Rafidain Journal of Science

https://rsci.mosuljournals.com

Vol. 33, No. 4, pp. 15-23, 2024

Enterococcus faecalis as a Risk Factor in Aerobic Vaginitis among Pregnant Women

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p-ISSN: 1608-9391
e -ISSN: 2664-2786

Article information

Received: 28/2/2024
Revised: 12/5/2024
Accepted: 22/5/2024

DOI: DOI: 10.33899/rjs.2024.185380

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ABSTRACT

Aerobic vaginitis (AV) manifests as an imbalance in vaginal flora, characterized by aberrant amounts of aerobic and intestinal pathogens, resulting in different degrees of vaginal infection. In this study, 120 vaginal swab samples were collected from diagnosed female patients, confirmed using clinical signs and history. The swabs were inoculated on blood and Chrome agar, The diagnosis depended on classical methods, and further confirmed by the Vitek-2 technique. The incidence of *Enterococcus faecalis* isolates was 18.33%. The research delved into antibiotic resistance, specifically vancomycin, levofloxacin, erythromycin, tetracycline, gentamicin, ciprofloxacin, and bacitracin, determined by Kirby-Bauer method using Mueller Hinton Agar. Results revealed high resistance to tetracycline and erythromycin (93%), contrasting with lower ratios for bacitracin (27%) and levofloxacin. gentamicin resistance was observed in 87%, while minimal resistance to vancomycin and ciprofloxacin (10%) was noted.

The study established a correlation between *E. faecalis* and AV in pregnant women, identifying it as a sole cause in adverse pregnancy outcomes. For precise diagnosis, 16S rRNA sequencing was employed, and the identified isolate, *E. faecalis*, was deposited in the Gene Bank database with specific accession number. Sequence similarity searches and phylogenetic analysis indicated 100% compatibility with various isolates globally, including those from Saudi Arabia, South Africa, Thailand, South Africa, Russia, Egyptian, India, China, Thailand.

Keywords: *Enterococcus faecalis*, Aerobic vaginitis, PCR, 16S rRNA sequence, antibiotics test.

INTRODUCTION

Enterococci are a type of Gram-positive bacteria that commonly inhabit the gastrointestinal tract of both animals and humans. In the past, *Enterococci*, such as *E. faecalis* and *E. faecium*, were considered harmless organisms that coexist with humans. However, recent studies have confirmed that *Enterococcus* can indeed cause infections and exhibit pathogenic behavior. One concerning aspect of *Enterococcus* its ability to possess both intrinsic and acquired antibiotic resistance genes. This characteristic makes *Enterococcus* a significant reservoir of resistance genes, leading to the emergence of multidrug-resistant (MDR) strains. As a result, treating infections caused by MDR *enterococci* becomes challenging because the available therapeutic options are limited (Zhao *et al.*, 2024). Aerobic vaginitis (AV) results from an imbalance in vaginal flora, characterized by an abnormal composition containing aerobic and intestinal pathogens, along with varying degrees of vaginal inflammation and immature epithelial cells. Inflammatory changes in AV are attributed to pathogens such as *E. faecalis*, *Escherichia coli*, *group B streptococcus*, and *Staphylococcus aureus*. *E. faecalis* is the most frequently isolated pathogen, accounting for 18.3% of cases and it's consistent with a study conducted by (Ayoub and Zaid, 2013). The pathogenic impact of aerobic bacteria like *E. faecalis* has been linked to adverse outcomes including spontaneous abortion premature birth puerperal sepsis abscesses and urinary tract infections (Jahić and Cerovac, 2022). Among *Enterococcus* species, *E. faecalis* and *E. faecium* are the most prevalent healthcare-associated pathogens worldwide. They have intrinsic resistance to multiple classes of antimicrobials such as cephalosporins, aminoglycosides, lincosamides, and trimethoprim-sulfamethoxazole. Additionally, enterococcal species possess a remarkable capability to acquire new resistance determinants due to the flexibility of their genome (Aung *et al.*, 2023). The pathogenic mechanism of these bacteria relies on their ability to colonize the mucous membranes and then cause pathological changes in the host through toxic activity by inducing an inflammation process and avoiding the host's immune defense mechanisms (Al-Dobardani *et al.*, 2023). The objective of this study was to provide a reference for future research and early diagnosis during pregnancy. Future research in this field can provide insights regarding the mechanisms of aerobic vaginitis-induced adverse pregnancy outcomes in humans and ways to prevent their occurrence.

MATERIALS AND METHODS

Collection of vaginal samples

Vaginal swab samples were obtained from pregnant women experiencing symptoms of aerobic vaginitis from 120 patients. These women attended the maternity, gynecological, and children's hospital from October to December 2023, with ages ranging between 20 and 45 years. Following proper instructions, the swabs were collected after washing the external genitourinary organs of the participants. This research was subjected to ethical considerations, and it was approved by the Committee of Ethical Standards in the college (Number 247, Date 13/9/2023), in line with the form issued for this purpose by the Iraqi Ministry of Health. After collection, the swabs were placed in sterile transport media, specifically Amies transport medium. This medium helps maintain the viability of bacteria present on the swab during transportation to the laboratory. Upon receipt, the samples were, underwent to wet preparation examination for an initial assessment of the samples, including the identification of any bacteria. The samples were cultured within two hours of receipt, culturing involves transferring the swab samples onto appropriate culture media that support the growth of bacteria, this step aims to isolate and identify the specific bacteria or fungi responsible for the aerobic vaginitis infections.

Culture and identification

All the swabs were inoculated in Todd Hewitt Broth with Gentamicin (8 ug/mL) and Nalidixic Acid (15 ug/mL) which was used for the selective enrichment of *E. faecalis*, especially from genital specimens. then the samples underwent culture on blood and selective agar media Hi Chrome strep

B agar medium, followed by phenotypic identification at the species level using traditional bacteriological and biochemical techniques, as outlined by (Manero and Blanch, 1999).

Morphological and microscopic diagnosis

The bacterial isolates were initially identified by observing their morphological and cultural characteristics on blood agar and Chrom agar. The colonies were incubated at 37°C with 10% CO_2 for 24 and 48 hours. Various traits such as colony color, size, texture, height, edge shape, and ability to lyse blood were recorded. The identification process included evaluating beta hemolysis on blood agar, the presence of blue colonies on Chrom medium, and other relevant features. Subsequently, the isolates were fixed on a glass slide, gram stain dye was applied to differentiate the bacteria based on their shape, arrangement, and staining properties by microscopic examination using an oil lens.

Vitec 2 bacterial diagnosis

The Vitek 2 device, developed by BioMerieux, is a widely used and highly accurate tool for bacterial diagnosis. It utilizes a panel of 64 biochemical tests, including the gram-positive card (GP) to identify bacterial species. With a diagnostic accuracy rate of 99%, the Vitek 2 device plays a significant role in the field of microbiology by accurately identifying bacterial strains.

DNA extraction

The Genomic DNA Micro Extraction Kit (Geneaid, Korea) was utilized to extract DNA from pure bacterial colonies, following the manufacturer's instructions. The concentration and purity of the extracted DNA was determined using nanodrop to assess the quality of the samples for downstream applications.

Genotype identification using 16S r DNA

Species-level genetic identification was performed using the 16S rDNA region with the primer set (F5–AGAGTTTGATCCTGGCTCAG-3, R5–GGTTACCTTGTTACGACTT-3) (Nagara *et al.*, 2017).

The PCR premix, primers, and extracted DNA were thawed at 4°C and briefly vortexed to ensure proper mixing. A 25 µl 10µM PCR mixture was prepared, consisting of 12.5 µl 10µM of PCR premix, 1 µl 10µM of each forward and reverse primer, 3 µl of DNA template, and 15 µl of sterile deionized distilled water. Amplification of the DNA was carried out using a thermocycler PCR device according to the PCR protocol (Nagara *et al.*, 2017). The amplification was performed in a DNA thermocycler system (TECHNE, USA), starting with an initial denaturation step at 95°C for 5 minutes. This was followed by 30 cycles of denaturation at 95°C for 60 seconds, annealing at 55°C for 60 seconds, and extension at 72°C for 60 seconds. The amplification was concluded with a final extension step at 72°C for 10 minutes. To visualize and confirm the presence of the amplified DNA, electrophoresis was conducted using a 1.5% agarose gel stained with safe dye. The PCR product was run at 50 volts for 45 minutes. The presence of band on the gel using an ultraviolet (UV) transilluminator to capture the DNA bands under UV light.

Investigation of gene sequence

Gene sequencing investigation took place in biotechnology laboratories in Korea. Genetic homology analysis was conducted using the BLAST software.

Antimicrobial susceptibility

The antimicrobial susceptibility of the bacterial strains was evaluated using the disk diffusion method on Muller Hinton agar, following the guidelines provided by the Clinical and Laboratory Standards Institute (CLSI, 2019). Several antimicrobial agents were tested, including Gentamicin (10µg), Vancomycin (10µg), Erythromycin (10µg), Levofloxacin (5µg), Tetracycline (10µg), Bacitracin (10µg), and Ciprofloxacin (10µg). The agar plates were incubated at 37°C for 24 hours, and the results were subsequently read. The zones of inhibition surrounding the disks were measured and interpreted according to established criteria. Specifically, a zone of inhibition ranging from 15 to 20 mm indicated susceptibility, while a zone measuring between 10 to 14 mm suggested moderate

susceptibility. Conversely, a zone below 10 mm was indicative of resistance. These findings align with the study conducted by (Xia *et al*., 2011) and contribute to our understanding of the antimicrobial susceptibility patterns of the tested bacterial strains.

RESULTS AND DISCUSSION

The primary objective of the sample collection was to isolate and identify *E. faecalis*. Under the microscope they are seen as oval in shape which may be either single, arranged in pairs, or chains Fig. (1). On blood agar, the bacteria presented as small, white, or milky yellow colonies producing hemolysin that degrading red blood cells forming beta hemolysis on petri plates Fig. (2), When cultured on Hi Chrome agar medium, the colonies appeared as spherical, blue structures measuring 2mm in diameter. Notably, each colony featured a white point at its center, accompanied by blue zones surrounding it Fig. (3). (As mentioned by Jaafar, 2022) Isolates were inoculated into the GP cards, which were then run on the VITEK 2 Compact system (bioMérieux, France), VITEK 2 identified 13/14 (99.2 %) of the isolates at the genus and species levels respectively (Table 1).

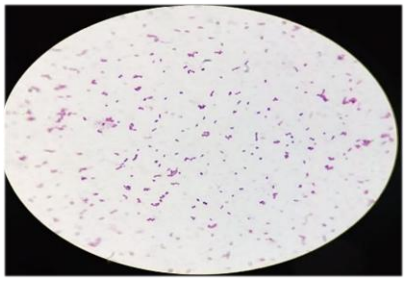

Fig. 1: *E. faecalis* under 100X magnification.

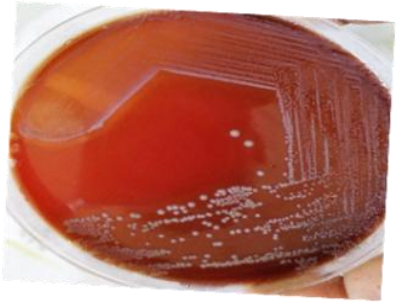

Fig. 2: *E. faecalis* on blood agar medium.

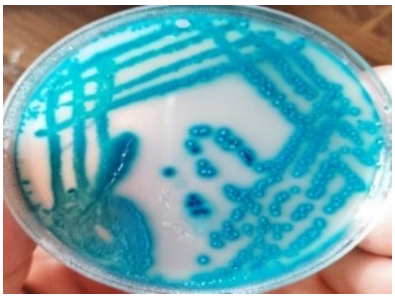

Fig. 3: *E.faecalis* on chrome agar medium.

Table 1: The results of vitek-2 system revealing the diagnosis of *E. faecalis*

bioMérieux Customer:

Dr.Radhwan Al-Jammas LAB.
Microbiology Chart Report

Printed November 8, 2022 4:36:53 PM GMT-06:00

Patient Name: s/3, .
Location:
Lab ID: 361

Patient ID: 361
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Enterococcus faecalis
Source: swab

Collected:

Comments:	

Identification Information	**Analysis Time:**	2.60 hours	**Status:**	Final
Selected Organism	99% Probability	**Enterococcus faecalis**		
	Bionumber:	104002721753471		
ID Analysis Messages				

Biochemical Details

2	AMY	+	4	PIPLC	-	5	dXYL	-	8	ADH1	-	9	BGAL	-	11	AGLU	-
13	APPA	-	14	CDEX	-	15	AspA	+	16	BGAR	-	17	AMAN	-	19	PHOS	-
20	LeuA	-	23	ProA	-	24	BGURr	-	25	AGAL	-	26	PyrA	+	27	BGUR	-
28	AlaA	+	29	TyrA	+	30	dSOR	+	31	URE	-	32	POLYB	+	37	dGAL	-
38	dRIB	+	39	lLATk	-	42	LAC	-	44	NAG	+	45	dMAL	+	46	BACI	+
47	NOVO	+	50	NC6.5	-	52	dMAN	+	53	dMNE	+	54	MBdG	+	56	PUL	-
57	dRAF	-	58	O129R	-	59	SAL	+	60	SAC	+	62	dTRE	+	63	ADH2s	+
64	OPTO	+															

The results of antibiotic sensitivity test for 14 *E. faecalis* isolates by using the disk diffusion assay as in Fig. (4).

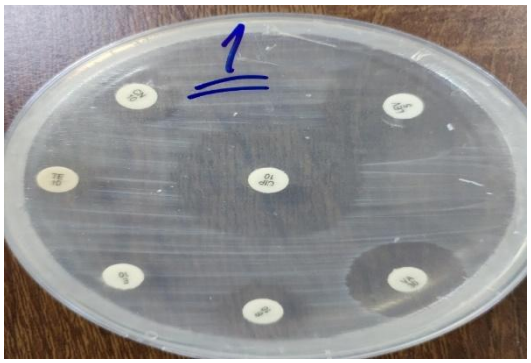

Fig. 4: *E. faecalis* antibiotic resistance.

The results of the antibiotic sensitivity test using the disk diffusion assay revealed a high resistance among the three bacterial strains. Notably, 93% of isolates demonstrated resistance to both Tetracycline and Erythromycin. In contrast, resistance ratios were lower for Bacitracin (27%) and levofloxacin, while Gentamicin resistance was observed in 87% of cases. Interestingly, the isolates exhibited minimal resistance to Vancomycin and Ciprofloxacin as 10%. As in Fig. (5).

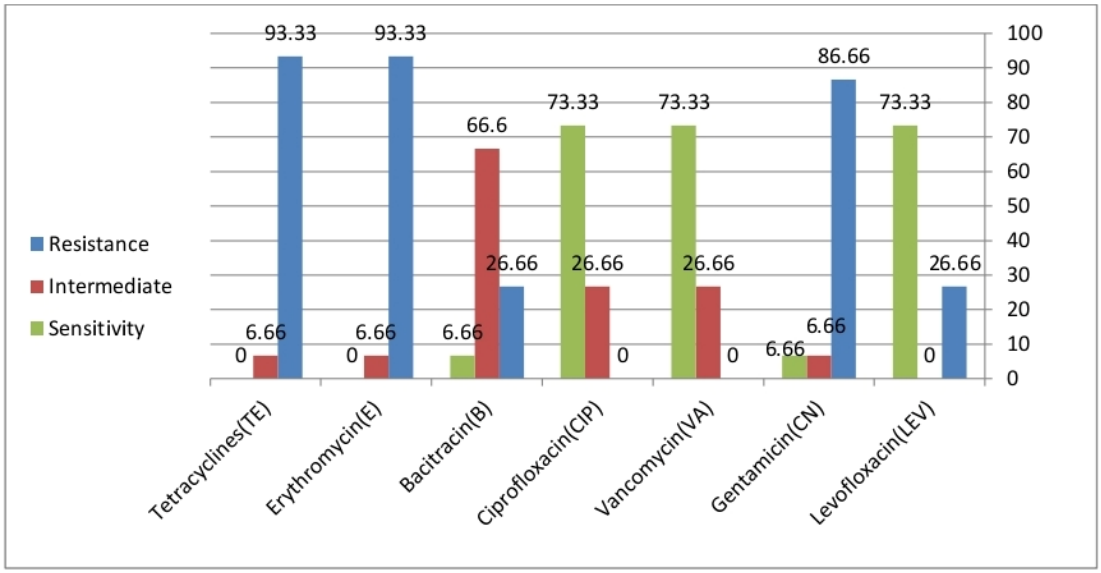

Fig. 5: Disk diffusion assay for *Enterococcus faecalis*.

Bacteria causing genitourinary diseases can evade antibiotics and the immune system by colonizing inside epithelial cells of the genitourinary tracts, this ability leads to about 80% of recurrent infections being caused by the Initial infecting strain (Flores-Mireles *et al*., 2015). Recent evidence indicates that additional uropathogens, such as *E. faecalis* and *Group B Streptococcus* (GBS), exhibit the capacity to invade host urothelial cells. Notably, their mechanisms of cell invasion differ, with *E. faecalis* relying on biofilm production, while GBS is thought to internally localize through the release of a pore-forming toxin (β-hemolysin/cytolysin), causing cellular injury and creating a point of entry (Ognenovska *et al*., 2022). Excessive and inappropriate antibiotic use is believed to heighten the risk of antibiotic resistance, *E. faecalis* bacteria possessing biofilm-forming capabilities are considered more resistant to antibiotics (Arason and Sigurdsson, 2010). In a Kenyan In other study discovered that approximately 40% of isolated *Enterococci* were resistant to penicillin

and ampicillin, with only 10% showing resistance to Aminoglycosides, research revealed *E. faecalis* highest sensitivity to Vancomycin (100%), Nitrofurantoin (94.23%), Doxycycline (90.38%), Ampicillin (86.65%), and Gentamycin (88.46%) (Mousavi *et al.,* 2020). Notably, Metronidazole, effective against bacterial vaginosis, should not be used for AV due to *E. faecalis'* natural resistance, potentially leading to increased infection for pregnant individuals, Clindamycin is a preferable option Fluoroquinolones like Ciprofloxacin and Ofloxacin can treat AV effectively as they minimally impact normal vaginal flora, facilitating swift recovery (Han *et al*., 2015).

After isolation and diagnosis using the Vitek-2 system, molecular analysis was performed using PCR, specifically targeting the 16S rRNA gene. This involved examining the results of DNA segment amplification and conducting agarose gel electrophoresis. The positive result for the *E. faecalis* gene was confirmed by electrophoresing a 1.5% agarose gel stained with safe dye for 45 minutes at 50 volts. Additionally, the agarose gel was photographed using an ultraviolet (UV) transilluminator. The electrophoresis results of the 16S rRNA gene showed a clear band at approximately 1500 bp of ladder, confirming the successful amplification of the target gene. Fig. (6).

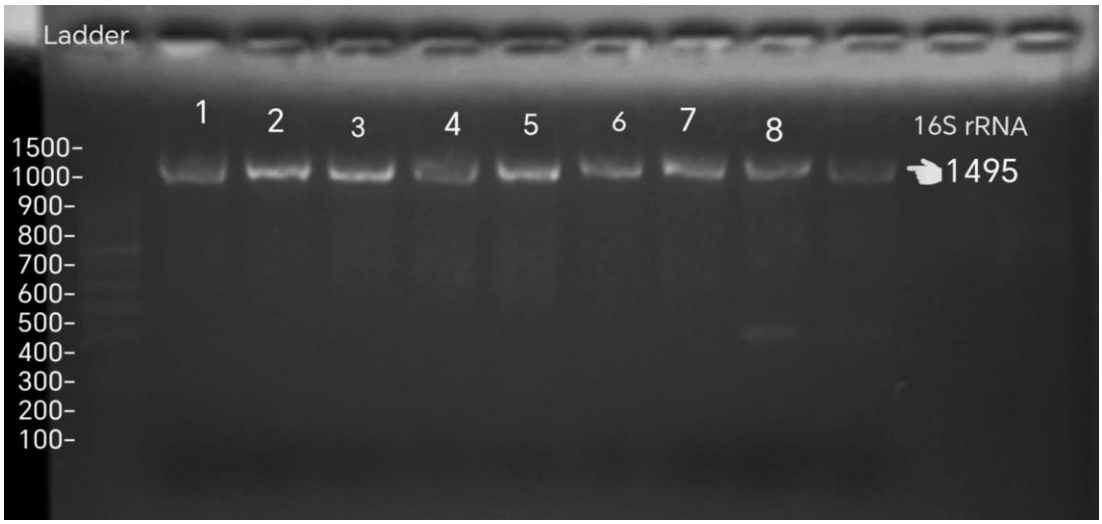

Fig. 6: Agarose gel electrophoresis showing amplification of 16s rRNA close to 1500 bp fragment Using 1500bp DNA Ladder.

The 16S rRNA gene is highly valuable in the field of microbiology for the precise diagnosis of bacterial isolates and the identification of new strains. Its sequences are especially crucial when dealing with slow-growing, fastidious, and rare bacteria. Moreover, this test is considered more accurate compared to alternative diagnostic methods (Woo *et al.,* 2008). According to accession number for *E. faecalis* PP069570 ،PP070529 ،PP082536 ،PP082537 ،PP084055 ،PP084095، PP084267 ،PP085181 ،PP085216 ،PP086971 ،PP086973 ،PP086979 ،PP086982 ،PP086973.

Phylogenetic analysis

Following the molecular diagnosis confirmation via examination of the 16S rRNA gene, the nucleotide sequence was determined. The obtained results were then compared on NCBI, and using BLAST software, the molecular findings of the isolated bacteria revealed a matching ratio of 99% *E. faecalis* KSA, Greece and India the Isolates were also registered in Gene Bank with the name *E. faecalis* strain ABAMMICRO and the code PP084095.1 showed below in Fig. (7) and (Table 3). Phylogenetic tree of Enterococcus faecalis ABAMMICRO isolates based on partial 16S rDNA gene

sequences. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches.

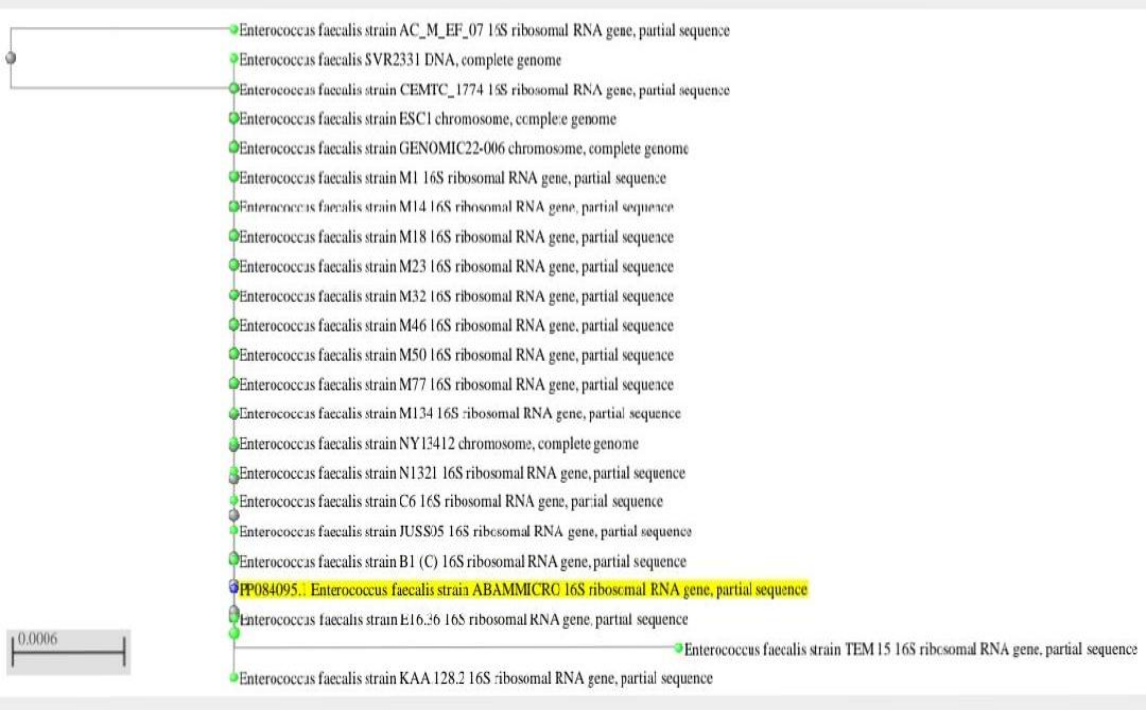

Fig. 7: Matching results of *E. faecalis* ABAMMICRO by using BLAST software.

Table 3: Matching ratios of *E. faecalis* ABAMMICRO with diagnosed isolates which are registered in NCBI gene bank

NO.	Accession	Country	Source	Host	Compatibility
1.	ID: KT598432.1	South Africa	*Enterococcus faecalis*	Surface water	100%
2.	ID: MT589112	Saudi Arabia	*Enterococcus faecalis*	Newborn infant stool	99%
3.	ID: ORO98620.1	Greece	*Enterococcus faecalis*	…….	99%
4.	ID: ORO18318.1	Russia	*Enterococcus faecalis*	Galleria melonella `Midgut`	99%
5.	ID: OO826456.1	Egypt	*Enterococcus faecalis*	Chronic kidney patients	99%
6.	ID: CP122480	India	*Enterococcus faecalis*	Stool from pig handler	99%
7.	ID: AP026714.1	Japan	*Enterococcus faecalis*	Urine	99%
8.	ID: OP889552.1	China	*Enterococcus faecalis*	*Arborophila rufipectus*	99%
9.	ID: ORO84122	Bangladesh	*Enterococcus faecalis*	Fish interstine	100%
10.	ID: OQ202175.1	Thailand	*Enterococcus faecalis*	Diabetic foot ulcer	99%
11.	ID: CP115992.1	China	*Enterococcus faecalis*	Foetoses	99%

CONCLUSION

Aerobic vaginitis (AV) is implicated in adverse pregnancy outcomes such as premature delivery, abortion and stillbirth, primarily linked to *E. faecalis* infection. Preserving a healthy vaginal biofilm during pregnancy is vital to controlling opportunistic infections, which may result in poor pregnancy outcomes. Optimal treatment involves antibiotics that have little consequence on the normal beneficial microbiota, such as *Lactobacillus* species, while eradicating *E. faecalis*, *Streptococcus agalactiae*, *S. aureus*, and *E. coli*, all of which have been implicated in the etiology of AV. Future research should focus on unraveling the human-specific mechanisms leading to AV-induced adverse pregnancy outcomes, enabling preventive measures. Additionally, efforts are

needed to enhance early diagnosis and effective treatment for AV during pregnancy, aiming to offer evidence-based medical guidance, safeguarding the health of both pregnant women and fetuses.

ACKNOWLEDGMENT

We extend our heartfelt gratitude to Mosul University and the College of Science for their unwavering oral and scientific support. Special thanks also to Rafidain Journal of Science for their invaluable assistance in publishing our research.

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المكورات المعوية البرازية كعامل خطر في التهاب المهبل الجرثومي بين النساء الحوامل

عبد الله محمد عبد **اميرة محمود الراوي**

قسم علوم الحياة/ كلية العلوم/ جامعة الموصل

الملخص

يهدف البحث إلى دراسة علاقة التهاب المهبل الجرثومي (Aerobic vaginitis) لدى النساء الحوامل مع جرثومة المعويات البرازية *Enterococcus faecalis* يظهر هذا الالتهاب بفعل عدم توازن في تكوين البكتيريا النافعة في المهبل، ويتميز بارتفاع مستويات غير طبيعية من الجراثيم الهوائية والمعوية في المهبل، مما يؤدي إلى درجات متفاوتة من العدوى المهبلية. انتشار التهاب المهبل يتراوح بين 12% و23.7% في النساء غير الحوامل اللاتي يعانين من أعراض الالتهاب، ومن 4% إلى 8% خلال فترة الحمل، مع زيادة في خطر الإصابة بالأمراض المنقولة جنسياً. من الكائنات المسببة للمرض تشمل *Enterococcus faecalis*، *Escherichia coli*، *Staphylococcus aureus* و*Streptococcus agalactiae*. تم جمع 120 عينة من مسحات المهبل من نساء حوامل وتأكيد التشخيص باستخدام العلامات السريرية والتاريخ الطبي. تم زرع المسحات على وسط أكار الدم المطبوخ ووسط الكروم الانتقائي، وتم تشخيصها باستخدام الطرق التقليدية والتأكيد الإضافي باستخدام تقنية Vitek-2 compact system. تم عزل جرثومة *Enterococcus faecalis* بنسبة 18.33%. قامت الدراسة أيضًا بتحليل مقاومة الجراثيم لعدة مضادات حيوية وهي vancomycin وLevofloxacin وErythromycin وTetracycline وGentamicin وCiprofloxacin و Bacitracin باستخدام تقنية تحديد التركيز الحد الأدنى للتثبيط (MIC) باستخدام وسط مولر هنتون اكار، أظهرت النتائج نسبة مقاومة عالية لـ Tetracycline وErythromycin (93%)، بينما تباينت نسب المقاومة إلى Levofloxacin وBacitracin (27%)، وكانت نسبة مقاومة الـ Gentamicin 87%، بينما لوحظت نسبة مقاومة ضئيلة بالنسبة لـ vancomycin وciprofloxacin (10%). خلصت الدراسة إلى وجود علاقة بين هذه الجرثومة والتهاب المهبل الجرثومي (AV) لدى النساء الحوامل محددة إياها كسبب في نتائج الحمل السلبية. تم تشخيص الجرثومة باستخدام تقنية 16S rRNA كوسيلة تشخيص أكثر تخصصية، حيث تم تسجيل 14 سلالة جديدة في بنك الجينات NCBI وكان إحدى هذه السلالات بإسم *Enterococcus faecalis* ABAMMICRO كما تم استخدام نظام BLAST وطريقة NB(Neighbor-joining) لتحديد التشابه في التسلسل الجيني ورسم الشجرة التطورية لهذه الجرثومة المعزولة وأظهرت النتائج عزلتنا الجرثومية متطابقة بنسبة 99% مع العديد من العزلات العالمية من ضمنها *E. faecalis* KSA, India and Greece.

الكلمات الدالة: المكورات المعوية البرازية، التهاب المهبل الجرثومي، التشخيص الجزيئي، اختبار المضادات الحيوية.