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Isolation and Molecular Characterization of Bacterial Vaginosis and Human Papilloma Virus among Saudi Females Under Reproductive Age in Al-Madinah Al-Monawara Region, KSA

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صدق الله العظيم

Dedication

This work is dedicated to

my father who taught me the meaning of life.

To my mother who taught me the meaning sacrifice..

to my husband and my sons whom taught me the meaning of love.

to my brothers and sisters and to my friends.

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ABSTRACT

Bacterial vaginosis (BV) is an infection that occurs when the balance of bacteria in the vagina is altered. It is a common condition affecting millions of women. Although the syndrome is curable, relapse rates are high. The infection can have gynaecological and obstetric complications. Hence BV may has a role in cervical cancer. On the other hand, different theories suggested the infection with papilloma virus, especially genotypes that belong to high-risk human papilloma virus (HR-HPV) group to be one of the most important causes of uterine and cervical malignancy and premalignancy lesions in human.

Two groups of Saudi females were included in this study. Group A include one hundred and nineteen (n= 119) Saudi females under reproductive age attending Maternity and Children Hospital (MCH) , at Almadenah Almonawarh from April 2016 to May 2017. High vaginal swap (HVS) was taken for culture, biochemical tests and PCR. On the other hand, two hundred an d thirty eight (n=238) cervical scrapings in LBC (liquid based cytology) were collected in group B, processed for PCR targeting HPV as well as BV and cytological examination.

For the first part of the study in which it was aimed to determine the frequency of *G. vaginalis* among group A. The results confirm the existence of vaginal infection in 63.8% of the study population as

follows: *G. vaginalis* in (20/76) 26.3%;(7/46) 15.3% were pregnant while (13/30) 43.3% were non-pregnant. *streptococcus* spp. was reported in (27.6%), *Candida* spp. were in (46.1%). *G. vaginalis* were found to be more frequent in age group (20-29) followed by age group (30-39) and (less than 20 and more than 40years).

The result of PCR in this group confirmed the presence of *G. vaginalis* among enrolled subjects with a ratio (20/119) 17% from the study subjects. Regarding group (B) population, PCR was performed to examine the presence of HR-HPV different genotypes as well as bacterial agents (*G. vagialis, P. lacrimalis* and *L.iners*) .

Low incidence of precancerous epithelial lesions was observed among enrolled patients 13 (5.5%) with the following distribution: high-grade squamous intraepithelial lesion in 5 cases (38.5%), low-grade squamous intraepithelial lesion in 4 (30.8%) and four cases (30.8%) as a typical squamous cells of un determined significance. Nevertheless, HR-HPV was detected only in 6 (2.5%) cases and overall prevalence of HR-HPV in abnormal Pap smears was (2/13)15.4%. On the other hand, HPV was also detect in (4/238) 1.9% among smears that were registered free from any type of malignancy (NILM).

On the other hand, only 170 samples in LBC were available to detect bacterial agent by PCR (the rest was lost during transportation). The

results showed (21/170) 12.4% of the study population were suffered from BV. Low incidence of *G.vaginalis* and *P. lacrimalis* population was observed (2.3%) for each pathogen. While *L. iners* was identified among 7.6% among the study. Co-infection with HPV was detected in two patients one of them was HPV type 52 and other was type 58 .Both of them elicited with no epithelial changes.

On the other hand two of six positive HPV have epithelial change but there is no bacterial vaginosis. The study concluded very low prevalence of HR-HPV in routine cervical screening samples among suspected Saudi women, weak relation between HPV and the incidence of cervical neoplasia was also observed. Additionally there is no association between BV and HPV in uterine cervical neoplasia .

Low incidence of bacterial vaginosis in group B (21/170) 12.4% This is due to indirect place for bacteria (cervical) not original site for this bacteria).This study may direct the clinicians and researchers to look for other suspected HPV genotypes in this regards and the high occurrence of BV among Saudi females.

ملخص الدراسة

التهاب المهبل الجرثومي عبارة عن خمج يحدث عندما يتغير توازن البكتيريا في المهبل وهو حالة شائعة تصيب ملايين النساء وعلى الرغم من أن المتلازمة قابلة للشفاء ، إلا أن معدلات الانتكاس عالية. يؤدي التهاب المهبل الجرثومي الى مضاعفات امراض النساء والولادة. لذلك قد يكون لالتهاب المهبل الجرثومي دور في سرطان عنق الرحم. من ناحية أخرى ، أشارت نظريات مختلفة إلى أن العدوى بفيروس الورم الحليمي البشري، خاصة الأنماط الوراثية التي تنتمي إلى مجموعة فيروس الورم الحليمي البشري عالية الخطورة هي واحدة من أهم أسباب الأورام الخبيثة في الرحم وعنق الرحم وعقيرات ماقبل الاورام.

في هذه الدراسة تم إدراج مجموعتين من النساء السعوديات. المجموعة (أ) تشمل مائة وتسعة عشر (ن = 119) نساء سعوديات في عمر الإنجاب حضرن لمستشفى صحة الأمومه والطفولة ، في المدينة المنورة في أبريل 2016 إلى مايو 2017. تم اخذ مسحة من اعلى المهبل للتزريع والاختبارات الكيمو حيوية وتفاعل البلمرة المتسلسل الزمني. من ناحية أخرى ، تم جمع مائتين وثمانية وثلاثين (ن = 238) مسحة من عنق الرحم (في السائل الخلوي) في المجموعة (ب) لمعالجتها عن طريق تفاعل البلمرة المتسلسل الزمني الذي يستهدف فيروس الورم الحليمي البشري وكذلك بكتريا التهاب المهبل الجرثومي والاختبارات الخلوية.

بالنسبة للجزء الأول من الدراسة التي استهدفنا فيها معدل حدوث القاردينلا المهبلية بين المجموعة (أ) تؤكد النتائج الاصابة بالتهابات المهبل في (119/76) 63,8%من مجتمع الدراسة على النحو التالي :القاردينلا المهبلية (76/20) 26,3% منهم (46/7) 15,3%حوامل بينما (30/13)43,3% غير حوامل. ذكرت البكتريا العقدية في27,6%،المتفطرة البيضاء 46,1%. وجدت القاردينلا المهبلية اكثر حدوثا" في الفئة العمرية العمرية (20-29) تليها الفئة العمرية (30-39) ثم الفئة العمرية (أقل من 20 وأكثر من 40سنة. اكدت نتائج تفاعل البلمرة وجود القاردينلا المهبليه بين عناصر الدراسة بمعدل معتبر 17%119/20 من مجتمع الدراسة.

فيما يتعلق بالمجموعة(ب) اجري تفاعل البلمرة للكشف عن الانماط الجينية المختلفة كذلك العوامل البكتيرية (القاردنيلا فاجيناليس،اللاكتوباسلس اينرس والببتونيفيلس لاكريميليس). لوحظ انخفاض حدوث العقيرات الظهارية المسببة للتسرطن بين المرضى المسجلين 13 (5.5 ٪) مع التوزيع التالي: عقيرة حرشفية عالية الدرجة داخل الطبقة الظهارية في 5 حالات (38.5 ٪) ، عقيرة حرشفية منخفضة الدرجة داخل الظهارة في 4 (30.8 ٪) وأربعة حالات (30.8٪) كخلايا حرشفية نموذجية ذات دلالة غير محددة. ومع ذلك ، تم الكشف عن فيروس الورم الحليمي البشري فقط في 6 (2.5 ٪) من الحالات والانتشار الشامل لأنماط فيروس الورم الحليمي البشري عالية الخطورة في مسحات عنق الرحم غير الطبيعية كان 15.4 ٪ (13/2).

من ناحية أخرى ، اكتشف فيروس الورم الحليمي البشري أيضا في 1.9 ٪ (238/4) بين المسحات التي تم تسجيلها خالية من أي نوع من الورم الخبيث . من ناحية أخرى 170 عينة فقط في المجموعة ب توفرت لكشف العوامل البكتيرية عن طريق تفاعل البلمرة (فقدت بقية العينات اثناء نقلها)، أظهرت النتائج أن (170/21) 12.4 ٪ من مجتمع الدراسة كانوا يعانون من التهاب

المهبل الجرثومي . لوحظ انخفاض معدل حدوث القاردينلا والاكريميليس2,3 % للممرضتين في حين تم تحديد اللاكتوباسلس اينرس 7,6%. تم الكشف عن الاصابة المشتركة مع فيروس الورم الحليمي البشري في اثنين من المرضى واحد من الانواع فيروس الورم الحليمي البشري 52 والآخر كان نوع 58 . كلاهما تم استنباطها دون أي وجود تغييرات في الخلايا الظهارية . من ناحية أخرى اثنان من فيروس الورم الحليمي البشري الإيجابي الجنس لديهم تغيير في الخلايا الظهارية ولكن ليس هناك التهاب مهبل بكتيري.

خلصت الدراسة إلى انخفاض معدل إنتشار فيروس الورم الحليمي في مسحات عنق الرحم الروتينية لدى النساء السعوديات المعرضات للاصابة. بالإضافة إلى ذلك ، لوحظ أيضا" العلاقة الضعيفة بين فيروس الورم الحليمي البشري ومعدل حدوث اورام عنق الرحم بينما لاتوجد علاقه بين التهاب المبل الجرثومي وفيروس الورم الحليمي في الاصابة بسرطان عنق الرحم وجود معدل بسيط من التهاب المهبل الجرثومي في المجموعة ب نتيجة لان العينات مأخوذة من مكان غير مباشر للبكتريا(عنق الرحم). هذه الدلراسة قد توجه الاطباء والباحثين ليبحثوا عن انماط جينية اخرى لفيروس الورم الحليمي البشري بهذا الصدد وارتفاع حدوث التهاب المهبل الجرثومي عالميا فضلا الي النساء السعوديات.

PREFACE

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LIST OF ABBREVIATIONS

μL	Micro liter
TBE buffer	Tri base, Boric acid and EDTA buffer
HIV	Human Immunodeficiency Virus
SED	Sexually Enhanced Disease
STD	Sexual Trans meted Disease
BV	Bacterial vaginosis
KOH	Potassium hydroxide
CIN	Cervical intraepithelial neoplasia
A.vagina	*Atopobium vagina*
G.vaginalis	*Gardenerlla vaginosis*
P.lacrimalis	*Peptoniphilus Lacrimalis*
L. iners	*Lactobacillus Iners*
HR(HPV)	High Risk Human Papilloma Virus
PCR	Polymerase Chain Reaction
STIs	Sexual Trans meted Infections
HSV	Herpes Simplex Virus
MCH	Maternity and Children Hospital
HVS	High Vaginal Swab
LBC	Liquid Base Cytology
ASC-US	Atypical squamous cells of undetermined significance
LSIL	low-grade squamous intraepithelial lesion
NILM	Negative for intraepithelial lesion or malignancy
HSIL	high-grade squamous intraepithelial lesion

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CHAPTER ONE

INTRODUCTION AND OBJECTIVES

1. Introduction

The precise pathophysiology and epidemiology of Bacterial vaginosis (BV) , as well as the optimal medical management of the condition, are far from clear, with much of this lack of understanding occurring as a direct result of the difficulty in establishing a diagnostic standard for the syndrome (Ferris *et al.*,2004).

The syndrome of BV was first characterized using clinical criteria and simple laboratory tests applied to vaginal samples (Amsel *etal.*,1983). Together, this constellation of evaluations became known as the "Amsel criteria." A diagnosis of BV requires that at least 3 of 4 Amsel criteria be positive (abnormal gray discharge, pH of >4.5, a positive amine test, and presence of epithelial "clue" cells). Although generally regarded as a relatively specific method for identifying patients with BV, Amsel scoring requires considerable clinical acumen and has been demonstrated to be relatively insensitive (Schwebke,2000). A more accurate approach to BV diagnosis was proposed in the early 1990s (Schwebke,2000) and involved the use of semi-quantitative evaluation of vaginal microflora (0 to 3, normal; 4 to 6, intermediate; and 7 to 10, abnormal) based on observation of different bacterial morphotypes in Gram-stained

preparations of vaginal samples. This so-called “Nugent score” (NS) has since considered as the gold standard for BV diagnosis (Nugent *et al.*,1991).

Cervical cancer is a major global public health problem affecting socio economically deprived populations (Sankaranarayanan *et al.*, 2009). It is ranked the seventh most prevalent cancer and the second most common cancer in women after breast cancer globally (Pisani *et al.,* 2002). It is the second leading cause of cancer death in women worldwide with more than 270000 deaths reported every year. Over 80% of these deaths occur in developing countries (Muchiri *et al.,* 2006; Kent, 2010).

Cervical intraepithelial neoplasia (CIN) is a histological term used to describe full thickness epithelial change. It is divided for convenience and ability into three categories CINI, CINII, and CINIII (Bancroft and Gamble, 2002).

Studies have demonstrated that, with the probable exception of a very rare type of highly differentiated squamous cell carcinoma, Human Papilloma virus (HPV) is responsible for 99.7% of all invasive carcinomas of the cervix (Walboomers *et al.*, 1999; Sonc *et al.*, 2001). HPV infection plays an etiological role in the development of cervical cancer, and most of cervical cancers contain HPV DNA (McMurray *et al.*, 2001).

Genital HPV types have been classified into categories of low oncogenic risk (e. g types 6 and 11), and high oncogenic risk types (e. g types 16 and 18). Infection with high oncogenic risk types of HPV constitute of major risk factor and is necessary for the development of pre-cancerous neoplastic lesions and or of cancer of cervix. Combined HPV 16 and 18 cause approximately 70% of cervical cancer and high grade of cervical dysplasia-infection with low risk HPV types rarely if ever, progresses to cervical cancer as evidenced by the fact that single infection (without high risk HPV types) have never found in CIN II and invasive disease (Ostor *et al* ., 1993).

A survey of HPV types in invasive cervical cancer in 22 countries around the world revealed that HPV 16 accounted for 54% of the cancer associated HPV types followed by HPV18 (15%), HPV 45 (9%) and HPV 31(6%) .Thus, these four types account for 84% of all cancer associated HPV types (Basch *et al*., 1995).

The most important risk factor in the development of cervical cancer is infection with a high-risk strain of human Papilloma virus. The virus cancer link works by triggering alterations in the cells of the cervix, which can lead to the development of cervical intraepithelial neoplasia, which can lead to cancer.

Women who have many sexual partners (or who have sex with men who had many other partners) have a greater risk (American Cancer Society, 2006; Marrazzo, *et al.*, 2001).

The American Cancer Society provides the following list of risk factors for cervical cancer: human papilloma virus (HPV) infection, smoking, HIV infection, *Chlamydia* infection, dietary factors, hormonal contraception, multiple pregnancies, exposure to the hormonal drug diethylstilbestrol (DES) and a family history of cervical cancer There is a possible genetic risk associated with Human histocompatibility (HLA) Antigen encoded by the B locus on chromosome 6 (HLA-B7). (American Cancer Society, 2006).

Recently times are being made to develop prophylactic vaccines against high-risk HPV types. For developing countries, primary prevention by vaccination may provide the only real hope of reducing the incidence and mortality from cervical cancer. Of the two HPV vaccine in late stage development, Merck's quadrivalent vaccine, Gardasil protects against four HPV types and was approved for use in female patients from 9 to 26 years of age (Muchiri *et al*., 2006).

Screening of cervical cancer is a public health initiative is based on the assumption that prevention is better than cure and that early diagnosis allows for treatment while primary pathologic process is still reversible. Screening tests should ideally be inexpensive, specific, sensitive, and free

risk, they should also enjoy a high level of acceptability to the population (Muchiri *et al.*, 2006).

As abnormal microflora can produce carcinogenic nitrosamines and stimulate the release of cytokines, such as interleukin-1b, it has been suggested that BV may be important in the development of cervical cancer (Behbakht *et al.*,2002).Carcinogenic nitrosamines increase the probability 0f DNA damage and an altered cytokine profile may reduce immune's system ability to eliminate HPV infection. The changes may create a conducive environment for cancer development (Pavic *et al.*, 1984). The Centers for Disease Control and Prevention (CDC), which is a part of the principal agency in the United States government for protecting the health and safety of all Americans and for providing essential human services, have included BV on their list of emerging infectious diseases (CDC, 2002) .

1.2 Hypothesis of the Study

Human papillomavirus (HPV) infection, especially HPV-16 and HPV-18, plays a major role in the etiology of cervical cancer, but HPV alone is not sufficient to induce cancer. We propose that squamous cell cervical cancer is caused by an interaction of oncogenic viruses and exposures to other factors. Hence, infection by BV may induce this type of cervical cancer.

1.3 Rationale

The association of BV and development of cervical cancer is still unclear and conflicting. Some studies show the association of bacterial vaginal infection with cervical cancer and other studies show no association at all.

The Centers for Disease Control and Prevention (CDC), have included BV on their list of emerging infectious diseases.

In fact, still, not all of HPV infection leads to cervical cancer, suggesting that other cofactors could be present in the development of malignancy, therefore, BV is to be taken into consideration.

1.4 Research Questions

- What is the real percentage of *G. vaginalis* among Saudi women infected with BV?
- What other pathogenic organisms that may shared in causing BV among Saudi females?
- Is there any association between bacterial vaginosis (BV) and Human papilloma virus (HPV) among Saudi women?
- Does bacterial vaginosis act as co-factor in the development of cervical cancer?
- What is the most prevalent HR-HPV Genotypes that affect Saudi females ?

1.5 Objectives of the Study

1.5.1 General Objective

This study aims to detect the frequency of bacterial vaginosis and human papiloma virus in primary cervical screening using conventional methods and PCR technique.

1.5.2 Specific Objectives

- To determine the prevalence of *G. vaginalis* among Saudi infected females.
- To isolate and identify pathogenic organisms that cause BV among Saudi females
- To detect cytological patterns in vaginocervical smears using liquid base cytology and Gram's stain for BV.
- To use a PCR technique for diagnosis of BV.
- To detect human papilloma virus (HPV) genotypes using PCR techniques.
- To find any relations with risk factors and frequency of BV.

CHAPTER TWO
LITERTURE REVIEW

2.1 Bacterial Vaginosis

2.1.1 Definition

Bacterial vaginosis (BV) is a vaginal infection that occurs when the equilibrium of the natural flora in the vagina is altered. It is the most common cause of abnormal vaginal discharge (Wilson *et al.*, 2005; Donders, 2010), affecting millions of women of reproductive age annually. Although the syndrome is curable with antimicrobials such as metronidazole and clindamycin, relapse rates are high. BV may be asymptomatic (Donders, 2010) but is more commonly associated with vulvovaginal symptoms such as discharge, itch, malodor and discomfort. These are common complaints of women, occurring most commonly during and shortly after menstruation, at a time when the vaginal pH tends to be elevated compared with other times in the menstrual cycle (Melvin *et al.*, 2008). The reason for the rise in pH is unclear, but there is evidence of a temporary disturbance of the vaginal microflora and an increased incidence of BV and candidal infection around the time of menstruation (Melvin *et al.*, 2008; Eschenbach *et al.*,2000). There is also increasing evidence that the pathogenic effects of BV are not confined to the lower genital tract (Swidsinski *et al.*, 2013) and that the microbial correlation of BV involves a dense, highly structured polymicrobial

biofilm, primarily consisting of *Gardnerella vaginalis*, strongly adhering to the vaginal epithelium (Swidsinski *et al.*, 2005).

2.1.2 Historical Background

The first description of the normal bacterial flora of the vagina was published in 1892 by Doderlein (Doderlein, 1892) that was later called "Doderlein's bacilli". These facultative anaerobic Gram-positive bacteria have been shown to be part of a group of bacteria generally referred to as *Lactobacilli* and, in bacterial taxonomy (Hillier *et al*., 2008), they are classified into the genus *Lactobacillus*. In1895, Kronig (Kronig, 1898) reported a motile rod, which he believed normally occurred in the vaginas of pregnant women. This was probably the first description of a bacterium that is ,to day, known as *Mobiluncussp* (Hjelm *et al*., 1981; Spiegel and Roberts ., 1984; Durieux and Dublanchet 1980). In addition, Curtis was able to isolate the curved anaerobic bacterium from a woman with puerperal fever (Curtis, 1913). Curtis stated that the normal vaginal content is dominated by *Lactobacilli* and that the presence of anaerobic rods correlates to vaginal discharge (Curtis, 1914). This shift in vaginal flora was also reported in 1921 by Schroder (Schroder, 1921).

Schroder divided the vaginal discharge into three types. The first type was dominated by *Lactobacilli*, the second type consisted of a mixture of *Lactobacilli* and other bacteria, and *Lactobacilli* were absent in the third

type. Later, the term non-specific vaginitis was used to describe this syndrome because in contrast to *trichomoniasis* and candidiasis, it was impossible to identify a specific agent that caused the vaginitis. In 1955, Gardner and Duke (Gardner and Dukes 1955) isolated *Haemophilus vaginalis*, later called *Gardnerella vaginalis*, from women with non-specific vaginitis. Gardner and Duke named this syndrome *Haemophilus vaginalis vaginitis*. When investigators in other parts of the world started to use a more selective culture medium containing human blood, it was shown that many clinically healthy women could harbor *G. vaginalis*, albeit at much lower concentrations, without contracting *Haemophilus vaginalis vaginitis*, (Totten *et al.*, 1982; Hillier *et al.*, 2008). In 1984, at the second international meeting on the syndrome, the term bacterial vaginosis was coined and given the definition: a replacement of the *Lactobacilli* of the vagina by characteristic groups of bacteria accompanied by changes in the properties of the vaginal fluid. The term quickly found universal acceptance (Mardh and Taylor 1984).

2.1.3 Epidemiology of Bacterial Vaginosis

BV is the most common vaginal infection among women in their reproductive years (Donders, 2010; Morris *et al.*, 2001). BV is also the most common cause of vaginal discharge and malodor (Mania-Pramanik *et al.*, 2009). Generally, it is estimated that 1 in 3 women will develop the

condition at some point in their lives. Its prevalence ranges between 4.9 and 36% in developed countries (Henn *et al.*, 2005). An increased risk for the development of BV has been shown with surgery and pregnancy where it is estimated that 15 to 20% of pregnant women have BV (Alfonsi *et al*., 2004). Other studies have re-ported the prevalence of BV among non-pregnant women to range from 15 to 30%, and have reported that up to 50% of pregnant women have been found to have BV (Laxmi *et al.*, 2012). The prevalence of BV varies around the world. (Kenyon *et al.,* 2013) conducted a systematic review on the global epidemiology of BV. The BV prevalences were found to vary considerably between ethnic groups in North America, South America, Europe, the Middle East and Asia. Although BV prevalence is, in general, highest in parts of Africa and lowest in much of Asia and Europe, some populations in Africa have very low BV prevalences and some in Asia and Europe have high rates. Which might be due to various factors such as socio-demographic characteristics, sexual practices and hygiene behavior . BV is often linked to sexual behavior , and the epidemiological profile of BV mirrors that of established sexually transmitted infections (STIs) (Verstraelen *et al.*, 2010). There is, however, not conclusive evidence whether BV pathogenesis involves sexual transmission of pathogenic micro-organisms from men to women. *Gardnerella vaginalis* carriage and BV occurs rarely with children, but has been observed among adolescents (even

sexually non-experienced girls), contradicting that sexual transmission is a necessary prerequisite to disease acquisition (Verstraelen *et al.*, 2010). Although male-to-female transmission cannot be ruled out, there is little evidence that BV acts as an STD. BV is therefore rather considered as a sexually enhanced disease (SED), with frequency of intercourse being a critical factor (Verstraelen *et al.*, 2010).

2.1 .4 Etiology of Bacterial Vaginosis

The etiology of BV is poorly understood and remains a subject for debate. BV can arise and remit spontaneously or develop in to a chronic or recurrent disease (Donders, 2010). There are no proven individual predisposing factors exclusive to BV (Henn *et al.*, 2005). Risk factors that have been associated with BV include having multiple sex partners, a new male sex partner, sex with a woman, early age at first intercourse, frequent vaginal douching, use of vaginal foreign bodies or perfumed soaps, cigarette smoking and lack of vaginal *Lactobacilli* (Cherpes *et al.*, 2008). Although BV has never been proven to be sexually transmitted, it has an epidemiological profile consistent with that of a sexually transmitted infection (STI) (Henn *et al.*, 2005), although it is better described as asexually enhanced disease (SED). It is more common among women who have an STI or who use intrauterine devices . (Fethers *et al.*, 2008; Wilson *et al.*, 2007). Women who have never had

sexual intercourse may also be affected. BV may sometimes affects women after menopause. The decrease in oestrogen levels in premenopausal and postmenopausal women has been linked to an abnormal vaginal flora of 35 and 70%, respectively when compared to the normal flora (Wilson *et al.*, 2007). It has also been shown that amenorrhoea lowers the risk of BV as the absence of blood maintains vaginal pH, low and stable around pH 4.5. Subclinical iron deficiency (anaemia) is a strong predictor of BV in pregnant women (Verstraelen *et al.*, 2005), especially in developing countries. A longitudinal study published in 2006 showed a link between psychosocial stress and BV independent of other risk factors (Verstraelen *et al.*, 2005). It is generally a knowledged that vaginal *Lactobacilli* play an essential role in maintaining an environment that limits the growth of pathogenic microorganisms in the vagina (Mania-Pramanik *et al*., 2009). It has been suggested that the presence of oestrogen and *Lactobacillus* are needed to achieve an optimal vaginal pH of 4.0 to 4.5 (Melvin *et al.*, 2008; Suresh *et al.*, 2009). After puberty under the influence of oestrogen, glycogen is deposited in the vaginal epithelial cells, which is metabolized by vaginal epithelial cells to glucose (Suresh *et al.*, 2009). *Lactobacilli* produce lactic acid from glucose, keeping the vagina at an acidic pH (Suresh *et al.*, 2009). Some species of *Lactobacilli* produce hydrogen peroxide which is toxic to various microorganisms (Suresh *et al.*, 2009). Bacterial

vaginosis is therefore characterized by an alteration of the normal acidic *Lactobacilli*-predominant vaginal ecosystem to a vaginal milieu dominated by mixed anaerobic bacteria flora with an accompanying increase in pH (Geva *et al.*, 2006).

The complex etiology of BV of a continuum of changes in the vaginal flora, rather than a single pathogen infection (Morris *et al.,* 2001), includes a log10-fold increase in the numbers of facultative anaerobes (Srinivasan *et al.,* 2008) and a concurrent loss of indigenous *Lactobacillus*-predominant vaginal microflora. The development of a more anaerobic environment inhibits the growth of *Lactobacilli*. Srinivasan and Fredricks (2008) give a complete overview on the vaginal flora in BV from a microbiological and molecular perspective.

It is unknown whether the loss of *Lactobacilli* precedes the BV infection or is a result of the infection (Mania-Pramanik *et al.,* 2009). Furthermore, it is not known whether the change in flora is not the result of an as yet unidentified etiological factor, suggesting that the altered flora is actually a downstream event of BV (Nansel *et al.*, 2006). The overgrowth by the facultative anaerobes is associated with an increase in protease production especially carboxy peptidase which leads to the breakdown of peptides to amines which in an environment of higher pH can become volatile. Due to the flexible nature of the disease process, the host

response in BV should be considered, although most work has been performed on the changes in microbial flora. It was initially believed that inflammation is absent during BV (Morris *et al*., 2001).

2.1.5 Complication of Bacterial Vaginosis

More important than symptoms are complications associated with BV. These appear to be related to an increased risk of susceptibility to STIs including infection with *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, HSV-1 and -2, and an increased risk of HIV acquisition, and to an adverse outcome of pregnancy (Geva *et al.*, 2006). BV has been shown to increase the risk of gynaecological and obstetric complications such as preterm labour and delivery, chorioamnionitis, post-caesarean endometritis, post-abortion pelvic inflammatory disease and cervicitis. Several groups had found that bacterial vaginal flora has an impact on these complications (Johnson *et al.*, 1985), while other studies disproved some of these findings. The leading hypothesis concerning these associations is that absence of protective *Lactobacilli* increases biological susceptibility of acquiring an STI upon exposure (Alfonsi *et al*., 2004). However, the temporal nature of the association between BV and acquisition of STIs remains an ongoing discussion. Although there is evidence favoring the plausibility that BV also incurs an elevated risk for HPV acquisition (Khan *et al.*, 2007), this also remains a matter of debate.

Since the 1970's BV has been associated with pelvic inflammatory disease in the absence of *Chlamydia* or *Neisseria gonorrhea* (Morris *et al.*, 2001). Finally, there is also a potential link between BV and an increased risk of HIV infection (Mania-Pramanik *et al.*, 2009).

A cochrane study (McDonald *et al.*, 2011) found that the administration of antibiotics during pregnancy for overgrowth of abnormal bacteria in the birth canal does not reduce the risk of babies being preterm. A more recent Cochrane review confirmed this finding that antibiotic treatment can eradicate bacterial vaginosis in pregnancy, but that the overall risk of preterm birth was not significantly reduced (Brockhurst *et al.*, 2013). Furthermore, it has been shown that BV increases the risk of miscarriage between 13 and 24 weeks (Donders, 2010), the risk of babies being preterm and 40% elevated risk of low birth weight (Morris *et al.*, 2001).

2.1.6 Classification Systems

2.1.6.1 Spiegel Classification

In the Spiegel classification system, *Lacto bacillus* morphotypes and *Gardnerell a*morphotypes are detected and classified as 1+, 2+, 3+ , and 4+ according to the amount of the bacteria seen using Gram stained smears with a magnification of 1000 x. A microscopically detectable change in vaginal micro flora, from the *Lacto bacillus* morphotypes, with

or without *Gardnerella* morphotypes (normal), to a mixed flora with few or no *Lactobacillus* morphotypes (BV), is used in the diagnosis of BV. The presence of *Lactobacillus* morphotypes in low numbers (1+ to 2+) is interpreted as being consistent with BV. If the *Gardnerella* morphotypes outnumber the *Lactobacillus* morphotypes, this is also consistent with BV, even if the *Lactobacillus* morphotypes are present. If *Lactobacillus* morphotypes are present alone, the sample is interpreted as being normal (Spiegel *et al.*, 1983).

2.1.6.2 The Nugent Classification

Nugent *et al.*, 1991 developed a more objective scoring system for the diagnosis of BV based on observed morphotypes. Today, the Nugent scoring is the most frequently used laboratory based diagnostic tool for detecting bacterial vaginosis and it is considered as the gold standard. Nugent's scoring is employed along with Gram stained smears (1000 x magnification), using oil immersion. This results in a point estimation system (0 to 4 points)that is used to rate the amount of different bacterial morphotypes present in the samples. The presence of more than 30 *Lactobacilli* morphotypes per vision field earns 0 points, whereas the absence of *Lactobacilli* morphotypes earns 4 points. The amount of small bacteria present in the sample is also rated on a point system (from 0 to 4 points), but the points are assigned in the opposite way. The presence of

more than 30 small bacteria per vision field earns 4points and the absence of small bacteria earns 0 points. The existence of curved rods earns an additional 1 or 2 points, depending on the amount of curved rods in each field of vision. When the points are added together, a total score of 0-3 is considered normal; a score of 4-6 is classified as intermediate, and a score of 7-10 is consistent with BV (see Table 1). The scores from zero to ten do not represent a ratio scale. The variable amount of bacteria is rated on an interval scale, but is categorized with the categories BV/intermediate/normal, on an ordinal scale. (Nugent et al., 1991).

Table 1. The Nugent's scoring system

Score	*Lactobacillus* Morphotype /Vision field	*Gardnerella*Morphotype /Vision field	Curved bacteria Morphotype/Vision field
0	>30	0	0
1	5-30	<1	1-5
2	1-4	1-4	>5
3	<1	5-30	
4	0	>30	

Scores 0-3= Normal flora, 4-6= Intermediate flora, 7-10= BV(Nugent *et al.*, 1991).

The Nugent's scoring system has shown a high inter- and intra-observer reliability. However, questions still remain that require discussion (Forsum *et al.*, 2002). Forsum *et al*. emphasized the need for a standardization of interpretation for the basic morphotypes that play a central role in a diagnosis using Nugent's classification (Forsum *et al.*,

2008). Moreover, the field size of the microscope has an influence on the results (Larsson *et al.*, 2004), which is another issue of concern. In Nugent's classification, the presence of only 30 *Lactobacilli*/small bacteria per vision field counts, so both the area of the microscope images and the thickness of the smear make a difference.

2.1.6.3 The Hay/IsonClassification

The Hay/Ison is a classification/categorization system that is used for both Gram stained smears and PAP-stained smears (Hay *et al.*, 1992). In the Hay/Ison classification, vaginal flora is divided into the following three different categories: normal, intermediate, and BV. In this classification system, an estimation of the amount of the bacterial morphotypes is not done; instead a subjective evaluation of the relationship between the amounts of bacteria is conducted. The field size of the microscope does not have an influence on the results (Larsson *et al.*, 2004).

Table 2 The Hay/Ison classification

	Lactobacilli morphotypes	Gardnerella morphotypes
Normal (group 1)	Many	Few
Intermediate (group 2)	Equal amount	Equal amount
BV (group 3)	Few	Many

(Hay *et al*., 1992).

2.1.6.4 The Ison/Hay Classification

In the Ison/Hay classification system, the categories normal, intermediate, and BV are used. However, the following two categories are added: 0 (relatively empty smear) and 4 (dominance of *Streptococcus* morphotype) (Ison *et al.*, 2002). The categories 0 and 4 are added in an attempt to make the categorization more true to what is observable in clinical practice, as opposed to what might be hypothesized in relation to the concept of BV. However, what can be observed in reality must sometimes be seen as different types of entities. The Hay/Ison and Ison/Hay classification systems can be used on slides stained with different staining methods and also on smears with no stains.

Table 3. The Ison/Hay classification

	*Lactobacilli*morphotypes	*Gardnerella*morphotypes
Group 0	None	None
Normal (group 1)	Many	Few
Intermediate (group 2)	Equal amount	Equal amount
BV (group 3)	Few	Many
Group 4 .A lot of gram positive streptococci	None	None

(Ison *et al*., 2002)

2.1.7 Diagnosis of Bacterial Vaginosis

2.1.7.1Amsel Criteria

Clinical diagnosis using Amsel criteria requires that at least three of the following four criteria are met: first, a vaginal pH of greater than pH 4.5; second, the presence of clue cells in the vaginal fluid when examined in a wet mount; third, a milky homogeneous vaginal discharge (There must not be any granular elements; the fluid must be completely homogenous), and finally, positive Whiff (Sniff) test. Vaginal pH was measured by rolling the swab over a pH strip immediately after swabbing and the resulted color is translated into the pH value. Clue cells are epithelial cell studded with Gram variable *coccobacilli*, it was detected by microscopic examination of wet mount which was made by mixing the vaginal

discharge from the swab with a drop of saline. A homogenous milky discharge was detected by examination after speculation. Whiff test was done by mixing the vaginal discharge from a swab with a drop of 10% KOH a positive result is a "fishy" amine odor (Amsel *et al*.,1983).

After rolling the vaginal swab on a microscopic slide, it was left to dry then Gram stained with Gram staining protocol, then it was read by a single experienced microbiologist to eliminate the possibility of inter-observer difference. Slides were read according to Nugent score as follows: Morphotypes were counted as the average number of bacteria in 10-20 oil immersion field. The Nugent score was calculated by assessing for the presence of large gram-positive rods (*Lactobacillus* morphotypes decrease in *Lactobacillus* scored as 0 to 4), small Gram-variable and Gram-negative rods (*G. vaginalis* and *Bacteroides* morphotypes; scored as 0 to 4), and curved gram-variable rods (*Mobiluncus spp*. morphotypes; scored as 0 to 2), After the amount of each morphotype detected on the smear was graded it was then allocated a score as shown in Table (1). Then total score calculated from 0 to 10. A score of 1-3, considered normal, a score of 4-6 considered intermediate (means an intermediate state between normal and BV) and a score of 7 to 10 was consistent with BV. This method is considered the gold standard for diagnosis of BV(Nugent *et al*., 1991).

2.1.7.2 Culture

Isolation of *Gardnerella vaginalis* cannot be used to diagnose BV because it can be cultured from the vagina of more than 50% normal women . In research studies a high concentration of *Gardnerella vaginalis* is associated with the presence of BV (Watts *et al.*,1990 ; McDonald *et al.*,1997).

2.1.7.3 BV Blue Test

The Osom BV blue test ® provided by Gryphus Diagnostics, L.L.C. is a chromogenic test done by immersing the swab into the tube containing IBX 4041 (chromogenic substrate), and incubated at 37°C for 10 minutes and then, 1-2 drops of BV Blue developer solution (Na OH solution 40 mg/ ml) was added. Development of intense blue color within 3 minutes indicated the test as positive (Akhter *et al.*,2010).

2.1.7.4 PCR-Based Test

PCR-based tests are being used for molecular diagnosis of BV, mostly based upon molecular quantification of *G. vaginalis and A. vaginae* (Fredricks *et al.*,2005). These two species co-occur in the large majority of BV patients, and the possibility of combining the detection of these

two species for an accurate diagnosis of BV has been suggested (Catwright *et al*., 2012 ; Shipitsyna *et al*.,2013).

2.1.8 Treatment of Bacterial vaginosis

Treatment of BV is difficult for several reasons, the most compelling of which is that clinical cure, after various treatment regimes, is not universally successful (Oduyebo *et al*.,2009;Senok *et al*., 2009). The reported four-week cure rates vary between 60-70% and recurrence rates are high (Larsson and Forsum 2005). Thus, treatment failures continue to haunt both BV patients and their doctors. The plethora of metronidazole, tinidazole, and clindamycin preparations used for eradication of the BV-associated flora includes tablets, vaginal cream, vaginal pills, vaginal gels, topical slow-release cream, and oral tablets, but systematic studies of the optimal preparation for delivery of the drug are lacking (Senok *et al*., 2009).

2.1.9 Characteristics of microorganisms, associated with (BV)

2.1.9.1*Gardnerella vaginalis*

Is a Gram-variable facultative anaerobic bacterium and was one of the first organisms to be associated with BV. The abilities of *G. vaginalis* to form a biofilm and produce prolidase, sialidase, β-galactosidase, and vaginolysin may play a role in the pathogenesis of this condition (Forsum

et al.,2005; Donati *et al.*,2010) . In recent years, the application of culture-independent techniques has revealed the ubiquitous nature of *G. vaginalis*. Due to the common occurrence of this microorganism in healthy women, the role of *G. vaginalis* as a BV diagnostic marker has been challenged. (Lamont, *et al.*,2011; Srinivasan *et al* 2010; Zozaya, *et al.*, 2010; Fredricks, *et al.*,2009; Sha, *et al.*,2005). Even though the presence of *G. vaginalis* bacteria in the vaginal milieu signifies an occurring disturbance, the concomitant detection of *G. vaginalis* with other BV-associated microorganisms is more indicative of BV (De Backer *et al.*,2007 ; Menard *et al.*,2010; Bradshaw *et al.*,2006).

2.1.9.2 *Lactobacillus iners*

Is a rod-shaped facultative anaerobic non-spore forming Gram-positive bacteria. It is a common member of human-associated bacterial microflora. Along with *L. gasseri*, *L. crispatus*, and *L. jensenii*, it is considered to be one of the four major vaginal *Lactobacillus species*. Physiologically, *L. iners* is different from other vaginal *lactobacilli* as it is less prone to hydrogen peroxide production and also is the most fastidious microorganism compared with the other *Lactobacillus species* (Lamont, *et al.*,2011; Forsum, *et al.*,2005) . *L. iners* can be detected in both healthy and disturbed vaginal microflora including BV (Srinivasan *et al* 2010; Zozaya *et al.*,2010; Fredricks *et al.*,2007) . *L. iners*'

dominance, along with the depletion of other *Lactobacillus species*, indicates that the vaginal microflora may be in a transitional stage between abnormal and normal (Tamrakar *et al.*,2007 ; Jakobsson, and Forsum 2007; Verstraelen *et al.*,2009).

2.1.9.3 *Peptoniphilus lacrimalis*

Peptoniphilus are Gram positive anaerobic cocci that were formerly classified in the genus *Peptostreptococcus*. They are non-saccharolytic, use peptone as a major energy source and produce butyrate (Ezaki, *et al.*,2001).

2.2 Infections That Can Lead to Cancer

Since the start of the 20th century, it's been known that certain infections play a role in cancer in animals. More recently, infections with certain viruses, bacteria, and parasites have been recognized as risk factors for several types of cancer in humans. Infections can raise a person's risk of cancer in different ways. For example:

• Some viruses directly affect the genes inside cells that control their growth. These viruses can insert their own genes into the cell, causing the cell to grow out of control.

• Some infections can cause long-term inflammation in a part of the body. This can lead to changes in the affected cells and in nearby immune cells, which can eventually lead to cancer.

• Some types of infections can suppress a person's immune system, which normally helps protect the body from some cancers. Any of these changes might lead to a higher risk of cancer. (American Cancer Society 2014)

2.2.1 Oncogenic Viruses and Cervical Neoplasia

2.2.1.1 Human Papilloma Viruses (HPVs)

Human papilloma viruses (HPVs) are a group of more than 150 related viruses. They are called papilloma viruses because some of them cause papillomas, which are more commonly known as warts. Some types of HPV only grow in skin, while others grow in mucous membranes such as the mouth, throat, or vagina. A few types of HPV are the main causes of cervical cancer, which is the second most common cancer among women worldwide. HPVs also have a role in causing some cancers of the penis, anus, vagina, and vulva. They are linked to some cancers of the mouth and throat, too. (American Cancer Society 2014).

2.2.2 Bacteria That Can Lead To Cancer

2.2.2.1 *Helicobacter pylori*

Stomach cancer is not common in the United States, but it's one of the more common types of cancer worldwide. Long-term infection of the stomach with *Helicobacter pylori(H. pylori)* can cause ulcers. It can also inflame and damage the inner layer of the stomach. Some of these changes could lead to cancer over time, especially cancer in the lower part of the stomach. *H. pylori* infection is also linked with some types of lymphoma of the stomach. (American Cancer Society 2014)

2.2.2.2 *Chlamydia Trachomatis*

Chlamydia trachomatis is a very common kind of bacteria that can infect the female reproductive system as well as other parts of the body in both men and women. Some studies have found that women whose blood tests showed past or current *chlamydia* infection may be at greater risk for cervical cancer than women with negative blood test results. (American Cancer Society 2014)

2.3 Cervical Cancer

Cervical cancers is a cancer malignant of the cervix or within the cervical area. It may form in the interior lining of the cervix, junction of the

vagina and the uterus. (Saonere 2010). Cervical cancer begins to develop in the cells around the cervix. Pre-cancerous cells which are described as cervical intraepithelial neoplasia (CIN), squamous intraepithelial lesion (SIL) and dysplasia. The pre-cancerous cells cancer can fully grow into cancer. There are two main forms of cervical cancer namely squamous cell carcinoma and adenocarcinoma, of these types 80% to 90% of the cervical cancers are due to the squamous cell carcinoma which begin where the exocervix joins the endocervix. Cervical adenocarcinoma develops from the mucus-producing gland cells of the endocervix. (ACS, 2010.) In some cases some of the cancers can be as a result of a combination of both squamous cells carcinoma and adenocarcinoma, the carcinoma is known as adenosquamous carcinoma or mixed carcinoma. In some women precancerous cells go away with no treatment what so ever while others turn into true invasive cancers. (ACS, 2010).

2. 3.1Bacterial vaginosis and Cervical Cancer

The association of BV and development of cervical cancer is still remain unclear and conflicting. Some studies show the association of bacterial vaginal infection with cervical cancer (Platz-Christensen *et al.*,1994) and other studies show no association at all (Peters , *et al* .,1995). However, the possibility still exist that BV is in some way associated with the development of CIN, as a cofactor to human papillomaviruses (HPV).

2.3.2 Pathogenesis

The pathogenesis of BV in cervical cancer is complex. BV is not attributable to a single causative organism but it's due to immense overgrowth of composite of flora including *G.vaginalis, Peptostreptococci, Bacterioides spp., Mobiluncusspp., Mycoplasma* and *Ureaplasma urealyticum. Fusobacterium* and *Atopobium vagina* are also commonly found in BV. Inflammation plays a little role since this disease is due to the disruption of the vaginal microenvironment rather than a true tissue's infective state. This overgrowth shifts the *Lactobacillus*-predominated vaginal ecosystem to micro environment with anaerobic bacteria dominance. This conclusion is corroborated by experimental studies conducted in humans and animal models that demonstrated upon vaginal inoculation with a single vaginosis-causing bacterial species, BV will rarely occur. The factors that lead to anaerobes bacterial overgrowth remain uncertain. Increased availability of the substrates, raised in pH and loss of *Lactobacillus spp*. were implicated for BV because the vagina of normal women were predominantly inhabited by *Lactobacillus* spp. that produced hydrogen peroxide which play an important role in suppressing the overgrowth of anaerobes either directly or by producing a hydrogen-halide complex catalyzed by naturally-occurring cervical peroxidase. Following the anaerobic bacteria overgrowth, there will be an elevated

polyamine production by the anaerobes, enhanced by the decarboxylases which broke down the vaginal peptides into polyamines. These polyamines along with vaginal organic acids (acetic and succinic acids) are cytotoxic to the vaginal cells, leading to the vaginal cell's exfoliation manifesting as vaginal discharge which is malodorous and volatile due to its high amine content. It has been hypothesized that BV results in cervical cancer through an increase in vaginal nitrosamines and altered cytokine profiles (Pavic 1984). The presence of raise nitrosamines in vagina will lead to higher probability of DNA damage and change in cytokine profiles will cause altered response of immune system to clear up HPV infection. Therefore, BV will interact with HPV infection with the consequence of higher risk of developing cervical cancer among those with BV and HPV co-infection than those with a mono-infection. However, other infections for instance *Candida* was not associated with the development of CIN or cervical carcinoma (Engberts, *et al* ,2006). Never the less, the causal relationship between BV-HPV interaction and cervical cancer development may not be entirely infallible since the causation may be bidirectional due to reverse causality. Owing to the relative immunodeficiency state in advanced cancer stage, BV thrives and therefore seems to be antecedent factor of cervical carcinoma while still fact the opposite is true. It's equally coherent to postulate that BV-HPV infection is a bona-fide cause of cervical carcinoma. Unless there's a

properly executed and methodologically sound cohort study, the temporal order between BV-HPV and cervical carcinoma cannot be fully ascertained. Besides that, BV is diagnosed by a multitude of diagnostic criteria. The most common ones used are Amsel and Nugent diagnostic criteria. However, several studies for example two studies conducted in Netherlands, have used a unique diagnostic system called KOPAC to diagnose BV which was subsequently termed as vaginal dysbacteriosis, a completely different concept since its diagnosis is based on microscopic appearance rather than clinical Amsel or Nugent criteria. Therefore, the heterogeneity of diagnostic criteria further hampered the unifying conclusion on the causative potential of BV as cervical carcinoma's etiology (Sha *et al*., 2005).

2.3.3 Vaccines Against HPV

Vaccines are now available to help protect against infection from the main cancer causing HPV types. These vaccines are approved for use in females from age 9 up to their mid-20's. Some HPV vaccines have also been approved for use in boys and young men.(American Cancer Society 2014).

The vaccines can only be used to help prevent HPV infection – they do not stop or help treat an existing infection. To be most effective, the

vaccines should be given before a person becomes sexually active (has sex with another person). Because the vaccines are still fairly new (first approved in 2006), and it often takes decades for cancer to develop, it's not yet known how well they will protect against it, or exactly which types of cancers they might help prevent. These vaccines and others like them are being studied further.(American Cancer Society 2014) **.**

2.3.4Implication to Treatment

The mainstay of treatment in carcinoma of the cervix is radical radiotherapy and concurrent chemo-radiotherapy. The radiotherapy includes a combination of external beam radiotherapy followed by intra cavitarybrachy therapy. (American Cancer Society 2014) .

CHAPTER THREE

MATERIALS AND METHODS

3.1 Type of the Study

3.1. 1 Study Design

A Cross sectional laboratory based study was conducted aimed to provide evidences about bacterial vaginosis and its association with uterine cervical Neoplasia in human papilloma virus positive cases in Al-Madinah Al-Monawarah Region**.** Collection of Samples was taken from enrolled patients during the period from April 2016 to May 2017.

3.1.2 Study Area

Different Hospitals and clinical centers in Almadina Almunwarah Reigon were included in the study.

3.1.3 Study Population

Group A

This group was consisted of one hundred and nineteen (n= 119) Saudi females under reproductive age attending Maternity and Children Hospital. All candidates were presented with signs and symptoms of bacterial vaginosis. Thus high vaginal swab (HVS) was taken from all patients.

Group B

Two hundred and thirty-eight women (n=238) attended Maternity and Children Hospital (MCH) in Al-Madinah Al Munawarah and Yanbu Poly

clinics with different ages (15-80 years) and gynecological symptoms (menorrhagia, urine incontinence, vaginitis and other vaginal diseases).

3.1.4 Ethical Clearance

A confidential written consent was obtained from every participant. Approval to conduct this research project was sought from the Ethical Committee of Ministry of Health, KSA.

3.1.5 Data Collection

Data were collected by using a standard data questionnaire eliciting basic social and risk factors for cervical cancer: name, age, , area, education, occupation, date of marriage, smoking, husband smoke, LMP (last menstrual period), get any contraceptive (It's type, duration), presence of any sexual transmitted disease and it's type, clinical symptoms, previous abnormal cytology, presence of discharge and it's color, presence of grossly visible lesion and the clinical symptoms (Appendix I).

3.1.6 In clusion and Exclusion of Study group

All Saudi females in Al-Madinah Al-Monawarah Region was included in his study and their age ranged from 15 to 80 years and suffering from clinical symptoms of BV. Other nationality was excluded and age less than 15 and more 80.

3.2 Diagnostic Approach for Group A

The study targeted women under reproductive ages (n, 119; 75 pregnant and 44 non -pregnant) attending Obstetrics and Gynecology Clinic and Emergency Unit in Maternity and Children Hospital in Al-Madinah Al-Munawarah, Saudi Arabia. Sampling was carried out according to the WHO regulations. The swab was placed into Amies transport media (ATM) is the mineral salt base semi-solid media designed as a holding medium for maintaining viability of anaerobic bacteria. which was used for anaerobic culture and making smear (wet preparation and Gram's stain). The swab was processed in the Microbiology Laboratory within 4 hours.

3.2.1 Conventional Methods for Identification

Different biochemical tests including: Whiff test, Catalase test, Oxidase test, HVS Culture on 5% Human blood agar and Gram's stain were used to identify target organisms.

3.2.1.1 PH of Vaginal Fluid

The vagina is normally slightly acidic with a PH of 3.8-4.2, swab of the discharge was put onto litmus paper to check its acidity . PH greater than 4.5 is considered alkaline and suggestive of bacterial vaginosis.

3.2.1.2 Whiff Test (10% KOH)

Swab containing vaginal secretion was placed in a test tube containing 0.5 cc of 10% KOH reagent. A characteristic fishy odor is considered positive whiff test and suggestive of Bacterial Vaginosis.

3.2.1.3 Clue cells

The presence of clue cells (epithelial cells that are coated with bacteria) on wet amount(drop normal saline on slide containing vaginal discharge) visualized under microscope suggestive of bacterial vaginosis.(Appendix IV).

3.2.1.4 High Vaginal Swab Culture

The sample was streaked on 5% Human blood agar under aseptic conditions, then incubated aerobically for 24 hours, at 37°C.*Gardnerella vaginalis* produce beta haemolytic on 5%human blood agar. (Appendix IV).

3.2.1.5 Gram's Stain

Vaginal swab was smeared onto a clean microscopic slide, left to dry completely and heat fixed. Smear was stained by Gram's Stain . Gram negative to Gram-variable coryne from coccobacillary to bacillary forms,

suggestive *G.vaginalis.*

3.2.1.6 Catalase Test

Several colonies of the isolated organisms were immersed with the use of wooden stick in about 2–3 ml hydrogen peroxide solution (H_2O_2) in a test tube .The presence of bubbles indicated positive results. *G.vaginalis* is catalase negative. (Appendix IV).

3.2.1.7 Oxidase Test

A piece of filter paper was soaked with few drops of oxidase reagent. A colony of the test organism was then smeared on the filter paper using a wooden stick. The change of color immediately to dark purple indicated positive results. *G.vaginalis* is Oxidase negative. (Appendix IV).

3.2.2 Molecular Identification for Bacteria (Group A&B)

Nucleic Acid Amplification Techniques (NAAT) are diagnostic methods based on the amplification of Bacterial DNA. Polymerase Chain Reaction (PCR) was used as a rapid and accurate diagnostic tool for detection of Bacterial vaginosis.

3.2.2.1DNA Extraction

DNA was extracted by using DNA extraction kit according to the manufactures' instructions (Sacace Biotechnologies- Italy).

3.2.2.2 Primers Sequence

Specific primers were used to amplify sequences of the bacterial gene that found in *G.vaginalis* which isolated from culture (group A).Also *G.vaginalis* and other bacterial genes (*L. iners and P.lacrimalis*) which extracted from cervix samples in liquid base cytology (group B) (170 samples from 238 in LBC were available to detect bacterial agent by PCR (the rest was lost during transportation).

3.2.2.3 Preparation of Master Mix

Before starting master mix preparation, bench was disinfected using 70% ethanol before preparation of each batch. PCR was done by end-point PCR. The amplification was done using TECHNE® Ltd peltier thermal cycler (Germany), (Appendix IV). DNA amplifies was done using Maxime PCR Premix kit (iNtRON, Korea) (Appendex V)The PCR assay was carried out in a total volume of 25 µL of mixture containing 2 µLMaxime PCR Premix containing 1X PCR buffer, 1.5 mM MgCl2, 200 µM of each dNTP, and 1 U Taq DNA polymerase, 1 µL from 0.2mM forward primer and 1 µL from 0.2mM reverse primer was added (2 µL),

5 µL of template DNA and 18 µL of (nuclease free water).The contents of master mix were vortexed after addition of each item .In negative control 5µl of sterile distilled water was added, while DNA extracted .

3.2.2.4 PCR Amplification

The reaction mixtures were then placed in the thermal cycler TECHNE® ltd Peltier Thermal Cycle) that carried out the following PCR program: initial denaturation step at 95°C 10 minutes for one cycle followed by repeating cycles of denaturation (30 seconds at 95°C), annealing (30 seconds at 58°C) and extension (30 seconds at 72°C) for 40 cycles, followed by a 7 minutes final extension step at 72°C.

3.2.2.5 Loading of Samples and Electrophoresis

Agarose gel (2%) was prepared (Appendix II), mixed well and poured on to the casting tray. After solidification comb was gently removed and enough electrophoresis buffer was added to the tank to cover the gel (Appendix II) (about 1 mm of depth), the top of the wells were submerged.10µl of PCR product from each sample were delivered into the well. 5µl of DNA ladder (marker) length 100 bp ladder with fragments ranging from 100 bp to 1000 bp were added to one well in each run to estimate the size of tested DNA sequence.(Appendix VI). The gel

electrophoresis apparatus was connected to a power pack (Serva Blue Power 500, Germany).(Appendix IV). The electrophoresis was performed at 50 V for 30 minutes.

3.2.2.6 Visualization of PCR Product

After electrophoresis period the gel tray was removed from the electrophoresis apparatus and the buffer was discarded, target DNA fragments specific for bacteria viewed under ultraviolet transilluminator (SYNGENE, UK)(Appendix IV). Lastly the gel was transferred for photography documentation.

Table (4) Primers used for detection *of G. vaginalis L. iners,P.lacrimalis*

	primers Sequence	Product size bp	D.W for 100pm/ul
G.vaginalis	F 5′- GGGCGGGCTAGAGTGCA -3′ R 5′-GAACCCGTGGAATGGGCC -3′	210	320
L. iners	F 5′- 5' ACAGGGGTAGTAACTGACCTTTG-3′ R 5′- ATCTAATCTCTTAGACTGGCTATG-3'	571	300
P.lacrimalis	F 5-'AAGAGACGAACTTAGAGATAAGTTTT -3′ 'R 5′- CACCTTCCTCCGATTTATCAT-3′	186	F:290 R:300

Primers were imported from Macrogen, Korea (Appendix III)

3.3 Cytological Method Group B

3.3.1 Pap Stain

Papanicolaou stain (also Papanicolaou's stain and Pap stain) is a multichromatic staining histological technique developed by George Papanikolaou, the father of cytopathology. Pap staining is used to differentiate cells in smear preparations of various bodily secretions; the specimens can be gynecological smears or other materials containing cells. Pap staining is a very reliable technique. As such, it is used for cervical cancer screening in gynecology. The entire procedure is known as Pap smear. In 1995, Platz- Christensen *et al*.reported that PAP smears could be potentially as useful an instrument for clinical diagnosis of BV as Amsel's criteria (Platz-Christensen *et al*.,1995), while others expressed doubt (Lamont *et al*., 1999; Prey, 1999).

Vaginocervical fluid was collected by using modified cyto-brush and it was rinsed into a labled vial containing 10 ml of PreservCyt® transport medium according the manufacturer' instructions (Appendix IV).The samples were then processed using Beckton, Dickson; PrepStain Slide Processor/TriPath Imaging Inc, 2005. Finally, vials containing the residual sample was frozen until used.

3.4 Detection of HPV

HPV detection, was done by using HPV kit according to the manufacturer's instruction (Sacace Biotechnologies).(Appendix VII)

HPV High Risk Typing Test is based on three major processes: sample preparation, multiplex amplification of DNA using specific HPV primers and detection of the amplified products on agarose gel. Each PCR-mix-1 tube contains primers directed against regions of four HPV types and b-globin gene used as Internal Control.

3.4.1 DNA Extraction

DNA was extracted by using DNA extraction kit according to the manufactures' instructions (Sacace Biotechnologies- Italy).

3.4.2 PCR Amplification

In the amplification protocol, three sets of PCR premix-1 were used to check for the presence or absence of 12 possible genotypes (PCR mix-1 "16-35", PCR mix-1 "18-59", and PCR mix-1 "52-66") . Polymerase chain reaction (PCR) was adopted in 25 µl according to (Sacace Biotechnologies), in a PCR tube containing 5 µL of PCR-mix-1, 10 µL of 2.5 PCR buffer, 0.5 TaqF polymerase and 10 µL of template DNA were mixed. Also, control tubes were prepared to be included in the reaction. Amplification of the target sequences was conducted using PCR machine

(SYNGENE, UK) only when temperature reaches 95°C and start the following program (Table 5) .

Table 5. PCR program used in the amplification of HPV different types

Step	t5C	Time	Cycles
1	95 °C	Pause	
2	95 °C	15 min	1
3	95 °C	30 sec	42
	63 °C	30 sec	
	72°C	30 sec	
4	72°C	1 min	1
5	10°C	Storage	

3.4.3 Interpretation of the Results

PCR protocol was carried out according to Sambrook *et al.* (2001). Finally, the target ampilicons were monitored under UV light on 3% agarose gel, with reference to Table(6 and7) to compare the products sizes.

Table 6. Lengths of specific amplified DNA fragments of suspected HPV

Mix-1 (52 – 66)		**Mix-1 (18 – 59)**		**Mix-1 (16 – 35)**	
304 bp	HPV66	395 bp	HPV59	280 bp	HPV35
325 bp	HPV65	75 bp	HPV45	227 bp	HPV33
240 bp	HPV58	340 bp	HPV39	520 bp	HPV31
360 bp	HPV52	425 bp	HPV18	325 bp	HPV16

Table 7. Results for controls

Control	Which step of test is controlled	Specific bands in the gel 267-325 bp	Specific bands in the gel 723 bp	Interpretation
Neg. control	DNA isolation	No	No	Valid result
DNA - buffer	Amplification	No	No	Valid result
Internal control	Amplification	No	Yes	Valid result
HPV C+	Amplification	Yes	No	Valid result

CHAPTER FOUR

RESULTS

4.1 Group A

4.1.1 Epidemiological Findings

This is a cross-sectional laboratory base study in which one hundred and nineteen (n=119) patients from Maternity and Children Hospital (MCH) suffering from bacterial vaginosis were enrolled. (Figure1).

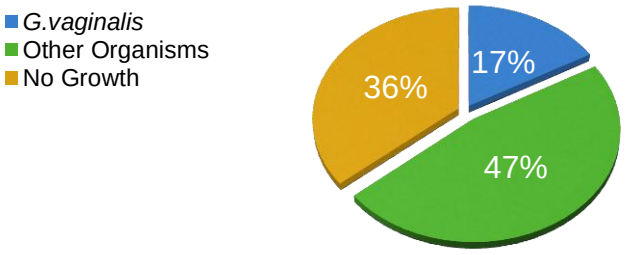

Figure1. Distribution of different pathogenic organisms among enrolled subjects.

4.1.1.1 Distribution of *G. vaginalis* and Age Groups

All candidates with *G.vaginalis* were classified into four age groups age group were found to be more affected (20-29) 50% followed by age group (30-39) 30% and (less than 20 and more than 40 years10% as shown in Figure 2.

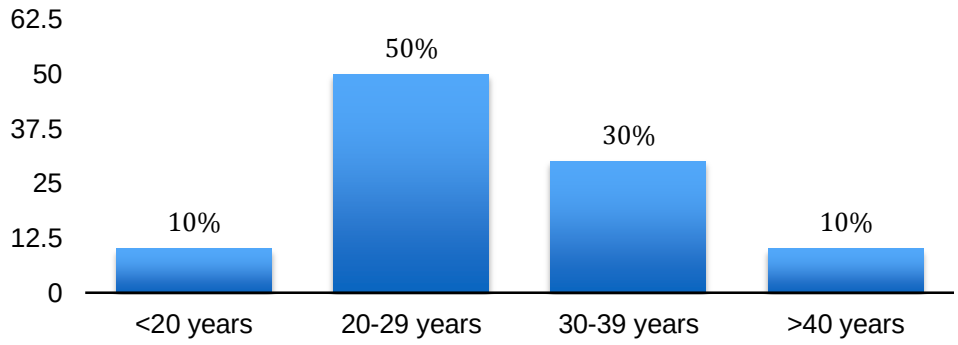

Figure 2. Distribution of *G.vaginalis* among patients according to age groups

4.1.1.2 Vaginal Infection and Pregnancy

In this study 75 pregnant women were included (75/119, 63.0%) as shown in Table 8 and Figure 4.

Table 8. Vaginal Infection and Pregnancy

Infection	Pregnant	Non pregnant	Total	Percentage
Vaginal infection	46	30	76	63.9
No infection	29	14	43	36.1
Total	75	44	119	100

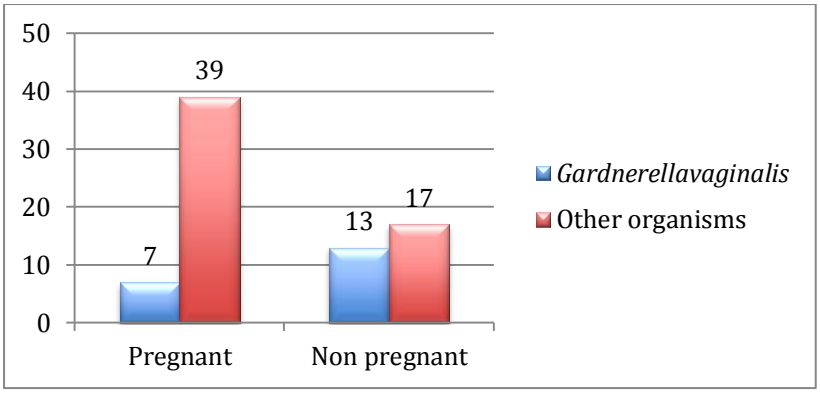

Figure 3. Frequency of *G. vaginalis* among Pregnant women**.**

4.1.2 Bacteriological Findings

4.1.2.1 Frequency of the Isolates

The data obtained in this study confirmed clearly the existence of vaginal infection among 76/119 (64.7%) subjects, 20/76 (26.3%) was *G.vaginalis* out of them eight as co-infection with other organism. On the other hand organisms other than *G.vaginalis* were detected in this study these include: *Candida albicans* 35/76 (46.1%) out of them four as co-infection with *Streptococcus* spp., 21/76 (27.6%) out of them four as co-infection with *Candida albicans* as shown in (Table 10 and Appendix IV).

Table 9. The Frequency of Isolated Organisms among Enrolled Subjects**.**

Number	Growth	Frequency	Percentage
1	*Gardnerella vaginalis*	20	26.3
2	*Candida albicans*	35	46.1
3	*Streptococcus species*	21	27.6
Total		76	100

4.1.2.1 Results of Gram's stain and other Biochemical Tests

The use of direct and indirect Gram's stain from high vaginal swab beside other important biochemical tests in this regards play important role in the identification of the causative agents (Appendix IV).

4.1.3 PCR Results

Polymerase chain reaction was used to confirm the conventional results. From the 20 specimens which were tentatively identified as *G. vaginalis* were directly subjected to PCR. In this regards *G.vaginalis* were detected among (17%)20/119 from the enrolled subjects (n, 119) , which show a band typical in size (210 bp) to the target gene as indicated by the standard DNA marker (Figure 7).

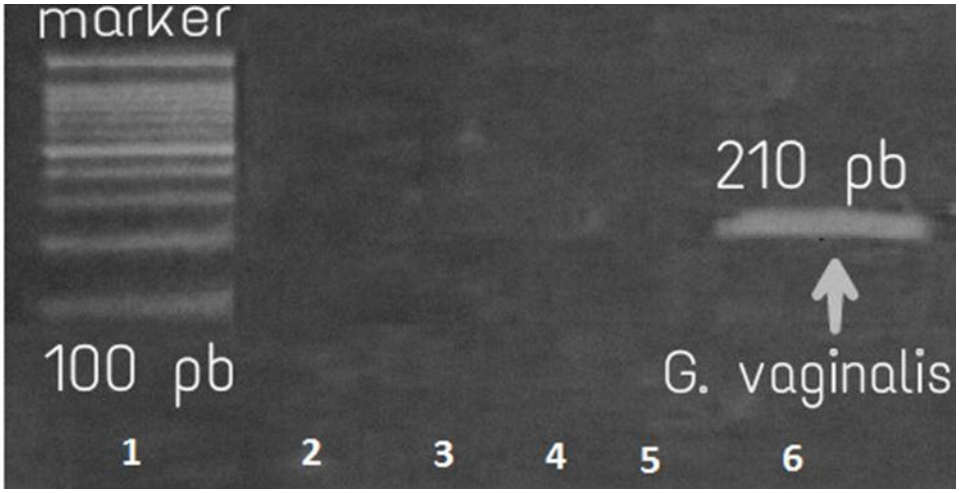

Figure 4. 2% agarose gel electrophoresis of PCR products: lane 1: DNA marker (1000 bp); lane 2: negative control; lanes (3, 4, 5) samples showing negative results; lane 6: sample showing positive result for *G. vaginalis* gene (210 pb).

4.2 Group B

4.2.1 Cytological Examination

Two hundred and thirty-eight (n=238) cervical smears were examined cytologically and assessed for the presence of HPV. The ages of the study population were from 15 to 80 years, with a mean of (39.7 ± 1.1 years); additionally, age groups 31-40 and 41-50 were found to be the most frequent in the study. The cytological investigation revealed that the incidence of abnormal epithelial cells is low constituting 13 (5.5%)13/238 cases which are distributed as follows; ASCUS in 4 (1.7%) cases, LSIL in 4 (1.7%), HSIL in 5 (2.1%) cases and the majority were in the age group (41-50) and (51-60). Among the enrolled subjects, 225/238, (94.5%) were reported free from any type of cervical cancer cells, among which 88/238

(37%) were cells with inflammations, 13/238 (5.5%) cells with Estrogen effect, 11/238 (4.6%) cells with Progesterone effect, 7/238(2.9%) vaginalis and the remaining 106 (44.5%) were normal cells . (Table 12).

4.2.2 Detection of HPV

HPV was detected in only six cases (2.5%) 6/238, with two of them present in patients with epithelial change (2/13) hand, the remaining four genotypes were detected within cells that appeared free from any types of neoplasia as follows; HPV genotype 52 was identified in two patients with inflammatory condition and genotype 33 was present in one case with inflammation. However, HPV-58 was confirmed in women with normal smears (Appendix IV and Tables 10,11,12 and 13).

Table 10. Distribution of HPV genotypes among enrolled subjects.

HPV genotype	Cytological diagnosis			Total
	Normal	Inflammation	HSIL	
HPV 16	0	0	2	2
HPV 33	0	1	0	1
HPV 52	0	2	0	2
HPV 58	1	0	0	1
Total	1	3	0	6

Table 11. Cytological results and age groups normal epithelial cells

Age group	Normal epithelial cells				Total
	Normal	Inflammation	Hormonal effect	Candida/ Trichomonas	
Less than 20	2	0	2	0	4
30-20	26	15	10	2	53
31-40	34	28	6	2	70
41-50	30	33	6	3	72
51-60	15	5	0	0	20
More than 60	6	4	0	0	10
Total	105	85	24	7	231

Table 12. Cytological results and age groups abnormal epithelial cells +HPV

Age group	Abnormal epithelial cells +HPV				Total
	ASC-US	LSIL	HSIL	HSIL+ HPV	
Less than 20	0	0	0	0	0
30-20	2	0	0	1	3
31-40	0	1	0	0	1
41-50	1	2	1	1	5
51-60	1	1	1	0	3
More than 60	0	0	1	0	1
Total	4	4	3	2	13

Table 13. Cytological results and age groups normal epithelial cells +HPV

Age group	Normal epithelial cells + HPV		Total
	Normal +HPV	Inflammation +HPV	
Less than 20	0	0	0
30-20	1	1	2
31-40	0	1	1
41-50	0	0	0
51-60	0	1	1
More than 60	0	0	0
Total	1	3	4

Figure 5. 3% agarose gel electrophoresis of PCR amplicons of HPV from cervical smears. Lanes: L= 1000 bp ladder; C1= positive control for HPV33 (325 bp); C2= positive control for HPV 16 (227bp); N= negative control; 1, 5 negative samples; 2,4 = HPV16 positive samples; 3= HPV33 positive sample.

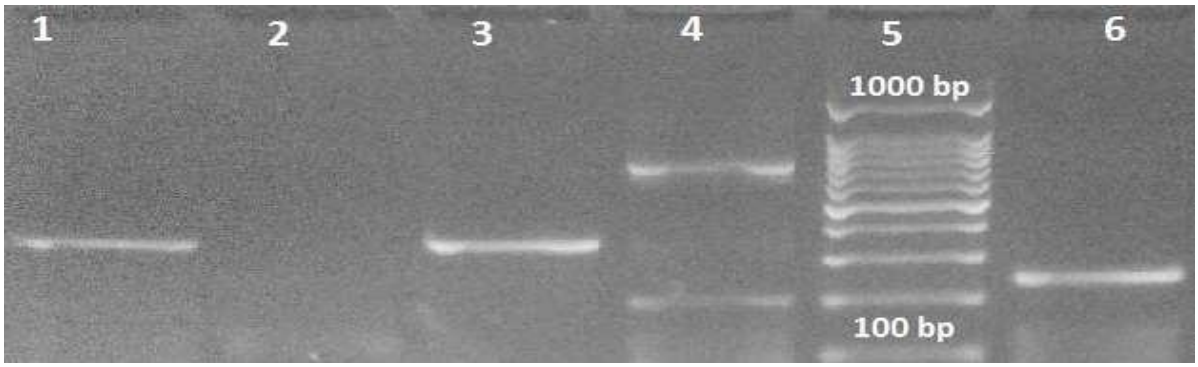

Figure 6. 3% agarose gel electrophoresis of PCR amplicons of HPV from cervical smears. Lanes: 5= 1000 bp ladder; 1= positive control for HPV 52 (360bp); 2=negative control; 3 = HPV52 positive sample; 4= internal control (723bp); 6= HPV58 positive sample (240 bp).

4.2.3 Detection of Bacterial Agents

Among the study subjects (n=170) our findings suggested different frequencies of target pathogens as follows: (4/170, 2.4%) *Gardinella vaginalis*, (4/170, 2.4%) *P. lacrimalis* and (13/170,7.6%) *L. iners* highest frequent as shown in and Table 14. Figure 4,7,8.

Table 14. Distribution of bacterial vaginosis among enrolled subjects

	PCR results	Frequency	Percentage
1	*Gardnerella vaginalis*	4	2.4
2	*Peptoniphius lacrimalis*	4	2.4
3	*Lactopacillus iners*	13	7.6
Total		21	12.4

PCR detected *G.vaginalis,* in (2.4%) of 170 clinical isolates, which show a band typical in size (210 bp) to the target gene as indicated by the standard DNA marker also it detected *L.iners* 7.6% 571 pb and *P. lacrimalis* 2.4% 186 pb Figure 7.12.13.

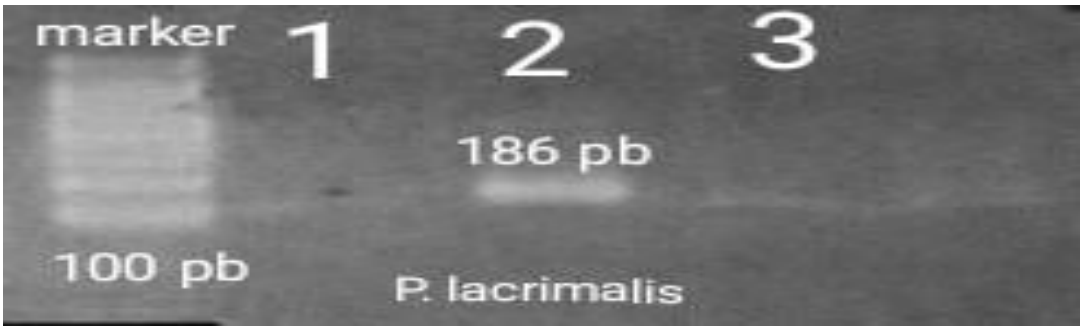

Figure 7 . 2% agarose gel electrophoresis of PCR product lane 1: negative control lane 2: sample showing positive results for *P.lacrimalis* (186pb), lane 3: sample showing negative result .

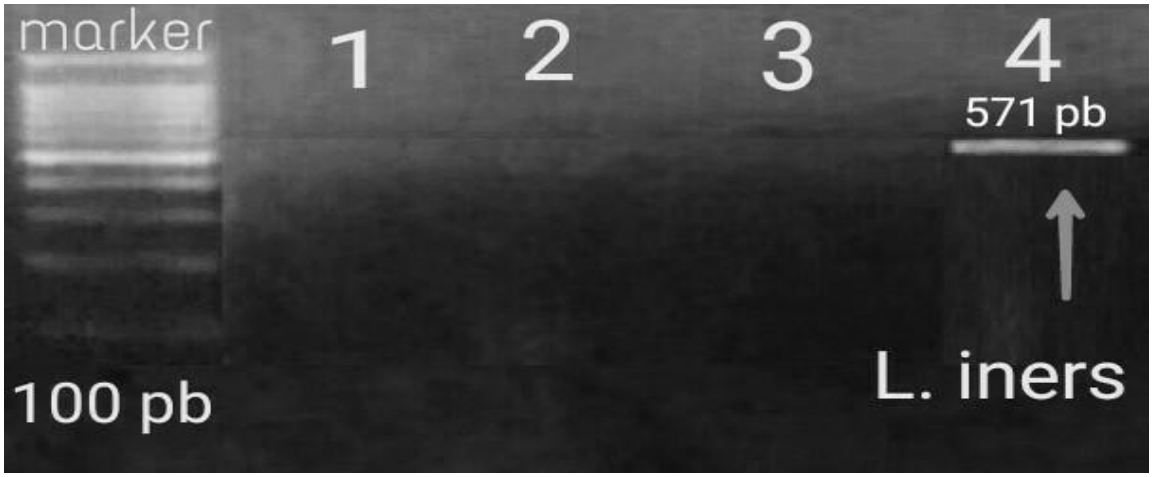

Figure 8. 2% agarose gel electrophoresis of PCR products: lane 1: negative control; lanes (2,3) samples showing negative results; lane 4: sample showing positive result for *L.iners*(571pb).

4.2.4 4 Bacterial vaginosis versus HPV

Bacterial vaginosis detect in twenty one women 12.4% two of them only have HPV 9.5% type 52 and 58 (Table 14).

Table 15 . Bacterial vaginosis and HPV positive case

HPV genotypes	Epithelial change	Bacterial vaginosis
16	2	0
33	0	0
52	0	2
58	0	0
Total	2	2

CHAPTER FIVE

5. DISCUSSION

For the first part of the study in which we aimed to determine the frequency of *G. vaginalis* among women in reproductive age with sings of bacterial vaginosis .

The results confirm the existence of vaginal infection in 63.8%76/119 of the study population, similar results were obtained in Riyadh, KSA by Tahany *et al.*, (2013) who reported 61.3% among enrolled women and this is an alarming of increased incidence of vaginal infection in KSA. Also, similar results were reported in Ghana by (Gloria and Daniel, 2013) as they confirmed that 66% of their studied populations were presented with vaginal infection.

The results also showed that the frequency of *G. vaginalis* were (26.3%) 20/76 of women with vaginal infection. In Riyadh, KSA, Tahany *et al.*, (2013) reported that 80.4% of enrolled women were infected with *G. vaginalis*. I suggested that high frequency of *G. vaginalis* in Riyadh because its large city and contain a lot of people from different Race. In England (69%) of infection with bacterial vaginosis was reported by (Jolly, 1983) and in America (87%) were reported by (Alla *et al.*, 2001).This may be du to lake of Islamic religious awareness regarding

sexuality issues in this areas. Similar results were listed in Ghana by (Gloria and Daniel, 2013), (28%) and in Bangladesh (25.5%) by (Begum *et al*., 2011), moderate to slightly high results were reported in Portugal by Debora, *et al.,* (2014) who found an infection rate of (36.9%).

In this study, the results of *Candida* spp. were (46.1%) 35/76. Lower percentage was obtained in Riyadh, KSA by (Tahany *et al.*, 2013) which was (16.3%) and in Ghana by (Gloria and Daniel, 2013) was (16%). However, in Tanzania, identical results (45%) were reported by (Namkinga, *et al*., 2005). This result shows the high prevalence of vaginal candidiasis in women of Al-Madinah Al-Munawarah , KSA. Isuggested that high prevalence of vaginal candidiasis due to our study group which contain pregnant women. Low immunity of pregnant woman increased occurrence of candida infections.

Streptococcus spp. was reported in (27.6%) 21/76 of this in the study population. This disagrees with both the low percentage (2.2%) found in Riyadh, KSA by (Tahany *et al.*, 2013) and the high percentage (58.7%) which was reported from Greece by (Tansarli, *et al*., 2013).This differences may be due to ethnic reasons.

In this study, the age group (20-29 years) was the one with the highest percentage of infection (50%) then (30-39 years) was with the moderate percentage (30%), and the lowest percentage was observed in the older

group (more than 40 years, 10%) and younger group (less than 20 years), (10%). That means age could be considered as a risk factor in the distribution of the bacterial vaginosis caused by *Gardnerella vaginalias*. A similar result was reported by (Tahany *et al.*, 2013) that the age group (21-30 years) had the highest percentage (52.2%) and in age group (31-40) had the moderate percentage (30.4%) but differs in age groups (less than 20 years and more than 40 years), which had the percentages (4.4% and 13%) respectively.

In this study bacterial vaginosis caused by *Gardnerella vaginalis* in pregnant women represent (15.3%)7/46 and in non-pregnant women represent (44.3%)13/30. Identical finding was reported in Bulgaria by (Raina *et al.*, 2013) who showed that in non-pregnant women the percentage was (44.3%) but it was different in pregnant women where the percentage was (41.1%). That mean there is increase of BV in non-pregnant women more than pregnant women.

Throw the questionnaire we found that BV was more common in educated women and in most women who used the IUD (intra uterine device) as a contraceptive and who are more likely to use vaginal douching. All BV infections are also detected in women whom their husbands was smoking.

Concerning the second part of the study, the incidence of abnormal epithelial cells is low, constituting 5.5% (13/238) of screened cases. This result is not far from that of Altaf (2008), who studied cervical neoplasia among Saudi women in the period from 1990 to 2004. Here, the author found that the incidence of abnormal pap smears was 4.7% of study group (5132 patients) and also reported the classification of abnormal pap smears in the study as follows; atypical squamous cell (2.4%), atypical glandular cells (1.1%), low scale squamous intraepithelial (0.6%) and high scale squamous intraepithelial (0.4%). Different malignant groups were observed which include: adenocarcinoma of the cervix, neuroendocrine carcinoma and squamous cell carcinoma.

This study showed that the most of precancerous lesions were distributed in the age group (41-50) followed by (51-60) years. This result totally disagree with Sawaya *et al.* (2000) who studied the percentage of cervical changes in a three years longitudinal study, their results indicated that the target cervical abnormalities were found to be predominant among women under 30 years old followed by age group of 30-49 and 50-64 years old respectively, while women over 65 years old showed the lowest frequency. Nevertheless, Saudi Cancer Report (SCR, 2011) showed evidences that cervical neoplasia among Saudi females were mostly found among age group of 15-44 years old. Our findings also suggested

that HPV-16 was detected among women of the age group 21-30 and 41-50 while HPV-33 was isolated at age group 51-60 years old. These results are not far from that of Bruni *et al.* (2010), who recorded cervical cancer among Saudi females to be most likely concentrated among the age group (41–45 years) followed by the age group (56–60 years) as has been observed in other communities.

One of the major concerns of this project is the estimation of the prevalence of HPV among suspected women. Different studies estimated that the prevalence of HPV among suspected females may exceed 99% worldwide (De Sanjose *et al*., 2010), whereas in KSA and other similar regions the ratio between HPV and cervical cancer is scanty (Hussain *et al*., 2012; Khorasanizadeh *et al*., 2012; Hammouda *et al.*, 2011; Alsbeih *et al.*, 2011; Al-Badawi *et al*., 2011).

On the other hand, many researchers noted that the situation of cervical neoplasia among Saudi women is comparable and the participation of HPV in this phenomenon is also within the normal range worldwide (Ghazi, 2014). The majority of these studies suggested that the most frequent genotypes were HPV 16 and 18 respectively (Alsbeih *et al.*, 2011; Al-Badawi *et al*., 2011). Furthermore, multiple infections caused by more than one genotype were recorded among Saudi females.(Ghazi *et al* .,2013) One important finding is the presence of epithelial cell

changes among 11 (84.6%) cases with the absence of any HPV genotypes, a result that was previously suggested by many researchers worldwide. Farnsworth (2011) observed significant prevalence (8%) of squamous carcinoma among Australian females identified negative for HPV. Similarly, Poljak *et al.* (2009) with the agreement of Tjalma *et al.* (2013) proved the occurrence of 12.6% of cervical cancer cases without any evidence of the existence of HPV as causal organism. This may provide evidences to adjust and standardize all the available diagnostic kits to be more reliable for a wide spectrum check of HPV genotypes (Sin *et al*., 2014). One more additional point, is the presence of HPV among 4 (30.8%) cases with normal cytological smears, which may be an inquiry to search for the any suspected participation of HPV in the initiation of future abnormalities among Saudi females, especially if we consider the appearance of these non-classical genotypes; 33 and 52, among cells with inflations. Similar findings which prove the role of these new genotypes were reported in this regard in the Middle East communities (Darnel *et al.,* 2010) and elsewhere (Bruni *et al.,* 2010; Sawaya *et al.,* 2000).

Based on the previously mentioned studies, this research finding totally agrees with the fact that the occurrence of cervical neoplasia among Saudi females is scanty compared to different part of the world. On the other hand, this study proved the existence of new genotypes beside HPV

16 (HPV 33 (16.7%) 1/6, HPV 52 (33.3%)2/6 and HPV 58 (16.7%)1/6 which were not previously observed in this region. However, Darnel et al., 2010, suggested that HPV 33 could be the most dominant genotype responsible for this condition in the Middle East followed by genotypes 18 and 16 respectively (Darnel *et al.*, 2010). These findings prove that there are genotypes other than 16 and 18 in charge of causing cervical cancer among Saudi women, a point that should be considered by researchers and specialists in the medical field, putting into consideration the impressive scanty distribution of HPV in this region.

On the other hand, the results reflected low ratio of bacterial vaginosis 21/170 (12.4%) as follow :*G.vaginalis* 4/170 (2.4%) , *P.lacrimalis* 4/170 (2.4%) and *L. iners* 13/170 (7.6%) among group B (cervical samples). This low ratio of Bacterial vaginosis result from the indirect site of the sample Cervical not original site for this bacteria so it is found in low numbers. BV detect in twenty one women two of them only have HPV 2/21 (9.5%)type 52 (31years) and 58 (28 years) but there is no epithelial change. On the other hand two of six positive HPV have epithelial change but there is no bacterial vaginosis .So I suggested that there is no association between BV and HPV in uterine cervical neoplasia .Different studies proved the association between bacterial vaginosis and cervical cancer (Platz-Christensen, *et al*., 1994; Barrington *et al.*,1997; Schiff *et*

al., 2000) cervical cytological abnormalities are found significantly more often in women with a disturbed vaginal flora, suggesting a possible link between BV and the development of cervical cancer. while other authors proved contrary findings in which they announced for negative relationship. Bacterial vaginosis is not important in the etiology of cervical neoplasia (Peters *et al* .,1995).

However, the possibility still exist that BV is in some way associated with the development of CIN, as a cofactor to human papillomaviruses (HPV). Therefore, BV was taken into consideration in series of studies on CIN (Sweet *et al*., 2000; Behbakht *et al* 2002 ; CDC; Pavic *et al* 1984; Platz-Christensen *et al* 1994; Peters *et al* .,1995; and Callahan *et al* ., 2003). In previous studies, the prevalence of BV in patients varies; 32-64% from STD clinics, 12-25% from gynecology outpatients clinics, 10-26% from antenatal clinics (Spiegel *et al* .,1980; Gravett *et al*.,1986; Eschenbach *et al*., 1988 and Discacciati *et al* .,2006) in their reports indicate the similarity of BV prevalence in both,18% of women with squamous intraepithelial lesion (SIL) and 12% of women without SIL. However, a higher rate of BV (33%) was found among women with high-grade SIL (Discacciati *et al* .,2006). Besides, study in Japan by Mikamo *,et al* (Mikamo *et al* .,1999) indicates that 50% of *Gardnerella vaginalis* and 80% of BV including other co-existing bacterial species was detected

in cervical cancer patients. The different prevalence of BV among these studies might be due to that different technique, diagnostic criteria and clinician's opinions in each study (Peters1995; Sikstrom *et al.*, 1997; Castle *et al* .,2001; Mao *et al* ; Boyle *et al.*, 2003; da Silva *et al.*, 2004; Watts *et al* ; Samoff ,*et al* .,2005; Figueiredo *et al* 2008; Verteram *et al;* Nam *et al* and Rahkola *et al.*, 2009). For example, the prevalence obtained using Nugent'scriteria was consistently higher as opposed to studies using clinical "Amsel criteria" (EvyGillet *et al* ., 2011). The study concluded very low prevalence of HR-HPV in routine cervical screening samples among suspected Saudi women. Additionally, weak relation between HPV and the incidence of cervical neoplasia was also observed.

5.1Conclusions

Referring to the results and findings obtained in this study we can conclude the following:

Among women with signs and symptoms of BV in group A; The results confirm the existence of vaginal infection and high frequency of *G. vaginalis* among saudi women in reproductive age**.** Age could be considered as a risk factor in the distribution of the bacterial vaginosis caused by *Gardnerella vaginalis*.

The result of PCR in this group confirmed the presence of *G. vaginalis* among enrolled subjects . *Gardnerella vaginalis* in pregnant women was low compared with non-pregnant women .Indirect smoking , vaginal douching,and IUD increased the incidence of BV.

Our results suggested decreasing prevalence of precancerous epithelial lesions in routine cervical screening samples, with scanty occurrence of HR- HPV compared with findings elsewhere.

There are genotypes other than 16 and 18 in charge of causing cervical cancer among Saudi women (HPV 33 HPV 52 and HPV 58). HPV-16 was detected among women of the age group 21-30 and 41-50 while HPV-33 was isolated in age group 51-60.

Among women with signs and symptoms of BV in group B; The results reflected low ratio of *G.vaginalis*, *P.lacrimalis* and *L. iners* among enrolled subjects. Low results due to the site of the samples (cervical) differ from group A(vaginal).

Bacterial vaginosis detect in twenty one women two of them only have HPV type 52 and 58 without evidence of epithelial changes. Also two of sex positive HPV have epithelial changes but without bacterial vaginosis infection. Here we found no association between BV and HPV in uterine cervical neoplasia.

5.2 Recommendations

For prospective future work we recommend the following:

Further researches on BV focus pathogenesis and treatment might be important to expand our knowledge on BV and its association with cervical cancer.

Further researches may concentrate on other HR-HPV and/or LR- HPV genotypes, which are currently not proved for this region.

The association of BV in cervical cancer among women worldwide requires a compulsory screening and treatment in different clinical conditions.

Staying away from the smoke source and minimizing the vaginal douching may be reduces BV.

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Appendix I
questionnaire

SHENDI UNIVERSITY

Bacterial Vaginosis and its Association with Uterine Cervical Neoplasia in Human Papilloma Virus Positive Cases in Al-Madinah Al-Monawarah Region, KSA

Name:……
……
Age:…….
……
Resident:.……
……..
Education:……
……
Occupation:……
……
Date of
marriage:……..……
Number of normal vaginal
delivery:…….....……
Do you use any contraceptive
:……...……

The type of contraceptive:

Pills ☐ Injection ☐ IUD ☐

Duration of uses of contraceptive……

Do you smoke: Yes ☐ No ☐

Do your husband smoke: Yes ☐ No ☐

Date of LMP:----------

Presence of STD: Yes ☐ No ☐

What is the type of

STD:……

What are the clinical symptoms of STD you

have:……

…

itching ☐ burning ☐ vaginal discharge ☐

bad odor ☐ Change of color ☐

Presence of visible lesion: Yes: ☐ No ☐ :

The clinical symptoms of this

lesion…….

Is there any previous abnormal cytology:

Yes: ☐ No ☐:

Resistant……..

Appendix II

Molecular reagents

10 X TBE buffer

Formula in grams per liter

Tris base…….108 gm

Boric acid…….…….…..55gm

EDTA……..…...40 ml of 0.5M

Deionized water……..……..…1 liter

Preparation

Amount of 108 gm. Tris base were weighed and added to 55gm of boric acid and 40 ml of 0.5M EDTA then dissolved into 1 liter deionized water pH 8.0.

1X TBE buffer

Formula in ml per liter

10 X TBE…….10 ml

Deionized water…….90 ml

Preparation

Ten ml of 10 X TBE buffer was added to 90 ml deionized water and heated until completely dissolved.

Ethidium bromide solution

Formula in grams per 1ml

Ethidium bromide……10 mg

Deionized water……I ml

Preparation

Twenty milligrams of ethidium bromide powder were dissolved into 1000 µl deionized water, and kept into brown bottle.

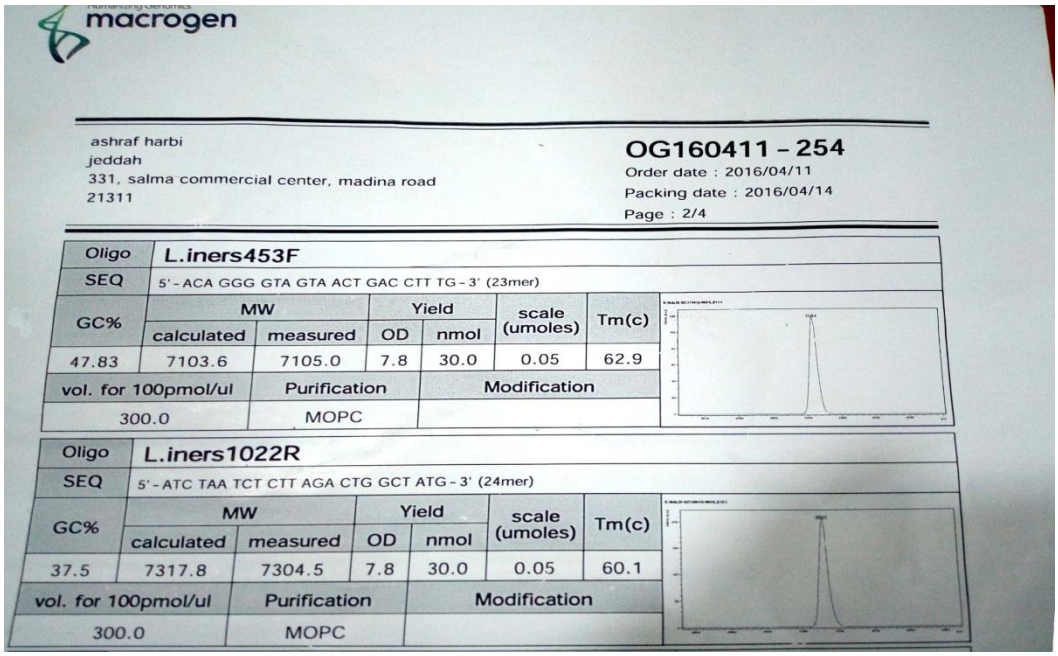
Agarose gel preparation

Amount of 2 gm of agarose powder dissolved by boiling in 100 ml 1X TBE buffer, then was cooled to 55°C in water bath, then,

1.5 µl of Ethidium bromides stock (10 mg/ml) per 100 ml gel solution for a final concentration of 0.5 ug/ml were added, mixed well and poured on to the casting tray that has been taped up appropriately and was equipped with suitable comb to form well in place. Any bubbles were removed and the gel was allowed to set at room temperature. After solidification, the comb was gently removed and the spacer from the opened sides was removed.

the opened sides was removed.

Appendix III
Primers



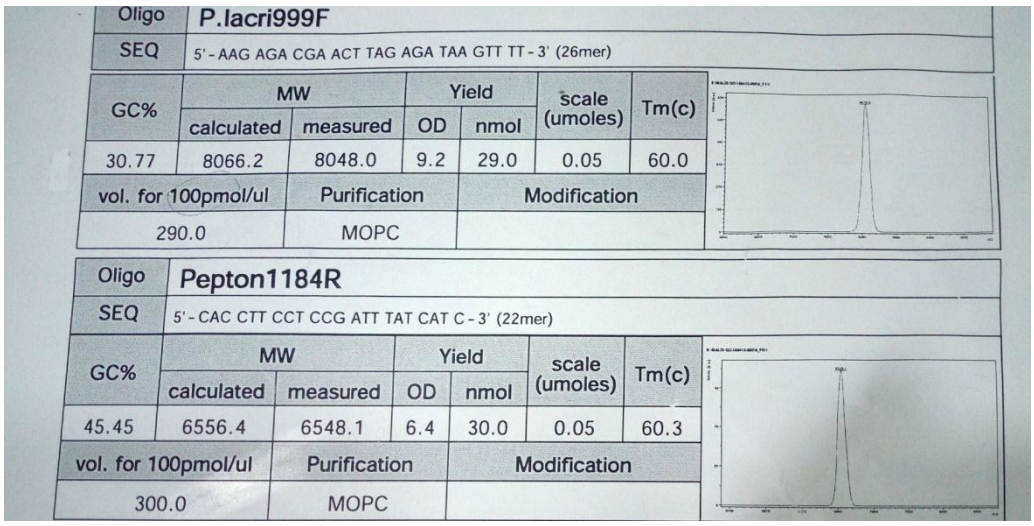
macrogen

ashraf harbi
jeddah
331, salma commercial center, madina road
21311

OG160411 – 254
Order date : 2016/04/11
Packing date : 2016/04/14
Page : 2/4

Oligo	L.iners453F						
SEQ	5'-ACA GGG GTA GTA ACT GAC CTT TG-3' (23mer)						
GC%	MW		Yield		scale (umoles)	Tm(c)	
	calculated	measured	OD	nmol			
47.83	7103.6	7105.0	7.8	30.0	0.05	62.9	
vol. for 100pmol/ul	Purification		Modification				
300.0	MOPC						

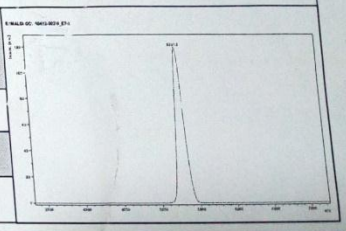
Oligo	L.iners1022R						
SEQ	5'-ATC TAA TCT CTT AGA CTG GCT ATG-3' (24mer)						
GC%	MW		Yield		scale (umoles)	Tm(c)	
	calculated	measured	OD	nmol			
37.5	7317.8	7304.5	7.8	30.0	0.05	60.1	
vol. for 100pmol/ul	Purification		Modification				
300.0	MOPC						



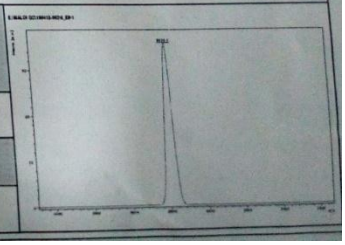
Oligo	P.lacri999F						
SEQ	5'-AAG AGA CGA ACT TAG AGA TAA GTT TT-3' (26mer)						
GC%	MW		Yield		scale (umoles)	Tm(c)	
	calculated	measured	OD	nmol			
30.77	8066.2	8048.0	9.2	29.0	0.05	60.0	
vol. for 100pmol/ul	Purification		Modification				
290.0	MOPC						

Oligo	Pepton1184R						
SEQ	5'-CAC CTT CCT CCG ATT TAT CAT C-3' (22mer)						
GC%	MW		Yield		scale (umoles)	Tm(c)	
	calculated	measured	OD	nmol			
45.45	6556.4	6548.1	6.4	30.0	0.05	60.3	
vol. for 100pmol/ul	Purification		Modification				
300.0	MOPC						

Oligo	G.vag_644F						
SEQ	5' - GGG CGG GCT AGA GTG CA - 3' (17mer)						
GC%	MW		Yield		scale (umoles)	Tm(c)	
	calculated	measured	OD	nmol			
70.59	5316.4	5301.3	6.2	32.0	0.05	59.6	
vol. for 100pmol/ul	Purification		Modification				
320.0	MOPC						



Oligo	G.vag_851R						
SEQ	5' - GAA CCC GTG GAA TGG GCC - 3' (18mer)						
GC%	MW		Yield		scale (umoles)	Tm(c)	
	calculated	measured	OD	nmol			
66.67	5549.6	5530.1	6.4	32.0	0.05	60.7	
vol. for 100pmol/ul	Purification		Modification				
320.0	MOPC						



Appendix IV

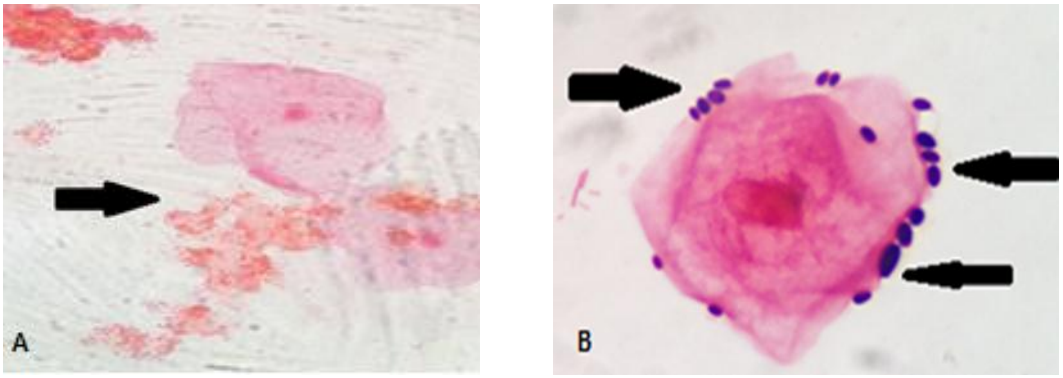
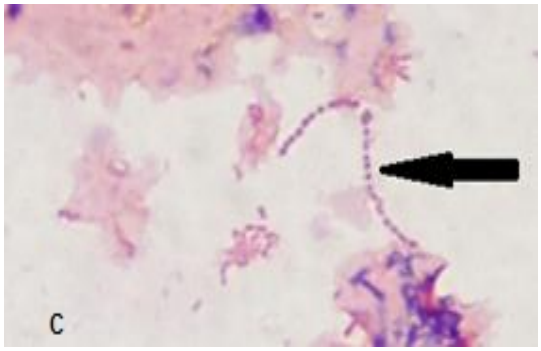


Figure 9. Direct Gram's stain from high vaginal swab of: A: *G.vaginalis* showing Clue cells; B: *Candida albicans* surrounded epithelium cell.



C: Direct Gram's stain from high vaginal swab *Streptococcus* spp. neighboring to the epithelium cell.

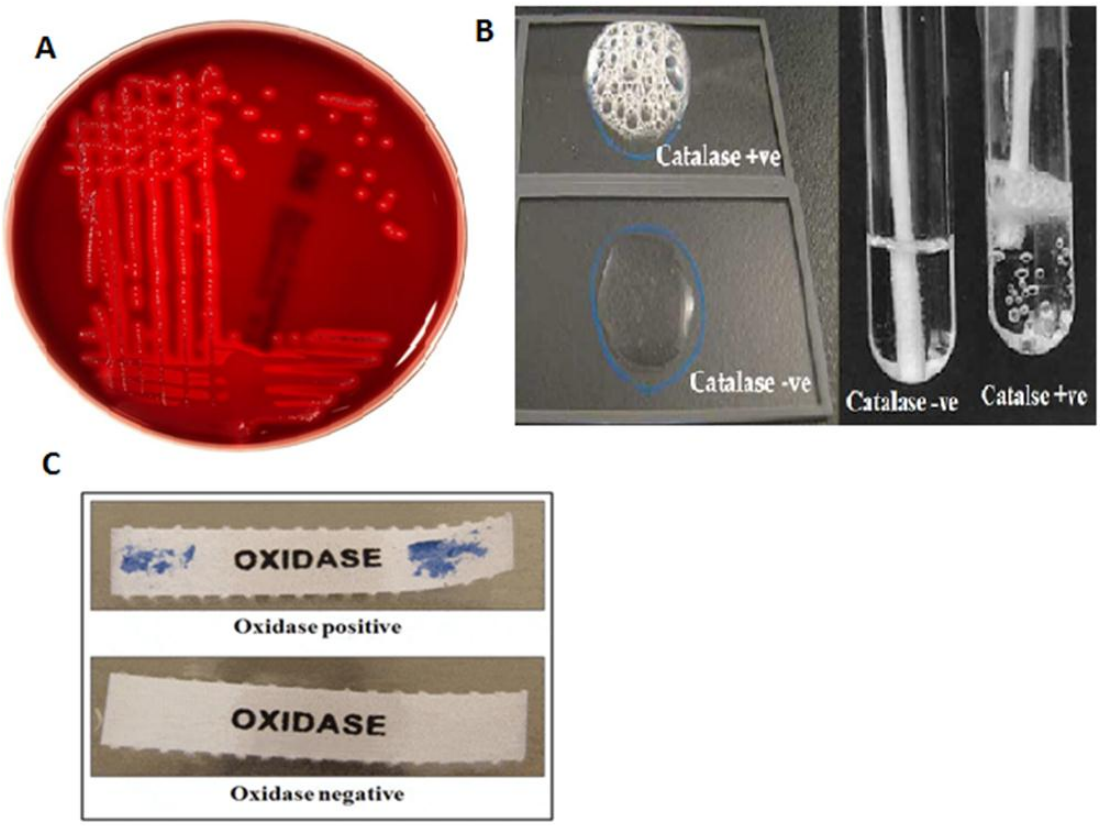


Figure 10.A: an overnight growth of *G.vaginalis* in Blood Agar producing beta hemolysis. B: catalase test ; right (positive result) and left (negative result) C: oxidase test with the presence of dark purple color

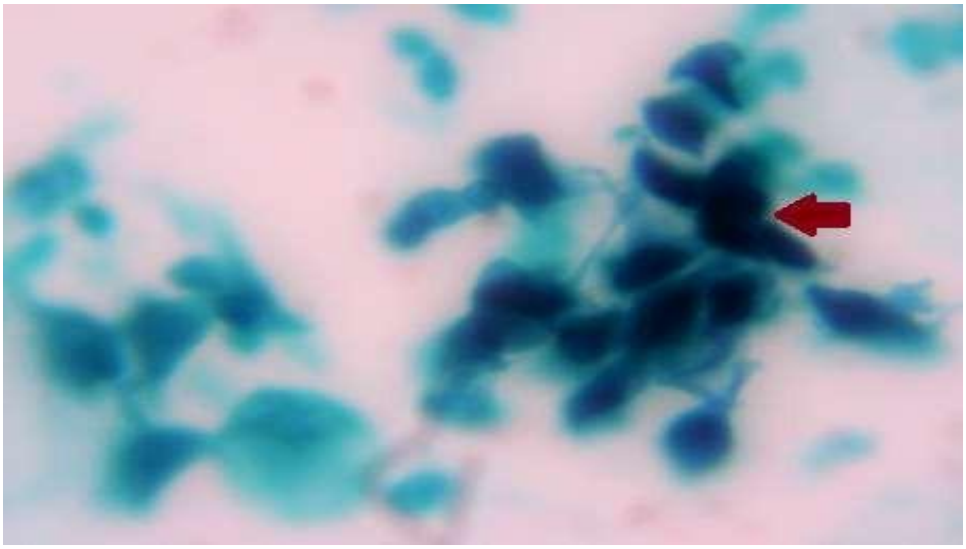


Figure 11. Cervical smear of 48 years old patient with HSIL, (HPV genotype 16), syncytial cluster of hyperchromatic cells with increased nuclear/cytoplasmic ratio and irregular nuclear membrane (liquid-based preparation, Thin Prep,X40).

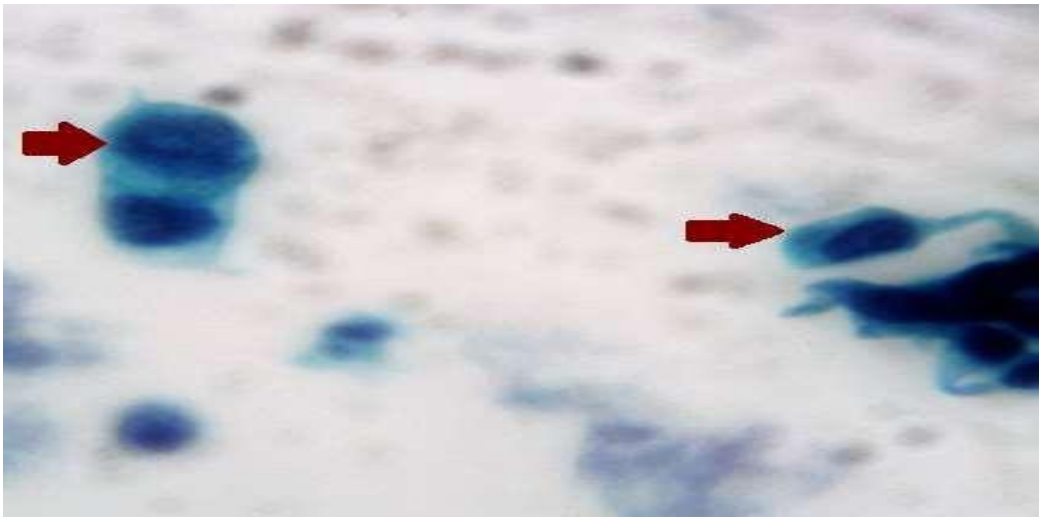


Figure 12. Cervical smear of 30 years old patients with HSIL, (HPV genotype16), enlarged cells with increased nuclear/cytoplasmic ratio with granular chromatin and slightly irregular nuclear membrane (liquid-based preparation, Thin Prep, X40).

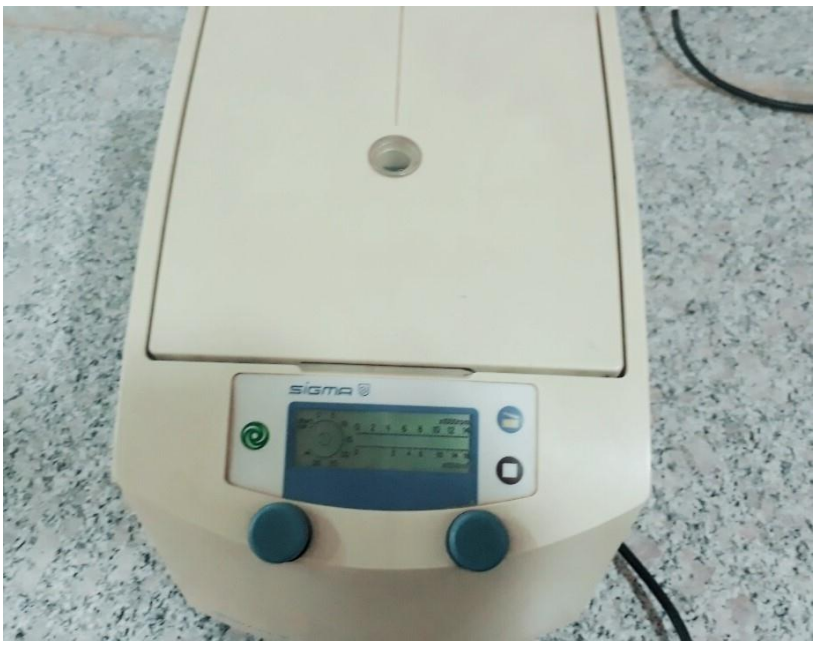


Figure (13) Microcentrifuge device

Figure (14) Thermocycle device

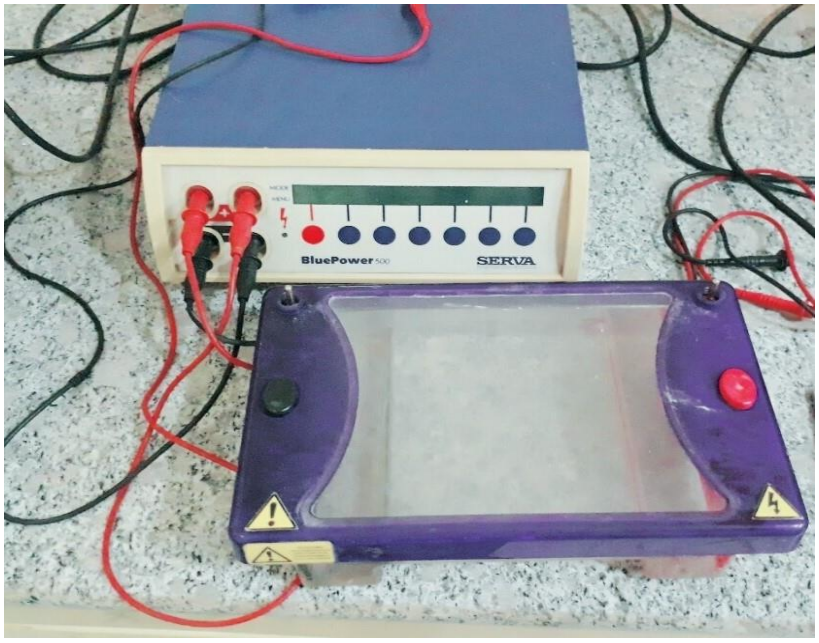


Figure (15). Gel electrophoresis and power supply device

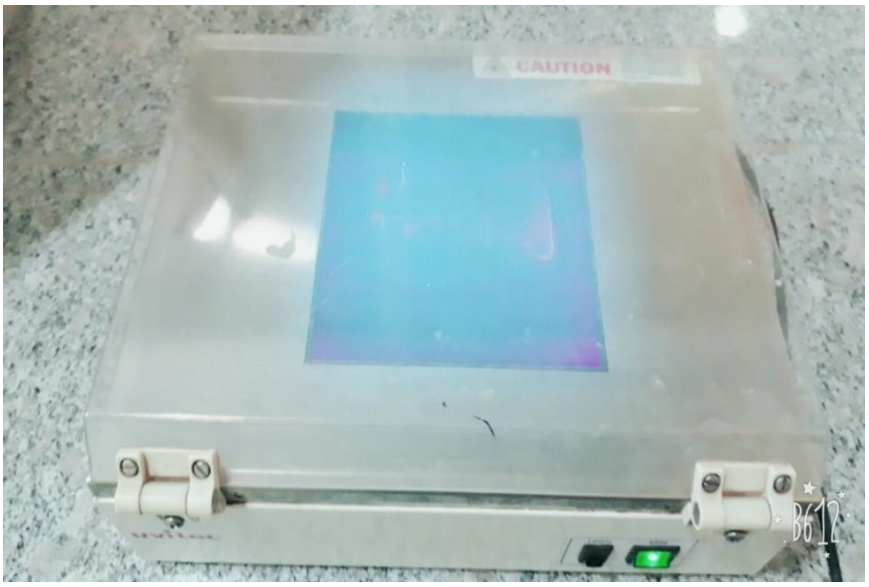


Figure (16). UV Light transilluminater device

Figure(17) Liquid Base Cytology

Appendix V

Maxime PCR PreMix Series *Research Use Only*

ISO 9001/14001 Certified Company

Maxime PCR PreMix Kit (*i*-Taq)

for 20μl rxn / 50μl rxn

Cat. No. 25025(for 20μl rxn, 96 tubes) **Cat. No. 25026**(for 20μl rxn, 480 tubes)
Cat. No. 25035(for 50μl rxn, 96 tubes)

DESCRIPTION

iNtRON's Maxime PCR PreMix Kit has not only various kinds of PreMix Kit according to experience purpose, but also a 2X Master mix solution.
Maxime PCR PreMix Kit (*i*-Taq) is the product what is mixed every component: *i*-Taq™ DNA Polymerase, dNTP mixture, reaction buffer, and so on- in one tube for 1 rxn PCR. This is the product that can get the best result with the most convenience system. The first reason is that it has every components for PCR, so we can do PCR just add a template DNA, primer set, and D.W.. The second reason is that it has Gel loading buffer to do electrophoresis, so we can do gel loading without any treatment. In addition, each batches are checked by a thorough Q.C., so its reappearance is high. It is suitable for various sample's experience by fast and simple using method.

STORAGE

Store at -20°C; under this condition, it is stable for at least a year.

CHARACTERISTICS

- High efficiency of the amplification
- Ready to use: only template and primers are needed
- Stable for over 1 year at -20℃
- Time-saving and cost-effective

CONTENTS

- Maxime PCR PreMix (*i*-Taq, for 20μl rxn) 96 (480) tubes
- Maxime PCR PreMix (*i*-Taq, for 50μl rxn) 96 tubes

Component in	20 μl reaction	50 μl reaction
i-Taq™ DNA Polymerase(5U/μl)	2.5U	5U
dNTPs	2.5mM each	2.5mM each
Reaction Buffer(10x)	1x	1x
Gel Loading buffer	1x	1x

Note : The PCR process is covered by patents issued and applicable in certain countries. iNtRON Biotechnology does not encourage or support the unauthorized or Unlicensed use of the PCR process. Use of this product is recommended for persons That either have a license to perform PCR or are not required to obtain a license.

PROTOCOL

1. Add template DNA and primers into Maxime PCR PreMix tubes (*i*-Taq).
   Note 1 : Recommended volume of template and primer : 3μl~9μl
   Appropriate amounts of DNA template samples
   - cDNA : 0.5-10% of first RT reaction volume
   - Plasmid DNA : 10pg-100ng
   - Genomic DNA : 0.1-1ug for single copy

   Note 2 : Appropriate amounts of primers
   - Primer : 5-20pmol/μl each (sense and anti-sense)
2. Add distilled water into the tubes to a total volume of 20μl or 50μl .
   Do not calculate the dried components

Example **Total 20μl or 50μl reaction volume**

PCR reaction mixture	Add	Add
Template DNA	1 ~ 3μl	2 ~ 4μl
Primer (F : 10pmol/μl)	1μl	2 ~ 2.5μl
Primer (R : 10pmol/μl)	1μl	2 ~ 2.5μl
Distilled Water	16 ~ 17μl	44 ~ 41μl
Total reaction volume	**20 μl**	**50 μl**

Note : This example serves as a guideline for PCR amplification. Optimal reaction conditions such as amount of template DNA and amount of primer, may vary and must be individually determined.

3. Dissolve the blue pellet by pipetting.
   Note : If the mixture lets stand at RT for 1-2min after adding water, the pellet is easily dissolved.
4. (Option) Add mineral oil.
   Note : This step is unnecessary when using a thermal cycler that employs a top heating method(general methods).
5. Perform PCR of samples.
6. Load samples on agarose gel without adding a loading-dye buffer and perform electrophoresis.

SUGGESTED CYCLING PARAMETERS

PCR cycle		Temp.	PCR product size		
			100-500bp	500-1000bp	1Kb-5Kb
Initial denaturation		94℃	2min	2min	2min
30-40 Cycles	Denaturation	94℃	20sec	20sec	20sec
	Annealing	50-65℃	10sec	10sec	20sec
	Extension	65-72℃	20-30sec	40-50sec	1min/Kb
Final extension		72℃	Optional. Normally, 2-5min		

EXPERIMENTAL INFORMATION

- ***Comparison with different company kit***

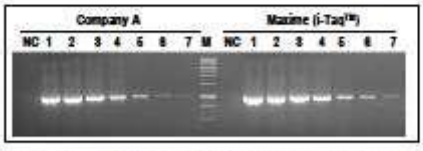


Fig.1. Comparison of Maxime PCR PreMix (*i*-Taq) and Company A's PreMix system by amplifying 1 Kb DNA fragment.
After diluting the λDNA as indicates, the PCR reaction was performed with Maxime PCR PreMix (*i*-Taq) and company's A product.
Lane M, SiZer-1000 DNA Marker; lane 1, undiluted λDNA; lane 2, 200 ng λDNA; lane 3, 40 ng λDNA; lane 4, 8 ng λDNA; lane 5, 1.6 ng λDNA; ; lane 6, 320 pg λDNA; lane 7, 64 pg λDNA; lane NC, Negative control

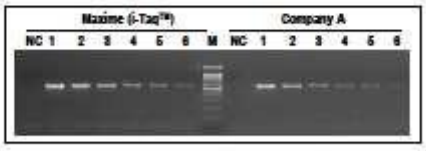


Fig.2. Comparison of Maxime PCR PreMix (*i*-Taq) and Company A's PreMix system by amplifying 570 bp DNA fragment (GAPDH).
Total RNA was purified from SNU-1 using easy-BLUE™ Total RNA Extraction Kit (Cat. No. 17061). And then, the first strand of cDNA was synthesized using Power cDNA Synthesis Kit (Cat. No. 25011). After diluting the cDNA mixture as indicates, the RT-PCR reaction was performed.
lane M, SiZer-100 DNA Marker; lane 1, undiluted cDNA; lane 2, 1/2 diluted cDNA; lane 3, 1/4 diluted cDNA; lane 4, 1/8 diluted cDNA; lane 5, 1/16 diluted cDNA; lane 6, 1/32 diluted cDNA; lane NC, Negative control

iNtRON BIOTECHNOLOGY
www.intronbio.com / info@intronbio.com
T. (0505)550-5600 / F. (0505)550-5660

Appendix VI

For research purpose only. Not for use in diagnostic procedures for clinical purposes. For IN VITRO USE ONLY.

ISO 9001/14001 Certified Company

SiZer™ DNA Markers

DESCRIPTION

iNtRON supplies a wide range of products for accurate size and mass estimations (quantitation) of nucleic acid fragments. Nucleic Acid Markers are available for sizing linear, or supercoiled DNA and single-stranded RNA fragments. A variety of these markers are available in the ready-to-use SiZer™ formats.

SiZer™ DNA Markers are ideal for determining the size of double-stranded DNA from 60~10,000bp base pairs. The SiZer™ DNA Markers consist of 7 ~ 15linear double-stranded DNA fragments. Several fragments are present at increased intensity to allow easy identification. All fragments are precisely quantified and mixed during the production.

For 5 µl loading, all fragments except typical band DNA fragments are 40 ng. The typical band of DNA fragments is 100 ng. These ladders are pre-mixed with loading dye and are ready to use.

All DNA Markers can be stained with RedSafe™ Nucleic Acid Staining Solution, ethidium bromide (EtBr) or other DNA stains.

CHARACTERISTICS

- Ideal for determining the size of DNA
- Stable for more than 12 months at - 20 ℃
- Ready to use without any handlings.

KIT CONTENTS

Product	Contents	Cat. No.
SiZer™-20 DNA Marker	0.3 ml	24071
SiZer™-50 plus DNA Marker	0.5 ml	24072
SiZer™-100 DNA Marker	0.5 ml	24073
SiZer™-1000 DNA Marker	0.5 ml	24074
SiZer™-1000 plus DNA Marker	0.5 ml	24075
SiZer™-15K DNA Marker	0.5 ml	24076
SiZer™- λDNA/HindIII DNA Marker	0.5 ml	24077

STORAGE

- Store at 4℃ and stable for more than 6 months. For more stable use, should be eliquoted and then stored at -20℃. (stable for more than 12 months)

GENERAL USE

- No DNase and RNase detected.
- Load 5 µl per each well of Agarose gel.

QUALITY CONTROL

Well-defined bands are formed during agarose gel electrophoresis. The DNA concentration is determined spectrophotometrically.
The absence of nucleases is confirmed by a direct nuclease activity assay.

ELECTROPHORESIS

- The 5 µl of ladder DNA was loaded, and then electrophoresed for 1hr at appropriate concentration of gel

PRODUCT USE LIMITATION

This product is developed, designed and sold exclusively for research purposes and in vitro use only. The product was not tested for use in diagnostics or for drug development, nor is it suitable for administration to humans or animals.

NOTICE BEFORE USE

- Do not heat before loading
- For quantification, adjust the concentration of the sample to equalize, it approximately with the amount of DNA in the nearest band of the ladder.
- Visualize DNA by staining RedSafe™, ethidium bromide (EtBr), or other DNA stains.

DETAIL INFORMATION

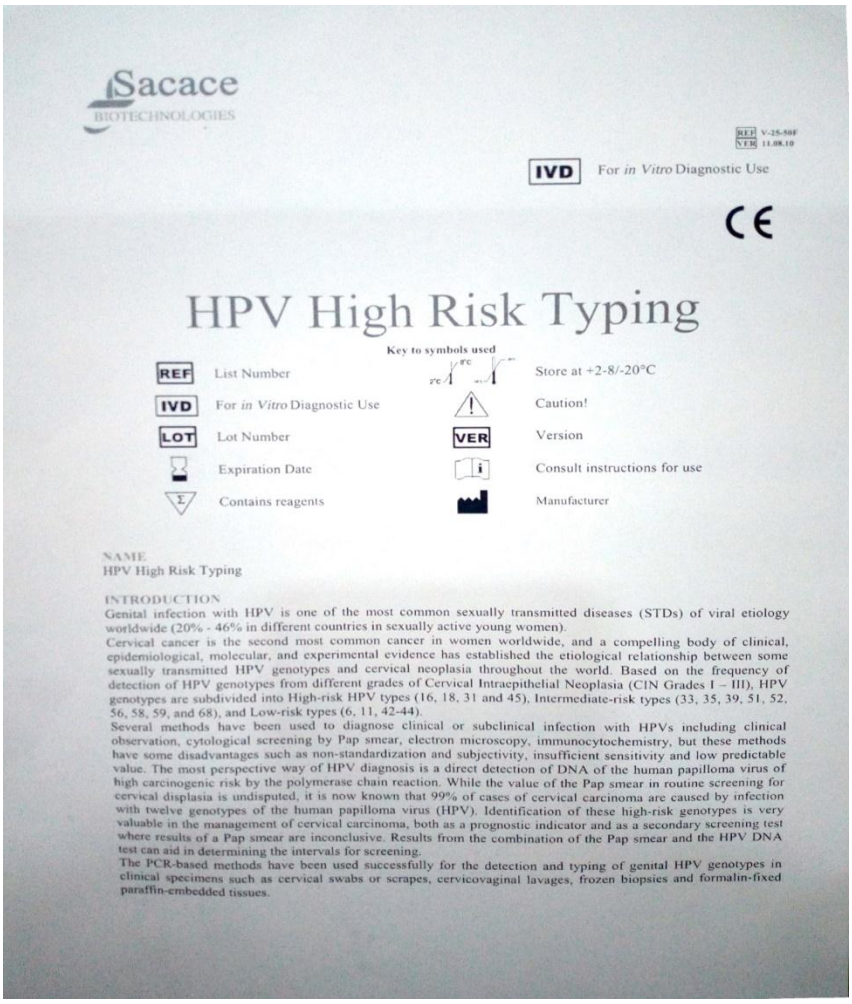
	Size range (bp)	Conc. (ng/µl)	Typical bands	Other bands	Loading Vol.	Band number	Contents
SiZer™ -20	60-300	128	100ng/5µl	40ng/5µl	5µl	13	60,80,100, 120,140, 160, 180,200,220, 240,260, 280,300
SiZer™ -50plus	50-500	128	100ng/5µl	40ng/5µl	5µl	13	50,100,150, 200, 250 300, 400,500, 600, 700,800,900,1000
SiZer™ -100	100-1500	100	100ng/5µl	40ng/5µl	5µl	11	100,200,300, 400, 500, 600, 700,800, 900, 1000,1500
SiZer™ -1000	250-10000	120	100ng/5µl	40ng/5µl	5µl	12	250, 500, 750, 1000,1500, 2000, 3000,4000, 5000, 6000,8000, 10000
SiZer™ -1000 plus	100-10000	144	100ng/5µl	40ng/5µl	5µl	15	100,200,300, 400,500, 700, 1000,1500,2000, 3000,4000,5000, 6000,8000, 10000
SiZer™ -15K	250-15000	85	125ng/5µl	50ng/5µl	5µl	7	250, 1000, 2500, 5000, 7500, 10000, 15000
SiZer™ - λDNA/HindIII	125-23130	100	350ng/5µl	-	5µl	8	125, 564, 2027, 2322, 4361, 6557, 9416, 23130

RELATED PRODUCTS

Product Name	Cat.No.
RedSafe™ Nucleic Acid Staining Solution (20,000x)	21411
DNA-spin™ Plasmid DNA Extraction Kit	17096/17097/17098
MEGAquick-spin™ Total Fragment DNA Purification Kit	17286 / 17287/17288
Maxime™ PCR PreMix (i-StarTaq)	25165
Maxime™ PCR PreMix (i-pfu)	25185

www.intronbio.com info@intronbio.com **iNtRON Biotechnology, Inc.**

Appendix VII



Sacace
BIOTECHNOLOGIES

REF V-25-50F
VER 11.08.10

IVD For *in Vitro* Diagnostic Use

CE

HPV High Risk Typing

Key to symbols used

REF	List Number		Store at +2-8/-20°C
IVD	For *in Vitro* Diagnostic Use		Caution!
LOT	Lot Number	VER	Version
	Expiration Date		Consult instructions for use
Σ	Contains reagents		Manufacturer

NAME
HPV High Risk Typing

INTRODUCTION
Genital infection with HPV is one of the most common sexually transmitted diseases (STDs) of viral etiology worldwide (20% - 46% in different countries in sexually active young women).
Cervical cancer is the second most common cancer in women worldwide, and a compelling body of clinical, epidemiological, molecular, and experimental evidence has established the etiological relationship between some sexually transmitted HPV genotypes and cervical neoplasia throughout the world. Based on the frequency of detection of HPV genotypes from different grades of Cervical Intraepithelial Neoplasia (CIN Grades I – III), HPV genotypes are subdivided into High-risk HPV types (16, 18, 31 and 45), Intermediate-risk types (33, 35, 39, 51, 52, 56, 58, 59, and 68), and Low-risk types (6, 11, 42-44).
Several methods have been used to diagnose clinical or subclinical infection with HPVs including clinical observation, cytological screening by Pap smear, electron microscopy, immunocytochemistry, but these methods have some disadvantages such as non-standardization and subjectivity, insufficient sensitivity and low predictable value. The most perspective way of HPV diagnosis is a direct detection of DNA of the human papilloma virus of high carcinogenic risk by the polymerase chain reaction. While the value of the Pap smear in routine screening for cervical displasia is undisputed, it is now known that 99% of cases of cervical carcinoma are caused by infection with twelve genotypes of the human papilloma virus (HPV). Identification of these high-risk genotypes is very valuable in the management of cervical carcinoma, both as a prognostic indicator and as a secondary screening test where results of a Pap smear are inconclusive. Results from the combination of the Pap smear and the HPV DNA test can aid in determining the intervals for screening.
The PCR-based methods have been used successfully for the detection and typing of genital HPV genotypes in clinical specimens such as cervical swabs or scrapes, cervicovaginal lavages, frozen biopsies and formalin-fixed paraffin-embedded tissues.