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Prevalence of *Staphylococcus* spp. and *Candida* spp. in pregnant and non pregnant women in genital tract with detection of *nor*A and *sdr*M genes

A Thesis

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By

Mustafa Salam Abdulkhaleq Noaman
B.Sc. Biology/Diyala university
(2017)

Supervised by

Assist. prof.Dr. Muthana A. Al-Mahdawi

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Signature:
Name:
Title: Assist. Prof. Dr. Muthana A. Almahdawi
Date: / /
( )

In view of the available recommendations, I forward this thesis for debate by the examining committee.

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Name:Prof. Dr. Ibrahim Hadi Mohammed
Head of Department of Biology
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Title: professor
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Signature:
Name:Dr. Hameed Salman Khamees
Title: Professor
Date: / /2020
*(**Chairman**)*

Signature:
Name: Dr. Zahraa Jaafar Jameel
Title: Assist. Professor
Date: / /2020
*(**Member**)*

Signature:
Name: Dr. Muthana A. Al-mahdawi
Title: Assist. Professor
Date: / /2020
*(**Supervisor**)*

Signature:
Name: Dr. Walaa Najm Abood
Title: Assist. Professor
Date: / /2020
*(**Member**)*

Approved by the council of the Faculty of College of Science, University of Diyala

Signature:
Name: Dr. Tahseen Hussein Mubarak
Title: Professor
Date: / /2020

*(**Dean of the College of Science**)*

Dedication

To the source of Love & tenderness
My mother

To the source of my life
My father

To the flowers of my life
My dear brothers and sister

Mustafa Salam Abdulkhaleq Noaman

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Summary

A cross sectional study was conducted to assess the prevalence of Gram positive bacteria and fungi that cause infections among female genital tract. This study included 500 vaginal swabs that were collected from women in Al-Batoul Teaching Hospital in Diyala-Iraq for the period from October 2019 to February 2020. The samples included 333 pregnant and 167 non-pregnant women with age group ranging from 13 – 59 years old. The results of the routine diagnostic test, that was confirmed by using VITEK comparte 2 system showed that there was a diversity of bacterial and fungi species, where the *S. aureus* bacteria had the highest rate of isolation in 23.8%, followed by *E. coli*, *S. epidermidis*, *Klebsiella spp*., *pseudomonas spp*., and *S. saprophyticus* (16.3%, 12.6%, 9.3%, 2.8% and 1.8% respectively). *C. albicans* was the predominant fungi isolated which was (21.9%) followed by *C. tropicalis*, *C. parapsilosis*, *C. glabrata* and *C. krusei* (4.2%, 3.7%, 2.8% and 0.50% respectively). The investigation of virulence factors showed that *S. aureus* can produce haemolysin with percentage of 90%. The results showed that (62.7%) of *S. aureus* isolates have the ability to adhere on the surface of epithelial cells while *S. epidermidis* and *S. saprophyticus* can adhere by (77.7% and 75% respectively). In this study, the results showed a variation of resistance to the antibiotics, Trimethoprim was the most resisted antibiotic by *Staphylococci* (53.6%) followed by Erythromycin and Trimethoprim/sulfamethoxazole (48.7% and 46.8% respectively), while 97.5% of bacterial isolates were sensitive to Imipenem, followed by Vancomycin and Clindamycin (68.2% and 67% respectively). The results showed that Caspofungin was the most effective antifungal against *Candida spp*. (85.9%), while Fluconazole was the least effective antifungal against *Candida spp*. by (63.3%). The results of the molecular study showed that there is a wide distribution of *nor*A (94.54%) and *sdr*M (90.90%) genes

among MDR isolates which harbor the characteristic of resistance to three or more antibiotic classes.

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Table of Abbreviations

No.	Symbole	The meaning
1	ABC	ATP binding cassette
2	AFST	Antifungal susceptibility test
3	AST	Antimicrobial susceptibility test
4	BV	Bacterial vaginosis
5	BW	Buffer wash
6	CA	Chromogenic agar
7	CONS	Coagulase negative staphylococcus
8	COPS	Coagulas positive staphylococcus
9	CV	Cytolytic vaginitis
10	EMB	Eosin methylene blue
11	MATE	Multidrug and toxic compound extrusion
12	MDR	Multi drug resistance
13	MFS	Major facilitator superfamily
14	MRSA	Methicillin resist S.aureus
15	MSA	Mannitol salt agar
16	MSSA	Methicillin sensitive *Staphylococcus aureus*
17	NPV	Negative predictive value
18	PBS	Phosphate buffer saline
19	PCR	Polymerase chain reaction
20	PIV	Pelvic inflammatory disease
21	PPV	Positive predictive value
22	RND	Resistance nodulation division

23	**SMR**	**Small multidrug resistance**
24	**TEA**	**Tris acid Ethylenediamineteracetic acid**
25	**TMA**	Trimetylamine
26	**TV**	**Trichomonas vaginalis**
27	**VISA**	**Vancomycin intermediate S.aureus**
28	**VVC**	**Vulvovaginal candidiasis**

CHAPTER ONE

Introduction

Chapter 1. Introduction

The natural Microbial flora of the vagina is currently considered as an important factor to protect the vagina from different types of causing the pathogenic diseases, where there are many types of microorganisms, the most important of which are lactic acid bacilli that contribute to maintaining the acidity of the vagina throughout the production of acid by fermenting to the collagen in the lining cells. These mechanisms seem to help preventing various types of pathogenic bacteria from settling in the vagina and causing infection as well as producing many inhibitors such as hydrogen peroxide H2O2 bacterocin (Nardis *et al.,* 2013; Petrova *et al.,* 2015), vaginitis occurs because of the excessive growth of bacteria, Increase in vaginal secretions, most often, it smells odors or fish and the secretions are white or gray and cause heartburn when urinating and itching (Donders *et al.,* 2014; *Sharma et al.,* 2014). Bacterial vaginosis B.V is known as an imbalance in vaginal microorganisms with a decrease in the number of Lactobacilli, where most cases begin with the formation of Biofilm which helps in the growth and prosperity of opportunistic bacteria (Bennett, 2015). Vaginal infection increases the risk of early birth among pregnant infected patient women. The infected patient women aged between (14-49) years affected where the B.V is more common in infected patient women of reproductive age (Queenan *et al.,* 2012). Vaginitis may sometimes affect infected patient women after menopause and iron deficiency may be associated with bacterial vaginosis at an early stage of pregnancy (Verstraelen *et al.,* 2005). The existence of female partner increases the risk of B.V with rate 60% where the bacteria were isolated associated with the occurrence B.V of the male genitalia and transmitted after intercourse from female to male (Amaya-Guio *et al.,* 2016). Vaginal conditions could be diagnosed using known Amsel's clinical criteria

including pH measurement, vaginal discharge appearance, clue cells on microscopy and whiff test (Amsel *et al.,* 1983). However, these requirements alone can't identify the cause of vaginal illness (Anderson *et al.,* 2004). According to the Centers for Disease Control and Prevention, there are various guidelines can be used for the diagnosis of vaginal infections, such as wet mount microscopy, direct Gram staining, potassium hydroxide preparation, routine culture, Papanicolaou smear, or DNA probes (CDDEP, 2015).The infection caused by *Candida* species is termed as vulvovaginal candidiasis (Maganti, 2011). *Candida spp.* is the most causative agents of vulvovaginitis in women (Mohammed *et al.,* 2017).It has almost 150 strains from which about 50% of all infections are caused by *Candida albicans,* but there are at least four other pathogenic species of this fungus, namely *Candida glabrata, Candida krusei, Candida parapsilosis and Candida tropicalis* (Singh *et al.,* 2017). An inflammation of the vagina is termed vulvovaginitis, (James *et al.,* 2006).Recently, the incidence of systemic candidiasis which is caused by *C. albicans* has increased. Usually, for treatment of systemic fungal infections, azoles such as fluconazole are used, but one of the biggest problems faced in the clinical practice is the emergence of resistance because of mutations occur for most of these azoles drugs currently used. Therefore, the most urgent challenge in pharmaceutical research is the discovery and the development of new antifungal from plant or microbial sources. (Alireza and Fahimeh, 2013). The immune system mechanisms for defensing against fungal infections are numerous, and range from protective mechanisms that were present early in evolution (innate immunity) to sophisticated adaptive mechanisms that are induced specifically during infection and disease. (Blanco and Garcia, 2008). Vulvovaginal candidiasis is common in adults, (70-75%) of women are affected by this infection at least once in their lives (Sobel, 2007). Pregnant women or women who used contraceptives usually

suffered from Vulvovaginal candidiasis, infection appears to be red ulcers with characteristic edges with thick white or yellow secretions with burning and pain in infection area (Boyd, 1998). Multidrug resistant (MDR) is a vexed term, from 1980 it was used to mean "resistant to multiple agents" without the number or types of agents being specified, more recently, the European Centre for Disease Prevention and Control (ECDC) has attempted to formalize the term as "resistant to three or more antibiotic classes" (Hawkey *et al.*, 2018).

This study was aimed to:

1- Determination of the prevalence of bacterial vaginosis caused by gram positive bacteria using the clinical criteria and the culture-dependent diagnosis.

2- Determination of the prevalence of fungal infections of female genital tract.

3- Determination of the accuracy of the diagnostic tests.

4- Antimicrobial susceptibilities of the isolates that cause the infection.

5- Detection the genes that are responsible for bacterial resistance to antibiotics for the MDR isolates.

CHAPTER TWO

Literature Review

Chapter 2. Literature Review

2.1 Vaginal infections

Worldwide, total disability-adjusted life years (DALYs) due to gynecologic diseases increased from 9292.8 thousands in 2005, to 10255.1 thousands in 2015 (kassebaum *et al.,* 2016). Vaginal infections with bacterial vaginosis, vulvovaginal candidiasis and trichomoniasis are consider a global health problem for women (Go *et al.,* 2006). Vaginitis can be defined as an inflammation and infection of vagina commonly encountered in clinical medicine (Yenidunya *et al.,* 2012). Diverse spectrums of pathogenic microorganisms were observed in the vaginal microflora. Bacterial vaginosis, candidiasis and trichomoniasis are responsible for majority of vaginal infections in women during the reproductive age (Spinillo *et al.,* 1997; Yenidunya *et al.,* 2012).

Abnormal vaginal discharge, itching, burning sensation, irritation and felling discomfortable are frequent complaints among patients attending obstetrics and gynecology clinics. However, a number of vaginal infections present with few or no symptoms (Adeyeba *et al.,* 2003). Vulvovaginal candidiasis (VVC) consider one of the most frequent infections in women of reproductive age. Approximately 75 % of adult women have at least one episode of vaginitis by candida during their life time (Adeyeba *et al.,* 2003; Yenidunya *et al.,* 2012). Unfortunately, about 40 – 50 % of women who had a first episode is likely to present a recurrence and 5% may present a form of "recurring" characterized by at least three or more episodes of infection per year (Prospero, 2014).

Trichomonal vaginitis (TV) is the most common sexually transmitted disease (Prospero, 2014). It is caused by a parasitic protozoan *Trichomonas vaginalis* (Yenidunya *et al.,* 2012). Globally, TV affects approximately 57–

180 million people, with the majority living in the developing countries (Chalechale and Karimi, 2010). However, in the most cases TV is asymptomatic. In women, TV affects more frequently between 20 - 40 years old and is quite rare before puberty and postmenopausal age (Prospero, 2014). The symptoms of TV are mainly characterized by vaginal discharge with gray or greenish-yellow fluid rather frothy, foul-smelling, intense itching, edema cervix redness, the sensation of itching, dyspareunia and postcoital bleeding, pelvic pain and urinary symptoms (Yenidunya *et al.*, 2012 ; Prospero, 2014).

Bacterial vaginosis (BV) is the most common cause of abnormal vaginal discharge among women of reproductive age. The prevalence of BV is about 30 % in women of reproductive age (Yenidunya *et al.*, 2012; Prospero, 2014). BV is characterized by raised vaginal pH and milky discharge in which normal vaginal flora (Lactobacilli) is replaced by a mixed flora of aerobic, anaerobic and microaerophilic species. Anaerobic organisms like *Gardnerella vaginalis, Prevotella spp., Mycoplasma hominis, Mobiluncus spp.* colonize vagina predominantly in BV (Prospero, 2014; Lamichhane *et al.*, 2014).

Gonococcal infections are the second most common prevalent sexually transmitted bacterial infections causing substantial morbidity worldwide each year. Gonorrhea is a potent amplifier of the spread of sexually transmitted human immuno deficiency virus (HIV) (Hng *et al.*, 2009). Various studies across the world have shown that women with BV are more likely to be co-infected with, *T. vaginalis, N.gonorrhoeae* and HIV (Lamichhane *et al.*, 2014). Aerobic vaginitis has been identified for a smaller proportion of women whose microbiota (lactobacilli) is dominated by facultative anaerobic or aerobic bacteria especially *S.aureus*, group B streptococci, *E.coli* and *Klebsiella spp* (Lamichhane *et al.*, 2014; Prospero, 2014).

Vaginal infections are associated with a significant risk of morbidity in women. If untreated they can lead to pelvic inflammatory disease (PID), which can cause long-term sequelae, such as tubal infertility, ectopic pregnancy, reproductive dysfunction and adverse pregnancy outcomes (e.g., preterm labor and delivery and low birth weight). Cervical dysplasia, increased risk of postoperative infection, HIV and Herpes simplex virus (HSV) -1 acquisition and transmission are also resulted from vaginal infections(Castellano *et al.,* 2010 ; Yenidunya *et al.,* 2012 ; Eshete *et al.,* 2013). Moreover, BV propagates viral replication and vaginal shedding of the HIV and HSV-2 (Trabert and Mirse, 2007). Investigators have also reported epidemiologic associations between trichomonas infection and subsequent cervical neoplasia and carcinoma (Saleh *et al.,* 2014). Various etiologies of vaginal infection results in a number of gynecologic complications and amplify HIV and HSV-1 transmissions. (Trabert and Mirse, 2007)

2.2 Vaginal normal flora

The vaginal flora was first reported by Albert Döderlein, as early as (1892). Döderlein found that the microflora was homogenously colonized with Gram-positive rods, which were designated the name "Döderlein's bacilli". Later, these bacilli have been identified as *Lactobacillus spp.* Lactobacilli which consider the predominant micro-organisms of the vaginal microbiota and play a major role in the maintenance of a healthy urogenital tract by preventing the colonization of pathogenic microorganisms. In healthy women, the vaginal microflora is dominated by Lactobacillus species, at a level of 10^7 -10^8 CFU g^{-1} of fluid, which exert a significant influence on the microflora of the environment (Boris and Barbes, 2000).

Lactobacilli play an important role in maintaining the female genital tract by some mechanisms, such as producing lactic acid that decreases vaginal pH

and prevents the occurrence of pathogenic bacteria, hydrogen peroxide, bacteriocin and surface binding protein which in turn can reduce the risk of bacterial vaginosis. When lactobacilli decrease, there is a reduction of hydrogen peroxide and consequently, overgrowth of facultative anaerobes occurs and bacterial vaginosis happens as facultative anaerobes can displace lactobacilli then rise in vaginal PH (Stojanović *et al.,* 2012). Furthermore, changes in innate immunity are responsible for the conversion of normal vaginal flora such as lactobacilli to facultative anaerobes that cause bacterial vaginosis (Redelinghuys *et al.,* 2016).

In consequence, a depletion of vaginal lactobacilli has been directly associated with an increase in the incidence of the genital and urinary tract infections (Pavlova *et al.,* 2002). The Lactobacili have been shown to produce biosurfactants and collagen binding proteins that function in inhibiting the pathogens adhesion and displace the pathogensm (De Gregorio *et al.,* 2020).

2.3 Anatomy of vagina

The vagina is a fibromuscular virtual tube, designed as the female copulatory organ. It extends from the vestibule, between the labia minora, and the uterus, the anterior urethra and bladder, and the posterior anal canal and rectum (Larsson *et al.,* 1977). Depending on rectum and bladder content, the vagina describes a (90°) angle with respect to the uterus axis, ascending posterosuperiorly. Considered as a parallelepiped, the vagina is composed of a posterior wall, separated from the rectum by the rectouterine pouch and from the anal canal by the fibromuscular structure of the perineal body. Microscopic examination reveals that the vaginal structure comprises [1] the inner mucosal epithelial stratum, [2] a lamina propria containing thin-walled veins, [3] the intermediate muscularis stratum, and [4] the external adventitial layer, the mucosal epithelium is hormone-dependent (estrogen) and changes

during the menstrual cycle, having the potential for a basal, nonsexual, moisture, Glycogen is fermented by Döderlein's bacillus, lowering the vaginal pH. The lamina propria of the mucosa contains blood vessels contributing to the diffusion of the vaginal fluid across the epithelium, elastic fibers, lymphatic vessels, and nerves (Jannini *et al.,* 2006).

2.4 Amsel's clinical criteria

In clinical practice, the most commonly used criteria are the clinical criteria set by Amsel. The diagnosis is positive for BV if it meets at least three of the four criteria. These criteria are: 1) presence of a typical discharge; 2) pH > 4.5, 3) positive whiff test; and 4) presence of clue cells in the wet smear;(Amsel *et al.*, 1983)

These criteria are:

1. A typical discharge, with BV, is thin. A normal discharge is floccular.
2. The pH is measured using paper the pH indicator. The sensitivity of pH-measurement for BV diagnosis is high but it has low specificity.

The study by Hallén and Co-workers found a 98.8 % sensitivity and a 71 % specificity

(Hallen *et al.,* 1987). However, in the study by Amsel, only 81% of the women with BV had a pH >4.5(Amsel *et al.*, 1983). In a study by Eschenbach and Co-workers, 97% of the women with BV had an elevated pH(Eschenbach *et al.*, 1988).

3. An increase in pH quickly releases amines, such as trimetylamine, which dissolve as an acid when the pH is low in the discharge.

A trimetylamine sniff test / whiff test is performed to detect odor by adding a drop of 10-20 % potassium hydroxide (KOH) to the speculum discharge, or by placing a drop of discharge on a slide and microscope and adding a drop of 10-20 % potassium hydroxide mixture and then smelling,

TMA is responsible for the smell of spoiled fish and can be detected in women with BV in vaginal specimens. Smelling directly from the speculum is known as a whiff test, a method proposed by Gardner and Duke in 1955(Gardner and Dukes, 1955). Amsel *et al*. introduced the addition of potassium hydroxide to the sniff test in 1983 (Amsel *et al.*, 1983).

Reported sniff test studies indicate a very high sensitivity and specificity. Hallén and Co-workers study's showed a 95 percent sensitivity and a 100 percent specificity (Hallén *et al.*, 1987). In a study by Thomason and Co-workers, the sensitivity was 91.1% and the specificity was 61.2%(Thomason *et al.*, 1990).

4. The fourth criterion are the wet-mount clue cells. A small proportion of the vaginal discharge is mounted on a microscope slide and one drop of saline is applied. The specimen is then covered with a glass cover and examined under a microscope. When the patient has BV, a significant number of *Gardnerella* morphotype bacteria cover some of the epithelial cells. Clue cells are vagina epithelial cells whose boundaries are difficult to see because there are so many bacteria found on the cell surface. The clue cell was one of the clinical criteria Gardner and Duke introduced. These cells provided the clue to the diagnosis (Gardner and Dukes, 1955).

Amsel's clinical criteria are one of the gold standards for diagnosing BV (Eschenbach *et al.*, 1988). In treatment studies, it is common to say that the patient is "cured" if none of the four Amsel's clinical criteria are present, and "improved" if the patient has one or two of the Amsel's criteria (a typical discharge and a pH> 4.5). If the patient meets the third and the fourth criteria (a positive whiff test and the presence of clue cells), treatment is considered to have failed(Larsson and Forsum, 2005).

2.5 Bacterial vaginitis

Bacterial vaginosis is a syndrome that caused by a certain group of bacteria that change in the vaginal environment as a result of the replacement of the natural vaginal flora caused by the absence of lactobacilli producing hydrogen peroxide with the growth of mixed microorganisms such as aerobic, anaerobic, and vaginal permanent bacteria as anaerobic bacteria make up 1% of the vaginal flora in natural women (Bodean *et al.*, 2013). There is also an increase in the pH of the vaginal environment, as it reaches more than 4.5 and repeated colic occurs in the vagina due to repeated sexual intercourse and the use of vaginal washings and smoking, which causes a reduction in the normal flora of the vagina, It is replaced by an overgrowth of the aerobic and facultative anaerobic bacteria (Ronald *et al.*, 2011).

The most important symptoms of vaginitis in affected women is the feeling of discomfort, itching, irritation and redness, and intense vaginal discharge with an unpleasant odor that resembles fish smell and increases after sexual contact (Hantoushzadeh *et al.*, 2010). It is one of the most common problems that accompany women during the childbearing period (Allsworth and Peipert, 2011), the use of contraceptives and hormone therapy are factors that increase the severity of bacterial vaginosis (Curran, 2010), and that candidiasis, urinary tract infection, frequent sexual intercourse and multiple partners increase the chances of bacterial vaginosis (Verstraelen *et al.*, 2012), vaginal discharge is one of the most common symptoms in cases of vaginal infections (Sobel *et al.*, 2012). The secretions may be caused by physiological factors, as they are usually secreted from the bartholin gland, the endometrium, the cervix, or the fluid in the fallopian tubes, along with the cells of the vaginal walls, including those caused by inflammatory diseases or some inflammatory disease. These secretions are affected by hormonal

changes during the menstrual cycle of adult women (Van Schalkwyk and Yudin, 2015).

The change in the physical and chemical properties of these secretions leads to an impact on the types of organisms present in the vagina and their abundance. Vaginal secretions are anti-bacterial for many bacteria, depending on the components of these secretions (Hickey *et al.*, 2011)

2.5.1 Vaginitis caused by Gram positive bacteria

The quantitative and qualitative bacteriological studies are performed on the normal bacterial flora of the vaginal and cervical sites, lactobacilli, diphtheroids, staphylococci, streptococci, and occasionally members of enterobacteriaceae were the dominant aerobic group of bacteria. (Levison *et al.*, 1977).The dominant anaerobic groups are composed of Gram positive bacilli which include lactobacilli, peptostreptococci (which now incorporate peptococci), and Bacteroidaceae. Group B streptococci (GBS) constitute a potentially important subgroup within the streptococci (Ledger *et al.*, 1975).

2.5.1.1 *Staphylococcus spp.*

Alexander Ogston is considered the first person to call this bacterium staphylococcus in (1882) when he first saw it in the pus found in the abscesses, and then Rosenbach isolate it and grew it in pure culture in (1884), as described two types of strains belonging to this genus which are *Staphylococcus aureus* and *Staphylococcus albus* which currently known as *Staphylococcus epidermidis* (Liu *et al.*, 2011). The bacterial colony is (0.5–1.5) mm in diameter, occurring singly, in pairs, in tetrads, in short chains (3-4) cells, and characteristically dividing in more than one plane to form irregular grapelike clusters. Gram positive, nonmotile, nonflagellate, nonspore-forming cell wall contains peptidoglycan and teichoic acid. Usually uncapsulated or limited capsule formation. *Staphylococcacea* are aerobic or

facultative anaerobes grows well in medium containing 10% NaCl, poorly in 15% NaCl (Duval *et al*., 2010). This bacteria is classified into two major groups which are coagulase positive staphylococci (COPS) represented by *Staphylococcus aureus* which is medically considered the most dangerous type , and coagulase negative staphylococci (CONS) represented by other types of staphylococci which *Staphylococcus epidermidis* medically represents the highest percentage among CONS , this is due to its possession of many virulence factors and its ability to form a biofilm on various surfaces (Namvar *et al.,* 2014).

Scientific classification of bacteria: (Holt *et al.,* 1994).

Domain:	Bacteria
Kingdom:	Eubacteria
Phylum:	Firmicutes
Class:	Bacilli
Order:	Bacillales
Family:	Staphylococcaceae
Genus:	*Staphylococcus*
Species:	*aureus* *epidermidis* *saprophyticus*

2.5.1.1.1 Coagulase positive *Staphylococcus*

2.5.1.1.1.1 Staphylococcus aureus

S. aureus are cocci that forming irregular grape-like clusters. They are non-motile, non-sporing and catalase positive. They grow rapidly and abundantly under aerobic conditions. On blood agar, they appear as glistening, smooth, entire, raised, translucent colonies that often have a golden pigment. The colonies are 2-3mm in diameter after 24hr incubation and strains are frequently β-haemolytic. Colonies may reach up to 6 to 8 mm in diameter after 3 days incubation. There are currently 2 subspecies of S. *aureus*; these are *S. aureus* subspecies *aureus* and *S. aureus* subspecies *anaerobius. Staphylococcus aureus* may be associated with severe infection and it is important to distinguish it from the opportunistic coagulase negative staphylococci. In routine laboratory practice, the production of coagulase is frequently used as the sole criterion to distinguish *S.aureus* from other staphylococci. It is also important to note that coagulase negative strains of *S. aureus* have been reported. Multi resistance to antibiotics has most often been associated with Meticillin resistant strains MRSA (Foster, 2002). *Staphylococcus aureus* produces virulence factors such as protein A, capsular polysaccharides and α toxin. Some strains of *S. aureus* produce toxic shock syndrome 1 toxin (TSST-1), Panton-Valentine Leucocidin or other toxins (Klein *et al.*, 2020).

2.5.1.1.2 Coagulase negative *Staphylococci* (CONS)

Coagulase negative staphylococci (CONS) are normal commensals of the skin, anterior nares, and ear canals of humans, they have long been considered as nonpathogenic, and were rarely reported to cause severe infections, However, as a result of the combination of increased use of intravascular devices and an increase in the number of hospitalized

immunocompromised patients, CONS have emerged as a major cause of nosocomial bloodstream infections, they are opportunistic pathogens which lack many of virulence factors associated with *S. aureus* (Becker *et al.*, 2014). There are more than 30 species of CONS. The taxonomy of these coagulase negative staphylococci (CONS) fall into clusters based on 16s rRNA sequences (Takahashi *et al.*, 1999).

2.5.1.1.2.1 Staphylococcus epidermidis

Staphylococcus epidermidis are approximately (0.5 to 1.5) µm in diameter and arranged in grape-like clusters. They are facultative anaerobes that can grow by aerobic respiration or by fermentation however some strains may not ferment. They form greyish-white, raised, circular, smooth, glistening, and translucent to slightly opaque, cohesive colonies approximately 1–2mm in diameter after overnight incubation, and are non-haemolytic on blood agar, they grow well at NaCl concentrations up to 7.5%, poorly at 10% and fail to grow at 15%.

They are either susceptible or slightly resistant to lysostaphin and are resistant to lysozyme. *S. epidermidis* is sensitive to Novobiocin, and this test distinguishes it from *Staphylococcus saprophyticus*, which is also coagulase negative, but Novobiocin resistant (Szemraj *et al.*, 2019).

2.5.1.1.2.2 Staphylococcus saprophyticus

They are positive for catalase and urease tests while they are negative for motility, coagulase, nitrate reduction and oxidase tests. They grow well on 10% NaCl agar, but only 11-89% strains tolerate 15% NaCl. Colonies appear as raised to slightly convex, circular, usually entire, 4.0 to 9.0mm in diameter, smooth, glistening, and usually opaque. Colony pigment is variable; however, most strains are not pigmented or might have a slight yellow tint which increases in intensity with age.

Two subspecies for *S. saprophyticus* exist: *S. saprophyticus subsp. bovis* and *S. saprophyticus subsp. saprophyticus*. The latter is more commonly found in human UTIs. *S. saprophyticus subsp. saprophyticus* is distinguished by its being nitrate reductase and pyrrolidonyl arylamidase negative while *S. saprophyticus subsp. bovis* is nitrate reductase and pyrolidonyl arymamidase positive (Hájek *et al.*, 1996). *S. saprophyticus* is resistant to the antibiotic novobiocin, a characteristic that is used in laboratory identification to distinguish it from *S. epidermidis*, which is also coagulase negative but novobiocin sensitive (Kloos, 1999).

2.6 Vulvovaginal candidiasis

Cervico-vaginal yeast infection is also known as genital candidiasis (Sobel, 2007; Ibrahim and yehia, 2017). It is a common gynecological problem in women of childbearing age (Kamath *et al.,* 2014). The infection occurs when there is overgrowth of the yeasts, mostly *Candida* species (Altayyar *et al.,* 2016). Candida species are usually coexisting with *Lactobacillus spp*. in the vagina. There is a balance between Candida, normal bacterial flora, and immune defense mechanisms, when this balance is disturbed; colonization is replaced by infection (Babić and Hukić, 2010). Under some conditions, such as reduced immunity, prolonged antibiotic therapy, use of steroids, pregnancy, use of oral contraceptives and diabetes, *Candida spp*. may become pathogenic and cause candidiasis (Grigoriou *et al.,* 2006).

Candida albicans is responsible for the largest number of symptomatic episodes of vaginal candidiasis (Ghaddar *et al.,* 2020). Non-albicans spp. are most commonly represented by *C. tropicalis, C. glabrata,* and *C. krusei*. Accurate species identification is important for the treatment of the Candida infections, as the non-albicans species of Candida continue to be increasingly

documented (Kazemi *et al.*, 2013). Nowadays, large varieties of *Candida spp.* identification procedures are available. Chromogenic media have been developed to produce rapid yeast identification, these media contain chromogenic substrates that react with enzymes secreted by microorganisms producing colonies with various pigmentations, and these enzymes are species specific, allowing organisms to be identified to the species level by their color and colony characteristics. Chrom agar *Candida spp*. has been shown to allow differentiation of Candidal yeasts by color and morphology (Mehta and Anupama, 2016).

The etiology of Candidal vulvovaginitis or vaginal thrush is *C. albicans* which is the most common cause (> 90%) of vaginitis. While the minority of these infections, is caused by non-*C. albicans spp*. (< 10 %), including *C. glabrata, C. krusei, C. parapsilosis and C. tropicalis* (Jombo *et al.*, 2010).

2.6.1 Candida species

2.6.1.1 *Candida albicans*

Candida albicans is an opportunistic pathogen. It is the normal flora of skin, oral cavity, gastrointestinal tract, the vaginal and the urinary tracts that has been known as "commensal" (Ferreira *et al.*, 2010). It accounts for up to (80 %) of *Candida spp*. which isolated from sites of infection and has been reported as a causative agent of all types of candidiasis. Environmental isolation has been usually from sources contaminated by human or animal waste such as soil, polluted water, plants and air (Kreger-van Rij, 1984).

2.6.1.2 *Candida tropicalis*

Candida tropicalis is a major causative agent of septicemia and disseminated candidiasis, especially in patients with lymphoma, leukemia and diabetes. It is the second most frequently encountered medical pathogen next

to *C. albicans*, and has been also found as part of the normal human mucocutaneous flora (Rippon, 1988).

2.6.1.3 *Candida parapsilosis*

Candida parapsilosis is an opportunistic human pathogen that may cause both superficial cutaneous infections and systemic disease. Other clinical manifestations include endophthalmitis and fungemia. Environmental isolation has been made from intertidal and oceanic waters, pickle brine, cured meats, olives, normal skin, and faces (Kreger-van Rij, 1984).

2.6.1.4 *Candida glabrata*

Candida glabrata is often the second or third most common cause of candidiasis after *C. albicans* (Wingard, 1995). The widespread and increased use of immunosuppressive therapy together with broad-spectrum antimycotic therapy increase the frequency of mucosal and systemic infections caused by C. glabrata (Pfaller, 1996). It has been isolated from patients with septicemia, pyelonephritis, pulmonary infections, endocarditis and hyperalimentation (Rippon, 1988).

2.6.1.5 *Candida krusei*

Candida krusei has been regularly associated with some forms of infant diarrhoea and sometimes with systemic disease. It has also been reported to colonize the respiratory, gastrointestinal, urinary tracts and patients having granulocytopenia. Environmental isolation has been made from beer, milk products, skin, faces of animals (birds) and pickle brine (Kreger-van Rij, 1984).

2.6.2 Pathogenesis and virulence factors of *Candida spp*.

Pathogenesis is the ability of microorganism to infect the host and produce disease that was facilitated by a number of virulence factors (Casadevall, 2007). There is a high incidence of candidiasis in

immunodeficient patients and knowledge of the virulence factors is important to comprehend the several ways by which Candida spp. avoid antifungal therapy and the immune response to the host causing infection. The infection can be cutaneous form (affecting on the mucosa) or generalized, affecting deep tissues as heart, lung, liver and brain (Batista *et al.,* 1999) Virulence factors facilitate adherence, cell multiplication at the mucosal surface followed by the production of proteinases and phospholipases that damage the tissues and cause an inflammatory response (Casadevall, 2007).

2.6.2.1 Adhesion and biofilm formation

The primary event in Candida infection is its adherence to host cells or medical devices surfaces through presence of specific proteins on its cell-wall called adhesin (Chandra *et al.,* 2001). Adherence of *Candida spp.* is seen as an essential early step in the establishment of the disease followed by cell division, proliferation, and subsequently biofilm development (Figure 2-1) (Ramage *et al.,* 2006). Biofilm formation is a potent virulence factor for a number of *Candida spp.* resulting in enhances adherence, as it confers significant resistance to antifungal therapy by limiting the penetration of substances through the matrix and protecting cells from host immune responses (Mukherjee and Chandra, 2004).

C. albicans biofilm formation is associated with the dimorphic switch from yeast to hyphal growth, and the biofilm structure involves, generally, two distinct layers: a thin, basal yeast layer and a thicker, less compact hyphal layer. It was supposed that the formation of mature biofilms and subsequent production of extracellular matrix was strongly dependent upon strain, species and environmental conditions (pH, oxygen, medium composition) (Jain *et al.,* 2007).

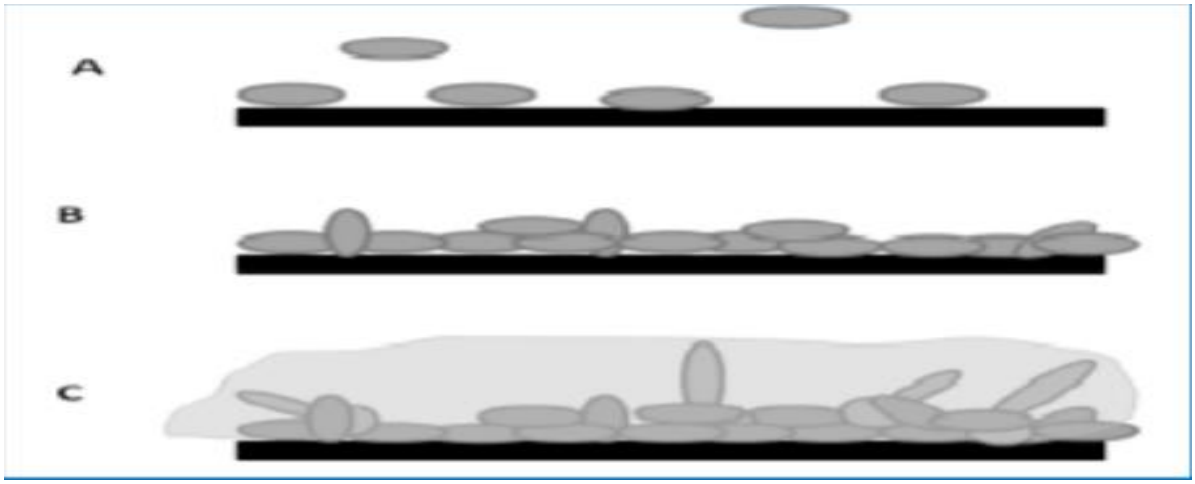

Figure 2-1 adhesion and biofilm formation of *Candida*

Stages of biofilm formation of *Candida spp.* (A) Initial adhesion to surface; (B) Formation of basal microcolony layers of *Candida* cells; (C) Mature biofilm of *Candida* constituted by cells and extracellular matrix (Carina, 2010).

2.6.2.2 Hemolytic activity

Pathogenic microorganisms can grow up in the host by consuming haemin or hemoglobin as a source of iron. The haemolysins were used by *Candida spp.* for degrading hemoglobin and extracting the elemental iron from host cells. Thus, haemolysins is consider a key of virulence factors enabling pathogen survival and persistence in the host (Luo *et al.*, 2001).

2.6.2.3 Germ tube formation

Candida albicans is a multi-morphic opportunistic fungal pathogen of humans. The ability to change morphology between ovoid yeast forms and several filamentous forms (germ tubes, pseudohyphae, and true hyphae) contributes to *C. albicans* virulence, The ability of *C. albicans* to cause both systemic and mucosal infection witnesses the elasticity of this fungus to inhabit quite diverse host niches (Berman, 2012). In fact that necessarily requires expression of different fungal survival factors for specific adaptation to those specific niches and the acidic vaginal pH and the type of feeding

medium appear to be the most suitable conditions for many gene expression and enzymatic activity (Alwaeli *et al.*, 2020).

2.6.3 Identification of Candida spp.

2.6.3.1 Sabouraud dextrose agar

Sabouraud dextrose agar (SDA) is the most frequently used primary medium for isolation of *Candida spp.* (Odds, 1991). Which, although permitting the growth of Candida, suppresses the growth of many species of oral bacteria due to its low pH (Marsh and Martin, 2009). Typically SDA is incubated aerobically at 37 °C for 24– 48 hr and Candida develops as cream, smooth, pasty convex colonies and differentiation between species is rarely possible (Baveja, 2010).

2.6.3.2 Crystal violet staining

Distinct features of yeasts can be identified by observing their morphology after staining with crystal violet (Aslanzadeh and Roberts, 1991). Summarizes the phenotypic characteristic of some common species of pathogenic candida after staining with crystal violet as showed in table 2-1 (Ellis *et al.*, 2007).

Table 2-1 Shape of *Candida spp.*

Attribute	Blastoconidia shape	Blastoconidia size(µm)
C. albicans	Spherical to subspherical	2-7 x 3-8
C. glabrata	Ovoid to ellipsoidal	3.4 x 2
C.famata	Ovoid to broadly ellipsoidal	3.5-5 x 2-3.5
C. norvegensis	Ovoid	2-3.5 x 3.5-5
C. parapsilosis	globose to ovoid	2-3.5 x3-4.5
C. kefyr	Short-ovoid to long-ovoid	3-6.5 x 5.5-11
C. krusei	elongated to ovoid	2-5.5x 4-15
C. guilliermondii	Spherical to subspherical	2-4x 3-6.5

(Ellis *et al.*, 2007).

2.6.3.3 Candida chromogenic agar

CHROMagar candida medium contains chromogenic substrates that react with enzymes secreted by microorganisms producing colonies with different pigmentation. These enzymes are species specific, allowing organisms to be identified to the species level by their color and colony characteristics. Thereby, it is considered as a differential medium for candida yeasts (Mehta, R. and Anupama, S.W., 2016). The manufacturer currently advertises its product for the ability to detect and differentiate three species of candida, *C.albicans* by growth as pale green colonies, *C. tropicalis* by growth as steel blue colonies, *C. krusei* by growth as pink and white to pink color for other yeast species after incubation for 48 hr at 37 °C (Odds and Bernaerts, 1994).

2.6.4 Geographic distribution of *Candida* species

The frequencies of candidemia due to the 5 most common species of Candida in the 4 geographic areas participating in the SENTRY Program are shown in Table (2-2, 2-3). *C. albicans* was most common in EUR (52.5%) and least common in NA (United States and Canada; 42.7%), whereas *C. glabrata* was most common in NA (24.3%) and least common in Latin America (LATAM) 7.1%. *C. parapsilosis* and *C. tropicalis* were more common than *C. glabrata* in LATAM (24.3% and 17.0% vs 7.1%, respectively). *C. tropicalis* was also a frequent cause of candidemia in the APAC region (14.1%). *C. krusei* was more common in NA (2.9%) and EUR (3.0%), and other miscellaneous species of Candida were common in Asia Pasific (APAC) 7.3% and 7.3% for NA (Pfaller *et al.*, 2019).

Table 2-2 Distribution of Candida species from 1997 to 2016

(Pfaller *et al.*, 2019).

		% by Species				
Year	No. Tested	CA	CG	CP	CT	CK
1997–2001	5067	57.4	16.0	12.3	9.1	2.5
2006–2008	2647	51.2	15.9	16.8	10.7	2.1
2009–2011	4080	45.3	18.9	17.6	10.0	2.6
2012–2014	4928	46.3	19.3	15.1	8.6	3.2
2015–2016	3653	46.4	19.6	14.4	8.3	2.8

Abbreviations: CA, *C. albicans*; CG, *C. glabrata*; CK, *C. krusei*; CP, *C. parapsilosis*; CT, *C. tropicalis*.

Table 2-3 Geographic distribution of Candida species

(Pfaller *et al.*, 2019).

		% by Species				
Region	No. Tested	CA	CG	CP	CT	CK
APAC	1314	46.0	17.9	12.9	14.1	1.8
EUR	5964	52.5	16.0	15.4	7.5	3.0
LATAM	1629	43.9	7.1	24.3	17.0	2.0
NA	6401	42.7	24.3	14.8	8.0	2.9
Total	15308	46.7	18.7	15.9	9.3	2.8

Abbreviations: APAC, Asia-Pacific; CA, *C. albicans*; CG, *C. glabrata*; CK, *C. krusei*; CP, *C. parapsilosis*; CT, *C. tropicalis*; EUR, Europe; LATAM, Latin America; NA, North America.

2.7 Bacterial and fungal resistance

Bacterial resistance can be define as the ability of the bacteria to multiply in the presence of drug concentrations that are mostly inhibitors of the same species or equal to the maximum achievable concentration during the therapeutic use. Recently, the rising of resistant bacteria is a growing public health problem that is mainly linked to antibiotics incorrect use. One of the connected problems consists of the increasing complexity of medical treatment and the growing amount of costs that is estimated as $30 billion annually (Goyal *et al.*, 2019). Other important issues are the over or incorrect prescription, the patients not finishing antibiotic treatments, the overuse in livestock and fish farming, and the lack of hygiene and poor sanitation and infection control in hospitals and clinics (Goyal *et al.*, 2019). There are two types of resistances: intrinsic and acquired (Katzung *et al.*, 2018). Previous studies have already demonstrated that patients' noncompliance towards antibiotic prescription is an emerging issue and that it is mainly linked to misunderstanding of the treatment (Tong *et al.*, 2018). Evidence has been already highlighted, in form of a call to action, against the indiscriminate use of antibiotics for animal agriculture and fish farming (Martin *et al.*, 2015), and regarding the strict necessity to manage healthcare associated infections that remain one of the biggest causes of death in most countries (Haque *et al.*, 2018).

Strategies to deal with increasing bacterial resistance include accurate knowledge of the microbial component that feeds infections (Patini *et al.*, 2018 ; Martellacci *et al.*, 2019) and the use of biological agents for their control (Patini *et al.*, 2019).

In medicine and dentistry, the prescription of antibiotics in recent decades has been the subject of debate since many authors presented results of

studies in which a reduction of prescription duration resulted in substantial equal effects in terms of infection resolution and postoperative adverse effects onset (Khariwala *et al.,* 2016). Since the World Health Organization (WHO) clearly reported that incorrect or over-prescription of antibiotics is one of the main causes of the development of resistance and since the abovementioned "short course therapies" are in contrast with pharmacological appropriate posology, it is plausible to hypothesize that these prescriptions have effects on the onset of bacterial resistance. For this reason, the purpose of the present review was to evaluate the existing literature about the role of different antibiotic regimens administered for therapeutic and prophylactic purposes in provoking bacterial resistance (Goyal *et al.,* 2019).

2.7.1 Antibiotics

Antibiotics are known as chemical compounds produced by microorganisms that prevent the growth of other microorganisms by killing or inhibiting (Coumes-Florens *et al.,* 2011). Also, antibiotics are known as special or modified by products of metabolism that have activity against different groups of microorganisms (Jacob, 2015) .Antibiotics can be divided according to their effect into two types of antibiotics, either they have a bacteriocidal effect, such as penicillins, or they are growth inhibitors and bacteriostatic bacteria such as tetracyclines (Mayer, 2010). Also, antibiotics can be divided, depending on the spectrum of their effectiveness, into broad spectrum antibiotics such as gentamycin, and they work against Gram positive and Gram negative bacteria narrow-spectrum Narrow spectrum antibiotics such as Penicillin as they affect a specific group of microorganisms (Tortora *et al.,* 2010).

2.7.2 Mechanisms of Antibiotic Resistance

In general, antibiotics function by binding to a cellular target such that an important biochemical process is blocked. Bacteria may resist the action of antibiotic by a variety of mechanisms, which are summarized here. These include reduction of the intracellular concentration of the antibiotic by increased efflux or reduced permeability, inactivation of the antibiotic by hydrolysis or modification, modification of the target to prevent antibiotic binding, and metabolic bypass of the cellular process blocked by the antibiotic as showed in figure 2-2 (Bonev and Brown, 2019).

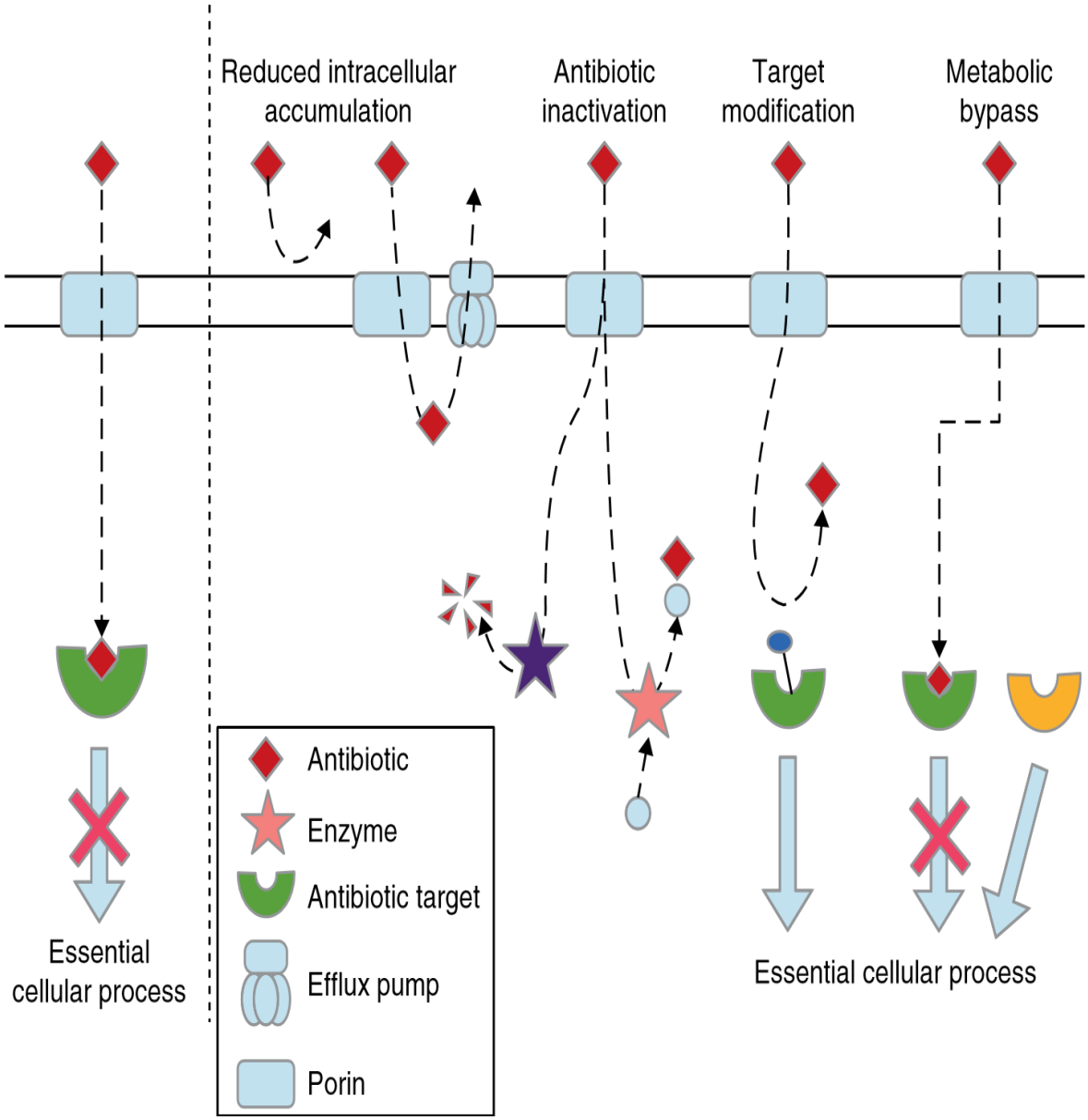

Figure 2-2 Mechanisms of bacterial resistance

(Bonev and Brown, 2019).

2.7.2.1 Reducing the concentration of drug

2.7.2.1.1 Increased efflux

All bacterial genomes encode multiple efflux pumps, which extrude a variety of compounds from the cell (Bonev and Brown, 2019). Efflux pumps are ancestrally ancient proteins and their original function is often unknown, but some are known to export naturally occurring molecules that are toxic to

the cell (Piddock, 2006). In addition, functional efflux pumps have been shown to be important for other cellular processes, such as virulence and biofilm formation in particular (Alvarez-Ortega *et al.,* 2013). Efflux pumps play a major role in determining the intrinsic level of susceptibility of a bacterial species to a particular drug but can also cause further clinically important antibiotic resistance when they are over-expressed. This can occur *via* mutations in local or global regulators (Baranova *et al.,* 1999), or by acquisition of insertion sequence (IS) elements that act as strong promoters upstream of efflux pump genes (Boutoille *et al.,* 2004). Alternatively, new pump genes can be acquired on mobile genetic elements, for example, the *mef* and *msr* genes that encode macrolide transporters in Gram-positive bacteria (Butaye *et al.,* 2003), while some efflux pumps have a narrow specificity, such as *Tet* pumps that confer high level resistance to tetracyclines, others known as multidrug efflux systems (Thaker *et al.,* 2010)

There are five known classes of multidrug efflux systems, summarized here. **1)** RND, resistance-nodulation-division family; **2)** MATE, multidrug and toxic compound extrusion family; **3)** SMR, small multidrug resistance family; **4)** MFS, major facilitator superfamily; **5)** ABC, ATP binding cassette superfamily(Piddock, 2006).

Major facilitator superfamily (MFS) pumps are the largest group of solute transporters and are responsible for most efflux-mediated resistance in Gram-positive bacteria (Poole, 2005), although they are also found in Gram-negative bacteria (Saidijam *et al.,* 2006). They consist of a single polypeptide chain with 12 or 14 membrane spanning domains, with substrate efflux powered by the proton motive force. As an example, several members of this

family cause clinically relevant resistance in *Staphylococcus aureus*. *nor*A confers resistance to fluoroquinolone antibiotics. (Floyd *et al.*, 2010).

2.7.2.1.2 Decreasing of permeability

The intracellular concentration of an antibiotic can be reduced by preventing its entry into the cell. This mechanism is particularly relevant in Gram-negative bacteria as the outer membrane forms an efficient permeability barrier. Gram-positive bacteria tend to be less intrinsically tolerant to antibiotics than Gram negative bacteria as they do not possess an outer membrane so are less able to control their permeability. However, reduced permeability to some drugs has been documented. For example, vancomycin intermediate *S. aureus* (VISA) produce a thickened cell wall. As vancomycin functions by binding peptidoglycan precursors and preventing crosslinking, this thickened cell wall sequesters the vancomycin, increasing the concentration required to permeate through the cell wall and weaken its structure sufficiently to cause cell lysis (Shariati *et al.*, 2020).

2.7.2.2 Drugs inactivation / modification

Antibiotic resistance mediated by degradation or inactivation of an antibiotic before it reaches its target is achieved by either hydrolysis of a key structural feature of the antibiotic, or modification of the antibiotic structure by transfer of a chemical group (Bonev and Brown, 2019).

2.7.2.3 Changing the target

Some Gram-positive bacteria have developed resistance to glycopeptide antibiotics such as vancomycin by an unusual antibiotic target modification strategy (Bonev and Brown, 2019).

2.7.2.4 Acquiring an alternative route

An alternative mechanism employed by some bacteria is to find a way to carry out of the cellular process normally blocked by the antibiotic, despite binding of the drug to the target, the inhibited step of a metabolic reaction can be by passed by acquisition of an alternative enzyme that can carry out the required reaction but is not inhibited by the antibiotic. Alternatively, the target enzyme can be overproduced, such that the concentration of drug required for complete inhibition is increased (Bonev and Brown, 2019). A high-profile example of a metabolic bypass resistance mechanism is seen in *β*-lactam resistance in methicillin-resistant *S. aureus* (MRSA). The *β*-lactam antibiotics act by inhibiting the transpeptidase reaction catalyzed by peptidoglycan cross-linking enzymes known as penicillin binding proteins (PBPs) ,This leads to cell lysis as the bacterium grows and the unlinked cell wall is unable to counter the internal osmotic pressure (Katayama *et al.*, 2000).

2.8 Distribution of efflux pumps genes

According to the data of the distribution of studies on efflux pump genes which showed that the highest distribution belong to the Asian countries (63.8%, n = 23), followed by the European country (16.6%, n = 6) and American countries (13.8%, n = 5). The lowest distribution relates to African countries (5.2%, n = 2). The identification and expression of efflux pump genes not find in Australian studies. The distribution of *nor*A, *nor*B, *nor*C, *qac*A/B and smr genes is evaluated in most studies. The frequency of these genes was studied in most Asian countries. Data from Asia regions indicated that among chromosomal genes *nor*A (75%), *nor*B (60%), *mep*A (35%) and *mde*A (33%) are most prevalent in five studies. The figure 2-3 showed the distribution of efflux pumps genes among *S. aureus* (Hassanzadeh *et al.*, 2020).

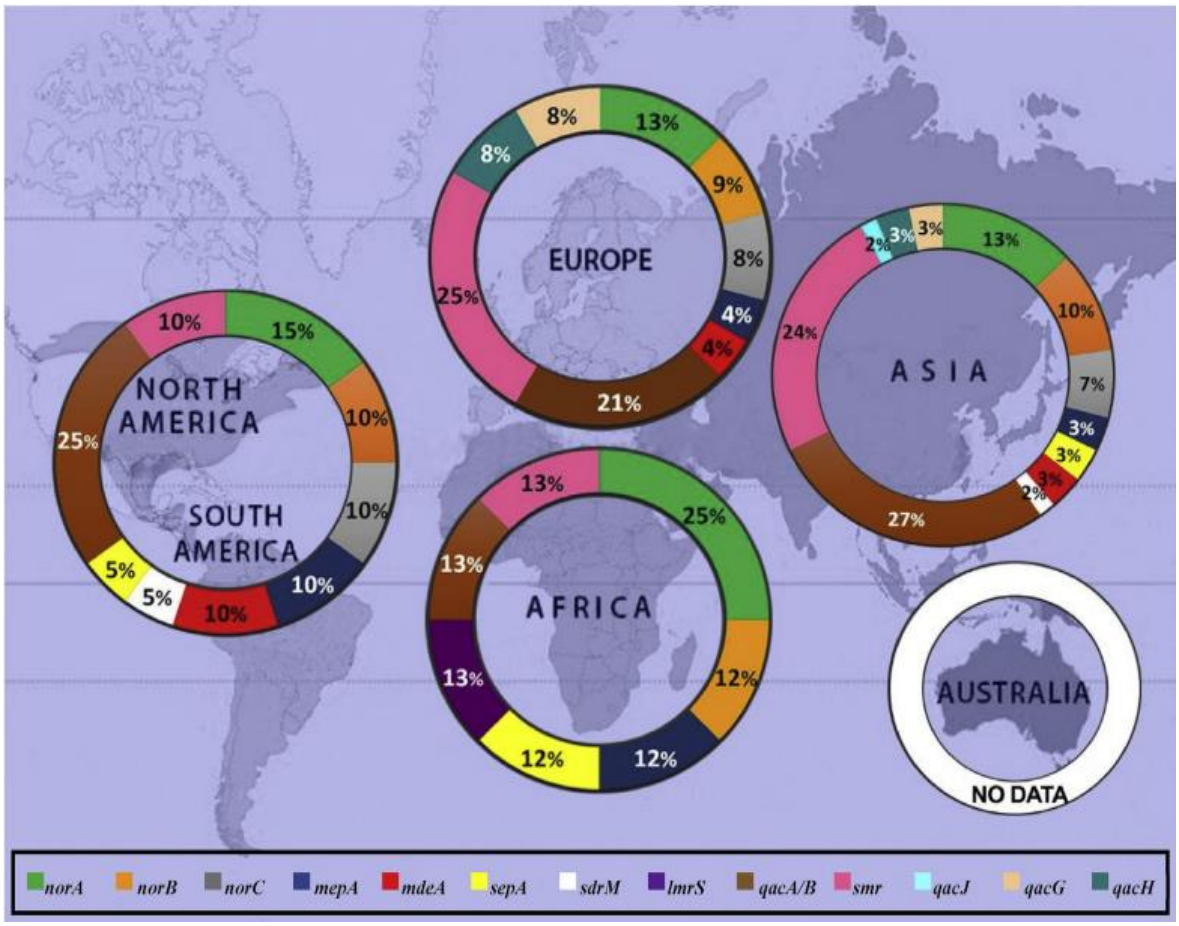

Figure 2-3 Distribution of efflux pumps genes among *S. aureus* (Hassanzadeh *et al.*, 2020).

CHAPTER THREE

MATERIAL AND METHODS

Chapter 3. Materials and methods

3.1 Materials

3.1.1 Apparatuses

All apparatus used in this study are mentioned in the table (3-1):

Table 3-1) Apparatus

NO.	Apparatus and equipment	Company	Origin
1	Autoclave	Wincom	China
2	Bunsen burner	Wincom	China
3	Candle jar	Oxoid	USA
4	Cold centrifuge	Eppendorf	Germany
5	Compound light microscope	VWR	USA
6	Incubator	JRAD	Syrian
7	Laminar Air Flow Hood		
8	Magnetic Stirrer with Hot Plate	VWR	USA
9	Micropipette	VWR	USA
10	Mixer	ICEN	China
11	Nano-drop	Eppendorf	Germany
12	Oven	JRAD	Syrian
13	PH Meter	vicometer	China
14	Refrigerator	National	Japan
15	Sensitive Balance	WANT	China
16	Spectrophotometer	Apple	Japan
17	Vortex	VWR	USA
18	Water bath	VWR	USA
19	Water Distillatory	Wincom	China

3.1.2 Equipment's

All equipment used in this study are mentioned in table (3-2)

Table (3-2) Equipment's

NO.	Material	Company	Origin
1	box slide	shanghai	China
2	cotton	Kardelen	Turkey
3	cotton swabs	AFCO	Jordan
4	Cover slip	AFCO	Jordan
5	Cylinder	Sigma	USA
6	Disposable Loops	Himedia	INDIA
7	Disposable speculum	shanghai	China
8	face mask	Nantong Jinshuo	China
9	filter paper	stonylab	USA
10	Flask	Sigma	USA
11	gloves	ROYAL	Malaysia
12	parafilm	Bemis	USA
13	petri dish	AFCO	Jordan
14	plastic pasteur pipette	AFCO	Jordan
15	rack	Sigma	USA
16	screw cap	Sigma	USA
17	slide	AFCO	Jordan
18	Transport Swabs	AFCO	Jordan
19	Tube	AFCO	Jordan
20	washing bottle	Sigma	USA
21	wax pen	AFCO	Jordan
22	wooden stick	AFCO	Jordan

3.1.3 Chemical and biological materials

All chemicals and biological materials used in this study are mentioned in table (3-3).

Table (3-3) Chemical and biological materials

NO.	Materials	Company	Origin
1	Ethanol 70 %	Aljoud	Iraq
2	KOH	CDH	India
3	H2 SO4	CDH	India
4	OIL immersion	CDH	India
5	Distal water	MED	Germany
6	Barium Chloride (BaCl2.2H2O)	BDH	England
7	Ethylene Diamin Tetra Acetic acid (EDTA)	HIMEDIA	INDIA
8	Glycerol	Fluka	Switzerland
9	Hydrochloric acid (HCL)	BDH	England
10	Hydrogen peroxide H2O2 (6%)	CDH	INDIA
11	Oxidase reagent	Himedia	India
12	Sodium chloride (NaCl)	BDH	England
13	Sodium hydroxide (NaOH)	CDH	INDIA
14	Gram stain	AFCO	JORDAN
15	AB Blood		

3.1.4 Antibiotics and antifungals

All antibiotics and antifungals are mentioned in table (3-4)

Table (3-4) Antibiotics and antifungals

No.	Antibiotic	Symbol	Conc. µg	company	country
1	Ofloxacin	OFX	5	Mast	UK
2	Vancomycin	VA	30	Mast	UK
3	Imipemem	IMI	10	Mast	UK
4	Erythromycin	E	15	Mast	UK
5	Gentamycin	GM	10	Mast	UK
6	Levofloxacin	LEV	5	Mast	UK
7	Ciprofloxacin	CIP	5	Mast	UK
8	Trimethoprim/sulfamethoxazole	TS	1.25/23.75	Mast	UK
9	Trimethoprim	TM	5	Mast	UK
10	Tetracycline	T	30	Mast	UK
11	Nitrofuration	NI	300	Mast	UK
12	Clindamycin	CD	2	Mast	UK
13	Chloramphenicol	C	30	Mast	UK
14	Clarythromycin	CLA	15	Mast	UK
15	Novobiocin	NOVO	5	Mast	UK
No.	**Antifungal**		**Conc. µg**	**company**	**country**
1	Caspofungin	CAS	5	Conda	Spain
2	Amphotericin B	AMB	50	Conda	Spain
3	Fluconazole	FLU	10	Conda	Spain

3.1.5 Culture media

All culture media used in the study are mentioned in table (3-5)

Table (3-5) Culture media

NO.	Culture media	Company	Origin
1	Blood agar	MAST	UK
2	Brain Heart Infusion broth (BHIB	MAST	UK
3	Eosin Methylene Blue (EMB) agar	MAST	UK
4	MacCkonky agar	MAST	UK
5	Sabouraud agar	MAST	UK
6	Mannitol Salt agar	MAST	UK
7	Candida Chromogenic agar	MAST	UK
8	Nutrient broth	MAST	UK

3.1.6 Molecular materials, Primers, and Instruments

All kits, primers and instruments used in the study are mentioned in tables (3-6), (3-7) and (3-8) respectively.

Table (3-6) Molecular Kits

Kits	Company	Origin
ABIOpure™ Total DNA	ABIOpure	USA
Agarose, Ethidium Bromide Solution (10mg/ml), GoTag Green Master Mix, Nuclease Free Water, TAE 40X, Quantiflor dsDNA System.	Promega	USA
Absolute Ethanol	ROMIL pure chemistry	UK
Primers	Macrogen	Korea

Table (3-7) Primers

Primer name	Sequence	Annealing Temp. (C)	Product size
norA-F	5-TTCACCAAGCCATCAAAAAG-3	60	620 bp
norA-R	5-CTTGCCTTTCTCCAGCAATA-3		
sdrM-F	5-CAACATGGCATTGGTTATTCTAC-3	55	186 bp
sdrM-R	5-ACAGCTGTTGGTTTAATAAAGC-3		

(Lin *et al.*, 2020)

Table (3-8) Molecular instruments

NO.	Instruments	Company	Origin
1	1.5 ml, 0.5 ml, and 0.2 ml Tube	JET BIOFIL	Singapore
2	Centrifuge	Fisher Scientific	USA
3	Gel Imaging System	Major Science	Taiwan
4	Micro Spin Centrifuge	My Fugene	China
5	Micropipette	Human	Germany
6	Microwave Oven	GOSONIC	China
7	OWL Electrophoresis System	Thermo	USA
8	Quantus Fluorometer	Promega	USA
9	Refrigerator	TEKA	Spain
10	Thermal cycle	BioRad	USA
11	Vortex	Quality Lab System	England
12	Water bath		China

3.2 Methods

3.2.1 Ethical consideration

A valid consent was obtained from hospital administrators. Also, all participants and health care providers were informed about the purpose of the study. Benefits were discussed; women were informed that the study posed no risks to their health. Participation in the study was voluntary and that participants have the right to withdraw at any time without giving any reason and without affecting their care or health. Measures were taken to ensure confidentiality through coding the data, and participants were informed that data collected will be used only for the purpose of the study, and oral consent was obtained.

3.2.2 Study design

This study was designed as a cross-sectional study. Clinical samples were collected from female patients admitte to the out-patient clinics of Gynecology and Obstetrics, in Al-Batoul Teaching Hospital for Gynecology and Children's in Diyala Governorate during the period from October 2019 to February 2020.

3.2.3 Subjects of the study

A total of 500 pregnant and non-pregnant women were included in this study, 500 patients subjected for sampling which included high vaginal swabs each woman. Each woman was underwent-detailed history regarding age (the age of women ranged from 13 to 59 years old), symptoms of infection, previous history of contraceptive and treatment received, patients having vaginal bleeding, and unmarried women are excluded from sampling.

3.2.4 Preparation of solutions and reagents

3.2.4.1 Preparation of solutions

3.2.4.1.1 Gram stain

Gram stain was ready- made by AFCO company– Jordan.

3.2.4.1.2 MacFarland standard solution

The solution of tube NO. 0.5 was prepared according to (Baron *et al* .,1994) by mixing 0.05 ml of barium chloride with 9.95 ml of concentrated sulfonic acid in which result in turbidity approximately equal to bacterial cells density of 1.5×10^8 cell/ml.

3.2.4.1.3 Phosphate buffer solution

Solution preparation was done by diluting 100 ml phosphate buffer in 900 ml of distilled water, then sterilized by autoclave, after that was kept at 4^oC until been used for samples collection (Forbes *et al*., 2007).

3.2.4.1.4 Human epithelial suspention

The epithelial cell was prepared by discarding a midstream urine sample taken from a woman without a urinary tract infection at a speed of thousand revolutions per minute for five minute, after that the sediment was washed with buffer phosphate saline, and then the cells suspended with the same BPS (Hagberg *et al.*, 1981).

3.2.4.2 Preparation of reagents

3.2.4.2.1 Catalase

This reagent was used at a concentration (3%). H_2O_2 diluting in D.W. (v/v) and stored in a dark container, to identify catalase producing bacteria (Forbes *et al.*, 2007).

3.2.4.2.2 Coagulase

This test was used to differentiate *Staphylococcus aureus* (positive) from coagulase-negative *staphylococci* (negative). The test was done by emulsifying several colonies of staphylococci in 0.5 ml of plasma in a tube to give a milky suspension, then tube was incubated at 35°C for 4 hrs. After incubation period, tubes were checked for clot formation (Forbes et al., 2007).

Coagulase (Clumping factor) test was accomplished to investigate bound coagulase and free coagulase according to (Forbes *et al.*, 2007) as follows:

Slide coagulase test (Slide method) the bounded coagulase was detected by mixing the suspected colony with one drop of saline then spread on clean slide followed by the addition of one drop of human plasma. Clot developing considered a positive results while negative results retested by tube method.

Coagulase test (tube method) free coagulase enzyme was detected by mixing single colony with 1ml of human plasma (diluted by normal saline 1:6) in a test tube and incubated at 37 °C. The result was recorded after 1-4 h. the positive result represented by a clot development; the tube with negative result was reinsulated for 24h and examined again.

3.2.4.2.3 Oxidase

It was readily-prepared containing tetramethyl –ρ- paraphenylene diamine dihydrochloride, stored in a dark container and cool place (Forbes *et al*., 2007).

3.2.5 Preparation of culture media

3.2.5.1 Blood agar

An enriched bacterial medium which encourages the growth of most types of bacteria. prepared as the manufacturer's instructions, 37.5 mg of blood agar base was applied to 1000 ml of distilled water and coated with

cotton, then sterilized by autoclave(Forbes *et al*., 2007). The medium was then cooled to about 50 C and 5% of fresh human blood was added, 15ml was dispensed to sterile petri dishes aseptically, then stored at 2-8 ºC in sealed plastic bags to prevent moisture loss. This medium has been used for the primary isolation of bacteria and to determine their ability to hydrolyse blood(MacFaddin, 2000).

3.2.5.2MacCkonky agar

Selective and differential media used to distinguish gram-negative bacteria and separate lactose-fermenting bacteria such as *E. colli, Klebsiella spp., Enterobacter spp.,* which appear pink-colored, and non-lactose-fermenting bacteria such as *Proteus spp., pseudomonas spp. Salmonella spp., Shigella spp.,* appearing in a pale or yellow colour Prepared according to the manufacturer's instructions (5.1 grams of medium was suspended in 100ml of distilled water) and boiling to dissolve the medium completely, then sterilized by autoclave at 15 lbs. pressure,121 ºC, for 15minutes, and cooled to 50 ºC, then mixed well before pouring into sterile petri plates.(Carroll *et al*., 2015). The inhibitory action of crystal violet on the growth of gram-positive organisms allows the isolation of gram-negative bacteria. Incorporation of the carbohydrate lactose, bile salts, and the pH indicator neutral red permits differentiation of enteric bacteria on the basis of their ability to ferment lactose (James *et al.,* 2020)

3.2.5.3Chocolate agar

When blood agar is heated, the red cells are lyzed and the medium becomes brown in colour. It is referred to as chocolate agar and supplies the factors required for the growth of *H. influenzae*. It is also used to culture nutritionally demanding pathogens such as *Neisseria meningitidis* and *Streptococcus pneumoniae*. It wasPrepared as described for blood agar except

after adding the blood, heat the medium in a70 C, it becomes brown in colour. This takes about 10–15 minutes during which time the medium should be mixed gently several times. Allow the medium to cool to about 45 C, remix and dispense in sterile petri dishes as described for blood agar (Cheesbrough, 2006). Chocolate agar plates were incubated at 35–37∘C up to 48hrs in a 5% CO2 in anaerobic jar.

3.2.5.4 Sabouraud dextrose agar (SDA)

Sabouraud dextrose agar was prepared according to the manufacturer instructions, 65 gm was suspended in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilized by autoclaving, dispensed in petri dishes and kept at 4 ºC until use.

3.2.5.5 Mannitol salt agar

Mannitol salt agar was prepared according to the manufacturer instructions, 111 gm was suspended in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving, dispensed in petri dishes and kept at 4 ºC until use. The mannitol salt agar plate is inoculated for the isolation of the *Staphylococci*.

This medium was used as a selective medium for isolation of Staphylococci and differentiation of *S. aureus* (MacFaddin, 2000).

3.2.5.6 Chromogenic agar

CHROMagar candida medium was prepared according to the manufacturer instructions, 48 g of powder was suspended in 1000 ml of sterile distilled water. Heat to boiling until completely dissolved.

3.2.5.7 Eosin Methylene Blue

This media was prepared according the manufacturing company by suspending 35.96 gm in 1000 ml (Collee *et al*., 1996)**.**

3.2.5.8 Mueller Hinton agar

This media was prepared according the manufacturing company by suspending 38 gm in 1000 ml (Collee *et al*., 1996; Forbes 2007).

3.2.5.9 Nutrient broth

This media was prepared according the manufacturing company by suspending 13 gm in 1000 ml (Collee *et al*., 1996).

3.2.5.10 Brain heart infusion broth- glycerol media

This medium attained by mixing 15 ml of glycerol with 85 ml of brain heart infusion broth and autoclaved at 121ºC for 15 minutes .It was used for preservation of bacterial isolates as stock for long time (Forbes *et al*., 2007). Brain-heart infusion broth was made according to the manufacturing company by dissolving 37gm in 1000 ml of distilled water and autoclaved at 121ºC for 15 minutes (MacFaddin, 2000).

3.2.6 Sterilization methods

3.2.6.1 Wet heat sterilization methods (autoclave)

All media and solutions were sterilized by autoclaving at 121 ºC at 15 pound/inch² for 15 min

3.2.6.2 Dry heat sterilization methods

All glasses were sterilized by electric oven at 180 ºC for 2 hrs.

3.2.7 Samples collection

After obtaining the permission from the females for examination and sampling, patient was rested in lithotomy-dorsal position. Inspection was done for any lesion and vaginal/cervical discharge. The vaginal swab samples were taken by using a special machine to open the vaginal called “sim „s speculum” then insert sterile cotton swab to 2 cm in distance inside the vagina and spin gently toward the wall of the vaginal before pulling swab and then

placed in the carrier media (stuart transport media) until arrival to the lab and work on them (Holt *et al.,* 1994). The speculum was withdrawn. The sample was labeled with the patient's data and number. All informations of patients were noted in the questionnaire (appendix A)

3.2.8 Diagnosis

3.2.8.1 Amstel's criteria

The clinical diagnosis of bacterial vaginosis was made when at least three out of four Amsel's criteria were present (Amsel *et al.,* 1983). These criteria are as follows:

3.2.8.1.1 Hydrogen ion potential

The pH has been determined directly with the use of narrow range (3.5-6) pH strips (Himedia/India) placed on the speculum after removing from vagina, or by touching the swab directly on to the pH strip (WHO, 2013). The colour change was matched with a colour coded guide provided by the manufacturer. A pH greater than 4.5 was considered as positive BV clinical criteria.

3.2.8.1.2 Vaginal discharge

An evaluation of the nature of the vaginal discharge was made by the clinician during pelvic examination. Discharge was reported as positive for BV if it thin, homogenous, and with milky colour (Easmon *et al.,* 1992).

3.2.8.1.3 Clue cells

The swab from vagina was extracted into 0.2 ml of physiological saline then a drop of this extract was placed on a clean glass slide and covered with a coverslip and examined at 40X magnification with a light microscope for the presence of clue cells (> 20% of epithelial cells with indistinct borders due to adherent bacteria) (Money, 2005). Clue cells were also detected during examination of Gram stained smears.

3.2.8.1.4 Whiff test

A drop of 10% potassium hydroxide was placed on a glass slide and the swab with vaginal fluid was stirred in the KOH drop and immediately evaluated for the presence of a fishy odour which indicates a positive result (Money, 2005).

3.2.8.2 Direct wet mount

The vaginal swabs are directly examined to check the presence of each of the clue cell, yeast cell, WBCs, bacteria and epithelial cell by working stuck to the sample by using an aqueous salt solution (normal saline) where the drop of stuck is placed on glass slide and covered by the cover slide and examined under the high power field of the microscope (Atlas, 1995).

3.2.8.3 Specimens culturing and incubation

The vaginal swabs are cultivation on blood agar, MacConky agar, chocolate agar and sabouraud dextrose agar to diagnose all kinds of microbes could exist within it, then incubated at 37 ° C for 24-18 hours, and incubated dishes that did not appear to grow within 24 hours for another 24 hours before counting negative result.

3.2.8.4 Phenotypic characterization

The morphological characteristics of the developing colonies were observed in their forms, color and surface of the colonies, the presence of distinctive odors, their strength and translucency, the pattern of blood hemolysis on the blood agar medium, and the lactose fermentation of the MacConky agar medium(Winn *et al* ., 2006). The shape, color and texture of fungal colonies were examined on SDA agar (Deorukhkar *et al.*, 2014).

3.2.8.5 Gram stain

The slide was placed on a rack of stain and the specimen covered with crystal violet and left to stand for 1 minute then it was washed briefly in tap

water shaking off the excess. The specimen was covered with iodine solution and let stand for 1 minute. It was washed with water and shaking off the excess. The slide was tilted at 45° angle and decolorized with the acetone-alcohol solution until the purple colour stopped running then washed immediately with water shaking off excess. The specimen was covered with neutral red and let to stand for 30 seconds to 1 minute. It was washed with water, shaking off excess, and gently blotted dry. The smear was ready to be read by using oil immersion lens (Macfddin, 2000).

3.2.8.6 Biochemical tests

Specific biochemical tests were done to reach to the final identification (Forbes *et al*., 2007). As:

3.2.8.6.1 Catalase

By using wooden stick, a small amount of colony growth was transfer to the surface of a clean slide then a drop of 3% H_2O_2 was added. A positive result was indicated by evolution of oxygen bubbles (Forbes *et al.*, 2007).

3.2.8.6.2 Coagulase

This test was used to differentiate *Staphylococcus aureus* (positive) from coagulase-negative staphylococci (negative). The test was done by emulsifying several colonies of staphylococci in 0.5 ml of plasma in a tube to give a milky suspension, then tube was incubated at 35°C for 4 hrs. After incubation period, tubes were checked for clot formation (Forbes *et al.*, 2007).

3.2.8.6.3 Oxidase

A piece of filter paper was moistened with several drops of oxidase reagent, then a small portion of the colony to be tested was picked up by wooden stick and rubbed on the filter paper, changing the colour to blue or purple within 10 seconds indicated a positive result (Forbes *et al.*, 2007).

3.2.8.6.4 Novobiocin sensitivity test

This test was used to distinguish among *Staphylococcus spp*. An overnight bacterial culture broth was spread over a plate of Mueller-Hinton agar by sterile swab. A disc of Novobiocin 5µg was placed on the agar medium. Then the plates were incubated at 37ºC for 24 hours and the inhibition zone around the disc was measured. Most *Staphylococcus spp*. showed a large zone of inhibition over 15mm in diameter, while other species like *S.saprophyticus* and *S.xylose* shows a much smaller inhibition zone or grows right up to the disc. (Collee *et al.,* 1996). Novobiocin is an antibiotic interfering with the unpackaging and repackaging of DNA during DNA replication and the bacterial cell cycle. Novobiocin binds to DNA gyrase, and blocks adenosine triphosphatase (ATPase) activity (Macfaddin, 2000)

3.2.8.6.5 Germ tube

The inoculum of yeast cells obtained from an isolated colony was suspended in 0.5 ml of human serum in a small tube (blood sample was centrifuged at 1500 rpm for 15 min and the serum was obtained), and then incubated at 37 ºC for 2-3 hrs. A drop of serum was transferred to a slide, covered by cover-slip, and then examined microscopically under an oil immersion lens for the presence of germ tubes ((Deorukhkar *et al.*, 2014).

3.2.8.6.6 Growth of candida spp. at 45C

Growth at 45ºC has been considered a useful test for the differentiation of *C. dubliniensis* (no growth) from *C. albicans* (growth). The medium (SDA) was inoculated with candidal colonies and incubated at 45ºC for 24 hrs. (Pinjon *et al,* 1998).

3.2.8.7 Detection of virulence factors

3.2.8.7.1 Hemolysis

Inoculate blood agar with pure bacterial culture and incubated in 37 C to 24 hours, the emergence of transparent regions around bacterial colonies developing indicates the susceptibility of bacteria to secretion hemolysin (Dulczak and Kirk, 2005).

3.2.8.7.2 Adhesion

Bacterial cell suspension (0.5 ml) prepared from transferring pure colonies from the 24-hour-old bacterial culture to 4 ml of physiological saline and mix it with 0.5 ml of human epithelial suspension prepared in paragraph (3.2.4.14), the mixture was incubated at 37 º C for one hour with stirring every ten minutes. After the incubation period had ended, the cells were washed four times using a physiological saline solution, with the use of a centrifuge at speed (1000 revolutions / min) for five minutes each time to get rid of non-sticking bacteria, then a drop of the final suspension was taken and placed on a clean glass slide, then left to dry at laboratory temperature, and then fixed by passing it over the flame quickly, after drying and fixing the slide, it was stained with a gram stain. Control slide were prepared from physiological saline and the epithelial cells only (Dulczak and Kirk, 2005).

3.2.8.8 Culturing on selective media

3.2.8.8.1 Mannitol salt agar

The mannitol salt agar plate is inoculated for the isolation of the staphylococci. The generally avirulent *Staphylococcal* species can be differentiated from the pathogenic *Staphylococcus aureus* because the latter is able to ferment mannitol, causing yellow coloration of this medium surrounding the growth. (James *et al.*, 2020).

3.2.8.8.2 Eosin Methylene Blue

Lactose and the dyes eosin and methylene blue permit differentiation between enteric lactose fermenters and non-fermenters as well as identification of the colon bacillus, *E.coli.* The *E.coli* colonies are blue–black with a metallic green sheen caused by the large quantity of acid that is produced and that precipitates the dyes onto the growth's surface. Other coliform bacteria, such as surface. *Enterobacter aerogenes,* produce thick, mucoid, pink colonies on this medium. Enteric bacteria that do not ferment lactose produce colorless colonies, which because of their transparency appear to take on the purple color of the medium. This medium is also partially inhibitory to the growth of gram positive organisms, and thus gram-negative growth is more abundant(James *et al.*, 2020).

3.2.8.8.3 Chromogenic agar

Candida Chromogenic agar is an alternative chromogenic formulation to the traditional media for the detection and isolation of Candida spp. In the medium Glucose is the fermentable carbohydrate providing carbon and energy. Peptone provides nitrogen, vitamins, minerals and amino acids essential for growth. Chloramphenicol is an antibiotic which aids in isolating pathogenic fungi from heavily contaminated material, as it inhibits most contaminating bacteria. It is a recommended antibiotic for use with media due to its heat stability and wide bacterial spectrum. The chromogenic mixture allows the identification and differentiation of all three species of *Candida albicans, Candida tropicalis and Candida krusei* by producing easy-to-read results in one plate, since they present different colored colonies, Bacteriological agar is the solidifying agent. Each isolate was cultured on SDA at 30°C for 48 h. After this, they were seeded on CHROMAgar™ Candida (Chromagar Microbiology) and incubated at 30°C for 48 hrs. The CHROMAgar™ allows selective yeast isolation, identifying colonies of

C. albicans, *C. dubliniensis*, *C. tropicalis* and *C. krusei* by morphology and color reaction (Hospenthal *et al*., 2006) .The strains were identified according to the manufacturer's instructions, which define *C. albicans* or *C. dubliniensis* as green colonies, *C. tropicalis* as steel blue colonies, *C. krusei* colonies as showing rose color and rough aspect, and the other species as white colonies.

3.2.8.8.4 Identification of bacterial isolates by using VITEK 2 system

VITEK-2 system was used in this study for diagnosis of the Gram positive isolates, with a very high degree of accuracy and this device includes 64 biochemical tests which used to diagnose bacteria with an accuracy of up to (98%) and the result of the examination takes about 8 h or less, as well as a test of bacterial sensitivity to antibiotics (Pincus, 2011).which involved many steps as follows:

I- Preparation of bacterial suspension:

A sterile swab was used to transfer a number of the bacterial colonies of a pure culture and separately suspended in 4 ml of sterile saline in clear plastic test tubes. The turbidity was then adjusted up to 2.0 optical densities.

II-Inoculation of identification card:

Identification cards were inoculated with the isolates suspension using an integrated vacuum apparatus. A test tube containing the suspension of the isolates were placed into a special rack (cassette), the identification card was placed into the neighboring slot while inserting the transfer tube into the corresponding suspension tube. The cassette can accommodate up to 10 tests or up to 15 tests. The filled cassette was placed either manually or transported automatically into a vacuum chamber station. After the vacuum was applied

and air was re-introduced into the station, the isolates suspension was forced through the transfer tube into micro-channels that fill all the test wells.

III- Card sealing and incubation:

Inoculated card was passed by a mechanism, which cuts off the transfer tube and seals the card prior to loading into the carousel incubator. The carousel incubator can accommodate up to (30) or up to (60) cards. All card types are incubated on-line at (35.5 + 1.0) ºC. Each card is removed from the carousel incubator once every 15 minutes, transported to the optical system for reaction readings, and then returned to the incubator until the next reading time. Data were collected at 15 minute intervals during the entire incubation period.

3.2.8.9 Maintenance

3.2.8.9.1 Short time maintenance

The yeast isolates were maintained for short period ranged from one to three weeks on slant agar using screw caped test tubes and stored at 4 C˚ (Collee *et al*., 1996).

3.2.8.9.2 Long time maintenance

Isolates were stored for long period in medium containing 15% glycerol at low temperature the medium was prepared by adding 1.5 ml of glycerol to 10ml brain heart infusion broth, then it was dispensed into small screw capped bottle and sterilized by autoclave. After cooling, the medium was inoculated with one pure isolated colony and incubated at 37C˚ for 18 hrs. The tubes were stored in freeze at -20C˚ (Colle *et al*., 1996).

3.2.8.10 Antimicrobial susceptibility test

3.2.8.10.1 Antibacterial

The sensitivity test procedure was done according to (CLSI, 2020) as the following steps: -

1. Mueller-Hinton agar plates were used for the rapidly growing species in the Kirby- Bauer process. The solvent was sterile in the plates and had a depth of around 4 mm.

2. Pure culture has been used as inoculum; (2-4) related colonies have been selected and transferred to around 5ml of standard sterile saline. To get an average number equal to ($1.5x\ 10^8$) cell/ml, the turbidity of microbial suspension was compared with the turbidity of the McFarland Standard.

3. The sterile cotton swab was immersed into the standard inoculum, streaking was performed 3 times on the entire agar surface of the plate with the swab, rotary the plate between each line at 50 degrees. The inoculum had been allowed to dry with a lid in place for 5-10 minutes.

4. Using disk dispenser (MAST™), antibacterial disks were applied on MHA (Mast™).

5. The plates were subsequently incubated at 37 ^oC and analyzed 18-24 h. Inhibition zones were measured, and the zones' diameters were reported to the nearest millimeter (Moreno *et al.*, 2006)

3.2.8.10.2 Susceptibility to antifungal agents tests

A suspension was prepared by picking five to six colonies from the SDA culture plate of ~1 mm diameter from a 24-hour old culture of Candida species. Colonies were then inoculated in 5 mL of sterile saline, and its turbidity was adjusted to 0.5 McFarland standards visually. A sterile cotton wool swab was moistened in the adjusted inoculum suspension, and then, excess fluid was rinsed by rolling the swab on the inside surface of the tube above the fluid surface. Müller-Hinton agar (MHA) surface was streaked to

make a lawn of the isolate. Antifungal susceptibility testing was undertaken by the disk diffusion method. Using disk dispenser (MAST™), fluconazole disk (10 µg), Caspoungin (5 µg) and Amphoterici B (50 µg) antifungal discs (Condalab™) were applied on MHA (Mast™) as recommended by the Clinical Laboratory Standard Institute (CLSI) document M27- A4.

3.2.8.11 Molecular methods

3.2.8.11.1 Extraction

Genomic DNA was isolated from bacterial growth according to the protocol of ABIOpure extraction as the following steps:

•For pellet recover, 1ml of overnight culture for 2min at 13000 rpm. Supernatant then discarded.

• The cell pellet was re-suspended completely in 200µl of Buffer CL.

• For protein digestion and cell lysis, 20µl of Proteinase K solution (20 mg/ml) was added to 200µl of buffer CL and cell pellet, then the tube mixed vigorously using vortex and Incubated at 56˚C for 30 min, for farther lysis incubated 30min at 70˚C.

• After incubation, 200µl of buffer BL was added to sample then the tube mixed vigorously using vortex and Incubated at 70˚C for 30 min.

• From absolute ethanol 200µl was added to the sample, pulse-vortex to mix the sample thoroughly.

• All of the mixtures were transferred to the mini-column carefully, then centrifuge for 1 min at 6,000 x g above (>8,000 rpm), and the collection tube was replace with a new one.

• From buffer BW 600µl was added to the mini-column, then centrifuge for 1 min at 6,000 x g above (>8,000 rpm) and the collection tube was replaced with a new one.

• From buffer TW 700µl was applied. Centrifuge for 1 min at 6,000 x g above (>8,000 rpm). The pass-through was discarded and the mini-column was reinserted back into the collection tube.

• The mini-column was Centrifuge at full speed (>13,000 x g) for 1 min to remove residual wash buffer, then the mini-column was placed into a fresh 1.5 ml tube.

• From buffer AE 100µl was added and Incubated for 1 min at room temperature, then centrifuge at 5,000 rpm for 5min.

3.2.8.11.2 Quantitation of DNA

Quantus Fluorometer was used to detect the concentration of extracted DNA in order to detect the quality of the samples for downstream applications. For 1 µl of DNA, 199 µl of diluted Quanty Flour Dye was mixed. After 5min incubation at room temperature, DNA concentration values were detected.

3.2.8.11.3 Primers preparation

Primers in table (3-9) were supplied by Macrogen Company in a lyophilized form. Lyophilized primers were dissolved in a nuclease free water to give a final concentration of 100pmol/µl as a stock solution. A working solution of these primers were prepared by adding 10µl of primer stock solution (stored at freezer -20 C) to 90µl of nuclease free water to obtain a working primer solution of 10pmol/µl.

Table 3-9) primers preparation

	Primer name	Vol. of nuclease free water (µl)	Con. (pmol/µl)
1	norA-F	300	100
2	norA-R	300	100
3	sdrM-F	300	100
4	sdrM-R	300	100

3.2.8.11.4 Reaction setup and thermal cycling protocol

The PCR calculation:

Table (3-10) PCR Component Calculation

No.of Reaction	**55**	**rxn**	**Annealing temperature of primers**	**60 and 50**	**No. of Reaction**
Reaction Volume /run	**20**	**µl**	**Number of Primers**	**2**	**Reaction Volume /run**
Safety Margin	**5**	**%**	**No. of PCR Cycles**	**30**	**Safety Margin**

Master mix components	Stock	Unit	Final	Unit	Volume	Master mix components
					1 Sample	55.05
Master Mix	2	X	1	X	10	550.5
Forward primer	10	µM	1	µM	1	55.1
Reverse primer	10	µM	1	µM	1	55.1
Nuclease Free Water					6	330.3
DNA		ng/µl		ng/µl	2	
Total volume					20	
Aliquot per single rxn	18µl of Master mix per tube and add 2µl of Template					

Table (3-11) PCR Program

Steps	**°C**	**m: s**	**Cycle**
Initial Denaturation	95	05:00	1
Denaturation	95	00:30	30
Annealing	55 or 60	00:30	
Extension	72	00:30	
Final extension	72	07:00	1
Hold	10	10:00	

3.2.8.11.5 Agarose gel electrophoresis

After PCR amplification, agarose gel electrophoresis was adopted to confirm the presence of amplification. PCR was completely dependable on the extracted DNA criteria.

3.2.8.11.5.1 Solutions

1 X TAE buffer, DNA ladder marker, Ethidium bromide (10mg / ml).

3.2.8.11.5.2 Preparation of agarose

• 100 ml of 1X TAE was taken in a beaker.

• 1 gm (for 1%) agarose was added to the buffer.

• The solution was heated to boiling (using Microwave) until all the gel particles were dissolved.

• 1µl of Ethidium Bromide (10mg/ml) was added to the agarose.

• The agarose was stirred in order to get mixed and to avoid bubbles.

• The solution was allowed to cool down at 50-60C°.

3.2.8.11.5.3 Casting the horizontal agarose gel

The agarose solution was poured into the gel tray after both the edges were sealed with cellophane tapes and the agarose was allowed to solidify at room temperature for 30 minutes. The comb was carefully removed, and the

gel was placed in the gel tray. The tray was filled with 1X TAE-electrophoresis buffer until the buffer reached 3-5 mm over the surface of the gel.

3.2.8.11.5.4 DNA loading

PCR products were loaded directly. For PCR product, 5µl was directly loaded to well. Electrical power was turned on at 100v/current for 75min. DNA moves from cathode to plus anode poles. The ethidium bromide stained bands in gel were visualized using gel imaging system.

3.2.8.12 Diagnostic characteristic

In order to describe the performance of diagnostic test, the sensitivity, Specificity, positive and negative predictive values of Amsel's criteria, the following characteristics were calculated (Forbes *et al.*, 2007):

3.2.8.12.1 Sensitivity

The percentage of individuals with the particular disease for which the test is used in whom positive results are found, i.e.:

Sensitivity = True positives / (True positives + False negatives)

3.2.8.12.2 Specificity

The percentage of individuals who do not have the particular disease being tested and in whom negative results are found, i.e.:

Specificity = True negatives / (True negatives + False positives)

3.2.8.12.3 Positive predictive value

The percentage of true positive test results based on the prevalence of the disease in the population studies, i.e.:

PPV = True positives / (True positives + False positives)

3.2.8.12.4 Negative predictive value

The percentage of true negative test results based on the prevalence of the disease in the population studies, i.e.:

NPV = True negatives / (True negatives + False negatives)

3.2.8.13 Statistics analysis

All data of the current study were analyzed by using word and excel 2013.

CHAPTER FOUR

RESULTS AND DISCUSSION

Chapter 4. Results and discussions

A total of 500 vaginal swabs were collected from 333 pregnant women and 167 non pregnant women and the age group ranging from 13 to 59 years old. Samples were collected from these women who admitted to the out-patient clinics of Gynecology and Obstetrics in Al-batol Maternity and Pediatrics Teaching Hospital during the period from October 2019 until February 2020.

4.1 Amsel′s Clinical Criteria

The diagnosis of BV in all patients in the clinical setting is based mainly on fulfillment of three out of four criteria which described by Amsel (presence of clue cells, elevated pH, positive KOH test and abnormal discharge) as shown in table 4-1. The prevalence of BV according to Amsel′s criteria was 25.6% while it was 27.6% when using culture dependent methods.

Amsel′s criteria can be performed somewhat easily and in short time, but they have several disadvantages. Firstly, Lactobacillus species, the keyword in any definition of BV, are completely ignored in these composite criteria. Secondly, Amsel′s criteria are either subjective (discharge and amine odor) or potentially difficult to judge (clue cells). Thirdly, Amsel′s criteria are combination of clinical and laboratorial observations, i.e. discharge and pH are observed clinically while fishy odour and clue cells are tested in the laboratory, thus there is a need for both a clinician and a technician at the same time (Jakobsson, 2008). Also, the bacteria that cause BV can’t be identified when the diagnosis is carry out by Amsel′s criteria therefore the treatment used could be wrong and lead to an exacerbation of BV.

Table 4-1 Sensitivity, specificity, Positive predictive value, and Negative Predictive Value of Amsel's creteria

Amsel's criteria	No. positive	No. negative	Sensitivity%	Specificity%	PPV%	NPV%
PH >4.5	234	266	92.30 %	71.42%	56.41%	95.86 %
Discharge	407	93	95.07 %	23.82%	33.10%	92.47 %
Clue cells	128	372	63.38 %	89.38%	70.31%	86.02 %
Whiff test	98	402	38.73 %	87.98 %	56.12 %	78.35 %

4.1.1 Vaginal discharge

The most sensitive clinical criterion was vaginal discharge (table 4-1), which had a sensitivity of 95%. The vaginal discharge has also high NPV (92.47%) while the specificity and PPV was low at (23.82 % and 33.10% respectively). Indeed there is a variation in the ability of clinicians to detect the discharge (Eschenbach, 1988). Abnormal discharge is associated with other infections rather than BV such as trichomoniasis and candidiasis (Adler *et al.*, 2004). On the other hand appearance of vaginal fluid may be altered by several factors including sexual intercourse and douching (Easmon *et al.*, 1992; WHO, 2013).

4.1.2 Vaginal pH

The vaginal pH also had high sensitivity (92.30%) since all patients with BV had elevated pH more than 4.5 (table 4-1). although, vaginal pH had high NPV (95.86%). At the same time vaginal pH had moderate specificity and PPV (71.42% and 56.41% respectively).

PH of vaginal discharge can be raised in response to several factors or during different situations. Vaginal pH may elevate above 4.5 at the time of menstruation. In the current study investigated women were at different stages

of their menstrual cycle. In addition, semen have a pH between 7 and 8 and during sexual intercourse the vaginal pH may rise as a result of the effect of seminal fluid, in our study 46 out of 300 (15.3%) women without BV but with elevated pH.

Other causes of increased vaginal pH may include infections such as trichomoniasis, atrophic vaginitis (Sobel, 1997), and desquamative inflammatory vaginitis (Forbes *et al.,* 2007). Also it was found that 25% of women with a pH above 5 had coccoid aerobic vaginitis (Donders *et al.,* 2011). pH may rise if the sample contains blood or if it was close to cervix, where cervical mucus has alkaline pH (Hay, 2002). In addition, douching also increases the vaginal pH (Brotman *et al.*, 2008).

Results of vaginal pH estimation showed that 41% of specimens from subjects without BV had a pH under the normal range at 3, this condition may due to cytolytic vaginosis. The results shows that 71.7% of women without BV had low pH.

Hu and Co-workers, 2014 reported that the percentage of pregnant women that have had CV was 81.80%. This may be as a result of the increased level of glucose during pregnancy, hence an increase in the numbers of lactobacilli. Cytolytic vaginosis is sometimes misdiagnosed as candidiasis because it produces symptoms similar to that of VVC (Puri, 2020). Cytolytic vaginosis may be confused with BV if the dominant Lactobacillus species is *L.iners* because it stains Gram negative and its cell morphology is rather coccobacillar than bacillar (Dong-hui *et al.*, 2009). The exact mechanisms leading to fragmentation or cytolysis of vaginal epithelia are not known (Cerikcioglu and Beksac, 2004). The treatment of CV is achieved by reducing the number of lactobacilli through rising the vaginal pH, where treatment may involve douching with sodium bicarbonate solution (Suresh *et al.,* 2009).

Amsel and Co-workers, 1983 in their original article defined a pH more than 4.5 as one of the four criteria for the diagnosis of bacterial vaginosis. This clearly excluded pH values that are equal to 4.5. Other studies have used different pH cut-off values (Hawes *et al.,* 1996; Hauth *et al.,* 2003). These variations have an effect on the overall performance of the test and also on the prevalence of the disease being investigated.

4.1.3 Clue cells

The presence of clue cells in stained smears was the most specific criterion among the other clinical criteria (Table 4-1), they had a specificity of 89.38% as they have not been detected in the most subject without bacterial vaginosis. This test had moderate sensitivity 63.38%, and it had moderate predictive values (PPV, 70.31% and NPV, 86.02%). The microscopic analysis of clue cells is sometimes difficult, and this criterion was applied differently from mere existence to occurrence on 20% of the epithelial cells (Eschenbach *et al.,* 1988).

4.1.4 Whiff test

KOH test or whiff test for the detection of fishy odor associated with BV had have high specificity 87.98% and high NPV 78.35% (Table 4-1), while the sensitivity was low and the PPV was moderate (38.73% and 56.12% respectively). This test, like other Amsel's criteria, is also subjective and depends on the investigator variation in the ability to detect the characteristic amine odor. Infection with *T.vaginalis* may give positive result for whiff test (Egan and Lipsky, 2000). Also false positive KOH tests can occur in women whose have had sexual intercourse recently (Spiegel, 1991). In addition, when whiff test gives a positive result, the sample become without amine odor due to volatility of amines quickly and completely (Hay, 2002).

4.2 Culture-Dependent Method

A total of 214 isolates belonged to 11 different microorganisms have been identified. The positive culture shows that 82 (38.3%) were gram positive bacteria represented by *Staphylococcus spp*. which include 51 (23.8%) isolates of *S. aureus*, 27(12.6%) isolates of *S. epidermidis* and 4 (1.8%) isolates of *S. saprophyticus* ; 61(28.5%) isolate were gram negative bacteria which include 35(16.3%) isolates of *E. coli*, 20 (9.3%) isolates of *Klebsiella spp*. and 6 (2.8%) isolates of *Pseudomonas spp.* ; and 71 (33.1%) isolates of *Candida* spp. which include 47 (21.9%) isolates of *C. albicans*, 9 (4.2%) isolates of *C. tropicalis*, 8 (3.7%) isolates of *C. parapsilosis*, 6 (2.8%) isolates of *C. glabrata* and 1 (0.5%) isolate of *C. krusei* (figure 4-1)

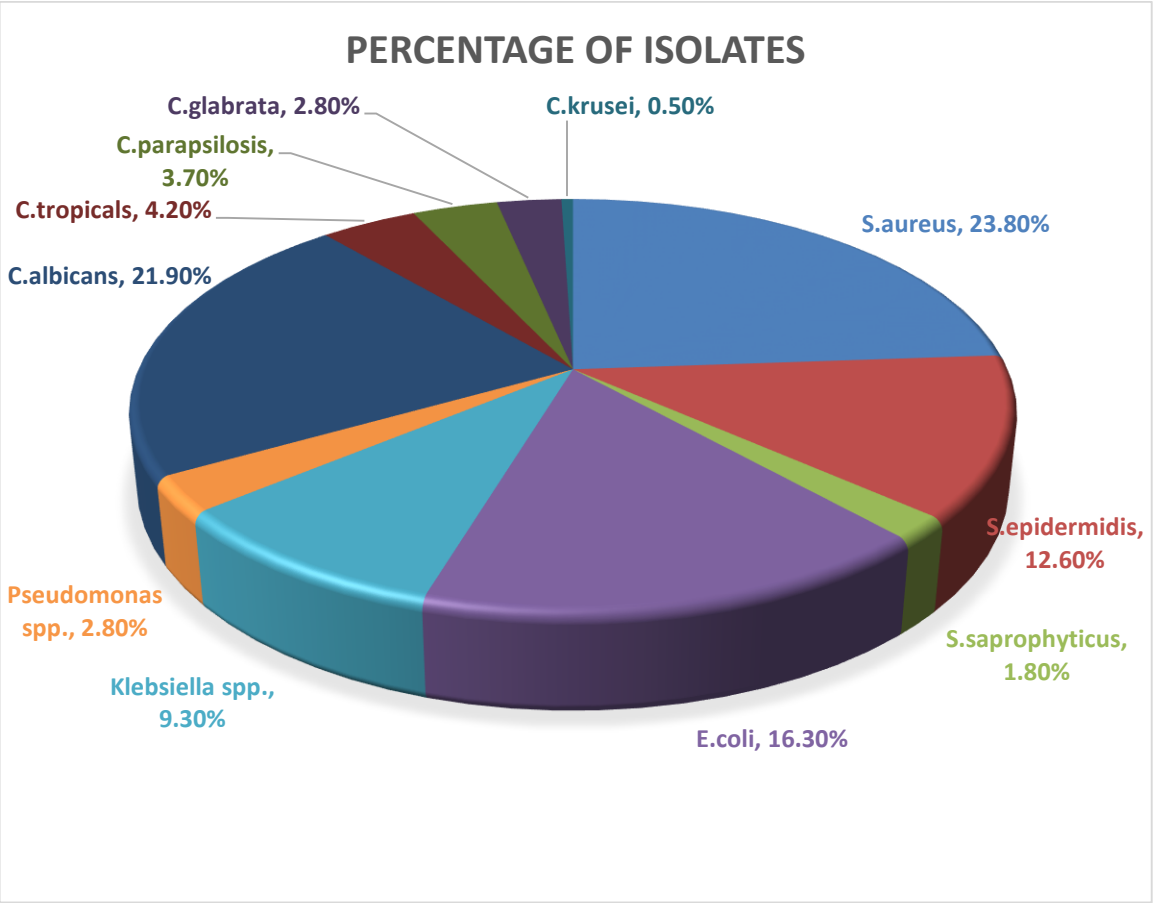

Figure 4-1 Percentage of isolates

4.2.1 Isolation and identification of G+ve bacteria

Many of biochemical and morphological tests were performed to identify the microbes isolated during our study .The results showed that 82 (38.3%) of the total isolate were *Staphylococcus* spp. As shown in the figure 4-2.

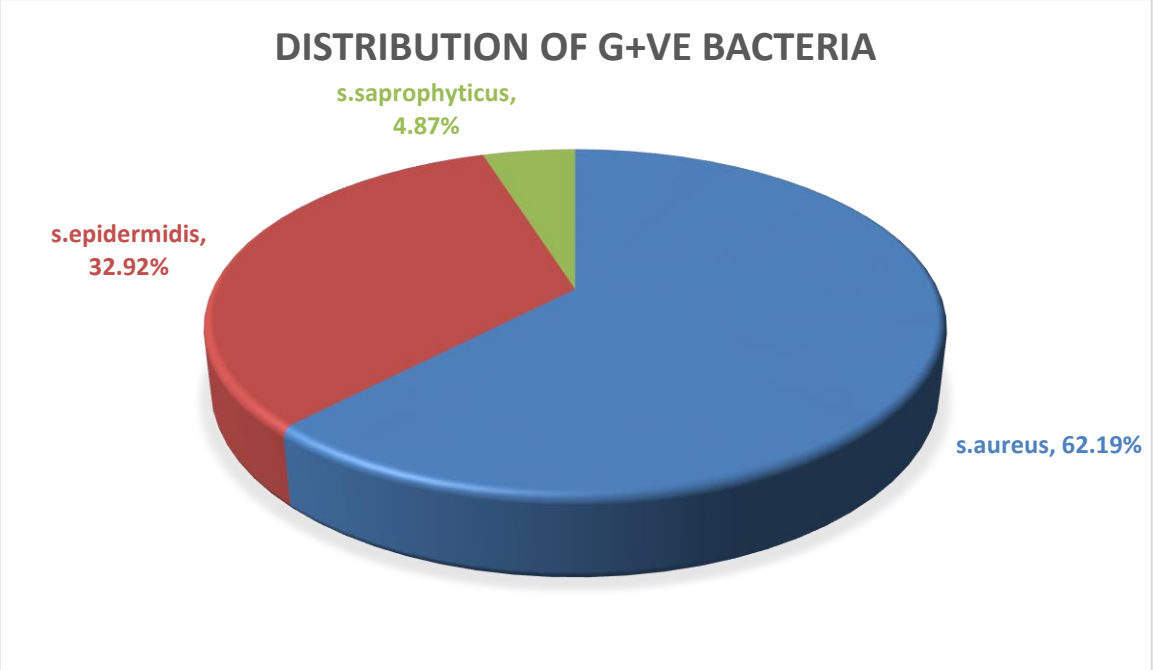

Figure 4-2 distribution of G +ve bacteria

S. aureus, the most frequently isolated Gram-positive bacterium in rate of 62.19% (51 isolates) among *Staphylococcus*, the results shows that 23.8 % of the total isolates were *S. aureus*. Our results agree with Bitew *et al.*, (2017) which was exactly 23.8%, in despite of these results were similar, the number of the samples of his research was 210. The results disagree with Farhan, (2019) and Tumuhamye *et al.*,(2020) Who found the percentage of *S. aureus* in vagina (10.46% and 8.2% respectively). Gram positive cocci (group B *Streptococci* and *Staphylococcus aureus*) have been implicated in a condition known as “aerobic vaginitis”, which differs from bacterial vaginosis and defines as a disruption of the lactobacillary flora, accompanied by sings of inflammation, and the presence of a rather scare predominantly aerobic

microflora composed of enteric commensals and pathogens (Donders *et al.*, 2002). These organisms have been found to be associated with some neonatal and maternal conditions, such as miscarriage, chorioamnionitis, perinatal and maternal postpartum septicemia, premature rupture of the membranes, and preterm birth (Larsen and Monif, 2001; Donders *et al.*, 2011).

S. epidermidis was the second spread of the staphylococcus bacteria by 27(32.92%) isolates and its percentage from the total isolates was 12.6%, the results disagree with Farhan, (2019) who found the percentage of *S. epidermidis* in vagina was 20% and with Wajeh, (2017) which was (8.6%).

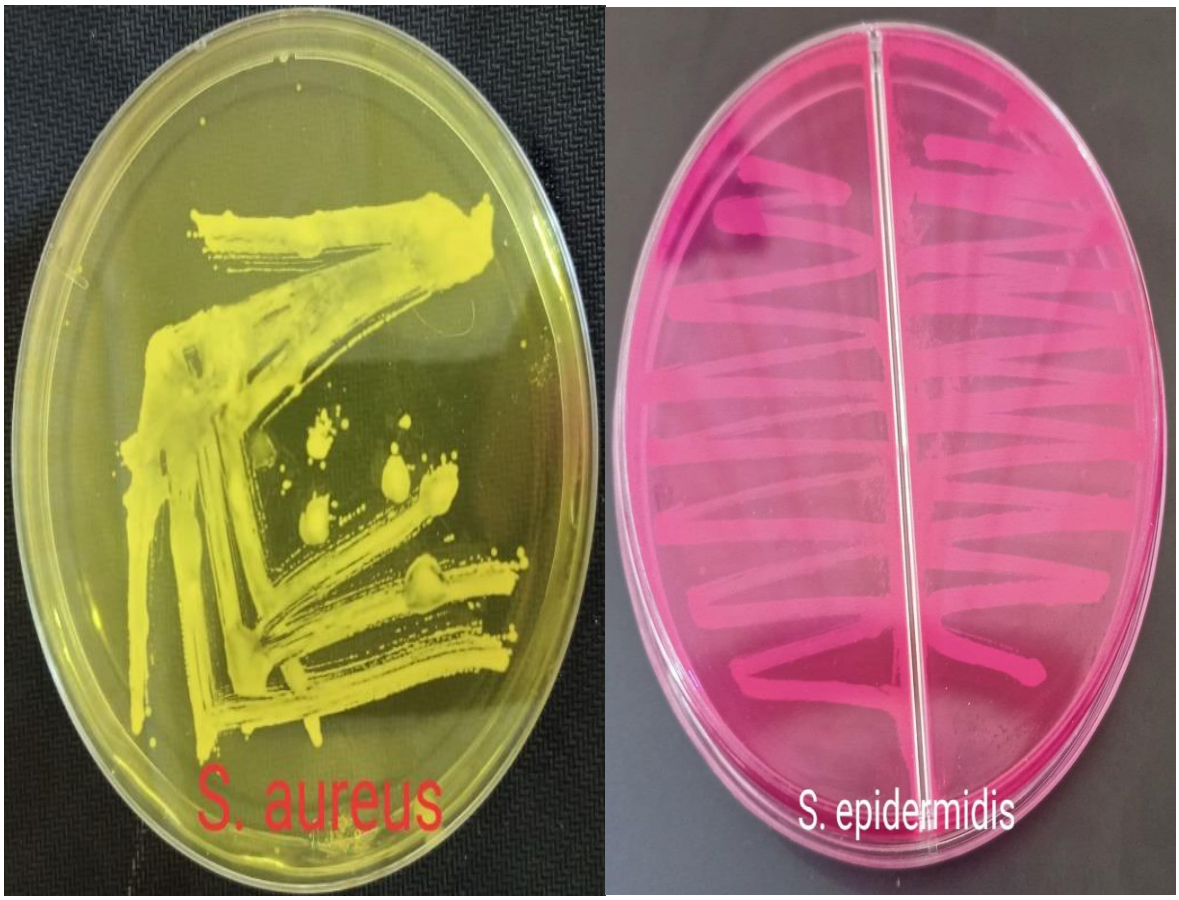

Figure 4-3 *S. aureus* and *S. epidermidis* on Mannitol salt agar

Novobiocin resistance *S. saprophyticus* was the least frequency bacteria among staphylococcus by 4 (4.87%) isolates and its percentage from the total isolates was 1.8%, this results disagree with Farhan, (2019) which was 7.97% as shown in table 4-2.

Table 4-2 Biochemical tests of *Staphylcoccus spp.*

Test / Bacteria	Gram stain		Catalase		Coagulase		Oxidase		Novobiocin resistance		Mannitol fermentation		hemolysis	
	R	%	R	%	R	%	R	%	R	%	R	%	R	%
S.aureus	+	100%	+	100%	+	100%	+	100%	+	100%	+	100%	+	90%
S.epidermidis	+	100%	+	100%	-	0%	-	0%	-	0%	-	0%	-	0%
S.saprophyticus	+	100%	+	100%	-	0%	-	0%	+	100%	-	0%	-	0%

4.2.1.1 Virulence factors investigation

4.2.1.1.1 Adhesion

The results of the adhesion test on *Staphylococcus spp*. to epithelial cells showed that 68% of staphylococcus bacteria were positive (table 4-3). The adhesion ratios for each type of staphylococcus were different, which was 62.74%, 77.77%, 75% for *S. aureus*, *S. epidermidis, and S. saprophyticus*, respectively.

Table 4-3 Adhesion of Staphylococcus isolates

Type of isolate	Total number	percentage
S. aureus	**32**	**62.74%**
S. epidermidis	**21**	**77.77%**
S. saprophyticus	**3**	**75%**
Total	**56**	**68%**

Adhesion is the first step for the onset of infection, as the pathogen's attachment to the host's cells provides protection from the host's defense mechanisms such as vaginal secretions and urine flow (Mappley *et al.*, 2011). Adhesion process occurs by means of fimbria, which helps the bacteria to adhere to the host's cells and work on their stability (Yeoman *et al.*, 2010). The ability of bacteria to adhere depends on the presence of receptors specific to the fimbria present in the cell membranes (Jacobsen *et al.*, 2012). The walls of the vagina are covered in the female reproductive system with a layer of mucus, and this layer constitutes an effective barrier against bacterial invasion as this layer is constantly changing due to various factors such as hormones that lead to peeling and erosion of the outer surfaces of the epithelial cells and this helps in the arrival of pathogens such as bacteria to the vaginal wall and adhesion and injury events (Hernández *et al.*, 2014).

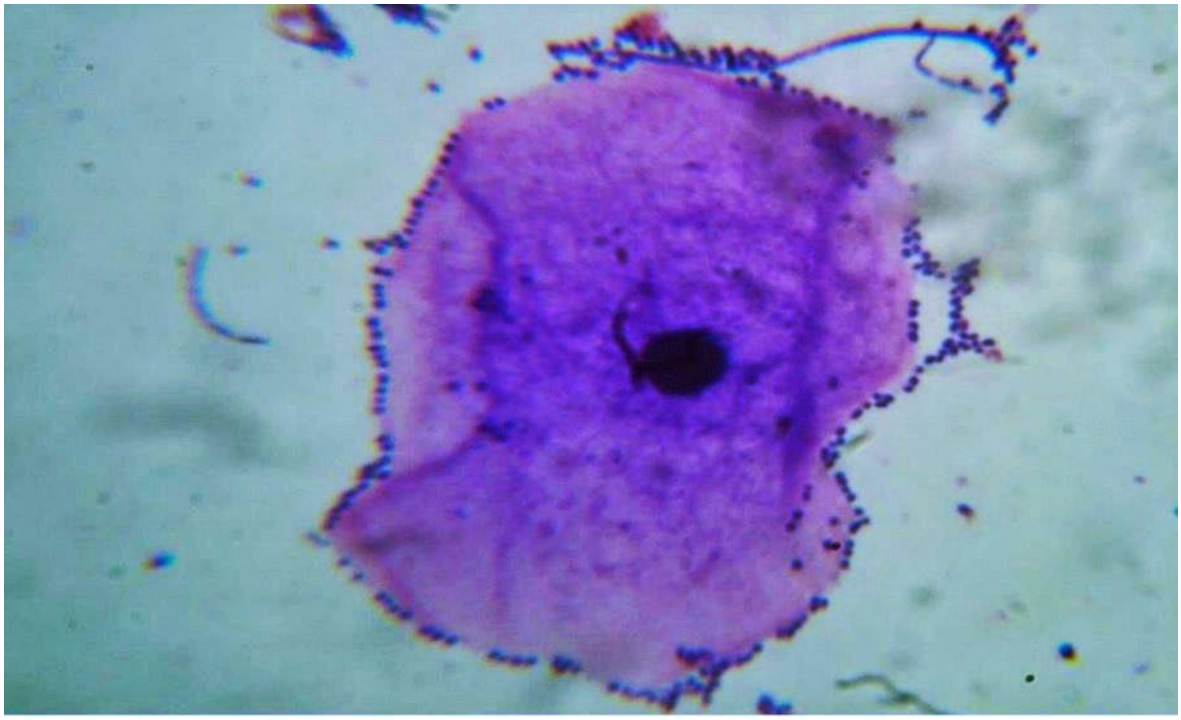

Figure 4-4 epithelial cell covered with Gram-positive Staphylococci (1000X)

4.2.1.1.2 Hemolysis

The results are shown in table (4-2) showed that 90% (46 isolates) of *S. aureus* produced hemolysin enzyme which was ß-hemolysis, 44% (12 isolates) of *S. epidermidis* didn't produce ß-hemolysis on blood agar. *S. saprophyticus* did not produce any hemolytic activity. The current results disagree with the results of Mona, (2011) which was 40% of *S. aureus* produced hemolysin on blood agar.

4.2.2 Isolation and identification of *Candida spp.*

In this study, 71 isolates of *Candida spp.* were isolated from women with VVC. The types and numbers of these isolates, showed in (figure 4-5), which were identified by using the Chromogenic agar and Biochemical tests. *C. albicans* (66.19 %) was the most commonly identified specie , followed by *C. tropicalis* (12.67%), *C. parapsilosis* (11.26%), *C. glabrata* (8.45%) and *C. krusei* (1.40%).This results was agreed with (Roudbarmohammadi *et al.*, 2016 ; Roudbary *et al*.,2013) who indicated that *C. albicans* was the most dominant species compare with other species.

Habibeh and Co-workers in 2013 have shown that *C. albicans* with 53.64% was the major causative agents of vulvovaginal candidiasis , this because *C. albicans* have a high ability to adherence on epithelial cells and its ability to produce germ tube in infected tissue ,and high product to protein digestive enzymes and phospholipase enzymes (Markus *et al*.,2002).

Among the 20 788 Candida isolates submitted for testing from 1997 to 2016, 46.9% were *C. albicans*, 18.7% were *C. glabrata*, 15.9% were *C. parapsilosis*, 9.3% were *C. tropicalis*, 2.8% were *C. krusei*, and 6.5% were miscellaneous *Candida spp.*. The rank order of the 5 most common species varied slightly over time, although *C. albicans* was the predominant species each year. Notably, the frequency of *C. albicans* decreased steadily from

57.4% in 1997–2001 to 46.4% in 2015–2016. *C. glabrata* was the most common non-albicans species overall and showed a steady increase from 16.0% in 1997–2001 to 19.6% in 2015– 2016. *C. parapsilosis* was third in rank order and increased in frequency from 12.3% in the 1997–2001 time period to 17.8% in 2009–2011. The frequency of *C. tropicalis* and *C. krusei* isolates was more stable over time, ranges of 8.3%–10.7% and 2.1%–3.2%, respectively (Pfaller *et al.*, 2019).

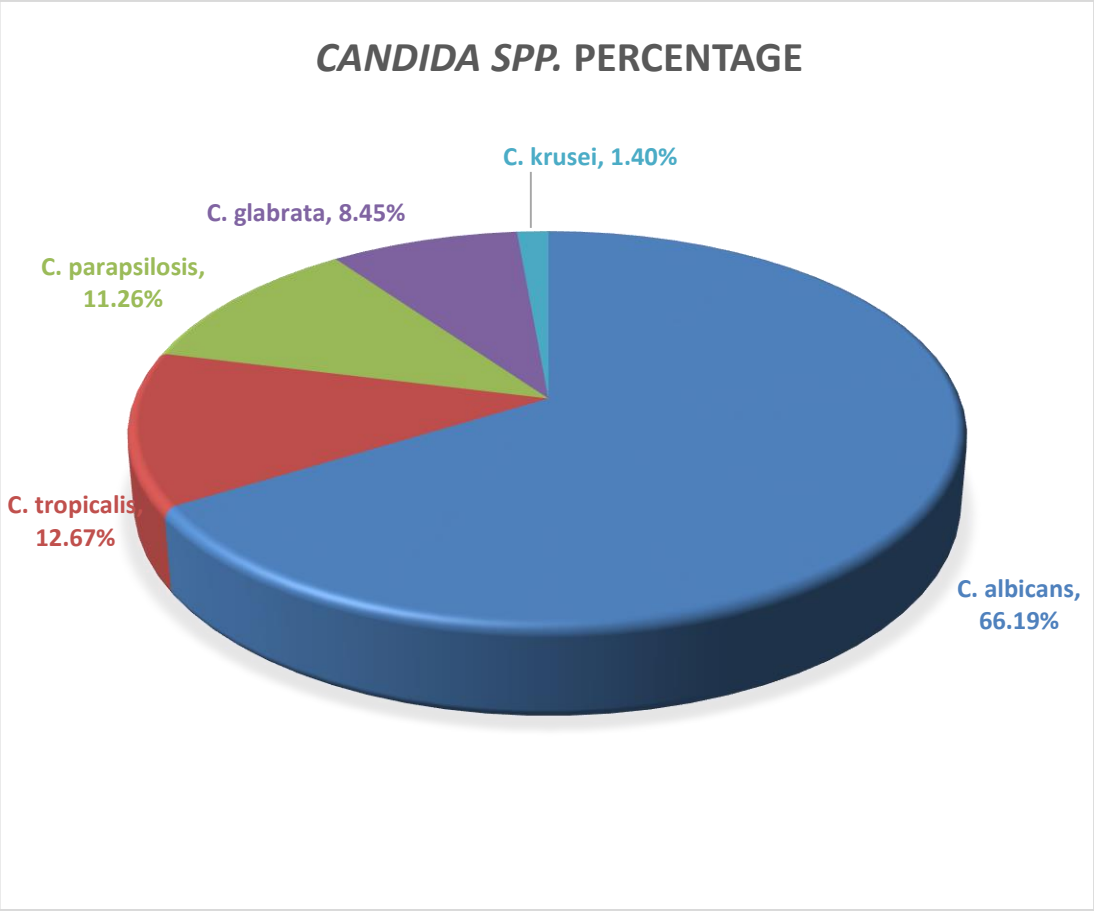

Figure 4-5 *Candida spp.* percentage

Table 4-4 *Candida spp.* on chromogenic agar

Test / Candida	Chromogenic agar		Germ tube		Growth at 45C	
	Result	%	Result	%	Result	%
C.albicans	Light green	100%	+	100%	+	100%
C.tropicalis	blue	100%	-	0%	-	0%
C.parapsilosis	White to cream	100%	-	0%	-	0%
C.glabrata	purple	100%	-	0%	-	0%
C.krusei	Pink	100%	-	0%	-	0%

Candida albicans formed short one piece germ tubes (table 4-4) which discriminated them from non albicans species; there was no restriction at the point of attachment to the yeast cells. These tubular extensions signify an early stage in the formation of true hyphae. The germ tubes were formed within two hours of incubation and this is a distinctive diagnosis characteristic of *C. albicans* differentiates it from other fungi. Other yeasts usually do not form germ tubes within this 3 hour, these results agree with this of Najwan and Co-workers in 2017, when they said that "All *C. albicans* strains were germ tube test (GTT) positive when tested directly from the colony, and all non-albicans species were GTT negative when tested directly from the colony".

4.2.2.1 Phenotypic methodes

On Sabouraud dextrose agar, colonies were White to cream colored, smooth, glabrous and yeast-like in appearance. Microscopic morphology showed spherical to subspherical budding yeast-like cells or blastoconidia. Gram's stains of smears showed gram-positive (dark blue/purple colour) budding yeasts without pseudohyphae. Gram-positive yeast cells are able to retain crystal violet stain due to thick peptidoglycan layer in the cell wall.

Pseudohyphae were elongated cells formed from blastospores (budding cells). A germ tube test was carried out in this study to differentiate *C. albicans* from *Candida* spp. This germ tube test is widely known as presumptive test for identification of *C. albicans*. When the suspected *C. albicans* was inoculated with human serum the formation of germ tubes was seen as long tube like projections extending from the yeast cells. There was no constriction at the point of attachment to the yeast cells. The germ tubes were formed within two hours of incubation in *C.albicans* (Sudbery *et al*., 2004).

4.2.2.2 Chromgenic agar

The color used to identify the *Candida spp.* is detailed in (Table 4-4). On Chromogenic agar (CA), all 47(66.1%) *C. albicans* isolates produced green smooth-type colonies after incubation for 24, 48 and 72 h (figure 4-6). Yucesoy and Co-workers in 2005 found that 100% sensitivity and specificity for *C. albicans* with CA after incubation for 48 h. This agreed with the finding in the present study, and the present study found CA to be useful for the presumptive identification of non- *C. albicans* species. This is similar to Agrawal *et al*., (2014) who demonstrated that the chromogenic agar medium can be explored for its potential as a chromogenic growth medium to assist in making an identification of not only *Candida* species but also other emerging medically important non *Candida* yeast species, based on the development of colored colonies. The color of *C. albicans* colonies on Chromogenic Candida agar was green, while *C. tropicalis* was blue and *C. krusei* was pink fuzzy, *C. glabrata* was purple and *C. parapsilosis* was white cream. The identification of clinically relevant yeasts by chromogenic medium is highly reliable and can be used as an accurate alternative to conventional identification methods (Agrawal *et al*., 2014).

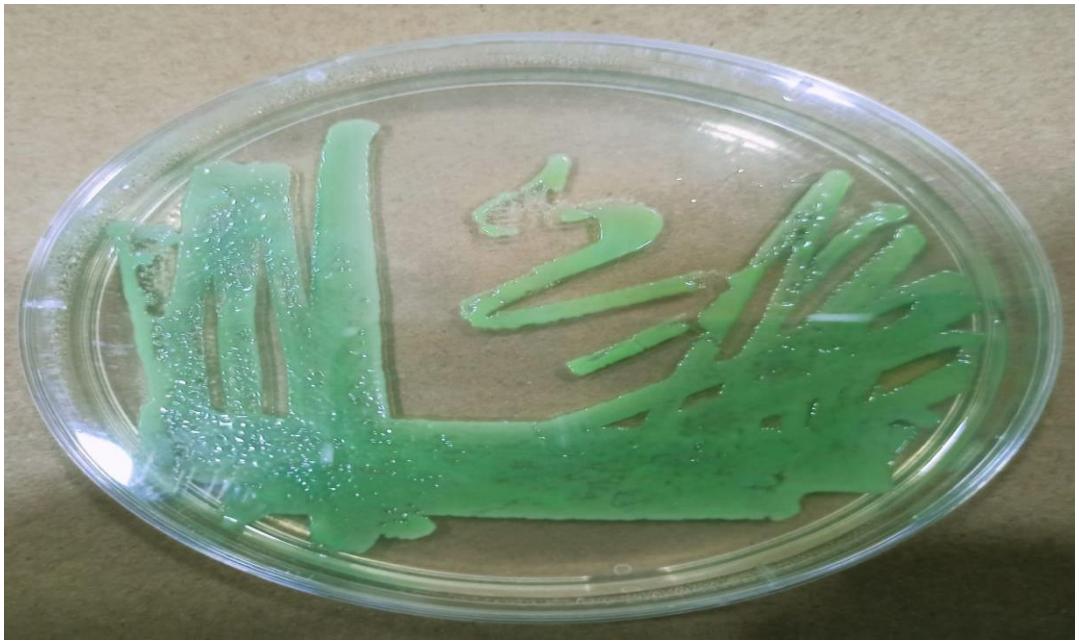

Figure 4-6 *Candida albicans* on chromogenic agar

4.3 Study the relationship between parameters and isolates

Several indicators were noted when samples were taken, and these indicators were studied and their relationship to the presence of vaginitis, several indicators showed a strong relationship with the occurrence of vaginitis, as shown below.

4.3.1 Distribution of vaginitis among age group

The results of the current study showed that the highest rate of infection with BV was in the age second age group (21-30) which was 50%, followed by the third age group, first age group, fourth age group and fifth age group, which the rate of infection was 26.5%, 12.1%, 9.7% and 1.2%, respectively (figure 4-7). Kamel and Co-workers in 2019 found that 41% of BV was in the age group of (21-30), while it was (51%) in the age group of 31-40 and 7.7% for the age group above 40 years. AL-Aouadi and Co-workers in 2016 found that the highest rate of infection by BV was in the age group of (20-29 and 30-39) by (36.9% and 28.2%) respectively.

On the other hand the results of VVC of the current study showed that the highest rate of infection with VVC was in the age second age group (21-30) which was 49.2%, followed by the third age group, first age group, fourth age group and fifth age group, which the rate of infection was (30.9%, 9.8%, 9.8% and 0% respectively). This findings was agreed with Kamel and Co-workers in 2019 who found that the highest rate of VVC was in the age group of 21-30 and 31-40 which was 47.4% for each age group.

Age is one of the factors responsible for the variation in the normal flora of the vagina and causes the periodic emergence of some pathogenic organisms. The reason for the high rates of vaginal infections in the age groups 21-30 years and this may be due to the fact that these ages represent the early years of marriage in which the Sexual activity increases, as well as the proliferative hormones reaching their highest levels, and the physiological and chemical changes that occur in the woman's body at this stage of the woman's life. As for advanced ages from the age of 40 and over, sexual activity decreases and the pH of the vagina returns to low acidity (AANP, 2010).

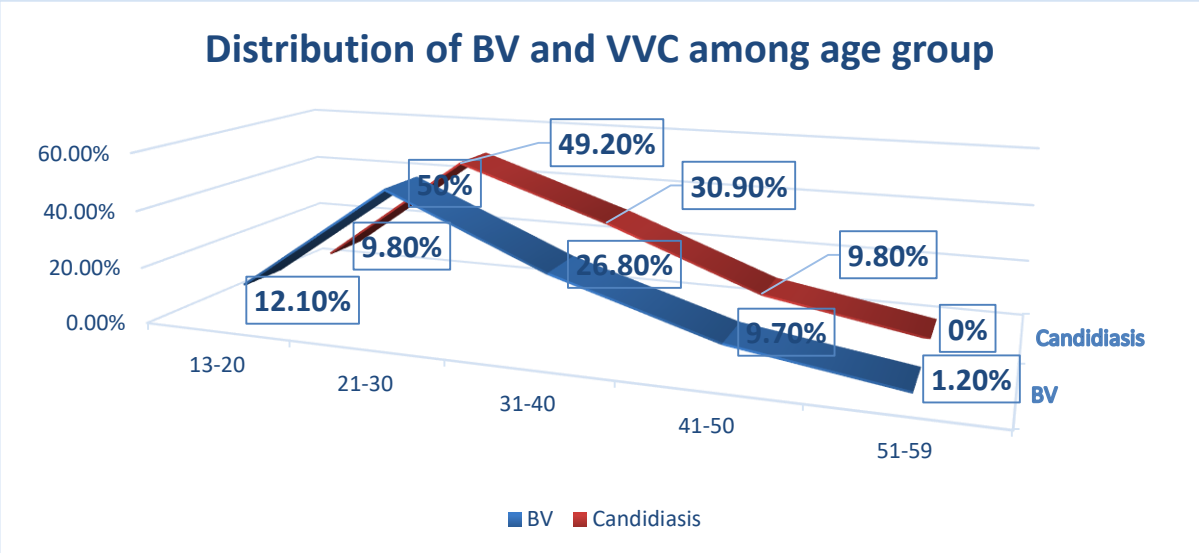

Figure 4-7 Distribution of BV and VVC among age group

4.3.2 Distribution of vaginitis between pregnant and non-pregnant women

The results of the current study showed that there is no notable difference in the infection with BV between pregnant and non-pregnant which was 53% and 47% respectively (figure 4-8). This findings is disagree with AL-Aouadi and Co-workers in 2016 who found that the highest rate of BV was in pregnant women by 67.39% while it was 32.61% in non-pregnant women.

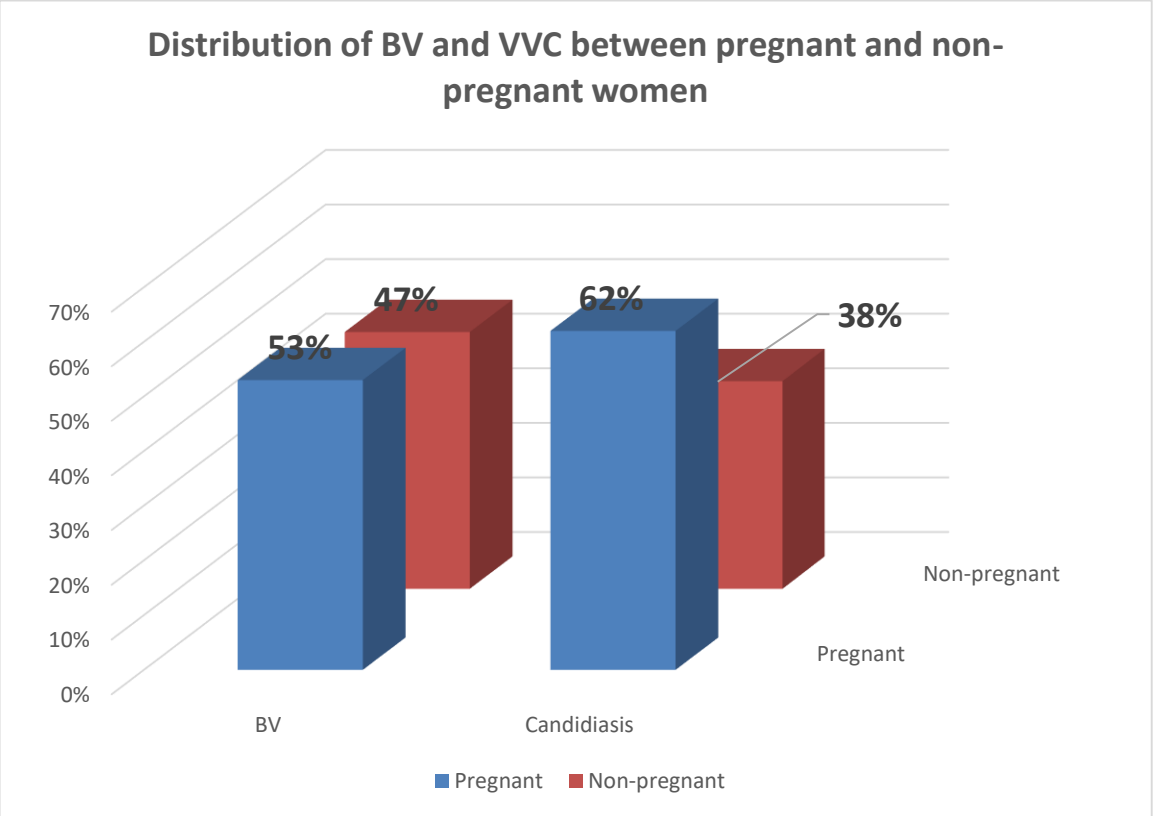

Figure 4-8 distribution of BV and VVC between pregnant and non-pregnant women

In the present study some differences were observed in the incidence VVC between pregnant and nonpregnant women; pregnant women recorded highest percentage 62 % (figure 4-8). VVC is commoner and more difficult to eradicate during pregnancy. This is probably due to high level of reproductive

hormones during pregnancy which provides an excellent carbon source for growth of *Candida* spp. (Jindal and Aggarwal, 2007).

4.3.3 Relationship between vaginitis and education level

The results of the current study showed a strong relationship between education level and the incidence rate of infection with BV and VVC. The results showed the higher the level of education the lower the incidence of infection as shown in figure 4-9. This result was agreed with Bitew and Co-workers in 2017 who also found that the education level has the effect of increasing awareness and reducing infections of vagina. Lack of education has been found to be significantly associated with bacterial vaginosis .However, our finding like other studies contradicted this conclusion. In the present study, bacterial vaginosis was higher among subjects having an education level of primary and secondary school compared to illiterate patients (Bahram *et al.*, 2009)

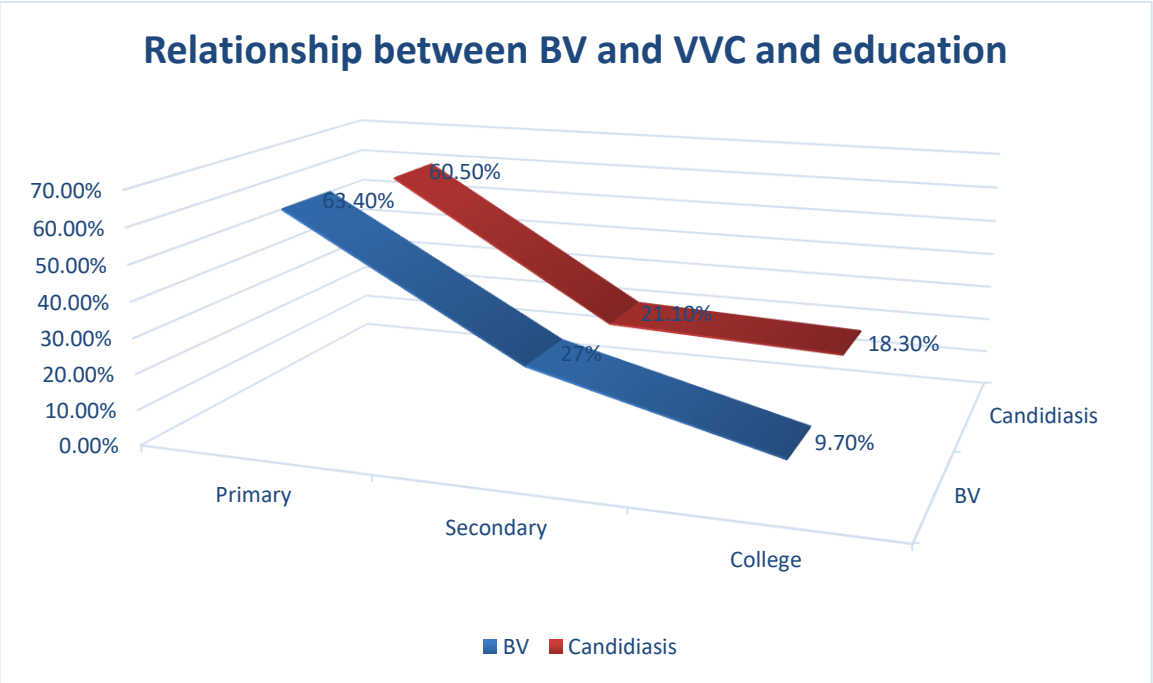

Figure 4-9 Relationship between BV and VVC and education

4.3.4 Relationship between vaginitis and status of living

The results of the current study indicated that the bacterial infection rate was 51.3% in the countryside and 48.3% in the city, while fungal infections were distributed 45% in the countryside and 55% in the city as shown in figure 4-10.

Among the reasons for the high rate of infection in the countryside compared to urban areas may be due to the lack of interest in public health, poor health and cultural awareness, and the high prices of medicines (CDDEP, 2015).

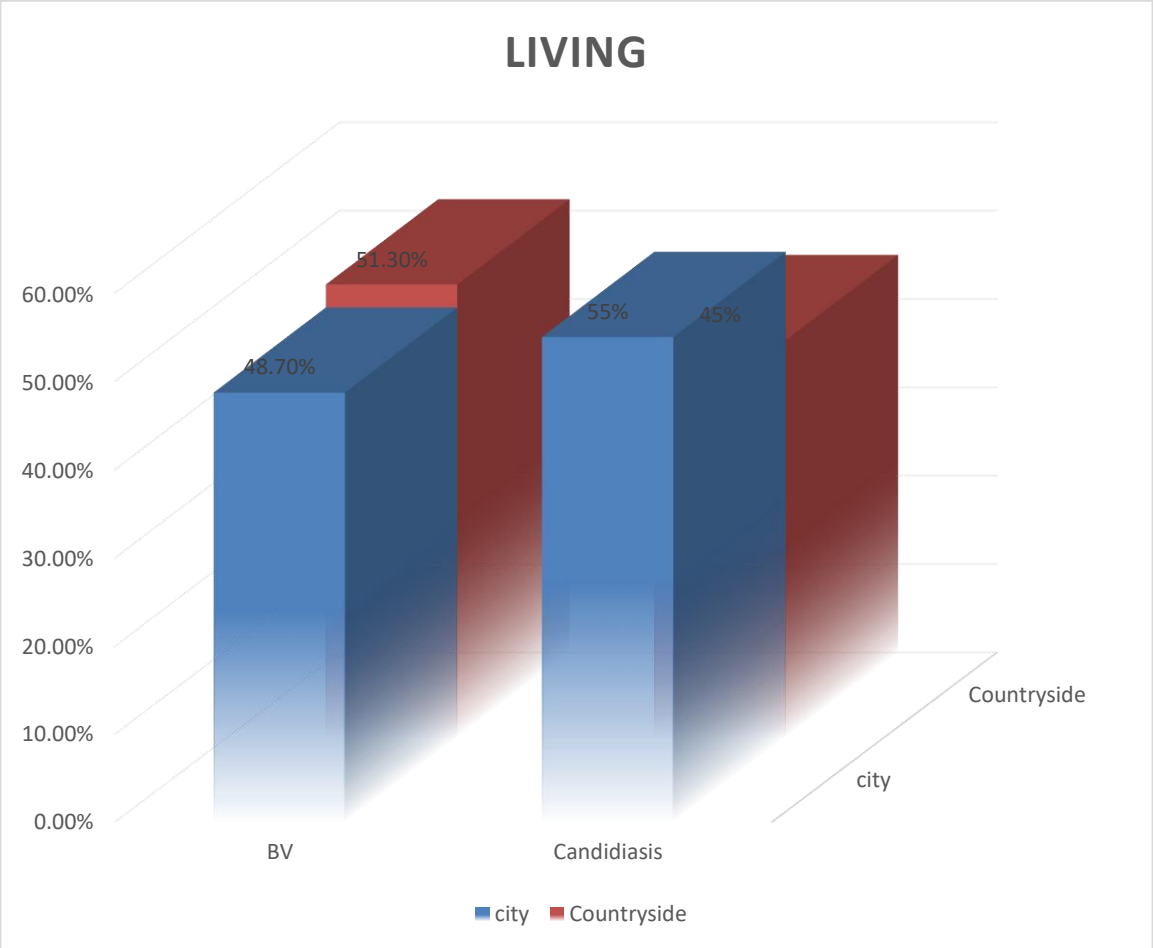

Figure 4-10 Relationship between BV and VVC and living

4.3.5 Relationship between vaginitis and douching

The present study found a strong relationship between BV and vaginal douching as shown in figure 4-11, the same relationship was reported by other studies (Mashburn, 2012; Kenyon *et al.*, 2013; Ness *et al*., 2002; Schwebke *et al*., 2004).Vaginal douching may change the vaginal flora, reduce the amount of *Lactobacilli*, and create environment that promotes excessive anaerobic growth (Smart *et al*., 2004). Although douching may be a consequence of symptoms of BV (i.e. vaginal discharge and malodor), or a current sexual transmitted diseases (Bruce *et al*., 2000).

Other studies disagreed with this finding like (Harikarnpukdee *et al.*, 2004 ; Demba *et al*.,2005) who recorded no association between vaginal douching and the development of BV, and they registered that the influence of douching depends on several factors e.g. douching frequency, reasons for douching, and preparation of solutions.

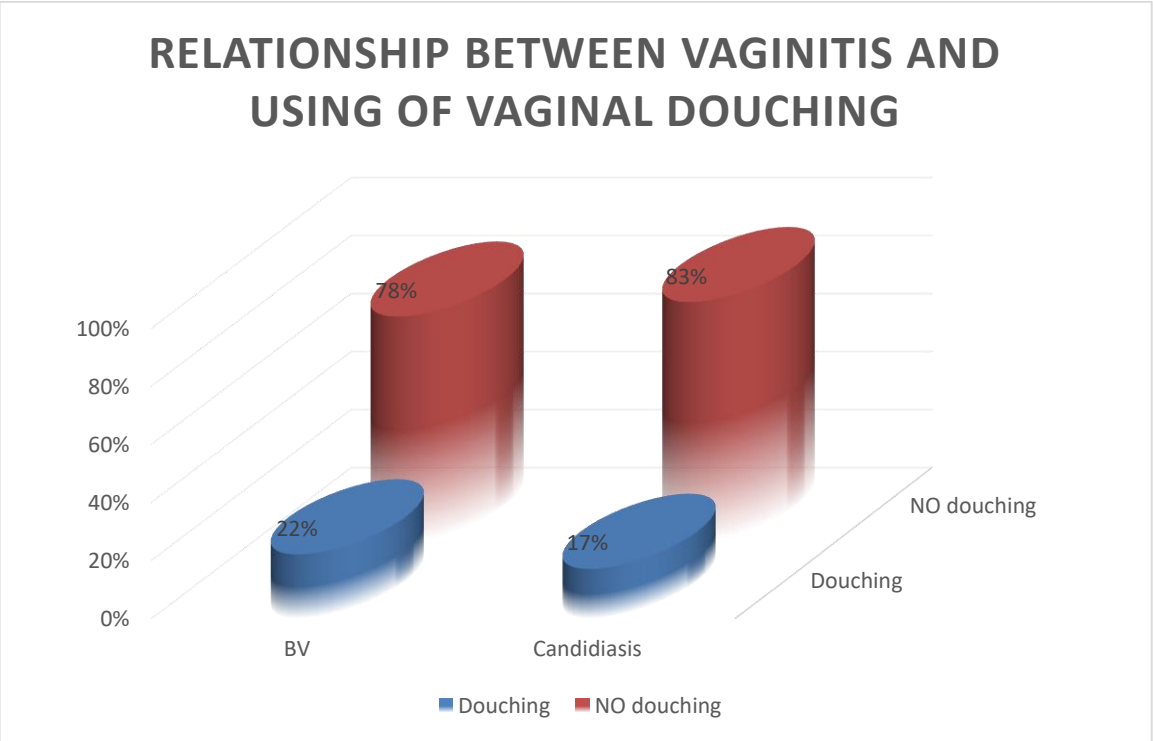

Figure 4-11 Relationship between vaginitis and vaginal douching

4.3.6 Relationship between vaginitis and pH

The results of the current study showed a strong relationship between pH and incidence rate of infection. Both BV and VVC have the highest rate of infection at pH=5, while the incidence rate of infection decrease when the pH elevate as shown in figure 4-12. The result of the examination was the pH elevated in women with bacterial vaginosis and, while pH decrease in women infected with VVC (Hussein and Razzaq, 2018), in the healthy adult female, each mL of vaginal fluid contains more than 10^5 lactobacilli, principally *Lactobacillus crispatus* and *Lactobacillus jensenii* (Giorgli *et al* ., 1987), normal lactobacillary flora is considered an important host defense, these organisms elaborate hydrogen peroxide, which limits the growth of anaerobic bacteria and other pathogens (Hawes *et al* ., 1996). Such lactobacilli also maintain the normal acidity of the vagina at a pH of around 4 to 4.5 .

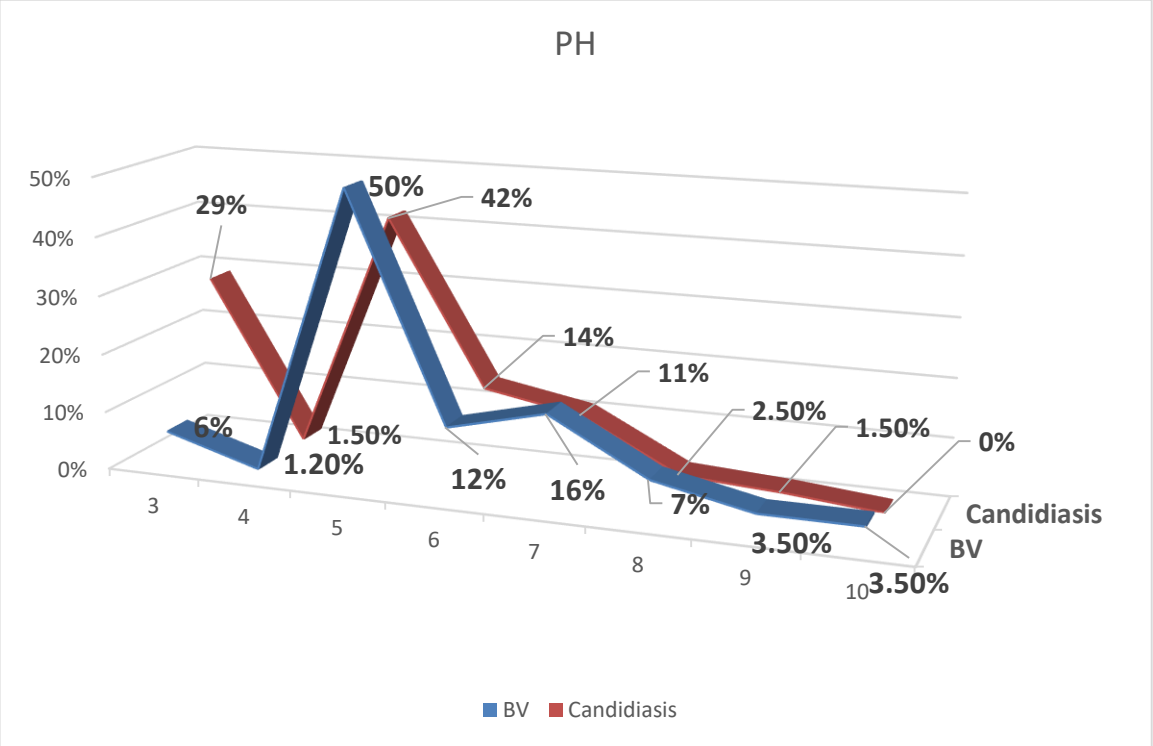

Figure 4-12 Relationship between vaginitis and pH

4.4 Antimicrobial susceptibility tests by disk diffusion

4.4.1 Antibacterial susceptibility test of *S. aureus*

In the current study, two classes of cell wall synthesis inhibitors were tested and the results showed (figure 4-13) that the S. *aureus* isolates were resist to **imipenem** by 4% and sensitive to it by 96%, this finding was agree with (Abdelaziz *et al.,* 2014; Wrood and Hanaa, 2017) which the sensitivity was 92% and 88.88% respectively and disagree with Al-Suadi, (2010) which the sensitivity was 100%. Resist to **vancomycin** by 43% and sensitive to it by 56%, this finding was disagree with (Al-Suadi, 2010; Abdelaziz *et al.,* 2014) which the sensitivity was 38.46%, 92% respectively.

In this study, five classes of protein synthesis inhibitors include nine antibiotics which *S. aureus* isolates were resist to **chloramphenicol** by 31% and sensitive to it by 64%. Resist to **clindamycin** by 47% and sensitive to it by 43%, this finding was disagree with Mulu *et al.,* (2015) which the resistance was 33% and also disagree with Bitew *et al.,* (2017) which the resistance was 11.1% and the sensitivity was 86.1%. Resist to **erythromycin** by 47% and sensitive to it by 25%, this finding was agree with (Mulu *et al.,* 2015; Bitew *et al.,* 2017) on the part of the resistance which was 50% and disagree with Bitew *et al.,* (2017) in the part of sensitivity which was 41.7%. Resist to **clarithromycin** by 31% and sensitive to it by 50%. Resist to **gentamycin** by 19% and sensitive to it by 60%, this finding was agree with Al-Suadi, (2010) on the part of sensitivity which was 53.84% and disagree with (Abdelaziz *et al.,* 2014 ; Bitew *et al.,* 2017) which the sensitivity was 100 and 80.6% respectively, on the other hand the resistance result to this antibiotic was close to Bitew *et al.,* (2017) which was 11.1% and disagree with Mulu *et al.,* (2015) which was 33%. Resist to **ciprofloxacin** by 29% and sensitive to it by 52%, this finding was agree to the results of (Mulu *et al.,* 2015; Bitew *et al.,* 2017) which the resistance was 33% and 22.2%

respectively, on the other hand the sensitivity was agree with Al-Suadi, (2010) which was 58.3% and disagree with Wrood and Hanaa, (2017) who found that all isolates of *S. aureus* were sensitive to ciprofloxacin (100%). Resist to **ofloxacin** by 33% and sensitive to it by 56%. Resist to **levofloxacin** by 27% and sensitive ti it by 56%. Resist to **tetracycline** by 41% and sensitive to it by 54%, this finding was disagree with (Mona, 2010 ; Mulu *et al.,* 2015 ; Bitew *et al.,* 2017) who found that the resistance was (60%, 67% and 63.8% respectively), while the results of our study was agree with Al-Suadi, (2010) on the part of sensitivity which was 50% and disagree with the results of Bitew *et al.,* (2017) who found that the sensitivity of *S. aureus* to tetracycline was 11.1%.

One class of DNA synthesis inhibitors was tested on *S. aureus* isolates which were resist to **nitrofuration** by (41%) and sensitive to it by (41%).

One class of a biochemical pathway (folic acid synthesis) inhibitors include two antibiotics were tested on *S. aureus* isolates which were resist to **trimethoprim by** 54% and sensitive to it by 35%. Resist to **trimethoprim / sulfamethoxazole** by 50% and sensitive to it by 45%, this results was disagree with Bitew *et al.,* (2017) which the resistance was 19.4% and 58.3% for the sensitivity as shown in figure 4-13.

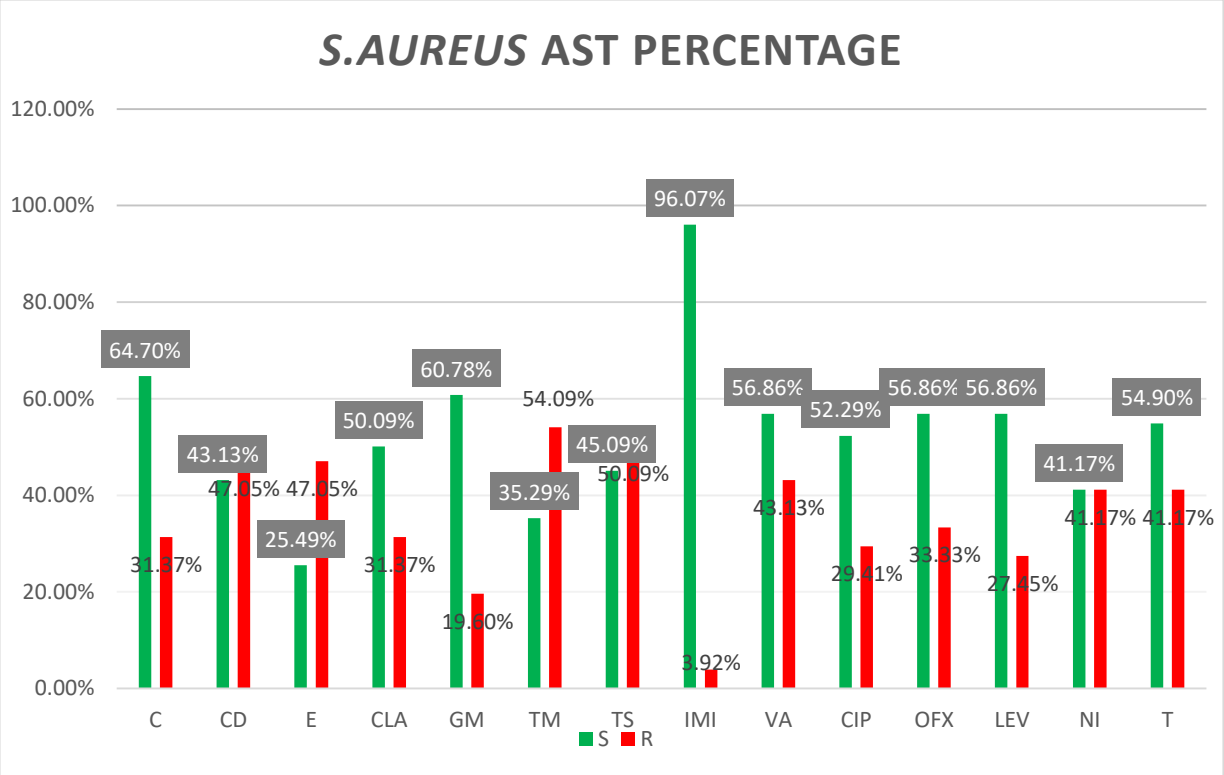

Figure 4-13 *S. aureus* antibacterial susceptibility test (AST)

4.4.2 Antibacterial susceptibility test of Coagulase negative Staphylococci CONS

In the current study (figure 4-14), two classes of cell wall synthesis inhibitors were tested and the results showed that *S. epidermidis* and *S. saprophyticus* isolates were resist to **imipenem** by 0%, this result was disagree with the findings of Al-Suadi, (2010) who found that (83.3%) of CONS were sensitive to imipenem. Resist to **vancomycin** by 12.9% and sensitive to it by 87.0%, this result was disagree with Al-Suadi, (2010) who found that 25% of CONS were sensitive to vancomycin.

In this study, five classes of protein synthesis inhibitors include nine antibiotics which *S. epidermidis* and *S. saprophyticus* isolates were resist to **chloramphenicol** by 22.5% and sensitive to it by 70.9%. Resist to **clindamycin** by 25.8% and sensitive to it by 54.8%. Resist to **erythromycin** by 22.5% and sensitive to it by 25.8%. Resist to **clarithromycin** by 19.3%

and sensitive to it by 51.6%. Resist to **gentamycin** by 22.5% and sensitive to it by 74.1%, this result was disagree with Al-Suadi, (2010) who found that 58.3% of CONS were sensitive to gentamycin. Resist to **ofloxacin** by 38.7% and sensitive to it by 48.3%. Resist to **levofloxacin** by 45.1% and sensitive ti it by 51.6%. Resist to **ciprofloxacin** by 38.7% and sensitive to it by 45.1%, this result was agree to the result of Al-Suadi, (2010) who found that 50% of CONS were sensitive to ciprofloxacin. Resist to **tetracycline** by 41.9% and sensitive to it by 48.3%, this result was disagree with Al-Suadi, (2010) who found that 66.6% of CONS were sensitive to tetracycline.

One class of DNA synthesis inhibitors was tested on CONS isolates which were resist to **nitrofuration** by 32.2% and sensitive to it by 54.8%.

One class of a biochemical pathway (folic acid synthesis) inhibitors include two antibiotics were tested on CONS isolates which were resist to **trimethoprim by** 51.6% and sensitive to it by 45.1%. Resist to **trimethoprim / sulfamethoxazole** by 38.7% and sensitive to it by 45.1%.

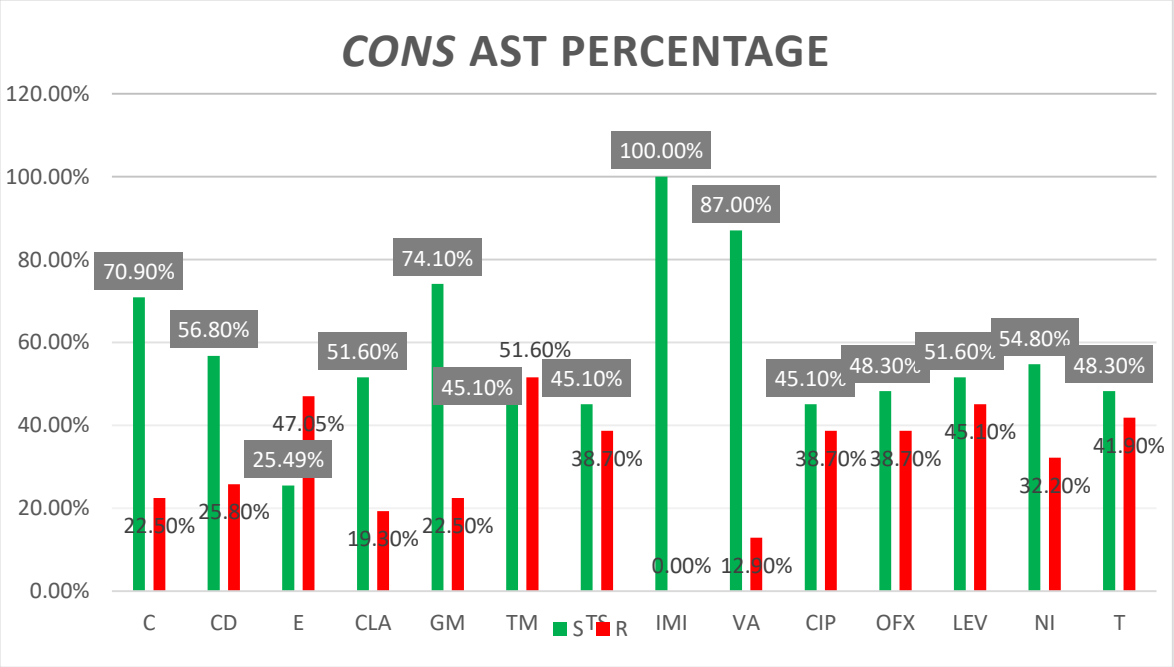

Figure 4-14 CONS antibacterial susceptibility test (AST)

4.4.2.1 MDR isolates

The international definition of MDR is complex, unsatisfactory and hinders the setting and monitoring of improvement programmes. Multidrug resistant (MDR) is a vexed term. From 1980 it was used to mean 'resistant to multiple agents' without the number or types of agents being specified. We use the more recently definition of multi-resistance by European Centre for Disease Prevention and Control (ECDC) which has attempted to formalize the term as 'resistant to three or more antibiotic from three classes (Hawkey *et al.*, 2018).

The results showed that 55 isolates, at a rate of 67%, bear the multiple resistance characteristic of 3 to 11 antibiotics out of the total number of total 14 antibiotics. *S. aureus* was the most MDR bacteria isolated which was 61.8% of the total MDR isolates while *S. epidermidis* consist 36.36% of MDR isolates and *S. saprophyticus* consist 1.8% of MDR isolates as shown in figure 4-15.

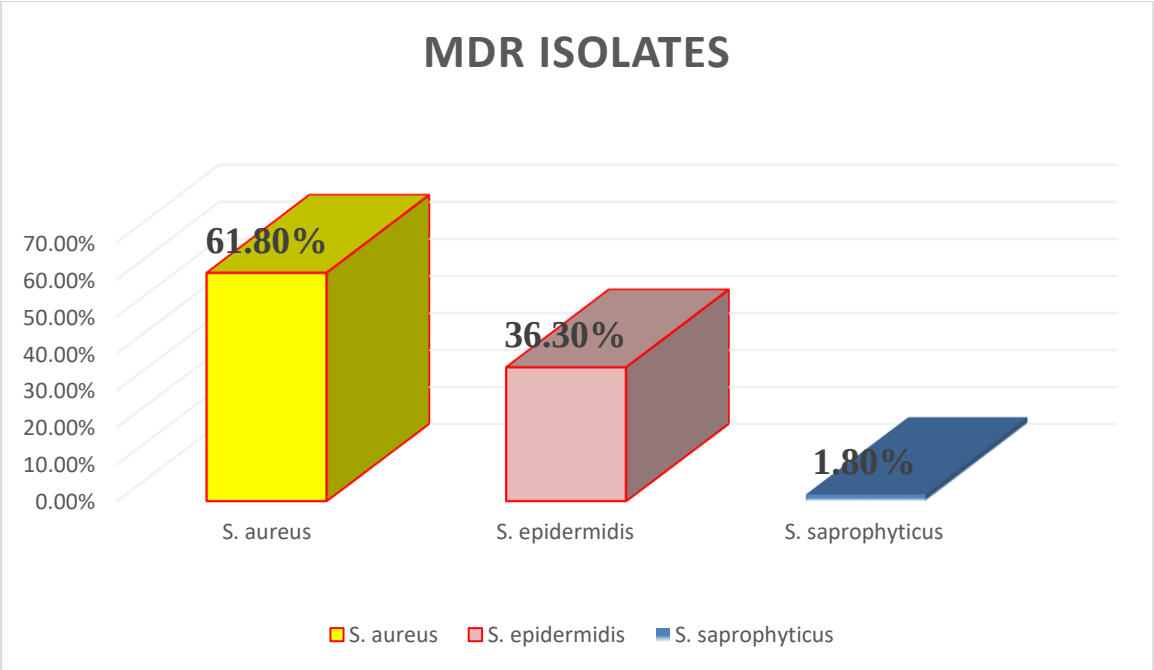

Figure 4-15 MDR isolates of *Staphylococcus spp.*

4.4.3 Antifungal susceptibility tests (AFST)

Susceptibility of *Candida spp*. was analyzed and the results showed (figure 4-16):

Fluconazole (FLU) was sensitive in 36.6% of isolates, and resistant in (63.3%) of isolates, this finding is disagree with (ElFeky *et al.*, 2016) who found that (77.8)% of isolates were sensitive to fluconazole and consistent with (Khan *et al.* 2018) who found that (33.3%) of *Candida spp.* were sensitive to FLU and (62%) of isolates were resist to FLU.

Caspofungin (CAS) was sensitive in 85.9% and resist in 14% of *Candida spp.* isolates.

Amphotericin B (AMB) was sensitive in (77.4%) and resist in (22.5%) of *Candida spp.* isolates, this result is disagree with (ElFeky *et al.* 2016) who found that AMB was the most effective antifungal used in his study which the sensitivity of AMB was 98.4%,

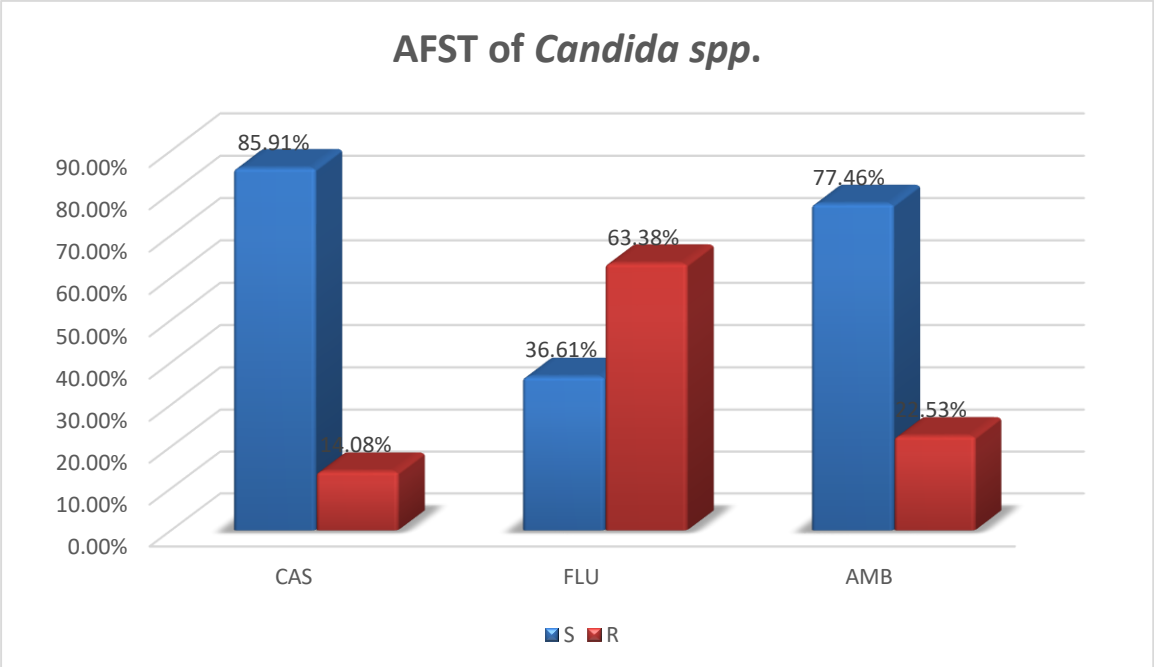

Figure 4-16 Antifungal susceptibility test (AFST) of *Candida spp.*

4.4.3.1 Antifungal susceptibility tests (AFST) of *C. albicans*

Caspofungin was sensitive in 37 (78.7%) and resist in 10 (21.2%) of *C. albicans*, this result was agree with Subramanya *et al.*, (2017) who found that 85.4% of isolates were sensitive to CAS and disagree on the part of resistance which was 6.3%. (Subramanya *et al.*, 2017).**Fluconazole** was sensitive in 34 (72.2%) of *C. albicans*, and resistant in 13 (27.6%) of *C. albicans*, this result was disagree with (ElFeky *et al.* 2016 ; Khan et al. 2018) who found that 89.5% and 37.7% of *C. albicans* were sensitive to FLU and 10.5 % and 53.3% of *C. albicans* were resist to it respectively, it is also disagree with Subramanya *et al.*, (2017) who found that 23% of *C. albicans* were sensitive to FLU and 48% were resist. **Amphotericin B** was sensitive in 11 (23.4%) and resist in 36 (76.5%) of *C. albicans*, this result was disagree with ElFeky *et al.*, (2016) who found that 100% of *C. albicans* were sensitive to AMB, it is also disagree with Subramanya *et al.*, (2017) who found that 97.9% of *C. albicans* were sensitive to AMB and 2.1% were resist (figure 4-17).

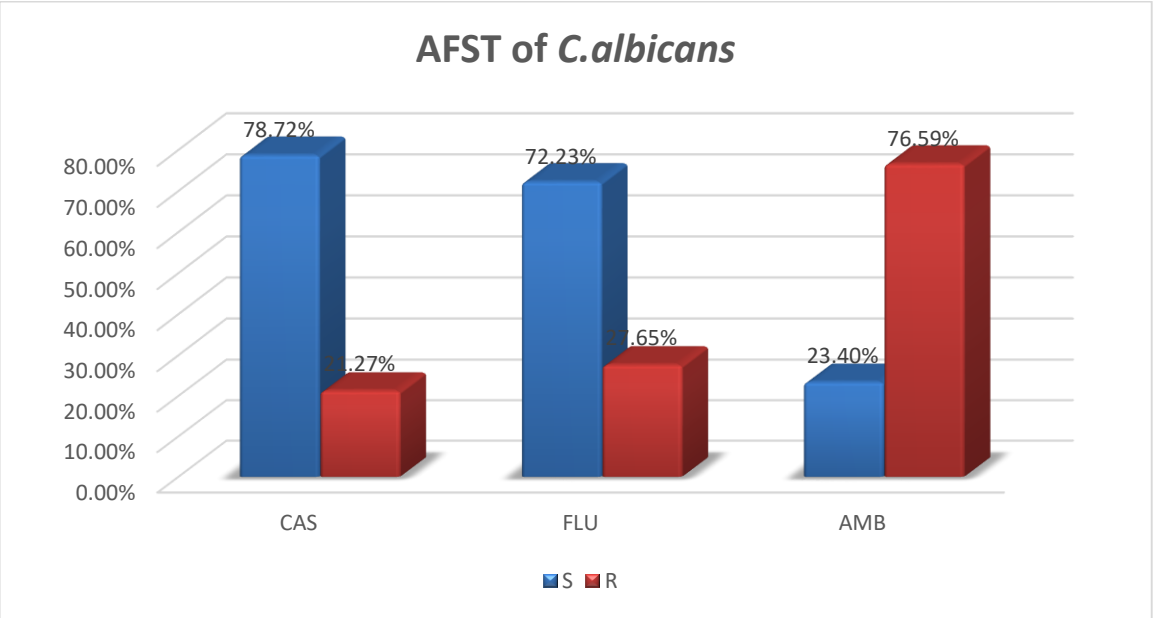

Figure 4-17 Antifungal susceptibility test (AFST) of *C. albicans*

4.4.3.2 Antifungal susceptibility tests (AFST) of *C. tropicalis*

Caspofungin was sensitive in 9 (100%) and resist in 0 (0%) of *C. tropicalis*, this result was agree with Subramanya *et al.*, (2017) who found that 100% of *C. tropicalis* were sensitive to CAS.

Fluconazole was sensitive in 9 (100%) of *C. tropicalis*, and resistant in 0 (0%) of *C. tropicalis*, this result was disagree with ElFeky *et al.*, (2016) who found that 100% of *C. tropicalis* were sensitive to FLU, it is also disagree with Khan *et al.*, (2018) who found that 61.1% of *C. tropicalis* were sensitive to FLU and 38.8% were resist to FLU , it is also disagree with Subramanya *et al.*, (2017) who found that 36.4% of *C. tropicalis* were sensitive to FLU and 54.6% were resist.

Amphotericin B was sensitive in 7 (77.7%) and resist in 2 (22.2%) of *C. tropicalis*, this result was disagree with ElFeky *et al.*, (2016) who found that 100% of *C. tropicalis* were sensitive to AMB (figure 4-18).

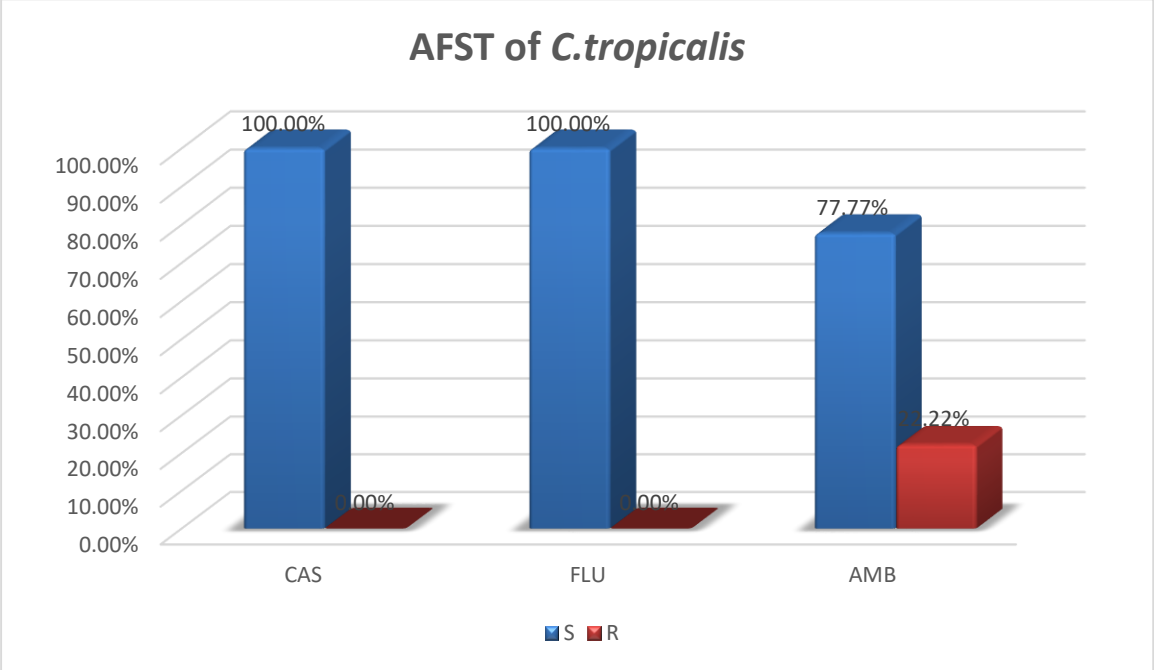

Figure 4-18 Antifungal susceptibility test (AFST) of *C. tropicalis*

4.4.3.3 Antifungal susceptibility tests (AFST) of *C. parapsilsis*

Caspofungin was sensitive in 8 (100%) and resist in 0(0%) of *C. parapsilosis.*

Fluconazole was sensitive in 5 (62.5%) of *C. parapsilosis*, and resistant in 3 (37.5%) of *C. parapsilosis*, this result was agree with ElFeky *et al.*, (2016) who found that 60% of *C. parapsilosis* were sensitive to FLU and 20% of *C. parapsilosis* were resist to it.

Amphotericin B was sensitive in 4 (50%) and resist in 4 (50%) of *C. parapsilosis*, this result was disagree with ElFeky *et al.*, (2016) who found that 80% of *C. parapsilosis* were sensitive to AMB and 20% of *C. parapsilosis* were resist to it (figure 4-19).

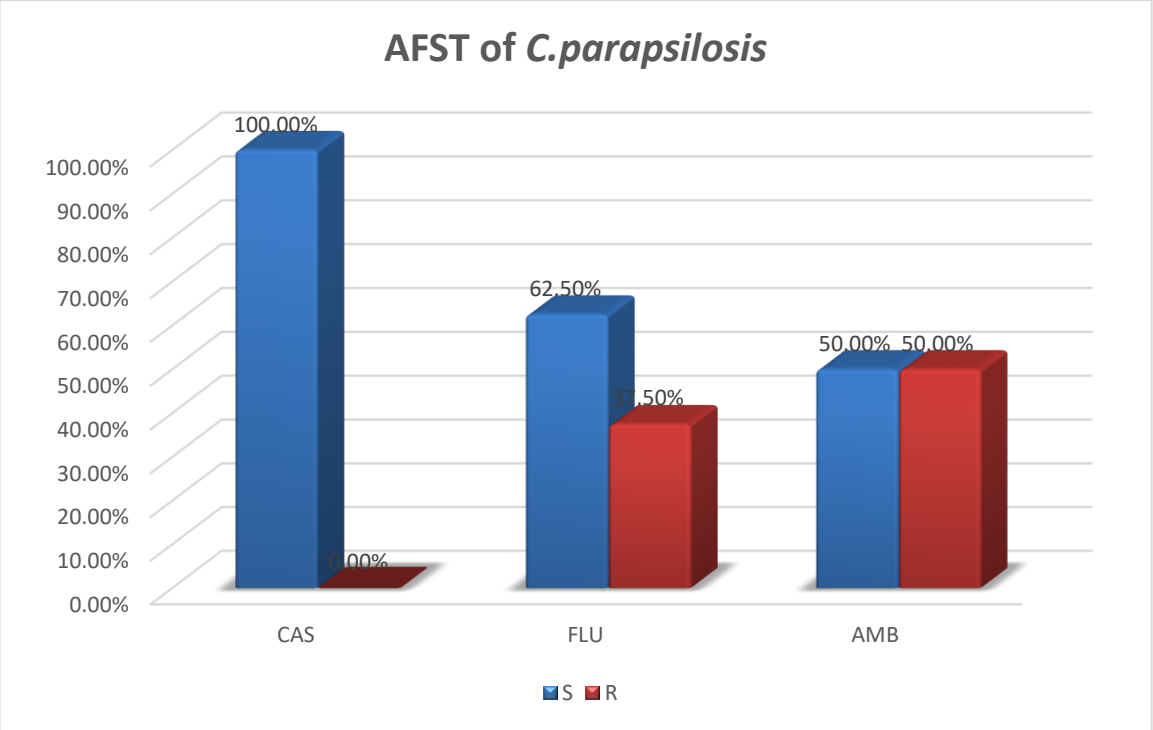

Figure 4-19 Antifungal susceptibility test (AFST) of *C. parapsilosis*

4.4.3.4 Antifungal susceptibility tests (AFST) of *C. glabrata*

Caspofungin was sensitive in 6 (100%) and resist in 0 (0%) of *C. glabrata*, this result was agree with Subramanya *et al.*, (2017) who found that 100% of *C. glabrata* were sensitive to CAS.

Fluconazole was sensitive in 6 (100%) of *C. glabrata* and resistant in 0 (0%) of *C. glabrata*, this result was agree with ElFeky *et al.*, (2015) who found that 50% of *C. glabrata* were sensitive to FLU and (0%) of *C. glabrata* were resist to it and agree with Khan *et al.*, (2018) who found that 100% of *C. glabrata* were sensitive to FLU, it is also disagree with Subramanya *et al.*, (2017) who found that 66.7% of *C. glabrata* were sensitive to FLU and 22.2% were resist.

Amphotericin B was sensitive in 4 (75%) and resist in 2 (25%) of *C. glabrata*, this result was disagree with ElFeky *et al.*, (2016) who found that 100% of *C. glabrata* were sensitive to AMB (figure 4-20).

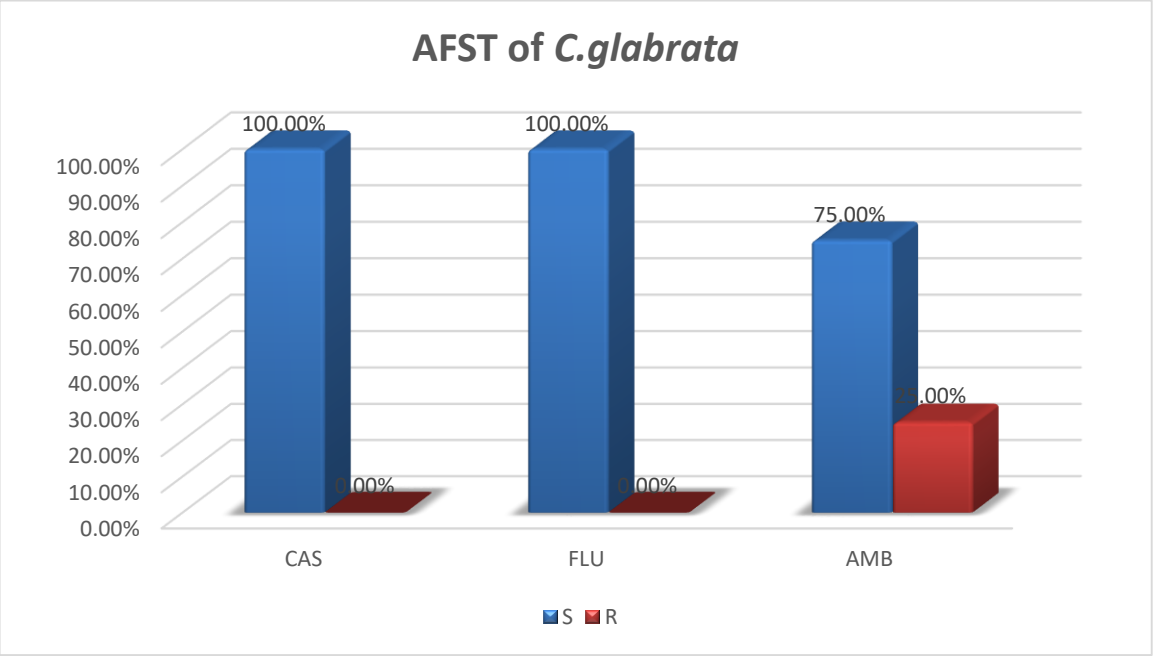

Figure 4-20 Antifungal susceptibility test (AFST) of *C.glabrata*

4.4.3.5 Antifungal susceptibility tests (AFST) of *C. krusei*

Caspofungin was sensitive in 1 (100%) and resist in 0 (0%) of *C. krusei,* this result was disagree with Subramanya *et al.,* (2017) who found that 66.7% of *C. krusei* were sensitive to CAS and consistent with him in the part of resistance which was 0%.

Fluconazole was sensitive in 0 (0%) of *C. krusei*, and resistant in 1 (100%) of *C. krusei*, this result is consistent with ElFeky *et al.*, (2016) who found that 20% of *C. krusei* were sensitive to FLU and 40 % of *C. krusei* were resist to it and consistent with Khan *et al.*, (2018) and Subramanya *et al.*, (2017) who found that 100% of *C. krusei* were resist to FLU.

Amphotericin B was sensitive in 1(100%) and resist in 0 (0%) of *C.krusei*, this result is consistent with ElFeky *et al.*, (2016) who found that 100% of *C. krusei* were sensitive to AMB and disagree with Subramanya *et al.*, (2017) who found that 33.3% of *C. krusei* were sensitive to AMB and 66.7% were resist (figure 4-21).

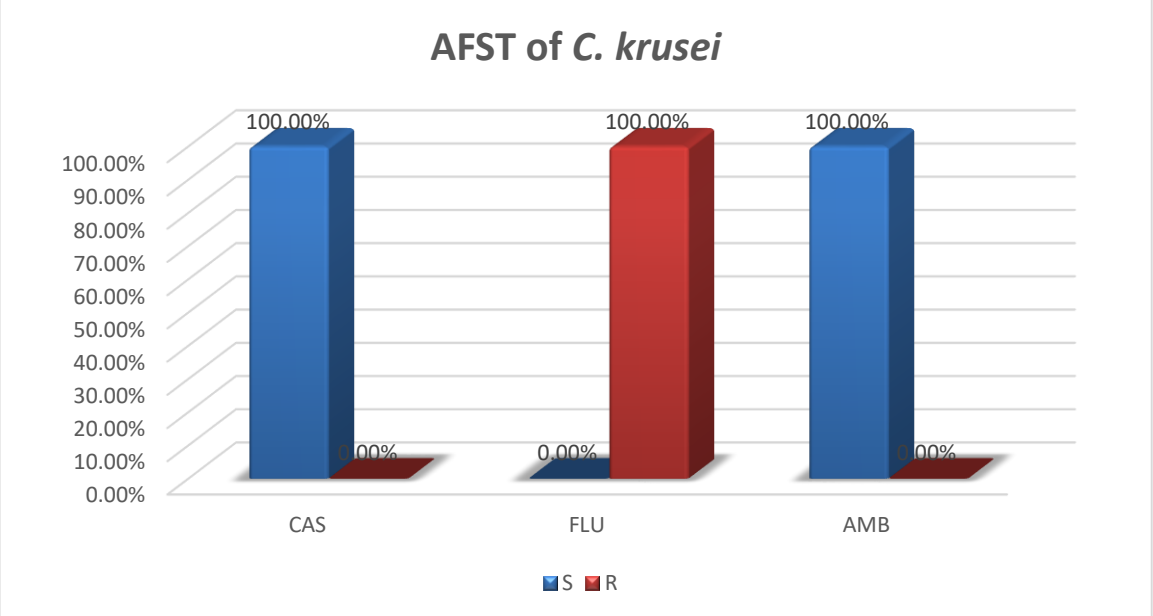

Figure 4-21 Antifungal susceptibility test (AFST) of *C. krusei*

4.4.3.6 Comparative study of AFST among *Candida spp.*

The results of the current study showed that Caspofungin was the most effective antifungal against *Candida spp.*, the resistance for *C. albicans* was 21.27% while it was 0% for non-albicans species. Fluconazole was the second most effective antifungal against Candida species, while Amphotericin B was the least effective antifungal (table 4-6).

Table 4-5 Comparative study of AFST among *Candida spp.*

Antifungal / *Candida spp.*	Caspofungin		Fluconazole		Amphotericin B	
	S (%)	R (%)	S (%)	R (%)	S (%)	R (%)
C. albicans	**78.72**	**21.27**	**72.23**	**27.65**	**23.40**	**76.59**
C. tropicalis	**100**	**0**	**100**	**0**	**77.77**	**22.22**
C. parapsilosis	**100**	**0**	**62.50**	**37.50**	**50.0**	**50.0**
C. glabrata	**100**	**0**	**100**	**0**	**75.0**	**25.0**
C. krusei	**100**	**0**	**0**	**100**	**100**	**0**

The echinocandins class of antifungal agents acts by inhibition of the synthesis of 1, 3-β-D-glucan in the fungal cell wall (Perlin, 2015). All 3 available echinocandins—Anidulafungin, Caspofungin, and Micafungin possess fungicidal activity against most species of Candida, including those resistant to polyenes and to azoles (Aguilar-Zapata *et al.*, 2015). Caspofungin, Micafungin, and Anidulafungin were approved for clinical use by the US Food and Drug Administration (FDA) in 2001, 2005, and 2006, respectively, and are now recommended as first line agents for treating (Pappas *et al.*, 2016).

Pfaller and Co-workers in 2019 showed that the resistance to 1 or more of the echinocandins was distinctly uncommon among isolates of *C. albicans* (0.0%–0.1%), *C. parapsilosis* (0.0%–0.1%), *C. tropicalis* (0.5%–0.7%), and *C. krusei* (0.0%–1.7%). Resistance to caspofungin (3.5%) was most prominent among *C. glabrata* isolates. No trend toward increasing resistance was seen over time for any of these species, although *C. tropicalis* exhibited an increase in resistance in 2015–2016 compared with previous years.

In this study, we found an emergence of resistance of *C. albicans* to caspofungin therefore, the most urgent challenge in pharmaceutical research is the discovery and the development of new antifungal which would be effective against all *Candida spp.*

The fluconazole resistance profile of the 4 most common species of Candida ranged from 0.3% (*C. albicans*) to 8.1% resistant (*C. glabrata*). *C. krusei* is not listed as it is considered intrinsically (100.0%) resistant to fluconazole, *C. krusei* is not listed as it is considered intrinsically (100.0%) resistant to fluconazole (Pappas *et al.,* 2016). The low fluconazole resistance rate among *C. albicans* isolates was consistent with previous reports (Lockhart *et al.*, 2012) and showed very little change from 0.2% in 1997–2001 to 0.1% in 2015–2016. Resistance to fluconazole showed an increase from 1997 through 2014 for *C. glabrata* (8.6% to 10.1%) and *C. tropicalis* (2.5% to 4.9%), with a slight decline for both species in 2015–2016, possibly due to increased use of echinocandins over fluconazole in those years (Bailly *et al.,* 2016). Given past recommendations to use fluconazole as first line therapy for *C. parapsilosis* infections (Pappas *et al.*, 2009), it is notable that fluconazole resistance increased over time for this species, while in our study we found that 37.5% of *C. parapsilosis* were resist to fluconazole.

Previously, (Pfaller *et al.*, 2019) investigators and others found echinocandin resistance in 8.0%–9.0% of fluconazole-resistant isolates of *C. glabrata* (Healey *et al.*, 2016). In the SENTRY Program from 2006– 2016, (they noted co-resistance in fluconazole-resistant isolates of *C. glabrata* (5.5%–7.6%) and in fluconazole-resistant isolates of *C. tropicalis* (1.9%–3.6%) and *C. krusei* (0.0%–1.7%).

In our study, we found that 21.27% of fluconazole-resistant *C. albicans* were resist to caspofungin, while other non-albicans species were 0%.

4.5 Molecular study of MDR isolates

4.5.1 Detection of *nor*A and *sdr*M genes in MDR isolates

In our study, fifty five MDR isolates of Staphylococcus species were detected for the presence of *nor*A and *sdr*M genes. The results of the current study showed that 94.54% of MDR isolates have *nor*A gene and 90.90% of MDR isolates have sdrM gene as shown in figures (4-22 and 4-23).

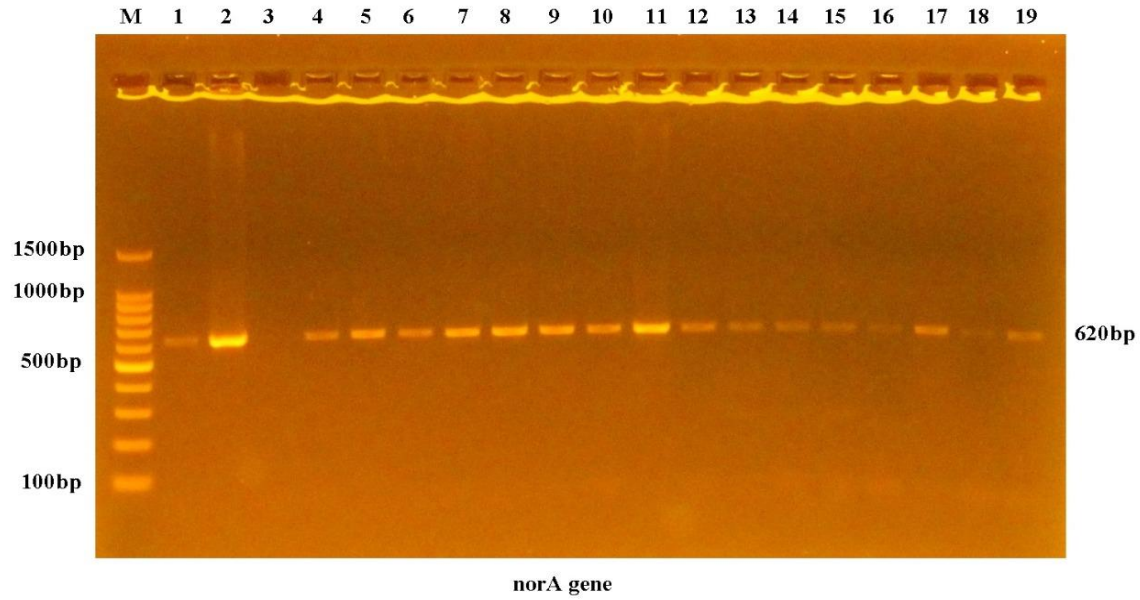

Figure 4-22: Results of the amplification of *nor*A gene of bacterial samples were fractionated on 1% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes 1-19 resemble 620 bp PCR products.

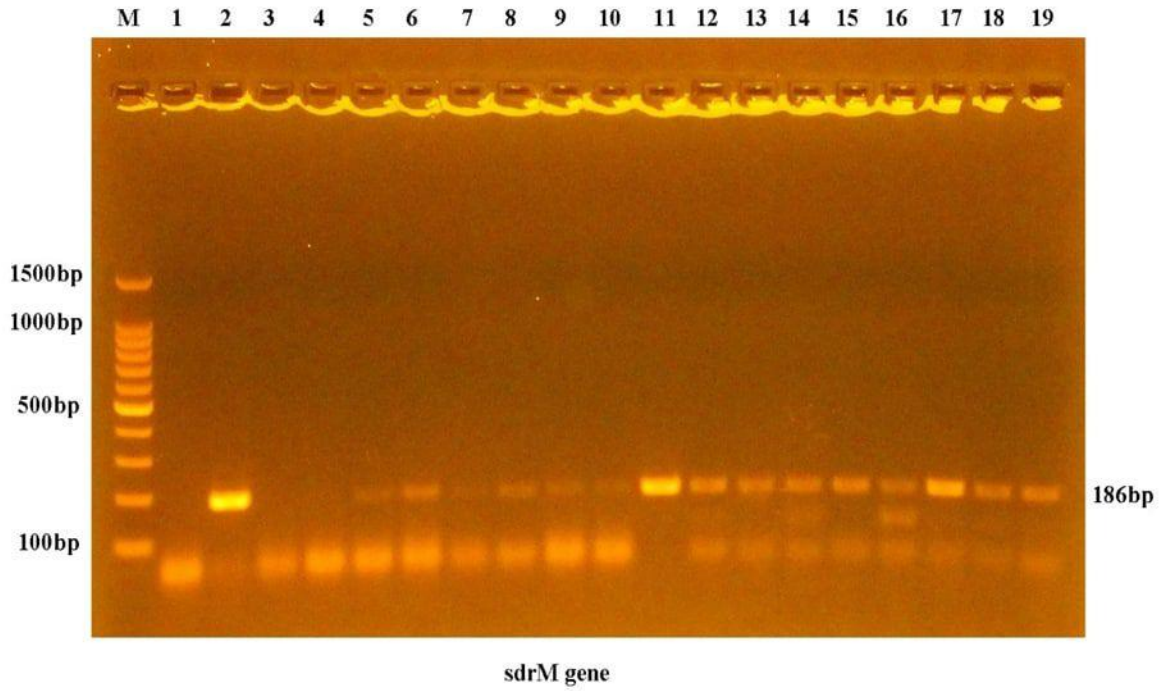

Figure 4-23 Results of the amplification of *sdr*M gene of bacterial samples were fractionated on 1% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes 1-19 resemble 186 bp PCR products.

4.5.2 Distribution of *nor*A and *sdr*M genes among *S. aureus*

In our study, we found that the distribution of *nor*A and *sdr*M genes are widely spread among *S. aureus* isolates that harbor the characteristic of MDR. The results showed that 32 (94.1%) of 34 of MDR *S. aureus* isolates have *nor*A gene while only two isolates didn't have the *nor*A gene. On the other hand, *sdr*M was found in 32 (94.1%) of MDR *S. aureus* isolates (table 4-7).

Table 4-6 Distribution of *nor*A and *sdr*M genes among MDR *S. aureus*

A	B	Name of isolate	*nor*A	%	*sdr*M	%	A.R
2	516	*S. aureus*	+		+		5
5	24	*S. aureus*	+		+		5
13	308	*S. aureus*	+	**94.1%**	+	**94.1%**	8
14	311	*S. aureus*	+		+		9
15	333	*S. aureus*	+		+		12
18	176	*S. aureus*	+		+		5
19	168	*S. aureus*	+		+		6
20	334	*S. aureus*	+		+		11
21	222	*S. aureus*	-		-		9
22	113	*S. aureus*	+		+		9
23	102	*S. aureus*	+		+		7
24	121	*S. aureus*	+		+		8
25	127	*S. aureus*	+		+		10
27	95	*S. aureus*	+		+		4
28	489	*S. aureus*	+		+		9
29	90	*S. aureus*	+		+		5
32	454	*S. aureus*	+		+		7
33	52	*S. aureus*	+		+		8
34	66	*S. aureus*	+		+		3
35	69	*S. aureus*	+		+		9
36	39	*S. aureus*	-		-		4
37	38	*S. aureus*	+		+		4
38	36	*S. aureus*	+		+		9
39	35	*S. aureus*	+		+		3
40	496	*S. aureus*	+		+		10
41	32	*S. aureus*	+		+		7
42	29	*S. aureus*	+		+		4
44	22	*S. aureus*	+		+		5
45	19	*S. aureus*	+		+		5
46	11	*S. aureus*	+		+		6
48	6	*S. aureus*	+		+		5
51	257	*S. aureus*	+		+		7
52	41	*S. aureus*	+		+		5
55	54	*S. aureus*	+		+		6

A= No. of isolate in gel electrophoresis / B= No. of isolate in samples/ A.R= No. of antibiotics resisted by the isolate

4.5.3 Distribution of *nor*A and *sdr*M genes in CONS isolates

The results of the current study showed that 20 (95.2%) of MDR coagulase negative *Staphylococcus* have the *nor*A gene and 18 (85.7%) of these isolates have the *sdr*M gene (table 4-8).

Table 4-7 Distribution of *nor*A and *sdr*M genes among CONS

A	B	Name of isolate	*nor*A	%	*sdr*M	%	A.R
1	410	*S. epidermidis*	+		-		7
3	445	*S. epidermidis*	-		-		6
4	436	*S. epidermidis*	+	**95.2%**	-	**85.7%**	5
6	400	*S. epidermidis*	+		+		5
7	393	*S. epidermidis*	+		+		6
8	370	*S. epidermidis*	+		+		7
9	357	*S. epidermidis*	+		+		6
10	353	*S. epidermidis*	+		+		7
11	395	*S. epidermidis*	+		+		4
12	498	*S. epidermidis*	+		+		5
16	212	*S. epidermidis*	+		+		6
17	207	*S. epidermidis*	+		+		6
26	101	*S. epidermidis*	+		+		7
30	70	*S. epidermidis*	+		+		9
31	100	*S. epidermidis*	+		+		5
43	22	*S. epidermidis*	+		+		5
47	10	*S. epidermidis*	+		+		5
49	5	*S. epidermidis*	+		+		3
50	3	*S. epidermidis*	+		+		3
54	94	*S. epidermidis*	+		+		8
53	34	*S. saprophyticus*	+		+		4

A= No. of isolate in gel electrophoresis / B= No. of isolate in samples/ A.R= No. of antibiotics resisted by the isolate

The distribution of *nor*A gene is evaluated in most studies. The frequency of this gene was studied in most Asian countries. Data from Asia regions indicated that among chromosomal genes *nor*A (75%) is most prevalent in five studies (Hassanzadeh *et al.,* 2020).

Among isolates from African countries, the highest distribution of the efflux pump genes belonged to *sep*A (95.3%), *mep*A (89.4%), *nor*A (86.4%) and *qac*A/B (40.5%) respectively. The prevalence of efflux pump genes among MRSA and MSSA isolates were detected in Pretoria in which *nor*A was 98.9%. Based on Iranian reports, *nor*A (64.3%) was the most frequently found among MRSA isolates, whereas significantly decreased in MSSA isolates. Based on evidence efflux pumps significantly contributed to the multidrug resistance in MRSA. In European Studies, the prevalence of *nor*A was 23.3% (Hassanzadeh *et al.,* 2020).

The chromosomally encoded MFS efflux pump genes *nor*A and *sdr*M were evaluated by most studies. Based on a few reports in Hassanzadeh *et al.,* article, chromosomal MDR pump genes contribute to fluoroquinolones resistance in *S. aureus*. The obtained results from Asian countries are summarized as follows: In India, the expression of *nor*A was reported (61.5%) of MRSA isolates (Chaudhary *et al.,* 2014). In Chinese studies, more than 90% of MRSA isolates were positive for each (*nor*A and *sdr*M) genes (Liu *et al.,* 2015). The results of Iranian studies showed that *nor*A was the most frequent gene (76.45%) detected (Hassanzadeh *et al.,* 2017; Ghaderkhani *et al.,* 2017).

A Study in Iraq, the frequency of *nor*A had been detected about 47% in *S. aureus* isolates (Mohammed *et al.,* 2015).

According to these studies in the different geographical region, the presence of antibiotics induced a various pattern of efflux pump genes, which prevent triggering and transferring antimicrobial resistant genes. Therefore, the frequency and expression of these genes should be investigated more, which is important in design of next generation antibiotics and inhibitors to improve their performance (Hassanzadeh *et al.,* 2020).

The MDR chromosomal efflux pump has been reported to have a broader efflux range than effluxes that harbored by plasmid, some of efflux pump harbored by mobile genetic elements that acquired by reservoir allowing resistant spreading, the contribution of antibiotic resistant by MDR presented at different levels and restricted by expression level of efflux genes; intrinsic level provides the basal level of gene expression that conferring resistant, acquired resistance through mutation at regulatory elements cause reverse repression and phenotypic resistant represented by the presence of special inducers promoting gene expression (Blanco *et al.*, 2016; Jang, 2016).

A single efflux pump can confer resistance to various antibiotics families or specialized toward single type of antibiotics such as the staphylococci tetracycline/fatty acid exporting by *tet*K and *tet*L pumps, or can confer resistant to set of biocide compounds, dyes, antibiotics, and even heavy metals such as *nor*A, *nor*B, *nor*C, *qac*A/B, *lmr*S and *mep*A in *S. aureus* (Jang, 2016; Blanco *et al.,* 2016; Chovanova *et al.,* 2015).

The *nor*A gene was first isolated from a norfloxacin-resistant clinical isolate by screening of a library of chromosomal DNA in plasmid pBR322 for an ability to confer resistance to *E. coli* (Kaatz *et al.*, 1990). norA is normally present in many (and perhaps all) strains of *S. aureus*; has a homolog in other staphylococcal species, including *S. epidermidis* (Yamamoto *et al.*, 1990).

CONCLUSIONS AND RECOMMENDATIONS

Conclusions

1- Amsel's criteria can be performed somewhat easily and in short time but it has also disadvantages.

2-*S. aureus* was the most commonly identified bacteria among *staphylococcus spp.*

3- *C. albicans* was the most commonly identified fungi among candida spp.

4- In Diyala province the highest infection in women with BV and VVC occurred in the age group of (21-30) and lower infection in women with BV and VVC in the age group of (51-59) years old.

5- There is a good relationship between using vaginal douching and the absence of vaginal infections.

6- The higher the level of education, the lower the incidence rate of infection.

7- The highest incidence of BV and VVC among women was at a pH level of (5).

8- Imipenem was the most effective antibacterial against bacteria (*staphylococcus spp.*) and trimethoprim was the lowest effective antibacterial used in the current study.

9- Caspofungin was the most effective antifungal against *candida spp*. and fluconazole was the lowest effective antifungal used in the current study.

10- *nor*A and *sdr*M genes are responsible for a wide range of antibiotics resistance.

Recommendations

Through the findings according to this study we recommended the following:

1- The need to pay attention to the necessary tests when the emergence of symptoms of genital tract infection female, especially culture and non-description of treatments for patients without a sensitivity test in order not to allow the emergence of strains of resistance between microorganisms causing the infection of the reproductive system .

2- Spread health awareness among women and urge attention to personal hygiene to reduce the spread of infection of the female reproductive system

3- Due to the prevalence of female genital tract infection among women, we propose do extensive studies on the female genital tract infection and the factors that can help it to occur.

4- Early diagnosis is very important to detect the suitable drugs.

5- Further research would be necessary to determine the mechanisms that are involved in the process of virulence in *Candida* spp. and the genes that responsible for antifungal resistance.

6- Studying other types of vaginitis that are less highlighted, such as cytolytic vaginosis, aerobic vaginitis, and lactobacillosis; and their association, in addition to BV, with adverse gynecological and obstetric outcomes such as infertility, miscarriage, and preterm labour.


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APPENDIX

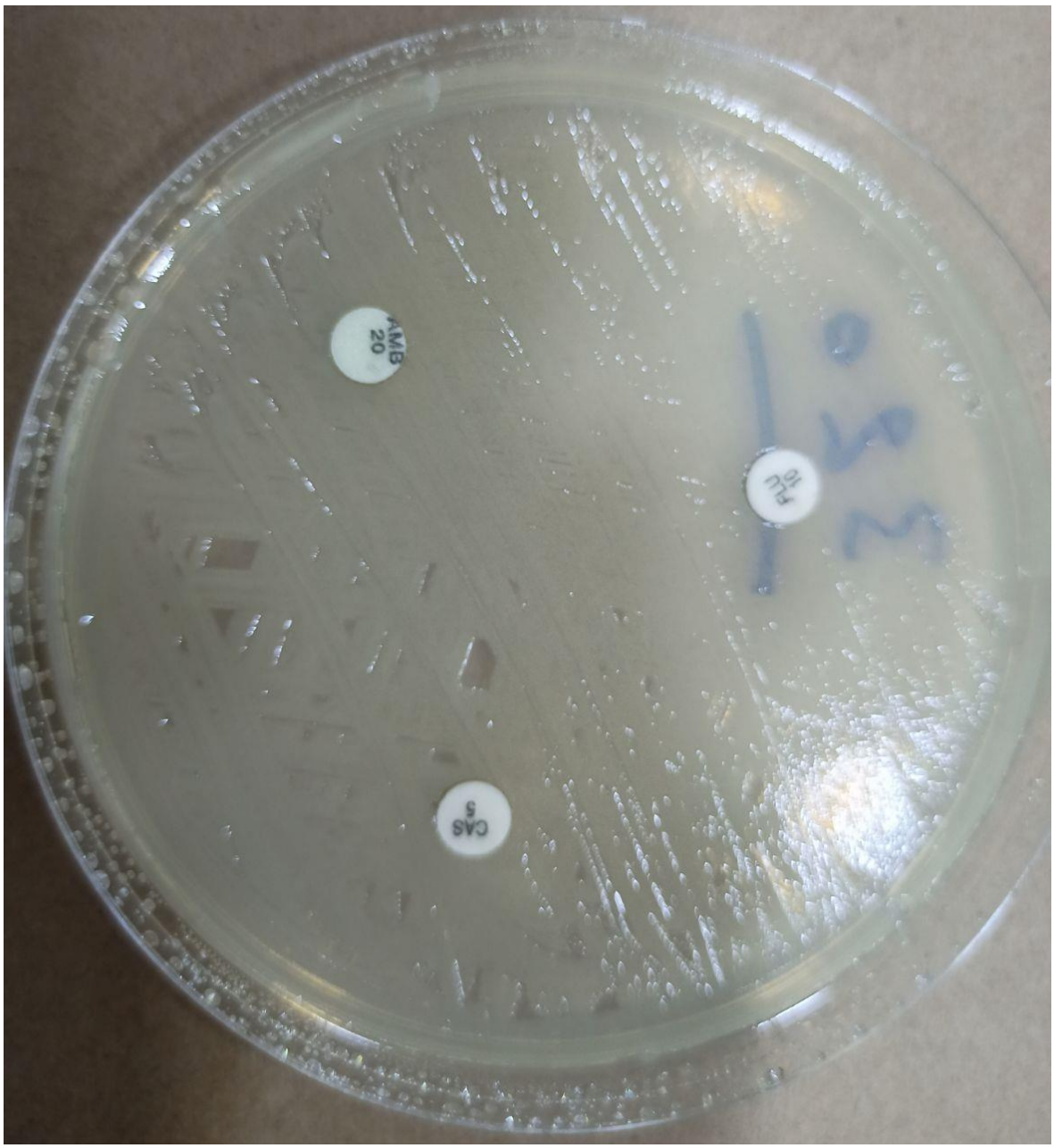
Appendix A

Questionnaire

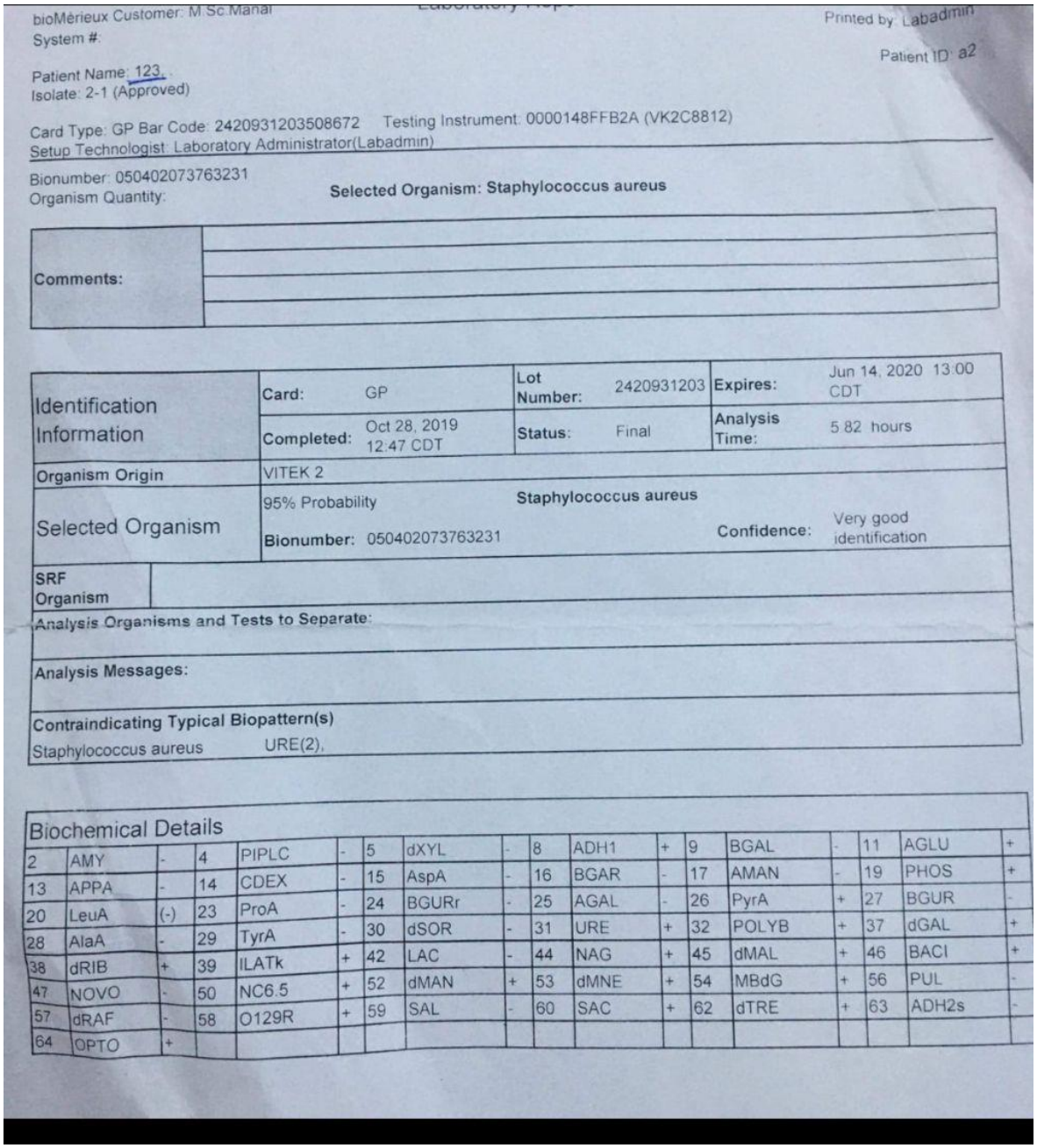
Name :		**Case no.**		**Age:**		**PH**	
Pregnant		**Non-pregnant**		**Non-fertile**			
Abortion	**Yes**	**No.of abortion**	**No. of natural birth:**	**No. of caesarean birth :**			
	No						
Accommodation	**Rural**	**Education :**	**Primary**		**Pre-infection**	**Yes**	
			Secondary			**No**	
	Urban		**Collage**				
Vaginal discharge	**Yes**	**Symptoms**	**Burning**	**Itching**	**Treatment**	**Yes**	
	No		**Pelvic Pain**	**Redness**		**No**	
Type of treatment :		**Contraception**	**IUD**		**Using of washing vaginal solution**	**Yes**	
			Tablets			**no**	
			Injection				
Vaginal ulceration	**Yes**	**Menopause**	**Yes**		**Surgery or cosmetic surgery**	**Yes**	
	No		**No**			**No**	
Diabetes mellitus	**No** / **Yes**	**Type I**		**Type of specimen**	**Vaginal swab**		
		Type II					
Date collection					**Cervical swab**		

Appendix B

Antifungal susceptibility test of *Candida spp.*



Identification of S. aureus by VITEK



bioMérieux Customer: M.Sc.Manal
System #:
Printed by: Labadmin
Patient ID: a2
Patient Name: 123
Isolate: 2-1 (Approved)

Card Type: GP Bar Code: 2420931203508672 Testing Instrument: 0000148FFB2A (VK2C8812)
Setup Technologist: Laboratory Administrator(Labadmin)

Bionumber: 050402073763231
Organism Quantity:

Selected Organism: Staphylococcus aureus

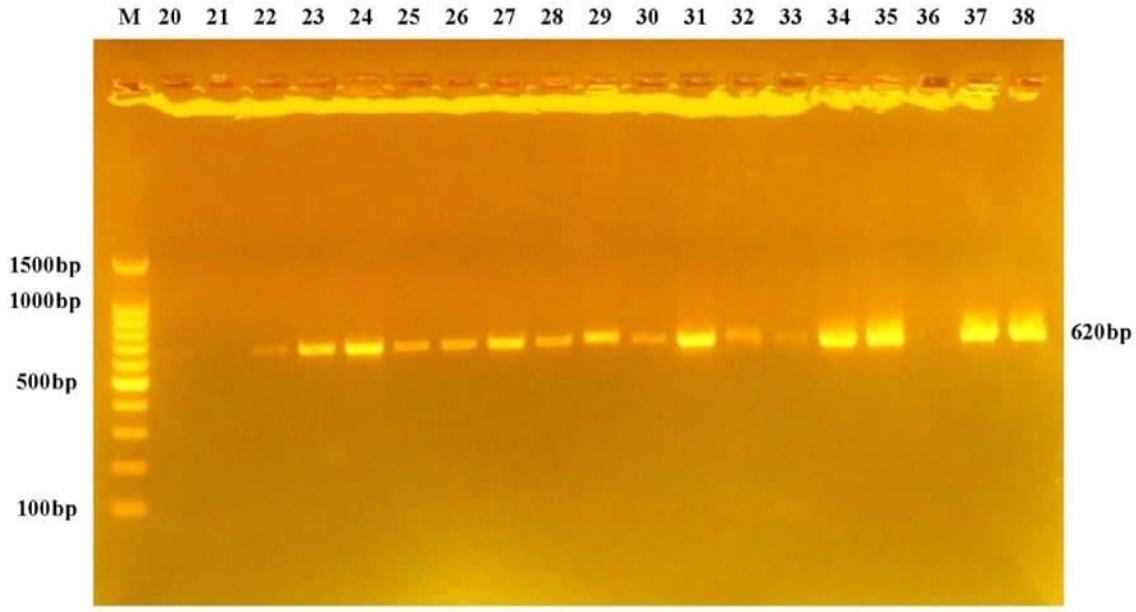
Comments:	

Identification Information	**Card:** GP		**Lot Number:** 2420931203	**Expires:**	Jun 14, 2020 13:00 CDT
	Completed: Oct 28, 2019 12:47 CDT		**Status:** Final	**Analysis Time:**	5.82 hours
Organism Origin	VITEK 2				
Selected Organism	95% Probability	**Staphylococcus aureus**			
	Bionumber: 050402073763231			**Confidence:**	Very good identification
SRF Organism					
Analysis Organisms and Tests to Separate:					
Analysis Messages:					
Contraindicating Typical Biopattern(s) Staphylococcus aureus URE(2),					

Biochemical Details

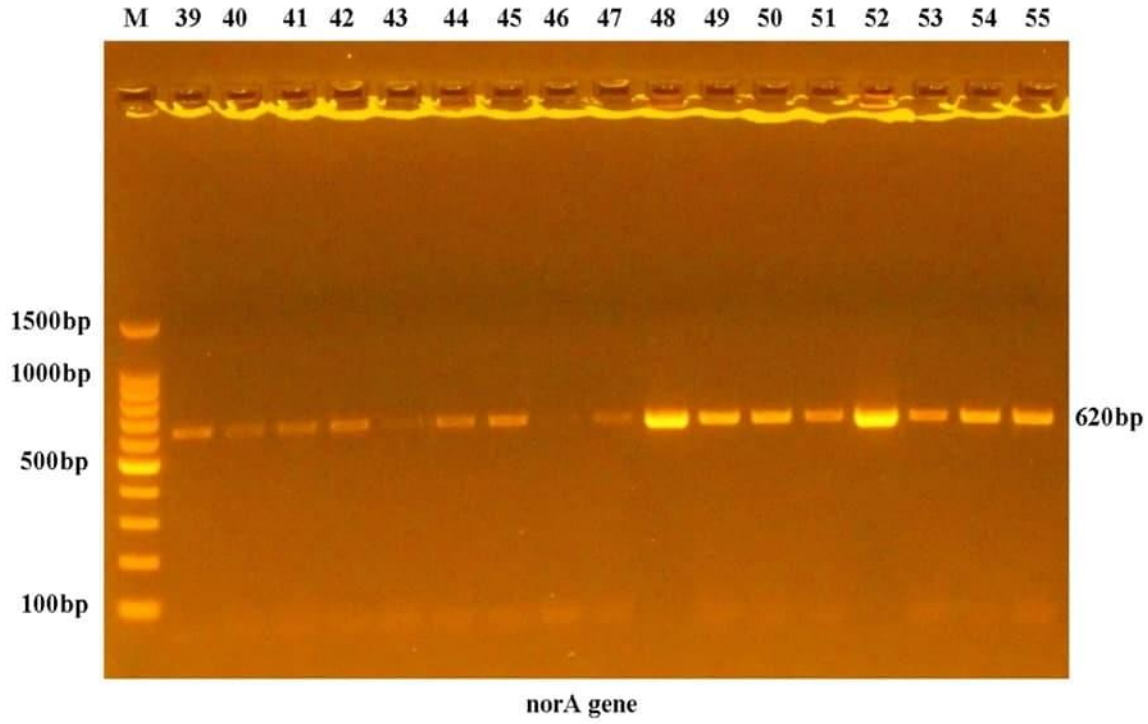
2	AMY	-	4	PIPLC	-	5	dXYL	-	8	ADH1	+	9	BGAL	-	11	AGLU	+		
13	APPA	-	14	CDEX	-	15	AspA	-	16	BGAR	-	17	AMAN	-	19	PHOS	+		
20	LeuA	(-)	23	ProA	-	24	BGURr	-	25	AGAL	-	26	PyrA	+	27	BGUR	-		
28	AlaA	-	29	TyrA	-	30	dSOR	-	31	URE	+	32	POLYB	+	37	dGAL	+		
38	dRIB	+	39	ILATk	+	42	LAC	-	44	NAG	+	45	dMAL	+	46	BACI	+		
47	NOVO	-	50	NC6.5	+	52	dMAN	+	53	dMNE	+	54	MBdG	+	56	PUL	-		
57	dRAF	-	58	O129R	+	59	SAL	-	60	SAC	+	62	dTRE	+	63	ADH2s	-		
64	OPTO	+																	

*nor*A gene on gel electrophoresis 20-38

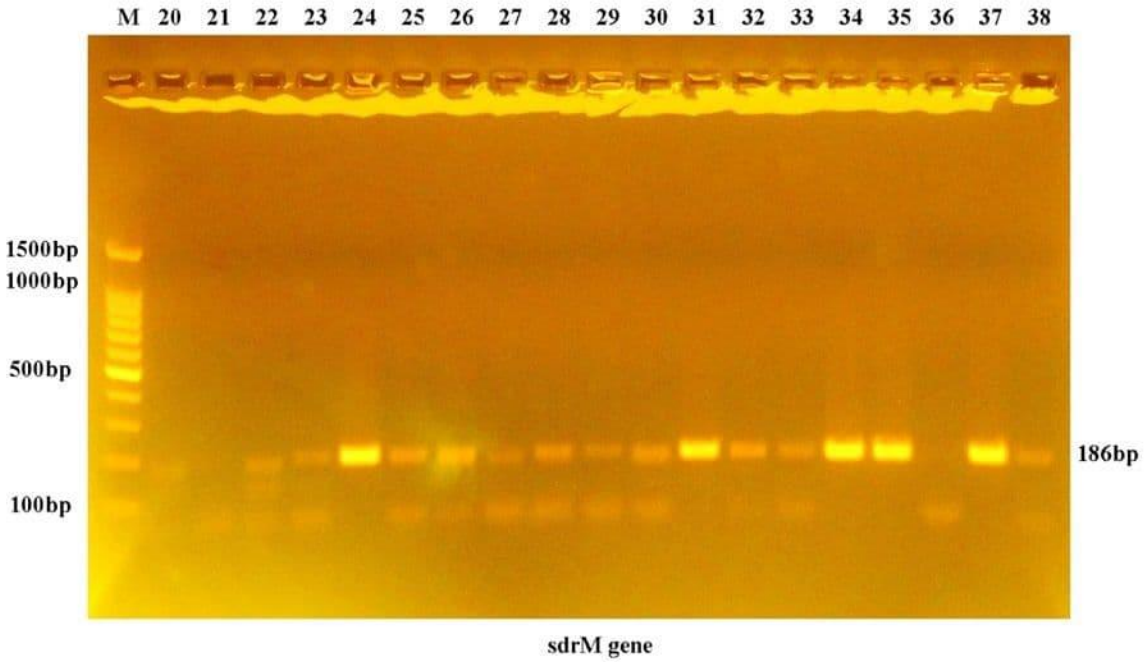


norA gene

*nor*A gene on gel electrophoresis 39-55

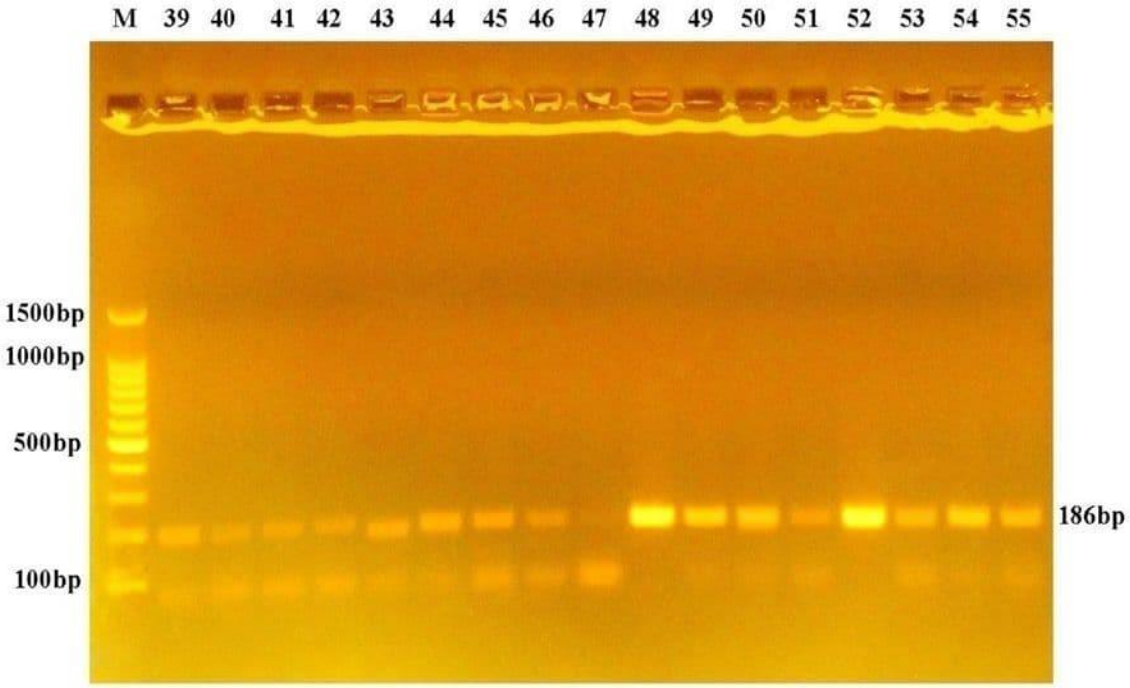


*sdr*M gene on gel electrophoresis 20-38



sdrM gene

sdrM gene on gel electrophoresis 39-55



sdrM gene

الخلاصة

أجريت دراسة مقطعية لتقييم انتشار البكتريا الموجبة لصبغة كرام والفطريات التي تسبب التهابات في الجهاز التناسلي الانثوي. جمعت 500 مسحة مهبلية من سيدات في مستشفى البتول التعليمي للفترة من تشرين الاول 2019 إلى شباط 2020. توزعت العينات بين 333 حامل و 167 غير حامل والفئة العمرية 13-59 سنة. أظهرت نتائج الاختبار التشخيصي الروتيني والتي اكدت باستخدام نظام VITEK comparte 2 ، وجود تنوع في الأنواع البكتيرية والفطرية ، حيث سجلت بكتيريا *S. aureus* أعلى نسبة عزل بواقع (23.8٪) ، تليها بكتريا *E. coli* و *S. epidermidis* و *Klebsiella spp.* و *pseudomonas spp.* و *S. saprophyticus* والتي كانت بواقع (16.3٪ ، 12.6٪ ، 9.3٪ ، 2.8٪ ، 1.8٪) على التوالي. كانت *C. albicans* الأكثر عزلًا بنسبة 21.9٪ يليها *C. tropicalis* و *C. parapsilosis* و *C. glabrata* و *C. krusei* بنسبة (4.2٪ ، 3.7٪ ، 2.8٪ ، 0.50٪) على التوالي. أظهرت نتائج دراسة بعض عوامل الضراوة أن العزلة *S. aureus* تنتج الهيموليسين بنسبة 90٪. أظهرت النتائج أن (62.7٪) من عزلات بكتريا *S. aureus* لديها القدرة على الالتصاق بسطح الخلايا الطلائية البشرية بينما كانت نسبة الالتصاق لبكتيريا *S. epidermidis* و *S. aprophyticus* (77.7٪ و 75٪) على التوالي. أظهرت النتائج تبايناً في المقاومة للمضادات الحيوية قيد الدراسة من قبل العزلات ، كان Trimethoprim أكثر المضادات الحيوية مقاومة من قبل البكتيريا بنسبة (53.6٪) / Erythromycin and Trimethoprim sulfamethoxazole بنسبة (48.7٪ و 46.8٪) على التوالي ، بينما كان Imepenem أكثر المضادات الحيوية حساسية من قبل البكتيريا بنسبة (97.5٪) يليها فانكومايسين وكليندامايسين بنسبة (68.2٪ و 67٪) على التوالي. أظهرت النتائج أن Caspofugin كان أكثر مضادات الفطريات فعالية ضد المبيضات بنسبة (85.9٪) ، بينما كان الفلوكونازول أقل مضادات الفطريات فعالية ضد المبيضات. بنسبة (63.3٪). أظهرت نتائج الدراسة الجزيئية للعزلات البكتيرية أن هناك توزيعًا واسعًا للجين norA (94.54%) و sdrM (90.90%) بين عزلات MDR التي تحتوي على خاصية المقاومة لثلاث فئات أو أكثر من المضادات الحيوية.

جمهورية العراق

وزارة التعليم العالي و البحث العلمي

جامعة ديالى

كلية العلوم

نسبة انتشار المكورات العنقودية والمبيضات لدى النساء الحوامل والغير حوامل في الجهاز التناسلي مع التحري عن جين *norA* و *sdr*M

مجلس عمادة كلية العلوم / جامعة ديالى كجزء من متطلبات نيل درجة الماجستير في علوم الحياة

من قبل الطالب

مصطفى سلام عبدالخالق نعمان

بكالوريوس علوم حياة / جامعة ديالى

(2017)

بأشراف

أ. م. د. مثنى عبدالقادر المهداوي

1442 ه 2020 م