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Detection of Antibiotics - Resistance Bacteria among Diabetic Foot Ulcer Patients in Khartoum State – Sudan 2022

الكشف عن البكتيريا المقاومة للمضادات الحيوية بين مرضى قرحة القدم السكرية في ولاية الخرطوم – السودان 2022

A Thesis Submitted in Partial Fulfillment of Requirement for the Degree of Master in Medical Laboratory Sciences (Microbiology)

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الآيـــــة

قَالَ تَعَالَىٰ:

﴿ فَتَعَٰلَى ٱللَّهُ ٱلْمَلِكُ ٱلْحَقُّ ۗ وَلَا تَعْجَلْ بِٱلْقُرْءَانِ مِن قَبْلِ أَن يُقْضَىٰٓ إِلَيْكَ وَحْيُهُۥ ۖ وَقُل رَّبِّ زِدْنِى عِلْمًا ﴿١١٤﴾ ﴾

سورة طه – الآية (١١٤)

Dedication

My humble effort is dedicated to

My loving **Father** and **mother**

My sweet **brothers** and **sisters**

To teachers everywhere those teach so other minds can grow

Acknowledgements

First and for most thanks to ALMIGHTY ALLAH for giving me strength, health and determination to accomplish this research work.

I would like to thank my supervisor Dr. Nasr Mohammed Nasr for continuous guidance throughout the path of this study.

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Abstract

Diabetic foot infection is a severe complication being faced by a large number of diabetic patients. Caused by many types of bacteria some of these are resistant to antibiotics ;thus lead to spread of lesions in the deep tissues may increase the risk of the amputation. The early detection and proper treatment of infection is essential to limit this complication.

This is descriptive cross sectional study was conducted in Khartoum State, Sudan during the period from May 2022-September2022, aimed to determine the frequency of antibiotic resistant bacteria among diabetic foot infection patients to find most risk factors associate with generating resistance to antibiotic.

Seventy swab samples were taken from lesion of diabetic patients. Identification of Bacteria by using different biochemical tests and antibiotic susceptibility test was performed. Out of seventy samples processed 66(94%) of them showed growth. The results showed that 66 patients sample (29/66) (44%) were diabetic foot ulcer patients have bacteria sensitive to antibiotics, compared to (37/66) (56%) with antibiotic resistant bacteria.

There was no significance association between antibiotics resistance and gender with p.value (0.144), and age with p.value (0.477). Also no significance association with type of diabetes with p.value (0.582).

The most isolated organism was Gram-negative bacteria the most common one is *Pseudomonas*.sp (15) (22.7%).The isolated bacteria showed varying susceptibility pattern to the antibiotics used and the most resistance found in gram-positive bacteria by *Staphylococus.aureus* (14) (25.8%) against Penicillin .

Finding of this study indicate high frequency of bacterial strains resistant to antibiotics in diabetic foot ulcer which may increase the risk of amputation.

المستخلص

تعد التهابات القدم السكريه من المضاعفات الشديده التي يواجهها عدد كبير من مرضى السكري. والتي تسببها انواع عديدة من البكتيريا وبعضها مقاومة للمضادات الحيويه وبالتالي تؤدي الى انتشار الافات في الانسجة العميقة التي قد تذيد من خطر البتر.الاكتشاف المبكر والعلاج المناسب ضروري للحد من انتشار التطور.

كانت هذه دراسه مقطعية اجريت في ولاية الخرطوم–السودان خلال الفترة من مايو2022 الى سبتمبر2022 وتهدف الى تحديد وتيرة الاصابة بالبكتيريا المقاومة للمضادات الحيوية بين مرضى قرحة القدم السكرية والعثور على اكثر عوامل الخطر المرتبطة بتوليد البكتيريا المقاومة للمضادات. اخذت 70 عينة من جروح مرضى السكري وتم التعرف عليها باختبارات كيميائية حيوية مختلفة وتم اجراء اختبار الحساسية للمضادات الحيوية .

واظهرت النتيجة من اصل 70 عينة تمت معالجتها اظهر (94%)66 منهم نمو بكتيري .ومن 66 عينة (66/29)(44%) غيرمصابين بالبكتيريا المقاومة للمضادات مقارنة ب(66/37)(56%) مصابين بالبكتيريا المقاومة للمضادات . لم يكن هناك ارتباط بين البكتيريا المقاومة وجنس المرضى و نوع السكري وكذلك علاقة سلبية مع العمرحيث كان معدل الاصابة بالبكتريا المقاومة موجود في كل الفئات العمرية.

اكثر البكتيريا المعزولة هي سالبة الجرام واكثرها الزائفة الزحارية(15)(22.7%) اظهرت البكتيريا المعزولة انماط حساسية متفاوتة للمضادات الحيوية المستخدمة كما ان اعلى نسبة مقاومة كانت ضد البنسلين بواسطة البكتيريا موجبة الجرام البكتيريا العنقودية الذهبية(14)(25.8%).

وفي الختام تشير هذه الدراسة الى وجود سلالات بكتيرية مقاومة للمضادات الحيوية قد يصعب علاجها والتي قد تذيد من خطر البتر.

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List of abbreviations

ADA	-	American Diabetes Association
AMR	-	Antimicrobial Resistance
CLSI	-	Clinical Laboratory Standards Institute
DFI	-	Diabetic Foot infection
DFU	-	Diabetic Foot Ulcer
DM	-	Diabetes mellitus
FDA	-	Foods and Drug Administration
GDM	-	Gestational Diabetes Mellitus
KIA	-	Kligler's Iron Agar
MDR	-	Multi-drug Resistance
MIC	-	Minimum Inhipetary Concentrations
NCCLS	-	National Committee clinical laboratory stander
VRE	-	Vancomycin Resistance Enterococci

CHAPTER I

INTRODUCTION

CHAPTER I

INTRODUCTION

1.1. Background

Diabetes mellitus (DM) is one of main problems in health systems and a global public health threat that has increased dramatically over the past two decades (Shahbazain *et al*., 2013).

With the prevalence of (DM) a number of new complications related to the health of patients have been witnessed in the recent decades. On top of it, diabetic foot infection (DFI) is a severe complication being faced by a large number of diabetic patients consisting of lesions in the deep tissues (Zhang *et al*., 2016).

Moreover foot wounds are the most common diabetes-related complication often leading to hospitalization, around 15% of diabetic patients experience foot ulcer once in their life time. (Yazdanpanah *et al*., 2015).

The development of ulcer on the foot is related to the trauma that disrupts the protective skin envelope on the foot, leading to the bacterial colonization of the underlying subcutaneous tissues (Padros 2018).

However, infection is caused by the overgrowth of microorganisms in these areas leading to the destruction of tissues. Therefore, (DFI) might result in the amputation of the lower limb due to healing failure (Yazdanpanah *et al*.,2015).

Foot infection in person with DM is often initially treated empirically. The empirical antibiotics used are usually meant for broad-spectrums organisms' coverage or according to local antibiogram study. Hence, therapy directed at known causative organisms may improve the outcome .many studies have reported on the bacteriology of DFIs over the past 25 years, but the results have varied, and they have often been contra directory (Citron *et al.*, 2007).

False diagnosis of DFI leads to unnecessary overuse or misuse of antibiotics. Furthermore, the types of pathogens and drug resistance rate of DFI are arising dramatically, due to the widespread use of broad-spectrum antibiotics and variations in antibiotic resistance genes (Boyanova and mitov., 2013).

Therefore study on the local causative organisms and antibiograms of DFIs an essential tool for better management of diabetic foot patients. (Lipsky *et al.*, 2004).

The current study is aimed at discussing the prevalence of antibiotic resistance among patients with diabetic foot ulcers.

1.2. Rationale

Foot infections are among the most common lower extremity complication in the (DM) population (excluding neuropathy), second only to foot ulcers in frequency (Lavery *et al.,* 2003).

As the incidence of (DM) is increasing globally, complications related to this endocrine disorder are also mounting and (DFIs)is an important cause of morbidity and mortality in patients with DM.DFIs affect one in10 patients with DM during their life time (Lipsky *et al.*,2004).

Infection may be caused by pathogenic bacteria originating from the external environment as well as by bacteria forming physiological microflora of the skin (e.g. *Staphylococcus epidermidis*, *Staphylococcus aureus*).

Pathogenic microflora is often transferred unconsciously by medical personnel and materials and substances used for treatment (Citron *et al.,* 2007).

Usually ulcerations contain mixed flora, consisting of several strains of bacteria. Most often these are aerobic bacteria and some strains anaerobic (Sopata *et al*., 2006).

Antibiotic resistance occurs when bacteria change in response to the use of these medicines. Bacteria, not human or animals become antibiotic-resistant. These bacteria may infect humans and animals, and the infections cause are harder to treat than those caused by non-resistant bacteria. Antibiotic resistance lead to higher medical costs, prolonged hospital stays, and increase mortality (Loukas *et al.*, 2021)**.**

Diabetes is now common and major health problem in sudan .The estimated prevalence of diabetes in urban areas in north sudan was thought to be around 19% in comparison with 2.5% in rural regions.like other developed and developing countries, high prevalence of uncontrolled diabetes (85%) is noted in sudanese individualswith type 2 diabetes.prevalence of diabetic foot ulcer was 18.1% and the risk of development of diabetic foot ulcer is increased with duration of diabetes more than 10 years.(Elmadhoun.,2016).

In this regard,applying this study to have more data about the detection of antimicrobial resistance bacteria among diabetic foot ulcer patients those who starting treatment and those who relapsed due to cutting or fail of treatment to control antibiotic resistance is one of the controlling strategy besides Observing treatment process.

1.3. Objectives

1.3.1. General objective

To determine the frequency of the antibiotics resistance bacteria among diabetic foot ulcers.

1.3.2. Specific objectives

1-To isolate and identify the bacterial pathogens from wounds infection in diabetic patients.

2-To carry out susceptibility testing for isolated bacteria to antibiotics (disk diffusion method).

3-to determine the association between the frequency of antibiotic resistance among diabetic foot ulcer and the possible risk factors of diabetes (age, gender, type of diabetic).

CHAPTERII

LITERATURE REVIEW

CHAPTERII

LITERATURE REVIEW

2.1. Diabetes mellitus

2.1.1. Definition

DM is a group of metabolic diseases characterized by chronic hyperglycemia resulting from defects in insulin secretion, insulin action, or both. Metabolic abnormalities in carbohydrates, lipids, and proteins result from the importance of insulin as an anabolic hormone. (American diabetes Association, 2014).

2.1.2. Classification of diabetes mellitus

The classical classification of diabetes as proposed by the American Diabetes Association (ADA) in 1997 as type 1, type 2, other types, and (GDM) is still the most accepted classification and adopted by ADA. (Wilkins, 2009).

Type1diabetes is characterized by destruction of the pancreatic beta cells, leading to absolute insulin deficiency. This is usually due to autoimmune. However, the absence of pancreatic auto antibodies does not rule out the possibility of type1 diabetes. Some patients with absolute insulin deficiency have no evidence of autoimmunity and have no other known cause for beta cell destruction. They are said to have idiopathic or type 1diabetes mellitus. (Chang *et al*., 2019).

Type 2 diabetes by far the most common type of diabetes in adults and is characterized by hyperglycemia and variable degrees of insulin deficiency and resistance. It is a common disorder whose prevalence rises markedly with increasing degrees of obesity. Insulin resistance and insulin deficiency can arise through genetic or environmental influences, making it difficult to determine the exact cause in an individual patient. In addition, hyperglycemia itself can impair pancreatic beta cell function and exacerbate insulin resistance (chang *et al*., 2019).

2.1.3. Pathogenesis

Lack of insulin drives the mobilization often energy stores from muscle, fat, and the liver. Glucose accumulates in the blood, causing hyperglycaemia. In the kidneys, the glucose reabsorption mechanism becomes saturated and glucose appears in the urine.

Glucose within renal tubules draws waterin by osmosis, leading to osmotic diuresis.The raised plasma osmolality stimulates the thirst centre.Overtime; diabetes damages capillaries and markedly accelerates atherosclerosis. (Cavanaugh *et al*., 2005).

2.1.4. Diabetic foot ulcer

Patients with DM are prone to multiple complications such as (DFU) that has shown an increasing trend over previous decades (Cavanaugh *et al*., 2005).

In total, it is estimated that 15% of patients with diabetes will suffer from DFU during their lifetime. Although accurate figures are difficult to obtain for the prevalence of DFU, the prevalence of this complication ranges from4% 27 %(Bakri *et al*., 2012).

The pathophysiology of the diabetic foot is complex; however, the infection is generally caused by the disturbance in the host such as neuropathy, immunopathy and arteriopathy and other factors related to patients. (Spichler *et al.,* 2015).

The development of ulcer on the foot is related to the trauma that disrupts the protective skin envelope on the foot,leading to the bacterial colonization of the underlying subcutaneous tissues.

The management of (DFU) including local wound care use of mechanical of floading, treatment of infection, and indications for revascularization.(Padros *et al.*,2018).

2.1.4.1. Etiology

Risk factors that can lead to foot wounds in patients with diabetes include loss of protective sensation due to neuropathy, prior ulcers or amputations, foot deformity leading to excess pressure, external trauma, infection, and the effects of chronic ischemia, typically due to peripheral artery disease.(Chang *et al*.,2019).

2.1.4.2. Ulcer classification

The first step in managing (DFU) is assessing, grading, and classifying the ulcer. Classification is based upon clinical evaluation of the extent and depth of the ulcer and the presence of infection or ischemia,which determine the nature and intensity of treatment.To assess for ischemia ,all patients with (DFU) should have ankle-brachial index and toe pressure measurements. (Boulton *et al*., 2008).

2.1.4.3. Risk factors

Several risk factors are predictive of ulcers and amputation. Early recognition and management of risk factors is important for reducing morbidity of (DFU).

Most risk factors are readily identifiable from the history or physical examination; the most important are previous foot ulceration, neuropathy (loss of protective sensation), foot deformity, and vascular disease.

The significance of these risk factors was confirmed by the results of community-based study of 1300 type 2 diabetic patients (Padros *et al.*, 2018).

2.2. Antimicrobial resistance

2.2.1. Definition

Resistance is the ability of a bacteria against the antagonizing effect of an antibacterial agent upon reproduction prevention or bactericidal. The development to resistance to antibiotics in bacteria often develops as a result of unnecessary and inappropriate use of antibiotics. Through the intense use of antibiotics, resistant microorganisms have emerged over the years, and problems were started to be experienced for the treatment of these infections emerged with these resistant microorganisms. Today, on the one hand trying to develop new drugs, on the other hand, there are difficulties in treatment as a result of development of resistance to these drugs rapidly. The development of resistance to antibiotics is a major public health problem in all over the world. (Salih, 2013).

2.2.2. Prevalence of Antimicrobial resistance

The increasing prevalence of (AMR) coupled with the dry antimicrobial development pipeline threatens the success and continuation of clinical medicine as we know it. This threat decreases the ability to successfully treat numerous infectious diseases while simultaneously increasing health risks for vulnerable patients. Medical procedures, such as hipreplacements, organ transplants, chemotherapy, hemodialysis and care for preterm infants may become too risky or impossible due to untreatable community-acquired ("nosocomial") infections. Common infectious diseases may once again result in death. (Per *et al.*, 2004).

Antibiotic resistance occurs when bacteria change in response to the use of these medicines. Bacteria, not human or animals become antibiotic-resistant. These bacteria may infect humans and animals, and the infections cause are harder to treat than those caused by non-resistant bacteria. (Lukas *et al.*, 2021).

2.2.3. Types of resistance to antibiotics

The main four types of resistance to antibiotics develop:

Natural (Intrinsic) resistance

Acquired resistance

Cross-resistance

Multi-drug resistance and pan-resistance (Salih, 2013).

Natural (Intrinsic, Structural) resistance: This kind of resistance is caused by the structural characteristics of bacteria and it is not associated with the use of antibiotics.It has no hereditary property.It develops as result of the natural resistance or the microorganisms not including the structure of the target antibiotic, or antibiotics not reaching to its target due to its characteristics. For example, Gram-negative bacteria vancomycin does not passing the outer membranes or Gram-negative bacteria is naturally resistant to vancomycin. Similarly, L-form shape of bacteria which are wall-less forms of the bacteria, and the bacteria such as cell wall-less cell *Mycoplasma* and *Ureaplasma* are naturally resistant to beta-lactam antibiotics that inhibit the cell wall synthesis. (Salih, 2013).

Acquired resistance: As result of the changes in the genetic characteristics of bacteria, anacquired resistance occurs due to its not being affected from the antibiotics it has been responsive before. This kind of resistance occurs due to mainly structures of chromosome or extra chromosomal (plasmid, transposon,etc) (Salih, 2013).

Cross resistance: Some microorganisms which are resistant to a certain drug that acts with the same or similar mechanism and also resistant to the drugs. This condition is usually observed in antibiotics whose structures are similar: such as resistance between erythcephalosporins and penicillins. However, sometimes it can also be seen in a completely unrelated drug groups. There is an example of cross-resistance between erythromycin-lincomycin.This may be chromosomal or extra chromosomal origin (Salih, 2013).

Multi-drug resistance and pan-resistance: Multidrug-resistant organisms are usually bacteria that have become resistant to the antibiotics used to treat them. This means that a particular drug is no longer able to kill or control the bacteria. In appropriate use of antibiotics for therapy resulted in the selection of pathogenic bacteria resistant to multiple drugs.Multidrug resistance in bacteria can be occurred by one of two mechanisms.First,these bacteria may accumulate multiple genes, each coding for resistance to single drug. This type of resistance occurs typically on resistance (R) plasmids. Second type of resistance, namely multidrug-resistance may also occur by the increased expression of genes that code for multi-drug efflux pumps,enzymatic inactivation,changes in the structure of the target.(Salih, 2013)

2.2.4. Mechanisms of Resistance to Antibiotics

The changes that occur in the receptor that connected to the drug and the region of the connection. Connection of the antibiotics' target areas is different.

Enzymatic inactivation of antibiotics: Most of Gram-positive and Gram-negative bacteria synthesize enzymes that degrade antibiotics. Enzymes include chloramphenicol and erythromycin.Reduction of the inner and outer membrane permeability.

Flush out of the drug (Active Pump System): Resistance developing through the active pump systems mostly common in tetracycline group of antibiotics.Using an alternative metabolic pathway. (Salih, 2013)

Mechanisms in these bacteria have evolved rapidly, owing to the presence of selective pressures. Their defense mechanisms against antibiotics involve the production of antibiotic deactivating enzymes, such as the several classes of β-lactamases or aminoglycoside emodifying enzymes, changes inantibiotic targets, and reduction of intracellular antibiotic concentration, either by limiting the entrance of the antibiotic or facilitating its expulsion. Due to the devastating results of infections caused by these pathogens, appropriate management of such cases is essential. For the clinician, it is important to know and understand the mechanisms of resistance employed by these pathogens, in order to select appropriate antibiotic treatment, especially in cases where the pathogen is known but the antibiogramis still pending. (Peter *etal*., 2020).

Vancomycin and related glycopeptides are drugs of last resort for the treatment of severe infections caused by Gram-positive bacteria.

Vancomycin was long considered immune to resistance due to it is bactericidal activity based on binding to the bacterial cell envelope rather than to protein target as is the case for most antibiotics. (Ivo GBoneca and Gabriela Chiosis., 2003)

However, vancomycin resistance has emerged, first in *enterococci* and, more recently, in *S.aureus*. Furthermore, they focus on strategies that have been developed or are undercurrent investigation to overcome infections caused by vancomycin-resistant strains. Among these are glycopeptides derivatives with higher potency than vancomycin, small molecules that resensitise bacteria to the antibiotic and novel non-glycopeptides antibiotics. These agents are targeted to interfere with protein and/or peptidoglycan (PG) synthesis and integrity or with membrane permeability. Whilst most of these agents are still in clinical or preclinical development, some have entered the clinic and currently represent the only option for treating (VRE). (Kirsten Nunez, 2019).

Staphylococci are Gram-positive aerobic organisms. *S.aureus* is the most pathogenic; it typically causes skin infections and sometimes pneumonia, endocarditis, and osteomyelitis. Treatment is usually with penicillinase-resistantbeta-lactams, but because antibiotic resistance is common, vancomycin or other newer antibiotics may be required. Some strains are partially or totally resistant to all but the newest antibiotics, which include Linezolid, Tedizolid, Quinupristin/ Dalfopristin, Daptomycin, Telavancin, Dalbavancin, oritavancin, Tigecycline, Eravacycline, Omadacycline, Delafloxacin, Ceftobiprole, Ceftaroline, and Lefamulin. (Mohamed and Keith.,2017).

2.3. Previous studies

In the study carried in India by (AMJ, 2015) to identify the spectrum of multi-drug resistance bacteria associated with diabetic foot infection one hundred patients sample processed and 82 yields positive cultures 20 organism (24.4%) were gram-positive and 62 organisms (75.6%) were gram-negative. *S.aureus* (24.4%) and *E.coli* (24.4%) were the most common isolated organisms followed by *P.aeruginosa* (17.1%), *K.oxytoca* and *Citrobacter sp* each (12.1%) and *Proteus sp* (9.8%).the Gram-negative organisms (53.6%) of the organisms extended spectrum beta lactamase producer with highest production by *E.coli*.(AMJ., 2015)

Another studies conducted in china there were 11,483 diabetic patients with an average age of 60.2 ±10.1 years and a mean course of 10.6 ± 5.0 years between 2010 and 2019, covering most geographical regions of China. The prevalence of Gram-positive bacteria (43.4%) was lower than that of Gram-negative (52.4%).

The most prevalent pathogens isolated were S.*aureus* (17.7%), *E.coli* (10.9%),*P.aeruginosa* (10.5%), *K.pneumoniae* (6.2%), *S.epidermidis* (5.3%), *E.faecalis* (4.9%), and fungus (3.7%). The prevalence of polymicrobial infection was 22.8%. Gram-positive bacteria were sensitive to Linezolid, Vancomycin,and Teicoplanin. More than 50% of gram -negative bacteria was resistant to third-generation Cephalosporins, while the resistance rates of Piperacillin/Tazobactam, Amikacin, Meropenem, and Imipenem were relatively low. Among the 6017 strains of the isolated organisms, 20% had multi-drug resistance (MDR).S.*aureus* (30.4%) was the most predominant MDR bacteria, followed by extended-spectrum b-lactamase (19.1%)(Fan *et al*.,2022).

In south of china other study conducted by (Xiaoying Xie , 2017). atotal of 232 isolates were detected from the 117 swab specimens collected from diabetic patients, including 207 (89.2%) bacteria and 25 (10.7%) funguses, totally 46 pathogens. In the bacterial infection, the proportion of gram-negative bacteria (54.1%, 112/207) was higher than gram-positive bacteria (45.9%, 95/207). *Enterobacteriaceae* was the main gram-negative bacteria (73.2%, 82/112), mainly including *Escherichia coli*, *E.cloacae*, and *K.pneumonia*, among which the predominant isolates were *K.pneumonia* (15.2%, 17/112). *Proteus* (18.8%, 21/112) and *Pseudomonas.sp* (14.3%, 16/112) followed. *Staphylococcus* (65.2%, 62/95) is the predominant pathogen in gram-positive bacteria, main of which was *S.aureus* (43.2%, 41/95), and followed by *Enterococcus* (20.0%, 19/95). *Candida* was the main pathogen in fungal infection, accounted for 68.0%. as the

representative of gram-positive cocci, *S.aureus* showed a high resistance rate to common antibiotics.high resistance rate to Penicillin was detected (92.3%, 36/39), followed by the Tetracycline (64.1%, 25/39). high resistance rates to the common antibiotics were detected in *Enterobacteriaceae*. Almost all the isolates were resistant to the Ampicillin (85.4%, 70/82), followed by the first/second generation Cephalosporin, including Cefazolin(72.0%, 59/82) and Cefuroxime (64.6%, 53/82), Low resistance rates were detected to Carbapenem (1.2%, 1/82), Cefoperazone-Sulbactam (7.3%, 6/82), the fourth generation Cephalosporin (8.5%, 7/82), and Tobramycin (8.5%, 7/82). (Xiaoying Xie *et al.*, 2017).

Another study conducted in Australia by McArdle in 2018 to identify the bacteria associated with diabetic foot infection all the patients with diabetic foot infections enrolled in Royal Darwin Hospital one hundred patients sample processed 40(40%) were gram-positive and most common organism is Methicillin resistant *S.aureus* 28(70%) 60 organisms (60%) were gram-negative, *E.Coli* 25(42%) and *p.aeruginosa* 19(31%), were the most common isolated organisms. The study highlighted that the ratio of diabetic patients is increasing all over the world leading to amputations in worst cases. (McArdle *et al*., 2018).

Another study conducted in south india concludes that most isolated organisms were Gram-positive such as *s.aureus* and *enterococcus*. And Gram-negative bacteria such as *P.aeruginosa* and *E.coli*. (Kathirvel *et al*., 2018).

The current study is aimed at discussing the prevalence of antibiotic resistance among patients with diabetic foot ulcers.

CHAPTER III

MATERIALS AND METHODS

CHAPTER III

MATERIALS AND METHODS

3.1. Study design

The study was descriptive-cross sectional study.

3.2. Study area and duration

Study was conducted in Khartoum State, during the period from May 2022 to September 2022.

3.3. Study population

Seventy diabetic foot ulcer patients involved to participate.

3.3.1. Inclusion criteria

Patients who diagnosed with diabetic foot ulcer.

3.3.2. Exclusion criteria

Diabetic patients were treatment with antibiotics.

3.4. Sample size

Seventy swab samples from diabetic patients were used and in this study.

3.5. Data collection

Samples were collected; Questionnaire was confirmed to demographic clinical and laboratory data.

3.6. Ethical consideration

Approval was taken from Ethical and Scientific Research Committee of Medical Laboratory Sciences College, Sudan University of Science and Technology and verbal consent was taken from all patients also clinical approval was taken.

3.7. Lab processing

3.7.1. Collection of specimens

Atotal of seventy specimens were collected from either abscess aspirates or wound swabs were collected with sterile cotton swabs and moistened with sterile normal saline, then labeled with patients name and age, and abscess specimens were collected with sterile syringes and all specimens were transferred during two hours to the laboratory.

3.7.2. Culture of specimens

Wound and abscess aspirates samples were cultured in Blood agar aerobically and unaerobically and MacConkey agar medium, incubated at37 ºC for 24hrs.

3.7.3. Identification of microorganisms

3.7.3.1. Colonial morphology

The growing colonies were morphologically examined for size, color, shape, and hemolysis on blood agar by observation of the zone around colony, clear zone alpha hemolysis, green zone beta hemolysis in blood agar media, and the colonies were examined on MacConkey for lactose fermentation, pink color indicate lactose fermentation, and yellow color indicate non lactose fermentation.

Table (3-1) Colonial morphology and Gram stain of isolates

Isolates	On Blood Agar	On MacConky Agar	Gram stain
S.aureus	Medium white to grey high convex beta hemolytic colony	Tiny colorless colony non ferment	Positive cocci in cluter
S.epidermidis	Medium white to grey high convex beta hemolytic colony	Tiny color less colony Non ferment	Positive cocci in cluster
Enterococcus.sp	Medium white to grey alpha or non henmolytic	Very tiny colony pink ferment	Positive cocci in pairs and chain
Pseudomonas.sp	Large grey high convex colony	Medium to large non ferment	Negative bacilli
E.coli	Medium white to grey medium convex colony	Medium pink colony lactose ferment	Negative bacilli
Klebsiella.sp	Large grey high convex colony	Large pink mucoid colony high convex ferment	Negative bacilli
Proteus.sp	Large grey colony with swarrming	Medium colorless non lactose ferment	Negative bacilli

3.7.3.2. Indirect gram stain

Dry smear was prepared by emulsifying colony in drop of physiological saline and spread evenly in clean dry slide, then allowed to dry, and then the smear was fixed by passing over the flame for seconds.

Crystal violet was added to cover fixed smear for one minute, then washed by tab water, lugol's iodine was added for one minute and washed off by tab water, then decolorized by using acid alcohol for15-20seconds and also washed by tab water, finally safranin

was added for 2 minutes and washed off by tab water then wiped the back of slide, let to dry and examined under microscope by oil immersion lens(x100) (Cheesbrough, 2000).

3.7.3.3. Biochemical tests

3.7.3.3.1. Catalase test

2ml of 3% hydrogen peroxide was transferring to sterile test tube and by using wooden stick apportion from growth of organism under test was added to release of air bubbles indicate positive result, no air bubbles indicate negative result. Positive results appear as formation of air bubbles but in negative result no air bubbles are formed. (Cheesbrough, 2000).

3.7.3.3.2. DNAse test

By using of sterile straight loop under aseptic condition the organism under test was inoculated in the DNAse agar1plate and making heavy spot, the plate were incubated at 37°C for overnight at incubator. In the end of incubation period the plate cover with hydrochloric acid, the presence of clear zone around the spot indicates positive result. (Cheesbrough, 2000).

3.7.3.3.3. Coagulase test (slide method)

This test used to differentiate between *S.aureus* (positive) from other *Staphylococci* (negative) the test was performed by emulsifying portion of colonies from pure growth in a drop of undiluted plasma.Formation of Clot indicate positive result.(Cheesbrough, 2000).

3.7.3.3.4. Oxidase test

Oxidase test is helpful in the identification of microorganisms having ability to produce cytochrome oxidase enzyme. The test helps to differentiate oxidase positive Pseudomonacea and negative Enterobacteriacea families. Cytochrome oxidase basedon the principle of transfer of electrons from donor (Electron transport chain) to final acceptor (oxygen) and reduction will takes place in the form of water. Cytochrome oxidase will oxidize the electron donor and the color will change to dark purple. This test is performed by impregnation of 1 percent tetra-methyl-p-phenylenediamine dihydrochloride acting as artificial electron donor into a filter paper and dried. The bacterial colonies are smeared on paper strip and check for color. Change within 10 sec. (Win *et al*., 2006).

3.7.3.3.5. Indole Test

Following test is helpful in the identification of to bacteria having the ability to produce tryptophanase enzyme. This enzyme wills convert tryptophan amino acid into indole gas. Thus gas can be checked by adding different reagents such as Ehrlich's reagent or Kovac's reagent. Kovac's indicators contain para-dimethyl amino benzaldehyde in isoamyl alcohol and conc HCl while Ehrlich's contain ethanol instead of isoamyl alcohol. Indole gas reacts with the reagent and the form red color which indicates positive test result. (Mac Faddin, 2000)

3.7.3.3.6. Urease test

Urea medium, whether broth or agar, contains urea and the phenol red as a pH indicator. Many organisms produce the urease enzyme, which catalyzes the splitting of urea in the presence of water to release ammonia and carbon dioxide. The ammonia combines with the carbon dioxide and water to form ammonium carbonate, which turns the medium alkaline, turning the indicator from its original orange-yellow color to bright pink. (Tille *et al*., 2014)

3.7.3.3.7. Motility test

To differentiate between motile and non-motile bacteria.Motility is a very important means of identification in the family Enterobacteriaceae.Motility by bacterium is mostly demonstrated in a semi solid agar medium. In semi-solid agar media, motile bacteria 'swarm' and give a diffuse spreading growth that is easily recognized by the naked eye. The inoculums are stabbed into the center of a semisolid agar deep. Bacterial motility is evident by a diffuse zone of growth extending out from the line of inoculation. The non-motile bacteria will only grow in the soft agar tube and only the area where they are inoculated. (Cheesbrough, 2000).

3.7.3.3.8. Kligler's Iron Agar (KIA)

Test employs a medium for the identification of Enterobacteriaceae, based on double sugar fermentation and hydrogen sulphide production. Phenol red is the pH indicator, which exhibits a colour change in response to acid produced during the fermentation of sugars.

Stab the center of the medium in to the deep of the tube to within3-5mm from the bottom. With draw the inoculating needle and streak the surface of the slant. Incubate aerobically at 35ºC for 24 hours. Fermentation of dextrose results in production of acid, which turns the indicator from red to yellow. Since there is little sugar i.e. dextrose, acid production is very limited and therefore a reoxidation of the indicator is produced on the

surface of the medium, and the indicator remains red. However, when lactose is fermented, the large amount of acid produced, avoids reoxidation and therefore the entire medium turns yellow.

The combination of ferrous sulphate and sodium thiosulphate enables the detection of hydrogen sulfide production, which is evidenced by a black color. Lactose fermenters produce yellow slants and butts. The high amount of acids thus produced helps to maintain an acidic pH under aerobic conditions. Tubes showing original color of the medium indicate the fermentation of neither glucose nor lactose. Gas production (acrogenic reaction) is detected as individual bubbles or by splitting or displacement of the agar by the formation of cracks in the butt of the medium. (Cheesbrough, 2000).

3.7.3.3.9. Citrate Utilization Test

This test used to differentiate among the Gram-negative bacilli in the family Enterobacteriaceae. The medium contains citrate as the sole carbon source and inorganic ammonium salts. Bacteria that can grow on this medium produce an enzyme, citrate-permeate, capable of converting citrate to pyruvate. Pyruvate can then enter the organism's metabolic cycle for the production of energy. Streak the slant back and forth with light inoculums picked from the center of a well-isolated colony.

Incubate aerobically at 35 to 37 ºC for up to24 hours

Observe a color change from green to blue along the slant. When the bacteria metabolize citrate, the ammonium salts are broken down to ammonia, which increases alkalinity. The shift in pH turns the bromthymol blue indicator in the medium from green to blue above pH 7.6. (Cheesbrough, 2000).

3.7.3.3.10. Bile esculin test

This test used to differentiate *Enterococci* and group D *Streptococci*, which are bile tolerant and can hydrolyze esculin to esculitin from non-group D *Viridians* group *Streptococci* which grow poorly on bile. The organism was inoculated on esculin agar slant, the slant were incubated at 35ºC with loose caps for 24 h. reaction was considered positive when one half or more of the medium was blackened.(Facklam *et al*.,2000).

Table (3-2) Biochemical tests of Gram positive bacteria

	Organism		
Biochemical test	*S.aureus*	*S.epidermidis*	*Enterococcus sp*
Catalase test	Positive	Positive	Negative
DNAase test	Positive	Negative	-
MSA	Yellow colonies (lactose ferment)	Pink colonies (non lactose ferment)	-
Bile esculin agar	-	-	Positive

Table (3-3) Biochemical tests of Gram-negative bacteria

	Organism			
Biochemical test	*E.coli*	*Klebsiella.species*	*Pseudomonas. species*	*Proteus .species*
Oxidase test	Negative	Negative	Positive	Negative
Citrate utilization test	Negative	Positive	Positive	Positive
Urease test	Negative	Positive	Negative	Positive
Indole test	Positive	Negative	Negative	Negative
KIA	Yellow butt Yellow slope cracking and no h2s	Yellow butt Yellow slope cracking and no h2s	Pink butt pink slope no cracking and no H2s	Pink butt pink slope no cracking and H2s production

3.7.3.4. Antimicrobial susceptibility test

Disk diffusion test

The disk diffusion susceptibility method is simple and practical and has been well standardized. The test is performed by applying bacterial inoculums of approximately1-2(10^8) CFU/ml to the surface of a large (150mm diameter) agar plate. Up to12commercially-prepared, fixed concentration, paper antibiotic disks are placed on the inoculated agar surface plates and incubated for 16-24 hours at 35 ºC prior to determination of results. The zone of growth inhibition around each of the antibiotic disks is measured to the nearest millimeter. The diameter of the zone is related to the susceptibility of the isolate and to the diffusion rate of the drug through the agar medium. The zone diameter of each drug are interpreted using the criteria published by the (CLSI), formerly the NCCLS) or those included in the US (FDA) approved product insert for the disks. The results of the disk diffusion test are qualitative in that a category of susceptibility (i.e., susceptible, intermediate or resistant) is derived the test rather than an MIC. However, some commercially-available zone reader systems claim to calculate an approximate MIC with some organisms and antibiotics by comparing zone sizes with standard curves of that species and drug stored in an algorithm. (Korgenski and Daly1998).

Disk diffusion test is the usual applicable method for assessing the antimicrobial susceptibility pattern in most in situations and hospitals. (Hossein ,2012).

3.7.3.5. Statistical Analysis

Categorical variables were described by number and percent (N, %). Chi-square test was used to compare between categorical variables. A two-tail $P < 0.05$ was considered statistically significant.

CHAPTER IV

RESULTS AND DISSCUTION

CHAPTER IV

RESULTS AND DISSCUTION

4.1. Results

Seventy swab and wound aspirate samples were collected from DFU patients. Most isolated organisms is Gram-negative bacteria 35(53%) compared with 31(47%) Gram-positive bacteria as in table (4-1).

Table (4-1) Distribution of Isolates according to Gram stain

Gram stain	Frequency	Percentage
Gram-negative bacteria	35	53%
Gram-positive bacteria	31	47%
Total	66	100%

Also the result showed the most isolated organism in Gram-positive bacteria was *S.aureus* 17(23.8%), followed by *Enterococcus.sp* 9(13.6%) and *S.epidermides* 3(4.5%).and the most isolated organisms in Gram-negative bacteria was *Pseudomonas.sp* 15 (22.7%) followed by *E.coli* 8 (12%), *Klebsiella.sp* 7(7%) and *proteus.sp* 5(5%) as in table (4-2).

Table (4-2) Frequency of clinically important microorganisms isolated from DFU

Organisms	Frequency	Percentage
S.aureus	17	23.8%
S.epidrmides	3	4.5%
Enterococcus.sp	9	13.6%
Mixed organisms	2	3%
Pseudomonas.sp	15	22.7%
E.coli	8	12%
Klebsiella.sp	7	7%
Proteus.sp	5	5%
Total	66	100%

Antibiotic Susceptibility Test of isolates (disc diffusion method) Table (4-3) was prepared showed resistance and sensitivity pattern of isolates to different antibiotics. Some bacteria were resistant to more than two antibiotics and some were resistant to at least two antibiotics, most resistance showed in penicillin by *S.aureus*.

The interpretation of each Gram-positive bacterium either resistant or susceptible to antibiotic is showed in table (4-3).

Table (4-3) Antibiotic Susceptibility Test of gram-positive bacteria

		Gram-positive bacteria			
Antibiotics		***S.aureus* (17)**	***S.epidermides* (3)**	***Enterococcus.sp* (9)**	**Mixed (2)**
Penicillin	S	2 (12%)	2(67%)	2(22%)	0(0%)
	R	15 (88%)	1(33%)	7(78%)	2(100%)
Amoxicillin/ Clavulinic acid	S	17 (100%)	3(100%)	9(100%)	1(50%)
	R	0(0%)	0(0%)	0(0%)	1(50%)
Ceftriaxone	S	17(100%)	3(100%)	9(100%)	1(50%)
	R	0(0%)	0(0%)	0(0%)	1(50%)
Clindamycin	S	14(82%)	3(100%)	6(67%)	1(50%)
	R	3(18%)	0(0%)	3(33%)	1(50%)
Gentamicin	S	15(88%)	3(100%)	5(56%)	1(50%)
	R	2(12%)	0(0%)	4(44%)	1(50%)
Ciprofloxacin	S	13(76%)	3(100%)	8(89%)	1(50%)
	R	4(24%)	0(0%)	1(11%)	1(50%)
Tetracycline	S	12(70%)	3(100%)	4(44%)	0(0%)
	R	5(30%)	0(0%)	5(56%)	
Cotrimoxazole	S	_	_	_	_
	R	_	_	_	_
Ceftazidime	S	17(100%)	3(100%)	9(100%)	1(50%)
	R	0(0%)	0(0%)	0(0%)	1(50%)
Cefotaxime	S	_	_	_	_
	R	_	_	_	_
Erythromycin	S	10(59%)	3(100%)	3(33%)	0(0%)
	R	7(41%)	0(0%)	6(67%)	2(100%)
Cefoxitin	S	13(76%)	3(100%)	7(78%)	0(0%)
	R	4(24%)	0(0%)	2(22%)	2(100%)

Antibiotic Susceptibility Test of Gram-negative bacteria, Some bacteria were resistant to more than two antibiotics and some were resistant to at least two antibiotics, most resistance showed in Clindamycin by *Pseudomonas.sp.*

The interpretation of each Gram-negative bacterium either resistant or susceptible to antibiotic is showed in table (4-4).

Table (4-4) Antibiotic Susceptibility Test of gram-negative bacteria

		Gram-negative bacteria			
Antibiotics		***Pseudomonas.sp*** **(15)**	**E.Coli (8)**	***Klebsiella.sp*** **(7)**	***Proteus.sp*** **(5)**
Penicillin	S	_	_	_	_
	R	_	_	_	_
Amoxicillin/ Clavulinic acid	S	15(100%)	7(88%)	5(71%)	5(100%)
	R	0(0%)	1(12%)	2(29%)	0(0%)
Ceftriaxone	S	6(40%)	0(0%)	3(43%)	5(100%)
	R	9(60%)	8(100%)	4(57%)	0(0%)
Clindamycin	S	5(33%)	_	_	_
	R	10(67%)	_	_	_
Gentamicin	S	11(73%)	6(75%)	7(100%)	5(100%)
	R	4(27%)	2(25%)	0(01%)	0(0%)
Ciprofloxacin	S	9(60%)	8(100%)	7(100%)	5(100%)
	R	6(40%)	0(0%)	0(0%)	0(0%)
Tetracycline	S	_	_	1(14%)	4(
	R	_	_	6(86%)	1
Cotrimoxazole	S	11(73%)	8(100%)	7(100%)	5(100%)
	R	4(27%)	0(0%)	0(0%)	0(0%)
Ceftazidime	S	7(47%)	8(100%)	4(57%)	5(100%)
	R	8(53%)	0(0%)	3(43%)	0(0%)
Cefotaxime	S	_	8(100%)	7(100%)	5(100%)
	R	_	0(0%)	0(0%)	0(0%)
Erythromycin	S	_	_	_	_
	R	_	_	_	_
Cefoxitin	S	_	_	_	_
	R	_	_	_	_

Males were (45/66) (68.2%) compared with females (21/66) (31.8%).Resistance in the females (9/37) (24%). While (28/37) (76%) in the males. There was no significance statistical association between gender and AMR with P.value 0.144.as in Table (4-2).

Table (4-5) Relationship between Gender and Antibiotics Resistance Bacteria

Gender	Resistance to Antibiotics	Sensitive to Antibiotics	Total	P.value
Males	28(76%)	17(59%)	45	0.144
Females	9(24%)	12(41%)	21	
Total	37(56%)	29(44%)	66	

The distribution of age groups in the study population are categorized in 4 groups, group (1): less than 20 years: represent 3%, group (2): 20- 40 years represent 22.7%, and group (3): 40- 60 years represent 33.3% and group (4) above 60 years represent 40.9% of the population. Age grouped above 60 years was most frequent 15 (41%) with AMR compared with 40-60 years age grouped 13(35%),20-40 years age group 9(24%) and less than 20 years age group 0(0%) they was no significance statistical association between age and AMR with P.value as0.477.As shown in Table (4-6).

Table (4-6) Association of Age and Antibiotics Resistant bacteria

Age	Resistance	Sensitive	Total	P.value
<20	0 (0%)	2 (7%)	2(3%)	0.477
20-40	8 (24%)	7 (21%)	15(22.7%)	
40-60	14 (35%)	8 (31%)	22(33.3%)	
>60	15 (41%)	12(41%)	27(41%)	
Total	37 (56%)	29 (44%)	66(100%)	

The population had their type of diabetes (75.8%) subjects were type 2 and (24.2%) subjects were type 1.resistance must frequent in type 2 diabetes mellitus with no significance Statistical association between type of diabetes mellitus and AMR with P.value 0.582as in Table(4-7).

Table (4-7) Association of type of diabetes and Antibiotics Resistance Bacteria

Type of diabetes	Resistance to Antibiotics	Sensitive to antibiotics	Total	P.value
Type 1	8 (11%)	8(38%)	16(24.2%)	0.582
Type 2	29 (89%)	21 (62%)	50(7508%)	
Total	37 (56%)	29 (44%)	66(100%)	

5.2. Discussion

Foot infection in person with DM is often initially treated empirically. The empirical antibiotics used are usually meant for broad spectrums organisms coverage or according to local antibiogram study. Hence, therapy directed at known causative organisms may improve the outcome. (Citron *et al.,* 2007).

In this study the overall frequency of antibiotics resistance is 37 (56%) patients from 66 (100%) isolate which is agreed with many studies which represent high prevalence of resistance to antibiotics among diabetic foot ulcer patients.

The most isolated bacteria is Gram-negative 35 (53%) with the most common isolate was *Pseudomona.sp*. 15(22.7).while gram-positive 31(47%) with the most common isolate was S.aureus 17(25.8). Some bacteria were resistant to more than two antibiotics and some were resistant to at least two antibiotics, most resistance Gram-positive bacteria showed in penicillin by *S.aureus and the* most resistance in Gram-negative bacteria showed in Clindamycin by *Pseudomonas.sp.* Similar to the study in India done by AMJ, (2015) represent 20/82 organism (24.4%) were Gram-positive and 62/82 organisms (75.6%) were Gram-negative .*S.aureus* (24.4%) and *E.coli* (24.4%) were the most common isolated organisms followed by *Pseudomonas.sp* (17.1%).(AMJ,2015).

And the study conducted in China by Fan du *et al*.,(2022). The prevalence of Gram-positive bacteria (43.4%) was lower than that of Gram-negative (52.4%). The most prevalent pathogens isolated were *S.aureus* (17.7%), *E.coli* (10.9%), and *Pseudomonas.sp* (10.5%). More than 50% of gram -negative bacteria was resistant to third-generation Cephalosporins, while the resistance rates of Piperacillin/ Tazobactam, Amikacin, Meropenem, and Imipenem were relatively low, *S.aureus* (30.4%) was the most predominant MDR bacteria. (Fan Du *et al*., 2022).

In a study in south china from 207 isolates the proportion of Gram-negative bacteria (54.1%, 112/207) was higher than gram-positive bacteria (45.9%, 95/207). *Enterobacteriaceae* was the main Gram-negative bacteria (73.2%, 82/112),mainly including *Escherichia coli*, *E.cloacae*, and *K.pneumonia*, *Staphylococcus* (65.2%, 62/95) is the predominant pathogen in Gram-positive bacteria, majority of which was *S.aureus* (43.2%, 41/95), and followed by *Enterococcus* .(Xiaoying Xie *et al.*, 2017).

Another study from one hundred patients samples processed 40(40%) were Gram-positive and most common organism was Methicillin resistant *S.aureus* 28(70%) 60

organisms (60%) were Gram-negative, *E.Coli* 25(42%) and *P.aeruginosa* 19(31%), were the most common isolated organisms.(McArdle *et al*.,2018).

Most organisms isolated were resistant to antibiotics was *S.aureus* 14(37.9%) followed by *Pseudomonas sp* 10(27%) then *Enterococcus.sp* 6(16.2%), *Klebsiella sp* 3(8.1%), mixed organisms 2(5.4%), *E.coli* 2 (5.4), and no resistance in *S.epidermides* and *Proteus sp* and not agree with (kathivel *et al*., 2018) which represent Gram-positive most isolated than Gram-negative.

There was no statistical association between frequency of antibiotics resistance bacteria and gender with p.value (0.144) also no statistically significance association with age with p.value (0.477), and type of (DM) with p.value 0.582. The other study cannot focus the association between the AMR and patients characteristic.

This variation in the result may be due to sample size which other studies run by larger sample of patients in comparison with this study or may be due to difference in the methods used for identification or difference patient's characteristic and geographic area.

CHAPTER V

CONCLUSION AND RECOMMENDATIONS

CHAPTER V

CONCLUSION AND RECOMMENDATIONS

5.1. Conclusion

The present study concluded that there was high frequency of antibiotics resistance and this frequent is reduce the success DFU control, with most isolated organisms is Gram-negative bacteria but most frequency of antibiotics resistance bacteria showed in Gram-positive bacteria. Also there was no significant association between frequency of antibiotics resistance bacteria and age, gender and type of diabetes. Morever the most resistance to antibiotics showed in Penicillin.

5.2. Recommendations

1- Early detection and identification of causative agents with antimicrobial susceptibility to isolates to select the proper antibiotics.

2-Treatment should be carefully managed to control the resistant types and should be followed up to avoid treatment cut.

3- More research on AMR with genotyping needed for further information and more research should be conducted (include large sample size) to collect more data about the AMR prevalence

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APPENDIXES

Appendix (1) questionnaire

بسم الله الرحمن الرحيم

Sudan University of Sciences and Technology

College of Graduate Studies

Detection of Antibiotics- Resistance Bacteria among diabetic foot ulcer patients at Khartoum State-Sudan2022

الكشف عن البكتيريا المقاومة للمضادات الحيويه بين مرضى قرحة القدم السكرية في الخرطوم–السودان 2022

This questionnaire related to patients of diabetic foot ulcer:

Patients NO: ----------

1/ Gender

A/ male ( ) B/ Female ( ).

2/Age

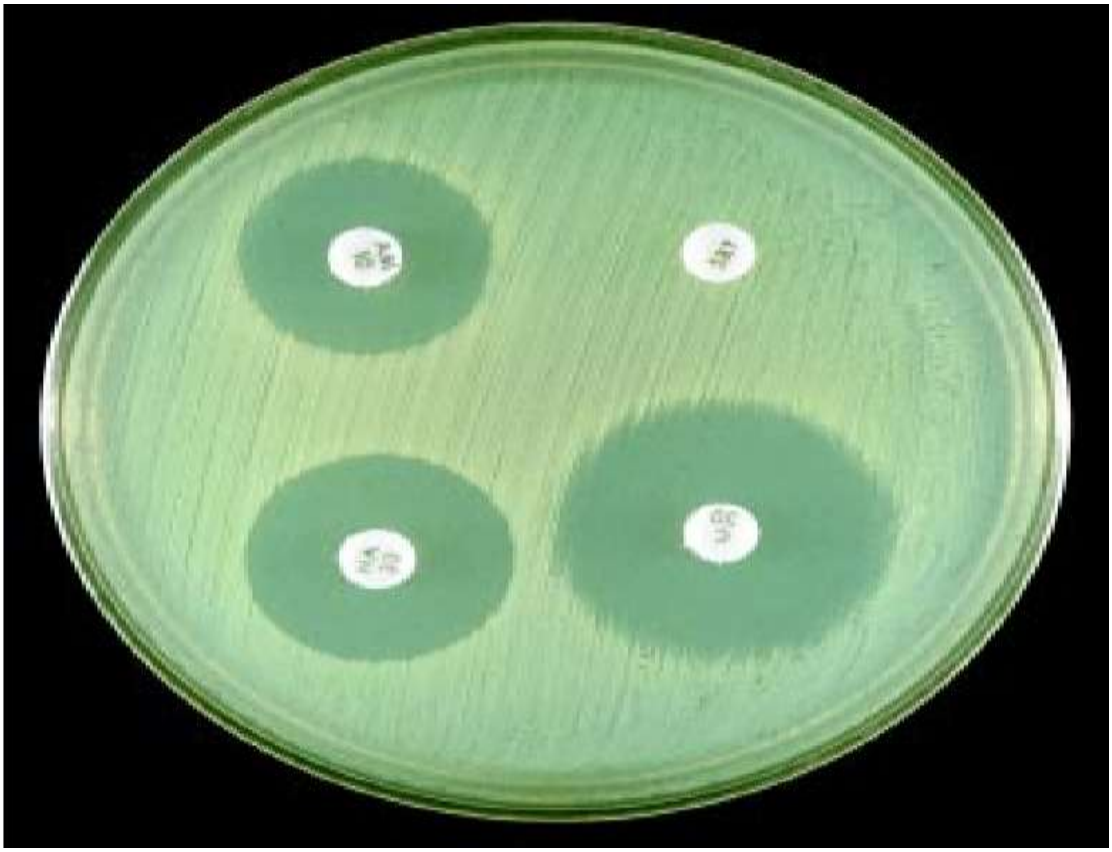
A/ <20 ( ) B/ 20-40 ( ) C/ 40-60 ( ) D/ >60 ( )

3/Type of diabetes

A/ Type 1 ( ) B/ Type 2 ( )

Appendix (2)

Mueller Hinton agar plate



Antimicrobial Susceptibility Test of isolates (disc diffusion method)