Isolation of Pathogenic Bacteria and Antimicrobial Profiling Patterns among Patients with Diabetic Foot Ulcer in Selected Hospitals in Addis Ababa, Ethiopia.

By:

Asegdew Atlaw (BSc)

Department of Microbiology, Immunology and Parasitology, School of Medicine, College of health Sciences, Addis Ababa University

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Advisors:

Dr. Yimtubezinash Woldeamanuel (MD, M.Sc, PhD, Associate Professor)

Habtamu Biazin (M.Sc, PhD Student)

Collaborator: Dr. Abdurezak Ahmed (MD) at TASH

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Asegdew Atlaw (BSc)

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Abbreviation and Acronyms

AAU	Addis Ababa University
ATCC	American Type Collection Culture
BAP	Blood Agar Plate
CLSI	Clinical Laboratory Standard Institution
CTX-M	Cefotaximase-Munich
CT	Computerize Tomography
COVID-19	Corona Virus Disease 2019
DFI	Diabetic Foot Infection
DFU	Diabetic Foot Ulcer
DM	Diabetes Mellitus
DKA	Diabetic Ketoacidosis
DMIP	Department of Microbiology, Immunology and Parasitology
DN	Diabetes Neuropathy
ESBL	Extended Spectrum of Beta-Lactamase
ESKAPE	*Enterococcus faecium, Staphylococcus aureus, Klebsiella Pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter species*
GN	Gram-Negative
GNB	Gram-Negative Bacteria
GP	Gram-Positive
GPB	Gram-Positive Bacteria
HHS	Hyperosmolar Hyperglycemic State
HGA1C	Hemoglobin A1C
IDSA	Infectious Disease Society of America
IWGDF	International Working Group on the Diabetic Foot
LDC	Lysine Decarboxylase
MAC	MacConkey
MDR	Multiple Drug-Resistance
MDROs	Multiple Drug-Resistance Organisms

MHA	Muller Hinton Agar
MRI	Magnetic Resonance Image
MRSA	Methicillin-Resistance Staphylococcus Aureus
MSSA	Methicillin-Sensitive Staphylococcus Aureus
NCDs	Non-communicable diseases
N-IFU	Neuro-ischemic Ulcer
NMDR	None Multiple Drug-Resistance
OUT	Operational unit of Taxonomic
PAD	Peripheral arterial Disease
PN	Peripheral Neuropathy
SIM	Sulfur-Indole-Motility
SOPs	Standard Operating Procedures
STGG	Skimmed milk Tryptone, Glucose, Glycerol
TASH	Tikur Anbessa Specialized Hospital
TCC	Total Contact Cast
TSI	Triple Sugar Iron
UK	United Kingdom
VRE	Vancomycin-Resistance Enterococci
WHO	World Health Organization

Abstract

Background: Infected diabetic foot ulcer (IDFU) is one of the challenging problems globally, associated with diabetes mellitus. It is a leading cause of lower extremities amputation, sepsis, and even mortality. Both gram-negative and gram-positive bacteria including anaerobic bacteria and fungi are considered potential causes of infections. **Objectives:** This study aimed to isolate potential pathogenic bacteria and their antimicrobial susceptibility pattern including phenotypical screening of extended-spectrum beta-lactamases (ESBLs) and carbapenemase-producers among IDFU in selected hospitals in Addis Ababa, Ethiopia.

Materials and Method: Institutional based cross-sectional study was conducted from November/2020 to May/2021 in selected hospitals in Addis Ababa, Ethiopia. A sterile swab was used to collect samples from foot ulcers while a sterile needle was used to collect pus aspirates. All isolates were identified by using routine bacterial culture, Gram-staining, and a panel of biochemical tests. For each identified bacterial species, antibiogram profiles were determined by the Kirby Bauer disk diffusion method based on the Clinical and Laboratory Standards Institute guidelines. Double disk and modified carbapenem (meropenem) inactivation methods were used for screening ESBL and carbapenemase based on CLSI 2020 guidelines. **Results:** Out of the 130 recruited participants, 88 (68.2%) were males and 42 (33%) were females. One hundred twenty-seven pathogenic bacteria were isolated, of which, the predominant bacteria was *S.aureus* 25.19% (32/127), followed by *Pseudomonas species* 18.89% (24/127). Gram-positive isolates were susceptible to Chloramphenicol, Clindamycin, and Amikacin. Gram-negative isolates were also sensitive to chloramphenicol, Aztreonam, and Amikacin. Overall, 92.9% (118/127) were multidrug-resistant. From the gram-negative bacteria, 53.94% (41/76) were extended-spectrum beta-lactamases (ESBLs)-producers, and 26.47% (19/68) were carbapenemase-producers. **Conclusion:** Bacteria that infect diabetic foot ulcers are multidrug-resistance. The results emphasize the importance of identification of the causative agent and antimicrobial susceptibility testing before the selection of appropriate antibiotic(s) to treat diabetic foot ulcers.

Key words: Diabetic Mellitus, Diabetic foot ulcer, Antimicrobial testing, Multidrug-resistance, ESBLs, Carbapenemase-producers, Addis Ababa, Ethiopia.

1. Introduction

1.1. Background

As per the World Health Organization (WHO) description, Diabetes is a chronic, metabolic disease characterized by elevated levels of blood glucose (or blood sugar), which leads over time to serious damage to the heart, blood vessels, eyes, kidneys, and nerves. It is caused by heterogeneous aetio-pathology including unsuccessful insulin secretion, insulin action, or both. As a result, there will be disturbances of carbohydrate, fat, and protein metabolism (WHO, 2019).

Diabetes is a chronic disease found worldwide and over time leads to serious damage to many organs and systems in the body. It is considered as a precursor to a host of other complications or long-term specific effects like retinopathy, cardiovascular disease, hypertension, nephropathy, neuropathy, diabetic foot ulcer, and much more (Gizaw *et al*., 2015).

Diabetic foot ulcer (DFU) is one of the most significant and devastating complications of diabetes mellitus (DM). It is well-defined as a foot below the ankle affected by ulceration/a full-thickness wound/ that is associated with neuropathy and/or peripheral arterial disease of the lower limb in a patient with diabetes (Apelqvist *et al*., 2000). As reported by different researches, the major risk factors for DFU development are diabetic neuropathy (90%), peripheral arterial disease (2-10%), and consequent traumas of the foot (Alexiadou and Doupis, 2012). Other risk factors include structural foot deformities and abnormalities, previous history of foot ulceration or amputation, poor glycemic control, and cigarette smoking. In addition, the danger of foot ulceration and limb amputation increases with age and the duration of diabetes.

Generally, based on several parameters (like extent, size and depth, location, presence of infection, and ischemia), DFU severity is classified into different categories by different groups such as Meggitt-Wagner, University of Texas Antonio classification system, Infectious Disease Society of America (IDSA) classification scheme, and International Working Group diabetic foot(IWGDF) (Noor *et al*., 2017). Meggitt-Wagner classification is

one of the most common DFU classification systems and it has six grades based on the depth of ulcer, presence of gangrene, and extent of tissue necrosis (Jain, 2012, Jain, 2015). Although peripheral neuropathy and peripheral arterial disease (PAD) is considered to be the most significant risk factors for DFU occurrence, microorganisms facilitate the severity of diabetic foot ulcers. As different articles currently show, greater than 50% of the ulcerated foot of diabetes patients are infected by different pathogenic microbes (Kwon and Armstrong, 2018). Infection is classically characterized by signs like inflammation (erythema, edema, heat, pain) and purulent discharge but in diabetic foot wounds, this is not typical due to ischemia and neuropathy. The Infectious Diseases Society of America (IDSA) and International Working Group on the Diabetic Foot (IWGDF) have put criteria (definition) to measure the severity of DFIs such as uninfected, mild, moderate, and severe infection *(Lipsky et al*., 2012, Lipsky *et al*., 2016).

Due to DFU, intact skin loses many of the protective barriers or mechanisms, and invasive microorganisms can easily enter through a wound portal. A variety of pathogenic microbes colonize diabetes foot wounds causing local tissue damage. Bacterial growth is enhanced by the presence of tissue ischemia (resulting in hypoxia) or necrosis, also hyperglycemia that affects host defenses (Jneid *et al*., 2017). Infections start with a minor problem but after a time they progress to unmanageable conditions (involving deep tissues, joints, or bones). DFU which occurs for a long period and with severe infection is usually associated with poly-microbial colonization (Noor *et al*., 2017, Jneid *et a*l., 2017).

Several authors from other countries reported that both aerobic and anaerobic including multidrug-resistance (MDR) pathogenic micro-organisms with the ability to form biofilm are isolated from foot ulcers in diabetes patients (Banu *et al*., 2015). Among Gram-positive bacteria; *Enterococcus spp, Peptostreptococcus spp, Veilonella species, Bacteroides species*, and *Clostridium perfringens* were identified (Charles *et al*., 2015, Patil *et al*., 2017). Gram-negative bacteria, such as: *Enterobacteriaceae* (*E. coli, Proteus Spp., K. pneumoniae, Shigella spp.*) and non-fermenters include *P. aeruginosa, and Acinetobacter spp.* were also identified (Najari *et al*., 2019, Hitam *et al*., 2019). Moreover, Fungi (like candida) are also prevalent and associated with delayed healing of DFU (Xie *et al*., 2017). The source of

these microbes is the environment, the neighboring skin, or other endogenous sources, including the gastrointestinal tract (Malone *et al.*, 2017).

1.2. Statement of the problem

Diabetic foot ulcer is one of the common complications of diabetes patients and can lead to a considerable burden of social, psychological, and economic burden on patients and the health sector (Moxey *et al*., 2010). DFU accounts for significant morbidity, mortality. Patients with DFUs were shown to have a 2.5-fold increased risk of death compared with diabetic patients without foot ulcers (Walsh *et al*., 2016, Chammas *et al*., 2016). As reported by the International Diabetes Federation, 9.1-26.1 million people will develop DFUs yearly (Armstrong *et al*., 2017). When the diabetes mellitus prevalence rose globally, the incidence of DFU significantly increased and the recent worldwide prevalence on average is about 6.4% (Zhang *et al*., 2017).

The major challenge of DFU once it occurs is that the increment of vulnerability rises for different pathogenic microorganism infections and other consequences that can result in severe and costly outcomes like infection, gangrene, osteomyelitis, amputation, and even death (Hitam *et al*., 2019). As reported by IDSA and other studies, infection of diabetic foot ulcers will increase the chance for amputation by 50% compared to patients with uninfected foot ulcers *(*Lipsky *et al*., 2012, Noor et al., 2015, Rice *et al*., 2014).

Diabetes-related hospital admissions are also most pronounced because of infected diabetic foot ulcers. Also major and minor (83% and 96% respectively) amputations have been done related to DFU which is aggravated by infection (Hicks et al., 2016). On the other hand, diabetic foot osteomyelitis development is seen in around 44-68% of patients admitted to hospital with a diabetic foot infection and is the principal cause of amputation among such patients (Van Asten *et al*., 2016).

Patients with infected DFU will develop systemic infection/sepsis and speed up the death of the patient. In developing countries, there are numerous multidrug-resistant (MDR) gram-negative bacteria including extended-spectrum beta-lactamase (ESBL) or carbapenemase-producing Enterobacteriaceae and MDR non-fermenters, which are becoming a serious

concern associated with diabetic foot infections especially in tertiary referral hospitals (Kwon and Armstrong, 2018). For example: in Nigeria, the Burden of diabetic foot ulcers accounted 24.9% and the majority of the ulcer is already infected with Wagner grade ≥ 3 (Ugwu et al., 2019).

Ethiopia is also one of the countries affected by diabetic foot ulcer. A systematic meta-analysis study indicated that the general national prevalence of DFU among Ethiopian diabetic patients was 11.27% (Mulugeta *et al.*, 2019). Other studies among specific areas, have shown that the prevalence is in Nekemte 17.9% (Bekele *et al.*, 2019), Gondar 13.6% (Mariam *et al.*, 2017), Arbaminch 15% (Deribe, B., *et al* 2014), and TASH 78% : (DFU with cellulitis 12.2%, DFU with toe gangrene 16.3%, DFU with foot gangrene 18.4%, only foot ulcer 31.1%) (Amogne *et al.*, 2011). Another more recent study showed that the prevalence of TASH was 26% (Yimam, 2017).

1.3. Literature Review

1.3.1. Global Burden of Diabetic Mellitus

Diabetes Mellitus (DM) is a chronic condition with a major impact on the lives and well-being of individuals, families, and societies throughout the world (Harding *et al.,* 2019). It is among the top ten causes of death in adults, and one of the four prioritized among non-communicable diseases (NCDs), targeted for action by world leaders. All types of diabetes can lead to complications in many parts of the body and can increase the overall risk of premature death. Heart attack, stroke, kidney failure, leg amputation, nerve damage, and vision loss are possible complications of DM. Poorly controlled diabetes in pregnancy also increases the risk of fetal death and other chronic complications.

Diabetes and its complications bring about a substantial economic loss to people, their families, to the health system, and the national economic system through direct medical cost and loss of work. The major cost-contributing factor is the rise in cost for insulin 1, which is increasingly prescribed (WHO, 2016). In 2017, global health expenditure on diabetes was estimated to be USD 727 billion (Ogurtsova *et al.*, 2017).

The prevalence of diabetes has been steadily increasing over the past few decades. The International Diabetes Federation (IDF) has reported the national, regional and global status of diabetes. In 2009, it was estimated that 285 million people had diabetes, increasing to 366 million in 2011, 382 million in 2013, 415 million in 2015, and 425 million in 2017. It caused 1.5 million deaths in 2012 and it was estimated 4 million deaths globally in 2017. In the United States, 2,813,503 resident deaths were registered in 2017, which is more deaths than in 2016 (69,255). The crude death rate of (in 2017) is higher than the 2016 rate (Kochanek *et al.*, 2019).

The worldwide diabetes prevalence in 2019 is estimated to be 9.3% (463 million people), rising to 10.2% (578 million) by 2030 and 10.9% (700 million) by 2045. The prevalence is higher in urban (10.8%) than rural (7.2%) areas, and in high-income (10.4%) than low-income countries (4.0%). Just under half a billion people are living with diabetes globally and the number is projected to increase by 25% in 2030 and 51% in 2045 (Saeedi *et al.*, 2019).

The top ten countries for the number of people with the highest number of diabetes in 2019 are Chine (116 million), India (77 million), the United States of America (31 million), Pakistan 19.4 million), Brazil (16.8 million), Mexico (12.8 million), Indonesia (10.7 million), Germany (9.5 million), Egypt (8.9 million), and Bangladesh (8.4 million) (Saeedi *et al.*,2019.

In Africa, there are studies that found high proportions of undiagnosed DM in different communities in African countries. A systematic review and meta-analysis on the burden of undiagnosed diabetes mellitus in adults have shown that the pooled prevalence of undiagnosed diabetes mellitus in the African population was 5.37%. The pooled prevalence from subgroup analyses indicated that undiagnosed diabetes mellitus in the urban population (8.68%) is twice higher than that in the rural population (3.93%) (Asmelash *et al.*, 2019).
Annual national direct costs of diabetes in Africa differed between countries and ranged from $3.5 to $4.5 billion per annum. Indirect costs per patient were generally higher than the direct costs per patient with diabetes. The most commonly included healthcare items were drug costs, followed by diagnostic costs, medical supply or disposable costs and

consultation costs. The highest burden due to the costs associated with diabetes was reported in individuals with the low-income group (Mutyambizi *et al.*, 2018).

The prevalence of diabetes varied across Ethiopia, ranging from 0.3% at Debre Berhan Referral Hospital to 7.0% in Harar town. Five studies distinguished between urban and rural diabetes prevalence and all found a higher prevalence in urban areas. On the other hand, Retinopathy, foot ulcers, neuropathy, nephropathy, hypertension, erectile dysfunction, are the listed chronic complication of diabetes mellitus. Diabetic ketoacidosis (DKA), hypoglycemia, hyperosmolar hyperglycemic state (HHS), are the listed acute complication of diabetes mellitus in Ethiopia (Abebe *et al.*, 2017).

The prevalence of unrecognized DM and impaired glucose metabolism were 3.1% and 14.8%, respectively. The risk of unrecognized DM was higher among male participants, age group of 35 years and above, and high waist circumference. On the other hand, participants who perform sufficient physical activity had low risk of undetected DM (Yunka *et al.*, 2020).

A study conducted in Tikur Anbesa Specialized Hospital showed that of 8048 admissions, 523 (6.5%) had DM; of these 72% patients had type 2 and 28% type-1 disease, with male sex (62%) and older age (median age 60 years) being features of type-2 disease. Main admission diagnoses for type 2 disease were diabetic foot ulcer/gangrene (39%) and cardiovascular disease (21%); for type-1 disease, it was diabetic ketoacidosis (62%). Hypertension, neuropathy, nephropathy, retinopathy and diabetic foot accounted for 85% of the 756 existing complications. Overall in-patient mortality was 21%. Of the 89 deaths, 77 occurred among patients with type 2 disease (Gizaw *et al.*, 2015).

A Systematic review and meta-analysis on diabetes mellitus and its association with hypertension in Ethiopia showed that the pooled prevalence of DM was 4.99%. Hypertension was significantly associated with DM. The analysis also reflected that the burden of DM in Ethiopia is considerable, and the association between diabetes and hypertension is significant (Tesfaye *et al.*, 2019).

1.3.2. Overview of pathophysiology of diabetic foot ulcer

The disease of the foot is one of the most frequent demoralizing complications of diabetes. As reported by several studies around 15-25% of patients will, at some point, develop foot ulceration and infection. One of the negative outcomes of diabetic foot ulcers is an amputation, which is the loss of part or all of the foot, or the foot plus a portion of the lower limb. Among patients with DFUs, 5-24% will have an amputation and the number of people who require amputation because of diabetes is growing worldwide (Nigam and Knight, 2017).

The pathophysiology of diabetic foot ulcers has several components that cause an emergence in diabetic patients (Rosyid *et a*l., 2017). The components are neuropathic, vascular, trauma and infection, and immune system. All the above constituents show a base relationship with the hyper glycemic state of diabetes (Nigam and Knight, 2017, Boulton et *al.*, 2018) (Figure 2).

More than 60% of DFU are the result of underlying neuropathy (Clayton *et al.*, 2009). One of the more commonly pronounced mechanisms of action is the polyol pathway (Oates, 2002, Dunlop *et al.*, 2000). In the development of neuropathy, the hyperglycemic state leads to an increase in the action of the enzymes aldose reductase and sorbitol dehydrogenase (Wolf *et al.*, 2004). This results in the conversion of intracellular glucose to sorbitol and fructose. The accumulation of these sugar products results in a decrease in the synthesis of nerve cell myoinositol, required for normal neuron conditions (Wolf *et al.*, 2004).

Additionally, the chemical conversation of glucose results in a depletion of nicotinamide adenine dinucleotide phosphate store, which is necessary for the detoxification of reactive oxygen species and the synthesis of the vasodilator nitric oxide (Oates, 2002, Kawanami *et al.*, 2016). There is a resultant increase in oxidative stress on the nerve cell and an increase in vasoconstriction leading to ischemia that will promote nerve cell injury and death (Figueroa-Romero *et al.*, 2008). Hyperglycemia and oxidative stress also contribute to the abnormal glycation of nerve cell proteins and the inappropriate activation of protein kinase C, resulting in further nerve dysfunction and ischemia (Kawanami *et al.*, 2016).

Neuropathy in diabetic patients is manifested in the motor, autonomic, and sensory components of the nervous (Alexiadou *et al*., 2012) (Figure 2)**.** Damage to motor neurons of the foot musculature may lead to an imbalance of flexors and extensors, anatomic deformities, and eventual skin ulcerations. In addition, motor neuropathy affects all the muscles in the legs, implying in protrusion abnormal bones, and normal architecture of the foot changed, distinctive deformity such as hammertoe and hallux rigidus (Rosyid *et al*., 2017)**.**

Damage to the autonomic nerve impairs sweat gland function, the foot may develop decreased ability to moisturize skin, leading to epidermal cracks and skin breakdown. Which results in, patient may not notice a foot ulcer due to decreased peripheral sensation (Boulton *et al.*, 2018).

Sensory neuropathy is usually fairly deep (>50%) before experiencing a loss of protective sensation that results in insusceptibility to physical and thermal trauma, thus increasing the risk of foot ulcer (Syafril, S., 2018) (Figure 2). Not only the sensation of pain and pressure is lost, but also the proprioception of the sensation of foot position also disappeared. Because the blood supply required to heal a diabetic foot ulcer is greater than that needed to maintain intact skin, chronic ulceration can develop (Aumiller and Dollahite, 2015). As trauma occurs at the affected site, patients are often unable to detect the insult to their lower extremities. As a result, many foot ulcers go unnoticed and progressively worsen as the affected area is continuously subjected to repetitive pressure and shear forces from ambulation and weight-bearing(Aumiller and Dollahite, 2015; Alexiadou *et al*., 2013).

Vascular changes or peripheral arterial disease (PAD) is another contributing factor to the development of foot ulcers in up to 50% of cases (Everett *et al.*, 2018) (Figure 2). It leads to diabetic foot ulcers correlate with hyperglycemia-induced changes in the peripheral arteries of the foot and began on the cellular level. Endothelial cell dysfunction leads to a decrease in vasodilators, and plasma thromboxane A2 levels become elevated. The result is vasoconstriction and plasma hyper-coagulation in peripheral arteries leading to ischemia and increased risk of foot ulcerations (Armstrong *et al.*, 2017).

Immune changes, which include reduced healing response in diabetic foot ulcers, such as increased T lymphocyte apoptosis and arrested of neutrophil chemotaxis by the hyperglycemia has been observed in patients with diabetic foot ulcer ((Aumiller and Dollahite, 2015) (Figure 2). Moreover, smoking, hypertension, and hyperlipidemia are other factors that are common in diabetic patients and contribute to the development of PAD (Armstrong *et al.*, 1998). Cumulatively, this leads to occlusive arterial disease that result in ischemia in the lower extremity and an increased risk of ulceration in diabetic patients.

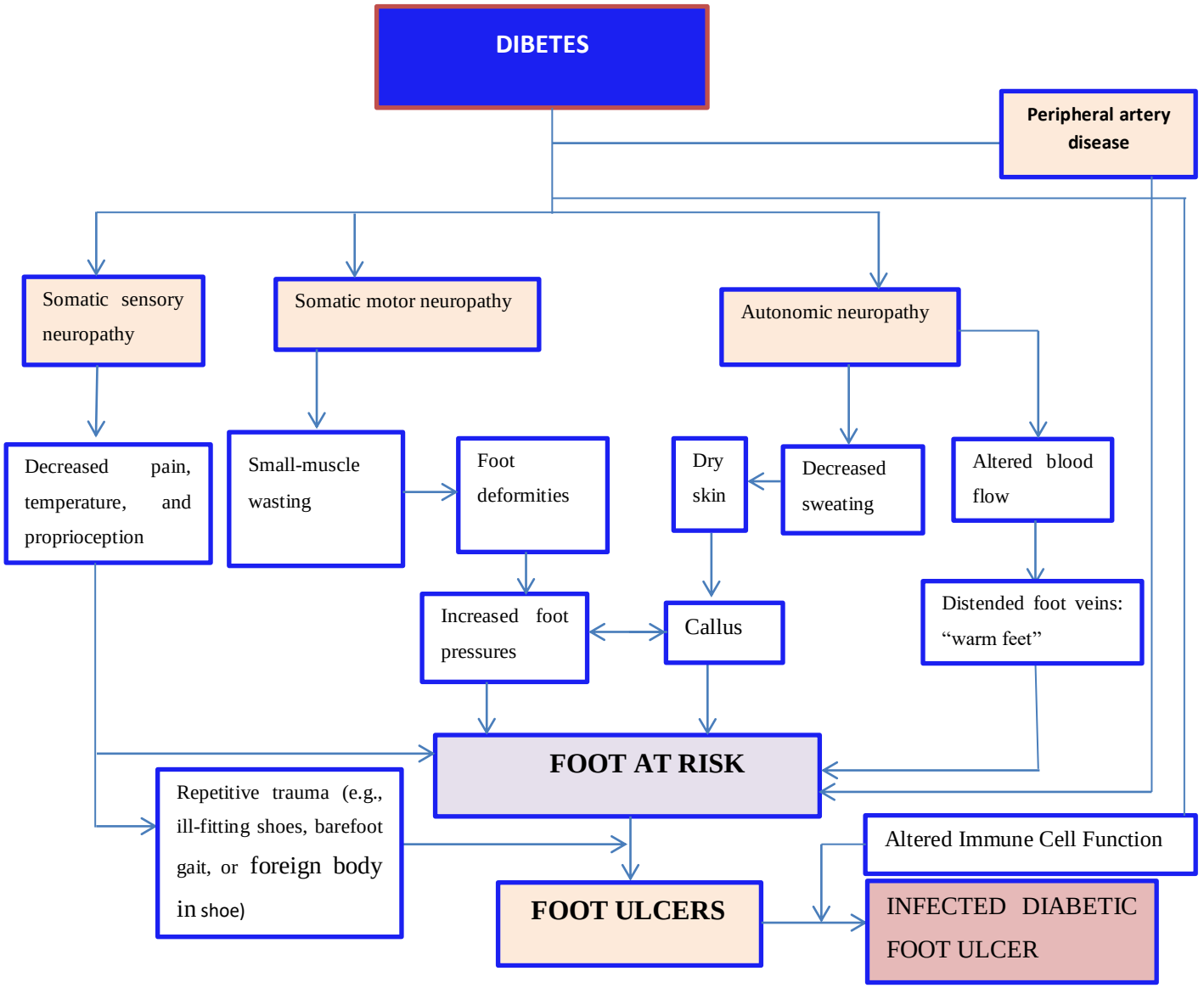

Figure 1: Pathways to diabetic foot ulceration and diabetic foot infection (Armstrong *et al.*, 2017; Boulton *et al.*, 2018).

1.3.3. Risk factor for diabetic foot ulcer and diabetic foot infection

The risk factors for foot ulcers in patients with diabetes include previous lower extremity amputation, history of a foot ulcer, anatomic foot deformity, peripheral vascular disease, diabetic nephropathy, poor glycemic control, and smoking (Aumiller *et al*., 2015, Lipsky *et al*., 2012).

The prevention of foot ulcers is important to avoid these devastating outcomes. Several studies have identified risk factors for diabetic foot ulceration, which include, among others, peripheral neuropathy, peripheral arterial disease, and foot deformity (Figure2) **(**Noor *et al*., 2017). The strongest predictors of ulceration are the presence of peripheral neuropathy and a history of ulceration, which shows that ulcers are often recorded up to 40% annually (Pound *et al*., 2005).

A total of 71 patients had a recurrent ulcer and significant independent predictors were the presence of minor lesions, barefoot peak pressure, day-to-day variation in stride count, and cumulative duration of past foot ulcers (Waaijman *et al*., 2014).
Research conducted in Australia on incidence and risk factors for developing an infection in patients presenting with uninfected diabetic foot ulcers indicated that the independent risk factors for developing diabetic foot infection included, DFUs healed deep DFUs, peripheral neuropathy, previous DFU history, foot deformity, female gender, and years of age (Jia *et al*., 2017).

In the presence of neuropathy, elevated plantar pressure during walking is another predictor of diabetic foot ulceration. However, studies on the sensitivity and specificity of barefoot plantar pressure to predict ulceration show that barefoot pressure, although important, is only a moderate predictor. This is probably because patients do not only walk barefoot in daily life but also wear shoes, in which the biomechanical conditions are different. Peak plantar pressures while walking barefoot are generally much higher than while wearing protective footwear that patients often are prescribed after healing of a plantar foot ulcer (Serena *et al*., 2019).

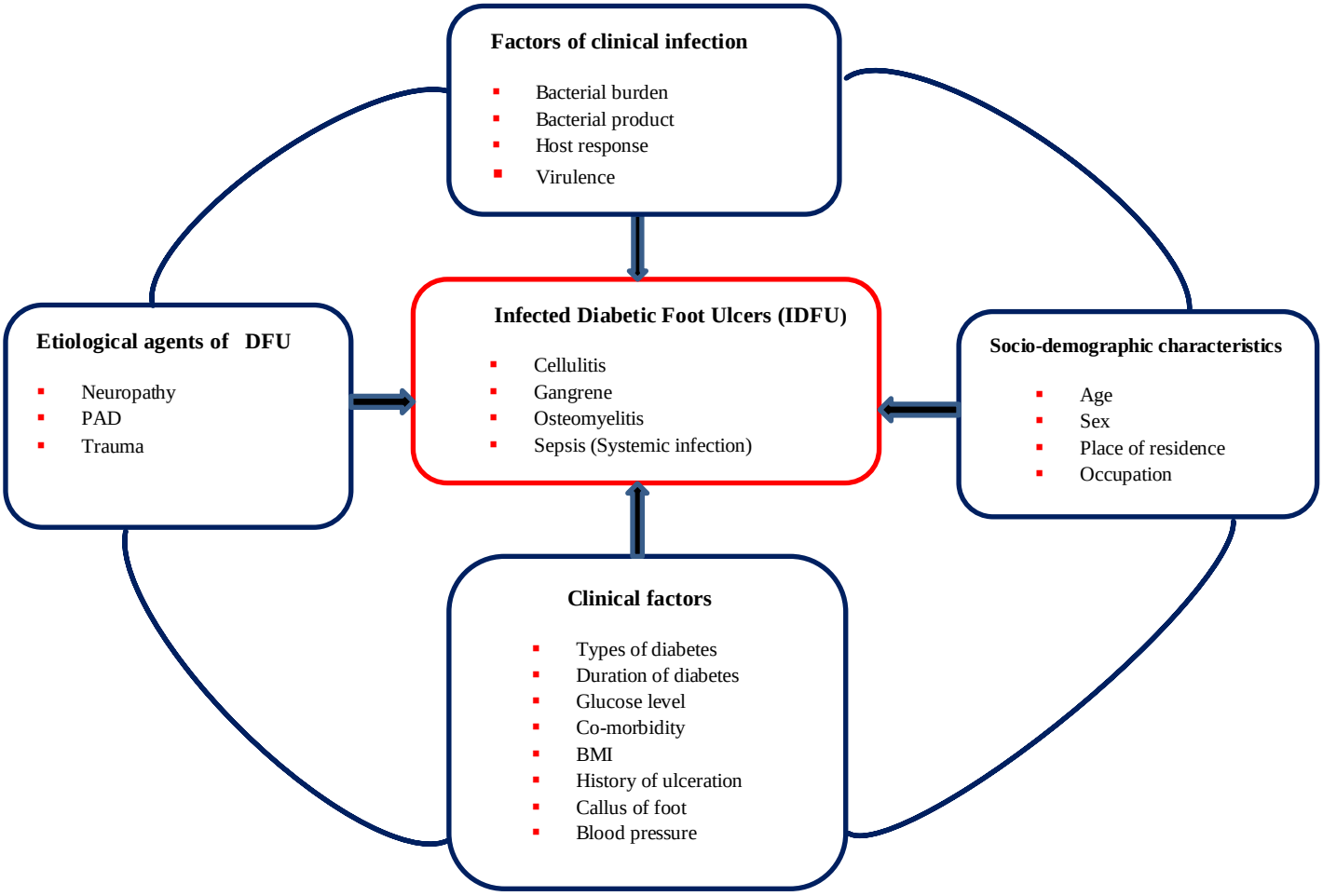

Figure 2: Conceptual framework showing possible predicators of diabetic foot infection (DFI)

1.3.4. Classification of diabetic foot ulcer and infection severity

An adequate description of ulcer characteristics, such as size, depth, appearance, and location, also provides the mapping of progress during treatment. The evaluation should determine the etiology of the ulcer and ascertain whether the lesion is neuropathic, ischemic, or neuro-ischemic. Several classification systems have been used until recently to assess the severity of diabetic foot lesions that attempt to incorporate different characteristics of ulcer including ulcer size, depth, ischemia, infection, and neuropathy (Ghotaslou *et al*., 2018).

In the study of DFU, one of the most commonly used classification systems is Wagner-Meggit system. Even though it was devised for dysvascular foot, it is in use for the past 25 years. It has a six-grade classification system, which takes criteria like the depth of ulcer, presence of gangrene, and extent of tissue necrosis. Wagner's grading does not take into consideration important clinical parameters such as ischemia, infection, and other co-morbid

factors but still it is one of the most widely used classification systems (Table 1) (Lipsky *et al.*, 2016).

Table 1: Wagner classification system of diabetic foot ulcer (Van Netten *et al.*, 2020; Lipsky *et al.*, 2016).

Grading	Description
0	Pre-ulcerative, with no open lesion or cellulitis
1	Superficial ulcer
2	Deep ulcer up to tendons and joint tissue
3	Deep ulcer with abscess, osteomyelitis, and joint sepsis
4	Localized gangrene of forefoot or heel
5	Gangrene of entire foot/global gangrene

International Working Group gives another classification system that gives risk information. It can predict people with diabetes who are at risk for foot problems. The risk assessment instrument developed by IWGDF on the diabetic foot has been shown to predict diabetic foot complications (Table 2) (Oyibo *et al.*, 2001; Van Netten *et al.*, 2020).

Table 2: Classification by International Working Group (Van Netten *et al.*, 2020; Noor et al., 2017).

Risk group	Description
Group 0	No neuropathy, no PVD
Group 1	Neuropathy, no-deformity or PVD
Group 2	Neuropathy and deformity and or PVD
Group 3	History Pathology

Abbreviations: PVD- Peripheral vascular disease

The University of Texas Antonio is another classification system that assesses diabetic foot lesions based on depth, wound infection, and presence of lower limb ischemia. In this system grading is done based on the depth of the lesion and stages are classified on the existence of ischemia, wound bio-burden, or a combination of both excluding neuropathy. There are grades zero up to three and stages A up to D. Wounds of higher grade or stages

are in the fear of healing without vascular repair or lower extremity amputation (Table 3) (Oyibo *et al.*, 2001).

Table 3: University of Texas Classification System (Parisi *et al.*, 2008; Noor *et al.*, 2017; van Netten *et al.*, 2020).

Stage	Grade			
	0	1	2	3
A	No open lesion	Superficial wound	Affected tendons/capsules	Affected bone/joint
B	With infection	With infection	With infection	With infection
C	Ischemic	Ischemic	Ischemic	Ischemic
D	Infection/Ischemia	Infection/Ischemia	Infection/Ischemia	Infection/Ischemia

The Infectious Disease Society of America (IDSA) classification scheme encompasses four progressive levels of infection based upon severity correlated to clinical findings. This classification scale, developed in 2004 and now widely accepted in many academic and clinical circles, was later validated and shown to predict clinical outcomes in a prospective observational study. Based on this scheme, mild infections are defined by less than two centimeters of erythema whereas moderate infections have greater than two centimeters of erythema (Lipsky *et al.*, 2012). Chronic infections are associated with systemic toxicity and/or metabolic instability. The disadvantage of using this classification is that it does not sufficiently clarify the local environment of the wound (Table 4).

Table 4: Diabetic foot infection classification schemes: Infectious Diseases Society of America (IDSA) **(**Ghotaslou *et al.*, 2018; van Netten *et al.*, 2020).

Description	IDSA	IWGDF
Wound without purulence or any manifestations of inflammation	Uninfected	1
≥2 Manifestations of inflammation (purulence or erythema, pain, tenderness, warmth, or induration); any cellulitis or erythema extends 52 cm around ulcer, and infection is limited to skin or superficial subcutaneous tissues; no local complications or systemic illness	Mild	2

Infection in a patient who is systemically well and metabolically stable but has ≥2 cm; lymphangitis; spread beneath fascia; deep tissue abscess; gangrene; muscle, tendon, joint, or bone involvement	Moderate	3
Infection in a patient with systemic toxicity or metabolic instability (e.g., fever, chills, tachycardia, hypotension, confusion, vomiting, leukocytosis, acidosis, hyperglycemia, or azotemia)	Severe	4

1.3.5. Bacteriological profile of diabetic foot ulcer infection

In the USA (Gardner *et al.*, 2013), a cross-sectional study was conducted on the Neuropathic diabetic foot ulcer microbiome using culture and 16S-rRNA gene sequencing methods. The most abundant operational taxonomic unit (OUT) was classified as *Staphylococcus spp* and was present in 49 of 52 DFU samples, comprising 29.6% of the total sequences and from this majority was *S. aureus* (96.5%), only 0.4% of the sequences were determined to be *S. epidermidis*. The second and third most abundant OTUs were *Streptococcus spp* (8.8% of the total sequences) and Lactococcus (3.9% of the total sequences), respectively.

A prospective cross-sectional study was carried out in South America (Kurup and Ansari, 2019) on identifying bacteriological profiles and other risk factors among diabetic and non-diabetic foot ulcer patients by culture technique. Two hundred fifty-four bacteria were isolated from 183 patients with an average of 1.4 organisms per lesion. Gram-negative bacteria (63.0%) were prevalent than gram-positive (37.0%). Among diabetic foot patients, *P. aeruginosa* (18.8%) was the most common isolate followed by *E. coli* (13.9%) among gram-negatives. Whereas MRSA (12.1%) followed by MSSA (7.9%) dominated among gram-positive bacteria. Around 42.1% (95% CI 34.8-49.6) of the infections were poly-microbial.

On the other hand, in Latin America (Vivanco *et al.*, 2017), descriptive-retrospective research was conducted on the bacteriological profile in diabetic foot patients using culture. From 70 patients, *E. Coli* 27 (69.28%), with a higher incidence degree were found followed *S. aureus* 25 (80%).

In England (Nelson *et al*., 2018), a cross-sectional study was documented on bacterial identification among infected diabetic foot ulcers by wound swab and tissue sampling. From this study the most frequently reported groups of pathogens were gram-positives cocci (70.6%) and gram-negative bacilli (36.7%); Enterobacteriaceae including coliforms (26.6%); obligate anaerobes (23.8%), and gram-positive bacilli (11.1%). Finally, they have concluded that the majority of pathogens were reported significantly more frequently in tissue than wound swab samples ($P<0.01$).

A study in Greek showed was done on antibiotic resistance in diabetic foot soft tissue infections (Demetriou *et al*., 2017). Among the 113 patients, 24 patients had negative tissue cultures. Both gram-negative bacteria and gram-positive bacteria were isolated from positive culture; 7 Proteus, 12 Klebsiella, 11 *Pseudomonas*, 8 *E. coli*, 9 *Enterobacter spp*, and 16 other gram-negative bacteria. Nine MRSA, 15 MSSA, 10 other *Staphylococci spp*, 6 *Streptococci spp*, and 8 *Enterococci spp* were also isolated. No high antibiotic resistance was reported. Piperacillin-tazobactam exhibited the lowest resistance in *Pseudomonas*, as well as in the other Gram-negative pathogens. In MRSA isolates, there was no resistance to anti-Staphylococcus agents. However, clindamycin, erythromycin, and amoxycillin/clavulanic acid exhibited high resistance in Gram-positive cocci.

A study in France (Hartemann *et al*., 2004) was done, on diabetic foot ulcers and multidrug-resistant organisms including risk factors. In 180 consecutive patients, wound cultures grew a single bacterial species in 50 (28%) wounds, two species in 64 (35%) wounds, three species in 36 (20%) wounds, and four species in 19 (11%) wounds. Cultures were sterile for 6% of the wounds. Among the 180 wounds, 29 (16%) grew MRSA and three (1.7%) ESBL-producing enterobacteria. MDROs are often present in severe diabetic foot wounds. Hygiene, measures or isolation precautions in the case of admission are among the factors for cross-transmission.

A prospective study was conducted in Brazil on 41 patients with DFI and 89 bacteria were isolated and 11 samples were reported as culture-negative (Perim *et al*., 2015). The most common isolated gram-positive bacteria were *S.aureus*, followed by *S.saprophyticus*, *S.epidermidis*, *S.agalactiae*, and *S.pneumoniae*. Among gram-negative bacteria, the most

commonly isolated were *Proteus spp*. and *Enterobacter spp*., followed by *E.coli*, *Pseudomonas spp*., and *Citrobacter spp*. Nine cases of MRSA had cefoxitin resistance, and among these isolates, three were resistant to vancomycin with the MIC technique. The antibiotic imipenem was reported as the most effective against both gram-positive and gram-negative bacteria.

A cross-sectional prospective study in Iran reported 92 bacterial isolates from 60 samples of diabetic foot ulcers with six(10%) of the patients reported as bacteria-free (Akhi *et al*., 2015). Predominant aerobic bacteria were *S. aureus* (28%) followed by Enterobacteriaceae family (24%) including *E. coli* (15%), *Citrobacter spp*. (4%), *Enterobacter spp*. (4%), and coagulase-negative *Staphylococcus spp*. (17%), *Enterococcus spp.* (15%), *P.aeruginosa* (7%) and *Acinetobacter spp*. (4%). In addition, 4% *Bacteroides fragilis* were obtained from anaerobic culture. All gram-positive isolates were susceptible to linezolid while all Enterobacteriaceae showed sensitivity to imipenem. Forty-three percent were resistant to vancomycin. ESBL-producers (31%) like Acinetobacter spp. (50%) followed by *E. coli* (36%), *P. aeruginosa* (33%) and *Enterobacter spp*. (25%).

A more recent cross-sectional study from the same country conducted on the bacteriology of moderate-to-severe diabetic foot infections in two tertiary hospitals showed that 95 aerobic bacteria were isolated from 105 specimens (Najari *et al*., 2019). *Staphylococcus spp*. and *E. coli* were the most frequent organisms isolated. MDR organisms constituted up to 48.4%, with 37.5% of isolated VRE, 48.8% of methicillin-resistant *Staphylococcus spp*., 77.8% of isolated ESBL-producing E. coli, and 66.7% of isolated MDR *Pseudomonas spp*. The minimum and maximum prevalence of resistance in Gram-negative bacteria were 17.6% and 87.5% for imipenem and ceftazidime, respectively. The prevalence of Gram-negative bacilli was higher in older patients (p value=0.039) and rose markedly in patients with a higher number of hospitalizations (p value=0.015).

In Malaysia, a retrospective cohort study was carried out on the significant association between polymicrobial diabetic foot infection and its severity and outcomes (Hitam *et al*., 2019). One hundred thirty-three microorganisms were isolated with 1.28 MOs/lesion. Sixty-two percent (n=83) gram-negative bacteria and 38% (n=50) gram-positive bacteria. Among

gram-negative, *Pseudomonas spp* (28%), *Proteus spp* (11%), *Klebsiella spp* (8%) and *E. coli* (4%). In addition, among gram-positive, *S. aureus* (54%) was predominant followed by Group B Streptococci (26%) and *Enterococcus spp* (6%). Thirty patients (28.8%) had poly-microbial infections. The association between the quantity of microorganisms and the severity of DFI was significant. Among severe DFI cases, 77.8% with poly-microbial received amputation compared to 33.3% with mono-microbial infection.

Twelve pathogens were identified from 33 patients in a study done in Indonesia. The most common bacteria on pus cultures was *K. pneumoniae* (33.3%), followed by *E. coli* (24.2%), *A. baumannii* (12.1%), and *S. aureus* (9.1%). Frequent susceptible antibiotics were Amikacin (88.8%), Imipenem (87%), Meropenem (84.6%), Erythromycin (75%), and Cefoperazone/ Sulbactam (68.9%) (Bulolo et al., 2018).

A retrospective analysis was carried out in Saudi Arabia (Al Ayed *et al*., 2018) on common pathogens and antibiotic sensitivity profiles of infected DFU, 134 pathogens were isolated from 126 patients. The most common gram-negative pathogen was *P. aeruginosa* (15.6%), followed by *Klebsiella spp* (6.7%). The most common gram-positive pathogen was *S. aureus* (35%), followed by Streptococcus (8.9%). The most widely accepted antibiotic to which the gram- negative bacteria was sensitive was gentamycin (20.1%), followed by ciprofloxacin (19%).

A 7 year prospective cohort analysis was done on potential risk factors and outcomes of infection with multidrug resistance among diabetic patients having ulcers in India (Zubair and Ahmad, 2019). Two hundred seventy-eight bacteria (255 aerobic, 21 anaerobic) were isolated, averaging 1.32 species per patient. 78.6% of patients had MDR infection and NMDR etiology was observed in 21.3%. Among the bacterial isolates, aerobic gram-positive cocci comprised of 34.5% and aerobic gram-negative bacilli for 65.7%. *E. coli* was the most common isolate, followed by *S. aureus*, *P. aeruginosa* 23.7%, and *Klebsiella spp*. Among the anaerobic bacteria isolated, *Peptostreptococcus spp* was the most common isolate, *Propionibacterium spp*, *C. perfringens*, *Bacteroides ureolyticus,* and *Eggerthella lenta*.

Another prospective study in India, was conducted on diabetic foot ulcers and biofilm formation by culture technique (Shashikala V., 2016). From 100 samples, 82 isolates were obtained. *S. aureus* and *E. coli* were the most commonly isolated organisms (24.40% each) followed by *P. aeruginosa* (17.07%), *Citrobacter spp*. (12.1%), *K. oxytoca* (12.1%) and *Proteus spp*. (9.76%). Overall, 20 (24.4%) were gram-positive and 62 (75.6%) were gram-negative bacteria. Thirty-eight (46.34%) of the isolates showed biofilm formation. *S. aureus* was the predominant biofilm former, with 14 (38.84%) of the isolates testing positive for biofilm formation. All 10 (100%) of the MRSA isolates were biofilm formers while only four (40%) of the MSSA isolates formed biofilm. The second highest biofilm formation was by *P. aeruginosa* (26.52%) followed by *Citrobacter spp*. (10.53%), *E. coli* (10.53%), *Proteus spp*. (10.53%) and *K.oxytoca* (5.26%).

More additional in India, prospective and observational hospital-based study was conducted on the bacteriological profile of diabetic foot ulcer with special reference to drug-resistant strains by tissue sampling and culture technique (Jain and Barman, 2017). One hundred eighty-five isolates were obtained from 150 patients. Among the isolates, gram-negative bacilli were isolated in 112/185 (61%) and gram-positive cocci in 73/185 (39%) cases. The most common isolate was *Staphylococcus spp*. 46 (25%), followed by *E. coli* (20%) and *Enterococcus spp*. (15%). Also 59/112 (53%) of the gram-negative bacilli were ESBL-producers, 19/46 (41%) were MRSA, and 5/27 (19%) were VRE.

A hospital-based retrospective study in China (Xie *et al.*, 2017) reported:- 232 isolates from the 117 specimens, including 207 (89.2%) bacteria and 25 (10.7%) fungi. The proportion of gram-negative bacteria was higher than gram-positive bacteria (54.1% versus 45.9%), in which Enterobacteriaceae (73.2%) and Staphylococcus (65.2%) were predominant, respectively. With an increase in Wagner's grades and IDSA/IWGDF grades, the proportion of gram-negative bacterial infection increased, especially *Pseudomonas*. Neuro-ischemic ulcer (N-IFU) was more susceptible to gram-negative bacterial infection. Additionally, with the aggravation of the wound and infection, the antibiotic resistance rates were increased. Gram-positive bacteria isolated in ischemic foot ulcer (IFU) showed more resistance than the N-IFU, while gram-negative bacteria isolates were on the opposite.

In another study in Southwest China (Wu *et al*., 2018), 555 strains were isolated from 354 samples, including 205 (36.9%) strains of gram-positive bacteria, 283 (51.0%) gram-negative bacteria and 67 (12.1%) fungal strains. In terms of distribution, patients with different Wagner grades had different bacterial composition ratios ($P < 0$ 01). Patients with Wagner grades 3–5 mainly had gram-negative bacteria. The specimens from chronic ulcer wounds were primarily gram-negative bacteria (54.2%), whereas fungi accounted for 14.4% of the infections. The distribution was significantly different from that of acute ulcers ($P < 0$ 01). The susceptibility tests showed that the *Staphylococcus* genus was more susceptible to vancomycin, linezolid, and tigecycline. Tobramycin was the most effective drug (97%) for the treatment of *E. coli*, followed by ertapenem (96.4%), imipenem (93.5%), and cefotetan (90%).

A descriptive observational study conducted in Karachi (Pakistan) at a tertiary care center reported that from 342 patients with diabetic foot infections, 671 aerobic bacteria were isolated. Poly-microbial infection was 56.87%. Gram-negative isolates were predominant 76.27%. *S. aureus* was most frequent among gram-positive bacteria 20.7% and *E. coli* 15.72% among gran-negative bacteria followed by *A. baumannii* 36 (5.24%). MRSA was found in 26.76% *S. aureus.* About 33.48% of antimicrobial-resistant isolates were observed including all *A. baumannii* strains were found to be MDR (Miyan *et al*., 2017).

In Libya (Elbaz *et al*., 2018), a cross-sectional study was conducted on antimicrobial-sensitivity patterns of *pseudomonas aeruginosa* isolates obtained from foot ulcer diabetes patients. Twenty-one strains of *P. aeruginosa* from 120 diabetic foot ulcers were detected. *P. aeruginosa* isolates exhibited multidrug-resistance to Ampicillin, Augmenting, Cefuroxime, Cefoxitin, Cefazolin, Ceftriaxone, Trimethoprim/sulfamethzole, Piperacillin. However, all isolates of *P. aeruginosa* were 100 % sensitive to Imipenem.

In Nigeria, a prospective study documented a 100% infection rate among subjects with 70.0% polymicrobial infections, and 97 isolates were obtained from 50 subjects with 1.94 isolates per subject (Ogba *et al*., 2019). The most prevalent was *S. aureus* 32 (32.9%), while the least one was *K. pneumonia* 10 (20.4%). Erythromycin was the most effective antibiotic

agent (65.6%) against *S. aureus* while gram-negative bacteria were more susceptible to augmentin (87.5%) and ciprofloxacin (75.0%).

Another study in Nigeria, a prospective cross-sectional hospital-based multicenter study found 218 isolates, 129 (59.2%) gram-negative bacteria, 59 (27.1%) gram-positive cocci and 29 (13.2%) anaerobic bacteria (Adeyemo *et al*., 2019). The top five aerobic bacteria encountered were *S. aureus*, *E. coli*, *P. aeruginosa*, *K. pneumoniae* and *Citrobacter spp*. The commonest anaerobes were *Bacteroides spp.*, and *Peptostreptococcus anaerobius.* Of the 93 IDFU cases, 74 (80%) were infected by MDR bacteria predominantly MRSA, ESBL-producing gram-negative bacteria, mainly of the CTX-M variety. Carbapenemase-producers encoded by *bla*VIM were only 4 (3.1%) among gram-negative bacteria.

In a prospective study in Egypt, mono-bacteria among 48.75% of patients and mixed bacterial growths among (56.08%) patients were isolated from 80 patients (Dwedar *et al*., 2015). Gram-negative bacteria (56.08%), were more commonly isolated compared with gram-positive bacteria (27.7%), while gram-negative anaerobes accounted for (8.1%), and Candida species (8.1%). The commonest isolate was *P. mirabilis* (16.8%) followed by *E. coli* (13.5%), MSSA (11.4%), *Pseudomonas spp* (10.8%), and MRSA (10.1%). Among the gram-negative bacteria 49% were ESBL-producers and 3.6% were carbapenemases producers while 46.8% *S. aureus* were MRSA. Overall 42.5% isolates were identified as multidrug-resistance.

In Ethiopia, a hospital-based retrospective study on diabetic foot disease was conducted (Amogne *et al*., 2011). Swab culture was positive in 92 out of 119 (77.4%) patients. The result yielded a wide range of bacteria with Klebsiella and Proteus as the most common. Others like *Providencia species*, *Citrobacter species*, *Morganella morganii*, *Acinetobacter species*, *Salmonella species*, *E.coli*, *Staphylococcus aureus*, and poly-microbial were also reported.

1.3.6. Diagnosis of potential bacterial pathogens

Diagnosis of diabetic foot infection starts with a clinical assessment that requires appropriate debridement to remove necrotic sections and callus to fully visualize the wound. As per

different group guidelines and recent review articles, diagnosis of infection is based on the manifestation of purulence or at least two classic symptoms or signs of inflammation (erythema, edema, warmth, tenderness, pain, or indurations). Secondary signs in the case of the neuropathic foot include friable or discolored granulation tissue, foul odor, and non-purulent discharges, delayed wound healing (Noor *et al.*, 2017).

Proper attention should be paid in evaluating risk factors of DFI, such as positive probe-to-bone (PTB), ulceration present for >30 days, a history of recurrent foot ulcers, a traumatic etiology, the presence of peripheral arterial disease in the limb involved, history of lower-extremity amputation, lacking protective sensation, renal insufficiency or a history of walking barefoot (Ghotaslou *et al.*, 2018).

An adequate description of ulcer characteristics, such as size, depth, and base, margins of the ulcer, appearance, and location is a necessary aspect in mapping the progress during treatment. A detailed assessment of the presence of granulation tissue or slough should be made on the floor of the ulcer to determine subsequent management (Lipsky *et al.*, 2004).
Patients with diabetic foot infection should be properly evaluated for arterial insufficiency and neuropathic condition on a structured schedule based on defined risk factors. The presence of fever, tachycardia, or tachypnea may indicate an infected wound.
Infection is well defined as invasion and colonization of pathogenic microbes in foot wounds causing local tissue damage favored by hyperglycemia mediated deranged host defenses. First, the infection starts as a slight problem and after time progress to involve deep tissues, joints, or bones, especially if unmanaged (Bowler *et al.*, 2001).

Discovering the microbial causative agent is an important aspect of controlling DFI. Due to hyperglycemia, foot ulcer in diabetics has lost many of protective barriers/ mechanisms of intact skin providing a gateway for invasive microorganisms. The presence of non-replicating pathogens is termed contamination, whereas DFU with fast-dividing microbes is colonized (Sadeghpour *et al.*, 2019). Critical colonization or transition state between colonization and invasion delays wound healing and altered microbial-host interactions increased virulence; diffused immune responses accelerate the process. Once a colonized

foot ulcer progresses to an infected wound, the microbiological analysis should be done to evaluate the underlying pathogen/s (Khan *et al.*, 2018).

Management of clinically evident diabetic foot infection requires appropriate systemic antibiotic therapy, which is best guided by isolating the causative pathogens. Proper specimen collection such as deep tissue samples can reveal the true flora and preferred over wound swab as later may reveal colonizing agents providing false results. A curettage or tissue scraping from the base of the ulcer provides a more accurate result if promptly sent for aerobic and anaerobic analysis (Sadeghpour *et al.*, 2019). Swab or tissue specimen obtained is evaluated for phenotypic testing as recommended by CLSI guidelines. This can be attained by the culture of a specimen using selective or standard growth media, along with antimicrobial sensitivity testing (Noor *et al.*, 2017).

1.3.7. Prevention and control of diabetic foot ulcer

Management of DFU follows multidisciplinary strategies as per different recent reviews that include education, feet examination, glucose control, debridement, offloading, treatment of infection, advancing dressing, and surgery (Aumiller *et al.*, 2015).

Education is considered a primary prevention method of DFU. Up to 50% of DFU, cases can be prevented by education. As recent reviews documented on DFU, education is the cornerstone to prevent DFU (Murugan *et al.*, 2018). The main aims of foot care education for diabetic patients are to prevent the occurrence of devastating foot ulcers and amputation. A wide range of involvement is to educate the patients toward better foot care to prevent ulcers. It is extremely suggested that diabetic patients with DFU be educated about risk factors and the importance of foot care, including self-inspection, daily foot hygiene, blood sugar control, monitoring foot temperature, and use of proper footwear. A combination of education with other care strategies is better to have an effective reduction of the incidence and morbidity of lower limb complications caused by DFU (Dorresteijn *et al.*, 2012).

Feet examination is essential in all diabetic patients and should be conducted on every visit. Any changes in the skin such as dryness, fissures, callus, deformities varicose veins, and nail changes should carefully be looked at by physicians. Palpation of the foot, palpation of

peripheral pulsations such as dorsalis pedis, and palpation of the femoral artery should be carefully examined as well (Alexiadou *et al*., 2012).

Glucose control is one of the most important factors to prevent morbidities and mortalities related to DFU. Disregarding control of blood glucose is the primary cause of DFU. One of the tests used to measure blood sugar is the HbA1C level that indicates the average blood glucose concentration over about three months. The higher the HbA1C level, the more glycosylation of hemoglobin in red blood cells will occur. Studies have indicated that blood glucose level > 11.1 mmol/L (equivalent to >310 mg/mL or an HbA1C level of >12) is associated with lower immunity since it decreases neutrophil function, including leukocyte chemotaxis. Uncontrollable blood glucose concentration also has been associated with more inflammatory responses suppression and decreasing host response to an infection (McMurry et al 1984; Aumiller and Dollahite, 2015).

Debridement is another type of DFU management protocol. It is described as the surgical removal of the dead tissue, foreign, and infected materials from the diabetic foot ulcer. Once the ulcer occurred, debridement is the first and the most important intervention in the management of the wound. The number of bacteria can be decreased by this procedure, and it stimulates the production of local growth factors and reduces pressure. Different types of debridement are used including surgical, enzymatic, autolytic, mechanical, and biological. Surgical debridement, which involves cutting away dead and infected tissues followed by daily application of saline moistened cotton gauze, has been pronounced as the most effective in the containment of DFU (Nigam *et al.*, 2017).

Another important method in the control of DFU is offloading also known as pressure modulation. It is more consideration for the management of neuropathic type ulcers in patients with DM. Studies have concluded the efficacy of this method in the healing of DFU (Katz *et al*., 2005). When choosing the proper method, the physicians have to consider many factors including the physical characteristics and the patient's compliance. Total contact casts (TCC) are the most effective offloading method for the management of the neuropathic DFU. Total contact casts is shaped carefully to the foot with a heel for walking (Boulton *et*

al., 2004). The cast is designed to decrease the pressure on the ulcer and distribute it over the entire surface of the foot. Thus, the ulcer will be secured.

Treatment of infected DFU is another step in the management of ulcers. The diagnosis of infection is first based on clinical appearance and basic symptoms (erythema, edema, pain, tenderness, and warmth). Specific treatment for microorganisms is very advisable to prevent cellulitis, osteomyelitis, and sepsis. MRI or CT scan is also the most advanced technology available to reject the possibility of abscess with osteomyelitis (Rebolledo et al., 2011).

Advancing dressing is another type of method in the control of DFU. Dressing suggests a moisturized balanced protection with protease sequestration and growth factor stimulation. Adding to these benefits, the antimicrobial, oxygen permeability and autolytic debridement help produce granulation tissues and stimulates re-epithelialization (Moura *et al*., 2013). As a result, each diabetic patient with DFU requires a specific type of dressing. To choose the appropriate dressing, the physician has to characterize the DFU, including the wound site, scar, depth, exudative vs. non-exudative, wound margins, presence of infection, and the dressing's conformability. In general, the wound dressing is categorized as passive, interactive, or active. While passive dressings are mainly used to protect the acute wounds as they absorb reasonable amounts of exudates and offer protection, the active and interactive dressings can modify the physiologic nature of the wound by promoting cellular and growth factors secretion (Dumville *et al.*, 2012).

Surgery, as documented in several DFU management guidelines, has played a major role in the control of foot ulcers as well as its prevention. It is not risk-free and generally, surgical procedures for DFU are classified as non-vascular, vascular, and in some cases amputation. Nonvascular surgery is further categorized as elective, prophylactic, curative, and emergent that aims to correct deformities (Lepantalo *et al*., 2011; Abou-Zamzam *et al*., 2007; Lipsky *et al*., 2016).

1.4. Significance of the study

Diabetic foot ulcer infections are poly-microbial in nature. Many complications of DFU which are caused by bacterial infections like osteomyelitis, limb amputation, sepsis, and even mortality can be minimized by giving due attention for identification and controlling of the infection. Since management of any infection is among the listed gold standard in the management of DFU, it is necessary to detect specific etiological agents and their susceptibility patterns. As a result, early management of diabetic foot ulcers with the appropriate antibiotic therapy can be given for a successful outcome. In addition, this calls upon a need for a well-planned bacteriological study of diabetic foot ulcers.

The outcome of this research will assist physicians by providing the most common microbial causes associated with diabetic foot infections. The antibiogram generated in this study can suggest to clinicians about the antibiotic choice in the study sites. Additionally, treatment failure, antimicrobial resistance, adverse events, and costs will be reduced significantly. Finally, this finding will put a base-line data for further similar studies on prevention and control and to give care for diabetic foot infection patients. Hence, this study was intended to determine the pathogenic bacterial of diabetic foot ulcers using optimal culture techniques and the antimicrobial sensitivity pattern of the isolates.

2. Objectives

2.1. General objective

- Isolation of pathogenic bacterial and their antimicrobial susceptibility pattern among patients with diabetic foot ulcer at selected hospitals in Addis Ababa, Ethiopia.

2.2. Specific objectives

- To determine the magnitude of bacterial infection among diabetic foot ulcer patients at selected hospitals in Addis Ababa, Ethiopia.
- To determine the antimicrobial susceptibility pattern of the bacterial isolates among patients with diabetic foot ulcers at selected hospitals in Addis Ababa, Ethiopia.
- To determine the magnitude of multidrug-resistant bacterial pathogens among patients with diabetic foot ulcers at selected hospitals in Addis Ababa, Ethiopia.
- To determine the magnitude of extended-spectrum beta-lactamases (ESBLs) and Carbapenemase-producers among the bacterial isolates.

3. Method and Materials

3.1. Study area

The study was conducted at Addis Ababa in three selected Governmental Hospitals: The first study site was Tikur Anbessa Specialized Hospital (TASH), which is a teaching hospital under AAU and the biggest referral hospital that gives a comprehensive health care service for greater than 500,000 patients per year through its twenty specialty clinics and five main inpatient service departments. Moreover, TASH has various Departments, faculties, and residents under specialty training in the school of medicine to provide patient care in the hospital (Negash *et a*l., 2021). Based on the registration log book, currently at DM clinic of TASH, 65-85 patients visit per day and weekly 1-4 DM patients with DFU are seen. Annually 10,000-12,000 DM patients were registered. Yekatit 12 hospital was the second study site, which is under the city government of Addis Ababa health bureau, and it became a medical college in 2011. This hospital currently serves as a research center for the college in addition to its medical service. The number of DM patients visiting the hospital was not documented separately. Menelik II hospital, which is also under the city government of Addis Ababa health bureau, was the third study site with around 50-65 patients visit per day at DM clinic.

3.2. Study design and study period

A hospital-based cross-sectional study was conducted in DM clinic of the three selected hospitals in Addis Ababa, Ethiopia. The study was carried out from November/2020–May/2021.

3.3. Population

3.3.1. Source population

The source population was all patients attending a diabetic center/clinic at the selected governmental hospitals in Addis Ababa.

3.3.2. Study population

All DM patients with diabetic foot ulcers visiting the DM clinic at the selected governmental hospitals during the study period.

3.4. Eligibility Criteria

3.4.1. Inclusion Criteria

All adult diabetic patients with diabetic foot ulcers, with ulcers greater than and equal to grade one Wagner's classification system, and those who have given informed consent were included.

3.4.2. Exclusion Criteria

All diabetic patients with the ulcer that was below grade one Wagner's classification system, and those who have taken antibiotics for their ulcers at the time of visiting diabetes clinics were excluded.

3.5. Sample size and Sampling procedure

3.5.1. Sampling size determination

By considering the rareness of the case, the sample size was taken by the duration of data collection from November 2020 to May 2021. Within seven months, the total sample was 130. Convenient sampling procedures were carried out to recruit the study participants.

3.6. Study variable

- Dependent variable
  - ✓ Bacterial isolates and antimicrobial susceptibility pattern
- Independent variable
  - ✓ Age
  - ✓ Sex
  - ✓ Place of residence
  - ✓ Types of diabetes

- ✓ Duration of diabetes
- ✓ Wagner's grades of ulcer (Grade1, Grade 2, Grade 3, Grade 4, Grade 5)

3.7. Measurement and data collection

3.7.1. Data collection

Data on demographic and clinical characteristics of patients were collected by a questionnaire. The questionnaire was initially prepared in English then translated into Amharic. The data collected includes age, sex, occupation, educational level, marital status, place of residence, Wagner's grades of ulcer, peripheral vascular disease, peripheral neuropathy, duration of diabetes, types of diabetes, and history of follow-up.

3.7.2. Sampling procedure, culture, and antimicrobial susceptibility test

3.7.2.1. Sample collection and transport

The sample was collected, (by nurses who are assigned at DM clinic), before initiation of antimicrobial therapy. Based on the appearance of foot ulcer or wound; swab sample, tissue sample, discharge/purulent(1-3ml) sample were collected by a sterile cotton swab, sterile scalpel, or dermal curette (curettage), a sterile needle or syringe, respectively. Stuart's transport medium was used for transporting swabbed and tissue samples within two hours. Aspirated samples were transported with the syringe in the icebox within two hours. Samples from each study site were transported to the postgraduate Bacteriology laboratory of the Department of Microbiology, Immunology, and Parasitology, Addis Ababa, University.

3.7.2.2. Sample Processing

Gram stain, culture, and a panel of biochemical testing have been done after the sample reaches the laboratory. Swab samples were inoculated directly on media. Aspirated/pus samples were first mixed thoroughly then inoculated on appropriate media. Tissue samples were mixed in normal saline to homogenize the tissues.

3.7.2.3. Culture and Identification

The culture was done on a Blood agar plate (BAP), MacConkey agar (MAC) and incubated at 35-37°C for 24 hours. If the growth of bacteria is seen after 24 hours, further processing was done but if growth was not seen, re-incubation for additional 24 hours was proceeded to confirm the presence of slow-growing isolates. Colonies were characterized by using standard techniques like hemolysis, morphology, color, mucoid and topography of the colony. Pathogenic Gram-positive bacteria like *Staphylococcus spp, Streptococcus spp, and Enterococcus spp* were differentiated using Gram stain, catalase test, coagulase test, bile esculin test, hemolysis on BA, DNase test. Mannitol salt agar was used to differentiate *S.aureus* from coagulase-negative *Staphylococcus species (*Figure 3).

Gram-negative bacteria like Enterobacteria, *Acinetobacter spp*, and *Pseudomonas spp* were differentiated after inoculation on MacConkey agar. Growth of lactose fermenter colonies (pink) and non-lactose fermenter colonies (pale) in MacConkey agar was strictly observed. Lactose fermenter colonies further confirmed by a battery of biochemical tests for *Escherichia coli*, *Klebsiella spp*, *Enterobacter spp*, *Citrobacter spp*, *Serratia spp*. On the other hand, non-lactose fermenter colonies were confirmed for identification of *Pseudomonas spp*., *Proteus spp*., *Acinetobacter spp*., and others by different biochemical test. Biochemical tests used for the differentiation of pathogenic Gram-negative bacteria were used: urease test, citrate utilization test, oxidase test, triple sugar iron (TSI) agar test, lysine decarboxylase (LDC) and sulfite-indole-motility (SIM) test (Figure 4).

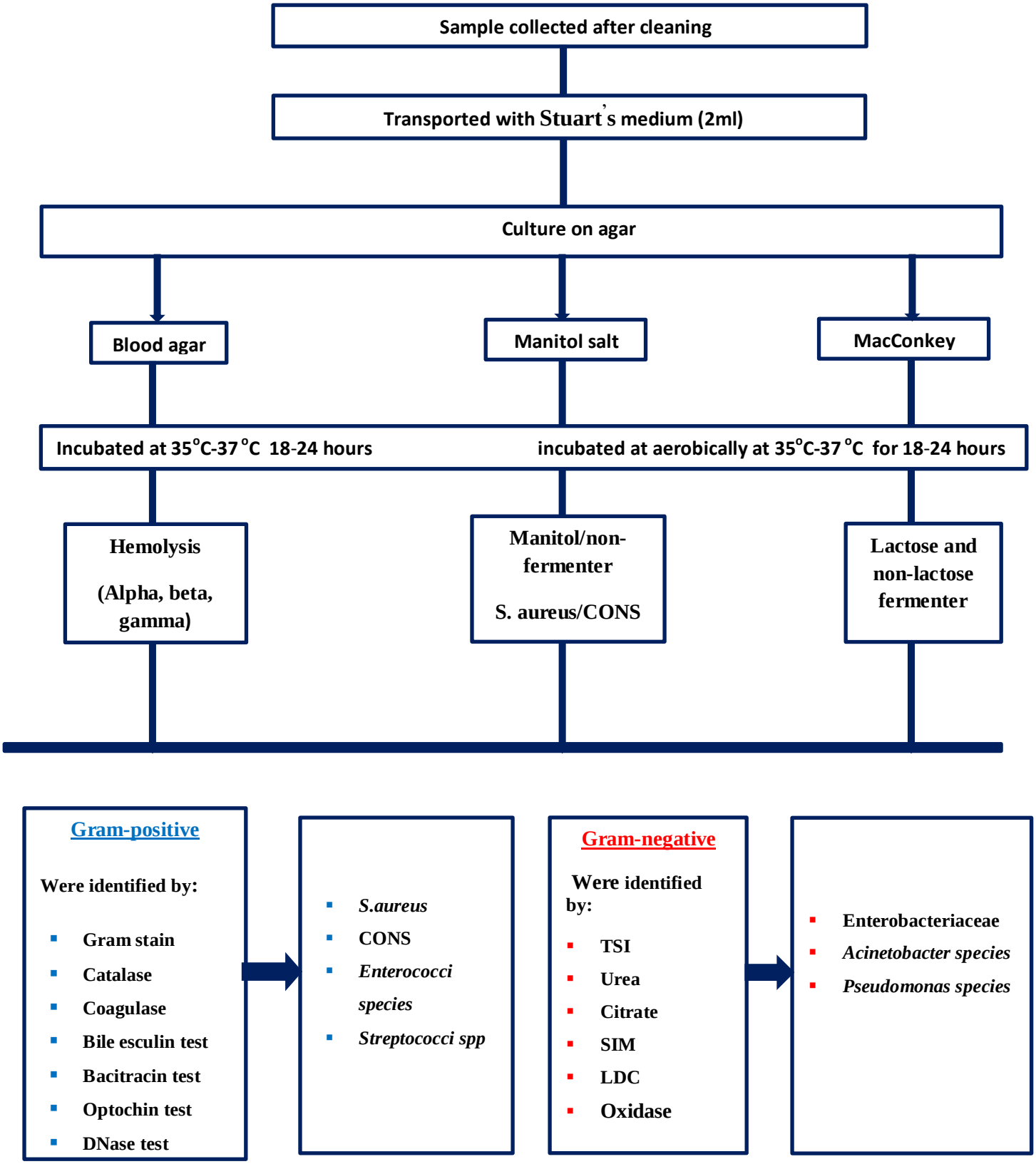

Figure 3: Flow chart for laboratory procedures

3.7.2.4. Antimicrobial susceptibility test of the isolates

Antibiotic susceptibility patterns of the potential pathogenic bacterial isolates were done with 21 antibiotics (Oxoid, UK) based on the Kirby Bauer's disc diffusion technique method on Mueller-Hinton agar (MHA) (Oxoid, UK) as described in the Clinical Laboratory Standard Institute (CLSI) guideline 2020 (CLSI, 2020). An inoculum for each isolate has been prepared by emulsifying colonies from an overnight pure culture in sterile normal saline (0.85%) in test tubes with the turbidity adjusted to 0.5 McFarland standards. The bacterial suspension was uniformly streaked on MHA plates using sterile swabs and left for 3 minutes before the introduction of the antibiotics.

For Gram-negative bacteria the following antibiotics were used (in μg/disk) : Ampicillin-Sulbactam (10/5), Amoxicillin and Clavulanic acid (10/10), Ceftriaxone (30), Cefotaxime (30), Ceftazidime (30), Amikacin (30), Polymaxin B, Gentamicin (10), Ciprofloxacin (5), Sulfamethoxazole-Trimethoprim (1.25/23.75), Cefoxitin (30), Cefepime (30), Piperacillin-Tazobactam (100/10), Tobramycin (30), Imipenem (10), Meropenem (10), Chloramphenicol (30), and Aztreonam (30) based on Clinical Laboratory Standards Institute (CLSI, 2020). For Gram-positive bacteria antibiotics (in μg/disk) Penicillin (10 units), Oxacillin (2), Gentamicin (10), Erythromycin (15), Ciprofloxacin (5), Doxycycline (30), Amikacin (30), Vancomycin (30), Cefoxitin (30), Sulfamethoxazole-Trimethoprim (1.25/23.75), Clindamycin (2) and Chloramphenicol (30) antibiotics were selected for susceptibility testing. Plates were incubated at 35 °C for 16-18 hours, and the diameters of the zone of inhibition were measured with Vernier caliper and results were interpreted according to the Clinical Laboratory Standards Institute standards (CLSI, 2020).
Double disks (cefotaxime 30μg, Cefotaxime-Clavulanic acid 30/10μg) and (Ceftazidime 30μg, Ceftazidime-Clavulanic acid 30/10μg) were used for phenotypically screening of ESBL-producers among pathogenic gram-negative bacterial isolates

ESBL test results interpretation: If the test isolates ESBL-producer, there will be $\geq$5 mm increase in zone diameter for either antimicrobial agent tested in combination with clavulanate versus the zone diameter of the agent when tested alone (For example, if CAZ zone=16; Ceftazidime-Clavulanic zone=21, the test isolate is ESBL-producer).

In addition, further screening for phenotypically carbapenemase-producers among pathogenic gram-negative bacterial isolates was done using the modified carbapenem inactivation method based on guidelines of CLSI, 2020.

Test result interpretation of modified carbapenem inactivation method

A. If the test isolate is carbapenemase positive, zone diameter will be 6-15 mm, which means that Meropenem susceptible *E.coli ATCC 25922* will grow around the disk, or presence of pinpoint colonies within 16-18 mm zone.
B. If the test isolate is carbapenemase negative, the clear zone diameter will be $\geq$ 19 mm, which means that Meropenem susceptible *E.coli ATCC 25922* will not grow around the disk.

3.7.2.5. Quality assurance of laboratory procedures

To ensure the quality of the result from different assays, internal quality assurance systems were placed for all laboratory procedures and double-checking of the results have been done. All the methods to be used were validated as fit for the purpose before use in the study and any amendment and/or modification were documented in the logbooks. Standard operating procedures (SOPs) were used for specific purposes for all laboratory procedures. Quality control strains of *Escherichia coli ATCC® 25922, Enterococcus faecalis ATCC® 29212, Pseudomonas aeruginosa ATCC® 27853, Staphylococcus aureus ATCC® 25923, K. pneumoniae ATCC®1705,* and *K. pneumoniae ATCC®1706,* were used to confirm the result of antibiotics, media and to assess the quality of the general laboratory procedure (CLSI, 2020). The quality of the reagents, antibiotic disk, and media used was checked regularly. The isolates were stored at ˉ80 °C in a broth containing skimmed milk, tryptone, glucose, and glycerol (STGG) at the postgraduate Bacteriology laboratory of the DMIP, Addis Ababa University.

3.8.2. Quality assurance

Collaborators at each study site and principal investigator discussed on how to collect the data and complete the questionnaire appropriately. The validity of the data collection instrument was kept by conducting a pre-test and using a structured questionnaire. All the

collected data were checked daily for completeness, accuracy, and consistency by the principal investigator.

3.9.3. Data processing and analysis

Data were entered into EpiData v.4.6.0.4 and cleaned and analyzed using SPSS Statistical Software version 25 (IBM company, Comp.soft-sys.stat.spss). Descriptive statistics such as frequencies and percentages were employed to report numerical summaries of findings. Patterns of quantitative values were presented using graph presentations and statistical tables.

3.9.4. Operational definitions

Diabetic Foot Ulcer (DFU): A foot below the ankle affected by ulceration that is associated with neuropathy and/or peripheral arterial disease of the lower limb in patients with diabetes (Apelqvist *et al.*, 2000).

Diabetic Foot Infection (DFI): Is a type of infection on the foot of diabetes patients with the non-typical clinical feature, because of glucose effects on the vascular, neurological, and immune systems that can compromise the local and systemic response to infection (Lipsky *et al.*, 2012).

Diabetes Neuropathy (DN): Is a disease affecting nerves (motor, sensory, autonomic) causing, impairment in sensations, movement, and other aspects of health depending upon the nerve affected due to hyperglycemia (Alexiadou *et al.*, 2012, Noor et al., 2015)).

Peripheral Arterial Disease (PAD): Is an atherosclerotic occlusive (loss of bleeding) disease of lower extremity due to hyperglycemia (Noor et al., 2015).

Wagner-Meggit Classification System: It is a six-grade classification system of DFU, which takes into consideration the depth of ulcer, presence of gangrene, and level of tissue necrosis (Jain, 2015, Lipsky et al., 2016).

3.9.5. Ethical consideration

Ethical clearance was first obtained from the Ethics and review committee of the Department of Microbiology, Immunology and Parasitology, College of Health Science,

AAU, and approved by the Department. Approval was also obtained from Addis Ababa public health research and emergency management directorate. The written official letter was distributed to each of the hospitals included in this study. Study participants were informed about the study and their consent sought, before the data collection, and confidentiality was maintained by omitting their names and personal identifiers throughout the study.

3.9.6. Dissemination of result

This result will be submitted to DMIP, AAU. The result of this study will also be disseminated to TASH and Addis Ababa public health research and emergency management directorate. It will also present in different scientific and international research meetings. It will be published in an international or national peer-reviewed journal.

4. Result

4.1. Socio-demographic data of the study participants

One hundred thirty (130) study participants were included (23 from TASH, 31 from Yekatit-12 hospital, and 76 from Menelik-II hospital). Out of the total, 88 (67.69%) were males and 42 (32.3%) were females. The majority of the participants were in the age group of 50-75 years. Only 4 participants were below 40 years, 35 participants between 41 and 50 years, 42 participants between 50 and 60 years, 30 participants between 61 and 70 years, 17 participants between 71 and 80 years, and 1 participant above 80 years. The study participants are residents of Addis Ababa (84.5%) and others came from a rural area (15.5%). Forty-six % of the study subjects were self-employed, 35.7 % were employed, and 17.8% were unemployed (Table 5).

4.2. Clinical data of the study participants

The mean duration of diagnosis of DM was 11.3$\pm$ 7.59 S.D. years (range: 2-50 years) with a minimum of 2 years and a maximum of 50 years. The type of DM that the study participants had was type-I in 51.9% (67/130), and 48.4% (63/130) participants had type-II. Most of the participants were taking insulin therapy 63.6% (82/130), anti-hyperglycemic agent 33% (43/130), and a combination of oral and insulin therapy in 3.9% (5/130). Hemoglobin A1c (HbA1c) data of participants was collected and the mean glycated hemoglobin value was recorded as 9.38%$\pm$ 2.65 S.D (range: 5.6-16%).

Regarding the co-morbidities in the study participants, hypertension in 54.3% (70/130), and kidney disease in 18.6% (24/130) were identified. Peripheral neuropathy (PN) and peripheral vascular disease (PVD) were found, 81.4% (104/130) and 17.69% (23/130), respectively. In addition, 19.4% (25/130) participants were cigarette smokers. All study participants were characterized for their leg skin texture, which is a well-known risk factor for the development of DFU among DM patients. Dry skin in 31.0% (40/130), moist skin in 40.7% (53/130), and cracked skin in 28.7% (37/130) was recorded.

The Meggitt-Wagner classification system was used to classify diabetic foot ulcers. The majority of study participants presented with grade three in 48.4% (62/130), followed by

grade two 33.59% (43/130), 14.8% (19/130) with grade four, three (2.3%) with grade one, and grade five in one (0.8%) participant. Thus, gangrenous ulcers (grade 4 and grade 5) constituted 15.3% (20/130) of all the ulcers and nine participants underwent amputation (Table 5).

Table 5: Socio-demographic and clinical data of the study participants

Characteristics	Categories	Frequency (n)	Percent (%)
Sex	Male	89	68.2
	Female	41	31.8
Age	<40	4	3.2
	41-50	36	27.2
	50-60	42	32.7
	61-70	30	29.5
	71-80	17	14.4
	≥ 81	1	0.8
	Total	130	100
Number of participants at study sites	TASH	23	17.69
	Yekatit-12 hospital	31	23.84
	Menelik-II hospital	76	58.46
Residence	Urban	110	84.61
	Rural	20	15.38
Type of diabetes	Type-I	67	51.53
	Type-II	63	48.46
Duration of diabetes	< 1 year	0	0
	1-10 years	75	53.38
	11-20 years	38	27.69
	21-30 years	16	12.30
	>31 years	1	0.76
HGBA1C	1-5 g/dl	0	0

	6-10 g/dl	75	57.69
	11-15 g/dl	43	33.07
	≥15 g/dl	2	1.53
Hypertension	Yes	70	53.84
	No	60	46.15
Kidney disease	Yes	24	18.46
	No	106	81.53
Peripheral Neuropathy (PN)	Yes	104	80
	No	26	20
Peripheral Vascular Disease(PVD)	Yes	43	33.07
	No	87	66.92
Leg skin texture	Dry skin	40	30.76
	Moist skin	53	40.7
	Cracked skin	37	28.46
Wagner's classification system	Grade 1	3	2.3
	Grade 2	45	34.61
	Grade 3	62	47.69
	Grade 4	19	14.61
	Grade 5	1	0.76
	Total	130	100

4.3. Bacterial isolates from diabetic foot ulcer infections

Out of 130 study participants, positive bacterial growth was observed in 92.3% (120/130) DFUs, and in 7.7% (10/130) participants, there was no bacterial growth seen after 24 hours incubation. A total of 68.33% (82/120) poly-bacterial growth was identified. The percentage of ulcers with poly-bacterial growth increased as the grade of ulcers increased. Of the 120/130 (92.3%) culture-positive cases, a total of 127 bacterial isolates were recorded with 41 gram-positive and 86 gram-negatives. The proportion of gram-negative bacteria 86 (67.7%) was higher than gram-positive bacteria 41(32.2%) (Table 6).

Table 6: Bacterial isolates among diabetic foot ulcer study participants

Bacterial profiles		Frequency	Percentage
Bacterial growth	Growth seen	120	92.3
	No growth	10	7.7
	Total	130	100
Gram staining	Gram-positive	41	32.28
	Gram-negative	86	67.71
	Total	127	100
Number of isolates per sample/case	Mono-bacteria	38	31.66
	Poly-bacteria	82	68.33
	Total	120	100

Among the isolated bacteria by aerobic culture, the most predominant bacteria were *Staphylococcus aureus* 25.19% (32/127), followed by *Pseudomonas species* 18.89% (24/127), and *Escherichia coli* 16.53% (21/127). Only 0.78% of study participants with diabetic foot ulcers harbored *Klebsiella oxytoca* (Figure 4).

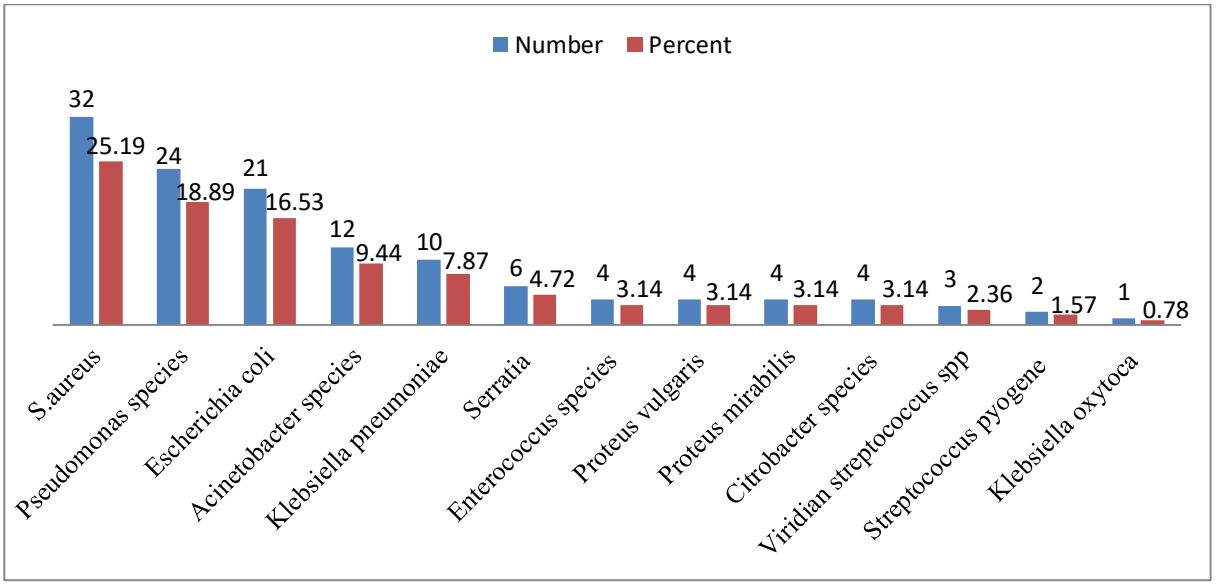

Figure 4: Magnitude of bacterial isolates among study participants

The majority number of culture-positive cases was observed in Wagner grade 3 diabetic foot ulcers 50.3% (64/127) followed by Wagner grade 2 26.7% (34/127). Overall infection was mono-bacterial in 31.66% (38/120) and poly-bacterial in 68.33% (82/120) of samples.

Staphylococcus aureus 32 (25.1%), *Pseudomonas species* 24 (18.8%) and *E.coli* (16.5%) were the most common isolates (Table 7).

Table 7: Distribution of bacterial isolates among Wagner classification of DFUs system

Isolated Bacteria	Wagner classification of DFUs, n (%)					Total
	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5	
Acinetobacter species	0	0	10(83.3)	2 (16.6)	0	12 (9.4)
Citrobacter species	0	1 (25)	2 (50)	1 (25)	0	4 (3.1)
E.coli	0	4 (19)	9 (42.8)	7 (33.3)	1 (4.7)	21 (16.5)
Enterococcus species	0	2 (50)	2 (50)	0	0	4 (3.1)
K.oxytoca	0	0	0	1	0	1 (0.8)
K.pneumoniae	0	1 (10)	5 (50)	4 (40)	0	10 (7.8)
Proteus mirabilis	0	2 (50)	1 (25)	1 (25)	0	4 (3.1)
Proteus vulgaris	0	2 (50)	0	2 (50)	0	4 (3.1)
Pseudomonas species	0	7 (29.1)	13(54.1)	4 (16.6)	0	24 (18.8)
S.aureus	1 (3.1)	11(34.3)	18(56.2)	2 (6.25)	0	32 (25.1)
S.pyogenes	1 (50)	0	1 (50)	0	0	2 (1.5)
Serratia	0	3 (50)	2 (33.3)	1 (16.6)	0	6 (4.7)
Viridian streptococcus species	0	1(33.3)	1 (33.3)	1 (33.3)	0	3 (2.3)
Total	2(1.5)	34(26.7)	64(50.3)	26(20.4)	1(0.8)	127 (100)

4.4. Antimicrobial profiles of isolated bacteria

4.4.1. Antimicrobial profiles of gram-positive isolates

In this study, the susceptibility patterns of each pathogenic bacterial isolates against both broad-spectrum and narrow-spectrum antibiotics were studied for 127 isolates. Among 41/127 (32.2%) gram-positive bacteria, all *S.aureus* and *Enterococcus species* isolates were resistant to Oxacillin, Penicillin, Cefoxitin, and Bacitracin except 18.7% (6/32) *S.aureus* which were susceptible to Oxacillin and Cefoxitin. Similarly, the majority of isolated *S.aureus* and all *Enterococcus species* were resistant to Gentamycin, Doxycycline,

Erythromycin, and Cotrimoxazole. However, all *S.pyogenes and Viridian streptococcus species* were sensitive to the majority of antimicrobial agents (Table 8).

The majority of *S.aureus* were sensitive to Amikacin 81.25% (26/32), Ciprofloxacillin 50% (16/32), Clindamycin 62.5% (20/32). Interestingly, all isolated *S.aureus* and *Enterococcus species* were found to be sensitive to Chloramphenicol (100%). 50% (2/4) Vancomycin-resistant *Enterococcus species* isolates have been noted (Table 8).

Table 8: Antimicrobial susceptibility patterns of isolated gram-positive bacteria

Antimicrobial tested		Susceptibility pattern of isolated gram-positive bacteria											
Classes	Antibiotics	*S.aureus* (N=32)			*Enterococcus spp* (N=4)			*S.pyogenes* (N=2)			*Viridian streptococcus spp* (N=3)		
		R N (%)	I N (%)	**S** N (%)	R N (%)	I	**S** N (%)	R	I N (%)	**S** N (%)	R N (%)	I N (%)	**S** N (%)
Penicillin	PEN	32 (100)	0	0	4 (100)	0	0	0	0	2 (100)	0	1(33.3)	2(66.6)
	OX	26 (81.2)	0	6 (18.7)	4 (100)	0	0	0	0	2 (100)	0	1(33.3)	2(66.6)
Cephamycin	CXT	26 (81.2)	0	6 (18.7)	4 (100)	0	0	0	0	2 (100)	0	1(33.3)	2(66.6)
Aminoglycosides	GEN	13 (40.6)	12 (37.5)	7 (21.8)	4 (100)	0	0	0	0	2 (100)	2(66.6)	0	1(33.3)
Aminoglycosides	AMK	3 (9.3)	3 (9.3)	26 (81.2)	2 (50)	0	2 (50)	0	0	0	0	0	3(100%)
Tetracycline	DO	22 (68.7)	4 (12.5)	6 (18.7)	4 (100)	0	0	0	0	0	2(66.6)	0	1(33.3)
Quinolones	CPR	10 ((31.2)	6 (18.7)	16 (50)	3 (75)	0	1 (25)	0	0	2 (100)	1(33.3)	0	2(66.6)
Sulfonamides	SXT	17 (53.1)	4 (12.5)	3 (9.3)	3 (75)	0	1 (25)	0	0	2 (100)	0	0	3(100)
Chloramphenicol	CHL	0	0	32 (100)	0	0	4(100)	0	0	2 (100)	0	0	3(100)
Macrolides	E	11(34.3)	11(34.3)	10(31.2)	4(100)	0	0	0	0	2(100)	0	0	3(100)
Lincosamides	DA	6 (18.75)	6 (18.75)	20 (62.5)	2 (50)	1	1 (25)	0	0	2 (100)	0	0	3(100)
Glycopeptide	VA				2 (50)	0	2 (50)	0	0	2 (100)	0	0	3(100)

Abbreviation: *PEN-Penicillin, OX-Oxacillin, CXT-Cefoxitin, GEN-Gentamycin, AMK-Amikacin, DO-Doxycycline, CPR-Ciprofloxacin, BC-Bacitracin, SXT-Trimethoprim-sulfamethoxazole, CHL-Chloramphenicol, E-Erythromycin, DA-Clindamycin, VA-Vancomycin*

4.4.2. Antimicrobial profiles of gram-negative isolates

The majority of *Escherichia coli*, *Klebsiella pneumoniae*, Serratia, *Proteus vulgaris*, *Proteus mirabilis, Citrobacter species*, and *Klebsiella oxytoca*, were sensitive to Chloramphenicol, Amikacin, and Ceftazidime. Amikacin, Aztreonam, Ceftazidime, were found to be the best drug for *Citrobacter species* (Table-9).

Table 9: Antimicrobial susceptibility patterns of isolated gram-negative bacteria

Antimicrobial tested		Susceptibility pattern of isolated gram-negative bacteria								
Classes	Antibiotics	***Acinetobacter species*** **(N=12)**	***Pseudomonas species*** **(N=24)**	***K.P*** **(N=10)**	***E.coli*** **(N=21)**	**Serratia** **(N=6)**	***P.mirabilis*** **(N=4)**	***P.vulgaris*** **(N=4)**	***Citrobacter species*** **(N=4)**	***K.oxytoca*** **(N=1)**
Aminoglycosides	AMK	1(8.33%)	11(45.8%)	3(30%)	11(52.3%)	4(66.66)	2(50)	2(50)	4(100)	1
	TOB	1(8.33%)	0	1(10%)	0	0	0	0	0	
Tetracycline	DO	0	0	0	0	0	0	1(25)	0	0
Quinolones	CPR	4(33.33%)	8(33.33%)	3(30%)	7(33.3%)	0	1(25)	1(25)	1(25)	1
Sulfonamides	SXT	1(8.33%)	2(8.33%)	0	0	1(16.66)	2(50)	0	0	0
Chloramphenicol	CHL	3(25%)	4(16.66%)	3(30%)	11(52.3%)	0	2(50)	2(50)	1(25)	1
Monobactam	ATM	3(25%)	10(41.6%)	8(80%)	12(57.1%)	3(50)	2(50)	3(75)	3(75)	1
Polymyxin	PB	0	1(4.16%)	0	1(4.7%)	0	0	0	0	0
Cephalosporins	CXT	0	0	0	1(4.7%)	0	0	0	0	0
	FEP	0	0	0	0	0	0	0	0	0
	CTR	0	2 (8.33%)	1 (10%)	4 (19.0%)	0	1 (25%)	2 (50%)	0	0
	CTX	1 (8.33%)	0	1 (10%)	3 (14.2%)	0	1 (25%)	1 (25%)	1 (25%)	0
	CAZ	3 (25%)	10 (41.66%)	3 (30%)	6 (28%)	1 (16.6%)	1 (25%)	2 (50%)	4 (100%)	0
Beta-lactamase inhibitors	TZP	2(16.66%)	3(12.5)	1(10%)	5(23.8%)	2(33.33)	2(50)	2(50)	1(25)	0
	SAM	0	0	0	0	0	0	0	0	0
	AUG	0	0	0	0	0	0	0	0	0
Carbapenems	IMI	6 (50%)	13(54.16%)	4(40%)	16(76.1%)	2(33.3%)	3(75%)	4(100%)	3(75%)	1
	MER	5 (41.66%)	15(62.5%)	6(60%)	15(71.4%)	3(50%)	3(75%)	3(75%)	4(100%)	1

Abbreviation: *AMK-Amikacin, TOB-Tobramycin, DO-Doxycycline, CPR-Ciprofloxacin, SXT-Trimethoprim-sulfamethoxazole, FEP-Cefepime, TZP-Piperacillin-Tazobactam, SAM-Ampicillin-Sulbactam, AUG-Augmentin, CTR-Ceftriaxone, CTX-Cefotaxime, CAZ-Ceftazidime, IMI-Imipenem, MER-Meropenem*

All gram-negative bacteria were resistant to Cefoxitin, Ampicillin-Sulbactam, Tobramycin, polymyxin b, Cefepime, and Augmentin. *Acinetobacter species* and *Pseudomonas species* were found to be the most resistant to all gram-negative antimicrobial agents including against Amikacin, Chloramphenicol, Aztreonam, Ceftriaxone, Ceftazidime, Imipenem, and Meropenem. More than half of the isolated gram-negative bacteria were resistant against Doxycycline, Cotrimoxazole, Piperacillin-Tazobactam, Ceftriaxone, Cefotaxime, Imipenem, and Meropenem (Table-10).

Table 10: Resistance patterns of isolated gram-negative bacteria

Antibiotic tested		Resistance pattern of isolated gram-negative bacteria								
Classes	Antibiotics	***Acinetobacter species*** **(N=12)**	***Pseudomonas species*** **(N=24)**	***K.P*** **(N=10)**	***E.coli*** **(N=21)**	**Serratia** **(N=6)**	***P.mirabilis*** **(N=4)**	***P.vulgaris*** **(N=4)**	***Citrobacter species*** **(N=4)**	***K.oxytoca*** **(N=1)**
Aminoglycosides	AMK	10 (83.33%)	11 (45.83%)	5 (50%)	5 (28.80%)	2(33.33%)	2 (50%)	2 (50%)	0	0
	TOB	12(83.33)	21(87.5)	7(700	19(90.47	5(83.33)	4(100)	3(75)	3(75)	1
Tetracycline	DO	11 (91.66%)	23 (95.83%)	10 (100%)	18 (85.7%)	6 (100%)	4 (100%)	3 (75%)	3 (75%)	1
Quinolones	CPR	8 (66.66%)	13 (54.16%)	4 (40%)	8 (38.0%)	5(83.33%)	2 (50%	2 (50%)	2 (50%)	1
Sulfonamides	SXT	10 (83.33%)	21 (87.5%)	9 (90%)	19 (90.4%)	5(83.33%)	2 (50%)	4 (100%)	4 (100%)	1
Chloramphenicol	CHL	8 (66.66%)	16 (66.66%)	3 (30%)	2 (9.5%)	6(100%)	2 (50%)	0	2 (100%)	0
Monobactam	ATM	7(58.33)	13(54.16)	2(20)	5(28.8)	2(33.3)	2(50)	3(75)	0	0
Polymyxin	PB	12 (100%)	24 (100%)	10 (100%)	21 (100%)	6(100%)	4 (100%)	4 (100%)	4 (100%)	1
Cephalosporins	CXT	11(91.6)	24(100)	10(100)	20(95.2)	6(100)	4(100)	4(50)	4(100)	1
	CFP	12 (100%)	24 (100%)	10 (100%)	21 (100%)	6(100%)	4 (100%)	4 (100%)	4 (100%)	1
	CTR	12 (100%)	20 (83.33%)	6 (60%)	11 (52.3%)	6 (100%)	3 (75%)	1 (25%)	2 (50%)	1
	CTX	11 (8.33%)	21 (87.5%)	8 (80%)	15 (74.4%)	6 (100%)	3 (75%)	1 (25%)	3 (75%)	1
	CAZ	7 (58.33%)	13 (54.16%)	4 (40%)	7 (33.3%)	3 (33.3%	3 (75%)	0	0	1
Beta-lactam inhibitors	TZP	6 (50%)	16 (66.66%)	4 (40%)	7 (33.3%)	2(33.33%)	2 (50%)	0	2 (50%)	1
	SAM	12 (100%)	24 (100%)	10 (100%)	21 (100%)	6(100%)	4 (100%)	4 (100%)	4 (100%)	1
	AUG	12 (100%)	24 (100%)	10 (100%)	21 (100%)	6(100%	4 (100%)	4 (100%)	4 (100%)	1
Carbapenems	IMI	6 (50%)	11 (45.83%)	6 (60%)	5 (23.80)	4(66.6%)	1 (25%)	0	0	0
	MER	7 (58.33%)	9 (37.5%)	4(40%)	6(28.5%)	3(50%)	1(25%)	1(25%)	0	0

Abbreviation: AMK-Amikacin, TOB-Tobramycin, DO-Doxycycline, CPR-Ciprofloxacin, SXT-Trimethoprim-sulfamethoxazole, FEP-Cefepime, TZP-Piperacillin-Tazobactam, SAM-Ampicillin-Sulbactam, AUG-Augmentin, CTR-Ceftriaxone, CTX-Cefotaxime, CAZ-Ceftazidime, IMI-Imipenem, MER-Meropenem

4.4.3. Multi-drug resistant isolates

Multidrug-resistance profiles of the organisms showed that of the 127 bacterial isolates, 92.9% (118/127) were MDR, that is resistant to more than two agents of antibiotic classes, whereas 7.08% (9/127) were non-MDR. There was resistance against all classes of antibiotics among *Acinetobacter species* and *Pseudomonas species* pathogens. However, the result of MDR pattern compared within species shown in Table 11.

Table 11: Multidrug-resistance pattern among bacterial isolates of DFUs participants

Bacterial isolates	R_0	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	R_9	**MDR**
S.aureus	0	1 (3.1)	3 (9.3)	4 (12.5)	8 (25)	5 (15.6)	5 (15.6)	3 (9.3)	3 (9.3)	0	28(87.5)
Enterococcus species	0	0	0	0	0	0	0	1 (25)	0	3 (75)	4(100)
S.pyogenes	2 (100)	0	0	0	0	0	0	0	0	0	0
Viridian species	0	0	0	1 (33.3)	1 (33.3)	1 (33.3)	0	0	0	0	3(100)
Gram-positive (Total=41)	2 (4.8)	1 (2.4)	3 (7.3)	5 (12.19)	9 (21.9)	6 (14.63)	5 (12.19)	4 (9.75)	3 (7.3)	3 (7.3)	**35 (85.36)**
Pseudomonas species	0	0	2 (8.3)	3 (12.5)	3 (12.5)	4 (16.6)	5 (20.8)	0	6 (25)	1 (4.1)	22(91.6)
E.coli	0	0	0	6(28.5)	6(28.5)	4(19.0)	3(14.2)	1(4.7)	1(4.7)	0	21(100)
Acinetobacter species	0	0	0	0	2(16.6)	2(16.6)	2(16.6)	1(8.3)	2(16.6)	3(25)	12(100)
K.pneumoniae	0	0	0	0	2(20)	4(40)	2(20)	0	1(10)	1(10)	10(100)
Serratia	0	0	0	0	0	0	4(66.6)	2(33.3)	0	0	6(100)
Proteus mirabilis	0	0	0	1(25)	0	0	2(50)	1(25)	0	0	4(1000
Proteus vulgaris	0	0	0	0	2(50)	1(25)	1(25)	0	0	0	4(100)
Citrobacter species	0	0	1(25)	1(25)	0	1(25)	1(25)	0	0	0	3(75)
K.oxytoca	0	0	0	0	1	0	0	0	0	0	1
Gram-negative (Total=86)	0	0	3(3.4)	11(12.7)	15(17.4)	16(18.6)	20(23.2)	5(5.81)	10(11.6)	5(5.81)	**83 (96.5)**
Total isolates (127)	2(1.57)	1(0.7)	6(4.7)	16(12.5)	25(19.6)	22(17.3)	25(19.6)	9(7.08)	13(10.2)	8(6.2)	**118 (92.9)**

Note: *R0-Sensitive for all classes of antibiotics, R1-Resistance for one class of antibiotics, R2-Resistance for two classes of antibiotics, R3-Resistance for three classes of antibiotics, R4-Resistance for four classes of antibiotics, R5-Resistance for five classes of antibiotics, R6-Resistance for six classes of antibiotics, R7-Resistance for seven classes of antibiotics, R8-Resistance for eight classes of antibiotic, R9-Resistance for nine classes of antibiotics, R10-Resistance for ten classes of antibiotics, MDR-Multidrug-resistance.*

4.5. ESBL-producers of gram-negative isolates

In this study, further phenotypic tests of ESBL-producers were carried out on 76 pathogenic gram-negative bacteria. Of 76 gram-negative bacteria, 53.9 % (41/76) were phenotypically noted as ESBL-producer and 46.05% (35/76) were non-ESBL-producer. The most ESBL-production rates were seen among *K.pneumoniae* 75% (6/8), *Acinetobacter species* 75%

(9/12), Serratia 75% (3/4), *Pseudomonas species* 64.3% (9/14), and followed by *E.coli* 57.8% (11/19), *Citrobacter species* 50% (2/4), *Proteus mirabilis* 25% (1/4).

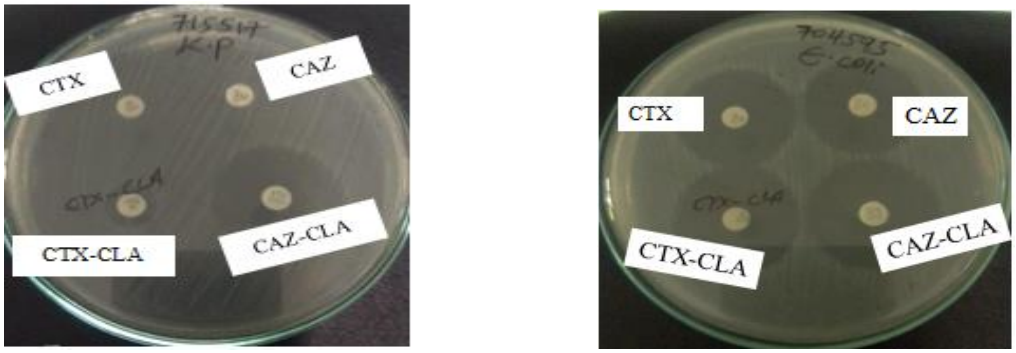

Figure 5: ESBL-producer of *Klebsiella pneumonia*, and non-ESBL- producer of *E.coli*, respectively

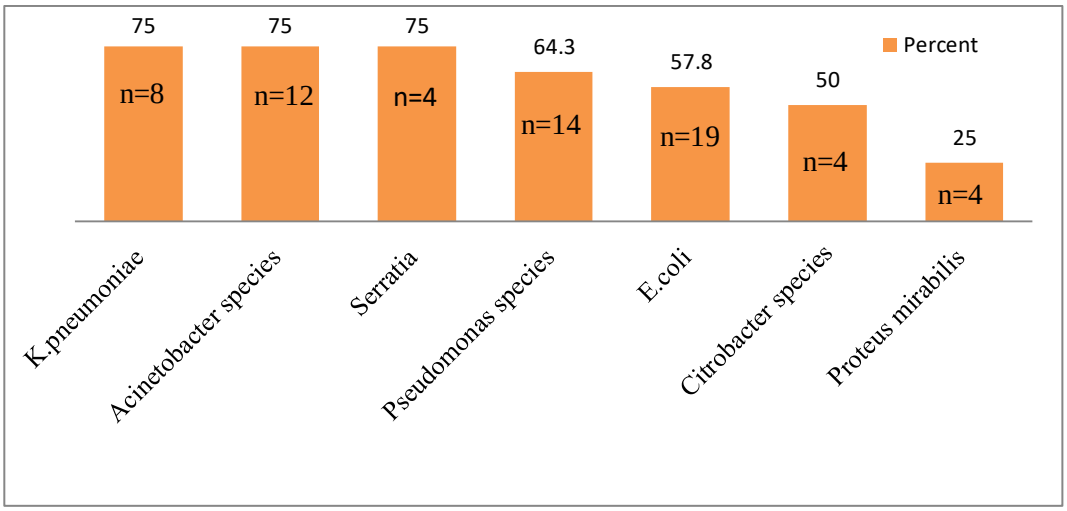

Figure 6: Magnitude of ESBL-producers among pathogenic gram-negative isolates

4.6. Carbapenemase-producers of gram-negative bacteria isolates

Phenotypical determination of carbapenemase-producer pathogenic gram-negative bacterial isolate also was another further analysis in this study. Based on the modified disk inactivation (meropenem) method (CLSI 2020 guideline), this additional carbapenemase test was done for 68 ESBL positive and Imipenem or Meropenem resistant gram-negative isolates. Out of 68 pathogenic gram-negative bacteria isolates, 27.9% (19/68) were carbapenemase-producer, whereas 73.53% (50/68) were non-carbapenemase-producer. The

most carbapenemase-producers rate was seen among *K.pneumoniae* 5/8, followed by *Serratia* 3/6, *Acinetobacter species* 4/10, *Pseudomonas species* 4/17, *E.coli* 4/19, *Proteus mirabilis* 0, *Proteus vulgaris* 0, *Citrobacter species* 0.

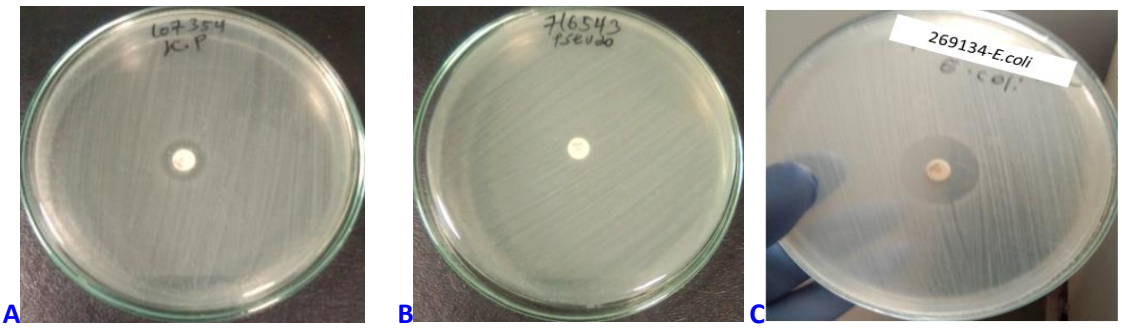

Figure-7: Carbapenemase-producer of *K. pneumoniae* (**A**), *Pseudomonas species* (**B**) *and non*-carbapenemase -producer of *E. coli* (**C**).

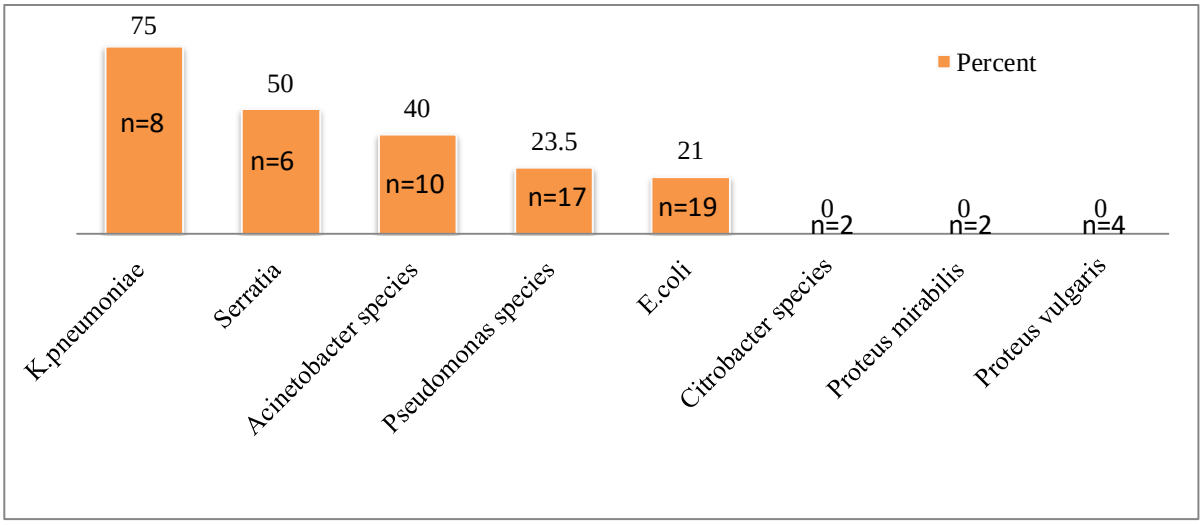

Figure 8: Magnitude of carbapenemamse-producers among gram-negative isolates

4.7. Discussion

A diabetic foot ulcer is an important complication of DM and if untreated DFU will become infected and lead to various other consequences such as gangrene, osteomyelitis, and amputation of the limb. Surgical intervention and treatment with an antibiotic regimen are the options used for the management of this infection. This study was conducted to determine the dominant pathogenic bacterial associated infection of DFUs and determine their antimicrobial susceptibility and resistance pattern in the three study sites.

In this study, bacteriological analysis of samples taken from DFU, showed that superiority of growth 92.3% than non-growth 7.69%, which is in agreement with another study conducted in Ethiopia, with growth 77.3%, no growth 22.7% (Amogne *et al.*, 2011), in other countries such as Egypt growth 81.7%, no growth 22% (Ismail *et al.*, 2021). More magnitude of poly-bacterial 68.33% than mono-bacterial 31.66%, which is supported by in other countries like Egypt poly-bacterial 56.08%, mono-bacterial 48.75% (Dwedar *et al.*, 2015).

The magnitude of gram-negative 71.6% greater than gram-positive isolates 34.16%, which is also in agreement with another study conducted in Ethiopia, gram-negative bacteria 88.55%, and gram-positive bacteria 7% (Amogne *et al.*, 2011), and in other countries such as Egypt gram-negative bacteria 56% greater than gram-positive bacteria 27.7% (Dwedar *et al.*, 2015), including recent study in the same country, gram-negative 79%, gram-positive 21% (Ismail *et al.*, 2021), Nigeria gram-negative 59.2%, gram-positive 27.1% (Adeyemo *et al.*, 2019), Pakistan gram-negative 76.27%, gram-positive 23.83%, poly-bacterial 56.87%, mono-bacterial 44.23% (Miyan *et al.*, 2017), India gram-negative 61%, gram-positive 39% (Kumar *et al.*, 2017), and in South America gram-negative 63%, gram-positive 37% (Kurup and Ansari, 2019).

The bacterial isolation rate and poly-bacterial content of the ulcer were increased with the increment of ulcer severity. This indicates that how much the organisms affect the healing process of DFU, which is supported by different published articles worldwide, like in

Nigeria (Otta *et al*., 2019), China (Xie *et al.*, 2017), India (Thanganadar *et al*., 2019), and recently in India (Shah *et al*., 2021).

Among the overall pathogenic aerobic bacterial isolates in the current study, *S.aureus* 25.19% was predominant, but a previous study in Ethiopia *Klebsiella species* 23.9% and *Proteus species* 18.47% were the common isolates (Amogne *et al*., 2011), while in Egypt *P. mirabilis* (16.8%) was commonest (Dwedar *et al*., 2015), in Saudi Arabia *Pseudomonas species* 15.6% (Al Ayed *et al*., 2018), and in South America *Pseudomonas species* (18.8%). This variation may be due to sample size difference, geographical are difference, method of isolation difference. However, the present study agreed with other findings in different parts of the world, like in Kenya 17.5% (Mutonga *et a*l., 2019), in Nigeria 32.9% (Ogba *et al*., 2019), in India 24.42% (Aleem *et al*., 2021), 25% (Shah *et al*., 2021), in China 65.2% (Xie *et al.*, 2017), in Iran 28% (Akhi *et al*., 2015).

Also in this study, out of the gram-negative isolates, *Pseudomonas species* 18.89% was the most frequent followed by *E.coli*. These findings correlated with other studies. For example, in Libya 17.5% (Elbaz *et al*., 2018), in India 23.2% (Thanganadar et al., 2019), and 23.6% (Shah et al., 2021). In contrast to these findings, a previous study in Ethiopia reported no *Pseudomonas species* isolated from 92 cultured samples, whereas *E.coli* was isolated in 5.43% (Amogne *et al*., 2011), while in Pakistan the most common among gram-negative bacteria was *E.coli* 15.72% (Miyan *et al*., 2017). This variation might be due to the sample size differences from one study to another study, geographical area difference, isolation method. The current study, confirmed that both gram-positive and gram-negative aerobic pathogenic bacteria are considered as etiology and challenges for the management of DFU, and leads to infection, osteomyelitis, and possibly amputation of the limbs.

More significantly, the present study found that 92.9% of multidrug-resistance among different isolated pathogenic bacteria. This high drug resistance is somehow greater than study conducted in Nigeria 80% (Adeyemo et al., 2021), in India 51.3% (Jain et al., 2017), recently in the same country 78.6% (Zubair and Ahmad, 2019), in Iran 48.4% (Najari et al., 2019). The high multidrug-resistance in the present study might be due to poor hygienic practice in the study area, difference in the study population, indiscriminate use of

antibiotics. In this study, the majority of isolated *S.aureus* were resistant to Gentamycin, Doxycycline, Erythromycin, and Cotrimoxazole, whereas the majority of *S.aureus* were sensitive to Amikacin, Ciprofloxacillin, Clindamycin. Similarly, all *Enterococcus species* were resistant to Gentamycin, Doxycycline, Erythromycin, and Cotrimoxazole. Importantly, all isolated *S.aureus* and *Enterococcus species* were found to be sensitive to Chloramphenicol drug (100%). Vancomycin-resistant *Enterococcus species* was 50% in the current study, which is greater than the study conducted in India 19% (Jain *et al.*, 2017).

Of the 76 gram-negative bacteria, more than half of the isolates (53.95%) were ESBL-producers, which are consistent with the study documented in India (53%) (Jain *et al.*, 2017), in Egypt 49% (Dwedar *et al.*, 2015). In poor resource settings, the burdens of ESBL producing gram-negative bacteria are more prevalent among DFU patients. Some reports, from the Middle East and North Africa, showed that the ESBL-producing bacteria range from 11% to 53% (Jouar *et al.*, 2019). (For example, 46% in eastern India (Konar *et al.*, 2013), 42 % in Odisha, India (Otta *et al.*, 2019), 38% in Istanbul Turkey (Saltoglu *et al.*, 2018), 33% in Nigeria (Adeyemo, A. *et al.*, 2021). 31% in Iran (Akhi *et al.*, 2015). The current study indicated that gram-negative ESBL-producers in patients with DFU was high among *K. pneumoniae* 75%, *Acinetobacter species* 75%, Serratia 75%, *Pseudomonas species* 64.3%, and followed by *E.coli* 57.8%, *Citrobacter species* 50%, *Proteus mirabilis* 25%, which is somehow different with the study conducted in Iran that reported *Acinetobacter spp*. (50%) followed by *E. coli* (36%), *P. aeruginosa* (33%), and *Enterobacter species* (25%) (Akhi *et al.*, 2015).

Among 68 gram-negative bacteria in this study, 19 (27.9%) were positive for phenotypic screening of Carbapenemase. A high rating was recorded among *K.pneumoniae* followed by *Acinetobacter species*, *E.coli*, *Pseudomonas species,* and Serratia. The present study results are greater than the study conducted in Egypt with 3.6% were carbapenemase producers (Dwedar *et al.*, 2015), including a recent study in the same country which showed 11.7% (14/98) carbapenemase producers (Ismail *et al.*, 2021), and in Nigeria 4 (3.1%) carbapenemase producers (Adeyemo *et al.*, 2019).

5. Strength and Limitation

5.1. Strength of the study

- This study has further tried to analyze ESBL and carbapenemase-producers among pathogenic gram-negative isolates among patients with diabetic foot ulcers at governmental hospitals which was not previously documented in Ethiopia.

5.2. Limitation of the study

- The sample size was small due to the rareness of the cases during the study period, because of various reasons including COVID-19 pandemic which impacted the health-seeking behavior of the patients, and some patients might have also tried to manage their foot ulcers in their home by buying materials to care for from local Pharmacies.
- Molecular tests were not done as a confirmatory test for ESBL and carbapenemase-producers genes among gram-negative isolates

6. Conclusion

A diabetic foot ulcer can be infected by a variety of pathogenic and high number of multidrug-resistance organisms (MDROs), including ESBL and carbapenemase-producers. *S.aureus* was the predominant bacteria isolated from diabetic foot ulcers in the current study. Moreover in this study, the number of gram-negative bacteria was higher than gram-positive bacteria, and poly-bacterial infections were noted as the severity of the ulcer increase. The bacteria isolated were more susceptible to chloramphenicol, aztreonam, amikacin, clindamycin, and vancomycin in the current study. Based on this study, diabetic foot ulcers were infected by the majority of worldwide known highly antimicrobial-resistant pathogenic bacteria, which are known as ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter species*)

The results show an overall increase in bacterial resistance to antimicrobial agents and emphasize the importance of microbiological analysis and antimicrobial susceptibility tests before the selection of antibiotic(s) for management of diabetic foot ulcers.

7. Recommendation

All health professionals who are involved in the management of diabetic foot ulcers should depend on the result of microbiological analysis, and drug susceptibility tests before initiating treatment. Since these study focused only on pathogenic aerobic bacteria, further study including other micro-organisms such as anaerobic bacteria, Fungi will be recommended. Moreover, there should be an antimicrobial stewardship program for the treatment of infected diabetic foot ulcers, which can reduce the antimicrobial resistance burden.

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Annex- I: Information sheet to participants of the study

Information sheet and consent form

1. *Information sheet*

Hello, how are you? My name is _____. My colleague besides me is_____. This is an interview to be done with you for a study that is being conducted at postgraduate medical microbiology laboratory at Addis Ababa University, School of Medicine, College of Health Science and Department of MIP.

Title of the study

The title of the study is detection of bacterial profiles and their antimicrobial susceptibility patterns among patients with diabetic foot ulcer at selected hospitals in Addis Ababa, Ethiopia.

Propose of the study

The purpose of the study is to detect potential pathogenic bacteria and their antimicrobial susceptibility pattern from DFU in selected hospitals in Addis Ababa, Ethiopia.

What it will mean if you decide to take part in the study?

If you agree to participate in this study, you will participate in this interview in a private place. The interview will last for about 30-40 minutes and will be facilitated by my collaborators and me. During the interview, you will be asked to respond questions related to diabetic foot ulcer like duration of the ulcer, predisposing factors, and others.
During the interview, my coworker will write down what you say. The recorded data will not contain your names or other identifying information. They will just be labeled with a study number.

The results of this study will assist physicians and other health service providers by providing aetiological agents in order to manage diabetic foot ulcer among diabetes patients. It will also help to contribute in the subsequent efforts to improve prevention, diagnosis, treatment and support of diabetic foot ulcer patients.

Risks and discomforts

There is no possible risk associated with participating in this study. May be there is a little pain during sample collection from the ulcer which will be collected by professionals. You are free to decline answering any question that you do not wish to answer and you may leave our interview at any time you want to.

Confidentiality

All information obtained will be held securely and stored on paper, and computer files. No one except the interviewers will know that you took part in the study the answers that you give will be marked with a special study number only, and not your name. The data will protect information about you in this research to be the best of our ability.

Voluntary participation

Your participation is voluntary. You may withdraw from the interview at any time without giving a reason and without any penalty. If you have questions regarding this study or would like to be informed of the results after its completion, please do not hesitate to contact: Asegdew Atlaw, MSc student at Addis Ababa University, School of Medicine, College of Health Science and Department of MIP.

Cell phone: +251922562620

Email: **asegdew21@gmial.com**

Annex II. Consent Form

I have read the information sheet concerning this study (or have understood the verbal explanation) and I understood what will be required of me and what will happen to me if I take part in it. I also understand that may withdraw from this study without giving any reason at any time and without my families' or me being affected for my refusal.

May I continue the interview?

1. Yes _____Continue the interview
2. No _____Stop the interview and thank the respondent

Witness's signature certifying that the informed consent has been given

Witness's signature _____ Date _____

Amharic version (Information sheet and consent form)

የሚጀመር ቅፅ

ሄሎ እንዴት ነህ/ነሽ? የኔ ስም----------ከኔ ጋር አብረሽ የሚሩት---------:: ይሄ ከአንተ ጋር/ካንቺ ጋር/ የሚራ ቃለ-ምልልቅ ነዉ :: ጥናቱ የሚሄደዉ በአዲስ አበባ ዩንቨርስቲ ደህረ-ምረቃ የሜዲካል ሜክሮባዮሎጂ የህክምና ት/ቤት ቡፍ ሳይንስ ኮሌጅ ዉስጥ ነዉ፡

የጥናቱ ርዕስ

በአዲስ አበባ በተሚጡ ሆስፒታሎች ዉስጥ የስኳር ህመምተኛ ሆነዉ የእግር ቁስል ያላቸዉ ታካሚዎች ላይ ባክቴሪያዎችን ማግኘትና የተገኙትን ባክቴሪያዎች በየትኛዉ ሞደሀኒት ሊሞቱ እንደሚችሉ ሚጋገጥ ነዉ፡

የጥናቱ አላማ

በአዲስ አበባ በተሚጡ ሆስፒታሎች ዉስጥ አደገኛ ባክቴሪያዎችን ማግኘትና በየትኛዉ ሞደሀኒት ሊታከሙእንደሚችሉ ሚጋገጥ ነዉ፡

ምን ማለት ነዉ አንተ/አንቺ በዚህ ጥናት ዉስጥ ለመሳተፍ ብትወስን/ብትወስኚ?

በዚህ ጥናት ዉስጥ አንተ/አንቺ ብትስመማ/ሚ- በቃለ-ምልልቅ ዉስጥ አንተ/አንቺ ትሳተፋለህ/ትሳተፊያለሽ ማለት ነዉ፡ ቃለ-ምልልቁ የሚወስደዉ ጊዜ ከ 30-40 ደቂቃ ነዉ፡ በእኔና አብረሽ በሚሩ ባለሞያዎች ቃለ-ምልልቁ ይካሄዳል፡: በቃለ-ምልልቅ ሂደት ዉስጥ የተለያዩ ጥያቄዎች ከእግር ቁስሉ ጋር የተያያዙ ለምሳሌ የጊዜ ቆይታዉ፤ለቁስሉ ሚረጩ ምክንያት ካሉ እና የመሳሰሉት ጥያቄዎች ሲነሱ ማልስ ጥሰጣለህ/ትሰጫሽ፡:

የቃለ-ምልልቁ ምላሾች ይመዘገባሉ እና በሚዘገበበት ሰዓትም ስማ ሌሎች ማንነትን የሚገልፁ ነገሮች አይመዘገቡም፡ በጥናቱ ዉስጥ አንዱ ተሳታፊ ከአንዱ ተሳታፊ የሚለየዉ በቁጥር ብቻ ነዉ፡

የዚህ ጥናት ዉጤቱ የስኳር ህመምተኛ የእግር ቁስልን ለማከም ለመቆጣጠር ዶክተሮችንና የቡፍ አገልግሎት ሰጪዎችን ያግዛል፡:በተጨማሪም የዚህ ጥናት ዉጤቱ የስኳር ህመምተኛ የእግር ቁስልን አጥብቆ ለማከላከል፣ የላብራቶሪ ምርመራ ለሞደረግ፣ ድጋፍና እክብከቤ ለሞደረግ እስተዋፆ ያደርጋል፡:

በጥናት ዉስጥ በማተፍ ያሉ ጉዳቶች

በዚህ ጥናት ዉስጥ በማተፍህ/በማተፍሽ የሚጠር ምንም አይነት ጉዳትና ስጋት የለም፡ ምን አልባት ናሞዉ ከቁስሉ ላይ በሚሰደበት ሰዓት በጣም ትንሽ የሚል ህመም ሊሰማ ይችላል፡፡ ሜልስ ማስጡት በማፈልግበት/በማፈልጊበት ጉዳይ ላይ ሜልስ አለማስጡት ነፃነቱ አለህ/አለሽ፡፡ በማንኛዉም ሰዓት ከቃለ ማዉጀቱ ማዉጡት ይቻላል፡፡

ሚስጥርን ማስጠበቅ

ሁሉም የሚሰበሰቡ ሚጃዎች በዉረቀትና በኮምፒተር በጥንቃቄ እና ሚስጥርን በጠበቀ ሜልኩ ይቀመጣሉ፡ ቃለ-ማዉጀቱን ከሚካሂደዉ ሰዉ ዉጪ ማንም ሰዉ ጥናት ዉስጥ ማተፍህን/ማተፍሽን የሚዉቅ ሰዉ የለም፡ የማነት ስያሜዉም በተለየ የጥናት ቁጥር ይሆናል፡፡

በፍቃደኝነት ላይ የተመሰረተ ተሳትፎ

ተሳትፎዉ በፍቃደኝነት ላይ የተመሰረተ ነዉ፡ በማንኛዉም ሰዓት ከቃለ-ማዉጀቱ ያለምንም ምክንያትና ቅጣት ማቋረጥ ይቻላል፡፡ ማንኛዉም አይነት ጥያቄ ካለ እባክህ/እባክሽ ከማየቅ እንዳትቆጠብ/እንዳትቆጠቢ፡ ትያቄዉን በቀጥታ ለአሰግደዉ አጥላዉ (ማስተርስ ተማሪ በአዲስ አበባ ዩንቨርስቲ ህክምና ት/ቤት፣ ጤና ሳይንስ ኮሌጅ እና ማጆክሮባዮሎጂ፣ ኢሚዩሎጂና ፓራሲቶሎጂ ዲፓርትማት)፡፡

ስልክ ቁጥር፡- **+251922562620** Email:- **asegdew21@gmail**

በአሚኛ የስምማት ፎርም

ከዚህ ጥናት ጋር የተያያዘዉን የሚጃ ዉረቀት አንብቤዋለሁ (በቃል የተገለፀዉን በሚባ ተረድቼዋለሁ)፡፡ በጥናት ዉስጥ በማተፌ ከእኔ የሚጠበቀዉንና እኔ ላይ ምን ሊከሄድ እንደሚቸል ተረድቻለሁ፡ በተጨማሪም ያለምንም ምክንያት ከጥናቱ ማተፈን ማቋረጥ እንደምቸል በሚባ ተረድቻለሁ፡

ቃለ-ማዉጀቱን ማቅጨል እችላለሁ?

1. ቀጭል ቃለ-ማዉጀቱን----------አማስግናለሁ!!!
2. አቁም ቃለ ማዉጀቱን---------- አማስግናለሁ!!!

የስምማት ፎርሙስለመሟቱ እና ስማስጡቱ የሚረጋግጥ የእማኝ ፊርማ፡

የእማኝ ስም----------ፊርማ---------- ----ቀን----------

Introduction to the Interview

Thank you for deciding to participate in the interview and for coming to this session. Previously (on the statement of consent form), we have discussed briefly on the purpose of the research, how you were identified, and your part in the research study. Now I am going to have discussion with you on the relevant topic items. There is no right or wrong answers. All answers /responses/ ideas you provide are equally important and you are requested to respond honestly from your experiences and beliefs. I may interrupt and probe your ideas. Once again, I would like to tell you that what we are going to discuss is very confidential and it will be used only for the research.

Annex-III. Questionnaire

Part one: Socio-demographic		
Q. No	Questions	Answer
1.	Age	_____
2.	Sex	**1.** Female **2.** Male
3.	Marital status	**1.** Married **2.** Single **3.** Widowed **4.** Divorced
4.	Educational Level	**1.** Cannot read and write **2.** Can read and write **3.** Primary school **4.** Secondary school **5.** College graduate or above
5.	Occupation	**1.** Student **2.** Self employed **3.** Employed **4.** Unemployed
6.	Monthly income?	_____ birr

7.	Residence	**1.** Urban **2.** Rural
Part two: Clinical History		
8.	Duration of diagnose diabetes mellitus?	_____
9.	Where you got diagnosed about your Diabetes mellitus?	**1.** Government health center **2.** Government Hospitals **3.** Private clinic **4.** Private hospital
10.	What type of DM you have?	**1.** Type I **2.** Type II **3.** Specify others_____
11.	What type of treatment taking now?	**1.** Insulin therapy **2.** Anti-hyperglycemic agent **3.** Combination oral hyperglycemic agent and Insulin therapy **4.** Non-pharmacological regulation
12.	If Anti-hyperglycemic agent, which drug?	**1.** Metformin **2.** Daonil **3.** Both **4.** Others, specify_____
13.	Duration of non-pharmacological (diet and exercise) regulation?	_____
14.	Duration of anti-diabetic agents taken?	_____
15.	Are you taking medicine regularly?	**1.** Yes **2.** No
16.	Are you coming for follow up regularly?	**1.** Yes **2.** No
17.	Do you have hypertension?	**1.** Yes **2.** No
18.	If yes, are you taking medicine for?	**1.** Yes

		2. No
19.	If yes, What type of medicine?	**1.** _____ **2.** _____ **3**. _____
20.	Do you have kidney disease?	**1.** Yes **2.** No
21.	If yes, what type of kidney disease?	**1.** Chronic kidney disease (CKD) **2.** Acute kidney injury (AKI) **3.** Other, specify_____
22.	If you have kidney disease, are you taking medicine?	**1.** Yes **2.** No
23.	If yes, what type of medicine?	**1.** _____ **2.** _____ **3.** _____ **4**. _____
24.	Are you cigarette smoker?	**1.** Yes **2.** No
25.	Are you alcoholism?	**1.** Yes **2.** No
26.	Do you have peripheral neuropathy?	**1.** Yes **2.** No
27.	If yes, duration?	1. _____
28.	Do you have follow up neurologic clinic?	**1.** Yes **2.** No
29.	If yes, duration of follow up?	_____
30.	What is your skin texture?	**1.** Dry **2.** Moist **3.** Cracked
31.	Do you always wear good supportive shoes?	**1.** Yes **2.** No
32.	Do you wash and dry your feet especially between the toes?	**1.** Yes **2.** No

33.	If you have pain do you see your doctor?	**1.** Yes **2.** No
34.	Have you annual foot examination by professional?	**1.** Yes **2.** No
35.	Do you check fasting blood sugar?	**1.** Yes **2.** No
36.	If yes, how many times do you check?	**1.** every week **2.** every two week **3.** every month
37.	Have you ever received foot care education?	**1.** Yes **2.** No
38.	Do you avoid going bare foot outside indoor?	**1.** Yes **2.** No
39.	Do you check for foreign object in shoes before wearing them?	**1.** Yes **2.** No
40.	Do you have foot ulcer?	**1.** Yes **2.** No
41.	Have you ever given sample for microbiological analysis?	**1.** Yes **2.** No
Part three: Physical examination finding		
42.	Blood pressure?	_____
43.	Pulse Rate?	_____
44.	Weight?	_____
45.	Height?	_____
46.	Body mass index?	_____
47.	Presence of peripheral vascular disease?	**1.** Present **2.** Absent
48.	Presence of peripheral neuropathy?	**1.** Present **2.** Absent
49.	A type of diabetic foot ulcer by	**1.** No grading

	Wagner's grading system?	**2.** Grade 1 **3.** Grade 2 **4.** Grade 3 **5.** Grade 4 **6.** Grade 5
Part four: Laboratory findings		
50.	Fasting blood sugar	_____
51.	Hemoglobin A1c	_____
52.	Creatinine	_____
54.	Protein in the urine	_____

Annex-IV: **Laboratory Procedures of Sampling, Culturing, Biochemical testing, and AST from DFU.**

1. General Principles

Skin and soft tissue are infected by several variety of microorganisms which are reside on the skin and mucus membranes of the body, as well as those found in the environment (especially hospital acquired MDROs). The route of entry of these microbes to the body is through breaks in skin or mucus membranes, through wounds, which made by trauma or bites (exogenous), as complication of surgery or foreign-body (implants(endogenous), or they can be distributed to the tissues through the vascular system (hematogenous).

Superficial wounds, abscess/fluid, or tissue specimens from those collected from deep body sites all are should be distinguished by laboratory professionals. Most of time superficial wound and abscess specimens grow primary pathogens that can cause skin and soft tissue infections. *Staphylococcus aureus, Pseudomonas aeruginosa*, some members of the *Enterobacteriaceae*, beta-hemolytic streptococci, and anaerobes are considered as primary agents of skin and tissue infections. Also from deep wound and invasively collected abscess/fluid specimens and tissues much wider micro-organisms diversity can usually be recovered if the appropriate media and cultures are inoculated.

Mostly, acute wound infections are due to external damage to intact skin such as those produce during surgery or by trauma and bites. Nevertheless, chronic infections, such as decubiti or foot and leg ulcers, are associated with complications like impaired vascular flow or metabolic disorder disease (e.g., diabetes mellitus). Wound/ulcer colonization and then infection is often pronounced as polymicrobic, with both aerobes and anaerobes bacteria.
Best achievements of microbiological profile by culture techniques needs best type of specimen like tissue collected during surgery or aspirates taken through intact skin by needle and syringe or by fine-needle biopsy. If the skin surface and surgical areas are properly disinfected prior to specimen collection, the organisms present can be assumed the cause of infection.

Interpretation of microbial cultures taken from open skin or abscesses may be compromised, because of the fact that these wounds are often occupied with a large number of indigenous microbiota. Such cultures are indicated only if there are clear signs of infection or if a wound is failing to heal. Proper preparation of the wound prior to specimen collection can minimize contamination. After appropriate debridement and cleansing of the wound, the specimen should be obtained by biopsy from the leading edge of the lesion, where pathogens should be present and colonizing organisms are less likely to occur. Bacterial cultures of purulent material obtained by needle and syringe aspiration can also provide meaningful results. If an aspirate or tissue sample cannot be obtained, swab collections of exudate from the deep portion of lesions can be submitted. Swabs are the least appropriate specimen for microbiology analysis, because the organisms isolated may only be colonizing the area and not involved in the infective process. Tissues and aspirates are acceptable for anaerobic culture, as anaerobes can account for 38 to 48% of the total number of microbial isolates in wound specimens.

2. Specimen Collection Procedure for Aerobic Bacteria

1. Cleanse and debride the wound before obtaining specimen (s) for culture
2. Obtain an appropriate specimen for culture from infected wounds
3. Obtain a tissue specimen for culture by scraping with a sterile scalpel or dermal curette (curettage) or biopsy from the base of a debrided ulcer

4. Aspirate any purulent secretions using a sterile needle and syringe
5. Promptly send specimens, in a sterile container or appropriate transport media, for aerobic and anaerobic culture (and Gram stain)

Do not

1. Do not culture clinically uninfected lesion, unless for specific epidemiological purposes
2. Do not obtain specimen for culture without first cleansing or debriding the wound
3. Do not obtain specimen for culture by swabbing the wound or wound drainage

2.1. Specimen processing for culturing

A. Swabs

A**.** Place swab in 1 to 2 ml of broth and vortex.

B**.** Squeeze the swab against the side of the broth tube to express remaining fluid and then discard.

C. Inoculate aerobic plates and prepare smear for Gram stain as described for aspirates and pus.

D. Alternatively, the swab can be used for direct specimen plating. Always inoculate media from the least inhibitory to the most inhibitory.

E. Save broth in the refrigerator for up to 7 days for further testing, if indicated.

B. Aspirates and pus

A. Mix the specimen thoroughly.

B. If sufficient specimen is, collected; inoculate invasively collected aspirates to broth culture medium to make a 1:10 dilution. If the volume is small, omit broth culture.

C. Prepare smear for Gram stain by placing a drop of specimen on a slide and spreading it to make a thin preparation. If the aspirate fluid is clear, use the cytocentrifuge to concentrate the specimen for the smear.

D. If sufficient specimen is available, save a portion in the refrigerator for up to 7 days for further testing, if indicated.

2.2. Inoculation Technique of aerobic

1. Label plates with at least the identifying number and date of culture. If convenient, label at least one plate with the anatomic site and patient name.
2. Generally inoculate onto plate by touching specimen to one quadrant with a swab, pipette, or sterile forceps containing the specimen.
A. Sterilize the inoculating loop in the micro-incinerator for 5 to 10 s. Allow cooling. Alternatively, use separate, sterile disposable loops or sticks.
B. Streak with gentle pressure onto one-fourth to one-third of the culture plate using the sterile loop, or stick, with a back-and-forth motion several times and without entering the area that was previously streaked. Avoid touching the sides of the petri dish.
C. Turn the plate a quarter turn. Pass the loop through the edge of the first quadrant approximately four times, while streaking into the second quadrant. Continue streaking in the second quadrant without going back to the first quadrant.
D. Rotate the plate another quarter turn and repeat the above procedure until one or two additional quadrants are streaked.

2.3. Incubation conditions of aerobic

1. Incubate BAP, CNA or PEA, and CHOC in humidified incubator at 35 to 37^{o}c with 5% CO2. Incubate for a minimum of 48 h for open wound cultures and for 3 to 4 days for invasively collected specimens with no initial growth. Incubation may be extended to 7 to 14 days for invasive specimens (i.e., aspirated fluids and tissues) that remain culture negative after 3 to 4 days of aerobic incubation depending on the specimen source, organism of concern, or patient's clinical history.
2. Incubate MAC or EMB plates in ambient air at 35 to 37^{o}c, unless it is inconvenient to keep them separate from the rest of the culture in 5% CO2.
3. Perform a Gram stain on all specimens and use in the evaluation of culture.

3. Biochemical Tests

Biochemical tests are used to differentiate different organisms based on their genus and species characteristics. Biochemical tests are performed on pure culture. The following are some of the common biochemical tests used for differentiation of different bacteria.

3.1. Catalase test

This test is used to differentiate those bacteria that produce the enzyme catalase such as staphylococci from non-catalase producing bacteria such as streptococci.

Principle**:** Catalase acts as a catalyst in the breakdown of hydrogen peroxide to oxygen and water. An organism is tested for catalase production by bringing it in to contact with hydrogen peroxide. Bubbles of oxygen are released if the organism is a catalase producer. The culture should not be more that 24 hour.

Procedure

- Pour 2-3 ml of the hydrogen peroxide solution into a test tube.
- Using a sterile wooden stick or a glass rod, remove several colonies of the test organism and immerse in the 10% hydrogen peroxide solution.
- Look for immediate bubbling. Care must be taken when testing an organism cultured on a medium containing blood because catalase is present in red cells. If any of the blood agar is removed with the organism, a false positive reaction may occur.
  Result: Active bubbling indicate positive test and release of bubbles indicate Negative test.

3.2. Coagulase Test

This test is used to differentiate *Staphylococcus aureus,* which produces the enzyme coagulase, from *S.epidermidis* and *S.saprophyticus,* which do not produce coagulase.

Principle: Coagulase causes plasma to clot by converting fibrinogen to fibrin.

Material Required EDTA anticogulated human plasma. The plasma should be allowed to warm to room temperature before being used. Oxalate or heparin plasma can also be used. Do not use citrated plasma because citrate-utilizing bacteria e.g. Enterococci, *Pseudomonas* and *Serratia* may cause clotting of the plasma (in tube test). The plasma can be stored frozen in amounts ready for use.

Procedure:

- Place a drop of distilled water on each end of a slide or on two separate slides
- Emulsify a colony of the test organism (previously checked by Gram staining) in each of the drops to make two thick suspensions. Note: Colonies from a Manitol salt agar culture are not suitable for coagulase testing. The organism must first be cultured on nutrient agar or blood agar.
- Add a loopful (not more) of plasma to one of the suspensions, and mix gently.
- Look for clumping of the organisms within 10 seconds.
- Note: plasma is added to the second suspension. This is used to differentiate any granular appearance of the organism from true coagulase clumping

Results: Clumping within 10 seconds indicates *S. aureus* and no clumping within 10 second indicate negative for coagulase production.

Control

- Positive coagulase control ……..*Staphylococcus aureus*
- Negative coagulase control …… ……*E.coli* or *Staph epidermides*

3.3. Oxidase test/Cytochrome oxidase test

The oxidase test is used to detect bacteria that produce the enzyme cytochrome oxidase which catalyze oxidation of reduced cytochrome by oxygen molecule. It assist in the identification of *Pseudomonas, Neisseria, Vibrio, Brucella, and pasteurella species,* which are oxidase positive.

Principle: A piece of filer paper is soaked with a few drops of oxidase reagent. A colony of the test organism is then smeared on the filter paper.

Material Required Fresh Oxidase reagent (Tetramethyle-p-phenylenediamine dihydrochloride, 1%). Filter paper or oxidase regent strip

Procedure:

- Place a piece of filter paper in a clean petri dish
- add 2 or 3 drops of freshly prepared oxidase reagent,
- Using a piece of stick or glass rod (not an oxidized wire loop), remove a colony of the test organism and smear it on the filter paper.
- Look for the development of a blue-purple color within a few seconds.

Result: Blue – purple color shows positive oxidase test (Within 10 seconds) and No blue – Purple color shows negative oxidase test (Within 10 seconds). Note: Ignore any blue – purple color that develops after 10 seconds.

3.4. Urease test

This test is used to detect the enzyme urease, which breaks down urea into ammonia. Testing for urease enzyme activity is important in differentiating enterobacteria. Proteus strains are strong urease producers. Y. enterocolitica also shows urease activity (weakly at 35-37^{o}C).

Principle: The test organism is cultured in a medium that contains urea and the indicator phenol red. When the strain is urease producing, the enzyme will break down the urea (by hydrolysis) to give ammonia and carbon dioxide. With the release of ammonia, the medium becomes alkaline as shown by a change in colour of the indicator to pink-red. Two Ways of performing a urease test are using modified Christensen's urea broth and using a Rosco urease identification tablet.

Method

A. Urease test using Christensen's (modified) urea broth

- Inoculate heavily the test organism in a bijou bottle containing 3 ml sterile Christensen's modified urea broth
- Incubate at 35-37^{o}C for 3-12 h (preferably in a water bath for a quicker result).
- Look for a pink colour in the medium.

Results: Pink color shows positive urease test and no pink color negative urease test.

B. Urease test using a Rosco urease tablet

- Urease identification tablets are available commercially
- Prepare a dense 'milky' suspension of the test organism in 0.25 ml physiological saline in a small tube.
- Add a urease tablet, close the tube and incubate at 35-37^{o}C (preferably in a water bath for a quicker result) for up to 4 hours or overnight.
- Proteus and M. morganii organism give a positive reaction within 4 hours.

Results: Red/purple colour shows positive urease test and yellow/orange shows negative urease test

Control: Positive urease controi by *Proteus spp* and negative urease control by Salmonellae

3.5. Indole Test

The test detects the ability of an organism to produce indole from Tryptophan present in the medium. Testing for indole production is important in the identification of enterobacteria. Most strains of *E. coli, P. vulgaris, P. rettgeri, M. morganii, and Providencia species* are indole positive organisms.

Principle: The test organism is cultured in a medium, which contains tryptophan. Indole production is detected by Kovac's or Ehrlich's reagent which contains 4(p)-dimethylamino-benzaldehyde. This reacts with the indole to produce a red colored compound. Kovac's reagent is recommended in preference to Ehrlich's reagent for the detection of indole from enterobacteria.

Material required Kovac's or Ehrlich's reagent, bijou bottle/test tube

Method: An indole test can be performed:

- As a single test using tryptone water and kovac's reagent.
- As a combined beta-glucuronidase-indole test using a Rosco PGUA/indole identification tablet and kovac's reagent. This is useful when identifying E. coli.
- As a combined lysine decarboxylase-indole test using a Rosco LDC/indole identification tablet. This is useful in helping to identify salmonellae and shigella

Indole test using tryptone water and kovac's reagent.

- Inoculate the test organism in a bijou bottle containing 3 ml of sterile tryptone water.
- Incubate at 35 – 37 ^{o}C for up to 48 hours
- Test for indole by adding 0.5ml of Kovac's reagent.
- Shake gently.
- Examine for a red color in the surface layer within 10 minutes.

Results: Red surface layer shows positive indole test and no re surface layer shows negative indole test

Control**:** Positive control is *Escherichia coli* and negative control is *Klebsiella pneumoniae*

3.6. Citrate utilization test

The test detects the ability of an organism to use citrate as its only source of carbon. This test is one of several techniques used occasionally to assist in the identification of enteric bacteria.

Principle: Some bacteria can obtain energy in a manner other than by the fermentation of carbohydrate by using citrate as source of carbon. The utilization of citrate by a test bacterium is detected in citrate medium by the production of alkaline by-products. The medium includes sodium citrate as the sole source of carbon and ammonium phosphate as the sole source of nitrogen.

Material required Simmon's citrate medium/agar, Inoculating loop

Method:

- Prepare slopes of the medium in bijou bottles as recommended by the manufacturer (store at 2-8 ^{o}C)
- Using a sterile straight wire, first streak the slope with a saline suspension of the test organism and then stab the butt.
- Incubate at 35^{o} C for 48 hours
- Look for a bright blue colour in the medium

Results: Bright blue color shows positive citrate test and no change in colour of medium shows negative citrate test

Controls: Positive control with *Klebsiella pneumoniae* and negative control with *Escherichia coli*

3.7. Bile solubility test

This helps to differentiate *S.pneumoniae*, which is soluble in bile and bile salts, from other alpha-haemolytic streptococci (viridans streptococci) which are insoluble.

Principle: A heavy inoculum of the test organism is emulsified in physiological saline and the bile salt sodium deoxycholate is added. This dissolves S. pneumoniae as shown by a clearing of the turbidity within 10-15 minutes. Viridans and other streptococci are not dissolved and therefore there is no clearing of the turbidity.

Material Required: Sodium deoxycholate, 100 g/l (10% w/v), Physiological saline (sodium chloride, 8.5 g/l).

Method

- Although the bile solubility test can be performed by testing colonies directly on a culture plate or on a slide, a tube technique is recommended because the results are easier to read.

Tube method:

- Emulsify several colonies of the test organism in a tube containing 2 ml sterile physiological saline, to give a turbid suspension.
- Divide the organism suspension between two tubes
- To one tube, add 2 drops of the sodium deoxycholate reagent and mix
- To the other tube (negative control), add 2 drops of sterile distilled water and mix
- Leave both tubes for 10-15 minutes at 35-37^{o}C.
- Look for a clearing of turbidity in the tube containing the sodium deoxycholate.

Results**:** Clearing of turbidity shows *S. pneumoniae,* no clearing of turbidity shows probable not *S. pneumoniae*. There should be no clearing of turbidity in the negative control tube to which distilled water was added.

Controls: Bile solubility positive control is Streptococcus pneumonia and bile solubility negative control is Enterococcus faecalis

3.8. DNase test

This test is used to identify S. aureus, which produces Deoxyribonuclease (DNase) enzymes. The DNase test is particularly useful when plasma is not available to perform a coagulase test are difficult to interpret.

Principle: Deoxyribonuclease hydrolyzes deoxyribonucleic acid (DNA). The test organism is cultured on a medium, which contains DNA. After overnight incubation, the colonies are tested for DNase production by flooding the plate with a weak hydrochloric acid solution. The acid precipitates unhydrolyzed DNA. DNase-producing colonies are therefore surrounded by clear areas due to DNA hydrolysis.

Material Required DNase agar plate, Hydrochloric acid, 1 mol/L (1 N)

Method:

- Divide a DNase plate into the required number of strips by marking the underside of the plate.
- Using a sterile loop or swab, spot-inoculate the test and control organisms.

- Make sure each test area is labeled clearly.
- Incubate the plate at 35-37^{o} C overnight.
- Cover the surface of the plate with 1 mol/L hydrochloric acid solution.
- Tip off the excess acid.
- Look for clearing around the colonies within 5 minutes of adding the acid.

Results: Clearing around the colonies shows DNase positive strain and no clearing around the colonies shows DNase negative strain

Note: Some methicillin resistant S.aureus (MRSA) strains give a negative DNase test. Some coagulase negative staphylococci are weakly positive. Rarely, *S. pyogenes*, *Moraxella* and *Serratia species* may give a positive DNase test.

Controls: Controls positive DNase control is Staphylococcus aureus and negative DNase control is *Staphylococcus epidermidis.*

3.9. Esculin hydrolysis test

This test can be economically performed using a Rosco bile esculin tablet The test can be performed by placing a tablet on a blood agar plate inoculated with the test organism and incubating it at 35-37^{0}C overnight. A positive test is indicated by the tablet and colonies around it turning black/grey. A negative test is shown by the tablet remaining white and no change in colour of the colonies.

A Zone of inhibition may appear around the tablet. Alternatively, the test can be performed by making a dense suspension of the test organism in 0.25 ml of physiological saline in a small tube, adding a tablet, and incubating at 35-37^{o}C for 4 hours (or overnight). A positive reaction is shown by a black/grey colour in the medium. ***Note:*** An esculin hydrolysis can also be performed by incubating the test organism on bile esculin agar but this medium is expensive and only available in 500 g pack size.

3.11. Triple sugar Iron (TSI) & Hydrogen sulfide production (H_2S)

Looks at fermentation of glucose, lactose, and sucrose and checks if hydrogen sulfide and gas is produced in the process. Basically a pH indicator will change the color of the media in response to fermentation. The color change that occurs in the tube will indicate what sugar or sugars were fermented. The presence of a black color indicates that H_2S was produced. In this media, H_2S reacts with the ferrous sulfate in the media to make ferrous sulfide, which is

black in colour. To inoculate, use a needle to stab agar and then use a loop to streak the top slanted region. In addition to TSI media, KIA media can be used to determine if H_2S production.

Result:

- Slant color red ……..…….. does not ferment either lactose or sucrose
- Slant color yellow……..…Ferments lactose and/or sucrose
- Butt color red…….no fermentation of glucose
- Butt color yellow…….some fermentation of glucose has occurred and acid has been produced
- Cracks seen in the agar……Gas formed (bubbles occurred, or the entire slant pushed out of the tube).
- Blackening in the Butt…. ……..…..H_2S has been produced

Figure 9: TSI test

From left to right:

A. Uninoculated control

B. Red slant and red butt, no black color= no fermentation of glucose, sucrose or lactose. No Hydrogen sulfide produced

C. Red slant and black butt= no lactose or sucrose fermentation, H_2S has been produced

D. Red slant with yellow butt= no lactose or sucrose fermentation, Glucose is fermented, no H_2S has been produced

E. Yellow slant, yellow butt and black coloration= Lactose, sucrose and glucose fermented, and H_2S has been produced

F. Yellow slant, yellow butt and lifting and/or cracking of media, no black coloration= Lactose, sucrose and glucose fermented, H_2S has not been produced but gas has been produced

G. Yellow slant, yellow butt and no lifting and/or cracking of media, no black coloration= Lactose, sucrose and glucose fermented, H2S has not been produced nor has gas been produced

3.12. Sulfur-Indole-Motility (SIM) test

This is a differential medium. It tests the ability of an organism to do several things: reduce sulfur, produce indole and swim through the agar (be motile). SIM is commonly used to differentiate members of *Enterobacteriaceae*.

Sulfur can be reduced to H_2S (hydrogen sulfide) either by catabolism of the amino acid cysteine by the enzyme cysteine desulfurase or by reduction of thiosulfate in anaerobic respiration. If hydrogen sulfide is produced, a black color forms in the medium. *Proteus mirabilis* is positive for H_2S production. Bacteria that have the enzyme tryptophanase, can convert the amino acid, tryptophane to indole. Indole reacts with added Kovac's reagent to form rosindole dye which is red in color (indole +). *Escherichia coli* is indole positive. SIM tubes are inoculated with a single stab to the bottom of the tube. If an organism is motile than the growth will radiate from the stab mark and make the entire tube appear turbid. *Pseudomonas aeruginosa* and the strain of *Proteus mirabilis* are motile.

4. Antimicrobial Susceptibility Testing (AST) Procedures

4.1. General principles

A standardized inoculum of bacteria is swabbed onto the surface of a Mueller-Hinton agar (MHA) plate. Filter paper disks impregnated with antimicrobial agents are placed on the agar. After overnight incubation, the diameter of the zone of inhibition is measured around each disk. By referring to the tables in the CLSI standard, a qualitative report of susceptible, intermediate, or resistant is obtained.

4.2. Materials

4.3. Media and reagents

1. Agar plates (150 mm; depth, approximately 4 mm) , Store at 2 to 8^{o}c.
    A. Mueller-Hinton agar (MHA)
    B. MHA with 5% sheep blood (BMH)
3. Antimicrobial disks, Store with desiccant at 8^{o}c.

4.4. Supplies

1. Sterile cotton-tipped swabs
2. Sterile plastic pipettes
3. McFarland 0.5 turbidity standard

4.5. Equipment

1. Forceps
2. Ruler, template, or sliding caliper
3. Movable light source
4. Black nonreflecting surface (e.g., sheet of black paper or black counter surface)
5. Vortex mixer
6. 35^{o}C ambient-air incubator; CO2 incubation for some organisms.
7. Multidisk disk dispensing apparatus and container (optional)

4.6. Procedure of disk diffusion sensitivity testing

The validity of this technique depends on use of reliable Muller Hinton agar, discs of correct antimicrobial content and turbidity standard equivalent to McFarland's.

A. Bring agar plates and canisters of disks to room temperature before use.

1. Using a sterile wire loop, touch 3 – 5 well – isolated colonies of similar appearance to the test organism and emulsify in 3 – 4 ml to sterile physiological saline or nutrient broth.
2. In a good light, match the turbidity of the suspension to the turbidity standard (McFarland 0.5 turbidity). Mix the standard before use.
3. Using a sterile swab, inoculate a plate of Muller Hinton agar. Remove excess fluid by pressing and rotating the swab against the side of the tube above the level of the suspension.
4. Streak the swab evenly over the surface of the medium in three directions rotating the plate approximately 60^{o} to ensure even distribution.
5. With the Petri dish lid in place, allow 3 – 5 minutes for the surface of the agar to dry.
6. Using sterile forceps or multi disc dispenser, place the appropriate antimicrobial discs evenly distributed on the inoculated plate.

NB. The discs should be about 15mm from the edge of the plate and no closer than 25mm from disc to disc. No more than 12 disks on a 150-mm plate and no more than 5 disks on a 100-mm plate. Each disc should be lightly pressed down to ensure its contact with the agar. It should not be moved once in place.

7. Within 30 minutes of applying the discs, invert the plate and incubate it aerobically at 35^{o}C for 24 hours.
8. After overnight incubation, examine the control and the test plates. Using a ruler measure the diameter of each zone of inhibition in mm on the underside of the plate.

4.7. Interpretation of Zone Size

Using the interpretative chart of CLSI, interpret the zones sizes of each antimicrobial and report the organisms as 'Resistant', Intermediate (moderately sensitive) or 'Sensitive' (susceptible).

4.8. Reading of Unclear Result

1. Some Proteus strains make swarming growth in the area of inhibition zone. There is major inhibition zone and a thin growth of bacterial in the inhibited zone. in this case, ignore the thin growth and measure the size of the major inhibition zone.
2. Colonies in the inhibition zone: This is seen either in mixed culture or due to presence of few resistant strains of the test organism. In this case, repeat the test.
3. Over lapping of inhibition zone: This may be due the error during the placement of the discs. Repeat the test and place the disc properly.

4.9. Factors Influencing Zone Size in Disc Diffusion Test

1. Inoculum density (bacterial density): If the inoculum is heavy – the zone size will be falsely, reduced so sensitive strains will be falsely reported as resistance. If the inoculum is light – the zone size will become falsely large and resistant species are reported as falsely sensitive. Therefore, the turbidity of the inoculum should be exactly equal to the McFarland's standard.
2. Time of disc application: The discs should be placed 3 – 5 minutes after inoculating the media. In case of delay (>3 – 5') the result will be reduced zone size. So sensitive species are reported as resistant.

3. Temperature: At higher temperature, the bacterial growth is retarded and the whole media will appear as falsely sensitive. Moreover, if the temperature is low the bacterial growth is inhibited and the result is falsely increased zone of inhibition.

3. Depth of the agar: When the depth of the agar is, less than 4 mm there will be large zone of inhibition and when the media is thick or greater than 4mm then there will be reduced zone of inhibition.
4. Potency of the disk: - is the strength of the antimicrobial agent in the disc. If the potency is deteriorated due to different factors then there will be smaller zone of inhibition, which leads to falsely resistant result.

Double disks (Cefotaxime 30µg, Cefotaxime-Clavulanic acid 30/10µg) and (Ceftazidime 30µg, Ceftazidime-Clavulanic acid 30/10µg) will be used for phenotypically screening of ESBL-producers among pathogenic gram negative bacterial isolates

ESBL test result interpretation: If the test isolates ESBL-producer, there will be $\geq$5 mm increase in a zone diameter for either antimicrobial agent tested in combination with clavulanate versus the zone diameter of the agent when tested alone. (For example, if CAZ zone=16; Ceftazidime-Clavulanic zone=21, the test isolate is ESBL-producer).

In addition, further screening for phenotypically carbapenemase-producers among pathogenic gram-negative bacterial isolates will be using modified Carbapenem inactivation method based guidelines of CLSI, 2020.

Procedure of modified Carbapenem (Meropenem) inactivation method

1. For each isolate to be tested, emulsify a 1-µl of loopful of bacteria for enterobacterales from an overnight blood agar plate in 2ml tryptone soya broth (TSB)
2. Vortex for 10-15 seconds
3. Add a 10-µg meropenem disk to each tube using sterile forceps or single disk dispenser. Ensure the entire disk is immersed in the suspension.
4. Incubate at $35^{o}C \pm 2^{o}C$ in ambient air for 4 hours $\pm$15 minutes.
5. Just before or immediately following completion of the TSB-meropenem disk suspension incubation, prepare a 0.5 McFarland suspension (using the colony suspension method) of *E.coli ATCC 25922* in nutrient broth or saline.

6. Inoculate an MHA plate with *E.coli ATCC 25922* as for the routine disk diffusion procedure and make sure the inoculum suspension preparation and MHA plate inoculation steps are each completed with within 15 minutes. Allow the plate to dry for 3-10 minutes before adding the meropenem disks.
7. Remove the meropenem disk from each TSB-meropenem disk suspension using a 10-µl loop by placing the flat side of the loop against the flat edge of the disk and using surface tension to pull the disk out of the liquid. Carefully drag and press the loop along the inside edge of the tube to expel excess liquid from the disk. Continue using the loop to remove the disk from the tube and the then place it on the MHA plate previously inoculated with the meropenem susceptible *E.coli ATCC 25922* indicator strain. Disk capacity: four disks on a 100 mm MHA plate; eight disks on 150mm MHA plate.
8. Invert and incubate the MHA plates at $35^{o}C \pm 2^{o}C$ in ambient air for 18-24 hours.
9. Finally, following incubation measure the zone of inhibition as for the routine disk diffusion method.

Test result interpretation of modified Carbapenem inactivation method

C. If the test isolate is Carbapenemase positive, zone diameter will be 6-15 mm, means that meropenem susceptible *E.coli ATCC 25922* will grow around the disk, or presence of pinpoint colonies within 16-18 mm zone.
D. If the test isolate is Carbapenemase negative, clear zone diameter will be $\geq$ 19 mm, means that meropenem susceptible *E.coli ATCC 25922* will not grow around the disk.

4.9. Quality Control strains

1. *Escherichia coli ATCC® 25922 (*for Enterobacteriaceae)
2. *Staphylococcus aureus ATCC® 25923 (*for Gram-positive bacteria*)*
3. *Enterococcus faecalis ATCC® 29212(*for *Enterococcus spp)*
3. *Pseudomonas aeruginosa ATCC® 27853(* for *Pseudomonas spp)*
4. *K. pneumoniae ATCC®1705,* and *K. pneumoniae ATCC®1706*
4. *Escherichia coli ATCC® 35218* (for beta-lactam–beta-lactamase inhibitor combinations)

Annex-v: Declaration

I, the undersigned, declare that this Master of Science degree thesis is my original work, has not been presented for a degree in this or any other University and that all sources of materials used for the thesis have been duly acknowledged.

Name of PI: Asegdew Atlaw Woldeteklie (BSc, MSc. Candidate)

Signature_____ date of submission:_____

Advisor:

Dr. Yimtubezinash Woldeamanuel (MD, M.Sc, PhD, Associate Professor, Addis Ababa University)

Signature_____ date_____

Co-advisor

Habtamu Biazin Kebede (PhD student, Lecturer: Addis Ababa University)

Signature_____ date_____