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Vancomycin-Sensitive E. Faecalis Transferred to Resistant Isolates After Multiple Mixed Subcultures With Vancomycin-Resistant S. Aureus Isolated Women With Genital Tract Infection

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KEYWORDS

Vancomycin, transferred, vancomycin-resistant

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. Aim: The aim of this study is to investigate the molecular transferring of vancomycin-sensitive Enterococcus faecalis to resistance isolates following multiple mixed subcultures with vancomycin-resistant Staphylococcus aureus in women with genital tract infection. 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. The collected swabs were cultured on various selective media, including blood agar, MacConkey agar, Sabouraud's dextrose agar, azide blood agar, chromogenic agar (UTIC), and CHROM agar candida. Real- Interpretation of RT-PCR data relied on Ct values, which indicate the cycle number at which the fluorescence signal surpasses the baseline threshold. Higher Ct values correspond to lower gene expression or amplification, while lower Ct values indicate higher gene expression or amplification. The accuracy of gene amplification was assessed by the Ct values obtained from triplicate reactions. Results: A high percentage of isolates show resistance to several antibiotics, such as Ceftriaxone (71.43%), Penicillin G (82.86%), Vancomycin (85.71%), Levofloxacin (80%), Ampicillin (80%), Oxacillin (80%), Imipenem (80%), Chloramphenicol (80%), Gentamycin (100%), and Cefotaxime (100%). Among the cases of vancomycin-resistant E. faecalis, 18 isolates (60%) tested positive for the Van A gene, 8 isolates (26.67%) for the Van B gene, and 3 isolates (10%) for both Van A and Van B genes. Only 1 isolate (3.33%) tested negative for both genes. The study demonstrated that repeated mixed subcultures of vancomycin-resistant Staphylococcus aureus (S. aureus) with vancomycin-sensitive Enterococcus faecalis (E. faecalis) resulted in a notable change in the resistance status of both bacterial species over time. On the 4th to 10th days, all S. aureus isolates were vancomycin-resistant, while 100% of E. faecalis isolates remained vancomycin-sensitive. This pattern continued on the 22nd, 28th, and 34th days, with fluctuations in resistance percentages. By the 40th day, significant changes are observed, with the percentage of vancomycin-resistant S. aureus remaining stable at 80%, indicating a persistent resistance phenotype, and the percentage of vancomycin-sensitive E. faecalis decreasing from 100% to 20%, highlighting a substantial shift in resistance status over time.

1. 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 (BV), aerobic vaginitis (AV), 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.

2. 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),

Primers

The source of all primers used in this study was Macrogen® (Korea). The name, sequence and product size are given in table **(1).Table 3. 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
VanA	F-CAAGTCAGGTGAAGATGGATC R-GCAGAGTATTGACTTCGTTCA	629 bp	Newly Designed
VanB	F-GTATGGAAGCTATGCAAGAAG R-ACAAAGACAGGGTAGGTAAG	365 bp	Newly Designed

3. Methods

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 Table **Cycling program**

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

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.

4. Results and Discussion

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.

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

In the current study, a high percentage of isolates show resistance to several antibiotics, such as Ceftriaxone (71.43%), Penicillin G (82.86%), Vancomycin (85.71%), Levofloxacin (80%), Ampicillin (80%), Oxacillin (80%), Imipenem (80%), Chloramphenicol (80%), Gentamycin (100%), and Cefotaxime (100%). On the other hand, antibiotics like Ciprofloxacin (34.29%), Augmentin (54.29%), Nitrofurantoin (42.86%), and Amikacin (71.43%) demonstrate varying degrees of sensitivity, Table 4

Table 4: Antibiotics sensitivity of E. faecalis isolates causing vaginitis

Antibiotics	Sensitive		Resistant	
	No.	%	No.	%
Amikacin	10	28.57	25	71.43
Ceftriaxone	10	28.57	25	71.43
Penicillin G	6	17.14	29	82.86
Ciprofloxacin	23	65.71	12	34.29
Vancomycin	5	14.29	30	85.71
Augmentin	16	45.71	19	54.29
Levofloxacin	7	20	28	80
Ampicillin	7	20	28	80
Nitrofurantoin	20	57.14	15	42.86
Oxacillin	7	20	28	80
Gentamycin	0	0	35	100
Cefotaxime	0	0	35	100
Imipenem	7	20	28	80
Chloramphenicol	7	20	28	80

Table 4.13 reports the results of PCR detection of Van A and Van B genes in vancomycin-resistant E. faecalis isolates causing vaginitis, providing both the number and percentage of isolates positive for each gene or combination. Among the cases of vancomycin-resistant E. faecalis, 18 isolates (60%) tested positive for the Van

A gene, 8 isolates (26.67%) for the Van B gene, and 3 isolates (10%) for both Van A and Van B genes. Only 1 isolate (3.33%) tested negative for both genes. Table 5

Table 5: PCR detection of Van A and Van V genes of E. faecalis isolates causing vaginitis

Vancomycin resistant E. faecalis	No.	%
Van A gene positive	18	60
Van B gene positive	8	26.67
Van A and Van positive	3	10
Negative	1	3.33
Total	30	100

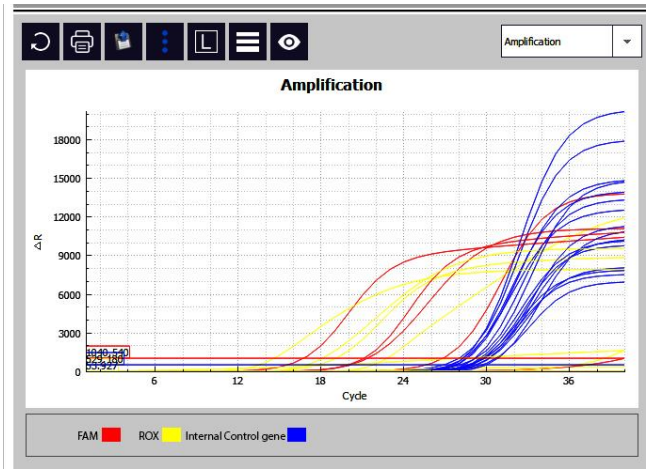

Figure 1: Detection of Van A gene by real time PCR

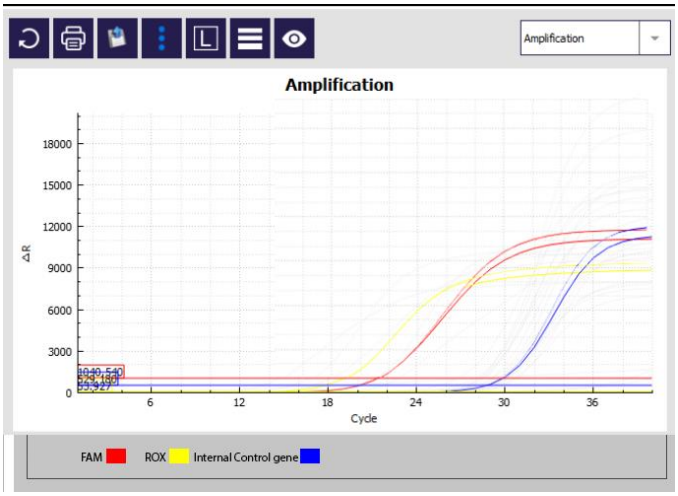

Figure 2: Detection of Van B gene by real time PCR

Table 6 details the conversion of vancomycin-sensitive E. faecalis to resistance isolates after multiple mixed subcultures with vancomycin-resistant S. aureus at different time points after the 1st mixed subculture. On the 4th to the 10th days, all S. aureus isolates are vancomycin-resistant, remained 100% resistance and 100% of E. faecalis isolates remained vancomycin-sensitive. As subcultures progress, on the 16th day, a shift occurs in the E. faecalis isolates, with 80% n demonstrated vancomycin resistance and 20% sensitivity. This pattern continued on the 22nd , 28th , and 34th days, with fluctuations in resistance percentages. By the 40th day, significant changes are observed. The percentage of vancomycin-resistant S. aureus remained stable at 80%, indicating a

persistent resistance phenotype. In contrast, the percentage of vancomycin-sensitive E. faecalis decreases from 100% to 20%, highlighting a substantial shift in resistance status over time. These results highlight the dynamic interplay between vancomycin-resistant S. aureus and vancomycin-sensitive E. faecalis over time.

Table 6: Transferring of vancomycin-sensitive E. faecalis to resistance isolates after multiple mixed subcultures with vancomycin-resistant S. aureus

Days after 1st mixed subculture	Mixed culture of S. aureus and E. faecalis							
	S. aureus				E. faecalis			
	Vancomycin resistance		Vancomycin sensitive		Vancomycin sensitive		Vancomycin resistance	
	No.	%	No.	%	No.	%	No.	%
4th day	5	100	0	0	5	100	0	0
10th day	5	100	0	0	5	100	0	0
16th day	5	100	0	0	4	80	1	20
22nd day	5	100	0	0	4	80	1	20
28th day	5	100	0	0	4	80	1	20
34th day	5	100	0	0	3	60	2	40
40th day	4	80	1	20	1	20	4	80

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.*, (11) when showed that vaginitis symptoms have multiple etiologies. In agreement with our funding, study done by Ali *et al.*, (12) showed that, the prevalence of *E. faecalis* among women with vaginitis was were 15.7%. Al-Kafajy *et al.*, (13) revealed that *Escherichia coli* were the most encountered frequency (37.2%) followed by *Staphylococcus aureus* with (27.9 %). *Enterococcus faecalis* (23.2%), *Gardnerella vaginali*s (16.2 %), and *Candida spp* (11.6 %) were less common. From a total of 602 vaginal swabs from pregnant women, Ghasemi *et al.*, (14) 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 ans the detection techniques applied (161). Daood *et al.*, (15) in similar study showed that *Enterococcus faecalis* forming 27 (21.2%) of the isolated bacteria (57.4%) of positive growth.

In the current study, a high percentage of isolates show resistance to several antibiotics, such as Ceftriaxone (71.43%), Penicillin G (82.86%), Vancomycin (85.71%), Levofloxacin (80%), Ampicillin (80%), Oxacillin (80%), Imipenem (80%), Chloramphenicol (80%), Gentamycin (100%), and Cefotaxime (100%). On the other hand, antibiotics like Ciprofloxacin (34.29%), Augmentin (54.29%), Nitrofurantoin (42.86%), and Amikacin (71.43%) demonstrate varying degrees of sensitivity. Antimicrobial susceptibility testing revealed that *E. faecalis* had a greater resistance pattern to most antibiotics tested, including cephalosporin and aminoglycoside. These results are similar to other studies done in earlier which that show most *E. faecalis* samples are resistant to most cephalosporin antimicrobials (16,17). In agreement with our finding, Ghasemi *et al.*, (14) indicated that, most isolates exhibit resistance to various antibiotics, while others show varying degrees of sensitivity, such as Ciprofloxacin, Augmentin, Nitrofurantoin, and Amikacin . Glycopeptide antibiotics remain the drug of choice for infections caused by resistant Enterococcus species (18). Vancomycin resistant Enterococci have emerged as important nosocomial pathogens in the last two decades throughout the world(19). Vancomycin resistant Enterococci are associated with many infections ranging from mild to life threatening. Extensive use of vancomycin to treat infections with MRSA has led to decreased susceptibility to vancomycin among Staphylococcus aureus. As of today very limited options are available for treating serious infections caused by vancomycin resistant Enterococci (VRE) and vancomycin resistant Staphylococcus aureus (VRSA) (196). In regard of vancomycin resistance, several studies reported that the most common isolated E. faecalis was with vanA genes (20,21). Most VRE outbreaks in human populations are attributed to the vanA and vanB gene

clusters (22,23). Nasaj *et al* (24) in similar study found that, vanA-type was the most common genotype seen among VRE strains and vanB is the second. Praharaj *et al* (25) indicated that, majority of the VRE isolates have the vanA gene, 3.3% of VRE isolates followed by vanB. In another study, the frequency of genes vanA and vanB among VRE strains were identified 85% and 37.5% respectively (26). In a study done by Sulaiman *et al* (27) , they showed that 88.89% of VRE strains were observed with *Van A* gene production 77.78% were observed with *Van B* gene production as detected by real-time PCR (P<0.001). Increase in the prevalence of VRE, especially E. faecalis, in different countries has been attributed mainly to the incidence and diffusion of vanA and vanB positive VRE, which exhibited some virulence factors such as *gelE* and *cylA* (28,29). The extracellular surface protein (esp), encoded by the chromosomal esp gene, found on pathogenicity island in multidrug-resistant pathogenic lineages of E. faecalis strains. Esp is a cell wall-associated protein which contribute to the colonization and persistence of E. faecalis strains in ascending infections of the urinary tract. In addition, Esp may participate in biofilm formation, and may also be involved in antimicrobial resistance (30). The study reveals that vancomycin-sensitive E. faecalis and vancomycin-resistant S. aureus isolates transition to resistance over time. From 4-10 days, all S.

aureus isolates are vancomycin-resistant, while E. faecalis isolates remain vancomycin-sensitive. By 40 days, the percentage of vancomycin-resistant S. aureus remains stable at 80%, while the percentage of vancomycin-sensitive E. faecalis decreases to 20%. The vanA, vanB, vanG, vanM and vanN operons are transferable between bacteria. The distribution of these van operons in enterococci has been reviewed by Ahmed and Baptiste (31). Previous research, such as the study by Christensen *et al* (32), has also demonstrated the ability of E. faecalis bacteria to develop resistance to vancomycin which is considered a last-resort antibiotic for treating multidrug-resistant bacterial infections. The observed transition of S. aureus isolates to vancomycin resistance over time may be attributed to the acquisition of resistance determinants, such as the vanA or vanB genes, through horizontal gene transfer or genetic mutations (33.34). Studies by Jones et al. (Year) have highlighted the role of genetic mechanisms in conferring vancomycin resistance in S. aureus strains. McGuinness *et al* (35), have investigated the *in vitro* transfer of vancomycin resistance from vancomycin-resistant Enterococcus (VRE) to Staphylococcus aureus, they found that the resistance was transferred through the horizontal transfer of the Tn1546 transposon containing vanA gene from VRE strains. The resistance was maintained even after overnight passages on MSA plates containing subinhibitory levels of vancomycin. In a parallel previous study De Niederhäusern *et al* (36), have documented that enterococci are an important source of glycopeptide resistance genes for Listeria monocytogenes via the transfer of movable genetic elements, confirming that the Tn1546 is carried by wide host-range plasmids able to overcome the species barrier. In 1992 Noble *et al* (37), have recognised that vanA gene transfer from Enterococci to Staphylococci was achieved through in vitro techniques and showed that the transfer could be achieved using vancomycin as a selective agent, but not using filter mating. The stability of vancomycin resistance phenotypes over time, particularly in S. aureus isolates, suggests the establishment of stable resistance mechanisms within the bacterial population(38,39). In the majority of the cases, the patients with VRSA infection were also infected with a vancomycin-resistant Enterococcus (VRE) isolated at the time of VRSA isolation (40). The high level of vancomycin-resistance in VRSA isolates therefore seems to involve the horizontal transfer of the Tn1546 transposon containing the vanA gene, frequently harboured in a plasmid belonging to the Inc18 plasmid family (40,41), from coinfecting VRE strains. Weigel *et al* (42), Severin *et al* (43) in two different experiments demonstrated the ‘‘in vitro’’ vancomycin-resistance transfer from clinical VRSA to the MRSA strain COL, reinforcing concerns about potential widespread of vancomycin resistance.

Conclusions

- Escherichia coli, Staphylococcus aureus, and Enterococcus species were among the most commonly isolated bacteria. Enterococcus faecalis isolates exhibited various virulence factors, with notable percentages.
- The majority of cases tested positive for the Van A gene, followed by the Van B gene. Co-occurrence of both genes was also observed in a subset of isolates.
- The study demonstrated the dynamic interplay between vancomycin-resistant S. aureus and vancomycin-sensitive E. faecalis over time. Following mixed subcultures, all S. aureus isolates remained vancomycin-resistant, while a notable shift occurred in E. faecalis isolates, with a gradual decrease in vancomycin sensitivity and an increase in resistance percentages over time.

Recommendations

- Continuously monitor antibiotic resistance patterns among Enterococcus faecalis isolates causing vaginitis to guide empirical treatment decisions and inform local antibiotic prescribing guidelines.
- Foster collaboration between microbiologists, infectious disease specialists, gynecologists, and pharmacists to develop comprehensive treatment guidelines for vaginitis management.
- Conduct longitudinal studies to assess the long-term outcomes and clinical implications of the dynamic interplay between vancomycin-resistant S. aureus and vancomycin-sensitive E. faecalis.

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