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REVIEW ARTICLE

The Molecular Identification of Agg and Hyl Genes of Enterococcus Faecalis Isolated from Different Clinical Samples

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Abstract:

Background: Enterococcus faecalis is a gram-positive, facultatively anaerobic, catalase-negative, oxidase-negative, non-spore-forming bacteria belonging to the genus Enterococcus frequently lives in the intestines of healthy people. It typically appears in pairs or chains of various lengths and its Microorganisms with mesophilic characteristics grow for ten – fifty four ◻ C and optimal temp comprised between 30 ◻ C and 35◻ C. They are also able to grow in a huge range of pH from 4.4 and 9.6 and in hyper salty media with 6.5% NaCl.

Objective: This study aims to molecularly detect some genes that are responsible for virulence factors (agg and hyl) by PCR technique.

Materials and Methods: 130 clinical specimens were acquired from patients who were admitted to the Maternity and Pediatric Teaching Hospital in Al-Diwaniyah City during the period from November 2022 to March 2023. Each specimen was tested for culture on multiple types of media (Blood agar, MacConkey, Bile Esculin agar, and Chromogenic ager) followed by biochemical testing (Catalase test and Oxidase test). After cultivation, the samples were identified by using the VITEK-2 compact system. The isolates were investigated genotypically for harboring virulence factors genes that include agg and hyl by molecular methods PCR.

Results: The VITEK-2 compact system revealed that only 12 isolates were identified as Enterococcus faecalis. The results showed the percentages of genes were (75%) and (41.6%) for agg and hyl respectively.

Conclusion: It has been found that the existence of several virulent factor genes in E. faecalis, such as egg and hyl, boosts this pathogen's pathogenicity, and amongst E. faecalis isolates, the agg gene exhibited a greater proportion, confirming the critical need to develop control the infection associated with the development and increase pathogenicity of this pathogen.

Keywords:

Enterococcus faecalis, virulence factors, PCR technique.

Introduction:

Enterococcus faecalis is one of the top three causes of nosocomial infections or hospital-acquired infections, and it accounts for around 80% of infections in people [1]. It is the most frequently isolated species from infections, causing a number of conditions including meningitis, endocarditis, bacteremia, and urinary tract infections (UTIs) [2].

E. faecalis may also produce virulence factors that contribute to their pathogenicity. It can accumulate multiple genetic elements encoding virulence factors and antibiotic resistance genes, as well as develop biofilm, which plays an important role in infections [3].

Aggregation substance, encoded by the agg gene, (AS) is a plasmid-encoded bacterial adhesion that responds to pheromones and efficiently connects donor and recipient bacteria to facilitate plasmid exchange. While the donor cell expresses the AS, the recipient cell's surface must also express the "binding substance," which is the corresponding ligand for AS that is encoded on the chromosome [4]. AS has also been shown to facilitate intracellular survival time in macrophages by promoting opsonin independent adhesion to and phagocytosis of E. faecalis by human macrophages. Human neutrophils and macrophage reactions to E. faecalis that express AS therefore appear to differ; however, it may be deduced that AS acts as a protective factor for the bacteria against the host defensive systems [5] .

The hyl gene encoding the hyaluronidase enzyme causes tissue damage by degrading the hyaluronic acid. Furthermore, disaccharides formed as a result of hyaluronic acid degradation may be a source of food for bacteria [6]. Hyaluronidase the

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spreading factor is primarily a degradative enzyme that affects hyaluronic acid; as a result of its action, it is linked to tissue injury. It is prevalent in nature and can be found in mammalian cells [7]. Hyaluronidase, a component of E. faecalis, aids in the dissemination of the bacteria and their toxins to the host tissue. The movement of more bacteria from the root canal to the periapical lesions will continue to be encouraged. In addition, hyaluronidase stimulates the production of toxins by other bacteria, which worsens the damage and inflammation. This circumstance is ideal for the growth of E. faecalis [8].

The Materials and Methods

The current study was conducted in the Maternity and Pediatric Teaching Hospital in AL- Diwaniyah City, during the period from November 2022 to March 2023. 130 samples were collected from two sites of infection (mid-stream urine and high vaginal swab) and immediately transported to the lab after being marked with the patient's information.

Midstream urine samples (95 samples) were collected in sterile screw-cap containers (4 - 5 ml) from patients with urinary tract infections and immediately subjected to aerobic culture on Blood agar and MacConkey agar medium to the general hospital laboratory.

High vaginal swabs were taken from women patients suffering from abnormal vaginal discharge, itching, burning, and lower abdominal pain. Gynecologists obtained swabs from 35 women. The target vaginal area smear had been observed by the gynecologists using a sterile un-lubricated speculum. vaginal sterile cotton-tipped swabs with Amies medium were used for culture. E. faecalis was identified according to its morphology with Gram stain and chains appearance for primary isolation after being cultured on Blood agar, MacConkey agar, Bile esculin agar, and m-EI chromogenic agar and incubated for 24 to 48 hrs. at 37□, followed by biochemical testing (Catalase test and Oxidase test, Growth at 42□). Moreover, as a final confirmation step out of 130 samples, only 16 samples were subjected to the VITEK-2 compact automated system.

Extraction of the DNA was carried out by E. faecalis isolates employing a commercial kit, and it was conducted depending on manufacturer instructions (TRANS, China). The primer sequences used to amplify genes encoding agg and hyl genes are listed in Table (1). The primers were resuspended by dissolving the lyophilized product of primers and preparing the stock primer by adding PCR water (free nuclease water) according to the instructions of the manufacturer as in Table (2). The PCR tubes were positioned in the thermal cycler and the conditions of the correct PCR cycling software parameters were changed according to each primer as in Table (3). The PCR products were analyzed according to the manufacturer instructions by agarose gel electrophoresis.

Statistical Analysis

A chi-square test (X2) was used for statistical analysis to evaluate the variables independence, alongside the IBM Statistical Package for Social Sciences (SPSS) software for statistics, Ver 32. Statistics were deemed significant at values lower than or equal to 0.05 [9].

Results

Out of 130 samples, 25 (19.2 %) isolates gave negative results for growth, 105 (80.7%) isolates gave positive results for growth, out of 105 positive culturing samples, and only 16 isolates (12.3%) were suspected to be E. faecalis

E. faecalis was identified according to biochemical and its morphology with Gram stain and chains appearance or apairs for primary isolation Table (4) after being cultured on Blood agar, MacConkey agar, Bile Esculin agar, m-EI chromogenic agar and incubating for 24 to 48 hrs at 37□.

Colony morphology and culture characteristics were observed macroscopically, most of the isolates produce α- hemolysis on Blood agar while the others do not produce hemolysis. All the isolates that grew on MacConkey agar appeared as lactose fermenters with deep pink-magenta colored colonies. On Bile Esculin agar all the isolates converted the color of media into black coffee brown (due to the hydrolysis of esculin). The identification of E. faecalis was performed by direct inoculation on m-EI chromogenic agar (recovery and distinction of E. faecalis), the isolates were given blue colonies.

As a final confirmation step, all the16 samples were subjected to a VITEK-2 compact automated system to confirm the finding. Table 5 illustrates the percentage of E. faecalis isolates from total samples, where only 12 (75%) isolates (according to the VITEK) were identified as E. faecalis. The percentage of E. faecalis isolated from midstream urine was (80%) while it was (66.6%) from high vaginal swabs. The findings of the current investigation revealed that 8 (80%) of the isolates of E. faecalis were found in urine samples and 4 (66.6%) were found in high vaginal swabs.

In all the 12 isolates of E. faecalis, the gene for aggregation Substances (agg) was positive in (75%) of the isolates, where each band showed up in the gene anticipated size (1553bp) for all the positive isolates as in Figure 2. PCR assays also showed that the gene for hyaluronidase (hyl) was positive in 5 (41.66%) of isolates, where each band showed up in the gene anticipated size of the gene (276bp) for all the positive isolates as in Figure 2.

Discussion

Although E. faecalis can live peacefully in the GIT of the human, when it grows unchecked in the gut or gains access to extra-intestinal sites, it can transform into an opportunistic pathogen. E. faecalis overgrowth in the GIT is often associated with antibiotic treatment and host inflammation, which can lead to subsequent translocation to other sites [10].

UTIs are a member of major microbial diseases with considerable economic impacts on society according to new studies, E. faecalis can infiltrate cells and form intracellular microbial communities in the bladder in addition to adhering to epithelial cells in the urinary tract [11]. The majority of Ur pathogens in healthy people come from rectal bacteria and reach the urinary tract through the urethra into the bladder. Uropathogens first attach to and populate related to the local proximal urethra via the ascending route [12]. Up to 50% of illnesses in individuals with established cystitis could ascent through into higher urinary tracts, and also, the majority of instances of pyelonephritis were associated with increased morbidity ascending from the bladder over all the ureter and then into the renal pelvis [13]. According to the findings of the current study, 8 (80%) isolates of E. faecalis were found in urine samples, which is similar to previous results [14] and [15], which isolated (73.4%) and (87.27%) of E. faecalis from urine samples respectively. An Iraqi

study [16] in Duhok, in 2014 and 2015 indicated that UTIs were caused by the dispersion of microbial species, and the main Uropathogens were E. coli (52%), followed by S. aureus (11%). While the findings of another study in Duhok showed that E. faecalis was the second most prevalent Uropathogen in this area [17].

E. faecalis can invade the female genital tract, especially in individuals with aerobic vaginitis, or after antibiotic therapy, vaginal colonization rises particularly in immunocompromised individuals and when the host microbiota changes, E. faecalis is linked to a wide range of illnesses. There is mounting evidence that connects enterococci to both bacterial and aerobic vaginitis. [18]. Healthy women usually have E. faecalis in their vaginal tract, and the prevalence of E.faecalis is higher in those who have aerobic vaginitis (AV) and commensal Lactobacillus spp. Malodor and discomfort are early signs of AV, but it can progress to more significant issues like PID, severe UTIs, and pregnancy difficulties.[19, 20].

The current study showed that 4(66.6%) E. faecalis isolates were found in high vaginal swabs. This finding was consistent with recent research [21, 22] which isolated (75.0%) and (65%) of elevated vaginal swabs of E. faecalis, respectively. A previous study in five hospitals in Kuwait showed that (9·0 %) of E. faecalis from a high vaginal swab were isolated [23]. Another study [24] revealed that enterococci were present in 8.14% of the women with vaginal discharge.

The present study showed that the gene for Aggregation Substances (agg) was positive in (75%) of the isolates, and previous investigations [25, 26] have shown a high prevalence of this gene in E. faecalis. In contrast, a previous study [27] did not find this gene in E. faecalis whereas other studies [28] reported that 74% of E. faecalis isolates harbored agg gene. The current PCR assays showed that the gene for hyaluronidase (hyl) was positive in 5 (41.66%) of the isolates, while in a study by [29], hyl was not found in E. faecalis but in contrast in the present study and some other studies, this gene was detected in less frequency among E. faecalis isolates. A study by [30] showed that the hyl gene was detected in only (4%) of all E faecalis isolates. Another studies carried out by [31, 32] showed the prevalence of hyl gene was (55.41),(66.7%), respectively. According to several studies, bacterium hyaluronidases contributed to the breakdown of the biofilm matrix elements and subsequently cell dispersal [33].

Conclusion

It had been found that the existence of several virulent factor genes in E. faecalis, such as agg and hyl, boosts this pathogen's pathogenicity, and amongst E. faecalis isolates, the agg gene exhibited a greater proportion, confirming the critical need to develop control the infection associated with the development and increase pathogenicity of this pathogen.

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Table 1: PCR primers for detection of biofilm formation genes

NO	Gene	Sequences of primer (5-3)		Product Size of pcr (bp.)	Reference
1	agg	F	5'-AAGAAAAAGA AGTAGACCAAC-3'	1553	Study design (NCBI)
		R	5'-AAACGGCAAG ACAAGTAAATA-3'		
2	hyl	F	5'-ACAGAAGAGCTGCAGGAAATG-3'	276	Study design (NCBI)
		R	5'-GACTGACGTCCAAGTTTCCAA-3'		

Table 2: Components of the PCR mixture

No	Mixture Contents	Volume (µl)
1	Master Mix	12.5 µL
2	Forward Primer	1.5µL
3	Reveres primer	1.5µL
4	Template DNA	5 µL
5	Distilled water	4.5 µL
Total		25

Table 3: PCR Thermo Cycling Conditions:

Gene	Temperature (℃) / Time					Cycle No.
	Inetial Denaturation	Cycling condition			Final Extention	
		Denaturation	Annealing	Extention		
agg	94	94/60	56	72/60	72	35
hyl	94	94/60	45	72/60	72	

Table 4: the characteristic and biochemical tests for E. faecalis:

Tests	Results
Gram Stain	Positive
Growth in 42 C°	Positive
Catalase	Negative
Oxidase	Negative
40% Bile tolerant	positive
Esculin hydrolysis	Positive
Lactose fermentation	positive
Azide tolerance	Positive

Table 5: Distribution of E. faecalis isolates according to the Vitic:

Source samples	No of samples	Suspected E. faecalis(%)	No.of E. faecalis(%) by VITEK-2
Midsream Urine	95	10 (10.52%)	8 (80%)
HSV	35	6 (17.14%)	4 (66.66%)
Total	130	16 (12.3%)	12 (75%)
Calculated X2	0.365		
Calculated P value	0.551*		