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Prevalence of Some Virulence Genes Among *Enterococcus faecalis* Bacteria Isolated From Urinary Tract Infection Patients

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Abstract: The human gastrointestinal system, vaginal tracts, and oral cavity are home to the common gram-positive, facultative anaerobic bacteria known as Enterococcus species. When recovered from clinical specimens of hospitalized patients, the most prevalent species of the broad genus Enterococcus bacteria—which contains over 50 species—are E. faecalis and E. faecium. This study aim to isolation and identifaction of *E.faecalis* depended on phenotype and molecular method, the phenotypic patterns using traditional, and then diagnosed it based on the genotypes and using specialized primers for 16srRNA and D-Ala:D-Ala ligase genes using polymerase chain reaction as well as molecular detection of *SprE* and *PAI* virulence genes and carrying out antibiotic susceptibility test . Between June and September 2024 , one hundred and forty five urine samples were collected from patients of different ages (from 26 to 60 years old) and of both sexes (males number 81 and female number 64) that suffering from UTIs that avoid from taking antibiotics for at least a week, Mid-stream urine samples were collected in sterilized screw-cap containers. To prevent any potential contamination, the specimens were gathered in the correct manner. Three Babylonian hospitals—Al-Hilla Teaching Hospital, Marjan Hospital, and Imam Al-Sadq Hospital—were the sites of sample collection, The result showed presence (*16srRNA and ddl*) genes in all isolation bacteria at 100% and *sprE*, *PAI* genes were performed by Conventional PCR amplification was 35.71 % , 21.42 % respectively . Antibiotic sensitivity tests were performed using Vancomycin, linezolid, ampicillin, chloramphenicol, nitrofurantoin and ciprofloxacin ,the result of antibiotic sensitivity tests showed very high resistant (96.42%) to ampicillin, whereas a few isolates exhibited strong ciprofloxacin resistance (85.71%) and vancomycin (82.14%), while some isolates showed moderate resistance (46.42%) to nitrofurantoin, and (57.14%) to chloramphenicol, and show low resistance to linezolid (10.71%).

Key words: *E.faecalis*, *sprE*, *PAI*, UTIs.

Introduction /

A common commensal bacteria in the human digestive system is Enterococcus faecalis. However, certain strains are probiotic, while others are associated with opportunistic infections, making it challenging to differentiate between these strains using conventional taxonomic methods [1]. This bacterium is observed single, under a microscope, it appears in pairs or short chains; facultative anaerobic, non-motile, and usually non-hemolytic, colonies on blood agar with a diameter of 1-2 mm. *Enterococci* generate lactic acid through fermentation without producing gasEnterococcus has negative catalase and oxidase reactions. A 6.5% saline medium with a pH range of 4.8 to 9.6 and an ideal temperature range of 35 to 37°C is suitable for its growth [2]. It possesses the capability to generate certain virulence factors [3]. *Enterococcus faecalis* can induce many nosocomial infections, including urinary tract infections (*UTIs*). Its pathogenicity is influenced by multiple virulence factors [4]. Although most *Enterococci* are anaerobic, several species require oxygen for survival. Enterococci cannot digest cellulose or pectin, and their metabolic route does not include nitrate reduction. They are a common, possibly dangerous species that have phenotypic tolerance or resistance to a variety of physical treatments and disinfectants [5]. *Enterococci* are among the primary contributors to severe healthcare-associated illnesses. Due to their inherent tolerance to various antibiotics, *Enterococci* can rapidly acquire substantial drug resistance via horizontal gene transfer [6]. Antibiotic resistance influences pathogenicity, making it more difficult to treat enterococcal infections medically because there aren't many effective treatment options [7]. By controlling adhesion and colonization, invasion of host tissues, and the production of poisons and enzymes, virulence factors play a role in disease [8]. The genus *Enterococcus* has a relatively brief history, largely connected to that of other Gram-positive cocci, particularly streptococci. Currently, 55 species of *Enterococci* have been documented based on *16S rDNA* sequences [9]. Numerous virulence factors are associated with the pathogenesis of E. faecalis. Microbial infections release extracellular chemicals called virulence factors, which allow them to get past the host's defenses and cause illness [10].

Material and methods

Sample collection

Between June and September 2024, 145 urine samples were obtained from patients with various age groups and both sex, suffering from UTIs that avoid from taking antibiotics for at least a week, Sterilized screw-cap vials were used to collect urine samples midstream. To prevent any potential contamination, the specimens were gathered in the correct manner. Three Babylonian hospitals—Al-Hilla Teaching Hospital, Marjan Hospital, and Imam Al-Sadq Hospital—were the sites of sample collection. each urine sample was placed in a disposable screw cap container used for collection of urine samples, labeled with sex, number and date of collection.

Isolation and Identification of *E. faecalis*

The VITEK-2 system, chromogenic agar, blood base agar, and bile esculin agar base were used to cultivate each specimen. They were then incubated for

24 hours at 37 °C and examined at the molecular level using a traditional polymerase chain reaction.

Molecular methods

DNA extraction

The DNA was extracted from E. faecalis isolates using the Favorgen® Genomic DNA Extraction Mini Kit (Korea). and utilize gel electrophoresis to determine DNA purity.

Diagnosis of *E.faecalis* using PCR technology

The polymerase chain reaction technique was used to diagnose and identify E. faecalis bacteria and some virulence genes using specific primers prepared according to the manufacturer's instructions, the composition of which includes specific sequences present in the bacterial genetic material. Table (1) describes the primers used for detection of (16srRNA, ddl E. faecalis, sprE, PAI) genes.

Table (1): Primer sequences and product size of the genes used in the study

Target gene	Nucleotide sequence (5'—3')	Product Size/ bp	Reference
16SrRNA	F: GGATTAGATACCCTGGTAGTCC	320	11
	R: TCGTTGCGGGACTTAACCCAAC		
Ddl	F:ATCAAGTACAGTTAGTCTTTATTAG	941	12
	R:ACGATTCAAAGCTAACTGAATCAGT		
SprE	F: GGT AAA CCA ACC AAG TGA ATC	300	13
	R: TTC TTC CGA TTG ACG CAA AA		
Pai	F: GAC GCT CCC TTC TTT TGA C	387	
	Nucleotide sequence (5'—3')		

Optimization of PCR master mix for amplification of (*16srRNA, ddl E.faecalis, SprE, PAI*) gene was accomplished after several trails; thus, the following mixtures were adopted for detect this genes of *E.faecalis* isolate as in Table (2).

Table (2): PCR master mix to detect the genes of *E.faecalis* isolates.

Component	25µL (Final volume)
Masret mix	12.5µl
Forward primer	10 picomols/µl (1 µl)
Reverse primer	10 picomols/µl (1 µl)
DNA	1.5µl
Distill water	9µl

Detection of *ddl E.faecalis, 16srRNA, SprE, PAI* genes by conventional PCR

Optimization of PCR program for amplification of (*16srRNA, ddl E.faecalis, SprE, PAI*) gene was accomplished after several trails; thus, the following program were adopted for *E.faecalis* isolates, as in Table (3), (4), and (5).

Table (3): PCR Program to detect *ddl gene* for *E.faecalis*

No.	Phase	Tm (C°)	Time	No. of cycle
1-	Initial Denaturation	94 C°	5min	1 cycle
2-	Final Denaturation	94 C°	1min	30 cycle
3-	Annealing	54 C°	45 Sec	
4-	Extension	72 C°	1min	
5-	Final Extension	72 C°	5 min	1 cycle

Table (4): PCR program to identify the *16srRNA* gene amplification by conventional PCR.

No.	Phase	Tm (C°)	Time	No. of cycle
1-	Initial Denaturation	94 C°	5min	1 cycle
2-	Final Denaturation	94 C°	1min	30 cycle
3-	Annealing	54 C°	1min	
4-	Extension	72 C°	1min	
5-	Final Extension	72 C°	5 min	1 cycle

Table (5): PCR program to identify the *SprE* and *PAI* genes amplification by conventional PCR.

No.	Phase	Tm (C°)	Time	No. of cycle
1-	Initial Denaturation	94 C°	3min	1 cycle
2-	Final Denaturation	94 C°	30 s	30 cycle
3-	Annealing	54 C°	30 s	
4-	Extension	72 C°	30 s	
5-	Final Extension	72 C°	5min	1 cycle

Antibiotics sensitivity test

A small number of identical bacterial colonies were taken from a new culture on an agar plate after 24 hours incubation 37C° and suspended until the turbidity reached 0.5 McFarland, which corresponded to 1.5×108 CFU/ml.

Bacteria are put on a Mueller-Hinton agar (MHA) plate, then antibiotic disks are added. After allowing the bacteria to develop overnight, transparent media surrounds the six disks (linezolid, ampicillin, ciprofloxacin, nitrofurantoin, vancomycin, chloramphenicol).

Results and discussion

Isolation and identification of *E.faecalis*

The isolated bacteria were determined to be Gram-positive cocci, which had spherical or oval forms and may be observed alone, in pairs, or in brief chains. *Enterococcus faecalis* was isolated based on the cultural characteristics of the colonies, including the shape, size, color, and texture of the colonies. Colonies of *E. faecalis* appeared on bile esculin agar in the form of small, transparent colonies with brown-black halos, and the color of the medium turned black, Bile Esculin Agar is mostly used to distinguish *Enterococcus* from *Streptococcus*. Members of the *Enterococcus* genus may thrive in 40% bile (oxgall) and hydrolyze esculin into glucose and esculetin. Esculetin interacts with ferric ions to form a black complex. indicating the presence of bacterial growth in the medium figure (1A). and On chromogenic agar medium figure (1B), small-sized bacterial colonies with a

transparent blue color appeared depending on the chromogenic substrates present in the medium, where one of the chromogenic substrates is cleaved by the *β-glucosida*se that it possesses *Enterococci*, leading to the formation of blue colonies.

A B

Figure: (1A) Colonies of *E.faecalis* on bile esculin agar after 24 hours of incubation at 37°C appear colonies with brown-black halos on bile esculin agar .

Figure: (1B) Colonies of *E.faecalis* on chromogenic agar after 24 hours of incubation at 37°C appear blue color on chromogenic agar.

Molecular methods for identification of *Enterococcus faecalis* isolate.

The findings of this study revealed that all E. faecalis isolates (100%)had the *ddl*, *16srRNA*, *SprE* and *PAI* genes, with respective product sizes of 941bp, 320bp, 300bp, and 387bp respectivelly (Figures 2,3,4,5).

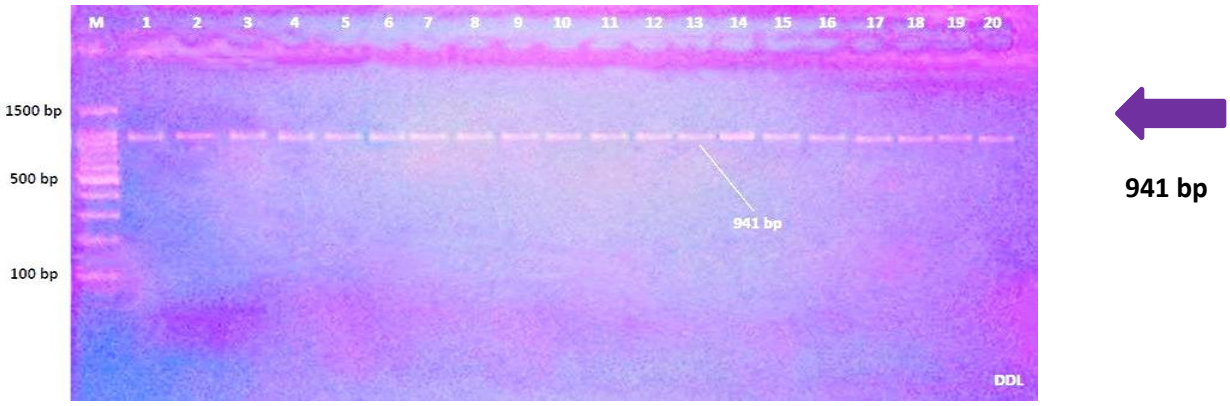

Figure (2): Gel electrophoresis of ddl gene of *Enterococcus faecalis* (from line 1 to line 20) using 1.5% agarose gel electrophoresis (80 volt for 1 hours).

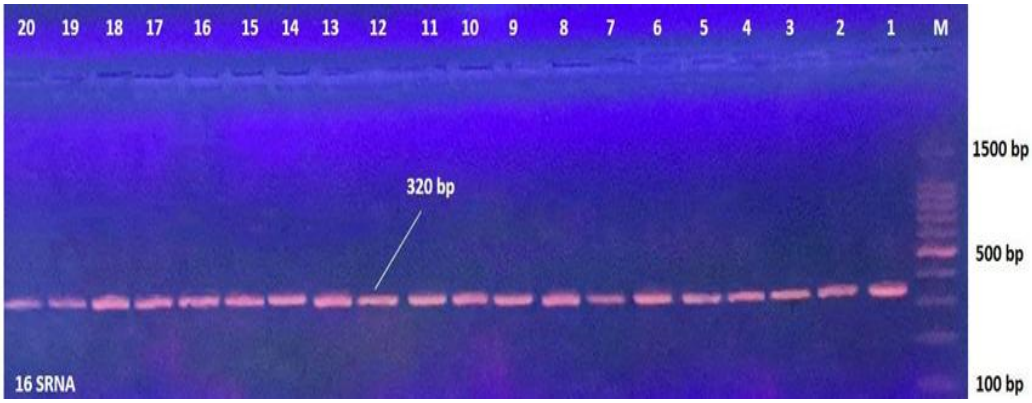

Figure (3): Gel electrophoresis of 16srRNA gene of *Enterococcus faecalis* (from line 1 to line 20) using 1.5% agarose gel electrophoresis (80 volt for 1 hours).

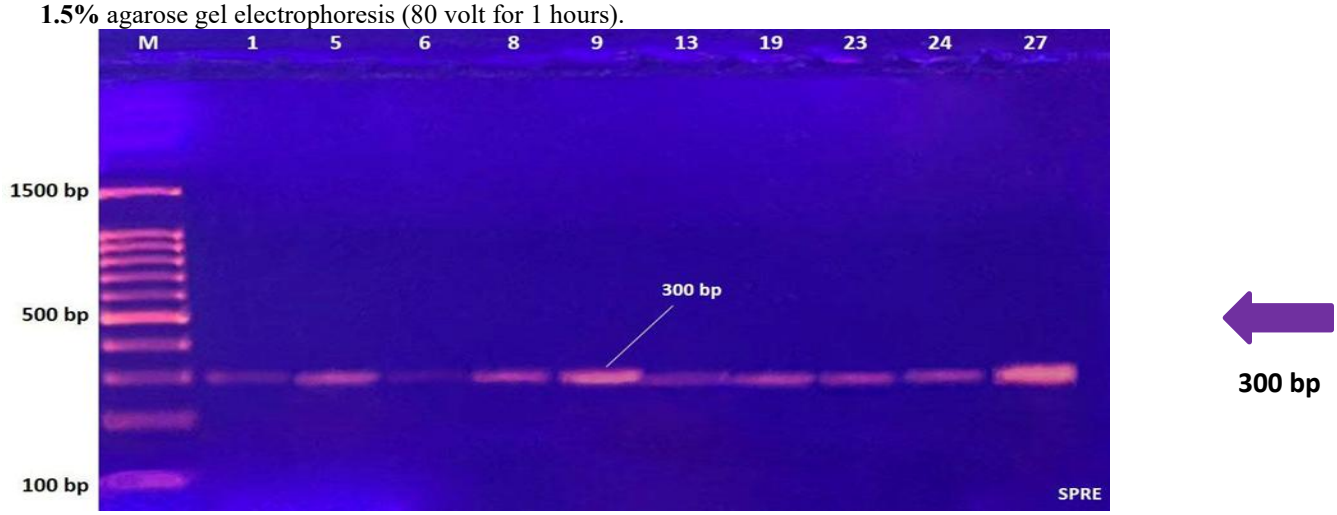

Figure (4): Gel Electrophoresis of *SPRE* (300 bp) Gene using 1.5% Agarose Gel Electrophoresis (80 volt for 1 hours).

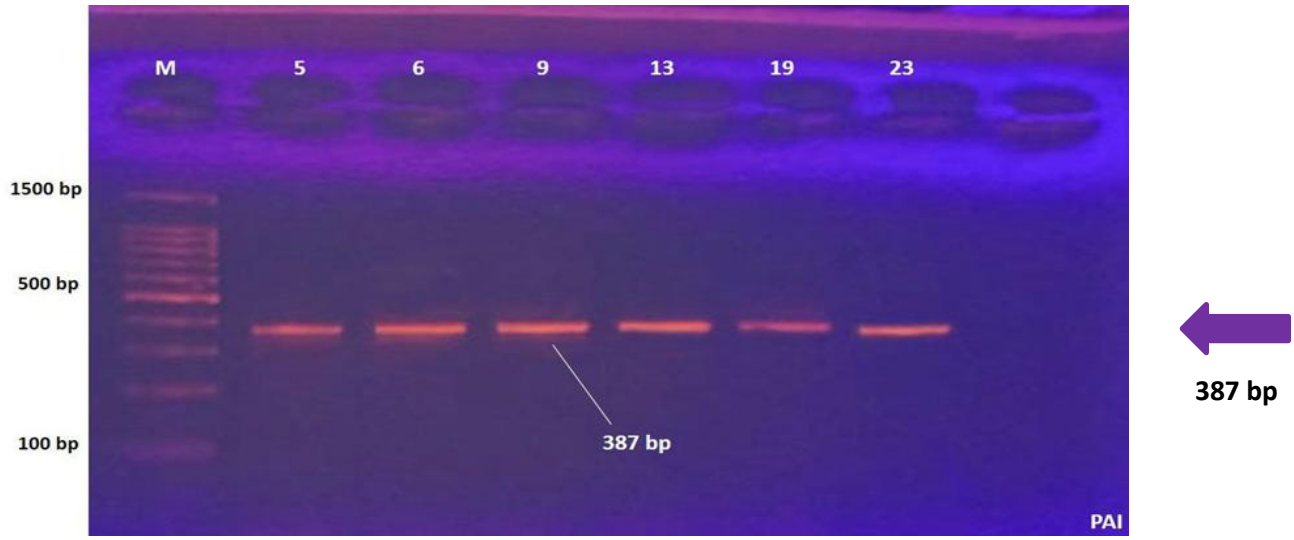

Figure (5): Gel Electrophoresis of *PAI* Gene Using 1.5% Agarose Gel Electrophoresis (80 volt for 1 hours).

The polymerase chain reaction technique was employed to diagnose and identify 28 isolates of *E. faecalis* utilizing specific primers prepared per the manufacturer's instructions (Macrogen), which comprise specific sequences found in the bacterial genetic material targeting the *16S rRNA* and *ddl* genes, And gel electrophoresis was used to assess DNA purity. The results indicated the presence of *16s rRNA* and *ddl* genes in all isolated bacteria at 100%. These findings are consistent with those of Azhar and Hussein [14]. Who used 16S rRNA to identify E. faecalis, similar to the findings reported by Mustafa et al [15]. Using the ddl gene for molecular identification of this bacteria. The SprE gene encodes a serine protease, is located just downstream of gelE, and is co-transcribed with it. This gene produces a 26-kDa serine protease that is secreted and shares similarities with Staphylococcus aureus V8 protease [16]. *SprE* facilitates the adhesion to extracellular matrix proteins, hence augmenting the capacity of *E. faecalis* to develop and reinforce biofilms [17]. The results of the current experiment are fairly comparable to those of Sachit and Bunyan [18]. 27.2% of the population carried the SPRE gene. Enterococcus faecalis has a 153-kb pathogenicity island (PAI) that contains several virulence components, including the Enterococcal surface protein (esp). Laverde Gómez [19]. Many clinical strains of E. faecalis include a significant pathogenicity island that encodes the Enterococcal surface protein (Esp), which is thought to improve biofilm formation and virulence [20]. The current study similar to Al- Hamdani and Tuwaij [21] that show Percentage of *PAI* gene was 23.8%. Based on the results obtained from the current research on *SprE* gene, it appears that these results Mismatched with Hashem and his colleague [4] They found that the percentage reached 87% of all isolates of *E. faecalis*. Also this current study disageerd with Yean [13] that show percentage of *SPRE* gene was 76% and *PAI* gene was 39.9%.

Antimicrobial susceptibility test

Antibiotic sensitivity tests were performed using *Vancomycin*, *linezolid*, *ampicillin*, *chloramphenicol*, *nitrofurantoin* and *ciprofloxacin*. The result of the current study and the Percentage of antibiotics show in Table (6).

Table (6): Percentage result for each antibiotic

Antibiotic	Sensitive (%)	Resistance (%)
Ciprofloxacin	4 (14.28)	24 (85.71)
Nitrofurantoin	15 (53.57)	13 (46.42)
Chloramphenicol	12 (42.85)	16 (57.14)
Vancomycin	5 (17.85)	23 (82.14)
Ampicillin	1 (3.57)	27 (96.42)
Linezolid	25 (89.28)	3 (10.71)

The results of the current study contradicts with studied by Molechan et al. [22] whose result indicate resistance to *ciprofloxacin* (4%) and *chloramphenicol* (10%) in South Africa. Also this study disagreed with Khdir [23] who showed that Nitrofurantoin Sensitivity was 100% in Kurdistan Iraq, and close to results in the same study towards *vancomycin* and *ampicillin* which showed (100%) resistant to bacteria. In another hand disagreed with study by Georges et al. [24] in Kenya which showed that isolates of *E. faecalis*

had no resistance to *vancomycin*. The sensitivity to the *Linezolid* in the present study was relevant to other studies reported by Akpaka et al. [25] in Caribbean countries and Karimi et al. [26] in Iran, of *E. faecalis* isolates who displayed 100%, 98% respectively was sensitivity to *Linezolid*.

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مدى انتشار بعض جينات الضراوة في بكتريا المكورات المعوية البرازية المعزولة من مرضى التهابات المسالك البولية

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وزارة الصحة، بغداد، العراق[1] ، معهد الهندسة الوراثية والتقنيات الاحيائية للدراسات العليا [2] ، جامعة بغداد [3] ، قسم علوم الحياه، كلية العلوم، جامعة بابل [5]

الخلاصة:

تعد المكورات المعوية البرازية هي واحدة من الأمراض البكتيرية الأكثر شيوعا التي تتطلب العلاج بالمضادات الحيوية هي التهابات المسالك البولية. تعد هذه البكتريا إيجابية الجرام، واللاهوائية اختيارية، وتعتبر واحدة من أكثر عدوى المستشفيات شيوعاً، مع ارتفاع معدل الوفيات، وقد تم الإبلاغ عن أن بكتيريا المكورات المعوية البرازية هي السبب الثاني الأكثر شيوعاً لعدوى المسالك البولية، والقدرة على التسبب في التهابات حادة بسبب امتلاكها ترسانة من جينات الفوعة , في الفترة ما بين حزيران 2024 وأيلول 2024، تم الحصول على (145) عينة بول سريرية من المرضى من كلا الجنسين وفئات عمرية مختلفة، تم جمعها من ثلاث مستشفيات في بابل (مستشفى الحلة التعليمي، مستشفى مرجان، مستشفى الامام الصادق)، تمت زراعة جميع العينات السريرية على أجار الاسكولين الصفراوي وأجار الكروموجينيك، ، ومن ثم تم إجراء التشخيص باستخدام نظام Vitek2 ، حيث تم اعتماد النتائج الإيجابية لجميع الاختبارات السابقة حيث تم الحصول على (28) عزلة بكتيرية من المكورات المعوية البرازية ، وتم إجراء اختبار الحساسية للمضادات الحيوية فانكومايسين، لينزوليد، الأمبيسيلين، الكلورامفينيكول، النيتروفورانتوين والسيبروفلوكساسين باستخدام طريقة الانتشار القرصي.حيث أظهرت النتائج أن البكتيريا كانت شديدة المقاومة للأمبيسيلين والسيبروفلوكساسين والفانكومايسين بينما كانت حساسة للغاية للينزوليد. تم استخراج الحمض النووي لجميع العزلات البكتيرية ثم تم استخدام جهاز الفصل الكهربائي لتقدير جودته , وتم إجراء الكشف الجزيئي لبعض الجينات على النحو التالي. تم تأكيد تشخيص البكتيريا باستخدام بادئات تشخيصيه للبكتريا لاثنين من الجينات التشخيصية (*16srRNA* و*ddl*) و التي أعطت نتائج مماثلة لتلك التي تم الحصول عليها باستخدام الطرق السابقة وكذلك الكشف الجزيئي لجينين الفوعة سيرين. البروتياز *SprE* والجزر المرضية *PAI* حيث أظهرت النتائج وجود جين *SprE* بنسبة (35.71%)، بينما كانت نتيجة جين *PAI* كانت (21.42%).