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In Vitro Investigation and Molecular Study of Normal Flora Isolated from Infant Stool against MDR and PDR Bacterial Vaginosis Among Women in Erbil City

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Abstract:

In women of reproductive age, bacterial vaginosis (BV) is the most common lower genital tract infection globally. A fishy malodor and a thin vaginal discharge are common symptoms shared by women with BV.

BV causes the vagina to become less acidic than normal, which leads to an increase in "bad" bacteria. Although women of any age can have BV, those in reproductive age (15–41 years old) are more vulnerable. BV cannot be spread through swimming pools or toilets, nor is it a sexually transmitted disease.Using Vitek 2 Compact technology, 50 pathogenic bacterial specimens were extracted from women who had been diagnosed with vaginosis. These specimens were then analyzed for species identification and antibiotic sensitivity profiling.In women with bacterial vaginosis, *S. aureus, E. coli,* and *K. pneumoniae* are the most often discovered bacteria .Antibiotic resistance profiling showed concerning patterns: 14% of the specimens showed signs of pandrug resistance (PDR), 25% showed signs of extended drug resistance (XDR), and 50% showed signs of multidrug resistance (MDR).Two approaches are used to assess the biofilm test. Congo red staining method and tissue culture plate method. 20 Beneficial bacteria strains were extracted from infant fecal and used as normal flora against pathogenic bacteria by method called agar well diffusion method, only 2 bacteria used (*K. pneumoniae and P. aeruginosa).*The efficiency of bactericidal treatment was assessed using zone of inhibition analysis. zones with a diameter of 24–28 mm. Additionally, these probiotic organisms were able to significantly reduce the biofilm formation capacity of both high and moderate biofilm-forming isolates., . Beneficial bacterial strains isolated from the intestinal flora of newborns show great potential as innovative treatment options for treating pathogens that cause vaginosis and are resistant to antibiotics. These organisms are interesting candidates for probiotic-centered interventions in treating persistent and treatment-resistant vaginosis infections because of their combination bactericidal and biofilm-inhibiting properties, as well as their biological origin and proven safety parameters.While integron genetic elements (classes I and II) were found in pathogenic specimens using multiplex polymerase chain reaction with percent 100%.

Keywords: Bacterial vaginosis, Biofilm, Normal flora, Multi-drug resistance MDR.

1 INTRODUCTION

Vaginitis is an inflammation of the vagina, and the most common causes of it are trichomoniasis, vulvovaginal candida, and bacterial vaginosis [1]. BV defined a change in the balance of the vaginal microflora, characterized by an increase in facultative and anaerobic bacteria in number and/or type, a decrease in lactobacilli, which are primarily species that produce hydrogen peroxide, and a rise in the pH of the vagina, is known as bacterial vaginosis [2]. Although many women report vaginal discharge and a fishy smell, up to 50% may not have any symptoms.[3]. Accoording to estimates, the prevalence of bacterial vaginosis varies significantly between countries, even within the same population. It can vary from 8% to 75% [4]. The bacteria that cause BV are primarily intestinal in origin. *Staphylococcus aureus(S.aureus)*,*Streptococcus agalactiae (S.agalactiae*), *Enterococcus species, E. coli,* and *K. pneumoniae* are the most often isolated bacteria in BV patients [5].

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After taking antibiotics, BV has a significant recurrence rate [6]. Since antibiotics haven't been able to permanently cure BV, many women and medical professionals are looking for other treatments [6]. Bacterial vaginosis is a common side effect of the available treatment strategies. Within 6–12 months of finishing antibiotic treatment, between 50% and 80% of women will experience another bout of bacterial vaginosis.[6]. Antimicrobial resistance, biofilm, reinfection through sexual partners, and the inability to restore a health-optimal vaginal microbiota are some of the hypothesized causes of this therapeutic failure [7]**.** It is common to find bacteria that are resistant to most of the antibiotics that are now on the market, and in certain cases, to all of them. These microbes are referred to as Extensively Drug-Resistant (XDR), Multidrug Resistant (MDR) and Pan Drug- Resistant (PDR). Bacteria diverse definitions are given to the diverse resistance patterns of MDR, XDR, and PDR bacteria. The terms MDR, XDR, and PDR represent acquired nonsusceptibility to at least one agent in three or more antimicrobial categories, nonsusceptibility to all agents in all antimicrobial categories, and nonsusceptibility to at least one agent in all but two or fewer antimicrobial categories (i.e., bacterial isolates retained susceptibility to only one or two categories), respectively [8]. A community of bacteria known as a biofilm is entrenched in polysaccharide intercellular adhesion (PIA), which enables the bacteria to adhere to both biological and non-biological surfaces, endure for extended periods of time, produce recurring and chronic infections, and strengthen resistance to antibiotics [9]. Using Congo red stain and a tissue culture plate methodology , is the most widely used method and is regarded as the standard strategy for detecting the formation of biofilms, while the other offers a straightforward qualitative method for detecting the formation of biofilms using the Congo Red Agar (CRA) method [10].The antagonistic impact or capability includes adherence to the intestinal wall, a decrease in the intestinal adhesion of pathogenic bacteria, aggregation and coaggregation, and the generation of antimicrobial compounds such as bacteriocins. Many lactic acid bacteria that are found in the intestinal tracts of humans and animals and are derived from dairy-fermented foods have been extensively described as having antagonistic properties to probiotic potential [11]. These bacteria produce lactic acid, hydrogen peroxide, and bacteriocin, which operate as a barrier against vaginal pathogens that cause vaginitis and compete with harmful bacteria in the vaginal ecosystem. Lactobacilli have also been demonstrated to increase vaginal acidity, which lowers the incidence of STIs and pregnancy-related illnesses [12]. Through the acquisition, storage, disposal, and use of reading frameworks in mobile elements known as cassettes, integrons-ancient structures-mediate the evolution of bacteria. In roughly 17% of the bacterial chromosomes, they are found [13]. Integrons have been regarded as natural expression cloning systems because they may integrate open reading frames and transform them into functional genes through site-specific recombination. There are three classes into which integrons fall [14,15]. Integrons are thought to be important contributors to the emergence and dissemination of antibiotic resistance, despite the fact that they are not mobile in and of themselves. among microorganisms that are Gram-negative bacteria They share several traits with one another, including antibiotic resistance integrons. For example, they typically move about and have a brief cassette sequence that is frequently encoded for antibiotic resistance [16,17].

2 MATERIALS AND METHODS

Study design and sample collection: This cross-sectional study was conducted on bacterial vaginosis and infant stools that annually attend the Maternity Teaching Hospital and Raparen Hospital. A total of 150 pathogenic bacteria were collected from high vaginal swab specimens of women with genital tract infections. A total of 20 normal flora bacteria were collected from infant stool.The patients were of different ages, Much information was taken directly from each patient using a special questionnaire sheet, and the information included : socio-demographic information like age, gender, pregnant and non-pregnant, chronic diseases, abortion history, history of previous bacterial vaginosis, symptomatic and asymptomatic, and was filled out by all patients.

Isolation and identification of microorganisms

Direct inoculation of the vaginal swab samples was performed on culture media: Blood agar and MacConkey agar were cultured aerobically for 24 to 48 hours at 37˚C. [18]**.** Pure colonies of isolated microorganisms were identified using morphological and culturing [19]. Species identification for pathogens was performed using the Vitek 2 compact system [20].

Antibiotic Susceptibility

Antibiotic susceptibility testing of all the bacterial isolates was performed using (AST-GP) and (AST-GN) cards of the VITEK 2 System according to the manufacturer's instructions (BioMérieux/ France). Antibiotics used in this study belong to 8 classes (10 antibiotics) for both Gram-positive bacteria and Gram-negative bacteria.[21].

Biofilm detection

All bacteria isolates were tested to detect biofilm formation using the Tissue culture plate method and the Congo red agar plate method.

Tissue culture plate method (TCPM)

All bacteria from the vagina were cultured using brain-heart infusion (BHI), which comprised brain-heart infusion broth (AOBOX, Beijing, China), 0.3% starch, and 0.3% glucose [22]. After being diluted to 1 x**10** FU/mL and adjusted to 0.5 McFarland (1.5 × 108 CFU/mL), the overnight growth was transferred to a 96-well plate (Falcon, Corning Inc., NY, USA). This was followed by an anaerobic incubation at 37°C (AnaeroPouch-Anaero, C-1, Mitsubishi Gas Chemical CO., INC., Tokyo, Japan). Following incubation, 1× phosphate buffer saline (PBS) was used to wash the wells, and the bacterial solution was disposed of (P1020, Solarbio, Beijing, China). 0.2 percent crystal violet (C8470, Solarbio, Beijing,

China) was used to stain the samples. They were then rinsed with 1× PBS and decolored with 95% alcohol. An enzyme marker at 580 nm was used to determine the eluate's OD value. The mean value of the negative controls, which contained solely sBHI, plus three times the standard deviation, was used to calculate the OD cutoff value (ODc). The OD value of the microtiter wells was then divided by the ODc to determine the biofilm forming capability. [23] All analyses were repeated three times on different dates.

Congo red agar plate method

37 g/L BHI broth, 10 g/L agar base, 50 g/L sucrose, 1 L water, and 0.8 g/L Congo Red indicator make up the Congo Red Agar medium. In addition to other media components, Congo Red indicator was made as a concentrated liquid and autoclaved for 15 minutes at 121°C before being added to cooled agar at 55°C. After that, the isolate was injected and incubated at 37°C for 24 hours. Colonies that were black demonstrated a positive outcome. Pink colonies indicated a negative outcome [24].

Detection of ESBL by Double Disc Diffusion Test (for BV)

The turbidity of a bacterial suspension was corrected to a McFarland standard of 0.5 The suspension was streaked on Mueller Hinton agar plates by CLSI recommendations. On one side of the plate, a disc containing Amoxicillin (20µg/ml) and Clavulanic acid (10µg/ml) (AMC) was positioned. A single disc containing 30 µg/ml of ceftazidime (CASG) was positioned on the plates, 1 cm from one edge to the other, from a disc containing amoxicillin and clavulanic acid. For 18 to 24 hours, the plates were incubated at 37˚C. Following incubation, a positive result was defined as an increased zone of inhibition between amoxicillin clavulanic acid and any one of the β-lactam discs [25,26].

AmpC Disk Test

A Mueller Hinton agar plate was covered with tested isolates whose turbidity was equal to 0.5 McFarland standards. Following an overnight incubation period, isolates exhibiting blunting of the ceftazidime zone of inhibition adjacent to the ceftriaxone disk or decreased susceptibility to each of them were screened as positive for AmpC β-lactamase production. Ceftazidime (30µg/ml) and Ceftriaxone (30µg/ml) disks were positioned 20 mm apart from center to center [27].

Isolation and identification of the normal flora

Twenty healthy newborns (17 boys and girls), of which 3 were delivered vaginally and 17 by cesarean section (fecal samples from 20 newborns aged from 1-9 weeks and at the Raparen Hospital-Erbil and were properly labeled and quickly transferred on ice to the lab for culturing and identification [29].The newborn infants who were found to be healthy were picked at random and then cultured on MacConkey agar (MA) for identification using the vitek 2 compact system [30].

Antibacterial activity of normal flora bacteria

The antibacterial activity of normal flora bacteria was detected against pathogenic bacteria by using method called agar well diffusion methods to detect the antagonistic effect.

Agar well diffusion methods

A common technique for assessing the antimicrobial activity of microbial extracts is the Agar well diffusion method [32]. The agar plate surface is infected by distributing a volume of the microbial in oculum throughout the whole agar surface, just like in the disk-diffusion approach. A volume (20-100 mL) of the antimicrobial agent or extract solution at the required concentration is then added to the well after a 6–8 mm sterile cork-borer or tip has been used to aseptically punch the diameter hole. Then, depending on the test microbe, agar plates are incubated under the appropriate conditions. Diffusing throughout the agar media, the antimicrobial ingredient prevents the tested microbial strain from growing [33].

Extraction of Genomic DNA

The genomic DNA was extracted from vaginal samples by using (the Beta Bayren extraction kit (Beta Bayern GmbH, Germany) and by following the instruction of the protocol of the manufacturer. The first step was weighing the frozen sample by analytical balance. The sample could not thaw until adding the lysis buffer (Inhibit EX Buffer) to the vaginal sample to avoid the degradation of the DNA. The extracted DNA was stored at −20 °C for less than six months until used for running the PCR. The purity of the extracted DNA was measured by the Nanodrop [34.35].

Amplification of bacterial vaginosis DNA by Multiplex PCR and primers

To identify and knowing molecular characterization of BV integron class I, II gene by Multiplex PCR. The integron gene represent a large genetic element. Most research studies use the same designed primer. Primers were used for amplification of (integron) gene; two sets of oligonucleotides were used, (IntI1-F: 5'-CCTCCCGCACGATGATC-3'; IntI1-R: 5'-TCCACGCATCGTCAGGC-3') and class II integron primers (IntI2-F:5'-GTAGCAAACGAGTGACGAAATG-3'; IntI2-R: 5'-CACGGATATGCGACAAAAAGGT-3') were synthesized commercially (Microgene, South Korea) [36].

Multiplex PCR condition

Due to its ability to simultaneously amplify numerous target DNA/RNA sequences from several diseases in a single reaction, multiplex PCR (mPCR) has become a revolutionary diagnostic tool. The reaction conditions included initial denaturation at 94 °C for 4 minutes, followed by 30 cycles of denaturation at 94 °C for 45 seconds, annealing at 55 °C for 45 seconds, and elongation at 72 °C for 55 seconds, with a final elongation of 8 minutes. Amplification products were stored at −20 °C. Primer design for class II integrons ensured similar annealing temperatures and distinguishable electrophoretic bands. Gradient PCR (51–60 °C) was used to optimize annealing temperature, and 55 °C was selected for

multiplex PCR. Five microliters of PCR products were loaded onto 2% agarose gel containing ethidium bromide, and electrophoresis was performed in 0.5× TBE buffer at 100 V for 30 minutes. Bands corresponding to class I and II integrons were visualized using a Kodak gel imaging system [37].

2.1 STATISTICAL ANALYSIS

Data from the questionnaire and laboratory investigation were analyzed using SPSS version 26. Descriptive statistics were used to summarize the data. Chi-square was used to assess differences in the proportions of BV.

3 RESULTS

A total of 150 HVS collected in Maternity Teaching Hospital, in Erbil City. only 50 have bacterial vaginosis 100%. As shown in Figure 1 the bacteria that collected from women with genital tract infection classified in two types gram positive bacteria (48%) and gram-negative bacteria (52%). Most predominate bacteria are *E. coli* and *K. pneumoniae* followed by *staphylococcus species, streptococcus agalactiae, P. aeruginosa, Enterobacter species and Enterococcus facials.* The age range of the participants was between 20-29 (16%), 30-39 (25%),40-49 (15%), And over 50 (2%) years age. All female with BV in this study married (92.0%) only about 8% percent was divorced . Table 1 shows that the pregnant women are less than non-pregnant women, As the percentage of pregnant women with BV is estimated to be 20% while its 80% of non-pregnant women's this ratio varies from country to country some countries may be have opposite results.

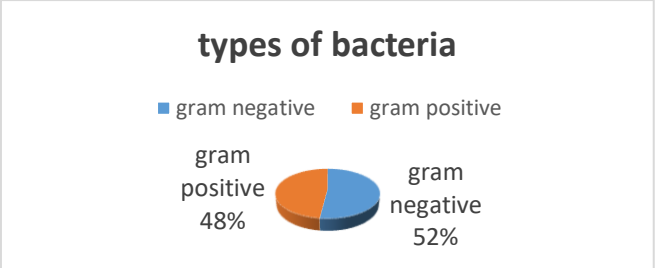


FIGURE 1. Total presences of bacterial vaginosis among positive result.

Table 1. Frequency of socio demographic characteristics of the patients with BV.

Variable	Category	Frequency	Percentage (%)
Sample collection	High Vaginal Swab	50	100.0%
Institution	Maternity Teaching Hospital	50	100.0%
Bacterial classification	Gram - positive bacteria	24	48.0%
	Gram -negative bacteria	26	52.0%
Material Status	Married	46	92.0%
	Divorced	4	8.0%
Age Groups	20-29	8	16.0%
	30-39	25	50.0%
	40-49	15	30.0%
	≥ 50	2	4.0%
Pregnant status	Pregnant	10	20.0%
	Non-pregnant	40	80.0%

The total sensitivity of all bacterial isolates was highest with at levofloxacin (56.0%) and The highest resistance was observed with clindamycin 72.0% with sensitive at 26.0% as shown in Figure 2 . 48.0% of isolates had (MDR), with the most common strains being E. coli (60.0%) and K. pneumoniae (53.8%). Pseudomonas aeruginosa exhibited 100% (PDR) and (XDR) traits. Furthermore, 2.0% displayed PDR phenotypes while 14.0% displayed XDR patterns.

A biofilm test was conducted on bacteria taken from women's using two methods tissue culture plate method (TCPM) with ELISA, Congo red stain method (CRSM). the result from first methods were more accurate than the second method. This finding shows high biofilm formation, with 41 (82%) producing strong biofilms and 5 (10%) producing moderate

biofilms. Only four isolates (8%) produced weak or non- biofilm and most bacteria that produce biofilm is *S. aureus* (Figure 3) However the second method showed black colonies indicating the presence of biofilm formation while colonies in red color indication of non-biofilm formation.

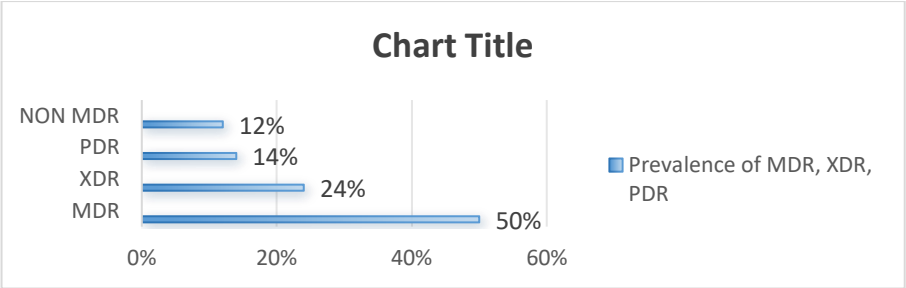


FIGURE 2. Prevalence of MDR, XDR, PDR phenotypes among bacterial vaginosis.

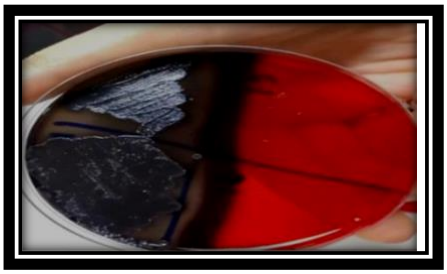


FIGURE 3. Biofilm formation by Congo Red Agar Method A: Black colonies indicate biofilm producers. B: Red colonies are non-Biofilm producers.

Table 2. Prevalence of ESBL Production in MDR, XDR, PDR, and Non-MDR Bacterial Isolates.

Bacteria isolates	Resistance pattern	Total (n)	ESBL positive	ESBL negative
Staphylococcus spp.	MDR	11	0 (0,0%)	11 (100.0)
	XDR	4	2(50.0%)	2(50.0)
	PDR	2	0(0.0%)	2(100.0)
	Non-MDR	1	0(0.0%)	2(100.0)
E. coli	MDR	4	3 (75.0%)	1(25.0%)
	XDR	4	4(100.0%)	0(0.0%)
	PDR	4	4 (100.0%)	0(0.00%)
	NON-MDR	4	0 (0.0%)	0(0.0%)
K. pneumoniae	MDR	6	6(100.0%)	0(0.0%)
	XDR	2	2(100.0%)	0(0.0%)
	PDR	1	1(100.0%)	0(0.0%)
	NON-MDR	3	1(33.3%)	2(66.7%)
S. agalactiae	MDR	3	0(0.0%)	3(100.0%)
E. faecalis	MDR/XDR	2/1	0(0.0%)	3(100.0%)
P. aeruginosa	PDR	1	1(100.0%)	0(0.0%)
Enterobacter spp.	MDR	1	1(100.0%)	0(0.0%)
Statistical analysis		P value 0.0007		

Chi-square testing demonstrated significant correlation between ESBL synthesis and resistance phenotypes ($p = 0.0007$). Higher resistance categories (XDR/PDR) showed increased β-lactamase production compared to MDR and susceptible isolates, indicating species-dependent enzyme expression pattern. According to Table 3, The test for ESBL shows that the gram-negative bacteria produce more ESBL than the gram-positive bacteria, *E. coli and K. pneumoniae* being the highest producers for ESBL enzymes with rates of 81.3% and 91.7%.

Table 3 shows the bacteria isolates from women with vaginal infection have the ability to produce AMPC B- lactamase, an important resistance mechanism linked with a reduction in β-lactam antibiotic susceptibility, it's important to mention that gram-negative bacteria produce more AMPC B-lactamase than gram positive bacteria such as *E. coli a*nd *K. pneumoniae* as shown in Table 4.

Table 3. Prevalence of Phenotypic AMPC detection among bacterial vaginosis.

Bacterial Species	Total (n)	AmpC Positive n (%)	AmpC Negative n (%)
Staphylococcus spp MDR (n=11) , XDR (n=4), PDR(n=2)NON-MDR (n=1)	18	1 (5.6)	17 (94.4)
E. Coli MDR (N=4) XDR(N=4) PDR(N=2) NON MDR(N=2)	12	12 (100.0)	0 (0.0)
K. Pneumoniae MDR(N=6) XDR(N=2) PDR(N=1) NON MDR(N=3)	12	11 (91.7)	1 (8.3)
S. agalactiae MDR(N=3) XDR(N=0) PDR(N=0) NON MDR(N=0)	3	0 (0.0)	3 (100.0)
E. faecalis MDR(N=2) XDR(N=1) PDR(N=0) NON MDR(N=0)	3	1 (33.3)	2 (66.7)
P. aeruginosa MDR(N=0) XDR(N=0) PDR(N=1) NON MDR(N=0)	1	1 (100.0)	0 (0.0)
Enterobacter spp. MDR(N=1) XDR(N=0) PDR(N=0) NON MDR(N=0)	1	1 (100.0)	0 (0.0)
Total	**50**	**27 (54.0)**	**23 (46.0)**
Statistical analysis	P-value < 0.001 (Chi-square test)		

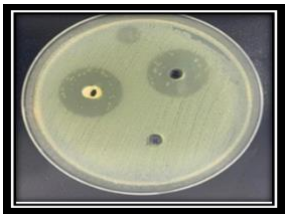
these findings show that MDR, XDR, and PDR bacterial isolates produce substantially more AMPC enzymes than non-MDR isolates. This implies that the AMPC enzyme is highly correlated with increased resistance levels, which could support the resistance mechanism in these bacteria.

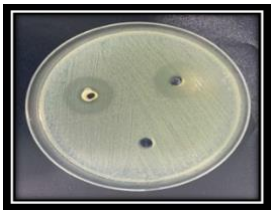
Table 4. Percentage of anti-biofilm activity of normal flora against MDR-XDR and PDR bacterial vaginosis.

Bacteria isolates	Resistance	Pre-treatment	Post-treatment (K)	Post-treatment (P)	% Reduction
S. aureus (1)	MDR	3.93	0.548	0.171	96.07%
S. aureus (2)	MDR	1.27	0.262	0.205	98.73%
S. aureus (3)	MDR	2.52	0.267	0.132	97.48%
S. aureus (4)	MDR	2.73	0.303	0.42	97.27%
S. aureus (5)	MDR	1.29	0.116	0.233	98.71%
S. aureus (6)	MDR	1.42	0.441	0.351	98.58%
S. hemolytic (7)	MDR	1.23	0.377	0.23	98.77%
S. hemolytic (8)	MDR	2.941	0.379	0.21	97.05%
S. hemolytic (9)	XDR	2.944	0.347	0.115	97.05%
S. hemolytic (10)	PDR	3.28	0.262	0.178	96.72%
S. saprophytic (11)	PDR	2.89	0.25	0.289	97.11%
S. saprophytic (12)	MDR	1.90	0.324	0.432	98.1%
S. saprophytic (13)	MDR	2.14	0.081	0.231	97.86%
S. epidermis (14)	XDR	2.37	0.133	0.203	97.63%
S. epidermis (15)	MDR	2.86	0.202	0.204	97.14%
S. epidermis (16)	MDR	2.50	0.152	0.21	97.5%
S. epidermis (17)	XDR	2.10	0.239	0.184	97.9%
S. pseudintermedius (18)	MDR	3.69	0.199	0.144	96.31%
E. coli (1)	MDR	3.44	0.214	0.315	96.56%
E. coli (2)	MDR	3.95	0.356	0.15	96.05%
E. coli (3)	MDR	3.88	0.093	0.256	96.12%
E. coli (4)	MDR	3.98	0.173	0.224	96.02%
E. coli (5)	XDR	3.67	0.137	0.273	96.33%
E. coli (6)	XDR	3.62	0.172	0.348	96.38%
E. coli (7)	XDR	3.26	0.325	0.225	96.74%
E. coli (8)	XDR	3.41	0.216	0.154	96.59%
E. coli (9)	PDR	3.90	0.215	0.11	96.1%
E. coli (10)	PDR	3.42	0.219	0.525	96.58%
Klebsiella (1)	MDR	2.75	0.172	0.426	97.25%
Klebsiella (2)	MDR	3.969	0.170	0.26	96.03%
Klebsiella (3)	MDR	3.991	0.137	0.223	96.00%

Klebsiella (4)	MDR	3.421	0.356	0.287	96.57%
Klebsiella (5)	MDR	2.943	0.325	0.253	97.05%
Klebsiella (6)	MDR	3.921	0.449	0.138	96.07%
Klebsiella (7)	XDR	3.682	0.207	0.151	96.31%
Klebsiella (8)	XDR	3.7	0.293	0.22	96.3%
Klebsiella (9)	PDR	1.657	0.211	0.371	98.34%
Klebsiella (10)	Non-MDR	1.235	0.162	0.322	98.76%
Klebsiella (11)	Non-MDR	0	0	0	0%
Klebsiella (12)	Non-MDR	1.273	0.131	0.282	98.72%
S. agalactiae (1)	MDR	1.559	0.475	0.352	98.44%
S. agalactiae (2)	MDR	2.214	0.248	0.289	97.78%
S. agalactiae (3)	MDR	1.949	0.21	0.18	98.05%
E. faecalis (1)	MDR	0.911	0.252	0.162	99.08%
E. faecalis (2)	MDR	0.53	0.223	0.225	99.47%
E. faecalis (3)	XDR	1.33	0.357	0.12	98.67%
P. aeruginosa (1)	PDR	2.12	0.280	0.149	97.88%
Enterobacter (1)	MDR	2.125	0.090	0.422	97.87%
Statistical analysis			$p < 0.000001$		

A





B

FIGURE 4. Effect of normal flora as an antimicrobial agent: A: Agar-well diffusion method on gram negative B: Agar-well diffusion method on gram positive.

Table 5. the impact of normal flora on pathogen bacteria by agar well diffusion method.

Pathogen bacteria	**Size of inhibition zone**		**Reactions**
	By *Klebsiella pneumoniae*	**By *Pseudomonas aeruginosa***	
S aureus MDR *(1)*	*18mm*	*17mm*	*Positive*
S. aureus MDR *(2)*	*11mm*	*13mm*	*Positive*
S. aureus MDR (3)	*29mm*	*33mm*	*Positive*
S. aureus MDR *(4)*	*12mm*	*19mm*	*Positive*
S. aureus MDR *(5)*	*22mm*	*29mm*	*Positive*
S. aureus MDR *(6)*	*10mm*	*23mm*	*Positive*
S. hemolytic (7) MDR	*12mm*	*15mm*	*Positive*
S. hemolytic (8) MDR	*19mm*	*17mm*	*Positive*
S. hemolytic (9) XDR	*35mm*	*32mm*	*Positive*
S. hemolytic (10) PDR	*20mm*	*25mm*	*Positive*
S. saprophytic (11) PDR	*20mm*	*18mm*	*Positive*
S. saprophytic (12) MDR	*11mm*	*14mm*	*Positive*
S. saprophytic (13) MDR	*17mm*	*16mm*	*Positive*
S. epidermis (14) XDR	*22mm*	*23mm*	*Positive*
S. epidermis (15) MDR	*7mm*	*9mm*	*Positive*
S. epidermis (16) MDR	*10mm*	*17mm*	*Positive*
S. epidermis (17) XDR	*19mm*	*24mm*	*Positive*
S.peudintermidus(18) MDR	*No zone*	*No zone*	*Negative*
Escherichia coli (1) MDR	*10mm*	*8mm*	*Positive*
Escherichia coli (2) MDR	*17mm*	*20mm*	*Positive*
Escherichia coli (3) MDR	*35mm*	*30mm*	*Positive*
Escherichia coli (4) MDR	*24mm*	*28mm*	*Positive*
Escherichia coli (5) XDR	*33mm*	*37mm*	*Positive*
Escherichia coli (6) XDR	*14mm*	*18mm*	*Positive*
Escherichia coli (7) XDR	*11mm*	*15mm*	*Positive*
Escherichia coli (8) XDR	*16mm*	*19mm*	*Positive*
Escherichia coli (9) PDR	*14mm*	*16mm*	*Positive*
Escherichia coli (10) PDR	*22mm*	*22mm*	*Positive*

Klebsiella pneumonia (1) MDR	*30mm*	*33mm*	*Positive*
Klebsiella pneumonia (2) MDR	*37mm*	*33mm*	*Positive*
Klebsiella pneumonia (3) MDR	*25mm*	*29mm*	*Positive*
Klebsiella pneumonia (4) MDR	*19mm*	*23mm*	*Positive*
Klebsiella pneumonia (5) MDR	*22mm*	*29mm*	*Positive*
Klebsiella pneumoni (6) MDR	*11mm*	*14mm*	*Positive*
Klebsiella pneumonia (7) XDR	*16mm*	*19mm*	*Positive*
Klebsiella pneumonia (8) XDR	*20mm*	*24mm*	*Positive*
Klebsiella pneumonia (9) PDR	*33mm*	*38mm*	*Positive*
Klebsiella oxytoca (10) NON MDR	*Non- zone*	*Non- zone*	*Negative*
Klebsiella oxytoca (10) NON MDR	*Non- zone*	*Non- zone*	*Negative*
Klebsiella oxytoca (11) NON MDR	*Non- zone*	*Non- zone*	*Negative*
Streptococcus agalactia (1) MDR	*18mm*	*15mm*	*Positive*
Streptococcus agalactia (2) MDR	*27mm*	*24mm*	*Positive*
Streptococcus agalactia (3) MDR	*22mm*	*19mm*	*Positive*
Enterococcus faecalis (1) MDR	*12mm*	*10mm*	*Positive*
Enterococcus faecalis (2) MDR	*31mm*	*29mm*	*Positive*
Enterococcus faecalis (3) XDR	*25mm*	*22mm*	*Positive*
Pseudomonas aeruginosa (1) PDR	*20mm*	*0*	*Positive*
Enterobacter spp. (1) MDR	*20mm*	*18mm*	*Positive*

(Table 5and Figure 4) shows that a clear antagonistic effect of non-pathogenic bacteria (*K. pneumoniae and P. aeruginosa)* on pathogenic bacteria, where bacteria from healthy newborns taken and their effect on bacteria taken from women with BV. After that another test conducted to confirm the effect of non-pathogenic bacteria on bacteria that causing BV. The anti-biofilm test showed that the pathogenic bacteria had a strong to moderate biofilm, due to effect of this of this bacterium the biofilm was reduced to weak or non-biofilm formation. In this study, the integron class I, II gene used in all gram negative and gram-positive bacteria by Multiplex PCR, the result showed the presence of this gene in all bacteria isolated from women with BV at rate of 100%.

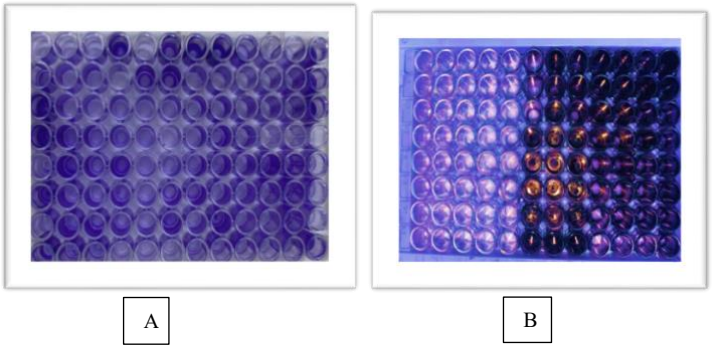
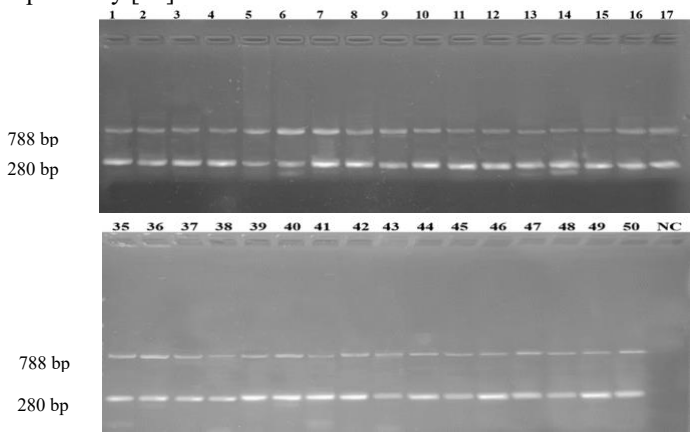


FIGURE 5. A: After treatment with crude normal flora, B: Before treatment with crude normal flora.

Integron genes I and II were employed in this study in all high vaginal swab samples using multiplex PCR. The results showed that the presence of these genes was present in all samples that were isolated from women with gastrointestinal tract infections at a percentage of 100%. This finding is unlike that of a study in Iran,Tehran.which showed that the presence of class I and II integrons showed that 70% of the isolates had class 1 integron, while class 2 integron was observed only in 3% of the strains [65]. Previous studies in other parts of the world also investigated the frequency of different classes of integrons. In a study conducted in 2008 by Farshad et al., 6.25% and 10.41% of strains had class 1 and class 2 integrons, respectively [66].



788 bp

4 DISCUSSION

From high vaginal swab samples of female patients with genital tract infection at Maternity Teaching Hospital in Erbil City,150 pathogenic bacteria were retrieved only fifty of them have BV. According to the results, gram-negative bacteria comprised 52% percent of pathogenic isolates, whereas gram-positive bacteria comprised 48% percent. gram-negative bacteria were more common in females with genital tract infection than gram-positive bacteria. The Both gram- positive and gram- negative bacteria were involved as causative agents of the infection (Figure1). The variation in the obtained results among published articles is considered a normal event, which may be due to several factors,such as geographic distribution, population size, data analysis, behavioral differences, and socioeconomic status .This finding unlike by [38]. and in Nepal by Ranjit et.al [39]. In this study, specifically 50%, belong to the age group of 31-40 years. This discovery may suggest that women within this specific age range exhibit a higher representation within the studied population .Moreover, it is noteworthy that the age cohort ranging from 21-30 years constitutes 16% and 41-50 years (30%) of the total sample, while the group aged over 50 years comprises 4% (table 1) the result Unlike with some study obtained by Bitew [40] and by [41] This may be attributed to an increased frequency of sexual intercourse in these women which may subsequently result in disruption of the protective physical barrier of vaginal mucosa and change of microflora [42]. The prevalence was higher among married women. The explanation for this observation was related to the fact that sexual relations, but also, the majority of patients in this study were married. Similar results were reported by [43]. In this study, the percentage of affected of pregnant women by BV is 20%, while among non- pregnant women it is 80% . This result varies from country to country and contrasts with finding obtained by [44] and [45. The high incidence of infection in pregnant women could be attributed to hormonal changes [46]. The differences between our results and others might be due to sample size, and our target populations were selected by physicians, only women patients with symptoms of vaginitis like abnormal vaginal discharge, itching, burning, and lower abdominal pain as shown in Table 1. Also found that the prevalence increased with gestational week, and this is related to our finding of increasing prevalence with trimester. We found the prevalence to be higher in the first trimester, which is unlike the finding obtained by [47]. The predominant gram-positive bacteria were *S. aureus* (18/50%)); on the other hand, the predominant gram-negative bacteria were *E. coli* (12/55%) and *K. pneumoniae* (12/45%) The results findings were in agreement with studies conducted in India *S. aureus* (41.07%),[48]. And Addis Ababa *E. coli* 43 (41%) and *K. pneumoniae* 28 (30.5%) [49]. As shown in figure 2, that Levofloxacin had the highest efficacy of all the studied antibiotics, with a sensitivity rate of 56.0% (28 out of 50 isolates). Levofloxacin was the most dependable treatment option in this investigation because it showed that over half of the bacterial isolates were responsive to it. And this finding is unlike that obtained in India by [50]. Clindamycin, on the other hand, demonstrated the greatest resistance rate of 72.0% and the lowest sensitivity rate of just 26.0%, suggesting a limited therapeutic impact against vaginal bacterial isolates. These findings emphasize how crucial it is to choose antibiotics according to patterns of local susceptibility in order to prevent treatment failure and the emergence of resistant bacteria, with study revealed that *Streptococcus agalactiae* has lower resistance to all classes, whereas *Pseudomonas aeruginosa* has higher resistance. The finding is not in agreement in Naples, Italy [51]. The current study found that 48.0% of the isolates were MDR, meaning they were resistant to at least one agent across three or more antimicrobial categories. *E. coli* and *K. pneumoniae* were the most common multidrug-resistant pathogens, affecting 60.0% and 53.8% of isolates, respectively. The most concerning example was Pseudomonas aeruginosa, which was 100% XDR and PDR, meaning it was resistant to every tested antimicrobial treatment. Furthermore, 14.0% of the isolates were found to be XDR. Despite the relatively modest overall rate PDR (2.0%), its existence highlights the possibility of significant therapeutic failures. The growing threat of resistant microorganisms is highlighted by these findings, which also emphasize the significance of prudent antibiotic usage and continuous resistance monitoring in clinical settings [52]. Similar results were reported by [53] A highly structured biofilm characterizes bacterial vaginosis. Clue cells are epithelial cells with a thick bacterial coating on their surface. The epithelium of healthy subjects did not exhibit adherent biofilms, in contrast to that of bacterial vaginosis [54]. We assessed (50) isolated bacteria using two screening techniques: the Congo red stain culture method and the tissue culture plate method (TCP) with ELISA to determine their capacity to form biofilms. Out of the 50 isolates as shown in Figure 3, identifying the biofilm formation factor, the tissue culture plate method is more sensitive than the Congo red stain method. This finding shows high biofilm formation, with 41 (82%) producing strong biofilms and 5 (10%) producing moderate biofilms. Only four isolates (8%) produced weak or biofilm. and were primarily observed in *Staphylococcus spp*., *E. coli,* and *Klebsiella sp*p. All isolates of *Streptococcus, Pseudomonas, and Enterobacter specie*s also produced strong biofilms. These results concurred with those published by Mathur et al. [55]. from India revealed that, in agreement with Bose et al., 53.8% of the 152 isolates examined and classified as biofilm producers by the TCP approach were strong and moderate biofilm producers. [56] from Pakistan, who found that the TCP approach identified 63.7% of the 110 isolates as (strong and moderate) biofilm makers. This suggests that the capacity to cling to objects and form biofilm is a crucial aspect of clinical isolate pathogenicity [57]. However, 46 isolates (92%) formed biofilm using the Congo red culture technique. Four isolates, or 8% of the total, failed to produce biofilm, as seen in Figure 3. This finding differs from that which was discovered in Turkey by [58]. The amount of biofilm formation in Enterococcus isolates varied. Because biofilm-forming bacteria are found in vaginal samples at high frequencies, our findings underscore the importance of biofilm in antibiotic resistance and treatment challenges. This study, which was published in India by [59]. These findings show that MDR, XDR, and PDR bacterial

isolates produce substantially more AMPC enzymes than non-MDR isolates. This implies that the AMPC enzyme is highly correlated with increased resistance levels, which could support the resistance mechanism in these bacteria.

Inhibitors of β-lactamases, such as clavulanic acid, sulbactam, or tazobactam, typically block extended-spectrum. β-lactamases (ESBLs). As a result, the use of β-lactam/β-lactamase inhibitor combos has become a possibility for treating infections caused by organisms that produce ESBL. Carbapenem-resistant organisms have also been linked to the rise in the usage of carbapenems to treat ESBL-producing pathogens.[60]. As shown in Table 3, The results show that 25 (48%) isolates of gram-negative bacteria were screened for ESBL production using the double disk diffusion method. The findings revealed that *K. pneumoniae* and *E. coli,* the two main producing bacteria, produced 81.3% and 91.7%, respectively. This is in contrast to [61]. They found that 72.6% of *E. coli* isolates in Baghdad, Iraq, produced positive ESBL results. However, Jarjees (2006) from Erbil, Iraq, observed that BV produced ESBL enzymes in *E. coli* at a rate of 86.07%. From Japan, on the other hand, showed that BV produced ESBL enzymes in *E. coli* at an 84.7 percent rate and *K. pneumoniae* at a 65.4% rate [62]. Table 6 shows that The agar well diffusion method was used to investigate the antibacterial activity of normal flora bacteria *[K.pneumoniae , P.aeruginosa]*. The inhibition zone of diameter of agar well diffusion method between 17mm-16mm for gram positive bacteria such as *S. aureus* and the inhibition zone of gram negative bacteria such as *E. coli* 24mm-28mm as a figure 5 however, there is no inhibition zone of *Klebsiella oxytoca and Staphylococcus pseudintermedius* so this finding demonstrated that normal bacterial flora works on multi, pan - drug, extremely drug resistances this is one of the most Significant virulence factor of pathogenic bacteria isolate accounting 50 out of 150 vaginosis in this study which is Similar result reported in Indonesia using Randu Honey against pathogenic bacteria [63.64].As shown in figure 4, the anti-biofilm activity was also found against biofilm producers among pathogenic isolates, which is one of the virulence factors of pathogenic bacteria. When crude normal flora bacteria were used to treat biofilm-producing isolates, the results showed that normal flora bacteria would reduce the biofilm formation of bacteria except Klebsiella oxytoca and Staphylococcus pseudintermedius. According to the study's findings, the majority of the bacterial vaginosis isolated from women produced strong biofilms. The normal flora bacteria *(K.pneumoniae* and *P.aeruginosa*) which was utilized as an anti-biofilm against these bacteria, significantly decreased their formation of strong biofilms to weak ones. The findings of this study are consistent with the research conducted by [54]. Only a small number of researchers have looked at how beneficial bacteria affect harmful bacteria and the formation of their biofilms. Data collected in the current investigation showed that normal flora bacteria crude extract had significant effects on bacterial vaginosis isolates, especially on the inhibition of biofilm development, and it has strong antibacterial properties against isolates that are MDR, PDR, and XDR. Antibiotic alternatives should be investigated since the biofilm mode of growth is characterized by protracted

CONCLUSION

Nowadays, controlling germs that are resistant to several drugs is one of the most difficult health-related issues. By using the microflora bacteria that produce beneficial effects, we liminated only pathogenic microorganisms that caused infection and fought their virulence factors. The multidrug resistance and extensively drug-resistant (XDR) and PDR and the biofilm formation factor of pathogenic bacteria isolated from female genital tract infections were the study's target virulence factors. The study's goal is to employ normal flora bacteria as a medicinal substance

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