Ministry of Higher Education &
Scientific Research
University of Diyala
College of Education for Pure Science

Variations in Blood Adhesion Genes and Ulcer Foot Bacterial Biofilm Genes: Effect of Copper Oxide Nanoparticles on Bacterial Biofilm

A Thesis submitted to
The Council of College of Education for Pure Science/ University of Diyala- in a Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biology

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1441 A.H **2019 A.D**

القرآن الكريم

وَقُلِ ٱعۡمَلُواْ فَسَيَرَى ٱللَّهُ عَمَلَكُمۡ
وَرَسُولُهُۥ وَٱلۡمُؤۡمِنُونَۖ وَسَتُرَدُّونَ إِلَىٰ عَٰلِمِ ٱلۡغَيۡبِ وَٱلشَّهَٰدَةِ
فَيُنَبِّئُكُم بِمَا كُنتُمۡ تَعۡمَلُونَ ١٠٥

صدق الله العظيم

(التوبة : 105)

Dedication

The origin of my existence in life... Father

The spirt in which my soul live... Mother

The source of my strength and vigor in life... Brothers

My endless joy and happiness in life ...Sisters

Everyone who has the effective trace in my Education

*To all these **DEARS** I dedicate my work*

MOHANAD

Acknowledgement

First of all, my thanks and compliment is to Allah whom without his mercy and assistance, this Dissertation would never have been finished.

This thesis never would have been completed without the cooperation and guidance of a great many people. Without going through reams of paper, I will list a small portion of the people who have made this body of work possible. I want to thank my advisor, **Prof. Dr. Ammar Ahmed Sultan**. **Dr. Ammar**, you have stimulated a collaborative atmosphere and fostered an attitude of excellence in this department for which I am grateful, as well as I would like I want to thank my advisor, **Prof. Dr. Asmaa M. Salih Almohaidi**, for steering me through four different projects during my research in College of Education for Pure Science /University of Diyala. Thank you, **Dr. Asmaa**, for patiently challenging me to accurately and thoroughly investigate a scientific problem. You have a wealth of knowledge that exceeds anyone I have ever known. I appreciate your insight and thoughtful, probing questions that consistently honed in on the crux of any problem that arose in my research. I am very thankful that I had the opportunity to work with you and hope that I have in some small way enriched your life as you have mine.

Special thanks goes to College of Education for Pure Science /University of Diyala, also go to Department of Biology in this College.

Thank you to all of my Ph.D. committee members.

"**Dr. ziad**", thank you very much for your endless discussions regarding the biophysics in this thesis. Your brain is a deep well and I enjoyed Working with you, to benefit from you in consolidating the principles of physics to the general science.

My thanks and appreciation to a friend of study and work and a lifetime companion assistant lecturer **Luay Qasim Abdulhameed**

Special thanks goes to **Dr. Anoar alkaeem** in Baquba Teaching Hospitals.

Special thanks and appreciation to **Dr. Elham Abdel Latif**, Ministry of Science and Technology for providing standard isolates

I would like to thank **Dr. Nadia Fadhil**, Paternity and Kinship division, at the Medico-Legal Directorate On her help and advice during work.

Special thanks goes to all my colleagues especially **Mohameed, Wisam, Saja, Eman** and **Lina.**

Finally, I wish to express my deep gratitude to **my family** who supported me morally and spiritually.

I will conclude by saying that all of the errors in this work are exclusively mine.

Mohanad

Certification

We certify that this thesis (**Variations in Blood Adhesion Genes and Ulcer Foot Bacterial Biofilm Genes: Effect of Copper Oxide Nanoparticles on Bacterial Biofilm)** was prepared by **Mohanad Waheeb Mahdi**, under our supervision at the Department of Biology, College of Education for Pure Science, University of Diyala, Iraq. As partial fulfillment of the requirements for the degree of Doctor of Science in Biology.

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Data: / /2019

Certification of examination committee

We, the members of the examination committee, certify that we have evaluate this thesis entitled (**Variations in Blood Adhesion Genes and Ulcer Foot Bacterial Biofilm Genes: Effect of Copper Oxide Nanoparticles on Bacterial Biofilm)** was prepared by **Mohanad Waheeb Mahdi**, and recommend for acceptance of the thesis partial fulfillment of the requirement for the degree of doctor of sciences in (**Biology)**.

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Summary

This study conducted on 100 Iraqi individual for 50 patients (30 males and 20 female), aged range between (43- 94) years that were diagnosed with Type 2 Diabetes Mellitus (T2DM) and foot ulcers, and 50 Healthy subject (27males and 23 female). Their ages ranged (27-66), who were periodic attended to the hospital of Baghdad and Baqubah, from February to December 2018. Questionnaire list (Appendix1) was prepared for T2 DM patients, and the controls who were characterized in terms of age, gender, family history of diabetes, duration of disease for patients, height (abdominal circumference), were which measured and recorded in cm, and body weight which was measured and recorded in Kg. The patients and controls were also determined for fasting blood glucose (FBG), glycated hemoglobin (HbA1c), lipid profile (TC, TG, HDL, LDL and VLDL), insulin, resistance insulin (HOMO IR), and Atherogenic index. In addition, the studies polymorphism for the adhesion gene and bacteria was done in the molecular biology laboratory in biology department College of Education for pure Science in the University of Diyala.

The aim of this study is to evaluate the relationship between Adhesion genes of diabetic foot ulcer and biofilm genes of bacteria, and on the other hand, the effect of copper oxide nanoparticle on isolated bacteria from foot diabetic ulcers.

The genetic polymorphism of L-selectin gene rs2205849 of the genotype AA showed high frequency ratio in patient group compared with control 67.5 and 56%, respectively with etiological risk factor 1.63 but non significant according to fisher's probability.

The results of the E-selectin gene polymorphism showed five SNPs in the E-Selectin Leu554Phe gene amplification region. The amplified portion that was extended from the end of intron 8 to exon 11 of the E-Selectin gene contains the

following locations: (rs5355 (G> A), rs5368 (C> T), Rs751151130 (G> A), (T> Del), and rs5367 (C> T)).

Present data showed significant increase in mean of biochemical characteristic Fasting blood glucose (FBG) , Glycohemoglobin (HbA1c), Total Cholesterol(TC), High Density Lipoproteins(HDL), Low Density Lipoproteins (LDL), Very Low Density Lipoproteins(VLDL), triglycerides (TG), insulin, HOMO IR, and Atherogenic Index in Patients (AIP) compared with control.

Results of the bacteriological showed of bacteria so that from positive and negative bacteria were selected for Gram stain: *S. aureus* and *P. aeruginosa*. Both represent a high recurrent infection of bacteria isolated from diabetic foot patients by 16 isolates and 10 isolates respectively.

The most common genotypes were identified in E-Selectin gene for five SNPs AG:rs5355, CC:rs5368, GG:rs751151130, TT:rs5367 and T> Del, the frequency of each genotype was 75%, 60%, 60% 70% and 77.5%, respectively. These results are corresponding with *cupA* gene variation's at position 2840275 and 2840436 where one mutation missense were recorded.

Nanoparticles (NPs) have been increasingly used as alternative antibiotics against bacteria. Nanotechnology may be especially helpful in treating bacterial infections. Antibacterial mechanisms of nanoparticles against bacteria and factors involved in the comparison. The result of nanoparticles on bacteria was assessed by studying the genetic variation in the bacterial genome before and after treatment. It is the aim of this study. Current results showed the genetic variation of bacterial adhesion genes: *icaA*, *icaD*, and *CupA* showing increased deletion. Because of the effect of copper oxide nanoparticles on the base of cytosine.

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List of Abbreviations

Abbreviation	Name
0,1,2,3-D	Zero, one, two, three-dimensional
ADA	American Diabetes Association
AIP	Atherogenic Index of Plasma
AP-1	Activator protein 1
APCs	Antigen presenting cells
BMI	Body Mass Index
CAD	Coronary Artery Disease
CETP	Cholesteryl Ester Transfer Protein
CHD	Coronary Heart Disease
CO	Central Obesity
CRA	Congo red agar
CRP	C-Reactive Protein
CuO	Copper oxide
CuO-NPs	Copper Oxide Nanoparticles
cup	chaperone-usher pathway
CVD	Cardio Vascular Disease
Cyto	Intracellular cytoplasmic tail
ECM	extra cellular matrix
ECs	Endothelial cells
EGF	Epidermal growth- factor
ELAM-1	Endothelial leukocyte adhesion molecule-1
EMA	Effective mass approximate
ERK	Extracellular signal-regulated kinase
ESAMs	Endothelial cell-selective adhesion molecules
EtBr	Ethidium bromide
ETS	Transformation-specific transcription elements
GlyCAM-1	glycosylation cell adhesion molecule
H.W.E	Hardy-Weinberg Equilibrium H.W.E
HDL-C	High Density Lipoprotein Cholesterol
HEV	high endothelial venules
HG-MCM	High Glucose-treated Macrophages
HOMA	Homeostatic Model Assessment
HPCs	Hematopoietic Progenitor Cells
HUVECs	Human umbilical vein endothelial cells
ica	Intercellular adhesion
ICAM-1	Intercellular Adhesion Molecule-1

ICE	IL-1β converting enzyme
IgSF	Immunoglobulin Superfamily
IKK	Inhibitory kB kinases
IKKβ	kinases IκB kinase-β
IR	Insulin Resistance
IRAK	IL-1R-associated kinase
IRS-l	Insulin receptor substrate-l
JAMs	Junctional adhesion molecule
LD	Linkage disequilibrium
LDL-C	Low Density Lipoprotein Cholesterol
LFA-1	Leukocyte function associated antigen
LOD	Logarithm of odds
MAPK	mitogen-activated protein kinase
MetS	Metabolic Syndrome
MIC	Minimum Inhibitory Concentration
MYD88	Myeloid differentiation factor 88
NCAMs	Neural cell adhesion molecules
NFAT	Nuclear factor of activated T-cells
NF-κB	Nuclear Factor Kappa B
NIK	NF-κB-induced kinase
OEGE	Online Encyclopedia for Genetic Epidemiology
OR	Odds Ratio
PCAD	Premature Coronary Artery Disease
PCAMs	Platelet-endothelial cell adhesion molecules
PSGL-1	P-Selectin Ligand Glycoprotein 1
RFLP	Restriction Fragment Length Polymorphism
RIFLE	Risk, Injury, Failure, Loss, and End-stage Kidney
SCR	sequence consensus repeats
SNPS	Single nucleotide polymorphisms
SSP	Sequence specific primers
STEM	science, technology, engineering and mathematics
TB	Tight binding
TEM	Transmission electron microscopy
TG	Triglyceride –Cholesterol
VCAM-1	Vascular cell adhesion molecule -1
VCAMs	Vascular cell adhesion molecule
VLDL-C	Very Low Density Lipoprotein -Cholesterol

1- Introduction

Diabetes is either inherited or acquired or both that make it as multi-gene diseases, and this applies to type 2 diabetes. Genetic susceptibility for diabetic with interaction of genetic and environmental factors create type 2 diabetes. The risk for developing the disease is 40% with one parent having type 2 diabetes, and 70% with both parents suffering from diabetes According to the American Diabetes Association 2016.

Studies have shown of 2115 non-diabetic patients followed for more than five years, the results showed that individuals with a family history of diabetes with FBG more than 5.5 with BMI over 30 had a 16-fold increased risk of type 2 diabetes. (Al-Hasnawi., 2017).

Cell adhesion molecules (CAMs) are located on the cell surface involved in binding with the extracellular matrix (ECM) or with other cells in the operation called cell adhesion (Khalili and Ahmad, 2015). Selectins (type I transmembrane proteins) are a family of mammalian vascular adhesion molecules involved in the tethering and deceleration of cells in lymphatic and bloodstream on endothelium capillary (Choudhary *et. al.* 2015). They play a great role in the passage of leukocytes into inflammation sites by mediating the first attachment and rolling of leukocytes on vascular endothelium before to integrin dependent extravasation and arrest (Natoni *et al.,* 2016). Selectins are related to DMT2, another local study showed E-selectin that plays a role in Iraqi patients with DMT2 (Al-mohaidi *et al.,* 2014; Al-Hasnawi *et. al.,* 2017). There are three types of selectin: P-selectin, E-selectin, and L-selectin (Wu *et. al.,* 2014). The genes for the selectins are located on chromosome number one (Kneuer *et.al.* 2006). which make patients more ready for the complication the disease.

Studies have indicated that different genetic variants (Single Nucleotide Polymorphisms, SNPs) of the inflammatory markers were correlated to different diseases including T2DM and others SNPs related to disease (Rodrigues *et al.,* 2016).

The cell walls of both Gram-positive and Gram-negative bacteria contain toxic components that are potent virulence factors and have central roles in the pathogenesis of bacterial septic shock, a frequently lethal condition (Silhavy *et al.* 2016). Considered adhesion of virulence factors for gram-positive and negative bacteria and the formation of biofilms of pathogens resistant bacteria to antibiotics. (Schroeder *et al.,* 2017). There is a strong correlation between the microbes and the replication of resistant bacteria (Munita and Arias, 2015). Increased association between Multiple drug resistance (MDR) bacteria and diabetic foot ulcer increases the risk of amputation (Yoga *et al.* 2006). Microbial resistance has dramatically increased during the last three decades, and spread around the world wide. Most Pathogenic bacteria have developed many ways to resist almost highly used antimicrobials. These pathogens can cause a wide range of superficial infections, and life-threatening infections in immunocompromised patients (Li and Webster, 2017).

There are therefore emergency needing to find new antimicrobial molecules, with an innovative chemical structure, (WHO, 2015) like Nanoparticles which they have great antimicrobial properties due to their three dimension in surface size to large size, (Yoga *et al.* 2006). Copper is one of the nanoparticles used in modern experiments: that depend on its easy oxidation, selectively processes dual DNA, allowing for mass volume control (Tauran *et al.* 2013). Copper surfaces or alloys can eliminate 99.9% of pathogenic bacteria in hours, including methicillin-resistant

Staphylococcus aureus, *Escherichia coli*, *P. aeruginosa*, *Monoclonal Listeria*, *intestinal Salmonella*, *Camylopactropylica*. (Sçnchez, 2016).

1-2 Aims of the study:

1- Study SNPs in human selectin L and E genes and sequencing of some segments of selectin genes.
2- To Evaluation the relationship between Adhesion genes of Diabetic Foot Ulcer patients and bacterial adhesion genes
3- To Evaluation the effect of nanoparticle on commonly isolated bacteria from Foot Diabetic Ulcers.

2. Literature Review

2.1 Genetic Variation

Genetic Variation is the difference in DNA sequences between individuals. Mutations and recombination are major sources of variation (Stapley *et al.,* 2017)

Genetic variability is continuously synthesized through mutations, but its persistence in the genome is determined by various environmental and genomic factors. Some of these factors leave an imprint on the sequential genetic variation across the entire genome, while others only affect local variation patterns (Wright, 2005). The whole genetic variability is influenced by environmental factors across populations, where the current population is descended from a few previous generations. A new mutation lasts on average for a longer period of time if it has a beneficial effect on subsequent generations leading to improve adaption. In addition, the overall range of genetic variations is strongly affected by mutations, which vary across the genome, and population size. Large populations tend to exhibit more variations than small populations because, they generate more mutations. (Loewe and Hill, 2010)

Simonti and Capra (2015) explained that most of the variants of deoxyribonucleic acid (DNA) in the human genome are through natural selection and are determined for three main reasons:

1- The main part of the genome, estimated at 97%, does not indirectly affect gene function, by regulating gene expression or the transcription process.

2- If a new variation occurs in 1.5% of the genome encodes a functional product (coding regions), it may not result in a change in amino acids it may be replaced by "synonym".

3- Variables that affect regulatory regions or coding regions may not influence amino acid changes (uninterrupted alternatives) over generations.

Interestingly, single nucleotide polymorphism (SNPs) has been associated with more complex variables with the emergence of multiple allele replicas in the population. The importance of gene selection and identification is based on the proposed heterogeneity of the disease where genes are pre-selected based on hypotheses and studied in the population (Duret, 2009).

Several important studies have shown that the use of gene variability in many diseases, such as cancer and diabetes in some circumstances, is biologically important in societies. In particular, SNPs contribute to identifying the phenotype of the gene image (Hofker *et al.*, 2014).

There are several factors together that have formed the diversity of genome and one of these key factors is the random transmission of mutations in genins or some variations or mutations that occur in codons with no effect on genome. The occurrence of these genomic variations in genius gives us a closer look at the history of genotyping in genome diversity (Zia and Moses, 2011).

2-1-1 Types of Genetic Variation

Polymorphisms (nucleotides polymorphisms) are differences in the DNA sequence. In one nucleotide these simple changes can be transversion or transitional and occur when the monoclonal nucleotide sequence differs from the normal range in at least one percent of the population. When single nucleotide polymorphism SNPs occur within a gene, they create different variables, or alleles, for the gene. SNPs are very rare, so sequences tend to pass unchallenged across generations (Robert and Pelletier, 2018).

2-1-2 Single-nucleotide Polymorphisms SNPs

SNP which is a unit of genetic differences has gained much attention because it is associated with complex diseases SNP has been used to distinguish individuals with healthy diseases. (Mirkovic, *et al.*, 2016).

SNP can be classified according to its location in the genome: in gene coding regions, in areas of Non-coding genes or in genetic regions. Most of these censuses occur in non-encoded areas. However, SNPs that occur in the coding region are considered important because they can affect a variety of important biological and molecular activities such as stability, expression level, and protein function. However, recent studies have shown that SNPs in unencrypted regions may still affect other activities such as gene binding, gene expression, and unencrypted RNA sequences. (Ramírezbello *et al.*,2017).

SNP occurs in each genome at a frequency of one in 1000 basis points. They may be responsible for the diversity of individuals, the evolution of the genome, the most common family characteristics such as differences between individuals in drug response, curly hair, common and complex diseases such as Parkinson's, mental disorders, heart and blood vessels, obesity, hypertension, diabetes, and cancer. SNPs may alter the amino acids encoded or can be muted (Ghagane *et al.* 2016).

Estimated 10 million SNPs, only 3-5% are presented in the coding area. Half of these alternatives cause the replacement of amino acids, and it has been suggested that 20% of SNPs can potentially damage proteins. Producing a neutral side-loading protein (Sunyaev *et al.*, 2001).

Type of Mutations Leading to Formation of SNPs

DNA sequences can be altered in several ways. Genetic mutations have varying effects on health, depending on where they occur and whether they alter the function of the underlying proteins. Types of mutations include or substation point (Witham *et al.*, 2011).

- Silent mutation: Silent mutations cause a change in the sequence of bases in the DNA molecule, but does not alter the amino acid sequence of the protein (Figure 2.1) (National Library of Medicine .,2019).

- Missence: This type of mutation is a changed in one pair of DNA bases that leads to the replacement of amino acid by another in the protein made by the gene (Figure 2.1).
- Nonsense mutation: A nonsense mutation is also a changed in one DNA base pair. Instead of substituting one amino acid for another, However, the altered DNA sequence prematurely signals the cell to stop building a protein (Figure 1). This type of mutation results in a shortened protein that may function improperly or not at all.

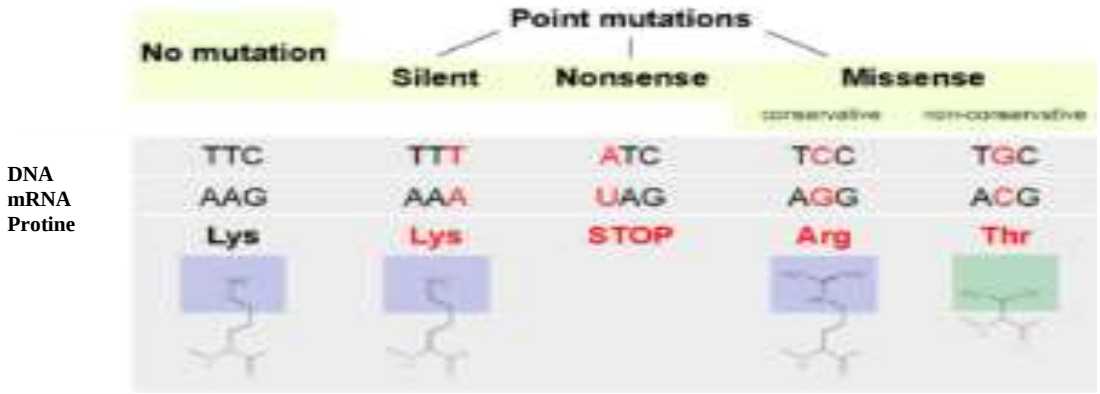


Figure 2.1: some mutations leading to SNPs(National Library of Medicine .,2019)

- **Insertion and Duplications**

Other common forms of genetic differences include insertions and deletions and one or more rules. It is estimated that 5-10% of the human genome is repeated once or more. These regions include both large portions of relatively large DNA ranging from 10 to 400 kilobytes (KB) multiplied several times throughout the genome, and a large category of short sections (1 to 5), frequently distributed and refined. The latter exhibit a relatively high percentage of insertion or deletion mutation, thus showing a multiform variation. This category of frequent repetition is important in human genetics (Sung *et al.*, 2016).

- **Frameshift Mutation**:

This type of mutations occurs when the addition or loss of DNA bases changes the gene reading frame. The reading frame consists of sets of 3 rules for each amino acid code. The frameshift mutation changes the set of these rules and changes the code for amino acids. The resulting protein is usually non-functional. Insertions, deletions, and duplications can be tire spikes (National Library of Medicine .,2019).

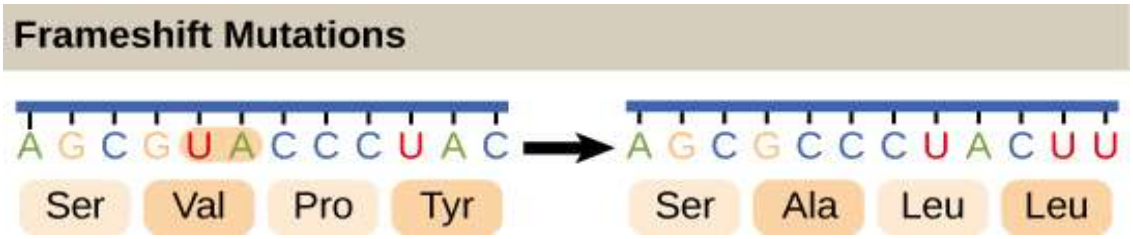


Figure 2.2: Frameshift mutation adds or deletes 1 or 2 bases. (National Library of Medicine .,2019)

- **Repeat Expansion**:

Recurrent nucleotides are short sequences of DNA that are repeated several times in a row. For example, trinucleotide replication consists of three-base sequences, and the nucleotide replication consists of two-base sequences. Or from multiple DNA sequence replication short (Paulson., 2018).

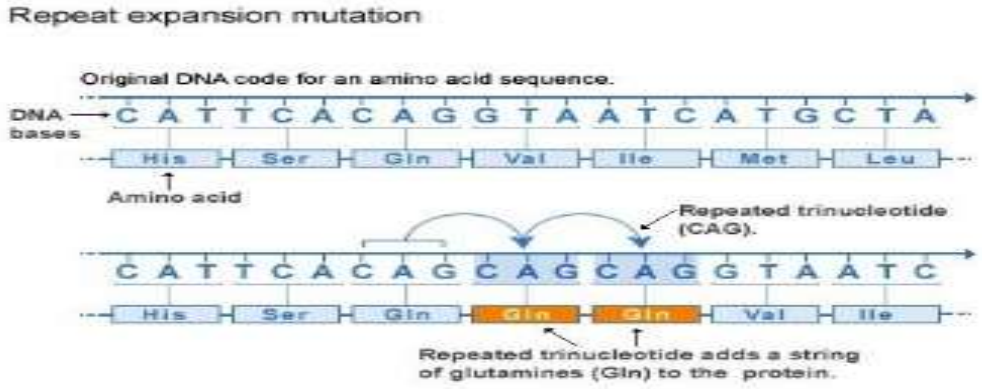


Figure2.3: Repeat expansions(National Library of Medicine .,2019)

2-1-3 Importance of SNPs

SNPs contribute to the adjustment of diseases through change or modification in the pathways of inherited diseases within families, such as cystic fibrosis, immunodeficiency diseases, and diabetes, (Which is caused by Various entering into the body) which gives of SNPs the importance of changing the metabolic pathway of the disease. (Jackson *et al.,* 2018).

SNPs are important in molecular genetics and molecular techniques because of its wide-scale availability of the genome and it's used as an indicator of early detection of diseases, in addition to the accurate mapping of target areas and genetic diversity (Jackson *et al.,* 2018).

2-2 Adhesion Molecule

There are many molecules between cells and extracellular matrix (ECM). These molecules consist of cell surface protein, which mediated the interaction between cells which called adhesion molecules. Four families of adhesion molecules available, which are: immunoglobulin-like adhesion molecules, integrins, cadherins and selectins (Ren *et al.*, 2011). Most of them are typical transmembrane, proteins that have cytoplasmic, transmembrane and extracellular domains. These proteins play many crucial functions in a cell's interface and environment, whether this environment is another cell, from a similar cell type or a different, extracellular matrix or even soluble elements at times. The adhesion molecules are widely distributed and almost every cell expresses cellular adhesion molecules (Grouleff *et al.*, 2015).

In the immune system, cell adhesion plays a critical role in initiating and sustaining an effective immune response against foreign pathogens. (Ren, *et al.* ,2011)

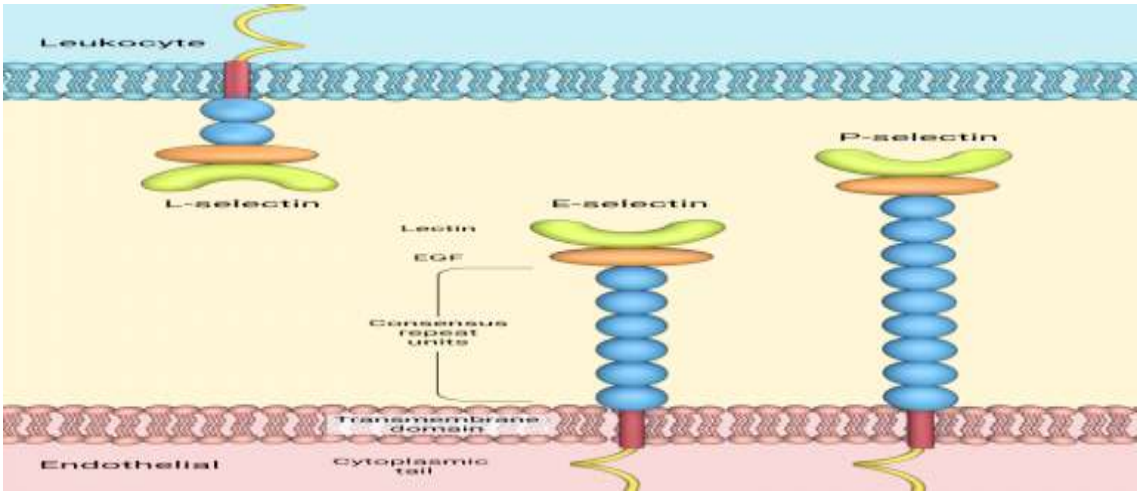


Figure 2.4 : Structure of Adhesion molecule (http://www.geneontology.org).

They have been identified as key players forming circles cellular cell. Adhesion is characterized by a high structural type that reflects their large functional diversity, many of them display functions such as cell conversion, cytoskeletal reorganization, or gene expression control. In another view, adhesion molecules play an important role in metastases and invasion of cancer cells (Farahani *et al.*, 2014; Missaire and Hindges, 2015), also linked to human mental disorders, such as schizophrenia, diabetes foot, bipolar disorder, autism spectrum disorders, mental retardation and depression (Missaire and Hindes, 2015).

2.3 Selectins

The name selectin comes from the words "selected" and "lectins," which are a type of carbohydrate-recognizing protein. (Kappelmayer and Nagy ,2017). It a glycoprotein that mediates the adhesion of hematopoietic cells to vascular surfaces and to each other known as ca^{++} -based glycoproteins (Elzubeir *et al.*, 2016). Selectin consist of 3 member's family, E-selectin expressed on the lining endothelial cells, P-selectin on the platelets and lining, and L-selectin on the leukocytes. Each one of the family composed of a transembrane of type I N-terminal, a lectin-dependent area of calcium, the epidermis A field such as growth factor (EGF), cross-membrane

(TM) and cyto-cell cytothelial (cyto), variable number of repeatable units (Helfrich *et al*., 2008).

Selectins mediate cell adhesion rolling through a dynamic process requiring rapid building and bond breaking under flow (McEver and Zhu 2010). Cells can receive chemokine receptor receptors as well as adhesion receptors that make cells slowly roll and then stop to decode blood vessels to slow tissue (Ley *et al*., 2007). The minimum determinant for the glucan factor to bind to selectin is the carbohydrate containing the X-Silyli antigen, which appears to be E-selectin associated with higher affinity (Kneuer *et. al*., 2006).

2-3-1 Selectins Structure and Expression

The Selectins family is involved in a unique extracellular regulation consisting of a lectin-dependent range of Ca, N, (EGF) - such as domain, sequence consensus repeats (SCR) and similar units of domains in complementary regulatory proteins, membrane field, cytoplasmic domain number Short [2-2] (Sun *et al*., 2015). The cytoplasmic tail of L-selectin is comparatively short, containing just 17 amino acid residues, but is protected across species (Steeber *et. al*., 2007).

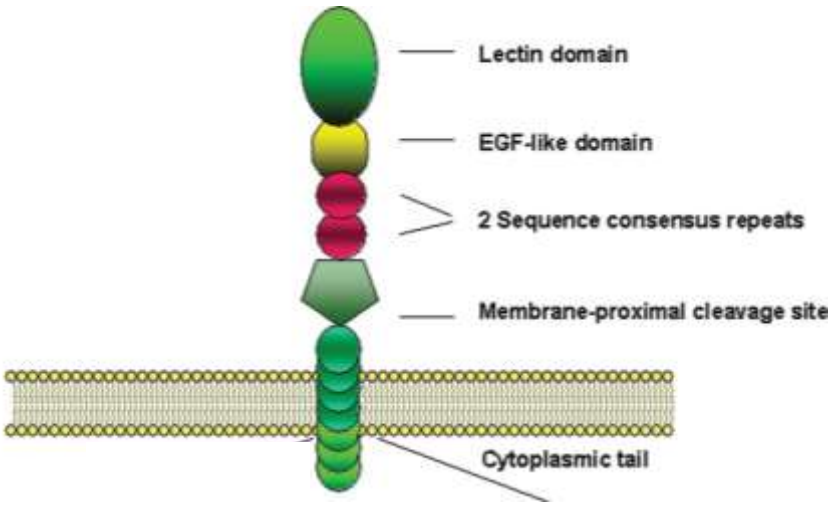


Figure 2.5: Structure of Selectins Leukocyte Selectin (L-Selectin), Endothelial Selectin (E-Selectin), and Platelet Selectin (P-Selectin) (Silva *et. al.*, 2017).

The family selectins are very similar in the lectin and EGF domains, indicating 65% of the amino acid sequence identity, with a lower serial identity (~ 40%) present in the SCR domains. E-and P-selectin human has six and nine SCR domains, respectively, while L-selectin contains two SCR domains. In contrast to extracellular domains, cytosolic domains show the little similarity between determinants (Kappelmayer *et al*., 2017).

These three types of slectin molecules are (40–60%) homologous at the nucleotide and amino acid level and share several features: a conserved (EGF)-like domain of (~30) amino acids, amino acid (120) NH terminal C-type (calcium dependent) lectin-binding domain, a short intracellular COOH-terminal domain, a single membrane spanning domain, and several amino acid (60) repeat sequences that share homology with complement regulatory proteins (Goligorsky *et. al.*, 2010).

The cytoplasmic domain of L-selectin has been shown to be important for optimal working even with its short length and to be involved in a number of processes ranging from leukocyte rolling, regulation of ligand binding action, and endoprolytic cleavage to signal transduction and cytoskeletal association (Steeber *et. al.*, 2007).

2-3-2 L-Selectin (Gene and Protein)

According to the genes encoding P-, E-, and L-selectin are localized to a cluster on the chromosome one long arm (1q) (National Center for Biotechnology Information NCBI). L-selectin (SELL, CD62) gene location on chromosome 1 (1q24.2), figure 2.6. The total length of the L-selectin gene 27kbp which is consisting of eight introns and nine exons (Liu *et. al.*, 2012).

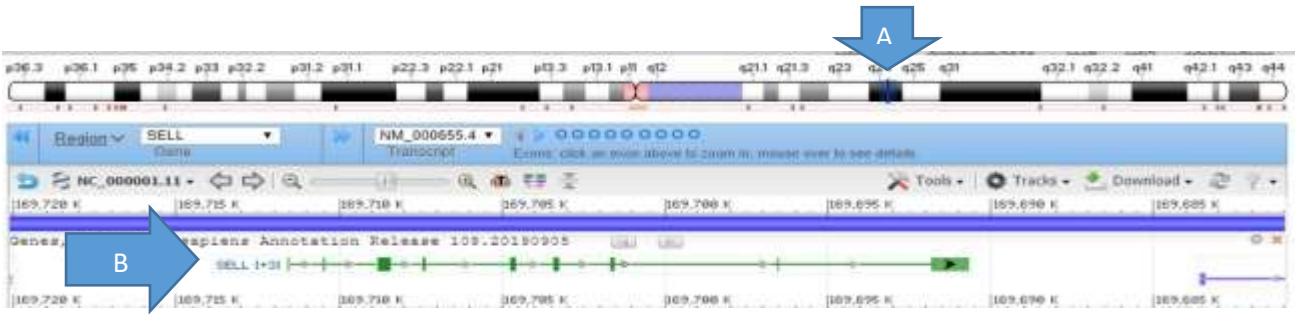


Figure 2.6: L-Selectin (SELL, CD62) A-Gene Location on Chromosome 1 (1q24.2), B-Gene Length, Gene Exon) https://www.ncbi.nlm.nih.gov, 2019).

The human L-selectin gene contains about 200 polymorphic encodings. Central oxons are encoded for many fields such as EGF domains play important roles in cell adhesion, oligomerization, and optimal supply of EGF. (Toma *et al*., 2017).

L-selectin is one type of protein across the membrane, and is linked to carbohydrate based bonds. Titrate containing one saccharide, three focusses and two, three silica acid, was found as a capping group in some sugary cells of superficial proteins. In addition, both tyrosine sulfate and carbohydrates can improve the binding of L-selectins with their bonds (Auvinen *et al.*, 2014).

Facilitating the accumulation of neutrophils along vascular endothelium at inflammatory sites by initiating neutrophil-neutrophil and neutrophil-endothlial interactions. L-selectin is a type C that binds separate carbohydrate structures to selected glycoproteins (Matala *et al*., 2001).

The migration process of lymphocyte between blood lymphatic system types is central to immune control (Rzeniewicza *et al*., 2015). After activation by chemical attractive materiales Leucocyte by L-selectin CD62L interact with endothelial vascular cells, and passage of immune cells to intravenosly infected tissues (Sardarian *et al*., 2014) Figure 2.7.

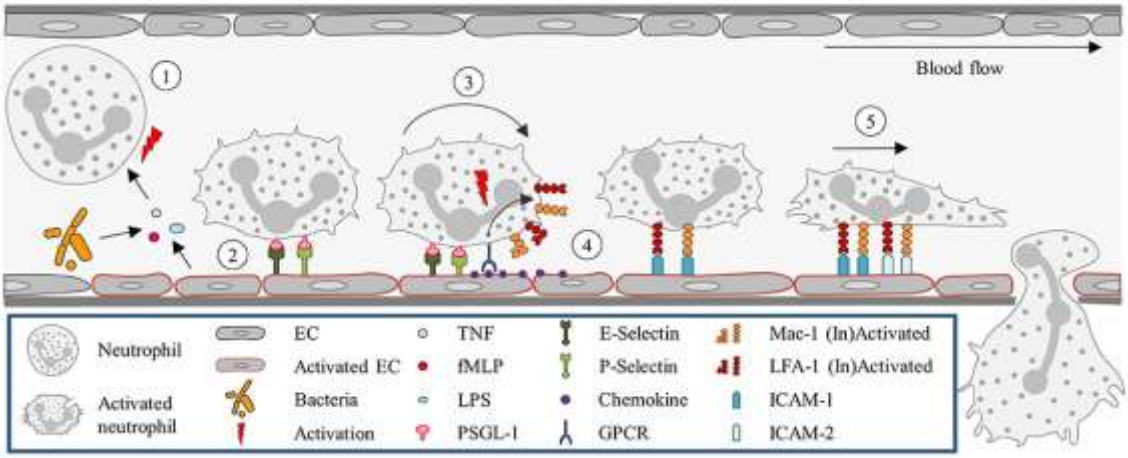


Figure 2.7: L-Selectin Function. Regulation of Neutrophil migration into tissues. (Maas *et al*., 2018)

2-3-2-1 L-selectin Ligand

Leucocyte initiated under conditions of hydrodynamic shear flow by a transient rolling interaction mediated by L-selectin expressed on their surface and carbohydrate ligands expressed on lymph node high endothelial venules (HEV). L-selectin ligands have a sulfated carbohydrate epitope present on most of them which inhibit the binding of lymphocytes to peripheral lymph node HEV. The first two L-selectin ligands identified from lymphoid tissues, glycosylation cell adhesion molecule (GlyCAM-1; a secreted ligand) and CD34 (a cell-surface ligand) (Khan, *et al*, 2002).

L-selectin-ligand binding on leukocytes makes changes in structural actin within cells and increased expression of adhesion molecule together, these sugary proteins transport the leukocyte response from the original and rolling imagination to permanent adhesion and eventual migration into the site of inflammation (Jones *et al*., 2015).

2-3-2-2 Single Nucleotide Polymorphisms (SNPs) of L-Selectin

L-selectin gene contains different SNPs one of themis rs2205849, which Polymorphism −642C>T (rs2205849) in the promoter region has shown a consistent association with some autoimmune and viral diseases, (Sandoval-Pinto *et al*., 2015).

The (Sandoval-Pinto, *et.al*, 2017) study showed that the polymorphisms-642C> T (rs2205849) and 725C> T (rs2229569) were associated with changes in gene expression, and affinity ligand and an increased risk of heart disease and blood vessels.

2-3-3 E-Selectin (Gene and Protein)

SELE gene is composed of 14 exons located at position 1q24.2 (Figure 2.8), the glycoprotein in the cell 11kDa (also known as the endothelial adenocarcinucleotide (ELAM-1), a family member of the CD62-like E antigen (CD62E) and SELE).

Expressed glycoprotein on the surface of endothelial cells according to neonatal cytokines IL-1. Tumor necrosis agent and bacterial lipopolysaccharide, supporting circulating leukocytes at inflammation and tissue injury sites (Khazen *et al*., 2009).

Figure 2.8**:** E-Selectin (SELE, 6401) A-Gene Location on Chromosome 1, GRCh38.p13, B- Gene Length, Gene Exons. (https://www.ncbi.nlm.nih.gov, 2019).

E-selectin is selected in the circulatory system (E-selectin soluble: sE-selectin) or flows quickly after activation (depending on the cell type), sE-selectin serum bypass is a sign of inflammation, selectin is higher among

individuals with chronic inflammation compared to their healthy counterparts (Wodok *et al*., 2018).

It is found that the expression of E-Selectin is controlled through the action of the transcription factor NF-κB after the exposure to IL-1β or TNF-α (Kang *et al.,* 2016; Wang *et al.,* 2001). Three NF-κB sites are identified in the human E-selectin gene that has a primary role for the supreme promoter activity following cytokine exposure.

NF-κB is a heterodimer composed of50-kDa (p50) and 65-kDa (p65) subunits that subsist in the cytoplasm of many cells, such as endothelial cells (The inactive form that bound the inhibitory protein I-κB (IκB)) (Baldwin, 1996).

The activation of NF-κB by TNF-α initiates the activation of IκB kinases α and β (IKKα and IKKβ) which phosphorylate IκBα and IκBβ, at the two conserved NH2-terminal residues which in turn activates them for quick poly-ubiquitination accompanied by degradation by the 26S proteasome (DiDonato *et al.,* 1997; Zandi *et al.,* 1997; Rahman *et al.,* 1998). This leads to the release of NF-κB dimer that motivate the transcription of genes contributed to inflammatory and immune responses including the E-selectin gene (Wang *et al.*, 2001).

Furthermore, it is found that activations of c-Jun-NH2- terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK), pathways are important for the induction of endothelial cell E-selectin expression by smooth muscle cells, (Chiu *et al.,* 2007), as well as in High Glucose-treated Macrophages (HG-MCM) (Chen *et al.,* 2011), while the induction of E-selectin expression by IL-1β and IL-6 produced by endothelial cell/smooth muscle cells is mediated by IL-1 receptor associated kinase (IRAK) and Glycoprotein-130 (gp130) (Chiu *et al.,* 2007). C-Reactive Protein (CRP) is found to have a pro-inflammatory effect by inducing high levels of

expression E-selectin in the endothelial cells of both umbilical vein and coronary artery (Pasceri *et al.*, 2000).

2-3-3-1 E- Selectin Ligands

Similar to all C-type lectins, E- Selectin binds to carbohydrate ligands in a calcium-dependent manner. All these ligands bind to the domin of lectin interfering with a single calcium symmetry site opposite to the location of the EGF domain on a surface region (Varki,1994; Kansas, 1996). Many ligands for E-selectin have been identified in different types of cells; including CD44 (Dimitroff *et al.*, 2001; Yago *et al.*, 2010), Macrophage-1 antigen Muc-1, Macrophage-2 antigen Mac-2 binding protein (Shirure *et al.*, 2012), death receptor-3 (Gout *et al.*, 2006) and ESL-1 (Dimitroff *et al.*, 2005; Geng *et al.*, 2012)

The 220-kD sialomucin-like protein P-Selectin Ligand Glycoprotein 1 (PSGL-1) is diagnostic on Hematopoietic Progenitor Cells (HPCs) as an E-selectin glycoprotein ligand with other proteins that serve as E-selectin ligands, all selectins recognize the tetrasaccharide sialyl Lewis x(sLe^X) bssides its isomer sialyl Lewis A($sLe^{A)}$ (Varki, 1994; Kansas, 1996; Dimitroff *et al.*, 2001; Gout *et al.*, 2008; Nimrichter *et al.*, 2008). Figure 2.9.

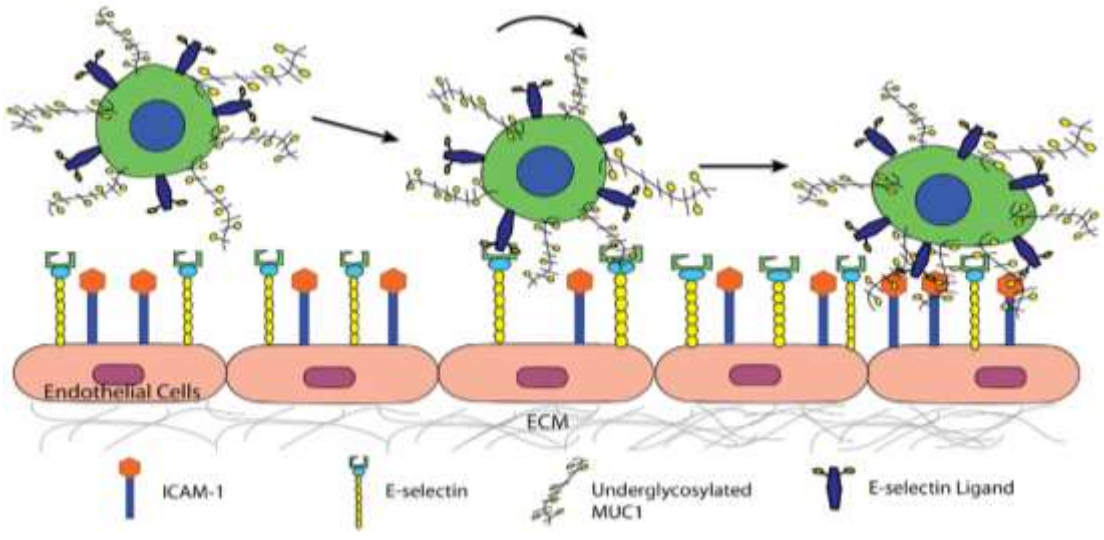


Figure2.9: E-selectin and Ligands–mediated slow rolling (Geng *et al.*, 2012).

2-3-4 Role of L-Selectin and E-Selectin in Adhesion of Circulating Leukocytes to Vascular Endothelium

Recruiting circulating leukocytes starting with recruit hematopoietic progenitors, or platelets to specific organs or to sites of infection or injury, is the rolling adhesion on vascular surfaces. Cells apply the multistep process in order to link drop down the vessel wall, slow arrest and then gather or emigrate into the underneath tissues (McEver and Zhu, 2010). A model of 3 steps describes leukocyte extra vacation during inflammation (Ley *et al.*, 2007; McEver and Zhu, 2010).

(1) Initiating linking and rolling on activated endothelia is mediated by L-selectin, P-selectin and E-selectin, which interact with (PSGL1) on the leukocytes.

(2) Activation, mediated by chemoattractants is generated by activated endothelium and tissue resident which result in integrin mobilization.

(3) Strong cell adhesion is mediated by leukocyte integrin binding to the immunoglobulin superfamily (IgSF) members on the endothelium. The interaction between the Intercellular Adhesion Molecule-1 ICAM-1, Vascular cell adhesion molecule (VCAM-1, CD106, and the integrins LFA-1, (very late antigen-4) VLA-4, and the leukocytes, provides the firm union required for the trans-endothelial migration, Figure 2.10.

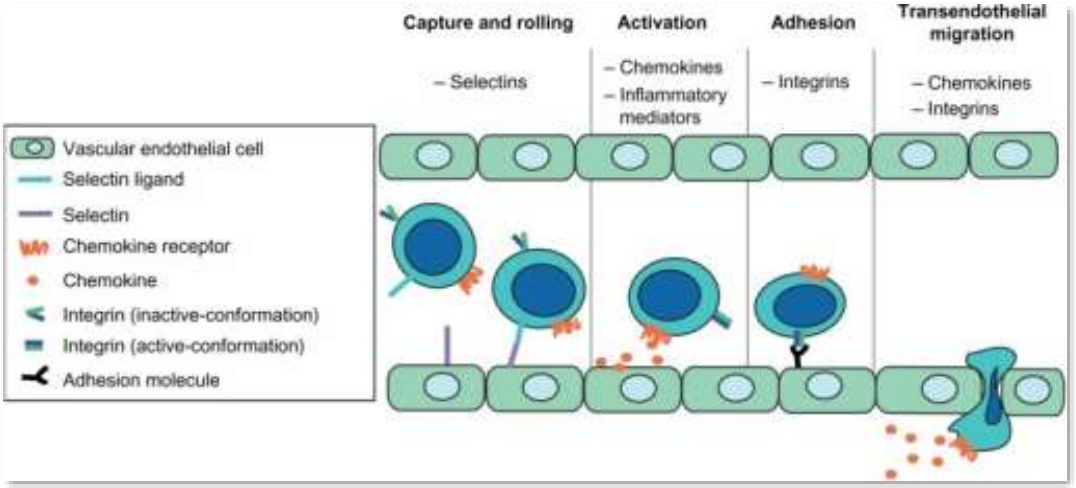


Figure2.10: Leukocyte Adhesion Cascade (Keshav and Wendt, 2015).

2-3-5 Selectin as Defense Mechanism against Microbes

At the site of injury, Macrophages that encounter microbes release interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF-a). These molecules activate the local endothelial cells to express the cell surface molecules: selectins and ligands for integrins. The chemokines produced in the tissue (eg, by the complement system) or by the endothelial cells are displayed on their surface bound to heparin sulfate glycosaminoglycans (Figure2.11) (Robert and Pelletier, 2018).

In acute (localized) inflammation, innate immune cells, including macrophages and neutrophils are recruited from the local blood vessels to the site of injury. Smooth muscle contraction causes the local blood vessels to constrict thus, increasing the local blood pressure. Concomitantly, the local permeability of the vascular endothelium to plasma increases, and the increased blood pressure forces the plasma into the tissue, bringing complement components and antibodies. Also loosening tight junctions facilitates the extravasation of leukocytes from the blood into the tissue; this process is called diapedesis. The leukocytes initially roll along the capillary wall under the sheer force of the blood flow (Pober and Sessa, 2014).

When leukocytes bind to the selectins and the proteoglycan-bound chemokines, their integrins are transformed into a high-affinity state and bind to the integrin receptors on the endothelial cells. After firm adhesion to the endothelial cells, the leukocytes migrate to the junctions between the endothelial cells and extravasate into the tissue (Muller, 2013).

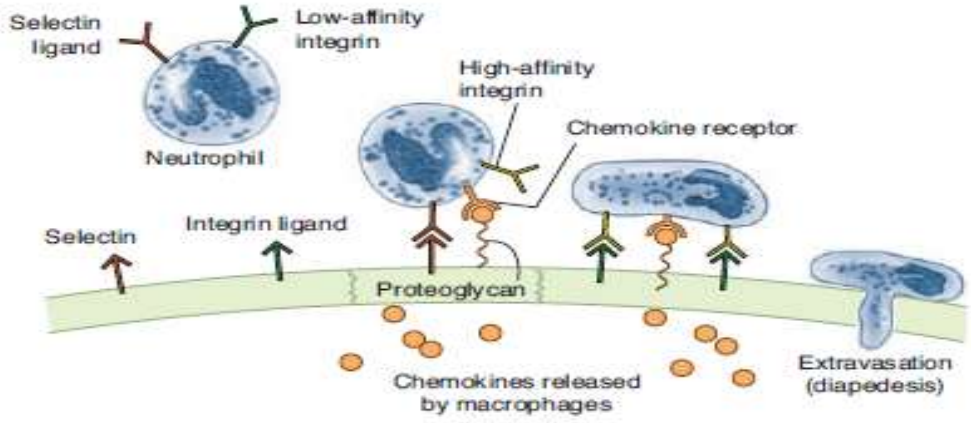


Figure2.11: Interaction of leukocytes with endothelial cells during leukocyte recruitment into tissues (Adapted from Abbas *et al.*, 2018).

Any an infectious microbe breaches an epithelium and enters the subepithelial tissue, resident macrophages recognize the microbe and respond by producing cytokines, (TNF) and (IL-1), act on the endothelium of small vessels at the site of infection. These cytokines stimulate the endothelial cells to rapidly express two adhesion molecules called E-selectin and P-selectin Circulating neutrophils and monocytes express surface carbohydrates that bind weakly to the selectins (Silva *et al.*, 2018).

The cytoskeleton of the leukocytes is reorganized, and the cells spread out on the endothelial surface. Chemokines also stimulate the motility of leukocytes (Filippi, 2016). As a result, the leukocytes begin to migrate between endothelial cells, through the vessel wall, and along the chemokine concentration gradient to the site of infected tissue. (De Oliveira *et al.*, 2016).

The sequence of selectin-mediated rolling integrin-mediated firm adhesion and chemokine-mediated motility leads to the migration of blood leukocytes to an extravascular site of infection area within minutes after the infected tissue. The same sequence of events is responsible for the migration of activated T lymphocytes into infected tissues. The accumulation of leukocytes at sites of infection with concomitant vascular dilation and increased leakage of fluid and proteins in the interstitial space of the tissue causing inflationammation. Inherited deficiencies in integrins and selectin

ligands lead to defective leukocyte recruitment to sites of infection and increased susceptibility to infections. These disorders are called leukocyte adhesion deficiencies. (Abbas., 2016) faciliting the bacterial and increased inflation process.

2-4 Foot Ulcer

Diabetes mellitus is a metabolic disorder that is characterized by high blood glucose levels for aprolonged period of time, resulted either because of the low insulin product from the pancreas, or because the cells are not responding properly to the produced insulin. This elevation in blood sugar levels might be associated with long term damage and failure of many body organs; like eyes, kidneys, heart and nerves (WHO, 2016). Millions of people are affected by diabetes mellitus all over the world. making it a famous global health problem (Singh, 2001). There are two main types of diabetes: Type one diabetes (T1DM), Type two diabetes (T2DM).

Diabetic foot is a common and costly complication of DM T2. In addition to causing serious morbidity, it is the largest number of diabetes associated with patients' hospital days, the most common and non-traumatic cause of amputation (Jeffcoate and Harding, 2003; Lipsky, 2004). Patients with diabetes also have common infections that include bones and soft tissues called stinky feet. In general, people with diabetes suffer from a more severe infection and take longer to recover from similar infections in other people (Suresh *et al.*, 2011). Foot ulcers were examined on the evidence of inflammation such as cellulitis, purulent hemorrhage, evidence of necrosis with or without osteoarthritis, and systemic toxicity (Stevens *et al.*, 2014). In addition, some studies suggest that the interactions of organisms in these multi-pesticide mixtures lead to the production of ferment factors, such as blood, protease, collagenase, as well as short-chain fatty acids that cause

inflammation that impedes wound healing, and contribute to the development of ulcer into deepest tissue. (Yao *et al*., 2005).

2-4-1 Susceptibility to Diabetes

Immunodeficiency and associated defects in leukocyte function have been implicated in the inherent susceptibility of the diabetic patient to infection (Alves and Casqueiro, 2012). Diabetics have inherent susceptibility to infection due to defects in leukocyte function resulting in the damaged pharynx, malignant cell dysfunction, and white cell chemical deficiency and adherence (Nather *et al.,* 2008). Many of the factors related to diabetes can weaken wound healing, including blood sugar fluctuation, hypoxia (hypoxia to wound) infection of impaired circulation may impair the ability of white blood cells to destroy pathogenic bacteria, and fungi increase the risk of infection, and the disease itself (Lavery *et al.,* 2007). The open pest allows for the introduction of microbes that thrive in the presence of a weak host response (Lipsky, 2001).

2-4-2 Classification of Diabetic Foot Ulcer

According to Wagner, the grading system depending on the depth of the wound and the extent of tissue necrosis (Sharma *et al*., 2006).

Grade 0: Preulcer. No open lesions skin intact; may have deformities, erythematous areas of pressure or hyperkeratosis.

Grade 1: Superficial ulcer. Disruption of skin without penetration of the subcutaneous fat layer. Superficial infection with or without cellulitis may be present.

Grade 2: Full-thickness ulcer. Penetrates through the fat to atendon, or joint capsule without deep abscess or osteomyelitis.

Grade 3: Deep ulcer which may or may not probe the to bone, with abscess, osteomyelitis, or joint sepsis. Includes deep plantar space infections or abscesses, necrotizing fasciitis, and tendon sheath infections.

Grade 4: Denotes gangrene of a geographical portion of the foot such as toes, forefoot or heel. The remainder of the foot is salvageable though it may be infected.

Grade 5: Gangrene or necrosis to the extent that the foot is beyond salvage and will require a major limb- or life-sparing amputation.

The types of organisms that because osteoarthritis are similar to those that cause autoimmune infections in the skin, subcutaneous tissue, fascia, or muscle, and are often multiple. Typical organisms include *S. aureus*, nodules, anaerobic, and gram-negative neurotransmitters (John and Ebright, 2005). The Wagner stages were with most of the two isolates and three. Moreover, it is difficult to tell whether microorganisms isolated from ulcers are pathogens or simply contaminants. (Candel *et al*., 2003; Crouzet *et al*., 2011). So that it is important to diagnose the type of infected bacteria by molecular accurate method quikly to used propel treatment (Al-Shmmary, 2016).

2-5-Diabetic Foot Ulcer and Microbial Infection

Loss of protective sensation resulting from peripheral sensory neuropathy is a very prevalent complication of diabetes and is a risk factor for both ulceration and amputation (Alves and Casqueiro, 2012). Motor and autonomic fibers are also typically involved with distal symmetrical neuropathy. The former will result in intrinsic muscle atrophy or drop foot as well as structural deformities, such as claw toes. High plantar foot pressures frequently result, which have also linked to foot ulceration (Raje and Dinakar,2015).

Developing a consequence of continued skin pressure over bony prominences; they lead to skin erosion, local tissue ischemia and tissue necrosis, and those are particularly susceptible to fecal contamination. 25% of foot ulcers have underlying osteomyelitis and bacteremia is also common (Dana and Bauman ,2014).

The bacteriology of foot ulcers is similar to that of some of the acute necrotizing soft tissue infections. The anaerobic and aerobic bacteria involved are likely to contribute to the deterioration of a lesion (Ki and Rotstein, 2008).

As in most wound types, *S. aureus* is a prevalent isolate in diabetic foot ulcers together with aerobes including *S. epidermidis*, *Streptococcus* spp., *P. aeruginosa*, *Enterococcus* spp. and coliform bacteria. With good microbiological, anaerobes have been isolated up to 95% of diabetic wounds. The predominant isolates being *Peptostreptococcus*, *Bacteroides*, and *Prevotella* spp. (Kwon and Armstrong, 2018).

Aerobic pathogens such as *S. aureus*, *P. aeruginosa*, and beta-hemolytic *Streptococci* are recognized for their ability to produce potentially destructive virulence factors and the clinical effects associated with *Clostridial* exotoxins are also widely acknowledged. However, many of the nonsporing gram-negative anaerobes that are often abundant (yet often invisible) in wounds also possess a wide variety of virulence factors that may impair wound healing. *Bacteroides*, *Prevotella* and *Porphyromonas* species are capable for expressing adhesion factors (capsular polysaccharide, fimbriae and hemaglutinin), tissue damaging exotoxins (proteases, collagenases, hyaluronidases, fibrinolysin, gelatinase, elastase, and condroitin sulfatase) and antiphagosytic factors (capsule, short chain fatty acids, and immunoglobulin A [IgA], IgM, and IgG proteases), all of which may contribute to the impairment of wound healing processes. In association

with aerobic microorganisms, the pathogenic potential of some Gram-negative anaerobes is often increased, and consequently, the combined effects of aerobes and anaerobes in wounds may produce a pathogenic effect that cannot be achieved by one type of microorganism alone (Citron *et al*., 2007).

Gram-positive bacteria, such as *S. aureus*, are the most common pathogens in mild and moderate infections not previously treated. (mainly hyperglycemia and arterial insufficiency) (Mazen, 2008).

Microbial synergy may increase the net pathogenic effect and hence the severity of infection in several ways (Akhi *et al*., 2015):

1) Oxygen consumption by aerobic bacteria induces tissue hypoxia and a lowering of the redox potential which favors the growth of anaerobic bacteria.

2) Specific nutrients produced by one bacterium may encourage the growth of fastidious and potentially pathogenic cohabiting microorganisms

3) Some anaerobes are able to impair host immune cell function and thus provide a competitive advantage to themselves and other cohabiting microorganisms.

Diabetic Foot Ulcers do not occur on their own, but usually follow this type of trauma, which may not be noticed by the patient. This shock may result from inappropriate shoes, bare feet, alien objects or hot water. (Sumpio, 2012). There is no doubt that the daily level of physical activity does not in itself predispose to new or recurrent foot ulcers. (Caputo *et al.*, 2005) In patients with diabetes, localized and systemic inflammation markers are often reduced as a result of terminal endings associated with vascular disease and immunosuppression. The affected foot may be painless

due to neuropathies and this may lead to unnecessary delay in the medical care request (Urbancic-Rovan, 2005)

Most bacteria present in diabetic foot ulcers are subject to fermentation metabolism. Fermentation is a type of metabolism other than oxidation. Carbon molecules are used as an energy source, and pyruvate or its derivatives are used as the receiver of the electron, leading to the generation of fermentation products such as alcohols or organic acids. Fermentation does not provide much energy for the cell such as airbreathing, but it is sufficient to save energy for a cell (Smith *et al.*, 2016). An example of this process is seen in *S. aureus*, a common bacterial member of the diabetic foot ulcer. Staphylococcus can be used either as respiration or aerobic fermentation, but fermentation is more common in diabetic foot ulcers (Dunyach-Remy *et al.*, 2016). The process of fermentation in *S. aureus*, produces lactic acid.

P. aeruginosa was responsible for 30% of pneumonia, 19% of urinary tract infections, and 10% of leukemia injuries (Lamas *et al.*, 2017). Their pathogenesis is based on the production and release of multiple virulence factors such as alginate, bili, and adipose plaques that respiration in *P. aeruginosa*. In addition, many factors of *P. aeruginosa* virulence, including toxins (exotoxin A and exoenzyme S), proteases (elastase, LasA proteases, and alkali protein), and hemolysins (phospholipase and rhamnolipid) (Andrejko and Mizerska-Dudka, 2012). pyocyanin, pyoverdine, hydrogen cyanide, as well as cell-related factors, such as polysaccharide and whip (Gellatly *et al.*, 2013), are produced by bacterial cells, which are regulated by a mechanism called quorum sensor (Williams and Cámara, 2009).

2-6 Some Virulence Factors Mechanisms of Compainy Diabetic Foot Ulcer:

Pathogenicity refers to the ability of microorganisms to cause disease in the host organism. Microbial pathology is expressed by its virulence: a term that indicates the degree of microbial pathogenesis (Spichler *et al.*, 2015). The virulence factor refers to these factors that enable the host to be infected and to improve the microbial potential in causing the disease. Viruses and bacteria are able to produce molecules that effectively inhibit the host's immune response, which may be associated with persistent inflammation and tissue damage (Cerqueira and Peleg, 2011).

Bacteria have multiple virulence factors, including those associated with the cell (Sarowska *et al.*, 2019). Alginate is another associated agent, the mannuronic and glucuronic acid, which is a mucosal form of biofilm, in which bacterial cells are submerged, thus protecting bacteria from the body's defenses such as phagocytes, lymphocytes, cirrhosis of the respiratory tract, antibodies (Daboor *et al.*, 2019).

2-6-1 Adhesions of Bacteria

In bacteria adhesives are bacterial components capable of binding to a wide range of surfaces, thus mediating interactions between bacteria and surface. The ability of bacteria to adhere to surfaces is the first step in the process of colonization in many outlets (Berne *et al*., 2015).

Their ability to colonize human skin and remove crusts also plays an important role in causing infection, epidemics and environmental perseverance (Cerqueira and Peleg, 2011).

An important facet in the interaction between *S. aureus* and its host is the ability of the bacterium to adhere to human extracellular matrix components and serum proteins. In order to colonize the host and

disseminate, it uses a wide range of strategies, the molecular and genetic basis of which are multifactorial, with extensive functional overlap between so the molecular adhesive components of adhesion are verey important for host pathogen. (Clarke and Foster, 2006)

The adhesion of *P. aeruginosa* to the epithelial cells lining the tissues and infected organs. The pili on the surface of the *P. aeruginosa* contributes with adhesion to the cell. It also plays a major role in the early settlement of infection in the mucous membranes of the urinary, reproductive, intestinal tract and respiratory tract of patients with cystic fibrosis (Bakshani *et al.*, 2018). *P. aeruginosa* is associated with endothelial cells of the upper respiratory tracts and epithelial cytoskeletal cells, and this association is made by special receptors of Galactose, Mannose, or Sialic acid present on the surface of the cytoskeletal cells The process of colonizing *P. aeruginosa* bacteria in the respiratory tract needs cilia (Chowdhury *et al.*, 2014). for successive propagation over the host cell surface (Bakshani *et al.*, 2018).

2-6-2 Biofilms in Wounds Foot Ulcer

Biofilms are found widely in nature and have been extensively studied in different human diseases for many years (Lu *et al*., 2019). Their presence is often associated with chronic wounds, also be involved in acute wound infection. Biofilms may be unperturbed by the antimicrobial or neutrophil attack and can survive a relatively harsh environment, resisting attempts at removal (Wolcott *et al*., 2008).

Chronic open wounds provide "fertile ground" for colonization with MDR bacteria and may support complex biofilm flora with large numbers of bacteria including aerobic Gram-positive cocci (*S. aureus*) various *Enterobacteriaceae, P. aeruginosa,* sometimes other non-fermentative Gram negative rods, and obligate anaerobes. Bacterial virulence factors and local conditions (necrotic tissue, ischemia, hyperglycemia, trauma, weakness)

may allow planktonic bacteria to invade surrounding living tissue to produce acute infections cellulitis or fasciitis (Lipsky *et al*., 2004; Wolcott and Dowd, 2011).

Biofilms are defined as organization of microorganisms that are attached to abiotic or abiotic surface. Biofilm formation is a multistage process that lets microbial cells adhere to the surface as shown in Figure 2.12, while the subsequent production of an extracellular matrix (containing polysaccharide, proteins and DNA) results in a firmer attachment (Stoodley *et al*., 2002). Bacterial cells embedded in this matrix communicate with each other to form coordinated group behavior mediated by a process called quorum sensing (QS) (Zhang and Dong, 2004). Sessile (biofilm-associated) increased resistance to antimicrobial agents (Hall and Mah, 2017). Biofilm formation is often caused resistance the antimicrobial drugs and an estimated 65-80% of all infections (Singh *et al*., 2017). When a cell switches to the biofilm mode of growth, phenotypic shift behavior appeared by expressed genes (Wei and Ma,2013). With availability of key nutrients, chemotaxis toward the surface, motility of bacteria, surface adhesions, and presence of surfactants (Cheng *et al*., 2019).

For example, *S. aureus* when growing within a biofilm has been resisting to antimicrobial to 100 times more than (Orazi *et al*., 2019).

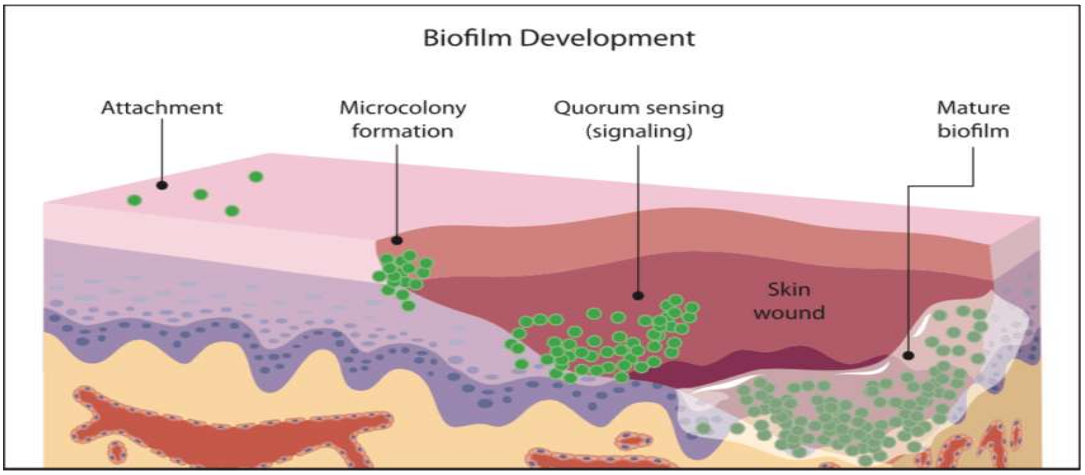


Figure 2.12: Development of biofilm

2-7 Genetic Study of Bacterial adhesion

2-7-1 *Staphylococcal* genes (*icaA and icaD* genes)

Biofilm formation is regulated by the expression of adhesion between the polysaccharide (PIA), which mediates the cell to cellular adhesion and is the product of the gene for *ica A,D,B* and *C* (Ammendolia *et al.*, 1999). Intercellular adhesion (*ica*), a position that consists of *ica A, D, B* and *C* genes. Proteins mediate the synthesis of PIA and polysaccharide adhesion in *S. aureus* (O'Gara and Humphreys, 2001). Genes *icaA*, and *icaD* have been reported to play an important role in the formation of biofilms in *S. epidermidis* (Yazdani *et al.*, 2006). Findings indicate an important role of *ica A* and *ica D* because of their strong ability to produce biofilm in a high proportion of clinical isolates (Al-Mahallawi *et al.*, 2009).

The common expression of *icaA* with *icaD* can significantly increase biofilm production (Cafiso *et al.*, 2004). Different studies had shown the critical role of gene *ica* as virulence factors in *Staphylococcus* infection (Rohde *et al.*, 2010). Genes *icaA* translates N-acetyl transferase, the enzyme involved in the synthesis of N-acetyl glucosamine oligomers from UDP-N-acetyl glucosamine. Furthermore, it has been reported that *icaD* plays a role in the maximal expression of N-acetyl glucosamine transferase, leading to the phenotypic expression of polysaccharide (Arciola *et al.* 2001). The expression of the *ica* genes, as a result of the formation of biofilms, is very variable between *S. aureus*. Therefore, Biofilm influenced by environmental signals and induced in response to external stress and inhibitory concentrations of certain antibiotics (Mertens and Ghebremedhin, 2013).

Figure 2.13: A- Gene *icaA* biofilm PIA synthesis N-glycosyltransferase: REGION: 2706338.2707576. B- Gene *icaD* biofilm PIA synthesis protein REGION: 2707540.2707845 (https://www.ncbi.nlm.nih.gov/2019).

2-7-2 *Pseudomonas aeruginosa cupA* genes

P. aeruginosa is an opportunistic bacterial pathogen that poses a major threat to long-term-hospitalized patients. The capacity of *P. aeruginosa* to form biofilms is an important requirement for chronic colonization of human tissues and for persistence in implanted medical devices. Various stages of biofilm formation by this organism are mediated by extracellular appendages, such as type IV pili and flagella. Recently three *P. aeruginosa* gene clusters termed (chaperone-usher pathway) cup based on their sequence relatedness to the chaperone-usher fimbrial. The *cupA* gene cluster, but not the *cupB* or *cupC* cluster, is required for biofilm formation on the abiotic surface (Vallet, *et al.*, 2004)

The components of a cup that is involved in the identification of these putative adhesins on the cell surface of *P. aeruginosa* suggost that this organism possesses a wide range of factors that function in biofilm formation. These structures appear to be differentially regulated and may function at distinct stages of biofilm formation, or in specific environments colonized by this organism (Jani, *et al.*, 2016).

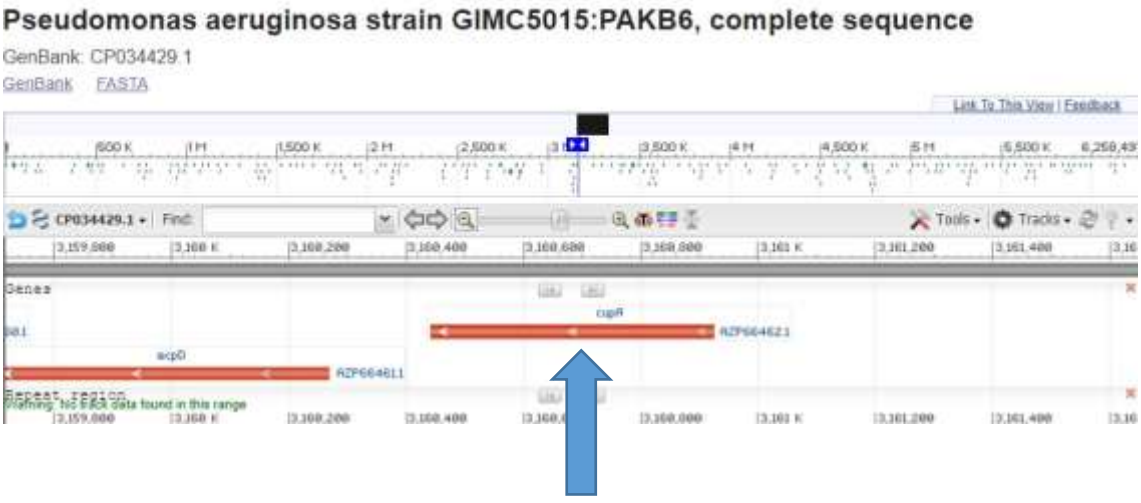


Figure 2.14: Gene *cupA genes* Length, (https://www.ncbi.nlm.nih.gov/2019.)

2-8 Multi-drugs Resistant (MDR) Bacteria in Diabetic Foot Ulcer

Increased association between Multi-Drugs Resistant (MDR) bacteria and diabetic foot ulcer increases the risk of amputation (Jain and Barman, 2017).

During the last three decades, microbial resistance has dramatically increased and spread around the world. Pathogenic bacteria have developed several ways to resist almost all antimicrobials used. These pathogens can cause a wide range of superficial infections, (but are also the cause of life-threatening infections in immunocompromised patients). Therefore, emergenciy is to find new antimicrobial molecules, with an innovative chemical structure, and, if possible an innovative mechanism of action. However, to reach his goal (Tascini *et al.*, 2011).

Scientists and researchers need to address the growing problem of antibacterial resistance to antibiotics and find alternative ways to reduce the risk of having similar antibiotic-like properties. Nanoparticles have been used because they have great antimicrobial properties due to their surface size to large size (Wang *et al.*,2017).

2-9 Nanotechnology

Nanotechnology is relatively a new science of study in which a set of sciences, including the STEM (science, technology, engineering and mathematics). Disciplines are involved to synthesize nanomaterials, about 1 to100 nanometers. At the nanoscale level, materials have distinct chemical, physical, optical, magnetic and electrical properties due to their large surface area to volume ratio (Chaturvedi *et al.*, 2012).

2-10 Nanoparticles:

Nanoparticles are of great scientific interest as they are effectively a bridge between bulk materials and atomic or molecular structures. A bulk material should have constant physical properties regardless of its size, but at the nano-scale this is often not the same. (Uskoković, 2013). The

properties of materials changed as their size approaches the nanoscale and as the percentage of atoms at the surface of a material becomes significant. For bulk materials larger than one micrometer the percentage of atoms at the surface is minuscule relative to the total number of atoms of the material.

The interesting and sometimes unexpected properties of nanoparticles are partially due to the aspects of the surface of the material dominating the properties in comparison with the bulk properties. Nanoparticles exhibit a number of special properties relative to the bulk material. Nanoparticles have a very high surface area to volume ratio (Foundations of Nanotechnology, 2015). This provides a tremendous driving force for diffusion, especially at elevated temperatures. The large surface area to volume ratio also reduces the incipient melting temperature of nanoparticles (Edward,2004). Moreover, nanoparticles have been found to impart some extra properties to various day-to-day products.

2-11 Nanoparticles Synthesis

Nanomaterials are not simply another step in the miniaturization of materials. They often require very different production approaches. There are several processes to create nanomaterials. Two main approaches are used in nanotechnology. In the "top-down" approach, Nano-objects are constructed from larger entities without atomic-level control. In the "bottom-up" approach, materials and devices are built from molecular components that assemble themselves chemically by principles of molecular recognition as shown in Figure (2.15) (Cabeza, 2016)

The top-down approach often uses the traditional workshop or microfabrication methods where externally controlled tools are used to cut, mill and shape materials into the desired shape and order. Micropatterning techniques, such as photolithography and inkjet printing belong to this category (Das *et al*.2000).

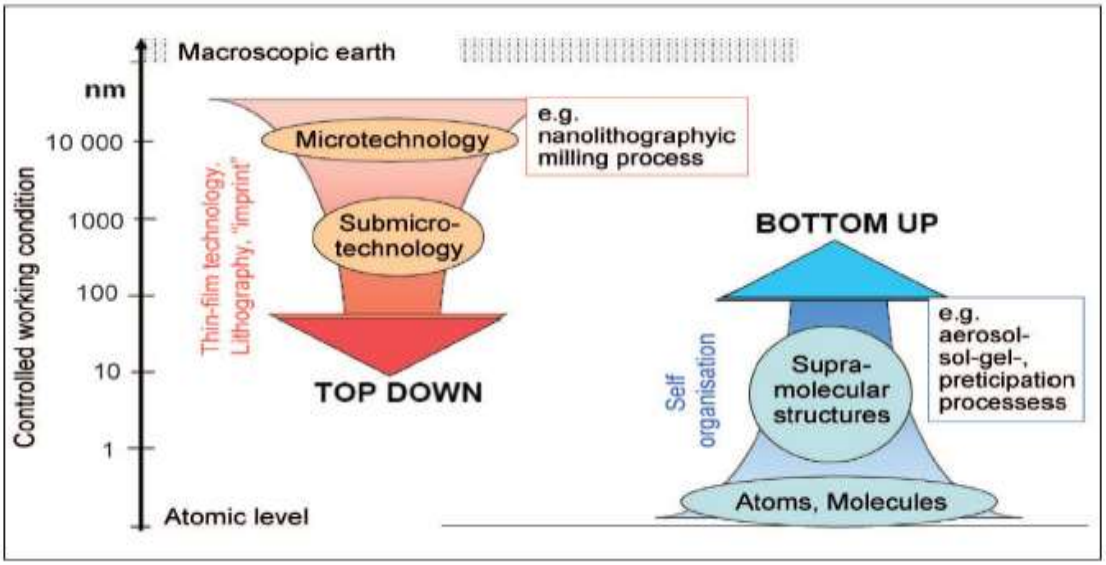


Figure 2.15: Top-Down and Bottom-Up Approaches for Nanomaterials synthesis

The biggest problem with a top-down approach (Ito and Okazaki,2000) is the imperfection of the surface structure. The conventional top-down techniques such as lithography can cause significant crystallographic damage to the processed patterns, and additional defects may be introduced even during the etching steps (Grill *et al*.2007). For example, nanowires made by lithography are not smooth and may contain a lot of impurities and structural defects on the surface. Such imperfections would have a significant impact on physical properties and surface chemistry of nanostructures and nanomaterials, since the surface over volume ratio in nanostructures and nanomaterials is very large. The surface imperfection would result in a reduced conductivity due to inelastic surface scattering, which in turn would lead to the generation of excessive heat and thus impose extra challenges to device design and fabrication. Bottom-up approach (Yang *atel*,.2010). Refers to the build-up of a material from bottom: atom-by-atom, molecule by- molecule, or cluster-by- cluster crystal growth, growth species, such as atoms, ions and molecules, after impinging onto the growth surface, assemble into crystal structure one after another.

For most materials, there is no difference in physical properties of materials regardless of the synthesis routes, provided that chemical composition, crystalline, and microstructure of the material are identical (Zhang and Lan, 2008) Of course, different synthesis and processing approaches often result in appreciable differences in chemical composition, crystallinity, and microstructure of the material due to kinetic reasons. Consequently, the material exhibits different physical properties.

Although the bottom-up approach is not new, it plays an important role in the fabrication and processing of nanostructures and nanomaterials. There are several reasons for this. When structures fall into a nanometer scale, there is little choice for top-down approach. The bottom-up approach also promises a better chance to obtain nanostructures with rewer defects, more homogeneous chemical composition, and better short and long range ordering. This is because the bottom-up approach is driven mainly by reduction of Gibbs free energy, so that nanostructures and nanomaterials such produced are in a state closer to a thermodynamic equilibrium state. On the contrary, top-down approach most likely introduces internal stress, in addition to surface defects (Thiruvengadathan *et al*,.2013).

2- 12 Nanoparticle Morphology:

Morphological characteristics to be taken into account are flatness, sphericity, and aspect ratio. A general classification exists between high- and low-aspect ratio particles. High aspect ratio nanoparticles include nanotubes and nanowires, in various shapes, such as helices, zigzags, belts, or perhaps nanowires with diameter that varies with length. Small-aspect ratio morphologies include spherical, oval, cubic, prism, helical, or pillar. Collections of many particles exist as powders, suspension, or colloids. Agglomeration and aggregation are mechanisms leading to the formation of planets, stone, soil (Buzea *et al*,.2007), and also nanoparticles. Here, loosely

attached primary particles (by van der Waals, capillary or electrostatic forces) are called agglomerates while particles held together by chemical/sinter forces (metallic, ionic or covalent bonds) are termed aggregates (Pratsinis, 2003).

2- 13 Preparation Methods of Nanomaterials

There are a large number of techniques available to synthesize different types of nanomaterials in the form of collides, clusters, powders, tubes, rods, wires, thin films. Some of the already existing conventional techniques to synthesize different types of materials are optimized to get novel nanomaterials and some new techniques are developed. (Paques *atel*,.2014).

There are various physical, chemical, biological, and hybrid techniques available to synthesize nanomaterials. The technique to be used depends the material interest, types of nanomaterial viz zero-dimensional(0-D), one-dimensional(1-D) or two-dimensional(2-D), their sizes and quantity **(**Rane *atel*,.2018).

2-13-1 Hydrothermal Method

Hydrothermal synthesis is a method of preparing a variety of materials such as metals, semiconductors, ceramics, and polymers. The process involves the use of a solvent under moderate to high pressure (typically between 1 atm and 10000 atm) and temperature(typically between 100º C and 1000º C) ,That facilitates the interaction of precursors during synthesis if the water is used as the solvent,the method is called "hydrothermal synthesis" .The synthesis under hydrothermal conditions is usually performed below the supercritical temperature of water (374 ºC).The process can be used to prepare many geometric including thin film, bulk powders, single crystals, and nanocrystals .In addition, the morphology[sphere (3D), rod(2D), or wire(1D)] of the crystals formed is controlled by manipulating the solvent super saturation chemical of interest concentration, a kinetic

control .The method can be used to prepare thermodynamically stable and metastable states including novel materials that can not be easily formed from others synthetic routes (Schubert and Hüsing, 2012).

2- 14 Estimated the Nanoparticles Size

In the nanoparticle research, there are several models to estimate the size of the nanoparticles depending on the character that measure (bandgap, structure).

In general, one can estimate the size from the following measurements

1. UV-Vis spectrum absorption.

2. X-ray diffraction (XRD).

3. Transmission electron microscopy (TEM).

2-14-1 Estimate the Size from UV-Vis Spectrum Absorption

To estimate the size of the nanoparticles from UV-Vis spectrum, it can be used two models:

1. Effective mass approximate model (EMA) using Brus equation Foundations of Nanotechnology 2015.

2. Tight binding model (TB) using guessing fitting which is derivative by Sapra and Sarma, 2004 very quickly. Indeed, Weimarn's law dictates that the final crystal size is inversely proportional to the initial degree of supersaturation. Mono disperse size distributions, wherein the particles have the same size within 5%, can be obtained by either; stopping the reaction (nucleation and growth) quickly at the point where all crystallites are roughl of the same size and disparity; or by supplying a constant reactant source to keep a supersaturated condition during the course of the reaction, so that all nuclei grow at the same rate (Tauc and Vancu.,1996).

2-14-2 Estimate the Nanoparticles Size from X-ray Diffraction Scherrer Equation

One can use the Scherrer equation to estimate the nanoparticles crystallite size as shown in equation (Khodair *et al*,.2019).

$$\mathbf{Dav} = \frac{\mathbf{k}\,\boldsymbol{\lambda}}{\boldsymbol{\beta}\,\boldsymbol{Cos}\,\boldsymbol{\theta}} \quad \textbf{.....(1)}$$

Where: -

λ: is Cu-k (alph) wavelength.

K: is shape factor is equt to 0.9

β: is the full-width at half maximum measured in radias.

θ: is the Bragg diffraction angle.

From this equation one can see that there is an inverse relation between D and β, this means when D is small, β will take a big value and gives the first sign for nanoparticles is the broadening of the peak of an X-ray diffraction pattern.

2-15 Properties of Copper oxide

Copper (II) oxide (CuO) or cupric oxide is the inorganic compound with the formula CuO. A black solid, it is one of the two stable oxides of copper, the other being Cu_2O or cuprous oxide. As a mineral, it is known as tenorite. It is a product of copper mining and the precursor to many other copper-containing products and chemical compounds (Wayne, 2002).

It is produced on a large scale by pyrommetallurgy used to extract copper from ores. The ores are treated with an aqueous mixture of ammonium carbonate, ammonia, and oxygen to give copper (I) and copper (II) ammine complexes, which are extracted from the solids. These complexes are decomposed with steam to give CuO.

It can be formed by heating copper in air at around 300 – 800°C:

Copper(II) oxide belongs to the monoclinic crystal system. The copper atom is coordinated by 4 oxygen atoms in an approximately square planar configuration shown as in figure (2.16) (Forsyth. *et al.*, 1991).

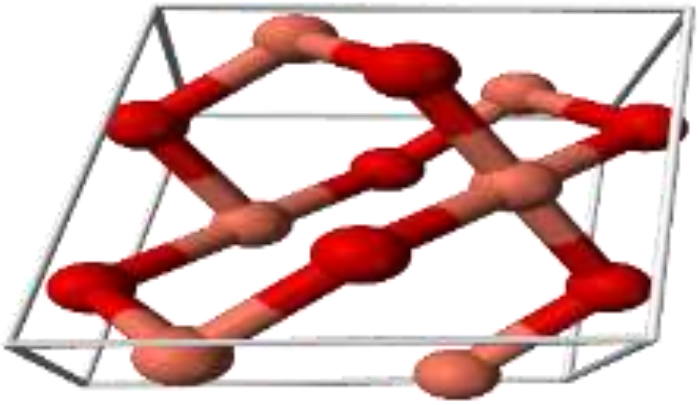


Figure 2.16: Monoclinic Structure of CuO (Forsyth. *etal.,* 1991).

2-16 Copper Oxide Production and its Antibacterial activity

2-16-1 History of Copper and its Antibacterial Activity

The time for the discovery of copper is unknown but is estimated at about 9000 BC in the Middle East (Tauc and Vancu,1996). Copper is the oldest metal used, used by Egyptians around 2000 BC as a wound and water sterilizer, copper has many properties such as good resistance to corrosion, light activity and antimicrobial activity (Sarma *et al.* 2004).

One of the most important applications of copper and copper compounds is killing bacteria, because of their diversity, low cost, necessary for humans at low levels and characteristics of the activity of biocides (Reyes-Jara *et al.* 2016).

Research on antimicrobial activity of Copper Oxide Nanoparticles (CuO-NPs) is limited. has antibacterial activity against a group of Gram-negative and gram-positive bacteria such as *S. aureus*, and *E. coli* (Martin *et al.*,2010).

2-16-2 Mechanisms of Copper Oxide Nanoparticles as Antibacterial

The precise mechanism of bacterial clearance by CuO is not clear. However, limited proposed mechanisms are reported, one of which is the Cu ions released from nanoparticles that come into contact with the bacterial cell membrane that damages the bacterial cell membrane (Martin *et al.,* 2010).

It has been suggested that diffused copper ions may lead to disruption of DNA structure through the interaction of ions with DNA molecules (Ahmad and Sardar,2013). Another proposed mechanism is "nanoscale effect" where (M. Heinlaan and coworkers) Reported that the volume of nanotube copper plays an important role in toxicity and, therefore, on the efficiency of disinfection (Von, 2006).

The most recent mechanism proposed is oxidative stress (ROS) can be caused by CuO-NPs, depending on the rate of CuO-NPs degradation, where ROS may damage the structure of the bacterial cell (Hena and Growther, 2016).

3. Materials and Methods

3.1 Materials

3.1.1 Equipment

Equipment used in this study are shown in Table 3.1.

Table 3.1: Equipments and their sources.

Equipment	Company	Country
Autoclave	Binder	USA
Deep freeze refrigerator	Artciko	Denmark
Digital camera	Sonyo	Japan
Electrophoresis constant power supply And Gel electrophoresis apparatus	Biotech-Fisher	Australia
Eppendorf centrifuge	Hettich	Germany
GeneAmp® PCR System 9700	Applied Biosystems	USA
Hot plate with amagnetic stirrer	Gallenkamp	England
Incubator	Binder	USA
Laminar air flow Hood	Binder	USA
Magnetic stirrer	Kamag RCT	Germany
Microfuge®18 Centrifuge	Beckman Coulter	USA
Micropipette (Automatic) 20, 100, 1000 µL	Human lab	Germany
Oven	Gallenhamp	UK
pH meter	Jenway 3320	UK
Sensitive balance	Precisa	Switzerland
Spectrophotometer	Applied Biosystems	USA
Transmission Electron Microscopy (TEM)	PHILIPS	Japan
Vitek 2	BioMerieux	(France)
Vortex Mixer	Griffin	Germany
Water bath	Memmert	Germany
Water distillatory	Gallenkamp	England
X-Ray Diffraction	Shimadzu Xrd-6000	Japan

3.1.2 Chemicals

Biological and chemicals materials used in the study and their sources are shown in Table 3.2.

Table 3.2: Biological Materials and chemicals and their sources:

Chemicals	Sources	Country
Absolute ethanol 99%	BDH	England
Acetone	Sigma Aldrich	(USA)
Agarose	Biobasic	Canada
$BaCl_2.2H_2O$	BDH	England
Boric acid, A.R.	HiMedia	India
Citric acid	BDH	England
Congo red stain	BDH	England
Copper chloride hydrate	Sigma Aldrich	(USA)
Copper nitrate trihydrate	Sigma Aldrich	(USA)
Copper(II) sulfate pentahydrate (CuSO4.5H2O)	Alfa Aesar	USA
Crystal violet	Fluka	Switzerland
Deionized sterile water D.W.	Bioneer	Korea
DNA ladder Marker (2000bp)	Alfah	USA
DNA Loading dye	Bioneer	Korea
DNA molecular size marker	Bioneer	Korea
EDTA	BDH	England
Ethidium bromide (EtBr)	BDH	England
Free Nuclease water	Bioneer	Korea
Gelatin	Sigma	Germany
Glacial acetic acid	BDH	England
Glucose	GFS	Canada
Glycerol ($C_3H_8O_3$)	Fluka	Switzerland
Go Taq® master mix	Bioneer	Korea
Gram stain reagents	Himedia	India
H_2SO_4	BDH	England
Hexamine	Sigma Aldrich	(USA)
Hydrochloric acid (HCL)	BDH	England
Hydrogen peroxide (H_2O_2)	BDH	England
Iodine	GCC	England
Kovac's Reagent	GFS	Canada
NaCl	BDH	England
NaH_2PO4	BDH	England
NaOH	BDH	England
Normal Saline (Sterile)	Pioneer	Korea
Peptone	Difco	China

Phosphate buffer saline (PBS)	Sigma	UK
Primers (lyophilized)	Alpha DNA	Canada
Sterile distilled water	Parenteral drugs	India
Sucrose	BDH	England
Sulfuric acid (H_2SO4)	BDH	England
Tris-base (hydroxymethyl amino methane)	Sigma	Germany
Tris-Borate-EDTA buffer (TBE buffer) 10X	Promega	USA

3.1.3 Chemical Kits

Kits used for clinical and laboratory diagnosis are shown in table 3.3.

Table3.3: The Chemical Kits Used in this Study:

Kits name	Company	Country	Catalogue Number
Cholesterol	Spinreact	Spain	Ref: MD41021
gSYNC™ DNA Extraction Kit	Geneaid Biotech	Taiwan	GS100/300
HbA1c	Boditech Med	Korea	CFPC-38
High Density Lipoprotein	Spinreact	Spain	Ref: 1001095
Insulin	Demeditec	Germany	Ref:DE2935
Fasting Blood Glucose	Spinreact	Spain	Ref:1001191
Triglyceride	Biolabo	France	Ref: 87319
Quantifiler®, Human DNA quantification kit	Applied Biosystems	USA	Ref 4343895
Vitek 2 compact system kit	Biomerieux	France	

3.1.4 Media

Table3.4: Culture Medium

Culture Medium	Company	Country
Agar-agar	Oxoid	England
Blood Agar Base	Oxoid	England
Brain-Heart Infusion Agar& Broth	Oxoid	England
MacConkey Agar	Oxoid	England
Mannitol Salt Agar	Biolab	Turkey
Mueller-Hinton Agar	Oxoid	England
Nutrient Agar	Oxoid	England
Nutrient Broth	Oxoid	England
Pepton Water	Himedia	India
Pseudomonas Ceramide agar	Oxoid	England
Simmone Citrate	Himedia	India
Tryptic soy broth	Himedia	India

3.1.5 Antibiotics used in Vitek 2

Table3.5: Antibiotics types used in this study:

Type Antibiotic	Symbols	Company (Origin)
Amikacin	AK	Biomerieux France
Amoxicillin/Clavulanic Acid	AMC	
Ampicillin	AM	
Cefalotin	CF	
Cefotaxime	CTX	
Cefoxitin	FOX	
Ceftazidime	CAZ	
Ciprofloxacin	CIP	
Gentamicin	CN	
Imipenem	IPM	
Nalidixic Acid	NA	
Ofloxacin	OFL	
Piperacillin / Tazobactam	TZB	
Ticarcillin	TIC	
Tobramycin	TM	

3.1.6 Disposable Materials

The disposable materials and their origin used in this study are shown in Table 3.6

Table 3.6: Disposable Materials

Disposable	Source	Country
Blood collection vacuum tube Gel and clot activator	Orsin	Shanghai
Blue tips (1000 µL)	JRL	Lebanon
Cotton	HDA	China
Disposable glass pasture pipette	Volac	England
EDTA tubes 5mL	AFCO	Jordan
Eppendorf tubes 0.2, 1.5 and 2 ml	Eppendorf	USA
Filter paper	MEHECO	China
Gauze	Cotton products	Iraq
Gloves without powder	Broche	Malaysia
Microtiter plate 96 well Polystyrene	Bio Basic	Canada
Parafilm	BDH	England
Petri dish	Afco-Dispo	Jordan
Plain test tubes 10 ml	SUN	Jordan
Plan tubes (different volume)	Afco-Dispo	Jordan
Polystyrene tubes	Difco	USA
Screw – capped tube	HDA	China
Syringe 10 ml	MEDECO	UAE
White tips (10µL)	Eppendorf	USA
Yellow tips (100µL)	FL-medical	Italy

3.2 Patients and Control Groups

The current study was conducted on 50 Iraqi patients (30 males and 20 females) diagnosed with Type Two Diabetes Mellitus(T2DM) having foot ulcer, their age range was between 43-94 years during February to December 2018. Patients group were attented to Educational Baqubah hospital / Diyala, and Specialized Centre of Endocrinology and Diabetes Baghdad/ AL-Russafa Health Directorate Iraq. They were periodic patients the Patients were told about the purpose of the study and interested volunteers were enrolled after obtaining their consent (Appendix1).

According to the American Diabetes Association 2016 guideline, the Patients were selected on the basis of criteria for diabetes used Fasting Blood glucose (FBG)≥126 mg/dL (7.0mmol/L), or glycosylated hemoglobin HbA1c ≥ 6.5% (48 mmol/mol) (ADA,2016). The results were confirmed by repeated testing (ADA, 2016).

The study excluded patients with various diseases such as kidney disease, chronic liver disease, malignant disorders, and thyroiditis. Diabetics under the age of 18 were excluded, those with less than 6 months of follow-up, or pregnant women. Topics were also excluded from the history of high blood pressure, coronary artery disease, endocrine dysfunction or those taking any lipid-modifying drug from the study.

Fifty healthy subjects (27males and 23 females) were included along with the patients group as a control group. This control group consists of non-diabetic healthy individuals according to the laboratory finding (FBG) (value <90mg/dL).

Both patients and the control groups were characterized according to age, gender, Educational level, family history of diabetes, duration of disease, Smoking, weight (kg), height (m), abdominal circumference (cm)

information have been installed for each person in a special form (Appendix2). Blood group, (FBG), (HbA1c) and lipid profile tests were performed for each subject in both groups as well as insulin concentration measurement tests.

3-3 Blood Samples Collection

Venous blood samples (8ml) were collected from each fasting subject after 12 hours fast, then divided into two aliquots: one for the biochemical tests (as serum) and the other aliquot was kept at -20°C for genotyping analysis.

3-3-1 Genomic DNA Extraction

After thawing the frozen blood samples at room temperature was extracted using DNA extraction kit (gSYNC ™). DNA extraction and purification using whole blood (fresh or frozen blood). The extraction steps done were in accordance with the group's guidelines Cat. No. GS100. The samples were then stored at -20 ºC until use.

3-3-1-1 Measurement of DNA Concentration and Purity

DNA concentration were Measure was measurement by adding 495 µl of the distilled water to 5 µl of the sample DNA and reading the UV absorption of the spectrophotometer using the optical density measurement (O.D) at 260-280 nm and apply the following equation:

The concentration of DNA (µg/ml) = the amount of absorbance at wavelength 260 nm of the sample x inverted dilution x 50, the purity = absorbance at wavelength 260 / absorbance at wavelength 280. (Maniatis *et al.*, 1982).

3-3-1-2 Agarose Gel Electrophoresis

- **Preparation of agarose gel**

1.10 ml of 10 X TBE pH 8.5 was added in a beaker.

2.Sterile double distilled water (90ml) was added to the TBE buffer to get 1X concentration.

3.One gram of agarose powder was added to the buffer mixture.

4.Solution was heated till boiling.

The solution was allowed to cool down to 40-50°C, then 5 µL of Ethidium Bromide dye was added and mix gently.

- **Casting of the Horizontal Agarose Gel**

1. The gel was assembled to cast tray and the comb was positioned at one end of the tray.
2. The agarose solution was poured into the gel tray and allowed to solidify at room temperature for 30 minutes.
3. The comb was carefully removed and the gel was placed in the electrophoresis chamber.
4. The chamber was filled with TBE-electrophoresis buffer 1X and reached approximately 1-2 mm over the surface of the gel.
5. DNA was mixed with loading buffer (ratio 5:1) and loaded in the wells of the agarose gel.
6. The cathode was connected to the well side (DNA side) of the unit and the anode to the other side.
7. The gel was run at 60 volts until loading dye migrated to a suitable distance from the well.
8. The DNA result was viewed by the gel Document analyzer system

Agarose gel preparation and casting was made according to Lee et al (2012).

3-3-1-3 Polymerase Chain Reaction

PCR technique were applied in this study including Sequence Specific Primers (SSP-PCR).

Three single nucleotide polymorphism were genotyped by designing specific primers set, then a PCR program was designed according to its specific primers characters.

-For designing primers, free online programs were used as below:

http://primer3plus.com/cgi-bin/dev/primer3plus.cgi and http://eu.idtdna.com/calc/analyzer In addition, And the primers blasting was made. https://blast.ncbi.nlm.nih.gov/Blast.cgi

3-3-1-3-1 Primers

Primers were provided by DNA Alpha/Canada as mentioned and listed in tables (3.7 and 3.9) DNA substitutes that were introduced in the form of a freeze-dried product were de-sterilized after short rotation depending on the manufacturer's instructions. The suspension of the stock to give a final concentration 100pmol/µl as a supply solution. Work solution with 10pmol/µl was made by adding 10 µl of the supply solution plus 90 µl of the nuclease free water depending on the procedure of each primer and stored at -20 ºC until use.

3-3-1-3-2 L-selectin

The L-selectin rs2205849 polymorphism at position–642 of the promoter region (c.725 C-T) was genotyped by SSP-PCR. Primer used for the amplification of this loci were as mentioned in table 3.7.

Table 3.7: Sequence of primer of L-selectin polymorphism rs2205849

Primers	Sequence	Tm	Size	References
F	5’- CCTCCTTCACTCATTGTTG -3’	51	550	Chen *et al.* (2007)
R	5’- AGGTACTTGTAGGCTCCC -3’	53		

The PCR reaction was performed for final 20 µL reaction volume by using 5 µL of 2X Go Taq® bioneer master mix, 2 µL of 10M of each primer (forward, reverse), 5µL of genomic DNA and the volume was made

to 20 µL, with nuclease free water. The reaction was carried out with GeneAmp® PCR System thermocycler as mentioned by the reference (Table 3.8):

The PCR products 550 bp of the targeted SNP, the results were visualized by 1% agarose gel electrophoresis stained with ethidium bromide (1.30 hr, 70 volts/cm) guided with 2000bp DNA ladder.

Table 3.8: PCR Program for L-selectin gen rs2205849 Genotyping:

Steps	Temperature (ºC)	Time	Cycles number
Initial denaturation	95	5 min.	1
Denaturation	94	30 sec.	30
Annealing	56	30 sec.	
Extension	72	45 sec.	
Final extension	72	4 min.	1
Hold	4	∞	∞

3-3-1-3-3 E-Selectin

Genotyping of E-Selectin Leu554Phe (rs5355) polymorphism was done by SSP- PCR The researcher designed the sequence of primers used for amplification of E-selectin rs5355 was shown in Table 3.9.

Table 3.9: Sequence of primers of E-Selectin polymorphism

Primers	Sequence	Tm	Size	References
F	ATTGCACAATTCCGGGAGGC	60	419	design by a researcher
R	TCTTCCTCATGACCTTTCTTCGT	59		
F	5'-AGCGCTACTTAGTTTTCAGCA-3'	58	490	
R	5'-CTTGGGAAACGTATTGCTGGA -3'	58		

The PCR reaction was performed for final 20 µL reaction volume by using 5 µL of 2X Go Taq® bioneer master mix, 2 µL of 10M of each primer (forward, reverse), 5µL of genomic DNA and the volume was made to 20 µL, with nuclease free water. The reaction was carried out with GeneAmp® PCR System thermocycler as mentioned by the (Tables 3.10 and 3.11).

Table 3.10: PCR Program for E-Selectin rs5355 Genotyping

Steps	Temperature (°C)	Time	Cycle number
Initial denaturation	95	4 min.	1
Denaturation	94	30 sec.	30
Annealing	58	30 sec	
Extension	72	45 sec	
Final extension	72	10 min.	1
Hold	4	∞	∞

Table 3.11: PCR Program for E-Selectin rs5368 Genotyping

Steps	Temperature (°C)	Time	Cycle number
Initial denaturation	95	5 min.	1
Denaturation	94	30 sec.	31
Annealing	58	30 sec	
Extension	72	45 sec	
Final extension	72	10 min.	1
Hold	4	∞	∞

3-3-1-4 DNA Sequencing

The DNA sequence analysis was performed by using Geneious software 11.1.5 and other software (for data sent by the Macrogen / Korea sequence service). A direct sequence analysis was performed using the front and back prefixes of each selected sample. NCBI BLAST was used to detect SNPs and any other change within genomic areas studied.

3-3-2 Clinical Methods

3-3-2-1 Body Mass Index Measurement

Body mass index (BMI) is a measure of relative weight based on height and mass of the individual. Bodyweight is measured in kilograms (kg) and divided by height in square meters. BMI is the commonly accepted indicator of adult lipid rating and is recommended for use with adolescents and children (Nuttall, 2015). The formula below to calculate BMI:

$$BMI = Weight\ (Kg) / [Height\ (m^2)]\ 2$$

The standard weight status classes associated with BMI ranges for adults are shown in the following table [3-12] (Moody and Neave, 2016).

Table 3.12: BMI ranges for adults BMI Weight Status *(*Moody and Neave, 2016)

Underweight	Below 18.5 kg/m²
Normal	18.5 - 24.9 kg/m²
Overweight	25.0 - 29.9 kg/m²
Obese	≥ 30.0 kg/m²
Morbidly obese	40 or more

3-3-2-2 Abdominal Circumference

Abdominal circumference was measured at the centre point between the iliac crest and the last rib, using measuring tape (Ma *et al.* 2007).

3-3-2-3 Biochemical tests

- **Fasting Blood Glucose (FBG) Test**

Fasting blood glucose was measured for each subject in the study by enzymatic colorimetric method, using spinreact kit (Ref:1001191) according to the manufactures' instructions.

- **Glycosylated HbA1c Test**

This test was performed using the Boditech Med HbA1c kit (ref: CFPC-38), with fluorescence immunoassay technology for all the study subject as mentioned in the instruction manual.

- **Lipid profile**

Lipid profile for each subject in this study was obtained by performing the following tests:

- **Serum Total Cholesterol Test**

This test was performed using the Spinreact cholesterol kit (enzymatic colorimetric method, Ref: MD41021) as described in the user manual.

❖ **Serum Triglyceride Test**

Triglyceride test glucose was measured for each subject in the study using the enzymatic reaction of GPO (glycerol phosphate oxidase) Biolabo kit (Ref: 87319).

❖ **Serum High Density Lipoprotein (HDL)**

High Density Lipoprotein Cholesterol for each subject in this study was obtained by using Spinreact cholesterol kit (Precipitant Reagent, Ref: 1001095) according to the manufactures' instructions.

❖ **Serum Low Density Lipoprotein (LDL)**

Serum Low Density Lipoprotein Cholesterol can be calculated mathematically from the total cholesterol, triglycerides and the HDL – cholesterol concentration in individual with triglyceride values < 425mg/dl (Friedwald *et al.*,1972).

LDL = Total cholesterol – (Triglycerides/5) – HDL cholesterol.

❖ **Serum Very Low Density Lipoprotein (VLDL)**

Serum Very Low Density Lipoprotein Cholesterol concentration was calculated by: using the following formula:

S. VLDL= Triglyceride /5 in mg/dl.

➢ **Atherogenic Index of Plasma (AIP)**

The Atherogenic index of plasma was calculated by the following equation:

Atherogenic Index = Total cholesterol/ HDL

➢ **Insulin Hormone Test**

This test was performed using Demeditec ELISA (solid phase sandwich principle), kit (Ref:DE2935) as mentioned in the instruction.

- **Homeostatic Model Assessment 2 (HOMA2)**

It is a process for quantifying insulin resistance and beta-cell function. It was named HOMA by Matthews *et al* (1985). The equation for insulin resistance include the relation between glucose and insulin as following:

HOMA-IR = (Glucose mg/dl× InsulinμlU/ml)/405

3-4 Copper Oxide Nanoparticles

3-4-1 CuO Nano Particles Preparation by Hydrothermal Method:

CuO nanoparticles prepared by. Equimolar amount (0.1M) of ($CuCl_2.2H_2O$) and hexamine ($C_6H_{12}N_4$). Dissolved in deionized water and stirred in magnetic stirrer to get a homogenous solution and transferred into 50 ml Teflon –lined stainless steel vessels (autoclave). The vessels were tightly sealed and heated to 120ºC for 5 hours, they were, then slowly cooled at room temp. Precipitated powders were washed several times using deionized water and absolute ethanol. The precipitates were sonicated for 5 min prior to filtering, annealed at 450 ºC for 1 hour, cooled to room temperature.

3-4-2 Characterization Measurements (Morphological and Structural) of Copper Oxide Nanoparticles.

Structural and optical properties to diagnose CuO-NPs have been investigated in this study by various devices to identify the synthesized CuO-NPs; carrying out some measurements as shown in the following techniques:

3-4-2-1 UV-Visible Absorption Spectroscopy

Investigating the optical Absorbance spectra of the CuO-NPs solution was measured by a double-beam UV-VIS spectrophotometer model

(Shimadzu-1). Absorption spectroscopy measurements were performed by suspending the nanoparticles in distilled water and the samples were sonicated for 15 minutes to ensure even the dispersion of particles before collecting the spectra. The nanoparticle suspensions were transferred to a quartz cuvette cell with 1 cm optical path for measuring the optical properties at room temperature. The wavelength range was recorded within the spectral from (295-1100 nm). Each spectrum was analyzed to determine the extinction maximum. For optical properties, absorption coefficient, transmittance measurements, and the determination were calculated and described in details (Olson *et al.*, 2015). The measurement of UV-VIS spectral analysis was performed in the Central Service Laboratory Ibn al-Haytham. College of Education for Pure Sciences (Ibn al-Haytham) / University of Baghdad

3-4-2-2 X-Ray Diffraction Spectrum (XRD) Technique

The X-Ray diffraction device type (XRD, 6000-Shimadzu X-ray Diffractmeter) was measured to investigate the crystal structure (crystal phases and to determine the crystallite size of the phase of copper oxide nanoparticles (Tsuji *et al*., 2012). X-ray powder diffraction studies were performed by suspending the nanoparticles and applying the suspension dropwise to the XRD well plate. The process was continued until a flat and even a layer of particles was achieved on the surface of the well plate.

The solution was dropped and dried on a glass substrate and was done utilized (Philips PW). The scan angle (2θ) was varied from (20º-70º) degrees at (10º min^{-1}). Diffraction signal intensity was recorded, and processed using Diffrac Plus. Phases present in the samples were identified with the search match facility available with high score software. Central Service Laboratory Ibn Al-Haytham. College of Education for Pure Sciences (Ibn al-Haytham) / University of Baghdad.

3-4-2-3 Transmission Electron Microscopy (TEM)

The transmission electron microscopy used to show the shape of CuO NPS, model CM10 PHILIPS, working voltage 60KV. The measurements were investigated in the Ministry of Science and Technology, Baghdad, Iraq.

3-5 Bacteriological

3-5-1 Collection of Samples

(50) bacterial isolates from Patients with a diabetic foot ulcer who attended the (Educational Baqubah Hospital /Diyala), and at the Specialized centre of Endocrinology and Diabetes (Baghdad/ AL-Russafa Health Directorate) Iraq. Samples identified as clinical surveys were collected according to the clinical provisions determined between February 2018 and December 2018.

3-5-2 Standard Strain

Standard isolates were obtained from the Ministry of Science and Technology / Microbiology Division and equipped with diagnostic kits.

Strain	Genetics	Source
Staphylococcusc aureus	Wild type	ATCC 6538
Pseudomnas aeruginosa	Wild type	ATCC 27853

3-5-3: Sterilization

Sterilized, glass tools that need to dry by the oven at t 180 degrees for 2 hours, culture media and some solutions were sterilized by autoclave at 121°C, 15 pound/inch2 for 15 minutes.

3-5-4 Solutions and Indicator

3-5-4-1 Solutions

- **Sodium Hydroxide Solution**

Different solutions and buffers were used and prepared in this study and were sterilized by autoclave. The pH of these solutions was adjusted by adding few drop either from 0.1 N of NaOH or 0.1 N HCl.

- **Physiological Solution (Normal Saline Solution 0.85%):**

It is prepared by dissolving 0.85gm of NaCl in 100 ml distilled water and it is sterilized by autoclave at 121°C for15 minutes (Barros *et al.*, 2012).

- **McFarland Standard Solution**

  Thr turbidity standard solution was prepared by adding 0.5 ml of barium chloride (1.175gm W/V barium chloride to one liter distilled water) to 99.5 ml of sulfuric acid (1%). Then the solution was transferred to screw capped tube, sealed tightly, and stored in dark at room temperature (Baron and Finegold, 1990).

3-5-4-2 Indicators

- **Catalase Reagent**

Hydrogen peroxide(H_2O_2) 3% was prepared from its stock solution in a dark bottle and it was used in catalase test (Harley and Prescott, 2007).

- **Oxidase Reagent**

In order to prepare this reagent, 0.1gm tetramethyl para phenylene diamine dihydrochloride was dissolved in 10 ml distilled water and instantly used (Harley and Prescott, 2007).

3-5-5 Preparation of Cultural Media

3-5-5-1 Ready-Made Media

All media were prepared listed below according to the manufacturing company instructions, pH was adjusted to (7.0) and they were brought to boil in water bath to dissolve all constituents completely, and then sterilized by autoclave at 121ºC for 15 min at 15 pounds per Square Inch, then dispensed into sterile Petri dishes or tubes as required and incubated for 24 hours at 37°C to ensure the sterility then stored at 4°C until use.

These media include the following:

- Brain Heart Infusion (BHI) agar and broth;
- MacConkey agar
- Mueller Hinton agar and broth
- Nutrient agar and broth
- Simmon's citrate agar
- Tryptic soya (TS) agar and broth
- Mannitol Salt Agar (MSA)

3-5-5-2 Laboratory Prepared Media

- **Blood Agar:**

Blood agar was prepared by adding 40gm of blood agar base to one liter of distilled water, then heated until boiling and sterilized by autoclaving at 121˚C, 15 pound/inch^2 for 15 min. After cooling to 45˚C it was supplemented with 5% of fresh human blood.

- **Cetrimide Agar**

Prepare to dissolve 45.3 gm of medium in 1000 ml of distilled water over 10 ml of glycerol and autoclaved at 121°C, 15 pounds/inch2 for 15 minutes, using this medium in the growth of Pseudomonas isolates and select a selective medium (Hashim,2013).

- **Peptone Water Medium:**

By adding 8 grams of peptone powder to one liter of distilled water, then dispensed into test tubes (5 ml), and autoclaved at 121˚C, 15 pound/inch^2 for 15 minutes. This medium was used to detect the presence of indole (can be detected by the addition of Kovacs' reagent) after hydrolyzation of tryptophan by bacteria that have tryptophanase enzyme.

- **Congo-red Agar:**

The CRA medium is composed of brain heart infusion broth (BHIB) (37 g/L), sucrose (50 g/L), and agar agar (15g/L) dissolved in 900 ml of

deionized water. Congo red stain was prepared by mixing (0.8 g/L) with 100 ml of deionized water vigorously. Thus, the concentrated aqueous solution was sterilized by autoclaving it separately from the other medium constituents. After cooling, the dye solution and medium reached 55°C. It was mixed and poured in Petri-dishes, and was left to solidify and use to detect biofilm formations (Freeman *et al*., 2010).

3-5-6 Identification of bacterial isolates

3-5-6-1 Biochemical tests

- **Oxidase Test**

Single isolated colony from overnight culture was transferred to of filter paper by wooden stick applicator, then 2-3 drops of oxidase reagent were added to the colony. The change in color to dark purple within 20-30 seconds indicates a positive result (Harley and Prescott, 2007).

- **Catalase Test**

This test was carried out by transferring one colony from overnight bacterial growth culture by a sterile wooden stick applicator and placed on a glass slide. Few drops of 3% hydrogen peroxide were added on the colony, mixed well and observed appearance of gas bubbles indicates a positive result (Brown, 2005).

- **Indole test**

Indole test was performed by inoculating peptone water medium with a loopful bacterial culture, and incubated at 37˚C for 48hrs. 0.5ml of Kovac's reagent was then added to the tube, the formation of red-ring between the medium and reagents after gentle shaking indicated a positive result (Harley and Prescott, 2007).

3-5-6-2 Morphological Examination

Identification *S. aureus* and *P. aeruginosa*

Pure colonies are selected on nutrient agar by using the sterile loop and sub-stabilized on the agar salt agar surface, which contains a high concentration of salt (NaCl 7%) and phenol red as an indicator. Then, plates are incubated at 37 ° C for 18 to 24 hours. Changes in colour from pink to yellow indicate positive results. Although non-fermented manitol bacteria produce small red or red colonies with no change in colour to the negative results indicated (Bachoon and Dustman, 2008). Changes in colour from transparent colour to green colour also indicate a positive result for *P. aeruginosa*. Bacterial isolates were identified by their ability to grow in medium Pseudomonas agar, medium cetrimide agar.

3-5-7 Diagnostic kits

Bacterial Identification using VITEK-2 System

The Vitek-2 compact system was used as an automated microbiology analyzer system utilizing growth-based technology for the identification of the bacteria in clinical samples to confirm the biochemical test. The system accommodates the colorimetric reagent card that was incubated and interpreted automatically using associated hardware and software advances. The samples were performed according to the manufacturer's instructions (BioMerieux-France).

VITEK2 system was also used for the diagnosis of the isolated bacteria. Stock culture strains were subcultured onto MacConkey agar plates. Bacterial suspensions matching to MacFarland 0.5 standard in 0.45% sterile sodium chloride solution was used. The turbidity of the bacterial suspensions was adjusted with a densitometer. The VITEK 2 ID-GN cards, AST-No. 12 cards, and bacterial suspension were manually loaded into the VITEK 2 system. Each test card was automatically filled

with a bacterial suspension, sealed, and incubated for 6 h. through this period, the cards were read by kinetic fluorescence measurement every 15 min. The VITEK 2 system software first analyzed the data and then reported the results automatically (Ling *et al.,* 2003).

3-5-8 Maintenance of bacterial isolates

- **Short Term Storage**

Short term maintenance for period ranged from one to three months was done by culturing the bacteria on nutrient agar slant using screw capped tubes and incubated at 37°C for 24 hr, then stored at 4°C (Harley and Prescott, 2007).

- **Long Term Storage**

To maintain bacterial isolates for a long period time (up three months), the process was carried out by culturing the isolated bacteria on media containing 20% glycerol at low temperature. The medium was prepared by adding 2ml of glycerol to 8ml of brain heart infusion broth, dispensed into a small screw-capped bottle and sterilized by autoclaving. After cooling, the tubes were inoculated by one pure isolated colony and were incubated at 37 °C for 24 hrs. The tubes were stored in deep freezing at -20°C (Vandepitte *et al*., 2003).

3-5-9 Phenotypic Detection

3-5-9-1 Selection of Antibiotics

The antibiotic was selected on the base of their widely prescription by the physician for the positive and negative bacterial infections based on Clinical and Laboratory Standards Institute (CLSI) guidelines using (Ceftriaxone): Antibiotic for foot ulcers used as a treatment in the Iraqi hospital.

3-5-9-2 Disc Diffusion Method

Antimicrobial susceptibility testing for all studied isolates was carried out using clinical laboratories (in vitro) (Kirby-Bauer). Disk diffusion method against antibiotics was followed as described by WHO (2003).

The drug concentration decreases the increase of the distances from the disc. At the point where the concentration of a drug is at a defined distance from the disc, the disc becomes unable to inhibit the growth of the test organism, a zone of inhibition is formed according. This test allows several antimicrobial agents to be tested on a single plate.

The most effective antibiotics for each bacterial isolates were determined. by CLSI (2013) criteria consider the following points:

1.Bacterial suspension was prepared by picking 1-2 pure isolated colonies grown on MacConkey agar plate and was suspended in 5 ml normal saline and vortexed to mix well. The turbidity was adjusted to approximately (1.5×108) CFU/ml by comparing the suspension with 0.5 McFarland turbidity standard tube.

2.A sterile cotton-tipped swab was used to obtain inoculums from the bacterial suspension. These inoculums were streaked on a Mueller-Hinton agar medium plate. The plate was rotated at 60° and was lawned in the same manner. The plate was then rotated another 60° and was swabbed again, and left about 10 minutes to dry and absorb the bacterial suspension.

3.The antibiotics were distributed on a plate surface medium, maximum of two discs per plate to avoid the overlapping of zones of inhibition by flamed sterile forceps. Then, they were incubated at 37°C for 24hrs under aerobic conditions. Two replicas were done for each isolate.

4.The results were read by using a metric ruler that measured the inhibition zone diameter (mm).

3-5-10 Biofilm Formation Assays

using two methods to detect and evaluate production of biofilm formation. with some modifications:

3-5-10-1 Culturing on Congo Red Agar plates (CRA) (qualitative biofilm production assay).

The morphology of the colonies and their phenotypic changes were studied using CRA medium as previously described by Freeman *et al*. (2010). The colonies were streaked on Congo red agar plates and were incubated aerobically for 24 hours at 37°C to obtain single bacterial colonies.

The CRA-positive isolates appeared as black colonies with a rough and dry crystalline consistency on CRA. They could be considered as strong evidence for the ability to from biofilm. CRA-negative isolates, on the other hand, remained red smooth colonies with occasional darkening at the center. An indeterminate result was indicated by the darkening of the colonies, but with the absence of a dry crystalline colonial morphology.

3-5-10-2 Microtiter Plates Method (MTP) (quantitative biofilm production assay)

This analysis was determined by the microtiter plate method and considered by Christensen *et al*., (1985). It was performed by a spectrophotometric method, which measures the total biofilm biomass (bacterial cells and extracellular matrix). The Biofilm formation assays and the standard test for detection of biofilm formation, and as follows (Eftekhar and Speert, 2009; Sharma, 2013) with some modifications: -

1) Stating briefly; each isolate was grown from fresh brain heart infusion agar plates. It was inoculated in 10 ml of a trypticase soy broth (TSB)

medium supplemented with 1% glucose and was incubated for 24 hrs at 37°C.

2) The overnight growth was diluted in a fresh medium with a ratio of 1:90 in TSB-1 % glucose.

3) 200 µl of diluted cell suspension was filled in each well of 96 a sterile well polystyrene flat-bottom microtiter plates (3 wells for each isolates). Besides, 200 µl aliquots of only TSB + 1% glucose were dispensed into each of eight wells of column 12 of microtiter plate to serve as a control (to check non-specific binding and sterility of media). The plates were covered with a lid and were incubated aerobically under constant conditions for 24 hrs at 37ºC.

4) After incubation, the contents of each well were removed and the 96 wells polystyrene were carefully washed. The wells were rinsed three times with phosphate buffer saline (PBS) (pH 7.2) to remove non-adherent cells free-floating (planktonic cells).

5) The attached bacteria in 96 well polystyrene microtiter plates were fixed with 200 mL of 99% methanol per well for 15 minutes. The plates were decanted and allowed to dry for 30 min at room temperature.

6) Bacterial cells which were adhered to the wells were stained with a crystal violet solution (1% v⁄v) and added to each well that was stained for 30 minutes at room temperature. Then, the plates were washed successively with D.W.

7) The microtiter plates were then inverted and tapped forcefully on a filter paper to remove any excess liquid. The microtiter plates were air dried for approximately 1hours.

8) The dye bound to the adherent cells was resolublized with 200 µl of 95% ethanol for 20 min, and the cells were fixed to the attached bacteria.

9) Optical Density (OD) of each well was measured at 630 nm and determined using ELISA reader. In addition, the turbidity was also measured at 570 and 600 nm prior to the staining of biofilm mass to compensate for the growth differences in the isolates.

10) Each assay was performed in triplicate and the average optical density was considered. The experiment was repeated three times.

11) According to their optical densities, the adherence capability of each bacterial cell was classified into the following four categories: non-adherent (−), weakly adherent (+), moderate adherence (++), and strongly adherent (+++) cells based on the absorbance of the bacterial film using the advance outlined by Stepanovi *et al*. (2000).

The optical density cut-off value (ODc) was considered as three standard deviations (SD) above the mean and OD the negative control, as shown in the following principle:

ODc = average OD of negative control + (3 × SD of negative control).

3-5-11 Antibacterial Activity Test of Copper Oxide Nanoparticles:

3-5-11-1 Determination of Minimum Inhibitory Concentration (MIC) of Copper Oxide (CuO) Nanoparticles by Tube Method (TM) (Chao *et al*., 2012).

The minimum inhibitory concentration (MIC) of CuO-NPs was determined by a method recommended in CLSI (2013) with some modifications as stated below:

The study isolates were cultured in a nutrient agar for overnight, and the sterile tubes were used; and each tube contains 9 ml of bacterial suspension in (TSB) with 1% glucose of approximately 1.5×10^8 cfu/ml; Taking one ml from each of CuO-NPs concentrations (0,25,50,75,100,500,1000 and 2000) µg/ml was taken after introducing it to the ultrasound bath apparatus to

ensure that the nanoparticles did not agglomerate. Then it was mixed with 9 ml of bacterial growth, and incubated aerobically in a shaker incubator for 24 hrs at 37°C. The bacterial growth in each tube was measured with spectrophotometer by reading optical density at 630 nm wavelength. The mean values of inhibition were calculated from the triple reading in each test. A broth tube with only bacteria isolates without CuO-NPs was used as control. and MIC was read as the lowest concentration of copper oxide nanoparticles at which there was a tube without any visible growth of the bacterial cells (Amiri *et al*. 2017).

3-5-11-2 Agar Well-Diffusion Method (ADM)

The inhibitory effects of the synthesized CuO NPs were determined by following a modified Kirby-Bauer disc diffusion method as briefed below: (Amiri *et al*. 2017).

A lawn of bacterial culture was organized by spreading 100 µl of bacterial suspension, which was compared to Mcfarland solution to get the right concentration cell density; 1.5 $\times 10^8$ cfu/ml of each isolate on a solid Muller Hinton agar; The plates were allowed to stand for 15 minutes to allow culture absorption; then the 6 mm diameter size wells were punched into the agar with the head of a sterile cork borer and using micropipette, the wells in each plate were loaded with 100 µl of different concentrations, (0,25,50,75,100,500,1000 and 2000) µg/ml of the suspension containing CuO-NPs by suspending the nanoparticles in nanopure water. The samples were sonicated for 30 minutes to ensure even dispersion. They did not agglomerate particles within the suspension and they were finally incubated for 24 hrs at 37°C. After the incubation period, the size of the inhibition zone around each well was measured. The average of the inhibition zone around the well contained less than the CuO-NPs concentration and the lowest inhibitory concentration.

3-5-12 Extraction of the Bacterial DNA by Molecular Methods.

Genomic DNA is extracted from bacteria culture from isolates of clinical specimens. Depending on traditional methods, it was purified from the bacterial cells using a DNA Mini Kit supplemented by the Genaid Kite, Korea). DNA chromosomes obtained as models for all PCR experiments were used. Gram-positive and Gram-negative bacteria protocol in used Cat. No**. GBB100**.

All subsequent steps for the PCR test described in section 3.3.1 were followed for DNA purity, Gel Electrophoresis, preparation of primers and the following table 3.13 showing Primer used to amplify Gene Biofilm Used in this Study.

Table 3.13: Primer used to amplify Biofilm Gene Used in this Study

Primer Name	Primer Sequence Oligo sequence F (5' → 3')	Primer Sequence Oligo sequence R (5' → 3')	Product size (bp)	Reference
S. aureus				**design by a researcher**
icaA	CAGAGGTAAAGCCAACGCAC	TTCCCTCTGTCTGGGCTTGA	870	
icaD	CCCAACGCTAAAATCATCGCTAAA	GATTCTCTCCCTCTCTGCCATT	390	
P. aeruginosa				
cupA	AGCGATAGAGGTTGGTGTC	TTCGATGATCGCCTGTTG	590	

3-5-13 Detection of some Adhesion factors genes

Staphylococcus spp. The isolates were examined for the presence of (*icaA*) (*icaD*) genes; these genes are responsible for producing the *S.areuse* adhesion factor. PCR is used to amplify (*icaA*) and (*icaD*) genes using specific primers. The reaction volume of the polymerase chain reaction is 20 µl reaction mixture containing 5µl DNA template. Optimal conditions are identified in the methodology. Several experiments have proved the purpose of reaching these conditions. It has been found that the best size of

the DNA template is 5 µl. 2µl of each primer forward and primer interface of (*icaA*), (*icaD*) with a concentration of 10 pmol / µl of each separated by a PCR mono-plex. In the reaction steps of the polymerase chain reaction, temperatures 94 ° C (*icaA*), 95 °C (*icaD*) are the beginning of an initial smoothing reaction for one session. When the product is electrically discharged, the best glow under ultraviolet light is a product reaction that contains a temperature of 55 ° C for *icaA* and 59 ° C for *icaD*, while other temperatures are not enough to obtain the desired glow. Therefore, these temperatures are supported for best results in many experiments. The conditions for amplification of the gene (*icaA*) as shown in Table (3.14) and the amplification conditions of the gene (*icaD*) as shown in Table (3.15).

The PCR products of genes (*icaA*) and (*icaD*) are analyzed by electrophoresis on a 2% agarose gel prepared, using the 2000-bp DNA ladder.

Table 3.14: The thermocycling conditions program (*icaA*) gene.

Steps	Temperature (°C)	Time	Cycles number
Initial denaturation	95	6 min.	1
Denaturation	94	30 sec.	32
Annealing	56	30 sec.	
Extension	72	45 sec.	
Final extension	72	4 min.	1
Hold	4	∞	∞

Table 3.15 The thermocycling conditions program (*icaD*) gene.

Steps	Temperature (°C)	Time	Cycles number
Initial denaturation	95	2 min.	1
Denaturation	94	30 sec.	30
Annealing	56	30 sec.	
Extension	72	45 sec.	
Final extension	72	4 min.	1
Hold	4	∞	∞

On PCR completion, the product is electrophoresed in 1% agarose. For electrolytes, 5 µl of PCR product is added in a 1% well of agarose gel. Then, 2µl of 2000 bp DNA ladder is transferred with dye loading in the first

well of the agarose gel to serve as a marker. The electrode is performed at 70 volts for approximately 120 minutes. The gel is then removed and the bands are imaged on the gel under the Tran UV light.

Detection of the gene *cup A*

P. aeruginosa the isolates are examined for the presence of a *cupA*. These genes are responsible for producing the *P. aeruginosa* adhesion factor. PCR is used to amplify gene (*cupA*) using specific primers. The reaction volume of the polymerase chain reaction is the 20 µL by using 5 µL of 2X Go Taq® bioneer master mix, 2 µL of 10M of each primer (forward, reverse), 5µL of genomic DNA and the volume was made to 20 µL, with nuclease free water. The amplification conditions of the gene (*cupA*) are shown in table 3.16. PCR products from the gene (*cupA*) are analyzed by electrophoresis on the 1% agarose gel, guided with a 2000bp step DNA ladder.

Table 3.16: The thermocycling conditions program (*cupA*) gene.

Steps	Temperature (°C)	Time	Cycles number
Initial denaturation	95	2 min.	1
Denaturation	94	1 min.	30
Annealing	58	1 min.	
Extension	72	2 min.	
Final extension	72	10 min.	1
Hold	4	∞	∞

3-6 Statistical Analysis

The Statistical Package for the Social Sciences - SPSS V.22 (2012) program was used to test the effect of clinical factors on the study parameters. Chi-square test was used to significant compare between percentage and Least Significant Difference –LSD test (ANOVA) or t-test was used to significant compare between means. An estimate of correlation coefficient between variables in this study.

WINPEPI computer program (version 11.63) was used to estimate the statistical significance of the p values that was calculated with Fisher's exact test as well as the ODD Ratio that was assessed by a special $\chi 2$ formula. Hardy-Weinberg Equilibrium was tested by chi-squared test that was done using OEGE - Online Encyclopedia for Genetic Epidemiology studies (Rodriguez *et al.,* 2009).

4. Results and Discussion

4.1 General description

This study conducted on 100 Iraqi subjects, aged range from 43 to 94 and divided to 50 patients (30 males and 20 female) and 50 health individuals.

Patients were diagnosed with T2DM having foot ulcers, who were periodic visit the hospital of Baghdad and Baqubah, from February to December 2018. The patients had been instructed about the purpose of the study and interested volunteers have been enrolled along with 50 healthy subjects (27 males and 23 female) that were taken as a control group. Diabetic patients were selected according to the World Health Organization 2016 guideline. All subjects were on fasting for at least nine hrs. when blood samples were collected. Venous blood samples (8ml) were collected from each subject and divided into two aliquots: one for the biochemical tests and the other aliquot was kept at -20°C for the genotyping analysis.

Cotton swabs were used for ulcer bacterial infection that identified by traditional and molecular analysis.

4.2 Genetic Study

4.2.1 Genomic DNA Concentration and purity in studied groups

Genomic DNA was extracted from whole blood samples of both patient and control groups using a DNA extraction kit (gSYNC™ DNA Extraction Kit, Geneaid).

Figure 4.1: Extracted Genomic DNA Bands from blood of patients detected on 1% agarose gel 60 volt/cm for 20 min.

The nucleic acid concentrations as well as purity ratios, were automatically calculated by spectrophotometer, results showed DNA purity (1.8 - 2).

4.2.2 Detection of L-Selectin rs2205849 Polymorphism Sequencing

Sixty-five DNA samples (25 healthy control and 40 patients) were sequenced. The amplification of DNA was amplified by PCR and gSYNC™, DNA Extraction Kit product produced by Geneaid and using a set of primers dedicated to the promotor of the gene L-Selectin previously designed by Chen *et al.* (2007). The results for the amplified DNA fragment are shown in Figure 4.2.

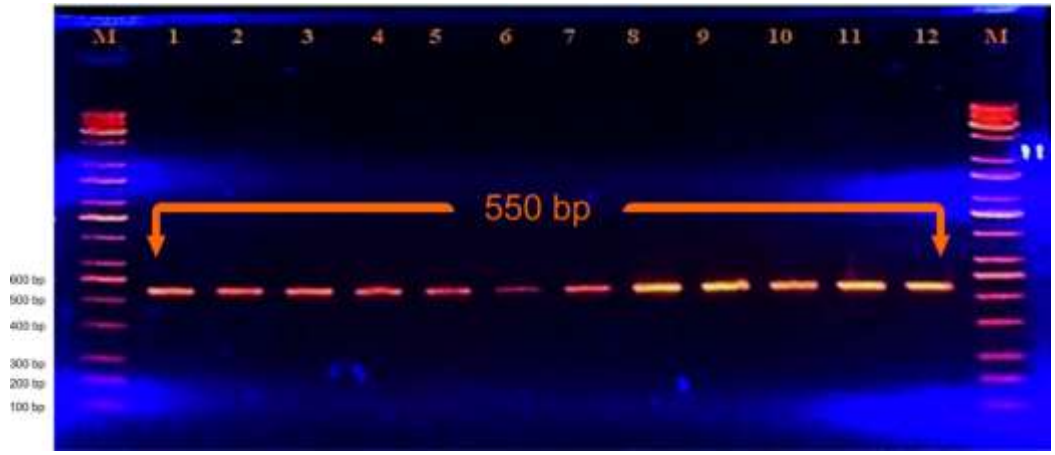


Figure 4-2 Electrophoresis of L-Selectin rs2205849 A >G Gene amplification products on 1% agarose gel and 70 volts for 1:30 h using DNA Ladder (100bp-2000bp). This is shown in well M and starts from 100 bp, and rest lane 1-12 represents gene bands of 550 bp

Table 4.1: Sequencing ID, Score, and Identities for L-Selectin Gene Promoter rs2205849

Accession	Identities	Score	Expect	Range
ID: NG_016132.1	408/411(99%)	741 bits(401)	0	4389 to 4798

Table 4.1 showed the expect for the nucleotide sequence for L-Selectin gene promoter rs2205849 which range from 4389 to 4798 nucleotide sequence of the Homo Sapiens L-Selectin gene was less than zero this

result indicated indicating strong significant (identical) (99%) match between the query sequence and reference sequence of NCBI (ID:NG_016132.1), with score 741 bits which also indicated great degree of similarity between both sequences.

Table4.2: Expected Frequencies Genotypes of L-Selectin rs2205849 A >G Using Hardy-Weinberg Equation

Groups			AA	AG	GG	A	G	X^2
Patients Genotypes	Observed	No.	27	9	4	63	17	4.3
		%	67.5	22.5	10	0.787	0.212	
	Expected	No.	24.81	13.39	1.81	Not detected		
		%	62.02	33.47	4.52			
Control Genotypes	Observed	No.	14	8	3	36	14	1.06
		%	56	32	12	72	28	
	Expected	No.	12.96	10.08	1.96	Not detected		
		%	51.84	40.32	7.84			

The expected frequencies of genotypes showed no significant differences ($X^2 < 3.84$) between observed and expected frequencies for both T2DM patients and the control group

The observed genotype frequencies in both homozygous genotype AA and GG patients and control groups were higher than those predicted by the Hardy-Weinberg Equilibrium H.W.E theory (Table-4.2) patients revealed significant levels of $\chi 2$ value; they were 4.3. While control not significant (1.06) so it not deviated from H.W.E these values for patients were not agree with H.W.E. The departure from H.W.E. may indicate that this locus may undergo to evolutionary selection in Iraqi population and the diabetic causes the departure.

The results of the current study were consistent with the results of the local study on diabetic patients, conducted from Saeed and Al-mohaidi (2017) showed a deviation from H.W.E which justified their results because of that sample belongs to partially closed population as Iraqi Arab population because consanguinity as the same ethnicity group showed high percentage in our population for culturing thought, as well as they are subjected to environmental variations. Environmental changes, with the

view point of natural selection, some genotypes are favored by a population, while they are not by others (Turnpenny, 2012). According to this fact, present allele frequencies of polymorphic systems have different frequencies in different populations even they are from the same ethnicity, differences by race in incidence for inflammation-diseases suggest that there may be underlying ethnic differences in inflammatory pathway genes (Reich *et. al.*, 2007).

Table4.3: Genotypes and alleles frequency of the rs2205849 A>G L-selectin polymorphism P values calculated with Fisher's exact test

Group Genotypes	Study groups		Odds Ratio	CI 95%	P value Fisher –test	preventive or etiological fraction
	Patient	Control				
A/A	27(67.5)	14(56)	1.63	0.57 to 4.64	0.364	26.10%
A/G	9(22.5)	8(32)	0.62	0.20 to 1.97	0.325	12.30%
G/G	4(10)	3(12)	0.81	0.15 to 4.75	0.849	2.20%
Alleles						
A n (%)	63(78.75)	36(72)	1.44	0.62 to 3.28	0.347	24.10%
G n (%)	17(21.25)	14(28)	0.69	0.30 to 1.60	0.347	8.60%

$p \geq 0.05$ is not significant

Table (4.3) showed the genotyping analysis according to sequencing for position 4487 rs2205849 A>G in gene L-Selectin. Genotype AA showed high frequency ratio in patient groups compared with control (67.5 and 56% respectively) with etiological risk factor 26.10% but non significant according to fisher's probability (0.364).

At the same time other homozygous genotype GG show low non-significant preventive risk in patient's comparing to control (10% and 12%) respectively, the same result recorded in heterozygous genotype (GA) which shows low significant 22.5% preventive association with disease in patients comparing to control (32%), so that it may refer to negative association between both GG, GA with disease.

On the other hand, high distribution of allele A in patients (78.75%) with etiological fraction 1.44 while allele G shows the high distribution in control 28% with preventive fraction 0.69 as a protective allele from the disease. This result consists of the result of both genotype GA and GG. From these result we can concluded that allele G may play a preventive role and individual with it not susceptible to diabetes.

The result of rs2205849 polymorphism may affect the variations of L-selectin. Gene levels of L-selectin AA genotype were significant more than (AG and GG genotypes) in patients with DMT2 compared with control. A local study showed heterozygous genotype possible risk factor in patients with DMT2 (Saeed and Al-mohaidi, 2017).

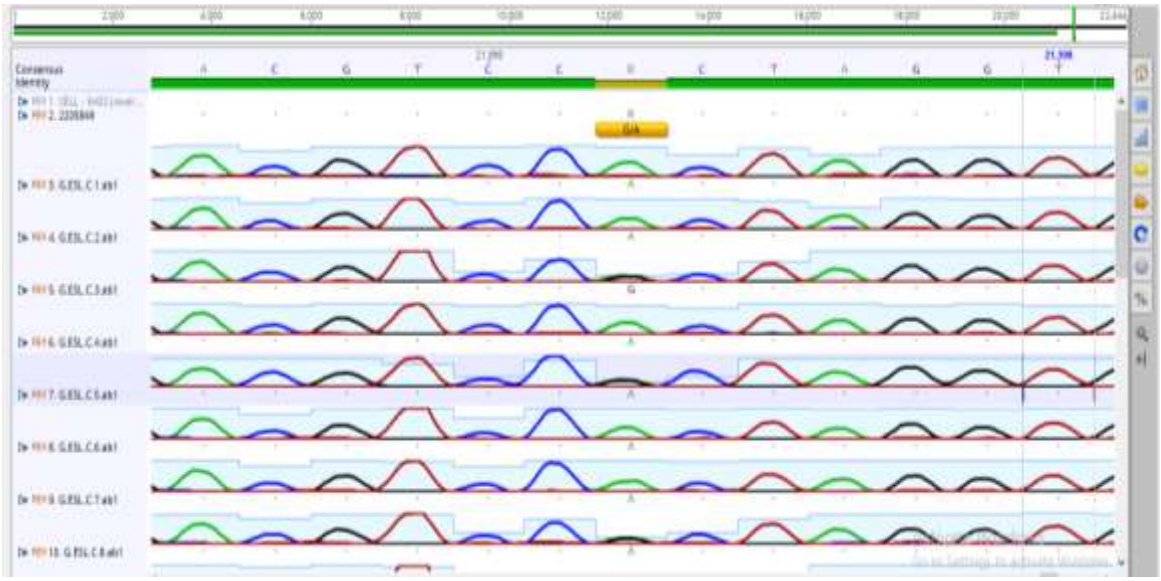


Figure 4.3: rs2205849 polymorphisms by Geneious software and genotypes showed (AA, AG, GG) in the samples study, R: Heterozygous

The sequence alignment revealed in figure 4.3 a transition A >G in some samples at the genomic location 21392 shown in figure (4.3) which represents L-Selectin gene promoter (rs2205849) polymorphism, however sequencing of genotypes at genomic location 21390 was AA, AG, and GG.

4.2.3 E-Selectin

DNA sequence was confirmed by blasting with NCBI (Table 4.4) The score for position targeted E-Selectin segment was 590 bits which indicates the great degree of similarity between both sequence, while the identities were 99.39 % due to the high polymorphic content of the target region, also the expect was less than zero indicating a strong match between the target sequence and the reference sequence of NCBI.

Two sites of the E-Selectin gene were amplified based on two types of SNPs, rs5355 and rs5368 respectively, that selected for the current study using novel set of primers designed by the researcher especially for this locus in order to obtain the required DNA sequence around the SNPs, the resulted fragment (419 and 490 bp) is shown in Figures (4.4 and 4.6).

4.1.3.1 Detection of E-Selectin gene rs5355 Polymorphism Sequencing

The nucleotide sequence of the E-Selectin rs5355 region blast was indicated in table (4.4). The score was 590 bits which indicates the great degree of similarity between both sequences as well as the expected result that was less than zero indicating a strong significant (identical) match between the query sequence and the with reference sequence of NCBI.

Table 4.4: Sequencing ID, Score, and Identities for the first targeted segment of the E-Selectin gene

Accession	Identities	Score	Expect	Range
NG_012124.1	99.39%	590 bits(319)	1e-171	19 to 343

Tables 4.5 and 4.6 show the information of the five SNPs detected in amplified region of the E-Selectin. The results indicated that the amplified fragment was extended from the end of the intron 8 of the E-Selectin gene along the region of the exon 11 of the E-Selectin gene.

Table 4.5: Position and Allele Information of the 5 SNPs from E-Selectin Gene Based on NCBI Assembly Data

SNP	RefSeqGene	Gene (ID)	SNP to RefSeqGene	Position
rs5355 (G>A)	NG_012124.1	SELE (6401)	Fwd	12351
rs5368 (C>T)	NG_012124.1	SELE (6401)	Fwd	11275
rs751151130 (G>A)	NG_012124.1	SELE (6401)	Fwd	11276
rs5367 (C>T)	NG_012124.1	SELE (6401)	Rev	11174
(T>Del)	NG_012124.1	SELE (6401)	Fwd	11145

RefSeqGene: genomic reference sequences, Gene (ID) 6401: species-specific gene identifier, Fwd: forward, Rev: reverse.

Table 4.6: Location, Type, and Effect, Frequency of the SNPs in the Targeted Region of the E-Selectin Gene

SNP	location	Type	Effect
rs5355 (G>A)	Exon 11	Substitution	L (Leu) > F (Phe)
rs5368 (C>T)	Exon 9	Substitution	H (His) > Y (Tyr)
rs751151130 (G>A)	Exon 9	Substitution	H (His) > R (Arg)
rs5367 (C>T)	Splice region before Exon 9	Substitution	No amino acid change
(T>Del)	Exon 9	Frameshift	H (His) > P (Pro)

The E-Selectin rs5355 (G>A) SNPs were amplified using a novel set of primers designed by the researcher especially for this locus in order to obtain the required DNA sequence around the SNP, the resulted fragment (419 bp) is shown in Figures (4-4).

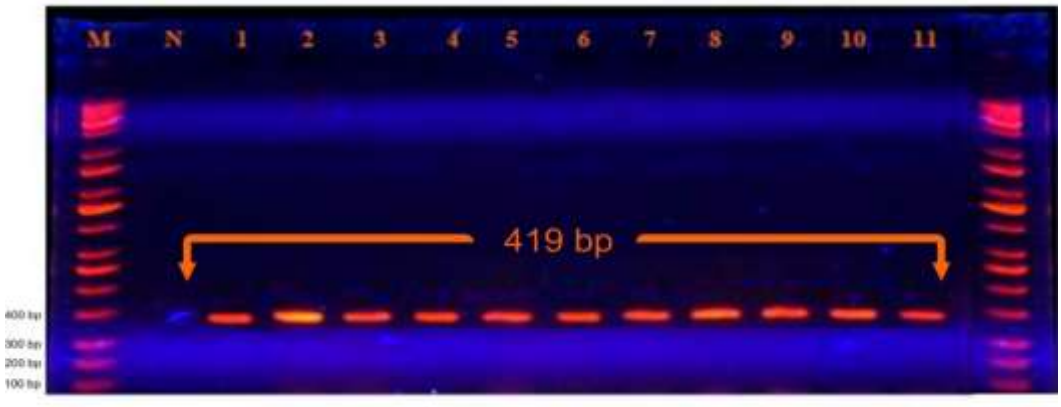


Figure 4-4 Electrophoresis of E-Selectin rs5355 G>A .Gene amplification products in 1% agarose gel and 70 volts for 1:30 h using DNA Ladder (100bp-2000bp). This is shown in well M and starts from 100 bp, lane N is the negative sample, and rest of well 1-11 represents gene bands of 419 bp (Primer designed by the researcher).

Table 4.7: Expected Frequencies Genotypes of E-Selectin rs5355 G>A Using Hardy-Weinberg equation

Groups			GG	GA	AA	G	A	X^2
Patients Genotypes	Observed	No.	8	30	2	46	34	11.43
		%	20	75	5	0.575	0.425	
	Expected	No.	13.22	19.55	7.23	Not detected		
		%	33.05	48.87	18.07			
Control Genotypes	Observed	No.	7	18	0	32	18	7.91
		%	28	72	0	64	36	
	Expected	No.	10.24	11.52	3.24	Not detected		
		%	40.96	46.08	12.96			

The expected frequencies of genotypes showed no significant differences ($X^2 < 3.84$) between observed and expected frequencies for both T2DM patients and the control group.

The observed genotype frequencies in both patients and control were higher than those predicted by the H.W.E theory (Table4.7). Both patients and control revealed significant levels of $\chi 2$ value; they were 11.43 and 7.91 respectively. These values were not agreeing with H.W.E. The departure from H.W.E. may indicate that this locus may undergo evolutionary selection in the Iraqi population.

From present study, it may suggest that this locus undergo evolutionary selection in Iraqi population made GA the common genotype. This locus has importance of being exon 11 region which may affect the transcriptional activity (Buckland, 2006; Knight, 2004).

Table 4.8: Genotypes and alleles frequency of the rs5355 G>A E-selectin polymorphism P values calculated with Fisher's exact test

Genotypes / Group	Study groups		Odds Ratio	CI 95%	P value Fisher –test	preventive or etiological fraction
	Patient	Control				
G/G	**8 (20)**	**7 (28)**	**0.640**	**0.20 to 2.17**	**0.460**	10.00%
G/A	**30 (75)**	**18 (72)**	**1.170**	**0.36 to 3.66**	**0.672**	10.70%
A/A	**2 (5)**	**0**	**0.2897**	**0.25 to 108.93**	**0.37**	4.90%
Alleles						
G n (%)	**46 (57.5)**	**32 (64)**	**0.76**	**0.36 to 1.58**	**0.525**	15.30%
A n (%)	**34 (42.5)**	**18 (36)**	**0.29**	**0.10 to 0.76**	**0.009**	51.30%

$p \geq 0.05$ is significant

According to sequence analysis for a position in gene E-Selectin rs5355 G >A. Genotype G A show non significant (Table 4.8)

Slightly high frequency (75%) in patients compared to control (72%) with etiological risk factors according to odds ratio 1.170 while both homozygo genotype GG and AA show non significant preventive fraction 10.00% and 10.70% with negative association with disease especially AA genotype present in patients without the GA genotype frequency in Iraqi present sample show high frequency in patients and control that may be made heterozygote common genotype in Iraq, population sample for rs5355 G >A.

No recorded in healthy individuals, all these results in agreement with allele frequency. Both alleles G and A Show an etiological fraction 0.76 and 0.29 respectively but allele A with significant fisher 0.009 this may indicate to an association between A allele in rs5355 G >A and a disease in Iraq Patients.

The E-Selectin rs5355 G1839A single nucleotide polymorphism results from a transition of the single base G to A in exon 10, when an SNP takes place in coding regions of genes, it may lead to substituting of the concerned amino acid in the protein structure with another amino acid which results in the alteration of protein activity (Ng and Henikoff, 2003). The resulted substitution of G to A at the position 1839 of the SELE protein results in the substitution of the (Leu) Leucine to (Phe) Phenylalanine, which leads to an augmentation in the ligand-binding function of the protein, as well as increasing the affinity for additional ligands, resulting in enhancing the cellular adhesion (Xiao *et al.,* 2012, Cai *et al.,* 2014).

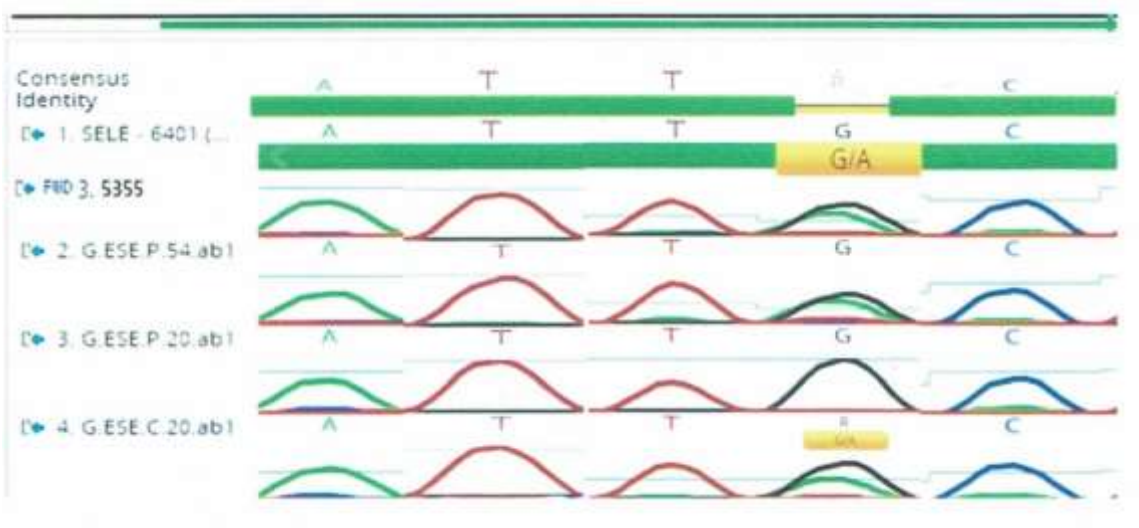


Figure 4-5: rs5355 G >A polymorphism by Geneious software and genotypes showed (GG, GA, AA) for samples study, R: Heterozygous.

4.1.3.2 Detection of E-Selectin gene rs5368 Polymorphism Sequencing

The E-Selectin rs5368 (C>T) SNP was amplified using a novel set of primers designed by the researcher especially for this locus in order to obtain the required DNA sequence around the SNP, the resulted fragment (490 bp) is shown in Figure (4-6).

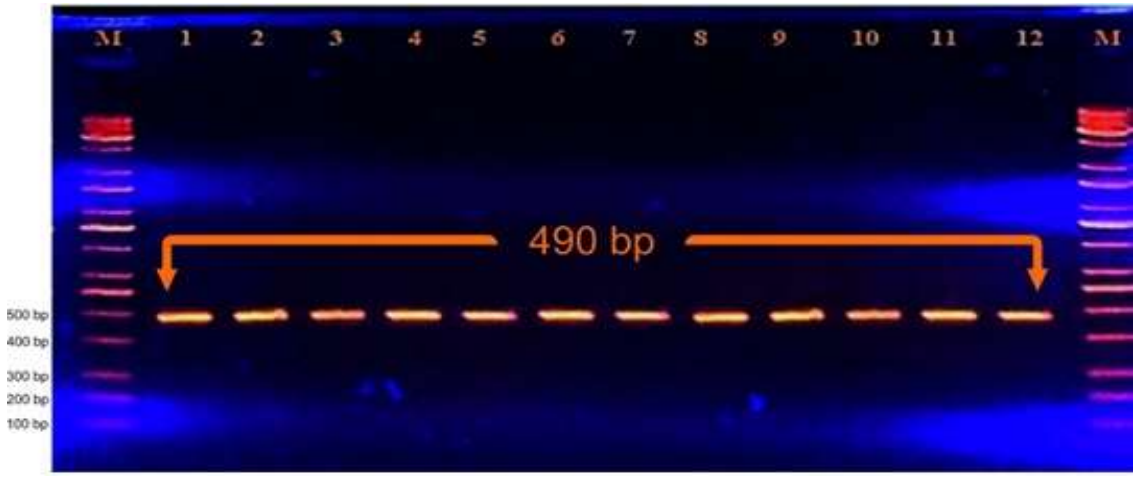


Figure 4-6 Electrophoresis of E-Selectin rs5368 C>T .Gene amplification products in 1% Agarose gel and 70 volts for 1:30 h using DNA Ladder (100bp-2000bp). This is shown in well M and starts from 100 bp, and rest of well 1-12 represents gene bands of 490 bp (Primer designed by the researcher).

In table 4.9 the patients and the analysis showed significant differences for all genotypes among observed and expected that mean present results not agree with H.E.W present deviation from H.W.E related with disease while the compaction showed that control agree with H.W.E.

Table4.9: Expected Frequencies Genotypes of E-Selectin rs5368 C >T Using Hardy-Weinberg equation

Groups			CC	CT	TT	C	T	X^2
Patients Genotypes	Observed	No.	24	10	6	46	34	5.57
		%	60	25	15	0.752	0.275	
	Expected	No.	21.03	15.95	3.03	Not detected		
		%	52.57	39.875	7.57			
Control Genotypes	Observed	No.	14	8	3	36	14	1.06
		%	56	32	12	72	28	
	Expected	No.	12.96	10.08	1.96	Not detected		
		%	51.84	40.32	7.84			

The expected frequencies of genotypes showed no significant differences ($X^2 < 3.84$) between observed and expected frequencies for both T2DM patients and the control group.

Table 4.10: Genotypes and alleles frequency of the rs5368 C >T E-selectin polymorphism P values calculated with Fisher's exact test

Genotypes / Group	Study groups		Odds Ratio	CI 95%	P value Fisher –test	preventive or etiological fraction
	Patient	Control				
C/C	24 (60)	14 (56)	1.18	0.42 to 3.29	0.703	9.10%
C/T	10 (25)	8 (32)	0.71	0.23 to 2.22	0.413	9.30%
T/T	6 (15)	3 (12)	1.29	0.29 to 6.89	0.862	3.40%
Alleles						
C n (%)	58(72.5)	36(72)	1.03	0.46 to 2.26	0.921	1.80%
T n (%)	22(27.5)	14(28)	0.98	0.44 to 2.19	0.921	0.70%

$p \geq 0.05$ is not significant

Table (4.10) showed the genotyping analysis for rs5368 C >T in gene E-Selectin. The present result, recorded high non significant frequency for CC Homozygous genotype in both groups patients and control with etiological risk factors (1.18). So there is a positive association between CC genotype and diabetes. TT genotype show non significant etiological effect 1.29. while heterozygous genotype show non significant preventive fraction 0.71 with a negative association with diabetes.

Allele C recorded similar frequency with patients (72.5%) and control (72%) with etiological risk factor 1.03 while allele T recorded high ration in control (28%) compared to patient groups (27.5%) with preventive fraction 0.98. So both alleles C and T facilitated the occurrence of disease.

The substitution of Histidine to Tyrosine that occurs due to the E-Selectin rs5368 (C468T) polymorphism was found to be associated with some clinical disorders (Kardia, *et al*., 2007; Wu *et al*., 2012; Wang *et al*., 2013; Liu *et al*., 2015; Hsieh *et al*., 2017). This may explain the potential consequences of these genetic variations in the E-Selectin gene. The analysis of the alleles and genotype distribution of the two detected polymorphisms is illustrated in the following figure.

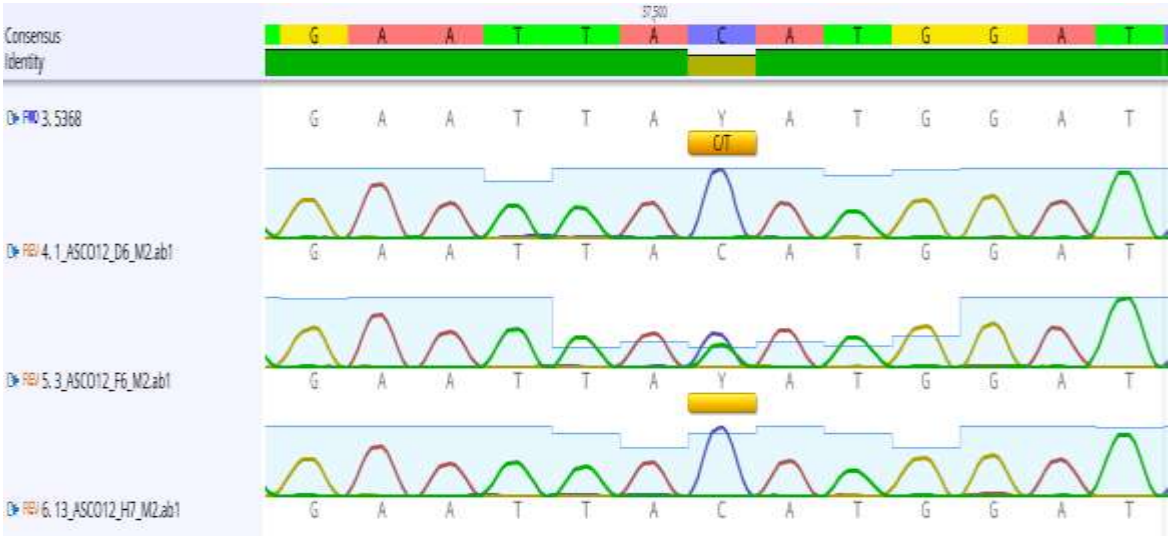


Figure 4-7: rs5368 polymorphisms by Geneious software and genotypes showed (CC, CT, TT) for samples study, Y: Heterozygous.

4.2.3.3 E-Selectin gene rs5367 Polymorphism Sequencing

According to Hardy–Weinberg equation for the expected frequencies of genotypes, no significant differences at ($p \geq 0.05$) between observed and expected frequencies for both patients and control group (Table 4.11).

The analysis of rs5367 SNP of splice region Exon 9 showed that genotype frequencies were in agreement with the Hardy-Weinberg equation.

Table 4.11: Expected Frequencies Genotypes of E-Selectin rs5367 T>C Using Hardy-Weinberg equation

Groups			TT	TC	CC	T	C	X^2
Patients Genotypes	Observed	No.	24	13	3	61	19	0.42
		%	60.0	32.5	7.5	76.25	23.75	
	Expected	No.	23.26	14.49	2.26	Not detected		
		%	58.15	36.2	5.65			
Control Genotypes	Observed	No.	18	5	2	41	9	2.6
		%	72	20	8	82	18	
	Expected	No.	16.81	7.38	0.81	Not detected		
		%	67.24	29.52	3.24			

The expected frequencies of genotypes showed no significant differences ($X^2 < 3.84$) between observed and expected frequencies for both T2DM patients and the control group.

Table 4.12: Genotypes and alleles frequency of the rs5367 T>C E-selectin polymorphism P values calculated with Fisher's exact test

Genotypes / Group	Study groups		Odds Ratio	CI 95%	P value Fisher –test	preventive or etiological fraction
	Patient	Control				
T/T	24(60.0)	18(72)	0.58	0.19 to 1.73	0.360	30%
T/C	13(32.5)	5(20)	1.93	0.59 to 6.83	0.330	15.6%
C/C	3(7.5)	2(8)	0.93	0.13 to 8.35	0.821	0.5%
Alleles						
T n (%)	61(76.25)	41(82)	0.7	0.28 to 1.70	0.449	24.2%
C n (%)	19(23.75)	9(18)	1.42	0.59 to 3.58	0.449	7.0%

$p \geq 0.05$ is not significant

Homozygous TT genotype was detected in 60.0% of T2DM patients, heterozygous CT genotype was detected in 32.5 % and homozygous CC genotype was detected in 7.5 %. The frequency of T allele was 76.25 % and the frequency the C allele was 23.75 %. In the control group, the TT genotype was detected in 72 % of subjects, CT in 20 % and CC in 8%.

The frequency of the T allele was 82 % and the frequency of the C allele was 18 %. The present results were consistent with previous study (Lachance,2010) which showed that the common allele T is that is considered as a dangerous agent in at least some cases, allele C the most protective. That may be related to sample size, the cultural of Iraqi population people who tend to inner consanguineous marriage.

The natural wild genotype of the most common allele of TT predominant genotype showed a non-significant difference in control compared with patients using a Fisher test and showed a protective genotype. Also heterozygous TC genotype showed a non-significant difference compared to control with patients and showed a genetic pattern associated with the risk to get disease, as well as the genotype CC showed a protective genotype. Current study - agree with a local study on patient's diabetes (AlHasnawi, *et a*l., 2017; Salman., 2018), various genetic factors, including single nucleotide polymorphisms, may play a role in the development of diseases. One nucleotide replaces another nucleotide of the gene, which occurs in the gene in a region near the gene, which plays a direct role in the disease. As occurring in region intron variant of rs5367 (Ng and Henikoff., 2003).

E-selectin polymorphism analysis showed high non-significant frequency of all genotypes in patients group when compared with the control group according to Fisher's exact test. The genotype TT recorded highly ratio in both groups patients and control (60-72%) which made it the common genotypes for this locus in the Iraqi population. Both homozygous genotype showed preventive fraction according to odds ratio (0.58, 0.93), while CT genotype according to odds ratio considers as an etiological fraction. The analysis of the allele impact, including both genotypes that containing C allele showed no significant association in patient group comparing with control (p= 0.449). According to OR, allele C tends to be a

risky allele with an environmental fraction (7.00%), while allele T tends to be a preventive allele (24.20%) (Table 4.12). Frequency of genotype TT, TC and CC significantly increase in the groups of patients compared with control, which is a reflection of the results achieved in other study conducted on Iraqi patients (Salman., 2018). While, Heterozygous TC genotype showed higher frequency in diabetic patients compared to control, which was consistent within another studies (Yoshida *et al*., 2001; Abu-Amero *et a*l., 2007).

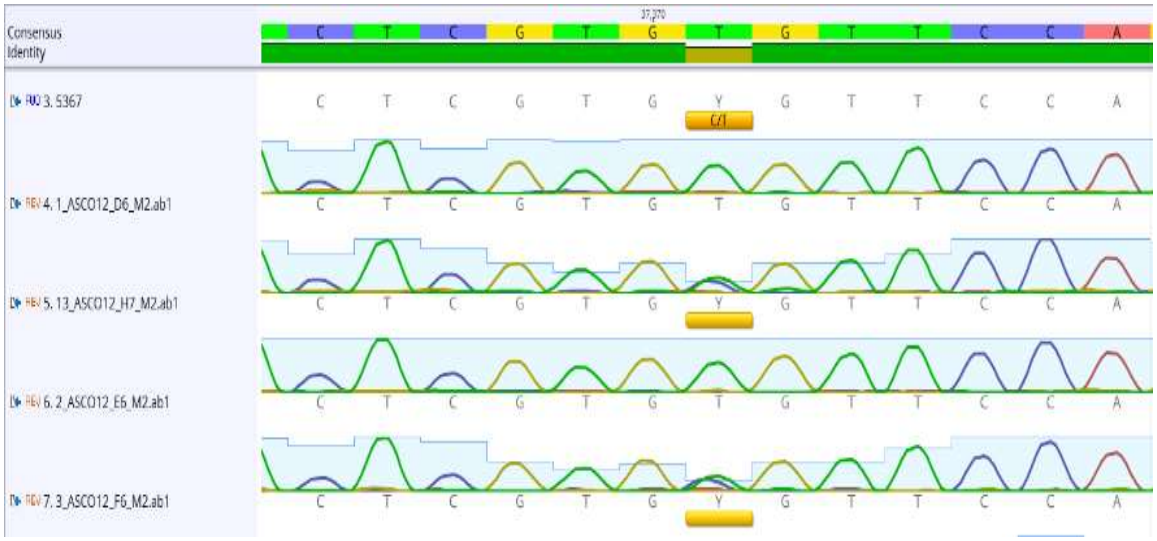


Figure 4-8: rs5367 T>C polymorphisms by Geneious software and genotypes showed (TT, CT, CC) in the studied groups, Y: Heterozygous.

4.2.3.4 E-Selectin rs751151130 Polymorphism Sequencing

The genotypes and allele frequency according to H.W.E. in table 4.13 showed that observed genotypes do differ significantly from expected, it means, that patients result not dieted from H.W.E. stable which means that disease not related with genotypes mean, while there are significant differences among all type of observed genotypes and expected frequency so the control result deviated from H.W.E. therefore this result needs further studies.

Table4.13: Expected Frequencies Genotypes of E-Selectin rs751151130 G >A Using Hardy-Weinberg equation

Groups			GG	GA	AA	G	A	X^2
Patients Genotypes	Observe	No.	28	10	2	66	14	0.72
		%	70	25	5	82.50	17.50	
	Expected	No.	27.23	11.55	1.22	Not detected		
		%	68.07	28.87	3.05			
Control Genotypes	Observe	No.	22	2	1	46	4	5.21
		%	88	8	4	92	8	
	Expected	No.	21.16	3.68	0.16	Not detected		
		%	84.64	14.72	0.64			

The expected frequencies of genotypes showed no significant differences (X^2 < 3.84) between observed and expected frequencies for both T2DM patients and control group

Polymorphism might be a result of multiple reasons which might include mutation, migration, and selection. Also the small population sizes. mating between genotypically similar individuals that increases homozygosity for the loci, the mating between close relatives also increases the homozygosity for the whole genome (Lewis and Knight, 2012).

Table4.14: Genotypes and alleles frequency of the rs751151130 G >A E-selectin polymorphism P values calculated with Fisher's exact test

Genotypes / Group	Study groups		Odds Ratio	CI 95%	P value Fisher –test	preventive or etiological fraction
	Patient	Control				
G/G	28(70)	22(88)	0.32	0.07 to 1.23	0.102	60.0%
G/A	10(25)	2(8)	3.83	0.82 to 27.40	0.078	18.5%
A/A	2(5)	1(4)	1.26	0.09 to 38.73	0.777	1.0%
Alleles						
G n (%)	66(82.50)	46(92)	0.41	0.11 to 1.28	0.157	54.3%
A n (%)	14(17.50)	4(8)	2.44	0.78 to 9.04	0.157	10.3%

$p \geq 0.05$ is not significant

E-Selectin rs751151130 polymorphism analysis showed a higher frequency of GG genotype in patient groups than control groups (70% vs. 88%), which showed statistical non significance (p= 0.102). On the other hand, the analysis of the allele impact, including both genotypes that containing G allele showed no significant association in patient group

compared with control (p= 0.157). The attributable fraction in patients group than the control group was 54.3% vs. 10.3% (Table 4.14):

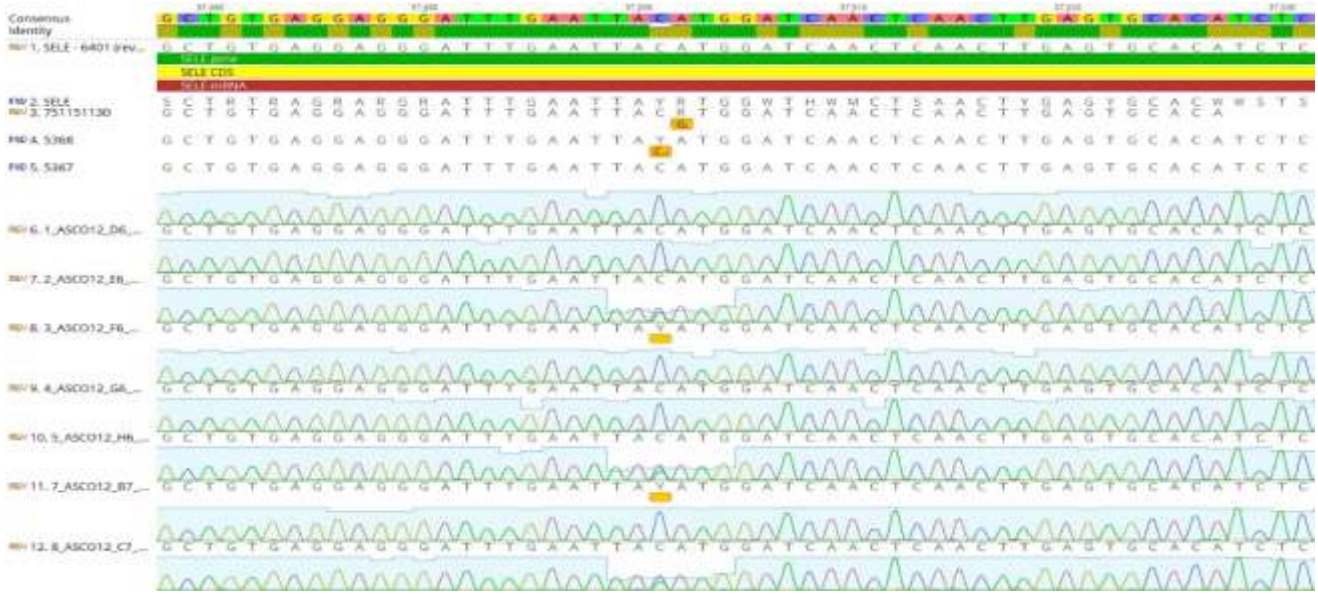


Figure 4-9: rs751151130 G >A polymorphism by Geneious software and genotypes (GG, GA, AA) in samples study, Y: Heterozygous.

4.2.3.5 Mutations in Exon 9 of the E- Selectin gene

Several processes were performed to process sequencing knowledge of amplification products and various samples, including refinement, alignment, and analysis with special sequences in the reference areas of the NCBI site. The sequences of E-Selectin gene are very similar to the 99% E-Selectin gene exon. Comparing the DNA sequences observed for these samples understudy with their stored reference sequences (Gen Bank acc. NG_012124.1). Using Snap Gene, a deletion mutation was identified and in most of the samples under study contained a deletion mutation of one of the nitrogenous bases T at 6408 or 11097 positions, as shown in figure 4.10, Table (4-15) shows that the incidence of mutation between patients and control (77.5% and 60%), respectively.

Table 4.15: Percentage of deletion mutation for E-Selectin gene

No. Groups	Frequency of Deletion 6408 Number of individuals	Percentage of variations
Patients 40	**31**	**77.5 %**
Control 25	**15**	**60 %**

Figure 4.10 DNA sequencing of Exon 9 reveals deletion mutation using SnapGene software.

This result represents that most of the Iraqi individuals within the present study showed that the amin bases have been deleted from their own E-Selectin gene studied segment. This state may be a special mutation that occur in the Iraqi population and need further studies to recorded this finding.

4.3 Clinical Features: Comparison between Patients and Control in the Clinical Parameters

The characteristics of both patients and control groups are listed in table 4.16. The results showed highly significant differences in all the Atherogenic index of plasma (AIP), Insulin and HOMA-IR, as well as the body mass index (BMI) and the Abdominal Circumference in patients group compared with the control group (4.16).

Biochemical properties (FBG- Fasting blood glucose, HbA1c-Glycohemoglobin, Total Cholesterol, HDL- high density lipoproteins, LDL-low density lipoproteins, VLDL- very low density lipoproteins, TG-triglycerides).

Table 4.16: Anthropological and Biochemical Parameters for study groups.

Parameters	Patients (Mean ± SE)	Control (Mean ± SE)
BMI (kg/m²)	26.458 ± 0.638	23.244 ± 0.418 *
Abdominal Circumference (cm)	89.060 ± 0.869	76.540 ± 0.799 *
FBS (mg/dl)	212.300 ± 4.863	101.740 ± 1.586 *
HbA1c (%)	9.660± 0.18	4.966 ± 0.70 *
Insulin µL/m	14.3±0.12	10.1±0.07 *
HOMA-IR	7.1±2.58	2.53± 1.50 *
Cholesterol(mg/dl)	202.980 ± 3.395	187.340 ± 3.083 *
TG(mg/dl)	214.500 ± 3.250	157.040 ± 2.579 *
HDL(mg/dl)	43.640± 1.054	65.140 ± 1.479 *
LDL(mg/dl)	116.440± 3.660	90.792 ± 3.549 *
VLDL(mg/dl)	42.900 ± 0.650	31.408 ± 0.516 *
AIP	4.795± 1.511	2.960± 0.869 *

* p≤ 0.001, BMI: body mass index, Insulin and HOMA-IR, HbAlc: glycated hemoglobin, TC: Total Cholesterol, TG: Triglyceride, LDL-C: Low-density lipoprotein cholesterol, HDL-C: High-density lipoprotein cholesterol, VLDC: Very low-density lipoprotein and AIP: Atherogenic Index of plasma.

These factors depending on the type of diabetes and severity of diabetes, glycaemic control, nutritional status, age, and other factors (Nema, 2008)., Furthermore, abdominal Circumference provides a good indicator for obesity and T2DM (Klein *et al.*, 2007). All chemical parameters increase (p≤ 0.001) significantly in Iraqi patients that agree with previous locally studies about Iraqi patients (Almohaidi,2015, Almohaidi ,2018). This elevation returns to other causes such as Lack of insulin secretion or defects in insulin resistance or disorder of pancreas and metabolism especially the saccharides in the liver (Abdulhameed, and Mahdi, 2018).

In the current study, it was observed that the HbA1C levels of patients were significantly higher (p≤ 0.001) than control. Previous studies consider that the HbA1C level as a good indication for the diagnosis of diabetes in patient's sera (Almohaidi *et al.* 2014). In present study, that serum total cholesterol is increased (p≤ 0.001) in group of diabetes

compared with the control, one of the possible reason that glycated hemoglobin may contribute with increasing cholesterol in the sera of diabetic patients (Virtanen,1993; Dahal *et al*. 2013), which they agreement the with previous report which emphasized that increasing lipid associated with increasing HbA1C (Kazim, 2011).

At the same time, LDL-C was increased ($p \leq 0.001$) in diabetes as compared with the control group and this result was similar to results (Mohammed *et al.*2011). Refer to same of the possible reason such as reduced of lipoprotein lipase activity which lead to reducing of TG catabolism and most converted VLDL-c into LDL-c which a play role large important during from reduction of the convert LDL-c in the live tissues (Robert, 2001; Abdullah *et al.* 2017). On the other hands, increased LDL-c result from defect linkage LDL-c with the receptors that stimulated with present insulin (Ismail *et al.*2011).

It is a well-known fact that low HDL-C is common in diabetes patients, a factor a strong factor for coronary heart disease (CHD) (Al-Mukhtar, 2005). Which is not consistent with the present study result showed a high level of HDL-C evident compared with the control group (Table 4.16). Serum triglycerides (TG) and VLDL-C are an increased ($p \leq 0.001$) in diabetic patients and this is in agreement with the result (Hussien *et al.*2013). That the most common lipid abnormality in DM is the which is known to be an independent risk factor of coronary heart disease (CHD) (Shrewastwa *et al.*2013). It is due to increase in VLDL-C synthesis and impaired VLDL-C catabolism (Manzato ,1994). Which results from reduced lipoprotein lipase activity which leads to increased TG and VLDL at the same time (Mohammed *et al.* 2011; Al-Qaesi *et al.*2013) due to insulin deficiency, Since the enzyme activity requires insulin for activation (Al-Qaesi *et al.*2013).

4.4 Bacteriological aspect

4.4.1 Distribution Isolate:

The present study included 50 patients with ulcers foot. Bacterial samples from ulcer showed different types of bacteria as mention in table 4.17. Among gram positive bacteria *S. aureus* has been chosen from negative *P. aeruginosa* was selected. Both of them represent the high frequent infection of isolated bacteria isolated from diabetic foot patients (16 and 10 respectively).

Table 4.17: Distribution of isolated bacteria from diabetic foot patients

Type of bacteria	Number of isolates from males patients	Number of female isolates	No.	Percentage
Gram positive				
Staphylococcus aureus	9	7	16	32
Staphylococcus epidermedis	2	-	2	4
Streptococcus pyogenes	2	1	3	6
Gram negative				
Pseudomonas aeruginosa	6	4	10	20
Escherichia coli	3	2	5	10
Klebsiella	4	1	5	10
Enterobacter	2	2	4	8
Proteus vulgaris	1	1	2	4
No growth	1	2	3	6
Total	30	20	50	

Males: 30: 60%
Female: 20: 40%

Have similar results to Abbas study (2017), as well as the (Mehta *et al*.,2014; Rajalakshmi and Amsaveni.,2012) studies, while it does not agree with the results of (Radji., 2014). 32.8% *P. aeruginosa*, 13 22.4% *E. coil* and 6.9% *S. pyogenes*.

It is clear from the above results that *S. aureus* bacteria are the main cause of diabetic foot injury and tissue damage, and such a result was recorded by a number of researchers (Radji., 2014. Perim., 2015). Some researchers believe Western countries in their studies on foot patients The development of clinical specimens results in the development of a single

pathogen, usually *S. aureus* or *S. Streptococcus* (Kosinski, and Lipsky., 2010). The wide variety of human diseases caused by the bacterium is due to its ability to release large amounts of extracellular products. Enzymes and toxins that increase the virulence and pathogenicity (Hildebrandt., 2015).

The results above revealed the number of the percentage of isolates were isolated in a high percentage of infection 30 (60%) male, 20(40%) female respectively as shown in table 4.17. The results were consistent with the study (Hayat *et al.*, 2011; Hena, and Growther., 2016) as confirmed by the dominance of infection in males. A study by Ossaneis *et al.,* (2016) showed that men were less self-care for the feet due to the following: they did not dry between the toes after bathing, and did not check the feet regularly, barefoot frequently, not trimmed nails. When compared to women.

4.4.2 Results of Identification

Bacterial isolates were diagnosed based on their microscopic characteristics by observing the shape, regularity and arrangement of their cells. Results showed microbial growth on differential media. The bacterial isolates were examined according to forbes *et al*., (2007) and showed differential and diagnostic results of the isolates under study as follows:

Catalase: The isolates were tested for catalase and most isolates had the ability to break down hydrogen peroxide (H_2O_2), converting it into water and oxygen gas. The appearance of these gas bubbles is evidence of the positive result. Except for *Streptococcus pyogenes*, the result was negative.

Oxidase: As for the oxidase test, all isolates were negative for this test because the bacteria did not have the ability to produce cytochrome

oxidase. Except for *P. aeruginosa*, the result is positive. (Brooks *et al.*, 2010).

Coagulase: Coagulase production test was carried out after mannitol sugar fermentation test where all isolates were able to convert liquid plasma into coagulant plasma due to the Coagulase enzyme which converts Fibrinogen to Fibrin (Dhakal and Mulvey., 2012). Host cells It is effective against many cell types (Nielubowicz and Mobley., 2010).

Mannitol salt agar: was considered a selective and differential media. The high concentration of salt selects for members of the genus *Staphylococcus*, since they can tolerate a high salt concentration. Mannitol salt agar also contains the sugar mannitol and the pH indicator of red phenol. If an organism can ferment mannitol, lactic acid is formed by the product that will cause the red phenol in the agar to turn yellow. (Götz *et al.*, 2006).

Citrate utilization: Gram-negative isolates were also tested for citrate consumption, which was a positive result for all isolates except *E. coli*, which was positive for indole testing. The colour of the medium containing bacteria after (24) hours of green to blue as a result of the consumption of jackets (Brooks *et al.*, 2010).

Indole test: Gram negative isolates were also tested for indole. All isolates were negative except *E. coli*, which was positive. A red ring appears within seconds of the addition of the Kovacs reagent, meaning that the bacteria possess Tryptophanase. (Brooks *et al.*, 2010).

Table4.18: Biochemical tests for the diagnosis of isolated bacteria from a diabetic foot ulcer

Type of bacteria	No.	Catalase	Oxidase	Coagulase	Mannitol salt agar	Citrate utilization	Indol
Staphylococcus aureus	16	+	-	-	+	/	/
Staphylococcus epidermedis	2	+	-	-	-	/	/
Streptococcus pyogenes	3	-	-	-	/	/	/
Pseudomonas aeruginosa	10	+	+	-	/	+	-
Escherichia coli	5	+	-	-	/	-	+
Klebsiella	5	+	-	-	/	+	-
Enterobacter	4	+	-	/	/	+	-
Proteus vulgaris	2	+	-	/	/	+	-

4.4.3 Identification of isolated bacterial by VITEK 2Compact device

The types of bacteria identified were:

Staphylococcus aureus, and *Pseudomonas aeruginosa*, appendix (3).

4.4.4 Detection of Biofilm Formation

In this study, determining for the ability of (12/16) from *S. aureus* and (7/10) from *P. aeruginosa* isolates for adherence and producing a slime layer (Biofilm formation) was experienced by using two conventional phenotypic methods to screen their ability to form biofilms. Direct methods allow the in situ observation of microbial colonization, including Congo-red agar (CRA), and microtiter plate (MTP) by ELISA reader. In addition, quantify the adherence and biofilm formation, it should be confirmed by genotypic characterization methods or by (PCR) methods to detect (*icaA* and *icaD*), and *cupA* genes can be useful to distinguish between colonizing or commensally *S. aureus* and *P. aeruginosa* respectively. They can lead to the early detection and management of potentially pathogenic isolates responsible for device-associated nosocomial infections. There are various methods for biofilm detection, and for evaluating the reliability of these

methods in order to determine the most suitable screening method, as shown below: -

4.4.4.1 Congo-red Agar(CRA) Method

The results of the current study showed that 12(75%) of *S. aureus* bacteria have the ability to produce biofilm. 4 (25b%) notability to produce the biofilm, 7(70%) of *P. aeruginosa* bacteria have the ability to produce the biofilm. 3(30%) notability to produce the biofilm. The bacterial colonies of the biochemistry have been shown to be black and have a dry crystalline density as shown in table 4.19. Structural and regulatory genes play an important role in biofilm formation and host colonization. The result genes affect colony aggregation by various mechanisms, including alteration of synthesis of transcriptional factors and regulation of extracellular polysaccharide production. (Chavez-Dozal *et al.*, 2015).

Congo red agar (CRA) is a planar, hydrophobic, diazo dye that is bound to lipids, proteins and to a broad range of other macromolecules. The dye interacts with the outer membranes and outer membrane proteins (David e*t al.*, 2005). Further, it is bound to amyloid like fibers which are recently detected cultures named phenol soluble modeling (Erskine *et al.*, 2018).

CRA method requires the use of a highly nutritious medium in this case, and a brain heart infusion broth with sucrose supplementation. Previous studies showed that the use of sugar supplementation (sucrose was normally used though similar results were obtained using glucose) is essential for the detection of slime production using the Congo red medium (Freeman *et al.*, 2010).

It was also noted by some studies that the samples grown in CRA enriched with sucrose showed significantly higher biofilm formation than those grown in CRA without sucrose (Na Cai *et al.* 2016).

CRA is selected in an attempt to improve its capability to identify the adhesion and biofilm formation by isolates using a specially prepared solid medium by product changes in the procedure and adjusting different physical parameters. Thus, the CRA method is simple, sensitive and fast to carry out and the results are usually based on the colony color produced, which ranges from red for non-biofilm producing strains to black for biofilm producing strains that are reproducible and have the advantage that colonies remain viable on the medium (Freeman *et al.* 2010). This method is used directly to recognize the production of exopolysaccharide, which is an essential requirement for biofilm formation (Kumer *et al.* 2012).

Table4.19. Biofilm producing of isolates

Producing of Biofilm	**Number of isolates *S. aureus***	**Percentage**	**Number of isolates *P.aeruginosa***	**Percentage**
positive isolates producing of biofilm	12	75	7	70
Non-productive isolates of biofilm	4	25	3	30
Total	16	100%	10	100%

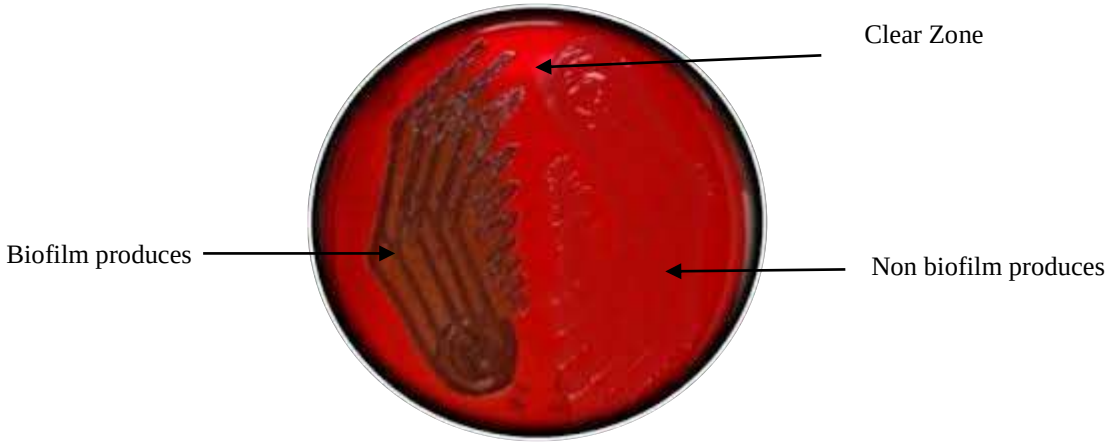


Figure 4.11: Detection test for the production of biofilms using Congo red method

4.4.4.2 Microtiter Plate Method (MTP)

The test tube method used to detect the ability of bacteria was used in the formation of biofilms. The isolates isolated from the patients with the foot ulcers showed their ability to form Biofilm. Table 4.20 shows the susceptibility of the bacteria to the production of the biofilms and the severity of its formation. and as follows:

A. Quantitative adhesion and production showed that the highest value of biofilm formation was 6(37.50%) and 5(50.00%), of isolates *S.aureus P. aeruginosa* respectively were strong adhesion and biofilm producers.

B- The 4(25.00%) and 1(10.00%) of isolates *S. aureus* and *P. aeruginosa* respectively created moderate adhesion and biofilm producer.

C- The (2.12.50%) and 1(10.00%) of isolates *S. aureus* and *P. aeruginosa* respectively the isolates gave weak biofilm producers.

D- The 4(25.00%)and 3(30.00%) of isolates *S. aureus* and *P. aeruginosa* respectively were non-biofilm producers. This result was determined according to the classification of bacterial adherence using the Christensen method.

Table4.20: Number and percentage of diabetic foot ulcers that have the ability to produce mud layer

Biofilm degree	**Isolates *S. aureus***		**Isolates *P.aeruginosa***	
Strong (+++)	6	(37.50%)	5	(50.00%)
Moderate (++)	4	(25.00%)	1	(10.00%)
Weak (+)	2	(12.50%)	1	(10.00%)
Non (-)	4	(25.00%)	3	(30.00%)
Total	16	100%	10	100%

Microtiter plate is an important tool for studding the early stages in biofilm formation. It allows the adhesion and formation of a biofilm on the wall and the microtiter plate is an important instrument for the study of the

early stages in biofilm adhesive and allows for the formation of a biofilm on the wall and bottom of a microtiter plate (O'Toole, 2011). It has been most widely used and was considered a standard test for the detection of biofilm formation because it allows a simple, more accurate and rapid way to quantify the contact cell attachment and biofilm formation of different bacterial strains (Khudhur, 2013).

The Microtiter plate method has the advantage of conomical quantitative technique for the identification of serious factors and typical culture conditions for biofilm formation in vitro. It is a higher sensitivity of 100% in the exposure of the positive strains (Stepanovic *et al.* 2000).

The Crystal violet staining that was used in this method is an essential dye known to bind to negatively charged molecules on the cell surface, as well as nucleic acid and polysaccharides. It gave an overall measure of the whole biofilm (Matz *et al.* 2005). The surface hydrophobicity of bacteria played an important role in the attachment to diverse polymers, such as polystyrene. It is related to the adherence of bacteria to the plastic surface, as is the case with catheters and prostheses (Magnusson, 1982).

The variation in biofilm thickness may be ascribed to differences in isolates ability to produce biofilm. The primary number of cells that might be successful for adherence or differences of the quality and quantity of auto-inducers quorum sensing signaling molecules (QS) system produced from each isolate (Brady *et al*. 2008; Beenken *et al*. 2010). The formation of biofilm plays an important role in the pathogenesis of pathogens; the development of these biofilms is support by the signal mediated QS system. The QS systems play an important role in the pathogenesis and regarding the role in biofilm formation of *S. aureus* and *P. aeruginosa* infections (Holling, 2014). Therefore, interference with QS may prevent the development of pathogenic bacterial biofilms.

Many genes affect aggregation of cooling by different mechanisms, which include alteration of synthesis of transcriptional factors and regulation of extracellular polysaccharide production. (Chavez-Dozal *et al.* 2015). The adhesion of bacteria and colonies on the biotic surfaces is the first step towards the formation of Biofilm. Biofilm resulting from the accumulation of bacteria is covered with a layer of polysaccharide and helps the bacteria to adhesion (Anderson *et al*. 2007).

4.5 Nanoparticles

4.5.1 X-ray Diffraction

X-ray diffraction analysis of CuO-NPs was prepared by the hydrothermal method at a temperature of 450°C. Figure (4.13) shows the result of X-ray diffraction. The angle of diffraction (2θ) is (35.515) and the corresponding is ($\bar{1}$11), where the crystallite size of the nanometer is amounted about (13.4nm) was calculated using the Scherrer equation. decreasing the temperature, the width of the peak and thus leads to a reduction in the Nanoscale size as shown in table (4.21). Its noticed that the average of crystallite size of CuO in nanostructure range and the patterns indicate that all of the nanoparticles have a polycrystalline and exhibited monoclinic phase according to (ICCD) card no. (05-0661).

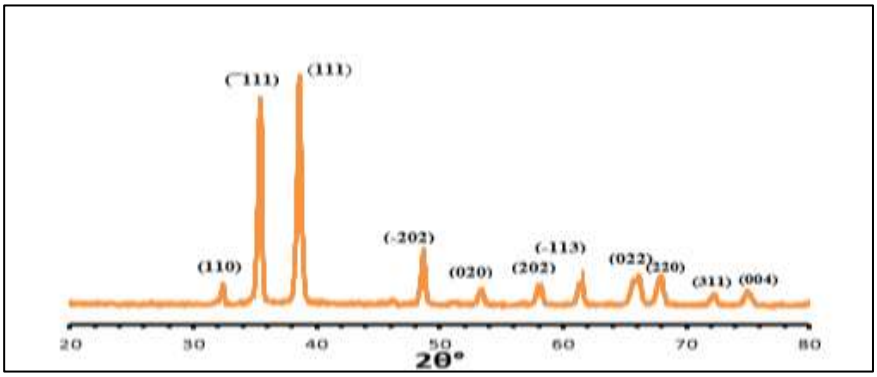


Figure.4.12: XRD spectra of CuO NPs prepared in the hydrothermal method

Table 4.21: XRD results of CuO prepared

Sample	2θ (deg)	FWHM(deg)	*hkl*
450°C	35.515	0.74111	$\bar{1}11$

4.5.2 Crystallite size

Table 4.22: variation Crystallite size of CuO prepared with annealing temperature

Sample	**Crystallite size**(nm)
450°C	13.4

4.5.3 UV-Visible of CuO-NPs

The optical properties of CuO nanoparticles have been studied by the UV-Visible spectrum, which shown in figure (4.14) UV-Visible spectroscopy is the most widely used technique to investigate the optical properties of the particles. The analysis was done in the range of (400-600) nm. The Figure shows that the absorption spectrum starts at 470 nm and reaches a maximum at 450 nm, the large energy gap is evidence that nanoparticles. This is a characteristic of nanomaterials, because the smaller the particle size the larger the energy gap. (Khan *et al.* 2017).

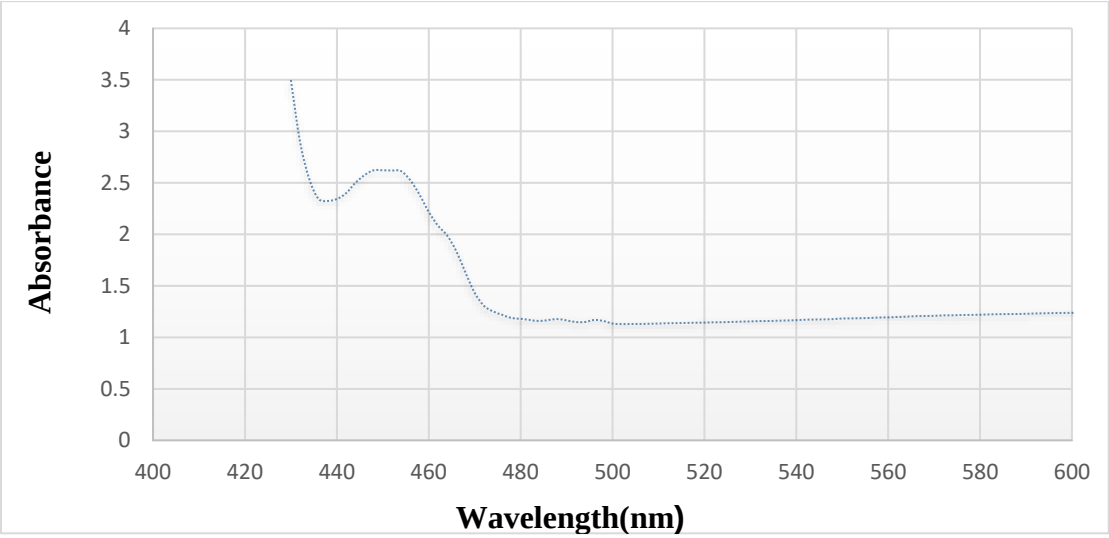


Figure4.13: Absorbance of CuO nanoparticles prepared by hydrothermal method at 450ºC

4.5.4 Morphological properties of CuO-NPs by Transmission Electron Microscopy(TEM)

The result of the TEM of the CuO-NPs prepared by the hydrothermal method at temperature 450 ºC as shown in figure (4.15) where the particle size was approximately (15) nm respectively. These indicate that the increased temperature was accompanied by an increase in nanosize. the size depends on the temperature value where the higher the temperature the larger the size of nanoparticles was analyzed and analyses by Image. The shape of nano particles is spherical. The average particle size determined by TEM image was very close to the crystallite size calculated from XRD results. Thus, the TEM results correlate well with XRD results.

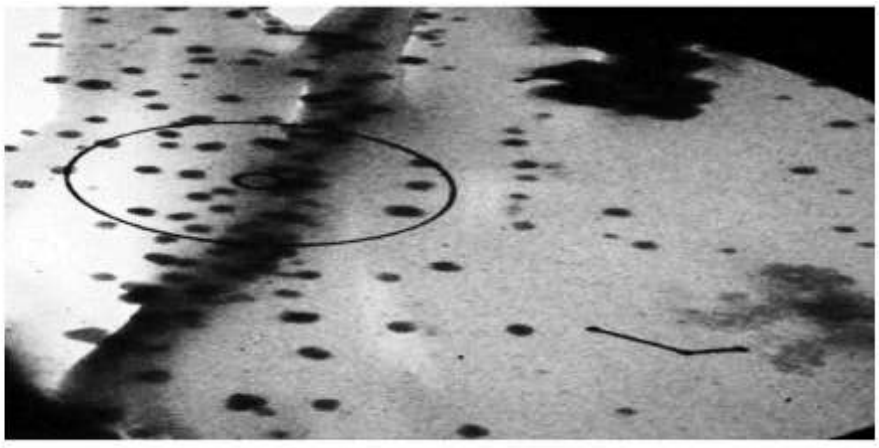


Figure 4.14: TEM image of CuO nanoparticles prepared by hydrothermal method at 450 ºC

4.5.5 Antibacterial activity of copper oxide nanoparticles

4.5.5.1 Determination of MIC results for CuO-NPs

MICs are the minimum inhibitory concentrations of CuO-NPs. A test was conducted against a number of stdied isolates. These isolates were identified using the tube broth dilution method according to the CLSI guidelines (CLSI, 2013) to gain a greater understanding of how volume affects antimicrobial activity. Eight tubes of CuO-NPs were tested with

different concentrations in the range of (0 to 2000) μg/ml. Other tubes were used to be control without NPs.

The MIC of CuO-NPs for antibacterial activities was introduced in the tube method to show the optical density (OD) of the isolates under study. Growth of bacterial culture in the presence of CuO NPs using the broth method compared with medium tube OD to control without NPs. The UV-VIS spectrophotometer was used in OD measurements of bacterial cultures in the liquid medium, and the wavelength at 630 nm was also measured.

Clearly the optical density decreased slightly with increasing concentrations of CuO-NPs in all bacterial isolates. This means that the effect of inhibiting the concentration of CuO-NPs was dependent on the method.

The results in figure 4.15 were shown that concentrations : 500, 1000 and 2000 μg/ml, were fatal to isolates *S. aureus* , (100μg/ml) were inhibitors and concentrations (75, 50,25and 0) μg/ml / ml were not effective respectively.

Figure 4.16 demonstrated that concentrations : (75, 100,500, 1000 and 2000) μg/ml, were fatal to isolates *P. aeruginosa* , (50 μg/ml) was inhibitos and concentrations (25 and 0) μg/ml were not effective espectively.

Concentration and volume are important factors affecting the antimicrobial properties of CuO-NPs. A wide range of synthetic compounds that exert antibacterial effects, but only some may be used as biocides to develop drugs or condoms. As shown in figure 4.16, the lowest concentration gave inhibition of (50μg/ml)concentration cells. Therefore, the concentration of CuO-NPs was most used to prevent the growth of study isolates. A single dose was used for biomaterials and to determine the effect of nanoparticles on genes in the present study.

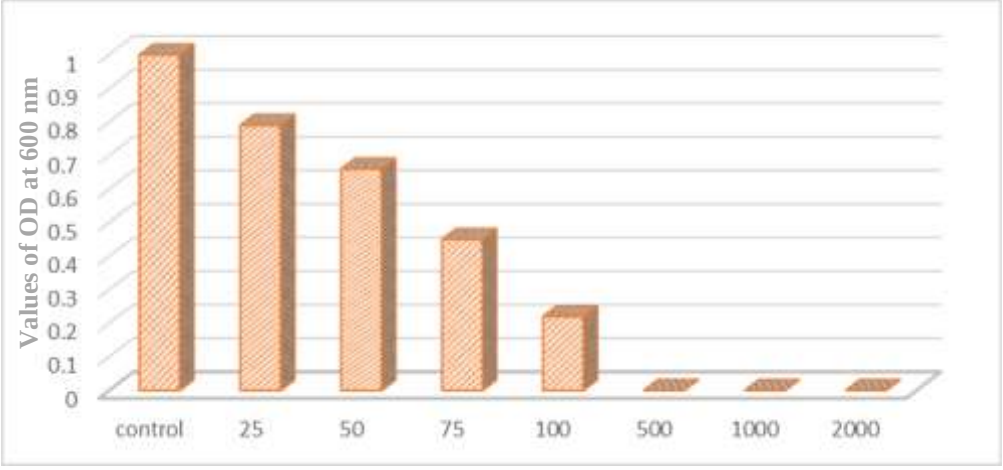


Figure 4.15: Effects of CuO NPs on Values of OD at 600 nm of *S. aureus*

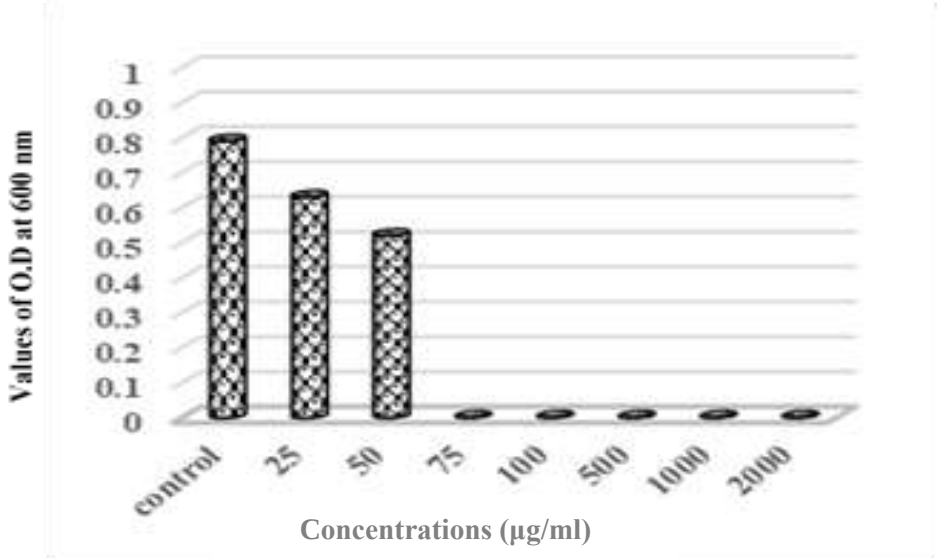


Figure4.16: Effects of CuO NPs on Values of OD at 600 nm of *P. aeruginosa*

The results of MIC showed that concentrations 100, 50µg/ml were sufficient as inhibitors of *S. aureus* and *P. aeruginosa* respectively . Figure (4.15 and 4.16) Concentration and volume are two important factors that affect the antimicrobial properties of CuO-NPs. Wide ranges of synthetic compounds exert an antibacterial effect, which is generated from the surface of copper oxide, In order to study CuO-NPs as new antimicrobial agents. The antimicrobial activity of CuO-NPs was determined against antibacterial . The antimicrobial capacity of CuO-NPs may be indicated on its small size 250 times smaller than the bacteria. This makes it easier to

adhere to the cell wall than the microorganisms that cause their destruction and lead to cell death (Amiri *et al.,* 2017). On the other hand, direct contact-dependent inhibition of planktonic bacteria might be the main killing mechanism by these nanoparticles, because antibacterial produced has biofilm, negatively charged of bacterial biofilm resist penetration of the nanoparticles. Could be the cause of higher concentrations and a longer duration of contact required for the elimination of biofilm producing bacteria. In addition, the moist or aqueous environment of the biofilm might increase the production of ROS by CuO-NPs that will increase ROS production by CuO-NPs, which was able to diffuse into the biofilm structure (Shrestha *et al.* 2010). Bacteria are in constant contact with ROS types, both during their life cycle and in their environment. These species cause damage to proteins, fats and nucleotides, adversely affecting the organism consequently all these interactions increase the bacterial cell lysis (Tauran *et al.* 2013).

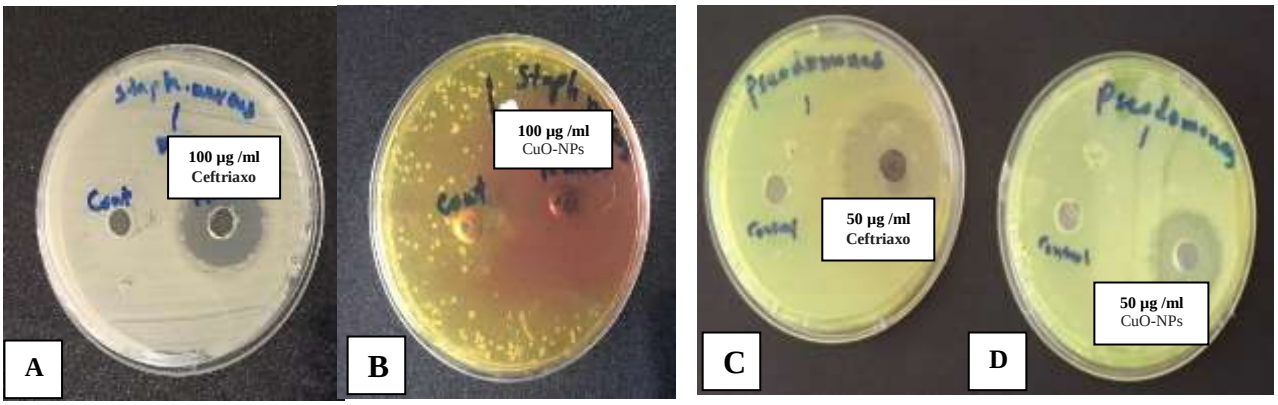
4.5.5.2 Agar Well Diffusion Method

MIC, and antibacterial activity of copper oxide nanoparticles against isolates were studied using the agar well diffusion method to show a clear inhibition zone around wells loaded with bacteria-induced CuO-NPs culture plates.

Results of the present study showed different concentrations similar to those used in the dilution method of tube broth As mentioned in section 4.5.5.1 of CuO-NPs synthesis when determining the MIC. The result was compared with that before using the method. In general, the inhibition of concentration observed as a result of using this method was significantly higher than other concentrations.

This result shows the highest antibacterial effect against studied isolates at concentrations (100 and 50). MIC of copper oxide nanoparticles was provided for antibacterial activities in the figure (4-17).

This means that the lowest concentration gave an inhibition diameter of 135nm is 100 µg/ml, which is the same for the MIC broth dilution method. The antibacterial activity of CuO-NPs was against clinical bacterial isolates when using well diffusion agar. Therefore, the concentration 100 µg/ml of copper oxide nanoparticles was the best use to inhibit the growth of isolates under study, the use of single dose in biofilm experiments and detection of the effect of nanoparticles in the genes of the present study.



***(Ceftriaxone): Antibiotic for foot ulcers used as a treatment in the Iraqi hospital**

Figure 4.17: MIC and Antibacterial Activity of CuO-NPs by Using Agar Well Diffusion method. **A**: *S. aureus* on medium Mueller Hinton agar and treated by Ceftriaxone as antibacterial, **B**: *S. aureus* on medium Mannitol Salt Agar and treated by CuO-NPs, **C**: *P. aeruginosa* on medium Cetrimide Agar and treated by Ceftriaxone and **D:** *P. aeruginosa* on medium Cetrimide Agar and treated by CuO-NPs.

The results of the current study were the approach with previously conducted studies on bacteria. For example, a study Alzubadiy and coworkers (2019) whose results showed the effectiveness of the use of copper nanoparticles against *P. aeruginosa*, as well as Mahdavi and coworkers (2013) showed that with the presence of CuO-NPs for both *Bacillus subtilis* and *E. coli*, the strain was significantly stabilized. They

concluded that CuO-NPs showed more antimicrobial activity for *B.subtilis* and *E. coli*. The results obtained by Cihan and coworkers (2015) showed that copper oxide nanoparticles had an inhibitory effect against pathogenic bacteria with a significant reduction in growth in different percentages of *S. aureus*, *E.coli* and *Serratia marcescens*, respectively.

Another study by Gordon and coworkers (2011) reported that the antibacterial activity of these CuO-NPs was found to be higher against *S. aureus*. Ismail and others made a similar observation. (2015) who used synthesized nanoparticles for rapid inhibition of *S. aureus* bacteria. In a study by Ramezani *et al.* (2017), they presented that the effects of CuO-NPs were tested against a strong biofilm that produced *P. aeruginosa* isolates.

4.6 Analysis of biofilm genes *icaA* and *icaD* and *cupA* in isolates

DNA extract was performed for all study biofilm isolates using the conventional PCR technique (molecular methods) in a unidirectional pattern with specific primers. This method was used to identify the genes responsible for the formation and formation of the biofilm in this study (genes *icaA, icaD* for *S. aureus* and *cupA* gene for *P. aeruginosa*).

The results of phenotype and genotype were compared. In this study to form a biofilm. PCR products appeared as a DNA band with 870, 390 and 590 of three genes *icaA, icaD* and *cupA*, respectively. The was confirmed by gel electrophoresis in 1% agarose stained with ethidium bromide, electrically filled at 70 volts for 1:30 hours and filmed under ultraviolet (UV) light as shown in figures 4-19, 4-20 and 4- 21.

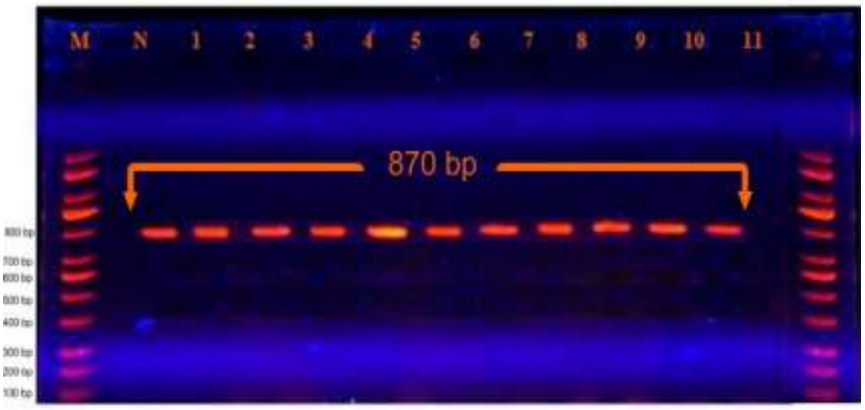


Figure4.18: PCR product of the *icaA* gene (870 bp), electrophoresis on 1% agarose at 70 volts. For 1:30 hours, DNA Ladder (100bp-2000bp) is shown in lane M and starts from 100 base pairs. The lane N represents the negative sample. The lane (1-11) represent the gene bundles in *S. aureus* isolates

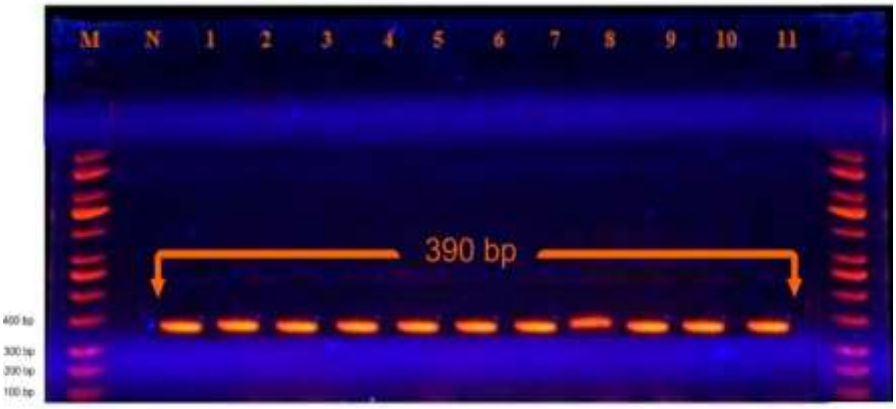


Figure 4.19: PCR product of the *icaD* gene (390 bp), electrophoresis on 1% agarose at 70 volts. For 1: hours, DNA Ladder (100bp-2000bp) is shown in well M and starts from 100 base pairs. The well N represents the negative sample. The lane (1-11) represent the gene bundles in *S. aureus* isolates.

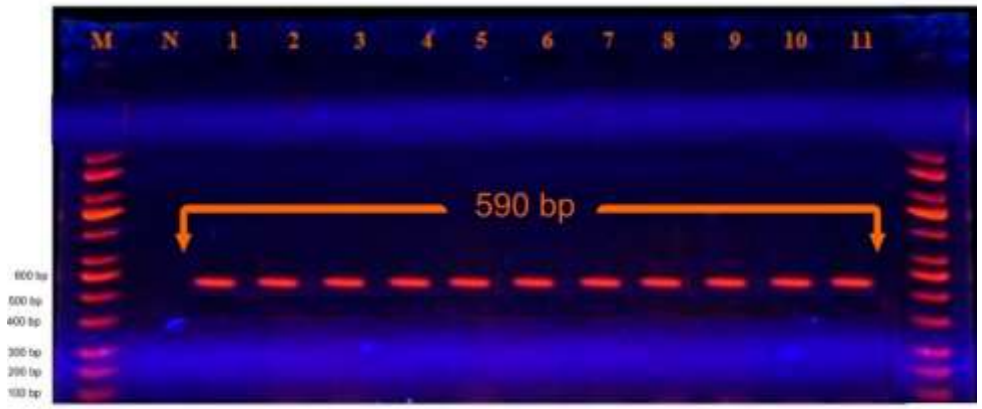


Figure 4.20: PCR product of the *cupA* gene (590 bp), electrophoresis on 1% agarose at 70 volts. For 1:20 hours, DNA Ladder (100bp-2000bp) is shown in well M and starts from 100 base pairs. The lane N represents the negative sample. The lane (1-11) represent the gene bundles in *P. aeruginosa* isolates.

4.7 Evaluation relationship among adhesion genes for Diabetic Foot Ulcer patients with Bacterial gene

This SNPs modification by substitution may enhance the adhesion activity that may lead to facilitate adhere of bacteria because the SNPs of selectin that may tends to increase the susceptibility to disease which will facilitate the attachment activity of bacteria to human and made infection.

The relation between genetic variation of human adhesion gene and bacteria adhesion molecules. The virulence power activity of bacteria depends in part of it on the adhesion gene. So the power of variation in adhesion human selectin gene with adhesion genes in bacteria as virulence genes, both of them may be facilitating the process of inflammation.

This finding goes with Testa *et al*. 2007, who said that various environmental factors trigger inflammation in the arterial wall, infection, and other factors.

Table 4.23 Relation between variation of adhesion genes in patients with T2D and biofilm gene *icaD* isolates *S. aureus* form Foot Ulcers

Human Gene	SNPs	Common genotype	Frequency ratio	Bacterial gene	Wild type	Mutant type	Location	Mutations Change amino acid	Type of mutation	Effect
L-Selectin	rs2205849	AA	67.5%	*icaA*	AAA	AGA	2706786	Arg> Lys	Substitution	Missense
E-Selectin	rs5355	AG A*	75%	*icaA*	ACT	ACA	2707471	Thr> Thr	Substitution	Silent
	rs5368	CC	60%	*icaA*	AGT	AAT	2706933	Asn > Ser	Substitution	Missense
	rs5367	TT	60%	*icaA*	ACT	ACA	2707471	Thr> Thr	Substitution	Silent
	rs751151130	GG	70%	*icaA*	AGT	AAT	2706933	Asn > Ser	Substitution	Missense
	D> T	Deletion T	77.5%	*icaA*	CAA	CGA	2707242	Arg> Gln	Substitution	Missense

* $p \geq 0.05$ is significant

The results in table 4.23 show variations in human L and E selectin with *S. aureus icaA* gene. The most common genotypes of human genes, as studied markers, and variance in associated bacteria were identified in terms of substitution of nitrogenous bases and the associated change in amino acid production. From the present study, the results showed that the

common genotype of L-selectin rs2205849 was AA at 67.5%, and variations in the *icaA* gene at position 2706786 pb. The substitution of nitrogenous Guanine in place of Adenine resulted in a mutation of the missense type, which changed the amino acid from Arginine to lysine.

The most common genotypes were identified in E-Selectin gene for five SNPs AG:rs5355, CC:rs5368, GG:rs751151130, TT:rs5367 and T> Del, each genotype of them have the highest frequency in patient's sample from Iraqi population was 75%, 60%, 60% 70% and 77.5%, respectively.

All these percentages correspond to variations in the *icaA* gene at position 2707471, 2706933 and 2707242 where two mutations (missense and Silent) were recorded. By substitution nitrogenous bases have produced different amino acids which can cause inflammation in the arterial system that can be incited by disparate mechanisms associate with the modifications made by bacteria moreover that modulation with an alteration in adhesion molecules may amplify the inflammatory, inflammation in foot ulcer is the main cause of amputation (Tuttolomondo *et al.* 2015).

Table 4. 24 Relation between variation of adhesion genes in patients with T2D and biofilm gene *icaA* isolates *S. aureus* form Foot Ulcers

Human Gene	SNPs	Common genotype	Frequency ratio	Bacterial gene	Wild type	Mutant type	Location	Mutations Change amino acid	Type of mutation	Effect
L-Selectin	rs2205849	AA	67.5%	*icaD*	AGA	AAA	27077835	Arg> Lys	Substitution	Missense
E-Selectin	rs751151130	GG	70%	*icaD*	AGA	AAA	27077835	Arg> Lys	Substitution	Missense

The results in Table 4.24 show variations in human selectin L and E genes with *S. aureus icaD* gene. The most common genotypes of human genes, studied markers previously in present study, with variance in isolated bacteria were identified in terms of substitution of nitrogenous bases and associated change in amino acid production. Data obtained from the program after alignment with a gene bank showed that the common

genotype of L-selectin rs2205849 was AA at 67.5%, and variations in the *icaD* gene at position 27077835 pb. The substitution of nitrogenous Guanine in place of Adenine resulted in a mutation of the missense type, which changed the amino acid from Arginine to lysine.

The most common genotypes were identified in E-Selectin gene for GG: rs751151130 SNPs with frequency of genotype was 70%. While variations in the *icaA* gene at position 27077835, as one mutation (missense).

These variations in patient's adhesion selectin gene and isolated bacteria from the same patients may indicate to association between genetic variation that may facilitate the infection.

Table 4.25Relation between variation of adhesion genes in patients with T2D and biofilm gene *cupA* isolates *P. aeruginosa* form Foot Ulcers

Human Gene	SNPs	Common genotype	Frequency ratio	Bacterial gene	Wild type	Mutant type	Location	Mutations Change amino acid	Type of mutation	Effect
L-Selectin	rs2205849	AA	67.5%	*cupA*	GGT	GAT	2840275	Gly> Asp	Substitution	Missense
E-Selectin	rs5355	AG A*	75%	*cupA*	GGT	GAT	2840275	Gly> Asp	Substitution	Missense
	rs5368	CC	60%	*cupA*	CTC	TTC	2840436	Leu> Phe	Substitution	Missense
	rs5367	TT	60%	*cupA*	GGT	GAT	2840275	Gly> Asp	Substitution	Missense
	rs751151130	GG	70%	*cupA*	CTC	TTC	2840436	Leu> Phe	Substitution	Missense
	D> T	Deletion T	77.5%	*cupA*	CTC	TTC	2840436	Leu> Phe	Substitution	Missense

* $p \geq 0.05$ is significant

The results in Table 4.25 show variations in the human and *P. aeruginosa (cupA)* gene. The most common genotypes of human genes, studied markers, and variance in associated bacteria was identified in terms of substitution of nitrogenous bases and associated change in amino acid production. Data obtained from the program after alignment with a gene bank showed that the common genotype of L-selectin rs2205849 was AA at 67.5%, and variations in the *cupA* gene at position 2840275 pb. The substitution of nitrogenous Guanine in place of Adenine resulted in a

mutation of the missense type, which changed the amino acid from Gly to Asp.

The most common genotypes were identified in E-Selectin gene for five SNPs AG:rs5355, CC:rs5368, GG:rs751151130, TT:rs5367 and T> Del, the frequency of each genotype was 75%, 60%, 60% 70% and 77.5%, respectively. This corresponds to variations in the *cupA* gene at position 2840275 and 2840436 where one mutation (missense) were recorded.

In Immunity response there is acritical step for migration of leukocytes depend on the L and E selectin, Leukocytes express L-selectin on their surfaces at the same time Endothelium of capillary vessels express E-selectin to let the selectin bind because Selectin – Selectin ligand interaction to pushed leucocytes on the surface of endothelium and recruit Lymphocyte for migration.

So any defect or variation be laying the all defaces mechanism.

Moreover, Mutations in bacterial virulence genes maybe increase the virulence process. Leading to accumulate and proliferate of bacteria becoming more pathogenesis.

4.8 Evaluate the effect of nanoparticle on isolated bacteria from Foot Diabetic Ulcers

4.8.1 Nucleotide Sequencing *icaA* gene from *S. aureus*

The sequences of the nitrogen bases were determined for isolates of *S. aureus* after reading the DNA sequences of the forward and reverse strands, recollecting the two strands, deleting the anomalies in either of them, analysis them and matching them to (NCBI) online at (www.ncbi.nlm.nih.gov). At the same time, Geneious Software has shown that reference sequences are very similar to *icaA* gene sequences 98 %. By comparing the observed DNA sequences of these samples with their stored reference sequences (Gen Bank: CP002388.1.1).

By analyzing the results of the sequencing of *icaA* gene according to the table 4.26 the data showed that (42.10%) 8 mutations substitution, 4 (21.05 %) Deletion mutations, 7 (36.84) Insertion mutations, this shows that the type and location of variations found in present sequence may result in different mutations. Some of these mutations lead to changes in the genetic codes and thus change in the amino acids consequently changing translation products. This finding considers as one of the most important reasons that may increase the resistance of bacteria depending on the new variation in the target gene (Hauser and Ozer., 2010).

Table 4.26: Mutations in the nucleotide sequence of *ica*A genes and related changes in proteins of *S. aureus* Gen Bank: CP002388.1.1

No. of sample	Wild type	Mutant type	Location	Change in amino acid	Type of mutation	Effect	Type of substitution
M NANO 1	AAA	AGA	2706786	Arg > Lys	Substitution	Missense	Transition
	AT-	ATT	2706789-2706790	Insertion T	Insertion	Ideation	Frameshift
	TAT	GAT	2840350	Leu > Ser	Substitution	Missense	Transversion
	AAG	GAG	2707529	Glu> Lys	Substitution	Missense	Transition
	TTC	TT-	2706793	Deletion C	Deletion	Frameshift	Deletion
	-GG	CGG	2706895-2706896	Insertion C	Insertion	Ideation	Frameshift
	TGC	TGC	2706976	Deletion C	Deletion	Frameshift	Deletion
	-TG	ATG	2707078-2707079	Insertion A	Insertion	Ideation	Frameshift
	ATT	AAC	2707272-2707273	Asn > Lie	Substitution	Missense	Transversion
	TAG	TCC	2707275-2707276	Asn > Lie	Substitution	Missense	Transversion
	GCT	CCT	2707277	Pro >Ala	Substitution	Missense	Transversion
M NANO 3	AGT	AAT	2706933	Asn > Ser	Substitution	Missense	Transition
	ACT	ACA	2707471	Thr> Thr	Substitution	Silent	Transversion
M NANO 4	AT-	ATT	2706789-2706789	Insertion T	Insertion	Ideation	Frameshift
	TTC	TT-	2706793	Deletion C	Deletion	Frameshift	Deletion
	TGC	TG-	2706976	Deletion C	Deletion	Frameshift	Deletion
	-TG	ATG	2707078-2707079	Insertion A	Insertion	Ideation	Frameshift
M NANO 7	TTC	TT-	2706793	Deletion C	Deletion	Frameshift	Deletion
	-GG	CGG	2706895-2706896	Insertion C	Insertion	Ideation	Frameshift
	CAC	TAC	2706956	Tyr >His	Substitution	Missense	Transition
	TGC	TG-	2706976	Deletion C	Deletion	Frameshift	Deletion
	-TG	ATG	2707078-2707079	Insertion A	Insertion	Ideation	Frameshift
	ATT	AAC	2707272-2707273	Asn > Lie	Substitution	Missense	Transversion
	TAG	TCC	2707275-2707276	Asn > Lie	Substitution	Missense	Transversion
	GCT	CCT	2707277	Pro >Ala	Substitution	Missense	Transversion

* M NANO: Isolation by treatment with CuO-NPs.

Variation affects the *icaA* gene by altering gene regulation and function. The above results show that different types of the mutations occurred in the gene when treated with CuO-NPs isolates, which may have related to the effect of genetic variation on the phenotype because they lead to a change in amino acids and then in the protein. Or bind to DNA nucleotides and open it to parts (Burgett *et al.*2012*)*. Nanoparticles can be smaller than bacterial pores especially nuclear pores, and therefore pass through the cell membrane, disrupt their function or interfere with DNA or protein synthesis (Bagchi *et al.*2013). The small size of CuO-NPs make it used as antimicrobial materials. Its availability makes it a perfect option as an antibacterial agent (Grass *et al.*2011*)* Nanoparticle size Should be below 100 nm, that will allow interaction between microbial membranes, enhancing NP effect even more.

Copper oxide generates toxic hydroxyl radicals (OH^-), that damage cell membranes of Gram-negative and positive bacteria (Sçnchez-Sanhueza *et.al,*2016). Copper Molecule working on abiotic surfaces showed that along with the rapid death of antibiotic-resistant strains, the destruction of plasmid genomic DNA has had an effect in preventing the spread of infection and gene transfer (Grass *et.al,*2011*;* Lok *et.al,*2007*).* Also Copper produce Hydroxyl radicals work as scavenger which eliminate the damaged DNA (Burkitt *et.al,*2000*)* Or maybe eliminate the weak region from DNA. The results in the above table indicated that the deletion mutation was repeated 4 times at the cytosine base, resulting in a breakthrough from Frameshift. The results also showed that a breakthrough in the type of deletion, also for the same nitrogen base. From previous finding cooper oxides looks promising alternative antibacterial materials with high effect as toxic materials by producing toxic hydroxyl radicals that damage cell membranes of bacteria (Sçnchez-Sanhueza *et al.*2016). Moreover, the most

effect noticeable from table 4.26 that Cytosine deleted much more than any other nitrogen base as evolutionary machine process, however this result agree with previous Iraqi work on *CupA* gene (Alzubaidy *et al.*2019*).* All previous findings emphasize that there is a real needing for more investigation in order to used CuO-NPs as promising alternative antibacterial materials.

4.8.2 Nucleotides Sequencing of *icaD* gene of *S. aureus*

By analyzing the results of the sequencing of the *icaD* gene according to the table 4.27, the data showed that 4(57.14%) mutations substitution, 3 (42.85%) Deletion mutations, this shows that the type and location of mutations found may result in different mutations.

Any DNA damage observed is due to the breakdown of dead cells in common oxidative lethality pathways (Grass *et al.,* 2011). The methods used to determine this was designed to detect small changes in the DNA which are then PCR amplified and also using mutagenicity assays (Espirito Santo *et al.,* 2011). We have observed, however, that the bulk of DNA in Staphylococcus is rapidly degraded upon exposure to copper by analysis of purified DNA and DNA fragmentation of intact cells in a wet fomite inoculum assay (Warnes *et al.,* 2010).

Table 4.27: Mutations in the nucleotide sequence of *icaD* genes and related changes in proteins of *S. aureus* Gen Bank: CP002388.1.1

No. of sample	Wild type	Mutant type	Location	Change in amino acid	Type of mutation	Effect	Type of substitution
* M NANO 1	AGT	AAT	2707697	Asn > Ser	Substitution	Missense	Transition
M NANO 1& M NANO 2	AGA	A-A	27077835	Deletion G	Deletion	Frameshift	Deletion
M NANO 2	GAA	GAG	27077839	Glu> Glu	Substitution	Silent	Transition
M NANO 1& M NANO 2	GAC	GA-	2707692	Deletion C	Deletion	Frameshift	Deletion
M NANO 1& M NANO 2	TTC	TT-	2707773	Deletion C	Deletion	Frameshift	Deletion
M NANO 1& M NANO 2	GTT	GTA	27077791	Thr> Thr	Substitution	Silent	Transversion
M NANO 2	AAA	AGA	27077829	Arg > Lys	Substitution	Missense	Transition

*** M NANO: Isolation by treatment with CuO NPs.**

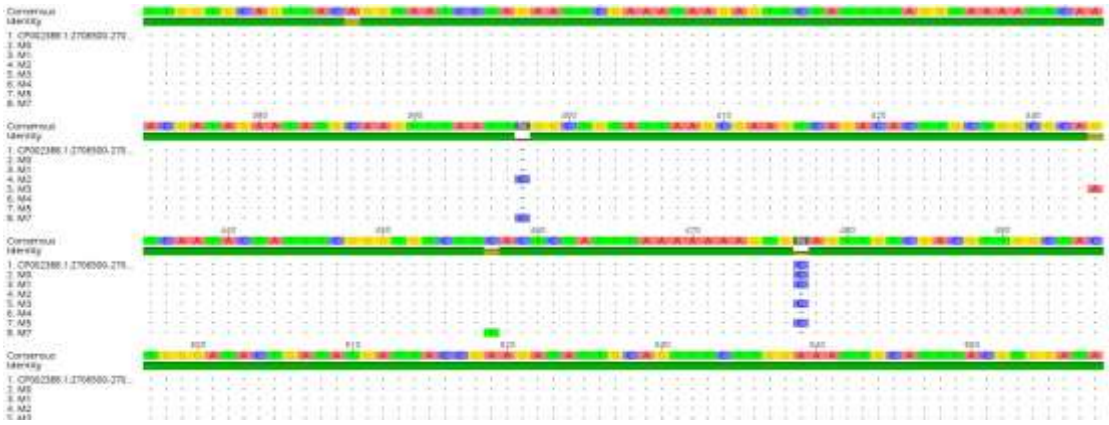


Figure 4.21: DNA sequences observed in four *S. aureus* isolates compared with the reference sequences of isolates *S. aureus* Gen Bank: CP002388.1.1. by Geneious Software

4.8.3 Nucleotides Sequencing *cupA* gene of *P. aeruginosa* isolates

The sequences of the nitrogen bases were determined for isolates of *P. aeruginosa* after reading the DNA sequences of the forward and reverse strands, recollecting the two strands, deleting the anomalies in either of them, analysis and matching them to (NCBI) online. And Geneious Software has shown that these sequences are very similar to *cupA* gene sequences 99 %. By comparing the observed DNA sequences of these samples with their stored reference sequences (Gen Bank: CP037925.1).

By analyzing the results of the sequencing of the *cupA* gene according (Table 4.28), the data showed that 4(36.36%) mutations substitution, 6 (54.54%) Deletion mutations and 1(9.09%) Insertion mutations, this shows that the type and location of mutations found may result in different mutations. Some of these mutations lead to changes in the genetic codes and thus change in the amino acids in translation, which are the most important reasons that increased the resistance of this gene (Burgett *et al.*2012*).*

Table 4.28: Changes in the nitrogen bases of sequences of isolates of *P. aeruginosa* Gen Bank: CP037925.1

No. of sample	Wild type	Mutant type	Location	Change in amino acid	Type of mutation	Effect	Type of substitution
M NANO	CTC	CT-	2840186	Deletion C	Deletion	Frameshift	Deletion
	CGC	- CG	2840238	Deletion C	Deletion	Frameshift	Deletion
	CCC	CTC	2840287	Pro> Ala	Substitution	Missense	Transition
	CAA	C - -	2840344 - 2840345	Deletion A	Deletion	Frameshift	Deletion
	TTG	TAG	2840350	Leu > Ser	Substitution	Missense	Transversion
	ACC	A - -	2840357- 2840358	Deletion C	Deletion	Frameshift	Deletion
	AGC	AG-	2840381	Deletion C	Deletion	Frameshift	Deletion
	CCC	-CC	2840382	Deletion C	Deletion	Frameshift	Deletion
	AAC	ACC	2840434	Asn> His	Substitution	Missense	Transversion
	C-T	CC T	2840438- 2840439	Insertion C	Insertion	Ideation	Frameshift
	CTC	GCA	2840444	Ser >Gly	Substitution	Missense	Transversion

*** M NANO: Isolation by treatment with CuO-NPs.**

Variation affects the *cupA* gene by altering gene regulation and function. The above results show that 84.61% of the mutations occurred in the gene when treated with CuO-NPs isolates, compared with 15.38% mutation recorded for isolation that were not treated with CuO-NPs, which may lead to the effect of the phenotype because they lead to a change in amino acids and then in the protein. Or bind to DNA nucleotides and open it to parts (Grass.; Rensing, and Solioz, 2011). Nanoparticles can be smaller than bacterial pores, and therefore pass through the cell membrane, disrupt their function or interfere with DNA or protein synthesis (Lok, and Chen, 2007).

Because of its small size, CuO-NPs make used as antimicrobial materials. Its availability makes it an important option as an antibacterial agent. Nanoparticle size it should not exceed 100 nm, allowing close interaction with microbial membranes, enhancing its effect even more. Copper generates toxic hydroxyl radicals that damage cell membranes of Gram-negative and Gram-positive bacteria (Sçnchez-Sanhueza *et al.*2016). that may make it promising alternative drug to treat foot ulcer infection for Iraqi diabetic patients instead of traditional non effective treatment by antibiotics in Iraqi hospital as mentioned by (AL-Shimmary *et al.*2016*)*

Copper Molecule working on abiotic surfaces showed that, along with the rapid death of antibiotic-resistant strains, the destruction of plasmid genomic DNA has had an effect in preventing the spread of infection and gene transfer (Warnes *et al.*2012*;* Bagchi *et al.*2013*).*

The results indicated in the above table that the deletion mutation was repeated 5 times at the cytosine base, resulting in a breakthrough from Frameshift. The results also showed that a breakthrough in the type of Ideation, also for the same nitrogen base. From previous finding cooper oxides looks promising alternative antibacterial materials with high effect

as toxic materials by producing toxic hydroxyl radicals that damage cell membranes of bacteria (Sçnchez-Sanhueza *et al*.2016). Moreover, the most effect noticeable from table 6 the deletion of Cytosine more than any other nitrogen base that needs more investigation.

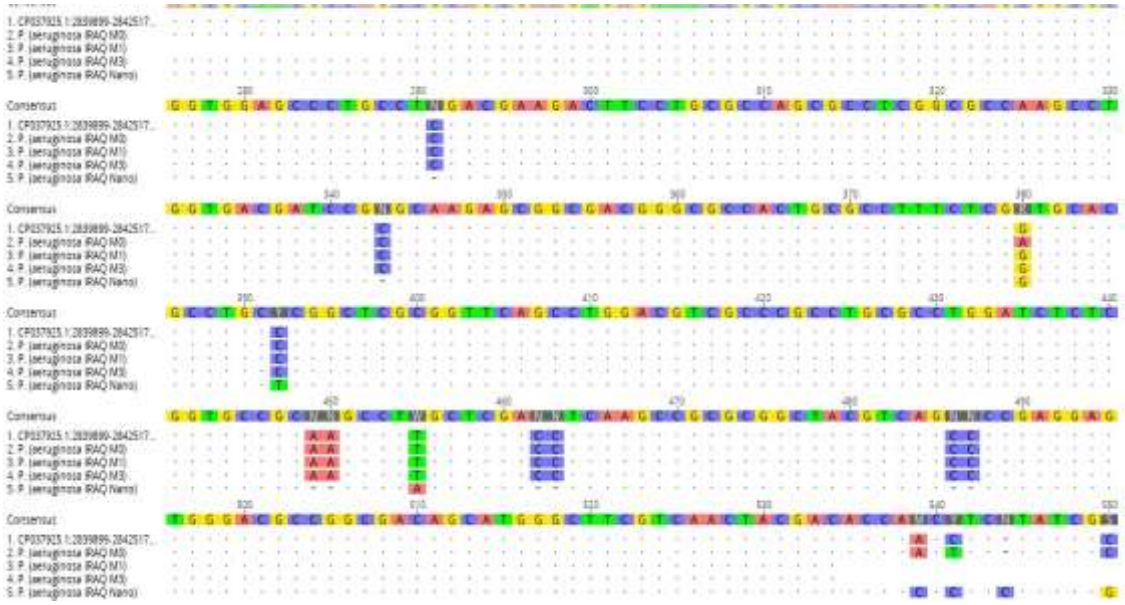


Figure4.23: DNA sequences observed in four *P. aeruginosa* isolates compared to the reference sequences isolates of *P. aeruginosa* Gen Bank: CP037925.1. By Geneious Software

Conclusions: -

1- L-Selectin Gene Promoter (rs2205849) is associated with the increased risk of Diabetic Foot ulcer the genotype AA and AG has a higher frequency in T2DM Iraqi patients, allele G show high distribution with as a protective allele from the disease.

2- E-Selectin rs5355 C>T Polymorphism, is associated with the increased risk of Diabetic Foot ulcer , the GA genotype has a higher frequency in Iraqi Diabetic Foot ulcer, and allele A with significant with Patients.

3- E-Selectin rs5368 C>T Polymorphism, alleles C and T facilitated the occurrence of disease of Diabetic Foot ulcers , the CC genotype has a higher frequency in Iraqi Diabetic Foot ulcer.

4- E-Selectin rs5367 T>C Polymorphism, showed that the common allele T is that is considered as a dangerous agent, allele C the most protective.

5- A deletion mutation of the nitrogen base T in exon 9^{th} at site 6408 is present in most of the present study.

6- High levels of total cholesterol, LDL, VLDL as well as BMI and the Abdominal Circumference are significantly associated with the occurrence and development of foot ulcer and can be used as screening tests for Diabetic Foot ulcer in Iraqi population.

7- Increased levels of insulin were associated with insulin resistance due to the presence of diabetes in the patients.

8- bacterial study showed gram positive bacteria *S. aureus* has been chosen and negative *P. aeruginosa* both of them represent the high frequent infection of isolated bacteria from Diabetic Foot ulcer patients.

9- The results showed a significant relationship between the *icaA* gene and rs5355 for the E-selectin gene.

10- Isolates collected from patients in various hospitals in Baghdad and Diyala were contains many mutations before being treated.

11- CuO-NPs was prepared by the hydrothermal method.

12- Present results illustrated the increasing deletion effect for CuO-NPs on the Cytosine nitrogen base.

Recommendations: -

1-Study the other segment of L-selectin and E-selectin genes of the molecular level with increasing size sample.

2-Study of the relationship of the E-selectin and L-selectin with other diseases in terms of immunological and molecular.

3-Examining the gene expression for each E-selectin and L-selectin, which may provide more information regarding the association with a foot ulcers in Iraqi patients.

4-Studding the sequence of genetic variation for bacterial adhesion genes *icaA*, *icaD*, *CupA* and for others types bacteria in a sample of the Iraqi population.

5-Preparing Nanoparticles by using a variety of preparation techniques including, physical, chemical and biological, developed a modulated method.

6-Effect of copper oxide nanoparticles on the Gene expression of virulence gene before and after treatment.

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Appendix (1)

موافقة عن عدم ممانعة للمشاركة في البحث

اسم الباحث الرئيسي. مهند وهيب مهدي الزبيدي

اسم المؤسسة :جامعة ديالى/ كلية التربية للعلوم الصرفة قسم علوم الحياة

اسم مشروع البحث: تغايرات جينات الالتصاق بين مرضى قدم السكري والبكتيريا المعاملة بالمواد النانوية

المعلومات: أني مهند وهيب مهدي الزبيدي، طالب دكتوراه في كلية التربية للعلوم الصرفة قسم علوم الحياة جامعة ديالى. اقوم بأجراء بحث عن تغايرات جينات الالتصاق بين مرضى قدم السكري والبكتيريا المعاملة بالمواد النانوية وذلك بالتحري عن الطفرات في جينات قيد الدراسة والتي قد تكون ذات علاقة بازدياد الاصابة بمرض القدم السكري في عينات الدم والمسحات المأخوذه من منطقة القدم التي استُحصلت ضمن الفحوصات الروتينية وبدون تعرض المرضى لأي خطر او أجراء او علاج إضافي غير المقرر لهم .

علماً أني سأوثق المعلومات الخاصة بكل مريض وسأفحص العينات ثم أقوم بخزنها لاحتمالية اجراء فحوصات اضافية عليها داخل البلد وخارجه مستقبلا. لك كل الحق (او ولي الامر) ان تقبل طواعية او ترفض الاشتراك في هذا البحث .

تأييد الموافقة :بعد قرأتي المعلومات أعلاه (أو قُرئت لي) وكانت الفرصة سائحة لي سؤال اي سؤال وسماع الاجابة، أوافق طواعية على الاشتراك في هذا البحث من خلال منح عيناتي من الدم والموافقة على خزنها او اجراء اي فحص مناسب عليها مستقبلاً داخل العراق وخارجه.

Appendix (2)

معلومات حول المصابين بالقدم السكري **رقم الإستبانة :( )**

اسم المريض : ..... العمر : ..... الجنس : .....
السكن : ..... الوزن : ..... فصيلة الدم .....
الطول : ..... الكتلة الجسمية : ..... محيط البطن : .....
المستوى الثقافي : ..... المستوى المعيشي : ..... رقم الهاتف : .....

*** مدة بدء الإصابة بالمرض :**

☐ **اقل من 5سنة** ☐ **اقل من 10 سنوات** ☐ **اقل من 15 سنة**

*** الطريقة التي تستعملها بالمعالجة :**

☐ **حميـة** ☐ **حبوب** ☐ **أنسولين تذكر الجرعة**

*** هل لديك أمراض أخرى مرافقة :**

☐ **بدانـة** ☐ **ارتفاع ضغط** ☐ **ارتفاع شحوم** ☐ **اخرى تذكر :**

*** هل أنت مدخن :**

☐ **أكثر من 20 سيجارة** ☐ **أقل من 20 سيجارة** ☐ **لا أدخن**

*** هل يوجد من أفراد أسرتك مصاب بالسكري :**

☐ **الوالد** ☐ **الوالـدة** ☐ **الأخوة أو الأخوات** ☐ **الزوجـة**

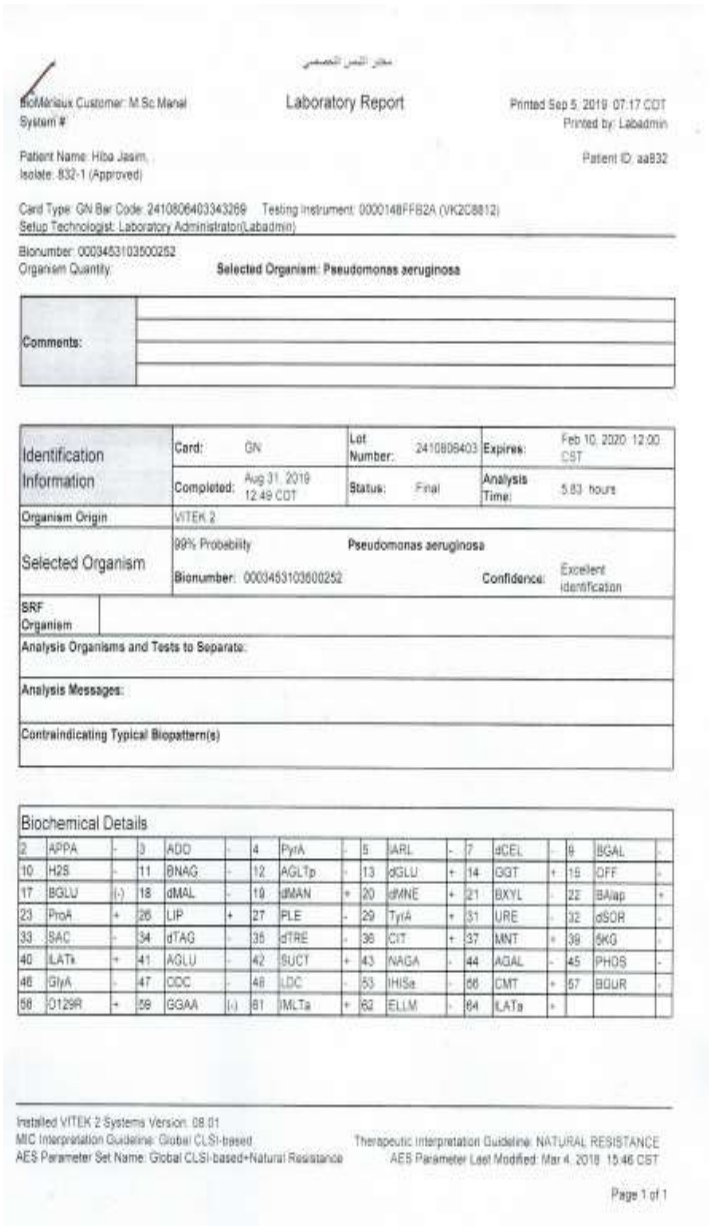
*** هل يحدث عندك بشكل متكرر :**

☐ **قرحات بالقدم أو تشققات** ☐ **فطور بين أصابع القدم** ☐ **إسهـال** ☐ **إمسـاك** ☐ **نوبـات إغمـاء**

*** هل تقوم بالعناية بالقدمين عن طريق :**

☐ **قص الأظافر بشكل مستقيم** ☐ **علاج الجروح والكدمات بشكل سريع** ☐ **علاج الفطـور** ☐ **تجفيف القدمين بعد الغسيل** ☐ **تجنب المشي حافياً** ☐ **تجنب لبس الأحذية الضيقة** ☐ **لا أقوم بكل ما سبق.**

Appendix (3)



مختبر النمس التخصصي

BioMérieux Customer: M.Sc.Manal
System #

Laboratory Report

Printed Sep 5, 2019 07:17 CDT
Printed by: Labadmin

Patient Name: Hiba Jasim,
Isolate: 832-1 (Approved)

Patient ID: aa832

Card Type: GN Bar Code: 2410806403343269 Testing Instrument: 0000148FFB2A (VK2C8812)
Setup Technologist: Laboratory Administrator(Labadmin)

Bionumber: 0003453103600252
Organism Quantity:

Selected Organism: Pseudomonas aeruginosa

Comments:	

Identification Information	Card:	GN	Lot Number:	2410809403	Expires:	Feb 10, 2020 12:00 CST
	Completed:	Aug 31, 2019 12:49 CDT	Status:	Final	Analysis Time:	5.83 hours
Organism Origin	VITEK 2					
Selected Organism	99% Probability		Pseudomonas aeruginosa			
	Bionumber:	0003453103600252			Confidence:	Excellent identification
SRF Organism						
Analysis Organisms and Tests to Separate:						
Analysis Messages:						
Contraindicating Typical Biopattern(s)						

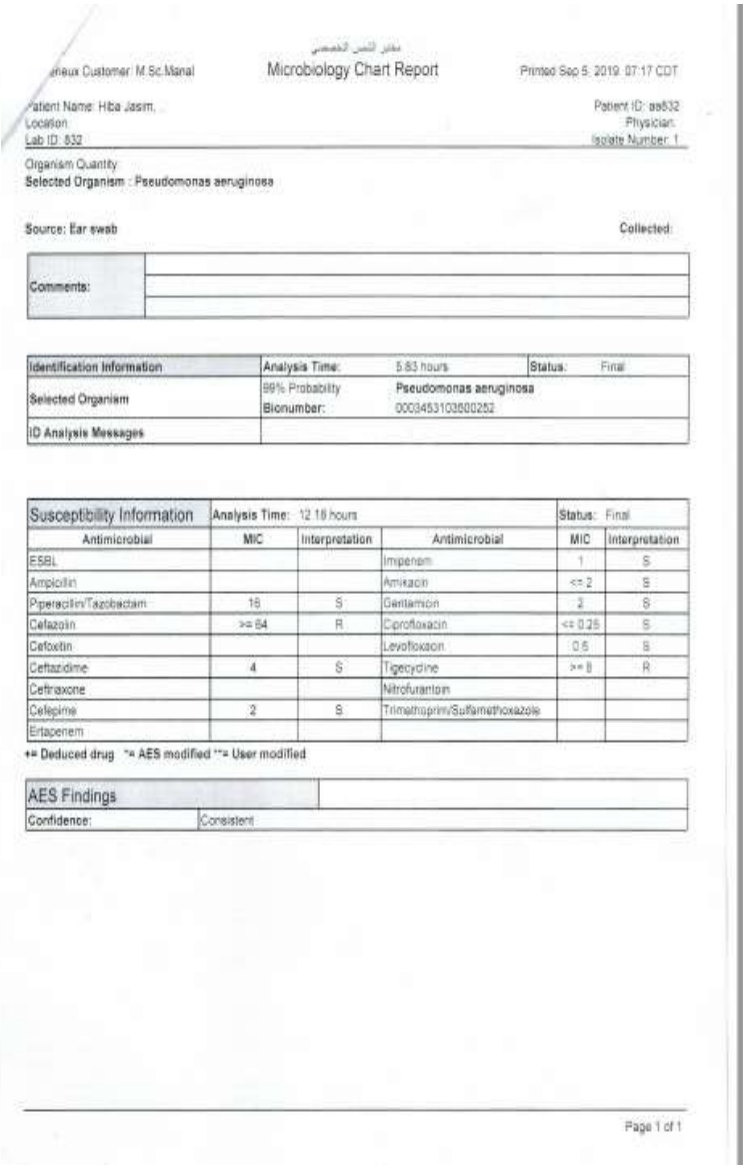
Biochemical Details

2	APPA	-	3	ADO	-	4	PyrA	-	5	ARL	-	7	dCEL	-	8	BGAL	-
10	H2S	-	11	BNAG	-	12	AGLTp	-	13	dGLU	+	14	GGT	+	15	OFF	-
17	BGLU	(-)	18	dMAL	-	19	dMAN	+	20	dMNE	+	21	BXYL	-	22	BAlap	+
23	ProA	+	26	LIP	+	27	PLE	-	29	TyrA	+	31	URE	-	32	dSOR	-
33	SAC	-	34	dTAG	-	35	dTRE	-	36	CIT	+	37	MNT	+	39	5KG	-
40	LATa	+	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	-	45	PHOS	-
46	GlyA	-	47	CDC	-	48	LDC	-	53	IHISa	-	56	CMT	+	57	BGUR	-
58	O129R	+	59	GGAA	(-)	61	IMLTa	+	62	ELLM	-	64	ILATa	+			

Installed VITEK 2 Systems Version: 08.01
MIC Interpretation Guideline: Global CLSI-based
AES Parameter Set Name: Global CLSI-based+Natural Resistance

Therapeutic Interpretation Guideline: NATURAL RESISTANCE
AES Parameter Last Modified: Mar 4, 2019 15:46 CST

Page 1 of 1



مختبر النمس التخصصي

BioMérieux Customer: M.Sc.Manal

Microbiology Chart Report

Printed Sep 5, 2019 07:17 CDT

Patient Name: Hiba Jasim,
Location:
Lab ID: 832

Patient ID: aa832
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Pseudomonas aeruginosa

Source: Ear swab

Collected:

Comments:	

Identification Information	Analysis Time:	5.83 hours	Status:	Final
Selected Organism	99% Probability	Pseudomonas aeruginosa		
	Bionumber:	0003453103600252		
ID Analysis Messages				

Susceptibility Information	Analysis Time: 12.18 hours			Status: Final	
Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
ESBL			Imipenem	1	S
Ampicillin			Amikacin	<= 2	S
Piperacillin/Tazobactam	16	S	Gentamicin	2	S
Cefazolin	>= 64	R	Ciprofloxacin	<= 0.25	S
Cefoxitin			Levofloxacin	0.5	S
Ceftazidime	4	S	Tigecycline	>= 8	R
Ceftriaxone			Nitrofurantoin		
Cefepime	2	S	Trimethoprim/Sulfamethoxazole		
Ertapenem					

+= Deduced drug *= AES modified **= User modified

AES Findings	
Confidence:	Consistent

Page 1 of 1

Laboratory Report

Printed: Jan 7, 2019 14:27 CST
Printed by: Labadmin

Selected Organism: Staphylococcus aureus

Identification Information	Card: GP	Lot Number: 2420047402	Expires: Sep 4, 2019 13:00 CDT
	Completed: Jan 7, 2019 04:32 CST	Status: Final	Analysis Time: 4.87 hours
Organism Origin	WTEK 3		
Selected Organism	99% Probability	Staphylococcus aureus	
	Bionumber: 1303052034643l1		Confidence: Acceptable identification

Biochemical Details

1	NAY	+	14	PRLC	-	27	4XYL	-	40	ADH1	+	53	MGAL	+	11	AGLU	-
13	GPPA	-	14	CDEX	-	15	AspA	-	16	BGAR	-	17	AMAN	+	18	PHOS	-
22	LeuA	-	23	ProA	-	24	BGUR	-	25	AGAL	+	26	PyrA	-	27	BGUR	+
28	AlaA	-	29	TyrA	+	30	dSOR	-	31	URE	-	32	POLYB	-	37	dGAL	-
38	GGT	+	39	LATk	+	42	LAC	-	44	NAG	-	45	dMAL	-	46	BACI	+
47	NOVO	-	50	NOS.5	+	52	dMAN	+	53	dMNE	+	54	MNE0	+	58	PUL	-
67	dRAF	-	68	0128R	-	69	BAL	+	83	DAC	+	82	dTRE	+	83	ADHGa	-
84	CATD	+															

Page 1 of 1

Microbiology Chart Report

Printed: Jan 7, 2019 14:28 CST

Selected Organism : Staphylococcus aureus

Identification Information	Analysis Time: 4.87 hours	Status: Final
Selected Organism	99% Probability Bionumber: 1303052034643l1	Staphylococcus aureus
ID Analysis Messages		

Susceptibility Information	Analysis Time: 13.90 hours			Status: Final	
Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
Oxacillin Screen	POS	+	Teicoplanin	<= 0.5	S
Benzylpenicillin	<= 0.03	S	Vancomycin	<= 0.5	S
Cefoxitin	<= 0.25	S	Tetracycline	<= 1	S
Ceftaroline	<= 0.5	S	Tigecycline	<= 0.12	S
Clindamycin	<= 1	S	Fosfomycin		
Nitrofurantoin	<= 0.12	S	Nitrofurantoin	<= 16	S
Ciprofloxacin	<= 0.25	S	Fusidic Acid	2	R
Inducible Clindamycin Resistance	NEG	-	Mupirocin		
Erythromycin	0.5	S	Rifampicin	1	S
Levofloxacin	<= 0.5	S	Trimethoprim/Sulfamethoxazole	<= 10	S
Linezolid	2	S			

<= Reduced drug '+ AES modified "+ User modified

AES Findings	
Confidence	Inconsistent

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الخلاصة

أجريت هذه الدراسة على 100 فرد عراقي اذ كانوا 50 مريضاً (30 ذكور و 20 إناث) تتراوح أعمارهم بين (94-43) سنة مصابين بمرض قرحة القدم السكرية، و 50 شخص من الاصحاء (27 ذكور و 23 إناث) تراوحت أعمارهم (66-27) ، الذين كانوا مراجعين دوريين للمستشفى في بغداد و بعقوبة ، من شباط إلى كانون الاول لعام 2018، تم إعداد قائمة الاستبيان (الملحق 1) لمرضى السكري، واخذت المعلومات من حيث العمر والجنس وتاريخ الأسرة من مرض السكري ، ومدة المرض ، والطول (محيط البطن)، ووزن الجسم الذي تم قياسه وتسجيله (كغم). ثم حدد للمرضى والاصحاء اختبارات: السكر الصيامي في الدم (FBG) ، الهيموغلوبين السكري (HbA1c) ، الملف الشخصي للدهون (TC، TG، HDL، LDL و VLDL) ، الأنسولين ، الأنسولين المقاوم (HOMO IR) ، ومؤشر تصلب الشرايين. بالإضافة إلى ذلك ، أجريت دراسات تعدد الأشكال لجين الالتصاق والبكتيريا في مختبر البيولوجيا الجزيئية في قسم الحياة كلية التربية للعلوم الصرفة في جامعة ديالى.

الهدف من هذه الدراسة هو تقييم العلاقة بين جينات الالتصاق بقرحة القدم المصابة بالسكري وجينات الأغشية الحيوية للبكتيريا ، ومن ناحية أخرى ، تأثير أوكسيد النحاس النانوي على البكتيريا المعزولة من قرحة مرضى السكري.

أظهر تعدد الأشكال الوراثية لجين L-selectin rs2205849 ان نسبة النمط الوراثي AA كانت عالية التردد في مجموعة المرضى مقارنة مع مجموعة الاصحاء وبلغت 67.5 و 56 ٪ ، على التوالي وبلغ مسبب عامل الخطر 1.63.

أظهرت نتائج تعدد الأشكال الجينية لجين E-selectin خمس مواقع في منطقة تضخيم جين E-Selectin Leu554Phe. اذ كان الجزء المضخم الذي تم تمديده من نهاية intron 8 الى exon 11 من جين E-Selectin ، يحتوي على المواقع التالية: rs5355 (G>A)، rs5368 (C>T)، rs751151130 (G> A)، (T> Del) ، و rs5367 (C> T).

أظهرت النتائج الحالية زيادة معنوية في متوسط اختبارات الكيمياء الحيوية: السكر الصيامي في الدم (FBG)، السكر التراكمي (HbA1c)، الكولسترول الكلي(TC)، بروتينات دهنية عالية الكثافة (HDL) ، بروتينات دهنية منخفضة الكثافة (LDL) ، بروتينات دهنية منخفضة الكثافة (VLDL) ، الدهون الثلاثية

(TG) ، الأنسولين ،(HOMO IR)، ومؤشر تصلب الشرايين(AIP) لدى المرضى مقارنة مع مجموعة الاصحاء .

أظهرت نتائج الجانب البكتريولوجي أنه تم اختيار بكتريا موجبة واخرى سالبة لصبغة للجرام: *S. aureus* و *P.aeruginosa*. وكلاهما يمثلان عدوى متكررة عالية من البكتيريا المعزولة من مرضى القدم السكرية بواقع 16 عزلة و 10 عزلات على التوالي.

تم تحديد الأنماط الوراثية الأكثر شيوعًا في جين E-Selectin لخمس مواقع AG: rs5355، CC: rs5368، GG: rs751151130، TT: rs5367 و T> Del ، كان تردد كل نمط جيني 75٪ ، 60٪ ، 60 ٪ 70 ٪ و 77.5 ٪ ، على التوالي. هذه النتائج تتوافق مع تباين جين *cupA* في المواقع 2840275 و 2840436 حيث تم تسجيل طفرة من النوع المغلظة.

أُستخدمت الجسيمات النانوية بشكل متزايد كمضادات حيوية بديلة ضد البكتيريا. قد تكون تقنية النانو مفيدة بشكل خاص في علاج الالتهابات البكتيرية وآليات مضادة للجراثيم من الجسيمات النانوية ضد البكتيريا والعوامل التي تشارك في المقارنة. تم تقييم نتيجة الجسيمات النانوية على البكتيريا من خلال دراسة التباين الوراثي في الجينوم البكتيري قبل وبعد المعاملة وهو الهدف من هذه الدراسة. وأظهرت النتائج الحالية التباين الوراثي لجينات الالتصاق البكتيري: *icaA* و *icaD* و *CupA* يتضح تأثير الحذف المتزايد من جراء تأثير اوكسيد النحاس النانوي على قاعدة السايتوسين .

وزارة التعليم العالي والبحث العلمي
جامعة ديالى
كلية التربية للعلوم الصرفة

التغايرات في جينات الالتصاق الدمية وجينات البايوفلم لبكتريا القدم السكري مع تأثير دقائق أوكسيد النحاس النانوية في جينات بايوفلم البكتيريا

أطروحة مقدمة إلى
مجلس كلية التربية للعلوم الصرفة جامعة ديالى - وهي جزء من متطلبات نيل درجة الدكتوراه فلسفة في علوم الحياة

من قبل
مهند وهيب مهدي الزبيدي

بكلوريوس علوم حياة / كلية التربية للعلوم الصرفة جامعة ديالى- 2009
ماجستير علوم حياة/ كلية التربية للعلوم الصرفة جامعة ديالى- 2012

بإشراف

أ.د عمار احمد سلطان
أ.د أسماء محمد صالح المهيدي

1441 هـ
2019 م