Open Access
Journal of the Faculty of Medicine Baghdad
Print ISSN: 0041-9419, Online ISSN: 2410-8057
DOI: https://doi.org/10.32007/jfacmedbaghdad3209
Research Article

Characterization of Gram-positive Bacteria Isolated from Gallstones of Iraqi Patients

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Received: Sept. 2025
Revised: Dec. 2025
Accepted: Dec. 2025
Published Online: Dec. 2025
Published: Dec. 2025

Abstract:

Background: Gallstone disease is a major global health problem, the prevalence of which depends on metabolic, genetic, and infectious factors. Recent reports point out that Gram-positive bacteria, mainly *Staphylococcus aureus* and *Enterococcus* species, are also involved in the development of gallstones via enzyme action and biofilm formation.

Objectives: To characterize Gram-positive bacteria within gallstones from Iraqi patients, evaluate their biofilm-forming capacity, and analyze the relationship between bacterial colonization, gallstone type, as well as cholesterol levels.

Methods: A total of 100 gallstones were obtained from 100 patients undergoing elective cholecystectomy between October 2024 and March 2025. Stones were aseptically processed for bacterial isolation and identification using selective culture media and the VITEK® 2 Compact System. Serum cholesterol levels were determined by enzymatic colorimetric assay. Biofilm formation was quantified via the 96-well microtiter plate method, and statistical correlations between gallstone type, cholesterol level, and bacterial presence were analyzed. Chi-square assessed the association between gallstone type and bacterial count, while Mann-Whitney U and Kruskal-Wallis tested differences in study variables.

Results: Cholesterol stones were more prevalent than pigment and mixed stones (57%, 40%, 3%), respectively. Bacterial growth was observed in 43% of gallstones, with *Enterococcus* species (31 isolates) predominating over *S. aureus* (12 isolates). Species-level identification revealed *E. faecalis* (n= 16), *E. faecium* (n= 9), and *E. gallinarum* (n= 6), marking the first reported isolation of *E. gallinarum* from gallstones. Cholesterol concentrations were significantly higher in sterile stones (median 235 mg/dl) compared to bacteria-positive stones (173-186 mg/dl). Biofilm analysis showed all *S. aureus* isolates as strong producers, whereas *E. faecalis* exhibited predominantly moderate-to-strong formation, while *E. faecium* and *E. gallinarum* displayed weaker capacities.

Conclusion: There is a significant interplay between microbial colonization and gallstone composition. Strong biofilm-producing Gram-positive bacteria, particularly *S. aureus* and *E. faecalis*, may facilitate stone maturation and persistence. The novel isolation of *E. gallinarum* expands the spectrum of biliary microbiota.

Keywords: Biofilm; Cholesterol; *Enterococcus*; Gallstone disease; *Staphylococcus aureus.*

Introduction

Gallstone disease (GSD) is a common digestive disease that has a great impact on health, economy, and society globally. The prevalence of GSD in Iraq is 20.5%, and a greater proportion of females (28.4%) than males (12.4%) were reported to be affected (1). Risk factors for gallstone formation include obesity, rapid weight reduction, and bariatric surgery (2). On a global scale, the prevalence of GSD is around 10% in adults from Europe and North America, but it ranges from 3% to 15% in Asia-Pacific areas (3). Gallstones are biochemically categorized as cholesterol, black pigment, and brown pigment stones. Cholesterol stones are more common among Western and urbanized dwellers, and they have been associated with metabolic risk factors such as obesity, hormone replacement therapy, and rapid weight loss. In contrast, pigment stones are more commonly related to chronic biliary tract infection or hemolysis (4, 5). The multifaceted etiology of GSD involves genetic susceptibility, hypersecretion of hepatic cholesterol, abnormal composition of bile, and gallbladder hypomotility (6). Newer studies suggest that microbial colonization may play a role in the pathogenesis of gallstone formation. Bacteria that produce β-glucuronidase and phospholipases in the gut and biliary tree can disturb bile homeostasis, enhance bilirubin deconjugation, and facilitate crystallization (7, 8). Gram-positive organisms, particularly *Enterococcus* spp. and also *Staphylococcus aureus*, have been isolated from gallstones and are known to form biofilms, secrete virulence factors, and adhere to biliary epithelium (9-

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12). Both exhibit strong biofilm formation on biliary surfaces, protecting from bile acids and antimicrobials (13). Their enzymatic arsenal, including β-glucuronidase and phospholipase, disrupts bile stability, accelerates bilirubin deconjugation, and promotes crystallization (14). Biofilms hinder antibiotic penetration and harbor drug-tolerant cells (15). Understanding the Gram-positive bacteria associated with gallstones is essential because these organisms may contribute to stone formation, persistence, and postoperative complications. Characterizing the isolates in Iraqi patients provides region-specific insight into microbial profiles that may influence diagnosis, treatment, and prevention strategies. This study aimed to characterize Gram-positive bacteria, specifically *Enterococcus* spp. and *S. aureus*, isolated from gallstones in Iraqi patients, evaluate their biofilm-forming abilities, and analyze their relationship with gallstone type and cholesterol content.

Patients, materials, and methods

Collection of specimens: One hundred gallstone samples were included from adult patients (age ≥18 years) who underwent an elective laparoscopic cholecystectomy for symptomatic gallstone disease in the Surgical Unit of Baghdad Teaching Hospital, the Medical City Complex and Al-Yarmouk Teaching Hospital in Baghdad, from October 2024 to March 2025. Patients younger than 18 years of age, pregnant patients, patients diagnosed with acute cholecystitis, cholangitis, or biliary pancreatitis at the time of operation, and those who received antibiotic therapy within 14 days before the surgery were excluded from the study. The gallstones were aseptically removed postoperatively, identified, and transported in sterile conditions to the microbiology laboratory for further processing. Ethical approval for the study was granted by the Ethics Committee at the College of Science/ University of Baghdad (Approval No. CSEC/0225/0021).

Serum cholesterol assessment in gallstone patients: Serum total cholesterol was measured by an enzymatic colorimetric method, CHOD-PAP. Serum samples were collected and processed by centrifugation at 3000 revolutions per minute for 10 minutes, then analyzed using a semi-automated clinical chemistry analyzer in accordance with the manufacturer's instructions (Biosystems diagnostic kits). Cholesterol levels were measured in mg/dl.

Bacterial isolation and identification: Eliminating possible external contamination, gallstones were surface-sterilized by immersion in 70% ethanol for five minutes. After removal from the ethanol, each stone was thoroughly crushed into a fine powder. A small portion of the pulverized material was then inoculated into brain heart infusion (BHI) broth to promote the growth of potential endogenous bacteria. Following aerobic incubation at 37°C for 24 hours, a loopful of the enriched culture was aseptically streaked onto a panel of differential and selective media, including blood agar, mannitol salt agar. Bile Esculin Azide Agar supplemented with sodium azide (commonly referred to as Pfizer agar) and HiCrome™ UTI agarfor bacterial isolation and presumptive identification. All culture media used in this study were supplied by HiMedia Laboratories (India). ensuring standardized and quality-controlled microbiological performance (16-18).

Identification of bacterial isolates by using the VITEK® 2 compact system: Bacterial identification was performed using the VITEK® 2 Compact system in accordance with the manufacturer's protocols. Initially, selected colonies from overnight culture were suspended in sterile saline to achieve a turbidity equivalent to 0.5 McFarland standard, ensuring standardized inoculum density. The prepared suspension was then aspirated into a sterile reagent cassette under controlled negative pressure. Following sample loading, the cassette was incubated within the VITEK® 2 instrument for approximately 6 to 8 hours to allow for comprehensive biochemical profiling. Upon completion, the system's integrated software automatically analyzed the biochemical reactions and interpreted the data using the ID-GP card for the identification of Gram-positive bacteria.

Biofilm formation assay: Bacterial biofilm was evaluated by the 96-well microtiter plate method. Bacterial suspensions adjusted to 0.5 McFarland standard were deposited in tryptone soy broth with 1% sucrose at 37°C for 24 h. Wells were rinsed fixed with methanol, stained and washed with crystal violet (0.1%), and decolorized by the use of ethanol (200 µl). The optical density was read at 570 nm, and biofilm formation was classified according to OD cut-off values as a non, weak, moderate, or strong producer (19,20).

Statistical analysis: The Chi-squared test was utilized to assess the relationship between the type of gallstone and the number of bacteria identified from the stones. The Mann-Whitney U test ($P < 0.0001$) and the Kruskal-Walli's test ($P < 0.0001$) was utilized to assess differences in nonparametric variables among study groups. A P-value ≤ 0.05 was considered statistically significant. Statistical analyses were performed with GraphPad Prism version 9.5.

Results

Gallstone type distribution: The analysis of gallstone indicated that cholesterol stones were the most common, comprising 57% of the cases (n=57), followed by pigmented stones at 40% (n=40). Mixed stones constituted the minimal proportion, accounting for only 3% (n=3), as illustrated in (Figure1 A, B and C), respectively. Comparison of prevalence revealed no significant difference between cholesterol and pigment stones ($P > 0.05$), whereas both cholesterol and

pigment stones were significantly more prevalent than mixed stones ($P < 0.0001$), as illustrated in (Figure 1D).

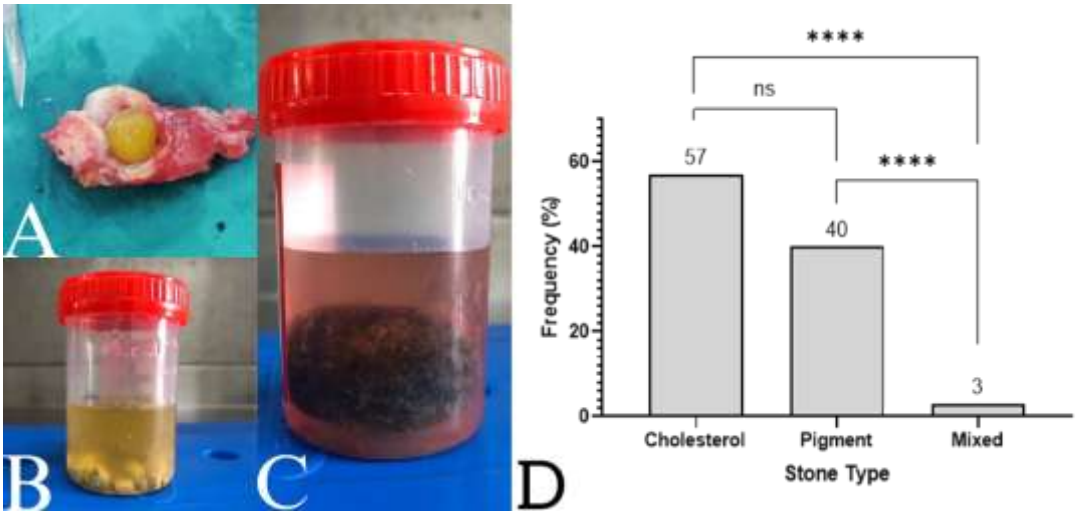

Figure 1: Distribution of gallstone. A) Cholesterol stones. B) Mixed stones. C) Pigment stones. D) Distribution of gallstone types in the present study.

Note: The four asterisks indicate levels of statistical significance "*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$".

Serum cholesterol profiles among patients with gallstones: The present study revealed that 60% of patients (n= 60) exhibited elevated serum cholesterol levels. of these 36% (n= 36) were classified as having borderline high cholesterol (200-239 mg/dl) whereas 24% (n= 24) exhibited high cholesterol levels (> 240 mg/dl). the remaining 40% (n= 40) demonstrated desirable cholesterol level (< 200 mg/dl).

Isolation and characterization

After processing the 100 specimens, 43 tubes showed bacterial growth, which is a positive rate of 43%. To selectively isolate these broths, they were sub-cultured onto mannitol salt agar and Pfizer agar. On mannitol salt agar, there were smooth, golden-yellow colonies with a round shape, which meant that acid was being made. The treated mannitol salt agar transformed from red to yellow among the 12 mannitol salt agar plates that were positive for *S. aureus* (12%). *Enterococcus* spp. produced diminutive grey-blackening esculin hydrolysis halos in 31 Pfizer agar plates (31%). these 43 culture-positive gallstones, mixed-type stones were discovered to be the only ones harboring *Enterococcus* spp. only and no other bacterial genus. Of the remaining 40 positive samples, *Staphylococcus aureus* was retrieved from 12 stones, and *Enterococcus* spp. was retrieved from the other 28. This distribution demonstrates the preponderance of enterococci species in most colonized stones and that *S. aureus* was limited to a subset of non-mixed stones. These identifications were confirmed with consistent coloration on HiCrome™ UTI agar, S. aureus as golden-yellow and *Enterococcus* spp. as turquoise-blue. Post-culture on 5% blood agar, β-hemolysis was observed in 8/12 S. aureus isolates (66.7%) and α-hemolysis in 10/31 *Enterococcus* isolates (32.3%), which is indicative of complete and partial hemolytic activity.

Accurate identification of clinical isolates using the VITEK® 2 compact system: Out of the total bacterial isolates obtained from gallstone specimens, *S. aureus* was identified in 12 samples out of 43 sample (27.9%). Additionally, members of the genus *Enterococcus* were detected in 31 sample out of 43 samples (72%), as confirmed by conventional culture on selective media and subsequently verified using the VITEK identification system. The VITEK results provided species-level resolution, allowing clear differentiation among *Enterococcus* spp.: *Enterococcus faecalis* (*E. faecalis*) was the most prevalent (n=16), followed by *Enterococcus faecium* (*E. faecium*) (n=9), and *Enterococcus gallinarum* (*E. gallinarum*) (n=6). This automated identification not only reinforced the colony morphology and biochemical profile observed on chromogenic and bile esculin agar but also facilitated accurate discrimination between closely related Enterococcal species.

Correlation between cholesterol levels and bacterial presence in gallstones: The present study investigated the correlation between cholesterol levels and bacterial colonization in gallstones. Figure 2 illustrats a comparison of cholesterol levels between gallstones exhibiting bacterial growth and those lacking it. The findings revealed a statistically significant disparity in cholesterol content, with stones devoid of bacterial growth displaying substantially elevated cholesterol levels ($P < 0.0001$). Data were presented as medians accompanied by interquartile ranges, emphasizing the

skewed distribution. The findings indicate a potential negative correlation between cholesterol levels and microbial colonization in gallstones.

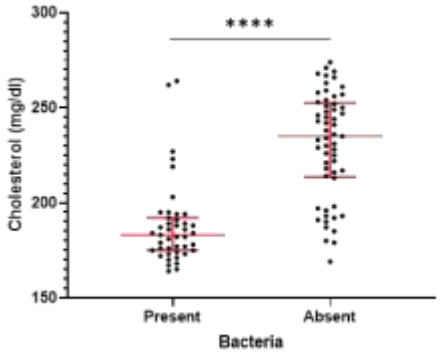

Figure 2: Cholesterol level according to presence or absence of bacteria isolated from gallstones.

Cholesterol levels in gallstones based on bacterial species: A significant difference in cholesterol levels was detected in gallstones infected with several bacterial species (Kruskal-Walli's test, *P* <0.0001). Data is presented as median ± interquartile range. Gallstones absent of bacterial development exhibited the greatest median cholesterol level (235 mg/dl), whereas stones with bacterial presence demonstrated much lower values, ranging from 173 to 186.5 mg/dL. Among the bacterial groupings, *E. faecium*, *E. faecalis*, *E. gallinarum*, and *S. aureus* have shown a correlation with reduced cholesterol levels in comparison to sterile stones. The data indicate that bacteria may be associated with the formation of gallstones, as illustrated in (Figure 3).

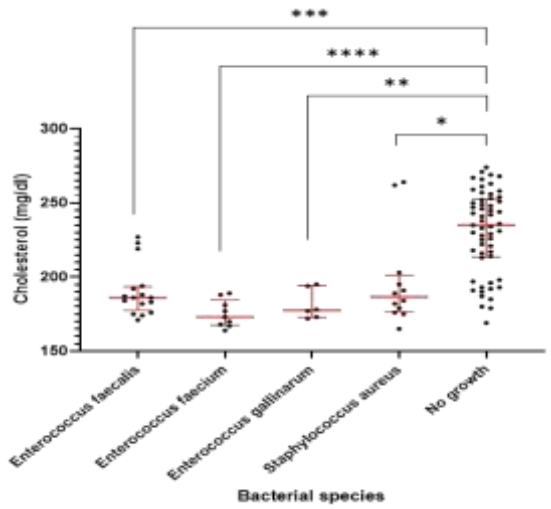

Figure 3: Cholesterol level distributed according to bacterial species isolated from the gall stone

Biofilm formation: In the present study, *E. faecalis* isolates exhibited a notable capacity for biofilm formation, with 30% classified as strong biofilm producers and 68% as moderate. In contrast, *E. faecium* isolates demonstrated moderate biofilm formation in 44% of the cases, while the remaining 56% were weak producers. All *E. gallinarum* isolates were categorized as weak biofilm formers. In comparison, *S. aureus* isolates uniformly displayed strong biofilm-forming ability, with 100% falling into the strong category. These findings are visually represented in Figure 4.

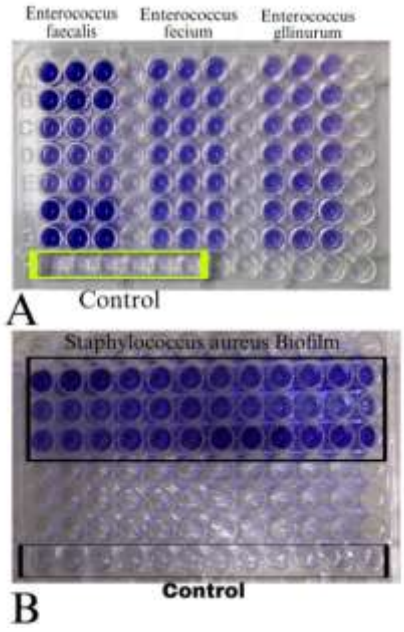

Figure 4: Biofilm formation using the 96-well U-shaped microtiter plate method. A) Represents *Enterococcus* isolates, while B) Corresponds to *S. aureus* isolates**.**

Discussion

In the present study, cholesterol gallstones accounted for 57% of cases, pigment stones (40%) and mixed stones (3%). The observations from international studies including United States, documented a predominance of mixed stones (80%) compared to pigment and cholesterol stones (10%) (21). in Nepal, reported a comparable ratio (44.74% cholesterol) (22). Western populations indicate a higher cholesterol stone prevalence, typically around 75% of cases (23). The elevated proportion associated with metabolic disorders such as dyslipidemia, obesity, and type 2 diabetes mellitus (24). Pigment stone, however, are more common in Middle Eastern and Southeast Asian populations, which may be attributed to disparity in prevalence of hemolysis along with parasitic infestation or dietary habits (25). Microbiological analysis in this study was compared to that of a Chinese study.. Notably, *E. faecalis* was among the most frequent isolates. examined 9,939 patients and found *E. coli*, *K. pneumoniae*, and *E. faecalis* to be the most prevalent isolates (3,033, 1,287, and 1,264 cases, respectively)

(26) previously published data from Iraqi populations recorded *S. aureus* in only 11.77% of gallstones (27). and others describing *E. faecalis* and *E. faecium* as frequent co-isolates in cholangitis cases (28). No global or local studies have reported the isolation of *E. gallinarum* from gallstones, making its identification in the present study a novel and pioneering finding. An inverse relationship was noted between bacterial colonization and cholesterol concentration within gallstones. Sterile stones exhibited significantly higher cholesterol content (median ~235 mg/dl) compared with bacteria-positive stones (173-186 mg/dl). This pattern supports prior metagenomic studies demonstrating that bacteria are more commonly detected in pigmented and mixed stones than in pure cholesterol stones. reported bacterial presence in the majority of pigment stones, whereas only a minority of cholesterol stones exhibited bacterial colonization (29). Such findings suggest that pigment stones often originate in the setting of biliary infection and stasis, with bacterial enzymes (e.g., β-glucuronidase) contributing to bilirubin precipitation. Our results support this hypothesis, indicating that cholesterol-rich stones are more likely to form in sterile bile due to metabolic supersaturation, whereas pigment stones are more strongly linked to microbial activity and bile biofilm development (29,30). Biofilm formation capacity varied among our isolates. All *S. aureus* strains were strong biofilm producers, while *E. faecalis* displayed predominantly moderate-to-strong biofilm formation (30% strong, 68% moderate). *E. faecium* exhibited weaker biofilm potential, and *E. gallinarum* was uniformly weak. These species-specific trends are consistent with known microbial behaviors; *S. aureus* is well-recognized for strong biofilm formation on host tissues and medical devices, while *E. faecalis* often forms substantial biofilm in clinical infections (31,32). The biofilm capabilities of these bacteria are of clinical significance, that biofilm-producing organisms are frequently associated with gallstone pathogenesis (11).

Limitations

The limitation of the present study was that only Gram-positive bacteria were investigated; the contribution to pigment stone formation made by Gram-negative microorganisms may have been underestimated. Additionally, the study was performed in two centers in Baghdad; therefore, findings cannot be generalized. Further large metagenomic studies in different populations would be required to describe comprehensively the total biliary microbiota spectrum of gallstone disease.

Conclusion

The present study demonstrated a correlation between gallstone type and bacterial colonization, as well as the influence of elevated cholesterol levels on bacterial growth within gallstones. Additionally, the study reported, for the first time, the isolation of *E. gallinarum* from gallstones.

Authors' declaration:

We confirm that all the Figures and Tables in the manuscript belong to the current study. Besides, the Figures and images, which do not belong to the current study, have been given permission for re-publication attached to the manuscript. The authors signed on ethical considerations. Approval-Ethical Clearance: The project was approved by the local ethical committee in (the College of Science, University of Baghdad, under reference number CSEC/0225/0021, dated February 11, 2025)**.**

Conflict of Interest: None

Funding: No specific grant from a public, private, or nonprofit funding organization was obtained for this study.

Data availability: Upon reasonable request, the corresponding author will make the data sets generated and/or analyzed during the current work available.

Authors' contributions

Study conception & design: Sameer A. Alash. Literature search: Sameer A. Alash , Ahmed M. Naji. Data acquisition & Data analysis: Sameer A. Alash , Ahmed M. Naji. Manuscript preparation, editing & review: Sameer A. Alash, Ahmed M. Naji.

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How to Cite this Article
Naji AM, Alash SA. Characterization of Gram-Positive bacteria in Gallstone among Iraqi Patients. J Fac Med Baghdad [Internet].2025 Dec. Available from: https://iqjmc.uobaghdad.edu.iq/index.php/19JFacMedBaghdad36/article/view/3209

التوصيف الميكروبي للبكتيريا موجبة الغرام في حصى المرارة لدى المرضى العراقيين

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الخلاصة:
الخلفية :تعد حصى المرارة من الاضطرابات الصحية العالمية المهمة، وتتأثر نسب انتشارها بعوامل استقلابية وجينية. وتشير الأدلة الحديثة إلى أن البكتيريا الموجبة لصبغة غرام، خصوصا المكورات العنقودية الذهبية وأنواع المعوية، تسهم في نشوء الحصوات عبر إفراز الإنزيمات وتكوين الأغشية الحيوية.
الأهداف :تهدف هذه الدراسة إلى توصيف البكتيريا موجبة الغرام المعزولة من حصى المرارة لدى المرضى العراقيين، وتقييم قدرتها على تكوين الأغشية الحيوية، وتحليل العلاقة بين الاستعمار الجرثومي وأنواع الحصى ومستويات الكوليسترول.
المنهجية :جمعت مائة عينة من حصى المرارة من مرضى خضعوا لاستئصال المرارة الاختياري في بغداد خلال المدة من تشرين الأول 2024 إلى آذار 2025. جرى التعامل مع العينات بطريقة عقيمة، وعرفت البكتيريا باستخدام الأوساط الانتقائية ونظام VITEK® 2 Compact قيم تكوين الغشاء الحيوي باستخدام طريقة الأطباق الدقيقة كما جرى قياس مستويات الكوليسترول في المصل بالطرق الأنزيمية.
النتائج :شكلت الحصوات الكوليسترولية 57%، تلتها الحصوات الصبغية 40% والمختلطة 3%. لوحظ نمو بكتيري في 43% من العينات، حيث غلبت عزلات المعوية (31 عزلة) على المكورات العنقودية الذهبية (12 عزلة). أظهر التشخيص النوعي وجود المعوية البرازية (16 عزلة) والمعوية البرازية (9 عزلات) والمعوية الدجاجية (6 عزلات)، مع تسجيل أول عزل المعوية الدجاجية من حصى المرارة. كان تركيز الكوليسترول أعلى بشكل ملحوظ في الحصوات المعقمة (235 ملغم/دل) مقارنة بالحاوية على بكتيريا (173–186 ملغم/دل). أظهرت عزلات المكورات العنقودية الذهبية قدرة قوية على تكوين الغشاء الحيوي، بينما أبدى المعوية البرازية قدرة متوسطة إلى قوية، في حين كان المعوية المنيومية والمعوية الدجاجية أضعف.
الاستنتاج :وجود تداخل مهم بين الاستعمار الجرثومي وتركيب حصى المرارة. وتشير النتائج إلى أن البكتيريا موجبة الغرام ذات القدرة العالية على تكوين الأغشية الحيوية، وبالأخص المكورات العنقودية الذهبية والمعوية البرازية، قد تسهم في نضوج الحصاة واستمرارها. كما يعد الكشف الأولي عن المعوية الدجاجية توسعا في الطيف الميكروبي الصفراوي.
الكلمات المفتاحية :حصى المرارة؛ المكورات العنقودية الذهبية؛ المعوية؛ الغشاء الحيوي؛ الكوليسترول.