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Some Virulence Factors for *Gardnerella vaginalis* in Babylon Province

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(قَالُواْ سُبْحَانَكَ لاَ عِلْمَ لَنَا إِلاَّ مَا عَلَّمْتَنَا إِنَّكَ أَنتَ الْعَلِيمُ الْحَكِيمُ)

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Sawsan

Dedication

To soul of my father…

To fountian of sympathy… my mother

To my way companion… my husband

To the light of my life…Reemas and Rawan

To the allegory of love and hope… my sisters and brother

To my friends…

Sawsan

Summary:

Vaginitis is the most common infectious disease of the female genital tract during the childbearing age. Common vaginal infections, namely bacterial vaginosis, *Gardnerella vaginallis* is one bacterial genus almost always associated with bacterial vaginosis.

A total of 200 vaginal swabs (100 for culture, 100 for Amsel's test) were collected from pregnant and non-pregnant women infected with bacterial vaginosis aged between (17-60) by the physician from hospitals in Babylon Province : Al- Zahraa hospital for maternity, Al- Exandria general hospital and outpatient clinic for the period from December 2021 to April 2022.

The wet smear prepared directly from the vaginal secretions and without staining showed the presence of clue cells covered with *G. vaginalis*. The result of culture, biochemical, and Gram stain present the percentage of samples positive for *G.vaginalis* was 56 (56%), including (7%), (26%), (13%), (8%), and (2%) isolated from < 20, (20-30), (31-40), (41-50), and >50 age, respectively.

Clinical diagnosis of bacterial vaginosis was considered positive if at least three of four criteria were seen, present 10 (17.8%) samples positive to four criteria and other samples positive to three of Amsel's criteria from the 56 samples.

When used polymerase chain reaction technique (PCR), the results of PCR amplification to specific *16SrRNA* primers (300 bp) of *G. vaginalis* isolates found only 12 (21.4%) from the 56 positive culture samples including (7.1%), (8.9%), (1.7%), (1.7%), and (1.7%) isolated from < 20, (20-30), (31-40), (41-50), and >50 age, respectively.

The virulence factors of *G. vaginallis* study by molecular method (vaginolysin, sialidase, and phospholipase C gene) and phenotypic method (biofilm and hemolysis). The results show only 4 (33%), 9 (75%), and 6 (50%) to *vly*, *sld* and *pho* gene, respectivily gave positive results from 12 *G. vaginalis* isolates . The biofilm formation of *G. vaginalis* study by Congo red agar, and Tissue culture plate methods. The results showed that all isolates were biofilm producers 12 (100%).

Antimicrobial susceptibility test towards eight antibiotics was determined using disc diffusion method. The isolates showed a variable levels of resistance to antibiotics used in this study, the isolates showed resistance to Metronidazole (100%), Clindamycin (92%), Moxifloxacin (0%), Penicillin G (42%), Ampicillin/Sulbactam (92%), Imipenem (0%), Meropenem (0%), and Piperacillin/Tazobactam (0%).

In this study appeared the relationship between biofilm formation and antibiotic resistant isolates 11/12 (92%) of resistant isolates to Clindamycin and Ampicillin/Sulbactam were recorded positive results for biofilm layer to both of them, 5/12 of resistant isolates to Penicillin G were recorded (42%) positive result for biofilm formation, while all resistant isolates to Metronidazole 12/12 (100%) were recorded positive results for biofilm.

The results of nucleotide sets are checked and confirmed by using (NCBI) – Basic Local Alignment Search Tool (BLAST analysis)-nucleotide blast-Search a nucleotide database using a nucleotide query online, which was a perfect program and gave the exact results of identity percentage with other world strains. Sequence alignment was performed by using 16SrRNA gene of *Gardnerella vaginalis* sequences databases information recorded in GenBank to find identity and similarity score

degrees of gene and compared with our local isolates, the results showed identity ranging from 78% to 83%, good query cover, and max score with other world strains of *Gardnerella vaginalis*. It was observed in the phylogenetic tree closely related between bacterial isolates.

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List of Abbreviations

Abbreviation	Meaning
16S rRNA	16 small ribosomal ribonucleic acid
Å	Angstrom
AV	Aerobic vaginitis
BHI	Brain heart infusion
bp	base pairs
BV	Bacterial vaginosis
CDC	Cholesterol-dependent cytolysin
^{o}C	Celsius degree
CDC	Centers for Disease Control and Prevention
CDCs	cholesterol-dependent cytolysins
CFU	Colony forming unit
CLSI	Clinical Laboratory Standard Institute
CO_2	Carbon dioxid
CRA	Congo red agar
dH_2O	Distilled water
DNA	DeoxyriboNucleic Acid
D.W	Distilled water
ELISA	Enzyme linked immunosorbent assay
EPS	Extracellular polymeric substances
g	Gram
GBS	Group B *Streptococcus*
GV	*Gardnerella vaginalis*
G+ve	Gram positive
G-ve	Gram negative
H_2O_2	Hydrogen peroxide
HIV	Human immunodeficiency virus
ILY	Intermedilysin
INY	Inerolysin
KD	Kilo Dalton

L	Liliter
LPS	Lipopolysacchrides
MEGA	Molecular Evolutionary Genetics Analysis
mg	Miligram
ml	Milliliter
mm	Millimeter
MR-VP	Methyl red- Voges –Proskauer
NAD	Nicotin amid dinucleotid
NCBI	National center for biotechnology information
NJ	Neighbor-Joining
nm	Nanometre
OD	Optical density
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
pH	Potential of Hydrogen
PLY	Pneumolysin
Pmol	Picomole
STIs	Sexually transmitted infections
STPs	Sexually transmitted pathogens
TBE	Tris-Borate-EDTA Buffer
TCP	Tissue culture plate
TSB	Tryptic soy broth
UV	Ultra violet
VLY	Vaginolysin
VVC	Vulvovaginal candidiasis
WBCS	White blood cells
µg	Microgram
µl	Microliter
µm	Micrometre

Chapter One

Introduction

1. Introduction

Bacterial vaginosis (BV) is the most common vaginal infection affecting women of childbearing age (Borgogna *et al*., 2021), and is a polymicrobial disorder of the lower genital tract characterised by an alteration in the vaginal microenvironment (dysbiosis) resulting in the loss of *Lactobacillus* species dominance, increase in vaginal pH and a dramatic overgrowth of pathogenic Gram negative and positive facultative and obligate anaerobic bacteria such as *Gardnerella, Atopobium, Bacteroides, Mobiluncus, Prevotella, Mycoplasma, Peptostreptococcus, Anaerococcus, Sneathia, Clostridium, Leptotrichia* species (Santos *et al*.,2018a; Morrill *et al*., 2020).

Bacterial vaginosis (BV) is characterised by foul-smelling vaginal discharge and may sometimes also be accompanied by dysuria, dyspareunia, burning and vaginal inflammation (Mendling and Holzgreve, 2022). Lactobacilli produce lactic acid and hydrogen peroxide that maintain a normal, acidic vaginal pH (3.5–4.5); the acidic environment inhibits the growth of pathogens and protects against infections (Amabebe and Anumba, 2018). A wide range of factors, including sexual habits, smoking and personal hygiene habits (Mendling 2016; Ranjit *et al*., 2018) , can increase the vaginal pH, making conditions unfavourable for Lactobacilli and allowing the growth of predominantly anaerobic bacteria such as *Gardnerella vaginalis*, resulting in BV(Amabebe and Anumba, 2018).

The BV is a major public health burden as it is associated with poor reproductive outcomes including preterm birth, low birth weight, miscarriage, failure of *in vitro* fertilisation, pelvic inflammatory disease, postpartum endometritis and increased risk of acquisition and

transmission of HIV and other sexually transmitted infections (STIs)(Redelinghuys *et al*., 2020; Ravel *et al*., 2021).

The *G.vaginalis*, a non-motile, catalase-negative, Gram variable facultative anaerobic coccobacilli (Jung *et al*., 2017; Morrill *et al*., 2020). It is involved, together with many other bacteria, mostly anaerobic, in bacterial vaginosis among women as a result of a disruption in the normal vaginal microflora (Chen *et al*., 2021).

The *G. vaginalis* possesses virulence factor that cause infection one of them its ability to produced biofilm facilitate their adherence to vagina epithelial cells. Adherence of *G. vaginalis* to host epithelium considered as initial steps in invasion (Wong *et al*., 2018). The various virulence factors of *G. vaginalis* are pili, microcapsule, surface hydrophobicity, adherence, vaginolysin, phospholipase C, protease and siderophores, sialidases and prolidases (Nisha *et al*., 2019).

Structured polymicrobial biofilm consisting of numerically predominant *G. vaginalis* and other incorporated bacterial species was detected in BV patients (Castro *et al*., 2019). The life cycle of biofilms comprises three phases, namely, attachment to a surface, secretion of polymeric matrix and aggregation of microbes for yielding mature biofilms, and dispersion by detaching from biofilms (Machado and Cerca, 2015; Jung *et al*., 2017). *G.vaginalis* is an important component of the human vaginal microbiome. It plays a key role in the pathogenesis of bacterial vaginosis (BV), the most common vaginal condition (Randis and Ratner, 2019).

The virulence factors of *G. vaginalis* include vaginolysin and sialidase that contribute to BV formation (Castro *et al*., 2018). Vaginolysin is a member of the cholesterol-dependent cytolysin family, which can lyse

avariety of human cells (Abdelmaksoud *et al*., 2017), by creating pores that alter cell integrity and shape, which is thought to disrupt local innate immunity, and thus, promote BV persistence (Nowak *et al*., 2018).

Gardnerella vaginalis (GV), an anaerobic pathogen that produces sialidase enzyme to cleave terminal sialic acid residues from human glycans, high sialidase activity is associated with preterm birth and low birthweight (Govinden *et al*., 2018).

Antibiotics, such as metronidazole or clindamycin, are recommended as first-line treatment for BV, but may be associated with antibiotic resistance, high rates of recurrence and poor patient treatment satisfaction(Mendling and Holzgreve, 2022). The sensitivity results of *G.vaginalis* isolates showed that clindamycin, vancomycin and ceftriaxone were highly sensitive, the next effective drugs were ampicillin, chloramphenicol and erythromycin, the maximum resistance found in case of cotrimoxazole and tetracycline, high resistance to metronidazole (Ara *et al*., 2017).

Aim of study

The study was designed with the aim to detection of *Gardnerella vaginalis* and some virulence factores as causing agent of bacterial vaginosis in women by the following objectives:

1. Isolation and identification of *G.vaginalis* by bacteriological and molecular(16SrRNA) methods.
2. Diagnosis some virulence factores such as vaginolysin, sialidase, phospholipase and biofilm formation.
3. Antibiotic susceptibility test for *Gardnerella vaginalis.*
4. Sequence of *G.vaginalis*.

Chapter Two

Literature Review

2. Literature Review

2.1. *Gardnerella vaginalis*

2.1.1. History and classification

Gardnerella is one bacterial genus always associated with BV, was first isolated by Leopold from the cervix swabs of women and the urine of men in 1953 (Leopold, 1953; Schellenberg *et al*., 2017). Later, it was found to be related to BV and named *Haemophilus vaginalis* by Gardner and Dukes in 1955 (Gardner and Dukes, 1955). It was initially placed in the *Haemophilus* genus, based on initial Gram negative staining and fastidious growth. The first electron micrographs by Criswell *et al*., (1972) revealed that *G. vaginalis* contains what appeared as a Gram negative cell wall structure. Initially, *G. vaginalis* seemed to possess gram-negative structures and was thought to contain lipopolysaccharide (LPS), but followup studies showed a lack of LPS (Sadhu *et al*., 1989).

It was later discovered that the organism does not require hemin, Nicotin amid dinucleotid (NAD) or other growth factors and thus was re-classified as *Corynebacterium* the former bacterial cells stained Gram positive and the cells morphologically resembled *Corynebacterium*, showing polar granules and club-like structures (Zinnemann and Turner, 1962, 1963). The cell wall structure, however, did not fit the well-known Gram-positive structure; the former *Haemophilus vaginalis* was shown to have a multilayered cell wall with components resembling that of Gram-negative bacteria (Criswell *et al*., 1972).

Following genetic analysis and DNA-DNA hybridization, it was discovered that *G. vaginalis* shared little sequence homology or biochemical characteristics with either the *Haemophilus* or

Corynebacterium genus. Therefore, a new taxonomic classification was proposed and was finally named after Dr. Gardner as *Gardnerella vaginalis* in 1980 (Piot *et al*., 1980). It was later shown that *G. vaginalis* does have a Gram-positive cell wall, but it takes on a Gram-negative staining pattern with the aging of the culture (Catlin, 1992).

The *G. vaginalis* typically stains as a Gram-variable coccobacillus, exhibiting both Gram-positive and Gram-negative cells. Electron microscopy indicated that the bacterium has a thin layer of peptidoglycan, typical of Gram-negative bacteria, but does not possess the outer cell envelope of Gram-negative bacteria. Chemical analyses established that the bacteria do not produce Gram-negative lipopolysaccharide, but rather the lipoteichoic acid of Gram-positive bacteria, confirming *G. vaginalis* is a Gram-positive bacterium that can also stain as Gram-negative due to its thin cell wall (Sadhu *et al*. 1989).

The *G. vaginalis* is difficult to be cultivated as β-hemolytic on media containing human or rabbit blood but not on sheep blood agar hemolysis is improved by anaerobic incubation. The observation that the diameters of colonies on Columbia agar base with 5% sheep blood this media called Vaginalis Agar (Lagace-Wiens *et al*.,2008), it was can be differentiated by beta-hemolysis on human blood bilayer Tween 80 agar, a Gram-variable reaction in Gram stains, and a negative catalase test (Piot *et al*. 1982).

Several studies have shown a relationship between these *G. vaginalis* strains and symptoms or severity of BV (Pleckaityte *et al*. 2012, Schellenberg *et al*. 2017).

On the basis of the measurement of the activities of three enzymes, i.e. β-galactosidase, lipase and hippurate hydrolase, *G. vaginalis* isolates

were assigned to eight different biotypes (Briselden and Hillier, 1990; Moncla and Pryker, 2009), the study of the researcher Udayalaxmi *et al*., (2011) was conducted to correlate the biotypes of *G.vaginalis* strains isolated from cases of bacterial vaginosis and their virulence factors. Adherence to vaginal epithelial cells, biofilm production, surface hydrophobicity, phospholipase C and proteas activity were tested on isolates. There is no any correlation between *G.vaginalis* biotypes and its virulence factors. Virulence factors expressed by *G. vaginalis* were not associated with a single biotype. And for the genetic relatedness between *Bifidobacterium* and *G. vaginalis* they putted in the same family, and dependents upon the molecular characterization (16SrRNA) they putting in this classification (Stackebrandt *et al*., 1997).

Domain : Bacteria

Lineage : Firmicutes

Class : Actinobacteria

Sub : Actinobacteridae

Order : Bifidobacteriales

Family : Bifidobacteriaceae

Genus : *Gardnerella*

Species : *vaginalis*

2.1.2. General characteristic of *G. vaginalis*

The bacterial cells are small, nonmotile, pleomorphic rods with average dimension 0.4×1.0 ~1.5μm, however, the length of some cells may reach up to 2–3 μm, non-spore-forming, they lack flagella, and they do not possess a typical capsule. Upon Gram staining, they can appear Gram variable (Onderdonk *et al*., 2016; Wong *et al*., 2018), due to thin cell wall Gram staining varies from positive to negative, therefore described as a Gram variable microorganism (Catlin, 1992). The cell size and morphology largely depend on their growth conditions and on their physiological state, this bacterium is immotile, with the cells frequently occurring in clumps in vaginal smears and when grown in liquid media (Turovskiy *et al*., 2011). The cellular surface of *G. vaginalis* is covered with fimbriae, which are responsible for the attachment of *G.vaginalis* to vaginal epithelial cells also known as clue cells (Qin and Xiao, 2022). *G. vaginalis* has fimbriae (pili) which are 3 to 7.5 nm in diameter covering the cell surface. Both fimbriae and exopolysaccharides are thought to be involved in attachment of *G. vaginalis* to the vaginal epithelium *in vivo* (Turovskiy *et al*., 2011).

2.1.3. Requirements, nutrition and metabolism

Gardnerella vaginalis is a facultative anaerobe and uses carbohydrates as its major energy source, this microorganism has a fermentative metabolism and acetic acid is a major product (Bratcher, 2018; Qin and Xiao, 2022). *G. vaginalis* grows well in microaerophilic conditions in 5-7% CO_2, optimum growth of *G. vaginalis* occurs at temperatures between 35 and 37^{o}C, however, growth can occur between 25- 45^{o}C. Optimum growth occurs at a pH between 6 and 6.5, no growth occurs at pH 4 and

slight growth occurs at pH 4.5 and 8 (Greenwood and Pickett, 1980; Chart, 2012). This micro-organism is fastidious, but it does not require either hemin or Nicotinamide Adenine Dinucleotide (NAD) for growth, as revealed by biochemical tests *G. vaginalis* is catalase, oxidase and β-glucosidase negative, it can ferment starch, dextrin, sucrose, glucose, fructose, ribose, maltose and raffinose resulting in the production of acetic acid as the major end product, in addition, some strains of *G. vaginalis* can also ferment xylose and trehalose, however, *G. vaginalis* is unable to ferment rhamnose, melibiose, mannitol, and sorbitol. Furthermore, *G. vaginalis* can hydrolyze hippurate but not gelatin or esculin, this microorganism is also positive for α-glucosidase activity and for β-hemolysis on human blood, but not sheep's blood (Turovskiy *et al*., 2011; Bratcher, 2018). The acquisition of iron by microorganisms plays a crucial role in the growth of most pathogens (Griffiths, 1991). Jarosik *et al*., (1998) studied the ability of *G. vaginalis* strains to acquire iron from the medium. Interestingly, all the strains they studied were able to acquire iron from ferric and ferrous inorganic substrates as well as hemin, haemoglobin, lactoferrin but not transferrin. Studies that analysed the genome of *G. vaginalis* revealed that the microorganism lacks enzymes in biochemical pathways that are involved in amino acid synthesis. It was predicted that *G. vaginalis* can synthesize some but not all purines and pyrimidine bases (Harwich *et al*., 2010).

2.2. Healthy vaginal ecosystem

Human vagina is covered by mucous membrane that is physiological barrier against foreign organisms, chemicals and other objects (Martin, 2012), the vaginal mucosa is made up of a stratified squamous

nonkeratinized epithelium covered by cervicovaginal secretion (Pekmezovic *et al*., 2019).

A healthy vaginal ecosystem is characterized by an intact vaginal epithelium and a microflora dominated by lactobacilli; *Lactobacillus* spp.; account for more than 95% of all bacteria present; also, these microorganisms provided defense mechanism against infection of bacterial vaginosis via maintaining the vagina an acidic pH (Sumati and Saritha ,2009 ; Kroon *et al*.,2018). In the ecosystem, a homeostatic and mutualistic relationship exists between the microbiota and its human host, the host provides a humid, nutritions, and warm habitat for the microbes, whereas the resident microbiota produces antimicrobial and anti-inflammatory factors. Thus, the first line of defense against nonindigenous microorganisms is established. Nevertheless, this balance can be broken by internal and/or external factors (Pekmezovic *et al*., 2019).

In normal vaginal flora, there are *Lactobacillus* species in 95% and facultative anaerobic and anaerobic microorganisms, including *Gardnerella vaginalis, Staphylococcus epidermis*, *Mycoplasma hominis*, *Streptococcal* species, *Bacterioides* species, *Prevotella bivius*, *Peptostreptococci* species, in 5% (Hillier *et al*., 1993; Wang, 2000), generally vaginal colonization with lactobacilli is believed to promote a protective environment since these bacteria prevent other microbes from colonizing the vaginal tract, using several mechanisms (Vaneechoutte, 2017; Kovachev, 2018), one of the best defense mechanisms studied is related to the production of lactic acid by the majority of *Lactobacillus* spp., which contributes to the maintenance of the vaginal pH below 4.5 (Tachedjian *et al*., 2017; Godha *et al*., 2018), lactobacilli are responsible

for reducing the pH via metabolizing glycogen from squamous cells to lactic acid, the resultant acid milieu that are provided protection against infection; such as bacterial vaginosis (BV) which is an over growth of predominantly anaerobic bacteria within vagina and a concomitant that lead to reduction or absence of lactobacilli (Smolarczyk *et al*., 2021). *Lactobacillus* also synthesizes bacteriocins, a type of antimicrobial peptides that can permeabilize the microbial cell membrane of pathogenic microorganisms (Stoyancheva *et al*., 2014). Furthermore, lactobacilli produce hydrogen peroxide that could act as a natural microbicide within the vaginal ecosystem (Atassi and Servin, 2010; Sgibnev and Kremleva, 2015). Furthermore, they can adhere to vaginal epithelial cells and compete with other microbial cells for binding sites (Carmo *et al*., 2016; Leccese Terraf *et al*., 2017). This ability of lactobacilli has an important role since the pathogen adhesion and colonization on the host cells often represent the first step of the infection process (Ribet and Cossart, 2015).

2.3.Vaginitis

Vaginitis is the most common infectious disease of the female genital tract during the childbearing age. Common vaginal infections, namely, bacterial vaginosis (BV), aerobic vaginitis (AV), vulvovaginal candidiasis (VVC), and trichomoniasis, can lead to the adverse obstetrical and gynecological outcomes, such as cervicitis, pelvic inflammatory disease, postoperative infection, intrauterine infection, peripartum infection, and neonatal infection (Workowski *et al*., 2021).

A healthy vaginal microbiota is considered to be significant for maintaining vaginal health and preventing infections. However, certain vaginal bacterial commensal species serve an important first line of

defense of the body. Any disruption of this microbial barrier might result in a number of urogenital conditions including aerobic vaginitis (AV) and bacterial vaginosis (BV)(Kaambo *et al*., 2018).

Vaginitis which is the vagina inflammation;that was been characterized by discharge of vaginal that are containing many white blood cells (WBCS); vulvar itching,; vulvar irritation,; odor of vagina; vaginal erythema;, dyspareunia; and dysuria (Bohbot *et al*.,2014 ; Barrientos-Durán *et al*.,2020).

Bacterial vaginosis is the condition that occurs when the balance of the vaginal flora (normal hydrogen peroxide (H_2O_2) producing *Lactobacillus* species) is dysregulated, and replaced by high concentrations of bad bacteria in the vagina like *Gardnerella vaginalis,Prevotella*, *Mobiluncus*, and *Bacteriodes* species (Koumans *et al*., 2002), and some aerobic bacteria occasionally *E. coli, S. aureus, E. faecalis*, and group B streptococcus (GBS) cause aerobic vaginitis (Onderdonk *et al*., 2016; Kaambo *et al*., 2018).

The diversity and bacterial species within the vagina can change rapidly in response to numerous endogenous and exogenous influences, reflecting the progression of vaginal infections. In healthy women, the vaginal microbiome changes according to age, pregnancy, menstruation, injury, and direct bacterial flora destruction (antibiotic usage, sexually transmitted infections, and vaginal irrigation)(Gajer *et al*., 2012), age, smoking habit, alcohol use, increased number of sex partners, menopausal period, use of hormonal contraception, and the presence of acute and chronic infections (Pál, 2005; Cherpes *et al*., 2008).

2.4. Diagnosis of bacterial vaginosis by Amsel test

There are four parameters used to determine the presence or absence of bacterial vaginosis (BV) according to Amsel's criteria :

1-Homogeneous Vaginal Discharge: Vaginal discharge of women with BV has a thin, homogeneous appearance, and a milk-like consistency, white discharge that smoothly coates the vaginal wall (Petersen *et al*., 2011; Nawani and Sujatha, 2013).

2- Elevated Vaginal pH: Normal pH value of vagina from 3.8 to 4.2 has regulatory and protectors mechanisms of vaginal environment, the change in the pH value indicates to presence of disbalance in the ecosystem of vaginal environment (Jahić *et al*., 2006). Elevation in vaginal pH ≥4.5 referred to as (BV), a pH of vaginal fluid more than 4.5 is a sensitive indicator of BV but of low specificity (Hemalatha *et al*., 2013; McKinnon *et al*., 2019).

3- Fishy Odour : The presence of a fishy odor when 10% potassium hydroxide was added to vaginal fluid from a woman with BV(Wariso *et al*., 2017). Fishy odor criterion is sometimes called the whiff test. One clinical indicator of BV is a ' fishy ' odor, this odor has been associated with increases in several biogenic amines that may serve as important biomarkers (Nelson *et al*., 2015). This phenomenon occurs not only when alkaline is added in the laboratory but also in vivo during intercourse when semen, which has an approximate pH of 7, is introduced into the vagina , whiff test is usually specific for BV but of moderate sensitivity (Spiegel, 1991).

4-Clue cells: Clue cells are vaginal epithelial cells which are so coated with bacteria that the cell border is granular. >20% of the epithelial cells

should have the appearance of clue cells for the test to be positive for BV (Livengood, 2009).

2.5.Virulence factor of *G. vaginalis*

2.5.1. Adhesion factors

Microbes employ a variety of strategies to adhere to abiotic and biotic surfaces, as well as host cell, microbes produce appendages (e.g. pili , fimbriae , flagella) and express adhesion proteins embedded in the cell wall or cell membrane, with adhesive domains targeting specific ligands or chemical properties (Beaussart *et al*., 2020).

The clue cell is a squamous epithelial cell which is covered by bacteria. First step of infection progress is adhesion of bacteria to epithelial cell through the adhesion molecules. In bacteria, pilis, lipopolysaccharide and biofilm have primary importance (Harwich *et al*., 2010).

Electron microscopy revealed fimbriae (pili) that are (3–7·5) nm in diameter cove ring the cellular surface of *G. vaginalis* which responsible for the attachment to vaginal epithelial cells (Turovskiy *et al*., 2011).

Presence of pili was demonstrated with electron microscopy in *G. vaginalis* and *Bacteroides* spp. Studies show that *G. vaginalis* has Type I pili (Demirzen *et al*.,2013).

Strands of exopolysaccharide produced by cells can be visualized using electron microscopy, which was involved with pili to confer adherence of *G. vaginalis* cells to vaginal epithelial cells (Turovskiy *et al*., 2011).

2.5.2. Vaginolysin

One of virulence factors is vaginolysin (VLY), a secreted protein toxin functioning as a hemolysin specific to human erythrocytes, neutrophils and endothelial cells (Gelber *et al*., 2008; Garcia *et al*., 2019).

Vaginolysin is a cholesterol-dependent cytolysin (CDC), most closely related to Intermedilysin (ILY) and Pneumolysin (PLY) produced in *Streptococcus spp*, which form membrane embedded pores of 250-300 Å (Gelber *et al.*, 2008; Dunstone and Tweten, 2012; Hotze and Tweten, 2012).

Gardnerella spp. secrete the toxins vaginolysin (VLY) which have structural and activity features attributed to cholesterol-dependent cytolysins (CDCs).The CDCs are produced by many pathogenic bacteria as virulence factors that participate in various stages of disease progression by forming lytic and non-lytic pores in cell membranes or via pore-independent pathways. The VLY is expressed in the majority of *Gardnerella* spp. isolates; less is known about the prevalence of the gene that encodes inerolysin (INY). The INY is a classical CDC; membrane cholesterol acts a receptor for INY. The VLY uses human CD59 as its receptor, although cholesterol remains indispensable for VLY pore-forming activity, VLY-induced damage occurs with high levels of membrane cholesterol (Pleckaityte, 2020).

Vaginolysin triggers the immune system, induces interleukin-8 production by human epithelial cells, and a specific secretory immunoglobulin A targeting the *G. vaginalis* vaginolysin, has been shown to be upregulated in the setting of bacterial vaginosis (Cauci *et al*., 2002; Pleckaityte, 2020).

Mutation of a proline residue which has been suggested to be necessary for the cytotoxicity of VLY potentiates the production of a

VLY toxoid which may be used for further development of vaccines (Gelber *et al*., 2008).

2.5.3. Sialidase

Sialidase (or neuraminidase) that enzymatically removes terminal sialic acid residues from different glycoconjugates which provides bacteria with nutrition and improves their ability for evasion of the host immune system and cellular interactions (Harwich *et al*., 2010). Several BV-associated species may produce sialidases. However, *Gardnerella* spp. are considered the main source of this enzyme in the cervicovaginal environment (Hardy *et al*., 2017b; Kurukulasuriya *et al*., 2021). Many strains of *G. vaginalis* are sialidase positive, with activity likely related to pathogenesis and virulence and potentially for survival in this environment (Lewis *et al*., 2013; Hardy *et al*., 2017b).

It is proteins component with 7500 KD molecular weight have a direct role in pathogenesis act by cleaves alpha-ketosidic linkages between glycosyl residues of glycoproteins, glycolipids and sialic acids, degrade the vaginal epithelial cell and inhibit the immune response by splitting the sialic acid residues in immunoglobulin IgM and IgA have an active role in both bacterial interaction and bacterial nutrition (Al-Zubaidi, 2012).

Gardnerella spp. have been proposed as the scaffold on vaginal mucosa for the attachment of other bacterial species, such as *Prevotella* and *Atopobium*, leading to biofilm formation (Hardy *et al*., 2015; Muzny and Schwebke, 2016). Thus, by cleaving the sialic acid of epithelial cells, sialidases may facilitate the adhesion of *Gardnerella* spp. to the underlying glycan-binding sites enabling biofilm formation (Varki and Gagneux, 2012; Castro *et al*., 2019).

Pregnancy problems such as premature birth in BV patients have been attributed to sialidase activity owing to its mucin oligosaccharides degradation activity (Cauci *et al*., 2008).

2.5.4 Phospholipase production

Phospholipases are ubiquitous hydrolases that catalyze the hydrolysis of phospholipids, a key component of eukaryotic cellular membranes. These enzymes are considered as an important virulence factors, as they help the bacterial pathogens in number of ways like host cell invasion, modulating the phospholipid content of their membrane. Also, these enzymes are crucial for the pathogenesis of certain bacteria because of their role in escape from the host defence mechanism, these enzymes are involved in the family of lipolytic enzymes that catalyze the hydrolysis of phospholipids into fatty acids and other lipophilic substances, and invade the host cells by destroying the phospholipids of cell membranes (Bandana *et al*., 2018).

Phospholipase C (Lecithinase or phosphatidylcholine phosphorylase) enzymatically hydrolyzes lecithin into phosphorylcholine and 1, 2-diglyceride and is another recognized virulence factor of this microorganism. Lecithin is converted to 1, 2-diglyceride by phospholipase C activity. lead to the production of prostaglandins and related compounds, bacterial production of this enzyme can lead to reproductive tract cell and tissue damage through direct and indirect mechanisms (Udayalaxmi *et al*., 2011).

2.5.5 Biofilm formation

Bacterial biofilms are complex surface attached communities of bacteria held together by self-produced polymer matrixs mainly composed of polysaccharides, secreted proteins, and extracellular DNAs. Bacterial biofilm formation is a complex process and can be described in five main phases:

1- Reversible attachment phase, where bacteria non-specifically attach to surfaces.

2- Irreversible attachment phase, which involves interaction between bacterial cells and a surface using bacterial adhesins such as fimbriae and lipopolysaccharide (LPS).

3-Production of extracellular polymeric substances (EPS) by the resident bacterial cells.

4-Biofilm maturation phase, in which bacterial cells synthesize and release signaling molecules to sense the presence of each other, conducing to the formation of microcolony and maturation of biofilms.

5-Dispersal/detachment phase, where the bacterial cells depart biofilms and comeback to independent planktonic lifestyle (Muhammad *et al*., 2020) figure (2-1).

Ahallmark of BV is the presence of a highly structured polymicrobial biofilm on the vaginal epithelium, presumably initiated by facultative anaerobes of the genus *Gardnerella*, which then becomes a scaffold for other species to adhere (Castro *et al*., 2020).

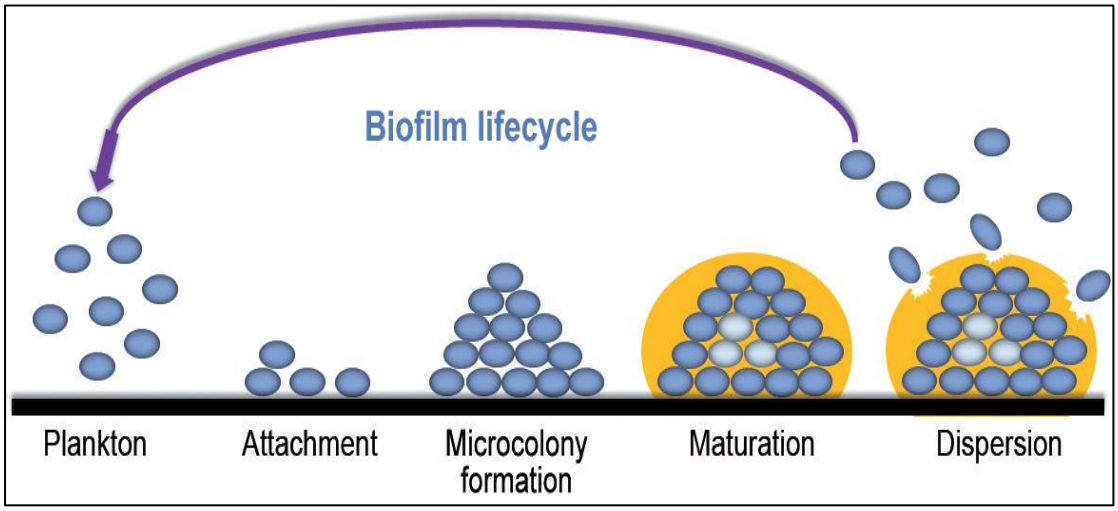

Figure (2-1) Stages of biofilm development (Santos *et al*., 2018b).

Gardnerella vaginalis (GV) has been implicated in BV development. Further, biofilm is accepted as one, if not the principle reason, for recurrent or recalcitrant BV, and GV has defined virulence factors that contribute to biofilm. The polymicrobial biofilms formed on vaginal epithelium play a crucial role in the pathogenesis of BV (Hardy *et al*., 2017a; Kunze and Larsen, 2019).

A biofilm can consist of a single microbial species or a combination of different species of bacteria, protozoa, archaea, algae, filamentous fungi, and yeast that strongly attach to each other and to biotic or abiotic surfaces (Costa-Orlandi *et al*., 2017; Raghupathi *et al*., 2018).

Biofilms are responsible for chronic illness and nosocomial infections, (Khatoon *et al*., 2018).

That bacterial biofilm may play a role in the pathogenesis of disease has led to an increased focus on identifying diseases that may be biofilm-related. Biofilm infections are typically chronic in nature, as biofilm-residing bacteria can be resistant to both the immune system, antibiotics, and other treatments. (Vestby *et al*., 2020).

2.6. Response of bacteria to antibiotic and drug

Antibiotics are compounds that target specific locations in a bacterium and to be effective, it targets a vital operation in the bacterium leading to either a stop to the cell's growth (bacteriostatic) or its death (bactericidal). There are many antibiotics available differing from each other by principle and mechanic of action (Sebastian *et al.*, 2021).

As the first-line of therapy for BV, the Centers for Disease Control and Prevention (CDC) recommends the use of oral or vaginally applied metronidazole (a DNA replication inhibitor) or clindamycin (a protein synthesis inhibitor) (Workowski and Bolan, 2015). Metronidazole, a derivative of nitroimidazole, is widely used. It may be administered orally at 500 mg twice a day for 7 days or applied intravaginally in the form of a 0.75% gel once a day for 5 days, intravaginal application of clindamycin at bedtime for 7 days is also a common treatment regimen, but relapse can reportedly occur, the relapse and recurrence of BV are the biggest challenges to current therapies; the recurrence rate is >50% mainly because of the development of a multispecies biofilm, with *G. vaginalis* being one of the dominant species (Machado *et al*., 2016).

Among the four major classes of erm genes (ermA, ermB, ermC and ermF) in different bacteria, ermA appears to be the primary gene responsible for clindamycin-associated antimicrobial resistance in *Gardnerella vaginalis* (Bostwick *et al*., 2016). The putative mechanism for tetracycline resistance is the presence of the *tetM* gene,which is found in tetracycline-resistant strains of *G.vaginalis* (Harwich *et al*., 2010).

The most well-characterized mechanism of resistance to metronidazole is the inactivation or deletion of genes with nitroreductase activity (Dhand and Snydman, 2009).

Hussin *et al*.,(2013) found that isolates of *G.vaginalis* were sensitive to ampicillin, gentamycin, cloxacillin, lincomycin, rifampicine, cefotaxime, erythromycin, and chloramphenicole, but isolates were resistance to neomycin, colistin, metroinazole, and nalidixic acid, which can be attributed to the exaggerated use in Iraq.

The use of ampicillin for the treatment of bacterial vaginosis has often been associated with failure to eradicate *G. vaginalis* or clinical cure. This is probably due to inactivation of ampicillin by the β-lactamases produced by vaginal anaerobes (Ara *et al*., 2017).

2.7. Molecular detection

The PCR is a simple and widely used process in which minute amounts of DNA can be amplified into multiple copies (Garibyan and Avashia, 2013).

Fredriks *et al*., (2007) reported to use other diagnostic methods for bacterial vaginal infection in addition to the previous methods (staining, Asmel criteria) which is the use of the polymerase chain reaction technique and the use of the *16S rRNA* gene, PCR detection of one or more fastidious bacterial species is a more reliable indicator of BV than detection of bacteria, such as *G.vaginalis*, previously linked to BV, highlighting the potential of PCR for the diagnosis of BV. The researcher Housseini *et al*., (2019) designed the primers of bacteria using the *16S rRNA* gene and DNA extracted from pure cultures of *G. vaginalis* bacteria in an polymerase chain reaction technique.

Mohammadzadeh *et al*., (2019) reported that 100% of *G. vaginalis* isolates were diagnosed using the *16S rRNA* gene of BV patients.

Janulaitiene *et al*., (2018) reported the diagnosis of virulence factors of *G. vaginalis* bacteria such as vaginlolysin and saildiase using specific primers and PCR technique. Other studies used real time PCR technique in molecular investigation of the bacteria *G. vaginalis*, as this technique is characterized by speed and accuracy in determining the target in the sample (De Backer *et al*., 2007; Biagi *et al*., 2009; Menard *et al*., 2010).

Chapter Three

Materials & Methods

3. Materials and Methods

3.1. Materials

3.1.1. Laboratory instruments and apparatuses

Laboratory instruments and apparatuses used in this study are illustrated in table (3-1).

Table (3-1) :Laboratory instruments and apparatuses

Item	Company	Origin
Anaerobic jar	BBL	England
Autoclave	Hirayama	Japan
Biosafety cabinet	Labogene	Denmark
Burner	Amal	Turkey
Digital camera	Samsung	Japan
Distillator	GFL	Germany
ELISA reader	Biotech	USA
Eppendorf tubes	Sterellin Ltd.	UK
Flask	Chemical-Lab	China
Gel documentation system	Biometra	Germany
Gel Electrophoresis apparatus	Cleaver scientific	UK
Glass slides	Sail brand	China
Incubator	Gallenkamp	England
Light microscope	Olympus	China
Medical cotton	Medicare Hygiene Limited	India
Medical gloves	Broche	PRC

Microcentrifuge	Hettich	Germany
Micropipettes	Top Dragon	Europe
Microscopic Cover slide	Gitoglas	China
Microwave	LG	Thailand
Millipore filters (0.22mm)	Sigem	Spain
Nano drop	Avans biotechnology corp	Taiwan
Oven	KampGallen	England
Parafilm	Bemis	USA
Pasture pipette	Proway	China
PCR thermocycler	Techne	USA
Petri dishes	Blasti lab	Lebanon
Plain tube	Dolphin	Syria
Platinum wire loop	Himedia	India
Refrigerator	Concord	France
Sensitive electric balance	Denver	Canada
Spectrophotometer	Apel	USA
Sterile swabs	Sigem	Spain
Sterile transport media swabs	Estmed	china
Tips	Biobasic	
Tissue culture Micro-titer plate	Holzel	Germany
Vortex mixer	Gemmy	Taiwan
Water bath	Memmert	Germany
Wooden sticks	Supreme	China

3.1.2. Chemical and biological materials

Chemical materials, biological materials, regents, stains and solutions used in the present study are illustrated in table (3-2).

Table (3-2) : Chemical and biological materials

Material	Company	Origin
Aceton	B.D.H	England
Agarose	Promega	USA
Barium chloride ($BaCl_2.2H_2O$)	B.D.H	England
Butanol		
Catalase reagent	Himedia	India
Congo red stain	Concorot	China
Crystal violet	Sigma	USA
DNA loading dye	Promega	
Ethanol absolute (95%)	Fluka chemika	Switzerland
Ethidium bromide	Biobasic	Canada
Glucose	Fluka	Switzerland
Glycerol	Sigem	USA
Growth supplement (Gentamicin sulfate, Naildixic acid, Amphotericin B)	Himedia	India
KOH (10%)	Schuchariot	Germany
Methyl red indicator		
Ninhydrin reagent	Fluka chemika	Switzerland
Normal saline	Pharmaline	Egypt
Nuclease free water	Promega	USA
Oxidase reagent	Himedia	India
Phosphate buffer		
Primers	Bioneer	Korea

Sucrose	Fluka	Switzerland
Sulfonic acid (H_2SO_4)	B.D.H	England
(TBE)Tris-Borate EDTA Buffer (10X)	Promega	USA
Urea	B.D.H	England

3.1.3. Commercial kits

Commercial kits were used in the present study are illustrated in table (3-3).

Table (3-3): Commercial kits

Type of kit	**Company**	**Origin**
DNA ladder (100bp ladder)	Promega	USA
Gram stain	Difco	
Green master mix	Promega	

3.1.4. Culture media

Culture media were used in the present study are illustrated in table (3-4).

Table (3-4): Culture media with the purposes

Medium	**The purpose**	**Company (Origin)**
Blood agar	Is an enriched, bacterial growth medium, isolation, identification and determine the type of hemolysis (Niederstebruch *et al*., 2017).	Himedia (India)

Brain-heart-infusion broth	This medium used to preserve the bacterial isolated as standard for a long time with 15% glycerol (Forbes *et al*., 2007).	Himedia (India)
Columbia blood agar base	This medium was used as a selective medium for the isolation of *G.vaginalis* (Gergova *et al*., 2013).	
Hippurate hydrolysis medium	This medium used for determining the ability of bacteria to produse amino acid (Glycin) as result of sodium hippurate hydrolysis (Lin *et al*., 1986).	
Kligler iron agar	It was used to determine the ability of bacteria to utilize carbohydrates supplemented with phenol red as the indicator (Forbes *et al*., 2007).	
MacConkey agar	is a selective and differential media. It is used in the differentiation of lactose fermenting from lactose non-fermenting gram-negative bacteria (Jung and Hoilat, 2020).	
Methyl Red (MR)/ Voges- Proskauer (VP) media	Used to determine whether the bacteria can ferment glucose and if it can produce acid (MacFaddin, 2000).	
Muller-Hinton agar	This medium used in the antibiotic	Oxoid (England)

	sensitivity test (MacFaddin, 2000).	
Tryptic soya broth	used for cultivation of bacterial isolates when it was necessary for biofilm detection (MacFaddin, 2000).	Himedia (India)
Urea agar	It was used to test the ability of bacteria to produce urease enzyme (MacFadden, 2000).	

3.1.5. Antibiotic discs

Table (3-5) presents the antibiotic discs employed in this study.

Table (3-5): Antibiotic discs

Antibiotic disc	Symbol	Disc Concentration (µg/disc)	Company (Origin)
β-lactam combinations			Himedia (India)
Ampicillin/Sulbactam	A/S	10/10	
Piperacillin/Tazobactam	PIT	100/10	
Carbepenem			
Imipenem	IMP	10	
Meropenem	MRP	10	
Fluoroquinolones			
Moxifloxacin	MO	5	
Lincomycin			
Clindamycin	CD	10	

Nitroimidazole			Himedia (India)
Metronidazol	MET	30	
Penicillins	P	10	
Penicillin G	P	10	

3.1.6. Primers

Primers used in the present study were listed in table (3-6).

Table (3-6): Study primers

Genes	Primer sequence (5′-3′)	Size (bp)	Reference
16SrRNA	F-5′GCTCAACCAGGCACAAAAACA3′ R-5′ TCCACGCCTAGTTGGGTCTA 3′	300	Mohammad-Zadeh *et al*., 2019
Vaginolysin (*vly*)	F-5′ GCACCAGATAGCCCAGCAGA3′ R-5′ TTCGGTGCCGTACTCATCCC 3′	540	
Sialidase (*sld*)	F-5′ AGCCCGCATATCCCGTATCG 3′ R-5′ GGACCTGGCCAACATGGAGT 3′	454	
Phospholipase C (*Pho*)	F-5′GCGTGCTCCGCTTCGATTAG 3′ R-5′ TCCGCGGTAACGCTTCTCTT 3′	421	

3.2. Methods

3.2.1. Scheme of study

Scheme of study illustrated in figure (3-1).

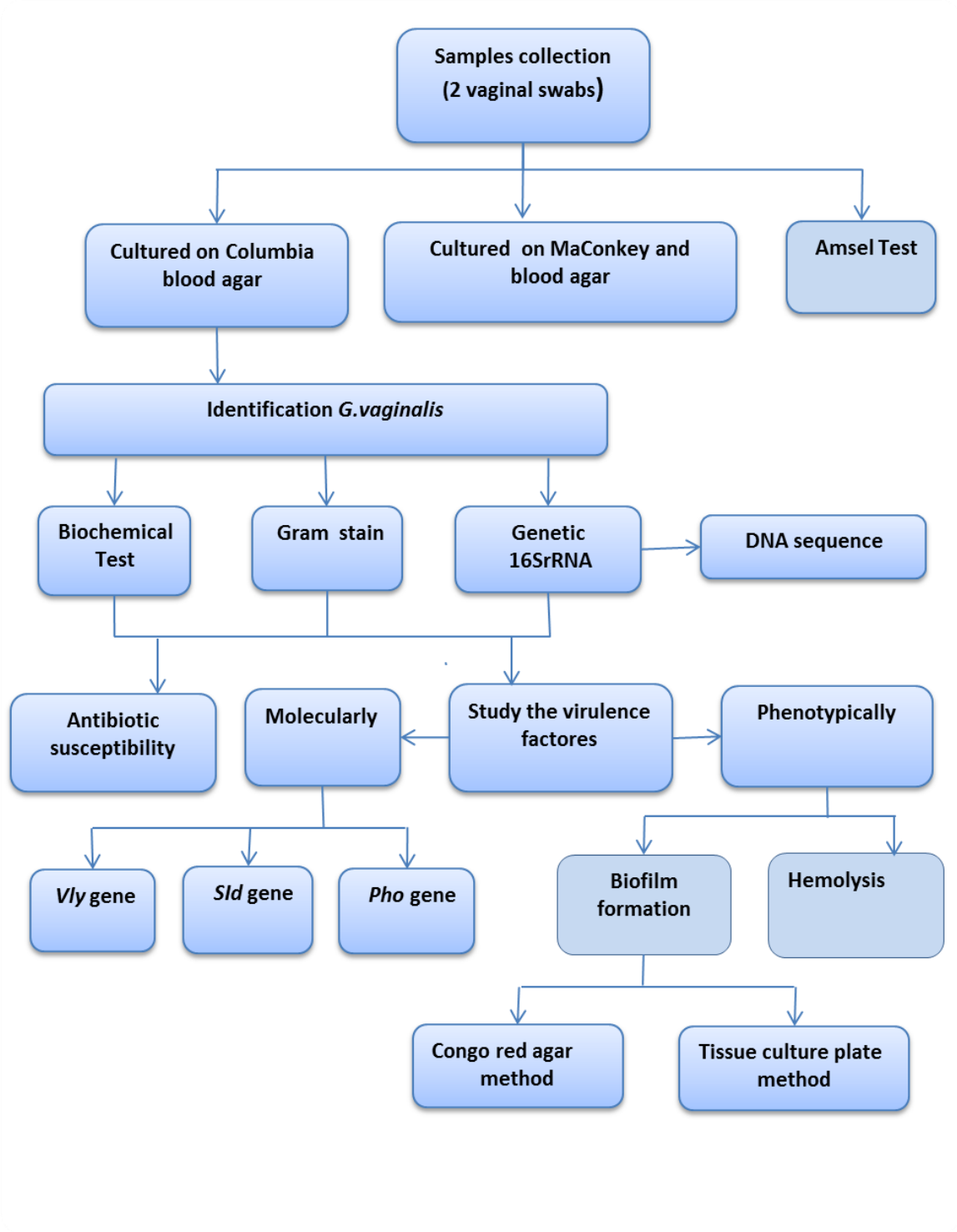

Figure (3-1): Scheme of study

3.2.2. Samples collection

Two hundred (100 for Amsel test , 100 for culture) vaginal swabs were taken from 100 pregnant and non-pregnant women (two high vaginal swabs from each woman) aged (17-60) years by the gynecologist with sterile cotton-tipped swabs was inserted in to the vagina to collect discharge from the lateral and posterior vaginal side were collected from hospitals in Babylon Province /Iraq: Al- Zahraa hospital for maternity , Al-Exandria general hospital and outpatient clinic during the period from December 2021 to April 2022.

3.2.3. Ethical approval

1-The study was done and the cases were collected after getting the agreement of the patients (verbal consent).

2- Approval of Babylon Science College Ethical committee.

3- Before starting the study , permission were taken from Babylon health presidency.

3.2.4. Preparation of solutions

3.2.4.1. Normal saline solution

Solution was prepared by dissolving 0.85 g of NaCl in 90 ml distilled water and further completed to 100 ml with D.W. then autoclaved at 121° C for 15 min and stored at 4° C until be used (Atlas,1995).

3.2.4.2. Phosphate buffer saline (PBS)

Buffer preparation performed by diluting 100 ml phosphate buffer in 900 ml of distilled water , then sterilized by autoclave, after that was kept at 4°C until using (Forbes *et al*., 2007).

3.2.4.3. Crystal violet solution (0.1%)

This solution was prepared by dissolving 0.1 gm of the material in 100 ml of D.W., filtrates by millipore filter and was kept in dark container (Collee *et al*., 1996).

3.2.4.4. McFarland standard solution (No. 0.5)

Solution was prepared according to Vandepitte *et al*., (2003) as follow:

A- Barium chloride solution $BaCl_2.2H_2O$

It was prepared by dissolving 1.175 g of $BaCl_2.\ 2H_2O$ in 100 ml distilled water.

B- Sulfonic acid solution H_2SO_4

It was prepared by adding 1 ml of H_2SO_4 to 99 ml distilled water. The tube solution of No. 0.5 was prepared by mixing 0.5 ml of (A) solution with 99.5 ml of (B) solution in which result in turbidity approximately equal to bacterial cells density of 1.5×10^8 cell/ ml.

3.2.4.5. Gel electrophoresis solutions

3.2.4.5.1. Ethidium bromide

Ethidium bromide dye (10 mg/ml) was prepared by dissolving 0.1 g of dye in 10 ml of sterile D.W and stored at 2-8 C° in a dark tube (Sambrook and Rusell, 2001).

3.2.4.5.2. Tris-Borate-EDTA (TBE)

Working buffer 1X TBE buffer was performed by mixing 100 ml from 10X TBE buffer to 900 ml of D.W (Sambrook and Rusell, 2001).

3.2.5. Reagents preparation

3.2.5.1. Oxidase reagent

This reagent was prepared 1g of tetramethyl-P-phenylenediamine dihydrochloride in 100 ml of D.W and was kept in dark container, oxidase reagent was used for determining the ability of bacteria to produce oxidase enzyme (Tadesse and Alem, 2006).

3.2.5.2. Catalase reagent

This reagent is hydrogen peroxide H_2O_2 (3 %) that was prepared from the stock solution and was kept in dark container, Catalase reagent was used for determining the ability of bacteria to produce Catalase enzyme (Tadesse and Alem, 2006).

3.2.5.3. Methyl red reagent

It was made by dissolving 0.1 g of methyl red in 300ml of 96 % ethanol, then adding D.W to produce 500ml, this reagent was used as an indication for the methyl red test (MacFaddin, 2000).

3.2.5.4. Ninhydrin reagent

It was prepared by dissolving 3.5 g of ninhydrin in 100 ml of mixture (aceton , butanol 1:1). This reagent was used for determining the ability of bacteria to produse amino acid (Glycine) as result of sodium hippurate hydrolysis (Winn *et al*., 2006).

3.2.6. Preparation of culture media

3.2.6.1. Columbia agar medium

This medium was used for the isolation of *G.vaginalis* from clinical specimens, it consists of :

- Columbia blood agar base.

- Human blood 5 %.

- *G.vaginalis* selective supplement.

- **Preparation of *G.vaginalis* selective supplement**

It was made by dissolving one vial of antibiotic (2 mg Gentamicin sulphate, 15 mg Nalidixic acid, 1 mg Amphotericin B) in 2 ml distilled water then sterilized by millipore filter. One vial is sufficient for 500ml of medium .

- **Preparation of Columbia blood agar base**

Forty four (44) g was suspended in 1000 ml purified/ distilled water. Heat to boiling to dissolve the medium completely.Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 45-50°C then adding *G.vaginalis* selective supplement and 5% of Human blood, mix together and distributed into Petri dishes then kept at 4°C until they used. This medium was used for detection of *G. vaginalis* (Gergova *et al*., 2013).

3.2.6.2. Blood agar medium

Blood agar medium was prepared by dissolving 40 g blood agar base in 1000 ml D.W. Heat to boiling and sterilize at 15 lbs pressure (121°C) for 15 minutes by autoclaving, cool to 45ºC and 5% of fresh human blood was added, mix well then pouring into petri dishes. It was used as

enrichment medium for the bacterial isolates and to determine their ability to hemolysis RBCs (Forbes *et al*., 2007).

3.2.6.3. MacConkey agar medium

MacConkey agar medium was made by dissolving 49.53 g in1000 ml distilled water, after heat medium to boiling. Sterilize at 15 lbs pressure at 121°C for 15 minutes by autoclaving. Cool to 45-50°C, and distributed into petri dishes, this medium was used to isolate Gram-negative bacteria by testing their ability to ferment lactose (MacFaddin, 2000).

3.2.6.4. MR-VP medium

MR-VP medium was mad by adding 17 g in in1000 ml distilled water,and distributed in to test tubes after boiling medium by heat, then sterilized by autoclave at 121°C for 15 minutes. This medium used to detect the partial and complete hydrolysis of glucose (MacFaddin, 2000).

3.2.6.5. Urea agar medium

This medium was made according to the manufacturing company by dissolving 24 gram urea agar base in 950 ml distilled water and sterilized by autoclave , then added 50 ml urea solution (40% sterilized by millipore filter) after cooling to 45°C, distributed into sterilized test tubes and allowed to solidify in a slant position. It was used to test the ability of bacteria to produce urease enzyme (Tille, 2017).

3.2.6.6. Tryptic soya broth

Tryptic soya broth was prepared according to the manufacturing company (30 g/L), autoclaved at 121ºC for 15 minutes and used for cultivation of bacterial isolates when it was necessary for biofilm detection (MacFaddin, 2000).

3.2.6.7. Brain heart infusion (BHI) broth

BHI broth was made according to the manufacturing company by dissolving 37 g in 1 liter of distilled water and autoclaved at 121ºC for 15 minutes (MacFaddin, 2000).

3.2.6.8. Kligler iron agar

The amount of 57.52 g was suspended in 1000 ml distilled water . Heat to boiling to dissolve the medium completly. Mix well and distribute into tubes . Sterilize by autoclaving at 15 lbs pressure (121 ° C) for 15 minutes, allow the tubes to cool in slanted position (Forbes *et al*., 2007).

3.2.6.9. Hippurate hydrolysis medium

It was prepared by dissolving 1 g of sodium hippurate in 100 ml of D.W, then sterilized by millipore filters (0.22mm) and distributed into sterile tubes (0.4ml) then kept at -20°C until be used.It was used for determining the ability of bacteria to produse amino acid (Glycine) as a result of sodium hippurate hydrolysis (Lin *et al*., 1986).

3.2.6.10. Maintenance medium

This medium performed by mixing 5ml of glycerol with 95ml of brain heart infusion broth and autoclaved at 121ºC for 15 minutes. It was used for preservation of bacterial isolates as stock for long time (Forbes *et al*., 2007).

3.2.6.11. Congo red agar

Congo red agar is medium consisting of brain heart infusion broth (37 g/l), sucrose (50 g/l), agar No. 1 (10 g/l), and Congo red (0.8 g/l). Congo red solution autoclaved it for 15 minutes at 121^{o}C. Finally, it was applied to autoclaved BHI agar supplemented with sucrose at a

temperature of 55^{o}C. The medium was used for detection of biofilm formation (Ruchi *et al*., 2015).

3.2.6.12. Mueller-Hinton agar

The amount of 38g of Mueller Hinton agar was suspended in 1L of distilled water, mix and dissolve them completely. Sterilize by autoclaving at 121 °C for 15 minutes. Pour the medium into the petri dish and wait for the medium to solidify this medium used in the antibiotic sensitivity test (MacFaddin, 2000).

3.2.7. Laboratory diagnosis

3.2.7.1. Wet smear

The wet smear of vaginal secretion was used to have been seen clue cells that was covered by bacteria (Catlin, 1992).

3.2.7.2. Direct staining

The direct staining conducted by using Gram stain of vaginal secretion to differentiate bacteria that was surrounded in clue cells (Catlin, 1992).

3.2.7.3. Diagnosis of bacterial vaginosis by Amsel's criteria

Depending on Amsel's criteria were diagnosed infected with bacterial vaginosis for all patient, Clinical diagnosis of bacterial vaginosis was considered positive if at least three of the following four criteria were seen: (Amsel *et al*., 1983)

1-Presence homogenous, white-milky vaginal discharge.

2-Vaginal discharge pH > 4.5 (examined by using pH strip).

3-Whiff test (amine odor test) doing by adding drops of 10% potassium hydroxide (KOH) to vaginal discharge on a glass slide, that give fishy odor as positive result.

4- Presence of clue cells examined by using microscope.

3.2.7.4. Culturing

Collected vaginal swabs were inoculated on different media, MacConky agar, Blood agar, Columbia blood agar supplemented with 5% fresh blood with the addition Nalidixic acid, Gentamicin, Amphotericin B for identification of bacterial isolates. The MacConkey agar, Blood agar were incubated aerobically at 37◦ C for 24 hours and Columbia blood agar was incubated anaerobically at 37◦ C in a candle jar for 36-72 hours (Ranjit *et al*., 2018).

3.2.7.5. Morphological and microscopical examination

After isolation of single colony was taken from each primary positive culture and identification depended on the morphology properties (colony size, shape, color, edge, pigment and texture). Then, single pure colonies were transferred to a microscopic slide, fixed well and stained with Gram stain to observe shape and reaction of cells were determined under a light microscope (Holt *et al*., 1994).

3.2.7.6. Biochemical tests

3.2.7. 6.1. Oxidase test

A single isolated colony was transferred from 24 hrs bacterial growth to a piece of filter paper by wooden stick. Hence, 2-3 drops of oxidase reagent were added on a filter paper. The positive result appeared within 20-30 sec. Indicating changing in color to dark purple (Forbes *et al*., 2007).

3.2.7.6.2. Catalase test

It was performed by transferring small portion of the colony on a glass slide by wooden stick and mixed with few drops of 3% H_2O_2, the evolution of bubbles of gas, indicates for positive test (Forbes *et al*., 2007).

3.2.7. 6.3. Urease test

Tubes containing slant urea agar medium were inoculated by bacterial isolates by stabbing, then incubated anaerobically at 37°C for 48 hours. The change the color of medium from yellow to pink indicates a positive result (Forbes *et al*., 2007).

3.2.7.6.4. Methyl red test

Tubes containing MR-VP broth were inoculated with bacterial colonies then incubated anaerobically at 37 °C for 28 hours, after that 5 drops of methyl red reagent were added. Shift of color to red indicates a positive result (MacFaddin, 2000).

3.2.7. 6.5. Hippurate hydrolysis test

Tubes containing sodium hippurate hydrolysis medium were inoculated with bacterial isolates and incubated anaerobically at 37 °C for 2 hours. After that 0.2ml of ninhydrin reagent were added, then Re-incubate for 10 minutes, appearance of a deep purple color indicates a positive result (Winn *et al*., 2006).

3.2.7.6.6. Kligler iron agar test

This test is used to detect the ability of bacteria to ferment sugars, glucose and lactose. Bacterial isolates were cultured on the Kligler iron agar by streaking on the surface and stabbing in buttom of the medium, then was incubated anaerobically at 37C° for 24 hr., the positive result

was noticed by changing the color of the phenol red indicator from red to yellow as a result for acid production from sugar fermentation, cracks and bubbles might appear in the medium at stabbing place according to gas production from sugar aerobic fermentation, black residue appeared at the butt of the tube as a result for the reaction between H_2S produced from anaerobic fermentation with ferrous sulphate in the medium (Forbes *et al*., 2007).

3.2.8 . Biofilm production

3.2.8.1. Congo red agar method

The CRA plates were inoculated with bacterial isolates and incubated at 37°C for 24 hours anaerobically. The presence of black colonies indicates biofilm formation, while the colonies of biofilm nonproducer remained pink or red in colour (Ruchi *et al*., 2015)

3.2.8.2. Tissue culture plate method (TCP)

1-A loopful of freshly cultured isolates was inoculated in 10 ml of trypticase soy broth with 1% glucose an incubated anaerobically for 48 hrs. at 37°C and then Bacterial suspensions were diluted 1:100 with fresh TSB medium.

2-Separate wells of a sterile polystyrene tissue culture plate, composed of 96 flat bottom wells, were filled by 200 µl of the prepared bacterial suspension and only sterile broth was used to ensure sterility and to identify non-specific binding.

3-The tissue culture plates were incubated for 24 hrs. at 37°C. After incubation, the plate was gently tapped to remove the content of the wells.

4-The wells were washed with 200 µl of PBS (Phosphate buffer solution) four times to remove any free bacteria present in the wells.

5-Sodium acetate (2%) were added to the wells and kept for 30 minutes in order to fix the biofilms formed by bacteria attached to the wells.

6-Staining of the fixed biofilms was conducted using crystal violet (0.1%). After 30 minutes, the wells were thoroughly washed by deionized water to remove any extra stain.

7-A 150µl of acetone: ethanol (2:8 v/v) mixture was added to dissolve bounded crystal violet.

8-A micro-ELISA reader (at 570 nm wave length) was used to measure the optical densities (OD) of stained bacterial biofilms.

9-Test was carried out in triplicate and average of three OD values was taken, optical densities values indicated bacterial adherence to the wells and biofilm formation.The results interpreted according to table (3-7). (Sultan and Nabiel, 2019).

Table (3-7) Classification of bacterial adherence and biofilm formation by TCB method

Optical densities values	Adherence	Biofilm formation
< 0.120	Non	Non/weak
0.120 – 0.240	Moderately	Moderate
> 0.240	Strong	High

3.2.9. Antibiotic susceptibility testing

3.2.9.1. Disc diffusion method

Kirby-Bauer method (Vandepitte *et al*., 2003) was used to carry the antibiotic susceptibility test as the follows:

1-The bacterial suspension was prepared by picked 3-5 colonies of each bacterial isolates from the culture and transferred to a sterile test tube containing 5 ml of normal saline and then it was compared with 0.5 McFarland standards (1.5×10^8 CFU/ml).

2-Sterile cotton swab was inserted into a tube containing bacterial suspension then it was rotated around and pressed against the inner walls of the tube to remove excess feed, then spread across petri dishes containing Muller-Hinton agar, then plates were left to dry.

3-Antibiotic discs used in the study mentioned in table (3-5) were placed on the inoculated plate using a sterile forceps.

4-Later the plates were inverted and incubated at 37ºC for 24 hours anaerobically.

5-Depending on CLSI (2021), the inhibition zones around the disks were measured by millimeter (mm) using a metric ruler.

3.2.10. Molecular identification

3.2.10.1. DNA extraction

DNA extraction was done according to boiling method (Mohran *et al*.,1998) as the following

1-A loopful of bacteria was suspended in 1 ml of distilled water in eppendorf tubes and mixed by vortex and resuspended in 200µl dH_2O.

2-Place eppendorf tubes in a boiling water bath for 10 min, placed on ice for 5 min, and then centrifuged at 10,000 x g for 5 min to separate the suspension.

3-Transfer the supernatant to new eppendorf tubes and use as DNA template for PCR.

3.2.10.2.Determination the purity and concentration of DNA

The concentration and purity of extracted DNA were evaluated by 1 µl of each DNA sample was applied in nanodrop to measure the optical density (O.D) at wavelength 260 nm and 280 nm. The ratio between the readings at 260 nm and 280 nm (OD260/OD280) was calculated to estimate the purity of the nucleic acids in the samples. Purity between 1.7 and 2.0 is usually accepted (Sambrook and Russell, 2001).

3.2.10.3. Primers preparation

1-The tube of lyophilized primers (Macrogen/Korea) were spined down before opening to prevent loss of pelleted oligonucleotide.

2-Amount of nuclease free water was added depending on manufacturer's instruction to obtain a final concentration of 100 pmol/µl (as a stock solution), stored in a deep freezer at -20°C until been used.

3-A working solution was prepared by adding 10µL of primer stock solution to 0.2 ml eppendrof tube that contains 90µL of Nuclease free water to obtain final concentration 10 pmol/µl, work solution was stored in the deep freeze until using in PCR mixture.

3.2.10.4. PCR reaction mixture

The polymerase chain reaction mixture for all genes in this study was set up for each gene alone in a final volume of 25 µl as mentioned below in table (3-8).

Table (3-8): Contents of the reaction mixture

Contents of reaction mixture	Volume (µl)
Master Mix	12.5
Forward primer	2
Reverse primer	2
Template DNA	5
Nuclease free water	Up to 25
Total	25

3.2.10.5. Amplification of genes

The polymerase chain reaction (PCR) is an enzymatic reaction used to *in vitro* amplification of target DNA with specific primers and a DNA polymerase, The extracted DNA, primers and master mix were vortex and centrifuged briefly to bring the contents to the bottom of the tubes, then placed in a thermocycler polymerase chain reaction. The polymerase chain reaction (PCR) was used to amplify (*16SrRNA*, *vly*, *sld*, *pho*) genes. PCR mainly consists of three consecutive stages (denaturation, annealing, and extension) of repetitive cycles to produce PCR product, amplification condition was carried out by Polymerase Chain Reaction (PCR) were listed in the table (3-9).

Table (3-9): Genes and PCR programs conditions

Gene	Stage	Temperature (°C)	Time	No. of cycles
16S rRNA	Initial denaturation	95	5 min	1
	denaturation	95	45 sec	30
	Annealing	59	50 sec	
	Extension	72	45 sec	
	Final extension	72	5 min	1
vly	Initial denaturation	95	5 min	1
	Denaturation	95	45 sec	30
	Annealing	62	50 sec	
	Extension	72	45 sec	
	Final extension	72	5 min	1
sld	Initial denaturation	95	5 min	1
	Denaturation	95	45 sec	30
	Annealing	62	50 sec	
	Extension	72	45 sec	
	Final extension	72	5 min	1
pho	Initial denaturation	95	5 min	1
	Denaturation	95	45 sec	30
	Annealing	61	50 sec	
	Extension	72	45 sec	
	Final extension	72	5 min	1

3.2.10.6. Agarose gel electrophoresis

Gel electrophoresis was used for the detection of PCR products which were visualized with the aid of ethidium bromide and UV transilluminator (Sambrook and Russel, 2001) as follows:

- A 1.5gm agarose was added to 100 ml of 1X TBE buffer to achieve a concentration of 1.5%.

- The solution was boiled (using microwave) until the agarose was completely dissolved and then the gel was allowed to cool at 45-50 °C.
- A 5µl of ethidium bromide (10mg/ml) was added to agarose solution, mixed well.
- Then the comb was fixed at one end of the tray for making wells used for loading DNA sample.
- The agarose solution was poured carefully in the tray, with avoiding air bubbles formation between the teeth of the comb after both the edges were sealed with cellophane tapes, the gel allowed to set at room temperature for 20-30 minutes.
- The comb was carefully removed and the gel were placed into the electrophoresis chamber filled with 1X TBE buffer that cover the surface of the gel until reached 3-5mm over the surface of the gel.
- The wells in agarose gel were loaded with 5µl of PCR product, and in one well, 5µl DNA ladder was mixed with 1µl of loading buffer to serve as a marker during electrophoresis run, whereas 5 µl of DNA extraction sample was loaded with 1µl of loading dye.
- Electrical power was turned on at 100v/mAmp for 75 minutes. The agarose gel was removed from the tank and visualized by a UV transilluminator documentation system and then photographed using a camera.

3.2.11. Sequencing of 16SrRNA gene

PCR products of 7 specimens of *G.vaginalis* were stored at -20 Cº, and then the nucleotide sequences of 16SrRNA genes(sanger sequencing) carried out by sending the specimens and primer to Macrogen Company in Korea.

3.2.12. Statistical analysis

Statistical Package for Social Sciences-version 20 (SPSS v20) was used for data input and analysis. Continuous variables presented as mean and discrete variables presented as numbers and percentages. Chi square test for goodness of fit used to test the significance of observed distributions. Chi square test for independence and used to verify the significance of observed associations. Findings with P value less than 0.05 was considered significant (Wayne, 2011).

Chapter Four

Results & Discussion

4. Results and Discussion

4.1. Study population

A total of 200 vaginal swabs (100 for Amsel test, 100 for culture) were collected from 100 pregnant and non-pregnant women (two high vaginal swabs from each woman) have bacterial vaginosis age between (17-60) collected by the physician from hospitals in Babylon Province : Al- Zahraa hospital for maternity, Al- Exandria general hospital and outpatient clinic for the period from December 2021 to April 2022.

A total of (100) patient were distributed in five groups according to the age < 20 years (10) patients, 20-30 years (45) patients, 31-40 years(27) patients, 41-50 years (14) patients, >50 (4) patients table (4-1).

Table (4-1): Distribution of patient according to the age

Age	Total NO.(%)
< 20	10 (10%)
20-30	45 (45%)
31-40	27 (27%)
41-50	14 (14%)
>50	4 (4%)
Total	100 (100%)

4.2. Identification of *G. vaginalis*

4.2.1. Wet smear

The wet smear prepared directly from the vaginal secretions and without staining showed the presence of clue cells covered with *G. vaginalis*. Wet mount microscopy was shown to be a superior approach for BV detection in a study that was conducted by Rumyantseva *et al.* (2015).

4.2.2. Direct staining

This test prepared from the vaginal discharges and staining with Gram stain showed presence of clue cells surrounded with *G. vaginalis* figure (4-1).

The direct stain of a vaginal swab is the laboratory method that is considered to be the gold standard for diagnosing BV. This method is utilized to assess the presence of rods (*G. vaginalis*), which are diagnostic of BV, as well as clue cells (squamous epithelial cells with granular appearance) (Gjatovska *et al*., 2020).

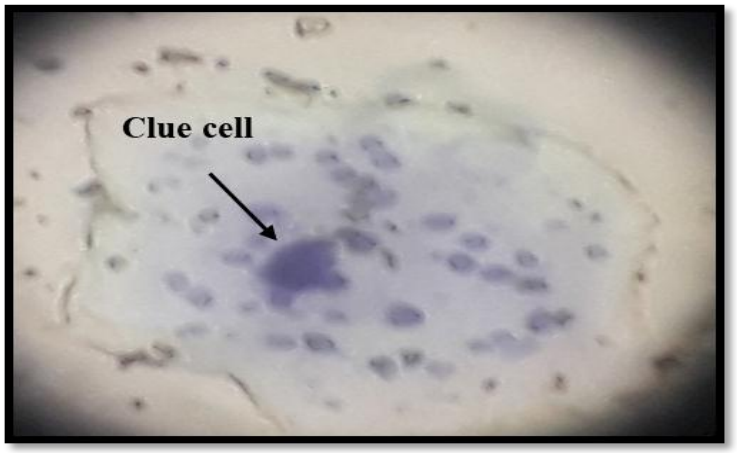

Figure (4-1) Direct staining of vaginal secretion, clue cell appear covered with *Gardnerella vaginalis*

4.2.3. Amsel test

Clinical diagnosis of bacterial vaginosis was considered positive if at least three of four criteria were seen, present 10 (17.8%) samples were positive to four criteria and other samples were positive to three of Amsel's criteria from the 56 samples table (4-2).

Table (4-2): Amsel criteria for patients

Character	Discharge	Whiff test	pH	Clue cell	No.(%)
1	+	+	+	+	10 (17.8%)
2	+	-	+	+	21 (37.5%)
3	+	+	-	+	11 (19.6%)
4	+	+	+	-	8 (14.2%)
5	-	+	+	+	6 (10.7%)
Total					56 (100%)

+ positive, - negative

Bacterial vaginosis is an imbalance in the ecology of the normal vaginal flora that is characterized by a depletion of lactobacilli and a multiplication of anaerobic bacteria. It most commonly presents clinically as a vaginal pH greater than 4.5, the presence of a thin and whitish homogeneous vaginal discharge, the finding of cells known as clue cells, and the presence of an amine odor upon the addition of (10%) potassium hydroxide (Bansal *et al*., 2019).

The presence of clue cells on saline wet-mount examination is the most specific and sensitive sign of bacterial vaginosis among the four Amsel's criteria microscopically, clue cells stand out from healthy vaginal epithelial cells due to their stippled and ragged appearance. The normal range for vaginal pH is between 3.8 and 4.2, and Amsel and colleagues found that a vaginal fluid pH that is higher than 4.5, vaginal pH more than

4.5 is a highly sensitive diagnostic marker for bacterial vaginosis, although it is not specific because other causes, such as semen, cervical mucus, menstruation, and trichomoniasis, can also increase vaginal pH (McGregor and McCullough, 2021).

Mohammadzadeh *et al*., (2015) shown that the Amsel criteria have a good level of both specificity and sensitivity in the diagnosis of BV. If there is no lab equipment available for the diagnosis of BV, this method can be used instead because it is highly effective, has low costs, and takes a short amount of time (due to the fact that it requires less equipment).

The Amsel criteria are the method that is considered to be the gold standard for the diagnosis of BV. This is especially true in developing countries where numerous criteria are used employed for the confirmation of BV. The clinical diagnosis and the results of a few simple laboratory tests are utilized for Amsel's criteria (Bhujel *et al*., 2021; Vieira-Baptista *et al*., 2021).

4.2.4. Culture identification

Gardnerella. vaginalis grow on Columbia agar supplemented with 5% fresh blood with the addition of Nalidixic acid, Gentamycin, and Amphotericin B, the colonies tend to be smooth, small colonies (hear pin) circular entire, glistening, and opaque colonies β- hemolysis surrounded with double hemolysis zone and some isolates was appeared non hemolytic, the addition of antibiotics were allowed to selective isolation of *G. vaginalis* that were described in figure (4-2).

Figure (4-2) : *Gardnerella vaginalis* growth on Columbia blood agar, the colonies appear smooth, small colonies (hear pin)

The *G. vaginalis* is a fastidious organism that needs an enriched medium for culture; in this study, Columbia agar that was supplemented with antibiotics and blood was used to promote the growth of this organism, this corresponds with de Souza *et al*., (2016).

4.2.5. Microscopic identification

Microscopic examination showed the cell arrangement by using Gram's stain. The smears of *G.vaginalis* bacteria stained with Gram stain showed the presence of small and heterogeneous bacilli of Gram-positive to heterogeneous forms of Gram stain, and they do not elongate into filamentous shapes and spread in the microscopic field figure (4-3). It was noted that increasing the incubation period of these bacteria led to the transformation of bacteria from Gram-positive to Gram-negative (Vieira-Baptista and Bornstein, 2019).

Gram staining technique is least expensive, requires the least time to perform, is more widely available than other laboratory methods and is the most interpretative of the laboratory method (Baruah *et al*., 2014).

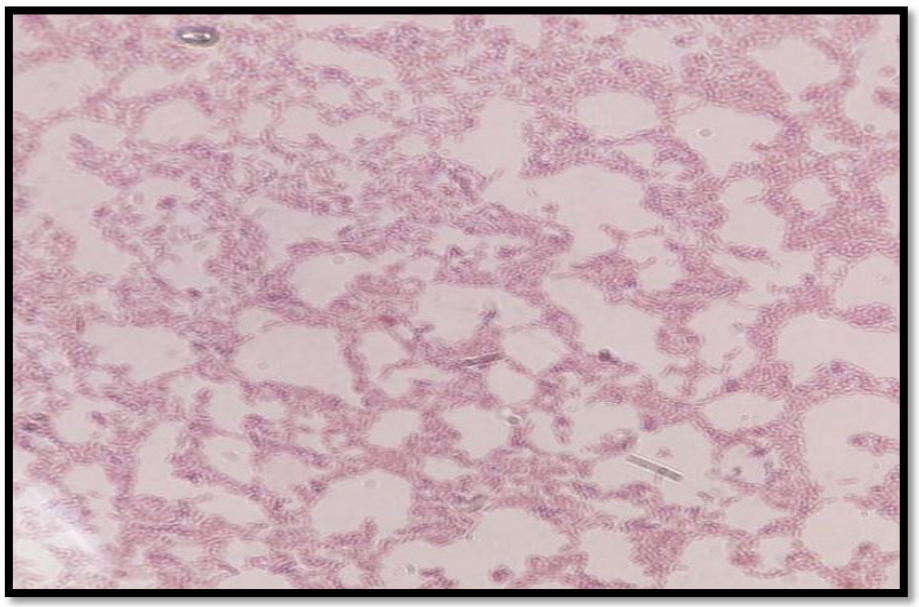

Figure (4-3): Appearance of *Gardnerella vaginalis* in Grams stains

4.2.6. Biochemical test

All the *G. vaginalis* isolates gave negative result of the oxidase test, which means it cannot produce cytochrome C, an enzyme of the bacterial electron transport chain which differentiated from genus that usually gives the positive result as a purple color, hippurate hydrolysis variable, β-blood hemolysis variable, urea hydrolysis negative, Kliglar iron agar test acid/acid, change color of slant and butt to yellow this means the fermentation of glucose and lactose table (4-3).

The isolates gave variable results to the catalase enzyme. The catalase enzyme breakdown of hydrogen peroxide into oxygen and water, when a small amount of an organism produces catalase, rapid elaboration of bubbles of oxygen (Cappuccino and Welsh, 2020).

Table (4-3) Biochemical test of *Gardnerella vaginalis*

Characteristics	Result
Oxidase	-
Catalase	±
Hippurate hydrolysis	±
β-Blood hemolysis	±
Urea hydrolysis	-
Methyl red test	+
Kliglar iron agar test	A/A

+ positive, - negative, ± variable, A/A: acid/acid

A total of 100 samples the result of culture, biochemical, and Gram stain present the percentage of samples positive for *G.vaginalis* was 56(56%), including (7%), (26%), (13%), (8%), and (2%) isolated from < 20, (20-30), (31-40), (40-50), and >50 age, respectively table (4-4).

Table (4-4): Distribution of *Gardnerella vaginalis* isolates according to the age

Age	No. of positive samples (%)	No.of negative samples (%)	Total (%)
< 20	7 (7%)	3 (3%)	10 (10%)
20-30	26 (26%)	19 (19%)	45 (45%)
31-40	13 (13%)	14 (14%)	27 (27%)
41-50	8 (8%)	6 (6%)	14 (14%)
>50	2 (2%)	2 (2%)	4 (4%)
Total	56 (56%)	44 (44%)	100 (100%)

P-value 0.810 > 0.05 is non-significant

The results of this study were comparable with the results obtained by Ali *et al*., (2017), who reported that *G. vaginalis* was isolated from (50%) of infection cases in Baghdad city by utilizing the culture method, while

more than the results obtained by other researchers in Iraq, Al-Muk and Hasony, (2001) found that the *G.vaginalis* isolates in a prevalence rate of (7.7%) were detected in Basrah city, Al-Sultany, (2012) found the frequency of *G. vaginalis* was (27.5%) from the culture in Babylon city, Al-Dhalmi, (2013) found *G. vaginalis* at a rate of (10%) in the city of Al-Kufa, Gatea *et al*., (2020) found that the isolation rate of *G.vaginalis* was (25.33%) obtained from miscarriage and non-miscarriage vaginosis women in Al-Hillah city and, Al-Alwani, (2008), who conducted research in the city of Al-Ramadi, the frequency among the culture was 27 percent, but less than the result abtained by Nisha *et al*., (2019) who found that 83.3% of the isolates of *G. vaginalis* came from women with BV.

This variation may be result from the geographic distribution, sample type, number of tested samples and uptake of the antibiotic. The difference in the prevalence sexually transmitted pathogens could be caused by a number of reasons; including environmental and socio-economic factors, accurate an gynecologist takes a sample from a patient, the method of diagnosis, the number of tested samples, the type of samples, and cultural factors (Ali *et al*., 2017).

The current results showed that women in the (20-30) and (31-40) age groups were more susceptible to infection, this finding may be related to the fact that most women have high rates of reproduction at these ages because reproductive activity is more active at these ages than at other ages, with hormonal changes acting as the primary causative factor and sexual activity.

Yuk *et al*., (2021) found the percentage of patients who tested positive for *G. vaginalis* was highest in the age group of (40-49) years old (37.8 %), followed closely by the age groups of (< 19) and (50-59) years old,

while Al-Rubaeaee, (2017) found that the (20-40) age group was susceptible to infection due to reproductive activity and hormonal shift (estrogen and progesterone level). This may be attributed to the fact that sexually active women, menstrual cycle, hormonal factors, and the use of contraceptives are all factors that play a role in the transmission of STPs. (Ali *et al*., 2017). Several studies found a relationship between age group and the progression of BV as well as the widespread distribution of *G. vaginalis* (Ranjit *et al*., 2018), this may be attributed to a number of factors that explain the role of age in the prevalence of BV, such as the interaction of behavioral, physiological, and immunological variables (Al-Daamy *et al*., 2019).

Table (4-5) showed that the pure culture of *G. vaginalis* isolates was (0%), whereas mixed *G. vaginalis* and other Gram-positive bacteria, *G.vaginalis* and Gram-negative bacteria were (19%, 37%), respectively. Also present the percentage of only Gram-positive bacteria or only Gram-negative bacteria was (12%, 21%), respectively, and (11%) of the samples was no growth figure (4-4).

Table (4-5): Distribution *Gardenella vaginalis* isolate among causes agents

Type of causes agent	Number	Percentage
Only *G. vaginalis*	0	0%
Only Gram- positive bacteria	12	12%
Only Gram- negative bacteria	21	21%
G. vaginalis and Gram-positive bacteria	19	19%
G. vaginalis and Gram-negative bacteria	37	37%
No growth	11	11%
Total	100	100%

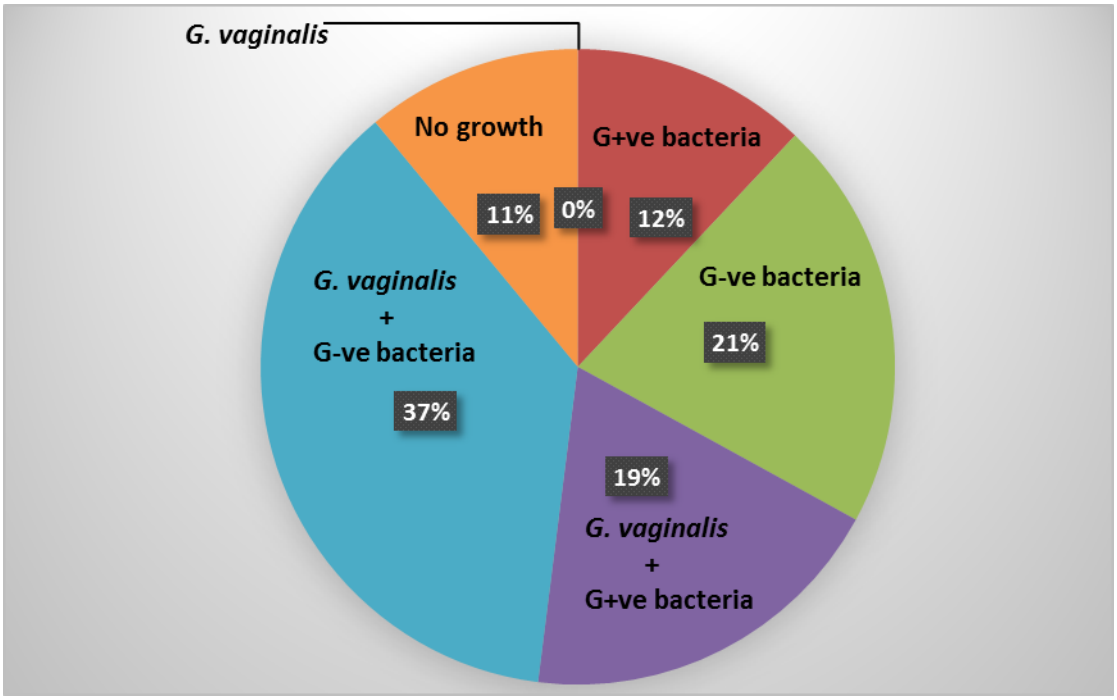

Figure (4-4): Distribution *Gardenella vaginalis* isolates among causes agents

It was found that mixed vaginitis caused by (*G.vaginalis* and Gram-positive) or (*G.vaginalis* and Gram-negative) led to adverse outcomes, but other studies found that mixed vaginitis is a complex vaginal symbiosis that is different from single vaginitis (Li *et al*., 2022), mixed vaginitis is when two or more kinds of vaginitis happen at the same time (Qi *et al*., 2021). The most common forms of mixed vaginitis are BV + VVC, BV + AV, AV + VVC, and VVC + AV + BV (Hillier *et al*., 2021). Research has demonstrated that mixed vaginitis is comparable to single vaginitis and similarly results in unfavorable gynecological and obstetrical outcomes (Abdul-Aziz *et al*., 2019). In addition, studies conducted *in vitro* and *in vivo* have shown that the colonization and virulence of microorganisms, as well as the immune response of the host, are enhanced in cases of mixed infection when compared with those observed in single infections. Furthermore, the clinical phenotypes and

adverse outcomes caused by microorganisms may be more severe in mixed infections than they are in single infections (Farrokhi *et al*., 2021).

4.2.7. Molecular identification

In this study used polymerase chain reaction was used technique (PCR), the results of PCR amplification to specific *16SrRNA* primers of *G. vaginalis* isolates after electrophoresis, a band of 300 bp when compared with ladder, was detected in only 12 (21.4%) from the 56 positive culture samples including (7.1%), (8.9%), (1.7%), (1.7%), and (1.7%) isolated from < 20, (20-30), (31-40), (41-50), and >50 age, respectively figure (4-5), table (4-6).

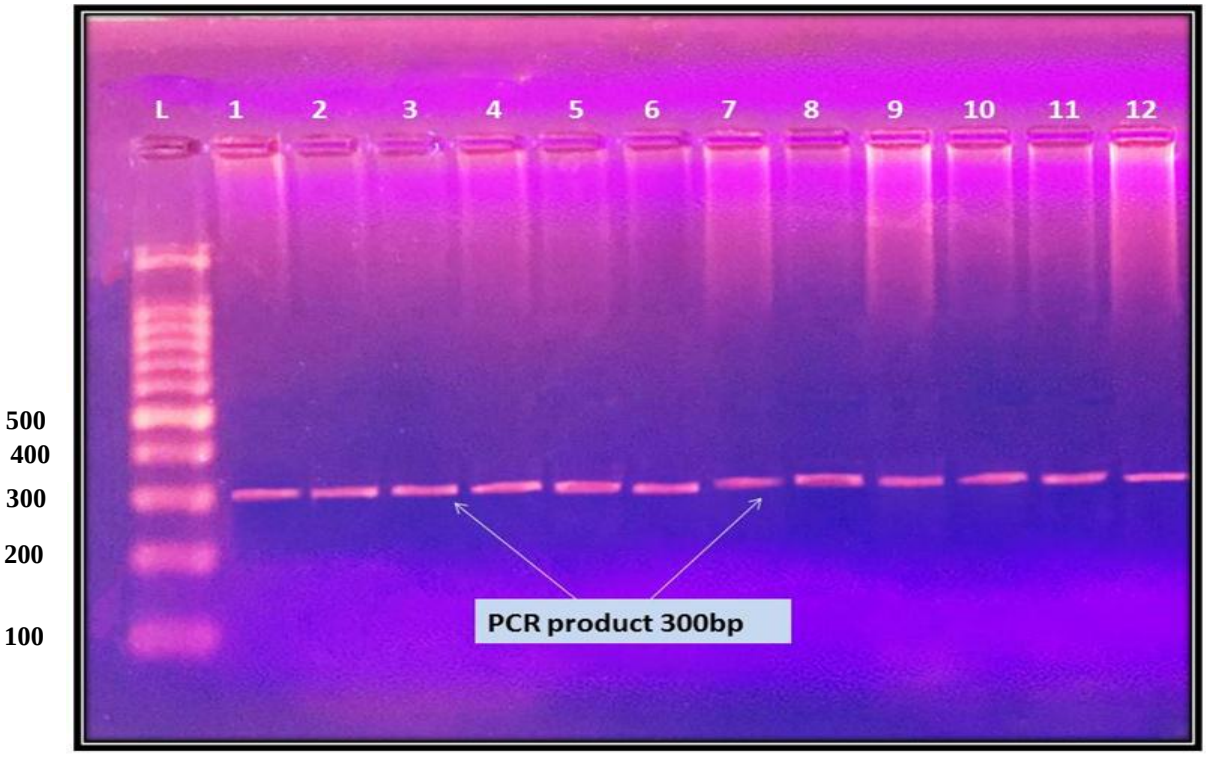

Figure (4-5): Agarose gel electrophoresis staining with ethidium bromide stains (1.5% agarose, 70volt for 60 min) for *Gardnerella vaginalis 16SrRNA* gene product (amplified size 300 bp) using DNA template of *Gardnerella vaginalis* isolates. Lane (L) molecular size marker for DNA molecules (1500-bp ladder). Lanes (1-12) show positive results

Table (4-6): Distribution of *Gardnerella vaginalis* isolates according to the age diagnosis by *16SrRNA*

Age	No. of positive samples (%)	No. of negative samples (%)	Total No.(%)
< 20	4 (7.1%)	3 (5.3%)	7 (13.5%)
20-30	5 (8.9%)	21 (37.5%)	26 (46.4%)
31-40	1 (1.7%)	12 (21.4%)	13 (23.2%)
41-50	1 (1.7%)	7 (12.5%)	8 (14.2%)
>50	1 (1.7%)	1 (1.7%)	2 (3.5%)
Total	12 (21.4%)	44 (78.5%)	56 (100%)

P-value 0.085 > 0.05 is non-significant

According to a study conducted by Menard *et al*., (2008), the sensitivity of the molecular diagnosis of *G. vaginalis* was found to be 95%, while the specificity was found to be 99%. The findings of these studies agree our results, demonstrating that molecular methods for detecting BV are more sensitive and specific than microscopy methods.

The result of this study less than the obtain by Gatea *et al*., (2020) result of PCR detection for *16SrRNA* gene of *G. vaginalis* obtained from miscarriage and non-miscarriage vaginosis women was (92.66 %), Al-Rubaeaee (2017) showed percentage (23.3%) gave positive results to *Gardnerella vaginalis* and, Pillay *et al*., (2020) found the *16SrRNA*gene of *G. vaginalis* was only amplified in 37 of the 137 samples analyzed (27.2%) in South African Women.

The findings of PCR showed that out of 27 cases of bacterial vaginosis, 21 cases (78%) were for *G.vaginalis*. The frequency of

vaginosis was highest in the age range of (18-23) years old, while it was lowest in the age range of (30-36) years old. (Housseini *et al*., 2019).

The results of the gel electrophoresis of the amplicon that was produced as a result of the amplification of the *16SrRNA* gene showed that the percentage of *G. vaginalis* among high vaginal swabs was 24 (63%) , the age group (15-25) years old had the highest proportion of *G. vaginalis* distribution (29%) followed by the age group (26-36) years (18.4%), and the age group (37-47) years had the lowest percentage (15.8%) (Al-Daamy *et al*., 2019).

Molecular techniques such as polymerase chain reaction (PCR) are utilized in the diagnostic process of a variety of diseases. However, in order to develop a simpler method that is applicable in clinical laboratories, particularly in developing countries, so simple PCR method was used to identify *G. vaginalis* in vaginal samples (Hashemi *et al*., 2021).

According to Sha *et al*., (2005) the Amsel criteria had a low predictive value for the diagnosis of BV. They also said that the PCR approach was substantially more accurate than Amsel criteria in determining the presence of this infection.

4.3. Molecular detection of *G. vaginalis* virulence factors

The polymerase chain reaction technique (PCR) was used for detection of specific virulence determinants, vaginolysine, salidase, phospholipase C genes.

4.3.1. Molecular detection of vaginolysin gene (vly)

The results showed that out of 12 isolates only 4 (33%) of *G. vaginalis* isolates gave positive results to *vly* gene with 540 bp when compared with the ladder as shown in figures (4-6).

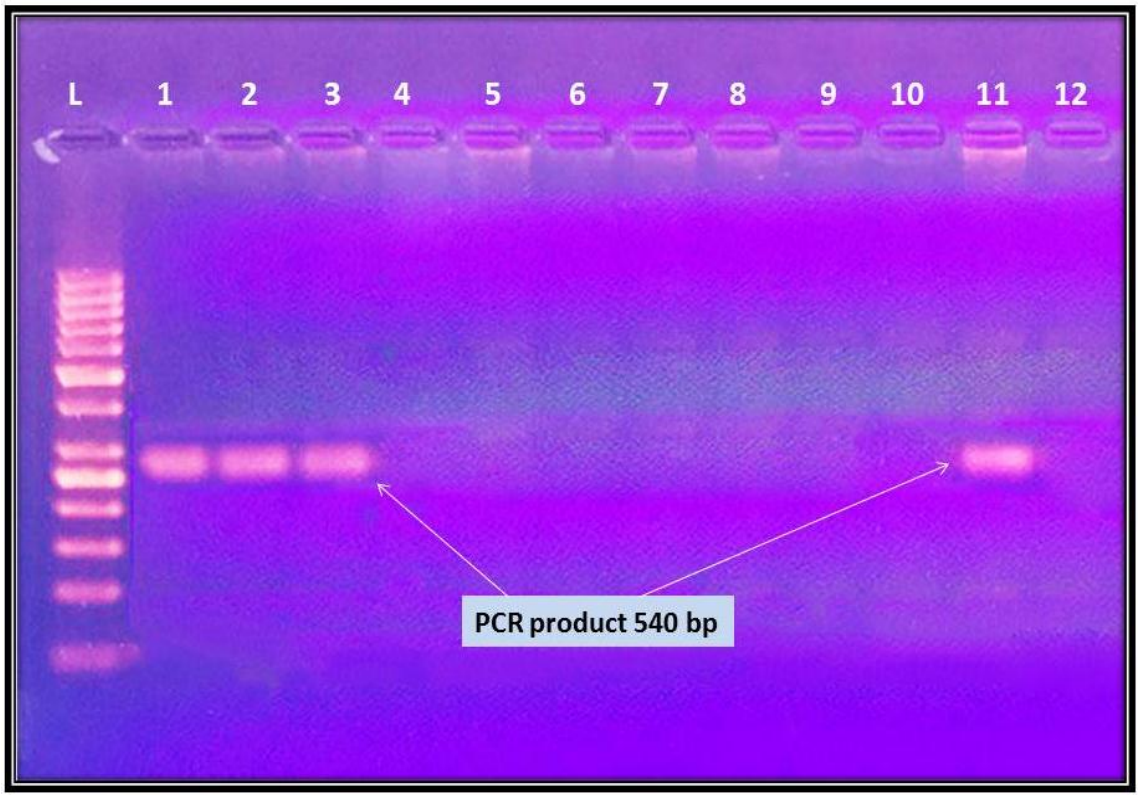

Figure (4-6): 1.5% agarose gel electrophoresis at 70 volt for 60 min after staining with ethidium bromide for vly PCR products. Lane (L) molecular size marker for DNA molecules (1500-bp ladder). Lanes (1, 2, 3, 11) showed positive results, the size of product is 540 bp

The result of this study was similar to the result abstained by other researchers Bunyan *et al*., (2018) who used molecular detection of vaginolysin gene that 12 samples (36.7%) were positive results and, Mohammadzadeh *et al*., (2019) found the prevalence of *vly* genes in BV-associated *G. vaginalis* was 10 (35.7%).

But less than the result found by de Souza *et al*., (2016) who detected *vly* gene in all *G. vaginalis* isolated from non-BV women and in(98.3%) of the bacteria from BV patients, Janulaitiene *et al*., (2017) found that the prevalence of *vly* genes in *G. vaginalis* isolated was (86.8%) by PCR.

Gardnerella vaginalis strains were able to create vaginolysin, a pore-forming toxin that was encoded by *vly* genes and led to the death of cells, vaginolysin, a toxin that is a member of the cholesterol-dependent cytolysin (CDC) family of toxins, promotes cell lysis by way of the complement regulating protein CD59 that is present in the cells that are the targets of the toxin (Wong *et al*., 2018)

Gardnerella vaginalis possesses some virulence factors that are already well recognized. One of these is the cytolytic toxin that functions as a hemolysin and is known as vaginolysin. This toxin is implicated in the pathogenesis of BV, which ultimately results in cell death (de Souza *et al*., 2016).

Randis *et al*., (2013) examined the cytotoxic activity of anaerobic bacteria linked to BV, it was discovered that only *G. vaginalis* was able to cause the lysis of vaginal epithelial cells, whereas the other bacteria under investigation did not result in any detectable cytological changes.

4.3.2. Molecular detection of sialidase gene (sld)

A total of 12 *G. vaginalis* isolates 9 (75%) isolates gave positive results for *sld* gene with molecular weight 454 bp in PCR amplification when compared with ladder, as shown in figure (4-7).

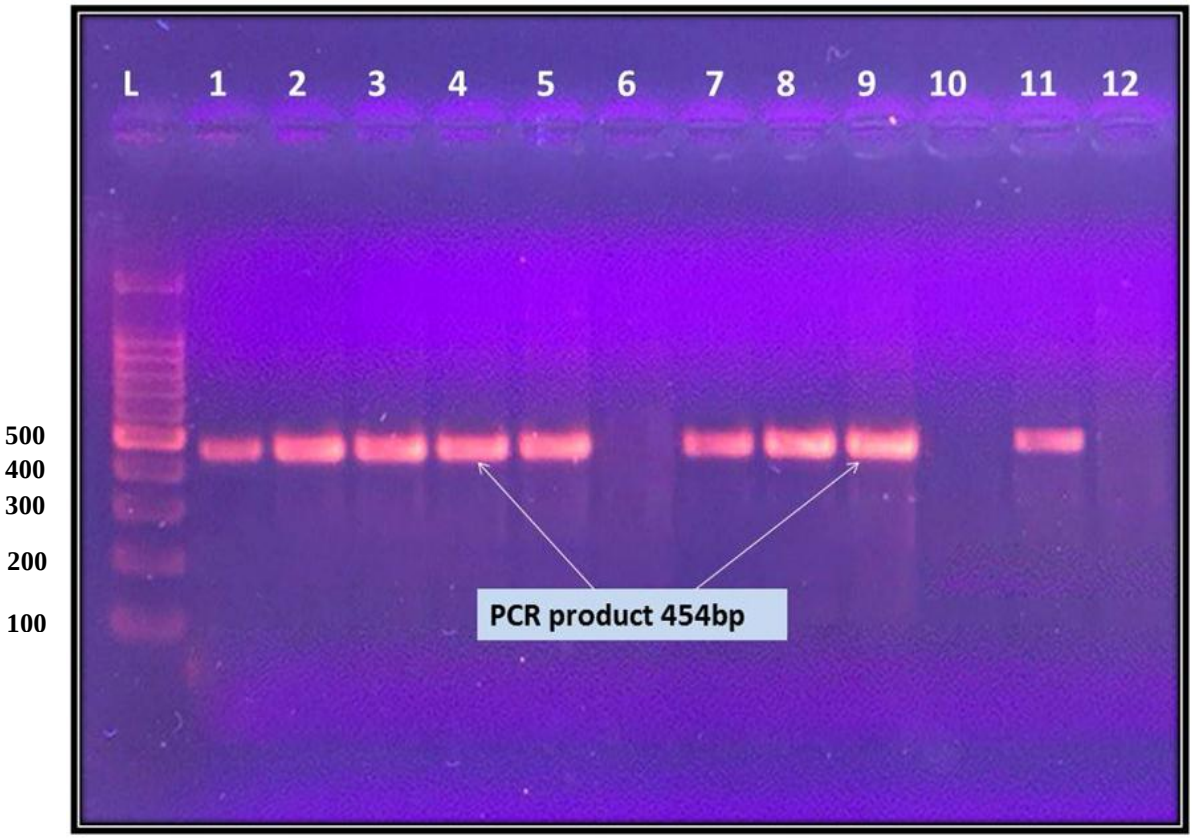

Figure (4-7): 1.5% agarose gel electrophoresis at 70 volt for 60 min after staining with ethidium bromide for sld PCR products. Lane (L) molecular size marker for DNA molecules (1500-bp ladder). Lanes (1, 2, 3, 4, 5, 7, 8, 9, 11) show positive results, the size of product is 454 bp

The result was consistent with the result of Hardy *et al*., (2017b), the putative sialidase A gene was found in 75% of the *G. vaginalis*-positive vaginal specimens. Also Shipitsyna *et al*., (2019) found the *G. vaginalis* sialidase gene was in (70%) of Russian women.

Also, Mohammadzadeh *et al*., (2019) found the prevalence of *sld* genes in BV-associated *G. vaginalis* was 19 (67.8%). *G. vaginalis* contains a virulence factor called sialidase, which is encoded by the *sld* gene and contributes to the establishment of BV (Qian *et al*., 2021).

The activity of sialidase has been suggested as a biomarker for BV. It has been independently linked to adverse pregnancy outcomes, such as uterine infections and premature births (Cauci and Culhane, 2011).

G. vaginalis an anaerobic pathogen, was responsible for the production of the enzyme sialidase, which cleaves terminal sialic acid residues of human glycans. Notably, a high sialidase activity level has been linked to premature birth as well as a low birthweight (Govinden *et al*., 2018).

4.3.3. Molecular detection of phospholipases C gene (pho)

The results showed that out of 12 isolates only 6 (50%) of *G. vaginalis* isolates gave positive results for *pho* gene with molecular weight 421bp PCR amplification when compared with ladder as shown in figure (4-8).

Mohammadzadeh *et al*., (2019) found the prevalence of *pho* genes in BV-associated *G. vaginalis* was 6 (21.4%). Al-Rubaeaee, (2017) detected the phospholipase gene in (50%) of the *G. vaginalis*-positive vaginal samples.

Phospholipases have the ability to cleave phospholipids found in eukaryotic membranes, which makes them an important factors of virulence. In addition, the significance that these enzymes play in evading the immune response of the host makes them very necessary for the pathogenicity of certain bacteria (Bandana *et al*., 2018). Phospholipase C may facilitate the release of arachidonic acid and prostaglandins, which contributes to the progression of the processes that result in premature delivery (McGregor and McCullough, 2021).

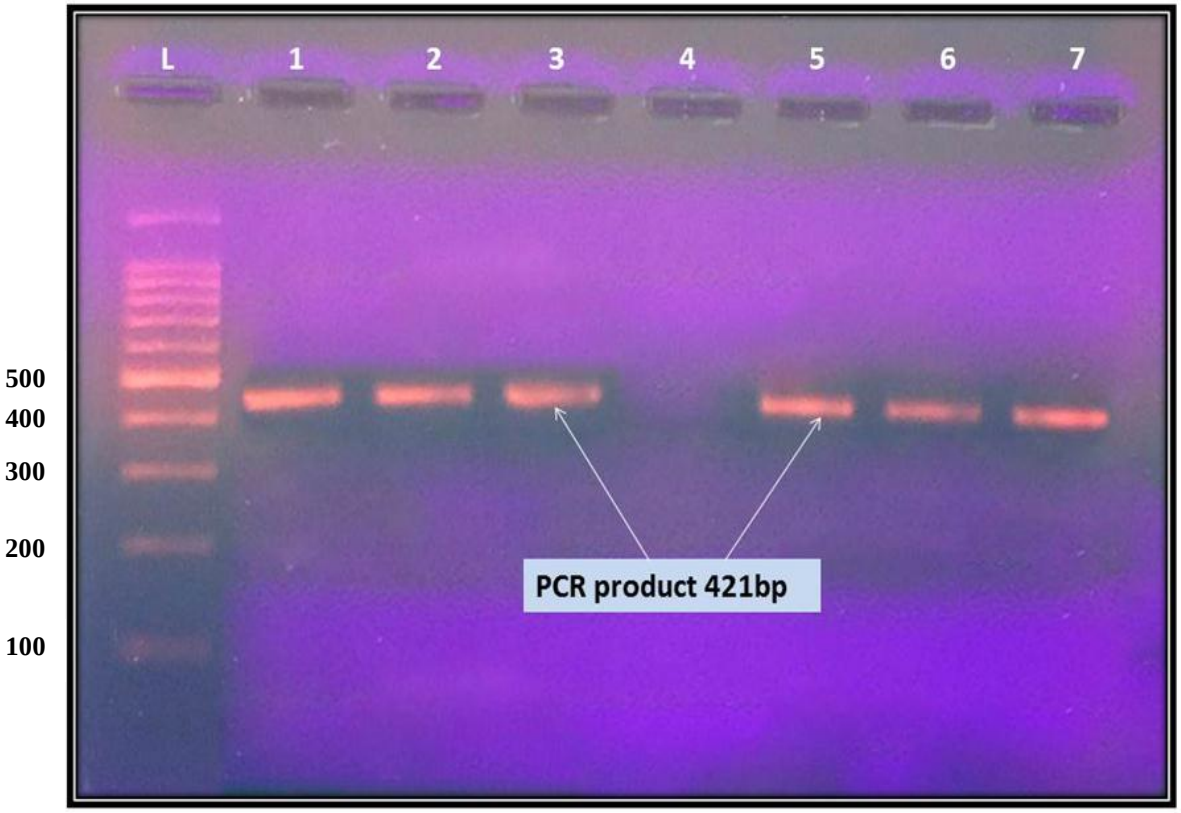

Figure (4-8): 1.5% agarose gel electrophoresis at 70 volt for 60 min after staining with ethidium bromide for pho PCR products. Lane (L) molecular size marker for DNA molecules (1500-bp ladder). Lanes (1, 2, 3, 5, 6, 7) show positive results, the size of product is 421 bp

The present study appeared that out of 12 isolates(3) of *G. vaginalis* isolates gave positive results for *vly* gene, *sld* gene and *pho* gene, (2) of *G. vaginalis* isolates gave positive results for *sld* gene and *pho* gene, (1) of *G. vaginalis* isolates gave positive results for *sld* gene and *vly* gene, (3) of *G. vaginalis* isolates gave positive results only for *sld* gene, (1) of *G. vaginalis* isolates gave positive results only for *pho* gene, (2) of *G. vaginalis* isolates gave negative results for *vly* gene, *sld* gene and *pho* gene table (4-7).

Table (4-7): Distribution virulence factors of *G. vaginalis*

No.	*vly* gene	*sld* gene	*pho* gene
1	**+**	**+**	**+**
2	**+**	**+**	**+**
3	**+**	**+**	**+**
4	**-**	**+**	**-**
5	**-**	**+**	**+**
6	**-**	**-**	**+**
7	**-**	**+**	**+**
8	**-**	**+**	**-**
9	**-**	**+**	**-**
10	**-**	**-**	**-**
11	**+**	**+**	**-**
12	**-**	**-**	**-**
Count(%)	(33%)	(75%)	(50%)

+positive, - Negative

4.4. Biofilm formation of *G. vaginalis*

4.4.1. Congo red agar method

A total of 12 isolates were tested for biofilm production by CRA, the results showed that all isolates were biofilm producers 12 (100%). Black colonies indicated biofilm production (the colour of colony changing from red to black on Congo red agar) figure (4-9).

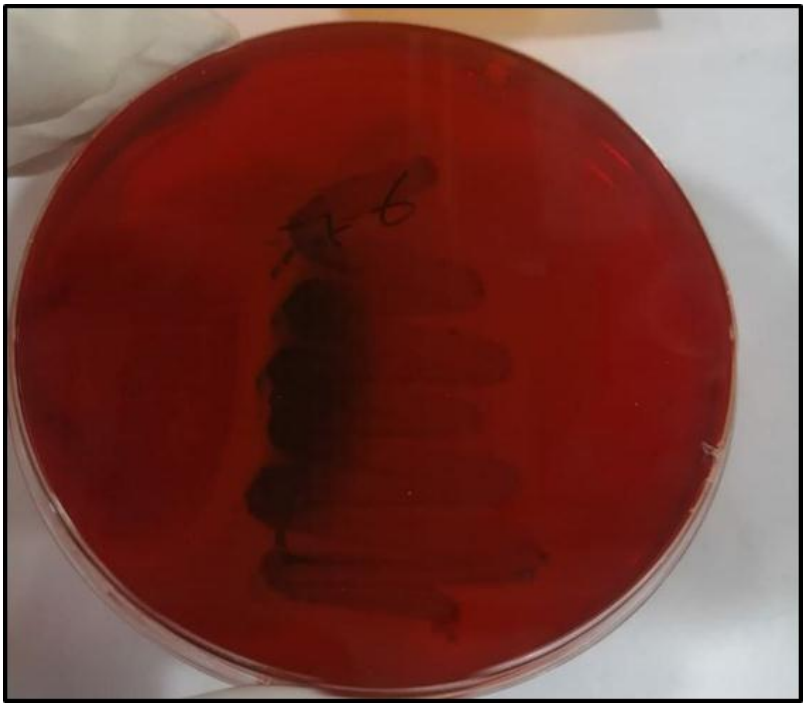

Figure (4-9) Production of biofilm in *Gardnerella vaginalis* on Congo red agar. The colonies appear black

The Congo red agar method is based on phenotypic characteristics, such as the morphology and color of biofilm-forming bacteria's colonies compared to those of non-biofilm-forming bacteria in the presence of Congo red dye.

The CRA method is simple to carry out, and the results are often dependent on the color of the colony that is created. The color of the colony can range from red for strains that do not produce biofilm to black for strains that do develop biofilm (Kaiser *et al*., 2013).

The biofilm serves as a barrier and protects the encapsulated bacterial cells from the primary antibiotic therapy, which frequently results in treatment failure and recurrent infection (Rosca *et al*., 2020).

4.4.2. Tissue culture plate method (TCP)

Biofilm formation of *G. vaginalis* isolates was detected by tissue culture plate method (TCP). Results showed that out of 12 *G. vaginalis* isolates all isolates were biofilm former 12 (100%) at moderate levels table (4-8).

Table (4-8) Production of biofilm in. *Gardnerella vaginalis*

Bacterial isolate No.	**Biofilm**			
	Strong	Moderate	Weak	% of biofilm Formation
***G. vaginalis* (12)**	0	12	0	100%

Tissue culture plate method (TCP) is regarded as the gold standard test for identifying the formation of biofilms (Hassan *et al*., 2011) . Crystal violet is a basic dye that is known to bind to negatively charged molecules on the cell surface as well as nucleic acids and polysaccharides, and thus gives an overall measurement of the whole biofilm. It has been used as a standard technique for rapidly accessing cell attachment and biofilm formation in a range of Gram positive and Gram-negative bacteria (Al-Dahmoshi, 2013).

A biofilm is a structured community of microorganisms in a self-produced extracellular matrix that adheres to the surface of epithelial cells, *G. vaginalis* has been found in abundance in the BV biofilm, and shedding of vaginal epithelial cells coated with the BV biofilm serves as a clue cell, *G. vaginalis* biofilms have the ability to adhere to epithelial cells and provide protective features, such as tolerance to hydrogen peroxide (H_2O_2) and lactic acid produced by lactobacilli, inhibition of

removal by the immune system, and antimicrobial resistance, which promote the recurring and chronic nature of bacterial vaginosis (Lev-Sagie *et al*., 2022).

Biofilm is a strong and dynamic structure that provides many benefits to its members, including adhesion/cohesion capabilities, mechanical properties, nutritional sources, metabolite exchange platform, cellular communication, protection and resistance to drugs (e.g., antimicrobials, antiseptics, and disinfectants), environmental stresses (e.g., dehydration and ultraviolet light), and host immune attacks (e.g., antibodies, complement system, antimicrobial peptides and phagocytes), and shear forces (Santos *et al*., 2018b).

The present result was similar to the result found by Al- Rubaeaee, (2017) who found that (100%) gave positive results for biofilm formation by *G. vaginalis* isolates but Nisha *et al*., (2019) was found (78.4%) of *G. vaginalis* isolates from women with BV gave positive results to biofilm production.

Verwijs *et al*., (2020) showed the high levels of *Gardnerella* species in biofilms have been linked to the failure of antibiotic treatment for BV patients.

4.5. Antibiotic susceptibility test

Antimicrobial susceptibility test towards eight antibiotics was determined using disc diffusion method. The isolates showed a variable levels of resistance to antibiotics used in this study, the isolates showed resistance to Metronidazole (100%), Clindamycin (92%), Moxifloxacin (0%), Penicillin G (42%), Ampicillin/Sulbactam (92%), Imipenem (0%),

Meropenem (0%) and Piperacillin/Tazobactam (0%) table (4-9), figure (4-10).

Overall the most active antibiotic for *G.vaginalis* isolates was Moxifloxacin, Meropenem, Imipenem and Piperacillin/Tazobactam were determine for 12/12 (100%). The least active antibiotics was Metronidazol 0(0%), Clindamycin and Ampicillin/Sulbactam 1/12 (8%) to both of them. Penicillin G was exhibited intermediate susceptibility 7/12 (58%).

Among the 12 tested isolates of *G. vaginalis*, 0 (0%) isolates was sensitive to Metronidazole, while 12 (100%) were resistant to this antibiotic. The resistance mechanism may be related to microbial community structure (biofilm layer formation) according to the result of this study, whereas 12/12 (100%) resistant isolates to Metronidazol were formed this biofilm layer *in vitro*.

Table (4-9) Percentage of antibiotic susceptibility of *Gardnerella vaginalis* isolates to eight antibiotics

Antibiotic	Sensitive (%)	Resistant (%)
Metronidazol	0 (0%)	12 (100%)
Clindamycin	1 (8%)	11 (92%)
Moxifloxacin	12 (100%)	0 (0%)
Penicillin G	7 (58%)	5 (42%)
Imipenem	12 (100%)	0 (0%)
Meropenem	12 (100%)	0 (0%)
Ampicillin/Sulbactam	1 (8%)	11 (92%)
Piperacillin/Tazobactam	12 (100%)	0 (0%)

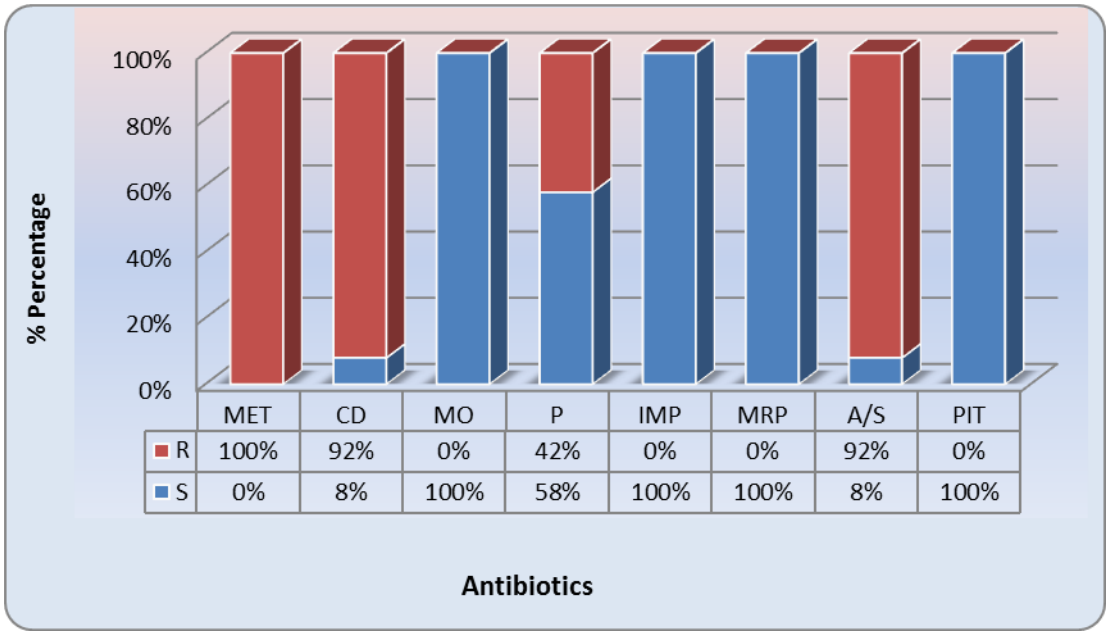

Figure (4-10) Percentage of antibiotic susceptibility of *Gardnerella vaginalis* isolates to eight antibiotics

Metronidazole(MET), Clindamycin(CD), Moxifloxacin(MO), Penicillin(p), Imipenem(IMP), Meropenem(MRP), Ampicillin/Sulbactam(A/S), Piperacillin/Tazobactam(PIT).

The resistant of Penicillin G to *G.vaginalis* isolates were determine for 5/12 (42%) isolates, and resistant to Clindamycin and Ampicillin/ Sulbactam 11/12 (92%) isolates to both of them, this high level resistance ratio was simultaneously with the ratio of biofilm formation layer for these isolates 92%, while the results of isolates showed the high level sensitive to Moxifloxacin, Imipenem, Meropenem and Piperacillin/Tazobactam 12/12 (100%), table (4-10), figure (4-11).

This study revealed the relationship between biofilm formation and antibiotic resistant isolates 11/12 (92%) of resistant isolates to Clindamycin and Ampicillin/Sulbactam were recorded positive results for biofilm layer to both of them, 5/12 of resistant isolates to Penicillin G

were recorded (42%) positive result for biofilm formation , while all resistant isolates to Metronidazole 12/12 (100%) were recorded positive results for biofilm figure (4-11).

Bhooshan *et al*., (2016) showed that all isolates of *G. vaginalis* were sensitive to imipenem and meropenem (100%). Taillandier *et al*., (2020) found that the antibacterial susceptibility tests revealed that the *G. vaginalis* bacteria were resistant to metronidazole but susceptible to penicillin G.

Moxifloxacin is more effective in BV-associated bacteria (*Gardnerella vaginalis*) than doxycycline, according to the *in vitro* susceptibility profile (Petrina *et al*., 2019).

de Souza *et al*., (2016) reported high resistance rate was observed for metronidazole (59.8%) of *G. vaginalis* isolates, high rates of susceptibility to clindamycin (93.1%) and Ampicillin/sulbactam (95.1%).

Younus *et al*., (2017) found the clindamycin and metronidazole, which are provided as the first line antibiotics for the treatment of BV in Malaysia, exhibited susceptibility to *G. vaginalis* isolates at a rate of (93.6%), these isolates also showed susceptibility to penicillin at a rate of (87.2%).

Table (4-10) Association antibiotic susceptibility and biofilm formation of *Gardnerella vaginalis* isolates

Antibiotices	***G.vaginalis* isolates, n=12**			
		No	**%**	**Biofilm formation n= 12**
Metronidazol 30µg/disk	S	0	0	0 (0%)
	R	12	100	12 (100%)
Clindamycin 10µg/disk	S	1	8	1 (8%)
	R	11	92	11 (92%)
Moxifloxacin 5 µg/disk	S	12	100	12 (100%)
	R	0	0	0 (0%)
Penicillin 10µg/ disk	S	7	58	7 (58%)
	R	5	42	5 (42%)
Imipenem 10µg/disk	S	12	100	12 (100%)
	R	0	0	0 (0%)
Meropenem 10µg/disk	S	12	100	12 (100%)
	R	0	0	0 (0%)
Ampicillin/ Sulbactam 10/10µg/disk	S	1	8	1 (8%)
	R	11	92	11 (92%)
Piperacillin/ Tazobactam 100/10µg/disk	S	12	100	12 (100%)
	R	0	0	0 (0%)
Total				
Count (%)	12 (100%)			12 (100%)

S : Sensitive , R : Resistant

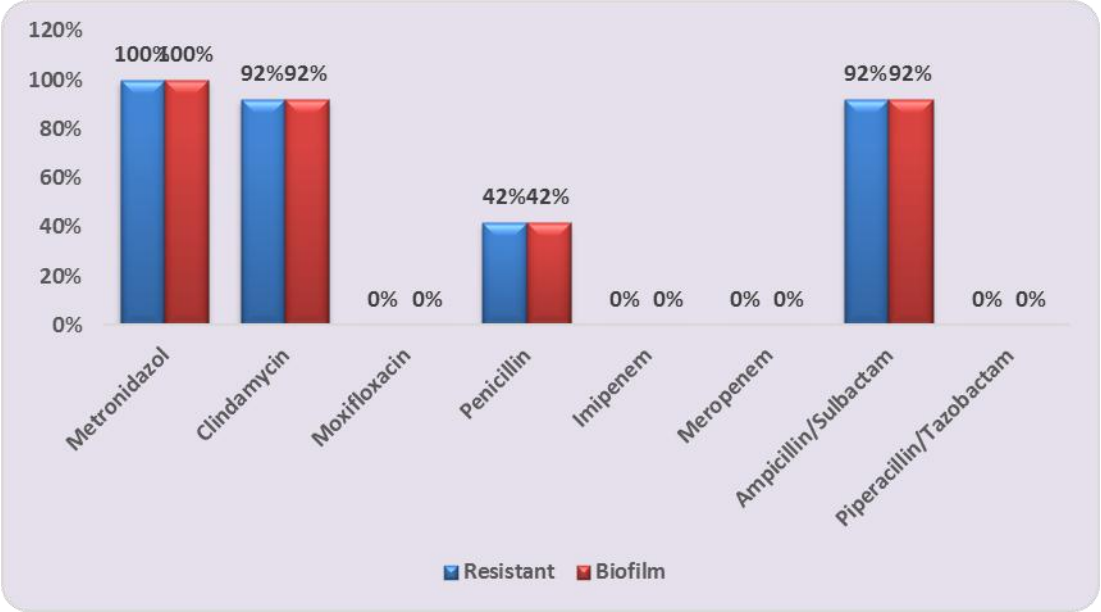

Figure (4-11) Relationship between number (%) of antibiotic-resistant and biofilm formation for *Gardnerella vaginalis*

Some strains of *G. vaginalis* have been discovered to be resistant to the antibiotic metronidazole, among the proposed resistance mechanisms are: A suppressed rate of drug activation within the cell due to its reduction, elevated DNA repair systems, increased activity of oxygen-consuming enzymes (such as catalase, peroxidase, and superoxide reductase), and accelerated clearance of the drug from the cell due to active efflux are all possible mechanisms of resistance (Löfmark *et al*., 2010).

Muzny and Sobel, (2022) explained the rate of BV recurrence after treatment is high; in many cases, it is greater than (60%). It is highly likely that the BV biofilm itself contributes to recurrent and refractory disease after treatment by limiting antimicrobial penetration.

The vaginal biofilm is a thick, multispecies biofilm in which *G. vaginalis* is the dominating species, standard antibiotics, such as metronidazole, are unable to completely remove the vaginal biofilm, which may explain the high recurrent rates of BV. An increase in the

transcription of genes that code for antimicrobial resistance was observed whenever *G. vaginalis* biofilms formed (Machado *et al*., 2016; Castro *et al*., 2017).

Bacterial resistance to antibiotics is primarily the result of a number of phenomena, including changes in the drug's target, bacterial impermeability to the antibiotic, and genetically associated changes (mutational events, genetic transfer of resistance genes through plasmids, and mutations of target genes) (Qi *et al*., 2016).

However, this is not the only factor contributing to the failure of antimicrobial therapy. In fact, one of the mechanisms of resistance employed by bacteria to survive in the presence of an antibiotic is their capacity to form communities known as biofilms embedded in an exopolysaccharide matrix (Cepas *et al*., 2019).

Biofilm production increased the antibiotic resistance of *Gardnerella* species probably through the following mechanisms (He *et al*., 2021):

1-The bacteria in the biofilm core consumed oxygen and nutrients, slowing the growth and metabolism of bacteria in the inner layer of biofilm. When bacteria were exposed to antibiotics, they developed increased tolerance, decreased susceptibility, and did not undergo genetic changes (Prax and Bertram, 2014).

2-The matrix acted as a barrier for relatively large antibiotic compounds, which slowed down the compounds' ability to penetrate the biofilm (Cerca *et al*., 2005).

3-The antibiotics were neutralized by the matrix components (Fux *et al*., 2005).

4-After the antibiotics were pumped out of the biofilm, they were destroyed by living bacteria (Van Acker *et al*., 2014).

Due to these mechanisms, biofilm-associated infections typically appear as persistent, progressive, chronic infections that are distinguished by relapses (He *et al*., 2021).

4.6. DNA sequencing

To obtain a trimmed sequence, each data sequence was trimmed from beginning to ending, according to normal waves. When compared to NCBI- Blast, this sequence has a high level of identity to other global sequence data. The waves produced by scanning the sequences indicated the strong and weak regions of the sequences, which are then trimmed, resulting in increased identity with global sequences at NCBI-Blasting.

The results of nucleotide sets were checked and confirmed by using (NCBI) – Basic Local Alignment Search Tool (BLAST analysis)-nucleotide blast-Search a nucleotide database using a nucleotide query online, which was a perfect program and gave the exact results of identity percentage with other world strains. Sequence alignment must be performed by using 16SrRNA gene of *Gardnerella vaginalis* sequences databases information recorded in GenBank to find identity and similarity score degrees of gene and compared with current local isolates.

The results of sequences alignment of the seven local isolates showed identity ranging from 78% to 83% (figure 4-12 to 4-18 and table 4-11), good query cover, and max score with other world strains of *Gardnerella vaginalis*.

Table (4-11): Alignment results of seven local *Gardnerella vaginalis* isolates with reference isolates retired from NCBI

Local Isolate	Reference of the isolate with highest percentage similarity(%)		
	Accession No.	**Similarity (%)**	**Country**
G. vaginalis No.1	MT644518.1	78 %	China
G. vaginalis No.2	MK968111.1	83 %	Iraq
G. vaginalis No.3	ON878197.1	81 %	China
G. vaginalis No.4	MK968111.1	82 %	Iraq
G. vaginalis No.5	JQ354973.1	80 %	Canada
G. vaginalis No.6	JQ354973.1	80 %	Canada
G. vaginalis No.7	MT644518.1	79 %	China

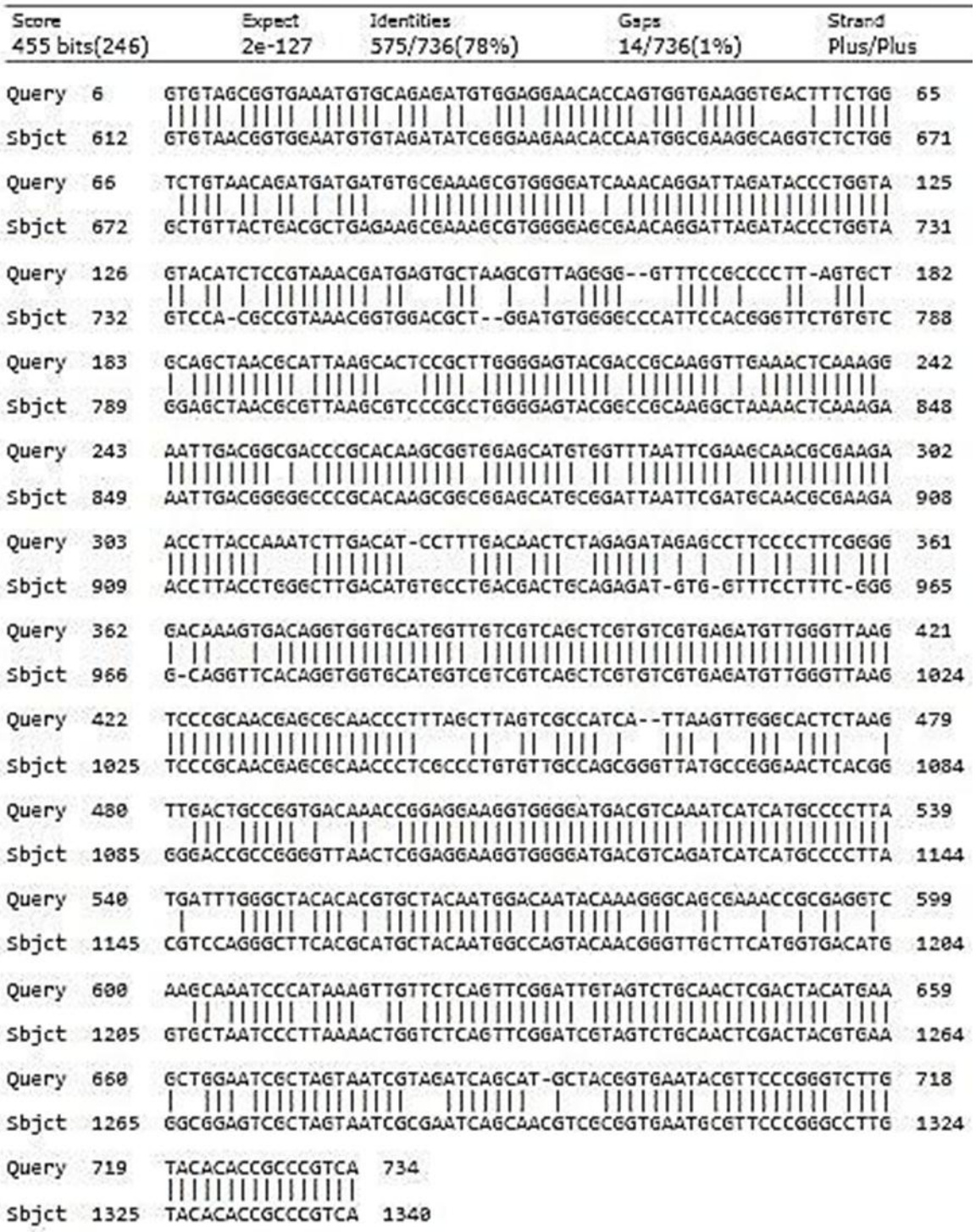

Figure (4-12): Basic Local Alignment of *Gardnerella vaginalis* 16SrRNA gene isolate No.1 with high similarity NCBI-BLAST *Gardnerella vaginalis* strain SD GV2 16S ribosomal RNA gene, partial sequence (accession number : MT644518.1 in GenBank)

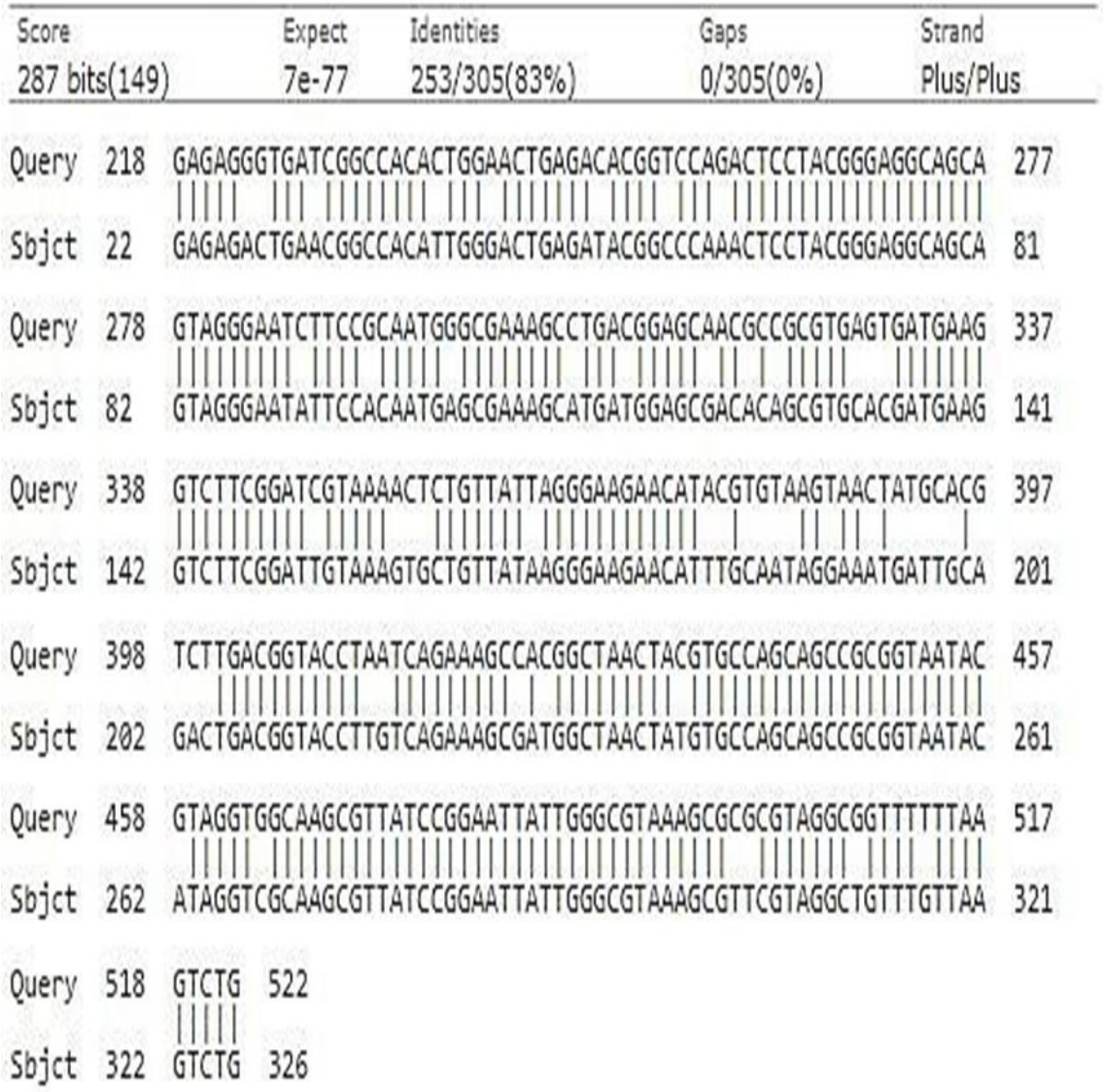

Figure (4-13): Basic Local Alignment of *Gardnerella vaginalis* 16SrRNA gene isolate No.2 with high similarity NCBI-BLAST *Gardnerella vaginalis* isolate IQ.BV No.2 16S ribosomal RNA gene, partial sequence (accession number : MK968111.1 in GenBank)

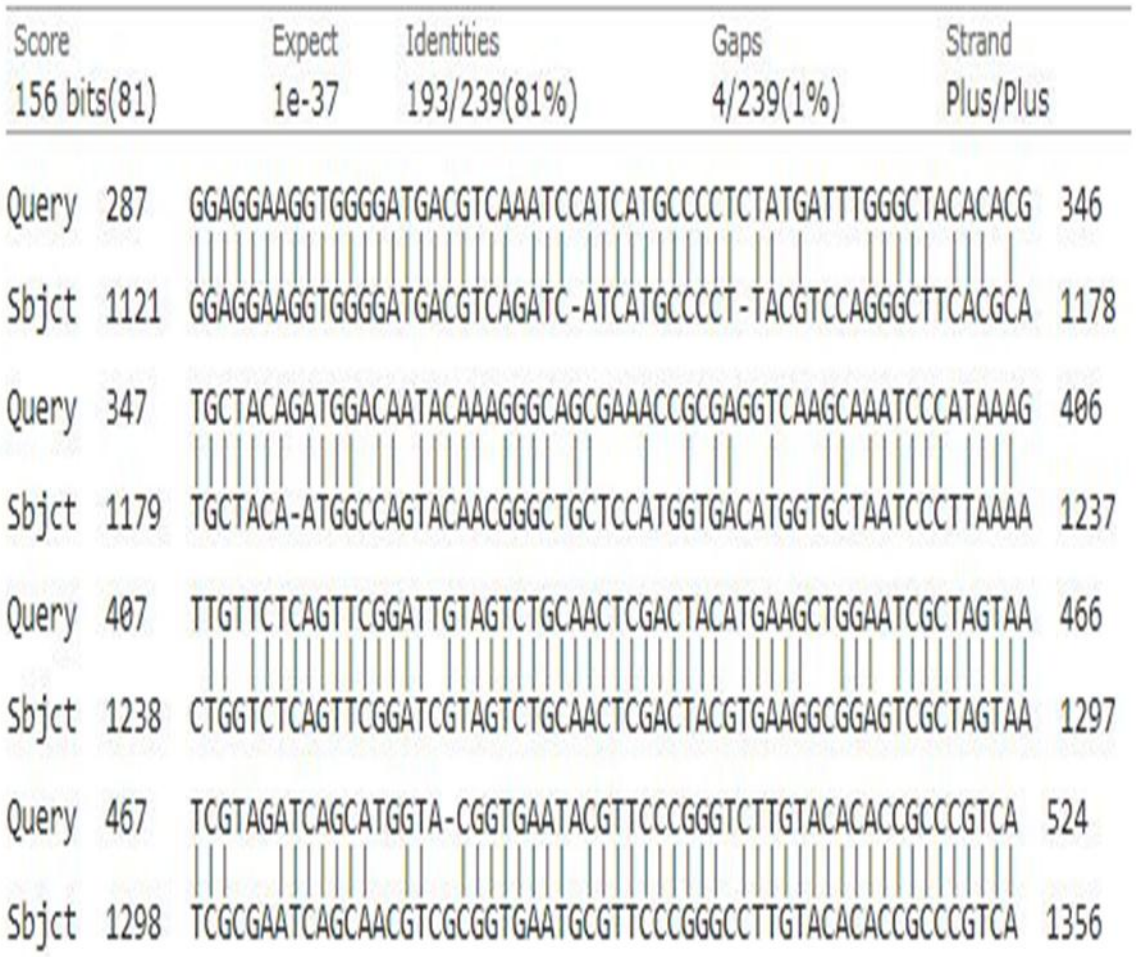

Figure (4-14): Basic Local Alignment of *Gardnerella vaginalis* 16SrRNA gene isolate No.3 with high similarity NCBI-BLAST *Gardnerella vaginalis* strain ZC14 16S ribosomal RNA gene, partial sequence (accession number : ON878197.1 in GenBank)

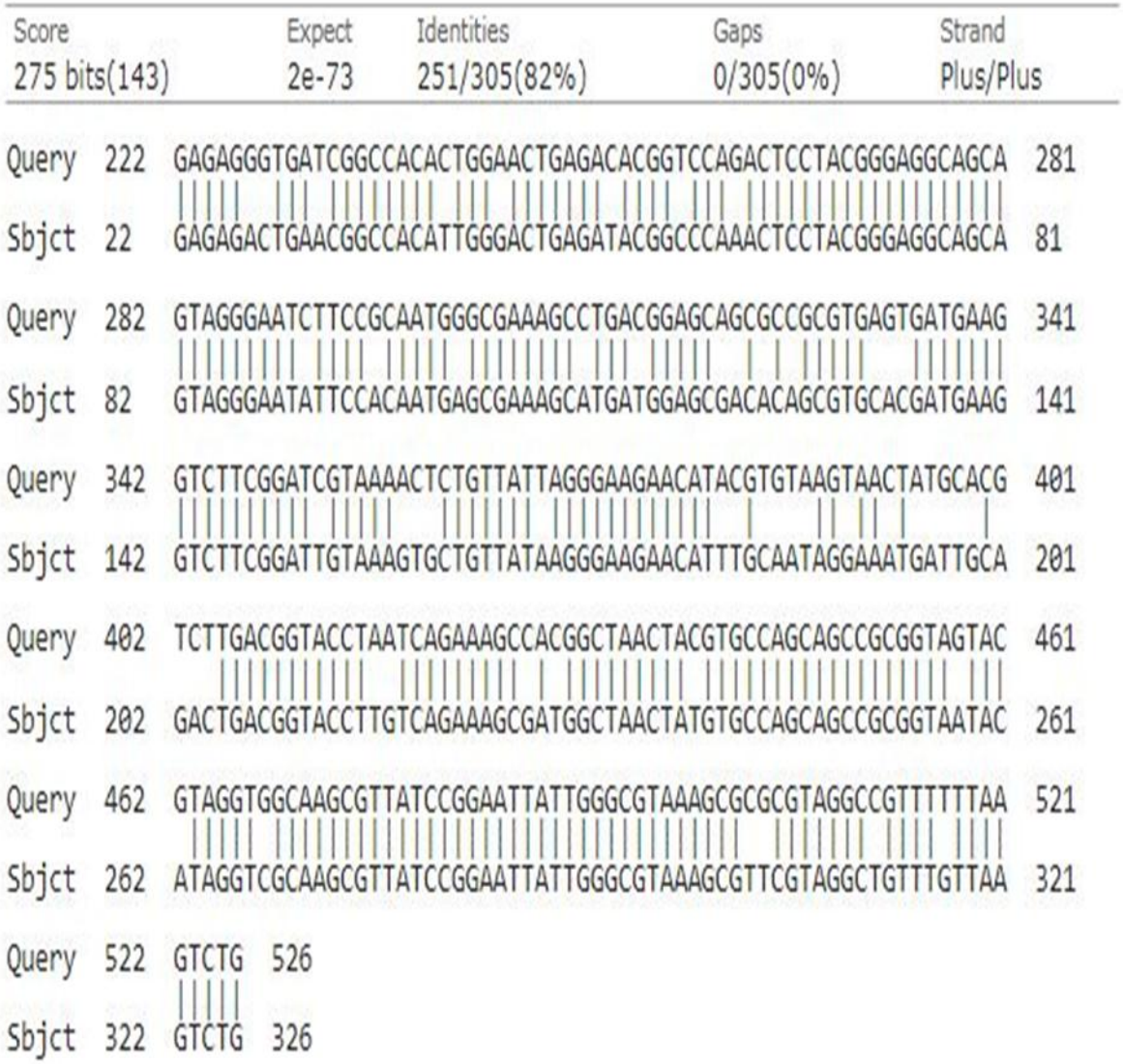

Figure (4-15): Basic Local Alignment of *Gardnerella vaginalis* 16SrRNA gene isolate No.4 with high similarity NCBI-BLAST *Gardnerella vaginalis* isolate IQ.BV No.2 16S ribosomal RNA gene, partial sequence (accession number : MK968111.1 in GenBank)

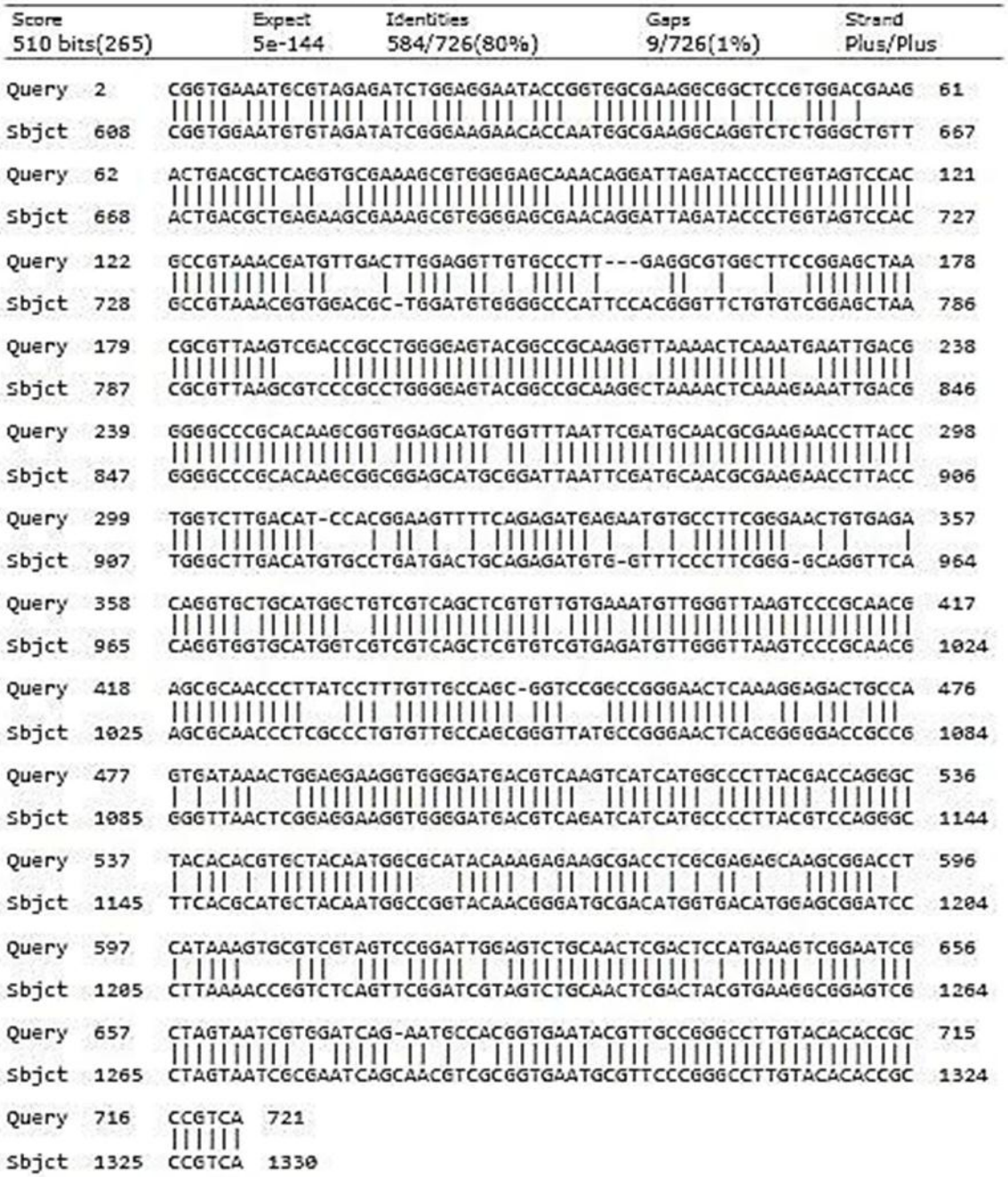

Figure (4-16): Basic Local Alignment of *Gardnerella vaginalis* 16SrRNA gene isolate No.5 with high similarity NCBI-BLAST *Gardnerella vaginalis* strain N153 16S ribosomal RNA gene, partial sequence (accession number : JQ354973.1 in GenBank)

Score	Expect	Identities	Gaps	Strand
510 bits(265)	5e-144	584/726(80%)	9/726(1%)	Plus/Plus

```
Query  3     CGGTGAAATGTGTAGAGATGTGGAGGAATACCGGTGGTGAAGGCGGCTCCGTGGACGAAG  62
             ||||| |||||||||| ||  ||| ||| |||  ||| |||||| | ||  ||| |
Sbjct  608   CGGTGGAATGTGTAGATATCGGGAAGAACACCAATGGCGAAGGCAGGTCTCTGGGCTGTT  667

Query  63    ACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCAC  122
             ||||||||| ||  ||||||||||||||||| ||||||||||||||||||||||||||||
Sbjct  668   ACTGACGCTGAGAAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCAC  727

Query  123   GCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTT---GAGGCGTGGCTTCCGGAGCTAA  179
             |||||||||| ||    | |||| || | |||| |      ||  |   |  |||||||||
Sbjct  728   GCCGTAAACGGTGGACGC-TGGATGTGGGGCCCATTCCACGGGTTCTGTGTCGGAGCTAA  786

Query  180   CGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACG  239
             |||||||||    |||||||||||||||||||||||||||| ||||||||||  |||||||||
Sbjct  787   CGCGTTAAGCGTCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGAAATTGACG  846

Query  240   GGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACC  299
             |||||||||||||||| |||||||| || ||||||||||||||||||||||||||||||
Sbjct  847   GGGGCCCGCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACC  906

Query  300   TGGTCTTGACAT-CCACGGAAGTTTTCAGAGATGAGAATGTGCCTTCGGGAACCGTGAGA  358
             ||| ||||||||    | || |  | |||||||| |  | | ||||||||  | |    |
Sbjct  907   TGGGCTTGACATGTGCCTGATGACTGCAGAGATGTG-GTTTCCCTTCGGG-GCAGGTTCA  964

Query  359   CAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACG  418
             ||||||| |||||||  |||||||||||||||| |||| ||||||||||||||||||||||
Sbjct  965   CAGGTGGTGCATGGTCGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACG  1024

Query  419   AGCGCAACCCTTATCCTTTGTTGCCAGC-GGTCCGGCCGGGAACTCAAAGGAGACTGCCA  477
             ||||||||||    ||| |||||||||| |||    |||||||||||||  || ||| |||
Sbjct  1025  AGCGCAACCCTCGCCCTGTGTTGCCAGCGGGTTATGCCGGGAACTCACGGGGGACCGCCG  1084

Query  478   GTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGACCAGGGC  537
             | | |||   |||||||||||||||||||||||||  |||||||| |||||||| |||||||
Sbjct  1085  GGGTTAACTCGGAGGAAGGTGGGGATGACGTCAGATCATCATGCCCCTTACGTCCAGGGC  1144

Query  538   TACACACGTGCTACAATGGCGCATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCT  597
             | ||| | ||||||||||||    ||||| | || ||||| | | || |    |||||| |
Sbjct  1145  TTCACGCATGCTACAATGGCCGGTACAACGGGATGCGACATGGTGACATGGAGCGGATCC  1204

Query  598   CATAAAGTGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCG  657
             | ||||    |||  ||| ||||| | |||||||||||||| | ||||| |||| |||
Sbjct  1205  CTTAAAACCGGTCTCAGTTCGGATCGTAGTCTGCAACTCGACTACGTGAAGGCGGAGTCG  1264

Query  658   CTAGTAATCGTGGATCAG-AATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGC  716
             |||||||||| | ||||| || | | |||||||| ||||||||||||||||||||||||||
Sbjct  1265  CTAGTAATCGCGAATCAGCAACGTCGCGGTGAATGCGTTCCCGGGCCTTGTACACACCGC  1324

Query  717   CCGTCA  722
             ||||||
Sbjct  1325  CCGTCA  1330
```

Figure (4-17): Basic Local Alignment of *Gardnerella vaginalis* 16SrRNA gene isolate No.6 with high similarity NCBI-BLAST *Gardnerella vaginalis* strain N153 16S ribosomal RNA gene, partial sequence (accession number : JQ354973.1 in GenBank)

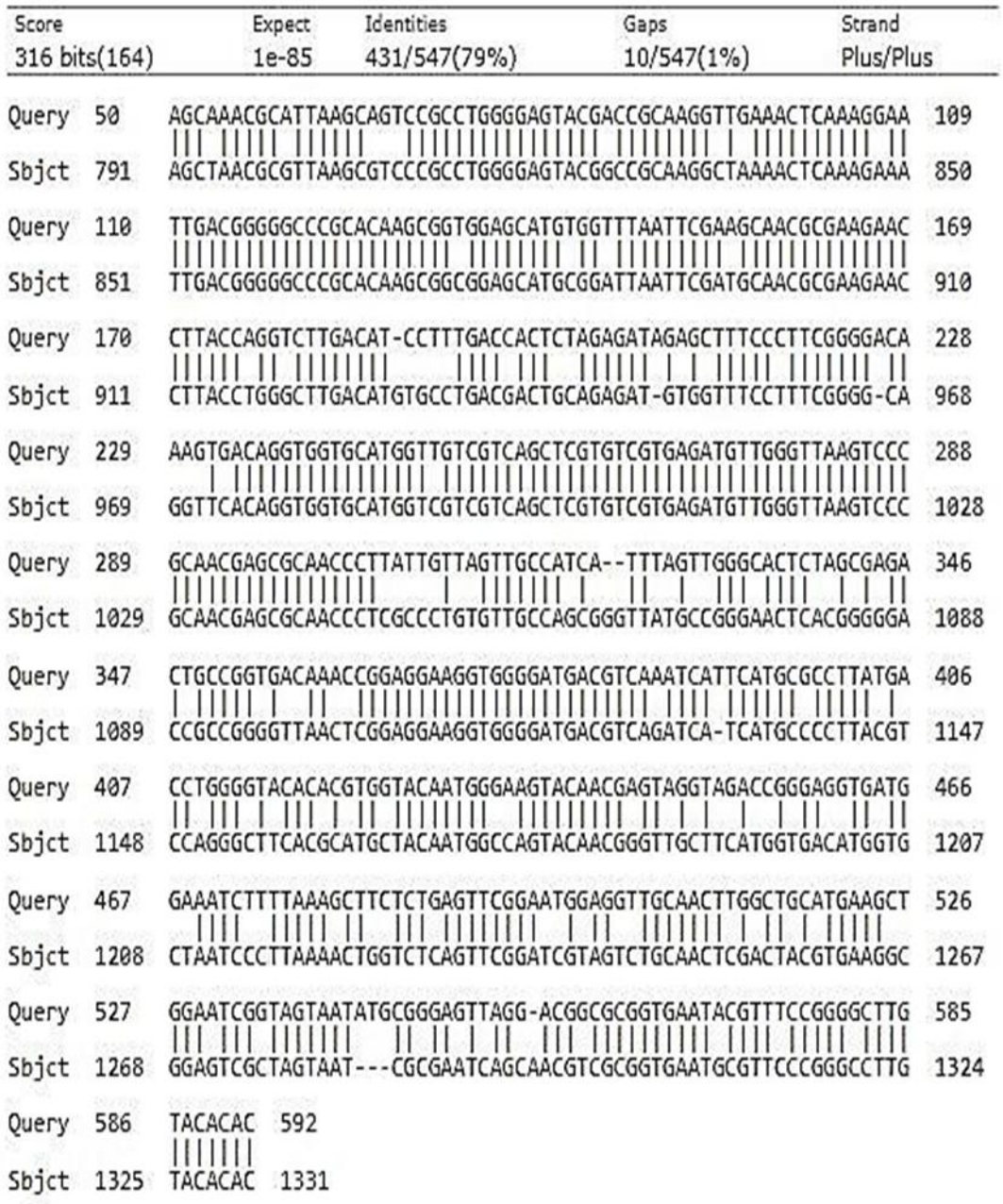

Figure (4-18): Basic Local Alignment of *Gardnerella vaginalis* 16SrRNA gene isolate No.7 with high similarity NCBI-BLAST *Gardnerella vaginalis* strain SD GV2 16S ribosomal RNA gene, partial sequence (accession number : MT644518.1 in GenBank)

The most common method for identifying bacteria is 16S rRNA gene sequence analysis. 16S rRNA gene sequence information has an expanding role in the identification of bacteria in clinical or public health settings. However, the data also clearly showed that it was not foolproof and applicable in each and every situation (Wang *et al*., 2007).

The 16SrRNA gene sequences have been the most often used housekeeping genetic marker in the study of bacterial phylogeny and taxonomy for a variety of reasons. The 16SrRNA gene is found in almost all bacteria, often as part of a multigene family or operon; the 16SrRNA gene's function has not changed over time, implying that random sequence changes are a more accurate measure of time (evolution); and the 16SrRNA gene (1,500bp) is large enough for informatics purposes (Janda and Abbott, 2001). Bacterial analysis by 16S rRNA has become popular because these sections of RNA are conserved and easy to sequence (Fukushima *et al*., 2002).

The 1.5-kilobase 16SrRNA gene sequence has been considered and widely used in bacterial taxonomy because it includes a high conservation region that has variable regions between species (Kox *et al*., 1995). The fact that the 16SrRNA gene can be sequenced readily is also significant. Bacterial identification can be improved by integrating molecular phylogeny with traditional approaches such as morphological, physiological, and biochemical features (Zhaolan *et al*., 2003; Li *et al*., 2006; Ma *et al*., 2008).

4.7. Phylogenetic analysis of local and world strains

The phylogenetic tree is drawn to scale, with branch lengths in the same units as the evolutionary distances used to infer the phylogenetic

tree. The dataset was cleansed of positions with gaps or missing data (Complete deletion option). MEGA X 10.2.4 is used to perform phylogenetic analysis.

There were 8 global taxa about 16SrRNA gene of *G.vaginalis* were downloaded from NCBI and submitted with 7 local sequences to Mega X 10.2.4 software to obtain the figure (4-19).

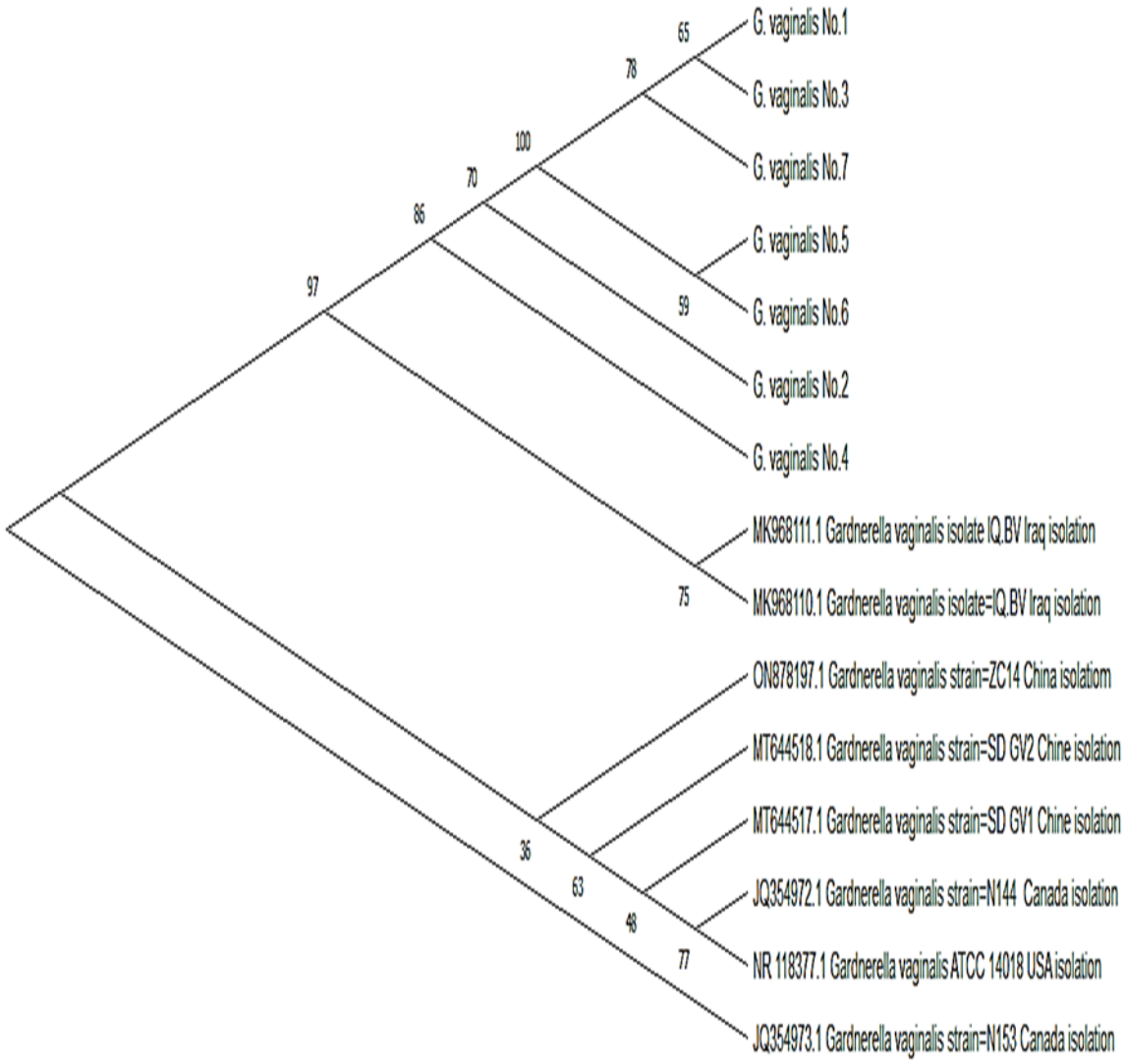

Figure (4-19): Phylogenic tree of 16S rRNA gene partial sequences of *Gardnerella vaginalis* of local and global sequences using neighbor joining bootstrap 1000 tree figure. Evolutionary relationships of 15 taxa. No.1 to 7 represent local isolates

The Molecular Evolutionary Genetics Analysis (MEGA) program is a desktop software that allows you to compare homologous gene sequences from various species or multigene families, with a focus on inferring evolutionary links and patterns of DNA and protein evolution. MEGA features a number of useful tools for assembling sequence data sets from files or web-based repositories, as well as tools for visualizing the results in the form of interactive phylogenetic trees and evolutionary distance matrices (Kumar *et al*., 2008). The first stage in the analysis was to align all of the sequences form 16S rRNA gene in this study with other world-wide references using MEGA X 10.2.4 's (Clustal W) program step. This program was shown to have a high degree of similarity with all world sequences, including the sequences used in this study. These (Clustal W) results were significant since they were directly utilized in the phylogenetic tree design.

The Neighbor-Joining (NJ) approach, which is a simplified version of the minimal evolution (ME) method, is used in this study to determine the close relationship between world and local sequences. Because it does not need the assumption of a constant rate of evolution, the NJ method yields an unrooted tree. An out group taxon is needed to find the root (Saitou and Nei, 1987; Rzhetsky and Nei, 1992).

A phylogenetic tree, also known as an evolutionary tree, is a branching diagram or "tree" that depicts the assumed evolutionary relationships among distinct biological species or other entities based on physical and/or genetic similarities and differences (Salahuddin and Khan, 2014). In 16srRNA gene phylogeny (Fig. 4-19), submitted 15 sequences, 7 sequences belong to local sequences and 8 sequences belong to global sequences obtained by download from NCBI they submitted to

a MEGA X 10.2.4 software program for getting phylogenic relationship among local and global sequences, after submitting these sequences to MEGA X 10.2.4 at the first time we found alignment by Clustal W, then use NJ method at bootstrap 1000, the local sequence of *Gardnerella vaginalis* No.2 and No.4 were closely related to each other, that genetically related to global sister sequences (MK968111.1 and MK968111.1) *Gardnerella vaginalis* isolates IQ.BV isolated in Iraq with good homology identity for both isolates. the local sequences *Gardnerella vaginalis* No.1 and No.3 were closely related to each other to form a sister sequence, which related to local sequences No.7. Also, the local No.5 and No.6 were closely related to each other to form sister sequences.

In clinical microbiology laboratories, 16S rDNA sequencing has been crucial in the precise identification of bacterial isolates and the discovery of new bacteria, much as the widespread use of PCR and DNA sequencing. In the case of bacteria with odd phenotypic profiles, uncommon bacteria, slow-growing bacteria, uncultivable bacteria, and infections that are culture-negative, 16SrDNA sequencing is crucial for bacterial identification. It offered insights into the causes of infectious diseases, which aids physicians in prescribing antibiotics, figuring out how long to treat patients for, and deciding how to practice infection control (Woo *et al*., 2008).

Conclusions & Recommendations

Conclusions

1-Moleculer diagnosis was more accurate than culture in diagnosis of *G. vaginalis*.

2-Bacterial vaginosis was amixed infection.

3-The most bacterial isolates of *G. vaginalis* have virulence factors including vaginolysine, salidase, phospholipase, hemolysis and biofilm.

4-All isolates of *G. vaginalis* produce biofilm.

5-*G.vaginalis* resistance to Metronidazole (100%), while sensitive to Moxifloxacin, Imipenem, Meropenem and Piperacillin/Tazobactam (100%).

6-There were closely related of *G. vaginalis* with isolates of Iraq, China and Canada as phylogenetic tree.

Recommendations

1-Further investigation is needed to show the prevalence of *G. vaginalis* in preterm labor due to various infection.

2-The needs to prepare vaccines for *G. vaginalis* bacteria because of the complications caused by this bacteria, especially on pregnant women and fetuses.

3-Studying the relationship between *G.vaginalis* bacteria and other bacterial species present in the vagina, as well as their relationship to diseases affecting the vagina.

4-Using Real–Time PCR to detect vaginal pathogen as the main causative agent depending on the copy number.

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الخلاصة

التهاب المهبل هو أكثر الأمراض شيوعًا في الجهاز التناسلي للأنثى خلال سن الإنجاب. الالتهابات المهبلية الشائعة، هي التهاب المهبل البكتيري، *Gardnerella vaginallis* هو جنس بكتيري مرتبط دائمًا بالتهاب المهبل البكتيري.

تم جمع ٢٠٠مسحة مهبلية (١٠٠عينة للزرع ، ١٠٠عينة لاختبارAmsel) من النساء الحوامل وغير الحوامل المصابات بالتهاب المهبل البكتيري الذين تتراوح أعمارهم بين (١٧-٦٠) من قبل الطبيبة وتم جمعها من مستشفيات محافظة بابل: مستشفى الزهراء للولادة، مستشفى الإسكندرية العام والعيادة الخارجية للفترة من كانون الاول ٢٠٢١ إلى نيسان٢٠٢٢.

بينت المسحة الرطبة المحضرة مباشرة من الإفرازات المهبلية وبدون تصبيغ وجود خلايا دالة مغطاة بـ *G. vaginalis*. كانت نتيجة الزرع والاختبارات الكيموحيوية وصبغة جرام تمثل النسبة المئوية للعينات الإيجابية لـ *G.vaginalis* كانت ٥٦٪ بما في ذلك ٧٪ ، ٢٦٪ ، ١٣٪ ، ٨٪ ، و ٢٪ معزولة عن عمر <٢٠ ، (٢٠-٣٠) ، (٣١-٤٠) ، (٤١-٥٠) و> ٥٠ ، على التوالي.

التشخيص السريري لالتهاب المهبل البكتيري يعتبر إيجابيًا إذا شوهدت ثلاثة معايير على الأقل من أربعة معايير ، وجدت ١٠ (١٧,٨٪) عينات إيجابية لأربعة معايير والعينات الأخرى إيجابية لثلاثة من معايير Amsel من ٥٦ عينة.

عند استخدام تقنية تفاعل انزيم البلمرة المتسلسل (PCR)، وجد ان نتائج التضخيم مع استخدام بادئات محددة 16SrRNA (٣٠٠ زوج قاعدي) لعزلات *G.vaginalis* هي فقط ١٢ (٢١,٤٪) من ٥٦ عينة موجبة للزرع تشمل (٧,١ ٪)، (٨,٩٪)، (١,٧٪)، (١,٧٪)، و(١,٧٪) معزولة عن عمر <٢٠، (٢٠-٣٠)، (٣١-٤٠)، (٤١-٥٠) و>٥٠ على التوالي.

درست عوامل الضراوة لـ *G. vaginalis* بطرق جزيئية (Sialidase,Vaginolysin, Phospholipase C gene) ومظهرية (الاغشية الحيوية وتحلل الدم) .النتائج بينت ٤ (٣٣٪) و ٩ (٧٥٪) و ٦ (٥٠٪) لجينات *vly* و *sld* و *pho* على التوالي أعطت نتائج إيجابية من ١٢ عزلة من عزلات *G. vaginalis*. تم احتبار تشكيل الغشاءالحيوي لبكتريا *G. vaginalis*

بواسطة أجار الكونغو الأحمر ، وطرق لوحة زراعة الأنسجة. أظهرت النتائج أن جميع العزلات كانت منتجة للغشاء الحيوي ١٢ (١٠٠٪).

تم اجراء فحص حساسية المضادات الحيوية تجاه ثمانية مضادات حيوية باستخدام طريقة انتشار القرص. أظهرت العزلات مستويات متفاوتة من المقاومة للمضادات الحيوية المستخدمة في هذه الدراسة ، وأظهرت العزلات مستويات متفاوتة من المقاومة للمضادات المستخدمة في هذه الدراسة، العزلات بينت مقاومة الى Metronidazole (١٠٠٪)، Clindamycin (٩٢٪)، Moxifloxacin (٠٪)، Penicillin G (٤٢٪)، Ampicillin/Sulbactam (٩٢٪)، Imipenem (٠٪)، Meropenem (٠٪) و Piperacillin/Tazobacta (٠٪).

في هذه الدراسة ظهرت العلاقة بين تكوين الأغشية الحيوية والعزلات المقاومة للمضادات الحيوية ١٢/١١ (٩٢٪) من العزلات المقاومة الى Clindamycin و Ampicillin/Sulbactam سجلت نتائج إيجابية لطبقة الغشاء الحيوي لكليهما ، ١٢/٥ من العزلات المقاومة الى Penicillin G سجلت (٤٢٪) نتيجة إيجابية لتكوين الأغشية الحيوية بينما كانت جميع العزلات المقاومة الى Metronidazole ١٢/١٢ (١٠٠٪)، سجلت نتائج إيجابية للغشاء الحيوي.

النتائج لمجموعة النيوكليوتيدات تم فحصها وتأكيدها باستخدام أداة بحث المحاذاة الاساسية، البحث في قاعدة بيانات نوكليوتيد باستخدام استعلام النيوكليوتيد عبر الانترنت، والذي كان برنامج مثالي وأعطى النتائج الدقيقة لنسبة التشابه مع السلالات العالمية الأخرى. تم اجراء محاذاة التسلسل باستخدام جين 16SrRNA من معلومات قواعد بيانات تسلسل *G.vaginalis* المسجلة في بنك الجينات للعثور على الهوية ودرجات التشابه للجينات ومقارنتها مع عزلاتنا المحلية، بينت النتائج التشابه يتراوح ٧٨٪ -٨٣٪ تغطية جيدة ودرجة قصوى مع سلالات العالم الاخرى من *G.vaginalis* . لوحظ في الشجرة التطورية ارتباطًا وثيقًا بين العزلات البكتيرية.

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

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بعض عوامل الضراوة لبكتريا *Gardnerella vaginalis* في محافظة بابل

رسالة مقدمة الى

مجلس كلية العلوم – جامعة بابل

وهي جزء من متطلبات نيل درجة الماجستير في العلوم/ علوم حياة

من قبل

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بكالوريوس علوم الحياة- كلية العلوم- جامعة بابل ٢٠٠٦

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