action as natural anti-infective agents [9].

## ■ PURPOSE OF THE STUDY

To optimize, characterize and biologically evaluate a glycolipid biosurfactant (BS) produced by *Lactobacillus plantarum* O91.

## ■ MATERIALS AND METHODS

### **Sample Collection and Isolation of Lactobacillus spp.**

A total of 160 samples were collected from two primary sources: dairy products and vaginal swabs from healthy Iraqi women. Dairy samples (1 g or 1 mL) were inoculated into

9 mL of MRS broth and incubated at 37 °C for 48 h under microaerophilic circumstances generated applying a candle jar. In addition, sequential dilutions were made via addition of homogenized material (1 mL) to 0.1% peptone water (9 mL) till the dilution achieved  $10^{-2}$ . Subsequent the protocol outlined [10], human samples of vagina were directly streaked onto plates of MRS agar and cultivated in a candle jar for 48 hours at 37 °C. Subsequent normal steps, resultant colonies were routinely sub-cultured on agar plates of MRS for additional isolation [11, 12]. Plates of MRS agar was utilized to preserve purified isolates till additional characterizations were accomplished.

### **Screening of Lactobacillus Isolates for BS Generation**

For encouraging intracellular generation of BS, every pure isolate was cultured in MRS broth (100 mL) and putted in incubator for 72 hours at 37 °C under anaerobic circumstances. Ten milliliters of the culture were centrifuged at  $10,000 \times g$  for fifteen minutes after the incubation period. The pellets were resuspended in 20 mL of phosphate-buffered saline (PBS, pH 5.0; 10 mM  $\text{KH}_2\text{PO}_4$ , 150 mM NaCl) after being twice cleaned with demineralized water. The cells were extracted by centrifugation after being gently stirred for 24 hours at room temperature. According to [13], the emulsification index determination (E24) was applied to the cell-free supernatant. After mixing two milliliters of supernatant with two milliliters of toluene, the mixture was vortexed for two minutes and left to stand for twenty-four hours. This method followed [14], whereas the following formula was applied to get the emulsification index:

$$\text{Emulsion Index (E24)\%} = \text{Height of emulsion layer} / \text{Total height of broth} \times 100.$$

### **Determination of Dry Cell Weight**

Ten milliliters of culture were centrifuged at  $8,500 \times g$  for twenty minutes in order to measure the biomass. According to [15] pellets were cleaned with phosphate buffer and dried in an oven at 80 °C to a consistent weight, represented as g/L.

### **Optimization of Culture Circumstances for BS Generation**

#### **Effect of Carbon Sources**

A mineral salt medium (MSM) consisting of 50 mL of various ingredients was enhanced with 0.5% (v/v) of several carbon sources, including whey, glucose, glycerol, fructose, or lactose. The ingredients included ammonium citrate (2 g/L), sodium acetate (5 g/L), magnesium sulfate (0.1 g/L), manganese sulfate (0.05 g/L), and potassium hydroxide (2 g/L). Media were inoculated with 2% ( $10^8$  CFU/mL; OD 0.5 McFarland) of the chosen isolate after sterilization and incubated at 37 °C, 120 rpm for 72 hours. After that, the biomass and BS yield were assessed.

#### **Effect of Nitrogen Sources**

To determine the optimal nitrogen source, the MSM medium comprising 0.5% lactose was supplemented with various nitrogen sources (0.1% w/v peptone, urea, malt extract, or yeast extract). Inoculum (2% v/v) was inoculated into every flask, which were putted in incubator for 120 h and 120 rpm at 37 °C. Then biomass and emulsification potency were analyzed.



### Effect of Initial pH

Prior to sterilization, media were set to values of pH of 4–8. Inoculum was inoculated into every flask, which were putted in incubator for 120 h and 120 rpm at 37 °C. Generation of BS was measured as described earlier.

### Effect of Incubation Time

For optimization of sources of carbon and nitrogen, cultures were putted in incubator for diversity of (24–120 h). At each time point, samples were collected for dry biomass and E24 determination.

### BS Extraction

The cultures were extracted after 120 hours of ideal growth conditions. After the cells were spun at  $10,000 \times g$  for 15 minutes, they were rinsed and redissolved in PBS at a pH of 5.0. They were then left to release extracellular BSs for 48 hours. Per [16], supernatants were isolated using a chloroform : methanol (2 : 1) mixture, chloroform alone, or methanol alone. After collecting the organic phase, it was dried at 40–45 °C and then weighed to find the extraction efficiency, following the methods outlined by [17, 18].

### Applications

#### Antibacterial Potency Assay

Following the protocol laid out by [19], the antibacterial potency was assessed using the agar well diffusion method. After 24 hours of growth, the test organisms were adjusted to a concentration of  $10^8$  CFU/mL (OD 0.5). One hundred microliters of BS, with concentrations ranging from one hundred to two hundred milligrams per milliliter, was produced in fifty percent DMSO and added to wells on Mueller-Hinton agar (the control DMSO alone). The areas of inhibition were measured in millimeters after incubation of the plates for 24 hours at 37 °C.

#### Anti-Biofilm Potency

Inhibition of biofilm was evaluated in accordance of [20]. Bacterial suspensions ( $1.5 \times 10^8$  CFU/mL) and BS solutions (100–200 mg/mL) were co-incubated in 96-well plates. A 99% methanol was applied to clean and fix the wells after 24h. Crystal violet (2%) was applied to stain biofilms, which were then cleaned, air-dried, and resolubilized utilizing acetic acid (33%). Absorbance was read at 595 nm. Assays were conducted in triplicate. Biofilm inhibition (%) was calculated applying:

$$\text{Anti-biofilm activity (\%)} = [1 - (\text{ODc} / \text{OD0})] \times 100,$$

where: ODc is the optical density of the well with a biosurfactant concentration and pathogen; OD0 is the optical density of the pathogen suspension with no biosurfactant (control). Duplicate assays was conducted and the mean of optical density was taken.

## ■ RESULTS

### Isolation and Identification of Bacterial Isolates

From dairy products and sources of human, 160 samples were cultivated on MRS agar, as selective medium for lactic acid bacteria. The samples were cultivated at 37 °C

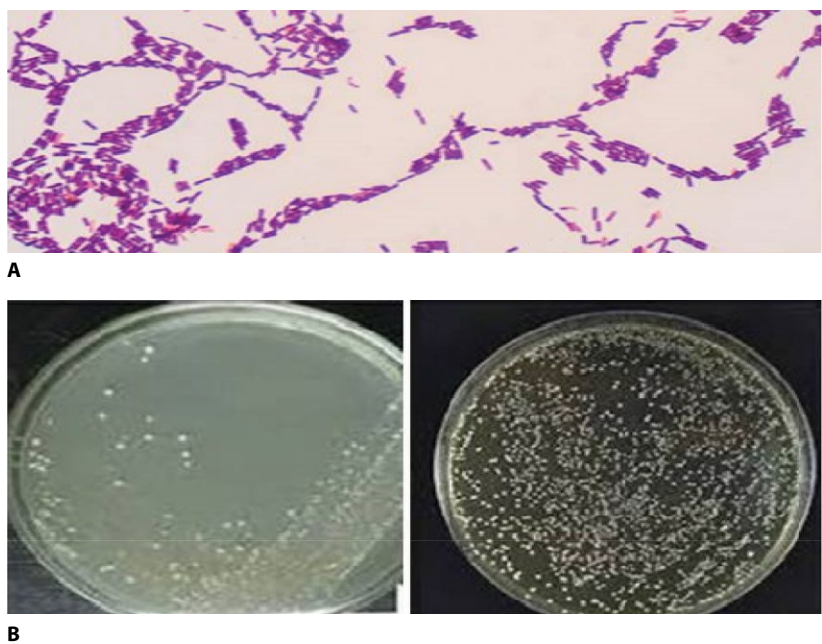


**Fig. 1. A – microscopic field of *Lactobacillus* cells, bacilli in shape and pairs in chain; B – the growth of *Lactobacillus* on MRS agar after 48 h of anaerobic incubation at 37 °C**

for 48 hours under microaerophilic conditions created by a candle jar (3–5% CO<sub>2</sub>). Ninety-two isolates showed characteristics typical of the species *Lactobacillus* after incubation. To verify their taxonomic identification, these isolates underwent a number of morphological, microscopic, and biochemical analyses. On MRS agar, colonies showed characteristic *Lactobacillus* shape, such as tiny (2–5 mm), smooth, convex, shiny, and non-pigmented appearances (Figure 1).

**Screening of *Lactobacillus* Isolates for BS Generation**

Ninety-two *Lactobacillus* isolates were first tested for the ability to manufacture BSs. Each isolate underwent preliminary screening to identify strains capable of producing

**Table 1**  
**Screening of *Lactobacillus* spp. for BS generation in MRS media after three days in shaker incubator (120 rpm) at 37 °C after 72 hrs**

| No. of isolates | Source | E24%  | Biomass g/l | Biuret test |
|-----------------|--------|-------|-------------|-------------|
| O3              | Dairy  | 53.9% | 3.9         | +           |
| O8              | Dairy  | 62.7% | 5.2         | +           |
| O12             | Dairy  | 55.8% | 4.2         | +           |
| O79             | Vagina | 59.3% | 4.8         | +           |
| O91             | Vagina | 68.7% | 5.7         | +           |
| O108            | Dairy  | 63.6% | 5.1         | +           |
| O147            | Vagina | 60.5% | 4.9         | +           |

Note: + = Green ring indicate for GL BS.



GL-type BSs. Eight of the isolates tested produced positive BS, as evidenced by the formation of a distinctive green ring in the biuret assay, which indicates the presence of GL. Among them, *Lactobacillus* O91 isolate had the greatest BS output and better emulsification efficacy, with an E24 value of 68.7% (Table 1).

**Optimization of Media Composition and Culture**  
**The Effect of Carbon Sources**

By adding 0.5% (w/v) of each carbon substrate to the generation medium, the impact of different carbon sources on BS production was evaluated. With an emulsification index (E24) of 69.5% and a corresponding biomass dose of 5.9 g/L, lactose supported the greatest BS output, as seen in Figure 2.

**Effect of Nitrogen Sources**

*L. plantarum* O91 was cultivated in medium enriched with different nitrogen substrates in order to ascertain the impact of various nitrogen sources on BS generation. Beef extract showed the greatest effectiveness, with a dry biomass of 6.2 g/L and an emulsification index (E24) of 72.4%. Urea, on the other hand, had the lowest emulsification effectiveness (38%), suggesting that it is suitable for inducing BS in this strain (figure 2).

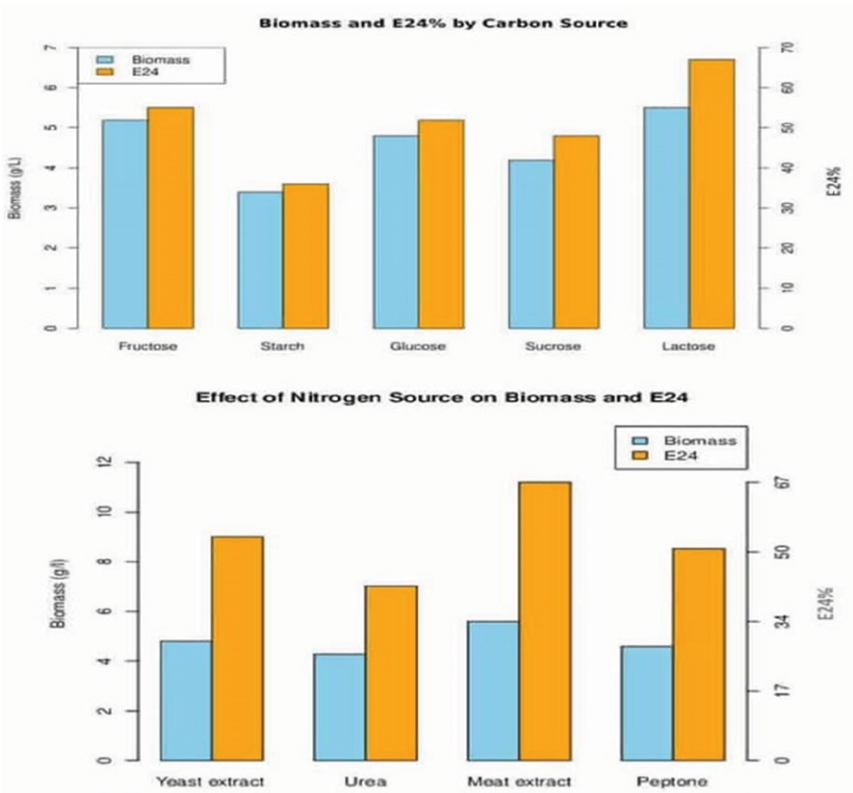


Fig. 2. Effect of carbon and nitrogen source

Circumstances Effect of PH

In order to determine how different starting pH levels affected BS production by *L. plantarum* O91, the MSM medium that had been tuned in earlier tests was changed to a variety of pH values. As illustrated in Figure 3, at pH 5, the strain achieved its maximum output of emulsification efficacy of 68.7% and biomass of 5.7 g/L.

Effect of Incubation Period

Various incubation periods (0–126 h) were evaluated to determine the optimal duration for biomass accumulation and BS synthesis by *L. plantarum* O91. After 72 hours of incubation, the strain showed its peak biomass output (6.2 g/L) and emulsification efficacy (E24=73.4%), as seen in Figure 3.

Extraction of Produced BS

Optimal conditions for BS formation applied to increase production. The isolate was grown on mineral salt medium (MSM) that had been adjusted to a pH of 5. It was

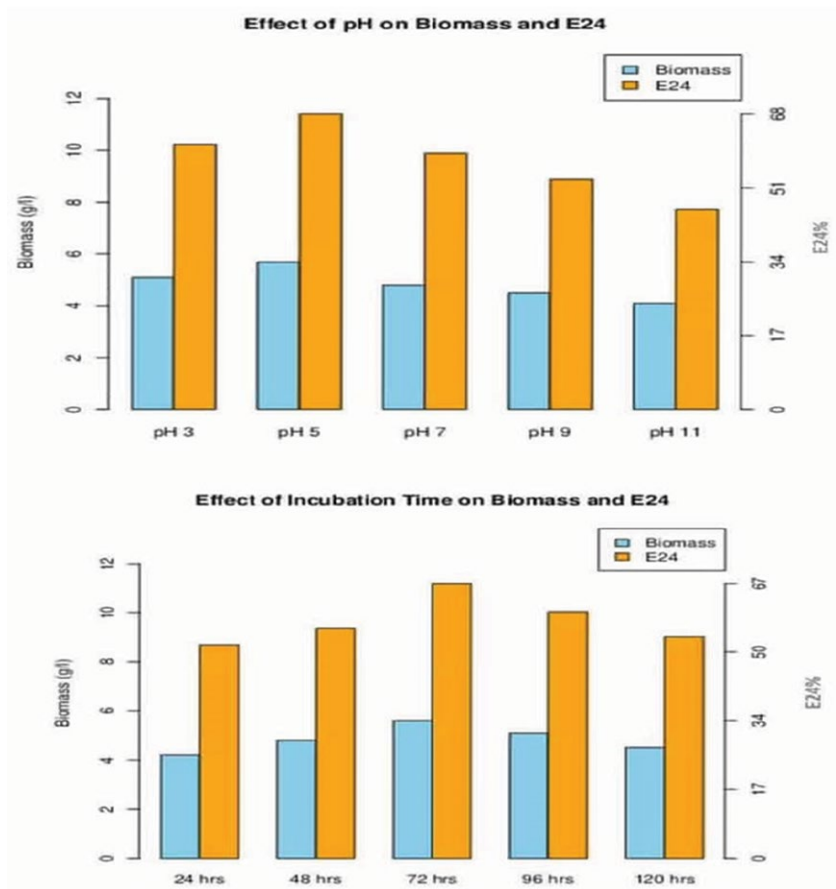


Fig. 3. Effect of PH and time



supplemented with 1% meat extract for nitrogen and 5% lactose for carbon. For 72 hours, cultures were incubated at 37 °C with constant agitation (120 rpm). After incubation, BS was taken out using an organic solvent extraction method that used a 2:1 mixture of chloroform and methanol. This method made the BS fraction mostly pure. We put equal amounts of culture supernatant and solvent combination in a separatory funnel and let them sit overnight to help with phase separation and solvent evaporation. After that, the residue was dried, and it gave 6.2 g/L of BS.

Found that *L. casei*-derived GLs efficiently inhibited numerous pathogenic bacteria, including *E. coli*, *P. aeruginosa*, *S. typhi*, *S. flexneri*, *S. aureus*, *S. epidermidis*, *Listeria monocytogenes*, and *L. innocua*. The antimicrobial profile observed in this study is consistent with these findings, lending credence to *L. plantarum* O91 GLs' ability to act as natural antimicrobial agents (figure 4).

**Table 2**  
**Anti-bacterial potency of GL produced by *L. plantarum* O91 toward pathogenic bacteria applying Well diffusion method**

| Pathogens/conc.      | Negative control | 100 mg/ml | 120 mg/ml | 140 mg/ml | 160 mg/ml | 180 mg/ml | 200 mg/ml |
|----------------------|------------------|-----------|-----------|-----------|-----------|-----------|-----------|
| <i>S. aureus</i>     | 0.0±0.00         | 4.5±0.71  | 11.5±0.71 | 18.5±0.71 | 22.5±0.71 | 25.0±0.00 | 27.0±0.00 |
| <i>E. coli</i>       | 0.0±0.00         | 4.3±0.71  | 10.5±0.71 | 13.5±0.71 | 15.0±0.00 | 18.5±0.71 | 23.5±0.71 |
| <i>P. aeruginosa</i> | 0.0±0.00         | 2.5±0.71  | 5.5±0.71  | 7.5±0.71  | 12.5±0.71 | 16.0±0.00 | 19.5±0.71 |
| <i>C. albicans</i>   | 0.0±0.00         | 3.5±0.71  | 7.5±0.71  | 12.5±0.71 | 16.0±1.41 | 18.0±0.00 | 21.5±0.71 |

**Anti-Biofilm Potency of GL Produced by *L. plantarum* O91**

The data showed a straight dosage-dependent trend, meaning that the anti-biofilm efficacy of the GL got better as the dosages got higher. At the greatest concentration tested, the BS significantly disrupted biofilms, reducing them by a factor of 70–79 in *S. aureus*, 59% in *E. coli*, 48% in *P. aeruginosa*, and 43% in *Candida albicans* (Figure 5).

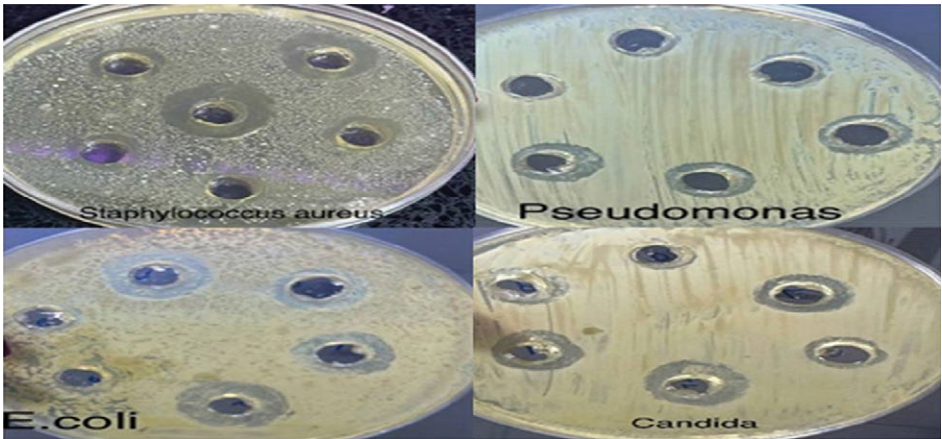


**Fig. 4. Antimicrobial potency of GL against pathogenic bacteria (*S. aureus*, *P. aeruginosa*, *E. coli* and *Candida*) by applying well diffusion method**

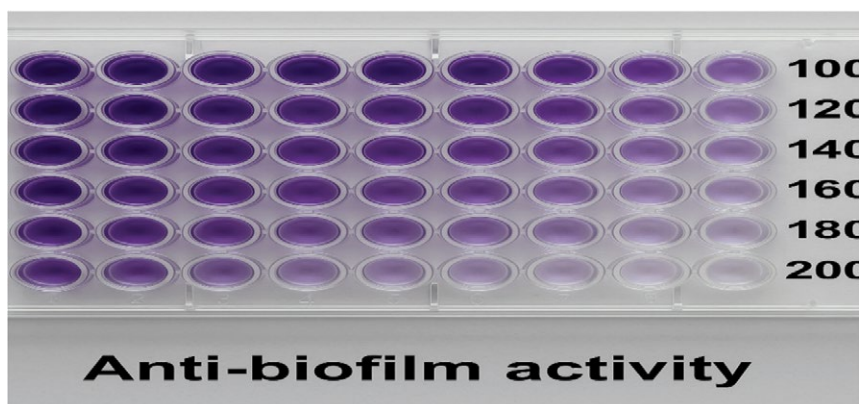


Fig. 5. Anti-biofilm potency of GL produced from *L. plantarum* O91

## ■ DISCUSSION

Gram-positive bacilli grouped singly, in pairs, or in short chains were visible under a microscope when submerged in 100× oil. According to typical descriptions of *Lactobacillus* spp., biochemical profiling showed consistent negative for oxidase, catalase, and indole assays. These results are consistent with the diagnostic standards provided by [21], who listed the phenotypic characteristics of isolates of presumed probiotic *Lactobacillus*.

The lead strain for subsequent optimization and characterization investigations was selected as O91 due to its high potency. This confirms what other studies have shown about the different BS-generating abilities of *Lactobacillus* species [22].

Fructose and glycerol, on the other hand, produced significantly lower emulsification activity of 45% and 47%, respectively. These findings show that *L. plantarum* O91 can metabolize a variety of carbon sources, but lactose offers the best conditions for cellular development and BS production. Which in turn propels microbial growth and the production of secondary metabolites, nitrogen is necessary for cellular metabolism [23].

Management of pH have vital functions through fermentation, as generation of BS declined considerably at values greater or lesser than this optimum range. For microbial BS pathways, optimal pH is critical. These outcomes are in accordance to preceding work representing these pathways of BS of microbial cells are sensitive to environmental parameters [24]. Generally speaking, *L. plantarum* growth and efficient production of GL BSs are both supported by maintaining an adequate pH.

After this, there was a noticeable drop in emulsification efficacy, especially after 126 hours. Nutrient depletion, the buildup of metabolic byproducts, and other adverse changes in the culture environment that prevent cellular growth and BS creation are probably connected to this decrease. Overall, the results show that BS production by *L. plantarum* O91 rises gradually with incubation period, starting about 48 hours into the early stationary phase and peaking between 65 and 72 hours. Productivity declines when the culture enters late stationary and decline stages following this optimal window. As seen, BS generation by *L. plantarum* O91 gradually raised during culture in lactose-containing media starting about 48 hours. This strain produces mostly cell-bound BS, according to the generation pattern. After around 72 hours of incubation, the BS output



kept increasing and peaked. These findings imply that BS biosynthesis in *L. plantarum* O91 begins during the exponential growth phase and continues for at least two days during the stationary phase. [25] found similar growth-linked generation kinetics and showed that numerous *Lactobacillus* species, such as *Lactobacillus casei* and *Lactobacillus brevis*, show raised BS synthesis during active growth. According to [26] secondary metabolites that obstruct emulsification or interfere with surfactant adsorption at the oil – water interface may cause BS production to stop in later stages of culture. There have also been reports of similar patterns in other microbes. According to [27], for example, *Acinetobacter calcoaceticus* RAG-1 enhanced emulsan production during the stationary phase. The idea of growth-linked generation was supported by [28], who found that applying olive oil as a substrate caused BS synthesis mostly during exponential growth. According to [29], rhamnolipid production in *P. aeruginosa* A3 also grew gradually, peaking at 108 hours and reached 10.6 g/L. All of these results support the hypothesis that the BS generated by *L. plantarum* O91 follows a growth-linked or early stationary-phase production pattern, which is in line with the behavior seen in a number of microbial species that create BS.

BSs have been shown to interact with microbial cell membranes, where they can change protein shape, compromise membrane integrity, and eventually cause cell lysis and intracellular metabolite release [30]. GLs are known for having a potent antimicrobial ability among the several classes of BSs. Rhamnolipids and lipopeptides are examples of compounds that have shown wide inhibitory effects toward bacteria and fungus [31]. In the present study, the antimicrobial potency of the GL BS produced by *L. plantarum* O91 was examined applying the agar well-diffusion method on Mueller – Hinton agar (MHA). The BS was tested toward four pathogenic organisms – *P. aeruginosa*, *S. aureus*, *E. coli*, and *C. albicans* – at dosages ranging from 100 to 200 mg/mL. Table 3 and show that the BS showed discrete zones of inhibition for all tested pathogens, with widths ranging from 11 to 27 mm. *S. aureus* observed the greatest zones of inhibition ( $4.5 \pm 0.71$  mm to  $27.0 \pm 0.00$  mm) at 100 mg/mL to 200 mg/mL, respectively, representing that the fraction of GL was notably efficient toward Gram-positive bacterial cells. Alike trends were observed in *E. coli*, whereas zones of inhibition raised from  $4.3 \pm 0.71$  mm to  $23.5 \pm 0.71$  mm in the interior of the similar range of dosage. *P. aeruginosa* achieved weaklier ( $2.5 \pm 0.71$  mm into  $19.5 \pm 0.71$  mm) although *C. albicans* zones of inhibition grew from  $3.5 \pm 0.71$  mm into  $21.5 \pm 0.71$  mm. The molecule of GL alone was responsible for the effectiveness, pathogen suspension without BS (as the negative control) did not display any hindrance. Gram-positive bacteria may be more susceptible due to structural variations in their cell walls. The outer membrane of gram-negative organisms serves as an extra barrier, preventing GL molecules from penetrating. Conversely, Gram-positive bacteria are more susceptible to BS-mediated harm because they have a larger yet more porous peptidoglycan coating [32]. This method of action is supported by the reported inhibitory effects, which point to peptidoglycan layer breakdown and interference with vital metabolic processes. Only a few research have demonstrated the antimicrobial activity of lactic acid bacteria-produced BSs [33].

The results show that *L. plantarum* O91 BS can prevent microbial adhesion and penetrate existing biofilms, reducing their structural integrity. Prevailing research has shown similar results to the new ones by [34] shows that species of *Lactobacillus* produce BSs that prevent *E. coli*, *S. aureus*, *S. arizonae*, and *L. monocytogenes* from forming biofilms. It was also demonstrated by [35] that a BS produced from *L. acidophilus* successfully

decreased the biofilm production of *Proteus mirabilis*. All things considered, these results raise the prospect that GLs produced by LAB could serve as natural anti-biofilm agents, which could have important ramifications for biology and medicine.

## ■ CONCLUSION

This study confirms *Lactobacillus plantarum* O91 as a potent producer of a glycolipid biosurfactant with significant antimicrobial and antibiofilm activities. Optimization of culture condition identified lactose, meat extract, PH 5, and 72 hr. incubation period as optimal for maximal biosurfactant yield. FTIR analysis verified the glycolipid structure of the purified compound. Biologically, the biosurfactant exhibited broad spectrum antimicrobial efficacy against major clinical pathogens, with *S. aureus* showing the highest sensitivity. Moreover, a strong concentration dependent antibiofilm activity was observed highlighting its ability to disrupt established biofilm and inhibit microbial adhesion. These findings emphasize the strain specific efficiency of *Lactobacillus plantarum* O91 and its potential as a sustainable, natural alternative to conventional antimicrobial and antibiofilm agents. Further in vivo and mechanistic studies are warranted to advance its biomedical and biotechnological applications.

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# Comparative Analysis of the Antibacterial Activity of Commercial Toothpastes Enhanced with a Biosurfactant against Dental Caries

**Conflict of interest:** nothing to declare.

**Authors' contribution:** Ola Sarmad Hamid – conceptualization, data curation, investigation, method, project administration, resources, software, writing – original draft and writing – review & editing; Nadhem Hasan Haydar – conceptualization, data curation, supervisor, writing – original draft and writing – review & editing.

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## Abstract

**Introduction.** Dental caries remains one of the most widespread chronic diseases affecting oral health and overall quality of life. Acidogenic bacteria demineralize tooth enamel by creating acid during carbohydrate fermentation causing of dental decay.

**Purpose.** To produce and characterize a biosurfactant derived from probiotic bacteria (*Lactobacillus* spp.) isolated from the oral cavity, and to assess its antibacterial properties.

**Materials and methods.** Biosurfactant was produced under optimum condition and characterized using FTIR spectroscopy and GC mass. The antimicrobial activity of the biosurfactant was assessed using the well diffusion method, at concentrations ranging from 100 to 200 mg/ml. The antibacterial potential of the commercial toothpastes was similarly evaluated using the same method. then study the synergistic effect between biosurfactant and each type of toothpaste.

**Results.** At concentrations ranging from 100 to 200 mg/ml result revealed significant inhibitory effects against *Streptococcus mutans*, with inhibition zones ranging from 4 to 26 mm. For *Staphylococcus aureus*, the biosurfactant demonstrated inhibition zones between 4 and 20 mm. The antibacterial efficacy of the toothpastes was significantly increased when the biosurfactant was added to the mix, even though the toothpastes alone had no bactericidal action against the oral bacteria. Notably, this combination produced inhibition zones of 23 mm against *S. mutans* and 22 mm against *S. aureus* for each type of toothpaste.

**Conclusion.** These findings highlight the possibility of biosurfactants produced by probiotic *Lactobacillus* spp. as effective antimicrobials in the fight against acid-producing bacteria. Furthermore, the findings suggest that the antimicrobial efficacy of these biosurfactants can be substantially augmented when used in conjunction with conventional oral hygiene products.

**Keywords:** lactobacillus, carries, antibacterial activity, toothpaste, biosurfactant



## ■ INTRODUCTION

Dental caries remains one of the most widespread chronic diseases globally, significantly affecting oral health and overall quality of life. Acidogenic bacteria, such as *S. mutans*, demineralize tooth enamel by creating acid during carbohydrate fermentation, and this is the main cause of dental decay. New and improved methods of oral hygiene, particularly toothpaste, have emerged as an essential part of the fight against the worldwide epidemic of dental cavities [1].

Because of their biocompatibility, anti-adhesive, and antibacterial qualities, biosurfactants – amphiphilic chemicals naturally produced by microbes – have recently attracted considerable interest. The effectiveness of these biological surfactants against biofilm-forming infections and cariogenic bacteria is encouraging. Thus, they're a greener option than the synthetic surfactants and additives found in regular commercial toothpaste, such as triclosan and sodium lauryl sulfate [2].

Toothpaste containing biosurfactants is very effective against oral infections because of this ability of multi way in kills bacteria. Their capacity to penetrate microbial cell membranes, increase permeability, and cause cell lysis is due to their amphiphilic structure. In addition, the communication pathway necessary for biofilm formation – quorum sensing – is disrupted by biosurfactants such glycolipids and lipopeptides. When it comes to avoiding dental plaque and the development of caries by inhibiting biofilm creation [3]. states that microbial biosurfactants are a safer and more selective alternative to broad-spectrum synthetic drugs since they have strong antibacterial action against *S. mutans* without disrupting the oral microbiota balance.

With the use of microbial biosurfactants, this study intends to compare and contrast the antibacterial effectiveness of various commercially available toothpastes. The research investigates the inhibitory effects against key caries-associated microorganisms and assesses how biosurfactant-enriched formulations perform compared to unmodified commercial products. In addition to being a useful scientific tool, this comparison method is crucial for directing the creation of safer and better long-term dental care solutions [4].

## ■ PURPOSE OF THE STUDY

To produce and characterize a biosurfactant derived from probiotic bacteria (*Lactobacillus* spp.) isolated from the oral cavity, and to assess its antibacterial properties.

## ■ MATERIALS AND METHODS

### **Preparation of Toothpastes**

One gram of toothpaste in nine milliliters of sterile distilled water is a common diluent for microbiological testing. Make sure there is consistency by mixing it completely in the original container before tasting. Another option is to move it to a new, sterilized glass container and mix it well. Put it somewhere cool, out of the sun. To prevent contamination or the evaporation of volatile components, keep sealed until ready to use.

### **Selection for Toothpastes**

Before you buy that new tube of toothpaste, there are a few things you should think about. Find out what's most important for your teeth and gums, and then pair it with the right toothpaste. Check for potential side effects, such as gum disease, alcohol sensitivity,

or allergic reactions. Commercial toothpastes available in Iraq were selected based on their active ingredients and numbered from 1 to 5. The active ingredients of the first toothpaste are Monofluorophosphate Sodium (MFP), the second paste (Herbal Ingredients), the third toothpaste (Potassium Nitrate), the fourth paste (Hydrated Silica), finally the fifth paste (Sodium Fluoride).

### **Antibacterial Activity of Toothpaste**

Two types of pathogenic bacteria (*S. mutans* and *S. aureus*) were used to test the antimicrobial capabilities of Five toothpastes based on active ingredients, the toothpaste (1) are Monofluorophosphate Sodium (MFP), the toothpaste (2) are Herbal Ingredients, the toothpaste (3) are Potassium Nitrate, the toothpaste (4) are Hydrated Silica, finally the toothpaste (5) are Sodium Fluoride. To find the toothpaste's inhibition zone width, the well diffusion method was used for evaluation. Muller Hinton broth was used to cultivate the harmful bacteria, which were then incubated overnight at 37 °C. Swabbing a small volume (0.1 ml) of each bacterial strain (O.D 600 nm) onto the Muller Hinton agar plates was done, and then wells were formed using cork borer. A hundred microliters of diluted toothpaste, consisting of 1 gram in 9 milliliters of water by weight, was put to each well. The plates were then left to incubate at 37 °C for a full day. An indicator of antibacterial efficacy was the inhibition zone.

### **Production of Biosurfactant**

The conditions for the production and extraction of biosurfactants were optimized. A volume of 1 mL of a specific bacterial isolate containing  $1 \times 10^8$  cells/mL (OD 600 nm) was added to 50 mL of MRS medium for fermentation. The cultures were incubated anaerobically for 72 hours at 35 °C. After incubation, the cells were harvested by centrifugation at 8,000 rpm for 20 minutes. After that, the cell pellet was rinsed twice with distilled water and resuspended in 10 mL of PBS. For four hours, while stirring gently, the suspension was left to incubate at room temperature. Centrifugation was used for 20 minutes to extract the bacterial suspension after the incubation period. For extraction, the liquid above the sediment was gathered. The supernatant was then mixed with a 2:1 (v/v) ratio of methanol and chloroform. Before letting it evaporate overnight, the mixture was shaken hard to blend everything well. At the liquid-liquid interface, a grayish precipitate was seen, which meant that biosurfactant was present [5].

### **Characterization of Glycolipid**

#### **The Spectrum of the Fourier Transforms Infrared (FTIR)**

The FTIR spectra of the biosurfactant could not be obtained without initially homogenizing the sample with KBr. Subsequently, data were collected within the wavelength range of 500 to 4000  $\text{cm}^{-1}$ . The ultraviolet spectra were recorded using a Shimadzu-I Raffinity-1 spectrophotometer. Analyzing the spectrum in terms of intensity and wave number offers a distinct perspective on the data [6].

### **Analysis of Biosurfactant with Gas Chromatography (GC) Technique**

Gas chromatography (GC) was used in compliance with the method described in to ascertain the fatty acid content of the biosurfactant [7]. A synopsis of the studies concerning the composition of fatty acids follows. A solution of 1 millilitre sulfuric



acid-methanol containing 10 milligrams of partly purified biosurfactant was heated to 90 degrees Celsius for 15 hours in order to produce acid methyl ester. The sulfuric acid was evaporated and the resultant hexane phase was recovered after adding 1 millilitre of hexane and thoroughly mixing. Using 1 millilitre of D.W., the hexane phase was combined. after the first meeting. The fatty acid methyl ester, which was extracted using hexane, was analysed using helium as a carrier gas on a Shimadzu 17-A gas chromatography column with a fused silica capillary (30 m × 0.25 mm, 0.25 µm film thickness).

### **Antimicrobial Activity of Biosurfactant**

Two types of pathogenic bacteria, *S. mutans* and *S. aureus*, were evaluated for their susceptibility to the antibacterial activity of a partially purified biosurfactant. The well diffusion method was employed to determine the inhibition zone diameter, indicative of the biosurfactant's antimicrobial potential. Muller-Hinton broth was used to cultivate the bacteria, which were incubated overnight at 37 °C. A 0.1 ml aliquot of each bacterial strain (at an optical density of 600 nm) was inoculated onto Muller-Hinton agar plates, and wells were created using a cork borer. One hundred microliters of each biosurfactant concentration, ranging from 100 to 200 mg/ml, was introduced into the wells. The stock solution of the biosurfactant was dissolved in dimethyl sulfoxide (DMSO). The plates were subsequently incubated at 37 °C for 24 hours. The antibacterial effect of the biosurfactant was evidenced by the formation of a clear inhibition zone around the wells [8].

### **Isolation of Pathogenic Bacteria**

From 1/12/2023 to 15/3/2024, 102 specimens of pathogenic isolates from carriers were obtained from various dental clinics in Iraq (Wasit) were used as indicators in the present investigation. Culturing in specific selective medium and other biochemical tests, including API 20E, were used to diagnosis all isolates.

### **Antibiotic Susceptibility Testing for Pathogenic Bacteria**

To determine antibiotic susceptibility, the disc diffusion method was used on Mueller Hinton agar that had already been seeded with the chosen indicator isolates. Various antibiotic discs, including rifampicin, amoxicillin, erythromycin, clindamycin, vancomycin, Gentamycin, Methicillin-Chloramphenicol, and ciprofloxacin, were utilized. A more antibiotic-resistant bacterial population is the desired outcome of antibiotic susceptibility testing.

### **Antibacterial Activity of Toothpaste with Biosurfactants**

The purpose of this study was to compare the effectiveness of several toothpastes based on ingredients, the toothpaste (1) are Monofluorophosphate Sodium (MFP), the toothpaste (2) are Herbal Ingredients, the toothpaste (3) are Potassium Nitrate, the toothpaste (4) are Hydrated Silica, finally the toothpaste (5) are Sodium Fluoride. when combined with a bio-surfactant in killing harmful germs. After adjusting to  $1 \times 10^8$  UFC/ml, 0.1 overnight growth of *S. aureus* and *S. mutans* was disseminated equally on the surface of Mueller-Hinton agar plates. Fifty microliters of a known concentration of glycolipid and fifty microliters of each toothpaste were added to the same well in every plate. As a control, we used wells that contained simply toothpaste. The inhibitory zones were measured after the plates were incubated at 37 °C for 24 hours.

Statistical Analysis

The statistical analysis, charts drawn, and tables were performed using Microsoft Excel 2019. The properties of the produced biosurfactants were characterized using FTIR spectroscopy. A Shimadzu gas chromatograph (GC) was used to analyze the biosurfactant. The results of GC showed statistical variation in the concentrations of the different compounds, allowing for an assessment of the effectiveness of the biosurfactant production process.

RESULTS

Antibacterial Activity of Toothpaste

Using the well diffusion method, this study tested in vitro antibacterial activity of various commercial toothpastes based on active ingredients, the toothpaste (1) are Mono fluorophosphate Sodium (MFP), the toothpaste (2) are Herbal Ingredients, the toothpaste (3) are Potassium Nitrate, the toothpaste (4) are Hydrated Silica, finally the toothpaste (5) are Sodium Fluoride, against two clinical isolates of multidrug-resistant *S. aureus* and *S. mutans* taken from the oral cavity (carious lesions). There were no inhibition zones seen in any of the tested items (different toothpastes), hence it was determined that they did not have antibacterial action (Figure 1).

Characterization of Glycolipid

Characterization of Produced Bio Surfactant FTIR Spectrum Analysis

The biosurfactant produced by *Lactobacillus* exhibited distinct absorption bands in its Fourier-transform infrared (FTIR) spectrum, indicative of a glycolipid structure (Figure 2).

Analysis of Biosurfactant with Gas Chromatography (GC) Technique

Table 1 demonstrates the identification of several sugar-lipid conjugates in the semi-purified glycolipid extract using gas chromatography-mass spectrometry (GC-MS) as show in (table 1).

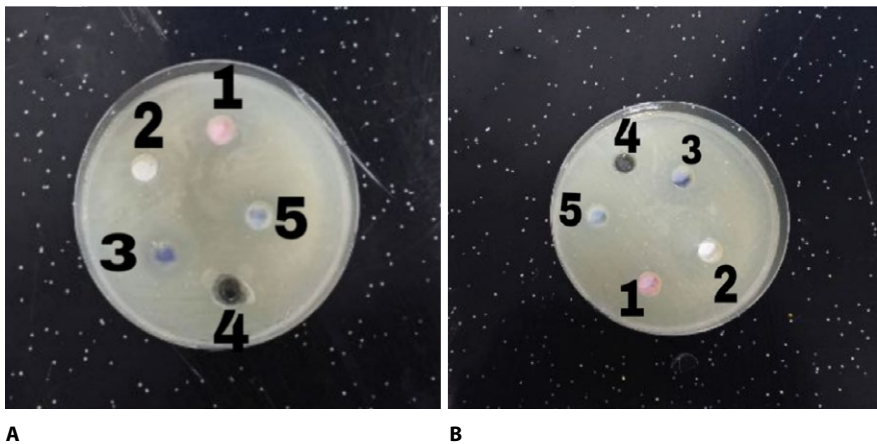


Fig. 1. A – antibacterial activity of different toothpastes against *S. mutans*; B – *S. aureus*

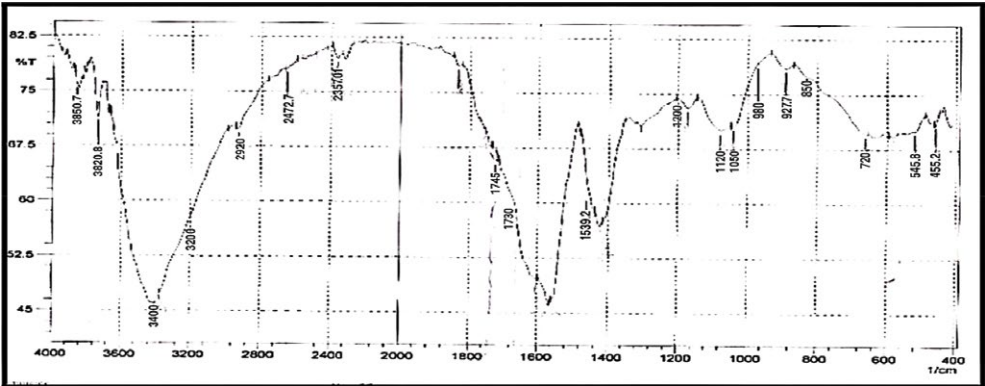


Fig. 2. FTIR spectrum analysis of biosurfactant

Table 1  
Analysis of biosurfactant with Gas chromatography (GC)

| No. | Retention time (min) | Compounds                                  | Molecular formula | Molecular weight (g/mole) | Area % | Relative Abundance % |
|-----|----------------------|--------------------------------------------|-------------------|---------------------------|--------|----------------------|
| 1   | 2.600                | Hexadecyl $\beta$ -D-glucopyranoside       | C22H44O6          | 404                       | 4.39   | 20                   |
| 2   | 4.830                | Octadecyl $\beta$ -D-galactopyranoside     | C24H48O6          | 432                       | 8.9    | 40                   |
| 3   | 6.343                | Stearyl $\beta$ -D-mannopyranoside         | C24H48O6          | 432                       | 6.32   | 30                   |
| 4   | 8.706                | Monogalactosyldiacylglycerol (C16:0/C18:1) | C43H80O10         | 756                       | 20.1   | 98                   |
| 5   | 10.098               | Digalactosyldiacylglycerol (C18:0/C18:1)   | C49H92O15         | 940                       | 7.2    | 36                   |
| 6   | 12.356               | Glycosyl ceramide (Glc-Cer, C18:0)         | C40H79NO8         | 717                       | 10.01  | 55                   |
| 7   | 14.982               | Sulfoquinovosyldiacylglycerol              | C45H84O12S        | 884                       | 12.5   | 68                   |

Antimicrobial Activity of Glycolipids

Glycolipid production by *Lactobacillus* spp. was investigated for its antimicrobial activity in vitro against two antibiotic-resistant clinical isolates (*S. aureus* and *S. mutans*) taken from the oral cavity. glycolipid had a notable antimicrobial effect against *S. aureus* and *S. mutans*, with inhibition zones ranging from 4 to 20 mm and 4 to 26 mm, respectively, at concentrations of 100 to 200 mg/ml as show in Figure 3 and table 2.

Isolation of Pathogenic Bacteria

There were 102 pathogenic specimens extracted from dental samples. The results of the various diagnostic methods used to determine the presence of the harmful bacteria, including 32 isolates of *S. mutans*, 54 isolates of *S. aureus* and 16 isolates of *Pseudomonas* spp.

Antibiotic Susceptibility Testing for Pathogenic Bacteria *S. Mutans* and *S. Aureus*

The disk diffusion technique, in accordance with CLSI 2024, was used to test for *S. aureus* and *S. mutans* using various antimicrobial drugs. Among the isolates tested,

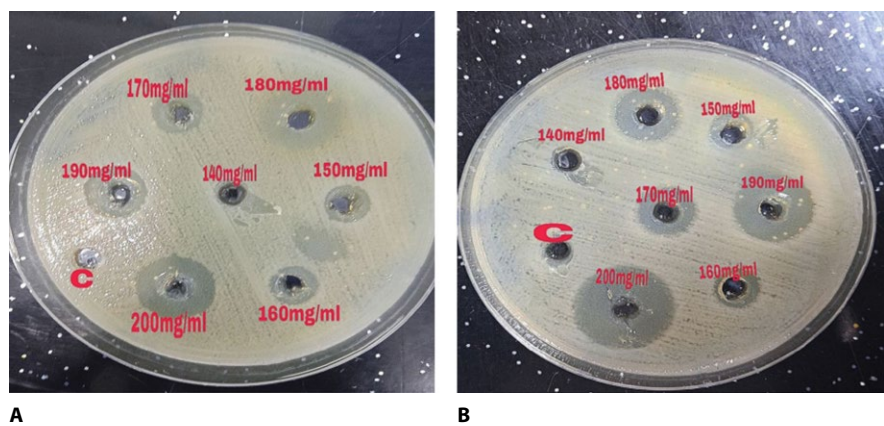


Fig. 3. Antimicrobial activity of Glycolipid against: A – *S. aureus*; B – *S. mutans*

Table 2  
Inhibition zone (mm) of pathogenic bacteria by glycolipid

| Biosurfactant (mg/ml) | <i>S. mutans</i> (mm) | <i>S. aureus</i> (mm) |
|-----------------------|-----------------------|-----------------------|
| 100                   | 0                     | 0                     |
| 110                   | 0                     | 0                     |
| 120                   | 0                     | 0                     |
| 130                   | 0                     | 0                     |
| 140                   | 0                     | 0                     |
| 150                   | 4                     | 4                     |
| 160                   | 5                     | 6                     |
| 170                   | 9                     | 7                     |
| 180                   | 12                    | 11                    |
| 190                   | 22                    | 15                    |
| 200                   | 26                    | 20                    |

77.8% were resistant to azithromycin, 55.6% to chloramphenicol, 48.1% to clindamycin, 46.3% to ciprofloxacin and gentamicin, and 44.4% to rifampin.

**Synergistic Antimicrobial Activity (Toothpaste with Bio Surfactant)**

The well diffusion method was utilized to test the in vitro antibacterial activity of three different commercial toothpastes based on active ingredients, the toothpaste (1) are Monofluorophosphate Sodium (MFP), the toothpaste (2) are Herbal Ingredients, the toothpaste (3) are Potassium Nitrate, the toothpaste (4) are Hydrated Silica, finally the toothpaste (5) are Sodium Fluoride. in combination with biosurfactant against two clinical isolates of multidrug-resistant *S. aureus* and *S. mutans* taken from the oral cavity (carious lesions). Figures 4, 5 indicate that, when combined, biosurfactants and four different types of toothpaste (Colgate, Sensodyne White glo, and Dabur) limit the growth of germs, forming a 23 mm zone for each type of toothpaste against *S. mutans* and a 21 mm zone against *S. aureus*. Where BS mean biosurfactant.

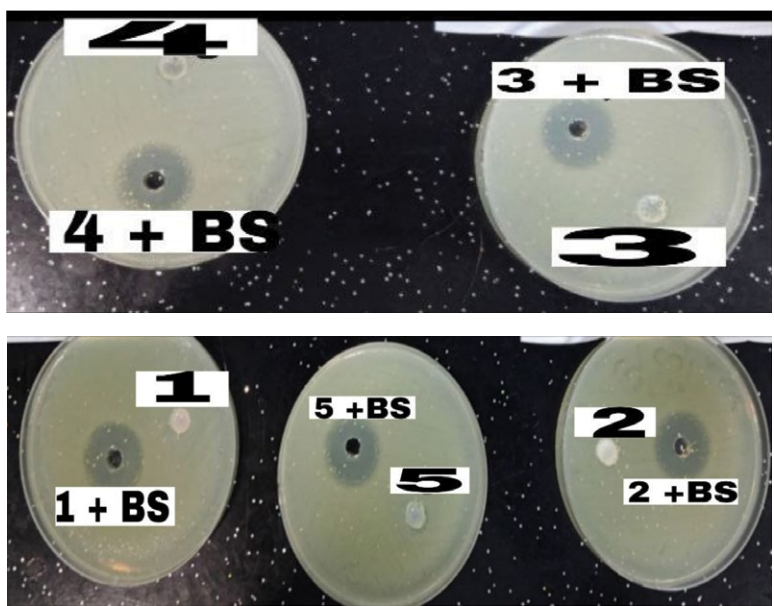


Fig. 4. Antibacterial activity of different toothpaste with biosurfactant against *S. aureus*

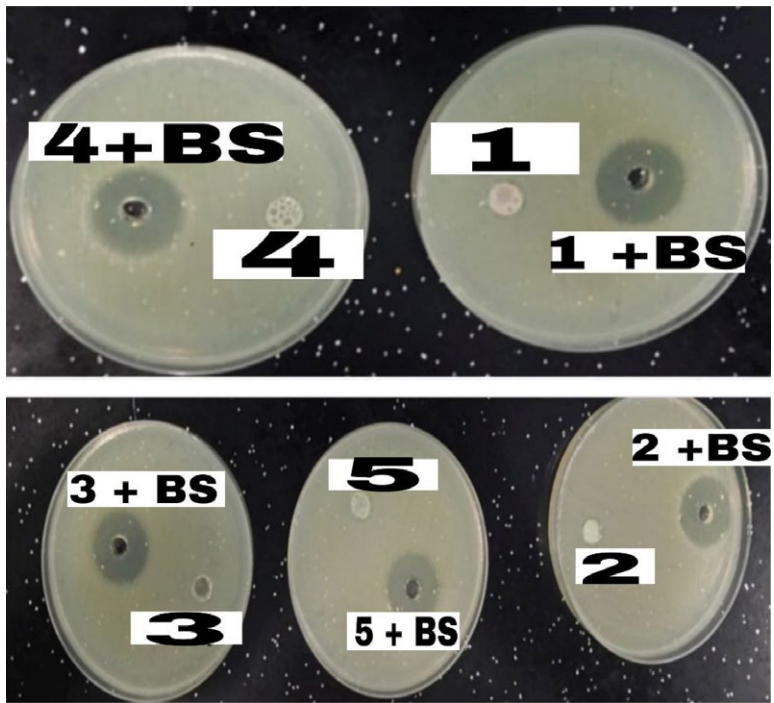


Fig. 5. Antibacterial activity of different toothpaste with biosurfactant against *S. mutans*

## ■ DISCUSSION

In this study, the different commercial toothpastes that were tested for their in vitro antibacterial activity using the well diffusion method. The tests were conducted against two clinical isolates of multidrug-resistant *S. aureus* and *S. mutans*, which were collected from the oral cavity (carious lesions). No inhibition zones were observed in any of the tested items, which led to the conclusion that they lacked antibacterial effect. Study by Unterbrink et al. (2024) show, those with severe caries or periodontal disease shouldn't rely on toothpaste as a treatment option on its own. On the contrary, they are best used as a complement to a full-scale dental care routine or as a preventative measure [9]. Even with sophisticated formulations like hydroxyapatite-based toothpastes, another research found that they only help with prevention and beginning remineralization, and not with treating gingivitis or severe caries [10]. Recent studies (2024) have demonstrated that the antibacterial activity of widely used commercial brands is limited. For instance, it was observed that Sensodyne toothpaste failed to inhibit the growth of bacteria and did not display any significant antibacterial properties [11].

Lastly, items for the mouth (such mouthwashes and toothpastes): Preventative care, not curative care, is their main focus. They play the following preventative roles: Minimizing the accumulation of microorganisms on dental plaque Achieving Fresh Breath Reducing gingivitis and other superficial gum diseases from occurring [12].

For characterization of biosurfactants, different techniques were utilized. Firstly, FTIR indicated that the stretching vibrations of the O-H bonds in the sugar moieties were characterized by a broad band within the range of  $3400\text{--}3200\text{ cm}^{-1}$ . The C=O stretching vibration corresponding to the lipid component was represented by a sharp peak at  $1740\text{ cm}^{-1}$ , while the CH stretching vibrations of the aliphatic fatty acid chains were observed as prominent peaks at  $2920$  and  $2850\text{ cm}^{-1}$  [13]. In the fingerprint region, C-O-C stretching vibrations in carbohydrates were associated with bands between  $1050\text{--}1120\text{ cm}^{-1}$ , whereas peaks in the range of  $850\text{--}950\text{ cm}^{-1}$  were attributed to C-H bending in the sugar rings [14]. A band at  $720\text{ cm}^{-1}$  suggested the presence of long-chain aliphatic fatty acids connected to carbohydrates [15]. The  $\beta$ -glycosidic linkages, indicative of sugar moiety attachment to the lipid backbone, were clearly identified by an absorption band near  $890\text{ cm}^{-1}$  [16]. Additionally, the existence of a well-ordered sugar moiety linked to the C-O-C backbone of the carbohydrate structure was further confirmed by a peak around  $545\text{ cm}^{-1}$  [17]. Collectively, these spectral features strongly support the conclusion that the biosurfactant is a glycolipid [18].

GC-mass technology was detected that Among the detected compounds, hexadecyl  $\beta$ -D-glucopyranoside was observed, indicating that a C16 fatty acid chain is covalently attached to a glucose molecule [19]. The presence of octadecyl  $\beta$ -D-galactopyranoside confirmed the attachment of a galactose moiety to a C18 fatty acid chain [20]. Additionally, aryl  $\beta$ -D-mannopyranoside was identified, suggesting the linkage of a mannose moiety to a C18 fatty acid chain [21]. Further analysis revealed the presence of sulfoquinovosyldiacylglycerol, which is a sulfonated quinovose sugar bonded to a fatty acid diglyceride [22]. The detection of digalactosyldiacylglycerol indicated the presence of two galactose units connected to fatty acid chains [23]. Glycosyl ceramide was also identified, consisting of a fatty ceramide conjugated to a glucose moiety (C18:0) [24]. The GC-MS results collectively identified a range of components, including lipid glycosides, galactolipids, glyceramides, and sulfated glycolipids, all of which are characterized



by the presence of sugar and lipid components. This chemical profile provides robust evidence confirming the glycolipid nature of the sample.

Table 2 and Figure 3 reveal that the highest concentration of glycolipid at which it was effective against *S. mutans* and *S. aureus* was 200 mg/ml. Key to the changes in membrane permeability properties is the effect of glycolipid concentration. An increase in permeability is caused by the detergent-like activity, which is proportional to the concentration of glycolipids in the membrane. A stronger impact is induced by higher concentrations [25].

The growth of *S. mutans* may be inhibited by glycolipids in many ways. They damage the bacterial cell membrane, which allows intracellular substances to flow out and eventually kills the cell. Because of their amphipathic nature, they may penetrate the lipid bilayer and cause it to become unstable and more permeable. In addition to preventing bacteria from adhering to surfaces and preventing the production of extracellular polymeric substances – essential for the establishment of the biofilm matrix – glycolipids impair biofilm formation. Glycolipids reduce bacterial colonization and persistence in the mouth by blocking the formation of biofilm [26].

*S. mutans*, *Streptococcus sobrinus*, *Lactobacillus* species, *Enterococcus faecalis*, and *Actinomyces naeslundii* are among the frequent oral pathogens that antimicrobial glycolipids show varying degrees of effectiveness against [27]. Furthermore authorized, which lessens plaque production and prevents germs from sticking to teeth [28].

Several antibiotic medications were tested for by employing the disk diffusion technique, in compliance with CLSI 2024, in order to detect *S. aureus* and *S. mutans*. By identifying the resistant strain, this test has the potential to determine the product's efficacy. That belongs to the genus *Staphylococcus*. The following percentages of antibiotic-resistant bacteria and viruses were found in the examined samples: 77.8% to azithromycin, 55.6% to chloramphenicol, 48.1% to clindamycin, 46.3% to ciprofloxacin and gentamicin, and 44.4% to rifampin. In a study conducted by Nakamura-Silva et al. (2024), it was shown that *Staphylococcus* isolates originating from root canal infections had a high degree of resistance to rifampicin, clindamycin, and azithromycin [29]. On the other hand, *S. mutans* isolates showed a distinct pattern of resistance. Among the antibiotics tested, 59.4% showed resistance to amoxicillin and clindamycin, while 56.2% showed resistance to erythromycin and methicillin. Among the medicines examined, gentamicin had the lowest resistance rate at 40.6%, while rifampicin and vancomycin both exhibited intermediate resistance levels at 53.1%. Ardila et al. [30] affirm that their research incorporates over fifty published studies on the subject of oral bacterial resistance to commonly used antibiotics in dentistry, including *S. mutans* and *Staphylococcus* spp., as well as to gentamicin, ciprofloxacin, erythromycin, vancomycin, and rifampin.

The in vitro antibacterial activity test using the well diffusion method was utilized on three commercial toothpastes (Colgate, Sensodyne White glo, and Dabur) in conjunction with biosurfactant. The test was conducted on two clinical isolates of multidrug-resistant *S. aureus* and *S. mutans*, which were obtained from carious lesions in the oral cavity. Colgate, Sensodyne White glo, Dabur, and biosurfactants all work together to inhibit microbial development; Figures 5 and 6 show that this is the case; against *S. mutans*, each brand of toothpaste forms a 23 mm zone, and against *S. aureus*, it is 21 mm.

There is increasing evidence suggesting that oral care products can be enhanced by incorporating biosurfactants. These surfactants possess antibacterial properties, which

help reduce tooth decay and plaque buildup [31]. Natural probiotic surfactants are a promising addition to toothpastes and mouthwashes due to their anti-adhesive efficiency and excellent compatibility with oral tissues [32]. Recent research has highlighted the importance of this approach in developing dental care products that include natural, non-toxic, and environmentally friendly ingredients [33]. A comprehensive study on the use of biosurfactants in medical applications has indicated that they are safer than common oral care chemicals [34]. Finally, systematic review confirms that biosurfactants are effective against *S. mutans* and superior to chemicals in terms of safety and antibacterial performance that approved Biosurfactants have high activity against caries-causing bacteria such as *S. mutans*, Anti-biofilm properties, Low cytotoxicity compared to chemical compounds such as triclosan and SLS and recommends the use of biosurfactants in oral products as safer and more effective natural alternatives [35].

## ■ CONCLUSION

The current study showed that commercial toothpastes containing a biosurfactant exhibited enhanced effectiveness against caries-causing bacteria such as *S. mutans*. The combinations of oral hygiene products and biosurfactants had the largest inhibition zones among all the formulations studied, suggesting a potential synergistic effect. In general, a better approach for preventing and controlling dental caries may be achieved by combining biosurfactants with existing commercial oral hygiene products.

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## Role of Antioxidants and Leptin in Insulin in Polycystic Ovary Syndrome Patients

**Conflict of interest:** nothing to declare.

**Authors' contribution:** Israa Faeck Ja'afar – conceptualization, data curation, investigation, method, project administration, resources, software, writing – original draft and writing – review & editing; Islam M. Fawzi – conceptualization, data curation, supervision, validation, visualization, writing – original draft; Safa Munthader Fawzi – conceptualization, investigation, method, validation, visualization, writing – original draft and writing – review & editing.

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### Abstract

**Introduction.** Polycystic ovary syndrome (PCOS) considered as the most common endocrine disorder that affect women in reproductive age, the key features is irregular menstruation, hyper androgenism and polycystic ovaries. The major abnormality that is associated with PCOS is the insulin resistance (IR), present in about 50–70%. Leptin decreases the secretion of insulin from pancreas and improves insulin sensitivity, while insulin increases the synthesis and release of leptin, but obesity and high blood glucose level dysregulate expression of leptin and the onset of obesity-related pathologies including PCOS.

**Purpose.** To study the role of leptin in follicular fluid and blood of PCOS patients. To find a relation between follicular fluid (FF) leptin and insulin with serum insulin, leptin and other hormones, as well as the relation between (FF) hormones with serum hormones and to know if there is change in IL6 level in those patients.

**Materials and methods.** 70 women were involved in this study, 35 as healthy control group and 35 patients with PCO. A full history was taken from all participant in the study. A thorough physical examination was done to all patients and control groups. In addition, body mass index (BMI) was measured for all as well as measurement of hip and waist circumference to estimate hip/waist ratio. 5ml of blood was aspirated from patients and control groups, serum was aspirated to measure Luteinizing hormone (LH), Follicular stimulating hormone (FSH), Estrogen and testosterone. In addition to estimation of leptin and insulin. Follicular fluid was taken from all participant for which estimation of leptin and insulin was done, as well as IL6. Abdominal and vaginal Ultrasound by a specialist gynecologist was done to all patients to prove the diagnosis of PCOS.

**Results.** Results showed that there was no significant difference between patient and control regarding fasting blood sugar P value was  $p=0.875$ , insulin in serum was  $p=0.296$ , insulin in follicular fluid was  $p=0.285$ . A significant difference observed between patient and control regarding leptin level in serum which was  $p=0.03$  as well as Leptin in follicular fluid  $p=0.01$ . Significant difference was observed in LH level ( $P=0.001$ ) between PCOS



women and the control group. The mean LH level in PCOS women was  $5.1 \pm 3.1$  mIU/ml and in control group was  $3.06 \pm 1.17$  mIU/ml. There was no significant difference in mean of FSH level in PCOS and control group. LH/FSH ratio in patient with PCOS was  $\geq 2$  in about 85% of them. IL-6 levels were notably higher in patients ( $35.6 \pm 4.01$  pg/ml versus  $1.25 \pm 0.02$ ) compared to control group. while the anti-oxidant capacity was lower in PCOS than control.

**Conclusion.** From this study its concluded that a significant inverse relation was found between serum insulin and serum leptin in both patient and control group. LH/FSH ratio in patient with PCOS was higher than control. The inflammatory marker IL6 was significantly higher in patients than control group, while the anti-oxidant capacity was lower in PCOS than control.

**Keywords:** PCOS, Leptin, FF, Insulin Resistance, IL6, oxidative stress

## ■ INTRODUCTION

PCOS considered as the most common endocrine disorder that affect women in reproductive-age (affecting 4–10%), with key features of irregular menstruation, hyper androgenism, and polycystic ovaries. Systematic screening of women according to the National Institutes of Health (NIH) diagnostic criteria estimated that 4–10% of women of reproductive age suffer from PCOS [1]. Although it was previously considered as a disorder of adult women, recent evidence suggests that PCOS is a lifelong syndrome, according to the Rotterdam diagnostic criteria, the prevalence of PCOS in adolescents varies between a minimum of 3% and a maximum of 26% [2]. However, the prevalence of the disease in children is still considered unknown [3]. The majority of women with PCOS presents some degree of insulin resistance, even in non-obese [4]. The resulting hyperinsulinemia causes an increase in the production of androgens as in the biologically active state [5]. The major abnormality that is associated with PCOS is the insulin resistance (IR), which increase the risk of type 2 diabetes. IR is present in about 50–70 percent of PCOS women independently of obesity. IR considered an important risk factor for the development of the metabolic syndrome in women with PCOS. It is known that IR progresses towards the development of compensatory hyperinsulinemia, which drives hyperandrogenemia in these women. Insulin resistance and compensatory hyperinsulinemia can be triggered by obesity with visceral fat accumulation [6, 7].

Fatty tissue secretes bioactive cytokines and adipokines, including adiponectin, leptin, and resistin, some evidence suggested an association between dysregulated expression of leptin and the onset of obesity-related pathologies including PCOS [8]. Long-term hyperinsulinemia has been shown to increase both expression of the ob gene and leptin, which is its product, both in vivo and in vitro studies. This may show some kind of interaction between leptin and insulin which may prove to be important [9]. Leptin is known to have many interactions in the female reproductive system [10]. Polycystic ovaries develop when excessive amounts of male hormones (androgens), particularly testosterone are produced by the affected ovary, this occur by either the release of high levels of insulin in the blood, excessive release of luteinizing hormone by the anterior pituitary in women who had sensitive ovary to this stimulus or decrease levels of sex-hormone binding globulin (SHBG) resulting in elevated level of free androgens. The

follicular development occurs from primordial follicles but due to the disturbed ovarian function this development has stopped at an early antral stage. The follicles appearing as a string of pearls 'along the ovarian periphery on ultrasound examination.

Oxidative stress (OS) is a condition that occurs as a result of an imbalance between the production of reactive oxygen species (ROS) and the body's ability to detoxify and neutralize them. It can play a role in a variety of reproductive system conditions, including polycystic ovary syndrome (PCOS), endometriosis, preeclampsia, and infertility [11].

Elevated interleukin-6 (IL-6) levels indicate chronic inflammation, a key component in PCOS pathogenesis, though its precise role and diagnostic potential remain unclear [12].

## ■ PURPOSE OF THE STUDY

To study the role of leptin in follicular fluid and blood of PCOS patients. To find a relation between follicular fluid (FF) leptin and insulin with serum insulin, leptin and other hormones, as well as the relation between (FF) hormones with serum hormones and to know if there is change in IL6 level in those patients.

## ■ MATERIALS AND METHODS

70 women were involved in this study, 35 as healthy control group and 35 patients with PCO.

Full history was taken from all participant in the study. A thorough physical examination was done to all patients and control groups. Body mass index (BMI) was measured for all participants as well as measurement of hip and waist circumference to estimate hip/waist ratio. 5ml of blood was aspirated from patients and control groups; centrifuged for 5 min at 3000 rpm, serum was aspirated to measure Luteinizing hormone (LH), Follicular stimulating hormone (FSH), Estrogen, progesterone, testosterone and IL6. In addition to estimation of leptin and insulin. Follicular fluid was taken from all participant for which estimation of leptin, insulin was done. Anti-oxidant capacity was measured; using (Antioxidant capacity total bioassay Kit).

All patients have done abdominal and vaginal Ultrasound by a specialist gynecologist to prove the diagnosis of PCOS.

### **Statistical analysis**

SPSS version 30 and excel 2024 was used to study the statistics, chi square used to determine association between two characteristics, with t-test to define the difference between two characteristics mean and correlation were done to assign the statistical linear association between two numerical variables, p value  $\leq 0.05$  considered as significant.

## ■ RESULTS

Seventy subjects were included in this study (35 patient and 35 control), their age ranged from 15–45 years. The mean age of patient was  $29.85 \pm 5.01$  and in control were  $31.60 \pm 5.20$ . No significant difference was found between patient and control regarding PCV ( $p=0.962$ ). The mean PCV in patient group was  $37.85 \pm 5.70$  and in control group was  $37.80 \pm 2.88$ . It was observed that the mean height in patient group was  $161 \pm 5.75$  cm and  $161.73 \pm 5.67$  cm in control group with no significant difference between them. The observed mean weight was  $78.09 \pm 16.15$  Kg in patient, versus  $77.02 \pm 16.3$  Kg



in control with no significant difference between them. The BMI index in patient was  $30.20 \pm 5.20 \text{ cm}^2/\text{kg}$  versus  $29.35 \pm 5.11 \text{ cm}^2/\text{kg}$  in control.

Table 1 illustrate the mean  $\pm$  Standard deviation of sex hormones in the studied groups.

**Table 1**  
Shows different sex hormone levels among patient with PCOS and control

| Parameters           | Patients group (N=35)<br>mean $\pm$ SD | Control group (N=35)<br>mean $\pm$ SD | P value |
|----------------------|----------------------------------------|---------------------------------------|---------|
| FSH (mIU/ml)         | 5.78 $\pm$ 2.06                        | 6.22 $\pm$ 1.83                       | NS      |
| LH (mIU/ml)          | 5.1 $\pm$ 3.13                         | 3.06 $\pm$ 1.17                       | 0.001   |
| Estrogen (ng/ml)     | 2475.13 $\pm$ 8.72                     | 554.15 $\pm$ 4.30                     | 0.005   |
| Progesterone (ng/ml) | 0.42 $\pm$ 0.51                        | 0.9 $\pm$ 0.01                        | 0.05    |
| Testosterone (ng/ml) | 5.44 $\pm$ 1.81                        | 3.01 $\pm$ 0.5                        | 0.05    |
| Prolactin (ng/ml)    | 19.69 $\pm$ 8.72                       | 18.5 $\pm$ 4.1                        | NS      |

The results shows a significant difference in relation to insulin level between patients and control, as well as a significant differences between serum leptin and FF leptin between patients and control table 2, In addition to a significant difference between serum leptin and FF leptin  $P \leq 0.05$ .

**Table 2**  
Comparison between patient and control regarding insulin and leptin in serum and follicular fluid

| Parameters                         | Patients group (N=35)<br>mean $\pm$ SD | Control group (N=35)<br>mean $\pm$ SD | P value |
|------------------------------------|----------------------------------------|---------------------------------------|---------|
| FBS, mmol/l                        | 5.11 $\pm$ 0.80                        | 5.08 $\pm$ 1.86                       | 0.875   |
| Insulin in serum, ng/ml            | 18.40 $\pm$ 3.21                       | 22.05 $\pm$ 2.05                      | 0.05    |
| Insulin in follicular fluid, ng/ml | 7.04 $\pm$ 8.04                        | 9.79 $\pm$ 9.80                       | 0.285   |
| Leptin in serum, ng/ml             | 86.51 $\pm$ 9.01                       | 92.65 $\pm$ 5.22                      | 0.05    |
| Leptin in follicular fluid, ng/ml  | 78.02 $\pm$ 8.11                       | 93.07 $\pm$ 11.01                     | 0.01    |

Correlation was done to measure the relationship between BMI, insulin and leptin in serum and in follicular fluid and are illustrated in table 3.

**Table 3**  
Shows different correlation in PCOS patient and control group

| Correlation between parameters                           | Patients                   | Control                   |
|----------------------------------------------------------|----------------------------|---------------------------|
| Serum leptin with serum insulin                          | $r = -0.2$<br>$p = 0.02$   | $r = -0.42$<br>$p = 0.02$ |
| Serum leptin with BMI                                    | $r = -0.06$<br>$p = 0.73$  | $r = -0.02$<br>$p = 0.91$ |
| Insulin in follicular fluid with leptin follicular fluid | $r = -0.09$<br>$p = 0.49$  | $r = -0.2$<br>$p = 0.43$  |
| Leptin in follicular fluid with serum insulin            | $r = -0.07$<br>$p = 0.06$  | $r = -0.04$<br>$p = 0.84$ |
| Insulin in follicular fluid with serum insulin           | $r = -0.002$<br>$p = 0.99$ | $r = 0.10$<br>$p = 0.6$   |
| Insulin in follicular fluid with BMI                     | $r = 0.2$<br>$p = 0.33$    | $r = -0.2$<br>$p = 0.35$  |
| Serum insulin with BMI                                   | $r = 0.02$<br>$p = 0.91$   | $r = -0.12$<br>$p = 0.54$ |

The inflammatory marker IL6 was significantly higher in patients with PCOS than control group  $35.6 \pm 4.01$  pg/ml versus  $1.25 \pm 0.02$  respectively, with a  $P=0.05$ .

The antioxidant capacity was  $0.25 \pm 0.01$  versus  $1.54 \pm 0.12$  in PCOS and control respectively, with a  $P=0.05$ .

## ■ DISCUSSION

The dysovulatory infertility PCOS, is characterized by chronic anovulation and hyperandrogenemia. These features manifest with advancement of age and gradual increase of adipose tissue, which are often linked to leptin and its receptor [5]. Obesity is common in more than the half of women with PCO. The central fat distribution (android obesity) exacerbates the risks of diabetes mellitus and cardiovascular disease. Although in this study there was no significant difference between patients and control regarding BMI and there was weak correlation between serum leptin and BMI which was  $30 \text{ kg/cm}^2$  ( $\pm 5.613$ ). The explanation for our higher incidence of overweight may be attributed to the food habits adopted in Iraq and to the lack of exercise among Iraqi women. In 2015 Royal College of Obstetricians and Gynecologists showed that LH may be elevated, with the LH:FSH ratio increased ( $>2$ ), with FSH normal; however, this is not part of the diagnostic criteria and may be normal. In this study there was significant difference between patients with PCOS and control group regarding serum LH. While there was no significant difference regarding FSH. Significant difference in serum estrogen between patients and control was found in this study, though its relation to the level of leptin is poorly understood as Jana Chacarbarti said in his study in 2016 [7]. Marfan Mohammad and Ashraf Olabi in 2016 were found that serum Leptin levels were correlated positively with BMI in Patient and control groups [8]. This result is in agreement with many studies have reported that leptin is correlated to BMI in both groups, in this study there's positive correlation between Serum leptin and BMI in patients group while negative correlation between theme in control group. A study in 2021 by Childs GV, et al. demonstrated higher leptin levels in PCOS subjects compared with control group and this goes with this study in which there is significant difference between patients and control regarding leptin level in serum and follicular fluid [9]. Several studies showed that leptin is correlated with insulin level in PCOS subjects [10], Yuanyuan Peng et al. in 2022 regarded elevated leptin as predictive marker of PCOS [13]. This study demonstrate negative correlation between insulin and leptin in follicular fluid in the control group, while positive correlation between them in patients group. The controversy could be attributed to the differences in the sample size, the study populations, and the heterogenic nature of the PCOS syndrome.

The elevated IL-6 levels in PCOS patients reported in this study aligns closely with previous research by Escobar-Morreale et al., who similarly documented increased IL-6 concentrations among women with PCOS, attributing this elevation to heightened inflammatory responses driven by insulin resistance [14].

ROS play a nuanced role in ovarian physiology, where controlled levels of ROS are essential for normal ovarian function. However, an imbalance in ROS production and antioxidant defenses can lead to OS, which may have detrimental effects on reproductive outcomes. They play roles in regulating key ovarian processes, including meiosis, which is essential for the development of mature oocytes; ovulation, where ROS participate in the signaling cascades that trigger the release of the mature egg; and corpus luteum maintenance and regression [15].



## ■ CONCLUSION

A significant inverse relation was found between serum insulin and serum leptin in both patient and control group. A significant increase in LH/FSH ratio between the two groups, as well as the anti-oxidant capacity was less in PCOS, IL6 level were higher in patients than control group.

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# Comparative Study of Hodgkin and Non-Hodgkin Lymphoma and their Impact on Treatment Outcomes

**Conflict of interest:** nothing to declare.

**Ethics statement:** the research was approved by the College of Science's review ethical committee at Al-Muthanna University. The article is published in author's edition.

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## Abstract

**Introduction.** Cells' neoplastic transformations which are primarily found in lymphoid tissues are called lymphomas. They are a heterogeneous group of T or B cell malignancies which can start in any body organ, although they typically start in lymph nodes.

**Purpose.** To evaluate changes in biochemical as well as hematological parameters and to identify genetic variations in CDKN2A gene in the patients with lymphoma.

**Materials and methods.** The current research looked at 50 samples, whose ages ranged between 15 and 80 years old. Biochemical, molecular, along with hematological testing have been used for analyzing the samples.

**Results.** The research revealed a considerable increase in hematological parameters at ( $P < 0.01$ ). A tumor suppressor gene called CDKN2A regulate the cell cycle through blocking cyclin-dependent kinases (CDKs), which stops uncontrolled cell division. In this study, the CDKN2A sequences were analyzed in twenty-five samples for controls (assigned A1 – A25) and an equal number of patients (assigned B1 – B25) to assess the possible association between CDKN2A and the onset of cancer.

**Conclusion.** The tumor suppressor gene CDKN2A controls cell cycle through blocking cyclin-dependent kinases (CDKs), which stops uncontrolled cell division.

**Keywords:** Hodgkin, Non-Hodgkin, lymphoma, CDKN2A, cyclin-dependent kinases

## ■ INTRODUCTION

In developed regions worldwide, people 70 years of age or older are diagnosed with no less than 50% of all cancers; such percentage is rising as populations age and life expectancy rises [1, 2]. In addition to being the 6th major prevalent cause of death for males and 7<sup>th</sup> for females, non-Hodgkin's lymphoma (NHL) is considered to be the 6<sup>th</sup> most widespread cancer [3]. Age increases risks of NHL, and over the past 20 years, NHL incidence was rising by 1% to 2% yearly, with the highest increase occurring in those over 60. White men aged between 75 and 84 years had the most change in incidence [4]. As a result, NHL is now a major contributor to mortality and morbidity in elderly population. One known



poor prognostic factor related to NHL is being older than 60. Patients older than 70 years had worse results compared to those between 60 and 70 years old, and overall survival declines with age [5]. No less than 65,000 new cases of Hodgkin lymphomas (HLs), which are lymphoid malignancies, are discovered all over the world each year. Approximately 1% of all de novo neoplasms occurring annually are HLs [6, 7]. In Western nations, HL is among the most prevalent malignancies among young individuals [8, 9]. It is considered as one of the histologically and etiologically heterogeneous diseases. Even though such peaks might differ by ethnicity and region, HL has bimodal distribution of age, with the first peak occurring throughout young adulthood (ages 15–35) and the second peak occurring in people who are over 55 [10, 11]. Through the production of proteins controlling cell cycle, tumor suppressor genes stop cells from proliferating out of control. Tumor suppressor genes, in contrast to the oncogenes, require the inactivation regarding the two alleles for contributing to the development of tumors. At gene locus, this usually leads to loss of heterozygosis. One nonfunctional or defective copy regarding tumor suppressor gene could be inherited by people with family history of tumor development, whereas the second copy is lost as a result of a somatic mutation. The two alleles are seldom lost due to somatic mutations alone [12]. Mutation in chromosome 9 CDKN2A gene, which codes for tumor suppressor genes p16 and p14arf, controlling the course of cell cycle, is present in almost 50% of such patients [13]. J was found to have c.225\_243del19 mutation on chromosome 9's CDKN2A gene through genetic testing. Given that it causes nonsense mutation in p16 tumor suppressor gene and its origin could be associated with common ancestor from early 18<sup>th</sup> century close to Leiden town, Netherlands, such particular mutation was referred to as p16-Leiden mutation in the past [14].

## ■ PURPOSE OF THE STUDY

To evaluate changes in biochemical as well as hematological parameters and to identify genetic variations in CDKN2A gene in the patients with lymphoma.

## ■ MATERIALS AND METHODS

### **Study Design**

Fifty samples of blood in this study. Twenty-five sample were obtained from patients with lymphoma. Also, twenty-five of sample were collected from healthy people. All participants undergone hematological, biochemical and molecular tests. They were between the ages of 25 and 75. The research has been carried out between April and August of 2025. The people were listed in Al-Hussein Teaching Hospital's Oncology Unit in the province of Al-Muthanna as having lymphoma.

### **Blood Sample**

Each one of the individuals had five milliliters of venous blood drawn, which has been then separated into serum tubes (2 milliliters) and EDTA polypropylene tubes (3 milliliters) kept in a refrigerator at –20 degrees Celsius. Blood in serum tubes has been centrifuged for separating the serum, which was then preserved in new plastic screw tip tubes as well as utilized for measuring the biochemical parameters, blood in EDTA tubes was utilized for performing differentiation WBCs, White Blood Cells (WBCs) count, sequence test, and platelets count.

**Hematological Parameters**

The complete blood count (CBC) regarding the controls and participants, which included the counts of differentiation WBCs, white blood cells (WBCs), and platelets, has been determined using a full set of control and calibrator reagents (Sysmax-Kx-21).

**Biochemical Parameter**

Serum creatinine, blood urea, alanine transaminase (ALT), along with aspartate aminotransferase (AST) were among the biochemical parameters measured using a complete system of control as well as calibrator reagents (Bio-Autolyzer).

**Molecular Methods**

**DNA Extraction and PCR Technique**

EasyPure® Blood Genomic DNA Kit (Cat.No.EE-121;Biotren) has been used for extracting DNA. OneTaq Quick-Load 2X Master Mix with Standard Buffer from New England BioLabs has been utilized for PCR according to manufacturer’s recommendations (NEB Protocol).

**Table 1**  
**PCR Amplification Program**

| PCR Step             | Temperature                          | Time   | Temperature |
|----------------------|--------------------------------------|--------|-------------|
| Initial denaturation | 94 °C                                | 30 sec | 1°          |
| Denaturation         | 94 °C                                | 30 sec | 35°         |
| Annealing            | Variables according to (primer’s TM) | 40 sec |             |
| Extension            | 72 °C                                | 1 min  |             |
| Final extension      | 72 °C                                | 5 min  | 1°          |
| Hold temperature     | 4 °C                                 | ∞      | –           |

**PCR Amplicons’ DNA Sequencing**

In accordance with sequencing company’s instruction manuals, resolved PCR amplicons have been sequenced commercially from reverse directions (Macrogen Inc. Geumchen, South Korea). For ensuring that the annotation as well as variances weren’t a result of PCR or sequencing artefacts, just clear chromatographs from ABI (Applied Biosystem) sequence files have been subjected to additional analyses. The obtained PCR fragments’ virtual positions as well as other details have been determined through comparing them with observed DNA sequences.

**Sequencing Data Interpretation**

With the use of BioEdit Sequence Alignment Editor Software v. 7.1 (DNASTAR, Madison, WI, US), results of sequencing that are related to PCR products have been aligned, edited and examined according to corresponding sequences in reference data-base. Every sequenced sample’s detected differences have been numbered in both PCR amplicons and the appropriate positions within reference genome. SnapGene Viewer version 4.0.4 (www.snapgene.com) has been used for annotating each one of the variants found within the gene under investigation.



### Checking the Identified SNPs

For determining its uniqueness, the identified SNPs have been uploaded to dbSNP (Single Nucleotide Polymorphism Data-base) server ([www.ncbi.nlm.nih.gov/projects/SNP/](http://www.ncbi.nlm.nih.gov/projects/SNP/)). A public repository for genetic variation both across and within species is dbSNP. The database is a repository related to submitted nucleotide variants and includes a wide variety regarding basic genetic polymorphisms. It is connected with data from the Human Genome Project, PubMed, GenBank, along with LocusLink, among other NCBI information sources. The server has been used to determine whether a specific SNP position had already been deposited in dbSNP server through checking and annotating it to its relevant reference genome.

### Deposition of Sequences to GenBank

The sequences that had been examined as well as evaluated have been uploaded to NCBI Bankit portal, and the server followed all of the guidelines provided by the server. For getting a distinct GenBank accession number for the sequences under investigation, the submitted sequence was submitted as nucleic acid sequences to NCBI.

### Statistical Analysis

SPSS version 24 is used for statistical analysis. Chi-square test has been utilized for looking for a link between categorical variables, Pearson correlation coefficient has been utilized for evaluating a correlation between two numerical variables, and ANOVA was utilized for determining the mean regarding data across more than two groups. Furthermore, significance level has been set at  $P \leq 0.050$ , while  $P \leq 0.010$  has been regarded as the very significant level.

## ■ RESULTS

Hematologically, a significant increase in PLT has been indicated in patients ( $p > 0.01$ ), particularly in those with lymphomas, which may be a reactive thrombocytosis often seen in malignancy. Eosinophil and WBC counts did not differ significantly between groups, though the WBC count trended lower in H.L patients, possibly indicating bone marrow suppression or disease-induced leukopenia. RBC counts and levels of Hb remained relatively stable between groups, although a statistically significant decrease in RBCs was noted in NHL patients ( $p = 0.032$ ), which could suggest mild anemia associated with chronic disease. A particularly striking observation was the significant reduction in neutrophil and lymphocyte percentages in patients compared to controls ( $p > 0.01$ ). These reductions may indicate immune suppression, either as a consequence of the malignancy itself or due to treatment effects. Moreover, basophil levels, absent in controls, were minimally increase in patients, though their clinical relevance requires further exploration (Table 2).

Biochemically, patients exhibited significantly elevated AST and urea levels ( $p > 0.01$ ), suggesting hepatic stress and possible renal impairment, both of which are common secondary effects of malignancy or chemotherapy. ALT levels were also increased in patients compared to controls, though significance varied among subgroups, with a mild elevation observed in N.H.L compared to H.L. Interestingly, creatinine levels showed a marked decrease in H.L patients compared to controls, while the N.H.L group exhibited a slight elevation ( $p > 0.01$ ), which may reflect different metabolic demands or degrees of systemic involvement in each lymphoma subtype (Table 3).

**Table 2**  
**Frequency distribution of some blood parameters levels according to type of lymphoma**

| Test        | Control (N=25) | H.L (N=13)    | N.H.L (N=12)  | P<br>value |
|-------------|----------------|---------------|---------------|------------|
|             | Mean±SD        |               |               |            |
| Eosinophil  | 0.16±0.20      | 0.09±0.009    | 0.1750.26     | 0.4        |
| PLT         | 103±20.27      | 309.23±132.92 | 322.90± 94.91 | <0.01      |
| WBC         | 9.188±3.25     | 5.89±3.70     | 8.29±3.80     | 0.1        |
| Basophile   | 0±0.00         | 0.03±0.016    | 0.03±0.016    | <0.01      |
| Neutrophils | 61.1±8.26      | 3.81±1.4      | 5.65±6.67     | <0.01      |
| Lymphocyte  | 27.64±7.10     | 1.52±0.60     | 1.73±1.27     | <0.01      |

**Table 3**  
**Frequency distribution of some biochemical parameter's levels according to type of lymphoma**

| Test     | Control (N=25) | H.L (N=13)  | N.H.L (N=12) | P<br>value |
|----------|----------------|-------------|--------------|------------|
|          | Mean±SD        |             |              |            |
| ALT      | 26.72±9.27     | 41.45±23.84 | 31.86±6.45   | 0.033      |
| AST      | 24.36±7.03     | 35.47±9.48  | 37.29±11.82  | >0.01      |
| CREATNIN | 30.0±10.19     | 0.60±0.15   | 0.75±0.49    | >0.01      |
| UREA     | 0.776±0.20     | 22.43±8.42  | 41.2±6.16    | >0.01      |

**Molecular Results**  
**DNA Amplification**

Genomic DNA has been extracted from the human whole blood samples for starting the molecular workflow. This has been confirmed by 1% agarose gel electrophoresis stained with ethidium bromide as well as observed under UV transilluminator. Lanes 1 through 25 showed well-defined and distinct bands, as seen in Figure 1, indicating effective high-integrity DNA extraction that is appropriate for molecular applications later on. Primers specific to CDKN2A gene, a tumor suppressor gene known to be crucial for the cell cycle regulation and commonly researched in cancer diagnostics, were then used in PCR experiment. The genetic analysis relies heavily on PCR, a selective and highly sensitive molecular technology enabling exponential amplification related to target DNA regions. An optimized annealing temperature of 57.3 °C has been used for amplification, producing a particular 771 bp product, as shown in Figure 2. CDKN2A gene was successfully as well as specifically amplified in all the tested samples, as evidenced by obvious, distinct bands in each lane (1–25) of PCR products resolved on 1.5% agarose gel. The reaction's specificity has been validated and the lack of contamination was confirmed when no amplification was found in negative control lane (N). The consistency and clarity of the bands across all positive samples demonstrate the reliability of the extraction and amplification procedures, highlighting the effectiveness of the PCR protocol for genetic screening and further molecular characterization.

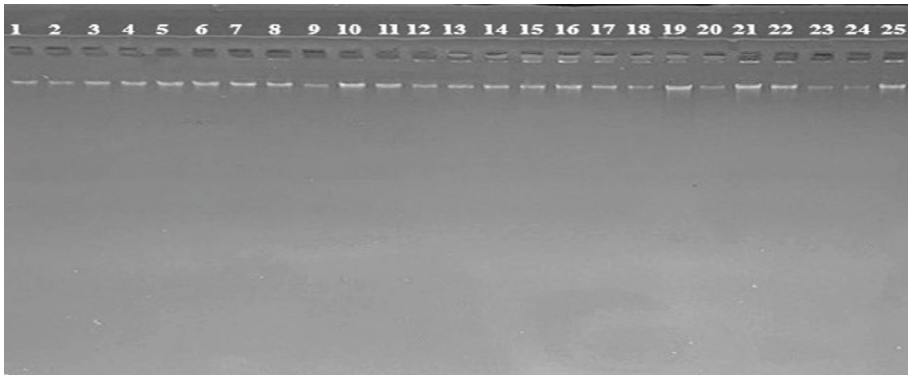


**Fig. 1.** After performing 1% agarose gel electrophoresis at 110 volts, ethidium bromide staining has been applied to the gel. The gel was visualized under a UV transilluminator, revealing clear bands corresponding to Human DNA molecules extracted from whole blood samples, Lanes (1–25) represent positive DNA extractions, indicated by distinct and well-defined bands

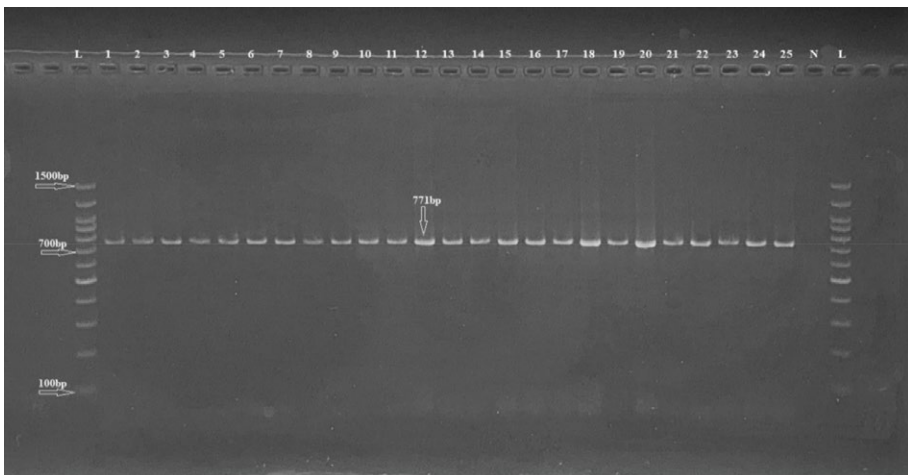


**Fig. 2.** For blood samples that exhibit 771 bp Primer TM at 57.3 °C, gel electrophoresis for PCR product related to CDKN2A gene primer (Agarose 1.5%, 15 min. at 110 voltage and subsequently dropped to 75 volts, 60 min.). Lane L: DNA ladder (1500–100) bp was visualized under ultraviolet light following ethidium bromide staining. Lanes 1–25 indicated positive outcomes, whereas Lane (N) denoted negative control

## ■ DISCUSSION

The chemotherapy has negative effects on non-target organs and alters immune responses, as has been documented in another research. Through lymph depletion, the therapy affects the hematopoietic compartment's homeostasis, impairing both adaptive and innate immune responses. Cells' ability to replicate hematopoietic DNA is compromised. White blood cell production is reduced and immune cell death is caused by the progressive depletion of the hematopoietic stem cells in bone marrow, which inhibits cell proliferation [15]. The observed decrease in neutrophils in this investigation

may be related to compromised hematopoiesis. Also, decline could be brought on by a somatic mutation in lymphoid progenitor cells, which alters the regulation of cell division, proliferation, and apoptosis (programmed cell death), it can also cause lymphocytes to accumulate in lymph nodes, spreading lymphadenopathy. It is possible that the cancerous cells (lymphoma cells) will enhance platelet adhesion, which will interfere with normal platelet function [16]. The fundamental units of the immune system are lymphocytes, which have the ability to cause cytotoxic cell death and cause cancer cells to release cytokines. Lymphocytopenia has a negative impact on the host's antitumor immune response, which in turn encourages tumor growth and lowers the prognosis for the patient [17]. Upon activation in response to endothelium injury, lysosomes, dense granules, and  $\alpha$ -granules release hundreds of proteins. Since these platelet-derived factors influence proliferative, angiogenic, inflammatory, and hemostasis in addition to hemostasis, activated platelets' release of cytokines may promote tumor development and angiogenesis. increased platelet activation within the potentially thrombogenic tumor [18].

These results may be due to the serum creatinine is influenced by low protein intake, liver illness, malnutrition, muscle atrophy, and hydration abnormalities – all of which are prevalent in cancer patients. Clinical importance arises from the failure to identify decreased kidney function [19]. Liver enzymes like ALT as well as AST are linked to various cancer types as well as the onset and prognosis of hepatocellular carcinoma and liver metastases. Raised ALT levels in hepatitis C virus-infected patients are thought to constitute a separate risk factor for hepatocellular cancer [20]. The liver is of high importance in metabolizing drugs as well as detoxifying the body, so any insult to the liver can lead to increased AST and ALT levels. Additionally, certain chemotherapy drugs are more hepatotoxic than others, further contributing to elevated liver enzyme levels during chemo radiotherapy [21].

Anticancer medications produce intra- and interstrand cross-links in bone marrow DNA by binding covalently with the DNA molecule. These cross-links can cause damage to DNA during replication and disrupt protein function [15].

Lately, two groups had discovered recurrent copy-number alterations (CNA) in tumor suppressors CDKN2A and TP53 that are generally linked to genomic instability and have a negative prognostic significance [22, 23]. Single nucleotide polymorphism (SNP) arrays as well as next-generation sequencing (NGS) regarding specific gene panels have served as the foundation for such initiatives. Whole genome sequencing (WGS) could examine the entire repertoire related to CNA, structural variations (SV), somatic mutations, and even mutational mechanisms implicated in the pathogenesis of cancer, in contrast to the aforementioned research [24].

The mechanistic bases related to frequent CNA that is seen in PTCL-NOS instances in bigger cohorts, such as deletions of CDKN2A, are probably provided by this indication [22, 23]. We found that CDKN2A deletions have been frequently focal and bi-allelic in the research. The inactivation pattern has been reported for CDKN2A in different hematological malignancies and is quite specific for driver tumor suppressors [25, 26].

One explanation could be that telomere-independent mechanisms could be the cause of melanomas in those with CDKN2A mutations. Telomere shortening and genomic instability could result from CDKN2A mutations that lead to uncontrolled cell proliferation as well as poor DNA repair [27]. In response to UVR, CDKN2A-deficient mice



showed elevated intracellular reactive oxygen species (ROS) levels and a decreased capacity to handle UVR-induced DNA damage [28]. Because DNA replication machinery cannot replicate the linear DNA ends, telomere DNA may be lost in the proliferating cells. In support of such theory, a recent research showed a substantial correlation between hypermethylation of CDKN2A promoters in breast cancer and telomere length shortening [29].

## ■ CONCLUSION

A significant increase in WBCs, differentiation WBCs, Platelets counts, liver enzyme levels (ALT & AST) and kidney function (blood urea and creatinine) was observed. Additionally, the tumor suppressor gene CDKN2A controls cell cycle through blocking cyclin-dependent kinases (CDKs), which stops uncontrolled cell division.

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## Evaluation of Oxidative Damage of Valproic Acid at Different Doses in the Liver of Male Rats

**Conflict of interest:** nothing to declare.

**Authors' contribution:** Zainab K. Ali – conceptualization, data curation, investigation, methodology, project administration, resources, software, visualization, writing – original draft and editing; Ibtehal M. Khudhair – conceptualization, investigation, methodology, project administration, resources, supervision, validation, visualization, writing – editing; Mustafa R. Oglah – conceptualization, investigation, methodology, project administration, resources, supervision, validation, visualization, writing – original draft.

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### Abstract

**Introduction.** Valproic acid is used to treat certain neurological disorders, such as bipolar disorder, epilepsy, and migraines. However, it carries the risk of side effects, such as liver damage.

**Purpose.** To examine the relationship between serum liver enzyme levels and the effects of valproic acid on oxidative and histological changes in male rats' livers at various doses.

**Materials and methods.** Twenty-four adult male albino rats were divided into four groups: a control group given distilled water, and three experimental groups treated with intraperitoneal valproic acid in doses of 125, 250, and 500 mg/kg/day for two weeks. Serum samples were analyzed for liver enzyme activity and antioxidant capacity, while liver tissue samples were examined for glutathione, malondialdehyde levels, and histological assessments.

**Results.** Valproic acid at high doses (500 mg/kg/day) significantly increased serum levels of alanine, aspartate, and alkaline phosphatase, decreased glutathione, and increased malondialdehyde ( $P < 0.001$ ). Group IV had extensive liver damage, including venous dilation and congestion, inflammatory infiltrates, disorganised hepatic cords, cellular swelling, and necrosis.

**Conclusion.** High-dose valproic acid caused oxidative damage and hepatotoxicity in male albino rats.

**Keywords:** valproic acid, oxidative stress, hepatic enzyme, hepatotoxicity, rats

### ■ INTRODUCTION

As an antiepileptic drug, valproic acid is useful for controlling both partial and generalised seizures. In addition to its function in migraine prevention, it has a number of clinical uses for other neuropsychiatric disorders, including bipolar disorder, social



phobia, and neuropathic pain [1, 2]. The liver was damaged by valproic acid through a series of interrelated processes [3, 4].

Valproic acid is converted into harmful metabolites like 4-ene-valproic acid, which directly damage hepatocytes [5, 6]. Valproic acid lowers glutathione, increasing oxidative stress and liver damage [7, 8]. Also, adversely impacts mitochondrial function by diminishing proton leaks and disrupting oxidative phosphorylation, resulting in reduced ATP production, which is crucial for sustaining cellular energy homeostasis [9–11]. Valproic acid causes hepatic steatosis by blocking carnitine palmitoyl transferase, which is the enzyme that moves long-chain fatty acids into mitochondria [12, 13].

This activates nuclear receptor-gamma, which causes enzymes like acyl-CoA thioesterase1 to be made more active [14, 15]. The changes make it easier for the body to absorb fatty acids and make triglycerides, which causes more fat to build up in liver cells. Keeping therapeutic drug levels is very important to lower the risk of hepatotoxicity that comes with valproic acid [11, 16, 17].

## ■ PURPOSE OF THE STUDY

To examine the relationship between serum liver enzyme levels and the effects of valproic acid on oxidative and histological changes in male rats' livers at various doses.

## ■ MATERIALS AND METHODS

### **Chemicals and Reagents**

Distilled water (PDPL: India), and valproic acid (Sigma-Aldrich, St Louts, MO, USA).

### **Experimental Animals**

This experiment used 24 [180–220 gm] male albino rats. The rats were moved from Gilgamesh University's College of Pharmacy's animal house and given 14 days to adjust. The subjects received pellets and water in plastic research cages.

### **Treatments and Animal Grouping**

This study used random sampling to divide rats into four six-rat groups. Group I, the negative control, received 1 ml/kg/day of distilled water orally. The other three groups received intraperitoneal valproic acid at 125, 250, and 500 mg/kg/day for 14 days to damage the liver.

### **Collection of Serum and Liver Tissues**

On day fifteen, rats were anaesthetized and injected with 50 mg/kg ketamine and 5 mg/kg xylazine. Blood was drawn from the right ventricle, incubated in gel tubes, and centrifuged to collect serum for measuring liver enzymes (ALT, AST& ALP). Liver tissue was homogenized to assess malondialdehyde (MDA) and glutathione (GSH) levels, with some slices preserved in 10% formalin for histopathological analysis.

### **Determination of Liver Function**

Colourimetric assay kits were used to measure the levels of aspartate aminotransferase (AST), alanine aminotransferase (ALP), and alkaline phosphatase (ALP) in accordance with the instructions provided by the manufacturer.

Determination of Oxidative Damage in Liver Tissue Homogenate

Liver tissue homogenate levels of oxidative damage (reduced GSH and MDA) are measured using the sandwich ELISA (Enzyme-Linked Immunosorbent Assay) technique (My BioSource) in accordance with the manufacturing procedure.

Histopathological Assessment

Liver tissue samples were fixed in 10% neutral buffered formalin for 24 hours, dehydrated, and embedded in paraffin. Thin sections (4µm) were prepared and stained with haematoxylin and eosin (H&E) for histological analysis, which was conducted using a light microscope to evaluate histopathological changes due to valproic acid.

Statistical Analysis

The analysis used SPSS [statistical packages for social sciences] version 16 to process data and present descriptive statistics as mean±SEM. One-way ANOVA was used to compare the four groups, and the Tukey test was used to analyse the results. The results' significance p-value.

RESULTS

The Impact of Varying Doses of Valproic Acid on Serum Liver Enzyme Levels

The administration of 125 mg/kg/day of valproic acid showed no significant effect on serum liver enzymes compared to the control group (P>0.05). In contrast, a dose of 250 mg/kg/day significantly increased serum levels of these enzymes (P<0.001). Additionally, a higher dose of 500 mg/kg/day also resulted in significantly elevated enzyme levels (P<0.001). As G1: negative control group; GII: positive control group, 125 mg/kg V.A; GIII: 250 mg/kg V.A; GIV: 500 mg/kg VA.

Table 1  
Serum Levels of Liver Enzyme (ALT, AST, and ALP)

| Group | N | ALT (nmol/ml) | AST (nmol/ml) | ALP (nmol/ml) |
|-------|---|---------------|---------------|---------------|
| G1    | 6 | 60±0.1 a      | 74±0.9 a      | 106±2 a       |
| GII   | 6 | 61±0.08 a     | 76±0.1 a      | 109±0.1       |
| GIII  | 6 | 151±0.1 b     | 205±0.9 b     | 136±0.9 b     |
| GIV   | 6 | 203±0.9 c     | 275±1 c       | 227±0.8 c     |

Note: dissimilar letters (a, b, and c) indicate a significant difference in liver enzyme levels between groups.

Hepatic Oxidative Damage Caused by Different Doses of Valproic Acid in Male Rats

This study indicates that group II shows no significant oxidative stress compared to the control group (group I), with GSH and MDA levels unchanged (P>0.05). In contrast, groups III and IV exhibited significant oxidative damage (P<0.005), particularly group IV, which displayed the most pronounced oxidative damage (P<0.001), with a GSH depletion of 6.1±0.2 µg/ml and MDA increase of 5.1±0.1 nmol/ml.

Histological Examination of Rat Hepatic Tissue Following Valproic Acid Treatment

The examination of samples at 400X magnification demonstrated that the control group's histological section displayed well-preserved hepatic architecture, characterised

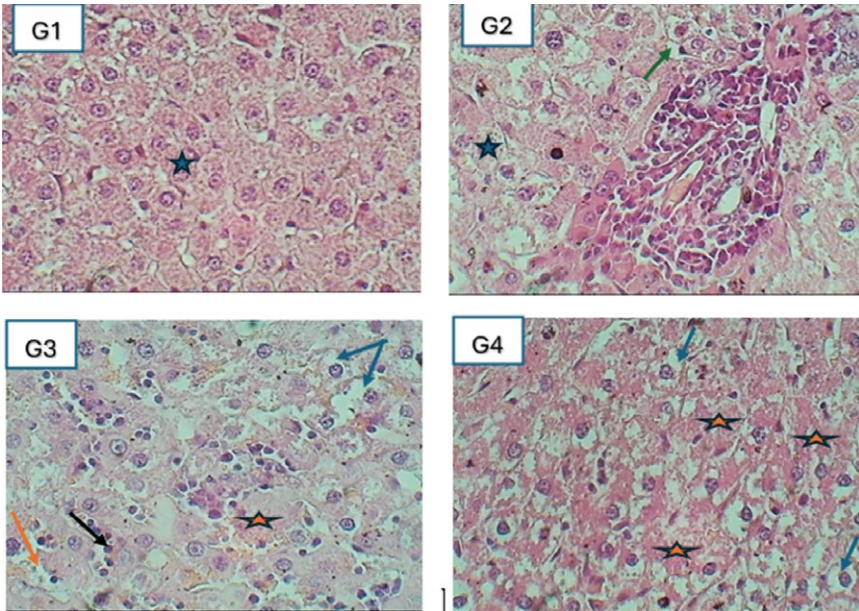


**Table 2**  
**Change (GSH And MDA) Levels in All Groups**

| Groups | Number of animals | GSH (µg /ml) | MDA (nmol/ml) |
|--------|-------------------|--------------|---------------|
| GI     | 6                 | 16±0.01 a    | 0.78±0.02 a   |
| GII    | 6                 | 15.9±0.01 a  | 0.9±0.01 a    |
| GIII   | 6                 | 13.6±0.1 b   | 1.6±0.1 b     |
| GIV    | 6                 | 6.1±0.2 c    | 5.1±0.1 c     |

by distinct lobules and polygonal hepatocytes, which suggests healthy cellular functions. No cell damage was found. In contrast, the 125 mg/kg/day group showed mild vacuolar degeneration, indicating early cellular stress, but no necrosis, fatty changes, or inflammatory infiltration.

The liver segment of a rat treated with 250 mg/kg of valproic acid showed mild to moderate degenerative alterations in hepatocytes, including cytoplasmic vacuolization, signs of hydropic degeneration, and slightly faded nuclei. While the general lobular architecture was preserved, there was mild dilation and congestion of hepatic sinusoids, and possible tiny inflammatory cell infiltration. Administration of high doses of valproic acid (500 mg/kg) in rats resulted in severe histopathological changes, including vacuolated hepatocytes with signs of cell injury such as pyknotic or karyolytic nuclei, extensive necrosis, and disruption of the hepatic parenchyma replaced by debris. Additionally, sinusoids were congested, with infiltration of inflammatory cells and early fibrotic changes observed in some regions. The general lobular structure is no longer distinguishable, and hepatocyte borders are obliterated (Figure, Table 3).



**Histopathological alteration in all groups. Blue asterisks: normal hepatocyte; green arrow: sinusoids dilation; back arrow: leukocyte infiltration; blue arrow: cellular swelling; red asterisks: necrosis**

**Table 3**  
**Histopathological alteration in all groups**

| Parameter                   | GI | GII | GIII | GIV |
|-----------------------------|----|-----|------|-----|
| Congestion                  | 0  | 0   | +    | ++  |
| Hepatic necrosis            | 0  | 0   | +    | ++  |
| Hepatic fibrosis            | 0  | 0   | 0    | +   |
| Inflammatory cells          | 0  | +   | +++  | +++ |
| Hepatic sinusoidal dilation | 0  | +   | +++  | +++ |
| Hepatic swelling            | 0  | +   | +++  | +++ |

Notes: 0 – nonobvious damage; + – mild hepatocellular lobules affected; ++ – moderate hepatocellular lobules affected; +++ – severe hepatocellular lobules affected.

■ DISCUSSION

In the present study, administration of valproic acid at doses of 250 and 500 mg/kg resulted in a significant elevation in liver enzymes [ALT, AST, &ALP], when compared to the control group. The presence of these enzymes is recognised as a reliable indicator of hepatic injury, with elevated levels indicating that higher doses of valproic acid may lead to hepatocellular damage [11].

The identified hepatotoxicity may be linked to oxidative stress, which is a key factor in drug-induced liver injury. Oxidative stress arises from an imbalance between the generation of reactive oxygen species (ROS) and the mechanisms of antioxidant defense [18–20].

Elevated enzyme levels at 250 and 500 mg/kg were associated with increased lipid peroxidation, as measured by MDA levels, and decreased antioxidant molecules, including glutathione [21–23].

Valproic acid causes oxidative damage in hepatic tissue, especially at high doses. However, liver enzymes did not change at 125 mg/kg, indicating that the liver's antioxidant systems could handle mild oxidative stress [11, 24, 25]. Also, cell membrane damage, enzyme leakage, and liver injury can result from high doses of the substance overwhelming antioxidant systems [26, 27]. Previous studies have shown that valproic acid causes hepatotoxicity through oxidative stress, mitochondrial dysfunction, and fatty acid metabolism disruption [9, 28].

Valproic acid's hepatotoxicity is largely due to oxidative stress. Due to an imbalance between ROS production and antioxidant defence system capacity. Valproic acid breaks down into reactive oxygen species (ROS), which damage lipids, proteins, and DNA. This process increases MDA and lipid peroxidation, indicating oxidative damage [29].

MDA levels above 250 and 500 mg/kg of valproic acid indicate membrane damage. However, reduced glutathione (GSH) is a key intracellular antioxidant that neutralises reactive oxygen species (ROS) and detoxifies lipid hydroperoxides as a cofactor for glutathione peroxidase [30–32].

GSH depletion due to ROS-induced utilisation is shown by the significant decrease in GSH levels at the same dosage. Fighting reactive oxygen species with glutathione depletes it, making it harder for the liver to handle oxidative damage and damaging cells. This cycle causes hepatocellular damage, as shown by increased liver enzymes and histopathological changes, due to increased ROS generation and lipid peroxidation (MDA) [33, 34].



The study found that 500 mg/kg valproic acid increases MDA and GSH. It appears that the mechanism is dose-dependent and that more ROS causes more oxidative damage. Oxidative stress can be managed well, and liver function kept at lower exposure levels with the 125 mg/kg dose [35, 36].

Examining liver tissue under a microscope showed histopathological alterations that varied according to the valproic acid dosage. The liver morphology was largely intact in the control and low-dose group (125 mg/kg), with normal hepatocytes and little inflammation. This finding is consistent with liver enzyme levels and stable oxidative stress indicators at this dosage [35, 37].

At a moderate dosage of 250 mg/kg, tissue biopsy indicated hepatocellular swelling, mild to moderate vacuolar degeneration, and sporadic infiltration of inflammatory cells in the portal regions. The changes that were seen are linked to higher MDA levels and lower GSH levels. This means that oxidative damage exists, which leads to lipid peroxidation and a lack of antioxidants. This, in turn, causes cells to swell and break down [38, 39].

Valproic acid treatment at 500 mg/kg caused extensive hepatocyte necrosis, inflammatory infiltration, and lobular architecture disruption in the liver [40, 41]. Histopathological changes indicate increased oxidative stress, as MDA levels rise and GSH falls. Biochemical data showing increased ROS production and decreased antioxidant defences suggest that valproic acid dose-dependently damages the liver through oxidative injury [42, 43].

## ■ CONCLUSION

High doses of valproic acid (500mg/kg/day) induced oxidative damage and hepatotoxicity in male albino rats. Also, the role of oxidative stress, GSH, and MDA in hepatic necrosis significantly affected hepatic function.

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## Antidiabetic and Nephrotoxic Effects of *Cladophora Crispata* Algae Extract in Experimentally Induced Diabetic Rats with Renal Dysfunction

**Conflict of interest:** nothing to declare.

**Authors' contribution:** BAN A. Esmael – conceptualization, data curation, investigation, method, project administration, resources, software, writing – original draft and writing – review & editing; Faris S. Kata – conceptualization, supervision, validation, visualization, writing – original draft; Ahmad M. Athbi – conceptualization, investigation, method, validation, visualization, writing – original draft and writing – review & editing.

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### Abstract

**Introduction.** *Cladophora crispata* is a green macroalga that contains multiple bioactive constituents and has been proposed as a candidate natural intervention for metabolic disorders.

**Purpose.** To evaluate the antidiabetic activity and renal impact of a methanolic *C. crispata* extract in an experimentally induced rat model combining diabetes and renal dysfunction.

**Materials and methods.** The algal biomass was collected from Basrah Governorate (Iraq), purified and taxonomically identified, then extracted using 70% methanol (Soxhlet) and chemically profiled by GC–MS to define the extract's constituent fingerprint. The in vivo experiment used 48 male albino rats maintained under controlled housing conditions, randomized into experimental groups (n=8/group). Diabetes was induced using streptozotocin, and renal toxicity was induced using gentamicin; following induction, rats received graded oral doses of the methanolic extract for the treatment period.

**Results.** Across outcomes, the induced positive control displayed marked hyperglycemia with reduced insulin, confirming successful diabetes induction, while extract-treated groups demonstrated glycemic improvement and partial restoration of endocrine function (glucose and insulin). Renal functional markers (urea and creatinine) were substantially elevated in the induced group and improved with extract administration, with dose-dependent patterns that suggested meaningful amelioration of renal dysfunction. Biomarker-level renal assessment (cystatin C and KIM-1) further indicated that filtration/injury-related indices were modulated by treatment, although normalization was not uniform across doses and markers. Oxidative status (MDA and SOD) also shifted with induction and treatment, supporting a redox-related mechanistic component alongside the observed metabolic and renal effects.

**Conclusion.** This study links chemical profiling with in vivo evidence that the methanolic extract of *Cladophora crispata* can meaningfully improve diabetes with renal dysfunction in rats. Induction caused severe hyperglycemia with reduced insulin and raised urea/

creatinine, confirming metabolic disturbance plus kidney injury. Treatment improved glucose control and increased circulating insulin.

**Keywords:** antidiabetic, nephrotoxic, *Cladophora crispata*, renal dysfunction, redox

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## ■ INTRODUCTION

Diabetic kidney disease (DKD) remains a leading driver of chronic kidney disease progression and end-stage kidney failure, arising from persistent metabolic stress, microvascular injury, and progressive tubulointerstitial damage that may not be fully captured by glucose indices alone [1]. A major barrier to early and mechanistically informative DKD assessment is that "functional" indices (e.g., creatinine/urea) may improve or fluctuate without parallel normalization of tubular injury biology. Biomarker work in diabetes supports the concept that tubular injury markers, including KIM-1, and filtration-sensitive markers, including cystatin C [2, 3].

Natural products remain of high interest as adjunct approaches in DKD because they can combine antihyperglycemic, antioxidant, anti-inflammatory, and anti-glycation actions within a single intervention framework. Seaweed-derived polysaccharides and phlorotannin-rich extracts have recently shown antidiabetic and nephroprotective signals in experimental diabetic nephropathy models, including improvements in glycemia, renal function markers, and oxidative stress profiles, while mechanistic in-vitro data support suppression of methylglyoxal/AGE-associated injury pathways and redox-sensitive signaling. Building on this rationale, the current study evaluates a methanolic extract of *Cladophora crispata* in a combined diabetic and renal dysfunction setting, using both functional (urea/creatinine) and mechanistic (cystatin C, KIM-1, MDA, SOD) endpoints to better distinguish functional improvement from persistent tubular injury and oxidative damage [4, 5].

Recent algal-based interventions support the metabolic rationale of the current work: an ethanol extract of *Chlorella vulgaris* significantly reduced blood glucose and the oxidative-stress marker MDA in alloxan-induced diabetic mice, indicating combined hypoglycemic and antioxidant activity [6]. In parallel, an alkaloid extract of *Spirulina platensis* improved dyslipidemia by lowering total cholesterol, triglycerides, LDL/VLDL and increasing HDL in hypercholesterolemic rabbits – relevant because dyslipidemia contributes to insulin resistance and worsened glycemic control [7].

## ■ PURPOSE OF THE STUDY

To evaluate the antidiabetic activity and renal impact of a methanolic *C. crispata* extract in an experimentally induced rat model combining diabetes and renal dysfunction.

## ■ MATERIALS AND METHODS

**Algal material (*Cladophora crispata*).** The green macroalga *Cladophora crispata* was used as the study biomaterial. Biomass was collected from water-mass algal mats in aquatic habitats in Basrah Governorate (Iraq), then cleaned, purified, identified, extracted, and chemically profiled prior to biological testing.

**Identification and purification of the *Cladophora crispata* isolate.** Axenic isolates were prepared following [8]. Samples were washed with sterile distilled water and



centrifuged at 3000 rpm for 50–90 s; the wash – centrifugation cycle was repeated  $\geq 12$  times, replacing the supernatant each time with sterile distilled water. Purity was checked microscopically and by culturing on nutrient agar with incubation at 37 °C for 24 h [7]. Purified biomass was freeze-dried and stored use.

For taxonomic identification biomass was centrifuged (3000 rpm) to concentrate algal material. The pellet was re-suspended in 5 mL distilled water, mounted on slides, and examined under a light microscope. Identification relied on morphological characteristics using standard taxonomic keys [10] and Algae Base.

**Preparation of *Cladophora crispata* methanolic extract.** Extraction followed [11] with minor modifications. Briefly, 40 g of freeze-dried powdered biomass was extracted with 800 mL of 70% methanol using a Soxhlet apparatus, then stored at –18 °C in tightly capped containers until use.

**GC–MS isolation and identification of chemical constituents in the methanolic extract.** Chemical constituents were profiled by gas chromatography – mass spectrometry (GC–MS).

**Physiological study in experimentally induced diabetic rats with renal dysfunction.** A rat model combining streptozotocin (STZ)-induced diabetes and gentamicin (GN)-induced renal toxicity was used. The *C. crispata* methanolic extract was administered orally during the treatment period. Serum biochemical parameters and ELISA-based biomarkers were measured to evaluate glycemic control, renal function/injury indices, oxidative stress markers, and liver enzyme activities.

**Laboratory animals and housing conditions.** Forty-eight male albino rats (170–230 g) were obtained from the College of Medicine, University of Basrah. Animals were housed under standard laboratory conditions

Dosing solutions were prepared based on [12]. The dry extract 0.032, 0.064, 0.128 or 0.192 mg was first dispersed in a few drops of Tween-80, then brought to volume with distilled water 8 ml to yield working preparations equivalent to 0.004, 0.008, 0.016 and 0.024 mg/mL/rat/day.)

**Experimental grouping and study design.** Rats were randomly assigned to experimental groups. Doses were calculated using an average body weight of 200 g. A negative control group received normal saline (injection) and distilled water orally for 28 days. An induced model was established using STZ followed by GN to generate diabetic nephrotoxicity [13]. Induced rats received oral *C. crispata* extract at 20 mg/kg (T1), 40 mg/kg (T2), or 80 mg/kg (T3) daily for 28 days; for a 200 g rat these corresponded to 4, 8, and 16 mg/day, respectively. A reference/positive-control regimen included oral extract administration at 80 mg/kg over the same period.

**Induction of diabetes in male rats using Streptozotocin (STZ).** After one week of acclimatization, diabetes was induced by intraperitoneal injection of STZ (Sigma, St. Louis, MO, USA) at 50 mg/kg (1 mL per 200 g body weight). animals with glucose  $\geq 300$  mg/dL were considered diabetic [14].

**Induction of renal toxicity in male rats using gentamicin (GN).** After confirmation of diabetes, nephrotoxicity was induced by intraperitoneal GN at 100 mg/kg daily for 10 days [15].

**Blood collection and serum preparation.** At the end of the experiment, rats were fasted for 12 h, anesthetized with chloroform inhalation, and dissected. Blood was collected via cardiac puncture serum was aliquoted into 1.5 mL tubes and stored at –80 °C until biochemical and biomarker analyses.

**Serum biochemical assays.** Serum biochemical parameters (glucose, urea, and creatinine) were quantified in all groups using commercial diagnostic kits and standard colorimetric/enzymatic methods: glucose was measured by the enzymatic method of [16] using Randox (UK) kits, renal indices were determined following [16] using Biolabo (France) kits, ELISA-based biomarkers (insulin, cystatin C, SOD, KIM-1, and MDA) were measured using BTLab (China) ELISA kits according to the manufacturer’s instructions and the ELISA approach described by [17], using a standard microplate ELISA reader.

**Statistical analysis.** Quantitative data were analyzed in GraphPad Prism v10.6.1 (GraphPad Software, USA). Results are presented as mean ± SD (n=8 per group), and group differences were tested using one-way ANOVA. When ANOVA was significant, post hoc multiple comparisons were performed (with compact letter display where applicable). Statistical significance was set at p≤0.05 (two-sided).

■ RESULTS

This table 1 is essential because it defines the chemical fingerprint of the tested extract and links biological effects to a characterized phytochemical profile. It reports the identified compounds with their retention times, molecular weights, molecular formulas, library identifications, and relative peak areas (%).

**Table 1**  
**Major GC–MS-identified chemical constituents of *Cladophora crispata* methanolic extract (selected 6 compounds)**

| Peak | Retention time (min) | Compound (Library/ID)                   | Molecular formula | Molecular weight (g/mol) | Area (%) |
|------|----------------------|-----------------------------------------|-------------------|--------------------------|----------|
| 10   | 23.115               | Hexadecanoic acid, methyl ester         | C17H34O2          | 270.45                   | 23.575   |
| 15   | 24.797               | 9-Octadecenoic acid, methyl ester, (E)- | C19H36O2          | 296.48                   | 21.381   |
| 3    | 20.999               | Methyl tetradecanoate                   | C15H30O2          | 242.40                   | 8.693    |
| 1    | 18.683               | Dodecanoic acid, methyl ester           | C13H26O2          | 186.29                   | 7.904    |
| 18   | 24.996               | Methyl stearate                         | C19H38O2          | 298.50                   | 5.183    |
| 17   | 24.907               | Phytol                                  | C20H40O           | 296.53                   | 2.345    |

This figure 1 provides the visual GC–MS output supporting the compound list presented in the chemical-constituent table. It demonstrates that multiple peaks were detected and matched to library spectra, strengthening confidence that the extract composition was analytically verified rather than inferred.

Induction produced marked hyperglycemia with a clear separation of the induced positive control from other groups. *C. crispata* lowered glucose across doses, with 40 mg/kg showing the lowest mean and strongest statistical separation, indicating the most pronounced antihyperglycemic response in this dataset. Insulin declined with induction, consistent with diabetic endocrine impairment, while all treated groups showed higher insulin than the induced group. The 20 mg/kg dose yielded the highest insulin mean, whereas 40–80 mg/kg brought insulin toward control-like values and 120 mg/kg remained elevated. The non-parallel ranking between glucose and insulin suggests that glucose improvement is not explained solely by insulin elevation and may also involve extra-pancreatic mechanisms (Table 2).

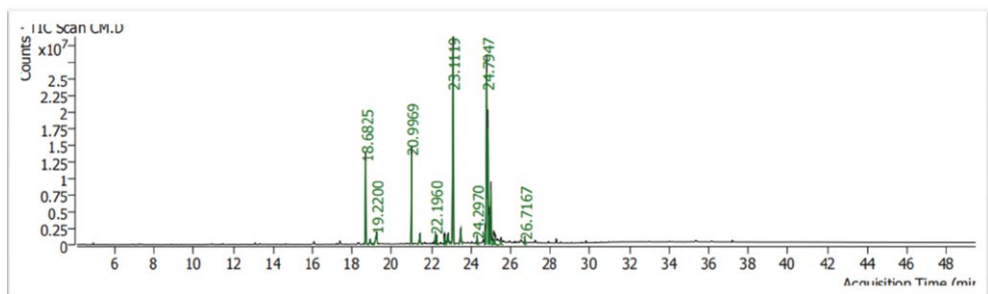


**Fig. 1.** GC–MS mass spectrum/trace of compounds detected in *Cladophora crispata* methanolic extract

**Table 2**  
**Glycemic and endocrine markers in diabetic rats with renal dysfunction (merged)**

| Group                    | Blood glucose (mg/dL), Mean±SD (Letter) | Insulin (mg/dL), Mean±SD (Letter) |
|--------------------------|-----------------------------------------|-----------------------------------|
| Negative control         | 154.333±23.278 (c, e)                   | 4.930±0.577 (c)                   |
| Induced positive control | 495.166±86.401 (a)                      | 2.555±0.358 (d)                   |
| Positive control (II)    | 218.200±15.477 (b)                      | 4.746±1.134 (c)                   |
| Treatment 1 (20 mg/kg)   | 156.850±21.496 (c, e)                   | 7.335±1.966 (a)                   |
| Treatment 2 (40 mg/kg)   | 120.100±29.893 (e)                      | 5.623±0.691 (b, c)                |
| Treatment 3 (80 mg/kg)   | 229.600±36.895 (b)                      | 5.503±1.094 (b, c)                |
| Treatment 4 (120 mg/kg)  | 190.700±31.183 (c, b)                   | 6.229±0.529 (a, b)                |

Note: different letters indicate significant differences among groups (means sharing ≥1 letter are not significantly different). One-way ANOVA with letter-based post hoc mean separation;  $p \leq 0.05$  ( $n=8/\text{group}$ ).

Renal dysfunction was evident after induction, with significant elevations in urea and creatinine, confirming impaired filtration and nitrogenous waste handling. *C. crispata* improved renal function markers in a dose-dependent pattern: urea normalized most strongly at 80–120 mg/kg (clustered with positive control II), whereas creatinine was restored most clearly at 40 mg/kg (statistically similar to the negative control). Adding cystatin C refined interpretation: 40–80 mg/kg reduced cystatin C toward/below control ranges, with 80 mg/kg giving the lowest value, while 120 mg/kg showed a paradoxical increase and formed a distinct group, raising concern at the highest dose. KIM-1 remained elevated with substantial overlap between induced and treated groups, indicating that functional improvement (urea/creatinine) did not translate into full normalization of tubular injury signaling within the study period (Table 3).

Induction increased MDA, supporting enhanced lipid peroxidation in the combined diabetic – renal injury state. *C. crispata* reduced MDA most clearly at 20 mg/kg (lowest mean), with intermediate doses trending toward control-like ranges, while 120 mg/kg showed a less favorable profile and overlapped with the induced group, suggesting diminished oxidative benefit at the highest dose. SOD was highest in the induced group, which is consistent with a compensatory upregulation under severe oxidative challenge rather than improved antioxidant "health". Treated groups shifted SOD toward intermediate values; 80 mg/kg approached the negative control by letter grouping, suggesting partial normalization of antioxidant defense demands. Interpretation is strongest when MDA

**Table 3**  
**Renal function and injury biomarkers (merged)**

| Group                    | Urea (mg/dL), Mean±SD (Letter) | Creatinine (mg/dL), Mean±SD (Letter) | Cystatin C (mg/dL), Mean±SD (Letter) | KIM-1 (mg/dL), Mean±SD (Letter) |
|--------------------------|--------------------------------|--------------------------------------|--------------------------------------|---------------------------------|
| Negative control         | 49.893±9.293 (c)               | 1.441±0.354 (c)                      | 15.638±1.652 (c, d)                  | 1.873±0.168 (b, d)              |
| Induced positive control | 145.799±24.488 (a)             | 3.337±0.802 (a)                      | 20.727±2.119 (b)                     | 2.379±0.487 (a)                 |
| Positive control (II)    | 30.305±6.581 (d)               | 1.815±0.389 (c)                      | 16.538±1.881 (c, d)                  | 1.529±0.049 (d)                 |
| Treatment 1 (20 mg/kg)   | 72.960±14.759 (b)              | 2.490±0.983 (b)                      | 17.561±1.145 (c)                     | 2.116±0.580 (a, b)              |
| Treatment 2 (40 mg/kg)   | 63.230±9.995 (b)               | 1.459±0.324 (c)                      | 14.731±2.751 (d, e)                  | 2.086±0.544 (a, b)              |
| Treatment 3 (80 mg/kg)   | 31.799±8.685 (d)               | 1.773±0.588 (c)                      | 13.077±2.149 (c)                     | 2.052±0.207 (a, b)              |
| Treatment 4 (120 mg/kg)  | 37.270±5.492 (d)               | 1.883±0.562 (c)                      | 23.147±3.012 (a)                     | 2.393±0.455 (a)                 |

Note: Different letters indicate significant differences among groups p≤0.05 (n=8/group).

and SOD are read together to distinguish reduced oxidative damage from compensatory enzyme responses (Table 4).

**Table 4**  
**Oxidative stress and antioxidant defense (merged)**

| Group                    | MDA (mg/dL), Mean±SD (Letter) | SOD (mg/dL), Mean±SD (Letter) |
|--------------------------|-------------------------------|-------------------------------|
| Negative control         | 2.027±0.369 (b, c)            | 2.762±0.593 (d)               |
| Induced positive control | 2.825±0.643 (a)               | 6.592±1.561 (a)               |
| Positive control (II)    | 1.959±0.565 (b, c)            | 4.591±1.082 (b, c)            |
| Treatment 1 (20 mg/kg)   | 1.881±0.506 (c)               | 4.449±0.916 (b, c)            |
| Treatment 2 (40 mg/kg)   | 1.991±0.338 (b, c)            | 5.264±1.428 (b)               |
| Treatment 3 (80 mg/kg)   | 2.290±0.533 (b, c)            | 3.780±0.881 (c, d)            |
| Treatment 4 (120 mg/kg)  | 2.451±0.388 (a, b)            | 4.649±1.057 (b, c)            |

Note: different letters indicate significant differences among groups; p≤0.05 (n=8/group).

■ DISCUSSION

The present study indicates that *C. crispata* methanolic extract exerts a clear antihyperglycemic effect in rats with experimentally induced diabetes combined with renal dysfunction. Blood glucose rose sharply in the induced model, while extract treatment reduced glucose, with the intermediate dose showing the most consistent improvement. This degree of correction may help limit downstream consequences of chronic hyperglycemia, including oxidative stress and microvascular injury relevant to diabetic kidney complications. The effect was not strictly dose-proportional, suggesting a non-linear response that may relate to biphasic bioactivity, counter-regulatory physiology, or dose-dependent absorption/clearance. Similar glucose-lowering activity has been reported for seaweed-based preparations in streptozotocin diabetes and diabetic nephropathy models, supporting multi-pathway actions [18, 19].

Improved glycemic control was accompanied by an endocrine shift in insulin. Diabetes induction lowered serum insulin, whereas all *C. crispata* – treated groups showed higher insulin, with the most evident rise at the lower dose. Because the strongest insulin increase did not coincide with the best glucose lowering, the extract likely acts through more than insulin stimulation alone, with plausible contributions from improved peripheral



insulin sensitivity, reduced hepatic glucose output, and altered carbohydrate digestion/absorption. This pattern is consistent with the broader seaweed literature, where the magnitude and direction of insulin change varies by model, extract composition, and dosing [18, 19].

Renal outcomes supported a protective signal. The induced diabetic–nephrotoxic group showed a pronounced rise in serum urea versus the normal control, confirming substantial renal impairment, while *C. crispata* reduced urea across doses, with higher doses shifting values closer to the control range. This dose separation suggests that part of the renal benefit may extend beyond glucose correction alone, especially because near-optimal urea improvement appeared at doses that were not necessarily optimal for glycemia. Similar patterns have been described in diabetic kidney injury studies using seaweed extracts or polysaccharides, often alongside improvements in oxidative stress indices and renal structure, supporting biologic plausibility for antioxidant/anti-inflammatory renoprotection [18, 20, 21].

When placed against supportive and opposing evidence, two points help interpret the current findings. First, some interventions report renoprotection with minimal glycemic improvement, implying kidney-directed anti-fibrotic/anti-inflammatory activity; in contrast, the present study shows clear glucose lowering, suggesting *C. crispata* may have dual metabolic and renal bioactivity. Second, the difference in "best" dose for glucose (lower – mid range) versus urea (higher range) may reflect mechanistic compartmentalization, where lower exposure preferentially supports metabolic control, while higher exposure more strongly targets renal oxidative stress, mitochondrial dysfunction, and inflammatory injury in combined STZ diabetes and nephrotoxic settings. Variations across studies in diabetes phenotype, renal insult type, extraction chemistry, and duration can further shift which endpoints dominate [3, 18, 22].

From a translational perspective, the overall pattern – glucose reduction, partial insulin restoration, and urea improvement at higher doses – suggests the extract may act across clinically relevant nodes, including pancreatic endocrine support, redox modulation, and preservation of renal functional integrity under toxic-metabolic stress. The non-linear dose response emphasizes the need for dose-finding and phytochemical standardization, particularly because renal impairment can alter pharmacokinetics and clearance [20, 23].

Consistent with renal dysfunction, the induced diabetic – renal injury group showed a marked rise in serum creatinine versus the negative control, indicating impaired filtration. *C. crispata* improved creatinine overall, with the 40 mg/kg dose bringing values close to the normal-control range and the highest dose also showing near-normalization, while the lowest dose remained only partly corrective and 80 mg/kg was intermediate. This supports a meaningful renoprotective effect, but also indicates that recovery is not strictly dose-proportional, a variability also reported across nephroprotection studies in drug-induced renal injury models [24].

Because creatinine can be influenced by non-renal factors, cystatin C adds interpretive sensitivity. Here, cystatin C increased in the induced group, decreased with 40–80 mg/kg, but rose again at the highest dose despite near-normal creatinine. This creatinine – cystatin C mismatch is clinically meaningful and may indicate incomplete protection or dose-related renal stress, consistent with reports of non-parallel biomarker behavior in renal impairment models [25].

KIM-1, reflecting proximal tubular injury/repair activity, did not move in parallel with filtration markers. KIM-1 increased in the induced condition and showed only modest shifts with treatment, with the 80 mg/kg group trending lowest and the highest dose remaining close to the induced profile. This suggests that functional indices can improve before tubular injury signaling fully normalizes within the study timeframe, and that clearer KIM-1 improvement may require longer exposure, different dosing, or additional mechanisms [26].

Oxidative-stress findings add mechanistic support and may help explain biomarker discordance. Induction increased MDA versus the negative control, while *C. crispata* lowered MDA most clearly at low/intermediate doses and less favorably at the highest dose. SOD was highest in the induced group, likely reflecting compensatory upregulation under severe oxidative load; treated groups shifted SOD toward levels closer to the negative control, consistent with reduced oxidative challenge. Together, lower MDA with SOD normalization supports an antioxidant contribution while also offering a plausible reason why tubular injury markers may lag behind functional recovery [27].

Our concurrent reductions in hyperglycemia and lipid peroxidation align with prior algal evidence showing that *Chlorella vulgaris* extract significantly decreased blood glucose and MDA in chemically induced diabetic mice [28]. This concordance supports the interpretation that algae-derived bioactives can modulate both glycemic dysregulation and oxidative stress, which is mechanistically relevant to DKD-related tissue injury.

GC–MS profiling in the current work provides a clearer chemical explanation for the changes observed in oxidative-stress indices (MDA and SOD). The methanolic extract appears lipid/terpenoid-enriched, with major constituents dominated by fatty-acid methyl esters (notably hexadecanoic acid methyl ester, 9-octadecenoic acid methyl ester, and octadecanoic acid methyl ester), alongside the diterpenoid alcohol phytol and the diterpene neophytadiene (GC–MS table). Structurally, these are highly hydrophobic molecules that can plausibly influence redox balance through (i) limiting lipid peroxidation propagation within membranes, (ii) modulating inflammatory/oxidative signaling, and (iii) supporting endogenous antioxidant defenses, which together aligns with the observed MDA reduction and the tendency of SOD to shift toward a more normalized state. This interpretation is consistent with the broader macroalgae literature, where terpenoids and fatty-acid derivatives are repeatedly highlighted as contributors to antioxidant and anti-inflammatory bioactivity in algal extracts [29].

Importantly, phytol itself has recent *in vivo* evidence of an antioxidant signature, including suppression of malondialdehyde and restoration of antioxidant enzymes including SOD, supporting its plausibility as one of the functionally relevant structures in the present extract [30]. Neophytadiene has likewise been summarized recently as a bioactive diterpene with antioxidant/anti-inflammatory potential and signaling relevance, which could contribute to the redox-linked improvements observed here, especially under diabetic – toxic renal stress [31] phenolics/polysaccharides) would strengthen causal attribution.

Taken together, the present study supports that *C. crispata* extract can improve glycemic control and renal functional indices in diabetic rats with renal dysfunction – most persuasively 40 mg/kg for creatinine normalization – while tubular injury signaling (KIM-1) remains less responsive within the same timeframe. Clinically, such discordance can reflect incomplete recovery or mixed glomerular – tubular phenotypes. The key



caution is the highest-dose rise in cystatin C alongside limited KIM-1 improvement and a less favorable oxidative profile, raising concern for a potential high-dose penalty under diabetic–renal impairment and supporting careful dose selection, longer follow-up, urine-based tubular panels, and histologic correlation in future studies [24, 25, 31].

## CONCLUSION

The links of chemical profiling with in vivo evidence that the methanolic extract of *Cladophora crispata* can meaningfully improve diabetes with renal dysfunction in rats. Induction caused severe hyperglycemia with reduced insulin and raised urea/creatinine, confirming metabolic disturbance plus kidney injury. Treatment improved glucose control and increased circulating insulin.

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# Acetyl Choline Down-Regulation and Up-Regulation due to the Effect of Pyridostigmine and Botulinum Neurotoxin Type-A in Experimental Model

**Conflict of interest:** nothing to declare.

**Authors' contribution:** Israa Saad Mustafa Ali – conceptualization, software, supervision, validation, visualization, writing – original draft and writing – review & editing; Ekhlas Hayyawi Hussein ALhatemi – conceptualization, investigation, method, project administration, resources, writing – original draft and writing – review & editing.  
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## Abstract

**Introduction.** Botulinum neurotoxin type A (BoNT-A) preparations are widely used as non-surgical, minimally invasive treatments to enhance and rejuvenate facial appearance. Pyridostigmine (PSG), an acetylcholinesterase (AChE) inhibitor, plays multiple roles in medical and pharmacological contexts by influencing acetylcholine (ACh) dynamics.

**Purpose.** To investigate how PSG and BoNT-A influence acetylcholine levels, focusing on their respective mechanisms of upregulation and downregulation of acetylcholine receptors.

**Materials and methods.** An interventional experimental study was carried out on twenty healthy adult male rabbits weighing between 1.5 and 2.5 kg. A Rabbit Nicotinic Acetylcholine Receptor (N-AChR) ELISA Kit was employed for analysis. The animals were randomly assigned to four groups (n=5 per group): a control group, a pyridostigmine-treated group, a BoNT-A-treated group, and a combination group receiving both BoNT-A and PSG.

**Results.** The BoNT-A group demonstrated the highest concentration of ACh receptors, whereas the PSG group exhibited the lowest. A statistically significant difference was observed among all groups ( $P < 0.0001$ ). BoNT-A administration induced an upregulation of AChRs, while PSG treatment resulted in receptor downregulation.

**Conclusion.** This study, the first of its kind conducted in Iraq, explored the interplay between PSG and BoNT-A on acetylcholine modulation. The findings indicate that BoNT-A promotes AChR upregulation, whereas PSG exerts the opposite effect by reducing receptor expression.

**Keywords:** botulinum neurotoxin type A, BoNT-A, acetyl choline, pyridostigmine, botox



## ■ INTRODUCTION

*Clostridium botulinum* is a spore-forming, rod-shaped, Gram-positive, anaerobic bacterium that exists widely in soil. Under favorable environmental conditions, its dormant spores can germinate and produce neurotoxins, collectively known as botulinum toxins [1, 2]. In therapeutic or cosmetic applications, extremely small doses of botulinum neurotoxin (BoNT) – ranging from picograms to nanograms – are injected to induce localized relaxation of targeted muscles, thereby decreasing excessive muscle tone or reducing glandular secretions [3].

In vitro studies show that BoNTA can interact with fibroblast growth factor receptor 3 (FGFR3) [4]. After binding, the neurotoxin is taken up by neurons through endocytosis and localized within recycling endosomes, where its C-terminal domain anchors into the endosomal membrane. Acidification of the endosomal lumen then triggers conformational changes that allow the light chain of the toxin to cross the membrane and enter the neuronal cytosol [5]. The disulfide bond linking the heavy and light chains is subsequently reduced, freeing the active light chain [2]. BoNT causes a temporary inhibition of neuromuscular transmission by attaching to presynaptic motor nerve terminals and blocking acetylcholine release, which results in reversible chemical denervation of affected muscles [6, 7]. Recovery occurs gradually as nerve terminals regain function and re-establish normal neurotransmission.

PSG, a reversible cholinesterase inhibitor, is primarily used to treat myasthenia gravis symptoms and to counteract non-depolarizing neuromuscular blockade [8]. It has also been employed as a protective agent against certain nerve toxins, such as during the Gulf War [9]. By inhibiting acetylcholinesterase, PSG prevents the breakdown of ACh in the synaptic cleft, thereby elevating its extracellular concentration at neuromuscular junctions [10]. This enhancement of ACh levels strengthens synaptic transmission and alleviates the muscle weakness seen in myasthenia gravis, effectively amplifying cholinergic signaling [11, 12].

Acetylcholine (ACh) is the principal neurotransmitter at cholinergic synapses and neuromuscular junctions within both the central and peripheral nervous systems. It plays a vital role in regulating posture, cognition, behavior, memory, and autonomic parasympathetic activity [13]. Its effects are mediated through two classes of cholinergic receptors: muscarinic acetylcholine receptors (mAChRs) and nicotinic acetylcholine receptors (nAChRs or AChRs), which are ligand-gated receptors expressed in both neuronal and non-neuronal tissues throughout the body [14, 15].

## ■ PURPOSE OF THE STUDY

To investigate how PSG and BoNT-A influence acetylcholine levels, focusing on their respective mechanisms of upregulation and downregulation of acetylcholine receptors.

## ■ MATERIALS AND METHODS

### **Study Design**

A prospective experimental study was conducted on forty adult male rabbits at the Pharmacology Clinic. The agents used in this research included *Clostridium botulinum* toxin type A (HUTOX, Global Co., Korea), PSG bromide (Mestinon, MEDA Pharma, Switzerland), and atropine sulfate (OMT Pharma, India). Quantification of nicotinic acetylcholine

receptors (nAChRs) was performed using a Rabbit nAChR ELISA Kit (NOVATEN Biosciences Inc., USA).

Animals

The study utilized forty healthy, mature male rabbits weighing between 1,000 and 2,000 g. All animals were housed under standard laboratory conditions with free access to food and water and were allowed a 7-day acclimatization period before experimentation.

Experimental Design

Rabbits were randomly assigned to four experimental groups (ten animals per group). Each treatment continued for four weeks as follows:

- Group-1 (Control): Received distilled water (4 mL/kg orally for 3 days), followed by an increased dose of 6 mL/kg for the remainder of the period, and were injected intramuscularly with distilled water (10 U/kg) into the right quadriceps muscle as a single dose.
- Group-2 (PSG): Administered PSG orally at 4 mL/kg initially, then increased to 6 mL/kg daily for 30 days.
- Group-3 (BoNT-A): Injected intramuscularly with BoNT-A (10 U/kg) into the right quadriceps muscle (single dose).
- Group-4 (BoNT-A + PSG): Treated with both BoNT-A (as in Group 3) and oral PSG (as in Group 2).

Statistical Analysis

All data were analyzed using SPSS software, version 20 (IBM Corp., Chicago, IL, USA). Descriptive statistics were presented as means, medians, standard deviations, and percentages where appropriate. Associations between categorical variables were assessed using the chi-square test, while one-way ANOVA was used to compare differences among groups. A  $P<0.05$  was considered significant.

RESULTS

The concentration of receptors in each group were shown in Table 1 and Figure 1. The highest level of ACh receptors  $33.7\pm6.913$  and  $31.6\pm2.951$  ng/ml was found in group D and group C. Whereas, the lowest level  $15.5\pm4.649$  ng/ml was found in group B, with highly statistical significant difference ( $P<0.0001$ ).

Table 1  
ACh receptor (ng/ml)

|           | ACh receptor ng/ml |            |            |            |
|-----------|--------------------|------------|------------|------------|
|           | A                  | B          | C          | D          |
| Mean±SD   | 23.7±4.547         | 15.5±4.649 | 31.6±2.951 | 33.7±6.913 |
| Median    | 23.5               | 16.5       | 32         | 32.5       |
| Min/Max   | 18/30              | 8/21       | 28/35      | 27/46      |
| P=≤0.0001 |                    |            |            |            |

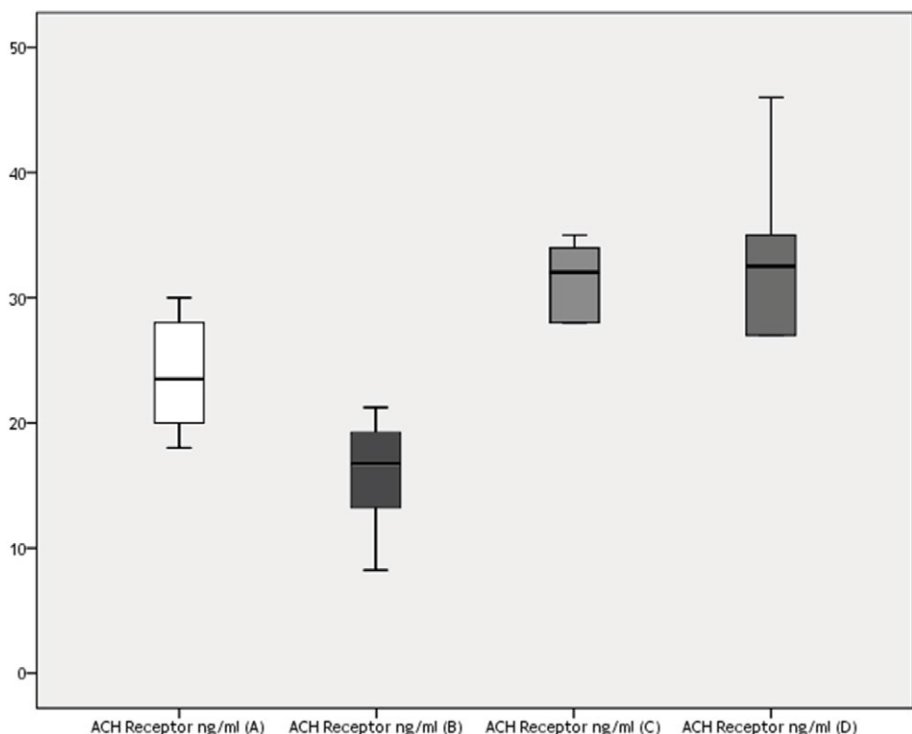


**Fig. 1. Box plot showed the concentration of ACh receptors in groups of the study**

**Table 2**  
**Post-Hoc-Tukey HSD (beta) comparison of ACh receptors**

| Pairwise comparisons | HSD  | P value |
|----------------------|------|---------|
| A:B                  | 7.9  | 0.0057  |
| A:C                  | 8.2  | 0.0039  |
| A:D                  | 10   | 0.0004  |
| B:C                  | 16.1 | <0.0001 |
| B:D                  | 2.1  | 0.78    |
| C:D                  | 18.2 | <0.0001 |

According to Post-Hoc-Tukey analysis, there were strongest significant differences among groups A:B, A:C, A:D, B:C and C:D ( $P=0.0057$ ,  $0.0039$ ,  $0.0004$ ,  $<0.0001$ ) respectively. However, no association recorded among group B:D ( $P=0.78$ ), as showed in Table 2 and Figure 2.

## ■ DISCUSSION

In this work, the greatest concentrations of acetylcholine receptors (AChRs) were detected in the groups treated with both PSG and BOTOX ( $33.7 \pm 6.913$  ng/ml) and with BOTOX alone ( $31.6 \pm 2.951$  ng/ml). The lowest concentration ( $15.5 \pm 4.649$  ng/ml) occurred

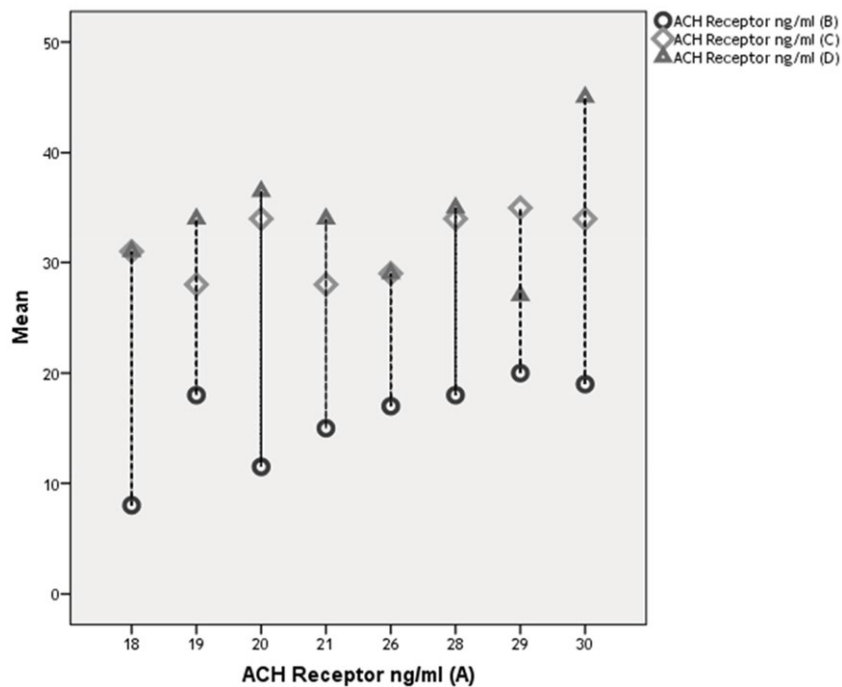


**Fig. 2. Post-Hoc-Tukey HSD of ACh receptors**

in the PSG-only group, and the differences among the groups were highly significant ( $P < 0.0001$ ). Previous work has indicated that PSG can alter AChR dynamics [16]. Consistent with this, earlier research demonstrated that extended exposure to high doses of PSG caused neuromuscular dysfunction within two to four weeks, shown by reduced specific tetanic tension and increased sensitivity to atracurium, whether or not AChR expression was reduced [17].

Our observations align with prior reports regarding acetylcholinesterase inhibitors. Studies by Herbstreit et al. [18], Kitajima et al. [19], and Eikermann et al. [20] described PSG-induced neuromuscular transmission changes and fade phenomena. In rat models, mild cholinergic effects such as increased salivation, tearing, and muscle fasciculations were observed following PSG administration [21].

In the present work, we recorded a dose-dependent elevation in nicotinic AChR (nAChR) levels across all treatment groups. This receptor upregulation may represent a compensatory response to the presynaptic inhibition caused by BOTOX. Our findings suggest that even a single injection of botulinum toxin can produce prolonged neuromuscular alterations lasting three to six months post-treatment. Although no evident structural denervation was observed, the toxin induced dose-dependent muscle weakness (as reflected by decreased muscle weight), corresponding with nAChR upregulation. These data are consistent with previous reports on the short-term neuromuscular impact of botulinum toxin [16, 22].

BoNT-A acts as a potent presynaptic blocker and is an established first-line therapy for multiple disorders, including strabismus, hemifacial spasm, cervical dystonia, limb



spasticity, cerebral palsy, migraine, hyperhidrosis, and overactive bladder [23]. Despite its efficacy, its pharmacological effects are temporary, requiring periodic reinjection for sustained therapeutic benefit in chronic conditions [24].

Byun et al. (2022) evaluated three BoNT/A formulations – Coretox®, Xeomin®, and Botox® – administered to the right hind limb of animals. They observed dose-dependent muscle weakness in the injected limb, while the contralateral side remained unaffected. Paresis was assessed using the digit abduction score (DAS), confirming that each formulation produced localized paralysis only at the injection site [25].

Post Hoc Tukey analysis in our experiment revealed highly significant group differences between (A:B), (A:C), (A:D), (B:C), and (C:D), whereas no difference was observed between (B:D). This pattern corresponds with findings from Morsch et al. [16], who noted that repeated injections into tibialis anterior and diaphragm muscles reduced AChR cluster intensity and area, especially in the diaphragm, suggesting dismantling of postsynaptic AChR clusters. PSG administration further intensified this loss, while control animals receiving human IgG showed no alterations. Similarly, Richtsfeld et al. [17] reported that prolonged PSG treatment caused neuromuscular dysfunction and possible AChR downregulation.

Regarding the duration of action, comparative studies have indicated no significant difference among BoNT/A formulations during the initial dosing cycle [25]. Morsch et al. [16] and Cole et al. [25] also found no differences in resting membrane potential across treatments, suggesting that reduced miniature endplate potential (mEPP) amplitude resulted from decreased postsynaptic AChR sensitivity rather than presynaptic activity changes. Thus, PSG, by prolonging acetylcholine persistence in the synaptic cleft, may synergize with anti-MuSK IgG to accelerate AChR cluster disassembly. Although cholinesterase inhibitors enhance AChR activation, excessive or chronic exposure can damage neuromuscular junction (NMJ) structure and function [26].

A clinical investigation involving post-stroke patients with hemi-spasticity or spastic foot drop found that repeated BOTOX injections produced greater benefits than the initial treatment cycle [27]. Repeated administration is essential for individuals with persistent muscle disorders [25]. Importantly, no significant evidence of antibody-mediated resistance was detected following multiple treatment cycles.

After discontinuation of PSG infusion, AChR levels increased, likely due to the reduction of acetylcholine-induced desensitization, resulting in restored neuromuscular responsiveness [17]. This may also relate to the reversal of agonist-induced receptor desensitization mechanisms observed with other receptor systems, such as adrenergic and opioid receptors [28]. The absence of direct allosteric binding of PSG to AChRs after withdrawal may further explain the recovery [17].

Our results show that PSG can contribute to AChR downregulation, while BOTOX triggers receptor upregulation. Although PSG pretreatment may offer protective effects against botulinum toxicity, further studies are required to evaluate its prophylactic use in humans [29].

## ■ CONCLUSION

This research represents the first investigation in Iraq examining the interplay between PSG and BOTOX on acetylcholine receptor modulation. We found that BOTOX promotes upregulation of AChRs, whereas PSG induces their downregulation.

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# Estimation of Antibiotic Residues in the Euphrates River Water in Nasiriyah City and their Environmental Impacts

**Conflict of interest:** nothing to declare.

**Authors' contribution:** Ahlam Faisal – conceptualization, data curation, investigation, methodology, resources, validation, visualization, writing – original draft and writing – review & editing; Afrah Maktoof – conceptualization, data curation, methodology, resources, validation, visualization, writing – original draft and writing – review & editing.

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## Abstract

**Introduction.** Antibiotics are emerging pollutants in aquatic environments, with significant environmental and health impacts.

**Purpose.** To study the seasonal variation in the concentration of antibiotics (tetracycline, gentamycin, and ciprofloxacin) in the surface water of the Euphrates River at three different locations in Nasiriyah.

**Materials and methods.** Water samples were collected during the summer and winter and analyzed using HPLC (high-performance liquid chromatography) to obtain accurate and reliable results.

**Results.** The results showed a significant increase in antibiotic concentrations during the summer compared to the winter, due to increased human and agricultural activity and higher temperatures, which reduce the natural degradation rate. Ciprofloxacin recorded the highest concentration at all locations, while gentamycin had the lowest concentration, reflecting its poor environmental stability and rapid degradation.

**Conclusion.** These results indicate clear pharmaceutical contamination of the Euphrates River waters in Nasiriyah, calling for periodic monitoring and improvements to water treatment systems, as well as rational use of antibiotics to protect the environment and public health.

**Keywords:** antibiotics, water pollution, Euphrates river, tetracycline, gentamycin

## ■ INTRODUCTION

The aquatic environment is key to the sustainable development of the global community and has critical environmental and economic importance [1]. Over the past few decades, one of the world's greatest concerns has been the shortage of fresh water. This is due to the acute shortage of fresh water, coupled with technological advances, population growth, and unplanned urbanization. Furthermore, runoff from agricultural

fields, as well as untreated domestic sewage and toxic industrial and health wastes, all contribute to the ongoing pollution of limited freshwater supplies [2]. Among these pollutants are antibiotics, which are chemicals that inhibit the growth of bacteria or reduce their numbers, such as those used in medical and veterinary treatments [3]. They function as natural compounds or chemicals that address various types of bacterial infections [4]. The administration of antibiotics for various independently diagnosed conditions or symptoms, along with the irregular or ongoing use of prescribed medical substances, is noted [5]. intensifies environmental issues in natural waters as a result of pollutants [6]. Despite substantial efforts to enhance wastewater treatment, existing technology has demonstrated inadequacy in eliminating the concentration of antibiotics found in wastewater. Consequently, substantial amounts of these antibiotics are released into aquatic environments. The discharge of these antibiotics into water bodies has significant environmental consequences and has frequently impacted human health through water consumption and use [7]. Pollutant levels in the sediments of the Euphrates River constitute the primary source of pollution, attributable to heightened human and industrial activities in the region [8]. These antibiotics significantly disrupt the aquatic environment, primarily by inducing hormonal imbalances. Hormonal medications, including birth control pills, disrupt the endocrine systems of aquatic organisms, resulting in masculinisation or feminisation. They contribute to antibiotic resistance, toxicity in aquatic organisms, behavioural alterations, and bioaccumulation, while also disrupting ecosystems and affecting the food chain in aquatic environments [9]. Antibiotics accumulate in aquatic organisms, resulting in substantial harm, particularly to fish tissue [10].

## ■ PURPOSE OF THE STUDY

To study the seasonal variation in the concentration of antibiotics (tetracycline, gentamycin, and ciprofloxacin) in the surface water of the Euphrates River at three different locations in Nasiriyah.

## ■ MATERIALS AND METHODS

**The Study Area:** The current study was conducted on the Euphrates River within the center of Nasiriyah city for the period from September 2023 to March 2024. Three sites were chosen to represent the beginning of the river's entry into the city center, a site in the middle of the river, and the third at the river's exit from the city, which are: S1 (Al-Zaytoun Bridge), S2 (Al-Zaytouniya), and S3 (near Al-Fadhiliya Bridge).

### **Study Area**

The Euphrates River is considered one of the main rivers in Iraq, with a length of 2775 km, and it is a type of long river in the world is also one of the largest rivers in the Middle East and has a chain of 27 fresh and warm rivers in the world. Its salinity also increases towards the south, and it is important for human and industrial use, and on its banks are spread orchards, palm trees, and field crops [11].

### **Sample Collection**

Water samples were collected using 2 litre polyethylene containers. Three samples were collected for each site and the samples were transferred to the laboratory to measure three antibiotics in the water (Tetracycline, Gentamicin, Ciprofloxacin).



### Extraction of Samples Water Sample

- Chemical analysis was conducted using the EPA1694 method established by the US Environmental Protection Agency, with certain modifications (EPA, 2007). Antibiotics of interest were extracted from water samples utilising a Dionex Auto TraceSPE280 (USA) equipped with an Oasis HLB cartridge. Water samples were obtained from a depth of 0.5 meters below the surface and preserved in 10-liter polyethylene containers.
  - The extraction of tetracycline from water samples commenced with the measurement of 100 ml of the sample, followed by the addition of 10 ml of methanol and citrate solution. The mixture was vortexed at high speed for 5 minutes and subsequently incubated at room temperature for an additional 5 minutes.
  - Centrifugation was performed at 3500 rpm for 10 minutes, and the extraction process was repeated with the addition of 2 ml of citrate buffer. The supernatant was filtered and subsequently loaded onto a solid-phase extraction (SPE) cartridge, which had been pre-conditioned with 10 ml of methanol and 4 ml of water. The cartridge containing the sample was rinsed with 5 ml of water, after which Tetracycline was filtered sequentially. Three millilitres of the sample were filtered using a 0.45  $\mu\text{m}$  cellulose filter paper, and 100 microlitres were injected into a high-performance liquid chromatography (HPLC) system [12].
  - Extraction of Ciprofloxacin and Gentamycin from water samples involves an initial incubation period of 30 minutes in darkness at room temperature. Following this, the extraction process commences with the addition of 10 ml of phosphate buffer, after which the tube is securely closed and placed in a shaker. The tube underwent agitation. Introduce 1 ml of phosphate solution, securely seal the tube, and position it in a shaker. Vigorously shake the tube for 10 minutes, followed by centrifugation at 4000 rpm for 10 minutes. Transfer the supernatant to a sterile 50 ml tube. Subsequently, introduce 10 ml of phosphate solution and agitate vigorously for a duration of 10 minutes using a shaker. The centrifugation process was conducted again.
- Centrifugation, combining the supernatants, and adjusting the pH of the complex extract to 7.5–8.0. This requires about 0.16 ml of 30% sodium hydroxide. Use a pH meter to check the pH and adjust it using 1% HCl or 1% NaOH [13].

### Analytical Methods

The final extracts of the samples were analysed utilising a high-performance liquid chromatography (HPLC) apparatus at the Ministry of Science and Technology, Department of Environment and Water.

#### HPLC Conditions

- Mobile phase = methanol:(70:30) D.W· column = C18-ODS (25 cm \* 4.6 mm)
- Detector (fluorescence) = Example= 310 nm, 420 Em, Flow rate = 1.2 ml/min

#### Automated and Quantitative Analysis

Tetracycline, Gentamicin, and Ciprofloxacin, a three antibiotics were analysed utilising a Syknn-S1122 HPLC system from Germany, which includes a high-gradient pump, an autosampler, a degasser, a hot oven, and a Shimadzu RF20F detector. The mobile phase A consisted of 0.1% formic acid, while phase B comprised acetonitrile in a volume/volume ratio of 30 : 70. The flow rate measured 0.7 ml/min. The excitation wavelength

for ciprofloxacin and gentamicin was 278 nm, with an emission wavelength of 160 nm, as measured by a fluorescence detector. Tetracycline was measured at an excitation wavelength of 515 nm using a UV detector. Chromatography was conducted at ambient temperature using a column measuring 250 05–18 mm and a volume of 10 µL. A 50 µL volume was injected into the column [14, 15].

Statistical Analysis

The data of the current study was statistically analysed by Statistical Package of Social Sienese version 26, based in using One way ANOVA and LSD at p. value <0.05 [16].

- Each p. value has two stars that indicate a high significant at p. value 0.01, p. value has one star indicate significant at 0.05, while p. value without star indicates a non-significant difference.
- Similar small letters above the means indicate the non-significant differences, while different letters indicate the significant differences.
- The LSD value is used for determining the significant differences between means in the ANOVA test, where we subtract any two means from the table and compare the result of the subtraction with the LSD value. If the value of the subtraction is equal to or higher than the LSD value, it indicates a significant difference, while if it is less, it indicates that there is non-significant difference.

Detection of Antibiotics

A Comparison between Level of Antibiotics in Water During Winter According to Positions. The current results were showed that the first water position was scored a significant increase the levels of all studies antibiotics, the tetracycline level was decreased significantly in both second and third positions, while the level of both gentamycin and ciprofloxacin were decreased significantly in the third water position, at p. value <0.05, as in the table 1.

Table 1  
A Comparison between level of antibiotics in water during winter according to positions

| Winter / water | Tetracycline            | Gentamycin              | Ciprofloxacin           |
|----------------|-------------------------|-------------------------|-------------------------|
|                | Mean±SD                 |                         |                         |
| Position 1     | 130.3±8.01 <sup>a</sup> | 91.53±3.75 <sup>a</sup> | 125.2±5.20 <sup>a</sup> |
| Position 2     | 70.63±5.70 <sup>b</sup> | 66.60±6.83 <sup>b</sup> | 103.0±8.70 <sup>b</sup> |
| Position 3     | 63.90±2.72 <sup>b</sup> | 51.53±3.88 <sup>c</sup> | 81.70±4.45 <sup>c</sup> |
| p. value       | <0.001                  | <0.001                  | <0.001                  |
| LSD            | 11.7                    | 10.0                    | 12.7                    |

A Comparison between Level of Antibiotics in Water during Summer According to Positions

The current results were showed that second water position was scored the high level of both tetracycline and ciprofloxacin, while the level of tetracycline decrease significantly in the third water position, and the ciprofloxacin decreased significantly in the other water positions, the levels of gentamycin increased significantly in the first water position and decreased significantly in third water position at p. value <0.05, as in the table 2.

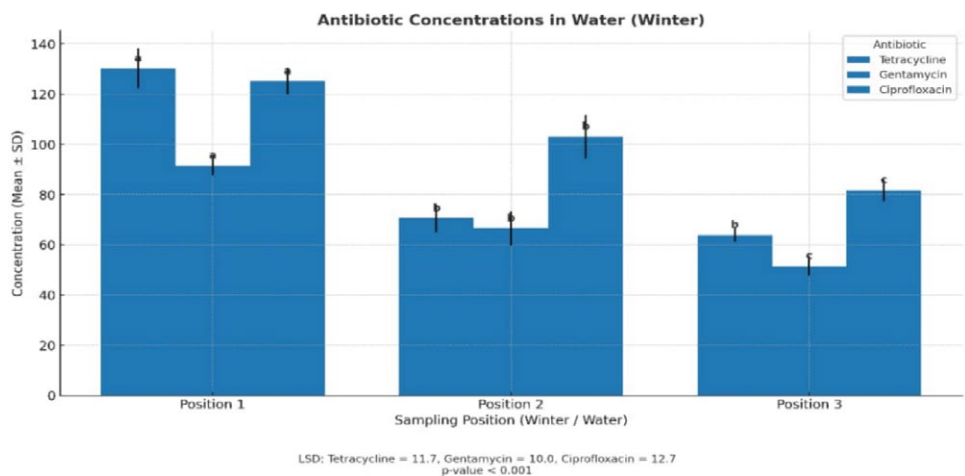


Fig. 1. Concentration of antibiotics in the Euphrates River water at the study sites in the winter season

Table 2  
A Comparison between level of antibiotics in water during summer according to positions

| Summer / water | Tetracycline | Gentamycin  | Ciprofloxacin |
|----------------|--------------|-------------|---------------|
|                | Mean±SD      |             |               |
| Position 1     | 215.8±3.00b  | 144.6±2.28a | 215.8±2.01b   |
| Position 2     | 133.2±2.91a  | 101.3±4.80b | 154.0±7.81a   |
| Position 3     | 104.7±3.45c  | 87.36±4.50c | 120.8±4.12b   |
| p. value       | <0.001       | <0.001      | <0.001        |
| LSD            | 6.25         | 8.01        | 10.4          |

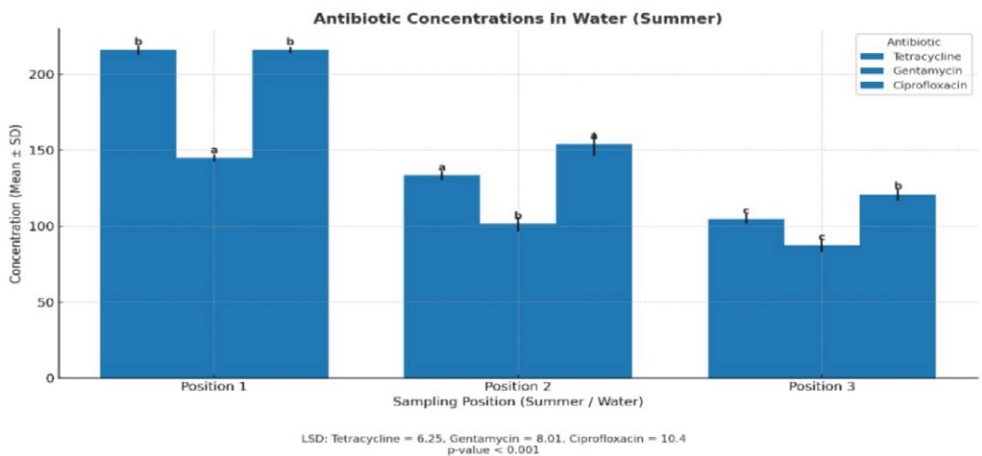


Fig. 2. Concentration of antibiotics in the Euphrates River water at the study sites in the summer

## ■ DISCUSSION

The results of the current study indicate a clear variation in the concentration of antibiotics (tetracycline, gentamicin, and ciprofloxacin) in water between winter and summer, with higher concentrations during the summer at all three sites. This seasonal trend is consistent with what has been reported by numerous global studies on the behavior of antibiotics in aquatic environments. In a comprehensive global study conducted by Wilkinson et al. [17], ciprofloxacin was detected in more than 60% of rivers in several countries, with the highest concentrations recorded in areas with high population density and weak environmental treatment systems, particularly in Asia and Africa. Concentrations of this antibiotic exceeded environmental limits in a large number of locations, indicating a risk of antibiotic contamination of surface waters. This is consistent with the current findings, which showed a significant increase in ciprofloxacin concentrations, particularly at Site 1.

A study by Xie et al. [18] in China found that tetracycline concentrations increased during the summer, due to increased agricultural use and higher temperatures that reduced the natural decomposition rate. This is consistent with the current findings, with tetracycline levels increasing at all three study sites during the summer. On the other hand, gentamicin recorded relatively lower concentrations compared to the other antibiotics. This was also observed in a study conducted in the Amazon River as conducted by Lima et al. [19], where its low concentrations were attributed to its poor stability in the aquatic environment and rapid degradation.

These results reflect the importance of temporal and spatial factors in studying antibiotic contamination of water and highlight the need for continuous seasonal monitoring, improved treatment systems, and reduced indiscriminate use of antibiotics.

## ■ CONCLUSION

Seasonal changes had an impact on the persistence of antibiotics present in the Euphrates River components. Ciprofloxacin recorded the highest value across all sites, followed by tetracycline, and finally, gentamicin, which recorded the lowest value.

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# Histological and Ultra Structural Observation of Dermal Fillers Effect on the Skin in Female Laboratory Rats

**Conflict of interest:** nothing to declare.

**Authors' contribution:** Afrah Ali Abdulameer – conceptualization, data curation, investigation, software, visualization, writing – original draft and writing – review & editing; Satar Abood Faris – conceptualization, method, project administration, resources, software, visualization, writing – original draft and writing – review & editing.

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## Abstract

**Introduction.** The use of dermal fillers, particularly hyaluronic acid fillers, is steadily increasing, leading to the emergence of various early and late complications following the injection process. Therefore, histological evaluation is a basic condition for understanding the response of skin tissues to fillers.

**Purpose.** To understand the histological changes of dermal fillers injected into the subcutaneous layer in laboratory rats over a range of time periods.

**Materials and methods.** This study was conducted on 50 female laboratory rats, divided into 5 main groups, each with a subgroup adopted as a control group. The main groups were injected with the dermal filler Hyaluronic Acid Neuramis Volume with Lidocaine in the subcutaneous layer at a dose of 0.1 ml for time periods of 13, 10, 9, 4, and 2 weeks, respectively, to compare tissue responses between different time periods after injection inside the body through histological and morphological examinations.

**Results.** The dermal filler caused different responses in the skin tissue during different time periods. The histological examination using a light microscope revealed that the skin tissue in the control group had a normal structure. The group that received the dermal filler treatment for two weeks displayed keratin layers in the epidermis, and thin collagen fibers in the dermis, along with macrophage infiltration and fluid accumulation. The group treated for 4 weeks displayed a layer of keratin in the epidermis. The keratin layer is above the epidermis, while the dermis has a branching network of collagen and infiltration of inflammatory cells, while the group treated for 9 weeks had mild hyperplasia in the deep stratum basale of the skin and the dermis was profuse with collagen in the papillary layer with infiltration of mononuclear cells and the presence of small blood vessels and thick connective tissue and elastic fibers, while the group treated for 10 and 13 weeks had all epidermal layers observed, while the dermis contains a regular network of collagen fibers and many hair follicles.

**Conclusion.** Hyaluronic acid injections stimulated the production of collagen fibers and new hair follicles, as well as the appearance of group of late histological complications represented by hyperplasia and granulation tissues. This requires conducting further studies using different dyes and different types of dermal fillers. Therefore, the current study revealed that their behavior in clinical practice is likely to be slightly different.

**Keywords:** dermal filler, ultra structural, female rats, hyaluronic acid, collagen



## ■ INTRODUCTION

Hyaluronic acid (HA) – containing dermal fillers, injected into the subcutaneous layer to treat signs of aging, have received approval with numerous positive results in medical or cosmetic clinical indications [1]. Studies have demonstrated that HA can deposit in the dermis and activate dermal fibroblasts [2]. According to Salih et al. [3], hyaluronic acid filler is made up of linear glycosaminoglycan, which is made up of 10,000 disaccharide units that contain N-acetyl-d-glucosamine and d-glucuronic acid. All HA dermal fillers are classified as monophasic gel phase fillers, such as the NEU series of fillers, which are similar to JUVX+ fillers, which can be distinguished from biphasic gel phase fillers, such as Restylane fillers. Optimal characteristics of monophasic fillers encompass excellent biocompatibility, enduring cosmetic outcomes, simplicity of administration, and little post-injection risks [3, 4]. Temporary hyaluronic acid fillers have a lifespan of 24–36 weeks, and when the appropriate amount is used, any further problems are really low. Therefore, any substance that is introduced into the skin tissue is classified as a foreign material, which initially presents a significant challenge to the immune system of the host [5].

Side effects of hyaluronic acid (HA) filler injection include pain at the injection site, redness, bruising, inflammation, edema of the skin, lumps after injection or shortly thereafter, and necrosis due to intra-arterial injection. Complications develop in less than two weeks [6]. Late complications include a cellular response in the surrounding tissues, which is characterized by dispersed macrophages and giant cells exhibiting chronic inflammation, an allergic reaction, and the formation of giant cell granulomas against the injected foreign material. This material, containing eosinophils, undergoes slow degradation by macrophages, leading to the migration of the filler and subsequent scarring [7]. The cause of granuloma formation or late inflammation is unknown, so histological predictions of possible late reactions to an individual filler cannot be made. Therefore, materials should be selected according to the patient's clinical needs, and the patient should be informed of the type of filler, whether it is long-lasting or absorbable. Another advantage of temporary fillers is that hyaluronidase can remove hyaluronic acid fillers [6].

Patients frequently struggle to recall the specific filler they received, and complications can arise when various doctors, including non-doctors, inject different fillers from different companies at different times, causing the same agents to behave differently due to variations in molecular size and production methods. This is the case for the HA products that are available in countless markets [5].

## ■ PURPOSE OF THE STUDY

To understand the histological changes of dermal fillers injected into the subcutaneous layer in laboratory rats over a range of time periods.

## ■ MATERIALS AND METHODS

There are 50 samples of female white rats examined, aged 12–16 weeks and weighing 200–250 grams. All experimental animals were in excellent health. They were placed in plastic cages equipped with water and sawdust in addition to providing food consisting of protein, corn, soybeans, and wheat. They were given free access to food freely and continuously and were left to acclimatize for one week before the experiment [8]. Then the laboratory rats were divided into five main groups: A, B, C, D, and E, each of which had

a secondary group that was considered a control group for the main groups, with a rate of 5 rats per group. The dermal filler injection Neuramis Volume Lidocaine (NVL) from (Meditox Inc., Seoul, South Korea) process was carried out for all groups at different time periods (2, 4, 9, 10, and 13 weeks, respectively) and at a dose of 0.1 mL in the subcutaneous layer. At the end of the experiment, skin samples were collected, prepared, and stained with hematoxylin and eosin (H&E) for histological examinations [9], using a light microscope at different magnification powers and using electron examination based on the methods of Luna and Bancroft and Gamble [10, 11]. Microscopic studies were conducted using a light microscope from Olympus, Japan, to determine the histological and cellular changes resulting from the injection of the dermal filler, as well as examination with a transmission electron microscope manufactured by TESCAN, China, to detect the resulting changes at the cellular level [12].

■ RESULTS

**Phenotypic Changes**

The current study found a set of phenotypic changes in female laboratory rats treated with hyaluronic acid dermal filler for different periods of time when compared to control groups (figure 1).

**Histological Examination of the Effect of Dermal Fillers on Skin Tissue**

The histological sections in the current study of female laboratory rats' skin in the control group showed the presence of skin tissue layers represented by the epidermis, dermis, and subcutaneous tissue, as shown in Figure 2A. The group of female rats treated with hyaluronic acid dermal fillers for two weeks showed the presence of keratin layers in the epidermis, while the dermis contained thin and branched collagen fibers, but there were areas suffering from collagen loss with infiltration of macrophages and fluid accumulation in the thickness of subcutaneous tissue with the presence of profuse and branched elastic tissue and hypertrophied muscle fibers, as shown in Figure 2B. The histological study of the female group treated with dermal fillers for 4 weeks showed a thin epidermis layer with a keratin layer on top, while the dermis had a branching network of collagen, but some of it suffered from collagen loss in the reticular layer, while the thickness of the subcutaneous

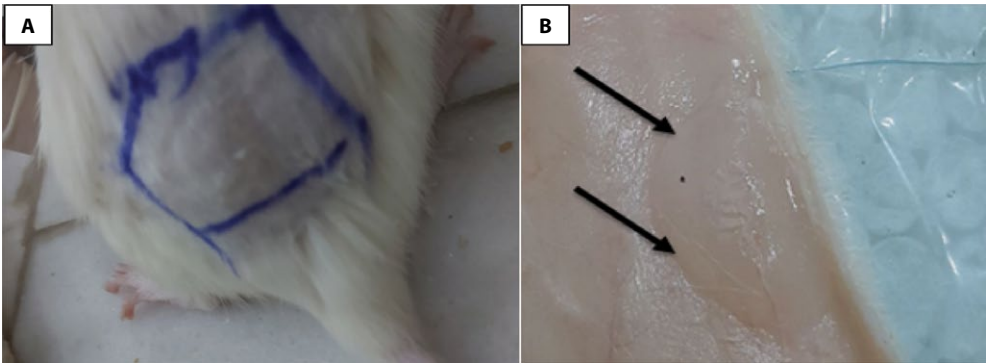


**Fig. 1. Shows the phenotypic changes in rats treated with hyaluronic acid filler (A) before dissection (B) after dissection**



tissue was characterized by the presence of thick adipose tissue and thick connective tissue with acidophilic fluid in the injection area, and infiltration of mononuclear inflammatory cells with a muscular layer between them, as in Figure 2C. In the 9-week-treated group, we observed a thin epidermis with mild hyperplasia in the deep stratum basale, a regular network in the dermis, abundant profuse collagen in the papillary layer, and the infiltration of mononuclear cells, while the tissue layer. The adipose tissue layer of the subcutaneous tissue is profuse with numerous small blood vessels and thick connective tissue with branched elastic fibers and hypertrophied muscle fibers, as in Figure 2D. The histological changes in the skin of females treated with dermal fillers for 10 weeks included the presence of all epidermal layers, which are stratum basale, stratum spinosum, stratum granulosum, and stratum corneum. The dermis contains a thick, regular network of branched collagen fibers and many hair follicles. The thickness of subcutaneous tissue was observed to be blood vessels with elastic connective tissue and hypertrophied muscle fibers, as in Figure 2E. The group that received treatment for 13 weeks demonstrated a noticeable thickness in the epidermis layers, specifically the stratum basale, stratum spinosum, stratum granulosum, and stratum corneum. These layers are composed of numerous layers of non-cellular, acidophilic keratin scales. The dermis, which is non-cellular and has hyperplasia, contains a thick network of closely packed collagen fibers in the papillary layer and reticular layers, which are packed with numerous hair follicles. In the subcutaneous tissue, there is a proliferating adipose tissue with peripheral nuclei, numerous blood vessels, elastic connective tissue, and thick muscle fibers, as depicted in the figure 2F.

### **Examinations Using Electron Transmission Microscopy**

The subcutaneous tissue of the control group of female rats underwent transmission electron microscopy (TEM) examination, revealing the presence of inactive fibroblast cells with a long elongated euchromatic nucleus and few organelles, including a few mitochondria in the cytoplasm and thin collagen fibers in the figure 3A. The group that received a two-week treatment with hyaluronic acid dermal filler revealed in the subcutaneous tissue layer the presence of a portion of the cytoplasm of immature fibroblast cells, which had been compressed by fluid accumulation. This cytoplasm included a well-developed rough endoplasmic reticulum and multiple vesicles, each containing a few collagen fibers. Additionally, the subcutaneous tissue layer revealed the nucleus of inflammatory cells and a small number of mitochondria in the figure 3B. While in the female group treated for four weeks, there was Four weeks nuclei of fibroblasts and inflammatory cells with cytoplasm full of granules and few collagen fibers, rough endoplasmic reticulum surrounded by regularly arranged collagen fibers with axial periodicity beside the cell in the figure 3C, microscopic examination showed that the group treated for 9 weeks had a prominent active fibroblast with a hyperchromatic nucleus, numerous mitochondria, a well-developed rough endoplasmic reticulum, two centrioles, multiple vesicles and collagen fibers in the figure 3D, while the group treated for 10 weeks had an active fibroblast with a hyperchromatic nucleus and a prominent nucleolus. Numerous mitochondria, well developed rough endoplasmic reticulum, wide multiple vesicles, thick elastic tissue and regular bands of collagen fibers in the figure 3E. In the 13-week treatment group, active fibroblasts with hyperchromatic nuclei and prominent nucleolus with numerous mitochondria and thick bands of collagen were observed in the figure 3F.

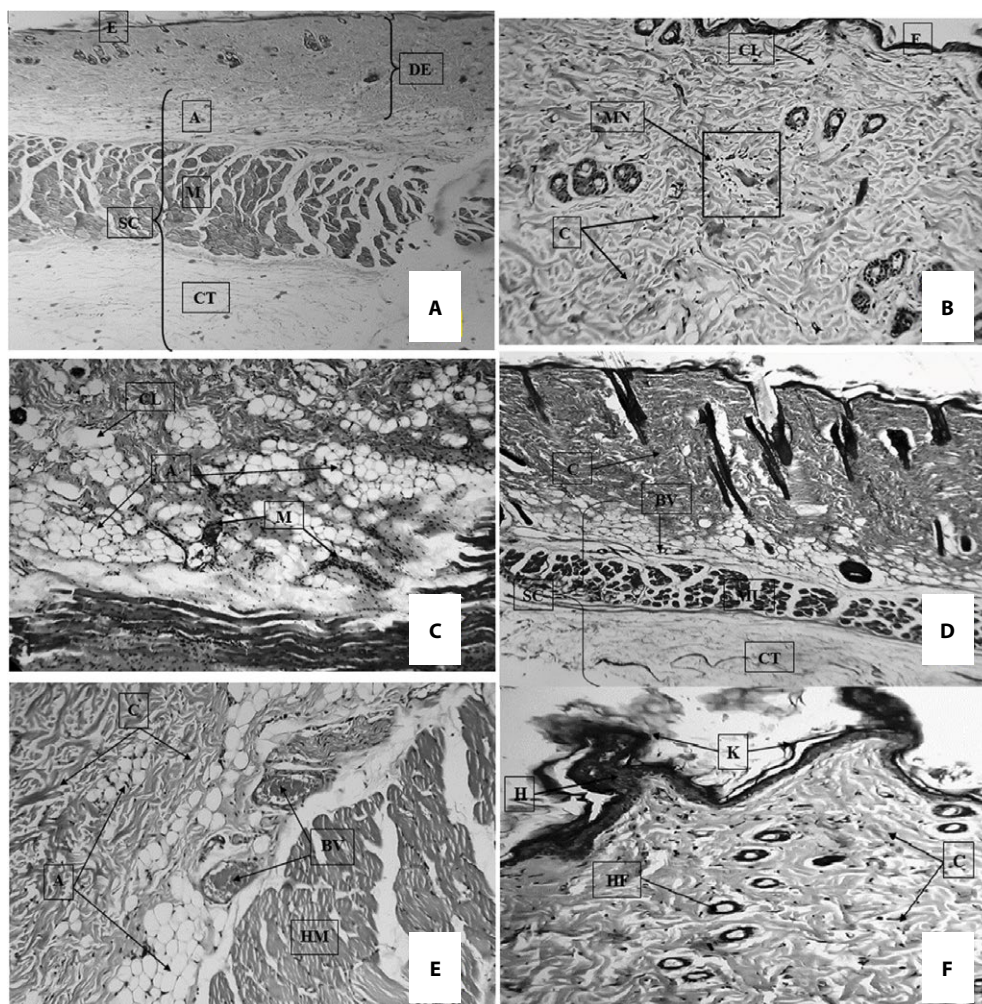


**Fig. 2. A – Control group female, showing the two layers of the skin, the epidermis and the dermis and the subcutaneous tissue (SC) contains superficial layer of adipose tissue (A), deep layer of loose connective tissue (CT) and band of muscle fibers (M) in between. 4X H&E. B – Female (2 week's injection). Showing the epidermis and the dermis. Infiltration of mononuclear cells (MN) in the dermis with thin and branched collagen fibers (C), but there are areas of dermis showed with collagen loss (CL) particularly in the papillary layer. 10X H&E. C – 4 weeks injection female. Higher magnification of the adipose tissue layer of the cutaneous tissue. There is profuse adipose tissue (A) with infiltration of mononuclear inflammatory cells (M) with CL in the reticular layer of the dermis. 10X H&E. D – 9 weeks injection female. There is thin epidermis, dermis with regular network of C with few spaces between them and increase in the thickness of SC which characterized by adipose tissue with small blood vessels (BV) and thick CT in deep and muscle fiber (MU) in between. 4X H&E. E – 10 weeks injection female – note thick, profuse and branched network of A with large BV and hypertrophied muscle fibers (HM). 10X H&E. F – 13 weeks injection female – showing thickening and hyperplasia (H) of the epidermis with an apparent covering acidophilic keratin scales (K) at the top and closely packed collagen fibers (C) in both the papillary and reticular layers of the dermis with many hair follicles (HF). 10X H&E**

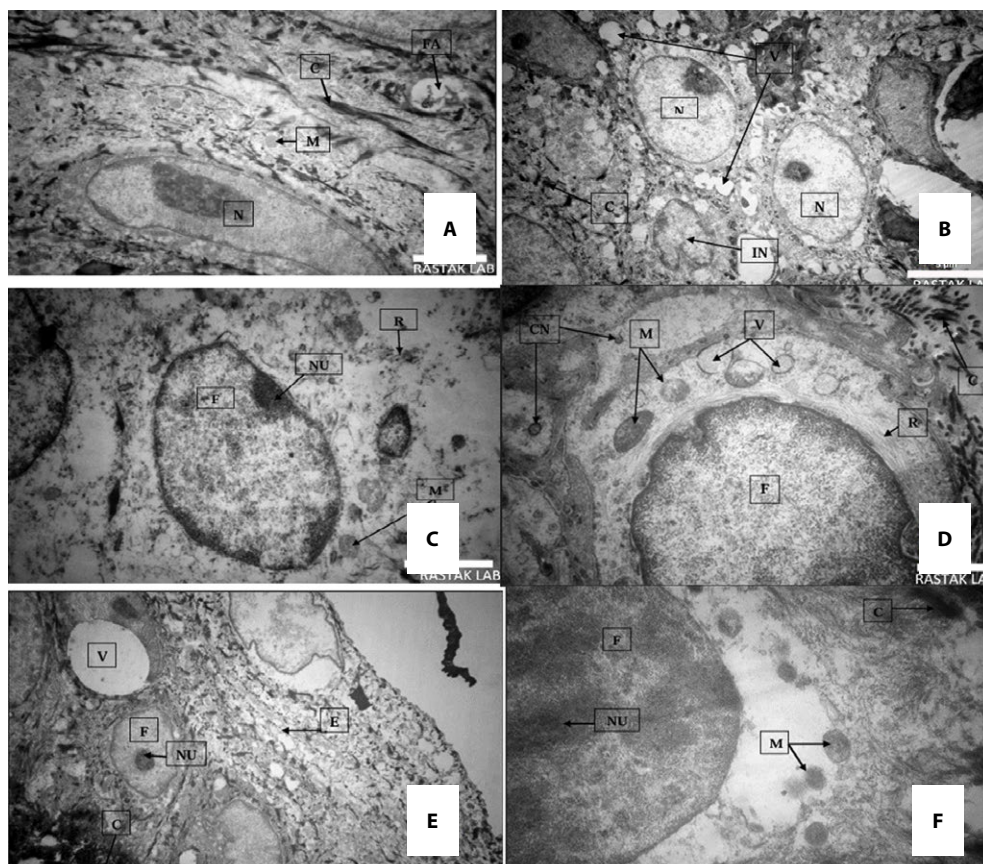


**Fig. 3.** A – an electron micrograph in the subcutaneous tissue of rat's skin. Note inactive fibroblast with elongated nucleus (N) and few organelles like few mitochondria (M) in the cytoplasm. Notice thin collagen fibers (C) and presence of areas of fluid accumulation (FA). (Control group, TEM X12000). B – an electron micrograph in the subcutaneous tissue of rat's skin showing immature fibroblasts with a prominent N. It contains well developed rough endoplasmic reticulum (R) and multiple vesicles (V). Notice few C. Also their inflammatory cell's nucleus (IN) (Two-week injection, TEM x 25000). C – an electron micrograph in the subcutaneous tissue of rat's skin. Note inactive fibroblast with flat hyperchromatic nucleus with prominent nucleolus (NU) and contains few M and rough R (Four-week injection, TEM X12000). D – an electron micrograph in the subcutaneous tissue of rat's skin. Showing a prominent active fibroblast with hyperchromatic N, numerous M, well developed rough endoplasmic reticulum and two centrioles (CN), and multiple vesicles (V) and collagen fibers. (Nine-week injection, TEM X12000). E – an electron micrograph in the subcutaneous tissue of rat's skin. Note an active fibroblast with prominent nucleolus (NU) and wide V. Note also thick elastic tissue (E) and regular bands of C. (Ten-week injection, TEM X25000). F – an electron micrograph in the subcutaneous tissue of rat's skin. Showing active fibroblasts (F) with hyperchromatic nucleus and NU with numerous mitochondria (M). Thick bands of C. (Thirteen-week injection, TEM X12000)

## ■ DISCUSSION

These results are consistent with what was mentioned by Hwang and Song [13], where his study was conducted on the HyaFilia product as a filler based on hyaluronic acid filler, which is a biphasic hyaluronic acid biphasic gel, as it was injected into the dorsal part

of the rat at a dose of 0.5 mL in the sub dermal layer, and it was observed that HA filler nodules at the injection site, then the average nodule volume increased to 132% on day 4 after injection and decreased slightly to 120% on day 7. As a result, the volume of injected HA filler doubled within 4 days of injection and then decreased slowly thereafter.

A range of side effects associated with filler injections sometimes appear, including injection site reaction, inappropriate depth, allergy to the product, long-term repeated treatment, infection, and necrosis [14]. Although most complications are temporary, there are some negative effects that last long and cause functional and cosmetic dysfunction. Necrosis is an effect caused by the direct injection of filler into a blood vessel [6]. Matarasso et al. [15] showed that HA filler injections cause a range of local effects, including bruising, swelling, granuloma formation, tissue necrosis, or vascular occlusion. Although HA fillers are stable and occupy space within tissues due to their ability to draw water from surrounding tissues, this is consistent with a study by Dunn et al [16]. The main symptoms include edema, erythema, pain, and itching.

The results back up what Signorini et al. [14] said: that most complications are mild and short-lived, and that serious adverse events are very rare. This is because injecting more hyaluronic acid changes the number of foreign body reactions caused by the filler components and bacterial proteins. This is because most hyaluronic acid fillers come from bacteria fermenting sugars, which can be a source of impurities. It does not agree with the study [17]. The signs and symptoms indicating the occurrence of vascular complications were severe pain, swelling, pustule formation, and necrosis. The main cause of tissue necrosis resulting from HA filler was intra-arterial injection [18].

The increasing use and continuous progress in surgical techniques over the past few years has determined the increasing demand for biocompatible materials for implantation in human tissues [19]. Few histological examinations are performed on animals and human volunteers, and no biopsy is taken [20]. Therefore, the current study was conducted on female laboratory rats, where the histological examination of the control group was observed to have a normal structure of the skin tissue represented by the presence of the epidermis with the dermis containing hair follicles and collagen fibers, this is consistent with Faris [21, 22].

Tissue sections from human skin that had 0.1 ml of HA filler injected intradermal showed a lot of macrophages, giant cells, and capillaries near the filler. The surrounding tissue cells didn't react much, but the macrophages broke down the filler slowly. The macrophages then ate a lot of filler particles Lemperle et al. [23], which led to more inflammatory cells entering the area and not many new collagen fibers being formed. Three months after injection, giant cells surrounded the pools containing the filler, and extracellular hydrolysis revealed the filler after six months. According to Lemperle et al. [24], some HA dermal fillers caused foreign body reactions and the infiltration of inflammatory cells, but tissue responses could recover over time. This fits with our results.

The study El-Khalawany et al. [22] conducted a histological examination that demonstrated alterations in the epidermis, superficial, middle, and deep dermis, as well as the subcutaneous layer treated with dermal fillers. These changes included the appearance of epithelial cells, inflammatory cells, neutrophils, and lymphocytes in the vicinity of the hair follicles. In the middle subdermis layer, a significant number of multinucleated giant cells surrounded the skin attachments. The histological examination of the hyaluronic acid filler after 4, 8, and 12 weeks revealed the appearance of inflammatory cells with



dense collagen fibers on the surrounding tissue. This response, resulting from a foreign body reaction, decreased after 8 weeks and disappeared by the twelfth week. Despite the absence of a chronic inflammatory reaction, there was minimal cellular infiltration into the material [25].

Our results align with the study Zarini et al. [26] that used Aquamid filler on female mice at 8 weeks of age. The study found no tissue reaction at the injection sites or abnormal behavior in the treated groups, despite the treated mice having a single well-defined skin cyst and intact upper epidermis. However, we detected some new blood vessels near the filler in the subcutaneous layer, accompanied by edematous edema, macrophages, and newly formed fibrous tissue during the 2–13 weeks. Meanwhile, the group treated for more than 57 weeks showed the presence of inflammatory cells in the skin tissue after 12 weeks, without any tissue lesions. According to histological results Aziz et al. [27], after one week of injection, the skin of rats in the HA dermal filler group exhibited normal epidermis and dermis with collagen fibrils, and the subcutaneous layer revealed numerous blood capillaries. After 8 weeks of injection, the skin in the treated group revealed thick collagen fibers in the papillary and reticular layers of the dermis, and the subcutaneous tissue exhibited a noticeable increase in thickness with numerous blood vessels, in line with our study. These results were consistent with some studies that observed an increase in collagen fibers at the site of filler injection [28].

Lowe et al. [29] studied HA Hylaform fillers injected intradermally at a dose of 0.1 mL. Microscopic examination showed normal skin with changes limited to the dermis and subcutaneous fat. The upper dermis had elastic degeneration and moderate infiltration of lymphocytes and plasma cells, along with a number of macrophages. We did not observe any eosinophil or giant cells. More pronounced fibrosis in the dermis accompanied the changes, which is consistent with our study. The study Huang et al. [30], which found a small amount of bleeding around the wound edge and a small amount of inflammatory cell infiltration around the hair follicles with inflammatory cell infiltration between the connective tissues, supports our histological results.

Skin flattening was observed at week 12 after injection of dermal filler with significant changes in the epidermis, dermis, and subcutaneous layers of aging mice, as the skin was rugged and became similar to the skin of the control group. Moreover, dermal filler injection contributed to dermal regeneration with mild infiltration of macrophages and lymphocytes [31]. This explains that dermal filler has an effect on skin tissue cell regeneration.

In treatment group 7, after one week with many stem cells present in the skin, it was proven that hyaluronan filler affects the adhesion, proliferation, differentiation, and migration of cancer stem cells, and this occurs through several newly discovered genetic signaling mechanisms that are linked to cellular receptors Solis et al. [32], in agreement with our study.

After injection, the dermis contained numerous fibroblasts, collagen fibers, and elastin fibers. It has been shown that HA filler is an important part of the epithelial-mesenchymal transition, which is when epithelial cells change into connective tissue cells [33, 34]. These changes boost the activity of the transforming growth factor- $\beta$  pathway, which tells fibroblasts and endothelial cells to make collagen and blood vessels to help wounds heal [35, 36]. All of these results indicate the activity of fibroblasts. It was noted that when HA filler is injected into separate areas within the damaged skin, it flows to areas containing

separate fibers, compressing the fibers in the injection area and creating a solid area similar to the natural dermis [33].

According to a study by Huang et al. [30], there was some bleeding around the edge of the wound and inflammatory cells moving into the area around the hair follicles and between the dermal connective tissues. This is in line with what Jia et al. [37] found about the migration of monocytes. There was an acute inflammatory response to the dermal filler HA-tyrosine/BDDE/HLC (HTBH) 4 weeks after it was injected into the skin of mice, according to a study Liu and Fan, [38]. This was seen under transmission electron microscopy (TEM).

## ■ CONCLUSION

HA dermal filler can cause different clinical effects and tissue reactions, including an inflammatory response that changes depending on how long the filler was injected and may depend on the recommended clinical indication and injection depth of locally available products. So, more research is needed to find out what the long-term effects of HA dermal fillers are and how the tissues around the injection site react using various staining and immunohistochemically methods.

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## Interleukin-10 (IL-10) Gene Polymorphism rs1800872 and Its Association with the Susceptibility and Progression of Osteoarthritis

**Conflict of interest:** nothing to declare.

**Authors' contribution:** Lamees M. Al-Janabi – conceptualization, data curation, investigation, methodology, project administration, resources, software, writing – original draft and writing – review & editing; Khalid Ali Zayer – conceptualization, data curation, investigation, methodology, project administration, writing – original draft and writing – review & editing; Ula A. Al-Sarraf – conceptualization, data curation, investigation, methodology, project administration, resources, writing – original draft and writing – review & editing.

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### Abstract

**Introduction.** Osteoarthritis is a common disease that affects older adults. It is linked to genetic factors and inflammation, and leads to chronic pain and high health-care costs.

**Purpose.** To determine the relationship between mutations and chronic diseases using genetic analysis in osteoarthritis patients and healthy people from southern Iraq.

**Materials and methods.** From 100 patients of southern Iraq whose average age between 40 to 60 years old samples collection and who were separated into two categories, group a: 50 osteoarthritic subjects (36 women and 14 men); group b: 50 apparently healthy persons (control group) (18 women and 32 men). The designing of primer is a special sequence of SNP (rs77245812) were used for discovering Specific two primers that which design across the PCR's Fast and imported from the Canadian IDT. The primers were obtained from the National Center for Biotechnology Information (NCBI).

**Results.** There are notable variations in the genotype distribution of the examined SNP between controls and patients with osteoarthritis (OA). 62% of OA patients had the AC genotype, compared to 80% of controls; 36% of OA patients and 20% of controls had the AA genotype, and 0.7% of both groups had the CC genotype. A significant correlation between genotype frequencies and OA status was found by statistical analysis using the chi-square test ( $\chi^2=8.85$ ,  $df=2$ ,  $p=0.01$ ).

**Conclusion.** A polymerase chain reaction (PCR) – based protocol using allele-specific primers is an effective tool for determining the genotype of this mutation. This may pave the way for personalized treatment strategies tailored to each individual's genetic makeup. The differences in the distribution of genotypes, especially with potential sex-related differences, also point to the complexity of the genetic factors in the disease and highlight the importance of studying interactions between alleles. In addition to the interaction between genes and the environment. Future research on the molecular mechanisms behind osteoarthritis may be guided by our findings, which highlight the importance of SNP genotyping in understanding genetic risk factors.

**Keywords:** osteoarthritis, genetic susceptibility, IL-10 rs1800872 SNP, sex-specific genetic variation, chronic pain



## ■ INTRODUCTION

The osteoarthritis is one of the highly popular and debilitating diseases, which affects the proportion of formation 80% of subjects those who are over the age 55 years. A person with a disease OA frequently had it has long-term pain and may cause severe disability and increases healthcare expenses [1, 2]. The cartilage of articular and the bone with subchondral wear, usage of the limb, overloads, and some metabolic diseases such as aging, inflammatory reactions, diabetes, as well as poor coordination and genetic diseases. have a role disease progression threshold of OA [3].

Use of genetic studies in high-volume pathological analyses in GWAS, researchers have recently made progress in unraveling the genetic basis of osteoarthritis, with 96 statistically independent risk variants. These variations explain the phenotypic diversity and are mostly linked to osteoarthritis of the knee and hip joints, and candidate gene studies concentrated on many groups of genes, including cartilage extracellular matrix (ECM) structural genes (e.g., COL2A1, COL9A3, COL11A1) [4, 5].

The interleukin type ten (InL-10) is multi-defense immune protein cytokine who mainly comes from human T-lymphocytes. One of where its impact is the formation reducer for the beginning of inflammation cytokines'. In case of the OA; InL-10 concentration that was showing been increased in tissues of joint that it had the affecting of anti-inflammatories [6, 7].

A defective release of anti-inflammatory and regulatory cytokines such as InL-10 may deal with a remarkable important in the obesity's distribution which mean a main hazard factor that occurrence and increases its intensity of OA [8].

## ■ PURPOSE OF THE STUDY

To determine the relationship between mutations and chronic diseases using genetic analysis in osteoarthritis patients and healthy people from southern Iraq.

## ■ MATERIALS AND METHODS

From 100 patients of southern Iraq whose average age between 40 to 60 years old samples collection and who were separated into categories:

- The group a: 50 osteoarthritic subjects, 36 women and 14 men.
- The group b: 50 apparently healthy persons (control group), 18 women and 32 men.

### **The Designing of Primer**

A special sequence of SNP (rs77245812) were used for discovering Specific two primers that which design across the PCR's Fast and imported from the Canadian IDT.

### **Polymorphism Genotype**

Polymorphism genotypes, especially Single Nucleotide Polymorphisms (SNPs), play important roles in determining an individual's predisposition to various diseases, including chronic conditions such as osteoarthritis (OA).

Advancements in genomic technologies have facilitated the relation between specific polymorphisms and disease phenotypes, supporting specific medical approaches. Recent studies posted at 2020 that have begun to unravel the complex relation between polymorphic genotypes and environmental factors, offering valuable insights into disease mechanisms and therapeutic target identification [9, 10].

PCR-specific expression of the interleukin 10 SNP gene (rs1800872), an enhanced method of thermal cycling that is more efficient and private. Complete denaturation of the template DNA is ensured by first denaturation at 94 °C for 180 seconds. One cycle consists of 30 seconds of denaturation at 94 °C, 30 seconds of annealing at 62–63 °C, and 30 seconds of extension at 72 °C. The target SNP region can be precisely bonded and smoked thanks to these characteristics. Complete elongation of all components is ensured by the final expansion at 72 °C for 600 seconds, increasing yield and product safety. Low copy number molds can be amplified with 35 cycles, which minimizes non-specific amplification. This procedure offers a strong foundation for precise SNP identification in genetic research, and the stability at 4 °C preserves the amplified products before further analysis (Table 1).

**Table 1**  
**PCR program**

| SNP                         | Initial Denaturation | Cycling conditions |                   |                | Final Extension | Hold | Cycle No. |
|-----------------------------|----------------------|--------------------|-------------------|----------------|-----------------|------|-----------|
|                             |                      | Denaturation       | Annealing         | Extension      |                 |      |           |
| Interleukin-10 (RS 1800872) | 94 °C / 180 sec      | 94 °C / u 30 sec   | 62–63 °C / 30 sec | 72 °C / 30 sec | 72 °C / 600 sec | 4 °C | 35 u      |

Table 2 lists the main criteria for determining the genotype of SNPs and IL-10 rs1800872. In the two front primers used, the specific primers FF and FF2 mark the alleles specifically designed to amplify alleles A and C, respectively.

The two primers differ by one nucleotide sequence at the 3’ end, and both primers have an ideal melting temperature (TM) of 62–63 °C, which is identical to that of the reverse primer in this way, the effective annealing process and targets are facilitated during the reaction of primers sequences. Electrophoresis is performed based on the expected size of the PCR primer reaction result of 240 base pairs, which confirms the accuracy of the amplification.

The primers were obtained from the National Center for Biotechnology Information (NCBI), which indicates their reliability and validity in genotyping tests. This well-thought-out approach is fully consistent with the best studies in molecular genetics research for the accurate detection of SNPs. It provides a strong basis for conducting studies linking the genotype and phenotype of an individual.

**Table 2**  
**Primers design**

| SNP                | Sequence (5’-3’) |                            | Allele | TM*      | PCR product Size (bp) | Source |
|--------------------|------------------|----------------------------|--------|----------|-----------------------|--------|
| IL-10 (RS 1800872) | F1               | 5- ATCCTGTGA CCCCCTGTA-3   | A      | 62–63 °C | 240 bp                | NCBI   |
|                    | F2               | 5- ATCCTGTGA CCCCCTGTC-3   | C      | 62–63 °C | 240 bp                |        |
|                    | R                | 5- CGTGGACAAATTGCCATTCCA-3 |        | 62–63 °C | 240 bp                |        |

Note: \* Temperature of melting.



Ethical Standards

All approvals were obtained in compliance with research ethical standards from the Thi-Qar Health Department in Iraq and the University of Thi-Qar College of Medicine (NO. 79 IN 2022). All samples are collected with the patients’ consent in official government health institutions

Statistical Analysis

The data were statistically analyzed using IBM SPSS software (V 27.0). Fisher’s exact test and the independent t-test were used for quantitative variables. In all reports, a p-value less than 0.05 is considered statistically significant.

RESULTS

There are notable variations in the genotype distribution of the examined SNP between controls and patients with osteoarthritis (OA). 62% of OA patients had the AC genotype, compared to 80% of controls; 36% of OA patients and 20% of controls had the AA genotype, and 0.7% of both groups had the CC genotype.

A significant correlation between genotype frequencies and OA status was found by statistical analysis using the chi-square test ( $\chi^2=8.85$ ,  $p=0.01$ ), suggesting a possible genetic influence on disease vulnerability. This finding is corroborated by allele frequency analysis, which revealed that whereas allele C was less common in OA cases (32%) than controls (40%), allele A was discovered with similar frequency in both OA patients (49%) and controls (50%).

The significance of allele C in the genetic susceptibility to OA is ( $p=0.01$ ) which indicates a significant difference in allele distribution (Table 3).

**Table 3**  
**Genotypes with alleles rate of inerleukin-10 (R.S 1800872) gene polymorphism in patient group and control group**

| Genotype                                    | OA           | Control      |
|---------------------------------------------|--------------|--------------|
| AC                                          | 62 (74%)     | 80 (74%)     |
| AA                                          | 36 (25.3%)   | 20 (25.3%)   |
| CC                                          | 2 (0.7%)     | 0 (0.7%)     |
| Total                                       | 100 (100.0%) | 100 (100.0%) |
| X <sup>2</sup> : 8.85, df: 2, P-value: 0.01 |              |              |
| Allele                                      | OA           | Control      |
| A                                           | 49           | 50           |
| C                                           | 32           | 40           |
| Total                                       | 81 (81.0%)   | 90 (90.0%)   |
| X <sup>2</sup> : 0.42, df: 1, P-value: 0.01 |              |              |

The table 4 shows the distribution of genotypes (AC, AA, CC) between the osteoarthritis (OA) patient group and the control group, with gender distinction. In the OA patient group, the AC genotype represented 16.0% of males and 46.0% of females, while the AA genotype represented 10.0% of males and 62.0% of females. The CC genotype was observed at a very low frequency in females (2.0%) and was not recorded in males. In contrast, in the control group, the distribution of the AC genotype was higher among males (48.0%) than females (32.0%), with 16.0% of males and 4.0% of females having the AA genotype,

and the CC genotype was not recorded in either males or females. This distribution illustrates differences between the sexes and between the two groups regarding the studied gene patterns, which may indicate a gender effect in the distribution of genotypes associated with osteoarthritis. Further statistical analyses are recommended to determine the significance of these differences and their association with disease risk.

**Table 4**  
**Gender distribution of interleukin-10 (RS 1800872) A/C SNP genotypes in patient groups and control subjects**

| Groups | AC                           | AA                          | CC                       | Total         |
|--------|------------------------------|-----------------------------|--------------------------|---------------|
| OA     | M 8 (16.0%)<br>F 23 (46.0%)  | M 5 (10.0%)<br>F 13 (62.0%) | M 0 (0.0%)<br>F 1 (2.0%) | 50<br>100.0%  |
| CON    | M 24 (48.0%)<br>F 16 (32.0%) | M 8 (16.0%)<br>F 2 (4.0%)   | M 0 (0.0%)<br>F 0 (0.0%) | 50<br>100.0%  |
| Total  | M=32<br>F=39                 | M=13<br>F=15                | M=0<br>F=1               | 100<br>100.0% |

Notes: X2: 6.27, df: 6, P-value: 0.01.

■ DISCUSSION

In recent years, advances in genetic research have highlighted the role of cytokine gene polymorphisms in the pathogenesis of osteoarthritis (OA). Osteoarthritis is a degenerative joint disease characterized by damage to articular cartilage and underlying bone. Interleukin-10 (IL-10) is an anti-inflammatory cytokine with variable expression levels due to single nucleotide polymorphisms (SNPs) within its gene, which significantly influences the occurrence and progression of osteoarthritis (OA) [11].

SNP rs1800872 is located in the promoter region of the IL-10 gene and affects cytokine production. variation in this SNP have been associated with OA, with specific alleles linked to reduced IL-10 production, this reduction may predispose individuals to inflammatory conditions that can worsen joint damage [12].

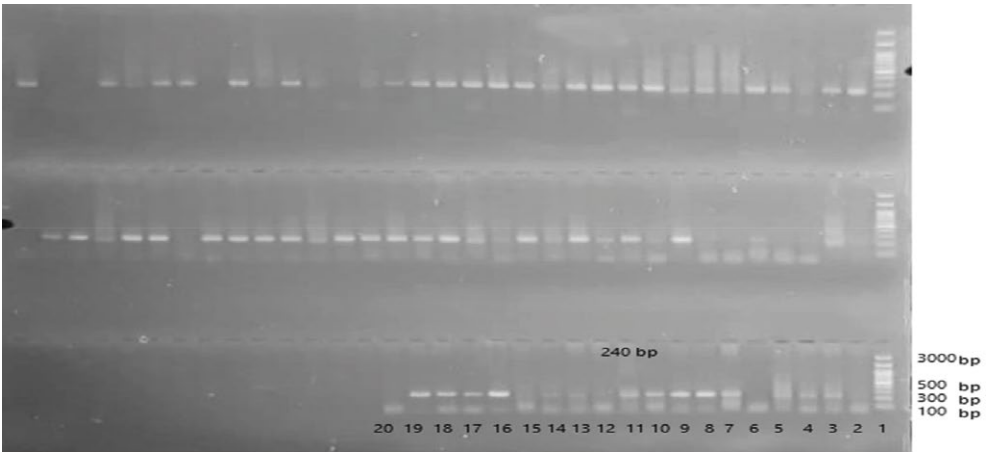


**Fig. 1. PCR amplification of interleukin-10 (RS 1800872) using primers to detect the A allele. Lane 1 shows a 100-base DNA ladder. Lanes 2-20 show PCR products (240 bases)**



The final extended extension step ensures complete assembly of the amplicons, which can then be subjected to genotyping methods such as sequencing or hybridization-based methods to determine the presence of the risk allele for SNP rs1800872 [13].

Other study has increasingly suggested that genetic markers like rs1800872 could be important in the prediction and customized treatment of OA. For instance, individuals with specific IL-10 genotypes might benefit from therapies that modulate inflammatory responses [14].

Interleukin-10 (IL-10), a gene located in the cytokine gene cluster on chromosome 1, plays a vital role as an anti-inflammatory cytokine. Its expression has been linked to several immune disorders, including osteoarthritis (OA), which is characterized by cartilage degradation and chronic pain [15]. The single nucleotide polymorphism (SNP) rs1800872 located in the promoter region of the IL-10 gene has received much attention due to its potential role in regulating IL-10 expression, which in turn may impact inflammatory operations within the joint.

Two alleles of this SNP that are associated with elevated levels "A" allele is frequently of IL-10 expressing and the linked to lower expression levels "C" allele. Researchers are working diligently to establish statistical relationships between these alleles and other factors related to susceptibility to osteoporosis, the type of response to treatment, and the progression of patients during the period of infection [16].

Advances in genotyping techniques since 2020 have allowed for precise identification of the genes using forward (F) and reverse (R) primer sequences specifically designed to amplify the region containing the names in this gene. As shown in the table, two forward primers (F1 with the A allele and F2 with the C allele) are used, along with a common reverse primer (R), to increase the size of a 240-base-pair (bp) product according to the National Center for Biotechnology Information (NCBI) guidelines. The melting temperatures of these primer sequences are carefully maintained between 62 and 63 °C to ensure fidelity during the polymerase chain reaction (PCR) amplification process [17].

The use of allele-specific primer sequences, combined with optimized PCR conditions, allows efficient discrimination between different rs1800872 alleles, which is critical for genetic studies related to osteoarthritis. This method takes advantage of the specificity achieved by aligning the melting temperatures of the primers with the annealing temperature specified in the PCR protocol, reducing the likelihood of non-specific binding and the formation of primer dimers [18].

These developments have led to a better understanding of the genetic predisposition to osteoporosis, with meta-analysis of recent genetic studies demonstrating a potential association between IL-10 polymorphisms and variability in osteoporosis presentation. This could potentially guide personalized therapeutic interventions (7). Also, considering that inflammation plays a role in OA pathogenesis, the SNP rs1800872 could serve as a marker to identify patients who may benefit from anti-inflammatory treatments targeted to modulate IL-10 pathways [19].

The osteoarthritis (OA) is a joint disease of degenerative type who represents a substantial burden on the global population, with multifactorial etiologies including biomechanical, biochemical, and genetic factors. The provided table compares the frequencies of certain genotypes and alleles between OA patients and controls, which could implicate genetic predisposition in the incidence and development of OA. According to the data, the AC genotype is found in a larger proportion of both OA patients and controls, suggesting it

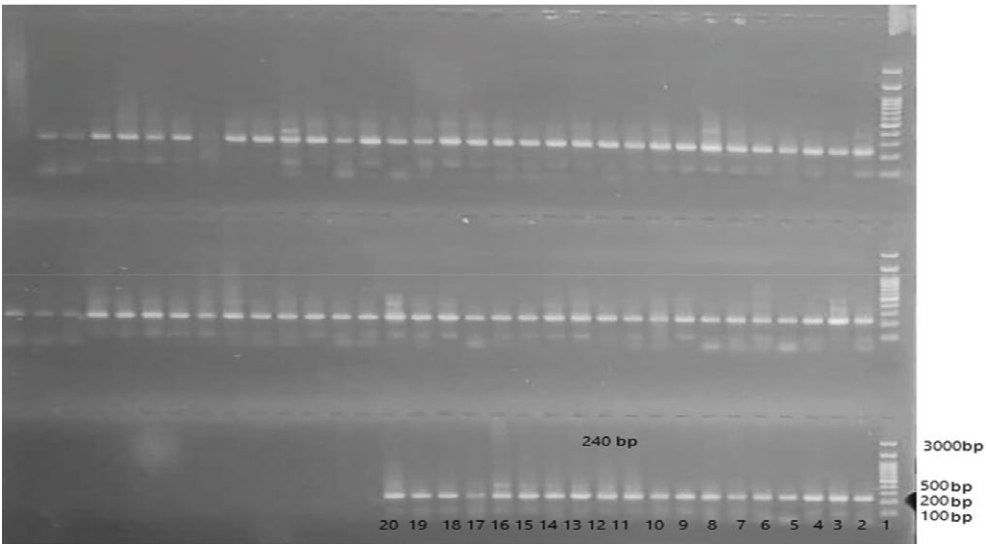


**Fig. 2. PCR amplification of interleukin-10 (RS 1800872) using specific primers for detection of the Allele C. Lane 1 represents a 100 bp DNA ladder. Lanes (2-20) represent PCR products (240 bp) from different patients**

may not be a specific risk factor. However, the Chi-Square statistic indicates a significantly variation in distribution of genotypes among groups despite proportional similarity, underscoring the complexity of genetic influence in OA. For the allele frequencies, the marginally greater distribution of allele A among OA patients against controls (49% vs. 50%) is not statistically significant when considered alone which could denote that the A allele, by itself, might not exert a strong influence on OA risk. Recent literature supports the notion that single genetic markers must be interpreted in the larger context of genetic networks and environmental interactions. A study showed on the genetic epidemiology of OA highlights specific genotypes might correlate with particular OA phenotypes or the severity of the disease, rather than OA susceptibility alone and emphasizes the considering important of the polygenic behavior of OA and fact that a multitude of SNPs each contribute a small amount to the overall genetic risk profile [20].

Furthermore, study expound on the idea that epigenetic modifications can change the effect genetic variants have on OA risk, indicating a layer of complexity beyond raw genotypic data. This reveals that environmental factors and lifestyle choices may modulate genetic predispositions and influence OA pathology [21].

Osteoarthritis (OA) is a prevalent form of arthritis disproportionately affecting millions worldwide, with females commonly having a higher incidence and severity of the disease. With the continuous advancements in genetic research, there is a growing interest in understanding the roles of specific genotypes in the predisposition to OA. The table provided presents the genotypic distributions among male (M) and female (F) OA patients and control (CON.) groups, which could suggest a role of sex in the genetic susceptibility to OA. Analysis of the table indicates disparities in genotype frequency between the sexes and between OA patients and controls. In the OA group, females exhibit a higher frequency

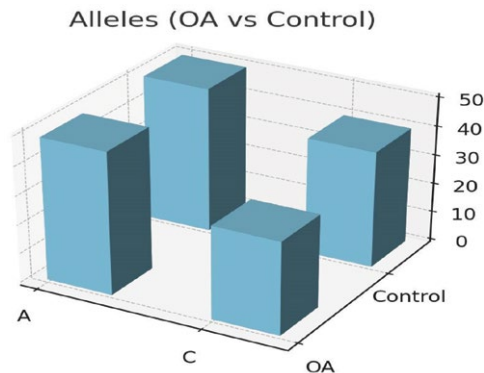


**Fig. 3. Genotype and allele distribution comparison**

of the AC genotype compared to males. Additionally, the AA genotype is notably more prevalent among female OA patients than male OA patients. This differential pattern suggests possible sex-specific genetic risk factors or interactions between sex hormones and genetic expressions influencing OA prevalence.

Recent literature post-2020 supports the investigation into sex differences in genetic studies of OA. A study by [22] investigated sex-specific genetic risks for OA, highlighting significant differences in the genetic architecture of OA between males and females. Interestingly, the control group data does not reflect the same pattern exhibited in OA patients, with males showing a higher frequency of the AC genotype than females. The absence of female controls with the CC genotype could suggest a very low prevalence or sex-specific protective factors. However, without a larger sample size and further statistical analysis, it is not possible to draw definitive conclusions from this data alone.

The PCR protocol described here is significant for elucidating the genetic risk factor of IL-10 rs1800872 SNP in OA. The association of this SNP with OA symptomatology, progression, and response to treatment underscores the importance of genetic testing in advancing personalized medicine. Future studies focusing on larger cohorts and diverse populations will be imperative to substantiate these findings and harness the potential of genotypic data to enhance outcomes for individuals with OA.

Utilizing allele-specific primers in PCR for genotyping the IL-10 rs1800872 SNP can help elucidate an individual's genetic susceptibility to OA and could potentially inform therapeutic strategies tailored to an individual.

The presence of a statistical difference in genotype distribution but not allele frequency implies a potential interaction between the two alleles in conferring risk, which should be further studied. The data alongside research findings suggest the need for a broader and more integrated approach to understanding the genomics of OA, focusing on gene with gene and gene with environment interactions and the possibilities role for epigenetics to fully elucidate the genetic contributions to OA pathogenesis and potential therapeutic targets.

Therefore, the data presented suggests that sex-specific genetic variations may be of interest in understanding OA's pathogenesis more comprehensively. Although statistical significance cannot be determined from the data alone, the evident disparities

in genotypic distribution warrant further investigation through larger scale, sex-specific genetic studies, and understanding their interaction with environmental influences and lifestyle factors to uncover new areas for OA risk management and treatment strategies.

■ CONCLUSION

It is important in the susceptibility and development of osteoarthritis (OA). A polymerase chain reaction (PCR)-based protocol using allele-specific primers is an effective tool for determining the genotype of this mutation. This may pave the way for personalized treatment strategies tailored to each individual's genetic makeup. The differences in the distribution of genotypes, especially with potential sex-related differences, also point to the complexity of the genetic factors in the disease and highlight the importance of studying interactions between alleles. In addition to the interaction between genes and the environment. Future research on the molecular mechanisms behind osteoarthritis may be guided by our findings, which highlight the importance of SNP genotyping in understanding genetic risk factors.

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# Histopathological Analysis of 200 Patients with Morphea

**Conflict of interest:** nothing to declare.

**Authors' contribution:** Fatema A. Al-Jaralla – conceptualization, data curation, investigation, method, project administration, resources, software, and writing – review & editing; Raghdaa S. Albanna – conceptualization, data curation, investigation, method, project administration, writing – original draft and writing – review & editing.

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## Abstract

**Introduction.** Morphea is a rare autoimmune fibrosing dermatitis with various clinical as well as histopathological features.

**Purpose.** To evaluate the different histopathological aspects of morphea.

**Materials and methods.** A retrospective observational study was conducted in Medical city hospital-Baghdad. Biopsies taken to morphea patients between the years 2016 and 2024 were included in this study. A histopathological review was conducted by 2 independent pathologists. There were 200 biopsy specimens from 200 patients; most of them did not receive topical or systemic treatment.

**Results.** The most common histopathological findings were collagen fibre changes 81%, thickened dermis 70%, replacement of adipose tissue by fibrosis 64%, hyperpigmented basal layer 58.5%, sweat gland caught up high in the dermis 56%, interstitial inflammatory infiltrate 51.5%, superficial and deep peri vascular inflammatory infiltrate 49.5%. Three histological stages were identified: early morphea 13.5% well established 70.5% and late burnt out morphea 16%.

**Conclusion.** This study provides a comprehensive description regarding the histopathological changes during different stages of morphea which might aid to improve our understanding of the nature of the disease and its progression.

**Keywords:** morphea, scleroderma, histopathology, fibrosing dermatitis, autoimmune disease

## ■ INTRODUCTION

Morphea is an uncommon autoimmune disease causes sclerosis and inflammation of the dermis and underlying soft tissue. Periods of inflammation combined with fibrosis are features of activity, and this eventually leads to a permanent pigment change and tissue loss [1]. Morphea incidence is estimated to be 2–3 per 100,000 in the United States [2]. However, morphea appears to be more common in Iraq. Since 1980, the prevalence of morphea in Iraq has risen steadily. According to Sharquie et al., female to male ratio was 1.5 : 1 and all ages were affected although young age group was most common [3]. Histopathologically, sclerosis, fibrosis, and atrophy of the epidermis and subcutaneous

tissue define morphea or circumscribed scleroderma [4]. The clinical picture of morphea correlates with histopathological features. A biopsy of the inflammatory boundary used to diagnose early active morphea reveals a perivascular infiltrate made up of lymphocytes and plasma cells, probably with the assistance of eosinophils and macrophages. When sclerotic skin in the centre of the lesion is examined, the dermis shows thicker and homogenized collagen fibers. As the sclerosis advances, it may be seen that the numerous collagen bundles surround eccrine glands and a few dermal blood vessels with narrow lumina and fibrotic walls [5].

■ PURPOSE OF THE STUDY

To evaluate the different histopathological aspects of morphea.

■ MATERIALS AND METHODS

The present work is a retrospective observational histopathological study that was conducted in the Center of Dermatology, Medical City, Baghdad-Iraq. Biopsy slides of patients presented with various types and stages of morphea were collected between the years of 2016 and 2024. A total of 200 slides belonging to 200 patients were included in this study, all of which had morphea in different locational body part.

All cases enrolled in this study were clinically and histopathologically diagnosed with morphea. Sections with adequate thickness were obtained from the centers of the lesions and were stained with hematoxylin and eosin. Two separate investigators examined the biopsy slides. Disagreement was resolved through debate and achieving a consensus. Different histopathological features were evaluated and recorded.

Based on our histopathological analysis, we propose a classification of morphea into three stages:

- Early Stage: Characterized by active inflammation with perivascular lymphocytic infiltrates and subtle collagen changes.
- Well-Established Stage: Marked by pronounced collagen deposition, dermal thickening, and persistent inflammatory infiltrate.
- Late (Burnt-Out) Stage: Defined by a reduction in inflammation with established fibrosis and atrophy.

■ RESULTS

A total number of 200 slides belonged to 200 patients with morphea were included in this work. Their ages ranged between 9–57 years presented clinically with various types of morphea. The slides have been systematically examined and the histopathological findings in morphea were recorded (Table 1).

The most common histologic features were collagen fibers changes (crowded, swollen, amorphous, homogenous, loss of normal fenestration) (81%), dermal thickening (70%), replacement of adipose tissue by fibrosis (64%), hyperpigmented basal layer (58.5%), interstitial inflammatory infiltrate (51.5%), a superficial and deep perivascular infiltrate (49.5%). Plasma cells neurotropism in 10 (5%).

Other accidental findings were focal calcinosis cutis and foreign body granuloma around hair follicle, each in one slide.



**Table 1**  
**List of histological findings in morphea cases**

| N  | Findings                                                                                        | No. of lesions (%) |
|----|-------------------------------------------------------------------------------------------------|--------------------|
| 1  | Collagen fibers changes (crowded, swollen, amorphous, homogenous, loss of normal fenestrations) | 162 (81)           |
| 2  | Thickened dermis                                                                                | 140 (70)           |
| 3  | Replacement of adipose tissue by fibrosis                                                       | 128 (64)           |
| 4  | Hyperpigmented basal layer                                                                      | 117 (58.5)         |
| 5  | Sweat glands caught up high in dermis and/or atrophic                                           | 112 (56)           |
| 6  | Interstitial inflammatory infiltrate                                                            | 103 (51.5)         |
| 7  | Superficial and deep perivascular inflammatory infiltrate                                       | 99 (49.5)          |
| 8  | Inflammatory changes limited to lower 2/3 of reticular dermis                                   | 78 (39)            |
| 9  | Adnexal structures replaced completely by fibrosis                                              | 67 (33.5)          |
| 10 | Atrophic epidermis                                                                              | 65 (32.5)          |
| 11 | Dermal vasculature affected by fibrosis                                                         | 45 (22.5)          |
| 12 | Hypertrophic epidermis                                                                          | 41 (20.5)          |
| 13 | Papillary dermis oedema (lichen sclerosis-like) due to progressive fibrosis                     | 40 (20)            |
| 14 | Presence of melanophages/ melanin incontinence                                                  | 28 (14)            |
| 15 | Telangiectasia in superficial dermal vessels                                                    | 25 (12.5)          |
| 16 | Line sign                                                                                       | 20 (10)            |
| 17 | Floating sign (inflammatory infiltrate surround collagen bundles)                               | 20 (10)            |
| 18 | Square biopsy (cookie cutter sign)                                                              | 15 (7.5)           |
| 19 | Plasma cells neurotropism                                                                       | 10 (5)             |
| 20 | Mild increase in dermal mucin                                                                   | 8 (4)              |
| 21 | Non specific / subtle changes                                                                   | 5 (2.5)            |
| 22 | Vacuolar interface                                                                              | 5 (2.5)            |
| 23 | Septal panniculitis                                                                             | 4 (2)              |
| 24 | Lichenoid interface                                                                             | 1 (0.5)            |
| 25 | Subepidermal blister                                                                            | 1 (0.5)            |

**Table 2**  
**Histological stages of morphea and their characteristics**

| Histological stage          | Histological characteristics                                                                                                                                                                                                                                                                                                                                                                                                                                                                            | No. (%)    |
|-----------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| Early morphea               | <ul style="list-style-type: none"> <li>Non-specific findings/subtle changes</li> <li>Acanthosis</li> <li>Epidermal hyperpigmentation with basal &amp;/or suprabasal melanosis without melanophages or melanin incontinence</li> <li>Slight superficial and deep perivascular lymphohistiocytic infiltrate</li> <li>Interstitial inflammatory infiltrate</li> <li>Floating sign</li> <li>Inflammation limited to lower dermis</li> <li>More eosinophilic collagen compared to normal collagen</li> </ul> | 27 (13.5)  |
| Well established morphea    | <ul style="list-style-type: none"> <li>Thickened dermis</li> <li>Homogenous amorphous collagen</li> <li>Replacement of adipose tissue</li> <li>Decreased adnexal structures</li> <li>Entrapment of sweat glands higher in dermis</li> </ul>                                                                                                                                                                                                                                                             | 141 (70.5) |
| Late &/or burnt out morphea | <ul style="list-style-type: none"> <li>Epidermal basal layer pigmentation</li> <li>Epidermal atrophy with melanophages</li> <li>Lichen sclerosis-like picture with progressive fibrosis</li> <li>Telangiectasia and edema of papillary dermis</li> <li>Parallel, straightened and compressed appearance of collagen</li> <li>Adnexal structures completely replaced by fibrosis</li> <li>Very sparse or no inflammatory infiltrate</li> <li>Dermal mucin deposition</li> </ul>                          | 32 (16)    |

Based on suggested histological criteria (Table 2), biopsy slides were classified into early, well established, and late &/or burnt out morphea.

Histological variants including, superficial morphea in 47 biopsies, morphea profunda in 11 biopsies, bullous morphea in one biopsy. Perineural plasma cell infiltrate was seen mostly in well-established lesions, and to a lesser degree in early lesions, and none was present in late lesions.

## ■ DISCUSSION

Morphea (scleroderma) is a connective tissue disease defined histologically by a range of alterations ending in dermal expansion with thicker collagen bundles parallel to the dermo-epidermal junction [6]. Remarkably, 14% of the studied biopsies exhibited melanin incontinence with the presence of melanophages, despite the absence of a typical interface dermatitis pattern. A similar observation was reported by Al Sharquie et al. (2020) [3]. We postulate that the prominent dermal inflammation seen in the early phase of morphea leads to an increase in both the number and caliber of dermal blood vessels. This vascular proliferation is associated with an upregulation of vascular endothelial growth factor (VEGF), a key mediator that may also promote melanogenesis [7]. Hyperpigmented epidermis appears to be more common in many inflammatory as well as tumoral lesions especially in Iraqi population. Strikingly, 14% of the studied biopsies showed melanin incontinence melanophages without the apparent interface dermatitis pattern. In fact, interface dermatitis was observed in only 3% of the studied samples. This observation might raise a question whether there is another explanation for the presence of melanin in papillary dermis other than interface dermatitis. Dermal pigment incontinence results when pigment-laden melanocytes or degenerated keratinocytes (either naturally aging or killed cells due to inflammation) fall into the papillary dermis [8].

Sharquie et al. have reported hyperpigmentations in morphea and mycosis fungoides among other diseases [9]. It's debatable whether hyperpigmentation has a causative relationship or an end result of the disease.

It is hypothesized that ultraviolet irradiation can trigger an angiogenic switch by upregulating proangiogenic factors – such as VEGF, basic fibroblast growth factor (b-FGF), and interleukin-8 (IL-8). VEGF, in particular, may enhance melanogenesis by binding to VEGF receptors on epidermal keratinocytes, thereby inducing the release of mediators (notably, arachidonic acid metabolites and plasminogen) that stimulate fibroblasts to produce collagen [3]. In parallel, both endothelial cells and fibroblasts secrete alpha-melanocyte stimulating hormone, which acts on melanocortin receptors on keratinocytes and melanocytes, promoting excessive melanin deposition [10].

Lichen sclerosis – like features were observed in histologically active/well established morphea as well as burnt out cases, a finding not uncommon in Iraqi patients with morphea. In a retrospective study involving 472 morphea patients from Germany (381 adults with a mean age of 46 years), lichen sclerosis was histopathologically confirmed in 5.7% of cases. Although the coexistence of morphea and lichen sclerosis is well documented, the precise relationship between the two remains contentious. Some investigators have argued that lichen sclerosis represents a superficial, subepidermal form of morphea, whereas others maintain that the conditions are distinct. Supporting a potential common genetic predisposition, both disorders have been associated with other autoimmune diseases, familial cases, and even concordance in monozygotic twins [11].



And an association of lichen sclerosis with HLA-DQB1 haplotypes, which have also been associated with SSc. The coexistence of the two conditions at the same body site, sometimes in a segmental or Blaschkoid distribution, and of histopathological specimens in which the features of both diseases have been found.

Supports the concept of a common aetiology Evidence supporting a common aetiology is derived from observations of lesions displaying a mental or Blaschkoid distribution and histopathological specimens in which features of both conditions are present [11].

The phenomenon of perineural plasmotropism may be overestimated or obscured by more prominent histological features in well-established lesions. Its diagnostic utility in early morphea is further compromised by the inconsistent presence of nerves in routinely obtained tissue sections. It is plausible that the anatomical distribution of cutaneous nerves – which frequently travel alongside superficial and deep dermal vessels, forming neurovascular plexuses [12, 13] – obscures the distinct recognition of perineural inflammation as an independent histomorphological feature.

In this study, Perineural plasma cell infiltrate was seen in only 10% of cases, mostly in well-established lesions, and to a lesser degree in early lesions, and none was present in late lesions. However, previous studies have reported higher prevalence of perineural inflammation. Succaria et al. observed this feature in 49% of cases, and analyses of routinely stained sections have detected perineural inflammation in 58% of morphea samples. Furthermore, Fleischmajer et al. documented perineural cellular infiltrates in 13 of 108 cases (comprising 11 localized and 2 systemic scleroderma cases, approximately 12%) [14].

When identifiable, perineural inflammation can be categorized into two distinct patterns: a 'concentric' type, in which  $\geq 75\%$  of the nerve circumference is encircled by mononuclear inflammatory cells, and a 'marginal' type, where less than 75% of the nerve is involved. Although the concentric pattern may be regarded as independent of perivascular inflammation, the marginal pattern likely represents an extension of perivascular inflammatory processes given the close anatomical relationship between nerves and vessels [14]. In our study, perineural inflammation was marginal i.e. less than 75% of the nerve is involved. Whether this finding is truly helpful in establishing the diagnosis in early morphea is dependent on the presence of a nerve in the section studied and unfortunately that's not always feasible.

More recent ultrastructural studies have demonstrated that alterations in nerve morphology progress from early to late stages of scleroderma-associated peripheral neuropathy. In advanced localized SSc, ultrastructural damage to peripheral nervous system fibers is evident and exhibits a patchy distribution that corresponds with the regional presence of dermal fibrosis and oedema [15].

The diagnosis of morphea (localized scleroderma) can be established at scanning magnification using several key histopathological features [16]:

1. Cookie-Cutter Sign (CCS):

- Definition: Biopsy specimens exhibit straight/parallel lateral edges, creating a square or rectangular shape [16], this finding was detected in 15 (7.5%) of our slides.

Likely reflects the abrupt transition between fibrotic (diseased) and normal tissue, aiding rapid recognition under low magnification.

2. Line Sign:

- Definition: A sharp, straight, linear interface between the thickened sclerotic dermal collagen and subcutaneous adipose tissue [17].

In our study it Present in 20 (10%) of cases and associated with deeper, more diffuse inflammation. It suggests subcutaneous involvement, emphasizing the need for biopsies to include subcutis for accurate assessment [18].

### 3. Floating Sign:

- Definition: Histiocytes encircling or wrapping collagen bundles. floating sign has been reported in interstitial granulomatous dermatitis and borrelia infection [19]. Regarding our study, floating sign was observed in 20 (10%) of slides.

So that floating sign can Support morphea diagnosis when combined with other signs, though not exclusive to morphea.

### 4. High Eccrine Glands (HEG):

- Eccrine glands appear "elevated" high due to dermal fibrosis and excessive collagen compressing surrounding tissue [16], in our study one of the most common histopathological findings was sweat gland caught up high in dermis 112 (56%).

## ■ CONCLUSION

This study provides a comprehensive description concerning the histopathological changes during different stages of morphea which would might aid to improve our understanding of the nature of the disease and its progression.

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## Molecular Phylogeny of Intestinal Protozoan Parasites from Human and Housefly Isolates

**Conflict of interest:** nothing to declare.

**Authors' contribution:** Khalef W.F. – conceptualization, data curation, investigation, software, visualization, writing – original draft and writing – review & editing; Alrikaby N.J. – conceptualization, investigation, visualization, writing – original draft and writing – review & editing; Oleiwi A.H. – conceptualization, data curation, investigation, writing – original draft and writing – review & editing.

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### Abstract

**Introduction.** The housefly, *Musca domestica*, is a mechanical vector for numerous enteric pathogens, but conclusive molecular evidence linking human and housefly parasite isolates is often lacking.

**Purpose.** This study employed phylogenetic analysis of the small subunit ribosomal RNA (SSU rRNA) gene to investigate the genetic relatedness of intestinal protozoan parasites between human and housefly populations.

**Materials and methods.** Using the Neighbor-Joining method in MEGA 11, we constructed phylogenetic trees for isolates of *Entamoeba histolytica*, *Giardia lamblia*, *Microsporidium* sp., *Cryptosporidium* sp., *Cystoisospora* sp., and *Cyclospora* sp. The results demonstrated an exceptionally close genetic relationship between human and housefly isolates for all parasite species.

**Results.** The total genetic changes between isolates and their respective NCBI-BLAST reference sequences were remarkably low, ranging from 0.0010% to 0.0020%, corresponding to sequence identities of ~99.99% and ~99.98%, respectively. Phylogenetic trees revealed that human and housefly isolates formed tight, monophyletic clades with no host-based segregation for most species. A key finding was observed in *Cryptosporidium* sp. isolates, where the majority clustered with the zoonotic *C. parvum*, while a single human isolate (IQN.3) clustered with the anthroponotic *C. hominis*, indicating concurrent transmission cycles. Multiple sequence alignments further confirmed high nucleotide similarity and minimal substitution mutations.

**Conclusion.** These findings provide robust molecular evidence that houseflies harbor protozoan parasites genetically identical to those infecting humans, strongly supporting their role as a critical mechanical vector. This underscores the necessity of integrating fly control into public health strategies for managing intestinal protozoal infections.

**Keywords:** SSU rRNA, phylogenetics, *musca domestica*, mechanical vector, housefly

## ■ INTRODUCTION

Intestinal protozoan infections remain a significant global public health burden, particularly in regions with inadequate sanitation, poor hygiene, and limited access to clean water. Pathogens such as *Entamoeba histolytica*, *Giardia lamblia*, and *Cryptosporidium* spp. are leading causes of diarrheal morbidity and mortality worldwide, especially among children, the elderly [1], and immunocompromised individuals [2]. The transmission of these parasites occurs primarily through the fecal-oral route, via the ingestion of infectious cysts or oocysts present in contaminated food or water [3]. Difficulties are confronted in the detection of that parasite in patients' faces because of intermittent excretion of the parasite [4].

The synanthropic housefly, *Musca domestica*, is ubiquitous in human settlements and thrives in close association with organic waste, sewage, and feces. Due to its feeding and breeding habits, and its ability to move freely between filth and food sources, the housefly has long been implicated as a potential mechanical vector for a wide array of enteric pathogens [5]. Mechanical transmission involves the passive carriage of pathogens on the fly's exoskeleton, mouthparts, or in its digestive tract [6], with no biological development of the parasite within the fly [7]. While early studies culturally isolated parasites from flies, these methods alone cannot confirm that the parasites carried by flies are the same genotypes responsible for human disease, a critical link required to definitively establish the fly's role in transmission epidemiology [8].

Molecular phylogenetic analysis provides a powerful tool to bridge this evidence gap. The small subunit ribosomal RNA (SSU rRNA) gene is a highly conserved yet informative molecular marker widely used for elucidating evolutionary relationships and taxonomic classification of protists [9, 10]. Its mosaic of conserved and variable regions allows for robust multiple sequence alignment and the inference of genetic distances with high confidence. By comparing the SSU rRNA gene sequences of parasite isolates from humans and houseflies, it is possible to determine their genetic relatedness with precision.

This study was designed to molecularly characterize and compare the genetic diversity of six intestinal protozoan parasites – *Entamoeba histolytica*, *Giardia lamblia*, *Microsporidium* sp., *Cryptosporidium* sp., *Cystoisospora* sp., and *Cyclospora* sp. – isolated from human and housefly samples within a defined community. The primary objectives were to: (1) construct phylogenetic trees based on SSU rRNA gene sequences to infer evolutionary relationships, (2) quantify the genetic distance and sequence identity between human and housefly isolates, and (3) assess the epidemiological implications of these genetic findings for understanding the housefly's role in the transmission dynamics of these significant enteric pathogens.

## ■ PURPOSE OF THE STUDY

This study employed phylogenetic analysis of the small subunit ribosomal RNA (SSU rRNA) gene to investigate the genetic relatedness of intestinal protozoan parasites between human and housefly populations.

## ■ MATERIALS AND METHODS

### Sample Collection and Parasite Isolation

Human stool samples were collected from symptomatic individuals presenting with gastroenteritis at local clinics after obtaining informed consent. Houseflies (*Musca*



domestica) were captured using sweep nets from various peri-domestic locations, including food markets, waste disposal sites, and residential areas, within the same community. Flies were anaesthetized and identified taxonomically. Parasitic cysts and oocysts were concentrated from human stool samples using formalin-ether sedimentation and from fly surface washes and homogenates using sucrose flotation, following established protocols [5].

### DNA Extraction, PCR Amplification, and Sequencing

Genomic DNA was extracted from both external and internal fly samples using the Presto™ Stool DNA Extraction Kit (Geneaid, Taiwan), following the manufacturer's instructions. DNA concentration and purity were measured using a NanoDrop spectrophotometer (Thermo Scientific, UK).

Conventional PCR was performed to detect *Entamoeba histolytica*, *Giardia intestinalis*, *Microsporidium* sp., *Cryptosporidium* sp., *Cyclospora* sp., and *Cystoisospora* sp., targeting the small subunit ribosomal RNA (SSU rRNA) gene. The primers used are listed in Table 1.

**Table 1**  
**Primer sequences and product sizes for PCR detection of intestinal parasites**

| Parasite                     | Primer Sequence (5'–3')                                | Product Size (bp) | GenBank Accession |
|------------------------------|--------------------------------------------------------|-------------------|-------------------|
| <i>Entamoeba histolytica</i> | F: CTGGTTGATCCTGCCAGTATT<br>R: CACCAGACTTGCCCTCCAAT    | 555               | AB282658.1        |
| <i>Giardia intestinalis</i>  | F: GAATCAGGGTTCGACTCCGG<br>R: CGTTACGGCCGGGAATACG      | 504               | U09492.1          |
| <i>Microsporidium</i> sp.    | F: GATAGCTCCCACGTCCAAGG<br>R: CTCCTTGTTGTAGCTCCG       | 522               | PP625131.1        |
| <i>Cryptosporidium</i> sp.   | F: GAGTATGGTCGCAAGGCTGA<br>R: TGTGTACAAAGGCAGGGAC      | 530               | AY237630.1        |
| <i>Cyclospora</i> sp.        | F: AGTTGGATTCTGCTGTGGTCA<br>R: TCCTATCTCTAGTCGGCATAGT  | 423               | PQ835031.1        |
| <i>Cystoisospora</i> sp.     | F: GCGGTAATTCAGCTCCAATAG<br>R: GCCCTAACTTTCGTTCTTGATTA | 418               | LC377842.1        |

The PCR reaction was performed in a total volume of 20 µL, containing 5 µL of DNA template, 2 µL of each forward and reverse primer (10 pmol/µL), 12.5 µL of GoTaq® Green Master Mix, and 3.5 µL of nuclease-free water. Amplification was carried out under the following conditions: an initial denaturation at 95 °C for 5 minutes; followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing at 58 °C for 30 seconds, and extension at 72 °C for 1 minute; with a final extension at 72 °C for 5 minutes and an indefinite hold at 4 °C. PCR products were separated by electrophoresis on a 1.5% agarose gel stained with ethidium bromide, run at 100 V and 80 mA for 1 hour in 1×TBE buffer. A 100 bp DNA ladder was used as a size marker. Gels were visualized under UV light using a transilluminator.

### Phylogenetic and Sequence Analysis

The obtained sequences were edited and assembled using BioEdit software. The identity of the isolates was initially confirmed by performing a BLASTn search against the NCBI GenBank database. Reference sequences for each parasite species with high similarity scores were downloaded. Multiple sequence alignments for each parasite set (including human isolates, housefly isolates, and reference sequences) were performed

using the ClustalW algorithm implemented in MEGA software version 11.0.13 [11, 12]. The aligned sequences were used to infer evolutionary history using the Neighbor-Joining (NJ) method [13]. The robustness of the phylogenetic trees was assessed by bootstrap analysis with 1000 replicates. The total genetic change (p-distance) was calculated directly within MEGA 11. Sequence identity percentages were derived from the alignment data and BLAST results.

■ RESULTS

**Prevalence of Parasites in House Flies**

A total of five hundred flies were collected from (butchers, fish and chicken shops, garbage of houses, vegetable stores, and house) from Thi-Qar province. The present study showed that 300 (60.0%) of flies carrying parasite, while 200 (40.0%) no carrying parasite was observed as shown figure 1.

**Phylogenetic Tree Analysis**

The DNA sequencing method was carried out to identification genetic relationship of intestinal protozoal parasite between human and housefly based on small subunit ribosomal RNA gene.

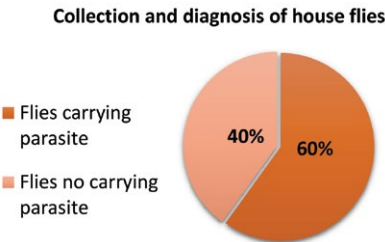


Fig. 1. Collection and diagnosis of house flies

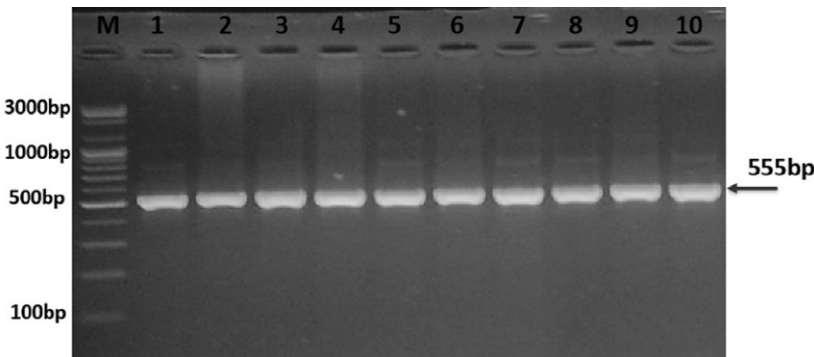


Fig. 2. Agarose gel electrophoresis image that show the PCR product analysis of small subunit ribosomal gene for detection of Entamoeba histolytica from house flies samples. Where, the Lane (M): DNA marker ladder (2000-100 bp) and the Lane (1-10) were show some positive Entamoeba histolytica at (555 bp) PCR product size

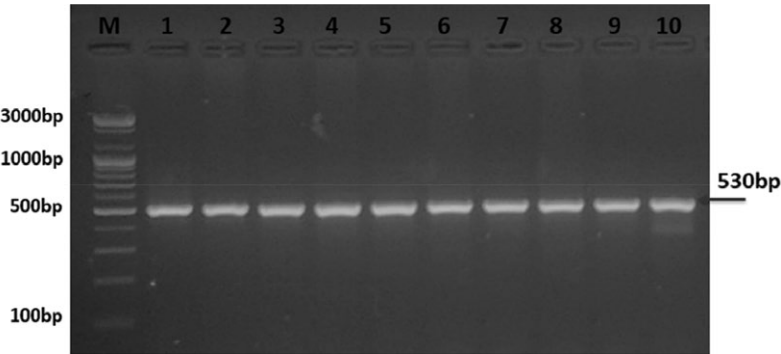


**Fig. 3.** Agarose gel electrophoresis image that show the PCR product analysis of small subunit ribosomal gene for detection of *Cryptosporidium* sp. from house flies samples. Where, the Lane (M): DNA marker ladder (3000-100 bp) and the Lane (1-10) were show some positive *Cryptosporidium* sp. at (530 bp) PCR product size

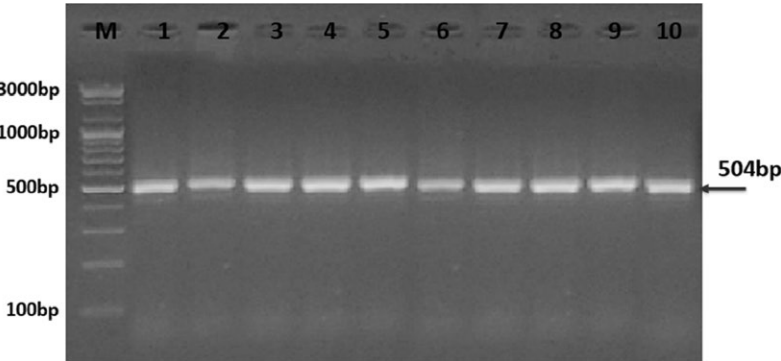


**Fig. 4.** Agarose gel electrophoresis image that show the PCR product analysis of small subunit ribosomal gene for detection of *Giardia intestinalis* from house flies samples. Where, the Lane (M): DNA marker ladder (3000-100 bp) and the Lane (1-10) were show some positive *Giardia intestinalis* at (504 bp) PCR product size

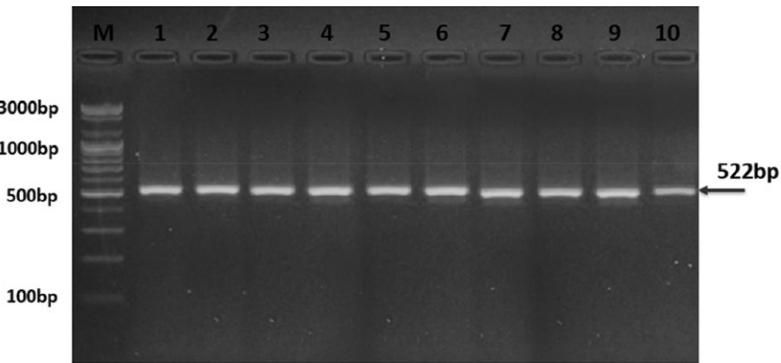


**Fig. 5.** Agarose gel electrophoresis image that show the PCR product analysis of small subunit ribosomal gene for detection of *Microsporidium* sp. from house flies samples. Where, the Lane (M): DNA marker ladder (3000-100 bp) and the Lane (1-10) were show some positive *Microsporidium* sp. at (522 bp) PCR product size

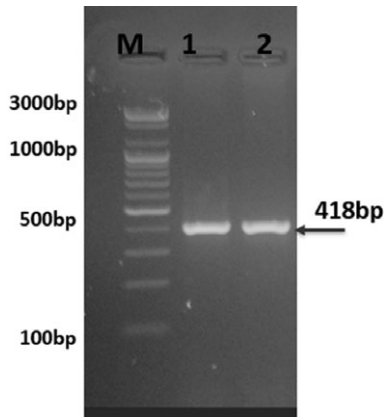


**Fig. 6.** Agarose gel electrophoresis image that show the PCR product analysis of small subunit ribosomal gene for detection of *Cystoisospora* sp. from house flies samples. Where, the Lane (M): DNA marker ladder (3000-100 bp) and the Lane (1-2) were show some positive *Cystoisospora* sp. at (418 bp) PCR product size

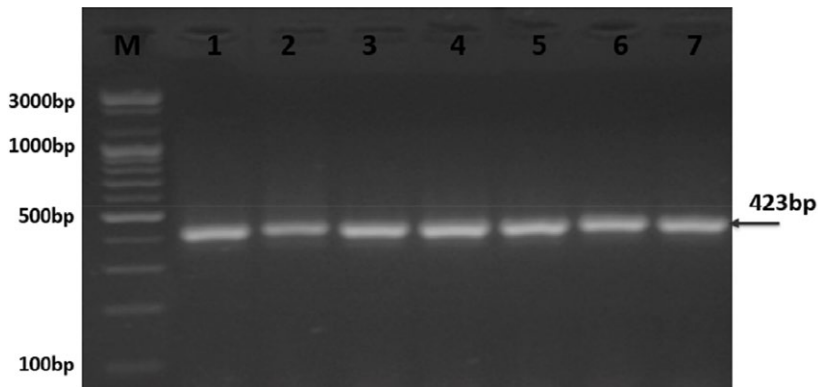


**Fig. 7.** Agarose gel electrophoresis image that show the PCR product analysis of small subunit ribosomal gene for detection of *Cyclospora* sp. from house flies samples. Where, the Lane (M): DNA marker ladder (3000-100 bp) and the Lane (1-10) were show some positive *Cyclospora* sp. at (423 bp) PCR product size

The phylogenetic trees were constructed to infer evolutionary relationships using the Neighbor-Joining method (MEGA 11 version). The *Entamoeba histolytica* human and housefly isolates showed a close genetic relationship to each other and were all closely related to the NCBI-BLAST *Entamoeba histolytica* reference isolate (AB282658.1) with total genetic changes of 0.0020%. The *Giardia lamblia* human and housefly isolates showed a close genetic relationship to each other and were all closely related to the NCBI-BLAST *Giardia lamblia* reference isolate (U09491.1) with total genetic changes of 0.0010%. The *Microsporidium* sp. human and housefly isolates showed a close genetic relationship to each other and were all closely related to the NCBI-BLAST *Microsporidium* sp. reference isolate (OR500624.1) with total genetic changes of 0.0010%. The *Cryptosporidium* sp. human and housefly isolates showed a close genetic relationship; however, the

human IQN.3 isolate was an exception, showing a closer relationship to the NCBI-BLAST *Cryptosporidium hominis* reference isolate (PQ834958.1), while all other isolates were closely related to the *Cryptosporidium parvum* reference isolate (AF112570.1) with total

| DNA Sequences                                          | Translated Protein Sequences               |
|--------------------------------------------------------|--------------------------------------------|
| Species/Abbrev                                         |                                            |
| 1. Entamoeba histolytica Human IQN.1 isolate           | TGATAAAGATAAATAC TTGAGACGATCCAGTTTGTATTAG  |
| 2. Entamoeba histolytica Human IQN.2 isolate           | TGATAAAGATAAATAC TTGAGACGATCCAAATTTGTATTAG |
| 3. Entamoeba histolytica Human IQN.3 isolate           | TGATAAAGATAAATAC TTGAGACGATCCAGTATGTATTAG  |
| 4. Entamoeba histolytica Human IQN.4 isolate           | TGATAAAGATAAATAC TTGAGACGATCCAAATTTGTATTAG |
| 5. Entamoeba histolytica Human IQN.5 isolate           | TGATAAAGATAAATAC TTGAGACGATCCTGTTTGTATTAG  |
| 6. Entamoeba histolytica housefly IQN.1 isolate        | TGATAAAGATAAATAC TTGAGACGATCCAAATTTGTATTAG |
| 7. Entamoeba histolytica housefly IQN.2 isolate        | TGATAAAGATAAATAC TTGAGACGATCCAGTTTGTATTAG  |
| 8. Entamoeba histolytica housefly IQN.3 isolate        | TGATAAAGATAAATAC TTGAGACGATCCAAATTTGTATTAG |
| 9. Entamoeba histolytica housefly IQN.4 isolate        | TGATAAAGATAAATAC TTGAGACGATCCAGTTTGTATTAG  |
| 10. Entamoeba histolytica housefly IQN.5 isolate       | TGATAAAGATAAATAC TTGAGACGATCCAAATTTGTATTAG |
| 11. AB282658.1 Entamoeba histolytica reference isolate | TGATAAAGATAAATAC TTGAGACGATCCAGTTTGTATTAG  |

**Fig. 8.** Multiple sequence alignment analysis of *Entamoeba histolytica* human and housefly isolates based on small subunit ribosomal RNA gene that aligned with NCBI-Genbank submitted *Entamoeba histolytica* reference isolate. The multiple alignment analysis was constructed using ClustalW alignment tool in (MEGA 11.0.13 version). The analysis showed the nucleotide alignment similarity and substitution mutations in small subunit ribosomal RNA gene among all alignment sequence

| DNA Sequences                                  | Translated Protein Sequences                   |
|------------------------------------------------|------------------------------------------------|
| Species/Abbrev                                 |                                                |
| 1. Giardia lamblia Human IQN.1 isolate         | AGGTC TGGTGCCAGCAGCCGCGGTAATTCCTGCTCGGGCGGGCGT |
| 2. Giardia lamblia Human IQN.2 isolate         | AGGTC TGGTGCCAGCAGCCGCGGTAATTCCTGCTCGGGCGGGCGT |
| 3. Giardia lamblia Human IQN.3 isolate         | AGGTC TGGTGCCAGCAGCCGCGGTAATTCCTGCTCGGGCGGGCGT |
| 4. Giardia lamblia Human IQN.4 isolate         | AGGTC TGGTGCCAGCAGCCGCGGTAATTCCTGCTCGGGCGGGCGT |
| 5. Giardia lamblia Housefly IQN.1 isolate      | AGGTC TGGTGCCAGCAGCCGCGGTAATTCAGCTCGGGCGGGCGT  |
| 6. Giardia lamblia Housefly IQN.2 isolate      | AGGTC TGGTGCCAGCAGCCGCGGTAATTCAGCTCGGGCGGGCGT  |
| 7. Giardia lamblia Housefly IQN.3 isolate      | AGGTC TGGTGCCAGCAGCCGCGGTAATTCAGCTCGGGCGGGCGT  |
| 8. Giardia lamblia Housefly IQN.4 isolate      | AGGTC TGGTGCCAGCAGCCGCGGTAATTCCTGCTCGGGCGGGCGT |
| 9. Giardia lamblia Housefly IQN.5 isolate      | AGGTC TGGTGCCAGCAGCCGCGGTAATTCCTGCTCGGGCGGGCGT |
| 10. Giardia lamblia Housefly IQN.6 isolate     | AGGTC TGGTGCCAGCAGCCGCGGTAATTCAGCTCGGGCGGGCGT  |
| 11. U09491.1 Giardia lamblia reference isolate | AGGTC TGGTGCCAGCAGCCGCGGTAATTCAGCTCGGGCGGGCGT  |

**Fig. 9.** Multiple sequence alignment analysis of *Giardia lamblia* human and housefly isolates based on small subunit ribosomal RNA gene that aligned with NCBI-Genbank submitted *Giardia lamblia* reference isolate. The multiple alignment analysis was constructed using ClustalW alignment tool in (MEGA 11.0.13 version). The analysis showed the nucleotide alignment similarity and substitution mutations in small subunit ribosomal RNA gene among all alignment sequence

| DNA Sequences                                       | Translated Protein Sequences             |
|-----------------------------------------------------|------------------------------------------|
| Species/Abbrev                                      |                                          |
| 1. Microsporidium sp. Human IQN.1 isolate           | CCGCGGTAATACCGACTTCAAGAGTGTGTATGAGAGATGC |
| 2. Microsporidium sp. Human IQN.2 isolate           | CCGCGGTAATACCGACTTCAAGAGTGTGTATGAGAGATGC |
| 3. Microsporidium sp. Human IQN.3 isolate           | CCGCGGTAATACCGACTTCAAGAGTGTGTATGAGAGATGC |
| 4. Microsporidium sp. Human IQN.4 isolate           | CCGCGGTAATACCGACTTCAAGAGTGTGTATGAGAGATGC |
| 5. Microsporidium sp. Human IQN.5 isolate           | CCGCGGTAATACCGACTTCAAGAGTGTGTATGAGAGATGC |
| 6. Microsporidium sp. Human IQN.6 isolate           | CCGCGGTAATACCGACTTCAAGAGTGTGTATGAGAGATGC |
| 7. Microsporidium sp. Human IQN.7 isolate           | CCGCGGTAATACCGACTTCAAGAGTGTGTATGAGAGATGC |
| 8. Microsporidium sp. Housefly IQN.1 isolate        | CCGCGGTAATACCGACTTCAAGAGTGTGTATGAGAGATGC |
| 9. Microsporidium sp. Housefly IQN.2 isolate        | CCGCGGTAATACCGACTTCAAGAGTGTGTATGAGAGATGC |
| 10. Microsporidium sp. Housefly IQN.3 isolate       | CCGCGGTAATACCGACTTCAAGAGTGTGTATGAGAGATGC |
| 11. Microsporidium sp. Housefly IQN.4 isolate       | CCGCGGTAATACCGACTTCAAGAGTGTGTATGAGAGATGC |
| 12. Microsporidium sp. Housefly IQN.5 isolate       | CCGCGGTAATACCGACTTCAAGAGTGTGTATGAGAGATGC |
| 13. Microsporidium sp. Housefly IQN.6 isolate       | CCGCGGTAATACCGACTTCAAGAGTGTGTATGAGAGATGC |
| 14. Microsporidium sp. Housefly IQN.7 isolate       | CCGCGGTAATACCGACTTCAAGAGTGTGTATGAGAGATGC |
| 15. OR500624.1 Microsporidium sp. reference isolate | CCGCGGTAATACCGACTTCAAGAGTGTGTATGAGAGATGC |

**Fig. 10.** Multiple sequence alignment analysis of *Microsporidium* sp. human and housefly isolates based on small subunit ribosomal RNA gene that aligned with NCBI-Genbank submitted *Microsporidium* sp. reference isolate. The multiple alignment analysis was constructed using ClustalW alignment tool in (MEGA 11.0.13 version). The analysis showed the nucleotide alignment similarity and substitution mutations in small subunit ribosomal RNA gene among all alignment sequence

| DNA Sequences                                                  | Translated Protein Sequences                                                  |
|----------------------------------------------------------------|-------------------------------------------------------------------------------|
| Species/Abbrev                                                 | * * * * * * * * * * * * * * * * * * * * * * * * * *                           |
| 1. <i>Cystoisospora</i> sp. Human IQN 1 isolate                | T T T T C C A G G A C T T T T A C T A T G A A A A T T A G A G T G T T T C A A |
| 2. <i>Cystoisospora</i> sp. Human IQN 2 isolate                | T T T T C C A G G A C T T T T A C T A T G A A A A T T A G A G T G T T T C A A |
| 3. <i>Cystoisospora</i> sp. Human IQN 3 isolate                | T T T T C C A G G A C T T T T A C T A T G A A A A T T A G A G T G T T T C A A |
| 4. <i>Cystoisospora</i> sp. Human IQN 4 isolate                | T T T T C C A G G A C T T T T A C T T T G A A A T T A G A G T G T T T C A A   |
| 5. <i>Cystoisospora</i> sp. Human IQN 5 isolate                | T T T T C C A G G A C T T T T A C T A T G A A A A T T A G A G T G T T T C A A |
| 6. <i>Cystoisospora</i> sp. Housefly IQN 1 isolate             | T T T T C C A G G A C T T T T A C T T T G A A A T T A G A G T G T T T C A A   |
| 7. <i>Cystoisospora</i> sp. Housefly IQN 2 isolate             | T T T T C C A G G A C T T T T A C T A T G A A A A T T A G A G T G T T T C A A |
| 8. JX025650.1 <i>Cystoisospora belli</i> reference isolate     | T T T T C C A G G A C T T T T A C T T T G A A A A T T A G A G T G T T T C A A |
| 9. OP837489.1 <i>Cystoisospora phillipsi</i> reference isolate | T A T T C C A G G G C T T T T A C T T T G A A A A T T A G A G T G T T T C A A |

[illegible]

genetic changes of 0.0010%. The *Cystoisospora* sp. human and housefly isolates showed a close genetic relationship to each other and were all closely related to the NCBI-BLAST *Cystoisospora belli* reference isolate (JX025650.1) with total genetic changes of 0.0020%.

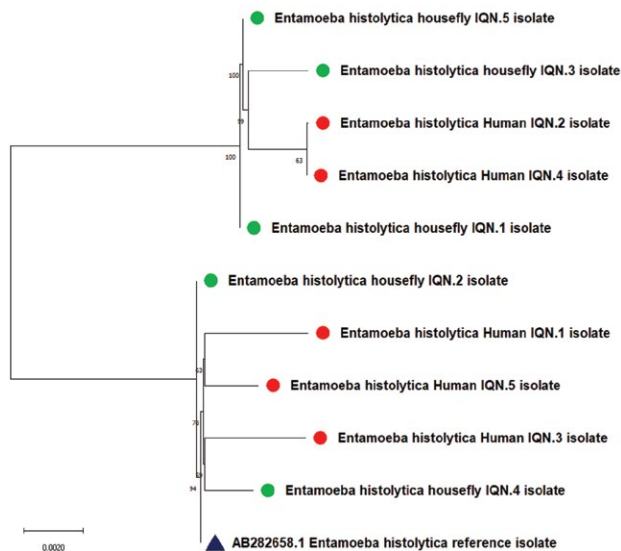


**Fig. 14. Phylogenetic tree analysis of based on small subunit ribosomal RNA gene that used for identification genetic relationship between *Entamoeba histolytica* of human and housefly isolates. The phylogenetic tree was constructed using the evolutionary history was inferred using the Neighbor-Joining method (MEGA 11 version). The *Entamoeba histolytica* human isolates were showed closed genetic related to and *Entamoeba histolytica* housefly isolates and all isolated related to NCBI-BLAST *Entamoeba histolytica* reference isolate (AB282658.1) at total genetic changes (0.0020%)**

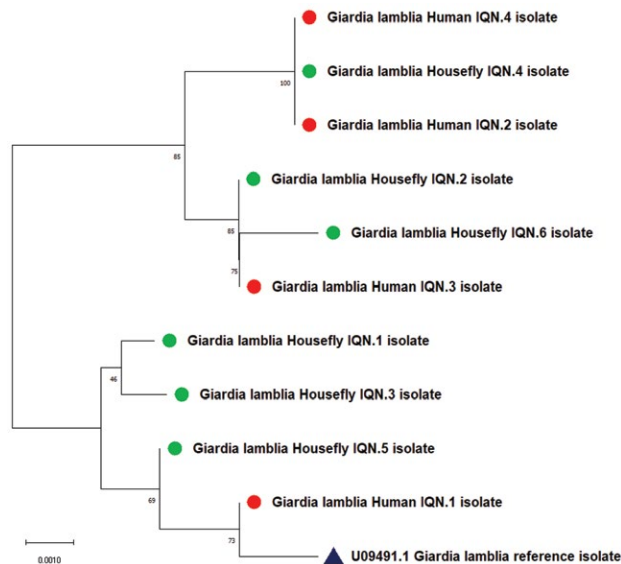


**Fig. 15. Phylogenetic tree analysis of based on small subunit ribosomal RNA gene that used for identification genetic relationship between *Giardia lamblia* of human and housefly isolates. The phylogenetic tree was constructed using the evolutionary history was inferred using the Neighbor-Joining method (MEGA 11 version). The *Giardia lamblia* human isolates were showed closed genetic related to and *Giardia lamblia* housefly isolates and all isolated related to NCBI-BLAST *Giardia lamblia* reference isolate (U09491.1) at total genetic changes (0.0010%)**

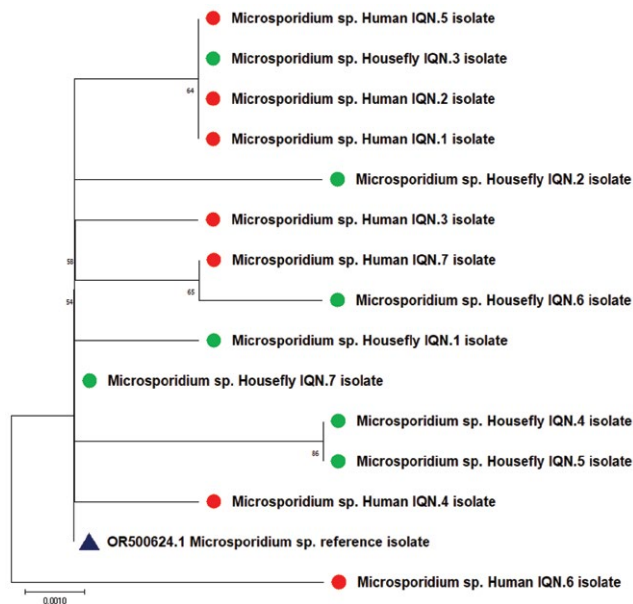


**Fig. 16. Phylogenetic tree analysis of based on small subunit ribosomal RNA gene that used for identification genetic relationship between *Microsporidium* sp. of human and housefly isolates. The phylogenetic tree was constructed using the evolutionary history was inferred using the Neighbor-Joining method (MEGA 11 version). The *Microsporidium* sp. human isolates were showed closed genetic related to and *Microsporidium* sp. housefly isolates and all isolated related to NCBI-BLAST *Microsporidium* sp. reference isolate (OR500624.1) at total genetic changes (0.0010%)**

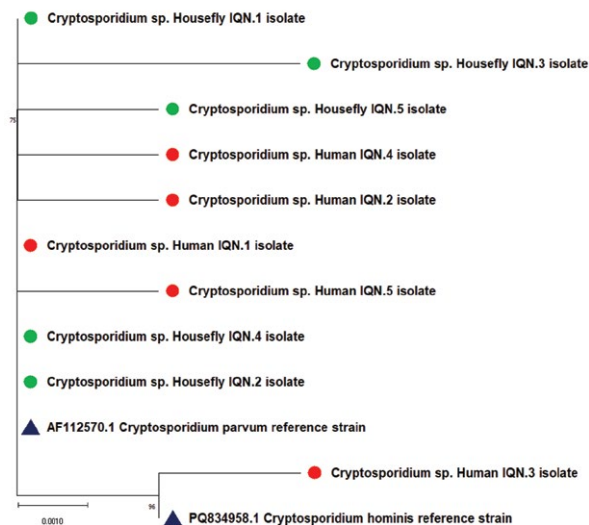


**Fig. 17. Phylogenetic tree analysis of based on small subunit ribosomal RNA gene that used for identification genetic relationship between *Cryptosporidium* sp. of human and housefly isolates. The phylogenetic tree was constructed using the evolutionary history was inferred using the Neighbor-Joining method (MEGA 11 version). The *Cryptosporidium* sp. human isolates were showed closed genetic related to and *Cryptosporidium* sp. housefly isolates and all isolated related to NCBI-BLAST *Cryptosporidium parvum* reference isolate (AF112570.1) except the *Cryptosporidium* sp. human IQN.3 isolate was showed closed genetic related to BLAST *Cryptosporidium hominis* reference isolate (PQ834958.1) at total genetic changes (0.0010%)**

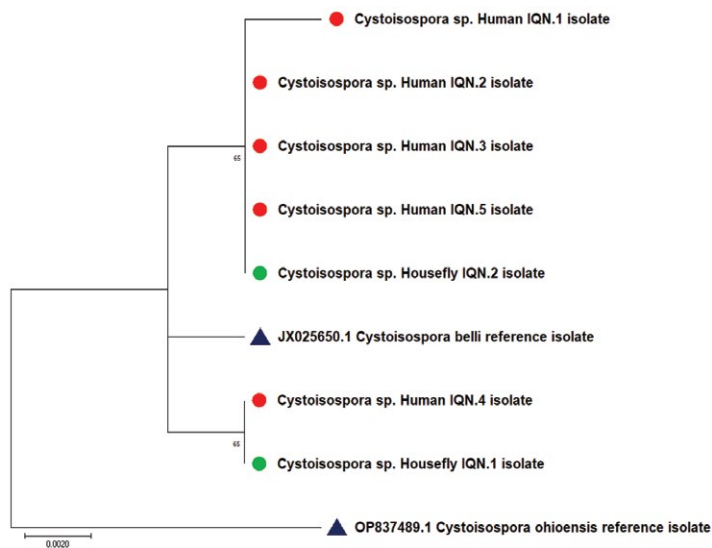


**Fig. 18.** Phylogenetic tree analysis of based on small subunit ribosomal RNA gene that used for identification genetic relationship between *Cystoisospora* sp. of human and housefly isolates. The phylogenetic tree was constructed using the evolutionary history was inferred using the Neighbor-Joining method (MEGA 11 version). The *Cystoisospora* sp. human isolates were showed closed genetic related to and *Cystoisospora* sp. housefly isolates and all isolated related to NCBI-BLAST *Cystoisospora belli* reference isolate (JX025650.1) at total genetic changes (0.0020%)

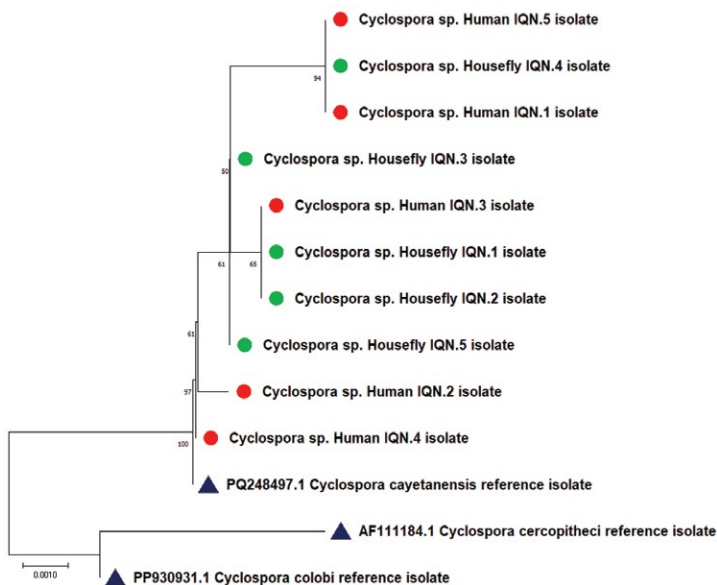


**Fig. 19.** Phylogenetic tree analysis of based on small subunit ribosomal RNA gene that used for identification genetic relationship between *Cyclospora* sp. of human and housefly isolates. The phylogenetic tree was constructed using the evolutionary history was inferred using the Neighbor-Joining method (MEGA 11 version). The *Cyclospora* sp. human isolates were showed closed genetic related to and *Cyclospora* sp. housefly isolates and all isolated related to NCBI-BLAST *Cyclospora cayetanensis* reference isolate (PP930931.1) at total genetic changes (0.0010%)

The Cyclospora sp. human and housefly isolates showed a close genetic relationship to each other and were all closely related to the NCBI-BLAST Cyclospora cayetanensis reference isolate (PP930931.1) with total genetic changes of 0.0010%.

The genetic similarity between human and housefly isolates as identified by NCBI-BLAST analysis.

All human and housefly isolates showed high genetic similarity and close phylogenetic relatedness based on NCBI-BLAST analysis as shown in table 2.

**Table 2**  
**NCBI-BLAST Homology Sequence Identity and Genetic Relationship Analysis of Human and Housefly Isolates**

| Parasite Species      | Isolate Source              | Closest NCBI-BLAST Reference Isolate (Accession No.) | Phylogenetic Branch Relationship                                  | Total Genetic Changes (%) | Sequence Identity (%)* |
|-----------------------|-----------------------------|------------------------------------------------------|-------------------------------------------------------------------|---------------------------|------------------------|
| Entamoeba histolytica | Human                       | AB282658.1                                           | Closed genetic relatedness; all isolates cluster together.        | 0.0020                    | ~99.98                 |
|                       | Housefly                    | AB282658.1                                           | Closed genetic relatedness; all isolates cluster together.        | 0.0020                    | ~99.98                 |
| Giardia lamblia       | Human                       | U09491.1                                             | Closed genetic relatedness; all isolates cluster together.        | 0.0010                    | ~99.99                 |
|                       | Housefly                    | U09491.1                                             | Closed genetic relatedness; all isolates cluster together.        | 0.0010                    | ~99.99                 |
| Microsporidium sp.    | Human                       | OR500624.1                                           | Closed genetic relatedness; all isolates cluster together.        | 0.0010                    | ~99.99                 |
|                       | Housefly                    | OR500624.1                                           | Closed genetic relatedness; all isolates cluster together.        | 0.0010                    | ~99.99                 |
| Cryptosporidium sp.   | Human (Majority) & Housefly | AF112570.1 (C. parvum)                               | Closed genetic relatedness; most isolates cluster with C. parvum. | 0.0010                    | ~99.99                 |
|                       | Human (IQN.3 isolate)       | PQ834958.1 (C. hominis)                              | Exception: IQN.3 clusters separately with C. hominis.             | 0.0010                    | ~99.99                 |
| Cystoisospora sp.     | Human                       | JX025650.1 (C. belli)                                | Closed genetic relatedness; all isolates cluster together.        | 0.0020                    | ~99.98                 |
|                       | Housefly                    | JX025650.1 (C. belli)                                | Closed genetic relatedness; all isolates cluster together.        | 0.0020                    | ~99.98                 |
| Cyclospora sp.        | Human                       | PP930931.1 (C. cayetanensis)                         | Closed genetic relatedness; all isolates cluster together.        | 0.0010                    | ~99.99                 |
|                       | Housefly                    | PP930931.1 (C. cayetanensis)                         | Closed genetic relatedness; all isolates cluster together.        | 0.0010                    | ~99.99                 |

■ DISCUSSION

Molecular detection and phylogenetic tree analysis can be used to directly diagnose gastrointestinal infection in people with parasites [14].

The findings of this phylogenetic investigation, rooted in the robust analytical power of the small subunit ribosomal RNA (SSU rRNA) gene, provide unequivocal molecular evidence that fundamentally advances our understanding of the transmission dynamics for several significant intestinal protozoan parasites, firmly establishing the synanthropic housefly (Musca domestica) not as a passive by stander but as an active and integral



mechanical vector within the local transmission cycle. The strategic selection of the SSU rRNA gene was critical to this conclusion, as its unique molecular architecture – featuring a mosaic of highly conserved regions flanking more variable domains – provided the dual advantage of universal priming sites for reliable PCR amplification across diverse eukaryotic taxa and sufficient sequence polymorphism to discern phylogenetic relationships at the species level, thereby enabling a consistent and comparable methodological framework for all parasites studied, from the amoebozoan *Entamoeba histolytica* to the various apicomplexans [9,10]. The resulting phylogenetic trees and genetic distance calculations revealed a pattern of breathtaking genetic homogeneity, with human and housefly isolates for *Entamoeba histolytica*, *Giardia lamblia*, *Microsporidium* sp., *Cystoisospora* sp., and *Cyclospora* sp. forming tight, monophyletic clades with negligible genetic divergence, evidenced by total genetic changes of only 0.0010% to 0.0020% and corresponding sequence identities of ~99.99% to ~99.98% relative to their reference sequences. This profound genetic stasis, particularly for a marker with a relatively slow evolutionary rate like the SSU rRNA gene, is not indicative of a poor choice of marker but rather serves as a powerful molecular signal of very recent common ancestry and ongoing, active transmission, effectively ruling out the possibility that the parasites from humans and flies represent separate, independently evolving populations and instead pointing towards a unified gene pool that is constantly being mixed [15, 16]. The most parsimonious explanation for this genetic conspecificity is the frequent and direct mechanical transfer of parasitic cysts and oocysts by houseflies, which, due to their synanthropic behavior, act as mobile bridges, passively carrying viable parasites on their exoskeletons and in their digestive tracts from fecally contaminated environments directly to human food and water sources, thereby creating a continuous feedback loop that perpetually mixes the parasite population between the reservoir and the host [5, 17]. This finding elevates the housefly from a mere nuisance to a critical target for public health intervention, necessitating that fly control be integrated into broader WASH (Water, Sanitation, and Hygiene) initiatives to effectively disrupt the transmission of these pathogens [18]. The nuanced exception of *Cryptosporidium* sp., where the majority of isolates clustered with the zoonotic *C. parvum* while a single human isolate (IQN.3) clustered with the anthroponotic *C. hominis*, further enriches our epidemiological understanding by revealing concurrent zoonotic and anthroponotic transmission cycles within the same community, demonstrating that houseflies are primarily involved in the zoonotic *C. parvum* cycle, likely through contact with animal feces, while also illustrating the complex interplay of multiple transmission pathways that can coexist [19, 20].

## ■ CONCLUSION

The high-resolution phylogenetic analysis of the SSU rRNA gene has successfully delineated a transmission landscape wherein the housefly functions as a key mechanical vector, ensuring the dissemination and genetic homogenization of intestinal protozoan parasites between unsanitary environmental reservoirs and human hosts, thereby perpetuating the cycle of infection and highlighting an indispensable target for future disease control strategies.

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