Comparative Analysis of Microflora and Screening of Isolated Phages against them in Pregnant and Non-Pregnant Females Suffering from Bacterial Vaginosis

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Comparative Analysis of Microflora and Screening of Isolated Phages against them in Pregnant and Non-Pregnant Females Suffering from Bacterial Vaginosis

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Dedicated to

My Beloved Parents

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CONTENTS

Title .... Page no

Acknowlegements vi

List of abbreviations 10

List of tables 11

List of figures figure no. 1

Abstract 2

Chapter 1: Introduction ***3***

Cha*pter 2: Literature Review* ***6***

2.1. Bacterial Baginosis (BV) 6

2.1.1. BV symptoms and pathology 7

2.1.2. BV and STDs 9

2.1.3. Factors involved in BVinfection 10

2.1.4. Diagnositic criteria 11

2.1.5. Epidemology 13

2.1.6. Treatments 15

2.2. Alternative therpeutic regimen: Bacteriophage therapy: 17

2.2.1. Biofilms and bacteriophages: 19

2.2.2. Applications of bacteriophages: 19

2.3. Objectives: 22

Chapter 3: Materials & Methods ***23***

3.1. Study population 23

3.2. Selection criteria 23

3.2.1. Inclusion criteria 23

3.2.2. Exclusion criteria 23

3.3. Sampling procedure....23
3.3.1. Patient consent....23
3.3.2. Collection of samples....23
3.3.3. Sample size....24
3.4. Materials....24
3.4.1. Recipes....24
3.5. Methodology....24
3.5.1. Sampling....24
3.5.2. Bacterial enrichment....24
3.5.3. Bacterial isolation....24
3.5.4. Morphological characterization....24
3.5.5. Biochemical characterization....25
3.6. Antibiotic resistance....26
3.7. Phage isolation and enrichment....26
3.8. Lytic activity of phages....26
3.9. Calculation of plaque formation potential....27
3.10. One step growth curve....27
3.11. Calculation of viral titer (pfu/ml)....28
3.12. Viral adsorption....28
3.13. Thermal stability of phages....28
3.14. ph stability of phages....29
3.15. Isolation of phage nucleic acid....29
3.5.1. Gel electrophoresis....30
3.16. Host range specificity....30
3.17. Molecular identification of bacteria....30

3.18. molecular identification of phages: 31

Chapter 4: Results *32*

4.1. Bacterial identification *32*

4.1.2. Characterization based on hemolysis on blood agar 34

4.1.3. Biochemical characterization 34

4.2. Prevalence of bacterial isolates among the BV positive pregnant samples 37

4.3. Antibiotic sensitivity 38

4.4. Phage isolation and enrichment 39

4.5. Phage lytic potential activtiy analysis 40

4.5.1. Spot test 40

4.5.2. Plaque assay 41

4.6. Growth curve analysis 42

4.7 Calculation of bacteriophage titer (pfu/ml) 46

4.8 Analysis of bacteriophage adsorption 48

4.9. Thermal sensitivity of phages 48

4.10. ph sensitivity of phages 50

4.11 Molecular identification of bacteria 52

4.12: Molecular identification of phages: 52

4.13: Genome isolation: 55

Chapter 5: Discussion ***56***

References *61*

Appendix-A *76*

Appendix-B *77*

LIST OF ABBREVIATIONS

BV	Bacterial Vaginosis
HPV	Human Papilloma Virus
HIV	Human Immunodeficiency Virus
AIDS	Acquired Immunodeficiency Syndrome
HSV	Herpes Simplex Virus
STD	Sexually Transmitted Diseases
AMR	Antimicrobial Resistance
TMA	Trimethylamine
HVS	High Vaginal Swabs
RTI	Reproductive Tract Infection
ID	Identification
PID	Pelvic Inflammatory Disease

LIST OF TABLES

Table no.	**Title**	**Page no.**
Table 3.1	Recipe table of media	81
Table 3.2	Recipe table of broths	81
Table 3.3	Gram staining solutions	82
Table 3.4	Catalase test reagents	82
Table 3.5	1% Chloroform Solution	82
Table 3.6	Glycerol Solution	82

LIST OF FIGURES

Figure no.	**Title**	**Page no.**
Figure 2.1	BV pathology	12
Figure 2.2	Nugent scoring criteria	15
Figure 2.3	Bacteriophage morphology	21
Figure 4.1(a)	Morphological characterization of bacterial isolates	34
Figure 4.2	Hemolysis of bacterial strains on blood agar plates	36
Figure 4.3	Gram staining of isolated bacteria	37
Figure 4.5	Catalase tests	38
Figure 4.8	Antibiotics sensitivity	41
Figure 4.9	Phage isolation	42
Figure 4.10	Spot Test	43
Figure 4.11	Plaque Assay	43
Figure 4.9	Spot Test	48
Figure 4.10	Plaque Assay	49
Figure 4.17(a)	FSEM analysis of phage lysate A8AnL1	55
Figure 4.17(b)	FSEM analysis of phage lysate A12AnB2(1)	56
Figure 4.17(c)	Analysis of FSEM phage adsorbed on bacterial	57
Figure 4.17(d)	FSEM analysis of phage lysate A33	58
Figure 4.18	Genome isolation of phages	59

LIST OF GRAPHS

Figure. no	Title	Page no.
Figure 4.1 (b)	Morphological Characterization of Microbial Isolates	35
Figure 4.1 (c)	Morphological Characterization of Microbial Isolates	35
Figure 4.4	Biochemical characterization by gram staining	38
Figure 4.6	Biochemical characterization by performing catalase test	39
Figure 4.7	Prevalence of bacterial isolates among expecting and non-pregnant females	40
Figure 4.12 (a)	Bacterial growth curve analysis	45
Figure 4.12 (b)	Bacterial growth curve analysis	45
Figure 4.12 (c)	Bacterial growth curve analysis	46
Figure 4.13 (a)	Bacterial growth curve with phage	43
Figure 4.13 (b)	Bacterial growth curve with phage	47
Figure 4.13 (c)	Bacterial growth curve with phage	48
Figure 4.14 (a)	Calculation of phage titer (pfu/ml)	49
Figure 4.14 (b)	Calculation of phage titer (pfu/ml)	49
Figure 4.14 (c)	Calculation of phage titer (pfu/ml)	50
Figure 4.15 (a)	Thermal sensitivity of phage	51
Figure 4.15 (b)	Thermal sensitivity of phage	51
Figure 4.15 (c)	Thermal sensitivity of phage A33	52
Figure 4.16 (a)	pH sensitivity of phage	53
Figure 4.16 (b)	pH sensitivity of phage	53
Figure 4.16 (c)	pH sensitivity of phage	54

ABSTRACT:

Bacterial Vaginosis (BV) is the infection of females' genital system. It is a polymicrobial infection which shifts the paradigm of vaginal healthy microbiome to facultative anaerobe. Globally, BV prevalence in females is about 21.2 million, in Pakistan its prevalence is 35.3%. BV leads to serious complications in pregnancy, facilitates the onset of sexually transmitted diseases (STDs) and cervical cancer. Clindamycin and Metronidazole antibiotics are used for the treatment of BV worldwide. However, the relapse rate of BV infection in females indicate resistant against antibiotics treatment. Probiotic therapy alone or in combination with antibiotics is an alternative option for the treatment of BV infection. Nevertheless, large number of studies failed to reveal significant effects of probiotic therapy in BV patients.

The pitfalls of currently available therapies against BV draw the attention of scientists to explore other treatment options. Phage therapy has shown a promising solution in this regard. The lytic activity of phages can be an effective strategy for combating antibiotic resistant bacteria. In addition to this advantage, phages are also host specific, safer, cost effective and easier to handle.

Current study was designed to investigate the BV microflora of pregnant and non-pregnant females and their sensitivity towards antibiotics. The samples were collected from Obstetrics and Gynaecology Department of KRL Hospital, Islamabad, Pakistan. The consent of the patients was obtained before sampling. Total 60 samples were collected using high vaginal swabs (HVS), 30 from pregnant while 30 from non-pregnant females. The sample containing HVS were streaked on three different media i.e., LB agar, MRS agar and Blood agar for the isolation and comparison of BV microbiota. Total 150 colonies and 10 different bacterial strains were isolated from collected samples. Morphological characterization showed no difference in the microflora of BV infected pregnant and non-pregnant females, however, the number of isolates were different in both groups. *Staphylococcus* and *Streptococcus* species were prevalent in both pregnant and non-pregnant females. While *Shigella flexneri* was present only in non-pregnant females and was detected by 16S rRNA sequencing. Sensitivity and efficacy of antibiotics against isolated microbiota was also observed. Surprisingly, all of the bacterial strains showed

resistant against metronidazole and clindamycin, except 35 bacterial strains which showed sensitivity against clindamycin.

Furthermore, isolation of phages was carried out from sewage water of slum areas of Islamabad, Pakistan. Lytic activity of isolated phages was examined against all isolates of BV samples. Three bacterial strains showed sensitivity to these isolated lytic phages. The 16S rRNA sequencing of these bacterial strains revealed that they belong to *Enterococcus feacalis* strain (QAUSD05), *Enterococcus spp*. (QAUEF03) and *Shigella flexneri* (HQ407262.1).

Bioactivity analysis of isolated phages was observed within a *pH* range of 1-11. Phages against *E. fecalis* and *Enterococcus* QAUEF03 showed their maximum activity at neutral *pH* 7 while the phage against *S. flexneri* showed its maximum activity at *pH* 9. Similarly, thermal sensitivity of phages was checked against *Enterococcus* strains, showed their highest activity at 55ºC and 70°C respectively. Phage of *S. flexneri* had an optimum activity at a temperature of 40ºC. Morphological identification of phages was performed by Scanning Electron Microscopy (SEM). SEM results showed that all phages were spherical in shape. In order to analyze the genome nature of phages, organic extraction method was employed. It was observed that all phages had DNA genome with the size range in between 15-20kbp. This show they might share lineage with *Podoviridae* family as per reviewed from the literature.

Overall, the current study provides the better understanding of BV microflora in pregnant and non-pregnant females. The isolation of phages provides a road map for the better treatment of BV infection microflora. Isolated phages can also be assessed to explore their potential to treat other bacterial diseases bacillary dysentery, shigellosis and urinary tract infections (UTIs) as well. Isolation of new phages against causative agents of BV, phages cocktails or combination with antibiotics may provide a pathway and a new hope to the world to combat the antibiotic resistance issue and highly effective treatment against BV infection.

Chapter 1:

Introduction:

Bacterial vaginosis (BV) is an infection of females' lower genital tract of reproductive age (Gillet et al., 2011). BV is a microbiological syndrome which causes a sudden change in females healthy lactobacilli microflora to opportunistic facultative anaerobes (Onderdonk, Delaney, & Fichorova, 2016). The symptoms of BV includes fishy odor, thin whitish grey discharge, pH>4.5, itching, burning and inflammation (Abdullateef, Ijaiya, Abayomi, Adeniran, & Idris, 2017; Hay, 2009).

BV is the most frequently reported infection of females throughout the world. Globally 21.2 million (29.2%) females of reproductive age are living their lives suffering from BV infection. In the third world countries, the rate of prevalence is 8-75% (Bitew, Abebaw, Bekele, & Mihret, 2017). BV incidence rate is high in black women 45-55%, followed by hispanic women 30%, and 20- 30% in Asian and Caucasian women (C. Kenyon, Colebunders, & Crucitti, 2013a). The rate of BV infection in Pakistani females is 35.3% (Habib & Siddiqui, 2016).

BV is related to number of obstetric and gynecological complications which includes spontaneous abortion, pelvic inflammatory disease (PID), preterm labour or delivery, wound infections of Caesarean, preterm fissure of membranes, chorioamnionitis, postpartum endometritis, and postsurgical infections (Yudin & Money, 2017). The epidemiological studies led to the discovery of various risk factors associated with BV. It is reported that females with BV infection are more prone to sexually transmitted diseases (STDs) and HIV as compared to healthy females (Hickey, Zhou, Pierson, Ravel, & Forney, 2012; C. Kenyon, Colebunders, & Crucitti, 2013b; C. R. Kenyon & Osbak, 2014; Mead, 1993).

The risk factors, onset and progression of BV disease is still a subject of debate (Cherpes, Hillier, Meyn, Busch, & Krohn, 2008). It is hypothesized that BV infection results in reduction of lactobacilli microflora in vagina, thus increase the chances of acquiring opportunistic pathogen and STDs infections. Some studies show that a person is at high risk of attaining HPV due to relapses of BV, however, these studies are also under

investigation (Gillet et al., 2011). The signs and symptoms representing BV are reported to be linked with varying biological markers. The onset of the disease symptoms indicate towards an altered hormonal level and pathogenic microbial infection such as *Candida*, *Trichomonas vaginalis* and *Nisseria gonorrhoeae* (Onderdonk et al., 2016).

Currently, most commonly used antibiotics are Metronidazole and Clindamycin to treat BV infection (Bostwick, Woody, Hunt, & Budd, 2016). They are given orally and locally in the form of tablets and gels. Metronidazole is incorporated orally or locally, both routes don't have significant results in complete elimination of the disease causing microbes (C. M. Mitchell, Hitti, Agnew, & Fredricks, 2009). Such frequent exploit of antibiotics causes resistance in bacterial strains which leads to the relapses of the BV within a time period of 06 months (Bradshaw & Sobel, 2016).

These limitations had triggered the researchers' attention towards other highly effective treatment, probiotic therapy. Probiotics maintain the vaginal microflora and keep pH balanced by a variety of lactobacillus strains given orally or intra-vaginally (Homayouni et al., 2014). Probiotics are used in combination with antibiotics or with different combinational therapies but the issue of recurrence still remains (Donders, Zodzika, & Rezeberga, 2014). This issue directs the attention towards another treatment called bacteriophage therapeutic.

After antibiotic resistance and failure of probiotic treatment against BV infection bacteriophages have gained a lot of attention for the control of bacterial infections worldwide (Elbreki et al., 2014). Bacteriophages are naturally occuring tiny viruses that kill bacteria. They are host specific and auto dosable. Many bacteriophages act as biocontrol agents against *Shigella, Staphylococcus, Klebsiella, Streptococcus, Escherichia, Pseudomonas,* and *Proteus* infections (Rhoads et al., 2009). According to Polish and Soviet research centers, 80-95% efficacy was seen in the cure of these infections (Alisky, Iczkowski, Rapoport, & Troitsky, 1998). In clinical trials of patients suffered from chronic otitis caused by *Pseudomonas aeruginosa* when treated with phages showed reduction in bacterial infection with no side effects (Wright, Hawkins, Änggård, & Harper, 2009). Russia, Poland and Germany are the main sites for the research and development of phages after manipulating and discovering new features (Parfitt, T. 2005). Phage therapy is

economically feasible with less time consumption and cost (Fenton, McAuliffe, O'Mahony, & Coffey, 2010; Matinkhoo, Lynch, Dennis, Finlay, & Vehring, 2011).

Some phages against UTIs and gynaecological pathogens have also been isolated. These bacterial strains include *E. coli, Staphylococcus* and *Pseudomonas* but, phage therapy against BV infection microflora is an untouched subject. Therefore, current study is designed to examine and compare the vaginal microflora of pregnant and non-pregnant females infected with BV disease. Furthermore, present study aims to isolate phages against multi drug resistant (MDR) strains of BV infection. Current study will assist in the better understanding of virulent microflora of BV infection, circulating in Pakistani females. Isolated phages will be helpful to devise new treatment regimens against BV infection.

Chapter 2:

Literature review

2.1. BACTERIAL VAGINOSIS (BV):

The human beings have millions or trillions of microbes in their body. The net weight of microbiota in human body is estimated as 2.5-3.0 kg. These microbes colonize in skin, vagina, urethral, oral, gastrointestinal, nasal, and body's other parts. Inhabited microbiota can be beneficial for humans or it may cause lethal infections to humans (Thursby & Juge, 2017). Scientists are trying to overcome these infections by different methods or manipulate these microorganisms for the benefits of human beings. However, Bacterial Vaginosis remains as a silent disease for many years but research on BV led to the discovery that its consequences are drastic (D. Machado, Castro, Palmeira-de-Oliveira, Martinez-de-Oliveira, & Cerca, 2016).

BV is a commonly described reproductive tract infection (RTI). The RTIs are the infections of upper and lower reproductive tract of women and men (Sloan, Winikoff, Haberland, Coggins, & Elias, 2000). Females are more prone to RTIs as compared to men. Uninfected men are less prone to get an infection from infected females as compared to uninfected females who can get an infection in higher frequency from infected males (Bhatia & Cleland, 2000). Even females have to face serious consequences of infections such as pelvic inflammatory disease (PID), infertility cervical cancer and ectopic pregnancy. RTIs infections are difficult to diagnose as they are asymptomatic in women (Durr-e-Nayab, 2005).

Hundred years ago, in 19th century, the Albert Doderlein (1892) describe the significance of Lactobacilli (Lepargneur & Rousseau, 2002). Premenopausal women in their normal vaginal secretions contain gram positive, long, rods. Glycogen is fermented by lactobacilli spp. and produce lactic acid. The vaginal health is maintained by lactic acid by developing acidic environment of the vagina i-e., < 4.5 pH (Fettweis, Serrano, Girerd, Jefferson, & Buck, 2012). During menstruation the pH is increased on second day (pH=6) while it decreases on 4th day to (pH=4). These vigorous changes in the vaginal environment may disturbed the ecology of vaginal microbiota (Andersen et al., 1994).

Women life from childhood to adult undergo a lot of hormonal changes which accord with the vaginal epithelium and microbiota. Glycogen is produced by the body for

storage purposes, in lumen of vagina it is used by vaginal microbes. The glycogen in lumen is metabolized by the elevated level of estrogen hormone produced by the estrogen receptors residing under the vaginal epithelial cells. These cells escalated growth, outcomes the expansion of glycogen during the mid-cycle stage of menstruation which results in the elevated level of estrogen (Kumar et al., 2011). Thickness of the epithelial cells layers occur due to the increased proliferation of cells (Patton et al., 2000). As the estrogen level increases, the amount of mucus also increases which is present in the vaginal epithelia. As viscosity of mucus decreases, estrogen level increased whose by-product is a watery discharge. These physiological changes in women concurrent with and naturally increase in the existence of lactobacilli species which prevail during the reproductive years (Kumar et al., 2011).

In 1894, Doderlain first time highlighted that certain women have pH > 4 and complaints about thin, and discharge of greyish white color. On investigation, it was found out that variety of different bacteria accumulated in vagina thus replacing the typical Lactobacilli rods. It was named as leucorrhea (Danielsson, Teigen, & Moi, 2011). In 1954 and 1955, Gardner and Dukes declared it as non-specific vaginitis and it was due to Sexually transmitted diseases (STDs). The main causative agent described by them was *Haemophilus vaginalis*, (Gardner & Dukes, 1954, 1955) then *Corynebacterium vaginale*, later named as *Gardnerella vaginalis,* purpled colored gram positive bacteria (Greenwood & Pickett, 1980). The infection was diagnosed by the "clue cells", epithelial cells of vagina is coated with pathogenic bacteria of BV. In 1983-1984, the name of nonspecific vaginitis was changed to Bacterial Vaginosis (Danielsson et al., 2011).

2.1.1. BV SYMPTOMS AND PATHOLOGY:

BV is thought to be the chronic source of vaginitis. Worldwide, 90 million cases of bacterial vaginosis were reported annually (Bhat, Kotigadde, & Shenoy, 2011). It is a poly-microbial synergistic syndrome. BV is a dysbiosis of normal aerobic vaginal microbiota to an anaerobic pathogenic microflora (Rao, Pindi, & Rani, 2016) The main causative agent of BV is still unknown but it is attributed that *Lactobacilli* that produces hydrogen peroxide (H_2O_2) are replaced with pathogenic anaerobic bacteria which are 100-1000 times more than the normal lactobacilli flora (Marrazzo, Thomas, Fiedler, Ringwood, & Fredricks, 2008).

Bacterial vaginosis (BV) have malodorous vaginal discharge. It is categorized by a thin greyish-white discharge which adhere to the wall of vagina, fishy odor, 6-7 pH, presence of clue cells, without inflammation, itching and burning (Gillet et al., 2011). In uninfected vagina mucin gel is present which is degraded by a *Bacterial Vaginosis Associated Bacteria (BVAB)* which contains a mucin degrading enzymes, it results in the production of heavy discharge through vagina (Olmsted, Meyn, Rohan, & Hillier, 2003). Anaerobic bacteria metabolize glycogen into volatize amines which gives fishy odor (Marrazzo et al., 2008). The reduction in the *lactobacilli spp.,* elevate vaginal pH. As the *lactobacilli* production lessen, the lactic acid, H_2O_2 and lactocin also obstruct (Kumar et al., 2011).

The known causative agent of Bacterial vaginosis are facultative anaerobes such as *Peptostreptococcus, Gardneralla vaginalis, Bacteroides*, *Bacterial Vaginosis Associated Bacteria (BVAB)*, *Mycoplasma hominis, Prevotella*, *Ureaplasma urealyticum, Mobiluncus* species (Hillier, 2005; Onderdonk et al., 2016; Rao et al., 2016). These polymicrobes produces metabolites which lessen the lactobacilli that produces lactic acid and shift towards the formation of short chain fatty acids (SCFA) during BV infection (O'Hanlon, Moench, & Cone, 2013; Yeoman et al., 2013). Cervicovaginal fluid (CVF) of infected female has acetate as a leading metabolite followed by other metabolites such as succinate, propionate and butyrate (Athanasiou et al., 2016; Stanek, Glover, Larsen, & Gain, 1992).

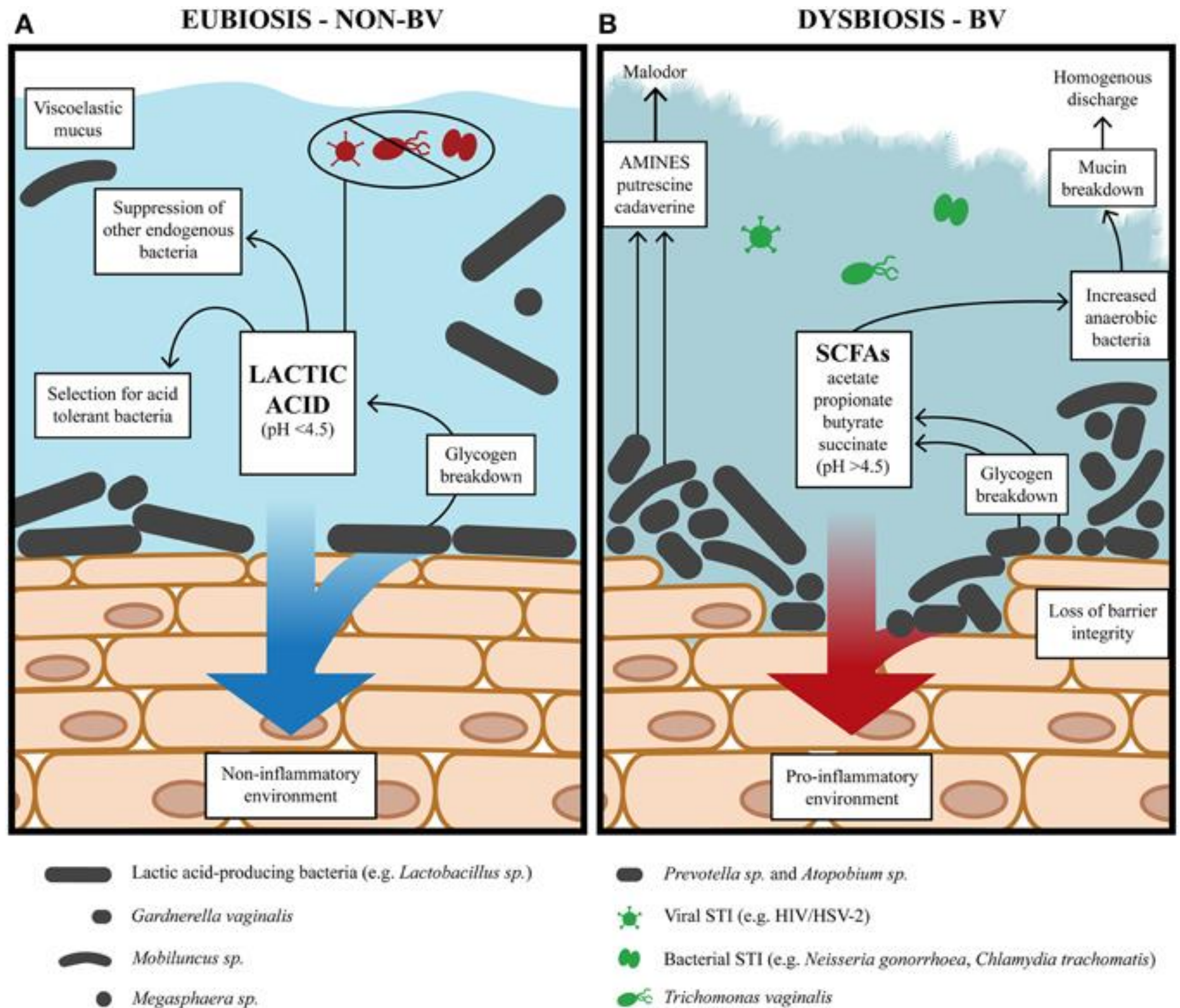


Figure 2.1*: This diagram illustrates the comparison between normal vagina and the bacterial infected vagina of females (a) shows the healthy vagina containing lactobacilli which produces lactic acid and maintain the pH < 4.5, thus suppressing pathogens growth (b) in case of BV, pathogens hoard prevents the production of lactobacilli thus increasing pH and SCFAs resulting in malodor and homogenous discharge. Adapted from (Aldunate et al., 2015).*

2.1.2. BV AND STDs:

BV has extreme clinical importance because of its obstetric and gynecological complications and high risks of attaining STDs. It is linked with spontaneous abortion, cuff cellulitis, premature rupture of membranes, salpingitis, preterm birth, PID, endometritis, infertility, miscarriages, chorioamnionitis, preterm labour, postpartum endometritis, urinary tract infection and halt infections after hysterectomy (Aldunate et al., 2015; Kumar

et al., 2011; Rao et al., 2016). BV is common in reproductive age of 14-44 years. In industrialized countries the prevalence of BV is 8-30% in women. The increase in infection also increase the STDs. For example, women infected with BV doubles the risk of HIV 60%. However, the females who are HIV positive and BV infected shed six folds more virus (Danielsson et al., 2011).

BV increases the chances of acquiring *Human Papilloma Virus (HPV)* **(Gillet et al., 2011),** *Trichomonas vaginalis* (Brotman et al., 2012), *Chlamydia trachomatis* (Gallo et al., 2012; Schwebke & Desmond, 2007)*, Neisseria gonorrhea* (Wiesenfeld, Hillier, Krohn, Landers, & Sweet, 2003)**,** *Herpes Simplex Virus-2 (HSV-2)* (Cherpes et al., 2005; Nagot et al., 2007) and *Human Immunodeficiency Virus (HIV)* (Atashili, Poole, Ndumbe, Adimora, & Smith, 2008; Cohen et al., 2012). BV pathogens produces metabolites and factors which provides an environment for the production of microbes which promotes STDs (Doerflinger, Throop, & Herbst-Kralovetz, 2014; Yeoman et al., 2013). According to studies, BVAB elevates vaginal pH which may help in the increase growth of acid-labile infectious pathogens of STDs and women are surge of contacting these infections (Z. Gong, Luna, Yu, & Fan, 2014). However, this increased obtaining of STDs such as HIV-1 and 2 escalate the viral amount and replication occur in cervicovaginal fluid (CVF) of women thus carrying this HIV to their male partners during sexual intercourse (Cohn et al., 2005; Coleman et al., 2007; C. Mitchell et al., 2013). Maintenance of eubiosis and dysbiosis is still a mystery and require further investigation and studies.

2.1.3. FACTORS INVOLVED IN BV INFECTION:

BV exists from asymptomatic to symptomatic infection. Epidemiological studies are improving our understanding about the cofounding factors which lead to the BV infection. These factors include predisposing factors, genetic factors,

2.1.3.1. PREDISPOSING FACTORS:

BV infection can occur by number of behavioral and socioeconomic risk factors such as cigarette smoking, douching, use of sex toys, multiple sexual partners, women sex with women (WSW) and sharing of sex toys within the women, lubrication with saliva, use of intrauterine devices (IUD) for contraception's, drug abuse, vaginal lubrication with petroleum jelly, early age intercourse, black ethnicity, oral sex, STDs or immune deficiency disorders like *HIV*, *HSV 1* and *2, Neisseria gonorrhoeae* and *Chlamydia*

trachomatis (Fethers, Fairley, Hocking, Gurrin, & Bradshaw, 2008; Seck et al., 2001; Srinivasan et al., 2010; Wiesenfeld et al., 2003). It is a well-established fact that occurrence of BV is much greater in black females (64%) as compare to white women (35%). It is hypothesized that the increased levels of stress are may be one of the reasons in higher incidence of BV in black ethnicity (Srinivasan et al., 2010).

2.1.3.2. GENETIC FACTORS:

Estrogen hormone have significant influence on the microbiota of vagina. During menopause the estrogen level decreases, which may lead in the reduction of lactobacilli and in some cases, this shift results in the urogenital infections. However, the mechanism of shift from healthy to pathogenic microbiota is still unknown but it is hypothesized that the genetic variations are responsible in the interruption of normal signaling of the innate immune system which may allow the growth of pathogenic bacteria. It is linked with the polymorphism of Toll like receptors (TLRs) genes which are linked in signaling. Black women, increase in pH and lessen of lactobacilli is explained by a hypothesis that an interleukin 1 of cytokine family i-e; IL1RN2 undergo allelic polymorphism in the gene intron (Fettweis et al., 2012). BV symptoms is different among women, this shows variable pathogenesis mechanisms and etiologies reflecting BV is exclusive to genetic determinant and microbiota (Aldunate et al., 2015). These studies still need a lot of work to understand the whole mechanism of BV infection whether it is genetic or environmental or both.

2.1.4. DIAGNOSTIC CRITERIA:

For a long time, the signs and symptoms of BV were ignored as it was considered a harmless condition, except for the heavy discharge and resulting discomfort. According to the latest research BV itself is not harmful but the factors associated with it are damaging.

Amsel et al. in 1983 invented a criterion for the identification of BV, it depends upon four symptoms, if three of them are present in women it is considered to be infected with BV. Thin, greyish white homogenous discharge, pH>4.5, 10% KOH addition on the discharge of vagina which results in the release of fishy amine odor, and clue cells determination with wet mount microscopy (Amsel et al., 1983).

The limitation in Amsel method for the detection of BV is; the positive KOH test also occurs if the semen is present in vagina. The vaginal secretions may be present due to the douching, intercourse or before and after the menstrual cycle can interpret as the BV

secretions. The *Candida* and *Trichomoniasis* sometimes give same appearance. pH of vagina is increased during menstruation or it may be enhanced because of the semen's presence. The presence of clue cells doesn't show that the person is infected with BV; they can also be occurred in the presence of degenerative cells or debris. Less amount of lactobacilli sometime attach to the epithelial cells of vagina (C. Kenyon et al., 2013a).

These limitation leads the development of more specific and sensitive methods for the recognition of BV bacteria. In 1991, Nugent established a Gram staining scoring system called as Nugent Scoring System (NSS) to determine the BV (Nugent, Krohn, & Hillier, 1991). At 1000x magnification these gram stains are assessed. The long positive purple colored gram rods are *Lactobacillus spp.,* scored as 0-4 as no infection. *Gardnerella vaginalis spp.,* small gram variable coccobacilli, *Bacteroides spp.,* small negative pink colored gram rods and *Mobiluncus spp.,* small gram variable rods are scored as 4-6 shows intermediate infection. A score of 7-10 is given to abnormal vaginal flora in which disappearance of Lactobacillus spp. along with the appearance of Gram-variable rods or curved Gram-negative and strict anaerobes (Beverly et al., 2005; Muzny et al., 2014). The precision and susceptivity of Amsel criteria is 83% and Nugent score is 89% (Schwebke, Hillier, Sobel, McGregor, & Sweet, 1996).

Table1: Nugent Scoring System (NSS) for Bacterial Vaginosis

ORGANISMS MORPHOTYPE	**SCORE**
Lactobacilli morphotype (gram positive bacilli)	0-4
Gardnerella and bacteroides spp. (gram negative coccobacilli)	4-6
Curved gram variable rods	7-10

Nugent score interpretation

1-4: Negative Bacterial Vaginosis

4-6: A person with slight infection; Intermediate score

7-10: Infected with Bacterial Vaginosis

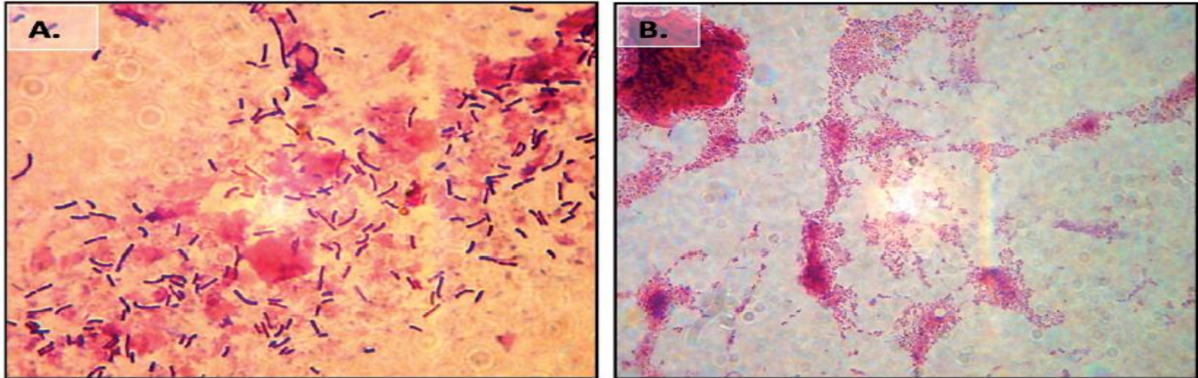


Figure 2.2: *NSS Criteria for BV: The vaginal smears are Gram stained (A) a non-infected normal woman with a Nugent score of 0. The figure depicts the dominancy of lactobacilli in vagina whereas figure (B) shows infected women smear having an abundance of bacteriocides and G. vaginalis spp. with a Nugent score of 10. Adapted from (Fischbach et al., 1993)*

Diagnosis of BV is still a challenge. Selection of different laboratory diagnosis methods demands more cost, complexity and the uninterpretable specimens. The other alternative methods established for the detection of BV are proline amino peptidase activity, polymerase chain reaction (PCR) and nucleic acid hybridization test. Recently sensor array, presence of RNA, and combinations of microbial products have been introduced for the detection of BV. However, these methods are costly and their specificities and sensitivities do not provide any advantage over the conventional methods. So, the methods Amsel and Nugent's scoring are up till now the best options for the detection of BV especially in developing countries (Rao et al., 2016; Verstraelen & Verhelst, 2009).

2.1.5. EPIDEMOLOGY:

Epidemiological studies conducted on BV show reduction in the *Lactobacilli spp.* Prevalence of BV is varied between and within the countries worldwide. BV prevalence rate is found to be higher in African countries in parallel to Asian and European population (Organization, 2008). Irrespective of geographical distribution white women are less prone to BV infection as compared to black women (Koumans et al., 2007). Globally, prevalence of BV in females is 20-30%, however, this prevalence is increased up to 50-60% in female sex workers and in pregnant females it ranges from 3.5-50% (Akinbiyi, Watson, & Feyi-Waboso, 2008; Krauss-Silva et al., 2014; Tolosa et al., 2006).

BV is a usual infection in United States with different frequency of varying race and ethnicity. The study conducted by National Health and Nutrition Examination Survey on public of U.S. in a time period of 2001-2004 using NSS vaginal smears. Females of aged 14-49 years shows 29.2% prevalence of BV but out of which only 15.7% women reported symptoms (Koumans et al., 2007). The prevalence in Turkey (23%), Norway (24%), Poland (19%). However, Australia, Indonesia and New Zealand show prevalence of BV with greater than 30%. Females of East and South Africa have greater rate of BV infection such as Gambia (37%), Mozambique (51%), Kenya (37%) and Lesotho (44%) (C. Kenyon et al., 2013a). On comparison females of Burkina Faso (West Africa) show 7% prevalence rate. The Sudan has low incidence of BV (Shobeiri & Nazari, 2006). Southern Iran represents (40%) while Egypt had 33% rate (Darwish, Makarem, Alnashar, & Hamadeh, 2005; Keshavarz, Duffy, Sadeghi-Hassanabadi, Zolghadr, & Oboodi, 2001). In Canada, native and indigenous women have 33% prevalence of BV. Prevalnce of BV in women of aged between 18-30 years, between mexian American women is (31.9%), non Hispanic black women (51.4%), non-Hispanic white women (23.2%), (Borgdorff et al., 2015), Hispanic (32%), whites (23%) and African-American (51%) (Yen et al., 2003).

High rates of BV have also seen in pregnant, infertile and HIV-positive women. The incidence of BV in pregnant females of Ethiopia is 19% while in Nigeria rate is 17%. However, the incidence of BV in sterile females of Qom and Iran was 70% (Ghiasi, Fazaeli, Kalhor, Sheykh-Hasan, & Tabatabaei-Qomi, 2014), the infertile women of Nigeria have high rates of BV (Durugbo, Nyengidiki, Bassey, & Wariso, 2015). The HIV positive women of India have a prevalence of BV 48% (Lallar, Nanda, & Nandal, 2015). In Bulgaria the BV incidence rate is 57% (Gergova, Strateva, & Mitov, 2013). Five different studies conducted in between 1995-2014 on women who sexed with another women (WSW) shows the incidence rate up to 25-50% (Vodstrcil et al., 2014). It is hypothesized that prevalence of BV in WSW is because of exchange in vaginal fluid (C. Kenyon et al., 2013a).

BV is reported to highly prevalent in Pakistani population as compared to STDs (Nayab, 2005).As per recent estimate, the incidence of BV in Pakistani female population ranges between 16-25 years is 35.3% (Habib & Siddiqui, 2016). While in pre-term labor, the frequency of BV was found to be 21% (Islam, Safdar, & Malik, 2009).

2.1.6. TREATMENTS:

Globally many approaches have been done by the scientists to prevent the recurrence of BV infection. These may comprise of antibiotics, synthetic drugs to probiotics. This section provides a detailed description of BV infection treatment with antibiotics and probiotic therapies.

2.1.6.1. ANTIMICROBIAL USED TO COMBAT BV:

To treat bacterial infections antibiotics are most preferrable option. Similarly, BV infected patients are treated with antibiotics. The antibiotics used to prevent the infection are metronidazole, tinidazole and clindamycin. However, most commonly used are metronidazole and clindamycin. Metronidazole is given in gel form that can be applied inside the vagina one time a day for continous 5 days or in oral form (500mg) for 7 days twice a day. However, clindamcyin is given once in a day in cream form (2%) for 7 days. The single and short courses of these antibiotics are less effective (Workowski & Bolan, 2015). Metronidazole are far better than clindamycin for *Prevotella spp*., *Anaerococcus tetradius*, *Finegoldia magna, Bacteroides spp.,* and *Peptoniphilus spp*. However, greater effects of clindamycin were seen on *Gardnerella vaginalis, Anaerococcus tetradius* and *Mobiluncus spp* (Petrina, Cosentino, Rabe, & Hillier, 2017).

The cure rate of metronidazole is 80-90% while women (50-70%) complaint about relapses within the time period of 4-6 weeks. However, relapse rate is 70% and the recurrence occur within the 90 days (Abbaspoor, Rabee, & Najjar, 2014). Even the use of metronidazole shows a lot of side effects including metallic taste, heartburn, and skin rash, neuropathy, vomiting, leucopenia, diarrhea, headache, and seizures and sometimes may even cause candida vaginitis (Fallah, Rabiee, & Moshtaghi, 2007). In 1967, tinidazole is used for the prevention of BV (Armstrong & Wilson, 2009). Tinidazole is a derivative of nitroimidazole. Both medicines show no differences in their efficacy and control of relapses (Schwebke & Desmond, 2011).

2.1.6.1.1. LIMITATION OF ANTIBIOTIC THERAPY:

The major hurdle in the cure of infections are antimicrobials resistance globally. Developed countries are also suffering from major antibiotic resistant problems. In US, *Staphylococcus aureus* resistance against antibiotics causes severe health problems in schools, hospitals, gymnasiums and other communal settings. The increased resistance of antibiotics is also occurred due to the combination of poor control practices of infections

specifically in hospitals and perfunctory usage of antibiotics. According to reports of U.S. hospitals, patients were given 190 million doses of antibiotics on a daily basis. However, some pathogenic bacteria are resistant for all approved antibiotics so there is a need to treat them with different methods including toxic drugs (Kutateladze & Adamia, 2010).

The major blemish of BV recurrence is antibiotic resistant of antibiotic which may be due to antimicrobial resistance (AMR) genes, pathogenic biofilms and non-genetic determinants of resistance (Marrazzo et al., 2008). The disease relapse rates are more than 50% and infection occur within the 6-12 months of treatment (Bradshaw et al., 2006; Nagaraja, 2008). As per another study, BVAB and biofilms are the possible factors for the recurrence of BV. BVAB persistence re-inoculation through sexual intercourse may increase the risk factors (Gray et al., 2009; Swidsinski, Dörffel, Loening-Baucke, Schilling, & Mendling, 2011). Clindamycin used for the treatment is resistant to anaerobic gram negative rods (Austin, Beigi, Meyn, & Hillier, 2005). According to whole metagenome sequence studies, in BV infection 4 clades of *G. vaginalis* were identified. Out of these 4 clades, 2 of them are resistant to metronidazole which is the another major cause of relapses in BV infected females (Balashov, Mordechai, Adelson, & Gygax, 2014; Schuyler et al., 2016).

High rates of recurrence intrigue researchers to direct attention towards alternative treatment such as extended and suppressive antibiotic regimens, combinations of first line regimens, and concomitant of oral and intravaginal probiotic therapies. However, these approaches are still under study (Bradshaw & Sobel, 2016). Limited knowledge and failures of treatment led scientists to move towards another treatments.

2.1.6.2. PROBIOTICS:

In 1907, Elie Metchnikoff first time described the term probiotics. He explained the importance lactobacilli, and re-growth of gastrointestinal flora (Sieber & Dietz, 1998). In 1989, Dr. R. Fuller described the significance of probiotics in sustaining a normal health in living organisms (Ouwehand, Salminen, & Isolauri, 2002). World Health Organization stated the "Live microorganisms when incorporated in a sufficient amount they implement a health benefits in the host". Basically it is a method in which live microorganisms are incorporated into a certain amount they produce positive effects on human health (Reid, Jass, Sebulsky, & McCormick, 2003). In BV, women vagina is reduced with lactobacilli,

so lactobacilli are incorporate orally or intra-vaginally for the colonization of these microbes, thus curing the BV or preventing its recurrence in women (Falagas, Betsi, & Athanasiou, 2007). Certain strains of Lactobacilli stop the growth of BVAB mainly by two methods: by hindering the bacterial attachment on the wall of vagina (A. n. Machado, Salgueiro, Harwich, Jefferson, & Cerca, 2013) and by manufacturing lactic acid (Boskey, Cone, Whaley, & Moench, 2001), bacteriocin (Aroutcheva et al., 2001) and H_2O_2 (Mastromarino et al., 2002).

According to some clinical trials, no promising results were seen in the cure of BV infection because of relapse issue and failure of biofilms removal (Falagas, Betsi et al. 2007) and any lactobacillus strains fail to grow and survive in lower female reproductive tract (FRT) (Bradshaw et al., 2012; Senok, Verstraelen, Temmerman, & Botta, 2009).

2.2. ALTERNATIVE THERPEUTIC REGIMEN: BACTERIOPHAGE THERAPY:

Since the discovery of antimicrobials, they are used to prevent the infections. Unfortunately, occurrence of bacterial resistance led the scientist to moves towards other therapies. One of the most promising therapy is bacteriophage therapy. In this therapy, viruses are used to kill bacteria (Örmälä & Jalasvuori, 2013). As viruses are natural predators of bacteria, they infect bacteria and destroy them. They are also known as "biocontrol agent" (Domingo-Calap, Georgel, & Bahram, 2016).

Bacteriophages were track down by Felix d'Herelle and Frederick Twort, they independently discover them in 1915 and 1917 respectively. However Twort neglected the work, d'Hérelle continued the research in phage therapy and done a lot of work on cholera and dysentery treatment (d'Hérelle, 2007). Western world halt the work on phages after the discovery of antibiotics. But Russia and Eastern Europe extent the work on phage therapy (Verbeken et al., 2014). The bacterial killers are being explored and investigated keenly at a number of research institutes in Russia, Poland and Germany. A variety of phages has been discovered as antibacterial against a wide variety of bacterial species such *as Staphylococcus, Streptococcus, Klebsiella, Pseudomonas, Proteus* and *Escherichia* to name a few, with a success rate of 80-90% (Alisky et al., 1998).

Phages can be isolated from any environment which supports the growth of bacteria. Phage population size is extremely high in all environments such as soil, water, sediments, in dead or living animals and plants. According to estimation terrestrial ecosystem has a population of 10^7 phages/gram of soil and sewage has 10^8–10^{10} phage/ml respectively (Ewert & Paynter, 1980; Schuch et al., 2013). The aquatic environment has 10^{31} of phage population (Parisien, Allain, Zhang, Mandeville, & Lan, 2008).

Phages have large number of advantages over antibiotics as they are very specific to their host and donot attack the normal body cells (Fenton et al., 2010). Phages are "auto dosable". Antibiotics are degraded in gastrointestinal tract before reach to the site of infection reducing their efficacy. However, the phages are removed when no more pathogens are present clearing the body without side effects. Bacteria may evolve resistance against phages but isolation of new phages can be possible (Matinkhoo et al., 2011). Phage therapy is economically feasible with less time and cost (Jin et al., 2012). The genome of the phages can be easily manipulated by biotechnological approaches for the better delivery and treatment of infections.

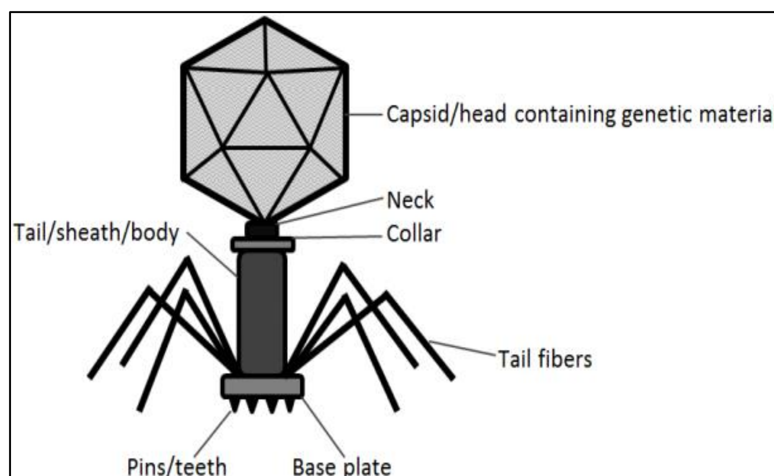


Figure2.3: *This figure depicts the morphology of phages that it is mainly constitute from three parts (i) head (ii) core (ii) tail. The head is made of proteins called capsid which contains the phage genome. While the tail fibers are used by phages for their attachment to the host cells.*

2.2.1. BIOFILMS AND BACTERIOPHAGES:

Biofilms protect pathogens from external environment or antibiotics through their polymeric extracellular matrices (Hathroubi, Mekni, Domenico, Nguyen, & Jacques, 2017; Parasion, Kwiatek, Gryko, Mizak, & Malm, 2014). In 1995, biofilms were reported for the first time (Doolittle, Cooney, & Caldwell, 1995). They are the major cause of trouble in medical field, while in humans they are responsible for pathogenic infections. To overcome the problem of antibiotic resistance, researchers are moving towards the phage therapy to eradicate the biofilms. Phages are independent of concentration their number increase with the increase in bacteria (Harper et al., 2014). Phages at the site of infection doubled themselves in such a way that they produces extracellular enzymes which destroy polymeric substances of biofilms (Kelly, McAuliffe, Ross, & Coffey, 2012). They insert themselves into the extracellular biofilm matrix that attach to the cells and macromolecules to destroy bacterial cells. Phages produce three types of enzymes to eradicate the biofilms (i) they put forth hydrolytic effect on the extracellular polymeric substances of biofilm (ii) they disrupt the bacterial capsules (iii) they destroy the bacterial cell wall (Lacroix-Gueu, Briandet, Lévêque-Fort, Bellon-Fontaine, & Fontaine-Aupart, 2005). Different studies on bacteriophages and biofilms shows promising results. The film form by staphylococcus strains show susceptibility to more than 50% when treated with bacteriophages. Another study shows the eradication of *P. aeruginosa* biofilms when treated with phages (Gabisoniya et al., 2016). The problem of antibiotic resistant to biofilms is treated by using phages, as they are easy to handle and shows little or no resistant against bacterial biofilms.

2.2.2. APPLICATIONS OF BACTERIOPHAGES:

For a long run bacteriophages are used and studied for different purposes. They are studied and implementing in bio-engineering, therapeutics, biotechnology and diagnostics etc. Phages are reproducible and can be used as phage cocktails. Even they a play critical role against bacterial resistance (Kutateladze & Adamia, 2010). Bacterial resistance against antibiotics is 10^{-5} per cell but for phages it is far less 10^{-7} to 10^{-8} per cell (Skurnik, Pajunen, & Kiljunen, 2007).

They are used for the prevention of various diseases but along with it, they can be used for the therapeutic interventions. Their high specificity permits them to be used for the detection and typing of various bacterial infections. Phage typing led researcher to

perceive conventional plaque formation, fluorescently labeled phages, modified phages when incorporated into genes their expression can be seen (Hagens & Loessner, 2007). For the identification of bacterial infection, FDA approved blood culturing employing phage infection can be used to check the availability of methicillin-resistant *Staphylococcus aureus* (MRSA) (Lu & Koeris, 2011). It is considered one of the most favorable gene delivery method (Barry, Dower, & Johnston, 1996). In this method, phage genome is removed and desired genome is encapsulated with phage protein coat, it saves the DNA from breakdown and the targeted gene can easily be delivered because the phage surface has specific proteins, which attaches itself on the surface of cell (Clark & March, 2006). The processing and targeting on the surface of phages are done by artificial covalent conjugation and phage display (Larocca et al., 1999).

Vaccines can also be injected with the help of phages; they can act as a vehicle. The phage genome acts a vehicle and the specific vaccine antigen sequence is implanted in its genome. Specific antigenic peptides are added into the phage protein in a process called phage display. Novel mimetopes and antigens can be concede when particular antiserum is used to screen phage display libraries (Clark & March, 2006). For the detection of vaccines, serums are used in the phage display libraries against specific diseases. According to some studies on animal's models whole phage particle can be used as vaccines by altering their genome after the incorporation of antigenic properties (Irving, Pan, & Scott, 2001).

These applications of phage therapy can be used by the scientists to medicate them for bacterial infections in humans, animals and plants.

2.2.2.1. PHAGES IN TREATING HUMAN INFECTIONS:

Various clinical trials of humans have been done in different time period. Patients of chronic otitis caused by *Pseudomonas aeruginosa* of phase I/II trial were treated with phages shows promising effects with the decrease in the concentration of bacteria with no side effects (Wright et al., 2009). Delmont laborites conducted study on antistaphylococcal bacteriophages. These phages when injected to humans through orally, subcutaneously intravenously, topically, and intranasally within a time period of 12 years, and they show promising results with little side effects (Kutter & Sulakvelidze, 2004). A phase I trial in

Texas has been conducted on wound patients by FDA approved drug. In this study, phage cocktail for *Staphylococcus*, *Pseudomonas aeruginosa* and *E. coli* were used and no safety concerns were seen (Rhoads et al., 2009). Phase II placebo-controlled double blind study was conducted with these phage cocktail, the single dose of phages was administered in a nanogram range. They were successful in eliminating a long term infection of *Pseudomonas aeruginosa* (Wright et al., 2009).

2.2.2.2. STATUS OF PHAGE THERAPY IN AGRICULTURE INDUSTRY:

In agriculture, bactericidal pathogens can be treated by phage therapy. According to different studies, bacteriophages were used to treat *Xanthomonas campestris*, *Ralstonia solanacearum* and *Erwinia amylovora* infections of spot and wilt in tomatoes and apple blossom respectively. Bacterial spots caused by *Xanthomonas pruni* on peaches and other diseases of peppers, cabbage, peaches and the mushroom patches caused by *Pseudomonas tolaasii* can be treated by phage therapy (Monk, Rees, Barrow, Hagens, & Harper, 2010; Schnabel & Jones, 2001). To make processed food or edibles, fresh and in texture they are treated by phages to decrease the amount of bacteria (Garcia, Rodriguez, Rodriguez, & Martinez, 2010) (Garcia, Rodriguez et al. 2010). Cheese can be protected by phages from *Salmonella enteritidis* (Modi, Hirvi, Hill, & Griffiths, 2001). Phages were applied to plants in synergy to other plant protection methods (Obradovic, Jones, Momol, Balogh, & Olson, 2004). After a successful experiment, the US company OMnilytics produces as commercial product "agriphage" which act as a biocontrol agent and is used to treat large number of infections and is available in markets of South and North America and recently licensed in Asia. Phages are considered as decontaminant for the removal of bacteria on food surfaces. Dutch company EBI isolated a bacteriophage with no toxicity and it is specific to Listeria (Listex P-100). It is used to treat the *L. monocytogenes* infections in stored refrigerated food (Carlton, Noordman, Biswas, De Meester, & Loessner, 2005)

2.2.2.3. BACTERIOPHAGE THERAPY IN ANIMALS:

Bacteriophage therapy is also used in the treatment of animals with a high success rate. In 1982, Smith and Huggins administered *E. coli* infection in mice intracerebally/ intramuscularly to check the activity of phages on them. Phage anti-K1 single dose when injected intramuscularly was much powerful in comparison to the many intramuscular doses of chloramphenicol, trimethoprimplus sulphafurazole, ampicillin and tetracycline

(Smith & Huggins, 1982). Study conducted by Biswas *et al* on a vancomycin resistant *Enterococcus faecium* (VRE) isolates against ENB6 phage. Mice models were injected with high dose of VRE to induce bacteremia. The mice were fully recovered with a single intraperitoneal injection of phage ENB6 (Biswas et al., 2002).

Cinetobacter baumanni (Soothill, 1992), *Pseudomonas aeruginosa, Klebsiella pneumoniae* (Chhibber, Kaur, & Kumari, 2008), *S. aureus* (Wills, Kerrigan, & Soothill, 2005) infections are treated with bacteriophage therapy in animal's models. All of them were effective and successful in treating diseases. Different studies on respiratory and ear infections were seen in animal models after the administration of bacteriophages with an auspicious result (Carmody et al., 2010).

In 2010, Korean Cheil Jedang Corporation used a product of bacteriophages "Bio Tector" in poultry to treat salmonellosis. According to Connerton *et al, Campylobacter jejuni* in the chicken gut is treated by phages (Carrillo et al., 2005). Yellowtail fish infection due to *Lactococcus garviaeis* also treated by bacteriophages. Bacteriophage-treated feed pellets were given to *Pseudomonas plecoglossicida* infected Ayu fish shows effective and positive results in treatment (Nakai & Park, 2002). Phage bioprocessing is used for increase in the shelf life of animal products (Greer & Dilts, 2002). Thus all of these findings open a new era of phage therapy to overcome bacterial infections in a most effective way.

2.3. OBJECTIVES:

The present study is focused on the isolation, characterization and comparative analysis of microflora of BV infected non-pregnant and pregnant females. Furthermore, bacteriophages were isolated and characterized against BV infection microbiota. This study will help in understanding the microbiota of BV infection, their prevalence in Pakistani female population. Current study will also provide a new path in the field of phage therapy to treat the BV and other bacterial infections, along with a suitable solution to antibiotic resistance which is now a day is a major global concern.

Chapter 3:

Materials & Methods:

This study was executed to collect samples of pregnant and non-pregnant females suffering from bacterial vaginosis (BV), and to study the causative agents of infection along with the isolation of bacteriophages against this microflora. The study was conducted in Atta-ur-Rehman School of Applied Biosciences (ASAB), NUST in collaboration with Gynecology and Obstetrics Department of Kahota Research Laboratories (KRL) Hospital, Islamabad, Pakistan. The approval was taken from both the organizations prior performing the study.

3.1. STUDY POPULATION:

Females attending KRL General Hospital positive for BV.

3.2. SELECTION CRITERIA:

3.2.1. INCLUSION CRITERIA:

a. Age range: 20-50.

b. Pregnant and non-pregnant females positive for BV.

c. All the women had normal blood glucose level and menstruation cycle.

3.2.2. EXCLUSION CRITERIA:

a. Unmarried and young girls.

b. The presence of more than one disease.

c. The use of any kind of antimicrobial oral or topical 30 days before the sampling.

3.3. SAMPLING PROCEDURE:

3.3.1. PATIENT CONSENT:

Patients who met the inclusion criteria, their consent were taken before collecting the BV samples. The consent contains the general information of the patient and its clinical history. The form is included in the appendices (Appendix-A).

3.3.2. COLLECTION OF SAMPLES:

The samples were collected randomly with the high vaginal swabs (HVS) by the gynecologist on duty. The samples were collected from the endo-cervical region of BV patients in KRL, Hospital and were taken to Virology Lab II, ASAB-NUST, Islamabad for further clinical research.

3.3.3. SAMPLE SIZE:

Total 60 samples were taken, from pregnant females 30 samples were collected while from non-pregnant females 30 samples were collected visited KRL Hospital from 6th October, 2017 to 5th April, 2018.

3.4. MATERIALS:

Growth media and solutions were made in distilled water within the lab. All the glass wares, media and solutions were autoclaved 20 minutes at 121°C for 15 psi and dried at 37°C.

3.4.1. RECIPES:

For making media, calculations were adjusted according to requirements and standard recipes were used. The recipe's details are included in Appendices (Appendix-B)

3.5. METHODOLOGY:

3.5.1. SAMPLING:

Using HVS, BV samples were collected from non-pregnant and pregnant females. Within the 2-3 hours of sample collection, samples were processed to avoid diminution of bacteria and contamination.

3.5.2. BACTERIAL ENRICHMENT:

Three different types of media i.e., Blood agar, Luria Bertaini (LB) agar and De Man, Rogosa and Sharpe agar were used for clinical research. These media were melted and poured in the petri plates and left at the room temperature to solidify in laminar flow hood to avoid contamination and moisture prior a day before sampling. The collected samples were streaked in quadrant or T-shape by swabs on three of the media plates and were labelled with their respective sample ID. The streaked petri dishes were incubated in aerobic and anaerobic environments in an incubator and temperature was pre-set at 37°C for 24 h.

3.5.3. BACTERIAL ISOLATION:

After 24 hours' bacterial colonies were observed on incubated plates. Pure colonies were achieved after repeated quadrant and T shape streaking for further analysis.

3.5.4. MORPHOLOGICAL CHARACTERIZATION:

On the basis of shape, color, and size hemolytic properties on blood agar media bacterial strains were isolated.

3.5.5. BIOCHEMICAL CHARACTERIZATION:

For biochemical characterization of bacteria, purified and isolated colonies were taken and distinguished on the base of following methods.

3.5.5.1. GRAM STAINING:

Gram Staining is an easiest technique to identify and characterize bacteria. Microscopic slides were taken and cleaned with xylene. The slides were labelled with their respective ID and a drop of water was added on them. With the help of inoculating loop isolated colonies was picked from streaked plates and immersed in a water drop to form a smear. After drying of smear they were heat fixed. Drop of Crystal violet (CV) stain was added for 1 minute on the smear and leave. To wash extra stain the smear was washed with water for 1 minute. Drop of Iodine (I) was added on the smear for 1 min. Iodine solution is used as a mordant which retain the CV-I complex stain. Again smear was rinsed with water. After this, few drops of 95% ethanol were added for 45 sec. Ethanol was used for the decolorization of the primary stain. Again washing was done and Safranin stain was added on smear for 1 minute. Slides were blotted on tissue papers and dried. Later observed under microscope at 40X and 100X magnification.

3.5.5.2. CATALASE TEST:

It is one of the main test used for the biochemical identification of a bacterium. It is used to differentiate aerobes and facultative anaerobes or for the identification of *Staphylococcus* and *Streptococcus*. The aerobic bacteria contain an enzyme which convert hydrogen peroxide (H_2O_2) into oxygen and water while anaerobic bacteria don't have catalase enzyme. The *staphylococcus* gives positive test with the formation of bubbles due to the production of oxygen, while streptococcus gives negative results. The basic purpose of this enzyme is to eliminate H_2O_2 and super oxides (toxics) accumulation in the bacterial cells which results in the death of cells. Flavoproteins are the enzymes that allow the production of these product while producing reduced amount of oxygen.

Prepared 3% H_2O_2 (Table 3.3) and add drop of this hydrogen peroxide on a microscopic slide. Take 24 h fresh bacterial culture of all the isolated colonies and mixed it with the H_2O_2 drops with inoculating loop. Within the 30-45 seconds oxygen bubbles formation was observed.

3.6. ANTIBIOTIC RESISTANCE:

Sensitivity of BV isolated bacterial colonies was checked with most commonly prescribed antibiotics used for the cure of BV infection. These antimicrobials are metronidazole and Clindamycin. Bacterial cultures were inoculated in LB medium for overnight. On the other side, LB agar plates were prepared by melting the medium and poured into autoclaved plates, and left for solidification. After 24h, fresh bacterial cultures were plated on LB agar petri dishes by continuous streak method and antibiotics were added on them by a disk diffusion method. The commercially prepared disks of metronidazole and clindamycin (Oxoid) were used. Plates were sealed by paraffin and incubated at 37°C for overnight.

3.7. PHAGE ISOLATION AND ENRICHMENT:

After the isolation and characterization of bacteria, phage isolation was carried out. Phages were isolated against collected bacterial isolates of BV infection. The sewage water sample was used for the isolation of phages. Collected water sample was kept for rest for few hours to settle down the sediments. Centrifugation was done for 15 minutes at 14,000rpm, pellet was discarded and supernatant was taken. The supernatant was passed through 0.22µm syringe filter. The mixture of 24h fresh bacterial cultures, 2X LB MgSO4 (table 3.4) enriched medium and centrifuged sewage water supernatant were taken in an 50ml falcon tube and placed in a shaking incubator for overnight at a temperature of 37ºC. On next day, after 24 hours, 1% chloroform (table 3.5) was added in the sample to break the bacterial cell wall. For disruption of bacterial cells, samples were centrifuged at 6000 rpm for 30 minutes. After centrifugation, pellet was discarded and supernatant was again passed through 0.22µm syringe filters. The filtrate was preserved at -20ºC for further use.

3.8. LYTIC ACTIVITY OF PHAGES:

Spot tests was carried out to examine the phages lytic activity. For this purpose, bacterial isolates were incubated in fresh LB broth at 37ºC for overnight in a constant shaking incubator along with that, LB agar plates were prepared with their respective specific labels. After 24h the bacterial samples were streaked on the solidified agar plates with continuous streak method. After streaking moderately warm soft agar (table 3.1) 3-5ml was poured on the plates. 20µl of phage lysate was taken through micropipette and

punched in the between the LB agar and molted soft agar. Plates were properly sealed and placed in an incubator of 37ºC for 24h.

3.9. CALCULATION OF PLAQUE FORMATION POTENTIAL:

Plaque assay was performed with agar over lay method. Serial dilutions of phage lysate were formed up to 10^{-20}. Fresh bacterial cultures (24h) 180 µl were incubated with 100µl of phage lysates dilutions for 15 minutes on constant shaking incubator at 37ºC. LB agar plates were prepared prior. The ten dilutions were used: 10^{-1},10^{-2},10^{-3} 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10}. After 15 minutes bacterial-phage mixtures was added on their respective labelled agar plates along with it molted soft agar 3-5ml, swirled the plates for evenly distribution of soft agar. Incubation of plates was carried out at 37ºC for overnight after sealing with paraffin. Later on, after 24h the plaques were calculated.

3.10. ONE STEP GROWTH CURVE:

First of all, bacterial log (exponential) phase was optimized. For this purpose, bacterial isolates were inoculated in LB broth medium and incubated at a temperature of 37ºC in water shaking incubator for overnight. After 24 hours, the optical density(OD) of overnight bacterial culture was taken at 600nm. Then 200µl of overnight bacterial culture was taken mixed with 500µl of fresh LB broth. Again OD was taken at 0 minute, the cultures were incubated again for 30 minutes and again OD was taken. This same procedure was done after every 30 minutes for the next 180 minutes to determine the log phase of bacteria.

To determine the phage burst size and latent phase Gong et al described one step growth method was used (Y. Gong et al., 1996). In this method, the overnight bacterial cultures were taken and inoculated in a fresh LB medium for 2h and incubated at 37ºC in constant shaker. After 2h, 180µl of bacterial cultures and 100µl of phage lysates were taken together and their optical density were taken at 600nm on 0 minute and mixtures were placed in shaking incubator for 5 minutes. After 5 minutes, again OD was taken at 600nm.The same procedure was done for the next 30 minutes to identify the maximum adsorption of phages. Experimental samples were carried out in triplicates along with control. The whole procedure was same except for controls, instead of phage lysate 100µl of LB enriched with $MgSO_4$ was used.

3.11. CALCULATION OF VIRAL TITER (pfu/ml):

Viral titer can be calculated by plaque forming unit (pfu) which is quantification of phage activity per ml. For this purpose, agar overlay method was used. Serial dilutions were prepared in LB $MgSO^4$ broth up to 10^{-20}. The dilutions used were 10^{-1},10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10} and a mixture of 100µl of phage lysate and 180µl of log phase bacterial culture were used and incubated in constant shaking incubator for 15 minutes at 37°C. The LB agar plates were prepared. After 15 minutes the bacterial-phage mixtures were added on their respective labelled agar plates and molten soft agar 3-5ml was added above the suspension and swirled. The glass plates were properly sealed and incubated in an incubator at 37ºC for overnight. After overnight incubation, pfu was calculated according to the formula mentioned below:

$$\frac{\text{Average no. of plaques} = \text{PFU/ml}}{\text{Dilution Factor X Volume}}$$

3.12. VIRAL ADSORPTION:

The adsorption of phages on bacterial cell was examined by magnesium (Mg^{+2}) ions and calcium (Ca^{+2}). Adsorption of phages were determined with 15mM of LB+$CaCl_2$ and 15mM LB+$MgSO_4$ media. The bacterial cultures against which the phages were isolated, were inoculated separately in LB+$CaCl_2$ and LB+$MgSO_4$ media (15mM) and incubated in a shaking incubator at 37°C temperature for overnight. Later, serially diluted phage lysates were assembled with the 24h bacterial suspension and incubation was given to the mixture for 15 minutes in shaking incubator at 37ºC. After 15 minutes, the molted soft agar and bacterial-phage suspension was mixed and added to the agar plates using the method called agar overlay method. The incubation was done at 37ºC for overnight.

3.13. THERMAL STABILITY OF PHAGES:

The phages lytic activity and inactivation were checked at different temperature. Aliquots of phages were prepared to incubate at 37ºC, 40ºC, 45ºC, 50ºC, 55ºC, 60ºC, 65ºC, 70ºC and 80ºC for 1hr. The incubated phage lysates activity was tested by spot tests by incubating the plates at 37°C for 24h.

3.14. pH STABILITY OF PHAGES:

The phages lytic activity was checked at various pH. The protocol used was proposed by Capra et al (Capra, Quiberoni, Ackermann, Moineau, & Reinheimer, 2006). LB broth of different pH i.e., 1, 3, 5, 7, 9, and 11 were made and autoclaved. The phage lysates were added in adjusted pH LB broths and incubated for overnight at 37ºC. Phages activity was checked by using spot test method.

3.15. ISOLATION OF PHAGE NUCLEIC ACID:

High titer phage lysates (20ml) were taken and 10µl RNase A (5mg/ml) and 10µl DNase I (10mg/ml) were added in them. The mixtures incubation was done at 37ºC in a constant shaker for overnight. After 24hr, the phage lysates were treated with 8ml of precipitant solution polyethylene glycol and sodium chloride (33% PEG 4000, 3.3M NaCl) to pellet the phage particles. After adding precipitant solution, the mixtures were gently mixed and stored for 1hr at -80ºC. After an hour, samples were centrifuged at 4ºC and 7,500 rpm for 1 hour and 20 min. Pellets were collected after discarding supernatants. Pellets were mixed in SM buffer (NaCl, $MgSO_4$. $7H_2O$, 1M Tris- Cl) by pipetting and transferred to 15ml falcon tubes and again centrifuged at 7,500 rpm and 4ºC for 15 minutes. The pellets were taken and resuspended in 600µl of SM buffer. The mixtures were transferred to 1.5ml Eppendorf tubes.

The phenol, isoamyl alcohol and chloroform was added to samples and gently mixed. The centrifugation of samples was done at 4ºC and 12,000 rpm for 15 minutes. Upper aqueous layers were collected after centrifugation and transferred to new 1.5ml Eppendorf tubes. Equal amount of chloroform was mixed in samples and centrifuged at 12,000 rpm and 4ºC for 20 min. The upper aqueous layers were removed and transferred to new autoclaved 2ml Eppendorf tubes. An equal volume of 0.3 M sodium acetate and isopropyl alcohol were mixed and vortex. The Eppendorf tubes containing samples were stored for 10 min at -80ºC. After incubation, centrifugation was carried out at 12,000 rpm for 20 min and pellets were collected by discarding supernatants. For washing, 70% ethanol (700µl) was added and again centrifuged at 12,000rpm for 10min. The pellets were dried and dissolved in 50µl of TE buffer (1mM EDTA (pH 8.0) and 10mM Tris-Cl) (Haq, Chaudhry, Andleeb, & Qadri, 2012; Sambrook & Russel).

The samples were treated with RNase A and DNase I separately to determine whether the samples contain RNA genome or DNA genome. For this purpose, the 7µl genome containing samples were taken and 10µl RNase A (5mg/ml) and 10µl DNase I (10mg/ml) were added separately in Eppendorf tubes and at 37ºC incubated for 1 hr.

3.5.1. GEL ELECTROPHORESIS:

The genome size was confirmed by gel electrophoresis. To perform gel electrophoresis, 1.5% agarose (1.8g) was added in 1X TAE (120 ml). Heat the mixture in a microwave for 1:30-2:30 min until agarose was completely dissolved, allowed the solution to cool and 3ml of Ethidium Bromide was mixed to the gel solution and mixed. Transferred the solution in to the gel caster and placed the comb. After solidification of gel, the gel was transferred into buffer filled chamber containing 1X TAE buffer. Carefully removed the comb, loaded the samples (7 µl) and loading dye (3µl) mixture in to the wells of the gel along with the ladder of 1 kb and 100kb. Electrophoresis was done at 80 volts for 1 hr. The gel was seen on Gel documentation system and image was recorded.

3.16. HOST RANGE SPECIFICITY:

The phages host range of A12AnB2(1), A8AnL1, A33 were determined by using spot test technique. The samples were taken from the ASAB bacterial bank. The bacterial isolates were mixed in LB Broth at placed 37ºC in shaking incubator for overnight. On solidified LB agar plates, after 24 hour fresh bacterial cultures were streaked and 3-5ml of soft agar was poured on the plates. After 2-3 minutes when soft agar was solidified, the phage lysates (20µl) was punched in between the soft agar and LB agar by pipette. The plates were labelled, wrapped with paraffin and placed in incubator for overnight at 37ºC.

3.17. MOLECULAR IDENTIFICATION:

3.17.1. 16S rRNA INDENTIFICATION:

For molecular identification, samples were sent to Macrogen, Korea for 16S rRNA for sequencing. Before transporting samples were preserved in Glycerol solution. Glycerol stock was prepared according to table 3.6. The stock was added in labelled falcon tube and autoclaved for 15 minutes at 120psi. The bacterial isolates samples were inoculated in fresh LB broth and incubated 37ºC for overnight. After 24 h, bacterial samples were centrifuged and pellet of bacterial cells were taken while discarding the supernatant. The pellet was

then dissolved in LB media and glycerol suspension and transported to Macrogen, Korea for the sequencing and identification of bacteria.

3.18. MORPHOLOGICAL IDENTIFICATION OF PHAGES:

Scanning Electron Microscopy (SEM) was used for the morphological characterization of phages. Two types of samples were prepared for analysis. One with only phage lysate while the second one contained phages adsorb on 24hr fresh bacterial culture suspension. Phage lysates were serially diluted to 10^{-10}-10^{-15} dilution within LB broth. Sterile petri dish was taken and glass cover slips were washed with 100% ethanol in that petri dish. The cover slips were labelled and 50% glutaraldehyde (100µl) was added for 15 minutes. For removing precipitates 1X PBS was used. After washing, samples 80µl were added on the slides along with the 100µl of 50% glutaraldehyde for 20 minutes. Again washing was done by 1X PBS solution. The samples were dehydrated, by washing them with 20%, 40%, 60%, 80% and 100% ethanol respectively for 10 minutes. The samples were left for drying and packed in Virology Lab II, ASAB. These samples were taken to Institute of Space and Technology (IST), Islamabad where gold spluttering and magnification was carried out.

Chapter 4:

Results

4.1. BACTERIAL IDENTIFICATION:

4.1.1. MORPHOLOGICAL CHARACTERIZATION:

The bacterial colonies of Bacterial vaginosis infected females were identified according to their morphological characterization. The 60 samples were collected from both non-pregnant females and pregnant females. Out of 60 samples, 150 bacterial colonies were obtained which belongs to 10 isolates on initial identification tests. These isolates were pick out on the basis of their initial identification tests. The isolated microorganisms were different in their morphology (shape, colony formation, size, and color) and biochemical characterization (Gram staining, catalase properties). The identified isolates were Streptococcus, Staphylococcus, Enterococcus, Candida, Lactobacilli, Lactococcus, and Neisseria species.
Morphological properties of bacteria are shown in Figure 4.1

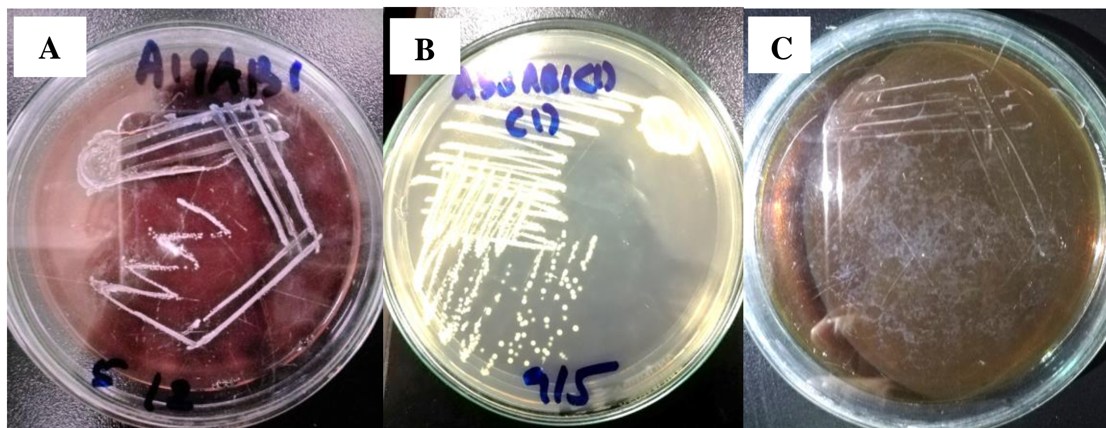


Figure 4.1 (a). Morphological characterization of bacterial strains: *This figure depicts the bacterial colonies growth on different media (A) on blood agar (B) on LB agar and (C) on MRS agar.*

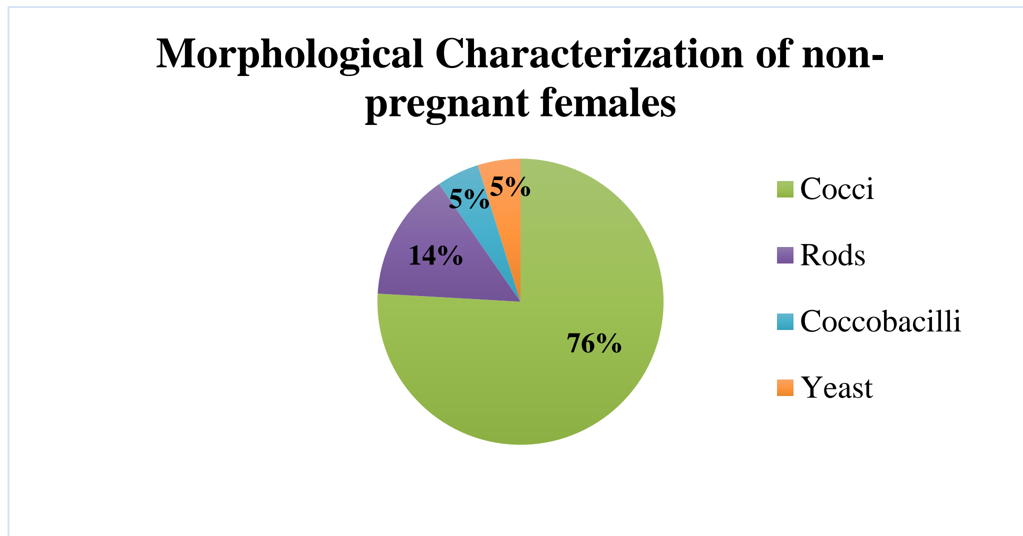


Figure 4.1 (b). Morphological Characterization of Microbial Isolates: *This graphs depicts the morphological characterization of bacterial isolates of non-pregnant females on the basis of shape. Bacterial isolates on the basis of their morphology shows 76% cocci were present in non-pregnant females followed by 14% rods, 5% coccobacilli and 5% yeast.*

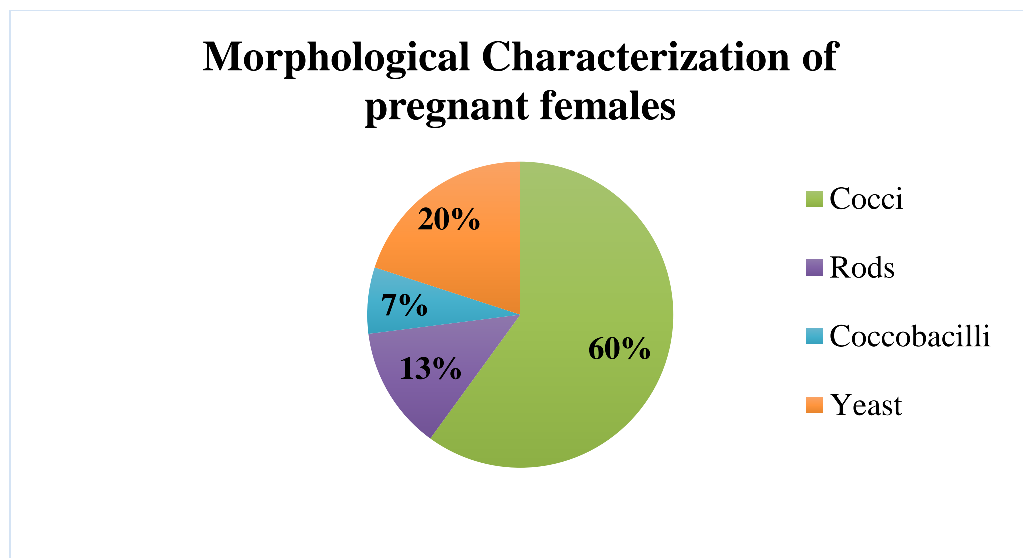


Figure 4.1 (c). Morphological Characterization of Microbial Isolates: *This graphs depicts the morphological characterization of bacterial isolates of pregnant females on*

the basis of shape. Bacterial isolates on the basis of their morphology shows 60% cocci were present in pregnant females followed by 20% yeast, 13% rods, and 7% coccobacilli.

4.1.2. CHARACTERIZATION BASED ON HEMOLYSIS ON BLOOD AGAR:

The BV infected pregnant and non-pregnant females' high vaginal swabs were streaked on blood agar plates to differentiate the colonies of the basis of their alpha, beta and gamma hemolysis and to identify fastidious microbes (Figure 4.2).

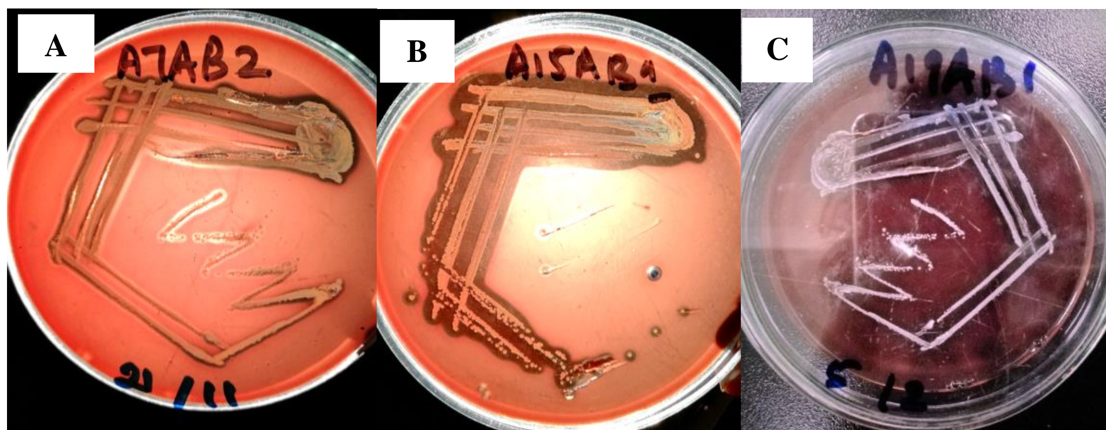


***Figure 4.2. Hemolysis by isolated bacterial strains on Blood Agar plates**: Bacterial isolated were streaked on blood agar plates to identify the hemolysis properties of isolates and to differentiate between the members of staphylococcus and streptococcus bacterial strains. Figure (A) and (B) shows beta hemolysis while (C) shows gamma or no hemolysis in pregnant and non-pregnant females.*

4.1.3. BIOCHEMICAL CHARACTERIZATION:

After morphological characterization, the bacterial isolates were further examined by biochemical testing such as Gram staining and Catalase test. For non-pregnant females, the gram staining results revealed 71 isolates were gram positive and 12 were gram negative. While in pregnant females, 65 isolated were gram positive (purple colored) and 5 were gram negative (pink colored) (Figure. 4.3 A-G).

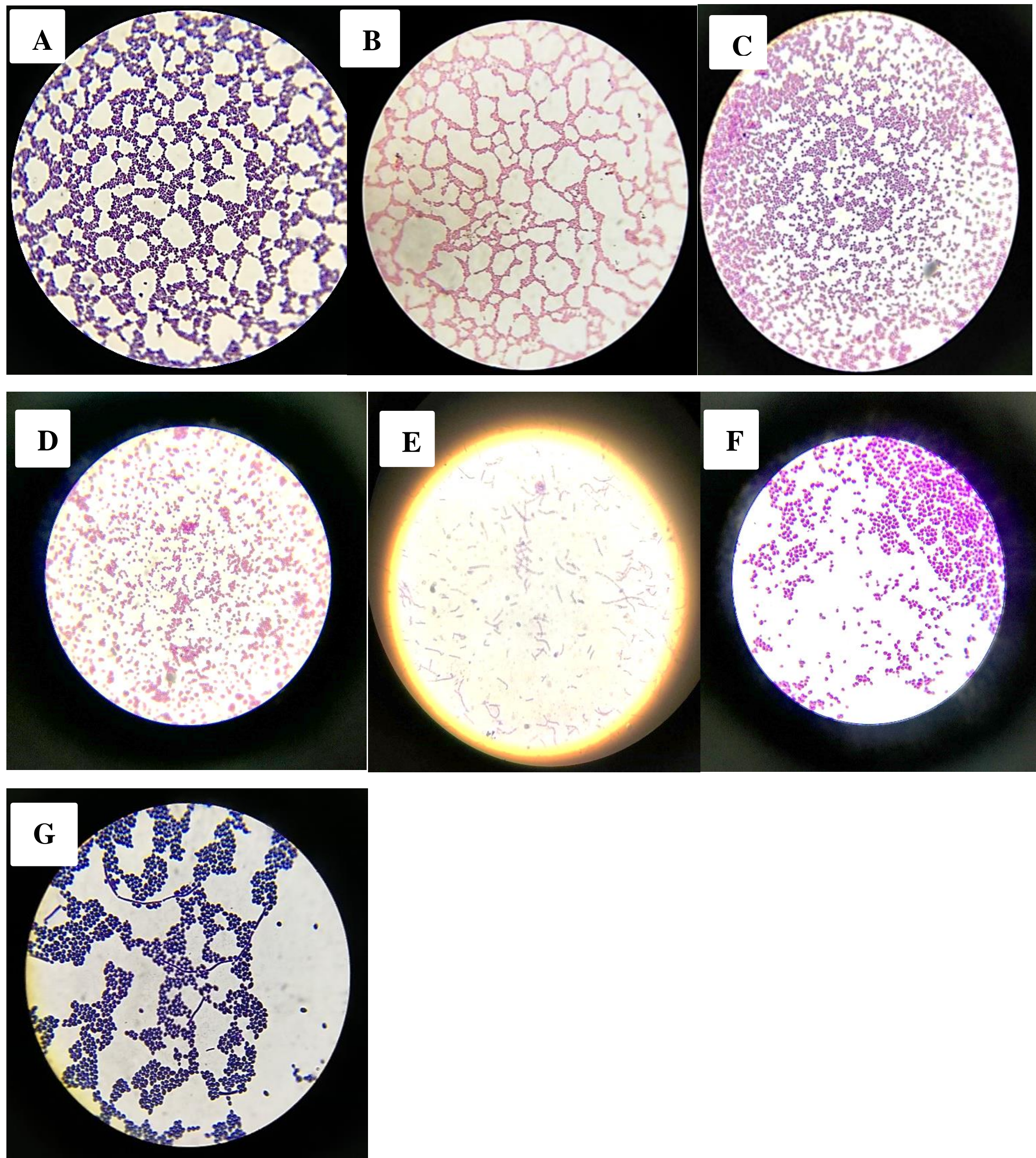


Figure 4.3. Gram staining of isolated bacteria: *This figure illustrates the bacterial morphology on the basis of gram staining. Figure (A) showed the Gram positive clustered cocci (B) Gram negative clustered cocci (C) Gram positive chains of cocci (D) Gram negative chains of cocci (E) Gram positive rods (F) Candida isolates (G) Mixed culture of Candida and Gram positive rods among pregnant and non- pregnant females.*

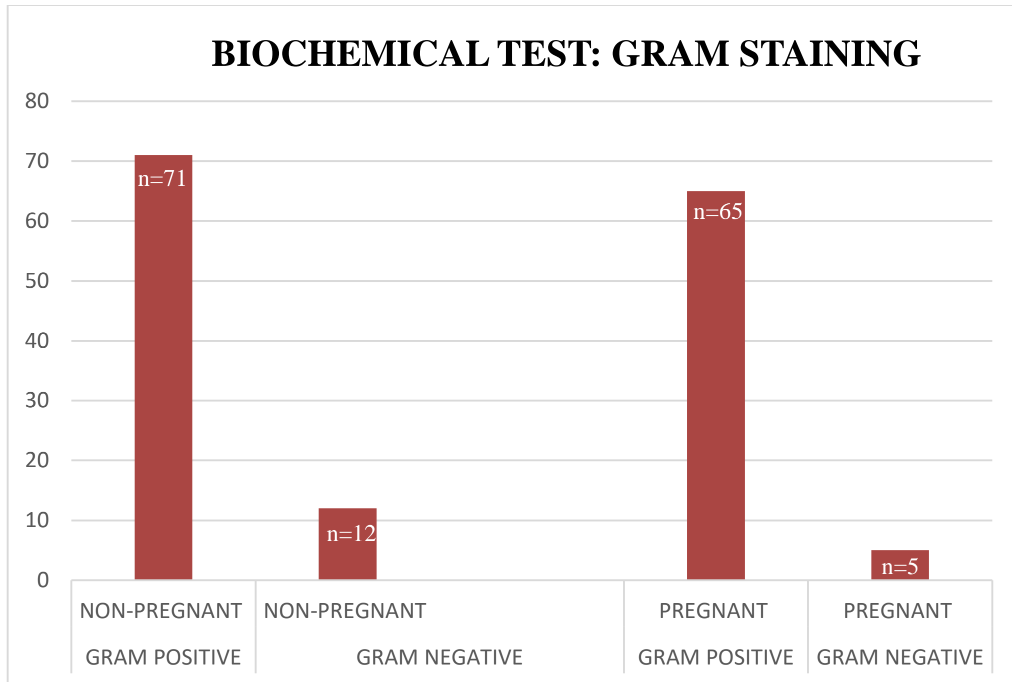


Figure 4.4. Biochemical characterization by Gram staining: *This graph shows the occurrence of bacterial isolates in non-pregnant females and pregnant females. Non-pregnant females have gram positive (n=71), while gram negative (n= 12) bacterial isolates. In comparison to non-pregnant, pregnant females' bacterial characterization shows gram positive (n=65) and gram negative (n=5) bacterial isolates.*

Catalase test was also performed on both infected pregnant and non-pregnant females bacterial isolates. The pregnant females had 69 catalase positive isolates while 4 catalase negative and the non-pregnant females had 74 catalase positive and 9 catalase negative isolates (Figure 4.4)

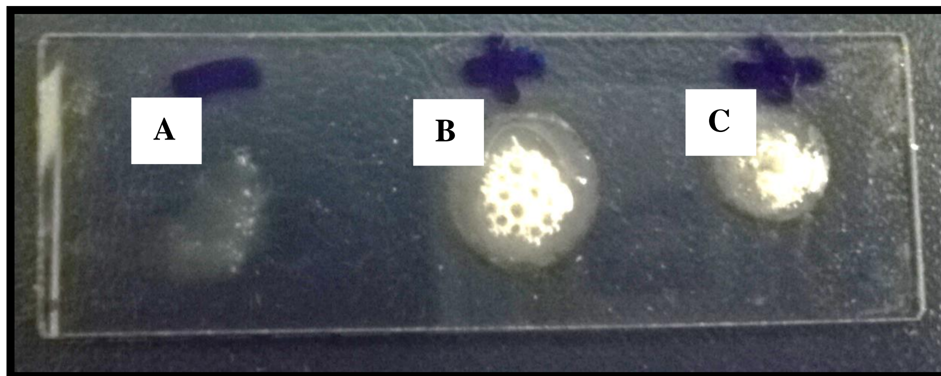


Figure 4.5. CATALASE TEST: *This test was performed on all bacterial isolates collected from both pregnant and non-pregnant to differentiate between the catalase positive and negative isolates. The isolates that form bubbles were catalase positive while with no bubble formation were seem to be catalase negative. (A) bacterial isolate was catalase negative while (B) and (C) were catalase positive.*

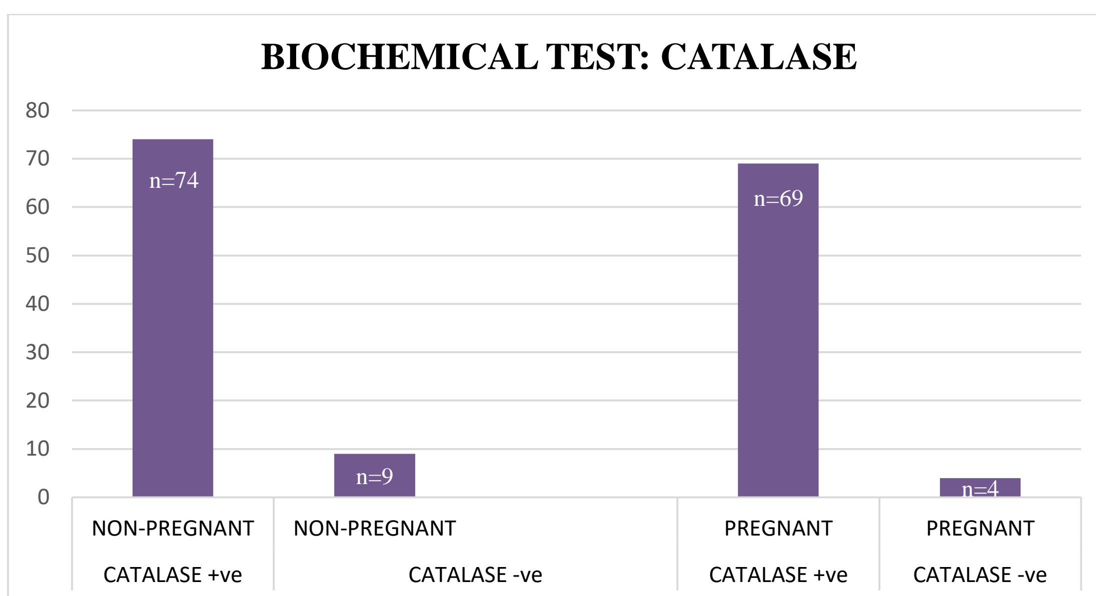


Figure 4.6. Biochemical characterization by performing Catalase test: *This graph shows the prevalence of bacterial isolates in non-pregnant and pregnant females. In non-pregnant females' catalase positive (n=74), while catalase negative (n= 9) bacterial isolates were present. In comparison to non-pregnant, pregnant females' bacterial characterization shows catalase positive (n=69) and catalase negative (n=4) bacterial isolates.*

4.2. PREVALENCE OF BACTERIAL ISOLATES AMONG THE BV POSITIVE PREGNANT SAMPLES:

The 150 bacterial isolates were obtained from both pregnant and non-pregnant females. Most abundantly present isolates were *Staphlococcus*, then *Streptococcus* followed by *Enterococcus, Candida, lactobacilli, Nisseria* and *Shigella* and exhibiting same pattern on both pregnant and no pregnant females.

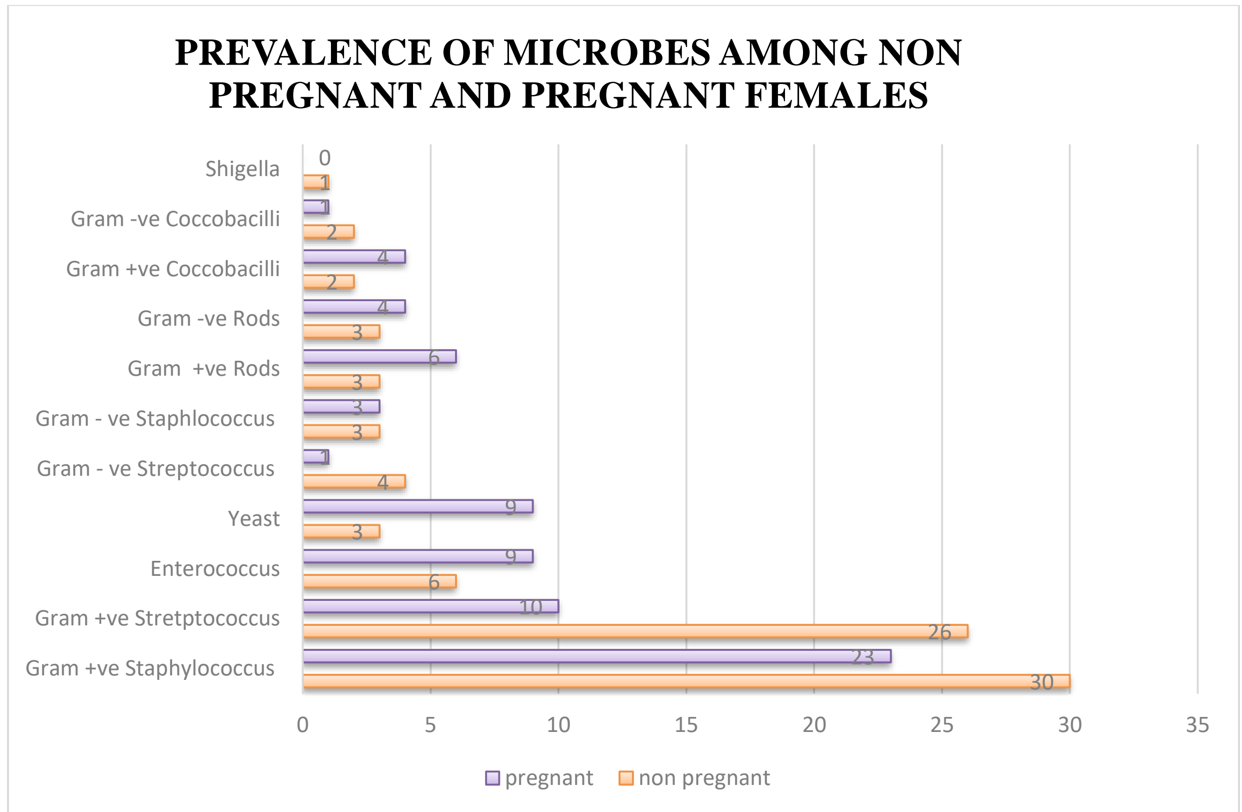


Figure 4.7: Prevalence of Bacterial Isolates among expecting and non-pregnant females: *Bar graph depicts prevalence of bacterial isolates in BV infection among females pregnant and non-pregnant. Staphylococcus was most abundantly prevailed in pregnant (33%) and non-pregnant (39%) women followed by Streptococcus (14%) in pregnant and (32%) in non-pregnant females. However, least prevalence isolate was Shigella with only 1 isolate in only non-pregnant female.*

4.3. ANTIBIOTIC SENSITIVITY:

The two commonly used drugs for BV infection treatment are metronidazole and clindamycin. These antibiotics were used to check the susceptibility of bacterial isolates. The disk diffusion was used to determine the antibiotic sensitivity (Figure 4.8). Surprisingly, all of the strains were found to be resistant against metronidazole. None of the bacterial strain showed activity against metronidazole. While clindamycin has shown sensitivity against few bacterial strains. Out of 150 bacterial strains 35 strains showed clear zones with disk diffusion method and 29 strains diameter was less than 25mm. According to antibiotic sensitivity chart if the zone of inhibition is less than <25mm, it must be

considered as negligible. The figures (A), (B), (D), (E) of bacterial strains showed complete resistant against antibiotics with no zone of inhibition. While, the figures (C) and (F) bacterial strains was insensitive to metronidazole but they were susceptible to clindamycin.

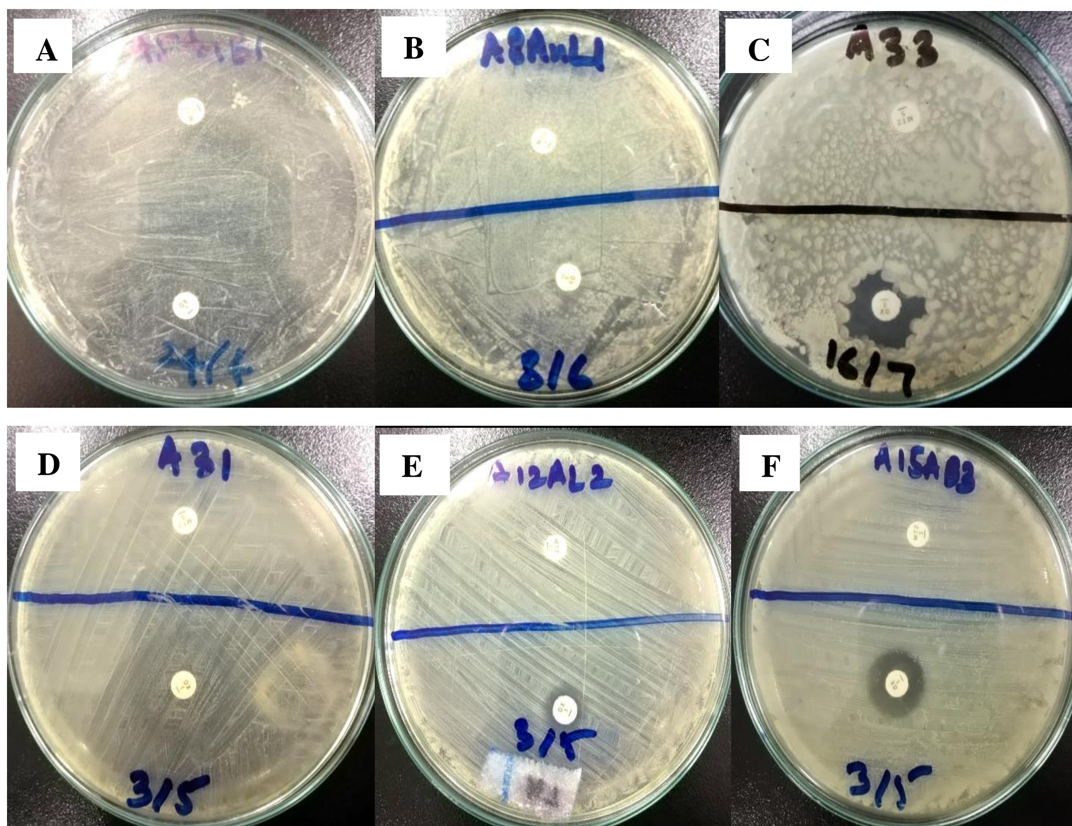


Figure 4.8. Antibiotic sensitivity: *Susceptibility of bacterial strains was checked against metronidazole and clindamycin. The figure (A, B, D and E) showed no cleared zones depicting antibiotic resistant while Figure (C and F) showed clearing zones against clindamycin only while resistant to metronidazole.*

4.4. PHAGE ISOLATION AND ENRICHMENT:

The isolation of phages was done by sewage water. The phage isolation protocol (Chapter 3 (3.3)) was followed to isolate the phage lysate. The isolated phage lysates were checked against all isolated bacterial strains (Figure 4.9). Out of all isolates 03 strains showed susceptibility against phage lysates i.e., A8AnL1, A12AnB2(1) and A33 (Figure 4.9 D, E and F) exhibiting clear zones.

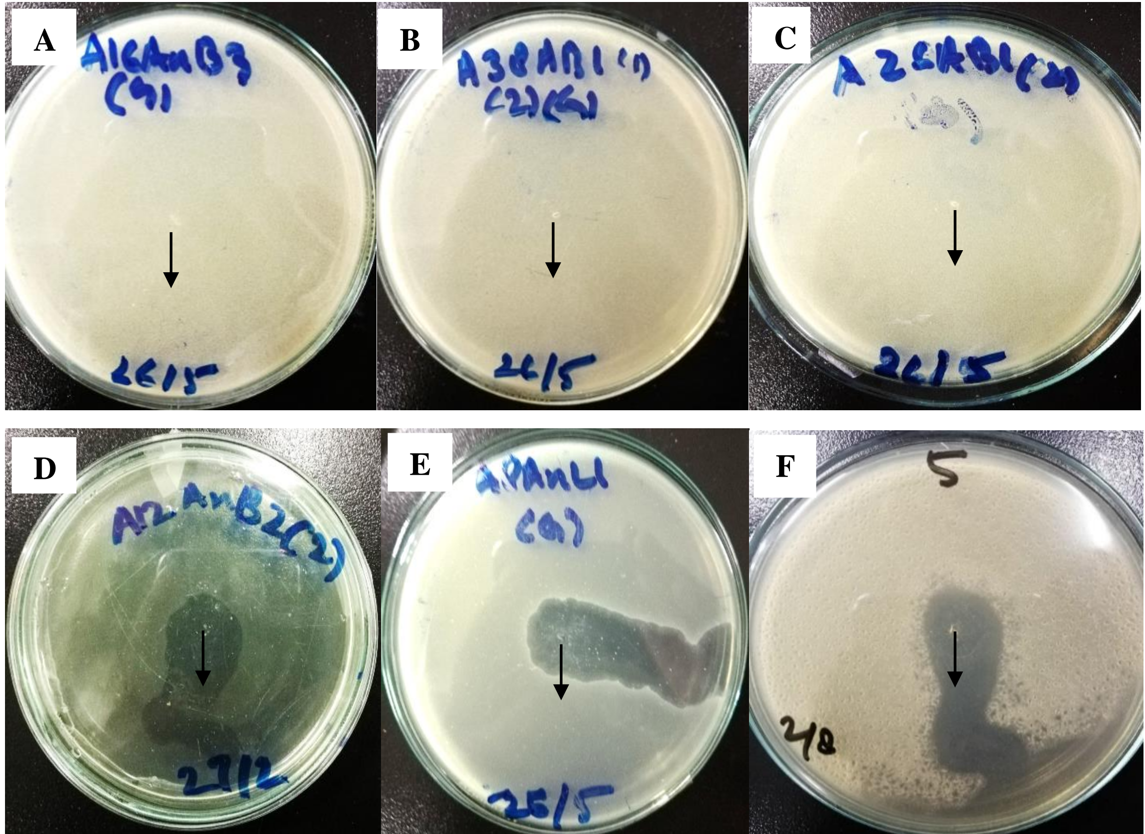


***Figure 4.9. Phage isolation:** These figures depict the isolation of phage against isolated colonies. Figure (A-C) shows no zone formation while D, E and F showed clear zone of phage lysate thus indicating positive results.*

4.5. PHAGE LYTIC POTENTIAL ACTIVTIY ANALYSIS:

The lytic activity of phages was tested, using spot test method and plaque assay method.

4.5.1. SPOT TEST:

The agar overlay method was used for the spot test against strains A8AnL1, A33 and A12AnB2(1). The spot test showed clear zone formation (Figure 4.10) with the phage lysates confirming the presence of respective phages.

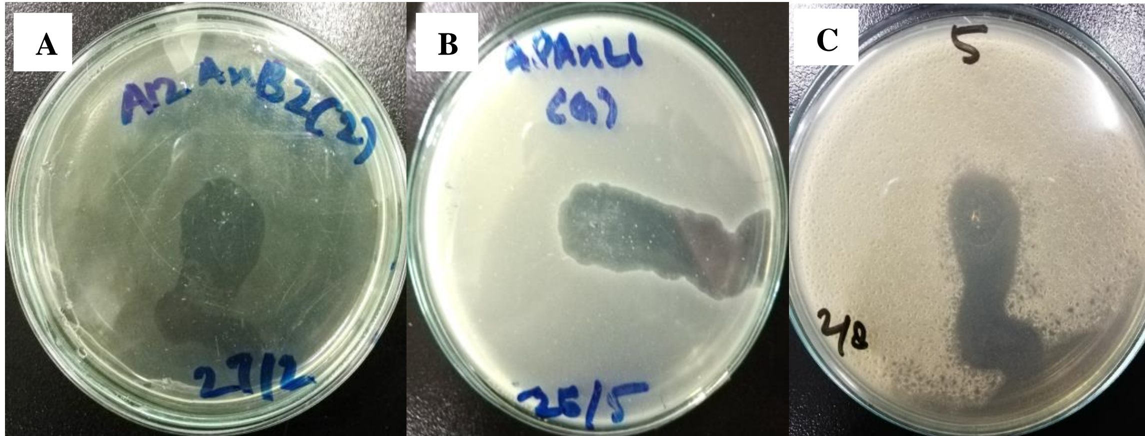
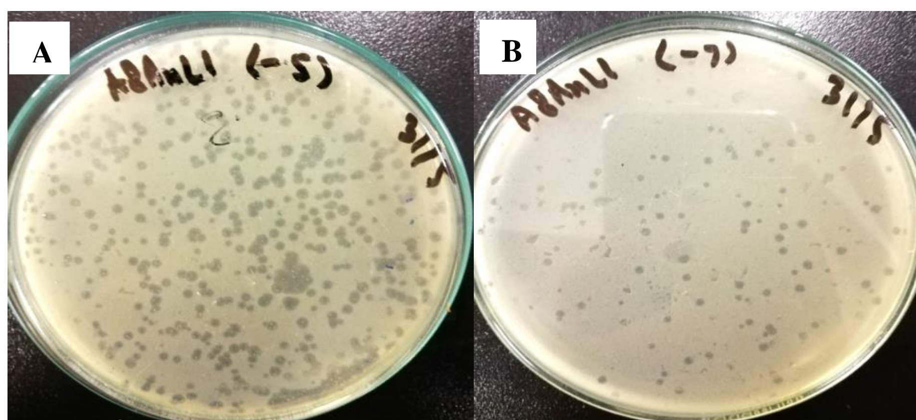


Figure 4.10. Spot Test: *Spot test was done to confirm the lytic activity of phages against A12AnB (1) (A), A8AnL1(B) and A33 (C) strains. The formation of clear zones against these bacterial strains were seen in figure (A), (B) and (C).*

4.5.2. PLAQUE ASSAY:

This method was done by making serial dilution of phage lysates up to 10^{-10}. Ten dilutions were used i.e.10^{-1}, 10^{-3}, 10^{-2}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, and 10^{-10} because further dilutions did not give any results. The diluted phage lysates were plated by agar overlay method after mixing with the log phase of bacterial culture. After the incubation time period the plates were observed for formation of plaque and counting of was done. The figure 4.11 shows the formation of plaques.



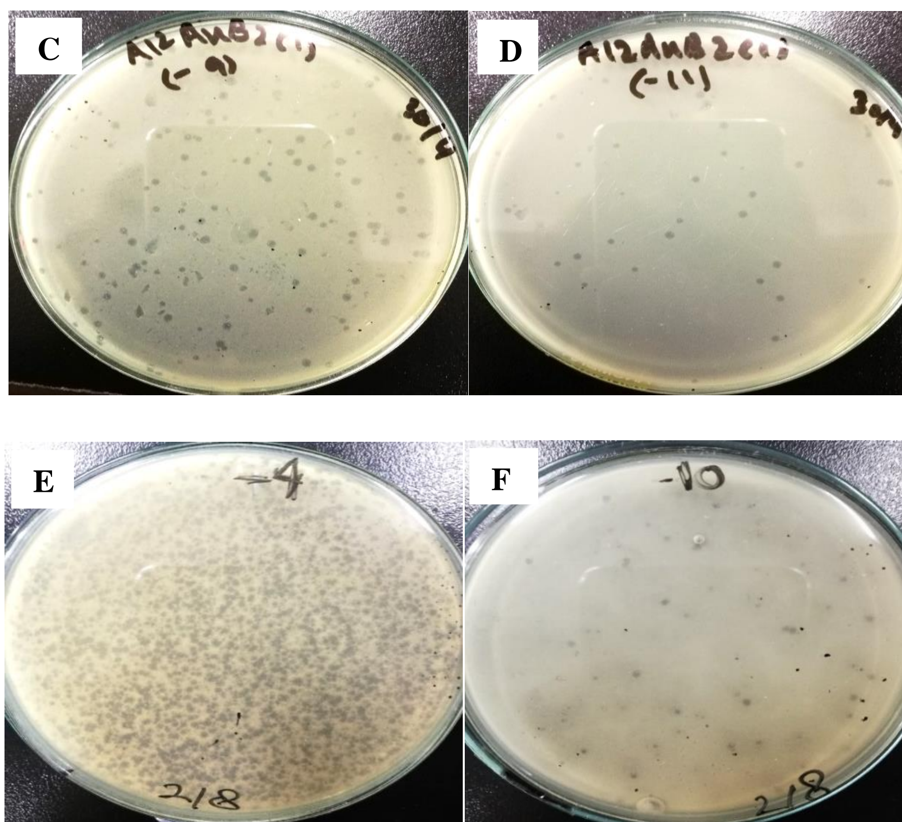


Figure 4.11. Plaque Assay: *Plaques assay were performed to check the lytic activity of phages against A8AnL1, A33 and A12AnB2(1) bacterial strains. The figures A and B showed lytic activity of A8AnL1 with the dilution of log 5 and log 7, while figures C, D, E and F showed the formation of plaques with A12AnB2(1) and A33 at a dilution log 9, log 11, log 4 and log 10 respectively.*

4.6. GROWTH CURVE ANALYSIS:

Growth curves were analyzed for both bacteria and phages. To determine the bacterial growth curves, the 24h fresh bacterial cultures were obtained and inoculated in fresh LB broth and placed at 37ºC in a constant shaker. At 600nm, optical density of bacterial cultures was taken at 0 minute and after every 30 minutes for 2 hours to determine the exponential phase of bacterial isolates represents in figure 4.12 (a), (b) and (c).

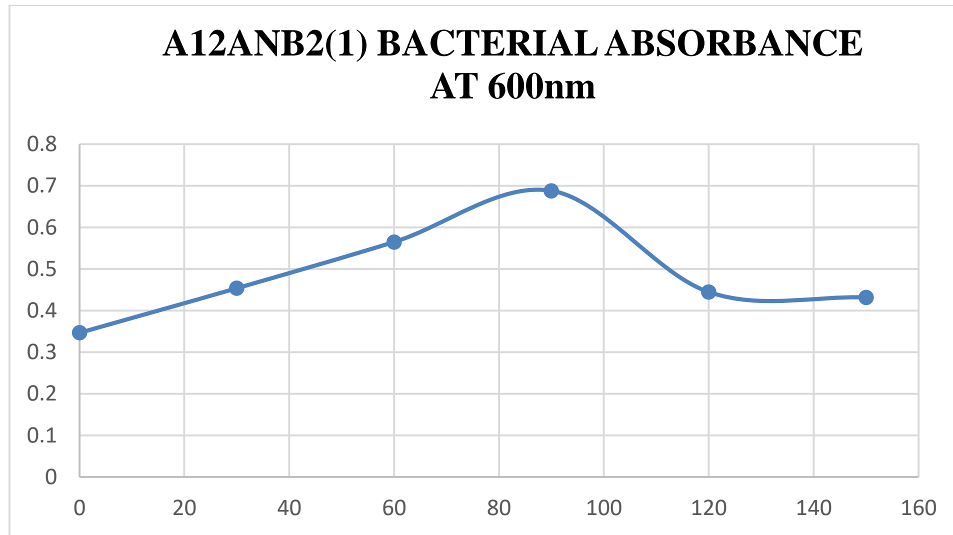


Figure 4.12 (a). Bacterial growth curve analysis: *This graph represents the life cycle of bacteria in all four phases of growth i-e. Lag Phase (0-30 min), Log Phase (30-60 min), Stationary Phase (60-90 min) and Decline Phase (90-120 min). This is a bacterial growth curve of sample A12AnB2(1).*

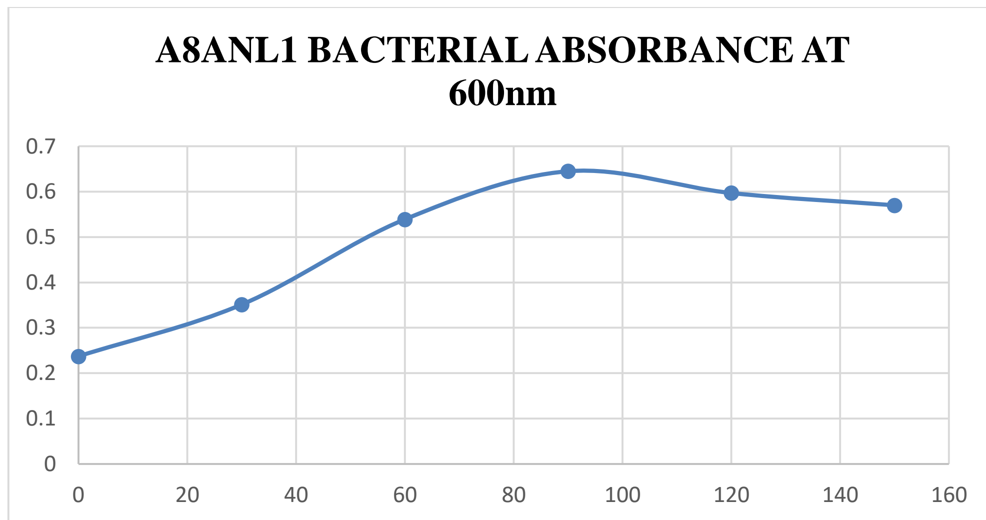


Figure 4.12 (b). Bacterial growth curve analysis: *This graph represents the life cycle of bacteria in all four phases of growth i-e. Lag Phase (0-30 min), Log Phase (30-90 min), Stationary Phase (90-120 min). This is a bacterial growth curve of sample A8AnL1.*

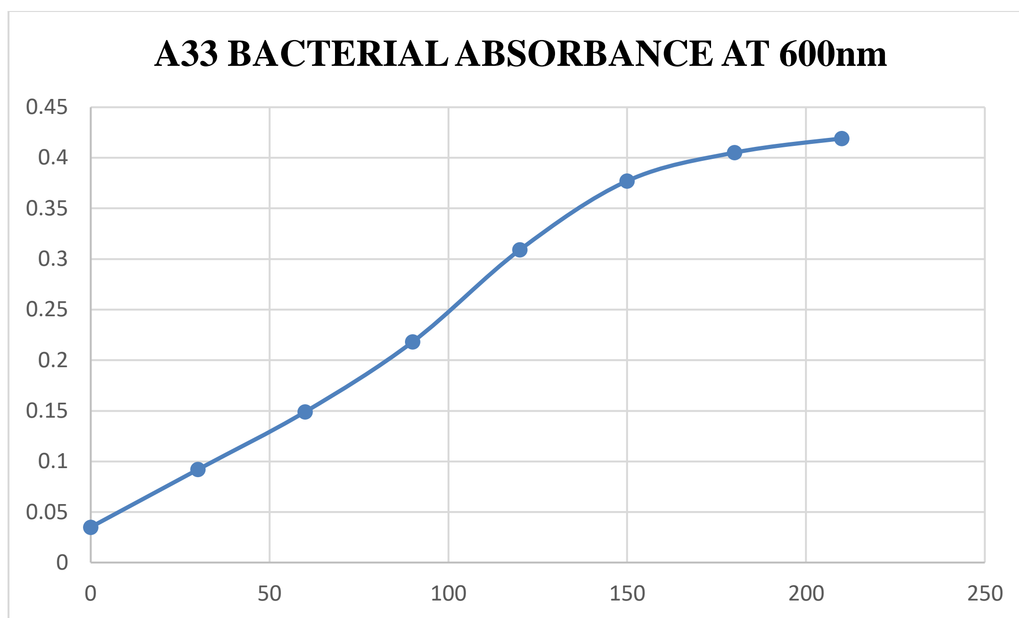


Figure 4.12 (c). Bacterial growth curve analysis: *This graph represents the life cycle of bacteria in all four phases of growth i-e. Lag Phase (0-30 min), Log Phase (30-90 min), Stationary Phase (90 -150 min). This is a bacterial growth curve of strain A33.*

The same experiment was performed to evaluate the growth curves of phage lysates. Formerly calculated time interval of fresh bacterial cultures were taken (180µl) and incubated with a phage lysates (100µl) with a constant shaking for 30 minutes at 37ºC. However, the experiment was done in two sets i.e. control and test. In case of control, 100µl of $MgSO_4$ enriched LB broth was used in replacement of phage lysate. The optical density at 600nm was calculated after every 5 minutes' interval to determine the time at which maximum phage adsorption occur. A noteworthy depletion was seen in bacterial growth after incubating bacteria with phage lysates in comparison to independent growth of bacterial isolates which is represents in figure 4.13 (a), (b) and (c).

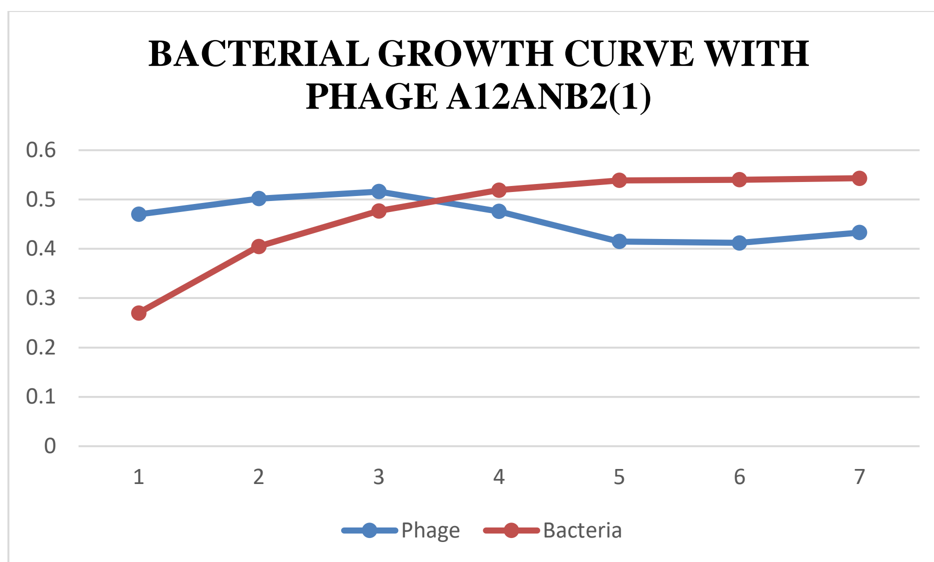


***Figure 4.13** (**a**). **Bacterial Growth Curve with phage:** This graphs represents the growth of bacteria with phage and its comparison when no phage lysate was added. The red line represents the bacterial growth of A12AnB2(1) without phage lysate. However, blue line represents when phage lysate was added to bacterial culture, it growth declines significantly. The OD of the bacterial growth was taken at 600nm.*

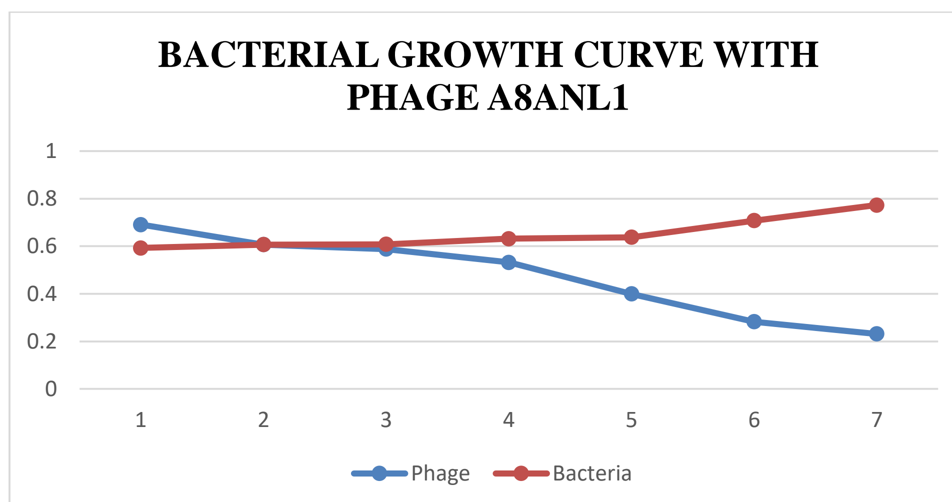


***Figure 4.13** (**b**). **Bacterial Growth Curve with phage:** This graphs represents the growth of bacteria with phage and its comparison when no phage lysate was added. The red line represents the bacterial growth A8ANL1 without phage lysate. However, blue line represents when phage lysate was added to bacterial culture, it growth declines significantly. The OD of the bacterial growth was taken at 600nm.*

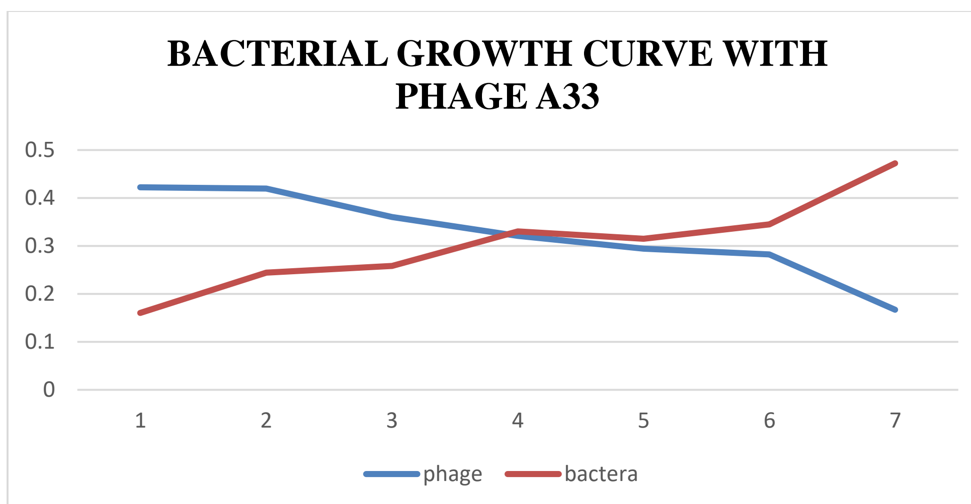


Figure 4.13 (c). Bacterial Growth Curve with phage: *This graphs represents the growth of bacteria with phage and its comparison when no phage lysate was added. The red line represents the bacterial growth A33 without phage lysate. However, blue line represents when phage lysate was added in bacterial culture, its growth declines significantly. The OD of the bacterial growth was taken at 600nm.*

4.7 CALCULATION OF BACTERIOPHAGE TITER (pfu/ml)

Plaque forming unit (pfu) of phages was determined by agar overlay method. This method was done by making serial dilution of phage lysates up to 10^{-20}. Ten dilutions were used i.e.10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10}, further dilutions showed no plaque assay count. These dilutions were made by diluting the phages sample with $MgSO_4$ enriched with LB broth. The diluted phage lysates were plated after mixing with the log phase of bacterial cultures and incubation of 15 minutes at 37ºC on shaking incubator. After the incubation time period of 24h at 37ºC the plaque formation was checked and plaques were counted by using the formula:

$$\frac{\text{Average no. of plaques}}{\text{Dilution factor x Volume}} = \text{PFU/ml}$$

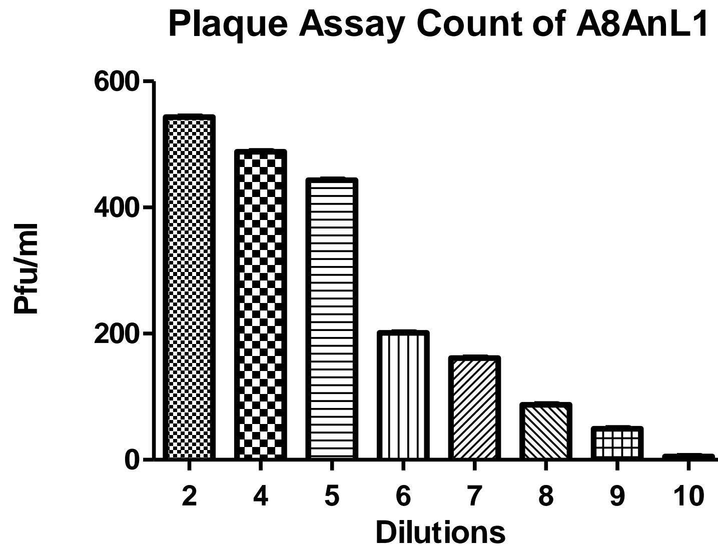


Figure 4.14(a): ***Calculation of Phage Titer (pfu/ml):*** *The graph shows the plaque forming unit (pfu) of the isolated phage. As the dilution factor was increased a decrease in the pfu/ml can be observed.*

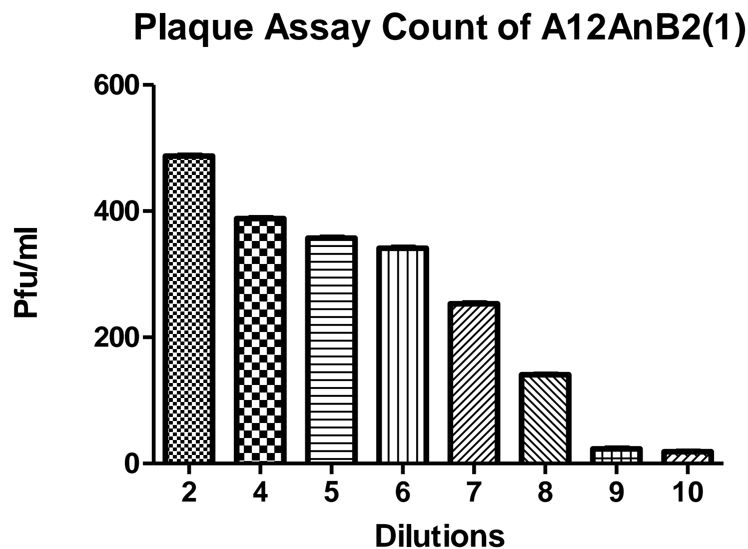


Figure 4.14(b): ***Calculation of Phage Titer (pfu/ml):*** *The graph shows the pfu of the isolated phage. As the dilution factor was increased a decrease in the pfu/ml can be observed.*

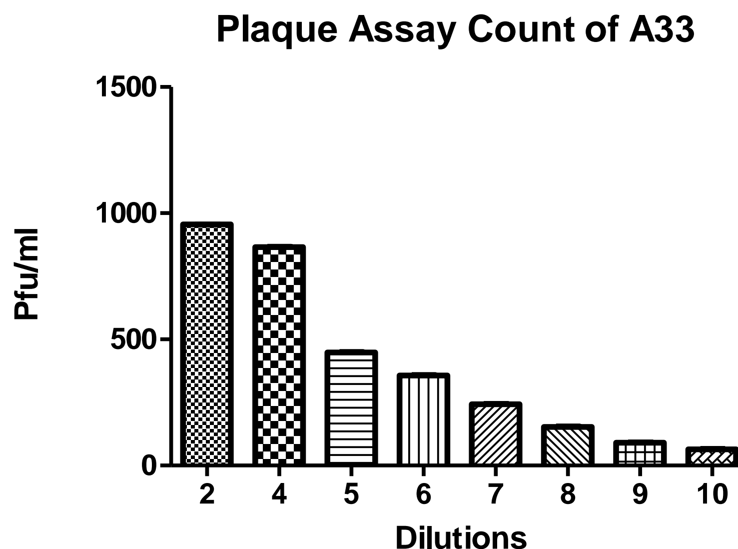


Figure 4.14(c): ***Calculation of Phage Titer (pfu/ml):*** *The graph shows the pfu of the isolated phage. As the dilution factor was increased a decrease in the pfu/ml can be observed.*

4.8 ANALYSIS OF BACTERIOPHAGE ADSORPTION:

The phages adsorption was determined by using calcium and magnesium ion adsorption effect. The bacterial isolates were incubated with phages in a 15mM of $CaCl_2$ and $MgSO_4$ and incubated for 24h at 37ºC after plating by agar overlay method. The pfu/ml was calculated after the incubation time period.

4.9. THERMAL SENSITIVITY OF PHAGES:

The lytic activity of phages was determined at different temperatures to evaluate optical temperature for phage activity. The phages activity was observed at 37ºC, 40ºC, 45ºC, 50ºC, 55ºC, 60ºC, 65ºC, 70ºC and 80ºC temperatures, after incubating the phage lysates for 1h at these temperatures. The spot test method was used to observe the lytic activity of phages against A8AnL1, A12AnB2(1) and A33 bacterial strains. The phage against A8AnL1 bacterial strain shows maximum activity 40ºC and minimum at 65ºC. The strain A12AnB2(1) showed maximum activity at 60ºC and zero lytic activity was observed on 70ºC and 80ºC. The third phage against A33 shows maximum lytic activity at 65ºC and minimum at 40ºC. The figure 4. 15 (a), (b) and (c) are described below.

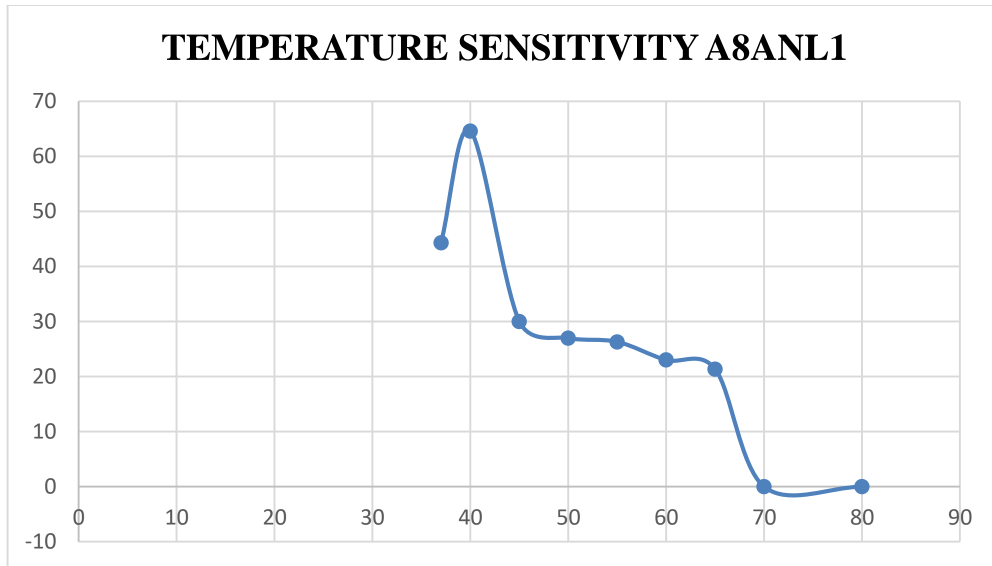


Figure 4.15(a). Temperature Sensitivity of Phage: *This graph represents the phage activity against A8ANL1 bacterial strain after treating it at various temperatures of 37ºC to 80ºC. Maximum activity was seen at 40ºC while minimum at 65ºC and no activity was seen at 70ºC and 80ºC.*

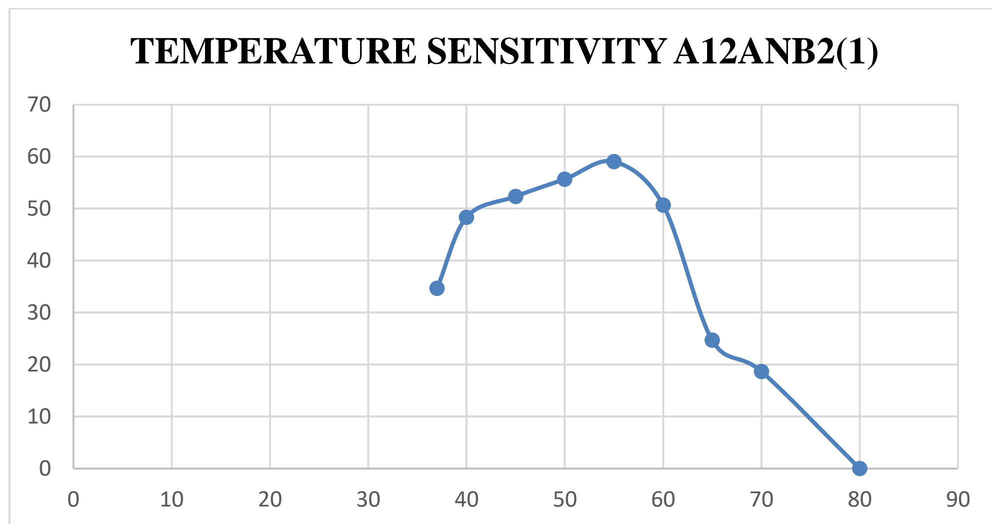


Figure 4.15(b). Temperature Sensitivity of Phage: *This graph represents the phage activity against A12ANB2(1) bacterial strain after treating it at different temperatures from 37ºC to 80ºC. The maximum activity of phage was seen at 55ºC while minimum at 70ºC and no lytic activity was observed at 80ºC.*

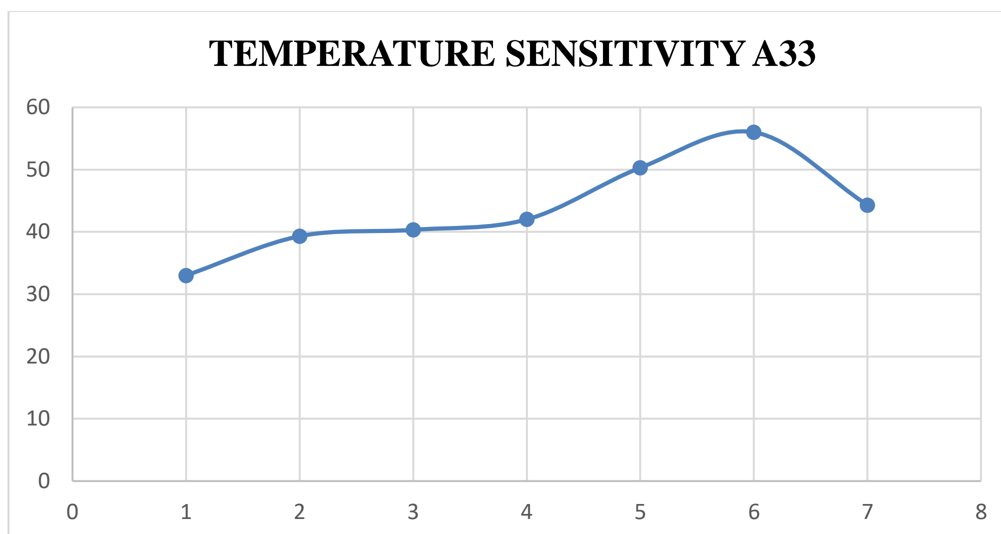


Figure 4.15(c). Temperature Sensitivity of Phage: *This graph represents the phage activity against A33 Bacterial strain at a temperature range of 37⁰C to 80⁰C. The maximum activity of phage was observed at 65⁰C while minimum was seen at 40⁰C.*

4.10. pH SENSITIVITY OF PHAGES:

The lytic activity of phages was determined at an optimal pH. The phage lysates were incubated for 24hr at 37⁰C with pH 1, 3, 5, 7, 9, 11. After incubation, the lytic activity of phages at each pH was determined by spot test technique against bacterial strains A8AnL1, A12AnB2(1) and A33. The lysates showed maximum activity at a pH 9 and minimum at pH 1 against A8AnL1 The maximum activity of phage A12AnB2(1) was shown at pH 7 and minimum at 5. The phage of strain A33 shows maximum at pH 7 and minimum at basic pH 11. The figure 4.16 (a), (b) and (c) illustrates the pH sensitivity.

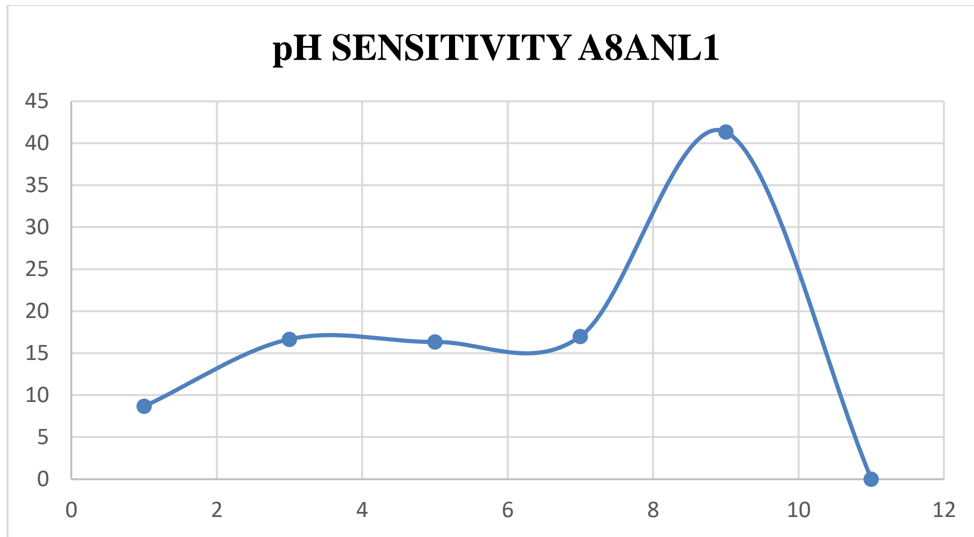


Figure 4.16(a). pH Sensitivity of Phage: *This figure illustrates the lytic activity of phage A8ANL1 at different pH i.e., 1, 3, 5, 7, 9, 11. The maximum activity was seen at pH 9 and the minimum activity at pH 1. While no activity was showed by the phage at pH 11.*

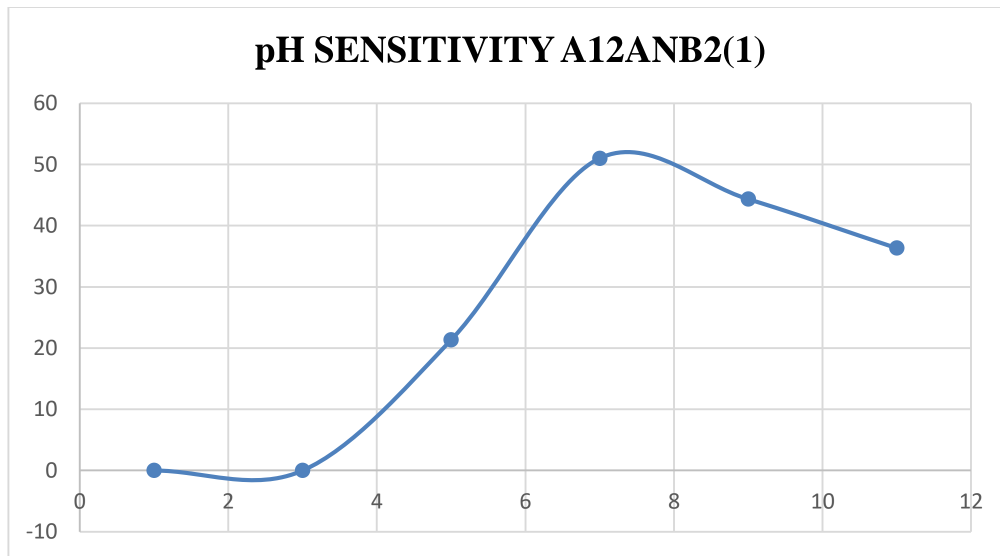


Figure 4.16(b). pH Sensitivity of Phage: *This figure demonstrates the lytic activity of phage A12ANB2(1) at different pH i.e., 1, 3, 5, 7, 9, 11. The maximum activity was seen at neutral pH 7 and the minimum activity at pH 5. The figure depicts the phage A12ANB2(1) showed no activity in acidic conditions.*

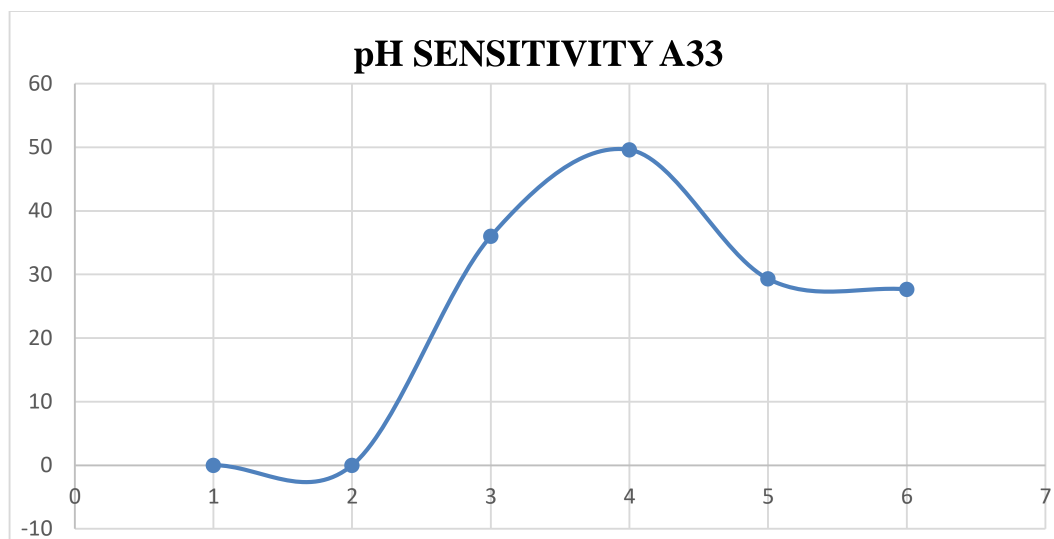


Figure 4.16(c). pH Sensitivity of Phage: *This figure demonstrates the lytic activity of phage A33 at different pH i.e., 1, 3, 5, 7, 9, 11. The maximum activity was seen at pH 7 and the minimum activity at pH 11. No activity was seen in highly acidic conditions.*

4.11 MOLECULAR IDENTIFICATION OF BACTERIA:

16S rRNA sequencing was done on 03 bacterial strains against which the phages showed lytic activity. Three samples were sent for sequencing to Macrogen, Korea. NCBI BLASTn was used for the analysis of the results of sequencing. The result of bacterial strain A12AnB2(1) showed similar homology with *Enterococcus feacalis* strain Cp1, bacterial strain A8AnL1 showed homology with *Shigella flexneri* strain T87 and Strain A33 homology matched with *Enterococcus sp*. QAUEF03.

4.12: MORPHOLOGICAL IDENTIFICATION OF PHAGES:

SEM was used for the morphological characterization of phages. Three samples were prepared each containing its own respective phage (Figure 4.17 a, b, c and d)**.** The samples were diluted to a concentration of 10^{-7} before adding on a coverslip in a LB Broth by serial dilution method. The samples were gold spluttered before viewed under the EM with the magnification of 20,000 to 50,000 at 20kV. The samples were prepared in Virology Lab II and processed in Material Lab in Institute of Space and Technology (IST), Islamabad.

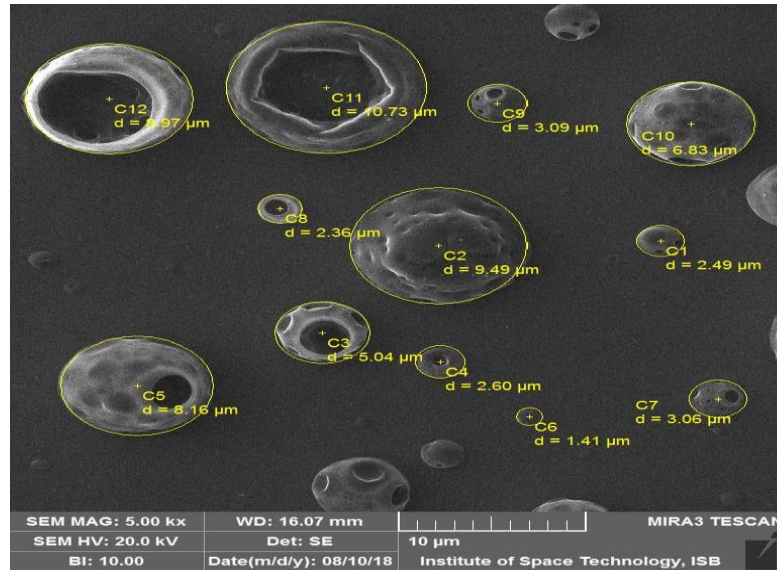


Figure 4.17 (a) SEM Analysis of phage lysate A8AnL1: *The SEM image shows the circular phage morphology of phage in the lysate. The sample was seen at 20kV at a working distance of 16.07mm (out of beam). The magnification was kept 10 micronmeter.*

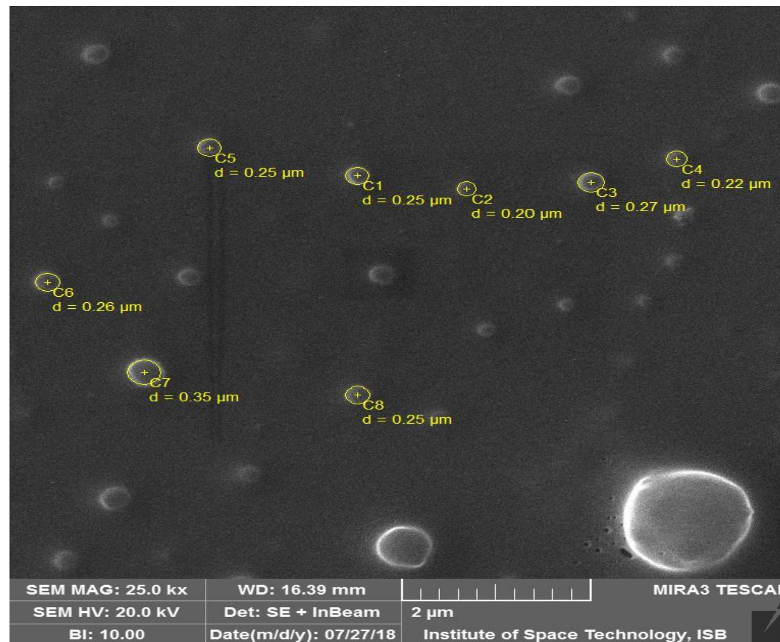


Figure 4.17 (b) SEM Analysis of phage lysate A12AnB2(1): *The SEM image shows the circular phage morphology of phage in the lysate. The sample was seen at 20kV at a working distance of 16.39mm (out of beam). The magnification was kept 2 micronmeter.*

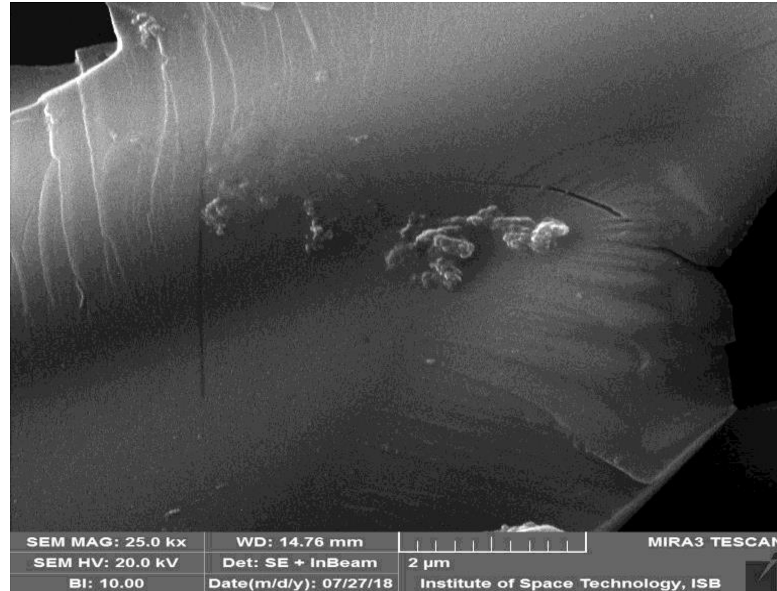


Figure 4.17 (c) Analysis of SEM phage adsorbed on bacterial cell: *The figure showed the lytic activity of the phage absorbed on bacterial cell wall. The deformation of the bacterial cell was seen in image. The sample was observed at 20kV at a working distance of 14.66mm (out of beam). The magnification was kept in 2 micronmeter.*

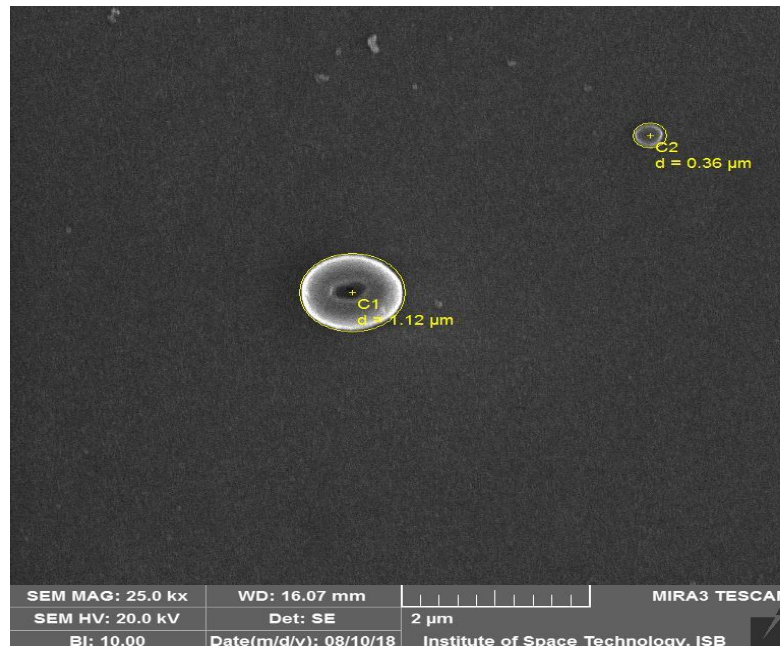


Figure 4.17 (d) SEM Analysis of phage lysate A33: *The SEM image shows the circular phage morphology of phage in the lysate. The sample was seen at 20kV at a working distance of 16.07mm (out of beam). The magnification was kept 2 micronmeter.*

4.13: GENOME ISOLATION:

The genome of all three phages were extracted to determine the family of phages. For the isolation of genome, organic extraction method was used. The 1.5% gel was used for the visualization of band and samples were run at 80V for 45 minutes with 1 kb ladder. The amplicons showed the size ranges in between 15-20kbp. The samples were treated separately with DNase I (1μ/μl) and RNase H (5μ/μl) to determine the nature of genome; whether the genome is DNA or RNA. The samples treated with DNase I showed no bands while samples treated with RNase H showed intact bands.

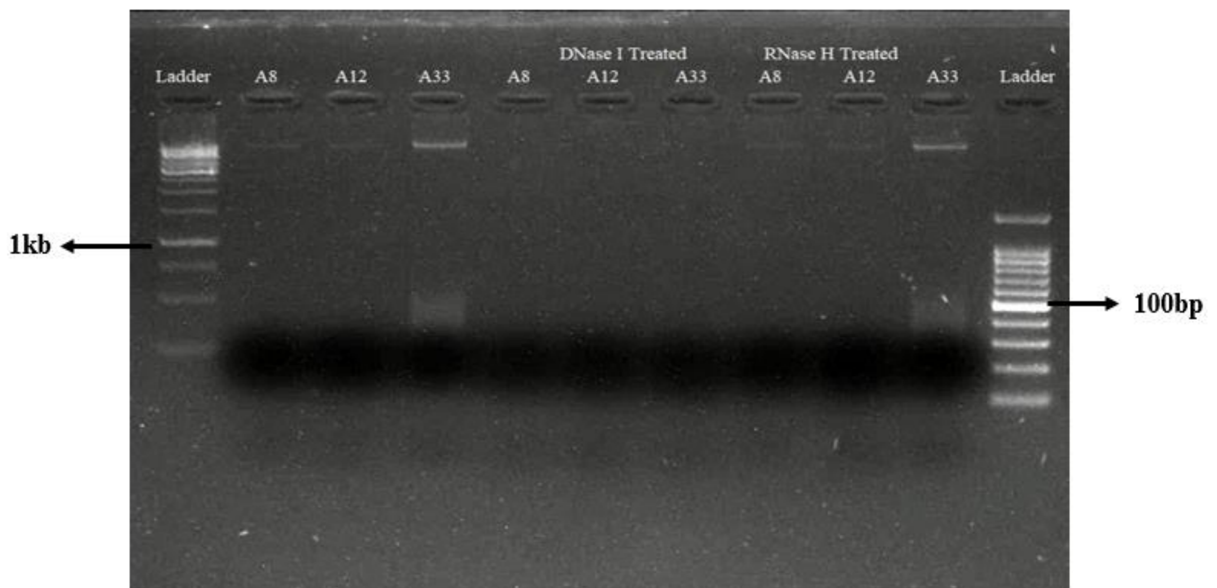


Figure 4.18: Genome isolation of phages: *This picture depicts the genome size of phage; it shows that three of the phages have genome size around 15-20kb while when treated in DNase I all of the bands degrade but when treated with RNase H none of the band disappear. It demonstrates that phages have DNA genome.*

Chapter 5:

Discussion

The healthy vaginal microflora protects the females' lower genital tract from infectious agents. Any commotion which shift the balance of vaginal microflora from lactobacilli to facultative anaerobes or the absence of lactobacilli is termed as Bacterial Vaginosis (BV) (Hillier, Krohn, Rabe, Klebanoff, & Eschenbach, 1993). BV is the most commonly reported infection and is responsible for the one-third of the vulvo-vaginal infections (Schwebke, 2009). Worldwide, the reported BV incidence is about 21.2 million. In Pakistan, the BV prevalence rate is 35.3%. BV is characterized by the abnormal vaginal discharge, itching, foul smell and pain in the pelvic region (Bradshaw & Sobel, 2016). BV infected females are more prone to preterm births, pelvic inflammatory disease and other chronic health issues. The BV disease itself is a silent infection but reported to develop sexually transmitted diseases (STDs) and HIV in infected females (Bautista et al., 2016). Similarly, other studies stated BV relation with the development of Herpes Simplex Virus 2 (HSV-2) infections and cervical cancer (Cherpes, Meyn, Krohn, Lurie, & Hillier, 2003).

The present study was conducted within the twin cities (Rawalpindi and Islamabad) of Pakistan, to isolate and determine the causative agents of BV and the comparison of BV microflora among pregnant and non-pregnant females. The samples were collected from KRL, Hospital, Islamabad, Pakistan. Total 60 samples were collected from pregnant and non-pregnant females infected with BV using high vaginal swabs (HVS). The 30 samples were collected from pregnant females while 30 samples were taken from non-pregnant females.

The bacterial isolates from BV infected samples were collected. The gram staining microscopic analysis revealed highest prevalence of *Staphylococcus* species (69%) followed by *Streptococcus* (46%)*, Enterococcus* (20%)*, Candida* (17%), *lactobacilli* (16%)*, G. vaginalis (10%), Neisseria* (2%)*, and Shigella* (2%) in both pregnant and non-pregnant females. In pregnant females (9%) and in non-pregnant females (4%) of the healthy lactobacilli were observed, while the remaining microflora were belonged to infectious agents of BV. This observation can be justify by the fact that the reduction of lactobacilli and the accumulation of pathogenic microbes are majorly responsible for BV (Africa, Nel, & Stemmet, 2014).

According to the literature the *Staphylococcus and Streptococcus* spp. are commensal organisms and makeup the natural flora of epithelium cells of human body. Under favorable conditions *Staphylococcus* and *Streptococcus* spp. surpass their healthy number and start acting like a pathogen (Cogen, Nizet, & Gallo, 2008). This fact further strengthens our data about the highest prevalence of *Staphylococcus* (69%) and *Streptococcus* (46%) spp. in the BV infected pregnant and non-pregnant females. Ample data suggest that BV is related with STDs and they share the common pathogenic microflora. *Neisseria* spp. is one of the reported STD (Shim, 2011). Current study results are in agreement with the presence of *Neisseria* spp. but its prevalence is not large enough to report a case of STD.

Antibiotics are used to treat the bacterial infections since ages (Bud, 2007). With the passage of time, bacteria evolved themselves and developed resistance against these wonder drugs (Ventola, 2015). Data of present study strongly support the fact of antibiotic resistance, the isolated bacterial strains of BV microflora of pregnant and non-pregnant females were resistant to Metronidazole and Clindamycin. The increase in the resistance against these antimicrobial has put the researchers and medical professionals into a difficult situation and urge them to find the alternative method to tackle the issue of resistance.

Globally, bacteriophage therapy is considered as a potential alternative treatment against infectious bacterial agents. This therapy is continuously providing evidence as a better treatment option as compared to antibiotics (Loc-Carrillo & Abedon, 2011). In bacteriophage therapy, lytic phages are more preferably and effectively used. Phages are host specific and only kill the selected host cells by misbalancing its cell metabolism, upsetting and rupturing of the host cell (Khalifa et al., 2016). Phage therapy effectiveness provides the idea of using bacteriophages to kill the causative agents of BV.

Phages are abundantly present in all environments in which living organisms can survive (Clokie, Millard, Letarov, & Heaphy, 2011). Sewage water samples are considered to be the richest source of bacteriophages (Tazzyman & Hall, 2015). In current study, the sewage water samples were collected from the slum areas of Islamabad, Pakistan for the isolation of phages against the BV isolated microflora.

Present study identifies the 03 phages; had shown promising lytic activity against the 03 different isolates of bacteria. The 16S rRNA sequencing showed their homology

with *Enterococcus feacalis, Enterococcus faecium* and *Shigella flexneri.* Isolation of phages from sewage water samples can be validated by the fact that *Enterobacteriaceae* family is widely present in the sewage water bodies (Gilmore, Lebreton, & van Schaik, 2013). The lytic activity of phages was observed against other isolated bacterial strains of BV, but our phages were strictly specific to their host cells. The lytic activity of phages was also observed against the bacterial strains other than BV microflora, but these phages showed their strict host specificity against the isolated strains.

Enterococcus spp. are widely associated with urinary tract infections (UTIs) (Habash & Reid, 1999; Jahić, Nurkić, & Fatusić, 2006; Sharami, Afrakhteh, & Shakiba, 2007). The evidence verified with the fact that BV infection increases the vaginal pH and provides the environment for the growth of uropathogens in vagina (Sumati & Saritha, 2009; Whiteside, Razvi, Dave, Reid, & Burton, 2015). *Enterococcus* is widely known for their ability to survive in extremely harsh conditions such as higher alkalinity (6.5% NaCl) or in glucose starvation. (Liu, Wei, Ling, Wang, & Huang, 2010; Saleh, Ruyter, Haapasalo, & Ørstavik, 2004). It is also reported that 75% of BV patients are also suffered from *Enterococcus* spp. infected UTIs (Amatya, Bhattarai, Mandal, Tuladhar, & Karki, 2013; Castro, Machado, & Cerca, 2016). The data presented here strongly support our finding of *Enterococcus* strains in BV infection among pregnant and non-pregnant females.

The other isolated phage in current study was against *Shigella flexneri* strain. They are enteric gram negative facultative rods. It is the major cause of bacilliary dysentery and shigellosis (Philpott, Edgeworth, & Sansonetti, 2000). It is transmitted through oral-fecal route, by come in contact with someone, ingestion of polluted food and water, travelling and man who have sex with man (MSM) (Puzari, Sharma, & Chetia, 2017).

Shigella is highly toxic, even the consumption of 10-100 organisms may cause the Shigellosis. Shigellosis is a watery diarrhea to dysentery (Philpott et al., 2000). S. *flexneri* is responsible for bloody diarrhea and bloody discharge from vagina in vulvovaginitis infection (Bayramoğlu, Aydın, Karagüzel, İmamoğlu, & Ökten, 2012). This fact is strongly in agreement with the idea of BV microflora containing *Shigella* strains as during sampling many females complained about inflammation and bloody discharged through vagina in extreme cases.

S. flexneri detection in laboratory is not easy because identification of gram negative rods from vaginal cultures is not an easy task. For the identification of *Shigella spp.*, the specific media are required for their growth such as selenite broth (SB), Salmonella Shigella (SS) agar and eosin-methylene blue (EMB). These media are not generally used for the identification of BV or other vaginal infection in hospitals which is the main reason why *Shigella* spp. is not significantly reported in vaginal infections (Öner, Özer, Turan, & Şakru, 2005). The other major reason for its least detection is that, *Shigella spp.* can only live for a few time period outside the human body and require intensive protection for its collection and transportation (Niyogi, 2005). This provides a strong agreement for current study in which a single strain of *S. flexneri* was observed.

S. flexneri is the major STD causing specie among all *Shigella spp. (Surawicz, 2007).* It is reported in homosexual and bisexuals individual who are involved in unprotected anal sex (Narayan, Galanis, & Group, 2016). Shigellosis increases the chances of the HIV infection in bisexual males or in their partners (Cresswell et al., 2015). The present study suggests that the BV increases the chances of STDs due to the involvement of sexually transmitted microbes in BV infection.

S. flexneri is resistant to sulphonamides, tetracycline, chloramphenicol, asciprofloxacin, amoxicillin, ampicillin and co-trimoxazole (Puzari et al., 2017). According to a molecular study of Faisalabad, Pakistan, *S. flexneri* is the frequently present as resistant infection in that region (Tariq et al., 2012). The current study provides an evidence of *S. flexneri* resistance against metronidazole and clindamycin.

Further studies were performed to evaluate the lytic activity of phages. The isolated phages against *Enterococcus* and *Shigella* strains showed their maximum lytic activity at pH 7 and 9 respectively. The maximum activity of phages at neutral or alkaline conditions strongly encourages the use of phages against *Enterococcus* and *S. flexneri* strains in treating BV infection. Other than BV infection, phage against *S. flexneri* can be used to treat diarrhea and Shigellosis in patients and *Enterococcus* phage in UTIs infections.

The isolated phages were also evaluated on the basis of their thermal activity on a wide range of temperature 37-80ºC. In current study, isolated phages showed their highest activity at 40ºC and 55ºC. This can be defined as the phages are not thermophiles and in

infected patients they can be used to destruct bacteria on normal body temperature at their maximum lytic activity.

Conclusion:

Current study provides the characterization and comparison of BV microflora among pregnant and non-pregnant females. The study also improves our understanding towards pathogens involved in BV infection. This attempt not only focuses on the microflora role in BV infection but also explicate the other better treatment options by identifying the pathogens. The isolation and biolytic activity analysis of phages against the causative agents of BV provides a promising alternative pathway to explore the potential therapeutic applications of isolated phages. The interactions between the BV microflora and phages in BV is still need to be explored. Phage therapy is giving us an approach to kill targeted bacterial species to treat infections without harming the natural flora of human body.

Future Prospective:

The following recommendation are proposed to be followed:

- The isolated phages can be used for the synthetic phage therapy as phagmids, for the better understanding of phages for treatment.
- The isolation of new phages for the other causative agents of BV can be done as an alternative treatment method.
- The phages can be used to check the host specificity for other pathogenic strains for better treatment of infections.
- Phages can be evaluated in cell lines as well as on animal models to elucidate safety in human infections.

REFERENCES:

Abbaspoor, Z., Rabee, Z., & Najjar, S. (2014). Efficacy and safety of oral tinidazole and metronidazole in treatment of bacterial vaginosis: a randomized control trial. *Pharmacol. Res, 4*, 78-83.

Abdullateef, R. M., Ijaiya, M. A., Abayomi, F., Adeniran, A. S., & Idris, H. (2017). Bacterial vaginosis: Prevalence and associated risk factors among non-pregnant women of reproductive age attending a Nigerian tertiary hospital. *Malawi Medical Journal, 29*(4), 290-293.

Africa, C. W., Nel, J., & Stemmet, M. (2014). Anaerobes and bacterial vaginosis in pregnancy: virulence factors contributing to vaginal colonisation. *International journal of environmental research and public health, 11*(7), 6979-7000.

Akinbiyi, A., Watson, R., & Feyi-Waboso, P. (2008). Prevalence of Candida albicans and bacterial vaginosis in asymptomatic pregnant women in South Yorkshire, United Kingdom. *Archives of gynecology and obstetrics, 278*(5), 463-466.

Aldunate, M., Srbinovski, D., Hearps, A. C., Latham, C. F., Ramsland, P. A., Gugasyan, R., . . . Tachedjian, G. (2015). Antimicrobial and immune modulatory effects of lactic acid and short chain fatty acids produced by vaginal microbiota associated with eubiosis and bacterial vaginosis. *Frontiers in physiology, 6*, 164.

Alisky, J., Iczkowski, K., Rapoport, A., & Troitsky, N. (1998). Bacteriophages show promise as antimicrobial agents. *Journal of Infection, 36*(1), 5-15.

Amatya, R., Bhattarai, S., Mandal, P., Tuladhar, H., & Karki, B. (2013). Urinary tract infection in vaginitis: a condition often overlooked. *Nepal Med Coll J, 15*(1), 65-67.

Amsel, R., Totten, P. A., Spiegel, C. A., Chen, K. C., Eschenbach, D., & Holmes, K. K. (1983). Nonspecific vaginitis: diagnostic criteria and microbial and epidemiologic associations. *The American journal of medicine, 74*(1), 14-22.

Andersen, P., Bucher, A., Saeed, I., Lee, P., Davis, J., & Maibach, H. (1994). Faecal enzymes: in vivo human skin irritation. *Contact dermatitis, 30*(3), 152-158.

Armstrong, N. R., & Wilson, J. D. (2009). Tinidazole in the treatment of bacterial vaginosis. *International journal of women's health, 1*, 59.

Aroutcheva, A., Gariti, D., Simon, M., Shott, S., Faro, J., Simoes, J. A., . . . Faro, S. (2001). Defense factors of vaginal lactobacilli. *American Journal of Obstetrics & Gynecology, 185*(2), 375-379.

Atashili, J., Poole, C., Ndumbe, P. M., Adimora, A. A., & Smith, J. S. (2008). Bacterial vaginosis and HIV acquisition: a meta-analysis of published studies. *AIDS (London, England), 22*(12), 1493.

Athanasiou, S., Pitsouni, E., Antonopoulou, S., Zacharakis, D., Salvatore, S., Falagas, M., & Grigoriadis, T. (2016). The effect of microablative fractional CO2 laser on vaginal flora of postmenopausal women. *Climacteric, 19*(5), 512-518.

Austin, M., Beigi, R., Meyn, L., & Hillier, S. (2005). Microbiologic response to treatment of bacterial vaginosis with topical clindamycin or metronidazole. *Journal of clinical microbiology, 43*(9), 4492-4497.

Balashov, S. V., Mordechai, E., Adelson, M. E., & Gygax, S. E. (2014). Identification, quantification and subtyping of Gardnerella vaginalis in noncultured clinical vaginal samples by quantitative PCR. *Journal of medical microbiology, 63*(2), 162-175.

Barry, M. A., Dower, W. J., & Johnston, S. A. (1996). Toward cell–targeting gene therapy vectors: Selection of cell–binding peptides from random peptide–presenting phage libraries. *Nature medicine, 2*(3), 299.

Bautista, C. T., Wurapa, E., Sateren, W. B., Morris, S., Hollingsworth, B., & Sanchez, J. L. (2016). Bacterial vaginosis: a synthesis of the literature on etiology, prevalence, risk factors, and relationship with chlamydia and gonorrhea infections. *Military Medical Research, 3*(1), 4.

Bayramoğlu, G., Aydın, F., Karagüzel, G., İmamoğlu, M., & Ökten, A. (2012). Two Cases of Vulvovaginitis Caused by Shigella flexneri and Shigella sonnei: a Case Report. *Balkan Medical Journal, 2012*(1).

Beverly, E. S., Chen, H. Y., Wang, Q. J., Zariffard, M. R., Cohen, M. H., & Spear, G. T. (2005). Utility of Amsel criteria, Nugent score, and quantitative PCR for Gardnerella vaginalis, Mycoplasma hominis, and Lactobacillus spp. for diagnosis of bacterial vaginosis in human immunodeficiency virus-infected women. *Journal of clinical microbiology, 43*(9), 4607-4612.

Bhat, G., Kotigadde, S., & Shenoy, S. (2011). Comparison of the methods of diagnosis of bacterial vaginosis. *Journal of Clinical and Diagnostic Research, 5*(3), 498-501.

Bhatia, J., & Cleland, J. (2000). Methodological Issues in Community-based Studies of Gynecological Morbidity. *Studies in family planning, 31*(4), 267-273.

Biswas, B., Adhya, S., Washart, P., Paul, B., Trostel, A. N., Powell, B., . . . Merril, C. R. (2002). Bacteriophage therapy rescues mice bacteremic from a clinical isolate of vancomycin-resistant Enterococcus faecium. *Infection and immunity, 70*(1), 204-210.

Bitew, A., Abebaw, Y., Bekele, D., & Mihret, A. (2017). Prevalence of bacterial vaginosis and associated risk factors among women complaining of genital tract infection. *International journal of microbiology, 2017*.

Borgdorff, H., Van Der Veer, C., Van Houdt, R., Alberts, C., De Vries, H., Bruisten, S., . . . van der Loeff, M. S. (2015). P06. 01 Women of dutch ethnic origin have lower prevalence of vaginal microbiome dysbiosis than women of other ethnic origin residing in amsterdam: BMJ Publishing Group Ltd.

Boskey, E., Cone, R., Whaley, K., & Moench, T. (2001). Origins of vaginal acidity: high D/L lactate ratio is consistent with bacteria being the primary source. *Human Reproduction, 16*(9), 1809-1813.

Bostwick, D. G., Woody, J., Hunt, C., & Budd, W. (2016). Antimicrobial resistance genes and modelling of treatment failure in bacterial vaginosis: clinical study of 289 symptomatic women. *Journal of medical microbiology, 65*(5), 377-386.

Bradshaw, C. S., Morton, A. N., Hocking, J., Garland, S. M., Morris, M. B., Moss, L. M., . . . Fairley, C. K. (2006). High recurrence rates of bacterial vaginosis over the course of 12 months after oral metronidazole therapy and factors associated with recurrence. *The Journal of infectious diseases, 193*(11), 1478-1486.

Bradshaw, C. S., Pirotta, M., De Guingand, D., Hocking, J. S., Morton, A. N., Garland, S. M., . . . Vodstrcil, L. A. (2012). Efficacy of oral metronidazole with vaginal clindamycin or vaginal probiotic for bacterial vaginosis: randomised placebo-controlled double-blind trial. *PLoS One, 7*(4), e34540.

Bradshaw, C. S., & Sobel, J. D. (2016). Current treatment of bacterial vaginosis—limitations and need for innovation. *The Journal of infectious diseases, 214*(suppl_1), S14-S20.

Brotman, R. M., Bradford, L. L., Conrad, M., Gajer, P., Ault, K., Peralta, L., . . . Ravel, J. (2012). Association between Trichomonas vaginalis and vaginal bacterial community composition among reproductive-age women. *Sexually transmitted diseases, 39*(10), 807.

Bud, R. (2007). Antibiotics: the epitome of a wonder drug. *Bmj, 334*(suppl 1), s6-s6.

Capra, M., Quiberoni, A. d. L., Ackermann, H.-W., Moineau, S., & Reinheimer, J. (2006). Characterization of a new virulent phage (MLC-A) of Lactobacillus paracasei. *Journal of dairy science, 89*(7), 2414-2423.

Carlton, R., Noordman, W., Biswas, B., De Meester, E., & Loessner, M. J. (2005). Bacteriophage P100 for control of Listeria monocytogenes in foods: genome sequence, bioinformatic analyses, oral toxicity study, and application. *Regulatory toxicology and Pharmacology, 43*(3), 301-312.

Carmody, L. A., Gill, J. J., Summer, E. J., Sajjan, U. S., Gonzalez, C. F., Young, R. F., & LiPuma, J. J. (2010). Efficacy of bacteriophage therapy in a model of Burkholderia cenocepacia pulmonary infection. *The Journal of infectious diseases, 201*(2), 264-271.

Carrillo, C. L., Atterbury, R. J., El-Shibiny, A., Connerton, P. L., Dillon, E., Scott, A., & Connerton, I. F. (2005). Bacteriophage therapy to reduce Campylobacter jejuni colonization of broiler chickens. *Applied and environmental microbiology, 71*(11), 6554-6563.

Castro, J., Machado, D., & Cerca, N. (2016). Escherichia coli and Enterococcus faecalis are able to incorporate and enhance a pre-formed Gardnerella vaginalis biofilm. *Pathogens and disease, 74*(3).

Cherpes, T. L., Hillier, S. L., Meyn, L. A., Busch, J. L., & Krohn, M. A. (2008). A delicate balance: risk factors for acquisition of bacterial vaginosis include sexual activity, absence of hydrogen peroxide-producing lactobacilli, black race, and positive herpes simplex virus type 2 serology. *Sexually transmitted diseases, 35*(1), 78-83.

Cherpes, T. L., Melan, M. A., Kant, J. A., Cosentino, L. A., Meyn, L. A., & Hillier, S. L. (2005). Genital tract shedding of herpes simplex virus type 2 in women: effects of hormonal contraception, bacterial vaginosis, and vaginal group B Streptococcus colonization. *Clinical Infectious Diseases, 40*(10), 1422-1428.

Cherpes, T. L., Meyn, L. A., Krohn, M. A., Lurie, J. G., & Hillier, S. L. (2003). Association between acquisition of herpes simplex virus type 2 in women and bacterial vaginosis. *Clinical Infectious Diseases, 37*(3), 319-325.

Chhibber, S., Kaur, S., & Kumari, S. (2008). Therapeutic potential of bacteriophage in treating Klebsiella pneumoniae B5055-mediated lobar pneumonia in mice. *Journal of medical microbiology, 57*(12), 1508-1513.

Clark, J. R., & March, J. B. (2006). Bacteriophages and biotechnology: vaccines, gene therapy and antibacterials. *Trends in biotechnology, 24*(5), 212-218.

Clokie, M. R., Millard, A. D., Letarov, A. V., & Heaphy, S. (2011). Phages in nature. *Bacteriophage, 1*(1), 31-45.

Cogen, A., Nizet, V., & Gallo, R. (2008). Skin microbiota: a source of disease or defence? *British Journal of Dermatology, 158*(3), 442-455.

Cohen, C. R., Lingappa, J. R., Baeten, J. M., Ngayo, M. O., Spiegel, C. A., Hong, T., . . . Delany, S. (2012). Bacterial vaginosis associated with increased risk of female-to-male HIV-1 transmission: a prospective cohort analysis among African couples. *PLoS medicine, 9*(6), e1001251.

Cohn, J. A., Hashemi, F. B., Camarca, M., Kong, F., Xu, J., Beckner, S. K., . . . Spear, G. T. (2005). HIV-inducing factor in cervicovaginal secretions is associated with bacterial vaginosis in HIV-1–infected women. *Journal of acquired immune deficiency syndromes (1999), 39*(3), 340.

Coleman, J. S., Hitti, J., Bukusi, E. A., Mwachari, C., Muliro, A., Nguti, R., . . . Lockhart, D. (2007). Infectious correlates of HIV-1 shedding in the female upper and lower genital tracts. *Aids, 21*(6), 755-759.

Cresswell, F. V., Ross, S., Booth, T., Pinto-Sander, N., Alexander, E., Bradley, J., . . . Richardson, D. (2015). Shigella flexneri: a cause of significant morbidity and associated with sexually transmitted infections in men who have sex with men. *Sexually transmitted diseases, 42*(6), 344.

d'Hérelle, F. (2007). On an invisible microbe antagonistic toward dysenteric bacilli: brief note by Mr. F. D'Herelle, presented by Mr. Roux. 1917. *Research in microbiology, 158*(7), 553.

Danielsson, D., Teigen, P. K., & Moi, H. (2011). The genital econiche: focus on microbiota and bacterial vaginosis. *Annals of the New York Academy of Sciences, 1230*(1), 48-58.

Darwish, A. M., Makarem, M. H., Alnashar, E. M., & Hamadeh, S. M. (2005). Screening for bacterial vaginosis in high-risk pregnancy: the experience of a developing country. *Acta obstetricia et gynecologica Scandinavica, 84*(5), 483-485.

Doerflinger, S. Y., Throop, A. L., & Herbst-Kralovetz, M. M. (2014). Bacteria in the vaginal microbiome alter the innate immune response and barrier properties of the human vaginal epithelia in a species-specific manner. *The Journal of infectious diseases, 209*(12), 1989-1999.

Domingo-Calap, P., Georgel, P., & Bahram, S. (2016). Back to the future: bacteriophages as promising therapeutic tools. *Hla, 87*(3), 133-140.

Donders, G. G., Zodzika, J., & Rezeberga, D. (2014). Treatment of bacterial vaginosis: what we have and what we miss. *Expert opinion on pharmacotherapy, 15*(5), 645-657.

Doolittle, M., Cooney, J., & Caldwell, D. (1995). Lytic infection of Escherichia coli biofilms by bacteriophage T4. *Canadian journal of microbiology, 41*(1), 12-18.

Durr-e-Nayab. (2005). Reproductive tract infections among women in Pakistan: an urban case study. *The Pakistan Development Review*, 131-158.

Durugbo, I. I., Nyengidiki, T. K., Bassey, G., & Wariso, K. T. (2015). Bacterial vaginosis among women with tubal factor infertility in Nigeria. *International Journal of Gynecology & Obstetrics, 131*(2), 133-136.

Elbreki, M., Ross, R. P., Hill, C., O'Mahony, J., McAuliffe, O., & Coffey, A. (2014). Bacteriophages and their derivatives as biotherapeutic agents in disease prevention and treatment. *Journal of Viruses, 2014*.

Ewert, D. L., & Paynter, M. (1980). Enumeration of bacteriophages and host bacteria in sewage and the activated-sludge treatment process. *Applied and environmental microbiology, 39*(3), 576-583.

Falagas, M., Betsi, G., & Athanasiou, S. (2007). Probiotics for the treatment of women with bacterial vaginosis. *Clinical microbiology and infection, 13*(7), 657-664.

Fallah, M., Rabiee, S., & Moshtaghi, A. A. (2007). Comparison between efficacy of single dose of tinidazole with a 7-day standard dose course of metronidazole in giardiasis. *Pakistan Journal of Medical Sciences, 23*(1), 43.

Fenton, M., McAuliffe, O., O'Mahony, J., & Coffey, A. (2010). Recombinant bacteriophage lysins as antibacterials. *Bioengineered bugs, 1*(1), 9-16.

Fethers, K. A., Fairley, C. K., Hocking, J. S., Gurrin, L. C., & Bradshaw, C. S. (2008). Sexual risk factors and bacterial vaginosis: a systematic review and meta-analysis. *Clinical Infectious Diseases, 47*(11), 1426-1435.

Fettweis, J. M., Serrano, M. G., Girerd, P. H., Jefferson, K. K., & Buck, G. A. (2012). A new era of the vaginal microbiome: advances using next-generation sequencing. *Chemistry & biodiversity, 9*(5), 965-976.

Fischbach, F., Petersen, E. E., Weissenbacher, E. R., Martius, J., Hosmann, J., & Mayer, H. (1993). Efficacy of clindamycin vaginal cream versus oral metronidazole in the treatment of bacterial vaginosis. *Obstetrics and gynecology, 82*(3), 405-410.

Gabisoniya, T., Loladze, M. Z., Nadiradze, M., Chakhunashvili, N., Alibegashvili, M., Tamarashvili, N., & Pushkina, V. (2016). Effects of bacteriophages on biofilm formation by strains of Pseudomonas aeruginosa. *Applied biochemistry and microbiology, 52*(3), 293-297.

Gallo, M. F., Macaluso, M., Warner, L., Fleenor, M. E., Hook, E. W., Brill, I., & Weaver, M. A. (2012). Bacterial vaginosis, gonorrhea, and chlamydial infection among women attending a sexually transmitted disease clinic: a longitudinal analysis of possible causal links. *Annals of epidemiology, 22*(3), 213-220.

Garcia, P., Rodriguez, L., Rodriguez, A., & Martinez, B. (2010). Food biopreservation: promising strategies using bacteriocins, bacteriophages and endolysins. *Trends in Food Science & Technology, 21*(8), 373-382.

Gardner, H. L., & Dukes, C. D. (1954). New etiologic agent in nonspecific bacterial vaginitis. *Science (New York, NY), 120*(3125), 853.

Gardner, H. L., & Dukes, C. D. (1955). Haemophilus vaginalis vaginitis: a newly defined specific infection previously classified “nonspecific” vaginitis. *American Journal of Obstetrics & Gynecology, 69*(5), 962-976.

Gergova, R. T., Strateva, T. V., & Mitov, I. G. (2013). Gardnerella vaginalis-associated bacterial vaginosis in Bulgarian women. *The Brazilian Journal of infectious diseases, 17*(3), 313-318.

Ghiasi, M., Fazaeli, H., Kalhor, N., Sheykh-Hasan, M., & Tabatabaei-Qomi, R. (2014). Assessing the prevalence of bacterial vaginosis among infertile women of Qom city. *Iranian journal of microbiology, 6*(6), 404.

Gillet, E., Meys, J. F., Verstraelen, H., Bosire, C., De Sutter, P., Temmerman, M., & Broeck, D. V. (2011). Bacterial vaginosis is associated with uterine cervical human papillomavirus infection: a meta-analysis. *BMC infectious diseases, 11*(1), 10.

Gilmore, M. S., Lebreton, F., & van Schaik, W. (2013). Genomic transition of enterococci from gut commensals to leading causes of multidrug-resistant hospital infection in the antibiotic era. *Current opinion in microbiology, 16*(1), 10-16.

Gong, Y., Trowbridge, R., Macnaughton, T. B., Westaway, E. G., Shannon, A. D., & Gowans, E. J. (1996). Characterization of RNA synthesis during a one-step growth curve and of the replication mechanism of bovine viral diarrhoea virus. *Journal of General Virology, 77*(11), 2729-2736.

Gong, Z., Luna, Y., Yu, P., & Fan, H. (2014). Lactobacilli inactivate Chlamydia trachomatis through lactic acid but not H2O2. *PloS one, 9*(9), e107758.

Gray, R. H., Kigozi, G., Serwadda, D., Makumbi, F., Nalugoda, F., Watya, S., . . . Kiwanuka, N. (2009). The effects of male circumcision on female partners' genital tract symptoms and vaginal infections in a randomized trial in Rakai, Uganda. *American Journal of Obstetrics & Gynecology, 200*(1), 42. e41-42. e47.

Greenwood, J., & Pickett, M. (1980). Transfer of Haemophilus vaginalis Gardner and Dukes to a New Genus, Gardnerella: G. vaginalis (Gardner and Dukes) comb. nov. *International Journal of Systematic and Evolutionary Microbiology, 30*(1), 170-178.

Greer, G. G., & Dilts, B. D. (2002). Control of Brochothrix thermosphacta spoilage of pork adipose tissue using bacteriophages. *Journal of food protection, 65*(5), 861-863.

Habash, M., & Reid, G. (1999). Microbial biofilms: their development and significance for medical device—related infections. *The Journal of Clinical Pharmacology, 39*(9), 887-898.

Habib, A., & Siddiqui, I. (2016). FREQUENCY OF BACTERIAL VAGINOSIS IN PATIENTS WITH VAGINAL DISCHARGE. *Khyber Medical University Journal, 8*(1).

Hagens, S., & Loessner, M. J. (2007). Application of bacteriophages for detection and control of foodborne pathogens. *Applied microbiology and biotechnology, 76*(3), 513-519.

Haq, I. U., Chaudhry, W. N., Andleeb, S., & Qadri, I. (2012). Isolation and partial characterization of a virulent bacteriophage IHQ1 specific for Aeromonas punctata from stream water. *Microbial ecology, 63*(4), 954-963.

Harper, D. R., Parracho, H. M., Walker, J., Sharp, R., Hughes, G., Werthén, M., . . . Morales, S. (2014). Bacteriophages and biofilms. *Antibiotics, 3*(3), 270-284.

Hathroubi, S., Mekni, M. A., Domenico, P., Nguyen, D., & Jacques, M. (2017). Biofilms: microbial shelters against antibiotics. *Microbial Drug Resistance, 23*(2), 147-156.

Hay, P. (2009). Recurrent bacterial vaginosis. *Current Opinion in infectious diseases, 22*(1), 82-86.

Hickey, R. J., Zhou, X., Pierson, J. D., Ravel, J., & Forney, L. J. (2012). Understanding vaginal microbiome complexity from an ecological perspective. *Translational Research, 160*(4), 267-282.

Hillier, S. L. (2005). The complexity of microbial diversity in bacterial vaginosis. *New England Journal of Medicine, 353*(18), 1886-1887.

Hillier, S. L., Krohn, M. A., Rabe, L. K., Klebanoff, S. J., & Eschenbach, D. A. (1993). The normal vaginal flora, H2O2-producing lactobacilli, and bacterial vaginosis in pregnant women. *Clinical Infectious Diseases, 16*(Supplement_4), S273-S281.

Homayouni, A., Bastani, P., Ziyadi, S., Mohammad-Alizadeh-Charandabi, S., Ghalibaf, M., Mortazavian, A. M., & Mehrabany, E. V. (2014). Effects of probiotics on the recurrence of bacterial vaginosis: a review. *Journal of lower genital tract disease, 18*(1), 79-86.

Irving, M. B., Pan, O., & Scott, J. K. (2001). Random-peptide libraries and antigen-fragment libraries for epitope mapping and the development of vaccines and diagnostics. *Current opinion in chemical biology, 5*(3), 314-324.

Islam, A., Safdar, A., & Malik, A. (2009). Bacterial vaginosis. *JPMA, 59*(9), 601.

Jahić, M., Nurkić, M., & Fatusić, Z. (2006). Association of the pH change of vaginal environment in bacterial vaginosis with presence of Enterococcus faecalis in vagina. *Medicinski arhiv, 60*(6), 364-368.

Jin, J., Li, Z.-J., Wang, S.-W., Wang, S.-M., Huang, D.-H., Li, Y.-H., . . . Chen, X.-D. (2012). Isolation and characterization of ZZ1, a novel lytic phage that infects Acinetobacter baumannii clinical isolates. *BMC microbiology, 12*(1), 156.

Kelly, D., McAuliffe, O., Ross, R., & Coffey, A. (2012). Prevention of Staphylococcus aureus biofilm formation and reduction in established biofilm density using a combination of phage K and modified derivatives. *Letters in applied microbiology, 54*(4), 286-291.

Kenyon, C., Colebunders, R., & Crucitti, T. (2013a). The global epidemiology of bacterial vaginosis: a systematic review. *American Journal of Obstetrics & Gynecology, 209*(6), 505-523.

Kenyon, C., Colebunders, R., & Crucitti, T. (2013b). The global epidemiology of bacterial vaginosis: a systematic review. *American journal of obstetrics and gynecology, 209*(6), 505-523.

Kenyon, C. R., & Osbak, K. (2014). Recent progress in understanding the epidemiology of bacterial vaginosis. *Current Opinion in Obstetrics and Gynecology, 26*(6), 448-454.

Keshavarz, H., Duffy, S., Sadeghi-Hassanabadi, A., Zolghadr, Z., & Oboodi, B. (2001). Risk factors for and relationship between bacterial vaginosis and cervicitis in a high risk population for cervicitis in Southern Iran. *European journal of epidemiology, 17*(1), 89-95.

Khalifa, L., Shlezinger, M., Beyth, S., Houri-Haddad, Y., Coppenhagen-Glazer, S., Beyth, N., & Hazan, R. (2016). Phage therapy against Enterococcus faecalis in dental root canals. *Journal of oral microbiology, 8*(1), 32157.

Koumans, E. H., Sternberg, M., Bruce, C., McQuillan, G., Kendrick, J., Sutton, M., & Markowitz, L. E. (2007). The prevalence of bacterial vaginosis in the United States, 2001–2004; associations with symptoms, sexual behaviors, and reproductive health. *Sexually transmitted diseases, 34*(11), 864-869.

Krauss-Silva, L., Almada-Horta, A., Alves, M. B., Camacho, K. G., Moreira, M. E. L., & Braga, A. (2014). Basic vaginal pH, bacterial vaginosis and aerobic vaginitis: prevalence in early pregnancy and risk of spontaneous preterm delivery, a prospective study in a low socioeconomic and multiethnic South American population. *BMC pregnancy and childbirth, 14*(1), 107.

Kumar, N., Behera, B., Sagiri, S. S., Pal, K., Ray, S. S., & Roy, S. (2011). Bacterial vaginosis: Etiology and modalities of treatment—A brief note. *Journal of pharmacy & bioallied sciences, 3*(4), 496.

Kutateladze, M., & Adamia, R. (2010). Bacteriophages as potential new therapeutics to replace or supplement antibiotics. *Trends in biotechnology, 28*(12), 591-595.

Kutter, E., & Sulakvelidze, A. (2004). *Bacteriophages: biology and applications*: CRC Press.

Lacroix-Gueu, P., Briandet, R., Lévêque-Fort, S., Bellon-Fontaine, M.-N., & Fontaine-Aupart, M.-P. (2005). In situ measurements of viral particles diffusion inside mucoid biofilms. *Comptes rendus biologies, 328*(12), 1065-1072.

Lallar, M., Nanda, S., & Nandal, R. (2015). Lower Genital Tract Infections in HIV-Infected Women: Can We Afford to Miss? *The Journal of Obstetrics and Gynecology of India, 65*(1), 45-49.

Larocca, D., Kassner, P. D., Witte, A., Ladner, R. C., Pierce, G. F., & Baird, A. (1999). Gene transfer to mammalian cells using genetically targeted filamentous bacteriophage. *The FASEB journal, 13*(6), 727-734.

Lepargneur, J., & Rousseau, V. (2002). Protective role of the Doderlein flora. *Journal de gynecologie, obstetrique et biologie de la reproduction, 31*(5), 485-494.

Liu, H., Wei, X., Ling, J., Wang, W., & Huang, X. (2010). Biofilm formation capability of Enterococcus faecalis cells in starvation phase and its susceptibility to sodium hypochlorite. *Journal of endodontics, 36*(4), 630-635.

Loc-Carrillo, C., & Abedon, S. T. (2011). Pros and cons of phage therapy. *Bacteriophage, 1*(2), 111-114.

Lu, T. K., & Koeris, M. S. (2011). The next generation of bacteriophage therapy. *Current opinion in microbiology, 14*(5), 524-531.

Machado, A. n., Salgueiro, D., Harwich, M., Jefferson, K. K., & Cerca, N. (2013). Quantitative analysis of initial adhesion of bacterial vaginosis-associated anaerobes to ME-180 cells. *Anaerobe, 23*, 1-4.

Machado, D., Castro, J., Palmeira-de-Oliveira, A., Martinez-de-Oliveira, J., & Cerca, N. (2016). Bacterial vaginosis biofilms: challenges to current therapies and emerging solutions. *Frontiers in microbiology, 6*, 1528.

Marrazzo, J. M., Thomas, K. K., Fiedler, T. L., Ringwood, K., & Fredricks, D. N. (2008). Relationship of specific vaginal bacteria and bacterial vaginosis treatment failure in women who have sex with women. *Annals of internal medicine, 149*(1), 20-28.

Mastromarino, P., Brigidi, P., Macchia, S., Maggi, L., Pirovano, F., Trinchieri, V., . . . Matteuzzi, D. (2002). Characterization and selection of vaginal Lactobacillus strains for the preparation of vaginal tablets. *Journal of Applied Microbiology, 93*(5), 884-893.

Matinkhoo, S., Lynch, K. H., Dennis, J. J., Finlay, W. H., & Vehring, R. (2011). Spray-dried respirable powders containing bacteriophages for the treatment of pulmonary infections. *Journal of pharmaceutical sciences, 100*(12), 5197-5205.

Mead, P. B. (1993). Epidemiology of bacterial vaginosis. *American journal of obstetrics and gynecology, 169*(2), 446-449.

Mitchell, C., Balkus, J. E., Fredricks, D., Liu, C., McKernan-Mullin, J., Frenkel, L. M., . . . Cohen, C. R. (2013). Interaction between lactobacilli, bacterial vaginosis-associated bacteria, and HIV Type 1 RNA and DNA Genital shedding in US and Kenyan women. *AIDS research and human retroviruses, 29*(1), 13-19.

Mitchell, C. M., Hitti, J. E., Agnew, K. J., & Fredricks, D. N. (2009). Comparison of oral and vaginal metronidazole for treatment of bacterial vaginosis in pregnancy: impact on fastidious bacteria. *BMC infectious diseases, 9*(1), 89.

Modi, R., Hirvi, Y., Hill, A., & Griffiths, M. (2001). Effect of phage on survival of Salmonella enteritidis during manufacture and storage of cheddar cheese made from raw and pasteurized milk. *Journal of food protection, 64*(7), 927-933.

Monk, A., Rees, C., Barrow, P., Hagens, S., & Harper, D. (2010). Bacteriophage applications: where are we now? *Letters in applied microbiology, 51*(4), 363-369.

Muzny, C. A., Sunesara, I. R., Griswold, M. E., Kumar, R., Lefkowitz, E. J., Mena, L. A., . . . Swiatlo, E. (2014). Association between BVAB1 and high Nugent scores among women with bacterial vaginosis. *Diagnostic microbiology and infectious disease, 80*(4), 321-323.

Nagaraja, P. (2008). Antibiotic resistance of Gardnerella vaginalis in recurrent bacterial vaginosis. *Indian Journal of Medical Microbiology, 26*(2), 155.

Nagot, N., Ouedraogo, A., Defer, M.-C., Vallo, R., Mayaud, P., & Van de Perre, P. (2007). Association between bacterial vaginosis and Herpes simplex virus type-2 infection: implications for HIV acquisition studies. *Sexually transmitted infections, 83*(5), 365-368.

Nakai, T., & Park, S. C. (2002). Bacteriophage therapy of infectious diseases in aquaculture. *Research in microbiology, 153*(1), 13-18.

Narayan, S., Galanis, E., & Group, B. S. (2016). Update on STIs: Are enteric infections sexually transmitted in British Columbia? *Canada Communicable Disease Report, 42*(2), 24.

Nayab, D.-e. (2005). Findings of the CBMS pilot study in Pakistan. *PEP working paper; 2005-08*.

Niyogi, S. K. (2005). Shigellosis. *The journal of microbiology, 43*(2), 133-143.

Nugent, R. P., Krohn, M. A., & Hillier, S. L. (1991). Reliability of diagnosing bacterial vaginosis is improved by a standardized method of gram stain interpretation. *Journal of clinical microbiology, 29*(2), 297-301.

O'Hanlon, D. E., Moench, T. R., & Cone, R. A. (2013). Vaginal pH and microbicidal lactic acid when lactobacilli dominate the microbiota. *PLoS One, 8*(11), e80074.

Obradovic, A., Jones, J., Momol, M., Balogh, B., & Olson, S. (2004). Management of tomato bacterial spot in the field by foliar applications of bacteriophages and SAR inducers. *Plant Disease, 88*(7), 736-740.

Olmsted, S. S., Meyn, L. A., Rohan, L. C., & Hillier, S. L. (2003). Glycosidase and proteinase activity of anaerobic gram-negative bacteria isolated from women with bacterial vaginosis. *Sexually transmitted diseases, 30*(3), 257-261.

Onderdonk, A. B., Delaney, M. L., & Fichorova, R. N. (2016). The human microbiome during bacterial vaginosis. *Clinical microbiology reviews, 29*(2), 223-238.

Öner, M., Özer, B., Turan, P., & Şakru, N. (2005). Dışkı kültürlerinde salmonella cinsi bakterilerin izolasyonunda uzamış inkübasyon süresinin etkisi. *Trakya Univ Tıp Fak Derg, 22*, 23-26.

Organization, W. H. (2008). Male circumcision: global trends and determinants of prevalence, safety and acceptability.

Örmälä, A.-M., & Jalasvuori, M. (2013). Phage therapy: should bacterial resistance to phages be a concern, even in the long run? *Bacteriophage, 3*(1), e24219.

Ouwehand, A. C., Salminen, S., & Isolauri, E. (2002). Probiotics: an overview of beneficial effects. *Antonie Van Leeuwenhoek, 82*(1-4), 279-289.

Parasion, S., Kwiatek, M., Gryko, R., Mizak, L., & Malm, A. (2014). Bacteriophages as an alternative strategy for fighting biofilm development. *Pol J Microbiol, 63*(2), 137-145.

Parisien, A., Allain, B., Zhang, J., Mandeville, R., & Lan, C. (2008). Novel alternatives to antibiotics: bacteriophages, bacterial cell wall hydrolases, and antimicrobial peptides. *Journal of Applied Microbiology, 104*(1), 1-13.

Patton, D. L., Thwin, S. S., Meier, A., Hooton, T. M., Stapleton, A. E., & Eschenbach, D. A. (2000). Epithelial cell layer thickness and immune cell populations in the normal human vagina at different stages of the menstrual cycle. *American Journal of Obstetrics & Gynecology, 183*(4), 967-973.

Petrina, M. A., Cosentino, L. A., Rabe, L. K., & Hillier, S. L. (2017). Susceptibility of bacterial vaginosis (BV)-associated bacteria to secnidazole compared to metronidazole, tinidazole and clindamycin. *Anaerobe, 47*, 115-119.

Philpott, D. J., Edgeworth, J. D., & Sansonetti, P. J. (2000). *The pathogenesis of Shigella flexneri infection: lessons from in vitro and in vivo studies.* Paper presented at the The Activities Of Bacterial Pathogens In Vivo: Based on Contributions to a Royal Society Discussion Meeting.

Puzari, M., Sharma, M., & Chetia, P. (2017). Emergence of antibiotic resistant Shigella species: A matter of concern. *Journal of infection and public health*.

Rao, S. R., Pindi, K. G., & Rani, U. (2016). Diagnosis of bacterial vaginosis: Amsel's criteria vs. Nugent's scoring. *Sch J Appl Med Sci, 4*(6C), 2027-2031.

Reid, G., Jass, J., Sebulsky, M. T., & McCormick, J. K. (2003). Potential uses of probiotics in clinical practice. *Clinical microbiology reviews, 16*(4), 658-672.

Rhoads, D., Wolcott, R., Kuskowski, M., Wolcott, B., Ward, L., & Sulakvelidze, A. (2009). Bacteriophage therapy of venous leg ulcers in humans: results of a phase I safety trial. *Journal of wound care, 18*(6), 237-243.

Saleh, I., Ruyter, I., Haapasalo, M., & Ørstavik, D. (2004). Survival of Enterococcus faecalis in infected dentinal tubules after root canal filling with different root canal sealers in vitro. *International Endodontic Journal, 37*(3), 193-198.

Sambrook, J., & Russel, D. Molecular cloning: A laboratory Manual. 3rd edn, 2001: cold spring harbor laboratory press, cold spring harbor, NY.

Schnabel, E. L., & Jones, A. L. (2001). Isolation and Characterization of FiveErwinia amylovora Bacteriophages and Assessment of Phage Resistance in Strains of Erwinia amylovora. *Applied and environmental microbiology, 67*(1), 59-64.

Schuch, R., Pelzek, A. J., Raz, A., Euler, C. W., Ryan, P. A., Winer, B. Y., . . . Xu, Y. (2013). Use of a bacteriophage lysin to identify a novel target for antimicrobial development. *PLoS One, 8*(4), e60754.

Schuyler, J. A., Mordechai, E., Adelson, M. E., Sobel, J. D., Gygax, S. E., & Hilbert, D. W. (2016). Identification of intrinsically metronidazole-resistant clades of Gardnerella vaginalis. *Diagnostic microbiology and infectious disease, 84*(1), 1-3.

Schwebke, J. R. (2009). New concepts in the etiology of bacterial vaginosis. *Current infectious disease reports, 11*(2), 143-147.

Schwebke, J. R., & Desmond, R. (2007). A randomized trial of metronidazole in asymptomatic bacterial vaginosis to prevent the acquisition of sexually transmitted diseases. *American Journal of Obstetrics & Gynecology, 196*(6), 517. e511-517. e516.

Schwebke, J. R., & Desmond, R. A. (2011). Tinidazole vs metronidazole for the treatment of bacterial vaginosis. *American Journal of Obstetrics & Gynecology, 204*(3), 211. e211-211. e216.

Schwebke, J. R., Hillier, S. L., Sobel, J. D., McGregor, J. A., & Sweet, R. L. (1996). Validity of the vaginal gram stain for the diagnosis of bacterial vaginosis. *Obstetrics & Gynecology, 88*(4), 573-576.

Seck, K., Samb, N., Tempesta, S., Mulanga-Kabeya, C., Henzel, D., Sow, P. S., . . . Delaporte, E. (2001). Prevalence and risk factors of cervicovaginal HIV shedding among HIV-1 and HIV-2 infected women in Dakar, Senegal. *Sexually transmitted infections, 77*(3), 190-193.

Senok, A. C., Verstraelen, H., Temmerman, M., & Botta, G. A. (2009). Probiotics for the treatment of bacterial vaginosis. *The Cochrane Library*.

Sharami, S., Afrakhteh, M., & Shakiba, M. (2007). Urinary tract infections in pregnant women with bacterial vaginosis. *Journal of obstetrics and gynaecology, 27*(3), 252-254.

Shim, B. S. (2011). Current concepts in bacterial sexually transmitted diseases. *Korean journal of urology, 52*(9), 589-597.

Shobeiri, F., & Nazari, M. (2006). A prospective study of genital infections in Hamedan, Iran. *Southeast Asian J Trop Med Public Health, 20*, 14-19.

Sieber, R., & Dietz, U.-T. (1998). Lactobacillus acidophilus and yogurt in the prevention and therapy of bacterial vaginosis. *International Dairy Journal, 8*(7), 599-607.

Skurnik, M., Pajunen, M., & Kiljunen, S. (2007). Biotechnological challenges of phage therapy. *Biotechnology letters, 29*(7), 995-1003.

Sloan, N. L., Winikoff, B., Haberland, N., Coggins, C., & Elias, C. (2000). Screening and syndromic approaches to identify gonorrhea and chlamydial infection among women. *Studies in family planning, 31*(1), 55-68.

Smith, H. W., & Huggins, M. (1982). Successful treatment of experimental Escherichia coli infections in mice using phage: its general superiority over antibiotics. *Microbiology, 128*(2), 307-318.

Soothill, J. (1992). Treatment of experimental infections of mice with bacteriophages. *Journal of medical microbiology, 37*(4), 258-261.

Srinivasan, S., Liu, C., Mitchell, C. M., Fiedler, T. L., Thomas, K. K., Agnew, K. J., . . . Fredricks, D. N. (2010). Temporal variability of human vaginal bacteria and relationship with bacterial vaginosis. *PloS one, 5*(4), e10197.

Stanek, R., Glover, D. D., Larsen, B., & Gain, R. E. (1992). High performance ion exclusion chromatographic characterization of the vaginal organic acids in women with bacterial vaginosis. *Biomedical Chromatography, 6*(5), 231-235.

Sumati, A., & Saritha, N. (2009). Bacterial vaginosis with special reference to anaerobes. *Indian Journal of Pathology and Microbiology, 52*(1), 56.

Surawicz, C. M. (2007). Shigella: A Sexually Transmitted Infection in Men Who Have Sex With Men. *Gastroenterology, 133*(5), 1737-1738.

Swidsinski, A., Dörffel, Y., Loening-Baucke, V., Schilling, J., & Mendling, W. (2011). Response of Gardnerella vaginalis biofilm to 5 days of moxifloxacin treatment. *FEMS Immunology & Medical Microbiology, 61*(1), 41-46.

Tariq, A., Haque, A., Ali, A., Bashir, S., Habeeb, M. A., Salman, M., & Sarwar, Y. (2012). Molecular profiling of antimicrobial resistance and integron association of multidrug-resistant clinical isolates of Shigella species from Faisalabad, Pakistan. *Canadian journal of microbiology, 58*(9), 1047-1054.

Tazzyman, S. J., & Hall, A. R. (2015). Lytic phages obscure the cost of antibiotic resistance in Escherichia coli. *The ISME journal, 9*(4), 809.

Thursby, E., & Juge, N. (2017). Introduction to the human gut microbiota. *Biochemical Journal, 474*(11), 1823-1836.

Tolosa, J. E., Chaithongwongwatthana, S., Daly, S., Maw, W. W., Gaitán, H., Lumbiganon, P., . . . Goldenberg, R. L. (2006). The International Infections in Pregnancy (IIP) study: variations in the prevalence of bacterial vaginosis and distribution of morphotypes in vaginal smears among pregnant women. *American Journal of Obstetrics & Gynecology, 195*(5), 1198-1204.

Ventola, C. L. (2015). The antibiotic resistance crisis: part 1: causes and threats. *Pharmacy and Therapeutics, 40*(4), 277.

Verbeken, G., Huys, I., Pirnay, J.-P., Jennes, S., Chanishvili, N., Scheres, J., . . . Ceulemans, C. (2014). Taking bacteriophage therapy seriously: a moral argument. *BioMed research international, 2014*.

Verstraelen, H., & Verhelst, R. (2009). Bacterial vaginosis: an update on diagnosis and treatment. *Expert review of anti-infective therapy, 7*(9), 1109-1124.

Vodstrcil, L. A., Walker, S. M., Hocking, J. S., Law, M., Forcey, D. S., Fehler, G., . . . Fairley, C. K. (2014). Incident bacterial vaginosis (BV) in women who have sex with women is associated with behaviors that suggest sexual transmission of BV. *Clinical Infectious Diseases, 60*(7), 1042-1053.

Whiteside, S. A., Razvi, H., Dave, S., Reid, G., & Burton, J. P. (2015). The microbiome of the urinary tract—a role beyond infection. *Nature Reviews Urology, 12*(2), 81.

Wiesenfeld, H. C., Hillier, S. L., Krohn, M. A., Landers, D. V., & Sweet, R. L. (2003). Bacterial vaginosis is a strong predictor of Neisseria gonorrhoeae and Chlamydia trachomatis infection. *Clinical Infectious Diseases, 36*(5), 663-668.

Wills, Q. F., Kerrigan, C., & Soothill, J. S. (2005). Experimental bacteriophage protection against Staphylococcus aureus abscesses in a rabbit model. *Antimicrobial agents and chemotherapy, 49*(3), 1220-1221.

Workowski, K. A., & Bolan, G. A. (2015). Sexually transmitted diseases treatment guidelines, 2015. *MMWR. Recommendations and reports: Morbidity and mortality weekly report. Recommendations and reports, 64*(RR-03), 1.

Wright, A., Hawkins, C., Änggård, E., & Harper, D. (2009). A controlled clinical trial of a therapeutic bacteriophage preparation in chronic otitis due to antibiotic-resistant Pseudomonas aeruginosa; a preliminary report of efficacy. *Clinical otolaryngology, 34*(4), 349-357.

Yen, S., Shafer, M.-A., Moncada, J., Campbell, C. J., Flinn, S. D., & Boyer, C. B. (2003). Bacterial vaginosis in sexually experienced and non–sexually experienced young women entering the military. *Obstetrics & Gynecology, 102*(5), 927-933.

Yeoman, C. J., Thomas, S. M., Miller, M. E. B., Ulanov, A. V., Torralba, M., Lucas, S., . . . Ho, M. (2013). A multi-omic systems-based approach reveals metabolic markers of bacterial vaginosis and insight into the disease. *PLoS One, 8*(2), e56111.

Yudin, M. H., & Money, D. M. (2017). No. 211-Screening and Management of Bacterial Vaginosis in Pregnancy. *Journal of Obstetrics and Gynaecology Canada, 39*(8), e184-e191.

APPENDIX-A

PATIENT CONSENT FORM

The study is intended for the analysis of causative agents of bacterial vaginosis through collection of vaginal swabs for the purpose of clinical research. It is ensured that the samples collected will be used solely for research purpose and nothing else.

Are you willing to participate in this research study? Yes/ No

Name of attendee: ______________________________

General information:

Age: ____________ Gender: ____________ Phone: ____________

City: ____________ Weight: ____________ Diagnosis: ____________

Sample ID: ____________

Symptoms:

____

____

____

____

Any therapy:

____

____

____

____

Information required:

Comorbidities: ______________________

Pregnancy: ______________________

Allergies: ______________________

Recurrent infection: ______________________

Financial Status: ______________________

Research Scholar

Ayesha Javed

MS Scholar

ASAB, NUST

APPENDIX B

Table 3.1: Recipe table of Media.

The table below shows the ingredients used for the preparation of different media used in the study.

Sr. No	MEDIA	INGREDIENTS	g/ml
1.	Luria-Bertaini (LB) Agar	Distilled water	100ml
		Yeast Extract	0.5g
		Tryptone	1g
		Agar	1,5g
		NaCl	1g
2.	Blood Agar	Distilled water	71.4ml
		Blood Base Agar	2.5 g
		Blood	5ml
3.	MRS Agar	Distilled water	400ml
		MRS Base Agar	11g
4.	Soft Agar (LB)	Distilled water	100ml
		Agar	0.5g
		Tryptone	1g
		NaCl	1g
		Yeast Extract	0.5g

Table 3.2: Gram Staining Solutions:

Sr No.	READY MADE SOLUTIONS
1.	Crystal Violet
2.	Iodine
3.	Absolute Ethanol
4.	Safranin

Table 3.3: Catalase Test Reagents (0.3% H_2O_2 Solution):

Sr. No.	COMPONENTS	µl/1ml
1.	H_2O_2	300µl
2.	Distilled water	Up to 1ml

Table 3.4: Recipe table of Broths:

Sr No.	BROTHS	INGREDIENTS	g/ml
1.	Luria-Bertaini (LB) Broth	Distilled water	100ml
		NaCl	1g
		Tryptone	1g
		Yeast Extract	0.5g
2.	Luria-Bertaini (LB) Broth enriched with MgSO4	Distilled water	100ml
		NaCl	1g
		Tryptone	1g
		Yeast Extract	0.5g
		$MgSO_4$	0.12g

Table 3.5: Chloroform (1%):

Sr. No.	COMPONENTS	ml/ml
1.	Chloroform	1ml
2.	LB Broth	100ml

Table 3.6: Glycerol Stocks:

Sr. No.	COMPONENTS	ml/ml

1.	Glycerol	50ml
2.	LB Broth	50ml