

دراسة بكتريولوجية و مناعية لتجرثم الدم البكتيري عند مرضى داء السكري في مدينة كربلاء

رسالة

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كجزء من متطلبات نيل شهادة الماجستير في الأحياء المجهرية
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Bacteriological and Immunological Study of Bacteremia in Diabetic Patients at Kerbala City

A Thesis

*Submitted to the College of Medicine/ Babylon
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requirements for the degree of Master in Medical
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بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

"يُؤْتِي الْحِكْمَةَ مَنْ يَشَاءُ وَمَنْ
يُؤْتِ الْحِكْمَةَ فَقَدْ أُوتِيَ خَيْرًا كَثِيرًا
وَمَا يَذَّكَّرُ إِلَّا أُولُو الْأَلْبَابِ"

صدق الله العلي العظيم
سورة البقرة / آية 269

الخلاصة

صممت الدراسة الحالية لدراسة حالات تجرثم الدم Bacteremia و قياس بعض المعايير المناعية عند مرضى داء السكري المراجعين لمستشفى الحسين (ع) في كربلاء للفترة من تشرين الثاني 2006 لغاية مايس 2007. تضمنت الدراسة 125 مريض تراوحت أعمارهم بين 17- 65 عاما كان 30 منهم مصابين بداء السكري من النوع الأول و 95 من النوع الثاني كما تضمنت الدراسة 55 شخص (أصحاء) كمجموعة سيطرة وبنفس المدى العمري.

جمعت عينات الدم من كلا المجموعتين (المرضى و الأصحاء) تم زرع عينات الدم لعزل البكتريا المسببة لتجرثم الدم كما تم تعيين عوامل الضراوة للعزلات البكتيرية (عامل الالتصاق، وجود الكبسولة، إنتاج أنزيمات التجلط ، تحلل الدهون ، تحلل الدم ، تحلل البروتينات وإنتاج البكتيريوسين) إضافة إلى إجراء فحص الحساسية لعدد من المضادات الحيوية. تضمنت الدراسة أيضا حساب أعداد الخلايا التائية بطريقة فحص E-rosette وقياس تركيز γ -IFN و IL-4 بطريقة ELISA وقياس مستوى الكلوبينات المناعية نوع IgG و IgM بطريقة الانتشار المناعي الشعاعي المفرد .

أظهرت النتائج أن نسبة تجرثم الدم البكتيري كانت (24%) في مرضى السكري. إن البكتريا الموجبة لصبغة غرام كانت هي السائدة (70%) في حين لم تتجاوز نسبة البكتريا السالبة لصبغة غرام 30% من حالات تجرثم الدم البكتيري عند هؤلاء المرضى إن *Staphylococcus epidermidis* كانت هي المسبب الرئيسي (61.9%) من حالات تجرثم الدم البكتيري المتسببة عن البكتريا الموجبة لصبغة غرام، بينما بكتريا *Klebsiella pneumoniae* هي السائدة (4:9) بين حالات التجرثم الدموي عند مرضى السكري المتسببة عن البكتريا السالبة لصبغة غرام. أظهرت نتائج اختبار الحساسية للمضادات الحيوية ان جميع العزلات السالبة والموجبة لصبغة غرام كانت حساسة لمضادي السيفوتاكسيم و التتراسايكلين .

كما ان الفحوصات المناعية أظهرت قلة عدد الخلايا التائية المكونة للشكل الزهري بشكل معنوي ($P < 0.05$) عند مرضى السكري النوع الأول و الثاني حيث كانت النسب (9.1% ، 10.63%) على التوالي مقارنة بالأشخاص الأصحاء (22.5%)، كما أظهرت النتائج أن تركيز ($\text{IFN-}\gamma$) هو الآخر قد انخفض بشكل معنوي ($P < 0.05$) عند نفس المرضى (0.285 وحدة دولية/ لكل مليلتر ، 0.313 وحدة دولية/ لكل مليلتر) على التوالي مقارنة بمجموعة السيطرة (0.860 وحدة دولية/ مليلتر) ، إلا أن مستويات (IL-4) انخفضت عند هؤلاء المرضى بشكل غير معنوي ($P > 0.05$) والتي كانت (7.050 بيكوغرام/ لكل مليلتر ، 7.703 بيكوغرام/ لكل مليلتر) على التوالي مقارنة بالأشخاص الأصحاء (13.650 بيكوغرام/ لكل مليلتر).

كما ان مستويات (IgG) ازدادت بشكل معنوي ($P < 0.05$) عند كلا النوعين من داء السكري مقارنة بمجموعة السيطرة غير ان مستوى IgM ازداد بشكل معنوي ($P < 0.05$) عند النوع الثاني 177.64 ملغم/ لكل مليلتر، وبشكل غير معنوي ($P > 0.05$) عند النوع الأول 145.78 ملغم/ لكل مليلتر مقارنة بمجموعة السيطرة 112 ملغم/ لكل مليلتر.

كما اظهرت النتائج ان نسبة الخلايا التائية المكونة للشكل الزهري في مرضى السكر الذين وجد عندهم تجرثم دموي 8.217% و تركيز $\text{IFN-}\gamma$ 0.109 وحدة دولية/ لكل مليلتر والذان انخفضا بشكل معنوي ($P < 0.05$) مقارنة بمرضى السكري الذين ليس لديهم تجرثم دموي (15.264% ، 0.577 وحدة دولية/ لكل مليلتر على التوالي) ، أما مستوى (IL-4) فقد انخفض بشكل غير معنوي ($P > 0.05$) لنفس المرضى، كما أظهرت النتائج ان تركيز الكلوبينات المناعية IgM و IgG قد ازداد بشكل غير معنوي ($P > 0.05$) (1865.24 ملغم/ لكل مليلتر ، 168.5 ملغم/ لكل مليلتر على التوالي) لنفس المرضى مقارنة بمرضى السكري الذين ليس لديهم تجرثم دم بكتيري (1626.996 ملغم/ لكل مليلتر ، 138.47 ملغم/ مليلتر على التوالي).

ABSTRACT

The present study designed to study the incidence of bacteremia and to measure some immunological parameters of diabetic patients after establishing the diagnosis via investigation and clinical diagnosis by physicians in Al-Hussein hospital in Kerbala city during the period from November 2006 until May 2007. A total of 125 patients with diabetes mellitus (30 type I and 95 type II) at age range (17-65 years), and (55) healthy persons as control subjects. Blood samples were collected from both patients and controls, blood culture was done for bacterial isolation and identification, virulence factors (colonization factor-I and III, capsule formation, coagulase, lipase, hemolysin, protease, and bacteriocin production) as well as antibiotic susceptibility tests were assessed for each isolate. This study included also the estimation of T-cells count by E-rosette test, interferon-gamma (IFN- γ) concentration, interleukin-4(IL-4)concentration by ELISA method and IgG and IgM concentration by single radial immunodiffusion (SRID)test.

The obtained results showed that bacteremia was observed in (24%) of the diabetic patients. Gram-positive bacteria were predominant 21:30(70%) than Gram-negative species 9:30(30%), *Staphylococcus epidermidis* was the main etiological agent (61.9%) of Gram-positive bacteremia, while *Klebsiella pneumoniae* was the main etiological agent (4:9) 44% of Gram-negative bacteremia in diabetic patients. Cefotaxime, tetracycline and trimethoprim-sulphamethazole antibiotics were the most effective drugs on both Gram-positive and Gram-negative.

Immunological tests show decrease T-cells count significantly ($p < 0.05$) in diabetic patients type I and type II (9.1%,10.63% respectively)

as compared with control subjects(22.55%).Concentration of IFN- γ was decreased significantly ($p<0.05$) in same patients(0.285I.U/ml ,0.313 I.U/ml) respectively as compared with control subjects(0.860 I.U./ml). Levels of IL-4 were decreased non significantly ($p>0.05$) in patients with diabetes type I and type II (7.050 pg/ml, 7.703pg/ml) respectively as compared with control subjects(13.650 pg/ml). The levels of IgG were increased significantly ($p<0.05$) in both types I and type II (1674.45mg/dL,2095.86mg/dL) respectively as compared with control subjects (1269.51mg/dL),but the levels of IgM were increased significantly ($p<0.05$) in type II (177.64mg/dL) and non significantly ($P>0.05$) in type I as compared with control subjects (112mg/dL).

In diabetic patients with bacteremia, T-cells counts (8.217%) and concentration of IFN- γ (0.109 I.U/ml) were decreased significantly ($p<0.05$)as compared with diabetic patients without bacteremia(15.246%, 0.577I.U/ml). The concentration of IL-4 was decreased non significantly ($p>0.05$) in diabetic patients with bacteremia (5.478pg/ml) as compared with diabetic patients without bacteremia (10.458pg/ml). Concerning, the levels of IgG and IgM were non significantly ($p>0.05$) increased in diabetic patients with bacteremia (1865.24mg/dL,168.5mg/dL)respectively as compared with non bacteremia diabetic patients (1626.996 mg/dL , 138.47 mg/dL) respectively.

We , the examiner committee , certify that we have read the thesis entitled (**Bacteriological and Immunological Study of Bacteremia in Diabetic Patients at Kerbala City**) and have examined the student (**Salim Hussein Hassan**) in its contents, and that in our opinion it is accepted as a thesis for the degree of **Master** of Science in **Medical Microbiology** with excellent estimation.

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List of Abbreviations

Abbreviations	Key
Abs	Antibodies
AGEs	Advanced Glycation Products
AK	Amikacin
ANOVA	Analysis of Variance
APCs	Antigen Presenting Cells
AX	Amoxicillin
β -cells	Beta cells
C. peptide	Connecting peptide
CFA	Colonization Factor Antigen
CFs	Colonization Factors
CIP	Ciproflaxin
CTX	Cefotaxime
D	Density
D.M	Diabetes Mellitus
D.W.	Distilled water
DKA	Diabetic Ketoacidosis
DNA	Deoxyribonucleic Acid
DO	Doxycycline
EAC-rosette	Erythrocyte Antibody Complement rosette
EDTA	Ethylene Di-amine Tetra acetic Acid
ELISA	Enzyme Linked Immuno-Sorbent Assay
E-rosette	Erythrocyte rosette
ETEC	Enterotoxogenic <i>E.coli</i>
FOX	Cefoxitin
G6PD	Glucose -6-phosphate Dehydrogenase
GAD	Glutamic Acid Decarboxylase

Abbreviations	Key
HIV	Human Immunodeficiency Virus
HNF-1 α	Hepatic Nuclear Factor-1 alpha
HRP	Hours Radish Peroxidase
hrs.	hours
I.U/ml	International Unit per milliliter
I.V.	Intra-Venous
IDDM	Insulin Dependent Diabetes Mellitus
IFN- γ	Interferon gamma
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
Igs	Immunoglobulins
IL-12	Interleukin- 12
IL-4	Interluekin-4
M.W.	Molecular Weight
MAbs	Monoclonal Antibodies
Mg/dL	Milligram per deciliter
MHC	Major Histocompatibility Complex
Min.	Minute
MRSA	Methicillin-Resistance <i>Staphylococcus aureus</i>
NA	Nalidic Acid
NCCLS	National Committee for Clinical Laboratory Standards
NIDDM	Non-Insulin Dependent Diabetes Mellitus
NK cells	Natural killer cells
nm	nanometer
NO	Nitric Oxide
OX	Oxacillin
PABA	Para-Amino Benzoic Acid

Abbreviations	Key
PBPs	Penicillin binding proteins
PBS	Phosphate Buffer Saline
PCF	Putative Colonization Factors
Pg/ml	Pico gram per milliliter
PMN	Polymorphous nuclear
r.p.m.	Round per minute
RBCs	Red Blood Corpuscles
rRNA	ribosomal Ribonucleic acid
SD	Standard deviation
sIgA	secretary Immunoglobulin A
SRBCs	Sheep Red Blood Corpuscles
SRID	Single Radial Immunodiffusion
SXT	Trimethoprim-sulphamethazole
Th1	T-cell helper 1
Th2	T-cell helper 2
TMB	Tetra Methyl Benzidin
TNF- α	Tumor necrosis factor alpha
tRNA	transport Ribonucleic acid
UDP-N-acetyl	Uridine Di Phosphate-N- acetyl
UTI	Urinary tract infection

Conclusions:

1- Gram-positive bacteria especially *Staphylococcus epidermidis* was the predominant bacteria that isolated from diabetic patients.

2- Cefotaxime and tetracycline were the most effective antibiotics that affect the growth of most Gram-positive and Gram-negative bacteria.

3- T-cell count was below the normal value in diabetic patients, also there was non significant difference between type I and II diabetes mellitus.

4- The levels of IFN- γ and IL-4 were below the normal value in diabetic patients.

5- The level of IgG concentration was increased significantly in both types of diabetic patients, while the level of IgM concentration was increased significantly in type II and increased non significantly in type I of diabetic patients as compared with control subjects.

6- T-cell counts and IFN- γ concentration were significantly decreased in bacterimic diabetic patients when compared to that of non bacterimic diabetic patients.

Recommendations:

- 1- Study the other microbial causative agents including viral, fungal and protozoa infections in patients with diabetes mellitus.
- 2- Apply to use the immunotherapies to enhance the immune response in diabetic patients.

Supervision Certificate

We certify that this thesis was prepared under our Supervision at the Department of Microbiology, College of Medicine in Babylon University as a partial requirement for the Degree of **Master in Medical Microbiology**.

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To

♥ *The memory of my father*

♥ *The kind heart : My mother*

♥ *My wife*

♥ *My flowers: MURTADAH and NAWRAS*

I dedicate this work.

Salim

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Salim

1-1 Introduction:

Diabetes mellitus (D.M.) is defined as an abnormal metabolic state in which there is glucose intolerance due to inadequate insulin action as well as the late development of many complications (Underwood,2004 ;Boon *et al.*, 2006). The World Health Organization recognizes two main forms of D.M type I and type II. Type I is usually due to autoimmune destruction of the pancreatic beta cells (β -cells) which produce insulin. Type II is a heterogeneous group of disorders characterized by tissue-wide insulin resistance and varies widely (Shareeff,1982;WHO,1999;Kasper,2005). Diabetic subjects probably have a higher risk of many infections. In addition, immunosuppression which occurs in those patients, because of increased sugar levels in blood stream and as a result of dysfunction of immune system make diabetic patients more incident for microbial infections, especially bacteremia (Joshi *et al.*,1999). Good metabolic control is a major factor in limiting the development and spread of infections and, most importantly, the development of diabetic complications which predispose to infections(Mitchell *et al.*,1998). Several factors could predispose diabetic patients to infections. These factors include: genetic susceptibility to infection; altered cellular and humoral immune defense mechanisms; local factors include poor blood supply and nerve damage, and alterations in metabolism associated with diabetes (James,2000).

Chronically ill and immunocompromized patients like D.M have an increased risk of bacteremia. They may also develop bacteremia and

fungemia(Everett and Hirschmann,1977; Vincent *et al.*,2004). In diabetic patients the incidence of bacteremia is higher because polymorphonuclear (PMN) cells are clearly influenced by the diabetic state. On the other hand, their antimicrobial function is inhibited by hyperglycemia due to inhibition of glucose -6- phosphate dehydrogenase (G6PD) (Thomson *et al.*, 2004; Moutschen, 2005). Another mechanism which can lead to increased prevalence of infections in diabetic patients is an increased adherence of microorganisms to diabetic and grow better in glucose, in addition to express different virulence factors (Geerlings and Hoepelman, 1999). Impaired host defense mechanisms such as impaired granulocyte function, decrease cellular immunity, impaired complement function and decreased lymphokine response may be influenced by glycemic control (McMahon and Bistran,1995; James,2000).

1-2 Aims of the study:

- I- Isolation and identification of bacterial species associated with bacteremia in D.M patients.
- II- Determination of bacterial virulence factors.
- III- Determination of the antibiotic profiles for these bacterial isolates.
- IV- Study of some humoral and cellular immunological parameters in diabetic patients with and without bacteremia.

1-3 History of D. M. :

The disease which resulted in a decrease in pancreatic hormone (insulin) was first mentioned in the early books of ancient Egyptian (1500B.C.). The Roman scientist Aritaius was the first who mentioned the term diabetes to describe this disease which means the fountain or urination with large quantities. However, Aritaius does not refer to the presence of sugar in urine in this disease (Engelgan, 2004).

In 1674, Thomas Willis referred to the sweetness of patient urine, and in 1774, Dobson proved that this sweetness was due to the presence of sugar in urine. Bokard in 1835 mentioned that the sugar “glucose” was the agent of sweetness, hence the designation “mellitus” was employed to describe the disease which means “the sweet taste of the urine” (Al-Soffi,2004).

The role of Arab scientists in this field was important and prominent, when they presented a comprehensive interpretation of this disease during the period (850-1250), and they referred to the accompanying thirst during the disease which was a result of abundant urination of the diabetic patients (Al-Rassam,2004). In 1906, a first pancreas extract was injected into a diabetic patients. In 1916 researchers invented a first assay for measurement of blood sugar. In 1921-1922 insulin was discovered and a first hormone was succeeded. After that laboratory-made insulin was produced commercially using genetically engineering bacterium (Al-Rassam,2004).

In 1980, the insulin pump was first discovered and employed. Nowadays, studies and researchers are still in a dramatic progress to find new solutions for this crucial disease(Alberti and Zimmer,1998).

1-4 Overview :

1-4-1 Diabetes mellitus (D.M): is a chronic disorder characterized by impaired metabolism of glucose and other energy-yielding fuels as well as the late development of vascular and neuropathic complications (Mitchell *et al* ;1998 , Boon *et al.*, 2006). Diabetes mellitus is a group of metabolic diseases characterized by high blood sugar (glucose) levels, which result from defects in insulin secretion, action, or both (Bennett and Plum,2004).

Normally, blood glucose levels are tightly controlled by insulin, a hormone produced by the pancreas (Alberti and Zimmer,1998). A common feature of D.M is a hyperglycemia that leads to damage of many organs in human body such a kidney, eyes and nervous system (WHO,1999 ;Charpenter *et al.*,2004 ; Boon *et al.*, 2006) .

1-4-2 Pancreas:

The pancreas is called the “hidden organ” because it is located deep in the abdomen behind the stomach. The discovery of a role for the pancreas in diabetes is generally ascribed to Joseph von Mering and Oskar Minkowski, who in 1889 found that dogs whose pancreas was removed developed all the signs and symptoms of diabetes and died shortly afterwards(Virtanen and Knip,2003). The normal adult pancreas contains about one million islets Langerhans within the organ. The core

of each islet consist of β -cells that produce insulin surrounded by a cortex of endocrine cells that produce other hormones including glucagon (α -cells) and somatostatin (δ -cells). Pro-insulin in the pancreatic β -cell is cleaved to release insulin and equimolar amounts of inert connecting peptide (C-peptide). Measurement of C-peptide can be used to assess endogenous insulin secretory capacity (Boon *et al.*, 2006).

1-4-3 Insulin :

In 1910, Sir Edward Albert Sharpey-Schafer suggested that people with diabetes were deficient in a single chemical that was normally produced by the pancreas he proposed calling this substance *insulin*, from the Latin *insula*, meaning island, in reference to the insulin-producing islets of Langerhans in the pancreas (Saeed and Al-Dabbagh,2003). Insulin is a protein hormone secreting from Langerhans islets of pancreas that represent about 1% of pancreas. It consists of 51 amino acids in two chains (α -chain consists of 21 amino acids and β chain that consists of 30 amino acids). The α and β chains are linked by disulphide bonds (Yonis and Ajoloni,1999 ; Relling ,2002).

Insulin has M.W 5750 Dalton and five minutes as half-life time (Laurence and Bennettem,1992), a short half-life of insulin is important for excess of its activity in target cells in the body (Bortol *et al.*, 1990). When insulin reaches to target cells binding with insulin receptors on the membrane that leading to receptor stimulating. Insulin receptor is a heterodimer with two α and β chains formed by disulfide bridges (Al-Soffi,2004; Charpenter *et al.*,2004).

1-4-3-1 Metabolic effects of insulin :

After eating a diet rich with carbohydrates, glucose level will be raised in blood stream and this leads to excess secretion of insulin that is helped to enter the glucose inside the cells especially muscle cells to be stored as a source of energy, hepatocytes as glycogen and in adipocytes in synthesis of lipids in the body (Malcongio and Tomlinson,1998; Kenneth , 2002).When the glucose levels in the portal vein falls to a sufficiently low value the pancreas emits glucagons from its alpha sites. This glucagon stimulates the liver to release stored glucose into blood stream. The liver subsequently does this by converting its stored glycogen into glucose and releasing it into the blood stream (Bronk,1999). It was believed that insulin inhibits phosphorylase enzyme that is involved in breakdown of liver glycogen into glucose(Maechler *et al.*,1993). In this way these two pancreatic sites (α and β islets) correct the level of blood sugar that is too low or too high (Niskanen *et al.*, 1998).

Glucose enters into muscle cells in two cases, first after two hours of diet and second in express exercise (Bishop *et al.*, 2000). In brain the effect of insulin is simple or absence to controlling of enter the glucose inside brain cells, which is characterized by high permeability to glucose through its membrane because glucose is the only source of energy and if blood glucose level decreased (20-50mg/dL), hypoglycemic shock and coma could occur (Diabetes care, 1998; Bennett and Plum,2004). While the effect of insulin on lipids metabolism is as follows:

a- Insulin catalyzes the glucose storage in the liver as glycogen form and the excess is converted into acetyl co-A which is the primary source to fatty acid synthesis (Loven *et al.*, 1986 ; Burke *et al.*, 1992)

b-It involves in activation of lipoprotein lipase in adipose tissue and this enzyme plays an important role in separation of lipoprotein into triglyceride and proteins, then triglycerides analyzed into glycerol and free fatty acids for simple absorbance (El-Hazmai and Warsy, 2000).

1-5 Classification of D.M:

Diabetes mellitus is classified on the basis of the pathogenic process that lead to hyperglycemia as a post to earlier criteria such as age of onset or type of therapy, the two board categories of D.M. are designated type I and type II (Kasper *et al.*,2005).

1-5-1 Type I :

Type I is characterized by disorder that has little or no insulin to prevent metabolic decompositions(Bennett and Plum,2004). This type indicates the processes of β -cell destruction that may ultimately lead to D.M. Type I is usually characterized by the presence of anti-glutamic acid decarboxylase (GAD), islet cell or insulin antibodies which identify the autoimmune processes that lead to β -cells destruction(Kuzuya and Matsuda,1997). This syndrome accounts for about 10% of diabetes (Tahhe,2005). The peak incidence of this form occurs in childhood and adolescence, but the onset may occur at any age, ranging from childhood to the ninth decade of life (Molbak *et al.*, 1994). These patients may also

have other autoimmune disorders such as Grave's disease, Hashimoto thyroiditis and Addison's disease (Betterle *et al.*, 1983).

1-5-2 Type II:

This is the most common form of diabetes and account 90% of diabetes characterized by variable degree of insulin resistance and impaired insulin secretion (Kasper *et al.*,2005). It is frequently undiagnosed for many years because the hyperglycemia is often not severe enough to provoke noticeable symptoms of diabetes (Mooy *et al.*,1995 ;Boon *et al.*, 2006). About 10% of type II require insulin treatment and ketoacidosis infrequent (Bierman *et al.*,1998 ; Mclaughlin,2003; Kasper,2005). Type II diabetes characteristically appears after the age of 40 years, and is associated with obesity (Bennett and Plum, 2004).

1-5-3 Gestational diabetes:

This type occurs in about 2% of pregnancy cases (Voty *et al.*,2002 ; Basu *et al.*,2003). About 30- 40% of gestational diabetes comment to type II diabetes through 3-10 years and rarely causes type I (Bronk, 1999 ; Bennett and Plum, 2004) .

1-5-4 Other specific types of D.M:

1-5-4-1 Genetic defects of β -cells function:

Several forms of the diabetic state may be associated with monogenic defect in β -cells function, frequently characterized by onset of mild hyperglycemia at an early age (generally before the age 25 years) (Byrne *et al.*, 1996 ; Clement *et al.*, 1996). The most common

form is associated with mutations on chromosome in a hepatic nuclear transcription factor (HNF-1 α) (Yamagata *et al.*, 1996). A second form is associated with mutation in glucokinase gene of chromosome. Glucokinase converts glucose to glucose- 6- phosphate, which is in turn stimulates insulin secretion by β -cells (Froguel *et al.*,1992).

1-5-4-2 Genetic defects in insulin action:

The metabolic abnormalities associated with mutations of the insulin receptor gene may range from hyperinsulinaemia and modest hyperglycemia to symptomatic diabetes(Taylor, 1992).

1-5-4-3 Diseases of exocrine pancreas:

Any process that diffusely injures the pancreas can cause diabetes. Acquired processes include pancreatitis, trauma, infection, pancreatic carcinoma and pancreatectomy (Larsen *et al.*, 1987).

1-5-4-4 Endocrinopathies:

Several hormones such as growth hormone, cortisol, glucagons and epinephrine antagonize insulin action. Diseases associated with excess secretion of these hormones can cause diabetes. This form of hyperglycemia typically resolve when the hormone excess is removed (MacFarlane *et al.*, 1997).

1-5-4-5 Drug or chemical induced diabetes:

Many drugs can impair insulin secretion. These drugs may not by themselves, cause diabetes but they may precipitate diabetes in persons with insulin resistance, examples of these drugs are nicotinic acid,

thyroid hormone, dilantin pentamidin and interferon alpha therapy (O'Byrne and Feely, 1990 ; Pandit *et al.*, 1993).

1-5-4-6 Viral Infections:

Certain viruses (rubella, cytomegalovirus and coxachie B virus) have been associated with β -cell destruction (King *et al.*,1983). The viral infections may be co-factors in causing diabetes (Kinp and Akerblom,1999).

1-6 The prevalence of D. M.:

The prevalence of D.M is difficult to determine because various standards are no longer acceptable have been used in diagnosis. Collected data show that approximately 150 million people have D.M worldwide ,and this number will double by the year 2030 , so as , in the world represent 2%-7% of population (Kucera *et al.*,2002 ;Boon *et al.*, 2006).The increase prevalence of diabetes is primary due to an increase in type II diabetes particularly in developing countries (Grundy *et al.*,1999 ; Voty, 2002). Approximately 90–95 % of diabetic patients have type II variety. The onset of type II usually precedes clinical diagnosis by several years. Type II diabetes accounts for 5-10 % of the U.S.A. population over the age of 60 years has diabetes (Goldfine and Goldfine , 2003).

In the Arab world, it had been estimated that 11-20% of population had D.M especially in oil producing gulf countries (Kashgari,2000). In Suadia the prevalence of diabetes increased from 6% in 1982 to 14% in

1992 (Saeed and Al-Dabbagh,2003). It has mentioned that 10 % of Iraqi population had D.M.(Tahhe,2005).

1-7 Symptoms of D. M. :

The clinical diagnosis of D.M. is often prompted by symptoms such as increased thirst, polyurea, recurrent infections, unexplained weight loss and in severe cases drowsiness and coma (WHO,1999 ;Engelgan,2004). In addition to that there are other symptoms like blurring of vision ,nocturia ,headache and fatigue (Charpenter *et al.*, 2004 ; Boon *et al.*, 2006).

1-8 Complications of D.M:

There are many complications occurs in diabetic patients include diabetic ketoacidosis (DKA), hypoglycemia which is the most frequent complications in type I diabetes (Charpenter *et al.*,2004; Achenbach *et al.*, 2004; Boon *et al.*,2006), microvascular complications, macrovascular complications (Panahloo and Yudkin, 1997), foot infection and the most important complications that include immunodeficiency and bacterial infections (bacteremia) (Gibbon and Habershaw,1995; Grayson ,1995).

1-8-1 Immunodeficiency:

One of the most complications of D.M is the immunodeficiency which result in greatly increased susceptibility to infections (Guyton and Hall,2006). Immunodeficiency is defect in the development and function of immune system result in an increased susceptibility to infections of certain infectious diseases (Abbas and Lichtman,2007).

The ability of the immune system to respond to pathogens is diminished in age extremes with immune responses beginning to decline at around 50 years of age (Awa *et al.*,2007). Immunodeficiency may be primary (congenital deficiency) or secondary (acquired deficiency) as in neonates, immunosenescences, pregnancy and elderly persons(Tewari *et al.*,1997)Immunodeficiency degree affected by the causes and their affect on the immune system like cancer disease which characterized with neutropenia with defect in humoral response characterized by decreasing levels of immunoglobulins in serum of these patients (Elting *et al.*,1997). In developed countries obesity ,alcoholism and illegal drug abuse are common causes of poor immune functions. In diabetic patients ,hyperglycemia impairs the immune system and also leads to the accelerated creation and accumulation of advanced glycation end-products (AGEs) that interact with monocytes and macrophages promoting the expression of more pro-inflammatory phenotypes (Grossi *et al.*,1996; Mealy,2000). Both old age and D.M are known to reduce immunity and increase the risk of infections indicating the importance of special attention to these high risk group (Chin ,2000). Concerning cellular innate immunity, most studies showed decreased function of polymorphonuclear (PMN) cells and diabetic monocytes /macrophages compared to the cells of control (Geerlings and Hoepelman,1999).

In humans, Halminen *et al.*,(2001) studied the expression profile of IFN- γ and IL-4 in blood of D.M and found that both to be significantly reduced as compared with normal controls. An alteration in the immune

responses to the pathogenic agents occurs in immunocompromized patients express a great degree of decrease in opsonization as a result of decrease the levels of specific antibodies, in addition to alteration of surface receptors of fragment constant (Fc) and third complement component (C3) that found on the surface of the phagocytes and this alternate the phagocytosis process and killing of microorganisms (Cheadle *et al.*,1996; Saeed and Castle,1998).Moreover, the hormonal disorders of diabetic patients causes an alteration of vascular permeability resulting in a decrease in the migration of phagocytes (Tater *et al.*,1985).

Regarding diabetic patients, succinate produced by both aerobic and anaerobic bacteria inhibits the chemotaxis of phagocytosis making the probability of infection five times greater than in non-diabetic patients (Al-Shahwani and Faqeeh ,2005). It was pointed that glycation induced alteration of T-cell immune competence. This provides a biochemical basis for the well recognized association between poorly controlled diabetes and bacterial infections (Sasaki *et al.*,1993). The hyperglycemic patients had been reported to highly susceptible to microbial infections, in addition binding of glucose to the biochemical active sites of complement components (C3) that inhibits the attachment of this protein to the microbial surface and there by impairs opsonization and phagocytosis functions (Chapel *et al.*,1999).

1-8-2 Bacterial infections associated with D.M:

1-8-2-1 Bacteremia: Bacteremia is the presence of bacteria in the blood stream. It may cause no symptoms and resolve without treatment, or it may produce fever and other symptoms of infection (Huber and Terezhalmay,2005). Several types of bacteria live on the surface of the skin or colonize the moist lining of the urinary tract, lower digestive tract and other internal surfaces. These bacteria are normally harmless as long as they are kept in check by the body's natural barriers and the immune system (Gale,2006). Gram-negative bacteremia secondary to infection usually originates from the gastrointestinal tract or the skin in patients with decubitus ulcers (Vincent *et al.*,2004).

1-8-2-2 Risk factors of bacteremia :

There are many conditions which increase the chance of developing bacteremia these include (Tenover and Hughes,1996):

- a- Immune suppression; either due to HIV infection, D.M. , cancer or drug therapy.
- b- Antibiotic therapy which changes the balance of bacterial types in the body.
- c- Prolonged or sever illness.
- d- Alcoholism or other drug abuse.
- e- Use of corticosteroids.
- f- Malnutrition.

g- Extreme ages.

h- Diseases or drug therapy that cause ulcer in the intestines, e.g chemotherapy for cancer.

The common immediate causes of bacteremia include (Thomson *et al.*,2004;Nowrouzian *et al.*,2007):

i- Drainage of an abscess , including an abscessed tooth.

ii- Urinary tract infections , especially in the presence of bladder catheter

iii- Decubitus ulcer (pressure sores).

iv- Intravenous procedures using unsterilized needles , including intravenous I.V. drug use.

v- Prolonged I.V. needle placement.

vi- Use of ostomytubes including gastrostomy.

The most common origin of the bacteremia are urinary tract (33%), gastrointestinal tract (18%) and respiratory tract (18.6%)(Cisterna,2001). It was found that the percentages of the most common bacteremia associated with D.M ranged between 15.7 % to 37 % (Akbar,2000 ; Cisterna *et al .*, 2001).

1-8-2-3 Etiology of bacteremia:

Berner *et al.*,(1998) mentioned that bacteremia represented 98% from blood stream infections, while Singh *et al.*,(1997) pointed to 81% from blood stream infections due to bacterial infections (bacteremia) and 19% due to viruses, parasites and fungi. The bacteria most likely to cause bacteremia include members of *Staphylococcus*, *Streptococcus*,

Pseudomonas, *Haemophilus* genera and *Escherichia coli* (Moutschen,2005).

Thomson *et al.*,(2004) reported that *Streptococcus pneumoniae* increased 1.5 folds in diabetic patients compared with individuals without diabetes. In Hong-Kong ,French *et al.*, (1990) mentioned that Gram-negative bacteria causing bacteremia in immunosuppressive patients represented 62.5%, and the percentage of *E.coli* ; *Klebsiella spp.* , *Pseudomonas spp.* and *Proteus spp.* were (27.8 % , 9.7 % and 4.4%) respectively, while Gram-positive bacteria represented 29.4% and the percentages of *Staphylococcus aureus* , β -hemolytic streptococci and *Streptococcus pneumonia* were (11,8 % , 4.8 % and 3.3 %) respectively. The *Staphylococcus aereus* and *Staphylococcus epidermidis* are most common causes of bacteremia in immunocompromized patients(Rupp *et al.*, 1999). Al-Thani (1999) studied bacteremia in immunocompromized patients in Qatar and he found that Gram-positive bacteria was predominate (59%) against Gram-negative bacteria (40%). Whereas in USA Gram-positive bacteria particularly *Staphylococcus aureus* has a higher incidence in bacteremia (27.3%) ,while Gram-negative bacteria particularly *E.coli* was (26.1%) in bacteremia of cancer patients (Miser *et al.* ,1981; Favero *et al.* ,1988). It was found that the incidence of bacteremia due to all microorganisms was increased two folds in diabetic patients while the incidence of Enterobacteriaceae bacteremia was increased three folds (Byran,1985). Gram-negative bacteria represented 51.1% of etiologic agents of

bacteremia in D.M patients which predominated by *E. coli* , *Klebsiella spp.* , *Proteius spp* and *Pseudomonas aeruginosa* (Macfarlane *et al* .,1986). The study reported by Cisterna *et al.*, (2001) found that the percentages of Gram-negative bacteria in D.M were as follows: *E. coli* (36%) ,*Pseudomonas aeruginosa* (2.71%) and *Proteus mirabilis* (2.65%). In another study bacteremia in diabetic patients was (4.8%) as a result of *E. coli*, *Salmonella typhi* and *Staphylococcus aureus* (Al-Wazni,2001). Oni *et al.*, (2000) mentioned that bacteremia due to Gram-negative bacteria in D.M were as follows: *Klebsiella spp.* (43%), *E. coli* (11.4%) and *Bacteriodes melaninogenious* was 4.15 %.

1-8-2-4 Diagnosis of bacteremia:

Blood is normally sterile so culturing of blood alone does not normally lead to the isolation of bacteria. Bacteremia is most commonly diagnosed by blood culture, in which a sample of blood is allowed to incubate with a medium that promotes bacterial growth. If bacteria are present in the blood stream at the time sample is obtained, the bacteria will multiply in the medium and can thereby detected (Thomson *et al.*,2004). Any bacteria that incidentally find their way to the culture medium will also multiply, for this reason blood cultures must be drown with great attention to sterile process (Brooks *et al.*, 2004). Growth in media is indicated by the presence of turbidity, gas bubbles, hemolysis of the blood in the culture bottle or even by observation of bacterial colonies suspended in the bottle (Washington,1975). Gram stained smears should be made from any broth that show visible signs of

growth, cultural characteristics, biochemical, serological and genetic analysis are widely used to confirm the diagnosis of bacterial isolates (Collee *et al.*, 1996).

1-8-2-5 Virulence factors of bacteria associated with diabetic patients:

Bacterial pathogens have developed many strategies for survival in higher organisms, which during their evolution have formed very sophisticated defense mechanisms. This defense system includes nonspecific reactions such as mechanical clearing of the mucosa, control of iron transfer, phagocytosis, elimination of bacteria by enzyme attack (e.g., by lysozyme), and activation of complement, as well as specific reactions involving antibodies and cells of the immune system. Pathogenic bacteria have worked out many different ways to overcome the host defense system. A number of biological features known as virulence factors are common to many bacterial species, although some of these are characteristic only for certain bacteria (Finlay and Falkow,1989). Common bacterial properties involved in the infection process include adhesion to epithelial surfaces, invasion (penetration) of host cells, intracellular multiplication of the pathogen, colonization of the cell tissue or transmission to a new susceptible host, production of enzymes which damage the host defense system, and synthesis of toxins (Hacker and Goebel,1987 ;Johnson,1991).

Virulence is the measure of pathogenicity of an organisms. The degree of virulence is related directly to the ability of the organism to

cause disease despite host resistance mechanisms; it is affected by numerous variables such as the number of infecting bacteria , route of entry into the body, specific and non specific host defense mechanisms and virulence factors of pathogenic bacteria employ the means by which they cause disease (Kenneth,2002):

a- Invasiveness, the ability to invade tissue, ability to bypass or overcome host defense mechanisms and the production of extracellular substances (invasions) which facilitate the actual invasive process

b- Toxigenesis, is the ability to produce toxins, both soluble and cell associated, which may be transported by blood and lymph. The most common virulence factors of bacteria are:

1-8-2-5-1 Capsule formation :

Capsule is a discrete detectable layer of polysaccharide deposited outside the cell wall of bacteria. It is known to protect bacteria from engulfment by phagocytes and from attack by antimicrobial agents. Since capsular substances are antigenic they can stimulate B-cells and produce antibodies (Abs) that can neutralize the effect of capsular substances and make the bacteria susceptible to phagocytic cells. This phenomenon does not find in immunocompromised patient because of decrease activity of B-cell to produce Abs required for the opsonization (Rajesh and Rutten,2004). Encapsulated strains of many bacteria are more virulent and more resistant to phagocytosis and intracellular killing than non encapsulated strains.(Oksur *et al.*,2005).

1-8-2-5-2 Hemolysin production :-

Many bacteria produce substances that dissolve red blood cells (RBCs) and called hemolysis. There are three types of hemolysis: alpha(α) hemolysis that is characterized by incomplete hemolysis and appear as greenish-darkening of the agar that contain RBCs, beta(β) hemolysis, that is a complete lyses of RBCs in the media, the area around and under the colonies are lightened and transparent, and gamma (γ) hemolysis, the RBCs in the media is unchanged and this is called non-hemolysis (Collee *et al.*, 1996 ; Ryan and Ray, 2004). In addition to destroyed RBCs, hemolysins destroys other cells (phagocytes) to protect bacteria from phagocytosis and facilitate the spreading in blood stream (Kenneth ,2002; Rajesh and Rutten,2004).

1-8-2-5-3 Coagulase production :

Coagulase is a cell associated and diffusible enzyme that convert fibrinogen to fibrin which cause clotting around bacteria lesions, which helps them persist in tissues. Coagulase also causes deposition of fibrin on the surfaces of individual staphylococci, which may help protect them from phagocytosis (Brooks *et al.*, 2004 ,Ryan and Ray , 2004) .

1-8-2-5-4 Protease production (gelatinase):

Proteases are enzymes that break down protein to primary elements (amino acids), gelatin is a protein derivative of animal collagen. Protease is a proteolytic enzyme which is often important in the invasiveness of microorganisms into the host tissues and considers as virulence factor as

a result of ability to break down immunoglobulins and complement components (Barrett *et al.*, 2003; Al-Rassam,2004).

1-8-2-5-5 Lipase production :

Lipases are enzymes that catalysis the hydrolysis of triglycerides and diglycerides to fatty acids and glycerol. Epithelial cells surface in human contain lipids, which hydrolyzed by lipase from many organisms that help in spreading of organisms through cutaneous and subcutaneous tissues and enhance colonization of the skin(MacFaddin,2000).

1-8-2-5-6 Colonization factors :

The first stage of microbial infection is the colonization that is mean establishment of pathogen at the appropriate portal of entry (Maria *et al.* , 2007). Colonization factors (CFs) include :

The first type is the type-I fimbriae enables the bacteria to bind to D-mannose residues on eukaryotic cell surfaces. Type 1- fimbriae is said to be mannose -sensitive since exogenous mannose blocks binding to receptors on red blood cells(Hagberg *et al.*,1981). The second type is the type III fimbriae, which are mannose-resistant fimbriae. This type of fimbriae is associated with their ability to hemagglutinate at presence of tannic acid-treated erythrocytes from several animal species (Old and Adegbola,1985). Colonization factors(CFs) and putative colonization factors (PCF) are proteins exposed on the surface of bacteria and are fimbrial (or fibrillar if they are especially thin). They promote attachment of the Enterotoxigenic *E. coli* (ETEC) to epithelial cells of the small intestine and therefore serve as virulence factors (Cassels and

Wolf,1995). Both epidemiological and challenge experiments in humans suggest that colonization factor antigen (CFA) are protective antigens such that immunity to CFA protects against challenge by other ETEC strains expressing the same CFA(Cravioto *et al.*,1990)

1-8-2-5-7 Bacteriocin production :

Bacteriocins are antibacterial proteins produced by bacteria. They differ from traditional antibiotics in having a relatively narrow spectrum of action and being lethal only for bacteria which are closely related to the producing strains (Riley and Gordon,1992). Many different bacteriocin groups have been described since and named after a species or genus of bacteria. The bacteriocin family includes a diversity of proteins in terms of size, microbial targets, mode of action, and immunity mechanism. The most extensively studied the colicins produced by *E. coli* (Braun *et al.*, 1994 ; Cramer *et al.*, 1995; Gouaux, 1997). Colicins gene clusters are encoded on plasmids and are composed of a colicin gene, which encodes the toxin; an immunity gene, which encodes a protein conferring specific immunity to the producer cell by binding to and inactivating the toxin protein; and a lysis gene, which encodes a protein involved in colicin release through lysis of the producer cell. The killing functions range from pore formation in the cell membrane to nuclease activity against deoxyribonucleic acid (DNA), ribosomal ribonucleic acid (rRNA) and transfer ribonucleic acid (tRNA) targets (Braun *et al.*, 1994 ; Gouaux ,1997).

Although colicins are representatives of gram-negative bacteriocins, there are differences found within this subgroup of bacteriocin family. *E. coli* encodes its colicins exclusively on plasmid replicons (Pugsley, 1984 ; James *et al.*, 1996). The bacteriocins (klebocins) of *Klebsiella pneumoniae* are found exclusively on plasmids (Al-Charrakh, 2005). The nuclease pyocins of *Pseudomonas aeruginosa* are found exclusively on chromosomes (Sano *et al.*, 1990).

Bacteriocins of Gram-positive bacteria are abundant and even more diverse as those found in Gram-negative bacteria (Tagg *et al.*,1976 ; Jack *et al.*, 1995). They differ from Gram-negative bacteriocins in two fundamental ways. First, the range of killing in Gram-positive bacteriocins can vary from relatively narrow as in the case of Lactococcin, which kills only *Lactococcus* ,to extraordinarily broad as in Nisin A, which have been shown to kill a wide range of organisms (Mota-Meira *et al.*, 2000). Secondly, the Gram- positive bacteria have evolved bacteriocin-specific regulation, whereas bacteriocins of Gram-negative bacteria rely only on host regulatory networks (Riley and Wertz,2002). Epidemiological investigations on bacterial colonization and disease have relied on bacteria marker systems. One of these important systems is bacteriocin typing (Edmondson and Cooke, 1979 ; Pal *et al.*, 1997).

1-8-2-5-8 Other virulence factors:

Urease is the virulence factor of many bacteria. Urea represents the main nitrogenous excretory product in humans and the majority of animals, urease catalyzes the hydrolysis of this compound to yield ammonia and carbon dioxide, which results in an increase in the urine pH (Clapham *et al.*,1990 ; Mobley and Hausinger,1989 ;Mobley *et al.*, 1995). Urease is considered as the primary cause of infection-induced urinary stones(Griffith *et al.*,1976; Clapham *et al.*,1990). Urease activity has been found in over 200 species of gram-negative and gram-positive bacteria (Mobley and Belas,1995; Mobley *et al.*,1995). This enzyme has also been implicated as a factor contributing to the pathogenicity of many bacteria including *Proteus*, *Providencia*, and *Morganella* species (Senior *et al.*,1980; Jones and Mobley,1987). The urease activity of these bacteria is used to distinguish them from other Enterobacteriaceae family members. This activity was constitutive in most *Proteus mirabilis* strains (Mobley and Hausinger,1989). The presence of flagella (organ of motility) on the surface of pathogenic and opportunistic bacteria has been thought to facilitate the colonization and dissemination from the initial site (Senior *et al.*,1980 ; Mobley and Belas,1995). Another aspect of this problem is associated with the important role of flagellin as a bacterial surface antigen (H antigen). Since flagellin is strongly immunogenic, it can be assumed that at least part of the immunoresponse of the host during the infection is directed against this antigen. Thus, the possible changes in flagellin antigenicity may enable

bacteria to escape the immunoresponse of the infected microorganisms (Belas,1994).

1-8-2-5-9 Antibiotic Resistance:

Resistance to antibiotic is considered as a virulence factor for the pathogenic microorganisms to cause the infections. Mechanisms of resistance to antimicrobials used to treat infectious diseases have been known since before antibiotics were introduced into routine clinical usage. Irresponsible and often overuse of antimicrobials have, however, exacerbated the problem by enriching for resistant bacterial populations at the expense of sensitive ones. With all too increasing frequency, so humans may face with important human pathogens displaying resistance to multiple antimicrobials (Stratton,2000; Brooks *et al.*,2004). The major mechanisms of antibiotic resistance are:

a- Enzymatic modification or destruction:

The predominant mechanism of resistance to β -lactams remains beta-lactamases, enzymes that inactivate the antibiotic by hydrolysing the β -lactam ring of the molecule (Livermore,1995; Piddock *et al.*,1997). Resistance arises from natural or mutational derepression of chromosomal genes or from acquisition of extra chromosomal genetic elements (plasmids or transposons) carrying the resistance genes. Similarly, bacterial resistance to aminoglycosides is primarily based on a chemical modification of the aminoglycosides which compromises binding of the agent to its ribosomal target. Resistance to

chloramphenicol is typically afforded by acetyltransferases (Stratton,2000).

b-Target alteration:

Changes in drug targets that interfere with or limit antibiotic interaction also prevent the bacteriostatic/bactericidal effects of these agents and, thus, promote resistance. The most common mechanism of resistance to macrolides, for example, involves modification of their target site on the ribosome, specifically methylation of an adenine residue in domain V of the 23S rRNA. β -Lactam resistance due to target site (i.e. penicillin-binding protein) alterations, especially in the streptococci, is also common. Resistance to methicillin in *Staphylococcus aureus* (MRSA) is generally due to production of a low-affinity penicillin-binding proteins (PBPs), which confers resistance to virtually all β -lactams (Jacoby,1996 ;Stratton, 2000). Vancomycin targets the D-ala–D-ala termini of the Uridine Di-Phosphate-N-acetyl(UDP-N-acetyl muramyl pentapeptide)precursor of peptidoglycan, ultimately interfering with peptidoglycan cross linking. Resistance arises as a result of the synthesis of abnormal pentapeptide precursors with lower affinity for vancomycin (Cormican and Jones,1996).

c-Impermeability:

In order for antibiotics to exert their bacteriostatic or bactericidal actions on bacteria they must access intracellular targets. This necessitates, in Gram-negative bacteria, that they cross the outer membrane, a substantial permeability barrier and thus, a major

determinant of antimicrobial resistance in these bacteria. Indeed, the outer membrane barrier explains, at least in part, the enhanced resistance of Gram-negative and Gram-positive organisms to many antimicrobials. The intrinsic resistance of many Gram-negative organisms to macrolides, for example, is probably explained by the limited permeability of this membrane to macrolides (Hartman and Tomasz,1981; Dawson and Coffey,2000).

d- Efflux:

Efflux as a means of antibiotic resistance is most commonly associated with the tetracyclines (e.g. TetA, TetB, TetK pumps) and the fluoroquinolones in both Gram-positive and Gram-negative bacteria. Tet genes are usually plasmid or transposon-encoded and thus, resistance arises from acquisition of these genes from external sources (Jarlier *et al.*,1996).

These pumps contribute to both intrinsic and acquired resistance, the latter arising from mutational hyperexpression of these chromosomally encoded efflux systems. Many of these and related efflux systems also provide for efflux of and thus, resistance to macrolides, β -lactams, aminoglycosides and tetracycline. Efflux-mediated resistance to macrolides has also been described in Gram-positive bacteria. Chloramphenicol resistance can also be afforded by efflux(Brooks *et al.*,2004).

e- Alteration of metabolic pathway: e.g. some sulfonamide-resistant bacteria do not require para-aminobenzoic acid (PABA) an important

precursor for the synthesis of folic acid and nucleic acids in bacteria inhibited by sulfonamides. Instead, like mammalian cells, they turn to utilizing preformed folic acid (Hall,1997; Murry *et al.*,2003).

1-8-2-6 Treatment of bacteremia :-

Bacteremia may cause no symptoms , but may be discovered through a blood test for another condition . In this situation it may not need to be treated ,except in patients especially at risk for infection, such as with immunodeficiency such as cancer, D.M and heart diseases (Gale,2006). No single specific therapy is available. The sulfonamides, ampicillin, cephalosporins, fluoroquinolones and aminoglycosides have marked antibacterial effects against the bacteria but variation in susceptibility is great and laboratory tests for antibiotic sensitivity are essential (Brooks *et al* .,2004). Carratala *et al.*,(1995) showed that 57% of viridans streptococci causing bacteremia were resistant to penicillin. Empiric long-term antibiotic therapy is no longer uniformly recommended for all cases of *Staphylococcus aureus* bacteremia, although experts disagree about the optimal length of therapy (Kim *et al.*,2003).

Al-Otaibi(2000) mentioned that cephalosporins reduce the occurrence of enterococcal bacteremia in diabetic patients.

2-1 Materials:-**2-1-1 Equipment and apparatuses :-**

List of equipment and apparatuses used through-out of the study.

Equipment	Company (origin)
Light microscope	Olympus, Japan
Autoclave	Portable, Japan
Incubator	Imperial III, Germany
Centrifuge	Anke, Europe S.A.
Electric balance	Sartorius, Europe S.A.
ELISA	Beckman, Germany
ELISA Mixer	Denley- England
ELISA Washer	Beckman , Germany
ELISA Shaker	Jean Robin- France
Refrigerator	Ishtar- Iraq
Automatic micropipette	Memmert , Germany
Hot air oven	Memmert, Germany
pH- meter	Inolab 720, Germany

2-1-2 List of chemicals and biological materials that used through-out the study:

i– Chemicals:

Chemicals and biological materials	Company, Origin
Ethanol (95%)	BDH, England
HCl (10 N)	BDH, England
HgCl ₂	BDH, England
Glucose	BDH, England
D- mannose	BDH, England
Acetic acid (10%)	BDH, England
NaOH (1 N)	BDH, England
NaCl	BDH, Norway
Trisodium citrate	BDH, England
H ₂ O ₂	BDH, England
Ficoll-Hyplaque(Lymphoprep,1.077 D)	Nywneel- Norway
Glycerol	BDH, England
Phosphate buffer saline(PBS-pH=7.2) tablets	BDH, England
Indian ink	BDH

ii– Stains:

Gram's stain set	Crescent, Saudi
Geimsa stain	Crescent, Saudi

iii– Ready made culture media:

These culture media were prepared according to the manufacture instructions and autoclaved at 121°C, 15 pound/ inch² for 15 minutes.

Culture media	Origin
Brain heart infusion broth	Mast diagnostic/U.K
Brain heart infusion agar	Mast diagnostic/U.K
Nutrient broth	Mast diagnostic/U.K
Nutrient agar	Mast diagnostic/U.K
Blood agar base	Mast diagnostic/U.K
MacConky agar	Mast diagnostic/U.K
Müller Hinton agar	Mast diagnostic/U.K
Manitol Salt agar	Mast diagnostic/U.K
Kligler's Iron agar	Difco, England
Api- 20 E	(BioMerieux- France)

iv– Antibiotic disks that are used through-out the study:

Type	Abbreviation	Concent. mg	Origin
Amikacin	(Ak)	30 mg	Bioanalyse, Turkey
Amoxicillin	(Ax)	25	Bioanalyse, Turkey
Oxacillin	(ox)	1	Bioanalyse, Turkey
Cefotaxime	(CTX)	30	Bioanalyse, Turkey
Cefoxitin	(Fox)	30	Bioanalyse, Turkey
Ciproflaxin	(CIP)	5	Bioanalyse, Turkey
Doxycycline	(Do)	30	Bioanalyse, Turkey
Nalidic acid	(NA)	30	Bioanalyse, Turkey
Trimethoprim- Sulphamethazole	(SXT)	1.25+23.75	Bioanalyse, Turkey

v– Immune reagents kits that are used through-out the study:

- i-** IFN- γ kit detected by ELISA (Biosource, Europe, S. A).
- ii-** IL- 4 kit detected by ELISA (Biosource, Europe, S. A).
- iii-** IgG and IgM endoplates of single radial immunodiffusion(SRID) test (Biomaghrab, Tunis).

2-1-3 Solutions**2-1-3-1 Phosphate buffer saline PBS (pH= 7.2):**

This buffer was used in E-rosette test. It was prepared by dissolving one buffered tablet in 100 ml distilled water(D.W) and sterilized by autoclaving (121 °C, 15 pound/ inch², for 15 minutes) in accordance with the instructions of manufacturer company (BDH) and was kept at 4 °C.

2-1-3-2 Alsever's solution (pH= 6.1):

This solution was used as anticoagulant, preservative and transport medium for sheep red blood corpuscles (SRBCs). It was prepared by dissolving the following materials in 1200 ml of D.W. (Lewis *et al.*, 2001).

Glucose	24.6 grams
Tri Sodium citrate	9.6 grams
Sodium chloride	5.1 grams

The pH was adjusted to 6.1 with drops of citric acid at concentration of 10% and then the solution was sterilized by autoclaving (Garvery *et al.*, 1977 ; Lewis *et al.*, 2001).

2-1-3-3 Normal saline (pH = 7.0) :

Normal saline was used in washing of SRBCs and for making the bacterial suspensions. It was prepared by dissolving 8.5 grams of sodium chloride (NaCl) in one liter of D.W. sterilized by autoclaving, and kept at 4°C. (Cruickshank *et al.*, 1975).

2-1-3-4 Ficoll- Hyplaque (Lymphoprep, 1.077 D):

This solution was used for the isolation of lymphocytes in E-rosette test. It was stored at 4 °C in dark bottles.

2-1-3-5 Suspension of sheep red blood corpuscles (SRBCs):

The SRBCs suspension was used in E-rosette test and prepared as follows:

Fresh blood was taken from jugular vein of sheep and put into sterilized bottle containing Alsever's solution in a proportion 1:1 volume. The blood-Alsever's solution mixture was centrifuged at 300g for 15 minutes, and the sediment (red corpuscles) was washed three times with normal saline. Finally, the red corpuscles were re-suspended in Alsever's solution to a final concentration (10%) (Garvey *et al.*, 1977).

2-1-3-6 D-mannose solution (0.1 M):

This solution was used in detection of colonization factor-I of the bacteria. It was prepared by dissolving 1.8 grams of D-mannose sugar in 100 ml D. W. (Ofek *et al.*, 1977).

2-1-3-7 Tannic acid solution (1%):

This solution was prepared by dissolving one gram of tannic acid in 100 D.W and dilute 1/200 and used in bacterial heamagglutinin (colonization factor-III)(Ofek *et al.*, 1977).

2-1-4 Culture media:

The culture media that were prepared during this study:

2-1-4-1 Blood agar media (B.A) :

Prepared by suspending 37 grams of blood agar base in one liter D.W. heating to make mixture and sterilizing by autoclave at 121°C, 15 pound/inch² for 15 minutes and supplemented with 10% of fresh human blood after cooling to 45°C. Final pH was adjusted to 7.1. It was used for cultivation of the fastidious bacteria and detection of hemolysine production (MacFaddin,2000) .

2-1-4-2 Brain heart infusion broth(BHIB)with5% glycerol:

This medium was prepared by adding 5 ml of glycerol to 95 ml of prepared BHIB and then sterilized by autoclave at 121°C, 15 pound/ inch² 15 minutes. BHI agar medium supplemented with 5% glycerol was prepared by adding 2% agar to the above mentioned medium. This medium was used in bacteriocin production (Al-Charrakh,2005).

2-1-4-3 Gelatin agar medium :

Prepared by suspending 13 grams of nutrient broth medium in one liter D.W and addition of 30 grams of gelatin, heating and sterilizing by autoclave. This medium was used for testing the ability of bacteria to liquefy gelatin (Su *et al.*, 1991).

2-1-4-4 Egg- Yolk agar Medium :

This medium was used to detect the ability of bacteria to produce lipase enzyme, prepared by suspending 7.4 grams of blood agar base in 200 ml D.W heating and sterilizing by autoclave and then supplemented with 20 ml of yolk-normal saline mixture after cooling to 45°C (Cruickshank *et al.*, 1975).

2-1-4-5 Semi- Solid medium:

Prepared by adding 0.5% agar to nutrient broth, and used for detecting the motility of bacteria (Collee *et al.*,1996).

2-1-5 Biochemical reagents :**2-1-5-1 Catalase test reagent :**

Prepared by dissolving 30 grams of H₂O₂ in 100ml of D.W (MacFaddin, 2000).

2-1-5-2 Gelatinase test (Friezer's Solution):

This solution was prepared as follows (MacFaddin, 2000):

- a- Addition of 20 ml of concentrated hydrochloric acid (HCl) to 50 ml of D.W.
- b- Dissolving 15grams of HgCl₂ in the above mixture by gently shaking and completing the final volume to 1000ml of D.W.

2-1-5-3 Oxidase test reagent :

The reagent was prepared by dissolving one gram of tetra methyl-P- Paraphenylene diamine dihydrochloride in 100ml of D.W. It was kept at dark reagent bottles until use (Collee *et al.*,1996; Brooks *et al.*,2004).

2-2 Methods:

2-2-1 Samples–Patients :

This study included a total of 125 diabetic patients as diagnosed by clinical physicians (35 Type I and 90 Type II) with age range (17-65 years) who attended Al-Hussein hospital in Kerbala during the period from November 2006 until May 2007. Case history of patients involving patient's name, age, type of therapy, and accompanying disease (Index-1) was recorded. Diabetic patients were no malignancy, no asthma, no cardiovascular accident, no heart failar and not take any antibiotics. In addition, the patients who were admitted in the hospital, the sample was taken before administration any antibiotics.

2-2-2 Healthy controls:

A total of (55) volunteers(no diabetes, no prolonged illness and not taking antibiotics) with age matching to the patients group to compare the results obtained for diabetic patients.

2-2-3 Samples collection :

Blood samples were collected from each patient and control (six milliliters of venous blood) and withdrawn by disposable syringe under aseptic technique. The blood samples were divided into three parts:

- I- One milliliter was immediately inoculated into sterilized blood cultures bottle that contain 10 ml of BHI broth for bacteriological investigation .
- II- Two milliliter were put in EDTA anti-coagulant tubes for E-rosette technique.

III- Three milliliter were out in sterilized plane tube and allowed to clot, then serum was separated by centrifugation at 300g for 15 minutes. The serum was stored by freezing until used for immunological tests.

2-3 Laboratory diagnosis:

2-3-1 Bacterial identification :

Bacterial isolates were identified at the level of species using traditional morphological and biochemical characterization according to (Holt *et al.*, 1994; Collee *et al.*, 1996 ;and MacFaddin, 2000; Murray *et al.*,2003).

2-3-1-1 Morphology of colonies on MacConky and blood agar medium.

2-3-1-2 Detection of capsule: Using negative stain that is carryout as follows:

- a-** A large loopful of undiluted Indian ink was placed on a clean glass slide.
- b-** A very small portion of solid bacterial culture was emulsified in the ink drop.
- c-** A clean grit-free cover- slip was placed on the ink drop.
- d-** The presence of capsule was examined with the oil immersion objective lens, and the highly refractive out-line of the bacterium was seen (Collee *et al.*, 1996).

2-3-1-3 Biochemical tests:**2-3-1-3-1 Catalase test :**

This test was used to detect the ability of bacteria to produce catalase enzyme. It was carried-out by mixing a single isolated colony transferred by woody stick with a drop of hydrogen peroxide (30% H₂O₂), the production of gas bubbles indicates a positive result (Cruickshank, 1975).

2-3-1-3-2 Coagulase test :

This test was used to detect the ability of an organism to clot plasma by the action of the enzyme coagulase. Coagulase slide method was used to detect the bound coagulase that is found on the surface of cell walls as following :

After emulsifying staphylococcal colony with a drop of sterilized normal saline on a clean slide, one drop of human plasma was added, then mixed gently, coagulase positive organisms became clumped after a few seconds. To compare the result control test was done by mixing saline and bacteria without plasma to ensure that the organisms do not clump spontaneously (Bennerman, 2003) .

2-3-1-3-3 Oxidase test :

To determine the presence of the oxidase enzyme. This test was performed by transferring a part of bacterial growth by sterilized woody sticks into filter paper saturated with recently prepared oxidase reagent(Tetramethyl P-Paraphenylene diamine hydrochloride).Turning the color from white to dark purple indicated the positive result (MacFaddin, 2000) .

2-3-1-3-4 Growth on Kligler's Iron agar (KIA) :

This test was used to determine the ability of an organism to attack specific carbohydrate incorporated in a basal growth medium, (with or without the production of gas) and to produce H₂S gas (appearance of black sediment at the bottom of the tube). This test was carried out by inoculating Kligler's iron agar slant medium with bacterial growth (24 hrs.) and incubated at 37°C for 24 hrs. The color changes in top slant and bottom and gas production were detected (Collee *et al.*,1996 ;MacFaddin, 2000) .

2-3-1-3-5 Api- 20E technique:

Api 20-E system consists of twenty microtubes containing dehydrated substrates. Microtubes were inoculated with a bacterial suspension; metabolism produces color changes that are either spontaneous or revealed by addition of reagents. The reactions are read according to the reading table (Index-2) and the identification is obtained by referring to the analytical profile.

2-3-2 Detection of virulence factors:**2-3-2-1 Coagulase factor test**

This test was carried out as described in (2-3-1-3-2) .

2-3-2-2 Lipase test :

Lipase test was carried out in Egg-Yolk agar medium to determine the ability of microorganisms to produce the enzyme lipase after inoculation of the medium agar, plates were incubated overnight at 37°C. The appearance of opaque pearly layer around the colonies indicates a positive result for this test (Collee *et al.*, 1996).

2-3-2-3 Gelatinase test :

It was carried out by inoculating of gelatin agar plate with bacterial isolate and incubated at 37°C for 24 hrs. Plates were flooded with mercuric chloride (Friezer's solution) as in (2-1-5-2) which causes an opacity in the medium with clear zones around gelatin-liquefying colonies (Collee *et al.*, 1996).

2-3-2-4 Haemolysin production :

Haemolysin production was carried out by inoculating of blood agar medium with bacterial isolate at 37°C for 24-48 hrs. An appearance of clear zone around the colonies referred to complete haemolysis (β -haemolysis) or greenish zone around the colonies referred to partial haemolysis (α -haemolysis), while no change referred to non-haemolysis (γ - haemolysis) (Doboy *et al.*,1980).

2-3-2-5 Haemagglutination test :

This test was carried out for detection of colonization factors. Two methods were used to determine the colonization factor I:
a- D-mannose inhibition test: This method as described by (Ofek *et al.*, 1977) was used to detect CF-I. This test was carried out as follows:

One volume of bacterial growth was put on glass slide and mixed with same volume of D-mannose solution (0.1M) as in (2.1.3.1.5), then same volume of human red blood cells suspension (3%) was added and allowed two minutes to observe the agglutination in absence of D-mannose solution which indicates a negative test and

not agglutination if present D-mannose solution referring to positive test.

b- The production of colonization factor III was detected by using tannic acid solution (instead of D-mannose solution) and same procedure was followed as described above (Ofek *et al.*,1977).

2-3-2-6 Detection of bacteriocin production :

Cup assay method that described by (AL-Qassab and AL-Khafaji,1992) was carried out for detection of bacteriocin production as follows:

I- All isolates were grown in brain heart infusion broth with 5% glycerol at 37°C for 18-24 hrs.

II- The growing bacterial isolates were heavily streaked on brain heart infusion agar with 5% glycerol and then incubated at 37°C for 18 hrs.

a- An *E. coli* strain (obtained from department of Microbiology College of Medicine/Babylon University was used as an indicator (sensitive) strain for detection of bacteriocin production by Gram-negative bacterial isolates (bacteriocin producers).

b- An *Staphylococcus aureus* (obtained from department of Microbiology College of Medicine/Babylon University) was used as an indicator (sensitive) strain for detection of bacteriocin production by Gram-positive bacterial isolates (bacteriocin producers).

III- Using sterile 5 mm cork borer, agar disks were cut from the cultured agar layer.

IV- The indicator strain was allowed to grow on nutrient broth for 2-3 hrs. in a shaking water bath at 37°C (to obtain 10^6 – 10^7 cells /ml).

V- A volume of 0.1 ml of indicator growth was spreader on nutrient agar plates and left to dry, then carefully, agar disks were transferred to the agar surface seeded with indicator strain and incubated overnight at 37°C.

VI- Sensitivity patterns were recorded, presence of inhibition zones around the agar disks indicated a positive result.

2-3-2-7 Antibiotic susceptibility test:

Five ml of nutrient broth medium inoculated with an isolated colony of test bacteria and incubated for 18-24 hrs. at 37°C, and then transferring 0.1 ml of the bacterial suspension has been spread on the surface of Müller–Hinton medium plate and left to dry. Antibiotic disks has been placed and incubated for 24 hr. at 37°C (NCCLS,2003a). The resulting zones of inhibition have been measured using a ruler and compared with the zones of inhibition determined by National Committee for Clinical Laboratory standards (NCCLS,2003b).

2-4 Immunological tests :

2-4-1 Erythrocyte–rosette formation (E-rosette test)

E-rosette means the clustering of sheep erythrocytes around a leukocyte or other cell. This test is used as a marker for T-lymphocytes of humans and most mammals. E-rosette test is a method used to identify, isolate and estimate the T-lymphocyte (Parslow *et al.*,2001).

This test was carried out according to (Burrell,1979; Madsen *et al.*, 1980 ; Frank, 1997; and Gengozian *et al.*,2002) as follows:

- a-** Three ml of lymphoprep (Ficoll-Hyplaque) was pipetted into centrifuge tube .
- b-** Two ml of freshly drawn blood were carefully layered onto the surface of centrifuge tube without mixing.
- c-** Centrifugation was carried out at 400g for 30 minutes at room temperature.
- d-** The leukocytes appeared as a fluffy white coating at the plasma medium interface, while the erythrocytes appeared on the bottom of the tube, the upper plasma layer was carefully collected and saved for further experiments.
- e-** The white cells were collected with a Pasteur's pipette and placed into another centrifuge tube.
- f-** Centrifugation was performed at 400 g for 30 minutes and the supernatant was discarded.
- g-** The preparation was washed three times in PBS at 400 g for 15 minutes.
- h-** The pellet was re-suspended in 0.5 ml of saline to which 0.05ml autologous plasma was added .
- i-** To another centrifuge tube containing 0.1 ml of plasma lymphocyte mixture, 0.1 ml of SRBCs (10%) was added and mixed gently.
- j-** The centrifuge tube containing the mixture was incubated at 37°C for 15 minutes, then centrifuged at 200 g for 15 minutes.

-
- k-** Incubation was carried-out for one hour at 4°C with supernatant stilled on the pellet .
- l-** The cells were resuspended, very gently by tilting the tube back and forth 2-3 times.
- m-** Blood film was prepared, fixed with ethanol for 10 minutes, and stained with Geimsa's stain for 15 minutes, the film was washed with D.W. dried in air and examined microscopically under oil immersion lens (100x).
- n-** One hundred lymphocytes were counted and the percent of rosette forming lymphocytes with three or more sheep erythrocytes adhered to it, were recorded.

2-4-2 Estimation of serum IFN- γ (Biosource Co.):

Principle: The biosource IFN- γ is a solid phase enzyme amplified sensitivity Immune assay (EASIA) performed on micro titer plate. Samples or standards containing IFN- γ react with capture monoclonal antibodies (MAbs-1) coated on the micro titer well and with a monoclonal antibody (MAb-2) labeled with horseradish peroxidase (HRP). After an incubation period allowing the formation of a sandwich: (coated MAbs-1-IFN- γ -MAb2- HRP).

The microtiter plate was washed to remove unbound enzyme labeled antibodies. Bound enzyme labeled antibodies are measured through a chromogenic reaction. Chromogenic solution (tetra methyl benzidin, TMB) is added and incubated. The reaction is stopped with the addition of stop solution (H₂SO₄, 1.8N) and the micro titer plate is then read at the appropriate wavelength. A standard curve is plotted

and IFN- γ concentration in a sample is determined by interpolation from the standard curve. The procedure of IFN- γ estimation: (according to the information supplied by Biosource Co.).

I- Reagent preparation;

a- Standards provided that prepare by addition 0.5 ml D.W to each one used to draw standard curve.

Standard	Concentration I.U/ml
0	0
1	0.6
2	1.3
3	3.9
4	7.9
5	22.5

b- Controls, prepared as in standards and there are two controls (control 1: 2.2 ± 0.6 I.U/ml ; control 2: 5.1 ± 1.3 (I.U/ml), used as internal laboratory controls.

c- wash solution, prepared by dilute 2 ml of the provide solution in 400 ml of D. W.

d- chromogenic solution, prepared by pipetting 0.2 ml of the concentrated chromogen into one vial of substrate buffer (21 ml) that is provided with the kit.

II- Assay procedure of IFN- γ estimation :

a- The required number of the strips was selected for the run.

b- The strips were secured into the holding frame.

-
- c-** 50 μ L of each standard or sample was pipetted into the appropriate wells.
- d-** 50 μ L of anti- IFN- γ conjugate was pipetted into all the wells.
- e-** The plate was incubated for two hours at room temperature on a horizontal shaker set at 700 r.p.m \pm 100 r.p.m.
- f-** The liquid from each well was aspirated.
- g-** The plate was washed three times by washing solution .
- h-** 200 μ L of freshly prepared chromogenic solution was pipetted into each well, within 15 min. following the washing step .
- i-** The plate was incubated for 15 minutes at room temperature on horizontal shaker set at 700 r.p.m \pm 100 r.p.m.
- j-** 50 μ L of stop solution was pipetted into each well.
- k-** Absorbance was read at 450 nm (reference filter 620 nm) within three hours.
- l-** The curve was drawn on linear graph paper, plotting the concentration of IFN- γ standards on horizontal axis and the absorbance on the vertical axis.
- m-** The IFN- γ concentration for unknown sample was read and content from the standard curve plotted in step (a).

2-4-3 Estimation of serum IL-4 level: (Biosource Co.)

Principle:

The assay is based on an oligoclonal system in which a blend of monoclonal antibodies (MAbs) directed against distinct epitopes of IL-4 are used. IL-4 that present in sample is react with capture monoclonal antibodies (MAb1) coated on the microtiter well and with

a monoclonal antibody (MAb2) labeled with HRP. After incubation period allowing the formation of a sandwich: (MAbs1–IL-4-MAb2-HRP), micro titer plate was washed to remove unbound enzyme labeled antibodies. Bound enzyme labeled antibodies were measured through a chromogenic reaction. Chromogenic solution was added and incubated. The reaction was stopped with the addition of stop solution (H₂SO₄) and the microtiter plate was then read at the appropriate wavelength. A standard curve was plotted and IL-4 concentration in a sample was determined by interpolation from the standard curve.

Procedure:

The procedure of IL-4 estimation: (according to the information supplied by Biosource Company) :

I- Reagent preparation :

a- Standards provided that prepare by addition 0.5 ml D.W to each one. These are used for drawing standard curve.

Standard	Concentration Pg/ml
0	0
1	14
2	41
3	125
4	460
5	1370

b- Controls prepared as in standards and there are two controls (control 1: 114 ± 22 Pg/ ml ; control 2: 371 ± 74 Pg/ml). These are used as internal laboratory controls.

c- Wash solution, prepared by dilute 2 ml of the provide solution in 400 ml of D.W.

d- Chromogenic solution, prepared by pipette 0.2 ml of the concentrated chromogen into one vial of substrate buffer that is provided with the kit .

II- Assay procedure of IL- 4 Estimation :

a- The required number of strips was selected for the run.

b- The strips were secured into the holding frame.

c- 100 μ L of solution B was pipetted into the appropriate wells for serum samples.

d- 100 μ L each sample, control or standard was pipetted into the appropriate wells.

e- 50 μ L of anti- IL-4 conjugate was pipetted into all the wells.

f- The plate was incubated for 2 hrs. at room temperature on horizontal shaker set at 700 r.p.m \pm 100 r.p.m.

g- The liquid was aspirated from each well.

h- The plate was washed three times by washing solution.

i- 200 μ L of freshly prepared chromogenic solution was pipetted into each well within 15 min. following the washing step.

j- The plate was incubated for 30 min. at room temperature on an horizontal shaker set at 700 r.p.m \pm 100 r.p.m.

k- 50 μ L of stop solution(H_2SO_4 1.8 N)was pipetted into each well.

l- Absorbance was read at 450 nm (reference filler 620 nm) within three hrs. and plotted on standard curve to calculate concentration of IL-4 in the serum.

2-4-4 Estimation of IgG and IgM Level by Single Radial Immunodiffusion (SRID) test :

Principle: The procedure consists in an immune precipitation in agarose between an antigen and its homologous antibody. It is performed by incorporating one of the two immune reactants (usually antibody) uniformly throughout a layer of agarose gel, and then introducing the other reactants (usually antigen) into wells duly punched in the gel. Antigen diffuses radially out of the well into the surrounding gel-antibody mixture, and a visible ring of precipitation forms. Ring diameters are measured by viewing device (ocular). Unknown concentration are determined from the tables supplemented with each type of plate (Dacie and Lewis, 1984).

Procedure:

a- Endo plates and the serum of (patients and controls) were removed from refrigerator. Reagent were equilibrated to room temperature.

b- Plate was removed from ziplock bag. After lid removed, the wells were inspected for moisture if moisture was present, uncovered plates were allowed to remain at room temperature until moisture evaporated. Sera of patients were thoroughly shaken by inversion pipette 5 μ L of the serum into the appropriate well, and putting wet cotton in the plate center.

c- Plates were incubated at room temperature for 48 hrs. to IgG and 72 hrs. for IgM test.

d- End point of diffusion was indicated by a sharp precipitation ring.

e- Diameter of ring was measured with 0.1mm precision by suitable device and then compared to the standard diameter to conclude the concentrations of serum immunoglobulin (Dacie and Lewis, 1984).

2-5 Statistical Analysis :

The mean, standard deviation(SD), and analysis of variance (ANOVA) test were calculated (Cochran,1974) for statistical analysis of the results of this study.

Table (3-13) The relationship between bacteremia and immunological parameters in diabetic patients

Testing groups	Total No. (%)	Immunological parameters				
		E-rosette* %	IFN- γ * I.U/ml	IL-4* Pg/ml	IgG* mg/dL	IgM* mg/dL
Bacterimic diabetic patients	30 (24)	8.217**	0.109**	5.478	1865.24	168.5
Non-bacterimic diabetic patients	95 (76)	15.246**	0.577**	10.458	1626.996	138.47
Total number	125 (100)					

* The mean value is calculated for both types of D.M with bacteremia

** The mean difference is significant at 0.05 level.

3-1 Age distribution of diabetic patients:

As shown in table (3-1) a total of one hundred twenty five diabetic patients were included this study. Type I was represented in 35:125 (28%) of diabetic patients their age ranged from (17-50 years), and type II was diagnosed as 90:125 (72%) with age range (40-65 years). These results agreed with other studies which found type I account for about 10–15% of diabetic patients, while type II is the most common form of D.M and represent 85-90% with the peak incidence occurs in over 40 years age (Boon *et al.*,2006). This suggests that D.M type I focused in childhood and young ages, while D.M type II occurred mainly in older ages. In addition, a fifty five normally health subjects were included as control groups .

Table (3-1) Distribution of patients according to age and type of diabetes

Testing groups	Total number	%	Age range (year)
Diabetes mellitus type I	35	28	17-50
Diabetes mellitus type II	90	72	40-65
Total number of patients	125	100	

3-2 Bacteriological study :

3-2-1 Blood culture :

Results of blood culture of clinical samples (table 3-2) revealed that positive bacterial blood culture indicating for bacteremia was observed in No.10 (28.57%) of D.M type I, and in 20 (22.2%) of type II. These results indicated the presence of bacteremia in both types of diabetic patients and this result within the range (15.7%-37%) of bacteremia associated with D.M that obtained by other investigators(Akbar,2000 ; Cisterna *et al.*,2001). Results also showed that bacteremia was increased significantly ($p<0.05$) in diabetic patients when compared with control subjects. This result agreed with Byran *et al.*,(1985) who mentioned that the frequent occurrence bacteremia among patients with diabetes represents a significant problem.

Table (3-2) Distribution of bacteremia among types of diabetic patients and controls

Testing groups	Total number	Number of bacteremia (%)	Number of non-bacteremia (%)
Diabetes mellitus type I	35	10 (28.57)	25 (71.43)
Diabetes mellitus type II	90	20 (22.2)	70 (77.8)
Total of patients	125	30 (24)	95 (76)
Controls	55	0	55(100)

3-2-2 Morphological distribution:

Table (3-3) show the distribution of bacterial isolates according to Gram staining. Gram-positive bacterial isolates appeared to be the predominant (70%) when compared to Gram-negative isolates which were isolated from only (30%) of diabetic patients. These results were in accordance with (Al-Thani,1999; Thomson *et al.*,2004; Moutschen,2005) who mentioned that Gram-positive bacteria were predominant agents of bacteremia in diabetic patients.

Table (3-3) Distribution of bacterial isolates according to type of Gram staining

Testing groups	Total No. of isolates	No. of Gram-positive bacteria	No. of Gram-negative bacteria
Diabetes mellitus type I	10	7	3
Diabetes mellitus type II	20	14	6
Total number	30	21:30 (70%)	9:30 (30%)

3-2-2-1 Gram-positive bacteria :

The result of cultural characteristics ,biochemical properties applied for Gram-positive bacteria (Index 3) revealed that these bacterial isolates belonged to the bacterial species expressed in (table 3-4) *Staphylococcus epidermidis* was the major Gram-positive isolate (61.90%), while *Bacillus cereus* (4.76%) was the less commonly

isolated Gram-positive species in diabetic bacteremia. These results agreed with (Rupp *et al.*,1999 ; VonEiff *et al.*,2001) who reported that *Staphylococcus epidermidis* was predominant agent of bacteremia in immuno-compromized patients.

Table (3-4) Gram-positive bacteria isolated from diabetic patients

Bacterial species	Total number	%
<i>Staphylococcus epidermidis</i>	13	61.90
<i>Streptococcus mitis</i>	4	19.05
<i>Staphylococcus aureus</i>	3	14.29
<i>Bacillus cereus</i>	1	4.76
Total of isolates	21	100

3-2-2-2 Gram-negative bacteria :

The identification of Gram-negative bacteria was based on Api-20E-system (index 2) and the cultural characteristics, biochemical properties of these bacteria (index 4) revealed that these isolates belonged to the bacterial species shown in (table 3-5) *Klebsiella pneumoniae* was the predominant type among other Gram-negative bacteria (44.4%),while the other bacterial types *E. coli*, *Citrobacter freundii* , *Proteus mirabilis* and *Pseudomonas aeruginosa* were isolated in low percentages. These findings agreed with the results of Oni *et al.*,(2000) who mentioned that the *Klebsiella spp.* represented (43%) of all Gram-negative bacteria that were able to cause bacteremia in diabetic patients. At the same manner, Al-Muslemawi(2007) showed

that *Citrobacter freundii* may cause bacteremia in immunocompromized patients including D.M.

Table (3-5) Gram-negative bacteria isolated from diabetic patients

Bacterial species	Total number of isolates
<i>Klebseilla pneumoniae</i>	4
<i>Escherichia coli</i>	2
<i>Citrobacter freundii</i>	1
<i>Proteus mirabilis</i>	1
<i>Pseudomonas aeruginosa</i>	1
Total	9

3-3 Virulence factors of bacterial isolates :

3-3-1 Colonization factor I and III (CF-I &III) :

Table (3-6) shows the results of virulence factors for bacterial isolates studied. The CF-I and III were detected (2-3-2-5) in all bacterial isolates which showed positive results with these factors. The CF-I contributes and aids the bacteria to adhere and multiply within eukaryotic cells. Bacterial adherence to host tissues is a complex process that, in many cases, involves the participation of several distinct adhesions, all of which may act at the same time or at different stages during infection (Ofek *et al.*,2002).

Table(3-6) Virulence factors of bacterial isolates recovered from diabetic patients

Bacteria isolate	coagulase	lipase	Hemolysin production	Gelatinase	Capsule formation	CF-	CF-III	Bacteriocin production
<i>S. aureus 1</i>	+	+	B	+	-	+	+	+
<i>S. aureus 2</i>	+	+	β	+	-	+	+	+
<i>S. aureus 3</i>	+	+	β	+	-	+	+	+
<i>S. epidermidis 1</i>	-	-	γ	-	-	+	+	-
<i>S. epidermidis 2</i>	-	-	γ	-	-	+	+	-
<i>S. epidermidis 3</i>	-	-	γ	-	-	+	+	+
<i>S. epidermidis 4</i>	-	-	γ	-	-	+	+	+
<i>S. epidermidis 5</i>	-	+	γ	-	-	+	+	+
<i>S. epidermidis 6</i>	-	+	γ	-	-	+	+	-
<i>S. epidermidis7</i>	-	-	γ	-	-	+	+	-
<i>S. epidermidis 8</i>	-	-	γ	-	-	+	+	-
<i>S. epidermidis 9</i>	-	-	γ	-	-	+	+	-
<i>S.epidermidis10</i>	-	+	γ	-	-	+	+	-
<i>S. epidermidis11</i>	-	-	γ	-	-	+	+	-
<i>S. epidermidis 12</i>	-	-	γ	-	-	+	+	+
<i>S. epidermidis 13</i>	-	-	γ	-	-	+	+	+
<i>Strep. mitis 1</i>	-	-	α	-	-	+	+	-
<i>Strep. Mitis 2</i>	-	-	α	+	-	+	+	+
<i>Strep. Mitis 3</i>	-	-	α	-	-	+	+	+
<i>Strep. Mitis 4</i>	-	-	α	-	-	+	+	+
<i>Bacillus cereus</i>	-	-	γ	+	-	+	+	+
<i>Klebseilla pneumoniae 1</i>	-	+	γ	-	+	+	+	+
<i>K. pneumoniae2</i>	-	+	γ	-	+	+	+	+
<i>:Kpneumoniae 3</i>	-	+	γ	-	+	+	+	+
<i>K. pneumoniae 4</i>	-	-	γ	-	+	+	+	+
<i>E.coli 1</i>	-	+	β	-	+	+	+	+
<i>E.coli 1</i>	-	+	β	-	-	+	+	+
<i>Citro.freundii</i>	-	+	γ	-	-	+	+	+
<i>Proteus mirabilis</i>	-	+	γ	+	-	+	+	-
<i>Psuedomonas auroginosa</i>	-	+	β	+	-	+	+	+

CF-I(colonization factor-I);CF-III(colonization factor-III); β -hemolysis(complete hemolysis); α -hemolysis(partial hemolysis); γ - hemolysis(Non-hemolysis)

Many pathogenic bacteria display polymeric adhesive fibers termed "pili" or "fimbriae" that facilitate the initial attachment to epithelial cells and subsequent successful colonization of the host (Ofek *et al.*,2002). Pili are virulence factors that mediate interbacterial aggregation and biofilm formation, or mediate specific recognition of host-cell receptors (Jonson *et al.*,2005). It is clear that pili play similar biological roles for commensal bacteria because they also have to colonize specific niches and overcome the host's natural clearing mechanisms. It is thought that commensal and some pathogenic *Escherichia coli* strains use type I pili or curli to colonize human and animal tissues (Maria *et al.*,2007).The expression of colonization factors by Gram-positive and Gram-negative bacterial isolates in the present study was in agreement with(Ofek *et al.*,1977; Maria *et al.*,2007).

3-3-2 Capsule formation :

All isolates of Gram-positive bacteria that isolated in the present study were non-capsule producers. Gram-negative bacterial isolates were also non-capsule producers except *Klebsiella pneumoniae* isolates (table 3-6) which exhibited a large and clear capsule when examined by negative staining method. Capsules are known to mediate specific or non-specific adherence of bacteria to particular surfaces, and to protect bacteria from engulfment by phagocytes and from attack by antimicrobial agents (Hyde ,2000 ; Brooks *et al.*, 2004).

Capsular components play important role in the severity of the infections that caused by many bacteria such as *Klebsiella pneumoniae* that have capsule which contain high component of sialic acid which inhibiting the activation of complement component, especially C3b (Williams and Tomas,1990 ; Podschun *et al.*,1992)

3-3-3 Coagulase production :

All isolates of *Staphylococcus aureus* bacteria were able to produce coagulase which is considered as a virulence factor for pathogenicity of these bacteria by clumping the fibrin around the bacteria (Hall,1991;Kenneth,2002). Possibly coagulase could provide an antigenic disguise if it clotted fibrin on the cell surface or could make the bacterial cells resistant to phagocytes or tissue bacterial target (Humphreys,2004).

3-3-4 Haemolysin production :

Table (3-6) also showed all isolates of *Staphylococcus aureus* (3:3) and *E. coli* (2:2) expressed β -hemolytic (complete lyses), in addition to one isolate of *Pseudomonas aeruginosa* which was also β -hemolytic, while all isolates of *Streptococcus mitis* (4:4) were α -hemolytic pattern(incomplete lyses) showing a greenish line around the bacterial colonies. All isolates of *Staphylococcus epidermidis* (13:13) and the single isolate of *B. cereus*, *C. freundii* and *Proteus mirabilis* were γ -hemolytic (non hemolytic) pattern, with no color change around the bacterial colonies. The production of hemolysin by *Staphylococcus aureus* is well known and considered as a main virulence factor for

these bacteria and it associated with increased severity of infections (Vergis *et al.*,2002).

3-3-5 Protease (gelatinase) production :

In this study, gelatin(2-1-4-3) was used as a protein for detection of protease activity in bacterial isolates. In Gram-positive bacteria all isolates of *Staphylococcus epidermidis* (13:13) were negative for this factor while *Staphylococcus aureus* isolates (3:3) were positive for this test, in addition to that, the single isolate of *B. cereus* was able to produce gelatinase. One isolate of *Streptococcus mitis* was also positive for this test. The single isolate of *Proteus mirabilis* and *Pseudomonas aeruginosa* were positive for gelatinase reaction, while all isolates of *Klebsiella pneumoniae*(4:4) and *E.coli* (2:2) were negative for this factor (table 3-6). Gelatinase is one potential virulence factor of many microorganisms because of its ability to breakdown immunoglobulins and complement components that make up the host defenses against microbial infections, and therefore enable the pathogen to invade the host tissues (Travis *et al.*, 1995 ; Poeta *et al.*, 2006).

3-3-6 Lipase production :

All isolates of *Staphylococcus aureus*(3:3) were positive for lipase enzyme production, while only (4:13)isolates of *Staphylococcus epidermidis* were positive for this factor. The single isolate of *B. cereus* was negative, and all isolates of *Streptococcus mitis* (4:4) were also negative for lipase production test (table 3-6). The results of lipase production test in Gram-negative showed that most isolates (8:9) were

positive to lipase production test. Host cell membranes contain lipids in their components, lipase enzyme will destroy this elements and aids the pathogen to penetrate the host tissue to develop the infections (Lisa *et al.*,1994 ; Bartels *et al.*, 2007) .

3-3-7 Bacteriocin production:

Cup assay method was used for detection of bacteriocin production, by which (20) isolates (66.67%) of total bacterial isolates (30 isolates) were able to produce bacteriocin and form a clear inhibition zones (12-22mm) on solid medium (table 3-6). These findings are in agreement with the results obtained by many researchers (Al-Qassab and Al-Khafaji,1992; Al-Dulami,1999; Al-Charrakh,2005) who found that cup assay method was the best method used for detection of bacteriocin-producers *Lactobacilli*, *E. coli*, and *Klebsiella pneumoniae* strains respectively. The importance of bacteriocin for virulence and pathogenicity of bacteria was controversial. Although the bacteriocin is essential for virulence and pathogenicity of the of *Enterococcus* in septicemia (Hancock and Gilmore, 2000; Al-Barzangi, 2001), because ,it was found that cytolysin of *Enterococcus faecalis* (possess both hemolysin and bacteriocin activities) promotes the appearance of this bacteria in blood indicating that the bacteriocin is essential for virulence of these bacteria in blood stream infections. By contrast several researches revealed that the bacteriocin activity is not essential for virulence and pathogenicity of the producing isolates(Opal *et al.*, 1988 ;Vidotto *et al.*, 1991).It was found that bacteriocin activity of

E. coli isolates is not essential for virulence and pathogenicity of the producing isolates, but it aids them in their competition (Vidotto *et al.*, 1991). Moreover, the ability of bacteriocin production in *E. coli* strains, isolated from urine of patients suffering of urinary tract infection (UTI) and from stool of healthy individuals, was tested. It was found that there was non significant difference in ability of these strains to produce bacteriocin, between those isolated from urine or stool samples, which indicates that the bacteriocin is not a virulence factor (Opal *et al.*, 1988). It was also found that in a mixed fermentation environment, production of bacteriocins may prove advantageous for a producer organism to dominate the microbial population (Graciela *et al.*, 1995) .

3-3-8 Other virulence factors:

In addition to virulence mentioned above, urease of *Proteus spp.* (index 4) has been implicated as a factor contribution to the pathogenesis of this bacteria. Urease catalyzes the hydrolysis of urea compound to yield ammonia and carbon dioxide, which results in an increase in the urine pH (Clapham *et al.*,1990). This enzyme has also been implicated as a factor contributing to the pathogenicity of many bacteria including *Proteus spp.* (Jones and Mobley,1987; Senior *et al.*,1980). The urease activity of these bacteria is used to distinguish them from other Enterobacteriaceae family members (Clapham *et al.*,1990). In general, motility is another virulence factor that detected by semi-solid media. The obtained results pointed to positive results of

motility in single isolate of *E. coli*, *Proteus mirabilis* and *Pseudomonas aeruginosa* (index 4).

The presence of flagella on the surface of pathogenic and opportunistic bacteria has been thought to facilitate the colonization and dissemination from the initial site (Senior *et al.*, 1980). The last observation is in contrast to what happens when anti-flagella antibodies (Abs) are added to cultures of a wild-type *Proteus mirabilis* strain. In this case, bacteria can not move because their swarming motility is inhibited by tethering of flagella due to binding of Abs. Swarming phenomenon can possibly protect bacteria from the action of secretory IgA (sIgA) during invasion of tissues (Mobley *et al.*, 1995).

3-3-9 Antibiotics resistance of bacterial isolates :

Results in (table 3-7) showed the antibiotic resistance of Gram-positive bacteria isolates of diabetic patients. Most isolates of *Staphylococcus epidermidis* (11:13) were highly resistant to amoxicillin and oxacillin, but they were highly sensitive to cefotaxime, amikacin, ciproflaxin and trimethoprim-sulphamethazole. They also showed low levels of resistance to nalidixic acid, tetracycline and ceftiofur. The resistance of *Staphylococcus epidermidis* to β -lactams is mediated by β -lactamase production (Humphreys, 2004). Also, Amyes (1983) expressed that *Staphylococcus epidermidis* exhibited resistance to many types of antibiotics and these resistance may be attributed to the R-plasmid acquired from pathogenic bacteria present in the site of infection. All isolate of *Staphylococcus aureus* (3:3) were resistant to

nalidic acid. Two isolates of them were resistant to amoxicillin and oxacillin, and only one isolate was resistant to cefoxitin, amikacin and tetracycline.

Table (3-7) Antibiotic resistance of Gram-positive isolates

Antibiotic type	Bacterial species (No.)							
	<i>Staph. epidermidis</i> (13)		<i>Staph. aureus</i> (3)		<i>Strept. mitis</i> (4)		<i>B. cereus</i> (1)	
	S*	R**	S	R	S	R	S	R
Amoxicillin	2	11	1	2	1	3	1	0
Oxacillin	2	11	1	2	1	3	1	0
Cefoxitin	10	3	2	1	3	1	1	0
Cefotaxime	13	0	3	0	4	0	1	0
Amikacin	13	0	2	1	2	2	1	0
Tetracycline	10	3	2	1	4	0	1	0
Doxycyclin	9	4	2	1	3	1	1	0
Ciproflaxin	13	0	3	0	2	2	0	1
Nalidic acid	8	5	0	3	2	2	0	1
Trimethroprim Sulphamethozole	13	0	3	0	3	1	1	0

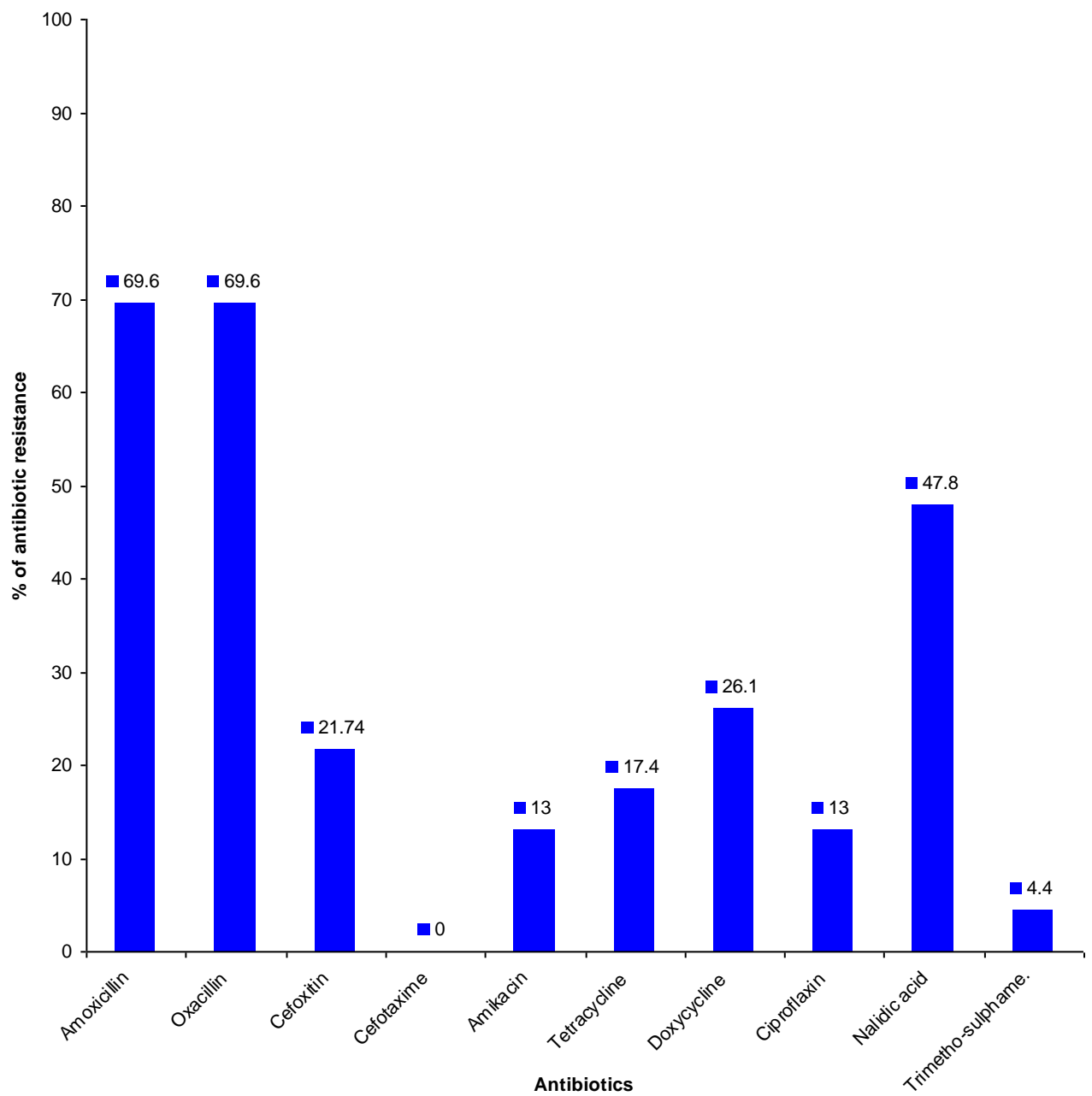
* Sensitive

** Resistant

Staphylococcus epidermidis bacteria had the highest number of multiresistant strains and these findings are in agreement with those isolated from clinical specimens (Davies and Stone,1986). *Staphylococcus epidermidis* may act as a reservoir for resistance which can be transferred to *Staphylococcus aureus*. The transfer of resistance among different genera of Gram-positive and Gram-negative bacteria and between *Bacillus* species and Staphylococci has been reported by many researchers (Schaberg and Zervos,1986; Mazodier and Davies,1991; Courvalin,1994). These results are similar to the findings obtained by Brook *et al.*,(1981) who showed, that *Staphylococcus aureus* isolates were resistant to amoxicillin (69.7%) and less resistant to cefotaxime, the oxacillin resistance rates found for *Staphylococcus aureus* and *Staphylococcus epidermidis* strains. Thus, a further significant increase in the rate of oxacillin resistance as was observed in the early 1990s was not registered. However, one may speculate that these rates would have been even higher if we had also included isolates from body sites other than blood, as was done in former studies (Voss and Kresken,1996; Kresken and Hafner,1999). Three isolates of *Streptococcus mitis* were resistant to amoxicillin and oxacillin, two isolates were resistant to amikacin, ciproflaxin and nalidic acid ,and only one isolate was resistant to cefoxitin, doxycycline and trimethoprim-sulphamethazole (table 3-7).The results above agreed with Carratala *et al.*,(1995)who mentioned that bacteremia due to viridans streptococci were highly resistant (77%) to penicillin's

products. Among the streptococcal species, the viridans streptococci may be the most important pathogens causing bacteremia and sepsis in immunocompromized patients (Kern *et al.*,1990). Until the 1980s,viridans streptococci were considered to be uniformly susceptible to β -lactam antibiotics, but resistance spread rapidly in the 1990s. In a recent study on the antimicrobial susceptibilities of viridans streptococci isolated from blood samples of immunocompromized patients in the Cologne area of Germany, only 81% of *Streptococcus mitis* strains were susceptible to penicillin G (Wisplighoff *et al.*,1999). Viridans streptococci are considered to be frequent causes of infection in immunocompromized patients (Kern *et al.*,1990;Carratala *et al.*,1995; Patrick,1999). This problem is exacerbated by the emerging resistance of streptococci to antimicrobial agents commonly used for empirical and prophylactic treatments in those patients. The increasing resistance of viridans streptococci to β -lactam antibiotics has been documented in neutropenic cancer patients by various investigators (McWhinney *et al.*,1993; Doern *et al.*,1996). The incidence of resistance has been associated with previous use of β -lactams and varies greatly among different institutions (Carratala *et al.*,1995). Results also found that the single isolate of *B. cereus* was sensitive to most antibiotics tested in the current study, and this result was confirmed by many researchers who found that *Bacillus spp.* isolates were highly sensitive to most antibiotics used in their studies (Weber *et al.*,1988). The results of antimicrobial susceptibility also showed that

(70%) of Gram-positive isolates were resistant to amoxicillin and oxacillin. They also showed intermediate resistance to nalidixic acid and high sensitivity to most antibiotics that used in the current study as in (figure 3-1). The high sensitivity of Gram-positive isolates for cefotaxime and ciprofloxacin confirmed by (Al-Hamawandi,2005), and therefore these drugs may be considered as a drug of choice for treatment of the infections that caused by this bacteria. The high sensitivity of Gram-positive isolates for trimethoprim-sulphamethazole in this study was disagreed with many researches (Cormican and Jones,1996 ; Jacoby,1996) which showed that many Gram-positive bacterial isolates showed resistant to trimethoprim-sulphamethazole.



Figure(3-1) Antibiotic resistance of Gram-positive bacterial isolates.

Table(3-8) showed the results of antibiotic resistance of Gram-negative bacterial isolates. All isolates of *K. pneumoniae* were resistant to amoxicillin and amikacin, three isolates of them were resistant to oxacillin, doxycycline, ciproflaxin and nalidic acid, and two isolates were resistant to cefoxitin ,only one isolate was resistant to cefotaxime, tetracycline and trimethoprim-sulphamethazol. These results agreed with other local studies(AL-Saeedi,2000;Al-Charrakh,2005) who mentioned that *K. pneumoniae* was highly resistant to amoxicillin and ampicillin by producing β -lactamases that render these isolates unsusceptible to most β -lactam antibiotics. *Klebsiella pneumoniae* showed multi-resistance to penicillins and most of the other antimicrobial agents tested. These results are in agreement with clinical strains reported by other studies(Jarlier *et al.*,1996; Al-Charrakh,2000). Amoxicillin, ampicillin and other penicillin derivatives frequently prescribed for pediatric patients usually predispose them to colonization with *K. pneumoniae*, is naturally resistant to these antibiotics (Ghysen,1991 ; Stock and Wiedemann,2001). Resistance to beta-lactam antibiotics in Gram-negative bacteria can be due to four mechanisms: Decreased permeability of the drug into the cell, hydrolysis of the drug by β -lactamase ,decreased affinity of the target penicillin-binding proteins (PBPs), or by pump-mediated resistance (Pidcock *et al.*,1997).

Table (3-8) Antibiotic resistance of Gram-negative isolates

Antibiotic	Bacterial species (No.)									
	<i>K. pneumoniae</i> (4)		<i>E. coli</i> (2)		<i>Citr. freundii</i> (1)		<i>P. mirabilis</i> (1)		<i>Pseudo. aeruginosa</i> (1)	
	S*	R**	S	R	S	R	S	R	S	R
Amoxicillin	0	4	1	1	1	0	0	1	0	1
Oxacillin	1	3	0	2	1	0	0	1	0	1
Cefoxitin	2	2	1	1	1	0	1	0	0	1
Cefotaxime	3	1	2	0	1	0	1	0	1	0
Amikacin	0	4	0	2	0	1	0	1	0	1
Tetracycline	3	1	1	1	1	0	1	0	1	0
Doxycycline	1	3	1	1	1	0	1	0	0	1
Ciproflaxin	1	3	0	2	1	0	0	1	0	1
Nalidic acid	1	3	1	1	0	1	1	0	0	1
Trimethoprim-sulphamethozole	3	1	2	0	1	0	1	0	0	1

* Sensitive

** Resistant

The major mechanism of resistance in bacteria causing clinically significant infection remains the expression of β -lactamases, of which there are several classes including plasmid-encoded and chromosomally-encoded enzymes (Bush *et al.*,1995; Frere,1995 ;Livermore,1998). Two isolates of *E. coli* recovered in the present study were resistant to oxacillin, amikacin and ciproflaxin but they were sensitive to cefotaxime and trimethoprim-sulphamethazole (Table 3-8). These results agreed with AL-Muhanna (2001) who showed that all isolates of *E. coli* were sensitive to cefotaxime and trimethoprim-sulphamethazole. Furthermore, AL-Hamawandi(2005) reported that all isolates of *E. coli* (100%) were sensitive to trimethoprim-sulphamethazole and ampicillin. This resistance could be interpreted depending on the fact that many strains of *E. coli* have acquired plasmids conferring resistance to one or more than one type of antibiotics, therefore antimicrobial therapy should be guided by laboratory result test of sensitivity (Chart,2004 ; Al-Hamawandi ,2005). The single isolate of *Citrobacter freundii* was resistant to amikacin although it is not used widely in treatment while others antibacterial having low level of resistance and normally used here in Iraq and taken by hands through the pharmacies and nalidixic acid, also, the single isolate of *Proteus mirabilis* bacteria was resistant to amoxicillin, oxacillin, amikacin and ciproflaxin (table 3-8). The single isolate of *Pseudomonas aeruginosa* was resistant to most antibiotics tested, and this result agreed with (Mahmoud,2001; Kiska and Gilligan,2003) who

showed that *Pseudomonas aeruginosa* isolated from blood-stream infections were resistant to 3rd generation cephalosporins and quinolones.

The main mechanisms for antibiotics resistance in *Pseudomonas aeruginosa* are: Production of β -lactamases, cephalosparinases, alteration of outer membrane and reduction of cellular permeability to prevent entry of antibiotic into the bacterial cell (Iyobo *et al.*, 1994 ; Norrby, 1996 ; James,1999).

Results from (Figure 3-2) revealed that Gram-negative bacteria isolated from diabetic patients, were highly resistant (100%) to a mikacin and (77.8%) to amoxicillin, oxacillin and ciproflaxin but they were highly sensitive to cefotaxime, tetracycline, and trimethoprim-sulphamethazole. However, the high sensitivity of these isolates to cefotaxime and trimethoprim-sulphamethazole may be due to the low number of Gram-negative bacterial isolates in the present study.

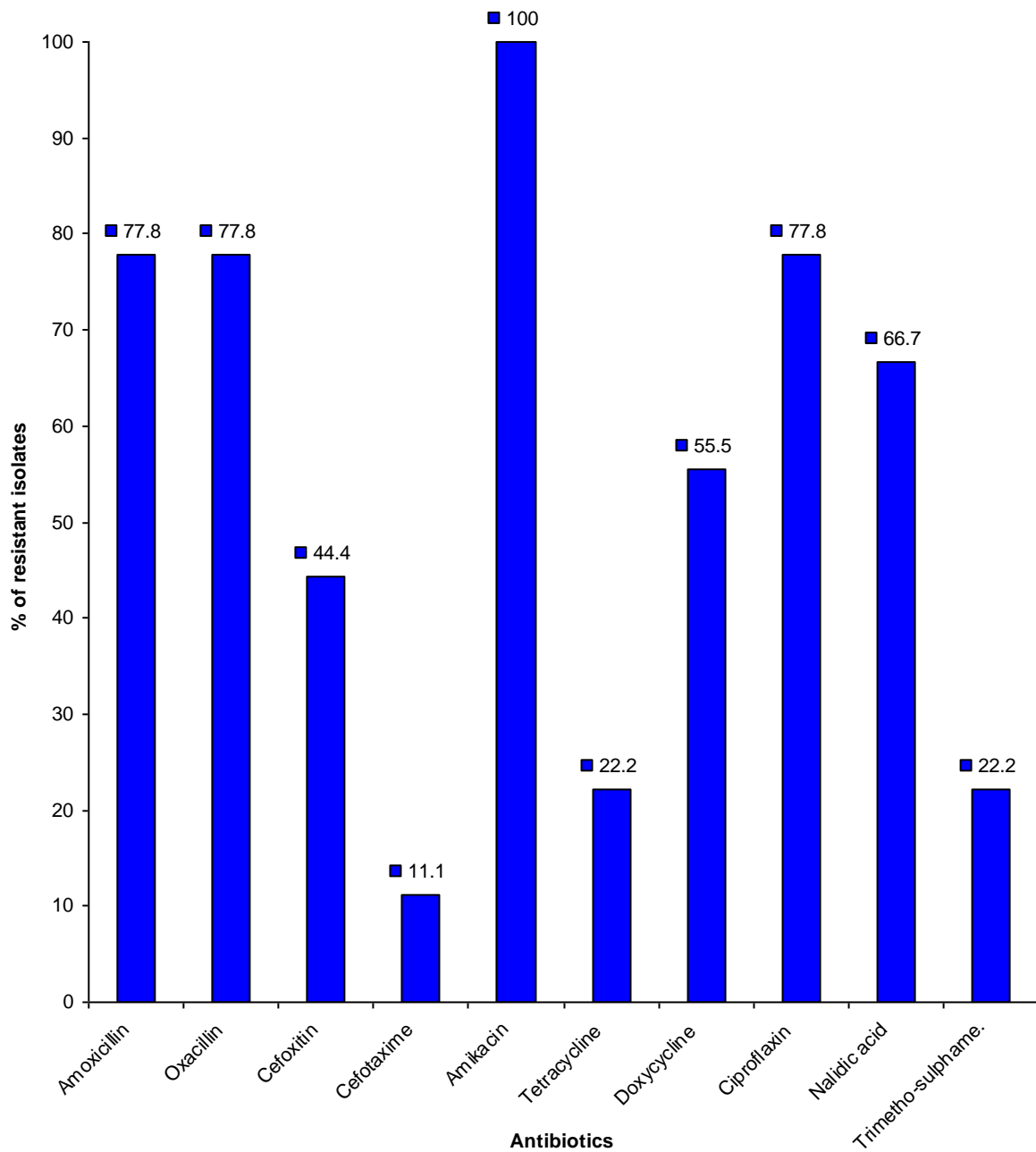


Figure (3-2) Antibiotic resistance of Gram-negative bacterial isolates.

3-4 Immunological Parameters :-

3-4-1- T-cell counts :-

E-rosette test was used to estimate T-cell count in D.M type I and type II, as well as control subjects. T-lymphocytes were considered E-rosette positive forming T-cells when three or more sheep erythrocytes adhere to it (Figure 3-3 a), otherwise they were considered E-rosette negative forming T-lymphocytes (Figure 3-3b).

E-rosette is a simplified common technique used for the quantitative assay of T-lymphocytes. It differs from erythrocyte-antibodies complement rosette (EAC-rosette) test which is used as a tool for quantitative assay of B-lymphocyte. The relationship is a linear between the number of rosette forming cells and the total number of lymphocytes (Weir, 1973; Al-Saadi, 2004).

Table (3-9) showed the results of E-rosette test, the mean value of E-rosette positive of T-lymphocytes in D.M type I was (9.1%), and in type II was (10.63%) which is less than the control subjects (22.55%). T-cell counts were decreased significantly ($p < 0.05$) in both types of diabetes patients, whereas the mean difference between both types (type I and type II) of diabetes patients was non significant ($P > 0.05$). These results indicate the effect of hyperglycemia on the proliferation and function of T-cells that leads to immunosuppression of cellular immunity in these patients and this results agreed with (Kotton, 2004) which pointed the main causes that may be responsible for subnormal T-cell levels which are acquired immune deficiency disorders including

D.M. , acute viral infections and congenital immunodeficiency disease. This decreasing in the T-cells greatly contributes to the impaired cell mediated immunity (Moretti,1992). This impairment is due to the helping role of T-cells in the activation of both arms of immune responses cellular and humoral (Th₁ and Th₂ cells) in addition to this direct roles by direct killing of foreign invaders by cytotoxic T-lymphocytes (Abbas, *et al.*,2000).

Several immunological abnormalities related to D.M. such as depletion of T-cells and defective of NK cells, also insulinopenia induced enzymatic defect have often been proposed to inhibit energy-requiring functions of phagocytes and lymphocytes (Moutschen *et al.*,1992).

Table (3-9) The T-cells count estimated by E-rosette test for diabetic patients and control subjects

Testing group	Mean value %	Standard error
Diabetes mellitus type I	9.1	1.16
Diabetes mellitus type II	10.63	0.817
Control subjects	22.55	1.16

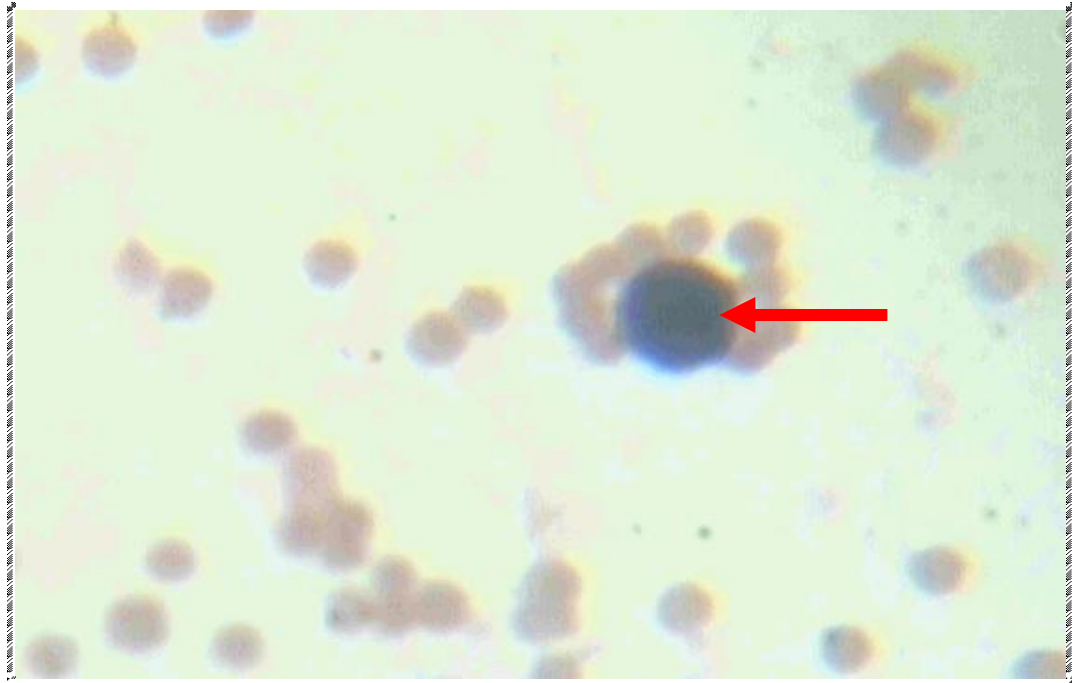


Figure (3-3a): Photomicrograph of an E-rosette positive T-lymphocyte
→1000X) .



Figure (3-3b): Photomicrograph of an E-rosette negative T-lymphocyte
→1000X) .

3-4-2 Levels of Interferon gamma (IFN- γ):

Table (3-10) showed the mean value of IFN- γ . In the diabetic patients type I it was (0.285 I.U/ml), type II was (0.313 I.U /ml) and control subjects was (0.860 I.U/ml). Thus, IFN- γ was decreased significantly ($p < 0.05$) in both types (type I and II) of diabetic patients as compared with control subjects. The results obtained indicates the presence of reduced cellular immunity in diabetic patients which is agreed with (Kukreja *et al.*, 2002 ; Tsiavon *et al.*, 2005 ; Puliti *et al.*, 2006) who stated that the IFN- γ was decreasing in diabetic patients. In this present work among two types (I and II) of D.M there is non significantly ($p > 0.05$) differences between them. IFN- γ is secreted from Th1 lymphocyte, NK cells and macrophage therefore the defect in these cells lead to decreased IFN- γ production that indicates reflects impaired host resistance in diabetic patients due to a dysregulation of cytokine network. IFN- γ enhances the microbicidal function of macrophages by stimulating the synthesis of reactive oxygen intermediates and nitric oxide (NO). It promotes the differentiation of naïve CD4⁺ T-cells to the Th1 subset and inhibits the proliferations of Th2 cells beside to activate neutrophils and stimulates the cytolytic activity of NK cells (Rajesh and Rutten, 2004; Doan *et al.* , 2005).

Table (3-10) Mean value of IFN- γ concentration in diabetic patients and control subjects

Testing group	Mean I.U./ml	Standard error
Diabetes mellitus type I	0.285	0.085
Diabetes mellitus type II	0.313	0.060
Control subjects	0.860	0.085

3-4-3 Interleukin 4 (IL-4) levels :

The mean values of IL-4 in D.M type I , D.M type II and control were (7.050 ,7.703 ,13.650 pg/ml) respectively as in table (3-11). The IL-4 levels were decreased non significantly ($p>0.05$) in both types of D.M in comparison to control subjects. These results agreed with Myer *et al.*,(1999) and Kukreja *et al.*,(2002) who mentioned that there was no significant reduction in the levels of IL-4 among diabetic patients as compared with controls. And Berman *et al.*,(1996), also mentioned the decreased production of IL-4 in human T-cells in diabetic patients. In addition, the levels of IFN- γ and IL-4 in blood of D.M patients showed a reduced values(Halminen *et al.*,2001).

Table (3-11) Mean values of IL-4 concentration in diabetic patients and control subjects

Testing group	Mean pg/ml	Standard error
Diabetes mellitus type I	7.050	2.523
Diabetes mellitus type II	7.703	1.784
Control subjects	13.650	2.523

3-4-4 Immunoglobulines concentration :

Table (3-12) shows the levels of IgG in type I , type II of D.M. and control subjects were 1674.45mg/dL,2095.86mg/dL and 1269.51mg/dL respectively. Also, the levels of IgM in the same groups were 145.78 mg/dL, 177.64mg/dL and 112mg/dL respectively. These results indicate the levels of IgG were increased significantly ($p < 0.05$) in both types of diabetic patients as compared with control subjects, while IgM levels were increased significantly ($P < 0.05$) in type II and non significantly ($p > 0.05$) in type I as compared with control subjects. These findings agreed with (Pietruska *et al.*,1989;Elting *et al.*,1997;and Gorus *et al.*,1998) who mentioned that the mean levels of IgM and IgG were higher in both types of diabetes mellitus patients.

Table (3-12) Mean values of the IgG and IgM concentrations in the serum of diabetic patients and control subjects

Testing group	Mean value of IgG (mg/dL)	Mean value of IgM (mg/dL)
Diabetes mellitus type I	1674.45	145.78
Diabetes mellitus type II	2095.86	177.64
Control subjects	1269.51	112

The changes in total of immunoglobulines concentrations were largely reversed under insulin therapy. They may reflect exposure to environmental triggers ,such as viral infections or to insulinopenia prior to clinical disease onset (Gorus *et al.*,1998). Because of recurrent infections to diabetic patients and continuous exposure to pathogens, immune system can be defended by secretion the primary and secondary response markers (i.e. IgM and IgG respectively) in order to defense against the infections (Abbas *et al.*,2000). Alteration in humoral immune response was less than in cellular immunity during immunosuppression process therefore the IgG and IgM levels in diabetic patients are higher than normal persons but not at the levels of IgG and IgM in persons suffering from microbial infections without diabetes. Diabetic might exhibit hypergammaglobulinemia (Al-Ardawi *et al.*,1994). At the time of diagnosis of D.M. , there exists IgM level increase with poor glucose control (Craig *et al .*, 2002).

3-5 Immune parameters of D.M patients with bacteremia:

The results that expressed in (table 3-13) show the relationship between bacteremia and immunological parameters studied in diabetic patients whether of type I or type II. E-rosette value which is used as a marker for T-cells count decreased significantly ($p < 0.05$) in bacterimic diabetic patients as compared with non bacteremic diabetic patients. T-cells in human body act as a major effector function for the activation of phagocytes and various lymphocytes, mainly by secreting cytokines that promote and regulate humoral and cell-mediated immune responses and inflammation. T-cells respond to antigen presented by antigen presenting cells (APCs) and activate the macrophages. In cell-mediated immunity, T-cells also act as direct cytotoxic cells where are able to kill cells that are showing a new or foreign on their surface mainly virus-infected cells, tumor cells or transplanted cells(Rahi,2003 ; Abbas and Lichtman,2007).

Mean value of IFN- γ concentration in diabetic patients with bacteremia was(0.109 I.U./ml), while in diabetes mellitus patients without bacteremia was 0.577 I.U/ml (table 3-13). These results refer to the significantly decreasing($p < 0.05$)in IFN- γ concentration in diabetic patients with bacteremia as compared with non bacterimic diabetic patients. Therefore, decreasing of IFN- γ concentration leads to increase the likelihood of the bacteremia in diabetic patients (Abbas *et al.*,2000).

Table (3-13) Relationship between bacteremia and immunological parameters in diabetic patients

Testing group	Total No. (%)	Immunological parameters				
		E-rosette * %	IFN- γ * I.U/ml	IL-4 * Pg/ml	IgG * mg/dL	IgM * mg/dL
Bacteremia	30 (24)	8.217 **	0.109 **	5.478	1865.24	168.5
Non-bacteremia	95 (76)	15.246 **	0.577 **	10.458	1626.996	138.47
Total No.	125 (100)					

- * The mean value is calculated for both types of D.M. with bacteremia .
 . * . * The mean difference is significant at 0.05 level .

Microbial products stimulate macrophages to produce tumor necrosis factor alpha (TNF- α) and IL-12 together these two cytokines then stimulate NK cells to produce IFN- γ (Bancroft *et al.*,1991). It plays an important role in the growth and differentiation of cytotoxic T-cells, activates NK cells and acts as a B-cell maturation factor. It regulates immunoglobulins (Ig) isotype production (Snapper and Paul, 1987; Murray, 1988). Concerning, the level of IL-4 concentration in bacteremic diabetic patients was (5.478pg/ml), and in non-bacteremic diabetic patients was (10.458 pg/ml) and these results indicate that the levels of IL-4 were decreased non significantly ($p>0.05$) in bacteremic diabetic patients when compared with non bacteremic diabetic patients. IL-4 has a differentiation activity leading to the production of immunoglobulin in addition to act as growth factor of B-cells (Roitt *et al.*,2001). It is produced by Th2 type of CD⁴⁺T-lymphocytes, following activation by antigen binding to the T-cell receptor and also produced by activated mast cells and basophiles (Hyde,2000). IL-4 down regulates the production of IFN- γ by Th1 CD4T-lymphocytes, on the B-cells, IL-4 has a growth factor activity mediated via the production of soluble CD23. On monocytes, IL-4 induces an increased number of major histocompatibility complex (MHC) class II antigens. By its pleiotropic activity, IL-4 is a key cytokine in the cytokine network that shows anti-inflammatory properties and is probably involved in mechanisms of allergy by production of IgE(Yokota,1988; Banchereau, 1990).

The levels of IgG and IgM in bacteremic diabetic patients were (1865.24 mg/dL and 168.5 mg/dL) respectively, while in non bacteremic were (1626.996mg/dL and 138.47mg/dL) respectively. These results were pointed to increase non significantly ($p > 0.05$) in both immunoglobulines levels in bacteremic diabetic patients as compared with non bacteremic diabetic patients.

Immunoglobulins increase during courses of bacteremia 10-15 folds than in normal patients. Thus, the non significantly increased levels of immunoglobulins in bacteremia diabetic patients is not high to the level of protection from infections, so there is non significantly differences between patients with bacteremia and controls without bacteremia. This may be due to shortage in the signaling of Th2 required for B-cell proliferation and differentiation into immunoglobulins-secreting cells (plasma cells) (Rich *et al.*, 2003).

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Index(1) Case Information sheet:

- Card number:**
- Patients name:**
- Age:** **Sex:** **Date:** / /200
- Home address :**
- Duration :**
- Treatment on Insulin () tablet ().**
- Laboratory diagnosis :**
 - * Blood culture :**
 - positive() negative().**
 - No. of isolate ().**
 - * T-cell count : (%).**
 - * IFN- γ Concentration (I.U/ml).**
 - * IL-4 Concentration (pg/ml).**
 - * IgG concentration (mg/dL).**
 - * IgM concentration (mg/dL).**

Index(2) Reactions reading table of APi-20 E system

**Index(3) Biochemical properties of Gram-positive bacteria that isolated
from diabetic patients**

Bacterial isolates	Catalase	oxidase	Coagulase production	Protease	lipase	Capsule	Hemolysis	Motility	Spore	Manitol fermentation
<i>Staph. aureus</i> 1	+	-	+	+	+	-	β	-	-	+
= 2	+	-	+	-	+	-	β	-	-	+
= 3	+	-	+	+	+	-	β	-	-	+
<i>Staph. Epidermidis</i> 1	+	-	-	-	-	-	γ	-	-	-
= 2	+	-	-	-	-	-	γ	-	-	-
= 3	+	-	-	-	-	-	γ	-	-	-
= 4	+	-	-	-	-	-	γ	-	-	-
= 5	+	-	-	-	+	-	γ	-	-	-
= 6	+	-	-	-	+	-	γ	-	-	-
= 7	+	-	-	-	-	-	γ	-	-	-
= 8	+	-	-	-	-	-	γ	-	-	-
= 9	+	-	-	-	-	-	γ	-	-	-
= 10	+	-	-	-	+	-	γ	-	-	-
= 11	+	-	-	-	-	-	γ	-	-	-
= 12	+	-	-	-	-	-	γ	-	-	-
= 13	+	-	-	-	+	-	γ	-	-	-
<i>Strept.mitis</i> 1	-	-	-	-	-	-	α	-	-	
= 2	-	-	-	+	-	-	α	-	-	
= 3	-	-	-	-	-	-	α	-	-	
= 4	-	-	-	-	-	-	α	-	-	
<i>B.cereus</i> 1	+	-	-	+	-	-	γ	*Nd	+	center

* Nd; non determined

Index(4) Biochemical properties of Gram-negative bacteria that isolated form diabetic patients

Bacterial isolates	Catalase	oxidase	gelatinase	lipase	capsule	hemolysin	motility	spore	Growth on KIA media
<i>K. pneumoniae</i> 1	+	--	-	+	+	γ	-	-	A/A
<i>K. pneumoniae</i> 2	+	-	-	+	+	γ	-	-	A/A
<i>K. pneumoniae</i> 3	+	-	-	+	+	γ	-	-	A/A
<i>K. pneumoniae</i> 4	+	-	-	+	+	γ	-	-	A/A
<i>E. coli</i> 1	+	-	-	-	+	β	+	-	A/A G
<i>E. coli</i> 2	+	-	-	-	-	β	-	-	A/A G
<i>Citrobacter freundii</i>	+	-	-	-	-	γ	-	-	A/A,G,H ₂ S
<i>Proteus mirabilis</i>	+	-	+	-	-	γ	+	-	K/K,G,H ₂ S
<i>Pseudomonas aeruginosa</i>	+	+	+	-	-	β	-	-	K/K

A/A : Acid/Acid ; A/A,G : Acid/Acid with gas ; A/A ,G,H₂S: Acid/Acid ,H₂S production ;

K/K,G,H₂S : Alkaline/Alkaline, with gas,H₂S production ; K/K : Alkaline/Alkaline

Index(5) Biochemical reactions according to API-20 E system of Gram-negative isolates that isolated from diabetic patients

Isolate No.	ONPG	ADH	LDC	ODC	CIT	H2S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	Proposed identification
1	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	<i>Klebseilla pneumoniae</i>
2	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	<i>Klebseilla pneumoniae</i>
3	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	<i>Klebseilla pneumoniae</i>
4	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	<i>Klebseilla pneumoniae</i>
5	+	-	-	-	-	-	-	-	+	-	-	+	+	-	-	+	-	-	-	-	<i>E. coli</i>
6	+	-	-	-	-	-	-	-	+	-	-	+	+	-	-	+	-	-	-	-	<i>E. coli</i>
7	-	-	-	-	-	+	-	-	-	-	-	+	+	+	+	+	+	+	+	+	<i>Citrobacter freundii</i>
8	-	-	-	+	-	+	+	-	-	-	+	+	-	-	-	-	-	-	-	-	<i>Proteus mirabilis</i>
9	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	<i>Pseudomonas auroginosa</i>

ONOG,beta galactosidase;ADH,arginine ;LDC,lysine decarboxylase ;ODC,ornithin decarboxylase ;CIT,citrate utilization ; H2S, H2S production ;URE, urease ;TDA, tryptophan deaminase ;IND, indol production ;VP, acetone production ;GEL,gelatinase ;GLU,glucose fermentation ; MAN, manitol ; INO,inositol ;SOR, sorbitol ; RHA,rhamnose ;SAC, sucrose ;MEL,melibiose ;AMY,amygdalin ; ARA,arabinose



CHAPTER ONE

INTRODUCTION
AND
REVIEW of
LITERATURES




CHAPTER TWO

**MATERIALS
AND
METHODS**



CHAPTER THREE

**RESULTS
AND
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CONCLUSIONS & RECOMMENDATIONS



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