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College of Sciences

Department of Biology

Molecular Characterization of Predominant Biofilm Forming Bacteria and Identification of Some Biochemical and Immunological Parameters Associated with Diabetic Foot Infection

A Thesis

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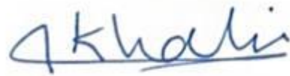
Signature: 

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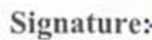
Signature: 
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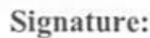
Signature: 
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Dedication

This work is reverently dedicated to:

Al-Imam Al-Mahdi (May Almighty Allah hasten the reappearance of him),

My country, Iraq, and all of its citizens.

All of the Iraqi martyrs, without whom we would never be here.

My spiritual inspiration, my darling father.

and My family.

Sarah 2024

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Summary

Summary

This study involved the isolation and identification of the most common species of bacteria responsible for diabetic foot infection along with their antibiotic susceptibility patterns and investigation of biofilm production of these bacteria as well as molecular detection of some virulence and antibiotic resistance genes .The current study, additionally, accompanied to find differences in the levels of some inflammatory markers including Toll like Receptor-2 (TLR-2), Interleukin-17A (IL-17A) and C-reactive protein (CRP) along with some antioxidants such as Superoxide dismutase (SOD) and glutathione (GSH) among type 2 diabetic subjects with and without diabetic foot ulcers and in healthy subjects.

A total of 142 swab samples were taken from diabetic ulcer patients checked in Al-Imam Al-Hasan center for endocrinology and diabetes and bacteriological examination was performed while 120 subjects enrolled in the current study for determination of biochemical and immunological markers divided into three groups :40 participants were type 2 diabetes mellitus patients with diabetic foot ulcer, 40 participants were type 2 diabetes mellitus patients without diabetic foot ulcer, and 40 participants were healthy control subject.

Cultivation of swab results in 23/142 negative culture and obtaining of high rate of poly-microbial infection reached to 82% while mono-microbial rates were restricted in 18% of which, 63/142 (44 %) isolates were Gram positive and 56/142 (39.4 %) were Gram negative bacteria. Out of 142 included subjects, 74/119 (62.18%) were male and 45/119 (37.18%) were female and most subjects that have the highest number of isolates were aged (55-64) years 33(27.7%) while subjects aged ≥ 75 years have the least number of isolates 18 (15 %). The biochemical and microscopical tests were applied and results in obtaining of *Staphylococcus aureus* 25 (21%) isolates, *Staphylococcus epidermidis* 18 (15%), *Streptococcus agalactiae*

15 (13%) and *Enterococcus faecalis* 5 (4%), while of Gram negative bacteria, *Klebsiella pneumoniae* isolates were 25(21%) , *Pseudomonas aeroginosa* 13 (11%) , *Proteus mirabilis*10 (8%) and *Escherichia coli* 8 (7 %) . Performance of susceptibility tests of *S.aurues* results in all isolated *Staphylococcus aureus* was maximum sensitive (84%) to Levofloxacin, while the lowest sensitivity (36%) was toward Azithromycin and Erythromycin , 40% of the sensitivity were toward Penicillin, Amoxicillin-Calvulonic Acid, Ceftazidime, Cefepime Cefotaxim, Ceftriaxone, Imipenem, Meropenem, Aztronem and Oxacillin, while the sensitivity of both Rifampin and Vancomycin were 52% and Ciprofloxacin was 48%. On the other hand, *Klebsiella pneumoniae* exhibited the highest resistance 100% toward Gentamycin and Levofloxacin followed by 96% resistance toward Ciprofloxacin and 92% resistance toward Amikacin. With respect to Pipracillin-Tazobactam, Cefotaxim, Ceftriaxone and Tetracyclin, resistance percent was 88% while Amoxicillin-Clavulanic acid, Cetazidime, and Cefepime were 84% resistant. Furthermore, *K.pneumoniae* has 76% resistance toward Imipenem and Meropenem and 68% toward Doxycycline and 56% with respect to Rifampin.

According to biofilm formation, tube method showed that *S.aureus* and *K. pneumoniae* were biofilm producers while microtiter plate method showed that 60% of *S.aureus* and *K. pneumoniae* obtained from Diabetic foot infection (DFI) in the current study were strong producers for biofilm while 40% of them were moderate producers.

PCR technique was used to detect that 60% of *S.aureus* contained *mec*A gene, 64% contained (*icaA*, *sea* and *erm*C) genes. Finally, 80% contained *ica*D gene. However, 88% of *K.pneumoniae* contained *mrk*D gene , 100% contained *Cps* gene, 12% contained *K1* gene and 60% contained *k2A* gene.

Summary

Concerning biochemical markers, mean Toll-like receptor 2 (TLR-2) levels in DFI patients was (7.36±1.85) ng⁄ml which was significantly higher than in DM patients and control groups ($p \leq 0.001$) while in interleukin-17A (IL-17A), the mean of levels in DFI patients were (123.7±33.52) ng⁄L which was significantly higher than DM and control groups ($P \leq 0.001$) and C-reactive protein (CRP) showed a higher rate in DFI patients (92.9±78.26) mg⁄L which was significantly higher than DM and control groups ($p \leq 0.001$).

Concerning the antioxidants, superoxide dismutase (SOD) activity in DFI group patients exhibited decreased levels significantly (241.4±35.61)(U ⁄ L) ($p \leq 0.001$) compared to DM and healthy individuals as well as glutathione (GSH) whereas the mean levels of this marker in DFI group patients decreased significantly (26.586±2.77) µmole⁄ml ($p \leq 0.001$) compared to DM and healthy individuals

The current study concluded that *S.aureus* is the most predominant of Gram positive bacteria in DFI while *K.pneumoniae* is the most prevalent of Gram negative bacteria and both of which are biofilm forming and high resistant for common antibiotics.

List of Contents

No.	Contents	Page
	Chapter One **Introduction**	
1-1	Introduction	1
	Chapter Two **Literatures Review**	
2-1	Literature Review	4
2-1-1	Diabetes mellitus (DM)	4
2-1-1-1	Types of Diabetes mellitus	4
2-1-1-2	Major complications of Diabetes mellitus	5
2-1-2	Diabetic Foot Infections (DFI)	5
2-1-2-1	Definition and pathophysiology of DFI	5
2-1-2-2	Classification of DFU	7
2-1-2-2-1	Wagner's classification system	8
2-1-2-2-2	IWGDF/IDSA classification	8
2-1-3	Bacteriological spectrum of DFI	10
2-1-3-1	Gram positive bacteria	11
2-1-3-1-1	*Staphylococcus aureus*	11
2-1-3-1-1-1	Characteristics of *S. aureus*	12
2-1-3-1-1-2	Virulence Factors of *S.aureus*	12
2-1-3-1-1-3	Molecular characteristics of *S.aureus*	13
2-1-3-1-1-4	Resistance of *S. aureus* to antibiotics	15
2-1-3-2	Gram negative bacteria	16
2-1-3-2-1	*Klebsiella pneumoniae*	16
2-1-3-2-1-1	Characteristics of *K.pneumoiae*	16
2-1-3-2-1-2	Virulence factors of *K.pneumoniae*	17
2-1-3-2-1-3	*Klebsiella pneumoniae* serotype	17
2-1-3-2-1-4	Molecular characteristics of *K.pneumoniae*	19
2-1-3-2-1-5	Resistance of *K.pneumoniae* to antibiotics	19
2-1-4	Biofilm	20
2-1-4-1	Factors that affect biofilm development	21
2-1-4-2	Components of biofilm	21
2-1-4-3	Biofilm formation of *Staphylococcus aureus*	22
2-1-4-4	Biofilm formation of *Klebsiella pneumoniae*	24
2-1-5	Immunological response	25
2-1-5-1	Innate immunity response to diabetic foot infection	25

Contents

2-1-5-2	Immunologic receptors	26
2-1-5-2-1	Toll-Like Receptor2 (TLR2)	27
2-1-5-3	Interleukins	28
2-1-5-4	C-Reactive Protein (CRP)	29
2-1-6	Oxidation and Free radicals	30
2-1-6-1	The role of Oxidative stress in DFI	30
2-1-6-2	Antioxidants system	31
2-1-6-2-1	Superoxide dismutase (SOD)	31
2-1-6-2-2	Glutathione (GSH)	32
Chapter Three **Materials and Methods**		
3-1	Devices and Equipment	34
3-2	Chemicals, kits and culture media	35
3-2-1	Antibiotics	37
3-3	Study design	38
3-3-1	Groups of the study	38
3-3-1-1	Inclusion Creteria of patients	38
3-3-1-2	Exclusion Creteria of patients	38
3-3-2	Ethical consideration	39
3-3-3	Types of sample	39
3-4	Methods	40
3-4-1	Culture media	40
3-4-2	Reagents and solutions used for bacterial identification	40
3-4-3	Isolation and identification of bacteria causing DFI	41
3-4-3-1	Differentiation of gram positive and gram negative bacteria	41
3-4-3-2	Isolation and identification of Gram positive bacteria	43
3-4-3-2-1	Isolation and identification of *Staphylococcus aureus*	43
3-4-3-2-2	Isolation and identification of *Staphylococcus epidermidis*	44
3-4-3-2-3	Isolation and identification of *Streptococcus agalactiae*	44
3-4-3-2-4	Isolation and identification of *Enterococcus faecalis*	45
3-4-3-3	Isolation and identification of Gram negative bacteria	45
3-4-3-3-1	Isolation and identification of *Klebsiella pneumoniae*	45
3-4-3-3-2	Isolation and identification of other Gram negative bacteria	46
3-4-4	Susceptibility tests of bacteria against antibiotics	46

Contents

3-4-5	Maintenance the bacterial isolates	47
3-4-6	Molecular characterization of predominant biofilm forming bacteria	47
3-4-6-1	Molecular characterization of *Staphylococcus aureus*	47
3-4-6-1-1	DNA extraction of *S. aureus*	47
3-4-6-1-2	Molecular characterization of *S. aureus* by polymerase chain reaction PCR	49
3-4-6-2	PCR assay	50
3-4-6-3	Gel electrophoresis	53
3-4-6-4	Detection of amplification products	53
3-4-6-5	Molecular characterization of *Klebsiella pneumoniae*	54
3-4-6-5-1	DNA extraction of *Klebsiella pneumoniae*	54
3-4-6-5-2	Molecular characterization of *Klebsiella pneumoniae* by polymerase chain reaction PCR	55
3-4-6-6	PCR assay	55
3-4-7	Investigation of biofilm formation	58
3-4-7-1	Investigation of biofilm formation by tube method	58
3-4-7-2	Quantification of biofilm formation by Microtiter plate	59
3-4-8	Determination of some immunological parameters in serum	60
3-4-8-1	Determination of Toll-like receotor-2 (TLR-2)	60
3-4-8-2	Determination of Interleukin-17A (IL-17A)	62
3-4-8-3	Determination of C-reactive protein (CRP)	64
3-4-9	Determination of some antioxidants concentration in serum	65
3-4-9-1	Evaluation of Superoxide dismutase (SOD) activity	65
3-4-9-2	Evaluation of Glutathione (GSH) concentration	66
3-4-10	Statistical Analysis	68
Chapter Four **Results and Discussion**		
4-1	Isolation and identification of bacterial species causing Diabetic Foot Infection (DFI)	70
4-1-1	Distribution of bacterial isolates according to sex	73
4-1-2	Distribution of bacterial isolates according to age	75

Contents

4-1-3	Distribution of polymicrobial and monomicrobial growth in DFI	76
4-2	Identification of bacterial species isolated from DFI	78
4-2-1	Identification of Gram positive bacteria	78
4-2-1-1	Identification of *Staphylococcus aureus*	78
4-2-1-1-1	Cultivation on Mannitol salt agar (MSA)	78
4-2-1-1-2	Biochemical tests	78
4-2-1-1-3	Identification of *S.aureus* by API STAPH kits	79
4-2-1-2	Identification of *Staphylococcus epidermidis*	79
4-2-1-3	Identification of *Streptococcus agalactiae*	80
4-2-1-4	Identification of *Enterococcus faecalis*	81
4-2-2	Identification of Gram negative bacteria	81
4-2-2-1	Identification of *Klebsiella pneumoniae*	81
4-2-2-2	Identification of *Pseudomonas aeruginosa*	82
4-2-2-3	Identification of *Proteus mirabilis*	83
4-2-2-4	Identification of *Escherechia coli*	84
4-3	Susceptibility tests of the most prevalent bacterial species in DFI toward antibiotics	85
4-3-1	Susceptibility tests of *Staphylococcus aureus*	85
4-3-2	Susceptibility tests of *Klebsiella pneumoniae*	89
4-4	Investigation of Biofilm forming Bacteria	93
4-4-1-1	Investigation of Biofilm formation of *Staphylococcus aureus* by tube method	93
4-4-1-2	Investigation of Biofilm formation of *Klebsiella pneumoniae* by tube method	93
4-4-2	Quantification of Biofilm formation by microtiter plate method	94

Contents

4-4-2-1	Quantification of Biofilm formation of *S.aureus* by microtiter plate method	94
4-4-2-2	Quantification of Biofilm formation of *K.pneumoniae* by microtiter plate method	97
4-5	Molecular identification of the prevalent bacteria	100
4-5-1	Molecular identification of *S.aureus*	100
4-5-1-1	Molecular detection of *mec*A gene	100
4-5-1-2	Molecular detection of *ica*A gene	103
4-5-1-3	Molecular detection of *ica*D gene	105
4-5-1-4	Molecular detection of *sea* gene	107
4-5-1-5	Molecular detection of *erm*C gene	108
4-5-2	Molecular identification of *K.pneumoniae*	111
4-5-2-1	Molecular detection of *mrk*D gene	111
4-5-2-2	Molecular detection of *Cps* gene	114
4-5-2-3	Molecular detection of *K1* gene	116
4-5-2-4	Molecular identification of *K2A* gene	118
4-6	Immune response	120
4-6-1	Determination of Toll-like Receptor 2 (TLR2) levels	120
4-6-2	Determination of Interleukine-17A (IL-17A) levels	123
4-6-3	Determination of C-Reactive Protein (CRP) levels	125
4-7	Antioxidants	127
4-7-1	Evaluation of Superoxide dismutase (SOD) activity	127
4-7-2	Evaluation of Glutathione (GSH) concentration	129
4-8	Odd ratio	131
4-9	Receiver Operating Characteristic Analysis	132
Conclusions and Recommendations		
4-1	Conclusions	136
4-2	Recommendations	136
	References	137
	Appendices	181

List of Figures

No.	Figure Title	Page
2-1	Causative factors of diabetic foot formation	6
2-2	Interaction between metabolic, anatomical and bacteriological factor in DFI	7
2-3	Schematic representation of *S.aureus* resistance mechaniams	15
2-4	Biofilm formation mechanism by *S.aureus*	23
2-5	Organization of the type 3 fimbrial gene cluster in *K.pneumoniae*	24
3-1	Isolation and identification of bacteria that infect DFI	42
3-2	Standard curve of TLR-2	62
3-3	Standard curve of IL17-A	64
3-4	Standard curve of GSH	68
4-1	The percentage of Gram positive and Gram negative bacteria isolated from DFI	70
4-2	Distribution of bacterial isolates obtained from DFI	72
4-3	The percentage of poly-microbial and mono-microbial infections in DFI	76
4-4	Electrophoresis of the PCR reaction product of *S. aureus* using the specific primer of the *mec*A gene (310bp) using 1.5% Agarose gel , 70 voltages for 50 minutes	101
4-5	Electrophoresis of the PCR reaction product of *S. aureus* using the specific primer of the *ica*A gene (151bp) using 1.5% Agarose gel ,70 voltages for 50 minutes	104
4-6	Electrophoresis of the PCR reaction product of *S. aureus* using the specific primer of the *ica*D gene (211bp) using 1.5% Agarose gel , 70 voltages for 50 minutes	106
4-7	Electrophoresis of the PCR reaction product of *S. aureus* using the specific primer of the *sea* (102bp) using 1.5% Agarose gel, 70 voltages for 50 minutes	109
4-8	Electrophoresis of the PCR reaction product of *S. aureus* using the specific primer of the *ermC* gene (572bp) using 1.5% Agarose gel ,70 voltages for 50 minutes	110
4-9	Electrophoresis of the PCR reaction product of *K. pneumoniae* using the specific primer of the *mrk*D gene	112

	(340bp) using 1.5% Agarose gel, 70 voltages for 50 minutes	
4-10	Electrophoresis of the PCR reaction product of *K. pneumoniae* using the specific primer of the *Cps* gene (418bp) using 1.5% Agarose gel, 70 voltages for 50 minutes	115
4-11	Electrophoresis of the PCR reaction product of *K. pneumoniae* using the specific primer of the *K1* gene (1238bp) using 1.5% Agarose gel, 70 voltages for 75 minutes	117
4-12	Electrophoresis of the PCR reaction product of *K. pneumoniae* using the specific primer of the *K2A* gene (531bp) using 1.5% Agarose gel, 70 voltages for 50 minutes	119
4-13	Receiver operating characteristics (ROC) curve analysis of TLR-2 levels in Patient and Control, The area under the ROC curve: 92%	134
4-14	Receiver operating characteristics (ROC) curve analysis of Toll-like levels in Patients for DFI, The area under the ROC curve: 74.6%,	135
4-15	Receiver operating characteristics (ROC) curve analysis of Toll-like levels in Patient for DM, The area under ROC curve: 67.3%	135

List of Tables

No.	Title of Table	Page
2-1	Wagner's classification of DFU	8
2-2	IWGDF/IDSA classification	9
2-3	Virulence factors of *Staphylococcus aureus*	14
2-4	Virulence factors of *Klebsiella pneumoniae*	18
2-5	Ingredients of biofilm	22
2-6	Types of TLRs that recognized Bacteria	26
3-1	Instruments and apparatus used in current study	34
3-2	Disposable materials used in this study	35
3-3	Chemicals and kits used in current study	36
3-4	Antibiotics discs used in susceptibility test	37

Contents

3-5	Primers with their sequences and amplicon sizes of *Staphylococcus aureus*	49
3-6	Master mixture components	50
3-7	PCR tube components of *Staphylococcus aureus* genes	50
3-8	PCR machine programming for *mec*A gene amplification	51
3-9	PCR machine programming for *icaA and icaD* genes amplification	51
3-10	PCR machine programming for *sea* gene amplification	52
3-11	PCR machine programming for *ermC* gene amplification	52
3-12	Primers with their sequences and amplicon sizes of *Klebsiella pneumoniae*	56
3-13	PCR tube components of *Klebsiella pneumoniae* genes	56
3-14	PCR machine programming for *mrk*D gene amplification	57
3-15	PCR machine programming for *Cps* gene amplification	57
3-16	PCR machine programming for *K1* and K2A genes amplification	58
3-17	Interpretation of biofilm by the microtiter plate method	60
3-18	Reagents components and concentrations of CRP kit	65
3-19	Amounts of blank and sample solutions used	65
4-1	Percentage of patients with DFI according to sex	74
4-2	Distribution of bacterial isolates according to age of patients with DFI	77
4-3	Results of morphological and biochemical tests for Gram positive bacteria isolated from DFI	79
4-4	Results of morphological and biochemical tests for Gram negative bacteria isolated from DFI	83
4-5	Susceptibility tests of *S.aureus* toward antibiotics	86
4-6	Susceptibility tests of *K.pneumoniae* toward antibiotics	90
4-7	Association between biofilm formation and antibiotic resistance of *S.aureus*	96
4-8	Association between biofilm formation and antibiotic resistance of *K.pneumoniae*	99
4-9	Genes distribution among *S.aureus* isolates obtained in this study	102
4-10	Association of biofilm genes and biofilm production ability of *S.aurues*	107

Contents

4-11	Gene distribution among *K.pneumoniae* isolates obtained in this study	112
4-12	Association of biofilm gene and biofilm production ability of *K.pneumoniae*	114
4-13	Mean difference of some Biomarkers among the Three Studied Groups	120
4-14	The effect of Age on the Toll Like Receptor-2 levels according to the three studied groups	122
4-15	The effect of Age on the IL-17A levels according to the three studied groups	124
4-16	The effect of Age on the CRP levels according to the three studied groups	126
4-17	Mean difference of some antioxidants among the Three Studied Groups	127
4-18	The effect of age on SOD activity according to the three studied groups	128
4-19	The effect of age on GSH concentration according to the three studied groups	130
4-20	Estimation the Associated of the analyzed factors in Diabetic foot infection disease two cases Compared to the control group	131
4-21	Receiver operating characteristic curve showing sensitivity and specificity of TRL-2 in DM patients compared to control	133
4-22	Receiver operating characteristic curve showing sensitivity and specificity of TRL-2	133

List of Abbreviations

Abbreviation	Description
ABR-Kp	Antibiotic-resistant *Klebsiella pneumoniae*
AGEs	advanced glycation end products
AMEs	aminoglycoside-modifying enzymes
API	Analytical profile index
ARA	arabinose
BPS	Phosphate Buffer saline
CAMPs	Cyclic Adinosine mono phosphate
CoNS	Coagulase Negative *Staphylococci*
cps	capsular polysaccharide synthesis

Contents

CRP	C-reactive protein
D.W.	Distilled water
DAMPs	Damage-associated molecular patterns
DFI	Diabetic foot infection
DFU	Diabetic foot ulcer
DM	Diabetes mellitus
DNA	Deoxy ribonucleic acid
DTNB	Dithiobis (2-nitrobenzoic acid)
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme linked immunosorbent assay
EPS	extracellular polymeric substances
F	Forward
GDM	gestational diabetes mellitus
GSH	Glutathione
HPR	Horseradish peroxidase
hvKp	Hypervirulent *Klebsiella pneumoniae*
ica	intercellular adhesin
IL-17A	Interleukin-17A
IWGDF	International Working Group on the Diabetic Foot
IDSA	Infectious Disease Society of America
LADA DM	latent autoimmune diabetes of the adult
LPS	Lipo-poly saccharide
MODY DM	maturity-onset diabetes of the young
MRSA	Methicillin Resistant *Staphylococcus aureus*
MSA	Mannitol salt agar
OD	Optical density
PAMPs	pathogen associated molecular patterns
PBP2a	penicillin-binding protein
PCR	Polymerase chain reacion
PEDIS	perfusion, extent/size, depth/tissue loss, infection and sensation.
PIA	polysaccharide intercellular adhesion
PMQR	plasmid-mediated quinolone resistance
PNAG	Poly-N-acetylglucosamine
PRRs	pattern recognition receptors
PSMs	phenol soluble modulins
QRDR	quinolone resistance determining regions
R	Reverse

Contents

RBC	Red blood cell
RNA	Ribonucleic acid
RNS	reactive nitrogen species
ROC	receiver operating characteristic
ROS	reactive oxygen species
SAg	super-antigens
SEls	Enterotoxin-like toxins
SINBAD	Site, Ischemia, Neuropathy, Bacterial Infection, and Depth
SOD	Superoxide dismutase
SPSS	Statistical Package for the Social Sciences software
T2DM	Type 2 diabetes mellitus
TBE	Tris Borate-EDTA Buffer solution
TCA	Trichloroacetic-acid
TE	Tris –EDTA Buffer solution
Th	T-helper
TLRs	Toll-like receptors
TSA	Tryptic soy agar
UTIs	Urinary tract infections
UV	Ultra violet
Wi-Fi	Wound, Ischemia, and foot Infection

Chapter One

Introduction

1-1 Introduction

Globally, diabetes lines among the top 10 reasons of mortality according to international Diabetes Federation Diabetes atlas by (Boyko *et al*; 2021). A recent study refers that the global diabetes prevalence in 2019 is estimated to be 9.3% (463 million people), increasing to 10.2% (578 million) by 2030 and 10.9% (700 million) by 2045 (Saeedi *et al*;2019) . On the basis of 2015 predominance statistics from the International Diabetes Federation, it is predictable that, yearly, foot ulcers develop in 9.1 million to 26.1 million people with diabetes worldwide and 1.0 million to 3.5 million in the United States only, have a history of foot ulceration (Armstrong *et al*; 2017). At least half of all amputations ensue in people with diabetes commonly because of an infected diabetic foot ulcer (Boulton *et al*; 2018). Diabetic foot ulcers are communal and caused by peripheral neuropathy and vascular disease in patient with diabetes. If they were left untreated, up to 50% of DFUs will progress an infectious complication. Osteomyelitis arises in 20% of DFIs. In addition, diabetes is the leading cause of non-traumatic lower limb amputation, with 20% of those with DFIs requiring amputation. Mortality is also elevated, with a 5-year mortality of about 40%; in patients with a history of amputation, mortality is raised to 60% (Mponponsuo *et al*;2021).

Diabetic foot infection (DFIs) is described as local detections of inflammation or purulence occurring at the position beneath the malleoli in a patient with diabetes (Paulson *et al* ;2018). Patients with diabetes are mostly vulnerable to foot infection as a result of neuropathy, vascular insufficiency, and reduced neutrophil function as mentioned by (Bader ; 2008). Suitable classification of wound severity is necessary in determining the requirement for hospitalization, antibiotic choice, surgical interference, and prediction, there for, multiple staging systems that include physical examination discoveries, signs of systemic inflammation, and ischemia have been suggested (Thurber *et al*; 2017) such as Wagner's classification, University of Texas classification, SINBAD classification, PEDIS

classification, Wi-Fi classification; etc. Wagner's classification was the most common, simplest and the most globally used system for diabetic foot ulceration as mentioned by Hobizal & Wukich (2012).

In order to control the diabetic foot infections and to avoid the amputation of the lower extremity, it is necessary to identify the bacterial spectrum of the DFI. In severe DFIs, patients confirmed a greater microbial variety which known as poly microbial infection (Radzieta *et al;* 2021) and in another study performed on 201 diabetic patients by Siddiqui *et al* (2021) , they found that 67.6% were poly microbial whereas 28.4% were mono microbial. Among the most prevalent bacteria that isolated from DFI, *Staphylococcus aureus* was the most predominant of Gram positive bacteria in many studies as a result of its numerous virulence factors such as α-hemolysine , leukocidin, exofoliative toxin and phenol soluble modulins (Dunyach-Remy *et al*; 2016). *Klebsiella pneumoniae* is also identified in DFI in many studies because of its virulence factors such as capsule, LPS, fimbriae and biofilm formation (Clegg and Murphy; 2016).

Biofilm is aggregates in which cells are frequently embedded in a self-produced matrix of extracellular polymeric substances (EPS) that are adherent to each other and/or a surface. It can invade chronic wound infections such as infected diabetic foot ulcers (DFUs), which establish a great clinical problem to patients (Hassan *et al*; 2022).

Innate immunity plays a vital role in the pathogenesis of type 2 diabetes and DFI. Subsequently the toll-like receptors (TLRs) are chief to innate immunity, it has been investigated that TLR2 homodimers and TLR2 heterodimers with TLR1 or TLR6 activate innate immunity by detection of damage-associated molecular patterns (DAMPs). Numerous DAMPs are produced during type 2 diabetes, so it may be theorized that TLR2 is involved in its development (Sepehri *et al;*2016) while modern studies, suggest that adaptive immune system, specifically Th17, produces IL-17A, plays a crucial role in the

pathogenesis of T2DM (Xia *et al*;2017). Furthermore, higher CRP levels might be considered as parameter in the overall valuation of T2DM risk (Stanimirovic *et al*;2022).

It is presently thought that oxidative stress plays a dynamic role in diabetic wound healing. An imbalance of free radicals and antioxidants in the body results in overproduction of reactive oxygen species (ROS) which lead to cell, tissue damage, and delayed wound healing. Therefore, decreasing ROS levels through antioxidative systems may reduce oxidative stress-induced damage to improve healing (Deng *et al*;2021).

The current study aims to determine the severity of biofilm forming bacteria that infect the diabetic foot infections in Kerbela city by isolation of these species and identifying them molecularly by the following objectives:

1. Collection of swabs from different grades of diabetic foot infection of both sexes and different age categories and cultivation of these swabs on appropriate culture media to determine the bacterial species infected to diabetic foot infection.

2. Identification of the main bacterial species that infect the diabetic foot infection by the available biochemical tests and determination of the strongest biofilm forming bacteria and performance of susceptibility test of the most prevalent biofilm forming bacteria against some available antibiotics.

3. Molecular identification of some virulence factors of the strongest biofilm forming bacteria and /or antibiotics resistance genes.

4. Collection of blood samples from diabetic foot patients of different age categories that previously described to evaluate of some biochemical parameters associated with diabetic foot infection such as enzymatic and non-enzymatic antioxidants and some other markers.

Chapter Two

Literatures Review

2-1 Literatures Review

2-1-1 Diabetes mellitus (DM)

Diabetes mellitus (DM) is a metabolic disease described by the incidence of chronic elevated blood glucose accompanied with impaired metabolism of carbohydrates, lipids and proteins (Conget; 2002). Insulin is a polypeptide hormone generally secreted by β cells in Langerhans islets of the pancreas and its main function is regulating glucose levels in the blood stream and prompts glucose storage in the liver, muscles, and adipose tissue, causing in overweight (Rahman *et al* ;2021). A resistance of insulin leads to high levels of blood glucose which called hyperglycemia, the clinical indicator of diabetes (Boyko *et al*; 2021).

2-1-1-1Types of Diabetes mellitus:

According to the American Diabetes association (2016), Diabetes mellitus can be divided into several types, Type 1 diabetes mellitus (Type 1 DM) which is characterized by the damage of insulin-producing beta cells in the pancreas, leading to whole insulin deficiency, Type 2 diabetes mellitus (Type 2 DM) which is characterized by insulin resistance which is increasingly mutual with reduced insulin secretion, gestational diabetes mellitus (GDM) which is diagnosed in the second or third trimester of pregnancy in women without a previous history of DM and finally other specific types: monogenic DM (neonatal DM, MODY DM (maturity-onset diabetes of the young), LADA DM (latent autoimmune diabetes of the adult), disorders of the exocrine pancreas (such as cystic fibrosis) drug-induced diabetes (glucocorticoids, antiretroviral, etc.).

2-1-1-2 Major complications of Diabetes mellitus:

The complications of DM can divided into two chief types the microvascular complications that effected on small vascular in each of retinal, peripheral nerve and kidney that can cause retinopathy, neuropathy and nephropathy disease, respectively and macro-vascular complications that effected on large vascular including Peripheral Artery Disease, Coronary Artery Disease and Cerebrovascular disease (Mezil *et al* ;2021).

2-1-2 Diabetic Foot Infections (DFI):

2-1-2-1 Definition and pathophysiology of DFI:

Infection is defined as an invasion and reproduction of microorganisms in host tissues that prompts a host inflammatory response, followed by tissue damage. Almost all DFIs arise in open wounds; as these are colonized with microorganisms thus DFI is defined clinically as the presence of indicators of an inflammatory process in any tissue beneath the malleoli in a person with diabetes mellitus. In persons with diabetic foot complications, signs and symptoms of inflammation may be screened by the presence of peripheral neuropathy, or peripheral artery disease or immune dysfunction. DFIs usually begin with a disruption in the protective cutaneous envelope, typically in a site of trauma or ulceration often in a person with peripheral neuropathy and commonly with peripheral artery disease (Lipsky *et al;*2020). Evidence of infection commonly contains typical signs of inflammation (redness, warmness, swelling, or pain) or pussy discharges, but can also include additional signs (such as non-pussy secretions, yellowed granulation tissue, undermining of wound edges and foul odor) (Lipsky *et al*; 2012).

The four main characteristics of the formation of diabetic foot ulcer as mentioned by (Deng *et al*; 2023) in figure No. 2-1 , are peripheral arterial disease, peripheral neuropathy, bacterial infection, and cell dysfunction whereas:

(A) Peripheral arterial disease is the most significant aspect in the development of diabetic foot. Severe ischemia of the skin of the lower limbs can cause ulcerated tissues becoming necrotic due to insufficient blood supply.

(B) Peripheral neuropathy can cause sensory, motor, and secretory dysfunctions in the skin of the lower limbs. These pathological alterations not only modify the physical mechanisms of the feet and lead to hurt of protective sensation but also lead to dry skin, which is not beneficial to the healing of diabetic wounds.

(C) Infection of wounds by various species of bacteria delays healing.

(D) The functional status of wound cells directly regulates healing quality.

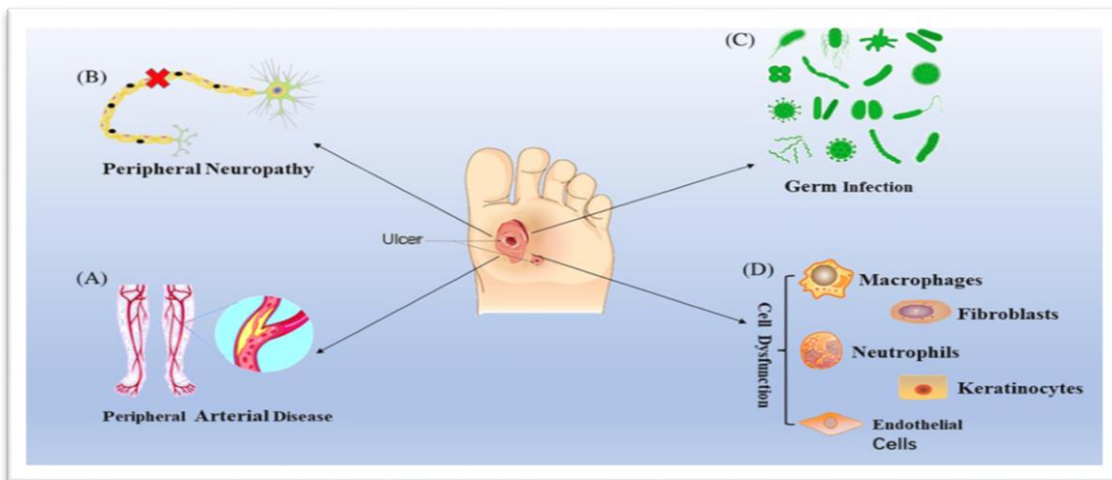


Fig. No. 2-1: Causative factors of diabetic foot formation

Dunyach-Remy *et al* (2016) illustrated in figure No. 2-2 the relationship between metabolic, anatomical and bacteriological factors that can lead to diabetic foot infection.

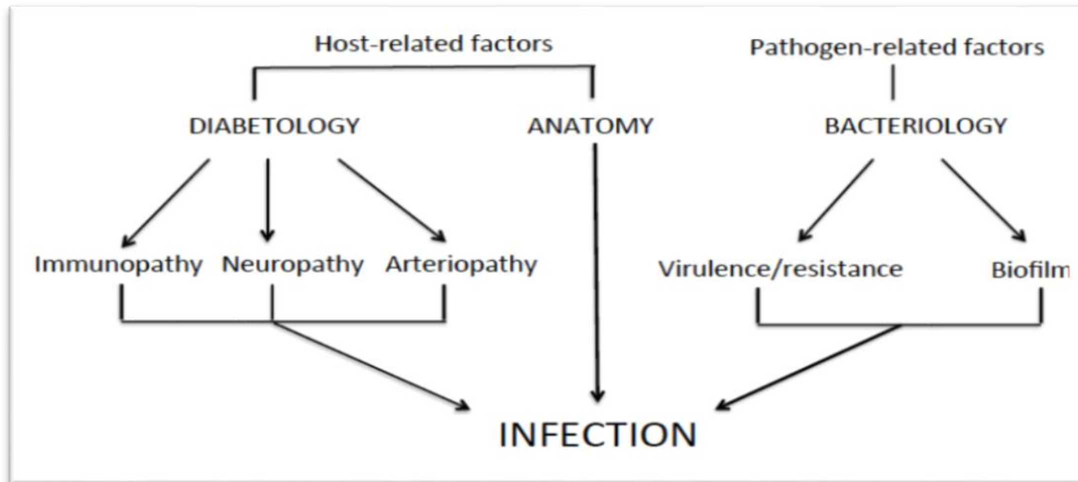


Fig. No. 2-2: Interaction between metabolic, anatomical and bacteriological factor in DFI

2-1-2-2 Classification of DFU:

A suitable classification system is important in defining ulcer features, which will help in planning strategies for the management of diabetic foot ulcers (DFUs), therefore; numerous wound classification systems are established on parameters such as the level of infection, neuropathy, ischemia, depth of tissue injury, and position, so many DFI classification systems have been suggested, but none is generally accepted (Ghotaslou *et al*;2018). There is a study done by (Monteiro-Soares *et al*; 2019) for International Working Group on the Diabetic Foot (IWGDF) included eight factors that were constantly and importantly interrelated to DFU outcomes that would establish the basis of a classification system:

1. Patient factors: End stage renal disease

2. Extremity factors: Peripheral artery disease; loss of protective sensation

3. Ulcer factors: Area; depth; site (forefoot/hind foot); number (single/multiple); infection.

DFU can be classified into several systems such as Wagner system, University of Texas system, The SINBAD system, PEDIS system, WI FI system and IWGDF/IDSA system.

2-1-2-2-1 Wagner's classification system:

As mentioned by (Hobizal *et al*; 2012) and (Shah *et al*; 2022), Wagner's classification is the simplest, best known and one of the most widely used and universally accepted for assessment and management of diabetic foot ulceration. It is consisting of six simplistic wound grades used to evaluate ulcer depth (grades 0-5) as illustrated in table No. 2-1

Tab. No. 2-1: Wagner's classification of DFU

Grade	Description
0	Skin intact but bony deformities lead to "foot at risk"
1	Superficial ulcer
2	Deeper, full thickness extension
3	Deep abscess formation or osteomyelitis
4	Partial Gangrene of forefoot
5	Extensive Gangrene

This classification does not include situation to loss of protective sensation, infection and ischemia and thus its efficacy may differ between countries. It is also too simplistic to offer predictive information at an individual level, including only two of the eight factors identified by the expert sheet (Monteiro-Soares *et al;* 2019).

2-1-2-2-2 IWGDF/IDSA classification:

It was first published in 2004 whereas some studies over the world have provided some proofs that severity of infection is related with greater levels of inflammatory markers (Ozer Balin *et al*;2019). The IWGDF/IDSA classification has numerous benefits, counting the most studies to confirm its use in diverse populations. It is comparatively easy for the clinician to use, requiring only a clinical examination and standard blood and imaging tests, aids in direct diagnostic and therapeutic decisions about infection, has no clear harms and has been broadly accepted by the academic community and practicing clinicians. Furthermore, other available classification schemes were not precisely advanced or

certified for DFIs (Monteiro-Soares *et al;*2019). Table No. 2-2 illustrated this classification.

Tab. No. 2-2 : IWGDF/IDSA classification (Lipsky *et al*;2019)

Grade	Manifestation
1 uninfected	**Uninfected**
	No systemic or local signs of infection
	Infected
	At least two of these items are present: - Local swelling or induration -Erythema > 0.5 cm* around the wound -Local tenderness or pain -Local increased warmth -Purulent discharge And no other reason(s) of an inflammatory response of the skin (e.g. trauma, acute Charcot neuro-osteoarthropathy, breakage, thrombosis or venous stasis)
2 mild infection	Infection with no systemic exhibitions involving: -only the skin or subcutaneous tissue (not any deeper tissues), and -any erythema present does not extend >2 cm** around the wound
3 moderate infection	Infection with no systemic manifestations, and involving: -erythema extending ≥ 2 cm* from the wound margin, and/or -tissue deeper than skin and subcutaneous tissues (e.g. tendon, muscle, joint, bone,)
4 severe infection	Some foot infection with related systemic signs (of the systemic inflammatory response syndrome [SIRS]), as exhibited by ≥ 2 of the following: -Temperature >38 °C or 90 beats/minute -Respiratory rate >20 breaths/minute
Add "(O)" after 3 or 4* **	Infection concerning bone (osteomyelitis)

Note: * Infection refers to any part of the foot, not just of a wound or an ulcer; ** In any direction, from the rim of the wound. The presence of clinically significant foot ischemia makes both diagnosis and treatment of infection considerably more difficult; *** If osteomyelitis is demonstrated in the absence of ≥ 2 signs/symptoms of local or systemic inflammation, classify the foot as either grade 3(O) (if ≥ 2 SIRS criteria)

2-1-3 Bacteriological spectrum of DFI:

Infection is best defined as an invasion and multiplication of microorganisms in host tissues that prompts a host inflammatory response, usually followed by tissue damage (Lipsky *et al*; 2020). DFIs arise mainly from skin ulceration associated with loss of protective sensation (peripheral neuropathy), altered foot architecture, and some forms of trauma (Kwon & Armstrong ; 2018). Though all wounds are colonized with microorganisms, the presence of infection is defined by ≥ 2 classic findings of inflammation or purulence. Infections are then classified into mild (superficial), moderate (deeper), or severe (supplemented by systemic signs or metabolic disorders) and this classification system, accompanied by a vascular valuation, determines which patients should be hospitalized, which may need a surgical interference, and which will require amputation (Lipsky *et al*; 2012). Although most DFIs are relatively superficial at presentation, microorganisms can extent closely to subcutaneous tissues, including fascia, tendons, muscles, joints and bones. The anatomy of the foot, which is divided into several isolated but intercommunicating sections, raises proximal spread of infection (Arago'n-Sa'nchez *et al*; 2012).

In DFI, the patterns of microbial infection are not constant. Bacterial profile revealed polymicrobial pattern and *Staphylococcus aureus* was the most frequent microorganism isolated as showed in a study done in Tanzania by (Chalya *et al;* 2011) and in another study performed by (Lipsky *et al*; 2012) , it has been approved that most DFIs are polymicrobial, with aerobic gram-positive cocci, and especially staphylococci, the most common microorganisms. Aerobic gram-negative bacilli were often pathogens in infections that were chronic or follow antibiotic treatment, and obligate anaerobes could be pathogens in ischemic or necrotic lesions while in KSA, exploration of microbial distribution revealed growth of bacteria in all diabetic foot wounds both aerobes with anaerobes as mentioned in a study done by (Aldhfyan *et al*; 2018).

In a study performed by (Kwon and Armstrong; 2018) , they approved that *Staphylococcus aureus* is the most common pathogen, with *Streptococcus*, *Enterococcus*, *Enterobacteriaceae* and *Pseudomonas* also prevalent in Korea and the most common isolates of bacteria were Methicillin Resistant *Staphylococcus aureus* (MRSA) ,Coagulase Negative *Staphylococci* (CoNS) , *Pseudomonas aeruginosa* , Methicillin Sensitive-*Staphylococci aureus* , *Klebsiella pneumoniae* , and *Escherichia coli* in a study done in India by (Aleem *et al* ; 2021) while Karuppiah *et al* (2022) approved in their study that pathogenic bacterial species including coagulase positive and coagulase negative *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella sp*., *Proteus* sp., *Pseudomonas* sp. and *Citrobacter* sp. were detected, among which *Staphylococcus aureus* was the main genus identified.

The most prevalent bacterial species that cause the diabetic foot infections:

2-1-3-1 Gram positive bacteria:

2-1-3-1-1 *Staphylococcus aureus*:

The staphylococci are gram-positive spherical cells, usually organized in grapelike irregular clusters. They grow readily on many types of media and are active metabolically, fermenting carbohydrates and generating pigments that differ from white to deep yellow. Some are normal flora of the skin and mucous membranes of humans; others cause purulence, abscess formation, pyogenic infections, and also lethal septicemia. The pathogenic staphylococci often hemolysis blood, coagulate plasma and produce extracellular enzymes and toxins. *Staphylococci* rapidly develop resistance to many antimicrobial agents, which causes therapeutic difficulties.

S. aureus is coagulase positive, which distinguishes it from the other species, and a major pathogen for humans. Its severity ranging from food poisoning or skin infections to severe life-threatening infections (Carroll *et al*; 2016).

2-1-3-1-1-1 Characteristics of *S. aureus*:

Staphylococci grow on most bacteriologic media under aerobic or microaerophilic situations. They grow quickly at 37°C but produce pigment best at (20–25°C). On solid media, colonies are curved, smooth, elevated, and shining. Colonies of *S. aureus* usually produces gray to deep golden yellow colonies; many colonies produce pigment only as a result of prolonged incubation. Various degrees of hemolysis are produced by *S. aureus* and sometimes by other species of *Staphylococci* . Medium to large (0.5-1.5 µm) colonies; smooth, entire, slightly raised, low convex, opaque; most colonies pigmented creamy yellow; most colonies beta-hemolytic (Carroll *et al*; 2016).

S. aureus produces colonies enclosed by a yellow halo on mannitol salt agar. In addition, small colony modifications of *S. aureus* on blood agar appear as small pinpoint colonies. Small colony variants could result from limited nutrients or other selective pressures and may return to the typical *S. aureus* phenotype following subculture. (Tille ;2014).

S.aureus produces spherical, gram-positive cells grouped together in irregular clusters .Gram stain must be applied on young cultures, because very old cells may lose their ability to retain crystal violet and may seem gram negative. (Tille; 2014). *Staphylococci* are about 1 µm in diameter, non-motile and non- spore former (Carroll *et al*; 2016)

2-1-3-1-1-2 Virulence Factors of *S.aureus*:

Table No. 2-3 describes the virulence factors of *Staphylococcus aureus* and their effects which are responsible of pathogenicity of this bacteria (Mahon ; 2019).

One of the most important virulence factors of *S.aureus* is enterotoxins which are extracellular products produced by a different *Staphylococcus aureus* strains and responsible for food poisoning in humans, contribution in toxic shock syndrome. There are

five identified members of the staphylococcal enterotoxin family, labelled as staphylococcal enterotoxins A-E (SEA-E). Each member has its exclusive biochemical and serological features, but shares significant homology in sequence, structure, and biological functions with other family members (Ren *et al*;1994). Enterotoxins were characterized by heat stable as well as pH- and proteases-resistant properties that share many mutual characteristics. They are non- glycosylated and single-chain proteins with a homologous and globular structure, as well as low molecular weight (19-30 kDa) (Argudín *et al*; 2012). The *sea* gene is encoded in a family of bacteriophage chromosomes and became a prophage, and the transcription of this gene is related with the life cycle of this prophage (Bokaeian *et al* ;2016).

2-1-3-1-1-3 Molecular characteristics of *S.aureus:*

Staphylococcus aureus genomes are about 2.8 Mbp in size and all strains have the same chromosome construction**.** The ‘core’ *S. aureus* genome is that portion of the genome which is present in all of *S. aureus* strains. This is in contrast to the accessory genome that is variably present. The core *S. aureus* genome is about 2.3 Mbp in size and comprises housekeeping genes, genes essential for growth and survival and numerous virulence genes including those encoding many of the microbial surface constituents recognizing adhesive matrix molecules ,such as protein A and the fibrinogen-binding proteins, also some exotoxins including α-hemolysin and the phenol soluble modulins (PSMs) **.**The accessory genome is composed of mobile genetic elements which include insertion sequences, plasmids, transposons, incorporated bacteriophages and genomic or pathogenicity islands that hold genes which can encode for proteins that paticipate to the virulence and appropriateness in a specific environment (Li *et al*;2010) (Chua *et al;*2013).

Tab. No. 2-3 : Virulence factors of *Staphylococcus aureus*

No.	Virulence factor		Function
1	Hemolysin	α	Damage RBC ,platelets and macrophage
		β	Damage the erythrocyte plasma membrane
		δ	Less toxic than other hemolysins
		λ	Associated with Panton-Valetine leukocidin
2	Panton-Valetine leukocidin		Polymorphonuclear leucocyte toxicity
3	β- lactamase		Enzyme that cleave the lactam ring of β-lactam antibiotics
4	Penicillin binding protein		Change the membrane binding protein
5	Hyaluronidase		Make the bacteria more spreadable in connective tissue
6	Lipases		Disrupt the lipid on the skin surfaces making it more vulnerable to bacterial entry to epidermal layers
7	Staphylocoagulase		Responsible for agglutination of plasma in coagulase test
8	Toxic shock syndrome toxin		A superantigen making the immune system hyper-active bind to IgG and prevent phagocytosis.
9	Protein -A		bind to IgG and prevent phagocytosis.
10	Enterotoxin A-E,G,J		Enterotoxin A, B, D heat stable, responsible of food poisoning Enterotoxin C and rarely G and J cause enterocolitis
11	Exofoliative toxin		Cause bullous impetigo

2-1-3-1-1-4 Resistance of *S. aureus* to antibiotics:

There are a number of mechanisms essential in pathogenic bacteria that makes it resistant in the presence of extreme circumstances and give it the ability to resist a large collection of important antibiotics and other toxic compounds. previously, the usage of antibiotics for a long period have been observed to explode a number of biochemical and genetic mechanism in bacteria that lets it to change the damaging effect of antibiotics found within their instant environment. Clones of bacteria with acquired or natural resistance features have been used constantly as a method of evolutionary response to the use of antibiotics. It is well-known that the attainment of antibiotic resistance mechanism ascended because of genetic events causing changes in the primal bacterial genome such as deletion or replacement of a single nucleotide base and multiplication of a single number of a gene. On the other hand, the most important means of persistence of resistance gene, is the horizontal transfer of mobile genetic elements such as transposons and plasmids both within bacteria of the same or different species. Figure No. 2-3 illustrates different antibiotic classes and resistance mechanisms of *S.aureus* (Bitrus *et al*;2018).

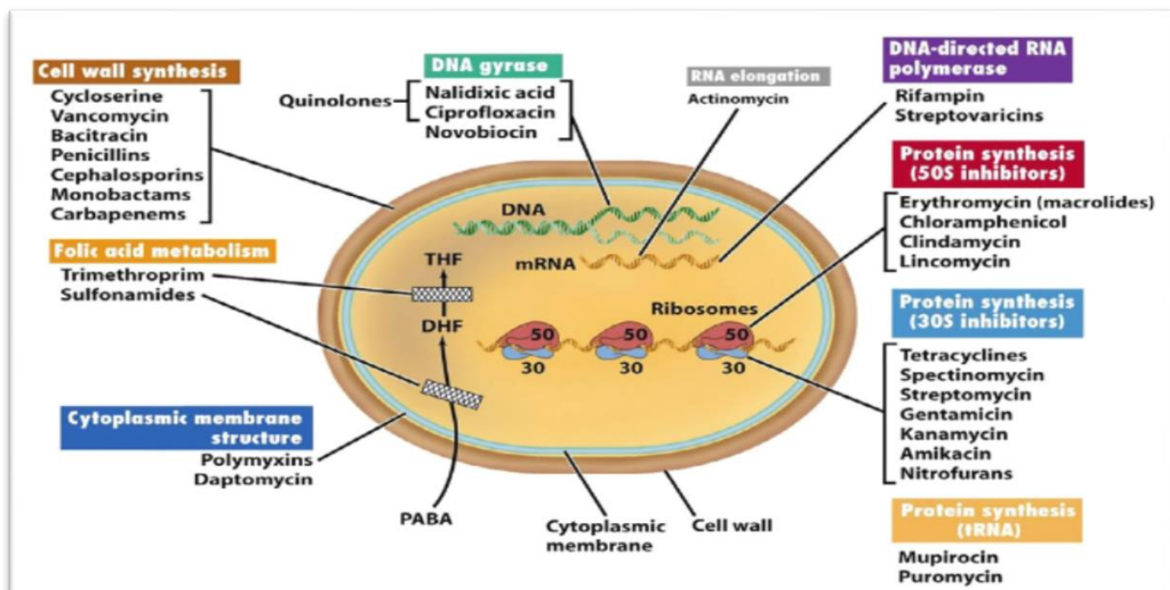


Fig. No. 2-3: Schematic representation of *S.aureus* resistance mechaniams

2-1-3-2 Gram negative bacteria:

2-1-3-2-1 *Klebsiella pneumoniae*:

Klebsiella pneumoniae is gram negative bacillus present in the respiratory tract and feces of about 5% of normal individuals. It causes small proportion (~1%) of bacterial pneumonias. *K. pneumoniae* can produce broad hemorrhagic necrotizing consolidation of the lung (Carroll *et al;*2016). *K. pneumoniae* is the most commonly isolated species that has the discrete feature of having a large polysaccharide capsule. *K. pneumoniae* is a common cause of lower respiratory tract infections among hospitalized patients and in immunocompromised individuals such as newborns and elderly patients. Another infection commonly related to *K. pneumoniae* involving immunocompromised hosts are wound infections, UTIs, liver abscesses, and bacteremia (Mahon ;2019).

Klebsiella pneumoniae belongs to the *Enterobacteriaceae* and is divided into:

1. Opportunistic: acts as opportunistic pathogens, infecting critically ill and immunocompromised patients.

2. Hypervirulent (hvKp): infecting healthy people in community sites and causing severe infections including pyogenic liver abscess, endophthalmitis, and meningitis.

3. Antibiotic-resistant (ABR-Kp) sub-types: Third group of *K. pneumoniae* encode carbapenemases, making them highly antibiotic-resistant. These strains serve as opportunists but are extremely difficult to treat (Martin and Bachman;2017).

2-1-3-2-1-1 Characteristics of *K.pneumoiae* :

Cultivation of bacteria on blood agar and MacConkey agar and incubation them for 24 hours resulted in large, regular, round, smooth, elevated, wet, and cream yellow colonies. There was no hemolysis on the blood agar and the colonies were smooth, round,

moist, and raised colonies while large, pink, regular, round, smooth, and raised colonies grew on MacConkey agar (He *et al*;2022).

All *Enterobacteriaceae* have the same microscopic morphology; therefore, Gram staining is not significant for the probable identification of *Enterobacteriaceae* (Tille; 2014). The Gram staining showed that the isolate was gram-negative bacillus. Single or short-chain variation could be observed by microscopic examination (He *et al*;2022).

2-1-3-2-1-2 Virulence factors of *K.pneumoniae*:

Table No. 2-4 summarizes the virulence that involved in *K.pneumoniae* pathogenesis (Clegg and Murphy; 2016) One of the most important virulence factors of *K.peumoniae* is capsule. the capsule, is synthesized by gene products from the capsular polysaccharide synthesis (*cps*) locus. In the bacterium, the capsule deliberates resistance against the bactericidal activity of antimicrobial peptides, complement, and phagocytes. Over 70 capsular serotypes have been reported for *K. pneumoniae*. Among these, strains with the K1 and K2 capsular serotypes, which mainly cause liver abscess and belong to particular clones are known to be hypermucoviscous or (HV) (Ko; 2017).

2-1-3-2-1-3 *Klebsiella pneumoniae* serotype:

Capsular serotypes K1 and K2 strains of *K. pneumoniae* have more pathogenicity. These serotypes have shown more resistance against the bactericidal effect of serum and phagocytic consumption associated with other serotypes. Therefore, the presence of K1and K2 capsular serotypes of *K. pneumoniae* in clinical samples may be a sign of serious infections (Akbari and Asadpour ; 2015).

Fang *et al* (2007) mentioned that the *mag*A gene was described for the first time in 2004 and exposed that the invasive *K. pneumoniae* strains had higher levels of hypermucoviscosity and *mag*A, while mutant strains lacking *mag*A lost their

exopolysaccharide also the *K2A* gene of capsule gene cluster *K. pneumoniae* has been used as a very dedicated technique to distinguish the capsule *K2* serotype.

Tab. No. 2-4 : Virulence factors of *Klebsiella pneumoniae*

No.	Virulence factor	Role in pathogenesis
1	Capsule	Inhibit and escape from phagocytosis by host cell, prompts dendritic cell maturation, neutralizes antibacterial activity of host defense
2	LPS	O-antigen offers serum resistance
3	Siderophore	Scavenge essential iron for survival, hypermucoviscous phenotypes have been related to increasing iron-binding activity
4	Urease	Limited role in precipitation of inorganic salts leading to catheter encrustation
5	Type 1 fimbriae	Involved in the formation of intracellular bacterial communities
6	Type 3 fimbriae	Important for biofilm formation on biotic and abiotic surfaces, role in biofilm formation on urinary catheters in vivo remains to be elucidated
7	Biofilm formation	Formation promotes resistance to host killing and antimicrobials, experimentally shown to be facilitated in part by fimbriae and capsule
8	Antibiotic resistance	Carbapenem- resistance prevent many treatment options

Diabetes is the most mutual immunocompromised state and makes patients susceptible to all necrotizing infections. Occurrence of diabetic patients for lower leg infections may elucidate the occurrence of necrosis in the lower extremities as the most common site. *Klesiella pneumoniae* necrosis cases from East Asia and the South East Asian region occurred mostly in diabetics, while from Western and European regions a

comparable number of cases have presented in diabetic patients as in non-diabetic patients (Rahim *et al*;2016) . *Klebsiella pneumoniae*, recognized for its affinity to occupy, quickly progresses to adjacent body parts and even distant sites. However, presence in skin necrosis may occur by two ways: either by direct bacterial invasion into subcutaneous tissue or more commonly, by the haematogenous way which may occur by metastatic invasion of bacteria from primary septic foci such as distant liver abscess, or by intestinal bacterial transportation into the circulation accelerated by immunosuppressive states such as liver cirrhosis. Pain, swelling, erythema, soreness, crepitus, bullae, skin necrosis, rapid progression despite antibiotic therapy, and severe sepsis or septic shock were the most features of *Klebsiella pneumonia* necrosis (Rahim *et al*;2019) .

2-1-3-2-1-4 Molecular characteristics of *K.pneumoniae*:

The *K.pneumoniae* genome involves a single circular chromosome and two plasmids. The genome size is 5,491,870 bp containing 57.38% GC and two plasmids of size 211,813 bp and 172,619 bp of GC% content 52.4% and 52.6%, respectively (Rafiq *et al*;2016).

2-1-3-2-1-5 Resistance of *K.pneumoniae* to antibiotics:

K. pneumoniae showed heterogenicity of resistance to antibiotics constructed on gender or specimen type. PCR detection of genes coding for porins and efflux pumps revealed different genotypes and strong correlation between antibiotics resistance profiles and investigated genes. The majority of explored *K. pneumoniae* was resistant to various antibiotics, with ampicillin, piperacillin, cefuroxime, cefepime, cefazolin and aztreonam being the least effective for *K. pneumoniae* while tigecycline and imipenem had the most advantageous profile (Lagha *et al*;2021). *K. pneumoniae* has been usually considered a daunting pathogen avoiding defense mechanisms. The roles of capsule represented by limiting the activation of inflammatory responses, inhibiting the bactericidal action of

complement and CAMPs, and abolishing phagocytosis by neutrophils and macrophages are perfect examples of *Klebsiella* secrecy strategies. Reduced expression of porins to avoid complement activation, and the role of the LPS O-polysaccharide to limit complement deposition on the bacterial surface are other examples of this *Klebsiella* stealth activities (Bengoechea and Pessoa; 2019).

2-1-4 Biofilm:

It has long been known that microbial species that existing in natural environments generally associate with surfaces where they together produce adhesive materials that help to preserve their locus long enough to offer a chance to develop complex structured communities or biofilms. Free microbes, known as planktonic cells, are considered to represent the form by which diffusion to other appropriate positions is confirmed and new biofilms are established (Watnick and Kolter; 2000). Ever later microorganisms were established to be the causative agents of infectious diseases during the late nineteenth century, the isolation, identification and control of planktonic microorganism derived from acute infections has dominated medical microbiology (Cooper; 2010).

One of the key explanations why DFUs are so hard to heal is associated with the existence of biofilms which stimulate wound inflammation and a remarkable deficiency of response to host defenses/treatment routes, which can lead to disease progression and chronicity. In fact, suitable treatment for the exclusion of these microbial communities can avoid the disease development and, in some cases, even prevent more serious results, such as amputation or death. However, the recognition of biofilm-associated DFUs is difficult because of the deficiency of approaches for diagnostics in clinical settings (Afonso *et al*; 2021).

Biofilm can be defined as a bacterial assemblage which exists in a matrix offering protection from antimicrobials and host defenses (MG and J; 2019). Biofilm is also defined

as aggregates of microorganisms in which cells are often embedded in a self-produced matrix of extracellular polymeric substances (EPS) that are adherent to each other and/or a surface (Ismail *et al*; 2022).

2-1-4-1 Factors that affect biofilm development:

Even if certain ingredients are mutual to all biofilms, the involvement of the host relative to the microorganisms, such as immunologic components and the physical location, has an effect on the biofilm structure. Numerous key environmental and cultural characteristics affect the selection of multispecies biofilm residents. These features including organism attachment efficacy, nutritional resources, substrata, and environmental shear pressure or force. Shear stress, maybe the most important characteristic, affects the morphology and dynamic performance of the biofilm. Steady-state kinetics of the organisms inside the biofilm can be completely changed by physical factors such as high shear, and the shear rate will determine the rate of destruction of cells and of the matrix from the biofilm (Mahon; 2019).

2-1-4-2 Components of biofilm:

Biofilms are hydrated, with fluid-filled network running throughout them. The fluid-filled channels assist the exchange of nutrients and carry away wastes. In addition, motile microorganisms can be found swimming in the aqueous portion of a biofilm. Table No. 2-5 lists some frequently ingredients of biofilms.

The 3D design of the mature biofilm often has 3 layers. The outermost layer is exposed to high concentration of nutrients and oxygen. It comprises the most active organisms, which chiefly look like the structure and activity of their planktonic complements. These organisms are mostly closely united, with selective pressures. Even though, they are also part of the EPS, the organisms can slough off and initiate biofilm formation downstream.

The next layer of the biofilm construction is an intermediate layer. Organisms in this layer regulate their metabolic activity, but they obviously have the ability to use nutrients and exchange genes, and have the potential for multiple drug resistance. They benefit from that arrangement, and it is not by chance. Finally, the innermost layer of cells is attached to the basic layer and is the earliest part of the biofilm. These microorganisms are the least metabolically active, and this is where most persevere cells are situated. Mostly, the innermost layer represents the inheritance for future populations that transfer genes laterally (Mahon; 2019).

Tab. No. 2-5: Ingredients of biofilm

Components	Percentage of matrix
Water	≤ 97%
Microbial cell	2-5% (many species)
Exopolysaccharide	1-2%
Protein	< 1-2%
DNA and RNA	< 1-2%

2-1-4-3 Biofilm formation of *Staphylococcus aureus*:

Bacterial biofilms do severe injury to the diabetic foot ulcer (DFU) because they play a critical role in infection invasion and proliferation. *Staphylococcus aureus*, the major Gram-positive bacteria in diabetic foot infection (DFI), is often contributing in colonization and biofilm formation. It was detected that *S. aureus* bacteria isolated from DFU wounds were more prone to form biofilms than those from non-diabetic patients. Moreover, it was found that advanced glycation end products (AGEs) stimulated the biofilm formation of *S. aureus* in clinical isolates in vitro, including methicillin-resistant strain. Analysis of biofilm ingredients verified that the biofilms formed mainly by cumulative extracellular DNA (eDNA) release (Xie *et al*;2020).

Creation of the extracellular polysaccharide, termed ‘Poly-N-acetylglucosamine (PNAG)’ in *S. aureus* is currently the best understood mechanism of biofilm development. PNAG is synthesized by enzymes encoded by the *ica* (intercellular adhesin) operon (Fitzpatrick *et al*; 2005). Figure No. 2-4 illustrates the *icaADBC* operon which is subject to the control of the product of the *icaR* gene, which is encoded upstream. *Ica*R, which is itself is under the control of various regulators and environmental conditions, binds in two dimers to the *icaADBC* promoter region, suppressing *icaADBC* transcription. *Ica*A and *Ica*D, two membrane proteins, synthesize a growing poly-GlcNAc chain from activated precursor GlcNAc units. This chain is distributed by the membrane protein *Ica*C, although *Ica*C has also been risked to be involved in PIA, poly saccharide intracellular adhesion O-succinylation. *Ica*B is an enzyme that is involved to the bacterial outer surface and presents positive charges in the otherwise neutral PIA molecule by de-acetylation of some GlcNAc residues. The cationic character is vital for surface attachment and functionality of PIA (Nguyen *et al*; 2019).

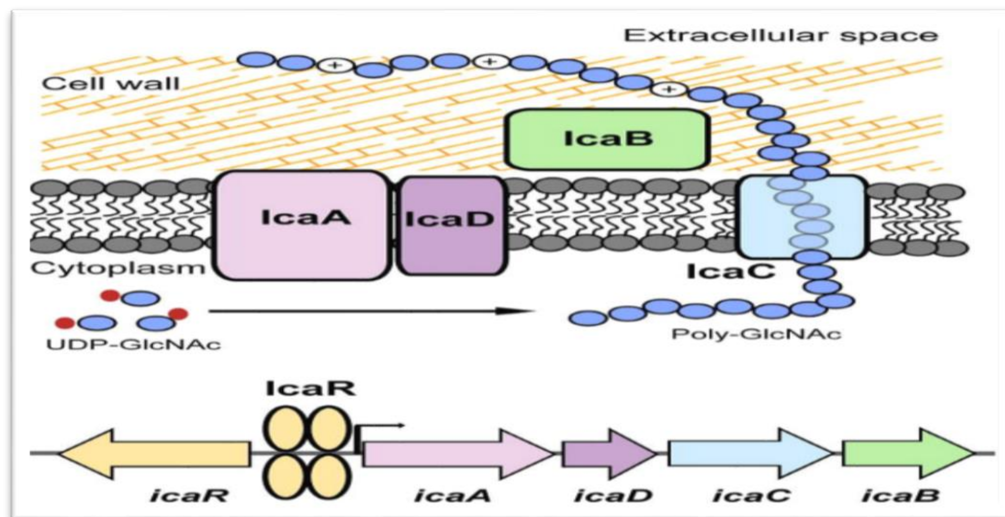


Fig. No. 2-4 : Biofilm formation mechanism by *S.aureus*

2-1-4-4 Biofilm formation of *Klebsiella pneumoniae:*

The ability to form biofilms is a vital virulence feature for numerous microorganisms, including *Klebsiella pneumoniae*. It is expected that 65-80% of bacterial infections are biofilm associated. A great number of factors contribute to biofilm establishment and maintenance in *K. pneumoniae*, which highlights the significance of this mechanism for the bacterial appropriateness (Guerra *et al*; 2022). Type-3 fimbrial genes are very vital to colonize, occupy and persevere *K. pneumoniae* and significant for biofilm formation on biotic and abiotic surfaces. *mrk* gene cluster consists of five genes (*mrk* A, B, C, D and F) (Muhsin *et al*; 2022). The expression of type 3 fimbrial adhesin-encoding gene (*mrk*D) was related with biofilm formation as well as resistance to variable antibiotics among *K. pneumoniae* clinical isolates (Elbrolosy *et al*; 2020). As illustrated in figure No. 2-5, the *mrk* cluster is adjacent to a three-gene cluster which encodes gene products that reveal amino acid affinity to other bacterial proteins required in c-di-GMP sensing and modulation thus, the transcriptional polarization of the *mrk* genes is indicated by the arrowheads. The *mrk*A promoter (P*mrk*A) has been mapped previously, and the location of the P*mrk*J and P*mrk*H promoters is established upon nucleotide sequence analysis (Johnson and Clegg; 2010).

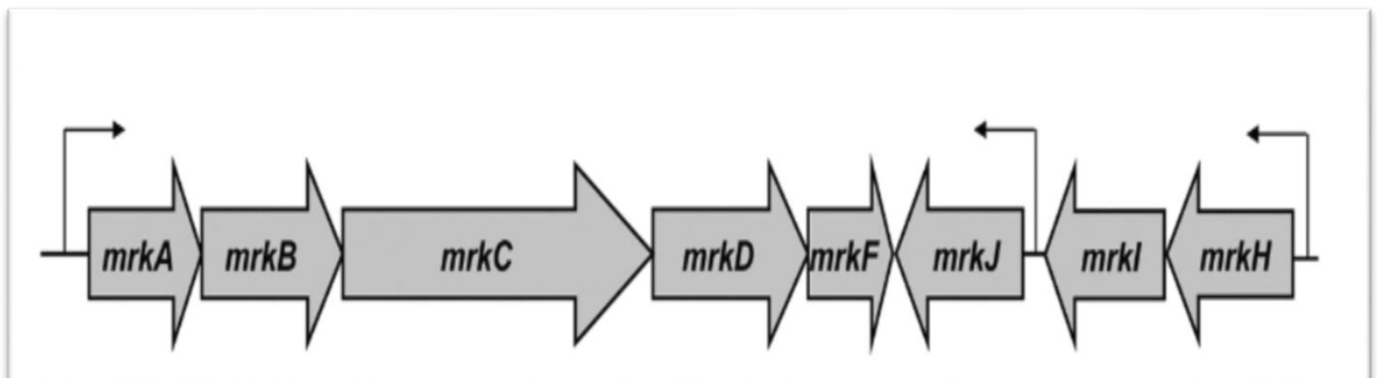


Fig. No. 2-5: Organization of the type 3 fimbrial gene cluster in *K.pneumoniae*

2-1-5 Immunological response:

As the immune system guards the host against pathogens, it uses diverse recognition systems to efficiently eradicate the invading pathogen or its products. A response produced against a potential pathogen is called an immune response (Carroll ; 2016). The immune system denotes to an assemblage of cells, chemicals and processes that serve to protect the skin, respiratory passageways, intestinal tract and other areas from foreign antigens, such as microbes (organisms such as bacteria, fungi, and parasites), viruses, cancer cells, and toxins. The immune system can be having two "outlines of defense": innate immunity and adaptive immunity (Marshall *et al*; 2018).

2-1-5-1 Innate immunity response to diabetic foot infection:

Inflammation can be defined as an elementary way in which the body reacts to infection, irritation or other injury, the key characters are being redness, warmth, swelling and pain. Inflammation is now documented as a type of nonspecific immune response and it is the basic process whereby tissues of the body respond to injury .The innate immune system is comprised of several different cellular constituents such as neutrophils, eosinophils, basophils, mast cells, monocytes, macrophages, dendritic cells, natural killer cells, gamma delta T cells, B-1cells .Rather than organizing a non-specific pro-inflammatory or phagocyte response, cell activation, pathogen recognition and a specificity of the innate immunity is deliberated by the presence of specific receptors expressed by these immune cells termed pattern recognition receptors (PRRs) (Stankov; 2012).

Toll-like receptors (TLRs) are the main pattern recognition receptors of the innate immune system and these receptors are extremely preserved in recognizing subtle molecular components of invading pathogens termed pathogen associated molecular patterns (PAMPs), such as lipids, lipo-poly saccharide, proteins and nucleic acids. Additionally, the recognition of microbial PAMPs by PRRs results in activation of precise

signaling pathways and a diversity of cell dependent responses, including pro-inflammatory cytokine release, phagocytosis and antigen presentation (Portou; 2019).

2-1-5-2 Immunologic receptors:

The innate immune system is an evolutionally preserved host defense mechanism against pathogens. Innate immune responses are originated by pattern recognition receptors (PRRs), which distinguish microbial constituents that are vital for the survival of the microorganism, among them, Toll-like receptors (TLRs) are talented in recognizing organisms ranging from bacteria to fungi, protozoa, and viruses, and they serve as a major role in innate immunity (Bauer *et al* ; 2008).

Toll-Like Receptor (TLR) family is an important group of receptors through which innate immunity distinguishes invasive microorganisms. TLRs are key molecules for microbial eradication, such as the staffing of phagocytes to infected tissues and consequent microbial killing (Bauer *et al*; 2008). Recognition of pathogen-associated molecular patterns by TLRs activates signaling actions that prompt the expression of effector molecules, such as cytokines and chemokines, guiding the adaptive immune responses (Wifi *et al*;2017).

There are numerous types of TLRs associated with bacterial ligand as illustrated in table No. 2-6 (Bauer and Hartmann; 2008).

TLRs are located either at the cell surface (TLRs 1,2, 4, 5,6) or in the intracellular compartment (TLRs 3, 7, 8, 9) primarily on exosomes and endoplasmic reticulum (Portou; 2019).

Tab. No. 2-6: Types of TLRs that recognized Bacteria

Bacterial component	Species	TLR usage
Lipo poly saccharide(LPS)	Gram-negative bacteria	TLR4

Diacyl lipopeptides	Mycoplasma	TLR2/TLR6
Triacyl lipopeptides	Bacteria	TLR2/TLR1
Peptidoglycans	Gram-positive bacteria	TLR2
Lipoteichoic acid	Gram-positive bacteria	TLR2/TLR6
Phenol-soluble modulin	*Staphylococcus aureus*	TLR2
Glycolipids	*Treponema maltophilum*	TLR2
Atypical LPS	Non-entero bacteria	TLR2
Flagellin	Flagellated bacteria	TLR5
CpG DNA	Bacteria	TLR9
Not determined	Uropathogenic bacteria	TLR11

2-1-5-2-1 Toll-Like Receptor2 (TLR2):

TLR2 is an important extracellular participant in mammalian toll family of leucine rich receptors. It is identified to be a signaling receptor for many microbial products including whole gram positive bacteria and mycoplasma (Wifi *et al*;2017).

In addition to exogenous microbial PAMP ligands, TLRs are activated by a variety of endogenous ligands produced as a result of tissue and cellular injury called damage associated molecular patterns (DAMPs). These are usually concealed from recognition, however following injury they are produced or exposed, prompting a TLR mediated inflammatory response. Additionally, DAMPs serve as danger signals, produced by injured tissues, notifying the immune system of damage (Portou; 2019).

The microbial infection elicits complex interactions between the pathogen and the host through the presentation of PAMP to the innate immune system. TLR-2 will recognize lipo-teichoic acid which is a key component of the cell wall of Gram-positive bacteria . This can lead to the activation of a transcription response program

followed by the secretion of cytokines, chemokines, and nitrogen monoxide (NO) so, that dysregulation in inflammatory response and the production of cytokines have an significant role in the development of multiple organ dysfunction in sepsis (Wen *et al*;2016).

The hyperglycemic status and elevated free fatty acids introduces pro-inflammatory cytokines in diabetes and this interaction is a suggestion for the immune system to join in that pathway. TLRs in and around the foot ulcer contribute in controlling the functions of the innate immune system and the production of inflammation. Continued and aggravated production of cytokines results in continuous inflammatory responses and weakening of wound healing which can cause extensive tissue damage to diabetic wounds which also can lead to amputations (Yehualashet; 2020).

2-1-5-3 Interleukins:

Interleukins are set of cytokines that expressed by leucocytes and they interact between cells of the immune systems and are able to stimulate cell growth, differentiation, and functional activation (Khadka ; 2014). Interleukins are assigned to each family established on sequence homology and receptor chain similarities or functional characteristics (Akdis *et al*;2016). The classification of interleukins is very varied and the division of interleukins established on the way they interact with lymphocytes, the organization of the molecules themselves, the arrangements of the receptors for these molecules, their functions and pro-inflammatory or anti-inflammatory properties (Brocker *et al*; 2010).

Interleukin -17 (IL-17), a pro inflammatory cytokine secreted by T-helper (Th)17 cells, has been accompanying with autoimmune diseases. The IL-17 family contains six structurally related cytokines, IL-17A through IL-17F. IL-17A, the ideal member of this family that has pro-inflammatory role in autoimmune diseases (Qiu *et al*; 2021).

Recent studies propose that adaptive immune system, particularly T lymphocyte, plays a key role in the pathogenesis of T2DM. One certain type of T lymphocytes named Th17, which is a subset of T helper cells secrets IL-17. There is a great evidence that the pro-inflammatory interleukin 17 (IL-17) playing a major role in patients of diabetes mellitus and its complications (Xia *et al*; 2017).

Chronic wounds exhibit continual inflammation with evidently delayed healing. The interleukin-17 family has been occupied as a group of pro-inflammatory cytokines in immune-mediated diseases in the gut and connective tissue, as well as inflammatory skin conditions. Skin wounding prompts the production of IL-17A, IL-17F, and IL-17C from keratinocytes and leukocytes (Hadian *et al*; 2019).

2-1-5-4 C-Reactive Protein (CRP):

C-reactive protein is acute-phase inflammatory protein, an extremely preserved plasma protein, that amplifies up to 1,000-fold at sites of infection or inflammation and manufactured chiefly in liver hepatocytes but also by smooth muscle cells, macrophages, endothelial cells, lymphocytes, and adipocytes. Resently, there is an increasing proof that CRP plays significant roles in inflammatory processes and host responses to infection including the complement pathway, apoptosis, phagocytosis, and the production of cytokines, particularly interleukin-6 and tumor necrosis factor-α (Sproston and Ashworth; 2018).

CRP levels elevate very quickly in response to trauma, inflammation, and infection and drop just as rapidly with the resolution of the condition. Therefore, the measurement of CRP is broadly used to display several inflammatory states. CRP attaches to damaged tissue, to nuclear antigens and to certain pathogenic organisms in a calcium-dependent mode. The function of CRP is linked to its role in the innate immune system, it activates complement, binds to Fc receptors and serves as an opsonin for various pathogens.

Interaction of CRP with Fc receptors result in the generation of pro-inflammatory cytokines that improve the inflammatory response. CRP distinguishes changed self and foreign molecules based on pattern recognition. So, CRP is thought to act as an investigation molecule for transformed self and certain pathogens. This recognition offers early defense and leads to a pro-inflammatory signal and activation of the humoral, adaptive immune system (Du Clos ; 2000).

2-1-6 Oxidative stress and Free radicals:

Oxidation is a major biological process for energy production in the body. One of the vital problem is the inclination of the oxygen molecule to generate free radicals (Mathew *et al*; 2011).

Free radicals are small diffusible molecules that are greatly reactive because of their unpaired electron. Free radicals were primarily believed to be oxygen centered radicals called reactive oxygen species (ROS) but also comprise a subgroup of reactive nitrogen species (RNS) and are all a product of usual cellular metabolism (Ifeanyi; 2018). Free radicals can harmfully modify lipids, proteins and DNA and have been concerned in aging and a number of human diseases. Lipids are extremely prone to free radical damage causing in lipid peroxidation that result in adverse modifications. Free radical ravage to protein can result in loss of enzyme activity. Damage to DNA can lead to mutagenesis and carcinogenesis (Devasagayam *et al*; 2004).

2-1-6-1 The role of Oxidative stress in DFI:

Oxidative stress can lead to insulin resistance, β-cell dysfunction and late diabetic complications (Evans *et al;* 2003). Reactive oxygen species (ROS) are vital regulators of several stages of wound healing. Definitely, low levels of ROS are needed for the match against external damage (Dunnill *et al*; 2017). Nonetheless, extreme oxidative stress on tissues and the reduction of antioxidant facility lead to redox imbalance, which is a chief

cause of non-healing diabetic wounds (Sanchez *et al*; 2018). Scientific studies explored that non-healing diabetic wounds are penetrated by the extremely oxidizing situation, which is associated with hyperglycemia and tissue hypoxia, that result in delayed wound repair and people with long-term type 2 diabetes have major declines in the antioxidant enzyme activity (Dworzański *et al;* 2020). Ulcers, as it known, are a lower-extremity complication of diabetes with high repetition rates. Oxidative stress has been recognized as a crucial factor in impaired diabetic wound healing. Hyperglycemia prompts accumulation of intracellular ROS and progressive glycation end products, initiation of intracellular metabolic pathways, such as the polyol pathway, and Protein Kinase-C signaling leading to suppression of antioxidant enzymes and compounds. Excessive and uncontrolled oxidative stress declines the function of cells concerned with wound healing process, leading to chronic non healing wounds (Zhang *et al*; 2021).

2-1-6-2 Antioxidants system:

To neutralize the effect of free radicals, the body is provided with another set of compounds called antioxidants which are produced either endogenously or obtained from exogenous sources and comprise enzymes like superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase, minerals such as Se, Mn, Cu and Zn, and vitamins such as vitamin A, C and E. In a healthy body, pro-oxidants and antioxidants sustain a ratio and a shift in this ratio towards pro-oxidants gives rise to oxidative stress. Human antioxidant defenses have developed to protect biological systems against oxidative stress (Patekar *et al*; 2014).

2-1-6-2-1 Superoxide dismutase (SOD)

Superoxide dismutase (SOD) are metallo-enzyme which play a chief role in antioxidant defense against oxidative stress in the body. SOD supplement may therefore activate the endogenous antioxidant mechanism for the neutralization of excessive free-

radical and be used in a range of pathological settings (Rosa *et al*; 2021). SOD has three isoforms in mammals: the cytoplasmic Cu/Zn SOD (SOD1), the mitochondrial Mn SOD (SOD2), and the extracellular Cu/Zn SOD (SOD3), all of which require catalytic metal (Cu or Mn) for their activation (Fukai and Ushio-Fukai; 2011). Typically, superoxide anion $O_2^{\bullet-}$ and ROS play a double role. At the physiological equilibrium condition, they are a by-product of O_2 reduction, required for cell signaling, but at the pathological condition they are considered harmful, as they can encourage disease and apoptosis, necrosis and autophagy cell death. The role of superoxide dismutase is an enzyme that is responsible for the exclusion of most of the superoxide manufactured in living organisms. Also, the toxicity prompted by superoxide and derived radicals is valuable in the oxidative death of microbial pathogens, which are consequently overwhelmed by specialized immune cells, such as neutrophils or macrophages, during the activation of innate immunity. (Andrés *et al*; 2023). However, elevated lipid oxidation consequent to diabetic conditions of foot ulcer prompts an over-expression of lipid peroxidase, SOD and catalase activity signifying a compensatory mechanism by the body to avoid additional tissue damage in the subjects (Bhattacharyya *et al*; 2019).

2-1-6-2-2 Glutathione (GSH):

Glutathione (GSH) is a tripeptide (γ glutamyl – cysteinyl – glycine) containing glutamate, cysteine and glycine, it may be present as the reduced (GSH) or oxidized (G-S-S-G) form and can therefore play a role in some oxidation–reduction reactions. It has many functions : the reduced form of glutathione that has a free sulfhydryl (-SH-) group acts as a redox buffer adjusting the redox condition of the cell, GSH helps in maintaining the enzymes in an active status by inhibiting the oxidation of sulfhydryl (-SH-) group of enzyme to disulfide (-S-S-) group, GSH plays a key role in detoxification by reducing H_2O_2 the harmful by-product of metabolism. Finally, GSH is required in transport of amino acids across the cell membrane of the kidney and intestine (Naik; 2017).

Glutathione (GSH) causes the removal of free radicals or drop in hydrogen peroxide level on status of oxidative stress (convert hydrogen peroxide to water). Decrease in the reduced GSH level has been stated in the erythrocyte of diabetics. Decrease in the level of GSH arises due to the competition between aldose reductase and glutathione reductase for NADPH, a cofactor, and increased oxidative stress (increased ratio of NADH/NAD) (Farhood *et al*; 2019). It is well known that diabetic individuals have an enlarged level of oxidative stress and free radical formation in their tissues. Similarly, their blood and tissues are noticeable by low glutathione levels. A reasonable hypothesis is that the increased oxidative stress reduces the glutathione of tissues, the latter being the major intracellular antioxidant responsible for lessening the free radicals. There is good indication that a declining glutathione antioxidant system is responsible, at least in apart, for the detected cardiovascular disease seen in diabetics (Bagherion *et al*; 2014).

Chapter Three

Materials and Methods

3-Materials and Methods:

3-1 Devices and Equipment:

- Instruments and apparatuses:

Table No. 3-1 shows the instruments and apparatus that were used in this study:

Tab. No. 3-1 : Instruments and apparatus used in current study

No.	Apparatus	Manufacturer
1	Analytical Balance	Denver-Germany
2	Autoclave	Labtech-Korea
3	Bunsen burner	Jenway-Germany
4	Cooling ultracentrifuge	MSE –England
5	Digital camera	ATTo-Japan
6	Distiller	GFL-Germany
7	ELISA reader	Huma Reader HS-Germany
8	ELISA washer	Thermo fisher-Germany
9	Hot plate	Biocote-England
10	Incubator	Binder-Germany
11	Laminar flow cabinet	Jeio-Tech-Korea
12	Light microscope	Motic-Germany
13	Micro centrifuge	Biobase-China
14	Micropipettes	Human-Germany
15	Oven	Biobase-China
16	PCR machine	Edison,NJ-USA
17	pH-meter	Mauritius-Germany
18	Refrigerator	LG-Korea

19	Spectrophotometer	Tudor-Korea
20	Sensitive balance	Kern-Germany
21	Standard wire loop	Himedia-India
22	Thermo-shaker	Biosan-Germany
23	UV transilluminator	LKB-SWEDEN
24	Vortex	ROMA-Italy
25	Water bath	Biobase-China

- **Disposable materials**

Table No. 3-2 presents the disposable materials used in this study.

Tab No. 3-2 : Disposable materials used in this study

No.	Disposable materials	Manufacturer
1	Disposable slides	AFMA-Jordan
2	Disposal Syringes	Meheco-China
3	Eppendrof tubes	Bioneer-Korea
4	Gel tubes	Arth alrafidain for labs-Iraq
5	PCR tubes	Biofil-India
6	Petri dishes	AFMA-Jordan
7	Swabs with media	AFMA-Jordan

3-2 Chemicals, kits and culture media:

Table No. 3-3 shows the chemical materials, kits and culture media that used in current study:

Tab. No. 3-3 : Chemicals and kits used in current study

No.	Chemicals and kits	Manufacturer
1	1,2,3-benzenetriol	BDH-England
2	2-nitro benzoic acid	BDH-England
3	Absolute methanol	Sigma-USA
4	Agarose powder	Scharlau-England
5	Antibiotic discs	Liofilchem-Italy
6	API kit	Biomerieux- France
7	Barium chloride	BDH-England
8	Blood agar base	Himedia-India
9	Brain-Heart infusion broth	Himedia-India
10	Deionized sterile distilled water	BiONEER-Korea
11	DNA Ladder 100bp	GeneDirex-Taiwan
12	Ethanol	Sigma- USA
13	Ethidium bromide	Biobasic-USA
14	Genomic DNA extraction kit	Addbio-Korea
15	Glacial acetic acid	GCC-UK
16	Glutathione	BDH-England
17	Glycerol	BDH-England
18	Gram Stain	VSI-Iraq
19	Hydrochloric acid	Sigma-USA
20	Hydrogen peroxide	VSI-Iraq
21	Interleukin-17A kit	BT LAB-China
22	MacConkey agar	Himedia-India
23	Mannitol salt agar	Oxoid-England
24	Muller-Hinton Agar	Himedia-India
25	Nutrient Broth	Himedia-India
26	Phosphate Buffer saline (BPS)	BDH-England
27	Potassium dihydrogen phosphate	Fluka-Switzerland
28	Potassium hydroxide	VSI-Iraq
29	Sodium chloride	Thomas Baker-India

30	Sodium hydroxide	Schorlau-European Union
31	Sulfuric acid H_2SO_4	SEARLE-USA
32	TBE 10X Solution	Bioneer-Korea
33	Tetramethyl p-phenylene diamine dihydrochloride	BDH-England
34	Toll like receptor 2 kit	BT LAB-China
35	Trichloroacetic acid	BDH-England
36	Tris –EDTA Buffer solution (TE)	Biobasic-USA

3-2-1 Antibiotics:

The antibiotics used in the current study were manufactured by Liofilchem, Italy and include the following antibiotics mentioned in table No. 3-4.

Tab. No. 3-4 : Antibiotics discs used in susceptibility test

No.	Antibiotic name	Abbreviation	Conc. mcg
1	Amikacin	AMK	30µg
2	Amoxicillin _clavulanic acid	AMC	20/10µg
3	Azithromycin	AZM	15µg
4	Aztreonam	ATM	30µg
5	Cefepime	FEP	30µg
6	Cefotaxime	CTX	30µg
7	Ceftazidime	CAZ	30µg
8	Ceftriaxone	CRO	30µg
9	Ciprofloxacin	CIP	5µg
10	Doxycycline	DXT	30µg
11	Erythromycin	ERY	15µg
12	Gentamycin	GEN	10µg
13	Imipenem	IPM	10µg
14	Levofloxacin	LEV	5µg
15	Meropenem	MRP	10µg
16	Oxacillin	OXA	1µg
17	Penicillin	P	10µg
18	Piperacillin	PIP	100µg

19	Pipracillin / Tazobactam	TZP	100/10μg
20	Rifampin	RIF	5μg
21	Tetracycline	TET	30μg
22	Vancomycin	VAN	30μg

3-3 Study design:

The current study was designed as case-control study, involved 142 individuals, 40 subjects of foot ulcer patients with T2DM (DFI), 40 subjects of T2DM patient's without foot ulcer, and 40 participants apparently healthy control group. Samples were collected from Al-Imam Al-Hassan center for endocrinology and diabetes.

3-3-1 Groups of the study:

1. Type 2 diabetic patients with diabetic foot ulcer of 40 patients were collected from Al-Imam Al-Hassan center for endocrinology and diabetes.

2. Type 2 diabetic patients without diabetic foot ulcer of 40 patients were collected from Al-Imam Al-Hassan center for endocrinology and diabetes.

3. Control group comprised of 40 healthy individuals free of T2DM and DFI. They were chosen randomly from the general population.

3-3-1-1 Inclusion Criteria of Patients:

1. Patient with type 2 diabetic foot ulcer regardless to the stage of ulcer infection.

2. Age of subjects was >35 years old.

3. Type 2 diabetes mellitus without foot ulcer.

3-3-1-2 Exclusion Criteria

1. Patients aged less than 35 years.

2. Non-diabetic patients with foot ulcers.

3. Type 1 Diabetic Mellitus.

4. Pregnant women.

3-3-2 Ethical Consideration:

This study was approved by Ethical Committee at College of Science/ University of Kerbala. All participants involved in this work were informed and agreement obtained orally from each one before the collection of sample.

3-3-3 Types of sample:

• DFU Swab sample:

The specimens were collected by swab method whereas the wound was cleaned with distilled water or saline to remove surface contaminants and ulcer was scraped before sampling to get exudate then the swabs were passed over the wound area in a zigzag motion while twisting the swab so that the entire head of the swab comes into contact with the wound surface and then was passed from the center of the wound outward to the edge of the wound. The swabs were saved in a transport media and sent to laboratory as soon as possible. Bacterial culturing of the swabs was done in Blood agar and MacConky agar media.

• Blood sample

Seven milliliters of venous blood were drawn from all participants by using a disposable syringe. The blood was put into a gel tubes and centrifuged at 4000 xg in cooling cenrifuge to get serum. The serum was put into an Eppendorf tubes and stored at (-20˚C) until using it to estimate the Interleukin-17A, Toll-like receptor 2, C-reactive protein, Superoxide dismutase and Glutathion.

3-4 Methods:

3-4-1 Culture media :

Various culture media were used in the current study included Blood agar media, MacConkey agar media, Mannitol salt agar, Nutrient broth media, Brain Heart infusion broth media and Mueller Hinton agar media. The mentioned culture media were prepared according to the manufacturer instructions.

3-4-2 Reagents and solutions used for bacterial identification

- **Catalase reagent:**

Catalase test was used to check the presence of catalase enzyme, which catalyst the toxic substance hydrogen peroxide into water and oxygen (Tille; 2014).

- **Oxidase reagent:**

The reagent was prepared instantaneously by dissolving 1 gram of Tetramethyl P-phenylen diamine dihydrochloride in 100ml of distilled water in opaque bottle. This reagent was used to estimate the ability of bacteria to produce oxidase enzyme (Shields & Cathcart; 2010).

- **Gram stain:**

This stain was used for the morphological identification of bacterial isolates (Tille; 2014).

- **Normal saline:**

This solution was prepared by dissolving 0.85 gm of NaCl in 50 ml of distilled water then completing the volume to 100 ml of distilled water too.

- **McFarland Solution Tube No. 0.5:**

This solution was prepared as described by Tille (2014) which was composed of two solutions:

A _Barium chloride solution 1.175% : It was prepared by dissolving of 1.175 grams of barium chloride in an amount of distilled water then the volume was completed to 100 ml of distilled water.

B _Sulfuric acid solution 1%: It was prepared by addition of 1ml of sulfuric acid into 99 ml of distilled water.

McFarland solution tube No. 0.5 was prepared by adding of 0.5ml of solution A into 99.5 ml of solution B and mixing the mixture very well.

- **Crystal violet stain:**

This stain was prepared in two concentrations (0.1 and 0.5) % by dissolving of 0.1 and 0.5 gram of the stain in 10ml of absolute methanol then completing the volume to 100 ml of distilled water. The 0.1% concentration was used for estimating the formation of biofilm by tube method and the 0.5% concentration was used for the quantification of the biofilm.

3-4-3 Isolation and identification of bacteria causing Diabetic Foot Infections:

Figure No. 3-1 shows the isolation and identification of bacteria of the DFI briefly.

3-4-3-1 Differentiation of Gram positive and Gram negative bacteria:

➢ **Potassium hydroxide KOH test:**

This test is performed by mixing a full loop of colonies growing on blood agar media with a small amount of 3% KOH on a clean slide. The formation of viscous material indicates the positive result and the bacteria is gram negative (Dash & Payyappilli; 2016).

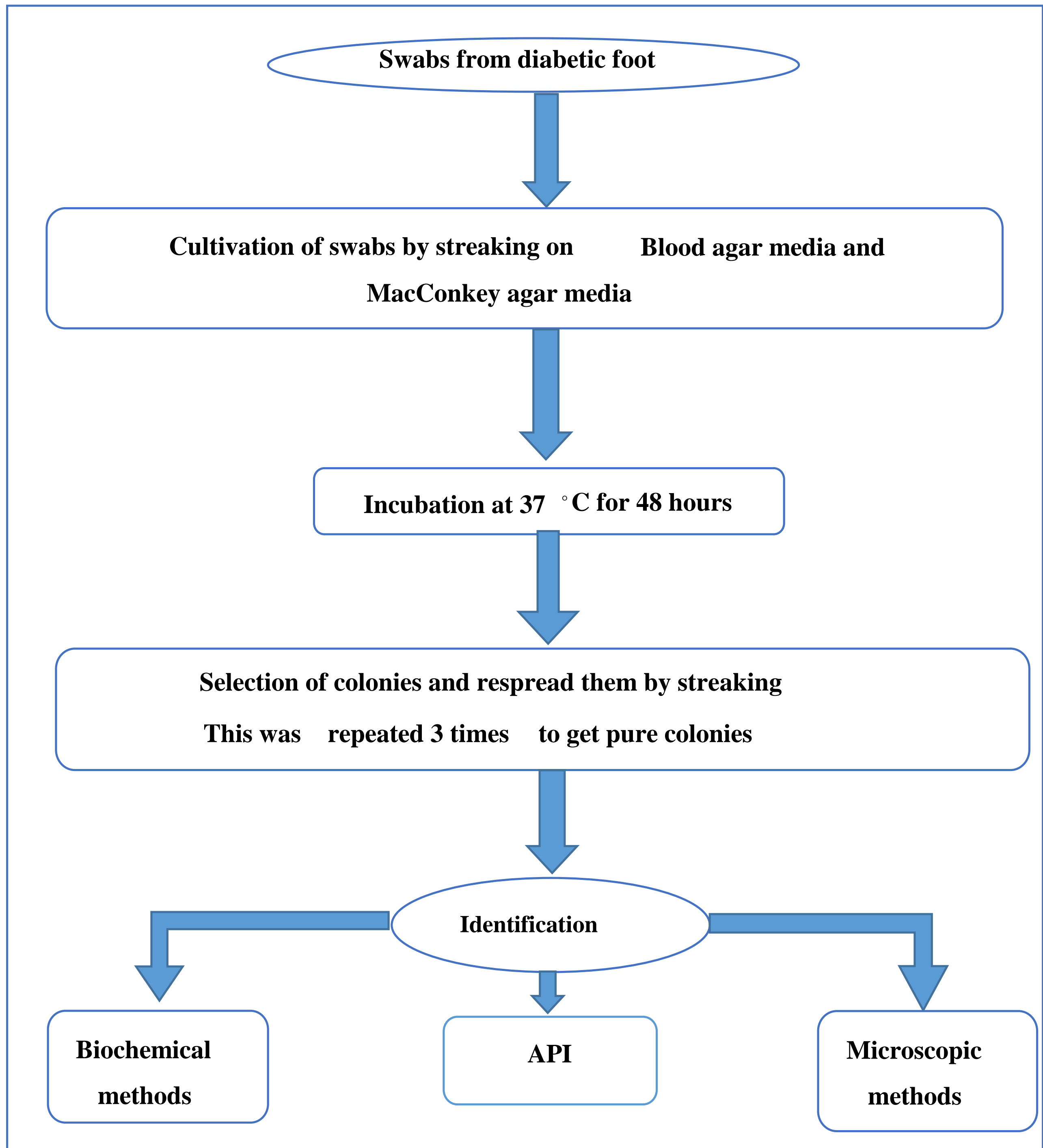


Fig. No. 3-1 : Isolation and identification of bacteria that infect DFI

3-4-3-2 Isolation and identification of Gram positive bacteria:

3-4-3-2-1 Isolation and identification of *Staphylococcus aureus*:

The colonies of *S.aureus* were notable on nutrient agar media after incubation for 24 hours at 37°C and it was identified by microscopical and biochemical methods.

- **Microscopic tests:**
  - **Gram stain:**

The colonies that were grown on blood agar media were selected and stained by Gram stain to identify the cells shape and aggregates.

- **Biochemical tests:**
  - **Catalase test:**

This test was performed by mixing a colony of bacteria of (18- 24) hours age with a drop of hydrogen peroxide 3% reagent on a clean slide. The appearance of bubbles indicate the positive result (Khatoon & Kalia ; 2022).

- **Oxidase test:**

A colony of bacteria of (18-24) hours of age was transported by a stick to filter paper and a drop of oxidase reagent was added. The conversion of colony color to the dark purple indicates a positive result (Shields & Cathcart; 2010).

- **Cultivation on Mannitol salt agar:**

This media was used to isolate the golden *Staphylococcus aureus* colonies and identify them by their ability to tolerate the salinity of the media and their ability to

ferment mannitol sugar and conversion the media color to yellow (Thomer *et al*; 2016).

- **Coagulase test:**

The samples were tested by using human plasma by putting 0.5 ml of plasma into sterile tubes then a bacterial colony of (18- 24) hours was transported by loop into plasma contained tubes and mixed well then incubated at (35-37) ˚C for 4 hours. The agglutination of plasma indicated a positive result. (Thomer *et al*; 2016).

- ***S.aureus* identification using API STAPH Kits:**

- **Inoculum preparation:**

Five colonies of grown bacteria on Mannitol salt agar were transferred to 5 ml of previously prepared phosphate buffer solution in a sterile test tube and vortexed to get a suitable turbidity then identification procedure was completed according to the constructions of the kit.

3-4-3-2-2 Isolation and identification of *Staphylococcus epidermidis:*

The colonies of *S. epidermidis* were distinguished on nutrient agar media after incubation at 37˚C for 24 hours then they were identified depending on microscopic and biochemical methods as described previously in *S.aureus* isolation and identification.

3-4-3-2-3 Isolation and identification of *Streptococcus agalactiae*:

The colonies of this bacteria were distinct when they grew on nutrient agar media after incubation period of 24 hours at 37˚C and the microscopic test and biochemical tests were performed as described in *S. aureus.*

3-4-3-2-4 Isolation and identification of *Enterococcus faecalis*:

The colonies of this bacteria were distinguished on blood agar media after 24 hours of incubation at 37°C then they identified as described in *S.aureus* isolation and identification.

3-4-3-3 Isolation and identification of Gram negative bacteria:

3-4-3-3-1 Isolation and identification of *Klebsiella pneumoniae*:

The colonies of *Klebsiella pneumoniae* were distinguished on MaCconkey agar media after incubation at 37°C for 24 hours then they were identified depending on the following microscopic and biochemical methods:

- **Microscopic tests:**
- **Gram stain**

The colonies that were grown on MaCconkey agar media were selected and stained by Gram stain to show the morphology and aggregates of the cells.

- **Biochemical tests:**
  - **Catalase and oxidase tests:**

These tests were applied as described previously in *Staphylococcus aureus* identification.

- **Identification of *Klebsiella pneumoniae* by using API20E**:
  - **Inoculum preparation:**

Five colonies of grown bacteria on MaCconky agar were transferred to 5 ml of previously prepared phosphate buffer solution in a sterile test tube and vortexed to get a suitable turbidity then identification procedure was completed according to the constructions of the kit.

3-4-3-3-2 Isolation and identification of other Gram negative bacteria:

The colonies of *Pseudomonas aeruginosa*, *Proteus mirabilis*. and *Escherichia coli* were distinguished on MaCconkey agar media then the microscopic and biochemical tests were performed. Identification by API 20 E was also conducted to identify the mentioned bacterial species as described in *Klebsiella pneumoniae* identification.

3-4-4 Susceptibility tests of bacteria against antibiotics:

Disc diffusion method was performed according to (Hudzick; 2009) to check the susceptibility of the most prevalent bacteria to the most traditional antibiotics as follows:

1. Four or five isolated colonies of the bacteria to be tested were transported by a sterile loop and suspend them in 2 ml of normal saline then vortex the tube to create smooth suspension.
2. The turbidity of this suspension were adjusted to a 0.5 McFarland standard.
3. A sterile swab was dipped into the inoculum tube and was inoculated to the dried surface of a Mueller-Hinton agar plate by streaking the swab over the entire agar surface in back-and-forth motion very close together to be distributed evenly then the plate was allowed to sit at room temperature 5_ 15 minutes, for the surface of the agar plate to be dry.
4. The appropriate antimicrobial disks were placed on the surface of the agar by sterile forceps and the plates were incubated for 18-24 hours at 37°C.
5. The inhibition zone sizes were measured to the nearest millimeter by using a ruler.

3-4-5 Maintenance the bacterial isolates:

The isolates were stored for short term by cultivation of them in a nutrient slant tubes and incubated for 18 hours at 37°C and stored in refrigerator and the cultures were renewed every month. For long term preservation, brain heart infusion broth was used with 15% glycerol support and keep the isolates in deep freeze until use.

3-4-6 Molecular characterization of predominant biofilm forming bacteria:

3-4-6-1 Molecular characterization of *Staphylococcus aureus*:

3-4-6-1-1 DNA extraction of *S. aureus*:

- **DNA extraction using bacterial genomic DNA extraction kit:**

Bacterial genomic DNA extraction kit prepared by addbio, Korea was used to extract bacterial DNA.

- **The used solutions:**
  - Lysozyme buffer 500 µL.
  - Lysozyme 50 mg/ L (20 µL).
  - Lysis solution 200 µL.
  - Proteinase K solution 20 µL.
  - Binding solution 200 µL.
  - Absolute ethanol 200 µL.
  - Washing 1 solution 500 µL.
  - Washing 2 solution 500µL.
  - Elution solution (100-200) µL.

- **Method**:

1. The overnight incubated colonies of *S. aureus* that cultured on Mannitol salt agar were suspended with 1 ml of distilled water and were centrifuged at 13000 rpm for 30 seconds in 1.5 ml Eppendrof tube.

2. The supernatant was discarded and 500 µl of lysozyme buffer and 20 µL of lysozyme were added then resuspension was applied by vortex.

3. The mixture was incubated at 37°C water bath for 1 hour then centrifugation at 13000 rpm was performed for 3 minutes and the supernatant was discarded.

4. Two hundred microliters of lysis solution and 20 µL of proteinase K solution were added and resuspended the cell pellet by vortexing then the mixture was incubated into a water bath at 56°C for 10 minutes.

5. Two hundred microliters of binding solution and 200 µL of absolute ethanol were added and mixed well by vortex then centrifuged at 13000 rpm for 3 minutes.

6. Five hundred microliters of supernatant were transferred into the upper reservoir of the spin column in 2 ml collection tube and centrifugation at 13000 rpm for 1 minute was performed.

7. Five hundred microliters of washing 1 solution were added to the spin column of collection tube and centrifuged at 13000 rpm for 1 minute.
8. Five hundred microliters of washing 2 solution were added to the spin column and centrifuged at 13000 rpm for 1 minute.

9. The spin column was dried by additional centrifugation at 13000 rpm for 1 minute to remove the residual ethanol in spin column then the spin column was transferred to new 1.5 ml micro-centrifuge tube and (100-200) µL of elution

solution were added to the spin column and let stand at least for 1 minute and centrifugation at 13000 rpm for 1 minute was performed to elute the genomic DNA.

10. DNA was stored at -20°C until use.

3-4-6-1-2 Molecular characterization of *S. aureus* by polymerase chain reaction PCR:

- **Primers that used in diagnosis of *S. aureus:***

The primers that used for identification of *S.aureus* were illustrated in table No. 3-5

- **Solutions used:**

A. preparation of stock primers:

The primers were prepared according to Macrogen, South Korea to get a final concentration of 100 pmoles/µL and stored at -20°C until usage.

Tab. No. 3-5 : Primers with their sequences and amplicon sizes of *Staphylococcus aureus*

Gene	Primers	Primer sequence 5'____3'	Product size (bp)	Annealing temperature	References
*mec*A	*mec*A-F *mec*A-R	GTAGAAATGACTGAACGTCCGATAA CCAATTCCACATTGTTTCGGTCTAA	310	50°C	Geha, *et al*;(1994)
*ica*A	*ica*A-F *ica*A-R	GAGGTAAAGCCAACGCACTC CCTGTAACCGCACCAAGTTT	151	60°C	Mahmoudi *et al*;(2019)
*ica*D	*ica*D-F *ica*D-R	ACCCAACGCTAAAATCATCG GCGAAAATGCCCATAGTTTC	211	60°C	Mahmoudi *et al*; (2019)

sea	*sea*-F *sea*-R	GGTTATCAATGTGCGGGTGG CGGCACTTTTTTCTCTTCGG	102	57°C	Mehrotra *et al*;(2000)
*erm*C	*erm*C-F *erm*C-R	GCTAATATTGTTTAAATCGTCAATTC C GGATCAGGAA AAGGACATTT TAC	572	53°C	Ghanbari *et al* ;(2016)

B. Master mix mixture:

Master mix was prepared according to Microgen, South Korea which contains the ingredients illustrated in table No. 3-6.

Tab. No. 3-6 : Master mixture components

Ingredients	23 µL reaction
Taq DNA Polymerase	50 U/ ml
Each: dNTP (dATP , dCTP , dGTP , dTTP)	400 µM
Tris-HCl (pH 9.0)	50 mM
$MgCl_2$	3 mM

3-4-6-2 PCR assay:

Primer solution was prepared in a 10 pmoles/µL by addition of 10 µL of stock solution to 90 µL of deionized sterile water then mixed well and stored in ice until usage while the stock solution of primers was stored in -20°C. The ingredients in table No. 3-7 should be involved into a PCR tube

Tab. No. 3-7: PCR tube components of *Staphylococcus aureus* genes

Ingredients	Concentration	Amount (µL)
Go Taq Green Master Mix 2X	-	10

*mec*A-F / *ica*A-F / *ica*D-F / *sea*-F / *erm*C-F	10 μM/μL	1.5
*mec*A-R / *ica*A-R / *ica*D-R / *sea*-R / *erm*C-R	10 μM/μL	1.5
Deionized water	-	7
DNA sample	-	3
Total volume	-	23

PCR machine was programmed as described in the following tables:

Tab. No. 3-8 : PCR machine programming for *mec*A gene amplification

Step	Operation	
1	1 cycle for 4 minute at 94°C for initial denaturation of templet DNA	
2	30 cycles include:	
	A	45 seconds at 94°C for denaturation of templet DNA
	B	45 seconds at 50°C for annealing of primers with templet DNA
	C	1 minute at 72°C for extension
3	1 cycle for 2 minutes at 72°C for final extension of DNA strands	

Tab. No. 3-9 : PCR machine programming for *icaA and icaD* genes amplification

Step	Operation	
1	1 cycle for 5 minute at 95°C for initial denaturation of templet DNA	
2	40 cycles include:	
	A	2 minutes at 95°C for denaturation of templet DNA
	B	20 seconds at 60°C for annealing of primers with templet DNA
	C	20 seconds at 72°C for extension
3	1 cycle for 5 minutes at 72°C for final extension of DNA strands	

Tab. No. 3-10 : PCR machine programming for *sea* gene amplification

Step		Operation
1	1 cycle for 5 minute at 94°C for initial denaturation of templet DNA	
2	35 cycles include:	
	A	2 minutes at 94°C for denaturation of templet DNA
	B	2 minutes at 57°C for annealing of primers with templet DNA
	C	1 minute at 72°C for extension
3	1 cycle for 7 minutes at 72°C for final extension of DNA strands	

Tab. No. 3-11 : PCR machine programming for *ermC* gene amplification

Step		Operation
1	1 cycle for 10 minute at 94°C for initial denaturation of templet DNA	
2	35 cycles include:	
	A	30 seconds at 94°C for denaturation of templet DNA
	B	30 seconds at 53°C for annealing of primers with templet DNA
	C	1 minute at 72°C for extension
3	1 cycle for 10 minutes at 72°C for final extension of DNA strands	

3-4-6-3 Gel electrophoresis:

- **Preparation of solutions:**

  - **Ethidium Bromide stain:**

This solution was used as it prepared by Biobasic, USA.

  - **Tris base boric acid EDTA (TBE buffer) 0.5X:**

This solution was prepared by addition of 50 ml of TBE stock solution (10X) manufactured by Bioneer Corp., Korea to 950 ml of distilled water.

  - **Agarose gel 1.5% preparation:**

This gel is prepared by dissolving 1.5 grams of agarose powder into 100 ml of TBE buffer 0.5X and was heated until boiling by microwave until agarose is dissolved completely then is cooled to 45°C and 10 mg/ml of Ethidium Bromide dye was added and mixed well with the gel and poured into the tray after putting the comb to form the wells and letting it to solidify then the comb was raised gently to get the wells which inside them, the PCR product was loaded and traveled by electrophoresis.

3-4-6-4 Detection of amplification products:

The amplicons were detected by travelling the samples on previously prepared agarose gel 1.5% whereas 5 µL of the samples were loaded to the wells and 5 µL of 100bp DNA ladder prepared by Genedirex, Taiwan. Then they travelled electrically at 70-volt voltage for 50 minutes then the gel was visualized by UV transilluminator to see the bands and assessment of molecular weight of the amplicons compared with the DNA ladder and finally photographed by using digital camera.

3-4-6-5 Molecular characterization of *Klebsiella pneumoniae*:

3-4-6-5-1 DNA extraction of *Klebsiella pneumoniae*:

- **DNA extraction using bacterial genomic DNA extraction kit:**

Bacterial genomic DNA extraction kit prepared by addbio, Korea was used to extract bacterial DNA.

- **The used solutions:**
  - ✦ Lysis solution 200 µL.
  - ✦ Proteinase K solution 20 µL.
  - ✦ Binding solution 200 µL.
  - ✦ Absolute ethanol 200 µL.
  - ✦ Washing 1 solution 500 µL.
  - ✦ Washing 2 solution 500 µL.
  - ✦ Elution solution (100-200) µL.
- **Method:**

1. The overnight incubated colonies of *Klebsiella pneumoniae* that cultured on MacConky agar were suspended with 1 ml of distilled water and were centrifuged at 13000 rpm for 30 seconds in 1.5 ml Eppendorf tube.

2. Two hundred microliters of lysis solution and 20 µL of proteinase K solution were added and resuspended the cell pellet by vortexing then the mixture was incubated into 56°C water bath for 10 minutes.

3. Two hundred microliters of binding solution and 200 µL of absolute ethanol were added and mixed well by vortex then centrifuged at 13000 rpm for 3 minutes.

4. Five hundred microliters of supernatant were transferred carefully into the upper reservoir of the spin column in 2 ml collection tube and Centrifugation at 13000 rpm for 1 minute was performed.

5. Five hundred microliters of washing 1 solution were added to the spin column of collection tube and centrifuged at 13000 rpm for 1 minute.

6. Five hundred microliters of washing 2 solution were added to the spin column and centrifuged at 13000 rpm for 1 minute.

7. The spin column was dried by additional centrifugation at 13000 rpm for 1 minute to remove the residual ethanol in spin column then the spin column was transferred to new 1.5 ml micro-centrifuge tube and (100-200) µL of elution solution were added to the spin column and let stand at least for 1 minute and centrifugation at 13000 rpm for 1 minute was performed to elute the genomic DNA.

8. DNA was stored at -20°C until use.

3-4-6-5-2 Molecular characterization of *Klebsiella pneumoniae* by polymerase chain reaction PCR:

- **Primers that used in diagnosis of *Klebsiella pneumoniae* :**

Table No. 3-12 shows primers that were used in diagnosis of *Klebsiella pneumonia*.

3-4-6-6 PCR assay:

As described in *Staphylococcus aureus*, the ingredients in table No. 3-13 were added into a PCR tube.

Tab No. 3-12 : Primers with their sequences and amplicon sizes of *Klebsiella pneumoniae*

Gene	Primers	Primer sequence 5'____3'	Amp. size (bp)	Annealing Tem.	References
*mrk*D	*mek*D-F *mrk*D-R	AAGCTATCGCTGTACTTCCGGCA GGCGTTGGCGCTCAGATAGG	340	60°C	Anis *et al* ;(2021)
Cps	*Cps*-F *Cps*-R	TATTCATCAGAAGCACGAGCTGGGAGAAGCC GTCGGTAGCTGTTAAGCCAGGGGCGGTAGCG	418	60°C	Booq *et al* ; (2022)
K1	*K1*-F *K1*-R	GGTGCTCTTACATCATTGC GCAATGGCCATTTGCGTTAG	1283	60°C	Abdul-Razzaq *et al*;2014
K2A	*K2*A-F *K2*A-R	CAACCATGGTGGTCGATTAG TGGTAGCCATATCCCTTTGG	531	60°C	Remya *et al;* (2018)

Tab. No. 3-13: PCR tube components of *Klebsiella pneumoniae* genes

Ingredients	**Concentration**	**Amount(μL)**
Go Taq Green Master Mix 2X	-	10
mrkD-F / *Cps*-F / *magA*-F / *k2A*-F	10 μM/μl	1.5
mrkD-R / *Cps*-R / *magA*-R/ *k2A*-R	10 μM/μl	1.5
Deionized water	-	7
DNA sample	-	3
Total volume	-	23

PCR machine was programmed as described in the following tables:

Tab. No. 3-14 : PCR machine programming for *mrk*D gene amplification

1	1 cycle for 5 minute at 95°C for initial denaturation of templet DNA	
2	35 cycles include:	
	A	30 seconds at 95°C for denaturation of templet DNA
	B	30 seconds at 60°C for annealing of primers with templet DNA
	C	5 minute at 72°C for extension
3	1 cycle for 10 minutes at 72°C for final extension of DNA strands	

Tab. No. 3-16 : PCR machine programming for *Cps* gene amplification

Step	Operation	
1	1 cycle for 5 minute at 95°C for initial denaturation of templet DNA	
2	30 cycles include:	
	A	30 seconds at 94°C for denaturation of templet DNA
	B	90 seconds at 60°C for annealing of primers with templet DNA
	C	1 minete at 72°C for extension
3	1 cycle for 7 minutes at 72°C for final extension of DNA strands	

Tab. No. 3-16 : PCR machine programming for *K1* and K2A genes amplification

Step	Operation	
1	1 cycle for 15 minute at 95°C for initial denaturation of templet DNA	
2	30 cycles include:	
	A	30 seconds at 94°C for denaturation of templet DNA
	B	90 seconds at 60°C for annealing of primers with templet DNA
	C	1 minete at 72°C for extension
3	1 cycle for 10 minutes at 72°C for final extension of DNA strands	

Gel electrophoresis was used as mentioned in *S.aureus* to get the amplification product then visualized by UV transilluminator (Green and Sambrook; 2012).

3-4-7 Investigation of biofilm formation:

3-4-7-1 Investigation of biofilm formation by tube method:

The formation of biofilm of *S.aureus* and *K.pneumoniae* was investigated by Bose *et al* (2009) with some modifications. The isolates of the two bacterial species were cultured in 10 ml of nutrient broth media by inoculating the media with loop full of each isolate independently and were incubated at 37°C for 24 hours then the cells suspension was drained and the tubes were washed by normal saline and left to be dried then stained with 0.1% of crystal violet and left for (5-10) minutes at room temperature then the tubes were turned over and let to dry. Finally, the appearance of a visible pigmented layer on the wall of tubes indicated a positive result.

3-4-7-2 Quantification of biofilm formation by Microtiter plate:

The method that described by Kirmusaoglu (2019) was performed with some modification to investigate the ability of bacteria to form a biofilm as the following:

- The young isolates were inoculated into 5 ml of a Brain-Heart broth and were incubated at 37°C for 24 hours.
- The bacteria were diluted with the same media and compared with a 0.5 standard McFarland solution.
- Two hundred microliters of diluted bacterial culture were transferred into every well in micro titer plate in a 4 duplicates for each isolate. Uncultured Brain-Heart infusion broth was added as a control into the wells then the microplate was incubated at 37°C for 24 hours after closing it tightly.
- The culture was drained and the wells were washed 3 times by normal saline.
- The microplate was dried in oven at 60°C for 30 minutes.
- Two hundred microliters of 0.5% crystal violet were added and were left for 15 minutes.
- The stain was drained and the wells were washed 3 times until the dye was disappeared then they were let to dry.
- Two hundred microliter of 33% of glacial acetic acid were added.
- The optical density was measured at a wave length of 630 nm by ELISA reader.
- The efficiency of isolates to form the biofilm was determined by comparing the optical density as described in table No. 3-17 .

Tab. No. 3-17 : Interpretation of biofilm by the microtiter plate method

Mean OD value	Biofilm formation
OD ≤ ODc	None
ODc < OD ≤ 2 × ODc	Weak
2 ×ODc < OD ≤ 4 × ODc	Moderate
4 × ODc < OD	Strong

*OD= Optical Density ODc= Optical Density of control

3-4-8 Determination of some immunological parameters in serum:

3-4-8-1 Determination of Toll-like receotor-2 (TLR-2):

This was determined by using of Human Toll-like receptor-2 ELISA kit as described below:

- **Assay Principle:**

The kit is an Enzyme-Linked Immunosorbent Assay (ELISA) manufactured by Bioassay Technology Laboratory, China. The plate in which was pre-coated with human TLR2 antibody. TLR2 present in the sample was added and bind to antibodies coated on the wells then biotinylated human TLR2 antibody was added and bind to TLR2 in the sample. Then Streptavidin-HRP was added and binds to the Biotinylated TLR2 antibody. After incubation unbound Streptavidin-HRP was washed away during a washing step by ELISA washer. Substrate solution was then added and color develops in proportion to the amount of human TLR2. The reaction was terminated by addition of acidic stop solution and OD was measured at 450 nm.

  - **Reagent components:** as illustrated in the leaflet of manufactured company.
  - **Standard solution preparation:**

As described in instructions, 120 µL of standard solution (48 ng/ml) was reconstitute with 120 µL of standard diluent to generate 24 ng/ml standard stock solution then serial dilutions were made by the same procedure to produce (12, 6, 3, 1.5) ng/ml solution and the standard diluent serves as the zero standard (0 ng/ml).

- **Wash buffer preparation:**

As mentioned in instructions, 20 ml of wash buffer concentrate 25x were added to deionized water to get 500 ml of 1x wash buffer.

- **Assay procedure:**

1. All reagents, standard solutions and samples were prepared as instructed at room temperature.
2. Fifty microliters of standard were added to standard wells and 40 µL of samples were added to sample wells and 10 µL of anti-TLR2 antibody to sample wells then 50 µLof streptavidin-HRP were added to sample wells and mixed well and the plate was covered with a sealer and incubated at 37˚C for 60 minutes in a thermo-shaker.
3. The sealer was removed and automated washing with wash buffer was applied for 5 times by ELISA washer then the plate was blotted onto paper towels.
4. Fifty microliters of substrate solution A were added to each well and 50 µL of substrate B were added to each well then the plate was covered with a sealer and incubated for 10 minutes at 37˚C in the dark.
5. Fifty microliters of stop solution were added to each well and the blue color was changed to yellow immediately.
6. The optical density value was determined immediately using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

Standard curve of TLR-2 was done using different concentration of TLR-2 starts from 0.000 ng/L and ends with 24.000 ng/ml at 450 nm as illustrated in figure No. 3-2 .

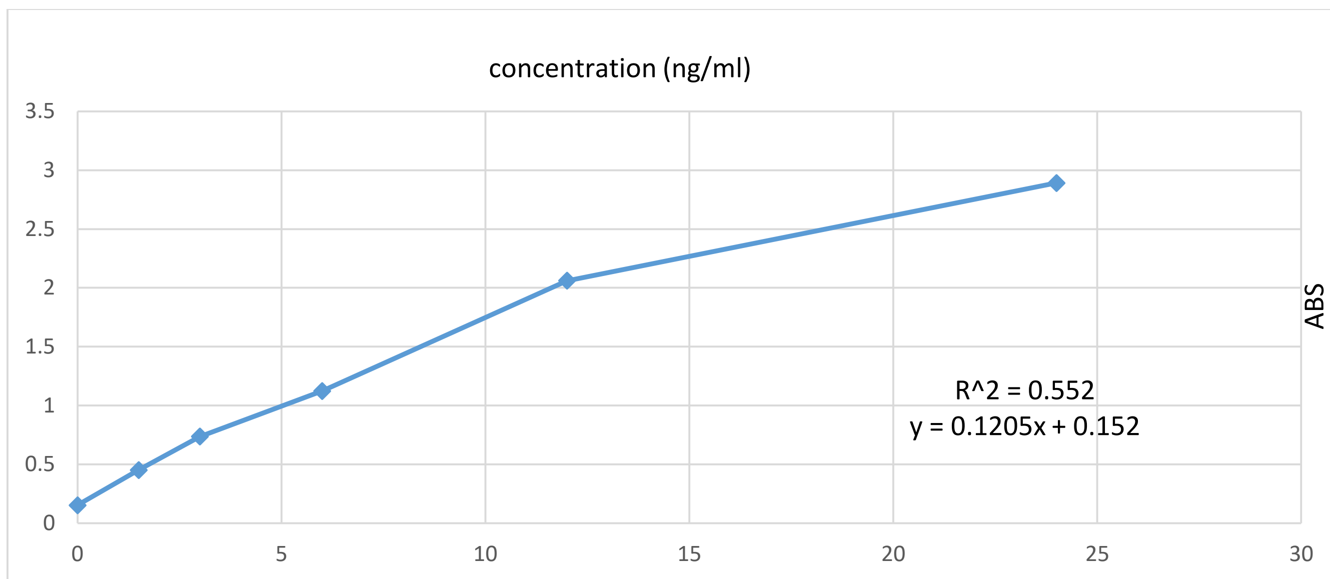


Fig. No. 3-2 : Standard curve of TLR-2

3-4-8-2 Determination of Interleukin-17A (IL-17A):

This was performed by using of Human Interleukin-17A ELISA kit as the following:

- **Assay Principle:**

As described in instructions, the kit is an Enzyme-Linked Immunosorbent Assay (ELISA) manufactured by Bioassay Technology Laboratory, China. In which, the plate was pre-coated with human IL-17A antibody. IL-17A present in the sample was added and bind to antibodies coated on the wells then biotinylated human IL17A antibody was added and bind to IL-17A in the sample. Then Streptavidin-HRP was added and binds to the Biotinylated IL-17A antibody. After incubation unbound Streptavidin-HRP was washed away during a washing step by ELISA washer. Substrate solution was then added and color develops in proportion to the amount of human IL-17A. The reaction was terminated by addition of acidic stop solution and OD was measured at 450 nm.

- **Reagent components:** as illustrated in the leaflet of manufactured company.

Standard solution preparation:

As described in instructions, 120 µL of standard solution (1280 ng/L) was reconstitute with 120 µL of standard diluent to generate 640 ng/L standard stock solution then serial dilutions were made by the same procedure to produce (320, 160, 80, 40) ng/L solution and the standard diluent serves as the zero standard (0 ng/L).

- **Wash buffer preparation:**

As mentioned in instructions, 20 ml of wash buffer concentrate 25x were added to deionized water to get 500 ml of 1x wash buffer.

- **Assay procedure:**

1. All reagents, standard solutions and samples were prepared as instructed at room temperature.
2. Fifty microliters of standard were added to standard wells and 40 µLof samples were added to sample wells and 10 µL of anti-IL17A antibody to sample wells then 50 µL of streptavidin-HRP were added to sample wells.
3. They were mixed well and the plate was covered with a sealer and incubated at 37°C for 60 minutes in a thermo-shaker.
4. The sealer was removed and automated washing with wash buffer was applied for 5 times by ELISA washer then the plate was blotted onto paper towels.
5. Fifty microliters of substrate solution A were added to each well and 50µL of substrate B were added to each well then the plate was covered with a sealer and incubated for 10 minutes at 37°C in the dark.
6. Fifty microliters of stop solution were added to each well and the blue color was changed to yellow immediately.
7. The optical density value was determined immediately using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

Standard curve of IL17-A was done using different concentration of IL17-A starts from (0.000, 40.000, 80.000, 160.000, 320.000 and 640.000) ng/L at 450 nm wavelength as illustrated in figure No. 3-3 .

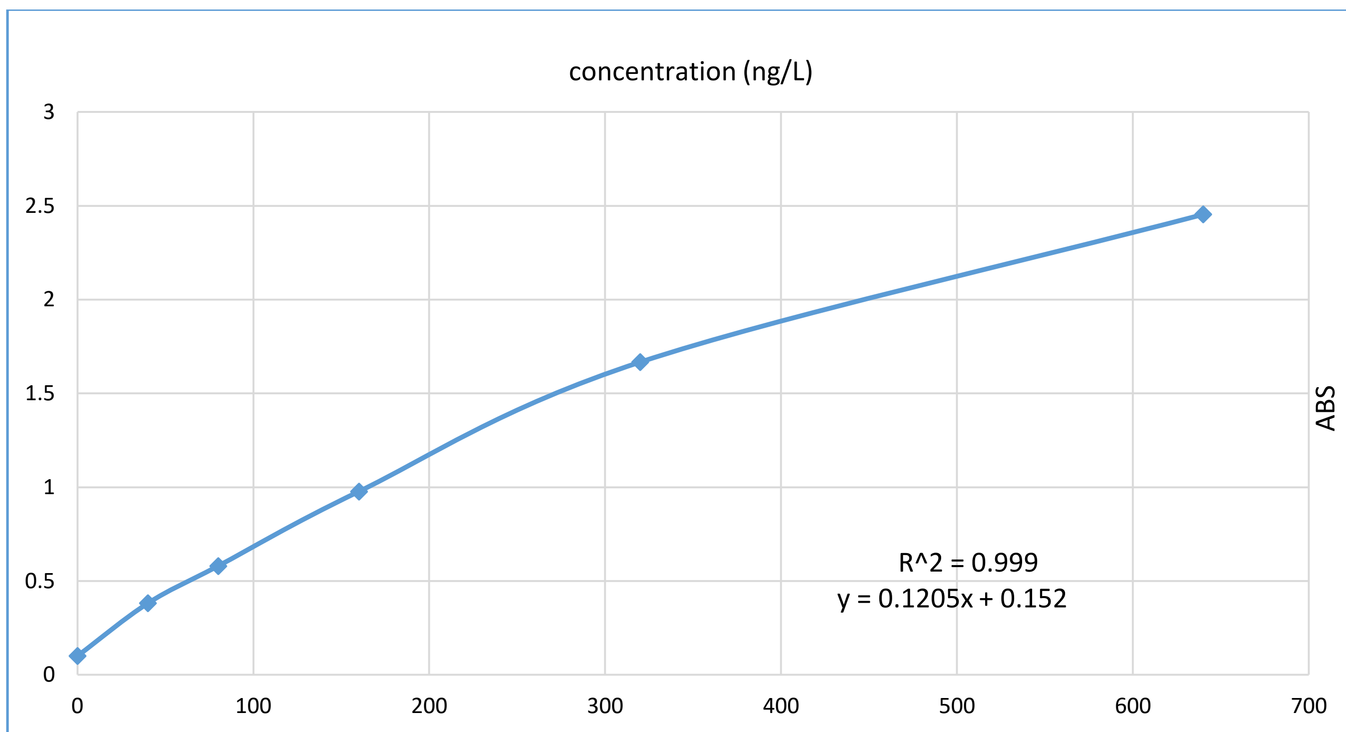


Fig. No. 3-3 : Standard curve of IL17-A

3-4-8-3 Determination of C-reactive protein (CRP):

This parameter was determined according to turbidimetry method by using of CRP kit prepared by Shenzhen Mindary Bio-Medical Electronics Co., China.

- **Reaction principle:**

Anti-human CRP antibody + CRP↔ Immunocomplex (agglutination)

Determination of the concentration of CRP through photometric measurement of immunocomplex between antibodies of CRP and CRP present in the sample, the absorbency increase was directly proportional to the concentration of CRP.

Tab. No. 3-18 : Reagents components and concentrations of CRP kit

R1	Tris buffer	100 mmol/L
	PEG	0.26 mmol/L
	Surfactant	<2% (m/v)
R2	Tris buffer	100 mmol/L
	Anti-human CRP antibody (goat)	Dependent on titer

Assay procedure:

Tab. No. 3-19 : Amounts of blank and sample solutions used

	Blank	Sample
Reagent 1	200 µL	200 µL
D.W	8 µL	_
Sample	_	8 µL

The above components were mixed, incubated for 3 minutes at 37°C, and the blank absorbance was read then 50 µL of Reagent 2 were added into blank and sample solutions and mixed thoroughly at 37°C and the absorbance was read again after 5 minutes, the CRP concentration of each sample was calculated by the analyzer automatically after calibration.

3-4-9 Determination of some antioxidants concentration in serum:

3-4-9-1 Evaluation of Superoxide dismutase (SOD) activity:

The activity of superoxide dismutase was determined by autoxidation of Pyragallol according to Marklund and Marklund, (1974) , SOD activity determination:

1. Tris buffer (pH 8.0): was prepared by dissolving 0.258 gm of tris and 0.111 gm of

Ethylenediaminetetraacetic acid (EDTA) in dH_2O and completing the volume to 100 ml.

2. Pyragallol solution (0.2 mM): was prepared by dissolving 0.0252 gm of pyragallol with 10 ml of HCl and completing the volume to 100 ml with dH_2O.

- **Procedure**

According to Marklund and Marklund (1974), reaction mix is consisting of 50 µl serum with 2 ml of tris buffer and 0.5 ml of pyragallol (0.2 mM) which absorbs light at 420 nm. Control solution contains the same materials except for the enzyme extract that was replaced by dH_2O. As a blank, dH_2O was used. One unit of enzyme is defined as the amount of enzyme that is capable of inhibiting 50% of pyragallol oxidation. SOD activity was calculated using the following equation:

$$SOD\ Activity\ (unit) = \frac{\frac{\%P}{50\%} \times R}{T}$$

Where:

- *%P:* percentage of the inhibition of pyragallol reduction

*Note: %P of every sample is calculated by comparing Δabs of the sample (X%) with Δabs of control (100%)

- *R:* Total reaction volume (2.55 ml)

- *T:* Time of reaction in minutes (2 minutes)

3-4-9-2 Evaluation of Glutathione (GSH) concentration:

- **Principle:**

Ellman's reagent 5,5 Dithio bis (2-Nitro benzoic acid) (DTNB) can be reduced easily by glutathione compounds to produce a yellowish compound of high optical density that has greater absorbance at 412 nm and directly proportional with glutathione concentration

(Ellman ; 1959). Procedure described by Moron *et al* (1979) to evaluate the GSH concentration in blood sreum whereas the following solutions have been prepared :

1. Sodium phosphate buffer (0.2M): which was prepared by dissolving of 2.4gm of sodium phosphate in an amount distilled water then completing the volume to 100 ml of distilled water and pH was adjusted at 8 .

2. Dithionitrobenzene solution (0.6 mM) (DTNB): It has been prepared by dissolving of 0.023 gm of DTNB in sodium phosphate buffer (0.2M) prepared above and after the completion of dissolving, the volume completed to 100 ml of the same buffer.

3. Trichloroacetic-acid (TCA) 5% was prepared by dissolving of 5 gm of TCA in an amount of distilled water then completing the volume to 100 ml.

4. Tris buffer solution (1.4 M): It was prepared by dissolving of 4.82 ml of Tris-base in 10 ml of EDTA-Na_2 (0.4 M) that prepared by dissolving of 1.489 gm of EDTA-Na_2 in 10 ml of distilled water then completing the volume to 100 ml and pH was adjusted to 8.9 by addition of 0.1N of hydrochloric acid.

- **Preparation of Glutathione standard curve:**

  The used solutions:

1. EDTA-Na_2 (0.2M): It has been prepared by dissoloving of 0.477 gm of EDTA-Na_2 in 100 ml of distilled water.
2. Standard glutathione solution (3 mg/ml): It has been prepared by dissolving of 0.0307 gm of glutathione in a total volume of 10 ml of EDTA-Na_2 (0.2 M).

The graduated concentrations of glutathione standard solutions were prepared, and the next volumes of a standard glutathione solution were added in test tubes (two tubes for every volume). Then, the suitable volume of EDTA-NA2 was added. Then 0.02 ml of DTNB was added for each tube, and 0.8 ml of Tris buffer was added the tubes were mixed well for 10-15 minutes and then centrifuged at 300 xg for 15 minutes, and the absorbance

was measured by spectrophotometer by using the blank solution to read the zero absorption at 412 nm then the sample absorbance has been read after 5 minutes of Ellman's reagent addition. GSH concentration has been obtained from the calibration curve using concentrations ranging from (0.0, 5, 10, 20, 30, 40 , 50 , 60 , 80, and 100) µmol/L that mentioned in Figure No. 3-4

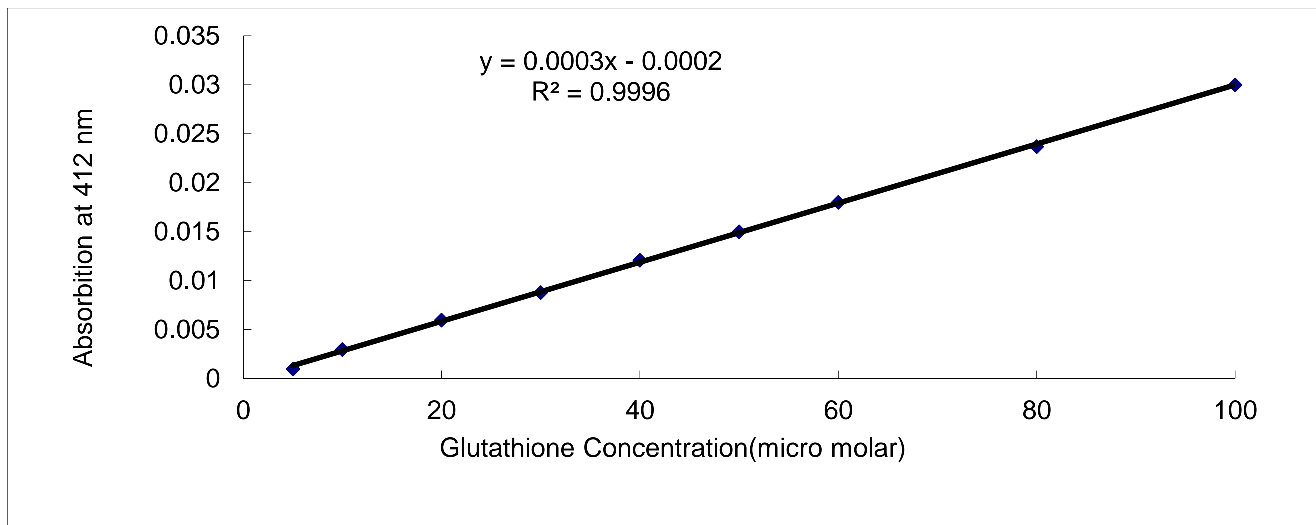


Fig. No. 3-4 : Standard curve of GSH

3-4-10 Statistical Analysis:

Information from the questionnaire from all participants were entered a data sheet and were assigned a serial identifier number. Multiple entry was used to avoid errors. The data analysis for this work was generated using The Statistical Package for the Social Sciences software, version 28.0 (IBM, SPSS, Chicago, Illinois, USA) and the Real Statistics Resource Pack software for Mac (Release 7.2) of the resource pack for Excel 2016. Copyright (2013 – 2020). Descriptive statistics was performed on the participants' data of each group. Values were illustrated by n (%) for categorical. The distribution of the data was checked using Shapiro-Wilk test as numerical means of assessing normality.

Significant differences in categorical variables among the parameters were confirmed through analytical statistical tests. Results of all hypothesis tests with *P*-values <0.05 (two-sides) were considered to be statistically significant.

The optimal threshold with high specificity and sensitivity for critical cases was detected using receiver operating characteristic (ROC) analysis. It was found out that all the values of P were two-sided, and a $P < 0.05$ was considered to be statistically significant.

Chapter Four

Results and Discussions

4- Results and Discussion

4-1 Isolation and identification of bacterial species causing Diabetic Foot Infection (DFI):

Swab samples were cultured on Blood and MacConkey ager media by streaking method and the Petri dished have been incubated at 37 ˚C for 48 hours , the isolation procedure resulted in obtaining of 23 (16.2 %) dishes without any growth whereas 119 bacterial colonies have been distinguished on the plates. Then 3 % KOH test has been performed to distinct between Gram negative and Gram positive bacteria and the application of this test results in obtaining of 63 (44.4 %) isolates of Gram positive and 56 (39.4 %) of Gram negative bacteria as mentioned in figure No. 4-1 .

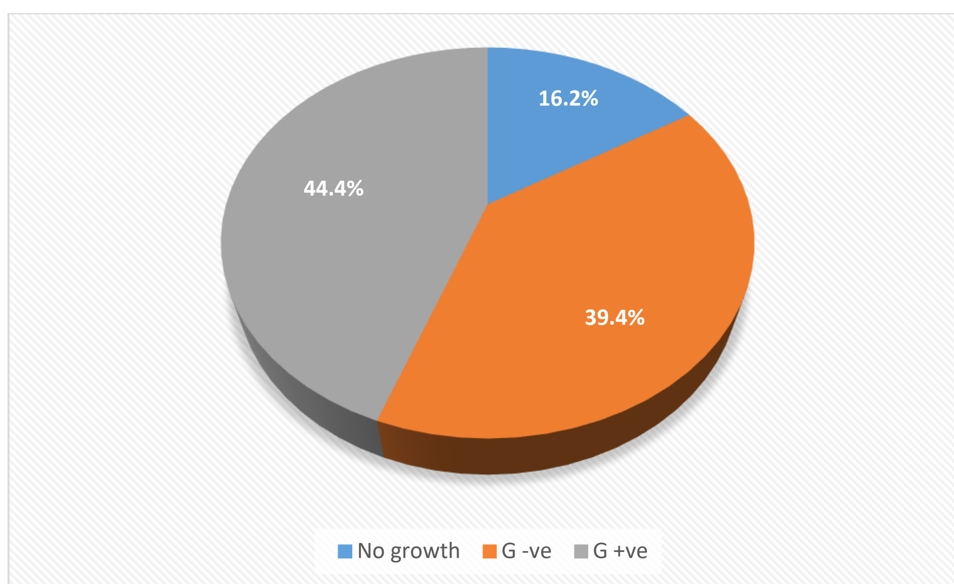


Fig. No. 4-1: The percentage of Gram positive and Gram negative bacteria isolated from DFI

Gram positive was the most prevalent bacteria in current study where they formed 44 % of the total isolates and this agree with numerous studies such as those done by Palomo *et al* (2022) and Shi *et al* (2023) whereas the percentages of

incidence of Gram positive bacteria were (68.1 and 52.3) % respectively in their studies that carried out in Brazil and China, respectively too.

The current findings disagreed with several studies, of those, Gram negative were the most prevalent bacteria (Adeyemo *et al*, 2019; Rahman *et al*, 2021 and Du *et al*, 2022) whereas Gram negative percentages were (59.2, 75.9 and 52.4) % in Nigeria, Bangladesh and China respectively.

This variance may be related to more frequent diabetic foot and the improper use of antibiotics in the developing countries. It was documented that Gram positive bacteria were prevalent in acute DFIs, whereas patients who had chronic wounds or had recently undertaken antibiotic therapy were at an enlarged risk of infection with Gram-negative bacteria (Lipsky *et al*; 2004).

The biochemical and microscopical tests were applied to identify the exact bacterial species obtained from DFI and the isolation procedure results in the predominance of *Staphylococcus aureus* 25 (21%) isolates, *Staphylococcus epidermidis* 18 (15%) , *Streptococcus agalactiae* 15 (13%) and *Enterococcus faecalis* 5 (4%) ,while of Gram negative , *Klebsiella pneumoniae* isolates were 25(21%) , *Pseudomonas aeruginosa* 13 (11%) , *Proteus mirabilis* 10 (8%) and *Escherichia coli* 8 (7 %) as mentioned in figure No. 4-2 .

The above results agreed with Ogba *et al* (2019) who obtained *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *E. coli* and *Klebsiella pneumoniae* in a percentage of (31.9 ,24.7, 17.5, 13.4 and 12.4) % respectively in a study performed in Nigeria while in another study done by Du *et al* (2022) it was reported that the most predominant pathogens isolated were *Staphylococcus aureus* (17.7%), *Escherichia coli* (10.9%), *Pseudomonas aeruginosa* (10.5%), *Klebsiella pneumoniae* (6.2%), *Staphylococcus epidermidis* (5.3%) and *Enterococcus faecalis* (4.9%) in their study which was carried out in China. In contrast, Rahman *et al* (2021) in Bangladesh also found that *Escherichia Coli* (51.9%)was the most

prevalent bacteria followed by *Staphylococcus aureus* (24.1%), *Proteus* spp. (16.7%), *Pseudomonas aeruginosa* (5.6%) and *Klebsiella* spp (1.9%).

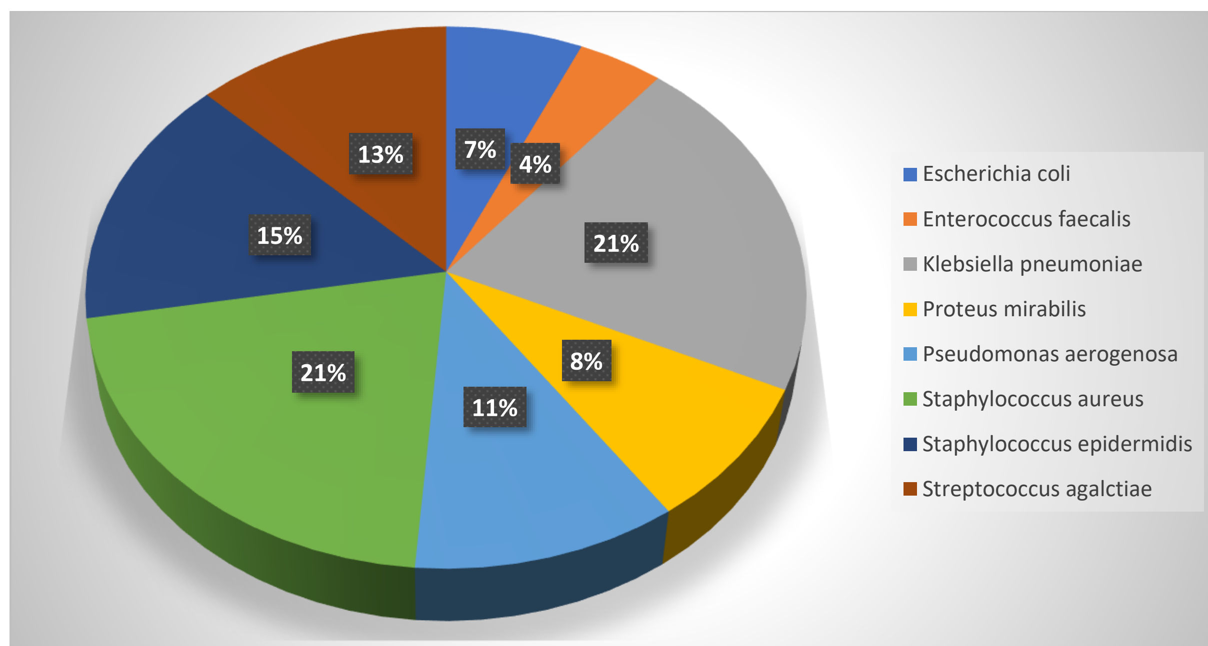


Fig. No. 4-2: Distribution of bacterial isolates obtained from DFI

This variation in bacterial profiles isolated from patients with DFUs could be attributed to variation in sample collection method, geographical region, treatment therapy and severity of infection (Al-Rubeaan *et al*; 2015). Banu *et al* (2015) documented that the bacterial nature of DFU infection is associated with the duration of the ulcer and previous antibiotic intake. In spite of the difference of bacterial distribution, *S.aureus* still the most prevalent in many studies of different countries such as in Australia (71.8%) (Commons *et al*; 2015) and China (17.7%) (Du *et al* ;2022). *Staphylococcus aureus* tends to colonize the skin or mucosal surfaces of diabetic patients, which can secrets a broad variety of enzymes and toxins such as proteases, lipases, nucleases, hyaluronidases, haemolysins (alpha, beta, gamma, and delta), and collagenase which cause host tissues more favorable for bacterial growth and tissue invasion (Shettigar and Murali ; 2020).

Klebsiella pneumoniae belongs to the *Enterobacteriaceae* family. It chiefly affects patients with compromised resistances to cause stark complications. It is an actual problem for patients with diabetes mellitus leading to “diabetic foot” infections and osteomyelitis (Podschun and Ullmann, 1998). As soon as infection is proven, *K. pneumoniae* produces a biofilm that permits evasion of the host’s defenses (Akers *et al;* 2014). Besides, phagocytosis by polymorphonuclear granulocytes is intensely delayed, as *K. pneumoniae* has an outer protective polysaccharide capsule, a key factor of their subsequent pathogenicity. The capsule overwhelms complement components, particularly C3b (Domenico *et al*, 1994; Diago-Navarro *et al*, 2014). Among many other pathogenicity features, bone adherence is credited to adhesion production that may be fimbrial, or non-fimbrial (Malhotra *et al;* 2014).

4-1-1 Distribution of bacterial isolates according to sex:

In current study, with exclusion of no growth swabs, males were the most vulnerable to diabetic foot infection (62.18 %) compared with females (37.81%) as shown in table No. 4-1 . These results consist with Aleem *et al* (2021) findings who documented that males and females formed (66 and 34) %, respectively of total diabetic foot infection patients in India while Jain and Barman (2017) in India found that males and females formed (81 and 18) % from total diabetic foot infection patients. Higher male incidence may be due to the higher level of outdoor physical activity with insufficient and inappropriate feet care among males in comparison to females. This result might probably have credited to the higher level of outdoor activity, have better admission to health care skills, deficiency of foot care and poor acquiescence to foot care tradition as compared to females. Additionally, male prevalence in DFU could be related to several factors such as gender-related variation in life styles, inadequate hygiene, kind of foot wear, and professional jobs that impose the feet to tolerate more pressure as a result of work and make them

more exposed to trauma (Al-Rubeaan *et al*, 2015 ; Amjad *et al*, 2017). A previous study found that male sex and poor glycemic control are independent risk factors for DFI (Shakil and Khan; 2010). Similar trends have been denoted in other reports, and authors have proposed that men are more probable to work outdoor which eventually elevates the risk to foot trauma and injury (Patil and Mane; 2017). Additionally, the propensity of women to take more concerning for medical care and personal cleanliness, higher percentage of women was present in the glycemic controlled group rather than men so more women in such group displayed non-infected wounds compared with men (Alhubail *et al*; 2020). However, this result is disagreeing with Jeber and Saeed (2013) who have found that the percent of infected foot findings in a local study whereas 46.6% were males and 53.3% were females.

Tab. No. 4-1 : Distribution of patients with DFI according to sex

Gender	**No. of patients**		***P*-value**
	No.	%	
Male	74	62.18	<0.001
Female	45	37.82	
Total	119	100%	

Almost several studies were reported an increased frequency of DFU in males than in females. Numerous studies reported that maximum number of DFU commonly occur within the age group of (40–60) years. Both male gender and age were thought to be as risk factor for DFU. Male prevalence was explained in several previous studies due to outdoor activity of them, difficult physical activity, be situated at higher risk for trauma, higher alcohol expenditure, elevated smoking behavior, better access to health-care settings, less obedience to foot care performance. The older patients with longer time of diabetic mellitus will have reduced immunity, nutritional insufficiencies, and were at risk for the occurrence of certain complications such as peripheral neuropathy and vascular diseases which

might lead to foot ulceration that distinguished by poor healing over a long period of time (Amjad *et al* ,2017 ; Neama *et al* ,2018; Marzoq *et al ,*2019 and Saleh and Hadi ,2019). In addition to several factors, such as professional activities and lifestyle that would lead the feet to tolerate more pressure have changed, and thus are more commonly seen in middle-aged and older people (Anvarinejad *et al*; 2015). The explanations for this may be that skin softens with age and can simply break even with unimportant trauma. The ability of the cells to proliferate declines as age progresses, response to stress and also immune response is diminished (Anand *et al*; 2016).

4-1-2 Distribution of bacterial isolates according to age:

Concerning the age groups, the range of patients age registered in this study was 35 to ≥ 75 with mean age (57.73± 13.05) and most subjects that have the highest number of isolates were aged (55-64) years 33 (27.7%) while subjects aged (≥ 75) years has the least number of isolates 18 (15 %) as shown in table No. 4-2 .

This distribution of isolates in current study is consist with Kadhim, (2021) findings in a local study who reported that all of the DFU patients were older than 41 years. Ogba *et al* (2019) reported that subjects aged (50–59) years had the highest number of isolates 37 (38.1%) whereas patients aged (70–79) years had the least number of isolates 15 (15.5%) in a study performed in Nigeria. In addition, this is in agreement with the document of Karmaker *et al* (2016) in Dhaka, Bangladesh with most of patients with average age of 58 years and older than 40 years. The study revealed that diabetics in their fourth and fifth decade of life were more susceptible to DFU also several previous studies documented that maximum number of DFU patients ensue within age range (40-60) years (Saleh and Hadi, 2019; Neama *et al*, 2018 and Anyim *et al,* 2019), respectively.

4-1-3 Distribution of polymicrobial and monomicrobial growth in DFI:

The poly-microbial infection rates in current study reached to 82% while mono-microbial rates were restricted in 18% as shown in figure No. 4-3 . Our findings are higher than those reported by Ogba *et al* (2019) where the poly-microbial infections reach to 72% in Nigeria. Globally, the number of isolates found in DFI varies widely. Poly-microbial infection rates have been found to be more prevalent in studies from a number of different countries including 83.7% in Portugal (Mendes *et al*; 2012) and 55.7% in Mumbai, India (Saseedharan *et al*; 2018) whereas others have reported higher rates of mono-microbial infections (Tiwari *et al,* 2012; Rahim *et al*,2016) in India and Pakistan, respectively. This alteration in number of isolates may be ascribed to diversity in DFU managing, through regular follow-up and better patient education and knowledge throughout the country in the recent year. In addition, DFI microbial etiology can alter due to differences in healthcare systems and standard protocols as well as ethnicity and health status of the patients studied (Al Benwan *et al*; 2012).

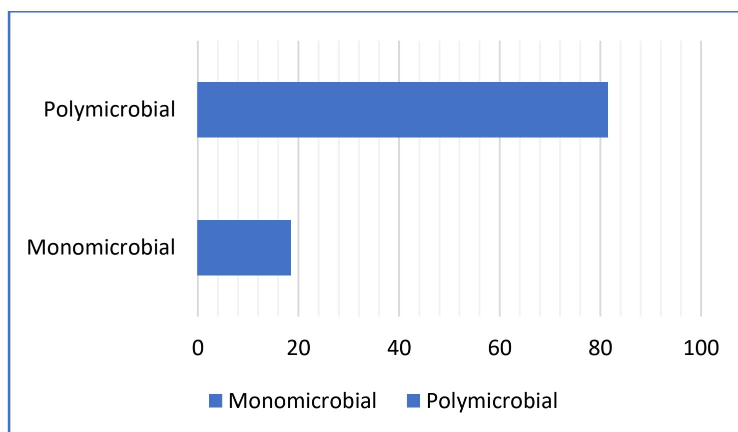


Fig. No. 4-3: The percentage of poly-microbial and mono-microbial infections in DFI

Tab. No. 4-2 : Distribution of bacterial isolates according to age of patients with DFI

Age group	No. (%) patients of DFI	No. (%) of isolates obtained from DFI								
		S. aureus	*S. epidermidis*	*S. agalactiae*	*E. faecalis*	*K. pneumoniae*	*P. aeruginosa*	*P. mirabilis*	*E. coli*	Total of isolates (%)
(35-44) years	26 (18.3%)	7 (28%)	3 (16.6%)	1 (6.66%)	1 (20%)	4 (16%)	2 (15.4%)	1 (10%)	2 (25%)	21 (17.6%)
(45-54) years	31 (21.83%)	8 (32%)	5 (27.7%)	2 (13.33%)	1 (20%)	5 (20%)	2 (15.4%)	2 (20%)	1 (12.5%)	26 (21.8%)
(55-64) years	40 (28.16%)	5 (20%)	6 (33.3%)	6 (40%)	1 (20%)	8 (32%)	3 (23%)	2 (20%)	2 (25%)	33 (27.7%)
(65-74) years	27 (19%)	4 (16%)	2 (11.11%)	4 (26.66%)	1 (20%)	5 (20%)	2 (15.4%)	3 (30%)	1 (12.5%)	22 (18.5%)
(≥75) years	18 (12.67%)	1 (4%)	2 (11.11%)	2 (13.33%)	1 (20%)	3 (12%)	4 (30.8%)	2 (20%)	2 (25%)	17 (14.3%)
Total	142	25 (21%)	18 (15%)	15 (13%)	5 (4%)	25 (21%)	13 (11%)	10 (8%)	8 (7%)	119

4-2 Identification of bacterial species isolated from DFI:

4-2-1 Identification of Gram positive bacteria:

4-2-1-1 Identification of *Staphylococcus aureus*:

The cultivation of swab gained from the diabetic foot infection on blood agar and the incubation of plates at 37 ˚C for 24 hours resulted in obtaining of round, convex, shiny, golden yellow colonies most of them could not grow on MacConky agar. Microscopically, the grown isolates appear as Gram positive cocci that can be suspected that bacteria belong to *Staphylococcal* genus which aggregate in clusters. The identification procedure results in obtaining of 25 isolates of *Staphylococcus aureus* and this agrees with the description of Levinson (2016). For the identification of *S.aureus* , the bacterial isolates cultivated on MSA and the biochemical tests would be applied.

4-2-1-1-1 Cultivation on Mannitol salt agar (MSA):

S.aureus colonies cultivated on MSA were opaque, shiny and yellow . MSA use to distinguish between *S.aureus* and other Gram positive cocci that are producing catalase. This media contain sodium chloride 7.5% that inhibits the growth of most bacterial species except *S.aureus* that could grow and ferment the mannitol sugar present in this media results in producing an acid that converts red phenol reagent color from pink to yellow (Tille; 2014).

4-2-1-1-2 Biochemical tests:

Results of biochemical tests for 25 isolates of *S.aureus* , revealed that all the isolates were producing catalase by their abilities to destruction of hydrogen peroxide (H_2O_2) and converting it to oxygen and water. Positive result appears as gaseous bubbles. When that 25 isolates were subjected to coagulase test, they gave positive results as they cause agglutination to plasma after incubation which agreed with Tille (2014). Table No. 4-3 describes the biochemical test results for all of the gram positive bacteria.

Tab. No. 4-3: Results of morphological and biochemical tests for Gram positive bacteria isolated from DFI

No.	Test	Results			
		S.aureus	*S.epidermidis*	*S.agalactiae*	*E.faecalis*
1	KOH test	_ Negative	_ Negative	_ Negative	_ Negative
2	Gram stain	+ Positive	+ Positive	+ Positive	+ Positive
3	Cellular appearance	Grape-like cells in irregular clusters	Spherical cells in irregular clusters	Spherical cells in chains	Spherical cells in pairs
4	Growth conditions	Facultative anaerobic	Facultative anaerobic	Facultative anaerobic	Facultative anaerobic
5	Catalase test	+ Positive	+ Positive	_ Negative	_ Negative
6	Oxidase test	_ Negative	_ Negative	_ Negative	+ Positive
7	Coagulase test	+ Positive	_ Negative	_ Negative	_ Negative

4-2-1-1-3 Identification of *S.aureus* by API STAPH kits:

S.aureus isolates were identified by API STAPH kits and the results that are documented in appendix No.1 show that the bacterial isolate gave positive results in GLU, FRU, MNE, MAL, MAN, PAL,VP, SAC, NAG, ADH and URE while gave a negative result in LAC, XLT, MEL, RAF and XYL while they gave a variable results in TRE, NIT and MDG. The results of API STAPH kits confirmed that the isolates belong to *Staphylococcus aureus.*

4-2-1-2 Identification of *Staphylococcus epidermidis*:

Cultivation of swabs on blood agar and incubation for 24 hours at 37 ˚C results in growing of 18 isolates of small to medium; opaque, gray-white colonies; most colonies non hemolytic; slime-producing strains are extremely sticky and adhere to

the agar surface (Tille; 2014). Microscopically, they appear as Gram positive cocci grouped together in clusters which indicate that bacteria belong to *Staphylococcus* genus (Levinson; 2016).

Biochemical tests of 18 *Staphylococcus* isolates revealed that they produce catalase which breakdown H_2O_2 and converts it into water and oxygen gas. Positive result appears as gaseous bubbles. Negative results were obtained for coagulase test for all the isolates which they are described in table No. 4-3 .

S.epidermidis isolates were identified also by API STAPH kits and the results that are illustrated in appendix No. 2 showed that the bacterial isolate gave positive results in GLU, MNE, MAL, MAN, PAL, SAC, ADH and URE while gave a negative result in FRU, TRE, MAN, XLT, MEL, RAF, VP and XYL while they gave a variable result in LAC and NIT. The results of API STAPH kits confirmed that the isolates belong to *Staphylococcus epidermidis.*

4-2-1-3 Identification of *Streptococcus agalactiae*:

The swabs also cultured on blood agar and incubation for 24 hours at 37 ˚C result in obtaining of 15 isolates which are small, transparent colonies and β-hemolysis to blood. Microscopically, they appeared as gram positive cocci grouped together in a long chain which indicate that bacteria belong to *Streptococcus* genus. (Levinson; 2016).

All of the 15 isolates gave a negative result in catalase test and coagulase which gave a prediction of *Streptococcus agalactiae* that illustrated in table No. 4-3 .

The 15 isolates *S.agalactiae* were identified by API 20 STREP kits and the results that are illustrated in appendix No. 3 showed that the bacterial isolate gave positive results in VP, HIP, βGUR , LAP, ADH, RIB, TRE and AMD while gave a negative result in ESC, PYRA, αGAL, βGAL, PAL, ARA, MAN, SOR, LAC, INU, RAF and GLYG. The results of API 20 STREP kits confirmed that the isolates belong to *Streptococcus agalactiae.*

4-2-1-4 Identification of *Enterococcus faecalis*:

After cultivation of swabs obtained from DFU on blood agar and incubation it at 37°C for 24 hours, five isolates were obtained as small, smooth, pale yellow or white colonies. Microscopically, they appear as gram positive diplococci which indicate that bacteria belong to *Enterococcus faecalis* (Tille; 2014).

Biochemical tests performed on the 5 isolates suspected to be *Enterococcus faecalis* display that bacteria were facultative anaerobic, catalase negative and oxidase positive as illustrated in table No. 4-3 .

The five isolates *E.faecalis* were checked also by API 20 STREP kits and the results that are illustrated in appendix No. 4 showed that the bacterial isolate gave positive results in VP, ESC, PYRA, LAP, ADH, MAN, SOR and TRE while gave a negative result in HIP, αGAL, βGUR, βGAL, PAL, ARA, LAC, INU and RAF while gave a variable result in AMD and GLYG. The results of API 20 STREP kits confirmed that the isolates belong to *Enterococcus faecalis.*

4-2-2 Identification of Gram negative bacteria:

4-2-2-1 Identification of *Klebsiella pneumoniae:*

The swabs collected from DFU were cultured on blood agar and incubated for 24 hours at 37° C , twenty five isolates of mucoid gray colonies were appeared while these isolates appear as large, mucoid light pink colonies when they have been grown on MacConky agar. Microscopically, they appeared as gram negative bacilli. These characteristics refer that isolates belong to *Klebsiella* genus. MacConkey agar considers as selective-differential media for *Enterobacteriaceae.* It considered selective because it contains crystal violet stain which inhibit Gram positive bacteria growth and contains bile salt which inhibit the growth of Gram negative bacteria except members of *Enterobacteriaceae* also it considers a differential media because it distinguishes between lactose fermented and non-fermented bacteria. It contains

lactose and red phenol as a pH indicator thus the colonies that ferments lactose sugar produces an acid that converts the color of the indicator into red (Mahon ;2019).

Application of biochemical tests on the 25 isolates suspected to be *K.pneumoniae* showed that the isolates were facultative anaerobic and gave negative results in catalase and oxidase as illustrated in table No. 4-4 .

The 25 isolates *K.pneumoniae* were identified by API 20 E kits and the results that are mentioned in appendix No.5 show that the bacterial isolate gave positive results in ONPG, CIT , URE, GLU, MAN, SOR, RHA, SAC, AMY and ARA while gave a negative result in ADH, LDC, ODC , H_2S , TDA, IND and GEL while gave a variable result in VP, INO and MEL. The results of API 20 E kits confirmed that the isolates belong to *Klebsiella pneumoniae* (Mahon ;2019)

4-2-2-2 Identification of *Pseudomonas aeruginosa:*

The cultivation of DFI swabs on blood agar and incubation them at 37° C for 24 hours resulted in the appearance of 13 flat colonies with metallic luster and serrated wavy margins surrounded by a green color with a grape smell also they have a transparent region around the colonies as a result of haemolysin production from bacteria that hemolysis the blood in the media (β- hemolysis) (Mahon ;2019). On MacConky agar they appear some pale colonies because of their incapacity of lactose fermentation. Microscopically, they appear as a Gram negative bacilli. These features indicate that bacteria are *Pseudomonas aerogenosa*. (Levinson; 2016).

Biochemical tests of the 13 isolates of *Pseudomonas* revealed that they gave a positive result for catalase, oxidase and KOH as showed in table No. 4-4 .

The 13 isolates *P.aerogenosa* were checked also by API 20 E kits and the results that are illustrated in appendix No. 6 displays that the bacterial isolate gave positive results in ADH , URE, GLU,VP, GEL, GLU, MEL, AMY and ARA while gave a negative result in ONPG, LDC, ODC , H_2S , TDA, IND, MAN, INO, SOR, RHA

Tab. No. 4-4: Results of morphological and biochemical tests for Gram negative bacteria isolated from DFI

No.	Test	Results			
		K.pneumoniae	*P.aeruginosa*	*P.mirabilis*	*E.coli*
1	KOH	+ Positive	+ Posative	+ Posative	+ Posative
2	Gram stain	_ Negative	_ Negative	_ Negative	_ Negative
3	Cellular appearance	Rod cells	Rod cells	Rod cells	Rod cells
4	Growth conditions	Facultative anaerobic	aerobic	Facultative anaerobic	Facultative aerobic
5	Catalase test	_ Negative	+ Posative	+ Posative	+ Posative
6	Oxidase test	_ Negative	+ Posative	_ Negative	_ Negative
7	Coagulase test	_ Negative	_ Negative	_ Negative	_ Negative

and SAC while gave a variable result in CIT. The results of API 20 E kits confirmed that the isolates belong to *Pseudomonas.aerogenosea.*

4-2-2-3 Identification of *Proteus mirabilis*:

The cultivation of swabs also on blood agar and incubation for 24 hours at 37° C results in obtaining of 10 gray colonies characterized by their swarming growth on the plate surface as waves and converting the blood into brown color with foul smell (Mahon ;2019) whereas the cultivation on macConkey agar, the colonies were pale with irregular margins. Microscopically, they were a Gram negative bacilli and all these features indicate that isolates belong to *Proteus mirabilis*. (Levinson; 2016).

Biochemical tests showed that *P.mirabilis* were facultative anaerobic, positive to catalase test, negative to oxidase test (Levinson; 2016). Results of *P.mirabilis* identification are illustrated in table No. 4-4 .

The 10 isolates of *P.mirabilis* were identified similarly by API 20 E kits and the results that are showed in appendix No. 7 displays that the bacterial isolate gave positive results in ODC, URE, H_2S and GLU while gave a negative result in ONPG, LDC, ADH, IND, MAN, INO, SOR, RHA, SAC, MEL, AMY and ARA while gave a variable result in CIT, TDA, VP and GEL. The results of API 20 E kits confirmed that the isolates belong to *Proteus mirabilis* (Mahon ;2019).

4-2-2-4 Identification of *Escherechia coli*:

The swabs were cultured on blood agar and incubated at 37˚ C for 24 hours, 8 isolates of gray smooth shiny colonies were obtained while the cultivation on MacConkey agar result in the appearance of flat, dry and pink colonies with enclose by a dark pink region as a result of bile salt deposition (Mahon ;2019). Microscopically, they were Gram negative bacilli and all these characteristics refer that isolates belong to *Escherichia coli*. (Levinson; 2016).

Biochemical tests on the 8 isolates gave a positive result of catalase test and negative result for oxidase test (Levinson; 2016) and the results summarizes in table No. 4-4 .

Checking up of the 8 isolates *E. coli* were applied also by API 20 E kits and the results that are mentioned in appendix No. 8 display that the bacterial isolate gave positive results in ONPG, LDC, IND, GLU, SOR and MEL while gave a negative result in ADH, ODC, CIT, H2S, URE, VP, GEL, TDA, INO and AMY while gave a variable result in RHA, SAC, MEL and ARA. The results of API 20 E kits confirmed that the isolates belong to *Escherichia coli*.

4-3 Susceptibility tests of the most prevalent bacterial species in DFI toward antibiotics

4-3-1 Susceptibility tests of *Staphylococcus aureus*:

In the current study, 25 isolates of *Staphylococcus aureus* were obtained from DFI swab which were the most predominant Gram positive bacteria among others. 15 (60%) of which were MRSA. For more accurate results, we labelled the 25 isolates as S1, S2, S3, S4, S5, S6, S7, S8, S9, S10, S11, S12, S13, S14, S15, S16, S17, S18, S19, S20, S21, S22, S23, S24 and S25 subsequently mentioned with details in appendix No. 9 .

Results shown in table No. 4-5 demonstrated that all isolated *Staphylococcus aureus* was maximum sensitive (84%) to Levofloxacin, while the lowest sensitivity (36%) was toward Azithromycin and Erythromycin.

Forty percentage of the sensitivity was toward Penicillin, Amoxicillin-Calvulonic Acid, Ceftazidime, Cefepime Cefotaxim, Ceftriaxone, Imipenem, Meropenem, Aztronem and Oxacillin, while the sensitivity of both Rifampin and Vancomycin were 52% and Ciprofloxacin was 48%.

In current study, 60% of *S.aureus* isolates were resistant to Penicillin, Pipracillin, Amoxicillin-clavulanic acid, Ceftazidime, Cefepime, Cefotaxim, Ceftriaxone, Imipenem, Meropenem and Aztronem that belong to β- lactam antibiotics. All of these isolates were (MRSA). These results agreed with Dwedar *et al* (2015) in Egypt who reported that all MRSA strains isolated from DFI were 100% resistant to anti-staphylococcal β-lactams, combined penicillins, 3rd generation of Cephalosporins and Cabapenems.

S.aureus can resist β-lactams by several methods such as the synthesis of β-lactamases which hydrolyzes the β-lactam ring of penicillin thereby execution it inactive (Lowey ;2003). Previous studies showed that β-lactam antibiotics have a

fatal effect on bacteria chiefly by two mechanisms: first, by binding to penicillin-binding protein (PBPs, i.e., cell wall mucin synthase), which suppresses cell wall mucin synthesis, interrupts the cell wall, and leads to bacterial enlargement and lysis; second, by prompting the autolytic enzyme activity of the bacteria, which resulted in autolysis and death (Matono *et al*; 2018).

Tab. No. 4-5: Susceptibility tests of *S.aureus* toward antibiotics

Bacterial susceptibility	**Antibiotics**																
	Penicillin	Pipracillin	Amoxicillin-Clavulanic Acid	Ceftazidime	Cefepime	Cefotaxim	Ceftriaxone	Imipenem	Meropenem	Aztronem	Azithromycin	Rifampin	Vancomycin	Ciprofloxacin	Levofloxacin	Oxacillin	Erythromycin
Sensitive (%)	40	40	40	40	40	40	40	40	40	40	36	52	52	48	84	40	36
Resistant (%)	60	60	60	60	60	60	60	60	60	60	64	48	48	52	16	60	64

In current study, 84% of *S.aureus* isolates were found to be sensitive to Levofloxacin while a study done by Turhan *et al* (2013) in which they reported that 95% of *S.aureus* isolated from DFI were sensitive to Levofloxacin in Turkey while 73.34% of *S.aureus* were sensitive to Levofloxacin according to Shareef *et al* (2017) in India. Levofloxacin belong to fluoroquinolones which are classified as bactericidal drugs. They inhibit the activity of topoisomerase II (gyrase) and topoisomerase IV enzymes, which are responsible for DNA super-coiling and recapitalization (Mlynarczyk-Bonikowska *et al*; 2022).

In the current study, 64% of *S.aureus* isolates were resistant to Erythromycin while Goh *et al* (2020) reported that 56% of *S.aureus* were resistant to Erythromycin in Malaysia and Neama *et al* (2018) in a local study reported that 70.7% of *S.aureus* were resistant to Erythromycin also Du *et al* (2022) in China reported that 67.6% of *S.aureus* were resistant to Erythromycin. On the other hand, 64% of *S.aureus* isolates were resistant to Azithromycin in our study. This is not in agreement with Du *et al* (2022) who reported that *S.aureus* isolated from DFI were 85% resistant to Azithromycin in a study performed in China.

Erythromycin and Azithromycin belong to Macrolides group and the resistance of bacteria to them can be illustrated by two mechanisms: first, by producing Estrase enzyme that disrupt lactone ring of the antibiotic or make an alteration in antibiotic structure by transferring of the functional group such as acyl, ribosyl, and phosphpryl through making an alteration in antibiotic structure by action of Glycocyl transferase. Second, through the modification of the antibiotic target by methylation of 23SrRNA (Kumar and Varela ;2013). The modification is carried out by the enzymes Adenylyl-N-methyltransferase (erythromycin ribosome methylation (ERM)), dimethylating adenine, which leads to resistance to all Macrolides (Mlynarczyk *et al ;*2010).

With respect to Rifampin, 52% of *S.aureus* isolates were sensitive in this study and that was approach to Chai *et al* (2021) findings who reported that 54.2% of *S.aureus* were sensitive to that antibiotic in China. Rifampin is a wide spectrum antibiotic against bacterial pathogens and its mode of action summarized by its ability to inhibit bacterial RNA-polymerase which can directly block the pathway of RNA elongation (Campbell *et al* ;2001).

Concerning Vancomycin, 52% of *S.aureus* isolates were sensitive to that antibiotic in current study whlie Atlaw *et al* (2022) reported in their study that 63% of *S.aureus* isolated from DFI were sensitive to that antibiotic in Ethiopia .

Vancomycin belongs to Glycopeptides which they are bactericidal and their mechanism of action is through inhibition the synthesis of peptidoglycan. Glycopeptides make bonds with the dipeptide D-Ala-D-Ala within GlcNAc-β-(1,4)-MurNAc-pentapetide, the precursor of peptidoglycan. These process occurs outside the cytoplasmic membrane (Zeng *et al* ;2016).

In current study, 52% of *S.aureus* isolates were resistant to Ciprofloxacin which is a close result to 57.14% that were resistant to Ciprofloxacin according to Aiswariya *et al* (2018) and with Du *et al* (2022) who reported that *S.aureus* were 50% resistant to Ciprofloxacin in India and China, respectively. Also Atlaw *et al* (2022) in Ethiopia reported that 50% of *S.aureus* isolated from DFI were resistant to Ciprofloxacin. Drug target mutations (DNA gyrase and DNA topoisomerase IV), mutations that reduce drug accumulation, and plasmids that protect cells from ciprofloxacin's lethal effects are the three mechanisms of ciprofloxacin resistance that have been discovered (Aslam *et al* ;2018).

With respect to Oxacillin, 60% of *S.aureus* in this study were resistant to this antibiotic. These results are not in agreement with Atlaw *et al* (2022) who documented that 81.2% of *S.aureus* isolated from DFI were resistant to Oxacillin in Ethiopia. Methicillin-resistant *S. aureus* (MRSA) have a major and durable problem to the treatment of infection by such strains. Resistance is frequently discussed by the acquirement of a non-native gene encoding penicillin-binding protein (PBP2a), with considerably lower affinity for β-lactams. This resistance permits cell-wall biosynthesis, the target of β-lactams, to sustain even in the presence of normally inhibitory concentrations of antibiotic. PBP2a is encoded by the *mec*A gene, which is carried by a discrete mobile genetic element (SCCmec) (Peacock and Paterson; 2015).

4-3-2 Susceptibility tests of *Klebsiella pneumoniae*:

Twenty-five isolates of *Klebsiella pneumoniae* were collected from DFI swab which were the most predominant Gram negative bacteria in the current study. The 25 isolates were labeled as K1, K2, K3, K4, K5, K6, K7, K8, K9, K10, K11, K12, K13, K14, K15, K16, K17, K18, K19, K20, K21, K22, K23, K24 and K25, respectively which were mentioned in appendix No. 10 .

Antibacterial susceptibility of *Klebsiella pneumoniae* was presented in table No. 4-6 . It was shown that the highest resistance 100% was toward Gentamycin and Levofloxacin followed by 96% resistance toward Ciprofloxacin and 92% resistance toward Amikacin. With respect to Pipracillin-Tazobactam, Cefotaxim, Ceftriaxone and Tetracyclin, resistance percent was 88% while Amoxicillin-Clavulanic acid, Cetazidime, and Cefepime were 84% resistant. Furthermore, *K.pneumoniae* has 76% resistance toward Imipenem and Meropenem and 68% toward Doxycycline and 56% with respect to Rifampin.

In current study, *K.pneumoniae* isolates were 88% resistant toward Pipracillin-tazobactam which are not in agreement with (Raheem *et al*; 2021 and Ali and Kamil ;2022) who documented that *K.pneumoniae* isolated from clinical sources were 100% resistant to Pipracillin-tazobactam in a local study . *K.pneumoniae* were resistant to Cefipime, Cefotaxim, Ceftriaxone and Ceftazidine in (84, 88, 88 and 84)%, respectively and that are not in agreement with Hamid *et al* (2020) who reported that *K.pneumoniae* isolated from DFI were 100% resistant to the mentioned antibiotics in Sudan. In the current study, *K.pneumoniae* isolates were 84% resistant to Amoxicillin-clavulanic acid while Hamid *et al* (2020) documented that *K.pneumoniae* were 90.1% resistant to the mentioned antibiotic in Sudan also it approaches to Aiswariya *et al* (2018) findings whereas *K.pneumoniae* resistance toward Amoxicillin-clavulanic acid was 81.25% in India.

Tab. No. 4-6: Susceptibility tests of *K.pneumoniae* toward antibiotics

Bacterial susceptibility	**Antibiotics**														
	Pipracillin-Tazobactam	Amoxicillin-Clavulanic Acid	Ceftazidine	Cefepime	Cefotaxime	Ceftriaxone	Imipenem	Meropenem	Amikacin	Gentamycin	Rifampin	Ciprofloxacin	Levofloxacin	Tetracycline	Doxycycline
Sensitive (%)	12	16	16	16	12	12	24	24	8	0	44	4	0	12	32
Resistant (%)	88	84	84	84	88	88	76	76	92	100	56	96	100	88	68

Numerous resistance mechanisms against β-lactams include modifications in the drug target site, reduced membrane permeability, and the activity of the drug efflux pump, but β-lactamases, especially extended spectrum β-lactamases (ESBLs) and carbapenemases are the most public features (Khalifa *et al;* 2021). This may be credited to the overuse of antibiotics or to the capacity of the microbes to grow as biofilms, or affected by genetic means, containing mutations and the horizontal transmission of resistance genes (Albu *et al*; 2018).

K.pneumoniae in this study were 76% resistant toward Imipenem and Meropenem while in a study performed by Oliveira *et al* (2022) in Portugal , it was clear that imipenem was effective against 32.4% isolates and meropenem was effective against 34.4% of *Klebsiella pneumoniae* isolated from clinical sources. This conclusion is not astonishing with the fact *Klebsiella spp*. is not only the chief reason of nosocomial infections but also a famous “collector” of multidrug resistance plasmids. As the most important carbapenemases, KPC enzymes

distribute mostly among *Klebsiella* spp., and result in reduced susceptibility to carbapenems (Tzouvelekis *et al*; 2012).

K.pneumoniae were 100% resistant to Gentamycin in our study and this result agreed with Rahman *et al* (2021) in Bangladesh who documented that *K.pneumoniae* isolated from DFI were 100% resistant to Gentamycin. Additionally*, K.pneumoniae* were 92% resistant toward Amikacin while Ali and Kamil (2022) documented that *K.pneumoniae* isolated from DFI were 100% resistant toward Amikacin in a local study. The best common mechanism of resistance to aminoglycosides includes aminoglycoside-modifying enzymes (AMEs). These enzymes include acetyltransferases, nucleotidyltransferases and phosphotransferases which vary in their capacity to modify aminoglycosides (Miró *et al*; 2012). Other mechanisms contributing to aminoglycoside resistance involve the upregulation of efflux pumps (Poole; 2004) and reduced intake of antibiotics into the bacterial cell (Nakamatsu *et al*; 2007). However, the production of 16S rRNA methyltransferase (16S-RMTase) is another important mechanism mediating resistance to approximately all clinically available Aminoglycosides (Doi *et al;* 2016).

K.pneumoniae were 100% resistant toward Levofloxacin and that result agreed with Liu *et al* (2022) whereas *K.pneumoniae* isolated from Diabetic foot ulcer were 100% resistant toward Levofloxacin in China. Also 96% resistance toward Ciprofloxacin showed by *K.pneumoniae* which are close to Ali and Kamil (2022) findings who reported that *K.pneumoniae* obtained from DFI were 100% resistant toward Ciprofloxacin in a local study. Resistance to fluoroquinolone is facilitated by numerous mechanisms. The main mechanism is the chromosomal mutation at quinolone resistance determining regions (QRDR) encoded by DNA gyrases (*gyr*A and *gyr*B genes) and topoisomerase IV (*par*C and *par*E genes) (Strahilevitz *et al*; 2009). The additional mechanism of resistance is plasmid-mediated quinolone resistance (PMQR) and this was first stated in 1998 in a clinical isolate of *K.*

pneumonia (Redgrave *et al*; 2014). The three PMQR mediators are the *qnr* proteins that protect the target enzymes encoding DNA gyrase and topoisomerase IV. Nevertheless another mechanism credited to fluoroquinolone resistance is the acc(6')-Ib-cr gene, encoding an aminoglycoside transferase which acetylates definite fluoroquinolones also. The *qep*A and *oqx*AB are specific efflux pump encoding genes that fling fluoroquinolone from bacterial cell, thus lead to resistance (Machuca *et al*;2014).

In current study, *K.pneumoniae* isolates were 88% resistant to tetracycline and this result is close related to Liu *et al* (2022) findings in China whereas they documented that *K.pneumoniae* isolated from DFI were 87.5 % resistant to tetracycline. Additionally, *K.pneumoniae* isolates were 68% resistant toward Doxycycline in the current study whereas resistance rate was 85% toward Doxycycline with respect to *K.pneumoniae* isolated from DFI in India (Wasnik *et al*; 2019).

The uncritical use of these antibiotics has led to antibiotic resistance. Tetracycline resistance is resulted from three mechanisms. First, overexpression of efflux pumps which decreases the cell's penetrability to antibiotics (Garcia *et al*; 2011). Second, proteins which guard ribosomes (S30 and S16) from tetracycline, modify the structure of these proteins, producing resistance to doxycycline and minocycline. Third, enzymatic alterations in antibiotics also result in resistance. The *tet*X gene result in antibiotic resistance due to tetracycline enzyme inhibition (Taylor and Chau; 1996).

K.pneumoniae isolates were 56% resistant toward Rifampin in current study and that result is agreed with HA *et al* (2016) in Egypt who reported that generally all *K.pneumoniae* isolates were resistant toward the mentioned antibiotic. Rifampin is an appreciated antibiotic for the treatment of mycobacterial and other infections. The clinically major resistance mechanism is mutation within a well-defined region of

the *rpo*B gene, which encodes the target of RIF, the β-subunit of bacterial RNA or RNA polymerase (Goldstein; 2014).

4-4 Investigation of Biofilm forming Bacteria:

4-4-1 Investigation of Biofilm formation by tube method:

Biofilm that is an adhesive exopolysaccharide is the main virulence factor causing biofilm-related infections. Tube method (TM) that is a qualitative test for recognition of biofilm producer microorganism, as a result of the incidence of visible film, is described by (Christensen *et al*; 1985) in USA.

4-4-1-1 Investigation of Biofilm formation of *Staphylococcus aureus* by tube method:

In the current study, all of the 25 isolates of *S.aureus* obtained from DFI were Biofilm formers compared with control tube. This result is agreed with Kashef *et al* (2022) who reported that 96.6% of *S.aureus* isolates were able to produce biofilm to variable degrees in Egypt while Manandhar *et al* (2018) in India reported that 34.4% of *Staphylococcus* isolates were found to be biofilm formers while 65.6% were biofilm non-producers according to tube method.

The tube method results showed 64% sensitivity and 74% specificity as compared to the genotypic assay consistence with the previous study (Oliveira and Cunha; 2010).

4-4-1-2 Investigation of Biofilm formation of *Klebsiella pneumoniae* by tube method:

All of the 25 isolates of *Klebsiella pneumoniae* obtained from DFI in current study were biofilm producers with respect to tube method. Moteeb (2008) found that 82.35% of *K.pneumoniae* isolates were biofilm producers in a local study and

17.65% were non producers according to tube method while Chilupuri *et al* (2021) found that 73.07% of *K.pneumoniae* isolates were positive for biofilm production in India. This divergence in results may be related to geographic distance, epidemiological variation, regional variation, antimicrobial-prescribing designs in hospitals and level of sanitization, criteria of the included patients, size of sample and duration of study (El-Badawi *et al*; 2017).

4-4-2 Quantification of Biofilm formation by microtiter plate method:

4-4-2-1 Quantification of Biofilm formation of *S.aureus* by microtiter plate method:

According to the microtiter plate method, 60% of *S.aureus* obtained from DFI in current study were strong producers for biofilm while 40% of them were moderate producers. The exact results with the distribution of bacterial isolates are mentioned in appendix No. 11 . This result is not in agreement with a study performed by Mamdoh *et al;* (2023), it has been found that 59% of *Staphylococcus* isolates were strong producers while 32.8% were moderate and 8.2% were weak producers in a study performed in Egypt while Mahmoudi *et al* (2019) in Iran reported that 58% of the *S.aureus* were strongly formers for biofilm , 22% were moderate producers , 14% were weak biofilm producer and 6% were non producers. In contrast, Liesse Iyamba *et al* (2022) in a study performed in Congo reported that 61.53% of *S.aureus* obtained from DFI were moderate producers of biofilm while Águila-Arcos *et al* (2017) found that (20, 44, 16, and 20)% of *S.aureus* isolated from clinical sources in Spain were strong, moderate, weak and non-producing biofilm producers, respectively.

With respect to the biofilm formation and antibiotics resistance, table No. 4-7 showed the association between the ability of *S.aureus* to produce the biofilm and their resistance toward the most traditional antibiotics.

Results revealed that 100% of the strong producers were resistant to Oxacillin and β-Lactam antibiotics. This is agreed with Gaire *et al* (2021) who reported that 100% of the strong biofilm producer *S.aureus* were resistant toward penicillin in Nepal and Pajohesh *et al* (2022) who found that all strong biofilm former isolates 100% presented resistance to penicillin and practically all were resistant to ampicillin and oxacillin 95.65% in Iran.

With respect to Erythromycin, our result revealed that 100% of strong producers were resistant to Erythromycin and this agree with Ghaderi *et al* (2020) who reported the same result in a study performed in Iran. Tahaei *et al* (2021) documented that resistance to Erythromycin and Rifampin was associated with biofilm positivity in their study in Hungary.

Regarding Vancomycin and Azithromycin, our result agreed with Iyamba *et al* (2022) in Congo who reported that 100% of strong *S.aureus* biofilm producers were resistant to Vancomycin and Azithromycin.

Resistance to Levofloxacin display a contrast result with Iyamba *et al* (2022) as our results reveal that strong and moderate biofilm producers of *S.aureus* have the highest percent of sensitivity to Levofloxacin and this conflicts with Iyamba *et al* (2022) who reported that 100% of *S.aureus* were resistant to Levofloxacin.

S.aurues showed variable rates of sensitivity to Rifampin which disagreed with Sultan *et al* (2022) in Indonesia who reported that all strains of *S.aureus* under study were sensitive and showed an early reduction in biofilm fitness after administration 8 μg/mL of rifampicin.

Biofilm production is major defense mechanism of bacteria that rises the founding and duration of infection. The ability of MRSA to produce a biofilm responsible for difficult-to-treat infections make MRSA a severe risk to human health, which is linked with tolerance of staphylococcal biofilms to antibiotics and can cause failure of antibiotic therapies. It was concluded that *S.aureus* isolated from

Tab. No. 4-7: Association between biofilm formation and antibiotic resistance of *S.aureus*

Antibiotics	**Biofilm formation ability**			
	Moderate n=10		**Strong n=15**	
	R No.(%)	**S No.(%)**	**R No.(%)**	**S No.(%)**
penicillin	0	10 (100%)	15 (100%)	0
Pipracillin	0	10 (100%)	15 (100%)	0
Amoxicillin-clavulanic acid	0	10 (100%)	15 (100%)	0
Ceftazidime	0	10 (100%)	15 (100%)	0
Cefepime	0	10 (100%)	15 (100%)	0
Cefotaxim	0	10 (100%)	15 (100%)	0
Ceftriaxone	0	10 (100%)	15 (100%)	0
Imipenem	0	10 (100%)	15 (100%)	0
Meropenem	0	10 (100%)	15 (100%)	0
Aztronem	0	10 (100%)	15 (100%)	0
Azithromycin	1 (10%)	9 (90%)	15 (100%)	0
Rifampin	4 (40%)	6 (60%)	8 (53.4%)	7 (46.6%)
Vancomycin	5 (50%)	5 (50%)	10 (66.7%)	5 (33.3%)
Ciprofloxacin	4 (40%)	6 (60%)	8 (53.4%)	7 (46.6%)
Levofloxacin	1 (10%)	9 (90%)	4 (26.7%)	11 (73.3%)
Oxacillin	0	10 (100%)	15 (100%)	0
Erythromycin	1 (10%)	9 (90%)	15 (100%)	0

clinical sources which have been produced biofilm were possessed *ica*A and *ica*D genes, with varied biofilm mass, signifying that these strains may moreover use other system to form biofilm (Kot *et al*, 2020 ; Azmi *et al*,2019).

4-4-2-2 Quantification of Biofilm formation of *K.pneumoniae* by microtiter plate method:

Sixty percentage of *K.pneumoniae* isolated from DFI in current study were strong producers of biofilm according to microtiter plate method while 40% of them were moderate producers. However, 62.5% of *K.pneumoniae* isolated from DFI in a local study were strong producers of biofilm and 37.5% were moderate producers as reported by Mahmood and Abdullah (2015). In another study performed in Indonesia, among biofilm producers, there were 26.95% isolates as strong, 28.74% isolates as moderate, and 29.94% *K.pneumoniae* isolates identified as weak biofilm producers (Nirwati *et al*; 2019). On the other hand, the biofilm analysis indicates that 32.5% *K. pneumoniae* isolates formed biofilm weakly, 21.6% isolates created moderately, and 20.4% isolates were strong producers of biofilms whereas 25.5% were non producers of biofilm in a study performed in Iran (Karimi *et al;* 2021).

Biofilm production is a vital feature in *Klebsiella pneumoniae* pathogenesis, ensuring increased resistance against environmental stressors and giving a reservoir for spreading and further gene exchange linked with antimicrobial resistance (Assoni *et al*; 2021). Numerous studies have discovered that type 3 fimbriae (*mrk*D gene) are essential in *K. pneumoniae* biofilm formation. Therefore, the *mrk*D gene may play major roles in forming biofilm (Jagnow and Clegg; 2003).

Regarding biofilm formation and antibiotics resistance, table No. 4-8 showed the association between the capability of *K.pneumoniae* to form the biofilm and their resistance toward the most traditional antibiotics. The current results revealed that all the biofilm producer isolates (strong and moderate) were resistant to the most traditional antibiotics which were similar to Shadkam *et al* (2021) findings who reported the highest resistance rates of strong and moderate biofilm *K.pneumoniae* producers to cefotaxime, cefepime, ceftriaxone, ceftazidime and ciprofloxacin in Iran while Nirwati *et al* (2019) reported that resistance rates of biofilm

K.pneumoniae producers were greater than non-biofilm producers to ampicillin, cefepime, ceftriaxone, gentamycin, amoxicillin-clavulanic acid, ciprofloxacin, Levofloxacin and meropenem in Indonesia. Antibiotic acquaintance is the most vital factor of antimicrobial resistance. The evolution of antibiotic resistance is comprising many factors such as the overuse of antibiotics in the hospital and in community. As a magnitude of the ability in purchasing antibiotics for free without recommendation, therefore, antibiotics are used disproportionately. In the health service setting, exhaustive and prolonged use of antibiotics are very probable as the main underlying factor in the widespread transmission of difficult-to-cure antibiotic-resistant nosocomial infections (Prestinaci *et al*; 2015). Biofilm production is a mechanism demonstrated by numerous microbes to survive in hostile conditions. The bacterial biofilm is an organized community of bacterial cells encircled in polymeric matrix and adherent to a surface. Microorganisms developing in a biofilm are inherently resistant to several antibiotics increasing the antibiotic resistance up to 1000 folds and great antimicrobial concentrations are needed to inactivate organisms growing in a biofilm. This can be as a result of the insufficient concentration of the antibiotics getting some areas of the biofilms and metabolic inactivity (accompanied by the incidence of active antibiotic degradation mechanisms contributing to stop the accumulation of the drugs up to an effective concentration) of the bacteria found at the base of the biofilms (Soto; 2014).

Tab. No. 4-8: Association between biofilm formation and antibiotic resistance of *K.pneumoniae*

Antibiotics	Biofilm formation ability			
	Moderate n=10		Strong n=15	
	R No.(%)	S No.(%)	R No.(%)	S No.(%)
Pipracillin / Tazobactam	10 (100%)	0 (0%)	12 (80%)	3 (20%)
Amoxicillin-calvulonic acid	9 (90%)	1 (10%)	12 (80%)	3 (20%)
Ceftazidime	10 (100%)	0 (0%)	12 (80%)	3 (20%)
Cefepime	9 (90%)	1 (10%)	12 (80%)	3 (20%)
Cefotaxime	10 (100%)	0 (0%)	12 (80%)	3 (20%)
Ceftriaxone	10 (100%)	0 (0%)	12 (80%)	3 (20%)
Imipenem	9 (90%)	1 (10%)	11 (73%)	4 (27%)
Meropenem	10 (100%)	0 (0%)	12 (80%)	3 (20%)
Amikacin	10 (100%)	0 (0%)	13 (87%)	2 (13%)
Gentamycin	10 (100%)	0 (0%)	15 (100%)	0 (0%)
Rifampin	3 (30%)	7 (70%)	11 (73%)	4 (27%)
Ciprofloxacin	10 (100%)	0 (0%)	14 (93%)	1 (7%)
Levofloxacin	10 (100%)	0 (0%)	15 (100%)	0 (0%)
Tetracycline	10 (100%)	0 (0%)	13 (87%)	2 (13%)
Doxycycline	8 (80%)	2 (20%)	9 (60%)	6 (40%)

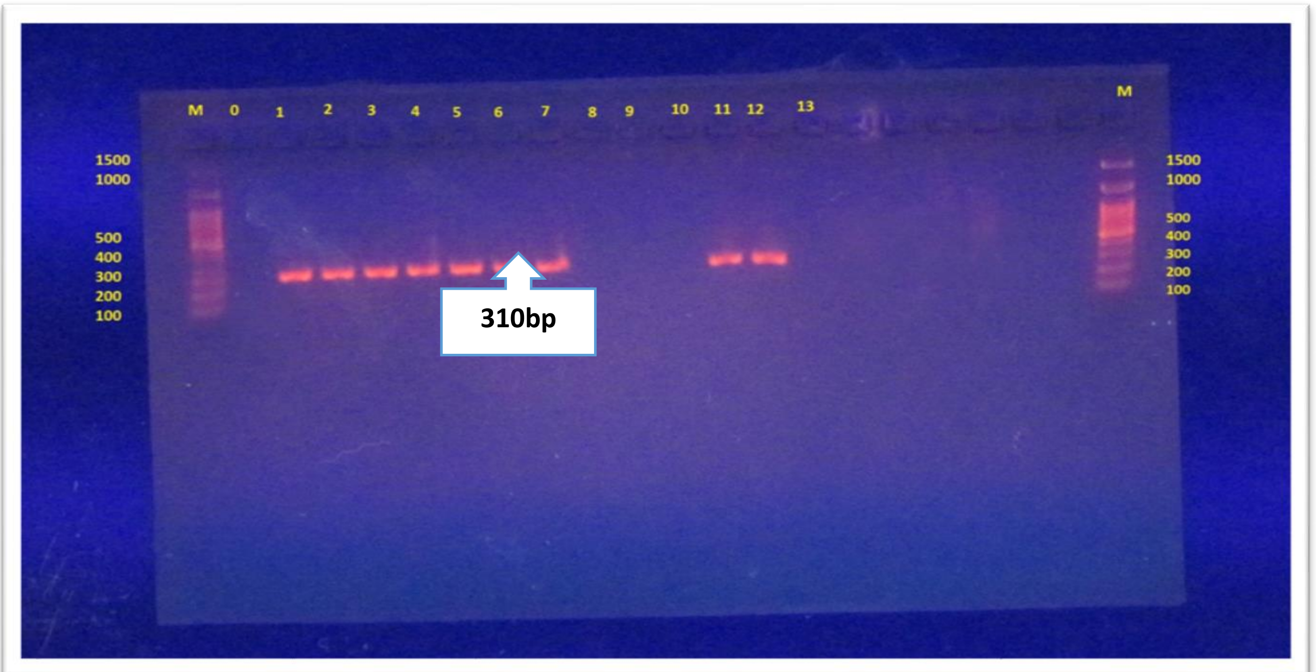
4-5 Molecular identification of the prevalent bacteria:

4-5-1 Molecular identification of *S.aureus*:

4-5-1-1 Molecular detection of *mec*A gene:

Rapid identification of methicillin resistant *Staphylococcus aureus* (MRSA) is necessary to give an early identification of this bacterium (Huang *et al*; 2008). Therefore, the alternative solution is the orientation towards the use of molecular methods using the PCR technique (Polymerase Chain Reaction), as the latter is characterized by being fast, sensitive, and highly specific, although it is expensive (Sakoulas *et al*; 2001).

In this study, the PCR technique was used to detect the presence of the *mec*A gene in *S. aureus* and the presence of this gene indicates that *S.aureus* are methicillin resistant (MRSA). Figure No. 4-4 shows the electrophoresis of the PCR products, through which it is clear that the primer of the *mec*A gene was successful in amplifying this gene through the appearance of a PCR product of 310 bp in size. This result agreed with Elhassan *et al* (2015) who could obtain the same result and they success in the electrophoresis of *mec*A gene of *S.aureus* obtained from different clinical sources in a study performed in Saudi Arabia . Upon examining the figure, it is clear that 15 isolates of the bacteria under study contain a gene *mec*A including S1, S2, S3, S4, S5, S6, S7, S11, S12, S14, S15, S16, S18, S20 and S24 as illustrated in table No. 4-9. Comparing of this isolates with susceptibility tests in appendix No. 9 , it has been found that mentioned isolates were resistant toward Oxacillin and β-lactam antibiotics. The methicillin resistance in *S. aureus* is linked with the presence of *mec*A gene encoding penicillin-binding protein (PBP2a) in mobile SCCmec (Dien Bard *et al;* 2014). The expression of *mec*A and PBP2a is a good sign for guessing oxacillin resistance in *S. aureus* (Sakoulas *et al;* 2001).



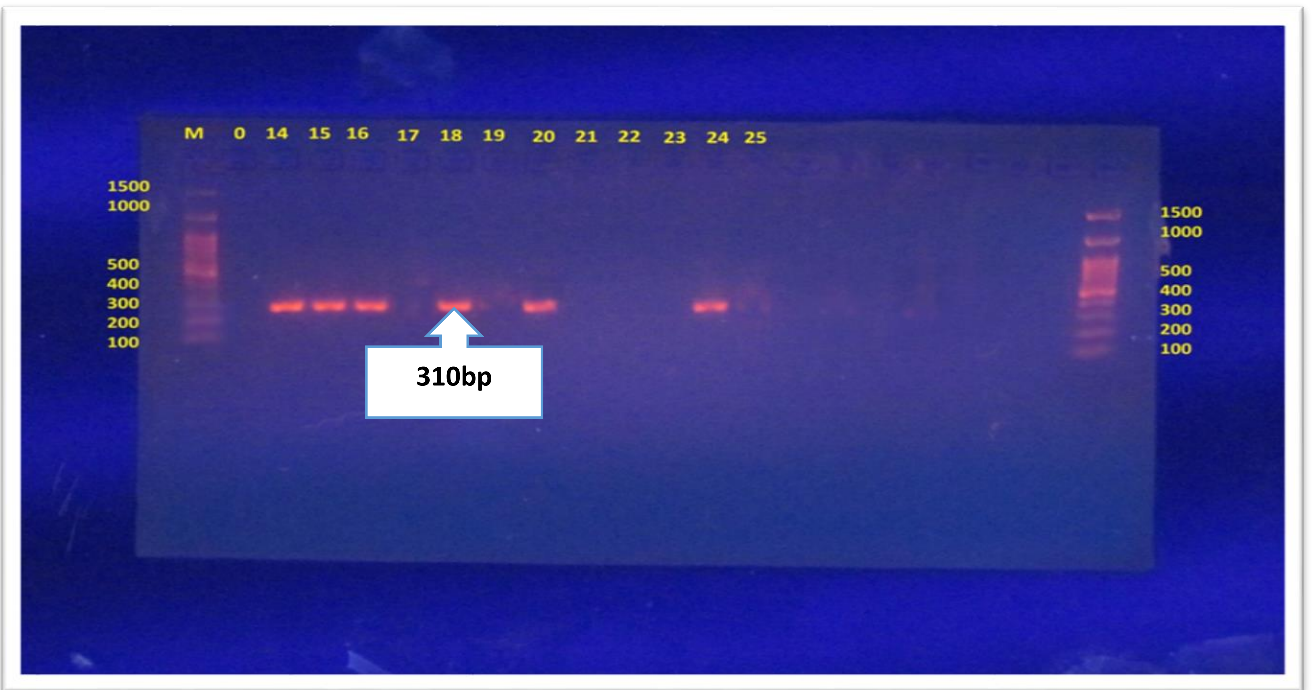


Fig. No. 4-4: Electrophoresis of the PCR reaction product of *S. aureus* using the specific primer of the *mec*A gene (310bp) using 1.5% Agarose gel , 70 voltages for 50 minutes

Tab No. 4-9: Genes distribution among *S.aureus* isolates obtained in this study

No. of isolate	*mec*A	*ica*A	*ica*D	*sea*	*erm*C
S1	+	+	+	+	+
S2	+	+	+	+	+
S3	+	+	_	_	+
S4	+	_	+	+	+
S5	+	+	+	+	+
S6	+	_	+	+	+
S7	+	_	+	+	+
S8	_	+	_	_	_
S9	_	_	+	_	_
S10	_	_	+	_	+
S11	+	+	+	+	+
S12	+	+	_	+	+
S13	_	+	+	_	_
S14	+	_	+	+	+
S15	+	+	+	+	+
S16	+	+	+	+	+
S17	_	+	+	_	_
S18	+	_	+	+	+
S19	_	+	+	+	_
S20	+	+	_	+	+
S21	_	+	+	_	_
S22	_	+	_	_	_
S23	_	_	+	+	_
S24	+	_	+	+	+
S25	_	+	+	_	_
No. and percentage	**15 /25 60%**	**16/25 64%**	**20/25 80%**	**16/25 64%**	**16/25 64%**

4-5-1-2 Molecular detection of *ica*A gene:

This study was also included the use of PCR technique to detect the presence of the *ica*A gene which responsible for biofilm formation. Figure No.4-5 shows that *ica*A primer was successful in amplifying this gene through the appearance of a PCR product of 151 bp in size in 64% of *S.aureus* isolates including S1, S2, S3, S5, S8, S11, S12, S13, S15, S16, S17, S19, S20, S21, S22 and S25 as illustrated in table No. 4-9. This result agreed with Mahmoudi *et al* (2019) who could obtain the same PCR product size through their electrophoresis of *ica*A gene in their study performed in Iran while Idbeis and Khudor (2019) results revealed that 100% of *S.aureus* enrolled in their study have possessed *ica*A of the same product size in a local study.

Regarding the strength of biofilm formation and the presence of *ica*A gene, our findings illustrated in table No. 4-10 revealed that 9 / 15 isolates (60%) of the strong biofilm producers contained *ica*A gene while 7 / 10 isolates (70%) of the moderate biofilm formers were possessed *ica*A gene while in a study done by Abdel-Shafi *et al*. (2022), it was reported that 47.37% of the strong *S.aureus* biofilm producers were possessed *ica*A gene.

The biofilm-involved polysaccharide of *S.aureus* is denoted to as the polysaccharide intercellular adhesion or (PIA) which has been well described. Hence, biofilm production is a crucial step in the pathogenesis of *Staphylococci* and depends on the expression of the *ica*ADBC operon involved in the synthesis of this polysaccharide intercellular adhesion (AL-Sheikh and Yosif; 2014). The *ica*A gene regulates the formation of exopolysaccharide (EPS) in biofilms. This EPS imposes the adhesion of the bacteria and can assist as a shelter against the host immune system and antibiotics treatment (Harapanahalli *et al;*2015).

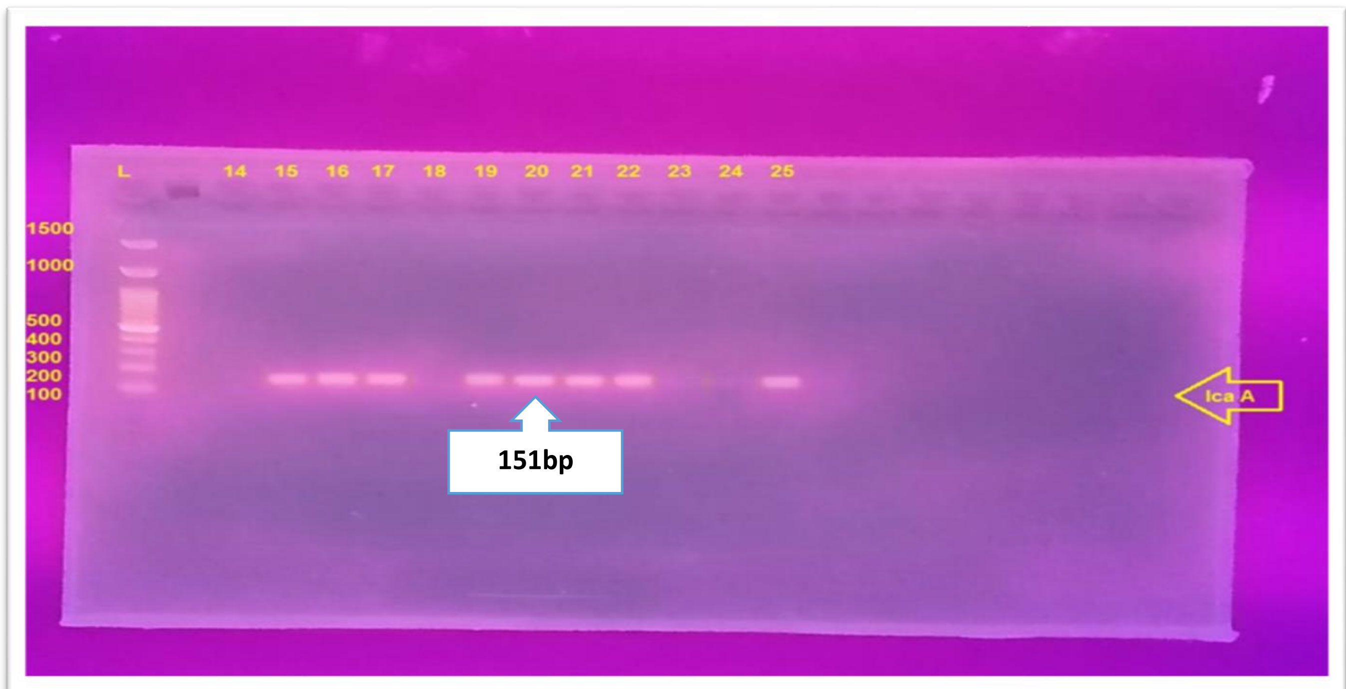


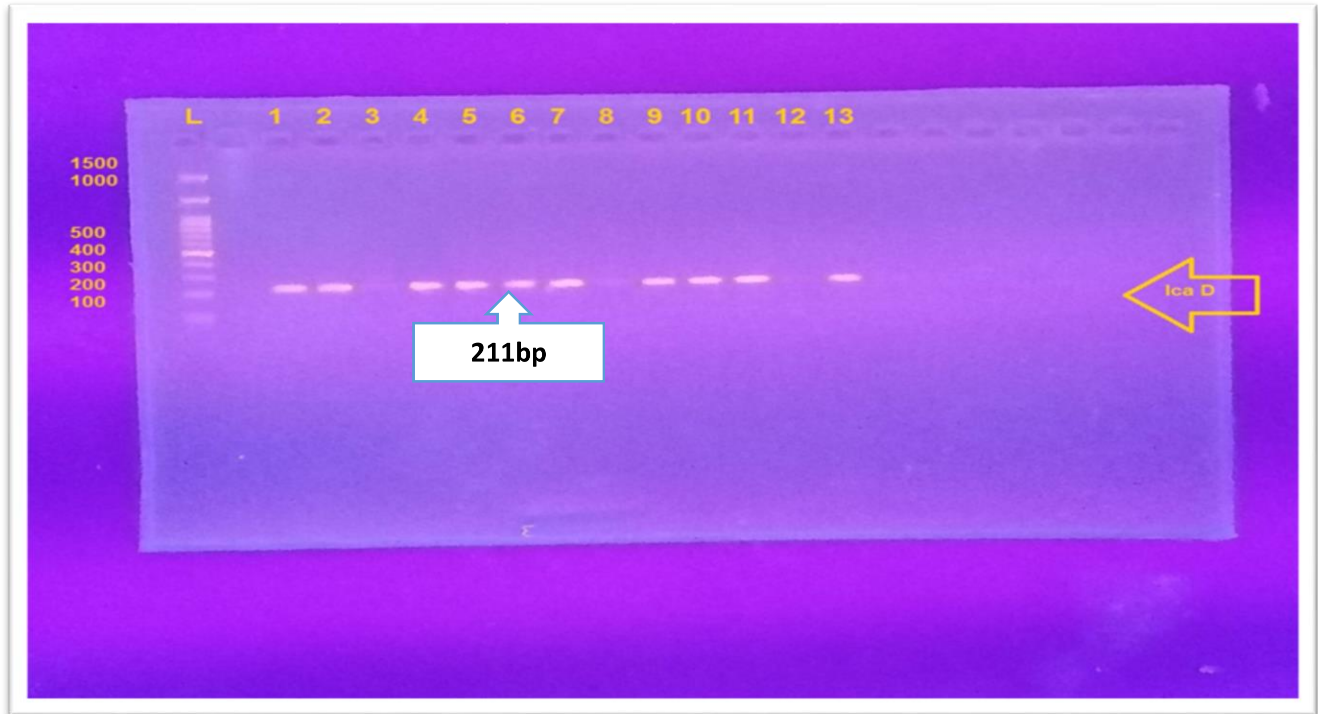
Fig. No. 4-5: Electrophoresis of the PCR reaction product of *S. aureus* using the specific primer of the *ica*A gene (151bp) using 1.5% Agarose gel ,70 voltages for 50 minutes

4-5-1-3 Molecular detection of *ica*D gene:

By using the PCR technique, the presence of *ica*D gene was detected which is responsible for biofilm formation. Figure No. 4-6 shows the *ica*D gene was successfully amplified that has been indicated by the presence of a PCR product size of 211bp in 80% of *S.aureus* isolates comprising S1, S2, S4, S5, S6, S7, S9, S10, S11, S13, S14, S15, S16, S17, S18, S19, S21, S23, S24 and S25 as illustrated in table No. 4-9. This result is in agreement with Mahmoudi *et al* (2019) who could have obtained the same PCR product size through their electrophoresis of *ica*D gene in their study in Iran.

With respect to the strength of biofilm production with the presence of *ica*D gene, our results were illustrated in table No. 4-10 reflects that among the strong biofilm producers, 12/ 15 (80%) of the isolates were containing *ica*D gene while 8 / 10 (80%) of the moderate biofilm producers were contained with *ica*D gene. Our result agrees with Mamdoh *et al* (2023) in Egypt who reported that 79.4% of strong biofilm producer were contained *ica*D gene and 75% of intermediate biofilm producers were contained *ica*D gene while Haddad *et al* (2018) documented that the highest rate 60.9% of moderate biofilm producers contained *ica*D gene and 65% of strong biofilm producers were possessed *ica*D gene in their study performed in Tunisia.

Biofilm is the origin for persistent or chronic bacterial infections and is deliberated to be a two-step process; first, the bacteria adhere to a surface, second, multiplying bacteria form a multilayered biofilm, which is associated with the formation of polysaccharide intercellular adhesion (PIA). This step is facilitated by intercellular adhesion (*ica*) locus, which consist of the *ica*A, *ica*B, *ica*C and *ica*D genes, and encoded the proteins involved for PIA and PSA (capsular polysaccharide adhesion), which are the significant biofilm components in *Staphylococcal* species (Yazdani *et al*; 2006).



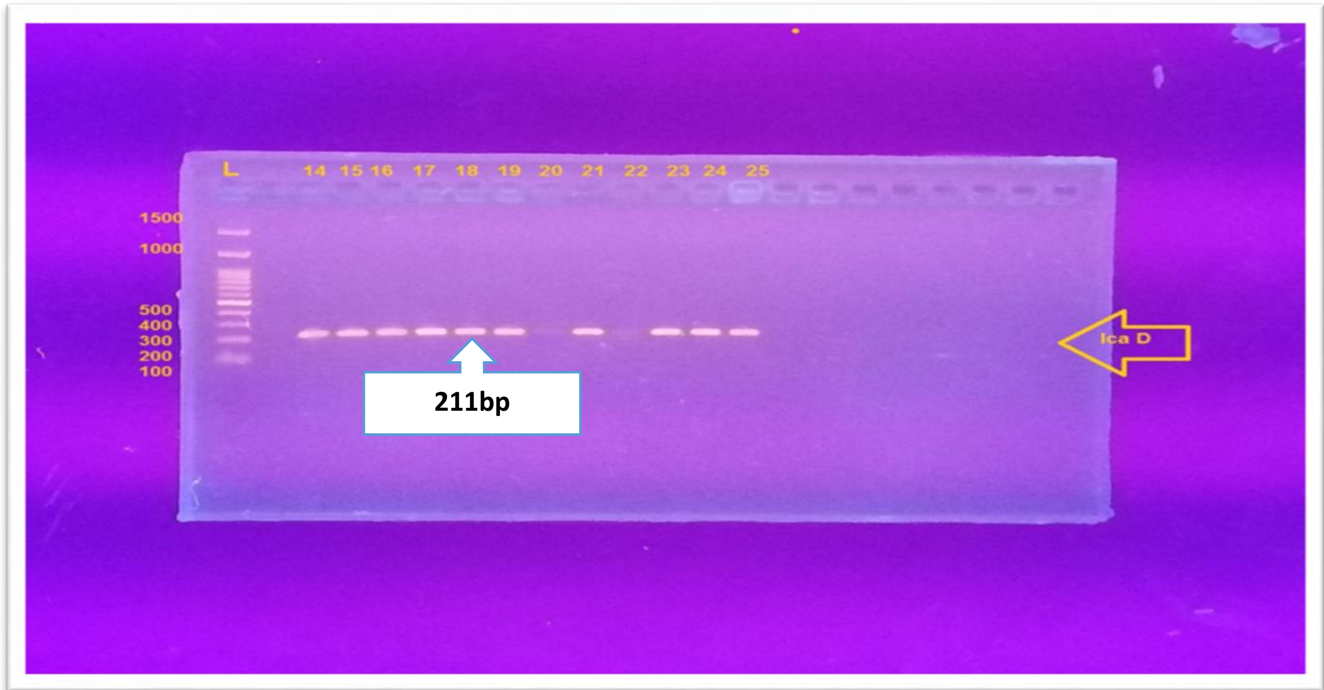


Fig. No. 4-6: Electrophoresis of the PCR reaction product of *S. aureus* using the specific primer of the *ica*D gene (211bp) using 1.5% Agarose gel , 70 voltages for 50 minutes

Tab. No. 4-10: Association of biofilm genes and biofilm production ability of

Gene	Biofilm formation ability		*P*-value
	Staphylococcus aureus		
	Moderate n=10	Strong n=15	
***ica*A**	70%	60%	0.01
***ica*D**	80%	80%	
Both	50%	40%	
Either *ica*A Or *ica*D	50%	60%	
Chi.q was significant at $p \leq 0.05$, n: number of cases			

4-5-1-4 Molecular detection of *sea* gene:

In this study, the presence of *sea* gene which is responsible for the production enterotoxin is detected by using the PCR technique. Figure No. 4-7 displays the successful amplification that has been distinguished by the appearance of bands related to *sea* gene PCR product size of 102bp in 64% of *S.aureus* isolates involving S1, S2, S4, S5, S6, S7, S11, S12, S14, S15, S16, S18, S19, S20, S23, and S24 as illustrated in table No. 4-9. Aljelehawy *et al* (2021) in a local study showed that 88% of *S.aureus* isolates contained *sea* gene of the same PCR product size while *sea* was the most commonly identified enterotoxin gene 81.08% in a study performed in China by Chen and Xie (2019).

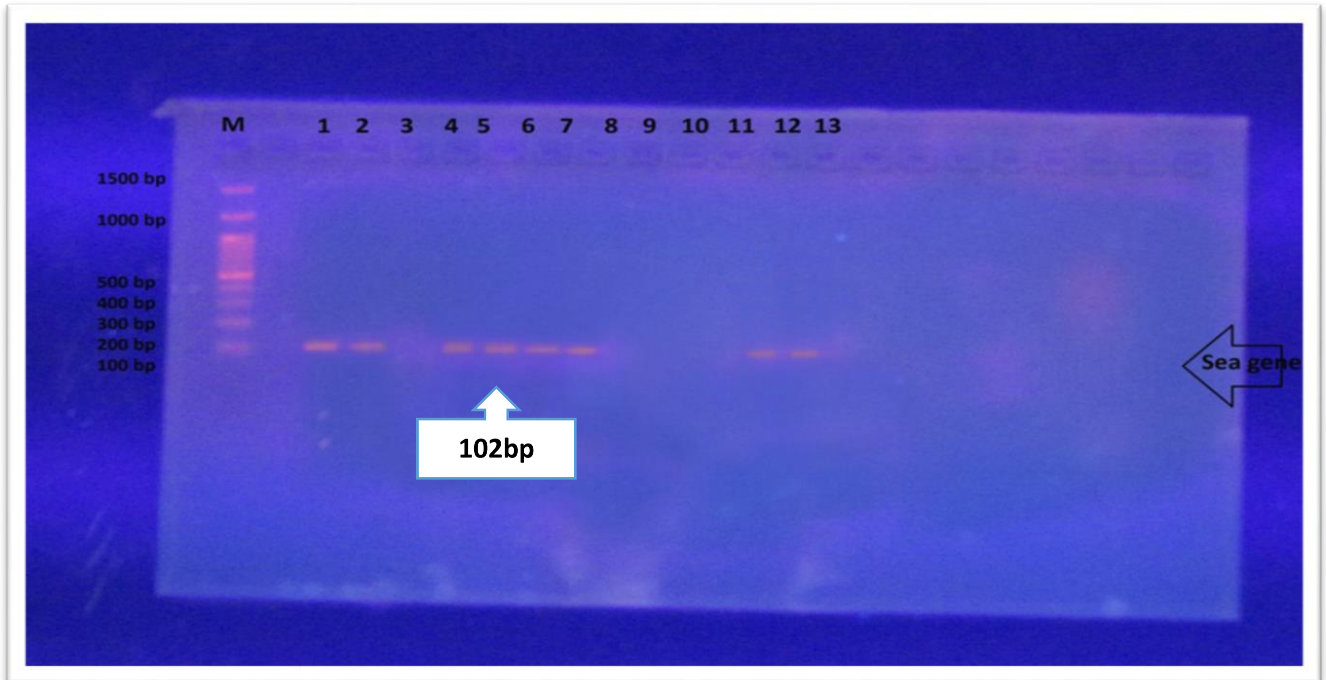
Enterotoxins are molecules of ~20–30 kD that related to the family of super-antigens (SAg). These molecules over-promote the production of cytokine from both T-lymphocytes and macrophages (Otto; 2013). The mechanisms by which staphylococcal enterotoxins work are not well known, but may comprise the activation of cytokine production,

eventually triggering cell death by apoptosis. They contribute considerably to chief illnesses (Spaulding *et al*., 2013 ; Vu *et al*., 2014). The popular of *S. aureus* isolated from DFU have the capability to release a large number of SAgs, particularly Staphylococcal Enterotoxins (SEs) and Staphylococcal Enterotoxin-like toxins (SEls) (Vu *et al*;2014). *sea* might have a chief role in atopic dermatitis by prompting the upregulation of adhesion molecules and provoking inflammatory responses in endothelial cells and keratinocytes (Lee *at al*; 2013).

4-5-1-5 Molecular detection of *erm*C gene:

By using PCR technique, the presence of *erm*C gene was detected. Figure No. 4-8 shows the successful amplification of *erm*C gene product size of (572bp) in 64% of *S.aureus* isolates obtained from DFI in the current study including S1, S2, S3, S4, S5, S6, S7, S10, S11, S12, S14, S15, S16, S18, S20 and S24 as illustrated in table No. 4-9. This was in agreement with Gushiken *et al* (2016) in Brazil who reported that 64.29% of *S.aureus* in their study contained *erm*C gene. In another study performed in Serbia by Aleksandra *et al* (2014), it was reported that 50% of *S.aureus* contained *erm*C gene.

The presence of *erm*C gene in our study was associated with resistance toward particularly Erythromycin and Azithromycin though as described in appendix No. 9 , it was cleared that all the isolates mentioned above were resistant toward Azithromycin and Erythromycin and this agrees with Sedaghat *et al* (2017) in Iran who reported that the most common erythromycin-resistant genes in *S. aureus* isolates were *erm*C. Modification of the ribosomal target site leads to a broad-spectrum resistance to macrolides, whereas efflux and enzymatic inactivation are of less importance. However; macrolides are considered by a resistance mechanism showing different phenotypic expression which is essential in their interpretation (Leclercq; 2002). The macrolide resistance genes are found on plasmids, transposons, and genomic islands and can be easily transferred horizontally between strains and species (Feßler *et al*; 2018).



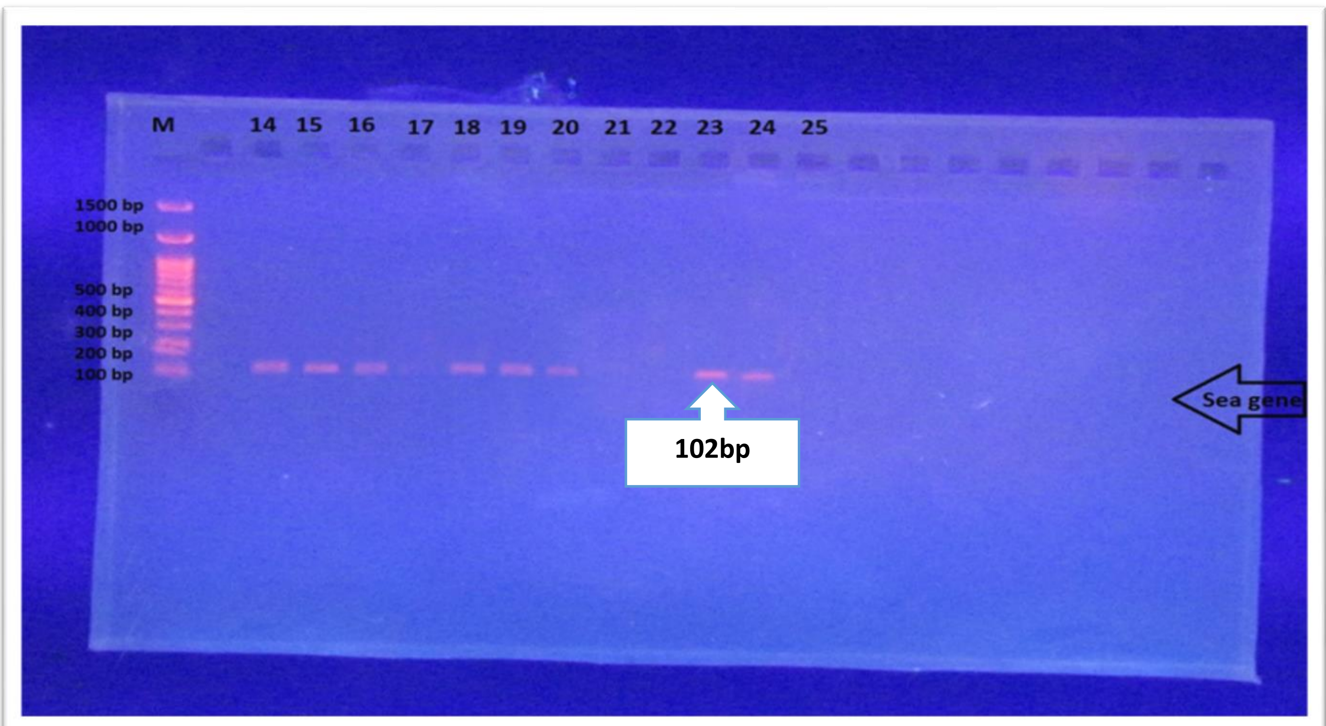
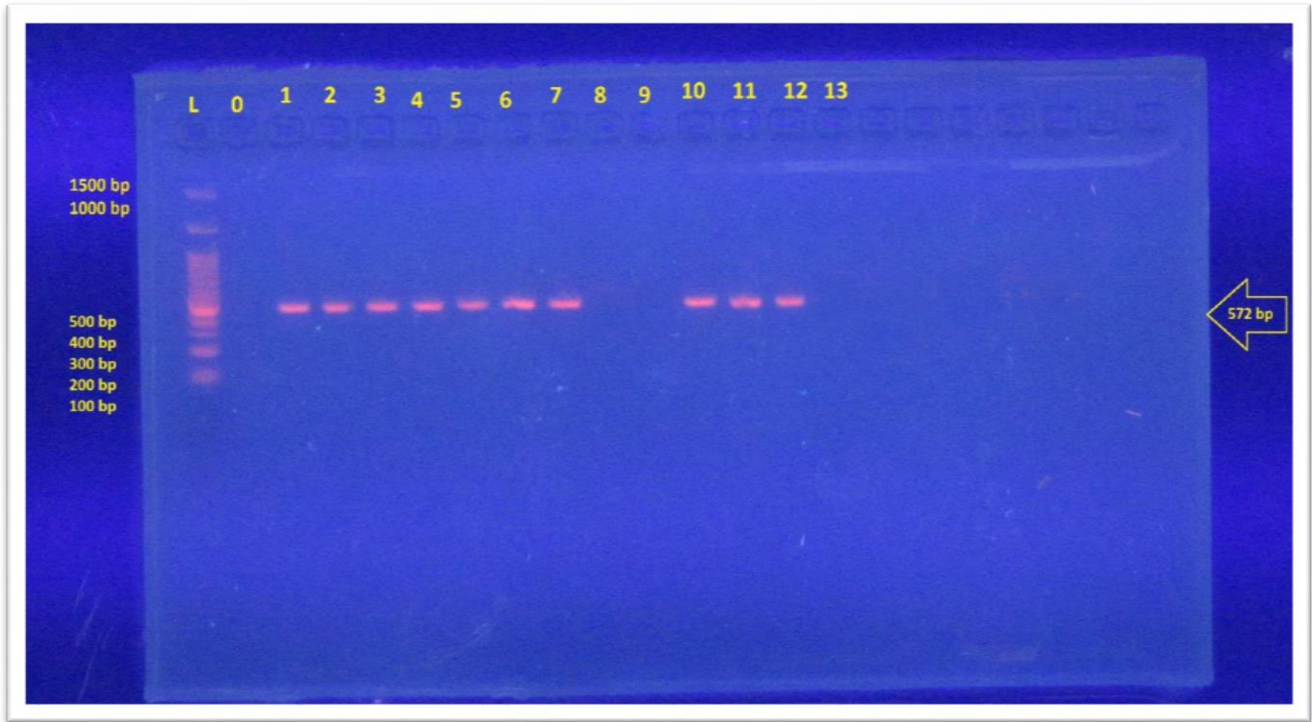


Fig. No. 4-7: Electrophoresis of the PCR reaction product of *S. aureus* using the specific primer of the *sea* (102bp) using 1.5% Agarose gel, 70 voltages for 50 minutes



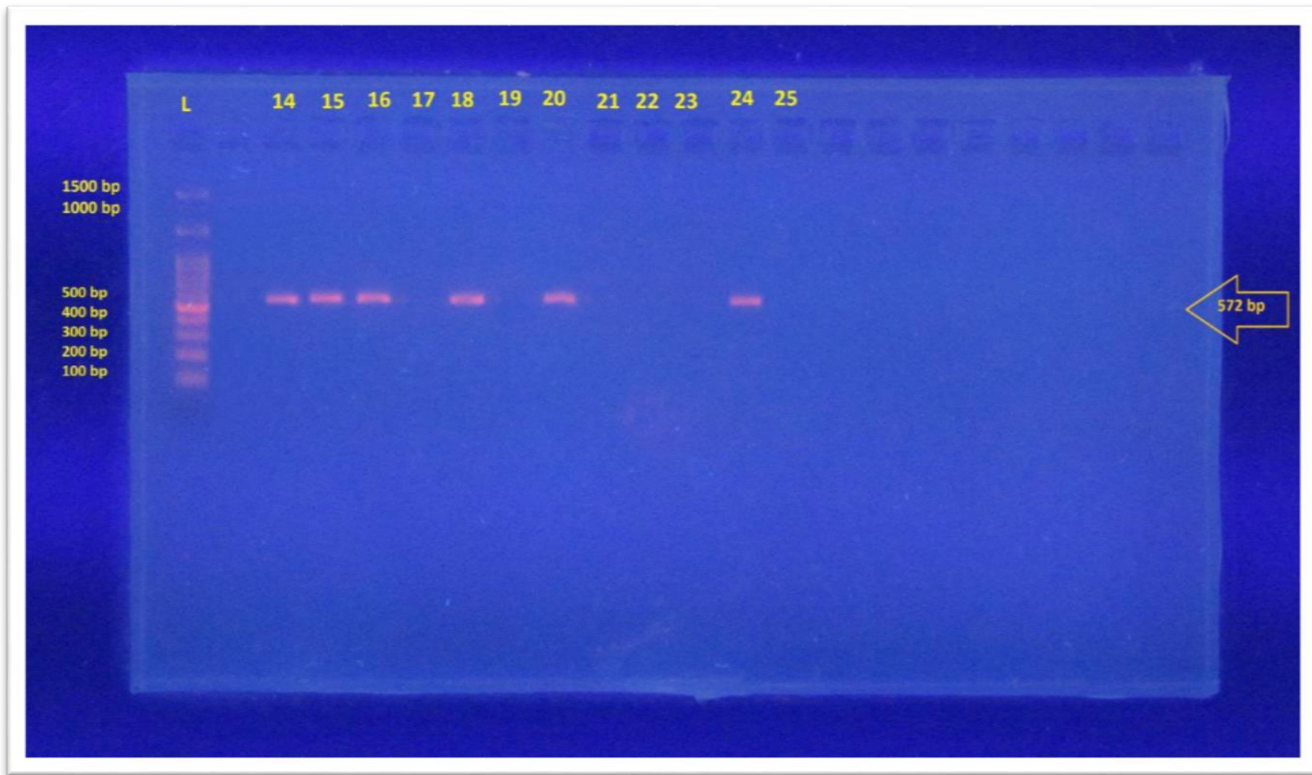


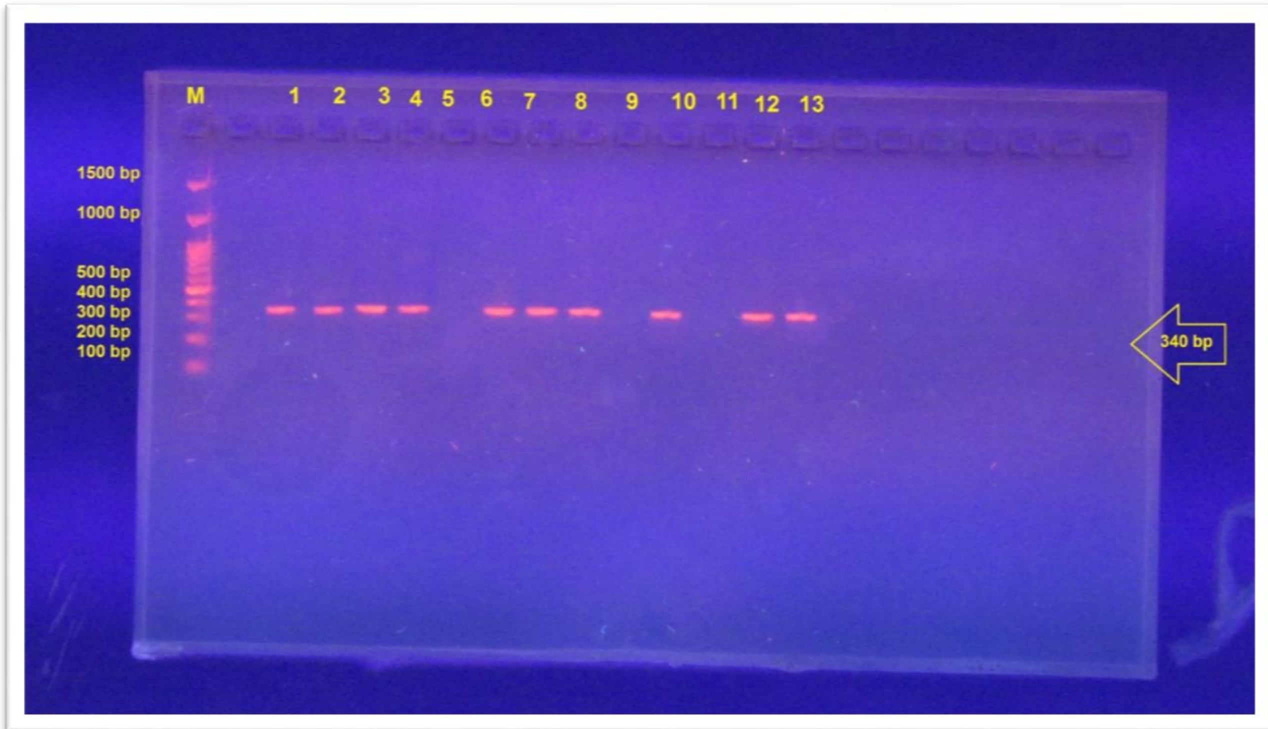
Fig. No. 4-8: Electrophoresis of the PCR reaction product of *S. aureus* using the specific primer of the *ermC* gene (572bp) using 1.5% Agarose gel ,70 voltages for 50 minutes

4-5-2 Molecular identification of *K.pneumoniae*:

3-5-2-1 Molecular detection of *mrk*D gene:

PCR was used in this study to detect the presence of the *mrk*D gene, which is one of the important genes used to identifying *K. pneumonia*. Figure No. 4-9 shows the electrophoresis of PCR products, which can be seen that the primer of the *mrk*D gene was successful amplifying this gene by producing a PCR product of 340bp in size involving 88% of *K. pneumoniae* isolates enrolled in this study including K1, K2, K3, K4, K6, K7, K8, K10, K12, K13, K14, K15, K16, K17, K18, K19, K20, K21, K22, K23, K24 and K25 as illustrated in table No. 4-11. Our results were similar to Rastegar *et al* (2021) and Anis *et al* (2021) who obtained a successful amplifying of *mrk*D gene of the same product size in Iran and Egypt respectively. Furthermore, Badger-Emeka and Emeka (2022) reported in their study performed in Saudi Arabia that 87% of *K.pneumoniae* isolates were included with *mrk*D gene which could also elucidate the high abilities of biofilm-production among them. In the current study, it is noticed that 12 / 15 (80%) of the strong biofilm producers were possessed *mrk*D gene and 10 /10 (100%) of the moderate biofilm producers have been included with *mrk*D gene as illustrated in table No. 4-12. This agreed with Mahmood and Abdullah (2015) in a local study reported that 100% of the strong *K. pneumoniae* biofilm producers isolated from DFI were possessed *mrk*D gene while Bakhtiari *et al* (2021) reported that 76.7% of the strong biofilm producers of *K. pneumonia*e were possessed *mrk*D gene however 50% of the moderate biofilm producers have *mrk*D gene in a study performed in Iran.

The capability to form biofilm isolates results in enlarged resistance to antibiotics, as a result, treatment failure, increasing treatment costs and increasing mortality (Moemen and Masallat; 2017).



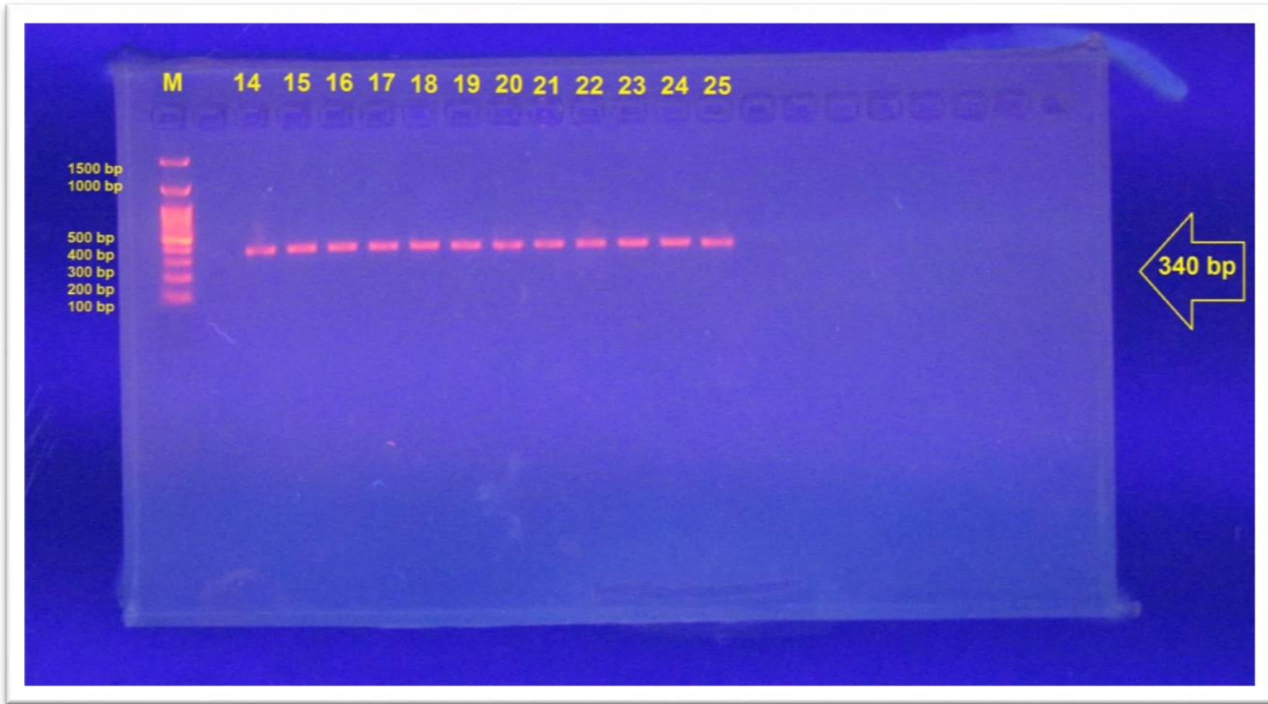


Fig. No. 4-9: Electrophoresis of the PCR reaction product of *K. pneumoniae* using the specific primer of the *mrk*D gene (340bp) using 1.5% Agarose gel, 70 voltages for 50 minutes

Tab. No. 4-11: Gene distribution among *K.pneumoniae* isolates obtained in this study

No. of isolate	***mrk*D**	***Cps***	***K1***	***K2A***
K1	+	+	_	+
K2	+	+	_	+
K3	+	+	_	+
K4	+	+	_	+
K5	_	+	_	_
K6	+	+	_	+
K7	+	+	_	+
K8	+	+	+	_
K9	_	+	_	_
K10	+	+	_	+
K11	_	+	_	_
K12	+	+	_	+
K13	+	+	_	+
K14	+	+	_	+
K15	+	+	_	_
K16	+	+	_	+
K17	+	+	+	_
K18	+	+	_	_
K19	+	+	_	+
K20	+	+	_	+
K21	+	+	_	+
K22	+	+	+	_
K23	+	+	_	_
K24	+	+	_	+
K25	+	+	_	_
No. and percentage	**22/25 88%**	**25/25 100%**	**3/25 12%**	**15/25 60%**

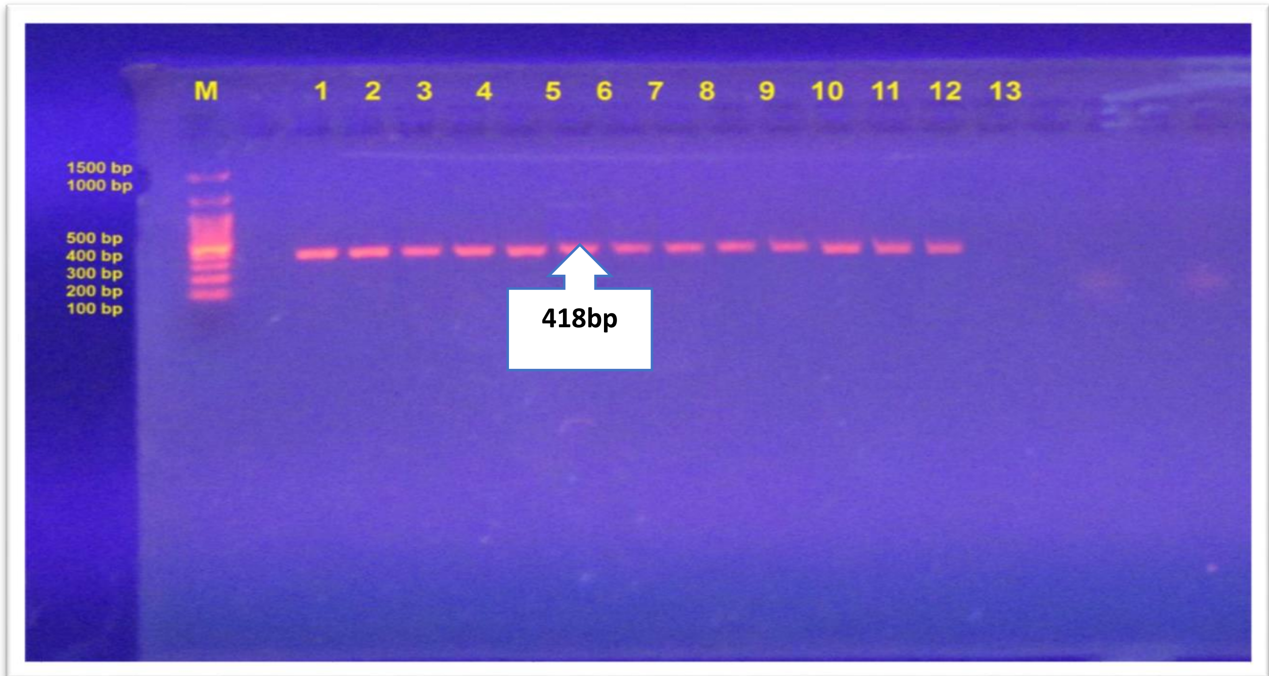
With respect to the association of biofilm formation strength and presence of *mrk*D gene in *K.pneumoniae*, table No. 3-12 illustrated this gene distribution among *K.pneumoniae* isolates.

Tab. No. 4-12: Association of *mrk*D gene and biofilm production ability of *K.pneumoniae*

Gene	Biofilm formation ability		*P*-value
	Klebsiella pneumoniae		
	Moderate n=10	Strong n=15	
***mrk*D**	100%	80%	**0.05**
Chi.q was significant at p ≤ 0.05, n: number of cases			

4-5-2-2 Molecular detection of *Cps* gene:

PCR was performed to verify the presence of the *Cps* gene in the studied strains using primers designed for this gene under optimal temperature conditions. Figure No. 4-10 displays the successful amplification of the *Cps* gene through the appearance of PCR product of 418bp in size in 100% of *K. pneumonia* isolates enrolled in the current study as illustrated in table No. 4-11 . Abdul-Razzaq *et al* (2014) and Akbari *et al* (2015) success in obtaining the same amplicon size of *Cps* gene in their study performed in Iraq and Iran, respectively. The presence of *Cps* genes in most isolated bacteria indicate that all these isolates can contain the genes of *Cps* biosynthesis as that stated by Lin *et al* (2011) in Taiwan. However, Campos *et al* (2004), reported that *K. pneumoniae* capsule polysaccharide (CPS) facilitates resistance to antimicrobial peptides and proteins by preventing the interaction of the agents with membrane targets. The capsule synthesis in *K. pneumoniae* is encoded by a gene found on the chromosomal operon, capsule polysaccharides (CPS) that enable the formation of the capsule (Pan *et al*; 2008).



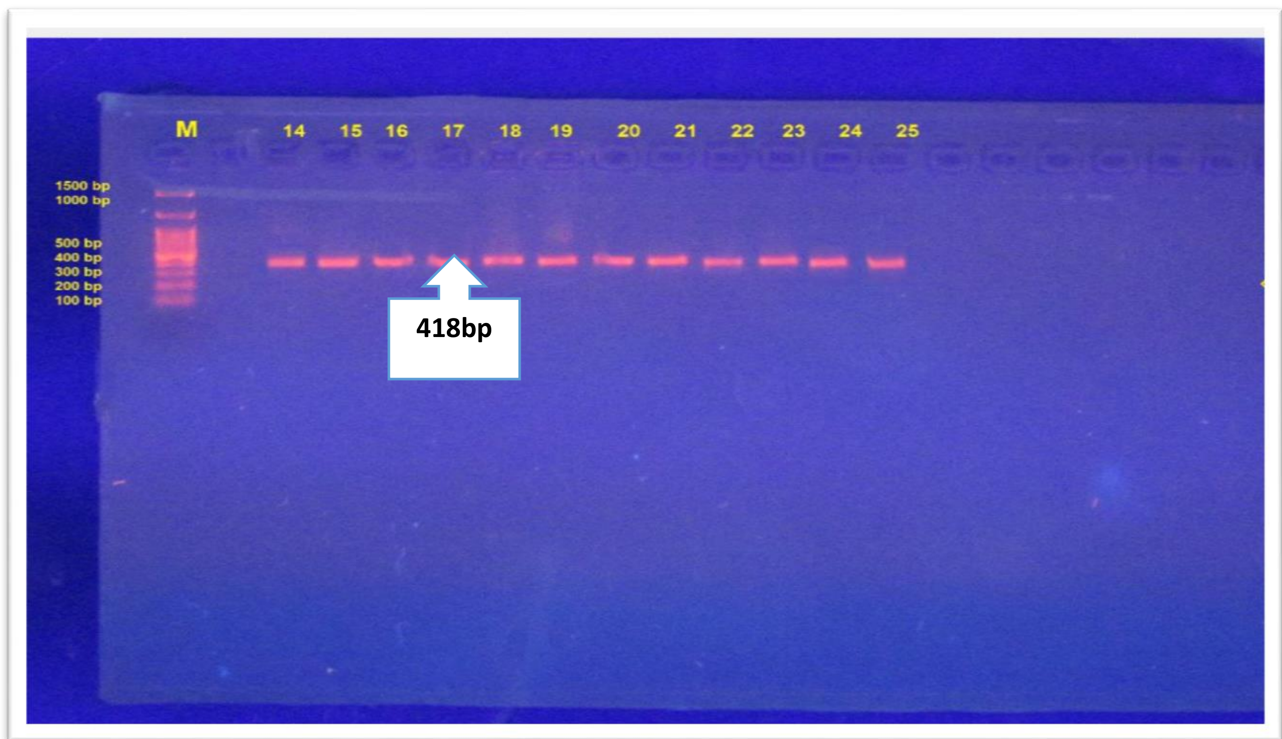


Fig. No. 4-10: Electrophoresis of the PCR reaction product of *K. pneumoniae* using the specific primer of the *Cps* gene (418bp) using 1.5% Agarose gel, 70 voltages for 50 minutes

Type 3 fimbria is a vital strip in the route of creating bacterial face structures, which directly and arduously have the ability of destroying rebellious responses and changing the exposed system in usual infections, to determine resistance, the most important factor being the growth prominence of bacteria. The core part structure to which bacteria familiarize to starvation and low oxygen environments causes bacterial growth to slow, dropping the efficiency of antibiotics that directly target metabolically active and dividing cells (Clegg and Murphy; 2016). CPS has a vital role in the connection of bacteria to epithelial and mucosal surfaces. It also guards the bacteria from serum opsonization and phagocytosis, thus it hides the bacteria from the host immune system. On the other hand, the capsule offers protection against hostile environmental conditions and decreases the permeability of antibiotics into the bacteria (Struve and Kropfelt*,* 2005 ; Burmølle *et al.*, 2008).

4-5-2-3 Molecular detection of *K1* gene:

Molecular technique particularly PCR was used in current study to verify the presence of *K1* gene in *K. pneumoniae* isolates under study. It has been found that only 12% of *K. pneumoniae* isolates possess *K1* gene of 1238bp in size including K8, K17 and K22 as mentioned in figure No. 4-11 and table No. 4-11. Abdul-Razzaq *et al* (2014) and Qassim and Khalid (2022) in a local study and Siu *et al* (2011) in Taiwan success to amplify the same amplicon size of *K1* gene. Close percent obtained by Qassim and Khalid (2022) whereas they obtained only 15.8% of *K1* gene from the *K. pneumoniae* isolates.

Fang *et al* (2007) in Taiwan termed the *mag*A gene for the first time in 2004 and exposed that the aggressive *K. pneumoniae* strains had higher levels of hyper-mucoviscosity and *mag*A, while mutant strains lacking *mag*A misplaced their exo-polysaccharide. Parallel to the *ma*gA capsule gene cluster of capsular serotype *K1* isolate, the *K2A* gene of capsule gene cluster *K. pneumoniae* could be used as a very specialized technique to recognize the capsule *K2* serotype (Chuang *et al;* 2006). The absences of mannose repeats on the capsule, avoiding it from being detected by macrophages. Mucoviscosity associated gene A (*mag*A) is only found in the *K1*capsule gene cluster, but

the chromosomal *K2* capsule associated gene A (*K2A*) is found in the *K2* serotype (Yu *et al.,*2006 ; Doo *et al*., 2008). *mag*A is a chromosomal gene that plays a crucial role in *Klebsiella* infections and is accompanying with production of a mucoviscous layer that makes *Klebseilla* resistant to phagocytosis (Guo *et al;* 2017).

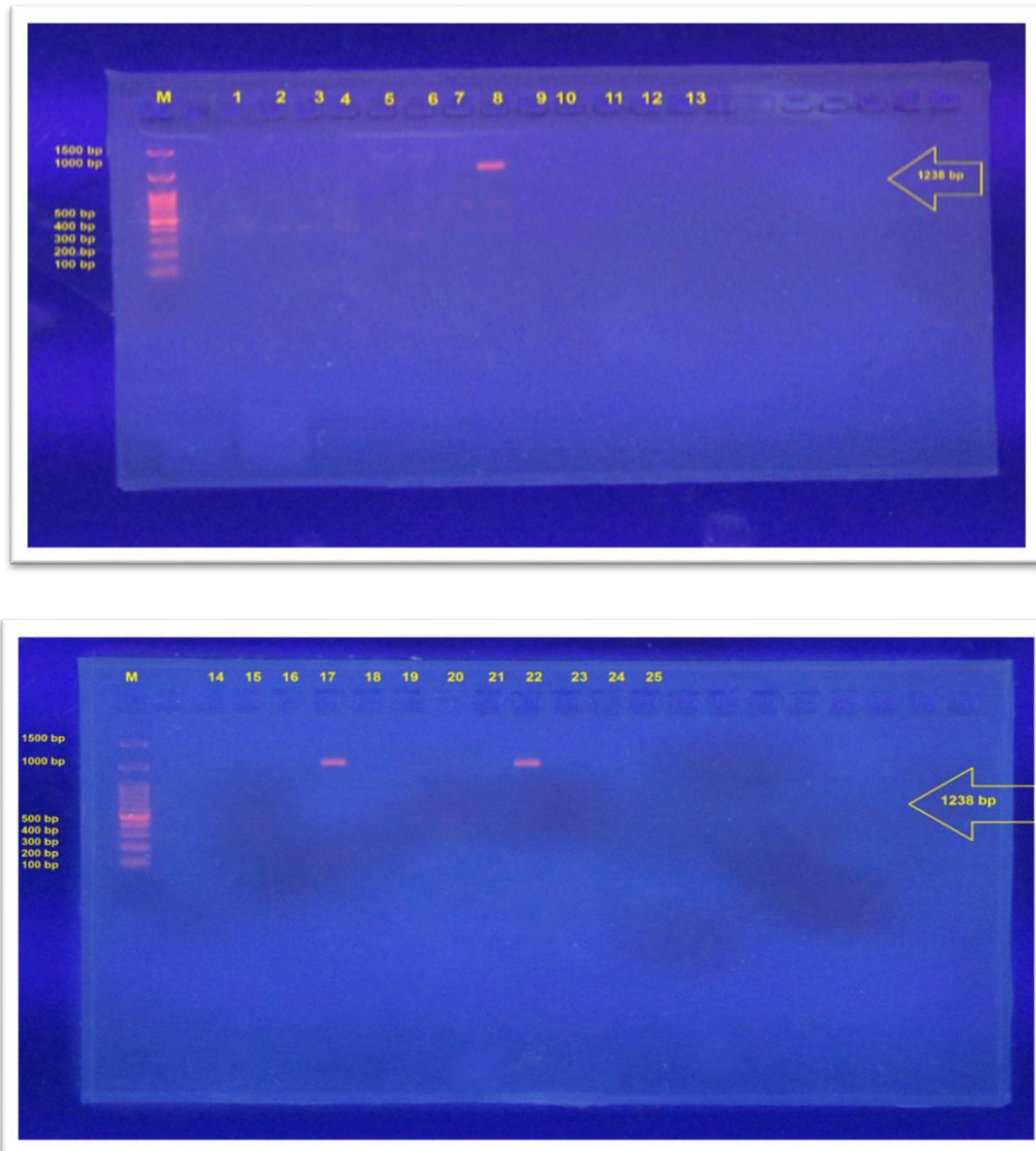
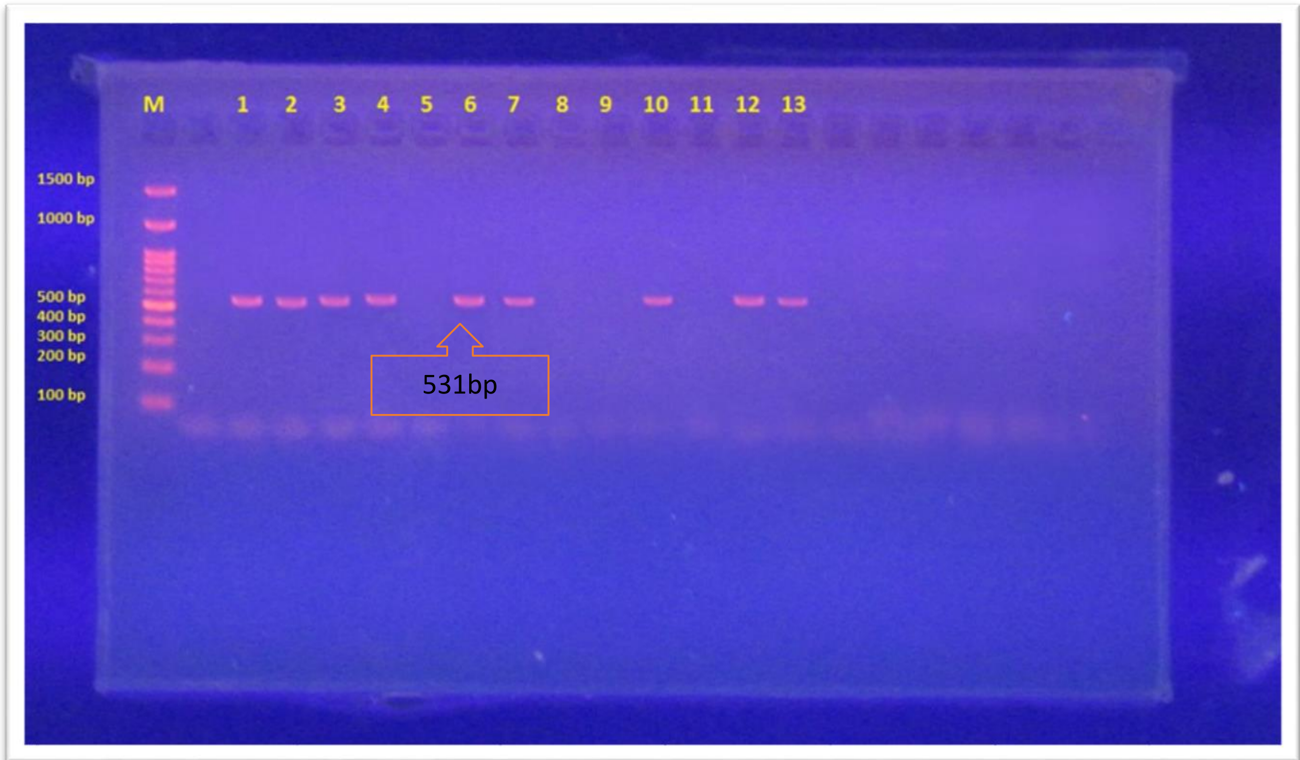


Fig. No. 4-11: Electrophoresis of the PCR reaction product of *K. pneumoniae* using the specific primer of the *K1* gene (1238bp) using 1.5% Agarose gel, 70 voltages for 75 minutes

4-5-2-4 Molecular identification of *K2A* gene:

In the present study, the molecular identification of serotype *K2A* was performed. Figure No. 4-12 shows that *K2A* gene was successful amplifying through the appearance of a PCR product of 531bp in size in 60% of *K. pneumoniae* isolates including K1, K2, K3, K4, K6, K7, K10, K12, K13, K14, K16, K19, K20, K21 and K24 as illustrated in table No. 4-11. Remya *et al* (2018) and Anis *et al* (2021) obtained the same results and success to amplify the amplicon of the same size of *K2A* gene in India and Egypt respectively while Alyassari *et al* (2019) in a local study obtained 72.72% of *K2A* gene of *K. pneumoniae* from clinical sources.

The chromosomal K2 capsule related gene A *(K2A*) for the *K2* serotype (Yu *et al.*, 2006 ; Doo *et al.*,2008) which isolates with capsule serotypes *K1* and *K2* are more resistant to phagocytosis than Non-*K1/K2* strains (Wang *et al*; 2012). The *K2A* gene of *K. pneumoniae* might be used as a specific diagnostic technique to classify the *Cps* of *K. pneumoniae* capsule K2 serotype, which matches to the *magA* region in the capsules gene clusters of *K1* isolate (Chuang *et al*; 2006). The progress of protuberant polysaccharide capsules associated with capsular serotypes *K1* or *K2* have been identified as the major virulence determinants for human hyper-virulent *K. pneumoniae* maybe because it appears to protect the bacteria from phagocytosis and prevent destruction by bactericidal serum factors (Russo and Marr; 2019).



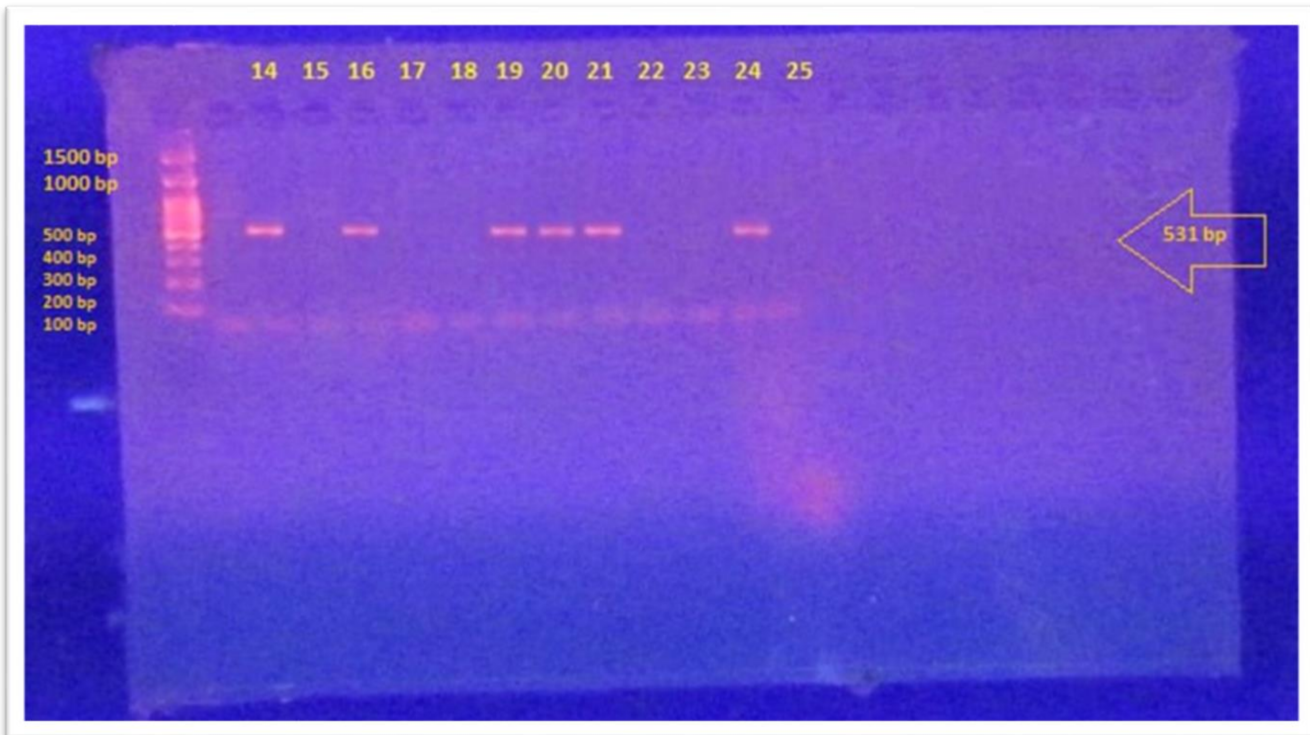


Fig. No. 4-12: Electrophoresis of the PCR reaction product of *K. pneumoniae* using the specific primer of the *K2A* gene (531bp) using 1.5% Agarose gel, 70 voltages for 50 minutes

4-6 Immune response

The current study was designed as case-control study, 120 individuals were included, 40 subjects of diabetic foot ulcer patients with T2DM (DFI), 40 subjects of T2DM patient's without foot ulcer, and 40 apparently healthy control group. Samples were collected during the period of 4 months (October, 2022 to January, 2023) from Imam Al-Hassan center for endocrinology and Diabetes.

4-6-1 Determination of Toll-like Receptor 2 (TLR2) levels

Patients with Diabetic foot infection exhibited an increasing level of TLR-2 compared to healthy control. Results indicated a significant difference in level of the mentioned TLR among groups under study as shown in table No. 4-13 where levels of TLR-2 in DFI patients were (7.36±1.85) ng/ml which was significantly higher than DM and control groups ($p \leq 0.001$).

Tab. No. 4-13: Mean difference of some biomarkers among the Three Studied Groups

Biomarker	DFI N=40 (mean±SD)	DM N=40 (mean±SD)	Control N=40 (mean±SD)	*P*-value
TLR-2 (ng/ml)	7.36±1.85*	6.46±2.09	4.74±0.92	<0.001
IL-17A (ng/L)	123.7±33.52	107.4±32.10*	91.78±13.58	<0.001
CRP (mg/L)	92.9±78.26*	9.16±3.00	7.47±2.79	<0.001

ANOVA was *: significant at p ≤ 0.05, Post hoc (LSD)

N: number of cases; SD: standard deviation; *: significant

The above results agreed with Dasu and Martin (2014) in USA who reported that TLR1, 2, 4, and 6 mRNA expressions were enlarged significantly in wounds of diabetic patients compared with non-diabetic wounds ($P < 0.05$). Another study performed in Ireland by Gupta *et al* (2017) documented that TLR2, 4–5 levels were raised in good glycemic control with complications (GCC) when compared to non-diabetic volunteers (NGT) while there was another study performed in Egypt by Wifi *et al* (2017) found that there was no statistical difference in the distribution of TLR2 between the 3 groups. Mohammad *et al* (2006) documented in their study performed in USA increasing TLR2 expression in bone marrow derived macrophage of non-obese diabetic mice while Creely *et al* (2007) in United Kingdom showed increased TLR2 expression in the adipose tissue of type 2 diabetes (T2DM) patients.

TLR-2 is recognized to be a signaling receptor for many microbial yields including whole Gram positive bacteria and mycoplasma (Flo *et al*; 2001) while a study by Ajuwon *et al* (2009) revealed that peptidoglycan derived from *Staphylococcus aureus* resulted in elevated TLR2 expression of adipocytes cell lines. Anti-infectious feature of TLR2 is apparent from the fact that the TLR2 underprovided mouse strain is more susceptible to infection with gram-positive bacteria such as *Staphylococcus aureus* (Kuo *et al*; 2013).

Concerning the age, our results revealed that the mean level of TLR-2 in DFI group was increased with increasing age specially in (55-64) as presented in table No. 4-14 . Likewise, the literature is strikingly variable concerning how TLR expression and signaling in peripheral leukocytes is affected with aging. Rises in TLR-2 expression and function have been reported by Simpson *et al* (2013) in Australia, as have declines in TLR-2 (Panda *et al*; 2010) in USA as well as no change with aging (Fulop *et al* ,2004 ; Nyugen *et al.*,2010) in Canada and USA respectively. Others have also shown reduced TLR function in aged mice in a study performed in Atlanta by Renshaw *et al* (2002).

Tab. No. 4-14: The effect of Age on the Toll Like Receptor-2 levels according to the three studied groups

Groups	(35 – 44) Years (mean±SD)	(45 – 54) Years (mean±SD)	(55 – 64) Years (mean±SD)	(65 – 74) Years (mean±SD)	(≥75) Years (mean±SD)
	Concentration (ng/ml)				
DFI	6.20±0.55	7.61±0.55*	8.68±0.55	7.21±0.60	7.12±0.60*
DM	6.01±0.55*	7.27±0.55	6.58±0.60	5.98±0.60*	6.49±0.55
Control	4.55±0.55	4.16±0.55	4.27±0.55	5.35±0.55	5.38±0.55
ANOVA was *: significant at p ≤ 0.05, Post hoc (LSD) SD: standard deviation; *: significant					

Previously, it has been reported that TLR miRNA analysis suggests altered expression with age, therefore; the functional impact of these alterations in transcriptional regulation of TLR adaptor molecules has yet to be confirmed (Dunston and Griffiths; 2010). In a study performed in united states by (Van Duin *et al;* 2007), it has been found that older adults have impaired responses to TLR1/2-specific stimulation, with decreased TLR1/2-induced TNF–α and IL-6 production in older adults when compared with younger participants.

4-6-2 Determination of Interleukine-17A (IL-17A) levels

Diabetic foot patients were displayed a rising range level of IL-17A compared to healthy individuals. Results demonstrated a significant difference in level of IL-17A among groups under study. The mean levels of IL-17A in DFI patients were (123.7±33.52) ng/L which was significantly higher than DM and control groups ($P \leq 0.001$) as shown in table No. 4-13 .

The current results were in agreement with Kadhim (2021) in a local study who demonstrated that her result showed significant increase in IL-17A serum level in DFU patients compared with control. On the other hand, AL-Sahi *et al* (2023) in a local study too reported a highly statistically significant difference ($P<0.01$) in levels of IL-17A in all diabetes cases groups when compared with a healthy control group and the level of IL-17 was highest in the group with (DFU) than in control. Also, the current results agreed with Parhi *et al* (2019) findings which stated that diabetic patients had a higher level of IL-17

as compared to the healthy controls and the level of IL- 17 in complicated diabetics was higher than the patients with T2DM without complications in India while in a study performed in Iran by Zareian and Mirzaii Dizgah (2014), it was reported that the serum concentration of IL-17 was significantly higher in the patients with T2DM than in the controls (P = 0.002) and in a study done in Turkey by Kaleli *et al* (2019), it has been documented a higher IL-17, IL-18, white blood cells, glucose and HbA1c in the diabetic group and diabetic foot group than in the control group.

IL - 17 A as a pro-inflammatory cytokine takes a dual function, prompting early immune responses against infections and contributing in autoimmunity and destructive inflammatory conditions. According to the earlier results, it was believed that high IL-17 levels in both DFI and DM groups may be accredited to the fact that IL-17 is a pro-inflammatory cytokine and elevated IL-17 levels in patients with DFI may be belong to the inflammation in the ulceration, weakening of skin safety, and numerous types of bacteria causing infections. One of the probable mechanism is that the binding of IL-17A with its receptor may improve the activation of metalloproteinase, hypertensive and vascular dysfunction. One more mechanism is through the activation of JAK/STAT pathway that result in hepatic insulin resistance, beta and liver cell apoptosis and miserable regulation of gluconeogenesis related molecules (Yousefidaredor *et al;* 2014). Higher levels of IL-17 in both DFI and DM groups paralleled with the control group gives an idea that IL-17 may be associated with diabetic disease and can be considered as a marker in the diagnosis of DM. Furthermore, a higher level of IL-17 in the DFI group proposes that IL-17 can be used as a pro-inflammatory marker for diabetic foot infection (Kaleli *et al*;2019).

Regarding the age, IL-17A showed a significant high level in increasing ages specially in (45-54 and 55-64) years, respectively as illustrated in table No. 4-15.

Tab. No. 4-15 : The effect of Age on the IL-17A levels according to the three studied groups

Groups	35 - 44 Years (mean±SD)	45 - 54 Years (mean±SD)	55 - 64 Years (mean±SD)	65 - 74 Years (mean±SD)	≥75 Years (mean±SD)
	Concentration (ng/L)				
DFI	119.57±10.98	144.00±12.03*	140.87±10.98	119.61±10.98*	94.42±12.03
DM	107.04±10.98	105.85±12.03	111.78±10.98*	119.30±12.03	93.01±10.95
Control	100.15±10.98	95.34±10.98	87.41±10.98	88.92±10.98	87.08±8.92
ANOVA was *: significant at $p \leq 0.05$, Post hoc (LSD) SD: standard deviation; *: significant					

Throughout aging, tissue-specific variations in the niche synergize with stem cell–intrinsic changes to contribute to the development of age-associated characters. IL-17 mediated signaling is heavily linked to the development of chronic inflammatory and autoimmune diseases also they found that the local environment of the aged skin interestingly looks like a low-level but persistent state of chronic inflammation that is suggestive of that in serious skin diseases (Kusumbe *et al*, 2016 ; Pentinmikko *et al,* 2019 and Koester *et al*, 2021).

Few studies have investigated the potential contribution of the retention of strong inflammatory responses to age-related disease. Retention of strong inflammatory responses with age, in the absence of counterbalancing and beneficial responses from the immune system, may dramatically enhance the disease indication. Inflammation is well established to be an amplificatory factor (Sfanos and De Marzo ; 2012). Previous data suggested that an aging immune system possibly promotes different diseases onset through induction of chronic inflammation, specifically interleukin signaling (De Angulo *et al;* 2013). studies have demonstrated that IL-17 becomes dysregulated with age and that the proportion of IL-17-producing cells is higher in aged mice than in young ones (Schmitt *et al;* 2013).IL-17A upregulation has been accompanying with various autoimmune and chronic inflammatory diseases such as diabetes (Kumar and Subramaniyam; 2015). Some recent studies have revealed that IL-17A is one of the crucial cytokines involved in the

progression of diabetes complications (Sindhu *et al,* 2017; Fores *et al* ,2018 and Qiu *et al,* 2021) .

4-6-3 Determination of C-Reactive Protein (CRP) levels

Patients with DFI revealed a massive increase in the mean CRP levels compared to the DM and control groups. Also, results indicated an increasing level of CRP with an increasing age range. Results in table No. 4-12 showed that the mean levels of CRP in DFI patients were (92.9±78.26) mg/L which was significantly higher than DM and control groups ($p \leq 0.001$).

The current results were consistent with Kadhim (2021) in a local study who reported that the results of CRP exhibited statistically significant elevation in DFI group compared with DM and healthy individuals also our results approaches to (Muhanedalnajer *et al*;2020) in a local study who verified that CRP levels was 103.11± 68.35 in DFI patients whereas was 8.95± 4.61 in Diabetic patients and 2.68 ± 1.7 in healthy individuals. Additionally, Xu *et al* (2022) in China support our results whereas they reported that CRP levels were higher in DFI patients compared with DM and healthy individuals.

C-reactive protein is acute phase protein and considered as the main inflammatory factor produced by liver during acute infection or inflammation. Plasma concentration could be increased as much as 1000 fold during injury and infection. Additionally, high level of CRP could predict the development of T2DM and cardiovascular disease (Festa *et al;* 2000). CRP is established as an important risk marker in diabetes. It is associated with the degree of glycemic control and also the different complications of diabetes (Mohan *et al ;*2005).

With respect to age groups involved in current study, CRP showed an elevated level with age progress especially at (55-64) years then declines in elderly patients as illustrated in table No. 4-16.

Tab. No. 4-16: The effect of Age on the CRP levels according to the three studied groups

Groups	(35 – 44) Years (mean±SD)	(45 – 54) Years (mean±SD)	(55 – 64) Years (mean±SD)	(65 – 74) Years (mean±SD)	(≥75) Years (mean±SD)
	Concentration (mg/L)				
DFI	85.97±18.40*	112.30±18.40*	129.37±18.40*	76.12±20.16*	60.76±20.16*
DM	8.47±18.40	9.72±18.40	11.24±20.16	8.82±20.16	7.55±18.40
Control	6.63±18.40	8.53±18.40	8.23±18.40	8.12±18.40	5.88±18.40

ANOVA was *: significant at p ≤ 0.05, Post hoc (LSD)
SD: standard deviation; *: significant

The above results approaches to Shaalan (2016) findings in a local study who reported that CRP levels in (50->60) years were higher than in younger DFI patients. Increased levels of inflammatory markers are generally associated with age-related diseases (Singh and Newman; 2011). Low-grade inflammation is also involved in the mechanism underlying age-related problems (Trollor *et al;* 2012). Immune system function generally declines with aging and is called immunosenescence. Immunosenescence is based upon three theories: the autoimmune theory based on the decreased ability to recognize between invaders and normal tissues, the immune deficiency theory based on the diminished effectiveness of the immune system, and the immune dysregulation theory based on the disruption of the regulation between multiple components of the immune system. Many investigators have reported that aging is associated with increased levels of pro-inflammatory markers such as CRP (Varadhan *et al*; 2014). Many people above 65 years of age suffer from various diseases, with an over 70 % prevalence of multiple chronic conditions in elderly populations (Bähler *et al;* 2015) .

Elevated levels of CRP in diabetic foot patients approved with fact that most of lesions are infected because wounds are a perfect place for bacteria to colonize and reproduce since raw tissue and exudate offer an excellent medium for bacterial growth (Shler *et al*; 2012).

Previous studies have been proposed that Type II diabetes may signify a disease of the innate immune system (Mutluoglu *et al*; 2011).

4-7 Antioxidants:

4-7-1 Evaluation of Superoxide dismutase (SOD) activity

Patients with Diabetic foot infection showed a decreased range level of SOD compared to healthy control. Results in table No. 4-17 indicate a significant difference in this biomarker level among groups enrolled in current study. SOD activity in DFI group patients decreased significantly (241.4±35.61) (U/L) ($p \leq 0.001$) compared to DM and healthy individuals.

Tab. No. 4-17: Mean difference of some antioxidants among the Three Studied Groups

Biomarker	DFI N=40 (mean±SD)	DM N=40 (mean±SD)	Control N=40 (mean±SD)	*P*-value
SOD (U/L)	241.4±35.61	160.44±66.78	307.90±45.71*	<0.001
GSH (µmole/L)	26.586±2.77	28.47±2.06	29.75±2.20*	<0.001

ANOVA was *: significant at p ≤ 0.05, Post hoc (LSD)
SD: standard deviation; *: significant

The above result disagreed with Bolajoko *et al* (2017) in Nigeria who reported that there are non-significant declines in SOD activity levels observed when DFU patients and controls were compared ($P > 0.05$) while in a local study performed by Moustafa and Omar (2017), it has been found that serum level of SOD was significantly decreased in patients with T2DM as compared with the control group $p < 0.001$. The decline in SOD activity observed in this study is comparable to the work of Bhatia *et al* (2003) who testified a significant decrease in SOD activity in DM subjects. In contrast, Bandeira *et al* (2012) when assessing the antioxidant enzymes, only SOD activity exhibited a significant

difference among the groups whereas diabetic patients had increased SOD activity compared to DM and the control.

Reactive oxygen species (ROS) are vital regulators of several phases of wound healing. Certainly, low levels of ROS are required for the fight against external damage (Dunnill *et al;* 2017). However, extreme oxidative stress on tissues and the decrease of antioxidant capability leads to redox imbalance, which is a major cause of non-healing diabetic wounds (Cano Sanchez *et al*; 2018). Clinical studies explored that non-healing diabetic wounds are penetrating by the highly oxidizing environment, which is associated with hyperglycemia and tissue hypoxia, which leads to delayed wound repair. People with long-term type 2 diabetes have significant reductions in the antioxidant enzyme activity (Dworzański *et al*; 2020)

Accordingly, serum Oxidative Stress biomarkers were recognized as potentially valuable tools in the diagnostics and management of the DFI (Rattan and Nayak ; 2008).

Concerning the age, our finding reveals that SOD activity was greater in (55-64) years then declines in elderly patients as illustrated in table No. 4-18.

Tab. No. 4-18: The effect of age on SOD activity according to the three studied groups

Groups	**(35 – 44) Years (mean±SD)**	**(45 – 54) Years (mean±SD)**	**(55 – 64) Years (mean±SD)**	**(65 – 74) Years (mean±SD)**	**(≥75) Years (mean±SD)**
	SOD activity (U/L)				
DFI	248.97±14.38	251.1±14.38	262.97±15.75	239.5±14.38	204.43±15.75
DM	190.85±14.38	191.75±14.38	189.76±15.75	125.45±15.75	104.39±14.38
Control	298.61±14.38*	338.6±14.38*	329.72±14.38	290.71±14.38*	281.85±14.38*
ANOVA was *: significant at p ≤ 0.05, Post hoc (LSD) **SD: standard deviation; *: significant**					

The above results disagreed with Moustafa and Omar (2017) in a local study, who reported that there was a significant negative fragile correlation between age and SOD in

patients group, as much as the age increase, the serum level of SOD decrease while Hisalkar *et al* (2012) reported that SOD drops in diabetic age group ≥50 years compared with age groups (30–39) and (40–49) years. Therefore, the results of Hisalkar *et al* (2012) showed that Oxidative Stress which influences the aging process that may be caused by a number of factors including increased free radical production, decreased antioxidant defense system, or a decreased removal or repair. Olędzki *et al* (2017) compared the activity of SOD and CAT in erythrocytes taken from young healthy people (aged 20–29) and older individuals (> 60 years of age). They reported reduced SOD activity in the older patients. Bartosz (2003) documented that the drop of SOD activity among aging women can be elucidated by enzyme inactivation by extra hydrogen peroxide, as well as by glycation of SOD molecules or reactions with lipid peroxidation products, the intensity of which increases with age.

4-7-2 Evaluation of Glutathione (GSH) concentration

In this study, the Glutathione concentration was decreased markedly in the DFI group compared to DM and control. Results in table No. 4-17 demonstrated a significant difference in this biomarker level among groups registered in current study. The mean levels of serum GSH in DFI group patients decreased significantly (26.586±2.77) µmole/ml ($p \leq 0.001$) compared to DM and healthy individuals.

The above results agreed with Muhanedalnajer *et al* (2020) in a local study who reported that GSH and total thiol levels were significantly lower in DFI as compared with control group while Aouacheri *et al* (2015) and Gawlik *et al* (2016) in Algeria and Poland, respectively reported that levels of GSH were significantly higher in patients with diabetes than in healthy control. Decreased level of GSH was also reported in other studies that observed that plasma GSH levels are lower in diabetic patients than control (Kalkan and Suher; 2013). In addition, a depressed GSH level was thought to be responsible for some of the metabolic disturbances seen in diabetic patients and subsequent to the onset of diabetic complications (Maritim *et al;* 2003).

In human, glutathione (GSH) reduces the action of free radicals for protection the body (Singh and Singh; 2017). Reduced glutathione (GSH), plays a chief role by guarding cells from oxidative damage by neutralizing the free radicals. Oxidative stress is being considered as a common pathogenic factor in diabetes mellitus which leads to reducing extracellular and intercellular antioxidant. GSH is a non-enzymatic antioxidant delays or prevents the oxidative process by different mechanisms. Antioxidant enzyme levels are particularly sensitive to oxidative stress and both increase and decrease these have been described in different disease states in where enhance of oxygen species is a cause or a significance of the diabetes mellitus (Lutchmansingh *et al*; 2018).

With respect to age, GSH showed decreased levels with aging as illustrated in table No. 4-19.

Tab. No. 4-19: The effect of age on GSH concentration according to the three studied groups

Groups	**35 - 44 Years (mean±SD)**	**45 - 54 Years (mean±SD)**	**55 - 64 Years (mean±SD)**	**65 - 74 Years (mean±SD)**	**≥75 Years (mean±SD)**
	Concentration (µmole/ml)				
DFI	27.03±0.89	27.16±0.89	26.62±0.98*	26.69±0.98	25.43±0.98
DM	29.87±0.89	29.65±0.89	28.54±0.98	27.5±0.98	26.82±0.89
Control	31.83±0.89	30.09±0.89	29.9±0.89	28.83±0.89	28.1±0.89*

ANOVA was *: significant at p ≤ 0.05, Post hoc (LSD)
SD: standard deviation; *: significant

Esther *et al* (1998) in a study performed in Netherlands reported that GSH contents decreased with age in both males and females. In age group (60–80) years, GSH content was significantly lower as compared with age groups (20–40) and (40–60) years in both sexes. Since high GSH is an essential factor in the detoxification of many compounds, these data indicate that the detoxification potential of the GSH system in lymphocytes may decrease with age in man.

Glutathione plays a critical role in many biological processes both directly as a co-factor in enzymatic reactions and indirectly as the major thiol-disulfide redox buffer in mammalian cells. Glutathione also provides a critical defense system for the protection of cells from many forms of stress. during aging, glutathione levels appear to decline in a number of tissues, thereby putting cells at increased risk of succumbing to stress (Maher ; 2005). Many previous studies have suggested that redox imbalances could have a pivotal role in the development and delayed healing of DF ulcers (Li *et al;* 2016). Accordingly, serum Oxidative Stress biomarkers were recently recognized as potentially valuable tools in the diagnostics and management of the DFI (Rattan and Nayak; 2008).

4-8 Odd ratio

Multinominal logistic regression was performed to analyze the association of the CRP and IL-17 A, TRL-2, GSH, and SOD with Diabetic foot infection disease and DM cases. It was found that the biomarkers (CRP and IL-17 A and TRL-2) showed a highly significant association in Diabetic foot infection disease (DFI) and represented as a risk factors factor (OR 55.058; 95% CI: (3.000-56.021) and OR: 1.035; 95% CI: (1.012-1.059)) and (OR 4.347; 95% CI: (2.257-8.371) respectively.

On the other and the level of GSH and SOD biomarkers is protected factors shown a highly significant in DFI (OR 0.616; 95% CI: (0.474-0.801) and OR: 0.961; 95% CI: (0.941-0.982))), respectively as illustrated in table No. 3-20 .

Tab. No. 4-20: Estimation the Associated of the analyzed factors in Diabetic foot infection disease two cases Compared to the control group

Variable	Groups	OR (Lower – upper)	P value
CRP			
	Control	1[a]	-
	DF	55.058 (3.000-56.021)	0.995
	DM	1.334 (1.099-1.619)	**0.004**
IL-17 A			
	Control	1[a]	**-**

	DF	1.035(1.012-1.059)	**<0.003**
	DM	1.040(1.016-1.064)	**0.001**
TRL-2			
	Control	1[a]	**-**
	DF	4.347 (2.257-8.371)	**<0.001**
	DM	3.998 (2.093-7.637)	**<0.001**
GSH			
	Control	1[a]	**-**
	DF	0.616 (0.474-0.801)	**<0.001**
	DM	0.708 (0.555-0.902)	**0.005**
SOD			
	Control	1[a]	**-**
	DF	0.961 (0.941-0.982)	**<0.001**
	DM	0.955 (0.934-0.976)	**<0.001**
p<0.05 considered significantly different, 1[a] : reference category is Control			

4-9 Receiver Operating Characteristic Analysis

ROC curve and AUC analysis for the TLR-2 for Patients compared to the control group. Results of the receiver operating curve (ROC) curve and AUC analysis for the TRL-2 as a possible diagnostic marker for Diabetic Patients are presented in table No. 4-21.

The results showed relatively good sensitivity and specificity (sensitivity = 81.4%, specificity = 97.6%) at a level = 5.87 in diagnosis patients in comparison with control. The p-values of the AUC are <0.001 and statistically significant. Youden's J statistics of the parameters in figure No. 4-13 confirm these results.

Further analysis was performed to check the diagnostic performance of TRL-2 toward DFI and DM cases. Results indicated that the TRL-2 level was more sensitive and specific in DFI patients. The sensitivity = 80%, specificity = 70%) at a level = 6.347 in comparison with control. The P-values of the AUC are <0.001 and statistically significant, as presented in table No. 4-22 and figure No. 4-14. These results agreed with Arbibe *et al* (2000) have shown the important role of TLR-2 in the pathogenesis of DM and its complication through the following mechanism: TLR2-mediated NF-kB activation in monocytes cells requires

Rac1, a key mediator of oxidative stress in monocytes. Thus, it appears that in diabetic wounds, TLR2 may have a key role in oxidative stress through Rac1 activation, leading to the activation of NF-kB and pro-inflammatory cytokines. Based on this hypothesis, the TRL-2 level was shown good Sensitivity and Specificity toward DFI patients.

Tab. No. 4-21: Receiver operating characteristic showing sensitivity and specificity of TRL-2 in DM (both DFI and DM) patients compared to control

ROC analysis	TRL-2
AUC	92%
Sensitivity	81.4%
Specificity	97.6%
P value	<0.001[S]
Cut off	5.87
Youden index	0.781
CI%(Lower- upper)	(0.860-0.973)
PPV	92.68%
NPV	88%
Accuracy	70%

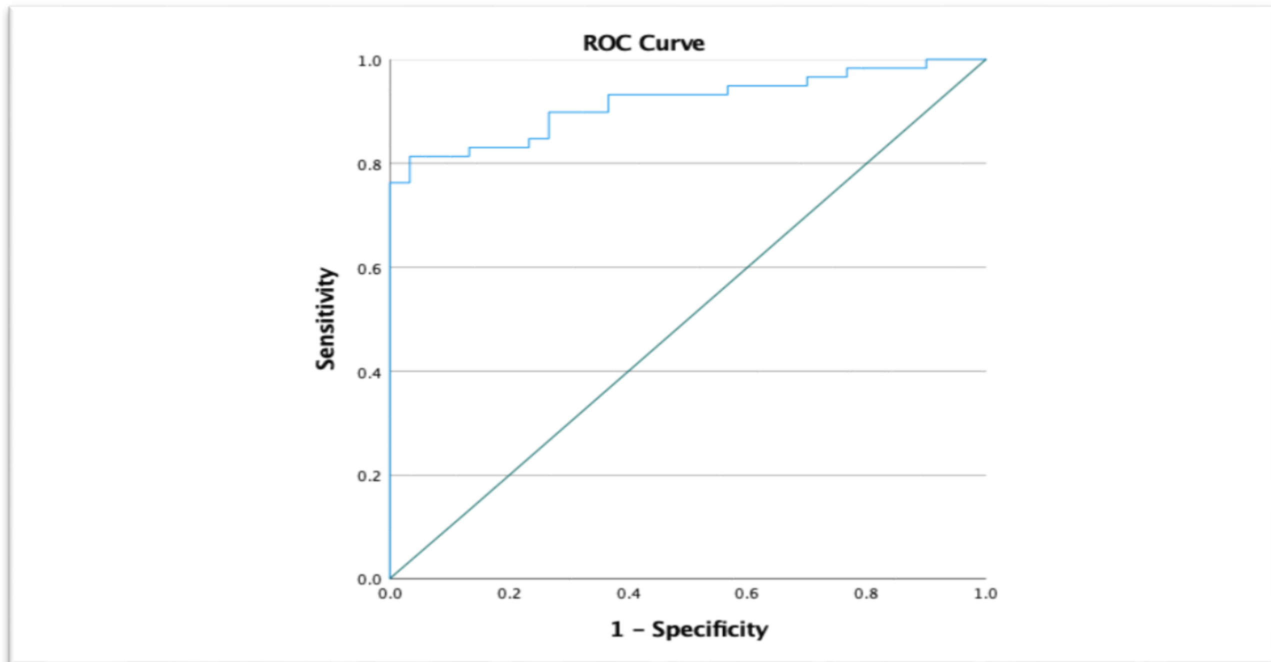


Fig. No. 4-13: Receiver operating characteristics (ROC) curve analysis of TLR-2 levels in Patient and Control, The area under the ROC curve: 92%

Tab No. 4-22: Receiver operating characteristic showing sensitivity and specificity of TRL-2

Test Result Variable(s)	DFI	DM
AUP	74.6%	67.3%
Sensitivity %	80%	79.3%
Specificity %	70%	65%
Youden index	0.461	0.36
Cut-off points	6.46	5.87
CI (95%)	(0.643-0.849)	(0.557-0.790)
P value	<0.001[S]	0.008[S]

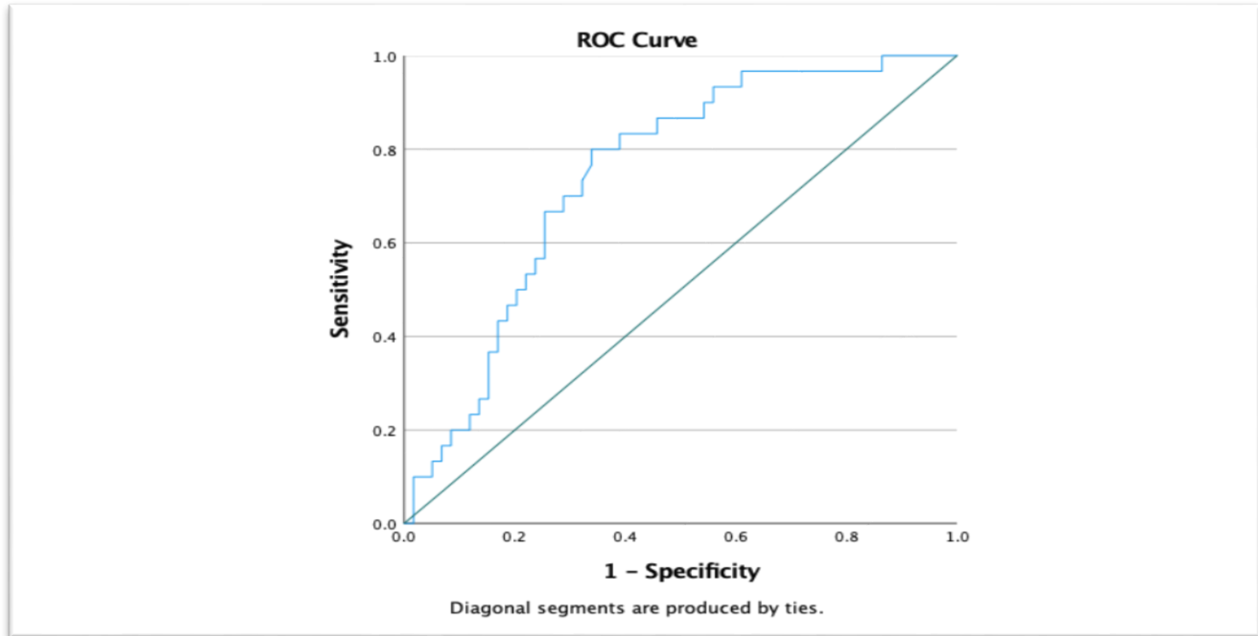


Fig. No. 4-14: Receiver operating characteristics (ROC) curve analysis of Toll-like levels in Patients for DFI, The area under the ROC curve: 74.6%,

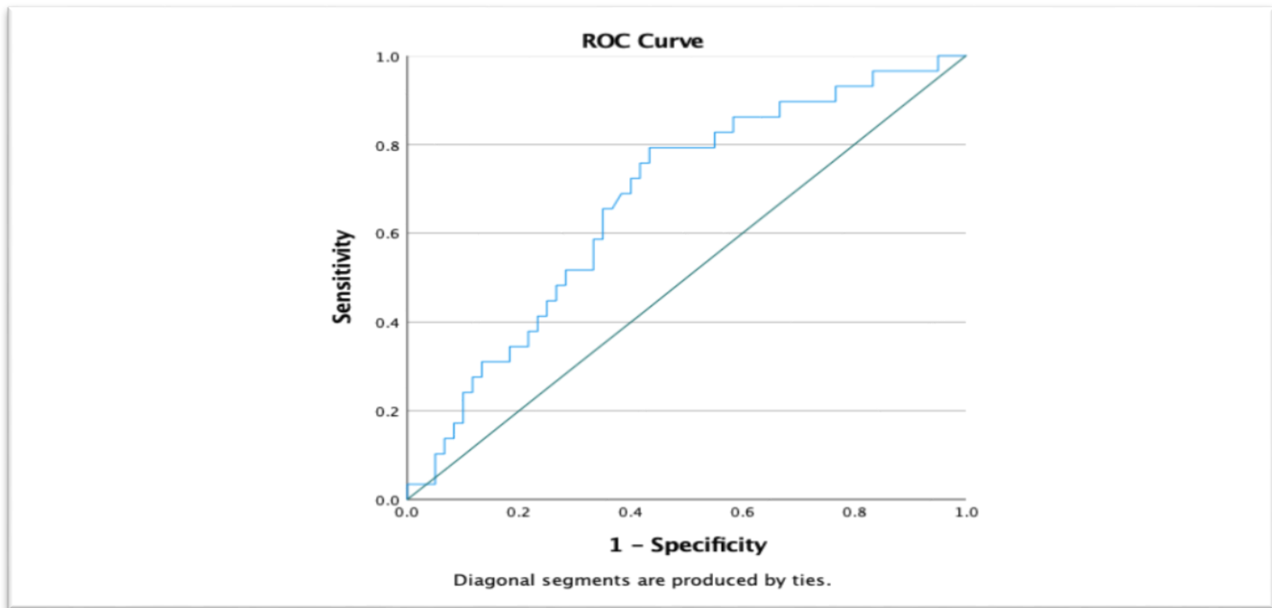


Fig. No. 4-15: Receiver operating characteristics (ROC) curve analysis of Toll-like levels in Patient for DM, The area under ROC curve: 67.3%

Conclusions and Recommendations

4-1 Conclusions:

The current study exposed the following:

1.The most prevalent Gram positive bacteria was *S.aureus* , 60% of which were MRSA whereas the most commonly isolated Gram negative bacteria was *K.pneumoniae*.

2. The presence of high resistance rate in *S.aureus* and *K.pneumoniae* against antibiotics and the incidence of high rate of biofilm strong producer in both prevalent bacterial species and lesser rate of moderate producers.

3. The presence of antibiotic resistance genes in *S.aureus* including *mec*A and *erm*C furthermore, the incidence of virulence genes involving *sea* , *ica*A and *ica*D while *K.pneumoniae* contained a virulence genes involving *mrk*D, *Cps*,*K1* and *k2A* genes

4. The Presence of significant elevated levels of immunological markers like TLR-2, Il-17A and CRP in DFI patients in comparison to DM and Control groups could support a positive role of these markers in T2DM pathogenesis while the lower levels of SOD and GSH in patients with DFI compared to DM and healthy control may consider these marker as a protective factor.

4-2 Recommendations:

1. Further studies require for anaerobic bacterial pathogenesis responsible for DFI and determine their antibiotic susceptibility pattern.

2. Further studies necessary for detection for more virulence and antibiotic genes.

3. Further studies require to evaluate the mechanism of drug resistance in bacteria to eradicate the evolution of totally drug resistance and partially drug resistance bacteria.

4. Further studies with larger sample size require to evaluate the association of some risk factors like sex, smoking and obesity with the development of DM and DFI.

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Appendices

Appendix No. 1: Diagnosis of *Staphylococcus aureus* by API STAPH kits

Test	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13
GLU	+	+	+	+	+	+	+	+	+	+	+	+	+
FRU	+	+	+	+	+	+	+	+	+	+	+	+	+
MNE	+	+	+	+	+	+	+	+	+	+	+	+	+
MAL	+	+	+	+	+	+	+	+	+	+	+	+	+
LAC	_	_	_	_	_	_	_	_	_	_	_	_	_
TRE	_	+	+	_	+	+	+	_	+	+	_	_	+
MAN	+	+	+	+	+	+	+	+	+	+	+	+	+
XLT	_	_	_	_	_	_	_	_	_	_	_	_	_
MEL	_	_	_	_	_	_	_	_	_	_	_	_	_
NIT	+	_	_	+	_	_	_	+	_	_	+	+	_
PAL	+	+	+	+	+	+	+	+	+	+	+	+	+
VP	+	+	+	+	+	+	+	+	+	+	+	+	+
RAF	_	_	_	_	_	_	_	_	_	_	_	_	_
XYL	_	_	_	_	_	_	_	_	_	_	_	_	_
SAC	+	+	+	+	+	+	+	+	+	+	+	+	+
MDG	_	+	+	_	+	+	+	_	+	+	_	_	+
NAG	+	+	+	+	+	+	+	+	+	+	+	+	+
ADH	+	+	+	+	+	+	+	+	+	+	_	+	+
URE	+	+	+	+	+	+	+	+	+	+	+	+	+

*** GLU=Glucose, FRU=Fructose, MNE=Mannose, MAL=Maltose, LAC=Lactose, TRE=D-Trehalose, MAN=Mannitol, XLT=Xylitol, MEL=Melibiose, NIT=Potassium nitrate, PAL=β naphthyl phosphate, VP=Sodium pyruvate, RAF=Raffinose, XYL=xylose, SAC=Sucrose, MDG=Methyl-αD glucopyranoside, NAG=N-acetyle glucosamine, ADH=L-Arginie, URE=urea**

Test	S14	S15	S16	S17	S18	S19	S20	S21	S22	S23	S24	S25
GLU	+	+	+	+	+	+	+	+	+	+	+	+
FRU	+	+	+	+	+	+	+	+	+	+	+	+
MNE	+	+	+	+	+	+	+	+	+	+	+	+
MAL	+	+	+	+	+	+	+	+	+	+	+	+
LAC	_	_	_	_	_	_	_	_	_	_	_	_
TRE	_	_	+	+	_	+	_	_	_	+	_	_
MAN	+	+	+	+	+	+	+	+	+	+	+	+
XLT	_	_	_	_	_	_	_	_	_	_	_	_
MEL	_	_	_	_	_	_	_	_	_	_	_	_
NIT	+	+	_	_	+	_	+	+	+	_	+	+
PAL	+	+	+	+	+	+	+	+	+	+	+	+
VP	+	+	+	+	+	+	+	+	+	+	+	+
RAF	_	_	_	_	_	_	_	_	_	_	_	_
XYL	_	_	_	_	_	_	_	_	_	_	_	_
SAC	+	+	+	+	+	+	+	+	+	+	+	+
MDG	_	_	+	+	_	+	_	_	_	+	_	_
NAG	+	+	+	+	+	+	+	+	+	+	+	+
ADH	+	+	+	+	+	+	+	+	+	+	+	+
URE	+	+	+	+	+	+	+	+	+	+	+	+

Appendix No. 2: Diagnosis of *Staphylococcus epidermidis* by API STAPH kits

Test	S1	S2	S3	S4	S5	S6	S7	S8	S9
GLU	+	+	+	+	+	+	+	+	+
FRU	–	–	–	–	–	–	–	–	–
MNE	+	+	+	+	+	+	+	+	+
MAL	+	+	+	+	+	+	+	+	+
LAC	+	–	–	+	–	+	+	–	+
TRE	–	–	–	–	–	–	–	–	–
MAN	–	–	–	–	–	–	–	–	–
XLT	–	–	–	–	–	–	–	–	–
MEL	–	–	–	–	–	–	–	–	–
NIT	+	–	–	+	–	+	+	–	+
PAL	+	+	+	+	+	+	+	+	+
VP	–	–	–	–	–	–	–	–	–
RAF	–	–	–	–	–	–	–	–	–
XYL	–	–	–	–	–	–	–	–	–
SAC	+	+	+	+	+	+	+	+	+
MDG	–	–	–	–	–	–	–	–	–
NAG	–	–	–	–	–	–	–	–	–
ADH	+	+	+	+	+	+	+	+	+
URE	+	+	+	+	+	+	+	+	+

Test	S10	S11	S12	S13	S14	S15	S16	S17	S18
GLU	+	+	+	+	+	+	+	+	+
FRU	–	–	–	–	–	–	–	–	–
MNE	+	+	+	+	+	+	+	+	+
MAL	+	+	+	+	+	+	+	+	+
LAC	–	+	+	–	+	–	+	+	+
TRE	–	–	–	–	–	–	–	–	–
MAN	–	–	–	–	–	–	–	–	–
XLT	–	–	–	–	–	–	–	–	–
MEL	–	–	–	–	–	–	–	–	–
NIT	–	+	+	–	+	–	+	+	+
PAL	+	+	+	+	+	+	+	+	+
VP	–	–	–	–	–	–	–	–	–
RAF	–	–	–	–	–	–	–	–	–
XYL	–	–	–	–	–	–	–	–	–
SAC	+	+	+	+	+	+	+	+	+
MDG	–	–	–	–	–	–	–	–	–
NAG	–	–	–	–	–	–	–	–	–
ADH	+	+	+	+	+	+	+	+	+
URE	+	+	+	+	+	+	+	+	+

Appendix No. 3: Diagnosis of *Streptococcus agalacitiae* by API 20 STREP kits

Test	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15
VP	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
HIP	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ESC	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
PYRA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
αGAL	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
βGUR	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
βGAL	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
PAL	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
LAP	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ADH	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
RIB	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ARA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
MAN	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
SOR	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
LAC	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
TRE	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
INU	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
RAF	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
AMD	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
GLYG	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

*** VP=Sodium pyruvate, HIP=hippuric acid, ESC=Escolin ferric citrate, PYRA=Pyraglutamic acid-β naphthylamide, αGAL=6-bromo-2-naphthyl-αD-galactopyranoside, βGUR=naphthoASBI- glucuronic acid, βGAL=2-naphthyl-βD-galactopyranoside, PAL=β naphthyl phosphate, LAP=leucine β naphthylamide, ADH=L-Arginie, RIB=ribose, ARA=arabinose, MAN=Mannitol, SOR=Sorbitol, LAC=Lactose, TRE=D-Trehalose, INU=inulin, RAF=Raffinose, AMD=starch, GLYG=glycogen**

Appendix No. 4: Diagnosis of *Enterococcus faecalis* by API 20 STREP kits

Test	E1	E2	E3	E4	E5
VP	+	+	+	+	+
HIP	–	–	–	–	–
ESC	+	+	+	+	+
PYRA	+	+	+	+	+
αGAL	–	–	–	–	–
βGUR	–	–	–	–	–
βGAL	–	–	–	–	–
PAL	–	–	–	–	–
LAP	+	+	+	+	+
ADH	+	+	+	+	+
RIB	+	+	+	+	+
ARA	–	–	–	–	–
MAN	+	+	+	+	+
SOR	+	+	+	+	+
LAC	–	–	–	–	–
TRE	+	+	+	+	+
INU	–	–	–	–	–
RAF	–	–	–	–	–
AMD	–	+	+	–	+
GLYG	–	+	–	+	–

Appendix No. 5: Diagnosis of *Klebsiella pneumoniae* by API 20 E kits

Test	K1	K2	K3	K4	K5	K6	K7	K8	K9	K10	K11	K12	K13
ONPG	+	+	+	+	+	+	+	+	+	+	+	+	+
ADH	–	–	–	–	–	–	–	–	–	–	–	–	–
LDC	–	–	–	–	–	–	–	–	–	–	–	–	–
ODC	–	–	–	–	–	–	–	–	–	–	–	–	–
CIT	+	+	+	+	+	+	+	+	+	+	+	+	+
H_2S	–	–	–	–	–	–	–	–	–	–	–	–	–
URE	+	+	+	+	+	+	+	+	+	+	+	+	+
TDA	–	–	–	–	–	–	–	–	–	–	–	–	–
IND	–	–	–	–	–	–	–	–	–	–	–	–	–
VP	+	+	+	–	+	+	+	+	+	+	+	+	+
GEL	–	–	–	–	–	–	–	–	–	–	–	–	–
GLU	+	+	+	+	+	+	+	+	+	+	+	+	+
MAN	+	+	+	+	+	+	+	+	+	+	+	+	+
INO	–	+	+	+	+	–	+	–	+	–	–	+	–
SOR	+	+	+	+	+	+	+	+	+	+	+	+	+
RHA	+	+	+	+	+	+	+	+	+	+	+	+	+
SAC	+	+	+	+	+	+	+	+	+	+	+	+	+
MEL	+	–	–	+	–	+	–	+	–	+	+	–	+
AMY	+	+	+	+	+	+	+	+	+	+	+	+	+
ARA	+	+	+	+	+	+	+	+	+	+	+	+	+

***ONPG=2-nitrophenyl-βD-galatopyrannoside, ADH=L-Arginie, LDC=L-lysine, ODC=L-omithine, CIT=citrate, H_2S= Sodium thiosulfate, URE=urea, TDA=L-tryptophan, IND=L-tryptophan, VP=Sodium pyruvate, GEL=gelatin, GLU= D-glucose, MAN=Mannitol, INO=inositol, SOR=Sorbitol, RHA=L-rhamnose, SAC=D-sucrose, MEL=D-melibiose, AMY=Amygdaline, ARA=L-arabinose**

Test	K14	K15	K16	K17	K18	K19	K20	K21	K22	K23	K24	K25
ONPG	+	+	+	+	+	+	+	+	+	+	+	+
ADH	–	–	–	–	–	–	–	–	–	–	–	–
LDC	–	–	–	–	–	–	–	–	–	–	–	–
ODC	–	–	–	–	–	–	–	–	–	–	–	–
CIT	+	+	+	+	+	+	+	+	+	+	+	+
H_2S	–	–	–	–	–	–	–	–	–	–	–	–
URE	+	+	+	+	+	+	+	+	+	+	+	+
TDA	–	–	–	–	–	–	–	–	–	–	–	–
IND	–	–	–	–	–	–	–	–	–	–	–	–
VP	+	+	+	+	+	+	–	+	–	+	–	+
GEL	–	–	–	–	–	–	–	–	–	–	–	–
GLU	+	+	+	+	+	+	+	+	+	+	+	+
MAN	+	+	+	+	+	+	+	+	+	+	+	+
INO	–	+	–	+	–	+	+	–	+	–	+	–
SOR	+	+	+	+	+	+	+	+	+	+	+	+
RHA	+	+	+	+	+	+	+	+	+	+	+	+
SAC	+	+	+	+	+	+	+	+	+	+	+	+
MEL	+	–	+	–	+	–	+	+	+	+	+	+
AMY	+	+	+	+	+	+	+	+	+	+	+	+
ARA	+	+	+	+	+	+	+	+	+	+	+	+

Appendix No. 6: Diagnosis of *Pseudomonas aerogenosa* by API 20 E kits

Test	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13
ONPG	–	–	–	–	–	–	–	–	–	–	–	–	–
ADH	+	+	+	+	+	+	+	+	+	+	+	+	+
LDC	–	–	–	–	–	–	–	–	–	–	–	–	–
ODC	–	–	–	–	–	–	–	–	–	–	–	–	–
CIT	–	+	+	+	+	–	+	+	+	+	+	+	+
H_2S	–	–	–	–	–	–	–	–	–	–	–	–	–
URE	+	+	+	+	+	+	+	+	+	+	+	+	+
TDA	–	–	–	–	–	–	–	–	–	–	–	–	–
IND	–	–	–	–	–	–	–	–	–	–	–	–	–
VP	+	+	+	+	+	+	+	+	+	+	+	+	+
GEL	+	+	+	+	+	+	+	+	+	+	+	+	+
GLU	+	+	+	+	+	+	+	+	+	+	+	+	+
MAN	–	–	–	–	–	–	–	–	–	–	–	–	–
INO	–	–	–	–	–	–	–	–	–	–	–	–	–
SOR	–	–	–	–	–	–	–	–	–	–	–	–	–
RHA	–	–	–	–	–	–	–	–	–	–	–	–	–
SAC	–	–	–	–	–	–	–	–	–	–	–	–	–
MEL	+	+	+	+	+	+	+	+	+	+	+	+	+
AMY	+	+	+	+	+	+	+	+	+	+	+	+	+
ARA	+	+	+	+	+	+	+	+	+	+	+	+	+

Appendix No. 7: Diagnosis of *Proteus mirabilis* by API 20 E kits

Test	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
ONPG	–	–	–	–	–	–	–	–	–	–
ADH	–	–	–	–	–	–	–	–	–	–
LDC	–	–	–	–	–	–	–	–	–	–
ODC	+	+	+	+	+	+	+	+	+	+
CIT	+	+	–	+	+	–	+	+	–	+
H_2S	+	+	+	+	+	+	+	+	+	+
URE	+	+	+	+	+	+	+	+	+	+
TDA	+	–	+	+	–	+	–	+	+	–
IND	–	–	–	–	–	–	–	–	–	–
VP	+	–	+	–	–	+	+	+	+	+
GEL	+	+	+	+	–	+	+	+	+	+
GLU	+	+	+	+	+	+	+	+	+	+
MAN	–	–	–	–	–	–	–	–	–	–
INO	–	–	–	–	–	–	–	–	–	–
SOR	–	–	–	–	–	–	–	–	–	–
RHA	–	–	–	–	–	–	–	–	–	–
SAC	–	–	–	–	–	–	–	–	–	–
MEL	–	–	–	–	–	–	–	–	–	–
AMY	–	–	–	–	–	–	–	–	–	–
ARA	–	–	–	–	–	–	–	–	–	–

Appendix No. 8: Diagnosis of *Escherichia coli* by API 20 E

Test	E1	E2	E3	E4	E5	E6	E7	E8
ONPG	+	+	+	+	+	+	+	+
ADH	_	_	_	_	_	_	_	_
LDC	+	+	+	+	+	+	+	+
ODC	_	_	_	_	_	_	_	_
CIT	_	_	_	_	_	_	_	_
H_2S	_	_	_	_	_	_	_	_
URE	_	_	_	_	_	_	_	_
TDA	_	_	_	_	_	_	_	_
IND	+	+	+	+	+	+	+	+
VP	_	_	_	_	_	_	_	_
GEL	_	_	_	_	_	_	_	_
GLU	+	+	+	+	+	+	+	+
MAN	+	+	_	+	+	+	_	_
INO	_	_	_	_	_	_	_	_
SOR	+	+	+	+	+	+	+	+
RHA	+	_	+	_	+	+	_	+
SAC	+	_	+	_	+	+	_	+
MEL	+	+	+	+	+	+	_	+
AMY	_	_	_	_	_	_	_	_
ARA	+	+	+	_	+	+	+	+

Appendix No. 9: Susceptibility tests of *Staphylococcus aureus* to antibiotics

Antibiotics	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13
penicillin	R	R	R	R	R	R	R	S	S	S	R	R	S
Pipracillin	R	R	R	R	R	R	R	S	S	S	R	R	S
Amoxicillin-clavulanic acid	R	R	R	R	R	R	R	S	S	S	R	R	S
Ceftazidime	R	R	R	R	R	R	R	S	S	S	R	R	S
Cefepime	R	R	R	R	R	R	R	S	S	S	R	R	S
Cefotaxim	R	R	R	R	R	R	R	S	S	S	R	R	S
Ceftriaxone	R	R	R	R	R	R	R	S	S	S	R	R	S
Imipenem	R	R	R	R	R	R	R	S	S	S	R	R	S
Meropenem	R	R	R	R	R	R	R	S	S	S	R	R	S
Aztronem	R	R	R	R	R	R	R	S	S	S	R	R	S
Azithromycin	R	R	R	R	R	R	R	S	S	R	R	R	S
Rifampin	R	R	S	S	R	S	S	S	S	S	R	S	R
Vancomycin	R	R	S	S	R	S	R	S	S	R	R	S	R
Ciprofloxacin	R	R	S	S	R	S	S	S	S	S	R	S	R
Levofloxacin	R	S	S	S	S	S	S	S	S	S	S	S	S
Oxacillin	R	R	R	R	R	R	R	S	S	S	R	R	S
Erythromycin	R	R	R	R	R	R	R	S	S	R	R	R	S

Antibiotics	S14	S15	S16	S17	S18	S19	S20	S21	S22	S23	S24	S25
penicillin	R	R	R	S	R	S	R	S	S	S	R	S
Pipracillin	R	R	R	S	R	S	R	S	S	S	R	S
Amoxicillin-calvulonic acid	R	R	R	S	R	S	R	S	S	S	R	S
Ceftazidime	R	R	R	S	R	S	R	S	S	S	R	S
Cefepime	R	R	R	S	R	S	R	S	S	S	R	S
Cefotaxim	R	R	R	S	R	S	R	S	S	S	R	S
Ceftriaxone	R	R	R	S	R	S	R	S	S	S	R	S
Imipenem	R	R	R	S	R	S	R	S	S	S	R	S
Meropenem	R	R	R	S	R	S	R	S	S	S	R	S
Aztronem	R	R	R	S	R	S	R	S	S	S	R	S
Azithromycin	R	R	R	S	R	S	R	S	S	S	R	S
Rifampin	S	R	R	S	R	R	S	R	S	S	R	R
Vancomycin	R	R	R	S	R	R	S	R	S	S	R	R
Ciprofloxacin	S	R	R	S	R	R	S	R	S	S	R	R
Levofloxacin	R	R	S	S	S	S	S	S	S	S	R	R
Oxacillin	R	R	R	S	R	S	R	S	S	S	R	S
Erythromycin	R	R	R	S	R	S	R	S	S	S	R	S

Appendix No. 10: Susceptibility tests of *Klebsiella pneumonia* to antibiotics

Antibiotics	K1	K2	K3	K4	K5	K6	K7	K8	K9	K10	K11	K12	K13
Pipracillin-Tazobactam	R	R	R	R	S	R	R	R	S	R	S	R	R
Amoxicillin-calvulonic acid	R	R	R	R	S	R	R	S	S	R	S	R	R
Ceftazidime	R	R	R	R	S	R	R	R	S	R	S	R	R
Cefepime	R	R	R	R	S	R	R	S	S	R	S	R	R
Cefotaxime	R	R	R	R	S	R	R	R	S	R	S	R	R
Ceftriaxone	R	R	R	R	S	R	R	R	S	R	S	R	R
Imipenem	R	S	R	R	S	R	R	R	S	R	S	R	R
Meropenem	R	R	R	R	S	R	R	R	S	R	S	R	S
Amikacin	R	S	S	R	R	R	R	R	R	R	R	R	R
Gentamycin	R	R	R	R	R	R	R	R	R	R	R	R	R
Rifampin	R	R	S	S	R	S	S	S	R	S	R	S	R
Ciprofloxacin	R	R	R	R	S	R	R	R	R	R	R	R	R
Levofloxacin	R	R	R	R	R	R	R	R	R	R	R	R	R
Tetracycline	R	R	R	R	S	R	R	R	S	R	S	R	R
Doxycycline	R	R	R	S	S	R	R	R	S	S	S	R	R

Antibiotics	K14	K15	K16	K17	K18	K19	K20	K21	K22	K23	K24	K25
Pipracillin-Tazobactam	R	R	R	R	R	R	R	R	R	R	R	R
Amoxicillin-calvulonic acid	R	R	R	R	R	R	R	R	R	R	R	R
Ceftazidime	R	R	R	R	R	R	R	R	R	R	R	R
Cefepime	R	R	R	R	R	R	R	R	R	R	R	R
Cefotaxime	R	R	R	R	R	R	R	R	R	R	R	R
Ceftriaxone	R	R	R	R	R	R	R	R	R	R	R	R
Imipenem	R	S	R	R	R	R	R	R	R	S	R	R
Meropenem	R	R	R	R	R	S	R	R	R	R	R	S
Amikacin	R	R	R	R	R	R	R	R	R	R	R	R
Gentamycin	R	R	R	R	R	R	R	R	R	R	R	R
Rifampin	S	R	R	S	R	R	S	R	S	R	R	R
Ciprofloxacin	R	R	R	R	R	R	R	R	R	R	R	R
Levofloxacin	R	R	R	R	R	R	R	R	R	R	R	R
Tetracycline	R	R	R	R	R	R	R	R	R	R	R	R
Doxycycline	R	S	R	R	S	R	R	R	R	S	R	R

Appendix No. 11: Quantification of Biofilm production of *S.aureus* by microtiter plate method

Staph.No.	OD.Mean	control	2*control	4* control	feature
1	0.3825	0.0624	0.1248	0.2496	strong
2	0.3075	0.0624	0.1248	0.2496	strong
3	0.34125	0.0624	0.1248	0.2496	strong
4	0.3147	0.0624	0.1248	0.2496	strong
5	0.339	0.0624	0.1248	0.2496	strong
6	0.3485	0.0624	0.1248	0.2496	strong
7	0.3435	0.0624	0.1248	0.2496	strong
8	0.15175	0.0624	0.1248	0.2496	moderate
9	0.1825	0.0624	0.1248	0.2496	moderate
10	0.1405	0.0624	0.1248	0.2496	moderate
11	0.4505	0.0624	0.1248	0.2496	strong
12	0.4225	0.0624	0.1248	0.2496	strong
13	0.1426	0.0624	0.1248	0.2496	moderate
14	0.3139	0.0624	0.1248	0.2496	strong
15	0.3465	0.0624	0.1248	0.2496	strong
16	0.49325	0.0624	0.1248	0.2496	strong
17	0.17125	0.0624	0.1248	0.2496	moderate
18	0.3495	0.0624	0.1248	0.2496	strong
19	0.14825	0.0624	0.1248	0.2496	moderate
20	0.3144	0.0624	0.1248	0.2496	strong
21	0.1465	0.0624	0.1248	0.2496	moderate
22	0.19525	0.0624	0.1248	0.2496	moderate
23	0.148	0.0624	0.1248	0.2496	moderate
24	0.4225	0.0624	0.1248	0.2496	strong
25	0.17525	0.0624	0.1248	0.2496	moderate

Appendix No. 12: Quantification of Biofilm production of *K.pneumoniae* by microtiter plate method

Kleb.No.	OD.Mean	control	2*control	4* control	feature
1	0.14125	0.0624	0.1248	0.2496	moderate
2	0.339	0.0624	0.1248	0.2496	strong
3	0.44225	0.0624	0.1248	0.2496	strong
4	0.14475	0.0624	0.1248	0.2496	moderate
5	0.31575	0.0624	0.1248	0.2496	strong
6	0.2865	0.0624	0.1248	0.2496	strong
7	0.15325	0.0624	0.1248	0.2496	moderate
8	0.14775	0.0624	0.1248	0.2496	moderate
9	0.347	0.0624	0.1248	0.2496	strong
10	0.44525	0.0624	0.1248	0.2496	strong
11	0.44575	0.0624	0.1248	0.2496	strong
12	0.14325	0.0624	0.1248	0.2496	moderate
13	0.4445	0.0624	0.1248	0.2496	strong
14	0.13925	0.0624	0.1248	0.2496	moderate
15	0.136	0.0624	0.1248	0.2496	moderate
16	0.2695	0.0624	0.1248	0.2496	strong
17	0.1485	0.0624	0.1248	0.2496	moderate
18	0.443	0.0624	0.1248	0.2496	strong
19	0.30175	0.0624	0.1248	0.2496	strong
20	0.14525	0.0624	0.1248	0.2496	moderate
21	0.443	0.0624	0.1248	0.2496	strong
22	0.5215	0.0624	0.1248	0.2496	strong
23	0.34375	0.0624	0.1248	0.2496	strong
24	0.13975	0.0624	0.1248	0.2496	moderate
25	0.42325	0.0624	0.1248	0.2496	strong

الخلاصة

اما بالنسبة لمضادات الاكسدة فقد اظهرانزيم Superoxide dismutase (SOD) انخفاضا معنويا في مرضى قرحة القدم السكري بمعدل U\L (241.4±35.61) مقارنة مع مرضى السكر من النوع الثاني والاصحاء واخيرا فان مستوى الكلوتاثيون (GSH) ابدى انخفاضا معنويا في مرضى قرحة القدم السكري و بمعدل (26.586±2.77) µmole\ml مقارنة مع مرضى السكر من النوع الثاني و الاصحاء.

و استنتجت الدراسة ان بكتريا *S.aureus* هي البكتريا الموجبة لصبغة كرام الشائعة في إصابات القدم السكري وان بكتريا *K.pneumoniae* هي السالبة لصبغة كرام الشائعة في تلك الإصابات و ان النوعين المذكورين مكونات للغشاء الحيوي و مقاومات لمعظم المضادات الحياتية المعروفة.

الخلاصة

Cefotaxim, Ceftriaxone, Imipenem, و Oxacillin كانت بنسبة 40% وكانت حساسيتها باتجاه المضادين Rifampin و Vancomycin بنسبة 52% اما حساسيتها باتجاه المضاد Ciprofloxacin فقد كانت 48%. و من جانب اخر , فان بكتريا *Klebsialla pneumoniae* أظهرت اعلى مقاومة (100%) تجاه المضادين Gentamycin و Levofloxacin يلي ذلك مقاومتها للمضاد Ciprofloxacin بنسبة 96% وكانت مقاومتها لمضاد Amikacin بنسبة 92% اما بالنسبة لمضادات Pipracillin-Tazobactam, Cefotaxim, Ceftriaxone, و Tetracycline فقد كانت مقاومتها بنسبة 88% و قاومت هذه البكتريا مضادات Amoxicillin-Clavulanic acid, Cetazidime و Cefepime بنسبة 84% فضلا عن مقاومتها للمضادين , Imipenem Meropenem بنسبة 76% و أخيرا قاومت هذا البكتريا مضاد Doxycycline بنسبة 68% و مضاد Rifampin بنسبة 56%.

تضمنت الدراسة الحالية الكشف عن انتاج العزلات البكتيرية للغشاء الحيوي بطريقة الانبوبة واتضح ان جميع العزلات المتحصل عليها من قرحة القدم السكري والتي تعود لنوعي *S.aureus* و *K.pneumoniae*كانت منتجة للغشاء الحيوي بينما اسفر التقدير الكمي لانتاج الغشاء الحيوي باستخدام طريقة المايكروتايتر ان 60% من كلا النوعين كانت قوية الإنتاج بينما 40% كانت متوسطة الإنتاج للغشاء الحيوي.

باستخدام تفاعل البلمرة المتسلسل PCR تم الكشف عن وجود جين *mec*A في 60% من عزلات بكتريا *S.aureus* و اتضح ان 64% من هذه البكتريا كانت حاوية على جينات *ica*A و *sea* و *erm*C و أخيرا فان 80% من هذه البكتريا كانت حاوية على جين *ica*D اما بالنسبة لبكتريا *K.pneumoniae* فان 88% منها تضمنت جين *mrk*D و 100% منها كانت حاوية جين *Cps* و 12% منها تضمنت جين *K1* وأخيرا فان 60% منها كانت حاوية على جين *K2A*.

أظهرت الدراسة الحالية ارتفاعا معنويا في مستويات TLR-2 في مرضى قرحة القدم السكري وبمعدل (7.36 ±1.85) ng\ml مقارنة مع مرضى السكر من النوع الثاني والاصحاء وأيضا هناك ارتفاع معنوي في مستوى IL-17A في مرضى قرحة القدم السكري وبمعدل (123.7±33.52) ng\L عند مقارنته بمرضى السكر من النوع الثاني و الاصحاء بينما كان مستوى C-Reactive protein (CRP) مرتفع بشكل معنوي في مرضى قرحة القدم السكري و بمعدل (92.9±78.26) mg\L مقارنة مع مرضى السكر من النوع الثاني والاصحاء.

الخلاصة

تضمنت هذه الدراسة عزل وتشخيص اهم الانواع البكتيرية المسببة لتلوث القدم السكري فضلا عن اجراء اختبار الحساسية لهذه الأنواع ضد اهم المضادات الحياتية المتوفرة وتضمنت الدراسة أيضا التحري عن انتاج تلك الأنواع البكتيرية للغشاء الحيوي مظهريا وأخيرا تم الكشف الجزيئي لبعض جينات الضراوة والمقاومة للمضادات الحياتية باستخدام تقنية تفاعل البلمرة المتسلسل فضلا عن تقدير مستويات بعض المؤشرات الكيموحيوية والمناعية للمرضى قيد الدراسة.

تم جمع 142 مسحة من مصابي قرحة القدم السكري المراجعين في مركز الامام الحسن (ع) للغدد الصم والسكري وتم زرع تلك المسحات على الأوساط الملائمة و اجراء الاختبارات البكتريولوجية لها فضلا عن جمع 120 عينة دم وتقسيمها الى ثلاث فئات : 40 من فئة مصابي قرحة القدم السكري مع داء السكر من النوع الثاني و 40 من مصابي داء السكر من النوع الثاني و 40 كانوا من الاصحاء.

من بين 142 مسحة تبين ان 74 (62.18%) كانوا من الذكور بينما 45 (37.18%) كانوا اناثا و تم الحصول على اكبر عدد من العزلات البكتيرية بنسبة (27.7%) من الفئة العمرية (64 -55) سنة بينما كان العدد الأقل من العزلات البكتيرية يعود الى المرضى في سن (≥75) سنة بواقع 18(15%) عزلة فقط. اسفرت عملية زرع المسحات عن الحصول على 23 زرع سالب بدون أي نمو بينما كانت النسبة الأعلى من العينات متعددة الاحياء المجهرية بواقع 82% و العينات ذات الكائن المجهري المفرد بنسبة 18%. ومن بين هذه العزلات فان 63 (44.4%) كانت موجبة لصبغة غرام و 56 (39.4%) منها كانت سالبة لصبغة غرام وبعد اجراء الاختبارات الكيموحيوية والمجهرية اتضح ان 25 (21%) تعود لجنس *Staphylococcus aureus* , *Staphylococcus epidermidis* 18 (15%), *Streptococcus agalactiae* 15 (13%), *Enterococcus faecalis* 5 (4%), *Klebsiella pneumoniae* 25(21%), *Pseudomonas aeruginosa* 13 (11%), *Proteus mirabilis*10 (8%) وأخيرا فان 8 عزلات (7%) كانت تعود لبكتريا *E.coli*.

تم اجراء اختبار الحساسية لبكتريا *S.aureus* و *Klebsiella pneumoniae* و اتضح ان عزلات بكتريا *S.aureus* أظهرت اعلى حساسية (84%) تجاه مضاد Levofloxacin بينما كانت اقل حساسية (36%) تجاه المضادين Azithromycin و Erythromycin و أظهرت نتائج الاختبار ان حساسية هذه البكتريا تجاه مضادات Penicillin , Amoxicillin-Calvulonic Acid, Ceftazidime, Cefepime, Meropenem, Aztronem

الخلاصة

جــــــــامعة كـــــربلاء

كلية العلوم

قسم علوم الحياة

التوصيف الجزيئي للبكتريا السائدة المكونة للغشاء الحيوي و تحديد بعض العوامل الكيموحيوية و المناعية المرافقة لاصابة القدم السكري

رسالة مقدمة

الى مجلس كلية العلوم / جامعة كربلاء

وهي جزء من متطلبات نيل درجة الماجستير في علوم الحياة

من قبل :

ساره محمد محسن

بكالوريوس علوم حياة – جامعة كربلاء ٢٠١٤

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كانون الثاني ٢٠٢٤ م **جمادي الآخر ١٤٤٥هـ**