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Molecular Detection of some Virulence Genes for *Enterococcus faecalis* Isolated from Women with Genital Tract Infection in Balad district

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ABSTRACT

Background: Vaginitis, characterized by inflammation of the vagina, is a prevalent health issue affecting millions of women worldwide which is often attributed to an imbalance in vaginal microbiota and is the most common cause of vaginal discharge in reproductive-aged women. This study was conducted to molecular detection of some virulence genes implicated in pathogenesis of *E. faecalis* in women with vaginitis in addition to molecular investigation of biofilm and vancomycin resistant genes.

Materials and methods: A cross-sectional study conducted in Tikrit City, Balad Province, from February 2023 to March 2024 involved 400 married, non-pregnant women aged 15-49 with vaginitis symptoms. These women sought consultation at Balad General Hospital's clinic and private clinics in the province. The study received approval from the council of the College of Medicine, Tikrit University. Bacterial isolation from vaginal samples was performed by gently inserting a swab into the vaginal opening, rotating it against the vaginal walls, and then carefully withdrawing it without touching the skin. Real- Interpretation of RT-PCR data relied on Cycle threshold values, which indicate the cycle number at which the fluorescence signal surpasses the baseline threshold.

Results: The study a spectrum of bacterial species involved. *Escherichia coli* was the highest isolated bacteria, 70(22.95%), followed by *Staphylococcus aureus* 55(18.03%), *Enterococcus faecium* (accounts for 50 cases (16.39%) of the isolated bacteria, *Klebsiella pneumoniae* and *Enterococcus faecalis*, each with 35(11.48%). *Staphylococcus epidermidis* was associated with 28(9.18%), *Staphylococcus saprophyticus* was 25(8.20%), and *Granulicatella elegans* was observed in 7 cases, (2.30%) of the isolated bacteria. Growth in 10% NaCl was seen in 65.71% of isolates, and protease production was evident in 62.86%.

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Introduction:

Vaginitis is an inflammation of vagina, also known as vulvovginitis when accompanied with inflammation of the vulva. Symptoms include vaginal discharge, pruritis, irritation, and erythema (1). The most common cause of vaginitis are bacterial vaginosis, aerobic vaginitis, candidal vaginitis, and trichomonal vaginitis (2). Also, number of different factors can effect on the health of vagina, include personal hygiene,hormones (particularly estrogen), pregnant women, diabetes, and allergies to spermicide or soaps (3). Bacterial vaginosis caused by excessive growth of facultative anaerobic bacteria a *Gardnerella vaginalis*, common symptoms include increase vaginal discharge usually white or gray in color with fishy odors(4). Aerobic vaginitis is a disturbance in the vaginal normal flora, caused by *Escherichia coli*, *Staphylococcus* e.g *staphylococcus epidermis*, *streptococcus agalactiae* and *Enterococcus faecalis*(5). Vaginal infections left untreated can lead to further complications, especially for the pregnant woman. For bacterial vaginosis, these include "premature delivery, postpartum infections, clinically apparent and subclinical pelvic inflammatory disease, [as well as] postsurgical complications (after abortion, hysterectomy, caesarian section), increased vulnerability to HIV infection and, possibly, infertility. Diagnosis is typically suspected based on a women's symptoms. Diagnosis is made with microscopy (mostly by vaginal wet mount) and culture of the discharge after a careful history and physical examination have been completed (1). This bacteria harbor a higher virulent strains such as a *gel E* (gelatinase) gene essential for degradation of polymerized fibrin, *esp* (enterococcal surface protein) gene associated with colonization and enhance biofilm formation, *cyl A* (cytolysin activator) gene which act as becteriocin against streptococcal strains and other Gram – positive bacteria, and *hyl* (hyaluronidase) gene which can hydrolyze the tissue of host cell, also have ability to formation of biofilms, is an important virulence factor, help bacteria to remain in human body for long time, and resistant to immune system defense(3). Treatment of *E.faecalis* infection is difficult because they acquired resistance against several antibiotics resulting from either DNA mutation or acquisition of new genes by gene transfer(6,7). This study was conducted to molecular detection of some virulence genes implicated in pathogenesis of *Enterococcus faecalis* in women with vaginitis in addition to molecular investigation of biofilm and vancomycin resistant genes.

Materials and methods

Across sectional study was carried out n Tikrit City (Balad Province) from In the period from the first of February 2023 to the end of march 2024. The study included 400 married, non-pregnant women with vaginitis , their age range from 15-49 years old, whom they attended to consultative clinic of Balad General Hospital and private clinics in Balad Province. Approval of the council of College of Medicine/ Tikrit University was obtain for the proposal of the study. Approval permission was presented to the director of Tikrit Health directorate. Questionnaire was developed by the researcher for the purpose of the study , including ages, number of children, residence, used contraceptive, drugs, asked women if suffered from (vaginal discharge, vulvar itching, bad odors).

The source of all primers used in this study was Macrogen® (Korea). The name, sequence and product size are given in:

Isolation of bacteria from Vagina:

Collection of vaginal swab included:

The swab package was partially opened, inserted the swab about 5 cm (2 inches) in to the vaginal opening, gently turn the swab for about 20 to 3o seconds while rubbing the swab against the walls of the vagina, then it was withdrawn the swab carefully without touching the skin and avoid the swab touching any surface before placing it into the collection tube([i]).

Samples culture:

The collected vaginal swabs were inoculated on to blood agar Macconkey agar and sabouraud,s dextrose then they were cultured on the selective medium azid blood agar, chromogenic agar (UTIC) and CHROM agar candida. The inoculated plates were incubated at 37ċ for 24 hours

Smear were prepared from primary culture colonies and stained by Gram stain then examined under light microscope to differentiated bacteria into gram- positive bacteria and gram- negative bacteria. Gram stain is almost always the first step in the identification of bacteria

Performing RT-PCR

Procedure

Any existing one-step qRT-PCR assay performed efficiently using standard cycling conditions may be converted to a fast, one-step qRT-PCR assay with KAPA SYBR FAST one-step qRT-PCR kits.

Preparation of qPCR master mix

1. The KAPA RT mix was kept on ice during use, and assembled reactions on ice to avoid premature cDNA synthesis.

2. PCR master mix containing the appropriate volume of all reaction components common to all or a subset of reactions to be performed.

3. Included a no template control (NTC) and no RT control (NRT) when necessary. The NTC would enable detection of contamination in the reaction components, while the NRT would enable detection of contaminating genomic DNA.

4. The required volume of each component was calculated based on

Interpretation

The data obtained from real time experiments were detected according to the Ct values which calculated from cycles and was proportional to the starting target copy number (logarithmic scale) used for amplification (the point that the fluorescence signal increased above baseline is the threshold cycle) which are inversely related to the amount of starting template that mean the high value of Ct refers to the low levels of gene expression or amplification gene, while low Ct value indicate high level of gene expression or high copy of gene amplification.

Amplification plots appeared when the fluorescent signal from sample is plotted against cycle number; however amplification plots include the accumulation of product through the period of qPCR experiment.

The amplification accuracy of gene product was noticed by the value of cycle threshold (Ct) for the triplicate reactions. The data obtained from real time experiments were detected according to the Ct values which calculated from cycles and was proportional to the starting target copy number (logarithmic scale) used for amplification (the point that the

fluorescence signal increased above baseline is the threshold cycle) which are inversely related to the amount of starting template that mean the high value of Ct refers to the low levels of gene expression or amplification gene, while low Ct value indicate high level of gene expression or high copy of gene amplification (Figure 3.6). Amplification plots appeared when the fluorescent signal from sample is plotted against cycle number; however amplification plots include the accumulation of product through the period of qPCR experiment.

Statistical analysis

Computerized statistically analysis was performed using SPSS version 26 statistic program. Comparison was carried out using Chi-square probability and correlation and for determination of probability value (P-value). The P value ≤ 0.05 was considered statistically significant, while for those which its P value was greater than 0.05 considered non-significant statistically.

Results

Table 3 illustrates the distribution of bacteria isolated from women with vaginitis, revealing a spectrum of bacterial species involved. Escherichia coli was the highest isolated bacteria, 70(22.95%), followed by *S. aureus* 55(18.03%), *Enterococcus faecium* (accounts for 50 cases (16.39%) of the isolated bacteria, *Klebsiella pneumoniae* and *Enterococcus faecalis*, each with 35(11.48%). Staphylococcus *epidermidis* is associated with 28(9.18%), Staphylococcus *saprophyticus* was 25(8.20%), and *Granulicatella elegans* was observed in 7 cases, (2.30%) of the isolated bacteria. (34.29%, 25.71%, and 28.57%, respectively). The Microtiter plate method similarly shows diversity in biofilm strengths, with 14.29%, 28.57%, and 40% classified as weak, moderate, and strong, respectively. It's noteworthy that a small percentage (11.43%) of isolates was negative for biofilm production in each method. Sensitivity and specificity calculations for each method further highlight their performance characteristics. The Congo Red and Tube methods demonstrate high sensitivity (88.57% and 100%, respectively) but vary significantly in specificity (100% and 13.79%, respectively). The Microtiter Plate method exhibits both high sensitivity (100%) and higher specificity (28.57%).

Table 4 presents the results of polymerase chain reaction (PCR) detection targeting the gelatinase (*gelE*) gene in enterococci. Among the positive cases for gelatinase production (n=22), a significant majority (86.36%) also tested positive for the *gelE* gene. Conversely, among the negative cases for gelatinase production (n=13), all were negative for the *gelE* gene.

Table 5 outlines the outcomes of polymerase chain reaction (PCR) detection targeting the cytolysin (*cylA*) gene in enterococci. In a cohort of 35 samples, 8 samples (22.86%) tested positive for the presence of the *cylA* gene, while 27 samples (77.14%) exhibited a negative result.

The endocarditis and biofilm-associated pilus (*Ebp*) operon is a component of the core genome of *Enterococcus faecalis* that has been shown to be important for biofilm formation, adherence to host fibrinogen, collagen and platelets, and in experimental endocarditis and urinary tract infection models.

Discussion

The majority of positive cultures of women with vaginitis reveal bacterial infections, constituting 277(75.89%) of the cases underscore the significance of bacterial vaginosis as a primary etiological factor in vaginitis. Bacterial vaginosis is characterized by an imbalance in the vaginal microbiota and pathogenic bacteria (3,5). The high prevalence of bacterial infections aligns with existing literature highlighting BV as one of the most prevalent vaginal infections among women of reproductive age (10). Escherichia coli, the most frequently isolated bacterium comprising 22.95% of cases, has been consistently implicated in vaginitis in previous research. While E. coli is primarily known as a gastrointestinal bacterium, its presence in the vaginal microbiota underscores its potential role in vaginal infections, particularly in cases involving fecal contamination or ascending infection from the perianal region. Studies have reported E. coli as a common pathogen in vaginal infections, emphasizing the importance of addressing hygiene practices and preventing fecal contamination to reduce the risk of E. coli-associated vaginitis(11,12). Manges *et al* (13) demonstrated that the most prevalent Gram positive bacteria were *S. aureus* (45.61%). The study was inconsistence with a study done in Erbil by Mohammed (14) who found a high percentage (46.21%) of *E coli* in his study. Enterococci are opportunistic pathogens commonly found in the gastrointestinal and genitourinary tracts and can cause various infections, including urinary tract infections and endocarditis. *E. faecalis* is associated with a wide spectrum of infections, particularly under immunocompromised states and during compositional shifts in the host microbiota. Our finding was comparable to a study of Shrestha *et al.,* (15) when showed that vaginitis symptoms have multiple etiologies. In agreement with our funding, study done by Ali *et al.,* (166) showed that, the prevalence of *E. faecalis* among women with vaginitis was were 15.7%. Al-Kafajy *et al.,* (17) revealed that *Escherichia coli* were the most encountered frequency (37.2%) followed by *Staphylococcus aureus* with (27.9 %). *Enterococcus faecalis* (23.2%), *Gardnerella vaginalis* (16.2 %), and *Candida spp* (11.6 %) were less common. From a total of 602 vaginal swabs from pregnant women, Ghasemi *et al.,* (18) indicated that, 49 (8.14%) isolates were identified as enterococci. Predominant species were respectively, *E. faecalis* 44 (89.8%), *E. faecium* 3 (6.1%), This inconsistency might be associated with difference among study participants, varied etiologies studied and the detection techniques applied (161). Daood *et al.,* (19) in similar study showed that *Enterococcus faecalis* forming 27 (21.2%) of the isolated bacteria (57.4%) of positive growth . In the current study, The majority of isolates exhibited various virulence factors, indicating a significant potential for pathogenicity. Notably, gelatinase production was prevalent in 62.86% of cases, while capsule production was observed in over half of the isolates, at 51.43%. Additionally, a substantial proportion of isolates demonstrated the ability to grow in 10% NaCl (65.71%) and produce proteases (62.86%). Other factors contributing to the virulence profile, such as growth in NaOH (40%), growth in peptone (42.86%), and lipase production (25.71%), were also notable. Several virulence and pathogenicity factors have been described from enterococci that enhance their ability to colonize patient's

tissues, increase resistance to antibiotics, and aggravate the infection outcomes (15,16,17). The findings of the study indicating a high prevalence of virulence factors among *E. faecalis* isolates align with several other studies investigating the pathogenic potential of this bacterium. For instance, a study conducted by Dahl *et al* (20) similarly found a significant proportion of *E. faecalis* isolates expressing gelatinase production, supporting the notion that this virulence factor is commonly present in clinical isolates. Additionally, Salim *et al* (21) reported comparable results regarding capsule production, highlighting its importance as a virulence factor contributing to the pathogenicity of *E. faecalis* strains. Secreted factors, such as gelatinase, autolysin A, and serine protease (SprE), are biofilm-associated factors that are involved in the degradation of host substrates, including collagen, fibrin, and certain complement components.

In agreement with our finding, Jubair (22) determined the types and frequencies of virulence factors expressed by enterococcal isolates and found that 77.3% of the isolates expressed protease production and 29.5% gelatinase production and also showed that most *E. faecalis* isolates had the ability to adhere to epithelial cells *in vitro*. It is worthy to mention that most of the previous studies also detect high rates of most *E. faecalis* isolates produce adherence factors (23-25). Enterococcal infections are often associated with the production of biofilms, assemblages of microbes enclosed in an extracellular polymeric matrix that exhibit cell-to-cell interactions These biofilms have been observed on catheters, diabetic ulcers, and wounds, resulting in severe infection (26). Biofilm producing bacteria are responsible for many recalcitrant infections and are difficult to eradicate (27). Enterococci are one of the causative organisms of UTI, biofilm formation allows the strain to persist in genitourinary tract for long time, and survival advantages conferred by biofilm include resistance to phagocytosis and antimicrobial agents. Shahi *et al.,* (28) indicated that 92.8% isolates of *E. faecalis* were biofilm producer (strong and moderate) by CRA method and 7.1% were weak biofilm production. Our results was in agreement with study obtain by Mohamad and El Shalakan (29) who found that (85.7%) of *E. faecalis* isolates were strong and moderate slimes producer on CRA plates. Microtiter plate method were found to be most sensitive, accurate and reliable screening method for detection of biofilm formation when compared to CRA methods, microtiter plate method was quantitative test method and it was considered the gold standard method for biofilm detection (26,27). Most of the studies recommend MTP method for general screening on biofilm formation (26,29). The current study, among the positive cases for gelatinase production (n=22), a significant majority (86.36%) also tested positive for the *gelE* gene. Conversely, among the negative cases for gelatinase production (n=13), all were negative for the gelE gene. In agreement with this finding, a similar study indicated that most of *E. faecalis* were with *gelE* gene (30). Kiruthiga *et al* (31) have indicated that *gelE* was the second most common detected in enterococci, more commonly in *E. faecalis* (85.39%), all the gelatinase producers (100%) harboured gelE gene while, the reverse was not true. Our results were in concordance with previous studies that have documented high gelatinase production in Enterococci as detected by

PCR (32,33). In concordance with previous reports, gelE was present as a silent gene in *E. faecalis* (77.8%), and *E. faecium* (58.6%) (185,186). Gelatinase encoded by *gelE*, is an extracellular zinc-endopeptidase/protease produced by *E. faecalis* that is capable of hydrolyzing gelatin, collagen, casein, hemoglobin, and other peptides. Gelatinase production in *E. faecalis* contributes to virulence which damages the host tissue facilitating bacterial migration and spread, colonisation and persistence by biofilm formation (30). The current study, 8 samples (22.86%) tested positive for the presence of the *cylA* gene. Kiruthiga *et al* (34) have indicated that *cylA* gene was detected in 41.43% of *E. faecalis* isolates. Different other studies were also found nearly similar finding (35,36). Cytolysin elaborated by hemolytic strains of E. faecalis contributes to virulence in in human infections. The cytolysin operon is a two-component system, lysin (L) encoded by cylL1, cylL2, cylM, cylB and an activator (A) encoded by cylA (37). A recent study found that Enterococcal surface protein, encoded by esp, is significantly higher among clinical isolates than faecal isolates and is associated with increased virulence, colonization and persistence in the urinary tract and biofilm formation (38). Aggregation substance, encoded by asa1, facilitates the conjugative transfer of sex pheromone gene-containing plasmids and enhances virulence (39). In addition, Kalode *et al* (36) have indicated that among *E. faecalis* isolates, 114 (79.72%) harbored *gelE* and 80 (55.94%) carried *cylA*. While Gousia *et al* (40) conducted several studies on the presence of virulence factors such as gelatinase, hemolysin, and Esp in patients with bacteremia caused by *E. faecalis*. They reported the prevalence of hemolysin and gelatinase in the isolated species were 17.9% and 47.4% respectively, that was inconsistent with the results of the present study and can be attributed to the method of sampling . In the current study, 26(74.29%) tested positive for the presence of the ebpB gene. In agreement with this finding, Hegstad *et al* (41) found that a high percentage of *Enterococcus faecalis* clinical isolates (94.59%) carried *ebpB* .

Conclusions

1- *Escherichia coli*, *Staphylococcus aureus*, and *Enterococcus* species were among the most commonly isolated bacteria. *Enterococcus faecalis* isolates exhibited various virulence factors, with notable percentages.

2- The study analyzed biofilm formation among *Enterococcus faecalis* isolates causing vaginitis, highlighting the prevalence of positive biofilm production. Additionally, a high percentage of isolates showed resistance to several antibiotics.

3- The presence of the gelatinase gene (*gelE*) was strongly associated with gelatinase production in enterococci.,

4- Detection of the cytolysin gene (*cylA*) in enterococci revealed a lower prevalence, with only a minority of samples testing positive for *cylA*. While *EbpB* gene was relatively common among the samples.

Recommendations

1- Further investigate the virulence factors exhibited by *Enterococcus faecalis* isolates to understand their

role in pathogenesis and disease progression in addition to the mechanisms underlying virulence factor expression and their impact on host-pathogen interactions.

2- Incorporate screening for genetic markers associated with virulence, such as the gelatinase gene (*gelE*) and cytolysin gene (cylA), into routine diagnostic protocols for enterococcal infections.

3- Continuously monitor antibiotic resistance patterns among *Enterococcus faecalis* isolates causing vaginitis to guide empirical treatment decisions and inform local antibiotic prescribing guidelines.

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TABLES

Table 1: The name, sequence and product size of primers used in this study Name of Primer Sequence Product size (bp) Reference**.**

Name of primers	Sequence	Product size(bp)	Reference
GelE	F-CTACCGACATGTTCCGTAAA R-GTTGGAAAAGGAGGCAATTTC	447 bp	Newly Designed
CylA	F-GTACACAAGTTGCTGGAGTAA R-AGTGGAGAAACTAGCGATGTA	505 bp	Newly Designed
EbpB	F-ATTAGTCACGGACAAGCATAC R-GTGCAGAGATTTCTTCCAAGA	278 bp	Newly Designed

Table 2: Cycling program

Step	Temp. (°C)	Time	Cycle
Reverse transcription	42 °C	10 min	Hold
Enzyme activation	95 °C	3 min	Hold
Denaturation	95.0 °C	15 sec	40
Annealing	53.0 °C	15 sec	40
Extension	72.0 °C	15 sec	40

Table 3: Distribution of bacteria isolated from women with vaginitis

Isolated bacteria	**No.**	**%**
E .coli	70	22.95
E. faecalis	35	11.48
S. aureus	55	18.03
S. saprophyticus	25	8.20
K. pneumonia	35	11.48
S. epidermidis	28	9.18
E. faecium	50	16.39
Granulicatella elegans	7	2.30
Total	305	100

Table 4: Distribution of *E. faecalis* isolates causing vaginitis according Biofilm formation by different methods

Biofilm production		**No.**	**%**
Method	**Result**		
Congo red method	Positive	31	88.57
	Negative	4	11.43
	Total	35	100
Tube method	Weak	12	34.29
	Moderate	9	25.71
	Strong	10	28.57
	Negative	4	11.43
Microtiter plate method	Weak	5	14.29
	Moderate	10	28.57
	Strong	14	40
	Negative	6	17.14

Table 5: PCR detection of enterococcal gene that encode for gelatinase (*gelE*)

Phenotypic gelatinase production	Gelatinase (*gelE*) gene			
	Positive		Negative	
	No.	%	No.	%
Positive (n:22)	19	86.36%	15	13.64%
Negative (n:13)	0	0	13	100 %

Table 6: PCR detection of enterococcal gene that encode for cytolysin (*cylA*)

cytolysin (*cylA*) gene	No.	%
Positive	8	22.86
Negative	27	77.14
Total	35	100

Table 7 : PCR detection of enterococcal *EbpB* gene

EbpB gene	No.	%
Positive	26	74.29
Negative	9	25.71
Total	35	100

FIGURES

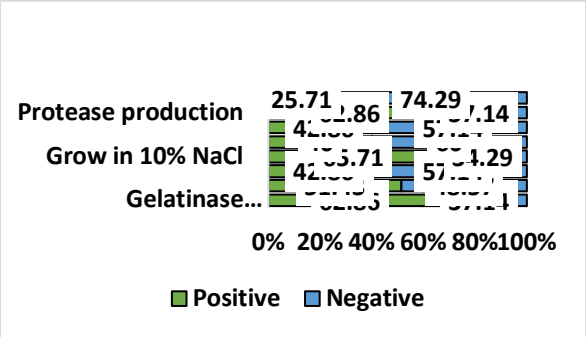

Figure 1: Distribution of *E. faecalis* isolates causing vaginitis according virulence factors

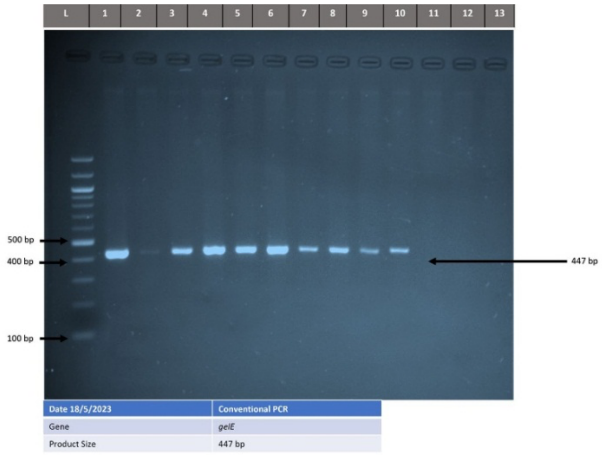

Figure 2: Gel electrophoresis for amplification of *gelE* gene using specific primers for E. faecalis.

Figure 3: Gel electrophoresis for amplification of cytolysin (*cylA*) gene using specific primers for *E. faecalis*.

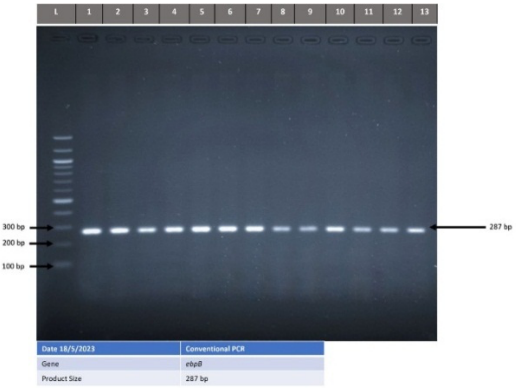

Figure 4: Gel electrophoresis for *EbpB* gene using specific primers for *E. faecalis*.