Isolation and identification of bacteria associated with diabetic foot infections

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DEDICATION

To the soul of my father, to my loving and supportive mother, wonderful brothers, unique sweet daughter, and all my teachers throughout my life.

With respect
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First and foremost, I would like to thank my Merciful Allah for giving me strength and health to do this work.

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I Would Like To Thank them All
Abstract

Bacteriological investigations were carried out for forty patients presented with diabetic foot wounds, during Jan. 2004 – Jan. 2005 at the Police University Teaching Hospital, Khartoum Teaching Hospital and at the Health & Medical services centre of the University of Khartoum. Swab samples were collected from thirty patients with diabetic foot wounds before and after surgical debridements. In addition 10 tissue samples were excised from infected amputated parts following surgery.

A total of 71 clinical bacterial isolates were identified to the species level on the basis of microscopical examination, Gram-reaction, cultural characteristics and biochemical tests.

These isolates represented nine genera including Gram-positive bacteria (42.25%) and Gram-negative bacteria (57.75). Poly-microbial infection was obtained in 60% of diabetic foot wounds (24 patients) and only 40% of diabetic wounds had mono-microbial aetiology (16 patients).

Mixed infection contained 2 to 6 isolates. However, the spectrum of microorganism isolated from superficial and deep tissue swab cultures were identical. In this investigation surface wound swabbing and deep tissue swabbing were found reliable procedure for the collection of sample for bacteriological studies from diabetic foot wound. Staphylococcus species were isolated at a rate of 28.17% and it represented the most common cause of diabetic food infection in this study. S. delephini a coagulase positive Staphylococci were isolated for the first time from diabetic wound (8.45%). Streptococci species were isolated at a frequency of (5.63%) and Coryne bacterium species at a rate of 8.45%.
The Gram-negative isolated included *E. coli* (12.67%), *Serratia marcescens* (7.04%), *Serratia plymuthica* (1.41%), *Klebsiella pneumoniae* (2.82%), *Proteus vulgaris* biogroup 3(1.40%) and *Proteus mirabilis* (9.86%). *Serratia marcescens* and *Serratia plymuthca* were reported for the first time to be involved in diabetic foot infection. *Serratia marcescens* was found to tolerate 4% glucose. Other Gram-negative bacteria include Vibrio species (8.45%), Aeromonas species (4.23%) and *Shewanella putrefaciens* (2.8%).

All our 71 isolates that include nine genera were resistant to penicillin, 83% were resistant to chloramphenicol and only 42.3% of the isolates were found sensitive to gentamycin. About 50% of *E. coli* and Staphylococci isolates were found resistant to gentamycin. All Streptococci isolate were resistant to various antibiotics used.
ملخص الأطروحة


جمعت العينات بأخذ مسحات من الجروح من 30 مريضا بالسكري وذلك قبل وبعد نظافة الجرح جراحياً. بالإضافة إلى 10 عينات من الأدغمة الحية. وقد تم عزل 71 عينة تنتتمي إلى تسع أنواع على أساس نتائج الفحوصات المخبرية والتفاعلات الصبغية والخصائص الإستنباتية والإختبارات الكيميائية الحيوية، حيث استُمِلت هذه العينات على البكتريا الموجبة الصبغة للجرام بنسبة (42.25%) والبكتريا سالبة الصبغة للجرام بنسبة (57.75%). وقد وجد أن 24 مريضاً بالسكري بنسبة (60%) تحتوي جروحهم على أكثر من عبتة واحدة و 16 مريضاً بالسكري بنسبة (40%) تحتوي جروحهم على عبتة واحدة فقط وفي كلا الحالتين وجد أن العبتات المعزولة إما من الجروح السطحية أو الأدغمة الحية المتشابهة.

في هذه الدراسة وُجد أن البكتريا موجبة الصبغة للجرام إشتملت على المكورات السيحية بنسبة (5.63%) والوديانية بنسبة (8.45%) والبكتريا العنقودية والأخيرة شكلت أكثر مسبب لالتهابات الجروح لمرضى السكري تواجداً بنسبة (28.17%) من المجموع الكلي للبكتريا الموجبة الصبغة للجرام. المكورات الدوليفية الموجبة تجعل البلازما ما عزلت للمرة الأولى من جروح السكري بنسبة (8.45%) في البكتريا سالبة الصبغة للجرام إشتملت على السريتيا البلازمية (12.67%) والسريتيا مار سينس (7.40%) والسريتيا بلمسيكا (1.41%) والكلبسيلا نيوموني (2.82%) وتوائف بنوعها بنسبة (4.14%). وقد تم عزل السريتيا مار سينس والسريتيا بلاميسكا للمرة الأولى من جروح السكري في السودان.

أما في البكتريا سالبة الصبغة للجرام فقد استُمِلت على جنس الفبريا بنسبة (8.45%) وجنين الأوروموناس بنسبة (4.23%) وأخيراً جنس الشوينيلا بنسبة (2.8%).

وقد وجد أن جميع 71 عبتة سجلت نسبة عالية من المقاومة ضد عقار البنسلين ونسبة 83% ضد عقار الكلورامفينيكول وأما نسبة 42.3% من مجموع العبتات لها حساسية ضد عقار الجنتاميسين ولكن السبحات سجلت نسبة عالية من المقاومة لكل مضادات الحيوية المستعملة في هذه الأطروحة.
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Introduction

Diabetes is considered as a major health problem facing the world as life threatening disease. It is a clinical metabolic syndrome characterized by hyperglycemia due to deficiency or diminished effectiveness of insulin (Kumar, 1994). The change leads in turn to the so-called complications of diabetes, frequently affecting the eye, kidney, nervous system and blood vessels (Frier et al., 1999).

Patients with diabetes are more likely than others to have problem with their feet. These problems can lead to dangerous infections of the foot. Recognizing and treating foot problems early can help diabetic patients to avoid serious complications. Diabetes affect the feet in two ways: It cause nerve damage and it can also cause hardening of blood vessels. Because of the abnormal increase of blood sugar in the blood, nerves of the body can be damaged. This is known as diabetic neuropathy. Diabetes also affects the blood vessels of the feet, causing them to narrow and this is known as diabetic vasculopathy. With less blood, the foot will not have enough oxygen and nutrients to heal and fight infections, causing the foot to become flatter. This creates new pressure areas that cause the skin to break down. When both the nerve and arteries are damaged, the diabetic patient might not notice simple wounds, which soon become seriously infected that can threaten the whole foot and even the leg. If not stopped, the infection can threaten the whole body. Redness, swelling and increased temperature of the foot are signs of infection.

Ischaemia, infection and neuropathy combine to produce tissue necrosis and gangrene in the extremities in diabetic patients. Once tissue damage has occurred in the form of ulceration or gangrene, infection remain
the main threat that rapidly takes hold in the diabetic foot and involve the muscles and progress to the bones. Excision of infected bone is needed if osteomyelitis develop and does not respond to antibiotic therapy.

Diabetic foot problem is the major cause of hospital bed occupancy by diabetic patients than for all other aspect of the disease combined. However, whenever irreversible arterial insufficiency is present, then it is often quicker and kinder to opt for early major amputation rather than to subject the patient to debilitating sequence of conservative procedure that fulminate in septicaemia and death of the patient. Nonetheless amputation in diabetes could be delayed or prevented by effective patient education, good medical supervision, nursing and appropriate antibiotic therapy. However, the choices of the right antibiotic therapy rely on proper isolation and identification of the causative microorganism and the performance of antibiotic sensitivity testing before commencement of antibiotic therapy.

The objectives of this study are as follows:

I. To isolate and identify bacteria associated with diabetic foot infection to the species level.

II. To determine the susceptibility of the various bacterial isolates to selected antibiotics commonly used in therapy of bacterial infections.
CHAPTER ONE

LITERATURE REVIEW
Chapter One
Literature review

1.1 Introduction

Diabetes mellitus is a disease known since antiquity (Tan, 2001). It was recognized far back in the history of medicine, it was described in ancient Egypt as a disease with excessive passage of urine. The first account of sweet urine was reported in Indian literature 500 years AD (diabetes insipidus). It was reported as a less harmful form of the diabetes.

Diabetes mellitus generally called "diabetes", derived from the Greek word diabetes: "dia" = through "betes" = to go "to pass through" and the Latin word "mellitus" = honey. This refers to the excretion of excess sugar in the blood into the urine, which is one of the main symptoms of diabetes. Diabetes is not a single disease but the pathological and metabolic state caused by inadequate insulin action, a feature common to all types is glucose intolerance. It is defined clinically as either a fasting plasma glucose level greater than 7.8 mmol/L (140 mg/dl) or a two hours post praedial plasma glucose greater than 11 mmol/L (200 mg/dl) (Frier et al., 1999).

Insulin is a major anabolic hormone. It promotes the uptake of glucose by cells and the formation of intracellular glycogen from glucose. It stimulates cells to utilize amino acid for protein synthesis rather for glycogenesis and it promotes the uptake of free fatty acids by adipose tissue (Frier et al., 1999). Therefore, lack of insulin results in a general catabolic state with loss of weight, hyperglycaemia, diminished protein synthesis, increased gluconeogenesis and hyperlipidaemia due to lipolysis in adipose
tissue. Although the renal threshold is usually raised, there is heavy glycosuria which results in an osmotic diuresis causing dehydration and thirst. In the liver, excess free fatty acids are converted via acetyl-Co-A into ketone bodies, which in the absence of available glucose, are metabolized for cellular energy. The ketone bodies (Acetoacetic acid, β hydroxybutyric acid and acetone) dissociate to produce hydrogen ions, with a resulting metabolic ketoacidosis. This complex of metabolic disturbances produces hyperosmolarity, hypovolaemia, acidosis and electrolyte imbalance, which have serious effects on the functions of neurons and results in one form of diabetic coma, ketoacidotic coma. The other major form, hyperosmolar non-ketotic coma, results from massive dehydration and profound hyperglycaemia in absence of ketoacidosis. Pathophysiological basis of the symptoms of the disease are shown in (Fig. 1).

Diabetes is now considered as most common global metabolic disease affecting more than 150 million people worldwide (about 6% of world population). It is the seventh leading cause of death in the world in 1995. World Health organization “predicts a rise to an alarming 300 million by 2025” a situation that is increasingly out stretching the health care resources devoted to diabetes (WHO 1999).

1.2 Classification of diabetes

Appropriate definition and classification of diabetes mellitus is essential for treatment and for orderly epidemiological and clinical research. Over 99% of cases of diabetes are caused by two diseases. Type 1 and 2 diabetes; type 2 diabetes is ten times more common than type 1 (Foulis, 2001) The principal differences between the two are given in (table 1).
Table (1) Classification of Diabetes*

<table>
<thead>
<tr>
<th></th>
<th>Type I diabetes</th>
<th>Type II diabetes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Onset under 40 years</td>
<td>Onset over 40 years</td>
<td></td>
</tr>
<tr>
<td>Thin patient</td>
<td>Obese patient</td>
<td></td>
</tr>
<tr>
<td>Affect 1: 40 of population liable to ketoacidotic coma</td>
<td>Affect 1:400 of population liable to hyperosmolar non ketotic coma.</td>
<td></td>
</tr>
<tr>
<td>Require insulin for therapy</td>
<td>Does not require insulin for therapy.</td>
<td></td>
</tr>
<tr>
<td>Concordance rate for monozygotic twins 40%</td>
<td>Concordance rate for monoozygotic twins 100%</td>
<td></td>
</tr>
<tr>
<td>Genetic link with class II MHC Ag</td>
<td>No genetic link with MHC Ag</td>
<td></td>
</tr>
<tr>
<td>Islet cell Ab present</td>
<td>Islet cell Ab absent.</td>
<td></td>
</tr>
<tr>
<td>Insulitis present</td>
<td>Insulitis absent.</td>
<td></td>
</tr>
<tr>
<td>B cells destroyed in pancreas</td>
<td>B cells not destroyed in pancreas</td>
<td></td>
</tr>
<tr>
<td>Islet amyloid absent</td>
<td>Islet amyloid present</td>
<td></td>
</tr>
</tbody>
</table>

* (Foulis, 2001)
Specific disease in which diabetes occurs as secondary event include chronic pancreatitis, haemochromatosis, cystic fibrosis, acromegaly, Cushing syndrome and non-insulin secreting islet cell tumors. In haemochromatosis excess iron is taken up by beta cells but not by other islet endocrine cells, resulting in inhibiting insulin synthesis. Gestational diabetes develops only during pregnancy. It occurs among women with a family history of diabetes (Coustan, 1993).

1.2.1 Type I diabetes (Insulin dependent diabetes mellitus)

In this disease insulin secreting beta cells are selectively destroyed in the islets but A, D and PP cells are preserved. The process of β cells destruction appear to take many years and the patient present clinically with diabetes when about 80% of β cells are lost (Foulis, 2001). Islets in which there is active β cell destruction are inflamed. The infiltrate consists mainly of lymphocytes with few macrophages.

1.2.1.1 Aetiology and pathogenesis

There is evidence that genetic factors, autoimmunity and possibly viral infection may all be involved.

1.2.1.1.1 Genetic factors

There is a significant link between type 1 diabetes and class II MHC genes – DP, D Q and DR in man. Persons carrying the DR3 allele have a relative risk of 5 times of developing type I diabetes. The figure for DR4 is 7 times and that of DR3/ DR4 hetrozyote is 14 times. There is even stronger association with DQ genes. However, the concordance rate between identical twins is only 40%, indicating the involvement of non-genetic
factors in the pathogen of the disease (Beckman et al., 2002 and Foulis, 2001).

1.2.1.1.2 Immunological feature

There is evidence for both humoral (islet cell Abs) and CMI directed against β cells. At present at least 80% of patients have circulating cytoplasmic Islet cell Abs. Type 1 diabetes thus join the group of organ specific autoimmune disease. Indeed, 15% of patient with type 1 diabetes also develop other organ specific autoimmune diseases such as thyroditis, pernicious anaemia, and autoimmune Addison’s disease (Foulis, 2001).

1.2.1.1.3 Viral infection

Up to 30% of patients presented with type 1 diabetes have serological evidence of recent or continuing Coxsackie B virus infection. This virus is known to be torphic for the endocrine pancreas. Other viruses which have been implicated are the mumps and rubella virus. The evidence to date suggests that as yet unidentified environmental factors (possibly viruses) act at a particular genetic subpopulation to stimulate autoimmunly directed against β cell. One finding that may be of relevance to this process is the β cells in type I diabetes, but in no other pancreatic disease, expresses the protein products of class II MHC genes. Such class II MHC molecules are necessary for Ag presentations to helper T cells, the cells which initiate the immune response. Thus, the islet β cells in type I diabetes may become APC, presenting cell specific Ags to which there is no tolerance e.g. insulin and Glutamic acid decarboxylase (GAD) (Abbass and Lichtman, 2000 and Roitt et al., 2001).
1.2.2 Type II diabetes (Non-insulin dependent diabetes mellitus)

The pancreas at clinical presentation of this disease does not show the same dramatic loss of beta cells as seen in type 1 diabetes. However, in about 70% of cases amyloid is present within islets. The chemical nature of the amyloid protein has now been determined. It consists of 37- amino acid peptide known variously as islet amyloid polypeptide, amylin or diabetes associated peptide. There is strong circumstantial evidence that this protein is produced by beta cells in both normal and diabetic subjects (Foulis et al., 2001).

1.2.2.1 Aetiology and Pathogenesis

Patients with type II diabetes, obese people and 25% of the normal population show resistance to the action of insulin. These people thus have to hyper secreted insulin to achieve metabolic homeostasis. While in many normal people, this possibly genetic, disorder may cause no illness, it is proposed that in a minority there is eventual beta cell exhaustion with falling insulin secretion and hence the development of type II diabetes, which is more common than type one and more benign. About 90 to 95% of people with diabetes have this type (Foulis et al., 2001).

1.2.3 Gestational diabetes

It also known as type III diabetes, it develops only during pregnancy. Thus both the pre-existing (but undiagnosed) diabetes and diabetes which has been precipitated by pregnancy are included in this.

Gestational diabetes occurs among women with a family history of diabetes. Impaired glucose tolerance in pregnancy is classified as gestational diabetes mellitus. In the majority of groups, glucose tolerance
returns to normal post-partum although risk of permanent type 2 diabetes is increased. Women who have had gestational diabetes have a 20 to 50 percent chance of developing type 2 diabetes within 5 to 10 years (Coustan, 1993).

1.3 Diabetes mellitus in Sudan

Diabetes mellitus in Sudan, as in many developing countries, constitutes a growing health problem with a major impact. It can be estimated from hospital records that the number of diabetic patients is increasing in all socio-economic classes. However, until recently no population based epidemiological studies were conducted among the adult population of northern Sudan (El Bagir et al., 1995). Most of the knowledge about diabetes mellitus comes from a few hospital – based studies.

In Sudan it was found that about 6% of the total population have diabetes mellitus and that 7% -10% of the patients attended the diabetic clinic were of Insulin dependent diabetes mellitus. Non-Insulin dependent diabetes mellitus accounted for 75% of type II diabetic patients attending an out patient diabetes clinic in Khartoum (El Mahdi et al., 1991).

1.4 Complication of diabetes

The complications of diabetes mellitus are severe and require a long time to develop in uncontrolled cases. These complications can be readily divided into acute complications, chronic complication and the mortality and morbidity of diabetes are due to the following complications.
1.4.1 Acute Complications

1.4.1.1 Hypoglycaemia

Blood glucose levels of less than 2 mol/L are usually associated with symptoms of sweating, tremor, hunger, confusion and ultimately coma. The coma due to lack of diabetic control is now a relatively rare cause of death.

Hypoglycaemia is a common side effect of insulin, it results from an imbalance of carbohydrates intake against insulin supply and the body’s metabolism of glucose (Frier, 1999).

1.4.1.2 Ketoacidosis

Diabetic Ketoacidosis (DKA) is a metabolic acidosis which develops in diabetic patients as a result of raised levels of circulating ketone bodies. It has been defined as severe uncontrolled diabetes where the total blood ketone concentration is greater than 5 mol/L or pH is below 7.2 (Souhman and Moxhan, 1990).

1.4.1.3 Non-Ketoacidosis Hyperosmolar Coma

Non- Ketoacidosis occurs when uncontrolled hyperglycaemia leads to dehydration, increased osmolarity and ultimately to coma (Souhman and Moxham, 1990).

1.4.2 Chronic (long-term) complications

1.4.2.1 Micro vascular complications

Diabetes mellitus is a disorder that primarily affects the microvascular circulation. In the extremities, micro vascular disease due to “Sugar-coated capillaries” limits the blood supply to the superficial deep structures. Pressure due to ill–fitting shoes or trauma further compromises the local blood supply at the micro-vascular level, predisposing the patient
to infection. The infection may involve the skin and soft tissues (Logerfo and Coffman, 1984).

1.4.2.1.1 Cardio vascular complication

This customary to speak of as diabetic macro-angiopathy is most commonly affecting large muscular arteries, while diabetic microangiopathy is affecting arterioles and capillaries. The former is simply atheroma, which tend to develop early and become severe in diabetics of either sexes. Thus, in addition to the fact that 50% of patients with type 2- diabetes have hypertension, may results in 80% of adult diabetic deaths, due to cardiovascular, cerebrovascular and peripheral vascular disease. In diabetic patients with peripheral vascular disease, the small muscular arteries of the lower leg and foot are commonly affected (Beckman et al., 2002). Therefore, a toe may be gangrenous in the presence of normal femoral and popliteal pulses due to the fact that relatively small vessels are narrowed by atheroma. In diabetic microangiopathy two types of lesion have been described:

1- A thickening of the basement membrane like material in capillaries.
2- Endothelial cell proliferation together with basement membrane thickening.

The cause of the microangiopathy is uncertain but affects diabetics of all types, appears to be related to the duration of the disease and it is probably aggravated to poor diabetic control, responsible for diabetic retinopathy and diabetic nephropathy (Frier et al., 1999)
1.4.2.1.2 Diabetic retinopathy

Diabetes can affect the eye in a number of ways. Retinopathy was significantly higher in diabetics with longer duration (i.e. over 10 years). Diabetic retinopathy is the commonest cause of the blindness under the age of 65 in development countries. Worldwide prevalence of retinopathy varies with duration of diabetes. It was generally found to be 20-40% among diabetics (Frier et al., 1999).

1.4.2.1.3 Diabetic nephropathy

Diabetes leads to thickening of the basement member of the glomeruli. The effect of the sustained elevation in the blood’s glucose level has a catastrophic effect on the kidney to develop end stage renal failure within years following diagnosis (Grefell and Watkins, 1996).

1.4.2.1.4 Diabetic neuropathy

Every part of the peripheral nervous system is vulnerable to diabetic involvement. The clinical neuropathy is a comparatively late complications, as manifest by numbness, tingling and partial loss of sensation and leading as indicated early to ulceration especially in foot (Foulis, 2001).

1.4.2.1.5 Other pathogenic effects

Trophic disturbances, such as ulceration of the fingers or toes and neuropathic arthropathy, may develop as complication of diabetic peripheral neuropathy. It is noteworthy that atheroma, diabetic microangiopathy, peripheral neuropathy and susceptibility to infections all tend to promote gangrene of the extremities in diabetes (Beckman 2002 and Frier et al., 1999).
Pregnancy in diabetics used to be associated with high incidence of toxemia and congenital abnormalities in the fetus. Now with progressive diabetic management aimed at achieving as good metabolic control as possible, the risk to mother and child have been considerably reduced (Frier et al., 1999).

1.4.2.2 Macro-vascular complications

Such as hypertension due to the effect of diabetes on the micro-vascular system. Impaired micro-vascular circulation hinders white cells migration into the area of infection and limits the ability of antibiotics to reach the site of infection in an effective concentration. Diabetes also accelerates macrovascular disease, which is evident clinically as accelerating atherosclerosis peripheral vascular disease. Atherosclerosis is more common, occurs at an earlier age and appears to progress more rapidly in diabetes (Beckman et al., 2002 and Frier et al., 1999).

1.4.3 Infection

There is increased susceptibility to bacterial and fungal infections. Boils, carbuncles and urinary tract infections, sometime complicated by pylonephritis and renal papillary necrosis, are of frequent occurrence and may precipitate into diabetic risk of tuberculosis especially in the lung and in insulin untreated, the disease tend to progress rapidly.

Most infections are contaminated by the patient’s own endogenous flora, which are present on the skin and mucous membranes. The usual pathogens on skin and mucosal surfaces are Gram–positive cocci notably Staphylococci and Streptococci, Gram–negative bacilli e.g. *Escherichia coli* and other anaerobic organism as indicated in (table 2).
Table (2) Pathogens commonly associated with wound infections and their frequency of occurrence

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Frequency (%)*</th>
<th>Frequency (%)**</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>20</td>
<td>51.7</td>
</tr>
<tr>
<td>Co agulase – negative stapylococci</td>
<td>14</td>
<td>-</td>
</tr>
<tr>
<td>Enterococci</td>
<td>12</td>
<td>-</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>8</td>
<td>18.6</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>8</td>
<td>9.4</td>
</tr>
<tr>
<td>Enterobacter species</td>
<td>7</td>
<td>-</td>
</tr>
<tr>
<td><em>Proteus mirabilis</em></td>
<td>3</td>
<td>17.4</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>Other Streptococci</td>
<td>3</td>
<td>2.9</td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>Group D streptococci</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>Other Gram – positive aerobes</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td><em>Bacteroids fragilis</em></td>
<td>22</td>
<td>-</td>
</tr>
</tbody>
</table>

* (Ghandour, 2001)

** (El Shafi, 1992)
1.4.3.1 Diabetic foot infection

Patients with diabetes are more likely than others to have problems with their feet. These individuals are predisposed to foot infection because of a compromised vascular supply secondary to diabetes. Local trauma and/or pressure (often in association with lack of sensation because of neuropathy), in addition to microvascular disease, may result in a variety of diabetic foot infections (Frykberg, 1998). The spectrum of foot infection in diabetes ranges from simple superficial cellulites to chronic osteomyelitis. Infections in patients with diabetes are difficult to treat because these patients have impaired microvascular circulation, which limits the access of phagocytic cell to the infected area and results in a poor concentration of antibiotic in the infected tissues. For this reason, cellulite is the most easily treatable and reversible form of infections in patients with diabetes (Lipsky et al., 2005).

Mortality is not common, except in unusual circumstances. The mortality risk is highest in patients with chronic osteomyelitis and in those with acute necrotizing soft tissue infections.

Diabetes affects the feet in two ways. It can cause nerve damage and it can cause hardening of the blood vessels (Logerfo and Coffman, 1984). Because of the abnormal increase of blood sugar in the blood, nerves of the body can be damaged. This is known as “diabetic neuropathy”. Diabetic neuropathy oftenly affects the nerves that supply sensation to the feet. This decreases the feeling of pain in the feet. Because of this, a diabetic patient may not feel a small cut or a blister. Decreased blood flow in the legs (arterial insufficiency), and decreased sensation (neuropathy) in the feet due to destruction of sensory nerves, can lead to bone malformations (charcot
foot), non-healing wounds, gangrene, infection and amputation of the foot or leg of a diabetic patient. An untreated cut could become infected, leading to gangrene.

Gangrene is a serious infection, which if not treated, could require an amputation, or cutting off, of part of the foot or the leg to save the rest of the lower extremity. A feeling of numbness or tingling in the foot may mean that the nerves are damaged. A cold, blue, or pale foot may mean that the foot has poor circulation (Armstrong et al., 1998b).

1.4.3.2 Common bacteria associated with diabetic foot infections

1.4.3.2.1 Gram–positive bacteria

1.4.3.2.1.1 Staphylococci

The staphylococci are Gram–positive spherical cells, usually arranged in grape-like irregular clusters, although organisms may appear as single cells, pairs or short chains (Murray et al., 1998). Staphylococci are nonmotile and do not form spores. They grow readily on most bacteriological media under aerobic or micro-areophilic conditions. They grow most rapidly at 37°C but form pigment best at room temperature (20-25°C). The genus Staphylococcus has at least 30 species; some are members of the normal flora of the skin and mucous membranes of humans; while cause suppurations, abscess formation, a variety of pyogenic infections and even fatal septicemia.

1.4.3.2.1.1.1 Staphylococcus aureus

*Staphylococcus aureus* is non motile, non sporing, aerobic and facultative anaerobic bacteria. It is catalase positive, usually oxidase negative and attack sugars fermentatively (Barrow and Feltham, 1993). It is
able to grow in a medium containing salt such as manitol salt agar, which used as a selective medium for isolation of Staphylococci that ferment manitol and colonies appear yellow to cream 1.2 mm in diameter.

*Staphylococcus aureus* is present on the skin and mucous membranes of humans. It’s an important pathogen in humans where it can cause a wide spectrum of diseases. The pathogenic *Staphylococcus aureus* often hemolyse blood, coagulate plasma and produce a variety of extra-cellular enzymes and toxins. Coagulase is an extra-cellular protein, which binds to prothrombin to form a complex called staphylothrombin (Todar, 2002). The protease activity characteristic of thrombin inactivated in the complex, resulting in the conversion of the fibrinogen to fibrin.

Coagulase is a traditional marker for identifying *Staphylococcus aureus* in the clinical microbiology laboratory. However, there is no overwhelming evidence that coagulase is a virulence factor, although it is reasonable to speculate that the bacteria could protect themselves from phagocytes and immune defenses by causing localized clotting (Todar, 2002). Moreover *Staphylococcus aureus* induced growth and virulence in the pigmenting anaerobes by providing essential growth factors (Bowler, 1999).

### 1.4.3.2.1.2 Streptococci

They are Gram–positive cocci, usually non motile, smaller than staphylococci in diameter, aerobic and facultatively anaerobic, catalase – negative, oxidase negative, attack sugars fermentatively without gas production and some may produce haemolysins (Barrow and Feltham, 1993).
1.4.3.2.1.2.1 *Streptococcus pyogenes* (Lancefield Group A)

They are Gram positive, spherical or oval cells, arranged in chain of varying length, each cell is about 1 \( \mu \text{m} \) in diameter, non motile, non spore forming, facultatively anaerobic and \( \beta \) - haemolytic (Barrow and Feltham, 1993). Among the pyogenic streptococci, *Streptococcus pyogenes* remains the major pathogen of man. Presence of \( \beta \) - haemolytic colonies on blood agar is due to the oxygen stable ‘S’ lysine. Many isolates, however, are only weakly haemolytic and anaerobic incubation often increases the extent of haemolysis with such strains (Barrow and Feltham, 1993). *Streptococcus pyogenes* secretes different toxins and enzymes that are associated with its virulence. These include streptolysin O which is oxygen labile, hyaluranidase which increases tissue permeability and leucocidin which destroys leucocytes (Barrow and Feltham, 1993).

1.4.3.2.1.2.2 *Streptococcus viridans*

It differs from *Streptococcus pyogenes* in that it is not beta-haemolytic on blood agar. And also it can grow at 45°C but not grow at 10°C. Viridans streptococci are isolated more often than any other bacteria from bacterial endocarditis, causing between 50 and 70% of all cases. They are also frequently isolated from patients with bacteremia and sepsis (Cheesbrough, 2000).

1.4.3.2.1.3 *Corynebacterium* Species

Corynebacteria are Gram-positive rods, non motile, non sporing, non acid fast, catalase positive, usually oxidase negative, aerobic and facultatively anaerobic. They do not attack sugar or they do so
fermentatively (Barrow and Feltham, 1993). These pleomorphic bacilli are seen as palisades or Chinese character.

1.4.3.2.1.3.1 Corynebacterium diphtheriae

They are Gram-positive rods, toxin–producing and pathogenic cause of diabetic infections. It can affect skin leading to serious complications when C. diphtheriae infects open wounds. (Wheat et al., 1986).

1.4.3.2.2 Gram–negative bacteria

1.4.3.2.2.1 Pseudomonas species

They are Gram-negative rods measuring 0.6 x 2.0 µm, non motile, aerobic, catalase and oxidase positive. The bacteria are strict oxidizer and non fermentative. Fluorescent diffusible yellow-green pigments may be produced by some species (Barrow and Feltham, 1993). Pseudomonas species grow in a wide variety of laboratory media, and they can live in temperature from 0°C – 54°C. They can use a very wide range of nutrients found in the environment even fluids and water. Pseudomonas species are non lactose – fermenting with pale colonies on MacConkey agar.

Pseudomonas species are obligate aerobe that grows readily in many types of culture media, sometimes producing a sweat or grape–like odour. It grows well at 37-42°C; its growth at 42°C differentiates it from other genera (Brooks et al., 1998).

1.4.3.2.2.1.1 Pseudomonas aeruginosa

It is the most common clinically significant species. They grow well on nutrient agar, the colonies were low convex, and cultures have disincitive nasty smell. Most strains produce pyocyanin pigment (yellow-
green) which colour the colonies and the surrounding nutrient agar medium with blue-green colour.

On Blood agar they were large, flat haemolytic. *P. aeruginosa* is frequently cultured from samples obtained from a draining sinus tract or deep penetrating ulcers in patients with diabetes (Cunha, 2004).

### 1.4.3.2.2.2 Enterobacteriaceae

Members of this family are Gram-negative bacilli. They are either non motile or motile with peritrichous flagella and do not form spores. All members were growing aerobically and are facultative anaerobes on a variety of non-selective (blood agar) and selective (MacConkey agar medium).

Enterobacteriaceae have simple nutritional requirements, ferment glucose, reduce nitrate, catalase-positive and oxidase-negative (Murray *et al.*, 1998). Some species (e.g *Escherichia coli*, *klebsiella pneumoniae* and *Proteus mirabilis*) are members of the normal commensal flora that can cause opportunistic infections.

#### 1.4.3.2.2.2.1 *Escherichia coli*

*Escherichia coli* is the common causative agent of diabetic wound infection (Murray *et al.*, 1998). On MacConkey agar most strains were lactose fermenting, have 2-4 mm colonies which are pink in colour, smooth, glossy and translucent. On blood agar, it produces small (1-4 mm in diameter) colonies that may appear mucoid and some strains are haemolytic (Barrow and Felthem, 1993).
1.4.3.2.2.2 *Klebsiella pneumoniae*

*Klebsiella pneumoniae* was also isolated and determined as an etiological agent of wound infection. On Nutrient agar the colonies are large, raised, moist and mucoid; they are lactose – fermenting on MacConkey agar. *Klebsiella pneumoniae* produce gas from glucose and is Methyl Red test and urease positive, whereas Voges Proskauer (VP) and potassium cyanide negative (Baker and Breach, 1980). On the other hand, Sleigh and Duguid, (1989) defined *Klebsiella pneumoniae* as non–motile, capsulated, lactose fermented and VP negative.

Barrow and Feltham (1993) and Cheesbrough (2000) classified *Klebsiella pneumoniae* into four subspecies based on DNA composition. The subspecies are *Klebsiella pneumoniae pneumoniae*, *Klebsiella pneumoniae aerogenes*, *Klebsiella pneumoniae ozaenae* and *Klebsiella pneumoniae rhinoscleromatis*.

Cheesbrough (2000) defined *Klebsiella pneumoniae* as VP variable, indole negative and H$_2$S negative. Morphology and cultural characteristics of Klebsiella species show that, they are thick bacilli, often slightly oval in outline, varying in size from 1 – 3 μm and 0.5 – 1.0 μm in width. They are capsulated and non sporulated (Barrow and Feltham, 1993). *Klebsiella pneumoniae* was reported to be instrumented in enhancing virulence of anaerobic bacteria (*Prevotella melaninogenica*) by providing succinate as an essential growth factor (Lev *et al*., 1971 and Maryrand and McBride 1980). Hence microbial synergy may increase the net pathogenic effect and the severity of infection (Bowler *et al*., 2001).
1.4.3.2.2.2.3 Proteus species

It was also isolated from wound infections, it is often a secondary invader of ulcer, burns and damaged tissues. Proteus are rod shaped organisms, commonly measuring 1 – 3 μm in length and 0.5 μm in width, but often showing considerable pleomorphism, occurring sometimes in coccal forms or as filaments measuring 10 – 20 μm in length.

Proteus cultures have a distinctive smell (fishy smell) with swarming on noninhibitory solid media such as nutrient agar and blood agar. On MacConkey agar they had non-lactose fermenting colonies. Most Proteus strains swarms with periodic cycles of migration producing concentric zones, or spread in a uniform film over moist surfaces of nutrient media. Optimal growth temperature is 37°C.

The organism is both aerobe and facultatively anaerobic, methyl red positive and acetoin negative. Proteus species are free living saprophytes in soil, vegetation, water and sewage, and are found in the intestine in many healthy persons. These organisms occur naturally in the environment of animals and man and particularly in the intestines, animal manure, human sewage, soil and water. In addition to this, they may also be found associated with abortion, otitis, peritonitis and dysentery in animals (Buxton and Fraiser, 1977). They occur also in infections of the urinary tract, wound and other sites (Sleigh and Duguid, 1990).

1.4.3.2.2.2.3.1 Proteus mirabilis

It is the main species of medical importance, it differs from Proteus species in that it is indole positive and cause wound infections. Many
strains of *P. mirabilis* produced bacteriocins (proticins) which has a lethal action against other strains.

Senior (1977) described a highly discriminating method of typing strains by determination of their proticin production and sensitivity. The method was made more discriminating when it was used in combination with O serotyping (Senior, 1977).

1.4.3.2.2.3 *Vibrio species*

They are Gram- negative straight or curved rods, motile by a single polar flagellum, aerobic and facultatively anaerobic. Catalase usually positive, oxidase positive and attack sugars by fermentation (Barrow and Feltham, 1993).

1.4.3.2.2.3.1. *Vibrio fluvialis*

It occurs in fresh, brackish and estuarine waters throughout the world. It’s associated with wound infections (Barrow and Feltham, 1993).

1.4.3.2.2.3.2 *Vibrio vulnificus*

*Vibrio vulnificus*, previously known as lactose – positive (L+) vibrious (Farmer, 1979) has been implicated occasionally in severe wound infections and septicaemia (Cunha, 2004).

1.4.3.2.2.3.3 *Vibrio mimicus*

*Vibrio mimicus* originally regarded as sucrose-negative variants. It has the same H antigen as *V. cholerae*, however, DNA hybridization and phenotypic characterization confirm its separate identity (Davis *et al.*, 1981).
1.4.3.2.2.4 Aeromonas species

They are Gram-negative rods, motile and non motile. Aerobic and facultatively anaerobic, catalase positive, oxidase positive, sugars attacked fermentatively and gas may be produced (Barrow and Feltham, 1993).

*Aeromonas hydrophilia* and *Aeromonas salmonicida* are implicated in wound infections (Cunha, 2004). *Aeromonas hydrophilia* is different from *Aeromonas salmonicida* on the production of acetion (VP-positive).

1.4.3.2.2.5 Shewanella putrefaciens

It is Gram-negative rods, motile, aerobic, catalase positive and oxidase positive. It is the only species of genus Shewanella known to occur in clinical material. This organism was once included in pseudomonas but was transferred because of its low G+C content of its DNA (Mac Donell and Colwell, 1985).

1.4.4 Diabetes and lower limb amputation

Amputation is perhaps the most feared and well recognized complication of diabetes by the general public. Amputation of a limb is one of the most devastating complications of diabetes, it has got many influences on all aspects of patient’s life, and to explore this impact health related quality of life (HRQL) measures were implemented (Frier *et al.*, 1999). Pathways leading to foot ulceration and amputation in diabetic foot disease are shown in (Fig.2).

For many patients, amputation is pivotal events that alter their quality of life; it poses serious threats to the social and psychological well being of the patient. Amputation of the lower limb places patients to an increased risk for functional disability and psychological distress.
FIG 2: Pathways leading to foot ulceration and amputation in diabetic foot disease. Inter-relationhips of etiological factors and principal clinical features are shown.

- Peripheral vascular disease
- Autonomic neuropathy
- Somato neuropathy
- Charcot arthropathy
- Diminished proprioception
- Reduced perception of pain
- Skin fissures
- Absent sweating
- Warm foot
- Distended foot veins
- Altered blood flow regulation
- Dry skin
- Limited joint mobility
- Chronic foot changes
- Weight bearing/pressure
- Reduced foot pulses
- Cold extremities
- Cold feet
- Rest pain
- Gangrene
- Infection
- Ulceration
- Amputation
Amputees with diabetes have a 50% chance of bilateral amputation, often due to increased wear on the remaining limb. At any time, perhaps 1 million patients with diabetes suffer from lower extremity ulcers around the world (Armstrong et al., 1998a).

Sudanese patients can handle amputation much better than those worldwide, because they are fortunate to have very strong social and family relations, which help them adapt and adjust to the new situation.

Loss of a part of the lower extremity is an unfortunate complication of diabetes. When the foot is neuropathic and there is an inadequate blood supply to the foot as a whole, local surgical measure with or without skin graft give rise to excellent result. However, when there is generalized vascular occlusive disease, local surgery is attendant by chronic ulceration and more radical surgery is likely to be required for cure (El Bagir, 1997).

1.5 Treatment of diabetic foot

The distinction between insulin – dependent diabetes mellitus (IDDM) and non – insulin dependent diabetes mellitus (NIDDM) at diagnosis is important to avoid unnecessary and costly insulin treatment, particularly in a country like Sudan. Difficulties with the diagnosis and classification of diabetes are frequently encountered, due to a severe lack of diagnostic materials and poor diabetes care services. Consequently, patients will receive inappropriate management, resulting in further deterioration of the metabolic status, which may accelerate the development of diabetic complications. So proper control, blood sugar level and patient’s education is important (El Bagir, 1997).
1.5.1 Antibiotics treatment

The use of antibiotics was a milestone in the effort to prevent wound infection. Treatment with antibiotics that has a broad spectrum of activity against Gram–positive and Gram–negative organisms, such as meropenem and gatifloxacin, are the best in treatment of bacterial infection (Cunha, 2000).

Grayson et al., (1995) reported that ampicillin and sulbactam in combination act as a betalactamase inhibitor. They are used for aerobes and against skin and enteric flora. Metronidazole (Flagyl), intravenous antibiotic, which is an imidazole ring–based antibiotic is active against various anaerobic bacteria and protozoa. It used in combination with other antimicrobial agents (Cunha, 2000). Levofloxacin is used in the treatment of infections caused by various Gram–negative organisms. Cefazolin is indicated as the first generation semisynthetic cephalosporin that arrests bacterial cell-wall synthesis, thus inhibiting bacterial growth. It is active against skin flora, including *S. aureus* (Gilman et al., 1980). Cunha, (2000) also reported that Clindamycin is successfully used for the treatment of serious skin and soft tissue Staphylococcal infections. Clindamycin is also effective against aerobic and anaerobic streptococci, except enterococci. Clindamycin is a semi-synthetic modification of lincomycin (Gilman et al., 1980). Its mechanism of action involves inhibition of protein synthesis and active against many strains of Staph-species, but may not inhibit methicillin resistant strains. Gentamycin is indicated as an important agent for the treatment of many serious Gram–negative bacillary infections. It has a broad antibacterial spectrum. It is very effective against *E.coli*, *Proteus mirabilis*, *Klebsiella pneumoniae*, Enterobacteria and *Pseudomonas*.
*aeruginosa*. Gentamycin is also active against most strains of *S.aureus* (Pratt, 1977). However, emergence of resistant microorganisms in some hospitals has become a serious problem and may limit the future use of gentamycin. In some hospitals, the nosocomial flora have undergone considerable alteration in susceptibility to this antibiotic during the last 25 years, with a gradual increase in resistance to gentamycin (Gilman *et al.*, 1980).

Tetracyclines are broad–spectrum antibiotics that have polycyclic structure. Tetracyclines are bacteriostatic, they inhibit protein synthesis by blocking the binding of aminoacyl tRNA to the mRNA – ribosome complex (Pratt, 1977).

1.5.2 Surgical treatment

1.5.2.1 Surgical debridement

Debridement involves removal of devitalized and contaminated tissue from wounds to expose healthier tissue and facilitate wound healing (Vowden and Vowden, 1999). Devitalized tissue provides a favourable environment for microbial growth and thus its removal will also reduce microbial load. Surgical debridement has been the mainstay of treatment of wounds of various etiologies. The rationale behind the necessity for periodic surgical debridement has been largely anecdotal but centers around the limitation of undermining, the promotion of an adherent wound margin and a favourable environment for epithelial cell migration, reduction of the bacterial burden and allows better penetration of topical antibiotics (Steed *et al.*, 1996). Surgical debridement in chronic osteomyelitis is the single most important therapeutic intervention to insure that debridement is
complete and that no further remnants of bacteria do exist because in chronic osteomyelitis, neither oral nor intravenous medications are effective without adequate surgical operation. If amputation is performed, physical therapy and rehabilitation may be involved (Cunha, 2004).

1.5.2.2 Bio-surgery

The current problem of antibiotic resistance and the difficulties in controlling MRSA (Methicillin Resistant *Staphylococcus aureus*) infection have led to increasing interest in maggot therapy of wound healing (Fig.3). Maggot therapy appears too successful because of its effect on pH and from mechanisms involving its secretions and its digestive system (Bunkis *et al.*, 1985). The maggots also have an effective debriding action and growth promoting effects.

Maggot therapy has been used for a wide range of wounds since its introduction as a therapeutic treatment for chronic pyogenic and tuberculous osteomyelitis (Baer, 1931). It also used to treat suppurative infections including abscesses, burns, gangrene and chronic leg ulcers (Bunkis *et al.*, 1985).

Maggots are classified within the Calliphoridae family, which includes Lucilia sericata, and used as a cost-effective alternation to conventional treatments or even surgery in the treatment of diabetic foot ulcer (Hall, 1995). They are facultative parasites, and have been chosen because the larvae develop rapidly and therefore act quickly in the wound (Fig 4). They do not invade internal organs, easy to rear and sterilize. Maggots prefer to feed on necrotic tissue and have therapeutic action by changing in the pH (Hall, 1995).
Fig. (3)

Maggots used to remove dead and dying material from an open wound on a patient's foot – and example of biosurgery

Bunkis et al., (1985)
Fig. (4) Maggot therapy in the management of the diabetic foot
Hall, (1995)
Bacterial growth is inhibited in the presence of maggot secretions, as compared with control group. This work was confirmed recently when live maggots were added to a suspension of *Staphylococcus aureus* (Thomas *et al.*, 1999). Moreover, Courtney *et al* (2000) found that maggot has an important role of debridement which is still regarded as important stage in wound healing; without it, wound healing may be delayed.

1.5.3 Treatment of neuropathic wounds

The treatment of foot problems is dependent on the underlying cause. Patients with neuropathy develop non-healing wounds because of abnormal pressure on a specific part of the foot. These wounds don’t hurt and are often unrecognized by the patients early in their development because of the decreased or absent sensation in the foot. These open wounds can then get infected leading to gangrene and consequently amputation. The treatment of these neuropathic wounds (malperforans ulcers) is supportive. Pressure from the wound surface must be relieved and infection prevented using the appropriate antibiotic and hence wound healing is promoted (Mueller *et al.*, 1989).

1.5.4 Treatment of wound due to diabetic vasculopathy

Non–healing wounds and gangrene frequently develop in diabetics due to inadequate blood flow in the legs and feet. Diabetics are prone to developing blockages in the arteries of the lower leg (calf). If the tissue of the foot has insufficient blood flow, minor scratches, crackes or abrasions of the skin may not heal and, in fact, may enlarge. They can also become infected leading to gangrene and amputation. Non–healing wounds due to
arterial insufficiency require improved blood flow in order to heal (Klingel et al., 2003).

This can be achieved through:-

1) Balloon angioplasty (ballooning) of a narrowing or blockage in an artery.
2) Surgical by pass around a blockage.
3) Therapeutic angiogenesis by the use of new experimental medicine to grow new blood vessels.
4) Rheopheresis.
5) Hyperbaric oxygen therapy.

These remarkable approaches are achieved through commitment to functional limb salvage and preventive medical intervention in diabetic patients at risk of limb loss (Klingel et al., 2003).

1.5.5 Treatment of lower extremity amputation

Lower extremity amputation (LEA) is the major cause of disability to patient who has diabetes mellitus. It is indicated as a last resort to save the foot, leg and the body from bacteria that fulminate in death of the patient. Therefore it is often quicker and kinder to opt for early major amputation rather than to subject patients to debilitating sequences of conservative procedures that fulminate in septicaemia and death of the patient. Nonetheless amputation in diabetic could be delayed or prevented by the previously described procedures (Bild et al., 1989).

1.6 Nutritional gangrene control

With no doubt, development of gangrene to a great extent is related to what the patient eats and how often he moves. In most cases, unhealthy
lifestyle, faulty diet and physical inactivity contribute to the production of degenerative changes in the body. At present there is strong evidence that exercise and stress reduction if done regularly are both protective to the cardiovascular system and supportive of the immune processes. The cornerstone of the nutritional method to control gangrene is a new product called. 'Gangrene Clearing Formula' which is a complex, synergistic formulation of selected nutrients supporting the entire circulatory system.

Nutritional factors are able to boost the body chemistry by:

a) Correcting possible deficiencies and / or imbalances.

b) Providing optimum condition for the proper functioning of the body’s own intrinsic ability to heal itself according to the orthomolecular nutrition.

As opposed to medical procedures, the nutritional method of controlling foot or leg gangrene is something done by the patients to get good results. In the other hand, although genetical factor predispose to type – 2 diabetes, nonetheless type – 2 diabetes is associated with poor diet and unhealthy lifestyle. In general nine out of ten cases of type – 2 diabetes could be prevented if people ate better, exercise more, stop smoking and adopted other healthy behaviours (Website 1).
CHAPTER TWO

MATERIALS AND METHODS
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2.1. Field investigation

In this study forty patients suffering from diabetic foot wounds were investigated. The study was carried out at: Police University Teaching Hospital, Khartoum Teaching Hospital and Health Medical Services Centre of the University of Khartoum. A questionnaire that accompanied this study is shown in appendix (1). Almost all patients had received a combination of antibiotics including metronidiazole.

2.2. Collection of samples

Swab samples were collected from thirty patients before and after debridement. Superficial swabs were collected from wounds surface or from superficial devitalized tissues removed by curettage during the process of debridement. Deep swabs were obtained from the deep wound surfaces following debridement. Ten tissue samples from ten patients were excised in small pieces immediately from amputated parts following surgery and kept in cooked meat media.

2.3. Transportation of samples

Samples were labeled and placed in an ice box, transported to the Department of Microbiology, at the Faculty of Veterinary Medicine, University of Khartoum with sufficient speed to avoid unnecessary delay or contamination prior the microbiology examination and all of them cultured within two hours after collection.
2.4. Culture Media

All media were dispensed under aseptic conditions in an aseptic preparation room provided with ultra-violet lamp, flame, phenol disinfectant and 70% alcohol for disinfecting the benches.

2.4.1 Solid Media

2.4.1.1 Nutrient agar (Oxoid Ltd.; England)

The medium was composed of 1gram lab-lemco powder, 2gram yeast extract, 5gram sodium chloride, 5gram peptone and 15gram agar. It was prepared by adding 28gram of dehydrated nutrient agar that was suspended in one litre of distilled water, steamed to dissolve completely, pH adjusted to 7.4 and sterilized at 121°C for 15 minutes. Then it was poured into sterile plates or distributed in a 10ml amount into sterile screw-capped bottles and allowed to set in slope position.

2.4.1.2 Blood Agar (Oxoid Ltd.; England)

The medium was composed of 10gram lab-lemco powder, 10gram peptone, 5gram sodium chloride and 15gram agar in one litre distilled water. Forty grams of blood agar base was suspended in one litre of distilled water, boiled to dissolve completely. The pH was adjusted to 7.3 and then sterilized at 121°C for 15 minutes. The base was cooled and the temperature was adjusted and kept at 45-50°C in a water bath. Then 7% of defibrinated sheep blood was added, mixed with gentle rotation and poured into sterile Petri-dishes in 20 ml volumes.
2.4.1.3. MacConkey agar (Oxoid Ltd.; England)

The medium consisted of 20 grams of peptone, 10 grams of lactose, 5 grams of sodium chloride, 5 grams of bile salt, 0.03 neutral red and 15 grams of agar in one litre of distilled water. In a litre of distilled water, 51.5 grams of dehydrated MacConkey agar were suspended, boiled to dissolve completely, the pH was adjusted to 7.4 and sterilized by autoclaving at 121°C for 15 minutes and poured in sterile plates in 20 ml volumes.

2.4.1.4 Starch agar (Oxoid Ltd.; England)

The medium consisted of 10 grams of potato starch, 50 ml of distilled water and 1000 ml of nutrient agar. The starch was triturated with water to a smooth cream, added to the molten nutrient agar, mixed, sterilized at 115°C for 10 minutes and dispensed into sterile Petri-dishes in 20 ml volumes.

2.4.1.5. Simmon’s citrate agar (Oxoid Ltd., England)

The medium composed of 0.2 grams of magnesium sulphate, 0.2 gram of ammonium dihydrogen phosphate, 1.0 gram of sodium ammonium phosphate, 2.0 grams of sodium citrate, 5.0 grams of sodium chloride, 0.08 grams of bromo-thymol blue and 15 grams of agar. Twenty three grams of dehydrated Simmon citrate agar were suspended in one litre of distilled water, boiled to dissolve completely, the pH was adjusted to 7.0 and sterilized by autoclaving at 121°C for 15 minutes. It was then poured into sterile screw-capped bottles and allowed to set in slope position.

2.4.1.6 Urea agar (Oxoid Ltd., England)

The medium was composed of 1 gram of peptone, 1 gram of dextrose, 5 grams of sodium chloride, 1.2 grams of disodium phosphate, 0.8 grams of potassium dihydrogen phosphate, 0.012 grams of phenol red and 15 grams of agar in one litre. An amount of 2.4 grams of urea agar base was suspended in 95 ml
of distilled water, steamed to dissolve completely, pH was adjusted to 6.8 and then sterilized by autoclaving at 115°C for 20 minutes. Cooling and keeping the temperature in water bath at 50°C, five ml of sterile 40% urea solution were aseptically added and mixed well. Then the medium was distributed in 10ml amounts into sterile containers and allowed to set in slope position.

2.4.1.7 Mannitol Salt Agar (Oxoid Ltd., England)

The medium consists of 1.0gram lab-lemco, 10.0gram peptone, 10.0gram mannitol, 75.0gram NaCl, 0.025gram phenol red and 15gram agar. The medium was prepared by dissolving 111grams of dehydrated medium powder in one litre of distilled water, sterilized at 121°C for 15 minutes and dispensed in 20ml amounts into sterile Petri-dishes aseptically.

2.4.1.8 Phenolphthalein Agar

The medium consists of 1000ml nutrient agar which melted and cooled to the temperature kept at 45-50°C in water bath. Then 10ml of 1% phenolphthalein phosphate solution was aseptically added, mixed and the medium was distributed into sterile Petri-dishes (Barrow and Feltham, 1993).

2.4.1.9 Lecitho-Vitellin (LV) agar

The medium consists of 100ml of Lecitho-vitellin solution (egg yolk emulsion) and 900ml of nutrient agar. The nutrient agar was melted and cooled to about 55°C before Lecitho-vitellin emulsion was added aseptically, mixed and 20ml volumes were dispensed into sterile plates (Barrow and Feltham, 1993).

2.4.1.10 Ammonium salt sugars (ASS)

The medium composed of 1 gram ammonium monohydrogen phosphate, 0.2gram potassium chloride, 0.2gram magnesium sulphate, 0.2
gram yeast extract, 20gram agar and 4 ml of 0.2% bromocresol purple solution (Barrow and Feltham, 1993).

The solids were added to one litre of distilled water and dissolved by steaming. The indicator was added and the medium was sterilized at 115°C for 20 minutes. The basal medium was allowed to cool to about 60°C and the appropriate sterilized solution of carbohydrate was added to give a final concentration of 1%. The medium was mixed thoroughly and distributed aseptically into sterile tubes which were inclined so that the medium sets as a slope.

2.4.1.11 Aesculin agar (Oxoid Ltd., England)

The medium composed of 1gram of aesculin, 0.5gram ferric citrate and 20gram agar were dissolved in 100ml peptone water and sterilized by autoclaving at 115°C for 10 minutes. Then each 10ml amounts was poured aseptically into sterile bottles and allowed to set in a slope position.

2.4.1.12 Casien Agar (Milk agar) (Oxoid Ltd., England)

The medium consists of 3gram yeast extract, 5gram peptone, 15gram agar and 10ml skim milk. Twenty four grams of solids suspended in one litre of distilled water, boiled to dissolve completely and sterilized by autoclaving at 121°C for 15 minutes after adjusting the pH to 7.2.

2.4.1.13 Reinforced Clostridial Agar (RCA) (Oxoid Ltd., England)

The medium consists of 3gram yeast extract, 10gram Lab Lemco, 10gram peptone, 1gram soluble starch, 5gram Dextrose, 5gram Nacl, 3gram Sodium acetate and 15gram agar. This medium was prepared by suspending 52.5gram of medium powder in 1litre of distilled water, mixed with magnetic stirrer and allowed to dissolve completely in boiling water and the pH was adjusted to 6.8± 0.1. The medium was then autoclaved at 121°C for
15 minutes, Cysteine hydrochloride 0.05% and Glucose 1% were added aseptically to the medium, mixed and distributed into sterile Petri-dishes in about 20ml each and stored at 4°C for used.

2.4.1.14 Diagnostic Sensitivity Test Agar (DSTA) (Oxoid Ltd., England)

The medium was composed of 10gram Protease peptone, 10gram veal infusion solids, 2gram dextrose, 3gram sodium chloride, 2gram disodium phosphate, 1gram sodium acetate, 0.01gram adenine sulphate, 0.01gram guanine hydrochloride, 0.01 gram uracil, 0.01gram xanthine, 0.00002gram aneurine and 12gram ‘ionagar’ No. 2. Fourty grams were dissolved in one litre of distilled water by boiling and autoclaved at 121°C for 15 minutes. The pH was adjusted to 7.4 and then distributed in 25ml volumes into sterile Petri-dishes in a levelling table.

2.4.2 Semi Solid Media

2.4.2.1 Motility Media (Oxoid Ltd., England)

The medium was composed of 13gram nutrient broth and 0.05gram Agar. It was prepared by adding 14.1gram of medium powder that was suspended in one litre of distilled water and boiled to dissolve completely. The pH was adjusted approximately to 7.2 and then the medium was distributed in 3.5ml volumes into test tubes containing Craigie tubes and sterilized at 121°C for 15 minutes.

2.4.2.2Hugh and Leifson’s (Oxidation and Fermentation Medium)

The oxidation fermentation medium was used to test the ability of organism to break down the carbohydrates either oxidatively or fermentatively. The medium was composed of 2gram peptone, 5gram sodium chloride, 0.3 gram potassium dihydrogen phosphate, 3gram agar and 0.2 % aqueous solution of bromocresol purple. The ingredients were
boiled to dissolve completely in 1 litre of distilled water, the pH was
adjusted to 7.1, filtered and the indicator was added and then sterilized by
autoclaving at 115°C for 20 minutes. Ten ml of 10% sterile glucose solution
was added aseptically to 90ml of the medium to give a final concentration
of 1%, mixed and 10ml of amounts were aseptically distributed into sterile
test tubes (Barrows and Feltham, 1993).

**2.4.2.3 Nutrient gelatin**

The medium consists of 3gram lab-lemco powder, 5gram peptone
and 120gram gelatine. The medium was prepared by adding 128grams to
one litre of distilled water, steamed to dissolve completely. The pH was
adjusted to 6.8 and poured in 5 ml volume into sterile Bijou bottles and
sterilized by autoclaving at 115°C for 15 minutes (Barrow and Feltham,
1993).

**2.4.3 Liquid Media**

**2.4.3.1. Nutrient broth (Oxoid manual, 1973)**

This simple medium contained the basic growth factors for most
bacteria. It was composed of 1gram lab-lemco, 2gram yeast extract 5gram
peptone and 5gram sodium chloride. The medium was prepared by
dissolving thirteen grams of dehydrated nutrient broth in one litre of
distilled water, then it was mixed well, the pH was adjusted to 7.4 and
sterilized by autoclaving at 121°C for 15 minutes. After sterilization the
medium was poured into sterile tubes in a 5ml amounts.

**2.4.3.2 Peptone Water**

This medium was used as base of carbohydrates utilization tests and
for other purposes. It was composed of 10gram peptone, 5gram sodium
chloride. It was prepared by dissolving fifteen grams of peptone in one litre
of distilled water, mixed well and distributed into 5ml amounts into test tubes. The pH was adjusted to 7.4 and sterilized by autoclaving at 121°C for 15 minutes (Barrow and Feltham, 1993).

2.4.3.3 Peptone water sugars

The solution of the specific sugar was prepared by dissolving 10 gram of sugar in 90ml of distilled water and then in 900 ml of peptone water. Ten ml Andrade’s indicator was added and the mixture was thoroughly mixed. The medium was distributed into sterile test tubes and the pH was adjusted to 7.1-7.3 and then sterilized by autoclaving at 115°C for 10 minutes. (Barrow and Feltham, 1993)

2.4.3.4 Nitrate broth

This medium was prepared by dissolving one gram of potassium nitrate in one litre of nutrient broth, distributed into tubes in 5ml volumes and sterilized by autoclaving at 115°C for 20 minutes (Barrows and Feltham, 1993).

2.4.3.5 Glucose Phosphate broth

The medium was used for methyl red (MR) test and Voges-Proskauer (VP) test. It consists of 5gram peptone, 5gram dipotassium hydrogen phosphate (K$_2$HPO$_4$) and 5gram glucose. It was prepared by adding the peptone and K$_2$HPO$_4$ in one litre of distilled water, mixed, steamed to dissolve, filtered and the pH was adjusted to 7.5. Glucose was added to give final concentration of 1%, mixed, distributed into sterile tubes in 5ml volumes and sterilized at 115°C for 10 minutes (Barrows and Feltham, 1993).

2.4.3.6 Robertson’s Cooked-Meat Medium

The medium was prepared by boiling 500grams of fat free minced ox
heart meat in 0.05 gram NaOH, strained through muslin and the meat particles were dried at a temperature below 50°C. Five grams of the dried meat were added to each screw-capped MacCarteny bottles and sufficient nutrient broth was added and sterilized at 121°C for 15 minutes (Barrows and Feltham, 1993).

2.5 Reagents

2.5.1 Hydrogen Peroxide

It was obtained from the British Drug House Chemical (BDH) Company, England, and prepared as a 3% aqueous solution and used for catalase test (Barrow and Feltham, 1993).

2.5.2 Tetra methyle-p-phenylenediamine dihydrochloride

It was manufactured by British Drug House was prepared as fresh 1% aqueous solutions and kept in a brown bottle in the dark and was used for oxidase test.

2.5.3 Methyl red

It was prepared according to Barrow and Feltham, (1993) by dissolving 0.04 grams of methyl red in 40ml ethanol and diluted with distilled water to 100ml. It was used in methyl red test (Barrow and Feltham, 1993).

2.5.4 Voges-Proskauer (VP) test reagent

It consists of 5% α-naphthol in ethanol as reagent (A) and 40% aqueous solution of NaOH as reagent (B). It is used for the detection of acetylmethylcarbinol production.

2.5.5 Nitrate test reagent

It was consisted of two solutions, solution (A): 0.8% sulphanilic acid in 5N- acetic acid. It was prepared by mixing 0.4ml of sulphanilic acid in
50ml of 5N-acetic acid. Solution (B): 0.5% dimethyl-α-naphthylamine in 5N-acetic acid (Barrow and Feltham, 1993).

2.5.6 Kovac’s reagent

It is composed of 5gram p-dimethylamino-benzaldehyde 75ml of amylalcohol and 25ml concentrated hydrochloric acid. Kovac’s reagent was prepared by dissolving the aldehyde in the alcohol by gentle warming in water bath (50-55°C), cooled and the acid was added, protected from light and stored at 4°C. It was used for detection of indole production (Barrow and Feltham, 1993).

2.5.7 Lugol’s iodine

It is composed of 5gram iodine, 10gram potassium iodide. They were dissolved in 100ml distilled water and used at 1/5 dilution (British Pharmacopoeia, 1963).

2.5.8 Lead acetate paper

It was prepared from a filter paper cut into strips of 5-10 mm wide and 50-60 mm long and impregnated with the hot saturated lead acetate solution, dried at 50-60°C and stored in screw-capped containers. It was used for detection of H₂S production.

2.6 Indicators

All indicators were prepared according to Barrow and Feltham 1993.

2.6.1 Andrade’s indicator

It was used in peptone water sugar medium. It was prepared by dissolving 5gram of acid fuchsin in one litre distilled water, and then 150ml 1N NaOH solution was added.

2.6.2 Bromothymol Blue

It was used for citrate medium and (O-F) medium.
A total of 0.2 gram of the powder was dissolved in 100 ml of distilled water.

2.6.3 Phenol Red

It was used for urea agar base medium as 0.2%.

2.6.4 Bromocresol Purple

It was obtained from the British Drug House (BDH) Chemical Company, England. It was prepared as 0.2% aqueous solution and used as indicator for other purpose.

2.6.5 Zinc Powder

It was obtained from British Drug House (BDH) Chemical Company, England. It was used for nitrate reduction test in five mg/ml culture.

2.7 Solutions

2.7.1 Normal Saline (0.85%)

It was prepared by dissolving 8.5 gram of sodium chloride in one litre distilled water and sterilized by autoclaving at 121°C for 15 minutes.

2.8 Methods of sterilization and disinfection

2.8.1 Sterilization

2.8.1.1 Dry Heat

2.8.1.1.1 Hot air oven

This method was used for sterilization of clean glassware, such as Petri-dishes, pipettes, test tubes, flasks, mortors and pestles.

2.8.1.1.2 Flaming

It was used for wire loops, straight wires and points of tissue forceps. Sterilization achieved by holding the object as near as possible to the flame until it became red hot.
2.8.1.2 Moist Heat

2.8.1.2.1 Autoclaving

Glassware such as MacCartney, bijou and universal bottles were sterilized in the autoclave at 15 pounds pressure for 15 minutes (121°C for 15 minutes). Media such as motility medium, nutrient broth, peptone water, O-F medium and nitrate broth were sterilized at 115°C for 20 minutes. Nutrient agar, blood agar, cooked meat medium and mannitol salt agar were sterilized at 121°C for 15 minutes whereas glucose phosphate broth, gelatin agar and aesculin broth were sterilized by autoclaving at 115°C for 10 minutes.

2.9 Cultural methods

2.9.1 Primary isolation

For isolation of aerobic bacteria the samples, collected in swabs were opened aseptically and inoculated on blood and MacConkey agar. For isolation of anaerobic bacteria, the samples collected in cooked-meat media were inoculated on blood agar and Reinforced clostridium agar.

2.9.2 Incubation of cultures

Inoculated plates for aerobic growth were incubated at 37°C for 24 hours, whereas plates for anaerobic growth were incubated at 37°C for 48 to 72 hours in anaerobic jar. Longer incubation period was used in identification tests media: urease, citrate and O-F media were incubated for up to 7 days. Voges-Proskauer Nitrate and peptone media were incubated for 48 hours. Sugar media were incubated for up to 7 days.

2.9.3 Purification of isolates

Primary isolates were sub-cultured on nutrient agar to obtain pure cultures.
The process was repeated till pure isolates were obtained and kept in nutrient agar slants for further identification.

2.9.4 Detection of pigment production

For pigment production nutrient agar plates were inoculated with the test organism, incubated for 24 hours at 37°C and then transferred and kept out at room temperature for observation for up to five days.

Colours were recorded as red, orange, yellow, brown, green and black or negative (i.e. no production of pigment).

2.9.5 Identification of isolated bacteria

2.9.5.1 Cultural characteristics

Cultures on solid media were examined with the naked eyes for growth and colonial morphology as well as any visible change in the media such as colour, shape, size and haemolysis in blood agar medium. Liquid media were examined with the naked eye for turbidity, pellicle formation, sediment and colour change. Plates which did not show visible growth were re-incubated and examined daily for up to seven days before they were regarded as negative.

2.9.5.2 Primary tests

2.9.5.2.1 Gram staining and microscopy

Gram’s stain was done according to the method described by Barrow and Feltham (1993). It was used to study morphology, shape and Gram staining reaction of each isolate. Sterile loop was used to prepare emulsion from single colony by adding it to a drop of normal saline on a clean slide. This smear was made and allowed to dry in air, fixed by flaming, placed on rack and flooded with crystal violet (basic stain) and stained for 2-3 minutes. Slides were washed in running tap water until the stain stop
running. Then the slide was covered with Lugol’s iodine (mordant) for 30 seconds, and washed in tap water. Decolourization was made with a few drop of acetone in no time. The slide was washed thoroughly in water, and counterstained with diluted carbol fuchsin for half a minute, washed and blotted to dry with a filter paper. The prepared slides were examined under the microscope using oil-immersion lens. Bacteria coloured red were labelled as Gram-negative organism while violet coloured bacteria were labelled as Gram-positive.

2.9.5.2.2 Catalase test

The test organism was grown on nutrient agar and one ml of 3% Hydrogen peroxide (H₂O₂) was added to the culture. Immediate evolutions of gas bubbles indicate catalase activity.

2.9.5.2.3 Oxidase test

The organism was grown on nutrient agar. A piece of filter paper 2cm in diameter was placed in a Petri dish and 2–3 drops of fresh 1% tetramethyl – p-phenylene diamine dihydrochloride were dropped on the paper. The test organism was taken by sterile glass rod and smeared across the surface of the impregnated paper. A positive reaction was indicated by development of dark purple colour within 10 seconds, while no colour change indicated negative reaction.

2.9.5.2.4 Motility test

The tested strain was stabbed into the motility medium using straight wire and incubated at 37°C for 48 hours. Motile bacteria spread outside the Criagie tube.
2.9.5.2.5 Oxidation Fermentation of glucose (O-F) test

The O-F test was used to determine whether a bacterium has the enzymes necessary for the aerobic break down of glucose (oxidation) and/or for the fermentation of glucose. Two tubes containing Hugh and Leifson’s medium were inoculated with the test organism by stabbing with straight wire. The medium in one of the inoculated tubes was covered with a layer of sterile liquid paraffin oil to a depth of one cm. The tubes were incubated and examined daily for up to 14 days. Change in colour to yellow in both opened and oil sealed tubes, indicated a fermenting organism, yellow colour in the open tube alone meant that the organism was oxidative.

2.9.5.2.6 Fermentation of sugars

The peptone water sugars were inoculated with test organism, incubated at 37º C and examined daily for acid production, which causes change in colour to pink. Durham’s tubes were also examined for the presence of gas production.

2.9.5.3 Secondary test

Identification to the species level is performed by additional biochemical test for each isolate.

2.9.5.3.1 Nitrate reduction

Nitrate broth was inoculated with the test organism, incubated at 37ºC for 2 up to five days. Then 1 ml of nitrate reagent solution A was added followed by one ml of nitrate reagent solution B. Positive reaction was indicated by the development of red colour. To tube not showing red colour within five minutes, powdered zinc was added up to 5 gram/ml of culture and allow standing. The development of red colour meant nitrate
was not reduced by the bacteria (i.e. negative) while absence of red colour meant the nitrate was reduced to nitrite and then further reduced to gases.

2.9.5.3.2 Indole test

Peptone water medium was inoculated with test organism and incubated for 48 hours. A volume of 0.5 ml of Kovac’s reagent was added and the tube was shaken gently and examined after one minute. A positive reaction for indole production was indicated by formation of a ring of pink colour at the interface.

2.9.5.3.3 Methyl red reaction

Glucose phosphate medium was inoculated with the test organism and incubated at 37º C for 48 hours, then two drops of methyl red solution was added and a positive reaction was indicated by the development of a red colour.

2.9.5.3.4 Voges – Proskauer (V-P) test

Glucose phosphate broth was cultured with the test organism and incubated at 37ºC for 48 hours, then one ml of this culture was transferred into a sterile bottle, to which 0.6 ml of 5% alcoholic solution of α- naphthol and 0.2 ml of 40% potassium hydroxide (KOH) were added. The mixture was then shaken and examined after 15 minutes to one hour in a slope position. A positive reaction was indicated by the development of strong red colour.

2.9.5.3.5 Aesculin hydrolysis

Aesculin agar was inoculated with test culture, incubated at 37ºC and examined daily for 7 days. Blackening around the colonies indicated positive reaction.
2.9.5.3.6 Gelatin hydrolysis or liquefaction

Nutrient gelatin medium was inoculated with a straight wire loop and incubated at 37°C for up to 14 days. Every 2-3 days, the inoculated medium was transferred to the refrigerator for 2 hours and then examined for liquefaction. Positive reactions were indicated by hydrolysis of the gelatin medium and loss of its gelling property, while negative reaction was indicated by no gelatin liquefaction.

2.9.5.3.7 Urease Test

A slope of urea agar medium was inoculated with the organism, incubated at 37°C and examined daily for up to 7 days. Change of colour to pink indicated positive reaction. No change in colour was considered negative.

2.9.5.3.8 Citrate utilization test

Simmon’s citrate medium was inoculated with the test organism incubated at 37°C and examined daily for 7 days. An alkaline reaction indicated positive which changed to blue colour. No change in colour was considered as negative.

2.9.5.3.9 Hydrogen sulphide production (H$_2$S)

A lead acetate paper was placed into the neck of the tube of the peptone water, cultured, incubated at 37°C and examined daily for 7 days. Blackening of the paper indicated positive reaction (Lead sulphate).

2.9.5.3.10 Starch Hydrolysis

Starch agar was inoculated with the test organism, incubated at 30°C for 5 days and the medium was flooded with Lugol’s iodine solution. Clear colourless zone indicated starch hydrolysis.
2.9.5.3.11 Digestion of casein

A plate of casein agar was inoculated with test organism, incubated at 37°C, examined for up to 14 days. Clearing of the medium around of the bacterial growth indicated positive reaction.

2.9.5.3.12 Lecithovitellin reaction

Lecithovitellin agar was inoculated by test bacteria and incubated for 5 days to examine the growth, opalescence under and around the colonies, was considered a positive reaction.

2.9.5.3.13 Phosphatase Test

Phenolphthalein phosphate agar was inoculated by test bacteria and incubated for 18 hours to obtain discrete colonies. Then 0.1ml of ammonia solution was placed on the lid of Petri-dish and the medium was inverted above it. Bright pink colonies indicated positive reaction.

2.9.5.3.14 Ammonium Salt Sugar Test

Ammonium salt sugar medium was inoculated by test bacteria and incubated at 37°C for 7 days, growth and acid production indicated positive reaction.

2.9.5.3.15 Coagulase test

2.9.5.3.15.1 Slide coagulase test

A colony of the test organism was emulsified in a drop of normal saline on a microscopic slide to produce a thick suspension. The suspension was mixed with a drop of undiluted plasma. A positive reaction was seen by clumping of suspension within five seconds only.

2.9.5.3.15.2 Tube coagulase test

Human or rabbit plasma (10%) in 0.5ml volume is mixed with 0.1ml of an 18-24 hours broth culture of test bacteria and incubated at 37°C.
Examination is carried out at 1, 3 and 6 hours for coagulation, if negative, the tubes were left at room temperature overnight and then re-examined.

2.10 Sensitivity test

The antibiotics sensitivity of each isolated organism was evaluated by using disc diffusion method (Cruickshank et al., 1975). Peptone water medium was inoculated at 37°C for 2 hours, and then about 1ml was poured onto diagnostic sensitivity test medium agar (DSTA). Excess fluid was removed by using Pasteur pipette and the plates were allowed to dry. Using sterile forceps, commercially prepared discs were placed on the plates and lightly pressed down to ensure the antibiotics disc were in contact with the agar. The discs used are shown in table 1. After 30 minutes of applying the discs, the plates were incubated aerobically at 37°C for 24 hours. Sensitivity to antibiotic was indicated by zone of inhibition around the disc. Using a ruler on the underside of the plate, the diameter of the zone was measured in mm. Results were scored according to zone diameter (Arcomex Arab company for Medical Diagnostics, Jordan).

Table 3: The antibiotics disc used in the Study:

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Disc potency</th>
<th>Antibiotic</th>
<th>Disc potency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>10μg</td>
<td>Gentamycin</td>
<td>10μg</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>10μg</td>
<td>Penicillin</td>
<td>1i μ</td>
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<tr>
<td>Cloxacillin</td>
<td>5μg</td>
<td>Streptomycin</td>
<td>10μg</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>5μg</td>
<td>Tetracyclin</td>
<td>10μg</td>
</tr>
</tbody>
</table>
CHAPTER THREE

RESULTS
Chapter Three

Results

3.1 Survey

Thirty swab and 10 tissue samples were collected from diabetic foot patients within the period Jan. 2004 ~ Jan. 2005. All patients have long history of diabetes. The samples were subjected to bacteriological examination by direct microscopy and culture.

3.2 Laboratory investigation

Bacteriological study of 40 patients with diabetic foot infections revealed polymicrobial aetiology in 24 (60%) and single aetiology in 16 (40%). The mixed infections contain 2 to 6 isolates. The total number of isolates was seventy one. The isolates represent nine different bacterial genera as shown in (Table 4). Three genera were Gram-positive and the others six genera were Gram-negative. All the isolates were aerobic or facultative anaerobic. Strict anaerobic bacteria were not isolated from tissue samples cultured under optimal anaerobic condition. All isolates were identified to the species level as shown in (Tables 5 - 13).

3.3 Gram-positive bacteria

Isolated organism were Staphylococcus spp. (28.2%), Streptococcus spp. (5.6%) and corynebacteria spp. (8.5%) as shown in (Table 4).

3.3.1 Staphylococcus species

The criteria used for the identification of the isolates were as fellows:

3.3.1.1. Morphology and staining

The organism appeared, in stained smears as Gram positive cocci, arranged in clusters. Staphylococci were non-motile.
3.3.1.2. Cultural characteristic

3.3.1.2.1. Blood agar medium

On primary isolation, the cultures were incubated at 37°C for 24 hours. Colonies produced were circular, smooth, opaque, low convex, soft and easily to be emulsified when touch by the wire loop. They may form a zone of haemolysis. Different strains of *Staphylococcus aureus* produced colonies with grey and cream colourless and beta-haemolysis (Fig. 5).

3.3.1.2.2. Nutrient agar

Within 24 hours incubation, *Staphylococcus aureus* produced round, smooth and low convex colonies. While *S. delephini* produced round, smooth, low convex and glistening opaque.

3.3.1.2.3 Mannitol salt agar

After 24 hours incubation at 37°C, colonies were round, smooth and low convex. Most strains fermented mannitol and produced yellow colour in colonies. This medium was used as selective media for Staphylococci.

3.3.1.3 Biochemical characteristic

All examined isolates were catalase positive and oxidase negative. Coagulase, urease, phosphatase and acetoin production were also positive. Acetoin was not produced by *Staphylococcus delephini* as is indicated in (Table 10).

3.3.1.4 Sugar fermentation test

All isolates of Staphylococci produced acid but no gas from glucose, maltose, lactose and trehalose but not from xylose and raffinose (Table 10).
3.3.2 Streptococcus species

3.3.2.1 Morphology and staining

Isolates of this organism were Gram-positive cocci, non motile, non capsulated, non-sporing, arranged in chains and some strains are capsulated.

3.3.2.2 Cultural characteristic

3.3.2.2.1 Blood agar medium

After 24 hours incubation at 37°C, *Streptococcus pyogenes* colonies were always dew-droplike surrounded with a narrow zone of complete haemolysis. Whereas *Streptococcus viridans* colonies were surrounded by an area of partial haemolysis and green-brown colour, it is described as alpha-haemolytic Streptococci (α – H.S.) as shown in (Table 10).

3.3.2.3 Biochemical reaction

The isolates of Streptococci were catalase and oxidase-negative. Indole and hydrogen sulphide were not produced and nitrate was not reduced as shown in (Table 10).

3.3.2.4 Sugars fermentation ability

All examined isolates attacked carbohydrates fermentatively and produced acid with no gas from glucose, sucrose, lactose and trehalose. They did not attack xylose and salicin (Table 10).

3.3.3 Corynebacterium species

3.3.3.1 Morphology and staining

The organism appeared, in stained smears as Gram-positive small rods, exhibiting a tendency to form clumps and palisade arrangement simulating Chinese letters. The organism was non-motile.
3.3.3.2 Cultural characteristic

3.3.3.2.1 Blood agar medium

On primary isolation, the cultures were incubated at 37°C for 48 hours. Growth was in the form of small tiny colonies (one mm in diameter). They were white, opaque and flat with matt surface. The colonies of *Corynebacteria diphtheria* were often surrounded by a narrow zone of haemolysis.

3.3.3.2.2 Nutrient agar

Growth obtained after 48 hours incubation and the colonies appeared small and splashed.

3.3.3.3 Biochemical reactions

All strains tested were positive to catalase, urease and utilized carbohydrates by fermentation. While *Corynebacterium pseudodiphtheritic- cum* do not attack carbohydrates. All tested organisms were negative for oxidase, indole and acetoin production as shown in (Table 10).

3.4 Gram–negative bacteria

3.4.1 Entero bacteriaceae

They include *Escherichia coli*, *Klebsiella pneumoniae* sub sp. *aerogenes*, *Proteus mirabilis* and *Proteus vulgaris* bio group 3 (Table 8).

3.4.1.1 *Escherichia coli*

3.4.1.1.1 Morphology and staining

Isolates of *E.coli* were Gram-negative rods, occurred singly, long and thin. They were motile and non-spore forming.
3.4.1.1.2 Cultural characteristic

3.4.1.1.2.1 MacConkey agar

Colonies after 24 hours incubation at 37°C were smooth, shiny, large (2-4 mm in diameter) and pink in colour. (Lactose fermenting) (Table 9).

3.4.1.1.2.2 Blood agar

Colonies after 24 hours incubation at 37°C, were white to yellowish, moist, glistening, opaque and circular with an entire edge. Some strains of *E. coli* are haemolytic.

3.4.1.1.2.3 Nutrient agar

Colonies after 24 hours incubation at 37°C, were small, white and low convex.

3.4.1.1.3 Biochemical reactions

All examined isolates were catalase and indole positive, reduced nitrate, hydrolyzed aesculin and MR positive. They were oxidase negative. Acetoin or hydrogen sulphide was not produced and gelatin was not liquefied (Table 9).

3.4.1.1.4 Sugars fermentation test

Isolates of *E. coli* produced acid with gas due to fermentation of glucose, lactose, fructose, maltose, xylose and mannitol (Table 9).

3.4.1.2 *Klebsiella pneumoniae*

3.4.1.2.1 Morphology and staining

Isolates of *Klebsiella pneumoniae* were Gram-negative rods, capsulated, short and thick. They were non-motile.
3.4.1.2.2 Cultural characteristic

3.4.1.2.2.1. MacConkey agar

Colonies after 24 hours incubation at 37°C were large (2-4 mm in diameter), mucoid and pink in colour (Lactose fermenting).

3.4.1.2.2.2 Blood agar

Colonies after 24 hours incubation at 37°C were large, mucoid and grey – white in colours.

3.4.1.2.3 Biochemical reactions

All examined isolates were catalase positive and oxidase negative. They were indole and MR negative. They utilized citrate and urease. Acetoin was produced by Klebsiella (Table 9).

3.4.1.2.4 Sugars fermentation test

Isolates of Klebsiella pneumoniae produced acid with gas due to fermentation of glucose, lactose, fructose, maltose, xylose and mannitol. Fermentation of dulcitol is variable (Table 9).

3.4.1.3 Proteus mirabilis

3.4.1.3.1 Morphology and staining

Isolates of Proteus mirabilis were Gram-negative rods. They were motile.

3.4.1.3.2 Cultural characteristic

3.4.1.3.2.1. Blood agar

Colonies after 24 hours incubation at 37°C were large and had swarming appearance. Most strains produce fishy smell and wavy culture.

3.4.1.3.2.2. Nutrient agar

Colonies after 24 hours incubation at 37°C were large, a fishy smell
and had swarming appearance.

3.4.1.3.3 Biochemical reactions
   All examined isolates were indole and oxidase–negative; they were catalase and MR positive. Hydrogen sulphide was produced and gelatin was not liquefied (Table 9).

3.4.1.3.4 Sugars fermentation test
   Isolates of *P. mirabilis* produced acid with gas due to fermentation of glucose, trehalose and xylose.

3.4.1.4 *Proteus vulgaris* bio group 3
3.4.1.4.1 Morphology and staining
   Isolates of *Proteus vulgaris* were Gram–negative rods, non sporing and non capsulated.

3.4.1.4.2 Cultural characteristic
3.4.1.4.2.1 MacConkey agar
   Colonies after 24 hours incubation at 37ºC were large and had swarming appearance. Most strains produce fishy smell and wavy culture. They are non-lactose fermenting.

3.4.1.4.2.2 Blood Agar
   Colonies after 24 hours incubation at 37ºC were large, swarming with a zone of haemolysis.

3.4.1.4.3 Biochemical reactions
   All examined isolates were indole and MR positive. They were catalase positive and acetoin was not produced.

3.4.1.4.4 Sugars fermentation ability
   All examined isolates attacked carbohydrates fermentatively and
produced acid with gas from glucose, sucrose and maltose. They did not attack lactose, mannitol and sorbitol (Table 9).

3.4.2 Serratia species

3.4.2.1 Morphology and staining

Isolates of *Serratia marcescens* and *Serratia plymuthica* were Gram–negative bacilli, non sporing and non–capsulated. They were motile.

3.4.2.2 Cultural characteristic

3.4.2.2.1 MacConkey agar

Colonies after 24 hours incubation at 37ºC were smooth, large and pink in colour.

3.4.2.2.2 Blood agar

Colonies after 24 hours incubation at 37ºC were black, moist and produced haemolysis (Fig. 6).

3.4.2.2.3 Nutrient agar

Colonies after 24 hours incubation at 37ºC were large, moist and low convex. They became pink or red in colour if they incubated at room temperature (Fig. 7).

3.4.2.3 Biochemical reactions

All examined isolates catalase positive and oxidase negative. They were indole and MR negative. They utilized citrate and urease. Acetoin was produced by Serratia (Table 9).

3.4.2.4 Sugars fermentation ability

Isolates of *Serratia marcescens* and *Serratia plymuthica* produced acid with gas due to fermentation of glucose, maltose, sucrose and trehalose (Table 9). The two isolated organisms were differentiated by the production
of acid due to fermentation of adonitol, glycerol and sorbitol by 
*S.marcescens* only. *S. marcescens* was able to grow in 4% glucose.

3.4.3 Pseudomonas species

3.4.3.1 Morphology and staining

Isolates of Pseudomonas were Gram–negative rods, motile, non–sporing and non – capsulated.

3.4.3.2. Cultural characteristic

3.4.3.2.1 MacConkey agar

Colonies after 24 hours incubation at 37ºC were large, pale yellow–lemon in colour and rough. They are non lactose fermenting (Table 11).

3.4.3.2.2 Blood agar

*Pseudomonas aeruginosa* colonies after 24 hours incubation at 37ºC were large, flat, irregular spreading and haemolytic in blood agar. The colonies produced yellow–green fluorescent pigments that diffused into the medium. While *Pseudomonas fluorescence* produced the same colonial appearance of *P. aeruginosa*, pigments are not produced and no haemolysis occurred in blood agar.

3.4.3.3 Biochemical reactions

All examined isolates were catalase and oxidase positive. *Pseudomonas aeruginosa* reduced nitrate, whereas *P. fluorescens* do not reduced nitrate. The two isolated organisms were differentiated by the growth of *P. aeruginosa* at 42ºC and *P. fluorescence* at 5ºC (Table 11).
3.4.3.4 Sugars fermentation ability

Isolates of *P. aeruginosa* and *P. fluorescens* attacked sugars by oxidation. But in ammonium salt sugars test, the two isolated organisms were differentiated by the fermentation of raffinose by *P. aeruginosa* only.

3.4.4 Aeromonas species

3.4.4.1 Morphology and staining

Isolates of Aeromonas species were Gram-negative rods, motile and some were non–motile.

3.4.4.2 Cultural characteristic

3.4.4.2.1 Blood agar

Colonies of *A. hydrophilia* after 24 hours incubation at 37°C were large, flat and haemolytic. Whereas *A. salmonicida* is non-heamolytic.

3.4.4.2.2 MacConkey agar

Colonies of *A. salmonicida* after 24 hours incubation at 37°C were large and pink (lactose–fermenting), whereas colonies of *A. hydrophilia* were non–lactose fermenting.

3.4.4.3 Biochemical reactions

All examined isolates were catalase and oxidase positive, reduced nitrate and were indole positive. Acetoin produced by *A. hydrophilia* but not produced by *A. salmonicida*.

3.4.4.4 Sugars fermentation ability

Isolates of *A. hydrophilia* and *A. salmonicida* produced acid with gas due to fermentation of glucose, sucrose, lactose and maltose (Table 12).
3.4.5 *Shewanella putrefaciens*

3.4.5.1 Morphology and staining

Isolates of *Shewanella putrefaciens* were Gram–negative rods, motile, non sporing and non capsulated.

3.4.5.2 Cultural characteristic

3.4.5.2.1 Blood agar

Colonies after 24 hours incubation at 37ºC were small, low convex and had haemolytic reaction.

3.4.5.2.2 Nutrient agar

Colonies after 24 hours incubation at 37ºC were small, low convex and had pink to orange colours (Fig. 8).

3.4.5.3 Biochemical reactions

All examined isolates were catalase and oxidase positive, reduced nitrate, lecithinase and were casein positive and gelatin was liquefied (Table12).

3.4.5.4 Sugars fermentation ability

Isolates of *Shewanella putrefaciens* produced acid from glucose in ammonium salt sugar.

3.4.6 Vibrio species

3.4.6.1 Morphology and staining

The organism appeared, in stained smears as Gram–negative rods. In wet smear they were motile with polar flagellum.
3.4.6.2 Cultural characteristic

3.4.6.2.1 MacConkey agar

Colonies after 24 hours incubation at 37ºC were large 2-3 mm in diameter, smooth and non lactose fermenting (Table 13).

3.4.6.2.2 Nutrient agar

Colonies of *V. fluvialis* and *V. vulnificus* after 24 hours incubation at 37ºC were large, whereas in *V. mimicus*, colonies appeared short and thick.

3.4.6.3 Biochemical reactions

All strains tested were positive to catalase, oxidase and indole. Nitrate was reduced to nitrite and acetoin was not produced (Table 13).

3.4.6.4 Sugar fermentation ability

Isolates of Vibrio species were variables for acid production. *V. vulnificus* and *V. fluvialis* attacked sugar such as sucrose and salicin by fermentation without gas production. Whereas *V. mimicus* did not attack these two sugars. *V. vulnificus*, *V. fluvialis* and *V. mimicus* did not attack xylose (Table 13).

3.5 Anaerobic bacteria

Ten tissue samples obtained from excised tissue or amputated parts cultured under optimal anaerobic condition produced no anaerobic growth. All samples were collected promptly after surgery in pre-boiled cooked meat media. However, in cultivation, control system of *Clostridium perfringnes* grew luxuriously.

3.6 Antimicrobial Sensitivity test

Sensitivity of the bacteria isolated from the diabetics to eight different antibiotics are shown in (table 14, 15, 16, 17, 18, 19, 20) by the
disk diffusion method. Thirty isolates (42.3%) were sensitive to gentamycin, while the majority of isolates (98.6%) were resistant to tetracycline. One the other hand seventy one isolates (100%) were resistant to penicillin (table 14).

Eight isolates of S. aureus (11.3%) were resistant to all eight antibiotics that were used in this study. While six isolates of S. aureus (8.5%) were resistant to ampicillin, penicillin and tetracycline but three of these six were sensitive to gentamycin and cloxacillin. One isolate was sensitive to cloxacillin and streptomycin, also one of these six was sensitive to chloramphenicol and the last one was sensitive to erythromycin (table 15).

Three isolates of E.coli (4.3%) were resistant to all antibiotics which used in this study. While six isolates of E. coli (8.5%) were resistant to ampicillin, penicillin, tetracycline, cloxacillin, erythromycin and chloramphenicol. Five of these six isolates were sensitive to streptomycin, whereas only one was sensitive to gentamycin (table 16).

All seven isolates of Proteus mirabilis (9.8%) were sensitive to gentamycin. Four isolates of them (5.6%) were sensitive to ampicillin and streptomycin. While two isolates (2.8%) were sensitive to ampicillin and only one isolate (1.4%) was resistant to ampicillin (table 16). The only one isolate of Proteus vulgaris bio group 3 was sensitive to gentamycin and streptomycin. Two isolates of Klebsiella pneumoniae were resistant to all antibiotics in this study.

All isolates of Serratia marcescens were found sensitive to gentamycin (Fig.9) and only (20%) of them showed sensitivity to erythromycin. All isolates were resistant to ampicillin, chloramphenicol,
cloxacilla, streptomycin, penicillin and tetracycline (table 17). *Serratia plymuthica* was resistant to all antibiotics in this study except gentamycin (table 17).

All isolates of *Pseudomonas aeruginosa* were resistant to the various antibiotics tested in this study except one isolate which was found sensitive to gentamycin (table 18). On the other hand *Pseudomonas fluorescens* was found resistant to all antibiotics tested.

The only one isolate of *Shewanella putrefaciens* was resistant to ampicillin, chloramphenicol, cloxacillin, erythromycin, penicillin, streptomycin and tetracycline, but was sensitive to gentamycin (table 18).

All isolates of *Aeromonas hydrophilia* were sensitive to ampicillin, gentamycin and streptomycin, whereas (50%) were sensitive to tetracycline. But all isolates were resistant to chloramphenicol, cloxacillin, erythromycin and penicillin. On the other hand the only isolate of *Aeromonas salmonicida* was resistant to all antibiotics showed in this study except gentamycin (table 18).

The two isolates of *Staphylococcus delephini* were sensitive to cloxacillin and one isolate was sensitive to gentamycin, but all of them were resistant to ampicillin, chloramphenicol, erythromycin, penicillin, streptomycin and tetracycline (table 15).

All isolates of *Corynebacterium diphtheria* were resistant to all antibiotics used in this study. On the other hand *Corynebacterium pseudodiphtheriticum* isolates (25%) were sensitive to ampicillin, erythromycin and gentamycin, but resistant to chloramphenicol, cloxacillin, penicillin, streptomycin and tetracycline (table 20).
The three isolates of *Streptococcus pyogenes* were resistant to all antibiotics in this study. This also applied for *Streptococcus viridans*.

All isolates of *Vibrio fluvialis* and *Vibrio vulnificus* were sensitive to gentamycin and resistant to ampicillin, chloramphenicol, cloxacillin, erythromycin, streptomycin and tetracycline (table 19). *Vibrio mimicus* was sensitive to streptomycin and *Vibrio alginolyticus* was sensitive to gentamycin and streptomycin but was resistant to ampicillin, chloramphenicol, cloxacillin, erythromycin, penicillin and tetracycline (table 19).

### Table (4): Types of bacteria isolated from Patient with diabetic foot wounds.

<table>
<thead>
<tr>
<th>Isolated organisms</th>
<th>Number of isolates</th>
<th>Percentage %</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus</em> spp.</td>
<td>20</td>
<td>28.17</td>
</tr>
<tr>
<td><em>Streptococcus</em> spp.</td>
<td>4</td>
<td>05.63</td>
</tr>
<tr>
<td><em>Corynebacteria</em> spp.</td>
<td>6</td>
<td>08.45</td>
</tr>
<tr>
<td><em>Serratia</em> spp. *</td>
<td>6</td>
<td>08.45</td>
</tr>
<tr>
<td>Other enterobacteriaceae</td>
<td>19</td>
<td>26.76</td>
</tr>
<tr>
<td><em>Vibrio</em> spp.</td>
<td>6</td>
<td>08.45</td>
</tr>
<tr>
<td><em>Pseudomonas</em> spp.</td>
<td>5</td>
<td>07.04</td>
</tr>
<tr>
<td><em>Shewanella putrefaciens</em></td>
<td>2</td>
<td>02.82</td>
</tr>
<tr>
<td><em>Aeromonas</em> spp.</td>
<td>3</td>
<td>04.23</td>
</tr>
</tbody>
</table>

* Species isolated for the first time from diabetics

Total number of isolate was 71.
Table (5): Staphylococci isolated from patient with diabetic foot wounds.

<table>
<thead>
<tr>
<th>Staph. spp.</th>
<th>No. of isolates</th>
<th>% from Staph. spp.</th>
<th>% from total isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staph. aureus</em></td>
<td>14</td>
<td>70</td>
<td>19.71</td>
</tr>
<tr>
<td><em>Staph. delephini</em></td>
<td>6</td>
<td>30</td>
<td>08.45</td>
</tr>
<tr>
<td>Total</td>
<td>20</td>
<td>100</td>
<td>28.16</td>
</tr>
</tbody>
</table>

* Species isolated for the first time from patient with diabetics foot wounds.

Table (6): Streptococci isolated from patient with diabetic foot wounds

<table>
<thead>
<tr>
<th>Strepto. spp.</th>
<th>No. of isolates</th>
<th>% from Strepto. spp.</th>
<th>% from total isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Streptococcus pyogenes</em></td>
<td>3</td>
<td>75</td>
<td>04.23</td>
</tr>
<tr>
<td><em>Streptococcus viridans</em></td>
<td>1</td>
<td>25</td>
<td>01.41</td>
</tr>
<tr>
<td>Total</td>
<td>4</td>
<td>100</td>
<td>05.64</td>
</tr>
</tbody>
</table>
Table (7): Corynebacterium species isolated from patient with diabetic foot wounds

<table>
<thead>
<tr>
<th>Corynebacteria species</th>
<th>No. of isolates</th>
<th>% from Coryn. spp.</th>
<th>% from total isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coryne. diphtheriae</td>
<td>3</td>
<td>42.86</td>
<td>4.23</td>
</tr>
<tr>
<td>Coryne. pseudodiphtheriticum</td>
<td>3</td>
<td>42.86</td>
<td>4.23</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>6</strong></td>
<td><strong>85.72</strong></td>
<td><strong>8.46</strong></td>
</tr>
</tbody>
</table>

Table (8): Enterobacteriae isolated from patient with diabetic foot wounds.

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of isolates</th>
<th>% from Enterobacteria</th>
<th>% from total isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serratia marcescens</td>
<td>5</td>
<td>20.00</td>
<td>07.04</td>
</tr>
<tr>
<td>Serratia plymuthica</td>
<td>1</td>
<td>04.00</td>
<td>01.41</td>
</tr>
<tr>
<td>E. coli</td>
<td>9</td>
<td>36.00</td>
<td>12.68</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>2</td>
<td>08.00</td>
<td>02.82</td>
</tr>
<tr>
<td>Proteus vulgaris bio group3</td>
<td>1</td>
<td>04.00</td>
<td>01.40</td>
</tr>
<tr>
<td>Proteus mirabilis</td>
<td>7</td>
<td>28.00</td>
<td>09.86</td>
</tr>
</tbody>
</table>
Table (9): Characters and biochemical properties of Enterobacteriae isolated from patient with diabetic foot wounds.

<table>
<thead>
<tr>
<th>Character</th>
<th>E.coli</th>
<th>Klebsiella pneumoniae sub sp. aerogenes</th>
<th>Proteus vulgaris bio group 3</th>
<th>Proteus mirabilis</th>
<th>Serratia marcescens</th>
<th>Serratia plymuthica</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shape</td>
<td>Rod</td>
<td>Rod</td>
<td>Rod</td>
<td>Rod</td>
<td>Rod</td>
<td>Rod</td>
</tr>
<tr>
<td>Motility</td>
<td>Motile</td>
<td>Non-Motile</td>
<td>Motile</td>
<td>Motile</td>
<td>Motile</td>
<td>Motile</td>
</tr>
<tr>
<td>Oxidase</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>OF</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>F</td>
</tr>
<tr>
<td>Yellow pigment</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Red pigment</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MacConkey’s growth</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Simmon utilization</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Urease hydrolysis</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Gelatin hydrolysis</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>H2S Production (pf Ac) paper</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Casien hydrolysis</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Acid from:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose (gas)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Adonitol</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Arabinose</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Dulcitol</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glycerol</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Inositol</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lactose</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Maltose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Mannitol</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Raffinose</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Salcin</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sucrose</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Trehalose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Xylose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Starch</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MR (37C)</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>MR (RT)</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>VP (37C)</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>VP (RT)</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Indole</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

RT = room temperature
Table (10): Characters and biochemical properties of Gram-positive bacteria isolated from patient with diabetic foot wounds.

<table>
<thead>
<tr>
<th>Test</th>
<th><em>Staph. aureus</em></th>
<th><em>Staph. delephina</em></th>
<th><em>Strepto. pyogenes</em></th>
<th><em>Strepto. viridans</em></th>
<th><em>Coryn. diphtheria</em></th>
<th><em>Coryn. Pseudo.</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Coagulase</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Novobiocin</td>
<td>+S</td>
<td>R-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Oxidase</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mannose</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Urease</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Sucrose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Mannitol</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fructose</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Trehalose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Xylose</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lactose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glucose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>VP</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Phosphatase</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Nitrate</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Maltose</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Haemolysis</td>
<td>β</td>
<td>α</td>
<td>β</td>
<td>α</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

ND = NOT DONE

+S = Sensitive

R- = Resistant
Table(11): Biochemical reactions of Pseudomonas species and *Shewanella putrefaciens*.

<table>
<thead>
<tr>
<th>Type of reaction</th>
<th><em>Pseudomonas aeruginosa</em></th>
<th><em>Pseudomonas fluorescens</em></th>
<th><em>Shewanella Putrefaciens</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram reaction</td>
<td>G-ve rods</td>
<td>G-ve rods</td>
<td>G-ve rods</td>
</tr>
<tr>
<td>Motility</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Oxidase</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>O-F medium</td>
<td>O</td>
<td>O</td>
<td>F</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Simmon’s citrate</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Urease</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Gelatin liquefaction</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Brown pigment</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Orange pigment</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Green pigment</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Casein hydrolysis</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tween 80 hydrolysis</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Growth at 5C</td>
<td>+</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>Growth at 42C</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Lecithinase production</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Carbohydrates (in ammonium salt medium) acid from</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Maltose</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Lactose</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sucarose</td>
<td>-</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>Xylose</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

O = Oxidative  F = Fermentative  ND = Not Done
Table (12): Biochemical reactions of Aeromonas species

<table>
<thead>
<tr>
<th>Type of reaction</th>
<th>Aeromonas hydrophila</th>
<th>Aeromonas salmonicida sup. species salmonicida</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram reaction</td>
<td>G-ve rods</td>
<td>G-ve rods</td>
</tr>
<tr>
<td>Motility</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Oxidase</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>O-F medium</td>
<td>-</td>
<td>F</td>
</tr>
<tr>
<td>Gas from glucose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Indole</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>VP</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Simmon’s utilization</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Urease</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Acid from:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sucrose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Salicin</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Xylose</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Table (13): Biochemical reactions of Vibrio species

<table>
<thead>
<tr>
<th>Type of reaction</th>
<th><em>Vibrio mimicus</em></th>
<th><em>Vibrio vulnificus</em></th>
<th><em>Vibrio fluvialis</em></th>
<th><em>Vibrio alginolyticus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram reaction</td>
<td>G-ve rods</td>
<td>G-ve rods</td>
<td>G-ve rods</td>
<td>G-ve rods</td>
</tr>
<tr>
<td>Motility</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Oxidase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Gas from glucose</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Growth with 0% NaCl</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Indole</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>VP</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Starch hydrolysis</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Acid from:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactose</td>
<td>ND</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Salicin</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Sucrose</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Xylose</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

ND = Not Done
Table (14) Antibiotic sensitivity of the various bacteria isolated from foot wounds of diabetic patients

<table>
<thead>
<tr>
<th>Type of Antibiotic</th>
<th>No. of sensitive isolates</th>
<th>%</th>
<th>No. of intermediately sensitive isolates</th>
<th>%</th>
<th>No. of Resistant isolates</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>9</td>
<td>12.7</td>
<td>4</td>
<td>5.6</td>
<td>58</td>
<td>81.7</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>1</td>
<td>1.4</td>
<td>11</td>
<td>15.5</td>
<td>59</td>
<td>83.1</td>
</tr>
<tr>
<td>Cloxacillin</td>
<td>5</td>
<td>7.0</td>
<td>0</td>
<td>0</td>
<td>66</td>
<td>92.9</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>3</td>
<td>4.2</td>
<td>4</td>
<td>5.6</td>
<td>64</td>
<td>90.1</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>30</td>
<td>42.3</td>
<td>0</td>
<td>0</td>
<td>41</td>
<td>57.7</td>
</tr>
<tr>
<td>Penicillin</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>71</td>
<td>100</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>15</td>
<td>21.1</td>
<td>1</td>
<td>1.4</td>
<td>55</td>
<td>77.5</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>1</td>
<td>1.4</td>
<td>0</td>
<td>0</td>
<td>70</td>
<td>98.6</td>
</tr>
<tr>
<td>Bacteria</td>
<td>Ampicillin 10μg</td>
<td>Chloramphenicol 10 μg</td>
<td>Cloxacillin 5 μg</td>
<td>Erythromycin 5 μg</td>
<td>Gentamycin 10 μg</td>
<td>Penicillin 10 μg</td>
</tr>
<tr>
<td>---------------------</td>
<td>-----------------</td>
<td>-----------------------</td>
<td>------------------</td>
<td>-------------------</td>
<td>------------------</td>
<td>------------------</td>
</tr>
<tr>
<td>Staph. aureus (1)</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Staph. aureus (2)</td>
<td>-</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>Staph. aureus (3)</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Staph. aureus (4)</td>
<td>-</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>Staph. aureus (5)</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>Staph. aureus (6)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Staph. delephini (1)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>Staph. delephini (2)</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Staph. delephini (3)</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

The cross corresponds to size of the size zonal inhibition of the organism according to (Arcomex Arab company for Medical Diagnostics).
- + 14 mm or less (resistant)
- ++ 15-16mm (moderately sensitive)
- +++ 17 mm or more (sensitive)
- 8 isolates of S.aureus were resistant
- 3 isolates of S.delephtini were resistant
- 3 isolates of Streptococcus pyogenes were resistant
- 1 isolates of Streptococcus viridans was resistant
Table (16) Sensitivity of *E. coli* and Proteus isolates to the various antibiotics tested

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Ampicillin 10μg</th>
<th>Chloramphenicol 10μg</th>
<th>Cloxacillin 5μg</th>
<th>Erythromycin 5μg</th>
<th>Gentamycin 10μg</th>
<th>Penicillin 10μg</th>
<th>Streptomycin 10μg</th>
<th>Tetracyline 10μg</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> (1)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>E. coli</em> (2)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td><em>E. coli</em> (3)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>E. coli</em> (4)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td><em>E. coli</em> (5)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td><em>E. coli</em> (6)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td><em>Proteus mirabilis</em> (1)</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td><em>Proteus mirabilis</em> (2)</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td><em>Proteus mirabilis</em> (3)</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Proteus mirabilis</em> (4)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Proteus mirabilis</em> (5)</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Proteus mirabilis</em> (6)</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td><em>Proteus mirabilis</em> (7)</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td><em>Proteus vulgaris</em> bio group 3 (1)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>+++</td>
<td>-</td>
</tr>
</tbody>
</table>

3 isolates of *E. coli* were resistant 2 isolates of *klebsiella pneumonia* were resistant
Table (17) Sensitivity of Serratia isolates to the various antibiotics tested

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Ampicillin 10μg</th>
<th>Chloramphenicol 10 μg</th>
<th>Cloxacillin 5 μg</th>
<th>Erythromycin 5 μg</th>
<th>Gentamycin 10 μg</th>
<th>Penicillin 10 μg</th>
<th>Streptomycin 10 μg</th>
<th>Tetracyline 10 μg</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Serratia marcescens</em> (1)</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Serratia marcescens</em> (2)</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Serratia marcescens</em> (3)</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>+++</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Serratia marcescens</em> (4)</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Serratia marcescens</em> (5)</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Serratia plymuthica</em> (1)</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>+++</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Table (18) Sensitivity of Psudomonas, Aeromonas isolates and *Shewanella putrefaciens*, to the various antibiotics tested

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Sensitivity to</th>
<th>Ampicillin 10μg</th>
<th>Chloramphenicol 10 μg</th>
<th>Cloxacillin 5 μg</th>
<th>Erythromycin 5 μg</th>
<th>Gentamycin 10 μg</th>
<th>Penicillin 10 μg</th>
<th>Streptomycin 10 μg</th>
<th>Tetracyline 10 μg</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pseudomonas aeruginosa</em> (1)</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Aeromonas hydrophilia</em> (1)</td>
<td></td>
<td>+++</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td><em>Aeromonas hydrophilia</em> (2)</td>
<td></td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td><em>Aeromonas Salmonicidnia</em> (1)</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Shewanella putrefaciens</em> (1)</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

- 3 isolates of *P. aeruginosa* were resistant
- 1 isolates of *P. fluorescens* was resistant
- 1 isolates of *Shewanella putrefaciens* was resistant
<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Sensitivity to</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ampicillin (10 \mu g)</td>
</tr>
<tr>
<td>Vibrio fluvialis (1)</td>
<td>-</td>
</tr>
<tr>
<td>Vibrio fluvialis (2)</td>
<td>-</td>
</tr>
<tr>
<td>Vibrio fluvialis (3)</td>
<td>-</td>
</tr>
<tr>
<td>Vibrio mimicus (1)</td>
<td>++</td>
</tr>
<tr>
<td>Vibrio vulnificus (1)</td>
<td>-</td>
</tr>
<tr>
<td>Vibrio alginolyticus (1)</td>
<td>-</td>
</tr>
</tbody>
</table>
**Table (20) Sensitivity of Corynebacteria isolates to various antibiotics tested**

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Sensitivity to</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ampicillin</td>
</tr>
<tr>
<td></td>
<td>10μg</td>
</tr>
<tr>
<td><strong>Corynebacteria diphtheriae (1)</strong></td>
<td>-</td>
</tr>
<tr>
<td><strong>Corynebacteria diphtheriae (2)</strong></td>
<td>-</td>
</tr>
<tr>
<td><strong>Corynebacteria diphtheriae (3)</strong></td>
<td>-</td>
</tr>
<tr>
<td><strong>Corynebacteria Pseudodiphtheriticum (1)</strong></td>
<td>-</td>
</tr>
<tr>
<td><strong>Corynebacteria Pseudodiphtheriticum (2)</strong></td>
<td>+++</td>
</tr>
<tr>
<td><strong>Corynebacteria Pseudodiphtheriticum (3)</strong></td>
<td>-</td>
</tr>
</tbody>
</table>
Fig. (5) *Staph. aureus* on blood agar medium with haemolysis
Fig. (6) *Serratia marcescens* on blood agar medium with pigmentation
Fig. (7) *Serratia marcescens* on nutrient agar medium with pigment formation
Fig. (8) *Shewanella putrefaciens* on nutrient agar medium with pigment formation
Fig. (9) *Serratia marcescens* sensitive to gentamycin
CHAPTER FOUR

DISCUSSION
Chapter Four

Discussion

Although micro-organisms are responsible for wound infections, widespread controversy exists regarding the mechanisms by which they cause infection and also their significance in non-healing wounds (Bowler et al., 2001). However, when clinical signs of infections are less evident, as is often the case in diabetic foot ulcer, greater emphasis has been placed on microbiological results (Armstrong et al., 1995). Our investigation aimed to isolate and identify aerobic and anaerobic bacteria that associated with diabetic foot infections. Aerobic polymicrobial population of 2 to 6 groups of bacteria were isolated in 60% of the samples collected and only 40% swab cultures produced monomicrobial aetiology despite the fact that almost all patient receive a combination of antibiotic including metronidiazole. This is in agreement with the finding of Anandi et al., (2004) who revealed polymicrobial aetiology in 64.4% and single aetiology in 19.6% in diabetic foot infection. Also our findings substantiate the previous work of Diamantopoulos et al., (1998). However, the absence of anaerobic growth from tissue samples was not due to technical errors in our cultivation system but rather due to the prior treatment of patients with metronidiazole that selectively kill anaerobic bacteria (Goldstein, 1996), and disturb microbial, aerobic-anaerobic synergistic interactions (Ingham et al., 1977). Nonetheless, in our cultivation control system, Clostridium perfringens grew luxurious. In chronic wound infection the process of wound healing always occurs despite the presence of microorganisms. Thus it is not the presence of microorganisms but rather their interaction with host that determine their influence on wound healing (Anne et al.,
2001). However, diabetic patients are immunocompromized and unable to clear invading microorganisms.

In view of polymicrobial nature of diabetic foot wounds, Karchmer and Gibbons, (1994), questioned the significance of mixed infection in wounds healing. However, the effect of specific type of microorganism on wound healing has been widely published (Bowler et al., 2001). As specific example aerobic pathogen such as *S. aureus*, *P. aeruginosa* and beta-haemolytic streptococci, have been more frequently cited as the cause of delayed wound healing and infection (Bowler et al., 2001). In this study we were able to demonstrate that diabetic foot wounds were associated with high incidence of mixed infection. This is in agreement with the previous finding of Deamantopoulos et al., (1998) and Cibele et al., (2004). However no single microorganism or group of microorganisms was found more determinate to wound healing than any other as reported by Trengove et al., (1996). Nonetheless, a significantly lower probability of healing was observed if four or more bacterial groups were present in any ulcer (Trengove et al., 1996). These observation and our findings support earlier view of Kingston and Seal (1990), who argued that all species associated with a microbial disease should be considered potentially synergistic, rather than a single species being causative, as commonly perceived.

It is intriguing to report that *Staphylococci delephini*, the coagulase positive, was isolated for the first time from diabetic foot and accounted for about 8.4% of sample collected (Table 5). Whereas *S. aureus* was recovered from 19.7% of samples cultured. This is in agreement with previous finding of Ghandour (2001), El Shafi (1992), Goldstein (1996) and Askar et
However the frequency of isolation of *Streptococci pyogenes* was 4.2% and *Streptococcus viridian* was 1.4%. This is in agreement with the results of Anne *et al.* (2001) and Votey and Anne (2005). On the other hand, the frequency of isolation Corynebacteria species was 8.4%, which is the same as reported by Wheat *et al.* (1986).

Moreover, the frequency of the isolation of entrobacteriaceae is comparable to recent work of Anandi *et al.* (2004) and Cibele *et al.* (2004). Also our finding confirmed the previous findings of Abd Elatti (2003) and Cunha (2004) who isolated *P. mirabilis* at a ratio of 9.8%. In contrast Ghandour (2001) reported a frequency of 3% only. *Klebsiella pneumoniae* was isolated from tissue sample (2.8%), substantiating results of Cunha (2004), who isolated this organism from necrotic tissue in diabetics. On the other hand *E. coli* has been reported as the main cause of chronic osteomyelitis in diabetics (Cunha, 2004). In our results the rate of *E.coli* isolation was (12.6%), similar to the previous report of Ali (1999), Cunha (2004) and Votey and Anne, (2005).

Other Gram-negative bacteria incriminated in diabetic foot infection were *P.aeruginosa* (5.6%) with in the same range as reported by Cunha (2004), Ghandour (2001), Anne *et al.* (2001) and Goldstein *et al.* (1996). In addition Vibrio species and Aeromonas species fall within the pathogens isolated in this study at a rate of 8.4% and 2.8% respectively. Cunha (2004) isolated these two species from diabetic foot wounds. Nonetheless, we report for the first time in the Sudan, the isolation of Serratia species at a rate of 8.4% Serratia *marcescens* has been considered as potentially pathogenic to insects (El Sanousi *et al.*, 1987) and involved in nosocomial infections.
In this study *Serratia marcescens* was isolated at a rate of 7%, it tolerates high level of sugar (4%) as indicated in the result and it is often the cause of diseased honey bee larvae (El Sanousi *et al.*, 1987). Recently Cibele *et al.* (2004) were able to isolate *Serratia marcescens* from diabetic foot infection in Brazil. Under the Sudan conditions, contaminated water might be the primary source of infection.

In this limited investigation 70% of the patients were male and 30% were females. The same sex ratio was reported by El Mahady (1991) who explained the low incidence of diabetic foot infection in females by the fact that females are more keen to care about their diabetes and less prone to have complication inducing factors than males.

In our study surface swab taken before debridement and deep tissue specimen taken towards the end of surgical debridement were found rewarding specimens. The profile of poly-microbial isolates from surface wound swabbing and deep tissue swabbing were identical. This led us to the conclusion that swab cultures accurately identify bacterial pathogens in diabetic foot wounds. This is in agreement with the previous finding of Slater *et al.* (2004), Johnson *et al.* (1995) and Laing (1994). Although the value of acquiring superficial swab samples has been seriously questioned (Perry *et al.*, 1991), the procedure is simple, inexpensive, non-vigorous, non-invasive and convenient for the majority of wounds including diabetic foot ulcers. Therefore, it is more likely that superficial wound fluid and tissue debris displayed a full spectrum of microorganism associated with diabetic foot infection. On the other hand swab sampling has been challenged on the basis that superficial microbiology does not reflect that of deep tissue (Perry *et al.*, 1991). However, studies by Bowler and Davis
(1999) have demonstrated the efficacy of swab samples, even for the isolation of anaerobic bacteria from a variety of acute and chronic wounds. Armstrong et al. (1995) observed no difference in the isolation rate of microorganisms from deep tissue and superficial in curettage in 112 diabetic ulcer infections. All these data substantiate our findings and swab sampling remains reliable to provide a meaningful data regarding microbial recovery.

Therapy of patient with diabetic foot wounds should be guided by culture of microorganism and antibiotic sensitivity of the isolates to achieve a better outcome. All the 71 isolates in this study were considered equally important in the causation of diabetic foot infection and in the delay of wound healing as has been proposed previously (Kingston and Seal, 1999). The results of antibiogram of the various isolates to the traditionally and commonly used antibiotics are shown in (tables 14-20). Only 42.2% of the isolates were found sensitive to gentamycin (aminoglycoside) in contrast to the previous report by Ali (1999), who described gentamycin as a drug of choice for treatment of diabetic foot wounds. All our isolates were found resistant to penicillin (100% resistant) and an emerging resistance of 83.1% to chloramphenicol was obtained, although Ali (1999) reported that all Gram-negative bacteria isolated from diabetic wounds were sensitive to chloramphenicol. However, Morales et al. (1999) reported an increased in vitro resistance of Streptococci to clindamycin, erythromycin and penicillin. Clinically Eron (1999) reported that cephalosporine, macrolides, clindamycin and semisynthetic penicillin, such as flucloxicillin and oxacillin are often the treatment of choice for chronic wounds.
CONCLUSIONS

The spectrum of microorganism isolated from superficial swab cultures and deep tissue specimen were identical. Therefore, swab cultures are valuable in identifying pathogens in diabetic foot wounds. In addition when surgical debridement is contraindicated or potentially traumatic to the patient, swab culture can be used to select appropriate antibiotic therapy. Polymicrobial infection was obtained in 60% of diabetic foot wounds and only 40% of the wounds had mono-microbial aetiology. *Staph. aureus* was shown to be the major cause of diabetic foot infection and for the first time *Staph. delephini* and *Serratia marcescens* were isolated from diabetic foot wounds. Other entrobacteriaceae, *E. coli*, Proteus and Klebsiella remain commonly associated with diabetic foot wounds. All our 71 isolates that falls in nine genera were resistant to penicillin and only 42.2% of the isolate were found sensitive to gentamycin. These are alarming results and the efficacy of these and other antibiotic should be reevaluated.
RECOMMENDATIONS

1. Surface wound swabbing and deep tissue swabbing are reliable procedures for collection of samples from diabetic foot wounds.
2. Future studies should focus on the role of *S. aureus* and other enterobacteriaceae including *Serratia marcescens* in the causation of diabetic foot wound infection and non healing wounds. Also future studies should consider the role of anaerobic bacteria in diabetic foot infection and the role of aerobic-anaerobic interaction in the maintenance of microbial synergy.
3. Antibiotic therapy should be guided with antibiograms results.
4. Studies on the emergence of antibiotic resistance in the bacteria commonly isolated from diabetic foot wound should be correlated to community consumption of antibiotics, following the WHO collaborating center for drug statistic methodology recommendation (2002).
5. Early aggressive surgical debridement may yields less microbiologically complex infections that might be controlled with less expensive narrow spectrum antibiotic therapy that may results in a less complex hospital course and improve the outcome.
REFERENCES


Appendix

Questionnaire

File No. …………………. Date & Time: ………………….

Name: ………………………………………………………………………………………………

Age …………………. Sex: ………………….

Address: ………………………………………………………………………………………………
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Clinical Diagnosis:
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…………………………………………………………………………………………………………

History of:

1. Diabetes: ……………………………………………………………………………………………

2. Wound infection:
   A) ……………………………………………………………………………………………
   B) ……………………………………………………………………………………………
   C) ……………………………………………………………………………………………

2. Antibiotics: ……………………………………………………………………………………………

3. Drug: ……………………………………………………………………………………………

Specimen Collection: ……………………………………………………………………………………………
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