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4. Conclusions and Recommendations

4.1 Conclusions:

- Diabetic foot infection was more common in patients of (60-70) years old.
- Polymicrobial pattern was the most common infection found in patients of diabetic foot .
- Gram (-) bacteria especially *Klebsiella pneumoniae* was the predominant pathogens in the diabetic foot infections, and *Staphylococcus aureus* was the most common of Gram (+) bacteria.
- All bacterial isolates were completely sensitive to imipenem except *Acinetobacter baumannii* that resisted all antibiotics used.
- Multi-drug resistance (MDR) pathogens were more commonly isolated from the diabetic foot infections.
- Local treatment of RIF gave more inhibitory effect on diabetic foot infections than the systemic treatment.
- Rifampicin solution that used for local treatment of patients gave the most efficient result on diabetic foot infections.

4.2 Recommendations:

- More studies are needed on other types of aerobic and anaerobic bacteria as well as fungi may exist in diabetic foot infections.
- The clinicians recommended to limit using systemic treatment, and Focus on local treatment against foot infections especially those caused by the multi-drug resistant pathogens.

1. Introduction and Literature Review:

1.1 Introduction:

Diabetic foot ulceration (DFU) or infection is one of the leading causes of human mortality and morbidity. It represents a severe complication of diabetes and the most common cause of diabetes associated hospital admissions (Lavery *et al.,* 2007). The number of cases associated with diabetic foot infection (DFI) has dramatically increased in the recent years. The main reason for this infection is the growing of diabetic population among younger groups.

Ulceration of the foot in diabetes is very common and frequently leads to the amputation of limps (Sharma *et al.,* 2006). The risk of the lower limps amputation is 17 to 41 times higher in the diabetics than in persons who do not have diabetes mellitus.

The major aetiologies of DFUs are neuropathy (nerve damage), peripheral vascular (arterial) disease and neuroischaemia (Lazarus *et al.,* 1994 and Levin, 1997). 40–70% of DFUs are caused by neuropathy, 15–24% by peripheral vascular disease and 15–45% by neuroischaemia (Frykberg *et al.,* 2000).

Approximately half of all foot wounds become infected over the course of diabetic therapy (Lavery *et al.,* 2007). Diabetic foot ulceration and its sequence (infection, gangrene and amputation) are associated with a reduced quality of life, high morbidity and premature mortality. It is predicted that the number of people with diabetes will rise from an estimated 171 million in 2000 to 366 million in 2030 (Wild *et al.,* 2004).

Initial therapy of diabetic foot infections is frequently empiric because reliable culture data is lacking. There is variability in prevalence of common bacterial pathogens isolated between Gram (+) and Gram (-) bacteria, as shown (Viswanathan *et al.*, 2002). The choice of empirical antimicrobial therapy is influenced by various factor such as severity of the illness (Wagner grading), the most likely type of causative organism, and coexisting complications. Host factors, for example co-morbid conditions, good glycemic control, concomitant renal and cardiovascular diseases can affect the need for hospital admission and choice of specific agents of their dosing intervals (Sharma *et al.*, 2006).

In diabetic foot ulcer, patients mortality is high and healed ulcers often recur, in addition the pathogenesis of foot ulceration is complex (Bano *et al.*, 2012).

Various methods, such as antibiotics and organic acids, are applied for treatment of diabetic patients suffering from foot infections. Using antibiotics is the most common one, but the way that the antibiotic be used to give better results needs to be more investigated, as well as trying other means for treatment of diabetic foot infection.

For that, this study was aimed to apply and select the most efficient method and way for treatment of diabetic foot infection, concentrating on local and systemic use of the most common antibiotic.

To achieve such goal, the following steps were used:

- Isolation and identification bacteria causing diabetic foot infection in patients.
- Assessing the *in vitro* susceptibility of bacterial pathogens to the commonly used antibacterial agents.
- Treatment of the diabetic foot infection patients with systemic and local treatment that chosen depending on the antibiotic susceptibility findings.
- Selecting the most effective treatment (local or systemic) against diabetic foot infection.

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1.2 Literature Review:

1.2.1 Diabetes:

1.2.1.1 History and definition:

Diabetes has been known to be a potentially lethal disease for more than thousand years (Dobson, 1968). It is chronic incurable disorder which can be managed but not cured .The history of diabetes has involved many contributions from over the world. The first mentioned is the medical condition that was distinguishable as diabetes was found in an ancient papyrus, discovered in 1862 by German Egyptologist George Ebers (Papaspyros, 1982).

In the fifth century, a reputed Indian Physician, Dr. Sushruta, for the first time recognized the two primary types of diabetes, one affecting very thin people and another form most often seen among the obese individuals (Leonid, 2009).

In 1869, a German researcher Paul Langerhans highlighted a special cluster of cells within the pancreas. Although the cells were recognized, the role they played in diabetes was not established.

Oskar Minkowski In 1889, performed experiments on removing the pancreases of dogs, thus inducing diabetes in the animals and definitively proving the significance of the pancreas (Leibowitz, 1972 ; MacLeod, 1978).

Edouard Laguesse in 1893, noticed the importance of the cells identified by Langerhans and in honor of Langerhans called (Islands of Langerhans). They are now known as islets of Langerhans (Leibowitz, 1972). In 1921, insulin was isolated by Frederick Banting and Charles Best from the pancreas of an animal and used to treat diabetes in humans (Leonid, 2009).

Identification of pancreas as the organ causing the diabetes led to the discovery of Islets of Langerhans and subsequently the isolation of insulin; one of the most significant milestones in diabetes research (Leibiger *et al*., 2010). Most recently hopes have been raised that a cure is imminent with advances in the transplantation of beta cells, stem cell therapies and research into gene therapy (Robertson *et al*., 2003 ; George, 2009 and Johnson and Luciani, 2010).

In the 21st century, the treatment options for patients with diabetes include transplantations of beta cells, stem cell transplants as well as transplants of organs such as pancreas (Gunaselli *et al*., 2010 ; Johnson and Luciani, 2010).

Genetic engineering was used to produce naturally occurring peptides that are able to stimulate the growth of insulin producing cells in the pancreas (Garcia *et al*., 2001). Advances in diabetes research are significant and much needed because diabetes is on the rise worldwide and is considered by some experts already to be at an epidemic level (Zimmet *et al*., 2001).

1.2.1.2 General effects of diabetes:

Diabetes have different effects on human health. These typically develop after many years (10–20), but may be the first symptom in people that not received a diagnosis before that time. The major effect is damage to blood vessels. Diabetes doubles the risk of cardiovascular disease (Emerging Risk Factors Collaboration, 2010) . The main macrovascular diseases are ischemic heart disease (angina and myocardial infarction), stroke and peripheral vascular disease. The capillaries also damages in Diabetes causes microangiopathy (Boussageon *et al.*, 2011).

Diabetic retinopathy, which affects blood vessel formation in the retina of the eye, can lead to visual symptoms, reduced vision, and potentially blindness. Diabetic nephropathy, the effect of diabetes on the kidneys, can lead to changes in the kidney tissue, loss of small or larger amounts of protein in the urine, and eventually chronic kidney disease requiring dialysis. Diabetic neuropathy, the impact on the nervous system, most commonly causing numbness, tingling and pain in the feet and also increasing the risk of skin damage due to altered sensation (Boulton, 2007). Together with vascular disease in the legs, neuropathy contributes to the risk of diabetes related foot problems.

1.2.1.3 Types of diabetes:

Rybka (2010) classified diabetes into two main types, diabetes mellitus and diabetes insipidus . Both diseases have the same symptoms of thirst and urination except that diabetes insipidus is caused by deficiency of the anttidiuretic hormone released by the pituitary glands

which directly affects water retention, hence diabetes insipidus is called the water diabetes. Diabetes mellitus on the other hand is called the sugar diabetes and is caused by the pancreas malfunctioning leading to insulin deficiency or a defect in the secretion of insulin or insulin resistance (David and Dolores, 2011).The research presented in this thesis is focused only on diabetes mellitus.

1.2.1.4 Diabetes mellitus disease (DM):

The centers for Disease Control and Prevention, (2011) classified diabetes mellitus into 3 main types: type 1, type 2, and gestational diabetes.

– **Type 1 diabetes mellitus (T1DM):** known as insulin dependent diabetes mellitus which is an autoimmune disease where the immune system destroys the insulin producing beta cells in the pancreas. This type of diabetes is also known as juvenile-onset diabetes and it can appear at any age below the age of 40 year.

– **Type 2 diabetes mellitus (T2DM):** The most significant type that known as non-insulin dependent diabetes mellitus. This type of diabetes is also known as late-onset diabetes and is characterized by insulin resistance and relative insulin deficiency. (this type will be more discussed below).

– **Gestational diabetes mellitus (GDM):** is the third type of diabetes which is found in pregnant women. The risk factors for GDM include a family history of diabetes, increasing maternal age and obesity (Landon, 2010).

1.2.1.5 Symptoms of Type 2 diabetes mellitus:

Characteristic symptoms of diabetes mellitus are the result of abnormal glucose metabolism. Due to insulin resistance, glucose does not reach the cells and accumulates in the blood, causing hyperglycaemia which is subsequently excreted in the urine (glycosuria). Glucose in the blood causes osmotic imbalance and hence causes frequent urination (polyuria) and because of the protein breakdown during gluconeogenesis in an attempt to provide more glucose to the cells there is weight loss, also polydipsia (increased thirst) and polyphagia (increased hunger) can develop rapidly (weeks or months) in type 1 diabetes, while they develop much more slowly in type 2 diabetes (Cooke and Plotnick 2008).

1.2.1.6 Complications of Type 2 diabetes mellitus:

Complication of diabetes affects almost all parts of the body. Patients with Type 2 diabetes mellitus (T2DM) have an increased risk of microvascular coronary heart disease, myocardial infarction leading to cardiovascular disease (Emerging Risk Factors Collaboration, 2010), peripheral vascular disease, neuropathy, nephropathy, retinopathy, cerebrovascular disease and diabetic foot ulcerations and infection. The consequences of infection are devastating and can led to amputation (Ousey and McIntosh, 2008). Boulton *et al.*, (2007) have suggested that infected foot ulceration precedes about 60% of lower extremity amputation in diabetic patients. This thesis take Type 2 diabetes mellitus and one of the complications, diabetic foot infections associated with it.

1.2.1.7 Diabetic foot infection (DFI):

Diabetic foot is one of the complications of diabetes and is the leading cause of hospitalization in diabetic patients(Frykberg *et al.,* 2007). Diabetic foot patient is characterized by several pathological complications such as neuropathy, peripheral vascular disease, foot ulceration and infection with or without osteomyelitis, leading to development of gangrene and even necessitating limb amputation (Anandi *et al.,* 2004 ; Khanolkar *et al.*, 2008). According to the Infectious Disease Society of America (IDSA) guidelines, infection is present if there is obvious purulent drainage and/or the presence of two or more signs of inflammation (erythema, pain, tenderness, warmth, or induration) (Lipsky *et al.,* 2004).

Diabetic patients have 25% risk for developing foot ulceration than other people (Singh *et al.,* 2005*).* Diabetic ulcers have 15 to 46 times higher risk of limb amputation than foot ulcers of other causes (Alavi *et al.,* 2007) Every year, more than a million diabetic patients require limb amputation (Khanolkar *et al.,* 2008).

1.2.1.7.1 Causes of diabetic foot infection:

The cause of diabetic foot begin from tissue damaging. Once the protective cutaneous barrier is breached, skin flora can gain access to the subcutaneous tissue, proliferate, and cause the host inflammatory response that classifies as infection (Lipsky *et al.,* 2004). Reduced sensation can substantially impair the patient's perception of touch, deep pressure, temperature, and joint position.

Peripheral vascular disease in diabetes characteristically affects vessels between the knee and the ankle (Bates and Aburahma 2004). Mechanical damage to poorly perfused (and often friable) tissues typically causes ulcers. The foot injury that initiates ulcers could result either from trauma or from mechanical stress that is repetitive (walking bare foot or in improper footwear).

Thermal injury (Dijkstra *et al.,* 1997) , foot deformity resulting in bony prominences (Robertson *et al.,* 2002) , restricted joint mobility (Zimny *et al.,* 2004) , poor foot care (Connor and Mahdi 2004) and bites from animals and vermin (Abbas *et al.,* 2005) all contribute to risk of ulceration and then infection. The impaired microvascular circulation in patients with diabetic foot limits the access of phagocytes favouring development of infection (Anandi *et al.,* 2004 ; Gadepalli *et al.,* 2006).

1.2.1.7.2 Pathophysiology of diabetic foot infection:

Several factors predispose diabetic patients to developing a DFI, including neuropathy, vasculopathy and immunopathy. Peripheral neuropathy occurs early in the pathogenesis of diabetic foot complications and considered the most prominent risk factor for diabetic foot ulcers (Reiber *et al.,* 1999). Diabetic patients with impaired protective sensation and altered pain response are vulnerable to trauma and extrinsic forces from shoe wear. Motor neuropathy causes muscle weakness and intrinsic muscle imbalance leading to deformation such as hammered or clawed toes

Autonomic dysfunction leads to changes in microvascular blood flow and arteriolar-venous shunting, diminishing the effectiveness of perfusion and elevating skin temperatures. With the loss of sweat and oil gland function, the diabetic foot becomes dry and keratinized which break more easily, leading to infection. Diabetic angiopathy is the most frequent cause of morbidity and mortality in diabetic patients (Joseph and LeFrock, 1987).

Macroangiopathy effect large vessels, which leads to peripheral arterial disease (PAD) of the lower extremities. Microangiopathy results in capillary basement membrane thickening, altered nutrient exchange, tissue hypoxia and microcirculation ischemia (Diabetes Care, 2003). All these are evidence supports that the patients at risk for ulceration and for wound healing.

Immunopathy explain that the impaired host defenses, because of hyperglycemia, have defects in leukocyte function and cause morphologic changes to macrophages. Bagdade *et al.*, (1974) demonstrated that leukocyte phagocytosis was significantly reduced in patients with poorly controlled diabetes.

Healing of the wound involves many processes which requires various cellular and inflammatory pathways including phagocytosis, chemotaxis, mitogenesis, collagen synthesis and the synthesis of other matrix components (Clark, 1996). In diabetic patients the cellular and the inflammatory pathways involved in wound healing are affected. Decreased chemotaxis of growth factors and cytokines impede normal wound healing by creating a prolonged inflammatory state.

Hyperglycemia with the presence of an open wound create a catabolic state. Negative nitrogen balance with little insulin caused by gluconeogenesis from protein breakdown. This metabolic dysfunction impairs the synthesis of proteins, fibroblasts and collagen (Inzucchi, 2006). Patients with diabetes tolerate infection poorly and infection adversely affects diabetic control. This repetitive cycle leads to uncontrolled hyperglycemia, that affecting the host's response to infection.

The pathophysiological mechanisms of diabetic foot infections are still a subject of controversy. So various hypotheses are proposed :

A. Deficiency of cell mediated immune mechanisms

In diabetes, hyperglycaemia alters leukocyte functions (neutrophils and fibroblasts), through hyperosmotic effects, and this breaking the healing cascade (Lazarus *et al.*, 1994).

B. Neuropathy and excessive pressure

Neuropathy predisposes the foot to infection. Loss of protective sensation (sensory loss) is a major cause of diabetic foot ulceration then infection, about 45–60% of all diabetic ulcerations considered to be neuropathic (Frykberg *et al.*, 2006). In the insensate foot, a number of factors increase the risk of ulceration and infection, including inappropriate footwear, trauma and repetitive stress. And angiopathy Influence the outcome (Edmonds and Foster, 2004 ; Armstrong *et al.*, 2007).

C. Anatomy of the foot

Motor neuropathy causes atrophy of the intrinsic foot muscles, altering the foot architecture leading to loss of integrity of the bone,which lead to bony prominence that lead to pressure and then trauma, which may end in osteomyelitis. Bacterial invasion result from the loss of integrity of the skin and causes trauma that results in infections (Tan and File, 1999).

D. Chronic nature of the lesion

Autonomic neuropathy causes dry skin and predisposes the skin to cracking. A foot with sensory neuropathy tends to suffer repeated injury, thus disrupting the skin integrity and providing a route for microbial invasion leading to an unhealed wound which then develops into a chronic ulcer (Bjarnsholt *et al.,* 2008). Additionally, the excess sugar lowers the resistance to infections which leads to a gangrenous ulcer with lower limb amputations (Gadepalli *et al*., 2006).

E. Hypoxia

The faulty healing response is affected due to the wound hypoxia caused by the microvascular and macrovascular changes within the diabetic patients (Yamasaki *et al.,* 2010 ; Kashiwagi, 2010). Due to a poor local perfusion by the host's hypermetabolic state and microbial cellular metabolism. Hypoxia promotes anaerobic subcutaneous infections and decreases the bactericidal activity of neutrophils.

F. Arterial disease

The microvascular defects lead to injury in small blood vessels leading to vasoconstriction. As the condition progresses the vascular abnormalities affect the membranes of the blood vessels leading to macrovascular conditions mainly peripheral vascular disease affecting the leg arteries. predictors of diabetic foot wound healing differed between patients with and without PAD, so these defined as two separate disease states and adverse effect of infection on healing was confined to patients with PAD (Prompers *et al.,* 2008). Arterial disease decreasing the blood supply to the wound and then the influx of endogenous and exogenous factors (antibiotics) involved in the fight against infection.

1.2.1.7.3 Clinical aetiology of diabetic foot infections:

A diabetic foot ulcer involves repeated infections due to aerobes, anaerobes or fungi individually or in combination. The infection starts locally with an ulcer affecting immediate surrounding skin with a purulent discharge and erythema. These signs can be missed due to the presence of neuropathy and ischemia which are the commonest risk factors to DFI (Boulton, 2007 ; Pendsey, 2010). The infection can become a spreading infection as the sepsis progresses to cellulitis. This spreading infection can become severe causing deep soft tissue damage. The deep tissue fills with pus and can cause abscess formation subsequently leading to tissue necrosis and severe bacteraemia and some time septicemia.

1.2.1.7.4 Classification systems of diabetic foot infection:

Several classification systems have been proposed and utilized for the assessment of diabetic foot ulceration (DFU) and DFI, but each system has different parameter for classification, so each one with its parameter will be discussed below in tables. There is no one universally accepted classification system. Classification can facilitate the treatment and can aid in the prediction of outcome (Frykberg, 2002).

A. Wagner classification system:

Wagner classification system is based on the depth of penetration, the presence of gangrene and the extent of tissue necrosis as shown below in table (1-1).

Table (1-1): Wagner classification system of Diabetic foot infection(DFI): (Wagner, 1987)

Wound grade	Description of grade
0	No lesion
1	Superficial ulcer
2	Deep ulcer
3	Abscess Osteitis
4	Gangrenous forefoot
5	Whole foot

The Wagner classification is one of the most widely use classification system, however, it does not consider two critically important parameters; ischemia and infection (Frykberg, 2002). So this classification is limited by its inability to recognize ischemia and infection as independent risk factors in all classification grades (Oyibo *et al*., 2001).

B. University of Texas diabetic wound classification:

The University of Texas diabetic wound classification system assesses the depth of ulcer penetration, the presence of wound infection, and the presence of clinical signs of lower-extremity ischemia (Oyibo *et al.*, 2001). The stages and grades of ulcers depth and ischemia are shown below in table (1-2).

Table (1-2): University of Texas Health Science Center classification system of Diabetic foot infection (DFI): (Oyibo *et al.*, 2001)

Grade / Stage	0	1	2	3
A	No open lesion	Superficial Wound	Tendon/ Capsule	Bone/Joint
B	With infection	With infection	With infection	With infection
C	Ischemic	Ischemic	Ischemic	Ischemic
D	Infection/ Ischemia	Infection/ Ischemia	Infection/ Ischemia	Infection/ Ischemia

This system uses the four grades of ulcer depth (0 to 3) and four stages (A to D), based on ischemia or infection, or both thus covering both the significant co morbidities of a diabetic foot ulceration(DFU).but it does not include measures of neuropathy or ulcer area. Oyibo *et al.*, (2001) evaluated 194 DFU, utilizing both Wagner and University of Texas classifications to compare patient prognosis. Their results revealed that the University of Texas grade had a slightly greater association with increased risk of amputation and prediction of ulcer healing, and it is a greater predictor of clinical outcome than Wagner's classification.

C. Curative health services (CHS) database:

The CHS wound database classifies wounds on basis of the rate at which anatomy of the wound was affected to the progression of infection as seen in table (1-3).

Table (1-3): Curative health services (CHS) wound grade scale of Diabetic foot infection(DFI): (Margolis *et al*., 2002)

Wound grade	Description of grade
1	Partial thickness involving only dermis and epidermis
2	Full thickness skin and subcutaneous tissues
3	Grade 2 plus exposed tendon, ligament and or joint tissue
4	Grade 3 plus abscess and or osteomyelitis
5	Grade 3 plus necrotic tissue in wound
6	Grade 5 plus necrotic tissue surrounding the wound

D. Infectious Diseases Society of America (IDSA) and the International Working Group on the Diabetic Foot (PEDIS system):

The Infectious Diseases Society of America published guidelines in 2004 (Lipsky *et al*., 2004) that subclassify infected diabetic foot wounds into the categories of mild (restricted involvement of only skin and subcutaneous tissues), moderate (more extensive or affecting deeper tissues), and severe (accompanied by systemic signs of infection or metabolic instability).

Similarly, the International Working Group on the Diabetic Foot has proposed the PEDIS classification system in table (1-4) (Schaper, 2004) which grades the wound on the basis of five features: perfusion (arterial supply), extent (area), depth, infection, and sensation.

Table (1-4): Clinical Classification System Proposed by the Infectious Diseases Society of America (IDSA) and the International Working Group on the Diabetic Foot (PEDIS system) for a Foot Infection in a Person with Diabetes. (Lipsky *et al*., 2004)

Clinical Manifestation of Wound	IDSA	PEDIS
No purulence or evidence of inflammation (i.e., erythema, pain, tenderness, warmth or induration)	Uninfected	1
Infected (≥2 of above) but any erythema extends ≤2 cm around ulcer & infection limited to skin/superficial subcutaneous tissues. No local complications or systemic illness	Mild	2
Infected patient who is systemically well & stable metabolically but has at least one of following: cellulitis >2 cm; lymphangitis; spread beneath fascia; deep tissue abscess; gangrene; muscle, tendon, joint or bone involved	Moderate	3
Infected patient with systemic toxicity or metabolic instability (e.g., fever, chills, tachycardia, hypotension, confusion, vomiting, leukocytosis, acidosis)	Severe	4

1.2.1.7.5 Microorganisms accompanying diabetic foot infections:

Diabetic foot infections are often complex and polymicrobial in nature (Alavi *et al.*, 2007), they contain aerobes, anaerobes and fungi that affecting the diabetic foot.

A. Aerobic bacteria in diabetic foot infections:

The microbiology of diabetic foot wound is complex. Human body has a vast number of bacteria living as normal flora with skin harboring many commensal. In a diabetic patient with poor immune responses, even a normal skin commensal can cause significant infection. Common skin commensal such as *Staphylococcus aureus* and *Staphylococcus epidermidis* are seen as pathogens in diabetic wounds (Citron *et al*., 2007). Other nasal commensal such as *Streptococcus* species have also been cultured from the diabetic wound clinical specimens. *Streptococcus* species rarely cause infection but can, in rare cases cause severe blistering cellulitis and tissue destruction (Loan *et al*., 2005).

Gram (–) aerobes such as the *Citrobacter sp., Escherichia coli, Pseudomonas aeruginosa, Acinetobacter* and *Serratia spp.* are examples of normal flora often cultured from a diabetic wound. There have been a number of studies showing the pathogenic nature of the Gram (–) bacteria in DFI (Viswanthan *et al.,* 2005 ; Abdulrazak *et al.*, 2005 ; Gadepalli *et al.*,2006 ; Citron *et al*., 2007 ; Bansal *et al.*, 2008). Although these are normal flora of the intestinal tract, they are isolated

from the diabetic foot ulcer as the patients may lack good hygiene due to their age or obesity (Cowen and Steels, 2004). These organisms often act in synergy with other bacteria and can cause severe infections (Ambrosch *et al.*, 2003).

B. Anaerobic bacteria of diabetic foot infections:

Anaerobes are often present as normal flora of the skin and mucous membranes. In states of distorted host defense or the skin integrity is disrupted, they can colonize and invade the vassal channels (Finegold, 1993).

In diabetic foot patients, due to ischemia and neuropathy with vascular inefficiency, tissue anoxia occurs which lowers the redox potential and favours the growth of anaerobic bacteria (Armstrong *et al.*, 2002; Stefanopoulos and Kolokotronis, 2004).

Within a wound environment, and in the presence of dead tissue, obligate anaerobes are amongst the dominant groups of microorganisms, despite the frequent exposure of the wound to air (Bowler *et al.*, 2001).

As the aerobic bacteria grow, they consume oxygen and create a more favorable environment for anaerobic bacteria. This has been demonstrated in studies involving communities of oral bacteria (Bradshaw *et al.*, 1996 ; Bradshaw *et al.*, 1998).

Most of the infections that harbour anaerobes have a foul odour, gas in the specimen and the location of infection is in proximity to a mucosal surface. There are specific major populations of bacteria evident in the

diabetic wound. Gram (+) anaerobes include the cocci (*Peptostreptococcus* spp.), which form part of the normal flora of humans, are the most frequently isolated from the clinical specimens. It includes the Gram positive spore forming (*Clostridium* spp.) along with other Gram positive non spore forming (*Propionibacterium* spp) (Citron *et al.,* 2007 ; Esposito *et al.,* 2008 and Baines *et al*, 2008). Most common Gram (–) anaerobes cultured from the DFI are *Bacteroides fragilis*, *Prevotella* species, *Fusobacterium* species and *Veillonella* species (Ge *et al*., 2002 ; Fille *et al.,* 2006).

C. Fungi in diabetic foot infections:

Fungi are also found as normal flora in the mucosal organs, skin, mouth and the digestive system. In certain situations, such as during illness, use of too many antibiotics and obesity fungi are capable of multiplying, also any damage to the skin can encourage fungal infections. These characteristics are often seen among the diabetic foot patients. Due to neuropathy and repeated trauma to the skin, fungi gain entry and multiply. One of the clinically significant fungi in DFI is *Candida* spp. It is a clinically significant fungus and has been shown to cause blood stream infections. *Candida* has also been shown to cause other deep seated yeast infections in immuno compromised diabetic foot patients (Bansal *et al*., 2008).

The most significant species of *Candida* isolated from diabetic foot are; (*C. tropicalis, C. albicans, C. guilliermondii and C. pseudotropicalis*),

these species which in association with other organisms become opportunistic (Mlinaric *et al.,* 2005). There are many host factors in patients suffering from diabetic foot infection which contribute to the pathogenicity of *Candida*. Hyperglycaemia is known to induce defects in the host granulocyte function, thereby leading to enhanced growth and tissue invasiveness, thus diabetic patients are at higher risk of systemic *Candida* infections (Heald *et al*., 2001).

1.2.1.٧.6 Polymicrobial occurrence in diabetic foot infections:

Diabetic foot infections are often complex and polymicrobial in nature (Lipsky *et al.,* 1990 ; Hunt, 1992 ; Bowler and Davies, 1999 ; Ge *et al.,* 2002 ; Vishwanathan *et al.,* 2002 ; Gadepalli *et al.,* 2006 and Alavi *et al.,* 2007). One of the largest surveys carried out, on a total of 825 patients, investigated the microbiological profiles of patients with mild and moderately infected diabetic foot ulcers. They revealed an average of 2.4 organisms recovered per wound.

In the infected diabetic foot ulcers, 75% had multiple microorganisms (Ge *et al.,* 2002). One of the most observations made in microbiological studies of diabetic foot ulcers is that the obligate anaerobes were never found alone, they were always isolated with aerobes signifying a relationship with each other and the polymicrobial nature of the infection (Finegold and Wexler, 1988 ; Hartemann *et al.,* 2004).

1.2.2 Treatment of diabetic foot infection:

Defining the microbiology of an infection is the first step to deciding the most appropriate antibiotic treatment. In general, while all wounds are colonized with microorganisms, only those that show clinical signs of infection require antimicrobial therapy. Systemic antibiotic therapy should be relatively narrowly targeted when possible, but broader spectrum or specially targeted therapy is often indicated when a patient has a clinically severe infection or is likely to be infected with a resistant pathogen. A moderate diet with low fat, salt and sugar along with exercise for overweight patients, was offered as a treatment to people with diabetes and still offered now (O‟Gorman and Krook, 2008 ; Bluher and Zimmer, 2010).

In the past few years, many studies have reported the results of treatments for DFIs (Lipsky, 2008). These include antimicrobial agents of various types, delivered in different ways, as well as several kinds of adjunctive treatments.

Unfortunately, there is still little or no evidence to support the effectiveness of many treatments. In fact, a recent systematic review of the effectiveness of antimicrobial treatments for diabetic foot ulcers summarized the results of papers published up until November 2002 (Nelson *et al*., 2006). The authors, after reviewing the 23 eligible randomized or controlled clinical trials, concluded that " the evidence is too weak to recommend any particular antimicrobial agent. Large studies are need of the effectiveness and cost effectiveness of antimicrobial interventions" (Bergin, 2006).

1.2.2.1 Systemic antimicrobials therapies:

Systemic antibiotics, mainly including the penicillins, cephalosporins, aminoglycosides and quinolones, can be used against a wide variety of Gram (+) and Gram (–) organisms (Vuorisalo *et al.*, 2009).

Several studies of systemic antibiotic therapy of DFIs have been published in the past few years. In light of the concern for Methicillin-Resistant *Staphylococcus aureus* (MRSA) infections. Lipsky *et al.*, (2004) ; Lipsky and Stoutenburgh, (2005) ; Citron *et al.*, (2007) ; Lipsky *et al.*, (2009) all of them compare two agent in a large group of patients with a DFI then have a clinical , microbiological outcomes and safety profile for each one. While these studies make it difficult to select any one agent as preferable to others, it is demonstrate the effectiveness of several new antibiotics. On the basis of these studies, linezolid, ertapenem and piperacillin/tazobactam have been approved by the FDA of the united state specifically for treating DFIs (but not for osteomyelitis).

Multidrug resistance is an increasing problem in isolates from DFIs, especially MRSA and extended-spectrum β-lactamase-producing Gram- negative bacteria. In two reports, the now rarely used polymixin agent colistin (alone or combined with other antimicrobials) was found to be effective in treating a series of diabetic patients with soft tissue or bone infections caused by multidrug-resistant *P. aeruginosa* (Tascini *et al.*, 2006).

One pharmacokinetic analysis of therapy with oral and parenteral route for the same agent in patients with a DFI found that a reduction in viable bacteria was reached significantly earlier with continuous intravenously (IV) infusion compared with intermittent dosing (Sedivy *et al.*, 2004).

A recent systematic review looked at randomized controlled trials of diabetic foot infections to determine what factors might be associated with treatment failure (Vardakas *et al.*, 2008). Among the 18 trials identified, the combined observed treatment failure rate was 23%. Comparing different regimens of antibiotics suggested that carbapenems were associated with fewer treatment failures, while MRSA infections, alone or as part of a polymicrobial infection, were associated with more treatment failures.

1.2.2.2 Adjunctive therapies:

Several therapies that are not directly antimicrobial have been used in conjunction with antibiotics or other treatments in an attempt to improve outcomes in DFIs. Certainly, all patients need supportive therapy, including optimal glycemic control , proper wound dressings, and fluid and electrolyte resuscitation for severely ill patients. Most patients also need some type of surgical procedure. Among the more widely used adjunctive treatments is systemic hyperbaric oxygen (Cimsit *et al.*, 2009).

It is difficult to interpret the results of the many published case series, but a systematic review of four randomized controlled trials with a total of 147 patients concluded that there was some benefit to the therapy, especially in reducing major amputations (Kranke *et al.,* 2004).

Another expensive new technology is granulocyte colony stimulating factor (G-CSF). A systematic review of the five published randomized controlled trials with a total of 167 patients found that the various regimens used afforded no improvement in resolving infection but they were associated with significantly fewer operative interventions (including amputations) (Cruciani *et al.,* 2005).

1.2.2.3 Topical antimicrobial therapies:

Topical antimicrobial therapy continues to be an appealing method for treating infected wounds. Several new silver based products shown to have broad spectrum effect against both the Gram (+) and Gram (–) organisms along with the yeasts and fungi (O‟Meara *et al.,* 2000). But a recent Cochrane systematic review that examined papers published through 2004 concluded that, "despite the widespread use of dressings and topical agents containing silver for the treatment of diabetic foot ulcers, no randomized trials or controlled clinical trials exist that evaluate their clinical effectiveness" (Bergin and Wraight, 2006). Similarly, there are few studies of the efficacy (or safety) of topical iodides in treating DFIs (Flynn, 2003). Investigational topical agents for treating DFI include antimicrobial peptides (Lipsky *et al.,* 2008) and super oxidized water solutions (Nelson *et al.,* 2006 ; Zahumensky, 2006). Studies to determine the usefulness of several of these new agents are currently being developed.

Investigators have tried a variety of antibiotic delivery mechanisms to treat open diabetic foot wounds. These include biodegradable materials, such as vancomycin impregnated calcium sulfate beads and gentamicin incorporated into collagen (Armstrong *et al.,* 2003 ;Heijink *et al.,* 2006). These devices can deliver high local antibiotic concentrations, for a sustained period of time with minimal systemic levels.

Another novel method of treating infected foot ulcers is the Biogun (Dang *et al.,* 2006 ; Lipsky, 2006). This device ionizes molecular oxygen and generates superoxide radical anions (O2 -) that have a bactericidal effect against microorganisms. In a study of 15 patients with MRSA colonization of a diabetic foot ulcer, this device eradicated the organism from 60%.

Honey, a topical agent that has been used for many years, has recently been promoted for treating MRSA infections (Molan and Betts, 2008).

A bacteriophage therapy is another example, through using viruses to kill the bacteria, but this method was fell out after the discovery of antibiotics (Shasha *et al.,* 2004). Weber-Dabrowska *et al.,* (2000) reported that 1300 patients infected, by multi resistant bacteria, recovery in 85% and transient improvement in another 11%. Determining which of these old or new remedies may prove useful in treating DFIs will require proper controlled trials.

1.2.2.3.1 Acetic acid (vinegar):

A. Definition of Acetic acid and vinegar:

Acetic acid is an organic compound with a chemical formula of CH_3CO_2H (also written as CH_3COOH or $C_2H_4O_2$). It is a colorless liquid with a distinctive sour taste and pungent smell. Despite that it is classified as a weak acid, but corrosive when concentrated. Vinegar is a liquid substance consisting mainly of acetic acid (CH_3CO_2H) and water. Other constituents of vinegar include vitamins, mineral salts, amino acids, poly phenolic compounds and nonvolatile organic acids (Morales *et al.*, 2002 ; Natera *et al.*, 2003).

FDA (2006) states that diluted acetic acid is not vinegar and should not be added to food products. The word "vinegar" derives from the old French *vin aigre*, meaning "sour wine". It is today mainly used in the kitchen as a general cooking ingredient, but historically, as the most easily available mild acid, it had a great variety of industrial, medical, and domestic uses, some of which are still promoted today.

B. Mechanism of action of vinegar:

The mechanism of action of acetic acid is through the acidification of a wound which increases the pO_2 and reduces the histotoxicity of ammonia which may be present. This acidification of a wound is, however, relatively short lived. Leveen (1973) stated that the wound does not maintain acidity for periods longer than about one hour and ,therefore, soaks would be required as a frequent replacement

D. Antimicrobial Uses of vinegar:

Vinegar was thought to be useful for treating infections in ancient times. Hippocrates (460-377 BC) prescribed it for curing pleurisy, fever, ulcers, and constipation. It was used by the ancient Egyptians to kill bacteria. When combined with honey to create oxymel, it was a common cough medicine in the ancient world. Vinegar also had multiple uses in ancient Babylon, where it was made from wine beginning around 5000 BC. The Babylonians used vinegar to preserve food and as a component of medicines (Myers and Richard, 2007).

As early as in 1916, elimination of *Pseudomonas* in superficial war wounds with the application of 1% acetic acid was reported. Again in 1968, a 5% solution of acetic acid was shown to be effective at eliminating *Pseudomonas aeruginosa* from infected wounds. A study with patients have venous leg ulcers showed that gauze dressings soaked with acetic acid were effective in decreasing the number of *Staphylococcus aureus* and Gram-negative rods (Hansson and Faergemann, 1995).

Milner (1992) reported absence of pain or discomfort as adverse effect for acetic acid use, upon using 5% acetic acid in treatment of 9 patients, none of them showed discomfort, two wounds lost *Pseudomonas* species within 2 days, and four within one week, and only one patient had grown bacteria after three weeks. Following eradication of *Pseudomonas*, the wounds were found to heal rapidly (Milner, 1992).

Pseudomonas cultured from wounds has been found to get inhibited by acetic acid in vitro (Sloss *et al.,* 1993). Some studies have suggested cytotoxic effects of acetic acid in vitro but clinically no such effects have been found (Drosou *et al.,* 2003).

It is possible that application of acetic acid may confer other benefits on the healing process as well as the removal of bacteria. When the effect of acetic acid on reepithelization was conducted on animal and human models, no negative impact on wound healing was detected (Kjolseth *et al.,* 1994).

Although acetic acid was initially delaying the reepithelization, but after the eighth day this effect disappeared and tensile wound strength was not influenced (Lineaweaver *et al.,* 1985)

In a study by Medina *et al.,* (2007), vinegar of 5% acetic acid concentration was found to have bactericidal activity against each of ; *Staph aureus, Listria monocytogenes, Salmonella Enteritidis, Escherichia coli* 0157:H7, and *Yersinia* sp. Such activity was attributed to its acidity.

At concentrations nontoxic to fibroblasts and keratinocytes (≤ 0.0025%), acetic acid solutions were unable to inhibit growth of *Escherichia coli*, *Enterococcus*, or *Bacteroides fragilis*. Only slightly effect was recorded by inhibiting growth of *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Rund, 1996).

1.2.2.3.2 Rifampicin (RIF):

A. Discovery and important of RIF:

Rifampicin (RIF) is a bactericidal antibiotic drug of the rifamycin group (Masters *et al.*, 2005). It is a semisynthetic compound derived from *Amycolatopsis rifamycinica* (formerly known as *Amycolatopsis mediterranei* and *Streptomyces mediterranei*) (Sensi *et al.*,1959). RIF is a broad-spectrum antibiotic that is used in the therapy of many infectious diseases. It has been used since 1968 to combat *Mycobacterium tuberculosis* (Campbell *et al.*,2001 ; Mick *et al.*, 2010). The cellular target of RIF is DNA-dependent RNA polymerases (RNAP).It has a high capacity to bind and inhibit (RNAP) through its specific interaction with the polymerase β subunit (Aboshkiwa *et al.*, 1995).

In 1957, a soil sample from a pine forest on the French Riviera was brought for analysis to the Lepetit Pharmaceuticals Research Lab in Milan, Italy. There, a research group discovered a new species which appeared immediately of great scientific interest since it was producing a new class of compound with antibiotic activity. The British Medical Journal, (1999) decided to call these compounds (rifamycins). Rifampicin is an intensely red solid, and the small fraction which reaches body fluids is known for imparting a harmless red-orange color to the urine , sweat and tears of users, for a few hours after a dose. Maximal concentrations in the blood are decreased by about a third when the antibiotic is taken with food (Sensi *et al.*,1959). Figure (1-1) show RIF structure.

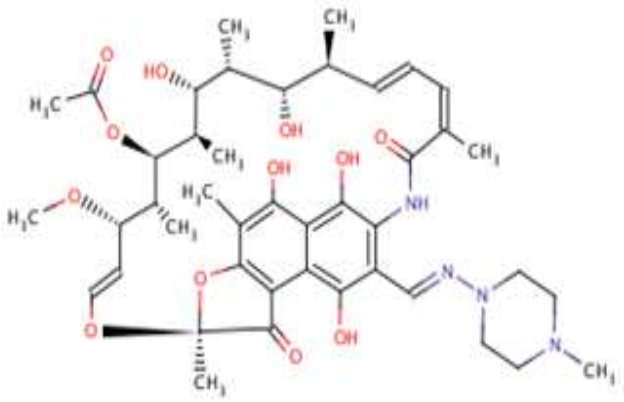


Figure (1-1) Chemical structure of Rifampicin. (Masters *et al.,* 2005)

B. Mechanism of action of RIF

Rifampicin inhibits bacterial DNA-dependent RNA synthesis by inhibiting bacterial DNA- dependent RNA polymerase (RNAP) through its specific interaction with the polymerase β subunit (Aboshkiwa *et al.*, 1995). Its binds near the RNAP active site at a protein pocket formed by the β subunit (Campbell *et al.*, 2001). Then blocks the initiation of transcription by preventing the synthesis of RNAs larger than dinucleotides. (Feklistov *et al.*, 2008) . RIF overlaps with the position of the third RNA nucleotide in the elongation complex (Korzheva *et al.*, 2000). These data strongly supported the initial hypothesis on the steric mechanism of RIF action (Campbell *et al.*, 2001 ; Feklistov *et al.*, 2008).

Resistance to rifampicin arises from mutations that alter the residues of the rifampicin binding site on RNA polymerase, resulting in decreased affinity for rifampicin (Campbell *et al.*, 2001 ; Feklistov *et al.*, 2008). The *rpoB* (gene that encodes the β subunit of bacterial RNA polymerase) is the site of mutations that confer resistance to the rifamycin antibacterial agents, such as rifampicin (Floss and Yu, 2005).

C. Antimicrobial uses of RIF

Rifampicin was introduced in 1967, as a major addition to the cocktail-drug treatment of tuberculosis, Hansen's disease and inactive meningitis, along with pyrazinamide, isoniazid, ethambutol and streptomycin (PIERS). It must be administered regularly daily for several months without break, otherwise, the risk of drug-resistant tuberculosis is greatly increased (Long, 1991). In fact, this is the primary reason it is used in tandem with the four mentioned drugs, particularly isoniazid (Erlich *et al.*, 1973). So monotherapy should not be used to treat these infections , it should be used in combination with other antibiotics, because resistance to RIF develops quickly during treatment,

In addition to its use in the treatment of methicillin-resistant *Staphylococcus aureus* (MRSA) in combination with fusidic acid, RIF is also used to treat osteomyelitis and prosthetic joint infections (Aboltins *et al.*, 2007 ; Mick *et al.*, 2010). Moreverو it is used in the prophylactic therapy against *Neisseria meningitidis* (meningococcal) infection, and recommended as an alternative treatment for infections with the tick-borne disease pathogens, such as in pregnant women or in patients with history of allergy to tetracycline antibiotics (Wormser *et al.*, 2006 ; Thomas *et al.*, 2009). Finally it has some effectiveness against vaccinia virus (Sodeik *et al.*, 1994 ; Charity *et al.*, 2007).

D. Adverse effects of RIF:

The most serious adverse effect related to rifampicin is the hepatotoxicity. Rifampicin is an effective liver enzyme inducer, promoting the up regulation of hepatic cytochrome P450 enzymes , increasing the rate of metabolism of many other drugs that are cleared by the liver through these enzymes. As a consequence, rifampicin can cause a range of adverse reactions when taken concurrently with other drugs (Collins, 1985).

For instance, patients undergoing long term anticoagulation therapy with warfarin have to be especially cautious and increase their dosage of warfarin accordingly (Stockley, 1994) . Failure to do so could lead to under treating with anticoagulation, resulting in serious consequences of thromboembolism. Rifampicin can reduce the efficacy of hormonal contraception, to the extent that the unintended pregnancies happen , this have been reported among users of oral contraceptives taking rifampicin in even short courses.

1.2.2.3.3 Probiotics:

A. History and definition:

The actual of probiotic concept belongs to Lily and Stillwell in 1965, after which probiotics are characterized as "microorganisms that promote growth of other microorganisms" (Lily and Stillwell, 1965).

In 1974, Parker talked about a food supplement for livestock and improve name of probiotics as "organisms and substances that helps the microbial ecosystem" (Parker, 1974).

Their importance was highlighted by Fuller in 1989 who described probiotics as live microorganisms with beneficial effects on host body, improving intestinal microbial balance (Fuller, 1989).

The universal meaning of the term "probiotic" was established by the World Health Organization (WHO) and the Food and Agriculture Organization (FAO) of the United States. These two organizations defined probiotics as "live microorganisms which when administrated in adequate amounts, have a beneficial effect on health of the host organisms" (Corcionivosch *et al*., 2010).

B. Probiotic microorganisms:

Probiotics are used for long times in food ingredients for human and also to feed the animals without any side effects (Holzapfel *et al*., 2001). Also probiotics are acceptable because of being naturally in the intestinal tract of healthy human and in foods (Çakır, 2003). The most commonly microorganisms used as probiotic preparations are shown in table (1-5).

Table (1-5): Microorganisms considered as probiotics (Holzapfel *et al*., 2001).

***Lactobacillus* spp.**	***Bifidobacterium* spp.**	**Other lactic acid bacteria**	**Nonlactic acid bacteria**
L.fermentum *L.delbrueci* *L. casei* *L.acidophilus* *L.amylovos* *L. crispatus* subsp. *bulgaricus* *L.gallinaru* *L. gasseri* *L. johnsonii* *L. paracasei* *L.plantarum* *L. reuteri* *L.rhamnoss*	*B. adolescentis* *B. animalis* *B. bifidum* *B. breve* *B. infantis* *B. lactis* *B. longum*	*Enterococcus faecalis* *Enterococcus faecium* *Lactococcu lactis* *Leuconstoc mesenteroides* *Pediococcus acidilactici* *Sporolactobacills inulinus* *Streptococcus thermophilus*	*Bacillus cereus var. toyoi* *Escherichia coli* strain nissle *Propionibacterim freudenreichii* *Saccharomyces cerevisiae* *Saccharomyces boulardii*

The most commonly utilized probiotic preparations include specific strains (of either alone or in combination) Lactobacilli, Streptococci and Bifidobacteria (Fuller, 1991), Lactobacilli are perhaps the most well known of these favorable microorganisms.

John *et al*. (1997) reported that these three genera are important components of the gastrointestinal flora. They are considered to be harmless, might be capable of preventing pathogenic bacteria, and essential for maintaining gut micro floral health.

In addition, Ducluzeau and Bensaada (1982) reported that *S. boulardii*, a yeast species similar to brewer's yeast, has demonstrated a direct antagonistic effect *in vivo* in mice against *Candida albicans, C. krusei* and *C. pseudotropicals* strains. Also, the experimental animals results showed that *S. boulardii* inhibited the action of cholera toxin on enterocytes (Dias *et al*., 1995).

Probiotics in theory can be composed of any live microbe. A large number of probiotics belongs to the *Lactobacillus* or *Bifidobacterium* genera. Also popular is *Saccharomyces boulardii* (yeast) (Sanders, 2009).

C. Mode of action of Probiotics:

Several mechanisms were suggested for the action of probiotics. These mechanisms are listed below briefly (Rolfe, 2000; Wadher *et al*. 2010).

- Production of inhibitory substances: Production of some organic acids, hydrogen peroxide and bacteriocins which are inhibitory to both Gram positive and Gram negative bacteria.
- Blocking of adhesion sites: Probiotics and pathogenic bacteria are in a competition, probiotics inhibit the pathogens by adhering to the intestinal epithelial surfaces by blocking the adhesion sites.
- Competition for nutrients: Probiotics inhibit the pathogens by consuming the nutrients which pathogen need.
- Stimulating of immunity: Stimulating of specific and nonspecific immunity may be one possible mechanism of probiotics to protect the host from intestinal disease.
- Degradation of toxin receptor: Because of the degradation of toxin receptor on the intestinal mucosa, it was shown that *S. boulardii*, a probiotic, protects the host against *Clostridium difficile* intestinal disease.

Some other studies on the mechanisms suggested suppression of toxin production, reduction of gut pH and attenuation of virulence (Fooks *et al*., 1999 ; Corcionivoschi *et al*., 2010).

Czerucka *et al*. (2007) found that several studies indicated two main mechanisms of action of *S. boulardii* against enteric pathogens: production of factors that compete with bacterial toxins and modulation of the host cell signaling pathways implicated in pro inflammatory response during bacterial infection.

E. Effect of probiotics on pathogenic bacteria:

Lactobacillus group of bacteria is famous for its uses as probiotic and in food preservation. It has own reputation due to its production of inhibitory compounds such as organic acid , hydrogen peroxide, diacetyl and bacteriocins (Brooks *et al*., 1998). Bacteriocins are proteins inhibiting other bacteria living in the same ecological place. So, *Lactobacilli* use it as a weapon for its survival (Todorov, 2009).

Moghaddam *et al*. (2006) reported that bacteriocins had been withdrawn special interest of microbiologist for the control of pathogenic bacteria. Some investigations had been declared the ability of bacteriocins to inhibit pathogenic bacteria like *E. coli*, *Pseudomonas* and *Klebsiella* (Raja *et al*., 2010). De Souza *et al*. (2005) reported that among the *Lactobacillus* species, *L. acidophilus* and *L. plantarum* had been extensively utilized as probiotics cultures in dairy and pharmaceutical products

In addition the antimicrobial effect of *Lactobacillus* spp., Vandenplas *et al*. (2008) demonstrated that *S. boulardii* had been a strong direct antagonist effect against a number of pathogens. *In vitro* studies had shown that *S. boulardii* reduced growth of *Candida albicans*, *E. coli*, *Shigella*, *Salmonella typhimurium*, *P. aeroginosa*, *S. aureus* (Czerucka *et al*., 2002).

3. Results and Discussion:

3.1 Isolation of bacteria:

A total of 67 swab samples were collected from patients referred to three hospitals in Baghdad suffering from diabetic foot infection (DFI). As shown in table (3-1), 31% of the samples were from sole of the foot, 24%, 17%, 12%, 9%, 4% and 3% from the big toe, 2nd toe, heel, 3rd toe, 5th toe and 4th toe, respectively. In this regard, Reiber *et al.* (1998) stated that DFI is developed at pressure points on the plantar surfaces, over the metatarsal heads, on the big toe, and on the heels.

Regarding gender of patients, same table shows that 64% of the samples were from males and 36% from females, Dhorod (2010) found almost similar findings when high percentage of diabetic foot infection was detected in males.

The age group of the diabetic foot patients were ranging between 28-75 yrs. Age group of 60-70 yrs was the most affected by diabetic foot infection such findings came in accordance to a study by (FryKberg *et al.*2000) when they found that diabetic foot infection was most common in age of 60-70 yrs. On the other hand, the duration of diabetes mellitus was between 4 to 35 yrs, while that of infection was from 1 week to 20 yrs.

Wagner classification system was used to classify the ulcer of the diabetic foot patients. Grade 2 (deep ulcer) was recorded in 33 patients, followed by grade 1(superficial ulcer), 3 (abscess osteitis) and 4 (gangrenous forefoot) with 21, 10 and 3 patients, respectively. While grade 0 (no lesion) and grade 5 (whole foot) were not recorded in any patient. These results are

almost similar to those of Dhorod (2010) who recorded that most of the bacterial isolates were obtained from grades 2, 3, 4 of the diabetic foot patients.

Table(3-1): Numbers and percentages of diabetic foot infection cases distributed according to site of infection and gender of patients.

Isolation source	Male		Female		Total	
	No.	%	No.	%	No.	%
Sole of foot	15	71	6	29	21	31
Big toe	12	75	4	25	16	24
2nd toe	7	64	4	36	11	17
Heel	5	62	3	38	8	12
3rd toe	3	50	3	50	6	9
5th toe	1	33	2	67	3	4
4th toe	0	0	2	100	2	3
Total	43	64	24	36	67	100

Very high percentage (71%) of infection from sole of the foot was recorded by male patients compared to only 29% for females. Infection of big toe, 2nd toe, and heel were also higher in males (75% , 64% , 62%), than in female (25% , 36% , 38%), respectively.

Adversely, cases of the 5th toe infection were more founded in female than in male patients with percentages of (67%) and (33%) , respectively. While the 3rd toe infection had the same occurrence percentage (50%) in both genders. On the other hand, the 4th toe infection was recorded only in female.

From the 67 foot infection cases of diabetic patients, only 60 gave positive results for bacterial occurrence when a total of 105 bacterial isolates was obtained from them (table 3-2).

Table (3-2): Numbers and percentages of bacterial isolates obtained from patients with diabetic foot infection (DFI).

Isolation source	DFI cases			No. of bacterial isolates
	Total	positive for bacteria		
		No.	%	
Big toe	16	13	81.25	24
2nd toe	11	10	90.90	17
3rd toe	6	5	83.3	8
4th toe	2	2	100	4
5th toe	3	3	100	5
Heel	8	8	100	14
Sole of foot	21	19	90.47	33
total	67	60	89.55	105

Highest occurrence of bacterial growth was recorded in the sole of patients foot when 33 of the 105 isolates were detected on it. Followed by the big toe, 2nd toe , heel , 3rd toe, 5th toe and the 4th toe with 24, 17, 14, 8, 5 and 4 isolates, respectively.

Table (3-3) contains distribution of the bacterial isolates from patients of the diabetic foot infection according to the number of bacterial types present on each foot site.

Table (3-3): Numbers of occurrence of bacterial types present on each patients foot site.

No. of bacterial types	Big toe	2nd toe	3rd toe	4th toe	5th toe	Heel	Sole of foot	Total	
								No.	%
One type	3	3	2	0	1	2	6	17	28
Two types	9	7	3	2	2	6	12	41	69
Three types	1	0	0	0	0	0	1	2	3
Total	13	10	5	2	3	8	19	60	100

One type of pathogenic bacteria was detected in 17 (28%) of 60 infected patients, while 43 (72%) of the patients were infected with more than one types (Polymicrobial infection) ; 41(69%) of them with two types and 2(3%) with three types of pathogens. On the other hand, no any bacterial type was detected in the rest (7, 10.45%) of the patients. Polymicrobial infection was also observed by several other studies such as (Wight-Pascoe *et al.,* 2001 ; Anandi *et al.,* 2004 ; Altrichter *et al.,* 2005 ; Shankar *et al.,* 2005 ; Alavi *et al.,* 2007). Adversely, Viswanathan *et al.,* (2002) and Raga (2007) detected only one type of bacteria in the patients of DFI. Differences in such matter may be related to the country where the study is carried on, and intensity of ulcer accompanying the infection (Gadepalli *et al.,* 2006 ; Senneville *et al.,* 2006).

In this study, Gram (-) bacteria were the predominant pathogens in the diabetic foot infections, Similar findings were also recorded by various studies such as (Shankar *et al.,* 2005 ; Gadepalli *et al.,* 2006 ; Alavi *et al.,* 2007 ; Raga, 2007 ; Ekta *et al.,* 2008). But in the studies of Mantey *et al.,* (2000), Dang *et al.,* (2003) and Diane *et al.,* (2007), Gram (+) bacteria was found to be the predominant organisms in the diabetic foot infections.

3.2 Identification of bacterial isolates:

The suspected bacterial isolates were identified, primarily, by the cultural and microscopic examinations, then to the species by the biochemical tests. Results obtained are illustrated as follows:

3.2.1 Cultural and microscopic characterization:

Identification of the suspected (105) bacterial isolates was performed at first depending on the characteristics of colonies grown on the surface of both MacConkey and Blood agar. Then identified depending on their Gram reaction and microscopic characteristics.

The suspected isolates were cultured on MacConkey agar medium due to the containing of bile salts and crystal violet which promote growth of *Enterobacteriaceae* and related enteric Gram negative rods, in addition to its suppresses of growth of Gram positive bacteria and some fastidious Gram negative bacteria. Lactose in this medium is the sole carbon source that differentiates between lactose-fermenting bacteria and non lactose-fermenting bacteria.

The lactose-fermenting bacteria is characterized by producing pink colonies due to the conversion of neutral red indicator dye when it is below pH 6.8. Adversely, the non-lactose bacterial growth appears colorless or transparent (Holt *et al*., 1994).

Blood agar is a bacterial growth medium containing 5% blood which is considered to be enrichment by providing a rich nutrient environment for many types of bacteria. It is also accounted as differential due its ability to distinguish pathogenic bacteria from others based on effect of their produced enzymes (known as hemolysins) which lyses the red blood cells through three types of hemolysis namely; alpha (zone of partial clearing , green, around the colonies), beta (complete zone of clearing surrounding the colony) and gamma (grow on blood agar with no hemolysis) (Atlas *et al*., 1995).

Regarding to the cultural characterization, color of the colonies were varied from pink to blue-green to creamy or colorless, their sizes were ranging from small to medium to large. Such characteristics of bacteria are suspected to be belonged to the species listing in table (3-5) depending on the characteristics described by Garrity (2005).

Gram staining procedure shows that 87 (82.86 %) of the isolates were Gram negative and 18 (17.14%) Gram positive.

Depending on the results of cultural and microscopic examination, it 27 of the isolates were suspected to belong to *Klebsiella*, 20 to *Pseudomonas*, 18 to *Staphylococcus*, 13 to *Escherichia*, 12 to *Proteus*, 5 to *Citrobacter*, 2 to each of *Acinetobacter*, *Enterobacter, Morganella* and *Pseudomonas*, while *Serratia* and *Aeromonas* were represented by only one colony each.

3.2.2 Biochemical characterization:

Results of the biochemical tests used for identification of bacterial isolates to the species are as shown in table (3-4).

Table (3-4): Results of the biochemical tests of bacterial isolates that obtained from diabetic foot infection patients.

Bacterial isolate	Biochemical test						
	IND	CIT	URE	CAT	OXI	KIA	H2S
Klebsiella pneumoniae	−	+	+	+	−	y/y	−
Pseudomonas aeruginosa	−	+	+	+	+	r/r	−
Staphylococcus aureus	−	−	−	+	−	y/y	−
Escherichia coli	+	−	−	+	−	y/y	−
Proteus mirabilis	−	v	+	+	−	r/y	+
Citrobacter freundii	+	+	+	+	−	y/y	+
Acinetobacter baumannii	−	+	v	+	−	r/r	−
Enterobacter cloacae	−	+	−	+	−	y/y	−
Morganella morganii	+	−	+	+	−	r/y	−
Pseudomonas fluorescence	−	−	−	+	+	r/r	−
Aeromonas hydrophila	+	v	−	+	+	y/y	+
Serratia marcescens	−	+	−	+	−	r/y	−

IND; Indole, CIT; Simmon citrate, URE; Urease, CAT; Catalase , OXI; Oxidase, KIA; Kligler iron agar, (-); negative, (+); positive, y; yellow, r; red.

The following bacterial isolates were negative for indole test: *Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus, Proteus mirabilis, Acinetobacter baumannii, Enterobacter cloacae, Pseudomonas fluorescence, Citrobacter freundii* and *Serratia marcescens.*While *Aeromonas hydrophila, Morganella morganii* and *E. coli* gave positive results.

Utilization of citrate is one of several important physiological test used to identify bacteria . *Klebsiella pneumoniae, Pseudomonas aeruginosa, Enterobacter cloacae, Citrobacter freundii, Acinetobacter baumannii, Serratia marcescens* and *Aeromonas hydrophila* isolates were positive for Simmon citrate test through their ability to use citrate as the sole of carbon source. But *E. coli, Pseudomonas fluorescence, Morganella morganii* and *Staphylococcus aureus* were negative to it, while *Proteus mirabilis* give variable reaction.

Regarding urease test, *Klebsiella pneumoniae, Pseudomonas aeruginosa, Proteus mirabilis, Citrobacter freundii* and *Morganella morganii* isolates gave positive results. Adversely, *Serratia marcescens, E.coli, Enterobacter cloacae, Pseudomonas fluorescence, Aeromonas hydrophila* and *Staphylococcus aureus* were negative to it. *Acinetobacter baumannii,* on the other hand, gave variable reaction.

All of the bacterial isolates obtained from diabetic foot infection patients were positive to the catalase test by producing gaseous bubbles after a drop of hydrogen peroxide was placed onto their colonies indicating positive results.

Klebsiella pneumoniae, Proteus mirabilis, E. coli, Citrobacter freundii, Staphylococcus aureus, Acinetobacter baumannii, Morganella morganii, Enterobacter cloacae, and *Serratia marcescens* were negative to the oxidase test. Adversely, *Aeromonas hydrophila, Pseudomonas fluorescence* and *Pseudomonas aeruginosa,* gave violet or purple color as indication of positive result.

Regarding KIA test, *Klebsiella pneumoniae, E. coli, Citrobacter freundii, Aeromonas hydrophila, Staphylococcus aureus,* and *Enterobacter cloacae* all have the ability to ferment both lactose and glucose with production of acid, that change the color of both slant and butt in KIA to yellow. But H2S production is found only in *Aeromonas hydrophila* and *Citrobacter freundii.*

Regarding ability of the isolates to ferment lactose and glucose in the , *Acinetobacter baumannii, Pseudomonas fluorescence* and *Pseudomonas aeruginosa* were unable to produce H_2S gas and the color of KIA medium remained red indicating that they are non-lactose fermenters. While *Morganella morganii, Serratia marcescens* and *Proteus mirabilis* fermented glucose but not lactose and turned the color of butt to yellow but the slant remained red. *Proteus mirabilis* was the only tested bacteria abled to produce H_2S gas.

When Api-20E system was used to confirm identification of bacterial isolates by the conventional primary and biochemical tests, 105 bacterial isolates belonging to twelve species were obtained. Their numbers and percentages are listed in table (3-5).

Table (3-5): Numbers and percentages of bacterial species isolates of diabetic foot infection patients identified by Api-20E system.

Species of bacteria	No.	%
Klebsiella pneumoniae	27	25.71
Pseudomonas aeruginosa	20	19.04
Staphylococcus aureus	18	17.14
Escherichia coli	13	12.38
Proteus mirabilis	12	11.47
Citrobacter freundii	5	4.76
Acinetobacter baumannii	2	1.90
Enterobacter cloacae	2	1.90
Morganella morganii	2	1.90
Pseudomonas fluorescence	2	1.90
Aeromonas hydrophila	1	0.95
Serratia marcescens	1	0.95
Total	105	100

Klebsiella pneumoniae was the most common species existed with a percentage of (25.71%), followed by *Pseudomonas aeruginosa* (19.04%), *Staphylococcus aureus* (17.14%) and *E. coli* (12.38%) . Similar results were obtained by Umadevi *et al.* (2011) when they found that *Klebsiella pneumoniae* was also the most occurred etiological agent in DFI patients and followed by the same above three species.

On the other hand, least occurrence of bacteria of the DFI patients was recorded by *Aeromonas hydrophila and Serratia marcescens* with a percentage of (0,95%) each, while other species occurred in between.

3.3 Antibiotics susceptibility of isolates:

Susceptibility of the bacterial isolates were examined towards 16 different antibiotics using disc diffusion method.

Results declared that**,** with exception of *Acinetobacter baumannii*, all bacterial isolates were completely sensetive to imipenem. In this regard, Livemore *et al*. (2001) found that imipenem have strong activity against most Enterobacteriaceae bacteria.

Werlinger and Moore (2004) investigated mechanism responsible for the resistance of clinical isolates of *E. coli* and referred it (may be) to the high-level expression of a plasmid-mediated β-lactamase, in combination with the loss of an outer membrane protein .

Susceptibility of most bacterial isolates to the amikacin was reported in this study, which is closed to that found by Paterson and Yu (1999) who reported high sensitivity to amikacin among bacterial isolates of their study. Umadevi *et al.*, (2011) also detected that the members of Enterobacteriaceae were found to be susceptible to amikacin.

Resistance of all isolates to the penicillin group and cephalosporin group also found in this study, and this may be related to isolates-possessing of β- lactamase enzymes (penicillinase and cephalosporinase) which are able to inactivate these antibiotics through cleaving β-lactam ring of the drug (Levinson and Jawetz, 2000). Another reason for the resistance is production of the extended spectrum ß-lactamase (ESBL). Nevertheless, some of the resistant isolates may be unable to produce ESBL. Production of other enzymes, such as AmpC ß-lactamases, capable of hydrolyzing the extended-spectrum cephalosporins could be the reason for resistance in non-ESBL producing isolates (Rice *et al*., 2003).

From the Enterobacteriaceae isolates, *E. coli* showed the highest resistance to the antibiotics used in the study. This may be because *E. coli* easily acquires the resistance factor from environment and easily resists penicillin derivatives drug like ampicillin (Wazait *et al*., 2003). Adversely, *E. coli* appeared sensitive to only imipenem and amikacin, results agreed with those of Ioana *et al*. (2010) who also reported high sensitivity of *E. coli* toward imipenem and amikacin.

Klebseilla pneumoniae was found to be resistant to the penicillin and cephalosporin groups which is closed to the finding of Stock and Wiedmenn (2001). In contrast, this bacteria was sensitive to the tetracycline. Marranzano *et al*. (1996) found that 90% of the *K. pneumoniae* isolates been investigated were sensitive to tetracycline.

Pseudomonas aeruginosa resisted penicillin, cephalosporin and chloramphenicol, but sensitive to aminoglycosids and carbapenem. In this regard, Gerri (2011) recorded similar finding. Nester *et al*. (2004) declared that *E. coli*, *Ps. aeruginosa* and *K. pneumoniae* can acquire resistance plasmid in a mixed culture. This explains further why most of these organisms are resistant to antibiotics.

Results declared that *Acinetobacter baumannii* were completely resistant to all 16 antibiotics which may be related to factors, such as Beta-lactamase, where this bacteria is known to produce at least one beta-lactamase (Higgins *et al*., 2013). Biofilm formation is a second factor where *A. baumannii* is able to form biofilms for its survival (Espinal *et al*., 2012). The formation of biofilms has been shown to alter the metabolism of microorganisms, then reducing their sensitivity to antibiotics. This may be due to the fact that fewer nutrients are available deeper within the biofilm. A slower metabolism can prevent the bacteria from uptaking an antibiotic or performing a vital function fast enough for particular antibiotics to have an effect. They also provide a physical barrier against larger molecules and may prevent desiccation of the bacteria (Worthington *et al*., 2012 ; Yeom *et al*., 2013). In addition, adherence of *A. baumannii* to epithelial cells of the outer membrane also involves in survival of bacteria (Choi *et al*., 2008).

Aeromonas hydrophila showed resistance to penicillin group and sensitive to aminoglycosids. Amy *et al*. (2011) reported similar results through their findings that penicillin and aminoglycosids group had no effect to *Aeromonas hydrophila.* While *S. aureus* found to be sensitive to amikacin, gentamycin and ciprofloxacin, and resistant to erythromycin and ampicillin. In this regard, Anguzu and Olila (2007) recorded similar finding.

3.4 Treatment of the patient:

Treatment of patients was started after identification of the bacterial isolates in each patients, by culturing swabs taken from the wounds before and after treatment.

Depending on the last recorded results and their microbiological responses to the treatment, the patients were classified into three categories: "Responders" (cured or improved) or "Nonresponders" (treatment failure). In case of eradication of all initial pathogens, it is called cured. Or improved, if at least one, but not all, of the initial pathogens were eradicated and no additional organisms were isolated. The nonresponder patients were considered to have "treatment failure" if all original pathogens of the initial wound cultures persisted, or if organisms other than the original pathogens appeared in the last wound culture.

3.4.1 Systemic treatment:

Antibiotic that used in treatment of diabetic foot patients was chosen after performing the susceptibility test for each bacterial isolates. From the 16 antibiotic used, the commonly used IPM was the most affected one, but in Iraq it is replaced by other antibiotics because it is unavailable at most of the drugs retail pharmacies.

As a result, *K. pneumonia, P. aeruginosa, P. mirabilis, P. fluorescence, A. hydrophila, S. marcescens, M. morganii, E. coli* and *C. freundii* showed high sensitivity to amikacin, while *E. cloacae* and *S. aureus* were more sensitive for ciprofloxacin. Adversely, *A.baumannii* was resistant for all antibiotics used.

Therefore, the first two antibiotic (AK and CIP) were chosen to be used through administration each of them to the patients depending on the occurrence of bacterial isolates. In case of patients having two isolates (one sensitive to AK and the other to CIP), the most affected one was chosen according to the diameter of the inhibition zone it gave.

Effect of systemic treatment of each of the above two antibiotics on the existence of bacterial causatives of diabetic foot infection was evaluated after patients used them for 3 weeks as shown in tables (3-6) and (3-7).

Table (3-6) Occurrence of bacterial isolates from diabetic foot infection patients before and after treatment with Amikacin (AK) antibiotic.

No. of patients	Bacterial isolates	
	Before treatment	**After treatment**
1	*K. pneumoniae*	No growth
2	*P. aeruginosa* *P. mirabilis*	*P. aeruginosa* *P. mirabilis*
3	*K. pneumoniae* *P. aeruginosa*	*K. pneumoniae* *P. aeruginosa*
4	*S. marcescens*	*S. marcescens*
5	*E. coli* *K. pneumoniae*	*E. coli* *K. pneumoniae*
6	*K. pneumoniae*	*K. pneumoniae*
7	*E. coli* *P. aeruginosa*	*E. coli* *P. aeruginosa*
8	*C. freundii*	*C. freundii*
9	*P. aeruginosa* *K. pneumoniae*	*P. aeruginosa* *K. pneumoniae*
10	*K. pneumoniae*	*K. pneumoniae*
11	*P. mirabilis*	*P. mirabilis*
12	*E. coli* *K. pneumoniae*	*E. coli* *K. pneumoniae*
13	*P. aeruginosa* *P. mirabilis*	*P. aeruginosa* *P. mirabilis*
14	*K. pneumoniae*	*K. pneumoniae*
15	*K. pneumoniae* *P. aeruginosa*	*K. pneumoniae* *P. aeruginosa*
16	*E. coli, K. pneumoniae* *S. aureus*	*E. coli, K. pneumoniae* *S. aureus*
17	*K. pneumoniae*	*K. pneumoniae*
18	*K. pneumoniae* *M. morganii*	*K. pneumoniae* *M. morganii*
19	*P. fluorescence*	*P. fluorescence*

Table (3-7) Occurrence of bacterial isolates from diabetic foot infection patients before and after treatment with Ciprofloxacin (CIP) antibiotic.

No. of patients	Bacterial isolates	
	Before treatment	**After treatment**
1	*K. pneumoniae* *S. aureus*	*K. pneumoniae* *S. aureus*
2	*P. aeruginosa* *S. aureus*	*P. aeruginosa* *S. aureus*
3	*A. baumannii*	*A. baumannii*
4	*S. aureus*	No growth
5	*K. pneumoniae* *S. aureus*	*K. pneumoniae* *S. aureus*
6	*P. aeruginosa* *S. aureus*	*P. aeruginosa* *S. aureus*
7	*K. pneumoniae* *S. aureus*	*K. pneumoniae* *S. aureus*
8	*S. aureus*	*S. aureus*
9	*K. pneumoniae* *S. aureus*	*K. pneumoniae* *S. aureus*
10	*P. aeruginosa* *S. aureus*	*P. aeruginosa* *S. aureus*
11	*S. aureus*	*S. aureus*

Nineteen out of the thirty patients, were treated with amikacin (AK). nine of them had only one type of pathogenic bacteria and rest of the patients (except one with three types) having two types.

When the remaining eleven patients were treated with ciprofloxacin, 4 of them had only one type of pathogenic bacteria and the other seven with two types. Moreover, three types pattern of pathogenic bacteria was not detected in any of the ciprofloxacin treatment.

Table (3-6) also shows that only one patient was cured while the others gave no response to the treatment when all the original bacterial isolates were appeared in the last wound culture. Similarly, table (3-7) showed the same results, when only one patient was cured and the rest had treatment failure.

Such results may be due to the abuse, especially the overuse of antibiotics in treatment of the diabetic foot infections that leads to elevate resistance of the causative bacteria to antibiotics (Rice *et al*., 1990).

Peterson (2002) reported that any latest or new drug use in clinics, take an average of 7 – 10 yrs before microorganisms can resisted it, and using antibiotics for much longer time as well as their oral route of administration also affect their rate of absorption into blood stream.

Bano *et al*., (2012) confirmed that multidrug resistant organisms (MDRO) infection is extremely common in hospitalized patients with diabetic foot ulcers, and this increasing prevalence of MDROs is disconcerting, because infection with these organisms limits the choice of antibiotic treatment and may lead to worse outcome. This is in accordance with the report of Hertamann *et al.*, (2004) who pointed out that prevalence of MDROs limits the choice of antibiotic . Also the high rates of antibiotic resistance observed may be due to the widespread usage of broad-spectrum antibiotics leading to selective survival advantage of pathogens. Severe wound grades 2 and 3 were significant independent risk factors for clinical failure in patients treated for a DFI in the study of (Lipsky *et al.,* 2007). Clinical failure was noticed in 23% of the patients with 2 and 3 grades, compared with 11% with a wound stage of 0 or 1.

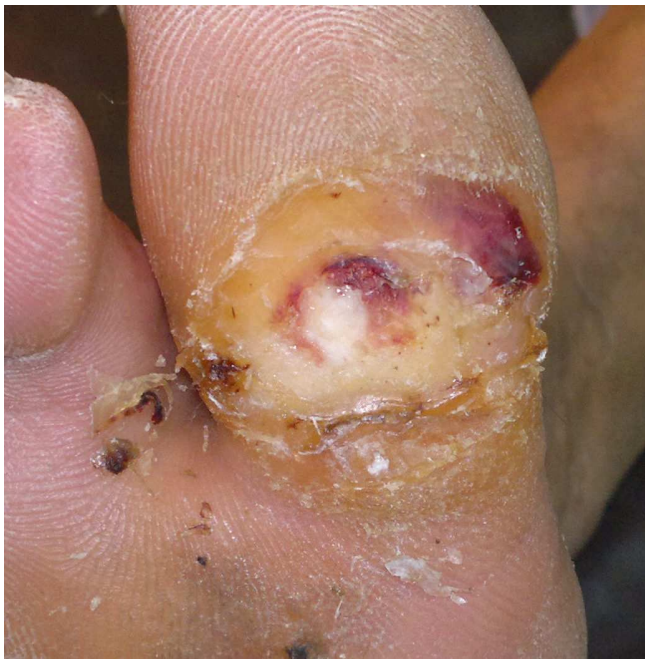
Analysis of data from randomized controlled trials on DFIs observed a treatment failure in 18 studies (Vardakas *et al.,* 2008). Isolation of MRSA was found to be a significant factor associated with treatment failure. Pittet *et al.*, (1999) showed that fever, prior hospitalization for DFI and gangrenous lesions were independent factors associated with treatment failure.

3.4.2 Local treatment:

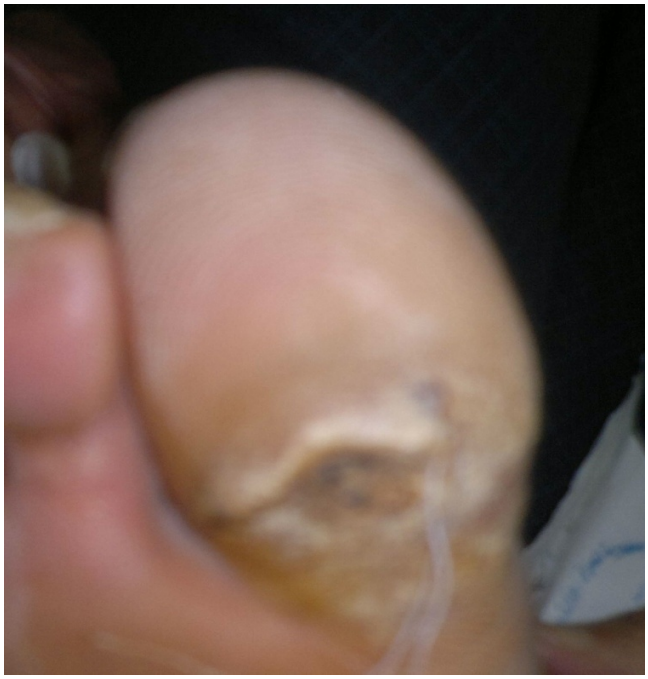
Three agents (acetic acid, rifampicin and probiotic) were used in the local treatment of the diabetic foot infections. A group of 10 patients was assessed for each agent and results of the bacterial isolates that appeared in each of the four week swabs are tabulated in table (3-8).

Table (3-8) Occurrence bacterial species after local treatment of diabetic foot infection patients with acetic acid.

N0.	Occurrence of bacterial isolates			
	first week	**second week**	**third week**	**fourth week**
1	*S. aureus* *P. mirabilis*	*S. aureus* *P. mirabilis*	*S. aureus* *P. mirabilis*	*S. aureus* *P. mirabilis*
2	*P. aeruginosa* *C. freundii*	*P. aeruginosa* *C. freundii*	*P. aeruginosa* *C. freundii*	*P. aeruginosa* *C. freundii*
3	*P. mirabilis* *E. coli*	*P. mirabilis* *E. coli*	*P. mirabilis* *E. coli*	*P. mirabilis*
4	*C. freundii* *P. mirabilis*	*C. freundii* *P. mirabilis*	*C. freundii* *P. mirabilis*	*C. freundii* *P. mirabilis*
5	*P. mirabilis* *K. pneumoniae* *P. fluorescence*	*P. mirabilis* *K. pneumoniae*	*P. mirabilis*	No growth
6	*E. coli* *M. morganii*	*E. coli* *M. morganii*	*E. coli* *M. morganii*	*E. coli* *M. morganii*
7	*K. pneumoniae* *P. aeruginosa*	*K. pneumoniae* *P. aeruginosa*	*K. pneumoniae* *P. aeruginosa*	*K. pneumoniae*
8	*P. mirabilis* *P. aeruginosa*	*P. mirabilis* *P. aeruginosa*	*P. mirabilis* *P. aeruginosa*	*P. mirabilis* *P. aeruginosa*
9	*S. aureus* *E. coli*	*S. aureus* *E. coli*	*S. aureus* *E. coli*	*E. coli*
10	*K.pneumoniae* *P. aeruginosa*	*P. aeruginosa* *K. pneumoniae*	*P. aeruginosa* *K. pneumoniae*	*P. aeruginosa* *K. pneumoniae*



Before

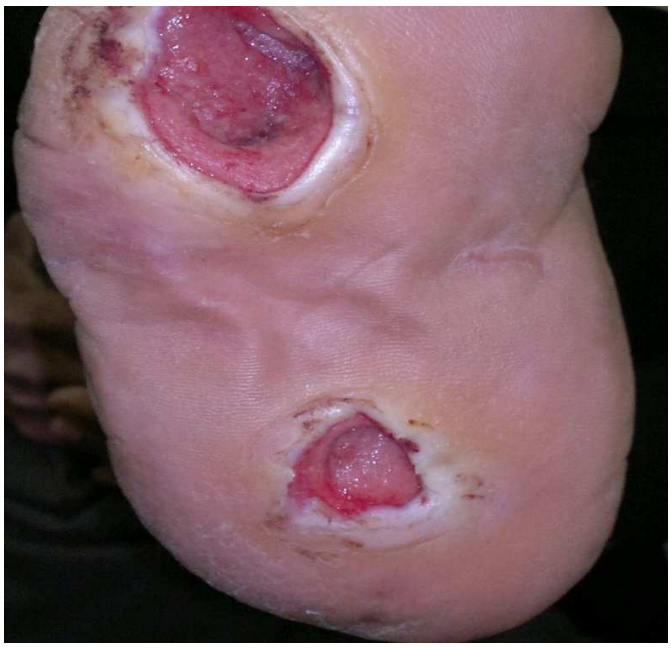


After

Figure (3-1): Photos of a patient with diabetic foot ulcer before and after treatment with acetic acid.

Table (3-9) Occurrence bacterial species after local treatment of diabetic foot infection patients with Rifampicin antibiotic.

N0.	Occurrence of bacterial isolates			
	first week	**second week**	**third week**	**fourth week**
1	*P. mirabilis* *P. aeruginosa*	*P. aeruginosa*	*P. aeruginosa*	No growth
2	*E. cloacae* *K. pneumoniae*	*K. pneumoniae*	*K. pneumoniae*	No growth
3	*P. mirabilis*	*P. mirabilis*	*P. mirabilis*	*P. mirabilis*
4	*C. freundii* *K. pneumoniae*	*C. freundii* *K. pneumoniae*	*C. freundii* (Few colony)	No growth
5	*S. aureus* *E. coli*	*E. coli* (Few colony)	No growth	No growth
6	*P. aeruginosa* *K. pneumoniae*	*P. aeruginosa* *K. pneumoniae*	*P. aeruginosa*	No growth
7	*E. coli* *K. pneumoniae*	*K. pneumoniae*	*K. pneumoniae*	No growth
8	*P. aeruginosa* *S. aureus*	*P. aeruginosa* *S. aureus*	*P. aeruginosa*	No growth
9	*K. pneumoniae* *S. aureus*	*K.pneumoniae* (Few colony)	No growth	No growth
10	*E. coli* *A. baumannii*	*A. baumannii*	*A. baumannii* (Few colony)	*A. baumannii* (Few colony)



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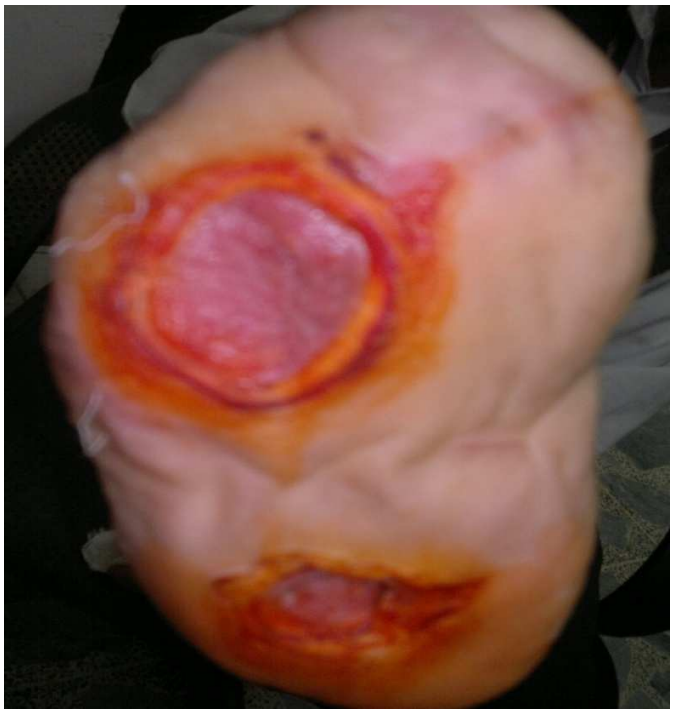
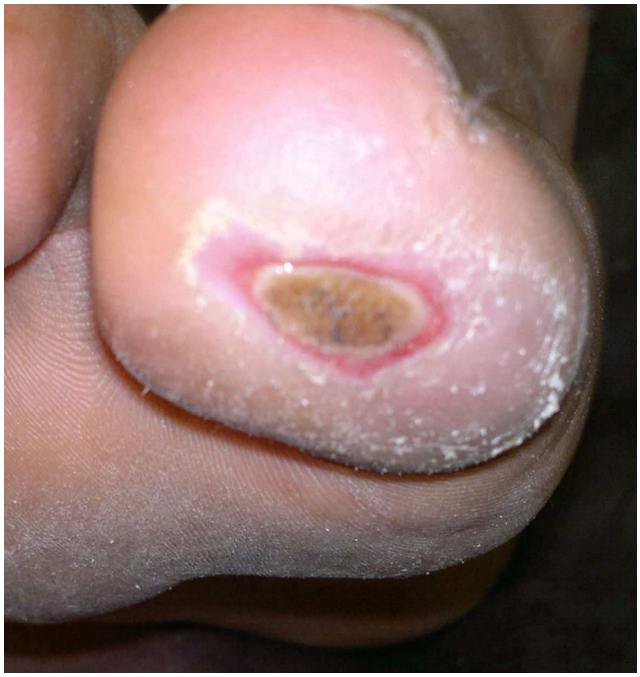


After

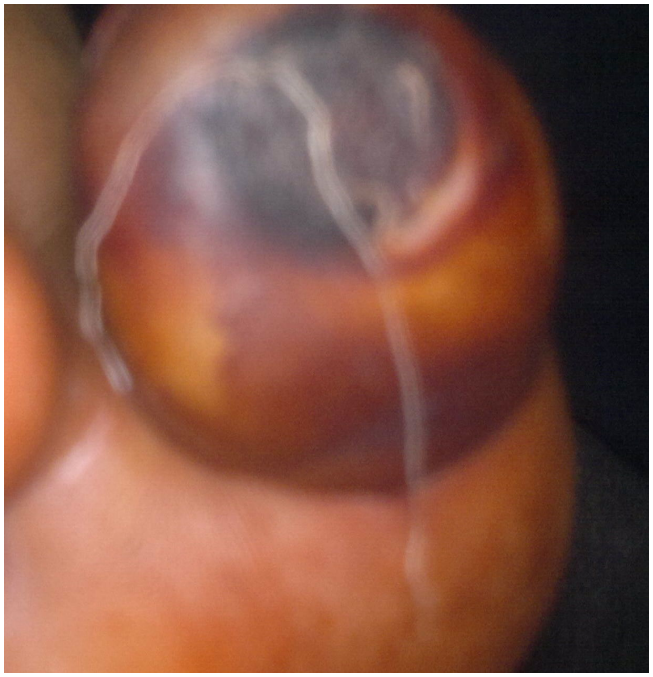
Figure (3-2): Photos of a patient with diabetic foot ulcer before and after treatment with Rifampicin.

Table (3-10) Occurrence bacterial species after local treatment of diabetic foot infection patients with probiotic (*Lactobacillus fermentum* and *L.delbrueci*).

N0.	Occurrence of bacterial isolates			
	first week	**second week**	**third week**	**fourth week**
1	*P. aeruginosa* *A. hydrophila*	*P. aeruginosa* *A. hydrophila*	*P. aeruginosa* *A. hydrophila*	*P. aeruginosa* *A. hydrophila*
2	*K. pneumoniae* *S. aureus*	*K. pneumoniae* *S. aureus*	*K. pneumoniae* *S. aureus*	*K. pneumoniae* *S. aureus*
3	*P. aeruginosa*	*P. aeruginosa*	*P. aeruginosa* *K. pneumoniae*	*P. aeruginosa* *K. pneumoniae*
4	*E. cloacae*	*E. cloacae*	*E. cloacae*	*E. cloacae* *P. mirabilis*
5	*E. coli*	*E. coli*	*E. coli*	*E. coli* (Few colony)
6	*P. mirabilis* *P. aeruginosa*	*P. mirabilis* *P. aeruginosa*	*P. mirabilis* *P. aeruginosa*	*P. mirabilis* *P. aeruginosa*
7	*P. mirabilis* *C. freundii*	*P. mirabilis* *C. freundii*	*P. mirabilis* *C. freundii*	*P. mirabilis* *C. freundii*
8	*S. aureus* *E. coli*	*S. aureus* *E. coli*	*S. aureus* *E. coli*	*S. aureus* *E. coli*
9	*K. pneumoniae* *P. aeruginosa*	*K. pneumoniae* *P. aeruginosa*	*P. aeruginosa*	*P. aeruginosa*
10	*E. coli* *K. pneumoniae*	*E. coli* *K. pneumoniae*	*E. coli* *K. pneumoniae* (Few colony)	*E. coli* *K. pneumoniae* (Few colony)



Before



After

Figure (3-3): Photos of a patient with diabetic foot ulcer before and after treatment with probiotics.

Results of treatment of DFI patients with acetic acid showed the infection was cured in only one patient that having three types of pathogenic bacteria (*P. mirabilis, K. pneumoniae* and *P. fluorescence*). By comparing the wound cultures, no bacterial growth was detected in the last swab. Improved status appeared in three patient, each of having two types of pathogenic bacteria in the first swab (*P. mirabilis* and *E. coli ; S. aureus* and *E. coli; K. pneumoniae* and *P. aeruginosa*). After treatment, some of the bacterial isolates disappeared, while only (*P. mirabilis* ; *E. coli* and *K. pneumoniae*) remained in them, respectively. Other patients showed no response to the treatment (treatment failure) when same pathogenic bacteria of the first infection appeared in last swab.

Proteus mirabilis, K. pneumoniae, P. fluorescence, E. coli, S. aureus and *P. aeruginosa* were disappeared from some patients. In this regard, Medina *et al.,* (2007) found that vinegar of 5% acetic acid concentration possessed bactericidal activity against each of *Staphylococcus aureus, Listeria monocytogenes, Salmonella enteritidis, E.coli* , and *Yersinia* sp. Such activity was attributed to its acidity. While Rund (1996) declared that acetic acid solutions with low concentrations have only slight effect of inhibiting growth of *Staphylococcus aureus* and *Pseudomonas aeruginosa* bacteria.

In the treatment of DFI patients with rifampicin , most of the patients were cured (the improvement was seen from the first swab and some of them showed no bacterial growth in the third swab and the last swap), while only one of them showed little response (improved) when the first cultures *E. coli* and *A. baumannii* were appeared, but in the second swab *E. coli* was disappeared and only *A. baumannii* remained.

One patients only had no response (treatment failure) to this antibiotic when *P. mirabilis* was found in all of the cultures. The significant effect of local antibiotics is probably related to a higher local concentration of antibiotics in the treated area (Heslop *et al.*, 2010).

In the treatment of DFI patients with probiotics, only one patient was improved but no patient was cured. However, *K. pneumoniae* was disappeared from *P. aeruginosa* and *K. pneumoniae* that found in the first culture. Treatment failure was seen in most of the patients. The explanation of such results may be that the concentrations of filtrates of the probiotics isolates were not enough to exhibit inhibitory effect on the pathogenic bacterial isolates. Low concentrations of microbial filtrates may result in the low concentration of the active compounds required to kill the pathogens (Barefoot and Kaenhammer, 1983).

After comparing the two ways of applying the pharmaceutical agents, local treatment was found to be the superior for treatment of diabetic foot infection. It is effective especially when accompanied by appropriate wound care as a therapeutic alternative to a broad-spectrum oral antibiotic agent. In addition, local treatment appears to be safe and may avoid the opportunity of resistant bacteria that can develop after oral systemic antibiotic therapy (Lipsky *et al.*, 2008). Local treatment has also the advantages of avoiding systemic adverse effects, providing increased target site concentration and allowing the use of agents not available for systemic therapy (Lio and Kaye 2004). They added that this is another reason made topical treatment be the best for treatment of diabetic foot infection. An acceptable topical anti-infective agent would need to demonstrate activity

against the spectrum of bacteria that are known to cause DFI, and it would need to avoid serious adverse effects, interference with wound healing, or induction of drug resistance.

2. Materials and Methods:

2.1 Materials:

2.1.1 Apparatus and equipments:

The following apparatus and equipments were used in this study:

Apparatus or equipment	Company (Origin)
Autoclave	Express (Germany)
Compound light microscopic	Olympus (Japan)
Digital balance	Ohans (France)
Electric oven	GallenKamp (England)
Incubator	GallenKamp
Laminar flow hood	Memmert (Germany)
Millipore filters	Sartorius (Germany)
Micropipette	Witey(Germany)
pH-meter	Radiometer (Denmark)
Water distiller	GLF (Germany)
Water bath	

2.1.2 Biological and chemical materials:

The following chemical and biological materials were used in this study:

Material	Company (Origin)
Ethanol (70%)	Local market (Iraq)
Peptone	BDH(England)
Hydrochloric acid	Sigma (USA)
Sodium chloride	Oxoid (England)
Sodium hydroxide	Merck (Germany)

2.1.3 Therapeutic agents:

The following therapeutic agents were used in this study.

Agent	Source
Vinegar (Acetic acid) 5%	Locally produced, Al-Badawy (Iraq)
Rifampicin 30mg/ml	Ajanta (India)
Probiotic (*Lactobacillus*) 1×10^7 cell	RAMEDA (Egypt)

2.1.4 Reagents and stains: Ready- to- use

- **Kovac's Reagent:** (Becton and Dickinson / Mexico)
- **Oxidase Reagent:** (Becton and Dickinson / Mexico)
- **Catalase Reagent:** (Becton and Dickinson / Mexico)
- **Gram stain kit:** (Fluka / Switzerland)

2.1.5 Antibiotics discs:

Antibiotic	symbol	Concentration (µg)
Amikacin	AK	30
Amoxicillin-clavulanic acid	AMC	30
Ampicillin	AMP	10
Aztreonam	AT	30
Cefotaxime	CTX	30
Chloramphenicol	C	30
Ciprofloxacin	CIP	5
Erythromycin	ERY	15
Gentamycin	GEN	10
Imipenem	IPM	10
Netilmicin	NET	30
Piperacilin	PIP	100
Tetracycline	TE	30
Ticarcillin-Clavulanic acid	TCC	75/10
Ticarcillin	TI	75
Vancomycin	VA	30

2.1.6 Culture media:

2.1.6.1 Ready-to-use media:

The following media were prepared and sterilized by autoclaving after adjusting pH as mentioned on their containers by the manufacturing companies:

Medium	Company (Origin)
Nutrient agar	Difco (USA)
Kligler Iron Agar (KIA)	
Simmon citrate agar	
Mueller Hinton	
Blood agar base	
MacConkey agar	Oxoid (England)
Urea agar base	Biolife (Italy)

2.1.6.2 Laboratory-prepared media:

The following media were prepared and sterilized as will be explained in item (2.2.3).

- **Blood agar medium**
- **Peptone water medium**
- **Urea agar base medium**

2.2 Methods:

2.2.1 Samples collection and cultivation:

Samples were collected from 67 patients of ages from 28 to 75 yrs suffering from diabetic foot infections who attended Al- Yarmook Teaching Hospital, Al-Kindy Teaching Hospital and the Specialist Center for Deaf Glands and Diabetes form the period of 4/11/2012 to 30/1/2013. Samples were taken from wounds by sterile disposable cotton swabs before returning to the transport medium. They were, then, cultured onto MacConkey agar and Blood agar plates before incubating at 37 ºC for 24 hrs. After incubation, bacterial colonies were subjected for identification as illustrated in item (2.2.5)

A questioner form (Appendix 1) was prepared for each patient to be filled with the name, sex, age, occupation, date of sampling, date of disease occurrence(D.M), site of infection, duration of infection, grade of infection, previous treatment and method of treatment.

2.2.2 Sterilizing methods (Baily *et al.*, 1990):

Three methods of sterilization were used:

2.2.2.1 Moist-heat sterilization (Autoclaving):

Microbial culture media, solutions, and reagents were sterilized by the autoclave at 121°C (15 Ib /inch^2) for 15 min unless otherwise stated.

2.2.2.2 Dry-heat sterilization (Oven):

Electric oven was used to sterilize glassware at 180°C for 3 hrs.

2.2.2.3 Membrane filtration:

Thermo labile components were sterilized by membrane filtration throughout millipore filters of pores size of (0.22) µm.

2.2.3 Preparation of solutions:

2.2.3.1 Normal saline: (Brown and Poxton, 1996)

It was prepared by dissolving 0.85 g NaCl in l00 ml of distilled water and sterilized by autoclaving.

2.2.3.2 Turbidity standard solution:

Ready - to - use McFarland No. 0.5 (1×10^8 cfu/ml) was applied to be compared with the bacterial isolates growth.

2.2.3.3 Rifampicin solution:

It was prepared by dissolving 1800 mg in 60 ml of distilled water to obtain a concentration of 30 mg/ml.

2.2.3.4 Probiotic solution:

It was prepared by dissolving one sachet in 60 ml of distilled water. (each sachet contained a mixture of *Lactobacillus delbruekii* and *Lactobacillus fermentum* in a concentration of $1x10^7$ cells for each).

2.2.4 Laboratory-prepared media:

2.2.4.1 Blood agar : (Atlas *et al*., 1995)

It was prepared by dissolving 40 g blood agar base in 1000 ml of distilled water before autoclaving. After cooling to 50 ºC, 5% of human blood was added to it, mixed well and distributed into sterilized Petri-dishes.

2.2.4.2 Peptone water : (Mackie and MacCartney, 1996)

This medium was prepared by dissolving 5 g of peptone in l00 ml of distilled water. It was distributed in test tubes (5 ml each) and sterilized by autoclaving, then stored at 4 ºC until use.

2.2.4.3 Urea agar base medium: (Atlas *et al*., 1995)

It was prepared by dissolving 24 g of urea base in 50 ml of distilled water, pH was adjusted to (6.5-7.0) and sterilized by autoclaving. Then 50 ml of 20% urea (previously sterilized by membrane filtration) were added aseptically, after that it was poured in sterile tubes and let to solidify as slant.

2.2.5 Identification of bacterial isolates:

Suspected bacterial isolates were primarily identified by microscopic and cultural examinations, then by the biochemical tests for final identification.

2.2.5.1 Cultural examination: (Garrity, 2005)

Colonies grown on the culture media were described according to their shape, size, margin, color, and odor.

2.2.5.2 Microscopic examination: (Atlas *et al*., 1995)

Gram staining was used to describe shape, Gram reaction, grouping of the cells of isolates.

2.2.5.3 Biochemical tests:

The following tests were performed according to (Macfaddin, 2000; Garrity, 2005).

2.2.5.3.1 Indole test:

Peptone water into the test tubes prepared in item (2.2.4.2) were inoculated with fresh culture of the bacterial isolates, separately, before incubated at 37 ºC for 24 hrs. A portion of 0.5 ml Kovac's reagent was added for each test tube. Appearance of red ring at the top of the broth indicates a positive result.

2.2.5.3.2 Simmon citrate test:

Simmon citrate slants were inoculated with the suspected bacterial

isolates, by streaking on the slant and incubated at 37ºC for 24 hrs. Appearance of growth and changing medium color from green to blue indicate a positive result.

2.2.5.3.3 Urease test:

Urea agar slants prepared as in item (2.2.4.3) were inoculated with the suspected bacterial isolates, then incubated at 37 ºC for 24 hrs. Changing medium color to purple - pink indicates a positive result.

2.2.5.3.4 Catalase test:

A single colony of the suspected bacterial isolates was placed onto a clean glass microscope slide with a sterile tooth pick, then a drop of hydrogen peroxide was placed onto the colony. Production of gaseous bubbles indicates the presence of catalase enzyme.

2.2.5.3.5 Oxidase test:

This test was done by using moisten paper with few drops of oxidase reagent. Cells from suspected isolates was picked up with a sterile wooden stick and smeared on the moisten paper. A positive results was detected by the development of a violet or purple color within 10 seconds.

2.2.5.3.6 Kligler iron agar (KIA) test:

Kligler iron agar slants were inoculated with the suspected bacterial isolates, then incubated at 37 ºC for 24 hrs. The results was read as follows:

- Alkaline/Alkaline → Red/Red
- Alkaline/Acid → Red/Yellow
- Acid/Acid → Yellow/Yellow
- H_2S production → Black precipitation
- Gas production → Bubbles formation

2.2.6 Api identification of isolates:

Reagents and indicators (IND,TDA, VP1-VP2), of the Api-20E system, were included in the kit of (Bio Mrrieux / France).The isolates were examined by this kit as following:

2.2.6.1 Preparation of the strips:

Five milliliters of tap water were placed into the Api incubation tray, then Api test strip was withdrawn from the sealed package and placed into the incubation tray.

2.2.6.2 Preparation of the inoculums:

Aseptically, one colony of each bacterial isolates grown on MacConkey agar was picked up with a sterilized loop and transferred to a test tube containing 5 ml of sterile normal saline solution. After shaking well, tube was recapped before comparing its turbidity with the McFarland No.0.5 solution.

2.2.6.3 Inoculation of strips prepare:

Bacterial suspension was transferred to the Api test strip by a sterile pasture pipette, the Api strip was tilted and the microtube was filled by placing the pipette tip against the side of the cupule, both the tube and cupule sections of the **CIT**,**VP** and **GEL** microtubes were filled. But the cupule sections of **ADH**, **LDC**, **ODC**, **H_2S** and **URE** microtubes were filled completely with sterilize mineral oil to prepare an-aerobic conditions. Then the test strip was incubated at 37ºC for 24 hrs. After incubation the reagents were added to **TDA**, **IND** and **VP** microtubes.

2.2.7 Maintenance of bacterial isolates: (Maniatis *et al*., 1982)

Bacterial isolates were primarily subcultured and incubated at 37 C for overnight before preserved as follows:

2.2.7.1 Short term storage:

Bacterial isolates were maintained for a period of few weeks on MacConkey agar in plates wrapped tightly with parafilm and stored at 4 ºC.

2.2.7.2 Medium term storage:

Slants of nutrient agar were inoculated with each of the bacterial isolate and incubation at 37°C overnight, before storing at 4°C for a few month.

2.2.8 Antibiotic susceptibility test:

The antibiotic susceptibility tests was performed as follow by using Kirby Bauer's disc diffusion method according to the Manual on Antimicrobial Susceptibility Testing (2004). Results were compared with Clinical Laboratory Standards Institute (CLSI) (2005).

- The inoculums were prepared by taken the isolated colonies from the growth of 18- 24 hrs agar plate, then suspended in the saline solution to match the 0.5 McFarland turbidity standard.
- A sterile cotton swab was dipped into the suspension, and pressed firmly on the inside wall of the tube to remove excess inoculums from the swab.

. The Mueller-Hinton agar plates were inoculated by streaking the swab over the entire agar surface. The inoculated plates were, then, placed at room temperature for 3 to 5 min to allow absorption of excess moisture, then the antibacterial disks were placed and pressed gently on the inoculated plates with a forceps to ensure contact with the agar.

· The inoculated plates were incubated at 37ºC for 18-24 hrs. After incubation, diameters (mm) of the inhibition zones were measured and compared with the standards of the CLSI.

2.2.9 Treatment of patients:

Treatment of the patients was carried out at Al-Kindy Teaching Hospital under the supervision of the surgeon Dr. Sadeq A. Al-Mukhtar for the period of 5/2/ - 5/3 /2013. At the beginning, the patients were divided into two groups (A ,B), each contained 30 patients. Systemic (oral) treatment was used for group A, and a local treatment for group B. And all patients must stop antibiotic use for 48hr before swabs.

2.2.9.1 Systemic treatment:

Systemic treatment was used to treat group A (30 patients). At the beginning, a swab was taken from each patient for identification of pathogenic isolates and testing their susceptibility for antibiotics.

Out of the sixteen used antibiotics in the susceptibility test, only one was used for each patients depending on it susceptibility toward the suspected causative bacterial pathogen. The antibiotic was administrated to the patient for 3 weeks, then a second swab was taken from each patient to determine effect of the systemic treatment on the diabetic foot infection.

2.2.9.2 Local treatment:

Group B was subdivided into three subgroups, each contained 10 patients. Each subgroup was treated locally with either acetic acid, rifampicin or probiotic, but with no systemic treatment.

The first subgroup was treated with acetic acid of 5% concentration obtained from local market as vinegar. Rifampicin solution with a concentration of 30 mg/ml, as prepared in item (2.2.3.3), was used for treating the second subgroup, while the last subgroup was treated with probiotic solution prepared in item (2.2.3.4).

After taken a swab from the patients and identifying the causative bacterial pathogen, each patient of the three subgroups (10 patients/ group) was supplied with the therapeutic agents, and he was asked to applied it locally by putting 4 ml on the diabetic foot ulcer twice a day for one week.

Another swab was taken from the diabetic foot ulcer, after one week, to detect the effect agents on the infecting bacteria. This procedure was repeated for four consecutive weeks. The suspected infecting bacteria were subjected to the cultural, microscopic and biochemical test for identification.

Appendix 1

Form of Infection

Diabetic Foot Patient

Name …….. **الاسم**……

Age ☐ **years** **Sex** ☐ **الجنس** ☐ **العمر** ☐ **سنة**

Occupation: …… **المهنة**.....

Date of the Disease (D.M) ……. **تاريخ الاصابة بالسكر** .....

Site of Infection: **موقع الاصابة:**

1. Pig toe ☐ ١. الاصبع الكبير ☐
2. 2nd toe ☐ ٢.الاصبع الثاني ☐
3. 3rd toe ☐ ٣. الاصبع الثالث ☐
4. 4th toe ☐ ٤. الاصبع الرابع ☐
5. 5th toe ☐ ٥.الاصبع الخامس ☐
6. Heel ☐ ٦. الكعب ☐
7. Other parts ☐ ٧. مناطق اخرى ☐

Duration of Infection ……. **تاريخ الاصابة**.....

Grade of Infection ……. **درجة الاصابة**.....

Previous Treatment ……..... **العلاج السابق**.....

Result of Swab C/S ……..... **نتيجة المسحة**.....

Method of Treat: **طريقة المعالجة:**

Rifampicin Solution ☐ **Acetic Acid** ☐ **Probiotic** ☐

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A Comparative Study on Treatment of Diabetic Foot Infection by Acetic Acid, Rifocin and Probiotics

By

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Abstract

Introduction: Diabetic foot infections are one of the most severe complications of diabetes. This study was aimed to determine the common bacterial isolates of diabetic foot infections and the in vitro antibiotic susceptibility then treatment.

Methods: A swab was taken from the foot ulcer, and the aerobic bacteria were isolated and identified by cultural, microscopic and biochemical test, then by api-20E system. After that their antibiotic susceptibility pattern was determined. Then local and systemic treatment was used to treat the diabetic foot patients.

Results: Bacterial isolates belonging to twelve species were obtained from diabetic foot patients. Gram (-) bacteria were the predominant pathogens in the diabetic foot infections, high percentage recorded by *Klebsiella pneumoniae* (25.71%). Polymicrobial infection was observed in 72% patients. Imipenem was the most affected antibiotic in susceptibility test, except for *Acinetobacter spp*. that resist for all antibiotic used, followed by amikacin and ciprofloxacin. Local treatment gave more inhibitory effect on diabetic foot infections than the systemic treatment.

Conclusion: High prevalence of multi-drug resistant pathogens was observed. Gram (-) bacteria especially *Klebsiella pneumoniae* was the predominant pathogens in the diabetic foot infections, and *Staphylococcus aureus* was the most common of Gram (+) bacteria. Local treatment was the best for treatment of diabetic foot infection patients.

Key word: Diabetic foot infections local treatment.

Introduction:

Foot ulceration or infection is one of the leading causes of human mortality and morbidity. It represents a severe complication of diabetes and the most common cause of diabetes associated hospital admissions (Lavery *et al.,* 2007). Diabetic foot is characterized by several pathological complications such as neuropathy, peripheral vascular disease, foot ulceration and infection with or without osteomyelitis, leading to development of gangrene and even necessitating limb amputation (Anandi *et al.,* 2004 ; Khanolkar *et al.,* 2008).

Diabetic ulcers have 15 to 46 times higher risk of limb amputation than foot ulcers due to other causes (Alavi *et al.,* 2007). It is predicted that the number of people with diabetes will rise from an estimated 171 million in 2000 to 366 million in 2030 (Wild *et al.,* 2004). Diabetic foot infections are often polymicrobial in nature (Gadepalli *et al.,* 2006 ; Alavi *et al.,* 2007**)**. The increasing association of multi-drug resistant (MDR) pathogens with diabetic foot ulcers is the most problem faced by the physician or the surgeon in treating diabetic ulcers without resorting to amputation (Yoga *et al.,* 2006).

Initial therapy of diabetic foot infections is frequently empiric because reliable culture data is lacking. There is variability in prevalence of common bacterial pathogens isolated between Gram (+) and Gram (-) bacteria, as shown (Viswanathan *et al.,* 2002). So, this study was performed to determine the common etiological agents of diabetic foot infections and their in vitro susceptibility to routinely used antibiotics. The treatment of patients with diabetic foot infections by local and systemic agents were also studied.

Methods:

Processing of specimens: A swab from the ulcer of diabetic foot patients was obtained. The specimens were taken immediately to the microbiology laboratory and processed without any delay. The specimens were inoculated on blood agar and MacConkey agar for isolation of aerobic bacteria. After 24 hours incubation at 37^{o}C, the bacterial isolates were identified based on standard bacteriological methods.

Antibiotic susceptibility testing: Antibiotic susceptibility testing was performed by Kirby Bauer's disc diffusion method according to National Community for Clinical Laboratory Standards, NCCLs, (2002). Amoxicillin-clavulanic acid, piperacillin, tetracycline, ciprofloxacin, gentamicin, amikacin, Cefotaxime, erythromycin, netilmicin, vancomycin, Ampicillin, Aztreonam, Chloramphenicol, Ticarcillin-Clavulanic acid, Ticarcillin and imipenem were tested for bacterial isolates .

Treatment of the patients: Treatment of the patients was carried out at the Hospital . At the beginning, 67 patients were divided into two groups (A ,B), each contained 30 patients, while the other 7 patients have no bacterial growth therefore they did not treated. Systematic (oral) treatment was used for group A, and a local treatment for group B. In the systemic treatment, a swab was taken from each patient for identification of pathogenic isolates and testing their susceptibility for antibiotics to chose the most affected one. The chosen antibiotic

was administrated to the patient for 3 weeks, then a second swab was taken from each patient to determine effect of the systemic treatment on the diabetic foot infection. In the local treatment, after taken a swab from the patients and identifying the causative bacterial pathogen, each patient was supplied with the therapeutic agents, and asked to use it. Another swab was taken from the diabetic foot ulcer, after one week, to detect the effect agents on the infecting bacteria. This procedure was repeated for four consecutive weeks.

Results:

From the 67 patients with diabetic foot, 64% were male and 36% were female. The age ranged from 28-75 yrs. On the other hand, the duration of diabetes mellitus was between 4 to 35 yrs, while that of infection was from 1 week to 20 yrs. A total of 105 bacteria were isolated from these patients. The bacteria isolated from the diabetic foot ulcers are summarized in table 1. One type of pathogenic bacteria was detected in 17 (28%) of 60 infected patients, while 43 (72%) of the patients were infected with more than one types (Polymicrobial infection) ; 41(69%) of them with two types and 2(3%) with three types of pathogens. On the other hand, no any bacterial type was detected in the rest (7, 10.45%) of the patients. Gram (-) bacteria were the predominant pathogens in the diabetic foot infections.

Species of bacteria	No.	%
Klebsiella pneumoniae	27	25.71
Pseudomonas aeruginosa	20	19.04
Staphylococcus aureus	18	17.14
Escherichia coli	13	12.38
Proteus mirabilis	12	11.47
Citrobacter freundii	5	4.76
Acinetobacter baumannii	2	1.90
Enterobacter cloacae	2	1.90
Morganella morganii	2	1.90
Pseudomonas fluorescence	2	1.90
Aeromonas hydrophila	1	0.95
Serratia marcescens	1	0.95
Total	105	100

Results of the antibiotic susceptibility testing declared that**,** with exception of *Acinetobacter baumannii*, all bacterial isolates were completely sensitive to imipenem. Susceptibility of most bacterial isolates to the amikacin was also reported in this study, From the Enterobacteriaceae isolates, *E. coli* showed the highest resistance to the antibiotics used in the study. Adversely, *E. coli* appeared sensitive to only imipenem and amikacin.

Pseudomonas aeruginosa resisted penicillin, cephalosporin and chloramphenicol, but sensitive to aminoglycosids and carbapenem. *Aeromonas hydrophila* showed resistance to penicillin group and sensitive to aminoglycosids. While *S. aureus* found to be sensitive to amikacin, gentamycin and ciprofloxacin, and resistant to erythromycin and ampicillin. Resistance of all isolates to the penicillin group and cephalosporin group also found in this study.

Discussion:

Diabetic foot ulcer is chronic and non-healing due to several factors such as neuropathy, high plantar pressures and peripheral arterial disease (Frykberg *et al.,* 2000). Such chronic long-standing ulcers are more prone for infection which delays the wound healing process In this study, Gram (-) bacteria were the predominant pathogens in the diabetic foot infections, Similar findings were also recorded by various studies such as (Shankar *et al.,* 2005 ; Gadepalli *et al.,* 2006 ; Alavi *et al.,* 2007 ; Raga, 2007 ; Ekta *et al.,* 2008). But in the studies of Mantey *et al.,* (2000), Dang *et al.,* (2003) and Diane *et al.,* (2007), Gram (+) bacteria was found to be the predominant organisms in the diabetic foot infections. One type of pathogenic bacteria was detected in 17 (28%) of 60 infected patients, while 43 (72%) of the patients were infected with more than one types (Polymicrobial infection). Polymicrobial infection was also observed by several other studies such as (Wight-Pascoe *et al.,* 2001 ; Anandi *et al.,* 2004 ; Altrichter *et al.,* 2005 ; Shankar *et al.,* 2005 ; Alavi *et al.,* 2007). Adversely, Viswanathan *et al.,* (2002) and Raga (2007) detected only one type of bacteria in the patients of DFI.

Results declared that**,** all bacterial isolates were completely sensitive to imipenem. In this regard, Livemore *et al*. (2001) found that imipenem have strong activity against most Enterobacteriaceae bacteria. Susceptibility of most bacterial isolates to the amikacin was reported in this study, which is closed to that found by Paterson *et al*. (1999) who reported high sensitivity to amikacin among bacterial isolates of their study. Umadevi *et al.* (2011) also detected that the members of Enterobacteriaceae were found to be susceptible to amikacin.

Resistance of all isolates to the penicillin group and cephalosporin group also found in this study, and this may be related to isolates-possessing of β- lactamase enzymes (Levinson and Jawetz, 2000). Another reason for the resistance is production of the extended spectrum ß-lactamase (ESBL) or other enzymes, such as AmpC ß-lactamases, capable of hydrolyzing the extended-spectrum cephalosporins (Rice *et al*., 2003).From the Enterobacteriaceae isolates, *E. coli* showed the highest resistance to the antibiotics used in the study. This may be because *E. coli* is easily acquires the resistance factor from environment and easily resisted penicillin derivatives drug like ampicillin (Wazait *et al*., 2003).

Results declared that *Acinetobacter baumannii* were completely resistant to all 16 antibiotics which may be related to factors, such as Beta-lactamase (Higgins *et al*., 2013). Biofilm formation is a second factor where *A. baumannii* is able to form biofilms for its survival (Espinal *et al*., 2012). In addition, adherence of *A. baumannii* to epithelial cells of the outer membrane also involves in survival of bacteria (Choi *et al*., 2008).

After comparing the two ways of applying the pharmaceutical agents, local treatment, especially rifocin, was found to be the superior for treatment of diabetic foot infection. It is effective especially when accompanied by appropriate wound care as a therapeutic alternative to a broad-spectrum oral antibiotic agent. In addition, local treatment appears to be safe and may avoid the opportunity of resistant bacteria that can develop after oral systemic antibiotic therapy (Lipsky *et al.,* 2008). Local treatment has also the advantages of avoiding systemic adverse effects, providing increased target site concentration and allowing the use of agents not available for systemic therapy (Lio and Kaye 2004). They added that this is another reason made topical treatment be the best for treatment of diabetic foot infection. An acceptable topical anti-infective agent would need to demonstrate activity against the spectrum of bacteria that are known to cause DFI, and it would need to avoid serious adverse effects, interference with wound healing, or induction of drug resistance.

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دراسة مقارنة على معاملة التهاب قدم مريض السكري بحامض ألخليك ومضاد الريفوسين والمعالجات الحيوي

الملخص:

المقدمة: اصابات القدم السكرية احدى اكثر المضاعفات الحادة لمرض السكر. هدفت هذه الدراسة الى تحديد العزلات البكتيرية الشائعة في اصابات القدم السكرية وفحص الحساسية للمضادات الحيوية ثم المعالجة.

الطرق:المسحة اخذت من قرحة القدم، وتم عزل البكتريا الهوائية وشخصت زرعيا ومجهريا وكيموحيويا وبعدة (Api-20E). وبعدها تم تحديد نمط الحساسية الدوائية. ثم تم استخدام المعالجة الموضعية والجهازية لمعالجة مرضى القدم السكرية.

النتائج:العزلات البكتيرية **العائدة ال ١٢ نوعا** تم الحصول عليها من مرضى القدم السكري. البكتريا السالبة لصبغة غرام

Summary

This study was aimed to treat diabetic foot infection patients by different methods and ways in order to select the best treatment. For such purpose, a total of 67 foot swab samples were collected from patients of both sexes who referred to three hospitals in Baghdad during the period of 4/11/2012 – 30/1/2013. Results showed that 105 bacterial isolates were obtained, and found after identification by cultural, microscopic, biochemical characterizations and Api-20E kit, to be belonging to the following 12 bacterial species: (*Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli, Proteus mirabilis, Citrobacter freundii, Acinetobacter baumannii, Enterobacter cloacae, Morganella morganii, Pseudomonas fluorescence, Aeromonas hydrophila* and *Serratia marcescens*).

Antibiotic susceptibility of bacterial isolates against 16 of the commonly used antibiotics was investigated through disc-diffusion method. Results declared that, generally, all isolates were resistant to penicillin and cephalosporin groups. Some of the isolates were sensitive to the amikacin and others to ciprofloxacin. On the other hand, all isolates were sensitive to imipenem except *Acinetobacter baumannii* which resisted all antibiotics used.

Local and systemic ways of treatment were applied on the diabetic foot infection to determine which of them gives better recovery. Two antibiotics were selected for using in the systemic treatment depending on their inhibitory activity in the antibiotic susceptibility test.

While the local treatment was performed by using three agents (vinegar of 5% acetic acid, rifampicin and probiotics represented by *Lactobacillus fermentum* and *L. delbrueckii*).

The two methods were compared by culturing wound swabs of the diabetic foot infection patients before and after treatment to detect whether all original pathogens of the initial wound culture were eradicated, still present or if organism(s) other than the original pathogens appeared in the last culture. Results showed that using of the chosen antibiotics by the systemic treatment had no effect on diabetic foot infections except on one patient who was cured. While local treatment with Rifampicin antibiotic was efficient by recording high inhibitory effect against the causative bacterial isolates. Acetic acid on the other hand, gave a slight inhibitory effect on bacterial isolates. While treatment by probiotics had almost no effect against the diabetic foot infection pathogens.

Supervisors Certification

We, certify that this thesis entitled **''A Comparative Study on Treatment of Diabetic Foot Infection by Acetic Acid, Rifampicin and Probiotics''** was prepared by **''Noorhan Sabih Resn''** under our supervision at the College of Science / Al-Nahrain University as a partial fulfillment of the requirements for the Degree of Master of Science in Biotechnology.

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Committee Certification

We, the examining committee certify that we have read this thesis entitled **"A Comparative Study on Treatment of Diabetic Foot Infection by Acetic Acid, Rifampicin and Probiotics"** and examined the student **''Noorhan Sabih Resn''** in its contents and that in our opinion, it is accepted for the Degree of Master of Science in Biotechnology,

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A Comparative Study on Treatment of Diabetic Foot Infection by Acetic Acid, Rifampicin and Probiotics

A Thesis

Submitted to the College of Science /Al-Nahrain University as a partial fulfillment of the requirements for the Degree of Master of Science in Biotechnology

By

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August: 2013 Shawal: 1434

بسم الله الرحمن الرحيم

اقْرَأْ بِاسْمِ رَبِّكَ الَّذِي خَلَقَ (١) خَلَقَ الإِنسَانَ مِنْ عَلَقٍ

(٢) اقْرَأْ وَرَبُّكَ الأَكْرَمُ (٣) الَّذِي عَلَّمَ بِالْقَلَمِ (٤)

عَلَّمَ الإِنسَانَ مَا لَمْ يَعْلَمْ (٥)

صدق الله

العلي العظيم

الآيات (١-٥) من سورة العلق

الإهداء...

إلى من علم هذا الكون

كيف يخط بأحلى لون...بلدي العراق...

إلى واحتي الخضراء حين تتصحر حياتي...الغالية أمي...

إلى سدي الذي يحميني حين تفيض بحار الحياة...الغالي أبي...

إلى من أحببته حتى امتلأ قلبي بحبه ومن أحيا لأجله ...زوجي علي...

إلى نور عيني من اشد بهم أزري...إخوتي وأخواتي ...

إلى كل من له الفضل في تعليمي

ابتداء من الذي علمني كيف أمسك القلم...

نورهان

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Noorhan

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List of Abbreviations

Abbreviation	Term
ADH	Arginine dehydrolase
CAT	Catalase
CHS	Curative Health Services
CIT	Citrate
CLSI	Clinical Laboratory Standards Institute
D.M	Diabetes mellitus
DFI	Diabetic foot infections
DFU	Diabetic foot ulcer
ESBL	Extended Spectrum Beta Lactamase
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
GDM	Gestational Diabetes mellitus
GEL	Gelatin
G-CSF	Granulocyte colony stimulating factor
IDSA	Infectious Disease Society of America
IND	Indole
IPM	Imipenem
KIA	Kligler Iron Agar
LDC	Lysin decarboxylase
MDRO	Multi Drug Resistant organism
MRSA	Methicillin resistant *Staphylococcus aureus*
ODC	Ornithin decarboxylase
OXI	Oxidase
PAD	Peripheral arterial disease
RIF	Rifampicin
RNAP	DNA dependant RNA polymerase
TDA	Tryptophan daaminase
T1DM	Type 1 Diabetes mellitus
T2DM	Type 2 Diabetes mellitus
UT	University of Texas
URE	Urease
VP1-VP2	Voges-Proskauer
WHO	World Health Organization

الخلاصة

هدفت هذه الدراسة لاستخدام طرائق وأساليب مختلفة لمعالجة التهابات القدم لمرضى السكري بغية اختيار المعاملة الأكفأ منها. وتم لهذا الغرض جمع ٦٧ نموذج مسحة قدم لمرضى من كلا الجنسين المراجعين لثلاث مستشفيات في بغداد خلال الفترة ٤ / ١١ / ٢٠١٢ إلى ٣٠ / ١ / ٢٠١٣. أشارت النتائج إلى الحصول على ١٠٥ عزلة بكتيرية، وجد بعد تشخيصها زرعيا ومجهريا وكيموحيويا أنها تعود إلى ١٢ نوعا بكتيريا هي: وباستخدام عدة (Api-20E) *Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli, Proteus mirabilis, Citrobacter freundii, Acinetobacter baumannii, Enterobacter cloacae, Morganella morganii, Pseudomonas fluorescence, Aeromonas hydrophila*and *Serratia marcescens*.

تم التحري عن حساسية العزلات البكتيرية اتجاه ١٦ مضاد حيوي شائع الاستخدام بطريقة انتشار القرص. عموما، فقد أظهرت النتائج إن جميع العزلات كانت مقاومة لمضادات مجموعتي البنسيلين والسيفالوسبورين. كما وكانت بعض العزلات حساسة للأميكايسين وبعضها الأخرللسبروفلاكسيسين. ومن جهة فقد كانت جميع العزلات حساسة للأمبنيم باستثناء العزلة *Acinetobacter baumannii* التي أبدت مقاومة اتجاه جميع المضادات المستخدمة.

استخدم أسلوبي المعالجة الموضعية والجهازية على التهابات قدم مرضى السكري للتحري عن الأسلوب الأفضل منهما في تحقيق الشفاء. اعتمادا على نشاطهما التثبيطي في اختبار حساسية العزلات للمضادات الحيوية، فقد تم اختيار مضادين حيويين لاستخدامهما في المعالجة الجهازية. فيما استخدمت في المعالجة الموضعية ثلاث أنواع من المواد العلاجية هي (الخل ذو تركيز ٥% حامض خليك و مضاد الريفامبسين ومعززات حيوية تمثلت بنوعين من بكتريا عصيات اللاكتيك هما *Lactobacillus fermentum* و *L. delbrueckii*).

تمت مقارنة النتائج التي أعطتها طرائق المعالجة والأسلوبين المعتمدين في العلاج وذلك بزرع مسحات الجروح المأخوذة من التهابات قدم مرضى السكري قبل وبعد المعاملة وذلك للتحري فيما إذا كانت جميع الممرضات البكتيرية التي كانت موجودة أساسا في الإصابة الأولية قد اختفت، أو أنها لازالت موجودة، أو أن هنالك نوع أو أكثر(من غير تلك التي كانت موجودة أساسا في الإصابة الأولية) قد ظهر في الزرع الأخير.

ويمكن الاستدلال من النتائج إن استخدام ألمضادات المختارة جهازيا (عن طريق الفم) لم يكن له تأثير ايجابي في معالجة التهابات القدم باستثناء مريضا واحدا تم شفاءه. بينما الاستخدام الموضعي لمضاد الريفامبسين أدى إلى إعطائه نشاط تثبيطي عال ضد جميع العزلات البكتيرية المسببة. وفي الوقت الذي أعطى حامض ألخليك تأثيرا تثبيطيا طفيفا على العزلات فلم يكن للمعززات الحيوية المستخدمة تأثيرا ملحوظا على البكتريا المسببة لالتهابات القدم لمرضى السكري.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم
قسم التقانة الإحيائية

دراسة مقارنة على معاملة التهاب قدم مريض السكري بحامض ألخليك ومضاد الريفامبسين والمعالجات الحيوية

رسالة

مقدمة إلى كلية العلوم/جامعة النهرين كجزء من متطلبات نيل درجة الماجستير في العلوم/ التقانة الإحيائية

من قبل

نورهان صبيح رسن

بكالوريوس تقانة إحيائية/ جامعة النهرين (٢٠١١)

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