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Scientific Research
University of Babylon
College of Medicine

Immunogenic Properties of Capsular Polysaccharides (CP5) against Methicillin Resistant *Staphylococcus aureus* in Rats

A Thesis

Submitted to the Council of the College of Medicine, University of Babylon, in Partial Fulfillment of the Requirements for the Doctorate Degree of Philosophy in Science / Medical Microbiology

By

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وَعَلَّمَكَ مَا لَمْ تَكُنْ تَعْلَمُ ۚ وَكَانَ فَضْلُ اللَّهِ عَلَيْكَ عَظِيمًا

صدق الله العلي العظيم

سورة النساء – من الآية 113

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Amel 2021

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Examination Committee Certificate

We, the examiner committee, certify that we have read this thesis entitled **"Immunogenic Properties of Capsular Polysaccharides (CP5) against Methicillin Resistant *Staphylococcus aureus* in Rats"** and have examined the student (**Amel Mohamed-Saed Mohamed-Jawad Al-Musawi**) in its content, and that in our opinion it is accepted as a thesis for the degree of by **Doctorate of Philosophy** of Science in Medical Microbiology with **Excellent** estimation.

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List of Abbreviations

Abbreviation	Meaning
AD	Atopic dermatitis
Agr	Accessory gene regulater system
AmpC	Molecular class C β-lactamases
APCs	Antigen-presenting cells
AST	Antibiotic susceptibility testing
B cell	Bone marrow cell
BSA	Bovine Serum Albumin
BSI	Blood stream infection
CA-MRSA	Community-acquired MRSA
CHIPS	Chemotaxis-inhibitory protein of *S. aureus*
CHIMs	Controlled human infection models
ClfA	Clumping factor A
CLRs	C-type lectin receptors
CLSI	Clinical and Laboratory Standards Institute
CoNS	Coagulase-negative Staphylococci
CRM197	a nontoxic recombinant mutant of diphtheria toxin
CPSs	capsular polysaccharides
CP5	Capsular polysaccharides 5 gene
CP8	Capsular polysaccharides 8 gene
CSF	Cerebrospinal fluid
DD test	Disc diffusion test
DCs	Dendritic cells
D.W	Distalalled water
Eap	Extracellular adherence protein
EDTA tubes	Ethylene di-amine tetra acetic acid tubes
ELISA	Enzyme Linked Immune Sorbent Assay
ESBL	Extended-spectrum beta-lactamase
Esx	Secretion system protein
FnbB	Fibronectin binding protein B
FnBPA	A domain of fibronectin-binding protein A
ETs	Exfoliative toxins
EU	Europe
G	Times gravity
GBS	Group B Streptococcus
Glycanp	Carbohydrate portion
GSK	GlaxoSmithKline
HA-MRSA	Hospital-associated MRSA
HEK-TLR2	TLR2-transfected human embryonic kidney cells
Hib	*Haemophilus influenzae* type B

Hla	α-hemolysin
HlaH35L	detoxified α-hemolysin
HRP enzyme	Horseradish peroxidase enzyme
ICU	Intensive care unit
ID	Identification
IFN-γ	Interferon-γ
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IL	Interleukin
ILC	Innate lymphoid cells
IL-4R	IL-4 receptor
LPS	Lipopolysaccharied
IsdB antigen	Iron-regulated surface determinant antigen
LukED	Leucocidin
LukSPV	Panton Valentine Leukocidin *Staph*
MAbs	Monoclonal antibodies
MDR	Multidrug-resistant
MenC	Meningococcal group C
*mec A*gene	Methicillin resistant *S. aureus A* gene
MGEs	Mobile genetic elements
MHCII	Major histocompatibility complex class II
MIC	Minimum Inhibitory Concentrations
MntC	Manganese transport protein C
MRSA	Methicillin resistant *S. aureus*
MSCRAMM	Microbial Surface Components Recognizing Adhesive Matrix Molecules
NDV3	NovaDigm Therapeutics
NIAID	National Institute of Allergy and Infectious Diseases
NLRs	NOD-like receptors
NMR	Nuclear magnetic resonance
NOD	Nucleotide oligomerization domain
NTHi	Nontypeable *Haemophilus influenzae*
OD	Optical density
OMPC	Outer membrane protein complex
PAMPs	Pathogen-associated molecular patterns
PBP2a	Penicillin Binding Protein 2A
PCR	Polymerase chain reaction
PDR	Pandrug-resistant
PGE2	prostaglandin E2
POC	proof of concept
PRRs	pattern recognition receptors
PVL	Panton-Valentine leukocidin

P –value	Probability –value
RIG-1	Retinoic acid-inducible gene (RIG)-I-like RNA helicases
rAls3p-N	Recombinant antigen (agglutinin like sequence 3 protein)
rAT/r rLukS-PV	Recombinant α-toxin and Panton Valentine Leukocidin vaccine
Rseb	Recombinant enterotoxin B
RPM	Centrifuge rotor speed
RP	Red pulp
SA4Ag	*S. aureus* vaccine 4Ag
SAE	*S. aureus* enterotoxin
SD	Standard deviation
SdrE, IsdA, SdrD, IsdB	A multicomponent surface protein
STEBVax	*S. aureus* enterotoxin B Vaccine
SpA	D domain of staphylococcal protein A
SPSS	Statistical Package for Social Science Software
StaphVAX	*S. aureus* vaccine
STAT3	Signal transducer and activator of transcription 3
STAT6	Signal transducer and activator of transcription 6
STRIVE	*Staphylococcus aureus* surgical In patient Vaccine efficiency
Th1	T helper type 1 cells
TLCs	Total Leukocytes Count
TLRs	Toll-like receptors
TNF- α	Tumor necrosis facter- α
TSST-1	Toxic shock syndrome toxin -1
TSLP	Thymic stromal lymphopoietin
TYK2	Tyrosine kinase 2
USUHS	Uniformed Services University of the Health Sciences
VRSA	Vancomycin-intermediate and resistant *S. aureus*
WBCs	White blood cells
WHO	World health organization
WP	White pulp
XDR	Extensively drug resistant

Dedication

I dedicate this work,

To the lord of all creatures (Allah)

To The messenger of peace

(The Prophet Mohammad and Kinsfolk Peace be upon them)

To the symbol of generosity (My Father and my Mother soul)

To my loving……(My brother soul and my sister soul)

To my dear supporters (My brother and my sister)

To the all symbol of martyrdom in Allah guideline

Amel
June, 2021

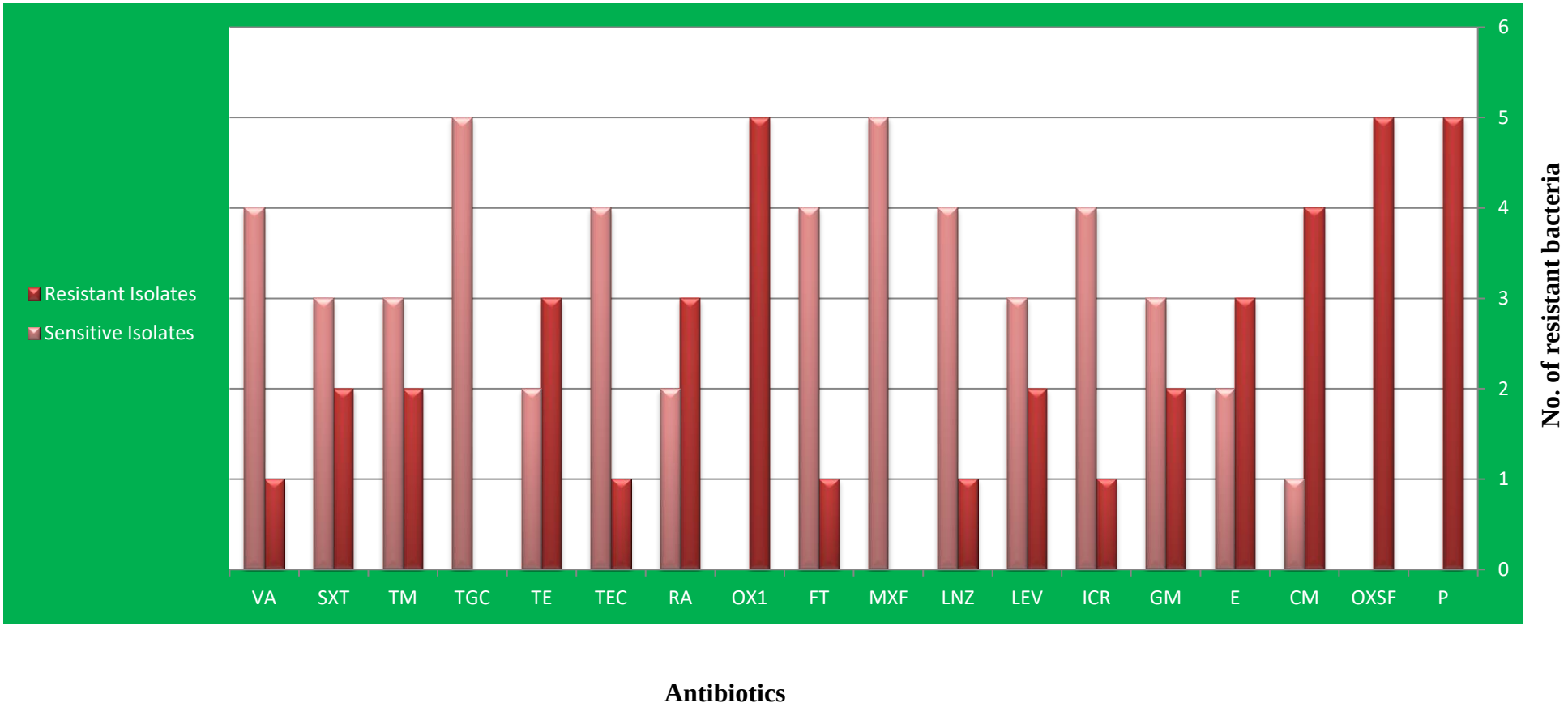


Figure 3-2: Antibiotic susceptibility pattern of MRSA isolated recovred from clinical samples.

VA Vancomycin, SXT Trimethoprim/Sulfamethoxazole, TM Tobramycin, TGC Tigecycline, TE Tetracycline, TEC Teicoplanin, RA Rifampicin, OX1 oxacillin, FT Nitrofurantion. MXF Moxifloxacin, LNZ Linezolid, LEV Levofloxacin, ICR inducible clindamycin resistance, GM Gentamicin E Erythromycin, CM Clindamycin, OXSF Cefoxitin screen, P benzylpenicillin.

Summary:

The present study aimed to investigate active immunization strategies using capsular polysaccharides (CP5) in protection against *Staphylococcus aureus* clinical isolates in animal models of infection in order to provide protection against various Methicillin Resistant *Staphylococcus aureus* in the acute fatal bacteremia model.

A total of 180 different clinical samples were collected from patients who admitted to the main to hospital in Babylon province; Hilla teaching hospital and Babylon teaching hospital for maternity and children in Al-Hilla city during a period from January till November 2020. Samples obtained included; blood, urine, cerebrospinal fluid, burns, wounds, and others (bronchial fluid, abscess).

Among 180 clinical samples, 46 (25.5%) gave positive growth on ordinary culture media while 134 (74.5%) gave no growth,46 (25.5%) gave positive growth on ordinary culture media, yielding 46 bacterial isolates, identified using biochemical tests, Vitek 2-system ID cards, and Analytical Profile Index (API) system.

Results also revealed that out of 46 culture positive samples recovered from patiants, high rate of isolation 16(34.7%) was found in urine samples, followed by 15 wounds (burns) samples (32.6%), blood 12 (26. 1%), Abscess 2 (4.3%), while only one sample (2.1%) from bronichal fluid showed positive growth.

Among 46 positive cultures of clinical samples, 22 (47.82%) were found to be Gram-positive and 24 (52.17%) as Gram-negative bacteria. Among 22 Gram-positive bacterial isolates 17 bacterial isolates belonged to Coagulase-negative Staphylococci (CoNS) and 5 were identified as *S. aureus*.

All 5 *S. aureus* isolates were tested for methicillin resistance using cefoxitin as indicator of MRSA as an initial screening test according to the guidelines of CLSI. Molecular detection of Staphylococcal cassette Chromosome ; *mecA* and

capsular polysaccharides (*CP5*) genes revealed that 5 isolates *S.aureus* carrying *mecA* gene and one isolate (A.MS isolate) had capsular polysaccharides (*CP5*) gene. Isolation, extraction and partial purification of capsular polysaccharide 5 (CP5) from local methicillin resistant *S. aureu*s isolates (A.MS isolate), the purification of CPs was carried out by previous methods, with modifications and mixture. As the first study in Iraq.

Chemical composition of the partial purified extract of *S. aureus* capsular polysaccharide 5 (CP5) from A.MS isolate was measured by UV- visible Specterophotometer. The results found that the absorption of CP5 at 206 nm was 0.279;Conc. 6 μg/ml , the absorption of protein (0.017) ;Conc. 3.4 μg/ml at 280 nm, and the absorption of Nucleic acid content (0.018) ;Conc. 3.6 μg/ml at 260 nm. Concentration of the partial extract of *S. aureus* capsular polysaccharide 5 (CP5) was 6 mg/L.

The present study aimed to investigate active immunization strategies using capsular polysaccharides (CP5) was emulsify with polysorbate 80(Tween-80) in animal models.

In the experimental group rats into *S. aureus* capsular polysaccharide (CP5) – Tween 80 group I (8 Female Rats treated intraperitoneally with *S. aureus* CP5 - Tween80 in two dose each dose 50 μg (10 μl) per animal, 7 days between first and second dose) and the control test group II (8 Female Rats treated intraperitoneally with phosphate buffer saline in two dose each dose (10 μl) per animal. Seven days between first and second dose).

After 3 weeks, then the rats were sacrificed and blood collected for hematological and serological analysis before challenge dose.

At 30 days after the first immunization for CP5, and control ; 16 rats divided into two groups (with 8 rats for each one) were challenged intraperitonially with a 0.5 ml inoculum containing 10^7 CFU *S. aureus* with 0.5 ml. The relative degree

of protection afforded by antigens was assessed by the number of rats surviving 7 days after infection, then the rats were sacrificed and blood samples were collected for blood culture, hematological, and immunological analysis, within 72 hours after the injection.

Blood samples were collected from all animals by heart puncture under general anesthesia (Chloroform) to detect total and differential leukocytes, bactericidal power of blood., interleukin-4 (IL-4) and., interleukin-12P40 (IL-12P40) were detect by ELISA technique, before and after challenge dose.

The hematological and immunologicall analysis results, before challenge dose; the results of Total Leukocytes Count (TLCs**)** among testing groups indicated not significant increasing the mean value in group I (11093 cells/mm^3) as comparing with group II (9650 cells/mm^3), at ($p < 0.05$) The results of differential leukocyte count indicated not significantly the percentage of two groups at ($p < 0.05$) level.

The levels of Interlukin-4 in the serum before challenge dose of animals groups appears significant increase in group I (4.775pg/mL), in comparison with group II (0.709 pg/mL), at ($p < 0.05$).

The results indicated not significant increase of Interluekin-12p40 level in the serum before challenge dose of animals groups ,in group I (7.496 pg/mL) as compared with group II (12.115 pg/mL), at ($p < 0.05$).

The results of the bactericidal power of blood from the viable bacteria *S. auerus* (MRSA) revealed that the rat animals that injected with viable MRSA (14 animals), were divided into two groups as described in chapter two. were blood culture done to assess the clearance percentage of blood to detect the effects of *S. aureus* capsular polysaccharide (CP5), on survive the viable bacteria (MRSA) in blood stream. The percentage of bactericidal power in group I was higher (71.5%) as compare with group II was (33.4%).

The hematological and serological analysis results, after challenge dose; The results of Total Leukocytes Count (TLCs**)** among testing groups indicated significant increasing the mean value in group I (9407cells/mm^3) as comparing with group II (4682 cells/mm^3) (The *P –value* < 0.05).

The levels of Interlukin-4 in the serum of animals groups after challenge dose appears indicated a significant increase in group I (8.6676pg/mL) in comparison with group II (2.8531 pg/mL) at ($p < 0.05$).

The results in the serum of animals groups after challenge dose indicated to not significant increase of Interluekin-12p40 level in group I (10.483 pg/mL) as compared with group II (5.792 pg/mL), at level ($p < 0.05$).

Furthermore, the histopathological examination results of spleen slices White pulp enlargement with lymphoid follicular changes, white pulp is diffused with red pulp, control group shrinks, eosinophils, plasma cells and lymphocytes infiltrate the cross section ,while the spleen slices of group I appeared, showing partial changes in the white pulp of the slices of the group, there were lymphoid follicles group I ,while the spleen slices of group II (control group) there is no change in the two spleen areas. The results are related to the effect of the conjugate of *Staphylococcus aureus* capsular polysaccharide (CP5) which stimulates the immune response against antigens entering the body.

We can conclude from the present study that the CP5(extracted from A.MS *S. aureus* isolate) showed improvement of the immune responses against MRSA antigen via enhance the levels of humoral immunity elements as well as histopathological changes that on spleen activity that stimulate immune responses against antigens that enter the body.

Introduction:

The emergence of antimicrobial resistance in *Staphylococcus aureus* posed a major veterinary and public challenge worldwide. *S. aureus* being a highly versatile pathogen can quickly acquire resistance genes. The development of resistance in bacteria predates the era of antibiotic use. However, resistance developments in *S. aureus* have been reported since the early 1940-ties, when penicillin resistant *S. aureus* was first reported. Ever since, this pathogen has gain global notoriety as the most common cause of nosocomial, community and livestock associated infection. The mechanism of resistance development in bacteria complicated the integration of a complex systems that included the efflux pump, alteration of drug target site, enzymatic inactivation and, mutation in drug target site and gene acquisition of resistance determinants through horizontal gene transfer (Bitrus *et al* ., 2018).

Many factors have been implicated in the development of antibiotic resistance, such as over and misuse of antibiotics mostly in developing countries; however, biofilm mediated drug resistance in bacteria is another major mechanism and it has been predicted that if the current treatment training continues unchanged, the infections caused by antibiotic-resistant bacteria would be a major cause of death in 2050 where the projected number of deaths will be around 10 million every year (O'Neill, 2016).

Methicillin resistant *S. aureus* (MRSA) representative a major problem. MRSA infections have increased levels of mortality, hospital stay, septic shock and subsequent infections. MRSA infections account for over 94,000 cases and 18,000 deaths annually in the United States and while estimates of its economic impact vary, it accounts for billions of dollars in expenditure in the United States as well as other countries (Uematsu *et al*.,2017) . Given its importance, the development of a vaccine and new antimicrobials to *S. aureus* is of high importance (Parker ,2018).

Both active and passive immunizations of *S. aureus* have been attempted, and all clinical trials have failed. These trials were based upon increased opsonophagocytic antibodies in animal models (mostly rodent) and in humans (Proctor, 2015).

Approximately 94% to 100% of *S. aureus* contains Capsular polysaccharides (CPs), and 85% of *S. aureus* CPs consists of CP5 and CP8 . Capsular polysaccharides are O antigens, which are low in molecular weight and immunogenicity. Furthermore, when united with proteins/nucleoprotein proteins or lipopolysaccharides, CPs can induce the production of anti-polysaccharide antibodies, thereby significantly enhancing immunogenicity (Li *et al* .,2018)

That study makes an interesting and important contribution to the future development of preventative therapies to *S. aureus* infection. Both previous and current major vaccine efforts and trials to *S. aureus* have been using protein and polysaccharide subunit vaccines. This study highlights the potential use of a live attenuated strain of *S. aureus* in the prevention of this important pathogen(Parker , 2018).

The Aim of this Study:

Aim and objectives

The aim of this study is to investigate active immunization strategies using capsular polysaccharides (CP5) in protection against *Staphylococcus aureus* clinical isolates in animal models of infection in order to provide protection against various MRSA in the acute fatal bacteremia model. thus, the administration capsular polysaccharides may direct the immune responses versus targets on the surface of bacteria and makes it more vulnerable against

immune responses and may be thereby increases the efficacy of immunogene in animal model.

This aim was achieved using the following objectives:

1.Isolation and identification of *Staphylococcus aureus* isolates recovered from different clinical samples in Babylon Province.

2. Determination of the antibiotic susceptibility testing of bacterial isolates using disk diffusion method and Vitek2 system according to CLSI guidelines.

3 Phenotypic detection of antibiotic resistance pattern among *Staphylococcus aureus* isolates .

4. Phenotypic and genotypic detection *Staphylococcus aureus* resistant to Oxacillin and cefoxitin, this strain must contain *mec A* gene to determine MRSA.

5. Genotypic detection *Staphylococcus aureus* ,this strain has contain capsular polysaccharides (CP5) gene.

6. Extraction and Partial purification of *Staphylococcus aureus* capsular polysaccharides (CP5) .

7. using *Staphylococcus aureus* capsular polysaccharides (CP5) as immunogen to evaluate the efficiency stimulation of immune response in laboratory animals; Albino Swiss rats (*Rattus norvegicus*). Studying the effect of the CP5 *Staphylococcus aureus* -Tween80 on immune response by using some immunological parameters :

A. Total and differential count of Leucocytes.

B. cytokines levels measureing (IL-4 ,IL-12 –p40).

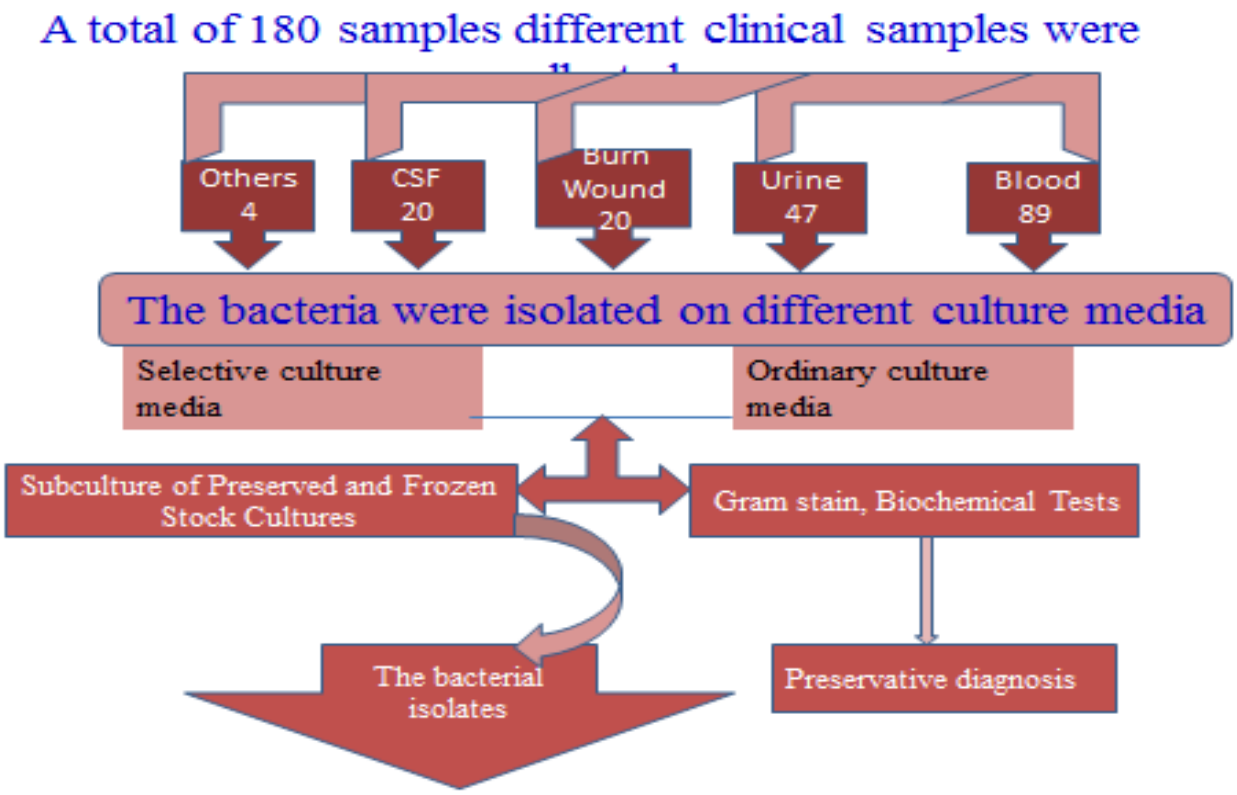
8. Detecting the bactericidal power(colonies forming unite) of blood in immunized laboratory animals.

9. Studying the histopathological changes in different spleen tissue of laboratory animals.

2- Materials and Methods

2.1 Study design, Patients, and Samples:

This study included two types of study design. Cross sectional study was used for studying human samples while case control was used for animal models study of the interface was achieved to investigate the active immunization strategies using capsular polysaccharides (CP5) in protection against *Staphylococcus aureus* clinical isolates in animal models of infection in order to provide complete protection against various MRSA in the acute fatal bacteremia model. thus, the administration capsular polysaccharides may direct the immune responses versus targets on the surface of bacteria and makes it more vulnerable against immune responses and may be thereby increases the efficacy of immunogene in animal model.



A total of 180 different clinical samples were collected from patients who admitted to Hilla teaching hospital , Babylon teaching hospital for maternity and

children in Al-Hilla city and Imam Al-Sadiq Teaching General Hospital. during a period of January till November 2020 .Exclusion criteria included the clinical samples taken from previously hospitalized individuals; and also from those who had received antibiotic therapy for more than seven days.

2.2 Ethical Approval

The study was conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki. It was carried out with patients verbal approval before sample was taken. The study protocol and the patients consent forms were reviewed and approved by the committee on publication ethics at college of Medicine, University of Babylon, under the reference No. BMS/0258/016.

2.3 Laboratory Animals

Sixteen of Albino Swiss females rats (*Rattus norvegicus*) supplied by College of Pharmacy / Karbala University, their ages were 8-9 weeks, and their weight 160-185 g were used to carry out the investigations of the present study in animal house of the College of Veterinary Medicine / Al-Qasim Green University, in which the temperature was 30 ±5°C, and a light: dark periods of 10:14 hours/day. The animals were housed in plastic cages with hard wood chips for bedding in an air- conditioned room and maintained on special pellets diet with free access to water during all experiments. Then they were given week time to get acclimatized with the laboratory condition before experimentation.

2.4 Materials:

The laboratory apparatus and equipment, chemical, biological materials, commercial kits, and culture media used with is study are illustrated in Table 2-1, 2-2, 2-3, 2-4 and 2-5 respectively.

2.4.1 Laboratory Instruments and Equipment:

The instruments and equipment used throughout this study are listed in Table 2-1.

Table (2-1): Laboratory instruments and Equipment

No.	Item	Company/Country
1	Autoclave	Stermite / Japan
2	Bact Alret System	BioMérieux /France
3	Benzen burner, Water bath, Incubator, Oven	Memmert / Germany
4	Centrifuge	Gemmy/Taiwan
5	Cooling Centrifuge, Incubator, Water Bath and Distillater, Eppindrof Tube (Different Size)	Memmert /Germany
6	DensiChek	BioMerieux R/France
7	Densitometer	BioMerieux R/France
8	Different size of millipore filter (0.22 µm, 0.45 µm) , 50 ml- Screw capped bottles, Wooden sticks	Proway /China
9	Digital camera	Sony/Japan
10	Distillator (Water distiller)	Cruma (USA)
11	EDTA tubes (ethylene di-amine tetra acetic acid (EDTA)) tubes	AFCO / Jordan
12	Electrophoresis and UV Transilluminator	Cleaver /U.K.
13	Gel tubes	AFCO / Jordan
14	Glass Slides	Beroslide /Germany
15	Glass beakers 50ml., 250ml., 500ml.,1000 ml., Conical flasks 250ml., 500 ml.	Hirschmann/ Germany
16	Graduated cylinders 500ml,1000ml	Hysil / U.K

17	Inoculating loop, forceps, PH indicator strips	Himedia / India
18	Lab safety cabinet	Cruma (USA)
19	Latex gloves, facial mask, head cap	Unimed / K.S.A.
20	Light microscope	Olympus / Japan
21	Microcentrifuge	Hettich /Germany
22	Micropipette, Microtiter plate	Dragon / USA
23	Pasture pipettes	Hirschmann/Germany
24	Pellicon cassette system (Millipore) with a membrane with cutoff of 10.000 Daltons	Sigma/ USA
25	Petri dishes	Dolphin Syria
26	Plastic Test tubes 10ml.	AFCO / Jordan
27	Refrigerator, Deep freezer	Concord/ Lebanon
28	Sensitive Electronic Balance	A and D / Japan
29	Sterile swab for streaking	Lab.Service / China
30	Syringes, Cotton	Babylon factory/Iraq
31	Thermocycler Apparatus	Agilent /U.S.A
32	UV- visible Specterophotometer	Shimadzu / Japan
33	Vitek 2 Compact Autoanalyzer	BioMerieux R/France
34	Vortex	Thermolyne /U.S.A

2.4.2 Chemical and Biological Materials:

The chemical materials used in this work are shown in Table (2-2)

Table 2-2: Chemical and biological materials

No.	Chemicals and biological materials	Company/country
1	Agar-agar	Himedia / India
2	Agarose	Bioneer/ Korea
3	Chloroform anesthesia	Sigma –Aldrich/ USA
4	Deionized water	Bioneer/ Korea
5	Deoxyribonucleate 5 oligonucleotido hydrolase (DNAase)	Promega/ USA
6	DNA Marker (100 bp Ladder with Loading Dye)	Promega/ USA
7	Ethidium bromide	Sigma/ USA
8	Ethanol (absolute and 70%), chloroforam	Fluka chemika/ Switzerland
9	Glycerol.	B.D.H / England
10	H_2O_2 3%	Tiba / Iraq
11	Lysozyme	Bioneer/ Korea
12	Maxime PCR PreMix(Master Mix)	Bioneer/ Korea
13	McFarland tube standard (0.5)	Mastgroup/England
14	N,N,N,N Tetramethyl-para-phenylene diamine dihydrochloride,	B.H.D / England
15	Normal saline specific for Vitek (PH=5.0-7.0;[Nacl]=0.45), ordinary normal saline.	Mexico /USA
16	Nuclease free water	Bioneer/ Korea
17	Phosphate buffered Saline(PBS Tablets)	Packed in Canada
18	Plasma solution	Blood bank
19	Proteinase K	Bioneer/ Korea
20	Ribonuclease (RNAase)	Geneaid/ U.K

21	Sodium Chloride	Turkey
22	Tris-Borate-EDTA Buffer (TBE buffer)	Promega/ USA
23	Tween 80 (poyxethyleneasorbate)	Sigma –Aldrich/ USA

2.4.3 Culture Media:

The culture media used in this work are shown in Table 2-3.

Table (2-3): Culture media used in this study

No.	Item	Company/Country
1	Blood agar base	Himedia / India
2	Brain heart infusion broth	
3	MacConkey agar	
4	Mannitol salt agar	
5	Nutrient agar	
6	Tryptic soy agar	Biolife/ Italia
7	Tryptic soy broth	

2.4.4 Antibiotics

2.4.4.1 Gram-Positive Antibiotic Cards by Vitek 2 Compact:

Table (2-4) Antibiotic cards by Vitek 2 compact used in the present study

Table (2-4): Vitek 2 AST P580 (Gram-Positive Susceptibility Card)

Antibiotic	Code	Con. of antibiotic	MIC interpretation		Reference
			Sensitive (≤ µg/ml)*	Resistant (≥ µg/ml)*	
Benzyl penicillin	P	0.125,0.250	0.03	0.5	BioMerieux® (France)
Cefoxitin Screen	OXSF	6	NGE	POS	
Clindamycin	CM	0.5,1,2	0. 5	4	
Erythromycin	E	0.25,0.5,1	0.5	8	
Fosfomycin	FOS	8,32	8	128	
Fusidic acid	FA	0.5,1,4	0.5	32	
Gentamicin	GM	8,16,64	4	16	
Inducible Clidamycin Resistance	ICR	CM0.5,CM/ E0.25/0.5	NEG	POS	
Levofloxacin	LEV	0.25,2,8	1	4	
Linezolid	LNZ	0.5,1,2	4	8	
Moxifloxacin	MXF	0.25,2,8	0. 5	2	
Mupirocin	MUP	1	2	8	
Nitrofurantion	FT	16,32,64	4	16	
Oxacillin	OX1	0.5,1,2	2	4	
Rifampicin	RA	0.25,0.5,2	1	4	
Teicoplanin	TEC	1,4,8,16	0.5	32	
Tetracycline	TE	0.5,1,2	4	16	

Tigecycline	TGC	0.25,0.5,1	0.12	2	
Tobramycin	TM	16,32,64	1	16	
Trimethoprim/ Sulfamethoxazole	SXT	8/152,16/304,32/608	2/38	4/76	
Vancomycin	VA	1,2,4,8,16	2	16	

***The values between brackets indicate the break-points recommended by CLSI, 2020

2.4.5 Prepared Kits:

Table (2-5): Prepared kits

Kit Types	Manufacturer (Origin)
Analytical Profile Index API Staph strips ViteK 2 system cards (ID)	BioMerieux (France)
ViteK 2 system cards (ID	
Vitek 2system AST Gram positive (AST-P580)	
Gram stain	AFCO/Jordan
Geneaid Genomic DNA Isolation Kit	Geneaid/ Korea
Interleukin-12 /p40(IL-12/p40)	Elabscience / USA
Interleukin - 4 (IL-4)	Elabscience / USA

2.5 Specimens collection:

2.5. 1. Burn Specimens:

Swabs were collected from burn, wound and abscess infections and placed in transport medium to deliver the sample to the laboratory and then inoculated on Blood agar and MacConkey agar and incubated at 37°C for 24 hrs.

2.5.2 Blood Specimens:

Using aseptic technique by applying Povidone iodine 2% and 70% alcohol at the site of vein puncture, 5 ml venous blood was drawn from the antecubital or femoral vein by the attending nurse. 2.5ml in children and 5ml in adults of blood according rate 1:5 was inoculated directly into blood culture bottles and incubated in Bact Alret System (BioMérieux /France). The positive sample was transferred and inoculated on Blood agar and MacConkey agar and incubated at 37°C for 24 hrs.

2.5.3 Cerebrospinal fluid (CSF) Specimen:

Generally, CSF is collected into three sterile tubes (12.5 ml) which do not contain anticoagulant. The tubes are numbered in the order in which they were collected and are then distributed to the appropriate laboratory for testing. One tube was sent to bacteriological analysis, after centrifugation 5000rmp/5 min, sediment usage to inoculated on different ordinary media then incubated at 37°C for 24 hrs to bacteriological identification.

2.5.4 Urine Specimens:

The patient was requested to collect the midstream urine in the given sterile wide mouthed plastic disposable container following aseptic precautions after cleaning the external genitalia with water and usage adhesive urine collection bag. The sample was inoculated immediately on ordinary culture media then incubated at 37°C for 24 hrs to bacteriological isolation.

2.5.5 Bronchial fluid specimens:

Samples were centrifuged at 5000 rmp/5 min and then inoculated immediately on ordinary culture media and incubated at 37°C for 24 hrs.

2.6.1 Preparation of Reagents:

The following reagents were prepared as described in (MacFaddin, 2000).

2.6.1.1 Catalase Reagent:

Hydrogen peroxide (3%) was prepared and used for detecting the ability of bacteria to produce catalase enzyme (MacFaddin, 2000).

2.6.1.2 Oxidase Reagent:

This reagent was prepared freshly in a dark bottle by dissolving 0.1 gm of tetramethyl *p*-phenyl diamine- dihydrochloride in 10 ml D.W. (MacFaddin, 2000).

2.7.1 Preparation of Buffers and Solutions:

Buffers and solutions which require sterilization were autoclaved at 121°C for 15 minutes. pH of the solutions were adjusted using 1M NaOH or 1M HCl.

2.7.1.1 Normal Saline Solution:

This solution was prepared by dissolving 0.85 gm of NaCl in 90 ml D.W. and further completed to 100 ml with D.W., autoclaved at 121°C for 15 minutes (Forbes *et al*., 2007).

2.7.1.2 Gram Stains Solutions:

The solutions were prepared according to the required microbiological methods. These solutions included: crystal violate, iodine, alcohol, and safranine (Forbes *et al*., 2007).

2.7.1.3Phosphate Buffer Saline

The solutions were prepared according to the required microbiological methods.

2.8.1 Preparation of Culture Media:

2.8.1.1 Synthetic Culture Media:

A group of culture media was prepared according to the instructions of the company and sterilized by autoclaving at 121^{o}C for 15 minutes.

2.8.1.2. MacConkey Agar Medium:

MacConkey agar medium was prepared according to the method recommended by the manufacturing company (Himedia, India). It was used for the primary isolation of most Gram-negative bacteria and to differentiate lactose fermenter from non-lactose fermenter (Forbes *et al*., 2007).

2.8.1.3 Nutrient Agar Medium:

Nutrient agar medium was prepared according to the method suggested by the manufacturing company (Himedia, India). It was used for the cultivation of the bacterial isolates when necessary (MacFaddin, 2000).

2.8.1.4 Nutrient Broth:

This medium was used to grow and preserve the bacterial isolates. This medium was prepared according to the method recommended by the manufacturing company (Himedia, India) (MacFaddin, 2000).

2.8.1.5 Mannitol Salt Agar Media:

Mannitol salt agar media was prepared according to the method suggested by the manufacturing company. It was used as a selective media for the isolation of Staphylococci and differentiation of *Staphylococcus aureus* (MacFaddin, 2000)

2.8.2 Semi-Synthetic Culture Media:

2.8.2.1 Blood Agar Medium:

It was prepared by adding 5 % human blood to previously sterilized blood agar base after cooling the medium to 45 °C and then poured into sterile petri dishes (MacFaddin, 2000).

2.8.2.2 Trypticase Soy Agar Medium

It was prepared according to the method suggested by the manufacturing company.

2.8.2.3 Trypticase Soy broth

Trypticase Soy broth was prepared according to the method suggested by the manufacturing company. It was used as a selective media for the enhancement of *Staphylococcus aureus* capsule production when was prepared by adding 5% NaCl (Cheng *et al*., 2017).

2.9 Isolation of bacteria from Specimens:

The cultivation of bacteria from infections at various body sites is accomplished by inoculating processed specimens directly onto artificial media. The media are summarized(Blood agar and MacConkey agar) and incubation conditions are selected for their ability to support the growth of the bacteria most likely to be involved in the infectious process. To enhance the growth, isolation, and selection of etiologic agents, specimen inoculate are usually spread over the surface of plates in a standard pattern so that individual bacterial colonies are obtained and semi quantitative analysis can be performed. A commonly used streaking technique. Using this method, the relative numbers of organisms in the original specimen can be estimated based on the growth of colonies past the original area of inoculation. To enhance isolation of bacterial colonies, the loop should be flamed for sterilization between streaking each subsequent quadrant. Streaking plates inoculated with a measured amount of specimen, such as when a calibrated loop is used to quantify colony-forming units (CFUs) in urine cultures, is accomplished by spreading the inoculum evenly over the entire agar surface . This facilitates counting colonies by ensuring that individual bacterial cells will be well dispersed over the agar surface.

2.10 Identification of bacterial Isolates :

Morphological Identification:

Its first step for identification of bacterial isolates was made by studying the culture, and macroscopic characteristics of the bacterial isolates like shape, color, and their reaction with Gram stain.

2.10.1 Biochemical tests:

2.10.1.1 Oxidase Test:

This test depends on the presence of certain bacterial oxidases enzyme that would catalyze the transport of electrons between electron donors in the bacteria and a redox dye (tetramethyl-ρ-phenylene-diamine dihydrochloride), the dye was reduced to a deep purple color.

A strip of filter paper was soaked with a little freshly made reagent, and the colony to be tested was picked up with a sterile wooden stick and smeared over the filter paper. A positive result was indicated by an intense deep purple color which appeared within 5-10 seconds (Forbes *et al*., 2007).

2.10.1.2 Catalase Test:

A small amount of bacterial growth was transferred by a sterile wooden stick onto the surface of a clean, dry glass slide, and one drop of 3% H_2O_2 is added to it. The formation of gas bubbles indicated the positive result (Forbes *et al*., 2007).

2.10.1.3 Coagulase Test:

The method of Benson (2001) was followed several colonies of bacterial growth were transferred with a loop to a test tube containing 5ml of brain heart infusion broth. The tube was covered to prevent evaporation and incubated at 37°C for 24 hrs. Then the tube mixed and centrifuged, 0.5ml of the supernatant was withdrawn and mixed with 0.5ml of human plasma, then incubated in the water bath at 37°C for 24 hrs.. If the plasma coagulates, the organism is

coagulase-positive. Some coagulations occurred in 30 minutes or several hours later.

Any degree of coagulation, from a loose clot suspended in plasma to a solid immovable clot, was considered to be a positive result, even if it takes 24 hrs. to occur.

2.10.1.4. Hemolysis Test:

Blood agar medium was streaked with a pure culture of bacterial isolate to be tested and incubated at 37ºC for 24-48 hrs. The appearance of a clear zone surrounding the colony is an indicator of β-haemolysis While the greenish zone is an indicator of α-haemolysis (Forbes *et al*., 2007).

2.10.1.5 Mannitol salt fermentation:

The ability of *S. aureus* to ferment mannitol can be detected *in vitro* on mannitol salt agar plates. The bacterial colony was streaked on mannitol plates and incubated for 24-48 hrs. at 37ºC. The changing color of medium from pink to yellow is an indication for a positive results (Benson, 2001).

2.10.2 Identification Process With Vitek2 System:

In clinical microbiology Vitek®2 used as an auto analyzer system for the identification (ID) and antibiotic susceptibility testing (AST) of the bacteria in clinical samples. However, the samples were achieved according to manufacture instructions as following:

A sterile plastic stick applicator used to take pure colonies from culture media and transfer a sufficient number of them to plastic test tubes. Test tubes contain about 3.0 ml of sterile saline to suspend the microorganism in Concentration of bacterial suspension in saline (0.45%-0.5%, pH 7) was checked by densitometer and was adjusted between the tolerances ranges before introducing the sample to the analyzer. After mixing by shaker in order to produce a homogenous suspension of bacteria, the turbidity of suspension was adjusted by adding proper amounts of saline or bacteria.

The density (turbidity) of the suspension was checked by using a calibrated turbidity meter called the DensiChek. This range for bacteria were between 0.50-0.63 McFarland.Vitek2® ID and AST cards were set up according to instructions given by BioMérieux®. All reagents and equipments needed for processing supplied by Manufacturer Company. All isolates introduced to the computer before processing and inoculated cards were processed in the instrument within 30 min of inoculation.

Identification (ID) and AST cards were loaded (inoculated) with bacterial suspensions using a vacuum chamber in machine. Test tubes containing the samples were placed into a cassette (a special test tube rack) and the identification card was placed in the neighboring place while inserting the transfer tube into the corresponding suspension tube. The cassette could accommodate up to 10 test tubes. The filled cassette was placed into a vacuum chamber station inside the vitek2 analyzer machine. The vacuum was applied then the air was recharged into the station, the bacterial suspension was forced through the transfer tube into micro-channels that filled all the test wells.

Inoculated cards were passed by a mechanism, which cut off the transfer tube and sealed the card prior to loading into the circular incubator. The incubator could accommodate up to 30 cards. All card types were incubated at 35.5 ± 1ºC. Each card was removed from the incubator once every 15 minutes, transported to the optical system for reaction readings, and then returned to the incubator until the next read time. Data were collected at 15-minute intervals during the entire incubation period.

2.10.3 Identification Process with API-Staph:

API Staph is a standardized system for the identification of the genera *Staphylococcus, Micrococcus* and *Kocuria*. This system uses miniaturized biochemical tests and a specially adapted data base. The complete list of those bacteria that it is possible to identify with this system can be found in the identification table at the end of this package insert.

The API Staph strip consists of 20 microtubes containing dehydrated substrates. These microtubes are inoculated with a bacterial suspension, Prepared in API Staph Medium, that reconstitutes the tests. During incubation metabolism produces color changes that are either spontaneous or revealed by the addition of reagents. The reaction are read according to the reading table and identification is obtained by referring to the Analytical Profile Index or using the identification software.

2.11 Preservation and Maintenance of Bacterial Isolates:

The bacterial isolates were preserved on nutrient agar slant at 4˚C. The isolates were maintained monthly by reculturing on new medium. Nutrient broth supplemented with 15% glycerol was used for long preservation and the isolates were maintained frozen at -20°C for several months (long term maintenance) (Forbes *et al*., 2007).

2.12 Subculture of Preserved and Frozen Stock Cultures:

Frozen stock cultures were sub-cultured on fresh blood agar plates, and then incubated in aerobic condition at 37°C for 24 hr. (Thomas, 2007)

Table 2-6: Reading the API Staph strips

Tests	Substrate	Reactions tests	Negative Results	Positive Results
0	No substrate	Negative control	Red	—
GLU	D-glucose	Positive control	—	Yellow
FRU	D-fructose	Acidification	Red	Yellow
MNE	D-mannose	Acidification	Red	Yellow
MAL	D-maltose	Acidification	Red	Yellow
LAC	D-lactose	Acidification	Red	Yellow
TRE	D-trehalose	Acidification	Red	Yellow
MAN	D-mannitol	Acidification	Red	Yellow
XLT	Xylitol	Acidification	Red	Yellow
MEL	D-melibiose	Acidification	Red	Yellow
NIT	Potassium nitrate	Reduction nitrate to nitrites	colorless-light pink	Red
PAL	β-naphthyl phosphate	Alkaline phosphate	Yellow	Violet
VP	Sodiumpyruvate	Voges Proskauer	colorless-light pink	Violat-pink
RAF	D-raffinose	Acidification	Red	Yellow
XYL	D-xylose	Acidification	Red	Yellow
SAC	D-saccharose	Acidification	Red	Yellow
MDG	methyl-αD-glucopyranoside	Acidification	Red	Yellow
NAG	N-acetyl-glucosamine	Acidification	Red	Yellow
ADH	L-arginine	Arginine DiHydrolase	Yellow	orange-red
URE	Urea	URUase	Yellow	red-violat

2.13. Antibiotic Susceptibility Testing

2.13.1 Detection of AmpC β-Lactamase

Phenotypic Detection of Methicillin Resistance

All 5 *S. aureus* isolates were tested for methicillin susceptibility using an initial screening test according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2020) The isolates were initially screened for Cefoxitin screen (Detection of MRSA isolates) to *S. aureus* isolates by Vitek 2 system.

2. 13.1.1 Initial Screening AmpC β-Lactamase (Cefoxitin Susceptibility):

All β-lactam resistant isolates were tested for cefoxitin susceptibility by using standard disk diffusion method (CLSI, 2020). The resistant isolates (≤21mm inhibition zone diameter) were consider as initially AmpC β-lactamase producers.

The distribution of antibiotics (used in Vitek-2 system) according to antimicrobial categories ,are illustrated in Table (2-7).

Table (2-7) Distribution of Antibiotics (used in Vitek-2 AST P580 system) according to antimicrobial categories.

Antibiotic agent	Code	Antibiotic agent categories.
Benzylpenicillin	P	Penicillin
Cefoxitin Screen	OXSF	Cephamycins
Clindamycin	CM	Lincosamides
Erythromycin	E	Macrolides
Fosfomycin	FOS	Phosphonic acids
Fusidic acid	FA	Fucidanes
Gentamicin	GM	Aminoglycosides

InducibleClidamycin Resistance	ICR	Lincosamides
Levofloxacin	LEV	Fluoroquinolones
Linezolid	LNZ	Oxazolidinones
Moxifloxacin	MXF	Fluoroquinolones
Mupirocin	MUP	Monoxycarbolic acid
Nitrofurantion	FT	Nitrofurantoins
Oxacillin	OX1	Cephamycins
Rifampicin	RA	Ansamycins
Teicoplanin	TEC	Glycopeptides
Tetracycline	TE	Tetracyclines
Tigecycline	TGC	Glycylcyclines
Tobramycin	TM	Aminoglycosides
Trimethoprim/Sulfamethoxazole	SXT	Folate pathway inhibitors
Vancomycin	VA	Glycopeptides
Antibiotic agent Categories No.		18

2.14. Molecular Materials:

Categorized and detailed down in **Table (2-9)**, the materials and kits employed in the molecular study. **Table (2-10)** contains all primer sets used in this study, with their sequences, PCR conditions and their amplicon size.

2.14.1 Preparation of 1X TBE Buffer:

The preparation of 1X TBE buffer was performed by dilution of a concentrated 10X TBE buffer, this dilution was accomplished as 1:10 (v/v); 1 volume of 10X TBE: 9 volumes of distilled water. This solution was used to prepare agarose gel and as a transmission buffer in electrophoresis process. Thus each 100ml of 10X TBE added to 900ml of sterile distal water to produce final concentration,1X TBE .

2.14.2 Rehydration of Primers:

Lyophilized primer pairs were rehydrated by DNA rehydration solution 1X (pH 8.0) Tris- EDTA buffer (TE-buffer). Initially, primer storage-stock tube prepared and then the working solution would prepared from primer stock tube. Consistent with the instructions of the producer (Bioneer / Korea), TE buffer was added to produce 300 picomole/microliter concentration of primer stock solution. The working solution prepared from stock as 1:10 (v/v) by dilution with TE buffer to get 10 picomole/microliter.

2.14.3 Preparation of Agarose Gel:

This gel was prepared by adding agarose powder in 1X TBE buffer to be dissolved by boiling, then it was left to cool to 50ºC. The dissolved amount of agarose powder is depending upon the aim for which agarose is used.

For DNA profile (visualization of the DNA after extraction), 1% agarose is used. While for visualization of PCR product (amplicon), 1.2% of agarose was employed. Ethidium Bromide stock solution with a concentration 10mg/ml was used. Only 5µl of this stock solution were supplemented to 100ml of melted agarose gel to get final concentration 0.5µg/ml (Sambrook and Russel, 2001). Then after the addition of ethidium bromide, mixed well and dispensed to the tray of gel electrophoresis **Table (2-8)**.

Table (2-8): Protocols of PCR reaction mixture volumes

PCR reaction mixture	Promega protocol (final volume 25µl)
Master mix 2X	12.5 µl
Primer forward (10µM)	2.5 µl
Primer reverse (10µM)	2.5 µl
DNA template	5 µl
PCR grade water	2.5 µl

Table (2-9): Molecular-Related Materials used in the curred study.

Item	Company	Country
-100 bp Ladder, consists of: 10 double-stranded DNA fragments ranging in sizes from 100 to 1,000 bp with 100 bp increments. The 500 and 1,000 bp bands are double to the intensity of other fragments and brighter, for easier identification and comparison of molecular weight). While all other fragments seem with equal intensity on gel. - Nuclease free water. - TE Buffer, 1X (pH 8.0) composed of 10mM Tris-HCl containing 1mM EDTA•Na2.	Bioneer Intron	Korea USA
-Blue/Orange Loading Dye, 6X containing: 1-0.4% orange G. 2-0.03% bromophenol blue. 3-0.03% xylene cyanol. 4-15% Ficoll. 5-10mM Tris-HCl (pH 7.5). 6-50mM EDTA (pH 8.0). It is used for loading DNA samples into wells and tracking migration during gel electrophoresis. -Ethidium Bromide Solution, (10mg/ml). - Primer pairs	Biobasic	Canada
Geneaid Genomic DNA Isolation Kit	Geneaid	UK
-Green master mix 2X Kit, consist of: 1-Taq DNA polymerase. 2- dNTPs, 400µM for each. 3-Tris-HCl (pH 8.5-9.0), 10 mM.	Promega	USA
4-KCl, 30 mM 5-$MgCl_2$, 3mM. 6-2eppendroffs of Nuclease free water 7-Stabilizer and tracking dye. - Agarose -TBE Buffer (Tris-Borate-EDTA), 10X (pH 8.3) Composition: 890mM Tris-borate, 890mM boric acid, 20mM EDTA.	Promega	USA

Table (2-10): The primer sequences and PCR conditions with their amplicons sizes(Bioneer, Korea)

Gene's Name	Primer Sequence (5´- 3´)	Size (BP)	Conditions	Reference
Antibiotic resistance-Coding Genes				
mecA	F(5-'TCCAGGAATGCAGAAAGACCAAAGC-3') R(5-'GACACGATAGCCATCTTCATGTTGG -3')	499	94°c 3min 1x 94°c 1.5min 55°c 1min 36x 72°c 1min 72°c 10min 1x Storage 4°c ; cold	(Al-Charrakh and Obayes,2014)
Virulence Factors toxins-Coding Genes				
Cp5	F (5`-GAA AGT GAA CGA TTA GTA GAA – 3`) R(5`-GTA CGA AGC GTT TTG ATA GTT-3`)	532	94°c 10min 1x 94°c 0.5min 45°c 0.5min 30x 72°c 2min 72°c 5min 1x Storage 4°c ; cold	(Mattar *et al.*, 2014)

In this study, *mecA* gene and CP5 gene were detected for all MRSA isolates by using a primer of PCR technique.

2.15. Extraction and purification of CP5:

The purification of CPs was carried out by a methods described, by Li *et al.*, 2018(index-1), Ahmadi *et al.*, 2019(index-2) with some modifications.

The combination was made using the two different methods for the extraction of the bacterial polysaccharides. The situation can be explained for the following reasons:

1-The difficulty of obtaining a single extraction method for polysaccharides, and the work took several months to reach to the most appropriate methods.

2-The difficulty of obtaining the materials and components needed to carry out the experiment, as most of them are prohibited from entering Iraq or for their high price.

There are other difficulties encountered in the present study such as the difficulty in obtaining basic materials to complete the research in terms of cost and time, like the transporter protein CRM197, and the materials for the conjugate process of all kinds in addition to the inability to obtain anti-CP5 CRM 197 ELISA kits that are specially made for the purpose of completing the research requirements.

The prohibition of the entry of standard bacterial isolates from the United States of America to Iraq, even after getting the agreement from a governmental medical institute within America. As well as the inability of suppliers inside Iraq to import several other materials related to the extraction process (for example: 0.05 M sodium metaperiodate -$NaIO_4$, ε-poly-L-lysine-agarose, etc.) make matters worse.

2.15.1: Isolation and partial purification of the *S. aureus* capsular polysaccharide.

Strain *S. aureus* mucoid was grown on Tryptic soy agar for 24 h at 37°C in a 1-liter fermentor, equipped with a pH titrator set to maintain pH 7.2. The culture was aerated with a rotating sparger and agitated. The culture medium optimal for capsule production and recovery was Tryptic soy broth modified by the addition of 5% NaCI.

1.Bacterial culture in Tryptic soy agar for overnight, than single colony was cultivated in 5 ml Tryptic soy broth for overnight and add to 1-liter Tryptic soy broth add 5% Nacl (TSB) medium for 2 days at 37° C. The medium was centrifuged and the bacterial cells were then suspended in PBS, followed by autoclaving at 121°C for 50 min and centrifugation at 5000g for 30 min.

2. The supernatant was collected and a second autoclaving-centrifugation cycle of the cell mass after resuspension with PBS was performed under similar conditions.

3. Two supernatants were combined, filtered through a 0.45 μm pore size membrane, and maintained overnight at 4°C.

4. The supernatants were filtrated in approximately 125 ml.

5. the following enzymes were added to the bacterial supernatants: 2.5mg of DNase , 6.25 mg of RNase, and 10 mg of lysozyme After overnight incubation at 37°C, the enzymes were added again the following morning. Six hours later, 25 mg of proteinase was added to the bacterial suspension, and the mixture was incubated at 37°C overnight.

6. The bacteria were removed by centrifugation at 10,000 x g and discarded. The supernatant was filtered through a 0.45-,um-pore-size membrane (Millipore) and precipitated with 30% ethanol (vol/vol) at 4°C overnight to partially remove nucleic acids and proteins.

7. The supernatant was brought to a concentration of 80% ethanol (vol/vol) and kept at 4°C overnight.
8. After centrifugation at 6,000 x g for 30 min, the precipitate was dissolved in deionized water, and kept at 4°C overnight.

9. An ultrafiltrate of this sample was prepared by passing the sample through a Pellicon cassette system (Millipore Corp., Bedford, Mass.) with membranes with a cutoff of 10,000 daltons (dialyzed extensively at 4°C).

10. The white, flocculent precipitate was suspended in approximately 100 ml of 0.05 M Tris buffer-1 mM MgCl2-1 Mm CaCl2, pH 7.3. The solution was digested overnight at 37°C with 2 mg of DNase, 10 mg of RNase, and 10 mg of lysozyme (Sigma). The enzymes were added again the following morning, follpwed by overnight digestion at 37°C with 10 mg of proteinase.

11. The sample was centrifugation at 6,000 x g for 30 min, the precipitate was dissolved in deionized water.

12. An ultrafiltrate of this sample was prepared by passing the sample through a Pellicon cassette system (Millipore Corp., Bedford, Mass.) with membranes with a cutoff of 10,000 daltons (dialyzed extensively at 4°C)

13. The crude capsular antigens was produced.

14. The crude capsular antigens was dry at 45°C.

15. Finally, purified CPs dialyzed against dH2O and lyophilized. Samples were stored at_ 20C until use.

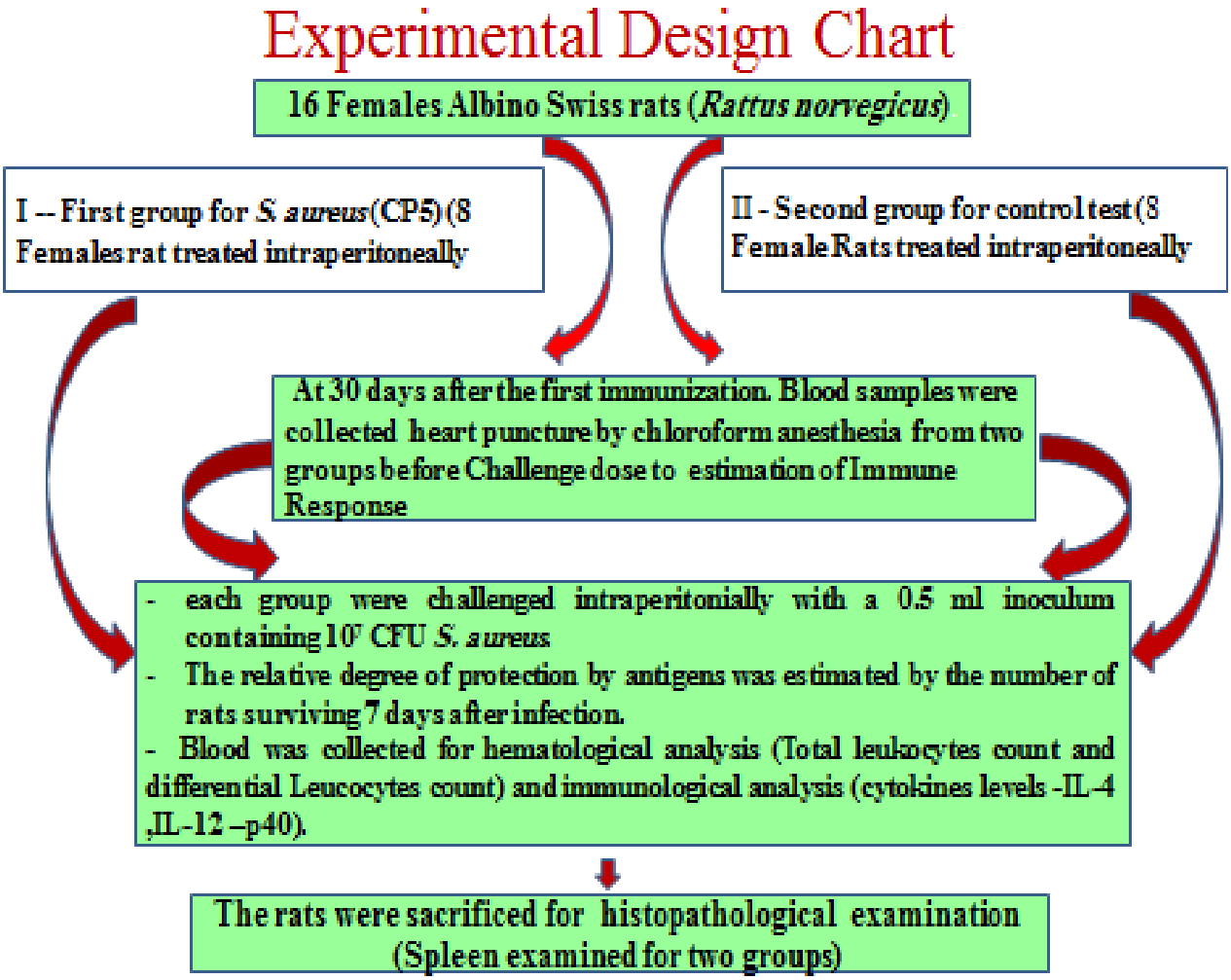
2.15.2: Characteristics of the *S. aureus* capsular polysaccharide(CP5) isolate

The *S. aureus* isolate haroured *mecA* gene and cp5 gene.

2.16: Bacterial Suspension Preparation

The *S. aureus* strains were inoculated into ordinary broth and incubated at 37°C with 250 rpm shaking overnight. The next day, The bacterial suspension was collected and centrifuged at 5000 rpm for 10 min. The cell pellet was then collected, washed 3 times with PBS, and then resuspended in PBS. the microorganism in 3.0 ml of sterile saline in test tube. The turbidity was adjusted to become equivalent to a McFarland No. 0.5. this Bacterial suspension used in later experimental.

2.17: Preparation material for immunological study :



2.17.1 *Staph aureus* capsular polysaccharide (CP5) solution preparation:

Purified CP5 was prepared as a 5 mg dissolved /mL solution (phosphate buffer saline)**.**

2.18 Experimental design

In the experimental group rats (n=16) were assigned randomly to 2 experimental groups (I , II) .

I - **First group for *S. aureus* capsular polysaccharide (CP5) :**

This group (8 Female Rats treated intraperitoneally with *S. aureus* capsular polysaccharide [24(v):1(v)] [(CP5)(96%) - Tween80) (4%)] in two dose each dose 50 μg (10 μl) per animal, 7 dayes between first and second dose).

II - **Second group for control test:**

This group (8 Female Rats treated intraperitoneally with phosphate buffer saline in two dose each dose (10 μl) per animal. Seven days between first and second dose).

2.19 Challenge of Immunized rats

At 30 days after the first immunization for CP5 and control 16 rats divided into 2 groups (with 8 rats for each one were challenged intraperitonially with a 0.5 ml inoculum containing 10^7 CFU *S. aureus* ,The relative degree of protection afforded by antigens was assessed by the number of rats surviving 7 days after infection, then the rats were sacrificed and blood 72h collected for hematological and serological analysis.

2.20.Estimation of Immune Response to Extracted CP5

2.20.1. Blood Sampling:

Four ml of blood samples were collected from each rat by heart puncture using a disposable syringe, 2 ml in EDTA (anticoagulant) tubes for white blood cell (WBC and differential count), other 2 ml of blood sample was placed in test tube

without anticoagulant and it was left in room temperature for clotting, then centrifuged at 3000vrpm for 5 min. The separated serum was divided into 100 µl in eppendroff tubes, and stored at - 20˚C. Each tube was used once to avoid repeated freezing and thawing for interleukins. For measured total WBC and differential leukocyte count used Automated hematological analyzer.

2.21. Immunological Parameters:

2.21.1. Estimation of (IL-12 /p40 and IL-4) by ELISA (Principle) :

The immunological parameters were included interleukin- 12p40 and interleukin -4(IL-12/P40 and IL-4). The principle of these testes have same principle according leaflets that provided with kits of manufacture Company (Elabscience Company), that briefed as the sample or standard containing these parameters ((IL-12/P40 and IL-4) reacts with captured monoclonal antibody (MAb-1) coated on the microtiter wells, then biotinylated Ab that specific for each one and with addition of monoclonal antibody (MAb-2) that labeled with horseradish peroxidase HRP enzyme, sandwiches were formatted. Excess unbound enzymes labeled antibody in microtiter wells were removed by well washing five times, and then solution of substrate was added to each well, a blue color appear. The reaction was stopped by addition of stop solution (Sulphuric acid) and the color turns yellow. At 450 nm wavelength, the optical density (OD) was measured and then the concentration was calculated by plotted on standard curve (Figure 2-2 and 2-3) that done with each test.

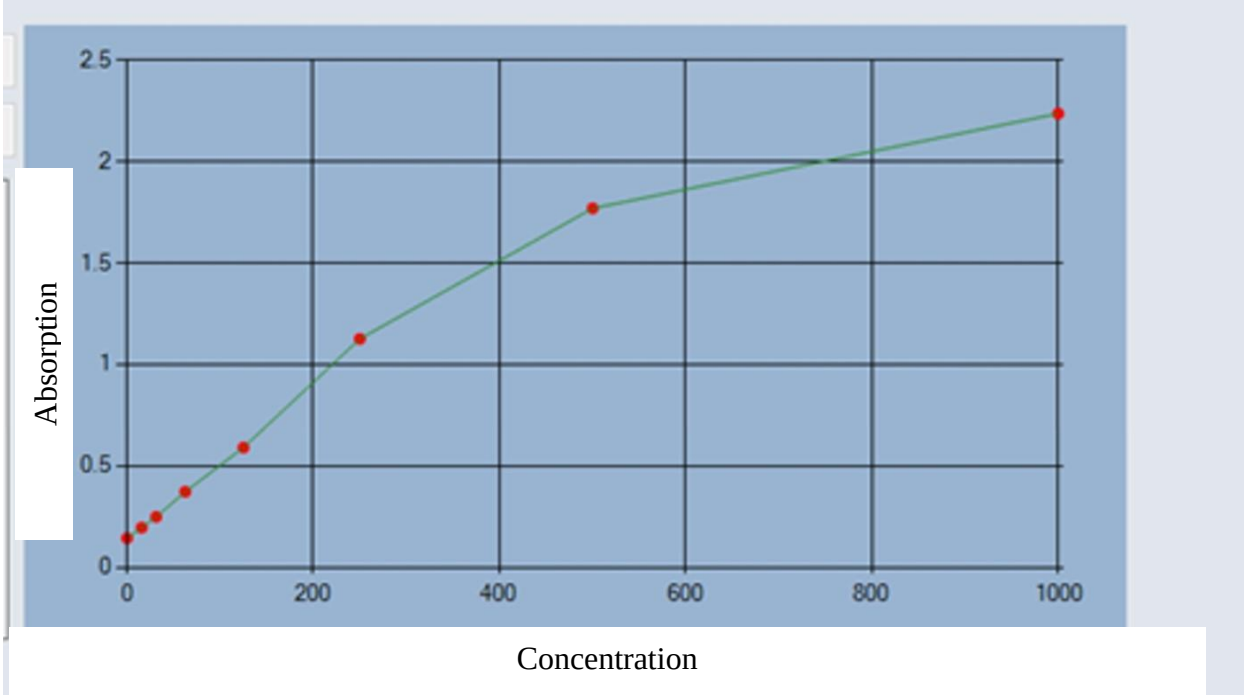


Figure 2-1 Standard curve of IL-4

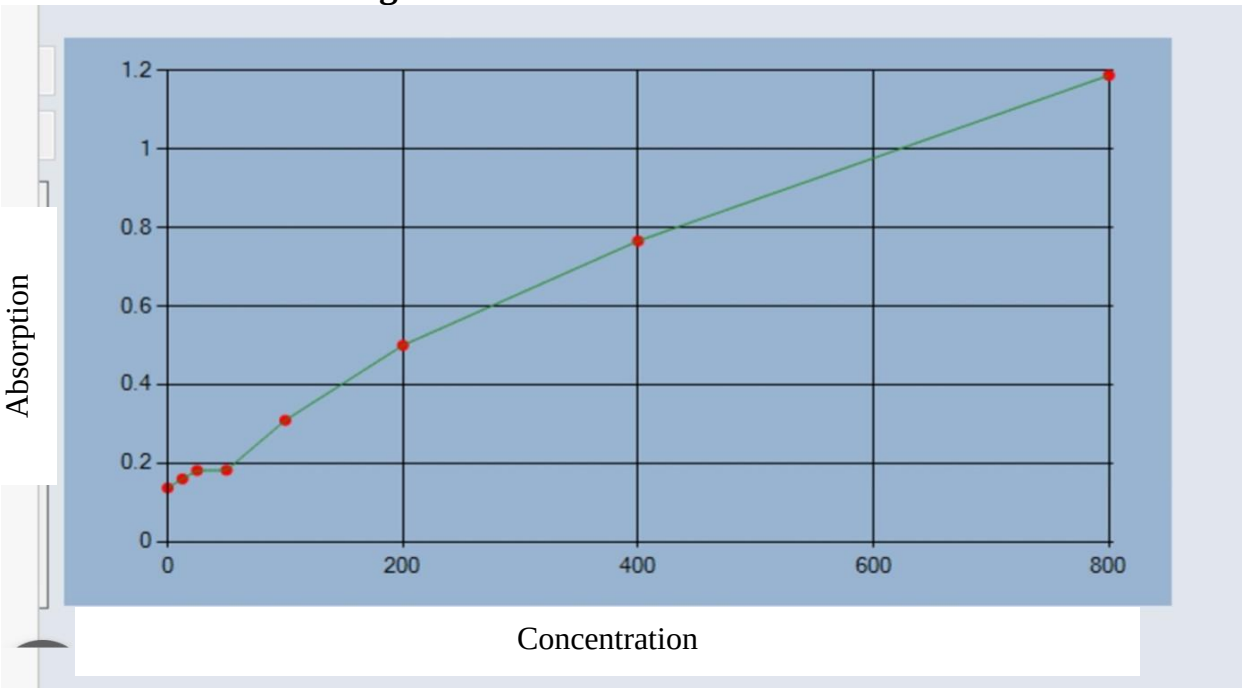


Figure 2-2 Standard curve of IL-12p40

2.22. Histopathological Section of Spleen

Two weeks after the post dose the rats were dissected by using sterile instruments and the organ(spleen) were removed. After that, the organs were transferred immediately to 10% formalin for a histopathological manipulation .

2.22.1.Preparation of 10 % Formalin Solution:

Formalin solution was used to keep the histological specimens which were taken from animals. It was prepared by adding 100 ml of formalin (37%) to 900 ml of D.W. to obtain 10% concentration of formalin.

2.22.2. Histopathology Examinations:

Histopathological is referring to the tissue microscopic examination in order to study the manifestation of disease and histopathological changes. In clinical medicine this refers to biopsy or surgical specimen examination after it has been processed (Slaoui and Fiette, 2011). The specimens of spleen, were prepared as below: (Gunasegaram, 2007).

The specimens were fixed in 10 % buffer formaldehyde solution immediately, and after 72 hours of fixation they were washed with tap water and processing was done by a set of upgrading alcoholic concentration (70% - 100%) for two hours in each concentration in order to remove the water from the specimens, and then clearance was done by xylol. The specimens were impeded with paraffin wax at 58° C, and blokes of tissue were made with paraffin wax and sectioned by microtome at 5 mm for all tissues. The prepared sections were stained with hematoxylin and eosin. The histopathological changes were observed under light microscope.

2.23. Statistical Analysis:

The data were presented in current study analyzed by Statistical Package for Social Science Software version ,Mean ± standard deviation (SD), analysis of variance (ANOVA) Statistical analysis performed using Statistical Package of Social Science (SPSS) program V. 20 analyzed descriptive statistics and Chi-square test ($\chi 2$) or Fisher exact test (typically used when sample sizes are small) used to determine the relationship between the variables, P value < 0.05 was considered statistically significant .

3. Results and Discussion

3.1:Distribution of bacterial isolates among clinical samples

The aim of this study was to isolate *Staphylococcus aureus* clinical strains that carrying *mecA* and capsular polysaccharides (CP5) genes, which can be further used as a candidate immunogenic compound against *S. aureus* infections.

Among 180 clinical samples collected, 46 (25.5%) gave positive growth on ordinary culture media while 134 (74.5%) gave no growth. The reason of negative culture may be attributed to fungal infection, viral infection, fastidious bacteria that might be lost during transporting or antibiotics usage before taking samples.

Regarding information taken from patients before samples collection such as whether patients received antibiotics before or not, the results found that 30 patients had received antibiotics before sampling and 16 of them were not.

Results also revealed that out of 46 culture positive isolates recovered from patiants, high rate of isolation 16 (34.7%) was found in urine samples, followed by wounds (burns) samples 15 (32.6%), while only one sample 1(2.1%) from bronichal fluid showed positive growth (Table 3-1).

Table (3-1): Distribution of positive growth isolates from patients according to the sources of samples

Sources of samples	No. of samples (%)	No. of positive growth (%)	No. of negative growth (%)
Blood	89 (49.4%)	12(26.1%)	77(57.4%)
Urine	47(26.1%)	16(34.7%)	31(23.1)
*CSF	20 (11.1%)	__	20(14.9)
Wounds (burns)	20 (11.1%)	15 (32.6%)	5(3.7)
Abscess	3(1.6%)	2(4.3%)	1(0.74)
Bronichal fuild	1(0.5%)	1(2.1%)	0(0%)
Total	**180 (100%)**	**46(25.5%)**	134 (74.5%)

*CSF (cerebrospinal fluid)

Among 46 positive cultures of clinical samples, 22 (47.82%) were found to be Gram-positive and 24 (52.17%) as Gram-negative bacteria. Among 22 Gram-positive bacterial isolates 17 bacterial isolates belonged to Coagulase-negative Staphylococci (CoNS) and 5 were identified as *S. aureus*.

3.2: Phenotypic and Molecular Detection of MRSA isolates

All 5 *S. aureus* isolates were tested for methicillin resistance using cefoxitin as indicator of MRSA as an initial screening test according to the guidelines of CLSI. The results revealed that all *S. aureus* isolates were found to be MRSA. For confirmation of MRSA isolates, *mecA* gene was detected for all cefoxitin - resistant *S. aureus* isolates by PCR using a primer designed by Al-Charrakh and Obayes (2014).

The susceptibility to oxacillin and cefoxitin results determined by conventional methods was compared with the results of the PCR assay using the specific primers of *mecA* resistance gene. A total of all isolates of *S. aureus* were employed in this study (Table 3-2).

Result in Figure (3-1) shows the electrophoretic patterns of the DNA products after PCR amplification using all these isolates, *mecA* was detected in all of cefoxitin - resistant *S. aureus* isolates .The present results showed that the phenotypic antibiotic susceptibility patterns were similar to those obtained by genotyping done by conventional PCR in which the gene *mecA* was detected in all cefoxitin resistant isolates

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MRSA detection is essential for appropriate patient care and infection control. Examination of MRSA can be done either phenotypically or genotypically. The gold standard to determine information about the genotype distribution of MRSA is to detect genes conserved constantly found. *mec*A gene is located within chromosome in a structure called Staphylococcal Cassette Chromosome (SCC*mec*) encodes mutant PBP2a or PBP2' of 76 kDa (Nasution *et al*., 2018) The presence of *mec*A gene is generally to indicate the potential resistance to beta-lactam group and used as a marker to identify MRSA. In this study, PCR product was shown as 499 bp amplicon in all resistant isolates using a primer designed by Al-Charrakh and Obayes (2014).

MecA gene encoded penicillin binding protein-2a (PBP2a), a mobile extrinsic genetic element that is carried on a genomic island (*SCCmec*). (Alkharsah, *et al*., 2018). PBP-2a has lower affinity for β-lactams compared to the typical penicillin binding protein-2 (PBP2) produced by methicillin susceptible *Staph aureus* (MSSA) because it prevents the active site from binding β-lactams (Hussain, *et al.*, 2019).

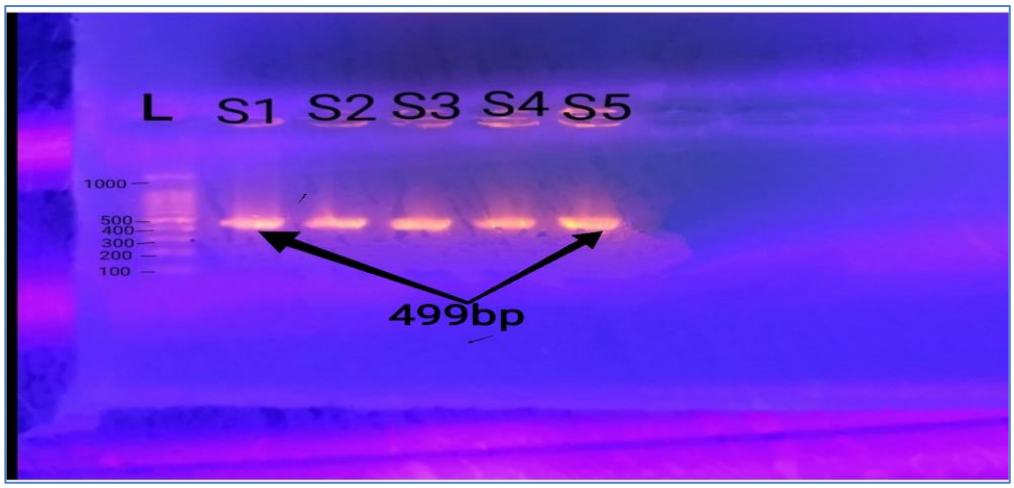

Figure (3-1): Gel electrophoresis of PCR amplification of the *mecA* gene from *S. aureus*, with the amplicon size 499 bp. PCR products were separated by electrophoresis in an 1.2% agarose gel. The electrophoresis was performed at 100 volt for 10 min and 80 volt for 40 min, marker DNA ladder (100-1000) bp.

3.3: Antimicrobial Susceptibility Testing

The antimicrobial susceptibility of MRSA isolates to different antibiotics was determined using MIC method according to the breakpoints recommended by CLSI (CLSI, 2020). Antibiotic resistance patterns (MDR, XDR, and PDR) were also determined using definitions of Magiorakos *et al*. (2012).

The Minimum Inhibitory Concentrations (MICs) values of *S. aureus* isolates. MRSA isolates were found to be moderately resistant to most of the antibiotics tested and all of them had MDR antibiotic resistance pattern (Table 3-2). **Figure 3-2** shows the distribution of MRSA isolates according to antibiotic susceptibility pattern. All *S. aureus* isolates were resistant to Oxacillin (OX1), Cefoxitin screen (OXSF), and benzyl penicillin (P), while they were sensitive to Moxifloxacin (MXF) and Tigecycline (TGC). However, they showed different degree of resistance towards other antibiotics used.

Table (3-2): Minimum Inhibitory Concentrations (MICs) of *Staphylococcus aureus* (MRSA) isolates

Antibiotic	Code	Isolate No.W001	Isolate No.W002	Isolate No.W003	Isolate NoAbscess 004	Isolate No.W005	MIC interpretation	
							Sensitive (≤ µg/ml)*	Resistant (≥µg/ml)*
Benzyl penicillin	P	+	+	+	+	+	0.03	0.5
Cefoxitin Screen	OXSF	+	+	+	+	+	NGE	POS
Clindamycin	CM	+	+	+	–	+	0.5	4
Erythromycin	E	+	+	+	–	+	0.5	8
Gentamicin	GM	–	+	+	–	–	4	16
Inducible Clidamycin Resistance	ICR	–	–	–	–	+	NEG	POS
Levofloxacin	LEV	–	+	+	–	–	1	4
Linezolid	LNZ	+	–	–	–	–	4	8
Moxifloxacin	MXF	–	–	–	–	–	0.5	2
Nitrofurantion Drug modified	FT	+	–	–	–	–	4	16
Oxacillin	OX1	+	+	+	+	+	2	4
Rifampicin	RA	+	+	+	–	–	1	4
Teicoplanin	TEC	+	–	–	–	–	0.5	32
Tetracycline	TE	–	+	+	+	–	4	16
Tigecycline	TGC	–	–	–	–	–	0.12	2
Tobramycin	TM	–	+	+	–	–	1	16
Trimethoprim/ Sulfamethoxazole	SXT	–	+	+	–	–	2/38	4/76
Vancomycin	VA	+	–	–	–	–	2	16
% of Resistance		**55.5%**	**61.1%**	**61.1%**	**23.5%**	**35.3%**		
Patterns of antibiotics resistance		MDR	MDR	MDR	MDR	MDR		

+(Resistant**), _(** Sensitive**)**

*****The values between brackets indicate the break-points recommended by CLSI, 2020

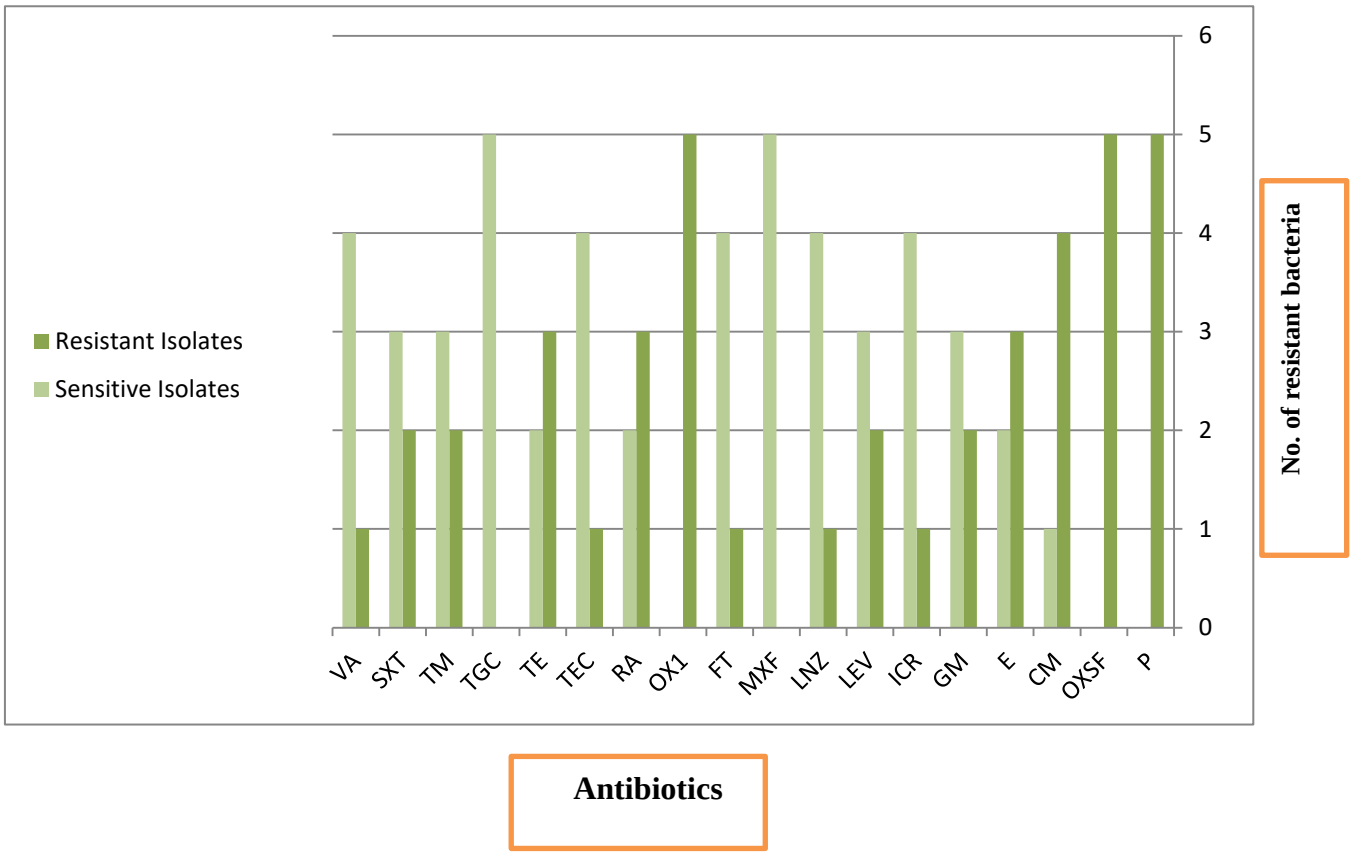

Figure 3-2: Antibiotic susceptibility pattern of MRSA isolated recovered from clinical samples.

Studies on MRSA surveillance in hospitals and communities represent the chief challenges of the healthcare setting .Unfortunately, penicillin resistant bacteria can spread in healthcare setting and in the community. Methicillin narrow spectrum semi-synthetic penicillin was introduced to overcome infections due to beta-lactamase-producing *S. aureus*. Strains isolated from the hospital are named as hospital acquired *S. aureus* (HASA) (Al-Musawi *et al*., 2020).

Oxacillin–resistant staphylococci are resistant to all beta-lactam antimicrobial drugs except newer cephalosporins with anti-MRSA activity. Thus, susceptibility or resistance to a broad spectrum of beta-lactam drugs may be decreased due to susceptibility tests such as penicillin, cefoxitin or oxacillin. Routine testing of other penicillins, cephems, beta-lactam/beta-lactamase inhibitor combination, or carbapenems is not recommended. A forecast of the presence of *mec*A-related oxacillin resistance in *S.aureus* and *S. lugdunensis*, examination of either cefoxitin disk diffusion or cefoxitin MIC tests can be used (Al-Charrakh and Obayes, 2014).

The number of mechanisms inherent in pathogenic bacteria that makes it resilient or strong in the presence of extreme conditions and confers it with the ability to resist quite a large compendium of important antibiotics and other toxic compounds are becoming extremely interesting. the use of antibiotics for a long period have been observed to evoke a number of biochemical and genetic mechanism in bacteria that allows it to maneuver the detrimental effect of antibiotics found within their immediate environment. Clones of bacteria with acquired or natural resistance characteristics have been used continuously as a form of evolutionary response to the use of antibiotics. It is a well-established information that the acquisition of antibiotic resistance mechanism occurred because of genetic events causing changes in the primordial bacterial genome such as deletion or substitution of a single nucleotide base and multiplication of a single number of a gene. However, the most important means of persistence of resistance gene, is the horizontal transfer of mobile genetic elements such as

transposons, integrons, and plasmids both within bacteria of the same or different species (Bitrus *et al*., 2018).

3.4: Deduction of capsular genotype by PCR technique.

Genomic DNA of *S. aureus* was used as a template for PCR amplification with of the CP5 gene.

In the present study, a total of 5 clinical isolates of *S. aureus* were tested for detection of capsular genotype by PCR technique (Figure 3-3), Capsular polysaccharides 5(CP5) was deducted in only one isolate (Isolate No Abscess 004) (A.MS. isolate) was derived from researcher name

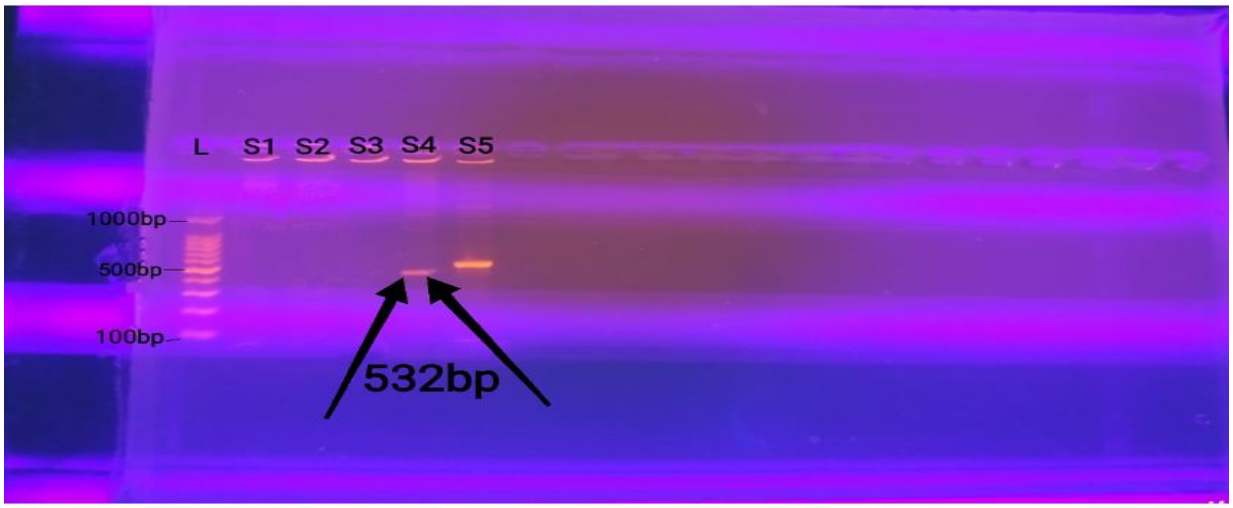

Figure (3-3):Gel electrophoresis of PCR amplification of the *CP5* gene from *S. aureus*, with the amplicon size 532 bp. PCR products were separated by electrophoresis in an 1.2% agarose gel. The electrophoresis was performed at 100volt for 10min and 80 volt for 40min, marker DNA ladder (100-1000) bp;

3.5: Capsular polysaccharides (CP5) Characteristics

Finally extraction and purification of CP5 according to our modification method was previously in chapter 2 as the first recorded , the purified CPs was dialyzed against dH_2O and then lyophilized to attain 6 mg from 1 liter of the growth culture. Samples were stored at -20° C until use.

Capsular polysaccharides (CPs) as vaccine candidates: The approach of targeting the capsular polysaccharides (CP) as vaccine candidates has been employed successfully against many bacterial pathogens, including *S. pneumoniae, H. influenzae*, and *N. meningitides* (Cheng *et al*., 2017). A polysaccharide capsule envelopes the surface of many bacterial pathogens and can confer resistance to phagocytic clearance by the host innate immune response, there by prolonged persistence of pathogen in the bloodstream of the host ,However, the resistance can be overcome by the opsonophagocytic antibodies targeting the capsule (Cheng *et al*., 2017).

In this study, CP5- Carrier protein conjugate [firstly a nontoxic mutant of diphtheria toxin, cross reacting mutant (CRM 197)] as Carrier protein conjugate with CP5 but used CP5 only was emulsify with Tween 80(as adjuvant) when absent CRM197.and conjugate material.

were protective against *S. aureus* bacteremia, Purified CP5 has humoral immunogenicity in animals. To relieve this, we conjugated CP5 -Tween 80 to enhances the immunogenicity of CP5 and to determine the optimal immunogenic dose, we actively immunized rats (as animal model) with two doses of CP5 with 50 μg per rat.

Li *et al* ., 2018 was reported that Thirty-six liters *S. aureus* cultures were subjected to high-pressure cracking, enzyme digestion and chemical removal, and membrane dialysis, which yielded a total polysaccharide amount of 348.64 mg. Glucose solutions at different concentrations were prepared in accordance with the phenol sulfuric acid method to generate a standard curve. Capsular

polysaccharide dilution was also in accordance with the phenol sulfuric acid method, the net yield of CP was 6.34 mg/L was presented in index (1).

Ahmadi *et al* ., (2019) reported that Strain SAl mucoid was grown for 24 h at 37°C in a 20-liter fermentor (LSL Biolafitte, Inc., Princeton, N.J.), equipped with a pH titrator set to maintain pH 7.2 and the capsular antigen (~500 mg) was chromatographed over a column (2.6 by 30 cm) of CM-Sepharose (Pharmacia), equilibrated with 0.01 M citrate buffer, pH 5.5, containing 0.01% merthiolate. The column eluate was monitored at 206 nm, as described above. Serologically active fractions not retained on this column were pooled, dialyzed, and lyophilized was presented in index (2).

Since there is a little or no immunological study in the world of *S. aureus* capsular polysaccharide (CP5) in rats (as animal model), according to our knowledge, this study is a first record of isolation and purification of the *S. aureus* capsular polysaccharide (CP5) in Iraq and considered as the first study of the immunological characteristics of *S. aureus* CP5 of rats in the world.

In the present study, given the limited output extracted, method was relied upon UV -Visible Spectrophotometer to detect the capsular polysaccharide (CP5) using a wavelength of 206 nm (Figure 3-4).

The final concentration of *S. aureus* capsular polysaccharide 5 (CP5) was 6 mg/L, can be deducted from A.MS isolate by UV- visible Specterophotometer using a wavelength of 206 nm. **(**Figure 3-4) and Chemical composition of the crude extract of *S. aureus* capsular polysaccharide 5 (CP5) (Table 3-3).

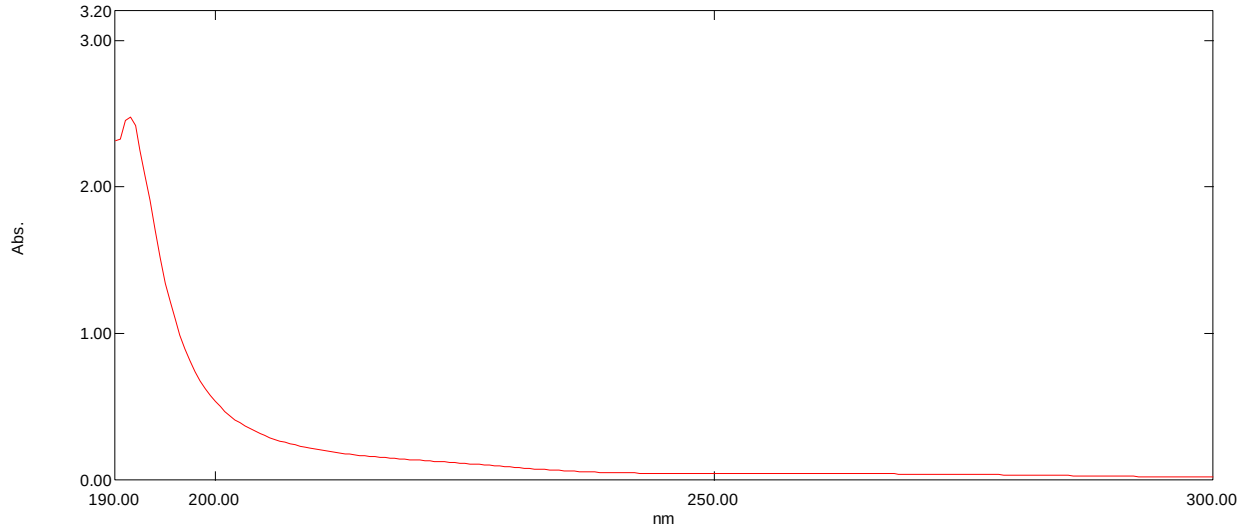

Figure 3-4a: crude extract of *S. aureus* capsular polysaccharide {CP5) detect by U.V Visible Spectrophotometer at wavelength of 206 nm. Abs; Absorption and n.m; Wave length

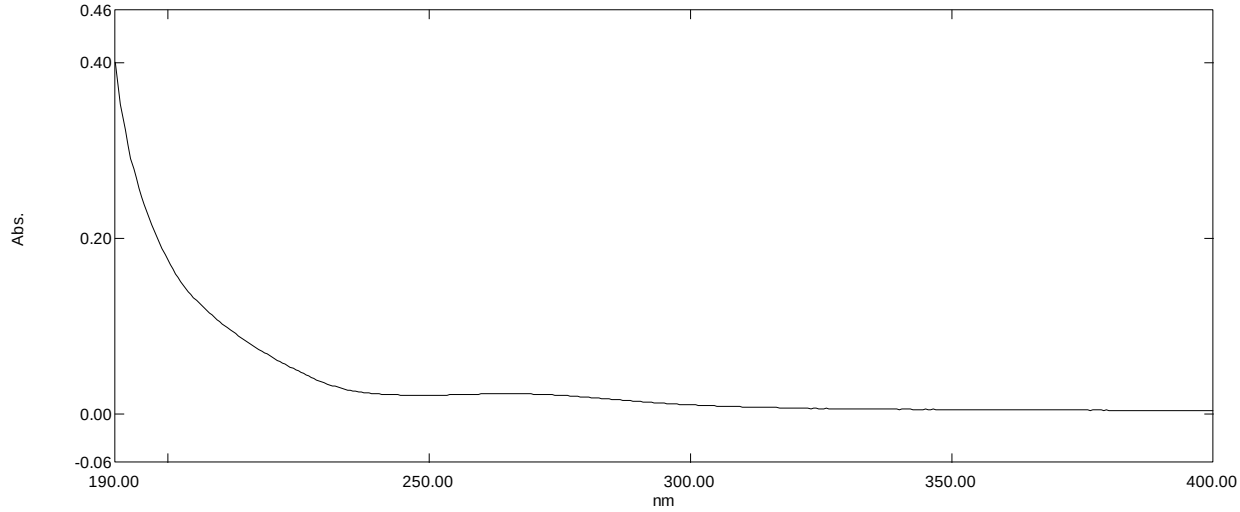

Figure 3-4b: Partial extract of *S. aureus* capsular polysaccharide {CP5) detect by U.V Visible Spectrophotometer at wavelength of 206 nm. Abs; Absorption and n.m; Wave length.

Table (3-3) Chemical composition of partial extract of *S. aureus* capsular polysaccharide 5 (CP5) from A.MS isolate by UV- visible Specterophotometer

The crude extract of *S. aureus* capsular polysaccharide 5 (CP5)	**Wave length(n.m)**	**Absorption /ml**	**Concenteration/ml**
Nucleic acid content	260	0.018	3.6 µg/ml
Protein content	280	0.017	3.4 µg/ml
Capsular Polysaccharide 5 Content	206	0.279	6 µg/ml

3.6: Hematological Parameters Results before challenge dose:

3.6.1 Total Leukocytes Count (TLCs) and Differential Leukocyte Count

The results of TLCs among testing groups indicated not significant increasing the mean value in group I (11093 cells/mm^3,) as comparing with group II (9650 cells/mm^3), at ($p < 0.05$) as in (Table 3-4).

The results of differential leukocyte count indicated not significant differences ($p < 0.05$) for the two groups as illustrated in (Table 3-4).

Table (3-4): Total leukocytes count (Mean & SD) and differential Leucocytes (Mean & SD) among testing groups before challenge dose.

Testing groups	**No.**	**Mean ± SD**			
		Total leukocytes count Cells/mm^3	**Lymphocytes (%)**	**Monocytes (%)**	**Neutrophil (%)**
Group I	8	11093 ± 1911	75.4± 9.398	15.72 ± 5.2409	5.62 ± 2.6659
Group II	8	9650 ± 3818	73.74± 3.388	15.92 ± 3.5238	8.44 ± 2.4805

Group I: *S. aureus* capsular polysaccharide (CP5) –Tween 80.

Group II: Control group (phosphate buffer saline).

The *P –value* is 0.2089, The result is no significant at $p < 0.05$

White blood cells (WBCs) or leukocytes play an essential role in immune defense, and include different subpopulations: neutrophil, eosinophil, and basophil granulocytes, monocytes, and lymphocytes. Leukocytes are produced and mature in the bone marrow, and, in the case of lymphocytes, in the lymphoid tissues. The number of leukocytes in the blood constitutes only a small percentage of the total population and undergoes wide vacillation. In the vasculature, a marginal pool and a circulatory pool of neutrophils are differentiated. The marginal neutrophils are attached to the endothelial cells, but detach and join the circulatory pool if blood pressure rises and the blood flow

velocity increases. Therefore, every change in blood pressure can result in a change in the amount of leukocytes present in the blood (Roland *et al*., 2014).

A complete WBC count is composed of the total number of leukocytes, the relative differential blood count, and the absolute differential blood count. Usually, the different subpopulations, as well as band and segmented neutrophils can be distinguished (Roland *et al*., 2014).

Said and Abiola (2014) found that the number value of total (WBCs in female rats is 1700-12150 cells/mm^3). Leukocytes in health and disease. The typical WBC count in rat is 3000 to 11,000 per microliter. They also revealed that lymphocytes are the predominant leukocyte in most strains of healthy rat, making up 82.6% to 93.4% of the WBC differential count. Neutrophils generally comprise 2.4% to 9.1% of the WBC count in rats female and are the most common granulocyte. Monocytes are the largest leukocyte and typically make up 3.5% - 8.4% of the total WBC count in rats female.

Vidya and his colleagues (Vidya *et al*., 2018) reported that Among pattern recognition receptors (PRRs), NOD-like receptors (NLRs) are involved in regulation of inflammatory and apoptotic responses; RLRs are associated with intracellular recognition of RNA virus replication; and the unique elements Toll-like receptors (TLRs) are involved in alarming the immune system against extracellular or endosomal pathogen-associated molecular patterns (PAMPs) like bacterial lipopolysaccharides, lipoteichoic acid, nucleic acids, TLRs act as natural adjuvants to vaccines that contain attenuated live or heat-killed viruses or bacteria. The role of TLRs is noteworthy in control of adaptive immune response through maturation of dendritic cells (DCs), induction of cytokines and co-stimulatory proteins expressions, and reversal of tolerance. Therefore, as natural adjuvants in vaccines, they help DCs in better antigen presentation, subsequently leading to a good immune response (Vidya *et al*., 2018).

3.7:Concentration of Serum Cytokine before challenge dose:

3.7.1. Concentration of Interlukin-4 in Serum of Rats:

The levels of Interlukin-4 in the serum of animals groups appears significant increase in group I (4.775pg/mL **,** in comparison with group II (0.709 pg/mL), at ($p < 0.05$) as in (Table 3-5).

Table (3-5): Interluekin-4 concentration (Mean ± SD) among the testing groups before challenge dose

Testing groups	No.	Mean value pg/mL	Standard deviation	P value
Group I	8	4.775	4.796	< 0.05
Group II	8	0.709	1.383	

Group I : *S. aureus* capsular polysaccharide (CP5) -Tween80

Group II : Control group (phosphate buffer saline).

The *P –value* is 0.0370, The result is significant at $p < 0.05$

In this study, the role of Interluekin-4 level is significant (when demonstration *S. aureus* capsular polysaccharide (CP5) injection free or conjugate with protein) because:

1. The capsular polysaccharides (CPS) of most pathogenic bacteria are T cell–independent antigens whose conjugation to carrier proteins evokes a carbohydrate-specific response eliciting T cell help. T cells recognize peptides bound to MHC molecules. Therefore, antigens that possess no peptide structure cannot be recognized by T cells. These antigens are called thymus-independent antigens and include lipopolysaccharide from the cell envelope of gram-negative bacteria and polysaccharide capsular antigens. These antigens may directly stimulate B cells to cause proliferation and secretion of antibody, or they may act as B-cell mitogens, directly causing mitosis regardless of the cell's antigenic specificity. The response to thymus-

independent antigens is generally weaker than the response to other classes of antigens, resulting in the secretion of lgM antibodies only and the absence of immunologic memory.

2. Interleukin-4 (IL-4) has many biological roles, including the stimulation of activated B- cell and T-cell proliferation; and the differentiation of B cell into plasma cells. It is a key regulator in humoral and adaptive immunity,IL-4induces B cell class switching to IgE, and up-regulates MHC classII production.

Interleukin-4 (IL-4) is an important cytokine that is responsible for the secretion of other cytokines. It plays a central role in regulating antibody production and humoral immune response to differentiate Th2 cells (Mehrbani *et al*., 2020).

The cytokines interleukin (IL)-4 and IL-13, signaling via the IL-4 receptor (IL-4R), composed type 2 immunity to helminth infections and toxins. Activation of epithelial and myeloid cells, and a transient neutrophils influx initiates type 2 immune responses, which are dominated by basophils, eosinophils, mast cells, B cell immunoglobulin E production, and type 2 T helper and T follicular helper cells. Interestingly, IL-4 and IL-13 can curtail chemotaxis and several effector functions of neutrophils in mice and humans. (Heeb *et al*., 2020).

Neutrophils are beneficial for type 2 immune responses and, simultaneously, type 2 cytokines restrict neutrophil effector functions, can be explained when considering the timing of events. During the initiation phase of a type 2 immune response, there is little or no type 2 cytokines present, and neutrophils are needed as a first wave of defense. Once the type 2 immune response is fully active, abundant IL-4 and IL-13 suppress

neutrophil effector functions, which at this stage—via neutrophil degranulation and NET formation—would cause excessive tissue damage. Thus, timed IL-4R signaling in neutrophils allows early influx but limits tissue damage by neutrophils during the "weep and sweep" phase of type 2 immunity (Heeb *et al* ., 2020).

3.7.2.Concentration of Interluekin-12p40 in Serum of Rats before challenge dose:

The results indicated a non-significant decrease of Interluekin-12p40 level in group I (7.496pg/mL) as compared with group II (12.115 pg/mL), at ($p < 0.05$) as illustrated in (Table 3-6).

In this study to understanding of Interluekin-12p40 level in group I when demonstration *S. aureus* capsular polysaccharide (CP5) injection free; Interluekin-12p40 have role in innate and adaptive immune response.

Table (3-6): Interluekin-12p40 concentration (Mean ± SD) among the testing groups before challenge dose

Testing groups	No.	Mean value pg/mL	Standard deviation	P- value
Group I	8	7.4967	7.1218	< 0.05
Group II	8	12.115	8.5303	

Group I: *S. aureus* capsular polysaccharide (CP5) -Tween80.

Group II : Control group (phosphate buffer saline).

The *P –value* is 0.332, The result is non-significant at $p < 0.05$

IL-12 consists of two subunits, which are linked by disulphide-bonds The smaller p35 monomer (35 kDa α-chain) is encoded on chromosome 3, while the gene for the larger p40 monomer (40 kDa β-chain) is located on chromosome 5 (Co-expression results in the formation of the biologically active p70 heterodimer (Ullrich *et al*., 2020)

Although p35 transcripts are found in many cell types, free p35 is not secreted without the p40 subunit. IL-12p40 is produced predominantly by activated monocytes, macrophages (MΦs) ,neutrophils and dendritic cells (DCs). It has been suggested that in rodents, but not in humans (Hamza *et al*., 2010).

Interluekin-12p40 (IL12p40) act as role interluekin-6 in pro-inflammatory cytokine , because the first massager formation in signal transduction cells during effecter binding with pattern recognition receptors (PRR) PRRs include Toll-like receptors (TLRs), Nucleotide oligomerization domain (NOD)-like receptors (NLRs), Retinoic acid-inducible gene (RIG)-I-like RNA helicases and C-type lectin receptors (CLRs). TLRs recognize extracellular or phagocytosed pathogen-associated molecular patterns (PAMPs) ;TLRs ,most of TLRs are linked to the expression of IL12p40 , while expression of IL12p35 is induced by only a limited subset of other receptors during regulation of IL12 secretion (Ullrich *et al*., 2020).

The biological activities of IL-12 are mediated via binding to a membrane receptor complex which is also composed of two subunits: IL-12R .1 and IL-12R .2. Both of the subunits are members of the class I cytokine receptor family ,which includes IL-6, IL-11, and leukocyte inhibitory factor related to glycoprotein gp (Hamza *et al*., 2010).

The p40 also couples with p19 to generate IL-23, having biological functions that are similar to as well as distinct from IL-12. While similar to IL-12, IL-23 augments the proliferation of Th1cells to produce more IFNγ ; contrary to IL-12, IL-23 is known to support the proliferation of memory T cells In addition to establishing heterodimers (IL-12 and IL-23), the p40 subunit is also released as p40 monomer (p40) and p40 homodimer (p40 2) (Mondala *et al*., 2020).

In contrast, IL- 12p40 homodimer, secreted by APC in the absence of p35 expression, and free p40 monomer do not mediate IL-12 activity but act as IL-12 antagonists. Here it is reported that prostaglandin E2 (PGE2), an inflammatory moderator with a previously known Th2-driving function, dose-dependently enhances the IL-12p40 mRNA expression and the secretion of IL-12p40 protein in human tumor necrosis factor alpha (TNF α)–stimulated immature dendritic cells (DCs) (Kalinski *et al*., 2001).

3.8:Challenge dose

3.8 .1 Bactericidal Power of Blood:

The rat animals that injected with viable MRSA strains (14 animals), were divided into two groups (as described in chapter two). Blood cultures were done to assess the clearance percentage of blood in order to detect the effects of *S. aureus* capsular polysaccharide (CP5), on survive the viable bacteria (MRSA) in blood stream. The results found that the percentage of bactericidal power in group I was higher (71.5%) as compared to group II was (33.4%) as illustrated in (Table 3-7).

Table (3-7): Bactericidal effect of blood on MRSA in testing groups

Testing groups	**No.**	**Number of Blood culture +ve**	**% of +ve blood culture**	**MRSA Clearance %**
Group I	7	2	28.5%	71.5%
Group II	7	4	66.6%	33.4%
Total	14	6		

Group I : *S. aureus* capsular polysaccharide (CP5) -Tween80.

Group II :: Control group (phosphate buffer saline).

3.9: Hematological Parameters Results after challenge dose:

3.9.1 Total Leukocytes Count (TLCs) and Differential Leukocyte Count .

The results of TLCs among testing groups indicated significant increase in the mean value in group I (9407cells/mm^3) as compared to group II(4682 cells/mm^3) ($P < 0.05$) as illustrate in (Table 3-8).

The results of differential leukocyte count indicated non-significant differences ($p < 0.05$) for the two groups as illustrated in (Table 3-8).

Table (3-8): Total leukocytes count (Mean ± SD) among the testing groups after challenge dose.

Testing groups	No.	M±SD			
		Total leukocytes count)Cells/mm^3 (p<0.05)	lymphocytes (%)	Monocytes (%)	Neutrophil (%)
Group I	7	9407 ± 3458	87.476 ±1.829	4.486 ±2,012	6.044 ± 1.1332
Group II	7	4682 ± 4558	84.796 ±10.4885	4.932 ± 2.4482	8.97 ± 1.1469

Group I : *S. aureus* capsular polysaccharide (CP5) -Tween80.

Group II : Control group (phosphate buffer saline).

The *P –value* is 0.0495 , The result is significant at $p < 0.05$

The immune response against *S. aureus* includes activation of both the innate and adaptive immune systems. As the first line of defense against infections, the innate immune response is rapidly activated by pattern recognition pathways that detect non-specific markers of microbial infection. A key result of this is activation of phagocytic cells such as macrophages and neutrophils. Neutrophils are recognized as a key component of the acute response and centrally important against *S. aureus*, as declared by the susceptibility of humans and rodant with

inherited and acquired neutrophil defects to deep-seated infections (Karauzum and. Datta, 2017)

Toll-like receptor (TLR) 2 on antigen-presenting cells (APCs) facilitates these cells to recognize peptidoglycan embedded lipopeptides and glycopolymers in the *Staphylococcus aureus* cell wall and mount an inflammatory response to this microbe. TLR2 signalling can also modulate immunity to *S. aureus* by inducing an interleukin (IL)–10 response in APCs. What determines the balance between proinflammatory and modulatory responses to *S. aureus* is unknown. they showed IL-10 response preferentially occurs upon CD14- and CD36-independent TLR2 signaling, triggering PI3K activation, and is restricted to monocytes and monocyte derived macrophages (MUs) (Frodermann *et al*.,2011)..

In contrast, monocyte-derived dendritic cells (DCs) produce mostly IL-12 and IL-23. The differential APC polarization induced by staphylococcal peptidoglycan translates into differential T helper responses: MUs primarily trigger IL-10 and weak IL-17 responses, whereas DCs trigger a forceful Th1/ Th17 response. Exploitation of TLR2 signalling plasticity by *S. aureus* may explain the wide range of outcomes of human encounters with this microbe (Frodermann *et al*., 2011).

Toll-like receptor 2(TLR2) activation depends on an active accessory gene regulator (Agr) system. *S. aureus* strains differ in their ability to stimulate TLR2. Some strains are almost unable to trigger TLR2 even in the existence of lipoproteins suggesting that other factors besides lipoproteins must have a role. Accordingly, we have found that *S. aureus* strain SA113 and its isogenic lipoprotein-deficient mutant did not differ much in their capacities to cause sepsis in wild-type and TLR2-deficient mice . In order to elucidate if and under which conditions TLR2 may be crucial in staphylococcal infections we compared different Gram-positive pathogens including several *S. aureus* strains for their

capacity to stimulate TLR2. SA113 extracellular factors in culture filtrates elicited only very weak interleukin (IL)- 8 release in TLR2-transfected human embryonic kidney (HEK-TLR2) cells (Hanzelmann *et al*., 2016).

Similar notes were made with other Gram-positive pathogens such as *Enterococci*, *Streptococcus pyogenes* and *Listeria monocytogenes*. When different MRSA strains were compared for their capacity to stimulate IL-8, it became obvious that hospital-associated MRSA (HA-MRSA) such as strains COL, Mu50 and N315 behaved like SA113 while highly pathogenic community-associated MRSA (CA-MRSA) such as USA300 and USA400 induced secretion of approximately tenfold higher amounts of IL-8, indicating that strong TLR2 stimulation is not a constant species-specific phenomenon but differs profoundly between individual strains and may be associated with high staphylococcal virulence Since CA-MRSA are distinguished from hospital related MRSA and SA113 by particularly strong activity of the global virulence regulator Agr, we compared the TLR2- stimulating capacities of CA-MRSA wild-type and isogenic Agr mutants (Hanzelmann *et al*., 2016).

3.10: Concentration of Serum Cytokine after challenge dose:

3.10.1 Concentration of Interlukin-4 in Serum of Rats after challenge dose:

The levels of Interlukin-4 in the serum of animals groups appears indicated a significant increase in group I (8.6676pg/mL) in comparison with group II (2.8531 pg/mL) at ($p < 0.05$) level as in (Table 3-9).

Table (3-9): Interluekin-4 concentration (Mean ± SD) among the testing groups after challenge dose

Testing groups	No.	Mean value pg/mL	Standard deviation	P value
Group I	7	8.6676	4.6568	< 0.05
Group II	7	2.8531	4.7414	

Group I : *S. aureus* capsular polysaccharide (CP5) - Tween80.

. Group II : Control group (phosphate buffer saline).

The *P –value* is 0.04633 , The result is significant at $p < 0.05$

In this study, the increase of Interluekin-4 (IL-4) level because the immune response against *S. aureus* activation of naïve CD4 T cells ; the Exogenous Pathway of Binding Peptides to Class II MHC Molecules.

lymphocytes respond poorly to IL-13. The expression of IL-4Rα (i.e., type I IL-4 receptor) plays thus a main role in lymphocyte responses to IL-4. The expression of IL-4Rα in naïve lymphocyte is relatively low and *in vitro*, a STAT5-dependent, STAT6-independent signal likely augments IL-4Rα expression, which then in an autocrine manner, further up regulates IL-4Rα expression,Th2 cells then express large amounts of IL-4Rα and are further stimulated *via* IL-4. In case of Th1 or Th17 cells, the lack of IL-4-positive signal inhibits the up regulation of IL-4Rα, but in the case of Th1 cells, for example, the differentiation does not ablate the ability of the cells to respond to IL-4. Interestingly, Th17 cells do express IL-13Rα1 (Junttila,2018)

Activation of naïve CD4 T cells in the existence of IL-4 via STAT6 signaling leads to the priming of Th2 cells. This subset of CD4 T cells is characterized by its signature transcription factor GATA-3, which promotes induction of Th2 cytokines that include IL-4, IL-5, and IL-13. Th2 cells play an important role in host defense against extracellular parasites, driving various aspects of cellular and

humoral immunity to promote parasite clearance and tissue repair. Their dysregulation contributes to allergic and atopic diseases of particular relevance to staphylococcal disease is atopic dermatitis (AD), a prevalent inflammatory skin disorder that is characterized by the overexpression of Th2 cytokines, which contribute to barrier permeability issues and other features of AD. Skin colonization and infection with *S. aureus* is almost a universal feature of AD (Hatice and Datta, 2017).

When IL-4 or IL-13 is released from T cells, cells carrying the receptors for these cytokines will respond. For IL-4 and IL-13, the unique utilization of the STAT6 transcription factor in the signaling they elicit allows them to implement specific functions on different cell types; IL-4 is the regulator of lymphocyte functions (Th2 differentiation and B-cell IgG1 and IgE class switch), whereas IL-13 is an effector cytokine, regulating smooth cell muscle contraction and mucus production in the airway epithelium, for example, in allergic asthma. thymic stromal lymphopoietin (TSLP) can induce the tyrosine phosphorylation of STAT6 (Junttila, 2018).

The adaptive immune response adventures in later during the course of infection, dependent on the presentation of bacterial antigens by antigen presenting cells and influenced by the cytokine environment generated by the innate response. Through T cell activation and B cell production of antibodies, the adaptive immune response targets specific bacterial antigens and can be recalled during subsequent infections to provide ‘memory’ against that particular pathogen. Antibodies and T cells can have direct activity against bacteria, but also amplify the activity of innate immune cells, e.g. by increasing phagocyte killing and recruitment. The prevalence of recurrent infections with *S. aureus* suggests the adaptive memory response is not completely effective, although it could be argued that the relative paucity of systemic infections despite the high rate of colonization may be evidence for its protective role. Understanding the contribution of the adaptive immune response in determining *S. aureus*

susceptibility may help identify risk factors and therapeutic strategies, and will be essential to harness for successful vaccine development (Karauzum and Datta, 2017).

Staphylococcus aureus. recent research has shown that bacterial wall compounds including lipoproteins ,but not peptidoglycans are centrally required for immune responses elicited in the context of arthritis. Indeed, *S. aureus* lipoproteins potently target macrophage TLR2 to induce chemokines secretion and to either trigger septic arthritis (if lipoproteins were administered alone) or to strengthen the clearance of bacteria in the case of infection . In the case of *S. agalactiae* among the many components proposed to play a role , lipoproteins are important mediators of its virulence (Reuschel *et al*., 2021).

Hence, it remains to be investigated whether the observed exacerbated effects of the *S. aureus* over the other bacteria are mediated by distinct properties of each bacteria lipoproteins and whether, in the context of perinatal infections, lipoproteins also may mediate a comparable effect to arthritis, e.g., by potentiating chemokine secretion and the resolution of infection **(**Reuschel *et al*., 2021).

The majority of T cells are comprised of CD4+ and CD8+ T cells that have long been recognized to be the major cellular arm of adaptive immunity. The major function of CD8+ T cells is to target intracellular pathogens by cytolytic killing of the infected host cell. Consistent with *S. aureus* being a primarily extracellular pathogen, a clear role for CD8+ T cells has not been reported, although CD8+ T cell activation can be detected during *S. aureus* infection and staphylococcal superantigen exposure. Naïve CD4+ T cells are polarized toward different effector functions depending on the cytokine environment in which activation of their TCR occurs. These helper T cell (Th) subsets are functionally characterized by their cytokine expression profiles (Murphy *et al*. 2014).

A percentage of these polarized cells will persist in the host as memory cells awaiting reactivation by subsequent antigen exposure. The role of these different subsets of effector CD4+ T cells in the context of *S. aureus* infection will be reviewed below. In addition to CD4+ and CD8+ T cells, more recently described subsets of T cells, such as γδ T cells, innate lymphoid cells (ILC), and NK T cells, give mainly to the innate immune response at mucosal sites rather than antigen-specific memory, although reports have suggested the potential for γδ T cells to contribute to a memory response under certain circumstances (Murphy *et al*. 2014; Karauzum and Datta ,2017).

3.10.2 Concentration of Interluekin-12p40 in Serum of Rats after challenge dose:

The results indicated not significant increase of Interluekin-12p40 level in group I (10.483 pg/mL) as compared with group II (5.792 pg/mL), at ($p < 0.05$) level as illustrated in (Table 3-10).

Table (3-10): Interluekin-12p40 concentration (Mean ± SD) among the testing groups after challenge dose

Testing groups	No.	Mean value pg/mL	Standard deviation	P- value
Group I	7	10.483	15.673	< 0.05
Group II	7	5.792	6.915	

Group I : *S. aureus* capsular polysaccharide (CP5) -Tween80

Group II : Control group (phosphate buffer saline).

The *P- value* is 0.5176. The result is no significant at $p < 0.05$.

In this study, the non-significant result at ($p < 0.05$) of Interluekin-12p40 level when injection pathogen (*S. aureus*) to occur infection in rats (as animal model) because :-

1. Mechanism immune response of *S. aureus* infection depend on TH2 and TH17 cells.
2. *Staphylococcus aureus*, sceration system display defects in host cytokine responses, specifically the production of interleukin-12 (IL-12) (p40/p70) (Anderson *et al*., 2017)

Interleukin 12 (IL-12) is the first member of the family described It is comprised of the IL-12p35 and IL-12p40 subunits and co-expression of both subunits in the same cell is required to secrete the disulfide-linked bioactive IL12p70 cytokine .Although it is secreted by a variety of hematopoietic cell types, the major physiological producers are antigen-presenting cells (APCs), such as DCs and macrophages. Binding of IL-12 to its high-affinity receptor (IL-12Rβ1/IL-12Rβ2) expressed on activated T cells, NK cells and DCs, activates TYK2 (tyrosine kinase 2), JAK2 and STAT pathways (Sun *et al*., 2015).

Also, IL-12p40 has been shown to act as a chemoattractant for (MΦs) and promotes the migration of stimulated dendritic cells .The p40 subunit is related with several pathogenic inflammatory responses such as silicosis, graft rejection and asthma, but it is also found to be protective in a mycobacterial infection model (Hamza *et al*., 2010).

The mechanisms where by the *S. aureus* ESS pathway tools its immune evasive strategies in the host were heretofore not known. Here, they showed that essE, encoding a membrane-associated protein, is required for *S. aureus* secretion of EsxA, EsxB, EsxC, and EsxD. EssE forms a complex with EsxC and with other components of the ESS pathway, including EssC, EssD, and EssI. In the accompanying paper this report that EssD is also secreted by the ESS pathway and that the protein bears a C-terminal nuclease domain (EssDC), whose activity

is inhibited by EssI in the bacterial cytoplasm. Here, that interaction with EssE in the cytosol of *S. aureus* is important for EssD stability. Unlike wild-type *S. aureus*, essE mutants display defects in host cytokine responses, specifically the production of interleukin-12 (IL-12) (p40/p70) and the suppression of, activators of TH1T cell responses and T cell chemotaxis, respectively .propose that essE-mediated secretion of protein effectors via the ESS pathway may enable *S. aureus* to manipulate host immune responses by modifying the production of specific cytokines (Anderson *et al*., 2017).

The production of IL-12p35 is pre-dominantly regulated at the translational level, transcriptional regulation is responsible for the amount of IL-12p40 expressed. The initial signal triggering IL-12 expression is the exposure of the above mentioned cells to bacteria, viruses, fungi or parasites. Pathogen associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) or CpG DNA ex-pressed or contained in such commensals or pathogens are recognized by pattern recognition receptors (PRRs) of the toll like receptor (TLR) family. This leads to the activation of several transcription factors regulating IL-12 production, most importantly NF-κB and interferon regulatory elements (IRFs) (Ullrich *et al*., 2020).

Interleukin-12 (IL-12) productions a critical role in the early inflammatory response to infection and in the generation of T helper type 1 (Th1) cells (1). IL-12 consists of a heavy chain (p40) and a light chain (p35) linked covalently by disulfide bonds to give rise to the so-called bioactive heterodimeric (p70) molecule (Mondala *et al*., 2020).

Staphylococcus aureus cells SAC induced p40 mRNA, which is shared by IL-12 and IL-23, in the presence or absence of IFN-γ at 2 and 5 hours after stimulation. The pattern of IL-12 p35 transcriptional expression induced by SAC was quite different from that of p40. IL-12 p35 mRNA was stimulated significantly only after IFN-γ priming plus SAC stimulation, similar to the

induction of p35 by LPS that requires IFN- γ and consistent with the dependence of optimal IL-12 p70 protein secretion on the presence of IFN-γ. Since IL-23 is important for IL-17 production and Th17 development, we also examined the induction of IL-23 p19 mRNA by SAC. In contrast to induction of p35, IL-23 p19 mRNA was induced directly by SAC and was not enhanced by IFNγ, instead a slight reduction of IL-23 p19 (Wang *et al*.,2012)

Neutralization of IL-12p40 at the time of vaccination significantly decreased the frequency of Th1 (Tbet+) cells and had no effect on the incidence of Th17). Neutralization of IL-12p40 reversed vaccine-related mortality during subsequent infection, supporting a deleterious role of vaccine-induced Th1cells in this model (Karauzum *et al*., 2017).

Interluekin-17 (IL-17) from either innate or adaptive sources plays an important role against *S. aureus* in murine models of infection at skin and mucosal sites. Induction of Th17 cells by vaccination can enhance protection at these sites, and also against bacteremia. Th17 cells perform to be potentially key players in immunity against *S. aureus*; however, their exact contribution to the control of human staphylococcal infection remains to be fully elucidated and their potential for autoimmune inflammation will need to be kept in check if they are to be targeted by clinical vaccines (Karauzum *et al*., 2017).

Vaccines offer protection against pathogens through multiple and diverse avenues. They do so indirectly by mobilizing the adaptive and innate immune responses. The activated immune system lymphocytes control the proliferation of the microbes, neutralize their toxic secretions or both. The process starts primarily with the creation of pathogen and toxin specific antibodies. The antibodies are formed either from the de novo B cells or from the clonal expansion of the memory B cells. The other effector cells involved are CD8+ and CD4+ T cells. The former, commonly referred as cytotoxic T lymphocytes

(CTL), induce apoptosis in the infected cells and bacteria by secreting granzymes and perforins in the extracellular matrix surrounding the pathogens. In a complementary role, the CD4+ T-helper (Th) lymphocytes assist and control the antibodies and T-cell response through their secreted cytokines (Barinov, *et al*., 2017; Shekhar, 2019).

There are many subgroups of Th cells, each with a unique role in the immune system. T-helper 1 (Th1) cells are the effectors against intracellular bacteria and other monocellular organism while Th2 cells help in removing the extracellular pathogens including bacteria and helminthes. Th17 is especially significant in the context of this research because it defends against the extracellular bacteria and fungi that colonize the epithelia of the skin and mucosa, e.g. *Staphylococcus aureus, Pseudomonas aeruginosa*, *Mycobacterium tuberculosis* and *Bordetella pertussis*, The Th17 effector cells produce interleukin-17 (IL-17), IL-22 and IL-26 in response to the mucosal inflammation. In addition, the B cells activate the C3a complement cascade of the innate immune system (Shekhar, 2019).

3.11: Histopathological Examination Results:

3.11.1 Spleen:

Histopathological examination results of spleen slices, white pulp enlargement with lymphoid follicular changes, white pulp is diffused with red pulp, control group shrinks, eosinophils, plasma cells and lymphocytes infiltrate the cross section ,while the spleen slices of group I* appeared, showing partial changes in the white pulp of the slices of the group, there were lymphoid follicles group I as shown in figure (3-5) while the spleen slices of group II (control group) there is no change in the two spleen areas, as shown in (Figure 3-6). The results are related to the effect of the conjugate of *S. aureus* capsular polysaccharide (CP5) which stimulates the immune response against antigens entering the body.

The spleen is divided by function and structure into the red pulp(RP) and white pulp (WP); in between these two regions is the marginal zone (MZ) in rodents and the perifollicular zone in humans. The WP is the primary immunologic region of the spleen in both species; however, the WP makes up less than a quarter of splenic tissue. The RP makes up the majority of the tissue and has an immune function distinct from that of the WP. Unlike lymph nodes (LNs), the spleen lacks afferent lymphatic vessels, and therefore all cells and antigen enter the spleen via the blood (Lewis *et al*., 2019).

3.11.2.Innate immune cell function and organization

Recognition of infection or host damage in the spleen activates a plethora of pattern recognition receptors (PRRs) on myeloid cells, which in turn induce requisite T cell activation signals on antigen presenting cells (APCs), cytokine secretion, and pathogen clearance in phagocytes. PRRs include Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-I-like RNA helicases and C-type lectin receptors (CLRs). TLRs recognize extracellular or phagocytosed pathogen-associated molecular patterns (PAMPs).

A number of cytosolic NLRs sense both microbial products that gain access to the cell interior (e.g,. intracellular flagellin) as well as host molecules released during states of cell stress or damage (e.g., extracellular ATP) called damage-associated molecular patterns (DAMPs). A number of CLRs are used as molecular markers for specific DC subsets in the spleen, such as Langerin and DC-SIGN, and help these particular subsets recognize carbohydrate moieties and internalize pathogens such as fungi and the exoskeleton of insects. PRRs are selectively expressed on different cell types in particular regions of the spleen, helping tailor the nature of both the early innate and subsequent adaptive immune response (Cerutti *et al*., 2013; Lewis *et al*., 2019).

3.11.3.Adaptive immune cell function and organization

T and B cells, the key effectors of the adaptive immune system, are present throughout the spleen. Their localization changes with activation state and is organized by expression of cell surface receptors and chemotactic gradients. B-2, follicular B cells are the canonical T- dependent antibody-producing B cells in the spleen . Naïve follicular B cells reside in WP follicles, but once activated, B cells rapidly cycle between the light and dark zone of the GC. CXCR5 drives B cells to the light zone, where they receive T cell help, whereas CXCR4 drives them into the dark zone, where they undergo rapid proliferation, class switching, and somatic hypermutation (Lewis *et al* ., 2019).

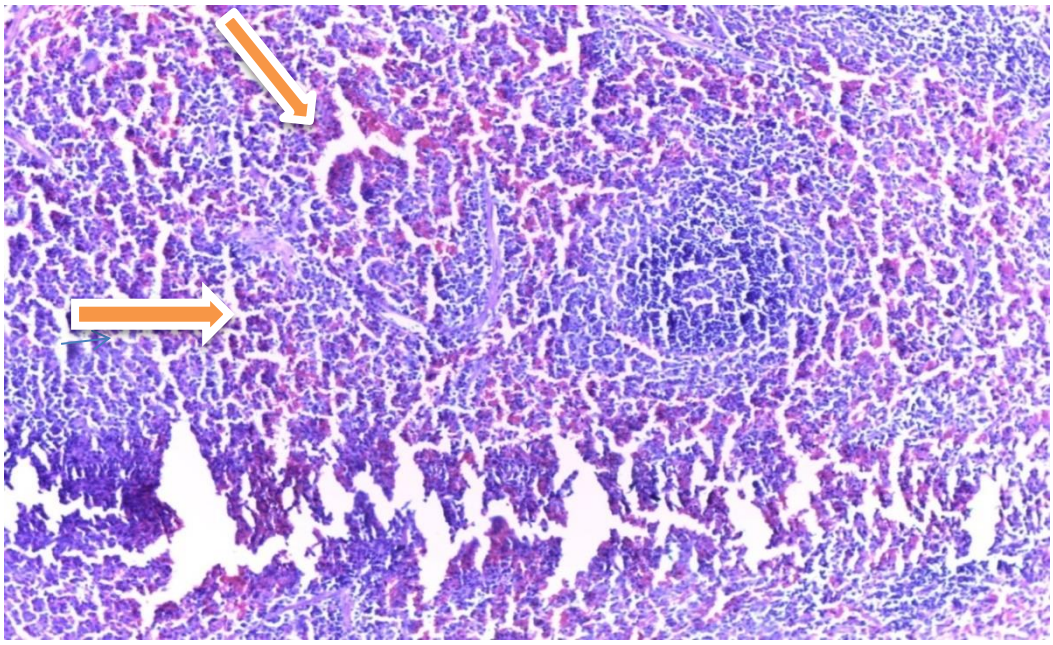

Figure (3-5 Group IA1) Histopathological section of spleen tissue of *S. aureus* capsular polysaccharide (CP5) group showing hyperplasia (partial expansion white pulp changes with lymphoid follicle) (H and E) low power; 10 X .

. : hyperplasia

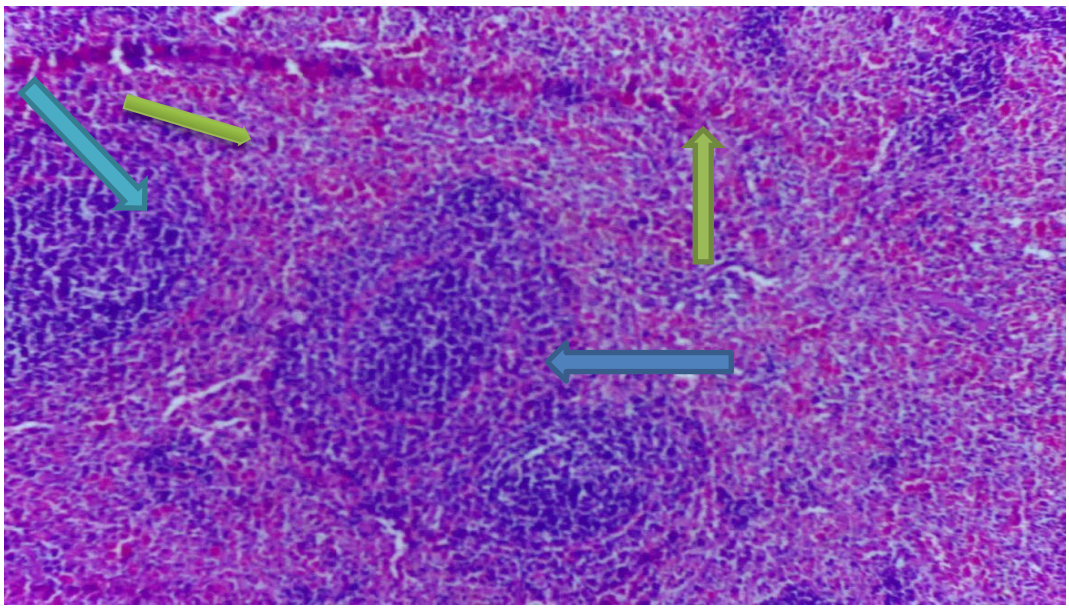

Figure (3-6 Group II B1) Histological section of spleen tissue of Control group showing hypoplasia (no expansion white pulp changes with lymphoid follicle) (H and E) low power; 10X.

Red Pulp , hypoplasia

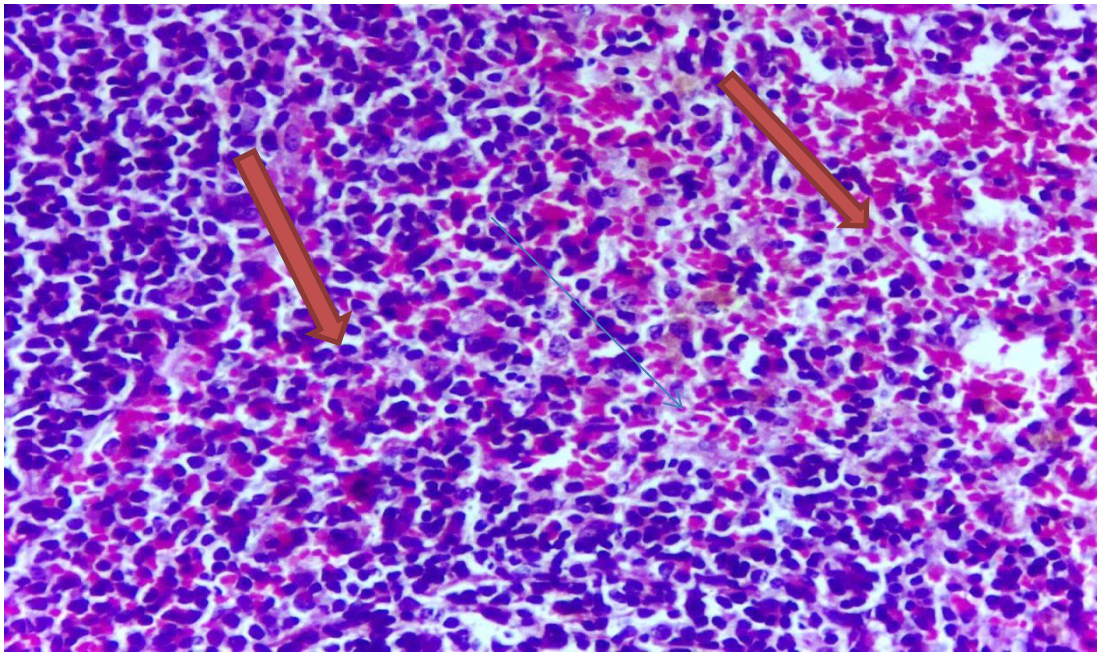

Figure (3-5 Group I A2) Histopathological section of spleen tissue of *S. aureus* capsular polysaccharide (CP5) group showing hyperplasia partial expansion white pulp changes with lymphoid follicle (H and E) high power; 40X; : hyperplasia

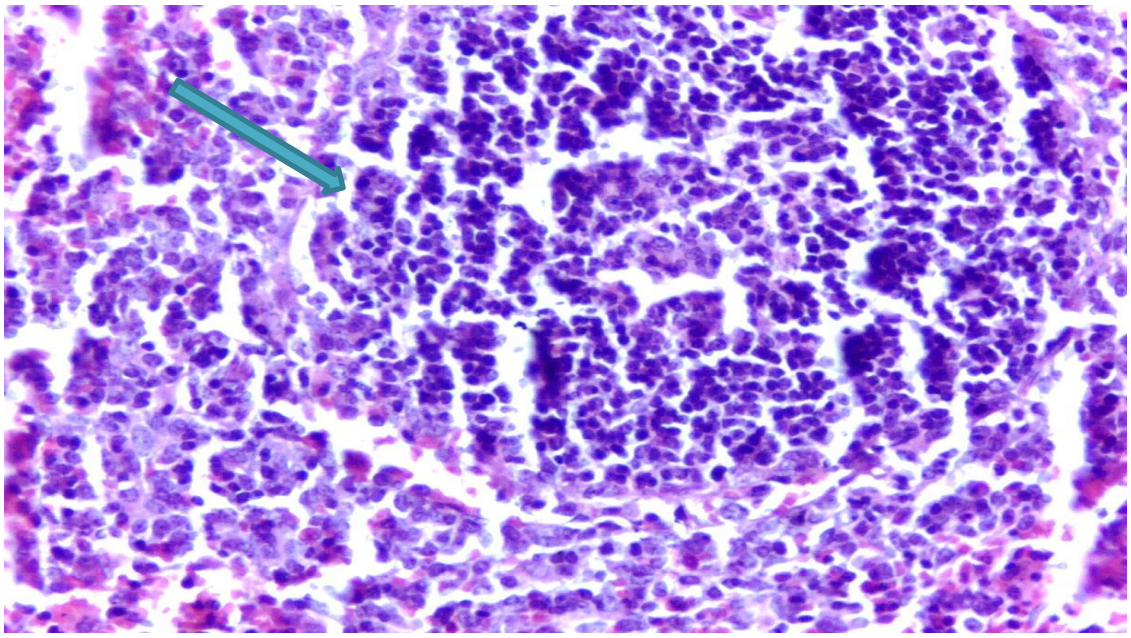

Figure (3-6 Group II B2) Histopathological section of spleen tissue of Control group showing hypoplasia no expansion white pulp changes with lymphoid follicle (H and E)high power; 40X.

hypoplasia

1.2 Literature Review:

1.2.1 Methicillin Resistance *Staphylococcus aureus* (MRSA):

The emergence of infections caused by drug-resistant bacteria is a serious and growing global health concern. Thus, significant efforts are being made in the development of new antimicrobial compounds with improved efficiency (Prestinaci *et al*., 2015; Mannaa *et al*., 2015).

However, despite these efforts, an increasing number of multidrug resistant bacteria consisting methicillin-resistant *S. aureus* (MRSA), extended-spectrum beta-lactamase (ESBL) producing Enterobacteriaceae, and carbapenem-resistant Gram-negative bacteria are being reported continuously (Nurjadi *et al*., 2015;; Al-Charrakh A.H., *et al*., 2016; Radhi and Al-Charrakh ., 2019).

Once, beta-lactams, aminoglycosides, fluoroquinolones, macrolides, and trimethoprim-sulfamethoxazole were considered effective antibiotics to treat infections caused by *S.aureus*. However, its abuse and misappropriation have caused resistance and up to 85% of isolates have been reported to be non-susceptible to most of these antibiotics in current clinical use (Santajit and Indrawattana, 2016).

In late years, antimicrobial resistance has become a major public health issue and MRSA strains which have developed resistance to all beta-lactam antibiotics including penicillins, cephalosporins (except ceftaroline and ceftobiprole), and carbapenems have been limited than 50%. The Centers for Disease Control and Prevention has reported 80,000 severe MRSA infections in the United States alone in 2011, with a rate of 11,000 deaths every year (Najafi, 2016). More than half of hospital-acquired infections are caused by *S. aureus* in most Asian countries (Al-Mohana *et al*., 2012; Centers for Disease Control and Prevention, 2013; Al-Charrakh *et al*., 2015). Similarly, in 2012,

MRSA was valued to have caused infections in over 75,000 patients leading to the death of more than 9,600 in the United States (Centers for Disease Control and Prevention, 2014). In the EU, the proportion of fatal cases is about 50,000 caused by multidrug-resistant staphylococci out of approximately 3 million nosocomial infection cases, as reported by the European Centre for Disease Prevention and Control (Wojtyczka *et al*., 2014).

A Chinese surveillance study reported *S. aureus* as one of the main pathogens causing Blood stream infections (BSIs), with more than half of the strains isolated being resistant to penicillin, erythromycin, cefazolin, and cefuroxime, whereas proportions of MRSA ranged from 30%–40% (Tian *et al*., 2018). In another study, conducted in 26 public hospitals in Hong Kong between January 2010 and December 2012, an increasing rate of MRSA was reported (You *et al*., 2017). In a recent meta-analysis report from Asia Pacific regions, the proportion of MRSA among all tested samples was reported to be up to 39% and the proportion of MRSA among all *S. aureus* isolates was reported to be up to 89% (Ansari *et al*., 2019). Multi-drug-resistant *S. aureus*, consisting MRSA, can easily spread from the hospital setting to the community and within the community and poses additional problems for infection control strategies (Gurunathan *et al*., 2014).

However, infection control programs have been implemented recently in several countries. In the United States, Europe, and many other countries, multiple infection control "bundles" such as allotting single rooms for MRSA-colonized or infected patients, targeted permission screening for high-risk patients and health care workers at high risk for infection with multi-drug-resistant pathogens, molecular typing of all MRSA strains, and decolonization of MRSA carriers, have been initiated and tested to control the spread. As a result of these strategies, a decreasing rate of MRSA has been reported.

However, the pattern of drug resistance still remains a magnificent challenge. Empirical treatment of presumptive *S. aureus* diseases with an alternative to the anti-staphylococcal beta-lactams such as clindamycin and trimethoprim-sulfamethoxazole, became widespread during the 1990s when community-associated MRSA was on the rise until 2000s (Ansari *et al*., 2019).

However, due to the overuse of these antibiotics, an increasing resistance continued to be reported and currently the resistance to these antibiotics posture a great a recent observational study on pediatric clinical cultures performed between 2005 and 2017 in the United States, a declining trend of MRSA from 41% to 27% over the study period, yet an increasing trend of clindamycin (from 21%–38% in MRSA and 5%–40% in MSSA) and trimethoprim-sulfamethoxazole (from 2%–13% in MRSA and relatively stable in MSSA) resistance were reported. (Ansari *et al*.,2019 ; Khamash *et al.,* 2018). Moreover, other studies have reported an increased incidence of MRSA as well as antibiotic resistance (Acree *et al* 2017; Enstrom *et al*., 2018; George *et al*., 2018).

Multiple styles have been implicated in the development of antibiotic resistance, such as over and misuse of antibiotics mostly in developing countries; however, biofilm-mediated drug resistance in bacteria is another major mechanism and it has been predicted that if the current treatment practice continues unchanged, the infections caused by antibiotic-resistant bacteria would be a major cause of death in 2050 where the expected number of deaths will be around 10 million every year (O'Neill *et al*., 2016).

To cope with these multi-drug resistance problems, several anti-staphylococcal drugs such as vancomycin, , linezolid, teicoplanin, tedizolid, daptomycin, tigecycline, ceftaroline, ceftobiprole, oritavancin, and dalbavancin have been suitable for treating the life-threatening infections caused by multi-drug-resistant *S. aureus*. Moreover, currently, in some

countries vancomycin and teicoplanin are the most commonly used drugs to treat MRSA infections (David and Daum, 2017). However, increased Minimum Inhibitory Concentrations (MICs) and reduced susceptibility to these antibiotics, lowly tissue penetration, and adverse reactions due to the use of these antibiotics, have been reported to cause a limitation of its use in clinical practice (Leong *et al*., 2018; Sabat *et al*., 2018).

Because of the emerging problem of resistance, the World Health Organization (WHO) has listed MRSA and recently emerged vancomycin-intermediate and resistant *S. aureus* (VRSA) as "high-priority" deadly bacterial pathogens (World Health Organization, 2017). To overcome the challenging situations in the management of multi-drug-resistant *S. aureus* infections, alternative therapeutic strategies are of utmost importance. (Ansari *et al*., 2019).

The constancy and worldwide spread of this pathogen is due to its' ability to rapidly acquire and defeat resistance and virulence determinants from other members of the genus *Staphylococci* through horizontal transfer of mobile genetic elements (MGEs) (Bitrus *et al*., 2017). Studies on whole genome sequence has revealed that the *S. aureus* genome is divided into a relatively stable core genome which is about 75-80% of the entire genome and a relatively less stable mobile genetic element (MGE) including of transposons, pathogenicity island, Staphylococcus cassette chromosomes, plasmids, bacteriophage and insertion sequence (Ansari *et al*., 2019). The MGEs in *S. aureus* are lineage specific and freely integrate, recombine and transfer in and out of genome via horizontal transfer They encode a wide array of resistance and virulence gene and immune avoidance genes, thus facilitating successful adaptation of MRSA and emergence of new and highly resistant and pathogenic clones (Ansari *et al*., 2019).

1.2.2 Development of antimicrobial resistance in *S. aureus*:

Staphylococcus aureus offers a better and more active model to understanding the complexity of the adaptive advancement of bacteria in the face selective antibiotic pressure. These pathogens have manifested a novel ability to speedily respond to the challenges posed by new antibiotics by the evolution of new antimicrobial resistance mechanisms. Resistance developments in these pathogens occur via alteration of the drug target site,

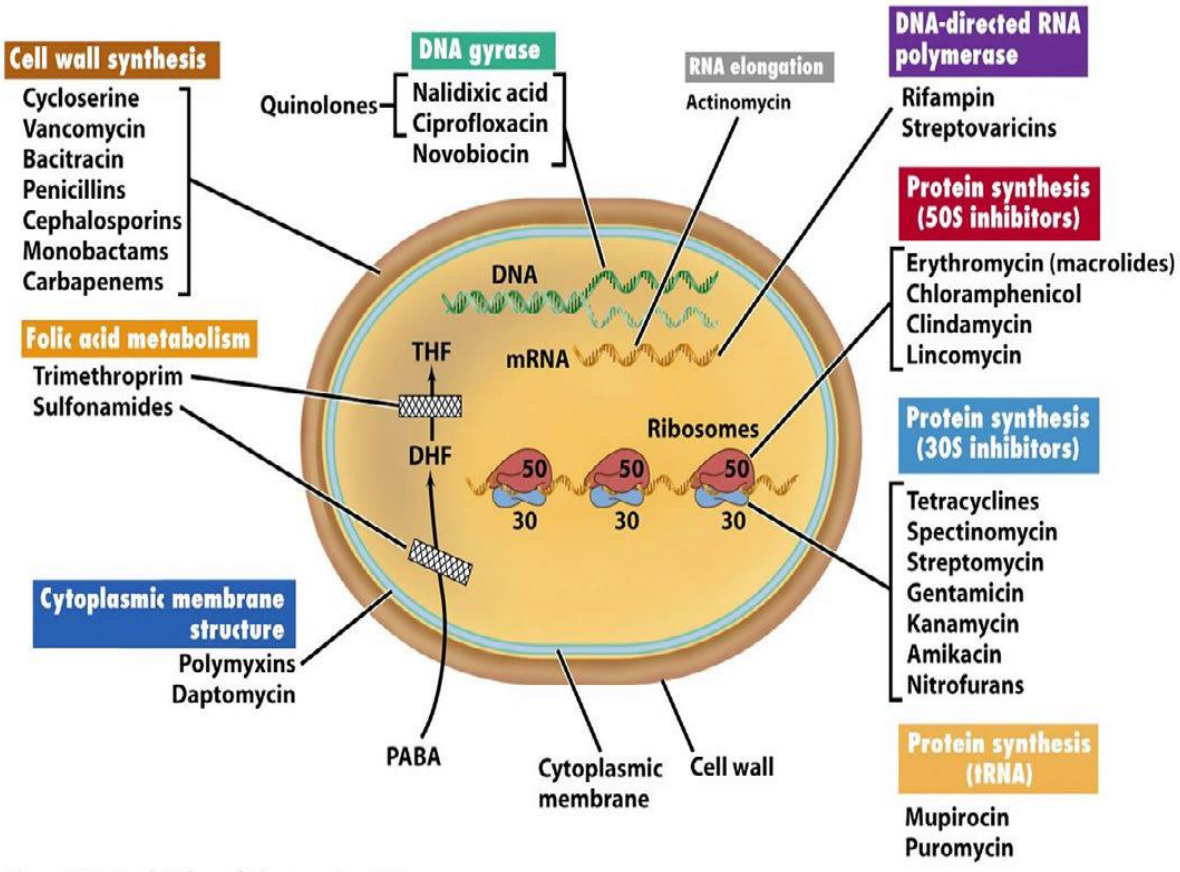

Figure 1-1: Schematic representation of antibiotic class and mechanism of antibiotic resistance in bacteria (Bitrus *et al*., 2018).

enzymatic inactivation of the antimicrobial agent, efflux pump and sequestration of the antimicrobial agent (Figure 1-1). Other resistance mechanisms have developed through acquisition of resistance determinants, position selection and spontaneous mutation (Bitrus *et al.*, 2017).

1.2.3 Virulence factors:

Staphylococcus aureus has battery of virulence factors. These factors facilitate the organism to be successful as pathogen that causes wide range of human and animal infections. Virulence factors help in attachment to host cells, breaking down the host immune shield, tissue invasion, causing sepsis and elicit toxin-mediated syndromes. This is the basis for persistent staphylococcal infections without strong host immune response (Kim *et al*., 2016). Based on their mechanism of action and role in pathogenesis, staphylococcal virulence factors are classified as represented in (Table1-1).

A summary of the various virulence factors of *S. aureus* that play a role in the pathogenesis of many types of infections can be seen in (Figure 1-2), as reported by (Sandi *et al*., 2015).

Table (1-1): Virulence factors of *S. aureus* and its characteristics (Kim *et al*., 2016).

Factors	Characteristics
Helping attachment to host tissues Microbial Surface Components Recognizing adhesive matrix molecules (MSCRAMM)	Cell surface proteins which interact with host molecules such as collagen, fibronectin & fibrinogen, thus, facilitate the tissue attachment. Staphylococcal protein A, fibronectin-binding proteins A and B, collagen-binding protein and clumping factor A & B belong to this family. They are also involved in host immune evasion
Breaking/evading the host immunity Polysaccharide microcapsule Protein A. Panton-Valentine leukocidin (PVL). Alpha-toxin (Alpha hemolysin). Chemotaxis-inhibitory protein of *S. aureus* (CHIPS):	 Resist the phagocytosis & killing by polymorphonuclear phagocyte. It binds to Fc portion of immunoglobulin, prevents opsonization, functions as super antigen & limits the host immune . PVL is found in most of community-associated MRSA (CA-MRSA) .PVL belongs to group of membrane pores forming proteins. It consists of two protein components (LukS-PV and LukF-PV) which act together as subunits and form porins on cell membrane of host cells, leading to leakage of cell contents and cell death. It was the first bacterial exotoxin to be identified as a cell membrane pore former which causes cell leakage and death . CHIPS is an extracellular protein which inhibits the chemotaxis functioning of neutrophil and monocytes
Tissue invasion Extracellular adherence protein (Eap) Proteases, lipases, nucleases, hyaluronatelyase, phospholipase C, metallo-proteases (elastase), and Staphylokinase.	 An exoprotein which binds to host cell matrix, plasma proteins & endothelial cell adhesion molecule ICAM-1. In addition to the roles of adhesion and invasion, it also has immune-modulatory activity . These extracellular enzymes cause tissue destruction and, thereby, help in bacterial penetration into tissues.
Induces toxinosis Enterotoxins	 *S. aureus* produces battery of enterotoxins which are potent gastrointestinal exotoxins. The

	Staphylococcal food poisoning is an intoxication which results from consumption of foods containing sufficient amount of preformed enterotoxins.
Toxic shock syndrome toxin -1 (TSST-1).	TSST-1 & some of enterotoxins are called as pyrogenic toxin super antigens. TSST-1 causes toxic shock syndrome especially in menstrual women .
Exfoliative toxins A and B.	Serine proteases which selectively recognize and hydrolyze desmosomal proteins in the skin. ETs cause staphylococcus-scalded skin syndrome, a disease predominantly affecting infants.

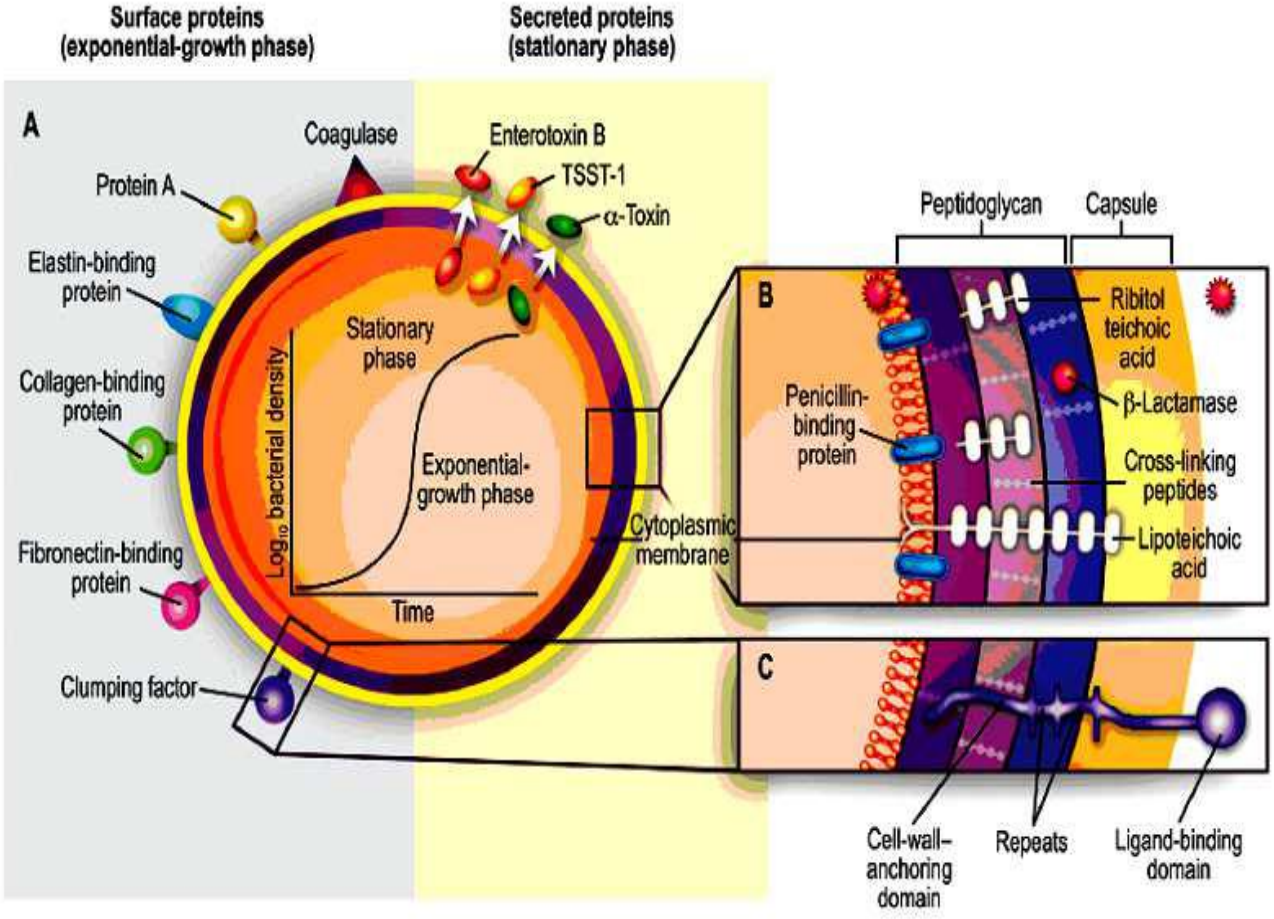

Figure (1-2): Pathogenic factors from a whole cell *S. aureus* of that have structure of surface protein (part A,B,C) known to have a role in *S. aureus* pathogenicity (Sandi *et al*., 2015).

1.2.4 Cytokines

Cytokines are a cell-signaling group of low molecular weight extracellular polypeptides/ glycoproteins synthesized by different immune cells, mainly, by T cells, neutrophils and macrophages, which are responsible to promote and regulate immune response (i.e. activity, differentiation, proliferation and production of cells and other cytokines). These polypeptides action on signaling molecules and cells, stimulating them toward sites of inflammation, infections, traumas, acting on primary lymphocyte growth factors and other biological functions(Ferreira *et al*,2018)

1.2.4.1 Interleukin-4 (IL-4)

The cytokines interleukin (IL)-4 and IL-13, signaling via the IL-4 receptor (IL-4R), orchestrate type 2 immunity to helminth infections and toxins. Activation of epithelial and myeloid cells, and a transient neutrophils influx initiates type 2 immune responses, which are conquered by basophils, eosinophils, mast cells, B cell immunoglobulin E production, and type 2 T helper and T follicular helper cells. Interestingly, IL-4 and IL-13 can curtail chemotaxis and several effector functions of neutrophils in mice and humans. This inhibitory role of IL-4 and IL-13 probably developed to limit tissue damage by neutrophils during type 2 immunity where a “weep and sweep” response aims at expulsion and decreased fecundity, instead of killing, of macroparasites immunity (Heeb *et al*, 2020).

1.2.4.2 Interleukin-12 p40 (IL 12p40)

As its first identified member, Interleukin-12 (IL-12) named a whole family of cytokines. In response to pathogens, the heterodimeric protein, consisting of

the two subunits p35 and p40, is secreted by phagocytic cells. Binding of IL-12 to the IL-12 receptor (IL-12R) on T and natural killer (NK) cells leads to signaling via signal transducer and activator of transcription 4 (STAT4) and subsequent interferon *gamma* (IFN-γ) production and secretion. Signaling downstream of IFN-γ includes activation of T-box transcription factor TBX21 (Tbet) and induces pro-inflamma-tory functions of T helper 1 (TH1) cells, thereby linking innate and adaptive immune responses. Initial views on the role of IL-12 and clinical efforts to translate them into therapeutic approaches had to be re-interpreted following the discovery of other members of the IL-12 family, such as IL-23, sharing a subunit with IL-12(Ullrich *et al* .,2020)

IL-12 is produced mainly by Dendritic cell Macrophages, monocytes, neutrophils, microglia cells and, to a lesser extent, by B cells; human but not murine B cells were found to produce IL-12 following CD40 ligation (Al-Mamouri ,L.S .2016).

1.2.5.1 Immunotherapy for *Staphylococcus aureus*:

Staphylococcus aureus causes severe disease in humans for which no licensed vaccine exists. A novel *S. aureus* vaccine (SA4Ag) is in development, targeting the capsular polysaccharides (CPs) and two virulence-associated surface proteins. Vaccine-elicited antibody responses to CPs are efficacious against serious infection by other encapsulated bacteria. Studies of natural *S. aureus* infection have also shown a role for TH17 and/or TH1 responses in protection. Single-antigen vaccines, including CPs, have not been effective against *S. aureus*; a multi antigen vaccine line is likely required. However, the influence of addition of protein antigens on the immune response to CPs has not been studied. Here, the immune response induced by a bivalent CP conjugate vaccine (to model the established mechanism of action of vaccine-induced protection against Gram-positive pathogens) was compared to

the response induced by SA4Ag, which contains both CP conjugates and protein antigens (Dupont *et al*., 2018).

Like many bacterial pathogens, *S. aureus* can evade detection by innate immune receptors by encapsulating itself with polysaccharides (Nanra *et al*., 2013) . Capsular polysaccharides (CPs) have been well recognized as effective targets for vaccines against encapsulated bacteria, such as *Streptococcus pneumoniae, Haemophilus influenzae,* and *Neisseria meningitidis*. As many as eight capsular serotypes have been identified in *S. aureus*, but the large part of disease-causing isolates express either capsular polysaccharide 5 (CP5) or CP8, making these attractive vaccine targets (Dupont *et al*., 2018). Expression of capsular polysaccharides, however, can be forceful during infection, and targeting additional protein antigens may be necessary for adequate protection (Dupont *et al*., 2018).

Two *S. aureus* surface proteins that have been identified as major virulence factors may serve as additional vaccine targets: manganese transport protein C (MntC) and clumping factor A (ClfA). MntC is included in the scavenging of manganese ions, which are important both as nutrients for *S. aureus* and as cofactors for superoxide dismutase, which enables *S. aureus* existence of the neutrophil respiratory burst. ClfA promotes binding of *S. aureus* to platelets and fibrogen, which is necessary for disease pathogenesis in several models of infection with this pathogen (Scully *et al*., 2015). Preclinical studies have demonstrated the ability of these antigens to improvement protection in animal models (Anderson *et al*., 2012). ClfA and MntC may therefore serve as valuable antigens to combine with capsular polysaccharide conjugates in a vaccine against *S. aureus* (Dupont *et al*., 2018).

1.2.6 Overview of Vaccine current effort:

A. Either vaccines currently available and their limitations or biological feasibility for vaccine development. Prior *S. aureus* infection does not provide protection against consequent infection, but infections among carriers are less severe, suggesting that some form of immunity does advance during prolonged colonization. Although all adults have pre-existing binding antibodies to *S. aureus* antigens, including capsule and clumping factor A (ClfA), these do not typically include functional antibodies that have opsonophagocytic or neutralizing properties, and therefore do not provide protection against infection (Giersinga *et al*., 2016).

There is precedence for the development of safe and effective bacterial vaccines that target single antigens or toxins, particularly capsular polysaccharides. The most prominent examples are the tetanus toxoid and pneumococcal conjugate vaccines. Application of these technologies to *S. aureus* is problematical by the bacterium's complex mechanisms of pathogenesis. *S. aureus* can comprise the normal human flora and, as such, has evolved a number of strategies to colonize and avoid the host immunity, including polymorphic expression of surface antigens and release of multiple redundant virulence factors (Golubchik *et al*., 2013).

These include iron acquisition factors such as IsdB, manganese uptake receptors such as MntABC, fibronectin binding proteins (ClfA, ClfB), polysaccharide capsule molecules (CP5 and CP8) and toxic shock syndrome toxin (TSST). To date, vaccine candidates have targeted individual cell surface components, such as the polysaccharide capsule, extracellular polysaccharides or cell wall associated proteins that support attachment, invasion or act as a receptor (e.g., hemoglobin for iron utilization). Although multiple vaccine candidates have shown promise through preclinical development in a range of

animal models, those that have reached late stage clinical testing have failed to demonstrate efficacy in human trials (Salgado-Pabón *et al*., 2014) .

B. General methodologies to vaccine development for this disease for low and middle income country markets *S. aureus* has not been viewed as a high-priority pathogen in low-income countries. However, based on the limited data avail-able, the incidence and mortality from multidrug-resistant *S. aureus* in these regions is likely significant, To date, only two vaccines have finished human efficacy, and neither have contemplated target populations or indications that are prevalent in low- and middle-income countries(Giersinga *et al*., 2016).

StaphVAX is a bivalent polysaccharide and protein-conjugated vaccine, directed against *S. aureus* capsular polysaccharide types 5 and 8 (CP5 and CP8), which are related with approximately 80% of *S. aureus* clinical infections. The candidate was evaluated in two phase III studies to prevent bacteremia in end-stage renal dialysis patients in the 3–54 weeks following immunization. In the first 40 weeks, bacteremia was reduced by 57% but efficacy dropped to 26% by week 54 (Giersinga *et al*., 2016).

A confirmatory Phase III study involving 3600 heamodialysis patients who were evaluated for bacteremia revealed no difference between vaccinated individuals and the placebo controls. The functional antibody titers induced by the vaccine in this second follow-up phase III study have not yet been made publicly available. Currently, then, the main reason for the second trial's failure is being attributed to manufacturing contradictions between different vaccine lots used for the two studies (Fattom *et al*.,2015) .

Development of the candidate has been discontinued. Another candidate, V710, elicits immunity against the cell-wall anchored iron scavenger protein IsdB, and was evaluated in a Phase III randomized controlled trial in

approximately 8000 adults listed for cardiac surgery. This trial was terminated when an interim analysis showed a statistically significant increase in mortality rate due to *S. aureus* infection and a significantly higher rate of other adverse events (Fowler *et al*., 2013).

Passive immunization strategies utilizing both polyclonal and monoclonal antibodies (mAbs) have been targeted for those who are immunocompromised and cannot mount an independent, strong immune response and for those at immediate threat of infection and do not have time to for an active immunization to take effect. Five antibody candidates have been developed and evaluated in late stage clinical studies; none have demonstrated efficacy (Fowler *et al*., 2014).

The focus has been on development of a prophylactic product that will protect against life-threatening *S. aureus* infections, but it is hoped that such a vaccine would also defend against all *S. aureus* infections including more commonly encountered skin and soft tissue infections. To date, however, no product has been shown to protect against any tested outcome (Giersinga *et al*., 2016).

Vaccines, Reverse Vaccinology, and Bacterial Pathogenesis(Figure 1-3)

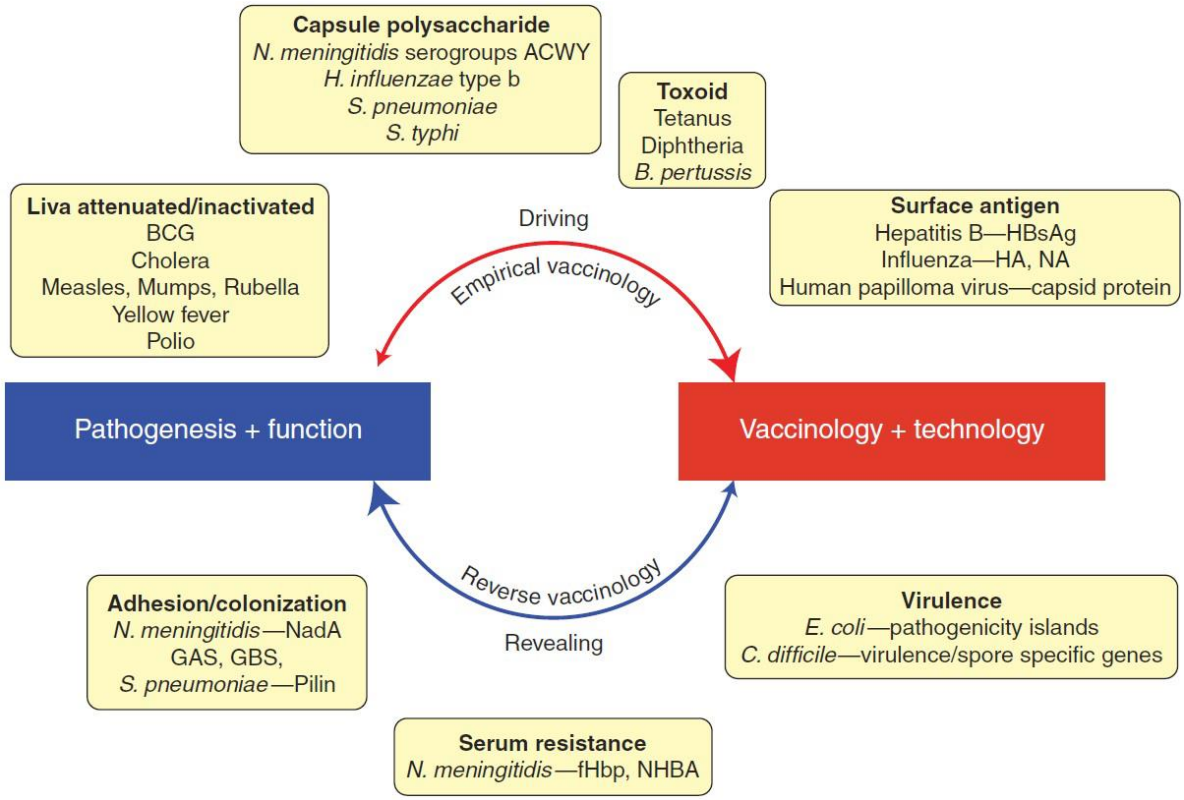

Figure 1-3: Empirical and reverse vaccinology. From "Vaccines, Reverse Vaccinology, and Bacterial Pathogenesis"**(**Sharma and Shekhar , 2019).

1.2.7 *Staphylococcus aureus* vaccine preclinical and clinical development: current state of the art:

1. **preclinical studies:**

About half of the analysed papers describe the preclinical phases of *S. aureus* vaccine candidates mainly using the murine model. This is a crucial stage in the development of immunization strategies, because a failure in this phase observably threatens any further research. GlaxoSmithKline (GSK) approached active immunization in mice and rabbits using the capsular polysaccharide antigens serotype 5 and 8 (respectively CP5 and CP8), responsible for cellular adhesion, and detoxified α-hemolysin (HlaH35L) that plays a crucial role in invasive

infections (Giersing *et al*., 2016, Reddy *et al*., 2017). The vaccine was produced by recombinant technology in *Escherichia coli*, procurement a bioconjugated and N-glycosylated protein (Wacker *et al*., 2014). Even though elicited antibodies in immunized animals were protective against bacteraemia and pneumonia, there was no further development of this study (Reddy *et al*., 2017).

CRM197 (a nontoxic recombinant mutant of diphtheria toxin)-conjugated polysaccharide antigens CP5 and CP8 have been valuated as vaccine candidates by Cheng *et al*. in a murine model of bacteraemia, lethal sepsis, and skin infection. Even if a noble antibody response was elicited and active immunization protected against staphylococcal bacteraemia, only the CP8-CRM component protected against dermonecrosis and neither CP5-CRM nor CP8-CRM protected against mortality in the sepsis model (Cheng *et al*., 2017).

A multicomponent surface protein (SdrE, IsdA, SdrD, IsdB) target vaccine was developed by Novartis (now GSK) and revealed a protection from lethal doses of *S. aureus* strains in mice (Reddy *et al*., 2017). The same company has recently formed an alum adjuvated vaccine, named 4C-Staph. It was targeted on four different antigens: the previously described HlaH35L in assortment with EsxAB, FhuD2, Csa1A. EsxAB is a fusion of two virulence secreted factors involved in abscess formation, FhuD2 is a lipoprotein involved in iron uptake, while the role of lipoprotein Csa1A is still not clearly understood (Mancini F, *et al*., 2016; Dayan *et al*., 2016). The advantageous effects of this quadrivalent vaccine have been shown in a murine model of joint and lung infections, with robust antibody response and CD4+ T lymphocyte

activation (Corrado *et al*., 2016). To date, there is no information on further development (Giersing *et al*., 2016;Reddy *et al*., 2017).

Another potential vaccine *S. aureus* antigen is the surface protein Clamping factor A (ClfA) that allows adhesion to several human tissues by fibrinogen binding. The successful preclinical study on ClfA opened the way to its request in multiple antigen vaccines, which are in advanced stages of development (Lacey *et al*., 2016; Dayan *et al*., 2016).

An equally successful preclinical act was not achieved by a recombinant vaccine (AT62, by the National Institute of Allergy and Infectious Diseases, USA) based on the α-hemolysin (Hla) subunit, that showed a weak activity in avoiding murine surgical wound infections, despite a robust antibody response. The Hla subunit seems nevertheless to be suitable for the development of multivalent vaccines (Adhikari *et al*., 2012).

An stimulating immunization target under evaluation, by the Pasture Institute of Iran and Pharmaceutical Sciences Branch of Islamic Azad University, is the Penicillin Binding Protein 2A (PBP2a) complicated in beta-lactam resistance due to target mutation. Vaccine based on PBP2a reduced the mortality rate and protected mice against lethal MRSA challenge (Haghighat *et al*., 2017). Other possible vaccine candidates are a mutant live *S. aureus*, unable to synthetize cell wall D-alanine (Moscoso *et al*., 2018) and a bivalent fusion vaccine based on the D domain of staphylococcal protein A (SpA) and the A domain of fibronectin-binding protein A (FnBPA), by the National Natural Science Foundation of China (Yang *et al*., 2018). Vaccination with the mutant live *S. aureus* resulted in a protective effect against *S. aureus* bacteremia in mice (Moscoso *et al*., 2018). The bivalent fusion vaccine showed a

protective efficiency in murine pneumonia and a skin abscess model (Yang *et al*., 2018).

Original studies reporting preclinical and clinical trials (where available) are listed in Tables (1-2) and (1- 3).

Table (1-2): Developing vaccines in preclinical phase studies (Redi *et al*., 2018).

Vaccine	Developer	Target antigen (type)	Valued immune response	Status and results	Original article reference
Glycovaxine	GSK	CP5/CP8/HlaH35L (recombinant)	Humoral	Completed, efficacy, no further development	Wacker *et al*., 2014.
PentaStaph	Nabi and USUHS	CP5/CP8/HlaH35L plus LukS-PV and wall teichoic acids (N.A.)	Humoral	Ongoing studies (sold to GSK), efficacy valuated separately for the each components	Schaffer and Lee, 2009.
N.A	N.A	CP5/CP8 (purified, CRM197 conjugated)	Humoral	Completed, elicited protection in mice against bacteremia, but not lethal sepsis; in the skin infection model, only conjugated CP8 protected against dermonecrosis	Cheng *et al.*, 2017.
N.A	Novartis (now GSK)	SdrE, IsdA, SdrD, IsdB surface proteins (recombinant, alum adjuvated	Humoral	Ongoing, efficacy, protection from lethality in mouse infection model	Stranger-Jones *et al*., 2006.
4C-Staph	Novartis (now GSK)	FhuD2, EsxAB, HlaH35L, Csa1A (purified, alum adjuvated)	Humoral and cellular	Completed, efficacy, reduction of murine lung infections and arthritis	Torre *et al*., 2015 ; Mancini *et al*., 2016.
N.A.	N.A.	ClfA (purified, or recombinant)	Humoral	Completed, efficacy, prevention of murine arthritis	Josefsson *et al*., 2001.
N.A.	NIAID	AT62 (recombinant from Hla)	Humoral	Stopped, scarce control of murine skin infections.	Adhikari *et al*., 2012.

N.A.	Pasture Institute of Iran and IAUPS	PBP2a (recombinant)	Humoral	Completed, efficacy, reduced mortality against bacteriemic MRSA infection	Haghighat *et al*., 2017.
N.A	N.A	D-alanine auxotrophic mutant (live mutant bacterium	Humoral and cellular	Completed, efficacy, reduction of abscesses formation in mice	Moscoso *et al*., 2018.
SpA-DKKAAFnBPA37-507 (SF)	NSFC	SpA/FnBPA (bivalent fusion vaccine, recombinant proteins)	Humoral and cellular	Completed, efficacy, reduction of pneumonia and skin abscesses in mice	Yang *et al*., 2018.

GSK: GlaxoSmithKline; CP: capsular polysaccharide antigens; Hla/AT: α-toxin; CRM: cross-reacting mutant, a nontoxic recombinant LukS-PV: Panton–Valentine leukocidin component S; N.A.: not available; Sdr: serine-aspartate repeat proteins; Isd: iron-regulated surface determinant; Fhu: ferric hydroxamate uptake; Esx: secretion system protein; Csa: conserved staphylococcal antigens; Clf: clumping factor; NIAID: National Institute of Allergy and Infectious Diseases, USA; IAUPS: Islamic Azad University, Pharmaceutical Sciences Branch; PBP: penicillin binding protein; NSFC:National Natural Science Foundation of China; Sp: staphylococcal protein; FnBP: fibronectin-binding protein**.**

2. Clinical studies:

Phase I Despite the efficiency obtained in the preclinical studies, some of the evaluated vaccine candidates did not suffer further development. A composed target vaccine (conjugated to tetanus toxin CP5/CP8 polysaccharides plus re combinant Hla/ClfA proteins) was developed by GSK, and it completed the phase I clinical trial (Dayan *et al*., 2016; Mohamed *et al*., 2017).

This vaccine elicited an increase in functional humoral antibody responses that could kill CP5-expressing strains in opsonophagocytic assays after a single dose, but an inefficient T-cell activation. No safety concerns arose during this study but this vaccine was not further developed (Levy *et al*., 2015; Giersing *et al*., 2016; Reddy *et al*., 2017).

A hypothetically hopeful immunization strategy was proposed by NovaDigm Therapeutics with the so called NDV3 vaccine. This vaccine consists of an alum adjuvated, recombinant antigen rAls3p-N (agglutinin like sequence 3 protein), a *C. albicans* surface protein that cross reacts with S. aureus (Lacey *et al*., 2016). NDV3 previously demonstrated a preclinical efficacy in reducing murine skin abscesses, so it was carried on phase I, showing safety and immunogenicity (Dayan *et al*., 2016). NDV3 is currently under study for the avoidance of Candida vaginitis (Giersing *et al*., 2016).

A cell wall vaccine, SA75 by Vaccine Research International, has shown good tolerability and safety during phase I, but it was not further developed (Giersing *et al*., 2016). Indeed, preclinical studies on similar types of cell wall vaccines showed controversial results, with sufficient immunogenicity only after intravenous injection, even if an efficient cellular and humoral response was observed in the murine model of skin and soft tissue infections (Selle *et al*., 2016, Zhang *et al*., 2017).

Secreted virulence factors have also been evaluated in phase I trials. Recombinant staphylococcal enterotoxins A and C1 by Integrated BioTherapeutics showed a safe profile (Roetzer *et al*., 2017). Moreover, Integrated Bio Therapeutics, in cooperation with the National Institute of Allergy and Infectious Diseases (NIAID), demonstrated a production of functional toxin-neutralizing antibodies in adults after immunization with STEBVax, an alum adjuvated recombinant enterotoxin B (rSEB) (Chen *et al*., 2016).

The SA4Ag vaccine by Pfizer involve four *S. aureus* virulence factors: CP5 and CP8 conjugated with diphteric toxoid plus recombinant-mutated ClfA and recombinant-mutated MntC (manganese transporter protein C). A previous use of an SA3Ag vaccine (lacking of MntC) and of SA4Ag showed an acceptable protection for both, but SA4Ag showed a more robust humoral immune

response (Esposito *et al*., 2016; Begier *et al*., 2017; Creech *et al*., 2017; Mohamed *et al*., 2017; Xu *et al*., 2018).

One of the most recent phase I trials was accompanied on the bivalent recombinant α-toxin and Panton Valentine Leukocidin vaccine (rAT/r rLukS-PV) produced by Nabi. It was investigated on healthy militarie personnel obtaining positive results in terms of safety and long-term immunogenicity (Landrum *et al*., 2017).

Phase II There are no ongoing phase II studies.

Phase II of the previously designated NDV3 by NovaDigm Therapeutics was stopped due to enrolment difficulties (Lacey *et al*., 2016). The use of the previously characterized recombinant staphylococcal enterotoxins A and C1 by Integrated BioTherapeutics is under evaluation for a phase II trial (Roetzer *et al*., 2017). SA4Ag (PF-06290510) is the only candidate tested in an ongoing phase IIb trial: the STRIVE (*Staphylococcus aureus* surgical In patient Vaccine efficiency) study aims to confirm the phase I results in a wider target population of adults receiving spinal surgery (Begier *et al*., 2017., Mohamed *et al*., 2017).

Phase III

Two phase III trials testing a purified CP5/CP8 conjugated with recombinant pseudomonal exotoxin A, StaphVax, by Nabi as well as a purified surface protein IsdB, V710 by Merck, were interrupted due to the absence of difference in the primary endpoint between vaccine and placebo for StaphVax and an increased mortality in exposed subjects for V710 (Giersing *et al*., 2016;; Reddy *et al*., 2017; Missiakas and Schneewind, 2016; Mohamed *et al*., 2017; Pozzi *et al*., 2017; Lacey *et al*., 2016). No other clinical phase III trial is ongoing or under evaluation. Likely manufacturing matters causing failure of StaphVax were hypothesized (Fattom *et al*., 2015; Dayan *et al.* 2016), but its

capsular polysaccharide antigens are further being evaluated within the PentaStaph vaccine (Redi *et al*., 2018).

Table (1-3): Developing vaccines in clinical phases (Redi *et al*., 2018).

Vaccine	Developer	Target antigen (type)	Valued immune response	Phase	Status and results	Key review or original article reference
GSK2392103A	GSK	CP5/CP8/Hla/ClfA (conjugated CP5/CP8 plus recombinant Hla/ClfA	N.A.	Phase I	Completed, no further development	Levy *et al*., 2015
NDV3	NovaDigm Therapeutics	rAls3p-N (C. albicans surface protein cross reacting with *S. aureus*; alum adjuvated)	Humoral and cellular	Phase I	Completed, safety and immunogenicity, stopped phase II due to enrolment problems	Schmidt *et al*., 2012
SA75	Vaccine Research International	Whole cell vaccine	Humoral and cellular	Phase I	Completed, safety and tolerability, no further development	Giersing *et al*., 2016
N.A.	Integrated BioTherapeutics	Enterotoxins A and C1, TSST (recombinant)	Humoral	Phase I	Completed, safety, evaluating possible phase II trial	Roetzer *et al*., 2016
STEBvax	Integrated BioTherapeutics and NIAID	Enterotoxin B (rSEB) (recombinant, alum adjuvated)	Humoral	Phase I	Completed, safety, demostrated production of toxin neutralizing antibodies	Chen *et al*., 2016
SA4Ag (PF-06290510	Pfizer	ClfA/MntC/CP5/CP8 (conjugated CP5/CP8 plus recombinant MntC/ClfA)	Humoral and cellular	Phase I	Completed, safety, robust immune response, ongoing phase IIb in adults receiving spinal surgery.	Creech *et al*., 2017; Begier *et al*., 2017; Frenck *et al*., 2017.
N.A.	Nabi	rAT(α-	Humoral	Phase I	Completed,	Landrum *et al*.,

		toxin)/rLukS-PV (recombinant)			safety, robust immune response.	2017
StaphVAX	Nabi	CP5/CP8 (purified and conjugated capsular polysaccharides)	Humoral	Phase III	Stopped, no differences between vaccine and placebo in end-stage renal patients	Fattom *et al.*, 2015
V710	Merck	IsdB (purified surface protein)	Humoral	Phase III	Stopped, increased mortality in vaccinated subjects postcardiotho-racic surgery	McNeely *et al*., 2014

GSK: GlaxoSmithKline; CP: capsular polysaccharide antigens; Hla/AT: α-toxin; Clf: clumping factor; Als3p: agglutinin like sequence 3 protein; TSST: toxic shock syndrome toxin; NIAID: National Institute of Allergy and Infectious Diseases, USA; Mnt: manganese transporter protein; LukS-PV: Panton–Valentine leukocidin component S; Isd: iron surface determinant.

1.2.8 Capsular polysaccharides (CPs) as vaccine candidates:

Bacterial capsule is an extracellular material, which can be microscopically visualized using special techniques, covering the bacterial cells. Several bacteria have been found to possess the capsules such as *E. coli, Neisseria meningitidis, Streptococcus pneumoniae, Haemophilus influenzae* as well as *S. aureus*. Bacterial capsules are composed of long polysaccharide chains known as CPs. Capsules are the bacterial structure first recognized by the immune system, therefore, encapsulated bacteria have developed an immune evasion property which is exploited in the development of vaccines (Ansari *et al*., 2019).

The CPs have been targeted as an effective vaccine candidate for the protection from many bacterial infections such as *S. pneumoniae*, *H. influenzae, and N. meningitides* (Trotter *et al*., 2008)

As many as eight different serotypes of capsules such as CP 1–8 (CP1 to CP8) have been found in *S. aureus*; however, the majority of the isolates

causing diseases have CP5 and CP8 which are the major effective vaccine targets (Ansari *et al*., 2019).

The expression of these CPs can be dynamic during infection, therefore, additional protein antigens are required for adequate protection(Nanra *et al*., 2009) .

In 2002, the first *S.aureus* vaccine StaphVAX, developed by Nabi Biopharmaceuticals, consisting of CP5 and CP8 conjugated to recombinant *P. aeruginosa* exoprotein A, was used as a vaccine candidate in patients receiving hemodialysis in its initial phase III clinical trials. However, the study failed to show a significant protective influence compared with placebo in a follow-up period of 3–54 weeks post vaccination. It was suggested that it may be due to many reasons such as the population targeted, production of the sub optimal conjugate, or varying conjugate manufacture between in the *S.aureus* bacteremia number in the follow-up period of 3–40 weeks post-vaccination was found in a subsequent trial (Ansari *et al*., 2019).

Based on this partial protection, Fattom *et al* conducted a similar study using StaphVAX in the same patient population receiving hemodialysis. The assessment of the protective efficiency in vaccine recipients vs placebo up to 35 weeks after receiving a single dose or up to 60 weeks after receiving one or two vaccine doses suggested no protection against *S. aureus* bacteremia (Fattom *et al.*, 2015).

The failure of this vaccine containing two single-antigens suggested that a multi-antigen vaccine containing several antigens might be efficacious. As a result, the first generation of multi-antigen vaccine containing three antigens (*S. aureus* three-antigen [SA3Ag]) such as CP5, CP8 conjugated to the CRM197 and ClfAwas designed (Rozemeijer *et al*., 2015).

Two types of vaccines namely, SA3Ag vaccine having CP5, CP8, and ClfA and *S. aureus* four antigen (SA4Ag) vaccine possessing CP5, CP8, ClfA, and recombinant P305A developed from a lipoprotein manganese transporter C (MntC) have been successfully developed by the researchers, which have exhibited superior immunogenicity compared to previous vaccines (Begier *et al*., 2017; Frenck *et al*., 2017; Creech *et al*., 2017).

The studies have revealed that the previous vaccines generated anti-staphylococcal antibodies capable of binding with *S. aureus* foremost to the uptake by phagocytic cells while the multi-antigen vaccines (SA3Ag and SA4Ag) are able of inducing high level of anti-staphylococcal antibodies that lead to the killing of *S. aureus* by increasing the phagocytosis of bacteria and were concluded to be safe with no significant increase in systemic adverse effects or local adverse effects in healthy adults (Ansari *et al*., 2019).

The partial success of the first phase trial stimulated the researchers to design a novel multi-antigen vaccine (SA4Ag) containing CP5 and CP8 conjugated with CRM197 (CP5-CRM197 and CP8-CRM197) together with MntC and ClfA antigens (Dupont *et al*., 2018).

A multicenter phase I /II trial study conducted in the United States evaluated the immunogenicity, safety, and tolerability of SA4Ag vaccine in healthy adult volunteers of 18–64 years of age when injected as a single intramuscular dose (Dupont *et al*., 2018).

The findings of a recent animal model study demonstrated that this vaccine could elicit cytokine production by naive peripheral blood mononuclear cells leading to the generation of anti-staphylococcal antibodies and memory B-cell response (Frenck *et al*., 2017).

A phase II/III study to evaluate the efficiency of the SA4Ag vaccine for the prevention of invasive *S. aureus* disease in patients between 18–85 years of age who have had elective spinal surgery is under way (Mohamed *et al*., 2017; Jansen *et al*., 2018).

The early stage of clinical trials inducing high levels of bacterial killing antibodies (Creech *et al*., 2017).

Capsular polysaccharides (CPs) in *S. aureus* can be seen in (Figure 1-4).

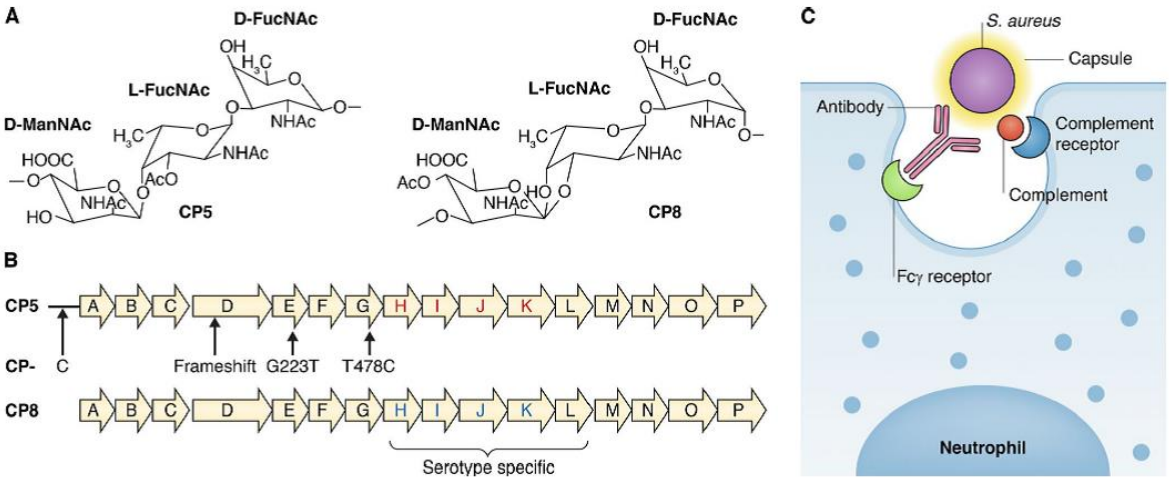

Figure (1-4) *S. aureus* CPs. (A) CP5 and CP8 have similar trisaccharide repeating structures that differ in glycosidic linkages and O-acetylation sites. (B) Genetic cap loci of *S. aureus* strains producing CP5 and CP8. Genes printed in color are responsible for the synthesis of serotype-specific modifications of CP. The diagram reveals the position of mutations in CP− strains of the *S. aureus* USA300 and USA500 lineages.(C) Diagram illustrating the hypothesis that capsule-specific antibody may induce OPA, inducing uptake of *S. aureus* by neutrophils via FcγR or complement receptors(Missiakas and Schneewind , 2016).

1.2.9 History of Conjugate Vaccines

Conjugate vaccines have been developed to induce a strong immune response against bacterial capsular polysaccharides (CPSs). CPSs are long polymers composed of many repeating units of simple sugars and serve as a protective external layer for many bacteria. Depending on the chemical

composition of the repeating unit (usually composed of one to seven monosaccharides). Bacteria can synthesize hundreds of chemically and immunologically different polysaccharides. Antibodies against the polysaccharides of many pathogenic bacteria, such as meningococcus, Hib, and pneumococcus, protect people from disease. Vaccines composed of purified polysaccharides against meningococcus and pneumococcus were developed in the 1970s. Inappropriately, those vaccines, while partially immunogenic in adults, were completely unable to induce an antibody response in infants and children, the population for whom the vaccines were mostly needed. The problem was solved in the 1980s when John Robbins and Rachel Schneerson at the National Institutes of Health in Bethesda, Maryland, and David Smith and Porter Anderson in Rochester, New York, independently figured out that, in 1929, it had been described that bacterial CPSs become very immunogenic when covalently linked to a carrier protein (Rappuolia *et al*., 2019) . And, thus, started working on aconjugate vaccine against Hib, which worked beautifully in infants and children. The Hib vaccine was licensedin1990 in the United States, and John Robbins, Rachel Schneerson, Porter Anderson, and David Smith received the AlbertL asker Award for Clinical Researchin 1996 for preventing meningitis in children (Albert and Lasker ,2018).

1.2.10 Mechanistic Considerations for Conjugate Vaccines (Product Characteristics that influence immune response):

The present knowledge of the mechanism of action of conjugate vaccines has been recently reviewed in (Figure 1-5) briefly (Avci *et al*., 2019). Many variables in the design, development, and production of glycoconjugate vaccines influence their immunogenicity and presumably their efficiency. The choice of saccharide size, carrier protein, conjugation chemistry, and formulation are some of the key decisions faced in every glycoconjugate

development program (Broker *et al*., 2016). Several of these factors have been proposed in the literature to explain why a given glycoconjugate vaccine or a regimen of such vaccines under performed in clinical studies(Avci *et al* ., 2019).

New glycoconjugate vaccines are developed based mostly on company specific design, know-how, expertise, and technology platforms, typically without benefit of solutions derived from the broader community experience. Moreover, introduction of new technologies arises sometimes from a need for product differentiation to create a competitive advantage or to navigate a complex intellectual property landscape. The result is sudden failures or inferiority of some new glycoconjugate vaccines in clinical trials and a limited number of successful, reliable glycoconjugate production technology platforms. As a consequence, confidence around the maintenance of product safety and efficacy for regulatory authorities and manufacturers relies closely on manufacturing process control and clinical and commercial product characterization without a clear understanding of the critical quality attributes of the products being made (Avci *et al* ., 2019).

Despite the commendable focus of academic and industry laboratories to bring forward new innovative or follow-on vaccine candidates as quickly as possible, the heart of the issue is the absence of available data to maintenance a community-wide basic understanding of the critical design features and attributes that predict optimal protective immune responses in the relevant populations. This challenge is further complicated due to the non-availability of reliable animal models that can correlate and predict clinical efficacy. Due to limited public information from the many previous successful and unsuccessful glycoconjugate vaccine programs as well as current active endeavors in the private sector, the optimal product characteristics of and

immunological responses to glycoconjugate vaccines are indistinct and will require significant efforts to define. We suggest below specific areas of focus for these collaborative efforts (Avci *et al* ., 2019).

1.2.10.1 Elucidation of the immune response mechanisms induced by glycoconjugate vaccines:

While glycoconjugate vaccines have provided great health benefits in controlling bacterial diseases, their chemical conjugations have often been empirically driven, with variably controlled production processes (e.g., conjugation) and analytical profiles, resulting in variably immunogenic glycoconjugate vaccine molecules. Process and quality consistency impact the composition of the vaccine product and in turn influence the immunogenicity and efficiency of glycoconjugate vaccines. One critical factor for augmenting glycoconjugate vaccine immunogenicity is our understanding of how glycoconjugate vaccines induce adaptive immune responses. While the traditional hypothesis suggests a peptide presentation to helper T cells (Costantino *et al*., 2011) a new exemplary proposes the presence of carbohydrate-specific T cells (i.e., Tcarbs) and their function in inducing adaptive immune responses in glycoconjugate immunization (Sun *et al*., 2019).

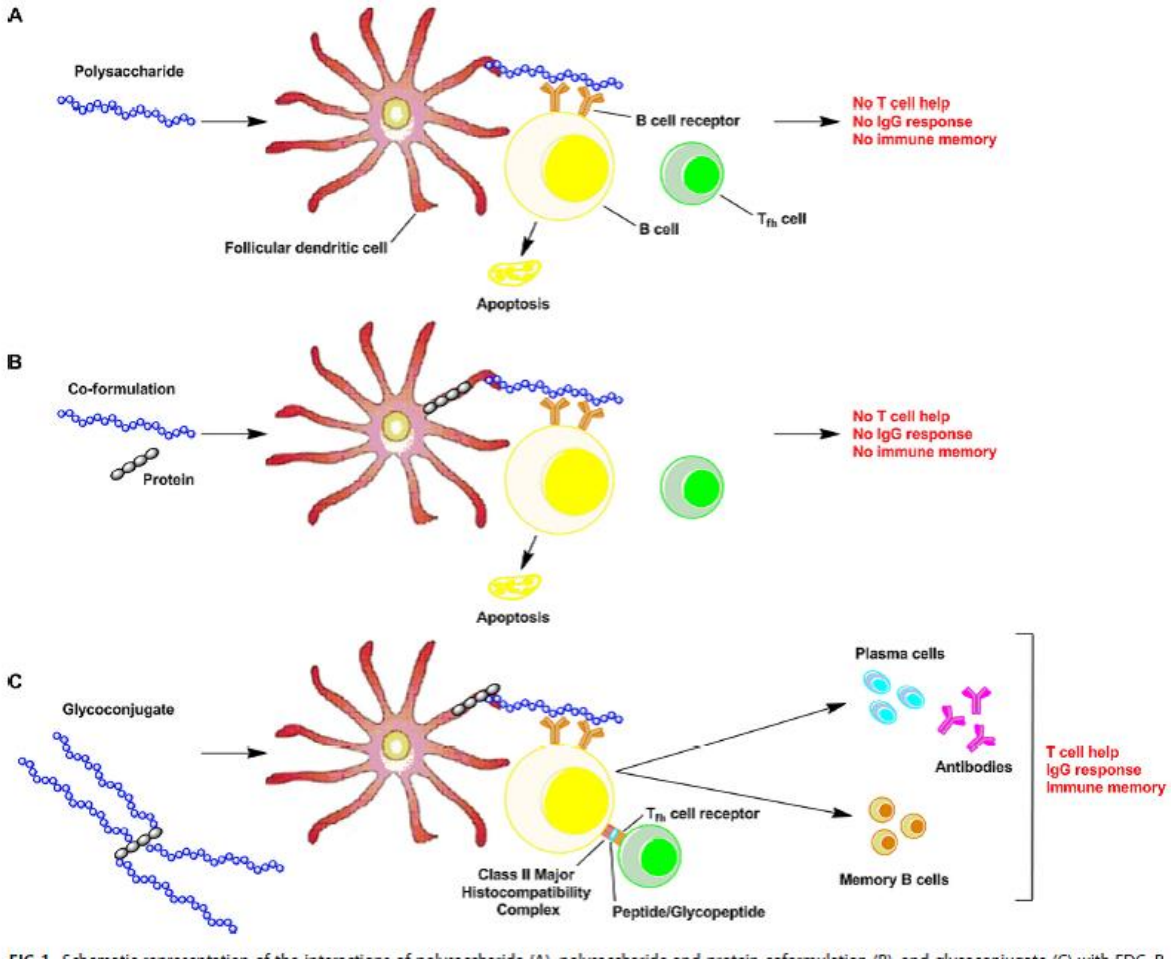

Figure (1-5): Schematic representation of the interactions of polysaccharide (A), polysaccharide and protein coformulation (B), and glycoconjugate (C) with FDC, B cells, and T h cells and the associated immune response. For polysaccharide and polysaccharide and protein coformulation, no IgG response and immune memory are induced because of the absence of T cell help. For glycoconjugate, the loading of peptide or glycopeptide into MHC and the engagement of TCR elicits an IgG response and immune memory (Avci *et al* ., 2019).

The hypothesis that led to the discovery of this new model was that carbohydrate antigens in their pure form do not bind to major histocompatibility complex class II (MHCII) and therefore are not presented effectively to T cells (Figure 1-5 A). However, when conjugated with a carrier protein, a peptide-bound, processed carbohydrate epitope (glycanppeptide) is generated in the endolysosomes of antigen-presenting cells (APCs). Through binding of the peptide portion to MHCII, the carbohydrate portion (glycanp) is existing on the APC surface for T-cell recognition (Figure 1-5C). Tcarb-

mediated immune responses induced by glycoconjugate immunization have been demonstrated to yield protective immunity in controlled in vivo model systems through either depletion (Avci *et al*., 2019) or adoptive transfer (Middleton *et al*.,2017) of epitope-specific CD4 T-cell populations. Most recently, it was demonstrated that polysaccharide structure commands mechanism of adaptive immune response to glycoconjugate vaccines (Middleton *et al* ., 2017).

In that study, four clinically important glycoconjugate vaccines were tested for their mechanism of action. Three of the four glycoconjugate vaccines tested induced adaptive immune responses regulated by Tcarbs. However, the meningococcal group C (MenC) conjugate vaccine immunity was predominantly limited to peptide-specific T cells. The explanation proposed for the lack of Tcarb stimulation by MenC conjugate was that MenC polysaccharide is substantially depolymerized in the endolysosomes, yielding small oligosaccharides (as small as a monosaccharide) that do not sufficiently mask the peptide in the MHCII binding groove and therefore do not elicit Tcarb responses. The relative contribution of peptide or glycan presentation models for glycoconjugate vaccine efficiency is yet to be fully explored. The current understanding based on the recent literature is that the glycan presentation is a natural result of the difficulty in cleaving the covalent/synthetic bond established between the glycan and the carrier protein and the slow or incomplete processing of the polysaccharide in the endolysosomes of antigen presentation cells. Thus, peptide-bound processed glycan epitopes are formed during processing in the endolysosomes to then be existed to helper T cells. To further expand the understanding of this mechanism of action , it would be important to characterize the presence of Tcarb in a definitive way by isolating more T carbs and by determining the

structure of the Tcarb bound to a glycopeptide by X-ray diffraction (Rappuoli *et al*., 2017).

The studies described above lay the groundwork for future investigations pertaining to elucidation of structural requirements for MHCII-dependent carbohydrate presentation; elucidation of molecular interactions yielding T-cell stimulation by epitopes generated from treating of glycoconjugate vaccines; and design and synthesis of structurally defined, knowledge-based, protective new generation glycoconjugate vaccines. For presenting products, glycoconjugate construction has been an empirically driven process of linking two molecules(carbohydrate and protein) without considering the molecular and cellular immune mechanisms critical for conjugate vaccine efficacy. A deeper understanding of these mechanisms (e.g., antigen uptake, processing, and T-cell activation) may also provide opportunities for optimizing novel chemical or biological conjugation technologies that have been recently described (Kapoor *et al*., 2018). Thus, delineatingT-cell-mediated immune activation pathways by glycoconjugate vaccines has inferences for using these vaccines to control or eliminate infectious diseases globally (Avci *et al* ., 2019).

1.2.10.2 Assessment of the impact of polysaccharide size, structure, functionalization, and conformation on immune responses to the conjugated polysaccharide:

It is commonly documented that oligosaccharide antigens with chain lengths longer than the minimal epitope length may behave as native polysaccharides. In certain cases, the protective epitope is nonlinear and the length of the polysaccharide sequences must be investigated for protective conformations in the context of the conjugate. This is further complicated by the need to define the role of labile side groups (e.g., O acetyl,

pyruvicacid,etc.) in the immunogenicity of the epitope. As an example, clinical evaluation of multivalent group B Streptococcus (GBS) conjugate vaccines has been ongoing for nearly 2decades (Buurman *et al*., 2019) but the optimal length for the GBS type III saccharide and the putative presence of a conformational epitope are still being debated (Carboni *et al*., 2017). In the clinic, they successes with glycoconjugate vaccines made with both oligosaccharides and polysaccharides, and efficacy induced by one or both seems to be polysaccharide dependent. As mentioned above in section i, it was demonstrated that polysaccharide structure can influence the specific mechanism of adaptive immune response to glycoconjugate vaccines (Sun *et al*., 2019). As conformational epitopes might be expected to be found, the field would benefit from improved molecular modeling of large polysaccharides. Finally, it would be useful to understand whether the size of the polysaccharide or the size of the conjugate makes the biggest influence on immune response (Avci *et al* ., 2019).

1.2.10.3 Evaluation of immune mechanisms by which carrier proteins alter the immune response:

Multiple carrier proteins have successfully been employed in licensed infant glycoconjugate vaccines (e.g., CRM197, tetanus and diphtheria toxoid, , and nontypeable *Haemophilus influenzae* (NTHi]) protein D). The optimal choice is unclear, although glycoconjugates using CRM197 and tetanus toxoid dominate the commercial markets. Additionally, immune response to nontraditional carriers based on conserved proteins (e.g., NTHi protein D) may also contribute to protection through their independent action as immunogens. they demand to develop a better understanding of T-cell responses induced by carrier proteins and differences between carrier proteins. Indeed, assuming that presentation of carbohydrate attached to carrier protein peptide to T cells via uptake to MHCII is a

systematic/universal process, it is likely that certain peptide fragments might be better than others. This requires discovering the carrier protein epitopes presented to and recognized by helper T cells from glycoconjugate processing in antigen-presenting cells. The principles of structural vaccinology highlighted by Bottomley *et al* (Bottomley *et al* ., 2016) should be applied to carrier protein design. Ultimately, carrier proteins should probably be designed in silico to achieve optimal T-cell presentation. Critically, confirmation in humans, and more specifically in the target human population (e.g., infants), is essential to evade optimizing to a specific animal model that may not correlate. This points to the need for great care in anticipating whether observations in animal models can directly inform proof of concept (POC) in humans (Avci *et al*., 2019).

1.2.10. 4. Assessment of the impact of conjugation platform on immune response:

An additional level of complexity in comparing glycoconjugate constructs stems from the variety of conjugation platforms (e.g., conjugation chemistry, multiple attachment versus single-point attachment of carbohydrate, presence/absence of linker, or sites of attachment on the carrier protein). From a broad overview of polysaccharide-protein conjugate vaccines, it appears that extremes of saccharide activation (very high or very low) have produced sudden results in conjugate immunogenicity. Inrelevant cases, the level of O-acetylation and/or sialylation must be assessed as it might be relevant to immunogenic epitope preservation and therefore guide the selection of conjugation methods (Berti *et al* ., 2018) .

Some chemistries lead to stability issues, while some polysaccharides are more amenable to conjugation by certain methods than others. Consistent presentation of carbohydrate epitopes is essential as shown with synthetic

oligosaccharides (Lisboa *et al*., 2017)., but the choice of conjugation chemistry is not observable for a given polysaccharide-carrier protein combination. Additionally, there are few published data to systematically evaluate the impact of linkers and sites of attachments on carrier proteins on immune response to saccharide haptens. Finally, new linkers and chemistries raise concerns due to the potential creation of immunogenic neoepitopes which may complicate the regulatory approval path for such products. For the field to really move toward a rational glycoconjugate design, they need to understand how details of antigen structure in the vaccine molecule influence immunogenicity in humans generally and ultimately in the target population for the specific vaccines. Quality attributes such as saccharide size, degree of saccharide activation, constraints on modifications of saccharide structure, use of linkers, density of attachment on carrier protein, or size spreading of conjugate particles appear important to control. New platforms (Kapoor *et al*., 2018) that allow the more precise control of such parameters should be used to develop test molecules for use in in *vitro*, in *vivo*, and ex *vivo* models to explore mechanisms of immune response to glycoconjugate vaccines. We do not know whether the Tcarb clone repertoire is dependent on the conjugation platform. The availability of well-defined test molecules for immunological experiments will be critical to our systematic evaluation of the impact of these quality attributes on the immune response to the conjugated haptens (Avci *et al*., 2019).

1.2.10.5 Development of a greater breadth of analytical tools to better characterize glycoconjugates:

Most conjugates are produced from large polysaccharides (average molecular weight more than 100 kDa), resulting in lattice-like conjugates (average molecular weight often more than 1,000 kDa), which limits how much can be learned with current analytical methodology. Addition of

adjuvants, surfactants, and other excipients makes characterization and stability observing more complex. Yet, new analytical tools are revealing more about these complex entities. Tools for physicochemical analyses, such as high-field nuclear magnetic resonance (NMR), particle-size distribution analyses, and perhaps cryoelectron microscopy can reveal structural details of glycoconjugates. They demand to apply high-performance physicochemical analyses, combined with cutting-edge immunological methods, to better understand the structure-function relationship for glycoconjugates to better inform fine-tuning of glycoconjugate design and production. If the use of a lattice glycoconjugate format is established to be immunologically superior to (e.g.) a single-point attachment glycoconjugate format for a given disease target, technologies enabling site-specific conjugation might be favored in order to enable in depth characterization of the glycoconjugate vaccine and better consistency of manufacturing. Ultimately, the connection of the results from these improved analytical tools to human immune response safety and efficacy is critical to advancing their understanding of the true critical quality attributes of these vaccines(Avci *et al* ., 2019) .

1.2.10.6 Development of better, more relevant in vivo or ex vivo models for evaluation of glycoconjugate vaccines:

It is obvious that research and development of glycoconjugate vaccines rely on animal models to establish scientific proof of concept that the vaccines can provide protection against the target pathogens. The animal models are by necessity focused on immune response to the vaccine and/or protection from challenge with the target pathogen, often with definition of surrogate markers of protection. However, these studies are often of imperfect relevance to human disease conditions and are unreliably predictive of the responses of the target human populations. Pursuit of “humanized”

animal or in vitro models as done in oncology with xenograft models or the use of *ex vivo* models (e.g., cellular models) for evaluation of antigen processing, presentation, and/or functional activity may be a useful addition to the current approaches. Regardless, testing in animals is an essential precursor to clinical trials, which are the first true opportunity to assess the immunogenicity/ efficacy of the vaccine candidate in the target human population. When likely, early use of new or enhanced glycoconjugate vaccines in controlled human infection models (CHIMs), such as those deployed for pneumococcal organisms (carriage) and typhoid (invasive disease), can assist in early-stage gating of vaccine candidates, depending upon the final target population (Avci *et al*., 2019).

1.2.10.7 Tailoring glycoconjugate vaccines for specific target populations:

Know that the immune systems of humans of different age groups are not functionally identical (Simon *et al*., 2015). The quality of the immune response to a given glycoconjugate vaccine is also dependent on the naive or primed exposure of the patient to the target pathogen. Considering this, they might increase efficacy by studying optimal dosing regimen and schedule or adjuvanting in clinical trials (Rappuoli *et al*., 2018). In addition, they still do not fully understand whether the Tcarb clones or the carrier-specific T-cell clones are triggered by a given vaccine candidate and how these events vary depending on the age of the getting population. This is only an example of fundamental understanding that we are currently lacking that limits our ability to truly optimize the efficacy of conjugate vaccines and tailor them to specific target populations (Avci *et al*., 2019).

Conclusions:

In the light of the previous data, the present study can conclude the following points:

1-Coagulase-negative Staphylococci (CoNS) were the most prevalent pathogens causing infection in patients.

2-All *S. aureus* isolates were Methicillin resistant *S. aureus* (MRSA); All contained *mec A* gene and all showed (MDR) pattern of resistance.

3 -Low rate of *S. aureus* having capsular polysaccharides (CP5) gene.

4.Extraction and Partial purification of *Staphylococcus aureus* Capsular polysaccharides (CP5) was successful modification method; this method as the first study in Iraq

5- Low growth blood culture in immunogenic group in laboratory animals, compared to control group after exposure to challenge dose.

6- Lymphocytes were the predominant leukocyte in most of healthy rat the WBC differential count, differ from neutrophils generally are the predominant leukocyte in human.

7-The results indicated not significant increase of Interluekin-12p40 level of *S. aureus* Capsular polysaccharides (CP5) before and after challenge dose.

8-The levels of Interlukin-4 in the serum of animals groups appears indicated a significant increase in *S. aureus* Capsular polysaccharides (CP5) before and after challenge dose.

9- We can conclude from the present study that the CP5(extracted from A.MS *S .aureus* isolate) showed improvement of the immune responses against MRSA antigen via enhancing the levels of humoral immunity elements as well as histopathological changes that on spleen activity that stimulate immune responses against antigens that enter the body.

Recommendations:

1- Conducting a controlled study have determined that SpA and capsule antigens could be targeted by either passive or active immunization as vaccine approaches to effectively target a broader range of strains with phenotypic adaptations involving different virulence determinants.

2- *Staphylococcus aureus* protein A (SpA) can be isolated and used for preparation of challenge dose to measure the immunogenic activity when conjugate with CP5 because the capsule produced by the SpA⁻ strains is enough to elicit antigen presenting cells (APC) uptake in the presence of capsule-specific antibodies.

3- Detection of polysaccharide and polysaccharide-protein conjugate with reverse phase chromatography and fourier transform infrared (FTIR) spectroscopy.

4- The phenol-sulphuric acid method can be used to determine carbohydrate concentration in *S. aureus* capsular polysaccharides (CP5) in high amount cultures usage.

5- Using of P glycoprotein inhibitor vit E.TPLS instead of Tween 80.

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الخلاصة :

تهدف الدراسة الحالية الى التحري عن الخواص المناعية والفعالية الوقائية للسكريات الكبسولية (CP5) في الوقاية من العدوى المتسببة عن بكتريا المكورات العنقودية الذهبية المقاومة للمثسلين في نماذج الحيوانات لغرض توفير حماية ضد مختلف الاصابات البكتيرية الحادة المتسببة عن الاصابة بكتيريا المكورات العنقودبة الذهبية المقاومة للمثسلين، من خلال حقن سكريات الكبسولة مباشرة لتحفيز الاستجابة المناعية (على عكس التخفيز بواسطة سطح البكتيريا) وذلك بزيادة فعالية التخفيز للمستضد في نموذج الحيوانات تحت الأختبار.

تم جمع 180نموذج لعينات سريرية مختلفة من المرضى الوافدين لعدد من المستشفيات الرئيسية في محافظة بابل (مستشفى الحلة التعليمي, مستشفى التعليمي للولادة والاطفال ومستشفى الامام الصادق التعليمي) خلال من الفترة كانون الاول الى تشرين الثاني 2020. وتم استثناء المرضى الذين سبق لهم الدخول للمستشفى مسبقا.

شملت النماذج المجموعة خلال الدراسة عينات الدم ,الادرار, سائل النخاع الشوكي, جروح الحروق، وغيرها ، بينت نتائج الدراسة الحالية انه من مجموع 180 عينة سريرية، فأن 46 منها (25.5%) اظهرت نمو بكتيري موجب على الاوساط الزرعية الاعتيادية، فيما لم تظهر 134 عينة سريرية (74.4%) نموا موجبا على الاوساط الزرعية

بينت نتائج الدراسة انه من مجموع 180 عينة سريرية تم الحصول على 46 عزلة (25.5%) بكتيرية مشخصة بواسطة الاختبارات الكيموحيوية , نظام Vitek2 و نظام أشرطة API. تم الحصول على العزلات البكتيرية من عينات الادرار 16 (34.7%), الحروق15(32.6%), الدم12(26.1%) , خراج 2 (4.3) والسائل القصبي 1(2.1%).

أظهرت نتائج الزرع البكتيري الحصول على 46 عينة سريرية ذات نمو بكتيري موجب , وأن كانت 22 عزلة (47.82%) منها كانت موجبة لصبغة كرام و 24 عزلة (52.17%) كانت سالبة لصبغة كرام. من مجموع 22(47.82%)عزلة موجبة لصبغة كرام ، وجد أن 17 عزلة كانت عائدة لمجموعة المكورات العنقودية السالبة لأنزيم الكوأوكيوليز Coagulase-negative Staphylococci (CoNS) وخمسة عزلات تم تشخيصها على أنها عائدة للنوع *S. aureus* .

اظهرت جميع العزلات الخمسة *S. aureus* نمط متعدد المقاومة لمقاومة المثسيلين باستخدام أقراص السفوكستين حسب CLSI ، وبالأعتماد على تقنية تفاعل البولميريز المتسلسل، اظهرت النتائج إمتلاك جميع العزلات الخمسة لجين *mecA* gene في حين أظهرت عزلة واحدة قابليتها على إمتلاك جين متعدد السكريات الخامس للكبسولة (CP5 gene) وهي العزلة (A.MS isolate).

تم العزل والاستخلاص والتنقية الجزيئة لمتعدد البولي السكريد الخامس من عزلات من *S. aureus* المحلية المقاومة للمثسلين والتي عرفت باسم الباحث (A.MS isolate) وقد الاستخلاص باستخدام طريقة مبتكرة ومحورة لأول مرة في هذه الدراسة من خلال التحوير والمزج بين طريقتين سابقتين.علما انها الدراسة الاولى في هذا المجال على مستوى العراق.

تم قياس المكونات الكيميائية لمستخلص التنقية الجزئية لمتعدد البولي سكرايد لكبسولة عزلة (A.MS isolate) بواسطة جهاز المطياف الضوئي المرئي للكثافة الضوئية وكانت االامتصاصية لمتعدد البولي سكرايد الخامس للكبسول هي (0.279) بتركيزl(µg/m 6) عند الطول الموجي 206 نانوميتر, للبروتين (0.017) بتركيز(3.4 µg/ml) عند الطول الموجي 280 نانوميتر, للحامض النووي (0.018) بتركيز(3.6 µg/ml) عند الطول الموجي 260 نانوميتر. علما ان التركيز لمستخلص خام لمتعدد البولي سكرايد لكبسولة عزلة A.MS (isolate) كان 6 ملي غرام/لتر.

تضمنت الدراسة الحالية في الجسم الحي *in vivo* التحري عن استراتيجية المناعية والفعالية لمتعدد البولي سكرايد لكبسولة (CP5) المستحلب بواسطة بولي سوربات 80 المجموعة الاولى I كانت لمتعدد البولي سكرايد لكبسولة عزلة (A.MS isolate) وتم حقن 8 حيوانات من انثى الجرذان بالغشاء البرويتوني على شكل جرعتين كل جرعة (10 µl) 50 µg لكل حيوان بواقع اسبوع بين الجرعتين. بينما كانت المجموعة II هي مجموعة التحكم او السيطرة وتكونت ايضا من 8 حيوانات من انثى الجرذان تم حقنها بمحلول ملح الفوسفويت المنظم ضمن الغشاء البرويتوني بواقع 10 مايكرولتر لكل حيوان ضمن كل جرعة بواقع اسبوع بين الجرعتين.

بعد ثلاثة اسابيع تم سحب دم لكافة الحيوانات لغرض اجراء تحاليل الدم لامراض الدم والفحوصات المناعية قبل اعطاء جرعة التحدي.

بعد 30 يوم من الحقن بالجرعة الاولى لمجموع الحيوانات البالغ عددها 16 والمقسمة الى مجموعتين (ثمانية حيوانات لكل مجموعة).تم حقن جميع الحيوانات المتبقية ببكتيريا المكورات العنقودية الذهبية المقاومة للمثسيلين بتركيز(10^7 CFU) وبحجم نصف مل لكل حيوان. وبعد ذلك يتم حساب درجة الحماية بتعداد عدد الحيوانات الباقية على قيد الحياة بعد اسبوع من الحقن البريتوني لها. وجمع نماذج الفحوصات لتحليل الزرع الدم تعداد كريات الدم وانواعها والفحوصات المناعية ضمن 72ساعة بعد الحقن.

تم سحب الدم من كافة الحيوانات من القلب تحت التخدير(الكلوروفوم) لاجراء تعداد لكريات الدم البيضاء وانواعها ,فعالية التصفية للدم, قياس تركيز الانترلوكين 4 وتركيز الانترلوكين 12 P40 قبل وبعد جرعة التحدي باستخدام تقنية المقايسة الامتصاصية المناعية للانزيم المرتبط قبل وبعد جرعة التحدي.

أظهرت نتائج تحاليل الدم والفحوصات المناعية قبل إعطاء جرعة التحدي عدم وجود زيادة معنوية بعدد كريات الدم البيضاء في المجموعة I متعدد السكريات للكبسولة (11093 cells/mm^3) مقارنة بمجموعة السيطرة II (9650 cells/$mm^{3)}$ بمستوى دلالة ($p < 0.05$) وكذلك اظهرت نتائج العد التفريقي لكريات الدم البيضاء عدم وجود فروقات معنوية بين المجموعتين عند مستوى دلالة ($p < 0.05$).

اما بالنسبة لمستوى اللانترلوكين الرابع (IL-4)، فقد اظهرت النتائج فروقات معنوية حيث كانت مجموعة كل من متعدد السكريات للكبسولة (4.775pg/mL) I مقارنة بمجموعة السيطرة II (0.709pg/mL) بمستوى دلالة $p < 0.05$.

الخلاصة

أظهرت نتائج دراسة مستوى الانترلوكين 12-p40 (IL-12-p40) عدم وجود فروقات معنوية في كل من مجموعة متعدد السكريات للكبسولة I (7.496pg/mL) مقارنة بمجموعة السيطرة II (12.115 pg/mL)عند مستوى دلالة ($p < 0.05$).

بينت نتائج فعالية تصفية الدم من العامل الممرض للمكورات العنقودية الذهبية (MRSA) كانت عالية بنسبة (71.5%) في مجموعة متعدد السكريات للكبسولة I مقارنة بمجموعة السيطرة II (33.4%) .

اظهرت نتائج تحاليل الدم والفحوصات المناعية بعد اعطاء جرعة التحدي وجود زيادة معنوية بعدد كريات الدم البيضاء في كل من المجموعة I متعدد السكريات للكبسولة (9407 cells/mm ومجموعة السيطرة II (4682 cells/mm^3) عند مستوى دلالة ($p < 0.05$).

اظهرت نتائج دراسة مستوى اللانترلوكين الرابع (IL-4) فروقات معنوية حيث كانت مجموعة متعدد السكريات للكبسولة I (8.6676 pg/mL) مقارنة بمجموعة السيطرة II (2.8531 pg/mL) عند مستوى دلالة ($p < 0.05$).

اما بالنسبة لمستوى الانترلوكين 12-p40 (IL-12-p40)، فقد اظهرت النتائج عدم وجود زيادة معنوبة في كل من مجموعة متعدد السكريات للكبسولة I (10.483 pg/mL) مقارنة بمجموعة السيطرة II (5.792 pg/mL) عند مستوى دلالة ($p < 0.05$).

على علاوة على ذلك، فقد أظهرت نتائج الفحوصات للمقاطع النسيجية للطحال على مستوى المجاميع اختلاف واضح في تمدد منطقة اللب الابيض للحويصلات اللمفاوية على حساب منطقة اللب الاحمر اضافة الى وجود الخلايا الحمضية والبلازما واللمفاوية . بينما كانت الحويصلات اللمفاوية متضيقة بمجموعة السيطرة II ولم تظهر اي تغير .واظهرت كل من مجموعة I متعدد السكريات للكبسولة تمدد جزئي لمنطقة اللب الابيض للحويصلات اللمفاوية على حساب منطقة اللب الاحمر. في حين لم يظهر هنالك اي تغير في مجموعة السيطرة II.

نستنتج من خلال هذه الدراسة وجود تأثير واضح لمتعدد السكريات للكبسولة العزلة A.MS) (isolate للمكورات العنقودية المقاومة للمثسيلين المستحلب بواسطة بولي سوربات 80 من خلال تحسين الاستجابة المناعية كمساعد مناعي ضد المكورات العتقودية المقاومة للمثسيلين وتعزيز عوامل المناعة الخلطية اضافة الى حدوث تغييرات نسيجية في الطحال ضد المستضد الداخل للجسم.

وزارة التعليم العالي والبحث العلمي
جـــــامعة بـــــابل
كليـــــة الطـــــب

الخصائص المناعية لمتعدد السكريات المحفظي ضد المكورات العنقودية الذهبية المقاومة للمثسلين في الجرذان

اطروحة مقدمة الى
مجلس كلية الطب /جامعة بابل
وهي جزء من متطلبات نيل شهادة دكتوراه فلسفة في العلوم/
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